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EVALUATION OF PHYLLOSPHERIC BACTERIAL COMMUNITY IN SOME MEDICINAL PLANTS AND THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT

Phyllospheric bacterial population from medicinal plants viz. *Withania somnifera*, *Lantana camara*, *Solanum nigrum* and *Ocimum basilicum* was examined. Endo and epiphytic bacteria were isolated on Luria Bertani (L.B.) agar medium from healthy tissues of medicinal plants. Different epiphytic and endophytic bacterial species showed different colonization frequencies. In epiphytic bacterial species, *Ensifer ahhaerens* had the maximum colonization frequency (67%), followed by *Azotobacter chroococcum* (54%), *Micrococcus lylae* (47%) and *Kurthia zopfii* (31%). While in case of endophytic study, *Agromonas oligotrophica* (100%), *Xanthobacter flavus* (75%), *Lampropedia hyalina* (46%), *Microbacterium lacticum* (40%), *Kurthia zopfii* (40%) and *Phenylobacterium immobile* (31%) showed maximum frequency. Furthermore, the antimicrobial activity of endophytes and their diversity in medicinal plants is concisely deliberated. Eight species of endophytic bacteria were found to produce antagonistic activity against pathogenic fungi and bacteria by plate assays. Results showed that endophytic bacterial isolates was antagonistic to fungi by agar diffusion method and bacteria by well diffusion technique. *In vitro*, the antimicrobial role gave fruitful results to open the way for complementary study in order to identify and purify the active compounds. In conclusion, endophytic bacteria from the four medicinal plants have antagonistic potential.

Keywords: Antagonistic activity**,** bacteria, endophytes, epiphytes, medicinal plants.

INTRODUCTION

Plants are in contact with different bacteria offered by the wind, provided via the water pattern, and enrolled to their roots and leaves from the soil. Many of these microbes are unable to start their life-cycle in connection to a living plant. The ultimate results plant-microbe communications is updated by variety and micro-organism genotypes and by the ecological perspective (Andrews and Harris, 2000). All land vegetation grows in friendly connection to multifaceted microbial areas. These affix to, and occupy, both rhizosphere and phyllosphere as epiphytes or endophytes. Plant-derived exudates and produced additional metabolites are suggested as a factor in motivating specific microbial colonization (Mukhtar *et al.,* 2010). The phyllosphere of plant leaf is a multifaceted terrestrial habitat which contained the diversity of many microorganisms as well as yeast filamentous bacteria and fungi. Microorganisms found on host surfaces are called epiphytes while if these make the colonies in the internal tissues of the hosts are called endophytes. The concurrence of theses microorganisms may show a vital part in health and protection of plant and also microbial biodiversity (Hawksworth and Rossman, 1997). Phyllospheric microbial communities enhance the defense of the host plants and improve their fitness in a given environment under certain stresses. The existence of epiphytic and endophytic microorganisms improves the plant growth, plant health and population dynamics (Hallmann *et al.,* 2007). Plant metabolites are novel antimicrobial compounds (Tan and Zou, 2001). Endophytes or epiphytes may fabricate naturally active plant products into secondary metabolites which are strong bacteriocide, anticancer and fungicides (Stierle *et al.,* 1993; Gunatilaka, 2006). Some endophytic bacteria isolated from younger radishes can be used as bio-control suppliers against phyto-pathogens (Seo *et al*., 2010). Castillo *et al*. (2002) analyzed that the endophytic *Streptomyces* sp. NRRL 30562 obtained from snake vine produces novel peptide medications that have extensive action against many pathogenic bacteria. Endophytic bacteria are viewed as prospective source to obtain anti-microbial agent for bio-control and medication use. Relevant literary works highlights strategies and strong proof for density-dependent connections among microbial tissues (Pierson *et al.,* 1998).

The objectives of this study were to (1) categorize the epiphytic and endophytic species of the bacteria and to calculate the frequency of colonization their range pattern related to leaves of four important medicinal plants and (2) to understand the role for medicinal potential of the different plants and their antimicrobial activity against different plant pathogens. We performed the *in vitro* antagonistic test against diversity of pathogenic micro-organisms to assess the strength of some endophytes of the selected plants which initially had shown the inhibitory tendency.

