CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Isolation and Identification of Endophytes from Traditional Medicinal Plant and Investigating their Pharmacological Activities

by

Eisha Aamir

A thesis submitted in partial fulfillment for the degree of Master of Philosophy

in the

Faculty of Pharmacy
Department of Pharmacognosy

2026

CERTIFICATE OF APPROVAL

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by

Eisha Aamir

(MPH243021)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Humaira Fatima	QAU, Islamabad
(b)	Internal Examiner	Dr. Reem	CUST, Islamabad

Dr. Muhammad Saalim
Thesis Supervisor
September, 2026

Dr. Mehboob Alam
Head
Dept. of Pharmacognosy
September, 2026

Dr. Muzaffar Abbas
Dean
Faculty of Pharmacy
September, 2026

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Acknowledgement

Acknowledgements In the name of Allah, the Most Gracious, the Most Merciful. All praise and gratitude belong to Almighty Allah (SWT), whose endless mercy, wisdom, and blessings granted me the strength, patience, and perseverance to complete this thesis. Peace and blessings be upon the Holy Prophet Muhammad (PBUH), whose noble character and timeless teachings on knowledge, integrity, and compassion continue to guide me in both my academic and personal life. I am sincerely grateful to the Capital University of Science & Technology (CUST), Islamabad, for providing a supportive academic environment and the resources essential for this research. My deepest appreciation goes to my respected supervisor, Dr. Muhammad Saalim, Department of Pharmacognosy, Faculty of Pharmacy, CUST, Islamabad, for his expert guidance, insightful feedback, and constant encouragement, which have been fundamental to the completion of this work. I would also like to thank my dear friend Dr. Mariam Saddique for her unwavering support, thoughtful discussions, and motivation throughout this journey. My sincere thanks to all the healthcare professionals and institutions who participated in this study, your cooperation and valuable input made this research possible. This thesis is dedicated to my beloved parents and cherished siblings. Your unconditional love, sacrifices, prayers, and belief in me have been the foundation of all my achievements. Finally, I acknowledge everyone who supported and encouraged me in any way. This milestone would not have been possible without you.

(Eisha Aamir)

Abstract

Berberis lycium (Sumblu) is a Himalayan medicinal shrub traditionally used for diabetes, jaundice, and inflammatory disorders is rich in alkaloids and phenolics but its fungal endophytes had never been isolated or evaluated pharmacologically in Pakistan. The aim of this study was to isolate and molecularly identify endophytic fungi *Berberis lycium* leaves, stem, and roots were collected from Kashmir, Pakistan and to evaluate the therapeutic potential of plant and endophytic extracts through in vitro antibacterial, and antifungal assays, in vivo, anti-inflammatory, testing, and in silico antidiabetic screening. Methanolic and ethyl acetate extracts are prepared by maceration and endophytes are isolated on Potato Dextrose Agar and identified via ITS sequencing and BLAST analysis. The known phytochemicals are docked against alpha-amylase and DPP-IV using PyRx and Schrodinger Glide followed by SwissADME and ProTox-3.0 profiling. The methanolic leaf extract gave the highest extraction yield (23.20%). Seven endophytes, four fungal and three bacterial, are recovered. NCBI BLAST confirmed isolate FBL1 as *Epicoccum latusicollum* (98.87%), FBS1 as *Fusarium oxysporum* (99.08%), and FBS2 as *Fusarium incarnatum* (99.62%). Disc diffusion based antibacterial profile showed that the methanolic root extract produced the largest inhibition zone (8.48 ± 0.30 mm) against *Escherichia coli* while the FBL1 biomass extract was most active against *Staphylococcus aureus* (9.15 ± 0.20 mm). The methanolic leaf extract showed the strongest antifungal activity against *Saccharomyces cerevisiae* (9.70 ± 0.20 mm), exceeding nystatin. In carrageenan-induced paw edema, the methanolic root extract (50 mg/kg) inhibited edema by 27.08% at 6 h, approaching diclofenac sodium's 30.56%. Docking identified Feselol (-9.8 kcal/mol, PyRx) and Cyanidin-3-rutinoside (-11.6 kcal/mol, Schrodinger) as the strongest DPP-IV inhibitors, surpassing sitagliptin (-8.8), while Camptothecin (-7.3) and Cyanidin-3-rutinoside (-10.1) ranked highest against alpha-amylase, five compounds satisfied all drug-likeness parameters. These findings establish *Berberis lycium* and its previously unreported fungal endophytes as validated sources of antibacterial, antifungal, anti-inflammatory, and antidiabetic lead compounds.

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Abbreviations

ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
BBB	Blood–Brain Barrier
BLAST	Basic Local Alignment Search Tool
BM	Biomass (extract)
CFU	Colony Forming Unit
CTAB	Cetyltrimethylammonium Bromide
DPP4 / DPP-IV	Dipeptidyl Peptidase-IV
Fsp^3 (F.Csp^3)	Fraction of sp^3-Hybridized Carbon Atoms
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
ITS	Internal Transcribed Spacer
MR	Molar Refractivity
nAHA	Number of Aromatic Heavy Atoms
NCBI	National Center for.Biotechnology Information
nHA	Number of Heavy Atoms
nRB	Number of Rotatable Bonds
PAINS	Pan-Assay Interference Compounds.
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Protein Data Bank / Potato Dextrose Broth
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SM	Secondary Metabolite (extract)

TE (buffer)	Tris-EDTA (buffer)
TPSA	Topological Polar Surface Area
WHO	World Health Organization

Chapter 1

Introduction

1.1 Background of the Study

The burden of infectious diseases and metabolic conditions like diabetes mellitus is continuously increasing globally. According to a report by the WHO, there are almost 422 million diabetic people throughout the world and this number is predicted to increase significantly in next few decades [1]. Simultaneously, many antibiotics became ineffective against many bacterial and fungal infections because of the increasing incidences of antimicrobial resistance, which posed an urgent need for the discovery and development of novel antibacterial and antifungal drugs from unexplored natural sources [2]. Natural sources such as medicinal plants and the endophytes associated with them serves as the most important reservoirs for novel drugs. From 1981 to 2 19, more than 5 percent of the total approved drugs are sourced from natural products, highlighting the significance of biological diversity as a source of novel pharmaceutical compounds [3].

Endophytes are microbes mainly bacteria and fungi and that live inside the endogenous tissues of living flora without causing any disease or producing any symptom of disease in them and spend at least a part of their life in their host [4]. They have the same habitat as that of their host and get exposed to the similar biosynthetic system as of their host tissue which often results in the synthwsis

of chemically identical secondary metabolites as produced by the host plant itself. The groundbreaking discovery of Taxol which an important anticancer drug, from the endophytic fungi *Taxomyces andreanae*, obtained from the bark of the Pacific yew tree (*Taxus brevifolia*) revealed the vast medicinal possibilities hidden within endophytic communities for the first time [4]. Following that discovery, scientists have extracted numerous bioactive substances from endophytes, such as alkaloids, polyketides, terpenoids, phenolics, peptides, and flavonoids, many of which demonstrate significant antibacterial, antifungal, anti-hyperlipidemic, antidiabetic, anticancer and anti-inflammatory properties [5].

Scientific studies estimate that there are between 3.5 and 5.1 million fungal species on the planet, but only a tiny percentage have been documented and classified [5]. A survey of studies released from 2 18 to 2 23 found 69 scientific papers on endophytes in medicinal plants, indicating that global interest in this area is increasing rapidly [6]. Penicillium, Fusarium, Chaetomium, Aspergillus and Pestalotiopsis are the most commonly isolated endophytes from medicinal plant hosts among fungal genera and are known for their abundant production of pharmacologically active compounds [5, 7].

Berberis lycium referred to as Indian barberry or Sumblu, is a coniferous shrub belonging to the family Berberidaceae, found naturally in the Himalayan highlands of Afghanistan, India, Nepal, and Pakistan. In South Asia, traditional communities have utilized various parts of this plant such as roots,.bark, stem and leaves, to address numerous health issues such as diabetes, jaundice, hepatitis, skin infections, fever, diarrhea, and joint pain [8]. The plant is chemically diverse, featuring the isoquinoline alkaloid berberine as its main component, along with palmatine, berbamine, oxyacanthine, camptothecin, protopine, and a variety of flavonoids and phenolic acids such as quercetin, caffeic acid, and chlorogenic acid [8, 9]. Berberine, in particular, has been thoroughly researched for its mechanisms of action related to antimicrobial, antidiabetic, and anti-inflammatory effects, including the activation,of AMP-activated,protein kinase and the,inhibition of alpha-amylase [10].

Despite the plant's known medicinal significance, research on *Berberis lycium*'s endophytic fungi is still quite limited. Bacillus species have been consistently

identified as the main colonizers in the limited investigations that have been done, which have only examined bacterial endophytes isolated from stem and root-tissues [11, 12].

Interesting issues concerning gene transfer between plant and endophyte are raised by the discovery that *Bacillus toyonensis* BAR1, which was isolated from the roots of the closely related species *Berberis aristata*, produced berberine itself [13].

Nevertheless, *Berberis lycium*'s fungal endophytes have never been identified, isolated, or assessed for possible medicinal uses in Pakistan. This crucial knowledge gap offers a substantial scientific opportunity as well as a strong rationale for the current study.

The current study aimed to isolate and identify endophytic fungi and bacteria from *Berberis lycium* leaves, bark, and roots collected in Kashmir, Pakistan, and to assess the therapeutic potential of their secondary metabolites using in vitro antibacterial and antifungal assays supplemented by in silico molecular docking of known phytochemicals against antidiabetic enzyme targets and an in vivo anti inflammatory investigation.

The work also provides a detailed computational drug likeness and ADMET profile of *Berberis lycium*'s key phytoconstituents, establishing a solid scientific foundation for future lead optimization and drug development.

1.2 Functional Definitions

1.2.1 Endophytes

Endophytes are microorganisms mainly bacteria and fungi that reside in the interior tissues of healthy plant hosts for at least part of their life cycle without causing any visible disease to them. They have a mutualistic or commensal relationship with the host and are distinct from epiphytes, which live on the plant's outer surface [4].

1.2.2 Endophytic Fungi

These are fungal microbes most commonly belonging to the phylum Ascomycota that reside the inside the plant tissues. They are isolated from plant tissues by surface sterilization and then culturing the internal tissues on selective media such as Nutrient Agar or Potato Dextrose Agar [5].

1.2.3 Secondary Metabolites

These are organic compounds produced by microorganisms that live inside the plant and are not directly necessary for growth or reproduction of plant but provide ecological benefits like competitive exclusion, resistance to pathogens, or signaling between species. Alkaloids, phenolic acids, flavonoids, terpenoids, polyketides and phenolic acids are the common secondary metabolites [5, 6].

1.2.4 Phytochemical Screening

It refers to assessment of extracts chemically to determine the presence or absence of major chemical classes of secondary metabolites using standard colorimetric and precipitation based reagent tests [8].

1.2.5 Molecular Docking

It is a computational method utilized to forecast the favored binding orientation and binding affinity of a small molecular ligand within the active site of a target protein. In this research, PyRx's AutoDock Vina and the Schrodinger Suite's Glide module are employed for docking against the antidiabetic enzymes such Alpha Amylase and Dipeptidyl Peptidase IV [10].

1.2.6 Minimum Inhibitory Concentration (MIC)

MIC is the smallest concentration of an antimicrobial agent that stops visible growth of a microorganism ounder specified in vitro conditions. It serves as a numerical indicator of effectiveness of antimicrobial agent [14].

1.2.7 ADMET Profiling

ADMET Profiling predicts the absorption, distribution, .metabolism, excretion and toxicity characteristics of chemical compounds. In the early drug discovery, tools like SwissADME and ProTox 3 are used to predict the in silico ADMET properties and to screen the compounds based on their pharmacokinetic suitability before validating it experimentally [15].

1.3 Aim of the Study

The main aim of this study was the isolation and identification of endophytes from a traditional medicinal plant *Berberis lycium*, commonly known as Sumblu, and to evaluate the the therapeutic potential of plant and endophytic extract through in vitro, in vivo and in silico techniques, which particularly focus on antibacterial, antifungal, anti inflammatory and antidiabetic activities.

1.4 Objectives of the Study

The objectives of this study are as follows.

i. To obtain the plant extract from different plant parts through maceration by using different solvents such as methanol and ethyl acetate and to calculate the yield of obtained crude extracts.

ii. To isolate the endophytes from different parts of *Berberis lycium* such as roots, stems and leaves.

iii. To use ITS region sequencing and phylogenetic analysis to identify the isolated endophytic fungi.

iv. To extract secondary metabolites from endophytic isolates using fermentation and solvent extraction techniques and to assess the yield of crude extracts.

v. To identify the main bioactive chemicals by performing initial phytochemical screening of plant and endophytic extracts.

vi. To perform in vitro antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* using the disc diffusion method to assess antibacterial potential of plant and endophytic extracts.

vii. To perform in vitro antifungal activity against *Saccharomyces cerevisiae* using the disc diffusion method to evaluate the antifungal potential of plant and endophytic extracts.

viii. To investigate the in vivo anti inflammatory activity of the methanolic extract of *Berberis lycium* using the carrageenan induced paw edema model in BALB/C mice.

ix. To assess the antidiabetic potential of known *Berberis lycium* phytochemicals by performing in silico molecular docking against the enzymes α-amylase and dipeptidyl peptidase IV and to perform ADMET and drug likeness profiling of the top ranked phytochemicals by using the SwissADME and ProTox 3.0 platforms.

1.5 Research Questions

The aim of the study was to answer the following research questions.

1. Which endophytic bacteria and fungi are present in *Berberis lycium* leaves, bark, and roots that are gathered from Kashmir, Pakistan?

2. Do the endophytic fungi that are isolated from *Berberis lycium* produce secondary metabolites that have coniderable antibacterial potential against *Staphylococcus aureus* and *Escherichia coli*?

3. Do *Berberis lycium* and its endophytic fungi extracts show antifungal efficacy against *Saccharomyces cerevisiae*?

4. Which known phytochemicals of *Berberis lycium* demonstrate the most advantageous in silico binding affinity against the antidiabetic targets α - amylase and dipeptidyl peptidase IV.

5. Do the top ranked phytochemicals from in silico research have pharmacokinetic and physicochemical characteristics similar to drugs that would encourage their development as medicinal agents?

6. Does the methanolic root extract of *Berberis lycium* exhibit considerable anti inflammatory activity against the carrageenan induced paw edema model in mice as compared to a standard drug?

1.6 Significance of the Study

This study has multiple significances. Firstly, it offers the first systematic isolation and characterization of fungal endophytes from *Berberis lycium* in Pakistan, filling a significant gap in knowledge about the endophytic communities of one of the most pharmacologically important plants in the Himalayan region. Prior research on *Berberis lycium* endophytes has only examined bacterial isolates from a small number of locations and plant sections, leaving the fungal endophytic community almost completely unexplored. [11, 12].

Secondly, by investigating natural sources for novel antibacterial, antifungal, anti inflammatory and antidiabetic agents, the study encounters two of the most important public health issues of the twenty first century i.e. the worldwide diabetes epidemic and antimicrobial resistance.

Berberis lycium endophytes are ideal candidates for therapeutic compound discovery because it has previously been demonstrated that the endophytes of medicinal plants in the genus Berberis produce berberine and other pharmacologically active compounds that reflect the therapeutic potential of the host plant [13].

Third, using rigorous scientific technique, the in vivo anti inflammatory study strengthens the evidence for ethnopharmacological claims by providing pre clinical biological confirmation for the traditional usage of *Berberis lycium* as an anti inflammatory agent [8].

The results of this study are expected to contribute to the broader scientific literature on endophyte pharmacology, natural product drug discovery, and the therapeutic potential of Himalayan medicinal plants.

Fourth, the comprehensive in silico component of this study provides the first dual target molecular docking analysis of *Berberis lycium* phytochemicals against α amylase and dipeptidyl peptidase IV, two clinically validated antidiabetic enzyme targets. The combined ADMET and drug likeness profile provides translational value by identifying which compounds are most suited for further pharmaceutical development [10, 15].

1.7 Scope of the Study

This study's scope is established by the subsequent boundaries and delimitations. The plant samples consisted solely of fresh leaves, bark, and roots of *Berberis lycium* obtained from one geographic area in the Kashmir region of Pakistan. Endophyte isolation was carried out utilizing conventional surface sterilization followed

by culturing on Potato Dextrose Agar with molecular identification relying on ITS region sequencing.

The in vitro antimicrobial evaluation was conducted two bacterial species: Staphylococcus aureus and *Escherichia coli* along with one fungal specie i.e. Saccharomyces cerevisiae through the agar disc diffusion technique. Determinations of minimum inhibitory concentration are excluded from the current study and are suggested for future research.

The in vivo study was limited to evaluating acute anti inflammatory activity by using the carrageenan induced paw edema model in BALB/C mice. Chronic inflammation models, antidiabetic animal studies, and clinical investigations are beyond the scope of the present work and constitute recommended directions for future research. The study does not include isolation, purification, or structural elucidation of individual active compounds from plant or endophytic extracts, which are proposed as a logical continuation of this work.

The in silico component encompassed molecular docking of known Berberis lycium phytochemicals retrieved from the literature and PubChem database against two antidiabetic protein targets, namely human α amylase and dipeptidyl peptidase IV, using PyRx and the Schrodinger Glide module ADMET profiling was conducted using SwissADME and ProTox 3 for the top ranked compounds only.

Chapter 2

Literature Review

2.1 Overview of Endophytes

Endophytes are the microorganisms commonly fungi and bacteria that inhabit the internal tissues of healthy plants without causing any harm or any visible symptoms of disease. They typically reside the intercellular spaces and vascular tissues where they live by making long-term associations with their host plants. Although these interactions are initially regarded as passive colonization, increasing evidence indicates that endophytes often form mutualistic relationships that benefit both partners. Endophytes can enhance plant growth in exchange of nutrients and a protected ecological niche, improve tolerance to environmental stresses, and protect the host against pathogens through the production of diverse bioactive secondary metabolites [4].

The occurrence of endophytes is considered a nearly universal phenomenon among higher plants, irrespective of their taxonomy or geographic distribution. However, the diversity and composition of endophytic communities vary considerably depending on plant species, tissue type, climatic conditions, geographical location, and environmental factors. Plants growing in biodiversity-rich ecosystems, such as tropical forests and mountainous regions, generally harbor more diverse endophytic populations than those from less diverse habitats [4]. These observations

suggest that both host characteristics and ecological conditions shape endophyte diversity, highlighting the importance of exploring medicinal plants from different environments to discover novel microorganisms with unique metabolic capabilities.

The pharmaceutical significance of endophytes lies in their remarkable ability to synthesize structurally diverse secondary metabolites with therapeutic potential. Comparative studies have demonstrated that endophytic fungi possess a greater capacity for producing novel bioactive compounds than many conventional microbial sources. For example, approximately 51% of biologically active metabolites isolated from endophytic fungi have been reported as previously unknown compounds, compared with 38% of compounds obtained from soil microorganisms [4].

This higher rate of chemical novelty emphasizes the immense potential of endophytes as reservoirs of new drug leads. Furthermore, although an estimated 3.5–5.1 million fungal species exist worldwide, only a small proportion has been isolated and characterized, indicating that the vast majority of endophytic diversity remains unexplored [5]. Consequently, medicinal plants continue to represent an important source for identifying new endophytic microorganisms capable of producing pharmacologically valuable metabolites.

Interest in endophytes as alternative source of natural products increased dramatically following the discovery that the fungal endophyte *Taxomyces andreanae*, isolated from *Taxus brevifolia*, could produce taxol, one of the most successful natural anticancer agents [4]. This finding fundamentally changed the perception of endophytes by demonstrating that microorganisms residing within plants could biosynthesize the same therapeutically important compounds as their hosts. Since then, numerous investigations have identified endophyte-derived metabolites with antibacterial, antifungal, antiparasitic, anticancer, antioxidant, anti-inflammatory, and antidiabetic activities. Notable examples include cryptocandin from *Cryptosporiopsis cf. quercina*, munumbicins produced by *Streptomyces* sp. NRRL 30562, and more recently discovered bacterial metabolites such as aureothin, aculeolatins, lobophorins, xiamycins, and lydiamycins, all of which have demonstrated promising pharmacological activities against a variety of infectious and

non-infectious diseases [4, 6].

Although these metabolites differ in their chemical structures and biological targets, they collectively demonstrate the exceptional biosynthetic potential of endophytic microorganisms and their growing importance in modern drug discovery.

Collectively, the available evidence suggests that endophytes represent one of the richest yet least explored sources of biologically active natural products.

Their widespread occurrence, remarkable chemical diversity, and ability to produce metabolites with activities comparable to those of their host plants make them attractive candidates for pharmaceutical research.

The diversity of endophytes isolated from different medicinal plants is summarized in Table 1. Building on this general understanding of endophytes, the following section examines their geographical distribution and diversity, providing insight into how environmental and host-related factors influence endophytic communities and their biosynthetic potential.

TABLE 2.1: Endophytes isolated from medicinal plants and their reported biological activities

Sr.	**Medicinal plant**	**Isolated endophytes**	**Reported biological activity**
1	*Dendrobium huoshanense*	*Streptomyces* sp.	Source of anticancer metabolites (huoshanmycins A–C).
2	*Cinnamomum cassia*	*Streptomyces* sp. HBQ95	Produces lydiamycins with antitubercular and antitumor potential.
3	*Lycium barbarum*	Eighteen bacterial endophytes	Enhanced plant growth and suppressed *Fusarium* spp. and *Verticillium dahliae*.
4	*Fagonia indica*	*Bacillus*, *Enterobacter*, *Pantoea*, *Erwinia*, *Stenotrophomonas*	Reported antioxidant, antibacterial, antifungal and antileishmanial properties.
5	*Berberis lycium*	*Bacillus cereus*, *B. thuringiensis*, *B. anthracis*	Displayed antibacterial and antifungal activities.

Table 2.1 continued from previous page

Sr.	Medicinal plant	Isolated endophytes	Reported biological activity
6	*Berberis aristata*	*Bacillus toyonensis*	Representative *Bacillus* endophyte associated with *Berberis* species.
7	*Berberis lycium*	*B. paramycoides*, *B. pseudomycoides*, *B. wiedmannii*, *B. subtilis*, *Calidifontibacillus erzurumensis*	Expanded the known diversity of bacterial endophytes; potential therapeutic relevance.
8	*Zanthoxylum gilletii*	*Fusarium*, *Chaetomium*, *Scopulariopsis*, *Trametes*	Illustrated fungal endophyte diversity.
9	*Markhamia lutea*	*Fusarium*, *Chaetomium*, *Scopulariopsis*, *Trametes*	Illustrated fungal endophyte diversity.
10	*Eucalyptus exserta*	*Phyllosticta*, *Eutypella*, *Lophiostoma*, *Pestalotiopsis*, *Chaetomium*	Demonstrated diverse fungal community with bioactive potential.
11	*Curcuma longa*	*Bacillus cereus*, *Pseudomonas putida* and other bacterial isolates	IAA production, phosphate solubilization, antibacterial and antifungal activities.
12	*Kennedia nigriscans*	*Streptomyces* NRRL 30562	Producer of broad-spectrum antibiotic munumbicins.

2.2 Endophytes in Asia

Asia is recognized as one of the most important regions for endophyte research because of its exceptional diversity of medicinal plants and its long history of traditional healing systems, including Ayurveda, Unani, and Traditional Chinese Medicine.

The extensive use of medicinal plants in these healthcare systems has encouraged researchers to investigate the microorganisms residing within these plants as potential sources of novel therapeutic compounds. This growing interest is reflected in a survey that reported 690 scientific publications on endophytes isolated from

medicinal plants between 2018 and April 2023, demonstrating the increasing global attention towards this field of research [6].

Studies conducted across different Asian countries consistently show that medicinal plants harbor diverse endophytic microorganisms capable of producing biologically active secondary metabolites. Although the therapeutic activities reported are similar, the nature of the metabolites varies according to the host plant, endophytic species, and environmental conditions. For example, endophytic Streptomyces isolated from *Dendrobium huoshanense* in China produced novel compounds known as huoshanmycins A–C, which exhibited promising anticancer activity against selected cancer cell lines [6]. Likewise, endophytes isolated from *Cinnamomum cassia*, an important medicinal plant used in Traditional Chinese Medicine, are found to produce lydiamycins with significant antitubercular and antitumor properties [6]. These findings indicate that despite originating from different medicinal plants, endophytes possess the ability to synthesize structurally diverse metabolites with important pharmaceutical applications.

The biological potential of Asian endophytes is not limited to the production of therapeutic compounds. In addition to their medicinal value, many endophytic microorganisms contribute to plant health by promoting growth and protecting plants against pathogenic microbes. Supporting this observation, Abdusamatov et al. conducted the first investigation of bacterial endophytes associated with Lycium barbarum in Uzbekistan and isolated 18 bacterial strains that not only enhanced plant growth but also inhibited several important phytopathogenic fungi, including Fusarium species and Verticillium dahliae, with inhibition zones of up to 20 mm [16]. Unlike the Chinese studies, which primarily emphasized the discovery of bioactive metabolites, this study highlighted the dual role of endophytes in both plant protection and sustainable agriculture. Such variations among studies may be attributed to differences in host plant species, geographical location, microbial diversity, and the specific objectives of each investigation.

Overall, the available evidence demonstrates that medicinal plants across Asia serve as valuable reservoirs of endophytic microorganisms with remarkable biosynthetic capabilities. While individual studies have focused on different host plants

and biological activities, they collectively support the view that Asian endophytes are promising sources of novel compounds with pharmaceutical and agricultural significance. However, a large proportion of medicinal plants in Asia remain unexplored for their endophytic diversity, indicating substantial opportunities for the discovery of new microorganisms and therapeutically important metabolites. The important endophytes isolated from Asian medicinal plants along with their reported biological activities are summarized in Figure 2.1.

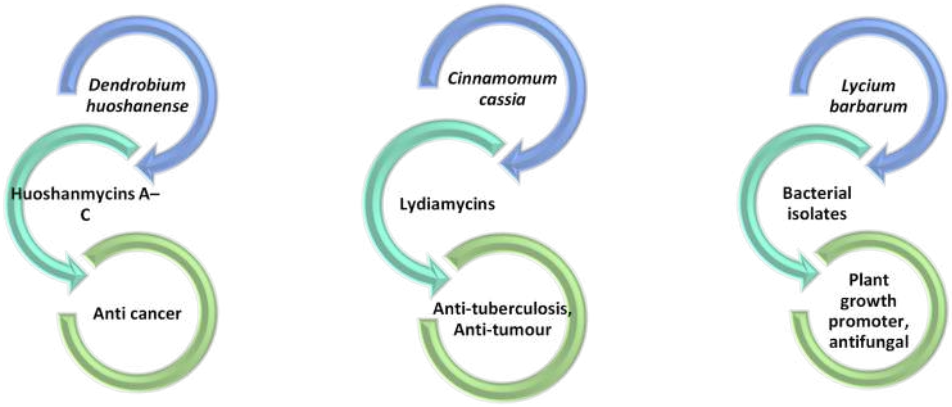


FIGURE 2.1: Plant name, isolated compound and its activity of some endophytes from Asian plants

2.3 Endophytes in Pakistan

Pakistan possesses a rich diversity of medicinal plants distributed across the Himalayan, Hindu Kush, Karakoram, and Sulaiman mountain ranges, where traditional herbal remedies have been practiced for centuries. Regions such as Khyber Pakhtunkhwa, Azad Jammu and Kashmir, and Gilgit-Baltistan are particularly known for their extensive use of indigenous medicinal flora. Despite this botanical wealth, the study of endophytic microorganisms associated with Pakistani medicinal plants remains relatively underdeveloped compared with neighboring countries.

Early investigations focused primarily on bacterial endophytes isolated from medicinal shrubs and herbs. The isolation of endophytic bacteria from the stems of

Fagonia indica, identifying members of the genera *Bacillus*, *Enterobacter*, *Pantoea*, *Erwinia*, and *Stenotrophomonas*. These isolates exhibited multiple biological activities, including antioxidant, antibacterial, antifungal, and anti-leishmanial effects, suggesting that Pakistani medicinal plants harbor endophytes with considerable pharmacological potential [17]. Similar findings have been reported in studies from other Asian countries, where *Bacillus* species frequently dominate endophytic communities and produce diverse bioactive metabolites.

Research on *Berberis lycium* has so far concentrated almost exclusively on bacterial endophytes. *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* were identified as the most active isolates, with all three strains demonstrating notable antibacterial and antifungal activities [11].

A subsequent study expanded the known diversity of bacterial endophytes in *Berberis lycium* by isolating *B. paramycoides*, *B. pseudomycoides*, *B. wiedmannii*, *B. subtilis* and *Calidifontibacillus erzurumensis* from root tissues [12].

The repeated recovery of *Bacillus* species from different plant parts and geographical locations suggests that this genus may have a particularly close ecological association with *B. lycium*. The isolation of *Bacillus toyonensis* from the roots of the related species *Berberis aristata* collected from the Kaghan Valley supported these observations, indicating that members of the genus *Bacillus* may commonly colonize *Berberis* species in the Himalayan region [13].

Although these studies collectively demonstrate the therapeutic potential of bacterial endophytes associated with *Berberis* species, several important gaps remain unresolved. Current research has largely ignored the fungal endophytic community of *B. lycium*, and the antidiabetic potential of its endophytes has received little direct investigation. In addition, sampling has been restricted to a limited number of geographical locations, making it difficult to determine whether the reported endophytic populations represent the full diversity associated with this medicinal plant [11, 12].

These limitations highlight the need for broader investigations encompassing different plant tissues, fungal endophytes, multiple biological activities, and wider

geographic sampling. Such gaps provide a strong rationale for the present study and lead naturally to the following discussion on the diversity of fungal and bacterial endophytes associated with medicinal plants.

2.4 Diversity of Endophytes

2.4.1 Fungal Endophytes

Fungal endophytes constitute the most extensively investigated group of endophytic microorganisms and are regarded as one of the richest natural sources of structurally diverse secondary metabolites.

Their widespread distribution across a broad range of plant species and ecosystems, together with their remarkable biosynthetic capabilities, has made them a major focus of natural product and pharmaceutical research. Most fungal endophytes belong to the phylum Ascomycota, although representatives of Basidiomycota have also been reported from various medicinal and non-medicinal plants [5, 7].

The predominance of Ascomycota is generally attributed to their superior adaptability, efficient colonization of internal plant tissues, and greater ability to produce bioactive metabolites that facilitate long-term associations with their hosts.

Despite differences in geographical location and host plant species, several fungal genera are consistently reported across independent studies. Among the most frequently isolated are *Fusarium*, *Pestalotiopsis*, *Chaetomium*, *Aspergillus*, *Penicillium*, *Talaromyces*, *Lophiostoma*, *Phyllosticta*, *Cladosporium*, and *Eutypella*, whereas members of *Basidiomycota*, such as *Trametes*, are encountered less frequently [7, 17].

The repeated isolation of these genera from diverse medicinal plants suggests that they possess effective strategies for host colonization and adaptation, enabling

them to establish stable endophytic associations under varying environmental conditions.

Comparative studies further demonstrate that although fungal diversity differs among plant species and ecological regions, certain genera repeatedly dominate endophytic communities. For instance, an investigation of *Zanthoxylum gilletii* and *Markhamia lutea* in Kenya recovered 24 fungal isolates, with *Fusarium* accounting for approximately 63% of the total population, followed by *Chaetomium*, *Scopulariopsis*, and *Trametes* [18]. In contrast, *Eucalyptus exserta* in South China identified 80 fungal isolates representing 13 genera, where *Phyllosticta* was the predominant genus, followed by *Eutypella*, *Lophiostoma*, *Pestalotiopsis*, and *Chaetomium* [7]. The contrasting dominance of *Fusarium* in one study and *Phyllosticta* in another indicates that endophytic community composition is strongly influenced by factors such as host species, tissue type, climatic conditions, geographical distribution, and isolation techniques. Nevertheless, the presence of genera such as *Chaetomium* in both investigations suggests that certain fungal taxa are capable of colonizing a wide range of plant hosts irrespective of environmental differences.

