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EVALUATION OF ANTIBACTERIAL EFFICACY OF CLOVE OIL AGAINST MULTIDRUG-RESISTANT CLINICAL ISOLATES

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ABSTRACT

The increasing emergence of multidrug-resistant (MDR) bacteria has become a major global healthcare challenge, limiting the effectiveness of conventional antibiotics. This study aimed to evaluate the antibacterial potential of clove oil against selected MDR bacterial strains isolated from clinical samples. A total of clinical specimens, including blood, urine, pus, and sputum, were collected from patients at different tertiary care hospitals. The isolated organisms were identified as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* using Gram staining and a series of biochemical tests such as oxidase, catalase, citrate, urease, and indole tests. Gram-positive cocci (*S. aureus*) appeared purple under microscopic examination, while Gram-negative rods (*E. coli* and *P. aeruginosa*) stained pink. The antimicrobial activity of clove oil was assessed through the agar well diffusion method. Different concentrations of clove oil (20 µL, 40 µL, 60 µL, 80 µL, and 100 µL) were tested to observe the zone of inhibition against each bacterial strain. The results demonstrated a concentration-dependent antibacterial effect. The maximum inhibitory zone for *S. aureus* was recorded at 10.5 mm, *P. aeruginosa* at 15 mm, and *E. coli* at 11 mm at the highest concentration of 100 µL. At lower concentrations, the antibacterial activity was significantly reduced, with *E. coli* showing no inhibition at 20 µL. These findings suggest that clove oil possesses significant antibacterial properties against MDR

	bacteria and can serve as a promising alternative or complementary approach to combat antibiotic-resistant infections. Further research and clinical trials are necessary to validate its efficacy and safety for therapeutic use.

INTRODUCTION:

Antibiotics are the basic need for treating microbial infections. Since antibiotics are discovered a medical belief has been established that chemotherapeutic agents are playing a significant role in eradication of infectious diseases. By the overuse of antibiotics now the whole world is facing a major therapeutic problem against multi-drug resistant bacteria (Abdullah, Hatem, & Jumaa, 2015). Non susceptibility or insensitivity to at the minimum one agent in three or more antimicrobial genre is known as Multidrug resistant (MDR). One bacteria get resistant against multiple drugs. Now at that time multidrug resistant organisms quartet worldwide. In last fifteen to twenty years the rate of infectious diseases is getting so high and the standard of public health is comparable in under developed countries as before the discovery of antibiotics (Van Duin & Paterson, 2016). Momentarily antimicrobial resistant set up a great threat to patient's cure that leading towards increment of morbidity rate and mortality rate and brutal loss to patient and the country economically and financially. The major bacteria that are getting resistance are *Pseudomonas aeruginosa, E.coli, Staphylococcus aureus, Enterococcus feacalis* and *Acinetobacter baumanni. E.coli and staphylococcus aureus* are most commons which are causing cholecystitis, furuncles, bacteremia, cholangitis, urinary tract infections, diarrhea and neonatal meningitis respectively. Antibiotic resistance could be a public health concern round the world. The amount of bacteria that are immune to antibiotics is increasing (Bassetti & Righi, 2013). The danger of antibiotic resistance is that treatable illnesses, like pneumonia, tuberculosis, or minor infections could become incurable. This might put a greater economic and emotional burden on families and on our healthcare system. Antibiotic resistance ends up in a decreased ability to treat infections and illnesses in people, animals and plants. This could cause the subsequent problem (Miyoshi-Akiyama et al., 2017). Presence of MDR is mainly found in in everywhere specially in food, animal and plants and also found its genes in human and become lethal for human beings. MDR affect human beings via spreading its multidrug resistant genes in environment (Andersson & Hughes, 2010). The major factor that are involve in spreading is the misuse and overuse of antibiotics. some other factors are the poor hygiene conditions, the quality of water used is very poor full of germs, poor disease control and prevention system,