MATERIALS AND METHODS

Medicinal Plants

Four medicinal plant species chosen for the study were *Withania somnifera* (Ashwagandha), *Lantana camara* (Lantana), *Solanum nigrum* (Makoo) and *Ocimum basilicum* (Niazbo) (Table 1). The fresh and healthy leaves of these plants were arbitrarily gathered from different locations around University of the Punjab, Lahore, Pakistan. The leaves from every plant were put independently into sterile container and then brought to laboratory for isolation of endophytic phyllosphere and epiphytic harmful bacteria.

Table 1. Detail of medicinal plants used in phyllospheric bacterial assessment.

Common name	Botanical name, family and description	Medicinal importance
Ashwagandha	*Withania somnifera* (L.) Dunal (Solanaceae) **Description:** A perennial shrub, usually 30-60 cm but can grow up to 170 cm.	Leaves, berries and tubers are used as sedative or calming agent, liver tonic, anti-inflammatory, antioxidant etc**.**
Lantana	*Lantana camara* L. (Verbenaceae), **Description:** It is a common low, erect or subscandent, vigorous shrub which can grow to 2 - 4 meters in height.	Leaves and roots are used to relieve itching, flu, colds, coughs, fevers, yellow fever, dysentery and jaundice, gonorrhea etc. Lantana oil is used for skin itches, leprosy and scabies.
Makoo	*Solanum nigrum* L. (Solanaceae) **Description:** Common herb or short-lived perennial shrub. It has a height of 30–120 cm.	The juice of the plant is effective in ulcers, dysentery, stomach complaints, fever, asthma, whooping cough and other skin diseases. The fruits are used as a tonic, laxative, appetite stimulant; and also for treating tuberculosis and mouth ulcers Its also inhibit growth of cervical carcinoma
Niazbo (Basil)	*Ocimum basilicum* L. (Lamiacaeae) **Description:** Common herb, grow to a size of 1-2 feet in height.	As folk remedy, it is used in enormous number of ailments, including boredom, cancer, convulsion, deafness, diarrhea, epilepsy, gout, impotency, insanity, nausea, sore throat, toothaches, and whooping cough and also used as an insect repellent.

Separation of endophytic and epiphytic bacteria

For isolation of epiphytic microflora, 10 grams leaves from each plant were soaked in 100 mL distilled water for one hour. Aliquot of 500 µL from leaf cleanse was plated on L.B medium (g L^{-1}): bacterial isolation was done by using the Beef Extract, 3.0; Peptone, 5.0m and Agar, 15.0. For endophytic micro-floral isolation, plant leaves (10 g) were washed by the running water followed by surface-sterilized for 1 minute in 70% ethyl alcohol, 3 minutes in 2% Sodium hypochlorite ($NaClO_2$) and two to three times washing in sterile distilled water. Blender was used to grind the sterile leaves and also used 100 mL water which was distilled sterile to form leaf solution. The mixture of the leaves (500 µL) was plated on medium plates. The sample inoculated plates were incubated at 37 °C for 24 hours. The bacterial colonies were counted and purified for classification.

Sørensen's quotient of similarity (*QS*) was designed for similarity of fungal /bacterial assemblages in leaf interiors and on leaf surfaces (Sørensen, 1948)

$$QS = 2a / (2a + b + c)$$

Where

a = Number of common species.

b & *c* = Numbers of spp. specific to the interior surface, correspondingly. The Sørensen index is an easy method to evaluate beta diversity, which range from a value of 0 where there is no varieties overlap between the communities to a value of 1 when exactly the same varieties are discovered in both communities (Mukhtar *et al.,* 2012). The comparative abundance/ frequency (%) of microbial strains separated by dilution plating was also measured as: (Number of colonies of a fungal species/ total count of fungal colonies) × 100.

