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In Vitro Antibacterial Activity of Spinacia Oleracea and Melilotus Indicus Used In Pakistani Folk Medicines against Some Specific Bacterial Strains

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Abstract: *The medicinal plants Melilotus indicus and Spinacia oleracea were collected from herb dealer in order to check antibacterial activity in opposition to different Gram-positive and Gram-negative bacterial strains. These medically important bacterial strains including Pseudomonas aeroginosa, Bacillus subtilis, Micrococcus luteus and Escherichia coli. Well diffusion method was used to check antibacterial activity of these plants. Spanacia oleracea showed better antibacterial activity against these bacterial strains but Melilotus indicus did not illustrate any results. The collected ethonomedicinal plants are used in folk medicines in treatment of osteoporosis, high blood pressure, cancer, and kidney and liver complaints*.

Keywords: *Melilotus indicus, Spinacia ole*racea, *Pseudomonas aeroginosa, Bacillus subtilis, Micrococcus luteus*

I. INTRODUCTION

Diversity of the plants has been utilized for the medicinal purposes as well as in herbalism. Scientists proved the variety of medicinal effects enclosed by few plants or their phytochemical constituents which are authorized by distinctive regulatory authorities such as United States Food and Drug Administration or European Food Safety Authority (EFSA, 2012).

According to the estimate of World Health Organization about 75-80% of the world's population exploits plant remedies partially or exclusively. Human have absolutely depend upon nature for their requirements such as for manufacturing of food, shelter, clothing, fertilizers and medicines (Cragg and Newman, 2005). We all are better aware of these plenty of hidden secrets of nature. Man has discovered some of its secrets since primordial era. How medicinal plants could cure or improve diseases or afflictions, these are the most effective discoveries among all others (Chevallier, 1996). According to a rough estimate there are approximately 250,000 to 500,000 plant varieties subsist on Earth. Numerous of them are all natural herbal (Boeheme, 1982). All of these herbs are enormously beneficial for the medication of any infectivity or illness and are relatively expensive. Most importantly, their pill forms are restricted to store for a year or further at room temperature.

Medicinal plants are considered to be most vigorous supplier of life for all human beings. Plants are best exploited as medicines (Samuelsson, 2004). The therapeutic characteristics of plant are much advantageous as remedy of various diseases. Primarily these medicines acquired crude drugs manner (Balick and Cox, 1997). These medicinal plants are hundred percent natural which is the best benefit of them. Currently these medicinal plants can impart superlative solution as the degree of various ailments is elevated. At the present time, multiple drug resistance is in progress which is because of random manipulation of commercial antimicrobial antibiotics that are most frequently used for the management of various maladies.

Additionally on host, these antibiotics are linked with undesirable consequences comprising hypersensitivity, immune-suppression and allergic reactions. These circumstances made obligatory for scientists to discover innovative antimicrobial substances. There is still essential to discover new and effective therapeutic agents as the distressing rate of antibiotics confrontation in bacteria of remedial worth has revealed. So in order to handle severe contagious instability from medicinal plants, advancement of substitutive antimicrobial drugs is needed (Agarwal, 1996).

Practically, for major health care, universally all cultures have depend upon medicinal plants historically and persistently. According to rough valuation, one-third of whole conventional medicines are for the healing of lesions or skin syndromes evaluate to 1-3% of modern drugs. Beneficial features of medicinal plants on skin comprises: abrasion therapeutics and any damage by fire (especially Aloe Vera); antifungal, antiviral, and antibacterial activity adjacent towards skin contamination for instance acne, herpes and scabies. Activity opposed to inflammatory/immune chaos distressing skin (e.g. psoriasis); and anti-tumor upgrading activity next to skin cancer (Mantle *et al*., 2001).

1.1. Characteristics of Plants:

1.1.1. Melilotus Indicus

Melilotus Indicus, occasionally marked wrongly as Melilotus Indica, named sweet clover (or sweet-clover), sour clover (sour-clover) in English, annual yellow sweet clover, small-flowered sweet clover, small-flowered melilot and small melilot in Indian and King Island. Melilotin Australia and New Zealand has yellow flowers and innate to northern Africa, Europe and Asia but adapted all over the world (Loring, 2010).

