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ASSESSMENT OF THE THERAPEUTIC EFFICACY OF METHICILLIN AND METHANOLIC EXTRACT OF FENUGREEK SEEDS

[a,b]Zainab Lanjar*, [a]Shamsuddin Bughio, [a]Rehana Buriro, [c]Ayaz Ali Memon, [d]Javad Ali Gadhi

[a] *Department of Veterinary Pharmacology, Sindh Agriculture University, Tandojam, Pakistan.*
[b] *Department of Veterinary Microbiology, SBBUVAS, Sakrand, Pakistan.*
[c] *National Centre of Excellence in Analytical chemistry, University of Sindh, Jamshoro, Sindh, Pakistan.*
[d] *Department of Veterinary Parasitology, Sindh Agriculture University, Tandojam, Pakistan.*

A B S T R A C T

Mastitis is one of the main diseases which negatively impact dairy animals, leading to decreased milk yield and quality and also huge economic losses. One of the key pathogens related to this disease is *Staphylococcus aureus*, with methicillin resistant strains (MRSA) making treatment more and more challenging. Objective: In the present study therapeutic activity of methanolic extract of *Trigonella foenum-graecum* (fenugreek) seeds against MRSA isolated from milk samples of buffaloes suffering from clinical mastitis were evaluated. Milk samples were collected from clinical mastitis involved buffaloes of Hyderabad district (n=100). Bacterial isolation performed by classical microbiological and biochemical methods. To identify methicillin-resistant isolates, the disc diffusion method for antimicrobial susceptibility testing was performed according to Clinical Laboratory Standards Institute guidelines. The methanolic extract of fenugreek seeds was screened for antimicrobial activities at different concentrations (50, 100, 150 and 200 mg/mL) by agar well diffusion method. Methicillin (100 µg/mL) and distilled water were used as a positive and negative control, respectively. Results indicated the isolation of *S. aureus* from 34% samples with MRSA positive in 20.6% isolates. Methicillin resistance was present in all MRSA isolates. As for the methanolic extract of fenugreek seeds, it did not show any inhibition zone in all examined concentrations. In conclusion the crude methanolic extract of *T. foenum-graecum* seeds was not shown sufficient therapeutic activity against MRSA isolates recovered from mastitic milk samples. More research must be done with different extraction processes and formulations.

Keywords: *Clinical Mastitis, Methicillin, Staphylococcus aures, Methanolic extract of Fenugreek seed*.

Corresponding Author: Zainab Lanjar
Email: zainab.makhdoom@yahoo.com

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INTRODUCTION

Mastitis is a significant global dairy challenge due to its detrimental impact on milk yield, milk quality and farm profitability. Mastitis is generally classified into two types: clinical mastitis (CM) and subclinical mastitis (SCM) (Maity & Ambatipudi, 2021). Clinical mastitis occurs and ranges from mild to severe, typically is characterized by udder pain, swelling of the udder, abnormal secretion of milk (dull) reduced output of milk (milk production) and on some occasions accompanied with fever systemic illness (Matthias & Angel, 2025). While subclinical mastitis does not have any obvious visible udder or milk changes, it is normally diagnosed based on a high somatic cell count as the result of leukocyte infiltration (Zaatout, 2021).

The etiology of mastitis encompasses a variety of bacterial species including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus* spp., and Escherichia coli. There can be other rare pathogens including *Pseudomonas aeruginosa*, *Mycoplasma* species, and *Mycobacterium* species involved (Shoaib et al., 2021, Ardicli et al., 2022). *S. aureus* is one of the most important udder pathogens of dairy animals among these pathogens. It generates clinical and subclinical mastitis and is frequently transmitted at the time of milking, causing lasting chronic infections. This challenges therapy even more so in the presence of multidrug resistance (Zigo et al., 2021). Methicillin-resistant

Staphylococcus aureus (MRSA) is of especial concern due to its multi-drug resistant nature, and represents an important public health and veterinary challenge (Palanichamy et al., 2022).

Fenugreek, also known as *Trigonella foenum-graecum* L., is an erect annual herb of the Fabaceae family. Seeds and leaves are abundant in bioactive ingredients such as proteins, lipids, alkaloids, flavonoids, fibers saponins, steroidal saponins vitamin and minerals. These constituents of phytochemicals are related to some biological effects which include antioxidant, antibacterial, antidiabetic and anticancer activities (Nagulapalli et al., 2017; Basu & Srichamroen, 2010). Previous studies have demonstrated that fenugreek extracts have some antimicrobial properties but depending on the used plant part and extraction method as well as the target organism's efficacy can vary (Al-Timimi, 2019). Sharma et al. Fenugreek seed extracts (2017) demonstrated greater antibacterial activity than Fenugreek leaf extract, while several authors describe using Fenugreek antibiotic effects against chosen pathogenic bacteria (Al-Timimi, 2019). In consideration of these findings, the current study was conducted to evaluate the in vitro antimicrobial efficacy of methanolic extract of fenugreek seeds against MRSA isolated from buffalo's milk afflicted with clinical mastitis.

