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Phytochemical and Synergistic Effects of Aerial Parts of *Olea Ferruginea* Royle Plant Extract against Nosocomial Bacterial Pathogens

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ABSTRACT

Due to the misuse of antibiotics drug resistance is increasing to herbal medicines are used as drugs nowadays. Northern Pakistan mountainous regions are full of medicinal plants. Among them Olea ferruginea is a native plant of northern areas of Pakistan. Those peoples who are admitted in hospitals, local health care centers, nursing homes etc are highly exposed to Methicillin-resistant Staphylococcus aureus (MRSA). The present study was conducted to evaluate the in vitro action of Methanol extract of O. ferruginea against MRSA. Confirmed strains were obtained from Khyber Teaching Hospital Peshawar and were brought to Microbiology Research Laboratory, Abasyn University Peshawar for further processing. Antibiotic sensitivity of the bacterial isolates of MRSA was checked on Mueller Hinton Agar (MHA) by using various antibiotics like cefoxitin, vancomycin, linezolid, erythromycin, doxycycline, clindamycin and levofloxacin. The phytochemical screening of the Olea ferruginea Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of various essential constituents i.e., flavonoids, Alkaloids, saponins, tannins. It has been revealed that the highest zone of inhibition (ZI) was produced against MRSA 1 (37±0.4 mm) followed by MRSA 2 (34±0.5mm) and MRSA 3 (29±0.5mm). Cefipime was used as

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positive control whereas dimethyl sulfoxide (DMSO) was used as negative control In susceptibility assay the linezolid showed highest activity i.e. 30.45±0.27 while linezolid and extract combination has no synergistic effect followed by vancomycin alone i.e. 16±0.5 mm while extract with vancomycin antibiotic has no synergistic effect as compare to alone antibiotic Doxycycline 15±0.17 mm combination has no synergistic effect Levofloxacin zone of inhibition 14±0.5 mm extract with Levofloxacin has not showed synergistic effect Minimum Inhibitory concentration is the least (minimum) concentration of extracts alone, and antibiotic + extracts combinations at different concentrations (5, 10, 15, 20 µl) plant extract alone and combination which prohibit the growth of microbe. MIC values of alone and antibiotic + extracts combinations were determined against MRSA at 5µl concentration. It recommended that alone extract of O. ferruginea can be used in treatment of nosocomial infection specifically against MRSA.

Keywords: Olea Ferruginea Royle, Medicinal Plants, Methicillin-Resistant Staphylococcus Aureus (Mrsa), Methanol Extract, Phytochemical Screening, Antibacterial Activity, Zone Of Inhibition, Minimum Inhibitory Concentration (Mic), Northern Pakistan, Nosocomial Infections

INTRODUCTION

Ethno-botany is a branch of science which deals with relation between plants and humans. This includes economic as well as medicinal uses. In different cultures the medicinal characteristics of plants have been researched and documented for years. Plants not only play a vital role in traditional medicine, but also play an important role in modern research such as phytochemical analysis. Based on this, synthetic drugs were developed. Medicative plants are a large class of plants with important economic significance, and their raw materials are used to synthesize drugs, perfume and cosmetics. Products made from these plants are a valuable source of income and are the main therapeutic discoveries of current era. However, due to the widespread use of antibiotics, people today are facing the problem of resistance to almost all pathogens (Howes & Houghton 2012). They also bring foreign currency to the country through exports. Pakistan has a very broad biodiversity, which comprises of 6000 total flora. In hilly regions of Pakistan i.e., Khyber Pakhtunkhwa (KP) and northern region of Hindukush–Himalayas of Pakistan more than 4000 plant species

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are found.

The use of plants by man is dated back to the origin of life on earth. In the beginning plant use was restricted to food, medicine and shelter but with the passage of time man explored the potential of plants for a number of other purposes. Hence, their dependency on plants increased both directly and indirectly. Wild plants have always been the matter of high concern and have always been used for their potential of human well-being. With the passage of time wild plants were cleared from their original habitat to replace the desired cultivated crops on large scale. This practice has always been affected by the availability of plants in their natural habitat and the way these resources are used by the local people are imperative. In developing countries medicinal plants provide a real alternative for primary health care. Due to the high cost of conventional allopathic medicine and inaccessibility of medicinal health care facilities especially in rural areas, the locals are compelled to rely on medicinal plants (Ali *et al.*, 2009).

