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Anti Bacterial Activity Of Leucaena Leucephala Seeds Extract Against Pathogenic Bacteria

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Abstract

Introducation and aim*. The development of urgent therapeutic alternatives is necessary to address the global problem of pathogenic bacteria, which can cause a wide range of diseases, from simple to opportunistic, and are resistant to many antibiotics. The most significant types of these bacteria are Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae. Medicinal plants are one type of alternative The antibacterial, antioxidant, and anticancer qualities of Leucaena leucocephala seeds have been utilized, and studies have validated their significance in lowering our reliance on antibiotics.* ***The aim was to*** *determined the antibacterial activity of aqueous ,hexan and ethanol extract of L. leucephala seed on s. aureus, E. coli and K. pneumonia Study the synergistic of ethanolic extract and antibiotics (CAZ/ CIP/OFX/) aganisit antibiotic_resistant bacteria.*

Material and methods: *In the Babylon Governorate, 120 bacterial isolates from various clinical specimens (urin, wounds, and brine) were described in this investigation between September 2024 and February 2025. Each isolate was cultivated on both MacConkey agar and blood agar. The identification of these isolates was carried out using a combination of methods, including microscopic examination, biochemical assays, and the VITEK automated system. The extracts were analyzed using GC-Ms(Gas Chromatography-Mass Spectrometry) , High-Performance Liquid Chromatography (HPLC) to identify and quantify phenolic compounds, tannins, and vitamin E. The antibacterial activity was assessed using the well diffusion method across a range of concentrations.*

Results *:three major pathogenic bacteria from 120 clinical isolates collected in Babylon Governorate: S. aureus (50 isolates), E. coli (35 isolates), and K. pneumoniae (35 isolates). S. aureus was prevalent in burn (50%) and wound (50%) samples, and also found in urine (33.33%). E. coli was notably high in urine (35.56%), with lower presence in wound (13.3%) and burn (5%) specimens. K. pneumoniae was exclusively isolated from urine samples (38.89%). Gram staining confirmed 50 positive isolates as S. aureus and 70 negative isolates (35 E. coli and 35 K. pneumoniae). Biochemical tests provided distinct profiles for the identified bacteria. All S. aureus isolates were catalase-positive and showed mannitol fermentation. Conversely, E. coli and K. pneumoniae were catalase-negative. The Indole test was positive only for E. coli, while S. aureus and K. pneumoniae were negative. For the Methyl Red (MR) test, E. coli was positive, and S. aureus and K. pneumoniae were negative. The Citrate test was positive for K. pneumoniae, but negative for S. aureus and E. coli. the antibacterial efficacy of L. leucocephala seed extract using different solvents (aqueous, ethanolic, hexane) against the isolated bacteria. The aqueous extract showed no inhibitory effect against S. aureus or the Gram-negative bacteria (E. coli and K. pneumoniae).*

Ethanolic extract exhibited significant inhibitory effects against S. aureus, with mean inhibition zone diameters ranging from 14.5 mm to 18 mm across various concentrations (25% to 100%). However, neither the ethanolic nor the hexane extracts showed any inhibitory effect against E. coli or K. pneumoniae, suggesting their active compounds may not penetrate Gram-negative bacterial cell membranes. The hexane extract showed limited efficacy against S. aureus, with a slight effect only at 25% concentration (16 mm inhibition zone). Ethanol was identified as the optimal solvent for extracting active compounds with potent antimicrobial properties, particularly against S. aureus. 50 isolates of S. aureus were tested against 11 common antibiotics. The highest resistance was observed against Cefuroxime (CX) at 54%, followed by Tetracycline (TE) at 30%, and Linezolid (LZ) at 26%. Moderate resistance was seen against Ciprofloxacin (CIP) at 24% and Azithromycin (AZM) at 22%. The isolates showed least resistance to Rifampicin (RA) and Levofloxacin (LEV) at 12% each, and Cefotaxime (CPT) at 6%. Conversely, S. aureus isolates demonstrated high sensitivity to RA (88%), Gentamicin

(CN) (84%), Ofloxacin (OFX) (80%), and CPT (74%). Combining the ethanolic extract of L. leucocephala seeds with certain antibiotics showed a synergistic effect against S. aureus. The extract significantly enhanced the activity of Ciprofloxacin (CIP), increasing its inhibition zone diameter from 13 mm to 30 mm, and Ofloxacin (OFX), increasing it from 10 mm to 25 mm. A less pronounced, but still suggestive, synergistic trend was observed with Cefazolin (CAZ), where the inhibition zone increased from 0 mm to 10 mm. This indicates that the extract can augment the effectiveness of certain antibiotics against S. aureus. GC-MS analysis of the ethanolic extract of L. leucocephala seeds identified 12 bioactive compounds, including Methyl linolelaidate, Hexadecanoic acid, methyl ester, and Diisooctyl phthalate. HPLC analysis revealed significant concentrations of phenolic compounds (263.9 mg/100gm) and a lower concentration of tannins (1.58%). Vitamin E was also detected at 0.36%. These compounds likely contribute to the observed antimicrobial activity, particularly the robust efficacy against S. aureus.

***Conclusions**: This study, conducted in Babylon Governorate, revealed the prevalence of S. aureus, E. coli, and K. pneumoniae as key causative agents in clinical infections. S. aureus was notably prevalent in burn and wound samples, while E. coli and K. pneumoniae were primarily isolated from urine. Traditional methods proved effective for bacterial identification. The ethanolic L. leucocephala seed extract showed potent inhibitory activity against S. aureus but no effect on Gram-negative bacteria. Importantly, the ethanolic extract demonstrated a synergistic effect when combined with certain antibiotics (e.g., ciprofloxacin, ofloxacin) against S. aureus, suggesting enhanced therapeutic potential. Phytochemical analysis confirmed the presence of bioactive compounds in the extract, supporting its therapeutic promise as an antimicrobial or an adjunct therapy against S. aureus infections.*

