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Original Article

In Vitro Antibacterial Activity of Ethanol Extracts of Neem, Papaya Leaves, and Garlic Compared to the Antimicrobial Agent Enrofloxacin against *Staphylococcus aureus*

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ABSTRACT

Mastitis is a significant health issue in buffaloes, commonly caused by *Staphylococcus aureus*, and is increasingly complicated by antimicrobial resistance. There is a growing interest in alternative therapies, particularly plant-based antimicrobials. **Objective:** To evaluate and compare the in vitro antibacterial activity of ethanol extracts of neem *(Azadirachta indica)*, papaya leaves *(Carica papaya)*, and garlic *(Allium sativum)* with enrofloxacin against S. aureus isolated from mastitis-infected buffalo milk. **Methods:** Fifty mastitis milk samples were collected and tested for the presence of *S. aureus* using cultural, staining, and biochemical techniques. Ethanol extracts were prepared from neem, papaya leaves, and garlic. The Minimum Inhibitory Concentration (MIC) of each extract and enrofloxacin was determined using serial dilution techniques. **Results:** *Staphylococcus aureus* was isolated from 24% of the samples. MIC values for neem, garlic, papaya, and enrofloxacin were 2.5 µg/µl, 0.312 µg/µl, 0.156 µg/µl, and 5 µg/µl, respectively. Papaya leaf extract showed the highest efficacy with the lowest MIC. All herbal extracts exhibited significant antibacterial activity, with statistically significant differences ($P<0.05$) among treatments. **Conclusions:** Ethanolic extracts of neem, garlic, and papaya demonstrated antibacterial activity against *S. aureus*, with papaya showing the highest potency. These findings suggest that herbal extracts could serve as effective alternatives or adjuncts to conventional antibiotics in managing buffalo mastitis.

INTRODUCTION

Bovine mastitis, an inflammatory response caused by physical damage or microbial infections in the udder tissue of the mammary gland, is a common disease that leads to substantial economic losses in the dairy industry due to decreased milk production and quality [1]. On average, mastitis costs are estimated at $147 per cow annually. Internal infections in the mammary glands caused by bacteria like streptococci, staphylococci, and coliforms are primary culprits. Bacterial invasion of the teat canal leads to toxin release, damaging milk-producing tissues

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and causing irritation. *Staphylococcus aureus (S. aureus)* is responsible for about 88% of these infections, significantly impacting dairy output. This issue is particularly severe in lactating buffalos, leading to substantial financial losses [2]. The most common mastitis-causing pathogens include *Streptococcus agalactiae, Staphylococcus aureus, Mycoplasma* spp., and others, with environmental pathogens such as *E. coli* and coagulase-negative staphylococci also playing a role [3].*Staphylococcus aureus,* a Gram-positive bacterium, is a significant zoonotic pathogen causing intramammary infections in dairy cattle [1]. Its increasing resistance to antimicrobial medications complicates treatment [4]. *S. aureus* resides on mucous membranes, skin, and in the noses of humans and animals [5]. The *Staphylococcus* genus includes both coagulase-positive and coagulase-negative species, with *S. aureus* being a notable coagulase-positive pathogen. Antibiotic resistance has escalated, necessitating alternatives to conventional treatments [6]. Contributing factors to this global issue include poor waste management, selection pressure, inadequate sanitation, overpopulation, extensive antibiotic use in agriculture, and wildlife dispersion [7]. The World Health Organization reports that over 80% of the global population relies on conventional medicine for various illnesses. However, multidrug-resistant bacteria's increasing resistance has intensified the search for effective alternatives [8]. Herbal plants play a crucial role in safeguarding health by combating harmful substances and oxidative stress [9]. *Azadirachta indica* (neem), a member of the Meliaceae family, is renowned for its therapeutic properties [10]. This tropical and subtropical tree has been used extensively in traditional medicine for various ailments. Neem contains numerous biologically active compounds with antioxidant, antifungal, and antibacterial properties [11]. Garlic *(Allium sativum* L.), from the Amaryllidaceae family, has well-documented health benefits and antimicrobial properties [12]. Its components, like allicin, exhibit broad-spectrum antibacterial activity [13]. *Carica papaya* L., known as papaya, has various therapeutic benefits and is used traditionally to treat multiple diseases. Its leaves contain bioactive compounds with antibacterial, antiviral, hypoglycemic, and anticancer properties [14]. Previous studies have demonstrated the antibacterial effects of ethanol extracts from papaya, neem, and garlic against various infections. Enrofloxacin, a second-generation fluoroquinolone, is widely used in veterinary medicine for its potent antibacterial activity against both Gram-positive and Gram-negative bacteria. It inhibits bacterial gyrase and topoisomerase IV enzymes. However, concerns over antibiotic resistance, particularly in intensive poultry production, have been raised [15].

