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FORMULATION AND EVALUATION OF HERBAL TOPICALS FOR BACTERIAL MASTITIS TREATMENT AND PREVENTION

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Abstract: Mastitis is one frequent disease observed in cattle causing a drastic loss in dairy and veterinary. However, antibiotics side-effects like Multi drug resistance calls for a safer and effective treatment. The objective of this project is to identify potential herbs having antibacterial effect against mastitis causing microorganism and formulate it into an ointment for topical application on infected udder. Development of herbal bar is aimed for prophylaxis of mastitis that can be used by the milkmen and also used for cleaning the cow udder. Fresh extracts and ethanolic extracts of *Tridax procumbens, Levisticum officinale, Aloe vera, Mentha spicata, Coriandrum sativum, Cynodon dactylon Trachyspermum ammi* and *Citrus limon* were used. The extract was incorporated into ointment and soap base. The products were tested for efficacy. Fresh extracts and ethanolic extracts of *Tridax procumbens, Levisticum officinale, Aloe vera, Mentha spicata, Coriandrum sativum, Cynodon dactylon Trachyspermum ammi* and *Citrus limon* were found to have antibacterial activity against *E. coli, Pseudomonas aeruginosa, Streptococcus agalactiae and Staphylococcus aureus* isolated from mastitis infected cows. This study represents a potential breakthrough in combating mastitis, offering relief to dairy farmers facing sudden economic losses. Given the disease's easily communicable nature, the developed herbal bar, boasting broad-spectrum antibacterial properties, could play a crucial role in disease control, especially as mastitis transmission commonly occurs through caretakers, water, or milking equipment. Such innovative product designs hold promise for safeguarding the dairy industry and veterinary sector in our country against unforeseen economic downturns. The product was subjected to antimicrobial and quality test for topical application. This natural formulation can be revolution to prevent mastitis.

Keywords: Antimicrobial activity, Cattle, Herbal extracts, Mastitis, Topical products.

1. INTRODUCTION

Mastitis, a pervasive disease in cattle, inflicts significant economic losses on the dairy industry globally, with Tamil Nadu standing as a prominent contributor to India's dairy sector[1]. Economic ramifications are profound, with mastitis-induced losses estimated at INR 1390 per lactation, translating to 38% of the dairy economy and half of all veterinary expenses[2]. Compounding the issue, conventional antibiotic treatments for microbial mastitis are fraught with challenges, including incomplete efficacy and potential side effects detrimental to both animal welfare and human health through consumption of contaminated dairy products. Traditional medicinal practices, leveraging the therapeutic potential of herbal remedies, offer promising alternatives to conventional treatments. Research in this domain aims to harness the innate antimicrobial properties of various medicinal herbs, such as *Tridax procumbens, Levisticum officinale, Mentha spicata, Coriandrum sativum, Cynodon dactylon, Aloe vera,* and *Citrus limon,* for the development of novel therapeutic formulations[3]. By exploring the synergistic effects of these botanical extracts, researchers strive to create potent formulations capable of combating multiple mastitis pathogens. The resulting products undergo rigorous quality and safety assessments to ensure compatibility for topical application in both cattle and humans. Moreover, the development of herbal bars with broad-spectrum antibacterial properties presents a promising strategy for controlling mastitis transmission, given its propensity for dissemination through caretakers, water sources, and milking equipment. Such innovative approaches hold immense potential to mitigate the economic burden of mastitis on the dairy industry while safeguarding animal welfare and public health. These efforts signify a crucial step towards sustainable disease management practices in dairy farming.

Among the array of highly contagious diseases afflicting cattle, including foot and mouth disease, haemorrhagic septicaemia, and peste des petits ruminants, mastitis stands out as a particularly concerning ailment for farmers. Mastitis, characterized by infection in the mammary gland tissue, manifests with both microbial and non-microbial origins. The severity of inflammation varies, classified into sub-clinical, clinical, and chronic forms, influenced by factors such as the causative pathogen's nature, the animal's age, breed, immunological health, and lactation status[2]. Research indicates a heightened incidence of mastitis infections during the monsoon season, though cases occur throughout the year[4]. Notably, animals in the lactation period of 30 to 90 days exhibit a significantly higher incidence rate, with approximately 83.3% of them affected. Moreover, a substantial proportion (75%) of animals in their first and second lactation cycles experience elevated rates of mastitis[5].