***Key words:** Bacteria , Antibiotics , Resistance ,Antibacterial activity ,leucaena leucephala seed*

INTRODUCTION

Most pathobacteria are a few microns in size and originate from a range of environmental sources, such as food, water, soil, animals, and human bodies. They are classified as membrane-bound organelles, which are prokaryotic bacteria without a nucleus or mitochondria. These sources can cause a number of diseases[1] . Numerous cells, tissues, organs, and bodies can be impacted by bacterial infections, which can result in a number of clinical illnesses[2] . Gram-positive, catalase-positive cocci that belong to the Staphylococcus ae family are the most common pathogenic bacteria. They are facultative, non-motile, spore-forming anaerobes with a diameter of 0.5 to 1.5 μm that typically form in clusters[3] . High salt concentrations can support its growth, and golden colonies are frequently formed. A wide range of temperatures is ideal for this facultative anaerobe to flourish in. Positive catalase and coagulase responses, novobiocin sensitivity, and mannitol fermentation are important characteristics for identification [4]. Bloodstream infections have a significant fatality rate, making S. aureus a major cause of infections in clinical settings[5] .

Gram-negative In addition to commensally inhabiting the gut, E. coli bacteria, which are members of the Enterobacteriaceae family, can cause a number of ailments, including major invasive disorders [6]. It is a major contributor to newborn meningitis and bacteremia in high-income nations motility is peritrichous and grows best at 37°C. Extended-spectrum beta-lactamase (ESBL) synthesis is a major resistance mechanism of multidrug-resistant E. coli bacteria[7] which provide a global treatment challenge and contribute to rising morbidity and death globally [8]. K. pneumoniae is a Gram-negative encapsulated bacteria that can infect different parts of the body[9] . This bacterium is widely found in nature and thrives in a variety of settings. It has the ability to colonize human mucosal surfaces, especially those in the gastrointestinal tract, asymptomatically. Nonetheless, K. pneumoniae is known to have the ability to cause a variety of diseases, making it a pathogen[10] .

The main components necessary for K. pneumoniae's pathogenicity are polysaccharide capsules and lipopolysaccharide. Additionally, K. pneumoniae has the capacity to create biofilms[11]. the quest for new sources of natural inhibitors that can increase the effectiveness of current treatments is increasingly critical. In this perspective, the plant L.leucocephala (Lam.) De Wit. of the family Fabaceae presents a promising subject of study. Other names for this perennial evergreen tree include Acacia leucocephala, Mimosa leucocephala, and L. glabrata [12]. is beneficial as a human food source because its seeds are high in proteins,

carbs, and fats. It can also be used in cooking and as a coffee substitute [13] .as well as for livestock feed and soil improvement. It may also have therapeutic uses. According to research, this plant's natural compounds may be a promising source of inhibitors that alter the way antibiotics work against bacteria that are resistant to multiple drugs. This could be done through mechanisms like bacterial cell wall modification, efflux pump inhibition, cell division inhibition, protein synthesis, and gene expression [14].

Aim

The aim was to determined the antibacterial activity of aqueous ,hexan and ethanol extract of L. leucephala seed on S. aureus, E. coli and K. pneumonia and Study the synergistic of ethanolic extract and antibiotics (CAZ/ CIP/OFX/) aganisit antibiotic_resistant bacteria.

Materials and Methods

Bacteria used in the study

S. aureus, E. coli and K. pneumonia

Plants used in the study

Seeds of *Leucaena leucocephala* were collected from the Al-Wardiya area, Babylon Governorate, Iraq, during the period spanning from 2024 to 2025**.**

preparation of Leucena lecocephala seed Extracts

L. leucocephala seeds were collected from Babylon Governorate, Iraq. Using a 1:10 (w/v) mixture of 100% water, 70% ethanol, and 70% hexane as solvents, 50 grams of powdered L. leucocephala seeds were macerated for 24 hours .A rotary evaporator was then used to evaporate the filtrate for 90 minutes at a temperature equivalent to the solvent's boiling point. Vial bottles were used to store the resultant extracts[15].

Detection of pathogenic bacteria from Diverse Clinical Isolates Using Traditional Methods

In a study involving 120 bacterial isolates collected across different age groups, over a period from September 2024 to February 2025, from various hospitals in Babylon Governorate The urine specimen used in this investigation was collected in a sterile, clean container and brought to the lab in a maximum of two hours. Burn and wound swabs (wound and burn specimens, which are retrieved from patients using a gel swab and sent to the laboratory) were gathered from patients who were admitted to Babylon hospitals. Before to being cultured on various media for the identification of E. coli, K. pneumonia, and S.aureus, such as blood agar, MacConkey agar, and Manitol salt agar, using a sterile loop spread on the surface of agar media, all specimens were grown on Brain Heart Infusion Broth Medium and incubated at 37 C° for 24 hours [16].Purified colonies were stored at -20°C in brain-heart infusion broth with glycerol [16]. Following the final diagnosis of theSpecimens , The patients were of both sexes and varying ages.

Biochemical Tests

Biochemical tests used in this study

Catalase Test : Added 1-2 drops of 3% H2O2 and mixed with bacterial colonies that have been moved from cultivation. A positive test result was indicated by the development of gas bubbles[17].

Oxidase Test:After being soaked in 1% oxidase reagent, filter paper was allowed to dry. Using a sterile inoculation loop, a freshly made pure culture was incubated for 18 to 24 hours on filter paper before being monitored for color changes. When exposed to oxidase reagent, organisms that are positive oxidase emit a purple hue[17] .

Mannitol Fermentation Test: Each bacterial isolate was inoculated with mannitol salt agar for this test, which was then incubated for 24 hours at 37°C. The sample was positive when the color turned yellow[17].

IMVC test

Indole test :A bacterial culture that had been evaluated overnight was added to peptide water medium, which was then incubated for 24 hours at 37 C°. Ten drops of Kovac's reagent were then added straight to the culture tube; a successful outcome is indicated by the red ring that forms on top of the broth following a gentle shake. The purpose of this test is to determine whether E. coli is capable of producing the tryptophanase enzyme, which hydrolyzes tryptophan to create ammonia, pyruvic acid, and indole[18]

Methyl red test:A bacterial culture was added to Methyl red-Voges proskauer medium, and the culture was cultured for 24 hours at 37 C°. The methyl red was then added in five drops. The medium hue of the appositive test shifted from yellow to red. The purpose of this test was to see whether bacteria could ferment glucose and ultimately create acid[18].

