CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

A Comparative Study of *Hippophae rhamnoides* and *Cichorium intybus* Found in the Karakoram Range of Gilgit Baltistan for Anticancer Potential Against Liver Cancer

by

Rukhsana Tabassum

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Dedicated to my beloved family who sacrificed their comfort for my PhD

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This is to certify that the research work presented in the dissertation, entitled "**A Comparative Study of *Hippophae rhamnoides* and *Cichorium intybus* Found in the Karakoram Range of Gilgit Baltistan for Anticancer Potential Against Liver Cancer**" was conducted under the supervision of **Dr. Erum Dilshad**. No part of this dissertation has been submitted anywhere else for any other degree. This dissertation is submitted to the **Department of Bioinformatics & Biosciences, Capital University of Science and Technology** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of **Biosciences**. The open defence of the dissertation was conducted on **June 20, 2025.**

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List of Publications

It is certified that following publication(s) have been made out of the research work that has been carried out for this dissertation:-

1. **Rukhsana Tabassum** and **Erum Dilshad** (2024). A comparative anti-cancer analysis of Iron Oxide nanoparticles of *Hippophae rhamnoides* and *Cichorium intybus* found in the Karakoram Range of Gilgit Baltistan against liver cancer targeting the RhoA gene. Drug Development and Industrial Pharmacy, 1–14. (doi.org/10.1080/03639045.2024.2400209).

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(Rukhsana Tabassum)

Abstract

Liver cancer is a serious health concern and its incidence is increasing worldwide. It is estimated that one million people will be affected by liver cancer annually by the year 2025 globally. The most common form of liver cancer is hepatocellular carcinoma, which accounts for more than 90% of cases, and the most prominent risk factor for this is HBV. HCC in men is continuously increasing in Pakistan and is thought to become the most common form of cancer in future. Some of the characterized Rho GTPases as RhoA are found to be involved in a variety of human cancers. Rho A is the small G protein in the Rho GTPase family which was found to be primarily involved in cytoskeleton of cell, cell polarity, cell cycle, cell migration and gene expression. Deregulation of Rho A signaling pathway is evidence to be closely linked to cancer progression, hence it is a major drug target for treating multiple malignancies. *Hippophae rhamnoides* and *Cichorium intybus* are grown in the mountains of the Karakorum range and have been used in traditional medicines since the beginning of time.

The basic aim of the current research work was to evaluate the extracts of *H. rhamnoides* and *C. intybus* and their Fe_2O_3 NPs against liver cancer cell line ($HepG_2$) by performing antiproliferative assays targeting the RhoA gene and apoptotic pathway genes and proteins. Plants were obtained from areas of Gilgit Baltistan such as central and upper Hunza, Ghizer district, and some areas of the Skardu division and their identification was done by the herbarium of Hazara University Pakistan. Fe_2O_3 NPs were synthesized using extracts of *H. rhamnoides* and *C. intybus* and characterized by UV-Vis spectroscopy, FTIR, SEM/EDS and XRD. MTT assay was used to study cytotoxicity against the $HepG_2$ cells. Real-time qPCR and ELISA were used for the gene and protein analysis respectively. An absorbance peak at 300 nm for *H. rhamnoides* and 289 nm for *C. intybus* nanoparticles was observed by UV-Vis analysis. The FTIR bands of *H. rhamnoides* Fe_2O_3 NPs suggested the presence of aldehydes, alcohols and polyols whereas bands of *C. intybus* Fe_2O_3 NPs suggested the presence of carboxyl groups, hydroxyl groups, alkynes and amines. EDS showed the composition of elements of both nanoparticles. The size of Fe_2O_3 NPs was found to be 27 ± 5nm for *H. rhamnoides* and $84 \pm$

4nm for *C. intybus*. XRD analysis confirmed the crystalline nature of synthesized nanoparticles.

The cytotoxic potential of Fe_2O_3 NPs synthesized from *H. rhamnoides* and *C. intybus* was assessed against the $HepG_2$ liver cancer cell line. The cytotoxic potential of synthesized nanoparticles and respective plant extracts was determined for distinct concentrations (20, 40, 60, 80 and 100 μM) and revealed promising results. During this experiment, $HepG_2$ cell line was exposed to plant extract and Fe_2O_3 NPs (20 to 100 μM) synthesized using the extract of *H. rhamnoides* and *C. intybus*. The IC50 value of 41.69 μM for *H. rhamnoides* and 71.04 μM for *C. intybus* Fe_2O_3 NPs compared to plant extract (78.10 μM and 96.03 μM for *H. rhamnoides* and *C. intybus* respectively) were found against $HepG_2$ cells. The gene expression and protein levels of RhoA were decreased in nanoparticle-treated cells as compared to the control group. The level of initiator caspase (caspase-9) and executioner caspases (caspases-3) and caspase-8 genes and proteins were also studied for $HepG_2$ cells treated with *H. rhamnoides* and *C. intybus* Fe_2O_3 NPs and plant extracts. The results presented that level of caspases genes and proteins was high in the cells treated with *H. rhamnoides* as compared to *C. intybus* NPs and extract in comparison to control group. This confirmed the role of nanoparticles in activation of cancer cell death by activating series of caspase reactions. The increased expression of these caspases (caspase 3, caspase 8 and caspase 9) at mRNA level and protein level showed that plant mediated Fe_2O_3 NPs have shown programmed cell death at remarkable degree. Moreover, it is also verified by this comparative study that *H. rhamnoides* have more potential to be used in the development of anticancer drugs as compared to *C. intybus*.

The polyphenols identified from the methanolic extract of *H. rhamnoides* and *C. intybus* using HPLC chromatography included gallic acid, salicylic acid, caffeic acid, kaempferol, rutin, quercetin, coumarin, ferulic acid, sinapic acid, vanillic acid, and chlorogenic acid. In the next step in silico predictions, molecular docking and molecular dynamic simulations were performed. There were five compounds selected for further study considering best vina score, cavity size and following Lipinski rule of five. ADMET properties were studied for drug-likeness of selected

compounds. RMSD values for each compound were also calculated and selected the value of best posed observed. The compound caffeic acid was found as a leading compound with adorable ADMET properties following Lipinski's rule of five. Caffeic acid may have the best potential to inhibit the RhoA protein because it stays intact and bound to the protein structure over the entire simulation run, indicating a strong interaction between the protein and the ligand, suggesting a potential inhibitory effect. Therefore, further studies are needed to investigate caffeic acid as a potential drug candidate for drug designing in future.

Keywords: *Hippophae rhamnoides*, *Cichorium intybus*, liver cancer, anticancer property, metallic nanoparticles, Rho GTPases, medicinal plants, bioactive compounds, Hepatocellular carcinoma.

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Abbreviations

ADMET	Absorption, distribution, metabolism, excretion, toxicity
AECHs	Atomic energy cancer hospitals
AFB1	Alpha toxin B1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AgNPs	Silver nano particles
CNS	Central Nervous System
CTCs	Circulating tumor cells
DNA	Deoxyribonucleic acid
EDS	Energy dispersive spectroscopy
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HPLC	High Performance Liquid Chromatography
ICI	Immune checkpoint inhibitors
MD	Molecular dynamics
MNPs	Magnetic nanoparticles
MRI	Magnetic resonance imaging
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

NAFLD	Nonalcoholic Fatty Liver Diseases
NASH	Nonalcoholic steatohepatitis
NMR	Nuclear Magnetic Resonance
ORR	Oral response rate
PAEC	Pakistan Atomic Energy Commission
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PTMs	Post translational modifications
RCT	Randomized controlled trial
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RNA	Ribonucleic acid
ROCK1	Rho associated coiled-coil-containing protein kinase 1
ROS	Reactive oxygen species
RTKN	Receptor tyrosine kinase
Rho GAPs	Rho GTPase activating proteins
Rho GDIs	Rho guanine nucleotide disassociation inhibitors
Rho GEFs	Rho guanine nucleotide exchange factors
SE	Standard error
SEM	Scanning Electron Microscopy
TACE	Trans arterial chemoembolization
TKI	Tyrosine kinase inhibitors
UV- vis	Ultra Violet Visible Spectroscopy
WHO	World Health Organization
XRD	X-ray Diffraction
ZnONPs	Zinc oxide nanoparticles

Symbols

μ	Mu
$\pm$	Plus Minus
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
Å	Angstrom

Chapter 1

Introduction

Cancer is one of the major causes of death globally, although many organizations in the world are working continuously and contributing hugely to combat this deadly disease [1]. According to the Statistics of GLOBACON in 2020 more than 19.3 million new cases of cancer were registered, and this figure seems to reach 28 million till 2040 [2]. Liver cancer is a worldwide health concern that is becoming more commonplace. By 2025, one million people are expected to be affected by liver cancer annually [3]. Mutations and epigenetic changes are the root causes of cancer, which is a multi-step illness. Normal cells have the basic capacity to develop into neoplasia, which acquires characteristics such as unchecked growth, angiogenesis, and resistance to cell death. Hanahan and Weinberg researched several important characteristics of malignant cells known as hallmarks of cancer, such as their ability to evade immune cells, alter metabolism, cause disruptions in the genome, and induce inflammation to support tumour growth [4, 5].

The most common form of liver cancer is hepatocellular carcinoma, which is a major liver tumor and is considered one of the global health challenges. It consists of 85 to 90% of primary liver cancers. In 80% of cases, HCC is caused by severe liver damage and cirrhosis [6]. The incidence rates of hepatocellular carcinoma are increasing in many countries such as Mongolia, China, Africa, Egypt, and Taiwan, and are estimated to continue increasing in the future [7]. The most significant risk factor for HCC is the hepatitis B virus (HBV), which is responsible for over

50% of cases. One of the top five most lethal cancers that is spreading quickly each year is liver cancer. It was shown that liver illnesses are typically more common in underdeveloped nations. Hepatitis B virus, hepatitis C virus, fatty liver disorders, liver cirrhosis linked to alcohol use, smoking, being overweight, diabetes, and an excess of iron in certain diets are risk factors for liver cancer [8]. The disease has a dismal prognosis; only 5 to 15% of individuals are eligible for surgical removal, and even then, this treatment is only appropriate for those who are in the early stages of the condition [9].

Previously, liver cancer was more common in Asians and Africans. However, due to the increase in the incidence of hepatitis C infection and cancers associated with alcohol and non-alcoholic steatohepatitis, it is increasingly widespread in Western countries [7]. Therefore, liver cancer has become a severe health issue globally. Usually, there are two types of cancers of the liver cancer. Primary liver cancer is caused by several cells in the liver. Hepatocellular carcinoma is the most predominant cancer, which consists of 85 to 90% of primary liver cancers. In above 80% of cases, HCC is caused by severe liver damage and cirrhosis [10]. Another kind of primary liver cancer is cholangiocarcinoma, which is found in the bile ducts of the liver and constitutes 10 to 20% of liver cancers [11].

Liver secondary cancer is due to metastasized circulating tumour cells (CTCs), rising from primary tumour organs other than the liver. The Recent methods to early detect CTCs of secondary liver cancer have been briefly presented by Rostami and colleagues [12]. Recently, many approaches have been proposed for the diagnosis of liver cancers. There are serological markers as the total -fetoprotein (AFP) and AFP-L3 are offered. Furthermore, radiology often practices imaging procedures such as ultrasound, computerized tomography (CT) scans, hepatic angiography and MRI, which are regularly used to diagnose liver cancers [13]. Over the past decade, several advancements have been made in treating HCC, especially in treating advanced diseases such as infectious diseases, neurological disorders, cardiovascular diseases, and cancer treatment. Resection and transplantation of the liver remain the basis of curative treatment. However, for patients not eligible for curative therapy, exciting progress has been made in the molecular and cellular

approaches for systemic therapy of HCC as immune checkpoint inhibitors (ICIs), tyrosine kinase inhibitors (TKIs), monoclonal antibodies [14]. In general, combining in vitro studies and in silico molecular target prediction approaches in future will help to find novel therapies to treat liver cancer and other types of cancers.

Progression and metastasis of cancer are the activities that involve important changes in the cells. It was observed that in tumour development there is a role of abnormal signalling of the Ras homology gene family called Rho GTPase. In the normal physiology of cell these acts as molecular switches and helps to regulate numerous cellular processes which includes adhesion, mobility, organization and cell division. It was found that in addition to varied roles in cells' normal physiology, Rho proteins were also found to have a significant effect on pathological processes as in cancer. The unstable functions of Rho GTPases show to effect almost every stage of cancer progression which specifically includes proliferation, invasion and metastasis. Some of the members of Rho GTPases as RhoA, Cdc42, and Rac1 are found involved in the cancer progression and metastasis of a variety of human tumors [15]

Small G proteins called Rho GTPases primarily regulate the cytoskeleton, polarity, morphology, vesicular motions, cell cycle, cell destruction, and gene expression in cells [16]. Numerous investigations have shown that these little proteins actively contribute to the development of cancer. As a result, Rho GTPases seem to be new targets for researchers studying cancer biology. Cancers exhibit significant alterations at the cell level during spread and progression. The most frequent alteration seen is the growth of tumour due to aberrant Rho GTPase signalling [17]. Therefore, determining how Rho GTPases function in HCC may aid in the search for a treatment for this severe cancer. RhoA is thought to be a possible therapeutic target in the future because it was recently discovered that small molecule inhibitors can target it in cancer [18].

RhoA is a small protein in the Rho GTPase family which is evidenced to be closely linked to cancer progression through invasion, microscopic satellite lesions, and differentiation [19]. It was found that RhoA and Rac1 become crucial in the angiogenesis of tumour as well as in the initiation, invasion and metastasis

of cancer. Because of potential roles of RhoA and Rac1 in cancer progression now this is an emerging topic of research for the research community specially in cancer biology. Additionally, some of the prior reports also showed the presence of an increased concentration of RhoA protein in some types of cancers as breast, lung and colon cancer. This proposes that a particular protein plays an important role in cancer invasion and neoplasm [20]. If we consider the role of RhoA in hepatocellular carcinoma, there is some evidence which shows the crucial role of this protein in the development of HCC [21], but future studies are needed which helps to discover novel therapeutic approaches to overcome the challenges of liver cancer.

The development of novel cancer therapies has received increased attention from the medical community in recent years. Since the 1960s, the efficacy of natural products in cancer treatment and other types of diseases has been widely acknowledged. Compounds with anti-cancer properties can be found in abundance in nature, particularly in plants and microorganisms. Similarly, natural chemical research based on plants has made tremendous strides in recent decades, resulting in the discovery of highly effective anticancer drugs. Numerous unique therapeutic medications for the treatment and prevention of cancer have been developed by scientists because of the abundance of beneficial plants available. The desire for natural substances as new anticancer pharmaceuticals has increased in response to the high toxicity of various cancer chemotherapy treatments, along with their unpleasant side effects and drug resistance [22].

Gilgit Baltistan is located in the north of Pakistan and spreads over the three highest mountain ranges including the Himalayas, the Karakoram range and the Hindukush. This area is gifted with tremendous natural resources, among them medicinal plants are frequently used in the cure and management of different diseases [23]. The local resources have been utilized by the ethnic groups in Gilgit Baltistan for centuries in their old traditional system of healing. New and emerging technologies include nanobiotechnology for nanomedicine development that aims to promote the anticancer efficacy of plant extract-based drugs by the controlled release of compounds and to investigate new methods for administration. The

identification of novel compounds in the plants is thought to represent a promising new avenue of investigation in the treatment of cancer [24].

Cancer is one of the main causes of death worldwide after cardiovascular diseases, it is considered one of the second leading causes of death in the world [25]. There is the use of many traditional and modern treatments such as chemotherapy, radiation and surgery to cure cancer. Since the use of these techniques is associated with many side effects, and toxicities linked with the use of conventional chemicals for cancer treatment. Instead of using conventional chemotherapy, there is development in the use of new effective drugs, with minimal side effects and plants are considered one of the important sources of these promising molecules. Hence plants play an important role for the treatment of different diseases, including cancer. It was found that between 25 and 28 per cent of modern medicines come directly or indirectly from plants, depicting the enormous potential of plants in the field used over the years. In addition, about 60 per cent of anticancer drugs are made from plant [26].

Since the dawn of time, humans have depended on the therapeutic qualities of plants. The majority of chemotherapy drugs are made synthetically or are made of substances that are refined and taken from plants. Herbal therapy is a useful substitute for traditional cancer care. Numerous investigations have been conducted on naturally occurring substances with cytotoxic properties that may be able to kill cancer cells. Owing to these benefits, medicinal plants have been studied and chosen for use in the creation of anti-cancer medications. The research of plant bioactive chemicals as potential anticancer agents has gained more attention recently [27]. Plant phytochemicals can fight cancer through processes such as promoting DNA repair, increasing the synthesis of beneficial enzymes that boost immunity, and inducing antioxidants [28].

Hippophae rhamnoides L is commonly known as sea buckthorns and Siberian pineapple. This is a deciduous shrub that belongs to Elaeagnaceae family. The fruits of this plant which are also called sea berries are orange or yellow, circular and are about 3-8 mm in size. The sea buckthorns grow naturally in North Western Europe, Central Asia and the Northern Himalayas. This plant is considered a

rich source of nutrients and bioactive compounds especially the berries and seeds are full of vitamins, minerals, fatty acids, free amino acids, organic acids, sugars, and volatile compounds. These are also known for their high vitamin C content making them the best source of vitamin C. Sea buckthorn is a medicinal plant and it has been used as a traditional healer for ages especially in some of the regions of Central Asia, and tribes in the Himalayan ranges. It is also used traditionally for the treatment as skin conditions, cardiovascular diseases, diabetes, inflammations and digestive issues, in many countries such as China, India and Romania [29, 30]. It was studied for anticancer activity by many researchers and found that polyphenols in *H. rhamnoides* can help to prohibit mutations such as those studied for breast cancer and liver cancer cell lines [31].

Locally the berries of this plant are used to treat blood pressure, asthma, ulcers, hepatitis, digestive disorders, and skin damage. Being a rich source of vitamins and minerals, especially some novel phenolic compounds it is drawing more attention from researchers. Sea buckthorn has been used as antidiabetic, ant inflammatory, anti-microbial, and anticancer activities. It was studied for anticancer activity by many researchers and found that polyphenols in sea buckthorn can help to prohibit mutations such as those studied for the human breast cancer cell line and human liver cancer cells [31].

There are unique bioactive compounds found in berries, also known as seaberries, as phenolic compounds, vitamins (specially vitamin C), unsaturated fatty acids, and phytosterols, like beta – sitosterol. The juices, jam and oil derived from these berries have been studied for their antioxidant, anti-inflammatory, and anticancer effects. The concentration of the compounds in fruits depends on, climatic conditions, size, maturity, and the methods used to process and store plant materials. Mature sea buckthorn produces berries which are yellow, orange, or red and fruit size ranges from 3 to 8 nm. Sea buckthorn berries consists of 68 percent pulp, 23 percent of seed, and 7.75 percent of outer skin [32]. In the berries of sea buckthorns, many compounds and sea oil are traced, as Li and colleagues studied the fruits of sea buckthorn rank among the utmost healthy and vitamin-rich fruits made by a plant. They are rich in flavonoids, and other antioxidants, vitamins,

(vitamins C, and E, B- carotene, and lycopene) phytosterols, polyunsaturated fatty acids (especially omega–7 palmitoleic acids), minerals (e.g., iron, calcium, etc.) and amino acids [33].

Cichorium intybus L is a biennial or perennial plant that belongs to the family Asteraceae previously called Compositae [34] grows wild in temperate zones of Europe, and South West Asia. It is also found in some parts of Australia, New Zealand and America. *C. intybus* usually grows in meadows, wastelands, track lands and lower mountain regions. There are multiple chemical compounds found in all parts of chicory, and enrich in vitamins and minerals. There are various types of vitamins found in this plant as vitamins B1, B2, C etc. and minerals as sodium, potassium, calcium, zinc etc. It was also found that this plant contains various sugars, flavonoids, proteins, terpenoids, and many other phenolic compounds. Chicory is traditionally used as an herbal medicinal plant in many parts of the world and is regarded as the most important plant in the Asteraceae family. Moreover, the extracts of this plant are studied for biological and medicinal properties such as anti-diabetic, anti-inflammatory, antioxidant and anticancer effects [35]. It is also studied for hepatoprotective effects [36]. In the traditional Ayurvedic and Unani medicine systems chicory seeds have been used extensively for therapeutic purposes [37]. Traditionally the extracts of this plant are used to treat liver disorders, cough relief, kidney disorders, diabetes, and digestive disorders. Cytotoxicity studies of *C. intybus* extracts have found antitumor potential. Some of the phytometabolites are studied for positive cytotoxic activities in vitro and in vivo studies, which proves the potential of chicory extracts for the discovery of antitumor drugs [38].

Metallic nanoparticles (in the size range of 1 to 100 nm) have captivated scientists for some time because of their wide range of potential uses in disciplines including medicine, agriculture and technology [39]. These metallic nanoparticles can be synthesized by elaborated physical and chemical processes but the biological method, which makes use of plants, is becoming the preferred way for the creation of efficient and safe nanomaterials. Biogenic MNPs have the potential to be used as a treatment due to their improved biological characteristics [40]. In short,

studying the medicinal plants of the mighty Karakorum by using nanobiotechnology can be an immense contribution to the field of medicine especially to treat liver cancer.

Creating, modifying and employing materials on the nanometer scale (between 1 and 100 nm) are essential to the field of nanotechnology [41]. There is an important area that must be considered is cancer treatment and the applications of nanobiotechnology which can help to improve existing therapeutic approaches. Human relied on the healing properties of plants ever since the beginning of time. Almost all chemotherapy medications are either synthetically produced or are compounds extracted and refined from plants. Approximately sixty per cent (60%) of all synthetic medications used in clinical settings are derived from plants, further demonstrating the importance of medicinal plants [42]. There is an urgent need for new cancer treatment in response to the increasing incidents of disease around the world. There is development in new cancer medication from plant's secondary metabolites, which is currently the subject of much scientific investigation [43]. In short, studying the medicinal plants of the mighty Karakorum by using nanobiotechnology can be an immense contribution to the field of medicine especially to treat liver cancer.

Green synthesis of nanoparticles is considered more beneficial as compared to traditional chemical synthesis because it costs less, offers minimum pollution, and improved environmental and human health safety. Phytochemicals and bioactive components of plants serve as reducing, capping, and stabilizing agents, making plant-mediated synthesis gentle environmentally friendly inexpensive, and time-efficient [44]. Taking these measures helps preserve our planet's natural resources and strengthen our economy over the long haul. Success in synthesizing biogenic nanoparticles from a wide variety of plants has been reported in several investigations. Green synthesis of metallic oxide nanoparticles is considered an important consideration due to an environment-friendly approach and showed a great deal of attention because this process does not involve harmful chemicals in its synthesized nanoparticles. This advance opens up new prospects in the fields of medicine, energy, and environmental research, which can promote the possibility of using

the abundant natural resources available for manufacturing nanomaterials without risk [45].

Recently metal oxide nanoparticles are considered as an important commercial utility, however, if we consider the toxicity of these nanoparticles, it's a crucial research concern. For this, the important solution is conforming low toxicity, by using the method of green synthesis of these nanoparticles. The biogenic synthesis of nanoparticles of metal oxide using plant extracts and microorganisms opens up immense prospects for the production of biomolecules and cost-effective nanoparticles which shows tremendous applications in the field of health sector. Compared to their bulk counterparts, nanoparticles have quite different properties and bioactivities [46].

The most researched substance among FDA-approved nanomedicines is iron oxide [47]. Magnetic iron oxide nanoparticles (MNPs) consisting of magnetite (Fe_3O_4) or maghemite (Fe_2O_3) have demonstrated efficacy as contrast agents, drug delivery vehicles, and thermal-based therapies at certain diameters [48]. This material has a very high efficiency for biological applications, including imaging, photothermal therapy, and diagnostics. Applications that require qualities that organic materials cannot provide are being filled by the biocompatibility and stability of inorganic materials like iron oxide [49]. Therefore, for the past few decades, researchers have focused a great deal of attention on these benefits in addition to Fe_2O_3 NPs' biocompatibility. In particular, Fe_2O_3 NPs can increase the therapeutic agents' permeability and stability across tissues, resulting in a prolonged circulation period. Thus, using Fe_2O_3-based nanocarriers offers a successful treatment with a lower dosage requirement for medication [50].

Fe_2O_3 NPs are regarded as some of the most prominent nanoparticles among various types of metal oxide nanoparticles. Several of the distinctive properties of these nanoparticles as biocompatibility, potent magnetic properties, low toxicity, biodegradability and catalytic activity have significantly aided their use in biomedical applications [51]. They have a broad spectrum of applications encouraging scientists to utilize them across various fields specifically for cancer treatment with improved delineation of the tumor microenvironment. These nanoparticles

have a minor band gap, and much surface area, and show stability in nature with low toxicity. Furthermore, biological production of Fe_2O_3 NPs is low cost as a result diverse medicinal plants are being used in the synthesis of nanomaterials with a wide range of uses. In a previous study, Fe_2O_3NPs were synthesized using the persimmon extract showing potent antibacterial activity and anticancer activity against $HepG_2$ cell lines [52]. Fe_2O_3 NPs have shown therapeutic potential for cancer treatment. Numerous advantages of synthesis of these NPs have been uncovered, such as stability across various shapes through eco-friendly approaches, magnetic behaviour, the ability of surface modifications, low toxicity and improved delineation of tumour microenvironment. Because of these properties, Fe_2O_3 is used in imaging cancer tissue as well as in site-specific targeting of cell and drug release against them [53]. Fe_2O_3 NPs are also used as nanocarriers to increase drug activity in combination therapy, as these nanoparticles are combined with chemotherapy drugs and can be used as safe drug carriers. There was a study conducted in which biosynthesized mesoporous Fe_2O_3 NPs loaded with the anticancer drug doxorubicin has successfully showed a prominent reduction of cancer cells [54]. Combining different functional groups into a single nanoparticle is one of the nanomedicine's difficulties that could yield the best outcomes for cancer treatment. The objective was successfully achieved by formulating folic acid targeted Fe_2O_3 NPs as the carrier which delivered plant-based anti-cancer drug curcumin against $HepG_2$ cancer cells [55]. Moreover, Fe_2O_3 NPs, functionalized with glucose and conjugated with coumarin were used against liver cancer ($HepG_2$) cell lines. The result revealed that there is higher cytotoxicity for liver cancer cells than for normal cells [56]. In a previous study hematite, Fe_2O_3 NPs were synthesized from the floral extract of *Callistemon viminalis* coated Fe_2O_3 NPs were also found very effective in halting $HepG_2$ cell line proliferation [57].

Human beings have experienced the anger of many chronic diseases form the past. Some of the traditional practices in the treatment of certain illnesses are considered effective in the reduction of the severity of disease. With the advancement in the development of chemistry, there is progress in the analysis of small molecules which helps to connect traditional knowledge to scientific knowledge in disease management. The process of drug discovery consists of many events, initially, there

is the identification and optimization of selected lead compounds (synthetic or natural) then there are preclinical studies and finally the clinical trials. Although there is continuous progress in drug development still constraints are there which affect the process hence there is a decline in approved drug molecules [58].

Using computer-simulated models, Insilco prediction aids in the development of biomedicines and the pharmacology of possible treatments. Molecular docking has grown importance as a component of Insilco's drug creation process in recent years. The interactions between biomolecules and small molecules in this method demonstrate a comprehension of binding sites and affinities as well as a workable mechanism of action. Therefore, it has been widely accepted that molecular docking can be used in the drug-designing process [59, 60]. Additionally, Insilco techniques help to identify the specific protein that is the target molecule and mediates anticancer activity, as well as in tracing the specific metabolites that are active.

The present study was designed for screening plants such as *Hippophae rhamnoides* and *Cichorium intybus* for their anticancer activities. The current study hypothesized that *Hippophae rhamnoides* and *Cichorium intybus* extracts and their Fe_2O_3 NPs may be effective drug candidates against the proposed drug target RhoA gene for the treatment of liver cancer. The main aim of the current research work was to explore the anticancer potential of Fe_2O_3 NPs synthesized using *Hippophae rhamnoides* and *Cichorium intybus* extract against liver cancer and to investigate the interactions between the identified polyphenols obtained by HPLC from the methanolic extracts of *H. rhamnoides* and *Cichorium intybus* and selected protein RhoA by in silico analysis. The objectives of the study included the synthesis of Fe_2O_3 NPs utilizing the extract of *Hippophae rhamnoides* and *Cichorium intybus*. Furthermore, to investigate the anticancer potential of synthesized nanoparticles against liver cancer by targeting the RhoA gene. It also included the determination of apoptotic role of synthesized nanoparticles and respective plant extract by targeting apoptotic pathway genes and proteins. The medicinal properties of some local plants of Gilgit-Baltistan have been known for centuries, but their potential as forerunners to the noble MNPs has received less attention. This study employs

the use of Fe_2O_3 NPs synthesized using the extract of *H. rhamnoides* and *C. intybus* found in the Karakoram Range targeting liver cancer. This study combined the therapeutic potential of plants with Fe_2O_3 NPs will offer an effective strategy for the treatment of liver cancer. **This is the first-ever report describing the comparative analysis of *H. rhamnoides* and *C. intybus* Fe_2O_3 NPs synthesis and their efficacy against liver cancer and describing screening of bioactive compounds of *H. rhamnoides* and *C. intybus* for computational analysis targeting RhoA protein and their efficacy against liver cancer.**

1.1 Gap Analysis

- Limited studies are available for the medicinal plants of the Karakoram range and their apoptotic role in the prevention of cancer.
- Less studies on the synthesis and evaluation of metallic oxide nanoparticles especially iron oxide nanoparticles of the extracts of plants *H. rhamnoides* and *C. intybus*.
- Lack of studies regarding the use of extracts of *H. rhamnoides* and *C. intybus* against liver cancer targeting the RhoA gene.
- Limited studies were found on the Insilco analysis of the polyphenols found in the extracts of *H. rhamnoides* and *C. intybus*.
- Fewer studies are available on the detection of polyphenols found in both plants and their use in computational analysis.
- Lack of studies regarding the preparation of iron oxide nanoparticles and their use against liver cancer targeting the RhoA gene.
- Lack of studies regarding the comparative study of *H. rhamnoides* and *C. intybus* against liver cancer targeting the RhoA gene.
- Lack of studies regarding the use of MNPs to study the local medicinal plants of Gilgit Baltistan.

1.2 Problem Statement

Cancer is considered one of the leading causes of global mortality, even though cancers at initial stages can be treated and many can be prevented and cured. The primary goal is to cure cancer or considerably prolong life. Available treatment options have adverse side effects. There is the necessity to identify novel compounds of medicinal plants with potential anticancer effects and having minimal side effects. New cancer treatments are urgently needed in response to the high toxicity of various cancer chemotherapy procedures with unpleasant side effects and resistance.

1.3 Research Questions

This research will answer the following questions.

Research question 1:
How much effective will be selected plants and green synthesized iron oxide nanoparticles from Karakorum range, in inhibiting liver cancer?

Research question 2:
How bioactive compounds of selected plants will be used to predict their therapeutic potentials for drug designing and computational approaches?

Research question 3:
Do iron oxide nanoparticles synthesized from the extract of *H. rhamnoides* and *C. intybus* are more effective than plant extract?

Research question 4:
Which plant extract from selected plants is more effective against liver cancer targeting the RhoA gene?

Research question 5:
Which metabolites among both plants are more effective to target RhoA protein when analysed by in silico methods?

Research question 6:
Does RhoA show an antiproliferative effect in liver cancer?

Research question 7:

How do iron oxide nanoparticles influence the expression of the Rho A gene associated with liver cancer?

Research question 8:

What is the comparative effect of iron oxide nanoparticles synthesized from the extract of *C. intybus* and *H. rhamnoides*?

Research question 9:

What is the difference in cytotoxicity potential of the synthesized iron oxide nanoparticles from both plants?

1.4 Aim and Objectives

The present research work focuses on the evaluation of *Cichorium intybus* and *Hippophae rhamnoides* plant extract against liver cancer by antiproliferative assays and computational approaches. It also involves the preparation of metallic nanoparticles from *Cichorium intybus* and *Hippophae rhamnoides* plant extract along with their characterization to focus on identifying their inhibitory role against liver cancer.

Research Objective 1:

To identify the antiproliferative role of *Hippophae rhamnoides* Buch and *Cichorium intybus* plant extracts and their respective iron oxide nanoparticles against liver cancer cell lines and their impact on the expression of liver cancer target genes, apoptotic pathway genes and proteins.

Research Objective 2:

Screening of flavonoids found in the extracts of *Hippophae rhamnoides* and *Cichorium intybus* and to analyze the role of identified flavonoids of *Cichorium intybus* and *Hippophae rhamnoides* Buch against liver cancer target gene through computational approaches.

1.5 Scope and Significance

There is an urgent need to design targeted therapies for cancers especially hepatocellular carcinoma which is considered the third leading cause of death globally. So, the need is to trace the genetic constituents playing a role in liver cancer, since targeting the members of Rho GTPase family and specially members of subfamily RhoA which plays a crucial role in the development of cancer. It was found through studies that there were very limited drugs designed to target RhoA which inhibits multiple Rho GEF. Although there are several inhibitors developed but have less clinical applicability. The current study will serve the purpose of detecting some of inhibitory compounds that could be serve to overcome the challenges of liver cancer. Moreover, green synthesizes nanoparticles act as nanocarriers and help to target the tumour cells. This method is eco-friendly and helps to minimize waste generated and is a sustainable process. This method will also help to develop novel cancer-targeted therapies with few side effects.

Nanoparticles are considered an ultimate platform for biomedical uses and play a much more important role in diagnosis and drug delivery systems. There are numerous applications of nanoparticles in the field of medicine as these are antimicrobial, antiproliferative, antiparasitic, anti-inflammatory, antidiabetic, antihypertensive, and anticancer effects.

Following is the significance of nanoparticles in the field of medicine especially anticancer effects.

(a) Utilizing medicinal plants and metallic nanoparticles, can lead to cost-effective treatment options for cancer compared to traditional methods.

(b) Combining medicinal plants with metallic nanoparticles results in synergistic effects, enhancing therapeutic potential.

(c) Medicinal plants as renewable resources, are sustainable practices for cancer treatment compared to other synthetic approaches.

(d) Metal-based nanoparticles work as carrier vehicles, which carry anticancer drugs/ compounds which result in increased effectiveness and efficiency of anticancer drugs and showing targeted delivery.

(e) Green synthesis can offer economically sustainable technology which can synthesize clean products, which will exert a significant influence on industrial development.

1.6 Applications and Innovations

The current study focuses on uncovering the bioactive compounds identified in some of the medicinal plants found in the Karakorum range, with the use of respective metallic nanoparticles as natural anticancer therapy. The basic and long-standing goal of this research work is tracing the particular natural compounds found in these plants for treatment and cure of liver cancer, which in future will help to explore novel diagnostic and therapeutic approaches against liver cancer. Following are some of the applications and innovations of current research work.

1. Anticancer potential of bioactive compounds of Sea buckthorn and *Cichorium intybus* and their metallic nanoparticles against liver cancer.

2. Gene targets of metallic nanoparticles synthesized from plant extracts.

3. Comparative study can help to show synergistic effects of combination of bioactive compounds from both plants that work together to enhance efficacy of therapy for liver cancer.

4. Personalized treatment plans can be developed by comparing the effects of these two plants on particular patients of liver cancer, based on the most effective plant-derived component.

5. By exploring different medicinal plants, we can identify the expression of liver cancer progression which helps in early diagnosis.

6. Metallic oxide nanoparticles conjugated with compounds from plants target cancer cell lines. This will allow for targeted delivery to cancer cells with reduce side effects and improved efficacy.

7. Green synthesized metallic nanoparticles can be used in combination with other therapies as chemotherapy and radiotherapy to enhance their effectiveness.

8. Innovations in the synthesis of iron oxide nanoparticles using plant extracts give rise to the development of nanocarriers with minimal toxicity and selective targeting of cancer cells.

The possible positive outcome of current research work will help the pharmaceutical industry and medical field to find biomarkers for diagnosis and treatment of liver cancer as well leading towards clinical trials.

Chapter 2

Literature Review

Liver cancer is a global health problem and its prevalence is increasing globally. It is predicted that one million people will be influenced by liver cancer per year by the year 2025 [61]. The most common type of liver cancer is called Hepatocellular carcinoma (HCC) which reports for more than ninety percent of cases, and the most important risk factor for this is the hepatitis B virus which comprises more than 50% of cases. Liver cancer is among the five top most deadly carcinomas that are rapidly increasing annually. It was found that generally there are more cases of liver diseases in developing countries. Risk factors for liver cancer include hepatitis B, virus hepatitis C virus, fatty liver diseases, cirrhosis of liver-related to alcohol usage, smoking, fatness, diabetes, and overloading of iron in various foods [62]. The prognosis of this disease is poor. For surgical removal only 5% to 15% of the patients are eligible, and, this can only be suitable for patients in the early stages [9]. Primary liver cancer is considered the seventh most prevalent cancer globally. Moreover, the incidence rate of liver cancer has been increasing in many countries and is expected to continue to rise over the next decade. There are two main histological kinds of primary liver cancer as first is HCC or hepatocellular carcinoma and the second is ICC or intrahepatic cholangiocarcinoma. HCC arise in the cells of liver most often due to the factors like oxidative stress, inflammation, and due to some of the primary liver diseases. While ICC appear in cholangiocytes that form intrahepatic duct of bile [63].

Patients' diet is much associated with the prevention, progress and treatment of cancer. A European study has shown that a diet loaded with fruits and vegetables is associated with a reduction in the risk of cancer [64]. The different naturally occurring compounds found in fruits, vegetables, and spices stimulates the repressing mechanism of cancer development and stimulate the progress associated with disease prevention. These bioactive compounds activate antitumor, antiproliferative, anti-inflammatory, and antioxidant activities which gives therapeutic options for new cancer treatment regimens. The liver shows an important role in detoxification process and the formation of hormone as well as carbohydrates, fats and protein metabolism. Many studies in China and the United States have examined the link between certain food patterns and the risk of liver cancer. These studies showed that vegetable diet patterns are linked with a lower risk of liver cancer [65].

There was the cohort study conducted in a multiethnic population which showed that the participants with healthy plant diets had a low risk of HCC incidents [66]. This healthy diet was comprised of several common food components, such as much use of fruits and vegetables, nuts, legumes, and grains and a lower use of red and packed meat although it is not specially developed for cancer prevention. There was a study conducted in which 8 flavonoids were examined and these have shown promising results in preventing the progression of hepatocarcinogenesis [67].

2.1 Epidemiology

Liver cancer is among the most prevalent cancers worldwide and is considered the fifth leading cause of cancer-related deaths globally. Moreover, in Asia and Africa, the mortality rate due to HCC is highest, but this is also increasing in the USA and different parts of Europe. According to the report of Surveillance Epidemiology End Results, in the USA there was a rapid increase in cases of HCC-related deaths in the early 2000s, and is predicted that liver cancer will tend to be the third leading cause of cancer-related deaths till 2040 if this tendency continues [68]. Only a few reports are showing the cancer registries of particular

populations in less-income and middle-income countries, making it hard to inspect present rates of prevalence of liver cancer and cancer afterwards. The absence of data makes it more difficult to understand the causes and occurrence of liver-related cancers and the scope of this disease and to organize interventional efforts that could be implemented.

For the incidence of liver cancer, gender disparity is observed in different parts of the world, where rates among males are two to threefold higher as compared to females. This gender disparity is not properly understood, but it was found that the risk factors for liver cancer are elevated in males as compared to females. It was hypothesized that some factors such as epigenetics, immune response, and differences in sex steroid hormones could be the reasons related to discrepant rates [69]. New High-rate areas although, have not much gender differences than other areas. Moreover, in Europe, it was observed that in countries where registries of liver cancer rates among males are four to five times higher than among females (e.g., in France, male to female ratio is =5:0) [70]. This gender disparity is yet not properly understood, but it was seen that the risk factors for liver cancer are higher in males as compared to females [71]. Hepatocellular carcinoma is on the rising trajectory in Pakistan, and think that it may emerge as the most common form of cancer in future. In Pakistan, the age-standardized rate is 7.6 per 100.000 individuals per year for males and 2.8 for females.

The main issue is lack of proper cancer registry in Pakistan so data can either be obtained from single center experiences or from scattered regional registries [72].

2.2 Risk Factors

Over 90% of HCC cases occur due to chronic liver diseases, as cirrhosis is considered the key risk factor for the onset of HCC [73]. Some of the other vital factors include alcohol consumption, HBV infection, or HCV infection, diabetes and obesity (Figure 2.1). The other less frequent factors include cirrhosis due to primary biliary cholangitis, and hemochromatosis, which are considered as a high risk of HCC and have much chance of its development in their life.