The pharmaceutical importance of fungal endophytes extends beyond their taxonomic diversity. Numerous studies have demonstrated that these microorganisms produce a broad spectrum of biologically active metabolites, including alkaloids, terpenoids, polyketides, peptides, flavonoids, quinones, and phenolic compounds, many of which exhibit antibacterial, antifungal, antioxidant, anticancer, anti-inflammatory, and antidiabetic activities [5]. The ability of fungal endophytes to synthesize metabolites that are structurally similar or even identical to those produced by their host plants further enhances their significance as sustainable alternatives for natural product production.

Consequently, fungal endophytes have become increasingly important in drug discovery programmes aimed at identifying novel therapeutic agents with improved efficacy and reduced environmental dependence on medicinal plant harvesting.

Overall, the available literature demonstrates that fungal endophytes exhibit considerable taxonomic diversity and exceptional biosynthetic potential. Although

the composition of fungal communities varies among medicinal plants and geographical regions, the consistent occurrence of several dominant genera across independent studies highlights their ecological adaptability and pharmaceutical relevance. This diversity not only contributes to the production of valuable bioactive metabolites but also provides a strong basis for continued exploration of fungal endophytes from medicinal plants.

While fungal endophytes have received substantial scientific attention, bacterial endophytes also represent an important component of the plant microbiome with unique biological functions and therapeutic potential, which are discussed in the following section.

2.4.2 Bacterial Endophytes

Bacterial endophytes constitute another important group of plant-associated microorganisms that play significant roles in plant growth, stress tolerance, and the production of biologically active metabolites. Although fungal endophytes have historically received greater scientific attention, recent studies have demonstrated that bacterial endophytes possess comparable pharmaceutical and agricultural potential. Both Gram-positive and Gram-negative bacteria have been isolated from a wide range of medicinal plants, with genera such as *Bacillus*, *Pseudomonas*, *Streptomyces*, *Burkholderia*, *Enterobacter*, *Pantoea*, and *Serratia* being among the most frequently reported [6, 17, 19]. These bacteria generally colonize plants through the root system before spreading to aerial tissues via the vascular network, allowing them to establish stable associations with different plant organs [12].

Among the reported bacterial endophytes, the genus *Bacillus* has emerged as one of the most dominant and extensively studied. Species including *B. cereus*, *B. subtilis*, *B. thuringiensis*, *B. pumilus*, *B. paramycoides*, *B. pseudomycoides*, *B. wiedmannii*, and *B. toyonensis* have been repeatedly isolated from medicinal plants and are recognized for their ability to synthesize a diverse range of bioactive metabolites, including lipopeptides, polyketides, antimicrobial peptides, and

plant growth regulators [11]. The frequent isolation of *Bacillus* species from different host plants and geographical regions suggests that these bacteria possess strong adaptive capabilities and establish effective symbiotic relationships with medicinal plants. Their ability to produce metabolites with antimicrobial as well as plant growth-promoting properties further enhances their pharmaceutical and agricultural significance.

Comparative studies from different medicinal plants consistently support the multifunctional nature of bacterial endophytes. For example, six bacterial endophytes isolated from the rhizomes of *Curcuma longa*, including *Bacillus cereus* and *Pseudomonas putida*, demonstrated multiple beneficial characteristics, such as indole-3-acetic acid (IAA) production, phosphate solubilization, and antibacterial and antifungal activities [19]. Endophytic bacteria isolated from *Fagonia indica*, including *Bacillus subtilis* and *Stenotrophomonas maltophilia*, effectively inhibited *Aspergillus* species and several pathogenic bacteria [17].

Although these investigations involved different medicinal plants and bacterial communities, both studies indicate that bacterial endophytes often exhibit multiple biological functions rather than a single therapeutic property. Such functional diversity is likely influenced by differences in host plant chemistry, environmental conditions, and microbial adaptation to specific ecological niches.

Besides *Bacillus*, members of the genus *Streptomyces* have also attracted considerable attention because of their exceptional capacity to produce clinically important secondary metabolites. Endophytic *Streptomyces* NRRL 30562 isolated from *Kennedia nigriscans* produces munumbicins, a group of broad-spectrum antibiotics, whereas *Streptomyces* sp. HBQ95 isolated from *Cinnamomum cassia* synthesizes lydiamycins with notable anticancer and antimycobacterial activities [6].

Overall, current evidence suggests that bacterial endophytes are indispensable components of the plant microbiome with functions that extend beyond plant colonization to include the production of valuable bioactive metabolites and enhancement of plant health. Although bacterial communities differ among medicinal

plants and geographical regions, recurring genera such as *Bacillus* and *Streptomyces* consistently demonstrate substantial pharmaceutical and biotechnological potential. The diversity of fungal and bacterial endophytes reported from medicinal plants is summarized in Figure 2.2. Understanding this diversity provides an essential basis for exploring the therapeutic activities of endophytes, which are discussed in the following sections.

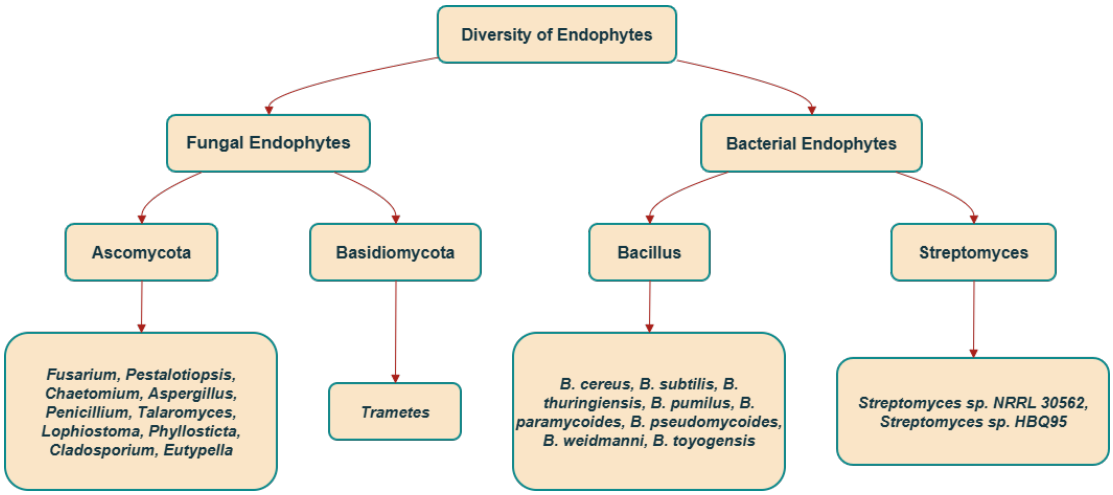


FIGURE 2.2: Plant name, isolated compound and its activity of some endophytes from Asian plants

2.5 *Berberis lycium*: Botanical Description, Ethnomedicinal Uses and Phytochemistry

2.5.1 Botanical Description

Berberis lycium is an evergreen, thorny shrub belonging to the family Berberidaceae and is one of the most important medicinal species of the genus Berberis. The plant generally attains a height of 3–4 m and is characterized by pale grey to whitish branches bearing yellow, trifurcate spines measuring approximately 10–20 mm in length. Its leaves are oblong to lanceolate, ranging from 2–6 cm in length and 6–12 mm in width, with a pale green upper surface and a greyish-white lower surface [20]. The leaf margins possess two to four small spines, a characteristic feature that distinguishes the species from many other members of the genus. The

plant produces pale yellow flowers measuring 6–8 mm in diameter, followed by oblong berries approximately 7–8 mm long that usually contain three to four seed [21].

Berberis lycium is widely recognized by different vernacular names across South Asia, reflecting its long-standing cultural and medicinal importance. It is commonly known as "Ishkeen" in Urdu, "Kashmal" in Hindi, and "Kawdach" or "Sumblu" in the Kashmir region [22]. The species is naturally distributed in Pakistan, India, Nepal, and Afghanistan, where it predominantly grows in the Himalayan mountain ranges and other high-altitude regions [23]. Its ability to thrive under harsh environmental conditions, including low temperatures, rocky soils, and limited water availability, demonstrates its remarkable ecological adaptability [23] Such environmental resilience may also influence the diversity of endophytic microorganisms associated with the plant, as ecological conditions are known to shape plant–microbe interactions and microbial community composition [24].

The plant reproduces through both self-pollination and cross-pollination, enabling successful propagation under varying environmental conditions [21]. Its wide geographical distribution, ecological adaptability, and extensive use in traditional medicine have made *Berberis lycium* an attractive species for phytochemical and microbiological investigations. These characteristics suggest that the plant may also harbor diverse endophytic microorganisms capable of producing bioactive metabolites with therapeutic significance.

2.5.2 Ethnomedicinal Uses

For centuries, *Berberis lycium* has occupied an important place in the traditional healthcare systems of Pakistan, India, Nepal, and other Himalayan regions, where different plant parts are routinely used to manage a wide range of human ailments. The roots, stem bark, leaves, fruits, and occasionally the whole plant are employed in traditional remedies for the treatment of jaundice, hepatitis, typhoid, fever, tuberculosis, diarrhoea, dysentery, skin infections, piles, scabies, kidney disorders, mouth ulcers, eye diseases, bone fractures, and inflammatory conditions [23]. The

extensive use of multiple plant parts for diverse therapeutic purposes suggests that *Berberis lycium* contains a broad spectrum of biologically active constituents capable of exerting various pharmacological effects.

Among the different plant parts, the root bark is considered the most therapeutically valuable in traditional medicine. It has been widely used for the management of dysentery, sore throat, gonorrhoea, and internal wounds, while aqueous preparations of the root bark are commonly applied externally for the treatment of boils, haemorrhoids, and other skin disorders [8].

In addition to these applications, the leaves and fruits are traditionally consumed by diabetic patients in Pakistan to help control elevated blood glucose levels and associated metabolic disorders, highlighting the plant's long-standing reputation as an antidiabetic remedy [8].

The continued use of *Berberis lycium* for diabetes management across different communities has encouraged scientific investigations into its hypoglycaemic properties and the identification of bioactive compounds responsible for these effects.

The diverse ethnomedicinal applications of *Berberis lycium* are largely attributed to its rich phytochemical composition, particularly the presence of the isoquinoline alkaloid berberine. Berberine has been extensively investigated and is reported to possess antimicrobial, antiprotozoal, antidiarrhoeal, antioxidant, anti-inflammatory, and antidiabetic activities, thereby providing scientific support for many of the traditional uses of the plant [8, 25].

Nevertheless, it is increasingly recognized that the therapeutic efficacy of medicinal plants may not depend solely on plant-derived phytochemicals. Emerging evidence indicates that endophytic microorganisms associated with medicinal plants can also synthesize biologically active metabolites, some of which are structurally similar or identical to those produced by their host plants. This possibility has generated growing interest in exploring the endophytic community of *Berberis lycium* as an additional source of pharmacologically important compounds.

Overall, the extensive traditional use of *Berberis lycium*, together with the growing scientific evidence supporting its biological activities, highlights its importance as a valuable medicinal plant.

These characteristics provide a strong rationale for investigating not only its phytochemical constituents but also its associated endophytic microorganisms, which may contribute to its therapeutic potential and offer new opportunities for natural product discovery.

2.5.3 Phytochemistry

The chemical make-up of *Berberis lycium* is very rich and diverse. The most important compound in the plant is the alkaloid berberine ($C_{20}H_{18}NO_4^+$), which belongs to the benzylisoquinoline family and has been studied for many pharmacological activities. A list of other alkaloids are found in the plant including camptothecin, salutaridine, koumidine, palmatine, luteanine, isomajdine, protopine, alpha-codeimethine, oxoglaucine and hydroxygardnutine [8, 26].

Berberis lycium also contains some steroids including sitosterol and β-sitosterol, flavonoids such as isorhamnetin, velutin and formononetin-7-O-glucoside. The anthocyanins of *Berberis lycium* include delphinidin-3-glucoside and cyanidin-3-glucoside that contribute to its antioxidant and anti-inflammatory activities. A large group of phenolic compounds has also been identified including vanillic acid, chlorogenic acid, quercetin, caffeic acid, syringic acid, gallic acid, coumaric acid, ferulic acid, bergeninand sinapic acid [11, 27]

Other unique alkaloids found specifically in Berberidaceae plants include berbamine, jhelumine, chinabine, karakoramine, punjabine, gilgitine, oxyacanthine and chenabine [11]. Some of these alkaloids have recently shown strong activity against the enzyme α-amylase, which is linked to blood sugar control. The plant also contains several important minerals such as sodium, potassium, zinc, iron, calcium and magnesium and these has been used to make silver and gold nanoparticles for medical applications.

2.5.4 Endophytes Isolated from *Berberis lycium*: Specific Strains Identified in Previous Studies

Despite the well-established medicinal importance of *Berberis lycium*, investigations into its associated endophytic microorganisms remain relatively limited. Compared with many other medicinal plants, only a few studies have explored the endophytic diversity of *Berberis lycium*, and these have focused almost exclusively on bacterial endophytes. Consequently, the microbial communities associated with this plant remain largely unexplored, particularly with respect to fungal endophytes and their therapeutic potential.

The researchers isolated twenty bacterial endophytes from internal stem and leaf tissues, using plant samples collected from Marghazar (Swat) and Murree, Pakistan. It compromises thirteen isolates from stems and seven from leaves. Molecular identification revealed that the three most biologically active strains belonged to *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis*, all of which demonstrated promising antibacterial activity [11]. This study provided the first evidence that *Berberis lycium* harbors endophytic bacteria capable of producing biologically active metabolites with antimicrobial potential.

Subsequently, Younis *et al.* expanded the understanding of *Berberis lycium* endophytes by investigating root-associated bacterial communities from plants collected in Nakyal, Kotli, Azad Jammu and Kashmir. Their study identified five bacterial species, namely *Bacillus paramycoides*, *Bacillus pseudomycoides*, *Bacillus wiedmannii*, *Bacillus subtilis*, and *Calidifontibacillus erzurumensis* [12]. Although the two investigations differed in sampling location and plant tissue, both consistently reported the predominance of *Bacillus* species. This recurring pattern suggests that members of the genus *Bacillus* may possess a strong ecological association with *Berberis lycium*, enabling them to colonize different plant tissues under diverse environmental conditions. Such host preference has also been observed in other medicinal plants, where *Bacillus* species frequently dominate endophytic bacterial communities because of their efficient colonization ability, stress tolerance, and capacity to produce a wide range of bioactive metabolites.

Additional support for the association between *Bacillus* species and the genus *Berberis* is provided by studies on the closely related medicinal plant *Berberis aristata*. *Bacillus toyonensis* was isolated from the root tissues of *B. aristata* collected from the Kaghan Valley, Pakistan. Besides producing the characteristic yellow pigment, this endophyte was capable of synthesizing berberine, carotenoids, and several other biologically important metabolites, highlighting its pharmaceutical potential [13]. The occurrence of metabolite-producing *Bacillus* species in both *Berberis lycium* and *Berberis aristata* suggests that these bacteria may play an important role in the chemistry and biological activity of *Berberis* species, although further comparative investigations are needed to confirm this relationship.

Although these studies have significantly improved our understanding of bacterial endophytes associated with *Berberis lycium*, several important knowledge gaps remain. To date, no comprehensive study has investigated the fungal endophytic community of this medicinal plant, despite fungal endophytes being recognized as prolific producers of pharmacologically valuable secondary metabolites. Furthermore, previous investigations have primarily emphasized antibacterial and antifungal activities, whereas other important therapeutic properties, particularly antidiabetic potential, remain largely unexplored. In addition, sampling has been confined to a limited number of geographical regions, making it difficult to determine the full diversity of endophytic microorganisms associated with *B. lycium*. The endophytes isolated from *Berberis* species are summarized in Figure 2.3. These existing gaps highlight the need for comprehensive investigations encompassing both fungal and bacterial endophytes from different plant tissues and geographical locations, thereby providing the scientific basis for the present study.

2.6 Methods Used for Isolation and Identification of Endophytes

The successful isolation and identification of endophytes depend largely on the methodology employed, as each step influences both the diversity and accuracy of

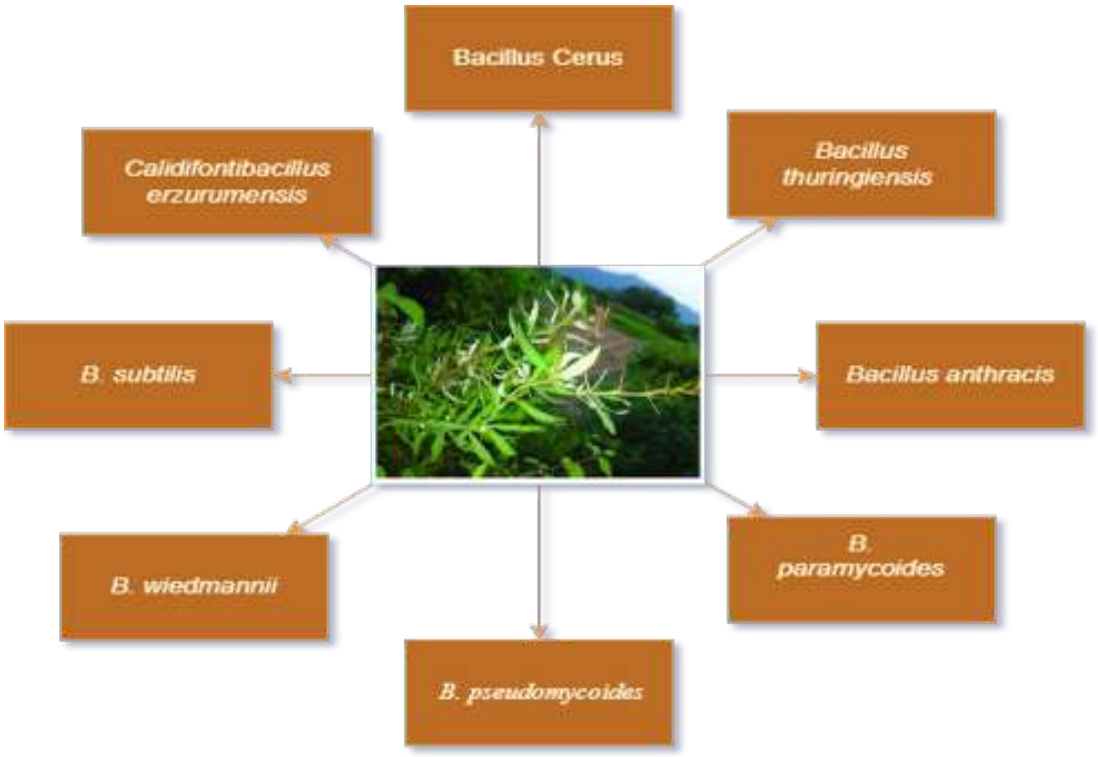


FIGURE 2.3: Endophytes isolated from berberis lycium

the microorganisms recovered. Although standardized protocols have been established over the past few decades, researchers frequently modify individual steps according to the host plant, tissue type, and the target group of microorganisms. Consequently, variations in isolation procedures often contribute to differences in the diversity and composition of endophytic communities reported among studies [4].

Surface sterilization is considered the most critical step in endophyte isolation because it eliminates epiphytic microorganisms while preserving the viability of microbes residing within plant tissues. Most studies employ a sequential treatment consisting of 70% ethanol, followed by 1–3% sodium hypochlorite, and several rinses with sterile distilled water [4, 11]. Although this protocol is widely accepted, the duration of exposure and disinfectant concentration are often adjusted according to the characteristics of the plant tissue. Softer tissues such as leaves generally require shorter sterilization times than roots or woody stems because prolonged exposure may damage internal tissues and reduce endophyte recovery [6]. Therefore, optimizing sterilization conditions is essential to achieve a balance

between effective surface decontamination and the survival of genuine endophytic microorganisms.

To ensure the effectiveness of surface sterilization, most investigators perform a sterility check by inoculating the final rinse water onto an appropriate culture medium. The absence of microbial growth confirms successful removal of surface contaminants, whereas colony formation indicates incomplete sterilization and necessitates repetition of the procedure [13, 19]. This validation step has become an important quality control measure because contamination by epiphytic microorganisms may lead to inaccurate identification of endophytes and misinterpretation of biological activities.

Following surface sterilization, small tissue segments are cultured on selective growth media to recover endophytic microorganisms. The choice of culture medium largely depends on the type of endophyte being investigated. For bacterial isolation, Tryptic Soy Agar (TSA) and nutrient agar are commonly employed because they support the growth of a broad spectrum of bacterial species within a relatively short incubation period [11, 12]. In contrast, fungal endophytes are generally isolated using Potato Dextrose Agar (PDA) or Sabouraud Dextrose Agar (SDA) supplemented with antibiotics such as streptomycin to suppress bacterial contamination [7, 18]. Fungal cultures usually require considerably longer incubation periods than bacterial cultures due to their slower growth rates. The repeated use of sub-culturing to obtain pure isolates is common to both groups and is essential for ensuring the reliability of subsequent morphological, molecular, and pharmacological investigations.

Although morphological and biochemical characteristics remain useful for preliminary characterization, molecular techniques have become the preferred approach for accurate identification of endophytes because they provide greater taxonomic resolution and reproducibility. For bacterial endophytes, amplification of the 16S rRNA gene using universal primers such as 27F and 1492R is widely regarded as the standard method for species identification [18]. Similarly, the internal transcribed spacer (ITS) region has become the accepted DNA barcode for fungal identification because it exhibits sufficient sequence variation to distinguish closely

related fungal taxa [18]. Compared with conventional identification methods based solely on colony morphology, DNA sequencing minimizes misidentification and allows reliable comparison of isolates with publicly available reference sequences in the NCBI GenBank database using the BLAST algorithm.

Phylogenetic analysis provides an additional level of confidence by revealing the evolutionary relationships between newly isolated endophytes and previously characterized species. Most studies construct phylogenetic trees using software such as MEGA, applying either the Neighbor-Joining or Maximum Likelihood algorithms with 1,000–2,000 bootstrap replications to assess the robustness of tree topology [19]. While both approaches are widely accepted, the Maximum Likelihood method generally offers greater statistical accuracy, whereas Neighbor-Joining is preferred for its computational efficiency, particularly when analyzing large datasets. Sequence similarities of approximately 98.65–99% with authenticated reference strains are generally considered sufficient for assigning bacterial isolates to the same species, although additional taxonomic evidence may occasionally be required for closely related organisms [13].

Following identification, the production and characterization of secondary metabolites represent the next major step in endophyte research. Culture conditions vary according to the type of microorganism and the metabolites of interest. Bacterial endophytes are commonly cultivated in liquid broth under shaking conditions to maximize biomass production, after which intracellular and extracellular metabolites are extracted using solvents such as methanol and ethyl acetate [11]. In contrast, fungal endophytes are frequently fermented on solid substrates, particularly sterilized rice medium, which has been shown to enhance secondary metabolite biosynthesis compared with liquid fermentation in many fungal species [7]. The extracted metabolites are subsequently analyzed using analytical techniques such as Gas Chromatography–Mass Spectrometry (GC–MS), enabling compound identification through comparison with spectral libraries including the NIST database [13].

Overall, advances in molecular biology, microbial cultivation, and analytical chemistry have substantially improved the isolation, identification, and characterization

of endophytes. Although standardized methodologies have enhanced reproducibility, differences in sterilization procedures, culture conditions, molecular markers, and analytical techniques continue to influence the diversity of endophytes recovered and the metabolites identified.

Consequently, careful selection and optimization of these methods remain essential for accurately exploring the therapeutic potential of endophytic microorganisms and facilitating the discovery of novel bioactive compounds.

2.7 Pharmacological Activities

2.7.1 Antibacterial Activity

The antibacterial potential of endophytes has been consistently demonstrated in medicinal plants from different geographical regions, highlighting their ability to produce diverse secondary metabolites with broad-spectrum antimicrobial activity. These metabolites inhibit bacterial growth through multiple mechanisms, including disruption of cell wall integrity, inhibition of DNA replication and protein synthesis, and damage to the bacterial cell membrane [5, 19].

Although both fungal and bacterial endophytes exhibit antibacterial properties, the level of activity varies depending on the endophytic species, host plant, and the nature of the bioactive compounds produced.

Among fungal endophytes, *Fusarium solani* (Ml.2) isolated from *Markhamia lutea* exhibited the strongest antibacterial activity, producing inhibition zones of 20.3mm against *Xanthomonas axonopodis* and 18.6 mm against *Pseudomonas syringae*, values comparable to those of the standard antibiotic chloramphenicol (20 mm) [18].

Likewise, *Chaetomium* sp. and *Lophiostoma* sp. isolated from *Eucalyptus exserta* demonstrated inhibition zones exceeding 10 mm against several bacterial pathogens, confirming that different fungal genera possess significant antibacterial potential despite differences in host plants and geographical origin [7].

Bacterial endophytes have shown similarly promising antibacterial activities. *Bacillus cereus* (LBL6) isolated from *Berberis lycium* produced inhibition zones of up to 19 mm against *Bacillus spizizenii* and exhibited broad-spectrum activity against several ATCC bacterial strains [11].

Comparable findings are reported for endophytes isolated from *Fagonia indica*, where all eight bacterial isolates inhibited *Klebsiella pneumoniae*, with *Bacillus tequilensis* showing the greatest activity against *Bacillus subtilis* [17]. Likewise, endophytic bacteria from *Curcuma longa*, particularly *Bacillus cereus* (ECL1) and *Pseudomonas putida* (ECL5), demonstrated effective antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [19].

In addition, *Bacillus toyonensis* BAR1 isolated from *Berberis aristata* inhibited multidrug-resistant *Enterobacter cloacae* and *Pseudomonas aeruginosa*, producing inhibition zones of 10 mm and 9 mm, respectively, with MIC values of 16 mg/mL against both pathogens [13]. Collectively, these studies indicate that endophytic microorganisms, particularly species belonging to the genus *Bacillus*, represent promising sources of antibacterial agents with potential applications against both susceptible and multidrug-resistant bacterial pathogens.

2.7.2 Antifungal Activity

Antifungal activity is one of the most extensively investigated biological properties of endophytes, largely because these microorganisms produce diverse secondary metabolites capable of inhibiting pathogenic fungi through multiple mechanisms. These include disruption of fungal cell membranes, inhibition of cell wall biosynthesis, interference with essential metabolic pathways, and suppression of spore germination, making endophytes promising alternatives to conventional antifungal agents in the face of increasing antifungal resistance [5].

Although both fungal and bacterial endophytes exhibit antifungal properties, their efficacy varies depending on the endophytic species, host plant, and the nature of the metabolites produced.

Among bacterial endophytes, *Bacillus* species isolated from *Berberis lycium* demonstrated notable antifungal activity, with *Bacillus thuringiensis* producing 60% inhibition of *Aspergillus niger* and *Bacillus anthracis* showing 56% inhibition of *A. flavus* at a concentration of 4 mg/mL [11]. Similar antifungal effects have been reported for bacterial endophytes from other medicinal plants. Endophytes isolated from *Lycium barbarum* inhibited *Fusarium* species with inhibition zones ranging from 5–15 mm and *Verticillium dahliae* with zones of 10–20 mm in approximately half of the tested isolates [16]. Likewise, all eight bacterial endophytes recovered from *Fagonia indica* exhibited activity against at least one fungal pathogen, with *Stenotrophomonas maltophilia* producing the largest inhibition zone (16 mm) against *A. niger* [19]. Comparable observations are reported for endophytes isolated from *Curcuma longa*, where all six bacterial strains inhibited the growth of *Fusarium solani* and *Alternaria alternata*, indicating that antifungal activity is a common characteristic of bacterial endophytes associated with medicinal plants [19].

Fungal endophytes have also demonstrated considerable antifungal potential. *Chaetomium* sp. and *Pestalotiopsis* sp. isolated from *Eucalyptus exserta* exhibited broad-spectrum antimicrobial activity against both fungal and bacterial pathogens, while the well-known metabolite cryptocandin, produced by *Cryptosporiopsis cf. quercina*, showed potent activity against *Candida albicans* and several dermatophytic fungi [4, 7]. Collectively, these findings suggest that both fungal and bacterial endophytes represent valuable sources of antifungal compounds with diverse mechanisms of action and potential applications in combating fungal infections and emerging antifungal resistance.

2.7.3 Anti-inflammatory Activity

Endophytes have emerged as promising sources of anti-inflammatory compounds because of their ability to synthesize a wide range of bioactive secondary metabolites, including alkaloids, flavonoids, terpenoids, phenolics, and peptides. These metabolites suppress inflammatory responses through multiple mechanisms, such

as inhibiting the production of prostaglandins, nitric oxide, pro-inflammatory cytokines, and reactive oxygen species by targeting key enzymes, including cyclooxygenase (COX), lipoxygenase (LOX), and phospholipase A_2. Interestingly, many of these metabolites are structurally similar to those found in their host plants, suggesting that endophytes may contribute to or complement the therapeutic properties traditionally attributed to medicinal plants [28].

Although direct investigations on the anti-inflammatory activity of endophytes associated with *Berberis lycium* are still lacking, substantial evidence supports the anti-inflammatory efficacy of the host plant itself. In an in vivo study, the methanolic extract of *Berberis lycium* significantly reduced carrageenan-induced paw edema, xylene-induced ear edema, and formalin-induced chronic inflammation in mice in a dose-dependent manner, producing effects comparable to diclofenac sodium [29].

The observed activity was attributed to the combined presence of berberine, flavonoids, phenolic compounds, and β-sitosterol, all of which are known to interfere with inflammatory signalling pathways and the synthesis of inflammatory mediators. These findings indicate that *Berberis lycium* contains multiple bioactive constituents acting through complementary mechanisms rather than relying on a single active compound.

Comparable results have been reported in in vitro investigations. Shruti et al. evaluated hydroethanolic extracts prepared from the roots, stems, and leaves of *Berberis lycium* using the protein denaturation assay and found that the root extract exhibited the greatest anti-inflammatory activity, with an IC_{50} value lower than that of diclofenac [30].

In contrast to studies emphasizing the role of berberine alone, the authors suggested that the superior activity of the crude extract resulted from synergistic interactions among multiple phytochemicals. This interpretation is supported by another study demonstrating that a berberine-rich extract significantly modulated inflammatory biomarkers, confirming the importance of berberine while also indicating that additional plant constituents may enhance its biological effects [30].

Together, these findings suggest that the anti-inflammatory activity of *Berberis lycium* is likely the result of the combined action of several bioactive metabolites rather than a single compound.

Considering that endophytes are capable of producing metabolites that are structurally similar or identical to those of their host plants, it is reasonable to hypothesize that endophytic microorganisms associated with *Berberis lycium* may also possess significant anti-inflammatory potential [31].

However, this possibility has received little scientific attention, representing an important research gap. Therefore, investigating the anti-inflammatory activity of endophytic metabolites may not only improve our understanding of the plant–microbe relationship but also provide sustainable alternative source of natural anti-inflammatory agents while reducing dependence on harvesting medicinal plants.

2.8 In Silico Evaluation of Phytochemicals from *Berberis lycium* for Antidiabetic Drug Discovery

2.8.1 Molecular Docking Studies of *Berberis lycium* Phytochemicals against Dipeptidyl Peptidase-4

Molecular docking has become an indispensable computational technique in contemporary drug discovery because it predicts the binding orientation and affinity of small molecules within the active site of target proteins. Compared with conventional experimental screening, docking offers a rapid and cost-effective approach for identifying potential lead compounds before laboratory validation. In recent years, molecular docking has been extensively employed to evaluate phytochemicals from medicinal plants against proteins involved in glucose metabolism, thereby accelerating the discovery of novel antidiabetic agents [32].

Among the various therapeutic targets for type 2 diabetes mellitus, dipeptidyl peptidase-4 (DPP-4) is one of the most extensively investigated enzymes. DPP-4 is responsible for the rapid degradation of the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which normally stimulate insulin secretion and suppress glucagon release following food intake. Inhibition of DPP-4 prolongs the activity of these incretins, resulting in improved glycaemic control with a relatively low risk of hypoglycaemia. Owing to this mechanism, synthetic DPP-4 inhibitors such as sitagliptin, saxagliptin, linagliptin, and vildagliptin have become established therapeutic agents for the management of type 2 diabetes mellitus [33].