Bovine mastitis, an inflammatory condition affecting the mammary gland tissue in dairy cows, represents a significant challenge for the dairy industry globally. The disease leads to considerable economic losses due to decreased milk production, veterinary expenses, and potential culling of affected animals. Various pathogens, including bacteria like *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli,* and *Pseudomonas aeruginosa,* contribute to the onset and progression of mastitis[3,4]. Traditional treatment approaches typically involve antibiotics, but concerns over antimicrobial resistance and residues in dairy products have prompted a search for alternative therapies.

In recent years, herbal treatments have emerged as promising candidates for managing bovine mastitis. Research has explored the antimicrobial and anti-inflammatory properties of medicinal plants in combating mastitis pathogens. For instance, extracts from plants such as *Tridax procumbens, Levisticum officinale,* and *Mentha spicata* have shown significant inhibitory effects against mastitis-causing bacteria[6,7]. These herbal remedies have demonstrated efficacy in reducing bacterial growth and alleviating inflammation in mammary tissues. Moreover, synergistic combinations of herbal extracts have been investigated for enhanced therapeutic outcomes in mastitis management. Formulations incorporating herbs like *Aloe vera, Coriandrum sativum,* and *Citrus limon* have exhibited potent antimicrobial activity against mastitis pathogens like *Staphylococcus aureus* and *Streptococcus agalactiae*[8,9]. Additionally, topical applications of herbal ointments and soaps have been developed to target mastitis infections directly on the udder surface, providing localized treatment without systemic antibiotic use. Despite the promising results of herbal treatments in bovine mastitis, further research is warranted to validate their efficacy, safety, and practical application in dairy farming. Nevertheless, these natural remedies offer potential advantages in reducing reliance on antibiotics and promoting sustainable management practices in dairy production.

The prevalence of major mastitis pathogens in India, such as *Staphylococcus* sp., *Streptococcus* sp., *Escherichia coli*, and *Pseudomonas* sp., varies significantly in the milk of dairy animals[10]. Previous studies have reported the incidence of *Staphylococcus* sp., *Streptococcus* sp., and *Escherichia coli* as 45%, 13%, and 14%, respectively[11]. Gram-negative bacteria are frequently isolated from acute clinical cases of mastitis; however, *Escherichia coli*, *Streptococcus agalactiae*, and *Staphylococcus aureus* are the most common bacterial agents and pose an increasing problem, particularly in low somatic cell count herds[9]. Estimating the losses associated with clinical mastitis is extremely challenging, as they stem from the costs of treatment, discarding, death, and decreased milk production. These losses significantly impact the dairy industry's economic viability[12].

About 20.5 million people in India depend upon livestock for their livelihood. Livestock contributed 16 per cent to the income of small farm households against an average of 14 per cent for all rural households. Livestock supports two-thirds of rural community. It also provides employment to about 8.8 per cent of the population in India.

Mastitis is the main cause of death in adult dairy cows. Some studies have been reported that the incidence of sub clinical mastitis ranged from 19.20 to 83% in cows. About 9.4 per cent of animals had clinical mastitis; 66.7-77.4 per cent had sub clinical mastitis. In India, about 70-80% economic loss has been attributed due to sub clinical mastitis alone.

Total direct economic loss due to acute, sub-acute, chronic and gangrenous mastitis in Tamil Nadu was found to be INR 1163.80, 1817.80, 3111.00 and 35085.60, respectively in which milk production loss constituted the bulk. The total annual economic losses due to mastitis was calculated to be 7165.51 crore rupees.

The ‘Holy Grail’ of treatment and prophylaxis remains vaccination. Udder health management has grown over the past years and easy quantification by data analysis using test results provided by regular dairy herd improvement (DHI) tests are available. Antibiotics are used to treat mastitis for more than fifty years. Antibiotic treatment creates residues into milk, and avoidance of residues developed is a vital facet of mastitis treatment. But still there is a lack of the most efficient, safe, and economical treatment.