poor access to quality. As the Rate of resistance is increased day by day in whole world, antibiotics are useless or less effective to treat such common infections like UTI, Sepsis, diarrhea and sexual transmitted diseases (Dzidic & Bedeković, 2003). Mainly antibiotic resistance was seen among these infections, so tackle such infections somehow alternative of antibiotics must be used. many of the herbs commonly used in world to tackle such infections as alternative to antibiotics. Among them clove oil is very beneficial effect against such MDR infections (Southon et al., 2020). Anti-microbial resistance is a serious worldwide issue that has held onto the underlying foundations of improvement. Antimicrobial opposition influences have invulnerable profile, tweaks with microorganism's wellness cost, and impacts the hereditary co-choice of safe species with their recurrence of reversibility potential. The biological processes of the organism are mainly involved for such a resistant element to battle the natural harmful circumstances (Basak, Singh, & Rajurkar, 2016). The inborn property of the microorganism, i.e., the normal obstruction of the organism, is an explanation of opposition development. The major causative component of obstruction advancement is likewise the recurrence of appearance of resistant microorganisms because of hereditary transformations or developmental level quality exchange (Arooj et al., 2025). Adjustments that occur in the medication related receptor and the area of the objective locales of the connection with the antibiotics agents are particular, these can be complicated chemicals and ribosomes. The most often distinguished obstruction predictable with varieties in the ribosomal target is in macrolide antibiotic agents (Nazir et al., 2025). The most famous models here are the evolvement of penicillin opposition due to the transformations of penicillin-restricting proteins beta-lactamase compounds in *Staphylococcus aureus, Streptococcus pneumoniae, Neisseria meningitides, and Enterococcus faecium* strains (Attia et al., 2016). The resistance mechanism occurred due to change in the permeability of outer and inner membrane of the cell which results decreased drug uptake in cell. As the permeability of membrane decreased which may be due to point mutations or insertional interruptions in coding sequences of proteins of resistant strains just like a specific mutation of porins called OprD, which is resistant to carbapenem in *pseudomonas aeruginosa* (Utchariyakiat, Surassmo, Jaturanpinyo, Khuntayaporn, & Chomnawang, 2016). Quinolone resistance is mainly due to depletion in outer membrane permeability. Clove oil is extracted from Eugenia caryophyllata of the Myrtaceae family. It has been utilized as a histological clearing specialist. Restoratively, it is generally utilized for alleviating toothache or cavity issues and in fragrant healing and as a germ-free in oral diseases (Rhayour, Bouchikhi, Tantaoui-Elaraki, Sendide, & Remmal, 2003). The admission of the oil gives carminative and hostile to plasmodic

properties. In the stomach, the impact is carminative, loosening up the gastric sphincter, and it energize eructation. Clove oil is also used in cooking for spice most used to give flavor to ginger bread pumpkin pie and in some soups and sauces (Verma, Karkun, & Siddiqui, 2015). Clove oil usually consist of different components including eugenol, β-caryophyllene and minimum level of benzyl alcohol. Eugenol to be 78%, with 13% β-caryophyllene (Pandey & Singh, 2011). Clove oil has important role as antibacterial, antifungal, insecticidal and cell reinforcement properties, and is utilized generally as an enjoying specialist and antimicrobial material in food. What's more, clove oil is utilized as a germ-free in oral diseases (Zwain). This natural oil has been accounted for to restrain the development of molds, yeasts and microbes. Clove (Syzygium aromaticum) is one of the most significant flavors that has been utilized for quite a long time as food additive and for the majority restorative purposes (HUSAIN, AHMAD, ASIF, & TAHSEEN, 2013). Clove is local of Indonesia yet these days is refined in a few areas of the planet remembering Brazil for the province of Bahia. This plant addresses one of the most extravagant wellspring of phenolic mixtures, for example, eugenol, eugenol acetic acid derivation and Gallic corrosive and forces extraordinary potential for drug, corrective, food and horticultural applications. The cell reinforcement and antimicrobial action of clove is higher than many natural products, vegetables and different flavors and ought to merit exceptional consideration. Another utilization of clove as parricidal specialist is a fascinating methodology to battle dengue which is a not kidding medical condition in Brazil and other tropical nations. Pharmacokinetics and toxicological investigations were additionally referenced (Nzeako, Al-Kharousi, & Al-Mahrooqui, 2006).

RESEARCH METHODLOGY: The present study was conducted using various clinical samples obtained from hospital patients, following all ethical and biosafety protocols. Samples included blood, pus, urine, and sputum, which were collected using standard aseptic techniques to avoid contamination and ensure sample integrity.

Sample Collection: Blood samples were collected through venipuncture after proper patient identification and disinfection of the puncture site. A sterile syringe was used to draw the required volume of blood, which was then transferred into appropriately labeled collection tubes for laboratory analysis. Pus samples were obtained directly from the infected site using sterile swabs, ensuring that the sample was taken from the deepest part of the wound to avoid contamination with surface flora. The swabs were immediately placed into transport media to preserve the viability of organisms. Midstream urine samples were collected in sterile containers after instructing the patients to perform proper hand hygiene and initial urine voiding before collecting the sample. Sputum samples were collected after instructing

patients to rinse their mouths, take deep breaths, and forcefully cough into sterile specimen cups to obtain deep respiratory secretions.