The search for naturally occurring DPP-4 inhibitors has increased considerably because plant-derived compounds generally exhibit structural diversity and may produce fewer adverse effects than synthetic drugs. Molecular docking studies have shown that several classes of phytochemicals, including alkaloids, flavonoids, phenolic acids, terpenoids, and glycosides, interact favourably with the catalytic residues of DPP-4. These interactions are mainly mediated through hydrogen bonds, hydrophobic contacts, π-π stacking, and van der Waals forces, all of which contribute to stabilization of the ligand–protein complex. Compounds capable of forming multiple interactions generally exhibit stronger binding affinities and are therefore considered more promising candidates for further biological evaluation [32].

Berberis lycium is recognized as a rich source of pharmacologically important phytochemicals, particularly isoquinoline alkaloids such as berberine, together with flavonoids, phenolic compounds, lignans, sterols, and terpenoids. Among these constituents, berberine has received the greatest scientific attention because of its well-established antihyperglycaemic activity. Experimental studies have demonstrated that berberine improves glucose homeostasis by activating AMP-activated protein kinase (AMPK), enhancing insulin sensitivity, reducing hepatic gluconeogenesis, and improving peripheral glucose uptake. In addition to these mechanisms, computational investigations have suggested that berberine can bind effectively within the active site of DPP-4, indicating that inhibition of this enzyme

may also contribute to its antidiabetic effects [34].

Besides berberine, several flavonoids and phenolic compounds identified in Berberis species have demonstrated encouraging docking performance against DPP-4. Comparative molecular docking studies have reported that polyphenolic compounds frequently exhibit strong binding affinities because their hydroxyl groups facilitate the formation of multiple hydrogen bonds with amino acid residues located in the catalytic pocket of the enzyme. Similarly, terpenoids and sterols contribute additional hydrophobic interactions that further stabilize ligand binding. These observations indicate that different classes of phytochemicals may inhibit DPP-4 through complementary interaction patterns rather than a single binding mechanism, highlighting the therapeutic potential of the chemically diverse constituents present in *Berberis lycium* [34].

Although many computational studies have reported favourable docking scores for natural compounds, direct comparison among studies should be interpreted cautiously. Variations in docking software, protein preparation methods, scoring algorithms, and search parameters frequently result in differences in predicted binding energies. For this reason, recent computational studies often employ more than one docking platform, such as AutoDock Vina and Schrödinger Glide, to improve prediction reliability.

Furthermore, docking scores alone cannot accurately predict biological efficacy, as compounds with excellent binding affinity may still possess poor pharmacokinetic characteristics or unacceptable toxicity profiles. Consequently, molecular docking is generally complemented by ADME and toxicity prediction tools, followed by experimental validation, to identify compounds with the greatest potential for drug development [32].

Overall, the available literature indicates that molecular docking is an effective strategy for prioritizing phytochemicals with potential DPP-4 inhibitory activity. The rich phytochemical composition of *Berberis lycium*, together with the established antidiabetic properties of several of its constituents, provides a strong scientific basis for computational evaluation of its metabolites against DPP-4.

Such studies not only facilitate the identification of promising lead compounds but also guide subsequent pharmacokinetic, toxicity, and experimental investigations required for the development of novel antidiabetic agents.

2.8.2 Molecular Docking Studies of *Berberis lycium* Phytochemicals against α-Amylase

Inhibition of carbohydrate-digesting enzymes has become an important therapeutic strategy for the management of type 2 diabetes mellitus because it reduces the rapid rise in postprandial blood glucose levels. Among these enzymes, α-amylase plays a key role in the initial hydrolysis of dietary starch into smaller oligosaccharides, which are subsequently converted into glucose by α-glucosidase. Therefore, inhibition of α-amylase delays carbohydrate digestion, slows glucose absorption, and contributes to improved glycaemic control. This mechanism forms the basis for clinically used α-amylase inhibitors such as acarbose, although their long-term use is frequently associated with gastrointestinal adverse effects, including abdominal discomfort, flatulence, and diarrhoea [35].

The limitations of currently available synthetic inhibitors have stimulated considerable interest in identifying naturally occurring α-amylase inhibitors from medicinal plants. Molecular docking has emerged as an efficient approach for screening phytochemicals against α-amylase by predicting their binding affinity and interaction patterns within the enzyme's catalytic site before experimental validation. Several studies have demonstrated that natural compounds inhibit α-amylase by forming hydrogen bonds, hydrophobic interactions, π–π stacking, and electrostatic interactions with catalytically important amino acid residues. The strength and number of these interactions largely determine the stability of the ligand–enzyme complex and influence the predicted inhibitory potential of the compound [32].

Berberis lycium contains a chemically diverse range of bioactive constituents, including alkaloids, flavonoids, phenolic acids, terpenoids, and phytosterols, many of which have been associated with antidiabetic activity. Among these compounds,

berberine has been extensively investigated because of its ability to regulate glucose metabolism through multiple mechanisms, including improvement of insulin sensitivity and suppression of hepatic glucose production [34].

Computational studies involving Berberis phytochemicals have further suggested that several alkaloids and flavonoids can establish stable interactions with the catalytic pocket of α-amylase, indicating their potential to inhibit enzymatic starch hydrolysis and reduce postprandial hyperglycaemia.

Comparative docking studies have shown that flavonoids generally exhibit stronger binding affinities than many other phytochemical classes because the presence of multiple hydroxyl groups facilitates the formation of several hydrogen bonds with catalytic residues of α-amylase. In contrast, terpenoids and phytosterols primarily contribute through hydrophobic interactions that stabilize ligand binding within the enzyme pocket. These findings suggest that different classes of phytochemicals inhibit α-amylase through complementary interaction mechanisms, highlighting the advantage of medicinal plants such as *Berberis lycium*, which contain multiple bioactive constituents capable of acting simultaneously [35].

Despite the encouraging findings reported in computational studies, docking results should be interpreted with caution because predicted binding energies are influenced by differences in docking algorithms, scoring functions, protein preparation methods, and ligand optimization procedures. Consequently, molecular docking is considered a preliminary screening technique that should be complemented by pharmacokinetic analysis, toxicity prediction, and experimental enzyme inhibition assays to accurately determine the therapeutic potential of candidate compounds [32].

Overall, the available evidence indicates that phytochemicals from *Berberis lycium* possess promising structural characteristics for interaction with α-amylase. Their ability to establish stable molecular interactions with the catalytic site supports further computational and experimental investigations aimed at identifying novel natural α-amylase inhibitors for the management of type 2 diabetes mellitus. The integration of molecular docking with ADME and toxicity prediction

further improves the identification of lead compounds with favorable pharmacological profiles.

2.8.3 ADME Prediction Using SwissADME

Although molecular docking provides valuable information about the binding affinity of a compound towards its target protein, successful drug development also depends on favourable pharmacokinetic properties. Many compounds with excellent docking scores fail during later stages of drug development because they exhibit poor absorption, limited bioavailability, rapid metabolism, or unacceptable distribution profiles. Therefore, early evaluation of absorption, distribution, metabolism, and excretion (ADME) has become an essential component of computer-aided drug discovery to identify compounds with the greatest potential for clinical success [15].

Among the available computational tools, SwissADME is one of the most widely used and freely accessible web servers for predicting the pharmacokinetic behaviour, physicochemical properties, drug-likeness, and medicinal chemistry characteristics of small molecules. Developed by Daina et al. the platform integrates several validated predictive models into a single interface, allowing researchers to rapidly evaluate lead compounds before experimental studies. Owing to its simplicity, accuracy, and comprehensive output, SwissADME has become a standard tool in the virtual screening of natural products and synthetic compounds.

SwissADME predicts several physicochemical properties that influence drug absorption and distribution, including molecular weight, lipophilicity (LogP), topological polar surface area (TPSA), hydrogen bond donors and acceptors, molecular flexibility, and water solubility. These parameters are subsequently assessed using established drug-likeness filters such as Lipinski's Rule of Five, Ghose, Veber, Egan, and Muegge criteria, which estimate whether a compound possesses characteristics consistent with orally active drugs although compounds violating one or more of these rules are not necessarily ineffective, compliance generally indicates a greater probability of favorable oral bioavailability.

In addition to physicochemical evaluation, SwissADME predicts important pharmacokinetic parameters including gastrointestinal absorption, blood-brain barrier permeability, P-glycoprotein substrate characteristics, cytochrome P450 enzyme inhibition, skin permeability, and bioavailability score.

One of its most distinctive features is the BOILED-Egg model, which graphically predicts passive gastrointestinal absorption and brain penetration based on molecular polarity and lipophilicity. This visual model enables rapid identification of compounds with desirable pharmacokinetic profiles and has been widely adopted in virtual screening studies involving natural products [36].

Recent computational studies investigating phytochemicals from medicinal plants routinely integrate molecular docking with SwissADME analysis because strong protein binding alone does not guarantee therapeutic efficacy. Several plant-derived compounds have demonstrated excellent docking scores against diabetic targets but are subsequently excluded because of poor gastrointestinal absorption, low bioavailability, or unfavorable metabolic characteristics. Consequently, combining docking studies with ADME prediction provides a more comprehensive strategy for prioritizing lead compounds for further biological evaluation [34].

Therefore, SwissADME has become an indispensable tool in contemporary drug discovery by facilitating the selection of compounds that exhibit not only strong biological activity but also favorable pharmacokinetic properties.

2.8.4 Toxicity Prediction Using ProTox-3

The identification of compounds with promising biological activity is only one aspect of successful drug discovery, as their safety profile is equally important. Many drug candidates fail during preclinical or clinical development because of unacceptable toxicological properties despite exhibiting excellent pharmacological activity. Therefore, computational toxicity prediction has become an essential component of modern computer-aided drug discovery, allowing potentially hazardous compounds to be identified before costly laboratory investigations are undertaken.

This approach not only accelerates drug development but also reduces the need for extensive animal experimentation.

ProTox-3 is the latest generation of the ProTox webserver developed for the in silico prediction of chemical toxicity. The platform integrates molecular similarity analysis with advanced machine learning algorithms trained on experimentally validated toxicological databases to predict a broad spectrum of toxicity endpoints.

Compared with previous versions, ProTox-3 provides improved predictive accuracy and expanded coverage, allowing the evaluation of 61 toxicity endpoints, including acute toxicity, organ toxicity, clinical toxicity, molecular initiating events, adverse outcome pathways (Tox21), toxicity targets, and several other toxicological parameters.

One of the principal features of ProTox-3 is the prediction of acute oral toxicity, expressed as the median lethal dose (LD_{50}), together with classification according to the Globally Harmonized System (GHS). Based on the predicted LD_{50} values, compounds are categorized into toxicity classes ranging from Class I (fatal if swallowed) to Class VI (relatively non-toxic).

Such classification enables rapid prioritization of safer compounds during the early stages of virtual screening and minimizes the progression of highly toxic molecules into experimental studies.

In addition to acute toxicity, ProTox-3 predicts several clinically relevant toxicity endpoints, including hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity, cytotoxicity, organ toxicity, and toxicity target interactions.

The platform also provides confidence scores for each prediction, allowing researchers to assess the reliability of the computational results. These comprehensive toxicity predictions facilitate early identification of safety concerns and support informed decision-making during lead optimization.

Recent computational drug discovery studies increasingly integrate molecular docking, SwissADME, and ProTox-3 because these approaches provide complementary information regarding efficacy, pharmacokinetics, and safety. While molecular

docking identifies compounds with strong binding affinity toward biological targets and SwissADME evaluates their pharmacokinetic and drug-likeness properties, ProTox-3 determines whether these compounds possess acceptable toxicological profiles. Consequently, compounds demonstrating favorable docking scores, satisfactory ADME characteristics, and low predicted toxicity are considered the most promising candidates for further in vitro and in vivo evaluation.

Natural products are frequently regarded as safer alternatives to synthetic drugs; however, numerous phytochemicals have been reported to exhibit hepatotoxic, mutagenic, or cytotoxic effects despite possessing significant biological activity. Therefore, incorporating ProTox-3 into the virtual screening workflow provides a more comprehensive evaluation of phytochemicals and improves the selection of lead compounds with greater therapeutic potential and acceptable safety profiles. This integrated computational strategy has become increasingly important in the discovery of novel antidiabetic agents from medicinal plants, including *Berberis lycium*.

Chapter 3

Methodology

3.1 Plant Collection, Extraction, Phytochemical Screening and Its Therapeutic Potential

3.1.1 Plant Sample Collection

Fresh and healthy leaves, bark and roots of *Berberis Lycium* were collected from Kashmir, authenticated by a plant taxonomist in Quaid e Azam University, and carried to the laboratory in sterile polythene sample bags. Samples were processed in less than 24 hours of collection to prevent contamination and degradation.

3.1.2 Sample Preparation and Extraction of Plant Metabolites

The leaves, stems and roots of the plant were separated, washed and dried up completely in the shade. These parts were grinded using the steel grinder to make the fine powder. The metabolites were extracted from roots, stem and leaves using ethyl acetate and methanol. The solvents were used in 1:10 with the powder [37]. Solvents were added to the powder and kept it macerated for 72hrs with occasional

shaking. The filtrate was collected and solvents were vaporized by using rotary evaporator and concentrated crude extract was obtained.

3.1.3 Preliminary Phytochemical Screening

Standard phytochemical screening was performed on ethyl acetate and methanol plant extracts. These extracts were tested for alkaloids, steroids, terpenoids, tannins, saponins, glycosides, proteins, free amino acids, carbohydrates, phenols and flavonoids.

These phytochemical screening tests were performed by using the method described in previous study [38].

3.2 Isolation of Endophytes and Therapeutic Applications of Its Secondary Metabolites

3.2.1 Isolation of Endophytes

Leaves, stem and roots were surface-sterilized by immersion in 70% ethanol for 1 min followed by immersion in 4% sodium hypochlorite for 5 min and 70% ethanol for 30 sec, final rinsing is done with sterile distilled water. Sterile leaf segments (1 cm^2) were placed on Potato Dextrose Agar (PDA) plates.

Plates were incubated at 28°C for 7-10 days. Emerging endophytes were subcultured to obtain pure endophytic isolates [39].

3.2.2 Preservation of Endophytes

Pure endophytic cultures were preserved in 30% glycerol stocks at -20°C and also maintained on PDA slants at 4°C for short-term use [40].

3.2.3 Molecular Identification of Endophytes

The fungal isolates were subjected to molecular identification by sequencing the internal transcribed spacer (ITS) region. PCR amplification and sequencing were performed commercially by Alpha Genomics (Islamabad, Pakistan) using universal fungal primers ITS1 and ITS4. The obtained nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI). Distance trees were generated using the NCBI Distance Tree of Results based on the Neighbor-Joining algorithm to examine the genetic relationships between the isolates and closely related reference sequences [41].

3.2.4 Fermentation and Extraction of Fungal Metabolites

Endophytes were cultured in 500 mL flasks containing 200 mL of Potato Dextrose Broth (PDB) for 21-28 days at 28°C under static conditions. Culture filtrates were extracted with ethyl acetate and methanol in a 1:1 using a separating funnel. The organic layer was concentrated using a rotary evaporator, and dried crude extract was stored at room temperature [42, 43].

3.3 Therapeutic Potential

The therapeutic potential of plant metabolites and endophytic secondary metabolites was analyzed using the crude extracts. The secondary metabolites of endophytes were extracted using Ethyl Acetate (EtOAc) and Biomass was extracted using Methanol (MeOH). The samples used for in vitro activities were given in the Table 3.1:

Table 3.1: Plant and Endophytic Extracts Samples

Endophytes		**Samples**
Biomass	FBL1	S1
	FBS1	S2

Table 3.1 continued from previous page

Endophytes		**Samples**
	FBR1	S3
	FBS2	S4
Secondary Metabolites	FBR1	S5
	FBL1	S6
	FBS1	S7
	FBS2	S8
Plant Part		**Samples**
Methanol Extract	Root	S9
	Stem	S10
	Leaves	S11
Ethyl Acetate Extract	Root	S12
	Stem	S13
	Leaves	S14

3.3.1 In Vitro Antibacterial Activity

3.3.1.1 Bacterial Strains

i. *Escherichia coli*

ii. *Staphylococcus aureus*

3.3.1.2 Method

i. **Disc Diffusion Method**

(a) Prepared AM19 media plates and inoculated them with bacterial suspensions standardized to 0.5 McFarland ($\approx 1 \times 10^8$ CFU/mL) using sterile cotton swabs.

(b) Sterile 6 mm filter paper discs were soaked with 20 μL of extract solution.

(c) Placed the discs on freshly inoculated AM19 plates.

(d) Incubated the plates at 37 °C for 24 hours.

(e) Measured the zones of inhibition and compared them with standard antibiotics [44].

3.3.2 In Vitro Antifungal Activity

3.3.2.1 Fungal Strains

i. *Saccharomyces cerevisiae*

3.3.2.2 Method

i. **Disc Diffusion Method**

(a) Prepared AM19 media plates and inoculated them with fungal spores or yeast suspension (0.5 McFarland standard).

(b) Sterile 6 mm filter paper discs were soaked with 20 μL of endophytic extract at the desired concentration.

(c) Placed the discs on freshly inoculated AM19 plates with the strain of *Saccharomyces cerevisiae*.

(d) Incubated the plates at 28–30 °C for 48–72 hours.

(e) After incubation, measured the zones of inhibition around the discs in millimeters using a caliper.

(f) Compared the results with positive controls (e.g., Nystatin 50 μg/mL) to assess antifungal activity.

3.3.3 In-Vivo Anti-inflammatory Activity

The in vivo anti-inflammatory activity of the methanolic extract of *Berberis lycium* was evaluated using the carrageenan-induced paw edema model in mice, following the method described in previous studies with slight modifications [29].

Male BALB/c mice weighing 25-30 g were acclimatized under standard laboratory conditions with free access to food and water before the experiment. The animals were randomly divided into six groups (n = 6).

Group I served as the normal control and received normal saline. Group II served as the negative control and received 1% carrageenan only.

Group III received diclofenac sodium (20 mg/kg, i.p) as the standard drug. Groups IV, V and VI received the methanolic extract of *Berberis lycium* roots at doses of 10 mg/kg, 30 mg/kg and 50 mg/kg body weight, respectively, by i.p injection.

One hour after treatment, acute inflammation was induced by injecting 0.1 mL of 1% w/v carrageenan solution into the subplantar region of the right hind paw of each rat.

Paw thickness was measured before carrageenan injection (0 h) and at 1, 4, and 6 hours after carrageenan administration using a digital Vernier caliper. The increase in paw thickness was considered as the degree of edema.

The percentage inhibition of paw edema was calculated using the following formula:

$$\text{Percentage Inhibition} = \frac{C - T}{C} \times 100$$

where,

C Mean paw edema of the negative control group.

T Mean paw edema of the treated group at the corresponding time point.

The results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-way analysis of variance (ANOVA) with GraphPad Prism (version 11.0.2). Differences were considered statistically significant at $p < 0.05$.

3.4 In Silico Studies on Known Phytochemicals From *Berberis Lycium*

3.4.1 In Silico Studies

The in silico approach allows prediction of the therapeutic potential of compounds produced by *Berberis Lycium*. The list of known phytochemicals of *Berberis lycium* was obtained from literature. The 3D structures of these compounds were downloaded from Pubchem. These ligands were then docked against alpha amylase and dipetidyl peptidase IV (DPP4) protiens for anti-diabetic potential of these compounds. The following workflow and tools were used:

3.4.1.1 Protein Data Bank

The Protein Data Bank (PDB) is an internationally recognized repository that provides experimentally determined 3D structures of biological macromolecules such as proteins, nucleic acids, and enzyme complexes. The 3D structures of human alpha amylase and dipetidyl peptidase IV (DPP4) proteins were retrieved from the PDB database. The PDB IDs were selected based on their resolution and with better PDB Validation. The PDB ID selected for human alpha amylase is 3BAI and 1NU6 for dipetidyl peptidase IV (DPP4) protein.

3.4.1.2 PubChem

PubChem is a publically accessible chemical database maintained by the National Center for Biotechnology Information (NCBI). It contains detailed information regarding chemical compounds, including molecular structures, physicochemical properties, biological activities, and three-dimensional conformations. All known ligand structures of *Berberis Lycium* were obtained from PubChem and downloaded as 3D Confirmation in SDF format.

3.4.1.3 ChemDraw Ultra 12.0

ChemDraw Ultra is a widely used software designed for generating accurate two-dimensional representations of chemical compounds. In the present study, ChemDraw Ultra 12.0 was utilized to draw the structures of phytochemicals that were unavailable in public databases. The software provides an efficient platform for generating chemically correct molecular structures and supports conversion into different file formats compatible with molecular modeling applications.

3.4.1.4 Chem3D Pro

Chem3D is a molecular visualization and modeling software used for converting two-dimensional chemical structures into optimized three-dimensional conformations. Ligand structures drawn on ChemDraw Ultra 12.0 were converted into 3D conformations. Geometry optimization, and energy minimization were performed to prepare ligands for docking.

3.4.1.5 Discovery Studio

Discovery Studio is a comprehensive bioinformatics and molecular modeling platform used for protein visualization, structure preparation, and interaction analysis. Target proteins were prepared using discover studio by removing water molecules, previously bound ligands, and other heteroatoms. Proteins were converted into .pdb format, ready for docking simulations.

3.4.1.6 PyRx

PyRx is an open-source virtual screening software that integrates AutoDock and AutoDock Vina for molecular docking studies. Ligand and protein files were imported into PyRx. Molecular docking is performed to predict the binding affinity and interactions of compounds with the target proteins. Virtual screening can be

performed if multiple compounds were tested simultaneously. Docked conformations were visualized, and binding poses, hydrogen bonds, hydrophobic interactions, and binding energies were analyzed.

3.4.1.7 Schrödinger Molecular Docking Suite

The Schrödinger Suite is an advanced computational chemistry and molecular modeling platform widely used in drug discovery and molecular docking studies. In the present study, Schrödinger was used for protein preparation, ligand optimization, receptor grid generation, and molecular docking using the Glide module. Protein preparation was carried out using the **Protein Preparation Module** in Maestro, where missing hydrogen atoms were added, bond orders were assigned, water molecules were removed, and the protein structure was optimized. Ligand preparation was performed using **LigPrep** to generate optimized 3D conformations, ionization states, stereoisomers, and energy-minimized structures. **Receptor grid generation** was carried out by taking the whole protein in the grid box. Molecular docking was performed using **Glide** in Standard Precision (SP) mode to evaluate ligand–protein interactions and docking scores. The docked complexes were analyzed for hydrogen bonding, hydrophobic interactions, π–π stacking, and binding orientations within the active site of the target proteins. The best docked poses were selected based on Glide score, binding interactions, and docking conformation stability.

3.4.1.8 Physicochemical Evaluation

The top 3 compounds are selected on the basis of docking scores both in PyRx and Schrodinger. ADMET studies were performed on these top 5 compounds for alpha amylase and DPP4 protein as well. The chosen compounds were subjected to ADMET Profiling using the Swiss ADME software developed by the SIB Swiss Institute of Bioinformatics for predicting ADME and drug-likeness properties of small molecules. It provides physicochemical, pharmacokinetic (GI absorption, BBB permeability, CYP inhibition), and medicinal chemistry parameters along

with bioavailability prediction. These parameters were evaluated extensively at physiological pH of 7.4. The toxicity studies were performed using ProTox-3.0 which is a web-based toxicity prediction platform that uses machine learning and similarity-based approaches to evaluate compound safety. It predicts acute oral toxicity, organ toxicity, toxicity endpoints, and toxicity pathways for early-stage drug safety assessment.

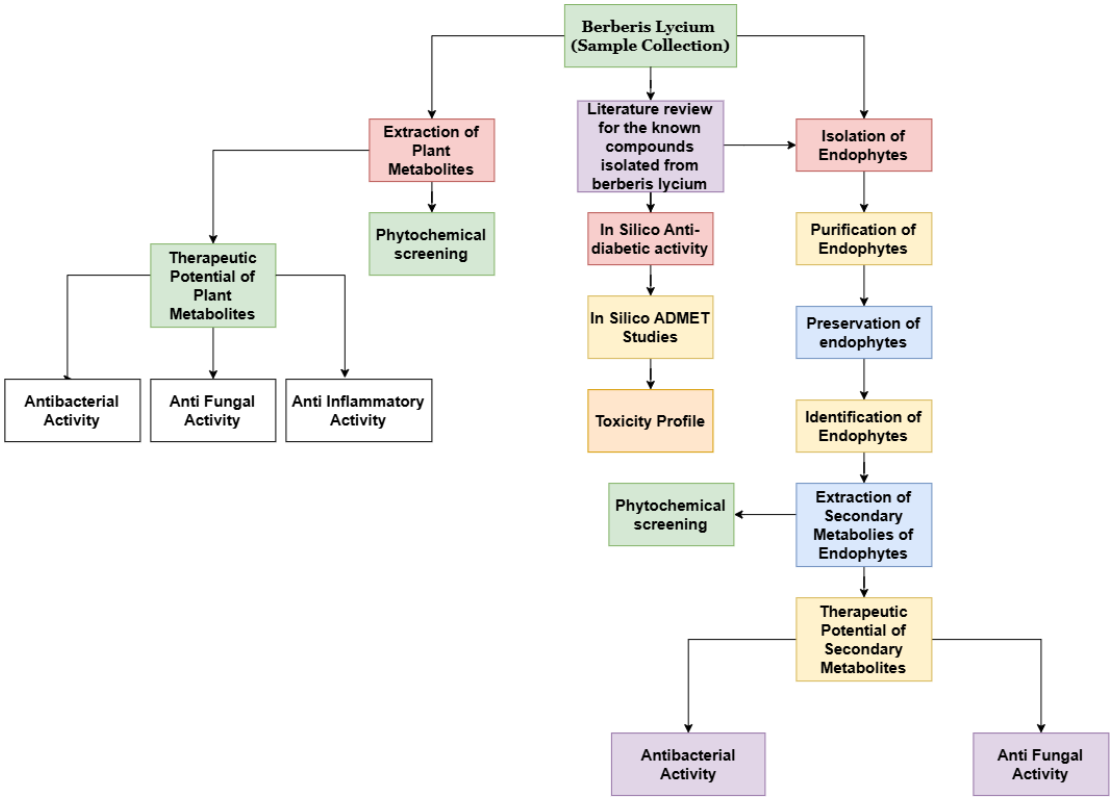


FIGURE 3.1: Flow chart of methodology of present study

Chapter 4

Results

4.1 Extraction Yield of *Berberis lycium* Extract

The percentage yield of ethyl acetate and methanolic extracts of *Berberis lycium* was calculated based on the dry weight of the crude extract obtained after solvent evaporation. Among the ethyl acetate extracts, the leaves produced the highest extraction yield (4.97%), followed by the stem (1.90%) and roots (1.73%). Similarly, the methanolic extracts showed the highest yield from the leaves (23.20%), followed by the roots (7.03%) and stem (3.80%). The results of extraction yield of *Berberis lycium* extracts are summarized in Table 4.1.

Overall, methanol produced substantially higher extraction yields than ethyl acetate for all plant parts. The highest extraction yield was observed for the methanolic leaf extract, indicating that the leaves contain a greater amount of methanol-soluble extractable constituents than the stem and roots. These constituents may include both secondary metabolites and other soluble plant components such as carbohydrates, pigments, proteins, and organic compounds.

TABLE 4.1: Extraction Yield of *Berberis lycium* extracts

Solvent	**Plant Part**	**Weight of Plant Material (g)**	**Weight of Dried Extract (g)**	**Percentage Yield (%)**
Ethyl acetate	Stem	20	0.38	1.90

Table 4.1 continued from previous page

Solvent	Plant Part	Weight of Plant Material (g)	Weight of Dried Extract (g)	Percentage Yield (%)
Ethyl acetate	Root	30	0.52	1.73
Ethyl acetate	Leaves	30	1.49	4.97
Methanol	Stem	20	0.76	3.80
Methanol	Root	30	2.11	7.03
Methanol	Leaves	30	6.96	23.20

4.2 Phytochemical Screeing of the Extracts

The phytochemical screening of the leaf, stem, and root extracts revealed notable differences in the distribution of secondary metabolites among the plant parts and extraction solvents as shown in table 4.2. No detectable phytochemical constituents were observed in either the methanolic or ethyl acetate extract of leaves. In contrast, the stem extracts contained alkaloids and steroids in both solvent systems, while flavonoids were detected only in the ethyl acetate extract.

The root extracts exhibited the richest phytochemical composition, showing the presence of alkaloids, steroids, terpenoids, carbohydrates, flavonoids, and proteins. Several important phytochemical groups, including saponins, tannins, glycosides, phenols, and free amino acids, were absent in all tested extracts.

These findings suggest that the roots serve as the primary reservoir of bioactive compounds and may be largely responsible for the biological activities observed in subsequent analysis.

TABLE 4.2: Phytochemical Screeing of the Plant Extracts

Tests	Leaf		Stem		Roots	
	Methanol Extract	Ethyl Acetate	Methanol Extract	Ethyl Acetate	Methanol Extract	Ethyl Acetate
Alkaloids	-	-	+	+	+	+
Steroids	-	-	+	+	+	+
Terpenoids	-	-	-	-	-	+

Table 4.2 continued from previous page

Tests	**Leaf**		**Stem**		**Roots**	
	Methanol Extract	**Ethyl Acetate**	**Methanol Extract**	**Ethyl Acetate**	**Methanol Extract**	**Ethyl Acetate**
Saponins	-	-	-	-	-	-
Tannins	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-
Phenols	-	-	-	-	-	-
Carbohydrates	-	-	-	-	+	+
Flavonoids	-	-	-	+	-	+
Proteins	-	-	-	-	-	+
Free amino acids	-	-	-	-	-	-

4.3 Endophytes

4.3.1 Isolation of Endophytes

4 endophytic fungi have been isolated from *Berberis lycium*, which are FBS1, FBS2, FBL1 and FBR1 as shown in the figure 4.1, figure 4.2, figure 4.3 and figure 4.4.

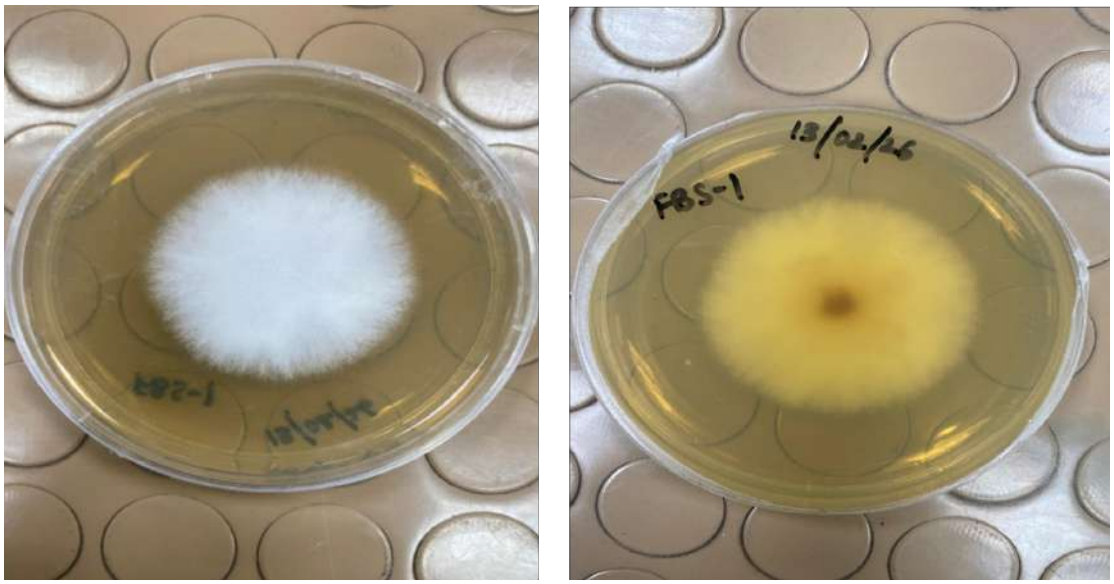


FIGURE 4.1: Endophytic fungi FBS1

FIGURE 4.2: Endophytic fungi FBS2

FIGURE 4.3: Endophytic fungi FBL1

FIGURE 4.4: Endophytic fungi FBR1

4.3.2 Identification of Endophytes

A total of seven endophytes were isolated from different tissues of *Berberis lycium*, comprising three bacterial isolates and four fungal isolates. The fungal isolates were designated as FBL1 (leaf), FBS1 (stem), FBS2 (stem), and FBR1 (root). The nucleotide sequences of these isolates were obtained in FASTA files and subjected to BLAST analysis, which identified isolate FBL1 as *Epicoccum latusicollum*, isolate FBS1 as *Fusarium oxysporum*, and isolate FBS2 as *Fusarium incarnatum*. The phylogenetic trees obtained through the NCBI Distance Tree of Results based on the Neighbor-Joining algorithm of isolate FBL1 demonstrated that it clustered closely with *Epicoccum latusicollum*, with 97% query cover and 98.87% percentage identification, confirming its molecular identification (Figure 4.5). Similarly, isolate FBS1 clustered with *Fusarium oxysporum*, with 99% query cover and 99.08% percentage identification (Figure 4.6), whereas isolate FBS2 grouped with *Fusarium incarnatum*, with 96% query cover and 99.62% percentage identification (Figure 4.7). The clustering patterns observed in the distance trees were consistent with the BLAST sequence similarity results and supported the taxonomic assignment of these fungal isolates.

