Berberis Species of Pakistan: A Rich Source of Phytochemicals with Reducing Potential for Green Synthesis of Metallic Nanoparticles and as an Alternate Host to Fungal Pathogens

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2022

Berberis Species of Pakistan: A Rich Source of Phytochemicals with Reducing Potential for Green Synthesis of Metallic Nanoparticles and as an Alternate Host to Fungal Pathogens

A PhD dissertation submitted in the partial fulfillment for the degree of Doctor of Philosophy (PhD) in Plant Sciences

By

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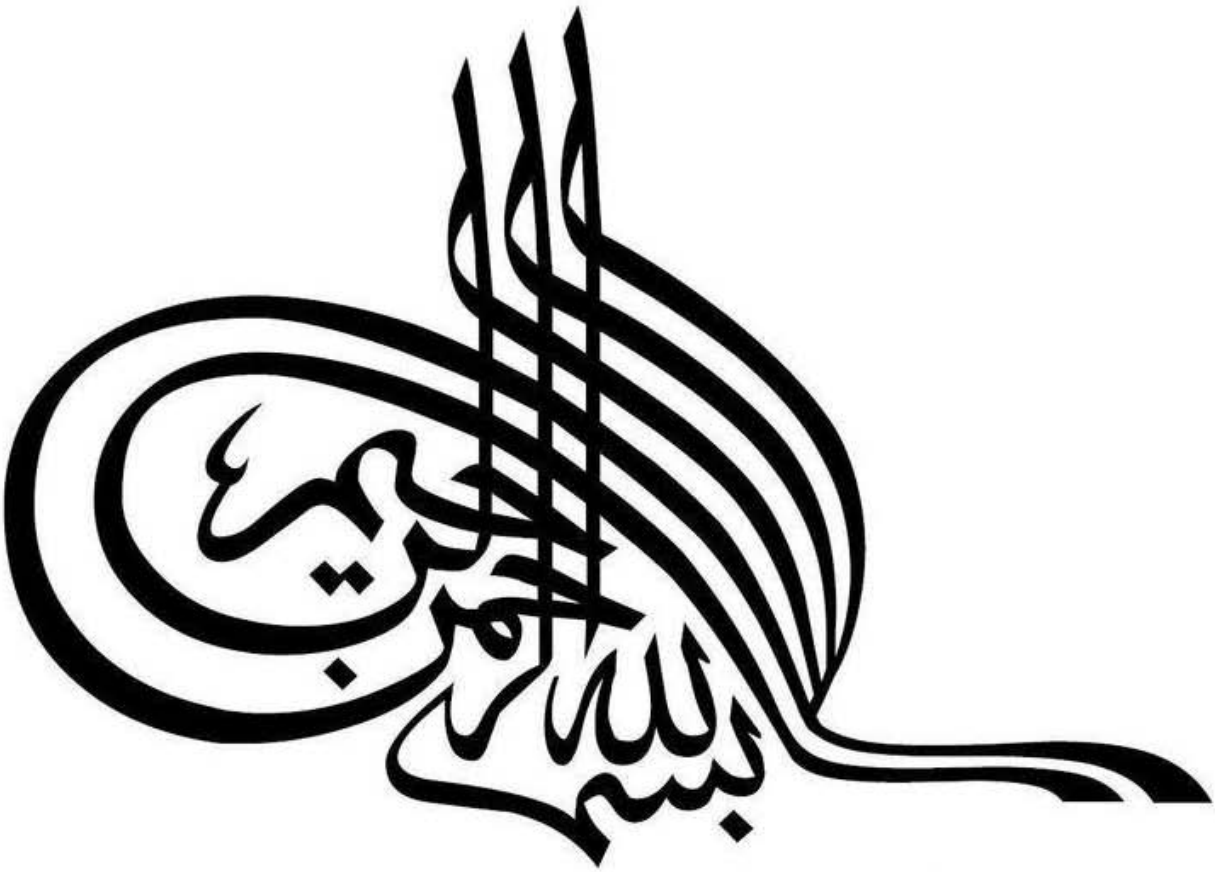
2022



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My Most Respected Mother, Sisters & Wife

&

To the Humanity that has been chastised by the Terrorism

&

To my most respectable supervisor Dr. Umar Masood Quraishi

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. Countless salutation upon the **Holy Prophet (Peace Be Upon Him)**, source of knowledge and blessings for entire creation, who has guided his Ummah to seek knowledge from cradle to grave and enabled me to win honor of life.

My special words of thanks should also go to my great supervisor **Dr. Umar Masood Quraishi** for always being so kind, helpful and motivating. He has been a living role model to me, taking up new challenges every day, tackling them with all his grit and determination and always thriving to come out victorious. His attitude of living every moment as it comes, making serendipitous observation and converting them to new possibilities, correlating ideas and understanding the obvious has helped me come a long way and will always guide me in future. His invaluable help of constructive comments and suggestions throughout the experimental and thesis works have contributed to the success of this research. His assistance during the presented work is gratefully acknowledged. I feel privileged to be his student once, a feeling that every student of his will second.

Most importantly, none of this would have been possible without the love and patience of my family to whom this dissertation is dedicated to, has been a constant source of love, concern, support and strength all these years. They have helped me financially, morally and spiritually. They always gave me words of encouragement. Special thanks to my sisters and fiancée **Sabiha Laila** for their endless love, prayers and encouragement.

I couldn't be thankful enough to **Dr. Karanjeet Sandhu, Dr. Rushen and Dr. Robert Park** from **Plant Breeding Inst., University of Sydney** for helping me during my 6 months scholarship under HEC programme IRSIP (Int. research support initiative programme) for which I am very thankful to HEC (Higher Education Commission), Pakistan.

I would like to thank **Dr. Mushtaq Ahmad,** Chairperson, Department of Plant Sciences, Quaid-i-Azam University, Islamabad for providing necessary support. I would also like to thank all my other teachers who even taught me a single word during my student life and research career.

No acknowledgement could ever adequately express my feelings to my affectionate and adorable It's my fortune to gratefully acknowledge the support of some special individuals. Words fail me to express my appreciation to **Dr. Zhaid Mehamood and Sadia Lateef** for their support and generous care. They were always beside me during the happy and hard moments

to push me and motivate me. Thank you doesn't seem sufficient, but it is said with appreciation and respect to all of them for their support, encouragement, care, understanding and precious senior ship. Our association goes a long way and they have been with me ever since then as friends and as companions. It's my good luck to have true seniors. The good time spent with them can never be erased from my memories.

My acknowledgement will never be complete without the special mention of my lab fellows, **Sadia Lateef, Luqman Bin Safdar, Fakhra, Rizwana Nawaz, Muhammad Arif, Saeedur Rehman, Said Akbar, Abbass Mastan, Jawad, Adil Hussain, Saeed Anwar, Minhas Elahi** and **Muhammad Anas** who have taught me the lab work and have lived by example to make me understand the hard facts of life.

Many friends Mehmood khan, **Mehran Ayub, Arif, Saeed Anwar, Dr. Abrar, Sajid Ali, Dr. Javed Iqbal, Dr. Musrat Ali, Dr. Iram Fatima, Dr. Benazir Hasan Abbasi, Nisar hazara, Munir Ahmed Khan, Dr. Mir Afzal, Dr. Niamatullah, Amjad uncle, Saeed kamoka and Ahsan Nawaz** have helped me stay sane through these difficult years. I greatly value their company and I deeply appreciate their belief in me. I am immensely thankful to **Dr. Amir Sultan, Dr. Syed Afzal Shah, Dr. Mushtaq** and **Dr. Javed Mirza** for helping me in plant identification. To all my friends who have lent a hand to complete this thesis, specially to **Luqman Bin Safdar, Dr. Javed Iqbal, Mahmood khan, Dr. Iram fatima, Dr. Musrat, Dr. Saleem** and **Muhammad Anas** for the words of encouragement and for helping me out with my simulations, thank you from the bottom of my heart. Words are short to express my deep sense of gratitude towards my following friends **Luqman Bin Safdar, Amir Kesrani, Jawed Abbassi, Javed jalbani, Dr. Afzal Shah, Maqsood Afridi, Waseem, Dr. Musrat, Gul Yaseen, Maria, Raoof tareen, Neamat, Saeed Baber, Imtiaz, Hafiz Bilal, Zain, Amal Baloch** and **Guddu Tea.** This list is endless. I owe my gratitude to all those people who have made this dissertation possible and because of whom my research experience has been one that I will cherish forever.

Siraj Uddin

PUBLICATIONS

S.NO	Title	Status	Journal Name	Impact Factor	Category	Year
1.	Green Synthesis of Nickel Oxide Nanoparticles from *Berberis* balochistanica Stem for Investigating Bioactivities	Published	Molecules	4.411	W	2021
2.	Green synthesis of nickel oxide nanoparticles using leaf extract of *Berberis* balochistanica: Characterization, and diverse biological applications	Published	Microscopy Research and Technique	2.117	W	2021

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ABBREVIATIONS

Abbreviations	Full form
BCR	*Berberis calliobotrys* Root
BRR	*Berberis baluchistanica* Root
BPR	*Berberis pachyacantha* Root
BOR	*Berberis orthobotrys* Root
BPrR	*Berberis parkeriana* Root
BBR	*Berberis baluchistanica* Root
BLR	*Berberis lyceum* Root
BPsR	*Berberis pseudumbellata* subsp. *Gilgitica* Root
BPrL	*Berberis parkeriana* Leaf
BPrS	*Berberis parkeriana* Stem
BPsS	*Berberis pseudumbellata* subsp. *Gilgitica* Leaf
BPsL	*Berberis pseudumbellata* subsp. *Gilgitica* Leaf
BBL	*Berberis baluchistanica* Leaf
BRL	*Berberis baluchistanica* Leaf
BOL	*Berberis orthobotrys* Leaf
BCL	*Berberis calliobotrys* Leaf
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
DPPH	2, 2-diphenyl-1-picrylhydrazyl
TAC	Total Antioxidant Capacity
TRP	Total Reducing Power
R	Correlation coefficient
IC	Inhibitory concentration
UNICEF	United Nations International Children's Emergency Fund
HPLC	High Performance Liquid Chromatography
ANOVA	Analysis of Variance
XLSTAT	Microsoft Excel® XLSTAT
NARC	National agricultural Research center
PCA	Principal Component Analysis
PCI	Principal Component 1
PCII	Principal Component 2

GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent
CNS	Central nervous System
Na	Sodium
K	Potassium
Fe	Iron
Mg	Magnesium
Zn	Zinc
Cu	Copper
Cr	Chromium
Ppm	Parts Per Million
AS	Absorbance of sample
AC	Absorbance of control
EDTA	Ethylenediamine tetra acetic acid
FTIR	Fourier transform infrared
HRBC	human red blood cells
MDR	MultiDrug-Resistant
PBS	Phosphate buffered saline
IC	inhibitory concentration
DNA	Deoxyribonucleic acid
Mg	Milligram
mL	Milliliter
µL	Microliter
LC	Lethal concentration
SG	speed of germination
KS	kanamycin sulfate.
RBC	Red blood cells
UTI	urinary tract infection
UV	Ultraviolet
R	Root
S	Stem
L	Leave

T	Treatment
C	Control
NiONPs	nickel oxide nanoparticles
BBS- NiONPs	*Berberis balochistanica* Stem-Nickel oxide nanoparticles
BBL- NiONPs	*Berberis balochistanica* leaf-Nickel oxide nanoparticles
XRD	X-ray diffraction
EDS	Energy dispersive spectroscopy
SEM	scanning electron microscopy
TAC	total antioxidant capacity
ROS	Reactive Oxygen Species
SDA	Sabouraud Dextrose Agar
BBS	Berberis balochistanica stem
TFC	total flavonoid contents
SSR	Simple Sequence Repeat
RFLP	Restriction Fragment Length Polymorphism
Ug99	Uganda 1999
US	United state
WSR	Wheat stem rust
WYR	Wheat strip rust
OSR	Oat stem rust
Berberis spp.	*Berberis* species
PCR	Polymerase Chain Reaction
CTAB	Cetyl Tri-methyl Ammonium Bromide
SDS	Sodium dodecyl sulfate
ITS	Internal transcribed spacer
EDTA	Ethylenediamine tetraacetic acid
TE	tris-EDTA
Taq	Thermus aquatic
V	Volte
H	Hour
Pg	*Puccinia graminis*
ng/ul	Nano gram per micro-litter

f.sp	formae speciales
Bp	Base pair

ABSTRACT

Berberidaceae family, is considered as one of the most beneficial in traditional medicine. In early 1900, this family was reported to be alternate host to various cereal pathogens. Thus, making the plants of this family both friend and foe. This dissertation illustrates both beneficial and damaging potentials of *Berberis* species of Pakistan. Exploring the traditional medical plants for bioactive compounds, has been pivotal part of modern medicine. Various bioactive compounds have been identified, characterized and utilized from *Berberis* species in recent past. Almost all of these studies focused on the bioactive compounds of the roots of *Berberis* species. Therefore, in the present study phytochemicals, nutritional and biological profile is carried out using various organs of the *Berberis* plant. In the present study, we aimed to analyse three parts (root, stem and leaf extracts) of eight selected *Berberis species* as alternate and suitable means for phytochemicals, nutritional, and antioxidant purposes through the conservational approach. There are limited scientific studies that confirm the detailed biological potential of *Berberis* species. Moreover, in present study we examined all parts of dominant *Berberis* species of Pakistan. The study illustrates the presence of significant concentrations of mineral elements, phytochemicals, and antioxidant potential. Thus, can be utilized as a natural antioxidant, antimicrobial and nutritional complements in herbal and food industries.

Spectroscopic profile revealed that all *Berberis* species comprise a rich source of elements in decreasing order *B. balochistanica* > *B. royleana* > *B. parkeriana* > *B. pseudoumbellata* > *B. pachyacantha* > *B. calliobotrys* > *B. lyceum* > *B. orthobotrys.* The HPLC results revealed that berberine was abundantly present in the root extracts of all selected species. Similarly, spectroscopic profile exhibited highest total phenolic (TPC) and total flavonoids contents (TFC) in *B. pseudoumbellata, B. royleana, B. pachyacantha* and *B. balochistanica.* In addition, all selected species showed antioxidant activity, but leaf showed best antioxidant potential as compared to roots and shoot. This is first report of essential nutrients and phytochemical compounds with high antioxidants activity indicating comparison between roots and shoots. These findings will be useful in the conservation of medicinal plants by discouraging the uprooting of underground parts for medicinal purposes.

To demonstrate the biological application of *Berberis* species, *B. balochistanica* plant with an astonishing amount of minerals and antioxidant activities was selected as model organism in present dissertation. *B. balochistanica,* is extensively used by local people as traditional medicine. Extensive utilization of the roots of this plant by local community has led

to depletion of this plant in Baluchistan province of Pakistan. Little is known about the phytochemical and biological significance of its alternative parts of these distinct species. Thus, this study was conducted using a rational approach by substituting the underground parts with renewable aerial parts to decrypt phytonutritional profile along with antioxidant, antibacterial, antifungal, antihaemolytic, DNA damaging, cytotoxic and phytotoxic potentials for comparison with roots. It was observed that all three plant parts; roots, stem, and leaves of *B. balochistanica* showed substantial antihaemolytic, DNA protecting and cytotoxic activities. Additionally, all parts particularly leaf extract retarded the bacterial growth of *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli*. Similarly, extracts of all parts reduced the mycelial growth of all tested fungal pathogens. Hence, these results signify a remarkable biological potential of *B. balochistanica*, especially its aerial parts. This study will recommend preserving of this endemic plant by discouraging the usage of its roots.

The utilization of plant natural material in bio fabrication of nanoparticles is recognized as a green technology. The present study revealed the importance of aerial parts of *Berberis* plants in the green synthesis of nanoparticles. The presence of valuable phytochemicals in stem and leaf extracts of *B. balochistanica* helped in stabilizing, capping, and reducing nickel salt into BB-NiONPs. The synthesis of nanoparticles was confirmed by various microscopic and spectroscopic techniques like UV visible, FTIR, XRD, EDX and SEM. Both stem and leaf extracts of *B balochistanica* successfully reduce and capped the NiONPs with fine size (31.44 nm and 21.00 nm) and crystalline rhombohedral shape. Both nanoparticles (BBS-NiONPs and BBL-NiONPs) were exposed to multiple *in vitro* and *in vivo* bioactivities to ascertain their beneficial biological applications. They exhibited strong antioxidant activities in terms of total antioxidant capacity (64.77 and 59.59 %) and 2, 2-diphenyl-1-picrylhydrazyl (71.48 and 69.98 %); and cytotoxic potential with IC_{50} (10.40 and 49.10 µg/mL). The synthesized nanoparticles restricted the bacterial and fungal pathogenic growth at 1000, 500 and 100 µg/mL. Additionally, these nanoparticles showed stimulatory efficacy by enhancing seed germination rate and seedling growth at 31.25 and 62.5 µg/mL. In aggregate, both extracts have a remarkable number of bioactive compounds which makes the green biosynthesis of NiONPs easy, economical, and safe. The biochemical potential of BBS-NiONPs BBL-NiONPs can be useful in various biomedical and agricultural fields, and could be used as nanomedicine and nano fertilizer.

On the other hand, the Barberry eradication program, initiated by federal and state cooperative plant disease control campaign in United States started in 1918. The aim of the

program was to eradicate common barberry (*B. vulgaris*) in efforts to reduce *Puccinia graminis f. sp. tritici* impact on wheat production. Barberry is an alternate host to various fungal pathogens of important cereal crops. Importantly, all these pathogens complete their sexual life cycles on Barberry plants. Thus, this plant plays a pivotal role to help in the emergence of new races after genetic recombination during the aecial stage. Different *Berberis* species also serve as a seasonal bridge for stem and stripe rust pathogen in Pakistan and neighbouring countries. In this study the diversity of rust on *Berberis* spp. was investigated at molecular and morphological levels. Simple Sequence Repeat (SSR) markers were used to investigate the presence of different rust on Barberry collected from four mountainous regions of Pakistan. Based on aecial growth and spore morphology, rust was divided into two groups *i.e.,* localized (*Puccina graminis*) and systematic (*Puccina arrhenatheri*). In total, 25 of 46 SSR markers were found to be useful for the screening of selected rusts collected from barberry. SSR analysis revealed three *Berberis* species namely *B. balochistanica*, *B. pachyacantha* and *B. lycium* as alternate hosts of wheat stem rust (WSR), while *B. lycium* was also identified as an alternate host of oat stem rust (OSR). However, no barberry was recognized as an alternate host of wheat yellow rust (WYR) in natural conditions. Overall, this study has confirmed that barberry serves as an alternate host for only stem rust in Pakistan.

Keywords: medicinal plants*; Berberis* species; Phytochemicals, Nutritional and biological potentials, Plant parts substitution; Green synthesis; NiONPs; Biological and Nano fertilizer applications; Wheat stem rust, Wheat strip rust, SSR markers, Aecium.

INTRODUCTION

From Neolithic era, necessities of human life like shelter, food and clothing have been derived from plants. Along with the basic requirements, these autotrophs have also been used to treat various diseases ranging from common cold to deadly cancer *etc.* Hence, medicinal plants have been the primary health care sources since the dawn of human civilization (Fierascu *et al.*, 2018). Plants containing bio-active ingredients with potent biological activities are recognized as medicinal plants. Approximately, 80 % of the world population depends on herbal plants for medicine. Herbal medicines are economical, easily available, and safe (Bodeker and Ong, 2005). Local communities use medicinal plants against different diseases such as common cold, typhoid, kidney pain, blood purification, joints pain, hair fall, chest infections, jaundice, pimples, toothache, high blood pressure, diabetes, cancer, snake and insects bites (Baloch *et al.*, 2013). In last few decades, synthetic drugs and antibiotics are getting great attention due to better development and targeted actions. However, due to high cost, toxic nature and increasing resistance of microbes against synthetic drugs, the medicinal plants are regaining strong position in traditional and modern medicinal system (Gupta and Birdi, 2017). These medicinal values of herbal plants are the consequence of the existence of various types of phytochemicals including alkaloids, polyphenols, flavonoids, tannins, steroid and terpenoids. Medicinal plants are rich in natural antioxidants, commercially utilized as nutritional supplements or as antioxidant additives (Škrovánková *et al.*, 2012). The phytochemical rich medicinal plants have great demand and are used as alternative natural drug agents in those areas where synthetic drugs are not available or affordable (Mustafa *et al.*, 2017). Pakistan is hosting a prosperous and distinct flora of nearly 5,700 plant species. Of 5,700 plant species , 2000 showed therapeutic characteristics (Ullah, 2017). Most of the species (70 %) are confined to one region, while remaining (30 %) are multiregional in nature. The extensive uses of medicinal plants by local inhabitants, venders, drug dealers and collectors jeopardize the medicinal plants of Pakistan (Shinwari, 2010). Nanotechnology is an emerging field with great potential in medical sciences. Nanoparticles made from herbal plants using green synthesis approach are gain more attention due to the presence of various bioactive compounds. For this purpose, various parts of plants such as leaves, stem, root, seed and fruits are used to synthesize various types of nanoparticles (Narayanan and Sakthivel, 2008). Plants are preferred in green synthesis of nanoparticle because they contain effective reducing agents (Ghazal *et al.*, 2021). For this propose, various parts of plants such as leaves, stem, root, seed and fruits have been used to synthesize various types of nanoparticles (Narayanan and

Sakthivel, 2008). *Berberis* species contains valuable ecofriendly secondary metabolites such as phenols, flavonoids, alkaloids, carotenoids and vitamins (Baloch *et al.*, 2013) which are useful for the green synthesis of nanoparticles because they serve as effective reducing agents during green synthesis. On the basis of their safe, nontoxic nature and presence of biomolecules, *Berberis* plant is one of the safest choices to be used in green synthesis of nanoparticles. Recently, different *Berberis* species have been used in green synthesis of various nanoparticles with potent biological properties (Arumugam *et al.*, 2021; Guo *et al.*, 2021).

Berberis species were reported to be the alternate host for various fungal pathogens including wheat rust pathogens in early 19th century. which lead to eradication campaign of Berberis from various parts of the Europe and America (Levine and Cotter, 1932; Kolmer *et al.*, 2007; Barnes *et al.*, 2020; Rodriguez-Algaba *et al.*, 2021).

1.1. Genus; *Berberis*

The genus, *Berberis*, belongs to family *Berberidaceae* with ~17 genera and 650 species (Rao *et al.*, 1998). This family was introduced by Jussieu A.L as "Berberides" and has been considered as the most aboriginal angiosperm (Jussieu, 1789; Brückner, 2000; Anup *et al.*, 2010). The distinguished feature of this genus is spiny stem with yellow wood, evergreen or deciduous, shrubby or small tree with yellow flowers (Rajasekaran and Pant, 2008; Mohi-Ud-Din *et al.*, 2021; Sobhani *et al.*, 2021).

Plants of genus "*Berberis"* are well known for their medicinal properties all over the world. Ethnobotanically, *Berberis* plants are used in treatment of sore eyes, healing of bones, internal injuries, curative piles, gonorrhoea, acute conjunctive, unhealthy ulcers and in

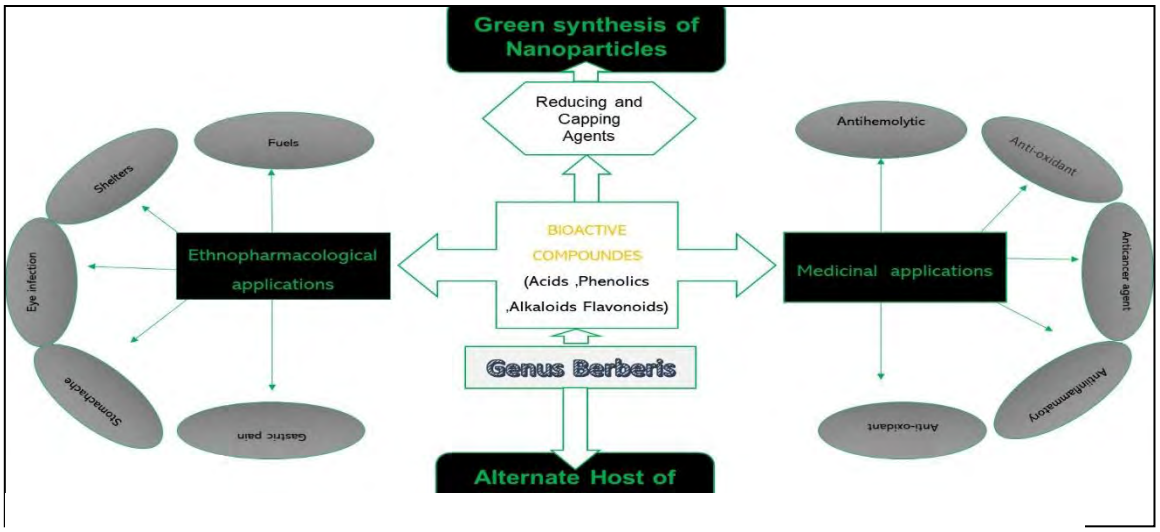

Figure 1: Beneficial and damaging potentials of *Berberis* species in various fields.

persistent ophthalmic (Rehman *et al.*, 2018). The plants of this family are famous for their medicinal properties with hidden medicinal traces in their fruits, barks and roots (Manan *et al.*,

2007). In literature, different parts of *Berberis* species have been reported to have bioactive compounds against chronic inflammatory disorders and have shown strong anti-convulsion, hypoglycemic, antioxidant, anticancerous, antihypertensive, anti-histaminic, anti-fatigue, anticoagulant, antidiabetic, osteolytic, antimicrobial, immuno-stimulant, hypotensive, CNS-depressant, anti-nociceptive and antimicrobial potentials (Ivanovska and Philipov, 1996; Ali *et al.*, 2013; Bibi *et al.*, 2014; Abushouk *et al.*, 2017; Fernández-Poyatos *et al.*, 2019; Pervez *et al.*, 2019).

1.1.1. Distribution of *Berberis* Species across Pakistan

About 650 species of *Berberis* have been widely distributed all over the biosphere, mainly Central Asia, West Asia, Southeast Asia, South and North America, Europe, and Africa. On the bases of their inhabitancy, Barberry plants are placed in three main groups namely Asiatic group (*B. aristate*), Mountainous group (*B. aquifolium*) and the European group (*B. vulgaris*) (Bhardwaj and Kaushik, 2012).

Pakistan is lying between longitude (60°55'-75° 30' E) and latitude (23° 45'- 36° 50' N) dominating 80,943 km^2 area at 0 to 8611 m altitude (Gilani *et al.*, 2014). Phytogeographically, Pakistan is divided into four regions namely; Irano-Turanian , Sino-Himalayan, Saharo-Sindian and Indian element (Ali and Qaiser, 1986). In Pakistan, 49 species of *Berberis* have been reported with taxonomically identification of 29 species (Supplementary Table 1) (Khan *et al.*, 2015). In Pakistan, the species belonging to respective genus mainly exist in mountainous areas of all provinces (excluding Sindh) at high altitude about 1400 m to 3500 m over sea level (Khan *et al.*, 2014; Khan *et al.*, 2016). Various *Berberis* species showed diverse pattern of distribution across the country (Table 1). The Northern area of Pakistan hosted most of the *Berberis* species (Jafri, 1975; Khan *et al.*, 2014; Khan *et al.*, 2015; Khan *et al.*, 2018). On the bases of distribution patterns, *Berberis* species are divided in to two categories (i) Single location and (ii) multiple locations. Khan *et al.* (2015) reported that 12 *Berberis* species are restricted to a single location including 4,4,3,1 species that are restricted to Gilgit Baltistan, Kashmir, Khyber Pakhtunkhwa and Balochistan respectively. While remaining species exist in multiple locations. For examples, *B. kunwarensis* is distributed over four zones *i.e.,* Gilgit Baltistan, Khyber Pakhtunkhwa, Kashmir and Punjab. Similarly, *B. lyceum* showed greatest distribution range including Balochistan, northern area of Gilgit-Baltistan, Murree and Kashmir (Khan *et al.*, 2014; Khan *et al.*, 2015; Khan *et al.*, 2016). In Pakistan, most of the *Berberis* species have been investigated for phytochemicals (Berberine,

Phenolic and flavonoid contents) and biological actions against numerous ailments. The details of some ethnopharmacologically important species are given below.

1.1.1.1. Berberis balochistanica

B. balochistanica is an endemic species distributed in Ziarat and Quetta district of Balochistan province (Khan *et al.*, 2015). It is locally called 'Zarch'. The local community uproot it and sell it in the local market. This species is locally used for digestion problems, stomach-ache, jaundice, cough, cold and other illnesses (Bibi *et al.*, 2014). The biochemicals characterized from root extracts of this species are baluchistanamine, aporphine, proaphorphin, benzylisoquinoline, pakistanine, pakistanamine and benzylisoquinoline alkaloid (Kakar *et al.*, 2012).

1.1.1.2. Berberis calliobotrys

B. calliobotrys is also known as 'Shin Zaralga'. In Pakistan. it is distributed across three zones *i.e.* Balochistan, Kashmir and Khyber Pakhtunkhwa (Khan *et al.*, 2015). Locally this species is used as a disinfectant for sore throat and intestinal pain (Singh *et al.*, 2007). The characterized phytoconstituents are chitraline, aporphine, pakistanamine, benzylisoquinoline, khyberine and pakistanine (Hussain *et al.*, 1980).

1.1.1.3. Berberis orthobotrys

B. orthobotrys is locally called 'Kishmal'. This species is used as a herbal drug to treat infections, wounds, piles, jaundice, kidney stones, diabetes, liver problems, swellings, sore throat, leucorrhoea, bleeding, uterine swellings, and other related complications (Khan and Khatoon, 2007; Akhtar *et al.*, 2008). These are distributed across two locations *i.e.*, Khyber Pakhtunkhwa and Gilgit-Baltistan (Khan *et al.*, 2015). Roots of *B. orthobotrys* contained

pakistanine, pakistanamine, dimer of kalashine and 1-0 methyl kalashine (Hussain and Shamma, 1980).

1.1.1.4. Berberis parkeriana

B. parkeriana is locally known as 'kala simbloo'. It is mainly reported in Khyber Pakhtoon khawa, Punjab and Gilgit-Baltistan (Khan *et al.*, 2015). It is traditionally useful for diabetic and various microbial diseases (Ali *et al.*, 2013).

1.1.1.5. Berberis pseudoumbellata sub species gilgitica

B. pseudoumbellata sub species gilgitica is also known as 'kashmal or kwaria'. It is limited to Gilgit-Baltistan and is usually used to treat diuretic astringents, intestinal disorders, jaundice, oxytocic, throat ache, and eye trouble (Singh *et al.*, 2007; Khan *et al.*, 2015). Main compounds present in *B. pseudoumbellata* are berberine, berbamine, jatrorrhizine, pisbenzy isoquinoline, ando oxyacanthine (Rajasekaran *et al.*, 2009).

1.1.1.6. Berberis chitria

B chitria is an endemic species, restricted to Kashmir. This species is comprising of important phytochemicals like; chitrian, berberine, berbamine, berlambine, berbamunine, columbamine, dihydro kaempferol, dihydropalmitine, quercetin, palmitine, glucose, and fructose. These active ingredients make this plant very useful for various clinical diseases such as cholera, ear and eyes diseases, diabetes, rheumatism, jaundice, fever, malarial fever, stomach disorders and skin diseases (Dutta and Panse, 1962; Lahiri and Dutta, 1967; Srivastava *et al.*, 2006).

1.1.1.7. Berberis pachyacantha

B. pachyacantha, is locally known as 'Simlu or Simu' (Jafri, 1975). It is widely distributed in temperate and semi temperate areas of Pakistan such as North-West Himalayas and Kashmir (Jafri, 1975).Various phytochemicals have been characterized in this species namely; berbamine, cyanidin- 3 glucoside, jatrorrhizine, oxyberberine, magnoflorine, isotetrandrine (Srivastava and Rawat, 2014).

1.1.1.8. Berberis lyceum

B. lyceum is one of the most distributed species of *Berberis* plant reported in Gilgit-Baltistan, Kashmir, North side of Khyber Pakhtunkhva, Punjab, and Balochistan. It is also known as 'Speen Kwaray'. It is used by the local inhabitants due to its antibacterial,

carminative, anticarcinogenic and ophthalmic ingredients (Khan *et al.*, 2016). This species is extensively studied and found to have numerous compounds such as baberine, berberine chloride, berbericine hydroiodide, berbericine hydrochloride, palmatine, diphenolic, jhelumine, gilgitine, kara-koramine, punjabine, sindamine, and umbellatine (Ikram, 1975; Landry *et al.*, 2021).

1.1.1.9. Berberis brandisiana

B. brandisiana is locally known as Shugloo. It is used by the local community for various illnesses like dysentery, sore throat, healing of wounds, and arthritis (Khan *et al.*, 2016). Various phytochemicals have been isolated from it, namely berbamine, berbernine, palmitine, thalifoline, reticuline, isoboldine, and apoglaziovine (Hussain *et al.*, 1980).

Table 1: Distribution of total (29) identified *Berberis species* (subspecies, varieties and forma) across Pakistan (www.efloras.org)

S No	Accepted Scientific Name	Location
1	*Berberis sp.*	Balochistan
2	*Berberis kashmirana*	Kashmir
3	*Berberis kunawurensis forma chitrioides*	Gilgit Baltistan, Kashmir, Khyber Pakhtunkhwa
4	*Berberis ulicina*	Gilgit Baltistan
5	*Berberis lyceum*	Khyber Pakhtunkhwa, Punjab, Balochishitan
6	*Berberis jaeschkeana*	Kashmir
7	*Berberis parkeriana*	Khyber Pakhtunkhwa
8	*Berberis jaeschkeana var. usteriana*	Kashmir
9	*Berberis huegeliana*	Kashmir
10	*Berberis jaeschkeana var. jaeschkeana*	Kashmir
11	*Berberis pseudumbellata subsp. Pseudumbellata*	Gilgit Baltistan, Khyber Pakhtunkhwa, Kashmir
12	*Berberis pseudumbellata*	Gilgit Baltistan
13	*Berberis royleana*	Khyber Pakhtunkhwa
14	*Berberis kunawurensis*	Kashmir
15	*Berberis glaucocarpa*	Kashmir
16	*Berberis pachyacantha subsp. Pachyacantha*	Khyber Pakhtunkhwa
17	*Berberis jaeschkeana*	Khyber Pakhtunkhwa, Kashmir
18	*Berberis pseudumbellata subsp. Gilgitica*	Gilgit
19	*Berberis pachyacantha subsp. Zabeliana*	Khyber Pakhtunkhwa, Punjab
20	*Berberis orthobotrys subsp. Capitata*	Gilgit Baltistan, Khyber Pakhtunkhwa
21	*Berberis brandisiana*	Punjab
22	*Berberis pachyacantha*	Khyber Pakhtunkhwa
23	*Berberis chitria*	Murree
24	*Berberis orthobotrys*	Kashmir
25	*Berberis balochistanica*	Balochistan
26	*Berberis calliobotrys*	Balochistan, Chitral
27	*Berberis brevissima*	Khyber Pakhtunkhwa
28	*Berberis aitchisonii*	Chitral
29	*Berberis vulgaris*	Gilgit Baltistan

1.2. Phytochemistry of *Berberis* Plant

With the emergence and development of new tools and methods, Medicinal plants are deeply investigated for the screening of bioactive and inert phytoconstituents with respect to its ethnopharmacological value. The non-nutritious substances, which protect the plants from various types of stresses, are acknowledged as 'phytochemicals. In this regard, more than 30,000 plants have been investigated for phytochemical constituents (Lattanzio, 2013). These plant protecting substances also provide protection to animals and human beings against various diseases (Savithramma *et al.*, 2011). The phytocompounds are separated into two main groups *i.e* primary and secondary metabolites (Kumar *et al.*, 2009). Primary metabolites contained simple biomolecules like carbohydrates, proteins, and lipids. While in secondary metabolites, other complex compounds like alkaloids, polyphenol, and terpenoids are included. These types of metabolites have significant role in plants growth, development and provide defence against biotic and biotic stresses (Overlingė *et al.*, 2021; Rizaludin *et al.*, 2021). The secondary metabolites like phenolic compounds and flavonoids make a great contribution in herbal and pharmaceutical drugs due to various functional groups like phenolic ring (C_6H_5OH), hydroxyl groups (-OH) and the carboxylic acid (-COOH) (Ribarova *et al.*, 2005;Atanassov *et al.*, 2021). These functional groups provide excellent antioxidant action to phytocompounds. As a result, phytochemicals exhibit strong biological activities like; antioxidant, antimicrobial, anti-inflammatory, anti-arrhythmic and antitumor activities (Rehman *et al.*, 2018). All these biological activities are directly proportional to the presence of above-mentioned functional groups. Phytochemicals with functional groups have an inhibitory effect against reactive oxygen species (ROS) by breaking the radical chain and chelating the metals in the body during stresses (Osawa, 1994). Recently, numerous phytochemicals have been isolated from different *Berberis* species such as *B. crataegina, B. integerrima, B. aetnensis* and *B. libanotica* possessing 22, 18, 26 and 37 bio-compounds respectively (Salehi *et al.*, 2019). All parts of *Berberis* species have been reported as rich sources of bioactive compounds. However, various parts have several types of phytoconstituents like stem and root extracts have almost same types of alkaloids including protoberberine, isoquinoline, and isoquinoline. While leaves of different *Berberis* species showed variation in the bio constituents containing acids and phenolic compounds (Bhardwaj and Kaushik, 2012). The main chemical constituents including alkaloids, terpenoids, flavonoids, anthocyanins, vitamins, sterols, carotenoids, lignins, lipids and proteins have been isolated from different barberry species (Khan *et al.*, 2016). The core alkaloids isolated from various *Berberis* plants are berberine, columbamine, berbamine,

khyberine, pakistanine, baluchistanamin, karachine pakistanamine, palmatine, oxyacanthine, and jatrorrhizine (Haji, 2010; Srivastava *et al.*, 2015; Khan *et al.*, 2016).

1.3. *Berberis* Plant; A Rich Source of Mineral Elements

Essential nutrients have certain key functions in the physiology and morphology of living organisms. Essential elements are characterized in two categories macro and micro-elements. Macro-elements are those elements which are required in surplus amount, while micro elements are crucial in trace amounts. The former ones have great contributions in structural and metabolic activities of cell, while later ones are vital for signalling and enzymes operations (Maiti *et al.*, 2016). The mineral elements reported to have a leading role in bioactive compounds synthesis by integrating in the structure of biomolecules, which ultimately lead to the biochemical reactions (Osae, 2001). Minerals have a significant role in the living body and the deficiency of minerals is one of the serious health issues in developing and underdeveloped countries (Batra and Seth, 2002). In a recent analysis of UNICEF report 2019, more than 200 million children under the age of five are facing growth problem around the globe, whereas about 340 million children are facing hidden hunger (Keeley *et al.*, 2019). Macro-elements are required in a substantial amount (more than 100 mg/day) for carrying out different functions of living organisms. Micro-elements are required in a trace amount and their daily intake level for living organisms is less than 100 mg/day (Aziz *et al.*, 2016). In human body, potassium and sodium are the essential elements, which regulate body fluid and electrolytes by utilizing transport protein pumps. These pumps sodium and proteins across the cell membrane. Electrolytes are essential for contraction of normal muscle fibres. Too much intake of sodium escalates blood pressure and may lead to urinary potassium loss. Whereas, potassium scarcity escalates blood pressure and suppresses muscle relaxation, which proceeds to muscle cramps and shoots up the risk of diabetes and hypertension and affects the nervous system (He and MacGregor, 2008). The Food and Nutrition Board of Institute of Medicine normally recommend 4,700 milligrams of potassium per day (Electrolytes and Water, 2004). Calcium is an important element, but plants absorb less calcium. Magnesium, a cofactor of various enzymes, is involved in the action of hormones that helps to regulate glucose level. Iron is also an important component of haemoglobin and is found in many human enzymes. High iron level can lead to liver toxicity, while its deficiency can lead to anaemia (Daram and Hayashi, 2005). Zn and Fe are essential constituents for all living cells. The permissible levels for Zn and Fe in edible plants are 27.4 and 20 ppm (Bogden and Klevay, 2000). However, they can also be toxic

in higher concentrations. The international permissible limits of the mineral elements are presented in Table 2.

Table 2: Recommended values of Mineral elements in medicinal plants (Salud and Organization, 2003; Nkuba and Mohammed, 2017).

Mineral Elements	**Recommended values (mg/kg)**
Na	2400
K	32500
Mg	60
Fe	148
Zn	38.6
Mn	31.9
Cd	0.12
Cu	5.67
Ni	1.05

Many researchers studied *Berberis* plants like roots and stem bark of *B. asiatica* (Swati et al., 2012), fruits of *B. vulgari*s (Akbulut et al., 2009), plant extracts of *B. vulgaris* and *B. integerrima* (Ardestani et al., 2013), and root extracts of *B. lyceum* (Ullah et al., 2013) as a rich source of macro and micro elements. Along with this, a negligible amount of toxic elements (Cu, Cr, Cd, Pb and Ni) were found in *B. lyceum* (Dastagir and Pervez, 2004).The variation in the mineral composition may depend on the genetic factor and habitat of the plant. Plants can absorb heavy metals from water, soil and air (Ercisli and Orhan, 2007).

1.4. Economic and Medicinal Importance of *Berberis* Species

Ethnobotanically, every part of *Berberis* plant has its own significance. Fresh, dried or extracts of different part of *Berberis* species are utilized locally (Rahimi-Madiseh *et al.*, 2017). The fruit is used in beverages, jam production, drinks, candy, syrups and other sweet products (Farhadi Chitgar *et al.*, 2017). While the leaf is used as flavouring agent in food and tea (Salehi *et al.*, 2019). Stem wood of *Berberis* is also used as natural dyes (Haji, 2010; Srivastava *et al.*, 2015). Along with phytochemicals, *Berberis* species are also popular due to their nutritional importance. Beside these, *Berberis* species are also known for their effectiveness in traditional herbal medicines from last 3000 years. For example, the roots, stem bark, leaves and fruits have been reported in Iranian, Ayurvedic and Chinese medicine. Numerous species of this genus have been extensively studied by many researchers for biochemical and pharmaceutical determinations. For Instance, Karimov (1993) reviewed 76 *Berberis* species and tabulated 129

alkaloids, whereas Ikram (1975) revised 24 *Berberis* species having medicinal and biochemical properties. Recently numerous new phytochemicals (22, 18, 26 and 37) were isolated from *B. crataegina, B. integerrima, B. aetnensis* and *B. libanotica* respectively (Salehi *et al.*, 2019). Mohi-Ud-Din *et al.* (2021) reported more than 40 *Berberis* species with folklore and pharmacological potential. Additionally, few species have been reported to be utilized as natural source of dyes (Haji, 2010; Srivastava *et al.*, 2015).

The species of *Berberis* genus has had common uses for human beings from early age like Assyrian people (Iraqi) in 668 BC used *Berberis* species for blood purification (Karimov, 1993). In traditional Indian medicine known as Ayurveda, the root and stem of common barberry has been widely used for several diseases like infections of ear, mouth and eye, indigestion, dysentery, vaginal diseases, reducing obesity and for snake bites (Khan *et al.*, 2016). The water boiled extracts of root and stem bark of some *Berberis* species have been used as local cure for skin diseases, jaundice, conjunctivitis, ulcers, bleeding piles, skin diseases, inflamed liver and inflamed spleen (Rajasekaran and Kumar, 2009).With the passage of time, this traditional knowledge of *Berberis* plant shifted in to pharmaceutical field due to its diversity, presence of bioactive compounds and pharmacological uses (Khamidov *et al.*, 1996). Different parts of other *Berberis* species have been used for various diseases including chronic inflammatory disorders and have shown strong anti-convulsion, hypoglycemic, antioxidant, anticancer, antihypertensive, anti-histaminic, anti-fatigue, anticoagulant, antidiabetic, osteolytic, antimicrobial, immuno-stimulant, hypotensive, CNS-depressant, anti-nociceptive and antimicrobial potentials (Ivanovska and Philipov, 1996; Ali *et al.*, 2013; Bibi *et al.*, 2014; Abushouk *et al.*, 2017; Fernández-Poyatos *et al.*, 2019; Pervez *et al.*, 2019) Similarly, in various studies, the roots of many species like roots of *B. aristata* (Singh and Kakkar, 2009), *B. lyceum* (Asif et al., 2007), *B. asiatica* (Srivastava et al., 2001), *B. aetnensis* (Iauk et al., 2007), *B. thunbergii, B. vulgaris* (Villinski et al., 2003), *B.integerrima* (Ashraf and Zare, 2015), *B. aetnensis* and *B. libanotica* (Bonesi *et al.*, 2013) were used for various medicinal purposes. These roots are being used for metabolic disorders, wound healing, antifungal, antibacterial, diabetes, phytochemical and for other biological activities. Khan et al. (2016) enlisted 12 *Berberis* species, the roots or bark of which are being used in ethnomedicinal and phytopharmacological fields locally. This study showed that using roots for medicinal

purposes increased the risk of depletion of plants. Several ethnobiological and scientific studies of numerous *Berberis* species have been reported (Manan *et al.*, 2007).

In stress conditions, the living body generates reactive oxygen species (ROS), which have adverse effect on the health of an organism. This ROS are various types of radicals such as hydroxyl radicals, superoxide anion radicals, singlet oxygen and hydrogen peroxide. In living body, these reactions are called oxidative damage or oxidative stress (Jackson *et al.*, 2001). Oxidative stress is one of the main causes of many diseases, because the free radicals produced in our body react with various molecules that can cause cancer, heart and inflammatory diseases (Makhaik *et al.*, 2021). The ROS production is mainly due to infectious pathogens, some metabolic processes, environmental and industrial pollutants, pesticides and synthetic drugs (Jakubczyk *et al.*, 2020). These free radicals are deactivated by the safeguard systems of antioxidants (Sen *et al.*, 2010). Antioxidants are defined as 'a substance that helps to prevent the living system from deterioration caused by reactive oxygen species'. The mechanism of an antioxidant is to neutralize or inhibit the free radicals by donating the hydrogen atom or an electron (Ozougwu, 2016). Antioxidants are of two types *i.e.*, enzymatic antioxidants like glutathione peroxidase, superoxide dismutase and catalase and non-enzymatic antioxidants which consist of ascorbic acid, carotenoids, α-tocopherol, glutathione and flavonoids (Krishnaiah *et al.*, 2011). The presence of high phenolic, flavonoid contents, and antioxidant capacity attribute to DNA protective capacity as these compounds neutralize the effects of free radicals on DNA, thus reducing DNA damage (Jun *et al.*, 2007; Dai and Mumper, 2010; Sevgi *et al.*, 2015). DNA is also a major target of ROS in most organisms. Furthermore, ionizing radiation, acridine dye and bleomycin may cause insertion, deletion or break the DNA strands (Altaf *et al.*, 2007; Sharma and Singh, 2021). Lack of this antioxidant may lead to a series of health problems in living systems. Therefore the alternate option is the supplement of antioxidant rich diet from natural plant resources (Knekt *et al.*, 1996). Plant based antioxidants act as a preventive medicines and protect the cells or tissues from any damage or diseases caused by reactive oxygen species (Umamaheswari and Chatterjee, 2009; Sarangarajan *et al.*, 2017). The phytochemicals like berberine alkaloids, phenolics, flavonoids and other bioactive compounds of *Berberis* species have strong biological activities *e.g.* antioxidant, antimicrobial, anti-inflammatory, anti-arrhythmic and antitumor (Rehman *et al.*, 2018). The presence of these phytochemicals with strong antioxidant properties has been reported in many *Berberis* species, such as *B. aetnensis*, *B baluchitanica*, *B. Lyceum*, *B. aristata* and *B. thomsoniana* (Campisi *et al.*, 2014; Akhtar and Mirza, 2018; 2018; Batool *et al.*, 2019). The

strong antioxidant potential of *Berberis* revealed its significance towards anticancer, antimicrobial, cardiovascular and other metabolic diseases. Some precious alkaloids were isolated from *Berberis* species such as berbamine and berberine which anti-leukemia, anti-inflammatory, anti-hyperglycemic, hepatoprotective, hypotensive and antioxidant properties (Tilaoui *et al.*, 2018).

Antimicrobial investigations of *Berberis* species revealed that, mostly crude extracts have inhibitory activities against all tested pathogens (Mohi-Ud-Din *et al.*, 2021). Interestingly, the extracts showed inhibitory activity against both gram positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Pseudomonas aeruginosa*). These cause systemic infections in patients with burn wounds, cystic fibrosis, acute leukemia, and organ transplants. After Escherichia coli, the most common gram-negative pathogen is Klebsiella pneumonia associated with a wide range of infections including urinary tract infection (UTI), intra-abdominal infection, pneumonia, pyogenic liver abscess (Bucić-Kojić *et al.*) and bloodstream infection (Podschun and Ullmann, 1998; Anderson *et al.*, 2014). *E. coli* causes intra-abdominal infection, urinary tract infection, and primary bacteremia (Eliopoulos and Bush, 2001). Similarly, many *Berberis* species have been studied and reported for their antifungal activities (Rehman *et al.*, 2018; Sharma *et al.*, 2018; Avci *et al.*, 2021). The variation in the efficiency of the various extracts against tested microbial strains would be due to the presence of different chemical constituents in the extracts. These activities are correlated with the phenolic, flavonoid contents, and berberine chloride present in the extracts, as these play a vital role against various microorganisms (Mori *et al.*, 1987).