Mastitis is a critical disease and is responsible for greater loss in dairy industry. Despite years of research no effective vaccine or medicines could be made commercially available. However, bovine mammary gland is a difficult target for antimicrobial treatment. When used antibiotics cause severe side effects like multi drug resistance. An eco-friendly and effective treatment has become the need of the hour and this study has put effects on developing a herbal concussion against mastitis pathogens to be incorporated into ointment for topical application. Another major reason for the spread of mastitis is improper hygienic management practices lead to the increased incidence of Mastitis at the farm level. To ensure good hygiene of both cow and the farmer who are in close contact with the cattle, an herbal bar is developed with antimicrobial activities. The present study could be an efficacious step to mastitis treatment over riding the several antimicrobial researches previously reported. Mastitis poses a significant threat to cattle health, resulting in substantial economic losses within the dairy industry. With Tamil Nadu contributing to 53% of dairy farming and producing 51% of the milk, it ranks among the top ten milk-producing states. Economic losses due to mastitis, estimated at INR 1390 per lactation, account for 38% of dairy economies and approximately 50% of veterinary expenses. Despite its impact, microbial mastitis lacks a complete cure or vaccine, and current antibiotic treatments carry risks of side effects for both animals and consumers of dairy products. Traditional medicine offers promising avenues, with ongoing research into herbal remedies for mastitis. This project focuses on investigating medicinal herbs and formulating an herbal solution targeting multiple mastitis-causing pathogens, initially for topical application in cattle and humans. The developed product underwent rigorous quality and safety assurance for both human and cattle application. This study represents a potential breakthrough in combating mastitis, offering relief to dairy farmers facing sudden economic losses. Given the disease's easily communicable nature, the developed herbal bar, boasting broad-spectrum antibacterial properties, could play a crucial role in disease control, especially as mastitis transmission commonly occurs through caretakers, water, or milking equipment. Such innovative product designs hold promise for safeguarding the dairy industry and veterinary sector in our country against unforeseen economic downturns.

2. MATERIAL AND METHODS

2.1.Collection of plants

A total of seven plants were used in this study. The leaves of *Tridax procumbens, Mentha spicata, Coriandrum sativum, Cynodon dactylon, Aloe vera* and *Citrus limon* fruit skin except *Levisticum officinale* (purchased from Euro Food Product, USA) were collected from surroundings in Tirupattur, Tamil Nadu.

2.2. Isolation of microorganism

Milk samples of five mastitis affected cows were collected aseptically and serially diluted and spread plated on to nutrient agar and LB agar plates. The plates were incubated at 37°C for 24 hours. The plates were observed for growth after incubation.

2.3.Preparation of herbal extracts

The collected plant parts were washed in 0.5% sodium hypochlorite solution and crushed aseptically to obtain fresh extracts. The extracts were labelled and maintained as SET I.

The plant parts were individually subjected to ethanolic extraction using separating funnel. Each plant (100g-200g) was extracted with 200ml – 400ml ethanol except *Levisticum officinale* which was extracted using 200ml – 400ml Ethanol-Chloroform (1:1). The crude extract obtained was weighed and stored for further tests.

2.4.Antimicrobial sensitivity test

The fresh extract and crude extracts were subjected to antimicrobial sensitivity test. Kirby-Bauer well diffusion method was employed using Muller Hinton Agar. The isolates obtained from infected cow were used as test pathogens. The culture plates were well diffused with the extracts and incubated at 37°C for 24 hours. The antimicrobial sensitivity was tested for individual extract and for mixtures. The extract/extract composition with better antimicrobial activity was selected for formulation of products.

2.5.Formulation of herbal concussion

The potent extracts were selected and 2% of each extract was mixed into a concussion to be added for product formulation.

2.6.Formulation of ointment

Method 1: To 100ml of virgin coconut oil, 100g each of all five herbs selected were added and simmered in heat for about 2-3 hours for efficient extraction. The oil was strained to sterile container. To the oil, 60g-80g of honey wax was added and heated to let the wax dissolve with the oil. This mixture was poured into containers and cooled to solidify.

