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IN VITRO EVALUATION OF ANTIMICROBIAL EFFICACY OF *AZADIRACHTA INDICA* (NEEM) EXTRACT AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATES FROM SUBCLINICAL MASTITIS IN CHOLISTANI CATTLE

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Abstract

Subclinical mastitis (SCM) is a disease of mammary glands which usually affects dairy cattle during lactation. It causes huge economic impact to the dairy industry in terms of less milk yield and increased treatment cost. Staphylococcus aureus is the primary pathogen associated with mastitis pathogens frequently isolated from infected cow's milk and possesses antimicrobial resistance against various antibiotics. Hence, current study was designed to detect SCM in lactating cows and to check in vitro antimicrobial efficacy of Azadirachta indica (A. indica) against S. aureus isolates of infected milk samples. For this purpose, 358 milk samples were tested through surf field mastitis test (SFMT) and further confirmed through bacterial culture on mannitol salt agar. Positive milk samples 163/358 (45.53%) were detected through SFMT and 45/163 (27.6%) were later confirmed through bacterial culture. Several risk factors such as age, parity number, diameter of teat apex, total milk yield and folded thumb milking were significantly (P<0.05) associated with occurrence of SCM. Efficacy of different concentrations of methanolic extracts of A. indica i.e. 500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml, 31.25mg/ml and frequently used antibiotics were checked against S. aureus isolates by disc diffusion method. The results revealed that S. aureus isolates were found sensitive to 500mg/ml concentration of A. indica extracts and to antibiotics including ciprofloxacin, septran and tylosin. While isolates showed resistance against the oxytetracycline, oxacillin and cefoxitin antibiotics. Hence, based on the results, it can be concluded that the methanolic extracts of A. indica can be used as an alternative therapy in treating the mastitis in cows.

Keywords: *Antibiotics, Azadirachta indica, Disc diffusion method, Subclinical mastitis, Surf field mastitis test, S. aureus*

INTRODUCTION

Mastitis, an inflammation of the mammary gland is one the most common diseases affecting dairy cows worldwide. About 60-70% of all antimicrobials administered on dairy farms are used to treat udder infections in dairy animals (1). Livestock industry plays vital role in agriculture sector and contributes significantly in terms of agriculture value and national GDP which is almost 62.68% and 14.36% respectively, in the economy of Pakistan (2). Pakistan is the 3rd largest milk producer country in the world but its dairy industry facing various disease challenges which hinder its progress by lowering milk yield (3-4). Approximately, 35 million people are engaged in rearing of farm animals which contribute about 11% share in the national GDP of Pakistan (5). Dairy animals play an important role in farmer's income and socio-economic growth (5). Several diseases effect milk production of dairy animals and mastitis is one of them which is most dangerous disease of dairy industry (5-6). The manifestation of mastitis can be seen in two forms; clinical and subclinical forms (7).

Sub-clinical mastitis is 15 to 40 times more prevalent than clinical mastitis and the annual economic losses associated with sub-clinical mastitis may exceed those caused by the clinical form (8). Mastitis is a complex multifactorial disorder of mammary glands in dairy animals. It causes the inflammation of mammary parenchyma which results in pathological alterations within the glandular tissues as well as chemical, physical and typically bacteriological alterations in the milk (9). More than 137 infectious causes of bovine mastitis have been identified and the most important ones are *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae*, coliforms and other streptococcus species (10). *Staphylococcus aureus* is mostly isolated pathogen from sub-clinical mastitis of dairy animals (11). *S. aureus* is primarily isolated using nutrient agar and mannitol salt agar as selective media in the laboratory.