Citrate Utilization Test: A positive result was shown by the medium's color changing from green to blue after a sterile loop loaded Simon's citrate slant agar with the tested bacterial culture and incubated it for 24 hours at 37 C°. The purpose of this test was to determine whether bacteria could use sodium citrate as a carbon source[18]

Preparation of Reagents and Solutions

McFarland Standard Solution

A pre-made 0.5 McFarland standard tube was employed in this investigation. This standard, which corresponds to a bacterial suspension concentration of roughly 1.5×10-8 colony-forming units per milliliter (CFU/ml), is used as a reference for turbidity. To determine the cell density of the bacterial suspensions under study, their turbidity was visually compared to this readily usable reference.

The Catalase Reagent

The ability of bacteria to create the enzyme catalyzes assessed for hydrogen peroxide was investigated using hydrogen peroxide synthesized at a concentration of 3% [17].

Vogas-Proskauer reagent

This substance consisted of two solutions: α-naphthol solution made by dissolving 5 gm of -naphthol in 100 ml of (95 %) ethanol, storing the solution in a dark bottle, and mixing it prior to use. 40 percent Potassium hydroxide solution made by dissolving 40 grams of KOH in 100 milliliters of deionized water and mixing the solution prior to use [18].

Methyl red indicator :This solution was prepared by dissolving 0.2 gm of methyl red in 300 ml of (95%) ethanol, and then the volume was completed to 500 ml by D.W[18].

Anti-Bacterial Activity

The antimicrobial activity of the plant extract was evaluated using a combined spread-plate and well diffusion method based on Egorov's method. Briefly, 0.1 mL of each bacterial suspension (standardized to a concentration of 1.5×10^8 cells/mL for all experiments) was uniformly spread onto the surface of Mueller-Hinton agar plates. Subsequently, wells of uniform diameter (6 mm) were aseptically formed in the agar utilizing a sterile cork borer. Following that, 0.1 milliliters of the plant extract was carefully added to each well and thoroughly mixed. In order to ensure that the extract was able to diffuse into the agar medium in an adequate manner, the inoculation plates were left at room temperature for a period of four to five hours. After that, the plates were kept in an incubator at 37 degrees Celsius for a week. After the incubation period had passed, a graduated ruler was used to measure the diameter of the inhibition zones that surrounded each well. This diameter was used to determine the degree to which the growth of bacteria was inhibited[19].

Antibiotics Susceptibility Test

Two ml of Muller Hinton Broth were inoculated with an isolated colony of the tested bacteria and then incubated for 18 hours at 37° C. The turbidity of bacterial suspension was then adjusted with the turbidity of McFarland (0.5) standard, equivalent to the concentration of 1.5×108 CFU/ml. 0.1 ml of bacterial culture that was streaked all over the surface of the Mueller-Hinton medium, then left to dry. A maximum of five antibiotic discs were placed on the plate. The plates were incubated for 24 hours at 37° C. The resulting zones of inhibition were measured by a ruler and compared with the inhibition zones determined by [20].

Antibacterial Susceptibility Testing of Seed Extract via Disk Diffusion Method

In the first part of the experiment, the disc diffusion method was used to evaluate bacterial susceptibility. A 100% concentration of the alcoholic seed extract was added to a Petri dish, followed by the addition of a calculated amount of Mueller-Hinton agar, which was then carefully mixed. After the medium solidified, the dish was inoculated with bacteria. Antibiotic discs (CAZ, , AZM, CIP, OFX) were placed at regular intervals

on the agar surface. The dishes were then incubated for 24 hours at 37°C. Subsequently, the resulting inhibition zones were measured and compared to those of the antibiotic discs used as controls[21].

Phtyto chemical analysis of ethanol extract of L. lecocephala seeds

Gas chromatography-mass spectrometry chromatographic conditions(GC-MS)

A thermal desorption TD-20 system, GCMSQP2010 Plus (Shimadzu, Nakagyo-ku, Kyoto, Japan), was used for GC-MS analysis. A mass spectrometer device using an RTx-5MS column (30 m × 0.25 mm × 0.25 μm) running in electron impact mode at 70 eV was interfaced to the gas chromatograph. The instrument's carrier gas, helium gas (99.99%), with a steady flow rate of 1.2 mL/min. At 80°C (isothermal for 4 minutes), the column's initial oven temperature increased by 5°C/min to 310°C. The flow rate was 1.21 mL/min, and the column pressure was 81.7 kPa. A mass scan from 40 to 650 m/z was used to prepare a mass spectrum at a 0.50 s scan interval [22].

Determination of total phenolic compounds

The total amount of phenolic compounds was determined in the ethanolic extract with a standard Folin - Ciocalteu reagent . The reaction mixture contained 100 µl of the extract, and 500 µl of the Folin-Ciocalteu reagent (Merck, Germany) and 1.5 ml of 20% sodium carbonate. The sample was then mixed on a vortex mixer and diluted with distilled water to the final volume of 10 ml. After 2 h reaction, the absorbance at 765 nm was determined and used to estimate the phenolic content using the calibration curve made with gallic acid (Sigma-Aldrich, Germany). The total amount of phenolic compounds was expressed in mg gallic acid equivalent (GAE) per g dry weight [23] .

Determination of total Tannins content

The sample extracts was 2mg mixed with water and ethanol (20 : 80) heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark-green solution indicates the presence of tannins. One ml of extract was added to two ml of sodium chloride (2%), filtered and mixed with five ml of 1% gelatin solution. A precipitate indicates the presence of tannin. And measurement absorption at = 540 nm[24].

Determination of vitamin E content

Dried plant material (0.5 g) was immersed in 20 ml of ethanol for 30 min in a water bath at 85°C. The solution was allowed to cool and then filtered into a separating funnel. Heptane (10 ml) was added, and the solution was shaken for 5 min. Then, 20 ml of 1.25% sodium sulfate was added and the solution was shaken again for 2 min, and allowed to separate into layers. Total tocopherols were determined by UV- VIS 545 nm . A volume of 0.5 ml of a-tocopherols in ethanol was processed in the same way as a sample, and used as a standard[25].

