

Research Article

Exploring the Potent Triad: Antibacterial, Antifungal and Phytotoxic Activities of *Ribes Himalense* Decne from Thandiani, Abbottabad, Pakistan

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Introduction

Abbottabad is named after Major James Abbott, the first deputy commissioner of Hazara (1849-1853). The area mostly lies on Eurasian land plate and Iranian plateau but the eastern part is close to the Indian subcontinent [1]. The geographic coordinates of the area are 73°13'E longitude and 34°09'N latitude, at an elevation of 1,256 m (40121 ft). Summers are pleasant with heavy monsoon season followed by snowfall in winters [2]. Thandiani is located in the moist temperate forest in Pakistan with rich plant diversity [3]. *Ribes himalense* Decne. is a member of family Grossulariaceae, represented by one ge-

Abstract

The current studies were conducted to highlight biological activities of *Ribes himalense* Decne. including antibacterial, antifungal and phytotoxic potentials. Antibacterial studies revealed that the inhibitory effect for ethanolic crude extract of leaves of *Ribes himalense* was 16mm, 16mm, 14mm and 17mm against *Escherichia coli*, *Proteus mirabilis*, *Streptococcus mutans* and *Bacillus subtilis* respectively. The ethanolic extracts of stem showed 15mm, 18mm, 13mm and 16mm against, *Escherichia coli*, *Proteus mirabilis*, *Streptococcus mutans* and *Bacillus subtilis* respectively. It was noted that the ethanolic extract of leaves *Ribes himalense* showed significant inhibition of 15mm against *Candida albicans* while the inhibition zone of stem was 12mm. It was tested and revealed that the aqueous ethanolic extract of *Ribes himalense* showed inhibition against the growth of *Lemna minor*, which was used as test plant during the research. The phytotoxic activities of the plant showed that 10µl extract caused 0 inhibition of growth and 10% by the leaves and stem respectively. At 100µl extract the growth inhibition was showed by 30% and 10% by the leaves and stem extracts of *Ribes himalense* respectively. It was further studied that at 100µl extracts the inhibition of growth was 70% and 100% by the leaves and stem respectively. Cytotoxic studies of the research plant were conducted and revealed that the ethanolic extract of stem is more cytotoxic than the leaves. At 500µg/mL, 1000 µg/mL and 2000µg/mL ethanolic extract of leaves showed about 90% cytotoxic effect while at 62.5µg/mL, 125µg/mL and 250µg/mL cytotoxic effects were 30%, 50% and 70% respectively. In case of ethanolic extract of stem at the concentration of 2000µg/mL, 1000µg/mL, 500µg/mL, 250µg/mL and 125µg/mL cytotoxic effect was 90% for each, while 80% cytotoxicity was observed at the concentration of 62.5µg/mL.

Keywords: Antifungal; Antimicrobial; Biological activities; Cytotoxic; Phytotoxic; *Ribes himalense*

nus and eight species in Pakistan. *Ribes himalense* Decne. can be found in Kalam, Gilgit Baltistan, Kaghan, Swat, Chagli Gali and Hazara. It is very commonly found in North Western Himalayas, between 2400-4000 m altitudes. Medicinal plant species have been widely used for many centuries across the globe [4]. Plant extracts are used in developing therapeutic medicines which work against wide range of microbes and infectious diseases [5]. Certain plant species contain polyphenols, alkaloids and volatile oils which are used as antibacterial agents. These plant extracts stop the growth of bacterial strains and kill them,