2.2.1 Infection of Hepatitis B Virus

HBV infection reports for more than 60% of cases of HCC in Asia, and Africa and 20 per cent of cases in the West. It was found that HBV which is a DNA virus incorporate the host genome, causing insertional mutations which activates oncogenes. The chances of occurrence of HCC have been observed to increase even if there are no chances of cirrhosis, although most of the patients suffered from hepatitis B virus-activated HCC, liver cirrhosis is observed. The huge incidence of HBV infection in East Asia led to the risk that HCC exceed the cost-effective threshold for men of about 40 years and in women of about 50 years, thus explaining surveillance programs. The rate of HCC has decreased in some regions of Asia although universal vaccination programs are being implemented [74].

2.2.2 Infection of Hepatitis C Virus

The Hepatitis C virus is the main source of HCC among the residents of North America, Europeans, and Japanese [75]. In this type of infection, HCC is mainly limited to cirrhosis or chronic liver damage because hepatitis C is an RNA virus that does not incorporate into the genome of the host. Most patients of HCV infection have been successfully cured with direct antiviral DNA treatment, which results in a 50 to 80% decrease in the cases of HCC [76]. Moreover, many patients belonging to ethnic minorities or those belonging to low socio-economic areas are still unaware of their infections and need to be tested for HCV.

2.2.3 Infection of Hepatitis D Virus

The virus of hepatitis D is also the RNA virus which needs surface antigens of the virus of hepatitis B for its replication and infectivity. It has been studied that about 20 to 40 million people are affected by HDV worldwide, which is correlated with a more chronic liver disease, which includes elevated fibrosis and the risk of cirrhosis than infection of HDV alone. In one of the most important studies, it was found that the prevalence of HCC was higher in the people who suffered from

the infection of acute hepatitis D virus or chronic infection of hepatitis D virus than among those who suffered from infection of HBV alone [77].

2.2.4 Role of Alcohol Intake

Alcoholic liver diseases, cirrhosis and HCC are caused by excessive use of alcohol. Currently, cirrhosis has been increased due to much consumption of alcohol and Non-Alcoholic Steatohepatitis (NASH). In a research investigation, it was found that the annual incidence of cirrhosis due to alcohol consumption in a population study has reached 1% while 2 to 3% in tertiary treatment centers and reported for more than 15 to 30% of the cases of HCC depending on the particular geographical region [78]. The increase in HCC is due to chronic alcohol consumption, for example, in many studies it was found that there is a rise in HCC among those hepatitis B carriers who use alcohol in contrast to individuals who do not use it. There is evidence that supports different alcohol-specific tumour-promoting mechanisms in patients.

2.2.5 NASH (Nonalcoholic Steatohepatitis)

NASH is another important cause of cirrhosis and is considered a basic step for HCC occurrence in patients suffering from obesity and diabetes mellitus. In most regions of the world, NASH is the most prevalent cause of cirrhosis, due to an increase in obesity. Since 2010, it was observed that the proportion of HCC has increased due to an increase in NASH, which currently represents 15-20% of the cases in the West [79]. Most of the studies showed that 25-30% of the cases related to HCC (due to NASH) occur even without cirrhosis, preventing the applications of inspections and programs aimed to target only patients with cirrhosis.

2.2.6 Aflatoxin

The aflatoxins are produced by Aspergillus fungi which deteriorates maize, trees of nuts, and some other types of foodstuffs present in the hot and moist environment

globally. The strongest aflatoxin, alfatoxinB1 (AFB1) causes hepatocarcinogenesis in human as well as in various animal species [80]. A meta-analysis study performed among Asians and Africans, where AFB1 is common, has predicted the population related possibility of liver cancer with alphatoxinB1 was 17% [81]. Contact to AFB1 can be lessened by several ways, such as change in the method of harvest grain and dumping methods and the addition of competitive species, etc. The most important demonstration of the reduction of AFB1 has been observed in China, where due to change in economic policy allows the substituting maize for rice in some of the highly endangered areas, these measures reduced contact with AFB1 in the particular population which is a step towards a decline in the liver cancer in the region [82].

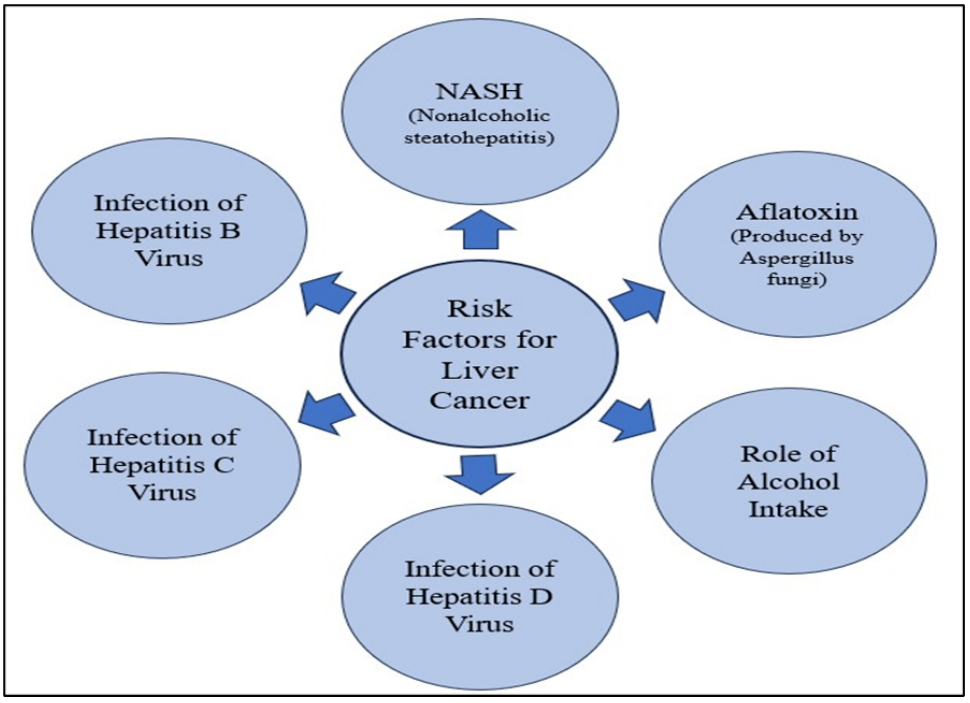


FIGURE 2.1: Risk factors associated with liver cancer [83]

2.3 Pathophysiology

The pathophysiology of HCC is considered an intricate approach consisting of multiphases. First step of the process is interaction of many factors which participate in the transfer of normal cells of liver into malignant cells which ultimately develop into HCC. Some of these factors are a corporation between some viral

and nonviral agents, genetical predispositions, microenvironment of cells, severe liver diseases and different immune cells. It was found that one of the key features of cancer is change in the microenvironment of cells which involves every phase during the progression of cancer. This involves basic transformation to the process of invasion and eventually to metastasis [84].

2.3.1 The Cells of Origin

The cell of origin of liver cancer is still questionable. Like other types of cancer, it may be of stem cells of the liver which shows amplification, or hepatocytes. Generally, the existence of stem cells and their role in the HCC itself is questionable. Furthermore, mature hepatocytes are long-living and have a significant proliferative ability against injuries. Much of mouse models aid to show that chances for HCC development mostly takes place in mature hepatic cells, but some other sources debate that these may be stem cells of liver [85]. Moreover, intrahepatic cholangiocarcinoma and tumours exhibit mixed morphology of HCC or cholangiocarcinoma which usually seems to originate from mature hepatocytes, depicting the concepts of metaplasia and plasticity of cells. This study confirms the concept that the morphology of tumors and epigenetics do not necessarily reflect the cell of origin [86].

2.3.2 Gene Mutations in HCC

Next-generation sequencing has facilitated in identification of cancer-causing genes called oncogenes or tumor suppresser genes, that are often changed in HCC. In more than 80% of HCC, it was observed that telomerase activation by TERT promotor mutation, chromosome translocation and viral insertion are important and potential drivers of alteration in genes. Studies revealed that the initiation of the Wnt–β-catenin signalling pathway in 30 to 60 per cent of the cases of HCC, produced by alteration in CTNNB1, encodes β-catenin, AXIN1, or APC (inhibitors of Wnt pathway) deactivation. Other common mutations can be found in TP53, RB1, CCNA2, CCNE1, PTEN, ARID1A, ARID2, RPS6KA3, or NFE2L2, that change

the cell cycle. Even cancer-causing gene mutation occurs at random, certain genes are linked to subclasses of molecular HCC which is defined by transcription profiles and histological phenotypes [87].

2.3.3 Molecular Alterations due to Infections of Viruses

The most common point for insertion mutations through HBV is located in the promotor of TERT, which leads to overexpression of telomerase, an enzyme that maintains the telomerase length [87]. Telomerase activation helps to prevent the chromosomes erosion during the process of aging which occurs physiologically in each particular cell division. Telomerase activation guards the cells from senescence and hence enhances the transformation of cell. Further frequent insertions which are linked with HBV have been traced to initiate oncogenes, which are involved in cell cycle control such as CCNE1 and CCNA2. If we consider the whole genome, these oncogenic changes result in replicative stress and complex rearrangements. It was studied that in a few HCC patients, adeno-associated virus 2 showed the same oncogenic mutations, with the most common spot for viral insertion in the TERT promoter, CCNE1 and CCNA2 [88]. These studies showed that those particular oncogenes which are stimulated by the infection of viruses actually forms basic promotors in the transformation of hepatocytes. In contrast, infection of the hepatitis C virus does not cause a strong and oncogenic effect and initiation of mutation is caused by chronic inflammation and oxidative stress.

2.3.4 Mutational Signatures and Role in HCC

In most cases, mutations occur throughout chronic illnesses of liver and cirrhosis. Hepatocytes acquire many genetic mutations and epigenetic changes that ultimately form the basis of HCC. Several factors that cause DNA mutations are linked with particular mutational signatures. The analyses of exome sequencing of the HCC genome recognized mutations in 22 and 24, specifically in Asian and African patients who were exposed to aristolochic acid (CTG trinucleotide A>T

mutation) and B1 aflatoxin (C>A mutation) [89]. The mutational signatures associated with the consumption of tobacco and alcohol are Signature 4 and Signature 16 [90]. It is not clear whether this observation can be interpreted into protective measures. Particular observations highlight the function of liver in detoxification of many metabolites that may harm the genome of liver cells by inducing passengers and drivers, which leads to oncogenesis.

2.3.5 Molecular categories of HCC

Findings based on genomic, epigenomic, histopathological, and immunological studies confirmed the evidence of the molecular and immunological classes of HCC. These molecular classes of the HCC are explained by the key molecular drivers and the pathways linked or depend on the immunological condition of the tumour [91]. These molecular levels link to specific genomic disruptions, clinical outcomes and histopathology. The class of proliferation consists of more than 48 per cent of HCC and is generally equipped in mutations in TP53 and also in amplifications of CCND1 else FGF19 [92]. In addition, it is a more prevalent form of HCC associated with HBV and has a bad prognosis. The class of proliferation further consist of two subclasses first one is the cell group of proliferation progenitor and the second is the proliferation–Wnt–TGFβ group [93].

2.3.6 Obesity Linked to HCC

Obesity is related to the prevalence of cancer in several organs of the body [94]. Obesity may cause precise variations which include changed immune functions and endocrine changes, which are the signs of characteristics of numerous cancers. Currently, some of the evidence in the West shows that fatty liver diseases are frequently the main source of HCC. Moreover, some other studies have shown that some particular mechanisms of the liver that promote HCC by NAFLD or NASH include metabolic and oxidative stress, altered immune function, inflammation responses, and changes in endocrine signalling mechanisms [95].

2.3.7 Oxidative Stress

Overloaded hepatocytes are characterized by the stress of endoplasmic reticulum and oxidative stress which causes inflammation and cell damage. A study conducted showed the cause of the HCC in mice induced by NASH the ER stress in hepatocytes of mice stimulates inflammatory signaling pathways, especially NF-κB and TNF, which leads to initiation of HCC [96]. However, these pathogenic mechanisms have not yet been demonstrated in liver cancer in humans. The impair fatty acid metabolism in liver cells damages DNA as elevated reactive oxygen species (ROS), the outcome of mitochondrial dysfunction. Furthermore, a change in the expression of metabolic enzymes can alter the ability of hepatocytes in repairing DNA damage.

Metabolic conditions also affect inflammatory signaling as a high level of IL-17 which is a tumor-boosting cytokine, is detected in NASH in humans. Moreover, in NASH there is much formation of lipids, but may also alter the formation of more abnormal lipids which act like oncometabolite [97]. For example, the constant activation of mTORC2 in liver cells of mouse rise the formation of the sphingolipid glucosylceramide, resulting in the rise of ROS production in cells resulting in HCC.

2.3.8 Immune Cell Infiltration of Fatty Liver

This is a histopathological characteristic of NASH. For basic research in pathogenesis and translational research, it is important to develop animal models which can accurately replicate human HCC [98]. Most of the models used for experiments proved that immune cells and cytokines has significant part in the pathogenesis of HCC. For example, in mouse models long NASH activates CD8+ T cells, causing hepatocyte damage which leads to HCC development. The Other types of immune cells which comprise B cells, T cells, natural killer cells, and various forms of myeloid cells are related to the pathogenesis of HCC initiated by NASH [99]. The mechanisms described above could promote HCC in the context of fatty liver diseases. However, their respective role to the HCC in humans is still unidentified. Chronic inflammatory HCC is a type of inflammation-related cancer, showing

more than 90 per cent of the strain of HCC due to inflammation and viral causes of hepatitis, use of alcohol, NAFLD and NASH. The microenvironment of immune cells is considered important in HCC pathogenesis.

2.4 Diagnosis and Screening

Most cases of HCC occur in identifiable patients i.e., chronic hepatitis B infections and cirrhosis, so most of the patients are diagnosed by surveillance. However, because of a lack of screening in some of the clinical practices, some of the patients may have an accidental liver mass identified in cross-sectional images or due to symptomatic advanced stages of HCC upon severe abdominal pain, loss of weight, or due to decline in liver function. It is estimated that this type of incidental diagnosis occurs in fifty per cent of cases globally, especially in developing countries. For proper diagnosis imaging techniques with abdominal ultrasound and high serum α-fetoprotein levels (>20 ng/ml), are applied. For lesions less than or equal to 1cm in diameter, quadruple-phase CT or dynamic contrast-enhanced MRI must be carried out.

Although most cases of HCCs can be diagnosed by imaging, it was found that about 10 per cent of the tumors have atypical characteristics. If there is such clinical doubt about HCC and its appearance is not detected by imaging, a biopsy or second contrast enhancement learning needs to be focused [100].

According to the guidelines of the scientific society, the screening for HCC must be performed every six months, as showed improved survival as compared to annual screening and no lower results than an interval of three months. With an increase in data, an abdominal ultrasound is the most commonly recommended screening method. There was data obtained from RCT conducted in China supporting the highest level of HCC surveillance. The mortality rate was minimized by 37 per cent in the total of 17, 920 patients subjected to surveillance as compared to those not screened for HCC [101].

2.5 Prevention and Management of HCC

The prevention of primary HCC is only possible with vaccination. It was found that there is a decrease in HCC cases due to the use of HBV vaccination. Likewise, there is a prominent reduction in the cases of HCC related to HCV infections due to the use of a new therapy called DAA therapy. A study conducted considering the data of Swedish registries [102] found that the use of aspirin reduced HCC from 8 per cent to 4 per cent after 8 years of follow-up. Some other studies also proved the effectiveness of consumption of coffee in the reduction of HCC cases. Due to valid data, there is a strong recommendation for the use of coffee in liver disorders, by the European Association for the Study of Liver (EASL). Apart from these studies, they also found benefits of use of vitamins A and K for chronic diseases of the liver.

There is more than eighty per cent chance of HCC in patients diagnosed with liver cirrhosis, so due to the health condition of particular patients, there are limited options for therapy. It was found that for patients having the early stages resection, transplantation and local ablation are the best options for treatment and TACE is recommended for patients at an intermediate stage. Patients having advanced stages are recommended for systemic therapy.

Surgical treatments including liver resection and transplantation, have long been the basis for anticancer therapy and provide the best results with survival of five to ten years respectively [103]. Hepatic resection is thought to be the treatment choice in patients with HCC without cirrhosis and post-operative liver decompensation is not an important issue. Another type of treatment called image-guided ablation is one of the potential treatment options for early-stage small HCC tumors [73]. Trans arterial therapy is also one of the treatment choices for patients with the intermediate stage of HCC as a meta-analysis study also showed a better survival rate of patients with particular therapy. External radiation therapy is also one of the successful therapies in HCC tumors with different sizes and stages of cancer.

2.6 Systemic Therapy for Hepatocellular Carcinoma

In recent years there has been advancement in the systemic therapy for hepatocellular carcinoma. This therapy is applicable for patients having advanced and intermediate stages of HCC and systemic therapy is advised when curative therapy is not possible. The US Food and Drug Administration (FDA) has currently approved six agents and adopted in guidelines of liver society at the international and national level since 2016 [100]. Following are the options for systemic therapy.

2.6.1 First Line Therapy

2.6.1.1 Atezolizumab + Bevacizumab

For many years sorafenib was used as the standard drug for the treatment of advanced stages of HCC, but after the IMbrave 150 trial it was found that atezolizumab and bevacizumab significantly improved overall survival than sorafenib hence these drugs are approved as first-line therapy for HCC metastasis [104].

2.6.2 Alternate First-Line Treatment

Sorafenib was approved in 2007 as the first oral tyrosine kinase inhibitor for therapy of advanced-stage HCC. A study was conducted in an Egyptian population to find the effectiveness of sorafenib in a trial study. It was observed that the patients receiving the treatment of sorafenib showed better results than the patients with supportive care [105].

2.6.3 Second-Line Therapeutic Options

Some of the second-line therapies are the following.

2.6.3.1 Regorafenib

For the patients treated with sorafenib, the other TKI, Regorafenib was recommended as second-line therapy for the patients of HCC in 2017 [106]. It was observed that the median OS increased to 10.6 months which was 7.8 months in the group of Placebo.

2.6.3.2 Cabozantinib

In January 2019, the FDA approved the use of cabozantinib in patients early treated with sorafenib [107]. In a trial it was found that median OS of patients taking cabozantinib was 10.2 months and it was 8.0 months in placebo-treated patients. Common side effects of this drug are skin reactions.

2.6.4 Combined Therapy Options

FDA approved the use of combined therapy consist of nivolumab and ipilimumab as a combine therapy of the treatment of HCC. This approval was based on the results of a cohort study in which the patients were given combined therapy of their two agents as prescribed. The outcome was positive as had 33% ORR and results long-lasting at least 24 months [108].

2.7 Status of Liver Cancer in Pakistan

Hepatocellular carcinoma is the type of cancer which is very prevalent and considered as the second major cause of death worldwide. HCC in men is continuously increasing in Pakistan and is thought to become the most common form of cancer in future. In Pakistan, standardized rate is 7.6 per 100.000 individuals per year for males and 2.8 for females. The risk factors for cancer of the liver in Pakistan include hepatitis B virus, hepatitis C virus and some other factors such as liver

cirrhosis, alcohol intake etc. It was found that in some regions there is a decline in hepatitis B-related cases due to vaccination campaigns.

In some of the remote areas of Pakistan, a lack of awareness about liver diseases is attributed to rise in the cases. The main issue is the lack of a proper cancer registry in Pakistan which is a basic source which helps to determine the risk factors and causes of particular disease. Because of a lack of proper health education and facilities the people who suffer from liver cancer usually present in an advanced stage and are deprived of proper treatment [109].

Cancer cases have rapidly increased in Pakistan in recent times as the World Health Organization also stated an increase in the prevalence of cancer cases in Pakistan. A study [110] conducted by Atomic Energy Cancer Hospitals (AECHs) considering data from 2019, liver cancer is among the five most common cancers in Pakistan in males it is 6.8% while in females it is above 3.9%.

Data obtained from cancer registries is a potent tool to monitor and evaluate cancer program outcomes. A cancer registry is a system for collecting patient's data which is than collected into a summary of particular patients. In this regard, Pakistan has its cancer institutes which are collectively known as Atomic Energy Cancer Hospitals (AECHs) which aid as the primary registries of most of the cancer patients in Pakistan.

The NIMRA hospital is an oldest cancer hospital in Pakistan which is built by the Pakistan Atomic Energy Commission (PAEC). This hospital is situated in the South Sindh province of Pakistan. This hospital is engaged in research studies to find cancer trends in the region. Recent data from the cancer registry showed a record of the prevalence of cancer from the year 2015 to 2021.

According to this study, it was found that the top cancers in males are head and neck, lungs and liver cancers, while in females these are breast, head and neck, and gynae tumors. Hence according to data liver cancer is the third most prevailing cancer in males and in females, this is the ninth most common cancer as shown graphically in Figure 2.2 [111].

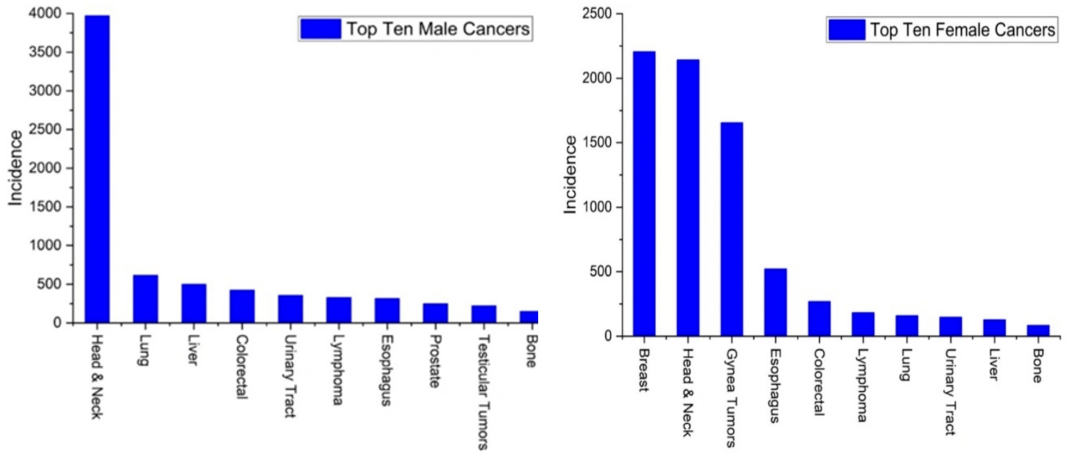


FIGURE 2.2: Top trending cancer from the data of NIMRA Hospital Sindh Pakistan [111]

2.8 Rho GTPase and Role in Cancer Progression

Cancer is a multistep disease caused by mutations and epigenetic variations. At the basic stage normal cells can transform into neoplasia, which gains some features such as uncontrolled proliferation, angiogenesis, and resistance to cell death. With the multiple divisions these cells are capable to invade the secondary sites and shows metastasis, an advanced stage of cancer. Some of the key features of cancerous cells called hallmarks of cancer are studied by Hanahan and Weinberg [112] as these cells escape from immune cells, can reprogram metabolism, generate inflammation to promote tumor [113]. Rho GTPases are small G proteins which function in the cytoskeleton of cells, polarity, morphology of cells, vesicular movements, cell cycle, cell degradation and expression of genes [114]. These small proteins help in cancer development and many studies found their active role in the progression of cancer. Due to this, Rho GTPases appear a novel targets in cancer biology for future researchers.

Progression and metastasis show substantial changes at the cell level in cancers. The common change observed is the development of tumor by the irregular signalling of Rho GTPase [17]. These proteins act as molecular switches regulate various cellular processes which includes adhesion of cell, motility, actin cytoskeletal

organization, and division of cell. Two protein families, the guanine nucleotide-exchange factors or Rhogefs and the GTPase activating proteins or Rhogaps can act upstream of Rho GTPases to control activation and deactivation [115].

In addition, to playing numerous roles in physiological processes, these proteins also have a strong effect on pathological processes such as cancer. In fact, the abnormal role of Rho GTPases can affect all cancer stages progression, as in proliferation, invasiveness, and metastasis of cancer cells.

It has been shown that several important Rho GTPases, like RhoA, Rac1, and Cdc42 are found engaged in progression and metastasis in many types of human cancers [116]. Compared to others, particularly Ras, which is mostly mutated in cancer, the mutations in the Rho family are very less frequent. Some exceptions persist as the latest few reports of mutations in Rho proteins, especially in Rac1, are found in some cancers such as melanoma and brain tumor [117].

Rho GTPases is a subfamily in the Ras superfamily with a size of 21 of 25 kDa and occurs in all the cells of eukaryotes. The first Rho GTPase protein was discovered in 1985 in the abdominal ganglia of aplysia. These consist of well-defined primary structures with 50 to 55 per cent sequence similarity.

However, Rho GTPase were later found that they function in actin cytoskeleton, and further studies showed that this helps to maintain cell polarity, vesicular transport, cell proliferation control and some other functions of cell biology [118].

In contrast, dysregulation in the functions of Rho GTPases results in various disorders for example T cell lymphoma mutations [119]. Furthermore, overexpressed proteins lead to high blood pressure, and irregular activation shows arthritis. HCC is the major cause of death around so there is a need to enhance the patient's chances of survival with chronic stages. For this, we need more studies and research work to understand the role and expression of rho proteins, especially during cancer [119]. However, the fundamental processes in which Rho GTPases are involved in the progression of HCC are still unclear.

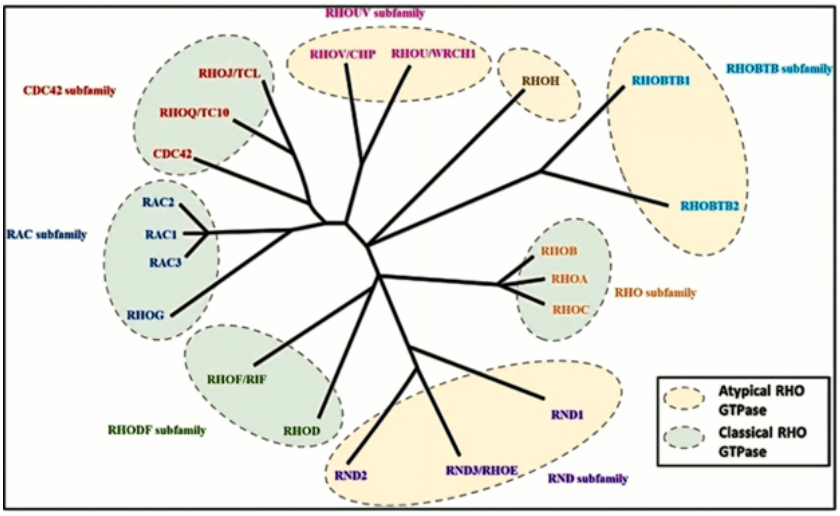


FIGURE 2.3: Phylogenetic tree (unrooted) of Rho GTPase family [21]

The phylogenetic tree of RHO GTPase (Figure 2.3) is unrooted and is built using Clustal Omega programs alignment of sequences (amino acids) of twenty Rho GTPase proteins. These are divided into 8 subfamilies, Rho, Rac, Cdc42, RhoDF, RhoUV, Rnd, RhoH and RhoBTB. These subfamilies are depicted toward the right in circles. GTPases are controlled by gene expression, location, phosphorylation, or stable condition of protein rather than by GEF or AP [21]. Moreover, the regulatory patterns of Rho GTPases are shown in Figure 2.4.

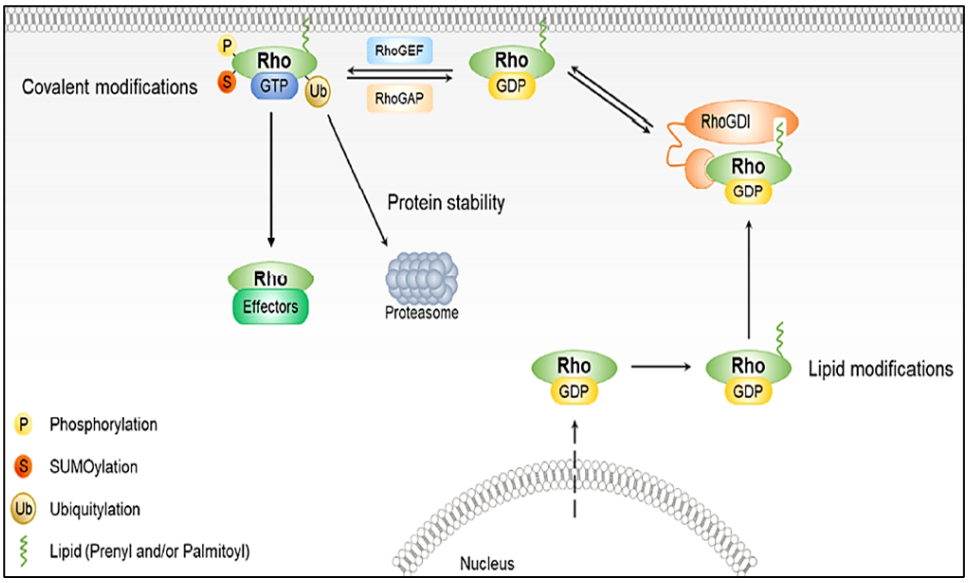


FIGURE 2.4: The above figure represents the mechanism of Rho GTPases regulation. Rho GEFs, Rho GAPs, and Rho GDIs regulate most Rho GTPases. Post-translational modifications as (lipid modifications, phosphorylation, ubiquitination and SUMOylating) are help in this process as PTMs of Rho GTPases work in the regulation of stability, and intracellular localization and convey signals to downstream effectors [120]

2.9 Signaling of Rho GTPase in Tumor Development

Uncontrolled growth of cancerous cells is attributed to cell cycle and metabolic dysregulation, properties of stem cells hold escape of cellular senescence and apoptotic evasion [121]. Rho GTPases are actively involved in the following processes.

2.9.1 Signaling of Rho GTPase in Basic Phases of Cancer

During the process of carcinogenesis, genetic changes in oncogenes and tumor suppressors can promote typical cells into cancerous cells. Altered cells exhibit properties such as abnormal proliferation of cells, apoptotic escape, and rapid growth. Tumor can be induced in mice if injected. Transformed cells also show changes in the cytoskeleton, obtaining fibroblastic forms, and prominent stress fibers in 2D substrates in which the interaction of Rho GTPase is essential. Many of these studies were due to influential research by Ridley and Hall, hence paved the way to understand that Rho GTPase is a molecular switch that manage cytoskeleton. Therefore, not surprising that some Rho GTPases are needed for cell transformation made by oncogenes or tumor suppressor deactivation [122].

2.10 Molecular Features of Rho GTPase

There are 20 Rho GTPases in humans which are classified into subfamilies according to homology in structure and mode of regulation [123]. The most important studied subfamilies are Rho (RhoA, RhoB, and RhoC). Rho GTPases are usually considered active when connected to GTP and inactive when linked to GDP, and their cycle between two forms is managed by protein families as Rho GEFs and Rho GAPs. These play an important role in the separation of bounded GDP from GTPases and the process of GTP hydrolysis [123].

2.11 Mutations in Rho Subfamily

In cancer, RhoA mutations are accumulated in important areas of contact with regulators and effectors, indicating that mutants differ in interactions, which leads to changes in the downstream signalling. Mutations in Y42 diminish the activation of PKN showing mDIA or ROCK1 signaling uninfluenced. RhoA mutation occurs in 15 to 25 per cent of diffuse gastric carcinomas studied and showed three major mutations, R5, G17, and Y42, with Y42C being the most common replacement. Y42C/S is also the most common point mutation in stomach adenocarcinoma [124]. Alterations in the codon of Y42 codon occur in Burkitt lymphoma with mutations in R5 residue. R5 mutations were found too in diffuse large B cell lymphoma [125].

2.12 Rho GTPase and Role in Different Types of Cancer

Rho GTPase are small G proteins which play a significant part in cancer invasion and metastasis. They affect the reorganization of the actin and cytoskeletal events in the cell for movements in the cell. These proteins have recently been regarded as biomarkers for cancer detection [126]. Rho GTPases regulate the morphology of cells by the cytoskeleton and involves to regulate the polarity of the cancer cells. These also show a significant role in cancer cell migration and proliferation.

2.13 Role of Rho GTPase in HCC

HCC is the fifth most prevalent cancer in the world and the third most common cause of cancer-related deaths worldwide. In addition, the annual incidence study found continual dysregulation of Rho GTPases in various cancers. The role of these proteins in the acquisition of malignant characteristics is well known and is contributed to modulating aggressive biological behaviours by affecting the

cytoskeleton structure of tumour cells (Figure 2.5). Since, Rho GTPases are involved in cell functions and are associated with all stages of cancer growth and progression, such as in angiogenesis, apoptotic resistance, invasion of tissues, and metastasis [127]. Thus, tracing the role of Rho GTPases in HCC can help to find the cure for this aggressive malignant disease.

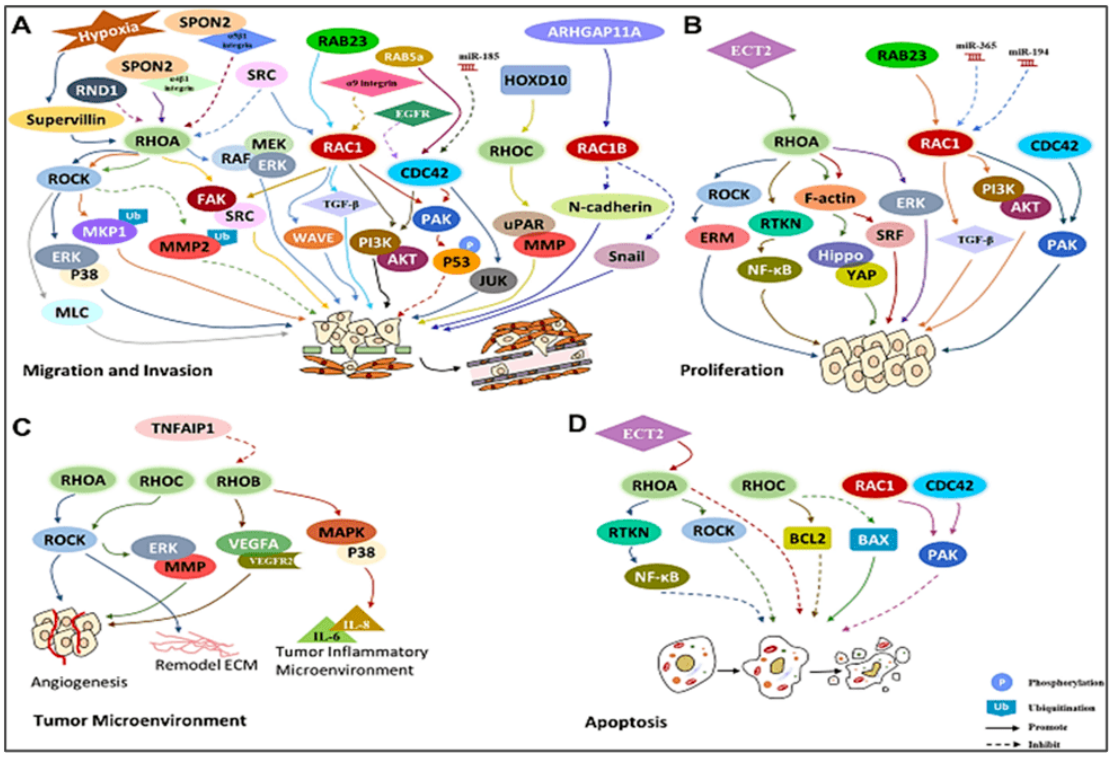


FIGURE 2.5: Role of Rho GTPase in HCC progression in numerous ways as (A) Role in cell migration and invasion, (B) proliferation of the cell, C tumours microenvironment (D) process of cell apoptosis[21]

2.13.1 Cell Migration and Invasion in HCC and Role of Rho

The ability to spread through metastasis is an essential part of cancer and an important aspect of predicting it. It is known that Rho GTPase helps to regulate cellular motility and therefore plays an important part in cancer cell migration, invasion and spread. There were many studies conducted which showed that upregulation of RhoA enhances the migration and breach of cells of HCC. For example, RhoA/ ROCK activates by upregulation of supervillain by hypoxia-induced,

thereby activating protein kinase (ERK)/p38 pathway thus promoting the migration and invasion of cells of HCC [98, 128]. Recent studies showed that this protein is over-expressed in HCC and is strongly associated with vein invasions and satellite lesions.

2.13.2 HCC Proliferation and Role of Rho GTPase

At the initial stages of HCC development, there is the process of uncontrolled cell proliferation which is an important step and it was found that the family of Rho GTPase functions in dysregulation of proliferation. As it helps to activate the ERM pathway through Rock1 to promote the proliferation of HCC cells. In addition, RhoA/Rhotekin stimulates the proliferation of cells of HCC by activating NF-B signalling. It was found that RhoA can also promote the proliferation of HCC cells and the progression of the cell cycle by increasing levels of proteins linked to the cell cycle, CDK1 levels and the proliferation of cell nuclear antigen by RTKN2 [129].

2.13.3 Role in HCC Microenvironment

The Rho GTPase family plays an essential role in inflammation and is involved in the microenvironment of HCC. Endothelial cells of tumor capillary exhibit abnormal elevated levels of RhoA which causes unusual mechanosensing and rapid angiogenesis. RhoA/ROCK is also able to modify ECM in the microenvironment of the tumor to promote the invasion of HCC cells [130].

2.13.4 Cell Apoptosis in HCC and Role of Rho GTPases

Apoptosis escape is important feature of cancer cells and Rho GTPase is found to participate in apoptosis. RhoA prevents the apoptosis of HCC cells by RTKN which helps to activate N-FB signaling. Furthermore, RhoA also block up apoptosis of cells of HCC by Rock2. This too prevents the apoptosis of HCC cells whenever activated by ECT2 [131].

2.14 Application of Natural Compounds in Liver Cancer

Piperine is an alkaloid found in black peppers, having antitumor, mutagenic, antioxidant, and antiproliferative activity. It slows the effect of lipid peroxidation and inhibits the effect of enzymes involved in the metabolism of drugs, including aryl hydrocarbon hydroxylase enzyme and UDP-glucuronyl transferase enzyme, contributing to improving the mechanism of drug bioavailability and bioactive compounds [132]. The main problem for various drugs is drug resistance, and how they are more effective in cancer treatment. The piperine concentrations were found to be toxic to cells of liver cancer and harmless to typical cells. Alkaloids inside the cells increase caspase-3 and caspase-9 activity inhibit catalase, and stimulate peroxide-driven, mitochondrial-mediated apoptosis. In addition, piperine inhibits the activity of receptor tyrosine kinase and the progression of HCC in humans; is induced through diethyl nitrosamine-induced HCC cells. Piperine increased the death rate of apoptotic cells [133]. A constituent of turmeric, called curcumin shows many biological effects on several diseases including cancer of the liver. The study showed that curcumin increased the potential of piperine activity for HCC caused by diethyl nitrosamine. In combination there were few structural, biochemical, and apoptotic variations occurred compared to only curcumin or placebo, inducing coactive activity in rat liver cells and blood serum.

Olive oil which is the basic source of oil used as dietary intake of Mediterranean region, contains a phenolic compound called oleocanthal. There is not any specific relationship between the consumption of extra-virgin olive oil and cancer, vascular disorders, Alzheimer's, and osteoporotic disorders. It was found that oleocanthal found in olive oil helps kill cancer cells and activates apoptosis. Oleocanthal was lethal to $HepG_2$, Huh7, and Hep3b, but typical liver cells of humans were found not harmed [134]. Furthermore, the bioactive compounds of allium show cancer-inhibitory features and are linked with a reduction in the risk of various cancers. It was found that allium extracts consist of various compounds of organosulfur and flavanols that help to prevent many stages of cancer. Diallyl sulfide is one of

the components that inhibits diethyl nitrosamine-induced hepatocellular adenoma and hepatocarcinogenesis [105].

Cnidium officinale Makino is a traditional herbal medicine in China to treat discomfort, inflammation, menstrual disturbance, and hypertension. Constituents from Cnidium have been found to reduce tumor angiogenesis and metastasis. When exposed to Maiko extracts there is a reduction in the viability of cell, and a rise in apoptotic bodies but the effect of these extracts was not evident for changing liver cells. There is an increase in the G0/G1 phases for $HepG_2$ cells. Treatment with extracts reduces the number of cells in the S phase showing a cell cycle arrest. This coexists in the upregulation of p53 and caspase–3 in a dose-dependent manner, with less expression of Bcl2, CDk4, and cyclin D [135].