This study aimed to compare the antibacterial effects of ethanol extracts from papaya, neem, and garlic with enrofloxacin against *Staphylococcus aureus* isolated from buffalo mastitis milk.

METHODS

This in vitro experimental comparative study was conducted to evaluate the antibacterial efficacy of ethanol extracts of neem, papaya leaves, and garlic against Staphylococcus aureus isolated from mastitis milk of buffaloes.

A total of 50 milk samples were selected based on availability and accessibility of clinically mastitic cases during the sampling period. Formal power analysis was not conducted due to practical constraints, but the sample size is comparable to previous similar studies. Samples were collected using a convenience sampling technique from buffaloes exhibiting clinical mastitis at various dairy farms in and around Tandojam. All samples were transported under cold chain conditions to the Central Veterinary Diagnostic Laboratory, Tandojam, for microbiological analysis.

Sterilization of Laboratory Equipment

Glassware, including Petri dishes, test tubes, pipettes, and conical flasks, was sterilized using standard procedures. Initially, all items were immersed overnight in a 1% hydrochloric acid (HCl) solution to eliminate grease and alkali residues. Subsequently, they were washed with a low-concentration solution of liquid detergent and disinfectant, followed by overnight rinsing in tap water and then four successive rinses in distilled water. The glassware was air-dried, wrapped in brown paper, and sealed with cotton plugs and aluminum foil. Sterilization was completed using a hot air oven at 165 °C for 2 h, followed by a 24 h cooling period. Plastic ware such as micropipette tips and Eppendorf tubes were sterilized in an autoclave at 121 °C under 15 psi for 20 min.

Culture Media Preparation

Blood agar was prepared by dissolving 4.8 g of dehydrated media in 100 mL of distilled water, followed by autoclaving at 121 °C for 15 min. After cooling, 5 mL of defibrinated sheep blood was added aseptically, and the mixture was dispensed into sterile Petri dishes. Plates were incubated at 37 °C for 24 h to confirm sterility. Nutrient agar was prepared using the Difco™ formula under similar autoclaving and plating procedures. Mueller-Hinton broth was prepared by dissolving 2.1 g of media in 100 mL of distilled water, autoclaved, and cooled for bacterial culture work.

Bacterial Isolation and Identification

Samples were initially cultured on nutrient agar and incubated at 37 °C for 24 h. Isolates were sub-cultured repeatedly on nutrient and blood agar plates to obtain pure colonies. Identification was performed based on colony morphology, Gram staining, and biochemical

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characteristics. Suspected Staphylococcus aureus isolates exhibited golden-yellow colonies, were Gram-positive cocci, and were confirmed using standard biochemical tests.

Gram Staining Procedure

A single bacterial colony was emulsified in a drop of distilled water on a glass slide and heat-fixed. The slide was sequentially stained with crystal violet (30 s), Lugol's iodine (30 s), and decolorized with 95% ethanol. A counterstain with carbol fuchsine was applied.Slides were rinsed between steps and air-dried before examination under an oil immersion objective.

Plant Material Collection and Extract Preparation

Leaves of *Carica papaya* (papaya) and *Azadirachta indica* (neem) were collected locally from Tandojam, while *Allium sativum* (garlic) bulbs were obtained from the local market. All materials were washed, shade-dried for approximately two weeks, and ground into a fine powder. For ethanol extraction, 20 g of powdered sample (dry weight basis) was soaked in 100 mL of 95% ethanol for 24 h at room temperature with intermittent shaking. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was evaporated to dryness under reduced pressure using a rotary evaporator. The dried extract residue was weighed to determine extraction yield and then reconstituted in distilled water to prepare a stock solution. Serial dilutions were prepared from this stock to achieve working concentrations ranging from 0.019 to 20 µg/µL. All concentrations reported represent micrograms of dry extract per microliter of solution. Enrofloxacin was used as a positive control and prepared by dissolving 20 mg in 20 mL of distilled water.

