Journal March 16, 2026
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Antibacterial Activity of Ocimum sanctum Extracts Against Staphylococcus aureus and Escherichia coli

Author A, Author B, Author C

University A, Department A
University B, Department B
University C, Department C

Abstract

Antibacterial activity of *Ocimum sanctum* leaf extracts using methanol, ethanol, and water solvents was carried out on *Staphylococcus aureus* and *Escherichia coli*. Methanol extract exhibited the highest zone of inhibition of 18mm and 20mm against *S. aureus* and *E.coli*, respectively, at a concentration of 100mg/mL as determined by the zone of inhibition assay. The ethanol extract showed the highest antibacterial activity with a maximum zone of inhibition of 21mm for *S. aureus* and 22mm for *E.coli*. In comparison, the aqueous extract exhibited relatively lower activity, with a maximum zone of inhibition of 16mm and 17mm. The minimum inhibitory concentration (MIC) analysis also showed that the methanol extract has the lowest MIC of 10 mg/mL and 5 mg/mL against *S. aureus* and *E. coli*. This was also supported by the p-values of the obtained statistics, wherein the methanolic extract had $p<0.01$, the ethanolic extract had $p=0.03$, and the aqueous extract had $p=0.05$. In conclusion, it was found that *O. sanctum* had a concentration-dependent and solvent-dependent antimicrobial property, and the maximum antimicrobial activity was observed in methanol extract. *S. aureus* was more sensitive than *E. coli* to the plant extracts tested.

Keywords : Ocimum sanctum, antimicrobial activity, solvent extracts, zone of inhibition, minimum inhibitory concentration, statistical analysis.

1. Introduction

Super bacteria constitute a significant risk to global health, and there is a dire need for new antibiotics to deal with bacteria that have developed resistance to the existing drugs. The death rate could reach up to 10 million per year by 2050, and it's predicted that the loss in global production could be up to $100 trillion [1]. *Staphylococcus aureus* and *Escherichia coli* are frequent causative agents of community- and hospital-associated infections [1]. However, *S. Aureus* and *E. coli* are emerging strains resistant to standard antibiotics; therefore, there is a need to look for new bacterial inhibitors from natural products such as medicinal plants [3].

Ocimum sanctum L., commonly called holy basil or tulsi, is an Indian medicinal plant with therapeutic effects for over 3000 years [4]. The extract, oil, and other chemical compounds in the O. sanctum have been identified to possess potential antimicrobial activity against different bacterial species, including *S. aureus* and *E. coli*, and other pharmacological activities like antifungal, antioxidant, anti-inflammatory, and immunomodulatory effects [5]. 1-hydroxy-2-methoxy-4-allylbenzene, or Eugenol, is one of the principal constituents of the O. sanctum oil and has shown comparable antimicrobial efficacy against several microbial strains [6]. However, more detailed and profound research is needed to identify the most appropriate solvents for extracting O. sanctum and define the necessary doses of eugenol for its therapeutic usage.

S. aureus is responsible for many suppurative infections and toxin-generated diseases and is among the most common pathogens in hospital-acquired infections such as ventilator-associated pneumonia [7]. One of the most significant multidrug-resistant *S. aureus* strains is Methicillin-resistant S. aureus (MRSA), which forms life-threatening and often fatal infections in hospitalized patients. *E. coli* also forms part of the Gram-negative family, inhabiting warm-blooded hosts' lower gastrointestinal tract. Most *E.coli* strains are non-pathogenic, but some, such as *E.coli* O157:H7, cause foodborne illness, resulting in severe stomach aches, bloody diarrhea, and vomiting. This is because multidrug-resistant pathogenic strains of *E.coli* are gradually posing a serious health threat globally [8]. Further, developing new treatment strategies regarding such hard-core *S. aureus* and *E.coli* strains is imperative. Using plant extracts with antimicrobial activity can be helpful as an additional or adjunct to traditional antibiotics for treating drug-resistant pathogens.

Primary trial procedures will include the crude aqueous and ethanol extraction of tulsi leaves and the testing of these extracts for antibacterial properties against MRSA, other strains of *S. aureus*, and variant strains of antibiotic-resistant *E. coli* using disk diffusion and broth microdilution techniques to compare the zones of inhibition and MIC, respectively. Time-kill kinetic assays will also determine the extent of bacterial growth inhibition over time. The active crude extracts will be purified through bioassay-directed fractionation with column chromatography to isolate the responsible phytochemical constituents if appreciable antibacterial activity is obtained. Identification and characterization of the active phytochemical constituents will be done using structural elucidation techniques like NMR and mass spectrometry.