2.15 Importance of Medicinal Plants

Traditional medicine is the way of using medicinal plants in the process of healing, before modern medicine. The use of ethnomedicine plays an important role in the primary health care of the locals and contributes greatly to the health cares of humans. The World Health Organization also focuses on traditional use of medicine in indigenous health practices and can use a single plant or in combination to treat acute illnesses. Since more than eighty per cent of people in rural areas of developing countries use traditional medicines for various diagnoses, thus, plants are an incredible source of promising pharmaceutical entities [136]. Human have relied on the healing properties of plants ever since the beginning of time. Almost all chemotherapy medicines are either synthetically produced or extracted and refined from plants. Herbal therapy is an effective alternative to conventional cancer treatment. Several studies have been made on naturally occurring compounds known to possess cytotoxic effects as having the potential to destroy cancer cells. Due to these advantages, medicinal plants have been investigated and selected for the preparation of cancer medicines [137].

Despite its frightening nature, cancer remains one of the world's leading healthcare issues, necessitating a preventative approach to its cure [138]. Chemotherapy may

be effective, but it comes with awful side effects. In contrast to conventional medicine, treatments that utilize plants and plant-derived products are simple, safe, environmentally friendly, inexpensive, quick, and low-toxic. Phytochemicals only harm cancer cells and have no negative impact on normal cells. Numerous signalling pathways contribute to carcinogenesis. Because phytochemicals can have beneficial effects on several target events, they are a promising component in the search for new anticancer medicines. Studies of these phytochemicals are still being conducted in the hopes of identifying useful therapies (those that can inhibit or halt the proliferation of cancer cells with few or no adverse effects). Many natural phytochemicals and their synthetic equivalents show promise as cancer treatments. Cancer treatment based on phyto molecules has made great strides in recent years [139].

2.16 *Hippophae rhamnoides* (Sea buckthorn)

This is a thorny shrub which belongs to the Elaeagnaceae family. Its fruits highly nutritious, packed with vitamins and minerals, and are widely utilized in the medicine and cosmetics industry (Figure 2.6). If we studied its traditional use, we found that this thorny shrub has been used in traditional medicine, in some regions such as Mongolia, China, Tibit, and some regions of Central Asia. Because of its important phytochemicals and pharmacological properties this plant has gained significant attention and has been studied extensively, with many phenolics isolated from this plant.

FIGURE 2.6: *Hippophae rhamnoides* (Sea buckthorn) plant with berries

There are unique bioactive compounds found in berries, also known as seaberries, such as phenolic compounds, vitamins (specially vitamin C), unsaturated fatty acids, and phytosterols, like beta-sitosterol. The juices, jam and oil derived from these berries have been studied for their antioxidant, anti-inflammatory, and anti-cancer effects. The concentration of the compounds in fruits depends on, climatic conditions, size, maturity, and process used to process and store plant materials [140]. Mature sea buckthorn produces berries which are yellow, orange, or red and fruit size ranges from 3 to 8 nm. The berries have a thin and waxy layer on the skin and are enclosed by a single sheathed seed enclosed by a cellular structure filled with juice. Sea buckthorn berries consist of 68 per cent pulp, 23 per cent seed, and 7.75 per cent skin. These berries are unpleasant in taste because of their high acid content and astringency. Many processes have been applying to minimize astringency, as frosting before additional processes and to mix to sweeter juices like apple and grapes. There are many compounds in the berries of sea buckthorn as studies showed that fruits of this plant rank among the healthiest and vitamin-rich. They are rich in flavonoids, and other antioxidants, vitamins, (vitamins C, and E, B- carotene, and lycopene) phytosterols, polyunsaturated fatty acids (specially omega–7 palmitoleic acid), minerals (e.g., Iron, Calcium, etc.) and amino acids [141].

This plant can tolerate drought, cold temperature, and high salinity. The fruits have numerous medicinal properties and are full of vitamin C, vitamin K, B, carotene as well as minerals as phosphorus, potassium, calcium, and magnesium. In addition, fruits contain, valuable oils, and are used in the cosmetic industry and nutraceutical [142].

2.17 Medicinal Value of *Hippophae rhamnoides*

H. rhamnoides is herbal remedy specially its fruits are rich in vitamins and minerals, and is drawing attention of scientists for its medicinal properties. If we consider the different bioactivities of sea buckthorn, it is found that it is studied for activities such as anti-cancer, anti-diabetic, anti-hyperlipidemic, anti-inflammatory,

as well as anti-microbial properties. This has been known for centuries for its medicinal properties and is considered a source of spectrum for bioactive compounds. Medicinal values of berries comprise, anti-microbial, anti-inflammatory, pain relief, enhances immune system and tissue regeneration. These berries are also considered to be a protection against cardiovascular diseases. *H. rhamnoides* has been used in most medicinal procedures which aim to combat cancer, heart alignments, ulcers, liver alignments, burns and brain illnesses [143].

2.17.1 Role in Cardiovascular Diseases

In fruits and vegetables naturally found polyphenols are called flavonoids. These flavonoids are abundantly found in *H. rhamnoides*. The richest flavonoids found in fruits and leaves are called isorhamnetin and quercetin [144]. Sea buckthorn flavonoids have profound effects in myocardial ischemia and reperfusions, oxidative damage and tumours. A study conducted by Pang and others in which the rats were administrated with a high amount of sucrose food, increased their systolic blood pressure, insulin level and triglycerides in the plasma, and angiotensin II in the heart and kidneys. Then experimental group was fed with food rich in sea buckthorn extracts. Results proved antihypertensive effects in which there was blocking of angiotensin II pathway and improved sensitivity to insulin. In another experiment rabbits fed with a diet rich in cholesterol, fed with 1ml of sea buckthorn oil from seeds daily for 30 days, reduced LDL, and a lesser atherogenic index showing an increase in HDL and vasorelaxant activity [145].

2.17.2 Role in Diabetes

A metabolic disorder of the endocrine system is called diabetes. In this disease patients are unable to use and produce insulin in the body properly, leading to high glucose in blood. Many studies were shown to investigate the potential of Sea buckthorn for diabetes treatment. A study conducted by Zhang et al., [146] examined the effect of aqueous extracts of sea buckthorn seeds on serum glucose, lipid profile, and antioxidant action of streptozotocin induced in diabetic Rats.

During the study rats were split into four different groups: which consists of normal control group, and a control group of diabetic rats. the diabetic group was provided with 5 mg per kg body weight of glibenclamide as a reference drug and the other group with diabetes was fed with 400 mg per kg body weight of sea buckthorn seed extracts. It was found that the seed extracts significantly reduced the level of serum glucose and nitric oxide (NO) in rats with diabetes. In addition, it was observed that there was an increase in the action of serum superoxide dismutase and the amount of glutathione. It indicates that sea buckthorns demonstrate hypoglycemic, hypotriglyceridemic, and antioxidant effects, suggesting that these plants can help prevent diabetic complications [147].

2.17.3 Anticancer activity of *Hippophae rhamnoides*

Various parts of *H. rhamnoides*, particularly fruits, known as seaberries, Siberian pineapple is well known for its exclusive bioactive compounds and vitamins mostly vitamin C, unsaturated fatty acids, and phytosterols such as beta-sitosterol. The berries of this plant are used to make juices, James, and oil, having valuable antioxidant, anti-inflammatory and anticancer properties. There are many phytochemicals present in *H. rhamnoides*, such as phenolic compounds, proanthocyanidins, curcumin, and resveratrol, which show potential effects in cancer chemoprevention [148]. It is well documented that a diet rich in phenolic compounds, particularly procyanidins and flavonoids is essential for low risk of cancer. It is further studied that a diet rich in phenolic compounds, especially, procyanidins and flavonoids, such as kaempferol, quercetin, and isorhamnetin protects the cell from oxidative damage results in genetic mutations that lead to cancer [149]. There was a study conducted by Kim and his colleagues [150] to investigate the effects of *H. rhamnoides* leaf extracts in the inhibition of the proliferation of Rat glioma cells. When treated with leaf extracts of *H. rhamnoides*, beside upregulating the expression of pro-apoptotic protein Bct-2-associatd X (bax) efficiently, also enhanced its localization in the nucleus. In another research the anticancer activity of *H. rhamnoides* was performed by an initiator (7,12 - dimethylbenz [a] anthracene) and promotor 12-0- tetradecanoylephorbol - 13 -acetate (TPA) induced carcinogenic effects

in mice. Important three polyphenols (catechin, gallocatechin, and epigallocatechin) and a triterpenoid (ursolic acid) of sea buckthorn extract are involved in the inhibition of TPA- induced inflammation [151].

2.18 *Cichorium intybus* L (Chicory)

C. intybus L. is a perennial herbaceous plant included in six members of the genus Cichorium which belongs to Asteraceae family (Figure 2.7). The plant is found in various regions of world as African regions, Asia, Europe, Australia, and North and South America. The leaf extract of this plant is mostly used as vegetable and powder roots as a substitute for coffee. Ethnomedicinal use of *C. intybus* is to treat diarrhea, liver disease, disorders of prostate and genital organs, lung disease, cough, malaria, and cancer [152]. Several studies have shown that phytochemicals of this plant are the potential source of healing effects, as drugs obtained from phytochemicals have shown promising results in healing tumor. *C. intybus* has various phytochemicals as guaianolides, 6-methoxy flavone, eudesmanolides, germacranolides, polyacetylene, sterol, delphinidin, and some novel compounds found in diverse ratios [153].

FIGURE 2.7: *Cichorium intybus* plant and seeds

Researchers studied the therapeutic efficacy of *C. intybus* phytochemicals in vitro and in vivo models of tumor. *C. intybus* medicinal properties show effective findings to trace its unique phytochemicals. This plant is effective for variety of diseases and the phytochemicals obtained also varied. It was found that the majority of compounds showed biological activities and few had several pharmacological properties. According to European literature, the traditional usage of Chicory roots causes relief in symptoms associated with digestive disorders and the loss of appetite. The extracts of plants are used to heal various symptoms and alignments. It was found that the juice has been found in folk medicine for the uterus and tumours. Different parts of the plant have an effective part in the treatment of disorders of the liver, and enlargement of the spleen, as a bitter tonic is beneficial for Jaundice, liver enlargement, etc. Traditionally, the roots of this plant are used to treat various diseases like liver disorders, inflammation of the urinary tract, and removal of gallstones [154].

2.19 Medicinal Value of *Cichorium intybus* (Chicory)

2.19.1 Anti-Microbial Activity

It was found that there is a difference in the antibacterial activity of chicory extracts in gram negative bacteria and gram-positive bacteria, as gram-positive bacteria are more susceptible. This difference in antibacterial activity may be due to cell wall composition of gram-negative bacteria which is hydrophilic and specially lipopolysaccharides which can prevent storage of phenolic compounds in particular cell membrane. It was found through various studies that the root extract of chicory has more potent antibacterial activity as compared to other parts of the plant. Additionally, it was observed that the phenolic compounds in chicory can disturb the cell wall and cell membrane, affecting their permeability, and releasing intracellular constituents which interfere with the function of the mitochondrial membrane [155].

2.19.2 Antioxidant Activity

C. intybus has much potential for being regarded as a natural substance for improving oxidative stress and liver injury caused by nitrosamine compounds. The antioxidant potential of chicory has been evaluated for potential use as an alternate to synthetic antioxidants in the food industry. The studies showed that there is significant relation among phenolic contents and antioxidant activity. In a study diabetic rats were administered of chicory leaf extracts with vanadium and it was observed that it efficiently modulated glucose metabolism and improved antioxidant activity [156].

2.19.3 Ant-inflammatory Activity

C. intybus has beneficial properties for the treatment of various skin disorders when used externally. The disorder which can be treated includes dermatitis, inflammation of mucous membrane and wounds etc. The *C. intybus* flower infusion is very effective in treating inflammation, and irritations of the skin and eyes because of its antiseptic and anti-inflammatory properties. They have nutritious properties as well. In addition, leaf extracts of *C. intybus* showed enhanced antioxidant potential which provides safety to the heart.

Furthermore, *C. intybus* has been shown to inhibit prostaglandin E2 and cyclooxygenase enzyme. The inhibition of TNF-α mediated cyclooxygenase induction by *C. intybus* root ethyl acetate extract has been studied in human colon carcinoma cells. It suppresses the synthesis of prostaglandin E2 in a dose-dependent manner [157]. Chicory also exhibits preventive effects against the ethanol induced immunotoxicity possessing an anti-inflammatory property. *C. intybus* roots display dose-dependent anti-inflammatory activity in the carrageenan-induced paw oedema model. It reduced the serum TNF-α, interleukin (IL)-6, and IL-1 levels resulting in rise in antioxidant activity in paw tissue. This suggests the anti-inflammatory and antioxidant effects of chicory roots may be facilitated by cytokines inhibition [157].

2.19.4 Anti-Cancer Properties

The study of cytotoxicity of *C. intybus* extracts has shown its anticancer potential. There are some novel constituents reported in this plant. Some of these are sterols, guaianolides, 6- 6-methoxy flavone, anthocyanin, delphinidin etc. Many of these bioactive compounds showed cytotoxic activity in vitro, and tumour inhibiting effects in vivo trials and clinical trials. These phytometabolites of *C. intybus* have demonstrated the potential of antitumor effects, as research conducted focused on structure and activity has further confirmed the biological activity of this plant. The cytotoxic effects of *C. intybus* have been studied in breast cancer (MCF-7), prostate cancer (LNCaP), leukemia cells, and renal adenocarcinoma [158]. There was the study conducted in mice in which the ethanolic extracts of *C. intybus* roots showed a prominent reduction in Ehrlich tumour carcinoma. It was found that there is increase in life span of mice with 500mg/kg/ per day intraperitoneal dose of particular extracts up to 70%. It was found that the aqueous-alcoholic macerate of the leaf of *C. intybus* also showed antitumor effects on melanoma C32 cell lines [159].

2.19.5 Anti-Diabetic Activity

Many studies found the antidiabetic effects of *C. intybus* in a study conducted by Pushparaj and his colleagues found the effect of Chicory plant alcoholic extracts on rats which were induced by diabetes with streptozotocin (STZ, 50mg/kg). These rats were being fed with plant extract (125mg/kg of body weight) once a day for 14 days. Results found that there is a reduction in 20 per cent of glucose, 91 per cent of triglycerides and 16 per cent of total cholesterol. Also, there was significant reduction in hepatic glucose 6-phosphatase activity, which helps to reduce the production of glucose by liver resulting in decrease in blood glucose level in the rats fed on chicory [160]. Sharma and colleagues studied to examine the effects of *C. intybus* on lipid peroxidase activity of both enzymatic and non-enzymatic. DMC was induced in rats by a single intraperitoneal injection of streptozotocin (40mg/kg). *C. intybus* seed extracts (250 and 500mg/kg) were

administrated orally one time a day for three weeks and it showed a significant capacity to restore blood glucose levels. The prominent necrotic changes in heart tissue which are caused by streptozotocin, were normalized when *C. intybus* extracts were administrated which indicates cardioprotective effects by inhibiting oxidative stress [161]. There is another compound found in this plant called cichoric acid which exhibits insulin-sensitive properties and, thus, can induce glucose tolerance in dose dependent manner. Furthermore, there are evidences suggesting that plant methanolic extracts definitely influences glucose transport without inducing adipogenesis [153].

In a study conducted by Chandra et al., aqueous chicory seed extracts were fed to rats with remarkably reduced levels of serum glucose and triglyceride [35]. Subsequently, the same author conducted a study in which 150 patients of type 2 diabetes mellitus, conformed reduction in inflammation, oxidative stress and hypertriglyceridemia when treated with *C. intybus* seeds.

2.20 Role of Nanotechnology in the Treatment of Liver Cancer

Scientists use nanotechnology to progress and enhance the efficacy of medicines used to treat cancer. This technique is composed of many nanocarrier drug delivery systems helping to reduce the amount of drug needed to increase the therapeutic effect, reduce toxicity, and extend the drug release time provided in only dose hence improving careful and particular targeting of cancer cells of the liver. In an in vitro experiment organic dye-doped, core-shell NPs are used, showing less leaching and photostable and covalently link with anti-human liver cancer mAbs. Moreover, silica NPs with fluorescent can specifically and efficiently search for $HepG_2$ cells of liver cancer [162]. A particular method is used in general chemotherapy drugs but some common problems such as toxicity due to the drug and resistance due to the efflux pump have been observed. This technique has been useful in many studies to show improved results for therapy of liver cancer. The study was conducted in

mice in which mesoporous and rattle-type structures of silica and docetaxel were added checking the efficiency of killing $HepG_2$ cells. In this method, 7 per cent of the docetaxel was related to free docetaxel used to kill cells of liver cancer. This remedy reduces the toxicity of ICR mice with a rise in antitumor activity and found a 15% improvement in tumor in mice which was transplanted subcutaneously by hepatocarcinoma [163].

Another study found that microspheres or microbeads loaded with drugs have reduced the quantity of doxorubicin required for liver tumors related to intra-arterial intake. The plasma concentration of nanospheres of doxorubicin ranges from 9 to 50 nmol/L, 70 to 85 percent less than in intra-arterial intake of doxorubicin. It was observed that the tumor necrosis was improved after an hour of treatment and reached an optimal level on the seventh day indicating further evaporation of the microsphere drug with minimal side effects [163]. Nanoparticles improve these results by adding another component to an agent. For example, lipid nanoparticles provide delivery of doxorubicin and curcumin which provides constant release over 48 hours and can cause synergy, such as much cytotoxicity, increased apoptosis and reduction of IC50. The doxorubicin/curcumin approach has been shown to inhibit the growth of tumors by synergistic inhibition related to free doxorubicin or curcumin [164, 165].

2.21 Green Synthesis of Metallic Oxide Nanoparticles

Metal and metal oxides are used to prepare nanoparticles, these metals include iron, zinc, silver, titanium, platinum, thallium, gold, silica, aluminium, etc. There are two primary approaches as top-down and bottom-up approaches (Figure 2.8). The top-down method includes techniques like lithography, laser ablation, ball mining, sputtering, electro-explosion, and etching. However, the bottom-up method is considered maximum productive method and involves the synthesis of Nps from simpler molecules [166]. Among other methods green synthesis approach is highly

recommended because this method is the most economical, eco-friendly, reliable and sustainable. This method needs no toxic chemicals, high temperature and pressure. Green synthesis has minimal influence on the health of humans and the environment. Currently, it is ideal method for nanoparticle production as it is low-priced process, use harmless raw material such as microorganisms, fungi, algae, bacteria, plant extracts etc. These comprise phytochemicals and biomolecules that help in the immediate decrease of metal ions. Bacteria and fungi typically need longer incubation period as related to phytochemicals for reducing metal ions. Plants on the other hand are considered well option in the synthesis of nanoparticles because they minimize the need for maintaining microbial culture. The biological method of synthesis of nanoparticles exhibits biocompatible properties, which makes them suitable for various biomedical applications including cancer therapy. In contrast, chemical routes often involve toxic reducing agents, which limits their potential in the biomedical field and poses risk to environment. The biological approach overcomes these issues by employing self-reducing agents [167].

Because of its focus on sustainability, protection, and human safety, green nanotechnology is an attractive and expanding area of study and development. When it comes to making, using, and disposing of chemicals, the green chemistry movement aims to minimize potential risks to people and the environment. A thorough understanding of the raw materials, particularly their preparation into nanoparticles and the subsequent bioactivities that are safe for humans and the environment is necessary for a successful implementation of this technology. This opens up the possibility of using the abundant natural resources available for the purpose of manufacturing nanomaterials without risk. The use of biological elements in the metallic NPs synthesis process as reducing, capping, and stabilizing agents is becoming more commonplace. Environmental friendliness and safety for usage around humans and animals are both improved by the introduction of microorganisms and plant products as reducing agents [168].

Creating, modifying, and employing materials on the nanometer scale (between 1 and 100 nm) are essential to nanotechnology. The unique physicochemical,

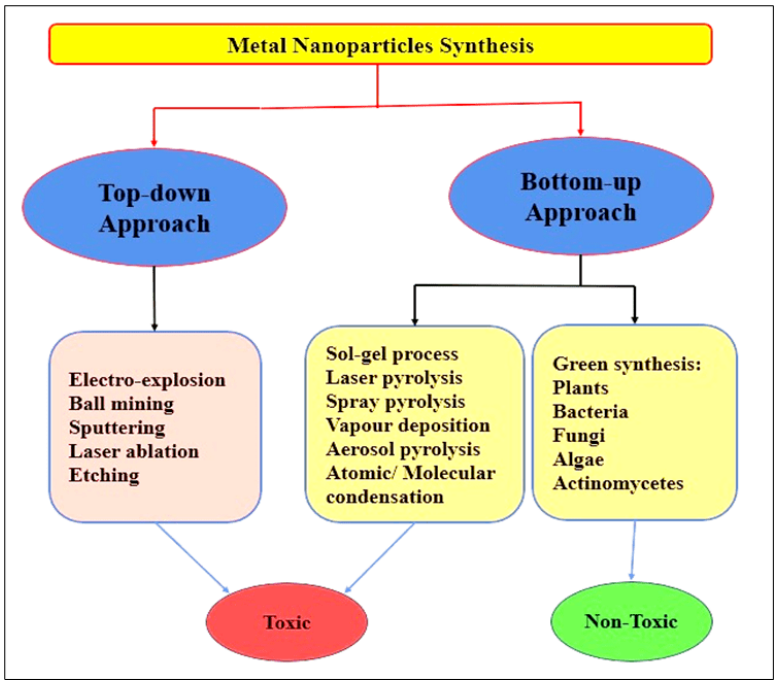


FIGURE 2.8: Different approaches to the synthesis of metallic nanoparticles [167]

optical, magnetic, and biological features of NPs have garnered much interest during the past few decades [169]. Bulk materials do not have the same qualities as finer-grained materials due to their microscopic size and volume compared to their surface area. As a result, researchers are investigating how these properties might be put to use for a wide variety of contexts, including but not restricted to pollution mitigation, water purification, agriculture, healthcare, and even space travel. Research into the advantages of MNPs, particularly those fabricated from noble metals like silver and gold NPs, has been extensive. These particles find use in fields as diverse as tissue engineering and healthcare as well as drug delivery and gene transfer [170]. In the next section, we'll take a glance at some of the most widely-used MNPs and how they're put to use.

2.22 Metal Oxide Nanoparticles

Nanoparticles (NPs) made of copper and copper (II) oxide have several applications in physics and materials science engineering. In addition to effectively

killing pathogens, their potent antibacterial qualities make them a desirable option. The usage of iron oxide nanoparticles (NPs) has been recognized in a wide variety of biological contexts, which includes gene therapy, stem cells, cancer, and atherosclerosis [171]. These NPs have been found to have a wide number of different applications beyond their original use as anticancer, antifungal, antibacterial, and targeted drug delivery agents. In particular, zinc oxide nanoparticles (ZnO NPs) are lauded for their ability to fight off cancer and microorganisms. Their benefits can be seen in applications as diverse as cosmetics, sewage treatment plants, and even food packaging. Food storage containers with ZnO NPs added as preservatives saw a significant reduction in the growth of bacteria and other microorganisms. Furthermore, ZnO NPs are much more toxic to bacteria than any other metal oxide NPs. Plants may be useful in the process of converting ions into MNPs due to their capacity to store hazardous metals. According to the literature various plant species and bioactive substances in the manufacture of MNPs, such as gold, silver, zinc, iron, copper, and platinum, cobalt, zirconium, cadmium, have been conducted [172].

2.23 Iron Oxide Nanoparticle

Iron oxide particles which are nanosized are studied for their potential applications in medical field as particularly in the diagnosis and therapy of several diseases. Interestingly it was found that the nanoparticles of Iron oxide are biodegradable, biocompatible, and nontoxic to human [173]. The other extra ordinary feature of iron oxide nanoparticles is their potential to join with some of the other biological molecules such as peptides, enzymes, nucleic acid, fatty acid, lipids and several metabolites. It was found that the presence of molecules immobilized on the nanoparticles show some superior properties and high reusability compared to counterpart which are immobilized [174, 175]. There are some of the unique properties observed in superparamagnetic iron oxide nanoparticles, as these are highly decomposable, have nontoxic effects and are biocompatible. The nanoparticles which have size less than 50 nm are considered suitable for endocytosis with use of drugs. Therefore, different synthesis methods as the sol-gel method, hydrothermal synthesis, co-precipitation method etc. are used to obtain iron oxide

nanoparticles with desired characteristics. Iron oxide nanoparticles can be synthesized biologically with living biota such as bacteria, algae, actinomycetes, fungi, viruses and plants. Currently, extensive research is conducted using plant-based iron oxide nanoparticles one of the possible reasons for this can be the fact that phytochemicals present in plants can reduce the ions of metals in less time as compared to bacteria, and fungi which need a lengthy incubation period. For the drug delivery, iron oxide nanoparticles can be conjugated with medicinal plants [176]. This is because of the ability to produce a formulation that yields multiple biological signalling pathways. There are various applications of iron oxide NPs in different fields as some are presented in the following diagram (Figure 2.9) [177].

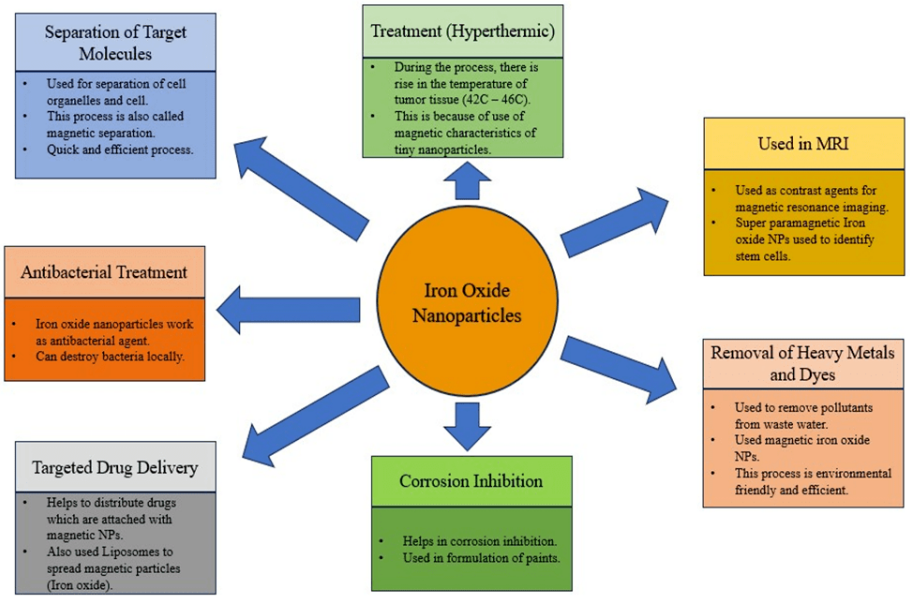


FIGURE 2.9: Applications of iron oxide nanoparticles [177]

Nanotechnology and nanoscience have become pioneering fields of research, offering a wide range of applications in both technological and scientific areas, particularly in medical sciences, biotechnology, biofilms and catalysis. Iron oxide nanoparticles stand out as some of the most prominent nanoparticles among various types of metal oxide nanoparticles. Some of the unique properties of these nanoparticles as biocompatibility, strong magnetic properties, low toxicity, and catalytic activity have significantly aided their use in biomedical applications [51]. Biosynthesis methods use harmless materials as plant extracts which are biocompatible and safe and act as potential reducing and stabilizing agents. There are many advantages of the synthesis of bio-based iron oxide nanoparticles as these are

cost-effective, eco-friendly, easy and fast synthesis, and non-toxic to the environment. Various parts of plants as leaves, stems, roots, seeds and flowers are used for the green synthesis of iron oxide nanoparticles. Phytochemicals (flavonoids, terpenoids, phenolics, carbohydrates and amino acids) in the plant extract are responsible for stabilizing and reducing iron oxide nanoparticles [178].

2.24 The Fortune of Cancer Cells Visible to NPs

There are various mechanisms involving the cytotoxic nature of metallic NPs, such as the formation of Reactive Oxygen Species (ROS), mitochondrial outer membrane permeabilization, activation of caspase-3 and breakage of DNA, all of which cause autophagy and necrotic death of cells of cancer [179]. The different sizes of nanoparticles small and large can enter into the cell differently. The larger nanoparticles enter via clathrin-mediated endocytosis and smaller nanoparticles enter via receptor-mediated contact with caveolin receptors located on the cell membrane (Figure 2.10).

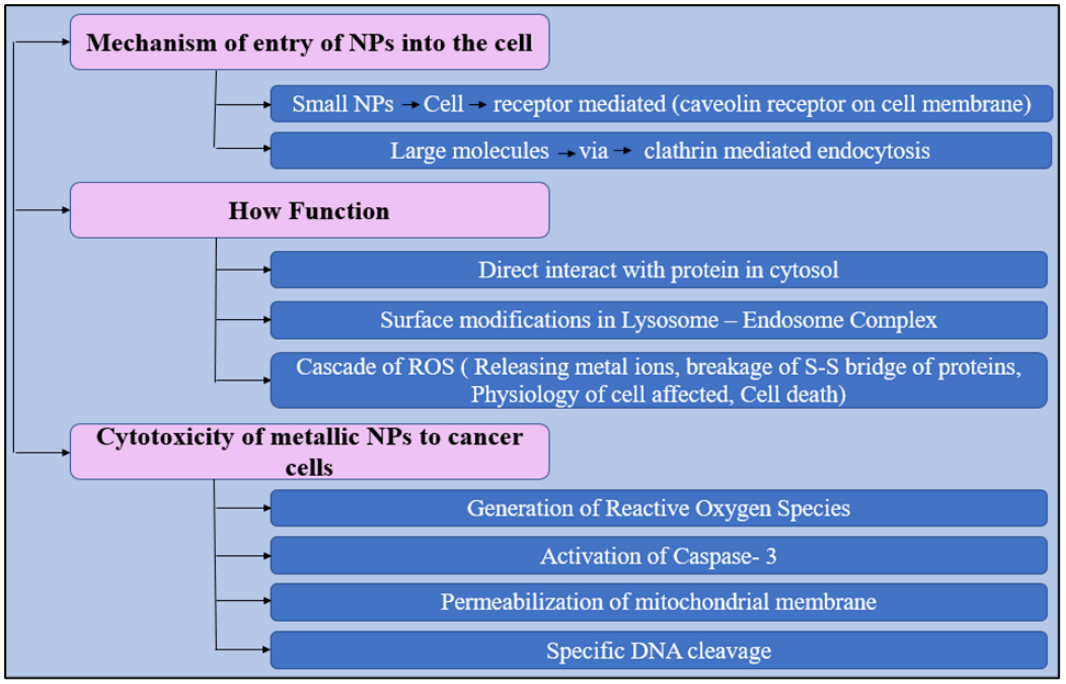


FIGURE 2.10: The fate of cancer cells exposed to nanoparticles [179]

These nanoparticles when once entered into the cell, take different routes to perform their functions, as either these show direct interaction with the protein or can experience surface modifications in the complex of lysosome-endosome, before being released into the cytosol. It was found that the nanoparticles moving inside cells activate a cascade of ROS and release metal ions that attach with the SH group of proteins breaking S-S bridges. Because of this cell physiology was affected and this way many signalling pathways got activated which leads to programmed cell death (Figure 2.11) [180].

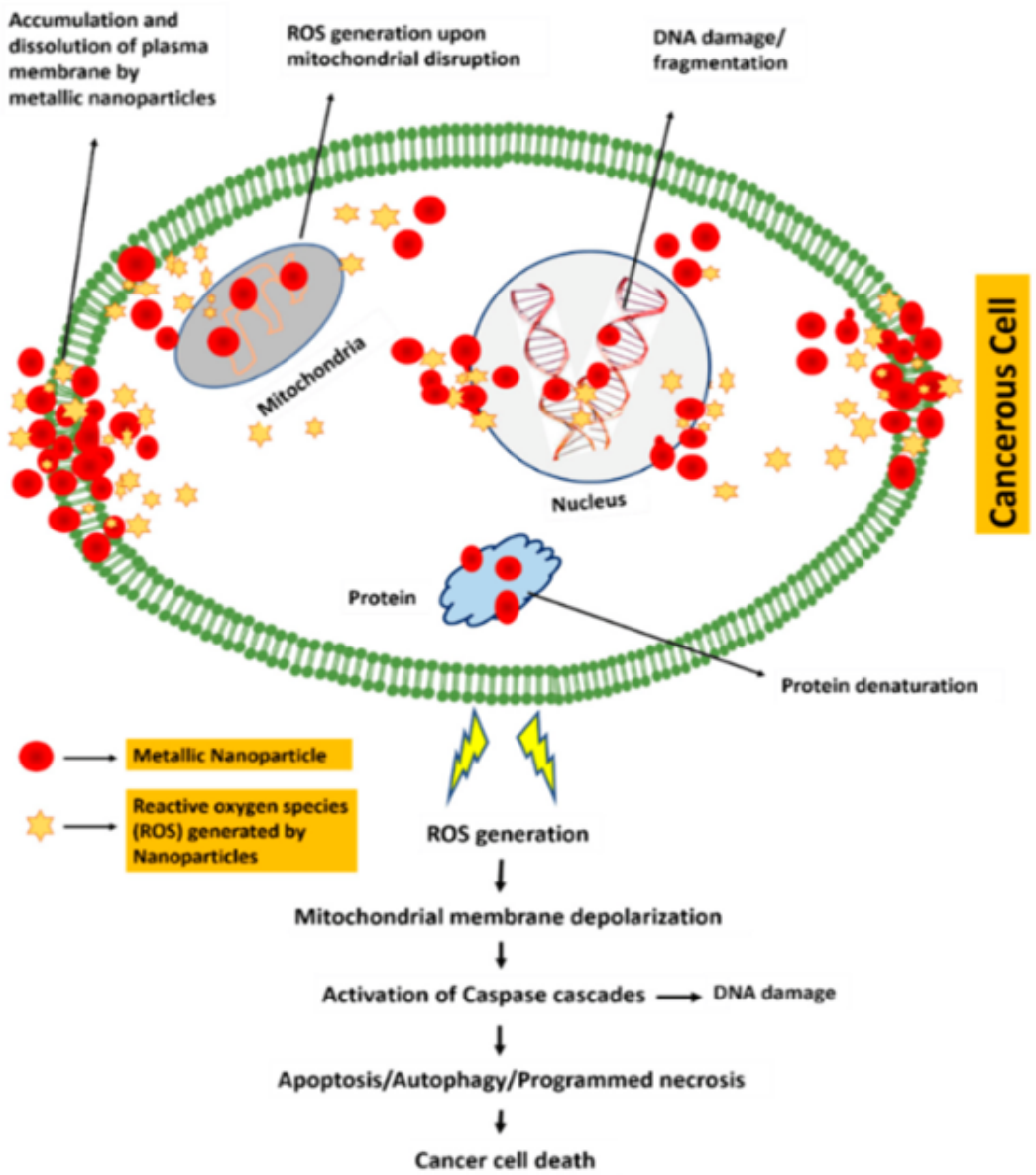


FIGURE 2.11: Mechanism of action of bio-synthesized metallic NPs initiating cytotoxicity in cancer cells [181]

2.25 Drug Designing and In-Silico Analysis

Since the beginning of time, human beings have witnessed the anger of several diseases. Some folklore practices are considered to reduce the harmful effects of health-related issues. At the beginning of the nineteen century, there was a development in chemistry which marked the discovery of small molecules as entities and bridged the scientific and folk knowledge into human health and disease management. The process of discovery of a drug consists of a series of events which help to know about the drug targets and natural and synthetic lead compounds are identified and optimized. In the next step there are preclinical studies and finally managed clinical trials [182] which are further divided into four phases. There is a crucial impact on the paradigm of drug discovery, when there were sudden and occasional outbreaks of diseases. Although there are many advancements in the sectors of biotechnology and pharmaceutical industries, the number of approved pharmaceutical groups is reduced due to product crises and drug attrition [183]. So, it is important to find other possible ways in drug discovery and procedures to combat various health effects and pandemic situations. The discovery of drugs is classified into two mechanisms conventional mechanism and the second one is reverse mechanism based on pharmacology.

2.25.1 Conventional Mechanism of Drug Development

Traditional drug discovery programs include a series of events as per the provisions of the U. S. Food and Drug Administration (FDA). Of the compounds which are selected as potential drug candidate, their validation and optimization and inspect for specific targets, only 0.0001 to 0. 001 percent were approved actually for human consumption and for marketing. Moreover, the cost for this entire procedure per molecule is about $2.6 billion, but the productivity crisis level stays relatively low [184]. In addition to cost-related restrictions, some other issues such as toxicity profiles, problems related to bioavailability, and because of deficiency of suitable model system there are translation failures. These issues are some of the main causes of the non-success of most drug-like molecules observed in the process of

drug development. For this, there is a need, not only to reduce the expenses of drug development procedures but to bring innovations in the improvements in drug productivity.

2.25.2 Drug Development (Reverse Pharmacology Based)

Reverse pharmacological methods are found important as compared to traditional approaches for the identification of drug candidates as well as for rational drug design. This method involves robust exploring of data and documentation of data which were found in clinical and preclinical studies. Moreover, helps in the translation of information obtained into the process of potential candidates for particular molecular targets. The main concept of reverse pharmacological drug discovery is based on the fact that rather than drug-based regimens, specific molecular targets are first identified with the use of high-speed molecular tools and genetics. In the next step, the target and ligands were optimized, and then the screening process was done for the identification of lead compounds concerning the target. At last, the drug candidates are studied for pharmacological activities. Reverse pharmacological-based drug discovery is considered the most efficient as it is less time-consuming (60 per cent less time) and less costly as compared to other classical approaches [185]. Hence reverse pharmacology-based drug development is an emerging technique which pioneered the discovery of potential drug candidates ensuring both safety and efficacy [184]. Following (Figure 2.12) are some of the steps of reverse pharmacological-based drug designing.

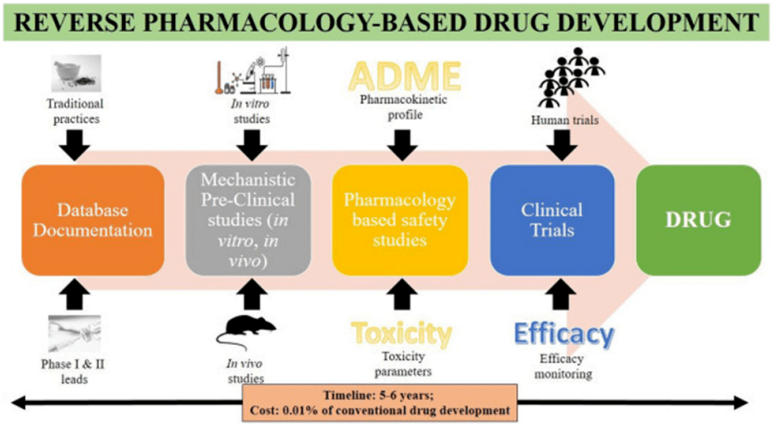


FIGURE 2.12: Steps of reverse pharmacological-based drug designing [186]

There is rapid development in the field of synthetic biology which includes the theory, programming, and application of computational modelling methods, predicting and explaining biological activities happening at the molecular level. Then in silico methodology is highly regarded as a beneficial tool for specific goals such as the identification of genes, and sequences to genomes, transcriptomes, proteomes, metagenome assemblies and de nova drug designing. In this respect, we can classify in silico methods into two classes. The first class also known as bioinformatics consists of data organization for accessing information, the development of statically, robust analytical tools and the interpretation and formulation of evolutionary hypothesis using data and analytical tools and the interpretation and formulation of evolutionary hypothesis using data and the interpretation and formulation of evolutionary hypotheses using data and analysis [187]. The second class the method of biological molecular structure, also known as biomolecular simulation is based on basic theoretical and chemical descriptions of particles (atoms and molecules) which are particularly interested in the movement, fluctuation and physical interaction of biological molecules such as proteins and peptides. Biomolecular simulations can be used to supplement in vitro and in vivo experiments by obtaining a molecular perception of biological processes and by analyzing simulated particles in atomic detail, new molecular interaction level, understanding and interpretation of experimental data can be added [188].

2.26 Molecular Docking

In recent years, molecular docking has become an important aspect of the development of biological drugs. This approach employs the prediction of connection between small molecules and proteins at the atomic scale [189]. This allows researchers to study the behaviour of small molecules such as nutraceuticals in target protein binding sites and to comprehend the essential biochemical processes of such interaction. This method relies on structure and needs a high-resolution 3D representation of target proteins acquired using methods such as x-ray crystallography, NMR spectroscopy or cryo-EM [190].