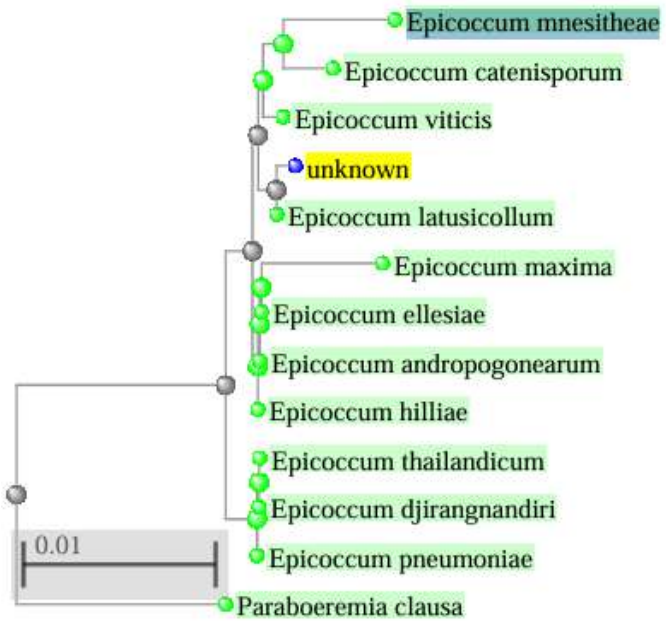


FIGURE 4.5: Neighbour-Joining phylogenetic tree based on ITS sequences showing the relationship of isolate FBL1 with closely related Epicoccum species.

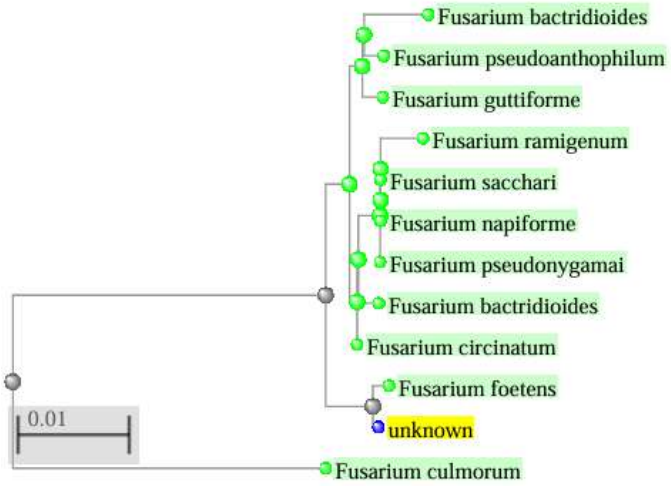


FIGURE 4.6: Neighbour-Joining phylogenetic tree based on ITS sequences showing the relationship of isolate FBS1 with closely related Fusarium oxysporum strains.

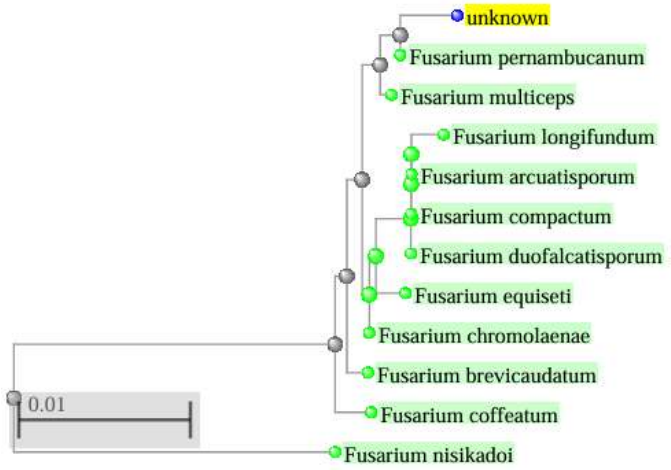


FIGURE 4.7: Neighbour-Joining phylogenetic tree based on ITS sequences showing the relationship of isolate FBS2 with closely related Fusarium incarnatum strains

The root isolate FBR1 did not yield a reliable ITS sequence suitable for species-level identification; therefore, molecular identification and phylogenetic analysis could not be performed for this isolate.

4.3.3 Fermentation and Extraction of Fungal Metabolites

The yields of biomass (BM) and secondary metabolite (SM) extracts obtained from the endophytic fungal isolates are presented in Table 4.3. Among the biomass extracts, FBS2 produced the highest yield (880 mg/L), followed by FBL1 (660

mg/L), FBS1 (520 mg/L), and FBR1 (460 mg/L). In contrast, the highest yield of secondary metabolite extract was obtained from FBL1 (320 mg/L), followed by FBS1 (180 mg/L), FBR1 (140 mg/L), and FBS2 (120 mg/L). Overall, the results showed that biomass yield and secondary metabolite yield varied among the fungal isolates, with FBS2 producing the highest biomass, while FBL1 produced the greatest amount of secondary metabolites.

TABLE 4.3: Yields of biomass extracts and secondary metabolites extract of endophytic fungi

Strains	**Yield of Biomass Extract (mg/L)**	**Yield of Secondary Metabolite Extract (mg/L)**
FBL1	660	320
FBS1	520	180
FBS2	880	120
FBR1	460	140

4.3.4 Phytochemical Screening of Fungal Metabolites

The qualitative phytochemical screening of the biomass (BM) and secondary metabolite (SM) extracts of the fungal endophytes showed clear differences in the phytochemical content among the isolates, as shown in Table 4.4. FBL1 contained only steroids and terpenoids in both extracts. FBR1 showed the highest number of phytochemicals, including alkaloids, steroids, terpenoids, phenols, flavonoids, and carbohydrates. FBS1 contained alkaloids and steroids in both extracts, while terpenoids were detected only in the biomass extract.

Similarly, FBS2 contains alkaloids, phenols and flavonoids in both extracts where as terpenoids were found only in the secondary metabolite extract. Glycosides, saponins, tannins, proteins and free amino acids were not detected in any of the fungal extracts.

Overall, FBR1 showed the richest phytochemical profile, indicating that it may have a greater ability to produce bioactive secondary metabolites than the other fungal isolates.

TABLE 4.4: Phytochemical Screeing of Endophytic Crude Extracts including biomass extracts and secondary metabolite extracts of FBL1, FBR1, FRS1 and FBS2.

Tests	**FBL1**		**FBR1**		**FBS1**		**FBS2**	
	BM Extract	**SM Extract**	**BM Extract**	**SM Extract**	**BM Extract**	**SM Extract**	**BM Extract**	**SM Extract**
Alkaloids	-	-	+	-	+	+	+	+
Steroids	+	+	+	-	+	+	-	-
Terpenoids	+	+	+	-	+	-	-	+
Saponins	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-	-	-
Phenols	-	-	+	+	-	-	+	+
Carbohydrate	-	-	-	+	-	-	-	-
Flavonoids	-	-	+	+	-	-	+	+
Proteins	-	-	-	-	-	-	-	-
Free amino acids	-	-	-	-	-	-	-	-

4.4 Therapeutic Potential

4.4.1 In Vitro Antibacterial Assay

The antibacterial activity of fungal biomass extracts, fungal secondary metabolite extracts and *Berberis lycium* plant extracts were tested against *Escherichia coli* and *Staphylococcus aureus* using the agar disc diffusion assay at concentrations of 2000, 1000, and 500 μg/disc. Ciprofloxacin (100 μg/disc) served as the positive control.

The zone of inhibition was measured in millimeters (mm), and the results were presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using ordinary two-way ANOVA followed by Tukey's multiple comparisons test, with comparisons made against the positive control.

Against *Escherichia coli*, inhibition zones ranged from 5.59 to 8.48 mm (Table 4.5). The biomass extract of FBL1 (S1) exhibited no detectable antibacterial activity at any tested concentration. In contrast, all remaining extracts produced measurable

zones of inhibition. Overall, fungal secondary metabolite extracts demonstrated greater antibacterial activity than the corresponding fungal biomass extracts.

Among the fungal extracts, the secondary metabolite extract of FBS2 (S8) produced the largest inhibition zone (8.40 ± 0.20 mm) at 2000 μg/disc, followed by the secondary metabolite extract of FBL1 (S6) (8.37 ± 0.15 mm) and the secondary metabolite extract of FBR1 (S5) (8.31 ± 0.25 mm). Several of these treatments exhibited significant differences compared with the positive control, as indicated in Table 4.5.

Amongst the plant extracts, the methanolic root extract (S9) showed the greatest antibacterial activity, producing zone of inhibition of 8.48 ± 0.30 mm at 2000 μg/disc. The methanolic leaf extract (S11) also demonstrated strong activity with the largest zone of inhibition of 8.43 ± 0.30 mm at 500 μg/disc. Similarly, the ethyl acetate stem (S13) and ethyl acetate root (S12) extracts produced maximum zone of inhibition of 8.22 ± 0.15 mm and 8.21 ± 0.35 mm, respectively. Significant differences relative to the positive control were observed for several plant extracts particularly at the higher concentrations.

The fungal biomass extracts generally exhibited lower antibacterial activity, with inhibition zones ranging from 6.62 ± 0.30 to 7.86 ± 0.30 mm, except for FBL1 (S1), which remained inactive throughout the experiment. Although greater inhibition was frequently observed at 2000 μg/disc, a consistent concentration-dependent trend was not evident for all extracts.

Against *Staphylococcus aureus*, all tested extracts demonstrated measurable antibacterial activity with zones of inhibition ranging from 6.68 to 9.15 mm (Table 4.6). Interestingly, the biomass extract of FBL1 (S1), which was inactive against *Escherichia coli*, exhibited the highest antibacterial activity against *S. aureus*, producing inhibition zones of 9.04 ± 0.25, 9.15 ± 0.20, and 8.66 ± 0.30 mm at 2000, 1000, and 500 μg/disc, respectively. These values are significantly different from those of the positive control.

Among the fungal secondary metabolite extracts, FBS1 (S7) produced the highest inhibition zone (8.19 ± 0.25 mm), followed by FBS2 (S8) (8.13 ± 0.20 mm).

The plant extracts also demonstrated appreciable antibacterial activity. The methanolic leaf extract (S11) exhibited the greatest inhibition (8.47 ± 0.30 mm), followed by the ethyl acetate root extract (S12) (8.42 ± 0.20 mm), ethyl acetate stem extract (S13) (8.34 ± 0.35 mm), and methanolic stem extract (S10) (8.23 ± 0.15 mm). Several of these treatments showed statistically significant differences compared with ciprofloxacin, as indicated in Table 4.6.

Comparison of the antibacterial activity against both bacterial strains demonstrated that the efficacy of the extracts depended on the target microorganism. *Escherichia coli* was generally more susceptible to the fungal secondary metabolite extracts and the methanolic root extract, whereas *Staphylococcus aureus* exhibited greater susceptibility to the biomass extract of FBL1 and several plant extracts. Notably, the biomass extract of FBL1 displayed a distinct strain-specific response, showing no detectable activity against *Escherichia coli* while producing the greatest inhibition against *S. aureus*.

This difference may reflect variations in bacterial cell wall structure, intrinsic susceptibility, and the spectrum of bioactive metabolites present in the fungal and plant extracts.

Overall, fungal secondary metabolite extracts demonstrated superior antibacterial activity compared with the corresponding fungal biomass extracts, suggesting that antimicrobial constituents are predominantly synthesized during secondary metabolism. Likewise, both methanolic and ethyl acetate extracts of *B. lycium*, particularly those prepared from the roots and leaves, exhibited considerable antibacterial potential.

Although higher extract concentrations generally produced larger inhibition zones, a consistent dose-dependent response was not observed across all treatments, indicating that the diffusion behaviour of crude phytochemical mixtures and possible interactions among their constituents may influence antibacterial activity in the agar disc diffusion assay.

These findings demonstrate that both fungal secondary metabolites and *Berberis lycium* extracts represent promising natural sources of antibacterial compounds

and warrant further phytochemical characterization and isolation of their active constituents.

TABLE 4.5: Antibacterial assay results against *Escherichia coli* expressed as Mean ± S.D (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. Asterisks indicate significant differences compared with the positive control (ciprofloxacin, 100 μg/disc). P values are indicated as: ns, not significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Samples	**Sample Description**	**C1 (2000 μg/ml)**	**C2 (1000 μg/ml)**	**C3 (500 μg/ml)**	**Control Ciprofloxacin (100 μg/ml)**
S1	Biomass extract of FBL1	–	–	–	7.14 ± 0.25
S2	Biomass extract of FBS1	$*7.86 \pm 0.30$	7.22 ± 0.25	7.20 ± 0.35	7.15 ± 0.20
S3	Biomass extract of FBR1	$*7.77 \pm 0.20$	6.81 ± 0.35	$*7.76 \pm 0.25$	7.27 ± 0.30
S4	Biomass extract of FBS2	$***6.62 \pm 0.30$	$*6.83 \pm 0.20$	$*6.79 \pm 0.15$	5.59 ± 0.35
S5	Secondary metabolites extract of FBR1	$*8.31 \pm 0.25$	7.69 ± 0.30	7.68 ± 0.20	7.52 ± 0.15
S6	Secondary metabolites extract of FBL1	$***8.37 \pm 0.15$	$**7.89 \pm 0.25$	7.75 ± 0.35	7.40 ± 0.20
S7	Secondary metabolites extract of FBS1	$*8.12 \pm 0.35$	8.02 ± 0.20	$*7.92 \pm 0.25$	7.68 ± 0.30
S8	Secondary metabolites extract of FBS2	$****8.40 \pm 0.20$	$****8.36 \pm 0.30$	$*7.54 \pm 0.15$	6.77 ± 0.25
S9	MeOH extract of Root	$****8.48 \pm 0.30$	$*8.33 \pm 0.15$	7.16 ± 0.25	6.91 ± 0.35
S10	MeOH extract of Stem	7.26 ± 0.25	$*7.77 \pm 0.30$	$** 7.70 \pm 0.20$	7.09 ± 0.15
S11	MeOH extract of Leaves	$***7.79 \pm 0.20$	$**7.97 \pm 0.35$	$****8.43 \pm 0.30$	6.72 ± 0.25
S12	EtOAc extract of Root	$****8.21 \pm 0.35$	$*** 7.96 \pm 0.25$	$** 7.76 \pm 0.20$	6.83 ± 0.30

Table 4.5 continued from previous page

Samples	**Sample Description**	**C1 (2000 μg/ml)**	**C2 (1000 μg/ml)**	**C3 (500 μg/ml)**	**Control Ciprofloxacin (100 μg/ml)**
S13	EtOAc extract of Stem	$***8.22 \pm 0.15$	$*7.61 \pm 0.30$	$*8.07 \pm 0.35$	7.13 ± 0.20
S14	EtOAc extract of Leaves	$*6.57 \pm 0.30$	$*7.67 \pm 0.20$	$** 8.09 \pm 0.25$	7.03 ± 0.35

TABLE 4.6: Antibacterial assay results against Staphylococcus aureus expressed as Mean ± S.D (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. Asterisks indicate significant differences compared with the positive control (ciprofloxacin, 100 μg/disc). P values are indicated as: ns, not significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Samples	**Sample Description**	**C1 (2000 μg/ml)**	**C2 (1000 μg/ml)**	**C3 (500 μg/ml)**	**Control Ciprofloxacin (100 μg/ml)**
S1	Biomass extract of FBL1	$****9.04 \pm 0.25$	$****9.15 \pm 0.20$	$****8.66 \pm 0.30$	6.68 ± 0.15
S2	Biomass extract of FBS1	7.44 ± 0.35	8.07 ± 0.25	8.13 ± 0.20	7.65 ± 0.30
S3	Biomass extract of FBR1	7.97 ± 0.15	7.85 ± 0.30	7.79 ± 0.25	8.02 ± 0.20
S4	Biomass extract of FBS2	7.99 ± 0.40	7.60 ± 0.35	$** 7.35 \pm 0.20$	8.09 ± 0.25
S5	Secondary metabolites extract of FBR1	7.81 ± 0.20	7.75 ± 0.15	7.56 ± 0.35	7.96 ± 0.30
S6	Secondary metabolites extract of FBL1	$**6.80 \pm 0.30$	$*7.05 \pm 0.25$	7.54 ± 0.15	7.62 ± 0.20
S7	Secondary metabolites extract of FBS1	6.90 ± 0.10	7.71 ± 0.30	$*** 8.19 \pm 0.25$	7.22 ± 0.35
S8	Secondary metabolites extract of FBS2	7.42 ± 0.35	$*** 8.13 \pm 0.20$	7.14 ± 0.40	7.26 ± 0.15

Table 4.6 continued from previous page

Samples	Sample Description	C1 (2000 μg/ml)	C2 (1000 μg/ml)	C3 (500 μg/ml)	Control Ciprofloxacin (100 μg/ml)
S9	MeOH extract of Root	7.93 ± 0.25	7.89 ± 0.30	7.77 ± 0.20	7.80 ± 0.25
S10	MeOH extract of Stem	$****8.23 \pm 0.15$	$****8.07 \pm 0.35$	$****7.97 \pm 0.30$	6.68 ± 0.20
S11	MeOH extract of Leaves	$**8.47 \pm 0.30$	$*8.25 \pm 0.15$	7.97 ± 0.25	7.65 ± 0.35
S12	EtOAc extract of Root	$*8.42 \pm 0.20$	8.37 ± 0.25	8.08 ± 0.35	7.84 ± 0.15
S13	EtOAc extract of Stem	$****8.34 \pm 0.35$	$****8.19 \pm 0.30$	$*7.49 \pm 0.20$	6.84 ± 0.25
S14	EtOAc extract of Leaves	$****7.78 \pm 0.25$	$****7.86 \pm 0.20$	7.21 ± 0.30	6.67 ± 0.40

4.4.2 In Vitro Antifungal Assay

The antifungal activity of fungal biomass extracts, fungal secondary metabolite extracts, and *Berberis lycium* plant extracts was evaluated against *Saccharomyces cerevisiae* using the agar disc diffusion assay at concentrations of 2000, 1000, and 500 μg/disc. Nystatin (10,000 IU/mL) served as the positive control. Antifungal activity was determined by measuring the diameter of the inhibition zones (mm).

The results were expressed as mean $\pm$ SD ($n = 3$), and statistical analysis was performed using ordinary two-way ANOVA followed by Tukey's multiple comparisons test, with comparisons made against the positive control.

No detectable antifungal activity was observed for the fungal biomass extracts of FBL1 (S1), FBS1 (S2), FBR1 (S3), and FBS2 (S4), the secondary metabolite extract of FBR1 (S5), the secondary metabolite extract of FBL1 (S6), or the methanolic root extract (S9), as no inhibition zones were detected at any of the tested concentrations (Table 4.7).

In contrast, the secondary metabolite extracts of FBS1 (S7) and FBS2 (S8), together with the methanolic stem (S10), methanolic leaf (S11), ethyl acetate root (S12), ethyl acetate stem (S13), and ethyl acetate leaf (S14) extracts, exhibited measurable antifungal activity against *Saccharomyces cerevisiae*. Among all tested samples, the methanolic leaf extract (S11) demonstrated the greatest antifungal activity, producing inhibition zones of 9.61 ± 0.35, 9.70 ± 0.20, and 9.26 ± 0.30 mm at 2000, 1000, and 500 μg/disc, respectively. These inhibition zones were significantly greater than those produced by the positive control at all tested concentrations (Table 4.7).

The secondary metabolite extract of FBS2 (S8) also exhibited pronounced antifungal activity, producing inhibition zones ranging from 8.53 ± 0.25 to 8.97 ± 0.20 mm. Significant differences compared with the positive control were observed at 2000 and 1000 μg/disc, whereas activity at 500 μg/disc was comparatively lower. Similarly, the ethyl acetate leaf extract (S14) demonstrated strong antifungal activity, with inhibition zones ranging from 7.92 ± 0.20 to 9.04 ± 0.25 mm, and exhibited statistically significant inhibition relative to the positive control at 1000 and 500 μg/disc.

The secondary metabolite extract of FBS1 (S7), methanolic stem extract (S10), ethyl acetate root extract (S12), and ethyl acetate stem extract (S13) displayed moderate antifungal activity, producing inhibition zones ranging from 7.11 ± 0.20 to 8.95 ± 0.15 mm. Among these, the secondary metabolite extract of FBS1 exhibited significantly greater inhibition at 500 μg/disc, while the ethyl acetate root and stem extracts demonstrated significant differences from the positive control at selected concentrations, as indicated in Table 4.7.

The positive control, nystatin, produced inhibition zones ranging from 7.09 ± 0.15 to 8.61 ± 0.20 mm under identical experimental conditions. Notably, the methanolic leaf extract (S11) consistently produced larger inhibition zones than the positive control at all tested concentrations, whereas the secondary metabolite extract of FBS2 (S8) and the ethyl acetate leaf extract (S14) also exhibited considerable antifungal activity.

Overall, the agar disc diffusion assay demonstrated substantial variation in antifungal activity among the tested extracts. Antifungal efficacy was restricted to selected fungal secondary metabolite and plant extracts, while fungal biomass extracts were largely inactive. The superior activity of the methanolic leaf extract and the notable inhibition exhibited by the secondary metabolite extract of FBS2 and the ethyl acetate leaf extract suggest that these extracts contain potent antifungal constituents. Although larger inhibition zones were generally observed at higher extract concentrations, a consistent concentration-dependent response was not evident for all extracts, indicating that diffusion characteristics and interactions among crude bioactive constituents may influence antifungal activity in the agar disc diffusion assay.

TABLE 4.7: Antifungal assay results against Saccharomyces Cerevisiae expressed as Mean ± S.D (n = 3). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. Asterisks indicate significant differences compared with the positive control (ciprofloxacin, 100 μg/disc). P values are indicated as: ns, not significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Sample	**Sample Description**	**C1 (2000 μg/ml)**	**C2 (1000 μg/ml)**	**C3 (500 μg/ml)**	**Control Nystatin (10000 IU/ml)**
S1	Biomass extract of FBL1	–	–	–	7.33 ± 0.20
S2	Biomass extract of FBS1	–	–	–	7.80 ± 0.30
S3	Biomass extract of FBR1	–	–	–	8.07 ± 0.15
S4	Biomass extract of FBS2	–	–	–	7.66 ± 0.25
S5	Secondary metabolites extract of FBR1	–	–	–	8.23 ± 0.35
S6	Secondary metabolites extract of FBL1	–	–	–	7.90 ± 0.20
S7	Secondary metabolites extract of FBS1	7.87 ± 0.30	8.26 ± 0.25	$***8.95 \pm 0.15$	7.89 ± 0.35
S8	Secondary metabolites extract of FBS2	$****8.97 \pm 0.20$	$**8.70 \pm 0.35$	$*8.53 \pm 0.25$	7.80 ± 0.15

Table 4.7 continued from previous page

Sample	Sample tion	Descrip-	C1 (2000 μg/ml)	C2 (1000 μg/ml)	C3 (500 μg/ml)	Control Nystatin (10000 IU/ml)
S9	MeOH Root	extract of	–	–	–	8.02 ± 0.30
S10	MeOH Stem	extract of	7.34 ± 0.15	7.24 ± 0.30	7.78 ± 0.20	7.76 ± 0.25
S11	MeOH Leaves	extract of	$****9.61 \pm 0.35$	$****9.70 \pm 0.20$	$****9.26 \pm 0.30$	8.09 ± 0.15
S12	EtOAc Root	extract of	$*7.96 \pm 0.25$	$****7.45 \pm 0.15$	8.49 ± 0.35	8.61 ± 0.20
S13	EtOAc Stem	extract of	8.59 ± 0.30	$*7.31 \pm 0.25$	$** 7.11 \pm 0.20$	8.02 ± 0.35
S14	EtOAc Leaves	extract of	$**7.92 \pm 0.20$	$****8.55 \pm 0.30$	$****9.04 \pm 0.25$	7.09 ± 0.15

4.4.3 In Vivo Anti-inflammatory Activity

The anti-inflammatory activity of the methanolic extract of *Berberis lycium* was evaluated using the carrageenan-induced paw edema model.

4.4.3.1 Mean Paw Thickness

The paw thickness is calculated at 0 hrs which is before giving the carrageenan injection and at 1 hr, 4hr and 6hr after the carrageenan subplanter injection. The total of 6 mice were present in each group, the mean of which are summarized in the table 4.8. The positive control group showed the greatest increase in paw thickness, with inflammation reaching maximum 4.10mm at 4 h and 3.60mm at 6 h.

TABLE 4.8: Mean Paw Thickness (mm)

Group	0 h	1 h	4 h	6 h
Negative Control	2.06	2.50	2.26	2.16
Positive Control	2.04	3.60	4.10	3.60

Table 4.8 continued from previous page

Group	0 h	1 h	4 h	6 h
Diclofenac Sodium (20 mg/kg)	2.00	3.44	2.90	2.50
Berberis lycium MeOH Extract (10 mg/kg)	2.20	3.52	3.03	2.65
Berberis lycium MeOH Extract (30 mg/kg)	2.58	3.65	3.37	3.07
Berberis lycium MeOH Extract (50 mg/kg)	2.45	3.63	3.10	2.63

4.4.3.2 Percentage inhibition of paw edema

The percentage inhibition of paw edema is calculated based on mean paw thickness value. The percentage inhibition is calculated at 4hrs and 6hrs given in the table 4.9. Diclofenac sodium (20 mg/kg) markedly reduced paw edema, showing 29.27% inhibition at 4 h and 30.56% inhibition at 6 h.

Among the extract-treated groups, the 50 mg/kg dose showed the highest anti-inflammatory activity (24.39% and 27.08% inhibition at 4 h and 6 h, respectively), followed by the 10 mg/kg dose (26.10% and 26.39% inhibition). The 30 mg/kg dose produced comparatively lower inhibition.

TABLE 4.9: Percentage inhibition of paw edema

Group	% Inhibition (4 h)	% Inhibition (6 h)
Diclofenac Sodium (20 mg/kg)	29.27	30.56
Berberis lycium MeOH Extract (10 mg/kg)	26.10	26.39
Berberis lycium MeOH Extract (30 mg/kg)	17.80	14.81
Berberis lycium MeOH Extract (50 mg/kg)	24.39	27.08

4.4.3.3 Two-way ANOVA results

Two-way ANOVA demonstrated significant differences among the groups followed by the Tuckey's multiple comparison test demonstrated in Figure 4.8.

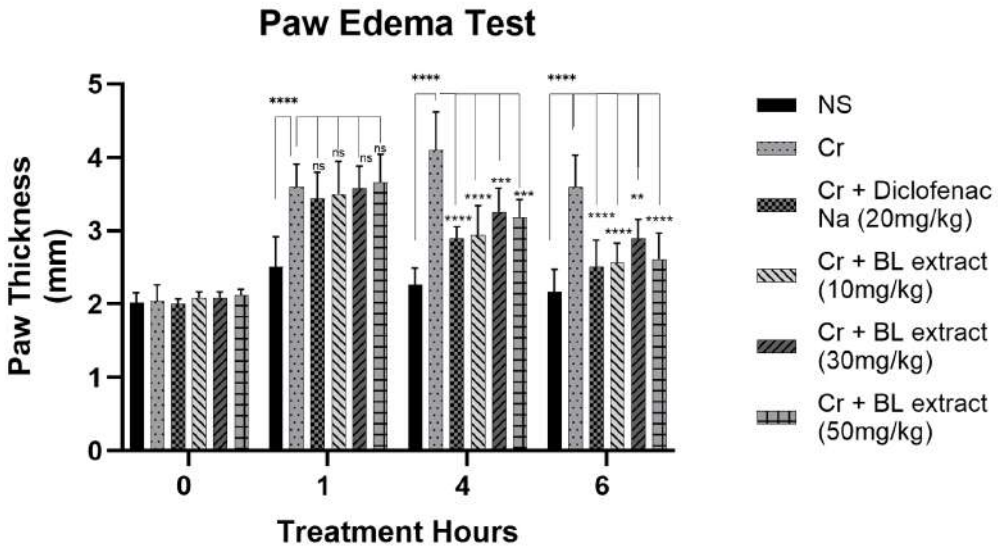


FIGURE 4.8: Anti-inflammatory effect of *Berberis lycium* extract on carrageenan induced paw edema. The results are expressed as Mean ± S.D (n = 6).It indicates extracts show promising decrease (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Overall, these findings indicate that the methanolic extract of *Berberis lycium* possesses significant anti-inflammatory activity, although its effect was slightly lower than that of diclofenac sodium.

4.5 In Silico Studies Result

The in silico investigation showed strong interactions between several identified phytochemicals and the selected antidiabetic targets.

4.5.1 DPP4 Receptor Binding

Against the DPP4 receptor, Feselol emerged as the best-performing compound in the PyRx study with score of -9.8 followed by Pelargonidin 3 rutinoside (-9.5) and Glycocholic Acid (-9.4). These compounds showed stronger binding than the reference drug Sitagliptin (-8.8) Similarly, Cyanidin-3-rutinoside exhibited the

strongest binding affinity in the Schrodinger analysis with score -11.625 indicating a highly stable ligand-receptor complex, followed by Peonidin-3- rutinoside (-9.890) and Delphinidin-3-glucoside (-9.256) as given in table 4.10.

TABLE 4.10: Docking scores of PyRx and Schrodinger against DPP4 receptor

PyRx		**Schrodinger**	
Ligand	**Binding Affinity (kcal/mol)**	**Ligand**	**Binding Affinity (kcal/mol)**
Feselol	-9.8	Cyanidin-3-rutinoside	-11.6
Pelargonidin 3 rutinoside	-9.5	Peonidin-3-rutinoside	-9.8
Glycocholic Acid	-9.4	Delphinidin-3-glucoside	-9.2
Cyanidin 3- rutinoside	-9.2	Malvidin-3,5-dihexoside	-8.7
Ginkolide B	-9.2	Cyanidin-3-lathyroside	-8.7
Peonidin 3 rutinoside	-9.1	Cyanidin-3-galactoside	-8.7
Delphindin 3-glucoside	-9.0	Pelargonidin - 3, 5 - diglucoside	-8.4
Luteone 7 - O - glucoside	-9.0	Pelargonidin-3-rutinoside	-8.1
Formononetin 7 - o - glucoside	-8.8	Carboxystrictosidine	-8.1
Sitagliptin	-8.8	Chlorogenic Acid	-7.9

4.5.2 2D Interaction Diagram for DPP4

The 2D interaction diagrams revealed detailed binding modes of the top phytochemicals with the DPP4 receptor.