Method 2: The ointment was also made with petroleum Jelly base. The petroleum Jelly was liquefied using water bath and to this crude extract of the samples namely *Tridax procumbens, Levisticum officinale, Mentha spicata, Coriandrum sativum, Cynodon dactylon, Aloe vera* and *Citrus limon* was added at a concentration of 1% each and mixed to make a uniform concussion without any phase separation. This mixture was added into containers and let to cool.

2.7.Formulation of soap

Glycerine soap base was purchased from local shop and was melted in double pot. To the base the herbal concussion prepared was added and stirred without bubbles. To the mixture a chunk (1cm2) of pears glycerine soap was added for fragrance. The mixture was cooled to 45°C and poured into a mould without bubble formation and cooled for 12-24 hours.

2.8.Antimicrobial assay of ointment and soap

The agar dilution method was employed to study the antimicrobial assay of the ointment and soap. The herbal soap and ointment of 1g each was dissolved separately to obtain a suspension. This was added to respective molten Muller Hinton agar plates, mixed and allowed to set. To these plates, the test mastitis pathogens were inoculated and incubated at 37°C for 24hours. The plates were observed for the presence or absence of microbial growth.

2.9.Quality tests for formulated products

Soap

The soaps were tested for pH and non-irritability as it is developed to be used topically on both cow and human skin.

Test of pH: The average pH should be in-between pH 7.0 – pH 10.0. For pH test the soap was lathered using distilled water and to the lather a pH paper/strip was dipped. The pH was determined using the standard chart.

Test for non-irritability: For non-irritability test, 10 voluntary individuals were subjected to use the soap and were observed for ten days for any irritation.

Ointment

The ointment was tested for pH, phase separation and non-irritability as it is formulated to be used topically.

Test of pH: The average pH should be in-between pH 7.0 – pH 10.0. For pH test the ointment (1g) was melted and a pH paper/strip was dipped into it. The pH was determined using the standard chart.

Test for non-irritability: For non-irritability test, 10 voluntary individuals were subjected to apply the ointment on the surface of palm and were observed for few minutes to few hours for any irritation.

Phase separation: The ointment after preparation was visually observed for phase separation. The stable ointment with proper preparation and mixing will not separate phases.

3. RESULTS

3.1.Isolation of microorganism

The isolation of microorganisms from bovine mastitis-affected cows is a critical step in understanding the etiology, pathogenesis, and management of this prevalent infectious disease in dairy cattle. (Table 1, 2).

3.2.Antimicrobial sensitivity of herbal extracts

It was observed (Table 3) that ethanolic extract of *Tridax procumbens* gave better sensitivity to *E. coli, Pseudomonas aeruginosa* and *Streptococcus agalactiae* as compared to commercial antibiotics, *Levisticum officinale* gave good antimicrobial activity against *Staphylococcus aureus, E. coli* and *Streptococcus agalactiae*. Mentha spicata act as a coolant however was observed to have high antimicrobial activity against *Streptococcus agalactiae* as compared to commercial antibiotics, *Coriandrum sativum* showed good antibiotic sensitivity against *Staphylococcus aureus* and *E. coli*, *Cynodon dactylon* gave better sensitivity against *Streptococcus agalactiae* and *Staphylococcus aureus* and *Citrus limon* had higher activity against *Streptococcus agalactiae, Staphylococcus aureus* and *E.coli, Aloe vera* showed activity against *E.coli* and *Staphylococcus aureus*. This as a mixture in the product gave much more antimicrobial activity as compared to commercial antibiotics. In comparison to herbal extracts, standard antibiotics (Tetracycline, Kanamycin, Chloramphenicol, and Gentamicin) consistently showed high sensitivity across tested strains, with Tetracycline and Chloramphenicol performing exceptionally well. Ethanol alone (99%) did not exhibit significant antimicrobial activity against the tested strains. These results suggest that while some herbal extracts show promising antimicrobial properties, their efficacy varies compared to conventional antibiotics. These findings underscore the potential of herbal extracts as alternatives or supplements to traditional antibiotics, emphasizing the importance of further studies to explore their clinical applicability and mechanisms of action in combating antimicrobial resistance.