METHODOLOGY

Sample collection

One hundred morbid milk samples were collected from buffaloes affected by clinical mastitis from various neighbouring dairy farms in the Hyderabad district. Samples were then collected in bijou bottles and stored until transporting to Central Veterinary Diagnostic Laboratory, Tandojam for further analysis. Processing of these samples were carried out for isolation and identification of methicillin-resistant *Staphylococcus aureus* associated with mastitis.

Animals with clinical mastitis were selected based on the visible swelling of the mammary gland, abnormal phenomenon in milk (Pus/blood streaks), and a decrease in milk yield (Abdalhamed et al., 2018). California Mastitis Test (CMT) adapted from Kandeel et al. (2018), where 3 mL of milk/quarter was mixed with CMT reagent using partitioned plastic paddle · Mild udder changes were further confirmed by the same test. The udders were washed with water and dried with cotton soaked in a 75% ethanol solution before milk samples were collected.

Isolation and phenotypic identification of bacteria

Milk samples were transported to the laboratory no later than one hour after collection. All samples were cultivated on mannitol salt agar and on blood agar (Oxoid, Hampshire, UK).Inoculated plates were incubated for 24 to 48 h at 37 °C. Bacteria were identified based on Gram stain, colony morphological characteristic, hemolytic pattern and biochemical characteristics as according to Mahlangu et al. (2018). The *S. aureus* isolates which were confirmed are stored at −20 ° C with tryptic soy broth (TSB; Becton Dickinson, Wokingham, UK) added by 20% of glycerol.

Disc diffusion method for antimicrobial susceptibility testing of MRSA

Methicillin susceptibility testing of confirmed *S. aureus* isolates was performed by the standard Kirby-Bauer disc diffusion technique on Mueller-Hinton agar (Merck, Darmstadt, Germany). Individual colonies from pure cultures were suspended in sterile normal saline to match the optical density of 0.5 McFarland standard (1.5×10^8 CFU/mL). Mueller-Hinton agar plates were inoculated with a bacterial suspension. Methicillin discs (5 µg) were placed, and the test was performed and read according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Weinstein & Lewis, 2020). The zones of inhibition were measured (mm) with Vernier calipers.

Collection of plant material

Fenugreek Seeds were collected from the local market in Hyderabad. The seeds were cleaned from dust and impurities with distilled water and dried in sunlight for roughly 5 hours. The seeds were ground with an electric grinder and kept in clean glass bottles until extraction for 24 hours after they dried.

Methanolic extract preparation of fenugreek seed

A total of 10 grams of seed powder was immersed in 100 mL 70% methanol and incubated at 37°C for a period 13 hours. Filtration of the mixture was then performed using Whatman No.1 filter paper and evaporation in a water bath at 100 °C for 8 hour to concentrate. The dried extract was kept at 4°C in an airtight container until used.

Antibacterial activity (Agar well diffusion method)

Antibacterial activity of Methanolic extract of fenugreek seed against Methicillin resistant *Staphylococcus aures* through well diffusion methods

The agar well diffusion method was used to evaluate the antibacterial activity of methanolic extract of fenugreek seed against methicillin-resistant Staphylococcus aureus. Using sterile swabs, a 0.1 mL aliquot of standardized bacterial inoculum (1.5×10^8 cells/mL) was then spread uniformly on the surface of Mueller-Hinton agar and allowed to dry. Wells were subsequently punched into the agar using a cork borer of 6 mm diameter (Abdullah, 2021). Square wells were then introduced with various alterations of methanolic

fenugreek seed extract at doses of 50, 100, 150 and 200 mg/mL. Distilled water was employed as the negative control and methicillin (100 µg/mL) was utilized as the positive control. After incubating the plates up to 37°C, inhibition zones were measured after incubation period of 24 hours by Vernier caliper.

RESULTS

Microbial Frequency in Mastitis Milk Specimen

From 100 clinical mastitis milk samples tested as culture-positive (65) or culture-negative (35). *Staphylococcus aureus* was the most frequently isolated organism (34 isolates, 52.3%) among positive samples. The next most common was *Staphylococcus epidermidis* with 17 isolates (26.1%), followed by *Streptococcus* species at 9 isolates (13.8%) and mixed isolates at 5 cases (7.6%), respectively as shown in Table 1. Gram staining, golden-yellow colonies on mannitol salt agar as shown in (Figure 1). β-hemolysis on blood agar and positive catalase and coagulase reactions were used to identify *Staphylococcus aureus* isolates from buffalo mastitis milk samples. Isolates were confirmed by biochemical testing to be *S. aureus* (Figure 2).

