Phytochemical Investigation and Biological Evaluation of Selected Medicinal Plants of Pallas valley, Kohistan

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QUAID-I-AZAM UNIVERSITY

ISLAMABAD, PAKISTAN

2022

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A THESIS SUBMITTED TO THE QUAID-I-AZAM UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PLANT SCIENCES

DEPARTMENT OF PLANT SCIENCES
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
2022

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Dedicated to my wonderful husband and dear father-in-law, my kids, my parents, and respected teachers whom all enable me to achieve this goal.

Acknowledgments

All praises belong to Almighty ALLAH, the most merciful, beneficent, creator, and source of all knowledge and wisdom, who enabled me to complete this grand task successfully. I also pay my respect to Hazrat Muhammad (Peace and Blessing of Allah Be Upon Him), His faithful companions, who are forever a genuine torch of guidance for humanity.

I felt highly privileged in taking the opportunity to express my deep gratitude to my respectable supervisor Prof. Abdul Samad Mumtaz, whose guidance, kind behavior, suggestions, moral support, and fruitful ideas from preliminary to the conducting level during the entire research work that have made it possible for me to complete this manuscript.

I am incredibly grateful to Prof. Kyung Hyun Choi for his keen interest, precious guidance, and expertise to strive for excellence in research. On many occasions, his advice worked to settle down the desperation associated with the overwhelming experience of being stuck at some point.

My sincere thanks to Prof. Dr. Mushtaq Ahmad, Chairman Department of Plant Sciences, Quaid-i-Azam University Islamabad, for providing facilities and possible support during the research activities. I take this opportunity to record my sincere thanks to all the respected teachers of BPS for their unceasing encouragement and suggestions.

I also felt my gratitude to my senior research fellows, especially Dr. Muhammad Maqsood Alam, Mr. Farooq Inam, Dr. Syed Afzal Shah, Dr. Atif Khalil, Dr. Hussain Badshah, Dr. Sajjad Iqbal, Dr. Afzal Mir, for their guidance, courage, and moral support during research work. I want to express my heartiest thanks to my sincere friends and lab fellows Waqar, Miss, Surat tun Nisa, Kaleem, Roma Waqar, Sumreen Dawood for their cooperation, support, suggestion and bucking me up whenever I lost hope.

Finally, profound gratitude to my beloved family members, especially my father M. Sharif, and mother Miss Parveen Akhtar, whose bundle of prayers and love has always given me the courage to cope with difficult situations and hard times. Their love, prayers, and encouragement kept me focused through the thick and thin of the journey. Thank you for believing in me. I want to express my deepest thanks to my parents-in-law Prof. Dr. Muhammad Azeem Farooqi, Fouzia Azeem, and my brothers-in-law, sister-in-law, and other family members. I appreciate your love, fellowship, support, and motivation. Thanks for being a part of my success story.

I am most grateful to my wonderful husband, Dr. Hafiz Muhammad Umer Farooqi, for his moral and emotional support during my Ph.D. studies. I would not have made it without you. Thank you, my cute sons, Muhammad Dawood Farooqi and Muhammad Yousuf Farooqi, and adorable daughter Sarah Farooqi for being excellent and patient kids when I was doing my research. Finally, I'd like to thank all participants of this research for their valuable participation, who constantly encouraged and remembered me in their prayers for my success.

Farzana Kausar

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LIST OF ABBREVIATIONS

MDR	Multidrug resistance
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
FRAP	ferric reducing antioxidant power
BCB	*β*-carotene bleaching
ACM	*Acer cappadocicum gled* Methanolic Extract
ACN	*Acer cappadocicum gled* n-hexane Extract
ACC	*Acer cappadocicum gled* Chloroform Extract
ACE	*Acer cappadocicum gled* Ethyl acetate Extract
ACB	*Acer cappadocicum gled* n-butanol Extract
PCM	*Prunus cornuta* Methanolic Extract
PCN	*Prunus cornuta* n-hexane Extract
PCC	*Prunus cornuta* Chloroform Extract
PCE	*Prunus cornuta* Ethyl acetate Extract
PCB	*Prunus cornuta* n-butanol Extract
QSM	*Quercus semicarpifolia* Methanolic Extract
QSN	*Quercus semicarpifolia* n-hexane Extract
QSC	*Quercus semicarpifolia* Chloroform Extract
QSE	*Quercus semicarpifolia* Ethyl acetate Extract
QSB	*Quercus semicarpifolia* n-butanol Extract
HCl	Hydrochloric Acid
HPAEpiC	Human pulmonary alveolar epithelial cells
HRPTEpiC	Human renal proximal epithelial cells
SDA	Sabouraud dextrose agar
MPS	Microphysiological system
CLSM	Confocal laser scanning microscopy
IC_{50}	Inhibitory concentration 50%
MIC	Minimum Inhibitory concentration
SD	Standard deviation
NMR	Nuclear magnetic resonance

ABSTRACT

The research interests in plant-based drug development due to the hidden potential of chemically diversified elements and their bioactive potential focused the researcher's efforts on the unexplored flora of this planet. The use of natural products to cure human ailments against deadly infections and diseases like cancer is not a new approach in this advanced era. Still, several plants are under pharmacological investigations/studies to provide new substances with particular medicinal effects. Therefore, this study evaluated the antimicrobial, antioxidant, and anticancer potential of *Acer cappadocicum Gled*, *Prunus cornuta,* and *Quercus Semecarpifolia,* along with phytochemical analysis through various biochemical tests. Plant extracts were prepared through the maceration method with organic solvents of different polarities. The extracts were subjected to antimicrobial appraisal through the agar well method against *Kelabsiella pneumoniae*, *Escherichia coli*, *Bacilus subtilis, Salmonella enterica,* and *Acinetobacter baumannii.* DPPH method was used to study antioxidant potential and to evaluate the anticancer effect of extracts MTS assay was used, and percentage cell viabilities were determined. The extracts' anticancer potential was studied against five cancer cell lines: HepG2, Caco-2, A549, MDA-MB-321, and NCI-H1437. Furthermore, toxicity studies were performed on alveolar and renal primary epithelial cells. The results showed that methanolic and butanolic extracts of *A. cappadocicum gled*. showed maximum antibacterial activity. A similar pattern against mycelial growth inhibition in plant-fungal pathogen by methanolic and butanolic extracts of *Acer cappadocicum gled*. However, human pathogenic fungi were not affected much by extracts, and only Rozae sp. showed moderate inhibition. For antioxidant assessment, results indicate that chloroform extracts of *A. cappadocicum gled* showed the strongest scavenging potential among extracts and cytotoxic evaluation. *A.cappodocicum* extracts exhibited significant (p=0.05) cytotoxic activity against A549, Caco2, and MDA-MB-231 cancerous cells. The results suggested the maximum activity obtained by n-hexane extract of *P. cornuta* against *A. baumanni, S. enterica,* and *E. coli* isolates followed by butanolic extract of *P. cornuta* against *A. baumanni* and *S. enterica* isolates. The results showed that *P. cornuta* reduced cell viability in the lung (A549) and breast (MDA-MB-231) cancerous cells. The extracts affected less in $caco_2$ cells compared to other investigated species. The evaluation of the antibacterial potential of *Q. semicarpifolia* was maximum with

methanolic extracts in all five strains and with butanolic extracts against *A. baumanni* and *K. pneaumonia* bacterial isolates. The *Quercus semicarpifolia* extracts performed well in Caco2 and MDA-MB-231 cancerous cells and reduced cell viability to 25% and 20%, respectively. The IC_{50} values demonstrated that the chloroform extract of *A. cappadocicum*, chloroform extract of *P.cornuta*, and methanolic extract of *Q. Semicarpifolia* showed potent antioxidant potential with 0.3795,0.4885 and 0.4003, respectively. The results suggested that the selected plants can be further assessed to treat bacterial infections and cancer disease with pharmacological and phytochemical perspectives. Among the selected plants, the inhibitory effect of *Acer cappadocicum Gled* extracts, good scavenging ability, and low cell viability of extracts against human cancer cell lines suggested that *Acer cappadocicum Gled* can be a potential material for natural product research.

1. INTRODUCTION

1.1. PHARMACOLOGICAL SIGNIFICANCE OF PLANT MATERIALS

Plants have been used since before recorded history and still fulfil our need to ensure better health for human beings. The detailed study of plants comprised of bioactivities and phytochemical analysis revealed phenolics profiles, saponins, tannins, quercetin, etc. Local plants are documented as sustenance for domestic and wild animals, nutritional and vitamin additives for people, and elements of many indigenous medicines in hilly terrain cultures [1]. Plants are used for nutritional purposes and for eliminating many health problems, and the therapeutic value of many plants remains undiscovered. About 80% of percent population in developing countries directly rely on plants for therapeutic purposes [2].

Plants are a significant source of herbal medicines [3]. Globally 119 drugs are derived from plants, and 74% were isolated during the chemical investigation from plants effective for human disease control [4]. Various studies on plants reported their biological potential as anti-inflammatory, anticarcinogenic, antibacterial, antifungal, antiviral, antimutagenic, and antiallergic [5]. In addition, the antioxidant property of plants is responsible for biological activities. Most importantly, the antimicrobial potential of plant extracts provides the basis for natural remedies [6].

Considering that almost 80 % of the world depends on traditional plants for disease cures. Nearly 70,000 plant species have been used for medicinal purposes. Examples of plant constituents in herbal medicines have been isolated and synthesized, like reserpine, taxol, and vincristine [7]. The pharmacological potential of plants is due to phytoconstituents or secondary metabolites. The medicinal properties result from metabolic pathways, the genetic structure of the plant, and environmental effects. The importance of medicinal plants in drug development is known to us, and humans have used them for different diseases from the beginning of human history. Traditional folk treatment from wild plants has always guided researchers to search for novel medications to develop healthy life for humans and animals. In addition, some medicinal plants are still obscured within the plant kingdom, which needs to be scientifically evaluated [7].

1.2 PRESENCE OF PHYTOCHEMICALS AS BIOACTIVE CONSTITUENTS IN PLANTS

The use of natural compounds obtained from microbes, animals, or plants has shown promising results to overcome bacterial resistance in microbes. These compounds showed various potent antioxidant, antibacterial and antifungal activities. Several plant-derived compounds with efficient antimicrobial potency can be classified into alkaloids, sulfur-containing compounds, terpenoids, polyphenols, etc., based on their chemical structures [8].

A) ALKALOIDS

Alkaloids are heterocyclic natural compounds with a variety of different structures. They are the natural inhibitors of bacterial growth. Examples of plant alkaloids are Piperine, Berberine, and isoquinoline alkaloid. Quinoline alkaloids such as Dictamnine, Kokusagine, and v. masculine. Reserpine is an indole alkaloid found in *Rauwolfia serpentina*. It has been found to increase antibiotic susceptibility in various bacteria, including *Staphylococcus* species, *Streptococcus* species, and *Micrococcus* species. In addition, multi-drug resistant (MDR) isolates of *A. baumannii* have shown remarkable susceptibility to risperidone. In addition to these sanguinarine alkaloids, Tomatidine is a steroidal alkaloid found in solanaceous plants, and Chanoclavine is a tricyclic ergot alkaloid in *Ipomoea muricata*. It has been shown to inhibit MDR *E. coli*. The steroidal alkaloid Conessine, derived from the bark of *H. antidysenterica*, is effective against various pathogenic bacterial isolates [8].

B) ORGANOSULFUR COMPOUNDS

There is extensive research on plant-derived sulfur-containing compounds' antimicrobial potential. Allicin (diallyl thiosulfinate) from Allium sativum and ajoene from garlic are organosulfur compounds. Capparales and Brassicaceae family members have been abundant in these phytochemicals and showed remarkable inhibitory effects against pathogenic bacteria.

Sulforaphane is a chemical found in various plants, including *Diplotaxis harra*. It's made from glucosinolates and has shown to have antitumor and antibacterial properties, particularly against *Helicobacter pylori*, a possible cause of stomach cancer. *S. aureus* and *Listeria monocytogenes* are also vulnerable to sulforaphane. [9].

C) PHENOLIC COMPOUNDS

Natural phenolic substances that demonstrated inhibitory effect include Resveratrol, baicalein from thyme (*Thymus vulgaris)*, Chinese skullcap (*Scutellaria baicalensis)*, and blue skullcap (*Scutellaria lateriflora)*, and Kaempferol, polyphenols (tannins), and anthraquinones. Chebulinic acid, originally isolated from black or chebulic myrobalan (Terminalia chebula), and Curcumin, which was acquired from Tumeric, demonstrated bactericidal efficacy against infections *S. aureus* and *E. coli* [10].

D) COUMARINS

Plants naturally produce coumarins. Many coumarins have properties like vasodilative, estrogen hormone production, decoagulant, pain-relieving, inflammation reduction, anticancer and antioxidant activities. For instance, coumarins and pyranocoumarins from the roots of *Ferula brachyloba (Boiss. & Reut.) Nyman*. Several coumarins, such as agasyllin, grandivittin, and aegelinol benzoate, showed antibacterial activity against infectious microorganisms. [8].

E) TERPENES

Terpenes are considered the most diverse group of phytochemicals, which also maintain the structure of cell membranes and perform multiple cell functions; for instance, photosynthesis uses carotenoids, and the electron transport chain uses quinones. They are most abundant in flowers (reproductive structures), fruits, vegetables, and foliage of plants. They are also an essential part of herbal resins, and many terpenes and their derivates impart defense against herbivores and pathogens [8].

1.3. PLANTS: A POTENTIAL NOVEL SOURCE OF ANTICANCER THERAPEUTICS

Globally cancer is the most common cause of human deaths and a vital problem to community health. This disease results in up to 70% of deaths globally, most rapidly in Africa, Asia, Central, and South America. Cancer is a broad term for uncontrolled cellular propagation, infiltration of the atypical cells into neighbouring tissues, and their proliferation to remote organs through blood and lymphatic systems. The process of cancer formation involves converting healthy cells to a pretumor wound which later grows into a fatal tumor in a multistep pathway. These alterations result from the

interlinkage between an individual's genetic structure and environmental causes, including physiochemical and biotic mutagenic substances [11].

1.3.1 EXTERNAL FACTORS OF CARCINOGENS

I. In physical factors, Radiation like UV and electromagnetic rays are primarily associated with skin and blood cancers [12].

II. In chemical factors, naturally occurring mineral compounds composed of soft and flexible fibers known as asbestos result in lung cancer and mesothelioma. The inhaled asbestos results in cancer growth in the lining of lungs, abdomen, or heart. Other examples include tobacco smoke, aflatoxins produced by certain fungi, and arsenic-contaminated water resulting in cancer proliferation in the lung, breast, bladder, and kidneys. [12].

III. In biological factors, viruses like papillomavirus, the hepatitis B virus, the hepatitis C virus, a few bacterial species, e.g., *Helicobacter pylori*, and cancer due to certain fish-parasitic flatworms, are a few more examples of biotic factors of cancer [12].

1.3.2 CANCER EPIDEMIOLOGY

The International Agency for Research on Cancer estimates that by 2030 the number of newly reported cancer cases will be 26 million, and the death rate will be 17 million per year. Cancer Research data in 2018 by the International Agency of Research on Cancer suggest that mortality due to cancer will be a vital cause of death worldwide in the twenty-first century. The most widespread cancer was respiratory and female breast cancer, about 11.6%, followed by colon and rectum cancer, almost 10.2 %, a prostate which is 7.1% in its prevalence, then stomach and liver cancer up to 5.7% and 4.7 %, respectively. The higher rate of cancer fatalities was associated with lung, colorectal, stomach, liver, and breast cancers. In males, the percentage death rate due to commonly diagnosed cancers was lung (20%), prostate (6.7%), colorectal (9.0%), stomach (9.5%), and liver cancer (10.2%). The percentage death rate of most frequent cancer in females was those of breast (15.0%), colon and rectum (9.5%), lung (13.8%), and cervix uterus (7.5 %) [13].

1.3.3 CANCER EPIDEMIOLOGY IN SOUTH ASIA

South Asian countries like Pakistan, India, and Sri Lanka, with a total population higher than 1,500 million, have a similar pattern for chronic disease prevalence. These similarities required a mutual approach to cancer disease control and the development of cancer control programs. In males, oral and lung cancer are most common, apart from Quetta in the far north, Larkana, and Chennai. Pharyngeal and laryngeal cancer is moderately high, followed by prostate cancer becoming more common in developed cities. In Pakistan, oral and breast cancers are number one and two on the cancer prevalence list. Ovarian cancer is also included in the five most common cancer types. With time, the increasing rate of breast cancer may affect cancer occurrence with increasing obesity and aging problem. The combined effort within South Asia is significant for cancer control in the region [14].

1.3.4 THERAPEUTIC TREATMENTS FOR CANCER

The therapeutic methods for cancer depend on their types, stage, and grade. Recent treatments include chemo & radiotherapy. The surgical method is used to remove tumor tissue-restricted to certain areas or widespread tumors for facilitating more effective chemotherapy. The therapy with radiation uses electromagnetic rays to damage cancer cells DNA. Antineoplastic agents or hazardous drugs in combination are a part of systemic chemotherapy treatment to prevent cancer proliferation by slowing or stopping the division of cancerous cells. But it is also used as a supportive approach to prevent disease recurrence after surgery or radiation therapy or to minimize the risk of re-occurrence of cancer after initial surgery or radiation treatment. Other cancer treatments are immune, hormonal, and Angio suppressive [15]. Treatment of cancer through immunotherapy involves strengthening the immune system to fight against cancer. Hormonal therapy inhibits the growth of hormone-related tumors which do not qualify for surgery or radiotherapy. In Angio suppressive therapy, angiogenic signals are emitted from the tumor to attain blood flow and result in shrunken tumors [15].

Despite various therapeutic methods and treatments, this disease remains a fatal problem and implies a surge for more efficient treatments. Therefore, more stress is given to the early diagnosis of disease and identifying more potent drugs to develop a targeted treatment. The researchers considered using ancient medicinal plants and

exploring wild plants to discover new anticancer drug sources as an essential alternative to recent cure methods [16]. Examples of such approaches are vincristine, paclitaxel, irinotecan, etoposide, and various notable anticancer drugs from natural sources [17]. Cancer is a fatal problem worldwide; despite so many efforts, synthetic chemotherapeutic agents have not satisfied the patients irrespective of their high-cost development. Since ancient times, phytochemicals have been used to treat human ailments. However, natural products have gained popularity over the last 3 decades for their significance in cancer prevention and medication. Besides this, there is growing evidence of phytochemicals as inhibitory substances of cancer proliferation and inflammation. Therefore, it signifies plant-based research for cancer control programs.

1.4 PLANTS: A POTENTIAL NOVEL SOURCE OF ANTICANCERS

Natural products have been the source of around 60% of modern cancer treatments, with the plant kingdom being the most prominent source. Some of them are Vinca alkaloids, Taxus diterpenes, Camptotheca alkaloids, and Podophyllum lignans. There are currently 16 novel plant-derived drugs in clinical trials, 13 in phase I or II and three in phase III. Flavopiridol, derived from the Indian tree *Dysoxylum binectariferum*, and meisoindigo, derived from the Chinese plant *Indigofera tinctoria*, have both been proven to have anticancer properties while being less harmful than traditional medications. Medicinal herbs are a popular alternative to drugs for cancer cure. Around 3000 plants have been suggested to possess anticancer potential [17]. Only a tiny portion of the plant kingdom has been studied for pharmacologically active plant compounds with cancer-fighting potential. As a result, it's possible that more research into rare and wild plants, as well as other less-explored settings, can lead to the discovery of several structurally innovative and mechanistically distinct cancer-fighting chemicals. Therefore, this study investigates the anticancer properties of a few plants and their possible utility in combating cancer.

1.4.1 MEDICINAL AND BOTANICAL USES OF SELECTED PLANT MATERIAL

Table 1-1. Traditional uses of selected plant species

Sr. No.	Plant Specie	Family	Habit	Medicinal Use	Botanical Use
1	*Acer cappadocicum Gled.* (Chin)	Sapindaceae	Small tree	Root extract is used in wound healing in animal foot diseases. Healing herb.	Fuel wood and Utensils making.
2	*Prunus cornuta* (Bhareet)	Rosaceae	Medium-sized tree	In small amounts stimulates respiration and improves digestion. Fruit is also used as energy and man tonic.	Oil from the kernel is a substitute for bitter almond oil.
3	*Quercus semicarpifolia* (Banjar)	Fagaceae	30m tall evergreen tree	Bark juice is used for muscular pain. Galls on the tree used for hemorrhage, dysentery & chronic diarrhea	Construction & fuelwood yield ggood-quality charcoal. Galls are a source of tannins used for dying purposes.

Table 1-2. Herbal efficacy of selected plant material established from literature.

Sr.	Plant Specie	Traditional Use	Literature Source	Reference
1	*Acer cappadocicum Gled.* (Chin)	Tonic for general health improvement	Book	Quattrocchi, U. (2012). World dictionary of medicinal and poisonous plants
2	*Prunus cornuta* (Bhareet)	Antimicrobial	Research article	MC Prohit et al, 2017.
3	*Quercus semicarpifolia* (Banjar)	Remedy for muscular pain, acute diarrhea, dysentery, hemorrhage & scabies	Books & research articles	Plants and people of Nepal (book by Manadhara N.P). A modern herb by Grieve (1984). Kala, Chandra Parkash. (2007). Local preferences of ethnobotanical species in the Indian Himalaya: Implications for environmental conservation. Current Science 93. 1828-1834

1.5. SELECTED PLANT SPECIES/ DESCRIPTION/ CYTOTOXICITY

1.5.1 *Acer cappadocicum Gled*

The trees are 20m in height with dark brown colored smooth bark. Twigs are glabrous and shiny. Leaves are deciduous, 5-15 cm across with 5-7 acuminate lobes, round sinuses, and entire margins. The base of leaves is subcordate-truncate, and leaves are glabrous except for nerve axils. Petioles are cylindrical, glabrous, and 3-15 cm in length. Flowers are pentamerous,6-8mm across, yellow and green. Sepals are oblong, 3-4 mm long. Stamens are 8 in number and inserted on a disc structure. The ovary is glabrous; styles are two and free. This Acer species is distributed in N.E. Turkey, Caucasus, N. Iran, the Himalayas to China, and Japan.

Figure 1.1 Morphology of *Acer cappadocicum Gled.*

The Genus *Acer* (Aceraceae) comprises 129 species that occupy the northern hemisphere areas and temperate areas of East Asian countries like China, Japan, South, North Korea, America, and Europe. Maple plants are traditionally famous for treating joint pain, bruising, renal issues, eye diseases, pain relief, and detoxifying agents. The genus Acer has been linked to pharmacological actions in over 40 species, 11 subspecies, and one variant. The Aceraceae family contains around 331 chemicals, including flavonoids, tannins, terpenoids, benzoic acids, glycosides, and alkaloids. The anti-inflammatory and hepatoprotective qualities of *Acer* species stated in traditional Aceraceae knowledge were corroborated by the biological activities of these plants. [18].

Wu Bi et al. (2016) [18] reported the traditional medicinal benefits of *Acer* species for the first time. Traditional Chinese medicine (TCM) has identified a variety of medical qualities of 20 *Acer* species, including body purification, rheumatism, and enhancing eyesight and used to treat sore throats, dyspepsia in children, high blood pressure, and tuberculosis, among other things. Traditionally, the roots, leaves, branches, twigs, or fruits have been utilized as a decoction or in tea form in combination with other TCM in the form of oil/food supplements. *Acer tataricum*

subsp. ginnala (Maxim), for example, was utilized to alleviate heat, improve eyesight, and preserve the liver. In Japan and Korea, the bark of *Acer maximowiczianum Miq*. has traditionally been used to treat eye and liver problems. The leaves of *Acer pictum Thunb* have traditionally been used to treat hemostasis. The sap of *Acer pictum Thunb* has traditionally been used to treat urinary, constipation, and gastrointestinal problems. In North America, twelve species of *Acer* have been used as a source of nutrition and medicine. The sap of *Acer* plants is consumed as maple syrup; a natural sweetener, e.g., *A. rubrum L.,* A. *Saccharum Marshall*, was used by the indigenous peoples of eastern Canada to treat breathlessness and coughing *A. pensylvanicum L*. was used to treat bronchitis, colds, cough, gonorrhea, and kidney dysfunction. [18].

1.5.2. ANTICANCER ACTIVITIES OF ACER SPECIES

Previously, various studies demonstrated the growth inhibition of cancerous cells by the extracts and compounds of Acer species. The underlying mechanism of inhibition suggested after research includes apoptotic and cell cycle arrest. Many active substances showed inhibition against B16 melanoma cells, most importantly glucopyranose and methyl gallate. Moreover, five different triterpenoid compounds from *A. mandshuricum Maxim* showed growth inhibition against four cancerous cells. In another study, the geraniin compound was isolated from *A. maximowiczianum Miq.* Inhibited the tumour numbers per mouse. Also, methanol extracts of *A. maximowiczianum Miq.* Exhibited anticancer effect in B16, HL60, and CRL1579 human melanoma cell lines. Acer species: *A. rubrum L*. and *A. saccharum Marshall* exhibited antitumor activity against various human cancerous and non-cancerous cell lines. *A. rubrum. L* exhibited potent inhibitory activity in colon cancer cell lines due to the presence of ginnalin A. Moreover, aqueous extracts of *A. tegmentosum Maxim*. studied by Kim et al. 2015 exhibited cytotoxic activity [18].

1.5.3. *Prunus cornuta*

A medium-sized tree (15 to 20 m tall) grows in temperate and subalpine areas. Leaves are simple, 8-10 cm long, oblong to lanceolate shape with toothed margins. inflorescence type is a drooping raceme with a 20 cm length. The flower symmetry is actinomorphic, 10 mm cross-sectionally with pale white coloration. The flowering

period of the plant extends from March to May. The oil from Kernel is an alternative to bitter almond oil locally [19,20].

Figure 1.2 Morphology of *Prunus cornuta*

1.5.4. ANTICANCER ACTIVITIES OF PRUNUS SPECIES

Prunus species have been investigated for their antibacterial, anti-inflammatory, and antioxidant properties [21]. Some Prunus species' cytotoxic and antitumor activities are remarkable, e.g., The *Prunus africana* (Hook.f.) Kalkman also known as African cherry, has been used as ethnomedicine to cure prostate cancer in the form of decoction in Uganda and has been patented in France for this cancer cure [22]. *Prunus spinosa* was tested for anticancer efficacy against human colorectal cancer (HCT116) in another investigation, colorectal adenocarcinoma (SW480), cervical (Hela), and lung (A549) cancer cell lines. The inhibitory effect of *P. spinosa* was up to 40% in HCT116 and SW480 concerning control [23]. Moreover, the anticancer activities of *Prunus domestica* were observed against colorectal carcinoma (Caco2) with IC_{50} 8.5µg/ml, breast cancer (MCF-7) with 43.5 µg/ml, and liver carcinoma (HepG2) with 23.1 µg/mL values [24]. Another Prunus species known as *Prunus mume* (Chinese plum or Japanese apricot) exhibited cytotoxic effects against B Lymphoma (BAL-M3), Melanoma (A375, SK-MEL28), lung cancer (H661, H157, H1299, A549),

Pancreatic cancer (PK1, PK54H), Liver cancer (HepB3), Colon cancer (SW480), Breast cancer (MCF-7, MDA-MB-468) and esophageal cancer (YES62) [25]. Furthermore, *Prunus serrulata var. spontanea* inhibited the growth of colon carcinoma (HT-29) with the highest inhibition (38.8%) at 500 µg/mL of methanolic extracts [26].

1.5.5. *Quercus semicarpifolia*

Q. semicarpifolia is a 20-meter-tall shrubby tree with tomentose young shoots and oblong to elliptical leaves. Leaves are glabrescent dorsally and rusty ventrally with leathery, forked vascular bundles and entire spinose margins. The 4-8 cm long male inflorescence, dense flowered, stamens 5-8 in number, filaments 2mm in length, and anthers are oblong with a glabrous surface. The nuts are 2.2-2.5 cm long with a glabrescent surface and dark brown when mature. The *Q. semicarpifolia* is distributed in Afghanistan and the Himalayas from Swat and Kashmir to Bhutan and China. It grows on 2400-3600 altitude [27,28]. *Quercus* species, commonly known as oaks, belongs to Family Fagaceae. They are widely distributed in temperate and tropical forests. Traditionally, they have been used to cure human diseases like asthma, hemorrhoid, diarrhea, gastric ulcers, and wound healing. Because of the presence of triterpenoids, phenolic acids, and flavonoids, they have exhibited anti-inflammatory, antibacterial, hepatoprotective, antidiabetic, anticancer, gastroprotective, antioxidant, and cytotoxic effects. Genus *Quercus* comprises 600 species worldwide and is mainly distributed in the Mediterranean, Asia, and North American basins. America and Asia are high-diversity areas of Quercus species, and Europe shows less diversity [28,29].