3.3.Antimicrobial assay of herbal concussion ointment and soap

The herbal concussion containing ethanolic extracts of *Tridax procumbens, Levisticum officinale, Mentha spicata, Coriandrum sativum, Cynodon dactylon, Aloe vera* and *Citrus limon* were prepared and incorporated into product. The antimicrobial assay was performed and the plates were observed after incubation of 24hrs at 37°C (Figure 1, Table 4, 5). The antimicrobial sensitivity assay of the product was observed and noted. The results from the antimicrobial assay of herbal soap, herbal ointment, and herbal formulation against various bacterial strains, along with the performance of Chloramphenicol (C) and Tetracycline (T) antibiotics, reveal insightful findings. The concussion gave higher antimicrobial sensitivity against the microorganisms. For *Staphylococcus aureus*, the herbal soap and ointment displayed similar efficacy, with inhibition zones of 30 mm and 30 mm, respectively, compared to Chloramphenicol's 22 mm. Against *Escherichia coli*, both the soap and ointment exhibited strong activity, with inhibition zones of 28 mm and 30 mm, respectively, surpassing Tetracycline's 25 mm. *Streptococcus agalactiae* was notably susceptible to the herbal formulation, showing a 33 mm inhibition zone, while the herbal ointment and soap also exhibited substantial activity with 32 mm and 37 mm zones, respectively. *Pseudomonas aeruginosa*, however, showed moderate susceptibility with inhibition zones ranging from 25 mm to 30 mm across the herbal products, while Chloramphenicol was effective against this strain with a 22 mm inhibition zone. The herbal formulations demonstrated competitive antimicrobial efficacy comparable to standard antibiotics, highlighting their potential as alternative treatments against these clinically significant pathogens. These findings underscore the importance of further investigation into the formulation's mechanisms and potential clinical applications in combating bacterial infections.

Furthermore, the study by Singh et al. (2016) emphasizes the significance of herbal concussions in addressing the challenge of multidrug-resistant pathogens, indicating their potential as valuable additions to the armamentarium against antimicrobial resistance.

3.4.Non-Irritability test

The herbal soap was tested for skin irritability for at least ten days. However, the ointment was applied for three days and the remaining days were observed for any after effects.

3.5.Phase separation

There observed no phase separation in the prepared ointment. Compared to formulations prone to phase separation, such as emulsions or suspensions, ointments typically exhibit greater stability due to their simpler composition and absence of dispersed phases.

3.6.Quantity of extract with bioactivity

The quantity of extract with bioactivity in skincare formulations plays a crucial role in determining the efficacy and safety of the final product (Table 6).

3.7.Ethics of research

No animal studies were performed as part of this research. Allergy testing on the researcher's own skin was conducted as part of this study. Given that the testing procedures were non-invasive and presented minimal risk, formal ethics approval was not sought.

4. DISCUSSION

The discussion delves into the potential of herbal formulations as an alternative treatment for mastitis, a prevalent and economically significant disease in dairy animals. Research by Bradley (2002)[13] and Contreras et al. (2007)[14] underscores the importance of isolating and identifying the microbial species responsible for bovine mastitis. By isolating microorganisms from mastitis-affected udder tissues or milk samples, veterinarians can accurately diagnose the infection and select appropriate antimicrobial treatments based on the susceptibility profiles of the identified pathogens[13]. A study by Sood et al. (2012)[15] demonstrated the antimicrobial potential of *Tridax procumbens* against *E. coli, Pseudomonas aeruginosa, and Streptococcus agalactiae*, corroborating the observation in your data. Comparison with commercial antibiotics could be supported by various studies such as that of Ahmed et al. (2015[16]), which compared the efficacy of plant extracts with standard antibiotics, showing the superiority of *Tridax procumbens* in certain cases. The antimicrobial activity of *Levisticum officinale* against *Staphylococcus aureus, E. coli,* and *Streptococcus agalactiae* can be supported by studies like Gulluce et al. (2003)[17]. Comparative analysis with commercial antibiotics might be reinforced by studies like those conducted by Rios and Recio (2005)[18], which evaluated the effectiveness of plant extracts against standard antibiotics. The antimicrobial activity of *Aloe vera against E. coli* and *Staphylococcus aureus* is supported by studies like Radha and Laxmipriya (2015)[19]. The antimicrobial activity of *Cynodon dactylon* against *Streptococcus agalactiae* and *Staphylococcus aureus* can be corroborated by studies such as Kumar et al. (2014)[20]. Antimicrobial activity against *Streptococcus agalactiae* could be supported by research such as that by Nascimento et al. (2000)[21], which explored the antibacterial potential of mint extracts. Research by Patel et al. (2017)[22] evaluated the antimicrobial activity of herbal concussions against various microorganisms, demonstrating their effectiveness. Another study by Mishra et al. (2019)[23] investigated the antimicrobial potential of herbal concussions prepared from multiple plant extracts, showing promising results in inhibiting microbial growth. The higher antimicrobial sensitivity observed with the herbal concussion could be attributed to several factors:

Concentration of active compounds: Herbal concussions often contain higher concentrations of active antimicrobial compounds compared to formulated products like ointments and soaps, which may result in increased efficacy.

Synergistic effects: Combining multiple plant extracts in a concussion may lead to synergistic interactions, enhancing their antimicrobial activity beyond what individual extracts might achieve.

Direct contact: Herbal concussions are applied directly to the affected area, allowing for better contact and penetration into microbial colonies, potentially leading to improved sensitivity. The findings from Saxena et al. (2018)[24] underscore the relevance of herbal concussions in clinical practice, suggesting their utility as effective antimicrobial agents against pathogens encountered in healthcare settings.

Research by Lodeiro-Martínez et al. (2019)[25] emphasizes the importance of prolonged testing periods to capture any delayed or cumulative effects that may arise from repeated exposure to skincare formulations. Conversely, the decision to apply the herbal ointment for three days followed by observation for remaining days for aftereffects reflects a targeted approach to assessing its immediate effects on the skin. Studies such as those by Akimoto et al. (2008)[26] and Lodeiro-Martínez et al. (2019)[25] have employed similar testing protocols to evaluate the acute effects of skincare products.

Research by Ghosh et al. (2015)[27] investigated the factors affecting the stability of pharmaceutical ointments, emphasizing the importance of proper formulation and manufacturing processes in preventing phase separation. Similarly, studies by Shah et al. (2017)[28] and Patel et al. (2018)[22] explored strategies for enhancing the stability and shelf-life of topical ointments, highlighting the significance of ingredient compatibility and homogeneity in minimizing phase separation. The absence of phase separation in the prepared ointment underscores its formulation quality and compatibility of ingredients. Studies by Chanchal et al. (2018)[29] and Kaul et al. (2019)[30] have highlighted the importance of optimizing the concentration of bioactive plant extracts in skincare formulations to enhance their efficacy against various dermatological conditions. The quantity of extract with bioactivity directly influences the therapeutic efficacy of skincare formulations. Research by Mishra et al. (2020)[23] demonstrated that increasing the concentration of bioactive compounds in herbal formulations resulted in improved antimicrobial activity against common skin pathogens.

CONCLUSION

Mastitis remains a complex disease and its management is a challenge. With the less effectiveness of antibiotics and its side effects, the implementation of herbal remedies could be of advantage. The present study developed a formulation of herbal soap for sanitation of the milkmen and cows promoting the prevention of mastitis without affecting the normal flora and herbal ointment for topical application on the udder of cow for treatment purposes. The product was found to be compatible for human use. The product was able to completely inhibit the growth of pathogens in 24-48 hours. The present study is the fruitful beginning to mastitis treatment and further this work is expected to be advanced into internal medicine formulation. A potent herbal formulation was developed utilizing leaf extracts of *Tridax procumbens, Levisticum officinale, Mentha spicata, Coriandrum sativum, Cynodon dactylon, Aloe vera,* and skin extract of *Citrus limon.* This formulation was then incorporated into an ointment designed for topical application on the udders of cows. Additionally, an herbal bar was formulated for prophylaxis and hygiene maintenance for both the cow and the farmer in close contact with diseased animals. Both the ointment and soap bar demonstrated effective antimicrobial activity against prevalent mastitis pathogens, including *Staphylococcus aureus, Streptococcus agalactiae, Pseudomonas aeruginosa, and Escherichia coli*. In tests comparing antibiotic inhibition rates, the herbal concoction exhibited 100% inhibition, while the herbal soap and ointment showed 100% inhibition against *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Streptococcus agalactiae,* with approximately 83% inhibition against *E. coli*. Moreover, rigorous safety and quality assessments revealed that both products maintained a safe pH level suitable for all skin types and exhibited no skin irritability, ensuring their suitability for widespread use. The products can be taken for further trials in animals and check for its efficiency.