4.5.2.1 Feselol

The 2d interaction diagram of Feselol binding with DPP4 receptor illustrated in Figure 4.9. Showed multiple stabilizing interactions, including conventional hydrogen bonds with residues such as ASP B501 and GLN B505, along with van der Waals contacts involving ASN B562, LEU B504, and PHE B559. Pi-Pi T-shaped and alkyl interactions further contributed to the stability of the complex, consistent with its strong PyRx binding affinity of -9.8 kcal/mol.

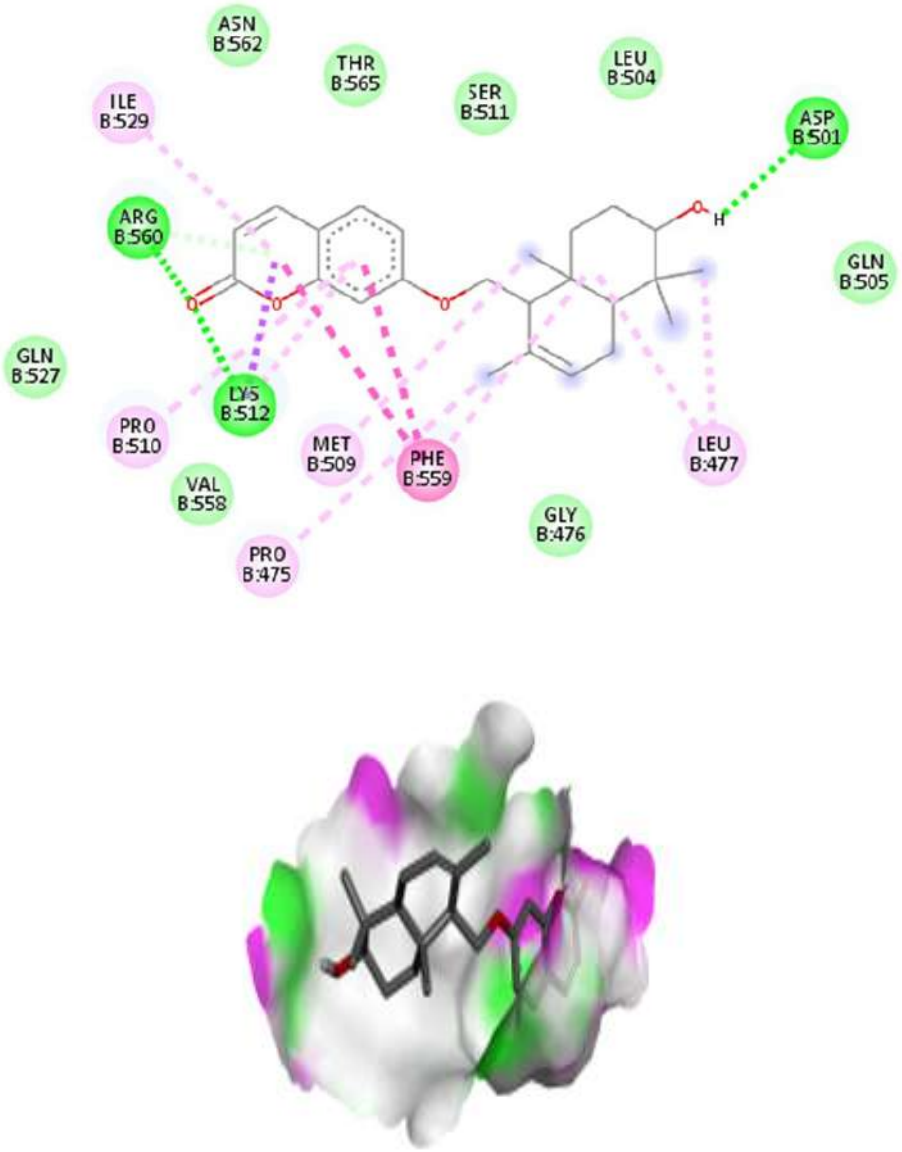


FIGURE 4.9: 2D and 3D Interaction of Feselol with DPP4

4.5.2.2 Pelargonidin 3 rutinoside

The interaction profile of Pelargonidin 3-rutinoside showed in Figure 4.10 illustrates prominent pi-pi stacking with aromatic residues such as TYR A547 and conventional hydrogen bonds with several polar amino acids including ASN A710 and GLU A205/A206. An unfavorable acceptor-acceptor interaction was noted near PHE A357; however, this was outweighed by extensive hydrophobic and hydrogen bonding networks, supporting its high ranking among the tested ligands.

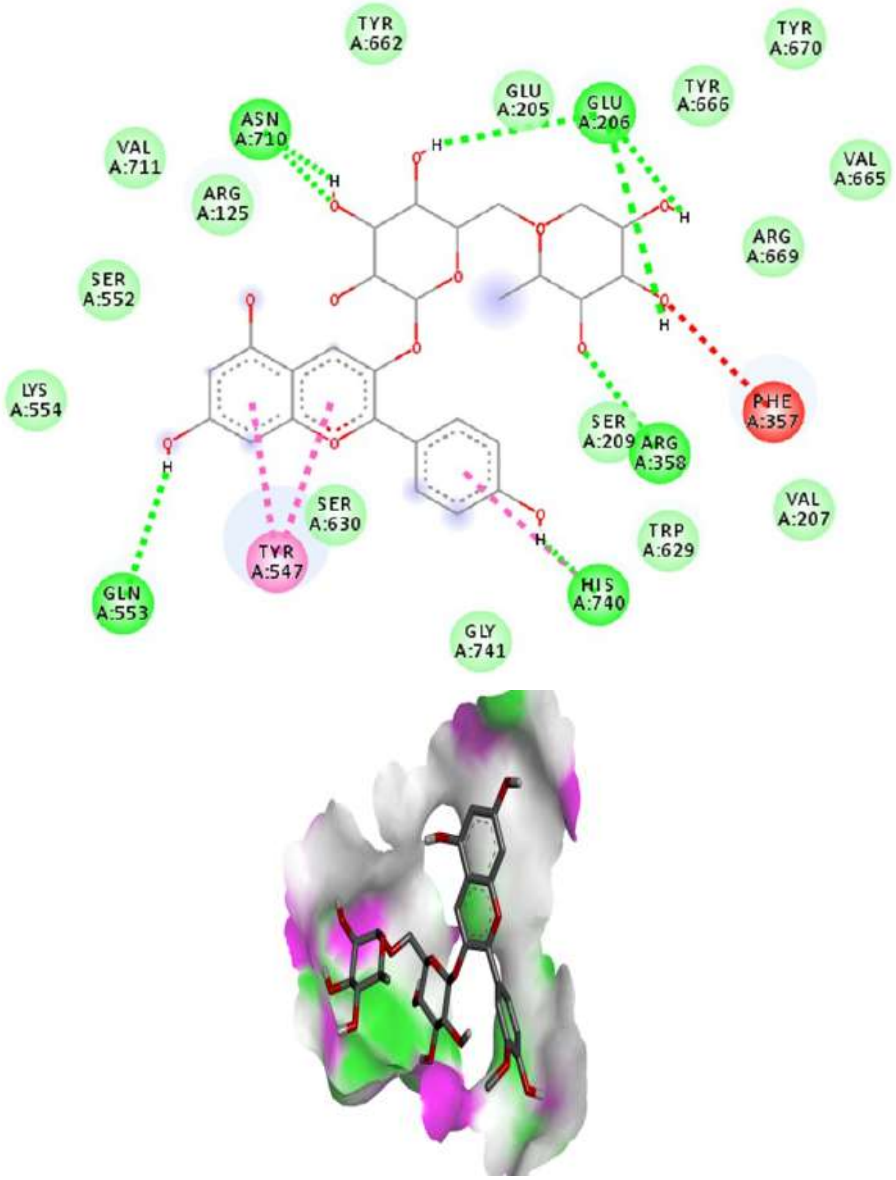


FIGURE 4.10: 2D and 3D Interaction of Pelargonidin 3 rutinoside with DPP4

4.5.2.3 Glycocholic Acid

Glycocholic Acid exhibited conventional hydrogen bonds with LYS A122, GLN A123/A153, and TRP A124 as shown in Figure 15, moreover pi-sigma and alkyl interactions are also present with PHE A240 and VAL A252 residues. These interactions highlight the compound's ability to engage both polar and hydrophobic regions of the DPP4 active site.

These interactions showed that the lead phytochemicals form highly stable complexes with DPP4 through a combination of hydrogen bonding and hydrophobic

contacts, often surpassing the reference inhibitor Sitagliptin in interaction density.

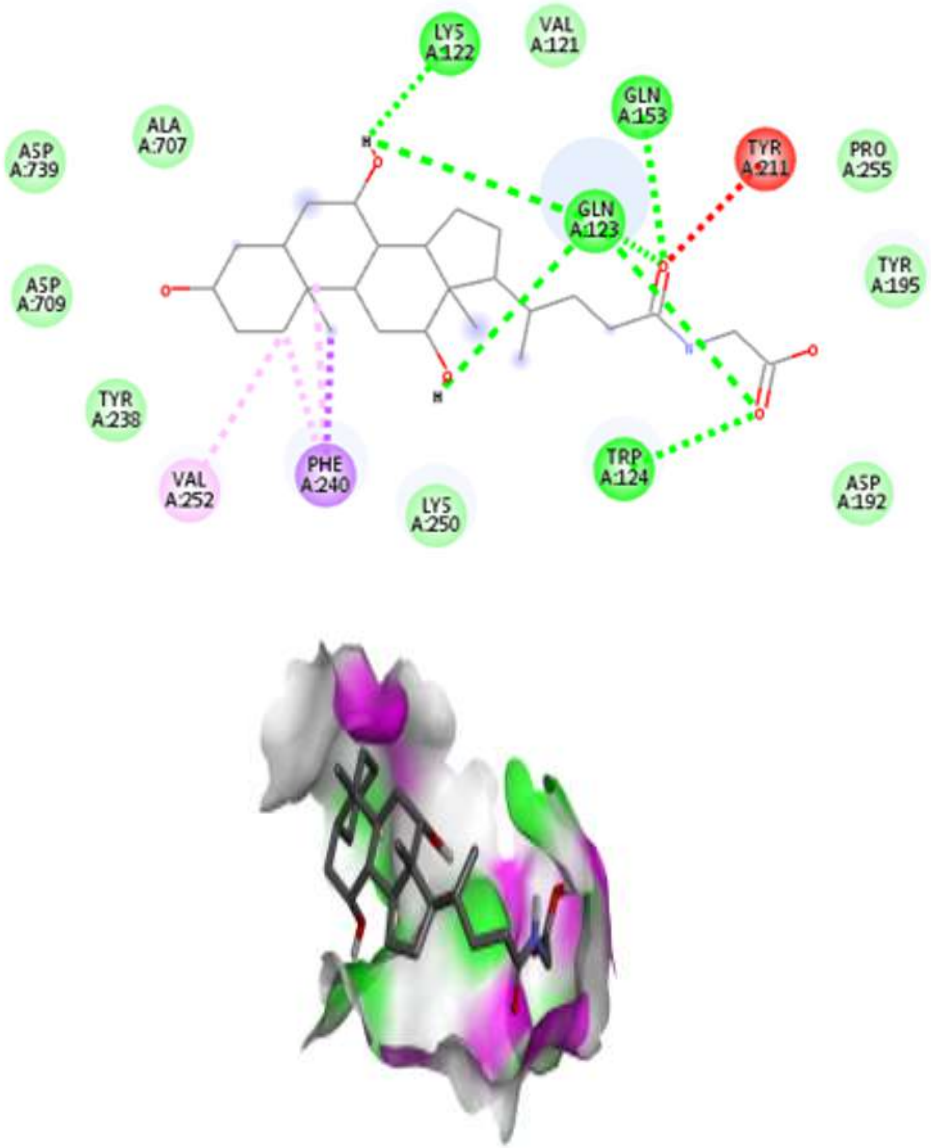


FIGURE 4.11: 2D and 3D Interaction of Glycocholic Acid with DPP4

4.5.2.4 Cyanidin-3-rutinoside

The 2D interaction diagram of Cyanidin-3-rutinoside with DPP4 is presented in Figure 16. The ligand formed hydrogen-bond interactions with residues including **GLN553, TYR662, GLU205, SER209,** and **ARG125**, along with π–π stacking with TYR547. Additional contacts with **TRP629, TYR631, ASN710,** and

VAL711 contributed to its binding within the receptor pocket. These interactions correspond with its strong Schrödinger docking score of **-11.625 kcal/mol**.

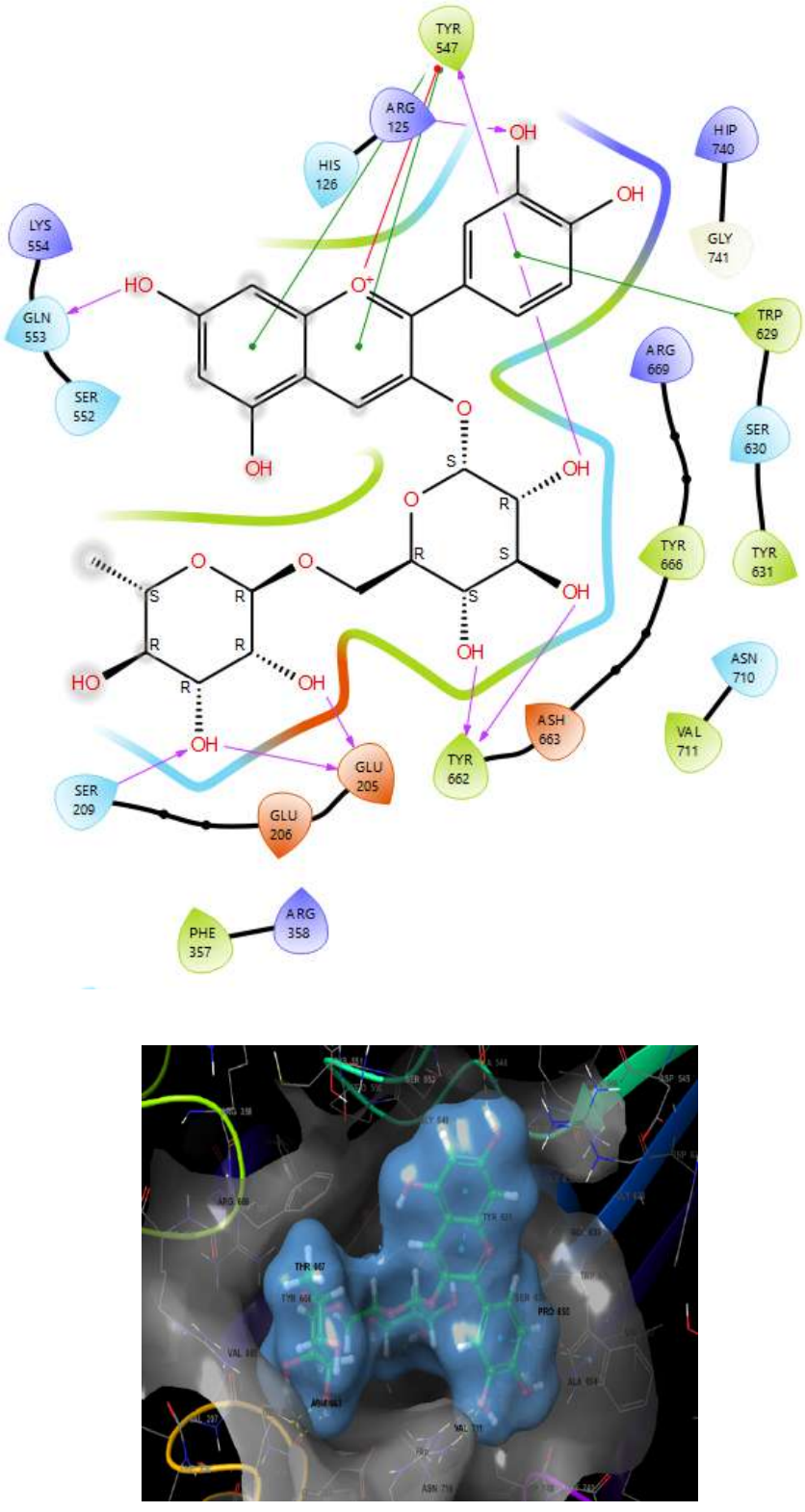


FIGURE 4.12: 2D and 3D Interaction of Cyanidin-3-rutinoside with DPP4

4.5.2.5 Peonidin-3-rutinoside

The 2D interaction diagram of **Peonidin-3-rutinoside** with the DPP4 receptor is presented in Figure 17. The ligand established hydrogen-bond interactions with residues including **ASP545, TYR547, TYR752**, and **ASP739**, along with hydrophobic contacts involving TRP629 and VAL546. These interactions indicate favorable accommodation of the ligand within the DPP4 binding pocket and support its strong predicted binding affinity of **-9.890 kcal/mol** in the Schrödinger analysis.

4.5.2.6 Delphinidin 3-rutinoside

Delphinidin 3-rutinoside formed several conventional hydrogen bonds with DPP4 residues, including **GLU117, ASP104,** and **LYS122**, which may contribute to anchoring the ligand within the binding pocket. Additional hydrophobic and pi-mediated interactions involving **TYR105, TYR120, TYR152,** and **TRP154** further supported ligand stabilization. Overall, the diverse interaction network indicates favorable accommodation of Delphinidin 3-rutinoside within the DPP4 binding site.

4.5.3 Alpha Amylase Receptor binding

For the α-amylase receptor, Camptothecin showed the top score of -7.3 in PyRx followed by again feselol (-6.9) and then by Pelargonidin (-6.8) as shown in Table 4.11. These findings provide computational evidence supporting the therapeutic potential of the studied phytochemicals in diabetes management. Cyanidin-3-rutinoside again displayed the most favorable docking score of -10.1966, followed closely by Cyanidin 3-lathyroside (-10.0195) and Pelargonidin 3,5-diglucoside (-9.58612). The consistently strong interactions observed for anthocyanin-based compounds suggest that they may contribute significantly to antidiabetic activity by inhibiting both DPP4 and α-amylase enzymes

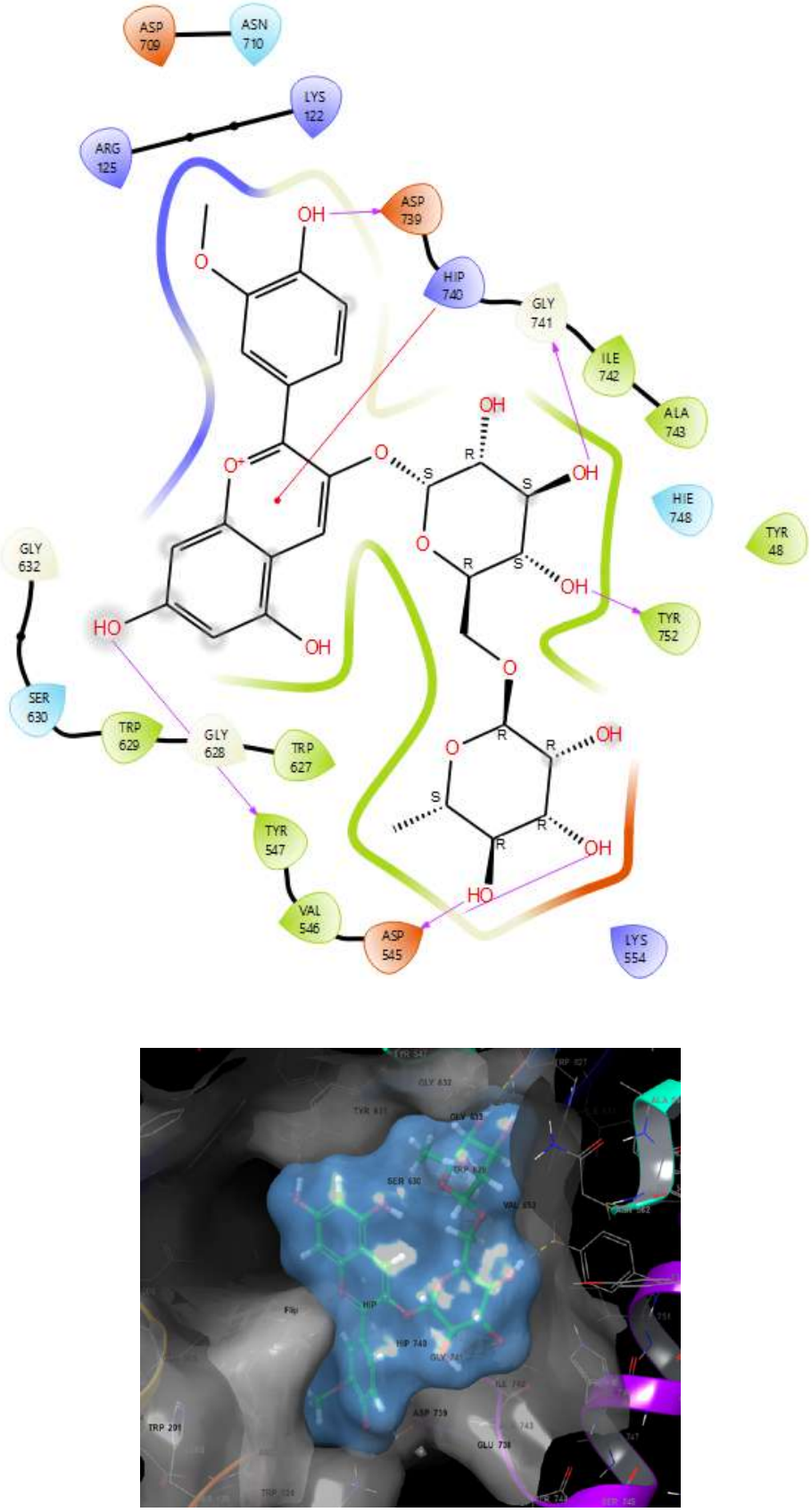


FIGURE 4.13: 2D and 3D Interaction of Peonidin-3-rutinoside with DPP4

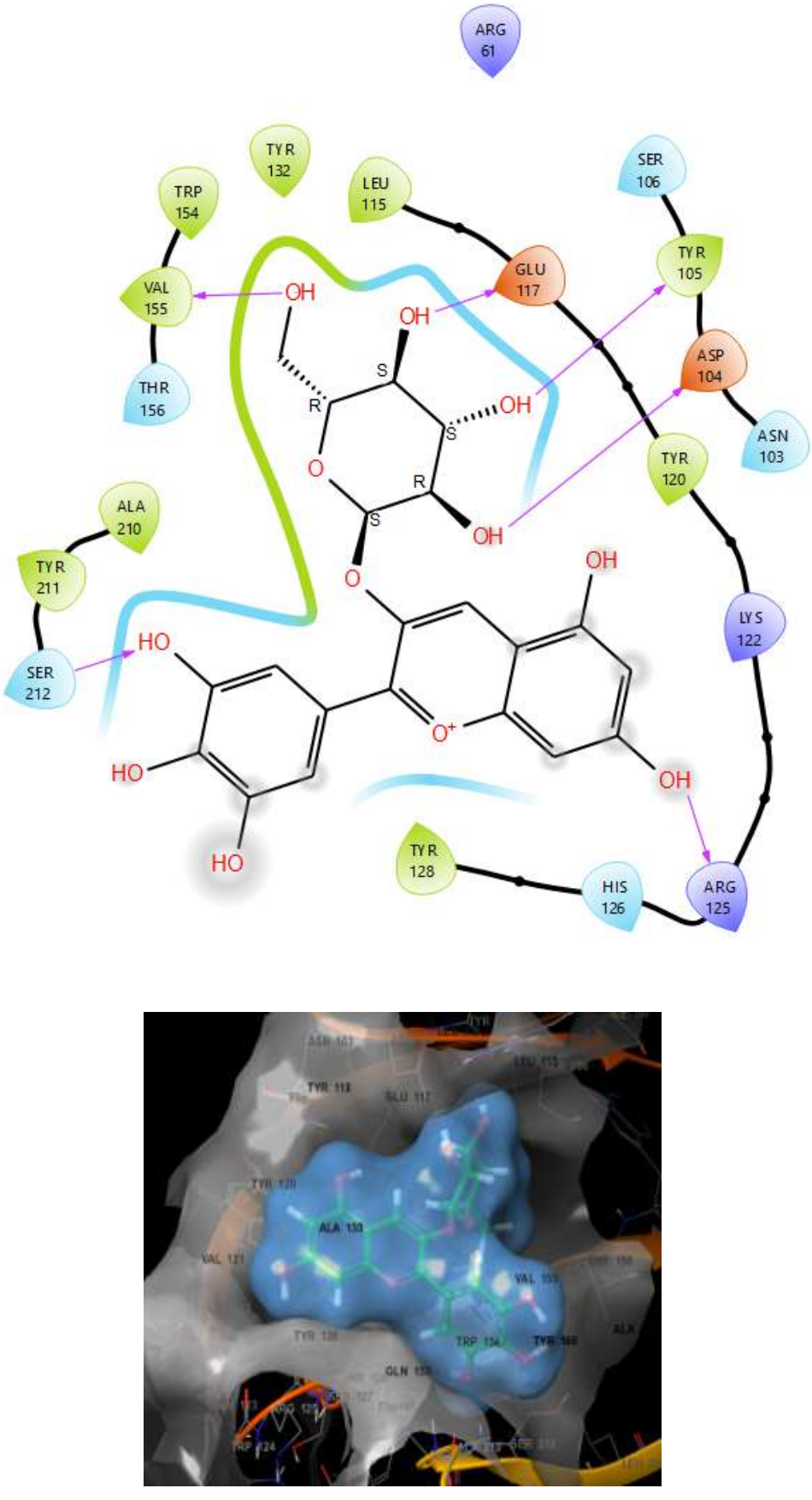


FIGURE 4.14: 2D and 3D Interaction of Delphinidin 3-rutinoside with DPP4

TABLE 4.11: Docking scores of PyRx and Schrodinger against alpha amylase receptor

PyRx		**Schrodinger**	
Ligand	**Binding Affinity**	**Ligand**	**Binding Affinity**
Camptothecin	-7.3	Cyanidin-3-rutinoside	-10.1966
Feselol	-6.9	Cyanidin 3-lathyroside	-10.0195
Pelargonidin	-6.8	Pelargonidin 3,5-diglucoside	-9.58612
Protopine	-6.7	Peonidin 3-rutinoside	-9.1572
Oxoglaucine	-6.5	Cyanidin-3-galactoside	-8.7518
Cafeic_acid	-6.4	Delphinidin 3-O-glucoside	-8.73689
Formononetin 7 - O - glucoside	-6.4	Malvidin 3-glucoside	-8.70696
Quercetin	-6.4	Pelargonidin 3-rutinoside	-8.39244
Velutin	-6.4	Pelargonidin 3-glucoside	-7.80049
Cyanidin-3-galactoside	-6.3	Carboxystrictosidine	-6.60484

4.5.3.1 Camptothecin

The 2D interaction diagram of Camptothecin with α-amylase in Figure 4.15 showed a network of stabilizing interactions within the enzyme's active site. These included a pi-anion interaction with ASP A300, an amide-pi stacked interaction with HIS A305, and multiple van der Waals contacts involving TRP A58, TRP A59, LEU A162, LEU A165, and TYR A62.

Additional pi-sigma and hydrophobic interactions with residues such as GLY A306 and ILE A235 further hold the ligand, explaining its top-ranked PyRx binding affinity of -7.3 kcal/mol. These interactions place Camptothecin effectively within the catalytic cleft of α-amylase.

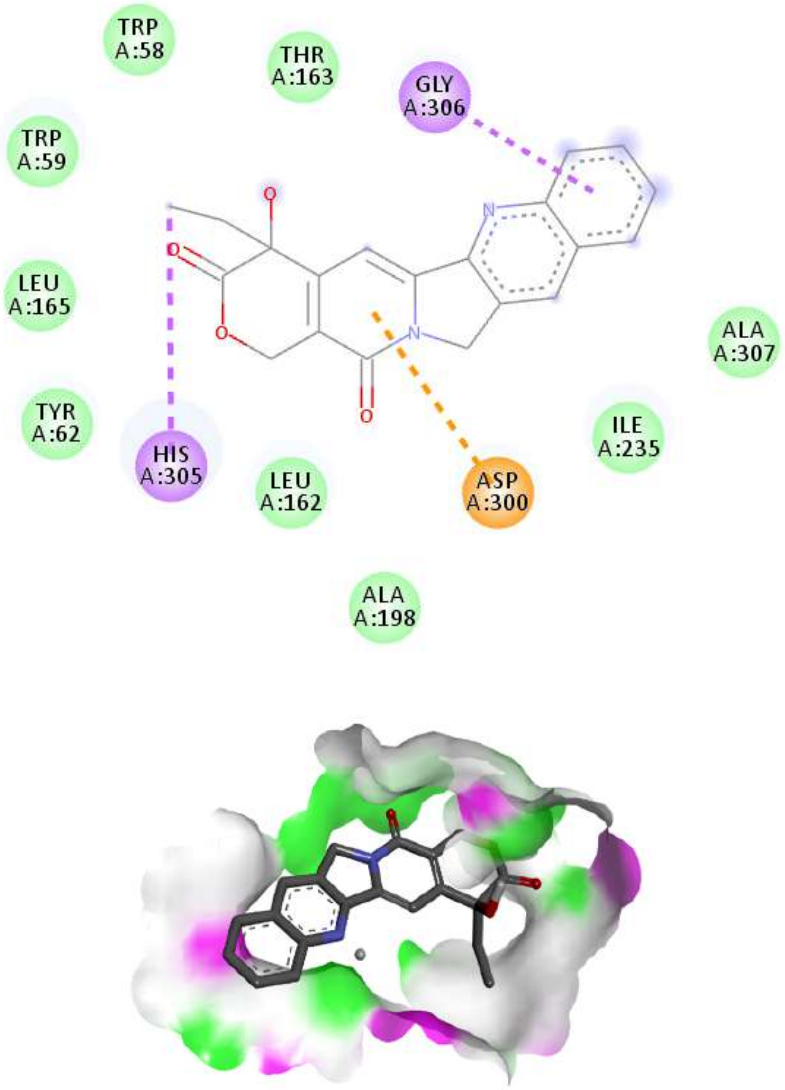


FIGURE 4.15: 2D and 3D Interaction of Camptothecin with alpha amylase

4.5.3.2 Feselol

Feselol interaction occurred through several conventional hydrogen bonds, with ASP A300, HIS A305, and GLN A63 amino acid residues which are complemented by pi-alkyl and van der Waals interactions with LEU A165, ILE A235, ALA A307, and other hydrophobic residues. The ligand's rigid scaffold allowed it to occupy the substrate-binding pocket efficiently, contributing to its strong binding score of -6.9 kcal/mol in PyRx and highlighting its potential as a dual inhibitor of both alpha amylase and DPP4 as shown Figure 4.16.