leaving no harmful effects on human health [6]. Phytochemical extractions are very helpful against bacterial infections [7] and it is necessary to find new antibiotics because certain drug-resistant drugs are causing havoc and epidemics [8]. Pathogenic fungi is causing severe threats to animal's and plant's health [9]. Currently research interest has been on peak to isolate useful extracts from plant species to fight against pathogenic fungi which possess antifungal activities with least side effects on human and animals health [10]. Phytotoxic activity inhibits the growth, development/germination of plant or plant parts. Many chemicals, particularly phenolic and their products, possess high phytotoxic effects [11]. Phytotoxic chemicals released by plant species may be useful in weed management, either solitary or in combination [12]. Allelopathy is the study where plant toxic allelochemicals are studied which poses negative effects on neighboring plants [13]. Many plants are used as effective organic herbicides which have sufficient phytotoxic potency [14]. The focus of pharmacological research today is to use plant extracts to cure and prevent acute and chronic diseases [15,16]. Many in-vitro and in-vivo experiments revealed the anti-inflammatory, anti-cancer and antioxidant properties of bioactive compounds of plants origin [17,18]. Medicinal plants and drugs obtained from medicinal plants have been using by human since ancient times [19]. Cancer is generally known as multicellular disease, characterized by uncontrolled cell division [20]. To reduce the toxicity and side effects of chemotherapy, alternative medicines are used. A number of chemicals have been identified from different parts of the plants which possess anti-cancer potentials, helpful in controlling the cancer [21]. An estimation shows that about 70% of the anti-cancer drugs are of plants origin and more than 3000 plant species have been identified with anti-cancer qualities [22].

A comprehensive literature review was made and it was concluded that there is no research work available on the antimicrobial, phytotoxic and cytotoxic activities of *Ribes himalense* Decne. therefore, the present work was designed to identify the phytochemical potentials of the plant species.

Material And Methods

Plant Collection, Preservation and Identification

Ribes himalense Decne. is a medium shrub which belongs to family Grossulariaceae. Plant specimens and vigorous stem and roots were collected from the research area. The identification was carried out by reviewing the available literature [23-25]. Plant specimens were pasted on standard herbarium sheets (17 x 11 inches) and deposited at Centre of Plant Biodiversity Herbarium for ready reference.

Field surveys and Geo-referencing

Detailed field surveys were performed between the months of August and September in 2023. *Ribes himalense* was sampled across the research area and GPS (Garmin Vista ETrex) was used to measure the coordinates of plant species. The distribution map was generated by ArcGIS software.

Biological Assay

PCSIR Laboratory Complex Peshawar was used to perform Biological assay as follow.

Drying and grinding

The leaves and stem of the plant species were carefully collected. Leaves and stem were washed and dried in shade at room

temperature. The dried leaves and stem were grinded with grinder separately and the powder were stored separately to avoid moisture and contamination.

Extraction

The standard procedure of Ahmad *et al.* [26] was adopted to prepare extracts. 255.8g of leaves and 363.2g of stem powder were taken in flasks and ethanol was added which was then incubated for 2 days with periodically shaking. The mixture was then filtered through a single layered filter paper. Equal amount of ethanol was added to the first extract, kept for a day and filtered again.

The extracts were combined and dried at 40°C in rotary evaporator to evaporate ethanol and obtain crude extract individually. The dried extracts were then stored in separate desiccators and kept at 2-8 °C for use. Finally, the crude extract was dissolved in 0.1% Dimethyl Sulfoxide (DMSO; 1 mg/μl 6), which is a non-toxic and universal solvent for many antimicrobial activities.

Antibacterial Assay

Material used during the procedure were, sterile borers, micropipette, sterile water, dimethyl sulphoxide, petri plates and bacterial strains. The antibiotic (Azithromycin & Ciprofloxacin) was used to cure bacterial infection. The bacterial strains used during studies are as follow. The microorganisms were put at 37°C in petri plates in nutritional medium and incubation was conducted for about 24 hours to increase the growth of microbes. Many microbial strains were grown in separate tubes.

Antibacterial screening

Antibacterial analysis was carried out using Agar well diffusion method. Plant extracts were dissolved in DMSO. For analysis Gram positive bacteria (*Bacillus subtilis*: clinical isolate & *Streptococcus mutans*: ATCC# 35668) and Gram negative bacteria (*Proteus mirabilis*: ATCC# 12553 & *Escherichia coli*: ATCC# 8738) were considered. The sub-cultured bacteria were placed in a saline medium for an incubation period of 30 minutes at 37 °C. Lawn was arranged through streaking on Mueller–Hinton Agar (MHA) plates by dipping sterile swabs of cotton in bacterial solution. Using sterile cork borer, wells of diameter 5 mm were formed and 100μL of sample were put in each. DMSO was used as solvent to prepare stock solution and Ciprofloxacin & Azithromycin were used as positive control. The plates were kept at 37°C for 24 hours after an incubation period of 2 hours in biosafety cabinet. The inhibition zone was calculated accordingly [27,28].