2.26.1 Molecular Docking Applications

Molecular docking is applied in the process of drug discovery. This process is used to identify the ligands which has the potential to be used as drug candidate. For this, there is a prediction of the binding affinity of small molecules to the target protein (Figure 2.13). A large number of databases could be screened to find particular small molecules which can be bound to the target protein showing high affinity [60]. Moreover, molecular docking can be used to predict ADMET properties of lead compounds. This can help to find the compound having adverse characteristics before the process of drug designing [191]. Molecular docking can also be used in the process of bioremediation which helps to estimate how well small molecules interact and bind to enzymes. These enzymes are used in the process of degradation of pollutants, and molecular docking helps in shaping inhibitors and activators of those specific enzymes involved in the process of remediation, hence enhancing their potential [192].

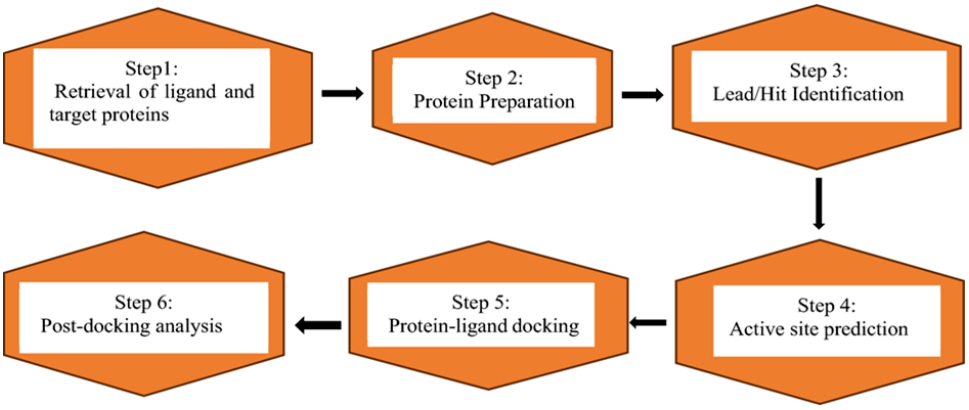


FIGURE 2.13: Steps of molecular docking [58]

2.27 Molecular Dynamic Simulation

Molecular dynamic simulation helps to acquire the relative motion of an atom or molecule based on the concept of Newton's equation of motion and acquire the appropriate kinetic property parameters by trajectory. MD simulations are used to target many body systems including nuclei and electrons, and there is the simulation of motions of the nucleus by computer, hence acquiring characteristics

and conformations of particular systems. Next, there is the connection of micro and macro systems with the help of computer services. In the molecular dynamic simulation, many disciplines such as physics, chemistry and biology are involved and play a distinct role [193]. Although this is considered an emerging technology its history is traced back to 1940 and the first ever gas MD simulation was done in 1957. The first MD simulation of protein was published in 1968 [194].

In addition to molecular docking, MD simulation is much more important to find the potential drug candidate using a robust virtual screening procedure, if the structure of the protein is reported. MD simulation adds to the information obtained from the results of the docking study and helps to trace the best and most potent lead molecule. It not only provides information regarding the interaction between the target protein and selected ligand with calculations (energy) but also conforms to the stability linked with the formation of complexes. These tools are considered essential in shaping the progress of potential drug candidates for definite pathological conditions [195].

In the process of drug discovery, MD simulation provides information (structural) of interaction drug-like candidates and target macromolecules with an assemblage of target protein conformation [196]. MD simulations work on the principle of algorithms of mathematics and quantum mechanics and also use the force field parameters (which merge different energy parameters such as electrostatic energy, binding energies, and wander wall energy) to anticipate the real action of the composed complex in the state of motion. The commonly used field force parameters for MD simulations are AMBER, CHARMM, etc. Although these force fields vary in the terms of use of parameters, results obtained by the use of these procedures are almost equal. More energy details on dynamic interactions obtained from energy calculations with Md simulation tools could play an important role in finding the potential of any drug-like molecule [197]. Current trends propose that both in silico methods as molecular docking and molecular dynamic simulations are actively considered to validate possible interactions between ligands and target proteins and to refine capable drug candidates [197].

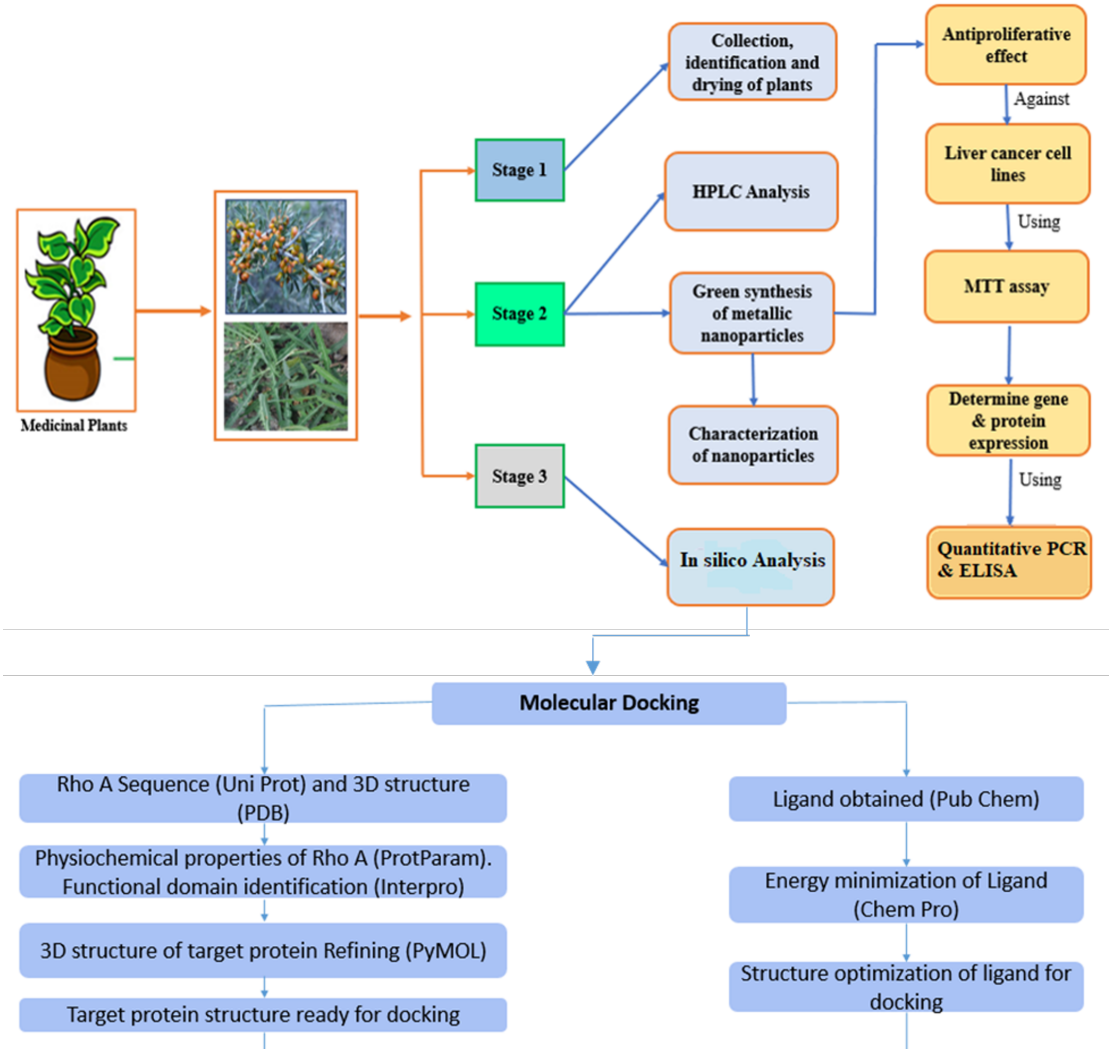
2.28 Applications of Molecular Dynamic Simulation

Molecular dynamic simulation can be employed to create stable protein variants, whereby the use of the simulation process, can find and analyze, the effect of mutations upon protein stability and dynamics of unstable residues. MD simulations can also be used to engineer functional regions and can find insight into protein folding and unfolding pathways [198]. One of the most efficient approaches for analyzing macromolecules in biology is the method of molecular dynamic simulation. Many of the studies showed that classical MD simulation plays a fundamental role in studying biological systems. This method is used to obtain various protein and nucleic acid combinations including the first attempts to simulate complicated events such as protein folding. These simulations often served as cost-effective and economical filters for the selection of a large number of candidates in particular contexts like ligand and protein design [199]. Development of new drugs is an area of interest where with the help of MD simulations can conduct different experiments. MD can also be useful in virtual screening by predicting, which chosen ligands are linked to targets. Virtual screening is typically conducted using traditional methods with an individual target protein structure using software of docking. Simulations can also be used to design drugs with the desired binding and dissociation kinetics of newly detected properties for effectiveness and safety. The efficiency of ligand binding to target is mostly linked with the time of residence, rather than binding affinity. There were several studies which found MD simulation-based methods of ligand classification found on their rate of dissociation [200].

Chapter 3

Materials and Methods

The overview of the methodology followed is given in Figure 3.1.



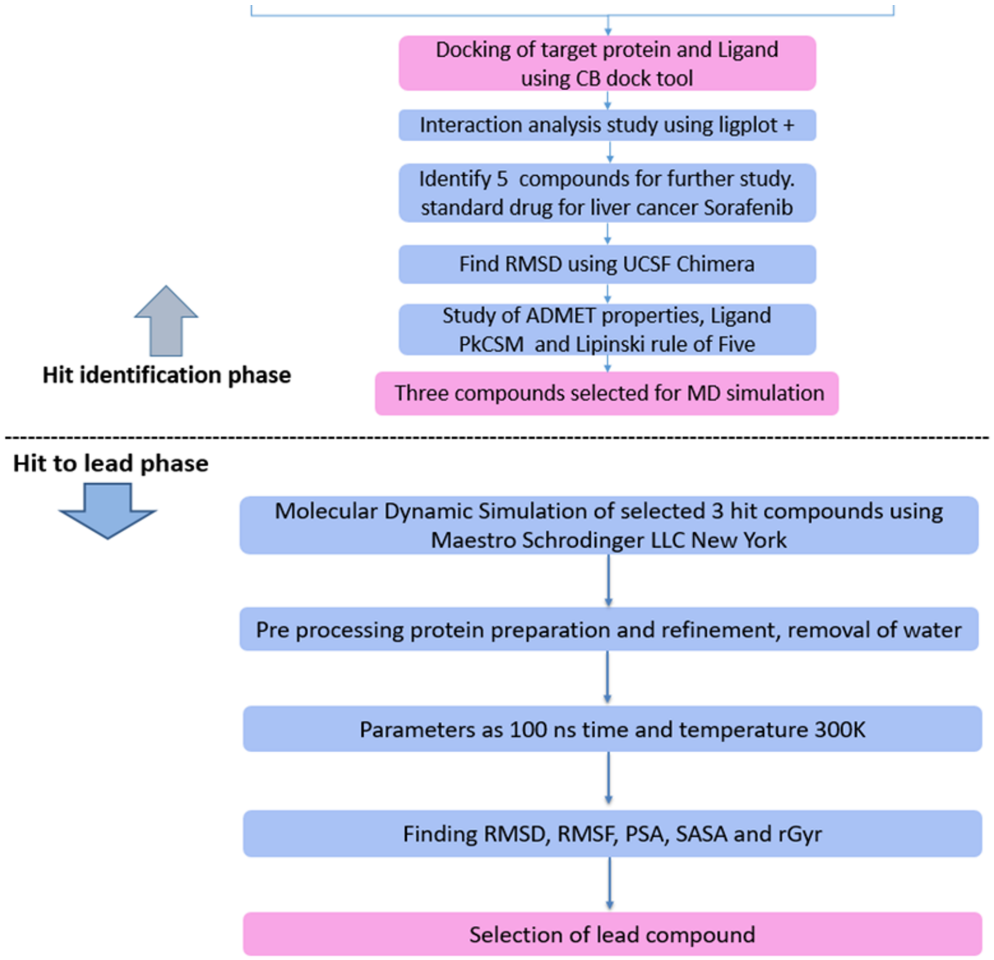


FIGURE 3.1: Proposed flow of overall methodology scheme of research work

3.1 Collection, Identification, & Drying of Plants

All the experiments were conducted according to national and international guidelines for conducting plant research. For the collection of plants, particular areas of Gilgit Baltistan such as central and upper Hunza, Ghizer district, and some areas of the Skardu division were targeted. To collect the medicinal plants of interest, help from some locals who have prior knowledge of the traditional use of these plants was sought. Both plants were identified from the herbarium of Hazara University Pakistan, with specimen voucher no FR-01 for *H. rhamnoides* L. and FR-02 for *C. intybus* L.

3.2 Plant Extract Preparation

For the plant extract preparation, initially, the seeds were dried at room temperature for 72 hours and then ground into powder form with the help of a mechanical grinder. Then the powder was extracted by soaking in distilled water and heated for 20 minutes with a magnetic stirrer at the rate of 200 revolutions per minute. The solution was cooled, filtered and stored at 4^oC for the synthesis of iron oxide nanoparticles. This process was conducted according to the recommended method with slight modifications [201]. All these procedures were conducted considering all national and international guidelines for plant research investigation including safe collection of plant material and voucher specimens' deposition in a public herbarium providing access to deposited material [202].

3.3 Synthesis of Fe_2O_3 Nanoparticles

Fe_2O_3 NPs of selected plants were prepared using precursor salt of respective metals (iron) and plant extract according to the reported methodology with slight changes. During this process 5 Mm solution of iron (II) sulfate heptahydrate ($FeSO4 \cdot 7H2O$) was prepared in distilled water and added to plant extract, in a ratio of 10:1, and kept the solution in the dark for 2 hours. The reduction reaction was monitored by a change in the colour of the solution. Then centrifugation was done thrice at 2000 rpm for 5 minutes. Afterwards, particles were washed with distilled water thrice. In the next step, samples were kept in a hot air oven (50-60 C^o) for drying. The dried powder was kept for further use [203].

3.4 Characterization of Nanoparticles

Physiochemical characterization is an important step in observing the biodistribution, effectiveness and safety of nanoparticles. During this, the functional properties of synthesized nanoparticles were assessed using different characterization techniques such as UV-visible spectroscopy, X-ray diffractometry (XRD), Scanning

Electron Microscopy (SEM), EDS, and Fourier Transform Infrared Spectroscopy (FTIR). The UV-Vis spectroscopy was done by placing the reaction mixture in quartz cuvettes and absorbance was measured in the range of 200 to 600 nm. Moreover, morphological details and size were attained by SEM (JEOL, JSM-6490 LATM) at the voltage of 20Kv (maximum) with the counting frequency of 2368 cps (maximum). The average size of synthesized nanoparticles was determined after finding the individual particle sizes in the field by ImageJ software [204]. To determine chemical composition, EDS was performed. For that purpose, dried Fe_2O_3 NPs were mounted on carbon tape and coated with gold sputtering for 2 minutes and then analyzed. To study surface chemistry of prepared nanoparticles FTIR was done. For this, nanoparticle solution was dried at the temperature of 75^{o}C and their dried powder was subjected to characterization in the range of 4000 - 400 cm-1 by using a KBr pellet strategy. The sample for XRD was made by taking a small amount of nanoparticle solution and drying it on a quartz plate (XRD D8 Advance Bruker Germany). The energy of the beam in the 10 - 20 KeV range caused the X-ray emission from the sample. The electron beam moved across the sample and the image was obtained from synthesized Fe_2O_3 NPs [205, 206]. Furthermore, the crystallite parameters of prepared nanoparticles were calculated using the following Debye Scherrer's relation,

$$D = 0.9\lambda/\beta \times cos\theta$$

where D represents crystalline domain size perpendicular to the reflecting planes, λ is the wavelength of incident X-ray (1.5406 Å), β is the angular full width at half maximum in radians, and is the angle of diffraction also called Bragg's angle.

3.5 Viability Assay to Determine Antiproliferative Effect

MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was used to assess the cytotoxic effect of iron oxide nanoparticle synthesized using the

plant extract of *H. rhamnoides* and *C. intybus*. Inside the cell, MTT is changed into blue-violet colour, water-insoluble, formazan.

This process is catalyzed by the enzyme of mitochondria called succinate dehydrogenase and happens only in the active mitochondria. The cause of this reaction is directly related to number of active mitochondria in the living cell. The greater value of absorbance rate means that there is more formazan is made which indicates the presence of more active mitochondria. Generally, MTT assay is used to trace cell viability and to estimate cell number [207].

To examine cytotoxicity and anticancer activity of nanoparticles of selected plant extracts, the human tumorigenic cell lines of liver cancer ($HepG_2$) (obtained from ATCC with ATCC number HB-8065TM) were used [208]. Some of the parameters followed to manage cell lines are as use of appropriate culture medium and conditions (temperature, humidity, CO2 level).

Also implemented quality control measures and stored cell lines in liquid nitrogen for long-term preservation [209]. Cytotoxicity of green synthesized nanoparticles of both plant extracts was determined via 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, during which cells were incubated at different concentrations (20,40,60,80 and 100 μg/mL) of plant extract and synthesized nanoparticles of selected plants extracts. The cells were treated with defined concentrations for an incubation period (24 hours, 48 hours) according to previous reports. The cells were seeded in 96 well plates for 24 hours containing 20 μL of RPMI growth medium.

Then the treated and control cells were incubated for 4 hours at 37 ^{o}C after adding 150 μL fresh medium and 50 μL of MTT solution. In the next step, the absorbance was measured at 560 nm on a microplate reader (Bio-TEK instruments). The anticancer activity was found by examining the in vitro half maximum concentration (IC50) as reported previously [210]. The following equation was used to calculate the cell viability of $HepG_2$ cells after treatment.

$$Percentagecellviability = \frac{Absorbanceoftreatment - Absorbanceofblank}{Absorbanceofcontrol - Absorbanceofblank} \times 100$$

3.6 Determination of Level of Gene Expression

Real-time qPCR which is also called quantitative PCR is the modification of the PCR approach that enables monitoring the advancement of PCR in real-time PCR itself. This is an enzymatic procedure that is applied in vitro to extend selected DNA regions, which helps to develop thousands to millions of copies of a particular DNA segment. For this process, the required ingredients include template DNA, primers, nucleotides (dNTPs), and DNA polymerase [211]. Besides improving accuracy, sensitivity and speed a key benefit of real-time quantitative PCR is that it provides a quantification relationship between the number of target sequences before the amplification and the number of amplicons assembled in the PCR cycle. This is very important to accurately quantify the target nucleic acid, which is vital in messenger RNA quantification to analyze gene expression [212]. Furthermore, post-PCR processes are not necessary to minimize the possibility of cross-contamination due to early amplicons. Hence this technique has transformed the identification and quantification of target nucleic acids leading to a broad spectrum of applications [213].

3.6.1 Procedure for Quantitative PCR

Total Ribonucleic acid was obtained from the treated cells by using the RNA extraction kit Pure-Link RNA mini kit (Thermo Fischer Scientific, catalogue no 12183018A) and real-time qPCR was performed to study the expression of targeted gene RhoA, bax and apoptotic genes (caspase 3, caspase 8 and caspase 9), according to reported methodology [214]. The experiment was managed with IC50 (concentration in μM) of synthesized nanoparticles and both plant extracts. Considering the kit's instructions, treated and untreated cells were washed with phosphate buffer saline (pH 7.2), and then 1 mL of RNX-plus solution (SinaClone, Iran) was applied to each cell. The prepared cDNAs, following kits (High-Capacity cDNA Reverse Transcription kit, catalogue no 4368814) instructions, were kept at -20^{o}C in preparation for Real-Time qPCR investigations after standard agarose gel electrophoresis method conformed to the cDNA synthesis. Real-time PCR

was conducted using a PCR system (7900HT Fast Real-Time PCR System) and TaqMan gene expression master mix (Applied Biosystems).

The reaction was performed in triplicates under the following conditions. Polymerase activation was done at 50C^o for 2 min, denaturation was done for 10 min at 94C^o followed by 40 cycles of denaturation at 94C^o for 15 sec. Afterwards annealing with synthesis was done for 1 min at 60C^o [215]. The β actin was used as a reference gene. It was expressed as a fold change from the β actin level and calculated using the ΔCt technique.

The following primers were used (Table 3.1).

TABLE 3.1: Primer sequences of the studied genes

Gene name	Primer sequences
RhoA	F: TCTGTCCCAACGTGCCCATCAT
	R: CTGCCTTCTTCAGGTTTCACCG
Bax	F: CCCGAGAGGTCTTTTTCCGAG
	R: CCAGACCATAGCACACTCGG
Caspase 3	F: TGCCTGTAACTTGAGAGTAGATGG
	R: CTTCACTTTCTTACTTGGCGATGG
Caspase 8	F: GACAGAGCTTCTTCGAGACAC
	R: GCTCGGGCATAGAGGCAAAT
Caspase 9	F: CTCAGACCAGAGATTCGCAAAT
	R: CTCAGACCAGAGATTCGCAAAT
β ***actin***	R: TTCCAGCCTTCCTTCTTG
	F: GGAGCCAGAGCAGTAATC

3.7 Determination of Selected Protein Expression

ELISA is a technique which is generally used to detect and quantify the target antigens present in a biological sample. Like other types of immunoassays, this technique relies on antibodies to assess the antigen using highly specific antibody-antigen interactions. During this process, the immobilization of the target antigen is carried out on the solid surface, and then the antigen-antibody complex

formed when bound to antibodies. We can analyze the antigen-antibody reactions through, colour change, light or through fluorescent signals acquired through enzyme-linked conjugate and an enzyme substrate. This will help to measure the target molecules concentration in a biological liquid or a biological system. This method is specific and is often used for research and diagnostic purposes, and the results obtained are rapid and high throughput [216]. ELISA kit (Thermo-Fisher Scientific) was used to measure protein levels of enzyme caspase 3, caspase 8 and caspase 9, (Catalogue No BMS 2012, BMS 2024, BMS 2025) bax (Catalogue No EEL030) and RhoA protein (Catalogue no PA5-116633) considering the instructions of manufacturer's protocol as triplicates. The experiment was managed with IC50 (concentration in μM) of synthesized nanoparticles of extract and both plant extracts. To prepare cell lysates, kit instructions were followed. There was the use of a secondary antibody (horseradish peroxidase-conjugated) to detect the proteins from the cell lysate that bind in particular to the primary antibody. The concentrations of proteins were estimated at 450 nm [214, 217].

3.8 Statistical Analysis

Each experiment was conducted in triplicate and the standard deviation along with standard error (SE) was calculated. A value of $p \leq 0.05$ was considered statistically significant, and quantitative data were presented as mean $\pm$ SE. To perform Two-way ANOVA, GraphPad Prism V.5 was used.

3.9 HPLC for the Determination of Polyphenols in Plant Extract

3.9.1 Collection and Preparation of Plant Material

Specific regions of Gilgit Baltistan, including the Ghizer district, parts of the Skardu division, and central and upper Hunza, were targeted for the collection of

flora. The chosen plant was identified using the specimen voucher number FR-01 for *H. rhamnoides* from the Hazara University of Pakistan herbarium. The seeds were first dried at room temperature for 72 hours to prepare the plant extract, and then they were ground into a powder using a machine grinder. 1gm of powdered seed and 10 mL of an 80/20 methanol/water solution were combined for the extraction process.

After two minutes of stirring the mixture, each extract was centrifuged for thirty minutes at 5000 rpm. A rotatory evaporator was used to evaporate the resulting methanolic extract. The following stage involved extracting three times with 1 mL of hexane using 2 mL of acetonitrile. 2 mL of methanol was added to this dried fraction.

The extracted material was subsequently subjected to HPLC analysis to identify any phenolic components. Similarly, the solution for standards was prepared by dissolving 100 mg in 1 mL of methanol [218].

3.9.2 Chemicals and Reagents Used and Preparation of Standard Stock Solution

The chemicals and reagents used in this process are methanol, distilled water, acetonitrile, formic acid and standards such as kaempferol, gallic acid, salicylic acid, chlorogenic acid, rutin, caffeic acid, HB acid, sinapic acid, coumarin, quercetin, benzoic acid, ferulic acid and vanillic acid. The standard stock solutions were prepared at a concentration of 1000 ppm and were synthesized in methanol.

For further use, the solutions were stored in appropriate containers and had to be kept at -20°C. To prepare the solutions of lower concentration, standard stock solutions were diluted (40, 20, 10 and 5 ppm). This helped to create a calibration curve which is used to quantify the analytes in the sample [219].

3.9.3 Plant Extract Preparation for HPLC Chromatography

For the plant extract preparation, initially, the seeds were dried at room temperature for 72 hours and then ground into powder form with the help of a mechanical grinder. For the extraction process, 2 grams of seed powder was mixed with 10 mL of solution of methanol/water (80/20). The solution was stirred for 2 minutes and centrifuged at 5000 rpm for 30 minutes. The obtained methanolic extract was evaporated using a rotatory evaporator. This fraction was dried and taken up by 2 mL of methanol. The prepared extract was further used for HPLC analysis and the determination of phenolic compounds [218].

3.9.4 Quantitative Analysis of Polyphenols using HPLC

HPLC is a technique which we use to analyze and characterize the extracts of plants, especially phenolic compounds. This method is efficient, with high resolution and high reproducibility. In general, we use this method for separation, quantification and the identification of polyphenols in the given plant extract. Generally used in the reverse phase involves three important points the column, elution solvent, and detector. Ther are the two variables which are used while separating the mixture as the stationary phase (column) and mobile phase (solvent). The solution for standards was prepared by dissolving 100 mg in 1mL of methanol [218].

The Perkin Elmar Flexar System, which includes an internal degasser, a diode matrix detector (PDA), and an Eclipse ODS Hypersil C18 Column (15 cm), was utilized for the HPLC analysis. Solvent 1 (water/formic acid, 0.1%) and Solvent 2 (acetonitrile/formic acid, 0.1%) made up the mobile phase. The injection volume was 20 μL, and the flow rate was 1 mL/min. To prevent damage to the column, further safety measures were implemented while the extracts and standards were meticulously filtered using Millipore membranes. To identify the peaks, retention times were compared to standards. A computer program viewed the signals that

the detector recorded [220]. With a specific retention time (Table 3.2), the analytes were determined.

TABLE 3.2: List of standards used in HPLC chromatography

Standards used	**Retention time (min)**	**Standards used**	**Retention time (min)**
Chlorogenic acid	2.880	Ferulic acid	12.460
Gallic acid	3.342	Salicylic acid	15.296
HB acid	6.756	Coumarin	16.085
Caffeic acid	7.494	Quercetin	16.954
Vanillic acid	7.687	Benzoic acid	18.306
Kaempferol	11.074	Rutin	23.989
Sinapic acid	12.237		

3.10 In Silico Analysis

In the next step in silico predictions were performed which helped to develop biomedicines, and explore the pharmacology of potential therapeutics using computer - stimulated models. In recent years molecular docking has become an important feature of Insilco drug designing.

This method consists of the interactions between biomolecules and small molecules, showing an understanding of binding sites and affinities, and feasible mechanism of action. Hence use of molecular docking in the process of drug designing has been well-accepted [60]. In silico approaches were also useful to indicate that particular protein, which is the target molecule and mediates anticancer activity and also useful to trace those particular metabolites which are in action.

In addition to molecular docking, MD simulation has received much importance in tracing potential drug candidates by the process of virtual screening. If the protein structure is known molecular dynamic simulation adds the information obtained from the results of molecular docking analysis and helps to find the best and most potent lead compound.

It can provide information about the interaction between the target protein and selected ligand through energy calculations and to conform stability due to the

formation of complexes. Both tools are considered useful in the development of potential drug candidates for certain pathological states. The flow chart of the methodology followed for computational analysis is given in Figure 3.1.

3.11 Molecular Docking

The basic purpose of molecular docking is to identify the best conformational interactions among target proteins and compounds (ligands). For this purpose, there must be the protein (target) and candidate ligand. During this study, RhoA is selected as the target protein and the ligands for this interaction are gallic acid, quercetin, caffeic acid, chlorogenic acid, HB acid, vanillic acid, sinapic acid, rutin, kaempferol and salicylic acid. For molecular docking there is the use of CB dock which is an online docking tool, which automatically finds binding sites, and simplifies the procedure [221]. Three parameters to be considered in docking results are vina score, cavity size and grid map size. These parameters help analyze the outcomes of dockings. Following are the important steps in performing molecular docking. For molecular docking following are steps followed.

3.11.1 Selection of Protein, Refining and Functional Domain Identification

The UniProt database (www.uniprot.org/uniprotkb) was used to pick the target protein's (RhoA) main sequence in FASTA Format (www.ncbi.nlm.nih.gov/gene). ProtParam tool (web.expasy.org/protparam) helped to forecast the characteristics as well as positively and negatively charged amino acids, molecular weight, instability index, and theoretical PI, the physical and chemical properties of a chosen protein [222]. RhoA's final 3D structure was obtained in PDB format from the Protein Data Bank (www.rcsb.org/structure). PyMOL (pymol.org/pymol-command-ref) software was used to refine the protein structure [223]. An online database called Interpro (www.ebi.ac.uk/interpro) was utilized to identify the functional domain of the target protein. Additionally, MODELLER v10.5

(salilab.org/modeller/)(was used to model the residues in the structure that were not previously modelled. The generated protein structure was applied to additional examination of molecular docking.

3.11.2 Ligand Preparation and Molecular Docking

The HPLC-identified polyphenols of both plants were obtained from the PubChem database [224]. Chem Pro ultra programme (chemistrydocs.com) (chem 3D v12.0.2) was utilized to minimize the energy consumption of these specific ligands. This is a crucial stage in the ligand preparation process because unstable ligands exhibit inconsistent vina scores after docking. Additionally, the drug likeliness of the chosen ligands from the PubChem database and the conventional medication sorafenib which is used to treat liver cancer was examined. The ligands that were chosen for this investigation underwent testing for the Lipinski rule of five, to find their drug likeness. ADMET characteristics were also examined to evaluate the molecule as a possible drug source and to aid in the successful drug discovery process. An online programme called PkCSM (biosig.lab.uq.edu.au/prediction) was utilized for this purpose and helped with drug screening based on drug score, toxicity, and drug likeliness [225]. The Ligplot Plus software (version v.1.4.5) (www.ebi.ac.uk/software/LigPlus/) produced a diagram of protein-ligand interaction. Ligplot Plus examined the hydrophobic interactions and H bonds of particular docked molecules and calculated the RMSD values. To find the values of RMSD, UCSF Chimera (www.cgl.ucsf.edu/chimera) was used.

For the Molecular docking CB dock, Yang Cao Lab (clab.labshare.cn/cb-dock) was used which is an online docking tool, which automatically finds binding sites [221]. Three parameters were considered in docking such as vina score, cavity size and grid map size. The virtual screening of the RhoA against the selected ligands (caffeic acid, gallic acid, and salicylic acid) was performed by GNINA (github.com/gnina/gnina), a molecular docking software that used convolutional neural networks (CNNs) for the scoring function [226]. Moreover, the complexes with the highest binding affinity score among the other complexes were shortlisted.

The interaction analysis was performed on these shortlisted complexes to highlight interacting residues within the domain regions.

Additionally, the 2D interactions of these shortlisted complexes were visualized using the Discovery Studio Visualizer (Biovia, D.S. (2019) Discovery Studio Visualizer. San Diego).

3.12 Molecular Dynamics Simulation

Molecular dynamic simulation is an in-silico approach which is used to study the behavior of molecules and their interactions. This method can help to analyze single molecules and also complex systems like protein-ligand interactions. MD simulations provide facts about the movement of atoms and molecules within a system giving insight into their dynamics. This is generally used for the analysis of the structure-to-function relationship of protein and protein-ligand complexes and helps study the stability of complexes at different nanoscale intervals and the fluctuations detected.

3.12.1 Molecular Dynamics Simulation of Shortlisted Complexes

The molecular dynamics (MD) simulations were performed utilizing Maestro 12.0 (version 12.0.012, Schrödinger, LLC, New York, NY), a robust molecular dynamics suite to observe the stability and flexibility of the shortlisted complexes [227]. Firstly, in the protein preparation wizard, the protein was subjected to preprocessing and refinement along with the removal of water beyond 5 angstroms. The SPC force field was applied as the solvent model in the system builder module, including salt ions. Subsequently, the system was loaded into the MD window module, where parameters were configured for 100 ns simulation duration at a default temperature of 300K. Eventually, the trajectory file generated from the simulation was imported into the simulation interaction diagram module for post-simulation analysis, along with the root mean square deviation (RMSD) and root

mean square fluctuations (RMSF) values. Moreover, three parameters were examined to assess ligand properties, such as Solvent Accessible Surface area (SASA), Polar Surface Area (PSA), and Radius of Gyration (rGyr). The binding capabilities of the molecules were observed throughout the simulation run by generating snapshots every 100th frame.

Chapter 4

Results

This chapter consists of the results of the study conducted to obtain the objectives designed for particular research work. In the first phase of the study, there was collection and identification of the plants. Then there was the preparation of the extracts of plants and the use of this extract for the synthesis of Fe_2O_3 nanoparticles and the use of different characterization techniques such as SEM, FTIR, EDS, UV-Vis spectroscopy and XRD to characterized nanoparticles formed. During this study, MTT assay was used to determine the cytotoxic efficacies of selected plant extract and nanoparticles synthesized from these plant extracts against liver cancer (HepG2) cell line. To examine the level of (RhoA) target gene expression real-time qPCR was done. The level of protein expression analysis was done using the ELISA technique. In the next phase of the study HPLC chromatography of the methanolic extracts of *H. rhamnoides* and *C. intybus* was performed. In the next step, Insilco prediction was performed, which consists of molecular docking and molecular dynamic simulations. These computational methods help study the drug likeness of the hit compounds found in both plants and identifying the lead compound having the potential to be used in the process of drug designing in future.

Objective 1: To identify the antiproliferative role of *H. rhamnoides* Buch and *C. intybus* plant extracts and their respective iron oxide

nanoparticles against liver cancer cell line and their impact on the expression of liver cancer target gene, apoptotic pathway genes and proteins.

4.1 Plant Identification and Extract Preparation

For the collection of plants, particular areas of Gilgit Baltistan such as central and upper Hunza, Ghizer district, and some areas of the Skardu division were targeted. To collect the medicinal plants of interest, help from some locals who had prior knowledge of the traditional use of these plants was sought.

Both plants were identified from the herbarium of Hazara University Pakistan, with specimen voucher no FR-01 for *H. rhamnoides* L. and FR-02 for *C. intybus* L.

4.2 Synthesis of Fe_2O_3 Nanoparticles

Nanoparticle formation was confirmed by the change in colour to dark brown which indicates the synthesis of Fe_2O_3 NPs of *H. rhamnoides* and *C. intybus* respectively. The formation of these nanoparticles was attributed to the reduction by the bioactive compounds of plants under study which carried out the reduction of metal ions and formed stable nanoparticles.

4.3 Characterization of Nanoparticles

Physiochemical characterization is an important step to observe biodistribution, effectiveness, safety, and the action of nanoparticles. The functional properties of synthesized nanoparticles were assessed using different analytical techniques such as UV-visible spectroscopy, X-ray diffractometry (XRD), Scanning Electron Microscopy (SEM), EDS, and Fourier Transform Infrared Spectroscopy (FTIR).

4.3.1 Fe_2O_3 Nanoparticles Analyzed by UV-Vis Spectroscopy

The process was additionally verified by the technique of UV-Vis spectroscopy. UV-Vis analysis showed the characteristic peak of Fe_2O_3 NPs synthesized using the extract of plant *H. rhamnoides* at 300 nm (Figure 4.1A). UV-Vis spectroscopy of Fe_2O_3 NPs synthesized from the extract of *C. intybus*, exhibited the characteristic absorbance peak at 289 nm (Figure 4.1B)

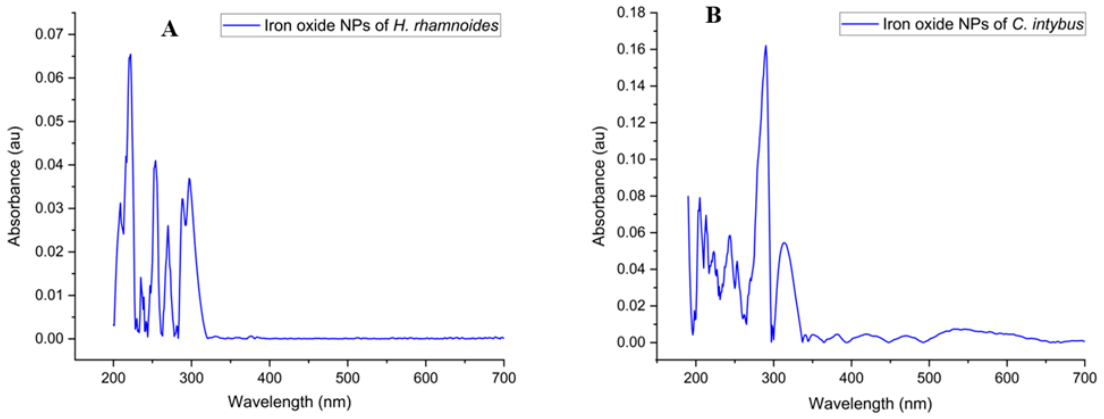


FIGURE 4.1: UV-Vis spectroscopy of *H. rhamnoides* (A) and *C. intybus* (B) Fe_2O_3 NPs

4.3.2 Fe_2O_3 Nanoparticles Verified by FTIR Spectrometer

FTIR spectroscopy was used to detect the functional groups present in plant extract, that bound to the surface of iron and involved in the synthesis of Fe_2O_3 nanoparticles. FTIR analysis was done by using an FTIR spectrometer, which helped to identify the numerous biomolecules present in the aqueous extract of *H. rhamnoides* and *C. intybus*. This characterization technique validated the presence of functional groups of active substances present in both plants responsible for the reduction and stabilization of Fe_2O_3 NPs. FTIR spectra revealed the characteristic absorption peaks related to particular functional groups present on the nanoparticle's surface. The major bands of FTIR spectrum of Fe_2O_3 Nps of *H. rhamnoides* (Figure 4.2) were seen at 3676, 2974, 1394,1250, 1055, and 403 cm-1. The minor bands were depicted at 2357, 2077, 1950, 1750, 891, and 479 cm-1.

In *H. rhamnoides* the major bands corresponding to 2973-2856 cm-1 indicated the presence of C-H group of aldehydes suggesting the presence of saturated compounds. Additionally, the absorption peak positioned at 1394 cm-1 attributed to the C-F stretch indicated alkyl and aryl Halides. The absorbance peak located at 2974 cm-1 corresponded to the O-H stretch indicating the presence of carboxylic acids in the FTIR spectra of *H. rhamnoides* Fe_2O_3 NPs. Likewise, the bands recorded between 650 cm-1-1000 cm-1 spectrum are due to the C-H group of alkanes. There is a band located at 2974 cm- in *H. rhamnoides* and 2922 cm- in *C. intybus* indicating the presence of aromatic rings.