Culture Media Preparation: For the isolation and cultivation of microorganisms, various culture media were prepared. Blood agar was prepared by dissolving the dehydrated media in distilled water, sterilizing it by autoclaving at 121°C for 15 minutes, and cooling it to 40-45°C before adding sterile defibrinated blood. The mixture was then poured into sterile Petri plates. CLED agar was prepared by dissolving 36 grams of media in 1000 mL of distilled water, boiling with constant stirring, autoclaving, and pouring into Petri plates after cooling to 50°C. Mueller-Hinton agar was prepared by suspending 38 grams of media in 1 liter of distilled water, autoclaving, and pouring into sterile plates under aseptic conditions. All prepared media were incubated for sterility checking before use.

Microscopic and Biochemical Identification: Initial identification was performed using Gram staining to classify bacteria into Gram-positive and Gram-negative categories based on cell wall characteristics. The procedure involved sequential application of crystal violet, Gram's iodine, alcohol decolorizer, and safranin, followed by microscopic examination under oil immersion. Further identification was achieved through a series of biochemical tests. The oxidase test was performed by applying bacterial colonies to oxidase reagent-impregnated filter paper and observing color changes. The catalase test was carried out by adding hydrogen peroxide to bacterial colonies and observing for bubble formation, indicating catalase activity. Citrate utilization was assessed using Simmons citrate agar, where a color change to blue indicated positive citrate metabolism. Urease activity was tested using Christensen’s urea agar, observing color changes to pink due to ammonia production. The indole test was conducted by inoculating tryptophan broth, incubating, and adding Kovac’s reagent; the formation of a red ring indicated indole production.

Antimicrobial Susceptibility Testing: The antimicrobial activity was evaluated using the agar well diffusion method. The bacterial inoculum was uniformly spread on Mueller-Hinton agar plates, and wells were aseptically punched using a sterile cork borer. Different volumes and concentrations of plant extract (e.g., clove oil) were dispensed into the wells. Plates were incubated at 37°C for 24 hours, after which zones of inhibition were measured to determine antimicrobial sensitivity. Minimal Inhibitory Concentration (MIC) was determined by inoculating bacterial suspensions into serial dilutions of antimicrobial agents and observing the lowest concentration that inhibited visible growth. Minimal Bactericidal Concentration (MBC) was determined by subculturing samples from the MIC tubes onto drug-free media and identifying the lowest concentration that produced a 99.9% reduction in bacterial

colonies. Serum bactericidal activity was also assessed by mixing patient serum with the bacterial isolate and determining the dilution at which 99.9% of organisms were killed. The presence of β-lactamase enzyme was detected using chromogenic cephalosporin substrates that change color upon hydrolysis of the β-lactam ring.

McFarland Standard Preparation: A 0.5 McFarland standard was prepared to standardize the bacterial inoculum. It was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate with 9.95 mL of 1% sulfuric acid, yielding a suspension visually comparable to bacterial turbidity standards used in antimicrobial susceptibility testing. Through these procedures, accurate isolation, identification, and sensitivity profiling of microbial pathogens were conducted, ensuring reliable data for antimicrobial evaluation.

RESULTS: In this study samples collected from different tertiary care hospitals including blood, urine, sputum and pus to look for different multidrug resistant bacteria of gram positive and gram negative class. Mainly three bacteria were selected *E.coli*, Pseudomonas aeruginosa and S.aureus. All these bacteria were verified by gram staining and different biochemical tests. To check antimicrobial resistance Antimicrobial sensitivity test was performed. After this clove oil was used by antimicrobial agar diffusion method to check effect of it on different MDRs.