1.5. Substitutional Approach by Using the Aerial Parts

Irrespective of its medicinal potential, these medicinal plants face serious difficulties due to misuse and over exploitation. Medicinal plants have two types of threats *i.e.,* general threats (increasing populations, urbanization, climatic, and biotic stresses) and scientific threats (unselective harvesting and uprooting of the medicinal plants for clinical and nutritional purposes). These threats results in the diminution of natural reservoirs (Ahn, 2017). According to International Union for Conservation of Nature and the World Wildlife Fund, more than 15,000 plant species are going to be extinct due to above mentioned threats (Raj *et al.*, 2019). While in Pakistan, around 64 medicinal plants and 636 other plant species are under threats of becoming endangered, because a large proportion of local community is dependent on these medicinal plants (Ullah, 2017). According to Majid *et al.* (2019), roots of medicinal plants are the most used part (36.17%) followed by leaves (29.79%) and other renewable parts (8.1%)

respectively. These problems will be reduced by adopting some strategies like, identification and development of conservation area, discouraging of uprooting of medicinal plants, promotion of awareness, cultivating of depleted plant species and usage of substitutional approaches by using the renewable and alternative aerial parts (Zschocke *et al.*, 2000). For substitutional strategies, the comparative studies with respect to its phytochemical and biological potentials of the same plant parts are essential. The substitutional strategies are less considered and only few reports are available about these issues. Zschocke *et al.* (2000) studied bulb, rhizome, and bark of *Eucomis autumnalis*, *Siphonochilus aethiopicus*, *Ocotea bullataand* and *Warburgia salutaris* plants respectively, and suggested the use of renewable parts as a substitute for bulbs, rhizomes, and bark of such species. In few other studies, this approach was applied to conserve the medicinal plants such as roots, corms, stem bark, and tubers were substituted with aerial renewable parts in *Aegle marmelos* (Sulaiman and Balachandran, 2013), *Hypoxis hemerocallidea* (Katerere and Eloff, 2008), *Curtisia dentate* (Shai *et al.*, 2009), *Pelargonium sidoides* (Lewu *et al.*, 2006) respectively. The stability between medicinal plants and medicinal drugs are necessary to reduce the depletion of some medicinal endemic plant such as *Berberis* species due to the uprooting of its underground parts. Srivastava *et al.* (2015) reported that, only root of more than 15 *Berberis* species have being collected and have been studied for ethnopharmacological purposes.

1.6. Nanotechnology: An Emerging Technology of Twenty First Century

In nanotechnology, the word nano is a Greek prefix which means vary small (10^{-9} m). Basically, nanoscience deals with matters at a nanoscale (1-100nm), and the technology which utilizes it in useful form in different fields is called nanotechnology (Mansoori and Soelaiman, 2005). This concept was first introduced by 'Richard Feymen' with title "There's Plenty of Room at the Bottom" (Feynman, 2018). Nanotechnology is one of the outstanding and fast-developing areas of science, which have capability to make particles at nano scale (1-100nm) in one dimension (Reddy *et al.*, 2019). In Nanotechnology, the consolidation, separation, and deformation of materials on an atomic or molecular level are considered. Nanotechnology is one of the emerging fields of science in twenty-first century. It is considered as an interdisciplinary field in which the material can be made at nano scale with innovation and enormous applications (Mansoori and Soelaiman, 2005). It is the combination of science, technology and engineering focusing on nanoscale, where researchers can control and manipulate the structure of materials at nano level. Two conditions are mainly focused in

nanotechnology, first is to control the shape and size at nanoscale and second one is the creation of novelty in those nanomaterials (Allhoff, 2009).

1.6.1.Nanoparticles

Nanotechnologies make the nanoparticles more inspiring regarding their

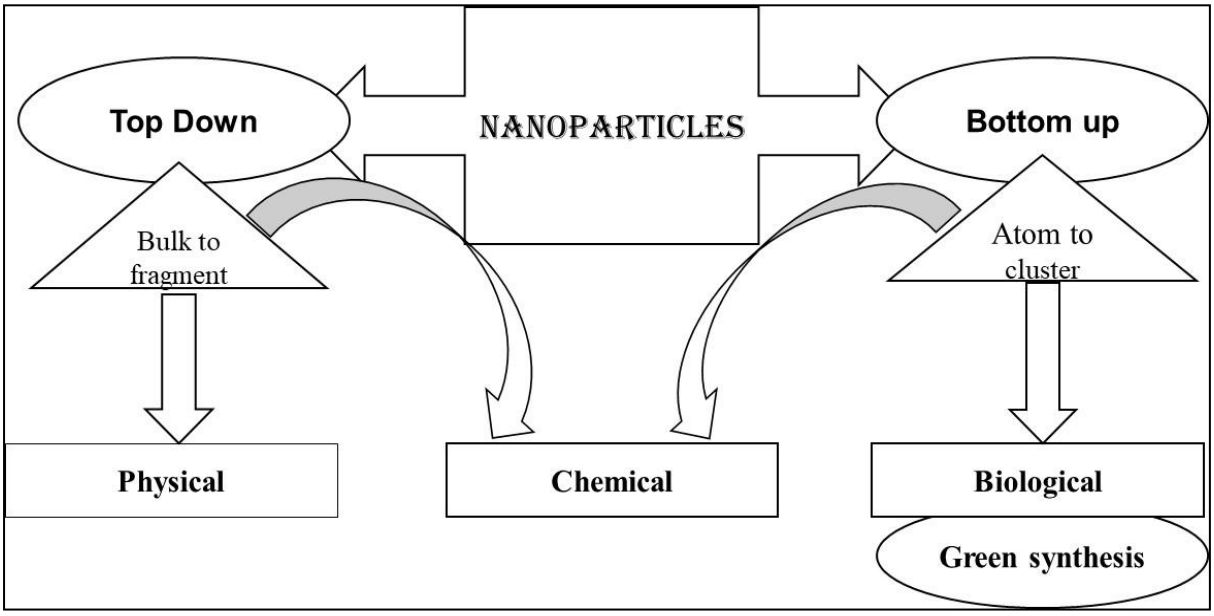

Figure 2: Synthesise of Metal Nanoparticles using various approaches.

physicochemical and biological properties as compared to bulk ingredients (Ghorbanpour and Wani, 2019). Due to nano size and high surface to volume ratio, the synthesized nanoparticles have great demands in multiple fields such as biomedical, pharmaceutical, industrial, commercial, mechanical, electrical, agricultural, and environmental fields (Ghorbanpour and Wani, 2019; Iqbal *et al.*, 2020). As, the causative agents of many infectious diseases are continuously developing resistance against available drugs like pesticides, fungicides, and other synthetic chemical compounds (Villa *et al.*, 2017). Nanoparticles have been extensively reported to have biological applications against various infectious diseases with potent biomedical potential against causative agents (Iravani, 2011). Nanoparticles can be synthesized via physical and chemical methods. These approaches are expensive at industrial scale and have potentially toxic effects on living beings and ecosystems (Anastas and Eghbali, 2010).

1.6.2. Synthesis of Nanoparticles: A "Green Chemistry" Model.

Generally, in physicochemical method; expensive, hazardous and harmful substances are utilized, which have great toxic effect on ecosystem (Nath *et al.*, 2013). To make nanoparticles less toxic, biologically safe and cost effective, researchers used 12 principles of green chemistry as reference guide to synthesize nanoparticles (Iqbal *et al.*, 2020). To synthesize

valuable nanoparticles, scientists have merged nanotechnology with green chemistry to fabricate environment-friendly nanomaterials using natural products like plants, bacteria, and fungi (Lateef *et al.*, 2016). The capability of a living system to utilize its intrinsic organic chemistry processes in remodelling inorganic metal ions into nanoparticles has opened up an undiscovered area of biochemical analysis. In the biogenesis of nanoparticles, ecologically recognized "green chemistry" model has been used for the fabrication of purified and nature-welcoming nanoparticles. Green synthesis concept seeks to avoid the unwanted toxic by-products by using nontoxic solvent and eco-friendly resources (Singh *et al.*, 2018). Plants and microorganisms have established the power to devour and accumulate inorganic metal ions from their neighbouring niche. The biological approaches in particular plant based is gaining great popularity due to its cheap, easy and eco-friendly behaviour (Rather *et al.*, 2020).

In green synthesis the metal salt is basically reduced and stabilized into metal nanoparticles by various phytoconstituents, which act as a caping agent (Iqbal *et al.*, 2020). Plants have numerous biomolecules such as alkaloids, steroids, flavonoids, phenolic, berberine, tannins, coumarins, carbohydrates and quinones (Srivastava *et al.*, 2004; Baloch *et al.*, 2013). These biomolecules have a role in reducing and capping the metal salts into nanoparticles. However, the size, structure and morphological nature of nanoparticles depends upon the biomolecules present in the plant extract (Rajeshkumar and Bharath, 2017). These biocompounds act as stabilizing, reducing and capping agents. For this purpose, various parts of plants, such as leaves, stems, roots, seeds and fruits have been used to synthesize various types of nanoparticles (Narayanan and Sakthivel, 2008). Plant based synthesized nanoparticles are more stable then bacterial or fungal assisted nanoparticles. Plant extracts are easily available with no culturing and preservation as compared to other biological methods like microorganisms required preservation and culture for growth (Hulkoti *et al.*, 2014). Autophyte-assisted synthesis of nanoparticles are categorized into main three groups *i.e.,* intracellular, extracellular, and phytochemical based green method. Among these three methods, the extracellular is relatively cheaper, easier and high yielding methods due to the presence of bioactive phytoconstituents, which act as reducing and stabilizing agent during nanoparticles synthesis (Mohammadinejad *et al.*, 2019).

A number of nanoparticles such as gold, silver, palladium, iron, cobalt, nickel oxide, copper oxide, cerium oxide, zinc oxide have been synthesized using plant extracts with numerous applications (Singh *et al.*, 2018; Yadi *et al.*, 2018). Recently in 2020, more than 20 plant mediated nanoparticles have been reported by Jadoun *et al.* (2021) and Singh *et al.* (2020).

Every biological system varies in its capabilities to supply metallic nanoparticles. However, not all biological organisms can produce nanoparticles due to their enzymatic activities and intrinsic metabolic processes. The advancement of eco-friendly methods in nanoscience has considerable worth to enhance their biological applications.

1.6.3. Characterization of Green Synthesized Nanoparticles

The synthesis of nanoparticles can be characterized by various microscopic and spectroscopic techniques (Kar *et al.*, 2014). Scanning Electron Microscopy (SEM) for screening of surface morphology along with composition of nanomaterials; and Transmission Electron Microscopy (TEM) is for internal makeup of nanoparticles are the prominent techniques to characterize nanoparticles (Mariam *et al.*, 2014). While spectroscopic approaches are used to characterise the size, shape, functional groups, movement, conductivity and other physical properties of synthesized nanoparticles. Spectroscopic techniques to characterize nanoparticles include; Ultraviolet–visible spectroscopy, which confirm the reduction of nanoparticles indicated by colour change (Pandian *et al.*, 2015); Fourier Transform Infrared Spectroscopy (FTIR) to examine the phytocompounds accountable for stabilization and reduction of nanoparticles (Kar and Ray, 2014); X-ray Riffractometer (XRD) is used to study purity, identification and quantitative evaluation of nanoparticles (Chen *et al.*, 2014); Energy Dispersion Analysis of X-ray (EDAX) and Dynamic Light Scattering (DLS) are useful for particle distribution and stability (Abbasi *et al.*, 2020; Iqbal *et al.*, 2020; Singh *et al.*, 2020; Jadoun *et al.*, 2021).

1.6.4. Mechanism of Phytofabricated Metal Nanoparticles Action

The emerging resistance against antibiotics and evolution of new infectious microbial species is the main challenge for researchers. Therefore, researchers are trying hard to develop nanomaterials with potent biological potentials against degenerative infectious diseases. It is reflected that strong antioxidant activities of green synthesized nanoparticles are due to the interaction and adsorption of antioxidant compounds from the extract onto the surface of synthesised nanoparticles (Khalil *et al.*, 2018). Nanoparticles have been reported with strong antimicrobial activities against both gram-positive and gram-negative bacterial strains (Khalandi *et al.*, 2017; Abbasi *et al.*, 2019; Dangi *et al.*, 2020; Singh *et al.*, 2020; Jadoun *et al.*, 2021). It corresponds with the fact that nanoparticles can penetrate inside the bacterial cell and obstruct metabolic activities (Nisar *et al.*, 2019). Similarly, nanoparticles have also been reported to have strong antifungal activities (Abbasi *et al.*, 2020; Ali *et al.*, 2020). The biogenic nanoparticles penetrate and enhance the permeability and generate reactive oxygen species

(ROS) inside the fungal cell which retards mycelial growth (Ali *et al.*, 2020). Due to the nano size, the nanoparticles displayed potent biological activities against various infectious pathogens. Nanoparticles have more attachment and penetration ability with the cell membrane of pathogens as compared to bulk materials (Ali *et al.*, 2020). However, the action mechanism of nanoparticles against microbial pathogens is still not clear and several research works are in progress to identify the exact mechanism. Various research reflects that the action of green synthesized nanoparticles is due to the interaction and adsorption of antioxidant compounds from the extracts onto the surface of synthesised nanoparticles (Nisar *et al.*, 2019). Some studies have represented that nanoparticles create a pit and gap on bacterial membrane and releases ions which interacts with metabolic cellular machinery, resulting into the inhibition of metabolic pathway and finally leads to cell death [**Figure 3**] (Kailasa *et al.*, 2019).

Recently, cytotoxic activity of nanoparticles was investigated against brine shrimp larvae. Nanoparticles retarded the larval movement due to the attachment and penetration inside the larval body, they reduced the metabolic activity and as a result, mortality occurred (Ates *et al.*, 2013). The dose-dependent mortality rate investigations have shown that nanoparticles can be used as anticancer drugs in biomedical fields (Khalil *et al.*, 2018; Abbasi *et al.*, 2020). The remarkable antioxidant, antibacterial, antifungal, and cytotoxic potentials of nanoparticles might be due to their nano gage dimension, precise surface area, the cohort of extra ions and adherence properties.

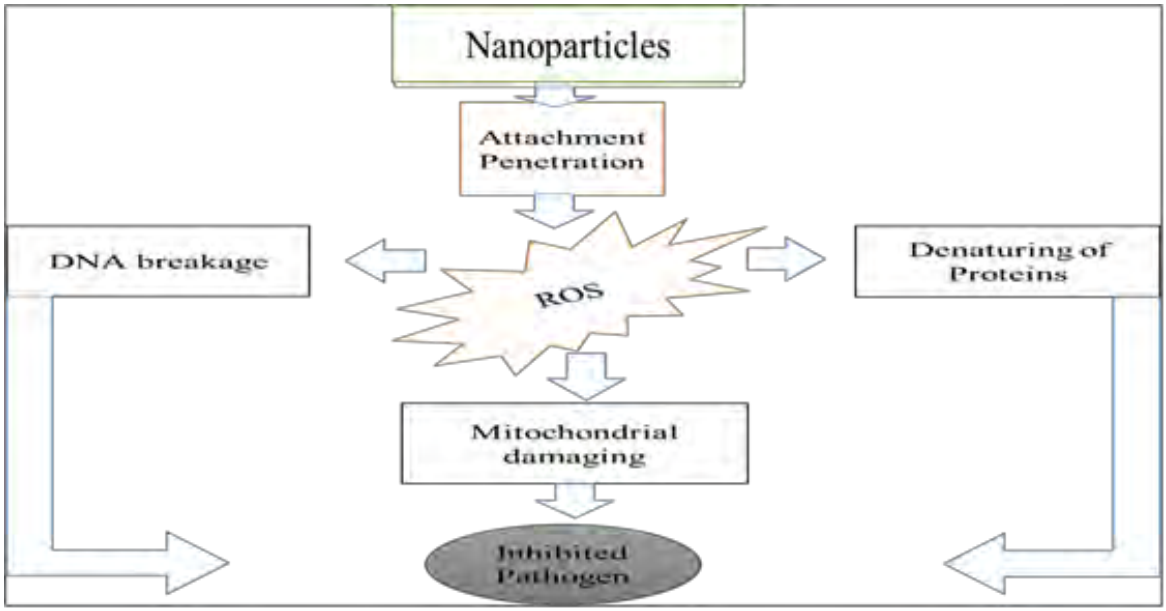

Figure 3: The schematic Action mechanism of biosynthesized Metal Nanoparticles.

Nanoparticles have also been used as bio-fertiliser in the agricultural field for enhancing nutrient uptake, breaking seed dormancy, and reducing the application of hazardous

agrochemicals (Younes *et al.*, 2020). Therefore, green synthesis of nanoparticles might be useful for controlled release of fertilizer, plant growth mediation and green alternative to agrochemicals. Numerous metal nanoparticles such as TiO_2, ZnO and Ag nanoparticles are used as biostimulators in the agricultural field (Prażak *et al.*, 2020). These positive response might be due to various factors such as infiltration of nanoparticles, releasing ions and making a suitable environment for oxygen and water uptake, hence breaking seed dormancy and promoting seedling growth (Dawood *et al.*, 2019; Younes *et al.*, 2020). These findings indicate that apart from their potential in the biomedical field, green synthesized nanoparticles could be effectively used in agricultural fields as nano fertilizers as well as stimulatory agents for plant physiology.

1.6.5. *Berberis* Species and Green Synthesis

As already detailed in sections **1.2** and **1.4** the importance of *Berberis* species as phytochemical rich plant with beneficial herbal agents. These valuable eco-friendly secondary metabolites are useful for the green synthesis of nanoparticles because they serve as effective reducing agents during green synthesis. Recently, this species gain attention of researchers due to its biochemical rich nature and wide distribution across the world (Ahmed *et al.*, 2008; Khan *et al.*, 2014). On the basis of their safe, nontoxic nature and presence of biomolecules; *Berberis* plant is one of the safest candidates to use in green synthesis of nanoparticles. Therefore, in last two years, different *Berberis* species have been used in green synthesis of various nanoparticles with potent biological Properties. The **Table 1** representing the number of species used in nanoparticles synthesise since 2020.

Table 3: Phyto fabricated nanoparticles using various parts of different *Berberis* species and their biological applications.

Nanoparticles	Plant Name	Part used	Applications	Reference
Silver	*B. asiatica*	Root	Antibacterial activity	(Dangi *et al.*, 2020)
Silver	*B. thunbergia*	Leaf	Anticancer	(Guo *et al.*, 2021).
Silver	*B. vulgaris*	Leaf	Anticancer activity	(Safipour Afshar and Saied Nematpour, 2020)
Silver	*B. vulgaris*	Leaf	Cytotoxic activity	(Safipour Afshar *et al.*, 2021)
Zinc oxide	*B. tinctoria*	Leaf, fruits	Multi-biological activity	(Arumugam *et al.*, 2021)
Zinc oxide	*B. aristata*	Whole plant	Antimicrobial activity	(Chandra *et al.*, 2019)
Zero-valent iron	Barberry	Leaf	Photocatalytic activity	(Samadi *et al.*, 2021)

1.7. *Berberis* species: Alternate Host to Fungal Pathogen

Food is one of the basic and fundamental needs of life and due to rapid increase in world population and urbanization, food demands are increasing exponentially (Gupta *et al.*, 2008; Wernicke, 2016). Agricultural crops are the prime source of the food. Wheat is one of the most important staple foods grown globally. It is grown on one sixth area of the land (89 countries) and provides staple food to one third of the population (Dončić *et al.*, 2019). Wheat also provides one-fifth of the protein and calorie need of the world population (Shiferaw *et al.*, 2013). While in Pakistan, wheat is in top position, growing at around 40 % of the land (9.199 Million hectares) with 26.57 million metric tonnes (Ali *et al.*, 2017). To meet the requirement of increasing world populations, the production of wheat should increase by 60 % till 2050 (Singh and Trethowan, 2007; Rosegrant and Agcaoili, 2010; Singh and Bowden, 2011). Wheat crops faces challenges such as climate change, increasing population and arising of the new strain of pathogens and pests (Rasheed *et al.*, 2020). Every year, considerable yield loss occurs due to biotic stresses, including rust pathogens that destroy the grain yield during epidemic or in susceptible varieties in seeded areas of crops (Johnston and Miller, 1934; Bashir, 2019).

Rust disease in cereal crops is caused by pathogen of subphylum *Puccinia mycotina*, order *Puccinales*, class Pucciniomycetes, and division Basidiomycota (Kirk *et al.*, 2008). It is reported that more than 8000 species of rust pathogens attack cereals including wheat, triticale, oat, rye, barley and other grasses (Brown and Hovmøller, 2002; Aime *et al.*, 2017). The most commonly reported pathogens across the globe are stem, stripe, crown and leaf rust on wheat, rye and barley caused by *Puccinia graminis* f.sp.*tritci* (Singh *et al.*, 2008; Berlin *et al.*, 2013), *Puccinia striformis* f.sp.*tritci* (pst) (Liu and Hambleton, 2010) *Puccinia coronate* and *Puccinia (triticinie, recondite and hordei)* respectively (Savile, 1984; Goyeau *et al.*, 2006; Singh *et al.*, 2006; Bolton *et al.*, 2008; Kirk *et al.*, 2008). The characteristics of these pathogens are to break the resistance of crops cultivars by producing enough spores which are easily disseminated by wind and have the potential to produce new races by sexual recombination, mutations and somatic recombination (Marsalis and Goldberg, 2016; Bhardwaj *et al.*, 2019). Epidemics caused by these diseases in wheat seeded regions are range from 20 to 100 % yield loss (Huerta-Espino *et al.*, 2011; Singh *et al.*, 2015). The different growing period of wheat provides an opportunity to complete the life cycle, as a result it can become epidemic associated with virulence against resistance genes in wheat (Zeng and Luo, 2008; Zeng *et al.*, 2014; Nsabiyera *et al.*, 2018; Sajjad *et al.*, 2018). The phyllo-sphere of cereal crops and Barberry can serve as a

habitat for biotrophic pathogens like rust. Rust pathogen of cereal and grasses are mostly heteroecious and macrocyclic consist of five spore stages *i.e.*, uredinial, telial, basidial, pycnial and aecial stages [Figure 4]. These rusts used wheat or other grasses for clonal reproduction while alternate host for sexual reproduction (Roelfs and Bushnell, 1985). Alternate hosts gain less attention, even though alternate hosts offer a platform to survive harsh winter season, provides inoculum for diseases development, makes new races and leads diverse rust populations through sexual reproduction (Zhao *et al.*, 2016).

Apart from its medicinal importance, *Berberis* spp. also serve as an alternate host to stem rust, stripe rust and other rusts species (Jin *et al.*, 2010). The importance of the *Berberis* spp. as an alternate host of *P. striiformis* f.sp. *tritici* in natural conditions in different areas are relatively unknown (Hovmøller *et al.*, 2011). Alternate hosts for stem and stripe rust is *Berberis* and mahonia species, while for leaf rust and crown rust alternate hosts are *Thalictrum* spp. and *Rhamnus* spp. respectively (Jackson and Mains, 1921; Large, 1940; Gäumann, 1959; Roelfs and Bushnell, 1985; Jin *et al.*, 2010). *Berberis* species, also serve as alternate host for other *Puccinia* species like *P. pygmaea, P. montanensis* and *P. brachypodii* (Cummins and Greene, 1966). The best way to overcome these diseases is to plant genetically resistant varieties (Singh *et al.*, 2016) and application of fungicides at right time *i.e.* flag leaf stage and development of new resistance varieties (Ali *et al.*, 2007; Ali *et al.*, 2009).

1.7.1. Types of Rust

1.7.1.1. Stem Rust *(Puccinia graminis* f.sp.*tritci)*

Stem rust or black rust is one of the worldwide spreadable and destructive fungal disease of cereal crops caused by *Puccinia graminis* f.sp.*tritci* (Roelfs, 1992). Stem rust is biotrophic pathogen reported on 365 different grass species by studying its urediniospores (LEONARD and SZABO, 2005). Stem rust is reported in humid and warm (15°C to 35°C) places of Africa, Asia, Europe, America, Middle East, New Zealand, and Australia where wheat is growing (Saari and Prescott, 1985; Singh *et al.*, 2008; Prank *et al.*, 2019). Stem rust produces pustule having brown or radish urediniospores on stems, leaves, glums and awn of cereal crops by spoiling grain size and lodge of the plant (Kolmer, 2005; LEONARD and SZABO, 2005). This pathogen can causes up to 80% loss of the wheat grain in susceptible wheat varieties across the globe (Bashir, 2019). Stem rust completes its life cycle on two different hosts *i.e.*, primary host (wheat or cereal crops) and alternate host (*Berberis spp*). The occurrence of both

hosts (alternate and primary hosts) is obligatory to complete life cycle by rust pathogen (Roelfs, 1982).

In 1959, more than 70 species of *Berberis* and some of *Mahonia* were reported as alternate hosts by Gäumann (1959). Sexual recombination occurs on the alternate host leading to produce new races, which become responsible for loss of grain yield (Dubin and Brennan, 2009). Plant breeders developed cultivars with SR resistance gene (*Sr31*) through green revolution during 20th century (Peterson, 2001). This resistance gene has been used globally in spring, winter and other varieties (Singh *et al.*, 2006; Singh *et al.*, 2011). After 3-4 decades the emergence of Ug99 in Uganda, breaks the resistance and virulence against resistance genes (Pretorius *et al.*, 2000; Jin and Singh, 2006). It is reported that 90% of the world wheat varieties were susceptible to this race (Singh *et al.*, 2011). Other races of this family were also found to have virulence against *Sr24, Sr36* and *SrTmp* (Jin *et al.*, 2008; Jin *et al.*, 2009; Pretorius *et al.*, 2010; Visser *et al.*, 2011; Newcomb *et al.*, 2016). To decrease their spores movement and new race development toward other Asian countries, four genotypes with *Sr2* genes were released in Afghanistan near the Iran border and many Ug99 resistant verities were also distributed in South Asian countries as an immediate remedy (Sharma *et al.*, 2013). Along this, *Sr2* gene is also used in CIMMYT, to protect against stem rust diseases in Mexico and in USA (Singh, 1993). Ug99 was reported in Africa, Yemen, Egypt and Iran, while in Pakistan, it has not been reported yet, but it is predicted that wheat growing countries of Asia are under threat (Singh *et al.*, 2015). The presence of Ug99 in Africa and Iran is due the presences *B. holstii* in eastern Africa (Singh *et al.*, 2015) and *B. vulgaris* in Iran (Rahimi-Madiseh *et al.*, 2017). The presence of *Berberis* plants indicated that sexual recombination and emergence of new races occur in these region (Hansen *et al.*, 2013). The emergence of this disease in Iran makes a challenge for wheat breeders and policy makers of other neighbouring countries, especially Pakistan to replace and modify all susceptible varieties recently growing in their countries. The genus *Berberis* is distributed nearly all over the world (Ahrendt, 1961; Rounsaville and Ranney, 2010) like 50% of *Berberis* species has been recorded in China (Ying and Chen, 2001) where *P. striformis* was also reported on *Berberis* species (Chen *et al.*, 2009; Zheng *et al.*, 2013; Wang *et al.*, 2016). Globally numerous *Berberis* species are reported as alternate hosts of stem rust (Jin, 2011; Zhao *et al.*, 2013) like *B. vulgaris*, on the other hand some are also reported as a non-host to stem rust like *B. thunbergii DC* (Levine and Cotter, 1932). In Europe, sexual reproduction of stem rust on barberry was confirmed in Sweden (Berlin, 2017). Similarly in UK, after 60 years wheat stem rust and alternative host were reported (Lewis *et al.*, 2018).

Waipara *et al.* (2005) first time observed *P. graminus* on flowers of *B. glaucocarpa* in New Zealand. Eradication program against common Barberry or *B. vulgaris* was started by US government from 1918-74 to control the stem rust pathogen and more than 500 million plants of common barberry were eradicated (Peterson Jr, 2003; Peterson, 2013). In Pakistan, 29 *Berberis* species have been reported so far (Khan *et al.*, 2014; Khan *et al.*, 2015). Some species of *Berberis* were reported to be susceptible to stripe rust (Mehmood *et al.*, 2019), but no information about stem rust is available yet.

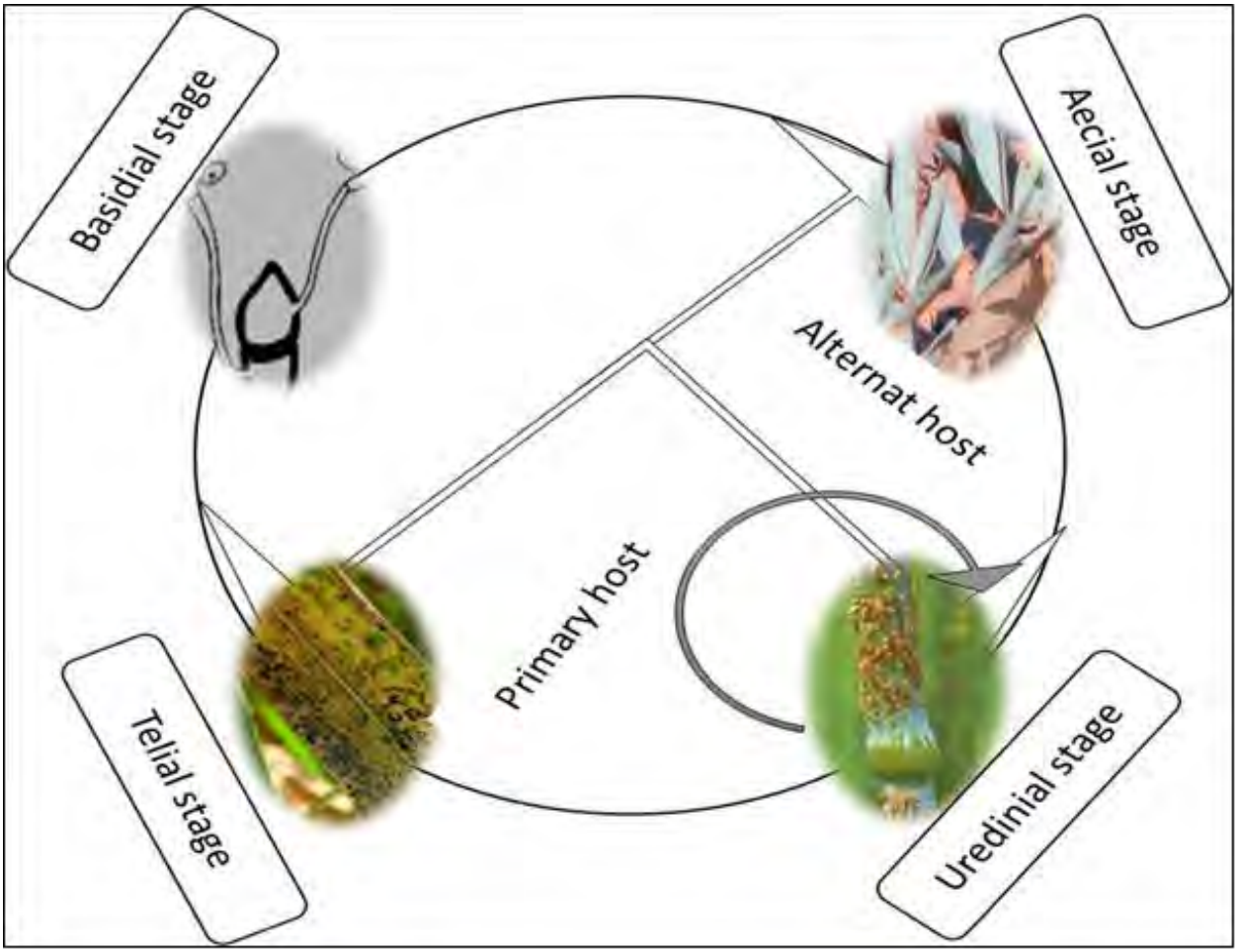

Figure 4: Life cycle of rust, showing the alternate (Barberry) and primary host (wheat) with four different phases**.**

In addition to wheat stem rust, various formae speciales of *P. graminis L.* also cause potentially serious diseases of oat, barley and rye (Eriksson and Henning, 1896). *P. graminis* has been subdivided in to formae speciales (f.sp) on the bases of rust parasitic adaptation towards cereal host (Eriksson and Henning, 1896). Certain fungi have the ability to host the grasses or cereals at genera levels and have ability to attack varieties of single species. On the basis of this adaptation, rust fungi are categorized subunits like subspecies and formae speciales level (Eriksson and Henning, 1896; Anikster, 1984). The formae speciales of stem rust also attack rye and barley (Leonard and Szabo, 2005). This finding was supported by the study of Leonard and Szabo (2005) and Johnson (1949) in which *P. graminis* f. sp. tritici and *P. graminis* f. sp. *Secalis* was found closely related while *P. graminis* f. sp. *avenae*, was found totally different from others. Berlin *et al.* (2012) observed high genetic diversity of oat stem rust in the presence of alternate host. Alternate host along with primary host generated more diverse races of rust as compared to primary host alone (Roelfs, 1982). Other formae speciales of stem rust showed narrow host range as compared to *P. graminis* f. sp. *Tritici* (Leonard and Szabo, 2005).

1.7.1.2. Stripe rust *(Puccinia striformis* f.sp.*tritci)*

Stripe rust or yellow rust is also globally important foliar and multiple-cycle disease of cereal crops caused by *Puccinia striformis* f.sp.*tritci* (pst) and have the ability to cause wheat loss in wheat growing area only under suitable conditions (Coakley, 1979; Rehman *et al.*, 2018). At a global level, about 5.5 million tons yield losses per year have been reported (Beddow *et al.*, 2015; Khanfri *et al.*, 2018). First, it was considered that proper moisture and low temperature area provide the platform for germination of uredospores on leaf, but now the fresh races are also adapted to higher temperatures at the equator (Moldenhauer *et al.*, 2006; Waqar *et al.*, 2018). Stripe rust was spread from Africa to Peninsula, Syria and Asia (Singh *et al.*, 2006), and the same pathway was followed by Ug99 to enter Iran (Singh *et al.*, 2008). The different growing period of wheat provides an opportunity to complete life cycle, as a result become epidemic and virulence against resistance genes in wheat like APR genes (Zeng and Luo, 2008; Zeng *et al.*, 2014; Nsabiyera *et al.*, 2018; Sajjad *et al.*, 2018). Pakistan also faces 13 stripe rust epidemics during different times with great yield losses. In northern side of Pakistan, stripe rust has become virulent against widely grown variety Inqlab-91 having *Yr 27* genes in 2005 (Rehman *et al.*, 2018). In 1995, stripe rust become epidemic against Pirsabak 85 and Pak 81 (Khan and Mumtaz, 2004). In Pakistan, virulence was observed for all *Yr* resistance genes and it is estimated that more than 70% of wheat producing areas are under threat to stripe

rust (Singh *et al.*, 2004; Bahri *et al.*, 2011). It clearly indicates that proper monitoring of stripe rust and screening of wheat varieties to identify resistance genes against new and existing stripe rust pathotypes needs to be mandatory (Rizwan *et al.*, 2010).

1.7.1.2.1. Stripe Rusts and *Berberis* Relationship

Stripe rusts have two life cycles *i.e.*, Pathogen life cycle and disease life cycle. For the last century, the life cycle of yellow rust has been a mystery for scientists (Jin *et al.*, 2010). Many researchers attempted to find the alternate host of stripe rust from 1890 to 1930 (Eriksson and Henning, 1894; Mains, 1933; Tranzschel, 1934; Straib, 1935; Rapilly, 1979). First time Mains (1933) and Rapilly (1979) rightly speculated that *Berberis* and *mahonia* species could serve as alternate hosts for yellow rust. In 2010, the above speculation were approved by Jin *et al.* (2010) that, *Berberis* spp are serving as an alternate host for the inoculation of wheat, oat, rye, blue grass and barley with aeceial samples of stripe rust (*P. pseudo striiformis*). Later, *B. vulgaris* (Xian-Ming *et al.*, 2012; Cheng and Chen, 2014; Rodriguez-Algaba *et al.*, 2014; Wang *et al.*, 2015; Wang *et al.*, 2016) and *Mahonia aquifolium* (Zhao et al., 2013; Kang et al., 2015; Wang et al., 2015), were reported to be susceptible to stripe rust pathogens. In China, 42 *Berberis* species were found susceptible against stripe rust under control conditions (Zhao *et al.*, 2013; Zhao *et al.*, 2016; Zhuang *et al.*, 2019). Now it is logical that new virulent races of stripe rust arises due to sexual reproduction on susceptible barberry plants (Jin *et al.*, 2010). In Pakistan, especially Himalayan region are considered as the centre of yellow rust pathogen with rich genetic diversity. Recently, Mehmood *et al.* (2019) proved that *Berberis* species and sub species of Himalayan region of Pakistan were susceptible to stripe rust under control conditions. While in China, (Xinjiang region) three *Berberis* species were reported as alternate host of yellow rust in control conditions (Zhao *et al.*, 2016; Zhuang *et al.*, 2019). Naturally, the alternate host like *M. aquifoliumas* and *Berberis spp*. of yellow rust is still not known, but the presence of rich genetic diversity of stripe rust in Asia like China and Pakistan, showed that natural genetic recombination is exist (Duan *et al.*, 2010; Ali *et al.*, 2014). In control conditions, many researchers confirmed that *Berberis* species serve as an alternate host for stripe rust (Wang *et al.*, 2002; Jin and Singh, 2006; Jin *et al.*, 2010; Cheng *et al.*, 2014; Rodriguez-Algaba *et al.*, 2014; Wang *et al.*, 2015; Tian *et al.*, 2016; Wang *et al.*, 2016; Mehmood *et al.*, 2019; Siyoum *et al.*, 2019; Zhuang *et al.*, 2019).

1.7.1.3. Leaf rust *(Puccinia triticinie)*

Leaf rust or brown rust are caused by *Puccinia triticinie* (Goyeau *et al.*, 2006; Bolton *et al.*, 2008), and usually infect leaves but have also been reported on glumes and awns (Marsalis

and Goldberg, 2016). A circular or oval yellow lesions on upper surface of leaf appeared, which become orange on maturation and released numerous spores from single lesion (Marsalis and Goldberg, 2016). This rust pathogen generally attacks on wheat, barley and rye as *Puccinia triticinie, P. hordei* and *P. recondita* f.sp. *secalis* respectively. Favourable conditions for leaf rust are mild days with enough moisture and 20-25 °C. This wide range adaptation to climate make this pathogen problematic which displayed new virulence races against wheat varieties (Huerta-Espino *et al.*, 2011; McCallum *et al.*, 2016).

Their main host is *Triticum* or *Triticale* species while, alternate host is *Thalictrum* spp and some others (Jackson and Mains, 1921; Craigie, 1927; Tranzschel, 1934; d'Oliveira, 1960; Sibilia, 1960). First time, *Ornithogalum* spp was reported as alternate host of leaf rust by Tranzschel (1914). Many searchers confirmed more than 30 species of this plant as alternate host. Similarly, *Thalictrum* spp. for *Puccinia triticinie* as an alternate host was confirmed in 1921 by Jackson and Mains (1921). Besides this, many researchers reported others species as alternate host of *Puccinia triticinie* like, *Isopyrum fumarioides* (Craigie, 1927), *Anchusa* spp (d'Oliveira and Samborski, 1966) and *Clematis* sp (Sibilia, 1960).

1.8. PCR Based Approaches for Rust Study

To control such pathogenic diseases, the easiest and most effective methods is conventional plant breeding and selection of resistance genes through breeding strategies, but it is time and labour intensive (Kerber, 1987; Sharma, 2003). From last decades, novel genetic tool have been introduced to overcome the above problems and to screen and develop a resistant and fruitful yield producing cultivars (Sharma, 2003; Landjeva *et al.*, 2007).

Rust pathogen of cereal and grasses are mostly heteroecious and macrocyclic consisting of five spore stages *i.e.,* uredinial, telial, basidial, pycnial and aecial stages [Figure 4] The obligatory lifetime and complicated life cycles make rust pathogens challenging to study. Therefore, DNA extraction from basidiospores, pycniospores and aeciospores is very challenging due to their existence on alternate host, low quantity of spores and their obligate nature. For these purposes, many approaches are trying to extract a good quality and quantity of DNA from collected samples. The extraction of good quality DNA totally depends on extraction methods. Different researchers adopted different approaches to extract DNA from fungus infected tissues or spores like urediniospores and aeciospores. Mostly CTAB method, is used by numerous researchers to extract good quality of fungal DNA (Murray and Thompson, 1980; Chen *et al.*, 1993; Berlin, 2017; Hu *et al.*, 2017). Nowadays, along with CTAB methods, commercially available KITS are used to extract DNA from fungal tissues or

spores. (Liu and Hambleton, 2010; Liu *et al.*, 2013; Rodriguez-Algaba *et al.*, 2014; Bergeron *et al.*, 2019).

The problems with extraction from fungal samples is that a limited amount of spores or infected tissues like as aeciospores or aecial cluster of rust pathogens (Drábková, 2014). Similarly, the cell wall of fungal tissues or spore is mostly comprised of chitin or other biochemical substances, which is not easily crushed. The other critical point is the grinding of materials like using pestle mortars and liquid nitrogen leads to loss and contaminations of the grinded materials (Drábková, 2014).

1.8.1. Molecular Markers for Screening of Fungal Population

After DNA extraction, the next step is the selection of affective molecular markers for identification and phylogenetic screening of fungal population. Molecular markers play a great role in population study of fungi. Numerous types of molecular marker are used by researchers to identify the fungal diversity. Commonly used markers for characterization of resistance genes in crop plants are RFLP (Hartl *et al.*, 1993; Nelson *et al.*, 1997), AFLP (Haile *et al.*, 2013), RAPD (Penner *et al.*, 1995), SSR (Peng *et al.*, 2000; Wang *et al.*, 2002), SCAR (Gold *et al.*, 1999; Liu *et al.*, 2012), STS (Naik *et al.*, 1998; Prins *et al.*, 2001), internal transcribed spacer (ITS) regions (Jasalavich *et al.*, 2000; Viaud *et al.*, 2000), beta tubulin and ATPase (Glass and Donaldson, 1995; Keeling *et al.*, 2000) and many others are used to study fungal population (Green *et al.*, 2004).

For *Puccinia graminis* f. sp. *Tritici* identification various types of primers were developed like Pgtfssr1-F/R primer (Wang *et al.*, 2011; Chen *et al.*, 2015), Pgtw (f)/ Pgtw (r) primer (Liu *et al.*, 2014), ITS1RustF10d and StdLSUR2a primers (Barnes and Szabo, 2007; Berlin *et al.*, 2012). Similarly, stripe rust specific markers were developed by different researchers like YRNT1/YRNT2 (Fraaije *et al.*, 2001), BAF6/BAF2 and SCAR primers *i.e.* , YR(f)/(r1) and YR(f)/YR(r2) (Lihua *et al.*, 2008), PSR/PSF primer (Zhao *et al.*, 2007), PST2 f/r primers (Wang *et al.*, 2008) and SCAR primers, *i.e.*, PSTF117/PSTR363 and TF114/TR323 (Gao *et al.*, 2016).

Among these markers, the ITS and SSR markers are mostly used for analysis of inter and intra specific species identification of phytopathogenic fungi (Henrion *et al.*, 1994; Pritsch *et al.*, 1997). For identification of the rust species, the ITS regions are best to be amplified while for variation among and within rust species, the SSR markers are better option to be used. For population genetics of rust fungi, a best set of markers are required like microsatellites marker. SSR markers have previously been used for populations study of rust pathogens (Barnes and

Szabo, 2007; Jin *et al.*, 2009; Zhong *et al.*, 2009; Admassu *et al.*, 2010; Berlin *et al.*, 2012; Berlin *et al.*, 2013; Berlin *et al.*, 2013; Berlin *et al.*, 2017). The advantages of these markers are their co-dominant, highly polymorphic and short size microsatellite motive with repeated nucleotide sequences (Enjalbert *et al.*, 2002; Thiel *et al.*, 2003; Varshney *et al.*, 2005; Guo *et al.*, 2007; Giraud *et al.*, 2008). The best and most feasible option to reduce attack by wheat pathogens is the molecular plant breeding tools to identify and develop resistant wheat varieties (Singh *et al.*, 2016). For this purpose, knowledge about crop pathogen diversity around the globe is essential (Brown and Hovmøller, 2002).

The presence of different *Berberis* species in Pakistan and their neighbouring countries may increase the emergence of new races and also serve as seasonal bridge for stem and stripe rust pathogen. However, Bhardwaj *et al.* (2019) reported that *Berberis* species as an alternate host in India have no role in epidemiology of stem and stripe rust on seasonal wheat crops. Berlin *et al.* (2017) reported that the role of sexual reproduction in rust pathogens virulence are relatively low in many regions of the world, but it may still have great role in new races development by genetic variation. Genetic study of resistance genes with a virulence genes in rust population on alternate hosts could be helpful for developing long resistance cultivars (Zhao *et al.*, 2016). Sexual reproduction of rust, leading to the production of new races, which become responsible for loss of grain yield in cereal crops and grasses (Dubin and Brennan, 2009). Alternate host gain less attention as compared to primary host, because uridnial and telial stages is considered economically important as it appears on cereal crops or primary host than aecial stage.

1.9.Aims and Objectives

Pakistan has been blessed with variable climates, from the high mountains of Himalayas to valleys of Karakoram, delta plains of five rivers to Vast agricultural plains and desert climate to coastal regions. In total 10 mountainous regions including Himalayas, Karakoram, Margalla, Hindu Kush, Safed Koh, Suleiman, Kirthar, Salt range and Toba-Kakari ranges. Species of family Berberidaceae are mostly adapted to mountainous regions. Keeping in view the diversity of climates of various mountainous regions of Pakistan, the present study was designed to explore the existing flora of family Berberidaceae in Pakistan for their potential use in medicine and as alternate host of cereal rust. Present study was designed to investigate phytochemical (berberine, phenolic and flavonoid), elemental contents **[Chapter 1. Berberis; A Medicinal Plant in Pakistan]** and biological applications of roots, leaves and stem extracts of selected Berberis species **[Chapter 2. Biological application of Berberis plant].** Additionally, role of Berberis in green synthesis of nanoparticles **[Chapter 3. Plant Mediated Green Synthesis of Metal Oxide Nanoparticles]** and an alternate host of rust under natural conditions **[Chapter 4. Berberis: An Alternate Host of Rust].**The key aims and objectives of this dissertation were:

The main objective of the study was to identify the geographic distribution of each *Berberis* species. In present dissertation, we examined the presence or absence of berberine contents, total phenolic and flavonoids contents of *Berberis* species of Pakistan. In addition, we also determined the mineral composition (Na, K, Mg, Ca, Fe, Zn, Cd and Cu) of all three parts (leaf, stem and roots) of these species. To access the medicinal importance of the *Berberis* species, we performed antioxidant, antimicrobial, anti-haemolytic, DNA damaging protection, toxicity, and phytotoxic potential of extracts of *Berberis* plants. Nanotechnology is an emerging field with multiple applications in agriculture, medicine etc. In this thesis, we also investigated the potential use of *Berberis* species in green synthesis of nanoparticles. As explained in introduction, roots of *Berberis* species have been reported to have various medicinal properties. The study was also designed to explore the medicinal importance of aerial parts, to ensure conservation of these endemic plants. On the other hand, it is well documented that *Berberis* species are alternate host to various fungal pathogen of import crops. In this dissertation we also examined the role of selected *Berberis* species as an alternate host of wheat rust in Pakistan.

CHAPTER 1

CHAPTER 1. BERBERIS; A MEDICINAL PLANT IN PAKISTAN

Introduction

Shelter, food, clothing, and medicine are the primary necessities of human civilization. Medicinal plants are those plants that show a rich source of bioactive compounds with curative effects. Medicinal plants are considered an easy to collect and rich source of disease curing compounds. As it has been reviewed in the introductory section, that more than 80% of the world population depends on medicinal plants, due to their medicinal values, biosafety and economic use compared to synthetic drugs (Bodeker and Ong, 2005). From the Neolithic era, human beings have been using their sense like taste, instinct, and experience (trial and error) to distinguish the herbal plant to treat their health problems. Consequently, human beings have had a close association with the medicinal plants since early times and still using the plants as a source of pharmaceuticals (Fierascu *et al.*, 2018). The use of medicinal plants as herbal drugs is increasing day by day all over the world due to the easy availability, convenience, affordability, and favourable effectiveness comparable to the typical artificial medicine.