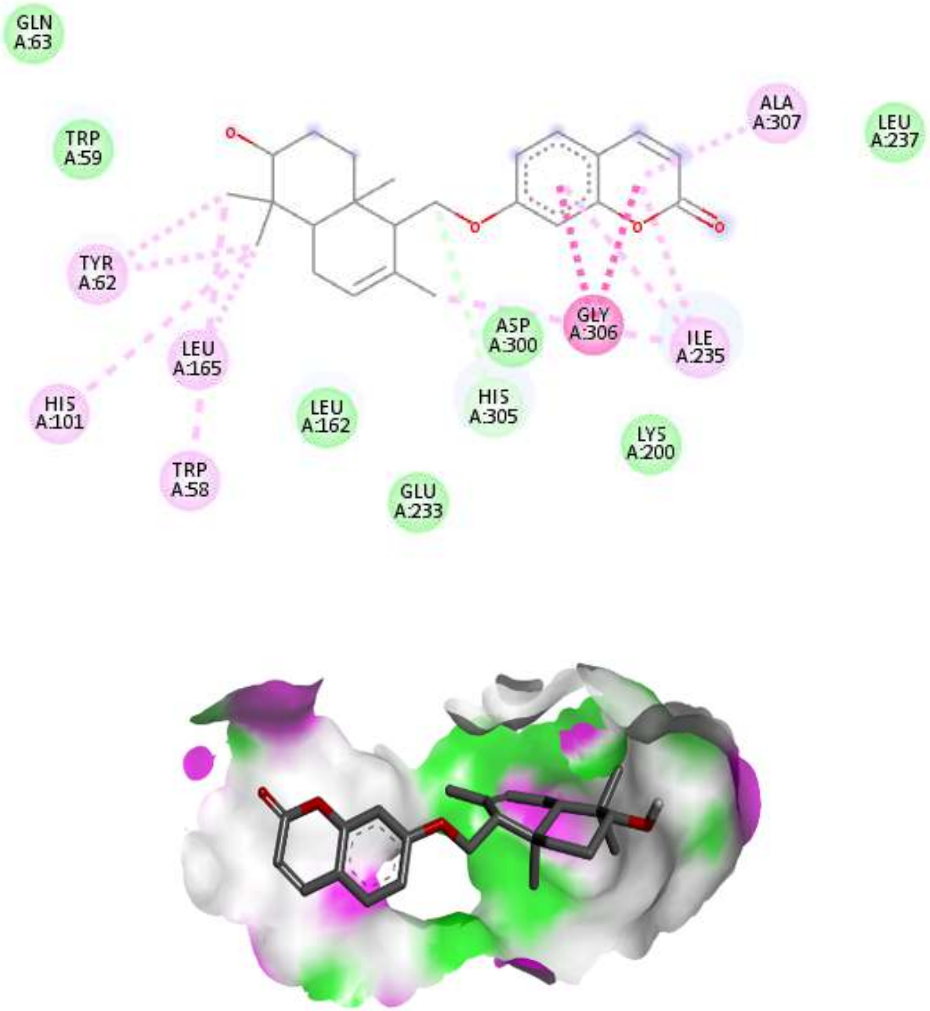


FIGURE 4.16: 2D and 3D Interaction of Feselol with alpha amylase

4.5.3.3 Pelargonidin

The final α-amylase interaction diagram (corresponding to another top Pelargonidin or anthocyanin derivative) showed a similar pattern of high interaction density. Key conventional hydrogen bonds are observed with ASP A300 and HIS A305, alongside carbon-hydrogen bonds and pi-alkyl interactions involving LEU A165, ILE A235, ALA A307, and GLU A233 as shown in Figure 4.17. The diagram emphasized how the hydroxyl groups on the anthocyanidin scaffold and the attached sugars enable extensive polar contacts, while the planar aromatic system engages hydrophobic subsites, reinforcing the structural basis for effective enzyme inhibition.

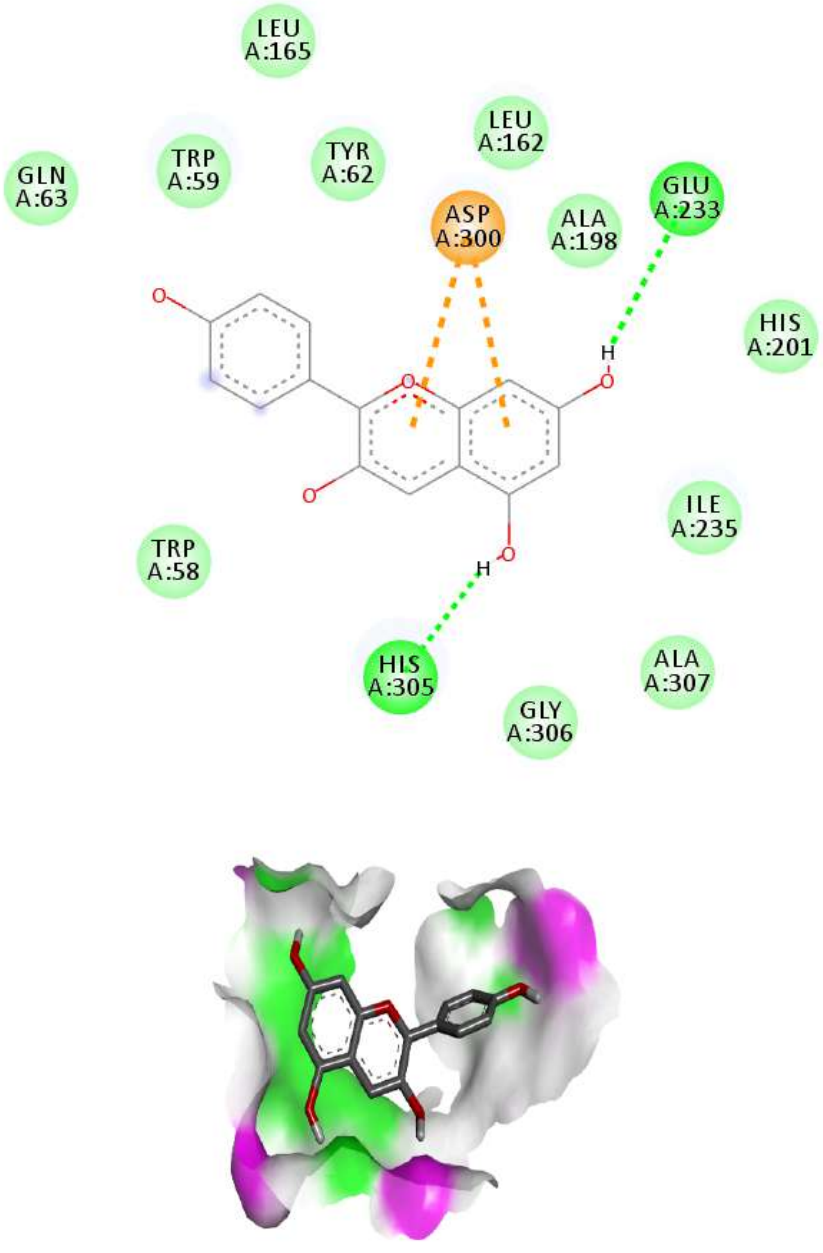


FIGURE 4.17: 2D and 3D Interaction of Pelargonidin with alpha amylase

4.5.3.4 Cyanidin 3-rutinoside

Cyanidin 3-rutinoside exhibited multiple hydrogen-bonding and hydrophobic interactions with the DPP4 binding site, involving residues such as **Lys 203, Glu 205, Tyr 62,** and **Gln 63**. Pi–pi stacking and hydrophobic contacts with aromatic residues further contributed to ligand stabilization within the binding pocket. The extensive interaction network suggests favorable accommodation of Cyanidin 3-rutinoside within the DPP4 active site.

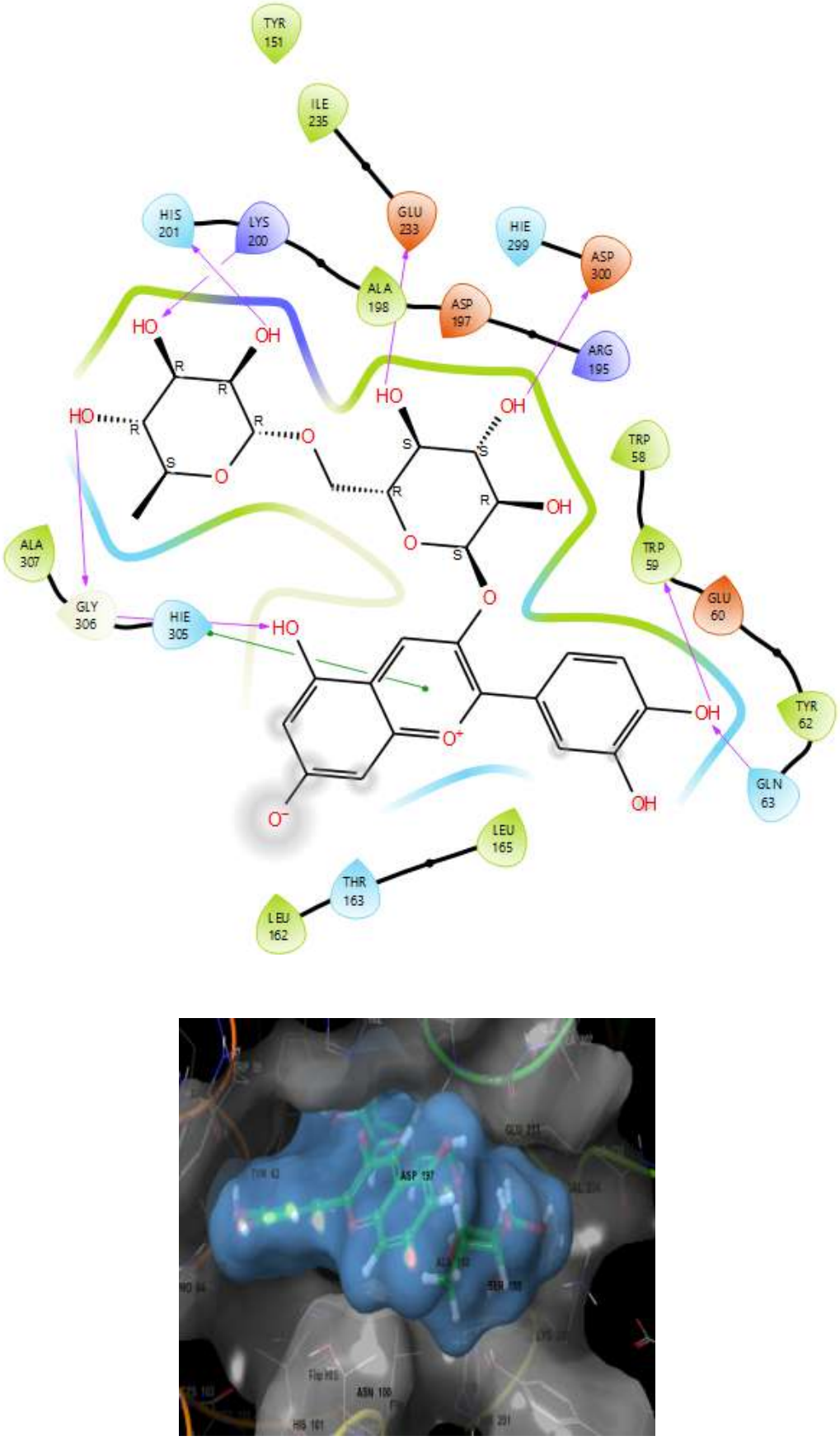


FIGURE 4.18: 2D and 3D Interaction of Cyanidin 3-rutinoside with alpha amylase

4.5.3.5 Cyanidin 3-lathyroside

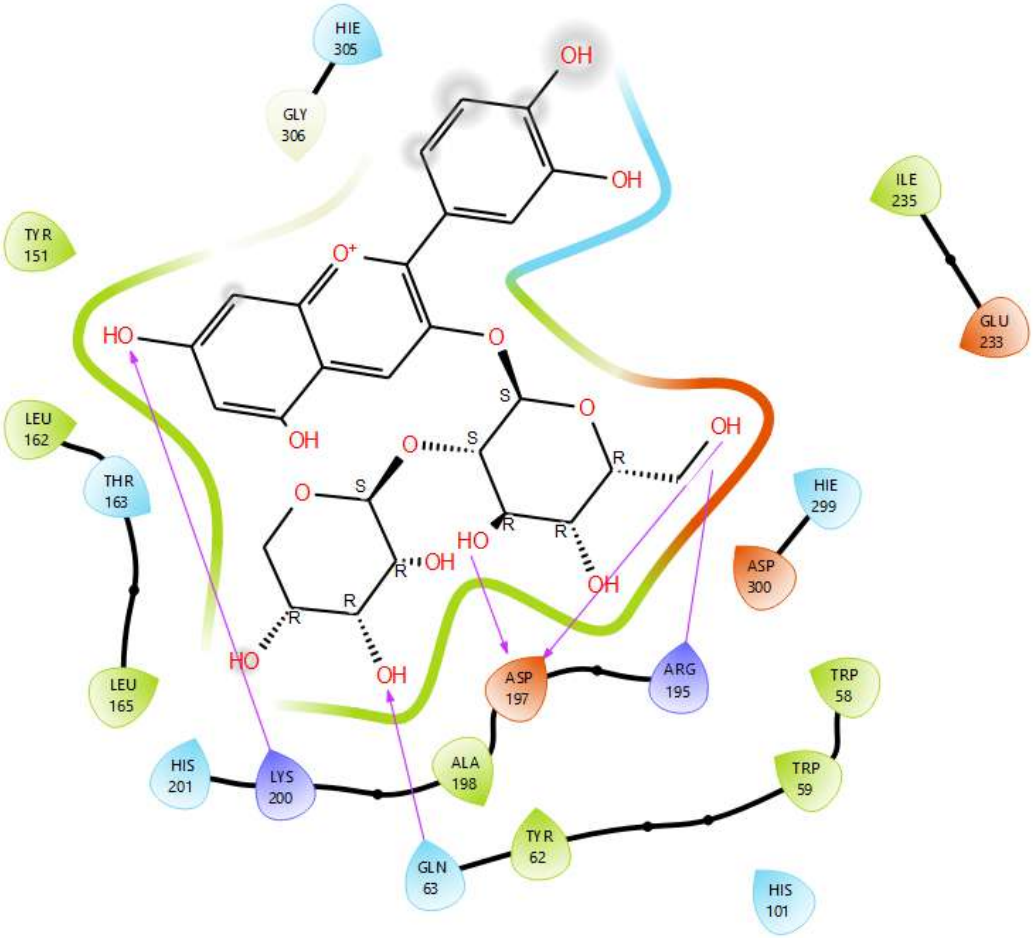
Cyanidin 3-lathyroside exhibited multiple hydrogen-bonding interactions with DP -P4, particularly involving **TYR151, THR163, ASP197,** and **LYS203** residues. Additional hydrophobic and π-mediated contacts with residues such as **TRP58, TRP59,** and **TYR62** may further contribute to stabilization within the binding pocket. Overall, the diverse interaction pattern indicates favorable accommodation of Cyanidin 3-lathyroside within the DPP4 binding site.

4.5.3.6 Pelargonidin 3, 5-diglucoside

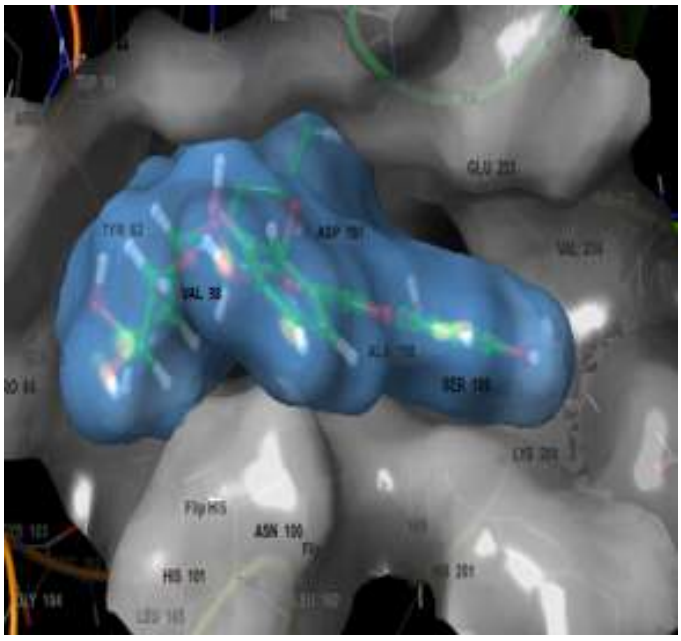
Pelargonidin 3, 5-diglucoside exhibited several conventional hydrogen-bonding interactions with DPP4 residues, including **ASP197, ASP300,** and **ASP356**, which may contribute to its stabilization within the binding pocket. Additional hydrophobic and π-mediated contacts involving **TYR62, TRP58, TRP59,** and **TYR151** further supported ligand–receptor association. Overall, the extensive network of polar and hydrophobic interactions suggests favorable binding of Pelargonidin 3, 5-diglucoside with DPP4.

4.5.4 Physicochemical Evaluation

The physicochemical properties of the selected phytoconstituents were evaluated using the SwissADME web server, and the calculated parameters are presented in Table 4.12. These parameters are important for predicting the drug-likeness, oral bioavailability, and pharmacokinetic behavior of the compounds. According to Lipinski's Rule of Five, compounds with a molecular weight below 500 g/mol, not more than five hydrogen bond donors (HBD), not more than ten hydrogen bond acceptors (HBA), and suitable lipophilicity are more likely to exhibit favorable oral bioavailability. Similarly, Veber's Rule emphasizes that compounds with ten or fewer rotatable bonds and a topological polar surface area (TPSA) below 140 $Å^2$ generally possess good intestinal absorption. Among the investigated compounds, Camptothecin, Feselol, Pelargonidin, Protopine, Oxoglaucine, and Glycocholic

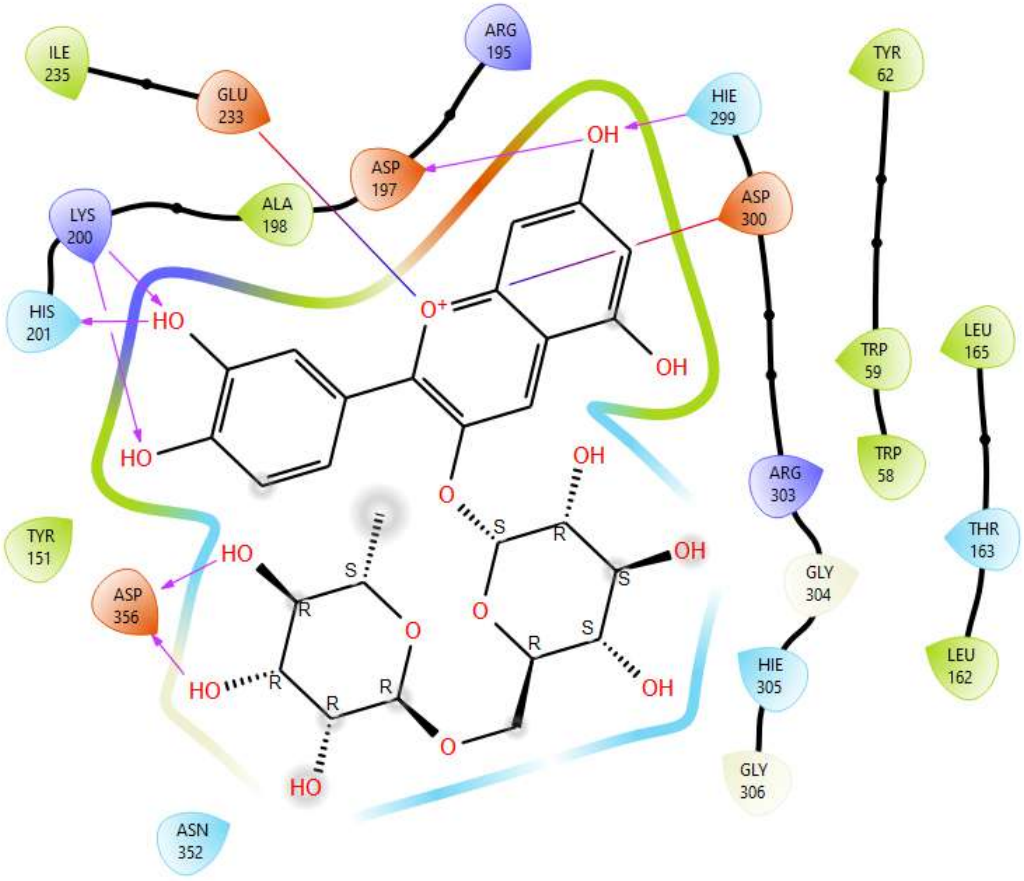


(a)

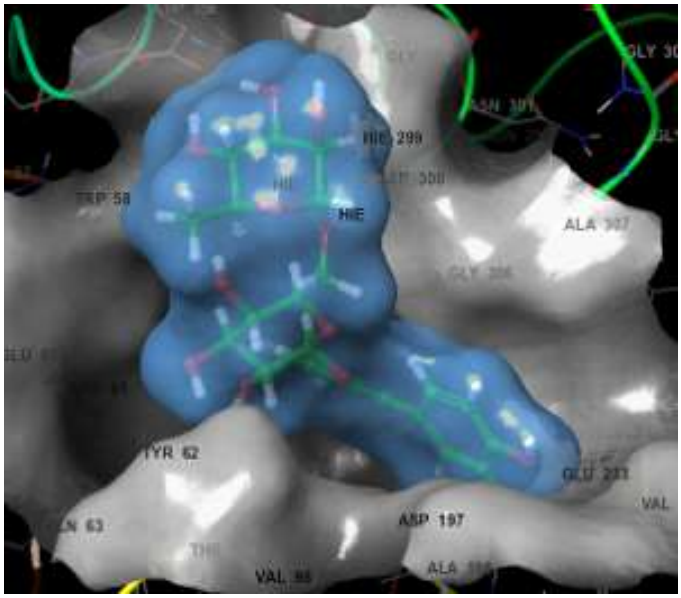


(b)

FIGURE 4.19: 2D and 3D Interaction of Cyanidin 3-rutinoside with alpha amylase



(a)



(b)

FIGURE 4.20: 2D and 3D Interaction of Peonidin 3, 5-diglucoside with alpha amylase

acid satisfied the recommended molecular weight criterion (<500 g/mol), whereas Pelargonidin 3-rutinoside (579.53 g/mol) and Cyanidin 3-rutinoside (630.98 g/mol) exceeded the acceptable limit, suggesting comparatively lower oral bioavailability.

The number of hydrogen bond acceptors and donors for most compounds remained within the acceptable range, while the glycosylated flavonoids Pelargonidin 3-rutinoside and Cyanidin 3-rutinoside possessed a higher number of hydrogen bond donors and acceptors due to the presence of multiple hydroxyl and sugar moieties.

The fraction of sp^3-hybridized carbon atoms (Fsp^3) varied considerably among the compounds. Glycocholic acid exhibited the highest Fsp^3 value (0.92), indicating a highly saturated molecular framework that is generally associated with improved three-dimensionality and potentially better pharmacokinetic properties.

Ginkgolide B (0.85) and Feselol (0.54) also demonstrated relatively high saturation, whereas Pelargonidin showed an Fsp^3 value of 0.00, reflecting its highly aromatic and planar structure. The number of rotatable bonds ranged from 1 to 7, with Glycocholic acid displaying the highest molecular flexibility (7 rotatable bonds), while Camptothecin, Pelargonidin, and Ginkgolide B contained only one rotatable bond, suggesting greater structural rigidity.

The number of rotatable bonds ranged from 1 to 7, with Glycocholic acid displaying the highest molecular flexibility (7 rotatable bonds), while Camptothecin, Pelargonidin, and Ginkgolide B contained only one rotatable bond, suggesting greater structural rigidity.

The topological polar surface area (TPSA) values ranged from 40.16 $Å^2$ for Protopine to 252.36 $Å^2$ for Cyanidin 3-rutinoside. All compounds except Pelargonidin 3-rutinoside, Cyanidin 3-rutinoside, and Ginkgolide B possessed TPSA values below 140 $Å^2$, indicating favorable membrane permeability and oral absorption. Overall, the physicochemical analysis suggests that most of the selected phytochemicals possess suitable molecular properties consistent with orally active drug candidates.

Overall, the physicochemical analysis suggests that most of the selected phytochemicals possess suitable molecular properties consistent with orally active drug candidates.

TABLE 4.12: Physicochemical Evaluation including MW, nHA, nAHA, F. Csp3, nRB, nHBA, nHBD, MR, TPSA

Ligands	**MW (g/mol)**	**nHA**	**nAHA**	**F. Csp^3**	**nRB**	**nHBA**	**nHBD**	**MR**	**TPSA (Å^2)**
Camptothecin $C_{20}H_{16}N_2O_4$	348.35	26	16	0.25	1	5	1	95.31	81.42
Feselol $C_{24}H_{30}O_4$	382.49	28	10	0.54	3	4	1	112.21	56.67
Pelargonidin $C_{15}H_{11}O_5^+$	271.24	20	16	0.00	1	5	4	74.15	94.06
Protopine $C_{19}H_{19}NO_4$	325.36	24	12	0.37	2	4	0	92.82	40.16
Oxoglaucine $C_{20}H_{17}NO_5$	351.35	26	16	0.20	4	6	0	96.58	66.88
Pelargonidin 3-rutinoside $C_{27}H_{31}O_{14}^+$	579.53	41	16	0.44	6	14	9	137.49	232.13
Glycocholic Acid $C_{26}H_{43}NO_6$	465.62	33	0	0.92	7	6	5	126.38	127.09
Cyanidin 3-rutinoside $C_{27}H_{31}ClO_{15}$	630.98	43	16	0.44	6	15	10	145.37	252.36
Ginkgolide B $C_{20}H_{24}O_{10}$	424.40	30	0	0.85	1	10	3	93.29	148.82

4.5.5 Pharmacokinetic Evaluation

The pharmacokinetic properties of the selected phytoconstituents were predicted using the SwissADME platform, and the results are summarized in Table 4.13. and Table 4.14. Parameters including gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, P-glycoprotein (P-gp) substrate status, cytochrome P450 (CYP) enzyme inhibition, skin permeation, and bioavailability score were evaluated to estimate the absorption, distribution, metabolism, and excretion characteristics of each compound.

Most compounds, including Camptothecin, Feselol, Pelargonidin, Protopine, Oxoglaucine, and Glycocholic acid, demonstrated high gastrointestinal absorption, suggesting good potential for oral administration. In contrast, Pelargonidin 3-rutinoside, Cyanidin 3-rutinoside, and Ginkgolide B exhibited low GI absorption, which may be attributed to their relatively high molecular weights and larger polar surface areas.

Blood-brain barrier permeability was predicted for Feselol, Protopine, and Oxoglaucine, indicating their potential to penetrate the central nervous system. Conversely, the remaining compounds were predicted to be BBB-impermeable, reducing the likelihood of central nervous system exposure and associated neurological effects and adverse effects.

Regarding transporter interactions, all compounds except Oxoglaucine, Pelargonidin 3-rutinoside, and Cyanidin 3-rutinoside were predicted to be substrates of P-glycoprotein (P-gp), suggesting that active efflux may influence their intestinal absorption and tissue distribution.

The predicted bioavailability scores ranged from 0.17 to 0.56. Pelargonidin 3-rutinoside and Cyanidin 3-rutinoside exhibited the lowest bioavailability score (0.17), whereas the remaining compounds demonstrated scores between 0.55 and 0.56, indicating moderate oral bioavailability.

The predicted skin permeation coefficients (Log Kp) varied from -5.95 cm/s for Protopine, indicating comparatively higher skin permeability, to -12.04 cm/s for Pelargonidin 3-rutinoside, suggesting minimal transdermal permeation.

Cytochrome P450 enzyme inhibition analysis revealed substantial differences among the compounds. Protopine and Oxoglaucine inhibited CYP1A2, CYP2C19, CYP2 C9, CYP2D6, and CYP3A4, indicating a greater potential for metabolic drug-drug interactions. Camptothecin inhibited CYP1A2, CYP2C9, and CYP3A4, while Pelargonidin inhibited CYP1A2 and CYP2D6.

In contrast, Feselol did not inhibit any of the evaluated CYP isoenzymes, suggesting a comparatively lower risk of metabolic interactions. CYP inhibition data for

Pelargonidin 3-rutinoside, Glycocholic acid, Cyanidin 3-rutinoside, and Ginkgolide B were unavailable.

Overall, the pharmacokinetic evaluation identified Feselol as the compound with the most favorable ADME profile due to its high gastrointestinal absorption, BBB permeability, absence of CYP inhibition, and acceptable oral bioavailability.

TABLE 4.13: Pharmacokinetic Evaluation including GI Absorption, BBB Permeation, Bioavailability, P-gp Substrate and Log Kp

Ligands	**GI Absorption**	**BBB Permeation**	**Bio availability**	**P-gp Substrate**	**Log K_p (Skin Permeation) (cm/s)**
Camptothecin	High	No	0.55	Yes	−7.19
Feselol	High	Yes	0.55	Yes	−6.49
Pelargonidin	High	No	0.55	Yes	−6.33
Protopine	High	Yes	0.55	Yes	−5.95
Oxoglaucine	High	Yes	0.55	No	−5.99
Pelargonidin 3-rutinoside	Low	No	0.17	No	−12.04
Glycocholic Acid	High	No	0.56	Yes	−7.97
Cyanidin 3-rutinoside	Low	No	0.17	No	−12.03
Ginkgolide B	Low	No	0.55	Yes	−9.16

TABLE 4.14: Pharmacokinetic Evaluation including CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 inhibition

Ligands	**CYP1A2 Inhibitor**	**CYP2C19 Inhibitor**	**CYP2C9 Inhibitor**	**CYP2D6 Inhibitor**	**CYP3A4 Inhibitor**
Camptothecin	Yes	No	Yes	No	Yes
Feselol	No	No	No	No	No
Pelargonidin	Yes	No	No	Yes	No
Protopine	Yes	Yes	Yes	Yes	Yes
Oxoglaucine	Yes	Yes	Yes	Yes	Yes
Pelargonidin 3-rutinoside	n/d	n/d	n/d	n/d	n/d
Glycocholic Acid	n/d	n/d	n/d	n/d	n/d
Cyanidin 3-rutinoside	n/d	n/d	n/d	n/d	n/d
Ginkgolide B	n/d	n/d	n/d	n/d	n/d

4.5.6 Drug Likeness

Drug-likeness assessment was performed using five widely accepted rule-based filters, namely Lipinski, Ghose, Veber, Egan, and Muegge, to evaluate the suitability

of the selected phytochemicals as orally active drug candidates. These computational filters assess molecular descriptors associated with oral bioavailability, permeability, and pharmacokinetic performance.

As shown in Table 4.15. Camptothecin, Feselol, Pelargonidin, Protopine, and Oxoglaucine successfully satisfied all five drug-likeness criteria, indicating favorable physicochemical properties comparable to those of marketed oral drugs. Glycocholic acid fulfilled Lipinski, Veber, Egan, and Muegge criteria but failed the Ghose filter, suggesting minor deviations from the ideal physicochemical space.

In contrast, Pelargonidin 3-rutinoside and Cyanidin 3-rutinoside failed all evaluated drug-likeness filters. Their high molecular weights, elevated polar surface areas, and excessive hydrogen bonding capacity likely contributed to these violations, indicating comparatively poor oral drug-likeness. Ginkgolide B satisfied Lipinski and Muegge rules but failed the Ghose, Veber, and Egan filters, primarily due to its high polarity and physicochemical characteristics.

Overall, the results suggest that Camptothecin, Feselol, Pelargonidin, Protopine, and Oxoglaucine exhibit the most favorable drug-like characteristics among the evaluated phytochemicals and therefore represent promising candidates for further pharmacological investigations.

TABLE 4.15: Drug Likeness Assessment including Lipinski, Ghose, Veber, Egan and Muegge.

Ligands	**Lipinski**	**Ghose**	**Veber**	**Egan**	**Muegge**
Camptothecin	Yes	Yes	Yes	Yes	Yes
Feselol	Yes	Yes	Yes	Yes	Yes
Pelargonidin	Yes	Yes	Yes	Yes	Yes
Protopine	Yes	Yes	Yes	Yes	Yes
Oxoglaucine	Yes	Yes	Yes	Yes	Yes
Pelargonidin 3-rutinoside	No	No	No	No	No
Glycocholic Acid	Yes	No	Yes	Yes	Yes
Cyanidin 3-rutinoside	No	No	No	No	No
Ginkgolide B	Yes	No	No	No	Yes

4.5.7 Medicinal Chemistry

The medicinal chemistry properties were predicted using SwissADME and are presented in Table 4.16. These parameters provide insight into potential assay interference, structural alerts, lead-likeness, and synthetic feasibility.

Most compounds generated no PAINS alerts, indicating a low probability of producing false-positive biological screening results. However, Protopine and Cyanidin 3-rutinoside each produced one PAINS alert, suggesting that these compounds may require careful interpretation during biological evaluation. Brenk analysis revealed no structural alerts for Camptothecin, Protopine, Oxoglaucine, and Glycocholic acid, whereas Feselol produced two alerts (coumarin and isolated alkene), Pelargonidin exhibited one charged oxygen/sulfur alert, and Pelargonidin 3-rutinoside, Cyanidin 3-rutinoside, and Ginkgolide B each generated one or more structural alerts.

