Research Article

Antimicrobial and Cytotoxic Potential of *Haplophyllum gilesii* (Hemsl.) C.C. from Northern Pakistan

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Abstract | Present study was aimed to enlighten the antimicrobial and cytotoxic activity of *Haplophyllum gilesii* (Hemsl.) C.C. a narrow endemic plants of northern areas of Pakistan. The antimicrobial potential of *H. gilesii* extracts in different solvents was assessed using agar well diffusion method against bacterial and fungal strains, while cytotoxic activity was studied in the methanolic extract using brine shrimp's lethality assay. All the extracts showed significant biological activity against Gram positive, Gram negative bacteria and selected fungal strains. Acetone, chloroform and methanolic extracts showed maximum activity i-e 39mm, 44mm and 35mm followed by ethyl acetate 27mm and n-hexane 26mm against microorganisms studied. Standard antibiotics were used as a positive control for bacteria and fungi respectively. The cytotoxic assay results showed that methanolic extracts of stem and root of *H. gilesii* had toxic effects on brine shrimp larvae with LD_{50} values of 116.72 µg/ml and 168.16 µg/ml respectively. The antimicrobial and cytotoxic parameters reported can be considered as quality standards of *H. gilesii* in herbal industry.

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Introduction

The genus *Haplophyllum* A. Juss. having 70 species; with a majority restricted to narrow ranges often as small as a single mountain e.g., a narrow endemic *Haplophyllum telephioides* growing in few mountainous areas of central Anatolia; *Haplophyllum viridulum* present in Fars province of Iran (Townsend, 1986).

Haplophyllum gilesii (Hemsl.) C.C. Townsend belongs to family Rutaceae, herbs to semi-shrub with simple leaves and creamy yellow flowers, shrub branching vigorously with height reaching up to 3 feet. It grows in dry habitats with patchy populations confined to only three localities of Karakoram-Himalayan range of Pakistan i.e. Chupo Das, Juglot and along Karakoram highway at Astore (Alam, 2009; Alam and Ali, 2010).

Many antibacterial agents are available in the market, which can be evolved by efforts of the amazing scientists. But nevertheless the microbes are challenging the scientists via growing the resistance to the presently available drugs. Plants are known to produce a selection of compounds to defend themselves against a wide variety pathogenic attack and therefore considered as potential source for various classes of antimicrobial agents. (Sridhar et al., 2012). The indiscriminate use of antimicrobial agents for the treatment of infectious diseases due to pathogenic microorganisms has developed resistance against bacteria, fungi and an extensive variety of

antibiotics (Cowan, 1999).

Haplophyllum species were used in Iraq for treatment of wounds. The decoction was used as a cure in stomachache for children, have an activity on central nervous system. The leaves of these plants were given to children as an infusion with vinegar for the treatment of convulsion and other nervous disorders. *Haplophyllum tuberculatum* was used traditionally in Algeria for many complains as antiseptic, for injuries and ulcers, as calming, hypnotic neurological, for infertility, diabetes, bloating, fever, liver disease, rheumatism, as vermifuge, for obesity, constipation, colon, diarrhea, gases, hypertension, menstrual pain, cardiac disease, scorpion stings, flu, vomiting, throat inflammation, tonsillitis, cough and loss of appetite. In the north of Oman, the juice expressed from the leaves was used as a remedy for headaches and arthritis. In Saudi Arabia, *Haplophyllum tuberculatum* was used traditionally for headaches and arthritis, to remove warts and freckles from the skin and to treat skin discoloration, infections and parasitic diseases. In Sudan the herb was used as an antispasmodic, to treat allergic rhinitis, gynecological disorders, asthma and breathing difficulties. (Al-Snafi, 2018).

No previous work has been done on the *H. gilesii* in Pakistan. In Pakistan this species has not yet been explored pharmacologically.Comprehensive pharmacological review of the other members of the genus *Haplophyllum* having vast antimicrobial, antioxidant, cytotoxic, cardiovascular and anti-inflammatory effects the present study was aimed to screen the antibacterial, antifungal and cytotoxic potential of *H.gilesii* (Hemsl.) C. C.

