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Antibacterial Effect of the Flavonoid Extracts from Organum Majorona Against Multidrug-Resistant Clinical Isolates

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Marjoram was used as a medicinal herb for a very long time. Colds, coughs, cramps, sadness, arthritis, chest congestion, muscle aches, cancer, ear infections, gastrointestinal problems, migraines, paralysis, and depression have all been treated using marjoram or marjoram oil**.** It has also been used as an aphrodisiac.
Aims: The objective of the current investigation was to determine which Gram positive and Gram negative bacteria that were resistant to drugs due to Extended Spectrum Beta Lactamase (ESBL) were susceptible to the action of *Origanum majorana* flavonoids.

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Study Design: Forty clinical samples were obtained from Basra Hospital for Child and Maternity and Abi Al-Khasib General Hospital. Swabs were obtained from patients with vaginal infections.
Methodology: The susceptibility of 17 isolates to 5 antibiotics was determined by disk diffusion method. the bacterial solution with physiological normal saline and inoculated into nutrient agar, supplemented with different concentrations of the Organum *majorona* flavonoid (100%, 75%, 50%, 25%).
Results: Indicated a significant difference ($p<0.05$) and LSD 15.4 in bacteria's responses to the flavonoid in *Organum majorona*. In order to compare isolates' antibiotic susceptibilities, the highest minimum inhibitory concentrations (MIC) for *Klebsiella pneumoniae* were found to be 30.5 mm in 100% concentration of *Organum majorona* flavonoid, this study indicate that *Enterococcus faecium* and *Staphylococcus haemolyticus* are more antibiotic-resistant than *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Furthermore, the luxS gene of *E. faecium* was amplified from the isolated DNA using PCR, the band of each amplified quorum sensing signaling molecule *luxS* gene was defined in 198 bp by comparing it to a normal molecular DNA ladder (1000 bp).
Conclusion: The growth of *Enterococcus faecium*, *Staphylococcus haemolyticus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* was suppressed by the flavonoid extraction from *Origanum majorana.*

Keywords: Isolation and identification; multidrug-resistant pathogen; Origanum majorana; quorum sensing luxS gene.

1. INTRODUCTION

Attention is now being directed towards biologically active components isolated from plant species that are used as herbal medicine, as they may produce a new potent source of antibacterial and antifungal activities. The widespread acquired bacterial resistance to antibiotics is one of the main causes of the bacterial resistance problem that the world is currently facing, posing a serious threat to global public health. The ability of plants to produce several secondary metabolites of somewhat complicated structures with antibacterial activity is linked to their antimicrobial qualities (Maiyo *et al.,* 2010; Erfan & Marouf, 2019).

The list of economically important plants in agriculture is gradually growing and includes species with chemical aromatic or medicinal properties or compounds that serve as sources of materials for food, chemicals, perfumes and animal industry. Previously the list only included animal feed, traditional food and fiber crops. One such plant is *Origanum Majorana* L (Abd Wahab, 2013). It is usually cultured in Mediterranean countries. Marjoram is a permanent shrub that has oppositely oriented dark green oval leaves, an oblique rhizome, hairy stalks resembling shrubs, and clusters of white or red flowers called bracts. The scent of marjoram leaves is quite appealing. The extremely aromatic leaves and flowers of marjoram, either fresh or dried, are frequently used as a spice to enhance flavor, increase sensory qualities and prolong the shelf life of various meals. Furthermore, it is a remarkable aromatic and medicinal plant with potent antioxidant and antibacterial qualities that protect against a variety of bacterial illnesses and mycotoxigenic fungi (Camo *et al.,* 2008). Marjoram extracts have been shown by certain writers to be effective in lowering lipid oxidation, color loss, and microbiological development in specific meat varieties. Due to its enormous potential for industrial and medicinal uses, it is regarded as a significant plant (Bassler, 2002 Bina & Rahimi, (2017).