Table 1: Percentage of bacterial species isolated from mastitis milk of buffalo.

Parameters	Buffalo	Percentage
Total sample	100	-
Culture positive bacteria	65	65
Culture negative bacteria	35	35
Staphylococcus aureus	34	52.3
Staphylococcus epidermis	17	26.1
Streptococcus species	9	13.8
Mix	5	7.6

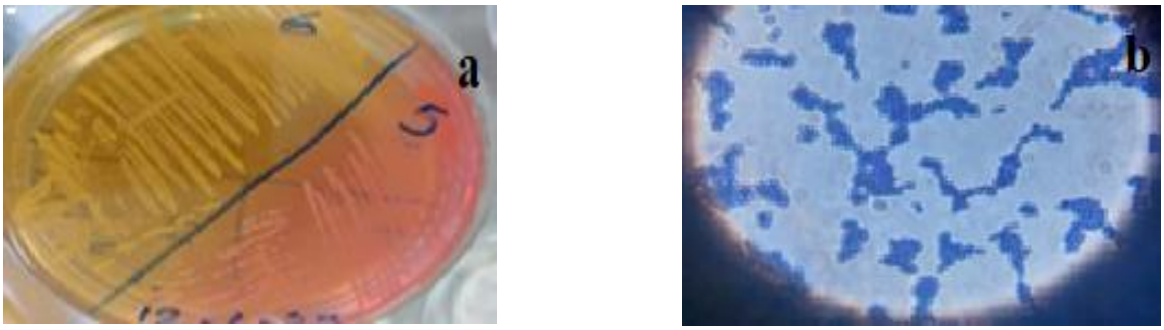


Figure 1: a) fermentation of mannitol salt agar (golden yellow) by *S. aureus* and b) morphology of *S. aureus*.

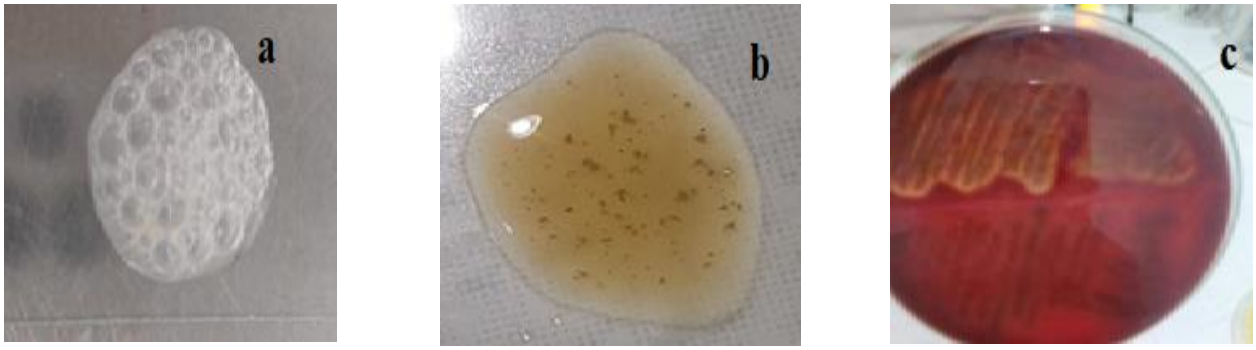


Figure 2: a) Catalyst, b) coagulase and c) β hemolysis positive S. *aureus*.

Methanolic extract of fenugreek seed

The syrup obtained after extraction was placed into Petri plates and dried in a water bath at 90 °C for eight hours to get dry powder as showed in Figure 3.

Antimicrobial susceptibility

As a result of antimicrobial susceptibility data from the 34 *S.*

aureus isolates, 7 methicillin-resistant isolates were classified as methicillin-resistant *S. aureus* (MRSA). Twenty seven (79.4%) were methicillin sensitive and 7 (20.5%) methicillin-resistant as showed in Table 2. recovery representing about ("%." All the MRSA isolates (n = 7) showed complete resistance to methicillin as showed in Figure 4.



Figure 3: a) Shows methanolic extract and b) dried powder of methanolic fenugreek seed.

Table 2: Antimicrobial susceptibility of MRSA from mastitis buffaloes.

Sample type	Staphylococcus aures isolates (n=34) Methicillin disk diffusion test: Resistance (%)	Sensitive (%)
Clinical mastitis milk	7 (20.5%)	27(79.4%)



Figure 4: Disk-diffusion test and inhibition zones of the Methicillin against MRSA *S. aureus*.