Antibacterial Susceptibility Testing

The antibacterial activity of the plant extracts and enrofloxacin was assessed using the microbroth dilution method in 96-well microtiter plates with Mueller-Hinton broth. Each well was inoculated with standardized bacterial suspensions and incubated at 37 °C for 24 h. Minimum inhibitory concentrations (MICs) were determined by visual inspection of turbidity, where the lowest concentration of extract or antibiotic that inhibited visible bacterial growth was recorded. All MIC values are expressed as µg of dry extract or drug per µL of solution. The bacterial inoculum was standardized to 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/mL) before use. Before performing ANOVA, the data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test to validate the assumptions of parametric analysis. MIC values were statistically analyzed using a one-way analysis of variance (ANOVA) to determine significant differences between treatments. The analysis was performed using Statistix software version 8.1(Analytical Software, Tallahassee, FL, USA), with the significance level set at $p < 0.05$.

RESULTS

This study aimed to evaluate the antibacterial efficacy of ethanol extracts derived from neem, papaya leaves, and garlic, in comparison with the antimicrobial agent enrofloxacin, against Staphylococcus aureus isolated from the mastitis milk of buffaloes. A total of 50 mastitis milk samples were collected and subjected to pathogen testing (Table 2).

Table 2: Number and Percentage Prevalence of *Staphylococcus aureus* Isolated from Mastitis Milk Of Buffalo

Bacterial Species	Total sample	Frequency (%)
Staphylococcus aureus spp.	50	12 (24)
Mixed Population	50	38 (76)

Among these, 12 samples (24%) were positive for S. aureus, while the remaining 38 samples (76%) were negative (Figure 1). Pathogens were identified based on their morphology, cultural characteristics, and staining reactions, with further confirmation obtained through biochemical tests.

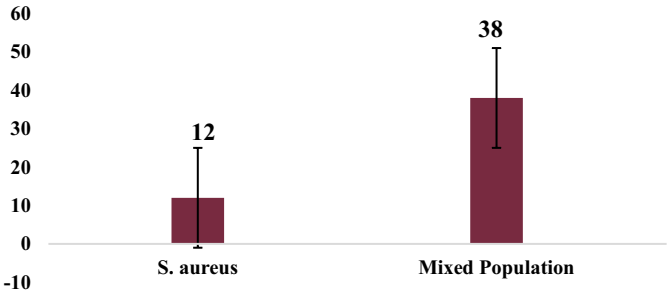

Figure 1: Number and Percentage Prevalence of *Staphylococcus aureus* Isolated from Mastitis milk of buffalo

Minimum inhibitory concentration of ethanol extract of papaya against *S. aureus*,

The susceptibility of Staphylococcus aureus isolated from buffalo mastitis milk was evaluated using a range of ethanol extract concentrations derived from papaya leaves. A concentration of 0.156 µg/µl was identified as the Minimum Inhibitory Concentration (MIC), as it effectively inhibited visible bacterial growth. Below this concentration, bacterial growth was observed, indicating reduced efficacy. At this MIC level (C8), 55.33% of the isolates showed susceptibility to the extract (Figure 2).

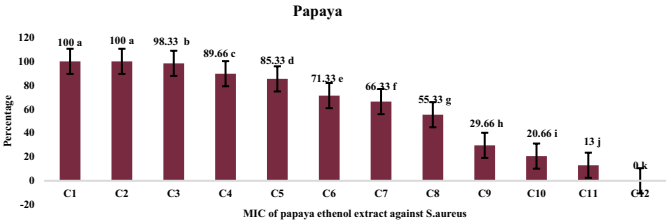

Figure 2: Shows MIC of isolated *Staphylococcus aureus* against different concentration of ethanol extract of papaya leaves

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Minimum inhibitory concentration of ethanolic extract of garlic against *S. aureus,* To ascertain the susceptibility of *Staphylococcus aureus* isolated from buffalo mastitis milk, a gradient of ethanol garlic extract concentrations (C1 = 20, C2 = 10, C3 = 5, C4 = 2.5, C5 = 1.25, C6 = 0.625, C7 = 0.312, C8 = 0.156, C9 = 0.078, C10 = 0.039, and C11 = 0.019 µg/µl) was employed. At lower concentrations, bacterial growth persisted, indicating resistance. However, at 0.312 µg/µl, visible growth inhibition was observed, and this concentration was identified as the minimum inhibitory concentration (MIC). At this MIC, 52.66% of the isolates exhibited susceptibility to the ethanol garlic extract (Figure 3).

