RESEARCH ARTICLE

Antimicrobial Efficacy of *Nyctanthes arbor-tristis* Linn. Leaf Extract against Common Pathogens of Sub-Clinical Mastitis in Cows

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Abstract

This study was conducted during December 2020 to June 2021 by screening 200 cows for sub-clinical mastitis by using California mastitis test and Whiteside test, and the antimicrobial efficacy of *Nyctanthes arbor-tristis* Linn (Jasmine, Parijatha) leaves extract was studied against mastitis pathogens. The *in vitro* efficacy of aqueous and methanolic extract of *Nyctanthes arbor-tristis* leaves and its combination was studied against a positive control, *i.e.,* antibiotic ciprofloxacin. The isolates of *Streptococcus agalactiae showed* maximum zone of inhibition against combination of aqueous and methanolic extract, while *S. aureus* and *Escherichia coli* showed maximum zone of inhibition against aqueous extract. The methanolic and aqueous extracts, and its combination of *Nyctanthes arbor-tristis* leaves had minimum inhibitory concentration (MIC) value 50 µg/mL and 25 µg/mL, against *Streptococcus agalactiae* and *Staphylococcus aureus,* respectively. The MIC values of methanolic and combination of both extracts against *Escherichia coli* was 50 µg/mL in comparison to aqueous extract, *i.e.,* 12.5 µg/mL. The aqueous extract had minimum bactericidal concentration (MBC) against *Streptococcus agalactiae* as 100 µg/mL. In methanolic and combination extract of *N. arbor-tristis* the highest value of MBC obtained for *Escherichia coli*, and *Streptococcus agalactiae* was 50 µg/mL each.

Keywords: Cattle, MBC, MIC, *Nyctanthes arbor-tristis* extracts, Sub-clinical Mastitis.

Ind J Vet Sci and Biotech (2022): 10.21887/ijvsbt.18.3.4

Introduction

Bovine mastitis is the most common and persistent inflammatory reaction of the udder tissue due to physical trauma or infections caused by microbial organisms. It is also the most costly disease of the dairy industry. Mastitis is commonly treated by administration of intramuscular or intravenous injection, or intra-mammary infusion of antibiotics such as penicillin, ampicillin, streptomycin, cloxacillin *etc*. (Bhosale *et al*., 2014). Pathogens have acquired resistance due to injudicious use of drugs, so many on-going studies are focused on treatment of mastitis by alternative methods and to replace antibiotics (Kalinska *et al*., 2019).

Nyctanthes arbor-tristis (Oleaceae) is a mythological plant and has high medicinal values in Ayurveda. One of the oldest systems is Ayurveda that uses plants and their extracts for treatment and management of various diseases. In India it is considered as one of the most useful conventional medicinal plant. It has several medicinal properties such as antibacterial, anti-helminthic, antipyretic, anti-inflammatory, antioxidant activities, hepatoprotective, anti-leishmaniasis, antiviral, antifungal, antihistaminic, antimalarial, and as an immunostimulant besides it is used as a laxative, in rheumatism, skin ailments and as a sedative (Rathee *et al.,* 2007; Narendhirakannan and Smeera, 2010; Bhalakiya and Modi, 2019). Crude extracts and isolated compounds from

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How to cite this article: Deepraj, Singh, J.P., Singh, S., Yadav, D.K., Ramakant, Niyogi, D., Joshi, R.K., Shrivastava, D.P. (2022). Antimicrobial Efficacy of *Nyctanthes arbor-tristis* Linn. Leaf Extract against Common Pathogens of Sub-Clinical Mastitis in Cows. Ind J Vet Sci and Biotech. 18(3), 16-20.

Source of support: Nil

Conflict of interest: None.

Submitted: 10/11/2021 **Accepted:** 15/01/2022 **Published:** 10/07/2022

the plant of *Nyctanthes arbor-tristis* (NAT) were shown to be pharmacologically active against inflammation, malaria, viral infection and leishmanisis. The antibacterial potential of NAT was evaluated on Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*) bacteria (Priya and Ganjewala, 2007). Methanolic extract of NAT leaves exhibits significant antibacterial activity against *S. aureus, Staphylococcus epidermis, Salmonella typhi* and *Salmonella paratyphi*-A with MIC value ranging between 1 and 8 mg/mL (Mahida and Mohan, 2007). No authentic literature is available on the effect of NAT extract on pathogens of sub-clinical mastitis (SCM) in cows. The present study was aimed to evaluate the *in vitro* antimicrobial efficacy of *N. arbor-tristis* Linn. leaf extracts against common pathogens of sub-clinical mastitis in cows.

