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Effect of Methanol Extract and Crud Alkaloids of Cloves Syzygium Aromaticum on *Staphylococcus aureus* and *E coli* Isolated from Vaginitis

Ala Jalal Ahmad[1], Sawar Ibraheem Maulood[2]

[1,2]Department of Biology, College of Science, Salahaddin University -Erbil, Erbil, Kurdistan Region, Iraq

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Abstract

Background: *Staphylococcus aureus* and *Escherichia coli* are two common bacterial species linked to vaginitis. These microbes can upset the delicate equilibrium of the vaginal microbiota, which can cause pain, irritation, and inflammation. An imbalance in the vaginal bacterial flora leads to a disturbance in the normal microbial balance, which in turn causes bacterial vaginosis (BV). Therefore, this research aimed to Identify the types of bacteria that cause vaginal infections in women and study the effect of methanol extract and crud alkaloids of cloves Syzygium aromaticum on them. **Methods:** The microbiological composition of cervical smears and vaginal swabs was examined for 50 random samples taken from a group of women at Hawler Hospital and private clinics in the period from October to December 2023. Bacteria associated with vaginitis were isolated and cultured using sterile swabs. Bacteria samples were identified through various biochemical tests. **Results:** According to the results, 44% of the samples were infected with *S. aureus* and 16% with *E. coli.* The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *E. coli* and *S. aureus* in the crude alkaloid extracts were constant at 2 mg/mL. These results indicate a consistent inhibitory effect against these two types of bacteria, and even bactericidal activity, especially against *E. coli*. **Conclusion:** This research concluded that *S. aureus* and *E. coli* are cervicitis-associated bacteria. In addition, clove extracts consistently exhibit bactericidal and inhibitory effects against *S. aureus* and *E. coli*, indicating the adaptability of these natural chemicals in targeting several types of bacteria associated with vaginal infections.

Keywords: Minimum Bactericidal Concentration**,** Minimum Inhibitory Concentration**,** *Staphylococcus aureus*, *Escherichia coli***,** Syzygium aromaticum**,** Methanol extract, Vaginitis.

INTRODUCTION

Staphylococcus aureus (S. aureus) and *Escherichia coli* (E. coli) are two common bacteria associated with vaginitis. These microbes can upset the delicate balance of the vaginal microbiota, which may cause pain, irritation, and inflammation, and subsequently, cause vaginal infections such as bacterial vaginosis (BV), vulvovaginal candidiasis (CVV), or aerobic vaginosis (AV).[1] The vaginal microbiome, which is mostly inhabited by Lactobacillus species, is a part of the human microbiome, which is a topic of growing scientific interest.[2] AV is a disorder that can result from disturbances in this microbiome, especially when aerobic bacteria like *S. aureus* and *E. coli* proliferate.[1] In addition, these types of bacteria constitute a global problem due to the spread of the diseases they cause and their resistance to drugs.[3,4] Furthermore, the digestive tract is often home to *E. coli*, which can spread to the vaginal area and cause infections. In addition, *S. aureus* may spread to internal tissues or the bloodstream and cause many serious infections, and thus the risk of the spread of these types of bacteria increases when their resistance to antibiotics increases.[5,6] Previous studies have indicated that the spread of *E. coli* and *S. aureus* in the vaginal environment disrupts the normal balance of the vaginal microbiome and contributes to the pathophysiology of vaginitis.[1,5,7,8] According to this, bacterial infections are one of the main causes of vaginitis, so it was necessary to understand the processes by which these bacteria cause vaginal infections to create effective therapeutic approaches. Moreover, there are between 5 and 10 million women around the world suffering from a common gynecological disease known as vaginitis, which is an inflammation of the vaginal tissues, and are seeking health care and treatment.[9]