1.1.2. Physical Characteristics

*Melilotus Indicu*s an annual emergent to 1 m (3ft 3in) by 0.6 m (2ft) and can fix nitrogen. Flowering season is from June to October and is hermaphrodite and the process of pollination is carried out by Bees. The tiny flowers are with thin, lengthened racemes and particular flowers are 3mm prolonged, resembles pea. It has alternate, 3 sharply-toothed leaves. It should be best in light (sandy), medium (loamy) and heavy (clay) soils with suitable PH neutral and basic (alkaline).

1.1.3. Classification

This plant involves in kingdom Planate and subkingdom Tracheobionta, super division Spermatophyte, division Magnoliophyta, and class Magnoliopsida. It is also inserted in subclass Rosidae, order Fabales, family Fabaceae, genus Melilotus Mill, species Melilotus Indicus and local name Sinji.

1.1.4. Medicinal Uses

Leaves and seeds of *Melilotus Indicus* are used for medicinal purposes such as:

- Antibacterial.
- Anticoagulant.
- Astringent.
- Emollient.
- Laxative.
- Narcotic.

Seeds exploit in bowel grievance and immature diarrhea (Khan, 2004).

1.1.5. Distribution and Habitat

It has broad national allocation in Micronesia, northern Africa, Europe, tropical Asia, UK US, South America, Australia and New Zealand (GRIN online & MMPLD Retrieved 2009-01-04).

1.2. Spinacia Oleracea

Spinach is an annual plant, *S. oleracea*, meant for succulent, edible leaves of this plant and prevalently cultured as a leaf vegetable.

1.2.1. Physical Characteristics

The leaves of *S. oleracea* are alternate, simple, and ovate to triangular, flat or curled, variable in size i.e.2-30 cm long and 1-15 cm broad. Larger leaves at the base and small leaves higher on the flowering stem. The flowers are inconspicuous, yellow-green having 3-4 mm diameter, maturing into a small hard dry lumpy fruit cluster with 5-10 mm. Contain several seeds and this plant get bigger to a height of 30 cm (Loring, 2010).

1.2.2. Classification

Spinach affixes in kingdom Plantae, division Magnoliophyta, class Magnoliopsida, order Caryophyllales, family Amaranthaceae, genus Spinacia, and species S. oleracea. It has binomial name Spinacia oleracea and local name Palak.

1.2.3. Nutritional value

S. oleracea once fresh, steamed, or quickly boiled has soaring nutritional value and full of antioxidants. Vitamins (A,E,C K and B2) , magnesium, manganese, folate, iron, , calcium, potassium, vitamin B6, folic acid, copper, protein, phosphorus, zinc, niacin, selenium and omega-3 fatty acids are calorific nutrients in it. Peptides termed rubiscolins have also been set up in spinach.

1.2.4. Medicinal Uses and Health Benefits

Spinach employs in the medication of constipation, anemia, high blood pressure, obesity, cancer, tumors, insomnia, osteoporosis, neuritis, dyspepsia and hypertension due to folate in them. It is also valuable for kidney and liver disorders. It has heavy amount of potassium but less intensity of sodium.

Its phytonutrients and pigments make easier to diminish skin cancer by shielding it from UV spoiling. It precludes the establishment of ulcers and other digestive sickness by shelter the mucous membrane of the stomach (Loring, 2010).

1.2.5. Diet

20% of the RDA of dietary fiber is offering in spinach one cup which can relieve digestion, constipation and maintains low blood sugar.

1.2.6. Cancer

Phytonutrients known as Flavonoids are present in large amount in spinach that has the property to retard the cell division in human stomach and skin cancer cells, they can also impart defense counter to prostate cancer.

1.2.7. Anti-Inflammatory

Neoxanthin and violaxanthin occur in extraordinary quantity in spinach and are two epoxy xanthophylls which are advantageous for controlling redness and soreness.