Morphological taxonomy of bacterial isolates

Isolated species was inoculated on LB agar plates and incubated at 37 °C for 24 hours then distinct individual colonies purified by streaking on a new agar plate. Identification of bacterial species was done by recording morphological characters, e.g., cell shape, growth rate, motility, colony color and colony morphology, The purified colonies were subjected to gram staining and characterized using biochemical tests and consulting the pertinent literature (Holt *et al.,* 2000, Koneman *et al.,* 1997, Benson, 1996).

Target pathogenic micro-organisms

The fungal and bacterial strains, *Fusarium oxysporum* (FCBP 1020), *Aspergillus nidulans* (FCBP 1121), *Alternaria alternata* (FCBP 1174), *Drechslera halodes* (FCBP 1133), *Pseudomonas syringae* (FCBP 010), *Corynebacterium minutissium* (FCBP 137), *Xenorhabdus luminescens* (FCBP 119) and *Xanthomonas axonopodis* (FCBP 001) were used for testing the antimicrobial activity. The microorganisms used in this investigation were obtained from the First Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences. The fungal strains were maintained at malt extract agar (MEA) and bacterial strains maintain on L.B agar media at 4 ºC. For the antimicrobial assay, seven days old fungal and one day old bacterial cultures were used.

Preparation of Bacterial Suspension

The antagonistic and pathogenic strains were grown on L.B agar media plates separately, incubated at 37±2 ºC for 24 h. The inoculum of each strain was prepared by adding 5 mL of a sterile saline solution (0.85% NaCl) to the Petri dishes. The cultures were scraped with a glass rod and the suspensions homogenized by agitation in a Vortex mixer. The amount of inoculum was measured in a spectrophotometer and adjusted with sterile saline solution (OD600 = 0.1 was equivalent to 1×10^8 colony forming units (CFU) mL^{-1} (Ali *et al.,* 2012).

Antifungal activity assay

Antifungal activity of bacterial strains against pathogenic fungi was determinate by agar diffusion technique (Khokhar *et al.,* 2011). For testing antimicrobial activity, malt-extract-agar (MEA) medium was used. After solidification of 9 cm Petri plate, agar surface was inoculated with 0.3 mL inoculum of antagonist bacteria. Bacterial suspension was spread evenly on the surface of medium. Then small disc from 7 days old fungal culture was taken by cork borer, each of 0.8cm diameters. Each petri dish was cultured in the center to check the inhibition activity of endophytic bacteria. Each fungal growth was measured after 5 days of incubation at 26 °C. Fungal growth without bacterial inoculum was used as control. The experiment was conducted in 3 replicates for each bacterial species.

Antibacterial activity assay

A bacterial suspension for antagonistic and pathogenic strains from 24 h old culture was used by well diffusion method (Ali *et al.,* 2012). Petri dishes (9 cm) containing L.B agar medium were surface inoculated with 0.3 mL of antagonistic bacterial inoculums. After 15 min inoculation, one well of 0.8 cm diameter was dug out in the agar medium, filled with 0.3 mL of bacterial suspensions. After 24 h incubation at 37 °C, the antibacterial effect was determined by measurement of the inhibition zone diameters. Three replicates of each treatment were also made and Penicillin (3 µg. mL^{-1}) was used as control.

Data analysis

Data were expressed as mean value ± SD. Data were analyzed by Duncan's Multiple Range Test ($P \leq 0.05$) to separate treatments means (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Frequency of phyllospheric bacteria

In the phyllosphere of different medicinal plants, eighteen bacterial species were isolated and identified (Table 2). On *W. somnifera* leaves, five ecto-bacterial (*Kurthia zopfii, Amphibacillus xylanus, Listeria monocytogenes,*