Almost 80% of the world′s population depends on plants remedies for its primary health care needs. The local people of the rural areas have good knowledge about the uses of plants and they prefer medicinal plants due to their easy availability and cheap therapy as compared to costly pharmaceuticals. Inhabitants of the remote areas have discovered the therapeutic activity of medicinal plants against certain diseases through their indigenous experiences. Number of studies have been carried out from the world on medicinal uses of plants among various indigenous communities. In neighboring countries of Pakistan, remarkable ethno-botanical work has been done in the past. While in all these studies qualitative approaches have been adapted to document ethno-botanical information. In Pakistan ethnobotany is getting matured with the passage of time and various studies have been reported from various parts of the country (Bibi *et al.*, 2014)

O. ferruginea Royle is a halophytic plant under conditions of salt stress, the plant increases its water content (becomes more succulent) and decreases the surface area of its leaves. However, seeds treated at high salinity levels recovered their germination potential after immersion in unsalted water. Chemical drugs have side effects so the tendency towards herbal medicine is increasing worldwide

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Commercially available chemical agents have unwanted effects like vomiting and diarrhea. Hence the search for atypical drugs continues and natural compounds extracted from plants can be used as alternative medicines (Mahdavi & Isazadeh, 2019)

O. ferruginea Royle is a native plant species which is found in hilly areas of India, Pakistan (Dir, Kashmir, and tribal area) and Afghanistan (Amjad, 2020). It is an ever-green tree which grows naturally and is also cultivated. It is an indigenous medicinal plant which belongs to family *Oleaceae*, locally known as Kahoo or Khoona in Pakistan. Medicinal plants are used to treat various ailments throughout the world. Such uses of plants vary from one region to another and are analyzed with the use of quantitative techniques (Hashmi *et al.*, 2015).

In the Holy Quran, olive tree is termed as a precious fruit. The name of olive tree is used seven times in the Holy Quran. The first plant in the Bible is the olive plant. the oil and tree of olive mentioned over 30 times in the Bible. The fruits and leaves of the olive are best sources of phenols, triterpenoids and flavonoids. An important therapeutic compound obtained from the leaf extract is oleuropein. There is not much difference between the activities of crude leaf extracts and extra virgin oils. The results indicate that compared to purified oil, the leaf extract is effective antibacterial agent and is cheap (Hussain *et al.*, 2014).

The whole parts of *O. ferruginea* are used as home remedy for the treatment of different types of diseases i.e diarrhea, dyspepsia, inflammation, antimicrobial fever, anti-diabetic ulcers, rheumatic swellings and nyctalopia (Mehmood *et al.*, 2018). Bacteria can cause severe infections in humans and other animals. For example, *S. aureus* has been found to cause superficial skin lesions and food poisoning. *Pseudomonas aeruginosa* is a nosocomial pathogen involve in high ratio of hospital-acquired infections (Malik *et al.*, 2015).

Plant synthesize and produce different types of secondary metabolites which possess antimicrobial activity. Formerly it was thought as secondary metabolites, not the products of the primary metabolic pathway, have no advantage to the plants who produced them. However, now it is believed that they contain vigorous functions.. To meet this need present work was carried out to scan the antimicrobial

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activity of a valuable medicinal plant such as *O. ferruginea*. Olive tree (*O. ferruginea* Royal), covers 8 million hectares in Mediterranean countries almost 98% of the world crop, is one of the most important fruit trees . These figures show its pronounced economic and social meaning and the probable aids to be derived from exploitation of any of its byproducts. The fruits and oil of Olea, important constituents of daily diet in a large part of the world's population, are widely studied for their alimentary use, whereas the leaves contain important secondary metabolites like oleuropein and oleacein, the former responsible for hypoglycemic activity .and the latter for hypotensive activity. It was also shown by several studies that leaf extract of olive has the ability to reduce the blood pressure in animals, prevent intestinal muscle spasms and relieve arrhythmia. Present work was aimed to investigate the phenolic contents, antimicrobial activity of *O. ferruginea* (Mehmood *et al.*, 2018).

Plants are recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. The biologically active compounds present in plants are called phytochemicals. These phytochemicals are derived from various parts of plants such as leaves, flowers, seeds, barks, roots and pulps. These phytochemicals are used as sources of direct medicinal agents. They serve as a raw material base for elaboration of more complex semi-synthetic chemical compounds. Since the phytochemicals cure diseases without causing any harm to human beings these can also be considered as "man-friendly medicines" (Banu & Cathrine 2015).

The potential of the phytochemicals have large scale pharmacological and biological activities such as antioxidant constituents (hydrolysable tannins, phenolic acid and flavonoids) of the plant materials for the care of health and protection from coronary heart diseases, cancer, anti-carcinogenic and anti-mutagenic effects. Varieties of herbaceous vegetables are protective against various diseases, particularly cardiovascular diseases. These herbaceous plants and species are harmless sources for obtaining natural antioxidants. Antioxidant constituents can delay or inhibit the oxidation of lipids and other compounds by inhibiting the propagation of oxidation chain reaction (Hussain *et al.*, 2011).