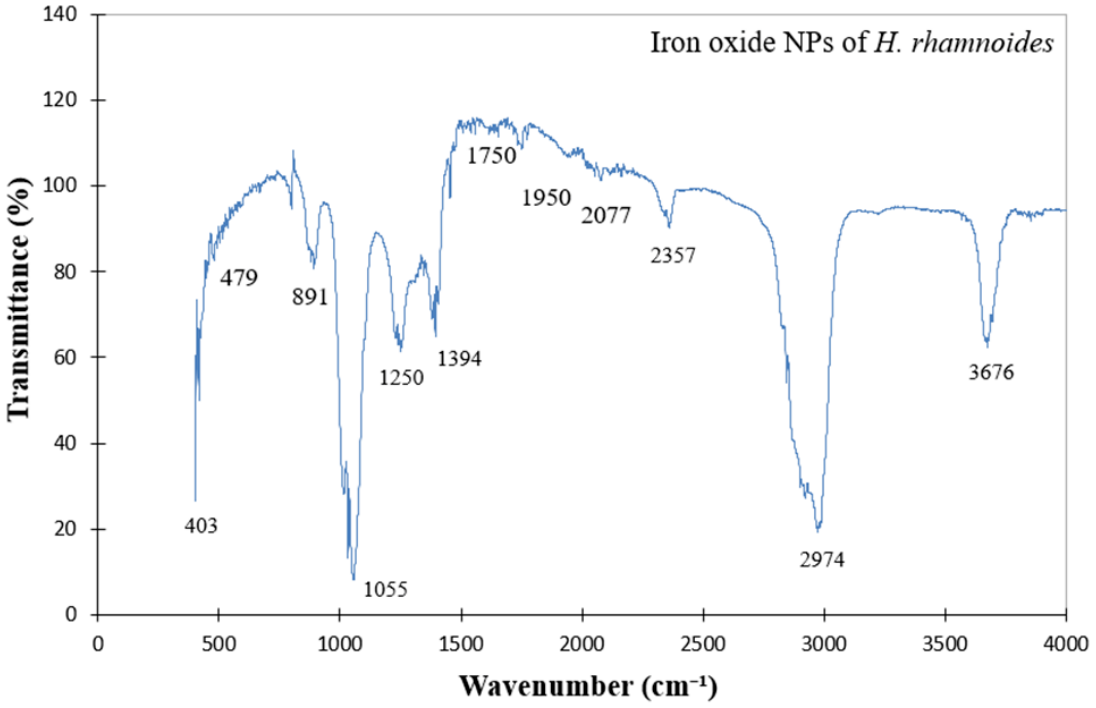


FIGURE 4.2: FTIR spectra of Fe_2O_3 NPs synthesized from *H. rhamnoides*

FTIR spectrum of Fe_2O_3 NPs synthesized from *C. intybus* showed the major bands at 3675, 3293, 2922, 1741, 1637, 1393, 1059, and 404 cm-1. Likewise, the minor bands were detected at 3293, 2360, 2160, 2034, 1393, 1305, and 711cm-1 as displayed in Figure 4.3. The major band 3675 cm-1 shows the N-H bending indicating the presence of proteins. The band present at 2922cm-1 depicted the presence of the O-H group indicating the presence of carboxylic acid, whereas the band at 3293cm-1 indicated stretching bond O-H of alcohols. A distinct band was observed at 1637 cm-1 indicating the presence of N- H bend of amines.

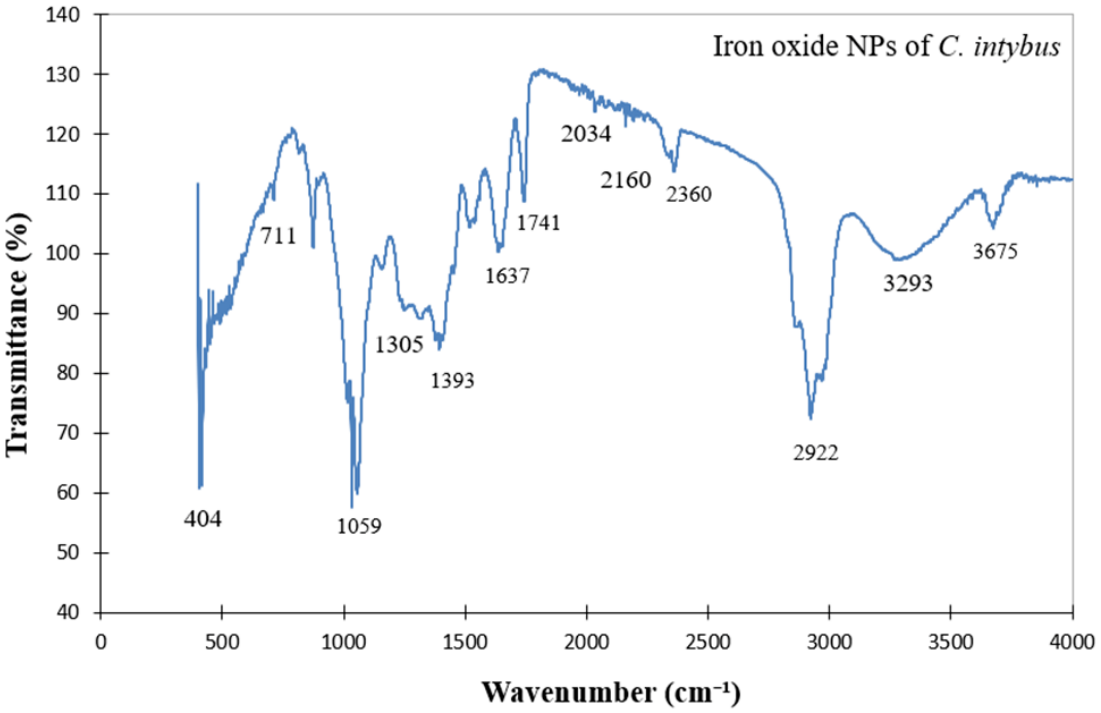


FIGURE 4.3: FTIR spectra of Fe_2O_3 NPs synthesized from *C. intybus*

4.3.3 SEM Analysis

Scanning electron microscopy (SEM) determined the morphological properties, size and shape of synthesized nanoparticles, during which electron beams of high energy are used to produce images. SEM images of Fe_2O_3 NPs of *H. rhamnoides* and *C. intybus* showed that the distribution of nanoparticles is steady and their size is in the nanoscale range.

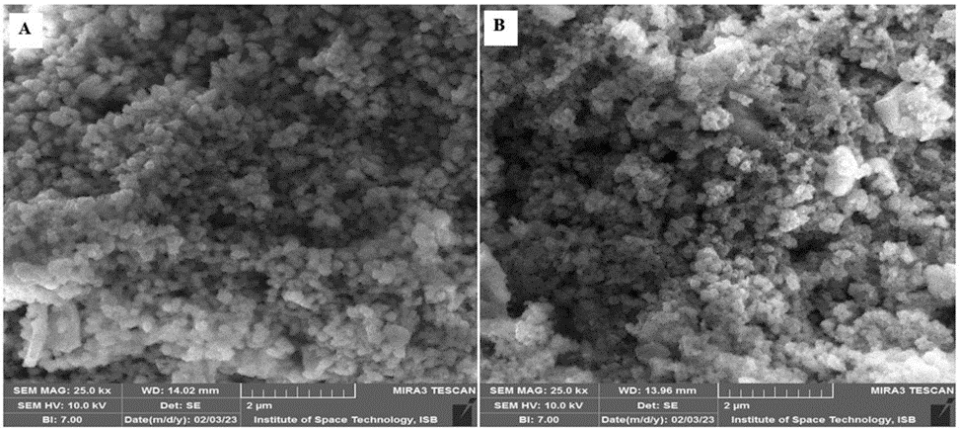


FIGURE 4.4: SEM analysis of *H. rhamnoides* (A) and *C. intybus* Fe_2O_3 NPs (B)

Most of the nanoparticles exhibited a defined shape and smooth surface with slight aggregations. These images also interpret a few of the nanocrystals with irregular shapes. The size of nanoparticles synthesized from *H. rhamnoides* was 27±5 nm in diameter (Figure 4.4A) and they were hexagonal as compared to nanoparticles of *C. intybus* which had a size range of 84±4nm (Figure 4.4B) and they were round.

4.3.4 Analysis by EDS

EDS is an analytical technique which is used to determine the elemental composition and distribution in synthesized nanoparticles. The chemical and elemental composition of the synthesized Fe_2O_3 NPs of both plants were determined using energy dispersive X-ray spectroscopy (EDS) besides SEM (Table 4.1). The EDS spectrum suggested that iron is the primary component in both samples along with oxygen without the presence of any contaminants (Figure 4.5A & B). The results confirmed the presence of iron in the particles and identified iron peaks in three different areas.

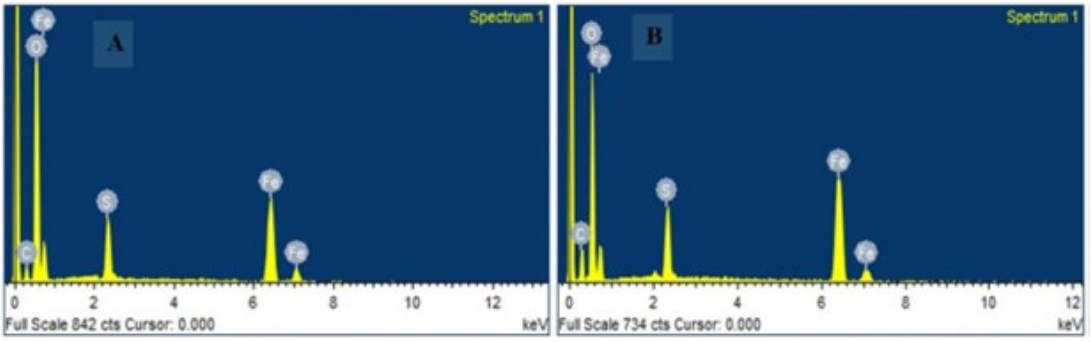


FIGURE 4.5: EDS analysis of *H. rhamnoides* (A) and *C. intybus* (B) Fe_2O_3 NP

TABLE 4.1: Elemental composition of EDS spectrum of *H. rhamnoides* and *C. intybus* Fe_2O_3 NPs

Elements	**Weight% in *H. rhamnoides* Fe_2O_3 NPs**	**Atomic% *H. rhamnoides* Fe_2O_3 NPs**	**Weight% in *C. intybus* Fe_2O_3 NPs**	**Atomic% *C. intybus* Fe_2O_3 NPs**
CK	17.6	29.74	08.19	14.48
OK	42.81	54.30	51.51	68.40

Table 4.1 continued from previous page

Elements	**Weight% in *H. rhamnoides* Fe_2O_3 NPs**	**Atomic% *H. rhamnoides* Fe_2O_3 NPs**	**Weight% in *C. intybus* Fe_2O_3 NPs**	**Atomic% *C. intybus* Fe_2O_3 NPs**
SK	5.86	3.71	6.34	4.20
FeK	33.72	12.25	33.96	12.92
Totals	100.00		100.00	

4.3.5 Fe_2O_3 Nanoparticles Characterized by XRD

XRD provides useful information about the phase nature, lattice parameters and crystalline structure of synthesized nanoparticles. This is a unique method to determine crystallinity of different compounds. The pattern of Fe_2O_3 NPs in XRD analysis was observed by using index POWDER-X software and matches with standard JCPDS, 77-1545 data. The result showed the diffraction peaks of Fe_2O_3 NPs of *H. rhamnoides* at degrees (2) 11.34^o, 17.94^o, 19.73^o, 28.85^o, 34.95^o, 43.38^o, 46.65^o, 56.35^o,60.41^o and 69.05^o while the X-ray powder diffraction peaks of *C. intybus* were found at 10.02^o, 12.58^o, 16.76^o, 20.46^o, 24.24^o, 32.53^o, 36.76^o, 39.95^o, 44.22^o, 50.76^o, 60.04^o, and 64.61^o (Figure 4.6A & B) which indicated the crystalline nature of nanoparticles. Furthermore, through Debye Scherrer's equation and broadening of Bragg's peaks, it was revealed that the approximate crystallite size of Fe_2O_3 NPs of *H. rhamnoides* was 8 nm and those of *C. intybus* were 12 nm.

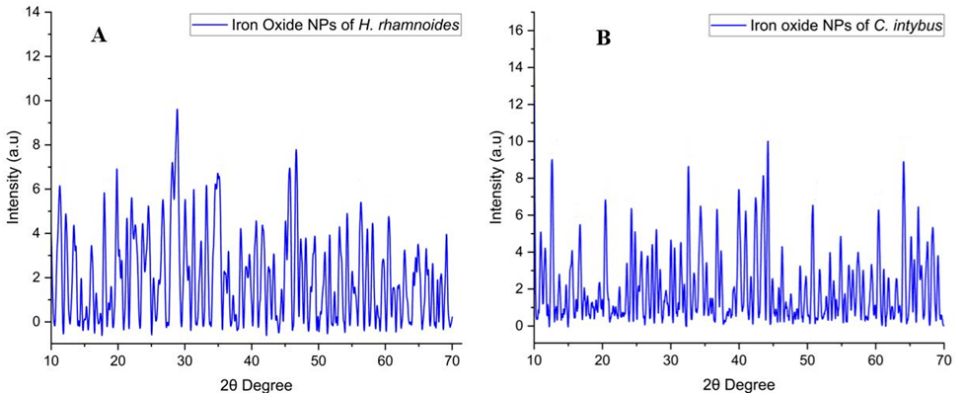


FIGURE 4.6: XRD analysis of *H. rhamnoides* (A) and *C. intybus* (B) Fe_2O_3 NP

4.4 Cytotoxicity Assay

The cytotoxic potential of Fe_2O_3 NPs synthesized from *H. rhamnoides* and *C. intybus* was assessed against the HepG2 liver cancer cell line. The cytotoxic potential of synthesized nanoparticles and respective plant extracts was determined for distinct concentrations (20, 40, 60, 80 and 100 µM) and revealed promising results. During this experiment, the HepG2 cell line was exposed to the plant extract and Fe_2O_3 NPs (20 to 100 µM) were synthesized using the extract of *H. rhamnoides* and *C. intybus*. The results showed the reduction of cell viability in dose dose-dependent manner, as with increased concentration of iron oxide nanoparticles, there was a decline in the cell viability (Figure 4.7). The IC50 values of *H. rhamnoides* and *C. intybus* nanoparticles were also found to be less (41.69 µM and 71.04 µM) compared to the IC50 values of their respective plant extracts (78.10 µM and 96.03 µM). These results also speculated that the plant extract of *H. rhamnoides* is more effective for cell cytotoxicity as compared to the extract of *C. intybus*. The data also found statistical significance with $P<0.0001$ (Table 4.2). The experiment included triplicates of each trial and S.E was calculated (indicated by error bars).

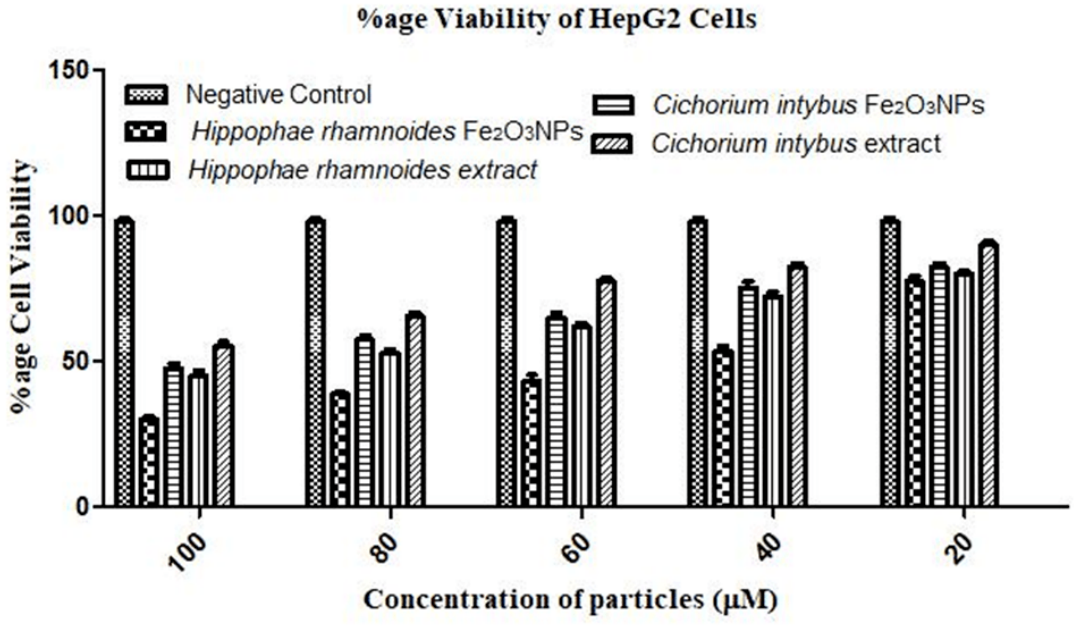


FIGURE 4.7: MTT assay determining cell viability of Fe_2O_3 NPs and plant extracts against HepG2 cell line

TABLE 4.2: Analysis of variance for factors affecting the viability of HepG2 Cells

Source of Variation	**Df**	**Sum of squares**	**Mean square**	**F Value**	**P Value**	**Significant**
Interaction	16	2616	163.5	26.60	<0.0001	Yes
Types of Nanoparticles	4	20040	5009	815.0	<0.0001	Yes
Concentration	4	8397	2099	341.5	<0.0001	Yes
Residual	50	307.3	6.147			

4.5 Gene Expression and Protein Analysis

RhoA gene which is primarily involved in cytoskeleton of cells, polarity, cell cycle, and cell degradation is a major drug target for treating multiple malignancies. The level of this gene significantly declined in the cells treated with Fe_2O_3 NPs and plant extract of both plant species as compared to untreated cells (Figure 4.8). *H. rhamnoides* plant extract and its NPs were found to show greater potential in deregulating the RhoA gene expression as compared to the nanoparticles and plant extract of *C. intybus*. This may be attributed to the fact that this gene is considered as oncogene and participates in carcinogenesis of liver cancer. These findings also proposed the upregulation of bax gene which is involved in apoptotic pathways (Figure 4.8). plant extract and Fe_2O_3 NPs of *H. rhamnoides* showed greater cytotoxicity by upregulating bax gene as compared to plant extract and NPs of *C. intybus* treated HepG2 cancer cells in comparison with the control group. The caspase 3, caspase 8 and caspase 9 showed higher expression as well which decipher the role of extrinsic and intrinsic pathway of apoptosis in nanoparticles mediated toxicity. Plant extract and Fe_2O_3 NPs of *H. rhamnoides* showed greater expression of caspase 3, caspase 8 and caspase 9 genes as compared to extract and NPs of *C. intybus*. These findings illustrated that *H. rhamnoides* showed more cytotoxicity by up-regulating the apoptotic pathway genes in comparison to *C. intybus*.

Moreover, ELISA was used to determine the protein levels of the aforementioned genes, following recommended instructions. The protein level of RhoA was found

to decrease while bax protein was found to be increased in treated cells as compared to those of untreated (Fig 4.9). The level of initiator caspase (caspase-9) executioner caspases (caspases-3) and caspase-8 protein was studied for HepG2 cells treated with *H. rhamnoides* and *C. intybus* Fe_2O_3 NPs and plant extracts. The results presented that the level of caspase proteins was high in the cells treated with *H. rhamnoides* as compared to *C. intybus* Nps and extract in comparison to the control group (Fig 4.9). This confirmed the role of nanoparticles in the activation of cancer cell death by downregulating the RhoA protein and upregulating the level of bax and caspase 3, caspase 8 and caspase 9 activating a series of caspase reactions. The increased expression of bax and these caspases (caspase 3, caspase 8 and caspase 9) at mRNA level and protein level showed that plant-mediated Fe_2O_3 NPs have shown programmed cell death at a remarkable degree. In the pathway of cell death, caspases are considered the main regulators having acritical role, as they are the main executioners of apoptosis and cell death in signal transduction. Moreover, it is also verified by this comparative study that *H. rhamnoides* has more potential to be used in the development of anticancer drugs as compared to *C. intybus*.

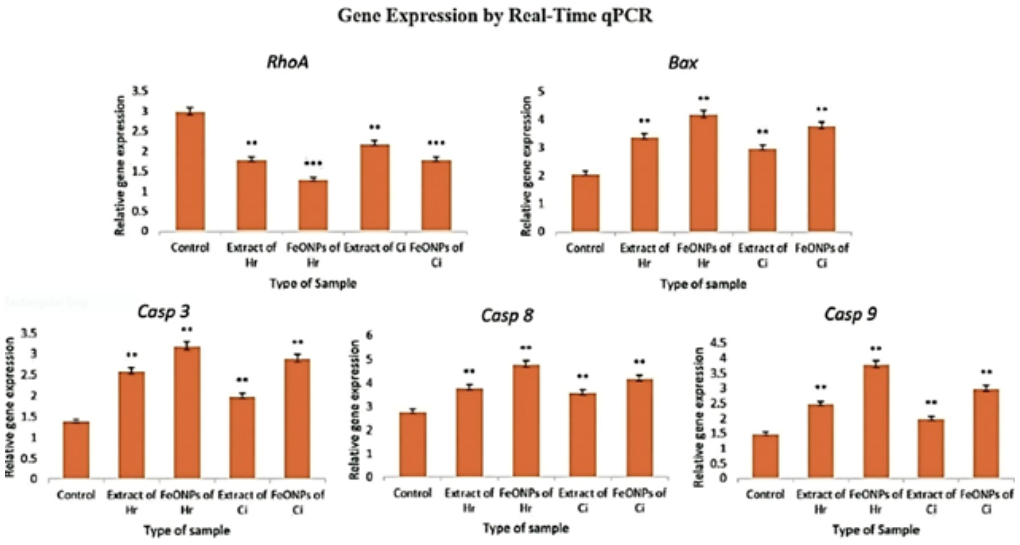


FIGURE 4.8: Gene expression studies by real-time q-PCR determining the level of RhoA, bax, caspase 3, caspase 8, and caspase 9. The experiment included triplicates of each trial and S.E. was calculated. The asterisk depicts the statistically significant difference in data compared with control plants at **P < 0.01 and ***P < 0.001. FeONPs= Fe_2O_3 NPs, Hr = *H. rhamnoides*, Ci= *C. intybus*

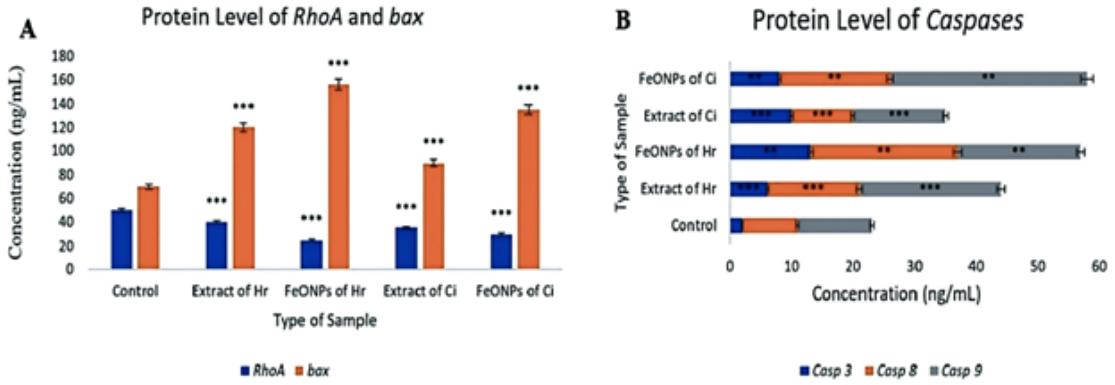


FIGURE 4.9: Determination of protein levels of RhoA, bax (A), and caspases (B) by ELISA. The experiment included triplicates of each trial and S.E. was calculated. The asterisk depicts the statistically significant difference in data compared with control plants at **P $<$ 0.01 and ***P $<$ 0.001. FeONPs= Fe_2O_3 NPs, Hr = *H. rhamnoides*, Ci= *C. intybus*

Objective 2: Screening of flavonoids found in the extracts of *H. rhamnoides* and *C. intybus* and to analyze the role of identified flavonoids of *C. intybus* and *H. rhamnoides* Buch against liver cancer target gene through computational approaches.

4.6 Quantitative Analysis of Polyphenols using HPLC

Using HPLC Chromatography, the methanolic extract of *H. rhamnoides* and *C. intybus* were studied. The HPLC profile of the investigated plant extract was examined using the standard polyphenols' retention period. The method relied on comparing the retention times of samples to those of standard retention times. The chromatogram (Figure 4.10) displays the standards that were utilized. This principle allowed for the identification of 12 polyphenols (Table 4.3), which included coumarin, gallic acid, quercetin, chlorogenic acid, HB acid, vanillic acid, sinapic acid, ferulic acid, rutin, kaempferol, and salicylic acid in the extract of *H. rhamnoides* (Figure 4.11). Furthermore, it was shown that salicylic acid had the third highest concentration (0.449 µg/mg) in the methanolic extract of *H. rhamnoides*, followed by coumarin (0.865 µg/mg). Chlorogenic acid had the highest

concentration in this extract (1.151µg/mg). Quercetin was the polyphenol with the lowest concentration (0.011 µg/mg) as shown in Figure 4.12. In a previous study to determine the phytochemical makeup of *H. rhamnoides* leaves and berries, many polyphenols were found. In comparison to the previous studies, some additional polyphenols as sinapic acid, and salicylic acid were also traced in the current study.

We can infer from the current and some earlier studies that the berries of *H. rhamnoides* have a high profile of polyphenols, which varies according to the climate, growth environment, and genetic background.

Likewise, there were 9 polyphenols (Table 4.4) identified in the extract of *C. intybus* these were gallic acid, quercetin, chlorogenic acid, HB acid, vanillic acid, benzoic acid, rutin, kaempferol, salicylic acid. These polyphenols as presented in chromatogram (Figure 4.13).

Moreover, the concentration was found for each component and it was found that the methanolic extract of *C. intybus* chlorogenic acid has the highest concentration (1.151µg /mg), The next concentration is of quercetin and then gallic acid is the third highest. The lowest concentration of the polyphenol was found in that of HB acid (Figure 4.14).

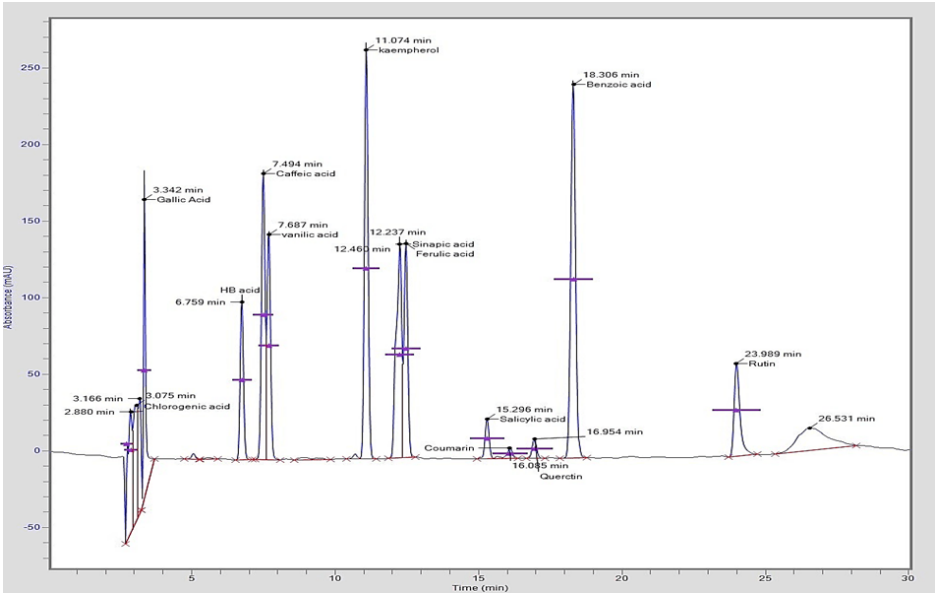


FIGURE 4.10: Chromatogram of the standard mixture used in HPLC Chromatography

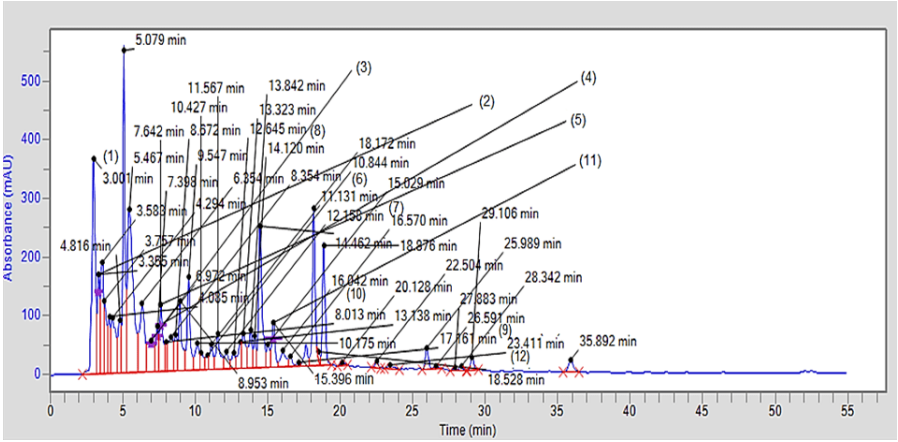


FIGURE 4.11: Chromatogram of the methanolic extract of *H. rhamnoides* at 275nm

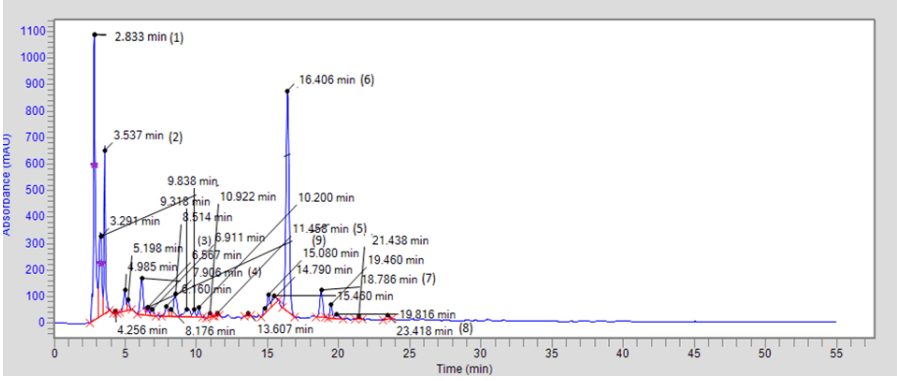


FIGURE 4.12: Chromatogram of the methanolic extract of *C. intybus* at 275 nm

TABLE 4.3: List of polyphenols found in methanolic extract of *H. rhamnoides* at 275 nm

S.#	Polyphenols found in ***H. rhamnoides***	Retention time (min)	Concentration µg /mg
1.	Chlorogenic acid	3.001	1.151
2.	Gallic acid	3.335	0.140
3.	Hb acid	6.972	0.057
4.	Caffeic acid	7.398	0.031
5.	Vanillic acid	7.642	0.084
6.	Kaempferol	11.131	0.215
7.	Sinapic acid	12.158	0.029
8.	Ferulic acid	12.645	0.022

Table 4.3 continued from previous page

S.#	Polyphenols found in *H. rhamnoides*	Retention time (min)	Concentration µg /mg
9.	Quercetin	17.161	0.011
10.	Salicylic acid	15.396	0.449
11.	Rutin	23.504	0.060
12	Coumarin	16.042	0.865

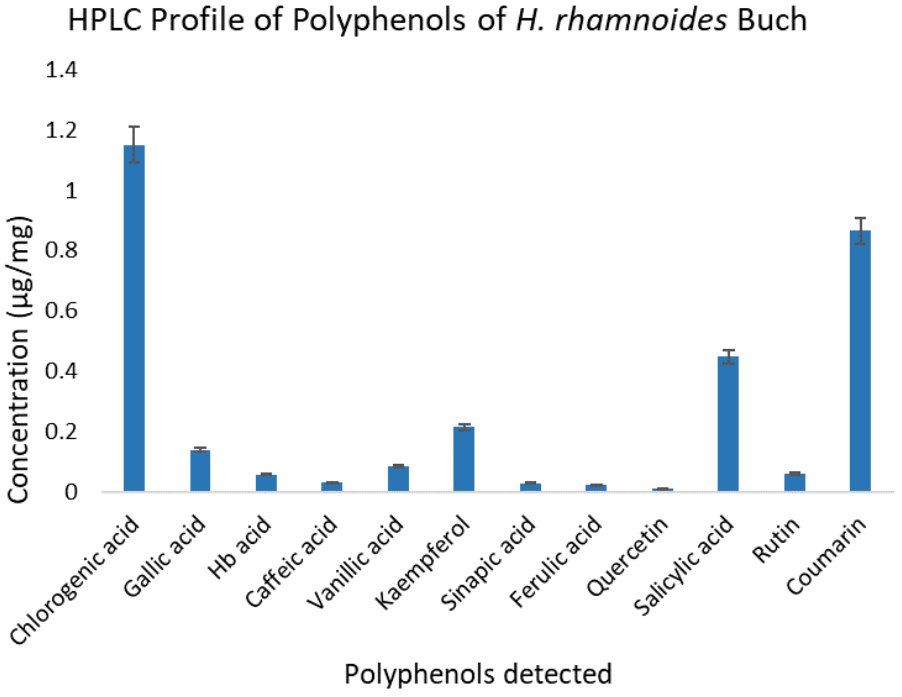


FIGURE 4.13: HPLC profile of polyphenols of *H. rhamnoides* methanolic extract

TABLE 4.4: List of polyphenols found in methanolic extract of *C. intybus* at 275nm

S.#	Polyphenols found in *C. intybus*	Retention time (min)	Concentration µg /mg
1.	Chlorogenic acid	2.833	1.8941
2.	Gallic acid	3.537	0.3358
3.	Hb acid	6.567	0.0155
4.	Quercetin	16.406	1.2312
5.	Vanillic acid	7.906	0.0233

Table 4.4 continued from previous page

S.#	Polyphenols found in *C. intybus*	Retention time (min)	Concentration µg /mg
6.	Kaempferol	11.458	0.0339
7.	Benzoic acid	18.786	0.0472
8.	Salicylic acid	15.086	0.2391
9.	Rutin	23.418	0.1040

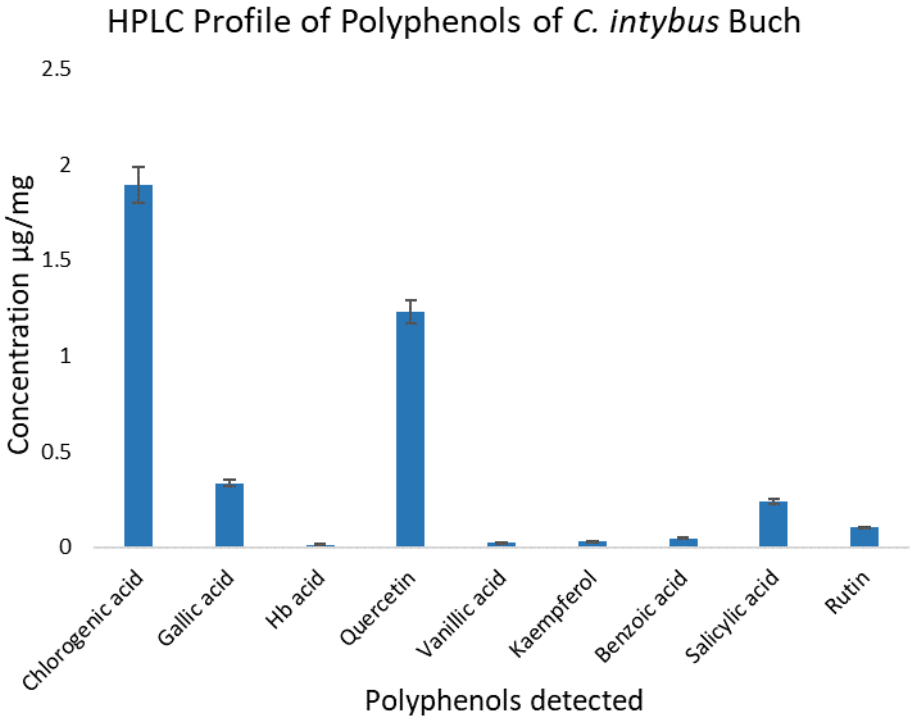


FIGURE 4.14: HPLC profile of polyphenols of *C. intybus* methanolic extract

4.7 In silico analysis using Molecular Docking

4.7.1 Protein Structure Analysis

The primary sequence of the protein RhoA was retrieved from the database uniport which has accession number P61586 and a residual length of 193 amino acids. An online tool ProtParam Expasy was used to study the physiochemical properties

of RhoA (selected proteins) as represented in Table 4.5. These properties include molecular weight, extension coefficient, instability index, positive and negative charged particles, theoretical PI, and GRAVY (grand average of hydrophobicity). Following is a primary sequence of the protein Rho A.
>sp | P61586 | RHOA_HUMAN Transforming protein RhoA OS = Hom sapiens OX = 9606 GN = RHOA PE = 1 SV = 1

TABLE 4.5: Physiochemical properties of selected protein RhoA studies using ProtParam tool

Physiochemical Properties	**Values**
Number of Amino Acids	193
Molecular Weight	5.83
Instability index (II)	51.73
Extension coefficient 1	18825
Extension coefficient 2	18450
Theoretical PI	5.83
Positively charged particles	29
Negatively charged particles	31
GRAVY	-0.368

4.7.2 Proteins 3D Structure Retrieval and Functional Domain Identification

The 3D structure of the RhoA protein was downloaded in pdb format (Figure 4.15A) with pdb id 4XH9 which was selected from the protein data bank (PDB). This protein molecule was used as a target molecule in docking with selected ligands. Rho GTPases are highly conserved GTPases and their dysregulation is associated with a range of abnormal cellular processes, targeting these proteins can minimize the severity of cancer progression. The structure of the target protein (RhoA) was visualized by using Pymol software as shown in Figure 4.15(B). Interpro an online database was used to identify the functional domains of RhoA protein. Conserved domains are involved in sequence/structure/relationship. This

protein contains mostly small GTP-binding domains (IPR005225 and TIGR00231) and other domains of small GTPase Rho family profile (PS51420) as shown in Figure 4.16.

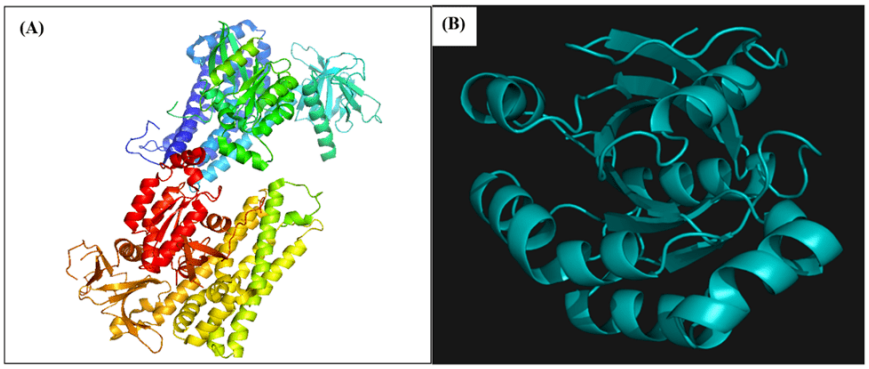


FIGURE 4.15: (A) 3D structure of RhoA protein retrieved from PDB. (B) RhoA protein visualized by Pymol.

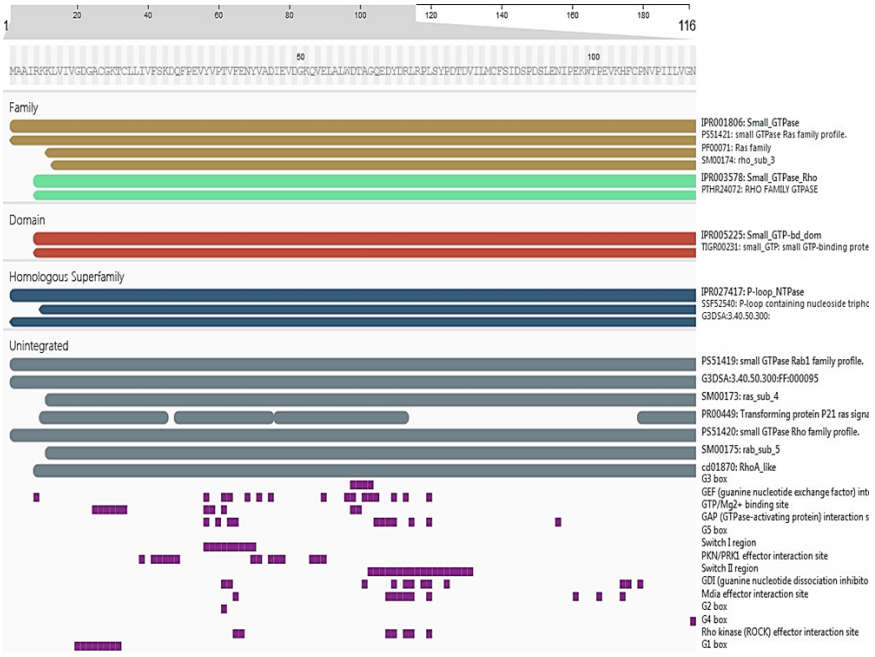


FIGURE 4.16: Rho A protein with functional domain identified using Interpro

4.7.3 Preparation of Ligands for Molecular Docking

The 2D structure and associated data for the ligands found in the plant extract of *H. rhamnoides* by HPLC were acquired from the PubChem database and are shown in Table 4.6 and the plant extract of *C. intybus* is shown in Table 4.7. The following stage involved minimizing the ligand's energy, which is crucial for getting

the ligand ready for docking because unstable ligands might have an impact on their vina scores. Therefore, ChemPro software (chem12) was utilized to minimize ligand energy.