Materials and Methods

Screening of Animals

The present study was conducted following ethical approval of IAEC during December 2020 to June 2021. A total 200 sub-clinical cases of mastitis in cows were screened from 4 blocks and 8 villages of Amethi and Sultanpur districts of Uttar Pradesh. Animals were screened based on cow-side tests namely California Mastitis Test (CMT) and White Side Test (WST). The physical examination of udder and teat was performed as per Schalm *et al*. (1971). The *in-vitro* efficacy of aqueous and alcoholic *Nyctanthes arbor-tristis* leaves extracts was studied against common mastitis causing organisms.

Preparation of Extract and Antibacterial Activity Test

Approximately 7 kg fresh leaves of *Nyctanthes arbor-tritis* were collected and dried at room temperature, powdered and stored. The extract of dried leaf powder of *N. arbor-tritis* was made in three different solvent system, *viz*., methanol, water and methanol plus water (7:3) by the Soxhlet extraction method and extracts were stored as pastes for testing the antibacterial activity.

The antibacterial activity test was performed by Kirby-Bauers disc diffusion method (Bauer *et al*., 1966) on Mueller Hinton agar (Hi Media, Mumbai, India) as per the CLSI guidelines (Clinical Laboratory Standards Institute, 2014). Sample extract used for the test was prepared for the concentration of 1 mg/mL in DMSO (Dimethyl sulfoxide) solvent, for each solvent extract. The discs were placed at respective position. Plates had two discs other than sample, one of the positive controls, that is antibiotic Ciprofloxacin at 20 µg/mL and 100% DMSO as a negative control. The test was carried out in duplicate and was repeated twice (Mahida and Mohan, 2007).

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial or drug that inhibits the visible growth of bacteria after overnight incubation (Levison, 2004), while minimum bactericidal concentration (MBC) is the lowest concentration of antibacterial agent required to kill a particular bacterium (Wiegand *et al.,* 2008). To determine the MIC value of all three extracts, broth micro-dilution method was applied (Wagenlehner *et al*., 2006). For each sample, *i.e.,* against one isolate, five different concentrations used were: 100, 50, 25, 12.5 and 6.25 µg/mL. For the preparation of initial concentration of 100 µg/mL, 400 µL of the sterile nutrient broth, 100 µL of the sample extract (1 mg/mL) and 500 µL of suspension of bacterial isolates (matched with 0.5 Macfarland standard) were used. From the tube having 100 µg/mL concentration, a serial dilution was performed to get other lower concentrations and volume of each tube was kept 1 ml. Based on the observation, the minimum concentration of the sample at which there was no visible growth, that is no turbidity in the tube, was taken as the MIC value of that sample and then 100 µL aliquot from these tubes was inoculated on the Nutrient agar media plates. The minimum concentration at which no colony growth appeared on the media plate was taken as the MBC value for that sample.

The Relative Percentage Inhibition (RPI) of the test extract with respect to positive control was calculated by using the following formula of Paluri *et al.,* (2012) : RPI=100 (X-Y)/(Z-Y), Where, X= Total area of inhibition of the test extract; Y=Total area of inhibition of the solvent and Z= Total area of inhibition of the standard drug.

Results and Discussion

A Total of 200 cattle screened from 4 blocks and 8 villages using CMT and WST revealed overall subclinical mastitis prevalence as 63.50 %, with 64% and 63% cases positive in Amethi and Sultanpur districts, respectively.

In-vitro Efficacy of Extracts of N. *arbour-tristis* (NAT) Leaves

The zones of inhibition recorded against aqueous, methanolic and aqueous-methanolic extracts of N. *arbour-tristis* (NAT) leaves and against positive and negative controls for *S. agalactiae*, *S. aureus* and *E. coli* are presented in Table 1 and Figure 1.

The results revealed that the isolates of *S. agalactiae* exhibited maximum zone of inhibition against positive control*, i.e.,* standard antibiotic Ciprofloxacin (19.16 ± 0.40 mm). Among the different extracts of NAT leaves the highest zone of inhibition was shown by aqueous-methanolic extract (11.50 ± 0.83 mm) followed by methanolic extract (10.33 ± 0.51 mm), and the least zone of inhibition was shown by aqueous extract (9.16 ± 0.75 mm) against *S. agalactiae* (Table 1).

Further, the comparison of the different extract and standard control antibiotic disc, revealed the highest zone of inhibition against the *S. aureus* by positive control, *i.e.,* standard antibiotic disc (20.66 ± 0.51 mm), and among the different extract of NAT leaves the highest zone of inhibition was shown by aqueous extract (15.16 ± 0.75 mm) followed by aqueous-methanolic extract of leaves (12.00 ± 0.89 mm) and the least one in methanolic extract (11.60 ± 0.81 mm) against the *S. aureus* (Table 1).

