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Evaluation of antibacterial effect of medicinal plant extracts on bacteria isolated from subclinical bovine mastitis

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Abstract

A cross sectional study was carried out from December 2018 to March 2019 in Thoothukudi town and its surrounding areas in order to determine the prevalence of subclinical mastitis and conducting antibacterial activity on the isolated pathogens. The study populations comprised of lactating dairy cows that were found in different age groups, parities and lactation stages. The milk samples were aseptically collected from a total of 120 quarters of teats from 30 apparently healthy cows. When the collected milk samples were screened by using the California Mastitis Test (CMT), 43.3 % of quarters and 53% of cows were found to be positive. The bacterial purification and identification depended on the morphology of colonies, catalase production, the response to Gram-stain and biochemical tests. The commonly recovered organisms were *Staphylococcus aureus*, CNS (Coagulase negative *Staphylococcus*), *Streptococcus uberis*, *Escherichia coli*, *Micrococcus* spp. and *Bacillus* spp. Among the isolated pathogens, *S. aureus*, CNS, *S.uberis*, *Bacillus* and *E. coli* were the most prevalent that accounted 55.47%, 15.74%, 12.7%, 11.89%,8.93% and 5.75%, respectively. The antibacterial activity against the isolated bacteria was evaluated by determining the disc diffusion method. The aqueous and ethanol extracts of selected medicinal plants (*Heliotropium curassavicum* and *Alternanthera sessilis*) were obtained by extraction in cold maceration method. Both the extracts were assessed for their antibacterial activity against isolated subclinical mastitis pathogens. The isolation of pure pathogens and testing their antimicrobial susceptibility are vital to effectively treat and control the disease

I. Introduction

Mastitis is a grave disease in dairy animals causing excessive economic losses due to decrease in milk yield as well as sinking its nutritive value. Bovine mastitis is caused by entry of bacteria in the mammary gland leading to inflammation. This dynamic disease, in which infection and inflammation wax and wane [1] is marked by physical and chemical changes in the milk, and pathological changes in the glandular tissue [2]. Of the 135 infectious agents associated with clinical mastitis episodes in dairy cattle, the most commonly isolated are *Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae,* and *Escherichia coli* [3] , [4]. *S. aureus* and *E. coli* are the most frequent causes of contagious and environmental clinical mastitis in dairy cattle, respectively [5], while *Klebsiella* is the most frequently found clinical mastitis pathogen in free-stall dairy cattle herds in Western Canada [5]. Many factors can influence the development of mastitis; however, Coagulase-negative *staphylococci* (CNS) were the most frequently isolated bacteria [6] and increased resistance for it was reported [7].

The conservative drugs used for the action of mastitis are of limited in types in emerging countries in general and in India specific due to this and other factors causal agents have showed adjustable degree of fight. Indians have been traditional users of plant derived medicines both directly and as an integral constituent of plethora of packages and practices of indigenous medicine. These plants and their extracts are being used in the pharmaceutical preparations of modern medicine, veterinary and in agriculture. This what guided our attention to the plant kingdom which might be a substitute cure when synthetic chemical compounds are unable to perform their role.

Heliotropium curassavicum L., belongs to the family Boraginaceae. It is perennial herb or prostrate creeper or erect shrub approaching 0.5m in height. The foliage and stem are fleshy, with the leaves spade shaped or thick and oval. The native place of this plant is tropic South America. It is also called salt heliotrope. It mostly occurs in Saline or alkaline flats, plains and meadows, usually along seashores, at the elevations of sea level 600 m. Mutagenic and carcinogenic activity of pyrrolizidine alkaloids have also present in this plant A tea is made from the dried leaves. The dried roots are ground to powder and applied to sores and wounds.