TABLE 4.6: Identified Ligands of *H. rhamnoides* and related information

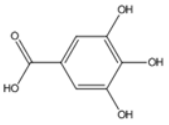
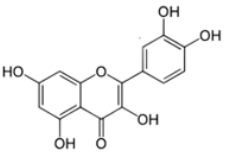
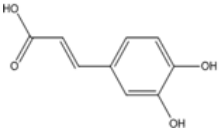
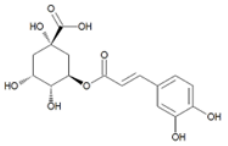
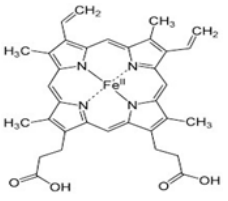
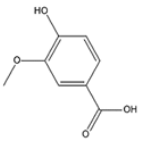
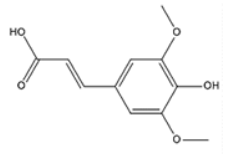
S. No.	**Ligands**	**CID no. of ligands**	**Molecular formula**	**Molecular weight**	**2D structure of ligand**
1	Gallic acid	370	$C_7H_6O_5$	170.12	
2	Quercetin	5280343	$C_{15}H_{10}O_7$	302.238	
3	Caffeic acid	689043	$C_9H_8O_4$	180.159	
4	Chlorogenic acid	1794427	$C_{16}H_{18}O_9$	354.311	
5	HB acid		Hb	614.16	
6	Vanillic acid	8468	$C_8H_8O_4$	168.148	
7	Sinapic acid	637775	$C_{11}H_{12}O_5$	224.212	

Table 4.6 continued from previous page

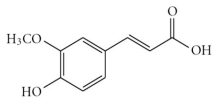
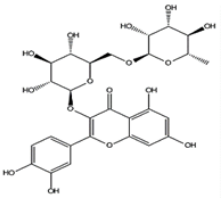
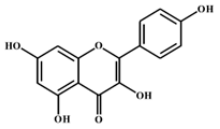
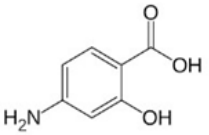
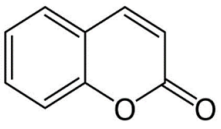
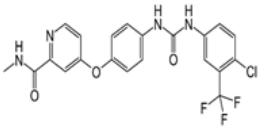
S. No.	Ligands	CID no. of ligands	Molecular formula	Molecular weight	2D structure of ligand
8	Ferulic acid	445858	$C_{10}H_{10}O_4$	184.186	
9	Rutin	5280805	$C_{27}H_{30}O_{16}$	610.521	
10	Kaempferol	5280863	$C_{15}H_{10}O_6$	286.239	
11	Salicylic acid	338	$C_7H_6O_3$	138.122	
12	Coumarin	323	$C_9H_6O_2$	146.145	
13	Sorafenib	216239	$C_{21}H_{16}CIF_3N_4O_3$	464.831	

TABLE 4.7: Identified Ligands of *C. intybus* and related information

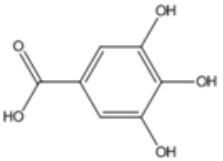
S. No.	Ligands	CID no. of ligands	Molecular formula	Molecular weight	2D structure of ligand
1	Gallic acid	370	$C_7H_6O_5$	170.12	

Table 4.7 continued from previous page

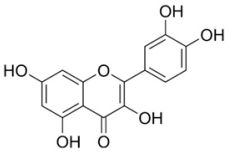
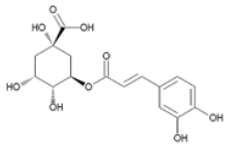
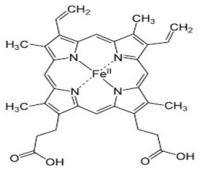
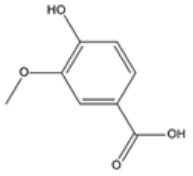
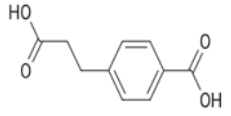
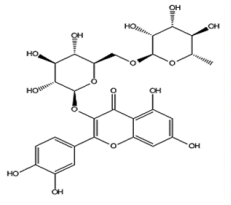
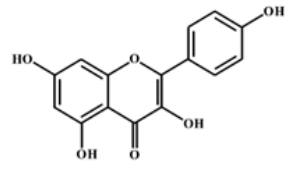
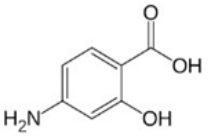
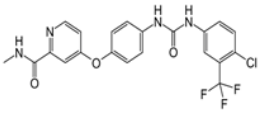
S. No.	Ligands	CID no. of ligands	Molecular formula	Molecular weight	2D structure of ligand
2	Quercetin	5280343	$C_{15}H_{10}O_7$	302.238	
3	Chlorogenic acid	1794427	$C_{16}H_{18}O_9$	354.311	
4	HB acid		Hb	614.16	
5	Vanillic acid	8468	$C_8H_8O_4$	168.148	
6	Benzoic acid	243	C_6H_5COOH	184.186	
7	Rutin	5280805	$C_{27}H_{30}O_{16}$	610.521	
8	Kaempferol	5280863	$C_{15}H_{10}O_6$	286.239	

Table 4.7 continued from previous page

S. No.	Ligands	CID no. of ligands	Molecular formula	Molecular weight	Molecular 2D structure of ligand
9	Salicylic acid	338	$C_7H_6O_3$	138.122	
10	Sorafenib	216239	$C_{21}H_{16}CIF_3N_4O_3$	464.831	

4.7.4 Molecular Docking

Finding the ideal conformational connection between target proteins and ligands was the main goal of molecular docking. The online docking tool CB-Dock was utilized for this investigation. This tool provided a 3D visualization of the results in five distinct positions (Figure 4.17).

Thus, as seen in Figure 4.17, the optimal stance with the lowest vina score was chosen. Taking into account many factors such as the optimal vena score, cavity size, grid map score, and the Lipinski rule of five (Table 4.8), the ligands quercetin, gallic acid, kaempferol, salicylic acid, and caffeic acid were chosen from the *H. rhamnoides* extract. Sorafenib, a synthetic drug used to treat liver cancer was also employed to compare the ligand's interaction with the target protein. Furthermore, no. of hydrogen bonds and hydrophobic interactions were also determined (Table 4.9). Likewise, there were 9 ligands identified in the methanolic extract *C. intybus* and 5 ligands were selected for molecular docking considering some of the features such as best vena score, cavity size grid map score and Lipinski rule of five (Table 4.8). These ligands were quercetin, chlorogenic acid, kaempferol, salicylic acid and gallic acid. For the comparison with the interaction of ligands, we also used a synthetic drug for liver cancer called sorafenib. The same online docking tool CB-Dock was utilized for this investigation. This tool

provided a 3D visualization of the results in five distinct positions (Figure 4.18). Thus, as seen in Figure 4.18, the optimal stance with the lowest vina score was chosen. Furthermore, no. of hydrogen bonds and hydrophobic interactions were also determined (Table 4.10).

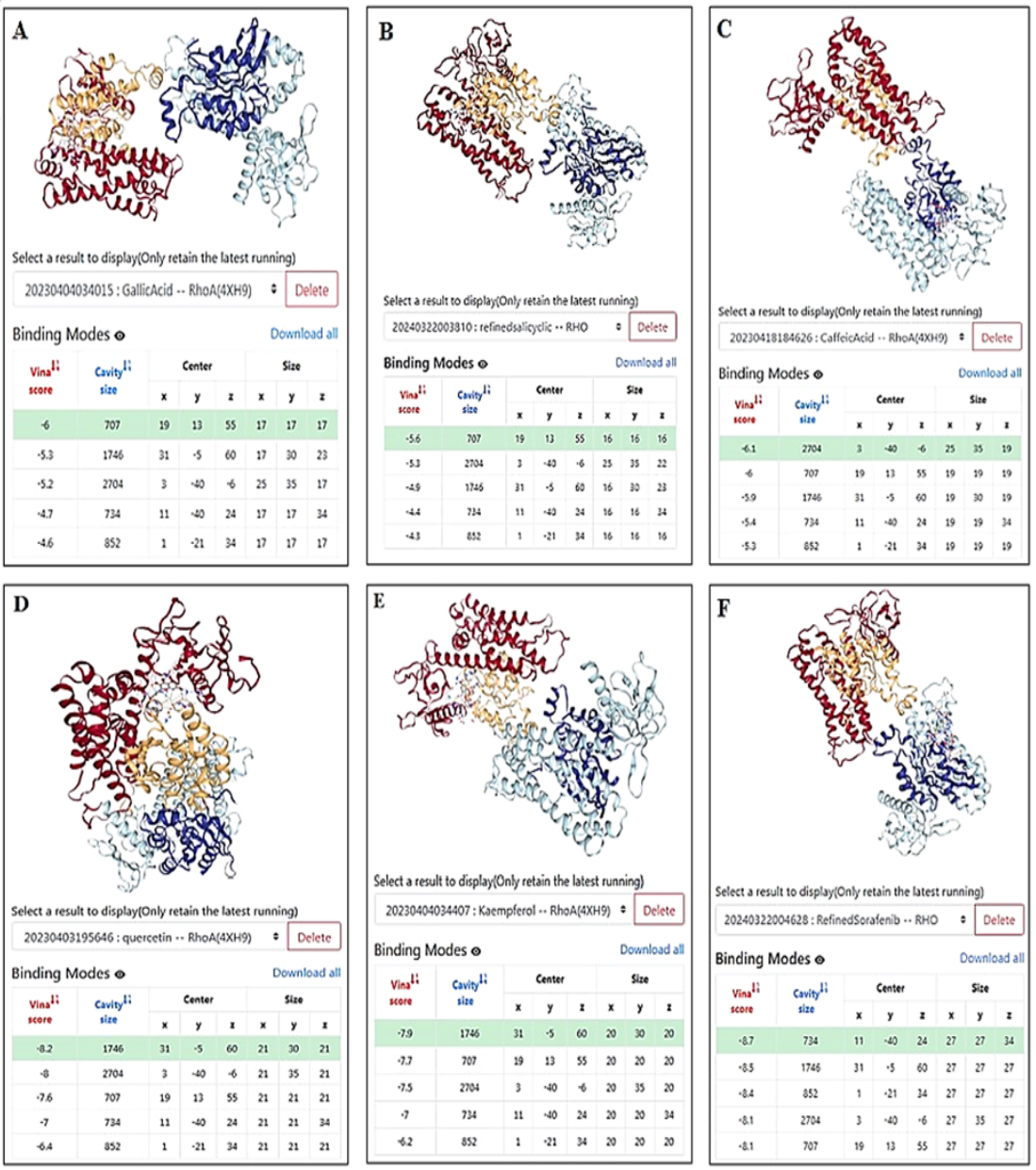


FIGURE 4.17: Best 3D visualization conformational poses of the interaction between the target protein and selected ligands from *H. rhamnoides*. A represents the interaction between gallic acid and RhoA, Likewise, B is salicylic acid and RhoA, C is the interaction between caffeic acid and RhoA, D is the interaction between quercetin and RhoA, E is the interaction between kaempferol and RhoA, and F is the interaction between sorafenib and RhoA

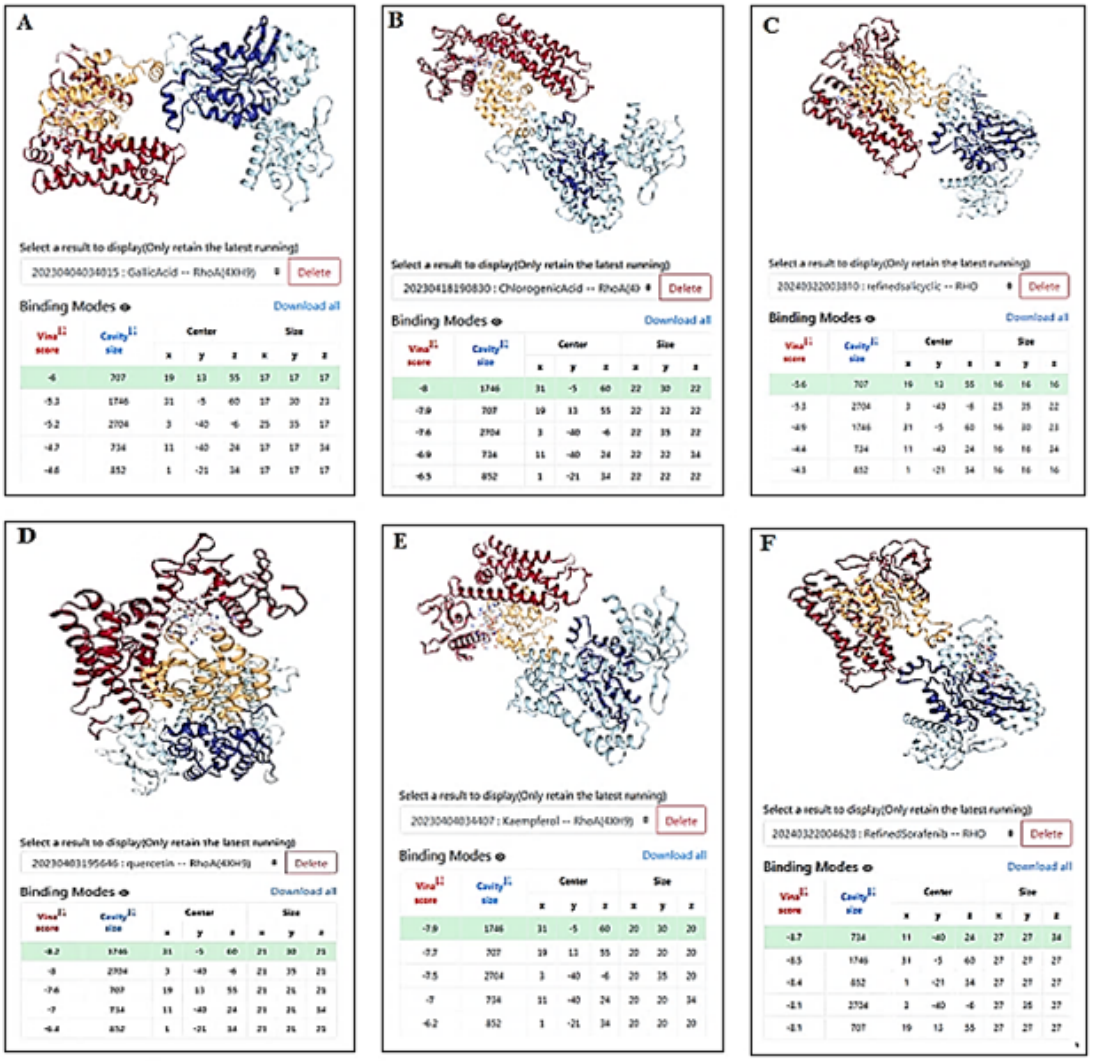


FIGURE 4.18: Best 3D visualization conformational poses of the interaction between the target protein and selected ligands from *C. intybus*. A represents the interaction between gallic acid and RhoA. Likewise, B is chlorogenic acid and RhoA, C is the interaction between salicylic acid and RhoA, D is the interaction between quercetin and RhoA, E is the interaction between kaempferol and RhoA, and F is the interaction between sorafenib and RhoA.

TABLE 4.8: Molecular docking outcomes of ligands (*H. rhamnoides* and *C. intybus*) with target protein.

S. No.	**Ligands**	**Vina Score**	**Cavity Size**	**Molecular Weight g/ mol**	**Grid Map**	**Max Energy Kcal/mol**	**Min Energy Kcal /mol**
01	Gallic acid	-6	707	170.12	55	-3.4919	-4.3164
02	Quercetin	-8.2	1746	302.238	60	14.9427	5.3551
03	Caffeic acid	-6.1	2704	180.159	35	1.9678	-4.2209
04	Chlorogenic acid	-8	1746	354.311	60	22.8823	9.8942
05	Vanillic acid	-5.8	2704	168.148	55	9.6551	3.8656

Table 4.8 continued from previous page

S. No.	Ligands	Vina Score	Cavity Size	Molecular Weight g/ mol	Grid Map	Max Energy Kcal/mol	Min Energy Kcal /mol
06	Coumarin	-5.6	707	146.145	60	9.5426	5.150
07	Sinapic acid	-5.6	2707	224.212	35	16.2128	3.8815
08	Ferulic acid	-5.8	1746	184.186	60	14.4263	5.4451
09	Salicylic acid	-5.6	707	138.122	60	5.6109	2.9688
10	Rutin	-9.7	2704	610.521	35	39.5842	-2.9537
11	Kaempferol	-7.9	1746	286.239	60	21.7643	6.4005
12	HB acid	-9.3	2704	614.16	24	70.3460	9.4645
13	Benzoic acid	-5.2	707	614.483	60	3.4983	1.1832
14	Sorafenib	-8.7	734	464.831	60	5.2519	-20.3147

TABLE 4.9: Results of the interaction of target protein, selected ligands of *H. rhamnoides* and synthetic drug Sorafenib using Ligplot Plus software

S. No	Ligands and drug	Binding energy	Number of hydrogen bonds	Amino acids	HBS distance	Hydrophobic interactions
1	Salicylic acid	-5.6	4	3	3.07	Glu54
					2.74	Arg5
					3.3	Lys7
					2.93	Leu294
						Leu302
						Lys301
						Val43
2	Quercetin	-8.2	6	5	3.03	Leu72
					3.05	Pro71
					2.98	Gly391
					2.7	Phe 106
					3.3	His 390
					3.12	
3	Gallic acid	-6	4	3	3.06	Lys301
					2.94	Ala56
					3.04	Leu294

Table 4.9 continued from previous page

S. No	Ligands and drug	Binding energy	Number of hydrogen bonds	Amino acids	HBS distance	Hydrophobic interactions
					2.88	Leu302
						Arg5
						Lys6
						Lys7
4	Kaempferol	-7.9	3	3	2.8	Gln490
					2.81	Pro101
					3.15	Glu102
						Phe106
						Asp67
						Arg68
						Leu388
						Lys98
						Glu97
5	Caffeic acid		3	3	2.89	Arg68
					2.91	Leu387
					2.99	Cys 389
						Leu388
						His 390
						Arg 70
						Asp67
						Phe106
6	Sorafenib	-8.7	1	2	2.99	Glu181
					3.07	Phe39
					2.99	Val35
						Pro36
						Lys164
						Val124
						Glu32
						Pro31
						Ser 26
						Val38

TABLE 4.10: Results of the interaction of target protein, selected ligands of *C. intybus* and synthetic drug sorafenib using Ligplot Plus software.

S. No	**Ligands and drug**	**Binding energy**	**Number of hydrogen bonds**	**Amino acids**	**HBS distance**	**Hydrophobic interactions**
1	Chlorogenic acid	-8	7	5	2.95	Asp67
					2.94	Pro71
					3.19	Glu361
					3.04	Arg68
					2.95	Phe 106
					2.84	Leu271
					3.14	Gly391
						His 105
						Cys389
						Lys367
2	Quercetin	-8.2	6	5	3.03	Leu72
					3.05	Pro71
					2.98	Gly391
					2.7	Phe 106
					3.3	His 390
					3.12	
3	Gallic acid	-6	4	3	3.06	Lys301
					2.94	Ala56
					3.04	Leu294
					2.88	Leu302
						Arg5
						Lys6
						Lys7
4	Kaempferol	-7.9	3	3	2.8	Gln490
					2.81	Pro101
					3.15	Glu102
						Phe106
						Asp67
						Arg68
						Leu388

Table 4.10 continued from previous page

S. No	Ligands and drug	Binding energy	Number of hydrogen bonds	Amino acids	HBS distance	Hydrophobic interactions
						Lys98
						Glu97
5	Salicylic acid	-5.6	4	3	3.07	Glu54
					2.74	Arg5
					3.3	Lys7
					2.93	Leu294
						Leu302
						Lys301
						Val43
6	Sorafenib	-8.7	1	2	2.99	Glu181
					3.07	Phe39
					2.99	Val35
						Pro36
						Lys164
						Val124
						Glu32
						Pro31
						Ser 26
						Val38

4.7.5 Use of Ligplot+ Software for Analysis of Docked Complexes

Ligplot + software was used for the analysis of docked complexes, which automatically generated a 2D representation of complex interfaces from the given PDB file input. As a result, we got an informative representation of the interactions (intermolecular) which includes H- bonds and hydrophobic interactions. H-bonds and hydrophobic contacts are depicted in Figures 4.19 and 4.20 for the selected complexes (*H. rhamnoides* and *C. intybus*). Considering diagrams H- bonds are shown in dashed lines, whereas hydrophobic contacts are represented with red arches with spikes.

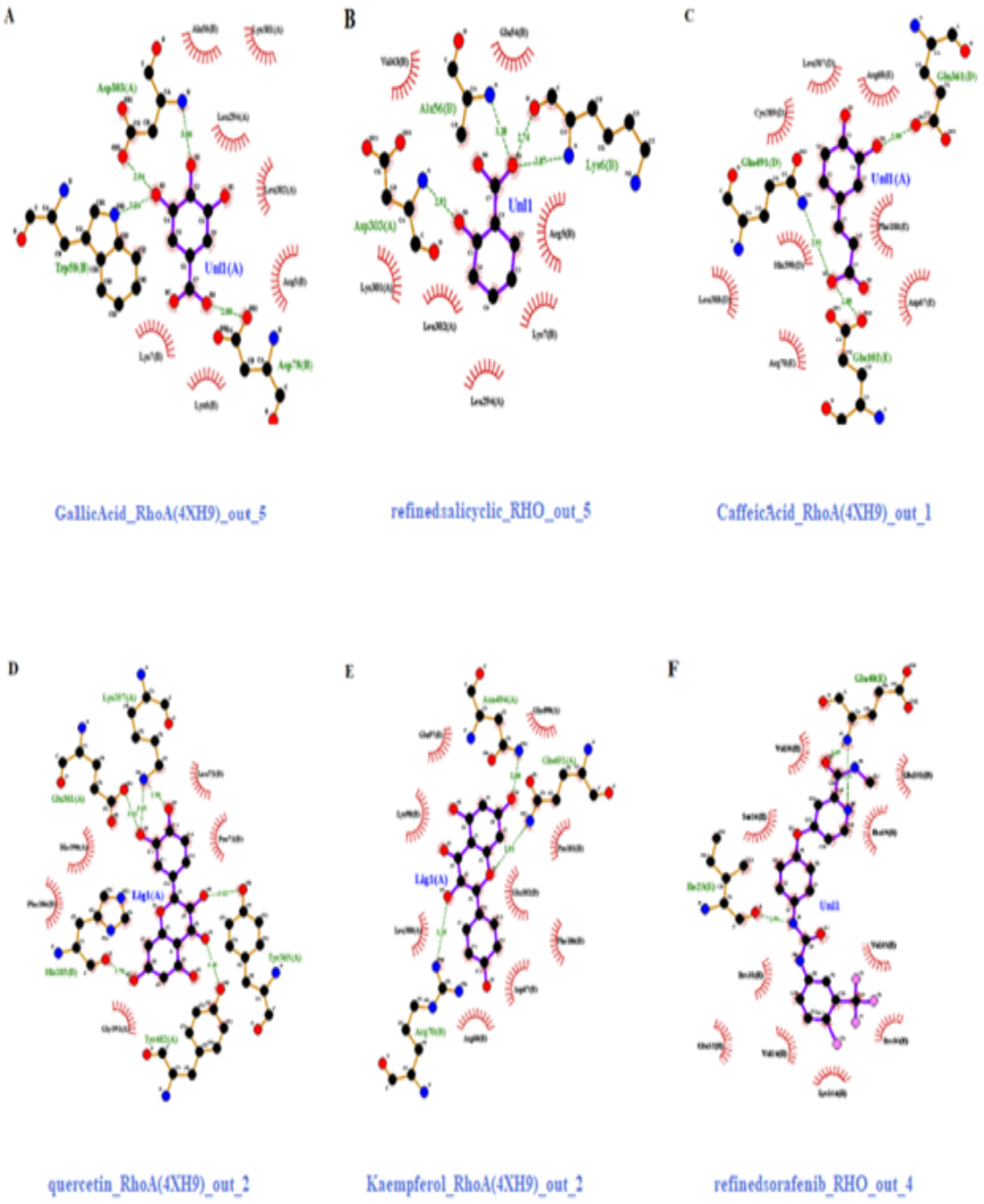


FIGURE 4.19: The Ligplot plus interaction results of best-docked ligands of H. rhamnoides with the target protein RhoA. The hydrogen bonds are presented with dashed lines in green colour, bond distance and hydrophobic interactions were represented as spiked red arches. These binding modes in A represent the image of the interaction between gallic acid and RhoA. Likewise, B, C and D are the interaction of salicylic acid, caffeic acid, quercetin and kaempferol while E depicts the interaction of the synthetic drug sorafenib with RhoA

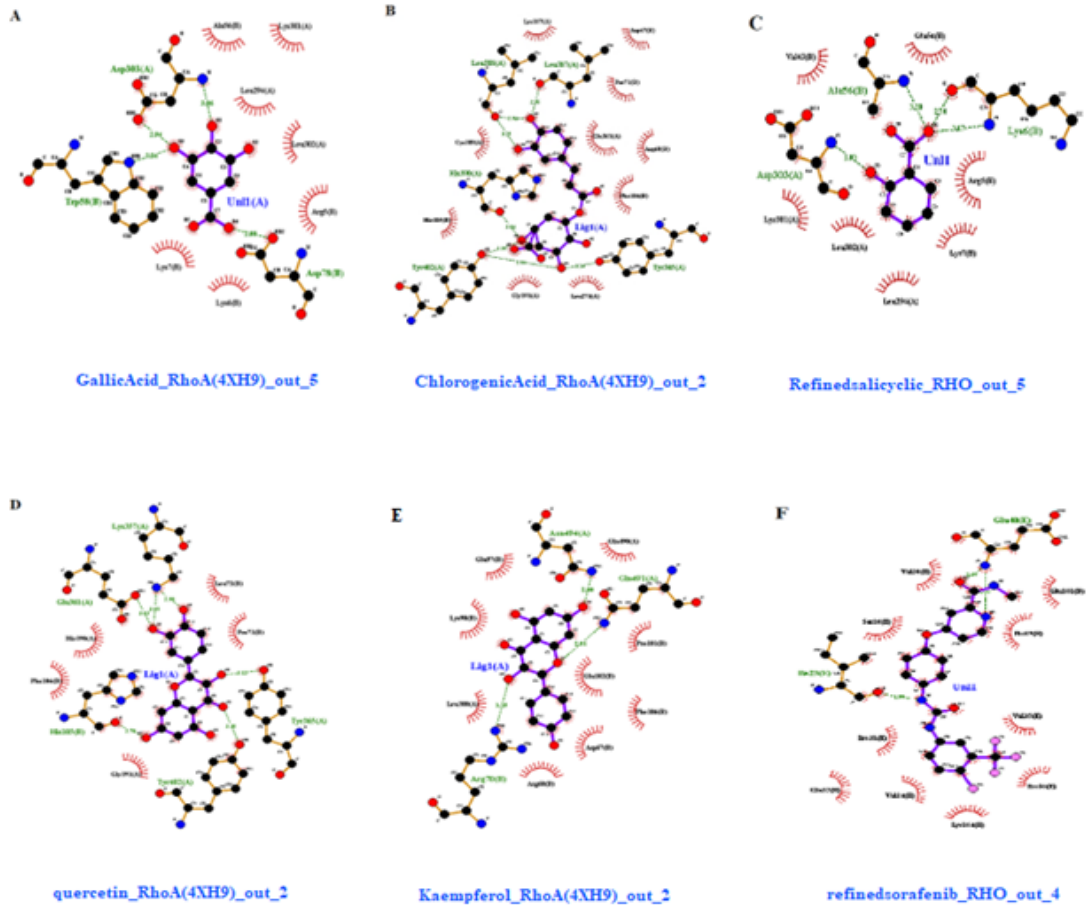


FIGURE 4.20: The ligplot plus interaction results of the best-docked ligand of C. intybus with the target protein RhoA. The hydrogen bonds are presented with dashed lines in green colour, bond distance and hydrophobic interactions were represented as spiked red arches. These binding modes in A represent the image of the interaction between gallic acid and RhoA, Likewise B, C, and D represent the interaction of chlorogenic acid, salicylic acid, quercetin and kaempferol while E depicts the interaction of the synthetic drug sorafenib with RhoA

4.7.6 Evaluating Molecular Docking through RMSD

Next, UCSF Chimaera software was used to examine the RMSD (root-mean-square deviation) values in the docking results (Figure 4.21 & Table 4.11). The RMSD values that are expected from interaction outcomes were used to quantify the system's deviation from its initial conformation and to analyze variations in docking postures. The average distance between the atoms in a protein and ligand structure that are overlaid was determined using RMSD. All of the poses were regarded in the current study as the ideal pose for illustrating the RMSD rules. The optimal positions for the salicylic acid, quercetin, gallic acid, kaempferol, and

caffeic acid from *H. rhamnoides* are shown in Table 4.11 with poses 2, 3, 3, 4, and 2 respectively. Additionally, the root-mean-square fluctuation, or RMSF, gave the average residual deviations and offered information on the protein's flexibility. It was believed that the values for RMSD couldn't be more than 4. An appropriate range for globular proteins is between 1 and 3A^o.

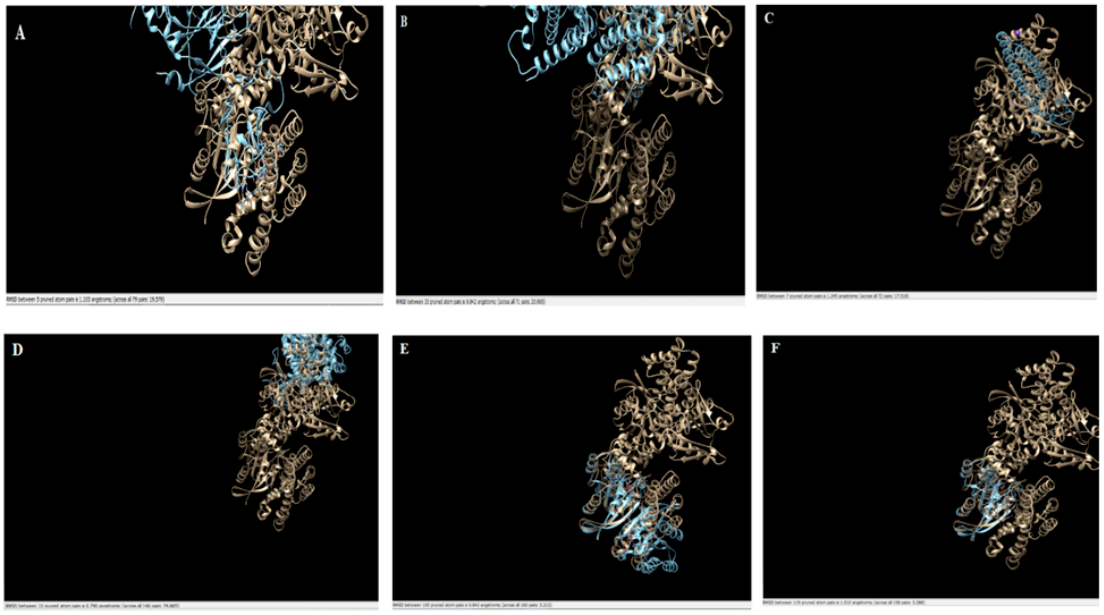


FIGURE 4.21: The best poses for RMSD values of selected ligands of *H. rhamnoides* using the software UCSF Chimera. Image A is the best pose for the RMSD value of gallic acid interaction with RhoA. Likewise, images B, C, D, and E, depict, the best poses for the salicylic acid, caffeic acid, quercetin, and kaempferol and image F represents the interaction of the synthetic drug sorafenib

TABLE 4.11: RMSD values of ligands of *H. rhamnoides* for 5 poses selected for docking

S. No.	**Ligands selected**	**Vina Score**	**RMSD Value (Pose 1)**	**RMSD Value (Pose 2)**	**RMSD Value (Pose 3)**	**RMSD Value (Pose 4)**	**RMSD Value (Pose 5)**
1	Salicylic acid	-5.6	1.304	0.842	1.034	1.096	0.988
2	Quercetin	-8.2	0.942	0.938	0.790	1.112	1.582
3	Gallic acid	-6.0	1.218	1.245	1.103	1.450	1.209
4	Kaempferol	-7.9	0.898	1.020	1.346	0.842	0.947
5	Caffeic acid	-6.1	0.971	0.750	1.286	0.939	1.128
6	Sorafenib	-8.8	1.010	1.107	0.986	0.874	0.996

Likewise, the ideal poses for selected ligands from *C. intybus* using the software UCSF Chimera as for salicylic acid, quercetin, gallic acid, kaempferol, and chlorogenic acid are shown in Figure 4.22. The finest pose for salicylic acid is pose 2, for quercetin its pose 3, likewise for the compound gallic acid the best pose is pose 3, for kaempferol the finest pose is pose 4 and for chlorogenic acid the best pose is pose 5 as depicted in Table 4.12.

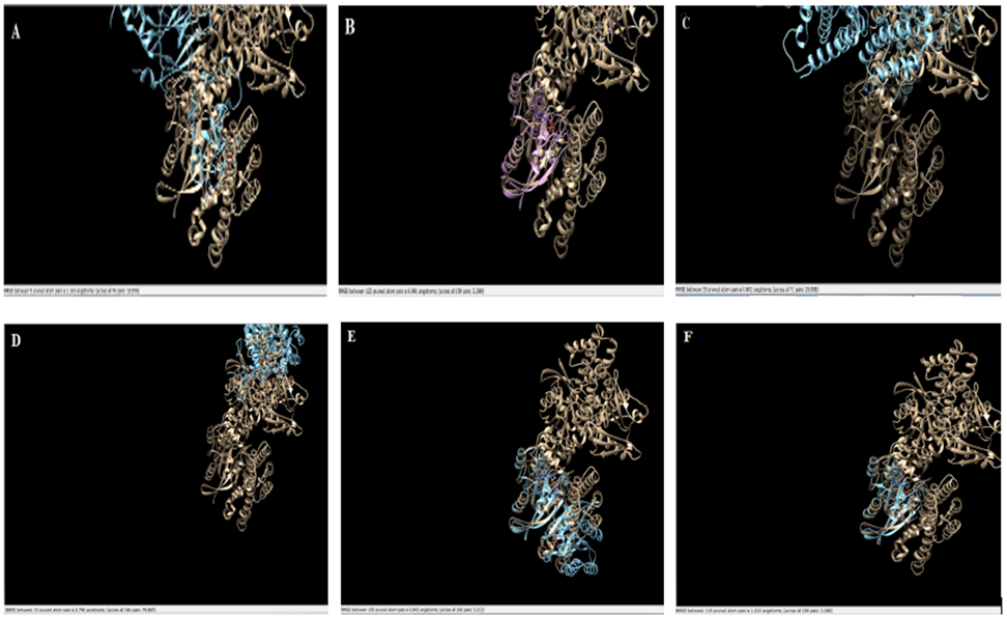


FIGURE 4.22: The best poses for RMSD values of selected ligands *C. intybus* using the software UCSF Chimera. Image A is the best pose for the RMSD value of gallic acid interaction with RhoA. Likewise, images B, C, D, and E, depict, the best poses for the compounds chlorogenic acid, salicylic acid, quercetin, and kaempferol and image F represents the interaction of the synthetic drug sorafenib.

TABLE 4.12: RMSD values of ligands of *C. intybus* for 5 poses selected for docking

S. No.	**Ligands selected**	**Vina Score**	**RMSD Value (Pose 1)**	**RMSD Value (Pose 2)**	**RMSD Value (pose 3)**	**RSMD Value (pose 4)**	**RSMD Value (Pose 5)**
1	Salicylic acid	-5.6	1.304	0.842	1.034	1.096	0.988
2	Quercetin	-8.2	0.942	0.938	0.790	1.112	1.582
3	Gallic acid	-6	1.218	1.245	1.103	1.450	1.209
4	Kaempferol	-7.9	0.898	1.020	1.346	0.842	0.947
5	Chlorogenic acid	-8	1.200	1.109	1.152	1.233	0.991
6	Sorafenib	-8.8	1.010	1.107	0.986	0.874	0.996

4.7.7 Bioactivity Analysis of Ligands and ADMET Properties Measurement

Five ligands and one standard medication were chosen from the drug Bank database in order to assess the characteristics of ADMET and analyze the bioactivity of the ligands (*H. rhamnoides*) as indicated in Table 4.13. Because of this, it's critical that selected ligands adhere to the Lipinski Rule of Five, which assesses the possibility that ligands will eventually function as an oral medication in humans. This rule states that the compounds that are chosen must fulfil certain requirements, the majority of which require the log P-value to be less than 5.

Other requirements of this rule are that the molecular weight of the specific chemical must be less than 500 Daltons and that there cannot be more than five hydrogen bond givers, and that there cannot be more than ten hydrogen acceptors. The ligands that meet the requirements of the five Lipinski rules are more likely to be more readily absorbed and bioavailable by the human body.

TABLE 4.13: ADMET properties of selected ligands of *H. rhamnoides* and synthetic drug sorafenib.

Drug likeliness properties	**Ligands and drug**					
	Gallic acid	Salicylic acid	Caffeic acid	Quercetin	Kaempferol	Sorafenib
Log P-value	0.5016	1.09	1.1956	1.988	2.2824	5.5497
Molecular weight	170.12	138.122	180.159	302.238	286.239	464.831
Hydrogen bond acceptor	4	2	3	7	6	4
Hydrogen bond donor	4	2	3	5	4	3
Bond (Rotatable)	1	1	2	1	1	5
Surface area	67.135	57.545	74.381	122.108	117.313	185.111
Water solubility	-2.56	-1.808	-2.33	-2.925	-3.04	-4.442
$CaCO_2$ Permeability	-0.081	-1.151	0.634	0.925	0.032	0.278
Intestinal absorption (Human)	43.374	83.887	69.407	77.207	74.29	83.628
Skin permeability	-2.735	-2.723	-2.722	-2.735	-2.735	-3.037

Table 4.13 continued from previous page

Drug likeliness properties	**Ligands and drug**					
	Gallic acid	Salicylic acid	Caffeic acid	Quercetin	Kaempferol	Sorafenib
P-glycoprotein substrate	No	No	No	Yes	Yes	Yes
P- glycoprotein I inhibitor	No	No	No	No	No	Yes
P- glycoprotein II inhibitor	No	No	No	No	No	No
VDss (Human)	-1.855	-1.57	-1.098	1.559	1.274	0.199
Fraction unbound (human)	0.617	0.563	0.529	0.206	0.178	0.059
CNS Permeability	-3.74	-3.21	-2.608	-3.065	-2.228	-2.007
BBB permeability	-1.102	-0.334	0.647	-1.098	-0.939	-1.684
CYP2D6 substrate	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No	Yes
CYP1A2 Inhibitors	No	No	No	Yes	Yes	Yes
CYP2C19 inhibitor	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	Yes
CYP2D6 inhibitor	No	No	No	No	No	Yes
CYP3A4 inhibitor	No	No	No	No	No	Yes
Total clearance	0.718	0.607	0.508	0.407	0.477	-0.219
Renal OCT2	No	No	No	No	No	No
AMES Toxicity	No	No	No	No	No	No
Max tolerated Dose (Human)	0.7	0.61	1.141	0.499	0.531	0.549
Herg I inhibitor	No	No	No	No	No	No
Herg II inhibitors	No	No	No	No	No	Yes
Oral Rat acute toxicity (LD50)	2.218	2.282	2.383	2.471	2.449	2.788
Oral Rat chronic toxicity (LOAEL)	3.06	2.483	2.092	2.612	2.505	1.198
Hepatotoxicity	No	No	No	No	No	Yes
Skin sensitization	No	No	No	No	No	Yes

Table 4.13 continued from previous page

Drug likeliness properties	**Ligands and drug**					
	Gallic acid	Salicylic acid	Caffeic acid	Quercetin	Kaempferol	Sorafenib
Minnow toxicity	3.188	1.812	2.246	3.721	2.885	0.189

The ligands selected were further studied for their pharmacokinetic properties (ADMET). These properties include absorption, distribution, metabolism, excretion, and toxicity which play an essential role in the selection of a compound as a drug candidate.

TABLE 4.14: ADMET properties of selected ligands of *C. intybus* and synthetic drug sorafenib.