Streptococcus pyogenes, *Streptococcus pyogenes*) and four endo-bacterial (*Caryophanon tenue, Phenylobacterium immobile, Xanthobacter flavus, Lampropedia hyalina*) species were isolated. *O. basilicum* supported *Azotobacter chroococcum, Micrococcus varians* and *Acinetobacter lwoffii* as epiphytic bacteria whereas *Xanthobacter flavus* and *Phenylobacterium immobile* were isolated from endo phyllosphere. *Agromonas oligotrophica* was only purified from endo phyllosphere and *Ensifer ahhaerens* and *Azotobacter chroococcum* from epi phyllosphere of *L. camara*. Three bacterial species (*Microbacterium lacticum, Kurthia zopfii, Azomonas insigins*) were recorded as endophytic and four (*Serratia marcescens, Micrococcus lylae, Arthobacter globiformis, Ensifer adhaerens*) as epiphytic bacteria in the phyllosphere of *S. nigrum*. Consequently, in this study the bacterial density or assemblages between epiphytic and endophytic areas was 0.00 by Sørensen's *QS* equation and shown in table 2. Therefore present results significantly revealed the survival on specific host or habitat between endo and epiphytic bacterial species.

Antifungal test

Eight endophytic bacterial species were screened for their antifungal activity against four pathogenic fungi (Table 3). Experimental results showed that all tested bacterial species show varying degree of antagonistic potential against fungal strains (Figure 1). *C. tenue*, L. *hyaline, A. oligotrophica* and *M. lacticum* showed effective antagonistic potential against *F. oxysporum.* Although in case of *X. flavus, C. tenue K. zopfii* and *A. oligotrophica* colony diameter of *A. nidulans* was effectively reduced. On the other hand, *L. hyaline* and *A. insigins* showed the highest antifungal activity against *A. alternate* with reduction of colony diameter up to 1.9 cm and 2.0 cm, respectively. In addition colony diameter of *D. halodes* was also effectively reduced by *P. immobile, X. flavus* and *L. hyaline*.

Table 2. List of Epi and Endophytic Bacterial species Isolated from Medicinal Plants

Name of weeds	Epiphytic species	Colony Frequency	Colony %age	Endophytic species	Colony Frequency	Colony %age	QS
Withania somnifera	*Kurthia zopfii*	06	31	*Caryophanon tenue*	01	08	0.00
	Amphibacillus xylanus	02	11	*Phenylobacterium immobile*	04	31	
	Listeria monocytogenes	02	11	*Xanthobacter flavus*	02	15	
	Streptococcus pyogenes	02	11	*Lampropedia hyalina*	06	46	
	Acinetobacter lwoffii	07	37				
Lantana camara	*Ensifer ahhaerens*	02	67	*Agromonas oligotrophica*	01	100	0.00
	Azotobacter chroococcum	01	33				
Solanum nigrum	*Serratia marcescens*	02	13	*Microbacterium lacticum*	02	40	0.00
	Micrococcus lylae	07	47	*Kurthia zopfii*	01	20	
	Arthobacter globiformis	03	20	*Azomonas insigins*	02	40	
	Ensifer adhaerens	03	20				
Ocimum basilicum	*Azotobacter chroococcum*	06	54	*Xanthobacter flavus*	03	75	0.00
	Micrococcus varians	02	18	*Phenylobacterium immobile*	01	25	
	Acinetobacter lwoffii	03	27				

Antibacterial test

Out of total eight endophytic bacterial species were screened for their antibacterial activity against four pathogenic bacteria. The inhibition zones ranged from 4.0 cm to 1.0 cm. Results demonstrated that all tested bacterial species show varying degree of antagonistic potential against bacterial strains (Figure 2). *P. immobile* exhibited most effective antagonistic potential with the inhibition zone ranged from 3.0-4.0cm against all pathogenic bacterial strains. In addition *L. hyaline* showed maximum zone against *P. syringae* (3.7 cm), *C. minutissium* (4.0 cm) and *X. luminescens* (4.0 cm) except *X. axonopodis* (2.9 cm). Whereas, *X. flavus* and *A. oligotrophica* were showed effective against *C. minutissium* only with zone of inhibition 3.5 cm and 3.0 cm, respectively. While in case of *C. tenue* was highly active against *C. minutissium* and *X. axonopodis* upto 3.7 cm and 3.9 cm diameters, respectively. Although *M. lacticum, K. zopfii* and *A. insigins* also showed effective antagonistic activity against *P. syringae, X. luminescens* and *X. axonopodis* (Table 4).