For *E. coli* also the highest zone of inhibition was shown by positive control (22.66 ± 0.51 mm), and among the different extract of NAT leaves the highest zone of inhibition was shown by aqueous extracts (20.50 ± 0.54 mm) followed by aqueous-methanolic extract (10.66 ± 0.51 mm) and methanolic extract (10.55 ± 0.83 mm) which were at par (Table 1). In an earlier study with methanolic extract of NAT leaves, the zones of inhibition reported for *S. epidermidis*, *S. aureus*, *Salmonella typhi* and *Salmonella paratyphi*-A were 11 mm, 4 mm, 2 mm and 7 mm, respectively (Mahida and Mohan, 2007).

Relative Percent of Inhibition

Among different extract of *N. arbor-tristis* leaves, namely aqueous, methanolic and its combination studied against. *S. agalactiae*, *S. aureus* and *E. Coli*: the maximum relative percentage inhibition (RPI) was recorded for aqueous extract of *N. arbor-tristis* leaves against *E. coli,* followed by *S. aureus* and it was least against *S. agalactiae* (Table 2). In methanolic extract, maximum RPI was shown by *S. aureus* followed by *S. agalactiae* and *E. coli*. In combination extract, maximum RPI was shown by *S. agalactiae* followed by *S. aureus* and *E. coli*. Aqueous extracts of the leaves of all the plants appeared to have less antibacterial activity than the methanol extracts (Parekh and Chanda, 2007), but in our study, aqueous extract had more antibacterial activity as compared to methanolic extract. This could be possible due to the presence of more bioactive phytochemicals in the aqueous extracts.

MIC and MBC Values of Different Extracts

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different extracts of NAT leaves recorded against *E. coli, S. aureus,* and *S. agalactiae* bacteria are shown in Table 3. The minimum

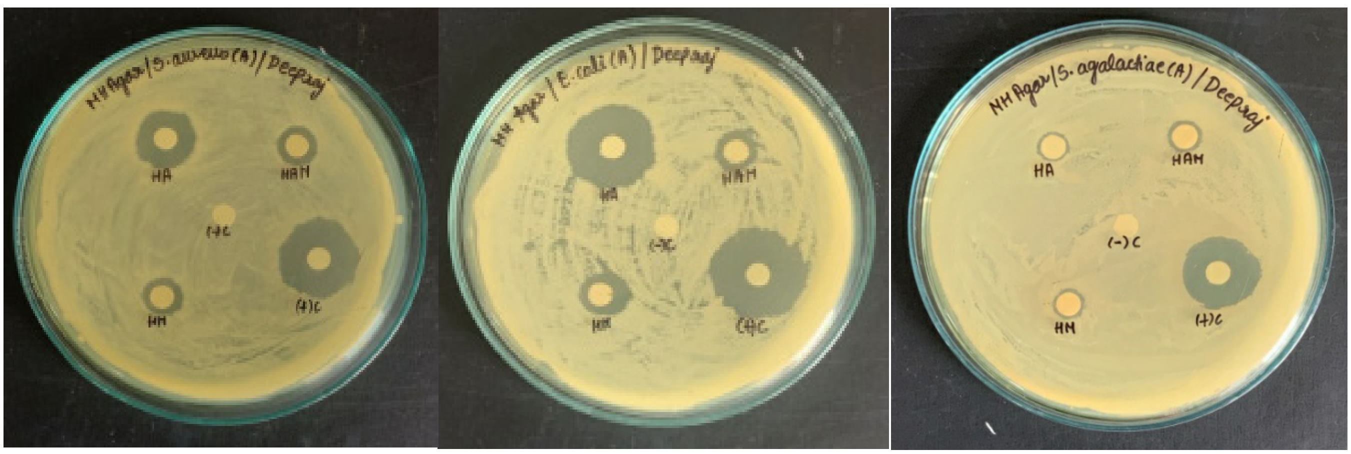

Fig. 1: Antibacterial activity of *N. arbor-tristis* leaves extract against *S. agalactia, S. aureus* and *E.coli* (*NA= *N. arbor-tristis* leaves aqueous extract; NAM = *N. arbor-tristis* leaves aqueous- methanolic extract; *N. arbor-tristis* leaves M = *N. arbor-tristis* leaves methanolic extract; +C = positive control (ciprofloxacin); -C = negative control)

Table 1: Comparison of *in vitro* antimicrobial efficacy of *N. arbor-tristis* discs with standard antibiotic agent Ciprofloxacin against *S. agalactiae, S. aureus* and *E. Coli*

			Zone of inhibition (mm)		
S. No.	*Name of Test extract*	*Concentration of disc*	*S. agalactiae*	*S. aureus*	*E. coli*
1	NA	1000 µg/mL	9.16 ± 0.75	15.16 ± 0.75	20.50 ± 0.54
2	NAM	1000 µg/mL	11.50 ± 0.83	12.00 ± 0.89	10.66 ± 0.51
3	NM	1000 µg/mL	10.33 ± 0.51	11.60 ± 0.81	10.55 ± 0.83
4	+C	1000 µg/mL	19.16 ± 0.40	20.66 ± 0.51	22.66 ± 0.51
5	-C	1000 µg/mL	Nil	Nil	Nil

NA- *N. arbor-tristis* aqueous; NAM- *N. arbor-tristis* aqueous & methanolic; NM= *N. arbor-tristis* methanolic extract, +C – Ciprofloxacin positive control, -C –DMSO negative control.