There are some herbal plants, such as cloves, that possess antimicrobial, antifungal, antibacterial activities, and anti-inflammatory activities because they contain flavonoids, alkaloids, and eugenol, which are effective therapeutic compounds.[10-13] Cloves (Syzygium aromaticum) are grown in Indonesia, India, Sri Lanka, and China, and their most important component is clove essential oil (CEO), which is considered antibacterial and antifungal.[11,12] In addition, CEO contains the main component eugenol, which has proven its effectiveness as an antimicrobial agent such as bacteria.[11] Cloves have been used in medicine as a treatment for many stomach ailments such as inflammation and diarrhea, and used as a sedative and analgesic.[12]

Natural extracts have drawn interest in the search for alternative therapies due to their possible antibacterial qualities. Therefore, the antibacterial activity of clove (Syzygium aromaticum)

methanol extract and crude alkaloid against a range of pathogens has been investigated.[9,14] Furthermore, investigating natural extracts such as clove methanol extract provides a viable path to alternative treatments for bacterial infections as these extracts contain phytochemicals that are highly effective against E. coli.[14] Eugenol, an antibacterial molecule, is one of the several bioactive chemicals found in clove extract.[15] Studies have indicated that methanol extract from cloves can suppress the growth of bacteria, which suggests that it could be used as a treatment for bacterial vaginitis.[12,14,16,17] Particularly, the antibacterial activity of clove methanol extract has been demonstrated to be susceptible to *E. coli* and *S. aureus*. These bacteria, frequently linked to vaginitis, could be the focus of complementary and alternative therapy approaches. Also, effective treatment of this frequent gynecological ailment requires an awareness of the roles played by *E. coli* and *S. aureus* in vaginitis.

Therefore, our study focused on investigating the possibility of using methanol extract and crude clove alkaloids (Syzygium aromaticum), which possess chemical compounds that effectively act against bacteria. Thus, testing its ability to control *E. coli* and *S. aureus*, which causes vaginal infections. As well as evaluating the antibacterial effects of these extracts associated with AV infections. Given the limitations in understanding the pathogens responsible for vaginitis in Hawler City, Iraq, this study seeks to clarify important information about the prevalence and importance of *E. coli* and other causative agents of vaginitis.

MATERIALS AND METHODS

Study design

The current study was conducted and its objectives were verified by collecting samples from women who visit the outpatient clinic at private clinics in Hawler Province in the Kurdistan Region of Iraq during the period from October to December 2023. The study was conducted on a group of women who were randomly selected.

Sample collection

In this study, 50 randomly selected participants were recruited from women. Specimens were collected for examination and to ensure a standardized and meticulous collection process, vaginal swabs and smears were obtained using a vaginal speculum to provide a clear view of the cervix. Swabs were carefully inserted into the posterior fornix and the upper part of the vagina, rotating gently to ensure comprehensive sampling. Great care was taken during withdrawal to minimize the risk of contamination. The collected swabs were then placed in

Amie's transport media for preservation and were promptly transported to the laboratory for further analysis.

Ethical considerations

The protocol for this study was approved by the Ethical Approval Committee of Salahaddin University, Kurdistan Region, Iraq (……) and the Institutional Review Board (IRB) of this university. The purpose of the study and the techniques that will be used were also clearly explained to the participants to obtain their informed consent to participate in the study.

Selection Criteria

Inclusion criteria included non-pregnant and non-menstruating women who visited the outpatient clinic in private clinics who had not recently engaged in sexual intercourse or used vaginal suppositories or antibiotics and had specific health problems and complaints, such as itching, discharge, lower back pain, infertility, vaginal repair, post-caesarian section follow-up, and others, were considered for participation. Exclusion criteria included pregnant and menstruating women who had recently engaged in sexual intercourse or used vaginal suppositories or antibiotics.

Bacterial isolates

Fifty vaginal samples, consisting of 22 *S. aureus* and 16 *E. coli* isolates obtained from patients with vaginitis, were collected aseptically using sterile swabs. After transferring the samples to the laboratory, they are analyzed according to the biochemical methods used by Cappuccino and Sherman.[18] The swabs were streaked onto selective media Mannitol Salt Agar (MSA) for *S. aureus* and MacConkey Agar for *E. coli* to facilitate the isolation of the respective bacterial species. Gram staining was performed to provide an initial differentiation between Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.[19] The swabs were cultured into Blood Agar (BA), MSA, MA, and Eosin Methylene Blue Agar (EMB). Cultured media were incubated at 37°C for 24 hours, further investigations were done using biochemical tests.