Table 3. Colony Diameter of Pathogenic Fungi in cm against Endophytic Bacterial species.

	Fungal Colony diameter (cm)							
Fungal Pathogens	Control	*C. tenue*	*P. immobile*	*X. flavus*	*L. hyalina*	*A. oligotrophica*	*M. lacticum*	*K. zopfii*	*A. insignis*
F. oxysporum	6.00±0.09c	1.60±0.14cd	2.30±0.06bc	3.90±0.05a	2.00±0.04b	1.80±0.16b	1.90±0.05d	3.50±0.16a	3.70±0.16a
A. nidulans	7.00±0.18a	1.80±0.07c	2.40±0.16b	2.00±0.08bc	2.70±0.08a	1.50±0.83bc	3.50±0.03a	1.90±0.18d	2.40±0.05c
A. alternata	6.50±0.05b	3.30±0.04a	3.70±0.05a	2.90±0.12b	1.90±0.14c	2.80±0.05a	3.00±0.01ab	2.90±0.12b	2.00±0.21cd
D. halodes	7.00±0.06a	2.50±0.15b	1.90±0.06c	1.40±0.11c	1.00±0.54cd	2.60±0.11ab	2.80±0.14c	2.50±0.06bc	3.00±0.08ab

* Values with different letters show significant difference (P0.05) as determined by Duncan's Multiple Range Test.

Table 4. Zone of Inhibition of Pathogenic bacteria in cm against Endophytic Bacterial species.

	Zone of inhibition (cm)								
Bacterial Pathogens	Control	*C. tenue*	*P. immobile*	*X. flavus*	*L. hyalina*	*A. oligotrophica*	*M. lacticum*	*K. zopfii*	*A. insignis*
P. syringae	1.00±0.05c	2.40±0.12c	3.80±0.04ab	2.70±0.08b	3.70±0.04b	1.80±0.05d	2.20±0.03c	1.80±0.03d	3.00±0.01a
C. minitissium	1.50±0.01a	3.70±0.08ab	3.30±0.15b	3.50±0.03a	4.00±0.07a	3.00±0.12a	2.00±0.21cd	2.00±0.05bc	2.70±0.19b
X. luminescens	1.20±0.04b	3.00±0.02b	4.00±0.21a	2.50±0.10bc	4.00±0.13a	2.20±0.10b	3.00±0.01ab	2.20±0.14b	3.00±0.13a
X. axonopodis	1.00±0.01c	3.90±0.16a	3.00±0.10bc	2.10±0.04c	2.90±0.09c	2.60±0.11bc	3.30±0.12a	3.70±0.09a	1.90±0.10c

* Values with different letters show significant difference (P0.05) as determined by Duncan's Multiple Range Test.