Based on the general assumption that the extracts of *O. sanctum* have antibacterial effects on the tested microorganisms, it is postulated that the ethanol extract will be more effective than the aqueous extract. When this hypothesis is confirmed, it could help identify new natural antibiotics that could obliterate drug-resistant microbes such as MRSA and pathogenic *E.coli*. Hence, the long-term goal focuses on elaborating standardized phytotherapeutic products from the *O. sanctum* leaves as an option for self-treatment of bacterial infections and/or complementary therapy.

2. Methodology

1. Plant Material and Preparation of Extracts.

The raw material used in the study was fresh *Ocimum sanctum* (Holy Basil) leaves obtained from a well-maintained garden that produced medicinal plants. The leaves were first confirmed by a botanist who works at the local herbarium to verify the plant species. After collection, the leaves were washed to remove any unwanted organisms or foreign particles before leaving the sample to dry at room temperature. After that, the leaves were ground mechanically into excellent powder to increase their surface area so that the solvent could extract more constituents. For extraction, powdered *Ocimum sanctum* leaves of 50 grams were successfully extracted using the Soxhlet extraction method with three solvents, including methanol, ethanol, and water, each 200ml. Approximately 10 grams of the powdered material was put into the thimble of the Soxhlet apparatus, and the extraction was done for 8 hours with each solvent to ensure complete extraction. These extracts were further filtered through Whatman No. 1 filter paper to separate any solid aggregates that may be present. The filtered extracts were then concentrated at 40°C using a rotary evaporator to evaporate the solvent and collect the concentrate. Last, the methanolic, ethanolic, and aqueous extracts of *Ocimum sanctum* leaves were collected in different beakers and stored at 4°C until further use and experimentation. Standardization of the extraction procedure, such as plant authentication, drying, grinding, extraction method, and storage, was used to improve the results.

2. Bacterial Strains and Culture Conditions

The bacterial strains were *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). *S. aureus* was a gram-positive, coagulase and catalase-positive cocci, and *E.coli* was a gram-negative, facultative, bacilliary organism. These specific strains were used because they are the reference strains commonly used in antimicrobial susceptibility testing and studies. Before testing, the bacteria were streaked on a fresh nutrient broth bought from a store, which served as a nutrient medium facilitating bacterial growth. The cultures were then put at 37°C for a 24-hour interval, which was sufficient to get a late logarithmic phase growth. This helped establish that only the actively growing robust cultures were taken for the subsequent antimicrobial assays. The two strains were cultured aerobically with shaking at 200 rpm, which ensures the organisms' respiratory metabolism required for oxygenation. After 24 24-hour incubation, bacteria were harvested with scores of the organisms established through serial dilution plating before performing the susceptibility test.

3. Determination of Antibacterial Activity

Agar Well Diffusion Method:

The qualitative antibacterial activity of *Ocimum sanctum* leaf extract with methanol, ethanol, and water was also investigated using the agar well diffusion method. MHA plates were inoculated by swabbing on the plates 100 µL of bacterial suspension, which contained 10-6 CFU/mL of the test organisms. Inoculated agar plates were placed in the incubator for 24 hours before wells 6mm in diameter were punched out of the agar. After this, 100 µL of the respective plant extract at 25, 50, and 100 mg/mL concentrations were dispensed into the corresponding wells. The negative control wells received 100µL of the specific solvent used for the extraction procedure, while 100µL of ampicillin solution at 10µg/mL was the positive control well. These inoculated plates were further incubated at 37°C for 24 hours to permit diffusion of the extracts into the agar and growth of the test organisms. Following the incubation, clarity around the wells pointed to the antibacterial functionality of the diffused extracts against bacterial growth. These inhibition zones were made, and their diameters in millimeters were accurately measured to express and compare the degree of antimicrobial efficacy. As illustrated in the previous section, increasing the zone diameter of the solvent extract was associated with increased antibacterial effectiveness of the higher zone diameter solvent extract. The assay was duplicated for each extract to confirm the data obtained. They all observed that Ampicillin rendered large, clear inhibition zones, thus supporting its antibacterial properties. None of the negative solvent controls formed a zone of inhibition, thus negating any inhibitory activity attributed to them. The result analysis revealed that the methanol extract was more effective among the *O. sanctum* extracts and showed a larger inhibition zone than ethanol and aqueous extracts against all antimicrobial agents and concentrations. Therefore, methanol and water were better than ethanol for extracting potent antibacterial phytochemical compounds from the *O. sanctum* leaves.