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CONFLICT OF INTEREST

There is no conflict of interest.

TABLES AND FIGURES

Table 1 Analysis of isolates from infected cow samples

Feature	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
No. of colonies in Nutrient Agar	TNTC	TNTC	TNTC	TNTC	TNTC
No. of colonies in SDA	Colonies not observed	Colonies not observed	Colonies not observed	Colonies not observed	Colonies not observed
Selective medium for Isolation					
Blood agar	Beta haemolytic	Beta haemolytic	Alpha haemolytic	Alpha haemolytic	Beta haemolytic
No. of Colonies, cfu/ml	9×10^6	61×10^6	10×10^6	22×10^6	40×10^6
MacConkey agar	Non-lactose fermentation	Lactose fermentation	Lactose fermentation	Non-lactose fermentation	Lactose fermentation
No. of Colonies, cfu/ml	50×10^6	60×10^6	77×10^6	23×10^6	10×10^6
Mannitol Salt Agar	Yellow colonies	Gold yellow colonies	Gold yellow colonies	Yellow colonies	Gold yellow colonies
No. of Colonies, cfu/ml	100×10^6	55×10^6	10×10^6	80×10^6	22×10^6
Eosin Methylene Blue Agar	Metallic sheen	Metallic sheen	Metallic sheen	Metallic sheen	Metallic sheen
No. of Colonies, cfu/ml	66×10^6	90×10^6	9×10^6	44×10^6	102×10^6
Pseudomonas isolation base agar	No growth	No growth	Blue-green colonies	Blue-green colonies	No growth
No. of Colonies, cfu/ml	-	-	6×10^6	12×10^6	-

TNTC = Too numerous to count, SDA = Sabouraud Dextrose Agar, CFU = colony forming unit, ml = millilitre

Table 2 Characterisation of the isolates

Feature	Unknow 1	Unknow 2	Unknow 3	Unknow 4
Gram staining	Gram Negative (Rod)	Gram positive (Cocci in Clusters)	Gram positive (Diplococci)	Gram negative (Curved Rod)
Motility test	**Motile**	Non-motile	Non-motile	**Motile**
Oxidase test	**Positive**	Negative	Negative	**Positive**
Catalase test	**Positive**	**Positive**	Negative	**Positive**
Urease test	Negative	Negative	Negative	Negative
Indole test	**Positive**	Negative	Negative	Negative
Methyl Red test	**Positive**	Negative	Negative	Negative
Voges Proskauer test	Negative	**Positive**	**Positive**	Negative
Carbohydrate (Glucose) Fermentation test	**Positive**	**Positive**	**Positive**	**Positive**
Citrate Utilization test	Negative	**Positive**	Negative	**Positive**
Interpreted organisms	*E. coli*	*S. aureus*	*S. agalactiae*	*P. aeruginosa*

Table 3 Antibiotic Sensitivity testing (Well diffusion method)