Morphological examination

Bacteria from suspected colonies were also stained with Gram stain for primary identification. Isolation and identification of *S. aureus* was done. The sample of *S. aureus* isolate was streaked on MSA and BA and the plates were incubated at 37°C for 24 hours. The appearance of golden yellow colonies on MSA and hemolytic colonies on BA were considered *S. aureus*.[20] *E. coli* was isolated by culturing samples into selective medium EMB agar and MacConkey Agar and

incubated at 37°C for 24 hours. Morphologically typical colonies producing metallic sheen and lactose fermentor had been selected for further identification.[19]

Biochemical examination

Four colonies from the pure culture of suspected bacteria were selected to be cultured and identified by the various biochemical tests. Confirmation of Genus, Staphylococcus was done by Gram staining and several biochemical tests including Catalase, Oxidase, Indole, Methyl red, and Voges- Proskauer tests. In addition, Nitrate reduction, acid from different sugars, and hemolysis on BA were also used for the identification of bacteria species. The suspected species of S. aureus was also confirmed by Mas-Ud.[20]

Plant Material

The Clove plant, sourced in the form of flower buds, was procured from the local Hawler Grand Bazaar specializing in medicinal plants. These flower buds were carefully transported to the University of Salahaddin to be prepared. In the laboratory, the flower buds undergo a careful preparation process. In addition, to enhance their longevity and facilitate subsequent processing, the clove flower buds were subjected to sunlight drying. This natural drying method aims to preserve the integrity of the plant and eliminate excess moisture. After that, the prepared clove plant was transferred to the laboratory for further analysis. Furthermore, the entire preparation process, undertaken in the year 2023, ensures that the clove plant maintains its botanical properties and serves as a reliable and standardized sample for subsequent scientific investigations. The utilization of locally sourced medicinal plants adds a valuable dimension to the study, considering the potential therapeutic properties associated with clove.

Methanolic extract of clove

The 10 g of powder of clove (Syzygium aromaticum) was weighted with the digital scale and transferred into 100 ml conical flasks. 15 ml of methanol was added. The conical flask was closed with foil paper and placed in a dark place for 5 days. Then the crude methanol extract was filtered by Whatman no.1 filter paper. Then methanol extract is concentrated at 80°C by using a water bath and then stored in the refrigerator at 40°C in a small and sterile plastic test tube.[14,21]

Alkaloid Extraction

10 g of powdered plant sample was extracted with 150 ml of methanol solvent in a conical flask for 8 hours. The solvent was removed at a controlled temperature of 40°C and using a low-pressure evaporator. The plant extracts were taken up in 10 ml of sulfuric acid (H_2SO_4) (2%) and passed through 3 × 50 ml of diethyl ether ($(C_2H_5)_2O$). It was separated from the oil

in the separation funnel. The pH of the aqueous acid solution was adjusted to 9 with Ammonia solution (NH_4OH). 3 x 50 ml chloroform was then added. The chloroform extract was dried in sodium sulfate and evaporated in a rotary evaporator under controlled temperature (40°C) and low pressure. The residue was stored at + 4°C for use in experimental studies.