Up To the 18th century, the medicinal properties of several plants, their methods of treatment and their effectiveness for human health were well-known, but biochemicals responsible for this efficacy remain unknown (Faridi *et al.*, 2010). After the invention of advanced scientific tools (microscopic and spectroscopic instruments), the concept of traditional medicine has been shifted to modern medicine (Salmerón-Manzano *et al.*, 2020). However, in underdeveloped countries, medicinal plants are used as alternative natural drugs agent in those areas where synthetic drugs are not available or affordable (Mustafa *et al.*, 2017). Through instrumentations, various types of valuable phytocompounds have been isolated and screened against various types of diseases. The phytochemicals are non-nutritious ingredients, which defend the plant from various types of biotic and abiotic accentuates (Savithramma *et al.*, 2011). These phytochemicals include alkaloids, polyphenols, flavonoids, tannins, steroids and terpenoids. In this regard, more than 3,00,00 plants have been investigated for phytochemicals purposes (Lattanzio, 2013).

These integrands are divided into two groups known as primary and secondary metabolites (Kumar *et al.*, 2009). Primary metabolites are representative of carbohydrates, proteins, lipids, while secondary metabolites consist of alkaloids, polyphenol and terpenoids. These metabolites are responsible for the proper growth, development and protection of plants

(Overlingė *et al.*, 2021; Rizaludin *et al.*, 2021). The secondary metabolites like phenolic compounds and flavonoids have a great contribution to herbal and pharmaceutical drugs (Ribarova *et al.*, 2005; Atanassov *et al.*, 2021). These herbal properties are directly proportional to the presence of functional groups (phenolic, hydroxyl and carboxylic acid). These functional groups have an inhibitory effect against reactive oxygen species by breaking the radical chain and chelating the metals in the body during stress (Osawa, 1994). The mechanism of antioxidants is to neutralize or inhibit the free radicals by donating the hydrogen atom or an electron (Ozougwu, 2016). Additionally, the antioxidant action exhibited strong biological activities like, antimicrobial, anti-inflammatory, anti-arrhythmic and antitumor (Rehman *et al.*, 2018).

Recently numerous phytochemicals have been isolated from different *Berberis* species such as *B. crataegina, B. integerrima, B. aetnensis* and *B. libanotica* having 22, 18, 26 and 37 bio-compounds respectively (Salehi *et al.*, 2019). All parts of *Berberis* species have been reported as a rich source of bioactive compounds. However, various parts have distinct types of phytoconstituents like stem and root extracts have almost the same types of alkaloids (berberine), while leaves of different *Berberis* species express variation in bio-constituents (acids and phenolic compounds) (Bhardwaj and Kaushik, 2012). The main chemical constituents includes alkaloids terpenoids, flavonoids, anthocyanins, vitamins, sterols, carotenoids, lignins, lipids and proteins have been isolated from different barberry (Khan *et al.*, 2016). The core alkaloids isolated from various *Berberis* plants are; berberine, columbamine, berbamine, khyberine, pakistanine, baluchistanamin, karachine pakistanamine, palmatine, oxyacanthine and jatrorrhizine (Haji, 2010; Srivastava *et al.*, 2015; Khan *et al.*, 2016). These phytochemicals exhibit strong biological activities like, antioxidant, antimicrobial, anti-inflammatory, antihaemolytic, anti-arrhythmic and antitumor (Rehman *et al.*, 2018). Apart from phytochemicals, the medicinal plant is also a rich source of mineral elements which serve as important constituents in several metabolic processes. The ethnobotanical, phytochemical knowledge and their biological application presented in the previous introduction chapter provides the foundation for understanding the medicinal significance of *Berberis* .

Pakistan hosts a prosperous and distinct flora of nearly 5,700 plant species. Of 5,700 plant species, 2000 showed therapeutic characteristics (Ullah, 2017). The native flora is distributed in various climatic zones (temperate, tropical, arid, and semi-arid). These medicinal plants are facing several challenges, like climate change, lack of knowledge, illegal plant

collection and uprooting of herbal plants. These challenges caused the depletion of native flora. More than 700 plants including 64 medicinal plants are endangered. Recently, the *Berberis* plant was reported as an endangered species, as its roots are extensively used in traditional and modern medicines by local inhabitants and pharmaceutical industries (Khan *et al.*, 2016; Islam *et al.*, 2021; Nazir *et al.*, 2021).

As review literature discloses the phytochemical and pharmacological importance of *Berberis* genus. This chapter evaluates the comparative study of elemental, phytochemical and antioxidative potentials of *Berberis* species distributed across the Pakistan. In vivo and in vitro biological applications of this genus will be assessed using *B balochistanica* plant as a model organism.

Research Articles

ARTICLE 1: COMPARATIVE PHYTOCHEMICALS AND ANTIOXIDATIVE STUDY OF GENUS *BERBERIS* IN PAKISTAN

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Received: date; Accepted: date; Published: date

Abstract

The genus *Berberis* is gaining attention due to its medicinal and nutritional properties in therapeutic and nutritional field. It also has to cope with scientific threats due to over exploitation of root and bark for medicinal purposes. In the present study, we aimed to analyse three parts (root, stem and leaf) of eight selected *Berberis* species as an alternate and suitable means for phytochemical, nutritional, and antioxidant purposes. Spectroscopic profile revealed that all *Berberis* species comprise of a rich source of elements in order *B. balochistanica* > *B. royleana* > *B. parkeriana* > *B. pseudoumbellata* > *B. pachyacantha* > *B. calliobotrys* > *B. lyceum* > *B. orthobotrys. B. balochistanica* showed highest amount of Sodium, magnesium, calcium, and zinc as compared to the other species. The HPLC analysis revealed that berberine was abundantly present in the root extracts and its concentration was found in descending order of BCR > BRR> BPR > BOR > BPrR > BBR > BLR > BPsR. The spectroscopic profile showed highest total phenolic (TPC) and total flavonoid contents (TFC) in *B. pseudoumbellata, B. royleana, B. pachyacantha* and *B. balochistanica.* All selected species showed antioxidant activity, the highest DPPH (2, 2-diphenyl-1-picrylhydrazyl) activity was recorded in BPrL,

BLR, BCR and BPsR, while TAC was found in descending order of leaves extracts > root extracts > stem extracts and TRP was observed in decreasing order of leaves extract > stem extracts > root extracts. In the end, Pearson correlation analysis (PCA) was performed which revealed strong positive correlation ($r \geq 0.5$) between DPPH activity and TPC and TFC of leaves extracts and weak correlation ($r = 0.033$) with berberine contents in root extracts. This correlation implies the role of phytochemicals in general antioxidant action of analysed aerial parts. These results have identified, for the first time, that along with roots, the aerial parts of all analysed species are rich in essential nutrients and phytochemical compounds with high antioxidant activity. These findings may be useful in the conservation of medicinal plants by discouraging the uprooting of underground parts for medicinal purposes.

Key words: *Berberis* plant; Mineral elements; Phytochemical screening; HPLC, Antioxidant; Alternate source.

1. Introduction

Globally, medicinal plants have been and still are used to cure ailments from colds to cancer (Fierascu *et al.*, 2018). The importance of medicinal plants is mainly due to the presence of biologically active, non-toxic and economical compounds (Bodeker and Ong, 2005). These chemical constituents includes several types of metabolites, which protect the plant from biotic and abiotic stresses (Zhao *et al.*, 2005; Crozier *et al.*, 2006). Owing to this natural defense properties, medicinal plants have attained considerable attention in pharmaceutical and food industries (Zhao *et al.*, 2005). Bioactive compounds primarily act as natural antioxidants that are commercially utilized as nutritional supplements or as antioxidant additives (Škrovánková *et al.*, 2012). With the emergence of growing technologies, novel plants are being explored to ascertain the presence of phyto-constituents with respect to their ethno-pharmacological values.

The genus *Berberis* belongs to the Berberidaceae family and comprises of ~17 genera and 650 species (Rao *et al.*, 1998). Pakistan is hosting 29 species that are distributed in mountainous regions of all provinces (Khan *et al.*, 2014). Morphologically, the genus *Berberis* consists of evergreen deciduous spiny shrub with yellow flowers and berry-type fruits (Mokhber-Dezfuli *et al.*, 2014). The medicinal properties of this genus are predominantly investigated due to the presence of various bioactive compounds. Which are mainly distributed

in different organs of the *Berberis* plant such as berberine in roots and polyphenol in leaves (Zuzanna Bober *et al.*, 2018). These phytochemicals have an inhibitory effects against reactive oxygen species and exhibit significant antimicrobial, anti-inflammatory, anti-arrhythmic and antitumor activities (Rehman *et al.*, 2018). Recently 18 - 37 compounds have been identified from *B. crataegina, B. integerrima, B. aetnensis* and *B. libanotica* (Salehi *et al.*, 2019).

Due to the presence of these phytochemicals, *Berberis* species have been exploited for chronic inflammatory disorders, hypoglycemic, anticancer, antihypertensive, anti-histaminic, anti-fatigue, anticoagulant, antidiabetic, osteolytic, hypotensive, CNS-depressant, anti-nociceptive and antimicrobial activities (Ivanovska and Philipov, 1996; Abushouk *et al.*, 2017). Beside these phytochemicals, this genus also comprises of nutritional elements which play a vital role in the living body. Deficiency of minerals is one of the serious health issues in developing as well as under-developed countries (Batra and Seth, 2002). In a recent analysis of UNICEF report 2019, more than 200 million children under the age of five are facing growth problems, while about 340 million children experience hidden hunger worldwide (Keeley *et al.*, 2019).

Keeping in view the ethno-medicinal importance of family Berberidaceae (Khan *et al.*, 2016; Majid *et al.*, 2020), the present study was designed to execute comparative analysis among different *Berberis* species that are observed in different areas of Pakistan. Hence, relative elemental analysis, phytochemical (berberine, phenolic and flavonoid) contents, and antioxidant potential of roots, leaves and stem extracts of selected *Berberis* species was carried out to prospect their bio-efficacy for use in food and pharmaceutical industries.

2. Materials and Methods

2.1. Collection of Plant Material

A total of eight medicinal species belonging to the genus *Berberis* were collected during June - August 2019 from different mountainous regions of Pakistan and their accession numbers were obtained from Herbarium of Quaid-i-Azam University, Islamabad and National

herbarium of NARC, Islamabad. Detail of selected species collected from various areas and their accession numbers are given in figure 1 and table 1.

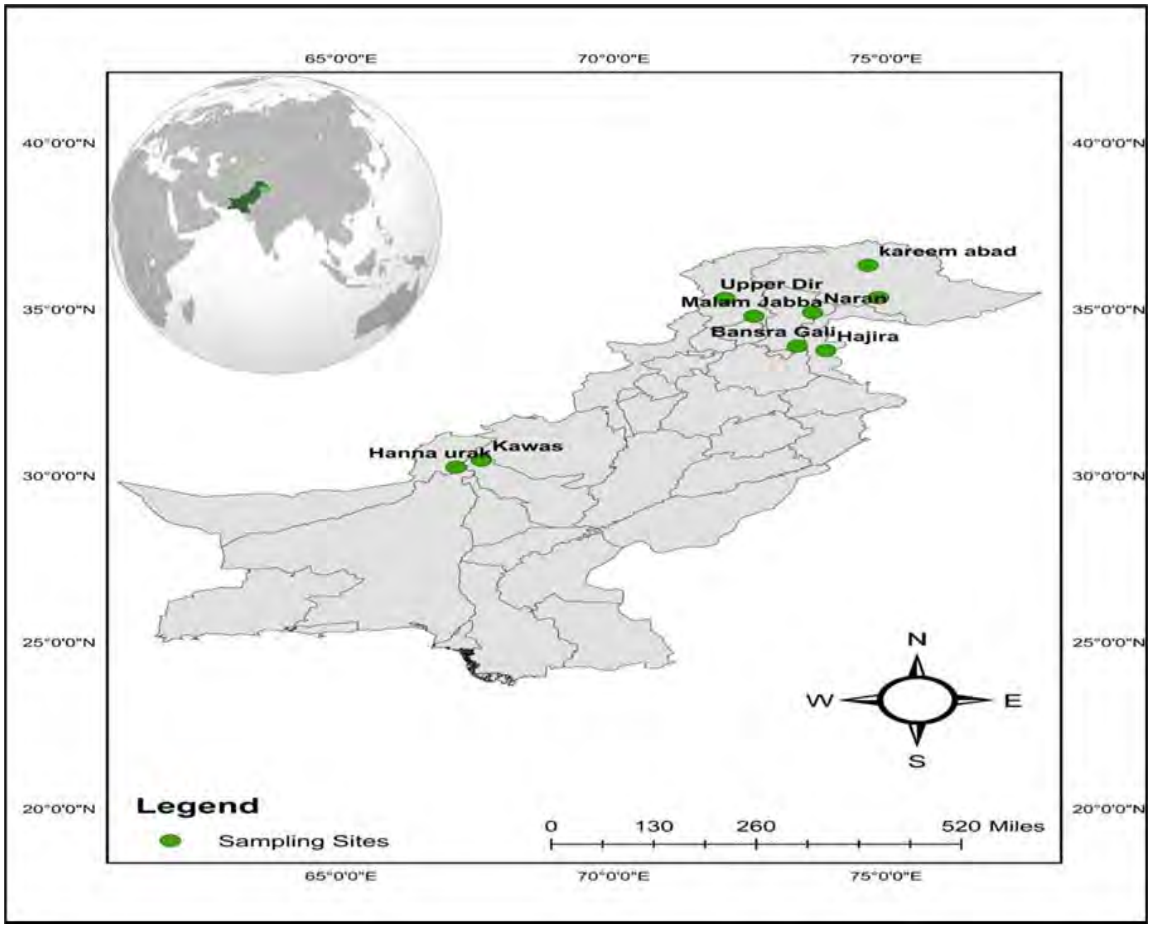

Figure1a. Distribution of selected *Berebris* species in paksitan .

Figure1b. Herbarium samples of selected *Berebris* species in paksitan .

Table 1. List of selected *Berberis species* along with their accession numbers, areas of collection and acronyms

S. No.	Species	Accession Numbers	Locations		Acronyms		
			Province	**District**	**Root**	**Leaves**	**Stem**
1.	*Berberis balochistanica*	100267	Balochistan	Quetta	BBR	BBL	BBS
2.	*Berberis calliobotrys*	100266	Balochistan	Ziarat	BCR	BCL	BCS
3.	*Berberis pachyacantha*	100433	Khyber Pakhtunkhwa	Mansehra	BPR	BPL	BPS
4.	*Berberis parkeriana*	131411	Khyber Pakhtunkhwa	Swat	BPrR	BPrL	BPrS
5.	*Berberis lyceum*	100269	Punjab	Murree	BLR	BLL	BLS
6.	*Berberis pseudumbellata* subsp. *Gilgitica*	131412	Gilgit-Baltistan	Hunza	BPsR	BPsL	BPsS
7.	*Berberis orthobotrys*	100432	Gilgit-Baltistan	Astore	BOR	BOL	BOS
8.	*Berberis chitrria*	131414	Azad Kashmir	Poonch	BRR	BRL	BRS

2.2. Extracts Preparation

The underground part (roots) and aerial parts (stem and leaves) of plants were washed, dried, powdered and then mixed with 200 mL of methanol. After 24 hours, the samples were incubated in a water bath at 40 °C for 3 hours followed by filtration using Whatman filter paper no. 1. The solvent was evaporated using a rotary evaporator (BUCHI Rotavapor R-220) and obtained crude extracts were placed in the refrigerator at 4 °C for further analysis (Harborne, 1973). For experimental analysis, the stock solution was prepared in methanol (95%) and dilutions were used in various parameters.

2.3. Elemental analysis

Dried powder of each plant sample (0.5 g) was digested in 8 mL of HNO_3: $HClO_4$ (3: 1 v/v) overnight and then heated until brown fumes turned to white. Afterwards, the digested mixtures were diluted with 25 mL of distilled water followed by filtration using Whatman no. 42 filter paper. Subsequently, these filtrates were subjected to the atomic absorption spectrophotometer (Spectra AA240 FS, Varian, New Jersey, USA) to analyze the calcium, potassium, sodium, magnesium, iron, zinc, cadmium, and chromium concentration.

2.4.Phytochemical analysis

2.4.1. Berberine quantification using HPLC method

HPLC was used for screening of berberine contents in three different organs of selected *Berberis* species. Ten microliters of plant extracts and standard (1000 µg/mL) were loaded on C18 reverse phase column. The flow rate, run time and column temperature were set as 1 mL/min, 20 minutes and 30 °C respectively. After several trials, the typical chromatogram was obtained using phosphate buffer (A) and acetonitrile (B) in a ratio of 50: 50 v/v and the eluted berberine was detected at 360 nm.

2.4.2.Determination of total phenolic and flavonoid contents using spectroscopic method

For total phenolic contents, 20 µL of selected extracts were mixed with 90 µL of Folin-Ciocalteu reagent and 90 µL of $NaCO_3$ solution in a 96-well plate. After incubation at room temperature for 60 min, the absorbance was measured. Gallic acid was used as a reference and results were expressed as mg GAE/g of the sample. Total flavonoids were estimated using the

Aluminium Chloride Colorimetric method with some modifications and results were expressed as mg of QE/g of the extract (Chang *et al.*, 2002).

2.5.Antioxidant analysis

2.5.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

Initially, reagent solution was prepared by adding 2.4 mg of DPPH (2, 2-diphenyl-1-picrylhydrazyl) in 25 mL of methanol. Then, 180 µL of reagent solution was added into 20 µL of test sample to make final 200 µL of reaction mixture. The mixture was incubated for 1 hour and absorbance was measured at 517 nm. In the end, radical scavenging activity was calculated using the following formula:

DPPH scavenging activity (%) = $Abs_{control} - Abs_{sample} / Abs_{control} \times 100$

2.5.2 Total antioxidant capacity (TAC)

The antioxidant capacity of selected species was evaluated by using phosphomolybdenum method and ascorbic acid was used as a standard (Prieto *et al.*, 1999). Plant extracts and reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate) were mixed and incubated at 95 °C for 90 minutes. Then the solutions were cooled, and absorbance was taken at 695 nm against blank. Reagent solution (1 mL) dissolved in appropriate volume of solvent were incubated and used as a blank. Antioxidant capacity was assessed using following formula:

Total antioxidant capacity (%) = $Abs_{control} - Abs_{sample} / Abs_{control} \times 100$

2.5.3 Total reducing power (TRP) assay

Each plant extract (2 mL) was mixed with 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (10 mg/mL) followed by the incubation at 50 °C for 20 minutes. Afterwards, 2 mL of tricholoroacetic acid (100 mg/L) was added and then centrifuged (3000 rpm) for 10 minutes to obtain the supernatant. Then, 2 mL of distilled water and 0.4 mL of ferric chloride were added in the supernatant and absorbance was taken after 10 minutes at 700 nm. Higher absorbance indicates higher reducing power of plant extract (Fejes *et al.*, 2000)

2.6.Statistical Analysis

The obtained results were interpreted as mean ± standard deviation of three replicates. For TPC and TFC, means were compared by using one-way ANOVA, followed by Tukey's test ($p < 0.05$) via statistix 8.1 software. IC_{50} values were determined in DPPH scavenging assay by using Graphpad prism software (Finney, 1952). Principal component analysis was

performed via XLSTAT to observe variability in the elements of tested extracts and Pearson correlation analysis was done to ascertain relationship between antioxidant assays and phytochemicals.

3. Results

3.1.Elemental analysis

Results of present study showed that all *Berberis* species comprise of a rich source of elements. The *Berberis* species with highest numbers of mineral elements were present in descending order of *B. balochistanica* > *B. royleana* > *B. parkeriana* > *B. pseudoumbellata* > *B. pachyacantha* > *B. calliobotrys* > *B. lyceum* > *B. orthobotrys.* Among the mineral elements, sodium (125.46 ± 0.56 ppm), magnesium (179.87 ± 0.72 ppm), calcium (696.17 ± 1.55 ppm) and zinc (43.21 ± 2.98 ppm) were identified highest in *B. balochistanica* (Quetta, Balochistan) as compared to the other species. However, potassium was found to be highest (527.09 ± 0.58 ppm) in *B. pseudoumbellata* which was collected from Hunza, Gilgit-Baltistan and iron was detected highest (339.59 ± 0.04 ppm) in *B. royleana* (Poonch, Azad Kashmir) (Figure 1). However, chromium and cadmium were not present in any extracts of studied species. Overall, roots and leaf extracts of *Berberis* species exhibited highest concentration of most of the elements as compared to the stem extracts (Table 3).

PCA biplot drawn between plants (active observations) and elements (active variables) revealed ~ 60.35 % of total variability achieved from axis 1 (39.48 %) and axis 2 (20.87 %) respectively. Species which lie close to the elements represented correlation between the species with respect to their concentration of elements. Moreover, elements lying on the right side of the axes (PC1) indicates minimum concentration while elements exhibiting maximum concentration are shown on the left side of axes (PC2) (Figure 2).

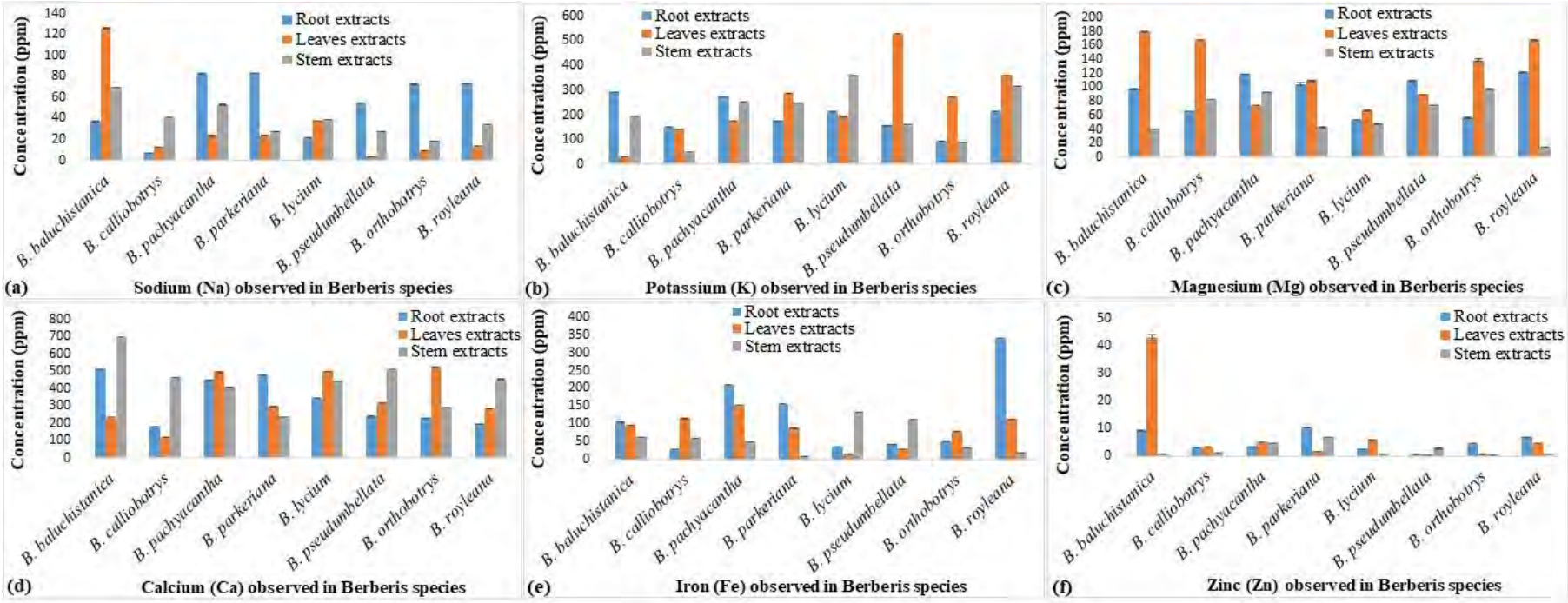

Figure 1: Concentration of various elements detected in roots, leaves and stem extracts of eight *Berberis* species collected from various areas of Pakistan (a) Sodium (b) Potassium (c) Magnesium (d) Calcium (e) Iron (f) Zinc. Data represents mean of three replicates and error bars indicates the standard deviation.

Table 2: Highest concentration of elements detected in eight *Berberis* species.

***Berberis* species**	**Concentration of elements**
B. balochistanica	Na (L), Mg (L), Ca (S), Zn (L)
B. calliobotrys	Mg (L)
B. pachyacantha	Na (R), Fe (R)
B. parkeriana	Na (R), Fe (R), Zn (R)
B. lyceum	K (S)
B. pseudoumbellata	K (L), Ca (S)
B. orthobotrys	Ca (L)
B. royleana	K (L), Mg (L), Fe (R), Zn (R)

Na: Sodium; K: Potassium; Mg: Magnesium; Ca: Calcium; Fe: Iron; Zn: Zinc; Cr: Chromium; Cd: Cadmium; L: Leaves; R: Roots; S: Stem

Table 3: Distribution of elements in different organs.

Elements	**Organ-wise distribution**
Sodium (Na)	Roots > Stem > Leaves
Potassium (K)	Leaves > Roots > Stem
Magnesium (Mg)	Leaves > Roots > Stem
Calcium (Ca)	Leaves > Stem > Roots
Iron (Fe)	Roots > Leaves = Stem
Zinc (Zn)	Leaves > Roots > Stem

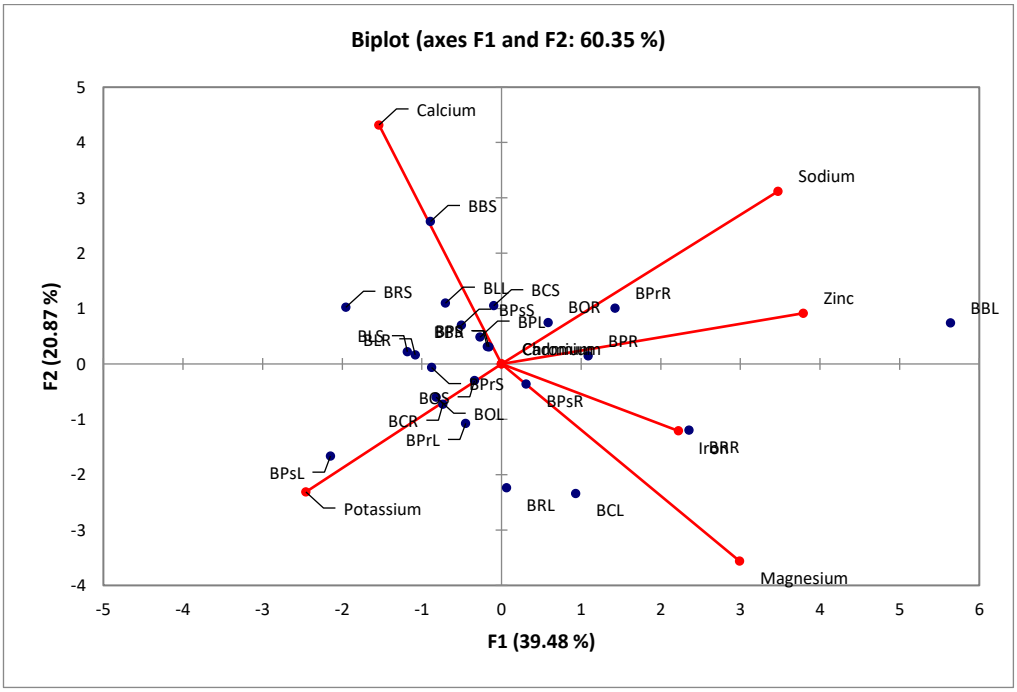

Figure 2: Principal Component Analysis (PCA) biplot for the elements present in roots, leaves and stem extracts organs of selected *Berberis* species.

3.2.Phytochemical analysis

Figure 3 shows the HPLC spectrum for the quantified berberine compound in different parts of examined species. Concentrations of berberine were estimated by comparing the retention time of all *Berberis* species with reference compound. Results revealed that berberine was abundantly present in the root extracts and its concentration was found in descending order of BCR (94504.63 ppm) > BRR (55721.02 ppm) > BPR (14500.67 ppm) > BOR (13387.40 ppm) > BPrR (3440.01 ppm) > BBR (2574.37 ppm) > BLR (2399.81 ppm) > BPsR (2312.54 ppm). In case of stem extracts, berberine was only detected in BPsS while among leaves extracts, berberine was observed in BRL (11265.84 ppm), BOL (1664.70 ppm) and BCL (976.59 ppm) (Table 4).

TPC was highest in the BPsL (70.374 ± 1.98 mg GAE/mg), BPrS (68.850 ± 2.76 mg GAE/mg) and BPS (58.178 ± 2.34 mg GAE/mg) and BRR (56.317 ± 2.66 mg GAE/mg) and BPsR (52.596 ± 2.33 mg GAE/mg). However, highest flavonoid contents were detected in BPsR and BPsL (41.401 ± 2.34 mg QE/g and 39.940 ± 2.44 mg QE/g) followed by BPrL (38.565 ± 1.42 mg QE/g) and BRL (37.789 ± 3.26 mg QE/g). Overall, *B. pseudoumbellata, B. royleana, B. pachyacantha* and *B. balochistanica* showed highest TPC and TFC (Figure 4).

Table 4: Berberine concentration observed in different *Berberis* species.

Name	Berberine concentration (ppm)		
	Root extracts	**Leaves extracts**	**Stem extracts**
B. balochistanica	2574.37	ND	ND
B. calliobotrys	94504.63	976.59	ND
B. pachyacantha	14500.67	ND	ND
B. parkeriana	3440.01	ND	ND
B. lyceum	2399.81	ND	ND
B. pseudumbellata	2312.54	ND	228.72
B. orthobotrys	13387.40	1664.70	ND
B. royleana	55721.02	11265.84	ND

Ppm refers to parts per million and ND mean not detected

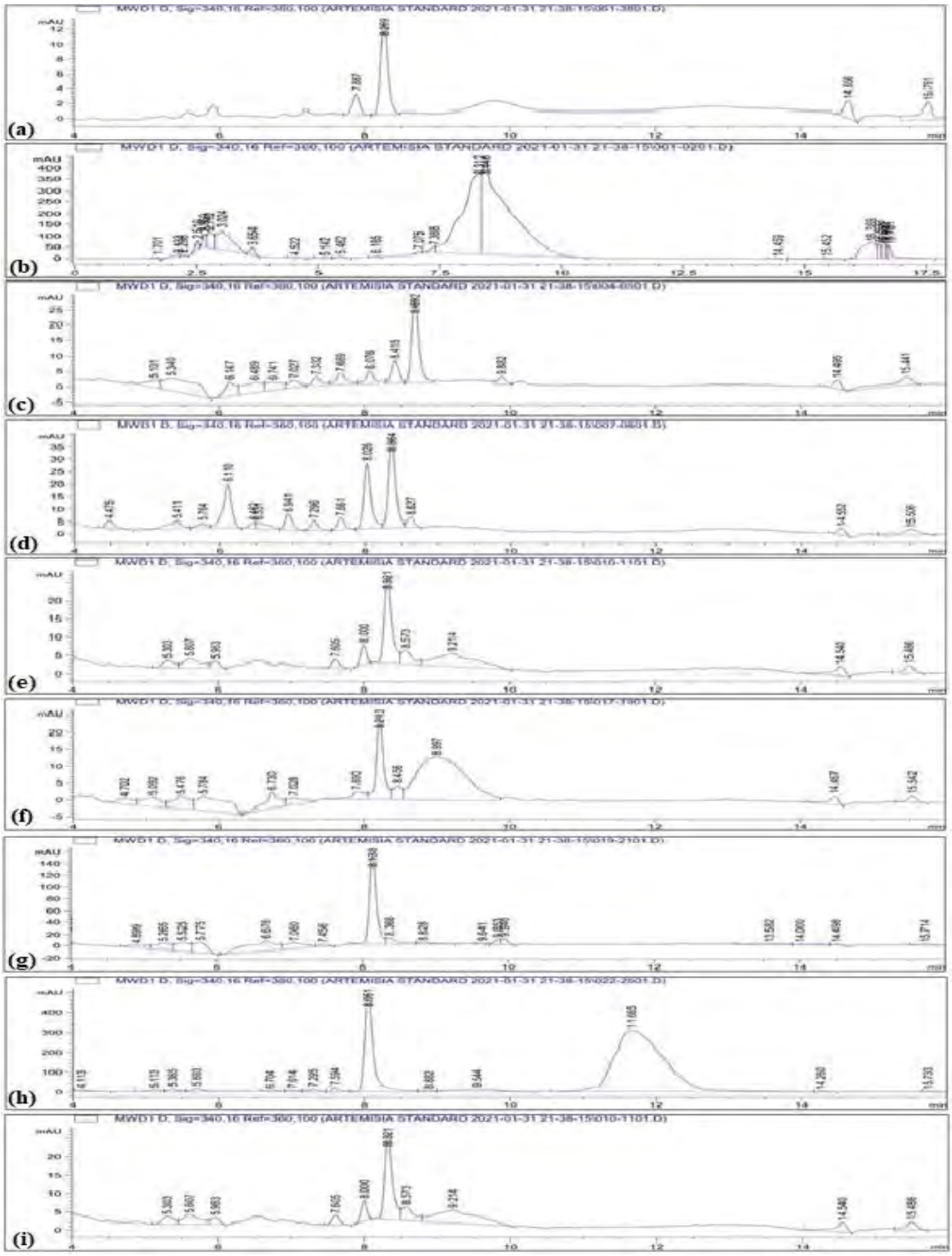

Figure 3. HPLC profile of roots of eight *Berberis* species indicating Berberine content. **(a)** Standard Berberine **(b)** *B. orthobotrys* **(c)** *B. balochistanica (***d)** *B. parkeriana* **(e)** *B. lycium* **(f)** *B. pseudumbellata* **(g)** *B. pachyacantha* **(h)** *B. royleana* **(i)** *B. calliobotrys*

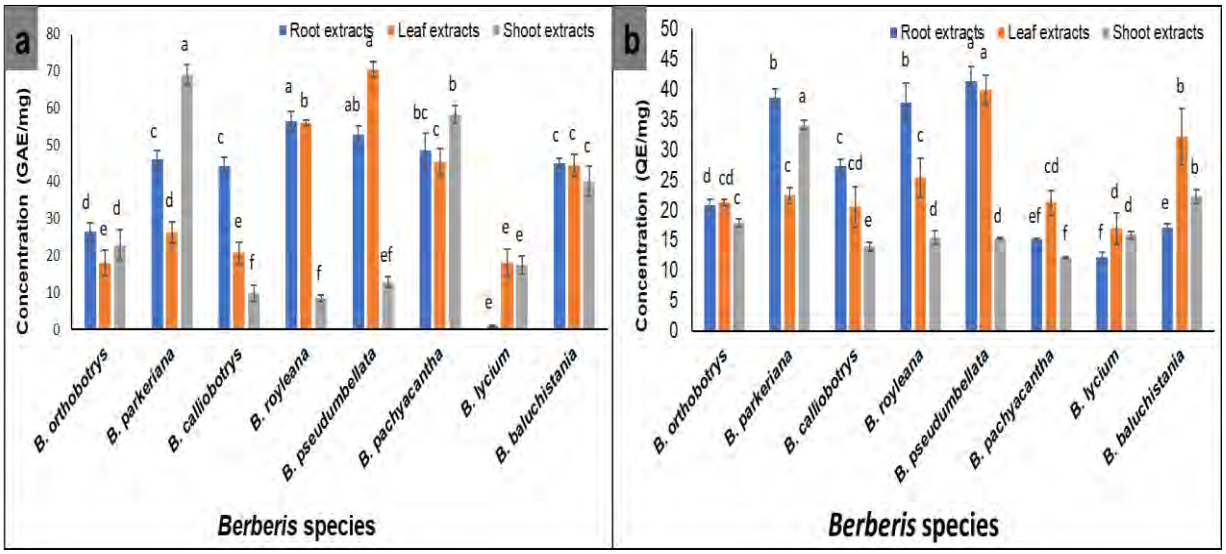

Figure 4: Phytochemical contentents (a)Total phenolic contents (mg GAE/g) and (b) Total flavonoid (mg QE/g) contents observed in the roots, leaves and stem extracts of eight *Berberis* species. Data represents the mean of three replicates and each letter (a-f) indicates significance at $P < 0.05$.

3.1.3. Antioxidant assays

Results of the DPPH assay revealed dose-dependent-response and percentage inhibition was increased with the rise in extract concentrations (250, 500 and 1000 µg/mL). IC_{50} values of root extracts were recorded in the range of 6.704 ± 1.832 µg/mL in *B. lycium* to 42.093 ± 2.475 µg/mL in *B. balochistanica* and while in case of leaves extracts, IC_{50} values ranged from 05.784 ± 1.399 µg/mL in *B. parkeriana* to 45.120 ± 3.986 µg/mL in *B. lycium.* However, stem extracts revealed lowest DPPH activity ranging from 12.598 ± 4.450 µg/mL IC_{50} value in *B. royleana* to 98.960 ± 6.850 µg/mL IC_{50} value in *B. pachyacantha.* Furthermore, highest DPPH activity was recorded in BPrL (5.784 ± 1.399 µg/mL), BLR (6.704 ± 1.832 µg/mL), BCR (8.200 ± 1.645 µg/mL) and BPsR (8.290 ± 1.80 µg/mL) (Figure 5a).

TAC was revealed highest in BBL (78.482 ± 1.078 mg/g) and BPsL (77.906 ± 0.812 mg/g) followed by BPsR (60.202 ± 2.88 mg/g) and BRR (55.856 ± 1.09 mg/g). However, stem extracts of selected species showed lowest TAC ranging from 11.922 ± 0.68 mg/g to 37.297 ± 1.383 mg/g (Figure 5b). Moreover, leaf extracts of *B. calliobotrys* (0.440 ± 0.06 mg/g) and *B. royleana* (0.433 ± 0.06 mg/g) exhibited highest reducing potential (Figure 5c). Among different organs, TAC was found in descending order of leaves extracts > root extracts > stem extracts and TRP was observed in decreasing order of leaves extract > stem extracts > root extracts.

In the end, Pearson Correlation Analysis was performed which revealed strong positive correlation ($r \geq 0.5$) between DPPH activity and TPC and TFC of leaves extracts and weak

correlation (r = 0.033) with berberine contents. Conversely, roots and stem extracts showed very weak correlation (r < 0.05) between DPPH and phytochemical contents. TAC displayed moderate correlation (r > 0.2) between TPC and berberine contents present in the *Berberis* root extracts while TRP indicated highly active correlation (r > 0.5) between TPC and berberine contents of stem extracts. However, all other extracts exhibited negative correlation with the phytochemicals (Figure 6).

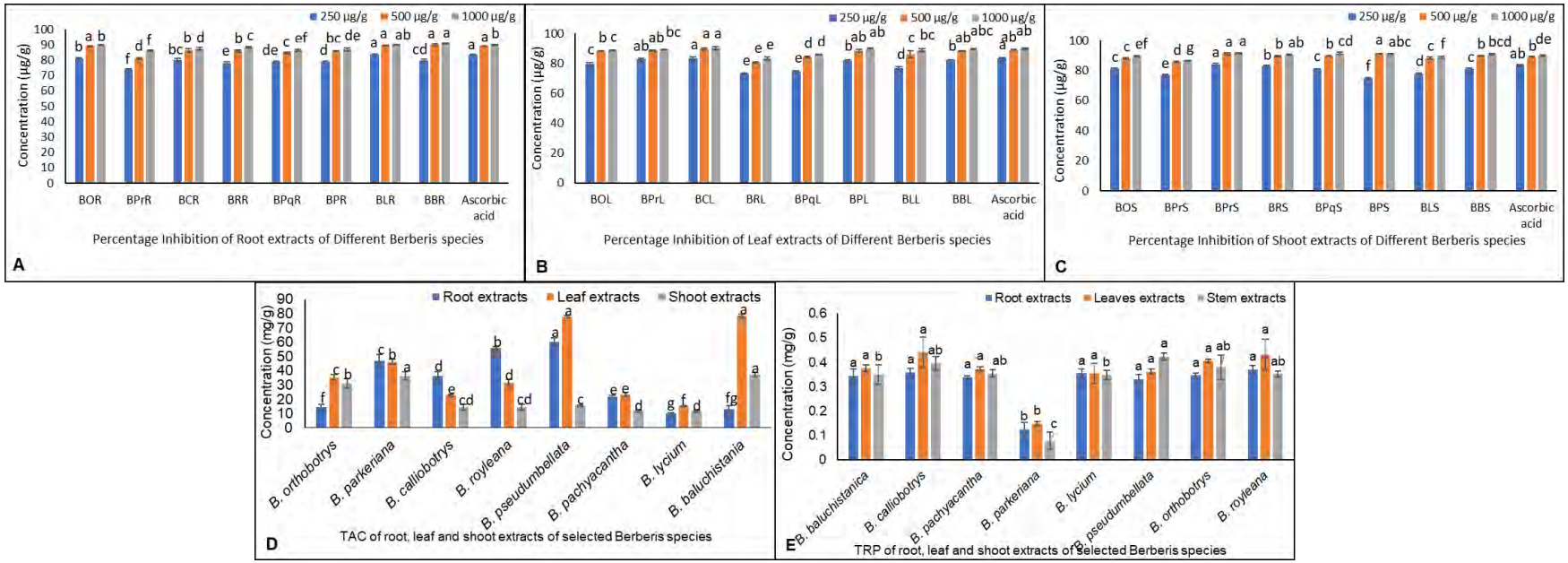

Figure 5: Antioxidant assays of root, leaves and stem extracts of eight *Berberis* species collected from different Provinces of Pakistan (A, B and C) DPPH radical scavenging activity of root, leaf and stem extracts (D) Total antioxidant capacity (E) Total reducing power assay

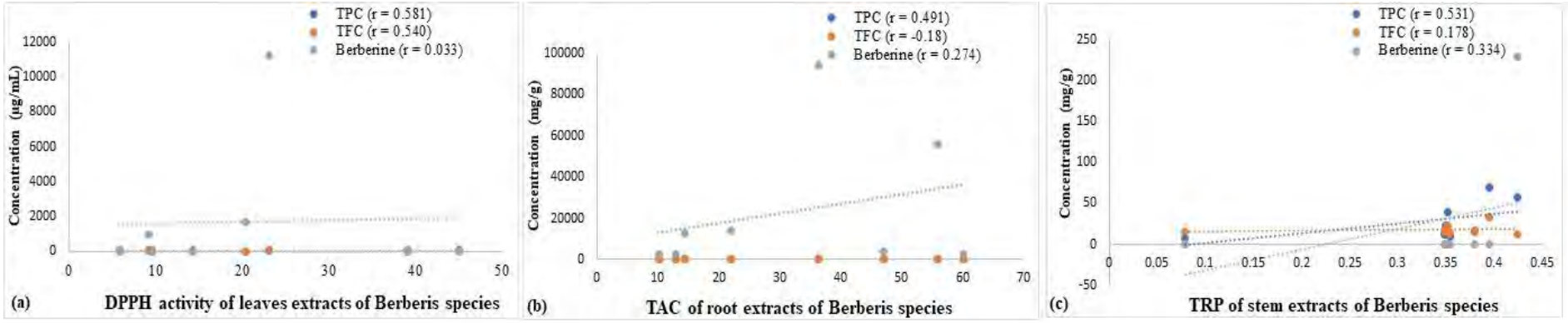

Figure 6: Correlation analysis of some selected extracts between antioxidant assays and phytochemicals. (a) DPPH activity of leaves extracts and phytochemicals (b) TAC of root extracts and phytochemicals (c) TRP of stem extracts and phytochemicals (r = Correlation Coefficient)

4. Discussion

Mineral elements are the integral components contributing to biochemical reactions, metabolic mechanisms and defense systems (Osae, 2001). Autotrophs like medicinal plants are a rich source of minerals and phytochemicals that have antioxidant potentials (Chakravarty and Ghosh, 2000). The introduction of wild plants into our diet will enhance the nutritional and phytochemical contents (Cernansky, 2015). In this study, eight *Berberis* species were investigated due to their immense ethnopharmacological usage by the local community. Result showed that roots, leaves and stem extracts of these species are rich source of six mineral elements excluding Cu and Cr. The distribution levels of mineral elements were observed in decreasing order of Ca > K > Fe > Mg > Na > Zn. Collectively, roots and leaf extracts indicated highest concentration of elements as compared to the stem extracts of these species. Among all species, the *B. balochistanica* and *B. royleana* showed highest concentration of elements as compared to the other species (**Table 6**). Such variability at species as well as organ level might be due to. the genetic factors, habitat and climatic conditions that affect these plants (Ercisli and Orhan, 2007). Parallel findings have been reported by many researchers who documented *B. asiatica* (2012), *B. vulgaris* (2009; Ardestani *et al.*, 2013), *B. integerrima* (Ardestani *et al.*, 2013) and *B. lyceum* (Ullah *et al.*, 2013) as a rich source of vital elements.

Macro-elements are required in substantial amounts (> 100 mg/day), whereas micro-elements are required in a trace amount (< 100 mg/day) to perform distinct functions of living organisms (Murray *et al.*, 2000). Normally, recommended dietary intake of Na, K, Mg and Ca are 3000, 3700, 350 and 1500 mg/day (Leśniewicz *et al.*, 2006). Zn and Fe are essential constituents for all living cells and their permissible level in edible plants is 27.4 ppm and 20 ppm (Bogden and Klevay, 2000). Calcium plays an important role in skeletal building and enzyme-mediated processes whereas potassium is required for the normal functioning of the

nerves, heart and muscles, sugar metabolism, acid-base balance and oxygen metabolism in the brain (Pirestani *et al.*, 2009). Our results were coherent with the previous findings of Shah *et al.* (2003) and Sood *et al.* (2010) who determined significant concentration of zinc (56.15 ± 0.01 µg/g), copper (95.67 ± 0.12 µg/g), potassium (161.42 ± 0.41 mg/100 g), sodium (14.5 ± 0.11 mg/100 g) and iron (2.61± 0.06 mg/100 g) in the leaves and fruits of *B. lycium*. Present studies also correlate with the earlier findings of Rahim *et al.* (2019) indicating the presence of iron, zinc, sodium, potassium in different concentrations in *B. balochistanica* extracts.

Magnesium is required for proper growth and function of bones and muscles while sodium is required for the regulations of the electrolyte, muscle contractions and the production of adrenaline and amino acids (Pirestani *et al.*, 2009). Similarly, zinc is involved in the metabolic process like synthesis and degradation of biomolecules and its concentration is highly effected by anti-nutritional compounds like oxalic acid and phytic acid (Organization, 2009). Iron is an integral part of cytochromes whereas cadmium is known for its toxicological properties (Macrae *et al.*, 1993). Present study revealed that the selected *Berberis* species possess all these essential elements and lack non-essential elements viz. cadmium and chromium. It can be concluded that *Berberis* species do not exhibit toxicological properties due to the absence of non-essential or toxic elements. Hence, these species can be preferably recommended to be use as a source of dietary elements as well as in preparing various drugs.

Some *Berberis* species have been inadequately examined with respect to the availability of bioactive compounds and their therapeutic potential. It has been perceived from a long time that only roots of *Berberis* speces are the vital source of phytochemicals with potent biological applications. However, the result of current study vividly displayed a plenteous diversity of elements along with TPC and TFC mainly in their stem and leaves also. Recently the HPLC method for berberine quantification has gained great momentum in the research field due to its easiness, rapidity, consistency and reproducibility (Kupiec, 2004).

The amount of berberine contents have been estimated in three parts (roots, stem and leaves) of eight *Berberis* species using HPLC method.

Comparatively, roots extracts showed prominent berberine peaks as compared to the stem and leaf extracts (supplementary figure 1). The maximum concentration of berberine was noted in BCR *i.e.* 94504.63 ppm while lowest amount was observed in BPsS (228.72 ppm). Present studies confirm the previous findings reporting highest amounts of berberine concentration in the root extracts of *B. asiatica* , *B.aristata, B. chitria* and *B.lycium* as compared to their stem extracts (Andola *et al.*, 2010; Srivastava and Rawat, 2014). Our results also correlate with the earlier findings in which significant berberine contents have been documented in the root extracts of *B. croatica* and *B. vulgaris* plants (Kosalec *et al.*, 2009). Through optimized method of HPLC, the obtained data revealed that the berberine contents greatly vary in these species that are present in different areas.

According to the previous literature, numerous species of barberry have been reported to exhibit potent biological properties specifically antiradical actions due to the presence of phytochemicals like berberine, phenolic and flavonoids contents (Končić *et al.*, 2010; Gundogdu, 2013). The current study disclosed that all selected species possess significant number of phytochemicals in all species in varying concentrations. Among all species, leaves and roots extracts of *B. psuedumbelleta* showed highest TPC and TFC. Inconsistency in the contents of bio-compounds (berberine, phenolic, flavonoids) of *Berberis* species might be due to multiple factors such as genetic makeup, harvesting period, storage and environmental conditions (altitude, mineral contents, pH, temperature, moisture etc) (Andola *et al.*, 2010; Eroğlu *et al.*, 2020). All-inclusive, selected species could be considered as a virtuous fount of natural antioxidant.