Materials and Methods

Extraction

The plant material (aerial parts) was washed thoroughly with distilled water, then dried (shade dry) and grinded to make fine powder with the help of an electrical grinder. About 600 grams of dried powder was soaked in 2 liters of methanol in the extraction flasks. This mixture was kept at 24°C in dark for one week and shaken twice each day. The methanolic extract was filtered with the help of Whatman filter paper No. 1 and residues were mixed with 500 mL methanol and the same procedure was repeated for three times. The filtrate was dried at 45°C under vacuum pressure in a rotary evaporator. Same technique was applied for the acetone, ethyl acetate, chloroform and n-hexane extracts. (Seidel, 2006; Handa, 2008). Crude extracts prepared in methanol, acetone, ethyl acetate, chloroform and n-hexane were further diluted in ethanol.

Antimicrobial assay

Selected concentrations of crude plant were 100mg/ml, 150mg/ml and 200mg/ml. Standard antibiotics (as shown in Table 1) have been used for positive control. Powdered drugs had been correctly weighed and dissolved in the appropriate dilutions to the desired concentration of 200mg/mL.

Agar-well diffusion method was employed to assess the antimicrobial assays. Mueller Hinton agar was used for media preparation (Carron et al., 1987).

Test microorganisms

Gram positive, Gram negative and fungal strains had been chosen on the basis of their clinical and pharmacological significance. The authenticated bacterial strains were acquired from Veterinary Research Institute (VRI) Peshawar and Department of Microbiology Hazara University Mansehra. Three strains of Gram-positive bacteria were *Enterococus faecalis*, *Bacillus subtilis* and *Staphylococcus aureus* and six strains of Gram-negative bacteria were *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Shigella flexeneri*, and *Salmonella typhi* and two fungal strains *Candida albicans* and *Candida glabrata*. Microorganisms had been cultured and maintained over nutrient agar media at 4^0C.

Cytotoxicity (Brine shrimp lethality assay)

Brine shrimp lethality assay was carried out by adopting the techniques as described by (Att-ur-Rehman et al., 2001) to discover the anticancer potential of the plant.

Requirements

Eggs of *Artemia salina* (Brine shrimp's eggs), Sea salt (3.8g/L), Distilled water (PH 7.4), Hatching tray, Aluminum foil, Micro pipette, vials, ethanol.

Hatching of brine shrimps eggs

The hatching tray was unequally divided by means of a perforated partition. Eggs had been sprinkled over the solution (Sea salt (3.8g) + 1000ml Distilled water) in small quantity and covered with dark carbon paper or

Table 1: *Mean zone of Inhibition 200mg/ml concentration.*

Test Microorganisms	Mean zone of Inhibition in mm + SD					
Fungi	**Methanol**	**Acetone**	**Ethyl acetate**	**Chloroform**	**n-hexane**	**Antibiotics**
Candida albicans	28 ± 1	37± 1.5	27 ± 2	37± 1	21 ± 1	Clotrimazole (30)
Candida glabrata	27 ± 1	29± 1.15	26 ± 1.5	44± 1.5	24 ± 0.5	Flucanozole (30)
Gram positive bacteria						
Enterococus faecalis	25.6± 1.5	30 ± 1.1	18 ± 0.5	27 ± 1.15	19 ± 0.5	Ampicillin (25)
Bacillus subtilis	28 ± 1	26 ± 2	22± 0.5	30 ± 1	27 ± 0.5	Ciproflaxacin (40)
Staphylococcus aureus	31 ± 1	39 ± 1.5	24± 1	23 ± 1	20 ± 1	Tetracyclin(25)
Gram negative bacteria						
Escherichia coli	29 ± 1.1	37 ± 1.5	22± 1	29 ± 1.5	25 ± 1	Cephalosporin (20)
Enterobacter cloacae	29 ± 1	21 ± 1.5	24± 1	23 ± 1.15	21 ± 0.5	Cephalosporin (20)
Pseudomonas aeruginosa	24.6± 1.1	34 ± 1	21± 0.5	21 ± 1	21 ± 0.5	Cephalosporin (35)
Vibrio cholera	27 ± 1	28 ± 1	21± 1	24 ± 1.5	21 ± 1	Tetracyclin (35)
Shigella flexeneri	31 ± 1	33 ± 1.5	23± 1	20 ± 1	19 ± 0.5	Cephalosporin (40)
Salmonella typhi	34.6 ±1.5	30 ± 1	19± 1.5	26 ± 0.5	20 ± 1	Azithromycin (35)