Antifungal assay

The standard methods after Haq *et al.* [29] was followed. *Candida albicans* was collected from clinical isolates and PCSIR Laboratory Complex Peshawar, and was used to study the antifungal activity. The materials used during this activity includes, SDA (Sabouraud, Dextrose, Agar), antifungal drugs (Clotrimazole, Ciprofloxacin & Azithromycin), Autoclave, Incubator, test tubes and micropipettes.

Phytotoxic Activity

Lemna minor L. was used to analyze the phytotoxic activities of *Ribes himalense* Decne. following protocols of Abbasi *et al.* [1]. Media composition was prepared (Table 2) and was used for the activity.

30 mg plant extract were mixed into 1.5 ml of ethanol to make stock solution. 10 plants of *Lemna minor* L. with three fronds were placed in 30 ml of medium. Concentrations (10 μ l, 100 μ l, 1000 μ l) were used for positive control medium, and extracts were put into the medium containing plants in petri dishes and were exposed to sunlight for seven days. Fronds number were examined after 7 days and contrast to the positive control. The procedure was repeated 3 times and percentage of inhibition seen was calculated by using following formula.

$$\text{Inhibition \%} = \frac{100 - (\text{Number of fronds in positive control})}{(\text{Number of fronds in negative control})} \times 100$$

Cytotoxic Activity

The cytotoxic activities of *Ribes himalense* Decne. was performed against brine shrimp nauplii, provided by PCSIR laboratories Complex Peshawar. 20 mg of plant extract was dissolved in 2 ml ethanol to prepare stock solution and procedures were adopted after Abbasi et al. [1] & Haq et al. [30]. 38 g of sea salts, dissolved in 1000 ml of distilled water and brine shrimp eggs were placed in container with perforated divider. The eggs were placed on the dark side of the container and the hatching nauplii migrated to the light side, later on. An estimated time of 2 days were given for the hatching eggs. Test tubes were filled with stock solution in different concentrations (62.5, 125, 250, 500, 1000 and 2000 μ g/mL) after 2 days and 10 freshly hatched shrimps were added to each test tube containing different concentrations of stock solution. Magnifying glass was used to see live and dead brine shrimps after 24 hours [31] [32].

Results

Field data were collected from the available sites of *Ribes himalense* Decne. during field visit.

The plant was found at 2 localities Thandiani-1 at 34°42' N latitude, 73°24' E longitude and Thandiani-2 at 34°42' N latitude and 73°47' E longitude at an altitude 1282 m and 1285 m respectively. The data was geo referenced with the help of GPS (Garmin Vista ETrex). Distribution, locality and sub locality maps were developed with the help of ArcGIS software (Table 1 & Figure 1).

Table 1: Showing bacterial strains, type and ATCC#.

S#	Strain which will be used	Type	ATCC#
1.	<i>Escherichia coli</i>	Gram negative	ATCC# 8738
2.	<i>Proteus mirabilis</i>	Gram negative	ATCC# 12553
3.	<i>Streptococcus mutans</i>	Gram positive	ATCC# 35668
4.	<i>Bacillus subtilis</i>	Gram positive	Clinical isolate