Statistical Analysis

All experimental data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS), version 26 (IBM Corp., Armonk, NY, USA). A two-way analysis of variance (ANOVA) was performed to test the significance of treatment effects. Whenever the ANOVA showed significant differences among means ($p \leq 0.05$), means were compared using Tukey's Honestly Significant Difference (HSD) test at the 5% level of significance to determine pairwise differences.The results are expressed as means ± standard deviation (SD), and means with different letters indicate statistically significant differences. Additionally, a One-Sample T-Test was used.

RESULTS

Detection of pathogenic bacteria from Diverse Clinical Isolates Using Traditional Methods

In Table 1, 120 bacterial isolates collected across different age groups, over a period from September 2024 to February 2025, from various hospitals in Babylon Governorate, the clinical specimens included burn and wound swap and urine. All isolates were cultured on MacConkey agar and blood agar The isolates were growing on both agar were transferred to Emb(Eosin Methylene Blue) medium. The appearance of a bright green color indicates the presence of E.coli bacteria, while the pink color of the isolates indicates K.

penumonia bacteria. As for the appearance of isolates only on blood agar, the isolate was transferred to manntiol medium. The color of the medium changed from red to yellow, indicating the fermentation of the bacteria in the Manntiol medium, which is S. aureus bacteria The number of isolates positive for Gram stain was 50, which are S.aureus, while the number of negative isolates was 70,(35) of which were E.coli isolates and (35)of K. penumonia isolates. The distribution of the major bacterial species isolated according to the specimen source. S. aureus was observed to be the most prevalent in burn (12) isolates representing (80%)and wound specimens (13) isolates, representing(86.6%)of the total isolates and urine specimens (25)isolates, representing 27.7%). In contrast, the occurrence of E.coli was particularly notable in urine specimens, with 30 isolates of each being identified, accounting for (33.3%) and burn (3) isolates representing (20%)and wound specimens (2) isolates, representing(13.3%)of the total isolates, K. pneumoniae coli was particularly notable only in urine specimens, with(35) isolates of each being identified, accounting for (38.89%)as showing in Fig1

Table1:Distribution of Major Bacterial Species by specimen Source

specimen Source	specimen number	S. aureus (n)	% of Total	E. coli (n)	% of Total	K. pneumoniae (n)	% of Total
Urin	90	25	27.7%	30	33.3%	35	38.89%
Wound	15	13	86.6%	2	13.3%	-	0.00%
Burns	15	12	80%	3	20%	-	0.00%
Total	120	50	38.46%	35	26.92%	35	26.92%

In Figure1,the Pathogenic bacteria on different media(A) show yellow colonies on mannitol salt agar with surrounding yellow medium.(B). gray to white colonies with clear zoon (3- hemolysis) around the colony on blood agar(C). K.pneuomnae on EMB .(D)\E.coli on EMB.

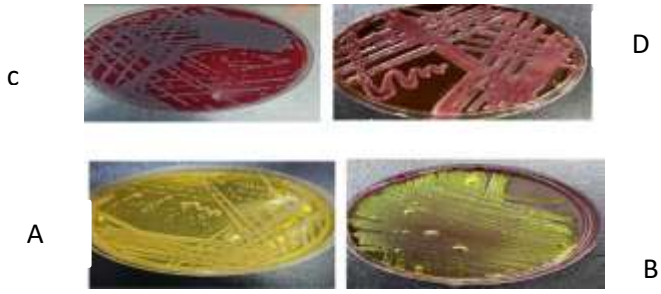


Fig .1.Pathogenic bacteria on different media

In Table 2,Detection Pathogenic bacteria by Biochemical Methods

In Figure 2,Catalase Test: All initial bacterial isolates, including S. aureus, showed a positive reaction, characterized by the rapid formation of bubbles upon the addition of hydrogen peroxide. However, E. coli and K. pneumoniae in the provided table showed a negative result .

Fig .2.catalase test of S.aureus.

Indole Test: The initial isolates tested negative, meaning they could not break down tryptophan to form indole. The reagent layer remained yellow or slightly cloudy, without the expected "cherry-red ring." E. coli, however, showed a positive result in the table, while S. aureus and K. pneumoniae were negative.

Methyl Red (MR) Test: Initial isolates tested positive. For specific strains, E. coli was positive, while S. aureus and K. pneumoniae were negative.

In Figure 3, citrate Test**:** Initial isolates tested positive, evidenced by a pH indicator color change consistent with alkaline byproducts of citrate metabolism K. pneumoniae was positive in the table, whereas S. aureus and E. coli were negative.

Fig.3 .Biochemical test (Simmon citrate test).

Mannitol Fermentation: S. aureus showed a positive result, while E. coli and K. pneumoniae were negative.

Table2:Biochemical Tests of Differentiation bacteria isolated from different specimens

Biochemical Test	S.aureus	E.coli	K.pneuominae
Catalase	+	_	+
Indole	_	+	_
Citrate	_	_	+
Mannitol Fermentation	+	_	_
Methyl Red	_	+	_

Anti-Bacterial Activity

The study looked into how different solvents affected how well L. leucocephala seed extract worked against diverse bacterial strains. At four concentrations,100%(0.1mg\ml), 75%(0.075mg\ml), 50%(0.05mg\ml), and 25%(0.025mg\ml),the effects of aqueous, ethanol, and hexane as solvents on inhibition zone diameters were assessed.

In Figure 5,Regarding Gram-negative bacteria, specifically E. coli and K. pneumoniae, none of the L. leucocephala seed extracts (aqueous, ethanolic, or hexane) demonstrated any inhibitory effect on their growth

across all tested concentrations. This suggests that the active compounds in the extracts may be unable to penetrate the complex outer cell membranes of Gram-negative bacteria, or that these bacteria possess specific resistance mechanisms that the extract cannot overcome .

the study revealed varying efficacy of L. leucocephala seed extract against the Gram-positive bacterium S. aureus, depending on the solvent used. The aqueous extract showed no inhibitory effect against S. aureus at any of the four concentrations (100%, 75%, 50%, 25%), recording a mean inhibition zone diameter of 0 mm in all cases (indicated by the letter 'a' in the table), confirming the lack of statistically significant differences and its ineffectiveness.