Hemolysins produced by *Staphylococcus aureus* are regarded as key virulent factors in the development of mastitis (12). Mastitis is the multifactorial disease and its susceptibility is linked with multiple factors such as age, lactation stage, parity, milk yield and udder anatomy (13). Disease incidence is mainly affected by management as well as environment associated factors (14). The detection methods of subclinical mastitis mainly include CMT, SFMT, somatic cell count and White side test (15). California mastitis test is mostly used in early detection and screening of SCM (16). Bacteria are mainly shed in the milk from the infected udder (17). The bacteria inhabit the mammary parenchyma, teat canals and cause teat lesions in the diseased cattle (18). Bacterial examination of milk samples can be used to identify infected udders in cows (19). *Staphylococcus aureus* causes both subclinical and chronic mastitis, leading to severe economic losses for dairy farmers (20). The primary treatment for mastitis typically involves administering antibiotics (tetracycline, streptomycin, penicillin, ampicillin and cloxacillin) through parenteral administration or intramammary infusion in cattle (21). *S. aureus* can develop antibiotic resistance like methicillin resistance seen in MRSA by acquiring the mecA gene which produces PBP2a crucial for cell wall synthesis (22). Resistance increases treatment costs and allows MRSA transmission to humans through animal product consumption or contact with contaminated animals nearly 60% of emerging human pathogens originating from animals (22).

Essential oils and plant extracts offer promising alternatives to synthetic drugs (23). In Pakistan, local healers use medicinal plants without complete knowledge of their active components (24). *A. indica* (Neem) contains hydroxypiralic, phytol and acetic acid which are a fast-growing evergreen tree native to the subcontinent. It has antibacterial and anti-inflammatory properties effective against udder infections (9). *A. indica* extract infusion decreases bacterial and somatic cell counts while increasing antioxidants and lymphocytes in milk (25). Antimicrobials of plants source had great treatment proficiency and also had therapeutic potential for infectious diseases but at the same time lessen numerous side effects which were linked with synthetic antimicrobials (10). The current study was conducted to assess the prevalence of sub-clinical mastitis, identify associated risk factors and evaluate the antimicrobial susceptibility of *S. aureus* isolates to the methanolic extract of *A. indica* in Cholistani cattle and their comparison with different antibiotics through *in-vitro* culture sensitivity test.

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of 358 lactating Cholistani cows were tested for sub-clinical mastitisusing the Surf field mastitis test (SFMT). The sample collection procedure started with fore-stripping, which involved 3 to 5 streaks of milk. This was followed by disinfecting the teats with a 0.25% iodine solution and drying them with a clean towel. Post-milking teat disinfection was conducted using a 0.5% iodine solution (26). Based on the SFMT analysis, 10ml of milk samples were collected aseptically after discarding the first few streams of milk following the standard protocol of the National Mastitis Council (27). The samples were stored in an icebox and shifted to laboratory of department of Clinical Medicine and Surgery at The Islamia University of Bahawalpur.

ISOLATION AND IDENTIFICATION OF *S. AUREUS* FROM SUB-CLINICAL MASTITIS

The mastitic milk samples were further processed for the isolation of *S. aureus* by culturing on mannitol salt agar (Oxoid®) Milk sample was spread on the differential media (mannitol salt agar) by using a sterile cotton swab. Then these agar plates were placed in an incubator set to 37°C for 24 to 48 hours. The developed colonies were selected and sub-cultured for purification. The purified colonies were morphologically identified using gram staining and biochemical tests (catalase and coagulase). The colonies of *S. aureus* formed on the agar plates were further evaluated for the morphology of bacterial colony. The phenotypic appearance of *S. aureus* colonies were identified by gram staining followed by bio-chemical test including catalase and coagulase test for confirmation (22).

RISK FACTORS

Association of different risk factors with occurrence of udder infections in cattle was determined. Age of lactating cattle, body weight, depth of udder, parity, teat length, teat apex diameter, distance from teat to floor, length of tail, type of floor, housing system, grimy legs, feeding calf, milking method and feeding system were recorded on specialized designed proforma. The length of teat, teat apex diameter was measured by using a vernier caliper while other measurements were taken by using the measuring tape (28).