1.2.8. Antioxidants

Antioxidants such as Beta-carotene, vitamin C and E, manganese, zinc and selenium that is present in spinach resist the beginning of Hypertension, osteoporosis and atherosclerosis.

1.2.9. Blood pressure

Peptides inside spinach are known to lower the blood pressure by slowing down the enzyme i.e. angiotensin I-converting enzyme.

1.2.10. Brain and Nervous Function

Higher concentration of Vitamin K in spinach is helpful in maintain healthy nervous system and brain function through the formation of sphingo lipids class of Lipids that are involved in making framework Myelin sheath that surrounds of our nerves.

1.3. Characteristics of Bacterial Strains used

1.3.1. Escherichia Coli

Escherichia Coli is normally shortened as *E. coli and is* Gram-negative, rod-shaped bacteria, which is brought into being in the intestine of warm-blooded creatures like endotherms. Many strains of E. coli never give harm to others but its little quantity brought about severe food poisoning in humans and sometimes dependable for recollection by reason of food contamination (Vogt *et al.,* 2005).

Its non-toxic strains exists as usual flora of gut, yielding vitamin K2 for the assistance of their hosts (Bently *et al.,* 1982) and grounds the pathogenic bacteria inhibition in the intestine (Hudault and Servin 2001).

1.3.2. Classification

E.Coli domain and kingdom both is Bacteria with phylum Proteobacteria. Its class is Gamma Proteobacteria and possesses order Enterobacteriales. This bacterium confined in family Enterobacteriaceae, genus Escherichia and species Escherichia coli (E. coli).

1.3.3. Characteristics:

These Bacterial strains are facultative anaerobes, Gram-negative, rod shaped, lactose fermenter and none sporulating.

1.3.4. Habitat and Transmission:

It is present in human colon, vagina and urethra. It transmits during birth, in neonate's causing meningitis and diarrhea by fecal-oral routs.

1.3.5. Diseases:

It causes UTI, sepsis neonatal meningitis, food poisoning, gastroenteritis etc

1.4. Bacillus subtilis:

Bacillus subtilis (hay bacillus or grass bacillus) is gram-positive or catalase-positive organism (Madigan *et al.,* 2005). It has a genus Bacillus. It's generally come with rod like appearance and can bear intense situation of atmosphere because of having endospore in them. Traditionally it's an obligate aerobe but it's not true about it to some extent (Nakano and Zuber, 1998).

1.4.1. Classification:

B.subtilis is included in domain Bacteria, phylum Firmicutes, and class Bacilli. It consist of order Bacillales, family Bacillaceae, genus Bacillus and species Subtilis.

1.4.2. Pathogenesis:

B.subtilis may infect food but is not regarded as initiator of disease. It infrequently causes food poisoning. It usually generates protein breakdown enzyme subtilis. Their spores can tolerate high temperature so its possible function is cooking food so reliable to form *ropiness*a sticky. These bacteria construct long-chain polysaccharides in spoil bread dough which gives them tough stability (Ryan and Ray, 2004).

1.5. Micrococcus luteus:

Micrococcus luteus having 0.05 to 3.5 microns in diameter and is a Gram Positive bacteria

1.5.1. Habitat and Transmission:

Micrococcus luteus has most common location i.e. mucous membranes of nasal cavities, upper respiratory tract, and the lining of the mouth. It is also noticed in dirt, soil and the air (Fleming, 1922).

1.5.2. Classification:

This *micrococcus luteus* can best fit in kingdom Bacteria and enclosed in phylum Actinobacteria. It possesses order Actinomycet and retaining family of Micrococcaceae. It includes in genus Micrococcus and species *M. luteus* with binomial name is Micrococcus luteus.