Quercus species have been used in gastrointestinal disorders such as diarrhea and hemorrhoids and as an antiseptic product in folk medicine. The bark of oaks exhibited numerous antiseptics and homeostatic properties to treat inflammation, gastro problems, toothache, and healing after skin burns. The resins of *Q. leucotrichophora A. Camus* were used to treat gonorrhea, asthma, hemorrhages, and powder of gallnut of *Q. infectoria* has been reported to treat uterine wall and ulcers. Acorns (fruit) of the *Quercus* species was considered an energy source to cure diarrhea, menorrhagia, obesity, and stomach ulcers [29]. Antioxidants, antimicrobials, anti-inflammatory, antidiabetic, hepatoprotective, gastrointestinal disease, skin disorder, antiobesity,

anticancer, and neurogenerative effects have been researched in the *Quercus* genus. All pharmacological effects are due to chemical components of the *Quercus* species, mainly triterpenoids, tannins, and flavonoids. Different studies show that acorns have solid antioxidant potential, which helps combat oxidative stress-related diseases like diabetes, cancer, cardiovascular and inflammatory diseases [29].

Figure 1.3. Morphology of *Quercus semicarpifolia*

1.5.6. ANTICANCER ACTIVITIES OF QUERCUS SPECIES

Several studies have confirmed the antitumor and cytotoxic effect of extracts of genus *Quercus* against different cancer cell lines. For Instance, methanolic and aqueous extracts of bitter oak, Caucasus oak, and Boz-Pirnal oak were evaluated against HepG2 Cells. Results showed that aqueous and methanolic extracts of Caucasus oak exhibited IC_{50} values of 165.291 µg/mL and 273.771 µg/mL, respectively, demonstrating the highest cytotoxic effect. Furthermore, ethanolic extracts of *Q. ilex* have shown cytotoxic effects at various concentrations from 250, 500, and 1000 mg/mL in a dose-dependent manner [29]. The triterpenoids isolated from *Q. robur* have been studied for cytotoxic effects against prostate (PC3) and breast adenocarcinoma (MCF-7) cell lines, Breast cancer cell lines (MCF-7) were most affected by triterpenoids compounds [30]. In addition to this, leaves extracts of *Q.*

resinosa were studied by single-cell electrophoresis assay on HeLa cells confirming that chemicals present in decoction were responsible for oxidative damage to DNA in human cells [31].

Moreover, *Q. incana* leaves were evaluated for apoptotic and antimetastatic activities against lung cancer cell lines. Results obtained indicated that cancer cell growth significantly reduced dose-dependent after treatment with botulin extracted from leaves of *Q. incana* [31]. Moreover, *Quercus suber L*. cork extracts induced apoptosis in HL-60 cells (human myeloid leukemia) [31].

1.6. ANTIBACTERIAL ACTIVITIES OF MEDICINAL PLANTS

Human ailments due to bacterial infections are a vital and all-time health issue worldwide. Several human disorders can be attributed to various bacterial isolates, such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Proteus vulgaris*. Furthermore, these bacterial isolates tend to develop resistance to antibiotics by modifying their genetic makeup. Also, toxicity and safety studies of antimicrobial agents are not adequately addressed. Thus, there is a huge necessity to develop an antimicrobial drug from natural sources against resistant strains [32,33].

Crude extracts have been investigated as antibacterial drugs against infectious diseases. Phytochemicals such as phenols, polyphenols, tannins, terpenoids, alkaloids, and flavonoids exhibited potential inhibitory activity against various bacterial species [33]. Various spectroscopic methods confirmed the plant chemicals. In another study, researchers studied the extracts of *Gnetum africanum* prepared in aqueous and ethanolic solutions. In *G. africanum*, results showed that plant elements (alkaloid, flavonoid, tannin, phenol, saponin, sterol, terpenoid, and cyanogenic glycoside) were presently responsible for antifungal and antibacterial activities against selected clinical isolates. In addition, different studies indicated that secondary metabolites from plants such as flavonoids, and glycosides possess *in vitro* antimicrobial potential [34].

In Pakistan, several medicinal plants have been investigated against microbes. The most reported Pakistani varieties with antibacterial potential against human pathogens were *Bergenia ciliata*, *Jasminum officinale*, and *Santalum album*. The aqueous

extracts of these plants were evaluated against *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Escherichia coli* using the agar well method [35]. In the late 1990s, plant extract usage in medical procedures increased. In developing countries, herbal medicines are still used to deal with health-related issues. Plant-derived compounds are a beneficial source of medicine for their diverse pharmacological and biological potential, including antibacterial activity [36].

Several other plants all over the world have been exploited for antibacterial potential. For example, *Cassia fistula* has been used to cure skin diseases, liver troubles, tuberculosis, and diabetes. Etc. Besides these pharmacological uses, *Cassia fistula* showed significant antibacterial activity, and its hydroalcoholic extracts have been investigated for new antimicrobial agents [37]. Another plant *Carpolobia lutea* usually grows in Asia and Africa, has been found to have antibacterial activity and anti-inflammatory, antimalaria, and gastroprotective effects [38]. In another study, *Cassia auriculata* exhibited solid inhibitory activity of bacterial strains by methanolic and chloroform extracts. Phytochemical screening of extracts showed that antimicrobial activity is probably linked to the presence of secondary metabolites [39].

1.7. ANTIFUNGAL POTENTIAL OF MEDICINAL PLANTS

Fungal pathogens causing human morbidities include *Aspergillus fumigatus* (pneumonia), *Fusarium spp*, *Penicillium marneffei*, *Coccidioides inmotis*, and *Histoplasma capsulatum*, *Candida spp*. (nosocomial infections). They caused about 88% of infections in the United States between 1980 and 1990. Furthermore, Candida albicans is responsible for nearly 3,400 cases of blood infections in 2013, USA [40]. Besides human fungal infections, plants suffer significant losses due to fungal diseases. For Instance, rust diseases due to *Puccinia spp*., *Fusarium* head blight because of *Fusarium graminearum*, and necrotrophic diseases by *Alternaria species* [41, 42]. Medicinal plants with antifungal potential have been reported all over the world. The methods used for this activity include disc diffusion, micro broth dilution, and percent spore germination inhibition tests. Literature showed that plants like *Solanum xanthocarpum* and *Datura metel* could inhibit the growth of fungal species.

Methanolic extracts of the plants mentioned above inhibited the invitro growth of *Aspergillus fumigatus*, *A. flavus*, and *A. niger* with 1.25–2.50 mg/mL MICs [43].

Recently, very few effective drugs have been available in the market with claimed antifungal treatment for plant diseases, animal fungal infections, and human fungal diseases. There is always a gap in the unmet demands of developing the antifungal product from natural flora with diverse chemical structures and a new way of action. The discovery of antifungal substances is essential to researchers due to the high emergence rate of fungal infections and resistance to recently used antifungal products [44]. A study by S. M. Mahlo et al., 2016 is an efficient example of plant extracts having potential antifungal activity against animal fungal pathogens, e.g., *Candida albicans* and *Cryptococcus neoformans* [44].

1.8 ANTIOXIDANT POTENTIAL OF PLANTS

Plant secondary metabolites have a protective effect on reactive oxygen species. Thus, the protective effect of bioactive compounds from plants over cancer or other chronic diseases can be attributed to their antioxidant-rich property and scavenging activities. Many epidemiological studies suggest that the decline in premature death due to cancer and other persistent disease is related to the intake of antioxidant-rich food. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) were produced during metabolic processes, and their production was countered by endogenous antioxidant systems. Sometimes, free radical levels increase at the cellular level and result in cell death or oxidative stress. Oxidative stresses have been recognized as responsible for cell component damage and initiating many diseases like liver function disorders, metabolic diseases, diabetes mellites, autoimmune diseases, and heart diseases. Thus, it becomes necessary to depend on an antioxidant-rich diet or supplements as therapeutic agents to combat oxidative stress [45]. Halliwell and Gutteridge defined antioxidants as a substance that can inhibit or slow the oxidation of any molecule even when present in low concentrations. Naturally, human beings have an antioxidant mechanism, and many anti-mutagenic functions originate from this property. Stabilization of free radicals most often occurs before they infect biological cells. But sometimes, the cellular level of free radicals exceeds so it becomes inevitable to intake antioxidants supplements or dietary antioxidants sources

[56]. Natural antioxidants have been extensively used in food, cosmetics, and pharmacy. Intake of food consisting of fruits and vegetables lowers the probability of lifelong diseases. Phenolics are considered for this physiological functioning of fruits and vegetables and give characteristic flavor and color to food. Plant polyphenols have been assumed to have health benefits against aging, cancer, and cardiovascular diseases. Therefore, natural antioxidants have become a significant area of research [47, 48].

Many chronic and acute diseases such as aging, diabetes, and neurodegeneration are caused by free radicals. Antioxidant substances such as phenolic acids, polyphenols, and flavonoids scavenge free radicals such as peroxide, hyperoxide, and lipid peroxyl [49]. Plants are a rich source of phenolics, flavonoids, tannins, and proanthocyanidins. The antioxidant constituents of plants offer protection against diseases. The intake of dietary antioxidants substantially reduced deaths from degenerative diseases [50].

Several medicinal plants have been previously studied for antioxidant potential. For example, *Lantana camara L.* (Verbenaceae) is an invasive plant with significant antioxidant potential. The methods used to evaluate antioxidant potential include DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay, Xanthine oxidase inhibition assay, Superoxide scavenging assay, and determination of total phenolics content [49]. Other complementary assays to analyze the extracts for their radical-scavenging activity include 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), superoxide anion (O_2^-), and nitric oxide (NO) radicals, for their ferric reducing antioxidant power (FRAP) and the capacity to inhibit lipid peroxidation by *β*-carotene bleaching test (BCB) [50].

1.9. HEPATOTOXIC EFFECT OF PLANT EXTRACTS ON LIVER-ON-CHIP

Chronic liver diseases result in 2 million deaths worldwide each year. In addition, due to hepatitis b, misuse of alcohol, metabolic syndromes, and obesity-like issues, the incidence of liver cirrhosis and hepatocellular carcinoma are a constant pressure-triggering element for hepatic disease prevalence [51]. Currently, vaccination, early screening, and antiviral treatments have substantially reduced liver ailments, but the lack of efficient in vitro models is one of the main hurdles in discovering new treatments for liver cirrhosis and carcinomas [51,52]. Organ-on-a-chip is a technology

that mimics the environment of the human organ system in vitro conditions. It is the mixture of biology, chemical, and material science fields which collectively engineered a biomedical device. It can be referred to as the "physiological organ system on a microfluidic chip." The microenvironment of the chip regulates the principle of the tissue system. It can predict the response of any external factor or drug with more precision when compared with other *in vitro* models [52,53]. To better understand human body functions, the direct approach involves an in vivo study of human models or animal models. Our body functions mainly depend on substances or molecules at the tissue, cells, and protein level. Therefore, it is impossible to understand the underlying mechanism of physiological processes only through *in vivo* studies. The expensive and low throughput in vivo testing pushes the biologist to test the substances on invitro cell culture models. 2D culture systems served as a platform for testing and toxicology studies for several years. However, drug testing in the 3D culture system provides more accurate and comparable results to the human tissue system concerning the combined effect of all microenvironmental factors in preclinical studies. That's why organ-on-chip technology is considered an advanced approach in drug testing and toxicology. Low reagents cost, controllable volumes and quick mixing, fast response or effect, and more precise physical and chemical elements gave an edge to this lab-on-chip approach in microfluidics [53,54].

2. AIMS AND OBJECTIVES

1. Primarily, to examine the inhibitory effects of various plant extracts prepared in different organic solvents against human cancer cell lines (gut, liver, lung, and breast), as well as human primary cell lines for cytotoxicity and safety study.
2. The present study was planned to conduct biochemical profiling of indigenous medicinal plants of Kohistan valley, Pakistan.
3. Qualitative phytochemical analysis of the selected plants for the confirmation of the presence or absences of various important secondary metabolites viz isoflavones, sesquiterpene lactones, phenolics, flavonoids, and non-alkaloids in extracts.
4. To evaluate various solvent extracts' *in vitro* antibacterial activity against clinical pathogens.
5. To assess various plant extracts' mycelial growth inhibition potential for selected fungal species.
6. Determination of antioxidant potential of selected plants.
7. Study of the effect of methanolic extracts of *A. cappadocicum* on HepG2 cancer cell lines in liver-on-chip model.

Overall, this study may provide the bioactivity details of wild plants with pharmacological potential.

3. MATERIALS AND METHODS

3.1 BACKGROUND ON PALLAS VALLY: STUDY AREA (GEOGRAPHY, POPULATION, DEMOGRAPHICS, AND ECONOMY)

The Himalayas mountains are the highest and steepest mountains in the world, spreading from Pakistan to Bhutan. Geographically they are divided into Eastern Himalaya (Nepal and Bhutan), Central Himalaya (India), and Western Himalaya (Pakistan and India held Kashmir). The climate of Western Himalayan mountains varies from tropical at the base and snowy at the elevations. The vegetation of the Western Himalayas includes temperate forests, pine forests, alpine shrubs, meadows in the North-western Himalayan, and conifer forests in the Western Himalayas. 70 to 80 percent of Indus River delta water is collected in the Western Himalayan from melting glaciers and snow. The Machiara National Park, Chitral Gol National Park, and Pallas valley are ecoregions of Western Himalaya [55].

Pallas valley is located at 72° 57′ longitudes, and 34° 44′ latitude and lies in the Kohistan district, Northwest Frontier Province (Figure 3.1). Approximately 60524 individuals and 1300 square kilometers with an altitude range from 7000-5200 m. The Pallas forests are a source of wood for construction, fuel, medicine, and trade. Climate conditions vary from sub-tropical at lower elevations to alpine at higher elevations with mixed temperate forests in between. Pallas valley represents the west Himalayan tract forests and is built under constraints of seasonal variations [56]. Pallas has several medicinal and commercial plants. Most importantly, *Saussurea costus* (Minyal), *Valeriana* sp. (Mushkbala), *Angelica glauca* (Chur), *Ajuga bracteosa* (Buti), *Rheum webbianum* (Chotyal), and *Skimmia anquetilia* (Namer). Commercially essential species are *Valeriana*, *Podophyllum*, *Saussurea costus* (Minyal), and *Viola Lilio*, *Acer cappadocicum gled*(chin), *Prunus cornuta* (Bhareet) and *Quercus semicarpifolia* (banjhor) [56]. The reported folk uses of *Prunus cornuta* are firewood, fodder, and medicine. The leaves of *Rhamnella gilgitica* are used in cooking, and the fruit is edible. *Parrotiopsis Jacquemontiana* is famous for skin and wound healing. *Acer cappadocicum Gled* is locally used for wood and fuel purposes [57,60].

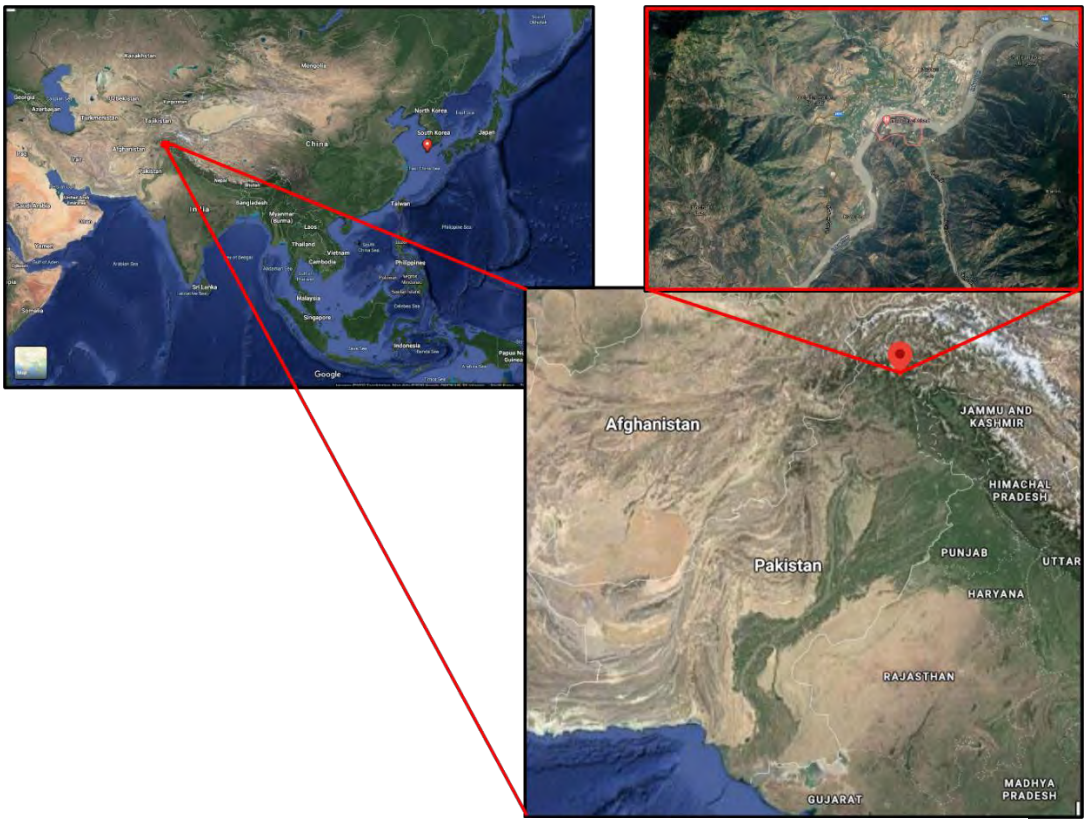


Figure 3.1 Location of Pallas valley, Kohistan, Khyber Pakhtunkhwa, Pakistan.

3.2. COLLECTION AND PREPARATION OF PLANT MATERIAL

For plant material collection, extensive field trips were made in Pallas valley, district Kohistan, Khyber Pakhtunkhwa. The coordinates of area were 36.6 ◦ N to 73.00 ◦ E. With the help of local people, fresh plant material of *Acer cappadocicum*, *Prunus cornuta,* and *Quercus semicarpifolia,* locally known as Chin, Bhareet, and Banjar plants, were collected during the spring season from March to May 2018 (Figure 3.2). In addition, biological authentication was performed by consulting plant taxonomists at the herbarium, Plant Sciences Department, Quaid-e-Azam University, Islamabad, Pakistan.

After collection, plant material was subjected to the removal of dust particles by brushing off extra debris and washed with distilled water. Next, the washed plant material was shade-dried (Figure 3.3). During drying, plant material was checked regularly for fungal infection or chances of any other contamination. Plants were then crushed and ground to a fine powder using a grinding mill (Figure 3.4). To avoid mixing one plant material with another, grinding mil was cleaned, washed with 70% ethanol, and dried properly. The powdered plant material was stored in resealable

zipper bags, appropriately labeled, and kept in a dark and cool place (below 10℃) for further use (Figure 3.4). An initial collection made by a previous student brought back some plants into ethnobotanical information. Hence, candidate plants were chosen after a literature review and the availability of plant material for detailed analysis. Finally, three plant species were selected for further studies. (Figure 3.5).

Figure 3.2 Collection of plant material; **(a)** *A. cappadocicum Gled* **(b)** *P. cornuata* and **(c)** *Q. semicarpifolia*

Figure 3.3. Air-shade drying of collected plant material; **(a)** *A. cappadocicum.* **(b)** *P. cornuata* and **(c)** *Q. semicarpifolia*

Figure 3.4. **(a)** Mechanical Grinding of plant material; **(b)** weighing; and **(c)** packaging of collected plant material

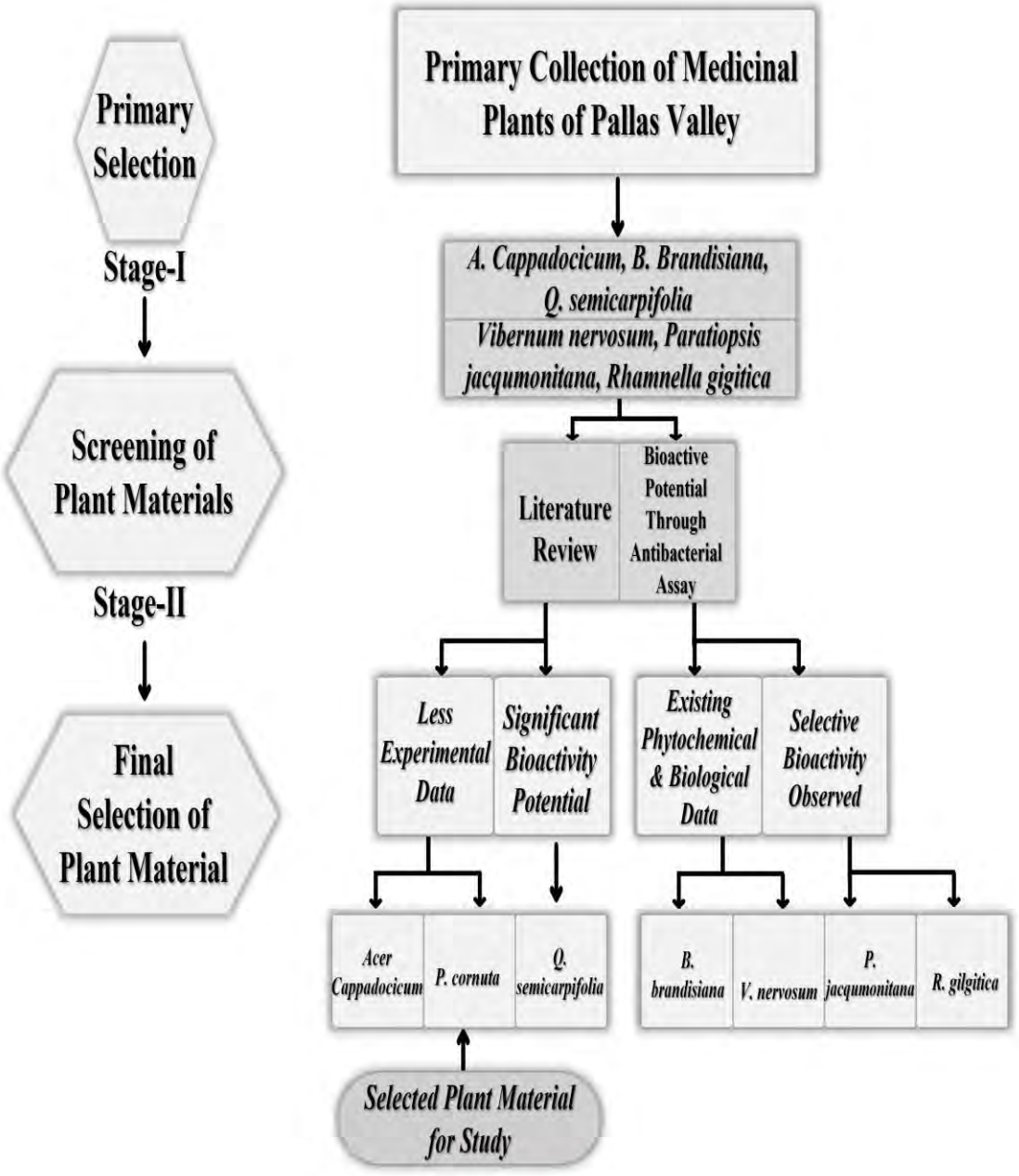


Figure 3.5. The schematic for the selection procedure of plant material

3.3. RESEARCH OUTLINE

The flowchart describes the basic experimental framework and research methodology followed in this study:

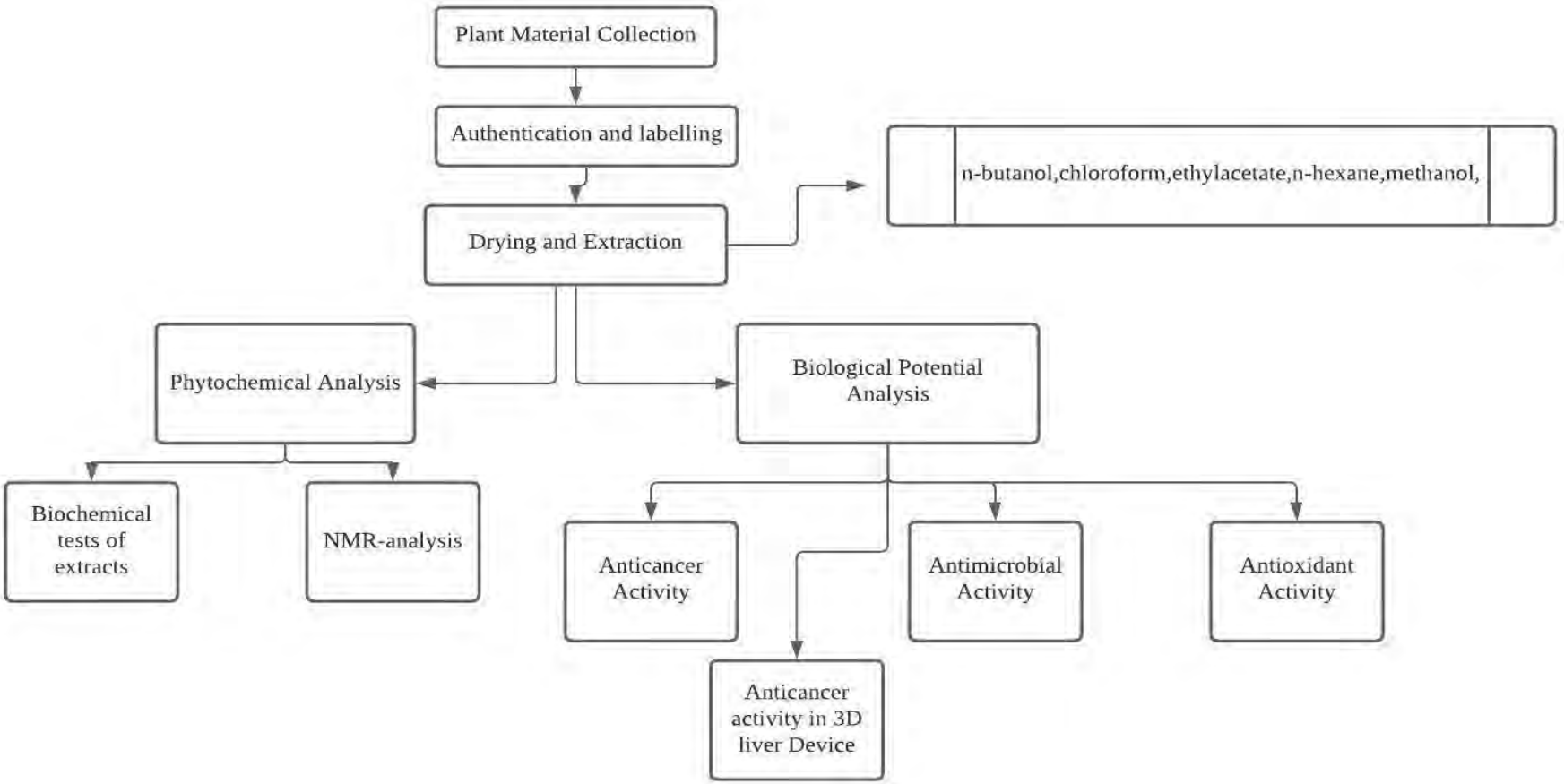


Figure 3.6. Schematic representation of research methodology

3.4. PREPARATIONS OF CRUDE EXTRACTS IN ORGANIC SOLVENTS

Extracts were prepared by the maceration method [58]. Dried leaves and branches were pulverized mechanically through a 60-mesh willy mill and soaked into solvents of different polarities (20 g/200ml) in conical flasks (250 ml), covered with cotton and aluminum foil. The material was placed on a shaker (Cat# CLS6791, Corning LSE Benchtop Shaking Incubator with Platform, Merck USA) for 72 hours at 200 rpm. After that, filter the residue with Whatman No.1 filter paper, and the filtrate was further subjected to evaporation at room temperature under shade. The concentrated extracts were stored at 4°C until further use (Figure 3.7).

All chemicals were of analytical grade. Absolute methanol, n-butanol, n-hexane, ethyl acetate, and chloroform were purchased from Sigma Aldrich. Silica gel from merk Germany, DPPH, ascorbic acid from Sigma Aldrich.

Figure 3.7. A generalized illustration of crude extracts preparation. It involves soaking plant material into the solvent of the desired polarity, followed by shaking for 72 hrs, filtration, and evaporation of filtrate.

Table 3-1: Plant extract labeling and solvents used

Sr. No.	Plant	Part Used	Solvent	Abbreviation
1	*Acer cappadocicum Gled*	Leaves & branches	Methanol	ACM
		Leaves & branches	n-hexane	ACN
		Leaves & branches	Chloroform	ACC
		Leaves & branches	Ethyl acetate	ACE
		Leaves & branches	n-butanol	ACB
2	*Prunus cornuta*	Leaves & branches	Methanol	PCM
		Leaves & branches	n-hexane	PCN
		Leaves & branches	Chloroform	PCC
		Leaves & branches	Ethyl acetate	PCE
		Leaves & branches	n-butanol	PCB
3	*Quercus semicarpifolia*	Leaves & branches	Methanol	QSM
		Leaves & branches	n-hexane	QSN
		Leaves & branches	Chloroform	QSC
		Leaves & branches	Ethyl acetate	QSE
		Leaves & branches	n-butanol	QSB

3.5. PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening of plant extracts was performed through various biochemical tests as follow [59,60,61,62,63,64,65].

A) DETECTION OF ALKALOIDS

i. Mayer's reagent test:

For this test 2 mL of conc. HCL was added to 2 mL of plant extract followed by adding Mayer's reagent drop by drop**.** The formation of white precipitates or green color indicates alkaloids.

ii. Heager's test:

Saturated picric acid (Heager's reagent) was added to 2 ml plants extract. Bright yellow precipitates confirm the alkaloids in test samples.