The term "Nosocomial" refers to any disease that a patient acquires during

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hospitalization. Recently, a new term "healthcare associated infections" is being used to refer to the types of infections caused by long-term hospitalization, which are the main risk factors for serious health problems and may even lead to death. Approximately 75% of the burden caused by these infections occurs in developing countries. If these pathogens are found in the body fluids or at a sterile body site, such as blood or cerebrospinal fluid an asymptomatic patients may be considered as infected. Infections acquired by health-care workers or hospital visitors can also be considered as nosocomial infections (khan *et al.*, 2015).

Annoyance from infectious diseases caused by drug/antibiotic resistant bacteria is common place in clinical management Oftentimes, newly added antibiotics control infections from resistant bacteria; however, over time, strains that are resistant to the new antibiotics also develop continually. Thus, in the continual process of drug development, several antibiotics, for example penicillin and their derivatives, have been introduced by the structural modification(s) of penicillin. It should be noted that the horizontal gene transfer of resistance character(s) causes movement to different bacteria of the same, similar or different heritage, so much so that progressively more antibiotic resistant bacteria emerge. For example, although it was once clinically known to be a harmless commensal, the Gram-positive (GP) bacterium *S. aureus* has now become drug resistant to a group of narrow-spectrum β-lactam antibiotics, the methicillin. The resistant strain, called methicillin-resistant *S. aureus* (MRSA), has become peripatetic and has caused grievous problems in surgical site infections, burn site infections, and urinary tract infections (UTI), which all lead to bacteremia in the lungs, septicaemia and innards, such as persistent lung infection and endocarditic, which cause morbidity and unexpected mortality (Swain *et al.*, 2015).

For MRSA, resistance is as a result of Methicillin-sensitive *S. aureus* (MSSA) strains that have acquired Staphylococcal Cassette Chromosome mec (SCCmec) which carries mecA gene. The gene encodes the penicillin-binding protein (PBP2a) which confers resistance to all β-lactam antibiotics. Vancomycin was previously the widely preferred drug for the treatment of MRSA infections. It is no longer the case with the emergence of *S. aureus* strains with reduced vancomycin sensitivity limiting

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the conventional treatment options for MRSA infections to very scanty expensive drugs. Presently, many researchers have reported the antibacterial activity of many plant extracts on MRSA. Hence, medicinal plants might be promising candidates for treatment of MRSA infections (Okwu *et al.*, 2019).

According to many studies the plant essential oils show many antimicrobial properties and hence can be used as possible tools in future against drug resistance properties of different microbes. For example, *in-vitro* use of essential oils from *Eucalyptus globulus and Thymus vulgaris* against methicillin resistant bacteria such as *Staphylococcus aureus* (MRSA) and other strains show significant results (Tohidpour *et al.*, 2010). Synergy occurs when two or more substances work together to provide a combined effect that is greater than the sum of their individual effects. It can be seen of as a natural "straight" method that has evolved over time to achieve more efficacy at a lower cost. Synergistic effects between herbal medicines and conventional pharmaceuticals or biochemical molecules have been discovered in this area. It's critical to recognize and capitalize on these interactions since any improvement brought about by such a process can be employed to cure human illnesses (Pezzani *et al.*, 2019).

The screening of crude plant extracts for synergistic interaction with antibiotics is expected to provide leads for the isolation of MDR inhibitors. The use of *Catha edulis* extracts at subinhibitory levels has been reported to reduce the minimum inhibitory concentration (MIC) values of tetracycline and penicillin G against resistant oral pathogens, *Streptococcus oralis*, *Streptococcus sanguis* and *Fusobacterium nucleatum*. A number of compounds with an *in-vitro* activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Polyphenols (epicatechin gallate and catechin gallate) have been reported to reverse beta-lactam resistance in Methicillin Resistant *Staphylococcus aureus* (MRSA). Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to have resistance modulating abilities on various antibiotics against resistant strains of *S. aureus* (Aiyegoro *et al.*, 2009)

Liaqat *et al.*, (2021) investigated the physicochemical, phytochemical, *in-vitro* anti-

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diabetic and anticancer potential of *O. ferruginea* bark. Primary and secondary metabolites were present in the extractants; glycol-saponins were found in the methanol extract, polyphenols and flavonoids were found in the chloroform extract and polysaccharides were found in the ethanol extract. The highest antidiabetic potential exhibited was by the chloroform extract and maximum alpha-amylase inhibition glucose uptake was done by ethanol extract. Cytotoxic activity against the HepG2 cells was shown by only ethanol extract which was dose-dependent. Hence the bark possessed *in-vitro* antidiabetic potential and anticancer/cytotoxic effects, attributable to the downfall ratio in the pro-survival signals of the Akt signaling pathway

Aim and Objectives

The study was designed with the aim of " Phytochemical and Synergistic Effects of aerial parts of *Olea ferruginea* Royle Plant Extract against Nosocomial Bacterial Pathogens while the objectives were to:

1. Prepare crude methanolic extract from aerial parts of *O. ferruginea* Royle plant and its phytochemical analysis.
2. Investigate the antibiogram pattern of MRSA strains isolated from Nosocomial infection.
3. Assess anti-bacterial potential and synergistic effects by using Minimum Inhibitory Concentration (MIC) and Fractional Inhibitory Concentration (FIC) of plant extract along with antibiotics against isolated MRSA strain.