Alternanthera sessilis L., belongs to the family Amaranthaceae. It is a perennial herb, many branched. Its habit and dimensions vary greatly depending on the humidity level, in dry conditions, it is erect and reach 30 cm in length; in wet conditions, it is prostrate, then erect with stems 10cm-1m long. In flooded areas, it is a floating herb reaching several meters in length. The plant occurs throughout tropical and subtropical regions. It contains the Alkaloids, Terpenoids, Tannins, Flavonoids, Phenolic compounds, Glycosides to observe phytochemical screening tests. *Alternanthera sessilis* include many medicinal properties. They are most effective in skin diseases. It cures asthma, lung diseases, Headache, to prevent the eye diseases. The present work aims to isolate and identify microorganisms causing bovine mastitis and evaluate the antibacterial activity of selected medicinal plant extracts against bacteria isolated from bovine mastitis.

II. Material and methods

Study Design

The cross sectional study was carried out between December 2018 to March 2019 in order to determine the prevalence of subclinical mastitis and to conduct antibacterial activity on the isolated pathogens. Purposive sampling method was used to select the cows according to the willingness of the owner. The sampling unit was each udder quarter, with apparently normal milk secretions.

Milk Sample Collection

The milk samples were aseptically collected from cows not treated early with either intra mammary or systematic antimicrobials agents. The sterile collection tube was used and the first stream of milk from each quarter was discarded. Later, the milk samples were kept in cold chain and transported to the laboratory (Table no.1 & 2).

Detection of Mastitis

The collected milk samples were further investigated by California Mastitis Test (CMT). This test was carried out as a screening test in order to diagnose the presence of subclinical mastitis in the collected samples according to the standard procedure of [8].

Isolation of Bacteria

Milk samples with the results of CMT ≥4 were examined for presence of bacteria. The samples were cultured on 5% sheep blood agar and MacConkey agar (MAC) plates. The plates were incubated under aerobic conditions at 37 °C for 18 h – 24 h. The bacteria were identified on the basis of colony morphology, responses to Gram stain, catalase production and biochemical properties according to the Quinn *et al.* [8].

Collection of Plants

Fresh plant parts were collected randomly from the gardens/tribal locations and villages of Tuticorin and Nagercoil district, Tamilnadu, India. The taxonomic identities of plants were confirmed by Dr. Soosai Raj, Assistant Professor, Department of Botany, St. Joseph's College, Trichy, Tamilnadu, India and the voucher specimen of the plants have been preserved at FA. Rabinant Herbarium St.Joseph's College Trichy. The collected plants were washed with running tap water, air dried, homogenized to a fine powder and stored in air-tight bottles at 4^{o}C.

III. Preparation of crude extracts

Solvent extraction

100 grams of dried plant material was extracted with 200 ml of ethanol kept on a rotary shaker for 24 h. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was composed and the solvent was faded to make the final volume one-fifth of the original size. It was kept at 4^{o}C in airtight jugs for extra studies.

Aqueous extraction

100 grams of dried plant material was extracted in distilled water for 6 h at slow heat. Every 2 h it was filtered through 8 layers of muslin cloth and centrifuged at 5000 g for 15 min. The supernatant was collected. This process was repeated twice and after 6 h the supernatant was focused to make the final size one-fifth of the unique size.

Disc Diffusion method

Mueller- Hinton agar plates were prepared aseptically to get a thickness of 5-6 mm. The plates were allowed to solidify and inverted to prevent the condensate falling on the agar surface. The plates were dried at 37ºC before inoculation. The organisms were inoculated in the plates prepared earlier, by dipping sterile swab in the previously standardized inoculums (Mac Farland), removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and streaking the swab all over the surface of the medium 3 times, rotating the plates through an angle of 60º after each application. Finally the swab was pressed round the edge of the agar surface. The inoculated medium was allowed to dry at room temperature with the lid closed. The discs were impregnated with 15µl of each of the extract and the plates were kept at room temperature for absorption of extract in the medium and then incubated at 37°C in the incubator for 24 hr. Diameter of the zones of inhibition were measured and compared with that for Ciprofloxacin.