B) DETECTION OF SAPONINS

Saponin detection test was performed with 2 ml distilled water added to 2 ml plant extract and shaken vigorously, the formation of a 1cm foam layer indicates saponins presence in the extract.[59]

C) DETECTION OF FLAVONOIDS

$FeCl_3$ test: Few drops of $FeCl_3$ solution were added to 1ml of plant extract samples. Flavonoid presence results in dark red precipitation [60].

D) DETECTION OF TANNINS

i. Alkaline reagent test:

The 2ml of plant extracts were subjected to 2ml of 1N NaOH, forming yellow to red ppt confirm tannins in the test sample.

ii. $FeCl_3$ test:

A volume of 2ml of 5% $FeCl_3$ was added to 1 ml of plant extract sample. The appearance of dark blue or greenish-black color confirms tannins in test samples [60].

E) DETECTION OF GLYCOSIDES

Keller Kiliani (cite this properly) test was performed for glycosides detection. First, a volume of 1 ml of glacial acetic acid was added to 1 ml of plant extract samples, followed by cooling, and adding 2 drops of $FeCl_3$. After that, carefully add H_2SO_4 along the walls of the tube. The reddish-brown ring at the junction of two layers indicates glycosides' existence.

F) DETECTION OF STEROLS

Salkowski test: 5 ml of chloroform and 2 ml of plant extracts sample was mixed in this test. Followed by careful addition of 1ml of conc. H_2SO_4 along the wall of the test tube. The reddish-brown color in the lower layer indicated sterol's presence in the test sample [59].

G) DETECTION OF PHENOLS

Ellagic test:

A few 5% glacial acetic acid drops were added to 1 ml of plant extracts. Then added a few drops of 5% $NaNO_2$. The muddy brown color revealed the existence of phenols.

H) DETECTION OF CARBOHYDRATES

Benedict's test was used for carbohydrate detection. A few drops of benedict's reagent/ alkaline solution of Cupric Citrate complex were mixed with test samples followed by boiling in a water bath. The reddish-brown precipitate indicated the presence of carbohydrates in the extract [60].

I) DETECTION OF PROTEINS

Xanthoproteic test: The volume of 1ml plant extracts was subjected to a few concentrated nitric acid drops. Yellow color formation revealed the presence of proteins in test samples [60].

J) DETECTION OF ANTHRAQUINONES

A few drops of 2% HCl were added to the plant extract solution. The formation of red precipitation indicated the anthraquinones in the test substance.

K) DETECTION OF PHLOBATANNINS

A few 10% ammonia solution drops were added to 1ml of plant extract sample. Pink color precipitation signified the existence of Phlobatanins in test samples.

Table 3-2. The methanolic crude extracts of plants were subjected to qualitative chemical tests to detect phytochemicals such as alkaloids, flavonoids, saponins, tannins, and carbohydrates [59,60,61].

Test	Procedure	Indication/detection
Alkaloids Detection		
Mayer's reagent test	For the identification of alkaloids, 2 mL of conc. HCl was added to 2 mL of plant extract followed by the drop-by-drop addition of Mayer's reagent**.**	The formation of white precipitate or green color appearance revealed the presence of alkaloids.
Hager's test	A few drops of saturated picric acid (Hager's reagent) were added to the 2 mL plant test extract.	The appearance of bright yellow precipitates revealed the existence of alkaloids in test samples.
Saponins		
	This test was performed by adding 2 mL distilled water to 2 mL plant extract and shaking vigorously for 5 min in a test tube.	The formation of foam layer (1cm) indicated the presence of saponins.
Flavonoids		
	A few drops of $FeCl_3$ solution were added to 1 mL f plant extracts samples	Flavonoid presence results in blackish-red precipitation.
Tannins		
Alkaline reagent test	Plant extracts (2ml) were added to 1N NaOH (2 ml) and mixed thoroughly.	The appearance of yellow to red precipitates confirmed the presence of tannins.
Ferric Chloride test	A volume of 2 mL of 5% $FeCl_3$ was added to 1 mL	The appearance of dark blue or greenish-black color

	of plant extract sample.	indicated the presence of tannins.
Glycosides		
Keller-Kilani test	Mixed the crude extract with glacial acetic acid (2ml) containing 1-2 drops of $FeCl_3$ solution (2%). Transferred the mixture into 2 ml of conc. H_2SO_4 test tube.	The formation of a reddish-brown ring at the interphase confirms the presence of cardiac glycosides in the mixture.
Sterols		
Salkowski test	5 mL of chloroform and 2 mL of plant extract sample were mixed in this test. Followed by careful addition of 1 mL of conc. H_2SO_4 along the walls of the test tube.	The reddish-brown color in the lower layer indicated sterols presence in the extracts sample.
Phenols		
Ellagic test	A few 5% glacial acetic acid drops were added to 1 mL of plant extract samples. Then added a few drops of 5% $NaNO_2$.	The muddy brown color revealed the existence of phenols.
Carbohydrates		
Benedict's test	A few drops of benedict's reagent/ alkaline solution of cupric citrate complex were mixed with test samples followed by boiling in the water bath.	Reddish-brown precipitate indicated the presence of carbohydrates in the test substance.
Proteins		

Xanthoproteic test	The volume of 1 mL plant extracts was subjected to a few drops of concentrated nitric acid.	Yellow color formation revealed the presence of proteins in test samples.
Anthraquinones		
	A few drops of 2% HCl were added to the plant extract solution.	The formation of red precipitation indicated the anthraquinones in the test substance.
Phlobatanins		
	A few 10% ammonia solution drops were added to 1 mL of plant extract sample.	Pink color precipitation signified the existence of Phlobatanins in test samples.

3.6. BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS

The crude plant extracts were subjected to various *in vitro* biological activities as described below.

3.6.1. ANTICANCER ACTIVITY OF PLANT EXTRACT

Human hepatocellular carcinoma cell line HepG2, human intestinal epithelial cell line Caco-2, A549, MDA-MB-231, and NCI-H1437 (Korea cell line bank, south Korea) were cultured in RPMI-1640 media supplemented with 10% FBS and 1% (v/v) penicillin and streptomycin solution. Cultured cells were kept at 37°C with 5% CO_2 in a cell culture incubator (Cat# I03-91-035, Lklab Korea, Republic of Korea). After 80 to 90% confluency, the cells were expanded up to 3 passages before seeding and washed with Dulbecco's Phosphate Buffer Saline (DPBS) (Cat# 14190144, Thermo Fisher, USA) to remove cell debris and metabolites before adding fresh media. Cells at 90% confluency were trypsinized with 0.05% Trypsin-EDTA solution (Cat # 25300054, Thermo Fisher, USA) and then suspended in freshly prepared RPMI-1640 media containing a specified concentration of FBS.

Human pulmonary alveolar epithelial cells (HPAEpiC) from ScienceCell Research laboratories were revived according to manufacturer instructions; the T-25 flask was coated with Poly-L-lysine (Sigma-Aldrich) at a concentration of 2µg/cm³ kept at 37°C for 24 hrs. Also, the flask was rinsed before adding culture medium containing alveolar epithelial cells (AEpiCM, Cat# 3201), minimal eagle medium, epithelial growth supplement (5ml),10% FBS, 1% Penicillin Streptomycin solution with 5% Carbon dioxide at 37°C. In addition, human renal proximal epithelial cells (HRPTEpiC) purchased from ScienceCell were cultured in Epithelial Cell Medium (EpiCM, Cat# 4101).

An MTS method assay was used to determine the number of viable cells [66]. The cells were kept in 96 well plates at a concentration of 2000 cells per well for 24 hrs. The cells were subjected to various concentrations (from 1 mg to 0.1ng/ml) of 100µl of serially diluted plant extracts for 48 hrs. For blank value, cells containing media only and for control anticancer drugs (doxorubicin and cyclophosphamide) were also incorporated into wells [32]. About 25 µl of MTS reagent (Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay, Cat# G3581, Promega, USA) was added and allowed the cells to incubate at 5% CO_2 and 37°C for 30 minutes. Measured the absorbance of cells at 490 nm by a multimode microplate reader (SpectraMax i3x) [67,68]. Percentage cell viability was also calculated [69] as follows:

$$\% \text{ age cell viability} = \text{Control -blank/sample -blank X } 100 \quad (1)$$

The IC_{50} value, the maximum concentration of extracts to inhibit 50% cell viability, was calculated by graphs [70]. All readings were performed in triplicate.

3.6.2. ANTIBACTERIAL ACTIVITY OF PLANT EXTRACTS

The strains selected were *Klebsiella pneumoniae* (82431), *Escherichia coli* (52321), *Bacillus subtilis, Salmonella enterica,* and *Acinetobacter baumannii*. The Department of Microbiology, Quaid-i-Azam University Islamabad. After overnight culturing of microbes at 37°C in nutrient broth, the cell suspension was homogenized and maintained to 0.5 McFarland standards ($5x10^5$ CFU/mL). For the antibacterial assay of the plant extracts, the agar well diffusion method was applied [71, 72]. The assay procedure is as follows.

1. The nutrient agar medium was prepared according to the manufacturer's instructions (Sigma-Aldrich) and autoclaved the media at 40 to 50℃.

2. After that, the freshly prepared media were poured into flat bottom Petri plates with 4mm depth and 90 mm diameter. Inoculated the solidified agar media surface using a sterile L-shaped glass rod with bacterial inoculum (0.2 ml). Five equidistant wells were made using a cork borer.

3. They were filled with about 80-100 µl of plant extracts at a 4 mg/ml concentration in test wells. Kanamycin and DMSO were used as a positive and negative control. Labelled properly by the name's crude extracts and controls, then agar plates were incubated at 37℃ for 24 hours. The assay was performed in triplicates. The zone of inhibition was measured (mm) from the top end of the well to clear zones.

3.6.3. ANTIFUNGAL ACTIVITY OF PLANT EXTRACTS

Antifungal activity of plant extracts was evaluated against fungal strains, i.e., *Rhizopus oryzae*, *Aspergilus flavous* (FCBP 0064), *Aspergilus niger* (FCBP 0198), and *Pythium* sp. [73,74]. The sample material was prepared by dissolving 40 mg of the extract in 1ml of DMSO stock solution. 2 mg of 'Terbinafine' was used in 1 ml DMSO solvent for positive control. Pure DMSO was used as a negative control. SDA (Sabouraud dextrose agar) medium was used for fungal cultures. Dissolved 6.5 g of SDA in 100 ml distilled water. Approximately 25 m medium was poured into Petri plates. After solidification, fungal cultures were streaked with sterile cotton buds on the entire agar medium surface. The plate edges were sealed with parafilm and incubated at 28 ℃ overnight. The mycelial inhibition of fungal strains was performed through the well diffusion method [72]. Equidistant wells were made in each Petri plate using a sterile cork borer. About 100µl of stock solution of plant extracts were added into wells and allowed to diffuse for 2hrs. Then the plates were sealed with parafilm and placed upside-down for 48 hours at 28 ℃. The zone of inhibition was measured as a cleared zone around wells. The experiment was performed in triplicate.

The Agar Slanting Method evaluated the percentage antifungal activity of crude extracts as previously reported [75] against fungal pathogens, i.e., *R. oryzae*, *Fusarium fujikuroii,* and *Pythium* sp. The selected fungal strains were cultured on

Sabouraud Dextrose Agar (SDA) and incubated at 37 ℃ for 24 h. The inoculum was poured into sterilized test tubes containing 4 ml of SDA. The test sample was made by dissolving 40 mg in 1 ml of DMSO. Approximately 65 ml of the sample was added to each test tube containing SDA. The media was solidified and incubated (37 ℃) for seven days. Terbinafine (2mg/ml) and DMSO were used as positive and negative controls, respectively (Fazli et al., 2012). After incubation, the linear growth inhibition was determined, and percentage inhibition was calculated using Eq. ().

$$\% \text{ inhibition} = 100 - \frac{\text{Linear growth (test)}}{\text{Linear growth (control)}} 100 \qquad (2)$$

3.6.4. ANTIOXIDANT ACTIVITY

Antioxidant activity was evaluated by preparing a stock solution of 40,000µg/mL of test plant extract in DMSO (40mg/ml). Further 10 working dilutions were prepared to range from 100 µg/mLto 1000 µg/mLby a stock solution using the serial dilution method. DPPH solution (0.1 mM) was prepared by dissolving 0.975 mg DPPH in 25 ml of methanol. Then it was placed on a magnetic stirrer for 30 minutes in the dark. For the assay, 190 µl of freshly prepared DPPH solution and 10 µl of each sample was mixed in 96 well microtiter plate. DMSO was used as a negative control, while ascorbic acid was used as a positive control. For Incubation, the reaction plate was wrapped with aluminium foil at 37°C for 30 minutes, after which absorbance was measured at a wavelength of 517 nm. The percentage scavenging activity was calculated as follows [76,77]:

$$\text{Scavenging activity \%} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \text{ X } 100 \qquad (3)$$

3.6.5. STATISTICAL ANALYSIS

All experiments were performed in triplicate. The results were described as a value of the mean. The p-value was determined by one-way ANOVA test using Prism software, and the value of $p=0.005$ was considered significant.

3.7 EFFECT OF METHANOLIC EXTRACT OF *ACER CAPPADOCICUM* ON LIVER-ON-CHIP CANCER MODEL

The Acer species have multiple traditional uses, such as treating rheumatism, bruises, hepatic disorders, eye disease, pain relief, and detoxification. In addition, several bioactivity analyses have confirmed the ethnomedical significance of Acer species toward hepatoprotective function [78]. Previously, the cytotoxic effect of *Acer cappadocicum* extracts on HepG2 (liver carcinoma) cells has been shown in *in vitro* studies [79]. In this study, the methanolic extracts of *Acer cappadocicum* were subjected to a hepatocellular carcinoma cell-based liver-on-chip cancer model of liver tumor microphysiological system (MPS) for analyzing cellular inhibition of hepatic carcinoma.

3.7.1. MICROFLUIDIC CHIP DESIGN

The microfluidic glass chip was designed with 56mm length, 41 mm width, and 1.1mm thickness of the top and bottom glass chips. The microfluidic channels of 300 µm in height and 800 µm in width were printed on top glass with a 3D printer. The lower and upper glass chip plates were held together with a magnetic chip holder's help. To overcome the leakage problem, the sealants of the silicon ring were placed in the magnetic chip holder. The chip design was automatically configured with simulation software (ANSYS fluent software). Sensor circuits and monitoring systems were also attached for non-invasive live observation (Figure 3.8) [80].

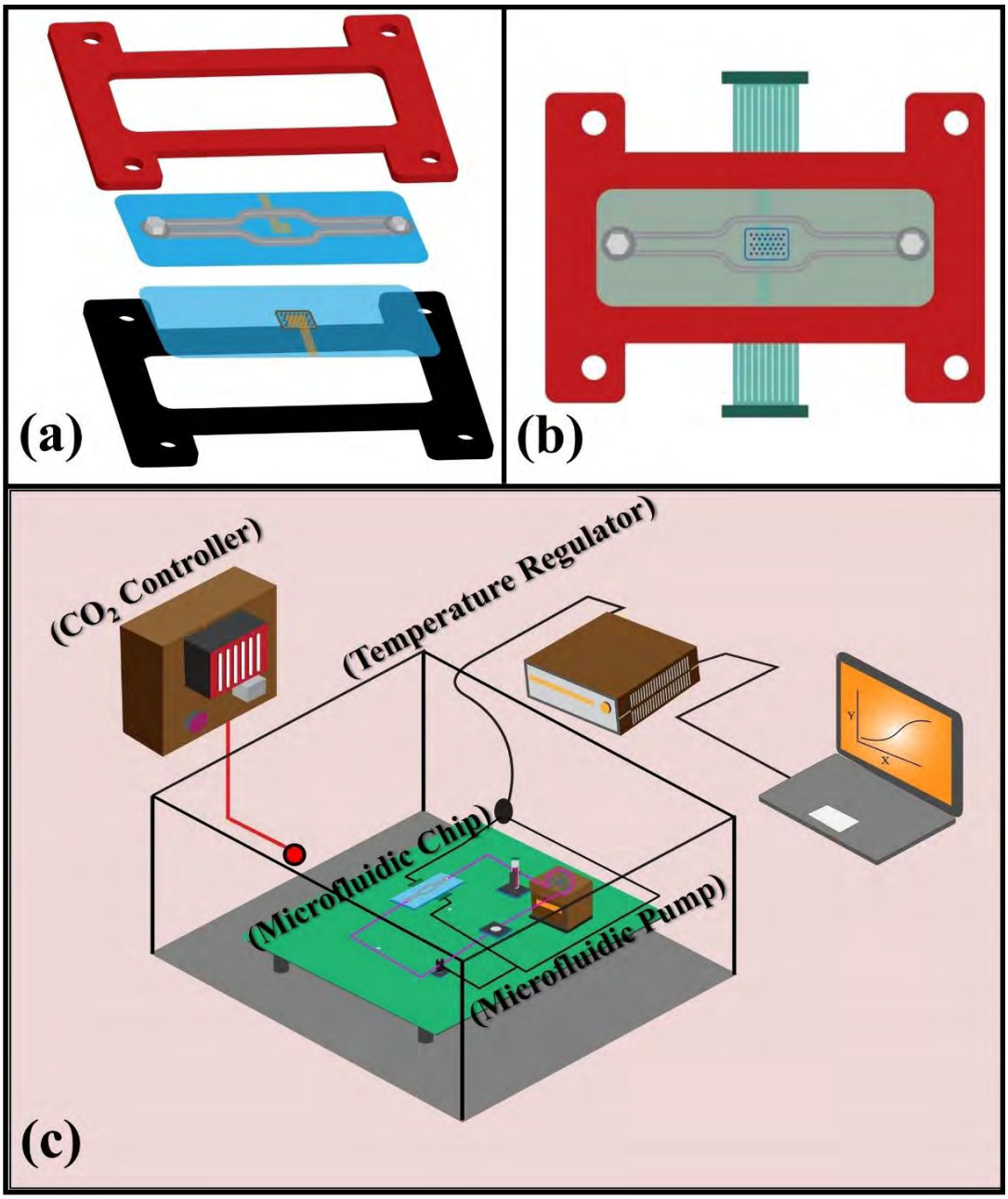


Figure 3.8. (a) Microfluidic chip components (3D printed chip holder in red and black, microfluidic glass chip consists of top and bottom glass chip with a rectangular area for cell seeding & culture **(b)** The assembled microfluidic chip (**c)** Microfluidic liver-on-chip platform and associated components (microfluidic peristaltic pump, cell culture media reservoir, microfluidic tubing, CO_2 and temperature controllers, computer for maintaining the environmental controls within the microfluidic platform.

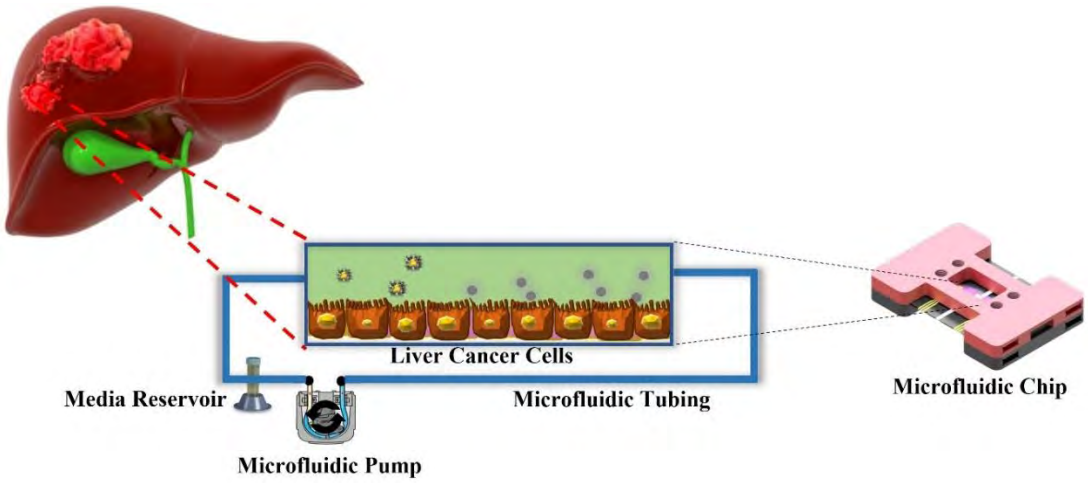


Figure 3.9. A schematic representation of the liver-on-chip device containing liver carcinoma (HepG2) cells

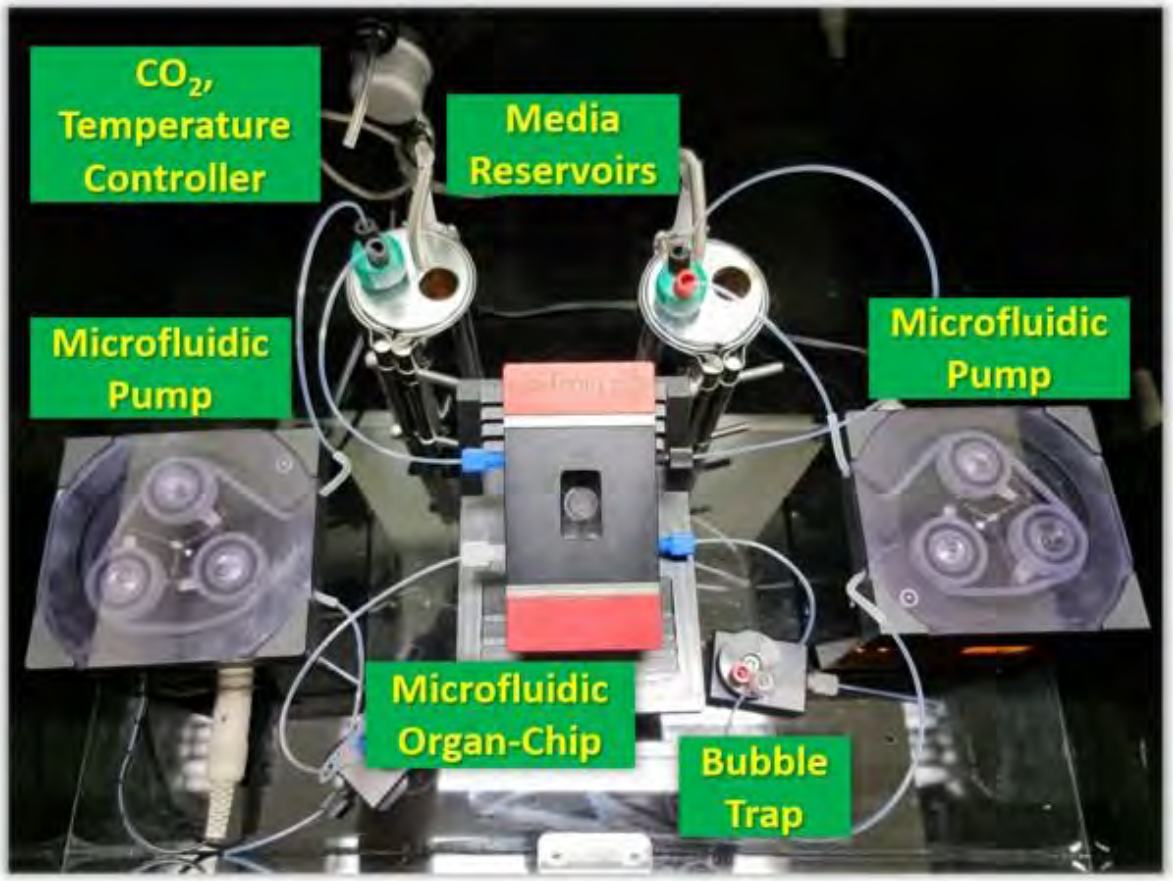


Figure 3.10 The real image of the experimental setup of the liver on-chip device and its associated components

3.7.2. CELL CULTURE AND CELL VIABILITY ASSAYS

The hepatocarcinoma cell lines were obtained from the Korea Cell Line Bank, South Korea. The HepG2 cells were maintained in DMEM cell culture media (11 965 092, ThermoFisher, USA), 10% fetal bovine serum (16000 044, ThermoFisher, USA), and 1% penicillin/streptomycin antibiotic solution (15 070 063, ThermoFisher, USA). The humidified environment (5% CO_2, 37°C) was provided to HepG2 cells. The cells were passage through thrice before seeding on the chip. The preheated DBPS solution was used for the periodic washing of cells. It removed the cellular metabolic waste and debris of cells. The cells were trypsinized with 0.05% trypsin-EDTA solution at 95% confluency. Subsequently, the cells pellet was suspended in DMEM for seeding the cells on the chip and, after that, sterilized the glass chip with isopropyl alcohol, followed by the three-time washing of the cell pellet with deionized water. Later, air-dried the cells and performed UV irradiation for 60 minutes in a biosafety cabinet. Then rat-tail collagen type I polymerization (5 µL) (cat# C3867-1VL, Sigma-Aldrich, USA) was performed on the cell culture area of the chip for 20 minutes. Afterward, the microfluidic chip was fixed in a 3D printed cell seeding kit and the chip was loaded with 1000 µL of HepG2 cell suspension (1.5×10^5 cell ml^{-1}).

The chip was then placed in humidified condition (cell culture incubator) for 24 hours for cells' attachment to the extracellular matrix (collagen). On day 2, we discarded the media and placed the chip in silicon gaskets of a 3D printed Chip holder. After that the second glass chip was placed on the upper portion of first chip. The upper glass chip had the media flow channel. The chip holder was tightened and placed into the microfluidic liver-on-chip experimental platform. The media flow was maintained with a peristaltic microfluidic pump, and 0.5 dyn cm^{-2} shear stress was applied to cells at the flow rate of 60 µL per minute. A media storage container (reservoir) of 2 ml was used, and a bubble trap was also placed to remove bubble formation in the microfluidic channel. A tubing system connected the liver-on-chip device with the cell culture media reservoir, bubble trap and liver-on-chip device. The chip was observed for periodic monitoring of monolayer formation by an in-house developed microscope. On day 3, the monolayer formation was confirmed by microscopic images (Figure 3.9, 3.10).

3.7.3. HEPATOTOXICITY EVALUATION IN LIVER-ON-CHIP CANCER MODEL AND LIVE/DEAD ASSAY

When after 72 hrs cell monolayer was formed in the liver-on-chip device, and various concentrations of plant extracts and standard drugs were applied to the device. Every experiment was performed thrice. First, the cells' inhibition or cytotoxicity was examined after 24 hrs with the help of a microscope. Then, cell viability was calculated for cells treated with extracts, cells treated with extracts vehicle solvent (DMSO), and cells treated with standard drug. Finally, the live/dead assay was performed with a staining solution (EZ-CYTOX, cat# EZ-1000, DAEILLAB Service, Korea) according to the manufacturers' instructions. Initially, we washed the cells with DPBS three times and then submerged them in EZ-Cytox solution (300 µL); after that, the chip was incubated in an incubator with 5% CO_2 at 37 ◦C for 1h. Followed by confocal laser scanning microscopy (CLSM) (Olympus, model# FV1200, Japan) at the excitation of 530 ~ 560 nm and emission of 590 ~ 620 nm. The images taken from the confocal microscope were analyzed for live and dead assays using ImageJ software (Version 1.52p, NIH, USA).

4. RESULTS

This chapter consists of four sections describing the details and qualitative and quantitative analysis of selected plant material research work. In every section, the results are described according to the numbering of plant material (plant 1, plant 2, plant 3). The first section revealed the biochemical substances of methanolic extracts of tested plant species and all extract types of *Acer cappadocicum Gled*. Section II is a significant part of biological activity, i.e., an anticancer assay of plant extracts, and entails the anticancer potential on various cancerous cells. The third section provides antimicrobial potential against different pathogenic species and antioxidant scavenging assay. Finally, section four includes cytotoxic data of selected plant extract on the *in vitro* organ-on-chip model. Following are the main sections covered in this chapter briefly.

Section I: Preliminary phytochemical investigation of selected plant species

Section II: Assessment of anticancer potential of plant extracts

Section III: Evaluation of antimicrobial and antioxidant potential of selected plants

Section IV: A short, novel study of the effect of *Acer cappadocicum Gled* on the liver-on-a-chip Model

Section I: QUALITATIVE PHYTOCHEMICAL INVESTIGATION OF SELECTED PLANT

4.1. PRELIMINARY PHYTOCHEMICAL ANALYSIS

The plant extracts prepared in methanol were assessed through phytochemical analysis. The extracts of *A. cappadocicum* showed the presence of alkaloids, tannins, glycosides, phenols, carbohydrates, quinones, anthocyanins, and proteins. However, sterols, phlobatanins, and terpenoids were not detected. Methanolic extract of *P. cornuta* revealed the presence of alkaloids, tannins, saponins, flavonoids, sterols, steroids, and terpenoids, while phenols, carbohydrates, anthocyanins, and quinones were not found. Similarly, the methanolic extract of *Q. semicarpifolia* contained: flavonoids, glycosides, sterols, steroids, saponin, and quinones. (Table 4-1).