Preparation and dilution of both extractions

To begin the experimental phase, a stock solution of both extracts was prepared. This was done by dissolving each extract, weighing 1 g, in 10 ml of its corresponding solvent, Dimethyl Sulfoxide (DMSO), and supplementing with 90 ml of nutrient broth (NB). Thus, several solutions were obtained with a final concentration of 10 mg/ml. Six solutions were prepared for each extraction process, each with different concentrations as follows: Test tube 1 (6 mg/ml): Mixed 1.2 ml of stock solution with 0.8 ml of NB; Test tube 2 (5 mg/ml): Combined 1 ml of stock solution with 1 ml of NB; Test tube 3 (4 mg/ml): Blended 0.8 ml of stock solution with 1.2 ml of NB; Test tube 4 (3 mg/ml): Incorporated 0.6 ml of stock solution with 1.4 ml of NB; Test tube 5 (2 mg/ml): Merged 0.4 ml of stock solution with 1.6 ml of NB; Test tube 6 (1 mg/ml): Mixed 0.2 ml of stock solution with 1.8 ml of NB.

Minimum Inhibitory Concentration Preparation for Methanolic Extract and Crude Alkaloid

Using broth culture media as a dilution medium, the MIC and MBC were determined. Stock solutions of the crude alkaloid and the methanolic extract were prepared for this procedure. A specific concentration of crude alkaloid and clove extract was mixed with 2 ml of liquid NB culture medium in individual test tubes. Subsequently, 0.1 ml of the microorganism culture suspension was added. Using a non-selective medium like BA, isolated colonies were chosen from an 18-24 hour agar plate for the inoculum preparation. To match a 0.5 McFarland turbidity standard, a straight broth suspension was made. As a result, the suspension of *E. coli* and *S. aureus* contained roughly 1 to 2 x 10^8 colony-forming units (CFU)/ ml After inoculation, each test tube contained roughly 5 x 10^5 CFU/ml of the adjusted inoculum suspension, which had been diluted in broth.[21,22] For a thorough evaluation of the antibacterial efficiency of the clove extracts against *E. coli* and *S. aureus*, the exact calculation of MIC and MBC values requires, also the careful fabrication of test tubes and standardized inoculum concentrations.

Statistical Analysis

In this study, a completely randomized design was used for data analysis. Duncan's Multiple Range Test was employed in the statistical analysis, which was carried out with SPSS software version 26 (SPSS Inc., Chicago, USA). This statistical method makes it possible to compare the means from many experimental groups, offering a solid assessment of the data and making

it easier to spot important variations between the variables under test. Selecting a fully randomized design improves the validity and dependability of the statistical results, guaranteeing a thorough and exact evaluation of the experimental outcomes.

RESULTS AND DISCUSSION

Three independent experiments produced consistent and encouraging results when clove methanol extracts and crude alkaloid extracts were used to test five isolated bacteria for MIC and MBC against *E. coli* and *S. aureus* from fifty vaginal samples as shown in Table 1. In previous studies, *E. coli* has been repeatedly identified as the prevalent pathogen among many pathogenic bacterial species.[3,5,23,24] Table 1 shows that most woman patients (76%) had positive bacterial cultures, with 44% being *S. aureus* and 32% being *E. coli*. Even though *E. coli* is normally a resident intestinal bacterium, certain strains can spread to other parts of the body and cause diseases.

Table 1: The number and percentage of *E. coli* and *S. aureus*

Vaginal infection (bacteria)	Number	Percentage %
S. aureus	22	44%
E. coli	16	32%

While the MBC values for *E. coli* stayed constant at 4 mg/ml, the MIC values for clove methanol extracts against *S. aureus* and *E. coli* were consistently observed to be 3 mg/ml and 4 mg/ml, respectively. The MIC and MBC values for *E. coli and S. aureus* in crude alkaloid extracts were consistently at 2 mg/ml for the two species as shown in Table 2. These results point to a consistent inhibitory effect against the two kinds of bacteria, and even bactericidal activity, especially against *E. coli*. The results of testing clove extracts to determine their minimum inhibitory concentration showed that these extracts possess antimicrobial activity against *E. coli* and *S. aureus*, as shown in Table 2. The antibacterial activities of clove extracts that have been detected are consistent with earlier research that has demonstrated the effectiveness of clove and its bioactive components, like eugenol, against a range of bacterial strains.[25] Strong antibacterial qualities have been demonstrated for eugenol, a main component

of clove, which lends credence to the consistency of the inhibitory effects seen in this investigation.[26] Furthermore, the consistency of MIC values throughout multiple trials highlights the repeatability and dependability of clove extracts' antibacterial activity.[27]