In recent years, the medicinal properties of plants have been investigated due to their potent antioxidant activities with minimum side effects and economic viability (Auddy *et al.*, 2003). In the present study, the examined species showed remarkable antioxidant activity which is strongly supported by the previous results of *Berberis* species such as *B. balochistanica* (Batool *et al.*, 2019), *B. lyceum* (Akhtar and Mirza, 2018), *B.*

aristata and *B. thomsoniana* (Bhatt *et al.*, 2018). Thus, it can be concluded that the significant activity displayed by leaves, roots and stem extracts of different *Berberis* species might be due to the presence of rich bioactive compounds (Osawa, 1994; Elzaawely *et al.*, 2007). Present results are also consistent with the study of Bhatt *et al.* (2018), who mentioned concentration dependent antioxidant activity ranging from 19.38 % to 98.47 % in different parts of *B. aristata* and *B. thomsoniana*. Statistical analysis was also done to highlight possible interactions between the phytochemicals and antioxidant assays.

All-inclusive, results revealed that stem and leaves of these plant species are equally invaluable in medicinal point of view. The extensive practice of utilization of *Berberis* roots as a traditional medicine will generate undue threats to its survival. Hence, this study supports the idea of using the aerial plant parts for industrial use, which will help in protecting this invaluable plant from becoming endangered. The three antioxidant models (DPPH, TRP and TAC) showed different results in all organs which can be justified by the differences observed in the phytochemical contents that are partially inter-related with these activities. Additionally, some researchers also correlated these disparities with the method, types of assays and experimental approaches used (Cao and Prior, 1998; Aremu *et al.*, 2011). It can be suggested that the polyphenolic contents existing in all analysed parts of *Berberis* species are responsible for antioxidant potential.

Nevertheless, it is not easy to correlate the current results of antioxidant studies with those of raw materials, as many other biological compounds might be involved in these activities. The antioxidant activities chiefly depend on the comprehensive chemical structure of active bio-constituents present in raw materials, therefore, in case of raw material like plant extract, the synergic interactions between biological compounds should be considered as an additional property. It also indicates that the isolation of individual compounds need to be fully accomplished in order to ascertain the property of that extract and/or compound.

In conclusion, all examined parts of all tested *Berberis* extracts showed significant concentrations of mineral elements, phytochemicals and antioxidant potential and hence can be utilized as a natural antioxidant, antimicrobial and nutritional complements in herbal and

food industries. Another notable finding is that the utilization of aerial parts could be initiated as a suitable and renewable source for the conservation of natural resources. This will help in preserving medicinal plants particularly the *Berberis* genus in Pakistan, which has been under a threat of extinction as roots are extensively used for traditional medicinal purposes.

As ***Chapter 1*** disclosed the comparative study of elemental, phytochemicals and antioxidative potentials of selected *Berberis* species of Pakistan. keeping in view the potent antioxidant potential with remarkable number of phytochemicals of these selected species- *in vivo* and *in vitro* biological application of this genus will be assessed in ***Chapter 2***.

CHAPTER 2

CHAPTER 2. BIOLOGICAL APPLICATION OF *BERBERIS* PLANT

Introduction

The results of *Chapter 1* indicate that the *Berberis* plant has an immense potential to be used in synthetic foods and drugs as all parts including roots, stem, and leaves of selected *Berberis* species are a rich source of essential nutrients and biomolecules (Osae, 2001). The main biomolecules in *Berberis* organs are alkaloids, polyphenols, flavonoids, tannins, steroids and terpenoids (Lattanzio, 2013). Additionally, biomolecules have significant biological properties in the body and provide defence against stresses (Osawa, 1994). The inhibitory potential of biomolecules provides medicinal integrity to plants (Ribarova *et al.*, 2005; Atanassov *et al.*, 2021). *Berberis* plants have been reported to possess strong biological activities like antimicrobial, anti-inflammatory, anti-arrhythmic and antitumor (Rehman *et al.*, 2018). The results of *Chapter 1* indicated that this plant has an immense potential to be used in synthetic foods and drugs as it is both nutrient-rich and safe.

Globally, *Berberis* species occupy a substantial position in many traditional medicines as well as in modern medicines due to the efficacious therapeutic powers. For example, the primitive Indian Ayurvedic medicines used barberry extracts as herbal ingredients against various illnesses like curing eye and ulcer diseases (Srivastava *et al.*, 2001). Similarly, the modern medicine system isolates and characterize various bioactive compounds like berberine, which are used against cancer and other infectious diseases (Srivastava *et al.*, 2015). Therefore, this genus is well recognized for hepatoprotective, cardiovascular, antimicrobial and anticarcinogenic activities (Srivastava *et al.*, 2015; Hewageegana and Arawwawala, 2020; Mohi-Ud-Din *et al.*, 2021).

Food is one of the basic, fundamental, and essential needs of life. The major concern for food security at the country level is the availability and preservation of foods. The demand for food protection is increasing day by day due to microbial spoilage of food (Gupta *et al.*,

2008; Wernicke, 2016). Therefore, food protection is one of the vital issues in food production companies. Various types of biotic factors are responsible for food spoilage like microorganisms. To cope with these microbes, various types of synthetic chemicals exhibiting antimicrobial potential have been used. However, usage of safe and nontoxic antimicrobial agents has been encouraged to control microbial attacks on foods. In the last decade, naturally occurring antimicrobial products achieved significant attention due to low side effects and potent antimicrobial activities. Different parts of the *Berberis* plant have been reported to possess significant antimicrobial activities against various clinical and food-related microbes. That is why, *Berberis* plant have remarkable demands in the food and pharmaceutical industries (Salehi *et al.*, 2019; Mohi-Ud-Din *et al.*, 2021).

Besides their biological application, the *Berberis* plant has been reported as an endangered species, as its roots are extensively used in traditional and modern medicines by local inhabitants and pharmaceutical industries (Khan *et al.*, 2016; Islam *et al.*, 2021; Nazir *et al.*, 2021). Therefore, an alternate solution is necessitated to protect this important medicinal plant. In Pakistan a total of 29 identified species have been identified, 14 species are restricted to a single zone, which makes these plants more vulnerable, as residents used their roots for numerous purposes like shelter, fuels and to control diseases. To save this genus in Pakistan, we should use alternate parts or organs which have parallel medicinal properties. Among *Berberis* species, the endemic species facing more pressure of eradication as limited to a specific area. For example, *B. balochistanica* is restricted to specific areas (Quetta and Ziarat) of the Balochistan province and their roots are extensively used in traditional and pharmacological fields (Kakar *et al.*, 2012; Abbasi *et al.*, 2013; Bibi *et al.*, 2014; Khan *et al.*, 2016; Pervez *et al.*, 2018; Batool *et al.*, 2019; Pervez *et al.*, 2019; Rahim *et al.*, 2019). These glitches will be abridged by adopting some strategies like identification and development of conservation area, discouraging of uprooting of medicinal plant, promotion of awareness, cultivating of depleted plant species and usage of substitutional approaches by using the renewable and alternative aerial parts (Zschocke *et al.*, 2000). This inclusive revision of *Berberis* species will help the scientific communities for further

assessment of this genus to create new developments with a substitutional approach in herbal fields.

Research Article

ARTICLE 2: CONSERVING ENDEMIC MEDICINAL PLANTS BY SUBSTITUTING THE RENEWABLE AERIAL PARTS AS AN ALTERNATIVE TO ROOTS: A CASE STUDY OF *BERBERIS BALUCHISTANICA*

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Highlights

- All three parts have been investigated for biological potentials.
- First time a rational approach was applied to protect this endemic plant by substituting the underground parts with renewable aerial parts.
- All three parts have been found with significant antibacterial, antifungal and anti-hemolytic potentials.
- Phytotoxic, cytotoxic and DNA protecting abilities of this plant have clearly showed that all parts have great bioactive compounds.

- These findings have suggested that all three parts of this plant have comparable medicinal potential.
- Most important finding of this study is that aerial parts of this plant have considerable invaluable properties which can be used as an alternate source of medical plant.

Abstract

Barberry plants are extensively used in ethnopharmacological fields, yet the population of endemic medicinal plant is under threats and a little is known about the phytochemical and biological significance of its alternative parts of these distinct species. In this study *B. balochistanica* was studied via a rational approach by substituting the underground parts with renewable aerial parts. The antihemolytic, DNA damaging, cytotoxic, phytotoxic, antibacterial and antifungal potentials of underground part and aerial parts were studied for comparison. Substantial reductions were reported in the haemolytic activity of heat stress (leaves 74.92%, roots 69.35%, stem 66.76%) at 1000 µg/ml. Our results showed that, leaf extracts inhibited the growth of *Staphylococcus aureus* (23 mm) and *Klebsiella pneumonia* (19.67 mm) and roots extracts inhibited *Escherichia coli* (19.67 mm). Similarly, extracts of all parts reduced the radish seedling growth and mycelial growth of all tested fungal pathogens. Furthermore, these results signified a remarkable nutritional and biological potential of *B. balochistanica*, especially its aerial parts and help to preserve the endemic plant by discouraging the usage of its roots.

Keywords: medicinal plants; *B. balochistanica;* Plant parts substitution; Conservation; Aerial parts; Nutritional and biological potentials

1. Introduction

The demand for herbal drugs is increasing constantly as they are safe and economical as compared to clinical drug therapy which can have side effects on health and environment (Bodeker and Ong, 2005). Since Neolithic era, herbal medicines and plants have been used to cure varius types of diseases (Fierascu *et al.*, 2018). Even today, approximately 80% of

the world population uses herbal medicines as a primary healthcare source. These estimates are higher in developing and under developed countries of the world (Ekor, 2014).

Irrespective of its medicinal potential, these medicinal plants face very serious difficulties due to misuse and over exploitation. Medicinal plants have two types of threats general threats (increasing populations, urbanization, climatic and biotic stresses) and scientific threats (unselective harvesting and uprooting of the medicinal plants for clinical and nutritional purposes) results in the diminution of natural reservoirs (Ahn, 2017). According to the International Union for Conservation of Nature and the World Wildlife Fund, more than 15,000 plant species are going to be extinct due to above mentioned threats (Raj *et al.*, 2019). These problems will be reduced by adopting some strategies like; identification and development of conservation area, discouraging of uprooting of medicinal plant, promotion of awareness, cultivating of depleted plant species and usage of substitutional approaches by using the renewable and alternative aerial parts of the plants (Zschocke *et al.*, 2000). The substitutional strategies are less considered and only few reports are available about these issues. Zschocke *et al.* (2000) studied bulb, rhizome and bark of *Eucomis autumnalis*, *Siphonochilus aethiopicus*, *Ocotea bullataand* and *Warburgia salutaris* plants respectively, and suggested to use renewable parts as a substitute of bulbs, rhizomes and bark of such species. In few other studies, this approach was applied to conserve medicinal plants by substituting roots, corms, stem bark, tubers with aerial renewable parts in *Aegle marmelos* (Sulaiman and Balachandran, 2013), *Hypoxis hemerocallidea* (Katerere and Eloff, 2008), *Curtisia dentate* (Shai *et al.*, 2009), *Pelargonium sidoides* (Lewu *et al.*, 2006) respectively. The stability between medicinal plant and medicinal drugs are necessary to reduce the depletion of some medicinal endemic plant such as *Berberis* species due to the uprooting of its underground parts. Srivastava *et al.* (2015) identified that only the root of more than 15 *Berberis* species is being collected and studied for ethno pharmacological purposes.

The genus, *Berberis*, is the largest genus of family *Berberidaceae,* with ~17 genera and 650 species, mainly shrubs, that are widely distributed in Asia, Europe, and America

(Ahrendt, 1961). In Pakistan, 29 different species have been reported in the mountainous ranges of the country (Khan *et al.*, 2015). Balochistan is the largest province of Pakistan with versatile ecological conditions. Several diverse flora and fauna are restricted to this province due to its spatial topography and climate (Tareen *et al.*, 2010). Local communities use medicinal plants against different diseases such as typhoid, edema, kidney pain, purification of blood, joints pain, hair fall, chest infections, jaundice, pimples, toothache, high blood pressure, diabetes, and snake and insects bites (Baloch *et al.*, 2013). *B. balochistanica* is an endemic species of Balochistan province, mainly in Kalat, Hanna Urak, and their allied areas, locally known as Zralga in Pashto and Zarchin in Brahui (Kakar *et al.*, 2012). Its root extracts are a rich source of secondary metabolites and are used as a beneficial herbal agent to cure cough, microbial infection, internal injuries of human beings and livestock (Zaidi *et al.*, 2012). Many antioxidants compounds are reported in this endemic plant such as flavonoids, alkaloids, vitamins, phenols, and carotenoids are useful in the reduction of cancer mortalities and coronary heart diseases (Baloch *et al.*, 2013), however, the mechanisms of action are not yet fully established (Benzie and Wachtel-Galor, 2011). The roots of *B.baluchistanica* are extensity studied for various purposes such as used for gentamicin-induced renal toxicity (Pervez *et al.*, 2018), antimicrobial and insecticidal activity (Kakar *et al.*, 2012), as food preservative (Abbasi *et al.*, 2013) and for phytochemical and biological studies (Baloch *et al.*, 2013; Batool *et al.*, 2019). Similarly, in various studies the roots of other species of this genus were used for various medicinal purposes like roots of *B. aristata, B. lyceum, B. aseiatica, B. aetnensis, B. thunbergii, B. vulgaris, B. aetnensis* and *B. libanotica* (Srivastava et al., 2001; Villinski et al., 2003; Asif et al., 2007; Singh and Kakkar, 2009; Bonesi et al., 2013) are being used for metabolic disorder, wound healing, antifungal, antibacterial, diabetes, phytochemical and for other biological activities. Khan et al. (2016) enlisted 12 *Berberis* species, with roots or bark, being used in ethnomedicinal and phytopharmacological

fields locally. These studies showed that using roots for medicinal purposes increases the risk of depletion of plants.

There are limited scientific studies that confirm the detailed biological potential of *B. balochistanica*. The present study described the prospective applications of *B. balochistanica* roots, stem, and leaves extracts for the treatment of microbial infections, inflammatory diseases, hemolysis, anticancer applications, and antioxidant applications, by analyzing their antihemolytic, antibacterial, antifungal, phytotoxic, and cytotoxic activities. The results obtained in the present study suggest that apart from its use in traditional medicine, the application of *B. balochistanica* extracts could be extremely useful in highly nutritional synthetic food supplements and in the composition of clinical drugs to treat deadly diseases such as cancer. Another notable important finding is that the aerial parts of *B. balochistanica,* possess equally invaluable medicinal potential, hence advising against the complete uprooting of the plant. This will help to preserve this endemic plant of Balochistan region, which has been under a threat of extinction as roots are used for traditional medicinal purposes.

2. Materials and Methods

2.1. Plant material

The medicinal plant *Berberis balochistanica* was collected from the mountainous regions of Hanna Urak, District Quetta, and Province Balochistan, Pakistan. After sampling, the plant was identified by examining the samples with the already present herbarium specimens and flora of Pakistan. A voucher specimen (RAW100268) was deposited at the National Herbarium, Islamabad, Pakistan.

2.2. Preparation of plant extract

The plant samples were washed thoroughly under running tap water and placed in a dark room at room temperature for up to one week and then dried in an oven for 28 h at 40°C. Dry powder of 20 g of each sample (dried roots, stem, and leaves) was added into 200 mL of methanol (95%) and continuously stirred on a stirrer for 12-18 h. After stirring, samples were transferred into water bath at 40°C for 2 h. The plant extracts were cooled and filtered three

times using Whatman filter paper to remove all types of course materials and evaporated using a rotary evaporator (BUCHI Rotavapor R-220). The plant extracts were kept in a refrigerator at 4°C for further studies (Harborne, 1973).

2.3. Antihemolytic activity

Six milliliters of human blood were collected from healthy human at dispensary of Quaid-i-Azam University Islamabad in EDTA vials and diluted with PBS (pH 7.4). After centrifugations at 1000 rpm for 12 min, the pellet was collected and washed three times by 0.2 M phosphate buffer, then diluted (4%) with phosphate buffer. one milliliter of diluted erythrocyte suspension along with 0.5mL PBS and 1 mL of the extract at different concentrations (1000, 500 and, 250 µg/mL) was used to study the hemolytic and anti-hemolytic activity of extracts against heat. After incubation, tubes were cooled and centrifuged at 2000 rpm for 10 min. The absorbance of the supernatants was estimated at 540 nm. The percentage of protection against heat induced hemolysis was calculated using the following equation:

$$\text{Inhibition \% of heat induced hemolysis} = \frac{1 - \text{OD sample}}{\text{OD control}} \times 100$$

2.4. Cytotoxic and phytotoxic assessment assay

To confirm the biocompatible nature of *B. balochistanica* plant extracts, cytotoxic and phytotoxic assessment assay was performed using *Artemia salina* larvae (Ocean Star, UT, USA) and radish seeds, respectively. Eggs of *A. salina* were incubated for 48 h under light at 30°C in sea water (3.8 g/L). Six different concentrations of plant extracts (10, 100, 500, 1000, 2500 and 5000 µg/mL) were added in different vials and their final volume was brought to 5 mL with the help of saline solution (sea water). After 24 h, ten mature shrimps were transferred to each vial and were incubated at 32°C for 24 h after which the living shrimps were counted (Meyer *et al.*, 1982). LC_{50} values and percentage mortality were calculated. For phytotoxic assessment, the radish seed assay method (Turker and Camper, 2002) was used. Three different concentrations of plant extracts (100, 250 and 500 µg/mL) were added in each petri plate containing sterilized filter paper (Whatman filter paper). All solutions were

evaporated and then distilled water (5 mL) was added. After sterilization, 15 seeds were placed in petri plate and incubated at 25°C in dim light. On the fifth day, root lengths were measured, and seed Seed germination indices data were reported using different index such as Germination capacity (final germination, percentage inhibition and the number of days required for 50% of the total number of seeds to have germinated) and germination rate (speed of germination) were calculated using previously published methods (Orchard, 1977; Bradbeer, 1988; Wardle *et al.*, 1991; Rossello and Mayol, 2002).

2.5. Antimicrobial activities

Bacterial and fungal strains were collected from the Department of Microbiology, Quaid-i-Azam University, Islamabad. Zone of inhibition and mycelium growth were investigated using well diffusion and food poison methods using four MDR clinical isolates and three phytopathogenic fungal stains respectively, against *Berberis* roots, stem, and leaves extracts. The clinical and fungal strains are *Staphylococcus aureus* (48755), *Pseudomonas aeruginosa* (23451), *Klebsiella pneumonia* (78501), *Escherichia coli* (30155), *Alternaria alternata*, *Aspergillus niger* and *Fusarium oxysporum*, respectively. Autoclaved Müller-Hinton agar and Sabouraud dextrose agar media (Oxoid CMO147) with different concentrations of plant extracts (500, 2500, and 5000 µg/mL) were used. Kanamycin and fluconazole were used as positive control. After incubation, the zone of inhibition was reported by measuring the diameter of clear zone around each well. The percentage inhibition of the mycelial growth was calculated by using the given formula.

$$\text{Inhibition \% age} = DC - DT / DC \times 100$$

Where DC is an average increase in mycelium growth in control and DT is an average increase in each treatment.

2.6. DNA protection assay

For DNA damaging assay, the method of Leba *et al.*, (2014) was used with slight modification. Two different genomes *i.e.,* plasmid DNA (alpha plasmid) and wheat DNA were used to study the protective capacity of tested plant extract against H_2O_2. Briefly, 1 µL

(Plasmid DNA) and 3 µL (wheat genomic DNA) were incubated with 1 µL of 1 mM FeSO4, 1 µL of 10% (v/v) H_2O_2, 3 µL of different concentration (5, 2.5, 1 and 0.5 mg/mL) of tested plant parts extracts. The final volume of the reaction mixture was made up to 20 µL with phosphate buffer (pH 7.0). All three types of DNA were exposed to Fenton's reagent alone and with different concentrations of plant extracts. After the incubation (37◦C for 1 h), the samples were loaded into a 1% (w/v) agarose gel containing 3 µL ethidium bromide and run in the gel electrophoresis apparatus. The gel containing DNA bands were photographed under UV light using a gel documentation system.

3. Results

3.1. Hemolytic and ant-hemolytic potential of *B. balochistanica*

It is important to know whether the medicinal plants are safe or not for pharmacological preparations. In the present study, all tested parts of *B. balochistanica* showed an exceptionally low hemolysis of red blood cells. The highest and lowest hemolytic percentage were exhibited by root or leaf extracts (2.90% and 1.91%) at 1000 µg/mL and 250 µg/mL, respectively (Figure 1 B). These results express that all the tested extract of *B. balochistanica* are non-hemolytic in nature, thus confirming the biosafety and biocompatibility of this plant to be used for drug making against diseases. Figure 1 A also shows the protective ability of *B. balochistanica* plant extracts against heat induced hemolysis. Remarkable reductions in

the red blood cells lysis by heat stress were observed in the presence of all tested extracts. All plant part extracts showed significant protection against heat (≥ 65%) stresses.

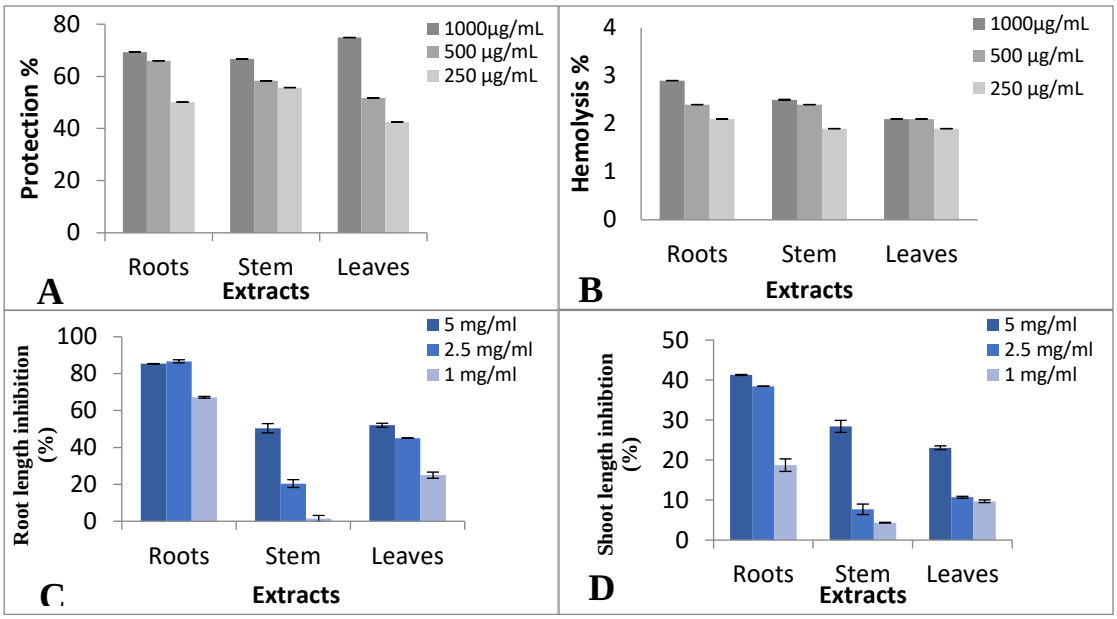

Figure 1. (A) Heat induced anti-hemolytic activity, (B) hemolytic activity of all three parts and (C and D) are percentage inhibition of roots and shoot length of radish seedling against *B. balochistanica* extracts (5, 2.5 and 1mg/mL).

3.2. Cytotoxicity effect

Both aerial and underground parts of *B. balochistanica* showed a significant brine shrimp cytotoxic activity. Lethal concentration (LC_{50}) in plant extracts was reported in ranges from 1.580 ppm to 9.084 ppm (Table 1). The experimental results conclude time and dose-dependent response. The percentage of death was observed highest (100%) at 1000 ppm in

all extracts of the plant. These results revealed the presence of cytotoxic compounds in all parts of *B. balochistanica.*

Table 1. Cytotoxicity Analysis of . *balochistanica*.

Brine shrimps' mortality		
Extracts	**LC50[a]**	**R^2**
Roots	1.58	0.96
Stem	3.37	0.91
leave	9.08	0.68

[a]LC50 refers to lethal concentration fifty

3.3. Phytotoxicity assessment- allelochemical nature

Phytotoxicity assay is one of the cheapest and simplest methods to determine the inhibitory or stimulatory potential of a plant species. Threat of weeds to crop losses is not a mystery they have been repeatedly reported in literature over the years. In the present study, the phytotoxicity of *B. balochistanica* plant extracts was investigated against radish plants. The total germination (final germination percentage), T_{50} (days required for 50% germination of total germinated seeds), speed of germination (SG), and percentage inhibition were assessed. The germination and inhibition percentages were less sensitive to the applied concentration of all extracts. Roots extracts showed a 16.67% toxicity at higher level, while stem and leaves extracts show nearly 10% inhibitory effects. Whereas the SG and T_{50} are retarded and delayed at applied concentrations of extracts. Among three extracts, root extracts showed a significant reduction and dilation of SG and T_{50} at all concentrations, as compared to the negative control and other extracts. In short, seed germination and inhibition were found to be less sensitive, while SG and T_{50} were found more sensitive in this study (Table 2). The extracts of all three parts were observed to significantly inhibit the root lengths of radish plant seedling when applied at different concentrations. Roots extracts showed a stronger inhibition (85.35 ± 0.12, 86.69 ± 0.82, and 67.20 ±0.47) at all concentrations, as compared to control and other parts extract. The impact on root length was also quite observable and significantly higher than shoot length (figure 3).

Table 2. Germination indices of radish plant seed against *B. balochistanica*.

Extract	T_{50}[a]			Final Germination (%)			Germination Speed			Inhibition Percentage (%)		
	Roots	**Stem**	**Leaves**	**Roots**	**Stem**	**Leaves**	**Roots**	**Stem**	**Leaves**	**Roots**	**Stem**	**Leaves**
5mg/ml	D3	D2	D2	83.33	90	90	6.42	6.64	8.63	16.67	10	10
2.5mg/ml	D2	D2	D1	96.67	90	90	7.48	8.06	9.38	3.33	10	10
1mg/ml	D2	D2	D1	93.33	93.33	93.33	6.92	8.60	11.13	6.67	6.67	6.67
Control	D1	D1	D1	100	100	100	12.25	12.25	12.25	0	0	0

[a]T_{50} refers to the days required for 50 % germination, where “D” stands for “days”.

3.4. DNA protection potential

The agarose gel electrophoresis result (Figure 2) showed the damage induced by Fenton's reagent on DNA in the presence and absence of *B. balochistanica* roots, stem, and leaves extracts at various concentrations (5, 2.5, 1 and 0.5 mg/mL). Two types of DNA (plasmid DNA and wheat genomic DNA) were used in this experiment. The agarose gel showed that, negative control (C-), which was untreated with Fenton's reagent has bright bands in both types of DNA, while in positive control (C+), DNA bands were not found on gel due to DNA degradation by Fenton's reagent. As the plant extracts were added along with Fenton's reagent, the DNA protection on gel appears with bright bands (Figure 2). These results revealed that all parts of *B. balochistanica* have a protective effect against damage induced by hydroxyl radicals on DNA.

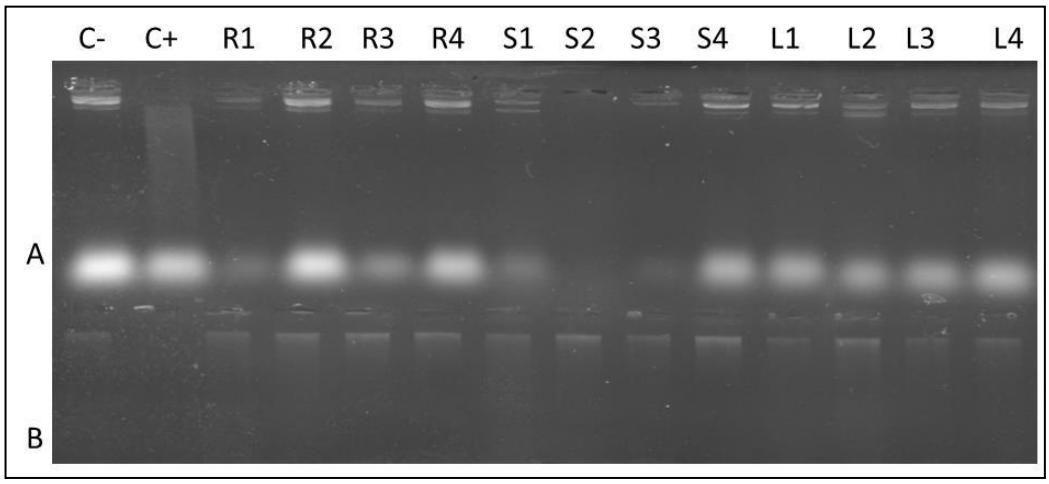

Figure 2. DNA protection assay of the *B. balochistanica* roots, stem and leaves. Whereas A and B refer to Plasmid DNA and Wheat DNA; Label C- and C+ showed negative control (Untreated DNA) and positive control (Treated DNA with Fenton reagent); while R1-4, S1-4 and L1-4 represented the DNA with Fenton reagent and different concentrations (5, 2.5, 1 and 0.5 mg/mL) of plant samples (roots, stem and leaves extracts, respectively).

3.5. Antibacterial analysis

The antimicrobial activity of *B. balochistanica* (roots, stem, and leaves) extracts was tested against four bacterial strains. The results were reported as a zone of inhibition (mm), as shown in Figure 3. Against gram-positive bacteria *Staphylococcus aureus*, all parts of *B. balochistanica* exhibited an inhibition at all applied concentrations. The maximum inhibition potentials were shown by the leaf extract (23.00 mm at 5mg/mL) while minimum inhibition was displayed by stem

extract (10.67 mm at 0.5mg/mL). On the other hand, only root and leaf extracts showed inhibition against all gram-negative strains. The maximum inhibition potential was shown by roots and leaf extracts against *Escherichia coli* and *Klebsiella pneumonia* (19.67 ± 0.58 and 19.67 ± 0.24 at 5mg/mL), respectively, while minimum inhibition potency was displayed by leaf extract against *E. coli* (11.33 ± 1.15 at 0.5mg/mL). All the gram-negative strains exhibit resistance against stem extract of *B. balochistanica,* as shown in figure 3. In this study, Kanamycin sulfate (10 µg/mL) was taken as positive control, which showed strong inhibition zones against all tested microorganisms, as compared to extracts.

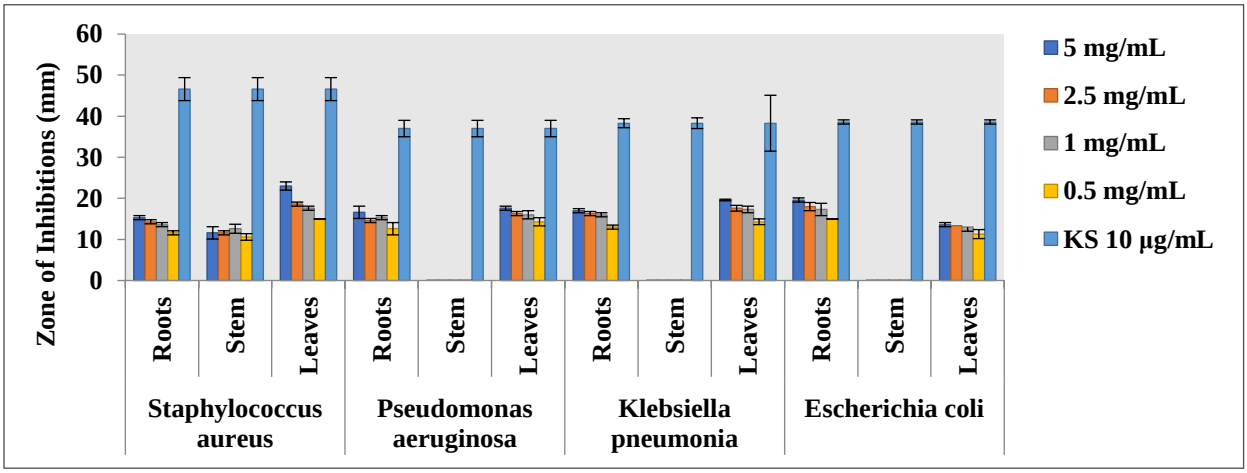

Figure 3. Antibacterial potencies of roots, stem and leaves extracts of *B. balochistanica* against four clinical isolates. KS refers to kanamycin sulfate. While mm represented the diameter in millimeter

3.6. Antifungal activities

The antifungal activities of *B. balochistanica* (roots, stem, and leaf) extracts were tested against three fungal pathogens by using the food poisoned method. This technique involved the interaction of extract and pathogens by observing the mycelium growth in the presence and absence of extracts (Figure 4). All extracts of plants exhibited inhibitory effect by retarding the mycelium growth of all tested pathogens. As compared to non-treated control, all extracts at all treatments displayed inhibitory activity against all fungal stains. The minimum inhibitory value was observed against *Aspergillus niger* (15.93%) by leaf extracts, while maximum inhibition (56.82, 53.41 and 50% 5 mg/mL) was exhibited by the stem, roots, and leaf extracts against *Alternaria alternata*, as compared to control. Similarly, *Fusarium oxysporum* growth was retarded

(48.13, 44.48 and 39.55% at 5 mg/mL) by roots, stem, and leaf extracts, respectively, as compared to non-treated control.

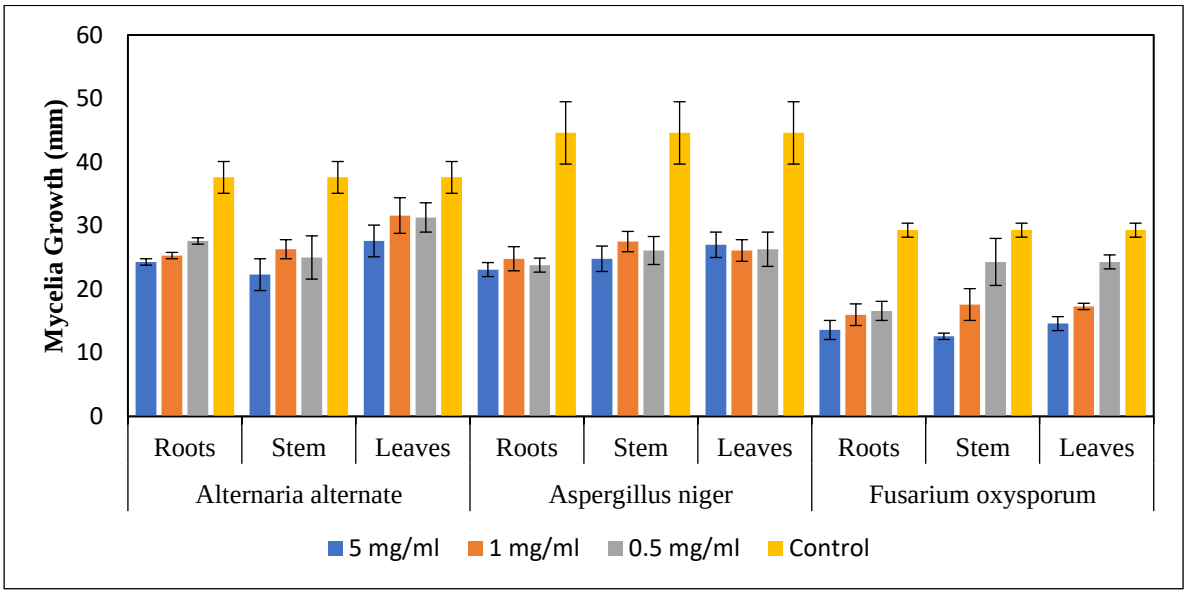

Figure 4. Antifungal evaluation of roots, stem and leaves extracts of *B. balochistanica* against three fungal pathogens. Control refers to non-treated (Sabouraud dextrose agar media) without extracts. While mm represents the diameter in millimeter.

4. Discussion

In recent years, the medicinal properties of plants have been investigated due to their potent antioxidant activities with minimum side effects and economic viability (Auddy et al., 2003). It is well known that phenolic compounds contribute directly to the antioxidant activity of plant extracts (Elzaawely et al., 2007). In the present study, the presence of total phenolic and flavonoids contents was reported for the first time in all three parts of *B. balochistanica,* where root extracts contained the highest phenolic and flavonoid contents followed by leaf and stem extracts (Chapter 1). Several species of *Berberis* have been reported for the presence of phenolic contents (Končić *et al.*, 2010; Gundogdu, 2013). Previous antioxidant studies on *B. balochistanica* reported the presence of phenolic and flavonoid contents, but only in extracts from roots (Baloch *et al.*, 2013; Batool *et al.*, 2019).

What happened consequently was that this medicinal plant, already endemic to Baluchistan region of Pakistan, started to become rare. The extensive use of the *B. balochistanica* roots as a traditional medicine, owing to its phytochemical potential, poses a great danger to its survival.

Results observed here indicated that stem and leaves of this plant are equally medicinally invaluable and uprooting it completely can be avoided.

Red blood cells are the most plentiful cells in human being having unique morphological and biological properties. Oxidative stress and other injurious substances like, heat, oxidative drugs, UV radiations may cause hemolysis of human red blood cells (HRBC) (Debnath *et al.*, 2013). Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators (Debnath *et al.*, 2013). It is known that the plants having flavonoids, flavonols and their constituents have potential to protect the HRBC from hemolysis (2016). Here, all tested parts (roots, stem, and leaves) show high protection of RBC against stress induced by heat. This high antihemolytic capacity of *B. balochistanica* was understandable from the fact that it contained a high amount of phenolic and flavonoid contents in all parts. This inhibition of heat and oxidative damaged of RBC membrane was considered to measure the mechanism of anti-inflammatory activity of *B. balochistanica* plant extracts. The plant extracts possibly stabilized membrane of red blood cells by inhibiting the release of active mediators and lytic enzymes of inflammation.

The brine shrimp lethality bioassay is a simple and inexpensive method for detecting the presence of bioactive compounds in plant extracts (Hamid *et al.*, 2011). Many researchers have reported that the plants having LC_{50} value below 20 µg/mL have antitumor and anticancer potential (Ali *et al.*, 2014). According to Meyer *et al.* (1982), plant extracts showing LC_{50} values lower than 1000 µg/mL are considered to have active plant constituents. Pai *et al.* (2012) reported that *B. aristata* has cytotoxicity effects against brine shrimp and has anticancer effects which were attributed to a higher presence of phenolic and alkaloid compounds. Consistently, in present study, all plant parts have high phenolic and flavonoid contents, and all tested extracts of plant showed strong cytotoxic effects against brine shrimp (table 1).

Many studies have reported that different germination indices are helpful in evaluating the allelopathic effects of any stress on seeds (Wardle *et al.*, 1991; Anjum and Bajwa, 2005). In the present study, radish seeds were observed to respond differently to the various extracts, which might be due to the presence of species dependent phytotoxins for seed coat permeability or seed size and seed structure(Batish *et al.*, 2006). The roots length of radish seedlings were significantly inhibited by *B. balochistanica* extracts (roots, stem, and leaves). These findings are similar to a

previous study of Rinez *et al.* (2011), where aqueous extracts of *Nicotiana glauca* Graham was tested against lettuce and radish plants, and 15-100% inhibition was reported due to the presence of phenolic contents. Several studies have reported phenolic compounds as potential candidates for phytotoxicity in allelopathic plants, due to their solubility in water (Xuan *et al.*, 2004; Batish *et al.*, 2006). Along with phenolic compounds, flavonoids are also reported as a phytotoxic candidate in nature (Bais *et al.*, 2003). The present study also indicated that root length was more sensitive (1.48 to 86.69%) than shoot length (4.35 to 41.30%), which could be used as an indicator for phytotoxicity. Rinez *et al.* (2011) also found root length to be a sensitive indicator of phytotoxic assay in their research.

The scavenging effect of *B. balochistanica* parts extract was studied on plasmid, human, and wheat DNA. All plant extracts showed remarkable DNA protection (Figure 4). Again, the presence of high phenolic, flavonoid contents, and antioxidant capacity attribute to DNA protective capacity as these compounds neutralize the effects of free radicals on DNA, thus reducing DNA damage (Jun *et al.*, 2007; Dai and Mumper, 2010; Sevgi *et al.*, 2015). Activity may also come from the presence of other antioxidant compounds such as carotenoids, vitamins, and others (Osawa, 1994; Javanmardi *et al.*, 2003).

Antimicrobial investigations of *B. balochistanica* revealed that mostly crude extracts have inhibitory activity against all tested pathogens, but the effect of Kanamycin sulfate is higher against all strains. Interestingly, the extracts showed inhibitory activity against gram positive skin opportunistic bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Pseudomonas aeruginosa*) that cause systemic infections in patients with burn wounds, cystic fibrosis, acute leukemia, and organ transplants. After *Escherichia coli*, the most common gram-negative pathogen is *Klebsiella pneumonia* associated with a wide range of infections; urinary tract infection (UTI), intra-abdominal infection, pneumonia, pyogenic liver abscess and bloodstream infection (Podschun and Ullmann, 1998; Anderson *et al.*, 2014). *E. coli* causes intra-abdominal infection, urinary tract infection, and primary bacteremia (Eliopoulos and Bush, 2001). The roots and leaf extracts showed inhibitory effects against both gram-positive and negative strains, but gram-negative strains showed resistance against stem extract of *B. balochistanica.* In short gram-positive strains were susceptible to all extracts while gram-negative were susceptible to only roots and leaves extracts and resistance to stem extract (figure5). The activities which have 14 mm or

more inhibition zone were considered as significant results (Riaz *et al.*, 2018). Here, roots and leaves extracts showed these meaningful results at higher concentrations (5 and 2.5 mg/mL) against *S. aureus*, *P. aeruginosa* and *K. pneumonia.*

Many *Berberis* species have been studied and reported for their antifungal activities (Hewageegana *et al.*, 2018; Rehman *et al.*, 2018), but these properties are mostly attributed to roots, also these have not been previously studied in *B. balochistanica.* In the present study, mycelial growths of all tested pathogens were markedly inhibited by all tested parts of plants (Figure 6). The variation in the efficiency of the various extracts against tested pathogenic strains is due to the presence of different chemical constituents in the extracts. These activities were correlated with the presence of phenolic, flavonoid contents, and berberine chloride present in the extracts, as these played a vital role against various microorganisms and infectious pathogens. Phenol and flavonoid compounds exhibited inhibitory effects against multiple viruses and bacteria and possess free radical scavenging and anticancer activity (Montoro *et al.*, 2005). The antimicrobial results of the present study also supported the idea of using the aerial plant parts for industrial use, which will help to protect this invaluable plant from becoming endangered.

5. Conclusions

This study provides comprehensive experimental evidence for *B. balochistanica*, an endemicplant of the Baluchistan region, to be used in clinical drugs and herbal medicine. The presence of phenols and flavanols were observed in all parts of the plant, which further contributes to a range of medicinal values such as antibacterial, antifungal, cytotoxic, phytotoxic, anti-hemolytic activities, and protection of DNA from oxidative stress and heat. An extremely useful finding of this study was that this extremely viable medicinal potential of *B. balochistanica* was not only present in its roots-stem and leaves also possessed equally invaluable properties. These results should also help in preserving this plant by discouraging its uprooting for medicinal purposes.

Author Contributions: Conceptualization, Siraj Uddin and Umar Masood Quraishi; Data curation, Siraj Uddin; Formal analysis, Siraj Uddin; Investigation, Siraj Uddin, Luqman Bin Safdar, Qurra-tul-Ain Safdar, Javed Iqbal; Methodology, Siraj Uddin, Luqman Bin Safdar, Qurra-tul-Ain Safdar, Javed Iqbal, Iram Fatima and Umar Masood Quraishi; Project administration, Umar Masood Quraishi; Resources, Umar Masood Quraishi; Supervision, Umar Masood Quraishi;

Validation, Luqman Bin Safdar Writing – original draft, Siraj Uddin and Luqman Bin Safdar; Writing – review & editing, Luqman Bin Safdar, Javed Iqbal Iram Fatima and Umar Masood Quraishi.

Funding: This research received no external funding.

Acknowledgments: The authors thank Professor Dr. Mushtaq Ahmad (Quaid-i-Azam University, Pakistan) for helping in the identification of plant specimen. This study was supported by Quaid-i-Azam University, Islamabad, Pakistan.

Conflicts of Interest: The authors declare no conflict of interest.

The ***Chapter 1*** and ***2*** disclosed the presence of phytochemicals with diverse biological application in all three parts of selected Berberis species. Results revealed that aerial parts have comparable number of phytochemicals (TPC and TFC) as roots and displayed strong antioxidant activities. As these biomolecules have functional groups (hydroxyl, phenolic rind etc.) which have reducing potentials. Therefore, these phytochemicals make this genus a candidate to use in green synthesis of nanoparticles. The next chapter describes the importance of Berberis aerial parts in green synthesis of metal Oxid nanoparticles with significant biological applications. Stem and leaf extracts of Berberis plants show strong reducing, capping, and stabilizing potentials, which successfully reduced the nickel nitrate slat in to fine (31 and 21 nm) nickel oxide nanoparticles with potent biologicals activity (**Uddin, Siraj,** Luqman Bin Safdar, Saeed Anwar, Javed Iqbal, Sabiha Laila, Banzeer Ahsan Abbasi, Muhammad Saqib Saif et al. "Green Synthesis of Nickel Oxide Nanoparticles from Berberis balochistanica Stem for Investigating Bioactivities." *Molecules* 26, no. 6 (2021): 1548) and (**Uddin, Siraj**, Luqman Bin Safdar, Javed Iqbal, Tabassum Yaseen, Sabiha Laila, Saeed Anwar, Banzeer Ahsan Abbasi, Muhammad Saqib Saif, and Umar Masood Quraishi. "Green synthesis of nickel oxide nanoparticles using leaf extract of Berberis balochistanica: Characterization, and diverse biological applications." *Microscopy Research and Technique* (2021).

CHAPTER 3

CHAPTER 3. PLANT MEDIATED GREEN SYNTHESIS OF METAL OXIDE NANOPARTICLES

Introduction

Nanotechnology is one of the emerging fields of science in the twenty-first century. It is considered an interdisciplinary field in which the material can be made at a nano scale with innovation and enormous applications (Mansoori and Soelaiman, 2005). It is the combination of science, technology and engineering focusing on a nanoscale, where researchers can control and manipulate the structure of materials at a nano level. For the synthesis of nanomaterial (1-100nm), various fields like materials, natural, biological, computational sciences and engineering combined (Zhang *et al.*, 2020). It relies on two main situations *i.e.*, to control the shape and size at the nanoscale and create innovation in those nanomaterials (Allhoff, 2009).

The synthesis of nanoparticles is carried out using various approaches like physical, chemical and biological. The physicochemical methods laden with lots of obstacles involving toxic solvents, harmful by-products, and excessive energy utilization. The uses of a biological field in nanotechnology created a new field, called nanobiotechnology. However, due to its safe and eco-friendly nature, biological materials have invited considerable attention (Singh *et al.*, 2016). Among biological, plants fabricated nanoparticles via the green synthesis approach is naturally safe, cost-efficient, and eco-friendly (Rather *et al.*, 2020). Plants can uptake inorganic ions (metals) from their local niche. In green synthesis, the inorganic metal salt is reduced and stabilized into metal nanoparticles by various phytoconstituents, which act as a capping agent (Iqbal *et al.*, 2020). These biomolecules have a great role in reducing and capping the metal salts into nanoparticles. However, the size, structure and morphological nature of nanoparticles depends upon the biomolecules present in plant extracts (Rajeshkumar and Bharath, 2017).

The nanomaterials have distinct functions ascribable to their latest or enhanced properties depending on the size, shape and distribution (Thakkar *et al.*, 2010). The synthesized nanoparticles are used in different fields including cosmetics, catalysis, drug and chemical industries, biomedical, electronics, food, transistors, space, mechanics, energy and environmental science

(Wang *et al.*, 2005; Zhang *et al.*, 2020; Uddin *et al.*, 2021). In this decade, the biomedical applications of green synthesized nanoparticles are expanded in several biological fields comprising drug and gene delivery, bioimaging, biosensors, and biostimulator (Zhang *et al.*, 2020). Recently several nanoparticles have been reported with potent cytotoxic effects and can be used as keen madicinal weapons versus multiple drug-resistant strains (Kalpana and Devi Rajeswari, 2018).

The genus *Berberis* containing valuable ecofriendly secondary metabolites such as phenols, flavonoids, alkaloids, carotenoids and vitamins (Baloch *et al.*, 2013), which are useful for the green synthesis of nanoparticles because they serve as effective reducing agents during green synthesis. Recently, these species gain the attention of researchers due to their biochemical rich nature and wide distribution across the world (Ahmed *et al.*, 2008; Khan *et al.*, 2014). Based on its safe, nontoxic nature and presence of biomolecules; the *Berberis* plant is one of the safest candidates to use in the green synthesis of nanoparticles. Therefore, in the last two years, different *Berberis* species have been used in the green synthesis of various nanoparticles with potent biological properties. Table 3 reviews literature representing the number of species used in nanoparticles synthesised since 2020.