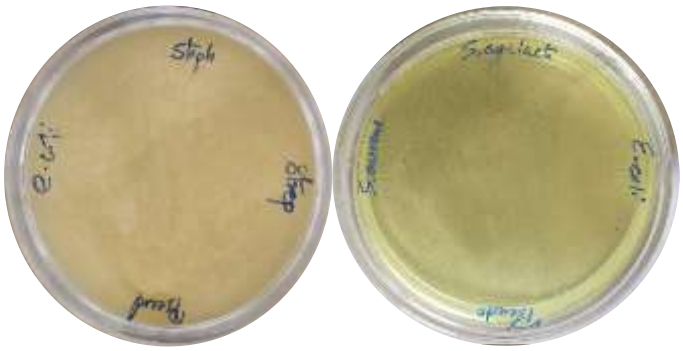
S. No.	Sample (100 µl)	Solvent	Zone of inhibition (mm diameter)				Resistant /Sensitive			
			S. aureus	*E. coli*	*S. agalactiae*	*P. aeruginosa*	*S. aureus*	*E. coli*	*S. agalactiae*	*P. aeruginosa*
1.	*Tridax procumbens extract*	Ethanol	18	20	19	20	S	S	S	S
2.	*Tridax procumbens*	Fresh	10	16	12	-	R	S	R	R
3.	*Cynodon dactylon*	Ethanol	18	11	21	10	S	R	S	R
4.	*Cynodon dactylon*	Fresh	10	9	16	-	R	R	S	R
5.	*Coriandrum satinum*	Ethanol	21	19	-	17	S	S	R	R
6.	*Coriandrum satinum*	Fresh	17	15	-	-	S	S	R	R
7.	*Mentha spicata*	Fresh	-	-	15	14	R	R	S	R
8.	*Mentha spicata*	Ethanol	16	12	30	22	S	R	S	R
9.	*Aloe vera*	Fresh	16	19	18	-	S	S	S	R
10.	*Aloe vera*	Ethanol	-	-	-	-	R	R	R	R
11.	*Levisticum officinale*	Ethanol + Chloroform	24	20	19	13	S	S	S	R
12.	*Citrus limon*	Ethanol	31	22	12	30	S	S	R	S
13.	*Citrus limon*	Fresh	25	19	09	20	S	S	R	S
14.	Tetracycline (T)*	Disc	18	30	33	13	S	S	S	S
15.	Kanamycin (K)*	Disc	19	22	10	08	S	S	R	R
16.	Chloramphenicol (C)*	Disc	22	15	32	-	S	S	S	R
17.	Gentamicin (GEN)*	Disc	17	16	18	18	S	S	S	S
18.	Ethanol	99%	07	-	-	-	R	R	R	R

Size of well = 05mm, R = Resistant, S =Sensitive, mm = millimetre, % = percentage, µl = microliter.

Table 4 Antimicrobial assay of herbal soap and ointment

Hours	**Herbal Soap**				**Herbal Ointment**			
	S. aureus	*E. coli*	*S. agalactiae*	*P. aeruginosa*	*S. aureus*	*E. coli*	*S. agalactia*	*P. aeruginosa*
12	No growth	Slight growth	No growth	No growth	No growth	No growth	No growth	No growth
24	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth

Figure 1 Antimicrobial assay of Herbal soap and Herbal ointment



Antimicrobial assay of Herbal Soap **Antimicrobial assay of Herbal Ointment**

Table 5 Antimicrobial sensitivity test of herbal soap and ointment against pathogens

Zone of inhibition (mm diameter)											
Herbal Soap				Herbal Ointment				Herbal Formulation			
S. aureus	***E. coli***	***S. agalactiae***	***P. aeruginosa***	***S. aureus***	***E. coli***	***S. agalactiae***	***P. aeruginosa***	***S. aureus***	***E. coli***	***S. agalactiae***	***P. aeruginosa***
31	35	37	28	30	28	32	30	30	30	33	25
Chloramphenicol (C)*											
22	16	30	-	22	16	30	-	22	16	30	-
Tetracycline (T)*											
17	25	22	13	17	25	22	13	17	25	22	13

mm = millimetre

Table 6 Composition of plant extract in ointment and soap

Herbal Plants	Herbal Ointment Contents Quantity (g) per 100g	Herbal Soap Contents Quantity (g) per 100g
Tridax procumbens	1.134	1.701
Mentha spicata	1.752	1.812
Coriandrum sativum	1.208	2.628
Cynodon dactylon	1.81	2.715
Citrus limon	1.01	1.362
Levisticum officinale	0.908	3.9
Aloe vera	2.6	1.5

g = gram

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