Table 2: Minimum inhibitory concentration (MIC) of clove methanol extract on E. coli and S. aureus concentrations in terms (mg/ml)

Bacteria		MIC	MBC
E. coli	1	3 mg/ml	4 mg/ml
	2	3 mg/ml	4 mg/ml
	3	3 mg/ml	4 mg/ml
	4	3 mg/ml	4 mg/ml
	5	4 mg/ml	5 mg/ml
S. aureus	1	4 mg/ml	5 mg/ml
	2	4 mg/ml	5 mg/ml
	3	4 mg/ml	5 mg/ml
	4	4 mg/ml	5 mg/ml
	5	4 mg/ml	5 mg/ml

The results of testing clove methanol extracts and crude alkaloid extracts to determine their minimum inhibitory concentration showed that these extracts possess antimicrobial activity against E. coli and S. aureus, as shown in Table 3. The study's findings support the possibility of using natural extracts as an alternative to bacterial vaginitis treatments. The demonstration of consistent and powerful inhibitory effects by clove methanol extracts and crude alkaloid extracts laid the groundwork for future research and possible pharmaceutical uses. The results of this study are consistent with what previous studies have confirmed that medicinal plant extracts can affect bacteria such as *E. coli* and *S. aureus* that are resistant to antibiotics, and this appears by increasing the size of the inhibition zone because these extracts have antibacterial properties.[28] These results open the way for new options to solve the problem of bacterial resistance to antibiotics, and can also help in finding a treatment for the bacteria that cause vaginitis. The MBC values shed light on the concentration necessary for bactericidal actions, whereas the MIC values show the concentration at which bacterial growth is suppressed. The stability of MBC values, particularly concerning *E. coli*, indicates that clove extracts may be able to efficiently kill as well as inhibit bacterial cells. To effectively treat bacterial infections, this dual action is essential.[29] Furthermore, the repeated tests show that crude alkaloid extracts and clove methanol extracts are effective antibacterial agents against *E. coli* and *S. aureus* that are isolated from vaginal samples. These results support the continued

efforts to investigate alternative therapies for bacterial vaginitis and highlight clove's potential as a source of natural antibacterial agents.

The specifics of the growth observations are shown in Table 2, where it was found that the ethanolic extract and crude alkaloid both had a MIC of 2 mg/ml. Comparable outcomes were noted for the MBC, where 3 mg/ml for the ethanolic extract and 3 mg/ml for the crude alkaloid were the MBC levels (Table 3).

Table 3: Minimum inhibitory concentration (MIC) of crude alkaloid extracts on *E. coli* and *S. aureus* concentrations in terms (mg/ml)

Bacteria		MIC	MBC
E. coli	1	2 mg/ml	3 mg/ml
	2	2 mg/ml	3 mg/ml
	3	2 mg/ml	3 mg/ml
	4	2 mg/ml	3 mg/ml
	5	3 mg/ml	4 mg/ml
S. aureus	1	2 mg/ml	3 mg/ml
	2	2 mg/ml	3 mg/ml
	3	2 mg/ml	3 mg/ml
	4	2 mg/ml	3 mg/ml
	5	2 mg/ml	3 mg/ml

The results showed that methanol extracts and alkaloids of cloves have clear antimicrobial activity against *E. coli* and *S. aureus*. This was done by testing specific concentrations of these extracts and inoculating them with bacteria to determine their MIC and MBC, as shown in Figures 1, 2, 3, and 4. Natural plant-derived chemicals, such as those in cloves, are thought to be artificially substituted for antibacterial molecules that have less adverse effects. The importance of studying medicinal plants and their effective role in treating many diseases caused by microbes has been generally emphasized.[30] A plant's ability to resist infection can be influenced by some variables, such as the amount of essential oil it contains and the technique used during extraction. From Figure 1 this result investigation with previous research compared different solvent extraction on spices and reported that the 80% ethanolic extraction method showed the highest inhibition towards both Gram-negative and Gram-positive bacteria.[31]