IV. Results

A total of 120 milk samples (30 healthy cows) were screened for mastitis by CMT. The prevalence of subclinical mastitis and normal healthy animals were 43.3%, and 53% respectively. Out of 120 cow's milk samples 150 isolates were obtained from 80 normal healthy and 70 subclinical cases of mastitis at various regions of Thoothukudi town. The commonly recovered organisms were *Staphylococcus aureus*, CNS (Coagulase negative *Staphylococcus*), *Streptococcus uberis*, *Escherichia coli*, *Micrococcus* spp. and *Bacillus*

spp. Among the isolated pathogens, *S. aureus*, CNS, *S.uberis*, *Bacillus* and *E. coli* were the most prevalent that accounted 55.47%, 15.74%, 12.7%, 11.89%, 8.93% and 5.75%, respectively (Table 3). They are considered the culturing of bacterial is a gold standard method. Gram-negative bacteria were identified by sub culturing on differential and selective media and tested to oxidase activity, acid production (glucose, lactose and sucrose fermentation), indole test, VP test and hydrogen sulfide production as National Mastitis Council' s guidelines as in Table no 4. The antibacterial activity of *H.curassavicum* and *A.sessilis* plant extracts that have antibacterial effect on mastitis isolated bacterial pathogens, the highest activity were observed with ethanol extracts of *H.curassavicum* compared to *A.sessilis* on Gram-positive bacteria *S. aureus, CNS, S. uberis* and *Bacillus* with DIZ values in range of 12, 10, 10 and 5 mm respectively. The aqueous extracts of both the plant extracts have shown less antibacterial activity (Table no 5). Evaluation of antibacterial activity (MIC) was carried out by micro dilution method for ethanol and aqueous extracts of *H.curassavicum* and *A.sessilis* on mastitis causing pathogens. The MIC value of *H.curassavicum* was found by ethanol extract exhibited the best antibacterial activity against Gram-positive bacteria.

V. Discussion

Mastitis in dairy cows is a serious problem as it is an economically devastating disease causing immense economic losses in dairy cows and bio health hazard to human worldwide especially in developing countries. The present investigation clearly indicated that uses of clinical inspection and CMT examination was a efficient diagnostic tool fordetection and differential of clinical and subclinical mastitis and apparently normal health cattle and this observation is in agreement with Gianneechini *et al*. [9]. They found that the screening of clinical and subclinical mastitis in animals by CMT is still the superior diagnostic tool. The results showed that the quarter was considered sub-clinically affected when positively by CMT. The high prevalence of subclinical mastitis in dairy cattle may be due poor hygiene and poor management in rural areas which the small or individuals dairy unit owners have no concept of subclinical mastitis, teat dipping, dry cow treatment and usually do not keep adequate herd records, this explain is in agreement with Fadlelmoula *et al.* [10].

The highest prevalent of *S. aureus* and *E. coli* may be due to transmission by teat-to-teat or cow-to-cow spread, possibly via by the Milker's hands under the lack of hygiene, these findings were in agreement with Das *et al*. [11], who considered these microorganisms as major etiological agents of clinical and subclinical mastitis worldwide. Gram-positive and catalase test positive distinguished between *Streptococci* and *Staphylococci*. The hemolytic designs and coagulase reaction with rabbit plasma were used to distinguish between *S. aureus* and CNS. Also, esculin hydrolysis and CAMP reaction distinguished between *S. agalactia* and added strep.

Due to increased indiscriminate and frequent use of those antibiotic in dairy animals leading to develop of antibiotic resistance bacteria which necessitates develop and search for novel sources as antimicrobial agents. Medicinal plant-derived mixes have increased extensive interest in the search of other antibacterial agents. They are safe and have a long history of use in traditional medicine for the conduct of communicable diseases. This activity may be attributed to the rich plant contents of active components such as alkaloids, terpenoids and tannins. The MIC for *A. sessilis* leaves extracts against Gram-positive bacteria particularly was found to be significantly active exhibiting the little potency with all solvents used and this confirms to the need for further study.

The current study highpoints on separation and identification of mastitis pathogens that are fundamental aspects of udder health control programs, milk quality and public health and food safety issues associated with food borne pathogens. Our results proved that the *H.curassavicum* and *A.sessilis* plant extracts can be used as antimicrobial agent as a natural alternative manner to some of the commonly used antibiotics in human and animals but further researches are still necessary to identify active compounds, effectiveness, toxicity, safety indices and clinical trials in treatment of infectious diseases.