In Table 3,Figure 5, the ethanolic extract exhibited a clear and statistically significant inhibitory effect on S. aureus growth across all concentrations. The mean inhibition zone diameters for the ethanolic extract were 18 mm at 100%, 14.5 mm at 75%, 16.5 mm at 50%, and 16.6 mm at 25%. The type indicate a dose-dependent relationship, with varying efficacy across concentrations as shown by the statistical significance of the letters (c, b, bc). The hexane extract, however, showed very limited efficacy against S. aureus, demonstrating a slight inhibitory effect only at 25% concentration, with a mean inhibition zone diameter of 16 mm (indicated by the letter 'b'). It had no effect at higher concentrations (100%, 75%, 50%), which recorded 0 mm (indicated by the letter 'a').

*Given the superior effectiveness demonstrated by ethanol as the optimal solvent for extracting active compounds from L.leucocephala, which yielded a consistently potent extract, it was decided to continue the research by focusing on this specific ethanolic extract. This decision is based on preliminary findings confirming ethanol's ability to achieve the highest concentration of biologically active components with broad antimicrobial properties, notably exemplified by its remarkable capacity to inhibit the growth of S. aureus. Consequently, the research was completed by focusing on studying the efficacy of the L.leucocephala extract prepared with ethanol as a solvent against S. aureus. The biochemical properties of this particular extract were meticulously investigated, the compounds responsible for its robust efficacy were identified.

Table 3. Effect of Different Solvents and Concentrations on the Antibacterial Efficacy (Mean ± S.D Inhibition Zone Diameter of L. leucocephala Seed Extract Against S .aureus

Con. / Solvents	100%(0.1mg\ml)	75%(0.075mg\ml)	50%(0.5mg\ml)	25%(0.025mg\ml)
Aqueous	**Mean±S.D**			
	0 **a**	0 **a**	0 **a**	0 **a**
Ethanol	18 ±1.5 **c**	14.5 ±1.3 **b**	16.5 ±2.0 **b**	16.6 ±2.1 **bc**
Hexane	0 **a**	0 **a**	0 **a**	16 ±2.3 **b**

*Different letters indicate statistically significant differences among the interaction treatments (Concentration × Concentration) based on Tukey's HSD test at $P < 0.05$.

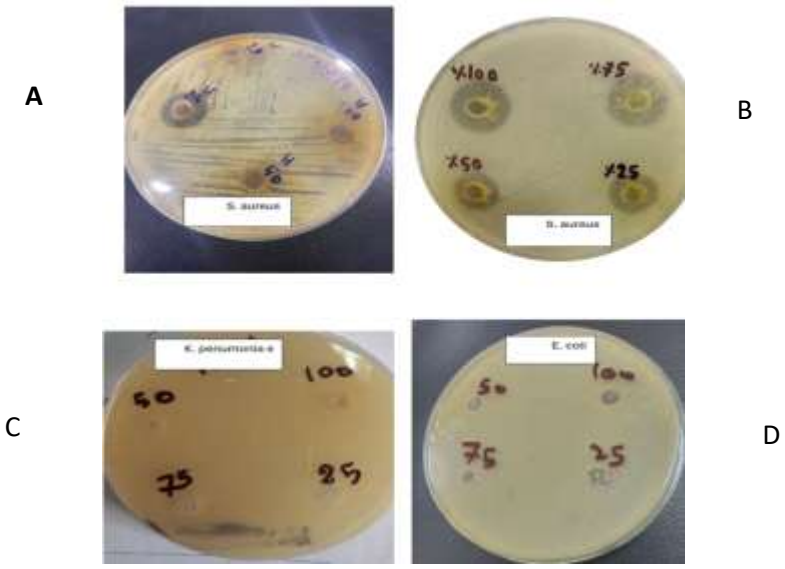
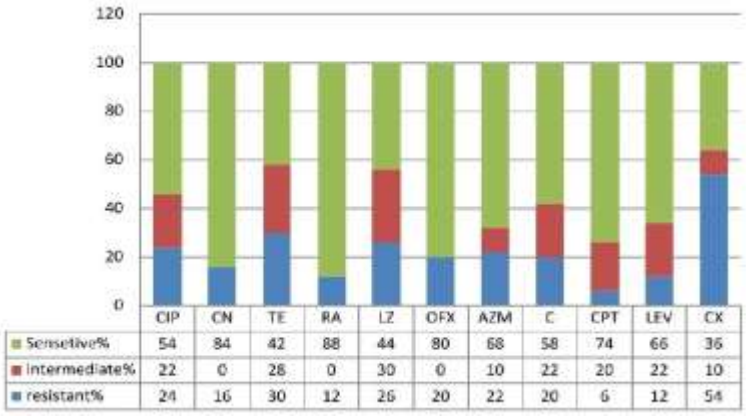


Fig .5 . A\anti-bacteria activity of hexane extract of L. lecpcephala seeds on S.aureus B\ anti-bacteria activity of ethanol extract of L. lecpcephala seeds on S.aureus
c\ anti-bacteria activity of ethanol extract of L. lecpcephala seeds on K.penumoniae
D\ anti-bacteria activity of ethanol extract of L. lecpcephala seeds on E.coli.

Antibiotic Susceptibility Test

In Figure 6,iIt is show,50 identified S. aureus isolates(1to50) are evaluate against 11 common antibiotics((CIP)Ciprofloxacin,(CN)Gentamicin,(TE)Tetracycline,(RA)Rifampicin(LZ) Linezolid,(OFX)Ofloxacin,(AZM)Azithromycin),(C)Chloramphenicol,(CPT)Cefotaxime,(LEV)Levofloxacin, (CX)Cefuroxime). Bacterial isolates exhibited varying resistance to the antibiotics under study. The highest resistance was notably observed against CX at 54%, followed by TE at 30%, and LZ at 26%. Resistance to CIP was found to be 24%, while AZM showed 22% resistance. Both OFX and C demonstrated 20% resistance, whereas resistance in CN decreased to 16%. The antibiotics to which the isolates showed the least resistance were RA and LEV at 12% each, and finally CPT at only 6%. On the other hand, the isolates were highly sensitive to RA at 88%, CN at 84%, OFX at 80%, and CPT at 74%. Good sensitivity was also observed towards AZM at 68%, LEV at 66%, C at 58%, and CIP at 54%. The antibiotics to which the isolates showed the least sensitivity were LZ at 44%, TE at 42%, and finally CX at only 36% .