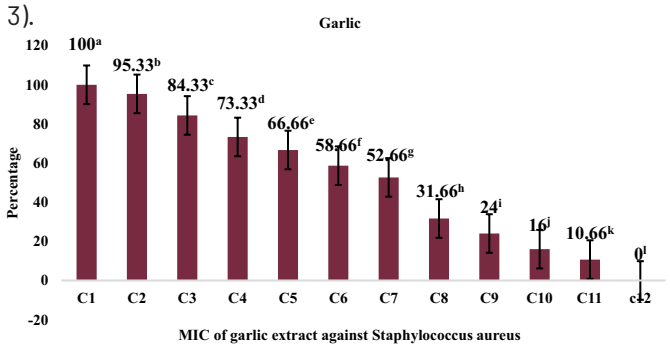

Figure 3: MIC of isolated *S. aureus* against different concentration of ethanol extract of garlic.

Minimum inhibitory concentration of ethanolic extract of neem against *S. aureus*, various concentrations of ethanol neem extract, ranging from C1 (20 µg/µl) to C11 (0.019 µg/µl), were employed to evaluate the susceptibility of *Staphylococcus aureus* isolated from buffalo mastitis milk. The extract inhibited visible bacterial growth at a concentration of 2.5 µg/µl, which was identified as the Minimum Inhibitory Concentration (MIC). Below this concentration, bacterial growth was observed, indicating resistance. At the MIC level, 51.33% of the isolates were found to be susceptible to the ethanol neem extract (Figure 4).

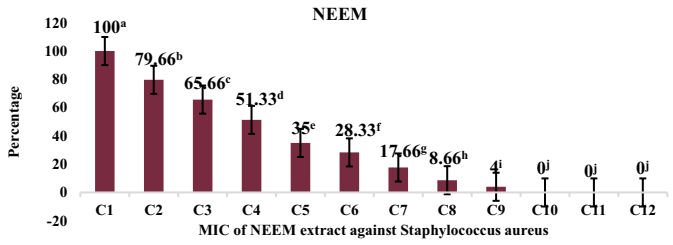

Figure 4: MIC of isolated *S. aureus* against different concentration of ethanol extract of neem leaves

Minimum inhibitory concentration of enrofloxacin against *S. aureus*, The susceptibility of Staphylococcus aureus isolated from buffalo mastitis milk was meticulously evaluated using enrofloxacin at a spectrum of concentrations, ranging from C1 (20 µg/µl) to C11 (0.019 µg/µl). The Minimum Inhibitory Concentration (MIC) was determined to be 5 µg/µl, at which visible bacterial growth was inhibited. According to the CLSI breakpoint for enrofloxacin, this MIC value indicates that 57% of the isolates were susceptible, while the remainder exhibited intermediate or resistant profiles. This threshold delineates the concentration required for effective bacterial inhibition (Figure 5).

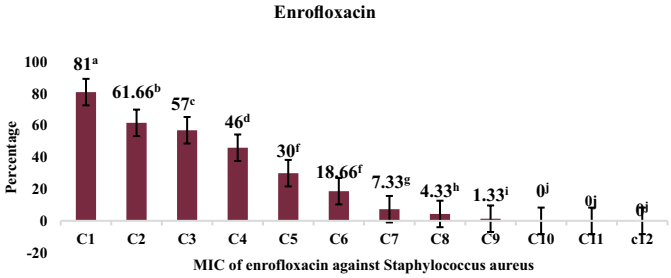

Figure 5: MIC of isolated *S. aureus* against different concentration of enrofloxacin.