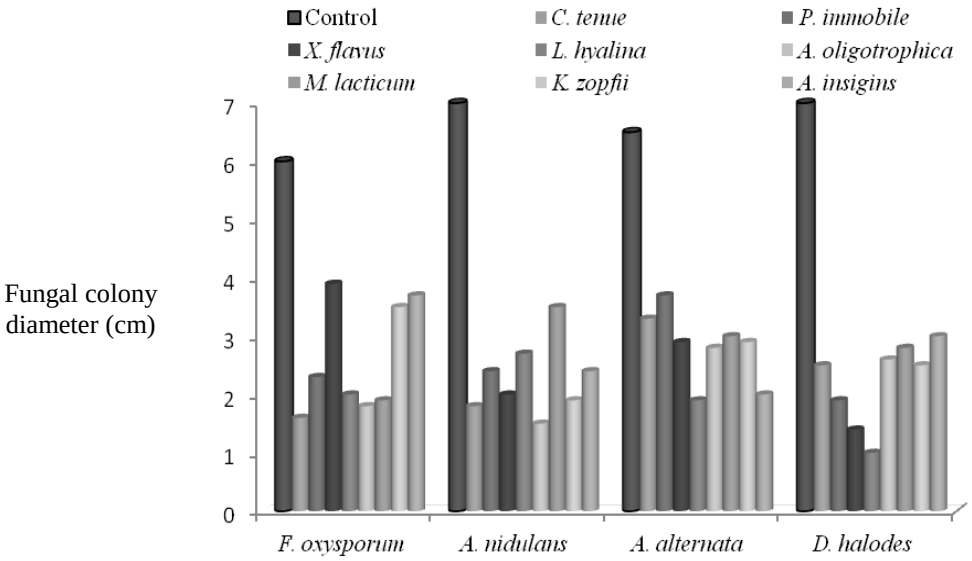

Fig. 1. Antagonistic potential of endophytic bacteria against fungal pathogens.

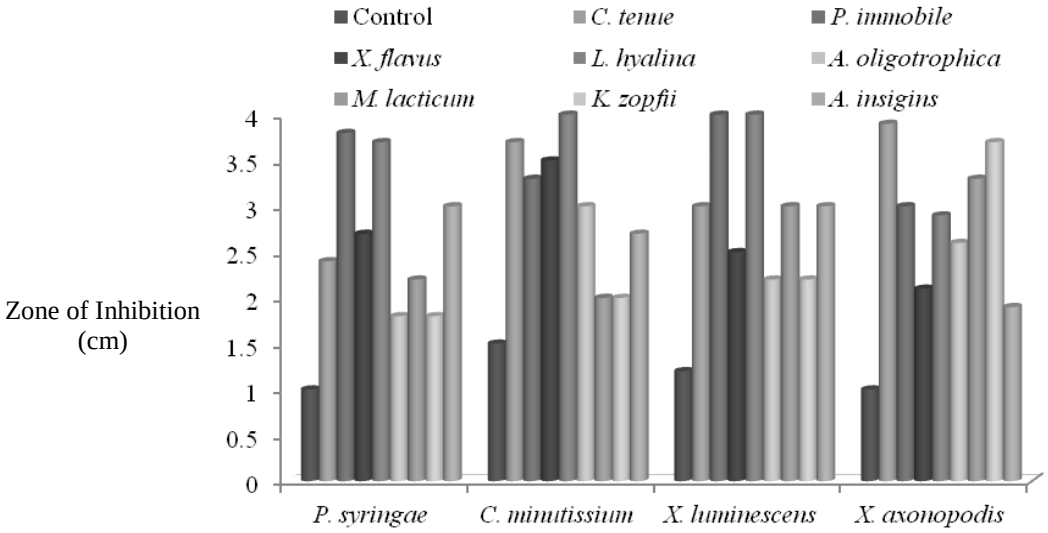

Fig. 2. Zone of inhibition of endophytic bacteria against bacterial pathogens.

Diverse communities of bacteria inhabit plant leaves and roots and those bacteria play a crucial role for plant health and growth. Moreover, Ryan *et al.* (2008) exhibited endophytic bacteria almost every plant on earth. The upper parts of plants as well as flowers, buds, stems, fruits and leaves give a habitation for the microbes named as phyllosphere. In addition, research on the diversity of endophytic microbes is essential for bio-technology and environmental studies. Because of the importance of many phyllosphere microbial population to healthy plant, there will likely be many practical applications that result from a better understanding of the interaction of microbes with plants and among themselves. This enhanced knowledge may play a role also to our understanding of the environment of human-pathogenic microbes on place surfaces and provide new ideas for the development of protection or control strategies to manage pre-harvest pollution of plants with enteric infection (Freiberg, 1999). In this study, eighteen culturable bacterial species associated with the phyllosphere of different medicinal plants were isolated and identified (Table 2). Previously thirteen endophytic bacterial species were identified from roots of *Panax* sp. by Cho *et al.* (2007). Moreover, a variety of bacterial genera was isolated from meristem of genus *Fragaria*. Additionally, diversity of epiphytic action-bacteria was identified from roots of *Triticum* spp. (Justin and Christopher, 2003). Species and number of bacterial strains different from each other and also from their host plants but also in endo and epi sphere. Currently, *Ensifer* strain show dominance over other bacterial endoflora and