Drug likeliness properties	**Ligands and drug**					
	Gallic acid	Salicylic acid	Quercetin	Kaempferol	Chlorogenic acid	Sorafenib
Log P-value	0.5016	1.09	1.988	2.2824	-0.6459	5.5497
Molecular weight	170.12	138.122	302.238	286.239	354.311	464.831
Hydrogen bond acceptor	4	2	7	6	8	4
Hydrogen bond donor	4	2	5	4	6	3
Bond (Rotatable)	1	1	1	1	4	5
Surface area	67.135	57.545	122.108	117.313	141.587	185.111
Water solubility	-2.56	-1.808	-2.925	-3.04	-2.449	-4.442
$CaCO_2$ Permeability	-0.081	1.151	0.925	0.032	-0.84	0.278
Intestinal absorption (Human)	43.374	83.887	77.207	74.29	36.377	83.628
Skin permeability	-2.735	-2.723	-2.735	-2.735	-2.735	-3.037
P-glycoprotein substrate	No	No	Yes	Yes	Yes	Yes
P- glycoprotein I inhibitor	No	No	No	No	No	Yes
P- glycoprotein II inhibitor	No	No	No	No	No	NO
VDss (Human)	-1.855	-1.57	1.559	1.274	0.581	0.199

Table 4.14 continued from previous page

Drug likeliness properties	**Ligands and drug**					
	Gallic acid	Salicylic acid	Quercetin	Kaempferol	Chlorogenic acid	Sorafenib
Fraction unbound (human)	0.617	0.563	0.206	0.178	0.658	0.059
CNS Permeability	-3.74	-3.21	-3.065	-2.228	-3.856	-2.007
BBB permeability	-1.102	-0.334	-1.098	-0.939	-1.407	–1.684
CYP2D6 substrate	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No	Yes
CYP1A2 Inhibitors	No	No	Yes	Yes	No	Yes
CYP2C19 inhibitor	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	Yes
CYP2D6 inhibitor	No	No	No	No	No	Yes
CYP3A4 inhibitor	No	No	No	No	No	Yes
Total clearance	0.718	0.607	0.407	0.477	0.307	-0.219
Renal OCT2 substrate	No	No	No	No	No	NO
AMES Toxicity	No	No	No	No	No	No
Max tolerated Dose (Human)	0.7	0.61	0.499	0.531	-0.134	0.549
Herg I inhibitor	No	N0	No	No	No	No
Herg II inhibitors	No	No	No	No	No	YES
Oral Rat acute toxicity (LD50)	2.218	2.282	2.471	2.449	1.982	2.788
Oral Rat chronic toxicity (LOAEL)	3.06	2.483	2.612	2.505	2.982	1.198
Hepatotoxicity	No	No	No	No	No	Yes
Skin sensitization	No	No	No	No	No	Yes
Minnow toxicity	3.188	1.812	3.721	2.885	5.741	0.189

The pharmacokinetic characteristics of the chosen ligands were investigated in more detail (ADMET). These characteristics like distribution, metabolism, excretion, absorption, and toxicity are crucial in determining if a molecule is a good

candidate for a medication. The PKCSM tool, which helps determine the pharmacokinetic characteristics of the ligands selected as therapeutic candidates, was utilized to investigate the ADMET features. Taking into account absorption properties (Table 4.13), which showed variations in the drug's and the chosen ligands' water solubility, the reference drug was less soluble in water than the other five selected ligands. Salicylic acid, caffeic acid, and gallic acid are the chemicals that were chosen because they are slightly more soluble in water than the other ligands. Some selected ligands had slightly higher $CaCO_2$ permeability than the synthetic drug when taking into account the absorption feature. Quercetin and caffeic acid had a higher $CaCO_2$ permeability than any other lead molecule. While caffeic acid is not a P-gp substrate and is not an inhibitor of either P-gp I or P-gp II, the synthetic medication sorafenib is a P-gp substrate, P-gp I inhibitor, and non-inhibitor of P-gp II. Similarly, quercetin is a substrate of P-gp and does not inhibit P-gp I or II. The remaining chosen lead compounds are all non-inhibitors of P-gp I and II, with the exception of kaempferol, which is a P-gp substrate.

When looking at the distribution properties, we find that the CNS permeability of sorafenib is in the region of -2, whereas other selected ligands such as caffeic acid have CNS permeabilities in the range of -2 and salicylic acid, quercetin, and gallic acid have CNS permeabilities in the range of -3. For other selected ligands as kaempferol, the range of CNS permeability is also -2. Similarly, while examining the unbound friction property in human plasma, several substances were shown to have a greater value than manufactured drugs. Notably, this was especially true for caffeic acid, gallic acid, and salicylic acid. This indicates traditional ligands such as salicylic acid, gallic acid, and caffeic acid are more effective than synthesized drugs. Furthermore, one of the key aspects in the investigation of ADMET properties is drug metabolism. Both the synthetic medication sorafenib and the selected ligands salicylic acid, kaempferol, quercetin, chlorogenic acid, and caffeic acid were not confirmed to be CYP2D6 substrates.

It is crucial to understand a drug's complete clearance to calculate dosage rates. Comparing our results to the reference drug, we found that there is a high overall clearance of quercetin, kaempferol, gallic acid, salicylic acid, and caffeic acid.

The Renal OCT2 substrate characteristic was absent from all of the chosen compounds. Similarly, we can determine the suggested medicine dose's tolerance limit by understanding the toxicity of a selected ligand.

The chemicals that were chosen for this study, such as gallic acid and caffeic acid, were determined to be far safer for humans than synthetic drugs and the other two ligands that were chosen. While the reference drug sorafenib itself shows Herg II inhibitor properties, all the chosen drugs fall into the category of No for Herg I and Herg II inhibitors.

The reference medicine has a higher oral rat acute toxicity property than the other chosen ligands, such as gallic acid, salicylic acid, and caffeic acid. The chosen ligands have a greater value (1.198) for oral rat chronic toxicity than sorafenib.

This indicates that the synthetic drug is more harmful than other particular combinations. The feature of hepatotoxicity was employed to investigate the toxic effect on the liver since certain substances, such as quercetin, gallic acid, and caffeic acid, do not have any toxic effects.

However, the reference medication falls into the category of yes, which indicates that it can demonstrate liver toxicity. All of the selected ligands have not exhibited any adverse reactions in the category of reactions, while sorafenib, a synthetic medication, has displayed some allergic reactions.

Similarly, five ligands from the extract of *C. intybus* and one standard medication were chosen from the drug bank database to assess the characteristics of ADMET and analyze the bioactivity of the ligands as indicated in Table 4.14.

The ligands such as gallic acid, kaempferol, salicylic acid, quercetin and chlorogenic acid were selected for further study considering bioactivity analysis and ADMET properties. The characteristics (ADMET) as distribution, metabolism, excretion, absorption, and toxicity were studied for each ligand selected and finally, two ligands from *C. intybus* gallic acid and salicylic acid were selected for further studies as hit compounds.

The ADMET properties of each hit compound (gallic acid and salicylic acid) are already described above under the heading, bioactivity analysis of ligands and ADMET properties measurement.

4.8 Molecular Dynamic Simulation

The molecular dynamics (MD) simulations were performed utilizing Maestro 12.0 (version 12.0.012, Schrödinger, LLC, New York, NY), a robust molecular dynamics suite to observe the stability and flexibility of the shortlisted complexes.

4.8.1 Structure Retrieval and Virtual Screening for MD Simulation

The RhoA protein's 3D structure was located and produced, including cleaning, minimization, and modelling. Notably, the protein has a length of 193 amino acids and has only been shown to have a domain, the tiny GTP-binding protein domain. Likewise, the ligand's structures (salicylic acid, gallic acid, and caffeine) were produced in the PDB format. The complexes with the highest binding affinities for each ligand were selected from the screening complexes (Table 4.15). On the other hand, the complex containing salicylic acid showed a binding affinity of -5.44 kcal/mol, the complex containing gallic acid showed a binding affinity of -6.01 kcal/mol, and the complex containing caffeic acid showed a binding affinity of -6.11 kcal/mol. Table 4.15 and Figure 4.23 lists the binding affinities of the screened complexes. According to this research, the RhoA protein and caffeic acid had the greatest binding affinity.

TABLE 4.15: Binding affinities of the RhoA and ligand complexes

Complex	**Affinities**
Caffeic acid	-6.11
Salicylic acid	-6.01
Gallic acid	-5.44

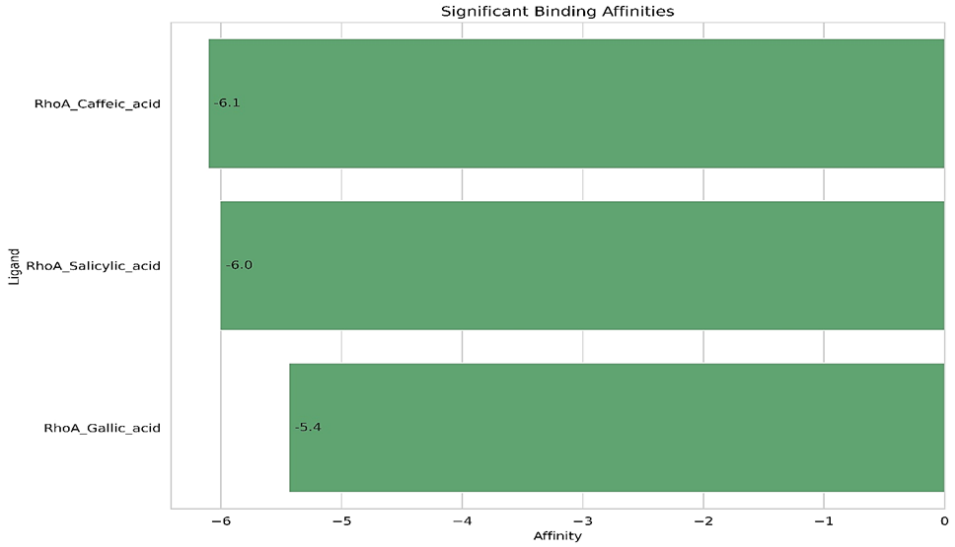


FIGURE 4.23: Binding affinities of the RhoA and ligand complexes

4.8.2 Interaction Analysis of Screened Complexes

To emphasize the connections and to pinpoint the interacting residues between the ligands and the RhoA protein, interaction analysis was carried out on top complexes (Figure 4.24 and Figure 4.25). The protein displayed interactions with caffeic acid at residues CYS (159), ASN (117), ALA (161), and LYS (118). Except for ALA (161), every interaction took place inside the small GTP-binding protein domain (5-159). The interactions were seen at residues SER (73) and ARG (70) in salicylic acid. Likewise, interactions were observed between gallic acid and residues ARG (70), PRO (71), and SER (73). Within the small GTP-binding protein domain (5-159), both ligands interacted in every interaction. Table 4.16 lists the interactions between proteins and ligands, along with the associated residues and domains. Figure 4.25 shows the 2D and 3D protein-ligand interactions of complexes. The zoomed view of the docked complex is depicted in Figure 4.26.

TABLE 4.16: Ligands binding with the residues and domain of the target protein

Complex	**Interacting residues**	**Domain**
Caffeic acid	CYS (159), ASN (117), LYS (118)	
Salicylic acid	SER (73), ARG (70)	Small GTP-binding protein domain (5–159)
Gallic acid	ARG (70), PRO (71), SER (73)	

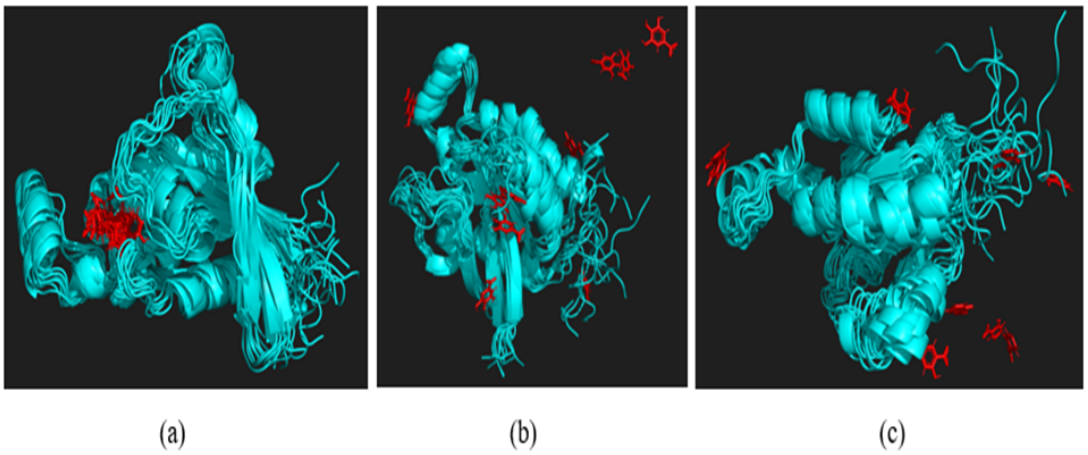


FIGURE 4.24: The MD simulation results. (a-c) Comparative visualization of trajectories of the caffeic acid-RhoA, gallic acid-RhoA, and salicylic acid-RhoA complexes, respectively

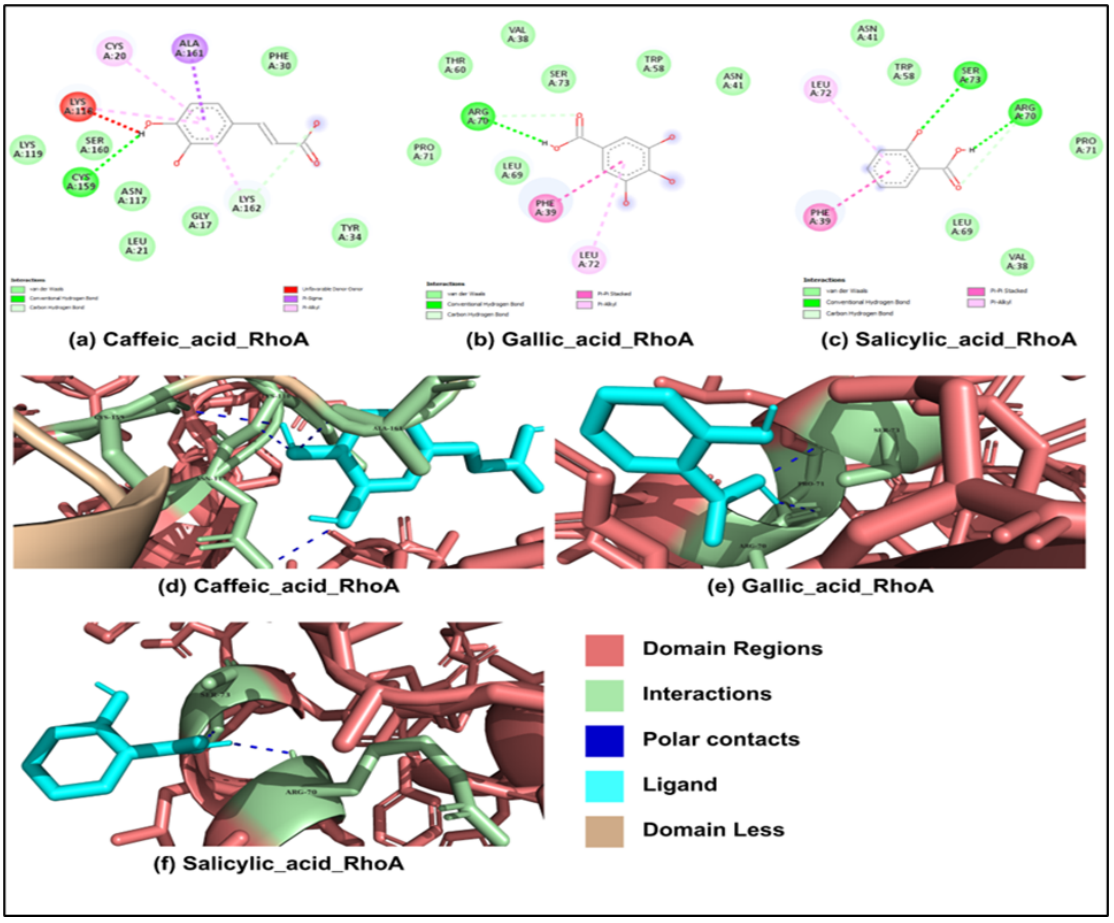


FIGURE 4.25: Interaction analysis of protein-ligand complexes. (a-c) 2D illustration of interactions of RhoA protein with the ligands. (d-f) 3D illustration of interactions of RhoA protein with the ligands within domain regions

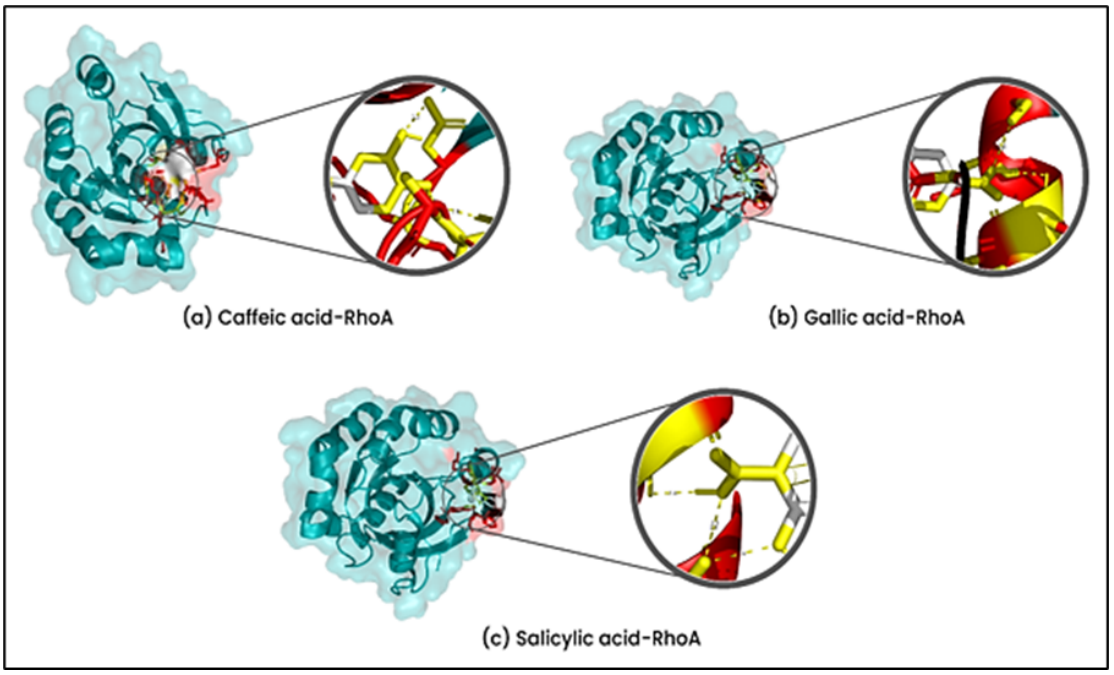


FIGURE 4.26: Zoomed view of docked complexes of RhoA protein

4.8.3 MD Simulations of Screened Complexes

The stability and flexibility of the leading complexes of gallic acid, salicylic acid, and caffeic acid were examined by molecular dynamics simulations. During the simulation period, the protein displayed an equilibrium state from 50.10 ns to 59.20 ns, for the caffeic acid. After the simulation, the ligand and protein had RMSD values of 4.36 Åand 3.17 Å, respectively. In addition, the protein and ligand had a minimal RMSD difference of 9.10 ns.

Additionally, throughout the simulation period, the gallic acid complex had an equilibrium point that moved from 60.90 ns to 85.10 ns. The protein and ligand had a minimal RMSD difference of 3.50 ns. The ligand and protein's RMSD values after the simulation were 17.43 Åand 6.38 Å, respectively. Finally, the protein demonstrated an equilibrium state with salicylic acid between 83.80 and 98.20 ns, whereas the ligand and protein's RMSD values after the simulation period ranged from 5.37 to 32.54 Å. The protein and ligand had a minimal RMSD difference of 8.50 ns.

The caffeic acid-RhoA complex, in contrast, had the lowest RMSD, indicating that caffeic acid binding imparts stability to a protein complex. Figure 4.27A shows the RMSD plots of all the proteins with their top-scoring compounds. Furthermore, as illustrated in Figure 4.27B, the gallic acid complex displayed a greater RMSF value in comparison to salicylic acid and caffeic acid, suggesting that this complex is more flexible than the other two.

Furthermore, compared to salicylic acid and caffeic acid, gallic acid had a noticeably higher PSA value, suggesting a greater aptitude for polar interactions such as hydrogen bonding and promoting stability (Figure 4.28A). Compared to gallic acid and salicylic acid, however, it was found that caffeic acid had a significantly lower SASA value, suggesting that the molecule has either become less stretched or more compact (Figure 4.28B). Last but not least, the caffeic acid displayed the greatest rGyr value (Figure 4.28C), suggesting a less compact or more elongated molecule with decreased stability as a result of increased flexibility.

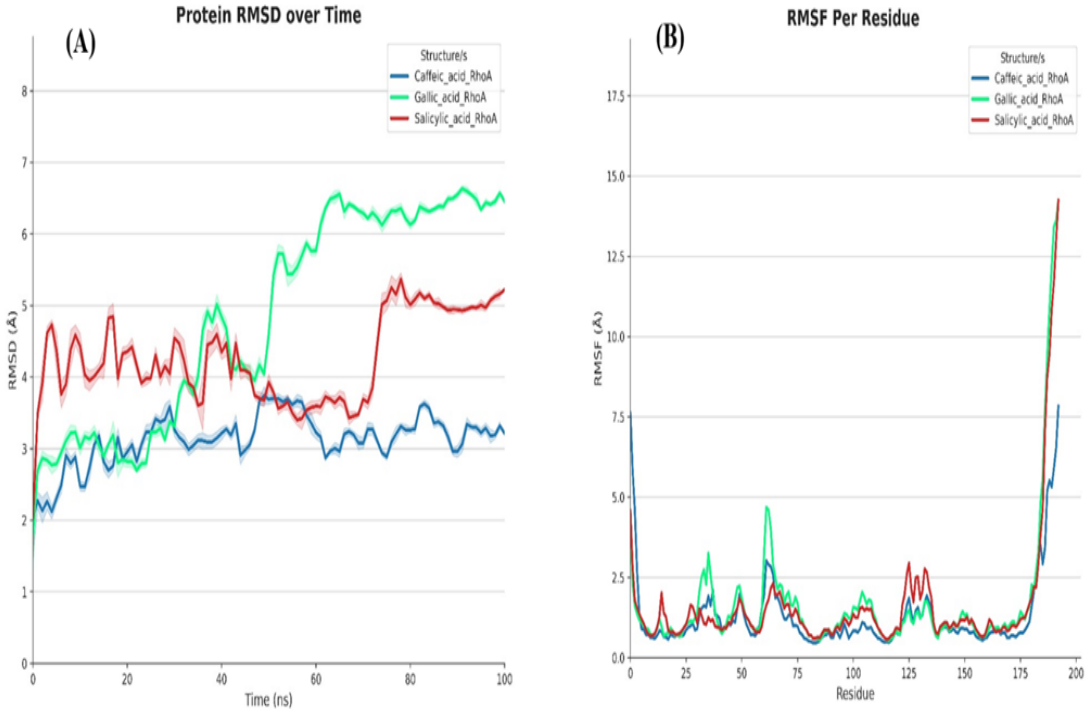


FIGURE 4.27: The above figure represents the RMSD of proteins over time (A). MD simulation illustration depicting the RMSF per residue for RhoA protein in complex with ligands (B)

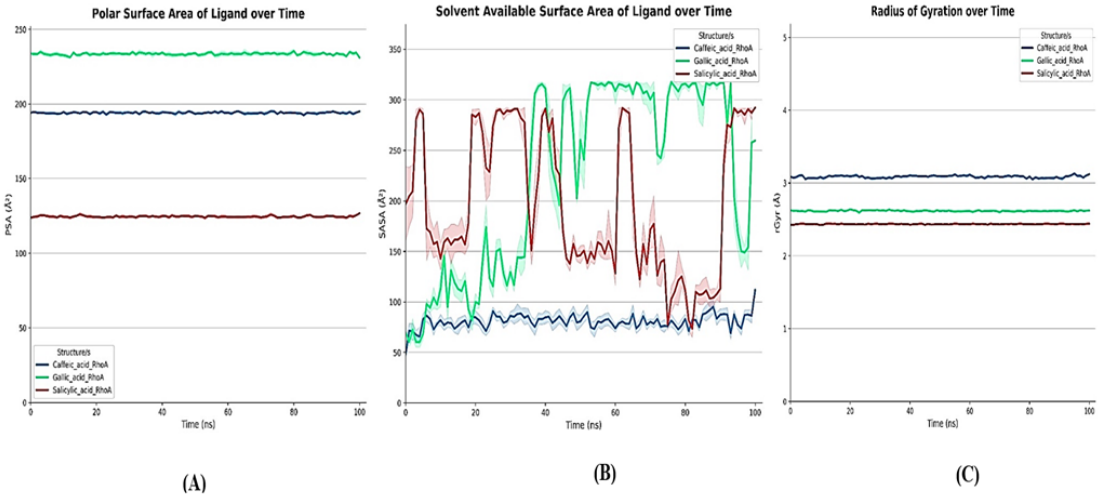


FIGURE 4.28: MD simulation results depicting the ligand properties including polar surface area (A), solvent available surface area (B), and radius of gyration (C)

These findings suggest that caffeic acid may have the greatest ability to inhibit the RhoA protein because it remains bound to the protein structure and intact throughout the simulation run, demonstrating a robust interaction between the ligand and the protein and raising the possibility of an inhibitory effect. Moreover, from the plant extract of *C. intybus* the ligands gallic acid and salicylic acid were selected as hit compounds. The stability and flexibility of the leading complexes of gallic acid, and salicylic acid, were screened by molecular dynamics simulations (Figure 4.29). All over the simulation time, the gallic acid complex had an equilibrium point that moved from 60.90 ns to 85.10 ns. The protein and ligand had a minimal RMSD difference of 3.50 ns. The ligand and protein's RMSD values after the simulation were 17.43 Åand 6.38 Å, respectively. Finally, the protein showed an equilibrium state with salicylic acid between 83.80 and 98.20 ns, whereas the ligand and protein's RMSD values after the simulation period ranged from 5.37 to 32.54 Å. The protein and ligand had a minimal RMSD difference of 8.50 ns. The gallic acid complex exhibited a greater RMSF value compared to salicylic acid and proposed that this complex is more flexible than the other two. Figure 4.29A shows the RMSD plots of gallic acid and salicylic acid. Furthermore, as shown in Figure 4.29B, the gallic acid complex displayed a greater RMSF value

in comparison to salicylic acid and suggesting that this complex is more flexible than the other two.

Furthermore, compared to salicylic acid, gallic acid had a noticeably higher PSA value, suggesting a greater aptitude for polar interactions such as hydrogen bonding and promoting stability (Figure 4.29E).

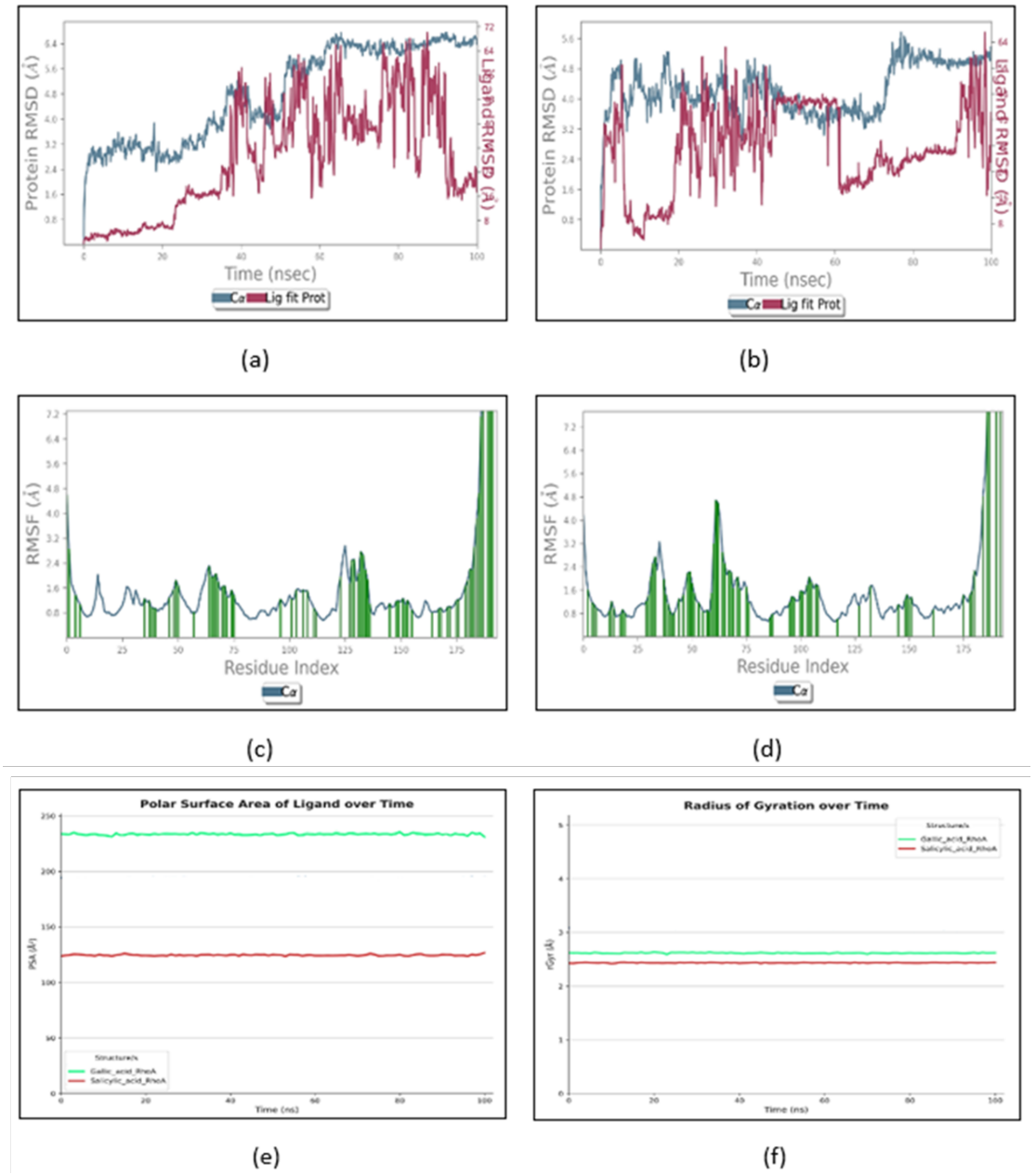


FIGURE 4.29: The MD simulation results; (a and b) RMSD of proteins in complex with gallic acid and salicylic acid detected in *C. intybus*. (c and d) MD simulation illustration depicting the RMSF per residue for RhoA protein in complex with ligand gallic acid and salicylic acid. (e and f) MD simulation results depicting the ligand properties as polar surface area and radius of gyration.

4.8.4 Comparison of Lead Compounds and Selected Drug

The standard drug and the chosen ligands were compared to determine the lead compound, taking into account several aspects such as interaction properties, docking values, and ADMET properties (RMSD and RMSF values). It was anticipated from the analysis of the data that the selected ligands, salicylic acid, gallic acid, and caffeic acid, were thought to be possible drug candidates. Given that it is the most stable and safest compound for humans, as well as having the lowest RMSD value during MD simulation, which indicates that it binds to protein complexes to impart stability, caffeic acid may be a viable option for future medication development.

Chapter 5

Discussion

Human have relied on the healing properties of plants ever since the beginning of time. Medicinal plants have been examined and chosen as therapeutic entities for many diseases specially for cancer treatment. *Hippophae rhamnoides* and *Cichorium intybus* are important medicinal plant grown in the mountains of Karakorum range and have been used in traditional medicines since the beginning of time. Because of important phytochemicals these plants gain much attention and investigated for pharmacological properties specially for anticancer potentials. Biotechnology has an essential role in drug delivery development and therapy especially for treatment of cancer. New technology which includes nanoparticles for the synthesis of nanomedicines, aims to enhance the anticancer activity of plant-based drugs by controlling the release of compounds and exploring new methods of administration. For this purpose, method of green synthesis has gained much intentness as stable, eco-friendly and sustainable process for the synthesis of a vast range of materials inclusive of metal oxide nanoparticles. The basic aim of current research work was to evaluate the extracts of *C. intybus* and *H. rhamnoides* plant extracts for their efficacy against liver cancer by performing antiproliferative assays targeting the RhoA gene and apoptotic pathway genes and proteins along with computational analysis. The present study was also aimed at synthesized nanoparticles encapsulated with the seed extracts of *Cichorium intybus* and *H. rhamnoides* and their characterization using UV-visible spectroscopy, SEM, FTIR and X-ray diffraction to study the cytotoxic and anticancer potential of these

metallic nanoparticles in comparison to plant extract against liver cancer cell lines (HepG2). During this research, in-vitro assays were used to determine the cytotoxic efficacies of selected plant extracts and nanoparticles synthesized from these plant extracts. In the next step, Insilco predictions were performed, which helped to develop biomedicines and explore the pharmacology of potential therapeutics using computer-stimulated models. Insilco approaches were useful to indicate that particular protein, which is the target molecule and mediates anticancer activity and also useful to trace those particular metabolites which are in action. Hence combining Invitro studies and Insilco molecular target prediction helped to find novel therapeutic agents against liver cancer.

5.1 Synthesis and Characterization of Nanoparticles

Nanotechnology has emerged as a highly active research field. Due to their wide applications in catalysis, electronics, sensing and medicine, nanoparticle synthesis has garnered significant attention in recent decades. [228]. Scientists have recognized the potential of biological organisms to reduce metal precursors since the nineteen century. Research has focused on biological methods due to the successful synthesis of nanoparticles using natural reducing, capping, and stabilizing agents, which avoids harmful chemicals and high energy consumption. There are several metals and their oxides which have been used to synthesized nanoparticles as silver (Ag), aluminum (Al), zinc (Zn), copper (Cu), silica (Si), gold (Ag), iron (Fe), etc. [229]. Metallic nanoparticles are nano in size with diameter ranging from 1-100 nm. These nanoparticles are gaining much importance due to applications in various fields (electronics, environmental cleanup, oil recovery, drug discovery, etc.). Due to their small size, and vast surface area, metallic nanoparticles have higher surface energy and characteristics than bulk materials [230]. Because of its focus on sustainability, protection, and human safety, green nanotechnology is an attractive and expanding area of study and development. When it comes to making, using, and disposing of chemicals, the green chemistry movement aims

to minimize potential risks to people and the environment. A thorough understanding of the raw materials, particularly their preparation into nanoparticles and the subsequent bioactivities that are safe for humans and the environment is necessary for a successful implementation of this technology. This opens up the possibility of using the abundant natural resources available for the purpose of manufacturing nanomaterials without risk. The use of biological elements in the MNP synthesis process as reducing, capping, and stabilizing agents is becoming more commonplace. Thus, green synthesis is considered a viable method for producing nanoparticles because of its biocompatible, nontoxic and environmentally friendly approach [231].

There are numerous types of iron and iron oxide nanoparticles synthesized using different plant extracts and parts of plants as seeds, stem, leaves, roots, bark and flowers [232]. These nanoparticles are usually used in many fields such as biotechnology, medicine, environment and photocatalysis and show remarkable properties such as having high surface area, environmental compatibility, superparamagnetic and non-toxicity. Iron oxide nanoparticles can be prepared using different methods (thermal decomposition, Sono chemical reaction, and hydrothermal applications) but these methods need specific equipment and are high-cost and low-yield approaches. There is use of toxic chemicals during these processes which results in waste solvents, which affect human health and the environment [233]. To overcome the negative impacts of these traditional methods, iron oxide can be synthesized using natural precursors as algae, bacteria, and plants. The type and parts of natural precursors (stem, cell, leaf, seed), extraction conditions and methods can change the properties of NPs [234]. The presence of numerous phytochemicals in plants behaves as reducing and capping agents in the preparation of NPs. The formation of brown colour is due to the interaction between the phytochemicals of selected plants and metal ions conforms to the formation of Fe_2O_3 NPs [235].

In this study, iron oxide nanoparticles were synthesized using the extracts of plants such as *H. rhamnoides* and *C. intybus* found in the Karakorum ranges of Pakistan. Metallic nanoparticles of selected plant extract were prepared using precursor salt

of respective metals (iron) and plant extract according to the reported methodology with slight changes. Nanoparticle formation was confirmed through the change in colour to dark brown which indicates the synthesis of Fe_2O_3 NPs of *H. rhamnoides* and *C. intybus* respectively. The formation of these nanoparticles was attributed to the reduction by the bioactive compounds of plants under study which carried out the reduction of metal ions and formed stable nanoparticles. There was a study conducted in which Fe_2O_3 were synthesized from the extract of Saccharum arundinaceum. These were used to assess the cytotoxic activity against brain glioblastoma cells, showing diminished activity at the concentration lower than 300 μg/mL [236].

The process was additionally verified by the technique of UV-Vis spectroscopy. This technique is commonly used for characterization of nanoparticles and has many advantages as speedy, simple, selectivity for different types of nanoparticles and affordable. UV-Vis analysis showed the characteristic peak of Fe_2O_3 NPs synthesized using the extract of plant *H. rhamnoides* at 300 nm. This result correlates with the range of characteristic peaks of Fe_2O_3 NPs i.e.300-600 nm. Furthermore, in a previous study, the Fe_2O_3 NPs synthesized from a plant extract of Bauhinia tomentosa showed a characteristic absorbance peak at 328 nm which was found to correlate with the present findings [237]. UV spectroscopy of Fe_2O_3 NPs synthesized from the extract of *C. intybus*, exhibited the characteristic absorbance peak at 289 nm. These spectra at the ideal wavelength specified the formation of Fe_2O_3 NPs. The results were validated by some of the previous evidence in which Fe_2O_3 NPs synthesized from a plant extract of Eucalyptus robusta showed an absorption peak at 290 nm [238]. Some previous reports have shown that an absorption wavelength of 200-800nm is considered suitable for characterized nanoparticles having a size range of 2-100 nm [239].

FTIR analysis was done by using FTIR spectrometer, which helped to identify the numerous biomolecules present in the aqueous extract of *H. rhamnoides* and *C. intybus*. This characterization technique validated the presence of functional groups of active substances present in both plants responsible for reduction and stabilization of Fe_2O_3 NPs. FTIR spectra revealed the characteristic absorption

peaks related to particular functional groups present on the nanoparticle's surface. The major bands of FTIR spectrum of Fe_2O_3 Nps of *H. rhamnoides* were seen at 3676, 2974, 1394, 1250, 1055, and 403 cm-1. The minor bands were depicted at 2357, 2077, 1950, 1750, 891, and 479 cm-1. In *H. rhamnoides* the major bands corresponding to 2973-2856 cm-1 indicated the presence of the C-H group of aldehydes suggesting the presence of saturated compounds. Additionally, the absorption peak positioned at 1394 cm-1 attributed to the C-F stretch indicating alkyl and aryl Halides. The absorbance peak located at 2974 cm-1 corresponded to the O-H stretch indicating the presence of carboxylic acids in the FTIR spectra of *H. rhamnoides* Fe_2O_3 Nps. Likewise, the bands recorded between 650 cm-1 -1000 cm-1 spectrum are due to the C-H group of alkanes.

There is a band located at 2974 cm- in *H. rhamnoides* and 2922 cm- in *C. intybus* indicating the presence of aromatic rings. The same results were obtained in a previous report [240]. FTIR spectrum of Fe_2O_3 NPs synthesized from *C. intybus* shows the major bands seen at 3675, 2922, 1741, 1637, 1393, 1059, and 404 cm-1. Likewise, the minor bands were detected at 3293, 2360, 2160, 2034, 1393, 1305, and 711cm-1. The major band 3675 cm-1 shows the N-H bending indicating the presence of proteins. The band present at 2922cm-1 depicted the presence of O-H group indicating the presence of carboxylic acid, whereas band at 3293cm-1 indicated stretching bond O-H of alcohols. A distinct band was observed at 1637 cm-1 indicating the presence of the N- H bend of amines. In a previous study, green synthesis of Fe_2O_3 nanoparticles from Mentha longifolia showed FTIR spectra in the range of 400-4000 cm-1 [241]. These results found parallel to one of the previous reports where the major bands were detected at 2260-3350 cm1 in iron oxide nanoparticles synthesized from a plant extract of Euphorbia tirucalli [242]. In another report, FTIR spectra was observed with distinct bands at 2067cm-1 and 1635cm-1 in iron oxide nanoparticles synthesized from plant extract of Teucrium polium. These bands corresponding to the -OH group indicating the presence of carboxylic acid and C=C group indicated the presence of organic compounds [243]. The occurrence of different functional groups in the FTIR spectra of both plants indicated the successful synthesis of Fe_2O_3 NPs by biomolecules.