PREPARATION OF DIFFERENT CONCENTRATIONS OF *AZADIRACHTA INDICA*

Neem leaves were collected and thoroughly washed with tap water to remove dust particles. These washed leaves were dried by spreading in a well-ventilated room away from direct sunlight for 2-3 weeks. The dried leaves were grinded to fine powder then stored in airtight bottle. Solvent extraction using methanol was used for preparation of different concentrations of *Azadirachta indica* (29). Methanolic extract of *Azadirachta indica* was serially diluted in distilled water for making different concentrations i.e. 500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml (30). Zone of inhibition (mm) was measured by using vernier caliper.

MINIMUM INHIBITORY CONCENTRATION OF METHANOLIC EXTRACT OF *AZADIRACHTA INDICA*

The antimicrobial efficacy was checked via disc diffusion method (31). For this purpose, the broth culture having 1×108 CFU/ml activated growth of *S. aureus* was spread on a solidified nutrient agar surface then three to four wells of four to six millimeter were made by borer aseptically. Then wells were then filled with *A. indica* extract. The petri-plates were incubated for 18-24 hours at 37°C. Finally zone of inhibition was measured. Minimal inhibitory concentration is the lowest concentration of extract that prevent visible growth of bacteria on plate after specified incubation period (10).

SUSCEPTIBILITY OF *S. AUREUS* AGAINST DIFFERENT ANTIBIOTICS

The susceptibility of different antibiotics including oxytetracycline, ciprofloxacin, tylosin, cefoxitin, oxacillin, septran (trimethoprim and sulfamethoxazole) (Bio-Analyze®) was checked against *S. aureus* by using disc diffusion technique.

STATISTICAL ANALYSIS

For data analysis Statistical package for the social sciences (SPSS-21) was used. Data on age of lactating cattle, body weight, depth of udder, parity, teat length, teat apex diameter, distance from teat to floor, length of tail were analyzed by Analysis of Variance (ANOVA) and t-test was used for the comparison of differences in means. Data on type of floor, housing system, grimy legs, feeding calf, milking method and feeding system were recorded in Microsoft Excel sheet and coded. The prevalence of SCM was calculated by taking average and Odds ratio was used to analyze the association of disease occurrence with risk factors. A significance level of $p<0.05$ was used for analysis.

RESULTS

The result showed 42.45% (163/358) SCM prevalence in Cholistani cattle based on SFMT. These 163 screened positive samples were subjected to bacterial culture on Mannitol Salt agar (Oxoid®) and 45 (27.6%) *S. aureus* isolates were confirmed on the basis of biochemical and morphological characteristics (Table I).

Table I. Prevalence of SCM in lactating cattle detected by two different tests

Sub-clinical mastitis	No. of animals tested	Number of positive animals	Prevalence
SFMT	358	163	45.53%
Bacteriological Culture	163	45	27.60%

Data regarding different risk factors showed that depth of udder, age, parity numbers and diameter of teat apex were recorded significantly ($p<0.05$) higher in mastitic than in the non mastitic cows. However, total milk yield, teat to floor distance and length of teats were found significantly ($p<0.05$) lower in infected cattle (Table II). Results of study revealed high occurrence of sub-clinical mastitis in cemented floor (23.86%), closed housing (17.58%), grimy legs (18.89%), feeding calf (15.21%), folded thumb for milking (27.96%) and stall feeding (19.10%) (Table III).

Table II. Comparison of different physical parameters of mastitic and non-mastitic cows

Parameters	Mastitic cattle (Mean±SD)	Non-mastitic cattle (Mean±SD)
Age of animals (years)	8.91±4.50	6.71±2.59*
Body weight of Animals (kg)	343.17±38.68	337.24±40.71
Length of Tail (cm)	98.96±5.27	99.08±4.92
Parity Number	3.83±2.63	3.04±2.14*
Total Milk yield (L)	7.79±1.44	9.46±1.82*
Depth of Udder (cm)	15.29±1.18	11.70±1.40*
Distance from teat to floor (cm)	33.49±2.90	44.13±2.31*
Diameter of teat apex (cm)		
Left Front	0.86±0.11	0.65±0.10*
Right Front	0.86±0.12	0.65±0.10*
Left Rear	0.84±0.11	0.69±0.11*
Right Rear	0.84±0.11	0.70±0.10*
Length of teat (cm)		
Left Rear	5.28±0.99	6.17±0.35*
Right Rear	5.28±0.98	6.09±0.32*
Left Front	5.91±0.52	6.84±1.10*
Right Front	5.86±0.54	5.81±1.12*