Agromonas strain in ectoflora of medicinal plants was observed. Consequently, *E. ahhaerens* had the maximum colonization frequency followed by *A. chroococcum, M. lylae, A. chroococcum* and *K. zopfii* in epiphytic case. On the other hand, endophytic study, *A. oligotrophica, X. flavus, L. hyaline, M. lacticum, K. zopfii* and *P. immobile* showed maximum frequency (Table 2). It is proposed that in this study the endophytic and epiphytic microbial population depends on the collection site and long distance of collection areas. Recently, many innovative microscopic tactics are introducing to study the identification of microbes and their gene expression (Andrews and Harris, 2000). In addition, Durgude *et al.* (2009); Baker *et al.* (2011) found dispersal of endophytic and endophytic microbes based on environment conditions and nutrients availability and these findings are match with our present results. In addition, four important medicinal plants viz; *W. somnifera, L. camara, S. nigrum* and *O. basilicum* were collected for the screening for novel anti-microbial compounds from endophytes. Recently, researchers studied on some medicinal plants for examined the endophytes (Yan *et al.,* 2011) but only a few were focused on endophytic bacteria (Mukhtar *et al.,* 2012; 2010). The current experiment indicated that multifaceted relations excited between experimented spp. in endophytic and epiphytic micro flora with relation to the host plants. The assessment of epiphytic and endophytic phyllosphere by microscopic gave important information on the frequency, distribution and development bacterial flora on leaves. The number of bacterial isolates of phyllosphere varied appreciably between experimented medicinal plants. In present study, table 3 and 4 show the inhibition of pathogenic microbes by endo-phytic bacteria isolated from medicinal plants. Additionally, antagonistic potential and inhibition zone of endophytic bacteria against fungal and bacterial pathogens respectively, also showed in Figure 1 and 2. Furthermore, *L. hyalina* and *P. immobile* exhibited significant results among eight strains and by the previous researches of Raja *et al.* (2011); Yan *et al.* (2011). Many antibacterial agents have been reported and identified from these strains. Phyllosphere bacteria support plant growth and arouse the colonization and tissues infection of plant pathogens (Rasche *et al*., 2006). According to Mukhtar *et al.* (2010) the physical environmental, leaf age, plant spp., availability, leaf position, and condition of immigrant inoculums which have been suggested for determining species of microorganisms in the phyllosphere. In the present study, diversity in epiphytic and endophytic bacteria depicted plant specificity. But in other study by Mukhtar *et al.* (2012) the endophyte assemblages of fir tree and its mistletoe parasite overlapped by less than 15%. Arnold *et al.* (2003) studies also supported the proof for plant liking within the epiphyte and endophyte community. The finding of plant associated microbes in present study are helpful for industrial and agricultural sector and strains of endophytic bacteria play important roles in the antimicrobial mechanism of plants and that they are significant resources for novel antimicrobial agents. In short, list of bacteria examined in this study have significant anti-microbial activity and these strains use as biocontrol agent against plant diseases. From the results, it is concluded that medicinal plants used has precise bacterial population by reference of endo phyllospere and epi phyllospere. The evidence for significant relations of microbial phyllosphere in habitation with agricultural crops may be changed to the condition of natural plant populations their quality and productivity. In an antimicrobial studies Siqueira *et al.,* 2011, Kharwar *et al.,* 2010, Gangadevi *et al.,* 2008, Radu and Kqueen, 2002 they were reported that endophytic bacteria may be considered as a potential source of antagonism. The endophytes produced the bioactive natural compounds which make available the new options to deal with the difficulty of diseases resistance growth by pathogenic bacteria. That is useful source of new therapeutic agents for the successful treatment of diseases in plants, animals and human. To seeing observations which indicated that endophytic bacteria produced by medicinal plants have pharmaceutical potential that can produce antimicrobial compounds. More studies needed to see the active compounds which produced using analytical chemistry and to find more concerning symbiotic role of these bacteria in medicinal plants in order to understand the benefits that these endophytes confer on these medicinal plants.

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