Table 2: *Brine shrimp lethality assay LD_{50} of Haplophyllum gilesii.*

S/No	Sample	No of deaths /30 larvae			LD_{50}
		100ppm	500ppm	1000ppm	
1	MeOH extract Hg (stem):	15	20	26	116.72
2	MeOH extract Hg (root):	13	21	23	168.16

aluminum foil to create darkness. The tray was placed under the lamp, when the eggs hatched out the larvae swam actively and migrated to the illuminated part of the tray.

Test sample preparation

Stock solution was prepared by dissolving 20mg of methanolic extract of a plant material (stem and root) in 2 ml of ethanol. From this stock solution 50, 250 and 500 ppm was transferred into the vials (3 vials/ concentration). Solvents in all the vials had been allowed to evaporate overnight and the residue was resolublized in 2ml of seawater. 10 larvae/vial had been placed using Pasteur pipette. The final volume was made upto 5 ml with seawater this made the final concentration of 100ppm, 500ppm, and 1000 ppm respectively, and incubated at 25^0-27^0 C for 24 hours under the light. Other vials had been supplied with solvent (ethanol) for negative control and reference cytotoxic drug for positive control. After 24 hours survivors in each vial had been counted with the help of magnifying glass.

Statistical analysis

Probit analysis was performed by using biostata software for calculation of LD_{50} values (Barkatullah et al., 2011).

Results and Discussion

The secondary metabolites produced by medicinal plants constitute a source of bioactive substances and nowadays scientific interest has increased due to the search for new drugs of plant origin.

Haplophyllum gilesii is an endemic plant native to Gilgit and Baltistan (Pakistan). No previous work has been reported regarding antimicrobial activities from Pakistan. The antimicrobial activity of the five extracts of the *Haplophyllum gilesii* were analyzed for three Gram positive, six Gram negative bacteria and two fungi by determining their zone of inhibitions values. All the extracts showed a significant activity against Gram-positive bacteria, Gram-negative bacteria and fungi. (Table 1)

Antibacterial activity

In antibacterial activity, the acetone extract was found significantly active against *Staphylococcus aureus*, *Enterococus faecalis* and *Bacillus subtilis* (Gram positive bacterial strains). Methanolic extract showed an excellent activity against *Staphylococcus aureus* followed by *Bacillus subtilis* and *Enterococus faecalis*. Chloroform extract exhibited very good activity

against *Bacillus subtilis* followed by *Enterococus faecalis* and mild activity against *Staphylococcus aureus*. Similarly, ethyl-acetate extract showed good activity against *Staphylococcus aureus* and *Bacillus subtilis* and mild activity against *Enterococus faecalis*. n-hexane extract showed good activity towards *Bacillus subtilis* and mild activity towards *Staphylococcus aureus* and *Enterococus faecalis* as shown in Table 1.