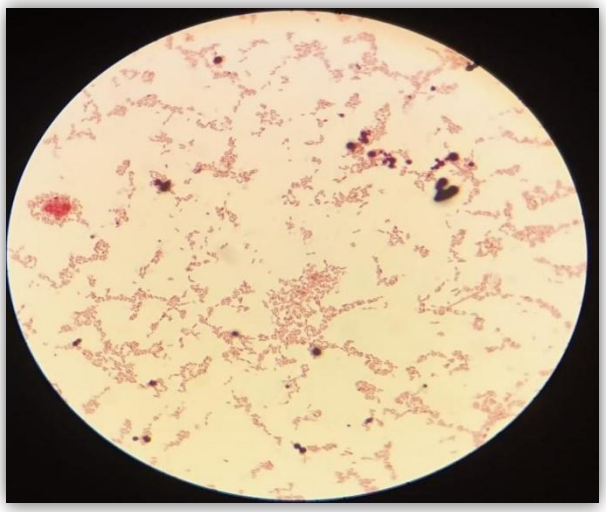

Figure 1: Shows Pink Color Gram Negative Rods Shaped Bacteria

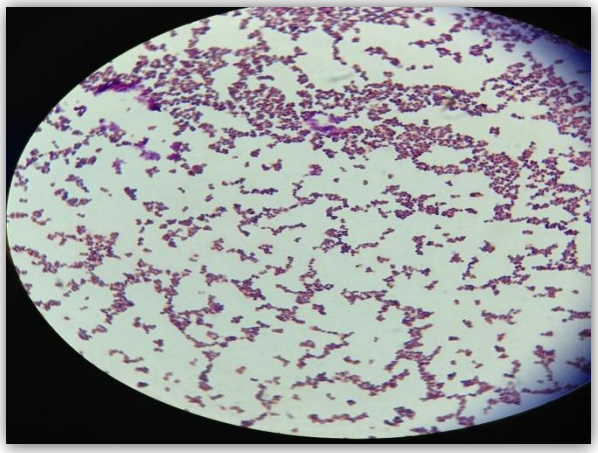

Figure 2: Shows Purple Color Gram Positive Cocci Shaped Bacteria

Gram staining was performed on all bacterial isolates to differentiate between Gram-positive and Gram-negative organisms. The Gram-positive bacteria appeared purple and were identified as cocci in shape, while the Gram-negative bacteria appeared pink and were rod-shaped. Based on microscopic examination, *Staphylococcus aureus* was identified as Gram-

positive cocci, whereas *Pseudomonas aeruginosa* and *Escherichia coli* were identified as Gram-negative rods.

Culture growth

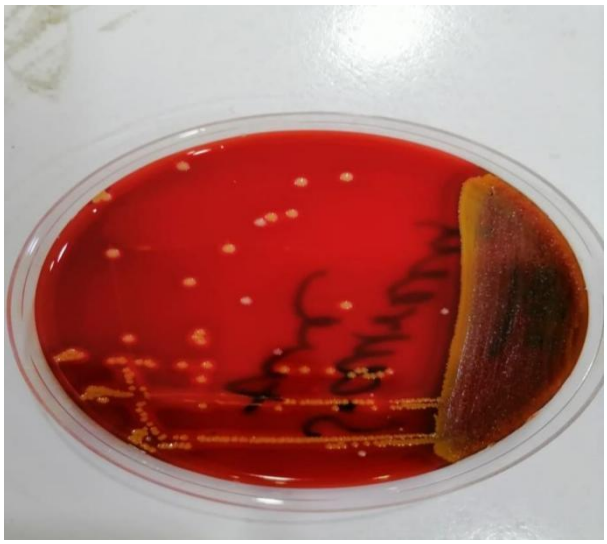

Figure 3: Shows S.aureus Colonies on Blood Agar

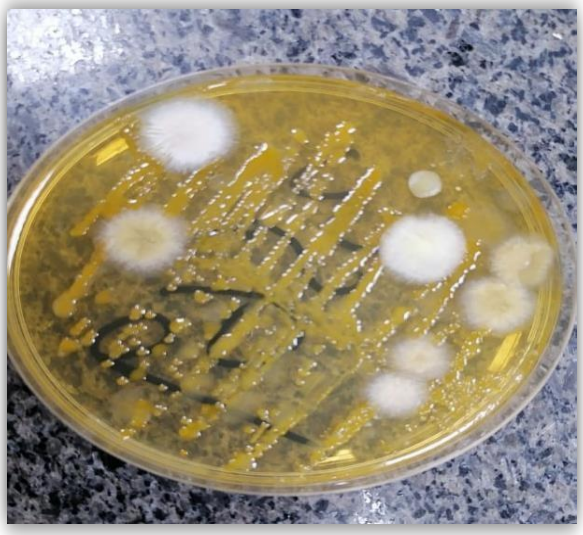

Figure 4: Shows E.coli Colonies on CLED Agar

Figure 5: Shows Pseudomonas aeruginosa Colonies in CLED Agar

After culturing of bacteria we got different type of colonies on different agars included blood and CLED agar. *S.aureus* shows golden color colonies on blood after incubation of 24 hours while *E.coli* shows yellowish colonies on CLED agar and *Pseudomonas aeruginosa* shows green color colonies on CLED agar.

