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INVESTIGATION INTO THE IMPACT OF CARVCROL EXTRACTS ON BACTERIA ISOLATES FROM OSTEOMYLITIS AFTER SURGERY

Tamara Ziyad Tariq
Biology Department College of Science / University of Mosul, Mosul City, IRAQ
tamara.23scp55@student.uomosul.edu.iq

Saba Abdul Salam Hamid Al-Sultan
2Microbiology Department, Ninevah College of Medicine, Ninevah University, Mosul City, IRAQ

Aws Ibrahim Sulaiman
Biology Department College of Science / University of Mosul, Mosul City, IRAQ

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Abstract: Carvacrol is one of the most ancient medicinal herbs. Carvacrol contains different alkaloids, flavonoids and saponins. In present studies the antimicrobial activity of Carvacrol alcoholic extract against Gram-negative bacterial and Gram-positive bacterial were determined by the microtiter plate method. Gram-negative bacterial and Gram-positive bacterial strains isolated from osteomyelitis patients after surgery in Mosul city , A total of 60 samples were collected from various sources in Mosul hospitals, during the period from January 10, 2024 , to May 1, 2024. The Aim of this study is determining the effectiveness and effect of alcoholic extract of carvacrol on bacterial isolates taken from a Osteomyelitis after surgery. Bacterial culture results on blood agar, MacConkey agar and nutreint agar revealed 54 positive samples for bacterial growth. Among these, Staphylococcus aureus 22 isolates (32.8%), Pseudomonas aeruginosa 12 isolates (17.9%), Enterobacter cloacae 5 isolates (7.5%) Klebsiella pneumoniae 4 isolates (5.9%), Escherichia coli 4 isolates (5.9%), Proteus mirabilis 3 isolates (4.5%), Streptococcus viridans 3 isolates (4.5%), Corynebacterium spp 2 isolates (3%). As for the following bacterial isolates, each of them constituted one isolate out of a total of 67 isolates, at a rate of 1.5% for each: Providencia spp. , Acinetobacter spp., Staphylococcus saprophyticus , Klebsiella oxytoca , Enterococcus spp. , Enterococcus faecium , Citrobacter freundii , Streptococcus groups C , Bacillus cereus , Enterobacter spp. , Acinetobacter baumannii , Morganella morganii, using vitek-2. High-performance liquid chromatography (HPLC) analysis of Oregano plant revealed the presence of active compounds such as Carvacrol, thymol. Plant extracts from carvacrol demonstrated a substantial inhibitory effect. Results: study indicated that plant extracts exhibited higher inhibition than antibiotics. The minimum inhibitory concentration (MIC) while the results of the lowest inhibitory concentration of carvacrol for the bacterial species under study showed that it had an effect at a concentration of 615 µg/ml against Escherichia coli, 307.5 µg/ml for the species Corynebacterium stratium, Morganella morganii and Pseudomonas aeruginosa, and the least effect was 76.875 µg/ml on Staphylococcus aureus, it gave less results than the antibiotic used Carvacrol (Absorbance was measured at Wavelength 600) .

Keywords: Osteomyelitis, Carvacrol, Bacterial isolation , Antimicrobial activity, HPLC

