Antibacterial activity of *Lallemantia royleana (Benth.)* indigenous to Pakistan

BY

Seemab Mahmood

2008-NUST-BS V& I-29

Atta-Ur-Rahman School of Applied Biosciences, ASAB

National University of Sciences & Technology, NUST

Islamabad, Pakistan

2012

Antibacterial activity of *Lallemantia royleana (Benth.)* indigenous to Pakistan

A thesis submitted in the partial fulfillment of the requirement for the degree of Bachelors

In

APPLIED BIOSCIENCES

BY

Seemab Mahmood

2008-NUST-BS-V&I-29

Atta-Ur-Rahman School of Applied Biosciences, ASAB

National University of Sciences & Technology, NUST

Islamabad, Pakistan

2012

National University of Sciences & Technology

FORM TH-1

BS (Hons). THESIS WORK

We hereby recommend that the thesis prepared by Seemab Mahmood, Titled: Antibacterial activity of *Lallemantia royleana (Benth.)* indigenous to Pakistan, be accepted in its present form to satisfy the thesis requirement of BS (Hons) degree.

Guidance & Examination Committee Members

Thesis supervisor:

1) Name: Dr. Muhammad Qasim Hayat
   Dept/Qualification: ASAB/PhD
   Address: ASAB, NUST, H-12 Islamabad. Signature: Qasim Hayat.

Thesis Committee Members

1) Name: Dr. Muhammad Ashraf
   Dept/Qualification: ASAB/Post Doc (Molecular cytogenetics)
   Address: ASAB, NUST, H-12 Islamabad. Signature:

2) Name: Dr. Nasar-um-minullah
   Dept/Qualification: ASAB/ PhD
   Address: ASAB, NUST, H-12 Islamabad. Signature:

Head of Department

15-06-2012
Date

COUNTERSIGNED

Date:

Date:

NUST Atta –ur- Rahman School of Applied Biosciences (ASAB), H-12 Islamabad

Dedicated to

My

Beloved

Family to whom

I owe

Everything that

Is

Mine.

ACKNOWLEDGEMENTS

All praises to Almighty Allah, The Creator of all the universe, The Omnipotent and origin of all the wisdom and knowledge. The Sustainor of al will, Who bestowed me to complete my research. All gratitude to the Holy Prophet (SAW) who guided me through the right path and made me realize for who I m and to whom I belong.

I feel honored to express my regards and admiration to my esteemed, skilled, dexterous and learned supervisor, Dr. Muhammad Qasim Hayat, for his keen supervision and thought-provoking discussions. I cannot express that how indebted I feel to have him guide me and showing me the sympathetic attitude. I deem myself to be auspicious to have worked under such an illustrious personality.

I m very thankful to Dr. Muhammad Ashraf, Principal and Professor, Atta-ur-Rahman School of Applied Bioscience (ASAB), for his hard work to keep the milieu friendly for research and study.

I would specially like to thank Shabina Ishtiaq, for helping me throughout my research study. She has been the example of a perfectionist to me.

I am grateful to my friends, Tehreem Jamil and Hafsa Zafar, for being there when I needed them and constantly providing me a helping hand in every situation. And I would like to appreciate all my class fellows for their cheerful company.

In the end I would like to thank my family without whom I would never have been who I m today. And to thank them to bear me during all the dim-witted situations.

Seemab Mahmood

TABLE OF CONTENTS

Title **Page No.**

Chapter-2

Literature Review 7

List of Acronyms

%	percentage
^{o}C	Degree Celsius
AMC	Amoxicillin/Clavulanate
AMR	Anti-Microbial Resistance
ASAB	Atta-ur-Rehman School of Applied Biosciences
Ca	Calcium
CDC	Centre for Disease & Control
Co	Cobalt
Cu	Copper
DMSO	Dimethyl Sulf-oxide
E. cloaceae	*Enterobacter cloaceae*
E.coli	*Escherichi coli*
ESACs	Extended-Spectrum Cephalosporinases
ESBL	Extended-Spectrum β-lactamase
g	gram
HEC	Higher Education Commision
K	Potassium
L. royleana	*Lallemantia royleana*
Li	Lithium
MBL	Metallo-β-lactamase
MDR	Multi drug resistant

Mg	Magnesium
mg/ml	milligram/milliliter
mm	millimeter
Mn	Manganeese
MRSA	Methicillin Resistant *S. aureus*
Na	Sodium
NDM-1	New Delhi metallo-β-lactamase 1
NUST	National University of Sciences & Technology
P. aeruginosa	*Pseudomonas aeruginosa*
Pb	Lead
pH	Power of H^+
PIMS	Pakistan institute of Medical Sciences
S. aureus	*Staphylococcus aureus*
TM	Trade Mark
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

List of Tables

List of Figures

ABSTRACT

Chemical isolates of many medicinal plants have been used as anti-bacterial agents worldwide. Due to increase in antibiotic resistance, it is the need of hour to look for the alternatives. In this research, the antibacterial activity of four extracts (methanol, ethanol, chloroform and aqueous) of *Lallemantia royleana* seeds were evaluated against four bacterial strains (*Escherichia. coli, Enterobacter cloaceae, Pseudomonas aeruginosa* and *Staphylococcus aureus*) for the first time by disc diffusion method. All organic extracts of *Lallemantia royleana* seeds displayed significant anti-bacterial activity against all the test bacteria. No antibacterial activity was observed in case of aqueous extracts. The chloroform extract exhibited highest anti-bacterial activity by giving greater values for zone of inhibition for all strains of bacteria. The seed of the plant demonstrated to be most potent against *S. aureus* infections and gastro-intestinal problems caused by *E. coli* and *E. cloaceae*. Results of the study have shown that *L. royleana* is the paramount candidate for phytochemical studies for the isolation of novel therapeutic compounds.

Chapter 1

INTRODUCTION

1.1 *LALLEMANTIA ROYLEANA*

In *Lamiaceae* family, *lallemantia royleana (Benth.)* is an important annual herb that belongs to the genus *lallemantia.* It is commonly known as "Lady's mantle" in English (Ahmad *et al.,* 2008), 'Tukhum-balango" in our local language (Urdu) and "Nazboo" in Sindhi. It is named Benth. after George Bentham who first described this plant.

1.2 MORPHOLOGICAL CHARACTERISTICS

Lallemantia royleana is an annual, unbranched, or branched herb from the base. Stems are erect, 5-30 cm in size and they are quadrangular, with a thick indumentum of petite hairs. Leaves of the herb are simple with a dimension of 15-20 x 7-15 mm. They also possess short eglandular hairs and speckled sessile oil globules. The herb is 6-8 flowered, have numerous bracts and inflorescence initiates in close proximity to pedestal of stem. The flowers are far-away or adjacent; linear-oblong similar in size to calyx. Calyx is tube-shaped, veined or ribbed and is of 6-7 mm in length with short hairs and oil globules. The upper lip has 3 ovate obtuse lobes; lower lip has 2 narrower lobes and teeth are evidently convergent in fruit and closing the mouth. Flowers are pale lilac, blue to whitish pink, 7-8 mm in size (eFloras.org).

Figure 1.1 *Lallemantia royleana (Benth.)* Plant or 'Tukhum-balangoo' (my.gardenguides.com)

Figure 1.2 *Lallemantia royleana (Benth.)* seeds (http://images.wellcome.ac.uk/)

The seed of *L. royleana* is brownish-black in color and they have a white blotch at the ending. They are about 3 mm in length, 1 mm in breadth and three-angled. Tasteless mucilage is formed by coating of seeds when they are saturated in water. (Morton, 1990).

1.3 GEOGRAPHICAL DISTRIBUTION

Pakistan is noticeably productive with reference to growth of *Lamiaceae* plants (Ali and Nasir, 1990). *L. royleana* is an annual plant that grows in sub-tropical areas worldwide. The geographic distribution of the *L. royleana* has been seen in European, Middle-Eastern and South-Asian countries especially in Iraq, Iran, India, Pakistan, Afghanistan and Turkey (Morton, 1990). It is also known to be cultivated in Russia, Tajikistan and China.

In Pakistan, it has been reported from Attock (Hayat *et al.*, 2008). Other habitat in Pakistan includes Chitral, Malakand, Hazara, Abbotabad, Swat, Mingora, Waziristan, Parachinar, Kohat, Rawalpindi, Jehlum, Quetta and Makran (http://www.tropicos.org).

1.4 ECONOMIC SIGNIFICANCE

Literature indicates that the *Lallemantia royleana* is a popular topic in phytochemical and ethno-botanical research. Numerous medicinal as well as industrial properties have been attributed to this plant.

1.4.1 Medicinal Applications

It is used worldwide because of its pharmacological properties. The form in which it is consumed frequently is the suspension of its seeds in water

which is used by people as stomach soother as well as a refreshing drink because of its cooling and sedative effect. Its ethno-botanical uses reported include the treatment of ailments such as abscesses, inflammation (Khare, 2007), respiratory problems, gastrointestinal infections (Naghibi *et al.,* 2005) and a lot more.

1.4.2 Industrial Applications

The wide ranges of industrial applications reported have practically been researched upon such as its use as a suspending agent (Abdulrasool *et al*., 2011), fat-replacers (Emadzadeh *et al*, 2011) and as enhancers of bioavailability of analgesic tablets (Kazim and Ibrahim, 2011).

1.5 OUTLOOK IN THE STUDY OF *L. ROYLEANA*

All the literature reporting the medicinal properties of *L. royleana* has been prepared by the surveys conducted among traditional healers or the local population who use it. Its medicinal properties are well known throughout the world (Akber *et al.,* 2011; Naghibi *et al.,* 2005).

In light of the literature reported, it is postulated that so far all the research has been carried out in determining its potential towards certain diseases and verifying them but they do not address its use as an antimicrobial agent. In sultry countries, 50% of fatalities occur due to infectious diseases (Atta-ur-Rehman, 2008). S*wift evolution of resistance proposes that most of the currently in-use drugs (antibiotics) may not be effective for much longer.* Thus, it appears useful to look for new methods in treatment of bacterial

infections. The increasing collapse of chemotherapeutics along with their potential side effects and resistance to antibiotic demonstrated by pathogenic bacteria requires the screening of *L. royleana* for its probable anti-bacterial activity.