Table 4-1: Qualitative phytochemical analysis of methanolic crude extracts of selected plants

Constituents	**Tests**	**ACM**	**PCM**	**QSM**
Alkaloids	Mayer's reagent test	+	+	+
	Hagar's test	+	+	+
Tannins	FCl_3 test	+	+	+
	Alkaline reagent test	+	+	+
Saponins		_	+	+
Flavonoids		+	+	+
Glycosides		+	+	+
Sterols		_	+	+
Phytosteroids		+	_	NA
Phenols		+	+	+
Carbohydrates		+	_	_
Anthraquinone		_	_	_
Phlobatanins		_	_	_
Terpenoids		_	+	+
Anthocyanin		+	+	_
Quinones		+	_	+
Proteins	Xanthoproteic test	+	NA	NA

Table: The qualitative indication of phytochemicals presents in plants metabolites extracts, + sign = present and - sign= absence.

The phytochemical screening of *A. cappadocicum* Gled extracts made in different solvents revealed the presence of alkaloids, glycosides, quinones, and proteins in all extracts. However, the presence of tannins was detected only in methanolic and butanolic extracts. Similarly, the presence of steroids in methanol and of phytosteriods in butanol and ethanol were detected (Table 4-2).

Table 4-2: Qualitative indication of phytochemicals present in different extracts of *Acer cappadocicum* Gled.

Constituents	Tests	ACB	ACC	ACE	ACM	ACN
Alkaloids	Mayer's Reagents Test	+	+	+	+	+
Tannins	$FeCl_3$ Test	+	-	-	+	-
	Alkaline Reagent Test	+	-	-	+	-
Steroids		-	-	-	+	-
Phytosteroids		+	-	+	-	-
Glycosides		+	+	+	+	+
Quinones		+	+	+	+	-
Proteins	Xanthoproteic Test	+	+	+	+	+
	Biuret Test	NA	NA	NA	NA	NA

Table: the qualitative indication of phytochemicals presents in *Acer cappadocicum* extracts, + sign = present and -sign = absence

Section II: Assessment of Anticancer Potential in *A. cappadocicum* Gled, *P. cornuta* and *Q. semicarpifolia*

4.2. ANTICANCER ACTIVITIES OF PLANT EXTRACTS

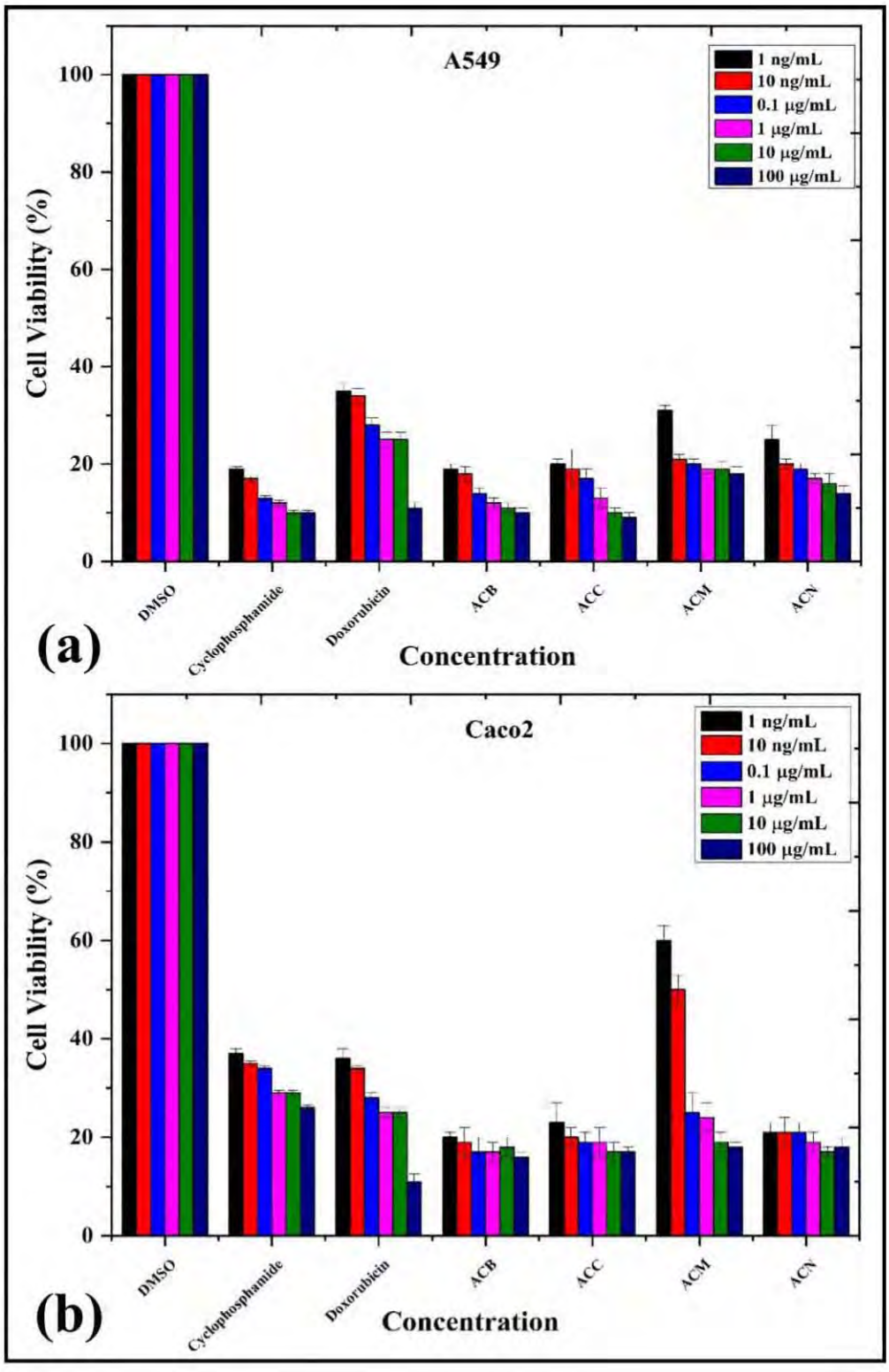
The cytotoxic activity of methanolic extracts was determined using an MTS assay in cancerous and normal cell lines exposed to various concentrations of extracts for 24 hours, and IC_{50} values were determined and expressed as percentage cell viability.

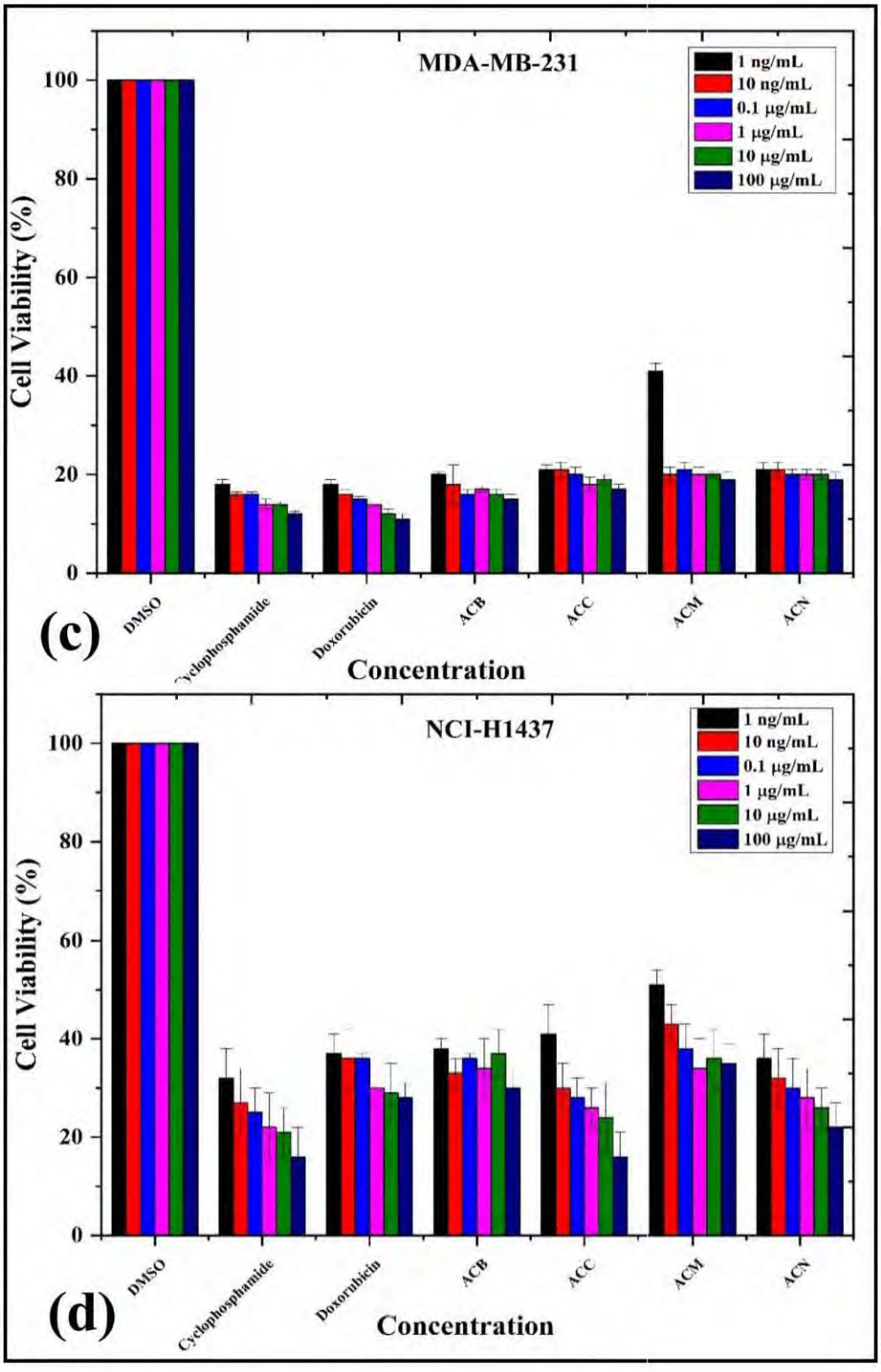
4.2.1. ANTICANCER ACTIVITY OF *ACER CAPPADOCICUM* GLED

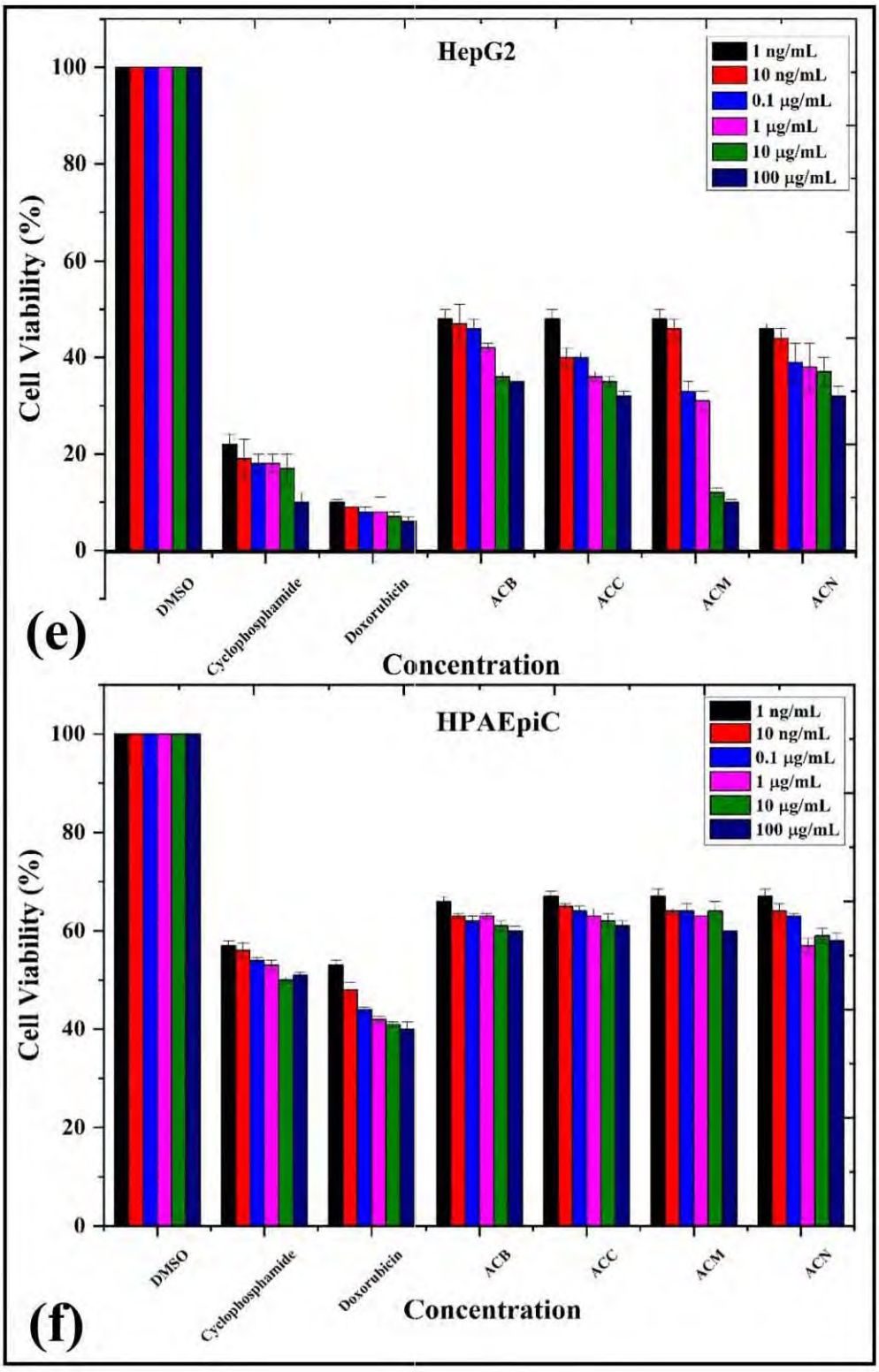
In the present study, *Acer cappadocicum* Gled first time has been evaluated for its biological activity and, most notably, for anticancer study. The extracts of *A. cappodocicum* Gled exhibited a potential inhibitory effect on cancer cells. Results clearly showed that *A. cappodocicum* extracts exhibited significant ($p = 0.05$) cytotoxic activity against A549, Caco2, and MDA-MB-231 cancerous cells (Figure 3.1). The low cell survival rate in these cell lines reflects a high rate of cytotoxicity, most notably in the case of A549 and Caco2 cell lines. The chloroform (ACC) and butanolic (ACB) extracts showed maximum anticancer activity than the standard.

MDA-MB-231 extracts (ACC, ACB, ACM, and ACN) behaved with the characteristic potential of cancerous cell growth inhibition, indicating that breast cancer cells are more prone to *A. cappodocicum* extracts. With *A. cappodocicum* extracts, HepG2 and NCI-H1437 cell lines exhibited a moderate cell survival rate (40-50%), indicating the cytotoxic effect of extracts on these cell lines. The reduction in cell survival of *n*-butanolic, chloroform, methanolic, and *n*-hexane extracts was in the order of MDA-MB-231> A549> Caco2 cells without much difference in concentrations. The extracts exhibited solid cytotoxic activity with 20-30% cell survival. In addition, the cell viability effect on NCH-H1437 chloroform extract showed maximum anticancer activity with low cell viability. In HepG2 cells, the methanolic extract showed the highest activity with a low survival rate of cells [37].

The plant extracts exhibited significant ($p<0.05$) cytotoxicity against the cancerous cell, while normal cells (primary lung alveolar and renal primary tubular epithelial cells) appeared less sensitive to the extracts (Figure 4.1).


A549
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
ACB
ACC
ACM
ACN
Concentration
(a)
Caco2
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
ACB
ACC
ACM
ACN
Concentration
(b)


MDA-MB-231
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
ACB
ACC
ACM
ACN
Concentration
(c)
NCI-H1437
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
ACB
ACC
ACM
ACN
Concentration
(d)


HepG2
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
ACB
ACC
ACM
ACN
Concentration
(e)
HPAEpiC
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
ACB
ACC
ACM
ACN
Concentration
(f)

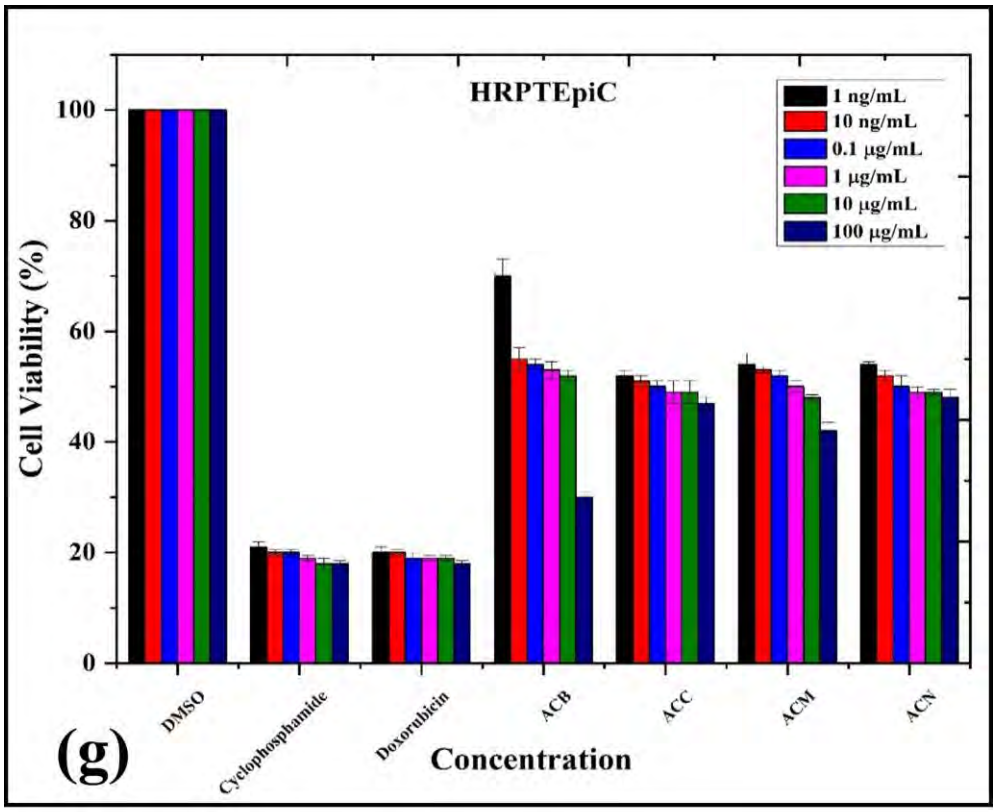


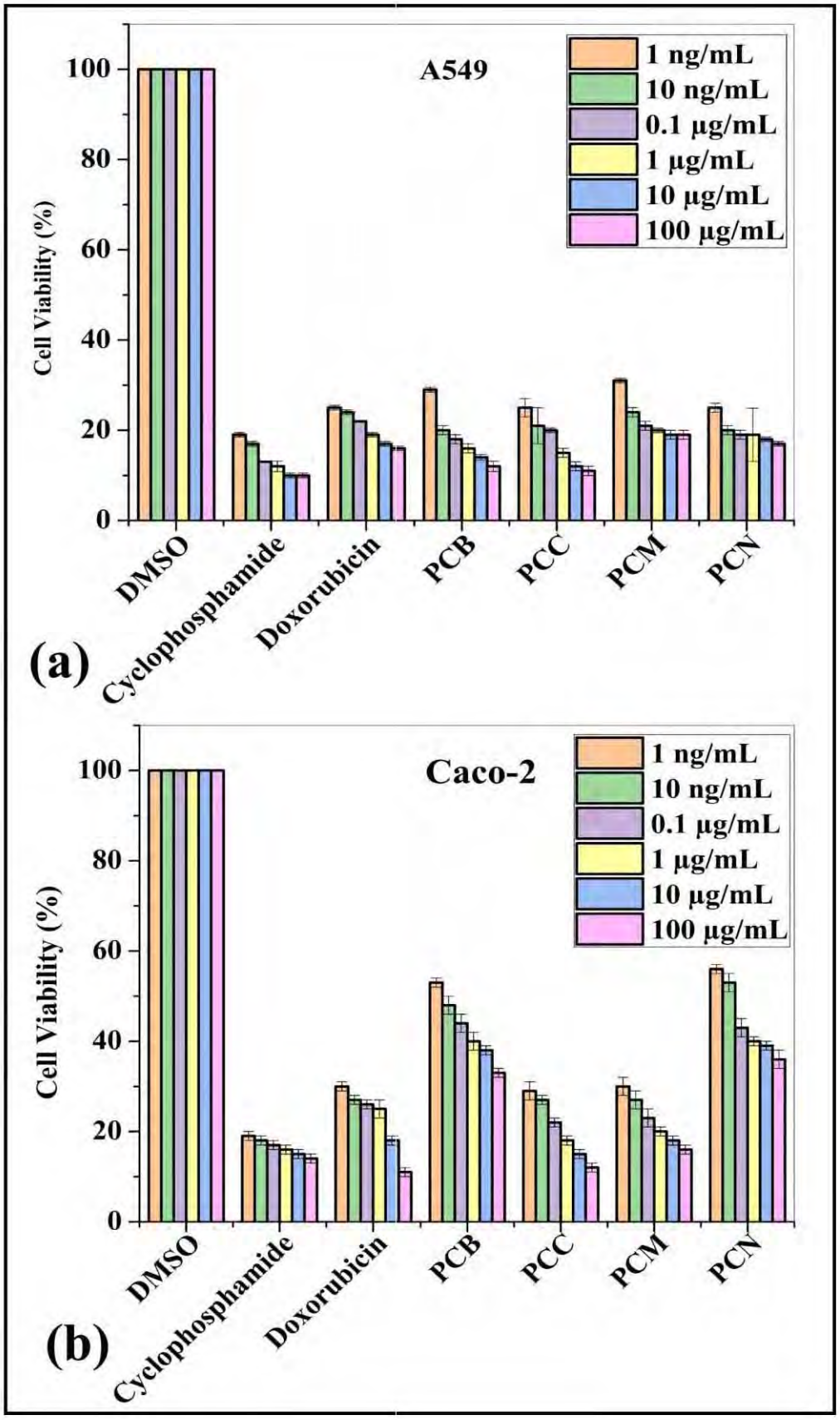
Figure 4.1. MTS assay Histograms represent the percentage concerning control cells (positive control: 30-40 % viable cells) after the exposure to:100 µg/ml,10 µg/ml, 1 µg/ml, 0.1 µg/ml, 10 ng/ml, 1ng/ml) of ACB, ACC, ACM, ACN extracts in A549 cells (a), Caco2 (b), MDA MB-231 (c), NCI-H1437(d), HepG2 (e) cancerous cell lines and HPAEpiC cells (f) and HRPTEpiC (g) cell lines. Data shows mean ± SE (n=3).

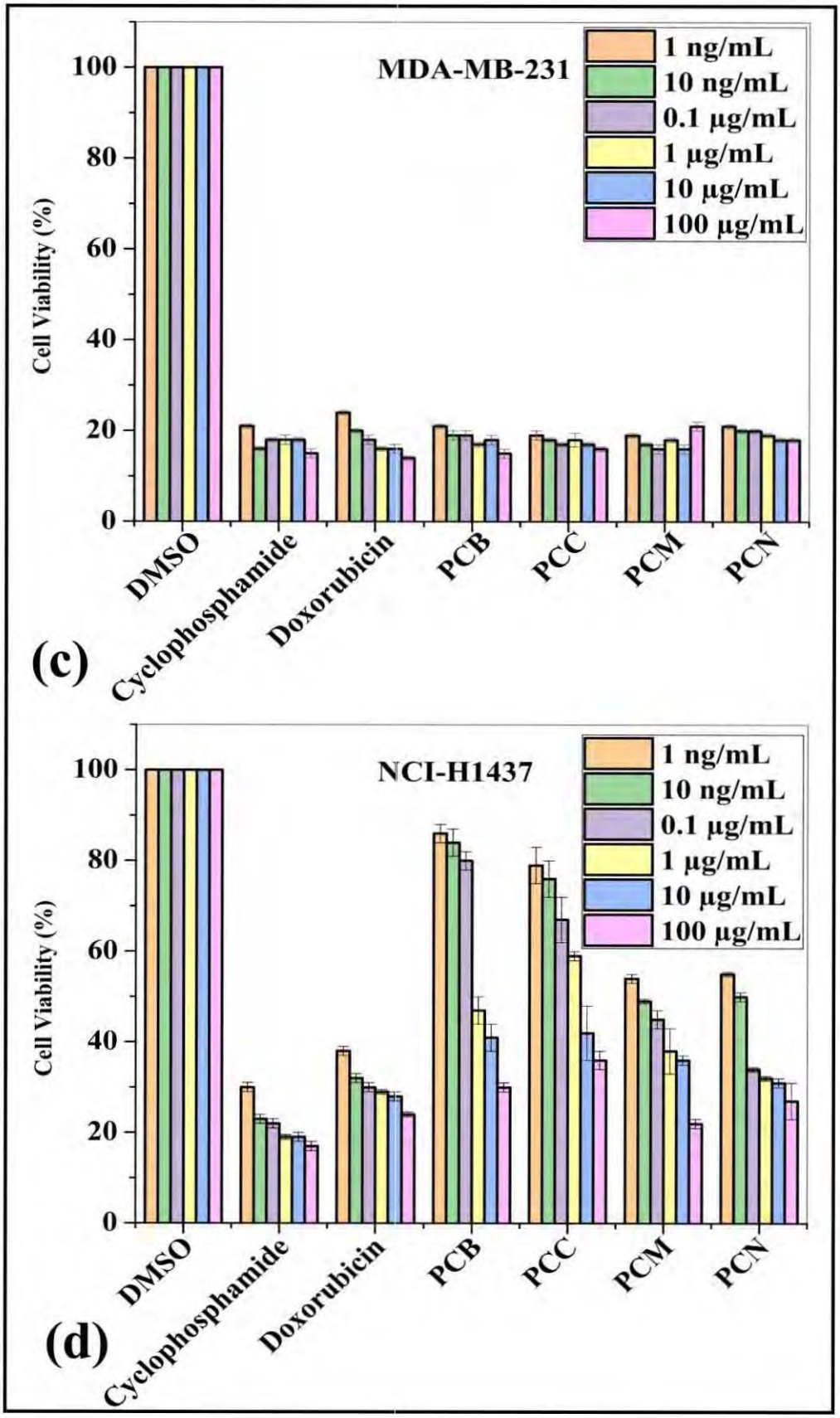
4.2.2. ANTICANCER ACTIVITY OF *PRUNUS CORNUTA*

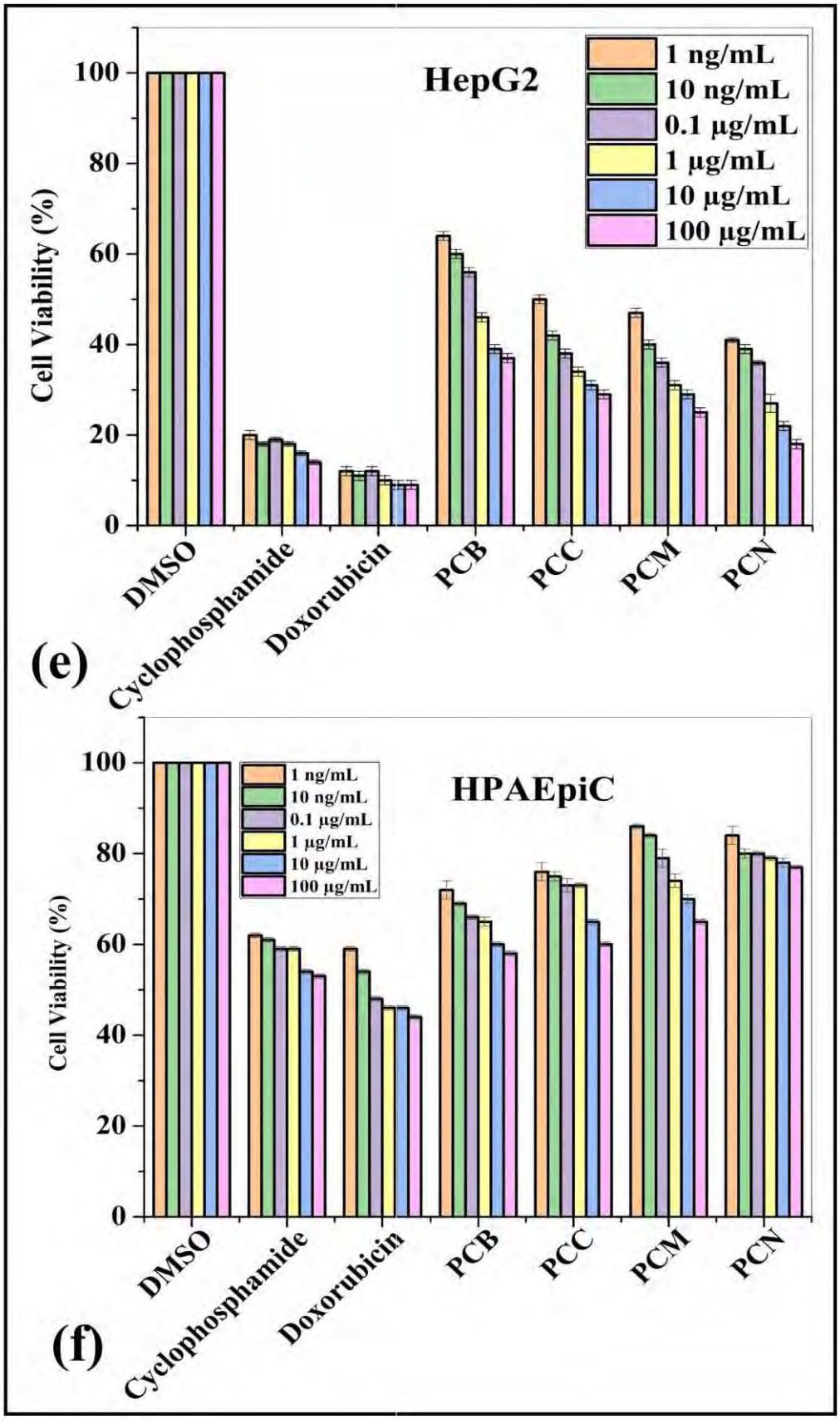
The inhibitory effect of *P. cornuta* extracts was highest against MDA-MB-231 and potent against A549 and Caco2 cells. Moreover, PC crude extracts showed moderate activity against HepG2 and NCI-H1437, indicating that higher concentrations are needed to have significant cytotoxicity in these cell lines. However, all extracts showed less inhibition of cell proliferation in NCI-HI437 cells and good inhibition in MDA-MB-231 (18-30%) compared with the standard drugs with 17 to 27 % cell viability (Table. 4-3). Further, the Percentage cell viability rate was 54 to 76 % in primary epithelial cells HPAEpiC and HRPTEpiC, providing safety data for this study, Figure. 4.2, f & g. In addition, extracts in different solvents showed a slightly different inhibition pattern against a specific type of cancerous cell lines. Chloroform extracts of *P. cornuta* plant altogether showed the highest cytotoxic effect in Caco2, A549, and MDA-MB-231 cancerous cells, also signifying the antibacterial activity results.