Introduction

The term is used to describe an infection or an inflammation of the bone when the root words osteon (bone) and maylo (marrow) combined with it is defined clinical state in which part of osseous skeleton is infected by microorganisms. The diseases classified as either acute or chronic. The infection with bacteria,fungi, and/or mycobacteria is depending on the length of time of infection or symptoms persist (1). S.aureus ,P.aerignosa ,E.coli, corynebacterium stratium , Morganella morganii , a significant opportunistic pathogen within Osteomylitis is accountable for and recognized as one of the major multidrug-resistant hospital pathogens by the Infectious Diseases Society of America. Global bacterial resistance to antibiotics is escalating, influenced by factors such as inappropriate clinical antibiotic usage, extensive application in the food production sector, and the unrestricted availability of antibiotics in many countries without prescriptions (2). Multidrug resistance (MDR), where microbes resist multiple drugs, is a growing concern, manifesting through natural resistance, genetic mutation, or acquired resistance from other species (3). As the need for antibiotic alternatives intensifies, exploring naturally occurring botanicals becomes crucial. Medicinal plants, historically and contemporarily, have played pivotal roles in medicine, offering bioactive secondary metabolites that facilitate healing (4). Carvacrol (CV) formed by a phenolic carbon structure, which is classified in monocyclic monoterpenes, is naturally derived from another monoterpene cymene and extracted, as one of the essential oils, from aromatic plants (5), is a medicinal herb with a rich therapeutic history, including anti-diabetic, anti-hyperlipidemic, and gastroprotective properties oregano plant contain diverse bioactive compounds such as Carvacrol and thymol, High-Performance Liquid Chromatography (HPLC) is employed to detect antimicrobial substances in Oregano plant, allowing for the separation, identification, and quantification of compounds in liquid samples. Recognized as a powerful analytical tool, HPLC is widely used for the qualitative and quantitative analysis of drug products and determining their stability (6) . The study aimed to determine the effect of Carvacrol extracts of Oregano plant on Gram-negative and Gram -positive bacterial species isolated from Osteomylitis patients after sergery in Mosul and to compare that with the effect of some antibiotics..

Methods

Sample Collection: A total of 60 samples were acquired from hospitals in Mosul. Specifically, 56 samples were obtained from Osteomylitis current study was conducted from January 10, 2024, to May 1, 2024, in Mosul . Immediate attention was given to the handling of the samples to ensure their integrity.

Ethics Statement: Ethical approval for this research was obtained from the Ministry of Health and Environment, Nineveh Health Directorate, with reference number (2550) on January 17, 2024.

Cultivation and Diagnosis of Samples: The collected samples were directly cultured on MacConkey medium Blood agar and nutrient agar and incubated at 37°C for 24 hours. Subsequently, isolates were purified using the streak plate method on MacConkey medium, followed by incubation at 37°C for an additional 24 hours. A single pure colony was initially subjected to standard bacteriological tests, encompassing assessments of colony morphology on MacConkey agar, Gram stain, catalase, and oxidase tests (7) . Phenotypic and microscopic characterization, along with chemical tests, were performed. Further diagnosis was carried out using the Vitek2 system, followed by molecular diagnosis through 16S rRNA analysis.

Collection and Preparation of Plant Extract: Oregano plant were procured from stores, dried, and ground using a household mixed.

Antibiotic susceptibility test:

The antibiotic susceptibility test was conducted using the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines from the year 2023.Staphylococcus aureus 22 isolates (32.8%); Pseudomonas aeruginosa 12 isolates (17.9%); Enterobacter cloacae 5 isolates (7.5%) Klebsiella pneumoniae 4 isolates (5.9%); Escherichia coli 4 isolates (5.9%); Proteus mirabilis 3 isolates (4.5%); Streptococcus viridans 3 isolates (4.5%); Corynebacterium spp 2 isolates (3%) As for the following bacterial isolates, each of them constituted one isolate out of a total of 67 isolates, at a rate of 1.5% for each: Providencia spp. ; Acinetobacter spp.; Staphylococcus saprophyticus ; Klebsiella oxytoca ; Enterococcus spp. ; Enterococcus faecium ; Citrobacter freundii ; Streptococcus groups C ; Bacillus cereus ; Enterobacter spp. ; Acinetobacter baumannii ; Morganella morganii. isolates were cultured on brain heart infusion agar. After an incubation period at 37°C for 18-24 hours, single colonies, approximately 3-5 in number, were selected and transferred into a test tube containing 4 ml of normal saline to produce a bacterial suspension. Activated the bacteria and were incubated at 37 close to tube (0.5) of the standard McFarland tubes for 24-18 hours (8). On the next day, a bacterial suspension was prepared, in which the bacterial count was equivalent to bacterial culture and placed in a plate containing the1.5 x 810 cells/cm² (9). A sterile cotton swab was immersed in the adjusted suspension, rotated several times firmly on the inside wall of the tube above the fluid level to eliminate excess inoculum, and then used to streak the entire surface of a Mueller-Hinton agar plate. This streaking process was repeated two more times, rotating the plate approximately 60° each time to ensure an even distribution of the inoculum. The plates were left to dry at room temperature for 15-20 minutes. Subsequently, antibiotic discs were placed on the agar using sterile forceps and pressed firmly. The plates were then incubated at 37°C for 18-24 hours. Following incubation, the diameter of the inhibition zones around the discs was measured using a metric ruler (mm), and the results were interpreted as sensitive, intermediate, or resistant based on the CLSI guidelines from 2023.