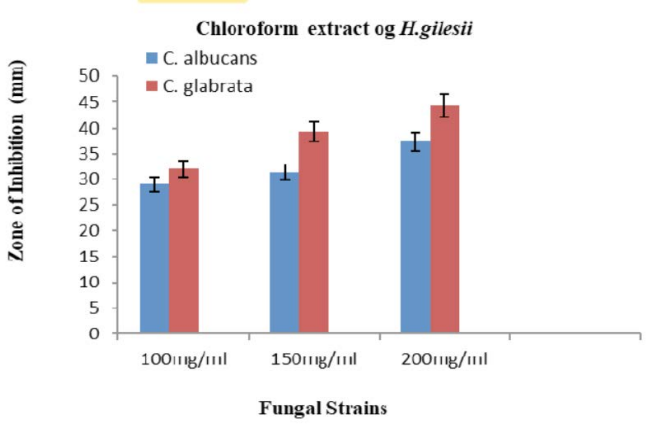

Figure 1: *Antifungal activity of Haplophyllum gilesii (Chloroform extract).*

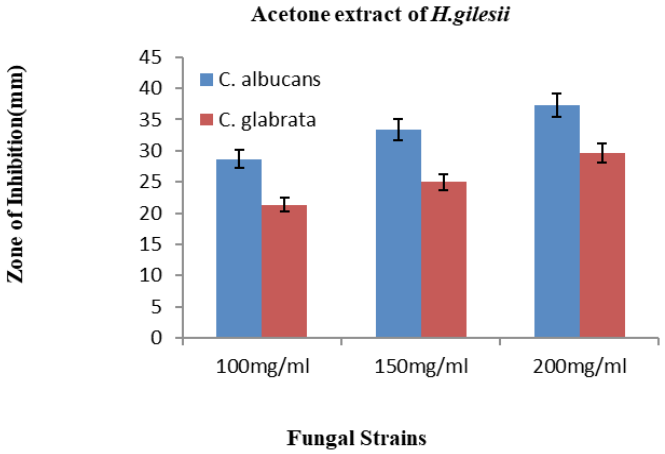

Figure 2: *Antifungal activity of Haplophyllum gilesii (Acetone extract).*

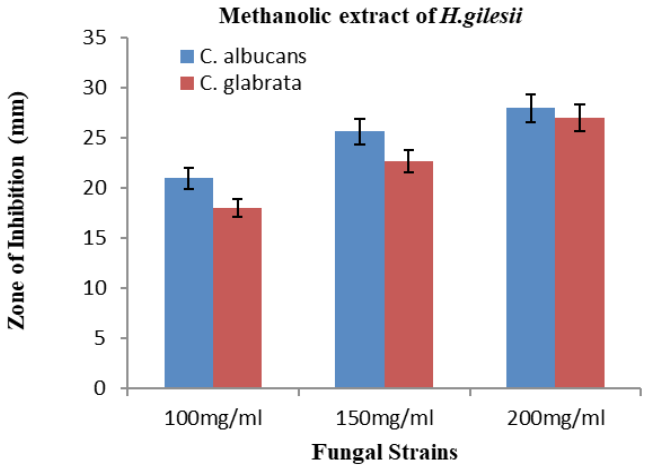

Figure 3: *Antifungal activity of Haplophyllum gilesii (Methanolic extract).*

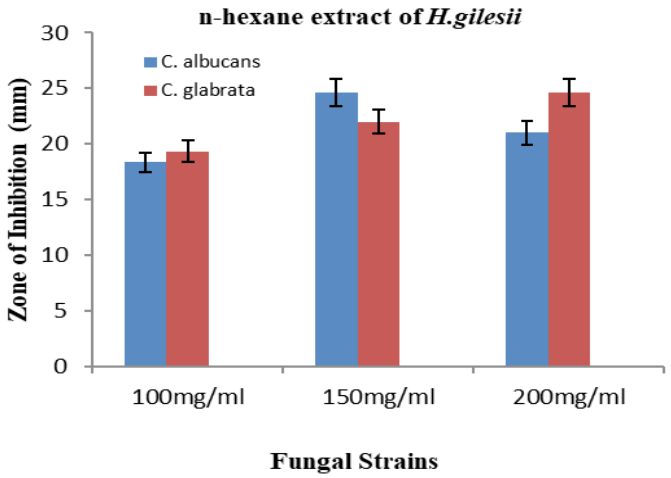

Figure 4: *Antifungal activity of Haplophyllum gilesii (n-haxane extract).*

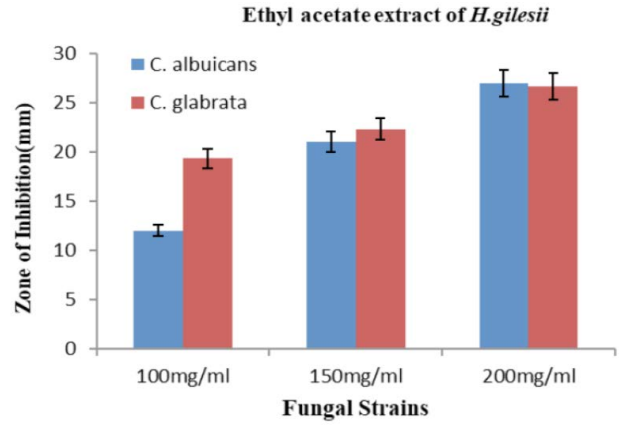

Figure 5: *Antifungal activity of Haplophyllum gilesii (Ethyl acetate extract).*