Table 4-3: Average (n=3) % age cell viability of PC plant extracts against human-derived cancerous cell lines and healthy cell lines at 100 µg/mL concentration.

Extracts	A549	Caco2	MDA-MB-231	NCI-H1437	HepG2	HPAEpiC	HRPTEpiC
PCB	26.85	42.60	19.00	67.71	50.30	64.85	58.85
PCC	26.85	20.50	18.00	58.14	37.30	72.28	52.28
PCM	29.14	22.30	26.57	47.71	34.60	78.85	63.28
PCN	22.00	44.50	19.71	39.14	30.50	80.71	53.28
QSB	38.80	29.71	29.28	46.42	35.71	71.74	56.00
QSC	36.60	30.42	22.42	35.85	33.00	75.71	54.85
QSM	38.80	34.42	28.57	48.85	31.85	76.14	61.85
QSN	30.80	24.00	20.28	68.71	23.85	64.14	54.00
Doxo.	20.50	20.80	19.00	27.42	10.85	49.57	19.57
Cycl.	14.42	17.14	18.00	20.71	17.85	57.14	18.71


A549
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
PCB
PCC
PCM
PCN
(a)
Caco-2
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
PCB
PCC
PCM
PCN
(b)


MDA-MB-231
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
PCB
PCC
PCM
PCN
(c)
NCI-H1437
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
PCB
PCC
PCM
PCN
(d)


HepG2
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
PCB
PCC
PCM
PCN
(e)
HPAEpiC
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability (%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
PCB
PCC
PCM
PCN
(f)

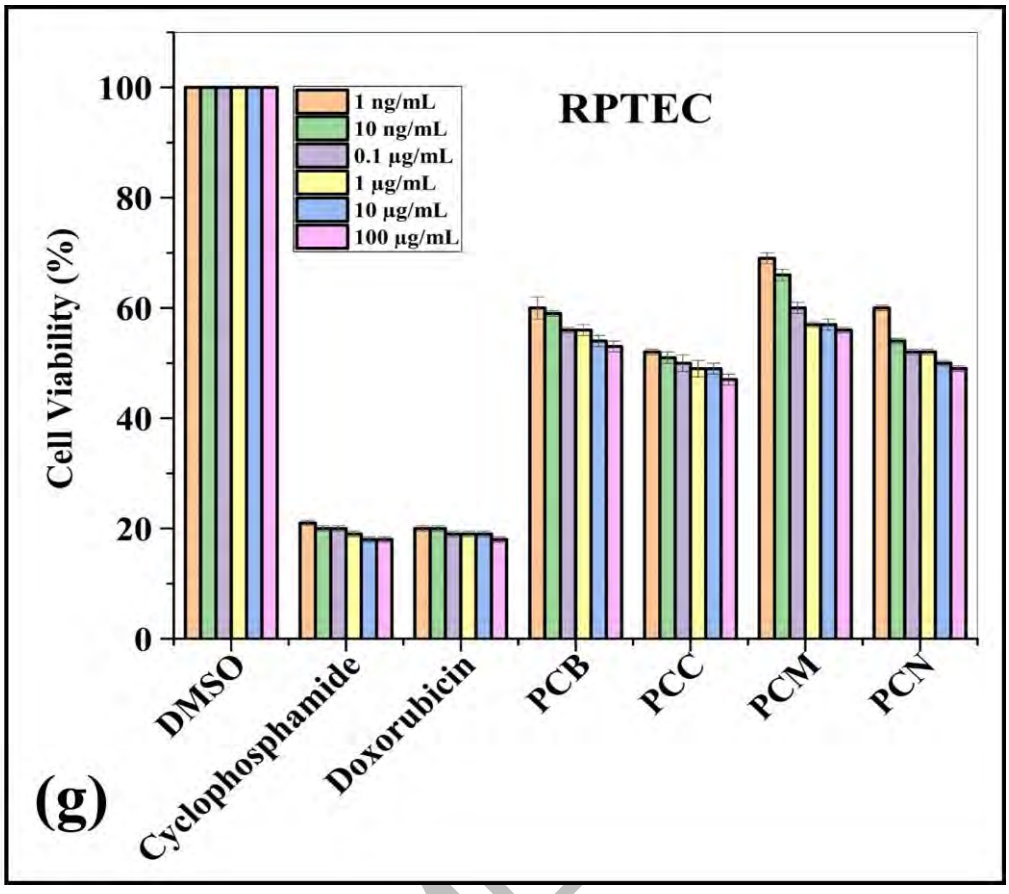
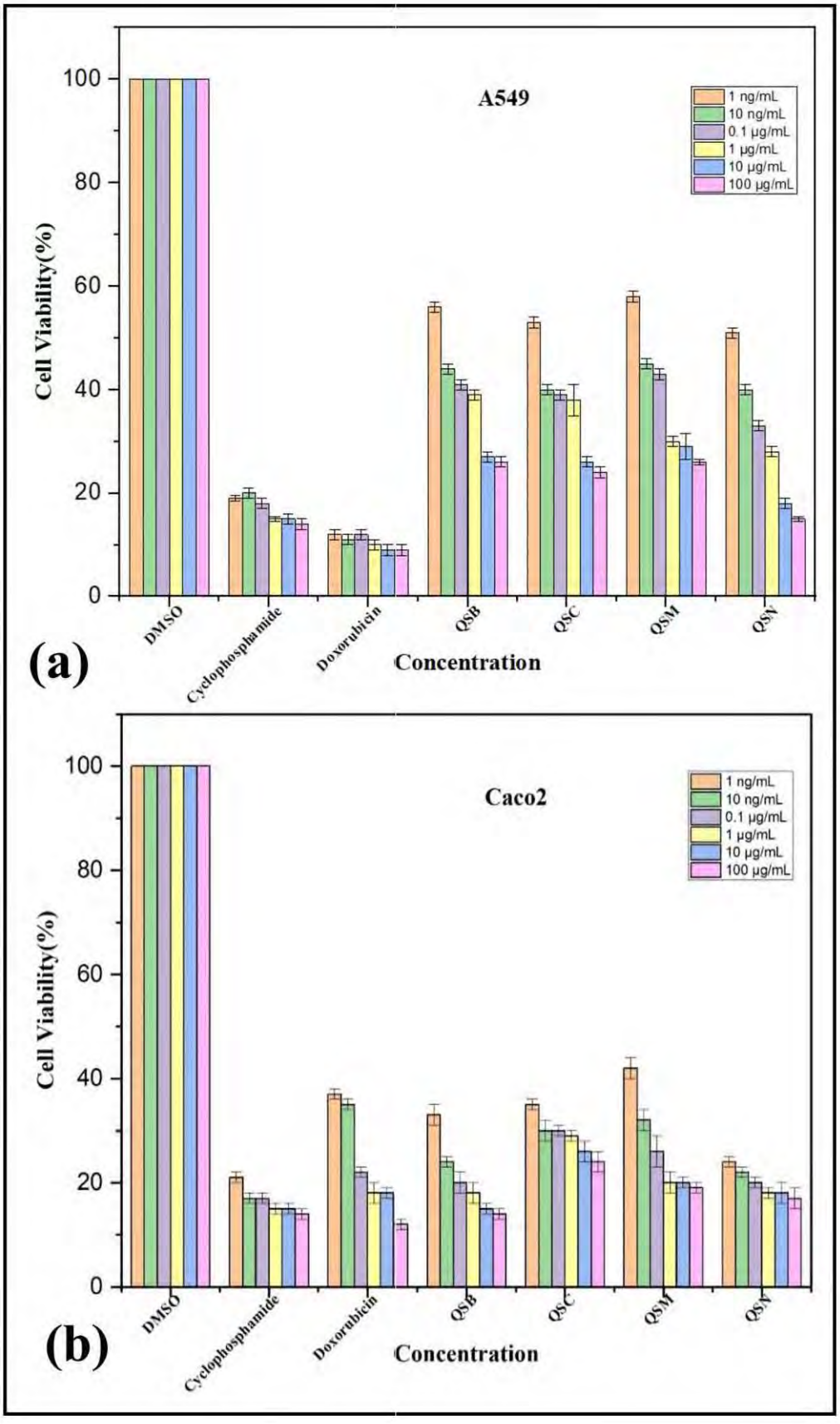


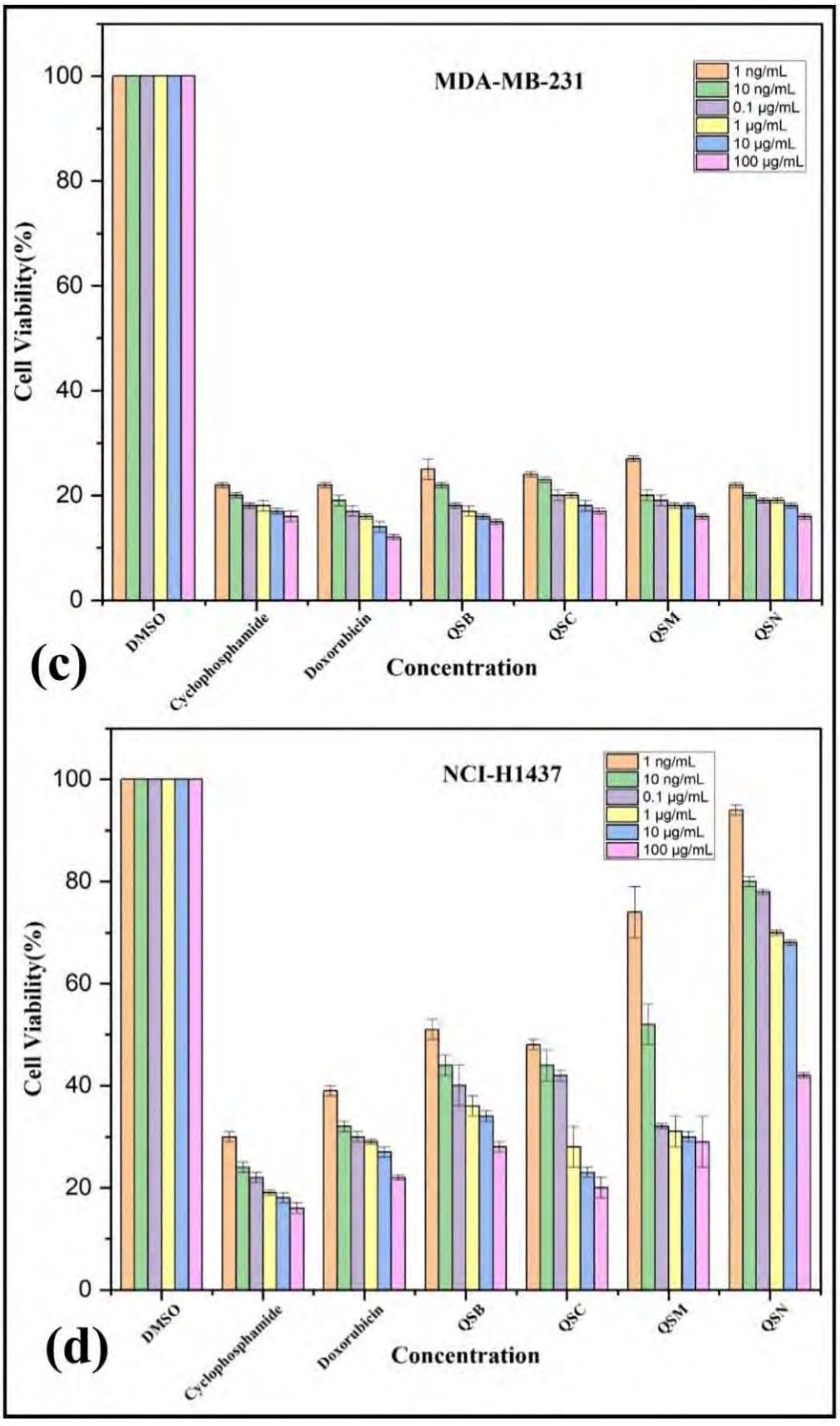
Figure 4.2. Cell viability: MTS assay Histograms represent the percentage concerning control cells (positive control: 30-40% viable cells) to:100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml,10 ng/ml,1 ng/ml) of PCB, PCC, PCM, PCN extracts in A549 cells (a), Caco2 (b), MDA-MB-231 (c), NCIH1437 (d), HepG2 (e) cancerous cell lines, and HPAEpiC (f) and HRPTEpiC (g) cell lines. Data shows mean ± SE (n=3).

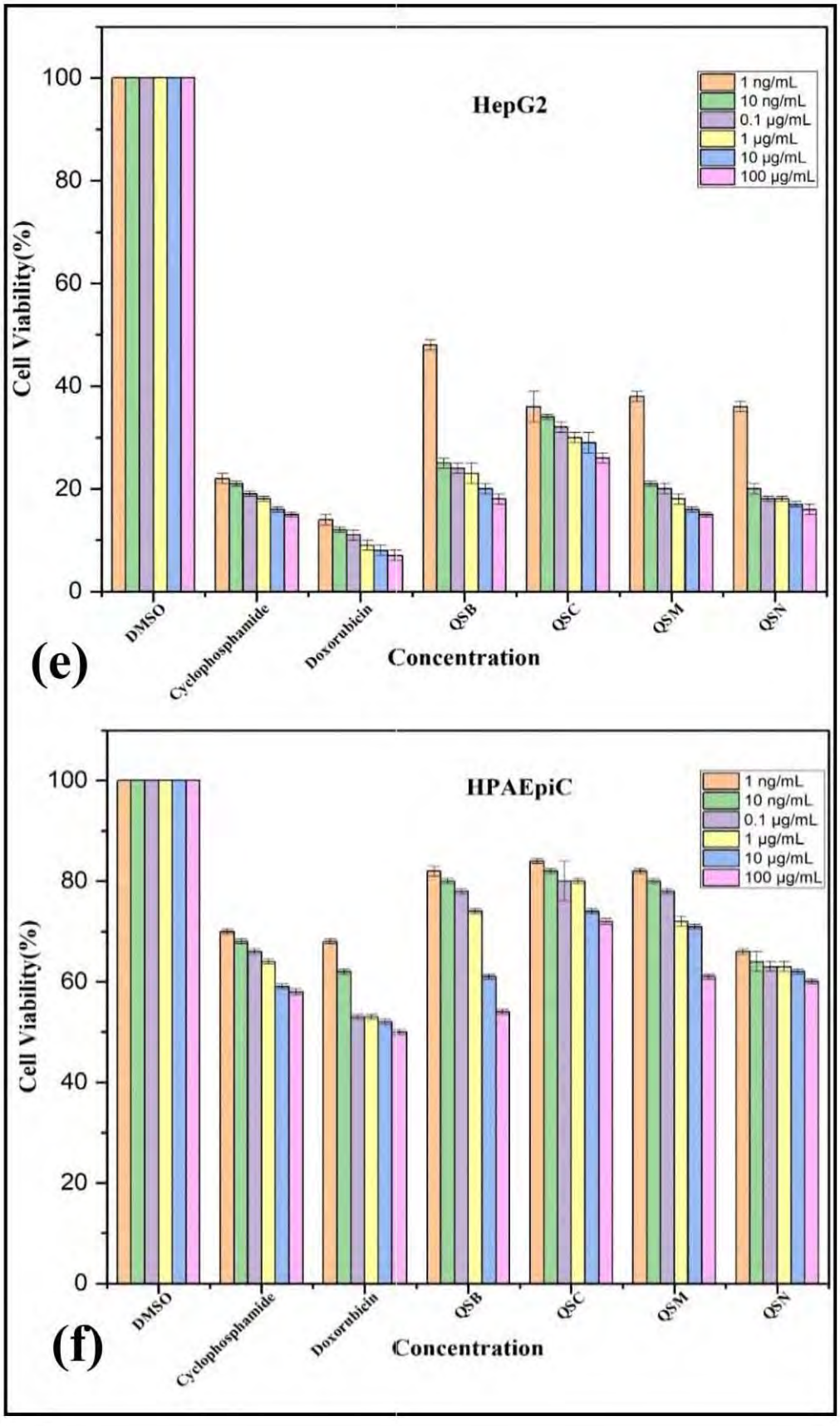
These findings also indicated that statistically significant (*p=0.001*) growth inhibition had been observed against A549 and MDA-MB-231(Figure 4.2). However, at lower concentrations, the effect of extracts is not statistically significant, particularly PCB and PCN extracts against Caco2 cells, all PC extracts against HepG2 cells, and PCB, and PCN extracts in NCI-H1347.

4.2.3. ANTICANCER POTENTIAL OF *QUERCUS SEMICARPIFOLIA*

In the case of QS extracts, a very similar pattern of anticancer activity was observed against MD-MB-231, and Caco2 establishing that extracts were more potent in these two cancerous cell lines, followed by A549 > HepG2 cells > NCI-H1437. Furthermore, butanolic and n-hexane extracts in the QS plant exhibited low cell viability, providing remarkable retardation of cancerous cell proliferation [26]. The anticancer activities of the extracts of the *Quercus semicarpifolia* have been presented in the Figure 4.3.


A549
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability(%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
QSB
QSC
QSM
QSN
Concentration
(a)
Caco2
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability(%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
QSB
QSC
QSM
QSN
Concentration
(b)


MDA-MB-231
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability(%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
QSB
QSC
QSM
QSN
Concentration
(c)
NCI-H1437
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability(%)
100
80
60
40
20
0
DMSO
Cyclophosphamide
Doxorubicin
QSB
QSC
QSM
QSN
Concentration
(d)


HepG2
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability(%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
QSB
QSC
QSM
QSN
Concentration
(e)
HPAEpiC
1 ng/mL
10 ng/mL
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Cell Viability(%)
0
20
40
60
80
100
DMSO
Cyclophosphamide
Doxorubicin
QSB
QSC
QSM
QSN
Concentration
(f)

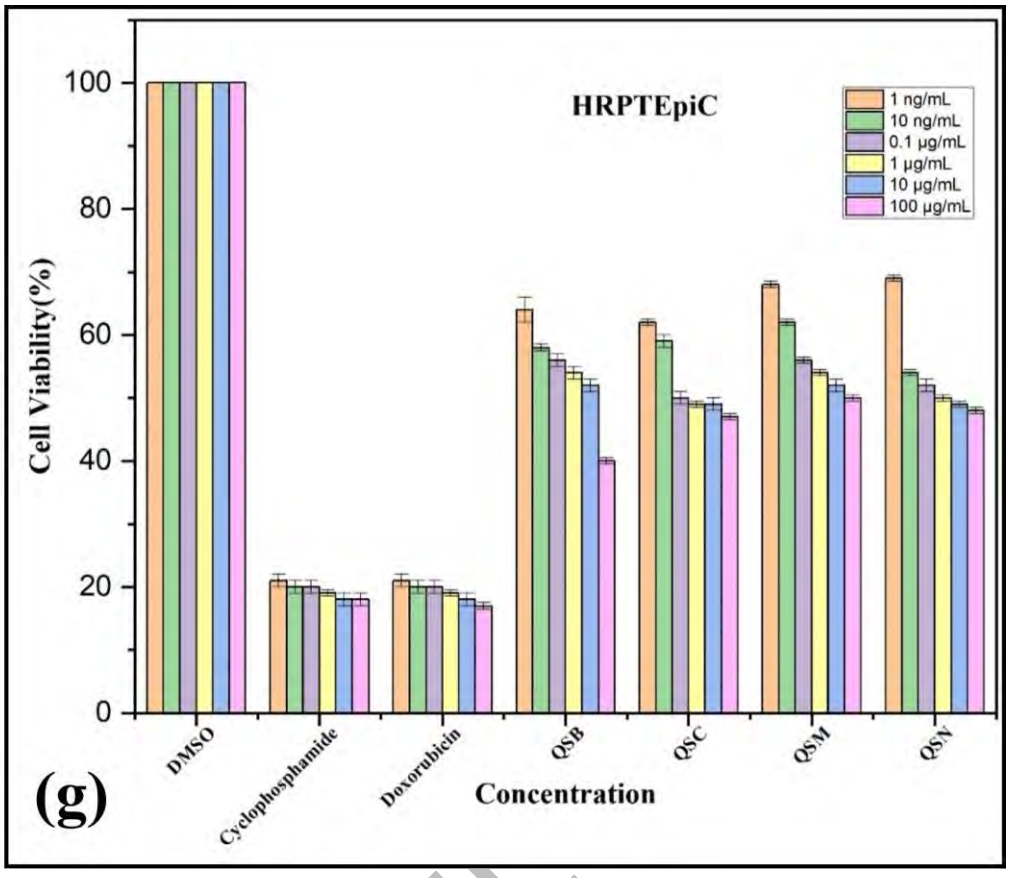


Figure 4.3 MTS assay Histograms represent the percentage of cell viability concerning control cells (positive cntrl:20-30 % viable cells) after exposure to: 1ng/ml, 10ng/ml, 0.1µg/ml, 1µg/ml, 10µg/ml, A549 cells (a), Caco2 (b), MDA-MB-231 (c), NCIH1437 (d), HepG2 (e) cancerous cell lines and HPAEpiC (f) and HRPTEpiC (g) cell lines. Data shows mean ± SE (n=3).

Comparing IC_{50} values of *P. cornuta* and *Q. semicarpifolia* among different cell lines showed that both plants are highly efficient as natural sources of cytotoxicity (Table 4-4). The PC extract showed lower IC_{50} values against A549 and MDA-MB-231 than the other three cell lines, indicating that extract could have a potential anticancer effect in these cell lines. The IC_{50} values of QS extracts were in order of Caco2 > MDA-MB-231 >A549 > HepG2 >NCI-H1437. The QS extracts suppress cancer growth in Caco2 and MDA-MB-231, suggesting that QS could be a potential anticancer agent in these cell lines.

Table 4-4: Cytotoxic activity expressed as IC_{50} (µg/mL) of plant extracts

Extracts	**IC_{50} Values (µg/ml)**						
	A549	**Caco2**	**MDA-MB-231**	**NCI-H1437**	**HepG2**	**HPAEpiC**	**HRPTEpiC**
PCB	35.54	40.9	37.27	38.27	39.6	46.38	46.00
PCC	34.38	45.90	38.72	37.97	42.2	46.56	46.05
PCM	33.22	45.3	36.484	41.74	42.9	45.26	45.83
PCN	31.84	40.53	38.17	42.28	43.93	45.71	45.65
QSB	45.91	34.60	38.31	41.85	44.83	42.20	46.03
QSC	43.68	32.31	38.43	43.31	43.17	42.58	45.58
QSM	44.74	33.68	37.52	42.06	45.80	42.33	45.80
QSN	45.69	35.66	38.29	35.05	45.79	44.26	45.81
Doxo.	45.4	35.68	45.66	45.54	46.17	55.35	45.95
Cycl.	46.4	37.66	45.77	43.63	49.28	47.71	46.52

Data are the means of triplicate.

4.3. SECTION III: EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT POTENTIAL OF SELECTED PLANTS

4.3.1. ANTIBACTERIAL EFFECT OF *ACER CAPPADOCICUM GLED*

In *A. cappadocicum* Gled. the antibacterial activity in the methanolic extract at a concentration of 40mg/ml exhibited the highest inhibition, i.e., a clear zone of 16.5 ± 1.0 mm antibacterial activity was observed against *B. subtilus*, *E. coli,* and *K. pneumonia,* and zones of 15.0 ± 1.0mm, 11± 2mm against S. *entrica* and *A. baumanni* respectively. On the other hand, the inhibition performance found with butanolic extracts was not too different as observed in methanolic extracts. In the latter case, the zone of inhibitions was 15.0 ± 1.0mm, 14.5 ± 1.0mm, and 15.0 ± 1.0mm against *B. subtilis*, *E. coli*, and *K. pneumonia,* respectively. Furthermore, the zone of inhibitions obtained with butanolic extracts against *S. enterica* was 11.5 ± 1.0mm and 16.0± 1.0mm. These zone of inhibition values were highest against *A. buamannii* among extracts (Table 4-5).

Table 4-5: Antibacterial activity of AC extracts against local strains of *E. coli*, *B. subtilis*, *K. pneumoniae*, *S. enterica*, and *A. baumannii*

Extracts	**Isolates**	***B. subtilis***	***E. coli***	***K. pneumoniae***	***S. enterica***	***A. baumannii***
		Zone of Inhibition (mm)				
ACB	1A	15	14.5	15	11.5	16
ACC	1B	9.5	10.5	10	0	14
ACE	1C	12	13.5	14.5	12	13
ACM	1D	16.5	16.25	16.5	15	11
ACN	1E	13	11	11.5	0	12
Positive		12	13	16	12	12
Negative		-	-	-	-	-

Zone of inhibition (mm) is expressed as mean, ACB; n-butanolic extract, ACC; chloroform extract; ACE; ethyl acetate extract, ACM; methanolic extract and ACN; n-hexane extract of *A cappadocicum.*

The antibacterial assay of *A. cappadocicum* Gled. against *B. subtilis*, *E. coli* and *K. pneumoniae* strains have been shown (Figure 4.4). On the other hand, the antibacterial activity of ACB and ACM extracts was maximum against the strains mentioned above.

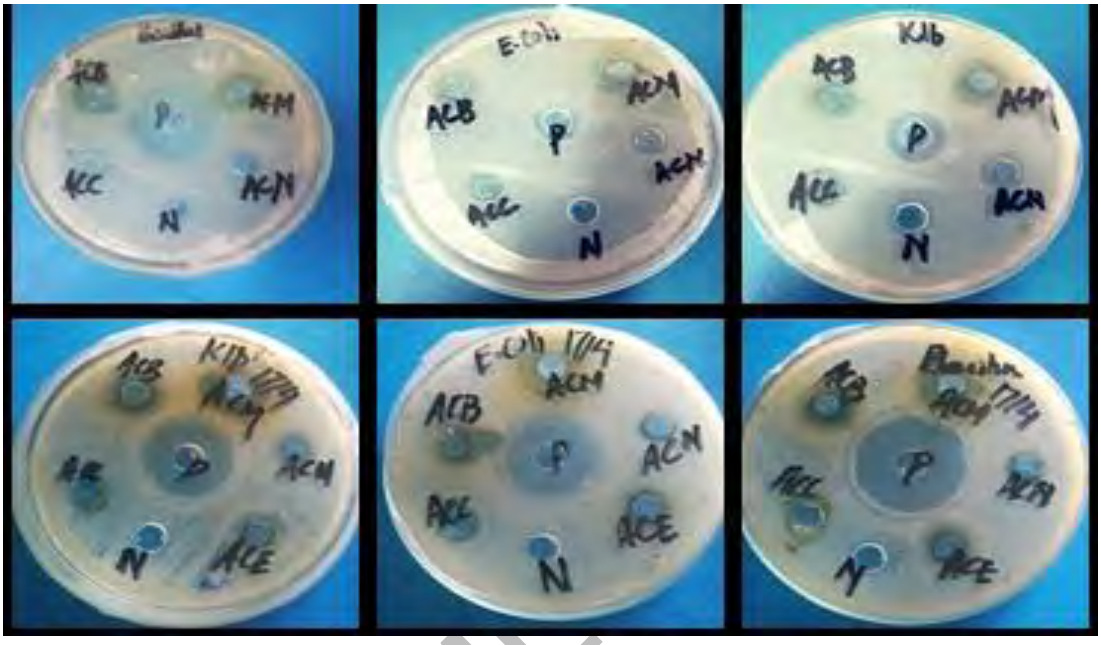


Figure 4.4. Antibacterial activity of *A. cappadocicum Gled* extracts against infectious bacterial species.