1.6 JUSTIFICATION OF PRESENT WORK

The extracts of *L. royleana* have a significant potential to be used in research of antibacterial compounds. It holds a great perspective for research by identifying its important compounds, isolating them and preparing their formulations to be used as synthetic drugs for the treatment of various ailments. Since natural antimicrobial products are effective and have ease of application, therefore they can be selected because of high therapeutic potential. They demonstrate an effective area to search for alternative and efficient compounds for treatment of infectious pathogens. Therefore in this research we unveiled the potential of indigenous seeds as an anti-bacterial agent.

1.7 AIM OF THE STUDY

Therefore our study aim to test the activities of different types of *Lallemantia royleana* extracts obtained from different extraction methods to screen for its antibacterial activity against certain bacterial species i.e. *Staphylococcus aureus, Pseudomonas aueroginosa, Escherichia coli* and *Enterobacter cloacae*.

1.8 OBJECTIVES

a. To obtain aqueous, ethanol, methanol and chloroform extracts of the seeds of *L.royleana.*

b. To screen the crude extracts for their antibacterial potencies via the disk diffusion test.

c. To screen for the different concentrations of the extracts that may confer antibacterial activity.

d. To compare the efficacy of the different extracts with themselves as well as with the antibiotic.

e. To compare the effectiveness of the extracts among different bacteria.

Chapter-2

LITERATURE REVIEW

2.1 MEDICINAL PLANTS

Since antiquity plants are exploited as therapeutic agents in both structured and non-structured forms such as Unani and folk respectively (Girach *et al* 2003). The awareness about medicinal plants and medical practices started spreading with the name of herbals in 15th century (Ghafoor et al., 2011). Herbalists from the ancient cultures believed that any part of plant resembling any body part was used for its ailment. (Baquar, 2001). Aromatherapy and aromatic herbs, for instance, were first used by Egyptians and Chinese civilizations in cosmetics and medicines (Burt, 2004; Songsong, 2011).

Medicinal plants comprises of active constituents in any of their parts like roots, bark, stem, and leaves, seeds and flowers which produce a curing response to the treatment of diseases. Out of 258,650 species only about 600-700 species are used worldwide both for their medicinal substances and as flavoring agents and natural pigments, in pharmaceutical, food, cosmetics and perfumery industries, (Shinwari, 2010). Medicinal and Aromatic plants being the vital among plants with economic importance have occupy themselves with a crucial role in lessening mankind's misery (Baquar, 2001). Other economic uses of medicinal plants include supplying fruits and vegetables,

browse for livestock and timber for fuel. They also contribute to rural health care and in poverty reduction from sale of processed products from herbal plants.

World Health Organization (WHO) reported that 80% of population in developing countries of the world takes benefit from traditional medicine as an important system for playing a pivotal role in curing ailments (Kamatou, 2006; Amiri, 2011). It is anticipated that local societies have used about ten percent (10%) of all flowering plants on Earth to treat a variety of contagions, although only one percent (1%) have gained appreciation by modern scientist. (Kafaru, 1994).

2.2 ETHNOMEDICINE IN PAKISTAN

Shinwari *et al.*, (2006) published a "pictorial guide of medicinal plants of Pakistan" in which he reports that more than 500 species of flowering plants are exploited as medicine. Medicinal plants have been reported from Galliyat areas of Khyber Pakhtunkwa (Ahmed *et al*., 2004). Important ethnomedicinal herbs have also been reported from Ayubia National Park, Abbottabad (Gilani *et al*., 2001) and from Utror and Gabral valleys, district Swat (Hamayun *et al*., 2005).

In the rural areas of Pakistan, elderly people apply their century's old knowledge of plant medicine as accountability to household and community members and to use plants to treat diseases (Shinwari, 2010). This knowledge is passed on to next generations. All sorts of diseases from headache to

Stomachic, to fever to cancer can all be cured from plants (Bhardwaj and Gakhar, 2005).

W.H.O reported a survey that 80%, 90% and 60% of the Indian, Bangladeshi and Pakistani population visit traditional healers for treatment (Gilani *et al*, 2001; Ahmed *et al*, 2004). The major means of treating various illnesses prior to the advent of Western medicine was the use of plants and their extracts for healing. The practice still prolongs, chiefly among rural society who might not have access to a hospital. It is not only the rural community, now a days, taking advantage of traditional medicines rather the other population also believes in their use. Ethnic groups believe that indigenous traditional medicines have played a vital role in the sighting of novel products from plants for use as chemotherapeutic agents.

2.3 *LAMIACEAE*

Lamiaceae (the "mint" family), also known as *Labiatae*, is a family of flowering plants that comprises of 250 to 258 genera and approximately 6,000 to 6,970 species across the world (Zomlefer, 1994; Mabberley, 1997). The old name of the family was *Labiatae* which was so specified because characteristically an upper lip and a lower lip are merged to form petals of flowers. The international panel of botanists recently agreed that all families of plants are represented with a distinctive plant of family and have the same ending (-aceae), so it is now called *Lamiacae*, subsequent to the variety of plant, Lamium (Dead Nettle) (theseedsite.co.uk/).

2.3.1 Characteristics of Family Lamiaceae

Nearly all members of the family have square stems; paired, opposite, simple leaves; and two-lipped, open-mouthed, tubular corollas (united petals), with five-lobed, bell-like calyxes (united sepals). Each single flower can generate four seeds which form at the base of the flower and develop inside the calyx. There is no seed pod. When the seeds are ripe, they merely roll out of the calyx (theseedsite.co.uk/).

2.3.2 Applications of Plants of Lamiaceae Family

The family has a multi-ethnic distribution and holds many plant species with culinary, ornamental and medicinal purposes;

2.3.2.1 Aromatic and culinary uses

Basil, oregano, parsley and many more belonging to this family are used as flavoring and aromatic agents in food (Naghibi et al., 2005). The essential oils specially add this charm to the family. The focal hub for domestication has been the Mediterranean region and Central/South-Western Asia and wild ancestors of this region promoted the family cultivation.

2.3.2.2 Medicinal uses

The *Lamiaceae* family of plants has been used since times immemorial as folk therapy for various health problems such as common cold, throat infections, acaricidal, psoriasis, seborrheic eczema, hemorrhage, menstrual disorders, miscarriage, ulcer, spasm and stomach problems (Takayama et al., 2011; Loizzo et al., 2010;. Ribeiro et al., 2010). Species of Labiatae family

are mainly used for digestive system problems like flatulence, diarrhea and dyspepsia and importantly for infections. Their use as anti-oxidant, anti-fungal, anti-bacterial, anti-inflammatory, anti-pyretic and anti-allergic has been established which debates for the more frequent use of these medicinal and culinary herbs (Naghibi *et al*., 2005). *Lamiaceae* species have anti-bacterial activities due to the presence of diterpenoids and tri-terpenoids compounds (Ulubelen, 2003).

2.3.2.3 Ornamental uses

Most of the plants can also be cultivated as ornamentals for example many species of *Salvia* are used for decoration. Big blue flowers of *Nepeta cataria* L., *N. grandiflora*, and *N. sibrica*, are spread worldwide as ornamentals (Naghibi *et al.*, 2005).

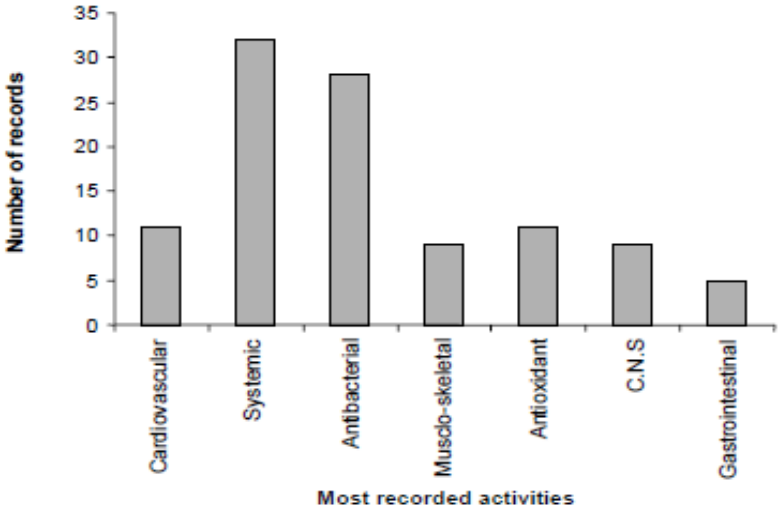

Figure 2.1: Assessed pharmacological activities in the main categories of health problems (Naghibi *et al*., 2005).

2.3.3 Pakistan's *Lamiaceae* plants

Pakistan is considered as hub of all the herbs and aromatic plants that have a potential to be used for rememdy (Hussain *et al*., 1988; Hussain *et al*., 2006; Erdemgil *et al*., 2007; Hussain *et al*., 2008). Medicinally or economically important flora is richly cultivated in Pakistan because of the favorable growth and climatic conditions. In Pakistan, basil, mint**,** thyme etc., grow as commercial crops (Wazir *et al*., 2004). A large number of other *Lamiaceae* species such as aromatic plants, herbs and medicinal plants grow wild in mountainous regions at different altitudes (Ali *et al*., 2000; Anwar *et al*., 2009a; Anwar *et al*., 2009b).

2.4 GENUS *LALLEMANTIA*

The following five species: *L. peltata, L. canescens, L. iberica, L. royleana* and *L. baldshuanica* represent the genus *Lallemantia.* These are distributed in Russia, China, Uzbekistan, India, Kazakhstan, Kyrgyzstan, Turkmenistan, Pakistan, Tajikistan, Afghanistan, South Western Asia, and Europe (Cao Shu, 1994). Some morphological characters vary between species. Considerable variations, chiefly useful at the intra-generic level were found in: stem length, leaves shape and margins, the shape and size of upper tooth of calyx, bracts and inflorescent shape and corolla calyx ratio (Talebi and Rezakhanlou, 2010).