*p< 0.05 indicates significant difference

Table III. Different managemental risk factors of mastitic and non-mastitic cows

Variables	Variable levels	Total no. of animals	Positive N	Positive %	95% C.I	Odds Ratio/ Reciprocal
Type of Floor	Cemented	88	21	23.86	15.84 - 33.59	2.30 / 0.44
	Non-cemented	75	9	12.00	6.01 - 20.88	
Housing System	Closed	91	16	17.58	10.77 - 26.43	1.71 / 0.59
	Semi-closed	72	8	11.11	5.30 - 20.01	
Grimy Legs	Yes	90	17	18.89	11.79 - 27.96	1.66 / 0.60
	No	73	9	12.33	6.18 - 21.42	
Feeding Calf	Yes	92	14	15.21	8.93 - 23.66	1.41 / 0.71
	No	71	8	11.27	5.37 - 20.27	
Milking Method	Whole hand	70	5	7.14	2.66 - 15.12	0.20 / 5.04
	Folded thumb	93	26	27.96	19.56 - 37.71	
Feeding System	Stall/grazing	74	8	10.81	5.15 - 19.49	0.51 / 1.95
	Stall feeding	89	17	19.10	11.93-28.26	

MINIMUM INHIBITORY CONCENTRATION OF *A. INDICA* EXTRACT

The results of antimicrobial efficacy of different methanolic concentrations of *A. indica* extracts against the *S. aureus* isolates was checked through *in-vitro* studies (Table IV). The mean of zones of inhibition against *S. aureus* isolates at concentrations of 500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml were 19.56±0.007 mm, 16.75±0.021 mm, 12.24±0.010 mm, 9.02±0.018 mm and 6.65±0.023 mm, respectively (Figure I). Minimum zone of inhibition of neem extract on *S. aureus* isolate was at 31.25 mg/ml concentration.

Table IV. Zone of inhibition of methanolic extract of different concentrations of *A. indica* against *S. aureus* isolates

Sr. No.	Bacterial isolates	*A. indica* extract concentration (mg/ml)	Zone of inhibition (mm) (Mean±SE)
1		500	19.56±0.007
2		250	16.75±0.021
3	*S. aureus*	125	12.24±0.010
4		62.5	9.02±0.018
5		31.25	6.65±0.023

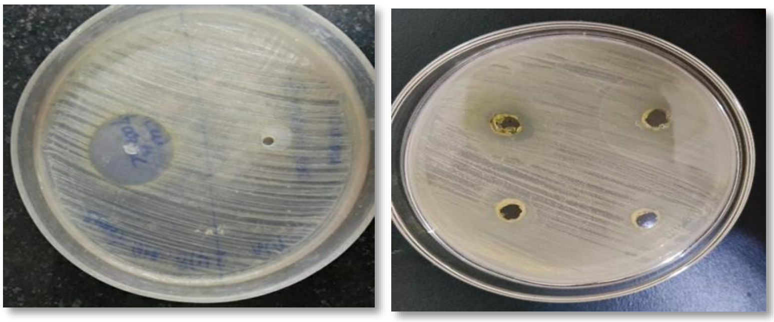

Fig. 1. Minimum inhibitory concentration of methanolic extract of *A. indica* showed maximum zone of inhibition at 500mg/ml (left side) and minimum zone of inhibition at 31.25mg/ml (right side)

ANTIBIOTICS SUSCEPTIBILITY OF DIFFERENT ANTIBIOTICS

Additionally, the different antibiotics susceptibility was also evaluated against *S. aureus* isolates. This study found that few anti-microbials including ciprofloxacin, Septran (trimethoprim + sulphamethoxazole) and tylosin were effective against *S. aureus* (Table V). But *S. aureus* had not showed sensitivity against other antibiotics such as oxytetracycline, oxacillin and cefoxitin (Fig. II).