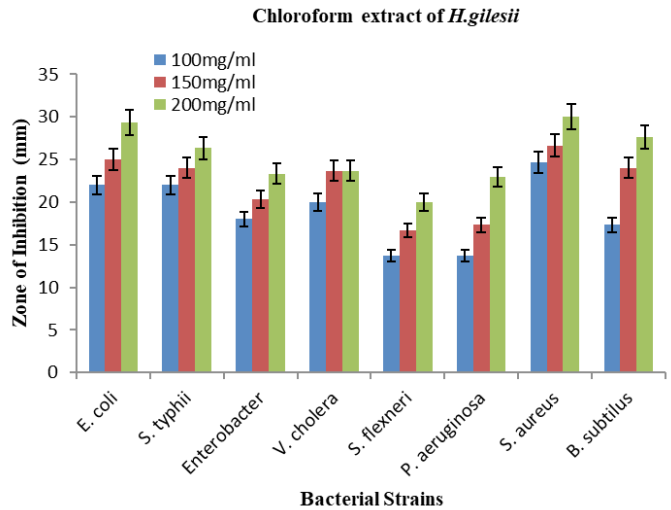

Figure 6: *Antibacterial activity of Haplophyllum gilesii (Chloroform extract).*

Among Gram negative bacteria acetone extract showed a most pronounced activity against *Escherichia coli* followed by *Pseudomonas aeroginosa*, *Shigella flexeneri*, *Salmonella typhi*, *Vibrio cholera* and *Enterobacter cloacae* respectively. Methanolic

extract showed an excellent antibacterial potential against *Salmonella typhi* followed by *Shigella flexeneri*, *Escherichia coli*, *Enterobacter cloacae*, *Vibrio cholera* and *Pseudomonas aeroginosa*.