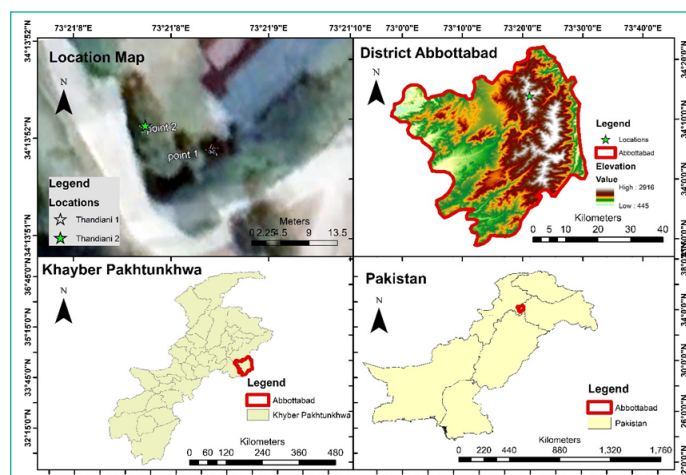


Figure 1: Location Map and geo-referencing of *Ribes himalense* in the study area showing 2 localities i.e., (Thandiani-1 and Thandiani-2).

Table 2: Composition of E-media.

S.No	Salt	Grams
1.	Ferric chloride	0.056
2.	Boric acid	0.0028
3.	Potassium hydrogen phosphates	0.68
4.	Potassium nitrates	1.6
5.	Copper sulphates	0.023
6.	Manganese chlorides	0.0036
7.	Calcium nitrates	1.17
8.	Ethylene diamine tetra acetic acids	0.0113
9.	Manganese sulphates	0.496
10.	Zinc sulphates	0.0023
11.	Sodium molybdates	0.0012

Biological Assay

PCSIR Peshawar was used to carry out biological studies of *Ribes himalense* Decne.

Antibacterial Assay

Four strains of bacteria i.e. *Escherichia coli*, *Proteus mirabilis*, *Streptococcus mutans* and *Bacillus subtilis* were used to study the antibacterial activities. To study the zone of inhibition two replicates were used each for leaves and stem with 100mg/ml concentration. The inhibitory zone for ethanolic crude extract of leaves of *Ribes himalense* was scored as 16, 16, 14 and 17mm against *Escherichia coli*, *Proteus mirabilis*, *Streptococcus mutans* and *Bacillus subtilis* respectively. Maximum inhibition zone of 17mm of leaves extract was noted against *Bacillus subtilis*. For the ethanolic extract of stem of the *Ribes himalense* Decne. also showed the antibacterial activity against *Escherichia coli*, *Proteus mirabilis*, *Streptococcus mutans* and *Bacillus subtilis* which is 15, 18, 13 and 16mm respectively. Maximum inhibition zone of 18mm of stem extract was noted against *Proteus mirabilis*.

Antifungal Assay

Candida albicans was used as a test organism for the antifungal activity of the crude extract of stem and leaves of *Ribes himalense* Decne. and it showed effective results. The ethanolic extract of plant leaves significantly inhibit *C. albicans* fungus with a zone of inhibition of 15mm. The inhibition zone for the stem extract was 12mm. Azithromycin, Ciprofloxacin and Clotrimazole were used as a standard for antifungal activity.

The comparative antimicrobial activity of ethanolic extracts of *Ribes himalense* Decne. and standard antibiotics Azithromycin, ciprofloxacin and Clotrimazole against test microorganisms is shown in the (Figure 2,3 & Table 4).

The ANOVA reveals that significant difference exists among the plant extracts and antibiotics. The leaves extract is more

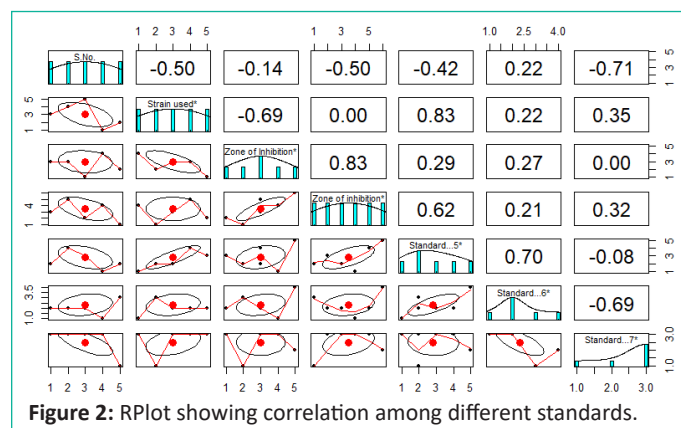


Figure 2: RPlot showing correlation among different standards.