ARTICLE 3: GREEN SYNTHESIS OF NICKEL OXIDE NANOPARTICLES USING LEAF EXTRACT OF BERBERIS BALOCHISTANICA: CHARACTERIZATION, AND DIVERSE BIOLOGICAL APPLICATIONS

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Received: 23 December 2020 | Revised: 16 February 2021 | Accepted: 1 March 2021

DOI: 10.1002/jemt.23756

RESEARCH ARTICLE

MICROSCOPY RESEARCH TECHNIQUE WILEY

Green synthesis of nickel oxide nanoparticles using leaf extract of *Berberis balochistanica*: Characterization, and diverse biological applications

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Review Editor: Chuanbin Mao

Abstract

In current report, nickel oxide nanoparticles (NiONPs) were synthesized using leaf extract of *Berberis balochistanica* (BB) an endemic medicinal plant. The BB leaves extract act as a strong reducing, stabilizing, and capping agent in the synthesis of BB@NiONPs. Further, BB@NiONPs were characterized using Uv–visible spectroscopy (UV–vis), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), Energy dispersive spectroscopy (EDS), scanning electron microscopy (SEM), and average size was calculated ~21.7 nm). Multiple in vitro biological activities were performed to determine their therapeutic potentials. The BB@NiONPs showed strong antioxidant activities in term of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and total antioxidant capacity (TAC) with scavenging potential of 69.98 and 59.59% at 200 µg/ml, respectively. The antibacterial and antifungal testes were examined using different bacterial and fungal strains and dose-dependent inhibition response was reported. Laterally, cytotoxic and phytotoxic activities were studied using brine shrimp and radish seeds. The result determined potential cytotoxic activity with LD_{50} value (49.10 µg/ml) and outstanding stimulatory effect of BB@NiONPs on seed germination at lower concentrations as compared to control. Overall, result concluded that biosynthesis of NiONPs using leaf extracts of *Berberis balochistanica* is cheap, easy, and safe method and could be used in biomedical and agriculture field as nanomedicine and nano fertilizer.

KEYWORDS
antimicrobial, *Berberis balochistanica*, cytotoxic, green synthesis, nano fertilizer, NiONPs, seed germination

1 | INTRODUCTION

Nanotechnology is one of the newly emerging scientific trends of 21st century and got sixth position as a promising and revolutionary technology in interdisciplinary field (Chandra et al., 2019). The word "nano" is originated from Greek meaning extremely small or dwarf. Nanoparticles (NPs) are typically clusters 1–100 nm in size. Due to the high "surface-to-volume ratio", nanoparticles (NPs) show unique and fascinating features such as thermal, optical, and catalytic activities. Based on their shape and structure, various terms are used for nanomaterials like nanotubes, nano cubes, nanoflower, core shell, and bimetallic (Iqbal et al., 2019; Khatami et al., 2018; Karthik et al., 2018).

NPs have gained significant attention and possess notable benefits in various fields such as electronic, mechanics, energy, industrial, agricultural, pharmaceutical, commercial, and biomedical fields (Chandra et al., 2019; Iqbal et al., 2018b, Haghniaz et al., 2021).

Different nanomaterials have been synthesized such as zinc oxide, cesium oxide, iron oxide, silver, gold, platinum, magnesium nanoparticles, Cr_2O_3 nanoparticles, etc. (Chandra et al., 2019; Behravan et al., 2019; Iqbal et al., 2018a, 2018b; Pugazhendhi et al., 2019; Sone et al., 2016). In recent studies, nickel oxide nanoparticles have been reported with remarkable position in multidisciplinary fields due to its wide band gap, small size, and its semiconductor properties (Abbasi et al., 2019a, 2019b, Abbasi et al., 2020a, 2020b; Iqbal et al., 2020a, 2020b). NiONPs have been used in batteries, super conductors, photocatalytic, and catalytic analysis (Mayedwa et al., 2018; Yang et al., 2018). Previous studies have demonstrated that NiONPs have several chemical and biological application such as adsorption of pollutants and dyes, anti-inflammatory, antimicrobial, anticancer, and cytotoxic potentials (Iqbal et al., 2020a, 2020b; Abbasi et al. 2019a, 2019b; Khalil et al., 2018). These inhibitory activities of NiONPs is due to releasing of ions and production of ROS which destroyed the structures and functions of cell (Khatami et al., 2018; Karthik et al., 2018). Along with this inhibitory activity of biogenic NPs, a significant stimulatory potential in field of agriculture have also been reported. Recently, application of NPs as nonfertilizer have gaining significant consideration in agriculture field due to its stimulatory potentials (Altindal & Altinda 2020). Plentiful NPs were reported with fruitful positive effect on seed germination, plant growth, and quality. For example, multiwalled carbon nanotubes (MWCNTs), SiO_2, ZnO, TiO_2, Fe/SiO_2, AgNPs, and CuO have been reported as a plant growth mediator (Shang et al., 2019). Regarding stimulatory effect, very little studies are available on NiONPs, whereas inhibitory activities on NiONPs were studied in various studies (Chaudhary et al., 2018; Faisal et al., 2013).

Various approaches are in practice for the synthesis of nanoparticles such as physical and chemical (Chandra et al., 2019; Khamlich et al., 2011). However, the physicochemical methods are economically not feasible due to more energy consumption and the presence of nondegradable toxic compounds limit their application specifically in biomedical fields (Chandra et al., 2019). Therefore, nanoparticle synthesis was shifted towards eco-friendly methods like green synthesis approach. Green synthesis of NPs is achieved using three important sources such as autotrophs (plant and algae) (Ovais et al., 2018), bacteria, and fungi (Iqbal et al., 2020a, 2020b). Plant mediated NPs synthesis is gaining significant momentum in interdisciplinary fields due to its easy availability and simplicity. It is reported that plants contain numerous valuable phytoconstituents which act as stabilizing, reducing, and caping agent during NPs synthesis (Khalil et al., 2018, Iqbal et al., 2020a, 2020b). In plant-based green synthesis, the extracts of plants parts are mixed with metal salts solution and allowed for synthesis of nanomaterials (Khalil et al., 2018). In green synthesis, various plant parts are utilized like leaf, root, bark, flower, fruit, and seeds (Iqbal et al., 2018a; Kalidhar, 1989). Plants are rich source of many phytocompounds such as flavonoids, phenolics, alkaloids, terpenoids, minerals, and vitamins which have strong pharmacological values against different kinds of ailments (Iqbal et al., 2018a; Ahmed et al., 2016; Ahn, 2017).

Berberis balochistanica is endemic species of family *Berberidaceae*, native to the mountainous regions of Baluchistan province, mainly in District Quetta, Kalat, and Ziarat (Kakar et al., 2012). Local communities use *B. balochistanica* against different diseases as a beneficial herbal agent to cure cough, purify blood, microbial infection, toothache, high blood pressure, diabetes, snake and insect bites, internal injuries of human beings and livestock (Bibi et al., 2014; Zaidi et al. 2012; Baloch et al., 2013). Further, *B. balochistanica* has been extensively studied for phytochemical and biological purposes, such as gentamicin-induced renal toxicity, antimicrobial and insecticidal activity, food preservative, cancer mortality, and coronary heart diseases (Pervez et al., 2018; Kakar et al., 2012; Abbasi et al., 2013; Baloch et al., 2013; Batool et al., 2019). This endemic plant is rich source of secondary metabolites such as flavonoids, alkaloids, vitamins, phenols, and carotenoids (Baloch et al., 2013). Many species of this genus have been used for green synthesis of NPs. Mehmood et al., (2016) synthesized Silver NPs using *B. valgarus* leaf and root extracts. Additionally, *B. aristate* has been used to synthesize ZnONPs (Harish et al., 2019). In present study, leaf extract of *B. balochistanica* plant was used to synthesize NiONPs. The synthesized BB@NiONPs were further characterized using UV–visible, FTIR, XRD, EDS, and SEM. Further, different in vitro biological potentials (antimicrobial, antioxidant, cytotoxic, and phytotoxic assays) were evaluated. The characterized NPs have all possible properties of an active NPs. To the best of our knowledge, *B. balochistanica* has been used for the first time for green synthesis of NiONPs and evaluated for biological and biofertilizer activities.

2 | MATERIALS AND METHODS

2.1 | Preparation of plant extract

The *B. balochistanica* plant leaves were collected, washed, and dried at room temperature. After cleaning, 40 g of leaves powder was added in 400 ml of autoclaved distilled water. The solution was place on a hot plate at 70–80°C for 2 hr under magnetic stirrer. After cooling, crud extract was filtered using three times using Whatman filter paper and supernatant was collected with pH 6.8. The leaf extract was stored at 4°C for future studies (Matinise et al., 2018).

2.2 | Nickel oxide nanoparticles synthesis

For synthesis of NiONPs, previously used protocols with slight modifications was used (Iqbal et al., 2019; Kaviyarasu et al., 2016; Sone et al., 2016). 20 ml of leaf purified extract was added to the solution of $NiNO_3$ (0.3 M) to synthesize NiONPs. The solution was subjected to heating at 60°C for 3 hr with proper stirring. The precipitated BB-NiONPs were washed three times by centrifugation for 25 min at 3000 rpm. Further, BB-NiONPs were dried at 60°C in incubator for

3 hr calcination using air furnace (KSL-1100X, MTI Corporation, China). The synthesize particles were characterized using different spectroscopic and microscopic techniques.

2.3 | Characterization of nickel oxide nanoparticles

Various spectroscopic techniques were applied to analyze the physical and chemical properties of prepared BB-NiONPs. The formation of nanoparticles of Nickel oxides were confirmed using UV-400 UV–Vis spectrophotometer (Germany) in a range of 200–800 nm. Fourier transformed infrared spectroscopy (FT-IR) with spectral ranges 400–4000 cm^{-1} were used to verify the capping and stabilizing properties of functional groups involved in BB-NiONPs synthesis (Alpha, Bruker, Germany). Thermally annealed properties of prepared samples were carried out using an X-ray diffractometer (PANalytical, Netherland) associated with a Cu radiation source. The corresponding size was analyzed by Scherrer equation.

$$D = k\,\lambda/\beta\cos\theta$$

where "D" is the crystalline size, "k" is the shape constant 0.9, "λ" is the X-rays wavelength its value is 1.5×10^{-10} m.

While morphology and particle size distribution of BB-NiONPs were examined by scanning electron microscopy (SEM) equipped with EDX machine (EM (NOVA FEISEM-450 applied with EDX detectors).

2.4 | Antioxidant assays of nickel oxide nanoparticles

The free radical-scavenging potential of synthesized Particles were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay using microplate reader (Ma et al., 2011). DPPH solution (2.4 mg) was prepared using 25 ml of methanol and kept in dark for 60 min and absorbance was measured at 517 nm using a microplate reader. Methanol and Ascorbic acid were taken as negative and positive control, respectively. The free radical scavenging activities of the synthesized particles were calculated using formula below:

$$\text{DPPH scavenging effect}\% = \frac{AC - AN}{AC} \times 100$$

where AC is optical density of control and AN is optical density of the NONPs.

The antioxidant activities of BB-NiONPs were further assessed by total antioxidant capacity (TAC) using phosphomolybdenum method (Prieto et al., 1999). Briefly, reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate) and BB-NiONPs were mixed and incubated for 90 min at 95°C. After cooling the absorbance was taken at 695 nm. The obtained results were expressed in microgram equivalent of ascorbic acid per milligram of test sample (μg AA/mg).

2.5 | Antibacterial activity of NiONPs using disc diffusion method

The screening of antibacterial activity of green synthesized BB-NiONPs was performed using disc diffusion method. The activity was performed against Gram-positive and Gram-negative strains *Staphylococcus aureus* and *Proteus vulgaris*, respectively. Different solutions of BB-NiONPs (100, 500, and 1000 μg/ml) were used in this assay. For this purpose, the agar was used for plating the bacteria to check the antibacterial activity of BB-NiONPs. When the agar solidified in plates then bacterial strains streaked out in them. After that, took paper discs and dip them in solutions of a synthesized BB-NiONPs. After discs sticking on plates, incubate all plates in incubator for overnight at 37°C. The bacterial growth was observed after incubation and bactericidal activity of BB-NiONPs was determined by measuring the diameter of ZOI around the discs. *B. balochistanica* leaf extract based synthesized BB-NiONPs were active in killing both Gram-positive and Gram-negative bacterial strains.

2.6 | Antifungal assay using poisoned food technique

The screening of Antifungal activity of BB-NiONPs was performed using poisoned food method (Mohana & Raveesha 2007). The activity was studied using different Phytopathogenic fungal pathogens such as *Alternaria alternate*, *Asperagalus niger*, *Penicillium spp*, and *Fusarium oxysporum*. Sabouraud dextrose agar media (Oxoid CMO147) were prepared and autoclaved for the growth of fungal strains. Different concentrations (100, 500, and 1000 μg/ml) of BB-NiONPs were mixed with SDA (Sabouraud Dextrose Agar) medium and shake properly. 5 mm in diameter filter disc of the 7-days-old culture of the above test fungus was placed at the center of the petri dish and incubated at 27°C for 5 days, the growth was measured in millimeter (mm). The SDA medium without the plant extracts served as control and fluconazole were used as a positive control. The Antifungal activity of BB-NiONPs was calculated in terms of percentage inhibition of mycelia growth using the given formula (Singh & Tripathi, 1999).

$$\text{Inhibition}\%\text{age} = MC - MT/MC \times 100$$

where MC and MT represent the average increase in mycelia growth in control and each treatment, respectively.

2.7 | Cytotoxic activity of BB-NiO nanomaterial against *Artemia salina*

The cytotoxic potential of biosynthesized BB-NiONPs was estimated using cytotoxicity assay against *Artemia salina* (Iqbal et al., 2019). The eggs of brine shrimp were added in the hatching chamber having artificial sea water (3.8 g sea salt in 1 L distilled water) followed by incubation for 48 hr at 30°C. After hatching 20 mature brine shrimps

were shifted to glass vials having different concentrations of BB-NiONPs (200–1 μg/ml) and volume was adjusted up to 5 ml by adding sea water. After 24 hr, alive brine shrimps were counted (Meyer et al. 1982). Percentage mortality and Lethality concentration LC_{50} values were calculated using GraphPad software, while DMSO and seawater was used as a negative control.

2.8 | Stimulatory and inhibitory bioassay of BB-NiONPs

The BB-NiONPs were estimated using phytotoxicity assay against radish seed (Turker and Camper, 2002). Sterilized seeds (15) were placed at equal distance in autoclaved petri plates having filter paper and different concentrations (1000, 500, 250, 125, 62.5, and 31.25 μg/ml) of BB-NiONPs were added in each petri plate. Finally, 5 ml distilled water was added in each plate. After incubation different index such as final germination, percentage inhibition and the number of days (T50) required to reach 50% germination of the total number of seeds were calculated according to Pérez & González (2006).

3 | RESULTS AND DISCUSSION

The phytochemical profile of *Berberis* species have been found rich source of bioactive compounds like alkaloids, polyphenol, flavonoids, vitamins, and minerals, which have significant biological activities and also used as food preservative and nutrient supplements (Batool et al., 2019). Many *Berberis* species have been reported with rich source of phytochemicals and substantial antioxidant activities like *B. balochistanica* (Batool et al., 2019), *B. lyceum* (Akhtar and Mirza 2018), *B. aristata* and *B. thomsoniana* (Bhatt et al., 2018). Recently, Salehi et al (2019) enlisted more than 15 *Berberis* species from 36 different studies having biological and microbial influence against different quantified infectious pathogens. The presence of bioactive compound, the antioxidative and antimicrobial properties make this genus valuable to use in green synthesis of nanoparticle. According to my knowledge and literature search, there is no data about chemical composition, antioxidant activity of leaf extract and their usage in green synthesis of nanoparticles. However, very limited reports are available about phytochemistry and antioxidant activity of root extract and this will be the first study about their leaf extract. Therefore, through this novel approach using aerial part (leaf extract) of *B. balochistanica* plant, as a reducing, capping agent and a superior alternative for green synthesis of NiO nanoparticles due to its, easy availability, renewability, low cost, biocompatible, and nontoxic nature.

3.1 | Structural and morphological characterization of the BB-NiONPs

The corroboration of BB-NiONPs were characterize by various techniques and the results were validated by preceding analysis. The initial step of synthesis was the color change when salt was added to plant extracts (Figure 1). Figure 2 represented the UV–Vis absorption spectrum of plant extracts and BB-NiONPs formations, as Nickel nitrate was reduced during leaf broth addition. The peak value at 210–230 nm indicated the absorption of metal ions while the sharpness of peaks represented stability and well dispersity of the nanoparticles. Additionally, FTIR spectra of BB-NiONPs with multiples peaks representing functional groups associated with active biomolecules (Figure 3). The FTIR profile of NiO displayed vibration at ~3309.75 for –OH groups, vibration at 2947.03 and 2833.47 indicating the C–H stretching, while others like at 1656.56, 1449.37, and 1018.90 are C=C and C–O stretching for aromatic ring and polyphenols. The presence of hydroxyl group clearly indicated that water molecules are adsorbed by NiONPs which confirmed the high surface area of the synthesized particles (Ezhilarasi et al., 2018; Kaviyarasu et al., 2016). This functional groups play vital role in stabilizing and reducing BB-NiONPs during green route. Similarly, FTIR spectra with stretching vibrations of Ni–O were also reported in previous study (Kaviyarasu et al., 2016; Mayedwa et al., 2018; Khalil et al., 2018; Chaudhuri & Malodia., 2017; Abbasi et al. 2019a, 2019b; Iqbal et al., 2019; Iqbal et al., 2020a, 2020b).

The crystalline nature of the BB-NiONPs was studied using XRD techniques. The clear and sharp peaks of BB-NiONPs on XRD profile represented the crystalline nature of BB-NiONPs and perfectly match with standard JCPDS file no. 01-075-0197. It represents the crystalline landscape of BB-NiONPs along with indexes of (111), (200), and (220) and 2 theta (2Ø) values; 37.15, 43.16, and 62.77 (Figure 4). The XRD profile confirm the Cubic crystalline nature of NiO Particles. The average crystallite size was 21.67 nm calculated by Scherrer's formula ($D = k\,\lambda/\beta\cos\theta$) as shown in Table 1. This pattern of NiONPs is also called bunsenite phase, which clearly signified the purity of synthesized NPs as already confirmed by Thema et al. (2016) using *Agathosma betulina* plant extract. This result has closed resemblance with already published XRD pattern using *Rhamnus virgata*, *Moringa aliefera*, *Vigna radiata*, and *Moringa oleifera* (Iqbal et al., 2019, Nasseri et al., 2016; Chaudhary et al., 2018; Ezhilarasi et al. 2016). Additionally, elemental composition of synthesized nanoparticles was studied by EDS and Ni and O were observed as shown in Figure 5. The EDS pattern accurately recognized the presence of Ni–O without any impurities in biosynthesized sample (Figure 5). However, some trace elements (Na, K, or S) are also observed, which is originated from biomolecules present in BBL extract. These trace elements have capping potential and attached to the surface of synthesized NPs (Ismail et al. 2016; Thema et al. 2016; Nwanya et al., 2015).

Furthermore, Zeta potential analysis of BB-NiONPs was determined and was found positive (+10 mV; Figure 6). This result indicated that the chances of particles agglomerated is high might be due to magnetic interaction and polymeric adherence among synthesize particles (Wang et al., 2008). Zeta-potential determined the physical properties like surface charge, surface functionality, and stability exhibited by nanoparticle in suspension. The Zeta potential dimension are based on particles movement under electric field. However, the environment and surface charge of the particles also have great effect on zeta potential measurement (Ahmed et al., 2016). Our result is consistent with the previous results using *Rhamnus virgata*-NiO, *Rhamnus*

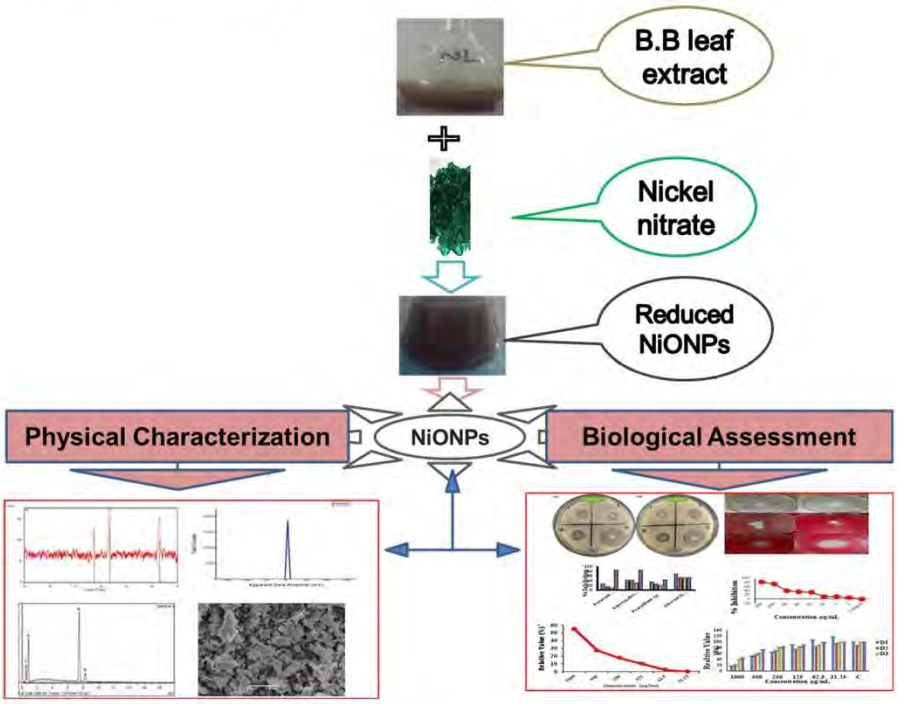

FIGURE 1 Physical characterization and Biological screening of green synthesized NiONPs using *B. baluchistanica* leaves extract and Nickel nitrate (precursor salt)

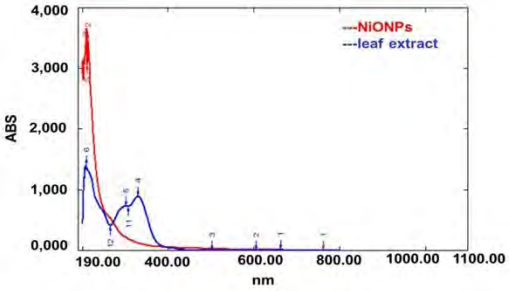

FIGURE 2 UV–Vis spectra of NiO and leaf extracts of *B. balochistanica*

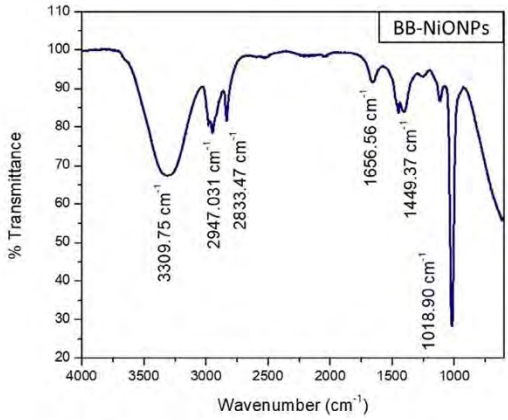

FIGURE 3 FTIR analysis of BB-NiONPs calcinated at 400°

triquetra-NiO (Iqbal et al., 2019; Iqbal et al., 2020a, 2020b; Manna & Bandyopadhyay 2017).

Along with structural profiles, the morphological features of BB-NiONPs were also evaluated by scanning electron microscopy (SEM). Figure 7 illustrated the typical SEM image of BB-NiONPs with modifications of ×1000, ×2500, ×5000, ×10,000, and ×30,000. The shape of nanoparticles was seemed to be highly agglomerated and these are basically the cluster of nanoparticles. This agglomeration might be due to the magnetic interaction and polymeric adherence nature of NPs (Iqbal et al., 2018a). In short, the leaf extracts of this medicinal plant are rich source of phytochemicals (Younis et al., 2018; Baloch et al., 2013; Batool et al., 2019) which act as fuel to make NPs having small size and high surface area.

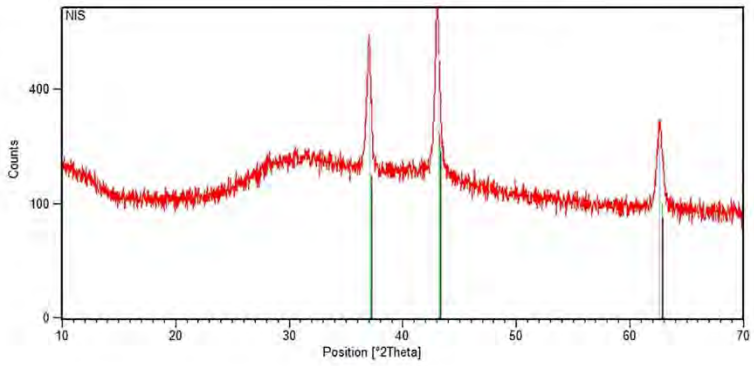

FIGURE 4 Characterization of green synthesized BB-NiONPs using X-ray diffractometer (XRD)

TABLE 1 Characterization of green synthesized NiONPs using X-ray diffractometer (XRD)

No	$2\theta_B$	θ_B	$2\Theta_1$	$2\Theta_2$	$\beta = \frac{2\theta 2 - 2\theta 1}{2} \times \frac{\pi}{180}$ (radian)	Interplanar spacing d (A°)	$D = \frac{0.9\lambda}{\beta \cos\theta B}$ (nm)	Miller indices (hkl)	Average size (nm)	Structure	JCPDS card no
1	37.15°	18.57°	36.75°	37.46°	0.00619	2.40755	20.97 nm	(111)	21.67	Cubic	ICSD ID 01-075-0197
2	43.16°	21.58°	42.73°	43.53°	0.00698	2.08500	20.79 nm	(200)			
3	62.77°	31.38°	62.36°	63.14°	0.00680	1.47432	23.25 nm	(220)			

Note: D is the crystallite size of the sample, *k* is the shape constant, and "λ" is the X-rays wavelength (1.5×10^{-10} m).

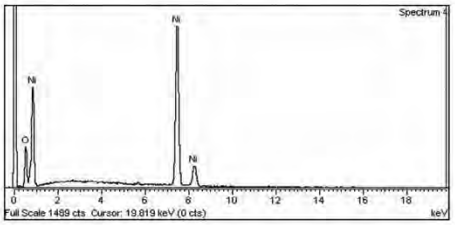

FIGURE 5 Elemental composition of BB-NiONPs using EDX analysis

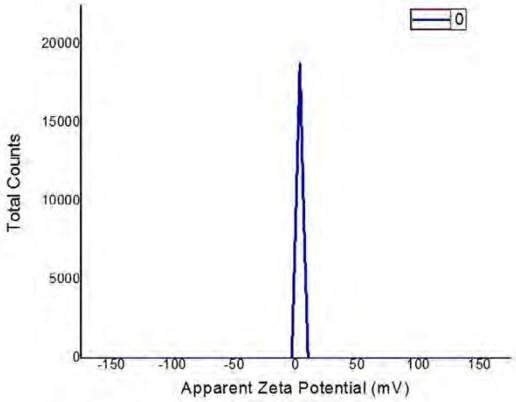

FIGURE 6 Zeta potential of BB-NiONPs

3.2 | Antioxidative potential of BB-NiONPs

The *B. baluchistanica*-NiONPs showed remarkable antioxidative potential via DPPH and TAC activity with percentage inhibition 69.98 and 59.59% at 200 μg/ml, respectively (Figure 8). In brief, dose dependent antioxidant response was reported in this experiment. Our finding is in agreement with previous studies in which NiONPs synthesized by leaf extract of *Rhamnus virgata* showed 70.36% DPPH and 51.43% TAC activities at 200 μg/ml (Iqbal et al., 2019).

3.3 | Antimicrobial potential of biofabricated BB-NiONPs

Some infectious diseases are series health problem globally as many pathogens became resistance against applied antibiotics. Therefore, to overcome the wide spread of infectious disease and to reduce the pathogens resistance against antibiotics, development of strong antimicrobial drug is very important (Iqbal et al., 2019; 2017; Khalil et al., 2017). The phytochemicals present in the complex form are the strong candidates for showing reducing and stabilizing potential in NPs synthesis and further acts as bactericidal and fungicidal activities. These biomolecules existing in the plants offer the defence against many microbes without any external sources. Therefore, it has naturally prevailing antioxidant and antimicrobial properties. Hence, when these biological

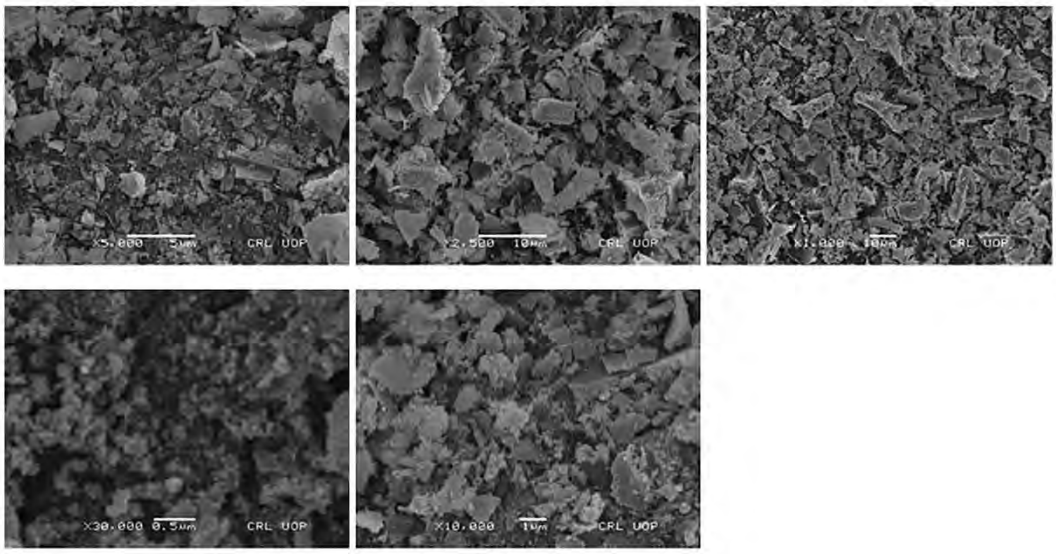

FIGURE 7 Characterization of BB-NiONPs using scanning electron microscope (SEM) at ×1000, ×2500, ×5000, ×10,000, and ×30,000

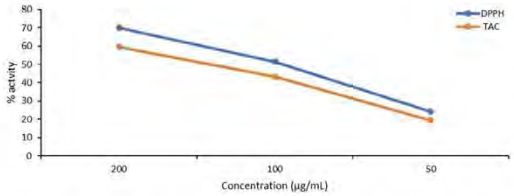

FIGURE 8 Antioxidant oxidant activity (DPPH and TAC) of synthesized BB-NiONPs by leave extract of *B. baluchistanica*. All values (triplicate) were measure by mean and *SD* (±)

entities combine with the metallic sources, they raise the antimicrobial behavior of biologically synthesized NPs.

In present study, the antibacterial characteristics of BB-NiONPs were investigated against Gram-positive (*S. aureus*) and Gram-negative (*P. vulgaris*) strains using varying concentrations (100, 500, and 1000 µg/ml) by disc diffusion assay (Table 2). Ciprofloxacin was used as positive control (Figure 9). The results indicated that biologically synthesized BB-NiONPs have higher antibacterial activity against both Gram-positive and Gram-negative bacterial strains.

Numerous bactericidal analysis of NiONPs are available while research study on fungicidal potential of nanomaterial are limited. In present study, the antifungal potential of BB-NiONPs (100, 500, and 1000 µg/ml) were investigated against *Alternaria alternata*, *Aspergillus niger*, *Penicillium sp*, and *Fusarium axysporum*. The rate of inhibition of fungal stains by various BB-NiONPs concentrations was varied; as *A. alternata* was more inhibited (52.5–70%) followed by *A. niger* (33.33–44.05%) then *Penicillium sp*. (22.37–36.84%) and *F. axysporum* (11.65–28.15%), respectively (Figures 10 and 11). Our findings are similar with earlier studies in which NiO nanoparticles showed inhibitory effect against bacterial and fungal pathogens (Iqbal et al., 2019; Chaudhary et al., 2018; Karthik et al., 2017). Along with this, it was also reported in this study that reference drug used in antibacterial and antifungal analysis showed strong inhibitory activities against test bacterial and fungal pathogens compared to BB-NiONPs. Overall, BB-NiONPs showed dose dependent response against tested pathogens. Our results are in correspondence with previous studies about reference drug and NiONPs (Iqbal et al., 2019; Karthik et al. 2017; Aisida et al., 2020). The mechanism of interaction of NiONPs with pathogens are not well known, but hypothetically, NPs enter in cytoplasm and disrupt the metabolic activities inside cell by oxidative and non-oxidative mechanism by releasing of free radicals (Chaudhary et al., 2018; Wang et al., 2017).

3.4 | Cytotoxic effect of BB-NiONPs on brine shrimp

The cytotoxic potential of BB-NiONPs was investigated against brine shrimp using brine shrimp lethality assay. Lethality assay is one of the easy and suitable method to screen the biological activities of any compound (Iqbal et al., 2019). Figure 12 shows the cytotoxic (% inhibition) potential of green BB-NiONPs at different concentrations (200–1 µg/ml). The cytotoxicity was observed in dose dependent manner as mortality (%) was increased with higher concentrations of BB-NiONPs. Noteworthy, in vivo lethality activities with LD_{50} value was

TABLE 2 Antibacterial activity of BB-NiONPs against *S. aureus* and *P. vulgaris*

Sample	Tested bacteria	Gram reaction	Zone of inhibition		Control	
			100 µg/ml	500 µg/ml	1000 µg/ml	Ciprofloxacin
NiO	*S. aureus*	*+ive*	14 mm	12 mm	16 mm	28 mm
	P. vulgaris	*−ive*	10 mm	16 mm	14 mm	26 mm

Note: All values (triplicate) were measure by mean and *SD* (±). Whereas *+ive* and *−ive* represented Gram-positive and Gram-negative bacterial strain, while mm refer to millimeter.

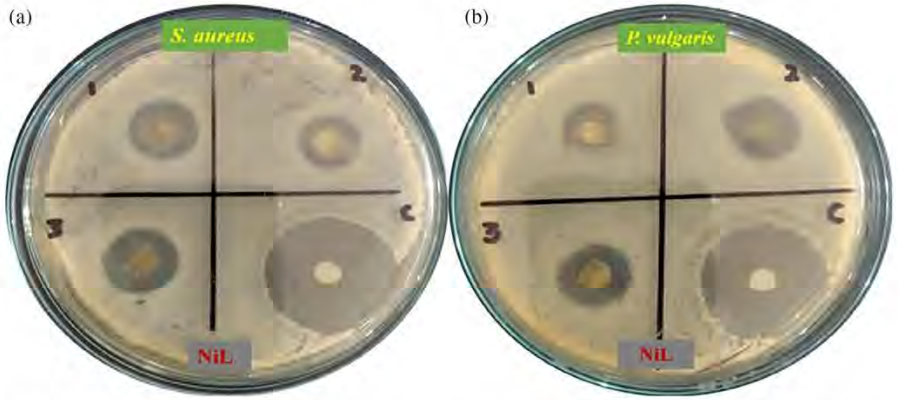

FIGURE 9 Antibacterial activity of BB-NiONPs against (a) *S. aureus*, (b) *P. vulgaris*, 100 µg/ml: 1, 500 µg/ml: 2, 1000 µg/ml: 3, Ciprofloxacin: C

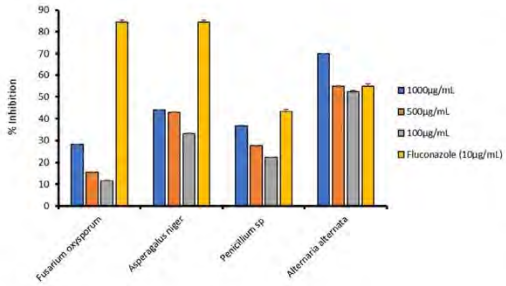

FIGURE 10 Antifungal activity of BB-NiONPs at different concentration (1000, 500, and 100 µg/ml). All values (triplicate) were measure by mean and *SD* (±)

calculated as 49.10 µg/ml. The result of present study reported dose dependent response of BB-NiONPs on applied concentrations (200–1 µg/ml; Figure 12). The cytotoxic effect of BB-NiONPs were in general category with IC50 value (49.10 µg/ml) which is in line with the results of earlier studies (Abbasi et al. 2019a, 2019b, 2020a, 2020b, Iqbal et al., 2019; Woodford & Livermore, 2009). The inhibitory effect of BB-NiONPs at applied concentrations (200–1 µg/ml) could be due to the absorbance of various functional molecules on the surface of BB-NiONPs from leaves extract of *B. balochistanica*.

3.5 | Stimulatory and inhibitory effect of BB-NiONPs on seed germination

Cytotoxicity or inhibitory potential have been extensively studied in previous research works, while only limited studies has been available on stimulatory potential of NPs. According to our literature review, the present study represented first time the bio stimulatory potential of BB-NiONPs. Phytotoxicity assay is the best assay for screening of stimulatory or inhibitory effect of nanoparticles. The phytotoxicity test of green synthesized BB-NiONPs were studied at different concentrations (1000, 500, 250, 125, 62.5, and 31.25 µg/ml) using radish seed. During analysis, it was distinguished that at lower concentration the germination was enhanced while at higher concentrations the germination rate was constrained (Figure 13a). Figure 13b shows that on first day of germination (D1), BB-NiONPs showed stimulatory effect by increasing relative germination rate 117.24 and 106.89% at lower concentration (31.25 and 62.5 µg/ml) as compare to control (not treated seed). At higher concentration final germination of seeds were delayed as a result, 50% of total seed germinations (T50) were not achieved at

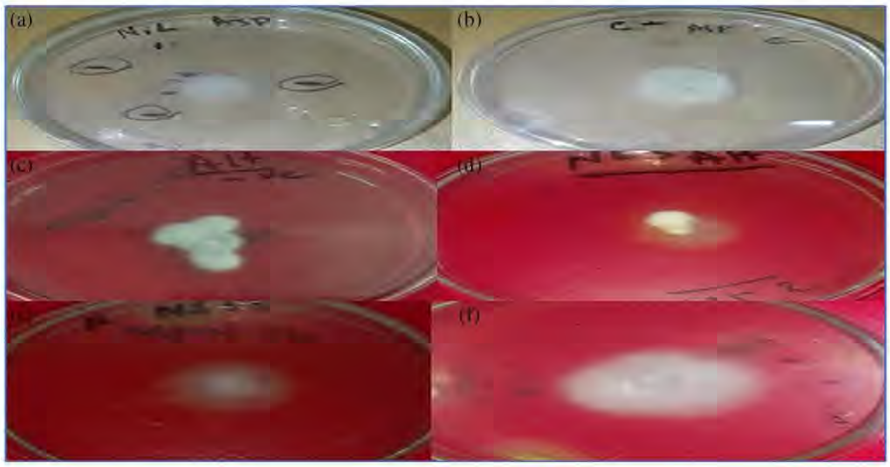

FIGURE 11 Antifungal activity of BB-NiONPs against fungal stains. Where (a), (c), and (e) represented the *Aspergillus nigu*, *Alternaria alternata*, and *Fusariem oxysporum* treated with BB-NiONPs. While (b), (d), and (f) refer to negative control

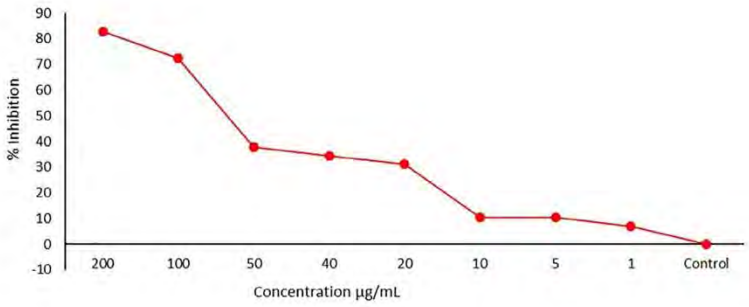

FIGURE 12 Brine shrimp lethality assay of BB-NiONPs against brine shrimp. All values (triplicate) were measure by mean and *SD* (±)

higher concentrations (1000 µg/ml) at day 4 (Figure 13b). Regarding stimulatory effect, very little studies are available on NiONPs, whereas inhibitory activities on NiONPs were studied in various studies (Chaudhary et al., 2018; Faisal et al., 2013). Recently, other nanoparticles like ZnO and TiO_2 nanoparticles were reported with stimulatory effect at lower concentration (Younes et al., 2020; Dawood et al., 2019). The enhancement in seed germination might be due to the capability of NiONPs to enter inside seed and trigger the dormancy breaking enzymes and accelerated oxygen and water uptake, a result seed germination started immediately (Younes et al., 2020). Usually, it was observed that, the concentrations of synthesized particles and their size have great toxic effect on seed germination by releasing ions and generation of reactive species (Abdel-Salam et al., 2018; Chaudhary et al., 2018).

The antimicrobial, cytotoxic, and phytotoxic potential of BB-NiONPs in present study is might be due to the reduced size, precise surface area, and generation of more ions from NiONPs. The generations of ions and adherence properties of NiONPs are accountable ROS production and oxidative stresses inside the cell, which ultimately demolished the membrane and metabolic activities of pathogen through genotoxic effect (Ezhilarasi et al., 2018; Aisida et al., 2020). This study also gave information about the stimulatory effect of BB-NiONPs at suitable quantity. Thus, it is indorsed that at lower concentrations the synthesized nanoparticles can be used as biostimulator by breaking the dormancy and accelerating the seed germination of those plant which have lengthy germination period and high dormancy.

In current study, BB-NiONPs displayed remarkable biological activities against various infectious pathogens. As compared to bulk materials, NPs have potential to attached and penetrated inside the pathogen cell membrane and alter the metabolic machinery (Ali et al., 2020; Iqbal et al., 2020a, 2020b). The mechanism of inhibitory action of synthesized NPs has not been yet clear. However, recent studies reflected that the action of inhibition might be due to the small size and high penetration power of nano materials as result the intracellular machinery become altered (Basak et al., 2014; Karthik et al., 2018). Briefly, the penetration of released Nickel ions inside the cell alters the membrane composition

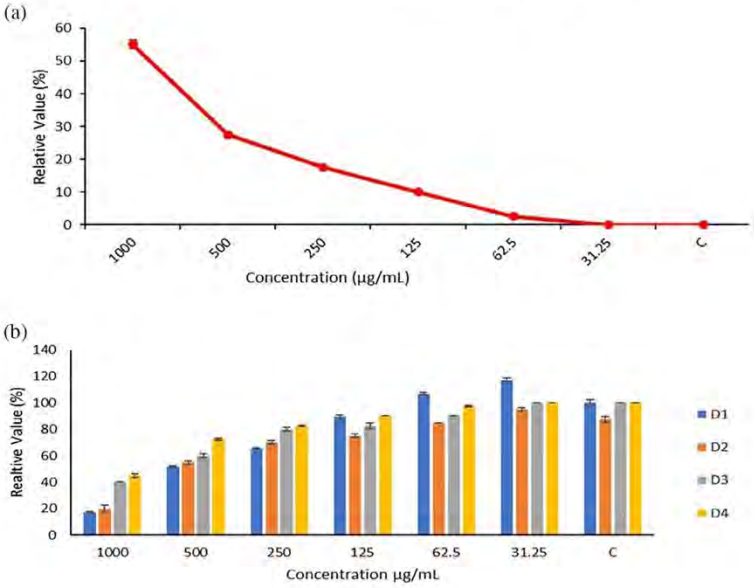

FIGURE 13 Phytotoxicity of BB-NiONPs using radish seed as model organism; (a) final germination and (b) Relative germination rate; T50 (The time required to reach 50% of the final germination). All values (triplicate) were measure by mean and *SD* (±). *C* refer to control (nontreated); while D1, D2, D3, and D4 represented the day first, day second, day third, and day forth of the seed germination against applied concentrations (1000–31.25 µg/ml) of BB-NiONPs and control

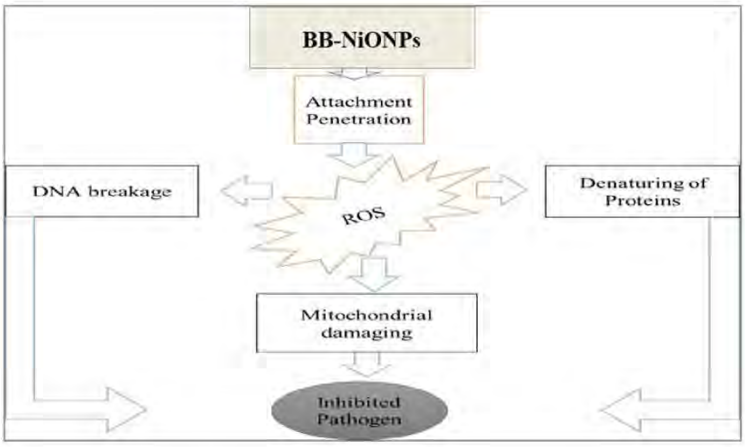

FIGURE 14 The schematic hypothetical antimicrobial mechanism of BBL-NiONPs

and caused leakage. After penetration, the BB-NiONPs damaged the cellular metabolic mechanism by damaging (DNA strand) and destroying the three-dimensional domains (proteins) and causing the osmotic and oxidative stresses (Figure 14; Srihasam et al., 2020).

4 | CONCLUSION AND FUTURE OUTLOOK

The current study deals with the biogenic synthesis of NiONPs using leaf extract of *B. balochistanica* plant. The synthesized NPs was confirmed by different spectroscopic and microscopic techniques. The prepared BB-NiONPs were also screened for different biological activities and it is reported that this nanoflower have outstanding antibacterial, antifungal, cytotoxic, phytotoxic, and stimulatory effect on seed germination. It is concluded that this biogenic BB-NiONPs is an easy, cheap, ecofriendly, and bio-stimulating mediator for biomedical and agriculture purposes. Finally, further in vivo, and extensive research are recommended to standardize the safety level first, then allowed to be used in pharmacological, biomedical, and agricultural fields.

ACKNOWLEDGMENTS

The authors thank Dr. Amir Sultan for the identification of plant specimens in National Herbarium, Islamabad, Pakistan. The authors thankful to Prof. of Plant physiology, Department of Plant sciences, Quaid I Azam University, Islamabad, Pakistan for providing research facilities and financial support for this research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Umar Masood Quraishi and Siraj Uddin conceived and designed the study. Siraj Uddin, Javed Iqbal, Banzeer Ahsan Abbasi, and Muhammad Saqib Saif performed the experiments. Siraj Uddin and Luqman Bin Safdar and Javed Iqbal analyzed the data. Siraj Uddin and Saeed Anwar performed statistical data analysis and visualizations. Siraj Uddin and Umar Masood Quraishi wrote the original draft. Siraj Uddin, Sabiha Laila, Luqman Bin Safdar, Banzeer Ahsan Abbasi, SAL, and Javed Iqbal reviewed and edited the manuscript. Umar Masood Quraishi and Siraj Uddin provided the resources. Muhammad Saqib Saif made valuable revisions and edited the manuscript. All authors read and approved the last version.

DATA AVAILABILITY STATEMENT

The authors declare that any needed data that can support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Uddin S, Safdar LB, Iqbal J, et al. Green synthesis of nickel oxide nanoparticles using leaf extract of *Berberis balochistanica:* Characterization, and diverse biological applications. *Microsc Res Tech*. 2021;84:2004–2016. https://doi.org/10.1002/jemt.23756

ARTICLE 4: GREEN SYNTHESIS OF NICKEL OXIDE NANOPARTICLES FROM BERBERIS BALOCHISTANICA STEM FOR INVESTIGATING BIOACTIVITIES

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Green Synthesis of Nickel Oxide Nanoparticles from *Berberis balochistanica* Stem for Investigating Bioactivities

Siraj Uddin [1,2], Luqman Bin Safdar [3,4,*], Saeed Anwar [1], Javed Iqbal [1,5], Sabiha Laila [6], Banzeer Ahsan Abbasi [1], Muhammad Saqib Saif [7], Musrat Ali [1], Abdul Rehman [8], Abdul Basit [9], Yong Wang [9,*] and Umar Masood Quraishi [1,*]

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Abstract: Green synthesis of nanomaterials is advancing due to its ease of synthesis, inexpensiveness, nontoxicity and renewability. In the present study, an eco-friendly biogenic method was developed for the green synthesis of nickel oxide nanoparticles (NiONPs) using phytochemically rich *Berberis balochistanica* stem (BBS) extract. The BBS extract was rich in phenolics, flavonoids and berberine. These phytochemicals successfully reduced and stabilised the $NiNO_3$ (green) into NiONPs (greenish-gray). BBS-NiONPs were confirmed by using UV-visible spectroscopy (peak at 305 nm), X-ray diffraction (size of 31.44 nm), Fourier transform infrared spectroscopy (identified -OH group and Ni-O formation), energy dispersive spectroscopy (showed specified elemental nature) and scanning electron microscopy (showed rhombohedral agglomerated shape). BBS-NiONPs were exposed to multiple in vitro bioactivities to ascertain their beneficial biological applications. They exhibited strong antioxidant activities: total antioxidant capacity (64.77%) and 2, 2-diphenyl-1-picrylhydrazyl (71.48%); and cytotoxic potential: Brine shrimp cytotoxicity assay with IC_{50} (10.40 µg/mL). BBS-NiONPs restricted the bacterial and fungal pathogenic growths at 1000, 500 and 100 µg/mL. Additionally, BBS-NiONPs showed stimulatory efficacy by enhancing seed germination rate and seedling growth at 31.25 and 62.5 µg/mL. In aggregate, BBS extract has a potent antioxidant activity which makes the green biosynthesis of NiONPs easy, economical and safe. The biochemical potential of BBS-NiONPs can be useful in various biomedical and agricultural fields.