	CIP	CN	TE	RA	LZ	OFX	AZM	C	CPT	LEV	CX
Sensetive%	54	84	42	88	44	80	68	58	74	66	36
intermediate%	22	0	28	0	30	0	10	22	20	22	10
resistant%	24	16	30	12	26	20	22	20	6	12	54

Fig. 6. Antibiotic Susceptibility Test

The Synergistic effect of Ethanol extract of L. leucephala seeds and antibiotic against S.aureus

In Figure 7,examine the mechanisms of action behind the interaction between ethanol extracts and specific antibiotics.Highlight case studies or research findings that demonstrate the successful use of ethanol extracts in clinical setting s. aureus. Specifically, the presence of the seed extract led to a substantial and statistically meaningful increase in the inhibition zone diameters for CIP (from 13 mm to 30 mm) and OFX (from 10 mm to 25 mm), demonstrating an enhanced antibacterial activity when the extract was combined with these antibiotics. While the increase in the inhibition zone diameter for CAZ in the presence of the extract (from 0 mm to 10 mm) suggests a potential synergistic trend, the effect was less pronounced compared to CIP and OFX. The statistically significant p-value therefore corroborates the visually evident enhancement of CIP and OFX activity against S. aureus in the presence of the seed extract.

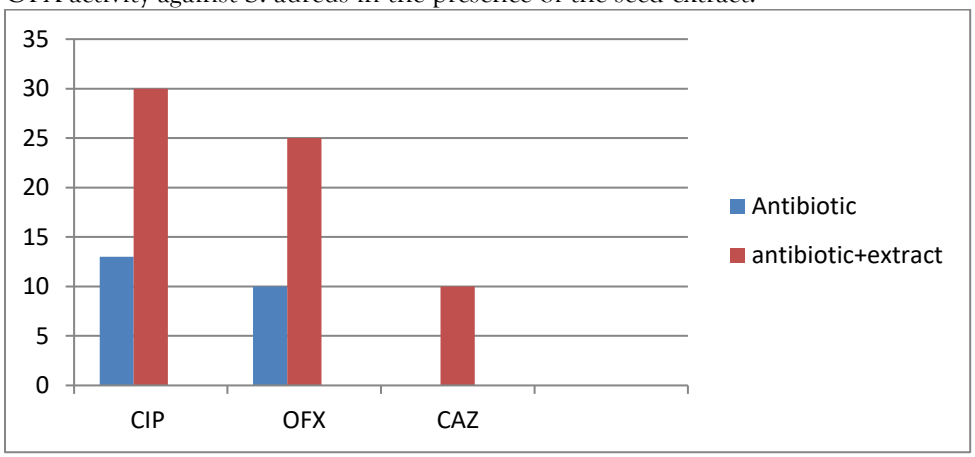


Fig. 7.the Synergistic effect of Ethanol extract of L. leucephala seeds and antibiotic against S.aureus

Phtyto chemical analysis of ethanol extract of L. lecocephala seeds

GC-Ms(Gas Chromatography-Mass Spectrometry)

In Table 3, GC-MS analysis 12 bioactive compound were identified in ethanol extract of L.lecocephala seeds as showing in Table3

Table 3: GC-MS of Ethanol extract of L.lecocephala seeds

N	Name	Quality
1	Methyl linolelaidate	99
2	Hexadecanoic acid, methyl ester	95
3	Diisooctyl phthalate	72
4	9-Octadecenoic acid (Z)-, methyl ester	68
5	Dodecamethylcyclohexasiloxane	64
6	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	64
7	2-Azanorbornane	50
8	Diisopropoxy-1,1,7,7,7-hexamethyl-3,5-bis(trimethylsiloxy)tetrasilox	46
9	F2A 13,14-H2 ME ESTER 3TMS	45
10	5-dimethyl-4-methylene-2-(trimethylsilyl)-1-cyclopenten-1-yl]methoxy	45

11	Butanedinitrile, 2,3-diethyl-2,3-dimethyl-, (R*,S*)-	37
12	Benzoic acid, 2,6-bis(trimethylsilyl)-, trimethylsilyl ester	28

High performance liquid chromatography(HPLC) of Ethanol extact of L.leucocephala seeds.

In Table 4, Furthermore, significant concentrations of phenolic compounds (263.9 mg/100gm) and a comparatively lower concentration of tannins (1.58%) were identifiedas, Vitamin E was also detected at 0.36%.

Table4: The amount of phenolic and tannin in seeds extract.

Total phenolic content (mg / 100 gm)	Total tannin content %
263.9	1.58

DISCUSSION

Important epidemiological information on the frequency of major pathogenic bacteria from a variety of clinical isolates in Babylon Governorate is provided by this study. The results demonstrate the important roles that K. pneumoniae, E. coli, and S. aureus play as the main causes of the illnesses under investigation. Particularly, the elevated levels of K. pneumoniae (38.89%) and E. coli (35.56%) in urine samples align with accepted medical microbiology concepts. These results are consistent with research that shows E. coli is the most frequent cause of simple urinary tract infections (UTIs)[16]. Furthermore, K. pneumoniae is known to play a major role in complex diseases linked to healthcare, such as urinary tract infections. This emphasizes how important these Gram-negative bacteria are to the pathogenesis of UTIs in the area under study. According to global trends, UTIs are the second most common disease after respiratory tract infections, and they are frequently made worse by unfavorable environmental factors and poor health. Regarding burn and wound infections, S. aureus was found to be the most prevalent isolate. This observation is in agreement with findings from other studies which similarly reported S. aureus as a dominant pathogen in burn and wound samples. Infection remains a major obstacle in the treatment of burn injuries, often characterized by a complex microbial landscape involving multiple species. The frequent association of multi-drug resistant bacteria with poorer clinical outcomes and challenges in antimicrobial therapy further emphasizes the importance of identifying prevalent pathogens like S. aureus in these wounds. While factors like cross-infection from medical staff, air, and materials are potential contributors, the consistent isolation of S. aureus highlights its endogenous or acquired presence in these specific infection types[26].