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Table 1: Areas of Milk sample collections in and around Thoothukudi town

S.No	Location	Type	Colour
1	Tuticorin	Nattumadu	White
2	Dheivaseyalpuram	Jersey	White
3	Dheivaseyalpuram	Sindhu	White
4	Settilampatti	Nattumadu	Pale Yellow
5	Eppodumvendran	Nattumadu	White
6	Athanoor	Sindhu	White
7	Kurukkusalai	Nattumadu	Pale Yellow
8	Meenachipatti	Jersey	White
9	Meenachipatti	Sindhu	White

Table 2: Characterization of Respondents and Herds Per Production in the Study Area

Parameter	No. of responses	Percentages
Sex		
Male	5	55.55
Female	4	44.44
Age		
21-30	-	-
31-40	9	100
41-50	-	-
Education		
Primary	9	100
Secondary	-	-
Cattle Bread		
Nattu Madu	4 (Tuty, Epvn, Kks, Sp)	44.44
Jercy	2 (Dsp, Mnp)	22.22
Sindhu	3 (Dsp, Mnp, Atn)	33.33
Lactating Cows	9	100
Milk Production		
(mean L/ day)	90 Lit.	-
Milking Frequency		
Once	0	-
Twice	9	100

Table 3. Examination of Cow's milk samples and isolated bacterial species

Sub Clinical Isolates (No. of Colonies)							
Place	**No. of colonies**	***S. aurus***	**CNS**	***S.uberis***	***Bacillus sp.***	***E.coli***	***Micrococcus***
Tuticorin	40	25 (62.5%)	-	10 (25%)	3 (7.5%)	-	2 (5%)
Dheivaseyalpuram	50	20 (40 %)	8 (16%)	5 (10%)	4 (8%)	10 (20%)	3 (6%)
Dheivaseyalpuram	45	30 (66.6%)	-	-	5 (11.11%)	5 (11.1%)	5 (11.1%)
Settilampatti	60	30 (50%)	10 (16.66%)	8 (13.33%)	5 (8.33%)	5 (8.33%)	2 (3.3%)
Eppodumvendran	55	25 (45.45%)	10 (18.18%)	15 (27.27%)	-	-	5 (9.09%)
Athanoor	50	30 (60%)	-	8 (16%)	5 (10%)	-	7 (14%)
Kurukkusalai	70	45 (64.28%)	10 (14.28%)	5 (7.14%)	5 (7.14%)	-	5 (7.14%)
Meenachipatti	50	30 (60%)	8 (16%)	-	10 (20%)	2 (4%)	-
Meenachipatti	60	30 (50%)	8 (13.33%)	5 (8.33%)	5(8.33)	5(8.33)	7(11.6)
Over all percentage	-	55.47	15.74	12.7	11.89	5.75	

Table 4. Biochemical characteristics of isolated bacteria

Bacterial Strains	Tests and Observation						Gram stain	Inference
	Indole	MR	VP	Citrate	Cattalse	Oxidase		
Strain 1	+	+	-	-	+	-	G-	*E.coli*
Strain 2	-	-	+	+	+	Variable	G+	*Bacillus*
Strain 3	-	+	+	+	+	-	G+	*Staphylococcus*
Strain 4	+	+	-	-	+	-	G+	*Streptococcus*

Table 5: Antimicrobial activity of crude extracts of *H.curassavicum* and *A.sessilis* against mastitis pathogens

Microbial pathogens	*H.curassavicum*		*A.sessilis*	
	Zone of inhibition (mm)			
	Ethanol	Aqueous	Ethanol	Aqueous
S. aureus	12	8	11	6
CNS	10	6	9	6
S.uberis	10	6	7	5
Bacillus sp	5	5	5	5
E.coli	5	5	5	-
Minimum Inhibitory Concentration (mg/ml)				
S. aureus	0.5	1.0	1.0	-
CNS	1.0	1.0	1.0	-
S.uberis	1.0	4.0	-	-
Bacillus sp	1.0	-	-	-
E.coli	4.0	-	-	-