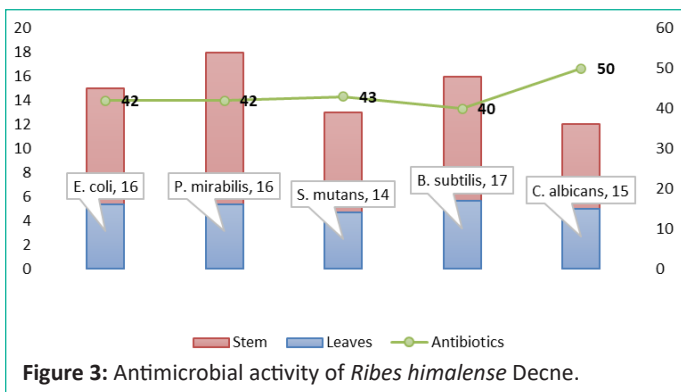


Figure 3: Antimicrobial activity of *Ribes himalense* Decne.

Table 3: Showing Plant Localities, Coordinates, Altitude and Distribution in Pakistan.

Plant species	Locality	Coordinates	Altitude	Distribution in Pakistan
<i>Ribes himalense</i> Decne.	Thandiani-1	34. 231129° 73. 352325°	2703m	Kaghan, Gilgit Agency, Swat, Kalam, Hazara, Changla Gali and Thandiani
	Thandiani-2	34. 23109° 73. 352420°	2704m	

Table 4: Showing strains used, zone of inhibition, concentration and standards.

S.No.	Strains used	Zone of inhibition(mm) at concentration (100mg/ml)		Standards		
		Leaves	Stem	Azith. (25µg)	Cipro. (25µg)	Clot. (30µg)
1.	<i>Escherichia coli</i> ,	16	15	30	43	NA
2.	<i>Proteus mirabilis</i>	16	18	41	43	NA
3.	<i>Streptococcus mutans</i>	14	13	31	43	NA
4.	<i>Bacillus subtilis</i>	17	16	22	40	NA
5.	<i>Candida albicans</i>	15	12	30	50	32.5

Table 5: ANOVA for antimicrobial activities of leaves and stem extracts.

SOV	d.f.	S.S.	M.S.	F-Value	P-Value
Microbes	4	96.889	24.222	1.3490	0.2750
Extracts	2	4874.178	2437.089	135.7290	0.0000
Interaction	8	197.378	24.672	1.3741	0.2476
Error	30	538.667	17.956		
Total	44	5707.111			

Coefficient of variation (CV) = 18.79%, α value @ 0.05

effective than stem extract while all the microbes are equally affected with the application of plant extract. Whereas, the interaction of both factor is non-significant.

Phytotoxic Activity

To evaluate the phytotoxic potential of the leaf extracts of *R. himalense* Decne. a growth bioassay was conducted against *Lemna minor* L. The aqueous ethanolic extracts of *R. himalense* Decne. markedly inhibited the growth of the test plant. The strength of the phytotoxicity of the plant extracts greatly varied by concentration. The growth of *Lemna minor* L. declined with increasing extract concentration (Figure 4,5). At 10 µl extract the growth of inhibition shown 0 and 10% by leaves and stem extract respectively. At 100 µl extract the growth of inhibition shown 30% and 10% by leaves and stem extract respectively. At 1000µl extract the growth of inhibition shown 70 and 100% by leaves and stem extract respectively. Paraquat solution was used as a positive control and growth inhibition of leaves and stem was 100% each. The negative control DMSO showed no phytotoxic activity as shown in the Table 6 (Figure 4 & 5).

Table 6: Showing extract concentration for phytotoxic activities, inhibition by leaves and stem of *Ribes himalense* Decne.