The biochemical identification tests provided crucial insights into the metabolic characteristics of the isolated bacteria, aiding in their differentiation and diagnosis.

The positive catalase test results, particularly those observed in S. aureus isolates, are consistent with what was mentioned by(Cappuccino and Welsh in 2020) [17].This test is considered a key diagnostic feature for distinguishing catalase-producing species. It is known for its ability to differentiate Staphylococcus species (catalase-positive) from Streptococcus species (catalase-negative) through the catalase enzyme's ability to break down hydrogen peroxide into oxygen gas and water, which is evident by the rapid formation of bubbles. The IMViC tests, a battery of examinations designed to differentiate members of the Enterobacteriaceae family, provided further characterization. The positive Methyl Red test result, along with positive citrate utilization as evidenced by alkaline byproducts, are important indicators of specific metabolic pathways within the isolates. The isolates tested belong to the Enterobacteriaceae family. The catalase test was positive, indicating the bacteria's ability to break down hydrogen peroxide. These findings align with the observations presented by Popovici ,(2024)[27].

Some studies have reported that the vast majority of E. coli strains are MR-positive, meaning that they are able to produce sufficient amounts of acid from glucose to cause a pH drop and a color change in the MR indicator. This observation aligns with findings from the current study, consistent with reports by [28].

The observed ineffectiveness of L. leucocephala seed extracts (aqueous, ethanolic, and hexane) against Gram-negative bacteria such as E. coli and K. pneumoniae across all tested concentrations suggests that the active compounds in the extracts may be unable to penetrate their complex outer cell membranes, or that these bacteria possess specific resistance mechanisms that the extract cannot overcome. This lack of efficacy against E. coli specifically contradicts a previous study by Rosida et al. (2017)[15], suggesting the need for further research to identify variables that might explain this discrepancy. While both studies utilized L. leucocephala seeds, the observed absence of efficacy against E. coli in our study might be attributed to the differing geographical origin of the plant, as the source[15] was in Indonesia, a distinct environment compared to our study's plant source. Plant varieties are known for their genetic and environmental variability, which influences the chemical composition of active compounds. Overall, the results of this study reinforce, and are consistent with, the findings of [29].

In contrast, the substantial variations in the antibacterial efficacy of L. leucocephala seed extracts against the Gram-positive bacterium S. aureus were highly dependent on the solvent used and its concentration. The ethanolic extract's clear and statistically significant inhibitory effect across all concentrations, with varying efficacy across concentrations as shown by the statistical significance of the letters (c, b, bc), indicates a dose-dependent relationship. These results highlight ethanol as the most effective solvent for extracting active compounds against S. aureus from L. leucocephala seeds. This partially aligns with previous research on the plant's chemical properties. For instance, the presence of phenolic content in the ethanolic extract of L. leucocephala seeds, believed to contribute to its effectiveness, is consistent with observations reported by Chowtivannakul et al. (2016)[30], whose research also indicated the presence of phenolic compounds within the seed extract of the same plant. Furthermore, the observed differences in extraction efficiency among various solvents like ethanol and hexane can be explained by variations in solvent polarity, which affects extraction capabilities[31] and the ability of certain solvents like ethanol to increase bacterial cell wall permeability [32].

The study highlights the susceptibility of S. aureus to the ethanolic extract. Given that S. aureus is a major cause of both hospital- and community-acquired infections [33]. these findings support the notion of using the extract as a potential alternative or adjunctive therapy. This effect is attributed to the presence of active compounds such as unsaturated fatty acids (e.g., oleic and linoleic acid), vitamin E, and palmitic acid, which are known for their antimicrobial properties. This hypothesis is consistent with other studies on natural compounds and their antimicrobial efficacy [34]. Antimicrobial resistance (AMR) has evolved into a major global public health threat, seriously jeopardizing our ability to successfully prevent and treat persistent diseases. The misuse and overuse of various antibacterial agents in healthcare and agriculture, along with bacterial evolution and horizontal gene transfer, are primary drivers of AMR [35]. Research has increasingly focused on S. aureus due to its significant role in a wide array of human infections, from superficial to life-threatening internal tract involvement, with a growing prevalence of S. aureus-associated urinary tract infections [36]. Antibiotics, being natural products produced by microorganisms to combat other microbes [37], are crucial in this fight.

The resistance patterns observed in the current study for S. aureus isolates generally align with global trends and previously reported data. The resistance of 12 S. aureus isolates to Ciprofloxacin (CIP) and 8 isolates to Gentamicin (CN) is consistent with findings reported by [38]. The high resistance rate of 54% (27 isolates) against Cefoxitin (CX) is notably consistent with Ali et al. (2021)[39]. Similarly, the 26% resistance rate (13 isolates) to Linezolid (LZ) is in agreement with findings by Yang et al. (2025)[40]. The results concerning Azithromycin (AZM) resistance are consistent with the mechanisms of macrolide resistance in S. aureus reviewed by Miklasińska-Majdanik (2021)[41], strengthening the current scientific understanding of resistance to this antibiotic class. Overall, the observed antimicrobial resistance patterns, including high rates of S. aureus resistance to penicillin, tetracycline, and ciprofloxacin, are consistent with observations made by Kim

et al. (2018)[42]. suggesting a similarity in resistance profiles. The consistency of S. aureus resistance to Tetracycline (TE) with Lubna et al. (2023) [43]further supports the prevalence of tetracycline resistance in staphylococcal bacteria.