Keywords: NiONPs; green synthesis; antioxidants; antimicrobial; cytotoxicity; nano fertiliser

Citation: Uddin, S.; Safdar, L.B.; Anwar, S.; Iqbal, J.; Laila, S.; Abbasi, B.A.; Saif, M.S.; Ali, M.; Rehman, A.; Basit, A.; et al. Green Synthesis of Nickel Oxide Nanoparticles from *Berberis balochistanica* Stem for Investigating Bioactivities. *Molecules* **2021**, *26*, 1548. https://doi.org/10.3390/molecules26061548

Academic Editors: Tetsu Yonezawa and Antonio Zuorro

Received: 14 January 2021
Accepted: 4 March 2021
Published: 11 March 2021

1. Introduction

Nanotechnology is revolutionising many industrial and technological fields due to the fact that it is possible with nanotech to orient material structures at extremely small scales, thereby extending the materials science toolkit. By using nanotechnology, materials can be

made lighter, stronger, more reactive, better electrical conductors and more durable, among countless other characteristics. The properties of nanoparticles (NPs) can be altered by changing their size at the nanoscale which provides the capacity to use them in multidisciplinary fields including medicine [1]. Up until now, a variety of metals and metal oxide NPs have been synthesised such as silver, gold, platinum, magnesium, iron oxide, caesium oxide and zinc oxide [1,2]. Among these prepared NPs, nickel oxide NPs (NiONPs) have fascinated scientists from multiple fields due to their easy synthesis, small size, wide bandgap and semiconductor properties [3]. NiONPs have been reported with numerous fruitful applications in batteries, sensors, superconductors, magnetic materials, photocatalytic and catalytic analysis [4]. In green synthesis, three main sources, i.e., autotrophs (plant and algae), bacteria and fungi are used [3]. Plant-mediated green synthesis is getting significant value in biomedical fields due to its simplicity, easy availability, ecofriendly nature and non-toxicity, and it eradicates the prerequisite of reducing agents and energy from the external environment. Studies show that plants contain numerous valuable bioactive compounds like alkaloids, polyphenols, flavonoids, terpenoids, vitamins and minerals, which play a vital role as stabilising, capping and reducing agents during phytofabrication of NPs [5]. These phytochemicals also have significant antioxidant, anti-microbial, anti-inflammatory, chemo-preventative and cytotoxic potentials [6]. During green synthesis, phytochemicals with strong antioxidant potentials adsorb onto the NPs surface and make them effective antioxidant, antimicrobial, and cytotoxic NPs [7]. Therefore, the green synthesis of NPs serves as a useful and eco-friendly approach. The focus of green synthesis is being rapidly shifted toward medicinal plants due to their rich biological potential [8].

Plants belonging to the family Berberidaceae are catching interest to prepare NPs with great medical and phytonutritional properties, some recent examples are: [9–11]. The endemic shrub, *B. balochistanica* (Zralga), belongs to the family Berberidaceae and is found in the Quetta, Ziarat and Kalat regions of Balochistan, Pakistan [12]. The decoction of the underground part (root) is used for the treatment of various diseases like coughing, fever, wound healing, internal injury, eye disease, rheumatism and other infections of human beings and livestock [13–17]. Recently, the roots of this plant were studied and various secondary metabolites like berberisinol, berberine, palmatine, 8-oxoberberine, oleanolic acid, β-sitosterol, gallic acid, phenols, carotenoids and vitamins were isolated [13,17]. These secondary metabolites in the root were found with remarkable antioxidant, antibacterial and antifungal potentials [17,18]. The presence of bioactive compounds with antioxidative and antimicrobial properties makes this plant valuable to use for the green synthesis of NPs. However, there is little data on the chemical composition and antioxidant activity of *B. balochistanica* stem extract and its usage in green synthesis.

The aim of this study was to characterise the phytochemical and antioxidant activities of stem extract of *B. balochistanica*. After confirmation of biomolecules with significant antioxidant activities, the extract was subjected to synthesise NiONPs. The synthesised NiONPs were characterised using UV-visible, Fourier transform infrared (FTIR), energy dispersion spectroscopy (EDS), X-ray diffractometer (XRD) and scanning electron microscopy (SEM) analysis. To evaluate the biological potential of BBS-NiONPs, different in vitro biological and biofertiliser activities including antimicrobial, antioxidant, cytotoxic, inhibitory and biostimulatory activities were assessed.

2. Results

2.1. Physical and Morphological Characterisation of BBS-NiONPs

The confirmation of BBS-NiONPs was observed by various techniques and the results were validated by preceding analysis. Figure 1a represents the UV-Vis absorption spectrum of BBS-NiONPs formations using stem extract of *B. balochistanica*. The peak value at 305.00 nm specifies the absorption of metal ions. Figure 1b represents the FTIR spectra with multiple peaks belonging to bio-constituents adsorbed by the synthesised NPs. The FTIR profile of NiO displayed vibration at 3309.75 cm^{-1} for -OH groups, at 2943.78 and 2831.77 cm^{-1} indicating the C-H stretching, while other multiple peaks at 1559.94,

1449.04,1415.19,1114.62 and 1021.53 cm^{-1} were related to C=C and C-O stretching for aromatic ring and polyphenols. The peak at 616.36 cm^{-1} displayed information about NiO in $NiNO_3$. The crystalline nature of BBS-NiONPs was analysed using the XRD technique. Sample NiONPs showed crystallite size (31.44 nm) and the peaks matched with JCPDS Card #: ICSD ID 00-022-1189. The plane of three clear peaks and interplanar spacing 'd' were (003) at 0.241nm, (012) at 0.2088nm and (104) at 0.1477 nm (Figure 1c). The XRD profile displayed a rhombohedral shape of synthesised NiO particles. The intense and sharp peaks exposed that NiONPs were successfully shaped in the stem broth of *B. balochistanica*.

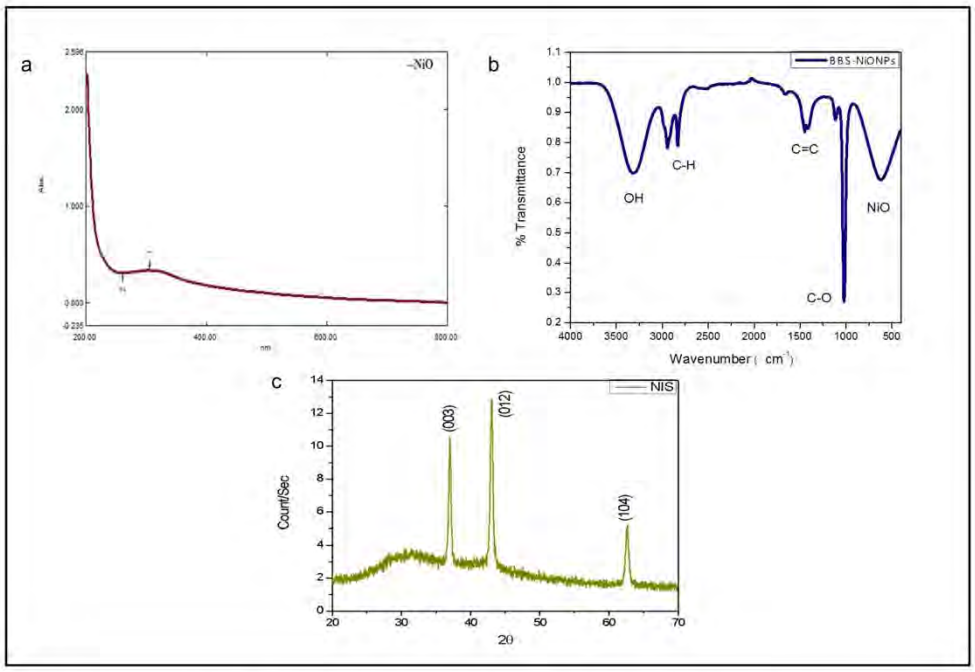

Figure 1. Spectroscopic profile of *Berberis balochistanica* stem (BBS)- nickel oxide nanoparticles (NiONPs). (**a**) UV-Vis spectrum of BBS-NiONPs. The peak value at 305.00 nm specifies the absorption of metal ions. (**b**) FTIR profile of BBS-NiONPs. Peaks indicate the presence of different functional groups. (**c**) XRD spectrum of BBS-NiONPs.

The elemental composition of synthesised nanoparticles was confirmed by EDS analysis. Figure 2a shows strong peaks of Ni (79.19%) and O (15.56%) by weight. The presence of carbon (4.50%) and potassium (0.74%) in graphs was attributed to grid support. Additionally, the morphological features of greenly invented BBS-NiONPs were assessed by scanning electron microscopy (SEM). Figure 2b illustrates the SEM profile of BBS-NiONPs confirming the highly agglomerated shape of the synthesised particles. The movement in suspension, for BBS-NiONPs, was studied by zeta-potential which was observed as 3.26 mV (Figure 2c).

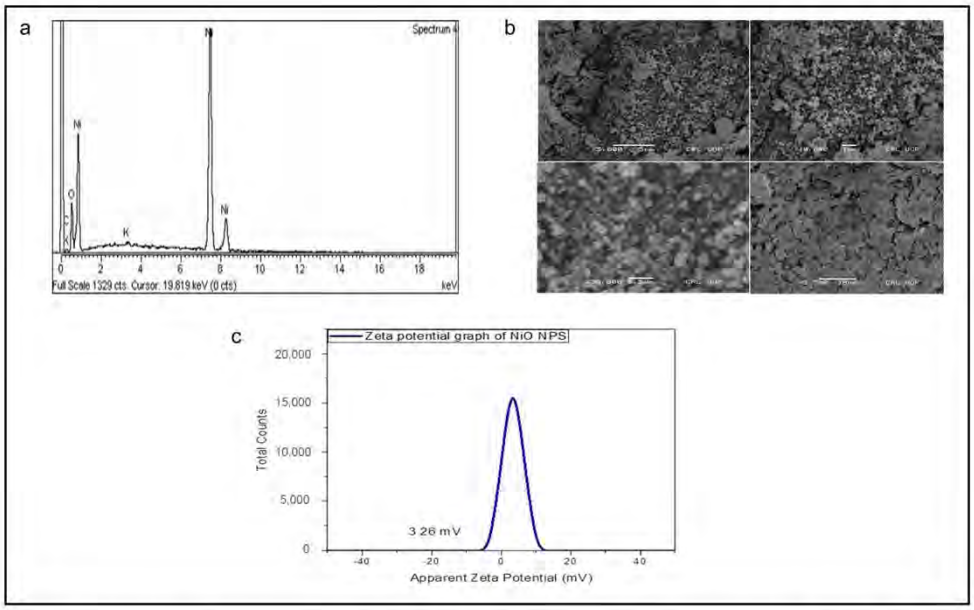

Figure 2. Spectroscopic and microscopic profile of BBS-NiONPs. (**a**) Energy dispersion spectroscopy (EDS) profile shows the elemental composition of BBS-NiONPs. Peaks are observed for Ni, O, C and K. (**b**) Micrograph of SEM shows the crystalline structure of BBS-NiONPs. (**c**) Zeta potential of BBS-NiONPs.

2.2. *Phytochemical and Antioxidant Analysis*

In the present study, the stem extract of *B. balochistanica* plant was first time analysed for total phenolic contents (TPC) and total flavonoid contents (TFC) (Figure 3a). The concentrations of TPC and TFC were 48.21 mg GA/g and 141.29 mg QE/g. The presence of an adequate amount of berberine compound was also observed using thin-layer chromatography (TLC; Figure 3b). Additionally, the presence of biological molecules in stem extract of BB was also confirmed by FTIR spectroscopy. The functional groups were separated on the basis of peak values. The spectrum profile, peak values and functional groups were compared with the IR standard chart as shown in Figure 3c and Table 1. The FTIR spectrum of BBS extract showed multiples peak values indicating the presence of phenols, alcohols, alkanes, alkenes, aromatic compounds, carboxylic acid and alkyl halides. Interestingly, the absence of peaks at 2220–2260 cm^{-1} indicated the absence of cyanide derivatives.

Table 1. FTIR analysis of *B. balochistanica* plant stem extract.

Peak Values (cm^{-1})	Strength [a]	Functional Groups	Interpretations
3306.95	Medium	OH	Phenol, Alcohol
2947.93–2834.58	Medium	C–H	Alkane
1655.05	Weak	C=C	Alkene
1449.31	Weak	C=C	Aromatic compounds
1114.57	Weak	C–O	carboxylic acids, alcohols
1016.4016	Strong	C–O	carboxylic acids, alcohols
587.88–522.90	Medium	C–Cl	Alkyl halides, Sulphur compounds

[a] Strength of peaks in the spectrum (500–4000 cm^{-1}).

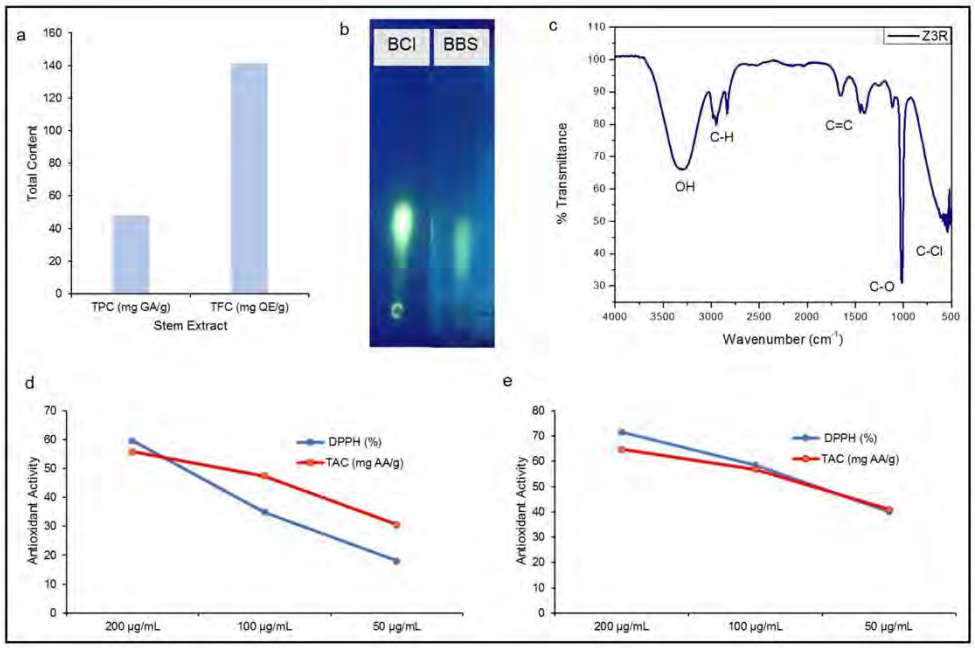

Figure 3. Phytochemical and Antioxidant analysis. (**a**) Total phenolic (mg GAE/g) and total flavonoid (mg QE/g) contents of stem extract. (**b**) Berberine in stem extract. BCl, berberine chloride; BBS, *B. balochistanica* stem. (**c**) FTIR spectrum of stem extract. (**d**) Antioxidant activities (DPPH and TAC) of stem extract. (**e**) Antioxidant activities (DPPH and TAC) of BBS-NiONPs. Numerical data are presented as mean ± standard deviation (n = 3).

Furthermore, the stem extract and green synthesised BBS-NiONPs were investigated for antioxidant potential via 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and total antioxidant capacity (TAC). At 200 µg/mL, both stem extract and BBS-NiONPs showed remarkable antioxidant activities with a percentage inhibition of 59.61 and 71.48% (DPPH) and 55.78 and 64.77% (TAC), respectively (Figure 3d,e). Because of the presence of these phytocompounds (TPC, TPC and berberine) and strong antioxidant activities, stem extract was selected as stabilising and capping agents for the synthesis of BBS-NiONPs in the current experiment.

2.3. Antibacterial and Antifungal Activity of Phytofabricated NiONPs

Figure 4a exhibits the antibacterial activity of phytofabricated BBS-NiONPs (100, 500 and 1000 µg/mL) against *Proteus vulgaris* and *Staphylococcus aureus* bacterial strains. BBS-NiONPs showed a dose-dependent response against both selected bacterial strains. Moreover, 10 µg Ciprofloxacin (positive control) was found more reactive than all the applied doses of BBS-NiONPs during antibacterial activities.

Antifungal analysis of BBS-NiONPs was carried out using different concentrations (50, 100, 500, 1000 µg/mL) against *Fusarium oxysporum*, *Aspergillus niger* and *Alternaria alternata*. Dose-dependent responses against all three analysed fungus strains were observed (Figure 4b). *F. oxysporum* was found less vulnerable at high concentration (1000 µg/mL) while *A. alternata* was found highly susceptible with a percentage inhibition of 71.25% followed by *A. niger* with a percentage inhibition of 39.51% at 1000 µg/mL.

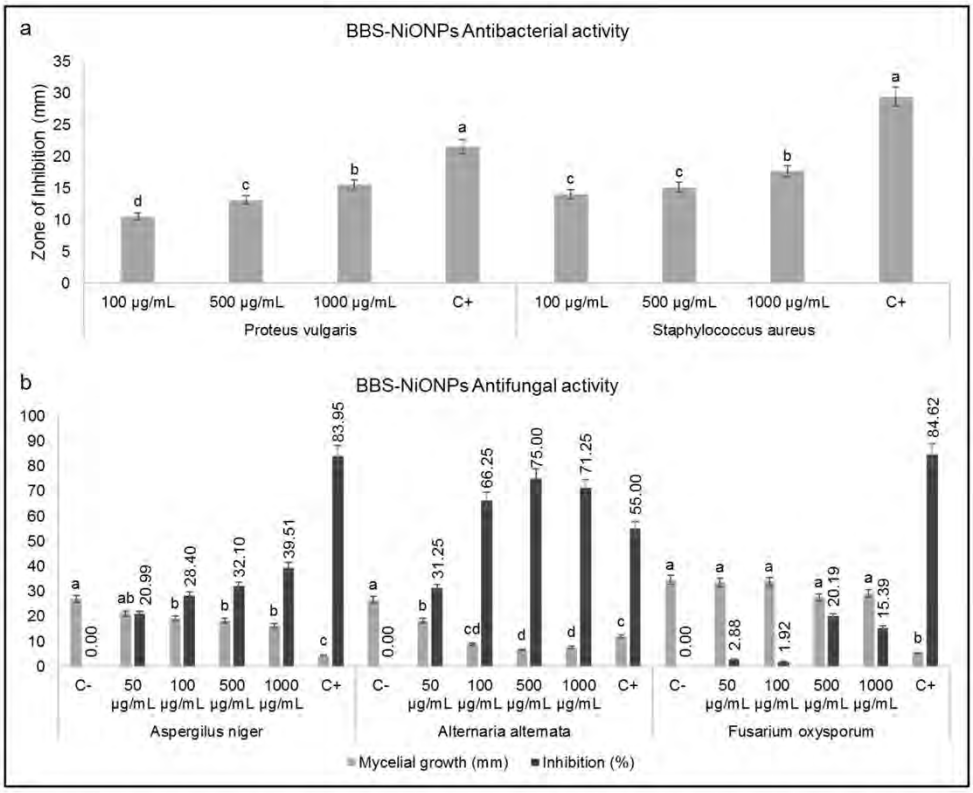

Figure 4. Antimicrobial potentials. (**a**) Antibacterial potential of BBS-NiONPs; C+ refers to Ciprofloxacin. (**b**) Antifungal potential of BBS-NiONPs. Letters indicate a significant difference ($p < 0.05$) between control and BBS-NiONPs treated samples. C+ refers to fluconazole. Numerical data are presented as mean ± standard deviation ($n = 3$).

2.4. Cytotoxic Potential of Biosynthesised BBS-NiONPs

The cytotoxic potential of biosynthesised BBS-NiONPs was examined against brine shrimp (BS) larvae. The BS larvae were exposed to BBS-NiONPs at different concentrations (200-1 µg/mL) for 24 h and a considerable dose-dependent cytotoxic response with IC_{50} value (10.40 µg/mL) was calculated (Figure 5).

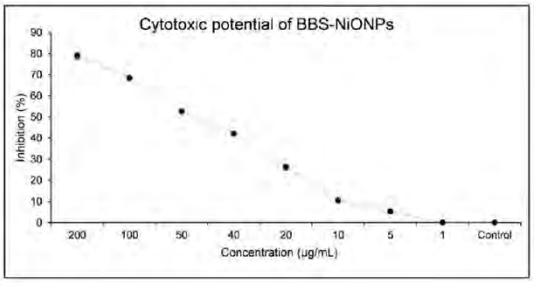

Figure 5. Brine shrimp mortality assays of BBS-NiONPs.

2.5. Inhibitory and Stimulatory Effect of BBS-NiONPs

Figure 6a signifies the percentage inhibition of seed germination at the applied concentration of BBS-NiONPs (31.25–1000 µg/mL). The percentage inhibition was observed in a dose-dependent manner. At lower concentrations (31.25–125 µg/mL), the seed germination was not inhibited, while at higher concentrations, the seed germination was inhibited as 16, 25 and 41% at 250, 500 and 1000 µg/mL, respectively. Furthermore, the relative germination rate (RGR) of seeds during the first two days was also more fascinating as at lower doses the germination started earlier in non-treated control. The seeds treated with BBS-NiONPs (31.25 and 62.5 µg/mL) showed 2–4% more germination than control (non-treated) after Day 1. After Day 2, the treated seeds (31.25 and 62.5 µg/mL) showed 3–8% stimulatory activity as compared to control (Figure 6b). In short, BBS-NiONPs showed stimulatory effects at lower doses and enhanced the speed of germination as compared to control (non-treated). Similarly, NiONPs also showed positive effects on the seedling growth of the radish plant. At lower concentrations (31.25 and 62.5 µg/mL), the seedling growth was improved by 13.86 and 7.92%, while at higher concentrations, the growth of the seedling declined by 33.17, 37.13 and 39.60% at 250–1000 µg/mL, respectively (Figure 6c,d).

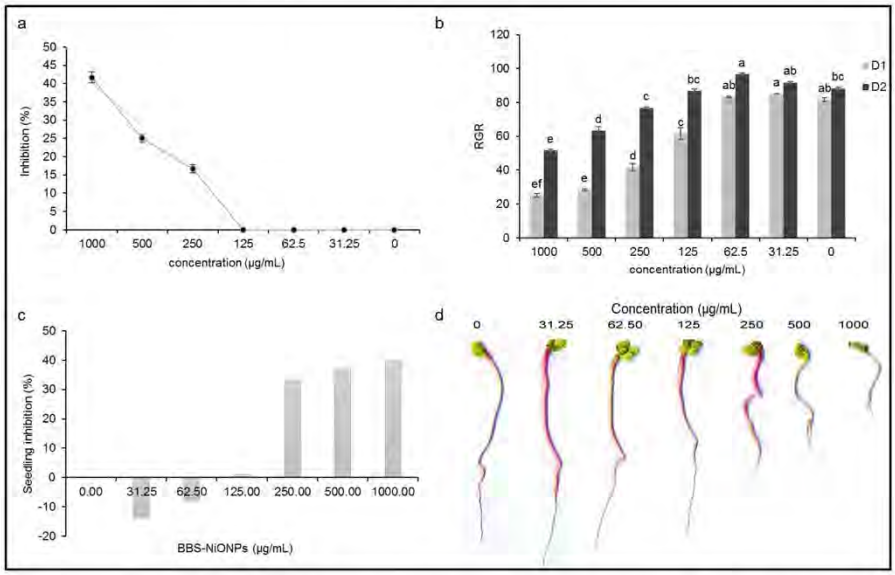

Figure 6. Effects of BBS-NiONPs on the seed germination and seedling growth of a radish plant. (**a**) Percentage inhibition (inhibitory effect) of seed germination against applied concentrations of BBS-NiONPs. (**b**) Relative germination rate (RGR) of seed (stimulatory effect) during first two days (D1 and D2). (**c**) Relative seedling growth rate (%). (**d**) Seedling length (mm) at applied concentrations. Letters indicate a significant difference ($p < 0.05$) between control and BBS-NiONPs treated samples. Numerical data are presented as mean ± standard deviation ($n = 3$).

3. Discussion

In *B. balochistanica* roots, many bioactive compounds such as phenolics, pakistanamine, proaphorphin, benzylisoquinoline, alkaloid, flavanols, berberine, oleanolic acid and gallic acid have been reported [13,14]. In the present study, the phytochemistry of BBS was investigated and remarkable antioxidant activities were noted. FTIR of BBS indicated the presence of multiple functional groups including phenols, alcohols, alkanes, alkenes, aromatic compounds, carboxylic acid and alkyl halides. These functional groups represent

secondary metabolites that act as a natural defense system and give medicinal properties to plants [19].

Based on the results of the present study on phytochemicals with potent antioxidant activities, the BBS extract was selected to use in the green synthesis of NiONPs. Previously, NiONPs have been synthesised by physicochemical and biological methods [20]. However, the biocompatibility and phytochemical potential of BBS-NiONPs indicate that utilising medicinal plants for green synthesis is a much better strategy to synthesise pure, safe, biocompatible and bioactive NiONPs. Although using medicinal plants for green synthesis provides rather effective NPs [11], it poses a threat to biodiversity due to the fact that many medicinal plants, such as *B. balochistanica* used in this study, are subject to extreme pressure due to their extensive use by local and homeopathic communities. Unlike Dangi et al. where they used *B. asiatica* roots for green synthesis [11], we synthesised NiONPs using *B. balochistanica* stem extract and found compatible, or even better, bioactivities of the synthesised NiONPs. These findings encourage using aerial parts of medicinal plants in green synthesis and other pharmacological fields—an approach that can help in the preservation of rare species.

Colour observation, spectroscopic and microscopic analysis characterised the biogenic BBS-NiONPs. The formation of BBS-NiONPs was verified by changing the colour from green to greenish-gray. The UV-Vis spectra at 305 nm, average size 31.44 nm, pure Ni and O elemental nature and rhombohedral agglomerated shape clearly validated the formation of BBS-NiONPs. Additionally, the multiple bands corroborate the presence of biomolecules on the surface of BBS-NiONPs which act as capping agents and the peak at 616.36 cm^{-1} is linked with Ni-O bond formation. These results show uniformity with previous reports [21–24]. The BBS-NiONPs showed agglomerated shape which represented the electrostatic interaction of the synthesised NPs with each other. It is reported that this happens due to the nano size, high surface tension, surface energy and different reducing phytochemicals in different plant extracts [25,26]. Due to this agglomeration, the dispersity of BBS-NiONPs became low in suspension.

The emerging resistance against antibiotics and evolving of new infectious microbial species are the main challenges for researchers. Therefore, the researchers are trying hard to develop nanomaterials with potent biological potentials against degenerative infectious diseases. The biogenic BBS-NiONPs showed astonishing DPPH radical scavenging and TAC antioxidant activities in a dose-dependent manner. As compared to BBS extract, BBS-NiONPs showed better antioxidant potential at all applied concentrations. Therefore, it is reflected that strong antioxidant activities of BBS-NiONPs are due to the interaction and adsorption of antioxidant compounds from the extract onto the surface of synthesised NPs [27]. Both bacterial isolates (gram-positive and gram-negative) were found susceptible to BBS-NiONPs. It coincides with the fact that NPs have the ability to penetrate inside the bacterial cell and obstruct metabolic activities [28]. BBS-NiONPs also showed antifungal response against tested fungal species. They inhibited mycelial growth in the following manner: *A. alternata* > *A. niger* > *F. oxysporum*. The BBS-NiONPs penetrate and enhance the permeability and generate reactive oxygen species (ROS) inside the fungal cell which retards mycelial growth [29]. The brine shrimp larvae motile movement was restricted by increasing the doses of BBS-NiONPs and ultimately mortality occurred within 24 h. This retardation of larval movement might be due to the attachment of NPs and when the NPs penetrated inside the larval body, they reduced the metabolic activity and, as a result, mortality occurred [30]. The dose-dependent mortality rate investigations have shown that the NPs can be used as anticancer drugs in biomedical fields [22,27,31]. The remarkable antioxidant, antibacterial, antifungal and cytotoxic potentials of BBS-NiONPs might be due to their nano gage dimension, precise surface area and adherence properties.

Due to the nano size, the BBS-NiONPs displayed potent biological activities against various infectious pathogens in the current study. NPs have more attachment and penetration ability with the cell membrane of pathogens as compared to bulk materials [29]. The exact mechanism of inhibitory activity of BBS-NiONPs is not clear. However, recent

studies show that this inhibitory potential of NPs is due to the penetration and interference of NiONPs with intracellular machinery. Briefly, the BBS-NiONPs released nickel ions which attached and penetrated inside the cell and caused leakage of the cell membrane. Inside the cell, the NiONPs generated ROS, which directly inhibited the cellular life machinery like breaking phosphate and hydrogen bonding of the DNA strand, destroying the three-dimensional structure of proteins and causing oxidative stress in the power house of the cell [32].

Recently, NPs have also been used as biofertilisers in the agricultural field for enhancing nutrients uptake, breaking seed dormancy and reducing the application of hazardous agrochemicals [33]. Therefore, green synthesis of NPs might be useful for controlled release of fertiliser, plant growth mediation and green alternative to agrochemicals. Numerous metal NPs such as TiO_2, ZnO and AgNPs are used as biostimulators in the agricultural field [34]. Significant reports are available on inhibitory potentials of NiONPs, while regarding stimulatory activities, little information is available [35,36]. In the present study, BBS-NiONPs increased the seed germination (2–8%) as compared to non-treated control, while at lower concentrations, the BBS-NiONPs showed nontoxic effects on seedling growth. In short, at a lower quantity, these green synthesised BBS-NiONPs are biocompatible and have the ability to speed up seed germination by breaking seed dormancy and can act as a growth-promoting agent. The positive response of seeds toward lower concentrations of BBS-NiONPs might be due to various factors such as infiltration of NPs, releasing ions and making a suitable environment for oxygen and water uptake, hence breaking seed dormancy and promoting seedling growth [33,37]. These findings indicate that apart from their potential in the biomedical field, green synthesised NPs could be effectively used in agricultural fields as nano fertilisers as well as stimulatory agents for plant physiology.

4. Materials and Methods

4.1. Collection of Berberis balochistanica Plant

B. balochistanica was collected during June-August 2019 from mountainous regions of Hanna Urak, Quetta, Baluchistan, Pakistan (30°16′ 28.45″ N, 67°10′ 50.43″ E). The specimen of the collected plant was identified by comparing it with already present herbarium specimens and flora of Pakistan. The voucher specimen (RAW100268) was deposited in the National Herbarium, Islamabad, Pakistan for reference study.

4.2. Preparation of B. balochistanica Stem Extracts

The BBS was washed and shifted to the oven for 10 h at 40 °C. Later, the crushed fine powder of the stem (20.66 g) was thoroughly mixed with 200 mL of distilled water. After stirring for 12 h, the BBS extract was incubated in a water bath at 40 °C for 2 h. The prepared extract was filtered three times using Whatman filter paper and centrifuged at 3000 rpm for 30 m to remove the remaining aggregates. For experimental analysis, the stock solution of BBS was stored at 4 °C. Primarily, we examined the phytochemical and antioxidant potential of BBS extract and then used it as a stabilising and reducing agent in the green synthesis of BBS-NiONPs.

4.3. Phytochemical Analysis of BBS

4.3.1. Berberine Analysis in BBS Extract

The presence of berberine in BBS extract was screened by TLC, 10 µL of Berberine chloride (10 µg/mL) was used as a reference compound. A small drop of BBS extract and the reference drug were drawn using a capillary tube on a preactivated TLC plate (8 × 8 cm). The TLC plate was put into the TLC tank having a mobile phase (methanol:ethyl acetate:acetic acid:water) (5:4:1:1, *v/v*). After separation, the TLC plate was dried and visualised by UV-Vis spectrum at 365 nm.

4.3.2. Total Phenolics and Total Flavonoids Contents Analysis

The TPC was determined in stem extract using Folin-Ciocalteu reagent [38]. In brief, 20 µL of root extract was mixed with 90 µL of Folin-Ciocalteu reagent, and then with 90 µL of $NaCO_3$ solution. After incubation at room temperature for 60 m, absorbance was measured. TPC was expressed as Gallic acid equivalents (mg GAE/g) of the sample. TFC was estimated using the Aluminium Chloride Colorimetric method with some modifications. TFC in stem extract was expressed as Quercetin equivalents (mg of QE/g) of the extract.

4.4. Green Synthesis and Physical Characterisation of BBS-NiONPs

4.4.1. BBS-NiONPs Synthesis

For the green synthesis of BBS-NiONPs, formerly used protocols with slight changes were used [27]. To synthesise BBS-NiONPs, 50 mL purified BBS extract solution was steadily added to the solution of $NiNO_3$ (0.3 M). The mixture was subjected to heating at 60 °C with proper stirring at 500 rpm for 3 h. The precipitated pellet of BBS-NiONPs was collected after centrifugation at 3000 rpm for 25 m and washed with distilled water (three times). The presumptuous pellet of BBS-NiONPs was incubated at 100 °C to entirely evaporate the remaining water. Finally, the synthesised NiONPs were physically and biologically characterised using different techniques.

4.4.2. Characterisation of BBS-NiONPs

The bio-reduction of $NiNO_3$ into NiONPs was confirmed by the colour changing and optical properties of this reduced solution were inveterate via absorption spectra using UV-400 UV-Vis spectrophotometer (Germany) at a scanning range of 200 to 700 nm. FTIR at scanning range 400–4000 cm^{-1} was used to verify the capping and stabilising properties of various functional groups associated with the green synthesis of NPs. Additionally, the structural, elemental, vibrational and morphological nature of BBS-NiONPs were studied using XRD, Raman spectroscopy and SEM (NOVA FEISEM-450 fortified with EDX apparatus). The size of biosynthesised NiONPs was calculated by the Scherrer equation.

$$D = k\,\lambda/\beta\cos\theta$$

4.5. Antioxidant Activity of BBS and BBS-NiONPs

4.5.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay

In this method, the in vitro free radical-scavenging potential of BBS and BBS-NiONPs at different concentrations (50, 100 and 200 µg/mL) was determined using a microplate reader [39]. Reagent solution was prepared by adding 2.4 mg of DPPH to 25 mL of methanol. The procedure involved the addition of 180 µL of reagent solution into 20 µL of the test sample to make 200 µL of the final reaction mixture. The mixture was subjected to a shaker followed by incubation for 1 h. Ascorbic acid was used as a reference antioxidant. DPPH solution without sample was taken as control and methanol was used as a blank solution. Finally, the absorbance of the control and tested samples was measured at 517 nm using a microplate reader to find radical scavenging activity using the following formula below:

$$\text{DPPH scavenging effect \%} = \frac{AC - AN}{AC} \times 100$$

where, AC and AN refer to the absorbance of the control and NPs at 517 nm.

4.5.2. Total Antioxidant Capacity

The TAC of BBS and BBS-NiONPs was evaluated by the phosphomolybdenum method [40]. NiONPs and the reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate) were mixed and incubated at 95 °C for 90 m. The solution was cooled, and the absorbance of the mixture was taken at 695 nm. TAC was calculated as µg/mg equivalent of ascorbic acid (µg AA/mg).

4.6. Anti-Microbial Analysis of BBS-NiONPs

4.6.1. Antibacterial Screening Using Disc Diffusion Method (DDM)

The disc diffusion method was used for antibacterial screening of green synthesised BBS-NiONPs against gram-negative (*P. vulgaris*) and gram-positive strains (*S. aureus*). Bacterial strains used in the present study were obtained from the culture bank (hospital isolates) of the Microbiology Laboratory, Faculty of Biological Science, Quaid-i-Azam University, Islamabad, Pakistan. Both strains were characterised by biochemical and cultural assessment [41]. Firstly, the Muller-Hinton agar was prepared, autoclaved and finally, the cooled media was poured into Petri plates. After solidifying, the bacterial strains were streaked out and a paper disc holding different concentrations of BBS-NiONPs (100, 500, 1000 µg/mL) and 10 µL of Ciprofloxacin (positive control) were put on bacterial cultures. The prepared plates were incubated at 37 °C overnight and bactericidal activity of plant-made BBS-NiONPs was observed for the zone of inhibition (mm) around the coated discs.

4.6.2. Antifungal Assay Using Poisoned Food Method (PFM)

The poisoned food method (PFM) was used to study the antifungal activity of BBS-NiONPs using different phytopathogenic fungal pathogens (*A. niger*, *A. alternata* and *F. oxysporum*). All fungal pathogens were obtained from Plant Pathology Laboratory, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan [29]. These fungal pathogens were cultured using autoclaved Sabouraud dextrose agar media (SDA; Oxoid CMO147). Different doses (100, 500 and 1000 µg/mL) of prepared BBS-NiONPs solution were mixed with SDA media, shaken properly, and poured into Petri plates for solidification. A disc of 7 days old fungal culture (5 mm) was put in the middle of the media plates. The non-treated SDA media and fluconazole-treated SDA media were used as the negative and positive controls, correspondingly. After incubation for 3 days at 27 °C, the mycelial growth against various concentrations of BBS-NiONPs was measured in millimetres and the percentage of inhibition was calculated by the given formula [42].

$$\text{Percentage Inhibition} = \text{FC} - \frac{\text{FN}}{\text{FC}} \times 100$$

where, FC and FN represent the average increase in fungal growth (F) in the control and each treatment (NPs).

4.7. Cytotoxic Assay of BBS-NiONPs by PFM

4.7.1. Brine Shrimp Cytotoxicity Assay (BSCA) of BBS-NiONPs

The cytotoxic effects of BBS-NiONPs were evaluated using the brine shrimp cytotoxicity assay. For this purpose, artificial seawater was prepared by using 3.8 g sea salt in 1 L distilled water in a hatching chamber having a partition. Eggs of brine shrimp (*Artemia salina*) were put in a covered portion of the chamber and incubated for 48 h at 30 °C. After hatching, 20 mature brine shrimp larvae were shifted to individual glass vials having various amounts of BBS-NiONPs (200-1 µg/mL) and the final volume was adjusted up to 5 mL by adding seawater. After 24 h, alive brine shrimps were counted [43]. Lethality concentration (LC_{50} values) and percentage mortality were calculated using GraphPad software.

4.7.2. Phytotoxicity Assay of BBS-NiONPs

The phytotoxic effect of green-made NPs was evaluated using the radish seed assay method (RSA) [44]. Different concentrations (31.25–1000 µg/mL) of BBS-NiONPs solution were introduced in each Petri plate containing sterilised filter paper (Whatman filter paper) and 15 seeds. The obtained data was measured as mean ± standard deviation (n = 3) and different seed germination indices (final germination, percentage inhibition) were calculated [45]. Finally, the seedling length was measured in mm.

4.8. Statistical Analysis

The obtained data were reported in triplicates and quantified as mean ± standard deviation. To check the inhibitory potential of BBS-NiONPs on bacterial, fungal and seedlings growth, a statistical valuation was performed by one-way analysis of variance. Multiple comparisons between means were estimated by LSD test at 95% confidence interval. All statistical analyses were performed using Statistix version 10. The cytotoxic activity of BBS-NiONPs was estimated by calculating the lethality concentration (IC_{50}) values using the probit analysis (GraphPad software, San Diego, CA, USA) [46,47].

5. Conclusions

The present study reports the green synthesis of BBS-NiONPs from the stem extract of the *Berberis balochistanica* plant. The presence of valuable phytochemicals with bioactive functional groups and potent antioxidants in stem extract helped in stabilising, capping and reducing nickel salt into BBS-NiONPs. The crystalline rhombohedral shape and fine size (31.44 nm) of BBS-NiONPs were confirmed by SEM and XRD techniques. Remarkable biological applications of BBS-NiONPs, like antioxidant, antimicrobial and cytotoxic potentials, were observed. BBS-NiONPs were also found as biostimulators in boosting up the germination frequency and seedling growth at suitable quantities. This indicates that they could be used as a substitute for synthetic chemicals in biomedical and agricultural fields. Moreover, they are also suitable for plants with high dormancy and slow seed germination. However, a further widespread investigation is recommended before introducing BBS-NiONPs into clinical and agricultural trials.

Author Contributions: Conceptualization, S.U.; Data curation, S.U. and S.L.; Formal analysis, S.A., M.S.S. and A.R.; Funding acquisition, Y.W. and U.M.Q.; Investigation, S.U., L.B.S., J.I., S.L., M.A. and A.R.; Methodology, S.U., J.I., B.A.A., M.S.S. and M.A.; Project administration, L.B.S., Y.W. and U.M.Q.; Resources, L.B.S., Y.W. and U.M.Q.; Software, S.U. and S.A.; Supervision, U.M.Q.; Validation, L.B.S., S.L., B.A.A. and A.R.; Visualization, S.U. and L.B.S.; Writing—original draft, S.U. and L.B.S.; Writing—review & editing, L.B.S., J.I., A.B., Y.W. and U.M.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data used for the present study has been provided within the manuscript. For further assistance, if required, please contact the corresponding author(s).

Acknowledgments: We thank Amir Sultan (National Herbarium, Islamabad, Pakistan) for helping in the identification of plant specimens.

Conflicts of Interest: The authors declare no conflicts.

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CHAPTER 4

CHAPTER 4. *BERBERIS:* AN ALTERNATE HOST OF RUST

Introduction

The pioneer of green revolution, ‘’Norman Borlaug’’ said that cereal disease like rust never sleeps (Derevnina and Michelmore, 2015). *Puccinia* is a type of pathogenic fungi, which attacks live plants to utilize their nutrients, reproduce and spread to attack the next host. Rust causing fungi are obligate pathogens, required living host for reproduction and development (Habib *et al.*, 2020). Rust diseaeses generate extremely comparable looking signs and symptoms on different host plants. Rust diseases have affinity towards a particular host, while some have ability to host many plants. In some cases different types of rust share common host plant (Lorrain *et al.*, 2019). Rust produces various types of spores at distinct stages of its life cycle with unique appearance. Mostly, rust complete its life cycle using different host plants with different seasonal stages. Living on different host plants is one of the greatest strategies of the rust to generate more spores with new adaptation and virulence. In harsh conditions, rust produces spores like teliospores with ability to survive outside the host plant (Lorrain *et al.*, 2019). There are four stages to complete life cycle: uredinial stage, telial stage, basidial stage and aecial stages. For example, stem rust and stripe rust complete their life cycles using two different hosts: primary host including cereal crops and alternate host such as barberry. The occurrence of both hosts (alternate and primary host) is obligatory to complete life cycle (Roelfs, 1982). Sexual recombination occur on alternate host leading to produce new races, which become responsible for loss of grain yield (Dubin and Brennan, 2009).

In previous decades, the emergence of Ug99 in Uganda, breaks the resistance and created virulence against resistance genes (Pretorius *et al.*, 2000; Jin and Singh, 2006). It is reported that 90% of the world wheat verities were susceptible to this stem rust race (Singh *et al.*, 2011). Ug99 was reported in Africa, Yemen, Egypt and Iran, while in Pakistan, it is not reported yet, but it is predicted that wheat growing countries of Asia are under threat (Singh *et al.*, 2015). The presence of Ug99 in Africa and Iran is due the presence of *B. holstii* in eastern Africa (Singh *et al.*, 2015) and *B.vulgaris* in Iran (Rahimi-Madiseh *et al.*, 2017). The presence of *Berberis* plants indicated that sexual recombination and emergence of new races occur in these regions (Hansen *et al.*, 2013). The emergence of this disease in Iran makes a challenge for wheat breeders and policy makers of

other neighbouring countries, especially Pakistan to replace all susceptible varieties presently growing in their countries.

Globally numerous *Berberis* species have been reported as alternate host of stem rust (Jin, 2011; Zhao *et al.*, 2013). In Europe, sexual reproduction of stem rust on barberry was confirmed in Sweden (Berlin, 2017). Similarly in UK, after 60 years wheat stem rust and alternate host were reported (Lewis *et al.*, 2018). Waipara *et al.* (2005) first time observed *P. graminus* on flower of *B. glaucocarpa* in New Zealand. Eradication program against common Barberry or *B. vulgaris* was started by US government from 1918-1974 to control the stem rust pathogen and more than 500 million plants of common barberry were eradicated (Peterson Jr, 2003; Peterson, 2013). In Pakistan, 29 *Berberis* species have been reported so far (Khan *et al.*, 2014; Khan *et al.*, 2015). Some species of *Berberis* were reported as susceptible to stripe rust (Mehmood *et al.*, 2019), but no information about stem rust is available in literature yet. Alternate host along with primary host generated more diverse races of rust as compared to primary host alone (Roelfs, 1982). In 1959, more than 70 species of *Berberis* and some of Mahonia ware reported as alternate host by Gäumann (1959).

Stripe rust or yellow rust is also globally important foliar and multiple-cycle disease of wheat crop caused by *Puccinia striformis* f.sp.*tritci* (pst) and have ability to cause yield losses in wheat growing areas under suitable environments (Coakley, 1979; Rehman *et al.*, 2018). At global level, about 5.5 million tons yield losses per year have been reported (Beddow *et al.*, 2015; Khanfri *et al.*, 2018). Pakistan also faced 13 stripe rust epidemics during different times with great yield losses (Rehman *et al.*, 2018). New information has been collected about stripe rust diversity at large global scale and is considered that Himalayan regions are the source and origin of diversity of stripe rust (Ali *et al.*, 2014; Ali *et al.*, 2014; Thach *et al.*, 2016; Walter *et al.*, 2016; Mehmood *et al.*, 2019). Many researchers attempted to find the alternate host of stripe rust from 1890 to 1930 (Eriksson and Henning, 1894; Mains, 1933; Tranzschel, 1934; Straib, 1935; Rapilly, 1979). Now it is understandable that new virulent races of stripe rust arises due to sexual reproduction on susceptible barberry plants (Jin *et al.*, 2010). In Pakistan, especially Himalayan regions are considered as the centre of yellow rust pathogen with rich genetic diversity. Recently, Mehmood *et al.* (2019) proved that *Berberis* species and sub species of Himalayan region of Pakistan were susceptible to stripe rust under control conditions. In control conditions, many researchers confirmed that *Berberis* species serve as alternate host for stripe rust (Wang *et al.*, 2002; Jin and

Singh, 2006; Jin *et al.*, 2010; Cheng *et al.*, 2014; Rodriguez-Algaba *et al.*, 2014; Wang *et al.*, 2015; Tian *et al.*, 2016; Wang *et al.*, 2016; Mehmood *et al.*, 2019; Siyoum *et al.*, 2019; Zhuang *et al.*, 2019). However, naturally the alternate host of yellow rust is still not known, but the presence of rich genetic diversity of stripe rust in Asia like China and Pakistan, showed that natural genetic recombination is exist (Duan *et al.*, 2010; Ali *et al.*, 2014).

Chapter 1, **2** and **3** disclosed the nature of Berberis plant with rich pool of minerals and phytochemicals which display a potent role in vivo and in vitro biological applications and green synthesis of NPs. Besides these valuable aspects. Present study (**Chapter 4**) give outline about undesirable characters of Berberis plant,which will open a new area for understanding and developing new strategies to evaluate Berberis plant for their pros and cons.

ARTICLE 5: INSIGHTS INTO THE VIRULENCE VARIATION OF *BERBERIS* SPECIES FROM PAKISTAN – ALTERNATE HOSTS TO WHEAT RUST PATHOGEN

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Abstract

Berberis species serve as an alternate host of the aecial phase of many rust species including wheat stem rust (WSR), wheat yellow rust (WYR) and oat stem rust (OSR). Barberry, as an alternate host, has recently gained attention due to the emergence of new races after genetic recombination during the aecial stage on barberry. Different *Berberis* species also serve as a seasonal bridge for stem and stripe rust pathogen in Pakistan and neighboring countries. The aim of this study was to identify the role of *Berberis* species and to examine the genetic diversity of rust on *Berberis spp*. at species and formae speciales levels collected from different areas of Pakistan using molecular and morphological techniques. Initially, PCR based approach was applied using simple sequence repeat (SSR) markers to investigate the presence of WSR, WYR, and OSR in 95 aecial samples grown under natural conditions. Based on aecial growth and spore morphology, rust was divided into two groups *i.e.,* localized (*Puccina graminis*) and systematic (*Puccina arrhenatheri*). For molecular study, DNA was extracted from infected leaf aecial lesion using different methods (CTAB, SDS and Kit) to avoid degradation. Kit extraction methods provide more satisfactory to extract DNA than CTAB methods. Positive control (DNA) of WSR, WYR and OSR were first screened using SSR markers and then Kits extracted DNA were successfully amplified by species and formae speciales specific SSR markers. In total, 25 of 46 SSR markers were found to be useful for the screening of selected rusts collected from barberry. SSR analysis revealed three *Berberis* species namely *B. balochistanica*, *B. pachyacantha* and *B. lycium* as alternate hosts of WSR, while *B. lycium* was also identified as an alternate host of OSR. However, no barberry was recognized as an alternate host of WYR in natural conditions. This study also showed the specificity of SSR markers at species and

formae speciales level. In conclusion, this study confirms that barberry serves as an alternate host for only stem rust in Pakistan.