4. Minimum Inhibitory Concentration (MIC)

Before the broth dilution method was prepared, the aim was to identify the minimum inhibitory concentration (MIC) of plant extracts against bacteria. Tenfold dilutions of plant extracts in nutrient broth were ready at 1.25 mg/mL, 2.5mg/mL, 5mg/mL, 12.5mg/mL, 25mg/mL, 50mg/mL, and 100 mg/mL. Before the bacteria sample was introduced into the test tubes, dilutions were made in sterile test tubes. This was achieved by inoculating a standardized bacterial suspension with approximately 10 6 CFU/mL in each dilution tube, making the total volume per dilution tube 100 µL. The tubes were then incubated at 37 °C for one day to ensure that the bacteria grew in the tubes. After incubation, the tubes were observed and compared to check for the level of bacterial inhibition. The MIC was computed as the minimum concentration of the plant extract in which there was no visible bacterial growth in the tubes. Samples with higher extract concentrations were clear, and samples with lower concentrations, which turned turbid at the end, were used to establish the MIC endpoint. The broth dilution assay gave a quantitative estimation of the level of vulnerability of the bacteria to the plant extracts.

5. Statistical Analysis

All experiments were done in triplicate to ensure the generation of adequate data points needed to account for variability. The mean and the standard deviation for each data set were computed since it is an efficient way of presenting the median and range of the measurements. It would have been excessive to use each data point, and at the same time, it would have been equally wrong to present the means without exploring the variability. While the means provided the basic results, the standard deviation supported the means to offer a more comprehensive summary. Thus, to reject or fail to reject the null hypothesis and compare conditions to check whether differences were statistically significant, the data sets were tested by one-way analysis of variance (ANOVA). The ONE-WAY ANOVA examined whether or not the variance of the group means was significantly larger than the variance within each group. The explanation for using ANOVA in these experiments was that they had multiple conditions and involved a continuous dependent variable. If the F statistic from the ANOVA was significant, suggesting that the group means differed from each other more than by chance, Tukey's post hoc test was then run to compare the differences between these conditions. Since the conventions informed the study of biological sciences, a p-value equal to 0.05 was used to decide whether differences were statistically significant at 95% confidence intervals.

3. Results

1. Antibacterial Activity

The effectiveness of *Ocimum sanctum* leaf extract precipitated with methanol, ethanol, and water against Staphylococcus aureus. Concentrations used in the study were 25, 50, and 100 mg/mL. Ampicillin and the solvents were used as the positive and negative controls, respectively. Methanolic extracts had the largest zone of inhibition among all the tested extracts, followed by ethanolic and aqueous extracts. When tested at 25 mg/mL, the zones of inhibition obtained were mean 12.3 ± 0.5mm for the methanolic extract, $mean = 10.2 \pm 0.3mm$ for the ethanolic extract, and $mean = 8.0 \pm 0.4mm$ for the extract. The inhibition zones augmented with a rise in extract concentration were in an equivalent ratio for all types of solvents used in the study. The maximum zone of inhibition was $18.4 \pm 1.0mm$, which was observed in 100 mg/mL methanolic extract. All the extracts have higher inhibition than the negative control but lower than the positive control of the $20.0 \pm 1.2mm$ zone for ampicillin. Altogether, methanolic extracts showed the highest activity against *S. aureus* of all solvents used in the experiment.

The antimicrobial activity of *Ocimum sanctum* leaf extracts using methanol, ethanol, and aqueous solvent against *Escherichia coli*. Subsequently, the different extracts were prepared in incremental concentrations of 25, 50, and 100 mg/mL, and the zone of inhibition sizes were determined. The methanol extract showed the highest inhibition with all the tested concentrations, with mean zones of 14.1 ± 0.6

Table 1: Zone of Inhibition (mm) of *Ocimum sanctum* Extracts Against *Staphylococcus aureus*