Acoholic Extract: The Oregano plant taken and ground in an electric grinder, then 40 grams were taken from them and placed in a Soxhelt device to extract a alcoholic extract. This extract was filtered with Whitman filter paper and this extract was concentrated using a rotary evaporator under reduced pressure at a 40C (10) The alcoholic extract liquid was stored at 4°C in closed bottles until use [13].

Preparation of Carvacrol Samples:

Preparation of Carvacrol Stock Solution:

1. Stock Solution: Dissolve carvacrol at a 1/10 ratio in dimethyl sulfoxide (DMSO) solvent.
2. Sterilization: Sterilize the solution using 0.45 µm syringe filters.

Preparation of Study Concentrations:

1. Stock Concentration: The stock solution concentration is 95.6 mg/mL (100%).
2. Dilution Series: Prepare study concentrations ranging from 12.5% (11.95 mg/mL) to 0.003% (2.9 µg/mL).
3. Culture Medium: Use Trypticase Soy Broth (TSB) enriched with 1% sucrose for the dilutions. (11)

Antibacterial Activity: Agar dilution method used for determining antimicrobial susceptibility, was employed. The antimicrobial agent was incorporated into a series of agar plates containing increasing concentrations of the agent to be tested. Inoculums of various microorganisms were simultaneously applied to the agar surface and incubated for 24-48 hours. A standardized inoculum was prepared, achieving 0.5 turbidity on the McFarland scale (1 × 108 colony-forming units (CFU) mL-1). Growth was measured and compared with the control. Mueller Hinton medium was used for its proven efficacy in routine susceptibility testing of non-fastidious bacteria (12)

Result and Discussion

Isolation and Diagnosis:

56 samples were obtained from osteomyelitis using swabs, and MacConkey medium revealed bacterial growth. Following isolation and diagnosis through the Vitek2 system and molecular methods,Staphylococcus aureus 22 isolates (32.8%); Pseudomonas aeruginosa 12 isolates (17.9%); Enterobacter cloacae 5 isolates (7.5%) Klebsiella pneumoniae 4 isolates (5.9%); Escherichia coli 4 isolates (5.9%); Proteus mirabilis 3 isolates (4.5%); Streptococcus viridans 3 isolates (4.5%); Corynebacterium spp 2 isolates (3%) As for the following bacterial isolates, each of them constituted one isolate out of a total of 67 isolates, at a rate of 1.5% for each: Providencia spp. ; Acinetobacter spp.; Staphylococcus saprophyticus ; Klebsiella oxytoca ; Enterococcus spp. ; Enterococcus faecium ; Citrobacter freundii ; Streptococcus groups C ; Bacillus cereus ; Enterobacter spp.; Acinetobacter baumannii ; Morganella morganii were identified.

Antibiotic Sensitivity Test (AST)

In this study, the results of the antibiotics test against bacteria isolated from cases of Osteomyelitis were based on the examination using the Vitek 2 compact technology, from bioMérieux Company, Also the test was performed manually to confirm, as well as adding some common types of antibiotics used by the orthopedic surgeons in the city of Mosul. In this study, the interpretive criteria for the diameter of inhibition zones for antibiotic susceptibility testing were adopted according to CLSI, 2023 (Clinical and Laboratory Standards Institute, 2023), as in the table (1.1)