S. No.	Extract Concentrations (µl)	Growth Inhibition by leaves (%)	Growth Inhibition by stem (%)
1.	10	0	10
2.	100	30	10
3.	1000	70	100
4.	Neg. Control Methanol	-	-
5.	Paraquat solution	100	100

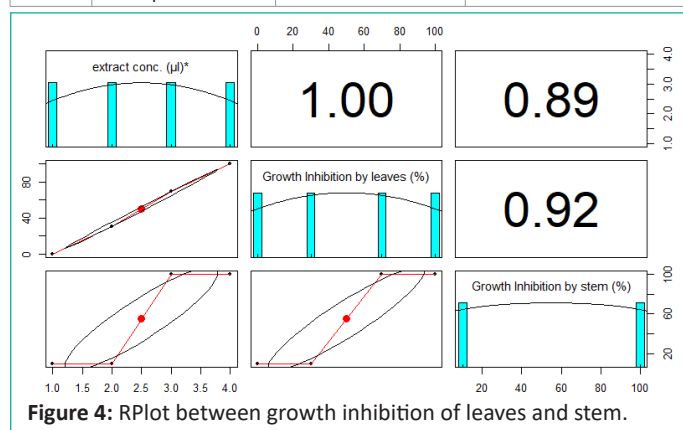


Figure 4: RPlot between growth inhibition of leaves and stem.

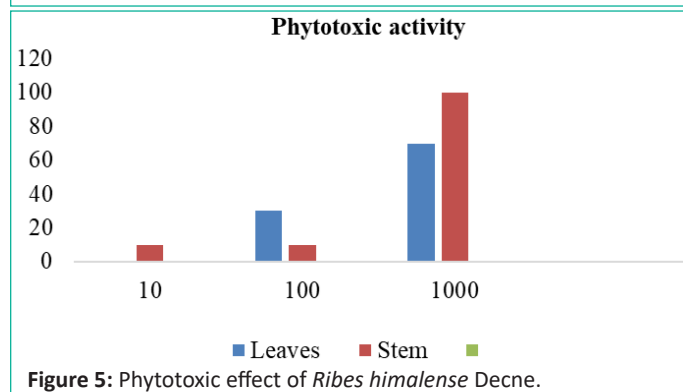


Figure 5: Phytotoxic effect of *Ribes himalense* Decne.

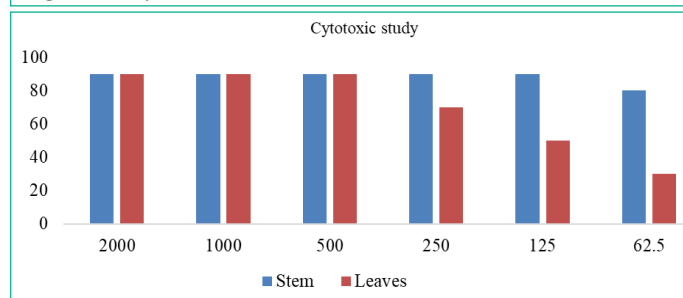


Figure 6: Cytotoxic effect of *Ribes himalense* Decne.

Table 7: Percent mortality of brine shrimps nauplii after 24h (µg/mL).

Sample	% mortality after 24h (µg/mL)					
	2000	1000	500	250	125	62.5
Leaves	90%	90%	90%	70%	50%	30%
Stem	90%	90%	90%	90%	90%	80%

Cytotoxic Activity

Brine shrimp larvae were the test organisms for the current cytotoxicity investigation. Cytotoxic studies of the *Ribes himalense* Decne. was carried out and the result showed that the ethanolic extract of the stem is more cytotoxic than the leaves. At 2000, 1000 and 500 µg/mL ethanolic extract of leaves showed 90% cytotoxic effect of each, at 250, 125 and 62.5 µg/mL cytotoxic effect was 70, 50 and 30% respectively. In case of ethanolic extract of stem at the concentration of 2000, 1000, 500, 250 and 125 µg/mL cytotoxic effect was 90% for each, while 80% cytotoxicity was observed at the concentration of 62.5 µg/mL (Table 7 & Figure 6).

Table 8: ANOVA for different concentrations of leaves and stem extracts for cytotoxic activity.