Today's antimicrobial medications originate from a variety of sources. Antibiotics are compounds that certain microbes naturally make through their metabolic processes. These substances have the capacity to either kill or inhibit other microorganisms (Rudramurthy, 2016). Some of microbes display antimicrobial resistance to numerous antimicrobial medicines, called multi drug resistance (MDR) (Magiorakos *et al.,* 2014). Resistance may result from a variety of biochemical and physiological mechanisms (Talaro, 2002; Nester *et al.,* 2004). The issue is that pathogenic bacteria and other microorganisms are highly erratic and have evolved many defense mechanisms against antibiotics and other antimicrobial medications. (Boucher *et al.,* 2013).

The antibacterial qualities of many plant species in various biological situations have been the subject of several studies. Since the majority of antibiotics come from bacterial or fungal sources, there hasn't been much attention paid to

employing plant chemicals as antimicrobials until lately. Due to the increase in antibiotic resistance and the realisation that an antibiotic's useful life is finite, a lot of research is being done these days on novel sources, particularly plant sources (Yazdanparast & Shahriyary, 2008). Furthermore, several of them are known to influence the cells adhesion characteristics, cell division and denaturation of protein this may aid in the treatment of thrombosis and cardiovascular illnesses (Yazdanparast & Shahriyary, 2008). In order to determine *Origanum majorana*'s antibacterial efficacy against multidrug-resistant bacteria obtained from clinical samples, this study was conducted.

2. MATERIALS AND METHODS

2.1 Data collection

Forty clinical samples were obtained from Basra Hospital for Child and Maternity and Abi Al-Khasib General Hospital. Swabs were obtained from patients with vaginal infections, and samples were sent to a lab for additional research.

2.2 Isolation of Bacteria

Nutrient Agar, Mannitol Salts Agar, Blood Agar and MacConkey Agar were prepared in 250 ml flasks and sterilized by autoclaving for 20 min. Spread 20ml of medium on the plate to solidify. Swabs were plated on the above medium and plates were incubated at 37°C for 24 hrs. Then check the plate for the presence of isolated colonies (Sirjana *et al.,* 2022).

2.3 Identification of Bacterial Isolates

The accuracy of the VITEK R 2 system in identifying bacteria isolated from Blood Agar, MacConkey Agar, Mannitol Salts Agar, and Nutrient Agar was assessed in this study.

2.4 Determination of Antibiotic-Resistant of Isolates

The susceptibility of 17 isolates to 5 antibiotics was determined by disk diffusion method (Van Dijk *et al.,* 2014). Add 0.1ml of bacterial inoculum to 100ml of nutrient broth, incubate at 30°C for 24 hrs. and then dilution of bacterial solution with physiological normal saline and using L-shape to spread bacteria on Muller Hinton Agar then put the antibiotic disc and incubated plates in the incubator 37 ºC for 24 hrs. and measure the diameter of the inhibition zone.

2.5 Flavonoid Extraction

After refluxing 35 g of powdered *Organum majorona* leaves and ethanol for 24 hours, the mixture was filtered through filter paper (Whatman No. 541), and 2% lead acetate was added to the water until the precipitate's components and structure were drained and separated (using sludge filtration paper Whatman No. 532). The precipitate was then washed with water, methanol, and ethyl acetate, in that order. When salt is dissolved in 50 milliliters of acetone and 10 milliliters of 2N HCl, it produces chloride, which is then filtered via filter paper (Whatman No. 540). the candidate is placed in a Petri dish at room temperature to dry, powder weight amorphous Which formed 3.502 g (Harbone & Baxter, 1993; Kandil *et al.,* 1994).

2.6 Determination of Minimum Inhibition Concentration of *Organum majorona* Flavonoid

Add 1ml of isolated bacterial inoculum to 100ml of nutrient broth, incubate at 30°C for 24 hrs., then dilute the bacterial solution with physiological normal saline and inoculated into nutrient agar, supplemented with different concentrations of the *Organum majorona* flavonoid (100%, 75%, 50%, 25%, 12.5) and incubated plates in the incubator at 37 ℃ for 24hrs. The diameter of the inhibition was measured and compared to the control (Roula *et al.,* 2010).