MATERIALS AND METHODS

This research study was carried at the Microbiology Research Laboratory (MRL), Abasyn University Peshawar.

3.1 Collection of Bacterial Strains

Already confirmed strain of MRSA was taken from Khyber Teaching Hospital Peshawar and was brought to Microbiology Research Laboratory, Abasyn University Peshawar for further processing.

3.2 Gram Staining

Gram staining is a technique which is used to differentiate bacteria on the basis of their cell wall structure. Gram positive bacteria stain blue to purple and gram

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negative bacteria stain red to pink.

Procedure

For the preparation of the smear, a clean slide was taken. After taking the slide, the smear was prepared by taking inoculum from the sample. The smear was air dried and heat fixed. Crystal violet was then poured and kept for about 30 sec to 1 min and rinsed with tap water. Gram's iodine was flooded for 1 min, and then washed. Then washed with 70% alcohol for about 10-20 sec and rinsed with water. At the end, counter stain (safranin) was added for about 1 min and then washed with distilled water or tap water. Then it was left to air dry and was observed under microscope. Red to pink colonies showed the presence of gram negative bacteria while blue to purple stained bacteria were gram positive.

3.3 Biochemical Tests

Different biochemical tests were performed for confirmation of organisms i.e. Catalase, Oxidase, Coagulase, TSI (Triple Sugar Iron), Urease, Indole and Citrate Tests as described by(Hemraj *et al.*, 2013)

3.4 Catalase Test

This test was used to identify microorganisms producing catalase enzyme. The enzyme produce bubbles (frothing) which indicates production of oxygen gas. The *Staphylococci* species are catalase positive while *Streptococcus* and *Enterococcus* species are catalase negative.

3.5 Oxidase Test

Oxidase test was used to determine bacteria which produce oxidase enzyme. The test was used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product.

3.6 Indole Test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. Tryptophan is an amino acid that undergo deamination and hydrolysis by bacteria that express tryptophanase and can convert amino acid tryptophan to its by-products. This test is used to differentiate among

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the families of *Enterobacteriaceae*. When indole is combined with Kovac's reagent (which contains hydrochloric acid and dimethyl-amino-Benzaldehyde in amyl alcohol). The solution turns from yellow to cherry red. Because amyl-alcohol is not water soluble, the red coloration will form an oily layer at the top of the broth.

3.7 Citrate Test

Citrate utilization test was used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium phosphate monobasic ($NH_4H_2PO_4$) is the sole fixed nitrogen source. Simon's citrate agar slants contain sodium citrate and ammonium ions. A pH indicator, bromomethyl blue is also included. Bromomethyl blue is green at PH < 7.0. Organisms that utilize citrate for energy, produce alkaline compounds as by-product. This test is used to differentiate among the gram negative bacilli in the family of *Enterobacteriaceae*.

3.8 Triple Sugar Iron (TSI)

Triple Sugar Iron is a differential medium that was used to differentiate enteric bacteria based on the ability to reduce sulphur and ferment carbohydrates based on physiological ability of organism, i.e. fermentation of glucose, lactose or sucrose for acid production, produce gas during fermentation and generate H_2O. If only glucose is fermented, acid produced in the butt will turn it yellow, but insufficient acid products are formed to effect the methyl red in the slant. However if either sucrose or lactose were formed, sufficient fermentation product turned both the butt and the slant yellow. If gas was formed during the fermentation, it caused the butt to form bubbles or as cracked. If no fermentation occurred, the slant and the butt remained red. Black precipitation in the butt revealed the presence of H_2S gas.

3.9 Urease Test

Urease test was performed to determine the bacteria's ability to breakdown the urea in the test tube. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Pink color indicates positive urease test.

3.10 Antibiogram Pattern

Antibiotic sensitivity of the bacterial isolates of MRSA was checked on Mueller

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Hinton Agar (MHA) by using various antibiotics like cefoxitin, vancomycin, linezolid, erythromycin, doxycycline, clindamycin and levofloxacin. The antibiotic susceptibility profiles were selected based on the Clinical and Laboratory Standards Institute (CLSI guidelines 2019).

3.10.1 *Culture Media Used*

The MRSA were cultured on the following media:

- MSA media: selective media for *S. aureus*
- MHA: for checking the sensitivity pattern of bacterial isolates
- Nutrient agar: used for sub culturing

Mueller Hinton Agar Media

Mueller-Hinton agar (MHA) is the best medium used for routine susceptibility testing using Kirby-Bauer disc diffusion method for non-fastidious bacteria (both aerobes and facultative anaerobes).