Biochemical tests:

Table 1: Shows results of different biochemical tests

Test name	*Pseudomonas aeruginosa*	*S.aureus*	*E.coli*
Oxidase	+	–	–
Citrate	+	+	–
Catalase	+	+	+
Indole	–	–	+
Urease	+	+	–

Antibiotic sensitivity evaluation after application of clove oil:

Figure 6: Zone of inhibition against E.coli

Figure 7: Zone of inhibition against Pseudomonas aeruginosa

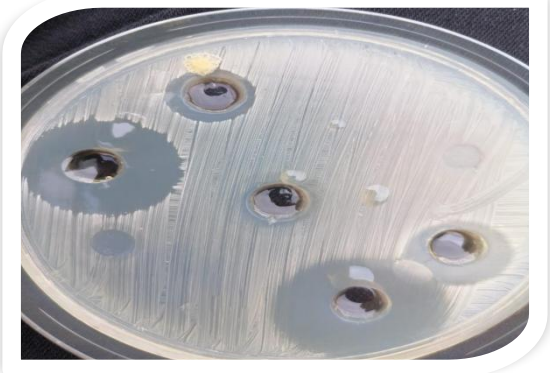

Figure 8: Zone of inhibition against S.auerus

Table 2: shows results of agar well diffusion method against S.aureus

Clove Oil concentration	Zone of inhibition
20µl	1.5mm
40µl	2mm
60µl	4mm
80µl	7mm
100µl	10.5mm

Table 3: Shows results of agar well diffusion method against Pseudomonas aeruginosa

Clove Oil concentration	Zone of inhibition
20µl	1mm
40µl	3mm
60µl	6.5mm
80µl	9mm
100µl	15mm

Table 4: shows results of agar well diffusion method against E.coli

Clove Oil concentration	Zone of inhibition
20µl	No zone
40µl	2mm
60µl	5mm
80µl	6.5mm
100µl	11mm

The antimicrobial activity was evaluated using the agar well diffusion method. After 24 hours of incubation, zones of inhibition were recorded. The maximum zone of inhibition was observed at 100 µL concentration, while the minimum zone was noted at 20 µL. Among the tested organisms, Escherichia coli exhibited the largest zone of inhibition measuring 11 mm, whereas Pseudomonas aeruginosa showed the smallest zone of inhibition measuring 1 mm.

DISCUSSION: The increasing emergence of multidrug-resistant (MDR) bacteria has raised significant global concern. According to recent studies, the World Health Organization (WHO) has published a priority list of 12 resistant bacteria that pose a serious threat to human health. Among these are *Pseudomonas aeruginosa* and Enterobacteriaceae, including *Escherichia coli*, *Klebsiella*, and *Proteus* species. These bacteria have developed resistance to multiple antibiotics, making treatment options increasingly limited. In this study, we focused on evaluating the antibacterial activity of clove oil against selected MDR bacteria including *E.*

coli, *P. aeruginosa*, and *S. aureus*. *E. coli* was the most sensitive organism to clove oil, exhibiting a maximum zone of inhibition of 11 mm, followed by *P. aeruginosa* with 10 mm at the highest concentration tested. These findings are consistent with previous studies where clove oil demonstrated zones of inhibition ranging from 10 to 19 mm against *E. coli*. One previous study reported a 12 mm zone at 15 mg/ml concentration, whereas in our study, a similar zone was achieved at 75 mg/ml concentration, indicating variability depending on experimental conditions and oil purity. Similarly, studies on *P. aeruginosa* have demonstrated significant sensitivity to clove oil. A prior study reported a 14 mm zone of inhibition at 100 mg/ml, while our study recorded a slightly higher inhibition zone of 15 mm at the same concentration. In the case of *S. aureus*, related research showed maximum zones of 14.7 mm, while our findings demonstrated a zone of 10.5 mm at 100 mg/ml. Comparative studies evaluating various essential oils revealed that while cinnamon oil exhibited the highest antibacterial activity, clove oil consistently showed strong inhibitory effects against MDR strains, supporting its potential as an alternative therapeutic agent.

CONCLUSION: Among these the most resistant bacteria is *E.coli* which is getting worst day by day and creating an alarming situation in whole world. The study revealed that clove oil has a promising antimicrobial activity against multidrug resistant bacteria including *E.coli, pseudomonas aeruginosa and S.aureus* and also we have seen *E.coli* is the most sensitive found against clove oil. At different concentrations we saw different activity of clove oil as we increase the concentrations we saw that the zone of inhibition is increasing. Mostly higher zone of inhibition is seen at 100 µl while minimum at 25 µl. Current study proving the worth of how effect is essential oils in making antibiotics especially clove oil shows great sensitivity and it's so overwhelming for the pharmaceutical scientist to use this product in making antibiotics against MDRs. For some extent this study through light on the importance of essential oils. By using these oils we can deal with the alarming situation of antibiotic resistant.

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