1.5.3. Pathogenesis:

Micrococcus is not an ordinary disease causing agent but *M.luteus* can originate to produce skin complaints that generate intensely itching, ulcers in organisms with compromised immune system like newborn babies or AIDS patients. It instantly provides the basis for severe troubles in patients with cooperative immune system for example septic shock, pneumonia, endocarditis or sepsis. It is also accountable for hospital acquired infections (Seifert *et al.,* 1995)

1.6. Pseudomonas Aeruginosa:

P.aeruginosa is an ordinary bacterium that is thought to cause ailments in animals, especially in humans. Its habitat is in water, skin flora and most probable in synthetic atmospheres in the entire world. They flourished best in common environments and less oxygen surrounding. They make colonies in numerous usual and simulated territories. It utilizes organ substances as their food. They used to damage injured tissues in animals or those having with less resistance. Widespread irritation and sepsis are the general symptoms of such illness. Its consequences are really fetal if their gathering arises in the lungs, urinary tract and kidneys (Balch *et al*., 1994).

1.6.1. Classification:

Its binomial name is Pseudomonas aeruginosa and belongs to the Bacterial kingdom. Its phylum is Proteobacteria having class Gamma Proteobacteria with the order of Pseudomonadales containing family Pseudomonadaceae including genus Pseudomonas and comprising species P.aeruginosa.

This bacteria is Gram-negative, gives their best growth in the presence of oxygen having rod shape with unipolar motility (Ryan *et al*., 2004). *P. aeruginosa* is considered to be the fortuitous disease causing agent in plants (Iglewski, 1996). *P.aeruginosa* is believed to be species category of the genus Pseudomonas (Anazai, 2000). *P. aeruginosa* transmitted through fecal contamination, Water and Aerosols etc. It causes UTI, Pneumonia, Sepsis and Nosocomial infections.

II. MATERIALS AND METHODS

2.1. Materials:

Spinacia oleracea and *Melilotus indicus*, Conical flasks, Funnel, Pipette, Spirit Lamp, Petri plates, Cotton, Bacterial Cultures (*Micrococcus luteus , Pseudomonas aeroginosa , Bacillus subtilis, Escherichia coli*), Colonies Pickers and Borer.

2.2. Extraction Procedure:

Selected medicinal plants i.e. *Melilotus Indicus* and *Spinacia Oleracea* were collected from Herb Dealer in district Mansehra. These plants were cleaned through flowing tap water, making air free and then same material was converted to fine powder in addition to storage in air tight bottles.

a. Cold water extract:

Cold water was used for immersing ten grams of every plant and revolved in rotatory mixer at 200 × g for 24 hrs. Then by filter paper all removed out material was strained followed by centrifugation at 5000 × g for 7 min. The 100% amount of the whole extract was present in supernatant. Then dilution of condensed extracts was prepared by a sequence of 80%, 60%, 40%, 20%, 10% dilutions through suitable volumes of disinfected refined water.

b. Hot water extract:

Removal of 10grams dried powder was done in purified water in the duration of 6 hours at sluggish temperature (40 ^{o} C). Then muslin cloth was used for its filtration subsequent to 2 hours in addition to centrifugation at 5000 × g for 7 min. Supernatants assortment was conducted. Reiteration of this mechanism was carried out two times and then condensation of supernatants was performed for the formation of the end volume one –fourth of the starting volume after 6 hours ,then 121 ^{o}C was autoclaved along with the storage (Parekh *et al., 2005).*

c. Methanol extract

Ten grams dried powder was added in 100 ml methanol and reserved rotator shaker at 200 × g for 24 hours. Muslin cloth was used for their filtration and then centrifugation was done 7 min at 5000 × g. Gathering of supernatant was done. Airtight bottles were used for the storage at 4 ^{o}C for additional learning (Parekh *et al*., 2005).

d. Antimicrobial activity evaluation (Microorganisms utilized):

Determination of antimicrobial activity was performed in contrast to *E.coli, B.subtilis, P.aeruginosa* and *Micrococcus luteus.*

Table1. Gram reaction and the diseases caused by the bacterial species utilized during current study

S. No	Microorganisms	Gram strain	Disease caused
1	***E.coli***	-ve	Food poisoning, UTI, neonatal meningitis, gastroenteritis
2	***B.subtilis***	+ve	Food poisoning.
3	***P.aeruginosa***	-ve	UTI, Wound infection in burn patients.
4	***M.luteus***	+ve	Skin infections, septic shocks or sepsis.

e. Culture media

Nutrient agar was used for culturing of E.*coli, P.aeroginaosa, M.luteus and B.subtilis*. Nutrient agar was used for the growth of bacterial cultures. Fortnight sub culturing of every cultures were taken place and then in the presence of oxygen these cultures were warm up in incubator for 24 hours at 37 ^{o}C.