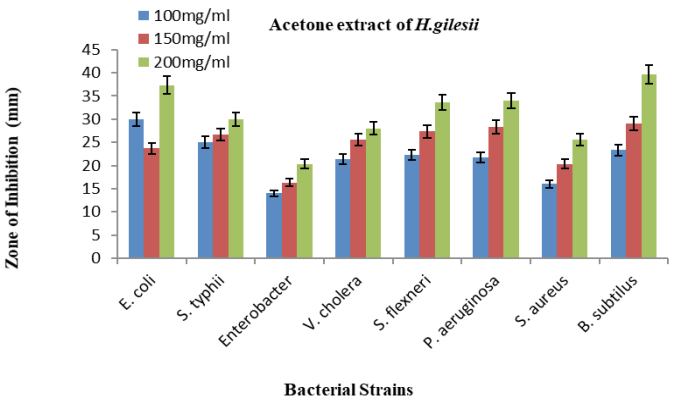

Figure 7: *Antibacterial activity of Haplophyllum gilesii (Acetone extract).*

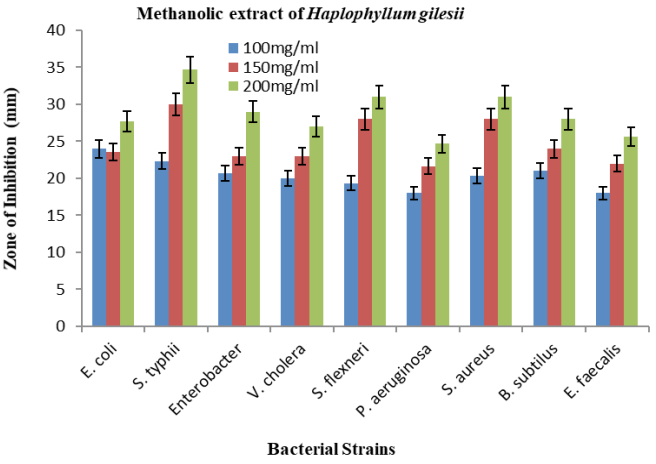

Figure 8: *Antibacterial activity of Haplophyllum gilesii (Methanolic extract).*

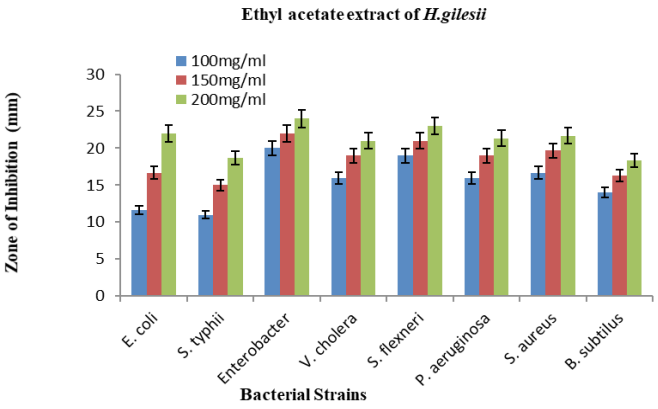

Figure 9: *Antibacterial activity of Haplophyllum gilesii (Ethyl acetate extract).*

Chloroform extract showed a significant activity towards *Escherichia coli*, *Salmonella typhi Vibrio cholera*, *Enterobacter cloacae*, *Pseudomonas aeroginosa* and*Shigella flexeneri*.