Antimicrobial Potential of Methanolic Extract of Fenugreek Seeds

To assess the antibacterial activity of methanolic fenugreek seed extract against methicillin-resistant *Staphylococcus aureus*, an agar well diffusion assay was performed. The extract was tested against various concentrations as well as methicillin which served as the positive control and distilled water served as the negative control. The indicator of antibacterial activity determined was the presence of inhibition zones around the wells.

No inhibition zone was noted for all tested concentration of the methanolic fenugreek seed extract including C_1 (50 mg/ml), C_2 (100 mg/ml), C_3 (150 mg/ml) and C_4 (200 mg/ml). Just as with the resistant isolates, methicillin (100µg/ml) produced no inhibition zone against sensitive isolates, and so as with distilled water. The results suggest that the extract tested in this study was unable to show antibacterial activity against MRSA under the experimental conditions employed here as shown in Figure 5.

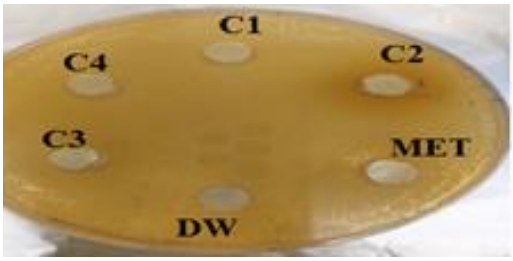


Figure 5: Growth of methicillin *staphylococcus aureus* treated methanolic extract of fenugreek seed.

Table 3: Antimicrobial activity of methanolic extract from Fenugreek seed against *methicillin resistance Staphylococcus aureus.*

	Distilled water	Methicillin (100µg/ml)	Various concentrations of Methanolic extract of fenugreek seed			
Parameter	Negative control	Positive control	C1 (200 mg/ml)	C2 (150 mg/ml)	C3 (100 mg/ml)	C4 (50 mg/ml)
			Zone of Inhibition (mm)			
Inhabition zone	R	R	R	R	R	R

R= resistant

DISCUSSION

The aim of this study was to assess the antibacterial activity of methanolic extract of *Trigonella foenum-graecum* seeds on methicillin-resistant *Staphylococcus aureus* isolated from buffalo mastitis milk. The extract concentrations tested did not exhibit a zone of inhibition in the assay. The resistant isolates also remained unaffected by methicillin and distilled water. Our results suggested that, under the conditions used in this study, fenugreek seed crude methanolic extract does not exert a direct bactericidal effect against MRSA.

The results of this study are consistent with some previous studies, but the literature is conflicted. Sharma et al. (2017) The methanolic extract of fenugreek exhibited higher activity than aqueous and acetonic extracts against *E. coli* and *S. aureus*, as well as leaf extract being more potent than seed extract. El Nour et al. Consequently, (2015) indicated that the methanolic fenugreek seed extracts at 250 mg/mL were not capable of inhibiting the growth of *Pseudomonas* spp., *S. aureus* or *E. coli* as mentioned in the same study, at 100 µL previously mentioned fenugreek seed aqueous and ethanolic extracts were also effective in producing a inhibition zones of 6.45 mm and 13.9 mm, respectively. Similarly, Subramaniam et al. (2021) evaluated the essential oil, boiled aqueous extract, and the ethanolic extract of *T. foenum-graecum* against *S. aureus*, *S. epidermidis*, *P. acnes* MRSA and MRSE through agar well diffusion with the observation as addition of boiled aqueous extract show greatest activity while essential oil & ethanolic showed no inhibiting power. Chua et al (2019) showed that ethanolic extracts of *T. foenum-graecum* are also ineffective against S. aureus. Well diffusion assays by Babaei et al. (2018) showed only a limited level of inhibition by the methanolic seed extract of fenugreek against MRSA.

The variations among the findings from different studies may be attributed to various and diverse factors including extraction solvent, plant part used, active compounds concentration, bacterial strain, and antimicrobial evaluation method. However, the crude methanolic extract did not show very encouraging activity which demonstrated that certain other extraction strategies and/or purified fractions might be needed to obtain bioactive antibacterial substances from fenugreek.

CONCLUSION

We conclude that *Staphylococcus aureus* is a significant bacterial cause of clinical mastitis in buffalo herds and a proportion of these isolates are methicillin-resistant. The 100% resistance of MRSA isolates to methicillin highlights the increasing problem of antimicrobial resistance in dairy herds and challenges associated with treating these infections. *Trigonella foenum-graecum* seeds crude +methanolic extract, evaluated at different concentrations, displayed no inhibitory effect on MRSA isolates. So, the extract was not shown to have significant in vivo antibacterial activity against methicillin resistant *S. aureus* associated with mastitis, under the conditions of this study. Future studies may explore other extraction methods, purified compounds and plant-based formulations and validate for better therapeutic candidates.

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