The histogram (Figure 4.5) demonstrates the *A. cappadocicum* Gled. extracts potential against various bacterial isolates. The antibacterial activity was maximum with methanolic extracts (ACM) followed by butanolic extract (ACB) compared to the positive control. In the case of *A. baumannii*, ACB showed the highest inhibitory activity. On the other hand, *S. enterica* isolate showed no susceptibility to chloroform (ACC) and N-hexane extracts (ACN) of *A. cappadocicum* Gled. The MIC values also sustained the potential of bacterial inhibition by ACB extracts with 8 µg/mL and ACM with 8 µg/mL and 16 µg/ml, as depicted in Table 4-6.

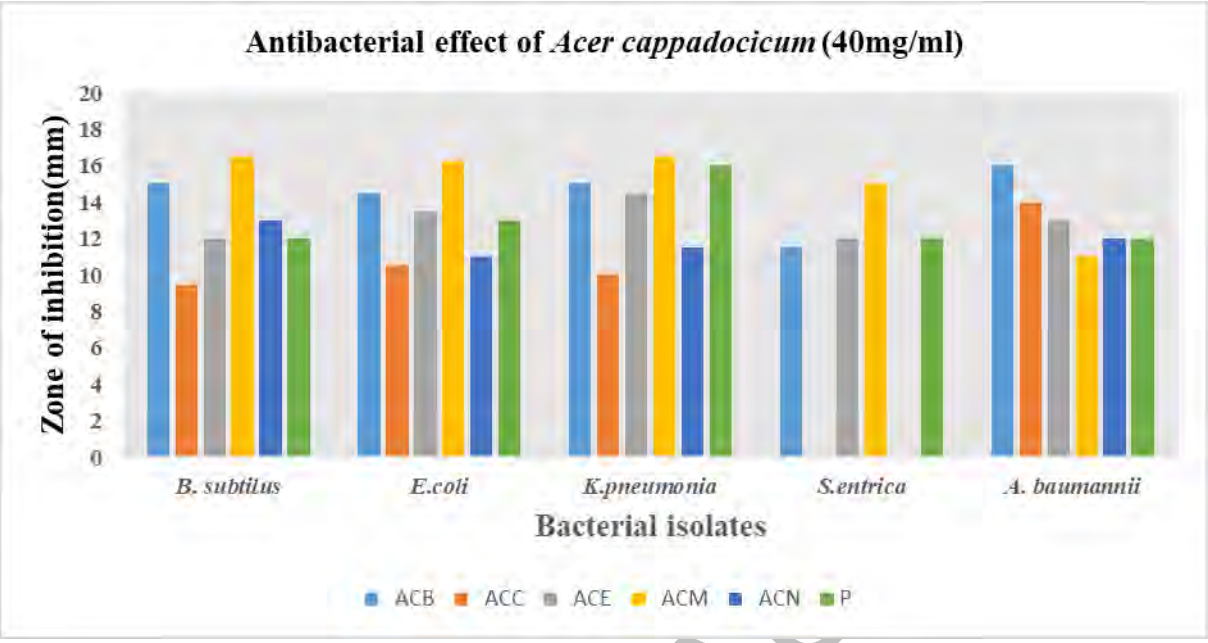


Figure 4.5. Histogram showing antibacterial activity of *Acer cappadocicum* extracts against selected bacterial isolates.

Table 4-6: MIC values of *Acer cappadocicum* extract against bacterial isolates.

Isolates	**Extracts**			
	ACB	**ACC**	**ACM**	**ACN**
B. subtills	8	128	8	32
E. coli	8	128	8	64
K. pneumonia	8	128	8	64
S. entrica	32	-	8	-
A. baumannii	8	64	16	32

Note: MIC= minimum inhibitory concentration, ACB; butanolic extract of *A. cappadocicum Gled*, ACC chloroform extract, ACM methanolic extract, ACN n-hexane extract, - = no zone of inhibition

4.3.2. ANTIBACTERIAL EFFECT OF *PRUNUS CORNUTA*

All PC extracts exhibited good antibacterial activity against selected strains. Maximum activity was shown by n-butanolic extract (PCB) against *A. baumanni* and *S.enterica* 16 and 14.5 mm zone of inhibition respectively, followed by PCN 15 and 15.5 in previously mentioned strains (Table 4-7).

Table 4-7: Antibacterial activity against local strains of bacterial strains by *P. cornuta* extracts

Extracts	**Isolates**	***B. subtilis***	***E. coli***	***K. pneumoniae***	***S. enterica***	***A. baumannii***
		Zone of Inhibition (mm)				
PCB	2A	12.5	11	12.5	14.5	16
PCC	2B	13	13	12	13	14
PCE	2C	12	11.5	13	13	13
PCM	2D	11	11	12	14	13
PCN	2E	12	14	11	15.5	15
Positive		12	13	16	12	12
Negative		-	-	-	-	-
Note: Highest activity;> 14, moderate activity; 12 -13mm. lowest activity; less than 11.						

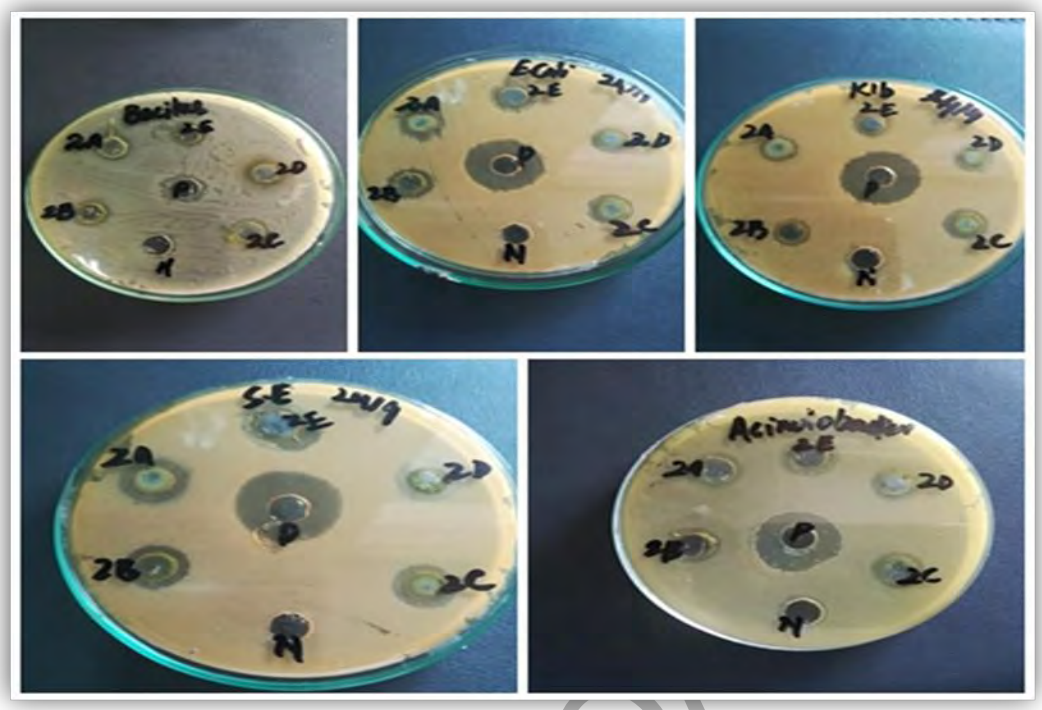


Figure 4.6. Antibacterial activity *Prunus cornuta* extracts against infectious bacterial species.

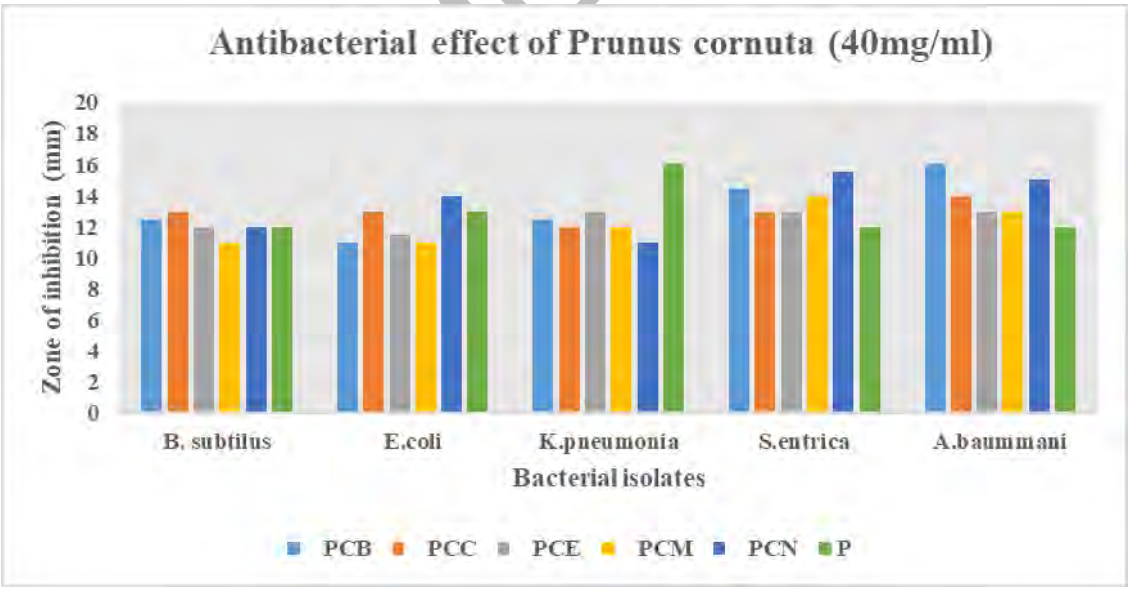


Figure 4.7. Histogram showing antibacterial activity of *Prunus cornuta* extracts against selected bacterial isolates.

The inhibitory potential of *P. cornuta* against selected bacterial strains has been illustrated (Figure 4.7). The butane extract of *P. cornuta* (PCB) showed maximum activity against *A. baumannii,* then against *S. enterica* and *B. subtilis,* and *K.*

pneumonia. The minimum antibacterial activity of butane extract made of *P. cornuta* was observed against *E. coli*. The chloroform extract of *P. cornuta* (PCC) produced maximum inhibition in *A. baumannii* and minimum in *K. pneumonia.* PCM and PCE extract showed a similar activity level against all tested bacterial strains and exhibited moderate to high activity, e.g., 12 to 14 mm. The investigated bacterial strains showed 2nd highest susceptibility to n-hexane-based extracts of *P. cornuta* (PCN) in the order of *A. baumannii* > *S. enterica* > *E. coli* > *B. subtilis* > *K. pneumonia* [Table 4-8].

Table 4-8: MIC values of *Prunus cornuta* extracts against local bacterial isolates.

Isolates	**Extracts**			
	PCB	**PCC**	**PCM**	**PCN**
B. subtills	32	32	256	64
E. coli	256	128	256	32
K. pneumonia	64	128	128	256
S. entrica	64	32	64	16
A. baumannii	16	16	64	16

Note: MIC = minimum inhibitory concentration, PCB; butanolic extract of *P. cornuta,* PCC chloroform extract, PCM methanolic extract, PCN n-hexane extract, - = no zone of inhibition.

4.3.3. ANTIBACTERIAL EFFECT OF *QUERCUS SEMICARPIFOLIA*

The antibacterial of the potential extracts of *Q. semicarpifolia* was illustrated in Table 4-9. The QSB extract showed maximum inhibition in *A. baumannii* and minimum in *S. entrica* with 15 mm and 8 mm. *E. coli* and *B. subtilis* showed similar susceptibility to QSB with a 12mm zone of inhibition. *K. pneumonia* also exhibited potent inhibition by QSB extract with 13mm. QSC showed high inhibition against *A. baumannii* (14mm), followed by *B. subtilis* and *E. coli* (12.5mm). QSE and QSM observed a similar inhibition pattern with a 16mm and 18mm zone of inhibition in *A. baumannii* and a 12.5 and 14mm zone of inhibition in *B. subtilis and E. coli,* respectively. QSM also showed significant potential against *K. pneumonia* with 15mm inhibition. The QSN showed the highest antibacterial activity against *A. baumannii* (16mm) and moderate activity against *K. pneumonia* (12.5mm); all other strains exhibited low susceptibility to QSN.

Table 4-9: Antibacterial activity against five different bacterial strains by *Q. semicarpifolia* extracts

Extracts	***B. subtilis***	***E. coli***	***K. pneumoniae***	***S. enterica***	***A. baumannii***
	Zone of Inhibition (mm)				
QSB	12	12	13	8	15
QSC	12.5	12.2	11	8	14
QSE	12	12.5	11	10	16
QSM	14	14	15	10	18
QSN	11	11	12.5	7	16
Positive	12	13	16	12	12
Negative	-	-	-	-	-

Note: Highest activity;> 14, moderate activity; 12 -13mm. lowest activity; less than 11.

The antibacterial zone of inhibition with *Q. semicarpifolia* extracts was shown in Figure 4.8 with positive control in the middle of the plate. The bar graphs (Figure 4.9) illustrated a higher susceptibility against *K. pneumonia* and *A. buamanni* strains by *Q. semicarpifolia* extracts. In addition, they validated the high inhibition potential of methanolic extract of *Q. semicarpifolia* (QSM) (Figure 4.8).

Figure 4.8. Antibacterial activity *Quercus semicarpifolia* extracts against infectious bacterial species.

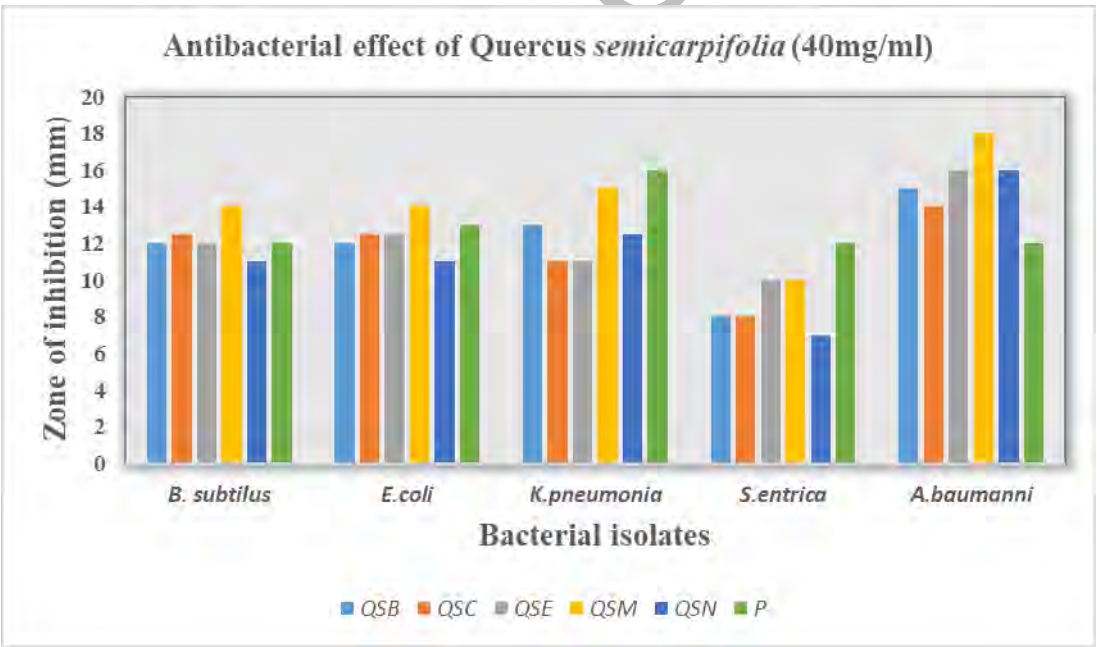


Figure 4.9. Histogram showing antibacterial activity of *Quercus semicarpifolia* extracts against selected bacterial isolates.

Table 4-10: MIC values of *Quercus semicarpifolia* extracts against local bacterial isolates.

Isolates	**Extracts**			
	QSB	**QSC**	**QSM**	**QSN**
B. subtills	64	64	16	128
E. coli	64	64	16	128
K. pneumonia	64	64	8	16
S. entrica	128	128	32	256
A. baumannii	16	16	8	8

Note: MIC = minimum inhibitory concentration, QSB; butanolic extract of *Q. semicarpifolia*, QSC chloroform extract, QSM methanolic extract, QSN n-hexane extract, - = no zone of inhibition

The lower MIC values by QSM extract showed high inhibitory potential after the antibacterial assay. Conversely, QSN extract exhibited a lesser potential to inhibit selected bacterial isolates.

4.4. COMPARISON OF ANTIBACTERIAL POTENTIAL OF AC, PC, AND QS METHANOLIC EXTRACTS WITH OTHER PLANT SPECIES OF PALLAS VALLEY

The results showed a preliminary screening of antibacterial activities of methanol extracts via the well diffusion method. ACM and PJM extracts showed maximum activity against bacterial strains. QSM followed it with moderate to good activity and PCM with the lowest activity (Table 4-11).

Table 4-11. Antibacterial Activity of Methanolic Extracts of different plants from Pallas valley.

Extracts	***E. coli***	***B. subtilis***	***K. pneumoniae***	***P. aeruginosa***	***MSA88***	***MRSA70***
	Zone of Inhibition (mm)					
ACM	20	22	21	10	17	18
BBM	18	19	18	17	-	-
PCM	11	11	12	13	-	-
PJM	22	23	56	12	17	17
RJM	17	15	21	-	-	-
QSM	18	15	20	11	16	14
VNM	-	14	15	-	-	-
Positive	13	12	16	12	12	12
Negative	-	-	-	-	-	-

Note: zone of inhibition (mm) expressed as mean of triplicates experiments, ACM; methanolic extract of *Acer cappadocicum,* BBM; methanolic extract of *Berberis brandsiana,* PCM; methanolic extract of *Prunus cornuta,* PJM; methanolic extract of *Paratiopsis jaqemontiana,* RGM; methanolic extract of *Rhamnella gilgitica,* QSM; methanolic extract of *Quercus semicarpifolia,* VNM; methanolic extract of *Vibernum nervosum.*

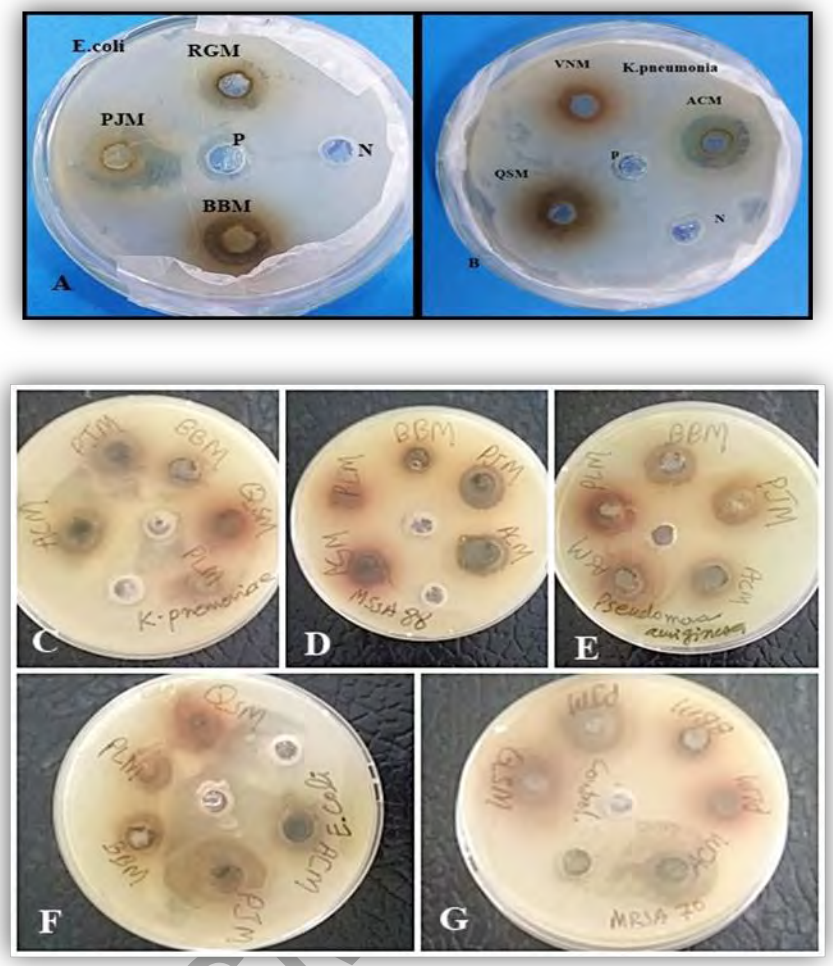


Figure 4.10. Antibacterial activity of methanolic plant extracts against **(A)** *E. coli*, **(B, C)** *K. pneumoniae*, **(D)** MSS8, **(E)** *P. aeruginosa*, **(F)** *E. coli*, **(G)** MRSA70

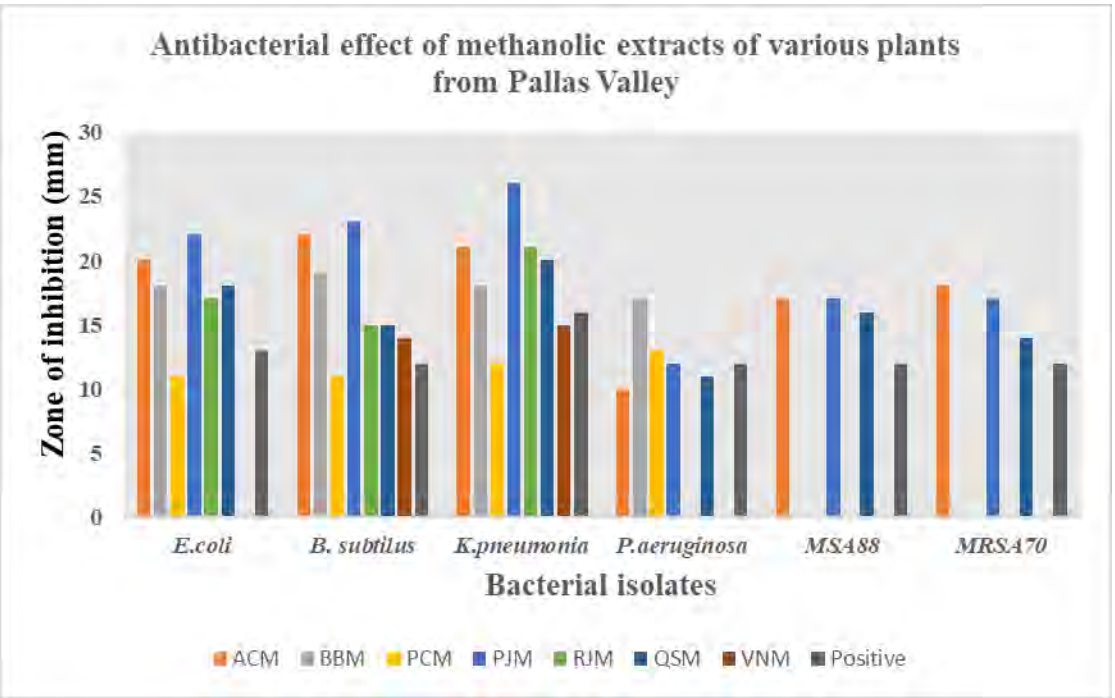


Figure 4.11. Histogram illustrating the antibacterial potential of methanolic extracts of various plants from Pallas valley against different bacterial isolates.

4.5. ANTIFUNGAL ACTIVITY OF PLANT EXTRACTS

The selected plant extracts were also tested for pathogenic fungal strains mentioned as *R. oryzae*, *A. falvus*, *A. niger,* and *Pythium* sp. The extracts showed no characteristic mycelial inhibition against above mentioned fungal species, except *R. oryzae*. The maximum inhibition against *R. oryzae* was exhibited by QSE (21mm), PCM (16.5mm), QSM (16.5mm), and ACE (16mm) (Table 4.12).

4.5.1. ANTIFUNGAL ACTIVITY BY ZONE OF INHIBITION (MM)

Table 4-12: Antifungal activity of *A. cappadocicum, P. cornuta,* and *Q. semicarpifolia* extracts

Extracts	**Fungal Isolates**			
	R. oryzae	***A. flavus***	***A. niger***	***Pythium sp.***
ACB	14	-	-	-
ACC	15	-	-	-
ACE	16	-	-	-
ACM	14	-	-	-
ACN	-	-	-	-
PCB	-	-	-	-
PCC	-	-	-	-
PCE	14	-	-	-
PCM	16.5	-	-	1.5
PCN	16	-	-	-
QSB	16	-	-	-
QSC	16	-	-	-
QSE	21	-	-	-
QSM	16	-	-	2.25
QSN	16.5	-	-	-
DMSO	-	-	-	-
Terbinafine	30	35	32.5	36

Note: Zone of Inhibition(mm), Values are expressed as the mean of triplicate experiments, -; means no activity, Extracts in butanol (ACB, PCB, QSB), chloroform (ACC, PCC, QSC), ethyl acetate (ACE, PCE, QSE), Methanol (ACM, PCM, QSM) and n-hexane (ACN, PCN, QSN).

Figure 4.12. Fungal inhibition on SDA media by plant extracts in butanol, chloroform, ethyl acetate, methanol, and n-hexane extracts. *Pythium sp.1, A niger, A. Flavous sp*. exhibited no significant inhibition except *Royzae sp*. with average of 15 % inhibition by butanol, chloroform, ethyl acetate, methanol, and 0.0 % by n-hexane extracts.

4.5.2 ANTIFUNGAL ACTIVITY BY MEASURING PERCENTAGE INHIBITION OF MYCELIAL GROWTH

Percentage inhibition of mycelial growth of fungal strains *F. fujikuroi*, *R. oryzae,* and *P. ultimum* by plant extracts are shown in Table 4-13. All *A. cappadocicum* extracts showed moderate activity around 40 to 50%. The lowest inhibition was 34% by chloroform extract against *F. fujikuroi,* and the highest was 56% against *P. ultimum* of *n*-hexane extract. Mycelial growth inhibition was significant in *R. oryzae* and *F.*

fujikuroi isolates. *R. oryzae* appeared susceptible to PCM and PCE extracts with 67 and 64 % mycelial inhibition, respectively. *Q. semicarpifolia* restricted the *R. oryzae* fungal growth up to 57% with QSM and QSB extracts. The maximum mycelial inhibition against *F. fujikuroi* pathogen was observed with PCC extract (59%) and QSE (54%).

Table 4-13: Percentage inhibition of mycelial growth of *F. fujikuroi, R. oryzae,* and *P. ultimum* by plant extracts.

Extracts	**Fungal Isolates**		
	F. fujikuroi	***R. oryzae***	***P. ultimum***
ACB	47	47	53
ACC	34	45	53
ACE	50	51	52
ACM	48	52	49
ACN	40	49	56
PCB	62	45	52
PCC	59	47	53
PCE	64	56	40
PCM	67	55	43
PCN	40	60	57
QSB	49	57	-
QSC	54	53	-
QSE	46	46	-
QSM	44	35	-
QSN	40	33	-
Positive Control	56	79	62
Negative Control	-	-	-

Note: %age mycelial inhibition expressed as mean ± SD (n=3); lower inhibition= 20-30%, moderate inhibition= 40 50%; high inhibition= 60-80%, Positive control; Terbinafine

The percentage inhibition of fungal isolates is shown in Figure 4.13. In this analysis, *P. cornuta* showed higher inhibition potential than *A. cappadocicum* Gled. and *Q. semicarpifolia. More specifically, F. fujikuroi showed high susceptibility to P. cornuta extracts* with methanol (PCM) and ethyl acetate (PCE) extracts. *On the other hand, A. cappadocicum* Gled inhibited growth in *P. ultimum* more effectively. At the same time, the butanol (ACB) and n-hexane (ACN) extracts have been less effective in inhibiting fungal growth as assessed using *P. ultimum* strain.

On the contrary, *Q. semicarpifolia* extracts did not show any potential activity against *P. ultimum.* All three plant species inhibited the *R. oryzae* isolate. For instance, QSM extracts exhibited a 35% inhibition, while PCN extracts performed better with 60% inhibition. The later values fall closer to those (79%) observed for positive control.

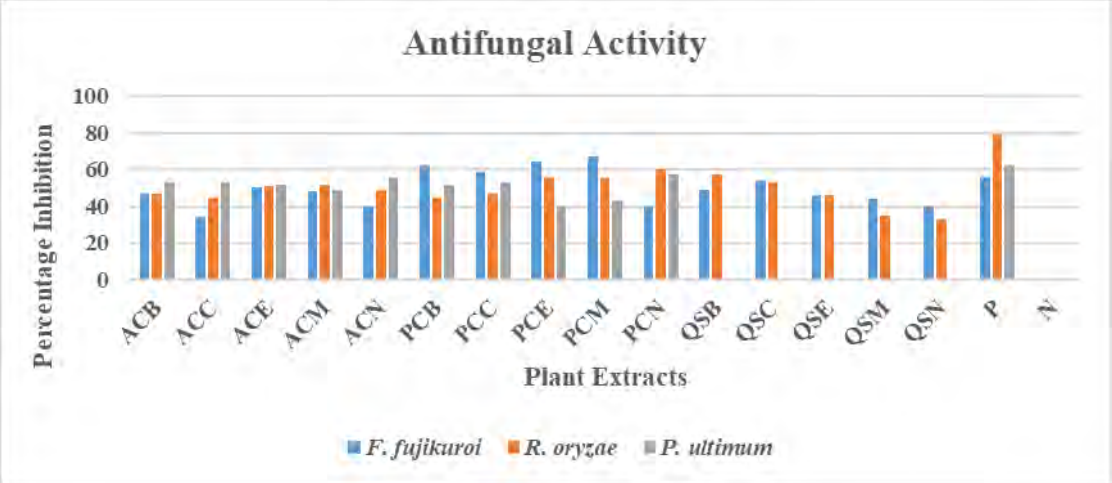


Figure. 4.13. Histogram showing the percentage inhibition of fungal isolates with extracts of *A. cappadocicum*, *P. cornuta,* and *Q. semicarpifolia*, P= Positive control, N= Negative control.