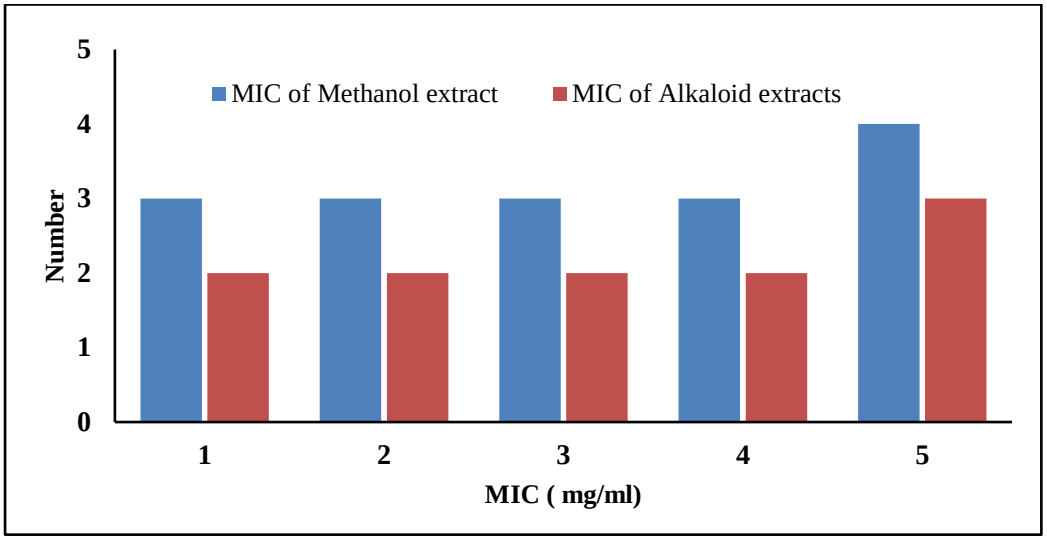

Figure 1: The difference between MIC for *E. coli* using methanol and alkaloid extracts of cloves.

For clove methanol extracts and crude alkaloid extracts against *E. coli* and *S. aureus* from vaginal samples, the repeated MIC and MBC assays have consistently shown strong antibacterial effects. The constancy of MIC values, namely at 2 mg/ml for crude alkaloid and clove methanol extracts against *E. coli* and 1 mg/ml for both extracts against *S. aureus*, highlights the dependability and repeatability of the detected inhibitory activity. The MBC values also suggest that clove extracts may have bactericidal properties, especially against *E. coli* as shown in Figure 2.