Lallemantia species have a wide range of species including medicine (Dinc *et al*., 2009). *Lallemantia iberica* seed has traditional uses as

reconstitute, stimulant, diuretic and expectorant (Samadi *et al*., 2007). The sap of *L. iberica* is used as tea. *Lallemantia royleana* seed when soaked in water can be used for fever, common cold, gastrointestinal problems, expectorant, given internally as a soothing agent during urinary problems and coughing (Naghibi *et al*., 2005).

2.5 *LALLEMANTIA ROYLEANA*

Lallemantia royleana, with a vernacular name of Tukhum-malanga, Tukhum-Balango, Balango or Balango shirazi, is a mucilaginous endemic annual herb belonging to the family Lamiaceae (Ghannadi and Zolfaghari, 2003). It is cultivated throughout Western Asia, Pakistan, India and Northern Iraq for its exceedingly medicinal and mucilaginous seeds which are used as remedial agent and added as appetizing ingredient in cooling drinks primarily by Muslims (Abdulrasool *et a*l., 2011).

2.5.1 Characteristics of *L. royleana* plant and nutlets

L. royleana is a hairy or nearly smooth annual herb. It is 15-45cm in height. The leaves of the herb are 2.5cm long. Lavender flowers are borne on long spikes in a swirl (Morton, 1990). The nutlets are about 3 millimeter in length, 1 millimeter in breadth, dark-brown to black in color, smooth, three angled and tapering towards the umbilicus which is marked by a tiny white spot (Abdulrasool *et a*l., 2011). They become coated with voluminous and translucent mucilage when moistened with water (Kazim and Ibrahim, 2011). The moistened nutlets are bland and somewhat spicy in taste (Naghibi *et a*l.,

2005; Abdulrasool *et a*l., 2011). According to Razavi *et al.,* (2011) Balangu seeds adsorb water quickly by the hydration process because of high mucilage content and produce a sticky, turbid and tasteless liquid, which can be used as a new basis of hydrocolloid in food formulations.

2.5.2 Compositional analysis of *L. royleana*

Balangu seed is reported to be rich in carbohydrates (61.74%), fibre (29.66%), oil (10.8%) and protein (0.87%) (Naghibi *et al.* 2005; Kazim and Ibrahim, 2011; Razavi and Karazhiyan 2009; Razavi *et al.,* 2011). Presence of carbohydrates gives it a cellulosic nature. Tannins are also reported to be present in *L. royleana* as well as low in moisture content (Ahmad *et al.,* 2008). The mineral content of *L. royleana* is shown in the table as reported by Ahmad *et al.,* 2008.

The presence of mineral contents and other compounds help to meet the recommended daily intakes of some macro and micro minerals and other dietary ingredients in human diet.

Table 2.1. Mineral content of *L. royleana* (Ahmad *et al.*, 2008)

Minerals	**Concentration (mg/kg)**
Na	**1711.21 ± 14.49**
K	**772.56 ± 10.26**
Li	**14.25 ± 3.16**
Cu	**30.10 ± 2.61**
Co	**85.40 ± 2.52**
Mn	**8.45± 0.95**
Pb	**1.01 ± 0.09**
Ca	**1930.80 ± 14.02**
Mg	**152.95 ± 7.65**

2.5.3 Ethnobotanical study on *L. royleana* seeds

The following table reports the potential effects produced by seeds

Table 2.2 Medicinal uses of *L. royleana* reported worlwide

Country	**Medicinal Uses**	**Reference**
Iran	Abscesses and Inflammation (poultice of seeds)	Khare, 2007
Jordan	Pneumonia, respiratory canals	Lev and Amar, 2002
Iran	Anti-thirst, sore-throat, constipation and cough	Amiri *et al.,* 2012
Iran	Fever, expectorant, common cold	Sairafianpuor, 2002; Naghibi *et al.,* 2005
Bangladesh	Low sperm-count, pre-mature ejaculation (fried and powdered seeds)	Akber *et al.,* 2011
Bangladesh	Dysentery, blood dysentery, diarrhea, stomach pain, carminative. (orally taken)	Akber *et al.,* 2011
Pakistan	Stomach warmness and intestinal problems (Attock)	Hayat *et al.,* 2008
Pakistan	Anti-emetic	Mohtasheemul *et al.,* 2012

Table 2.3 Other uses of *L. royleana* reported worldwide

Country	**Industrial uses**	**Reference**
Iran	Food hydrocolloid	Razavi and Moghaddam, 2011
India	Enhance bioavailability of analgesic tablets	Kazim and Ibrahim, 2011
Pakistan	Fodder	Durrani *et al.*, 2009; Haq *et al.*, 2010
Iraq	Suspending agent	Abdulrasool *et al*., 2011
Iran	Fat-replacer	Emadzadeh *et al*, 2011

I. The seeds can be used as fat replacers to formulate reduced calorie pistachio butter (Emadzadeh, 2011)

II. The seeds can enhance the bioavailability of analgesic tablets because they contain mucilage in the chemical constituents. Mucilage absorbs water and help in tablet disintegration (Kazim and Ibrahim, 2011).

2.6 ANTIBIOTIC RESISTANCE AND FUTURE PROBLEMS

The use of antibiotics has been beneficial to treat infectious diseases since 70 years. These drugs have significantly reduced sickness and casualty from contagious diseases since 1940s. CDC claims that recently the misuse and overuse of antibiotics has resulted into antibiotic resistant strains and jeopardized the essential drugs. The CDC approximates that nearly 2 million people in the United States get hold of an infection every year while in a hospital, resulting in 90,000 deaths. More than 70 percent of the bacteria that are the reason for these infections are resistant to at least one of the antibiotics frequently used to treat them.

Kumarasamay *et al.*, (2010) reports that Gram-negative *Enterobacteriaceae* are potentially a chief health predicament with carbapenem resistance. *Staph* infections are not the only problem because of the the methicilin resistant strains but infact 30% of pneumonia is caused by pencilin resistant *streptococcus pneumonia* (Tsidoras et al., 2001). Shuti *et al.*, (2011) reported resistance of *S. aureus* isolates in Nigeria to teicoplanin, vancomycin, phosphomycin, fusidic acid, rifampicin, daptomycin, mupirocin,

linezolid and tigecycline. 16%, 55% and 72% of isolates were resistant to oxacillin, tetracycline and trimethoprim/sulphamethoxazole (cotrimoxazole), respectively.

2.7 PLANTS AS ANTIMICROBIAL AGENTS

Medicinal plants are important anti-bacterial agents although they have also been acknowledged for their use as anti-oxidant, anti-diabetic, anti-tumor and anti-inflammatory. Plant extracts and volatile oils are used as raw drugs for their medicinal properties. According to Inayatullah (2009), synthetic drugs were preferred over natural drugs by researchers in the middle of 20^{th} century. However due to the side effects of synthetic drugs the trend is rising globally to shift assets from allopathic to traditional health care systems (Inayatullah, 2009; Jiang *et al.,* 2006). According to Shinwari (2010), the international bussiness for medicinal was US$ 62 billion in 2002 and an educated guess proposes that it will arrive at US$ 5 trillion by 2050 and as for China; the share in the traditional medicine is expected to improve to 15% from the existing 3% by the year 2010. The antimicrobial properties of following plants, herbs *Psidium guajava Linn. (*Guava*), Ocimum gratissimum Linn.(*Ocimum) and *Xylopia aethiopica A. Rich (*Xylopia*)* (Osei-Akosah, 2010) have been assessed.

2.8 *L. ROYLEANA* AS AN ANTIMICROBIAL AGENT IN PAKISTAN

The growing infectious disease burden in Pakistan is alarming as it is in other developed and developing countries and efforts to control such diseases

are hindered by the persistent boost in antimicrobial resistance (AMR) in the country. Keeping the problem in concern Pakistan initiated a project in collaboration with US which is HEC funded which focuses to Develop and Strengthen Capacity for Surveillance, Containment, and Diagnosis (http://sites.nationalacademies.org/PGA/dsc/pakistan/PGA_052681). Keeping in mind the ground realities while working for masses in Pakistan, it should be seen that a large population is poverty stricken. Therefore we need to work on alternative treatments that are cost effective.

Pakistan is basically an agricultural country and our exports include crop and livestock. 300 medicinal plants are traded by Pakistan and 12% flora is used in medicines. The main partners in the herbal market are India, China, USA and Japan (Ghafoor *et al.,* 2011). According to an approximation, 22 species of medicinal plants worth Rs.14.733 million were traded in 1990 while in 2002, this value ascended to more than Rs.122 million (Shinwari, 2010). To date, no study has been conducted on effectiveness of *Lallemantia royleana* as an antimicrobial agent. As the seeds of *L. royleana* have proven to be ethno-medicinally vital for other ailments therefore this makes it a aggressive contender for the practice. Its sedative effect and activity against gastrointestinal and urinary problems is another reason for the selection. Pakistan being the natural habitat of the plant helps to shift the economy in the favor of the people and thereby providing an economical treatment against infections. Ahmad, 2005 assessed the economics of cultivating *L.royleana* at

farmer's field. A total income of 16,000 per acre was obtained. However the use of fertilizers and high quality seeds increase the yield by 40-50%.

2.9 PATHOGENIC BACTERIA

Pathogenic bacteria and their toxins have been of vital concern for years now and continue to present a major menace to human health. Infectious diseases remain to be a considerable cause of morbidity and mortality worldwide, accounting for 50% of all deaths in tropical countries. The common bacterial infections range from skin infection to bubonic plaques and tuberculosis. Other serious bacterial diseases include cholera, diphtheria, pneumonia and diarrheal diseases. Since the discovery of the first antibiotic, penicillin, antibiotics are the most prescribed medications to treat the bacterial infections. *Despite the progress made in understanding and control of microorganisms, the emergence of antibiotic resistant strains and the side effects of drugs pose an enormous threat to public health.*

2.9.1 STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a pathogen which causes infections in hospital and community setting (Shitu *et al.,* 2011) and is responsible for inflammatory diseases, toxic shock syndrome and food poisoning. It is a facultative anaerobe, gram-positive, coccal bacterium within the family of *Staphylococcaceae* commonly found as part of the normal skin flora. It is predictable that 20% of human population is long term carriers of *S.aureus* (Kluytmans, 1997). The drug resistant strains of this specie are rapidly on rise

and efforts must be devoted to combat this. Understanding of these antibiotic resistant strains is crucial in order to combat the disease. The production of enterotoxin responsible for gastroenteritis also marks its importance for the study. Its Exotoxin is responsible for the Toxic-shock syndrome due to which it acquires more focus.