Table 2. Nutrient Agar Composition:

1	Extract (Beef)	1gm/l
2	Peptones	5gm/l
3	Agar	15gm/l
4	Glucose monohydrate	5gm/l
5	Extract(Yeast)	2gm/l
6	Sodium Chloride	5gm/l

f. Media Preparation:

The required quantity of Nutrient agar was added in pointed flask discretely. Flask was given high temperature on hot plate stirrer to dissolve the media completely. The media and all the glass-wares were sterilized by autoclaving at 15 psi and 121^{o}C temperature. Then in a laminar cabinet, media was transferred it inside petri plates subsequent to autoclaving. By preventing the concentration, germ free atmosphere was developed throughout discharging.

g. Agar diffusion assay:

Agar diffusion method was used for the estimation of antibacterial activity of *Spinacia oleracea* along with *Melilotus indicus* in opposite to various bacterial strains. The swabs were introduced with suspension formed by mixing of bacteria in 3ml of distilled water. Inoculum was introduced by spreading on the surface of solidified media horizontally and vertically by applying swab. In agar diffusion technique, formation of wells takes place by using cork borer (0.6cm) inside petri plates. Then by the assistance of uncontaminated micro pipette, the test compound was inoculated into the wells and the plates were kept warm in support of 24 hours at 37 $^{\circ}$C. With the help of scale, process of determining of diameter of zone of inhibition in millimeters had taken place in order to find out enlargement of microbes. This procedure was repeated three times and means values were accessible (Parekh *et al*., 2005).

III. RESULTS AND DISCUSSION

The antimicrobial activity of medicinal plants i.e. *Spinacia oleracea* and *Melilotus indicus* was examined in the current research. Four different species of bacterial strains which are mostly involved in common infections to human were used for the determination of antibacterial activity. From ancient times plants are important source for the development of new chemotherapeutic agents. Antibacterial activity is the first step towards the development of new agents. Regarding antiviral, antibacterial, antifungal, anthelminthic and anti-inflammatory properties of plants many reports have been available. (Mahesh and Satish. 2008)

The antibacterial activity of *Spinacia oleracea* and *Melilotus indicus* was investigated in different solvents. For this purpose extracts in three solvents used are Methanol, Cold water and hot water. The *Spinacia oleracea* extract was found to be effective in methanol and cold water. The methanol, hot water and cold water extracts of medicinal plants *Spinacia oleracea* show better activity of growth inhibiting against all of these bacterial strains. The highest activity was noted against Bacillus subtilis 1.76mm in cold water followed by E. coli 1.75mm in methanol. According to a research done in University of Azad Jammu and Kashmir, Muzaffarabad, Pakistan, the aqueous extract of fresh S. oleracea inhibited the growth of E. coli while on the other hand; the aqueous extract of fresh S. oleracea has minimum effect on the Micrococcus luteus. Similarly, the aqueous extract of discarded S. oleracea had no effect on the growth of M. luteus while the greater zone of inhibition was shown by E. coli (Nasim *et al*., 2012).

Melilotus indicus show no activity against these bacterial strains in the current study. According to a study done in Iran, highest MIC (minimum inhibitory concentration) of ethanolic and aqueous leaf extracts of *Melilotus indicus* against P.aeruginosa that is 497.4 mg/ml and similarly highest MIC of ethanol and aqueous seed extracts of *Melilotus indicus* against P.aeruginosa that is 498.5mg/ml (Javad Sharifi Rad *et al*., 2013).