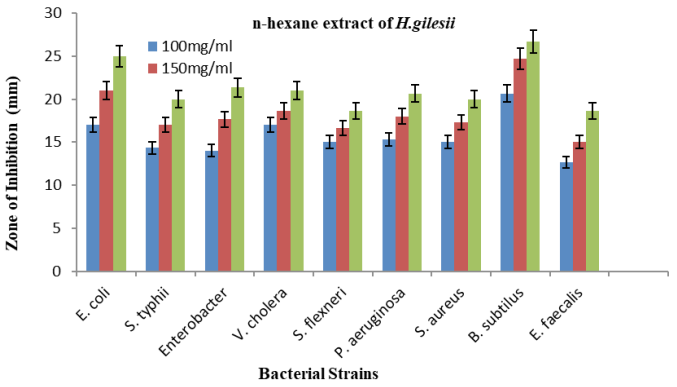

Figure 10: *Antibacterial activity of Haplophyllum gilesii (n-hexane extract).*

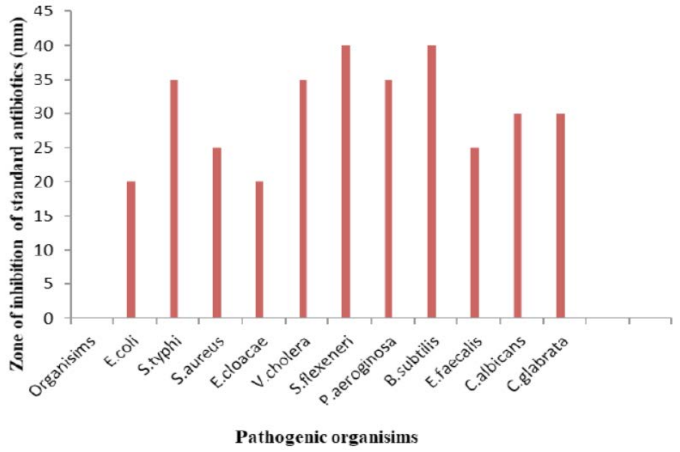

Figure 11: *Zone of Inhibition of standard antibiotics (Positive Control).*

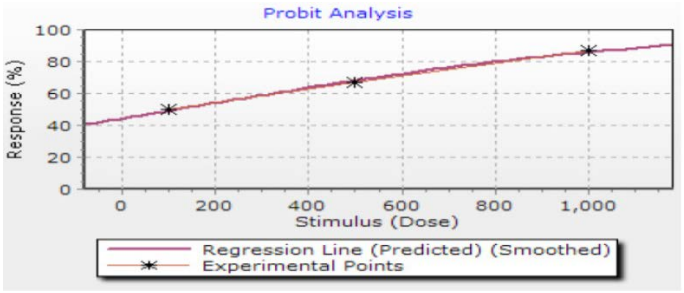

Figure 12: *Cytotoxic activity of Methanolic extract of H.gilesii (Stem).*

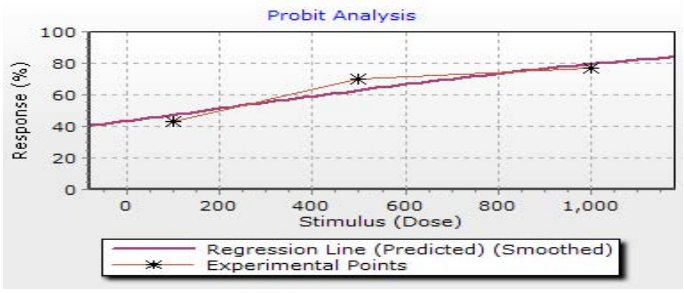

Figure 13: *Cytotoxic activity of Methanolic extract of H.gilesii (Stem).*

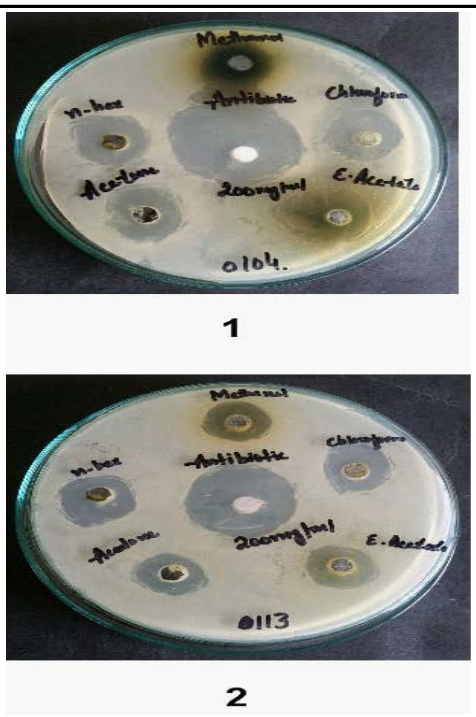

Plate I: *Antibacterial activity of Haplophyllum gilesii.*

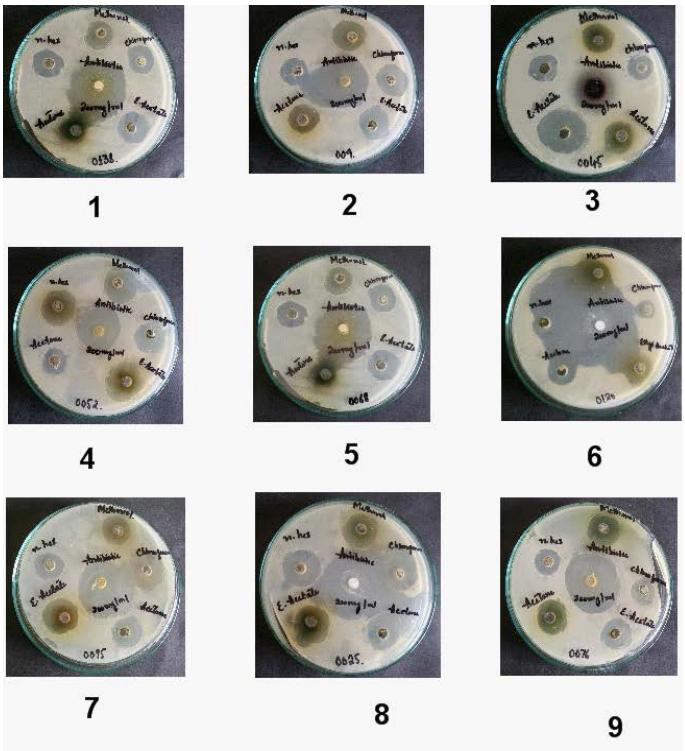

Plate II: *Antibacterial activity of Haplophyllum gilesii.*

Similarly, ethyl acetate extract exhibited a good activity against *Enterobacter cloacae*, *Shigella flexeneri* and *Escherichia coli* and moderate activity against *Pseudomonas aeroginosa*, *Vibrio cholera* and *Salmonella typhi*.

n-hexane extract showed moderate activity against *Escherichia coli*, mild activity against *Enterobacter cloacae*, *Pseudomonas aeroginosa*, *Vibrio cholera* and mid to weak activity against *Salmonella typhi* and *Shigella flexeneri* as shown in Table 1.