4.6 ANTIOXIDANT POTENTIAL OF SELECTED PLANTS

4.6.1 ANTIOXIDANT ACTIVITY OF *ACER CAPPADOCICUM GLED*

The IC_{50} value of butanolic, ethyl acetate, methanolic and n-hexane are 0.523 (ACB), 0.5146 (ACE), 0.5987 (ACM), and 0.5108 (ACN), respectively. Among all sample extracts, chloroform extract has the strongest scavenging activity.

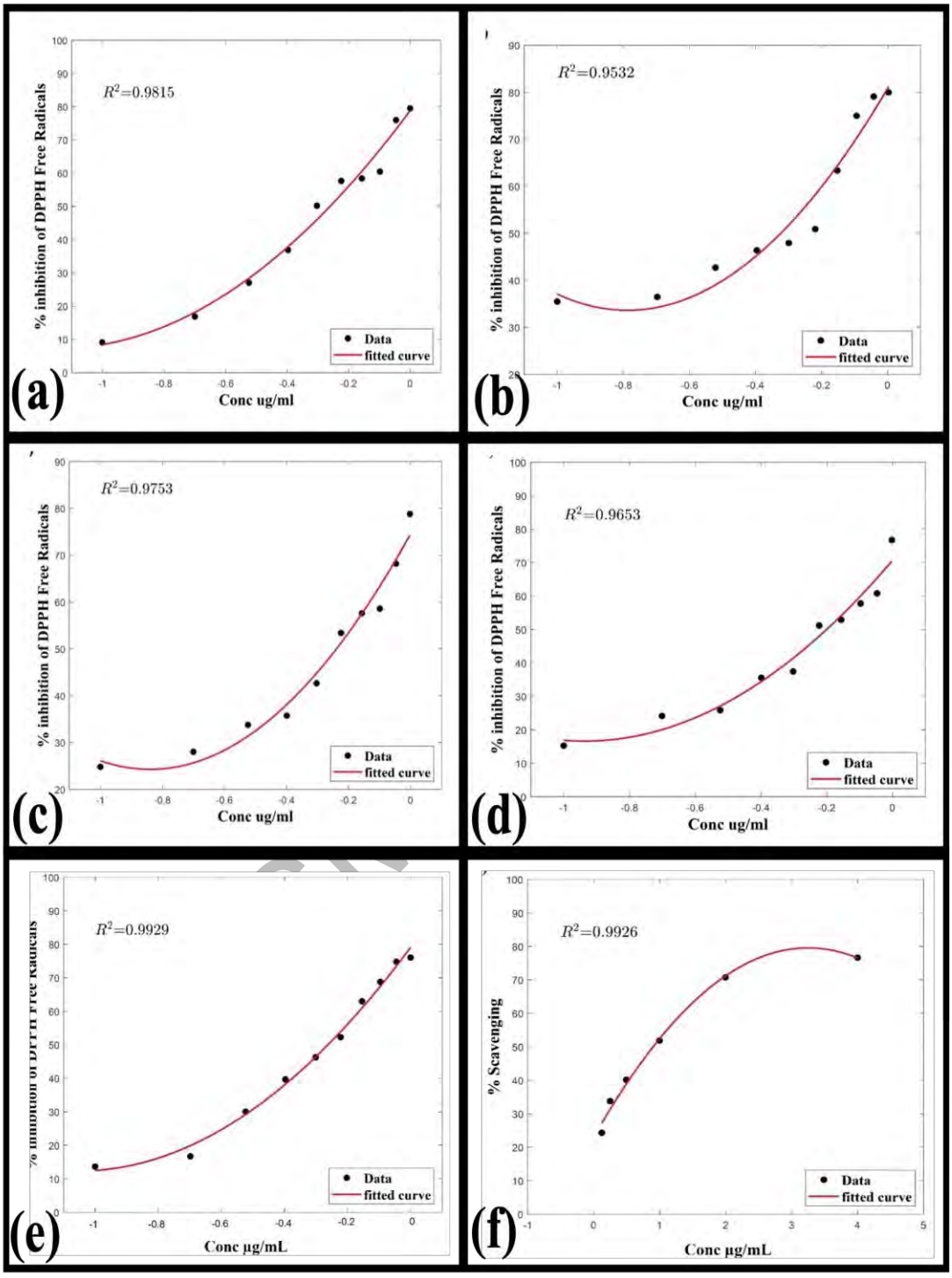


Figure 4.14. Free radical scavenging of plant extracts by DPPH method at different concentrations(µg/ml). **(a)** ACB extract, **(b)** ACC extract, **(c)** ACE extract, **(d)** ACM extract, **(e)** ACN extract, **(f)** standard (ascorbic acid)

The results of DPPH radical scavenging (%) at various concentrations are illustrated (Figures 4.14 a, b, c, d, e, f). From these figures, DPPH activity towards plant extracts

is demonstrated. As shown, the inhibition rate of extracts is concentration-dependent and expressed as IC_{50} value, which requires the concentration of samples necessary for 50% inhibition of DPPH by the extracts [33], which means the higher the concentrations of extract, the higher the activity of extracts was observed. At lower concentration (-1.0 µg/ml) exhibited similar activity (Figure 4.14 a, c, e). The ACC (Figure 4.14 b) showed maximum inhibition of DPPH.

Table 4-14: IC_{50} value of DPPH radical scavenging activity

Sample	**IC_{50} (µg/ml)**
ACB	0.5233
ACC	0.3597
ACE	0.5146
ACM	0.5987
ACN	0.5108
Ascorbic acid	15.67

4.6.2. ANTIOXIDANT POTENTIAL OF *PRUNUS CORNUTA*

The antioxidant potential of *P. cornuta* extracts is shown (Figure 4.15). Altogether, all extracts of *P. cornuta* have shown remarkable inhibition of DPPH radicals at different concentrations of plant extracts. The inhibition rate was 70-80% at higher concentrations. The results indicated that chloroform extract (PCC) has the strongest scavenging activity compared to other extracts. The IC_{50} value of sample extracts were: 4.004 µg/mL PCB), 0.4885 µg/mL (PCC), 5.225 µg/mL (PCE), 0.571 µg/mL (PCM) and 0.5134 µg/mL (PCN).

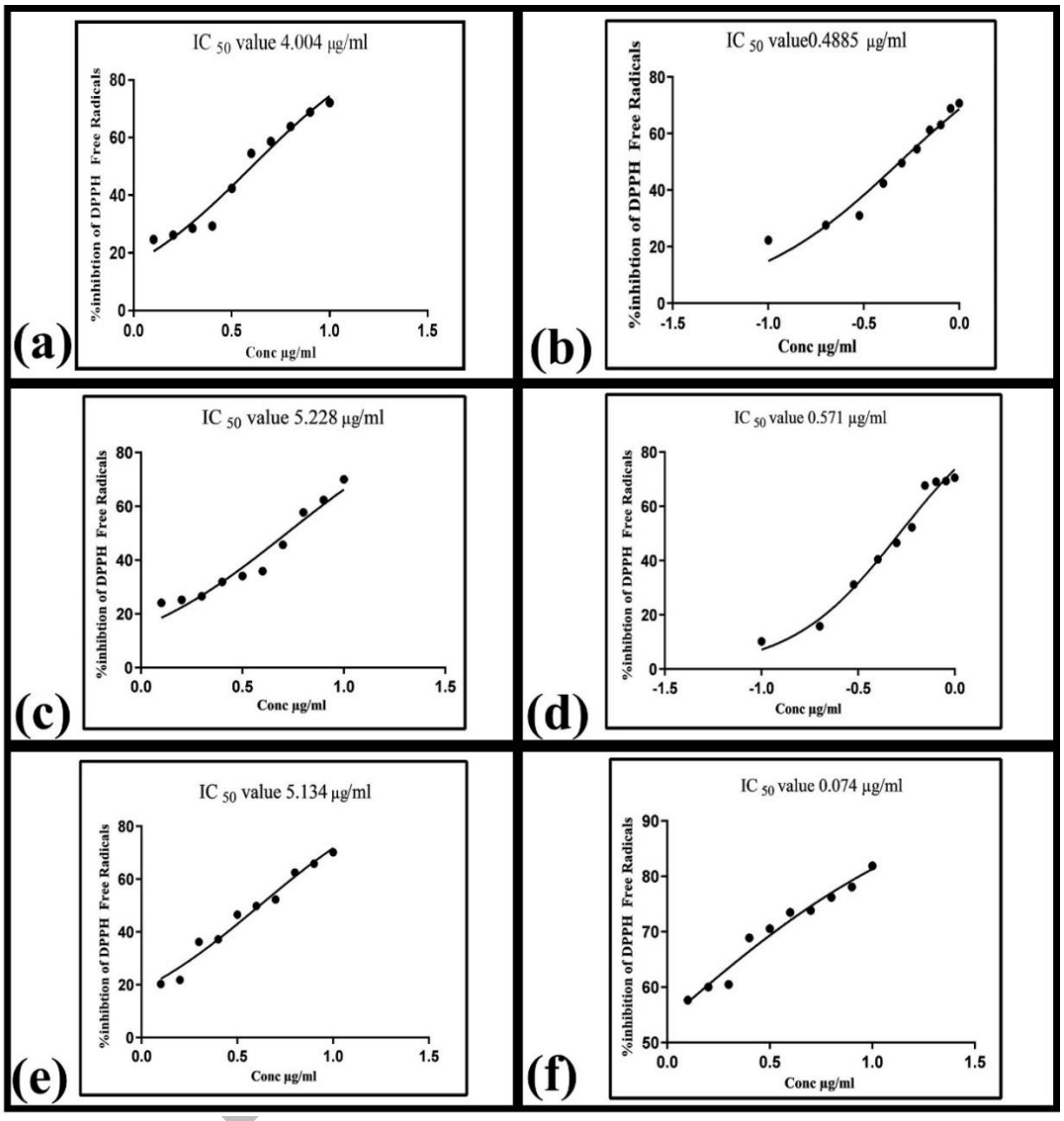


Figure 4.15 Free radical scavenging of plant extracts by DPPH method at different concentrations(µg/ml). Here **(a)** PCB extract, **(b)** PCC extract, **(c)** PCE extract, **(d)** PCM extract, **(e)** PCN extract, **(f)** standard (ascorbic acid)

Table 4-15: IC_{50} value of DPPH radical scavenging activity

Sample	**IC_{50} (µg/ml)**
PCB	4.004
PCC	0.4885
PCE	5.225
PCM	0.571
PCN	0.5134
Ascorbic acid	0.074

4.6.3 ANTIOXIDANT

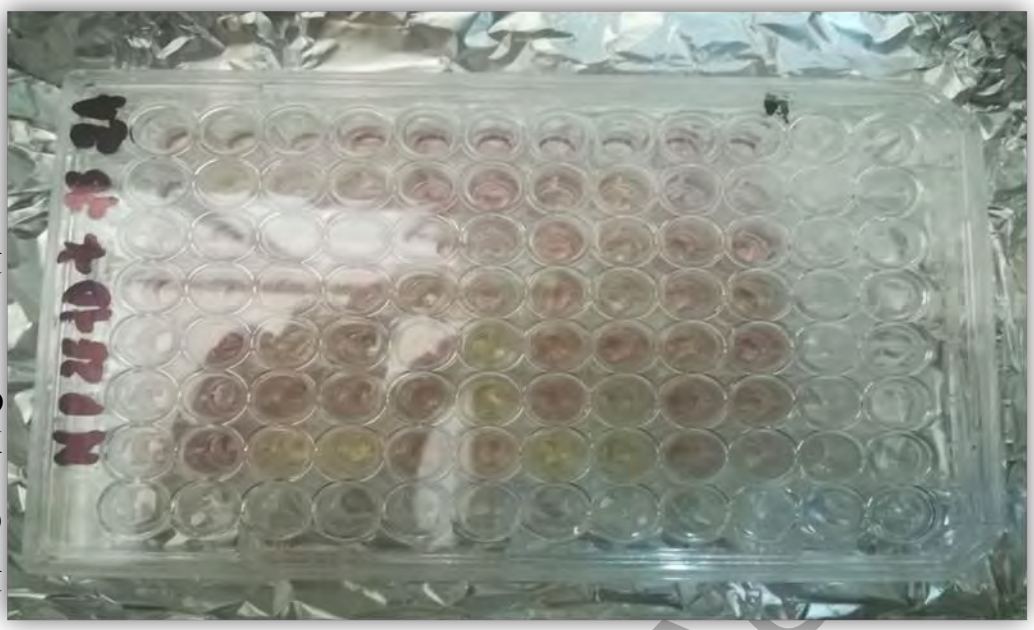


Figure 4.16. Free radical scavenging of plant extracts by DPPH method of *P. cornuta* extracts.

4.6.3. ANTIOXIDANT POTENTIAL OF *QUERCUS SEMICARPIFOLIA*

Antioxidant analysis of *Q. semicarpifolia* revealed a linear increase in the DPPH free radical inhibition with the increasing concentrations of plant extracts (Figure 4.17). In addition, the lower IC_{50} values of all extracts of *Q. semicarpifolia* reflected higher antioxidant potential. The order of inhibition was QSM > QSN > QSB > QSE and QSC (Table. 4-16).

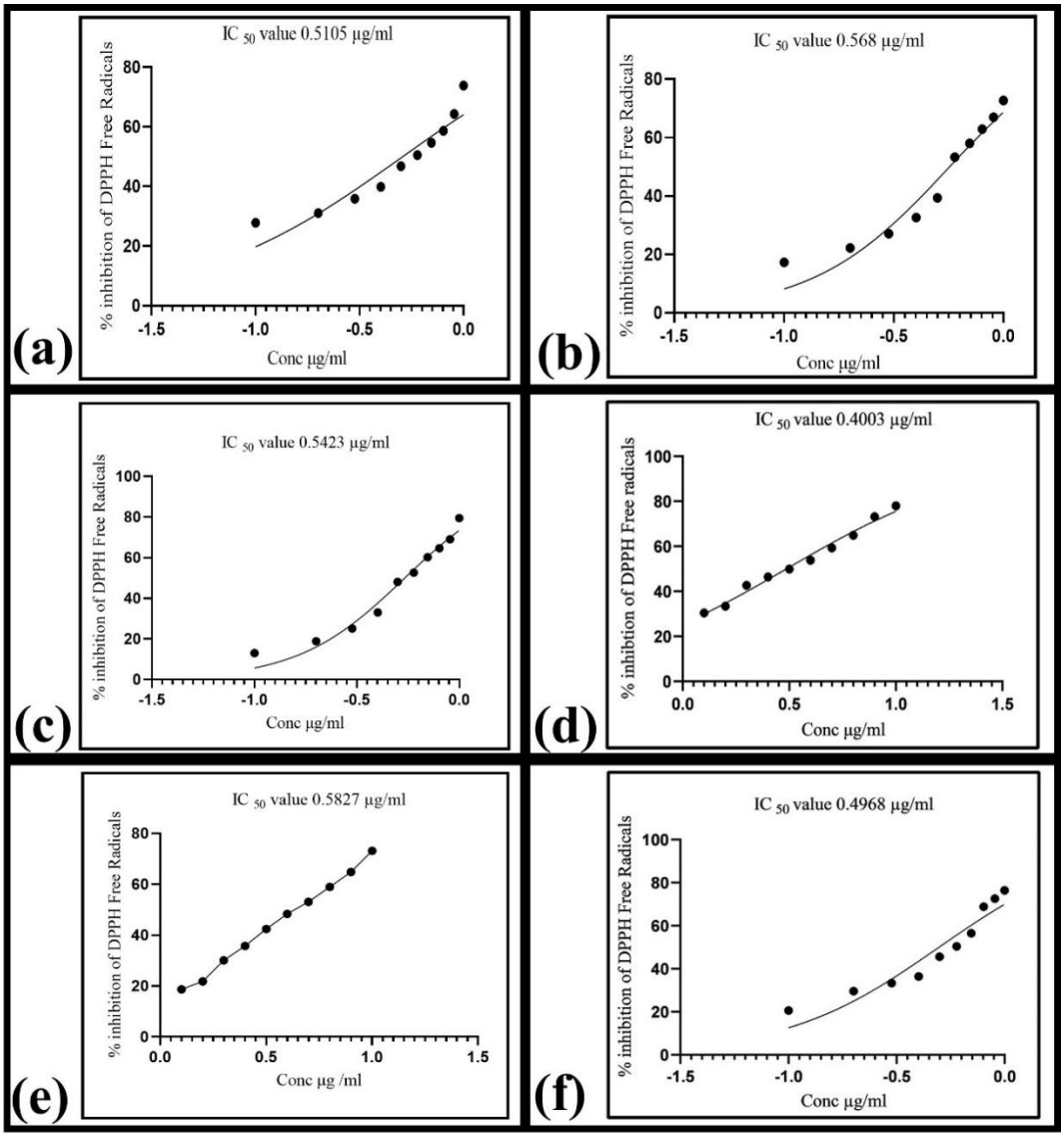


Figure 4.17. Free radical scavenging of plant extracts by DPPH method at different concentrations(µg/ml). **(a)** QSB extract, **(b)** QSC extract, **(c)** QSE extract, **(d)** QSM extract, **(e)** QSN extract, **(f)** standard (ascorbic acid)

Table 4-16: IC_{50} value of DPPH radical scavenging activity

Sample	**IC_{50} (µg/ml)**
QSB	0.5105
QSC	0.5680
QSE	0.5423
QSM	0.4003
QSN	0.5827
Ascorbic acid	0.4968

Section IV: EFFECT OF ACER CAPPADOCICUM GLED ON LIVER-ON-CHIP on liver-on-chip CANCER Model

4.7. COMPARATIVE ANALYSIS OF CELL VIABILITY

The evaluation of extracts of the Acer plant showed enhanced anticancer activity on the MPS system. The reduction of cell viability rate to 25.5 % at a high concentration (100ng/ml) in an MPS system was observed compared with the cell viability rate of 35.63 % in 96 well plates at the same concentration (Table.4-17).

Table 4-17: Percentage cell viability of methanolic extracts of *Acer cappadocicum Gled.* on MPS and 96 well plate

Sr. No.	Extract Concentration	Cell Viability of MPS	Cell Viability of 96 well plate		
		Average	S. D	Average	S. D
1	Control	92.4	1.93	90.4	2.5
2	DMSO	84.6	1.93	90	2.6
3	100 ng/ml	25.5	4.2	35.63	4.8
4	10 ng/ml	39	5	47	6.2
5	1 ng/ml	45	4.3	52	4.04

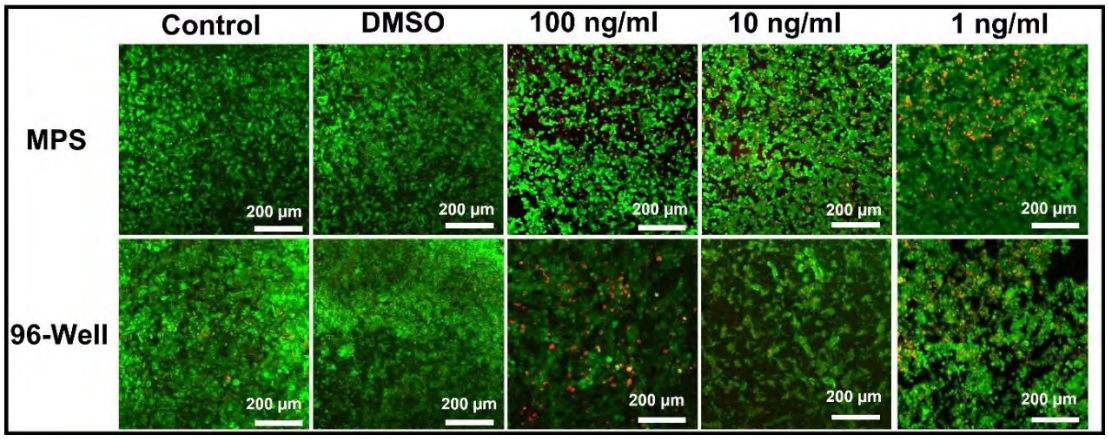


Figure 4.18. Confocal images of live/dead assay of HepG2 cancer cells from liver-on-chip cancer model after treatment with different concentrations of plant extract (100ng/ml,10ng/ml,1ng/ml) with negative and positive control on the organ-on-chip model (MPS system) and 96 well plate. Images were taken after 72 h of incubation at 37 °C.

Figure 4.18. illustrates that the methanolic extract of *A. cappadocicum* exhibited lower cell viability in the liver tumor MPS system than the conventional 96 well plate. In other words, plant extract showed better anticancer potential in the MPS system. The results suggested that the liver microphysiological system can be used for the cytotoxic assessment of plant extracts as an advanced clinical approach.

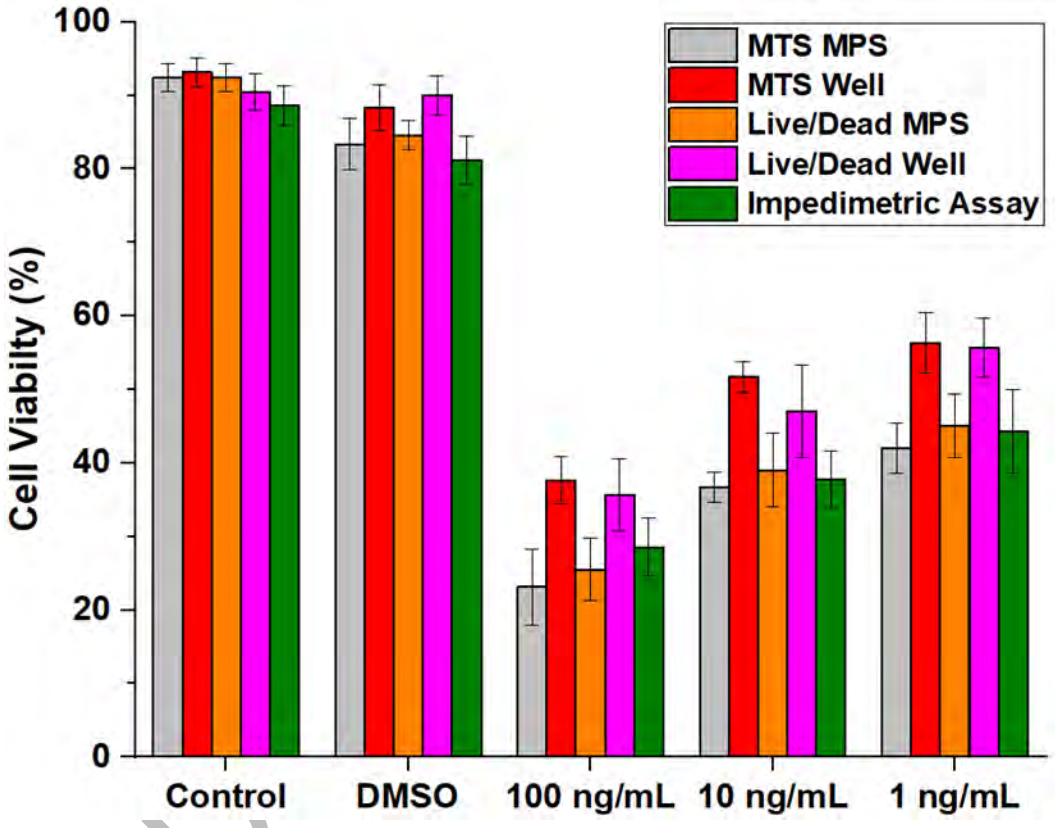


Figure 4.19. The percentage cell viability of different concentrations (100ng/ml,10ng/ml, and 1ng/ml) of methanolic extracts of *A. cappadocicum* within MPS (liver on chip model) and 96 well plates at the end of the experiment.

5. DISCUSSION

To the best of our knowledge, it is the first study addressing bioactivity potential, specifically the anticancer potential of Himalayan plants native to Pakistan. The reason for validating the anticancer potential of selected plant material is to highlight ethnobotanical knowledge of Pakistani flora and reproduce any medicinal agent from local flora. For this purpose, more than eight species of plants were collected from Pallas valley and screened for initial bioactivity potential through antibacterial assays. However, based on bioactivity findings and a literature survey, it has been reduced to three plants. As a result, only the plants with significant activity and limited published research work were selected for further investigations.

The traditional knowledge of indigenous plants is essential for pharmacological studies. The ethnopharmacological relevance of indigenous species is related to their geographic distribution and their phytochemical constituent's diversity with changing environmental conditions. It is crucial to characterize the new flora for their therapeutic potential, such as anticancer, antimicrobial, antioxidant, etc., to facilitate the drug discovery process [1,2,3,7]. Due to the global prevalence of infectious diseases and the reoccurrence of resistance varieties of pathogenic species of microbes and fungus, efforts towards a novel form of the inhibitory drug have been increased in the twentieth century. Moreover, the elevated rate of cancer disease worldwide and its painful side effects on the human body still questioning the current procedure used for its cure [11]. Therefore, plants are considered the best alternative for advanced drug discovery research or an herbal drug. The selected plant material consists of 3 species belonging to three different families, e.g., Aceraceae, Rosaceae, and Fagaceae.

The members of the Acer genus are distributed worldwide and confirmed for their medicinal uses through modern pharmacological studies [18]. The *Acer cappadocicum Gled.* first time has been evaluated for its biological activity and, most notably, for anticancer research. The extracts of *A. cappadocicum* exhibited a potential inhibitory effect on cancer cells. Results clearly showed that *A. cappadocicum* extracts exhibited significant ($p=0.05$) cytotoxic activity against A549, Caco2, and MDA-MB-231 cancerous cells (Figure.4.1). The low cell survival rate in

these cell lines reflects a high rate of cytotoxicity. Most importantly, in the case of A549 and Caco2 cell lines, chloroform (ACC) and butanolic (ACB) extract showed maximum anticancer activity compared with the standard. In MDA-MB-231, all extracts (ACC, ACB, ACM, and ACN) behaved with the characteristic potential of cancerous cell growth inhibition, indicating that breast cancer cells are more prone to *A. cappadocicum* extracts. With *A. cappadocicum* extracts, HepG2 and NCI-H1437 cell lines exhibited a moderate cell survival rate (40-50%), indicating the cytotoxic effect of extracts on these cell lines. Relative much higher cell viability for normal cells suggested that plant extracts were safe/nontoxic [38]. The studies have shown that phenolic-rich extracts of *Acer* species. Inhibit cell proliferation in tumor cell lines [39].

The findings of anticancer activity of *Acer cappadocicum Gled.* showed that the selected plant is highly effective against lung (A549), Caco2 (colon cancer/gut), and breast cancer cells (MDA-MB-231). Furthermore, a study by Hyun Ji Eo (2020) [81] validated the anticancer effect of *A. tegmentosum* on the same type of cancer cells, e.g., lung, breast, and liver cancer. The findings of their work showed that *A. tegmentosum* extract reduced the cell viability up to 30 % at 100 µg/mLin A549 (lung) and 33% in breast cells, which is quite close to the results of *A. cappadocicum* extracts cell viability rate up to 20-30% in A549 cells and 20 % in breast cancer cells, respectively. On the other hand, the *A. tegmentosum* extract reduced cell viability (%) in HepG2 cells to 12 % at 100µg/ml, whereas *A. cappadocicum* extracts reduced the cell viability to 40-50 %. Therefore, it can be due to the chemical constituents in both Acer species. The study results by Hyun Ji Eo (2020) also suggested that extracts of Acer species can be used as supplementary material for treating human cancer cells, particularly lung and breast cancer cells.

In another study [82], the effect of Acer species extracts ((*Acer saccharum Marsh*, *A. rubrum L.)* was investigated on Caco2 cells (human colon cancer). They confirmed that Acer species extracts inhibited the proliferation of Caco2 cells two-fold compared to normal cells, which is relevant to the results of *A. cappadocicum* extracts on caco2 cancel cells. Furthermore, it [82] also signifies the presence of phenolics in *Acer* species, which contribute to the anti-cancer effect of *Acer* species.