According to the CDC, methicillin resistant *S. aureus* (MRSA) accounted for nearly 60% of nosocomial infections in 2001, a figure that had nearly doubled over the previous decade. A study conducted on the epidemiology of *Staphylococcus aureus* colonization in nursing home residents reported that 62% residents were colonized with *S. aureus* confirming extranasal colonization with MRSA frequent among nursing home residents, chiefly among residents with an indwelling device (Mody *et al.*, 2007).

2.9.2 PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa (an opportunistic human pathogen) has been recognized increasingly for its ability to cause nosocomial infections. P. aeruginosa is an extremely adaptable organism that grows on a wide variety of substrates and alters its properties in reaction to modifications in the environment including the resistance where extensive antibiotics are used. It has emerged as a nosocomial multi-drug resistant pathogen across the globe and Asia (Lambert, 2002).

P. aeruginosa is a rod-shaped, aerobic gram negative bacterium within the family of Pseudomonadaceae and is ubiquitous not only in the natural environment but also in the hypoxic environment. The bacteria takes advantage of an individual's weakened immune system to create an infection. *P. aeruginosa* causes respiratory system infections, sepsis, gastrointestinal infections and a variety of systemic infections (http://www.ehagroup.com). Another reason of interest in the *P. aeruginosa* is the cause of infections in the patients of burn injuries and is a frequent colonizer of medical devices ().

A study focusing on the pathogenesis of *P. aeruginosa* in intensive care units over a ten year period showed its status to be 3.4% cases every year (Cuttelod *et al*., 2010). Recently, they have been frequently isolating strains of *P. aeruginosa* from various specimens from different patients in the hospital. *A study* describing the incidence rate, risk factors, and outcomes associated with *P. aeruginosa* bacteremia in a large Canadian health region indicated 29% mortality rate with pulmonary infection as the most important factor associated (Parkins, 2010). Beta lactam antibiotics and cephalosporins alone or in combination have been used to combat these infections but resistance to most antibiotics has already been noticed. Martinez-Rodriguez (2009) reported resistance of *P. aeruginosa* to carbapenems (imipenem and meropenem). This screening included the recently reported extended-spectrum cephalosporinases (ESACs) weakly hydrolyzing carbapenems. Resistance to meropenem and imipenem was observed in 78% and 87% of the isolates

respectively. A study indicates the increase in the number of MDR strains including metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa in a region called Scanidinavia which had low antimicrobial resistance before 1999 (Samuelsen, 2009). P. aeruginosa* often accumulates different resistance mechanisms, including ESAC production and loss of outer membrane protein, leading to carbapenem resistance (Martinez-Rodriguez, 2009).

2.9.3 ESCHERICHIA COLI

Escherichia coli is a rod shaped, gram negative, non-sporing, facultative anaerobe within the family *enterobacteriaceae*, resides in the intestine. *E. coli* and related bacteria comprises of 0.1% of gut flora (Eckburg *et al.*, 2005). These bacteria remain benign commensals as long as they do not get hold of genetic elements encoding for virulence factors. According to CDC some types of *E. coli* can cause diarrhea, while others cause urinary tract infections, respiratory illness and pneumonia, and other illnesses and some cause disease by making a toxin called Shiga toxin.

Diarrhea is one of the leading causes of death in children, accounting for approximately 2 million deaths each year globally (Bryce *et al.*, 2005). A study reported that Fluoroquinolone resistance, 86.8% of the total *E. coli* isolates, is emerging as a major type of antibacterial resistance, particularly among *E. coli* strains (Catteneo, 2007). Courpon-Claudinon (2011) carried out a study in which he reported decreased susceptibility to 3GC's in 3.8% of the strains. Extended-spectrum β-lactamase (ESBL), AmpC cephalosporinase and

OXA-type penicillinase phenotypes are most prevalent and thus are the main causes of resistance. According to Ortega (2012), amoxicillin/clavulanate (AMC) resistant *E. coli* isolates were collected from seven participant hospitals of Spain which showed a 9.3% of AMC resistance.

2.9.4 ENTEROBACTER CLOACAE

Enterobacter cloacae has come forward as an important nosocomial pathogen in the past few years which could cause a wide spectrum of infections including respiratory system disease, urinary tract infections, involving mostly immunocompromised individuals (Wisplinghoff et al., 2004; Galani et al., 2005). It is a gram-negative, rod shaped, facultative anaerobe which is clinically significant. Risk factors for infection of *E. cloacae* include use of broad spectrum cephalosporins and aminoglycosides.

According to Wang et al., (2012), 41.2% strains possessed one or two AmpC β-lactamase genes, and 29.8% isolates carried one or more broad-spectrum beta-lactamase genes. The presence of ESBLs, AmpC β-lactamase producing strains was associated with the compact susceptibility to carbapenems among *E. cloacae.* Hammami et al., (2009) reported that *Enterobacter cloacae* isolates collected at Charles Nicolle hospital in Tunisia showed that all strains were vulnerable to carbapenems. They were resistant to fluoroquinolones, gentamicin, tobramycin, and trimethoprim+sulfamethoxazole but variably resistant to netilmicin, amikacin, and tetracyclines. Dalben et al., (2008) 26 reports of outbreaks due to *E.*

cloacae in neonate patients: 52% were bloodstream infection outbreaks, of which 12.5% were related to multiple-dose prescriptions.

Chapter-3

MATERIALS AND METHODS

The research work described in this critique was carried out in Medicinal Plant Laboratory., ASAB, NUST, Pakistan.

3.1 COLLECTION AND PROCESSING OF SEEDS

Seeds of the plant were purchased from the local market of Gulrez & Bahria town, Rawalpindi. The taxonomic identity of the seed was confirmed by Taxonomist, Dr. Muhammad Qasim Hayat, Department of Applied Biosciences, NUST Islamabad, Pakistan. The seeds were then crushed and homogenized to very fine powder using a commercial blender (Westpoint, France). The powder was kept at room temperature in air tight bottles, wrapped up in aluminum foil to avoid contact with light.

3.2 SEED EXTRACT PREPARATION

The powdered seeds of *L.royleana* were used to prepare four extracts: aqueous (FINE water distilled water, Pakistan) extract and three solvent extracts i.e., ethanol (AnalaR® BDH laboratory supplies, England), Methanol (Scharlau, Scharlab S.L, Spain) and Chloroform (Scharlau, Scharlab S.L, Spain).

3.2.1 Aqueous extract

10g of fine powder was dissolved in 300ml of distilled water in a conical flask with its orifice covered with aluminum foil. It was then kept in

rotary shaker (Memmertt, Germany) for 3 days to ensure thorough mixing and the formation of a homogenized solution.

3.2.2 Solvent extract

10 g of fine powder was dissolved in 100 ml of the solvent (methanol, ethanol and chloroform) in a conical flask with its orifice covered with aluminum foil. It was then kept in rotary shaker (Memmertt, Germany) for 2 days to ensure thorough mixing and the formation of a homogenized solution.

3.2.3 Centrifugation and Filtration

The solutions were then transferred to 500ml centrifuge tubes and were centrifuged in the centrifuge machine (Eppendorf Centrifuge, 5810R, Germany) at 4000 rpm for 25 minutes. The supernatant was then collected and was filtered using the Whattman filter paper grade 4. The filtrates were then left to evaporate, to at least one-fourth of its initial volume, in a fume hood (Esco frontier laboratory, Singapore). Later it was stored at 4^{o}C before it was transferred to microfuge tubes (Eppendorf, Germany).

3.3 PREPARING DILUTIONS OF THE EXTRACTS

Microfuge tubes were weighed in the electric balance (Schimadzu AY220, Japan) before and after transferring the evaporated extract into them to determine the weight of the extract. A stock solution was then prepared for the evaporated extracts of each of the solvents as well as of the aqueous extract, by dissolving it in to 100% DMSO (Scharlau, Scharlab S.L, Spain). Dilutions of 100mg/ml, 50 mg/ml and 10 mg/ml were prepared in 100%

DMSO which were stored at 4^{o}C and were later applied on discs to be tested for anti-bacterial activity.

3.4 BACTERIA AND MEDIA

3.4.1 Bacterial strains

The antibacterial activity was examined for four bacterial strains, in vitro, for ethanol, methanol, chloroform and aqueous extracts of *Lallemantia royleana*. The bacterial strains that were used included gram-positive:

- *Staphylococcus Aureus*

And gram-negative:

- *Enterobacter Cloacae*
- *Pseudomonas Aeruginosa*
- *Escherichia Coli*

These bacterial cultures were obtained from Pakistan institute of Medical Sciences (PIMS), Islamabad, Pakistan. The bacterial strains must be in the log phase of growth for the best results. Therefore they were sub-cultured a day before the antibacterial assay was carried out. These bacteria were maintained on agar plates and were then stored at 4^{o}C

3.4.2 Preparation of Media

The agar used for bacterial culturing was Difco™ nutrient agar Becton & Dickinson, France. The composition of the nutrient agar purchased had

- 3g of Beef extract
- 5g of Peptone

- 15g Agar

The final pH of the agar maintained was 6.8 ± 0.2.

Media was usually prepared in the quantities 500ml or 250 ml. 11.5g and 5.75g of powder was suspended in 500ml and 250 ml of distilled water respectively. Thorough mixing was ensured and then it was heated with continuous agitation and boiled for 1 minute to completely dissolve the powder. The media was then autoclaved (Hirayama HVE-50, Japan) for 20 minutes at 121^{o}C. It was stored at room temperature after being autoclaved.

3.4.3 Preparation of plates

Agar solidified at room temperature therefore before preparing plates it was heated using a hot plate for 15 to 20 minutes until it was liquid again. Media was poured into the plates inside the laminar flow hood (Streamline Laboratory product, Singapore) to avoid contamination. The plates were then left ajar until agar solidified, to minimize condensation on the lid due to hot agar. As soon as the agar solidified the plates were covered, turned upside down and were stored at 4^{o}C.