SOV	d.f.	S.S.	M.S.	F-Value	P-Value
Concentration	5	6125.000	1225.000	639.1304	0.0000
Extracts	1	3025.000	3025.000	1578.2609	0.0000
Interaction	5	3725.000	745.000	388.6957	0.0000
Error	24	46.000	1.917		
Total	35	12921.000			

Coefficient of variation (CV) = 1.75%, α value@ 0.05

The ANOVA shows that there is significant difference among the different treatment means of concentrations and extracts from different portions (leaves and stem). Also, the interaction effect is significant. It reflects that increasing the concentration of plant extract up to certain level (500 $\mu\text{g}/\text{mL}$) will increase the cytotoxicity. The stem extract is more cytotoxic than leaves extract at low concentration (250 $\mu\text{g}/\text{mL}$ and below).

Discussion

Ribes himalense Decne. belongs to family Grossulariaceae which is represented in Pakistan by 1 genus and 8 species. It is found in Kaghan, Gilgit Agency, Swat, Kalam, Hazara, Changla Gali and Thandiani. Moreover, it is very common in NW Himalayas from 2400-4000 m [33]. During the present study the *Ribes himalense* was found at the two localities Thandiani-1 (2703m) and Thandiani-2 (2704m), showing considerable decrease in its population. During the field studies *Ribes himalense* was found at Thandiani-1 and Thandiani-2 during the geo-referencing at 2703m and 2704m altitude respectively. Geo-referencing of the *Punica granatum* in Dir district, Khyber Pakhtunkhwa, Pakistan has been carried out by Ali et al. [34]. Similarly, scientist *i.e.*, Ahmad et al. [35,36] carried out geo-referencing studies of some other important plant species from the other similar ecological zones.

Many researchers carried out work on the antimicrobial activities of various species. They reported that various parts of the plant are showing antimicrobial potential [37,38]. The antimicrobial activities of the *Aloe vera* were studied by Danish et al. [39] and Haq et al. [40], it was noted that the plant extract showed considerable results. It is noted during this study that the ethanolic extracts of leaves and stem of *Ribes himalense* Decne. showed more inhibitory impact on the test organisms. When both extracts were evaluated for antimicrobial potential, both shown different degrees of antimicrobial activity against the tested bacterial and fungal species. The extracts were compared with three standards antibiotics including azithromycin, ciprofloxacin and clotrimazole. The findings presented in this study are supporting the studies carried out by Muktar et al. [41] and Bereksi et al. [42]. The ethanolic extracts of plant leaves and stem significantly inhibit *C. albicans* which coincide with the findings of Rabie et al. [37] and Rong et al. [43].

The present study showed that the extract of *Ribes himalense* Decne. has phytotoxic potential. Similar findings are noted during screening of 81 species for their phytotoxic effects [44]. Results of this study showed similarity with the study of [45]. Cytotoxic activities are recorded both in case of leaves and stem extracts of *Ribes himalense* Decne. Like other plant species investigated by Khor et al. [46] & Nejad et al. [47] also showed cytotoxic activities including leaf extract of *Moringa oleifera*.

Conclusion

The ecological studies showed that the distribution range of the RH has reduced and facing severe ecological issues. The

population has been drastically decreased and no regeneration of *Ribes himalense* at any stage has been noticed in the research area and the plant has facing regeneration issues. The ethanolic extract of leaves and stem of the plant has shown considerable antimicrobial activities. The plant has shown phytotoxic and cytotoxic activity. The various parts of plant including leaves and stem have shown considerable phytotoxic and cytotoxic activities. The research area has rich phytodiversity and it is a tourist, picnic spot and due to anthropogenic activities, the flora of the area is facing severe problems. During the studies it was observed that habitat loss is occurring due to mass scale construction, widening and melting of excess roads. Which has resulted in floral and faunal habitat loss in the research area.

Author Statements

Author Contributions

Conceptualization, N.Z., A.U., and S.U.; software, S.U.; validation, N. Z., and S.U.; resources, N.Z., G. M. S., Z. A., M. S. and writing—original draft preparation, N.Z., S.U. writing—review and editing, A.U., S.U., K. R. and N. Z. visualization S.U., and N. Z.; supervision S.U., A. U., and N. Z.

All authors have read and agreed to the published version of the manuscript.

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