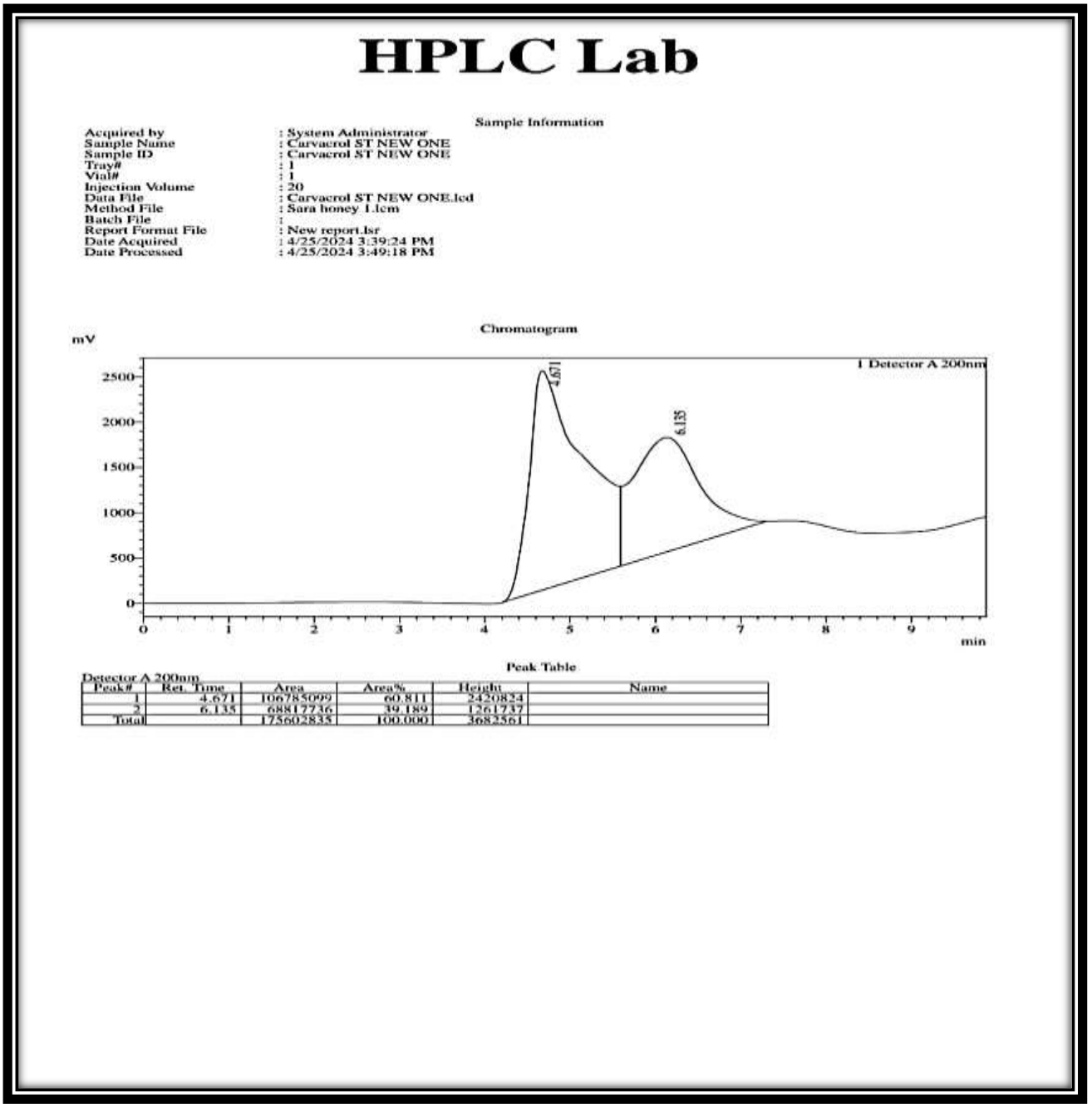
Table (1.1): Antibiotics used against types of bacteria isolated from cases of Osteomyelitis Antibiotic disc. The diameter of the Inhibition zone was measured in millimeters, and isolates were divided as either resistant, intermediate, or sensitive to antibiotics compared to the standard inhibition zone.