3.5 ANTIBACTERIAL ASSAY

The antibacterial activity of the extracts was determined by disc diffusion method. The assay was performed in the laminar flow hood cabinet (Streamline Laboratory product, Singapore). The UV was turned on 15 minutes before the experiment to avoid any contamination. The agar plates

were opened only inside the hood and nowhere else and so were the swabs and discs.

3.5.1 Inoculation of the agar plate

a. A colony was picked from the sub-cultured bacterial lawn using a sterile cotton swab by giving the swab a gentle touch on the bacterial surface growing on agar medium.
b. After the colony was picked up by the swab, the dried surface of the agar plate was inoculated by streaking the swab in back-and-forth motion, very close together, while moving across-and-down. The plate was rotated 45 degrees and the action was repeated four times. This ensured an even distribution of bacterial colony to allow the growth of a confluent bacterial lawn.
c. The swab was discarded in an appropriate container then.
d. As we had the four bacterial isolates to be tested by the four extracts therefore four plates for each bacterium were inoculated with it.
e. The plates were appropriately labeled i.e., four plates for each bacterium labeled after the four extracts Methanol, Ethanol, Chloroform and Aqueous along with the name of the strain. They were also labeled for the three dilutions (100mg/ml, 50mg/ml and 10mg/ml) of the extracts that were to be tested.
f. The experiment was performed in triplicate to minus out the extraneous activity due to confounding factors.

3.5.2 Placement of discs

a. Discs were prepared from Whattman filter paper Grade 4 of approximately 6 mm in size, the previous day.
b. Discs were then autoclaved (Hirayama, HVE-50, Japan) at 121^{o}C for 20 minutes.
c. A single disc was picked up by using a syringe (BD 5ml syringe, REF-305719, Becton & Dickinson, Pakistan) and the lid of the petri dish was removed. It was then placed on the agar inoculated with the bacterium, by syringes and P20 tips, on the points marked up for each dilution.
d. If the disc is misplaced then its position was adjusted using the tips. The syringe (BD 5ml syringe, REF-305719, Becton & Dickinson, Pakistan) must not touch the bacterial surface or else it contaminates all the discs while picking them up.
e. Two discs were placed on each point. This was repeated for all the plates.
f. A different syringe (BD 5ml syringe, REF-305719, Becton & Dickinson, Pakistan) was used for each bacterial strain. But a single syringe can be used for all petri dishes inoculated with single bacterium.

3.5.3 Pouring dilutions on the discs placed on the agar surface

a. The microfuge tubes containing dilutions of extracts were vortexed (Heidolph, Germany) before applying them on to the disc.

b. 40µl of 100mg/ml dilution of methanol extract was dispensed on the disc placed on the point labeled for 100mg/ml, on the respective plates i.e. all those labeled methanol for each bacterium.
c. This allowed the disc to absorb all the extracts applied and get impregnated with it.
d. Similarly 40µl of the other two dilutions (50mg/ml and 10mg/ml) were dispensed as well directly on the discs placed on the respective points for methanol extract.
e. The above process was repeated for the extracts of ethanol, chloroform and aqueous on their respective plates for each bacterium.
f. Antibiotics, Ampicilin and Kanamycin (obtained in powdered form, concentration prepared 100mg/ml), were used as a positive reference and 100% DMSO (Scharlau, Scharlab S.L, Spain) was used for negative reference.
g. The plates were then closed with the lids and they were kept in upside down position i.e. agar on the bottom side, for an hour to allow the liquid to be completely absorbed by the discs.
h. They were then incubated at $37^{o}C$ for 20 hours in the incubator (Memmertt, Germany).
i. The antibacterial activity was then evaluated by measuring the diameter in millimeters of the zone of inhibition of growth which includes the diameter

of the disc i.e. 6 mm, for the test organisms and they were thus compared with the controls.

Chapter-4

RESULTS

The antibacterial assay was performed by standard disc diffusion method. The antibacterial activity of methanol, ethanol, chloroform and aqueous extracts of *L. royleana* is reported in Table 1. While standardizing the activity of extracts, the three mentioned concentrations were found to be the effective ones because they showed anti-bacterial activity by inhibiting one or more organisms. It was considered that if all the extracts showed activity at concentration equal to or less than 100mg/ml would be the effective ones. The different concentrations (100mg/ml, 50mg/ml and 10mg/ml) of the extracts used, showed varying activities as illustrated in Table 1. All the three concentrations were tested against the four bacterial strains as already described in chapter 3.

No activity of aqueous extract was observed at any concentration. The inhibition zones ranged from 06.17 to 14.67 mm with all the concentrations of extracts giving varying activities against different bacteria. The activities were thus compared with positive and negative controls. All the experiments were repeated in triplicate and the results were expressed in standard deviation.

Table 4.1 Measurements of inhibitory zones obtained by extracts application.

Extracts/ Bacterial strains			***S. Aureus***	***E. Cloacae***	***E. Coli***	***P. Aeruginosa***
Methanol	**100mg/ml**		13.17 ± 2.08	12.00 ± 1.32	09.33 ± 1.04	11.83 ± 0.58
	50mg/ml		13.00 ± 2.78	10.17 ± 2.08	10.17 ± 0.76	09.00 ± 0.5
	10mg/ml		06.17 ± 5.39	12.33 ± 1.26	11.67 ± 2.36	07.00 ± 6.08
Ethanol	**100mg/ml**		13.83 ± 4.48	11.17 ± 1.04	11.17 ± 1.04	10.33 ± 1.26
	50mg/ml		12.67 ± 2.84	12.33 ± 3.82	12.83 ± 0.76	10.83 ± 1.44
	10mg/ml		12.33 ± 3.75	13.83 ± 1.26	10.00 ± 1.5	03.67 ± 6.35
Chloroform	**100mg/ml**		14.67 ± 0.58	14.00 ± 1.5	11.83 ± 3.79	10.67 ± 1.44
	50mg/ml		13.33 ± 2.93	11.83 ± 1.89	11.00 ± 1.32	13.67 ± 3.75
	10mg/ml		06.67 ± 6.29	11.67 ± 0.76	12.16 ± 0.76	10.50 ± 3.5
Aqueous	**100mg/ml**		00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
	50mg/ml		00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
	10mg/ml		00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
Controls	**+ive**	**Amp**	28	12	0	0
		Kana	22.5	35	25	19
	-ive	**DMSO**	0	0	0	0

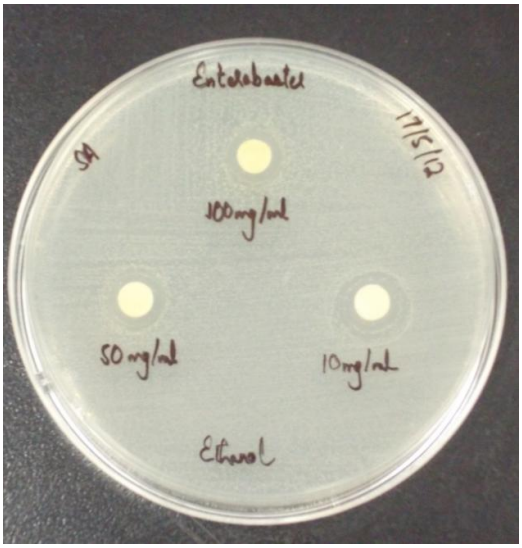

Plate A: Zones of inhibition of *E. cloaceae* by Ethanol extract

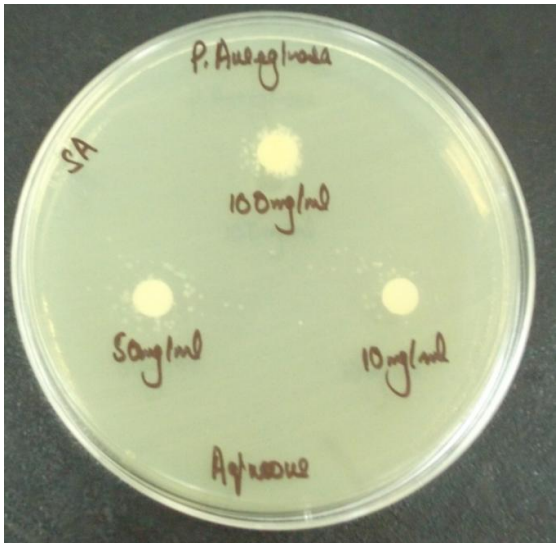

Plate B: Zones of inhibition of *P. aeruginosa* by Aqueous extract

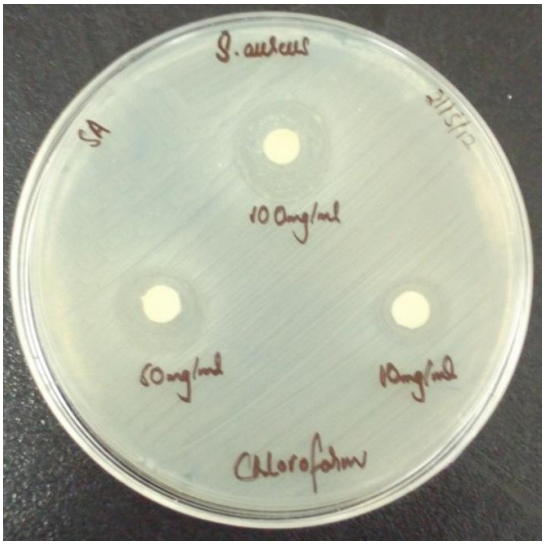

Plate C: Zones of inhibition of *S. aureus* by Chloroform extract

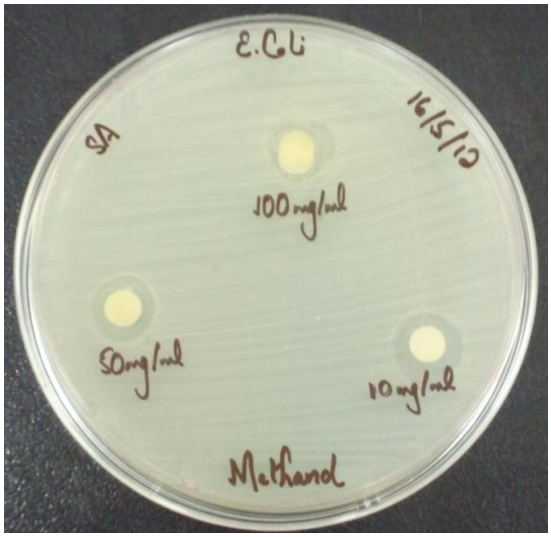

Plate D: Zones of inhibition of *E.coli* by Methanol extract

Figure 4.1. Plates A, B, C and D showing zones of inhibition for test bacteria at three concentrations (100mg/ml, 50mg/ml and 10mg/ml) of the four extracts (ehanol, aqueous, chloroform and methanol)