The activity of different extracts of *Spinacia oleracea* and *Melilotus Indicus* is given in tables.

Table3. Showing Cold water extracts of *Melilotus Indicus* leaves

S .No	Bacterial species	Zone of inhibition(mm)			Mean(mm)
1	Bacillus subtilis	0	0	0	0
2	Micrococcus luteus	0	0	0	0
3	P.aeruginosa	0	0	0	0
4	Escherichia coli	0	0	0	0

Table 4. Hot water extracts of *Melilotus Indicus* leaves

S. No	Bacterial species	Zone of inhibition(mm)			Mean(mm)
1	Bacillus subtilis	0	0	0	0
2	Micrococcus luteus	0	0	0	0
3	P.aeruginosa	0	0	0	0
4	Escherichia coli	0	0	0	0

Table 5. Showing Methanol extracts of *Melilotus Indicus* leaves

S.No	Bacterial species	Zone of inhibition(mm)			Mean(mm)
1	Bacillus subtilis	0	0	0	0
2	Micrococcus luteus	0	0	0	0
3	P.aeruginosa	0	0	0	0
4	Escherichia coli	0	0	0	0

Table 6. Showing Hot water extracts of *Spinacia oleracea leaves*

S.No	Bacterial species	Zone of inhibition(mm)			Mean(mm)
1	Bacillus subtilis	1.5mm	1.4mm	1.4mm	1.43mm
2	Escherichia coli	1.35mm	1.6mm	1.45mm	1.6mm
3	P.aeruginosa	1.45mm	1.5mm	1.4mm	1.4mm
4	Micrococcus luteus	1.46mm	1.3mm	1.46mm	1.45mm

Table 7. Showing Coldwater extracts of *Spinacia oleracea* leaves

S.No	Bacterial species	Zone of inhibition(mm)			Mean(mm)
1	Bacillus subtilis	1.9mm	1.7mm	1.7mm	1.76mm
2	Escherichia coli	1.3mm	1.5mm	1.7mm	1.45mm
3	P.aeruginosa	1.3mm	1.6mm	1.3mm	1.45mm
4	Micrococcus luteus	1.4mm	1.4mm	1.7mm	1.5mm

Table 8. Showing methanol extracts of *Spinacia oleracea* leaves

S.No	Bacterial species	Zone of inhibition(mm)			Mean(mm)
1	Bacillus subtilis	1.65mm	1.6mm	1.7mm	1.65mm
2	Escherichia coli	1.8mm	1.8mm	1.7mm	1.75mm
3	P.aeruginosa	1.4mm	1.4mm	1.3mm	1.45mm
4	Micrococcus luteus	1.4mm	1.4mm	1.4mm	1.43mm

Herbs have been considered as a supply of medicinal complexes from prehistoric ages. Medicine records and annals of civilization are mainly concerned with the identification of archives of utilization of herbal medicine in the management of various ailments. Numerous human diseases such as wound infections, typhoid, dysentery, jaundice, UTI and skin diseases can be cured by majority of parts of plants especially used in Tibbi, Unani, Allopathic systems of medicines.

IV. CONCLUSION

In present times, adjacent to various prepared antibiotics, a large amount of bacterial pathogens becomes evidence for resistance. Natural medicines are taken into account to be most effective. Just because of all these incentives, those bacterial pathogens are given priority for selection that instigates common infections. As test organisms four pathogenic strains of bacteria were operated i.e. *E.coli, Bacillus subtilis, M.luteus and P. aeroginosa* and along with this most importantly the sensitivity of plant extracts were analyzed. The studies accomplished on bacteria's concerning these plants are not only restricted to social originated infection and factors but these are also effectual in opposition to various microorganisms that were acquired from infections emerging in patients lying in hospital which resists countless medicines. Enhanced activity of growth inhibition against these strains can be exposed by Cold water, Hot water, Methanol extracts of *Spinacia oleracea.* Not any growth inhibition to these bacterial strains can be revealed by *Melilotus Indicus.*

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