The members of the genus prunus are famous for their nutritious fruits. Various species of this genus have been studied for antibacterial, antioxidant, and anticancer activities, for instance, *P. armenica*, *P. domestica,* and *Prunus serrulata var. spontanea,* etc. [83,84,85]. The results showed that *P. cornuta* reduced cell viability in the lung (A549) and breast (MDA-MB-231) cancerous cells. The extracts affected less in Caco2 cells compared to other investigated species. Bo-Bae Lee, (2007) [85] evaluated the effect of *Prunus serrulata var. spontanea* extracts on human colon cancer cells (HT-29). The outcome of this study confirmed that this species of Prunus genus inhibited the growth of colon cancer cells by 38.8% at the highest concentration. In the case of *P. cornuta* extracts activity on colon cancer cell lines(caco2), the cell viability was approximately 40 % with PCB and PCN extracts but extracts in chloroform and methanol showed high inhibition. A review by Christian Bailly (2020) [86] also confirmed the anticancer potential of *Prunus mume* (Rosaceae) extracts on lung, liver, colon, and breast cancer. Thus, emphasizing the anticancer role of members of genus prunus with the possible mechanism. Also, HepG2 cells showed moderate effect of *P. cornuta* extracts at low concentrations (1 ng/ml & 10 ng/ml). Evaluation of the anticancer activity of *Prunus spinosa. L* flower extracts in HepG2 cells (liver) showed a significant reduction in cancer cell growth above 50µg/mL [88]. This study shows that for effective inhibitory effect in HepG2 cells, higher concentrations of *P. cornuta* extracts are required. Nara observed a similar pattern of growth inhibition in breast cancer in a study on the phenolic enriched extracts of *Prunus avium. N lage*, (2020) [87]. *P. avium* extracts. Examples of phytochemicals in Prunus species that are considered related to the bioactivity of these plants are phenolics, anthocyanins, flavioniods [87]. HPLC-MS technique was used for phenolic confirmation in *P. mume* fractions. Similarly, the presence of glycosides (cyanogenic) and quercetins is also attributed to the cell death by Prunus extracts by Murati et al., 2019) [88]. The primary phytochemical analysis of *P. cornuta* also mentioned the occurrence of glycosides and quercetins (Table.4-1).

The *Quercus semicarpifolia* extracts performed well in Caco2 and MDA-MB-231 cancerous cells and reduced cell viability to 25% and 20%, respectively (Figure.4.3). This exceptionally higher inhibitory effect may be due to the presence of tannins, flavonoids, and glycosides (Table.4-1). This correlation of pharmacological effect on

cytotoxicity was confirmed in different species of genus *Quercus* [89,90]. In the lung (A549) cell line, cell viability was above 40 %, which is different behaviour from *P. cornuta* and *A. cappadocicum Gled*. Moreover, NCI-H1437 cells showed less inhibition by *Q. semicarpifolia* extracts. Another investigation on apoptosis of breast cancer by *Q. infectoria* extracts confirmed the dosed dependant inhibition of breast cancer, a similar outcome to *Q. Semicarpifolia* activity against cancerous breast cells (Sharba et al., 2020) [91]. Preliminary phytochemical investigation in *Q. infectoria* also confirmed the existence of tannins, flavonoids, saponins, phenolics, and volatile compounds. Perez et al. (2017) also confirmed the significant anticancer activity of *Q. robur* against breast adenocarcinoma cell line by the existence of triterpenoids [92]. Burlacu, E., (2020). mentioned that the main bioactive constituents are phenolics and glycosides in the Quercus species [9]. Elansary et al. also confirmed that extracts of *Q. macrocarpa and Q. robur* showed an inhibitory effect on human and breast cancer cells [93].

The essential findings of an anticancer investigation of selected plant species were exceptionally reduced cell viability in the lung (A549), Caco2, MDA-MB-231 cancerous cells by *A. cappadocicum Gled.,* A549 and MDA-MB-231 by *P. cornuta* extracts, Caco2 and MDA-MB-231 by *Q. semicarpifolia* extracts. There is significant cell death in the respective cell lines by these selected plant species. The more effective results suggested the selected plant material has a higher potential as an anticancer substance for drug discovery.

At lower concentration the effect of extracts is not statistically significant, particularly PCB and PCN extracts against Caco2 cells, all PC extracts against HepG2 cells, And PCB, PCN extracts in NCI-H1347 which points possible future research on the protective effect of extracts towards normal cells at a concentration less than 1 µg/ml. The moderate antiproliferative effect of PC extracts in HepG2 cells may demand a higher dose to produce effective inhibition, such as the significant inhibitory effect of *Prunus spinosa. L* flower extracts in HepG2 cells were used at 150- 200 µg/mL[28]. Most of the cell death in genus prunus was attributed to glycosides (cyanogenic), as reflected by the preliminary chemical characterization Table. 5 and to the existence of quercetins in the plant extracts [28]. Various anticancer drugs have been developed throughout time, but nowadays, resistance and side effects of cancer treatments

demand new, highly efficient anticancer substances with low toxic effects [29]. The results also illustrated that QS extracts efficiently inhibit proliferation in some cancer cells, as shown in Figure 4.3. The cytotoxic effect of the QS plant has been attributed to the presence of tannins, terpenoids, flavonoids, and glycosides. This correlation of pharmacological effect and chemical composition has also been reported in various Quercus species [30,24].

The limitations of anticancer activity were that to study the effect of extracts at very close concentrations (100 µg/ml,10 µg/ml, 1 µg/ml, 0.1 µg/ml, 10 ng/ml, 1ng/ml) so that the observed activity failed to show colossal variation across different concentrations. From the outcome of this study, it has been suggested to use plant extracts concentration with significant intervals so that the effect of extracts can be better evaluated at regular intervals of values. Another limitation was using an advanced NMR technique to validate extracts' chemical foundation of bioactivity. Due to lack of previous NMR literature about species and indications of some new complex compounds, which needed a time-consuming chemical study for further evidence and confirmation of that chemical substance, pushed this study towards fundamental phytochemical analysis.

The mechanism of anticancer activity may be mediated by reducing ROS species and the occurrence of phenolic and flavonoid compounds. The diversity in phytochemical constituents of selected plants could be a possible reason for effective anticancer activities against various cancer cells. However, the molecular basis of the antiproliferative mechanism is not evaluated yet. Further studies are required to delineate genetic pathways in these plants. For example, m RNA level, cell cycle arrest, or DNA apoptosis pathways of cancer cells after exposure to extracts can be studied for molecular analysis. Nara. N lage, (2020) [7] also demonstrated the expression of genes involved in cell survival by RT-PCR and calculated the level of mRNA.

The plant extracts' antibacterial analysis against pathogenic species was investigated by measuring the zone of inhibition (mm). *A. cappadocicum Gled* showed enhanced inhibition of bacterial isolates similarly to anticancer activity. The bar graph (Figure.4.5) showed that the butanolic extract (ACB) and methanolic extract (ACM)

have maximum antibacterial activity against all bacterial species. The MIC values also demonstrated that ACB and ACM have a high inhibition potential. (Table.4-6). Previous studies on *Acer syriacum* extracts have shown that the methanolic extracts *of A. syriacum* indicated the highest inhibition against selected bacterial isolates, including *K. pneumonia* and *E. coli*. The inhibition zones ranged from 10-25 mm diameter. Furthermore, the most active compound in Acer plants may be polyphenols including flavonoids, as suggested by Zhang et al.,2008 [94]. Extracts of *Acer pentapomicum* were tested for antimicrobial potential. The results showed that butanolic extracts have maximum activity against *P. aeruginosa* strain followed by ethyl acetate activity against *C. fruendii* with disc diffusion method [96].

The antibacterial investigation of *P. cornuta* extracts revealed that overall, all extract types have an excellent inhibitory effect against selected bacterial isolates (Figure 4.7). The histogram illustrated the maximum activity obtained by PCN (n-hexane extract) against *A. baumanni, S. entrica* and *E. coli* isolates, followed by PCB extract against *A. baumanni* and *S. entrica* isolates. Among different *Prunus species*, the dried juice extract of *P. domestica L.* showed 17-58% growth inhibition against tested 5 different bacterial strains, including *E. coli*, with 23-37% reduction of oxidative stress [97]. The methanolic extracts of *P. persica* showed effective inhibition against *B. subtilus, S. aureus,* and *K. pneamoniae* with 17 mm, 17 mm, and 24 mm zones of inhibition, respectively [98].

The evaluation of the antibacterial potential of *Q. semicarpifolia* was maximum with methanolic extracts (QSM) in all five strains and with butanolic (QSB) extracts against *A. baumanni* and *K. pneaumonia* bacterial isolates. An investigation on the antimicrobial potential of four different Quercus species extracts from turkey confirmed the highest activity by butanol extract of *Q. macranthera subsp. syspirensis, Q. cerris, Q. pubescens and Q. coccifera*. The phytochemical analysis of active extracts (butanol extract) detected flavonoids and condensed tannins. Also, hydrolyzable tannins were detected in all other extracts of *Quercus* species [95]. The comparative analysis of antibacterial activity with various plants of Pallas valley validated that the selected plant species are more biologically active in terms of their therapeutic potential (Table. 4-11). Hence, selected plant material showed better antibacterial, anticancer and antioxidant potential in all biological screening analyses.

The mycelial inhibition analysis was performed by agar well diffusion and slanting methods. The diameter of inhibition zones was not significant even by the active extracts of tested plant species. The *A. flavus, A. niger,* and *Pythium species* showed less susceptibility to tested plant extracts. The characteristic inhibition was only observed with *R. oryzae* strain in an order QSE > PCM, QSN > ASE, QSC, QSB.

F. fujikuroi, is a plant pathogen that causes bakanae disease in rice plants resulting in extraordinary elongation of diseased plants, eventually resulting in the death of the plant [28]. *R. oryzae* is an opportunistic human pathogen causing Mucormycosis and pulmonary infection in immunocompromised patients [29] and *P. ultimum,* a plant pathogen causing damping-off and root rot disease in a variety of economic plants [30]. Percentage inhibition of these strains was observed by selected plant material. However, the antifungal activity of *F. fujikuroi*, *R. oryzae,* and *P. ultimum* by *A. cappadocicum* extracts were less than 50%. *F. fujikuroi* appeared highly susceptible to *P. cornuta* extracts (60-70%), followed by *R. oryzae* PCN (60%), PCM (55%), and PCE (55%). *Q. semicarpifolia* extracts showed moderate inhibition in *F. fujikuroi* and *R. oryzae.*

Several epidemiological studies have illustrated a relationship between antioxidant-rich food and disease occurrence in humans. Evaluating natural antioxidants level has been performed through various in vitro antioxidant assays [99]. The IC_{50} values demonstrated that the chloroform extract of *A. cappadocicum* (ACC), chloroform extract of *P. cornuta* (PCC), and methanolic extract of *Q. Semicarpifolia* (QSM) showed potent antioxidant potential with 0.3795,0.4885 and 0.4003, respectively. Altogether, all extracts of *A. cappadocicum* showed superior inhibition of DPPH radical. Literature also confirmed that the Acer species are potential antioxidants, e.g., ethanolic and hot water extracts of Acer saccharum and Acer rubrum buds, showing a high level of total phenolic contents and antioxidants DPPH assay [100]. Another study showed up to 98% inhibition of DPPH by *Acer pseudoplatanus* and *Quercur robur* bud extracts which is quite in agreement with the results of *A. cappdocium* and *Q. Semicarpifolia* [101]. Liagliang Yao also demonstrated the presence of phenolic compounds and their good antioxidant potential by DPPH assay in *Acer saccharum Marsh*.

The limitations for the present study were the absence of spectroscopic analysis of potential extracts, which can provide more information about the chemical foundations of bioactivity and explain the research aims of the study in more detail. Key findings are the real potential of extracts' anticancer and antibacterial activity. There is a strong relationship between the antioxidant potential and anticancer activity of active extracts, which indicates the diversity of the phytochemical constituents of selected plant material. The high activity of *A. cappadocicum* was also confirmed on the *in vitro* liver tumor model of the microphysiological system, which is a unique and novel aspect of comparing the cytotoxicity of plant extracts with the current in vivo method. Therefore, it is recommended that phytochemical analysis of potential extracts be performed, which further involves isolation and the characterization of pure bioactive substances.

6. CONCLUSION

In the present study, three indigenous plants from Pallas valley were characterized for anticancer potential, bioactivity, e.g., antimicrobial and antioxidant. The first plant of study *A. cappadocicum Gled.* showed potent anticancer activity with chloroform and butanoic extracts in lung and breast cancer cells. Overall, Extracts of *A. cappadocicum* have shown a significant reduction *in vitro* cancer cell line proliferation. Similarly, *A. Cappadocicum* extracts demonstrated good antibacterial activity against human pathogens and moderate mycelial inhibition of plant pathogens, implying that it can be an efficient source to treat human and plant pathogens. The antibacterial zones are more precise with butanol and ethyl acetate extracts of *A. cappadocicum*.

With the second plant, *P. cornuta,* moderate bioactivity with bacterial species and fungal strains. The *P. cornuta* inhibited the bacterial species up to an average of 12 mm whereas showed good inhibitory activity of mycelial growth in *R. oryzae* with agar well method by methanolic, n hexane extracts, and F. *fujikurio* with agar slanting method. Out of 5 bacterial strains, two strains appeared more susceptible, e.g., *A. baumanni* and *S. enterica,* to extracts of *P. cornuta,* specifically with chloroform followed by n-hexane extract. Our study also confirmed that the *P. cornuta* extracts were cytotoxic to lung (A549) and breast (MDA-MB-231) cells. MDA-MB-231 showed high inhibition by the lowest percentage of cell survival. Also, *P. cornuta* extracts showed the nontoxic effect of plant extracts on normal HPAEpiC and HRTEpiC cells.

Bioactivity investigation of *Q. Semicarpifolia* extract exhibited good antibacterial potential with methanolic extracts and restricted maximum fungal growth in *R. oryzae* with ethyl acetate extract. Furthermore, the HepG2 and Caco2 cells appeared less sensitive to *Q. Semicarpifolia* extract than Prunus and Acer species. Additionally, the findings of this study revealed that there is significant inhibition of growth in breast cancer through *Q. Semicarpifolia* extracts. However, at cultured cells, *Q. Semicarpifolia* extracts only at 10-100 µg/mL exhibited considerable cytotoxic effect in Caco2, HepG2, and A549.

The plants under investigation also exhibited the excellent potential of antioxidant activity. Hence, they could be evaluated as nutraceutical sources except for *P. cornuta*. However, more safety data is required with *P. cornuta* species. Lastly, the organ-on-chip model can be a better alternative for plant cytotoxicity studies than conventional culture media due to the much closer fluidic microenvironment to the human body. Moreover, the effect of extracts was more pronounced on cancerous cells than normal cells. Altogether, extracts could be a new source in natural products research due to promising antimicrobial, antioxidant, and cytotoxic potential.

FUTURE RECOMMENDATIONS

- Further investigations are required to understand the underlying molecular mechanism and biochemical foundation of these plant extracts, which can provide the basis for these new natural sources on the drug ability list of pharmacognosy platforms.

- The pure substance isolation and characterization from *A. cappadocicum Gled* could be a practical approach for discovering potential anticancer constituents of this plant.

- After fractionation and purification, the selected plants should be explored to detect novel chemical substances for known bioactivity.

- Also, *in vivo* advanced studies on animal tumor models can be crucial in validating the potential therapeutic effect of extracts.

7. APPENDIX

Chemical compositions of test/ reagents used

SR Non	Test Reagents	Chemical composition
1	**Mayer's reagent**	Mercuric chloride (1.36g) + potassium iodide (5.00g) in 100 ml water
2	**Heager's reagent**	Saturated picric acid solution
3	**Keller Killiani test**	Glacial acetic acid (2ml) containing 1-2 drops of Fecl3 solution (2%). Transferred the mixture into 2ml of conc.H_2SO_4 test tube.
4	**Salkowski test**	5 mL of chloroform, 2 mL of plant extracts sample and 1 mL of conc. H_2SO_4 along the walls of the test tube.
5	**Ellagic test**	5% glacial acetic acid, 5% $NaNO_2$
6	**Benedict test**	alkaline solution of cupric citrate complex
7	**Xanthoproteic test**	Few drops of concentrated nitric acid

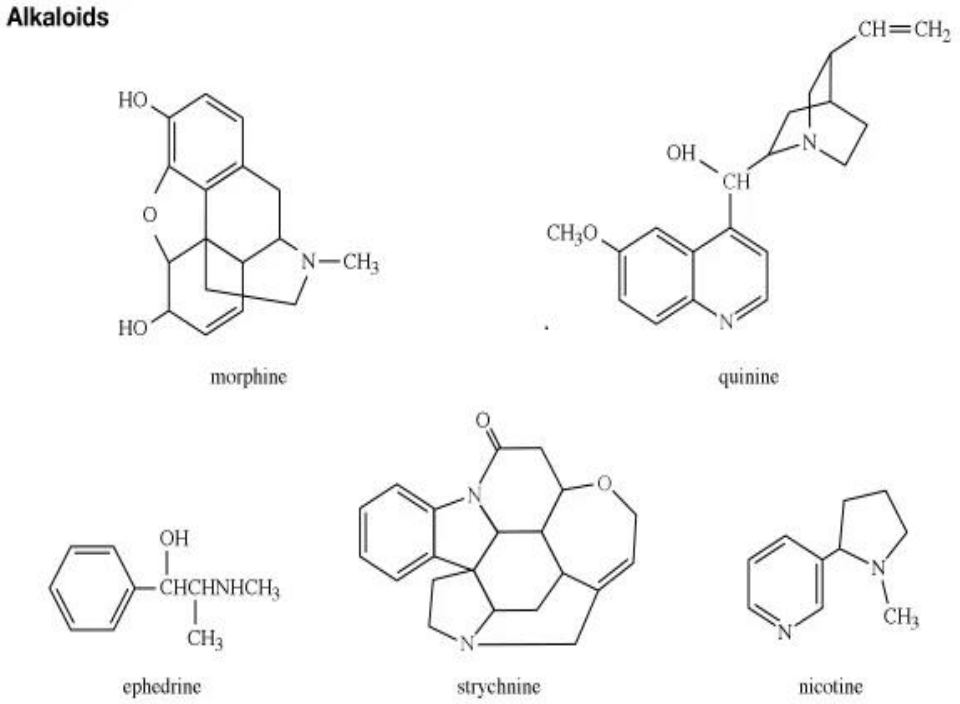
Description of tests of plant metabolites and their reagents used

Tests for metabolites	Added reagents	Expected results
Alkaliods	Heager's reagents (saturated picric acid solution)	Bright yellow ppt
	Conc. HCL + Mayer's reagent	Formation of white precipitation or green colour
Tanins	$FeCl_3$ (1%)	Blue coloration
Resins	Acetone + water	Turbidity
Saponins	Agitation	Formation of foam
Phenolics	$FeCl_3$(1%) + K3[$Fe(CN)_6$] (1%)	Green blue coloration
Flavonoids	KOH (50%)	Yellow coloration
Glycosides	Glacial acetic acid + $Fecl_3$ (5%) + H_2SO_4	Ring formation
Steroids	Chloroform + H_2SO_4	Brown ring

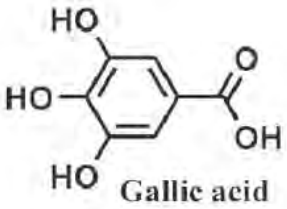
Phytosteroids	Chloroform + few drops of H_2SO_4	Bluish brown ring
Phenols	Glacial acetic acid (5%) + $NaNO_2$ solution	
Carbohydrates	Alpha-naphthol+ H_2SO_4	Purple ring
Anthraquinones	HCl (10%) + Chloroform + Ammonia (10%)	
Phlobatanins	HCl (1%)	
Terpenoids	1ml of 1% HCl, 2ml of plant extract (stand for 5-6 hr), 1ml of trill. Hill reagent.	Appearance of blueish green colour
Anthocyanins	2N NaoH	Bluish green colour
Quinones	1ml of conc. H_2SO_4	Appearance of red colour
Proteins	Conc.HNO_3	Yellow colour

Chemical Structures:

Alkaloids



Phenolics



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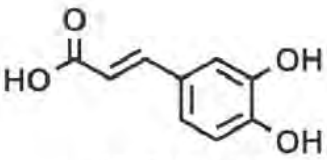

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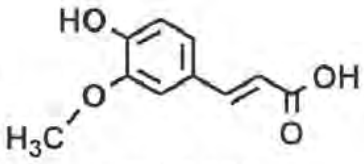

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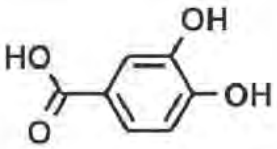
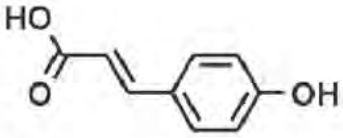
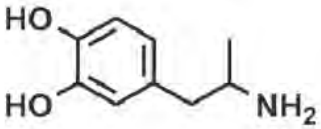


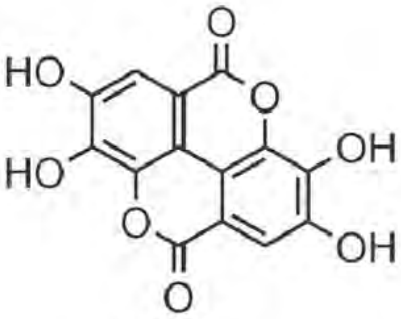
Protocatechuic acid



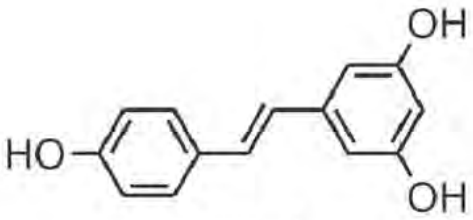
Coumaric acid



L-DOPA



Ellagic acid



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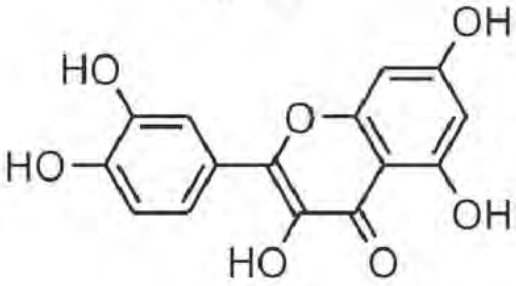

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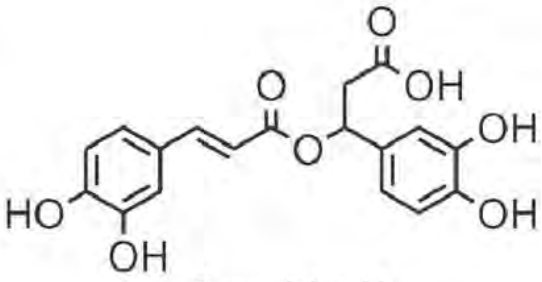
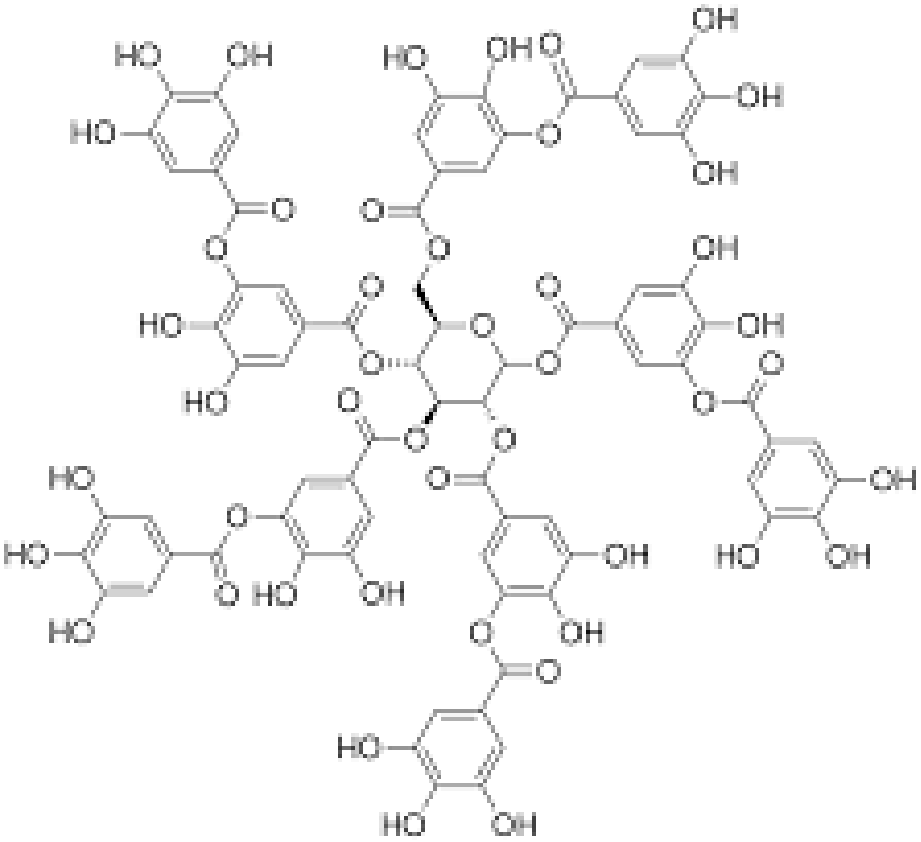
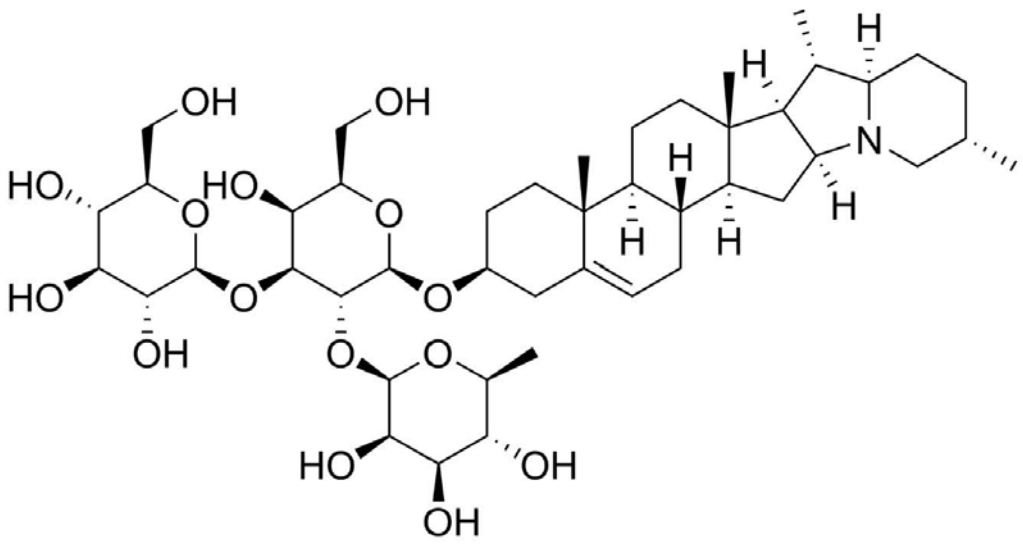


Rosmarinic acid

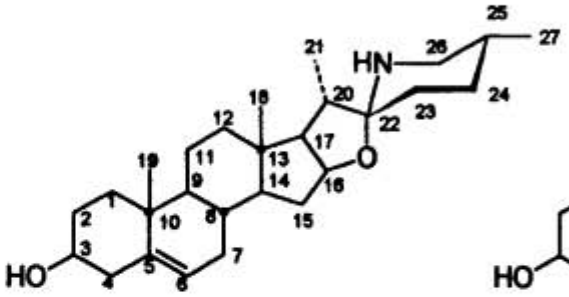
Tannins



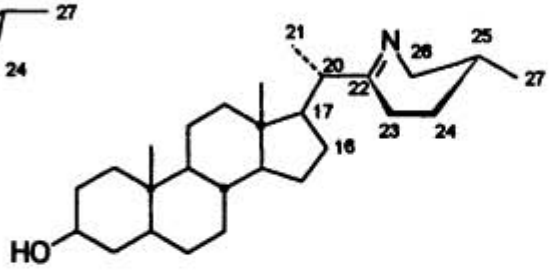
Saponins



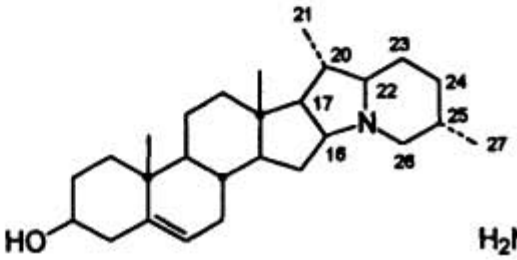
Steroid alkaloids in solanum plant



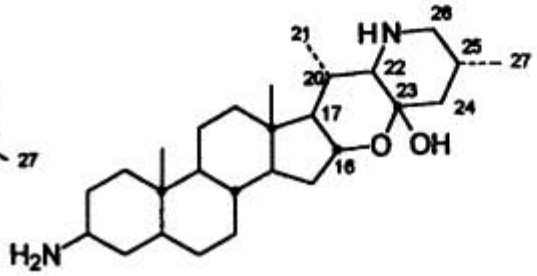
1 Solasodine



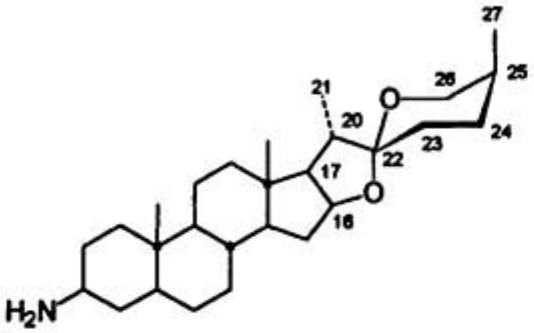
2 Solacongestidine



3 Solanidine



4 Solanocapsine



5 Jurubidine

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