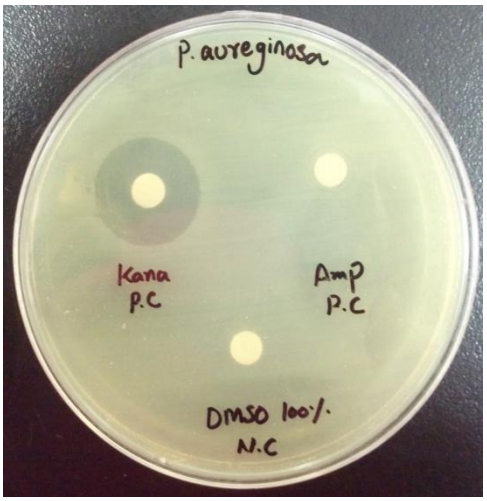

Plate A: *P. Aeruginosa* controls

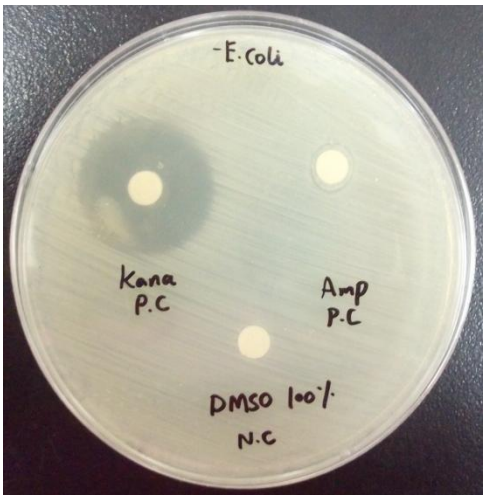

Plate B: *E. Coli* controls

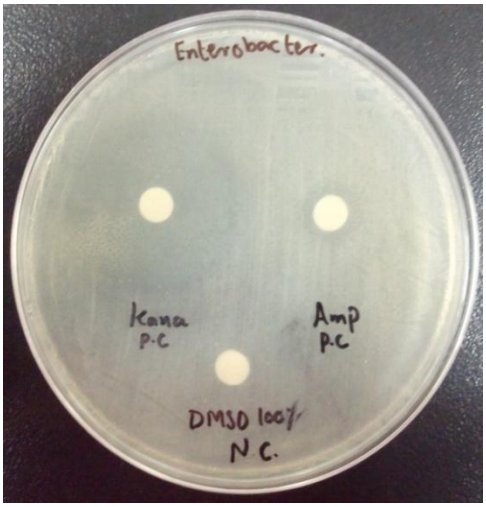

Plate C: *E. cloaceae* controls

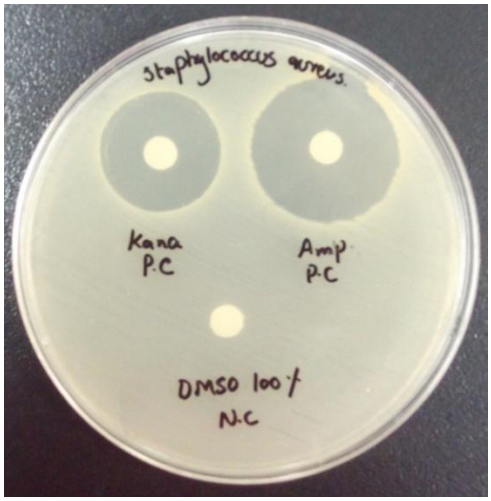

Plate D: *S. aureus* controls

Figure 4.2. Plates A, B, C and D show positive and negative controls for test bacterial strains. Positive controls: Kana= Kanamycin, Amp= Ampicilin. Negtive controls: 100% DMSO.

Chapter-5

DISCUSSION

Lallemantia royleana is known to posses various medicinal properties and people in Pakistan use it as a common soothing and refreshing agent as well as a herbal medicine. It is the common belief of people in Pakistan that soaked seed formulation is good for maintaining healthy digestive system in summer season as it has cooling effect on it (Naghibi *et a*l., 2005). Therefore, in local scientific literature (Razavi *et al.,* 2011; Amiri *et al.,* 2012) there is much focus on its ethno-botany and still the medicinal properties of *L. royleana* are not evaluated on scientific merits. Our research is first report on its anti-bacterial activity.

The bacterial strains we used in our study are known to cause enteric problems along with a large number of other diseases. In our research we verified the anti-bacterial activity of *L. royleana* that it can provide relief against enteric problems caused by *E. coli* and *E. cloacae*. It was also found that the *L. royleana* has potential to cure upper-respiratory tract infections caused by *P. aeruginosa. L. royleana* also showed positive anti-bacterial activity against *S. aureus* which is causative agent of staph diseases. In these cases our results agree with the ethno-medicinal studies of (Lev and Amar, 2002; Akber *et al.,* 2011; Hayat *et al.,* 2008).

The various extracts of *L. royleana* seeds have shown variability in anti-microbial activity. It was noted that methanol, ethanol and chloroform

extracts of the seeds possess a greater antibacterial potential for all the bacterial strains because the values for effective zones of inhibition were recorded greater. However, the aqueous extract showed no antibacterial activity at any concentration against any strain of bacteria used in this study. In contrast to our study, it is found interesting that local traditional healers use water as a solvent for their preparations (Hayat et al., 2008; Sairafianpuor, 2002). Distilled water doesn't have enough potential to extract out the medicinal compounds from the seeds. We agree with the general notion that organic solvents are physically more powerful to dig up the therapeutic composites.

As per interest, it was observed while preparing the aqueous extract that the powder didn't efficiently dissolved itself in water because of its mucilaginous activity. Therefore it was kept for shaking for a longer time as well as for evaporation as compared to other extracts, mentioned in chapter 3. As this study has never been conducted before, consequently we couldn't possibly sum up the behavior of aqueous extracts to give completely negative activity against all bacterial strains. A little growth, as shown in Plate 2. in the form of white dots, was also observed in case of almost all aqueous extracts against all bacteria.

In the present study we observed that chloroform extracts of *L. royleana* seeds exhibited highest and the lowest antibacterial activity against all bacterial strains. Among them the 100 mg/ml formulation of the chloroform

extract was the most potent one against *S. aureus,* giving the largest inhibition zone of 14.67 mm followed by *E. cloacae* (14 mm). In case of S. aureus, the same results were observed for all other extracts as well. As the concentration was reduced to 50mg/ml and 10mg/ml, the activity of the extract decreased respectively therefore indicating that a large percentage of the chloroform extract dissolved in 100% DMSO is more effectual as compared to lesser amount of extract suggesting the role of solvent in unleashing the useful compounds from the seeds.

E. coli gave a larger zone of inhibition at 10mg/ml (12.16 mm). It was observed in case of *E. coli*, that the lower concentrations of all extracts were more effective (See Table 4.1). For *E. cloaceae* the lowest concentrations of methanol and ethanol were more effective, 12.83 mm and 13.83 mm respectively, but at the same time the highest concentration of chloroform gave the large zone of inhibition as compared to other concentrations. *P. aeruginosa* was inhibited by chloroform extract by giving a smaller zone at 100mg/ml (10.67 mm) as compared to 50mg/ml (13.67 mm) suggesting 50mg/ml to be the more effective concentration against this bacteria. Except for methanol where it gave a larger zone at the highest concentration. The differences in the behavior of different extracts may be explained by the selectivity of the effectiveness of the type of compounds against particular bacterial strains.

For all the extracts, gram-positive *S. aureus* was found to be the most susceptible organism showing the maximum inhibition zone as compared to

other micro-organisms in case of all the extracts. However, the readings depict *P. aeruginosa* to be the least susceptible bacterial strain. Therefore we predict that *L. royleana* has activity against *P. aeruginosa* infections (nosocomial and respiratory) but to a little extent. This supports the work described by previous studies (Vlietinck et al, 1995; Rabe and Van Staden, 1997). So there is a requirement to determine the active compounds present in the seeds, to classify the compounds that might be more effective against this micro-organism (by being able to penetrate the cell wall or disrupt them) and to use a specific formulation of only those compounds in the drug synthesized. *L. royleana* seeds are reported to have an effective level of tannin content which is an important indication of the antibacterial activity observed (Ahmed *et al.*, 2007).

The most effective concentration of antibiotics was used as control so that the zone of inhibition is obtained. When compared to the controls, kanamycin and ampicilin nevertheless gave better results as compared to the extracts but the resistance has been reported. It was observed in our results as well that *P. aeruginosa* and *E.coli* were resistant to ampicilin. Therefore there is a great potential of all these extracts to be used in the synthesis of new drugs due to the rapid failure of all the antibiotics.

The antibacterial activity may be pinpointing of the presence of some metabolic toxins or broad-spectrum antibiotic compounds. However, Ahmad et al., also reported the presence of high level of Lithium content in *L. royleana*

which is considered to be essential for pharmacological properties of the seeds (Ahmad *et al.,* 2008).

5.1 Recommendations and Future Prospects

The seeds depicted efficient activity therefore it has great a potential for the screening of antiviral activity and antifungal activity. Identification and characterization of the secondary metabolites present in the plant, that are involved in the pharmacological properties is important so these compounds may be used in synthetic drugs. After our observed results, we can establish which extracts of *L. royleana* show antibacterial activity at what concentration which is fundamental knowledge for drug development and add to its therapeutic profile. It can also lead to development of drugs because they have more efficacies against antibiotic resistant strains and would be cost-effective for general population.