Lead-likeness evaluation demonstrated that Camptothecin, Pelargonidin, and Protopine fulfilled the lead-likeness criteria. Conversely, Feselol and Oxoglaucine failed due to molecular weight-related violations, while Pelargonidin 3-rutinoside, Glycocholic acid, Cyanidin 3-rutinoside, and Ginkgolide B each exhibited one or more violations, reducing their suitability as lead compounds.

Synthetic accessibility scores ranged from 2.97 to 6.50. Pelargonidin (2.97) showed the lowest score, indicating the easiest synthetic accessibility, whereas Cyanidin 3-rutinoside (6.50) displayed the highest score, reflecting greater synthetic complexity. Overall, the medicinal chemistry evaluation suggests that Camptothecin, Pelargonidin, and Protopine possess the most favorable balance between structural quality and synthetic feasibility.

TABLE 4.16: Structural alerts, lead-likeness, and synthetic feasibility

Ligands	**PAINS**	**Brenk**	**Leadlikeness**	**Synthetic Accessibility**
Camptothecin	0 alert	0 alert	Yes	3.84
Feselol	0 alert	2 alerts: cumarine, isolated _alkene	No; 1 violations: MW > 350	5.06

Table 4.16 continued from previous page

Ligands	PAINS	Brenk	Leadlikeness	Synthetic Accessibility
Pelargonidin	0 alert	1 alert: charged _oygen _sulphur	Yes	2.97
Protopine	1 alert: anil _di _aik _C	0 alert	Yes	3.31
Oxoglaucine	0 alert	0 alert	No; 1 violation: MW > 3.5	3.15
Pelargonidin 3-rutinoside	0 alert	1 alert	No; 1 violation(s)	6.43
Glycocholic Acid	0 alert	0 alert	No; 1 violation(s)	5.11
Cyanidin 3-rutinoside	1 alert	2 alert	No; 1 violation(s)	6.5
Ginkgolide B	0 alert	1 alert	No; 1 violation(s)	6.38

4.5.8 Water Solubility Predictions

Water solubility was predicted using the ESOL, Ali, and SILICOS-IT computational models, and the results are summarized in Table 4.17. These complementary models estimate aqueous solubility using different computational algorithms, providing a broader understanding of the compounds' dissolution characteristics.

According to the ESOL model, Pelargonidin 3-rutinoside and Cyanidin 3-rutinoside were predicted to be very soluble, while Camptothecin, Pelargonidin, Glycocholic acid, and Ginkgolide B were classified as soluble. Feselol, Protopine, and Oxoglaucine were predicted to be moderately soluble.

The Ali model produced comparable predictions, with most compounds classified as soluble or moderately soluble. Pelargonidin 3-rutinoside remained very soluble, whereas Oxoglaucine exhibited the lowest predicted solubility among all compounds.

Predictions generated by the SILICOS-IT model demonstrated greater variability. Pelargonidin, Pelargonidin 3-rutinoside, Glycocholic acid, Cyanidin 3-rutinoside, and Ginkgolide B were classified as soluble, whereas Camptothecin, Feselol, and Protopine were moderately soluble, and Oxoglaucine was predicted to be poorly soluble.

Overall, the three prediction models consistently indicate that most of the selected phytochemicals possess acceptable aqueous solubility, although Oxoglaucine may require formulation strategies to improve its dissolution characteristics.

TABLE 4.17: Water solubility predictions including Log S (ESOL), Log S (Ali), Log S (SILICOS-IT)

Ligands	**Log S (ESOL)**	**Solubility Class**	**Log S (Ali)**	**Solubility Class**	**Log S (SILICOS-IT)**	**Solubility Class**
Camptothecin	−3.49	Soluble	−3.07	Soluble	−5.83	Moderately soluble
Feselol	−4.18	Moderately soluble	−3.94	Soluble	−5.57	Moderately soluble
Pelargonidin	−3.49	Soluble	−3.90	Soluble	−3.24	Soluble
Protopine	−4.17	Moderately Soluble	−3.81	Soluble	−5.11	Moderately soluble
Oxoglaucine	−4.39	Moderately soluble	−4.55	Moderately soluble	−6.85	Poorly soluble
Pelargonidin 3-rutinoside	−1.37	Very soluble	−1.21	Very soluble	−1.0	Soluble
Glycocholic Acid	−3.3	Soluble	−3.93	Soluble	−2.66	Soluble
Cyanidin 3-rutinoside	−1.96	Very soluble	−2.1	Soluble	−0.41	Soluble
Ginkgolide B	−2.17	Soluble	−2.28	Soluble	−0.77	Soluble

4.5.9 Organ Toxicity

The organ toxicity profiles of the selected phytochemicals were predicted using Protox 3.0, and the results are presented in Table 4.18. Major toxicity endpoints

evaluated included hepatotoxicity, neurotoxicity, nephrotoxicity, respiratory toxicity, and cardiotoxicity.

Most compounds were predicted to be non-hepatotoxic, except Oxoglaucine, which showed active hepatotoxicity. Neurotoxicity was predicted for Camptothecin and Protopine, whereas the remaining compounds were predicted to be inactive. Nephrotoxicity was observed for Camptothecin, Feselol, Pelargonidin, Pelargonidin 3-rutinoside, Glycocholic acid, and Cyanidin 3-rutinoside, while Protopine, Oxoglaucine, and Ginkgolide B were predicted to be non-nephrotoxic.

Respiratory toxicity was predicted for all evaluated compounds, indicating that this endpoint should be considered during future experimental investigations. Regarding cardiotoxicity, only Glycocholic acid demonstrated predicted cardiotoxic potential, whereas all remaining compounds were predicted to be inactive.

Overall, Ginkgolide B exhibited the most favorable toxicity profile, showing inactivity for hepatotoxicity, neurotoxicity, nephrotoxicity, and cardiotoxicity, with only respiratory toxicity predicted. These findings suggest that while several compounds demonstrate promising pharmacokinetic and drug-likeness characteristics, further in vitro and in vivo toxicity studies are required to validate the computational predictions before therapeutic development.

TABLE 4.18: Organ toxicity predicted through protox 3.0 including hepatotoxicity, neurotoxicity, nephrotoxicity, respiratory toxicity and cardio-toxicity

Ligands	**Hepato Toxicity**	**Neuro Toxicity**	**Nephro Toxicity**	**Respiratory Toxicity**	**Cardio Toxicity**
Camptothecin	Inactive	Active	Active	Active	Inactive
Feselol	Inactive	Inactive	Active	Active	Inactive
Pelargonidin	Inactive	Inactive	Active	Active	Inactive
Protopine	Inactive	Active	Inactive	Active	Inactive
Oxoglaucine	Active	Inactive	Inactive	Active	Inactive
Pelargonidin 3-rutinoside	Inactive	Inactive	Active	Active	Inactive
Glycocholic Acid	Inactive	Inactive	Active	Active	Active
Cyanidin 3-rutinoside	Inactive	Inactive	Active	Active	Inactive
Ginkgolide B	Inactive	Inactive	Inactive	Active	Inactive

4.5.10 Toxicity End Points

The toxicity end points of the selected phytochemicals were predicted using the Protox 3.0 web server, and the results are presented in Table 4.19. The evaluated parameters included carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, ecotoxicity, clinical toxicity, and nutritional toxicity. These computational predictions provide valuable preliminary information regarding the potential safety profile of the compounds before experimental toxicological investigations.

Among the evaluated phytochemicals, Camptothecin, Feselol, Pelargonidin, Pelargonidin 3-rutinoside, Glycocholic acid, Cyanidin 3-rutinoside, and Ginkgolide B were predicted to be non-carcinogenic, whereas Protopine was the only compound predicted to exhibit carcinogenic potential. Regarding immunotoxicity, Camptothecin, Feselol, Protopine, Oxoglaucine, Pelargonidin 3-rutinoside, Cyanidin 3-rutinoside, and Ginkgolide B were predicted to be immunotoxic, while Pelargonidin and Glycocholic acid were classified as non-immunotoxic.

Mutagenicity analysis indicated that Protopine and Oxoglaucine were predicted to possess mutagenic potential, whereas all other compounds were predicted to be inactive for this toxicity endpoint. Cytotoxicity predictions showed that Camptothecin and Feselol were classified as cytotoxic, while the remaining phytochemicals were predicted to be non-cytotoxic.

Ecotoxicity was predicted for Feselol, Pelargonidin, and Oxoglaucine, whereas Camptothecin, Protopine, Pelargonidin 3-rutinoside, Glycocholic acid, Cyanidin 3-rutinoside, and Ginkgolide B were predicted to be non-ecotoxic.

Clinical toxicity predictions identified Camptothecin, Protopine, and Glycocholic acid as potentially clinically toxic, whereas Feselol, Pelargonidin, Oxoglaucine, Pelargonidin 3-rutinoside, Cyanidin 3-rutinoside, and Ginkgolide B were predicted to be clinically non-toxic. Nutritional toxicity was predicted only for Feselol, Pelargonidin, Pelargonidin 3-rutinoside, Cyanidin 3-rutinoside, and Ginkgolide B, while Camptothecin, Protopine, Oxoglaucine, and Glycocholic acid were predicted to be inactive.

Overall, the toxicity end point assessment suggests that Glycocholic acid possesses one of the most favorable toxicity profiles, exhibiting inactivity for carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, ecotoxicity, and nutritional toxicity, with only clinical toxicity predicted. Similarly, Pelargonidin demonstrated a relatively favorable safety profile, showing inactivity for carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, and clinical toxicity, although ecotoxicity and nutritional toxicity were predicted. In contrast, Protopine exhibited the highest number of predicted adverse toxicity endpoints, including carcinogenicity, immunotoxicity, mutagenicity, and clinical toxicity. These findings indicate that although several phytochemicals demonstrate promising pharmacokinetic and drug-likeness properties, comprehensive in vitro and in vivo toxicological studies are required to validate these computational toxicity predictions before considering further pharmaceutical development.

TABLE 4.19: Toxicity end points predicted through protox 3.0 including carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, ecotoxicity, clinical toxicity, nutritional toxicity

Ligand	**Carcinogenicity**	**Immunotoxicity**	**Mutagenicity**	**Cytotoxicity**	**Ecotoxicity**	**Clinical Toxicity**	**Nutritional Toxicity**
Camptothecin	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
Feselol	Inactive	Active	Inactive	Active	Active	Inactive	Active
Pelargonidin	Inactive	Inactive	Inactive	Inactive	Active	Inactive	Active
Protopine	Active	Active	Active	Inactive	Inactive	Active	Inactive
Oxoglaucine	Inactive	Active	Active	Inactive	Active	Inactive	Inactive
Pelargonidin 3-rutinoside	Inactive	Active	Inactive	Inactive	Inactive	Inactive	Active
Glycocholic Acid	Inactive	Inactive	Inactive	Inactive	Inactive	Active	Inactive
Cyanidin 3-rutinoside	Inactive	Active	Inactive	Inactive	Inactive	Inactive	Active
Ginkgolide B	Inactive	Active	Inactive	Inactive	Inactive	Inactive	Active

Chapter 5

Discussion

5.1 Extraction Yield of *Berberis lycium* Extract

The extraction yield of *Berberis lycium* varied markedly with both the extraction solvent and plant part, with methanol producing considerably higher yields than ethyl acetate in all samples. Among the investigated plant parts, the methanolic leaf extract exhibited the highest extraction yield (23.2%), whereas the ethyl acetate root extract showed the lowest yield (1.73%). These findings agree with the study where polar solvents extracted greater quantities of crude constituents from *Berberis lycium* root bark than semi-polar solvents because of their superior ability to dissolve polar phytochemicals [38].

Another study isolated several phytoconstituents from methanolic extracts of *B. lycium*, demonstrating the effectiveness of methanol in recovering a broad range of bioactive compounds [45]. The present findings are also consistent with the study which concluded that methanol is among the most efficient solvents for extracting polar secondary metabolites due to its high polarity and penetration into plant tissues [46]. Furthermore, some studies reported that extraction efficiency increases with solvent polarity because polar solvents dissolve phenolics, flavonoids, alkaloids and other hydrophilic constituents more effectively than moderately polar solvents such as ethyl acetate [47, 48]. Collectively, these studies support the

higher recovery obtained with methanol in the present investigation and suggest that the large yield observed in leaf extracts may be attributed to the abundance of methanol-soluble metabolites, chlorophylls, carbohydrates and other soluble cellular constituents.

Although the superiority of methanol observed in the present study agrees with previous reports, differences are noted regarding the plant part producing the highest extraction yield. It is reported in literature that approximately % yield from ethanol extracts of *Berberis lycium* root bark obtained by cold maceration, whereas the current study recorded a substantially higher yield from methanolic leaves [38]. Likewise, methanol produced higher extraction efficiency than ethyl acetate in *Berberis lycium* roots, although their work focused exclusively on root material rather than comparing different plant organs [49]. These differences may be explained by variations in plant part selection, solvent composition, extraction technique, geographical origin, harvesting season and environmental conditions, all of which significantly influence phytochemical accumulation and extraction efficiency.

The reason for difference in extraction yield is because it depends not only on solvent polarity but also on extraction temperature, particle size, solvent-to-solid ratio and extraction duration, and *Berberis lycium* contains numerous chemically diverse constituents whose recovery depends on extraction conditions [50, 51]. Therefore, the higher extraction yield obtained from methanolic leaf extracts in the present study likely reflects the combined influence of solvent polarity and the physiological status of the collected plant material rather than differences in solvent efficiency alone.

5.2 Phytochemical Screening of *Berberis lycium* Extracts

The phytochemical screening revealed considerable variation in the distribution of secondary metabolites among the different parts of *Berberis lycium*. The root

extracts exhibited the richest phytochemical composition, containing alkaloids, steroids, terpenoids, carbohydrates, flavonoids and proteins, whereas the stem extracts showed only alkaloids and steroids in both solvent systems, with flavonoids detected only in the ethyl acetate extract. In contrast, none of the tested phytochemical classes are detected in either methanolic or ethyl acetate leaf extracts. These findings are partly consistent with a previous study in which the presence of alkaloids, flavonoids, terpenoids and carbohydrates in the root bark of *B. lyceum* confirms that underground tissues are rich reservoirs of bioactive metabolites [38].

Berberine rich alkaloidal fractions are identified from *B. lycium*, highlighting roots as the principal storage site of isoquinoline alkaloids [51]. Comparable observations have also been reported in another study where higher concentrations of alkaloids and flavonoids are found in root extracts than in aerial parts [52]. The similarity between the present findings and these studies may be attributed to the physiological role of roots as storage organs, where plants accumulate defensive secondary metabolites to protect against soil borne pathogens and environmental stress.

However, the absence of detectable phytochemicals in the leaf extracts differs from several previous reports that identified phenolics, flavonoids and tannins in *Berberis lycium* leaves. Two studies reported measurable levels of phenolic compounds and flavonoids in methanolic leaf extracts, while another study observed that solvent polarity strongly influences the recovery and qualitative detection of secondary metabolites [11, 45, 52]. The processing conditions can substantially alter the composition of plant extracts [47, 50].

The discrepancy between the present findings and previous studies may therefore be explained by differences in geographical location, climatic conditions, harvesting season, plant maturity, post harvest drying methods and extraction procedures, all of which influence phytochemical biosynthesis and accumulation. In addition, qualitative phytochemical assays possess limited analytical sensitivity and may fail to detect metabolites present in low concentrations. Therefore, although the leaf extracts produced the highest crude extraction yield in the present study, the extracted material may have consisted predominantly of primary metabolites

such as chlorophylls, soluble carbohydrates, proteins and other non target compounds rather than the secondary metabolites evaluated during phytochemical screening.

5.3 Identification of Endophytes

The present study identified the fungal endophytes associated with *Berberis lycium* as belonging to the genera *Epicoccum* and *Fusarium* through ITS sequence analysis. Although studies specifically investigating endophytes of *Berberis lycium* are still limited, the isolation of these genera is not unexpected because both *Epicoccum* and *Fusarium* are among the most commonly reported fungal endophytes inhabiting medicinal plants. Similar observations have been reported which described *Fusarium* species as dominant endophytes capable of producing a wide range of biologically active secondary metabolites with antimicrobial, antioxidant and anticancer activities [53]. Literature highlighted that *Epicoccum* species frequently colonize healthy plant tissues and contribute to plant protection through the production of diverse bioactive compounds [54]. The predominance of these fungi in the present study also agrees with reports by Jia et al. (2016), who suggested that medicinal plants provide a favorable ecological niche for metabolically active endophytic fungi because of their rich phytochemical composition [55].

The Neighbor Joining distance trees generated using the NCBI Distance Tree of Results further supported the molecular identification of the isolates. In each tree, the query isolate clustered closely with its corresponding reference sequence, indicating a close genetic relationship. The short branch lengths separating the isolates from their nearest neighbors suggest limited sequence divergence within the ITS region and confirm that the isolates belong to the identified species. At the same time, the clear separation of the isolates from other related species within the same genus demonstrates sufficient genetic variation to distinguish them at the species level. Thus, the phylogenetic analysis complements the BLAST results by providing a visual representation of the evolutionary relationships between the isolates and previously characterized fungal species.

Among the identified fungi, *Epicoccum latusicollum* is of particular interest because members of the genus *Epicoccum* are recognized as prolific producers of structurally diverse secondary metabolites. These fungi have been reported to synthesize polyketides, diketopiperazines, flavonoid-like compounds, carotenoids, and other bioactive metabolites exhibiting antimicrobial, antioxidant, anti - inflammatory, antiviral, and cytotoxic activities (Nicoletti & Fiorentino, 2015).

Several studies have also demonstrated that *Epicoccum* species produce extracellular enzymes and metabolites that facilitate their adaptation to plant tissues while simultaneously protecting the host against invading pathogens [55]. Therefore, the isolation of *E. latusicollum* from *Berberis lycium* suggests that it may contribute to the therapeutic properties traditionally associated with this medicinal plant.

Similarly, species belonging to the genus *Fusarium* are among the most widely distributed fungal endophytes in medicinal plants. Although some *Fusarium* species are recognized as important plant pathogens, many non-pathogenic strains establish beneficial endophytic associations with their host plants. Endophytic *Fusarium* isolates have been reported to produce numerous pharmacologically important metabolites, including alkaloids, terpenoids, peptides, naphthoquinones, and polyketides with antibacterial, antifungal, antioxidant, anticancer, and antidiabetic activities [56]. The identification of *F. oxysporum* and *F. incarnatum* as endophytes of *Berberis lycium* therefore highlights their potential as alternative source of bioactive compounds that may contribute to the biological activities observed in the present study.

The occurrence of *Epicoccum* and *Fusarium* species in medicinal plants has been documented in numerous investigations. *Epicoccum* species have been isolated as endophytes from *Ocimum basilicum*, *Panax notoginseng*, *Artemisia annua*, and several other medicinal plants, where they exhibited strong antimicrobial and antioxidant activities [55].

Likewise, endophytic *Fusarium* species have been reported from *Withania somnifera*, *Catharanthus roseus*, *Taxus wallichiana*, *Tinospora cordifolia*, and *Curcuma longa*, producing metabolites with significant pharmaceutical applications

[57]. These observations indicate that medicinal plants provide unique ecological niches that support endophytic fungi capable of synthesizing valuable secondary metabolites.

In contrast, information regarding the endophytic fungal diversity of *Berberis lycium* remains scarce. Most previous investigations on *Berberis lycium* have focused primarily on its phytochemical constituents, such as berberine, berbamine, oxyacanthine, phenolic compounds, and flavonoids, which are responsible for many of the plant's reported pharmacological activities. Comparatively fewer studies have investigated the endophytic microorganisms associated with this species.

Therefore, the identification of *Epicoccum latusicollum*, *Fusarium oxysporum*, and *Fusarium incarnatum* from *Berberis lycium* adds new information to the existing knowledge of its endophytic microbiome and provides a foundation for future studies aimed at exploring the metabolic potential of these fungi.

It is noteworthy that although four fungal isolates are recovered, only three are successfully identified through ITS sequencing. The root isolate (FBR1) could not be assigned to a species because a reliable ITS sequence was not obtained. This may have resulted from poor DNA quality, PCR amplification failure, or insufficient sequence quality.

Future studies employing optimized DNA extraction methods or additional molecular markers, such as LSU rDNA, β-tubulin (BenA), translation elongation factor 1-α (TEF1-α), or RNA polymerase II (RPB2), may facilitate the accurate identification of this isolate and provide greater taxonomic resolution.

Overall, the combined use of ITS sequencing, BLAST analysis, and NCBI Neighbor Joining distance trees provided reliable molecular evidence for the identification of the fungal endophytes isolated from *Berberis lycium*. The identification of *Epicoccum latusicollum*, *Fusarium oxysporum*, and *Fusarium incarnatum* not only expands the current understanding of fungal endophyte diversity associated with this medicinal plant but also supports their further investigation as potential producers of therapeutically important secondary metabolites.

5.4 Fermentation and Extraction Yield of Endophytic Fungi

The fermentation results revealed considerable variation in crude extract production among the isolated fungal endophytes, with FBL1 producing the highest yield (32 mg/L), followed by FBS1 (18 mg/L), FBR1 (14 mg/L), and FBS2 (12 mg/L). Such variation is commonly reported in studies involving fungal endophytes and reflects the inherent metabolic diversity that exists even among closely related fungal strains.

Similar observations are reported, which demonstrated that individual endophytic fungi possess distinct biosynthetic capacities, resulting in significant differences in secondary metabolite production under identical fermentation conditions [56].

It is found that *Fusarium* isolates differ markedly in metabolite yield because of variations in their genetic makeup and regulation of biosynthetic gene clusters [53, 58]. The higher extraction yield obtained from FBL1 in the present study may therefore indicate greater metabolic activity or more efficient utilization of nutrients during fermentation.

On the other hand, the comparatively lower yields observed for FBS2 and FBR1 should not be interpreted as reduced biological potential. Previous investigations have shown that fungal strains producing smaller quantities of crude extract may synthesize highly potent metabolites in lower concentrations, resulting in strong biological activity despite lower biomass or extract yield [54, 57].

Fermentation conditions such as nutrient composition, incubation period, pH, aeration and fungal growth phase also play important roles in determining metabolite production and may account for differences between the present findings and those reported by other researchers.

Overall, the superior extract yield observed for FBL1 suggests that this isolate represents a promising candidate for further purification and characterization of bioactive secondary metabolites.

5.5 Therapeutic Potential

5.5.1 Antibacterial Activity

The antibacterial activity observed in the present study demonstrates that both *Berberis lycium* extracts and their associated endophytic fungal extracts possess measurable inhibitory effects against *Escherichia coli* and *Staphylococcus aureus*, although the magnitude of inhibition varied among plant parts, fungal isolates, extraction type, and bacterial species. The comparatively stronger activity of fungal secondary metabolite extracts than fungal biomass extracts agrees with previous reports indicating that endophytic fungi synthesize antimicrobial metabolites predominantly during the stationary phase of growth rather than accumulating them within the mycelial biomass [56, 57]. Similarly, the appreciable antibacterial activity of methanolic root and leaf extracts corresponds well with earlier investigations on *B. lycium*, where methanol efficiently extracted alkaloids, flavonoids, phenolic acids, and other antimicrobial constituents responsible for broad spectrum antibacterial activity [50].

The comparatively greater antibacterial activity of the root extracts observed in the present study is likely associated with the higher concentration of isoquinoline alkaloids, particularly berberine, palmatine, and berbamine, which are known to possess broad spectrum antimicrobial properties. Experimental studies have demonstrated that berberine inhibits bacterial growth by disrupting membrane function, interfering with nucleic acid synthesis, and impairing essential cellular processes, thereby reducing bacterial viability [59, 60]. These findings are consistent with the stronger antibacterial effects exhibited by the root extracts of *Berberis lycium* in the present investigation.

Although the plant extracts inhibited the growth of both test organisms, the inhibition zones are generally smaller than those reported for purified berberine or enriched solvent fractions. This difference is not unexpected because crude extracts contain a complex mixture of bioactive and inactive constituents that may dilute the concentration of antibacterial compounds. Furthermore, the diffusion of

phytochemicals through agar depends on factors such as molecular size, polarity, and solubility, which can limit the apparent activity of crude extracts. Similar observations are found which report that purified or enriched phytochemical fractions frequently produced greater inhibition zones than unfractionated herbal extracts despite originating from the same plant source [61, 62].

A notable observation in the present study was the variation in bacterial susceptibility, particularly the pronounced activity of the FBL1 biomass extract against *Staphylococcus aureus* and its lack of activity against *Escherichia coli*. Similar patterns have been reported for metabolites produced by endophytic fungi isolated from medicinal plants in which several endophytic fungal isolates exhibited greater antibacterial activity against *Staphylococcus aureus* than against *Escherichia coli* [7, 63]. The comparatively lower susceptibility of *Escherichia coli* may be attributed to the presence of an outer lipopolysaccharide membrane that acts as an effective permeability barrier, thereby reducing the penetration of many naturally occurring antimicrobial compounds [64].

The comparatively stronger antibacterial activity of fungal biomass extracts further supports the hypothesis that endophytic fungi are capable of synthesizing biologically active secondary metabolites with antimicrobial properties that contribute to the defensive capacity of their host plants. Similar findings have been reported in several studies where metabolites produced by endophytic fungi exhibited broad spectrum antibacterial activity against human pathogens. Endophytic fungi isolated from medicinal plants produced bioactive metabolites with pronounced antibacterial activity against both Gram positive and Gram negative bacteria [65]. Likewise, previous studies reported that metabolites isolated from endophytic fungi, including *Plectophomella* sp. and *Physalospora* sp., exhibited marked antibacterial activity, highlighting the considerable pharmaceutical potential of endophyte derived natural products [66].

The differences in antibacterial activity among the extracts are likely attributable to variations in the secondary metabolite composition of the crude extracts. Similar variability in antibacterial efficacy among crude endophytic fungal extracts

has been reported previously, indicating that the metabolite profile of each isolate plays a critical role in determining its biological activity [67, 68].

The absence of a clear dose dependent response among some of the tested extracts may be attributed to the nature of crude plant and fungal extracts as well as the limitations of the agar diffusion assay. Crude extracts contain complex mixtures of bioactive and inactive compounds, and increasing the concentration of the extract does not necessarily increase the concentration of the active antimicrobial constituents in a proportional manner. In addition, the diameter of the inhibition zone depends not only on antimicrobial potency but also on the ability of compounds to diffuse through the agar medium. Once diffusion reaches equilibrium or saturation, further increases in extract concentration may produce only minimal changes in inhibition zone diameter. Consequently, inhibition zones do not always increase proportionally with increasing extract concentration, even when biologically active compounds are present [14].

Overall, the findings of the present study indicate that both *Berberis lycium* and its associated endophytic fungi constitute valuable sources of antibacterial metabolites. The pronounced activity observed for fungal fermentation extracts suggests that endophytic fungi associated with this medicinal plant may provide promising lead compounds for the development of new antimicrobial agents. Further studies involving bioassay guided fractionation, structural characterization of the active metabolites, and investigations into their mechanisms of action are warranted to explore their pharmaceutical potential.

5.5.2 Antifungal Activity

The present study demonstrated that antifungal activity against *Saccharomyces cerevisiae* varied considerably among the endophytic fungal extracts and *Berberis lycium* plant extracts, indicating that antifungal metabolite production is highly dependent on both the fungal isolate and plant tissue. The methanolic leaf extract (S11) exhibited the highest inhibition, surpassing even the standard antifungal drug nystatin, while the secondary metabolite extract of FBS2 (S8) and

the ethyl acetate leaf extract (S14) also showed notable antifungal activity. These findings are consistent with previous reports demonstrating that methanolic extracts of *Berberis* species possess significant antifungal activity due to their abundance of alkaloids, flavonoids, and phenolic compounds, particularly berberine, which interferes with fungal cell wall integrity, membrane permeability, ergosterol biosynthesis, and mitochondrial function [69, 70].

Furthermore, endophytic fungi are increasingly recognized as prolific producers of bioactive secondary metabolites with potent antifungal properties, and differences in metabolite production among isolates largely account for the variability in antifungal activity observed in different endophytic extracts [71].

Similar observations have also been reported in literature, which found that methanolic extracts generally exhibit greater antifungal efficacy than less polar extracts because they recover a broader spectrum of bioactive metabolites. The strong activity observed in fungal secondary metabolite extracts further supports reports that endophytic fungi associated with medicinal plants synthesize antimicrobial metabolites analogous to those produced by their hosts [57].

However, the absence of activity in several biomass extracts suggests that many antifungal compounds are secreted extracellularly during secondary metabolism rather than being retained within fungal mycelia, a phenomenon previously described for endophytic fungi isolated from medicinal plants [56, 70].

A notable observation of the present study was that several extracts exhibited potent antifungal activity despite showing only moderate antibacterial effects, suggesting differences in the mechanisms governing fungal and bacterial susceptibility. This observation agrees with previous studies demonstrating that fungal cells possess unique membrane sterols and cell wall polysaccharides that serve as selective targets for numerous plant derived metabolites [72]. The superior performance of leaf extracts compared with root extracts against *Saccharomyces cerevisiae* differs from several antibacterial studies on *B. lycium*, where roots usually exhibit greater biological activity because of their higher berberine content. This discrepancy may indicate that antifungal activity in leaves is mediated by additional

metabolites such as flavonoids, anthocyanins or volatile constituents that interact more efficiently with fungal membranes [73].

Likewise, the good antifungal activity of FBS1 and FBS2 secondary metabolites is comparable with reports describing *Epicoccum* and *Fusarium* endophytes as prolific producers of polyketides, peptides and terpenoid derived antifungal compounds capable of inhibiting fungal growth [54, 74].

The lack of a clear concentration dependent response observed in some extracts may be explained by differences in compound diffusion through agar media, metabolite stability and synergistic interactions among multiple constituents within crude extracts rather than differences in intrinsic antifungal potency [75]. Overall, these findings highlight both *Berberis lycium* and its endophytic fungi as promising natural sources of antifungal agents that warrant further purification and mechanistic evaluation.

5.5.3 In Vivo Anti Inflammatory Activity

The present study demonstrated that the methanolic extract of *Berberis lycium* possesses significant anti inflammatory activity in the carrageenan induced paw edema model, with the 50 mg/kg dose producing 27.08% inhibition after 6 h, approaching the activity of the standard drug diclofenac sodium (30.56%).

The carrageenan induced inflammation model is widely accepted for evaluating acute inflammation because it involves an initial phase mediated by histamine and serotonin, followed by a later phase dominated by prostaglandins, nitric oxide, cytokines, and cyclooxygenase (COX)-2 activity. The significant reduction in paw edema observed at 4 and 6 h therefore suggests that the extract may interfere primarily with these late inflammatory mediators.

Similar anti inflammatory effects of *Berberis lycium* extracts have been reported in a previous study which demonstrated that methanolic extracts significantly suppressed carrageenan induced edema through the presence of alkaloids and phenolic compounds [76]. Likewise, it is summarized that berberine, the principal

alkaloid of *Berberis* species, suppresses NF-κB activation and reduces the production of TNF-α, IL-1β, IL-6 and prostaglandin E_2, thereby attenuating acute inflammatory responses. Comparable findings are also reported, which associated the anti inflammatory activity of medicinal plant extracts with flavonoids and phenolic constituents capable of inhibiting oxidative stress and inflammatory signaling pathways [19].

The statistically significant differences observed among treatment groups further support that the anti inflammatory effect of the methanolic extract was pharmacologically meaningful rather than occurring by chance.

Although the methanolic extract produced marked inhibition of paw edema, its activity remained slightly lower than that of diclofenac sodium, which is expected because diclofenac is a purified cyclooxygenase inhibitor with a well defined mechanism of action, whereas the plant extract represents a complex mixture of numerous phytochemicals present at varying concentrations.

The absence of a perfectly dose dependent response, with the 30 mg/kg dose producing lower inhibition than both 10 and 50 mg/kg, has also been reported in phytopharmacological studies and may result from differences in the absorption, metabolism and interaction of multiple constituents within crude extracts rather than reflecting reduced pharmacological potential [77].