Table 2: Showing the relative percent of inhibition

N. arbor-tristis leaves	*S. agalactiae*	*S. aureus*	*E. coli*
Aqueous extract	0.22	0.53	0.81
Aqueous-methanolic extract	0.34	0.33	0.22
Methanolic extract	0.29	0.31	0.21

Table 3: MIC and MBC values of the methanolic, aqueous extracts and its combination of *N. arbor-tristis* leaves against three bacterial isolates

		Methanolic extract		*Aqueous extract*		*Combination of aqueous and methanolic*	
Sr. No.	*Bacterial Isolate*	*MIC value (µg/mL)*	*MBC value (µg/mL)*	*MIC value (µg/mL)*	*MBC value (µg/mL)*	*MIC value (µg/mL)*	*MBC value (µg/mL)*
1	*S. aureus*	25	50	25	25	25	25
2	*S. agalactiae*	50	50	50	100	50	50
3	*E. coli*	50	50	12.5	12.5	50	50

inhibitory concentration of 25 µg/mL of methanolic extract was recorded against *S. aureus*, followed by 50 µg/mL for both *E. coli,* and *S. agalactiae*. The minimum bactericidal concentration of methanolic extract of *N. arbor-tristis* leaves against all the three microorganisms was 50 µg/mL. On the contrary, Mahida and Mohan (2007) reported high range of MIC (1-8 mg/mL) in the methanolic extract of NAT leaves, which exhibited significant antibacterial activity against *S. aureus, S. epidermids, S. typhi* and *S. paratyphi-A*.

The minimum inhibitory concentration (MIC) of aqueous extract was lower (12.5 µg/mL) for *E. coli* followed by *S. aureus* and *S. Agalactiae,* while MBC for aqueous extract against these pathogens followed the same trend with highest MBC value of 100 µg/mL for *S. Agalactiae*. The MIC and MBC values were found to be 25 µg/mL of methanolic and combination of aqueous and methanol extracts for *S. Aureus*, except MBC of methanolic extract, whereas it was 50 µg/mL for *E. coli* and *S. agalactiae* (Table 3). Chatterjee and Bhattacharjee (2007) reported that MIC values of the methanolic and aqueous extracts against *Staphylococcus aureus* were 62.50 and 72.50 mg/mL, respectively, while against *E. coli*, MIC values for the methanolic and aqueous extracts were 75.00 and 31.00 mg/mL, much higher as compared to present observations.

A lower MIC value indicates that less extract is required for inhibiting growth but not necessarily killing the pathogen; whereas MBC is the minimal concentration of test drug or extract necessary to kill bacteria. The MBC can be determined from broth dilution used for MIC tests by sub-culturing onto fresh agar plates that do not contain the test agent. The closer the MBC value to the MIC, the more bactericidal nature will be of the extract. Usually, if the ratio MBC/MIC ≤4, the effect is defined as bactericidal but if the ratio MBC/MIC >4, the effect is considered as bacteriostatic (Wiegand *et al.,* 2008).

Conclusion

The isolates of *S. agalactiae* exhibited maximum zone of inhibition in methanolic extract of *N. arbor-tristis,* whereas isolates of *S. aureus* and *E. coli* showed highest zone of inhibition in aqueous extract. Maximum relative percent of inhibition (RPI) was recorded for aqueous extract of *N. arbor-tristis* against *E. coli* isolated from mastitis cases. This study indicates that different concentrations of extract of *N. arbor-tristis* could be beneficial to control subclinical mastitis in cattle. Furthermore, to explore full potential of *N. arbor-tristis* for its antimicrobial activity and other clinical applications, molecular characterization and pharmacodynamics and pharmacokinetics studies are needed. In conclusion, antimicrobial efficacies of different extract of N. arbor-tristis was found to be effective in controling subclinical mastitis in cattle.

Acknowledgement

The authors are highly thankful to the Dean, C.V.Sc. & A.H, ANDUAT, Kumarganj, Ayodhya, for providing facilities required for research work.

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ANNOUNCEMENT: SVSBT-NS-2022

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