Table V. Anti-biogram patterns of *S. aureus* isolates

Sr. no.	Antibiotics	µg/disc orUnit/disc	Zone of inhibition diameter (mm)	Reference value (CLSI-2020)	Sensitive or Resistance
1	Tylosin (TY-30)	30	18.95	≥23- ≤13	Intermediate
2	Ciprofloxacin (CIP-5)	5	20.7	≥20- ≤15	Sensitive
3	Oxacillin (OX-1)	1	0	≥24 - ≤18	Resistance
4	Cefoxitin (FOX-30)	30	0	≥22- ≤21	Resistance
5	Septran (SXT-25) (Trimethoprim + Sulphamethoxazole)	25	19.5	≥16- ≤10	Sensitive
6	Oxytetracycline (T-30)	30	0	≥19- ≤14	Resistance

DISCUSSION

Mastitis is reported as the most prevalent disease in lactating cows that leads to substantial economic losses on a global scale (32). Subclinical mastitis possesses significant challenge for dairy animals nationwide with higher rate of prevalence mainly linked with area of location, management practices and overall herd health. Sub-clinical mastitis significantly impacts Cholistani cattle increasing the risk of clinical mastitis and reducing milk production (33). Results of the current study showed that prevalence of SCM was 42.45%

(163/358) in Cholistani cattle based on SFMT which was in close similarity with previously reported data (35.15%) (34), (31.53%) (35) and (49.0%) (36). However, higher rate of prevalence (76.9%) was also reported (37). Variation in mastitis prevalence may be due to management, treatment practices, regional differences and environmental microorganisms. Michira *et al.,* (2023), reported that this variation in occurrence of disease might be due to the difference in production systems (38). Based on bacteriological analysis prevalence of *S. aureus* SCM was 27.6% (45/163). Similarly, previous data had supported the current findings (20), 6% (39), 36.8% (40), 33.18% (41) and 15.8% (38). The prevalence and resistance patterns of *S. aureus* in subclinical mastitis may vary based on the geographical area and the specific population being investigated. *S. aureus* is the most prevalent pathogen of mastitis which can spread through milk handlers from one cow to another or from the environment to the animal depending on factors such as type of floor and udder hygiene (42).

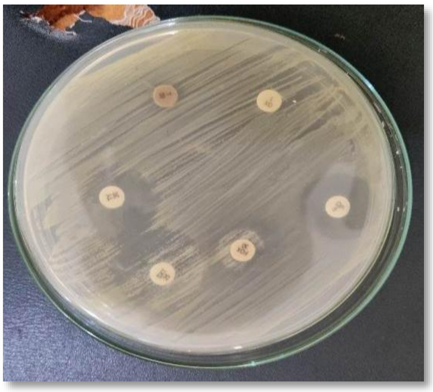

Fig. 2. Minimum inhibitory concentration of antibiotic discs showed ciprofloxacin, septran and tylosin were effective but oxytetracycline, oxacillin and cefoxitin not showed sensitivity against *S. aureus*.

In current study a significant association of age with occurrence of SCM was recorded. Sub-clinical mastitis was significantly (p<0.05) higher in older age animals than early age. This finding was similar with previously reported results (34, 35). Qayyum *et al.,* (2016), stated that higher prevalence of SCM in older age cattle might be due to relaxed sphincter muscles of teats which increased the entry of mastitic pathogens into udder (28). Contrarily, Mureithi *et al.,* (2016) reported that age of dairy cattle was not significantly associated the occurrence of SCM (42). Number of parity was significantly associated with occurrence of SCM in Cholistani cattle. Prevalence of SCM was significantly (p<0.05) higher in mastitic than non-mastitic cows. It might be due to the better efficacy of immune system of primiparous cattle as compared to multiparous cattle (43). Former types of cattle were more prone to mastitis over the time with extended time period of disease in multiparous cattle (44). Similarly, Barua *et al.,* (2014) reported that cattle with higher parity number were more productive and became more susceptible to infection with an increasing parity number (35).