For the preparation of the media, 38g of the media was suspended in 1L of distilled water. After suspending the mixture was heated and stirred to fully dissolve all components. The media was autoclaved at 121°C for 15 min. Once the nutrient agar has been autoclaved, it was allowed to cool while protecting it from solidifying. The cool media was poured into each plate and was left for solidification in sterile environment. Then the sample was inoculated and the plates were incubated at 37°C for 24 hrs. Jorgensen & Turnidge, 2015)

3.11 Collection of Plant

The aerial parts of *O. ferruginea* plant was collected in sterile polythene bags and was washed with running tap water several times to removed dust particles. It was identified in the Department of Botany, University of Peshawar. The plant was shade dried with no direct exposure to sunlight. The dried samples were crushed to powder by electric grinder. The powdered samples were then stored at room temperature and packed and sealed in clear polythene pouches (Hasan *et al.*, 2011) for future use.

3.12 Preparation of Plant Methanolic Extract

For crude methanolic extract preparation, *O. ferruginea* powder was dissolved in methanol and was kept for 7 days at room temperature with occasional mixing and

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shaking. After that all soluble components were filtered. The filtrate was evaporated through rotary evaporator at 45°C to obtain semi-solid crude extract (Kumar *et al.*, 2016).

3.13 Phytochemical Screening

For the presence of different active constituents i.e., alkaloids, tannins, flavonoids, saponin and steroids the extract was screened out by using standard procedures (Haseena & Jyothsna, 2019).

3.13.1 *Test for Alkaloids*

For assessing alkaloids, 2-3 ml of filtrate, Mayer's reagent and 1 ml of diluted HCl was added in a test tube and shaken. Formation of yellow precipitation indicated the presence of alkaloids.

3.13.2 *Test for Tannins*

To the extract, 5% $FeCl_3$ solution was added, the appearance of deep blue color confirmed the presence of tannins.

3.13.3 *Test for Flavonoids*

To 1 mL of the crude stock extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour appeared which became colourless on the addition of a few drops of dilute acid which indicated the presence of flavonoids

3.13.4 *Test for Saponins*

The saponin content was measured by dissolving 0.5 gram of extract in water and boiled. The height of the froth was used for the measurement of saponin in the sample.

3.13.5 *Test for Steroids*

By Libermann Burchard test steroids was detected. 2 drops of chloroform, 2ml of extract and 2ml of acetic anhydride was added to 1ml of concentrated sulphuric acid to the side wall of the test tube. The formation of a reddish ring at the contact zone of the two liquids and a greenish color in the separate layer was indicated the presence of steroids.

3.14 Evaluation of Antibacterial Activity of Extracts

For the assessment of antibacterial activity of plant extract well diffusion method was used. Bacterial broth culture was spread on the MHA plates using a sterile swab

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and wells were bored with sterile cork borer. Then stock solution of the crude extract was prepared by dissolving 3mg/ml of DMSO (<1%). From the stock solution about 100 µl was added to each well. As positive control Cefipime was used and as negative control DMSO was used. The plates were incubated at 37°C for 24 hrs and then the sensitivity is determined by measuring the zone of inhibition around the wells and antibiotic disk. The growth of the bacteria that are susceptible were inhibited around the extract well but those that are resistant were not showing inhibition (Essassi *et al.*, 2014).

3.15 Evaluation of Synergistic Effect

The prepared plant extract was measured in equal amount to the antibiotic disk and was inoculated in the antibiotic culture sensitivity disk as per the standard protocol. The antibiotics with the extract disc were incubated for 24 hrs at 37°C and the respective zone of inhibition was measured accordingly. This study was performed as triplicated.

3.16 Antibiotic, extracts and its combination (MIC) Assay

This technique was used to determine the lowest concentration of the extracts to inhibit the tested bacteria. For MIC determination a broth macro-dilution method was utilized for antibiotic, extracts alone, and antibiotic + extracts combinations at different concentrations (5, 10, 15, 20 µl). The bacterial cultures were transferred to sterile tubes. The extracts were incubated for overnight at 37°C. After incubation period the MIC of the extracts were considered positive in case of lack of any observable growth in tubes compared to blank control (Tin *et al.*, 2009).

3.17 Evaluation of Potentiation or Additive activities with selected Antibacterial Drugs

For the evaluation of additive, synergistic or antagonistic action, FIC (Fractional inhibitory concentration) & FIC Indices Fraction Inhibitory concentration indices (FICI) was determined using following formulas (Buyck *et al.*, 2015).

$FIC_{ab} = MIC_{(ab\ combination)} / MIC_{(ab\ alone)}$

$FIC_{extract} = MIC_{(extract\ in\ combination)} / MIC_{(extract\ alone)}$

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RESULTS

4.1. Phytochemical Analysis

Alkaloids

The phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of essential constituents Alkaloids.