2.7 Molecular Identification of Quorum Sensing Signaling Molecules (lux S) Gene from Enterococcus Faecium

Following bacterial isolate activation in Brain Heart Infusion Broth (BHIB), DNA extraction begins by collecting colonies and incubating them at 37˚ C for 24 hrs. according to the Geneaid Presto ™ Mini g DNA Bacteria Kit method and genomic DNA (template) of *E. faecium* was used to amplified *luxS* gene 198bp product by using a specific primer for this gene as sequence of oligonucleotides (5’ to 3’) of forward primers of *lux S* gene is GAGCACTTGACTGCCGAACT and the reverse is GCCACATTGTGTTTCATTGC .The thermal cycling conditions of PCR amplification are initial denaturation 94˚ C at 3 min,

denaturation 94˚C at 30 sec, annealing 60˚C at 1 min, extension 72˚C at 1.5 min. and final extension 72˚C at 7 min. (Megda *et al.,* 2018**).**

2.8 Statistical Analysis

The SPSS software was utilized to examine the data from this study. Chi-square analysis and one-way analysis of variance (ANOVA) were employed. A value of P<0.05 was regarded as statistically significant, a value of P<0.05 was regarded as non-significant, and a value of P<0.01 as highly significant.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Bacterial Isolates

Forty samples were collected from vaginal infections womens. all samples were cultured on nutrient agar, blood base agar, mannitol salt agar, and MacConcy agar medium with duplicate then sub culture on nutrient agar medium. Out of 20 samples, 7 (35%) were Gram positive (pure culture), whereas 10 (50%) were Gram negative (pure culture). All samples were grown in duplicate on nutrient agar, blood-based agar, mannitol salts agar, and MacConcy agar, and then sub cultured on nutrient agar. Of the 20 samples, 10 were Gram positive (pure culture) and (7) were Gram negative (pure culture). (Table 1) The isolates that gave gram positive and negative, rod, cocci, irregular or round, non spore forming and different colors. The Vitek 2 System's identification of bacterial isolates (Table 2). The vaginal environment is unusual in terms of bacterial colonization. It changes considerably over one's life as a result of developmental and hormonal changes. It is lined by stratified squamous epithelium, which regresses after maternal estrogen wears off. The vaginal flora in children is made up of skin commensals and stomach microorganisms. When a woman approaches menarche, lactobacilli take over the flora and her pH drops to around 4 (Nguyen *et al.,* 2024).

3.2 Antibiotic Resistant

The results show that *Enterococcus faecium* and *Staphylococcus haemolyticus* are more resistant to antibiotics than *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* (Table 3). This is because the cell walls of gram positive and negative bacteria vary in thickness, with gram positive having a cell wall that is about 20–30 nm and gram negative having a cell wall that is 8–12 nm. Gram positive bacteria also have a thick layer of peptidoglycan and a low amount of lipid, which gives them greater resistance to chemical compounds. (Cabeen & Wagner, 2005). Resistance in bacteria due to have energy driven drug efflux pumps that expel antibacterial drugs, bringing their intracellular concentration to sub or no inhibitory levels. A channel that actively exports antibacterial and other chemicals from the cell is what is known as an efflux pump (Piddock, 2006). Once the antibiotic has entered the organism through a porin channel, the efflux pump drives it back out. Because these transport proteins are often non-specific and able to pump a wide range of medications, they are sometimes referred to as multidrug resistant pumps. Primary active transport and secondary active transport are the two fundamental types of active efflux pumps (Poole, 2005). The hazardous bacteria *S. haemolyticus* has become resistant to almost all of the new antibiotics that have been created in the last 50 years. Because of its genome's flexibility, the bacterium can adjust to any environmental circumstance, including picking up genes that make it resistant to antibiotics and creating regulatory systems that allow it to adjust to rising drug concentrations (Hema-Ouangraoua *et al.,* 2021).