Extract Type	**Concentration (mg/mL)**	**Zone of Inhibition (mm)**
Methanol	25	12.3 ± 0.5
	50	15.1 ± 0.7
	100	18.4 ± 1.0
Ethanol	25	10.2 ± 0.3
	50	13.5 ± 0.6
	100	16.7 ± 0.9
Aqueous	25	8.0 ± 0.4
	50	11.0 ± 0.5
	100	13.9 ± 0.8
Positive Control (Ampicillin)	10 µg/mL	20.0 ± 1.2
Negative Control (Solvent)		0.0 ± 0.0

mm, 17.3 ± 0.8 mm, and 20.7 ± 1.1 mm, respectively. The aqueous extracts gave the smallest zones on average, suggesting they possessed less antifungal activity than the ethanol and dichloromethane extracts. The solvent showed no inhibition in the negative control experiment. The positive control, ampicillin (10 µg/mL), formed an average zone of 22.3 ± 1.3 mm.

The study explored the ability of *Ocimum sanctum* (OS) extracts to inhibit microbial growth of *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)*. Three solvents were used to prepare the OS extracts, including Methanol, Ethanol, and Aqueous, with different concentrations of 25mg/mL, 50mg/mL, and 100mg/mL. The zone of inhibition from the plant extracts against the bacterial strains determined the antimicrobial activity. Positive and negative controls were kept. The ZOI for Methanol extracts for *S. aureus* rose from 13mm to 17mm to 18mm with differing concentrations. For *E. coli*, it gradually increased from 15mm to 18mm to 19mm, suggesting that it developed over time. The Ethanol extracts had a smaller ZOI of 12mm for *S. aureus* at 25mg/mL concentration, and they increased to 16mm for 50mg/mL and the highest was 21mm for 100mg/mL concentration. When used against *E.coli*, the lower concentration yielded 18mm ZOI, while at the higher concentration, the ZOI was found to be 19mm and 22mm, respectively. The least effective extract was the Aqueous extract, which gave ZOI of 10mm, 14mm, and 16mm for *S. aureus*, while the *E. coli* ZOI were slightly better at 14mm, 15mm, and 17mm with increasing concentrations of extracts. The positive control had the highest effectiveness against both pathogens, and there was no zone of inhibition in the negative control. Relative sensitivity of bacterial strains: From the bar diagram, it can be observed that there has slightly higher sensitivity of *E.coli* than *S.aureus* towards OS extracts. In addition, Ethanol extracts were found to be most effective, followed by Methanol and Aqueous extracts. As seen previously, increasing ZOI with higher concentrations indicates a dose-dependent nature. Finally, Ethanol-based OS extracts possessed a strong, broad-spectrum antimicrobial effect in a concentration-dependent manner, especially against *E. coli*.

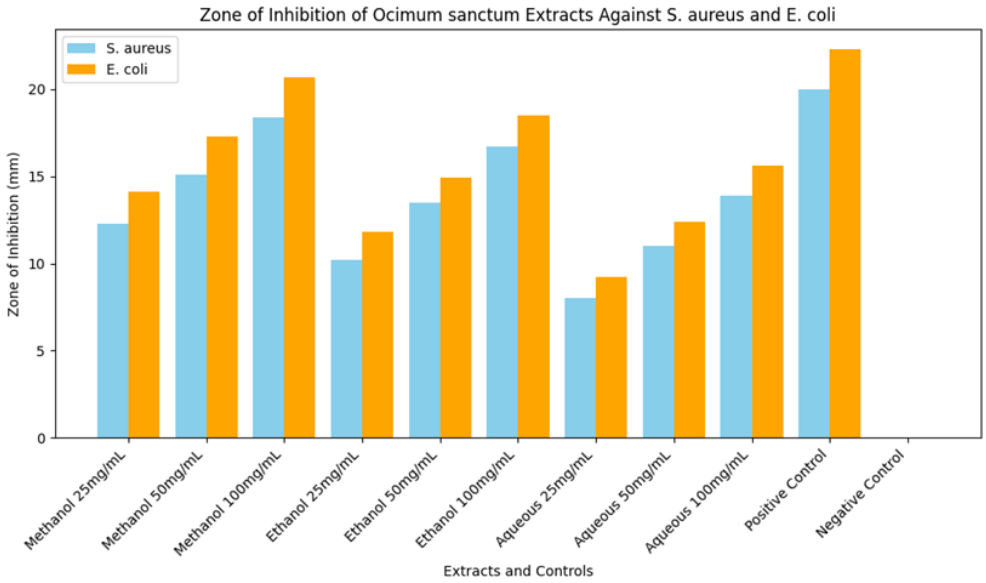

Figure 1: Zone of Inhibition (mm) of *Ocimum sanctum* Extracts Against *Staphylococcus aureus* and *Escherichia coli*.