Flavonoids

The phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of Flavonoids.

Tannins

The phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of Tannins.

Saponin

The phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of essential constituents Saponin.

Steroids

The phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of essential constituents steroids as shown in Table 4.1

Table 5.1: Phytochemical Analysis of *O. ferruginea* extract

Phytochemical	Detected/not detected
Alkaloids	+
Flavonoids	+
Tannins	+
Saponin	+
Steroids	+

4.2. Identification of Bacteria

Already confirmed strains of MRSA were taken from Khyber Teaching Hospital

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Peshawar and were brought to Microbiology Research Laboratory, Abasyn University Peshawar for further processing. They were processed according to the standard procedures i.e. Gram staining and Biochemical tests. The results of the gram staining and biochemical tests are shown in the Table 4.2.

Table 4.2: Bacteriological Assessment of Nosocomial MRSA Samples

Identified Organisms	Gram Staining	Citrate Test	Indole Test	Oxidase Test	Coagulase Test	Urease Test	Catalase Test	TSI test
S. aureus	+	+	-	-	+	+	+	**Acidic**

4.3. Antibacterial Activity

The antibacterial activity of the *O. ferruginea* was evaluated using 03 different *MRSA* bacterial species. The extract was administered at 100 µl concentration. The extract was screened for the antibacterial activity. It has been revealed that the highest ZI was produced against MRSA 1 (37±0.4 mm) followed by MRSA 2 (34±0.5mm) MRSA 3 and MRSA 4 (29±0.5mm) respectively while MRSA 5 (26±0.5mm) and MRSA 5 (25±0.4mm) Cefipime was used as positive control whereas DMSO was used as negative control. Results shown in Table 4.3 and Figure 4.3.

Table 4.3 Antibacterial Activity of the *O. ferruginea* against MRSA Bacteria

Bacteria	ZI (mm)	+ve control (FEP)	-ve control (DMSO)
MRSA 1	37±0.4 mm	12±0.5mm	0
MRSA 2	34±0.5mm	11±0.5mm	0
MRSA 3	29±0.5mm	0	0
MRSA 4	29±0.5mm	0	0
MRSA 5	(26±0.5mm)	0	0
MRSA 6	(25±0.4mm)	0	0

Key words: ZI=Zone of Inhibition,FEP= Cefipime

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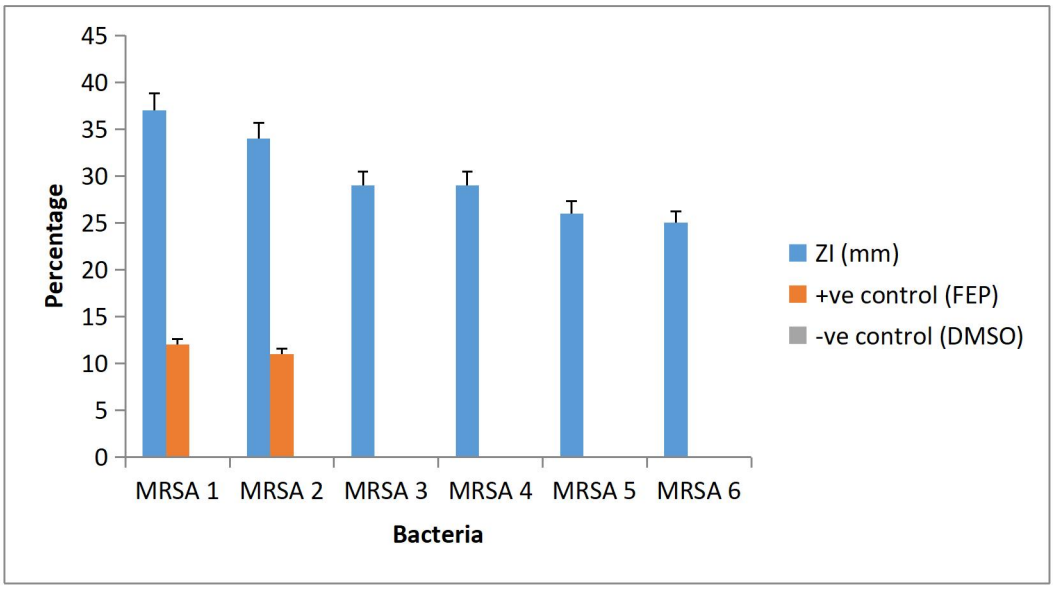


Figure 4.3 Antibacterial Activity of the O. Ferruginea Against MRSA Bacteria

5.4. Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory concentration is the least (minimum) concentration of extracts alone, and antibiotic + extracts combinations at different concentrations (5, 10, 15, 20 µl) plant extract alone and combination which prohibit the growth of microbe. MIC values of alone and antibiotic + extracts combinations were determined against MRSA at 5µl concentration respectively, Results are given in table 5.4 and figure 5.4