Keywords: *Berberis*, Pakistan, Wheat stem rust, Wheat strip rust, Oat stem rust, SSR markers, Aecium, Aeciospores, Kit, DNA.

1. Introduction

The global food demand is increasing dramatically due to growing population and habitat destructions (Gupta *et al.*, 2008; Wernicke, 2016). Agricultural crops are the prime source of food. Wheat is one of the major staple foods grown globally on one sixth area of the land (89 countries) and used by one third of the population as a source of food and income (Alexandratos and Bruinsma, 2012; Prosekov and Ivanova, 2018). Furthermore, wheat provides one fifth of the global population with proteins and calories (Shiferaw *et al.*, 2013). In Pakistan, wheat is grown on around 40 % of the land (9.199 million hectares) with 2.657 tones/ha average yield production (Ali *et al.*, 2017). Wheat productivity faces serious challenges like climate change, increasing population and arising of the new strain of pathogens and pests (Rasheed *et al.*, 2020). Every year, considerable yield loss occur due to biotic stresses, particularly rust pathogens that hampers the grain yield during epidemic or in rust susceptible varieties in wheat growing areas (Johnston and Miller, 1934; Bashir, 2019).

Rust, belong to subphylum *Puccinia mycotina*, order *Puccinales*, class Pucciniomycetes, and division Basidiomycota, causes severe yield losses in cereal crops (Kirk *et al.*, 2008). It is reported that more than 8,000 species of rust pathogens attack on wheat, triticale, oat, rye, barley and other grasses (Brown and Hovmøller, 2002; Aime *et al.*, 2017). The most commonly reported pathogens across the globe are stem, stripe, crown and leaf rust (on wheat, rye and barley) caused by *P. graminis* f. sp. *tritci* (Leonard and Szabo, 2005; Singh *et al.*, 2008; Berlin *et al.*, 2013), *P. striformis* f. sp. *tritci* (pst) (Liu and Hambleton, 2010), *P. coronate, P. triticinie, P. recondite*, and *P. hordei*, respectively (Savile, 1984; Goyeau *et al.*, 2006; Singh *et al.*, 2006; Bolton *et al.*, 2008; Kirk *et al.*, 2008). These pathogens possess the ability to break the resistance of crop cultivars by producing enough spores which are easily disseminated by wind and have the potential to produce new races by sexual recombination, mutations and somatic recombination (Marsalis and Goldberg, 2016; Bhardwaj *et al.*, 2019).

The most effective genes in wheat plant against stem, stripe and leaf rust include *Sr31*, *Yr9* and *Lr26* (Bhardwaj *et al.*, 2019). Recently, epidemic caused by these diseases in wheat seeded regions ranged from 20 to 100 % yield loss globally (Huerta-Espino *et al.*, 2011; Singh *et al.*, 2015). The different growing periods of wheat provide opportunity for pathogen to

complete its life cycle, and as a result become epidemic and virulence against resistance genes in wheat (Zeng and Luo, 2008; Zeng *et al.*, 2014; Nsabiyera *et al.*, 2018; Sajjad *et al.*, 2018). The phyllo sphere of cereal crops and Barberry serve as habitat for biotrophic pathogens like rust. Rust pathogens of cereal and grasses are mostly heteroecious and macrocyclic consisting of five spore stages *i.e.,* uredinial, telial, basidial, pycnial and aecial stage. These rusts use wheat or other grasses for clonal reproduction while alternate host for sexual reproduction (Roelfs and Bushnell, 1985). Until now alternate host has gained less attention, even though it offers platform for rust pathogen to survive harsh winter season, provides inoculum for diseases development, makes new races and leads to diverse rust populations through sexual reproduction (Zhao *et al.*, 2016).

Berberis is one of the most prominent genera having 650 species with distinguished features like evergreen dicot shrubs, distinct yellow flowers with three bracts and spine and yellow inner bark (Ahrendt, 1961; Landrum, 1999; Tiwari *et al.*, 2012; Khan *et al.*, 2014). This genus is considered as the most aboriginal angiosperm (Brückner, 2000) and is known for the herbal usage in Pharmacognosy (Kulkarni and Dhir, 2010). Out of the 49 species grown in Pakistan, 25 have been identified taxonomically (Khan *et al.*, 2015). Although they provide a great medicinal potential, *Berberis* spp. also serve as alternate hosts to stem rust, stripe rust and other rusts species (Jin *et al.*, 2010). Alternate hosts for stem and stripe rust are *Berberis* and *Mahonia* species, while for leaf and crown rust, these are *Thalictrum* spp. and *Rhamnus spp*., respectively (Jackson and Mains, 1921; Large, 1940; Gäumann, 1959; Roelfs and Bushnell, 1985; Jin *et al.*, 2010). However, the importance of *Berberis* spp. as alternate hosts of *P. striiformis* f. sp. *tritici* in natural conditions grown in different areas of Pakistan is relatively unknown (Hovmøller *et al.*, 2011).

Conversely, multiple races of stripe rust have been reported from barberry leaves in China under naturel conditions (Zhao *et al.*, 2013; Wang *et al.*, 2015). Studies from USA and some countries of Europe like Sweden have reported no associations between stripe rust and barberry under natural environment (Berlin *et al.*, 2013; Wang *et al.*, 2015; Zhao *et al.*, 2015; Lewis *et al.*, 2018). More than 45 species have been reported artificially as alternate hosts of stripe rust (Jin *et al.*, 2010; Zhao *et al.*, 2016; Zhao *et al.*, 2016; Mehmood *et al.*, 2019; LI *et al.*, 2021). In Pakistan, 7 species of *Berberis* have been reported as alternate hosts of stripe rust under controlled conditions (Mehmood *et al.*, 2019), however; under natural environment no study is available regarding strip rust on *Berberis* plant. *Berberis* species also serve as

alternate hosts for other *Puccinia* species like *P. pygmaea, P. montanensis* and *P. brachypodii* (Cummins and Greene, 1966).

The easiest and most effective methods to control such diseases include conventional plant breeding and selection of resistance genes through breeding strategies, but these techniques cause negative effects to the environment and are time consuming processes (Kerber, 1987; Sharma, 2003). In last two decades, novel genetic tools have been introduced to overcome the above problems and to produce the resistant and fruitful yield producing cultivars (Sharma, 2003; Landjeva *et al.*, 2007). Molecular genetic studies have indicated that the first step is the extraction of significant amount of genomic DNA from a limited amount of spores or infected tissues especially extracted from aeciospores or aecial clusters of rust pathogens (Drábková, 2014). A major challenge in doing that is that the cell wall of fungal tissue comprises of chitin, which is not easily broken down. The extraction of decent quality of DNA depends on the extraction methods. The DNA extraction from herbarium specimens and limited quantity of fungal tissues or spores (aecium or aeciospore) is challenging due to the fragmented or degraded DNA. Therefore, researchers have adopted different approaches to extract DNA from fungal infected tissue or spores (urediniospores and aeciospores). Researchers (Anikster, 1984; Liu and Kolmer, 1998; Justesen *et al.*, 2002; Barnes and Szabo, 2007) have used CTAB methods for DNA extraction. Various Kits have also been used by different researchers (Ali *et al.*, 2011; Demers *et al.*, 2017; Berlin *et al.*, 2018; Bergeron *et al.*, 2019; Rodriguez-Algaba *et al.*, 2021) to extract DNA from fungi especially rust pathogens. DNA extraction is critical for marker-based screening and identification of pathogenic strains which can help to identify resistant varieties.

Many types of molecular markers have been used by researchers to identify the fungal diversity. Commonly used markers for the characterization of resistance genes in crop plants have been identified (Rodriguez-Algaba *et al.*, 2014; Amom and Nongdam, 2017). Among these, ITS and SSR markers are mostly used for analysis of inter and intra specific species identification of phytopathogenic fungi (Henrion *et al.*, 1994; Pritsch *et al.*, 1997). For genetic diversity of rust fungi, a best set of markers are required like microsatellites markers. SSR markers have previously been used for the population study of rust pathogens (Barnes and Szabo, 2007; Jin *et al.*, 2009; Zhong *et al.*, 2009; Admassu *et al.*, 2010; Berlin *et al.*, 2012; Berlin *et al.*, 2013; Berlin *et al.*, 2013; Berlin *et al.*, 2017). The advantages of these markers are their co-dominant, high polymorphic and short size microsatellite motif with repeated

nucleotide sequences (Enjalbert *et al.*, 2002; Thiel *et al.*, 2003; Varshney *et al.*, 2005; Guo *et al.*, 2007; Giraud *et al.*, 2008).

The aim of this study was to identify the genetic diversity of *Berberis* species collected from different areas of Pakistan using molecular and morphological approaches and to evaluate their role in rust disease prevalence. For this purpose, PCR based approach was applied using simple sequence repeat (SSR) markers to investigate the presence of wheat stem rust, oat stem and wheat stripe rust under natural conditions.

2. Materials and Methods

2.1. Collection of Aecial samples

Rust samples from barberry plants were collected from Baluchistan, Khyber Pakhtunkhwa (KPK), and Punjab provinces during April to July in 2017, 2018 and 2019, and from Kashmir and Gilgit-Baltistan in Jun 2017. Five to ten sites were surveyed in each selected area and several collections were made from more than one bushes from the same site. The collected samples were placed in paper bags and kept in sealed plastic bags containing desiccant at 4 °C for later use. All samples collected from the same area were treated as one population. After sampling, the specimens were identified and deposited in the National Herbarium, Islamabad, Pakistan. The collected samples were stored in 70 % (v/v) ethanol according to Australian biosecurity law or quarantine department of Australia and were dispatched to the Plant Breeding Institute, University of Sydney for molecular fingerprinting.

2.2. Morphological analysis of infected leaf of Barberry plants

Collection of rust affected plants was carried out and then infected plants were preserved and submitted to the herbarium of Plant Sciences Department, Quaid-i-Azam University, Islamabad (Pakistan). The preserved rust spores were mounted in glycerin jelly and fixed as semipermanent slides. The spores were measured and photographed under a Leitz HM-LU compound light microscope. At least 30 spores were measured for each spore stage, including the smallest and the largest spores found.

2.3. DNA extraction by using CTAB and Kit-OmniPrep kit (GenoTech)

As rust pathogens are obligate parasites, as required living host therefore enough quantity of spores or lesions are difficult to obtain especially from alternate host in fact every aecium has a different genetic makeup. In this study, different DNA extraction methods were screened to obtain good quality and quantity of DNA from infected barberry leaves. Additionally, DNA was also extracted from the urediniospores of wheat stem rust as control. Leaf portion having visible aecial cups or spores were collected in plastic tube along with two steel beads (1.5 ml)

and were ground twice using tissue lyser II (QIAGEN) for 3 minutes at 25 rpm with alternate cold/heat shock (-20 /95 °C). The grinding method was same for all the extraction methods used in this study. DNA was extracted from using various approaches like CTAB base method and commercial kit for fungal DNA extraction with some modification.

DNA was extracted following the CTAB extraction methods of Liu and Kolmer (1998), Thach et al. (2016), Karaoglu et al. (2013) and Kankwatsa *et al.* (2018). Reagents required for all mentioned methods are shown in supplementary table 1. Briefly, extraction buffer (CTAB) of above-mentioned protocol was added to tubes containing crushed samples, followed by incubation, phase separation (phenol: chloroform: isoamyl alcohol), precipitation of DNA pellet with cold 100% (v/v) isopropanol with incubation at -20 °C for 30 minutes and finally the pellet was re-dissolved in tris-EDTA (TE) buffer.

Similarly, DNA was also extracted using OmniPrep kit (GenoTech), following the instructions provided by manufacturers with some modifications. During extraction, the velum of extraction buffers was adjusted according to the size of infected lesion on barberry leaf or quantity of spores used as the reference control. Kit methods consisted of lysis buffer for tissue lysis, chloroform for separation of phases, stripping solution for releasing of DNA, precipitation solution for precipitation of protein, cold 100% (v/v) isopropanol with incubation at -20 °C for 30 minutes for the precipitation of DNA and finally addition of TE buffer (Tris-EDTA) for re-dissolving of DNA. After extraction of DNA by CTAB and kit methods, the DNA were checked using gel (1 %) and were quantified using Spectrophotometer (ND-1000 V3.3.0).

2.4. PCR Analysis of Extracted DNA

The extracted DNA consisted of both host and rust pathogen DNA; therefore, the stock solution was diluted to 40 ng/µl and 10 ng/µl, while the control DNA was diluted up to 10 ng/µl. PCR reaction was run by using 2 µl DNA from both dilution (40 ng/µl and 10 ng/µl) per 10 µl reaction volume. DNA with 40 ng/µl was found with good amplification as compared to 10 ng/ µl dilution. Host amplification was evaluated using SSR marker as listed in Table 2. PCR was performed using 2 µl of genomic DNA (40 ng/µl) of infected leaf, 2 µl of 2x PCR buffer (NH_4 Reaction buffer, Bioline), 0.25 µl of each primer, 0.1 µl *Taq* DNA (Immolase DNA polymerase from Bioline) and 5.4 µl ddH_2O. The PCR reaction was carried out in a 96 well DNA thermocycler (Eppendorf Master cycler, Germany) using the standard PCR amplification profile: an initial denaturation for 10 minutes at 95 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 59 °C, 51 °C and 54 °C for WSR, WYR and OSR SSR primers

respectively, extension for 30 s at 72 °C and final extension for 7 min at 72 °C. The PCR product was separated using 3 % (w/v) agarose gel (Agarose I™, Amresco®, United States) at 100V for 1-1.5 h, using 5 µl of PCR product with 2 µl of loading buffer (Bioline). The DNA marker, Hyper ladder IV 100 lanes (Lot No. H4-106F Invitrogen, Australia) were used as standard reference and PCR products were visualized under UV light *via* Gel Doc™ XR+system (Bio-rad, Australia Pty. Ltd. Gladesville NSW). The alleles for each locus were counted manually using DNA marker Hyper ladder IV 100 lanes (Lot No. H4-106F Invitrogen, Australia) as standard reference.

2.5. SSR analysis and cross-amplification

In total, 46 SSR markers: 20 for wheat stem rust (WSR) (Kankwatsa *et al.*, 2018), 16 for wheat stripe/yellow rust (WYR) (2013) and 10 for oat stem rust (OSR) (2018) were tested using *P. graminis* f. sp. *tritici*, *P. graminis* f. sp. avenae and *P striformis* f. sp. *tritici* as control. Some of the SSR markers which gave clear amplicons on control were further used to amplify the DNA extracted using CTAB and kit methods from infected leaves of *Berberis* spp. The Kit protocols (Omni Prep kit) showing clear amplicons were further used for DNA extraction from collected samples. SSR markers which amplified the DNA samples of infected *Berberis* leaves were also evaluated on *P. graminis* f. sp. *tritici, P. graminis* f. sp. *avenae, P. striformis* f. sp. *tritici* and *P. graminis* f. sp. *secalis* to investigate the cross transferability**.**

3. Result

3.1. Berberis plant identification as an alternate host of rust

Barberry samples collected from same area were treated as one population. Among collection sites, Murree was found as a rich zone of infection, followed by Mansehra, Narran and Balochistan. In Kashmir and Gilgit-Baltistan, the ratio of infection or disease development was very low, and the size of lesion or aecia was small which could probably be due to the low temperature during the time of collection (Jun 2017). In total, 13 species were collected and 8 were identified as shown in Table 1, but only 5 *Berberis* species were exhibiting infected leaf (aecial). Out of 5 species, only three species including *B. lycium, B. pachyncanta* and *B. balochistanica* displayed PCR amplification using SSR markers.

Table 1. List of selected *Berberis* species along with their accession numbers and areas of collection.

Sr. no	Species	Accession Numbers	Locations	
			Province	**District**
1.	*Berberis balochistanica*	100267	Balochistan	Quetta
2.	*Berberis calliobotrys*	100266	Balochistan	Ziarat
3.	*Berberis pachyacantha*	100433	Khyber Pakhtunkhwa	Mansehra
4.	*Berberis parkeriana*	131411	Khyber Pakhtunkhwa	Swat
5.	*Berberis lyceum*	100269	Punjab	Murree
6.	*Berberis pseudumbellata* subsp. *Gilgitica*	131412	Gilgit-Baltistan	Hunza
7.	*Berberis orthobotrys*	100432	Gilgit-Baltistan	Astore
8.	*Berberis chitrria*	131414	Azad Kashmir	Poonch

3.2. Morphology of Aecium and Aeciospores on infected leaf

During field collection, three types of aecia were observed on the collected *Berberis* leaves. In most of the samples (Murree, Kashmir, Mansehra and Narran), aecia were cup shaped and gave yellowish orange expressions forming single or multiple confined spots. While the aecia observed on the samples collected from *B. balochistanica* were powdery with yellow to brown appearance covering the whole surface of infected leaf. The former one produced a cup like restricted spot (spots), while the later ones showed systematic appearance which also infected the whole branch of young *Berberis* leaf causing curled and stunted growth (Figure 1). In both types, the cup shape showed some differences like the confined aecia cups were embedded deeply in the infected leaf tissues at short distance from one another. On the contrary, the witches' boom like aecial on *B. balochistanica* leaf showed systematic appearance, infecting the whole leaf along with the young branches. These systematic appearances showed resemblance with the symptoms of *P. arrhenatheri*, with wider aecia on the surface of infected leaf. In the present study, small differences were observed in all analyzed aeciospores of rust on *Berberis.* Figure 2 represents the spore image of three taxa. The spore morphology of *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *Avenae* were almost the same

i.e., obvoid, while spores of *P. arrhenatheri* displayed oblong shape morphology. The spore length of *P. arrhenatheri* was also slightly larger than the other two taxa.

Figure 1. Localized aecia systematic lesions on infected leaf of *B. lycium* (A), *B. pachancantha* (B) and systematic lesions on infected leaf of *B. balochistanica* (C).

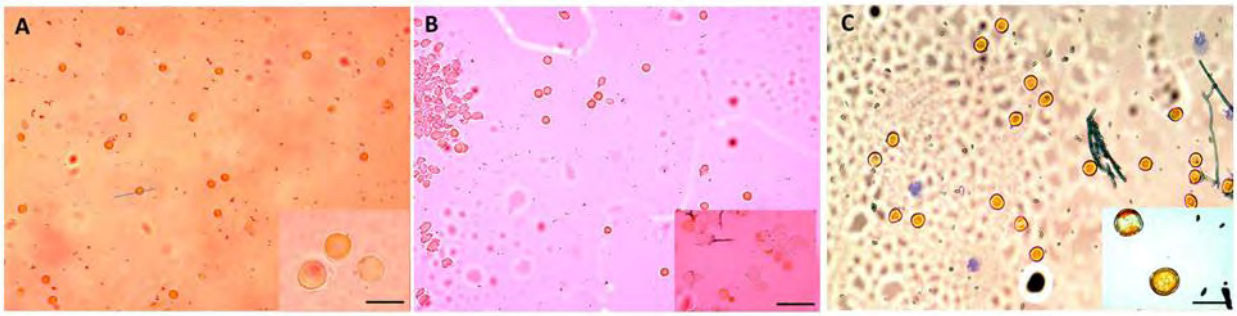

Figure 2. Aeciospores morphology of *P. graminis* f. sp. *tritici* (A)*, P. graminis* f. sp. (B) and *P. arrhenatheri* (C). Bar scale = 25 µm.

3.3. DNA extraction from *Berberis* leaf containing aecium

All the CTAB methods used in this study showed unsatisfactory results for DNA extraction from the infected leaves. All the samples revealed lower A260/280 ratios than the ideal range (1.8-2.0) with various CTAB methods, and the graphs were found in zigzag manner indicating degradation of DNA (Table 2). Additionally, none of the extracted samples were visible on gel, indicating that the quality of the extracted DNA was inadequate. Furthermore, none of the samples were amplified using SSR markers during PCR analysis (data not shown). Contrary to CTAB method, commercial Kit extraction showed satisfactory result as majority of the samples showed comparable A260/280 ratios within ideal range (1.8-2.0) and the graphs were perceived in smooth manner indicating negligible degradation of DNA (Table 2). It was also noted that the extracted product of both methods did not show any bands on gel electrophoresis as shown in figure 3.

Figure 3. DNA extraction from infected leaf (aecium) and control (urediniospores) using kit method. Where L and C represent the DNA ladder and Positive control (urediniospores), while 1, 2 and 3 refer to infected sample.

Table 2. Quantitative and qualitative analysis of DNA extracted from infected leaf (aecium) and control (urediniospores) using CTAB and kit methods.

Method used	**Sample Types**	**Concentration (ng/ul)**	**260/280**	**Band on GEL**
CTAB	Control (Pg)	1184.87	1.95	Yes
CTAB	Infected leaf	256.70	0.83	No
Kit	Infected leaf	162.30	1.93	No
Kit	Control	2868.26	2.11	Yes

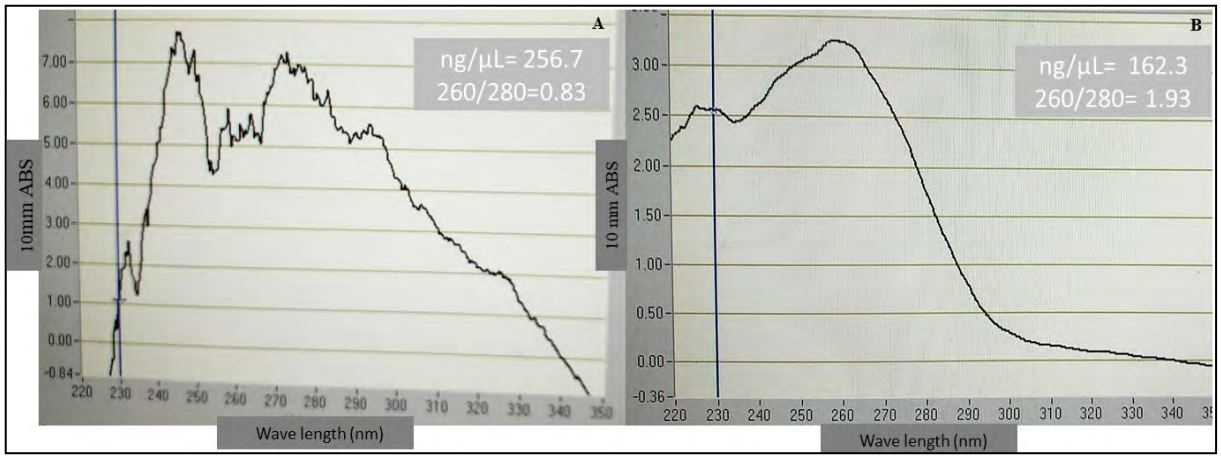

Figure 4. Graphical representation (Nano drops) of extracted DNA from infected barberry leaf using CTAB (A) and Kit (B) method.

3.4. Transferability of the SSR Markers

In total, 46 SSR markers: 20 for WSR (Kankwatsa *et al.*, 2018), 16 for WYR (2013) and 10 for OSR (2018) were tested using *P. graminis* f. sp. *tritici*, *P. graminis* f. sp. *avenae* and *P. striformis* f. sp. *tritici* as control. The markers which did not amplify the above-mentioned controls were not selected for further study. Out of the 46 SSR markers, only 25 (54.35 %) displayed the amplification. These 25 selected SSR markers were checked against seven isolates representing the *P. graminis* f. sp. *tritici*, *P. graminis* f. sp. *avenae*, *P. graminis* f. sp. *secalis* and *P. striformis* f. sp. *tritici* to investigate the cross transferability. Out of the 25, only 6 stem rust SSR markers showed correct amplification in all analyzed isolates. However, most of the WSR and OSR markers cross amplified the close related formae speciales like *P. graminis* f. sp. *tritici*, *P. graminis* f. sp. *avenae*. Some of these were revealed as very informative and showed species specific and formae speciales specific amplification including WSR (F2-21) and OSR (F2-03 and F3-09).

After cross amplification screening, only specific amplified primers were evaluated on 95 samples of rust collected from three different alternate hosts (supplementary table 2). Out of the 95 samples, only 50 samples were amplified by 10 WSR markers, 4 samples by two 2 OSR SSR markers, while no amplification was noted in case of WYR SSR markers. Out of the 12 SSR markers, 8 showed polymorphic (2-5 alleles) amplification and amplified 30 alleles (28 by WSR and 2 by OSR) for each marker used in the current study. Most of the samples amplified by WSR and OSR markers were collected from *B. lycium* located in Murree (Punjab). Only few samples of *B. balochistanica* (5) and *B. pachyncanta* (3) were amplified by WSR SSR markers only. Among the WSR SSR markers, the F2-21 SSR marker amplified 30.77 % of the samples followed by F9-41 (17.31%), F10-40 (13.46 %), F3-19 (11.54%), while F10-

21, F9-18, F7-29, F9-45 amplified 5.77 % samples (Table 3). Similarly, OSR SSR markers (F2-03, F3-09) amplified 5.77 and 3.85 % of analysed samples. In case of WSR SSR markers, the highest number of alleles was amplified by F2-21 with 0.17 allelic frequency, while the lowest number of alleles ware amplified by F9-44 and F8-15 with 0.03 allelic frequency. Additionally, OSR markers amplified only one allele with 0.03 allelic frequency as shown in Table 3. Contrary to stem rust SSR markers, yellow rust SSR markers indicated no amplification. Conclusively, the *Berberis* species collected from the three provinces of Pakistan are found as the alternate host of stem rust only. Furthermore, OSR were also reported in Punjab province on *B. lycium,* while SYR SSR markers did not amplify any sample collected from all three regions. This study clearly indicates that the studied plants serve as alternate hosts of stem rust and their formae speciales, but not for stripe rust.

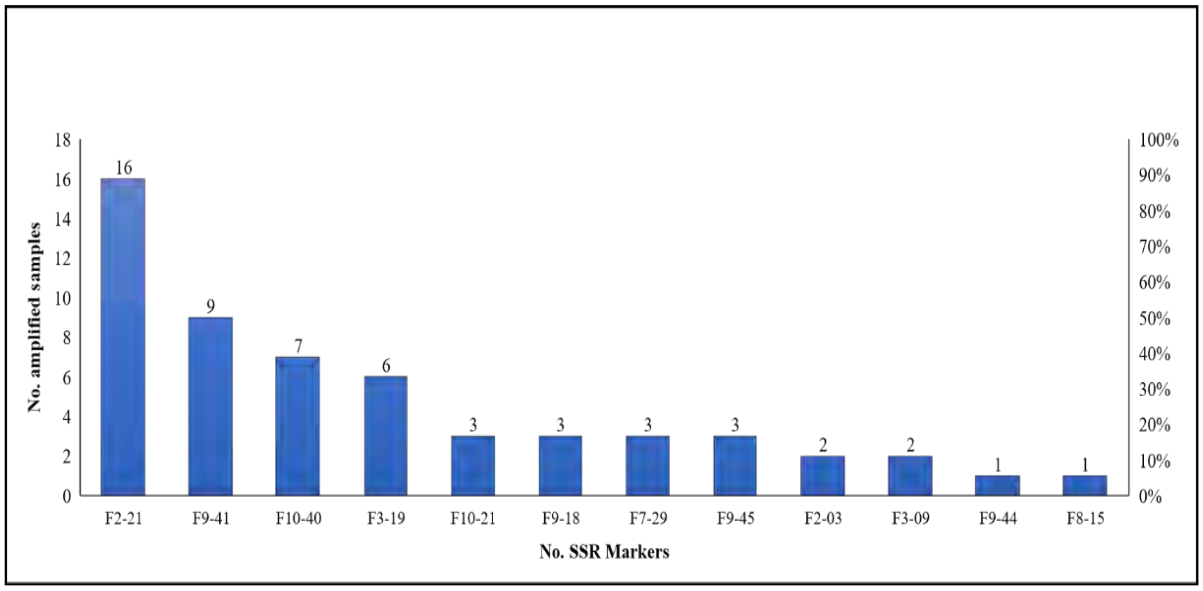

Figure 5. Number of samples amplified by SSR Markers (WSR and OSR).

Table 2. Polymorphic Nature of 12 SSR markers used to amplifies 95 Barberry samples collected from different provinces of Pakistan.

SSR Markers	No of alleles	Allele size (bp)	Allele frequency	No of samples amplified (%)	References
F2-21	5	300-400	0.17	30.77	(Kankwatsa *et al.*, 2018)
F3-19	3	350-450	0.10	11.54	(Kankwatsa *et al.*, 2018)
F9-41	3	120-190	0.10	17.31	(Kankwatsa *et al.*, 2018)
F9-44	1	330-350	0.03	1.92	(Kankwatsa *et al.*, 2018)
F8-15	1	300-330	0.03	1.92	(Kankwatsa *et al.*, 2018)
F10-21	3	290-320	0.10	5.77	(Kankwatsa *et al.*, 2018)
F9-18	3	300-390	0.10	5.77	(Kankwatsa *et al.*, 2018)
F7-29	3	390-450	0.10	5.77	(Kankwatsa *et al.*, 2018)
F10-40	4	300-400	0.13	13.46	(Kankwatsa *et al.*, 2018)
F9-45	2	270-290	0.07	5.77	(Kankwatsa *et al.*, 2018)
F2-03	1	350-300	0.03	3.85	(Gnocato *et al.*, 2018)
F3-09	1	170-190	0.03	3.85	(Gnocato *et al.*, 2018)

4. Discussion

4.1. Macroscopic and microscopic analysis of aecium and aeciospores

In Pakistan, 29 *Berberis* species have been reported in the flora of Pakistan (Khan *et al.*, 2015). In the present study, 13 species were collected and 8 of them were identified. Five of these were found with infected lesions (aecium) and the morphological analyses showed three types of rust (*P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *avenae* and *P. arrhenather*) on these *Berberis* species. Visual observation of the rust organs on different *Berberis* plants were observed. The symptoms vary from each other; some were localized at least indicating one infected spot and some showed at least 3-8 spots located at some distance from one another, while some were found with systematic manner covering the whole leaf and sometimes also invading veins and young stem. The localized symptoms were reported in northern area of Pakistan like Murree (*B. lycium*), Narran (*B. pachyncanta*), Kashmir (*B. chitrria*) and Sawat (*B. parkeriana*), while the systematic symptoms were observed on *B. balochistanica* located in Balochistan province (Quetta and Ziarat district). Similar symptoms were observed by (Berlin *et al.*, 2013) studying *Berberis* spp. in Sweden. According to previous literature, the single or multiple localized spots represented *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *avenae* aecial morphology, while the systematize symptoms in the form of witches' broom represented the aecial form of *P. arrhenatheri* (Berlin *et al.*, 2013). In this study, *P. graminis* f. sp. *tritici*

and *P. graminis* f. sp. *avenae* revealed insignificant differences. Moreover, it was also observed that the size of aecium on leaves varies which might be due to the existence of different stages of the rust disease (pu *et al 2020*). Additionally, obvoid spore morphology was displayed by *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *Avena,* while oblong shape morphology was represented by *P. arrhenatheri*. Obvoid shape indicated the presence of *P. graminis L,* while oblong shape aeciospore represented *P. arrhenatheri* (Sato and Sato, 1985; Berlin *et al.*, 2013). The spore length of *P. arrhenatheri* was slightly larger than the other two taxa. Parallel spore morphology has been reported in previous studies in China and Sweden (berlin at al 2013). Therefore, the identification of rust diversity using morphology will lead to erroneous result. To unravel this problem, it is necessary to conduct molecular study along with morphological studies. Berlin *et al.* (2013) also successfully differentiated various rust species (with same morphology) using molecular markers.

4.2. DNA extraction from infected *Berberis* leaf lesion

During this work, numerous modifications were made in the DNA extraction methodology to acquire a good quality and quantity of DNA for research progress. The problem with extraction from fungal samples was a limited amount of spores or infected tissues especially extracting from aeciospores or aecial clusters of rust pathogens (Drábková, 2014). Similarly, the cell wall of fungal tissue or spore was chiefly comprised of chitin or other biochemical substances, which is not easily crushed. The other critical point was the grinding of materials like using pestle mortar and liquid nitrogen leads to losses and contaminations of the ground materials (Drábková, 2014). Extracting DNA from fungi and their host (plant) is challenging and requires suitable approaches. Numerous studies showed that *Berberis* plants have many bio compounds particularly polysaccharides, alkaloids, phenols, protein and other secondary metabolites (Končić *et al.*, 2010; Gundogdu, 2013). These bioactive compounds not only reduce the DNA yields but also degrade the DNA. It is reported that isolation of a good quality and quantity of DNA from plants with extraordinary secondary metabolites can be challenging (Li *et al.*, 2001). Conventional DNA extraction methods are usually difficult to extract rust genomic DNA from infected leaf of *Berberis* plant. In the present study, many modifications were made to standardize the protocol. Firstly, the infected portion of leaf lesion was cut and ground using mixer mill along with cold/heat shock with alternate intervals. The kit method was then used to extract a reliable amount of DNA as compared to CTAB and SDS methods. The CTAB and SDS methods displayed degraded DNA on Nano drop analysis, while kit method produced a smooth graph with best 230/280 value. The smooth graph and 230/280

ratio reflected the efficacy of the modified kit protocol to produce enough DNA suitable for PCR based molecular studies (Devi *et al.*, 2013; Aboul-Maaty and Oraby, 2019)**.**

After this confirmation, only kits isolated DNA were successfully amplified by SSR markers. These clearly showed that kit-based extraction is suitable method to extract DNA from fungal tissue like aecium of rust. Various Kits have been employed by different researchers to extract DNA from fungi especially rust pathogen (Berlin *et al.*, 2017; Bergeron *et al.*, 2019). In short, the grinding along with cold/heat shock method easily break the fungal cell wall or spore and provide a contamination free DNA as compared to liquid nitrogen-pestle and mortar methods. Additionally, kits provide a pure DNA with high yield as compared to CTAB and SDS methods.

4.3. Marker based analyses suggest *Berberis* species in Pakistan to be the alternate host of stem rust, and not the stripe rust

SSR markers with high specificity were used to amplify the extracted DNA from rust on barberry plant. In total, 52.63 % samples were amplified by SSR markers related to stem rust, while no amplification was obtained by strip rust SSR markers. In the present study, majority of the samples were amplified by WSR SSR marker, followed by OSR SSR markers. Geographically, the WSR SSR marker amplified most of the samples that were collected from Murree region, while only 5 samples from Balochistan and 3 samples from KPK were amplified. Likewise, the OSR SSR marker just showed PCR product on samples collected from Murree. This clearly showed that, under controlled conditions, the aecia collected from *Berberis* spp. of three provinces of Pakistan are alternate host of stem rust only. Additionally, OSR were also reported in Punjab province on *B. lycium.* Stripe rust SSR marker did not amplify any aecial sample collected from different regions. This study clearly indicated that the studied Berberis plants serve as alternate hosts of stem rust and their formae speciales (OSR), but not for stripe rust. This finding has closed resemblance with the results of earlier studies, where Berberis plant was reported as an alternate host of stem rust and their formae speciales (Berlin *et al.*, 2013; Wang *et al.*, 2015). Recently, it was reported that East Asia, including Pakistan, has a low risk of WYR infection on Barberry, although there is a high risk of infection by WSR (Sinha and Chen, 2021). Some studies from China showed that under natural conditions barberry also serves as an alternate host of stripe rust (Zhao *et al.*, 2013; Wang *et al.*, 2015). Moreover, more than forty-five *Berberis* species have been reported as

alternate hosts of stripe rust during artificial conditions (Jin *et al.*, 2010; Zhao *et al.*, 2016; Mehmood *et al.*, 2019).

In Pakistan, the appearance of aecia on barberry leaves were dominant from May to July, while stem rust in wheat fields was seen in June till late July, which are consistent with the development of stem rust on barberry bushes. It is reported that the occurrence and epidemics of stem rust primarily depend on the presence of barberry bushes near the wheat field and precipitation during summer in that region (Wang *et al.*, 2015). Similarly, East Asia including Pakistan has a high risk of stem rust infection on barberry (Sinha and Chen, 2021). In the present study, no connection has been observed between stripe rust and *Berberis* plants in all three-province including Gilgit Baltistan and Kashmir. The absence of stripe rust on *Berberis* plants might be due to the early development of stripe rust in wheat field, as stripe rust in Pakistan appeared mostly during May before the aecium development on *Berberis* plant. The dry weather conditions during July and August in most area of Pakistan retards the development of rust on barberry. During the last three years of investigations, we did not find aecia on *Berberis* during late July. However, new aecia on *B. lycium* in Murree regions were found during late July, August and September. This might be due to the continuous precipitation prevailing in that area. These finding are also supported by SSR markers, which amplified most of the samples signifying WSR and OSR on *B. lycium*. Therefore, in Pakistan the *Berberis* plant is not a good alternate host for stripe rust in most of the regions in natural conditions. On the contrary, Mehmood *et al.* (2019) recently reported several *Berberis* species as alternate hosts of stripe rust in Pakistan under control conditions.

In short, the result of this study suggest that the selected *Berberis* species are significant hosts for stem rust and their formae speciales, but inappropriate hosts for stripe rust under natural settings owing to the dry environment and absence of young barberry leaf. According to earlier studies, the frequency of stripe rust infection on barberry leaf is very low *i.e.* up to 0.1 % (Wang *et al.*, 2015)**.**

5. Conclusion

The result of present study indicates that spore morphology and aecium appearance on the leaves of barberry are imperative tools to screen rust diversity. Along with molecular methods, the morphological features correspondingly have parallel significance in taxonomy and phylogenetic analysis of species and their biology. This study first time reports, through visual investigation, five *Berberis* species namely *B. balochistanica*, *B. pachyacantha*, *B. parkeriana*, *B. lycium* and *B. chitrria* as alternate hosts of rust in field. Using morphological

information, three different types of aecium (representing *P. graminis* and *P. arrhenatheri*) are reported with different types of aeciospores on different *Berberis* species.

Along with morphological study, rust species were also screened using molecular techniques. The Kit extracted DNA were successfully amplified by species and formae speciales specific SSR markers. Through SSR analysis, three *Berberis* pecies; *B. balochistanica, B. pachyacantha* and *B. lycium* were found as alternate hosts of stem rust, and *B. lycium* alone was reported as an alternate host of oat stem rust and wheat stem rust. Additionally, in this study no barberry was found as an alternate host of stripe rust in natural conditions. This study also signifies the specificity of SSR markers at species and formae speciales level. In short, our results confirmed that barberry is the main source of maintaining the stem rust populations in Pakistan.

CHAPTER 5

CHAPTER 5 . CONCLUSION AND FUTURE OUTLOOK

Berberis species have been used by mankind as medicine even in the early records of herbal drugs, on the other hand they also play a pivotal role in development of new races of important cereal crops. The species of this genus are reported to have important medicinal properties including tonic, antiemetic, anti-pyretic, antimicrobial, anti-inflammatory, hypotensive, anti-arrhythmic, anti-nociceptive, antioxidant, sedative, anti-pruritic, anti-cholinergic and cholagogic properties. The stem and root barks of *Berberis* plant have been extensively used against jaundice, diarrhea, to improve appetite, relieve upset stomach, high fever, hypertension, cholecystitis, dysentery, malaria, cholelithiasis, gall stones, leishmaniasis, ischemic heart diseases (IHDS), cardiomyopathies and cardiac arrhythmias, while leaves are used to make tea. In addition to its medicinal usage, berberis fruit juice is commonly used in Iran and Pakistan. *Berberis* fruit are also used in preparation of special dish with rice. While on the other hand, *Berberis* serves as an alternate host of rust as well. Literature review showed that rust is one of the major disease of cereals crops causing more than 50% of biotic losses. Therefore, both sides of this genus should be investigated for the development of new herbal drugs, isolation of new bioactive compounds with reducing powers and also screening the behavior of local Barberry towards rust in order to protect the staple food (wheat). In short, the phytopharmacological importance and pathological nature of *Berberis* is the prime need for a better understanding of this genus. Present study gives an outline that, the study of positive and negative aspects surrounding *Berberis* plant. This study has elaborated better understanding and development of new strategies to evaluate *Berberis* plant for medicinal purposes and to study their alternate nature towards rust to protect cereal crops.

In this decade, a new breakthrough was carried out by characterizing and isolating the bioactive compounds from various parts of some *Berberis* species. Although, studying only one part of many species or all parts of only one species limited the significance of this species. Therefore, phytochemical, nutritional, and biological profile should be carried out using various organs of *Berberis* plant. There are limited scientific studies that confirm the detailed biological potential of *Berberis* species. In present study, all examined parts of all tested *Berberis* species showed significant concentrations of mineral elements, phytochemicals and antioxidant potential. Hence, these can be utilized as natural antioxidant, antimicrobial and nutritional complements in herbal and food industries. Another notable finding is that the utilization of aerial parts could be initiated as a suitable and renewable source for the

conservation of natural resources. This will help in preserving medicinal plants particularly *Berberis* genus in Pakistan, which has been under a threat of extinction as roots are extensively used for traditional medicinal purposes.

In current study, thirteen *Berberis* species were collected from various provinces of Pakistan including Khyber Pakhtunkhwa, Punjab, Balochistan, Gilgit Baltistan and Kashmir regions. Out of thirteen, only eight species were taxonomically identified. After identification, three parts (root, stem and leaf) of these selected species were examined for their positive and negative attributes. All species showed significant amount of mineral elements, however *B.balochistanica* and *B.royleana* were found with highest number of minerals, while *B.lycium* was found with least numbers of mineral elements. Additionally, all parts showed substantial amount of total phenolic and flavonoid contents, but berberine contents were only reported in root parts of all species. Along with this, nutritional and phytochemical profile and strong antioxidants activities were revealed by all species. Leaf extract of *B. pseudohumbellata* and stem extract of *B. parkeriana* plant showed highest TPC and TFC, while lowest amount of TPC and TFC were reported in all parts of *B.lycium*. Similarly lowest TAC activities were observed in *B. lyceum* as well. While highest TAC activities were presented by *B. balochistanica* and *B. psuedohumbellata* leaf extracts. Overall, leaf showed remarkable antimicrobial activity in terms of DPPH, TRP and TAC.

After the nutritional and phytochemicals comprehensive outlines, it was necessary to investigate the biological applications of *Berberis* plant. Therefore, *B. balochistanica* plant was selected as model organism as this plant has been extensively used by local people due to easy availability in only two districts of Balochistan. Secondly, only one another species named *B. calliobuttrys* is reported in Balochistan province. So, all local communities of Balochistan province relay on this plant. As a result, this plant is now under depletion and endangerment. Thirdly, the maximum numbers of mineral elements with appropriate antioxidant activities were shown by all studied parts of this species.

The present study describes the prospective applications of various parts (roots, stem, and leaves extracts) for the treatment of microbial infections, inflammatory diseases, hemolysis, anticancer applications, and antioxidant applications. Present study clearly revealed that, all parts have potent antioxidant, antihemolytic, antibacterial, antifungal, anti-inflammatory, phytotoxic, and cytotoxic activities. The results obtained in present study suggested that apart from its use in traditional medicine, the application of barberry extracts could be extremely useful as nutritional supplements, food preservative and medication of

deadly diseases such as cancer. Another notably important finding is that the aerial parts of *Berberis* plant too, possess equally invaluable medicinal potential, hence advises against the complete uprooting of the plant. This will help in preserving this endemic plant, which has been under a threat of extinction as roots are used for traditional medicinal purposes. This study provides comprehensive experimental evidence using *B.balochistanica* plant as model organism. The presence of phenols and flavonoids in all parts of the plants and berberine contents only in roots contributes to a range of medicinal values such as antibacterial, antifungal, cytotoxic, phytotoxic, anti-hemolytic activities, and protection of DNA from oxidative stress and heat. DNA protecting nature and antihemolytic behaviors of this species is related to the presence of phytochemicals like phenolics and berberine contents. Brine shrimp assay revealed the cytotoxic potentials of this genus and confirmed the presence of bioactive compounds. This highly effective antioxidant, anti-hemolytic, anti-microbial and cytotoxic activity of this genus recommended the auspicious role in clinical and medical fields. Furthermore, these phytochemical rich species with strong antioxidant activities make this genus an excellent applicant to use as reducing and stabilizing agents in green synthesis of metal nanoparticles.

The present study revealed the importance of bioactive compounds in the green synthesis of BB-NiONPs from the stem and leaf extracts of the *B. balochistanica* plant. The presence of valuable phytochemicals with bioactive functional groups and potent antioxidants in stem and leaf extracts helped in stabilizing, capping, and reducing nickel salt into BB-NiONPs. The crystalline rhombohedral shape and fine size (31.44 nm and 21 nm) of BBS-NiONPs and BBL-NiONPs were confirmed by SEM and XRD techniques. Remarkable biological applications of both NiONPs, like antioxidant, antimicrobial and cytotoxic potentials, were observed. Both green synthesized NiONPs were also found as biostimulators in boosting up the germination frequency and seedling growth at suitable quantities. This indicates that they could be used as a substitute for synthetic chemicals in biomedical and agricultural fields. Moreover, they are also suitable for plants with high dormancy and slow seed germination. However, a further widespread investigation is recommended before introducing BB-NiONPs into clinical and agricultural trials.

The above concluding paragraphs revealed the miracles of genus *Berberis* for treating various types of illnesses. While on the other hands, *Berberis* plant hosts various types of deadly pathogens like rust. Rust is one of the seriously destroying pathogens of cereals crops including wheat. Therefore, this negative role of *Berberis* is needed to study along with its

medicinal role. As, Pakistan have 49 species of *Berberis* and Ug99 pathogen is already reported in neighboring countries like Iran. Therefore, in present study these aspects of *Berberis* were explored to find the role of Barberry as alternate host of rust in Pakistan.

First time, through visual investigation, five *Berberis* species namely, *B. balochistanica*, *B. pachyacantha*, *B. parkeriana*, *B. lycium* and *B. chitrria* were detected as an alternate host of rust in field. With the help of morphology, three different types of aecium (representing *P. graminis* and *P. arrhenatheri*) were reported with different types of aeciospores on different *Berberis* species. The result of present study indicated that spore morphology and aecium appearance on leaf of barberry is an imperative tool to screen rust diversity. Along with molecular methods, the morphological features correspondingly have parallel significance in taxonomy and phylogenetic analysis of species and their biology.

Along with morphological study, rust species were also screened using molecular techniques. For this purpose, a good quality and quantity of DNA is the primary step for PCR based molecular studies. Therefore, various methods (CTAB, SDS and Kits) were used to extract DNA. In present study, the kit with some modification provides enough DNA from aecial lesion to identify rust through PCR analysis. The Kit extracted DNA was successfully amplified by species and formae speciales specific SSR markers. Through SSR analysis, three *Berberis* species; *B. balochistanica*, *B. pachyacantha* and *B. lycium* were found as an alternate host of stem rust, and *B. lycium* alone was reported as an alternate host of oat stem rust along with wheat stem rust. Additionally, no barberry was found as an alternate host of stripe rust in natural conditions. This study also signifies the specificity of SSR markers at species and

formae speciales level. In short, our results confirmed that barberry is the main source of maintaining the stem rust populations in Pakistan.

Future Recommendations

The data obtained from this research works can be useful to develope, improve and isolate the natural bioactive compounds and screened for medicinal application and set as a biological marker. In future following recommendation are suggested as follow.

✓Identification of all *Berberis* species from multiple sites across Pakistan. Collection of various seasonal parts of *Berberis* according to developmental stages like flowering and fruiting stages should be done for correct identification. Identification of new sites for endemic species could be introduced for future regermination of this endemic threatened species.

✓Seeds of all Berberis species should be collected to conserve their germplasm for future applications.

✓A comprehensive profile of all parts along with local ethnopharmacological information should be collected and used as markers for clinal and food application of this genus.

✓Monitoring of threaten or endangered species and those endemic species which have been used from a long time for various purposes by local inhabitants. The new smart technology like GPS should be developed by Government to insure the exact nature of any species across the country.

✓Scientific communities should initiate various steps to conserve this genus from depletions by using various scientific approaches like tissue culture, seed germination and plantation of *Berberis* seedlings in new site and also establish the Nursery, which will provides barberry to markers and industries.

✓ Local people should be encouraged to use the aerial parts, as present study displayed their useful biological applications. These results should also help in preserving this plant by discouraging its uprooting for medicinal purposes. So, social awareness is essential step for controlling illegal practices of this species and pharmaceutical industries should discourage the uprooting of underground part of this plant.

✓Remedies and analytical methods built on the usage of nanoparticles are supposed to have valuable advantages for medication in the upcoming days. However, the nanoparticles also have side effects on health. Therefore extensive research with repeated trials is required to fix the safety levels and their application in medicines and other food industries.

✓Nickel oxide nanoparticles are used as a substitute for synthetic chemicals and should be used as seed germinator (nonfertilizer) for plants with high dormancy and slow seed germination. Therefore, field trial is strongly recommended.

✓Finally, further in vivo and extensive research is recommended to standardize the safety level first, and then allowed to be used in pharmacological, biomedical, and agricultural fields.

✓More studies are needed to identify these species and their relationship to rusts.