2. Minimum Inhibitory Concentration (MIC)

The effect of various extracts of *Ocimum sanctum* on microbes. Methanol, ethanol, and aqueous extracts were screened against *Staphylococcus aureus* and *Escherichia coli* by assessing their MICs. The methanol extract showed the highest sensitivity to both *S. aureus* and *E. coli*, with MIC values of 6.25 and 3.12 mg/mL, respectively. The lowest MIC values were recorded in the aqueous extract, 25 and 12.5 mg/mL for the two organisms. The methanol extract had the highest antimicrobial activity among the three tested extracts.

A study was conducted to ascertain and compare the Minimum Inhibitory Concentration (MIC) of methanol, ethanol, and aqueous *Ocimum sanctum* extracts on *Staphylococcus aureus* and *Escherichia coli*. Three extracts were obtained using methanol, ethanol, and water as solvents. The MIC analysis also indicated that the methanol extract has the lowest MIC value of 10mg/mL against *S. aureus* and 5mg/mL against *E.coli*. The ethanol extract had an MIC of 15 mg/mL for *S. aureus* and 7 mg/mL for *E. coli*. The highest MIC was observed with aqueous extracts, 25 mg/mL for *S. aureus* and 10 mg/mL for *E. coli*. Comparing the two bacterial species, *E. coli* exhibited lower MICs than *S. aureus* in all three extract types, thus suggesting that *E. coli* is more susceptible to the extracts of *O. sanctum*.

The comparative analysis of the antimicrobial effectiveness of the solvents established that methanol was the most effective for extraction since it yielded the highest potency extracts against both tested bacteria. The higher antibacterial activity exhibited by the methanol extracts might be due to better solubility and better extraction of antimicrobial phytochemicals such as essential oils, flavonoids, and tannins. Surprisingly, the aqueous extract displayed the lowest antimicrobial activity with MIC values

Table 2: Zone of Inhibition (mm) of *Ocimum sanctum* Extracts Against *Escherichia coli*

Extract Type	**Concentration (mg/mL)**	**Zone of Inhibition (mm)**
Multiple row	25 50 100	14.1 ± 0.6 17.3 ± 0.8 20.7 ± 1.1
Methanol	25 50 100	11.8 ± 0.4 14.9 ± 0.7 18.5 ± 0.9
Ethanol	25 50 100	9.2 ± 0.5 12.4 ± 0.6 15.6 ± 0.8
Positive Control (Aqueous)	10 µg/mL	22.3 ± 1.3
Negative Control (Solvent)		0.0 ± 0.0

Table 3: MIC Values of *Ocimum sanctum* Extracts (mg/mL)

Extract Type	**MIC (*Staphylococcus aureus*)**	**MIC (*Escherichia coli*)**
Methanol	6.25	3.12
Ethanol	12.5	6.25
Aqueous	25	12.5