Table 4.4: MIC of Alone, and Antibiotic + Extracts Combinations against *MRSA.*

Alone and Combination	**Stock solution 20mg/ml**
	MIC (5-20µl)
Extract	05
Extract with antbiotic	05

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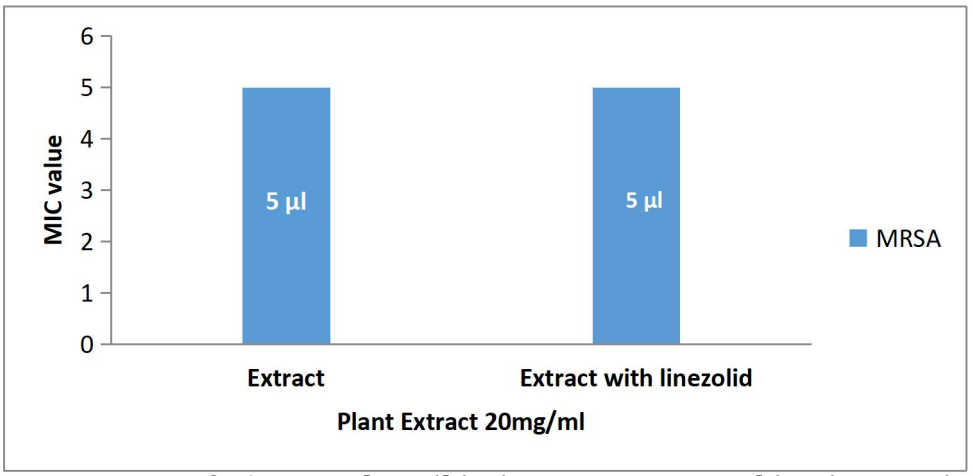


Figure 4.4: MIC of Alone, and Antibiotic + Extracts Combinations against MRSA

4.5. Fraction Inhibitory Concentration

The FIC of linezolid with extract against *MRSA*. The combination of this drug was used in different concentrations and FIC values were calculated. It is evident from the table that synergy was shown against *MRSA*. While when these drug and extract were applied on tested bacteria. Synergy was detected in all the cases.

FIC (ab) =MIC (comb.)/MIC (Alone)

Table: 4.5 Fraction Inhibitory Concentrations of Antibiotic and Extract against MRSA

Antibiotic + extract	FIC	Index	Remarks
linezolid +extract 20/ml	5	5	Synergy

5.6. Susceptibility of *MRSA* for extract in Combination with Antibiotics

In susceptibility assay the linezolid showed greater activity i.e. 30.45±0.27 while linezolid and extract combination has no synergistic effect followed by vancomycin alone i.e 16±0.5 mm while extract with vancomycin antibiotic has no synergistic effect as compare to alone antibiotic Doxycycline 15±0.17 mm combination has no synergistic effect Levofloxacin zone of inhibition 14±0.5 mm extract with Levofloxacin has no showed synergistic effect as shown in table 5.6

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Table 5.6 Susceptibility of *MRSA* for extract in Combination with Antibiotics

S. No	Antibiotics	*MRSA* zone of inhibition (mm)	
		Uncoated	Coated
1.	Cefoxitin	0	0
2.	Vancomycin	16±0.5	14±0.5
3.	linezolid	30.45±0.27	24±0.27
4.	Erythromycin	10	15
5.	Doxycycline	15±0.17	12±0.17
6.	Clindamycin	0	0
7.	Levofloxacin	14±0.5	12±0.5
8.	Cefipime	0	0