✓This study just focused on aecial stage, so in future relationship of all stages like survival of urediniospores, teliospore germination and basidiospore production and initiation of pycnial stage on *Berberis* should be studied. Along with this, wheat resistance, phenology of barberry and wheat fields are also needed to study in future.

✓Berberis susceptibility towards rust, stripe rust and *Berberis* relationship should be investigated in artificial conditions.

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Annexes

ANNEXES

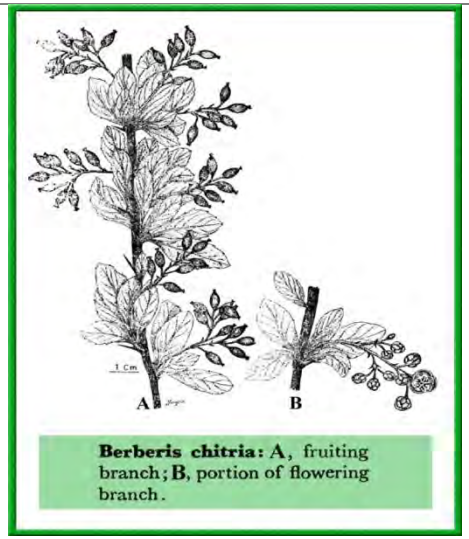
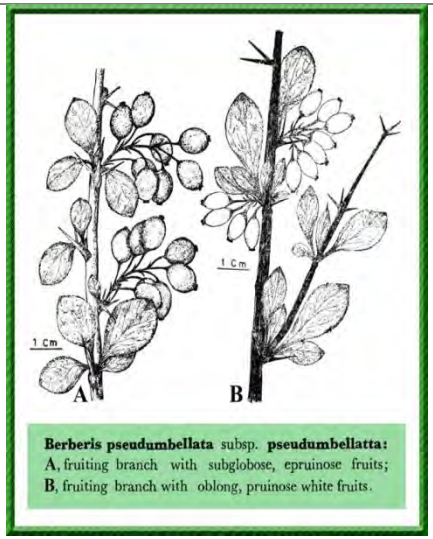
Berberis Species in Pakistan

Supplementary Table 1. List of taxonomically identifies Berberis species of Pakistan. (http://www.efloras.org/flora_page.aspx?flora_id=5)

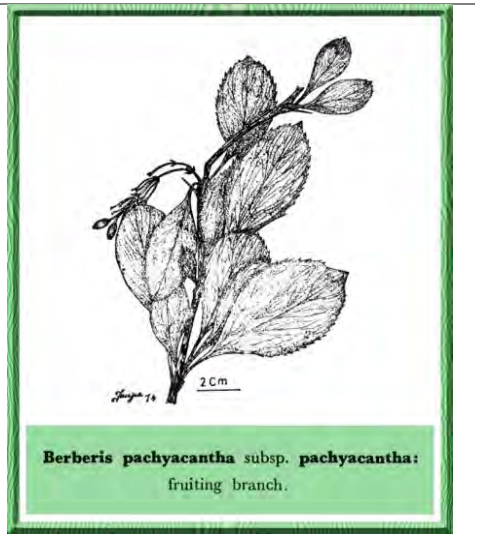
Sr #	*Species Name*	Picture	**Sr #**	Species Name	Picture
1	*Berberis lycium*		**14**	Berberis royleana	

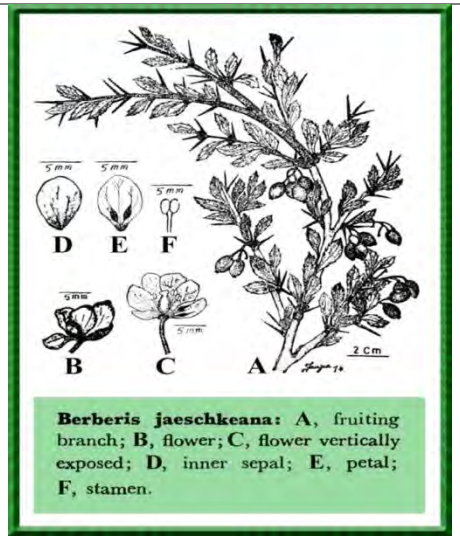
2	*Berberis baluchistanica*	 **Berberis baluchistanica:** **A**, flowering branch; **B**, fruiting branch; **C**, flower; **D**, outer sepal; **E**, inner sepal; **F**, petal.	**15**	Berberis orthobotrys	 **Berberis orthobotrys** subsp. **capitata:** **A**, fruiting branch; **B**, portion of fruiting branch showing panicled infructescence.
3	*Berberis aitchisonii*	 **Berberis aitchisonii:** **A**, fruiting branch with acute leaves and young fruits on longer inflorescence; **B**, fruiting and flowering branch with rounded leaf apices and shorter inflorescences.	**16**	Berberis ulicina	 **Berberis ulicina:** **A**, fruiting branch; **B**$_1$-**B**$_3$, leaf variation.

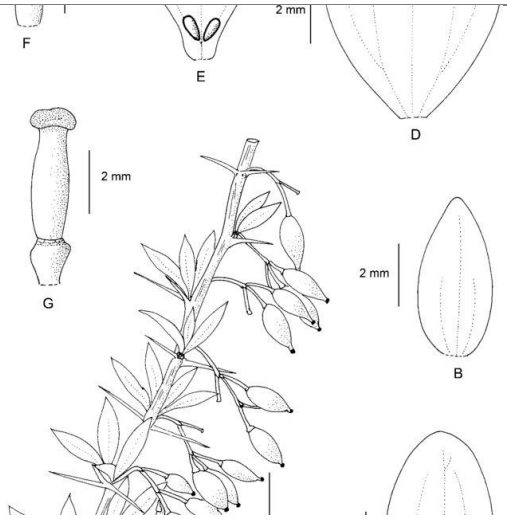
4	*Berberis brandisiana*	 **Berberis brandisiana:** fruiting branch.	**17**	Berberis parkeriana	 **Berberis parkeriana: A**, fruiting branch; **B**, leaf with green under-surface; **C**, leaf with somewhat pruinose-whitish under surface; **D**, flower.
5	*Berberis brevissima*	 **Berberis brevissima: A**, fruiting branch; **B**, fruit.	**18**	Berberis pseudumbellata	 **Berberis pseudumbellata** subsp. **gilgitica:** fruiting branch.

6	*Berberis calliobotrys*	 **Berberis calliobotrys:** **A**, flowering and fruiting branch; **B**, fruiting branch; **C**, flower.	**19**	Berberis pseudumbellata subsp. gilgitica	 **Berberis pseudumbellata** subsp. **gilgitica:** fruiting branch.
7	*Berberis chitria*	 **Berberis chitria:** **A**, fruiting branch; **B**, portion of flowering branch.	**20**	Berberis pseudumbellata subsp. pseudumbellata	 **Berberis pseudumbellata** subsp. **pseudumbellatta:** **A**, fruiting branch with subglobose, epruinose fruits; **B**, fruiting branch with oblong, pruinose white fruits.

8	*Berberis glaucocarpa*		**21**	Berberis kashmirana
9	*Berberis huegeliana*		**22**	Berberis pachyacantha subsp. pachyacantha



10	*Berberis jaeschkeana*	 **Berberis jaeschkeana:** **A**, fruiting branch; **B**, flower; **C**, flower vertically exposed; **D**, inner sepal; **E**, petal; **F**, stamen.	**23**	Berberis pachyacantha subsp. zabeliana	 **Berberis pachyacantha** subsp. **zabeliana:** fruiting branch.
11	*Berberis jaeschkeana var. jaeschkeana*		**24**	Berberis orthobotrys subsp. orthobotrys	

12	*Berberis jaeschkeana var. usteriana*		**25**	Berberis orthobotrys subsp. capitata	
13	*Berberis stewartiana*				

DNA extraction from aecium or infected leaf of Barberry.

Supplementary Table 2. Various Approaches used to Extract DNA from rust infected leaf of Berberis plant.

S.NO	Methods u	Extraction buffer	References
1	CTAB	2 % CTAB, 1M tris-HCL (pH8), 0.5M EDTA, 1.4 M NaCl and 1 % w/v polyvinylpyrrolidone	(Bailey et al., 2015)
2	CTAB	0.165 M Tris-HCl, pH 8.0; 66 mM EDTA, pH 8.0; 1.54 M NaCl; 1.1% CTAB, proteinase K at 50 µg/ml and 20% sodium dodecyl sulfate	(Liu and Kolmer, 1998)
3	CTAB	25 g L^{-1}D-sorbitol, 10 g L^{-1} N-lauroylsarcosine, 8 g L^{-1} CTAB, 0·8 M NaCl, 20 mM EDTA, 10 g L^{-1} polyvinylpolypyrrolidone, 0·1 M Tris, pH 8) and 5 μL proteinase K (10 mg mL^{-1})	(Thach et al. 2016),
4	CTAB	0.5 M EDTA (pH 8), 5 M NaCl, 1 M Tris-HCL (pH 8), Polyvinylpyrrolidone (40000 MW) and water	(Kankwatsa et al., 2018)
5	CTAB	1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 2.0% CTAB, 25 µg/ml Protein K, 20 mM EDTA, 0.5% sodium bisulfite, 1.0% 2-mercaptoethanol	(Chen et al., 1993)
6	OmniPrep (GenoTech	•Cold/heat beating (in present study) •Lysis buffer for tissue lysis, •Chloroform for separation of phases, •Stripping solution for releasing of DNA, •Precipitation solution for precipitation of protein •Isopropanol with incubation at -20 °C for 30 minutes for the precipitation of DNA (used in present study). •Addition of TE buffer for re-dissolving of DNA.	(Berlin et al., 2018) (With some modifications)

Cross-Species Transferability of SSR Markers

Supplementary Table 3. Cross-Species Amplifications of SSR Markers in *Puccinia* species.

S.NO	Markers	Locus	P. gramin	P. gramin	P. striifor	P. graminis f. sp. avenae	P. graminis f. sp. secalis	Negative Contr	s amplific	cific | ** Formae spec
SSR1	Wheat ste	F2-21		Yes		Yes			Cross	*
SSR2	Wheat ste	F8-15	Yes				yes		Cross	
SSR3	Wheat ste	F7-29	Yes	Yes		Yes	Yes		Cross	
SSR4	Wheat ste	F10-40	Yes	Yes		Yes	Yes		Cross	
SSR5	Wheat ste	F9-41	Yes	Yes	Yes	Yes	Yes		Cross	
SSR6	Wheat ste	F10-21	Yes	Yes		Yes	Yes		Cross	
SSR7	Wheat ste	F9-18	Yes	Yes			Yes		Cross	
SSR8	Wheat ste	F9-44	Yes	Yes			Yes		Cross	
SSR9	Wheat ste	F3-19	Yes	Yes			Yes		Cross	
SSR10	Oat stem	F1-20				Yes			Specific	**
SSR11	Oat stem	F2-03				Yes			Specific	**
SSR12	Oat stem	F2-42							N/A	
SSR13	Oat stem	F2-12				Yes			Specific	**
SSR14	Oat stem	F2-8				Yes			cross	
SSR15	Oat stem	F3-33							N/A	
SSR16	Oat stem	F2-17	Yes	Yes		Yes	Yes		cross	
SSR17	Oat stem	F3-9				Yes			Specific	**
SSR18	M1	Pgestssr 368	Yes	Yes		Yes	Yes		cross	
SSR19	M2	Pgestssr 024	Yes	Yes		Yes	Yes		cross	
SSR20	M3	Pgestssr 279	Yes	Yes		Yes	Yes		cross	
SSR21	M4	Pgestssr 109	Yes	Yes			Yes		cross	
SSR22	M5	Pgestssr 21	Yes						Specific	**
SSR23	M6	Pgestssr 021	Yes	Yes			Yes		cross	
SSR24	M7	Pgestssr 255							N/A	
SSR25	M8	PgtCAA 53	Yes	Yes			Yes		Cross	

PCR Based SSR molecular Analysis

Supplementary Table 4.1. PCR Based SSR molecular Analysis (Wheat Stem Rust SSR Markers) of Extracted DNA from infected leaf lesion of Berberis pieces.

S. No	Years	Province	Location	Host Name	F2-21	F6-31	F3-19	F9-41	F9-44	F8-15	F10-21	F9-18	F7-29	F8-2	F10-40	F9-45
1	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	2017	Punjab	Murree	*Berberis lycium*	300	N/A	N/A	N/A	N/A	N/A	N/A	N/A	430	N/A	N/A	N/A
3	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	2017	Punjab	Murree	*Berberis lycium*	380-400	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
9	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	2017	Punjab	Murree	*Berberis lycium*	300	N/A	N/A	140	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	400	
13	2017	Punjab	Murree	*Berberis lycium*	290-300	N/A	N/A	120-140	350	N/A	320	N/A	N/A	N/A	N/A	N/A
14	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	140		N/A	N/A	N/A	N/A	N/A	N/A	N/A
15	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
16	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
17	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
18	2017	Punjab	Murree	*Berberis lycium*	280	N/A	N/A	N/A	N/A	330	N/A	N/A	400	N/A	400	
19	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
20	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

21	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
22	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
23	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	450	N/A	N/A	N/A	N/A	N/A	N/A	N/A	390	
24	2017	Punjab	Murree	*Berberis lycium*	300	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
25	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
26	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	140	N/A	N/A	N/A	N/A	N/A	N/A	N/A	290
27	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
28	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
29	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
30	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	300	N/A	N/A	N/A	N/A
31	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
32	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
33	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
34	2017	Balochistan	Ziarat	*Berberis balochistanica*	310	N/A	400	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
35	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	350-390	N/A	N/A	N/A	290	N/A	N/A	N/A	N/A	N/A
36	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
38	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	270
39	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
40	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
41	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	140	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	2018	Punjab	Murree	*Berberis lycium*	370	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	140	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
46	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
47	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

48	2018	Punjab	Murree	*Berberis lycium*	310	N/A	400		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
49	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	390	290
50	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
51	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
52	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
53	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
54	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
55	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
56	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
57	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	450	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
58	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
59	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	360	N/A
60	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
61	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
62	2019	Punjab	Murree	*Berberis lycium*	300	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
63	2019	Punjab	Murree	*Berberis lycium*	350-380	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
64	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
65	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
66	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
67	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
68	2019	Punjab	Murree	*Berberis lycium*	400	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
69	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
70	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	400	
71	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	150	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
72	2019	Punjab	Murree	*Berberis lycium*	290	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
73	2019	Punjab	Murree	*Berberis lycium*	300	N/A	N/A	N/A	N/A	N/A	310	310	450	N/A	N/A	N/A
74	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

75	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
76	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	350-390	N/A	N/A	N/A	N/A
77	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
78	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
79	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	380	
80	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
81	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	150	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
82	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
83	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
84	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
85	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
86	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
87	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	540	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
88	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
89	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	190	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
90	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	310	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
91	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
92	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
93	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
94	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
95	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	400	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
96	Control	Australia	University of Sydney	*P. graminis f. sp. tritici*	300-350	300	380-400	130	330	300	300	300-350	390	270	300	280

Supplementary Table 4.2. PCR Based SSR molecular Analysis (OAT STEM RUST SSR Markers) of Extracted DNA from infected leaf lesion of Berberis pieces.

Years	Provience	Location	Host Name	F1-20	F2-03	F2-12	F3-33	F3-09
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	270-300	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	190
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A

2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2017	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	190
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A

2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lyceum*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A

2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	**270-300**	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachyacantha*	N/A	N/A	N/A	N/A	N/A
Control	Australia	University of Sydney	*P. graminis f. sp. avenae*	170-180	250-300	240		170

Supplementary Table 4.3. PCR Based SSR molecular Analysis (WHEAT STRIP RUST SSR Markers) of Extracted DNA from infected leaf lesion of Berberis pieces.

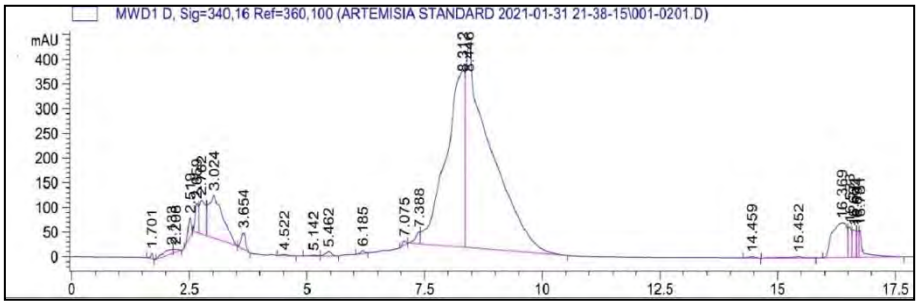
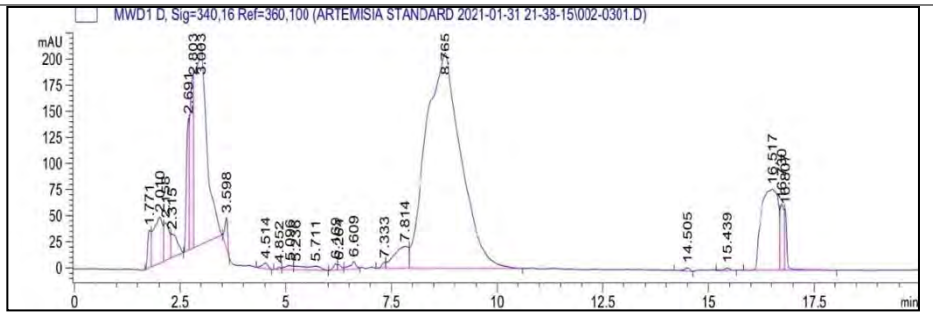
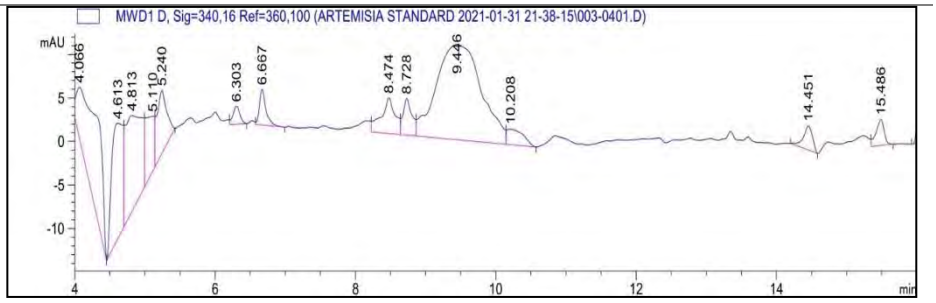
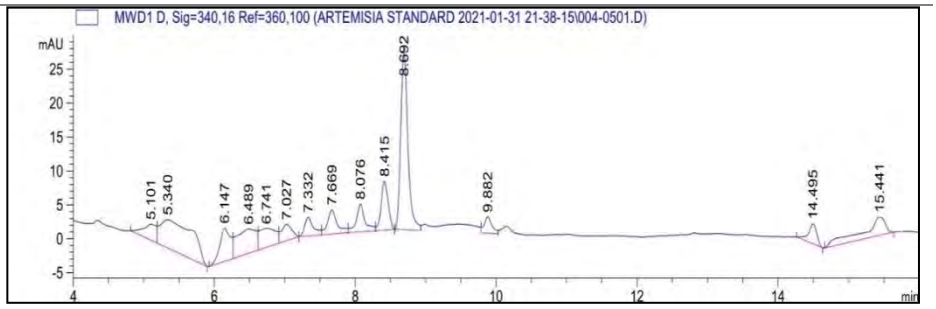
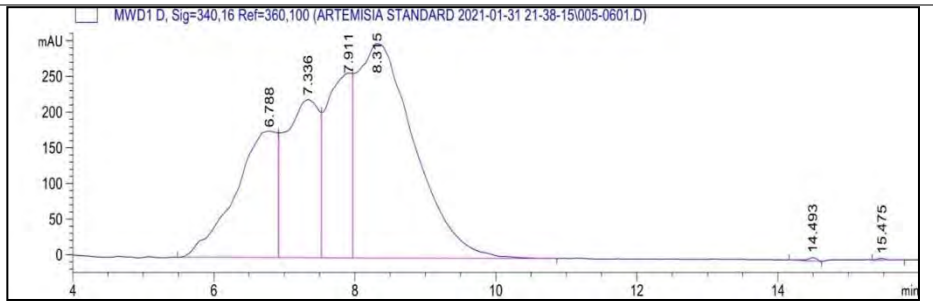
S No	Years	Provience	Location	Host Name	Sr11-39	Sr10-31	Sr10-7	Sr10-2	Sr11-21	Sr11-4	SR9-6	Sr10-13	Sr11-42	Sr14-37	SR10-22	SR15-31	SR9-11	SR10-19	SR10-6	SR10-7
1	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
9	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
13	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
15	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
16	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
17	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
18	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
19	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
20	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
21	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
22	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
23	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

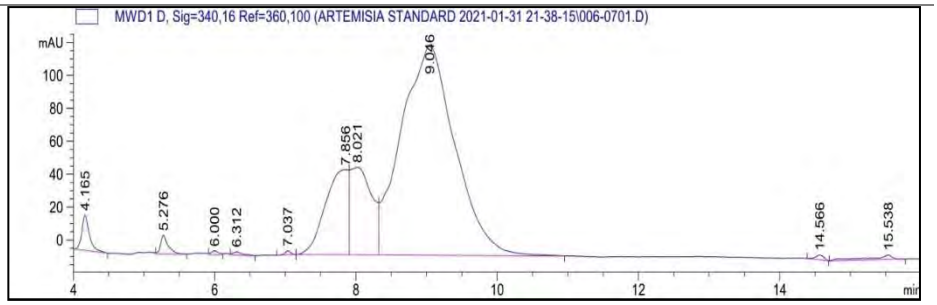
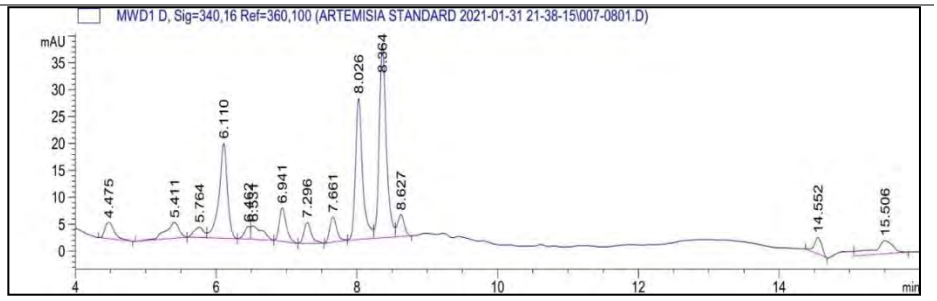
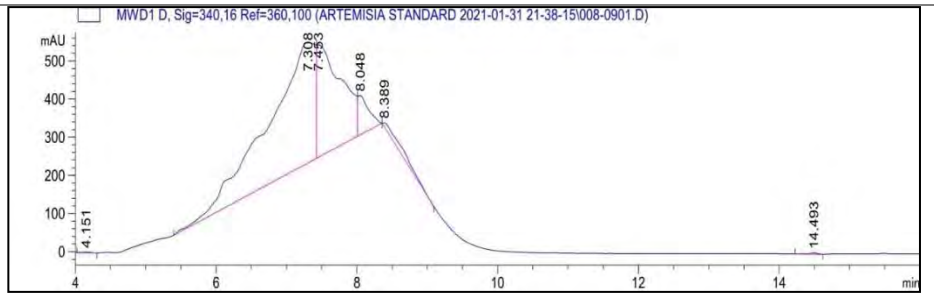
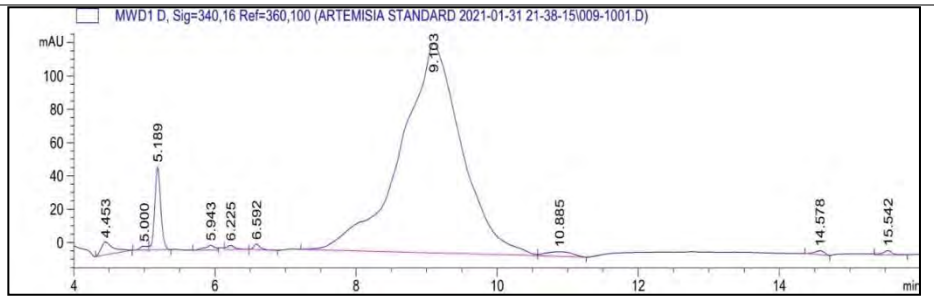
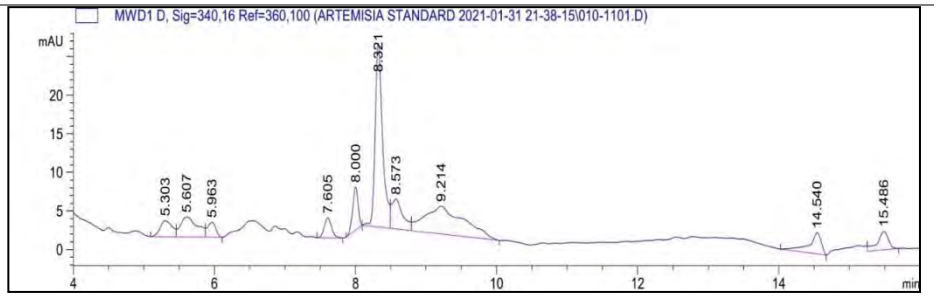
25	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
26	2017	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
27	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
28	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
29	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
30	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
31	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
32	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
33	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
34	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
35	2017	Balochistan	Ziarat	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
36	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	2017	Balochistan	Quetta	*Berberis balochistanica*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
38	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
39	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
40	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
41	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
46	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
47	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
48	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
49	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

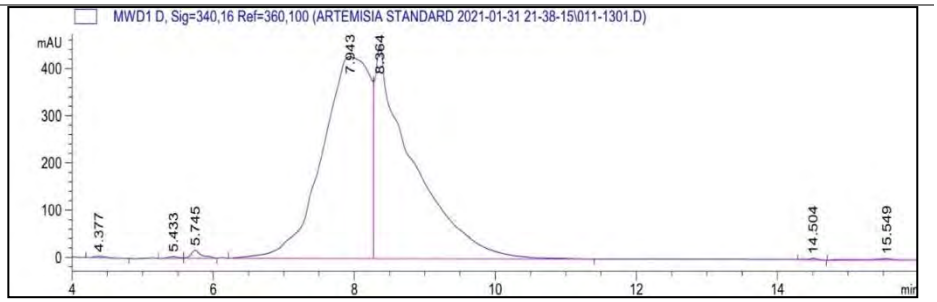
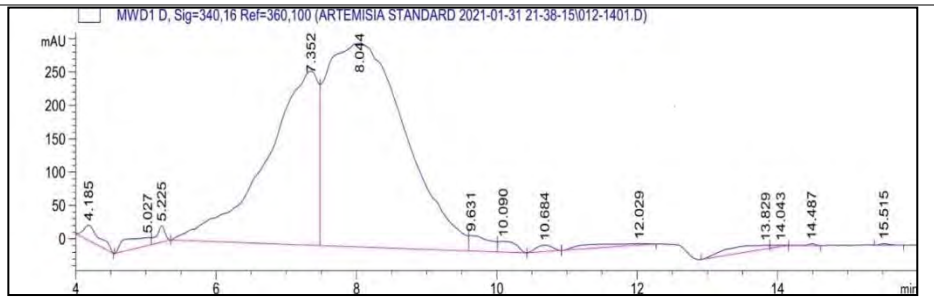
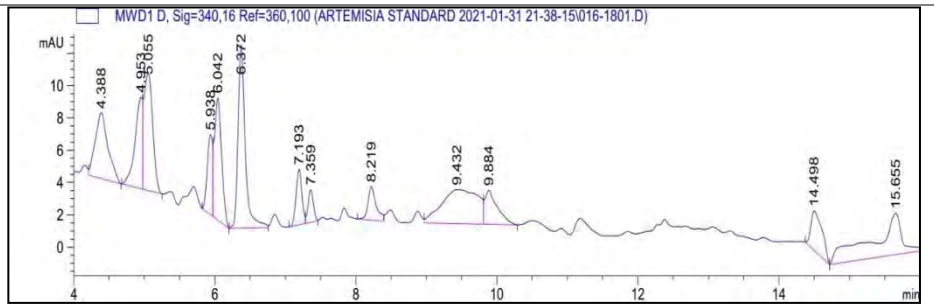
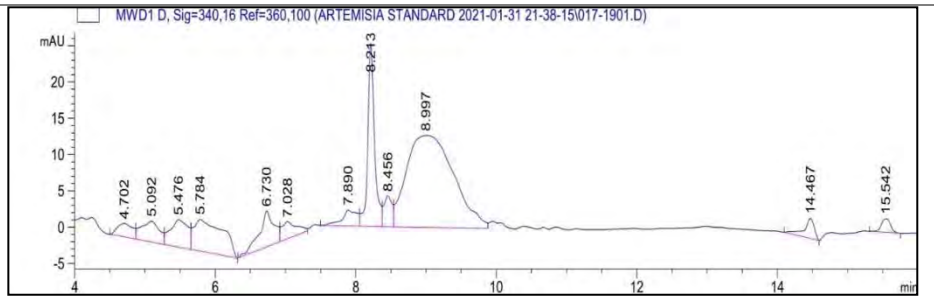
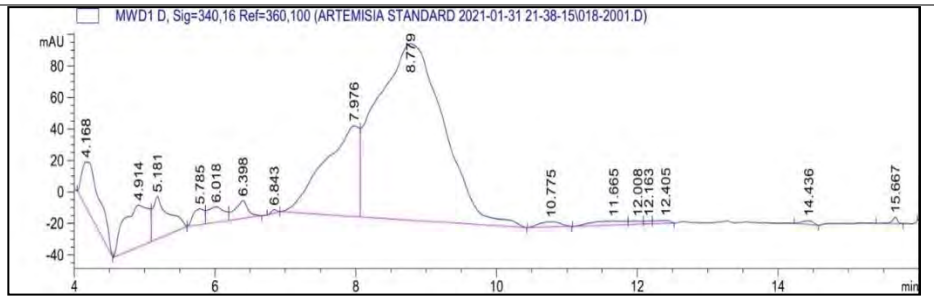
50	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
51	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
52	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
53	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
54	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
55	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
56	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
57	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
58	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
59	2018	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
60	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
61	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
62	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
63	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
64	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
65	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
66	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
67	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
68	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
69	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
70	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
71	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
72	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
73	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
74	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
75	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
76	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

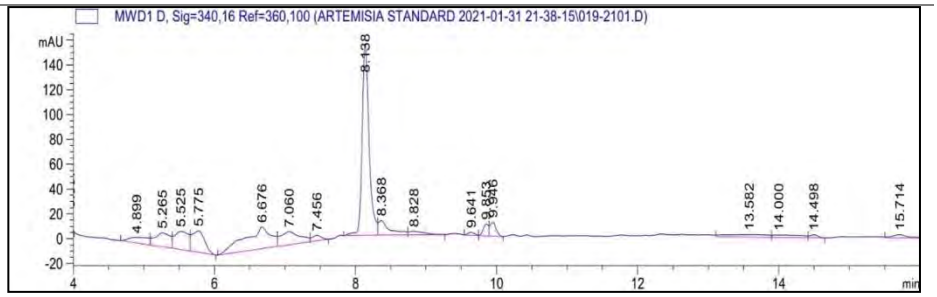
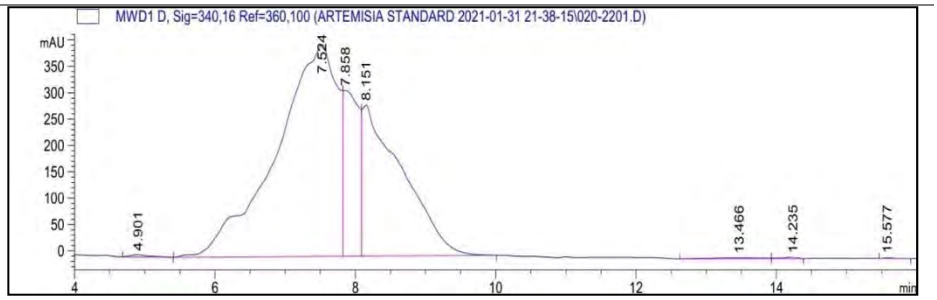
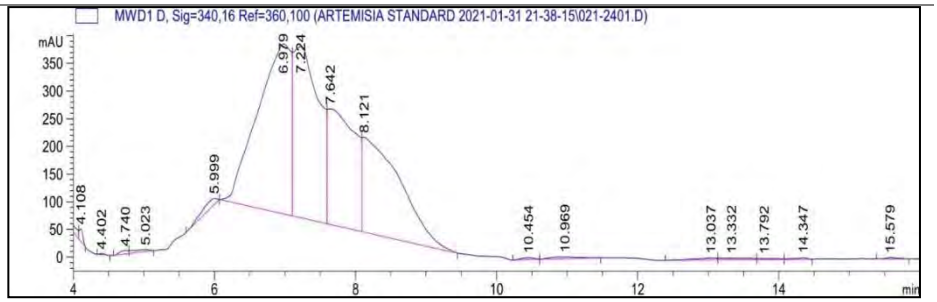
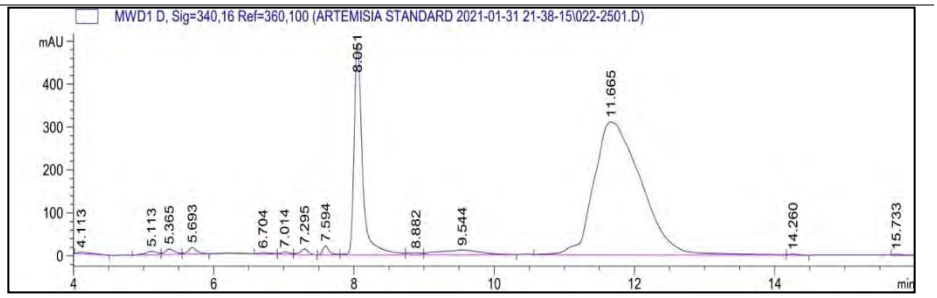
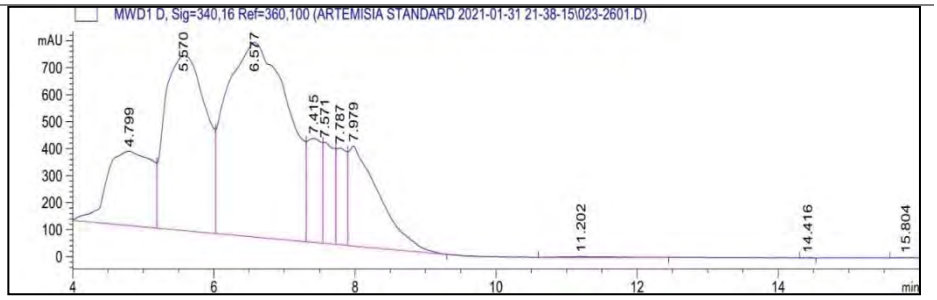
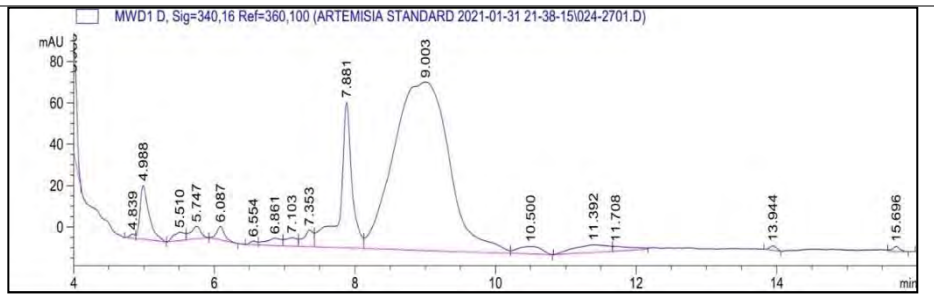
77	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
78	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
79	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
80	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
81	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
82	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
83	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
84	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
85	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
86	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
87	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
88	2019	Punjab	Murree	*Berberis lycium*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
89	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
90	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
91	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
92	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
93	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
94	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
95	2012	Khyber Pakhtunkhwa	Mansehra	*Berberis pachya cantha*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
96	Control	Australia	University of Sydney	*P. striiformis f. sp. tritici*	250	300	N/A	N/A	450	300	650	180	N/A	500	N/A	N/A	480	500	350	N/A

HPLC Profile of All Selected Berberis

Sr. No	Spiecie (Part)	HPLC profile
1	*B. orthobotrys* **(**Root**)**	
2	*B. orthobotrys* (Stem)	
3	*B. orthobotrys* *(*Leaf)	
4	*B. balochistanica* (Root)	
5	*B. balochistanica* (Stem)	

6	*B. balochistanica* (Leaf)	
7	*B. parkeriana* (Root)	
8	*B. parkeriana* (Stem)	
9	*B. parkeriana* (Leaf)	
10	*B. lyceum* (Root)	

11	*B. lyceum* (Stem)	
12	*B. lyceum* (Leaf)	
13	*B. calliobotrys* (Root)	
14	*B. calliobotrys* (Stem)	
15	*B. calliobotrys* (Leaf)	

16	*B.pachyacantha* (Root)	
17	*B. pachyacantha* (Stem)	
18	*B. pachyacantha* (Leaf)	
19	*B. chitrria* (Root)	
20	*B. chitrria* (Stem)	
21	*B. chitarra* (Leaf)	

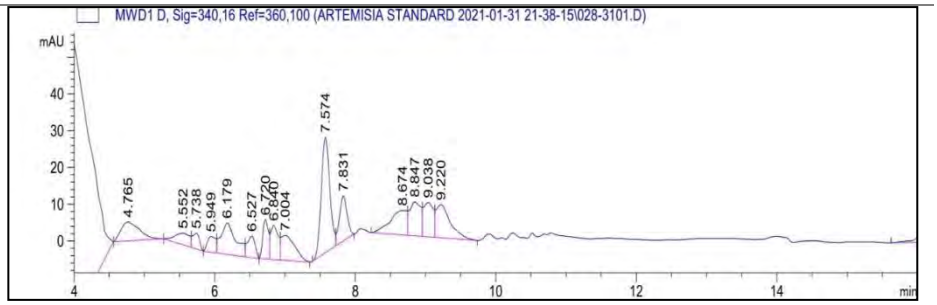
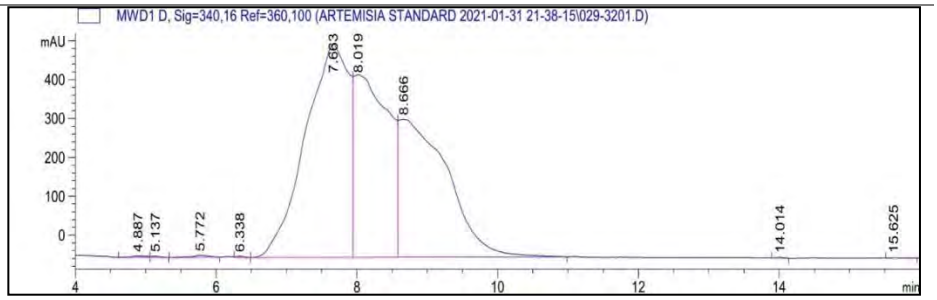
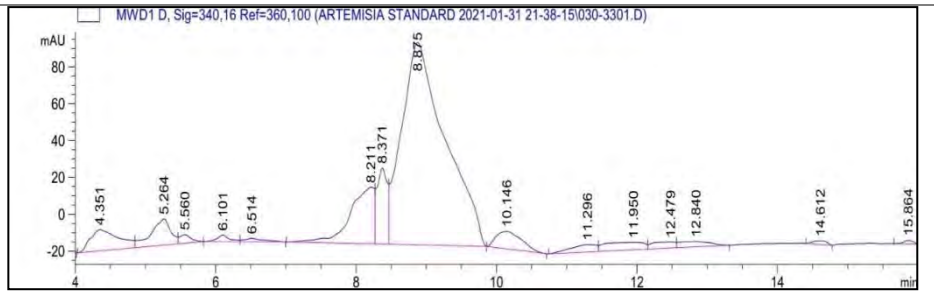
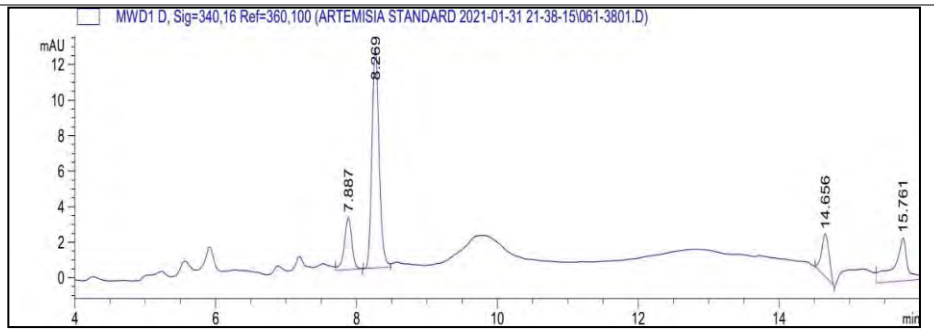
22	*B. pseudumbellata* subsp. *Gilgitica* (Root)	
23	*B. pseudumbellata* subsp. *Gilgitica* (Stem)	
24	*B. pseudumbellata* subsp. *Gilgitica* (Leaf)	
25	Standerd Berberine	

Figure S 1. HPLC Profile of All Selected Berberis species by investigating their Root, Stem and Leaf extract for the presence of Berberine contents.

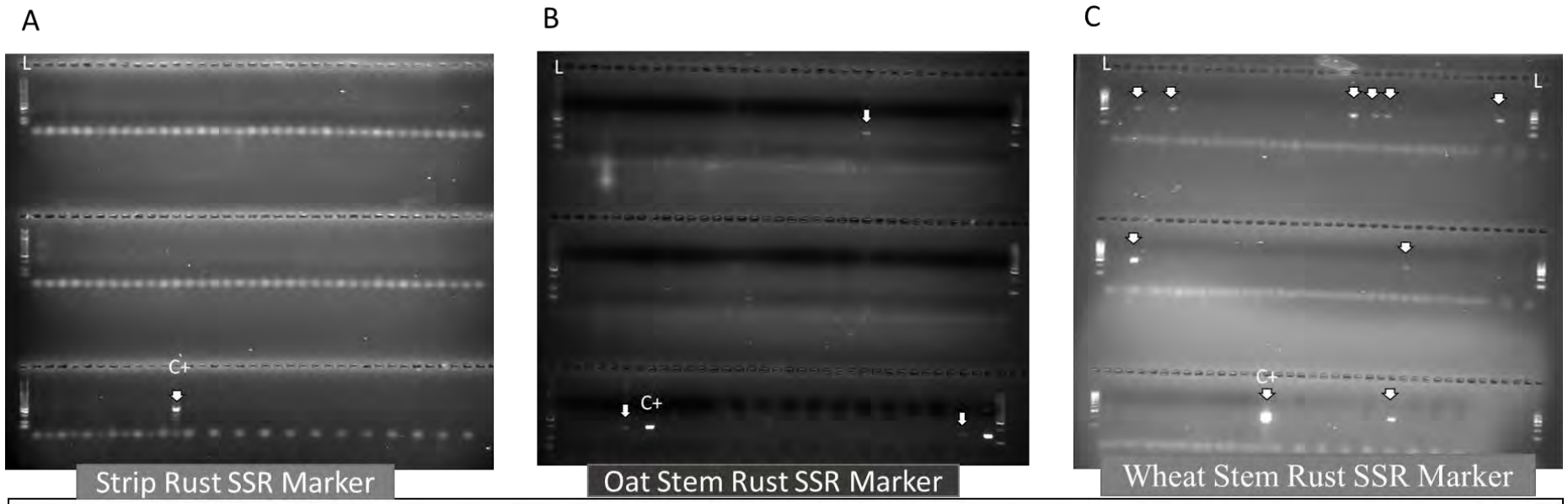


Figure S 2. SSR based Molecular study of Rust on Berberry; (A) Stripe rust specific SSR marker study (Sr11-39); (B) Oat stem rust SSR marker study (F9-3); (C) Wheat stem rust SSR marker study (F2-21). Where the arrow representing the Amplified PCR product by SSR markers

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Sesto Fiorentino, 5 January 2022

REVIEW OF DOCTORAL THESIS

***Author/Candidate*: Mr. Siraj-ud-Din**

***Title*: "*Berberis Species of Pakistan: A Rich Source of Phytochemicals with Reducing Potential for Green Synthesis of Metallic Nanoparticles and as an Alternate Host to Fungal Pathogens*"**

***Reviewer*: Prof. Alessandra Cincinelli**

The Thesis presents original research in the area of beneficial and damaging potentials of Berberis species of Pakistan. The candidate analysed root, sten and leaf extracts of eight selected Berberis species for phytochemical, nutritional and antioxidant purposes.

The thesis is well written, with only occasional minor grammatical and spelling mistakes.
The Thesis is very interesting to read and Figures and Tables are clear, useful and summarise the most important results.
The structure of the Thesis conforms to the principles and requests to the structure of scientific Thesis. The use of different fonts and structure of the text is proper and helps the reader to better orientation in the text.

The Thesis consists of an Introduction and five chapters. Introduction presents a useful description of Berberis species focusing on i.e. their distribution across Pakistan, economic and medicinal aspects, phytochemistry of Berberis plants, PCR based approach for rust studies. Objectives of the Thesis are also reported. Chapter 1 describes Barberies ad a medicinal plant in Pakistan; Chapter 2 is concerned with biological applications, Chapter 3 is dedicated to plant mediated green synthesis of metal oxide nanoparticles, while Chapter 4 focus on Barberis as an alternate host of rust. Conclusions are reported in Chapter 5 and are well and clearly described. In addition, References and Annexes are included at the end of the Thesis.

Dr. Alessandra Cincinelli
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The candidate has studied and used appropriate number of bibliography sources which are well quoted in the thesis. There is evidence of a deep theoretical knowledge and very good orientation in the problem discussed in the thesis.

The candidate has demonstrated the ability to understand a broad area of research and clearly identify some of potentials and problems in Barberis studies by developing novel solutions and approaches.

Thus, the fundamental objectives of the research have been well achieved, and the interesting results have been published in peer-reviewed journals such as Molecules, Microscopy Research and Technique and Scientific Report evidencing the importance of the research for the scientific community. The candidate was first Author of two of these international publications.

In Summary, my conclusion is that the PhD Thesis of Mr. Siraj-ud-Din presents original research results of large importance and I strongly recommend without hesitation that the candidate is awarded the doctoral degree.

Sincerely,

Alessandra Cincinelli

Berberis Species of Pakistan: A Rich Source of Phytochemicals with Reducing Potential for Green Synthesis of Metallic Nanoparticles and as an Alternate Host to Fungal Pathogens

Thesis submitted by Siraj-Ud-Din, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan, for the degree of PhD

The roots and shoots of members of the family Berberidaceae produce substantial quantities of secondary products many of which have been shown to have bioactive properties. In regions of Pakistan, Berberis species grow well and root extracts have been utilised by local populations as remedies for a host of medical conditions. The removal of root tissues often reduces the viability of the plant and part of this research was to examine whether stem and leaf extracts were also beneficial as a source for phytochemicals and antioxidants.

Plant tissues provide a natural source from which fabricate nanoparticles and the project also explored how aerial parts of Berberis species could be exploited to provide a way of stabilizing phytochemicals. The synthesized nanoparticles showed an ability to restrict the growth of bacterial and fungal pathogens and contribute to the generation of nanomaterials for agriculture and as fertilisers.

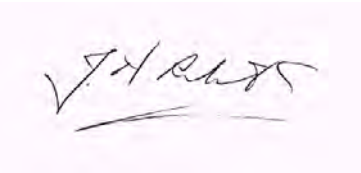
The third strand of the research was to examine the contribution that Berberis plants make as an alternate host for fungal pathogens of cereal crops. The approach taken was to use Simple Sequence Repeat (SSR) markers to explore infection of Barberry with different rust strains including stem rust and yellow rust.

The work has been carried out thoroughly and the thesis contains a substantial amount of interesting information and some of the data have already been published. Indeed, some of the chapters comprise publications which I applaud as a way of generating thesis content. Overall the thesis is well written and my only criticism is that the data described in the final chapter that explores the use of SSR markers needs to contain some images of the gels that have been utilised in the data presentation. This would confirm to the reader that the amplification of the DNA has been effective and that the size of the amplified products is as predicted. There are some typographical and grammatical errors that have been highlighted in the text and will need correction.

Overall the thesis comprises five main chapters followed by a section that contains reference citations. The first chapter provides an overview of the study that leads to the aims and objectives of the project. This is followed by four chapters that are written either in paper format or include the published papers themselves. Finally, there is a section that outlines the overall conclusions of the work and future recommendations.

In summary, this thesis represents a substantial body of work and the data collection carried out and the tools used to analyse the results have provided

the student with an excellent training in aspects of plant biology and analytical chemistry. The work shows clear evidence of scholarship and as a consequence I believe that the thesis is worthy of the award of a PhD.



Professor Jerry Roberts
Deputy Vice Chancellor Research and Enterprise
University of Plymouth

15 February 2022