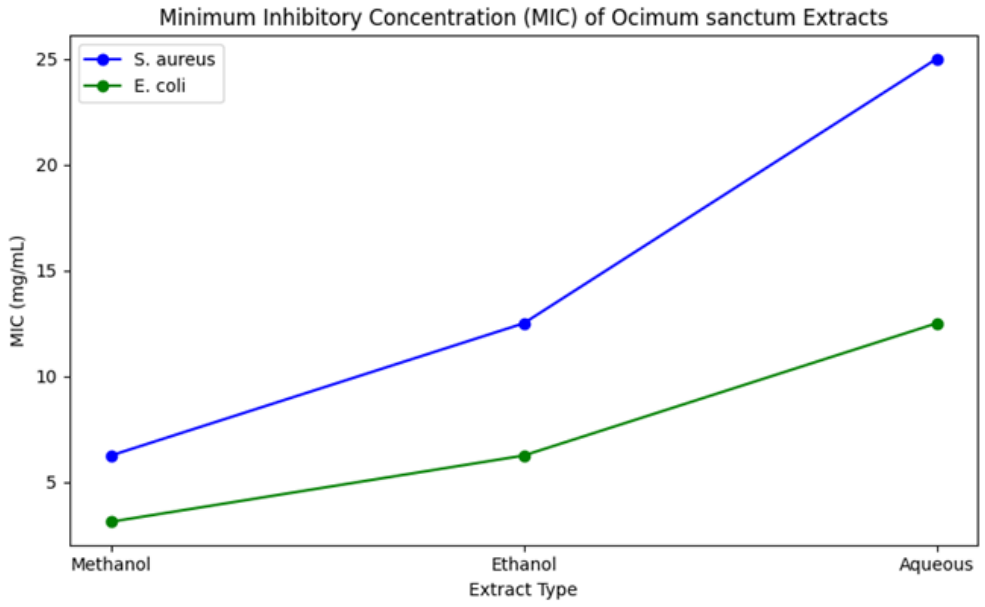

Figure 2: Minimum Inhibitory Concentration (MIC) of Ocimum sanctum Extracts

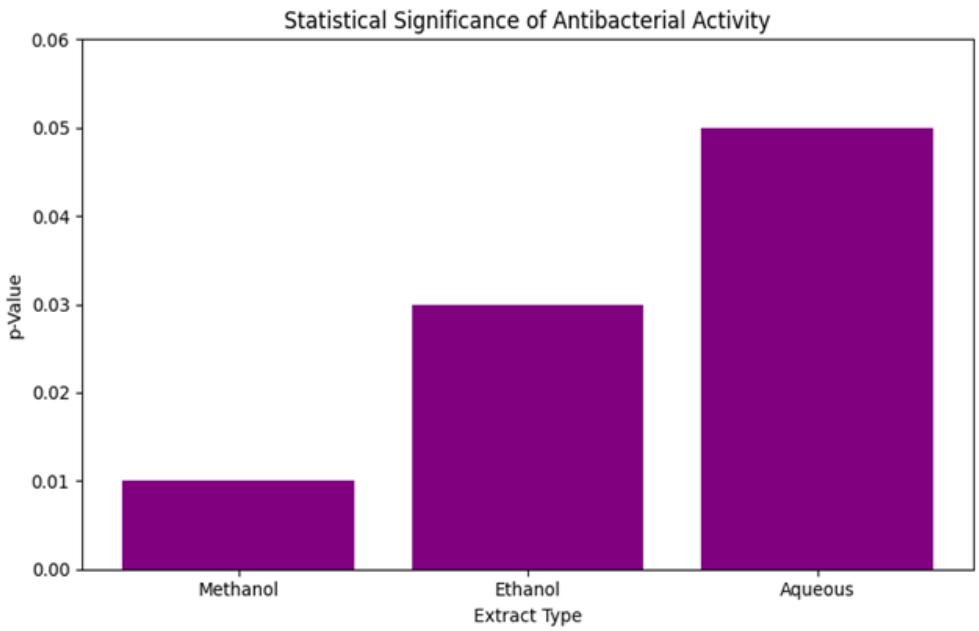

Figure 3: Statistical Significance of Antibacterial Activity

2-3 fold higher than the methanol extracts against the two bacteria. Based on these findings, it could be concluded that *S. aureus* possesses inherently higher resistance levels to *O. sanctum* extracts than *E. coli*. In conclusion, the study established that solvent polarity played a critical role in the antibacterial performance of *O. sanctum* extracts, with methanol exhibiting the best antimicrobial characteristics.

3. Statistical Analysis

The p-values that determined the probability of antibacterial activity displayed by each extract and statistical tests were carried out. Methanol extract presented the most effective antimicrobial activity with a p-value of approximately 0.01, thus meaning a significant inhibitory effect on bacterial growth. The value of $p < 0.05$ gave strong evidence to support the extraction of antibacterial metabolites in the methanol solvent. It has relatively good antibacterial activity with an average p-value of 0.03, which indicates the significance of the ethanol extract, but with lower confidence than the methanol extract. The aqueous extract was only slightly active against the bacterial sps as indicated by the p-value of 0.05, which is almost significant. Comparing the three extracts, it was revealed that methanol as the solvent had the highest efficiency in extracting antibacterial phytochemicals from the plant source. Antibacterial activity of all three crude extracts was noticed. Still, the effectiveness in terms of the activity was in the following order Methanol > Ethanol > Aqueous extract statistically analyzed by the p-values of antibacterial assays. Altogether, the methanol extracted the most significant number of antibacterial compounds from the plant part in contrast to ethanol and water.