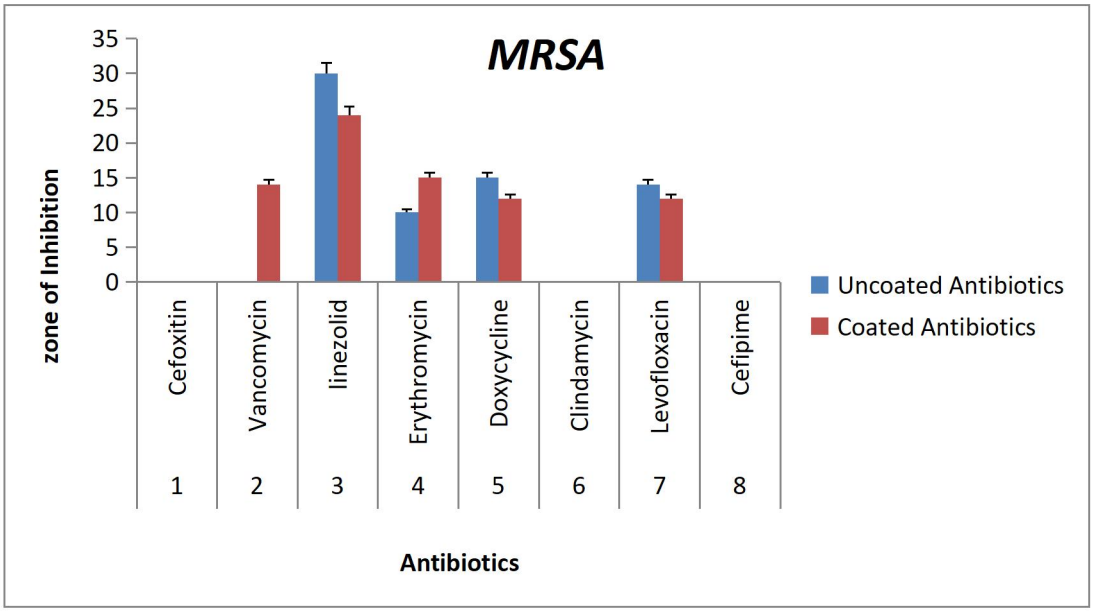


Figure 4.6 Susceptibility of *MRSA* for extract in Combination with Antibiotics

DISCUSSION

Methicillin-resistant Staphylococcus aureus (*MRSA*) infections occur in people who've been in hospitals or other health care settings, such as nursing homes and

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dialysis centers. The term "Nosocomial" refers to any disease that a patient acquires during hospitalization. Recently, a new term "healthcare associated infections" is being used to refer to the types of infections caused by long-term hospitalization, which are the main risk factors for serious health problems and may even lead to death. Approximately 75% of the burden caused by these infections occurs in developing countries. many researchers have reported the antibacterial activity of many plant extracts on MRSA. Hence, medicinal plants might be promising candidates for treatment of MRSA infections (Okwu *et al.*, 2019).

In the present study the phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The results showed the presence of various essential constituents i.e. Alkaloids flavonoids, tannins, saponins while in the previous study by Liaqat *et al.*, (2021) investigated the physicochemical, phytochemical, *in-vitro* anti-diabetic and anticancer potential of *O. ferruginea* bark. Primary and secondary metabolites were present in the extractants; glycol-saponins were found in the methanol extract, polyphenols and flavonoids were found in the chloroform extract and polysaccharides were found in the ethanol extract.

The antibacterial activity of the *O. ferruginea* was evaluated using 03 different *MRSA* bacterial species. The extract was administered at 100 µl concentration. The extract was screened for the antibacterial activity. It has been revealed that the highest ZI was produced against MRSA 1 (37±0.4 mm) followed by MRSA 2 (34±0.5mm) and MRSA 3 (29±0.5mm).

In the previous study by Malik *et al.*, (2015) studied the **a**ntibacterial Activity of Olive (Olea europaea) leaves extract using disc diffusion method against *S. aureus.* The results showed good antibacterial activity was shown by methanol extract of olive leaves with the average zone of inhibition 3-8mm Their findings showed that leaves of olive have interesting antibacterial activities. While in our study we performed well diffusion method against *MRSA* It has been revealed that the highest ZI was produced against MRSA 1 (37±0.4 mm).

Sudjana *et al.* (2009) *Olive* leaf extract was found to be most active against meticillin-resistant *S. aureus* (MRSA)], with minimum inhibitory concentrations (MICs) as low as 0.31–0.78% (v/v). In contrast, the extract showed little activity

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against all other test organisms (n = 79), with MICs for most ranging from 6.25% to 50% (v/v).. while in the current study Minimum Inhibitory concentration is the least (minimum) concentration of extracts alone, and antibiotic + extracts combinations at different concentrations (5, 10, 15, 20 µl) plant extract alone and combination which prohibit the growth of microbe. MIC values of alone and antibiotic + extracts combinations were determined against MRSA at 5µl concentration

CONCLUSIONS

From the present study it was concluded that the extract of *O. ferruginea* can be used in treatment of nosocomial infection specifically against *MRSA*. It has been revealed that the highest ZI was produced against MRSA 1 (37±0.4 mm). In susceptibility assay the linezolid showed greater activity i.e., 30.45±0.27. The phytochemical screening of the *Olea ferruginea* Royle Plant Extract was evaluated according to standard protocol. The presence of various essential constituents i.e., Alkaloids flavonoids, tannins, saponins and Steroids.

RECOMMENDATIONS

After conducting this study it is recommended that:

- *MRSA* were used for the evaluation of antibacterial activities. It is therefore, recommended to use other problematic species of bacteria and fungi.
- It is further recommended to synthesize nanoparticles by using plant extract.
- It is recommended to use other animal models for performing this activity.
- Furthermore, compound characterization can be done to recognize the biologically active compounds.
- The extract has been using methanolic extract. Other solvents can be used for the antibacterial activity.

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