CONCLUSION

In our study we report the antibacterial activity of *Lallemantia royleana* for the first time. The seeds of *L. royleana* showed promising antimicrobial properties indicating the potential for discovery of antibacterial principles. The present study supports the usage of the seeds of *L. royleana* by traditional healthcare practitioners and indicates that certain compounds might be responsible for antibacterial properties. Nevertheless, the chloroform extracts showed the maximum activity highlighting themselves to be the most potent and effective formulations for the treatment of pathogenic bacterial infections. *S. aureus* was the most susceptible organism to the activity of all these extracts, however, other bacterial strains showed equally effective inhibition as well. So we can expect that resistance in micro-organsisms will not be elicited by using these natural products as therapeutic agents. Consequently there is a need to perform phytochemical analysis on the seeds of *L. royleana,* which is a promising candidate for future research, for the determination of compounds that could be valuable for such activity and use them for the development of new drugs.

Chapter-6

REFERENCES

Abdulrasool, A. A., Naseer, A. A. and Rahi, F. A. (2011). Application of seed mucilage extracted from *Lallemantia royleana* as a suspending agent. Iraqi Journal of Pharmacological Science. Volume 20.

Ahmad, I., Hanif, M. A., Nadeem, R., Jamil, M. S. and Zafar, M. S. (2008). Nutritive evaluation of medicinal plants being used as condiments in South Asian Region. Journal of Chemicals Society Pakistan, Volume 30.

Ahmed, E., Arshad, M., Ahmad, M., Saeed, M. and Ishaque, M. (2004). Ethanopharmacological survey of some medicinally important plants of Galliyat areas of NWFP, Pakistan. Asian Journal of Plant Sciences, 3 (4): 410-415

Ali, M. S., Ahmed S, Ibrahim SA, Tareen RB (2005). Characterization and bioscreening of a new triterpenoid and a flavanone isolated from *Salvia nubicola* Chemical Biodiversity, 2: 910-916

Ali, M. S., Ahmed, W., Armstrong, A. F., Ibrahim, S. A., Ahmed, S. and Parvez, M. (2006). Guaianolides from *Salvia nubicola* (Lamiaceae). Chemical and Pharmaceutical Bulletin, 54(9): 1235-1238

Durani, M. J., Manzoor, M. and Irfan, S. (2009). Folk uses of some plants of Quetta, Pakistan. Pakistan Journal of Plant Sciences, 15 (1): 1-6

Lev, E. and Amar, Z. (2002). Ethnopharmacological surveys of traditional drugs sold in the Kingdom of Jordan. Journal of Ethnopharmacology, 82 (2-3): 131-145

Amiri, M. S., Jabbarzadeh, P. and Akhondi, M. (2012). An ethnobotanical survey of medicinal plants used by indigenous people in Zangelanlo district, Northeast Iran. Journal of Medicinal Plants Research, 6 (5): 749-753

Anwar, F., Hussain, A. I., Sherazi, S. T. H. and Bhanger, M. I. (2009b). Changes in composition and antioxidant and antimicrobial activities of essential oil of fennel (*Foeniculum vulgare* Mill.) fruit at different stages of maturity. Journal of Herbs, Spices and Medicinals Plants. 15: 1-16.

Anwar, F., Ali, M., Hussain, A. I. and Shahid, M. (2009). Antioxidant and antimicrobial activities of essential oils and extracts of fennel (*Foeniculum vulgare* Mill.) seeds from Pakistan. Flavour and Fragrance Journal. 24: 170-176.

Baquar, S. R. (2001). Textbook of Economic Botany (Ist Edition),Ferozsons (Pvt) Ltd., Lahore, Pakistan

Bhardwaj, S. and Ghakar, S. K. (2005). Ethnomedicinal plants used by the tribals of Mizoram to cure cut and wound. Indian Journal of Traditional Knowledge 4 (1): 75-80.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94 (3): 223-253.

Cao Shu BB. (1994). *Lallemantia* L. In: Chun XK [ed.], *Flora of China, Lamiaceae* 17: 133–134.

Cattaneo, C., Quaresmini, Cassari, G. S., Capucci, M. A., Micheletti, M., Borlenghi, E., Signorini, L., Re A., Carosi, G. and Rossi, G. (2007). Recent changes in bacterial epidemiology and the emergence of fluoroquinolone-resistant *Escherichia coli* among patients with haematological malignancies: results of a prospective study on 823 patients at a single institution. Journal of Antimicrobial Therapy. 61 (3):721-728

Courpon-Claudinon., Lefort, A., Panhard, X., Clermont, O., Dornic, Q., Fantin, B., Mentré, F., Wolff, M., Denamur, E. and Branger, C. (2010). Bacteraemia caused by third-generation cephalosporin-resistant *Escherichia coli* in France: prevalence, molecular epidemiology and clinical features. Clinical Microbiology and Infection. 17 (4): 557-565

Cuttelod, M., Senn, L., Terletskiy, V., Nahimana, I., Petignat, C., Eggimann, P., Bille, J., Prod'hom, G., Zanetti[1], G. and Blanc, D.S. (2010). Molecular epidemiology of *Pseudomonas aeruginosa* in intensive care units over a 10-year period (1998–2007). Clinical Microbiology and Infection. 17: 57-62

Cuvelier, M. E., Richard, H. and Berset, C. (1996). Antioxidant activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. Journal of American Oil & Chemists Society, 73: 645-652.

Dalben, M., Varkulja, G., Basso, M., Krebs, V. L. J., Gibelli, M.A., van der Heijden, I., Rossi, F., Duboc, G., Levin, A. S. and Costa, S. F. (2008) Investigation of an outbreak of *Enterobactercloacae* in a neonatal unit and review of the literature. Journal of Hospital infection. 70 (1): 7-14

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E. and Dethlefsen, .L. (2005). Diversity of the human intestinal microbial flora. Science, 308 (5728): 1635–1638.

Emadzadeh B., Razavi S. M. A., Hashemi M., Nassiri Mahallati M., Farhoosh R. (2011) Optimization of Fat Replacers and Sweetener Levels to Formulate Reduced- Calorie Pistachio Butter: A Response Surface Methodology. International Journal of Nuts and Related Sciences 2 (4): 37-54

Fujita, E. and Node, M. (1984). Diterpenoids of *Rabdosia* species. Progress in Chemistry of Natural Products, 46: 77-157

Ghafoor, A., Siddiqui, S. A., Jatoi, S. A. and Rabbani, M. A . (2011). Phytotherapy- Research gaps in Pakistan. Pakistan Journal of Botany, 43: 175-182

Ghannadi, A. R. and Zolfaghari, B. (2003). Compositional Analysis of the Essential Oil of *Lallemantia royleana* Benth. Journal of Flavour and Fragrance, 18: 237-239.

Gilani, A. H. and Atta-ur-Rahman (2005). Trends in ethnopharmacology. Journal of Ethnopharmacology, 100: 43-49.

Gilani, S. A., Qureshi, R. A. and Farooq, U. (2001). Ethnobotanical studies of Ayubia national park district Abbottabad, Pakistan. Online Journal of Biological Sciences, 1: 284-286

Girach, R. D., Khan, H., and Ahmad, M. (2003) Botanical identification of Thuhar, seldom used as unani medicine. Hamdard Medicus, XLVI (1): 27-33

Hamayun, M, Khan, M. A. and Hayat, T., (2005). Ethanobotanical profile of Utror and Gabral valleys, district Swat Pakistan.

Hammami, S., Boutiba-Ben Boubaker, I., Saidani, M., Lakhal, E., Ben Hassen, A., Kamoun, A., Ghozzi, R., Slim, A. and Ben Redjeb, S. (2012). Characterization and molecular epidemiology of extended spectrum beta-lactamase producing Enterobacter cloacae isolated from a Tunisian hospital. Microbiology Drug Resistance. 18 (1): 59-65

Hota, S., Hirji, Z., Stockton, K., Lemieux, C., Dedier, H., Wolfaardt, G. and Gardam, M. A. (2009). Outbreak of Multidrug-Resistant Pseudomonas aeruginosa Colonization and Infection Secondary to

Imperfect Intensive Care Unit Room Design. Infection Control and Hospital Epidemiology Journal, 30: 25-33

Hussain A., Sherma, O. P. V., Kumar. A. and Misra, L.N., (1988). Major Essential Oil- Bearing Plants of India. Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

Hussain A. I., Anwar, F., Bhathi, H. N. and Rashid, U. (2006). Phytochemical and *in vitro* anthelmintic screening of *Butea frondosa* and *Swertia chirata* from Pakistan. Journal of Chemical Society of Pakistan. 28 (1): 84-92.

Hussain, A. I., Anwar, F., Sherazi, S. T. H. and Przybylski, R. (2008). Chemical composition. Antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. Food Chemistry. 108: 986-995.

Inayatullah, S. (2009) Biological Evaluation of Some Selected Plant Species of Pakistan. Ph.D Thesis, Quaid-i-Azam University Islamabad-Pakistan.

Iwu, M. W., Duncan, A. R, Okunji, C. O. (1999). New Antimicrobials of Plant Origin. In: Perspectives on New Crops and New Uses (ed. Janick J). pp. 457-462, Alexandria, VA, ASHS Press.

Jiang, C., Chang, M., Wen, C., Lin, Y., Hsu, F. and Lee, M. (2006). Natural products of cosmetics: Analysis of extracts of plants endemic to Taiwan for the presence of tyrosinaseinhibitory, melanin reducing and

free radical scavenging activities. Journal of Food Drug Analysis, 14 (4): 346-352

Kafaru, E. (1994). Immense help formative workshop. In: Khan, R., Islam, B., Akram, M., Shakil, S., Ahmad, A., Ali, M. S., Sadiqui, M., Khan, A. U. (2008). Antimicrobial Activity of Five Herbal Extracts against Multi Drug Resistant (MDR) strains of Bacteria and Fungus of Clinical Origin. Molecules, 13.

Kamatou, G. P. P (2006) Indigenous Salvia speicies – An investigation of their pharmacological activities and phytochemistry. Ph.D thesis University of the Witwatersrand

Kazim, S. M. and Ibrahim, M. (2011). Enhancement of bioavailabilty using natural ingredients and estimation of drug content by orthogonal polynomials. International Journal of Chemical Sciences and Applications. 2: 100-107

Khare, C. P. (2007) Indian Medicinal Plants. An Illustrated Dictionary Springer-Verlag Heidelberg

Kluytmans, J., van Belkum, A. and Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clinical Microbiology Review. 10 (3): 505–520.