Figure 14: *Haplophyllum gilesii (Hemsl.) C.*

Figure 15: *Haplophyllum gilesii (Hemsl.) C.C.*

Al-Burtamani et al. (2005) performed the antimicrobial activities of the essential oils of *Haplophyllum tuberculatum* from Oman. They revealed that 10µl of *Haplophyllum tuberculatum* oil partly inhibited the growth of *Escherichia coli*, *Salmonella choleraesuis*, *Bacillus subtilis*, and *Candida albicans* which is comparable with that of 0.10 µg of gentamycin or 0.05µg of miconazole. While, the oil was ineffective against to *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. (Perez et al., 1999; Costa et al., 2000).

Antifungal activity

In current study, as shown in Table 1, chloroform extract showed the most promising activity against the *Candida glabrata* and *Candida albicans* while the

acetone extract showed the significant activity against both the fungal strains studied. Similarly, methanolic and ethyl-acetate extract showed a good activity against *C. glabrata* and *C. albicans*. n-hexane extract showed good to mild activity towards both the fungal strains as shown in the Table 1.

(Singh et al., 2002) reported that the antifungal assay, the oil of *Haplophyllum tuberculatum* confirmed weak antifungal activity against *Alternaria alternata*, *Stemphylium solani*, *Curvularia lunata*, *Fusarium oxysporium*, and *Bipolaris sp*. However, *Curvularia lunata* and *Bipolari* ssp. had been more liable to the poisoning effect of the oil at higher doses. The presence of monoterpene hydrocarbons in agar medium has been confirmed to inhibit the mycelia growth of *Curvularia pallescens* and *Fusarium oxysporium*. The antimicrobial activity of *Haplophyllum tuberculatum* growing in Libya was studied by (Sabry et al., 2016). Ethanolic extract of the aerial parts of *Haplophyllum tuberculatum* showed a significant anti-fungal activity against *Aspergillus fumigates*, *Geotricum candidum* and *Syncephalastrum racemosum* with (MIC 0.49, 0.12 and 1.95 µg/ml) (Al-Snafi, 2018).

Cytotoxicity

Current study reveals that methanolic extracts of stem and root of *Haplophyllum gilessi* showed a significant cytotoxic activity with LD_{50} values of 116.72 and 168.16 respectively as shown in (Table 2).

The shrimp lethality assay was proposed by (Michael et al., 1956) and further confirmed by (Vanhaecke et al.,1981; Sleet and Brendel, 1983). It was based on the capability to kill laboratory-cultured *Artemianauplii* (brine shrimp) larvae. Brine shrimp lethality assay is considered as a beneficial tool for preliminary assessment of toxicity. (Solis et al., 1993).

(Sabry et al., 2016) proposed the GC/MS analysis and cytotoxic activity of *Haplophyllum tuberculatum* essential oils against lung and liver cancer cells. Essential oils of *H. tuberculatum* at different concentrations (0-50 µg/ml) in DMSO were tested for cytotoxic activities against human tumor cell lines.

Conclusions and Recommendations

Our findings revealed that studied plant has significant antimicrobial and cytotoxic potential. On the basis of these results *H. gilesii* appears to be good and safe natural antimicrobial agent and brine shrimp lethality assay confirms that further studies can be done on various cancer cell lines, it could be of significance in human therapy as anticancer agent. Further studies should be done to search new compounds from *Haplophyllum gilesii*.

Author's Contribution

Saleha Ashfaq and Nazish Bibi, Performed the experimentation, calculations and draft writing. Manzoor Hussain, Supervised the project. Sabi-Ur-Rehman, Verified the analytical methods and Performed the result interpretation and calculation on biostata software. Jan Alam, Helped in plant Collection and identification. Muhammad Junaid, Supervised the findings in antimicrobial assay.

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