Prevalence of SCM in high milk producing cows was found significantly lower than non-mastitic cows. Contrarily, Barua*et al.,* (2014) reported higher SCM prevalence in cattle with higher milk production (35). Mureithi*et al.,* (2016), found significantly (p<0.05) close impact of milk yield on SCM occurrence (42). The lactating cows with high milk yield might be more susceptible to mastitis as their functional glandular tissue might be more prone to infection (45). Findings of current study revealed no significant association of live body weights and tail lengths with the prevalence of SCM. Yet, procedure of frequency analysis represented that higher body weights had significant association with the occurrence of SCM. These results were similar with previous report (28, 46).

Present study findings showed that animals with increased teat apex diameters and small teat lengths had higher prevalence of disease. These findings were in agreement with previous reports (47). These physical parameters may be associated with the entry of mastitis pathogens as they can easily enter the teat through increased apex diameters. This allows the pathogen to travel a short distance quickly and multiply in the udder parenchyma leading to inflammatory alterations associated with the mastitis (28).

Results of study revealed high occurrence of sub-clinical mastitis in cattle kept on cemented floor (23.86%) than non-cemented floor (12.00%). Type of floor had significant impact on the prevalence of SCM. Mekibib *et al.,* (2010), reported high prevalence of SCM in the soil floor and the dirty udder (48). This finding was similar with previous report of Mureithi *et al.*, (2016) who reported that dirty floor could be a possible source of the mastitis pathogen. Prevalence of SCM was higher (15.21%) in cattle with calf feeding than without calf feeding (11.27%) (42). It might be caused by the teat sphincter laceration by biting of calf. Barua *et al.*, (2014), reported that mastitis could be due to the entry of pathogens through the sphincter of teat after injury and laceration of udder (35). Animals kept on stall feeding (19.10%) and closed housing system (17.58%) had higher prevalence of disease. Similarly, factors of grazing were significantly related with disease occurrence (49). The reason of mastitis occurrence might be the close proximity of animals that increased the possibility of teat lesions. Moreover, teat lesions especially at openings of teats increased the risk of pathogens entry into udder finally resulting to infection of udder (28). It was stated that injury of teat canal caused an easy colonization of infectious agents which resulted mastitis (47).

Lactating cows with grimy legs (18.89%) had higher prevalence of SCM. Similarly, it was stated that grimy rear legs at lower parts had significant link with prevalence of this infection (50). This might be a result of the unhygienic conditions of hind legs and also the presence of grimy dung material which could assist in causing infection in dairy cattle (28). Current report showed the significant relationship of folded thumb for milking (27.96%) and occurrence of SCM in lactating cattle. This might be due to the damaging outcome on the teat canals during milking with the folded thumb which increased the possibility SCM occurrence (28). An efficacy of a specific antibiotic or drug for treating sub-clinical mastitis in Cholistani cattle could lead to the development of a targeted treatment strategy (33). Neem leaf extract had been found to possess antibacterial properties against multidrug-resistant pathogens. Several studies had indicated that neem exhibits antibacterial activity and was effective against *S. aureus* and other pathogens (51).

The results of antimicrobial efficacy of different concentrations of methanolic extracts of *A. indica* against the *S. aureus* isolates was checked through *in-vitro* study (Table IV). The results showed that *A. indica* at higher concentration (500 mg/ml) had highest zones of inhibition (19.56mm) against *S. aureus* isolates than the lower concentrations (250 mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml). Higher concentration of neem extract contains a higher quantity of active compounds such as azadirachtin, nimbin and nimbidin which are responsible for its antibacterial properties. These substances have the ability to damage bacterial cell membrane and leads to death or growth inhibition (52). In the case of *S. aureus* elevated levels of *A. indica* extract would yield a more substantial impact on the bacterium resulting in a broader inhibition zone. While lower concentrations of neem extract might exhibit a range of activities, although with reduced effectiveness compared to higher concentrations (30). Similar findings were reported previously (10, 53).