Conversely, the susceptibility profile highlighted key treatment options. The isolates exhibited the highest sensitivity towards Rifampicin (RA) at 88%, followed by Gentamicin (CN) at 84%, and Ofloxacin (OFX) at 80%. This spectrum of sensitivity underscores the differential response of Staphylococcus isolates to various antibiotics. The sensitivity of S. aureus to Ciprofloxacin observed in this study is close to the results of Hameed (2020),[44] who also reported low resistance to this antibiotic. The consistency in S. aureus sensitivity levels to antibiotics such as Ciprofloxacin (CIP), Chloramphenicol (C), and Levofloxacin (LEV) with findings reported by Nwankwo and Nasiru (2011)[45] reinforces the understanding that antibiotic resistance patterns in S. aureus can exhibit similar trends across different geographical regions and clinical contexts. Specifically, the 84% sensitivity to Gentamicin (CN) in this study compares favorably with the 73.4% sensitivity reported by Nwankwo and Nasiru (2011)[45].

Understanding the mechanisms of action for effective antibiotics provides further context. Gentamicin, an aminoglycoside, exerts its antibacterial effect by binding to the 16S rRNA within the 30S ribosomal subunit, disrupting mRNA translation and leading to non-functional protein synthesis [46]. Linezolid is an important antimicrobial against Gram-positive bacteria, including MRSA, and inhibits translation by binding to the 23S rRNA peptidyl transferase region [46]. Rifampicin (RA) is recognized for its potent antibacterial action, effectively targeting intracellular phagocytosed S. aureus and hindering its dissemination, making it valuable as an adjunctive therapy [48].

These findings provide crucial local epidemiological data on S. aureus resistance and sensitivity patterns, which are vital for guiding empirical antimicrobial therapy and informing infection control strategies in Babylon Governorate. In the face of the growing challenge of antibiotic resistance, the search for innovative strategies to restore their effectiveness is a pressing priority. One promising avenue lies in exploring the potential synergy between plant extracts and antimicrobial compounds. These studies aim to evaluate whether combining plant extracts, which contain a diverse array of biologically active molecules, can enhance the activity of antibiotics that have become ineffective against resistant bacterial strains. This concept has been supported by a study conducted by Cheesman *et al.*(2017)[49], the results of which demonstrated that the 100% concentration of the alcoholic extract enhanced the efficacy of certain antibiotics, albeit to varying degrees depending on the bacterial species and the antibiotic used [49]. The resistance phenomena can be effectively addressed by combining natural chemicals with currently available antibiotics in a synergistic fashion [50].The results of this study generally demonstrated that the 100% concentration of the ethanol extract enhanced the efficacy of certain antibiotics, although to varying degrees depending on the bacterial species and the antibiotic used. The statistical analysis revealed a significant difference between the seed extract and the antibiotics ciprofloxacin (CIP), ofloxacin (OFX), and ceftriaxone (CAZ) ($p = 0.021$, $p < 0.05$), indicating a notable interaction. This statistical significance is also backed up by the strong combined effect of the extract with CIP and OFX against S.aureus . Discuss the implications of enhanced antibiotic efficacy in combating antibiotic resistance.Explore the potential of combination therapy using natural extracts and antibiotics.Analyze how different bacterial species respond to ethanol extract and antibiotics in terms of effectiveness.Examine the mechanisms of action behind the interaction between ethanol extracts and specific antibiotics.Highlight case studies or research findings that demonstrate the successful use of ethanol extracts in clinical settings. aureus. Specifically, the presence of the seed extract led to a substantial and statistically meaningful increase in the inhibition zone diameters for CIP (from 13 mm to 30 mm) and OFX (from 10 mm to 25 mm), demonstrating an enhanced antibacterial activity when the extract was combined with these antibiotics. While the increase in the inhibition zone diameter for CAZ in the presence of the extract (from 0 mm to 10 mm) suggests a potential synergistic trend, the effect was less pronounced compared to CIP and OFX. The statistically significant p-value therefore corroborates the visually evident enhancement of CIP and OFX activity against S. aureus in the presence of the seed extract. Our current study's findings are highly

consistent with those reported by [22]. regarding the presence and identification of bioactive compounds in L. leucocephala seeds, as analyzed by GC-MS (Gas Chromatography-Mass Spectrometry). This mutual agreement underscores the rich phytochemical profile of the seeds and supports conclusions regarding their biologically active constituents.

These results support our hypothesis that the fatty compounds present in L. leucocephala seed extract, such as palmitic acid, may be responsible for the observed inhibitory effect on S. aureus growth. They also suggest that these compounds may be more effective against Gram-positive bacteria due to differences in bacterial cell membrane composition. This aligns with previous studies[22]. The presence of these compounds, such as phenols holds substantial scientific importance due to their known capacity to modulate bacterial growth and metabolism [51]. Furthermore, these compounds are recognized as natural antimicrobial agents, exerting their effects through diverse and complex mechanisms that contribute to inhibiting or eradicating microorganisms [52].

CONCLUSION

In this study provides crucial epidemiological information on the prevalence of major pathogenic bacteria isolated from various clinical specimens in Babylon Governorate. The findings highlight the significant roles of K .pneumoniae, E. coli, and S. aureus as primary causes of the investigated infections. Notably, S. aureus was highly prevalent in burn and wound samples, while E. coli was particularly conspicuous in urine specimens. K. pneumoniae was found exclusively in urine samples. The results demonstrate the effectiveness of traditional identification methods, including Gram staining and specific biochemical tests, in characterizing these bacterial species. Furthermore, the study revealed the promising efficacy of L. leucocephala seed extract, particularly its ethanolic form, in inhibiting the growth of S. aureus. However, the extract showed no inhibitory effect against the Gram-negative bacteria (E. coli and K. pneumoniae). Significantly, a substantial synergistic effect was observed when the ethanolic extract was combined with certain antibiotics (such as ciprofloxacin and ofloxacin) against S. aureus, suggesting the potential for enhanced therapeutic outcomes. Phytochemical analysis confirmed the presence of bioactive compounds like phenolic compounds and tannins in the ethanolic extract, which likely contribute to its antimicrobial activity. These findings underscore the therapeutic potential of L. leucocephala seed extract as an antimicrobial agent or as an adjunct therapy to enhance antibiotic effectiveness, particularly in combating S. aureus infections.

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