NO.	Antibiotic	Abbreviation	con. (ug)	Company	Origin
1	Azithromycin	AZM	15 µg	Bioanalyse	TURKEY
2	Tetracyclin	TE	10 µg	Bioanalyse	TURKEY
3	Cefoxitin	FOX	30 mcg	Bioanalyse	TURKEY
4	Levofloxacin	LEV	5 µg	Bioanalyse	TURKEY
5	Vancomycin	VA	30 µg	Bioanalyse	TURKEY
6	Amikacin	AK	10 mcg	Bioanalyse	TURKEY
7	Gentamycin	CN	10 mcg	Bioanalyse	TURKEY
8	Imipenem	IPM	10 µg	Bioanalys	TURKE

				e	Y
9	Ceftriaxone	CRO	10 µg	Bioanalys e	TURKE Y
10	Metronidazole	MET	30mcg	Bioanalys e	TURKE Y
11	Meropenem	MEM	10 µg	Bioanalys e	TURKE Y
12	AmoxicillinandClavulanicAc id	AMC	20and10 µg	Bioanalys e	TURKE Y

Qualitative and Quantitative Detection of Carvacrol Extract:

Following both qualitative and quantitative detection, the Carvacrol extract was subjected to High-Performance Liquid Chromatography (HPLC) analysis. The HPLC separation profile displayed various chromatographic peaks in the examined sample extract. It was observed that the Oregano plant extract contained Carvacrol and thymol in the alcoholic extract, as depicted in Figure No. (1). The study investigated the effect of the plant extract on bacterial growth, comparing its inhibitory effect with antibiotics. While the results of the lowest inhibitory concentration of carvacrol (Table 1.2)



for the bacterial species under study showed that it had an effect at a concentration of 615 µg/ml against Escherichia coli, 307.5 µg/ml for the species Corynebacterium stratium, Morganella morganii and Pseudomonas aeruginosa, and the least effect was 76.875 µg/ml on Staphylococcus aureus, it gave less results than the antibiotic used..Table 1.2 Minimal Inhibitory Concentration of Carvacrol (Absorbance was measured at Wavelength 600nm).

Table (1.2): **Minimal Inhibitory Concentration of Carvacrol**

Bacterial isolates	**MIC Concentrations (µg/ml)**					
	1230	615	307.5	153.75	76.875	38.43
Corynebacterium stratium	0.110	0.112	0.110	0.182	0.185	0.195

Morganella morganii	0.040	0.048	0.062	0.090	0.132	0.155
Escherichia coli	0.040	0.052	0.085	0.090	0.098	0.147
Pseudomonas aeruginosa	0.108	0.114	0.122	0.152	0.153	0.165
Staphylococcus aureus	0.125	0.127	0.132	0.136	0.138	0.197

Discussion

The indiscriminate use of antibiotics and synthetic antibacterial agents has led to the emergence of multidrug-resistant microbial strains, and some microbial strains exhibit reduced susceptibility to antibiotics (13). Natural therapeutic medications from various plant extracts have been developed to treat antimicrobial resistance (14). Traditional plant-based medicines have demonstrated high effectiveness in providing antimicrobial compounds. Phytochemical compounds help combat various infections caused by microorganisms. Plants are known to be rich sources of secondary metabolites, including terpenoids, tannins, alkaloids, and flavonoids, and these secondary metabolites are often responsible for plants' medicinal properties (15). Broth dilution techniques were used to determine the effect of plant compounds in inhibiting the growth of specific microorganisms 1 . Alcoholic extracts of Oregano plant are found to have significant antibacterial activity against several bacterial strains, including E.coli, S.aureus, Morganellla morganii, Corynebacterium stratium ,P.areginosa In another study, Carvacrol extract was found to have great antimicrobial properties

Conclusion

This study concludes that the alcoholic extract of oregano plant exhibits superior antimicrobial and anti-inflammatory activities compared to standard antibiotics and anti-inflammatory drugs. Consequently, the carvacrol extract holds potential as an alternative for treating inflammation resulting from Osteomylitis However, further investigations are essential to assess its viability for in vivo applications

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