Kumar, R. 2010. Early history of Jammu region: pre-historic to 6th century. Gyan Publishing House, 658 p.

Lambert, P. A. (2002) Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. Journal of the Royal Society of Medicine. 95: 22-26

Loizzo, M. R., Tundis, R., Conforti, F., Menichini, F., Bonesi, M., Nadjafi, F., Frega, N. G. and Menichini, F. (2010). *Salvia leriifolia* Benth (Lamiaceae) extract demonstrates in vitro antioxidant properties and cholinesterase inhibitory activity, Nutrition Research, 30 (12): 823-830.

Lu, Y. and Foo, L.Y. (2001). Antioxidant activities of polyphenols from sage (*Salvia officinalis*). Food Chemistry, 75: 197-202.

Mabberley, D. J. (1997). The Plant Book: a Portable Dictionary of the Vascular Plants, Cambridge University Press, Cambridge, UK.

Mody, L., Kauffman, C. A., Donabedian, S., Zervos, M. and Bradley, S. F.. (2007). Epidemiology of *Staphylococcus aureus* Colonization in Nursing Home Residents. Clinical Infectious Diseases. 46 (9): 1368-1373

Mojab, F., Poursaeed, M., Mehrgan, H.and Pakdaman, S. (2008). Antibacterial activity of *Thymus daenensis* methanolic extract. Pakistan Journal of Pharmacological Sciences. 21 (3): 210-213.

Morton, J. F. (1990). Mucilaginous plants and their uses in medicine. Journal of Ethnopharmacology, 29 (3): 245-66

Ali, S. I. and Y. J. Nasir. (1990) Flora of Pakistan. No. 192, 1 p.

Hayat, M. Q., Khan, M. A., Ahmad, M., Shaheen, N., Yasmin, G. and Akhter, S. (2008). Ethnotaxonomical Approach in the Identification of Useful Medicinal Flora of Tehsil Pindigheb (District Attock) Pakistan. Etnobotany Research and Applications, 6: 035-062

Naghibi, F., Mosaddegh, M., Motamed, S.M. and Ghorbani, A. (2005) Labiatae family in folk medicine in Iran: from ethnobotany to pharmacology. Iranian Journal of Pharmaceutical Research. 2: 63-79

Akber, M., Seeraj, S., Islam, F., Ferdausi, D., Ahmed, R., Nasrin, D., Nahar, N., Ahsan, S., Jamal, F. and Rahmatullah, M. (2011). A Survey of Medicinal Plants Used by the Traditional Medicinal Practitioners of Khulna City, Bangladesh. American-Eurasian Journal of Sustainable Agriculture, 5 (2): 177-195

Nickavar B, Mojab F, Dolat-Abadi R (2005). Analysis of the essential oils of two *Thymus* species from Iran. Food Chemistry, 90 (4): 609-611.

Nostro, A., Cannatelli, M.A., Crisafi, G. and Alonzo, V. (2005) The effect of *Nepeta cataria* extract on adherence and enzyme production of *Staphylococcus aureus*. International Journal of Antimicrobial Agents, 18: 583-5

Ortega, A., Oteo, J., Aranzamendi-Zaldumbide, M., Bartolomé, R.M., Bou, G., Cercenado, E., Conejo, M.C., González-López, J.J., Marín, M., Martínez-Martínez, L., Merino, M., Navarro, F., Oliver, A., Pascual, A., Rivera, A., Rodríguez-Baño, J., Weber, I., Aracil, B. and Campos,

J. (2012) Epidemiology and resistance mechanisms to amoxicillin-clavulanate in *Escherichia coli*: A Spanish multicenter study. Antimicrobial agents and Chemotherapy. 56 (6)

Osei-Akosah, E. (). Antimicrobial activity profile of the constituents of four Ghanaian aromatic medicinal plants. Ph.D thesis Kwame Nkrumah University of Science and Technology, Kumsai.

Parkins, M. D., Gregson D. B., Pitout, J. D. D., Ross, T. and Laupland, K. B. (2010). Population-Based Study of the Epidemiology and the Risk Factors for *Pseudomonas aeruginosa* Bloodstream Infection. Infection. 38:25-32

Pliego, M. P. C. (2007). Effect of natural antimicrobials against *Salmonella*, *Escherichia coli* O157:H7 AND *Listeria monocytogenes.* Ph.D thesis Texas A&M University.

Rabe, T. and Van Staden, J. (1997). Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 56: 81-87.

Razavi, S. M. A. and Karazhiyan, H. (2009). Flow properties and thixotropy of selected hydrocolloids, Experimental and modeling studies, Food Hydrocolloids, 23 (3): 908-912.

Razavi, S. M. A. and Moghaddam, T. M. (2011) Influence of different substitution levels of Lallemantia royleana seed gum on textural

characteristics of selected hydrocolloids. Electronic Journal of Environmental, Agriculture and Food Chemistry. 10: 2826-2837

Ribeiro, V. L. S., dos Santos, J. C., Bordignon, S. A. L., Apel, M. A., Henriques, A. T. and von Poser, G. L. (2010). Acaricidal properties of the essential oil from *Hesperozygis ringens* (Lamiaceae) on the cattle tick *Riphicephalus* (Boophilus) *microplus*, Bioresource Technology, 101 (7): 2506-2509.

Rodríguez-Martínez, J. M., Poirel, L. and Nordmann, P. (2009). Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa.* Antimicrobial agents and Chemotherapy. 53: 4783-4788

Samuelsen, O., Toleman, M.A., Sundsfjord, A. , Rydberg, J., Leegaard. T.M., Walder. M., Lia, A., Ranheim, T.E., Rajendra, Y., Hermansen, N.O, Walsh, T.R. and Giske, C.G. (2009). Molecular Epidemiology of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* Isolates from Norway and Sweden Shows Import of International Clones and Local Clonal Expansion. Antimicrobial agents and chemotherapy. 54:346-352

Stockwell, C., 1988. Nature's pharmacy. London, United Kingdom. Century Hutchinson Ltd.

Susy Hota, MD; Zahir Hirji, MHSc; Karen Stockton, MHSc; Camille Lemieux, MD, LLB; Helen Dedier, MLT; Gideon Wolfaardt, PhD;

Michael A. Gardam, MD, MSc. (2009). Outbreak of Multidrug-Resistant Pseudomonas aeruginosa Colonization and Infection Secondary to Imperfect Intensive Care Unit Room Design. Infection Control and Hospital Epidemiology Journal. 30: 25-33

Shinwari, Z. K., Rehman, M., Watanabe, T., Yoshikawa, Y. (2006). Medicinal and Aromatic Plants of Pakistan (A Pictorial Guide). Ph.D Thesis, Kohat University of Science and Technology, Kohat, Pakistan

Shinwari, Z. K. (2010) Medicinal plants research in Pakistan. Journal of Medicinal Plants Research, 4 (3): 161-176

Songsong Li. (2011) Enhancement of the antimicrobial activity of eugenol and carvacrol against Escherichia coli O157:H7 by lecithin in microbiological media and food. University of Tennessee, Knoxville

Stahl-Biskup E, Saez F (2002). Thyme. Taylor & Francis, London.

Stockwell, C., (1988). Nature's pharmacy. London, United Kingdom. Century Hutchinson Ltd.

Takayama., C., de-Faria, F.M., de Almeida, A.C.A., Valim-Araújo, Dd.AeO., Rehen, C.S., Dunder, R.J., Socca, E.A.R., Manzo, L.P., Rozza, A.L., Salvador, M.J., Pellizzon, C.H., Hiruma-Lima, C.A., Luiz-Ferreira, A. & Souza-Brito, A.R.M. (2011). Gastroprotective and ulcer healing effects of essential oil from *Hyptis spicigera* Lam. (Lamiaceae), Journal of Ethnopharmacology, 135 (1): 147-155.

Talebi, S. M. and Razekhanloy, A. (2010). The morphological study of the genus Lallemantia Fisch. Et Mey. (Lamiaceae) in Iran. Plant and ecosystem spring. 5: 3-20

Mohtasheemul, H., Salman, A., Ziauddin, A. and Iqbal, A. (2011). Antiemetic activity of some aromatic plants. Journal of Pharmaceutical and Scientific Innovation, 1 (1): 47-49

Ulubelen, A. (2003). Cardioactive and antibacterial terpenoids from *Salvia* species, *Phytochemistry,* 64 (2): 395-399.

Vlietinck, A.J., Van Hoof, L., Totte, J., Lasure, A., vanden Berghe, D., Rwangobo, P.C. and Mvukiyuniwami, J. (1995). Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. Journal of Ethnopharmacology, 46: 31-47.

Wang, F., Hu, Y., Hu, Z., Li, J., Tian, B. and Hu, Q. (2012). Characterization of drug-resistance and molecular epidemiology of Enterobacter cloacae among patients with clinical infections in a teaching hospital, China. African Journal of Microbiollogy Research. 6 (17): 3830-3835

Wazir, S. M., A. A. Dasti and J. Shah. (2004). Common medicinal plants of Chapursan valley, Gojal II, Gilgit-Pakistan. Journal of Research Science. 15(1): 41-43.

Wild, R. (1994). The complete book of natural and medical cures. Rodale Press, Inc, Emmaus, PA.

Zhang, H. J. and Li, L. N. (1994). Salvianolic acid 1: A new depside from *Salvia cavaleriei*. Planta Med, 60: 70-72

Zomlefer, W. B. (1994). *Guide to Flowering Plant Families.* Chapel Hill NC, University of North Carolina Press, North Carolina.

Sairafianpuor, M. (2002) Iranian Medicinal Plants and Antiparasitic Compounds: from Ethnobotany to Contemporary Scientific Evidence. Ph.D. Dissertion, Department of Medical chemistry, Royal Danish School of Pharmacy, Copenhagen.

Electronic Database Information

- eFloras.org
- http://www.tropicos.org
- http://images.wellcome.ac.uk
- my.gardenguides.com
- theseedsite.co.uk/)
- http://sites.nationalacademies.org/PGA/dsc/pakistan/PGA_052681
- http://www.ehagroup.com