Notably, *A. indica* leaf extracts displayed essential antibacterial properties (54). Antibacterial activity of neem leaf extracts could be due to the presence of phenolic compounds, carotenoids, ketones, triterpenoids, tetra-triterpenoids, azadirachtin, valavinoids and steroids (55). In current study minimum inhibitory concentration (MIC) of methanolic extract of *A. indica* leaves was 31.25mg/ml. Previous studies reported MIC of neem extract for *S. aureus* was 0.2mg/ml and 15.62mg/ml (10). Additionally, different antibiotic discs had showed different zones of inhibition 20.7mm (ciprofloxacin), 19.5mm (trimethoprim + sulphamethoxazole) and 18.95mm (tylosin) against *S. aureus* isolates (Table V). Shakuntala *et al.*, (2003) reported that maximum efficacy of ciprofloxacin and enrofloxacin whereas resistance of ampicillin and streptomycin was highest against pathogens linked with the genesis of mastitis in Meghalya (56). However, in current study *S. aureus* showed resistance against oxacillin, oxytetracycline and cefoxitin. Similar findings were reported previously (57-59). The variation in susceptibility of antimicrobial agents against *S. aureus* isolates in mastitis depended on region (60). According to current study, methanolic extracts could be used against *S. aureus* isolates in subclinical mastitis (53). It remained a valuable natural option due to its antibacterial properties especially in combating multidrug-resistant organisms where conventional antibiotics are non-responsive (53). However, there are limitations to using *A. indica* extracts for treating sub-clinical mastitis in Cholistani cattle (61). An important constraint to consider was that excessive doses of *A. indica* leaf extract might adversely affect liver and renal function in rats. Thus, precise dosing and vigilant monitoring were essential when administering neem extracts in cattle. The efficacy of *A. indica* extracts could fluctuate based on the specific extract used, its concentration and dosage and the severity of sub-clinical mastitis, moreover, various medicinal plants hold potential as effective sources of antibacterial agents against multidrug-resistant *S. aureus*, presenting valuable therapeutic opportunities (61, 62).

CONCLUSIONS

This study reveals a 45.53% prevalence of sub-clinical mastitis in Cholistani cattle based on SFMT with *Staphylococcus aureaus* found in 27.60% based on bacteriological culture on differential media. Data analysis on risk factors showed depth of udder, age, parity numbers and diameter of teat apex were recorded significantly ($p<0.05$) higher in diseased than healthy cows. However, total milk yield, teat to floor distance and length of teats were found significantly ($p<0.05$) lower in mastitic cattle. Also results of study showed high occurrence of sub-clinical mastitis in cemented floor (23.86%), closed housing (17.58%), grimy legs (18.89%), feeding calf (15.21%), folded thumb for milking (27.96%) and stall feeding (19.10%). The methanolic extracts of *A. indica* showed effective antibacterial activity against *S. aureus* suggesting its potential as a natural treatment option. Antibiotic susceptibility testing indicated variable resistance to some antibiotics underscoring the importance of careful antimicrobial management. Overall, addressing risk factors and utilizing natural antimicrobial alternatives can improve mastitis management in cattle. Further research should focus on the long-term effects and resistance trends.

Limitations:

Further investigation is necessary to determine the optimal dosing and formulation of neem extracts for the treatment of subclinical mastitis in Cholistani cattle. While, *A. indica* extracts show promise as a natural treatment option for this condition, additional research is needed to fully comprehend their therapeutic effectiveness and potential (61). Another concern regarding *A. indica* extracts is their potential side effects or interactions with other treatments i.e. *A. indica* extracts may not be suitable for use in breeding animals due to their prophylactic effects on certain species. Despite these challenges, *A. indica* extracts are becoming increasingly popular in veterinary treatment, with some companies offering animal and pet products derived from *A. indica*.

Conflict of Interest:

The authors declare that there are no conflicts of interest to disclose.

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