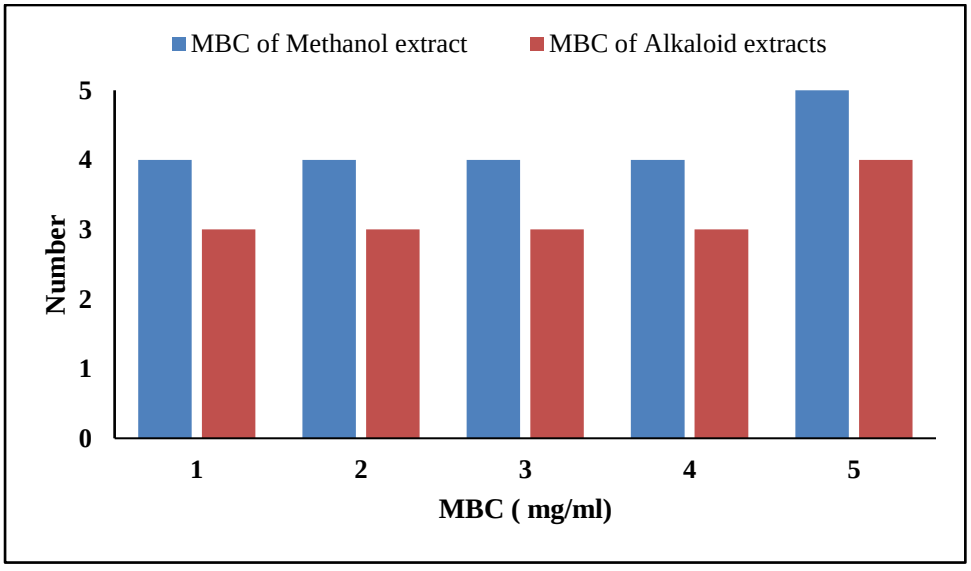

Figure 2: The difference between MBC for *E.coli* using methanol and alkaloid extracts of cloves

Simultaneously, the methanolic extract of cloves demonstrates a significant impact on *S. aureus*. This specific influence is highlighted in Figure 3 and Figure 4, showcasing the pronounced effect of the methanolic extract on this bacteria. The antimicrobial efficacy of a plant extract can be influenced by the extraction method and the choice of solvent. Varied methods and solvents used to extract plant compounds may result in distinct antimicrobial effects against specific microorganism species.[32,33]

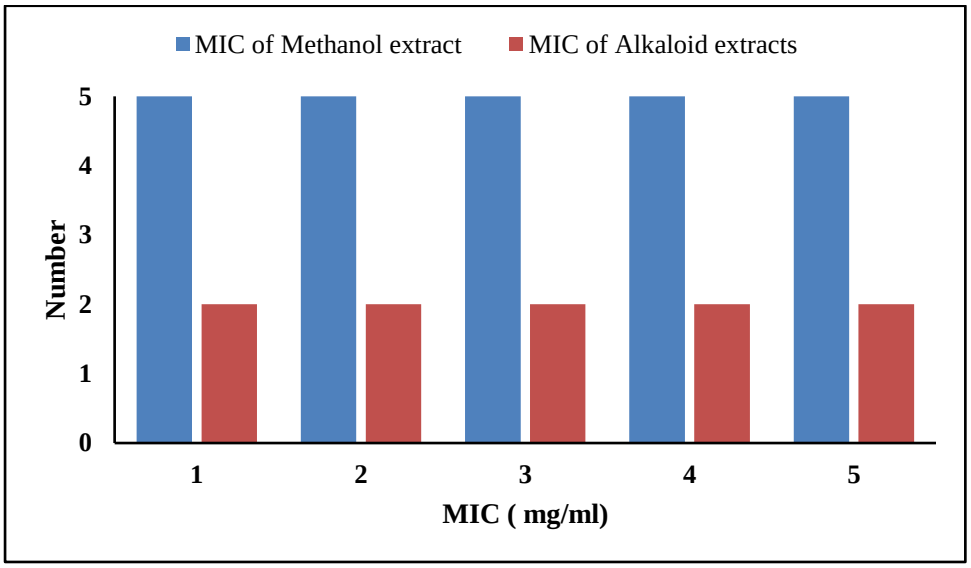

Figure 3: The difference between MIC for *S. aureus* using methanol and alkaloid extracts of cloves

The results of the dilution broth test showed the clove methanol extract and crude alkaloids' antibacterial properties. At the highest concentration tested (3 mg/ml) and at the highest concentration (4 mg/ml), the methanol extract demonstrated significant antibacterial activity against *E. coli* and *S. aureus* as shown in Figure 4. At the highest concentrations, the results showed that the crude alkaloids had antibacterial properties, and no bacterial growth was observed at less than 2 mg/ml.