Comparison of Minimum inhibitory concentration of ethanol extract of Neem, papaya leaves and garlic against *S. aureus*

To ascertain the comparative susceptibility of *Staphylococcus aureus* isolated from buffalo mastitis milk, various concentrations of ethanol extracts from neem, papaya leaves, and garlic (C1 = 20, C2 = 10, C3 = 5, C4 = 2.5, C5 = 1.25, C6 = 0.625, C7 = 0.312, C8 = 0.156, C9 = 0.078, C10 = 0.039, and C11 = 0.019 µg/µl) were employed. The MIC values for neem, papaya leaves, and garlic were determined to be 2.5, 0.156, and 0.312 µg/µl, respectively, with a statistically significant difference ($P<0.05$) observed among the concentrations. Resistance was noted at lower concentrations. The lowest MIC was recorded for the papaya leaf extract. ANOVA analysis revealed significant differences, further corroborated by LSD tests among the concentrations.

Comparison of Minimum inhibitory concentration of ethanol extract of Garlic, Neem and Papaya leaves with Enrofloxacin against *S. aureus*

The susceptibility of *Staphylococcus aureus* strains isolated from buffalo mastitis milk was assessed using ethanol extracts of neem, papaya leaves, and garlic at different concentrations, as well as enrofloxacin. The tested concentrations varied from C1 (20 µg/µl) to C11 (0.019 µg/µl). The minimum inhibitory concentration (MIC) values were determined to be 5 µg/µl for enrofloxacin, 2.5 µg/µl for neem, 0.312 µg/µl for garlic, and 0.156 µg/µl for papaya, as depicted in Figure 6.

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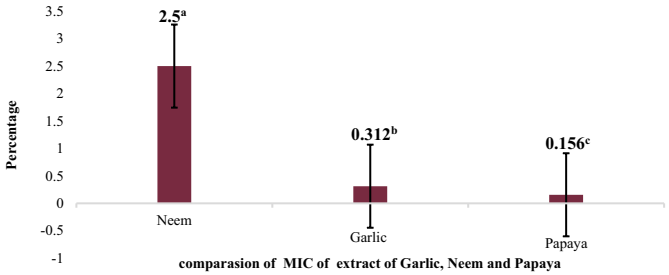

Figure 6: Comparative MIC of ethanol extract of neem, papaya and garlic against *S. aureus*

Herbal treatments demonstrated lower MIC values compared to enrofloxacin. Significant differences ($P < 0.05$) were observed among the concentrations tested, with MIC values of 5, 2.5, 0.156, and 0.312 µg/µl for enrofloxacin, neem, papaya leaf, and garlic extracts, respectively, and bacterial resistance noted at lower concentrations (Figure 7). Among the herbal extracts, papaya leaf extract exhibited the lowest MIC against S. aureus. Statistical analyses using ANOVA and LSD confirmed these significant differences ($p = 0.034$). Herbal treatments demonstrated significantly lower MIC values compared to enrofloxacin ($p = 0.021$), with papaya leaf extract exhibiting the highest efficacy ($p = 0.009$).

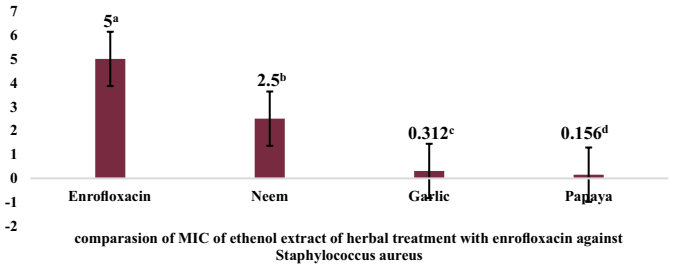

Figure 7: Comparative MIC of all herbal ethanol extract with enrofloxacin against *S. aureus*