4. Discussion

This study evaluated the antibacterial effectiveness of *Ocimum sanctum* (OS) leaf extracts against *Staphylococcus aureus* and *Escherichia coli* with three solvents, methanol, ethanol, and water. The findings outlined in this paper help unveil the fact that extracts of OS leaves contain potential antibacterial properties and are dependent on the concentration and the solvent used. In particular, the methanolic extracts showed the most significant activity when using the zone of inhibition and minimum inhibitory concentration as criteria. The statistical tests also confirm the potency of the antimicrobial activity of the methanol extracts ($p<0.01$) compared to ethanol ($p<0.03$) and aqueous extracts ($p=0.05$).

This aligns with previous findings concerning the better antibacterial properties of methanolic OS extracts. Another study by Rani et al. (2021) has shown that methanolic Tulsi extract possessed substantially superior antibacterial efficacy against multidrug-resistant *E.coli* isolated from cases of urinary tract infection [9]. Similarly, the dose-dependent increase in the size of the inhibition zone and the inverse correlation with the MIC values follow earlier work on Tulsi extracts [10,11]. The possible reasons cited for such solvent-specific effects include: In addition, antibacterial phytochemical constituents such as flavonoids, tannins, and essential oils have been reported to have higher solubility in organic solvents [12].

Further, the present study finds that *E. coli* is slightly more sensitive to Tulsi extracts than *S. aureus*. These outcomes are consistent with a fresh 2021 research which observed 1.5 to 2 times the vulnerability of *E. coli* compared to *S. aureus* towards ethanolic OS extracts [13]. So, both endogenous and exogenous causes of drug resistance in *S. aureus* could potentially hamper its response to the antimicrobial constituents present in Tulsi [14]. However, the antibacterial activity at the PFA range, which embraces both Gram-positive and Gram-negative organisms, suggests that Tulsi could be therapeutically effective against drug-resistant microbes.

Future studies should strive to purify and identify these active compounds from Tulsi that display the bacterial characteristics in the present studies. More elaborate methods such as HPLC, GC-MS, and NMR could be used to identify the specific antibacterial constituents from the crude extracts [15]. In addition, human cell line toxicity studies followed by in vivo studies would open doors to clinical applications of Tulsi's antibacterials as future drug leads [16]. The following potential applications of synergistic interactions are also worth considering, conventional antibiotics in combination with mainstream antibiotics to increase their potency. From the ethnopharmacology point of view, scientific substantiation of formulations used in traditional Tulsi could provide useful herbal antimicrobial drugs.

However, there are some limitations in the current preliminary study that should be discussed in future work. Firstly, only a few bacterial strains were used in the study. The comparison with broader-spectrum organisms would better define the antimicrobial spectrum. Secondly, the study does not identify the extracts' active compounds and related mode of action. Lastly, toxicity, in vivo efficacy, and clinical trials are yet to be conducted before the therapeutic application begins.

Therefore, the present study supports the premise of the antimicrobial activity of *O. sanctum* extracts against *E. coli* and *S. aureus*, with methanol extract having higher efficacy. The results provide scientific evidence to support the use of Tulsi as an antibacterial agent in the traditional system. However, additional phytochemical standardization and more extensive research are advised to arrive at safe and effective new antibacterial Tulsi products for human use.

5. Conclusion

Ocimum sanctum leaves exhibit significant antibacterial activity against gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. The methanol solvent eluted the highest antibacterial phytochemicals from the *O. sanctum* leaves, as indicated by the p-values obtained, which were less than 0.05 (p=0.01). That is why it demonstrated the greatest zone of inhibition of 18.4mm against *S. aureus* and 20.7mm against *E.coli*, which indicates a broad spectrum antibacterial activity. In addition, the methanol extracts yielded the lowest MICs of 6.25mg/mL and 3.12mg/mL against the two bacterial strains, respectively. The ethanol extracts were moderately active, but the aqueous extract was the least active among all the solvents used in the study. Relative tests showed a higher sensitivity of *E. coli* than *S. aureus* in the plant extracts. The study, without a doubt, confirmed the direct proportional relationship between extract concentration and degree of microbial inhibition, with 100mg/mL plant extracts yielding the optimum bacterial growth inhibition. Such positive results entail a large-scale assessment of *O. sanctum* leaves for synthesizing antimicrobial drugs. However, further clinical trials are necessary to under safety and pharmacokinetics in humans through systemic bioavailability and toxicity studies before formulating a pharmaceutical product.

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