Similar non linear responses have been described for herbal preparations containing synergistic and antagonistic phytochemicals that influence overall biological activity [78]. Previous investigations have also demonstrated that *Berberis* extracts exhibit anti inflammatory effects through multiple mechanisms, including inhibition of COX-2, lipoxygenase, inducible nitric oxide synthase (iNOS), and pro inflammatory cytokines, which may explain the sustained inhibition observed during the later phase of inflammation in the present study [79, 80].

Therefore, the present findings strengthen the evidence that *Berberis lycium* is a promising natural anti inflammatory agent and support further bioassay guided isolation of its active constituents together with mechanistic and chronic inflammation studies to validate its therapeutic potential.

5.6 In Silico Studies

5.6.1 Molecular Docking Analysis against the DPP 4 Receptor

The molecular docking investigation demonstrated that several phytochemicals identified from *Berberis lycium* exhibited favourable binding affinities toward the DPP-4 receptor, suggesting their potential as natural inhibitors of this clinically important antidiabetic target. In the PyRx analysis, Feselol emerged as the highest ranked ligand with a binding affinity of −9.8 kcal/mol, followed by Pelargonidin - 3 - rutinoside (−9.5 kcal/mol), Glycocholic acid (−9.4 kcal/mol), Cyanidin - 3 - rutinoside (−9.2 kcal/mol), and Ginkgolide B (−9.2 kcal/mol). All of these compounds showed binding energies equal to or better than the reference inhibitor Sitagliptin (−8.8 kcal/mol), indicating favourable interactions with the catalytic pocket of DPP-4. Similar AutoDock Vina studies have reported that naturally occurring flavonoids and anthocyanins generally exhibit docking scores between −9 and −11 kcal/mol against DPP-4, reflecting stable enzyme binding [82]. Among the identified compounds, Pelargonidin-3-rutinoside, Cyanidin-3-rutinoside, and Delphinidin-3-glucoside belong to the anthocyanin family, whose DPP-4 inhibitory potential has been well documented.

Previous studies have reported that anthocyanin glycosides interact more strongly with DPP-4 than their aglycones because the attached sugar moieties enhance hydrogen bond formation and ligand stabilization within the catalytic cavity [83].

The excellent docking scores obtained for these anthocyanins therefore agree with previous findings and support their contribution to the antidiabetic activity of *B. lycium*. In contrast, Feselol has not previously been investigated against DPP-4, making its superior docking score a novel observation. Likewise, Glycocholic acid has rarely been evaluated as a DPP-4 ligand, but its amphiphilic steroidal structure may facilitate both hydrophobic and hydrogen bond interactions, explaining its favourable binding affinity.

The Schrödinger Glide XP analysis also identified anthocyanin derivatives as the strongest DPP-4 ligands, although the ranking differed from that obtained using PyRx. Cyanidin-3-rutinoside exhibited the strongest GlideScore (−11.6 kcal/mol), followed by Peonidin-3-rutinoside (−10.8 kcal/mol) and Delphinidin-3-glucoside (−10.2 kcal/mol). These findings agree with previous Glide XP studies reporting superior docking scores for cyanidin, peonidin, and delphinidin glycosides due to extensive hydrogen bonding and favourable interactions with catalytic residues [84].

Chlorogenic acid showed a comparatively lower GlideScore (−7.0 kcal/mol), consistent with earlier reports suggesting that its smaller molecular size and lower interaction density reduce its predicted docking score despite its experimentally established antidiabetic activity. Thus, despite differences in numerical ranking, both docking platforms consistently identified anthocyanin glycosides as the most promising natural DPP-4 inhibitors.

The two dimensional interaction analysis further supported the docking results by demonstrating that the leading phytochemicals established multiple stabilizing interactions within the DPP-4 active site. Feselol formed hydrogen bonds with ASP501 and GLN505 together with van der Waals, alkyl, and π–π interactions, whereas Pelargonidin-3-rutinoside established hydrogen bonds with GLU205, GLU206, and ASN710 in addition to π–π stacking with TYR547. Glycocholic acid similarly interacted through hydrogen bonds with LYS122, GLN123, GLN153, and TRP124, while hydrophobic contacts with PHE240 and VAL252 further stabilized the complex. Previous studies have shown that ligands forming both hydrogen bond and hydrophobic interactions generally exhibit greater stability and lower binding energies than those relying on a single interaction type, providing a mechanistic explanation for the favourable docking scores observed in the present study [85].

Although some differences in ligand ranking are observed between PyRx and Schrödinger Glide XP, such variations are expected because the two platforms employ different search algorithms, ligand sampling strategies, and scoring functions. Similar discrepancies have been reported in comparative docking studies,

where both programs successfully identify active ligands despite differences in absolute docking scores [86]. Therefore, the consistent recognition of anthocyanin glycosides as high affinity DPP-4 binders across both docking platforms strengthens the reliability of the present computational findings. Nevertheless, molecular docking provides only theoretical predictions, and the promising phytochemicals identified in this study should be validated through enzyme inhibition assays and in vivo antidiabetic studies before their therapeutic potential can be confirmed.

5.6.2 Molecular Docking Analysis against the Alpha Amylase Receptor

Molecular docking analysis of the α-amylase receptor revealed that several phytochemicals associated with *Berberis lycium* showed favourable predicted interactions with the enzyme. Among the compounds evaluated using PyRx, Camptothecin demonstrated the most favourable binding affinity (-7.3 kcal/mol), followed by Feselol (-6.9 kcal/mol) and Pelargonidin (-6.8 kcal/mol). The binding behaviour predicted for Feselol is in agreement with earlier computational investigations reporting its interaction with α-amylase, suggesting that this compound may have affinity for the enzyme [81]. The relatively favourable score obtained for Camptothecin in the present analysis is also noteworthy and may justify further investigation of its potential α-amylase inhibitory activity. Pelargonidin showed a comparable predicted affinity, consistent with previous evidence indicating that anthocyanin compounds can interact with enzymes involved in carbohydrate digestion. Quercetin and Caffeic acid also exhibited favourable docking scores in the present study, in accordance with previously reported computational and experimental evidence of their α-amylase inhibitory potential. Collectively, these findings suggest that the investigated phytochemicals may interact with α-amylase, although docking scores represent theoretical predictions and cannot independently establish enzyme inhibition. Experimental enzymatic assays would therefore be necessary to confirm the inhibitory activity and determine the biological significance of these predicted interactions.

Similar AutoDock Vina studies identified several plant flavonoids with docking energies ranging from −6.3 to −8.2 kcal/mol against human pancreatic α-amylase, values comparable to those obtained in the present investigation [17]. Likewise, another study reported that anthocyanin derivatives isolated from *Musa paradisiaca* exhibited docking scores between approximately −6.8 and −8.5 kcal/mol against α-amylase, reinforcing the observation that polyphenolic phytochemicals possess favourable affinity towards this enzyme [82].

The slightly different binding energies reported among these studies are expected because docking scores are influenced by differences in receptor preparation, ligand optimization, grid box dimensions, software versions and scoring algorithms rather than solely reflecting differences in ligand potency.

The Schrödinger Glide analysis further highlighted the remarkable affinity of anthocyanin glycosides towards α-amylase. Cyanidin-3-rutinoside exhibited the highest GlideScore (−10.1 kcal/ mol), followed by Cyanidin-3-lathyroside (−10.02 kcal/mol), Pelargonidin-3,5-diglucoside (−9.5 kcal/mol) and Peonidin-3-rutinoside (−9.16 kcal/mol). These findings agree closely with the study, which experimentally demonstrated that Cyanidin-3-rutinoside inhibits pancreatic α-amylase with an IC_{50} of 24.4 μM and suggested that this anthocyanin delays carbohydrate digestion through direct enzyme inhibition [87]. It is demonstrated through molecular docking and enzymatic analysis that Pelargonidin-3-*O*-rutinoside possesses strong inhibitory activity against carbohydrate digesting enzymes because its glycosylated anthocyanin structure enables extensive hydrogen bonding interactions with catalytic residues. Although their study focused on α-glucosidase, the structural features responsible for enzyme binding are comparable to those observed for α-amylase in the present work [88]. It is investigated that several anthocyanins, including Cyanidin-3-rutinoside and Peonidin-3-glucoside, using both molecular docking and enzyme inhibition assays, and reported that these compounds bind directly within the α-amylase active site [89]. The study identified Glu233 as a key residue involved in ligand stabilization, while the present study similarly demonstrated extensive interactions of anthocyanin derivatives with residues located within the catalytic cleft, explaining their superior docking scores.

Collectively, these comparisons strongly support the present findings and indicate that glycosylated anthocyanins consistently exhibit greater affinity towards α-amylase than many other classes of phytochemicals because the presence of multiple hydroxyl groups and sugar moieties increases hydrogen bond formation and improves complementarity with the enzyme active site. The consistently high ranking of Cyanidin-3-rutinoside in both the present study and previously published investigations therefore highlights its potential as one of the principal antidiabetic constituents of *Berberis lycium*.

5.6.3 Physicochemical Evaluation

The physicochemical evaluation of the selected phytoconstituents revealed that most compounds possess molecular characteristics compatible with orally active drug candidates. Camptothecin, Feselol, Pelargonidin, Protopine, Oxoglaucine and Glycocholic acid satisfied Lipinski's molecular weight criterion and generally complied with the recommended limits for hydrogen bond donors, hydrogen bond acceptors and molecular flexibility. These findings are consistent with SwissADME analyses report who demonstrated that compounds meeting Lipinski's Rule of Five and Veber's criteria are more likely to exhibit favourable gastrointestinal absorption and oral bioavailability [15]. Lipinski and Veber emphasized that molecular weight below 500 Da, limited hydrogen bonding capacity, fewer rotatable bonds and TPSA below 140 $Å^2$ are key determinants of passive membrane permeability [90, 91].

Previous studies evaluated flavonoids using SwissADME and demonstrated that compounds satisfying Lipinski's Rule of Five and Veber's criteria exhibited favourable pharmacokinetic and drug likeness properties [92]. Comparable findings are also reported, where phytochemicals with acceptable physicochemical characteristics displayed promising ADME profiles and biological potential [17].

Furthermore, the relatively high Fsp^3 values observed for Glycocholic acid, Ginkgolide B and Feselol indicate greater three dimensional molecular architecture, which has been associated with improved drug likeness and clinical success. It

is proposed that compounds with higher saturation (high Fsp^3) generally possess improved solubility and reduced promiscuous binding compared with highly planar molecules [93].

Therefore, the favourable physicochemical profile of Feselol, together with its excellent docking performance against both DPP-4 and α-amylase, suggests that it represents one of the most promising lead compounds identified in the present study.

In contrast, Pelargonidin-3-rutinoside and Cyanidin-3-rutinoside exceeded the recommended molecular weight, hydrogen bonding and TPSA limits because of the presence of multiple hydroxyl groups and sugar moieties. Similar violations have frequently been reported for anthocyanin glycosides, which often display reduced predicted oral bioavailability despite possessing remarkable biological activities. It is noted that Lipinski's Rule of Five should be considered a guideline rather than an absolute requirement, particularly for structurally complex natural products [15].

Many clinically successful natural product derived drugs do not strictly satisfy conventional drug likeness rules but remain valuable therapeutic agents because of their potent biological activities [3].

Recent reviews further explained that anthocyanin glycosides generally exhibit high TPSA and molecular weight, which reduce passive intestinal diffusion but enhance target recognition through extensive hydrogen bond formation [94, 95]. This observation agrees with the present findings, where Cyanidin-3-rutinoside and Pelargonidin-3-rutinoside demonstrated the strongest docking affinities despite violating several physicochemical criteria.

Therefore, although glycosylated anthocyanins may exhibit comparatively lower predicted oral absorption, their superior receptor binding characteristics suggest that formulation approaches such as nano delivery systems or prodrug strategies could improve their pharmacokinetic behaviour while preserving their excellent antidiabetic potential.

5.6.4 Pharmacokinetic Evaluation

The pharmacokinetic prediction revealed notable differences in the absorption, distribution and metabolic characteristics of the investigated phytochemicals, emphasizing the importance of ADME profiling during early drug discovery. In the present study, Camptothecin, Feselol, Pelargonidin, Protopine, Oxoglaucine and Glycocholic acid exhibited high gastrointestinal absorption, whereas Pelargonidin-3-rutinoside, Cyanidin-3-rutinoside and Ginkgolide B showed low predicted GI absorption. Similar observations have been reported which demonstrated that in silico ADMET evaluation effectively distinguishes compounds with favourable oral absorption from those with poor pharmacokinetic characteristics before experimental validation [96]. Previous studies have reported that compounds possessing lower molecular weight and balanced lipophilicity generally exhibit superior intestinal permeability, whereas highly polar molecules show limited passive diffusion across biological membranes [97]. The low GI absorption predicted for Pelargonidin-3-rutinoside and Cyanidin-3-rutinoside agrees with previous reports describing glycosylated flavonoids as poorly absorbed because the attached sugar moieties markedly increase molecular weight and polarity, thereby restricting membrane permeability [98].

Furthermore, only Feselol, Protopine and Oxoglaucine are predicted to cross the blood brain barrier, whereas the remaining compounds are BBB impermeable. Similar findings have been reported which explained that BBB permeability is strongly influenced by molecular size, lipophilicity and hydrogen bonding capacity [99]. Therefore, the inability of the anthocyanin glycosides to penetrate the BBB is consistent with their elevated topological polar surface area and extensive hydrogen bonding potential observed in the present investigation.

The metabolism profile further differentiated the pharmacokinetic suitability of the selected compounds. Feselol demonstrated the most favourable profile by exhibiting high gastrointestinal absorption, BBB permeability and no predicted inhibition of CYP1A2, CYP2C19, CYP2C9, CYP2D6 or CYP3A4. This finding suggests a comparatively lower probability of metabolic drug drug interactions

and agrees with the observations which emphasized that compounds lacking CYP inhibitory activity are generally associated with improved metabolic safety and a lower risk of adverse pharmacokinetic interactions [100]. Conversely, Protopine and Oxoglaucine inhibited all major CYP isoforms evaluated, while Camptothecin and Pelargonidin inhibited selected CYP enzymes, indicating a greater possibility of altered drug metabolism during co administration with other therapeutic agents. Similar variability in CYP inhibition among phytochemicals has been reported by a previous study which demonstrated that natural products can significantly influence CYP mediated metabolism and P-glycoprotein transport depending upon their structural characteristics [101].

In addition, the present study predicted that most compounds are P-glycoprotein substrates, suggesting that active efflux mechanisms may influence their tissue distribution and oral bioavailability. Comparable findings have been reported that P-glycoprotein plays a central role in limiting intracellular accumulation of xenobiotics and many naturally occurring compounds [102]. Collectively, these findings indicate that although several phytochemicals demonstrated favourable pharmacokinetic behaviour, Feselol exhibited the most balanced ADME profile, making it the most promising candidate for further in vitro and in vivo pharmacokinetic investigations.

5.6.5 Drug Likeness and Medicinal Chemistry

The drug likeness assessment demonstrated that Camptothecin, Feselol, Pelargonidin, Protopine and Oxoglaucine fulfilled all five rule based filters (Lipinski, Ghose, Veber, Egan and Muegge), indicating that these compounds possess physicochemical properties compatible with orally active drug candidates. These findings are consistent with previous reports showing that successful drug candidates generally satisfy multiple drug likeness filters because balanced molecular weight, lipophilicity, hydrogen bonding capacity and molecular flexibility collectively improve oral bioavailability and pharmacokinetic performance. It is highlighted that compounds complying with Lipinski's Rule of Five exhibit a higher probability of

progressing through drug development but the drug likeness parameters remain essential for reducing late stage attrition during lead optimization [103]. Previous studies have reported that compounds occupying optimal chemical space possess greater "chemical beauty" and improved pharmaceutical potential [104].

In agreement with these observations, the present study identified Camptothecin, Pelargonidin and Protopine as highly drug like molecules. Conversely, Pelargonidin-3-rutinoside and Cyanidin-3-rutinoside failed all evaluated filters because of their high molecular weights, elevated topological polar surface areas and excessive hydrogen bonding capacity. Similar limitations of glycosylated flavonoids have been reported in studies evaluating anthocyanin derivatives, where attached sugar moieties substantially increase molecular size and polarity, thereby reducing oral drug likeness despite strong biological activity [105]. Glycocholic acid and Ginkgolide B exhibited only partial compliance with the evaluated rules, suggesting that although they retain pharmacological potential, further structural optimization may be required to improve their drug like properties. Overall, these findings indicate that simpler phytochemical scaffolds generally possess greater pharmaceutical suitability than highly glycosylated natural products, supporting previous observations reported in computational drug discovery studies.

The medicinal chemistry evaluation further strengthened the selection of promising lead compounds by identifying structural alerts, lead likeness and synthetic accessibility. Most investigated compounds produced no PAINS alerts, indicating a low probability of nonspecific biological activity or false positive screening results. These observations agree with Baell and Holloway, who developed PAINS filters to eliminate compounds capable of producing misleading bioassay outcomes and emphasized that naturally occurring compounds should not be rejected solely because they contain PAINS motifs, but instead require careful experimental validation [106].

In the present study, only Protopine and Cyanidin-3-rutinoside generated PAINS alerts, suggesting that their biological activities should be interpreted cautiously during future experimental studies. Likewise, Camptothecin, Pelargonidin and Protopine satisfied lead likeness criteria, whereas Feselol, Oxoglaucine and the

glycosylated flavonoids showed one or more violations, mainly because of molecular weight or structural complexity. Comparable findings are reported by which noted that natural products with relatively simple molecular architectures are more amenable to lead optimization than structurally complex metabolites [96]. Moreover, the lower synthetic accessibility scores observed for Pelargonidin, Protopine and Camptothecin indicate that these compounds may be synthesized or structurally modified more readily than Cyanidin-3-rutinoside and Ginkgolide B, whose higher scores reflect increased synthetic complexity.

Similar conclusions have been reported previously that computational medicinal chemistry assists in identifying compounds with favourable structural quality before costly laboratory investigations and combining medicinal chemistry filters with computational predictions substantially improves lead selection during early drug discovery. Collectively, the present medicinal chemistry analysis supports Camptothecin, Pelargonidin and Protopine as the top lead compounds for subsequent optimization and biological validation.

Although rule based filters are valuable for prioritizing compounds during early drug discovery, they should not be considered absolute determinants of therapeutic potential. Several clinically important natural product derived drugs violate one or more drug likeness rules yet remain pharmacologically effective because they utilize active transport mechanisms, undergo metabolic conversion or exhibit exceptional target affinity.

Therefore, although Pelargonidin-3-rutinoside and Cyanidin-3-rutinoside showed multiple violations in the present study, their excellent docking performance suggests that structural modification or advanced formulation strategies could improve their pharmaceutical potential without compromising biological activity. Conversely, the favourable balance of drug likeness, medicinal chemistry properties and molecular simplicity observed for Camptothecin, Pelargonidin and Protopine indicates that these compounds require comparatively less optimization before progressing toward experimental validation. These findings reflect the importance of combining docking studies with drug likeness and medicinal chemistry analyses to identify natural compounds possessing both high biological activity

and acceptable pharmaceutical characteristics, thereby increasing the probability of successful lead optimization and future therapeutic development.

5.6.6 Water Solubility Predictions

Water solubility is a crucial physicochemical parameter influencing drug absorption, dissolution rate and ultimately oral bioavailability. In the present study, the ESOL, Ali and SILICOS-IT models consistently predicted that most of the investigated phytochemicals possessed acceptable aqueous solubility, although some variability was observed among the three computational approaches. Pelargonidin-3-rutinoside and Cyanidin-3-rutinoside exhibited the highest predicted solubility, whereas Oxoglaucine demonstrated the lowest solubility, particularly in the SILICOS-IT model. These findings agree with previous observations which developed the ESOL model and reported that compounds containing numerous hydroxyl groups and sugar residues generally possess higher aqueous solubility than less polar molecules [107]. It demonstrated that SwissADME integrates multiple solubility prediction algorithms because no single computational model accurately predicts the behaviour of structurally diverse compounds [15]. The excellent solubility predicted for Pelargonidin-3-rutinoside and Cyanidin-3-rutinoside is consistent with previous reports describing anthocyanin glycosides as highly water soluble owing to their multiple hydroxyl groups and glycosidic substitutions [108].

However, despite their superior solubility, these compounds displayed poor drug likeness and limited gastrointestinal absorption in the present study. This apparent contradiction reflects the well established principle that high aqueous solubility alone does not guarantee efficient oral absorption, as excessive polarity may reduce passive membrane permeability. Therefore, an optimal balance between solubility and permeability remains essential for successful oral drug candidates.

The moderate to poor solubility predicted for Feselol, Protopine and particularly Oxoglaucine is also supported by previous investigations on lipophilic alkaloids and coumarin derivatives. It is reported that increased aromaticity and reduced hydrogen bonding capacity frequently decrease aqueous solubility despite favourable

biological activity [109]. The poorer solubility predicted for Oxoglaucine compared with the other investigated compounds may therefore be attributed to its relatively hydrophobic aromatic scaffold and lower capacity to interact with water molecules. Nevertheless, the agreement among the three prediction models for most compounds increases confidence in the computational findings, while the discrepancies observed for Oxoglaucine highlight the influence of different prediction algorithms on solubility estimation.

Similar inter model variation has been reported in which distinct computational methods frequently generate different absolute LogS values while maintaining similar overall ranking trends [110]. Consequently, although the present in silico analysis indicates favourable aqueous solubility for most phytochemicals, experimental solubility determination remains necessary to validate these predictions. Overall, the combined results suggest that anthocyanin glycosides possess excellent dissolution characteristics but comparatively poor permeability, whereas compounds such as Camptothecin, Pelargonidin and Feselol exhibit a more stable physicochemical profile that may be advantageous for subsequent pharmaceutical development.

5.6.7 Organ Toxicity

The in silico organ toxicity assessment demonstrated that most of the selected phytochemicals possessed a comparatively favorable safety profile, with hepatotoxicity being predicted only for Oxoglaucine, whereas Ginkgolide B remained inactive for all evaluated organ toxicities except respiratory toxicity. These results are coherent with previous computational toxicity investigations showing that many naturally occurring phytochemicals exhibit low predicted organ toxicity despite possessing significant biological activities. Studies employing Protox-II/Protox 3.0 have similarly shown that flavonoids, anthocyanins, and terpene derived compounds generally possess low hepatotoxic and cardiotoxic liabilities, making them attractive candidates for drug discovery [111, 112]. The absence of predicted hepatotoxicity for Feselol, Pelargonidin, Cyanidin-3-rutinoside, and Pelargonidin-3-rutinoside agrees with reports describing anthocyanins and related polyphenols as relatively

safe compounds with protective effects against oxidative liver injury rather than hepatotoxicity.

Likewise, the favorable toxicity profile observed for Ginkgolide B corresponds with previous pharmacological studies indicating low systemic toxicity and acceptable safety margins for ginkgolides during preclinical evaluations [113]. The predicted hepatotoxicity of Oxoglaucine, however, differs from some earlier reports describing aporphine alkaloids as moderately safe natural products. This discrepancy may result from structural differences among alkaloids, variations in prediction algorithms, or differences between computational prediction models and experimental biological responses. Such variations emphasize that computational toxicity prediction should be performed as an early screening tool rather than definite proof of toxicological behavior.

Neurotoxicity predictions identified Camptothecin and Protopine as potentially active, while nephrotoxicity was predicted for Camptothecin, Feselol, Pelargonidin, Glycocholic acid, Pelargonidin-3-rutinoside, and Cyanidin-3-rutinoside. These observations are partially supported by published studies demonstrating that camptothecin derivatives may induce neurological and renal adverse effects because of their potent inhibition of DNA topoisomerase I and subsequent effects on rapidly dividing cells [114]. Similarly, Protopine has been reported to interact with multiple neuronal signaling pathways, which may explain its predicted neurotoxicity despite its documented pharmacological benefits [115]. In contrast, anthocyanins such as pelargonidin and cyanidin glycosides are generally regarded as safe dietary phytochemicals with antioxidant and nephroprotective properties in experimental studies, making their predicted nephrotoxicity different from many biological reports. This inconsistency may arise because in silico models evaluate intrinsic structural alerts independent of dose, metabolism, antioxidant defense systems, and physiological repair mechanisms that influence toxicity in vivo. The universal prediction of respiratory toxicity across all compounds may also reflect conservative computational screening rather than confirmed biological toxicity, highlighting the necessity for experimental validation. Overall, these findings support the usefulness of Protox 3.0 as an initial toxicity screening platform while reinforcing that

definitive safety assessment requires integrated in vitro, in vivo and clinical investigations before these phytochemicals can be considered therapeutic candidates.

5.6.8 Toxicity End Points

The computational toxicity endpoint analysis indicated that most of the investigated phytochemicals exhibited a relatively acceptable safety profile, with only a limited number of predicted adverse effects. Among the evaluated compounds, Pelargonidin and Glycocholic acid demonstrated the most favorable toxicity patterns, whereas Protopine exhibited the greatest number of predicted toxicological alerts, including carcinogenicity, mutagenicity, immunotoxicity, and clinical toxicity. These findings are generally consistent with previous computational toxicology studies showing that naturally occurring flavonoids and anthocyanins usually possess lower predicted toxic potential than alkaloids because their antioxidant properties and long history of dietary exposure contribute to improved safety profiles [111, 112].

Similarly, investigations employing Protox-II and other machine learning based toxicity prediction platforms have shown that anthocyanin derivatives such as pelargonidin and cyanidin glycosides are generally anticipated to be non carcinogenic and non mutagenic, supporting the present observations [111, 116].

The predicted absence of carcinogenicity for Camptothecin, Feselol, Pelargonidin, Glycocholic acid, Cyanidin-3-rutinoside, and Ginkgolide B also agrees with published pharmacological evidence describing these compounds as biologically active but not inherently carcinogenic under normal therapeutic conditions. However, the carcinogenic and mutagenic predictions observed for Protopine differ from several experimental studies reporting its anti inflammatory, antioxidant, and neuroprotective activities with relatively low toxicity. These discrepancies are likely attributable to the structural alert based nature of computational models, which identify potential toxicophores without considering metabolic detoxification, dose dependency, or pharmacodynamic responses observed in biological systems.

The prediction of immunotoxicity for most compounds, including Feselol, Protopine, Oxoglaucine, Cyanidin-3-rutinoside, and Ginkgolide B, should be interpreted cautiously because computational platforms frequently classify compounds capable of modulating immune pathways as potentially immunotoxic. Previous studies have emphasized that in silico toxicity tools provide conservative predictions designed to minimize false negative safety assessments, often overestimating toxicity compared with experimental findings [15, 117]. Likewise, the predicted cytotoxicity of Camptothecin corresponds well with its well established anticancer mechanism involving DNA topoisomerase I inhibition, where selective cytotoxicity toward rapidly proliferating cells represents its desired pharmacological action rather than generalized toxicity [114].

In contrast, Feselol was predicted to be cytotoxic despite exhibiting favorable ADME and drug likeness characteristics, suggesting that its biological activity may require careful dose optimization during future investigations. The ecotoxicity predictions for Feselol, Pelargonidin, and Oxoglaucine may also reflect environmental hazard alerts rather than direct human toxicity, a distinction frequently highlighted in computational toxicology literature [118]. Overall, the present findings reinforce the value of Protox 3.0 as an efficient early stage toxicity screening tool while emphasizing that computational predictions should be complemented with in vitro, in vivo, and eventually clinical toxicological evaluations before these phytochemicals can be advanced as potential therapeutic agents.

Chapter 6

Conclusion

The present study was performed to isolate the endophytes from *Berberis lycium* and to evaluate the therapeutic potential of *Berberis lycium* and its isolated fungal endophytes by using microbiological, phytochemical, pharmacological and computational techniques. The present study was designed so as to explore that the medicinal properties of *Berberis lycium* mentioned in ethnopharmacology could be supported experimentally and whether the endophytic fungi isolated from it could be used as an alternative source for obtaining its pharmacologically active compounds.

The extraction of the plant and its phytochemical analysis showed that it contains a vast variety of chemical entities as its secondary metabolites, with the roots being the richest source of phytochemicals. Methanol produced the highest extraction yield for all parts of the plant as compared to ethyl acetate and proved to be a more efficient solvent than ethyl acetate. These findings support the ethnopharmacological importance of *Berberis lycium* (particularly its roots) as the richest source of biologically active compounds.

In total, seven endophytes are obtained from various plant sections, comprising four fungal isolates and three bacterial isolates. Molecular identification showed that the fungal isolates belong to the genera *Epicoccum* and *Fusarium*. To the best of our knowledge, this is the first study conducted on the isolation and molecular

identification of fungal endophytes from *Berberis lycium* collected from Kashmir, Pakistan. The variations observed in the yield of metabolites and phytochemical content among the fungal isolates suggest that these microorganisms have varied biosynthetic abilities and serve as potential sources of natural bioactive compounds.

The biological assessment further showed that both the plant extracts and fungal endophytic extracts have significant pharmacological effects. The methanol extract of roots exhibited the most potent antibacterial effect against *Escherichia coli* and *Staphylococcus aureus*, whereas various fungal extracts also inhibited the growth of bacteria. In the same way, both plant and fungal extracts showed antifungal potential against *Saccharomyces cerevisiae*, validating the antimicrobial capabilities of *Berberis lycium* and its related endophytes.

The *in vivo* anti inflammatory study demonstrated that the methanolic root extract significantly reduced carrageenan induced paw edema in mice in a dose dependent manner. The highest tested dose produced an effect comparable to the standard drug diclofenac sodium, providing scientific evidence for the ethnopharmacological use of *Berberis lycium* in the management of inflammatory disorders.

The computational studies further strengthened the experimental findings. Molecular docking identified several phytochemicals with strong binding affinity towards α-amylase and dipeptidyl peptidase IV (DPP-IV), two important therapeutic targets involved in diabetes mellitus. In addition, ADMET analysis predicted favorable physicochemical, pharmacokinetic, and safety profiles for the top ranked compounds, suggesting their suitability for further drug development.

Overall, the findings of this study successfully addressed the proposed research questions. The study established that *Berberis lycium* harbors previously unreported fungal endophytes, that both the plant and its fungal metabolites possess antibacterial and antifungal activities, that the methanolic extract exhibits significant anti inflammatory activity, and that several known phytochemicals of the plant possess promising antidiabetic potential supported by molecular docking and ADMET analysis.

Overall, the present study provides new scientific evidence supporting the medicinal value of *Berberis lycium* and highlights its fungal endophytes as promising reservoirs of therapeutically important secondary metabolites.

The integration of phytochemical analysis, endophyte isolation, biological evaluation, molecular docking, and ADMET prediction provides a comprehensive foundation for future natural product research and contributes to the discovery of novel compounds that may be developed into effective therapies for the management of infectious diseases, inflammation, and diabetes.

6.1 Future Prospects

The present findings provide several opportunities for further research. Separation and structural characterization of all bioactive compounds present in fungal extracts should be carried out using advanced analytical techniques such as MS, LC–MS, GC–MS and NMR spectroscopy.

The antimicrobial potential of both plant and fungal extracts should be further investigated by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) against a broad variety of clinically important microorganisms, including multidrug resistant pathogens. The antidiabetic potential predicted through molecular docking should be validated by *in vitro* enzyme inhibition assays against α-amylase, α-glucosidase and DPP-IV followed by evaluation in appropriate diabetic animal models.

Future metabolomic and genomic studies should compare the secondary metabolites produced by *Berberis lycium* and its fungal endophytes to determine whether the endophytes synthesize the same or structurally related bioactive compounds, particularly berberine and other medicinal alkaloids. Optimization of fermentation conditions including culture media, incubation period, pH and nutritional factors may enhance the production of valuable fungal secondary metabolites and enhance their pharmaceutical applications.

Molecular dynamics simulations should be performed for the most promising phytochemicals to assess the stability of ligand–protein complexes and to complement the molecular docking results.

Finally, comprehensive pharmacokinetic, toxicity, and mechanistic studies are required before the most promising plant derived or endophytic compounds can be considered for preclinical and clinical development. Such investigations may ultimately contribute to the development of safe, effective, and sustainable natural therapies for the treatment of infectious diseases, inflammation, diabetes and other chronic disorders.

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