DISCUSSION

Herbal medicine, an ancient medical practice, has been widely utilized across both developed and underdeveloped countries [16]. Literature indicates that approximately 50,000 plant species possess therapeutic properties [17]. Compounds found in plants like garlic, neem, and papaya have shown significant potential in treating infectious diseases. Their antibacterial properties make them valuable for addressing various medical conditions [18]. This study aimed to assess the antibacterial properties of ethanol extracts from neem, papaya leaves, and garlic at varying concentrations. These extracts were compared with enrofloxacin using the Minimum Inhibitory Concentration (MIC) method against *Staphylococcus aureus* isolated from buffalo mastitis milk. This study collected and analyzed 50 clinical mastitis milk samples from buffaloes under hygienic conditions. Among these, 12 samples (24%) were positive for *Staphylococcus aureus (S. aureus),* highlighting its prevalence as a mastitis-causing pathogen. The transmission of *S. aureus* is often linked to poor milking hygiene, particularly through the hands of milkers and towels used during milking. *S. aureus* is also a public health concern due to its entero-toxigenic strains, which can cause food poisoning in humans. Additionally, *Streptococcus* species, including *Streptococcus agalactiae,* have been identified as mastitis-triggering microorganisms, further emphasizing the need for effective control measures [19]. The Minimum Inhibitory Concentration (MIC) of various ethanol papaya leaf extract concentrations against *Staphylococcus aureus* was assessed. Resistance was observed at concentrations below 0.156 µg/µl, while the MIC of 0.156 µg/µl showed sensitivity in 55.33% of isolates. This supports previous findings that Carica papaya extracts possess antibacterial properties against S. aureus and *E. coli,* indicating potential for broad-spectrum therapeutic applications. Papaya leaf extract contains several bioactive constituents such as papain, alkaloids, and flavonoids, which contribute to its antibacterial activity. Papain, a proteolytic enzyme, is known to degrade bacterial proteins and disrupt microbial cell walls, while flavonoids can interfere with DNA gyrase and other essential bacterial enzymes [20]. The MIC of various ethanol garlic extract concentrations was evaluated against *Staphylococcus aureus*. Resistance was observed at doses below 0.312 µg/µl, while the MIC of 0.312 µg/µl showed sensitivity in 52.66% of isolates. This aligns with prior studies that found 2.5 mg/ml ethanol garlic extracts effectively inhibited S. aureus [21]. Previous studies noted MICs ranging from 0.14 to 0.63 µg/ml for garlic, demonstrating its potent antibacterial properties [22]. The key antibacterial compound in garlic is allicin, a sulfur-containing compound formed when garlic is crushed or chopped. Allicin inhibits thiol-containing enzymes in bacteria, disrupts metabolic functions, and interferes with quorum sensing and biofilm formation, thereby weakening the pathogen's resistance mechanisms. Similarly, ethanol neem leaf extract was tested for MIC against *S. aureus*, showing resistance at concentrations below 2.5 µg/µl. The MIC of 2.5 µg/µl was effective against 51.33% of isolates.Earlier research reported MIC values of 4–8 mg/ml for neem, with high efficacy at 8 mg/ml against *S. aureus* [23]. However, it is important to interpret the antibacterial efficacy of herbal extracts cautiously. Although the MIC values of plant extracts appear lower compared to enrofloxacin, this does not directly translate into superior clinical efficacy. Plant extracts are complex mixtures containing multiple bioactive compounds with variable concentrations, unlike purified antibiotics with defined pharmacokinetic and pharmacodynamic profiles. The variability in extract composition, potential interactions among constituents, and limited data on absorption, distribution, metabolism,

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and excretion limit the ability to predict in vivo effectiveness solely based on in vitro MICs.The findings indicate that both garlic and neem ethanol extracts possess significant antibacterial properties against S. aureus, corroborating previous research and highlighting their potential as alternative treatments for bacterial infections. Further exploration of these herbal extracts could lead to the development of effective antimicrobial therapies, especially in regions with limited access to conventional antibiotics. The inclusion of mechanistic insights into their action helps bridge the gap between ethnopharmacology and modern drug discovery. The MIC of enrofloxacin against Staphylococcus aureus was evaluated, revealing resistance at doses below 5 µg/µl. Enrofloxacin, with a MIC of 5 µg/µl, was sensitive to 57% of isolated organisms. Findings indicated dried leaf extract's efficacy against certain *S. aureus* and *Streptococcus pyogenes* strains resistant to common antibiotics like ofloxacin and nalidixic acid [24]. Enrofloxacin, frequently used in poultry, shows antibacterial activity similar to garlic's aqueous extract, effective against enrofloxacin-resistant *S. aureus* and E. coli [25].

CONCLUSIONS

All ethanol herbal extracts (neem, papaya leaves and garlic) yielded antibacterial activity against *S. aureus*. Papaya produced lowest minimum inhibitory concentration against isolated organisms. In comparison to herbal extracts, enrofloxacin exhibited higher MIC than all other treatments.

Authors Contribution

Conceptualization: BY, SB, RB, SA, VK
Methodology: JS, LK, QA
Formal analysis: MK
Writing, review and editing: AK

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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