The SEM analysis provided a detailed image of the nanoparticles synthesized revealing microstructure and surface morphology using an electron beam, scanning the surface of the sample. The images revealed that particles are well dispersed with a close compact arrangement with average particle size distribution. Scanning electron microscopy (SEM) determined the morphological properties, size and shape of prepared nanoparticles, during which electron beams of high energy is used to produce images. SEM images of Fe_2O_3 NPs of *H. rhamnoides* and *C. intybus* showed that the distribution of nanoparticles is steady and their size is in the nanoscale range. Most of the nanoparticles exhibit a defined shape and smooth surface with slight aggregations. These images also interpret a few of the nanocrystals with irregular shapes. The size of nanoparticles synthesized from *H. rhamnoides* was 27 ± 5 nm in diameter and they were hexagonal as compared to nanoparticles of *C. intybus* which had a size range of 84 ± 4 nm and they were round. These results correlate with the previous reports [244]. Previously, CuNPs synthesized from *H. rhamnoides* extract had a size range of 38 nm - 94 nm and were spherical mostly [245]. Recently, a study was conducted to synthesized the iron oxide nanoparticles from the extract of saline-stressed Zea mays in which the average particle diameter of SEM images varied from 2.22 to 27.83 nm [246]. In another study, Fe_2O_3 NPs were prepared using the extract of saffron and characterized using techniques as SEM, FTIR, XRD, and EDX. These green synthesized nanoparticles showed a particle size in the range of 24.27 to 46.27nm [247].

EDS is an analytical technique which is used to determine the elemental composition and distribution in synthesized nanoparticles. The chemical and elemental composition of the synthesized Fe_2O_3 NPs of both plants were found using energy dispersive X-ray spectroscopy (EDS) besides SEM. EDS spectrum suggested that iron is the primary component in both samples along with oxygen without the presence of any contaminants. This can be correlated with the study conducted previously in which synthesized iron oxide nanoparticles confirmed the formation of an EDS intensity peak, having an iron composition of 55 per cent and oxygen was 34.93 per cent [241]. The elemental composition of nanoparticles in the present study also showed the presence of some other elements as carbon and sulphur. The presence of these elements can be attributed to their presence in the

plant extract. The presence of carbon suggests the use of plant extract in the synthesis process, serving as the capping agent of Nps ensuring stability. These findings can be linked with the study in which EDS of leaf extract of V. leucoxylon contained some elements such as silicon, carbon and iron which indicate the presence of phytochemicals on the surface of nanoparticles [248].

The sulphur being referred to as the organosulphur compounds, is found in plant extract and functions as a capping agent in the synthesis of Fe_2O_3 NPs. Similar findings were also reported in a previous study [249]. XRD provided useful information about the phase nature, lattice parameters and crystalline structure of synthesized nanoparticles. The pattern of Fe_2O_3 NPs in XRD analysis was observed by using index POWDER-X software and matched with standard JCPDS, 77-1545 data.

The result showed the diffraction peaks of Fe_2O_3 NPs of *H. rhamnoides* at degrees (2θ) of 11.34^o, 17.94^o, 19.73^o, 28.85^o, 34.95^o, 43.38^o, 46.65^o, 56.35^o, 60.41^o and 69.05^o while the X-ray powder diffraction peaks of *C. intybus* were found at 10.02^o, 12.58^o, 16.76^o, 20.46^o, 24.24^o, 32.53^o, 36.76^o, 39.95^o, 44.22^o, 50.76^o, 60.04^o, and 64.61^o which indicated the crystalline nature of the nanoparticles. These findings can be linked with the study in which green synthesized iron oxide nanoparticles from a plant extract of *Phyllanthus niruri* showed similar distinct peaks in the XRD pattern [250].

A reduction in the intensity of peaks suggests the interaction of nanoparticles with biomolecules of the plant extract. This can be linked to a previous report [251]. There is variation in the diffraction peaks of both plants, as different plants extract contains a different concentration of phytochemicals, which function as reducing agents or stabilizers in nanoparticles. Hence difference in peak patterns of Sea buckthorns and *C. intybus* may attributed to differences in the plant extract composition, which results in distinct XDR peaks in both plants. These results of difference in the intensity of peaks can be linked with a previous study [251]. The variation in the peak pattern of Fe_2O_3 NPs of both plants may also be due to variations in the phase composition of particular NPs as found in the hematite

phase (α-Fe_2O_3), (maghemite γ -Fe_2O_3) and magnetite (Fe_3O_4) resulting in distinct XRD patterns with characteristic peaks. This is in close agreement with a previous finding in which the synthesized Iron oxide nanoparticles showed different phase composition [203].

5.2 Cytotoxicity Analysis by MTT Assay

Cancer is one of the most life-threatening issues in the world. According to the World Health Organization (WHO), there are 8.97 million deaths occurring annually due to cancer, making it the second largest cause of death after ischemic heart disease [25]. Despite many developments in this field, the current therapeutic options are still facing many difficulties. The most important challenge involves the absence of precise therapy and insufficient drug accumulation in cancer cells. consequently, unwanted side effects occur in non-target tissue especially the heart, bone marrow, nerve system, and gastrointestinal tract. Because of the intricate nature of tumors, it poses many challenges to their treatment strategies [252]. Nanomedicine which is combination of biotechnology, biomaterial and biomedicine, appears to be an optimistic hope to address these challenges. A nanoparticle-based drug delivery system has higher potency due to the increased half-life of vulnerable drugs, and the capacity to control and site-specific target of drug release [253]. Recently metallic nanoparticles have gained significant attention because of having potential to be used as multipurpose agents. Hence non-noble metal-based cancer therapies can progress towards more cost-effective treatments compared to the expensive chemotherapy options.

MTT assay was used to evaluate the cytotoxicity of plant extracts and iron oxide nanoparticles synthesized from plant extract. The cytotoxic potential of Fe_2O_3 NPs synthesized from *H. rhamnoides* and *C. intybus* was assessed against the HepG2 liver cancer cell line. The cytotoxic potential of synthesized nanoparticles and respective plant extracts was determined for distinct concentrations (20, 40, 60, 80 and 100 µM) and revealed promising results. During this experiment, the HepG2 cell line was exposed to a plant extract and Fe_2O_3 NPs (20 to 100

μM) synthesized using the extract of *H. rhamnoides* and *C. intybus*. The results showed the reduction of cell viability in dose dose-dependent manner, as with increased concentration of iron oxide nanoparticles, there was a decline in the cell viability. This means that there is concentration-dependent cytotoxicity as with the increase in the concentration of iron oxide nanoparticles there was a decrease in cell viability. This can be associated with observations from other studies [254]. The observed higher cytotoxicity is because of an elevated dose of nanoparticles, which leads to excessive production of reactive oxygen species (ROS). Oxidative stress induced by ROS damages cellular DNA, which leads to apoptosis and cell death [255]. These findings suggests that cancer cells are more delicate to ROS than normal cells and it is possible to target cancer cells through ROS-mediated processes [256]. The IC50 values of *H. rhamnoides* and *C. intybus* nanoparticles were also found to be less (41.69 μM and 71.04 μM) compared to the IC50 values of their respective plant extracts (78.10 μM and 96.03 μM). These results also speculated that the plant extract of *H. rhamnoides* is more effective for cell cytotoxicity as compared to the extract of *C. intybus*. In a particular study, the synthesis of iron oxide NPs from Punica granatum fruit peel extract also showed significant cytotoxic activity [257]. Moreover, Fe_2O_3 NPs were also found to be effective having cytotoxicity against breast cancer cells [258].

These results found that iron oxide nanoparticles synthesized from the extract of *H. rhamnoides* showed potent cytotoxicity towards HepG2 cell lines as compared to other plant. The increased cytotoxic potential is probably due to the presence of various phytochemicals of plant extract on the surface of nanoparticles, as it was found that these compounds can exhibit potent anticancer activity by ROS production which helps in phagocytosis [259]. Hence there is remarkable variation observed in cell cytotoxicity between Fe_2O_3 nanoparticle and plant extracts. These results also speculated that the plant extract of *H. rhamnoides* is more effective for cell cytotoxicity as compared to the extract of *C. intybus*. The disparity in cytotoxicity and ROS generation among Fe_2O_3 NPs synthesized from *H. rhamnoides* and *C. intybus* is probably due to the presence of various phytochemicals in each extract. It was found that polyphenols are major antioxidants studied for reducing tumour growth. The significant cytotoxic potential of *H.*

rhamnoides may be attributed to the presence of polyphenols, which may exhibit potent anticancer traits which are efficient in regulation of ROS generation [260]. It was found that treatments targeting ROS could serve as a novel approach to cancer therapies [256]. In a previous study, CuO nanoparticles were synthesized from the stem of *H. rhamnoides* in which IC50 values were found to be 48µg/mL and showed the inhibitory effects on the viability of HeLa cells of cervical cancer [245]. In a previous study, Fe_3O_4 Nps were successfully studied for cytotoxicity against Leukemia(Jurkat) cells when compared to some other cancer cell lines [261]. There was a study conducted in which the extracts of two plants were assessed for anticancer effects on oral cancer (KB) cell lines. The results showed that there is reduction in cell viability with the increase in extract concentration. The IC50 value of the ethanolic extracts of *Plectranthus amboinicus* was found as 53.0 µg/mL and the value of the ethanolic extract of *Glycyrrhiza glabra* was 43.6 µg/mL [262].

5.3 Gene Expression and Protein Analysis

Hepatocellular carcinoma makes up 90% of primary liver cancer and is considered one of the leading causes of cancer-related deaths globally. At present most patients of HCC often present with regional spread and metastasis of cancer, which leads to poor prognosis. The Rho family of GTPases include 20 members of small GTP binding proteins, each with molecular size ranging from 20 to 40 kDa. Rho GTPases regulate various biological processes primarily by remodelling actin and cytoskeleton. They alter between an active state when they bind to GTP and an inactive state when they bind to GDP, and this switching is regulated by three sets of proteins named GEFs, GAPs, and GDIs. Among Rho family proteins the Ras homology gene family member A which is called as RhoA, is the best-characterized protein. It was found that RhoA regulates many signal transduction pathways such as cell migration, gene expression, polarity of cell and cell cytoskeleton. In this study, the expression of target gene Rho A was studied with other apoptotic genes as caspase 3, caspase 8 and caspase 9. Real-time qPCR was used to study

the expression of target gene RhoA, bax and apoptotic genes (caspase 3, caspase 8 and caspase 9). This method is regarded as a reliable, accurate and effective approach for analyzing gene expression.

The RhoA gene is primarily involved in the cytoskeleton of cells, polarity, cell cycle and cell degradation. Dysregulation of RhoA signaling pathways can contribute to tumour development, hence is a major drug target for treating multiple malignancies. The level of this gene significantly declined in the cells treated with Fe_2O_3 NPs and plant extracts of both plant species as compared to untreated cells. *H. rhamnoides* plant extract and its NPs were found to show greater relative gene expressions in deregulating the RhoA gene expression as compared to the nanoparticles and plant extract of *C. intybus*. This may be attributed to the fact that variation in phytochemical (polyphenols) in both plant and varying biological activities of plant extract leads to differences in gene expression as polyphenols were found to have an impact on human health and have the potential to modulate gene expression [263]. *H. rhamnoides* phytochemicals may have potent anticancer properties leading to the deregulation of the RhoA gene. Some studies found RhoA acts as oncogene and participate in carcinogenesis of liver cancer. It is also reported that increased expression of RhoA is also found in hepatocellular, bladder, ovarian, breast, gastric, colon and lung cancer [264]. Moreover, RhoA was found to be crucial for cell motility, showing that this protein has a significant role in the invasive phenotype of the tumor cells. These results correlate with the previous findings in which the knockdown of RhoA led to inhibiting lung cancer cell migration and invasion [265]. It was also found that there is upregulation in many of Rho GTPase family members and have oncogenic activity and are detected in many types of human cancers [120].

The apoptotic role of plant extract and Fe_2O_3 NPs was also studied on different apoptotic genes. Apoptosis is the way of programmed cellular death, results in disassembly of intracellular components evading harm to surrounding cells [266]. These findings also propose the upregulation of bax gene which is involved in apoptotic pathway. Plant extract and Fe_2O_3 NPs of *H. rhamnoides* showed greater cytotoxicity by up-regulating the bax gene as compared to plant extract and NPs

of *C. intybus* treated HepG2 cancer cells in comparison with control group. The greater bax/bcl-2 gene ratio decreases the resistance faced by apoptotic genes and thereby inducing apoptosis. The bax gene was also found to have increased expression in HepG2 cells when treated with silver nanoparticles of Artemisia carvifolia Buch confirming its role in the programmed cell death [208].

Caspase 3, caspase 8 and caspase 9 showed higher expression as well which decipher the role of extrinsic and intrinsic pathway of apoptosis in nanoparticle-mediated toxicity. Plant extract and Fe_2O_3 NPs of *H. rhamnoides* showed greater expression of Caspase 3, Caspase 8 and caspase 9 as compared to extract and NPs of *C. intybus*. These findings illustrated that *H. rhamnoides* showed more cytotoxicity by up-regulating the apoptotic pathway genes in comparison to *C. intybus*. In prior research, the apoptotic role of Fe_2O_3 NPs was also evaluated against breast cancer cells [267]. Furthermore, another report indicated increased mRNA and protein levels of bax relative to bcl-2 against all the cancer types via producing reactive oxygen species (ROS) [268]. Apoptosis is a meticulously controlled evolutionarily preserved form of programmed cell death crucial for normal physiological functions like embryogenesis and adult tissue homeostasis. It is also widely known for its function as a system that suppresses cancer growth [269]. ELISA was used to determine the protein levels of aforementioned genes, following recommended instructions. The level of initiator caspase (caspase-9) executioner caspases (caspases-3) and caspase-8 protein was studied for HepG2 cells treated with *H. rhamnoides* and *C. intybus* Fe_2O_3 NPs and plant extracts. The results presented that the level of caspase proteins was high in the cells treated with *H. rhamnoides* as compared to *C. intybus* NPs and extract in comparison to the control group. This confirmed the role of nanoparticles in the activation of cancer cell death by activating a series of caspase reactions. The findings can be linked with a previous study in which there was an elevated level of caspases when treated with nanoparticle and extract from plant Fagonia indica [270]. The increased expression of these caspases (caspase 3, caspase 8 and caspase 9) at mRNA level and protein level showed that plant-mediated Fe_2O_3 NPs have shown programmed cell death at a remarkable degree. Phytochemicals are studied to regulate gene expressions leading to normal signal transduction pathways [271].

In the pathway of cell death, caspases are considered main regulators having a critical role as they are the main executioners of apoptosis and cell death in signal transduction [272]. The effector caspases -3/-7 are involved in the cleavage of certain precursors resulting in cell apoptosis. Caspases -8 and -9 are responsible for the activation of these effector caspases [273]. Previously, silver nanoparticles of Artemisia carvifolia were found effective against HepG2 cells by upregulating gene and protein levels of bax and caspase 3, 8 and 9 [208]. Moreover, it is also verified by this comparative study that *H. rhamnoides* has more potential to be used in the development of anticancer drugs as compared to *C. intybus*.

5.4 Phytochemical Screening by HPLC

Human have relied on the healing properties of plants ever since the beginning of time. Almost all chemotherapy medicines are either synthetically produced or compounds extracted and refined from plants. Herbal therapy is an effective alternative to conventional cancer treatment. Numerous studies have been conducted on natural compounds that exhibit cytotoxic properties such as the ability to induce cancer cell death. Owing to these benefits medicinal plants have been inspected and preferred for the preparation of cancer medicines. Recently there has been an increase in curiosity in the study of bioactive compounds of plants as an anticancer compound [27]. The phytochemicals of plants can prevent cancer by mechanisms such as stimulating the process of DNA repair, increasing the production of protective enzymes which enhance immunity and inducing antioxidant action [28].

H. rhamnoides is a thorny shrub which belongs to the Elaeagnaceae family. Its fruits are highly nutritious, packed with vitamins and minerals, and widely utilized in the medicine and cosmetics industry. If we study its traditional use, we find that this thorny shrub has been used in traditional medicine, in some regions such Mongolia, China, Tibit, and some regions of Central Asia. There are unique bioactive compounds found in berries, also known as seaberries, as phenolic

compounds, vitamins (specially vitamin C), unsaturated fatty acids, and phytosterols, like beta–sitosterol. The juices, jam and oil derived from these berries have been studied for their antioxidant, anti-inflammatory, and anticancer effects. The concentration of the compounds in fruits depends on, climatic conditions, size, maturity, and process used to process and store plant materials [274]. *Cichorium intybus* which is commonly known as chicory, is an herbal plant and belongs to the family Asteraceae grows wild in the temperate zone of Europe, South West Asia. There are multiple chemical compounds found in all parts of chicory, and enriched with vitamins and minerals. Traditionally this plant is used for the treatment of diabetes, digestive disorders, gastric ulcers and malaria. *C. intybus* is generally regarded as safe for use in both food and medical applications. FDA also has approved to enlist chicory extracts to be considered safe for use in food in the United States [275]. This plant was studied for various biological and pharmacological properties such as antidiabetic, antibacterial, antioxidant, anti-inflammatory, and antiproliferative effects [35].

To study the extracts (methanolic) of *H. rhamnoides* HPLC Chromatography was done. The retention time of the standard polyphenols is used to study the HPLC profile of the plant extract. The mechanism consists of comparing the retention time of samples to the retention time of standards. Based on this principle there are 12 polyphenols identified in the extract of *H. rhamnoides* these were gallic acid, quercetin, chlorogenic acid, HB acid, Vanillic acid, sinapic acid, ferulic acid, rutin, kaempferol, salicylic acid, caffeic acid and coumarin. Moreover, the concentration was found for each component and it was found that in the methanolic extract of *H. rhamnoides* chlorogenic acid had the highest concentration (1.151µg /mg). The next concentration is of coumarin and then salicylic acid is the third highest. The lowest concentration of the polyphenol found was that of quercetin. Various polyphenols were detected in a study conducted to find the phytochemicals composition of berries and leaves of *H. rhamnoides* [276]. In comparison to this study some more polyphenols as sinapic acid, and salicylic acid were also traced in this study. Based on this study and some of the previous findings we can assume that the berries of Sea buckthorns contain a high profile of polyphenols which show

variation according to climate and growing conditions as well as genetic background [140, 277]. In some of the early investigations, certain polyphenols were traced in the extract of *H. rhamnoides* [278, 279]. In another study, some of the flavonoids were detected in the pulp of two cultivars of *H. rhamnoides* [280].

To study the extracts (methanolic) of *C. intybus* HPLC was done as well. The retention time of the standard polyphenols is used to study the HPLC profile of the plant extract. The mechanism is based on comparing the retention time of samples to the standards. Based on this principle there are 9 polyphenols identified in the extract of *C. intybus*, these were gallic acid, quercetin, chlorogenic acid, HB acid, vanillic acid, benzoic acid, rutin, kaempferol, salicylic acid. Moreover, the concentration was found for each component and it was found that in the methanolic extract of *C. intybus*, chlorogenic acid had the highest concentration (1.151µg /mg), The next concentration was of coumarin and then salicylic acid was third highest. The lowest concentration of the polyphenol found was that of quercetin. In an early finding some of the poly phenols were detected in the extract of *C. intybus* [281, 282]. In another research finding some flavonoids were detected in the extract of *C. intybus* found in Romania [283]. Likewise, some more flavonoids were found in the aqueous extracts of *C. intybus* [284].

5.5 In Silico Analysis

The discovery of in silico drugs has progressed in recent years. Computational approaches have helped scientists to simulate chemical system, resolve 3D structures, optimize and develop new chemical compounds and analyze the atomic processes of drugs and natural molecules. New techniques have made the drug discovery process faster and easier than ever and several new compounds are now in clinical trials and some have received FDA approval [285]. In the discovery of new anticancer drugs there is much importance of computer aided drug design. It comprises the use of computational tools to predict potential drug candidates and biological targets. This will allow researchers to predict the potential drug candidates for several diseases as cancer [286]. For anticancer drug design, there are

now several ways which help to control tumour growth, and the process is divided into two paradigms first is structural-based drug design and second is ligand-based drug design.

Structural-based drug design requires 3D structural analysis of biological molecules. For this, there is the use of biomolecular spectroscopic methods such as NMR, and X-ray crystallography, which has significantly improved the structural information of therapeutic targets enabling remarkable advances in this area. In the structure-based approach, the 3D structure of the target protein is known and the interactions of all tested compounds are measured, then designing a new drug molecule having a high affinity to the target protein [287]. In ligand-based drug design, the 3D structure of the protein is not known, but ligand characteristics are known that bind to the target site. The pharmaceutical industry uses ligand-based drug discovery methods to explore new ligands with interesting biological activities and optimize drug pharmaceutical characteristics such as ADMET (absorption, distribution, metabolism, excretion, and toxicity) [285].

In the next step of a particular study, in silico predictions were performed, which helps to develop biomedicines, and to explore the pharmacology of potential therapeutics using computer-stimulated models. Insilco approaches will also be useful to indicate that particular protein, which is the target molecule and mediates anticancer activity and also useful to trace those particular metabolites which are in action. Hence in silico molecular target prediction will help to find out therapeutic agents against liver cancer targeting the RhoA protein. For these two computational approaches, molecular docking and molecular dynamic simulation were done.

Molecular docking is an Insilco method which is performed to study the interactions between ligands and targets. This procedure consists of the use of docking algorithms to spot minor compounds at the target active site, to search for the ideal conformation. Then the use of score functions can help to predict the ligand's affinity in that position [288]. Hence allows us to predict the lead compound with high binding affinity to target proteins or other biomolecules that play important roles in cancer biology.

The primary sequence of the RhoA protein was retrieved from the database uniport with accession no. P61586 and a residual length of 193 amino acids. An online tool ProtParam Expasy was used to study the physiochemical properties of RhoA. These properties included molecular weight, extension coefficient, instability index, positive and negative charged amino acids, theoretical PI, and GRAVY (grand average of hydrophobicity) [289]. The 3D structure of the RhoA protein was downloaded in pdb format with pdb id 4XH9 which was selected from the protein data bank (PDB) [290]. This protein molecule was used as a target molecule in docking with selected ligands. Rho GTPases are highly conserved GTPases and their dysregulation is associated with a range of abnormal cellular processes, targeting these proteins can minimize the severity of cancer progression [114]. The structure of the target protein (RhoA) was visualized by using the pymole software. Interpro an online database was used to identify the functional domains of RhoA protein. Conserved domains are involved in sequence/structure/relationship [291]. This protein contains mostly small GTP-binding domains (IPR005225 and TIGR00231) and other domains of small GTPase Rho family profile (PS51420).

There were 12 ligands (compounds) identified in *H. rhamnoides* as gallic acid, quercetin, caffeic acid, chlorogenic acid, HB acid, vanillic acid, sinapic acid, ferulic acid, coumarin, rutin, kaempferol and caffeic acid and from plant extract of *C. intybus* the ligands for this interaction are gallic acid, quercetin, caffeic acid, chlorogenic acid, HB acid, vanillic acid, sinapic acid, rutin, kaempferol and salicylic acid. The 2D structure and associated data for the ligands found in the plant extract by HPLC were acquired from the PubChem databases [292]. The following stage involved minimizing the ligand's energy, which is crucial for getting the ligand ready for docking because unstable ligands might have an impact on their vina scores. Therefore, ChemPro software (chem12) was utilized to minimize ligand energy.

Finding the ideal conformational connection between target proteins and ligands was the main goal of molecular docking [285]. The online docking tool CB-Dock was utilized for this investigation. This tool provided a 3D visualization of the results in five distinct positions. Thus, the optimal stance with the lowest vina

score was chosen [293]. Furthermore, no. of hydrogen bonds and hydrophobic interactions were also determined. Taking into account many factors such as the optimal vena score, cavity size, grid map score, and the Lipinski rule of five, the ligands quercetin, gallic acid, kaempferol, salicylic acid, and caffeic acid were chosen from the *H. rhamnoides* extract. Sorafenib, a synthetic drug used to treat liver cancer was also employed to compare the ligand's interaction with the target protein. Likewise, quercetin, gallic acid, kaempferol, salicylic acid, and chlorogenic acid were chosen from *C. intybus* extract.

In the next step, RMSD (Root-Mean-Square Deviation) values in docking results were checked using the software UCSF Chimera. The values of RMSD predicted in interaction results help examine the variation in docking poses and can measure the deviation of the system from its original conformation. The average distance between the atoms in a protein and ligand structure that are overlaid was determined using RMSD [294]. All of the poses were regarded in the current study as the ideal pose for illustrating the RMSD rules. Additionally, the root-mean-square fluctuation, or RMSF, gave the average residual deviations and offered information on the protein's flexibility. It was believed that the values for RMSD couldn't be more than 4. An appropriate range for globular proteins is between 1 and 3A^o [295].

Current computational studies focus on shaping drugs' pharmacokinetics, covering absorption, distribution, metabolism, excretion and toxicity. To predict compound's pharmacokinetics requires computational studies for better efficiency. The compounds screened from the plants are considered lead compounds if having adequate ADMET properties [296]. To evaluate the drug-likeness of a compound, it must follow the Lipinski rule of five which helps to predict the potential of a compound as an orally active drug in humans. According to this rule, a compound is likely to be considered drug-like if it obeys the criteria as molecular weight less than five hundred, log P value below five, no more than five hydrogen bond donors, and no more than ten hydrogen bond acceptors. This rule said that we can consider a compound as a drug candidate for oral use if it follows four of these rules [297].

For the analysis of the bioactivity of ligands and to measure ADMET properties, there was selection of five ligands and the standard drug from the drug bank data bank. For this, we have to consider that selected ligands have to follow the principle of Lipinski Rule of Five, which helps to determine the likelihood of compounds becoming orally active drugs in human [298]. This rule states that the compounds that are chosen must fulfil certain requirements, the majority of which require the log P-value to be less than 5. Other requirements of this rule are that the molecular weight of the specific chemical must be less than 500 Daltons and that there cannot be more than five hydrogen bond givers, and that there cannot be more than ten hydrogen acceptors. The ligands that meet the requirements of the five Lipinski rules are more likely to be more readily absorbed and bioavailable by the human body [299].

The pharmacokinetic characteristics of the chosen ligands were investigated in more detail (ADMET). These characteristics distribution, metabolism, excretion, absorption, and toxicity are crucial in determining if a molecule is a good candidate for a medication. The PKCSM tool, which helps determine the pharmacokinetic characteristics of the ligands selected as therapeutic candidates, was utilized to investigate the ADMET features [300]. Taking into account absorption properties, which showed variations in the drug's and the chosen ligands' water solubility, the reference drug was less soluble in water than the other five selected ligands. Salicylic acid, caffeic acid, and gallic acid are the chemicals that have been chosen because they are slightly more soluble in water than the other ligands. Some selected ligands had slightly higher $CaCO_2$ permeability than the synthetic drug when taking into account the absorption feature. Quercetin and caffeic acid had a higher $CaCO_2$ permeability than any other lead molecule. While caffeic acid is not a P-gp substrate and is not an inhibitor of either P-gp I or P-gp II, the synthetic medication sorafenib is a P-gp substrate, P-gp I inhibitor, and non-inhibitor of P-gp II. Similarly, quercetin is a substrate of P-gp and does not inhibit P-gp I or II. The remaining chosen lead compounds are all non-inhibitors of P-gp I and II, except kaempferol, which is a P-gp substrate.

When looking at the distribution properties, we find that the CNS permeability of

sorafenib is in the region of -2, whereas other selected ligands such as caffeic acid have CNS permeabilities in the range of -2 and salicylic acid, quercetin, and gallic acid have CNS permeabilities in the range of -3. For other selected ligands such as kaempferol the range of CNS permeability is also -2. Similarly, while examining the unbound friction property in human plasma, several substances were shown to have a greater value than manufactured drugs. Notably, this was especially true for caffeic acid, gallic acid, and salicylic acid. This indicates traditional ligands such as salicylic acid, gallic acid, and caffeic acid are more effective than synthesized drugs. Furthermore, one of the key aspects in the investigation of ADMET properties is drug metabolism. Both the synthetic medication sorafenib and the selected ligands salicylic acid, kaempferol, quercetin, chlorogenic acid, and caffeic acid were not confirmed to be CYP2D6 substrates.

It is crucial to understand a drug's complete clearance to calculate dosage rates. Comparing our results to the reference drug, we found that there is a high overall clearance of quercetin, kaempferol, gallic acid, salicylic acid, and caffeic acid. The Renal OCT2 substrate characteristic was absent from all of the chosen compounds. Similarly, we can determine the suggested medicine dose's tolerance limit by understanding the toxicity of a selected ligand [301]. The chemicals that were chosen for this study, such as gallic acid and caffeic acid, were determined to be far safer for humans than synthetic drugs and the other two ligands that were chosen. While the reference drug sorafenib itself shown Herg II inhibitor properties, all the chosen drugs fall into the category of No for Herg I and Herg II inhibitors. The reference medicine has a higher oral rat acute toxicity property than the other chosen ligands, such as gallic acid, salicylic acid, and caffeic acid. The chosen ligands had a greater value (1.198) for oral rat chronic toxicity than sorafenib. This indicates that the synthetic drug is more harmful than other particular combinations. The feature of hepatotoxicity was employed to investigate the toxic effect on the liver since certain substances, such as quercetin, gallic acid, and caffeic acid, do not have any toxic effects. However, the reference medication falls into the category of yes, which indicates that it can demonstrate liver toxicity. All of the selected ligands have not exhibited any adverse reactions in the category of reactions, while sorafenib, a synthetic medication, has displayed some allergic reactions.

Molecular dynamic simulation is an in-silico approach, used to examine that how molecules act and interact with each other. They can analyze single molecules as well as complex systems like protein-ligand interactions. These simulations provide information on how atoms and molecules move within a system giving insight into their dynamics. MD simulation is extensively used to analyze the structure-to-function relationship of protein and protein-ligand complexes and helps study the stability of complexes at different nanoscale intervals and the fluctuations observed. These have much applications such as used to predict binding energy between molecules, their interaction analysis, and to study the influence of physical factors like pressure, temperature, ions and water on molecular system. Moreover, they help study protein folding and unfolding dynamics [302]. The results of simulations are analyzed using some of the parameters such as RMSD (root mean square deviation) RMSF (root mean square fluctuations), the radius of gyration, free energy, H-bond dynamics and protein-ligand contact plots [287].

The MD simulations for the top complexes (caffeic acid, gallic acid, and salicylic acid) were conducted to observe the stability and flexibility. The caffeic acid revealed that the protein showed an equilibrium state from 50.10 ns to 59.20 ns during the simulation time. The RMSD values of the protein and ligand at the end of the simulation time were 3.17 Åand 4.36 Å. Moreover, the minimum RMSD difference between the protein and ligand was 9.10 ns. Furthermore, the gallic acid complex showed an equilibrium point from 60.90 ns to 85.10 ns during the simulation time. The minimum RMSD difference between the protein and ligand was 3.50 ns. At the end of the simulation time, the RMSD values of the protein and ligand were 6.38 Åand 17.43 Å. Lastly, the salicylic acid exhibited that the protein showed an equilibrium state from 83.80 ns to 98.20 ns, whereas the RMSD values of the protein and ligand at the end of the simulation time were 5.37 Åto 32.54 Å. The minimum RMSD difference between the protein and ligand was 8.50 ns.

Comparatively, the caffeic acid-RhoA complex showed the lowest RMSD, signifying the binding of caffeic acid dispenses stability to a protein complex. Additionally, the gallic acid complex showed an increase in RMSF value compared

to caffeic acid and salicylic acid, indicating the higher flexibility of this complex compared to the other two complexes. Moreover, the gallic acid demonstrated a notably higher PSA value than caffeic acid and salicylic acid, indicating an increased capacity for polar interactions, such as hydrogen bonds, and contributing to stability. In contrast, it was observed that caffeic acid showed a significant decrease in SASA value, indicating that the molecule has become more compact or less extended than gallic acid and salicylic acid. Lastly, the caffeic acid showed the highest value of rGyr, indicating that the molecule is more extended or less compact, implying lower stability due to increased flexibility. Based on these results, it can be observed that caffeic acid may have the best potential to inhibit the RhoA protein because it stays intact and bound to the protein structure over the entire simulation run, indicating a strong interaction between the protein and the ligand, suggesting a potential inhibitory effect.

The standard drug and the chosen ligands were compared to determine the lead compound, taking into account several aspects such as interaction properties, docking values, and ADMET properties (RMSD and RMSF values). It was anticipated from the analysis of the data that the selected ligands, salicylic acid, gallic acid, and caffeic acid, were thought to be possible drug candidates. Given that it is the most stable and safest compound for humans, as well as having the lowest RMSD value during MD simulation, which indicates that it binds to protein complex to impart stability, caffeic acid may be a viable option for future medication development.

Additionally, caffeic acid is expected to exhibit the strongest binding affinity with the RhoA protein during MD simulation. Caffeic acid is a polyphenol that is found in plants and has been shown to have antioxidant and anticancer properties [303]. Another study indicated that caffeic acid effectively treated cervical cancer through two distinct pathways, demonstrating the compound's potential to fight cancer in combination with other substances [304]. Serious research on the medicinal properties of caffeic acid revealed that it can prevent cell invasion and migration and may even lessen the spread of cancer [305]. Caffeic acid plays an important role in combating liver cancer as many in vivo and in vitro studies found

that this compound inhibits hepatic carcinoma by different mechanisms which include cell death, activation of caspases 9 [306]. Epigallocatechin a polyphenol is the type of catechin, which is studied for showing the effect on the Rho A gene suppressing its signalling in human hepatic stellate cell lines [307]. Dysregulation of the RhoA gene has been studied for hepatocellular carcinoma [19], presenting this gene as a possible therapeutic target. Therefore, in the future, caffeic acid, the lead compound, may be viewed as a promising therapeutic candidate to target Rho GTPases, specifically the RhoA.

Chapter 6

Conclusion

Liver cancer is a serious health concern and its incidence is increasing globally. It is predicted that one million people will be affected by liver cancer per year by the year 2025. The most common form of liver cancer is hepatocellular carcinoma (HCC) which consists of more than 90 per cent of cases, and the most prominent risk factor for this is HBV. Liver cancer is among the top five most deadly cancers rapidly increasing annually. HCC in men is continuously increasing in Pakistan and is thought to become the most common form of cancer in future. Rho GTPases are small G proteins and their basic functions is in cell cycle, cell degradation, vesicular movement and cytoskeleton of cell. It was found that abnormal signalling of Rho GTPases, is considered one of the hallmarks of cancer and will emerge as a novel therapeutic agent in future. Medicinal plants have been explored and chosen as therapeutic entities to treat many diseases especially for cancer treatment. *Hippophae rhamnoides* and *Cichorium intybus* are important medicinal plants grown in the mountains of the Karakorum range and have been used in traditional medicines since the beginning of time. Because of important phytochemicals, these plants gained much attention and were investigated for pharmacological properties, especially for anticancer potentials.

Biotechnology has tremendous contributions in the drug delivery and therapy specially for the treatment of cancer. New technology which includes nanoparticles

for the synthesis of nanomedicines aims to enhance anticancer activity of plant-based drugs by controlling the release of compounds and exploring new methods of administration. For this purpose, method of green synthesis has gained much intentness as stable, ecofriendly and sustainable process for the synthesis of a vast range of materials inclusive of metal oxide nanoparticles. The basic aim of current research work is evaluating the extracts of *C. intybus* and *H. rhamnoides* plant extracts for their efficacy against liver cancer by performing antiproliferative assays targeting RhoA gene and apoptotic pathway genes and proteins along with computational analysis.

The synthesis of well-defined Fe_2O_3 NPs has been accomplished by an efficient and reproducible green chemistry approach by using the extract of *H. rhamnoides* and *C. intybus*. The synthesized nanoparticles were found to be 27 ± 5 nm for *H. rhamnoides* and 84 ± 4 nm for *C. intybus*. Important phytochemicals of both plants were found to be involved in the reduction of iron metal to iron oxide nanoparticles. The current study reveals that *H. rhamnoides* and *C. intybus* found in the mountains of Karakoram range of Gilgit Baltistan have potent anticancer activity against liver cancer cell line $HepG_2$ presenting RhoA gene as a potential drug target. MTT assay showed a concentration-dependent decrease in cell viability of $HepG_2$ cells with downregulation of the RhoA gene and upregulation of bax and caspases enzymes showing apoptosis in liver cancer cells. However, the cytotoxicity potential of Fe_2O_3 NPs of *H. rhamnoides* was found to be higher as compared to nanoparticles and extract of *C. intybus*. This study confirms that Fe_2O_3 NPs synthesized from the extracts of *H. rhamnoides* and *C. intybus* could serves as promising potential anticancer agents in combating liver cancer in future. However, additional experimentation of synthesized NPs is needed to further confirm the efficacy and stability of plant-derived Fe_2O_3 NPs in exploring novel anticancer drugs against liver cancer.

This study also assessed the anticancer potential of *H. rhamnoides* and *C. intybus* polyphenols against liver cancer by focusing on the RhoA gene computationally. By utilizing HPLC chromatography, 12 polyphenols were found in the methanolic extract of *H. rhamnoides* and 9 were in *C. intybus*. Based on the vina score, grid

map score, and cavity size, five polyphenols were chosen for additional research utilizing in silico techniques such as molecular docking. Taking into account ADMET, properties, RMSD, and RMSF interactions, these polyphenols caffeic acid quercetin, kaempferol, gallic acid, and salicylic acid from the extract of *H. rhamnoides* and 5 poly phenols as chlorogenic acid, gallic acid, kaempferol, quercetin and salicylic acid from the extract of *C. intybus* were compared with the standard drug sorafenib. When choosing which ligand to investigate further as a potential lead compound, the Lipinski rule of five was taken into account. Among four other ligands that were chosen, caffeic acid was determined to be the most promising medication candidate due to its excellent ADMET characteristics, docking and MD simulation results. Taking into account all of the aforementioned characteristics, it was determined that caffeic acid could be a viable therapeutic option in the future for the treatment of liver cancer by targeting the RhoA gene.

There were 12 polyphenols identified in the methanolic extract of *H. rhamnoides* which include gallic acid, salicylic acid, vanillic acid, sinapic acid, ferulic acid, rutin, Hb acid, coumarin, chlorogenic acid, quercetin, kaempferol and caffeic acid. While in *C. intybus* 9 identified polyphenols were benzoic acid, gallic acid, vanillic acid, salicylic acid, chlorogenic acid, rutin, kaempferol, Hb acid and quercetin. The plant *H. rhamnoides* has higher activity and this can be attributed to the presence of caffeic acid in the plant. It can be combination of metabolites which is responsible for anticancer effect of both plants.

6.1 Future Recommendations

This study was performed to find the potential of plant extract-loaded iron oxide nanoparticles to target the RhoA gene in liver cancer. Despite that, there is still a need for more investigations and studies using animal models which will be helpful in further validation of this particular study. Some of the future recommendations are as follows.

1. Development of combined therapy as combining iron oxide nanoparticles with other therapeutic agents like chemotherapy drugs for synergistic effects.

2. Other types of cancers can also be targeted using iron oxide nanoparticles of plant extracts of *H. rhamnoides* and *C. intybus*.

3. Gene targets of metallic nanoparticles are used to target genes other than RhoA to analyze the efficacy of particular nanoparticles against genes other than RhoA.

4. Use of in silico analysis to apply nanoparticles loaded with plant extracts to individual patients based on their genetic makeup.

5. The extracts of *H. rhamnoides* and *C. intybus* can be used to target RhoA using other types of nanoparticles as gold, silver, zinc etc.

6. Comparative analysis of *H. rhamnoides* and *C. intybus* extract-loaded iron oxide nanoparticles can also be done for other types of cancers as breast cancer, lung cancer etc.

7. Designed nanoparticles can both diagnose and treat liver cancer, combining imaging and therapeutic functions.

8. The RhoA gene can be studied for its role in cancer development in other types of cancers as breast cancer, colorectal cancer etc.

9. The trials on animal models as well as clinical trials must be performed to observe RhoA gene as potential biomarker in liver cancer diagnosis.

10. Comparative analysis of the extract of *H. rhamnoides* and *C. intybus* nanoparticles for other types of metallic nanoparticles as silver, zinc and gold etc. can also be done.

11. More advanced techniques can also be applied to detect more bioactive compounds in both plants.

12. Synergistic effects of combining extracts of both plants can be studied, which will help to enhance treatment efficacy.

13. *In vivo* validation and clinical translation of findings can be conducted.

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