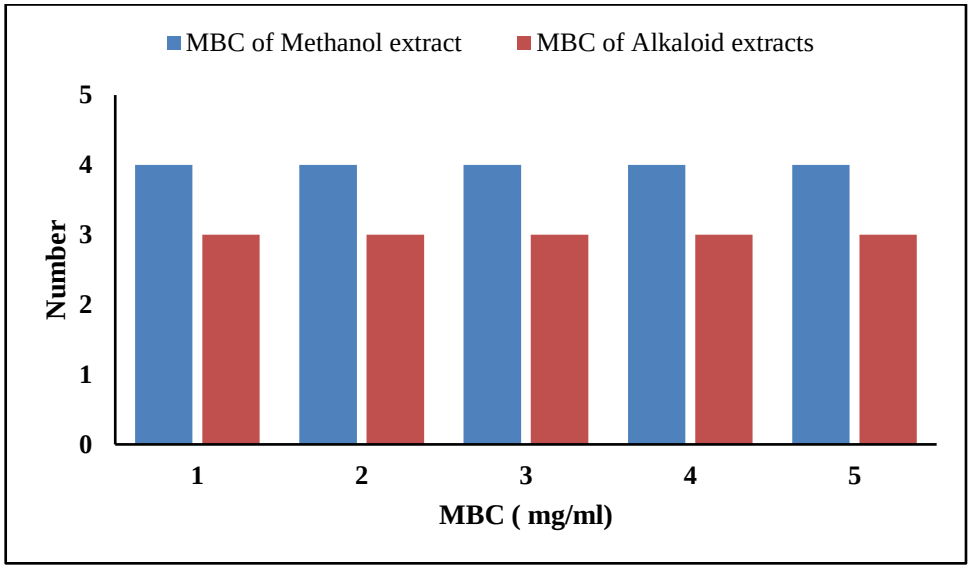

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Figure 4: The difference between MBC for *S. aureus* using methanol and alkaloid extracts of cloves

Finally, the results of this study demonstrated that crude alkaloids and clove extract, known for their potential antimicrobial characteristics, by broth dilution method for MIC and MBC analyses, have antibacterial activity specifically against *S. aureus* and *E. coli*. Because crude alkaloids had a higher concentration of bioactive chemicals, the results showed that they were more effective than methanol extract. Moreover, the results of our study are consistent with previous research that showed the effectiveness of cloves and their main component, eugenol, against a range of bacterial strains. The study thus adds another piece of evidence to the growing body of evidence proving the antibacterial qualities of clove extracts and demonstrates that methanol extract and crude clove alkaloids have an effect on *S. aureus* and *E. coli* isolated from vaginitis. The results also highlight the possibility of using natural extracts, especially those made from cloves, as effective alternatives for treating bacterial vaginosis, a common gynecological disease. The study's conclusions were strengthened by the fact that the mentioned MIC and MBC values for extracts remained stable over multiple experiments, which increases the possibility of further developing these extracts as medicinal substances that are effective in treating many bacterial diseases.

CONCLUSION

The opportunistic pathogens *S. aureus* and *E. coli* were the main focus of this investigation. These organisms are frequently isolated from women who report a variety of problems linked to inflammation of the cervix, which can be induced by infectious or non-infectious causes such as hormones, allergies, or trauma. Thus this research concluded that *S. aureus* and *E. coli*

are cervicitis-associated bacteria. In addition, clove extracts consistently exhibit bactericidal and inhibitory effects against *S. aureus* and *E. coli*, indicating the adaptability of these natural chemicals in targeting several types of bacteria associated with vaginal infections. Furthermore, the study emphasized the significance of dose considerations by highlighting concentration-dependent antibacterial effects. This study sheds light on the potential advantages of medicinal plants like clove and emphasizes how important it is to examine them. It highlights the importance of clove plants in resolving microbial issues and calls for more research into their characteristics. The findings of this study offer a strong basis for future research into the creation of natural treatments for bacterial vaginitis, even though more examination is necessary to examine the underlying mechanisms and their therapeutic applications. In conclusion, the antibacterial activity of crude alkaloid and clove methanol extracts highlights their potential as important tools in the continuous search for substitute therapies for bacterial illnesses.

Limitations of the study

No limitation in the study.

Conflict of interest

There are no conflicts of interest.

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