Table 1. The number and percentage of bacterial isolates from vaginal infections

Sex	Total No. of samples	Gram-positive	Gram-negative
Female	20	7 (35%)	10 (50%)

Table 2. Results of Vitek 2 system identification of bacteria and percentage

Vitek 2 system	No. of bacteria	Identify
Enterococcus faecium	2	99%
Staphylococcus haemolyticus	5	98%
Pseudomonas aeruginosa	2	99%
Klebsiella pneumoniae	8	99%

Table 3. Antibiotic test for isolates of inhibition (mm)

Samples	Nitrofurantionn 300 µg	Chloramphenicol 30µg	Norfloxacin 10µg	Meropenem 10 µg	Amikacin 10µg
Standard measures	≥14	≥18	≥17	≥17	≥17
Enterococcus faecium	R	R	S	R	R
Staphylococcus haemolyticus	R	R	S	S	R
Pseudomonas aeruginosa	R	S	S	R	S
Klebsiella pneumoniae	S	S	R	R	R

Resistant (R) Sensitive (S)

Table 4. Effect of minimum inhibitory concentrations (MICs ug/ml) of *Organum majorona* flavonoid on isolates (diameter of inhibition measured with millimeter)

Concentration of *Organum majorona*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus haemolyticus*	*Enterococcus faecium*
100	30.5	30	25.5	20
75	30	20.5	15	10
50	20.3	20	0	0
25	17.5	0	0	0
12.5	0	0	0	0

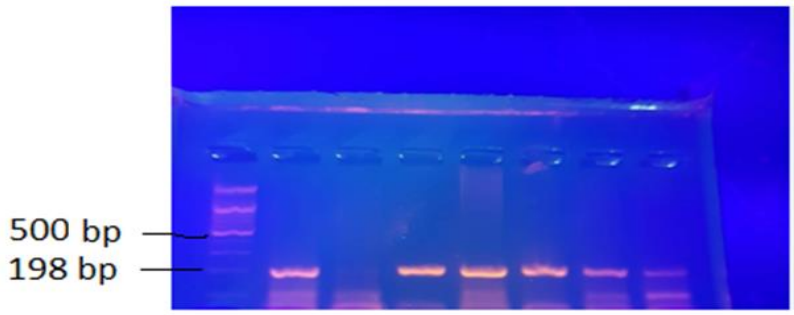

Fig. 1. Gel electrophoresis of PCR product of luxS gene (198bp) of Enterococcus faecium

3.3 Antibacterial Effect of the *Organum majorona* Flavonoid Extracts

Antibacterial varieties indicated significant differences for *Organum majorona* flavonoid at $p<0.05$ and LSD 15.4 as shown. Highest MIC by *Klebsiella pneumoniae* about 30.5 mm in 100% concentration of *Organum majorona* flavonoid compared with other isolates (Table 3). flavonoid extract from biological or toxic plants has a negative impact on the physiology of bacteria. Additionally, inhibitors of bacterial growth have an effect by influencing the object gene expression process, which in turn affects protein structure and leads to the development of important metabolic enzymes as well as the inhibition of quorum sensing (Mothana & Lindequist, 2005).

3.4 Molecular Identification of Quorum Sensing Signaling Molecules (lux S) Gene

The genomic DNA from isolates of *E. faecium* luxS were used to amplify the gene coded for the quorum sensing signaling molecules production using specific primer. The results illustrated in (Fig. 1) showed that all isolates of *E. faecium* give the positive result, the amplified fragment was about 198bp in size which was the same size for *luxS* gene this indicate all isolate had quorum sensing signaling molecules gene. The

luxS gene is a quorum sensing signaling protein that is involved in microbial communication. Signaling molecules, also known as autoinducers (AIs), are extracellular chemical signals that detect this communication. (Reading *et al.,* 2006; Megda *et al.,* 2018). These substances are created by growing bacteria and are then discharged into the surroundings. Bacteria respond in concert by expressing virulence genes, plasmid transfer, toxin synthesis, exopolysaccharide production and sporulation, biofilm growth, and antibiotic resistance when extracellular signals accumulate, suggesting the presence of relatively dense populations (Griffiths, 2005; Bassler, 2002).

4. CONCLUSION

Our findings show that the flavonoid extraction from *Origanum majorana* inhibited the development of *Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Staphylococcus haemolyticus*, and *Enterococcus faecium* at 100% concentration. Furthermore, biofilms worsen the multidrug resistance of antimicrobial medicines. Additionally, E. fecium's luxS gene encodes quorum sensing, a form of intercellular communication signal, and bacteria use community signalling to communicate with one another.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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