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Evaluation of *in vitro* Antibacterial Activities of *Vitexnegundo, Phyllanthusemblica* and *Cymbopogoncitratus* against Common Bacterial Pathogens Causing Subclinical Mastitis in Cows

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Abstract

The present study was undertaken to investigate the *in vitro* antibacterial activities of aqueous and ethanolic extracts of *Vitexnegundo, Phyllanthusemblica* and *Cymbopogoncitratus* for the treatment of bovine subclinical mastitis in cows. Milk samples were taken from the cows infected with subclinical mastitis and cultured aseptically. The bacterial pathogens isolated were *Staphylococcus aureus* and *Escherichia coli*. Antibacterial activities were assessed by agar well diffusion method. Aqueous extract of all the plant extracts showed better antibacterial property as compared to ethanolic extract against *Staphylococcus aureus* and *Escherichia coli*. At 500 mg/ml aqueous extract of *Vitexnegundo* showed the maximum zone of inhibition against *E. coli* (17.67±0.33 mm) followed by *Phyllanthusemblica* (17.33±0.33 mm) and *Cymbopogoncitratus* (16.33±0.33 mm). Aqueous extract of *Cymbopogoncitratus* at 500 mg/ml showed the maximum zone of inhibition against *S. aureus* (16.67±0.33 mm) followed by *Phyllanthusemblica* (16.33±0.33 mm) and *Vitexnegundo* (15.33±0.67 mm). Among the extracts, maximum activity was shown by aqueous extracts of *V. negundo* and *C. citrates* against gram-negative and gram-positive bacteria, respectively. Further studies focussing on isolation and characterization of bioactive compounds are needed to elucidate antibacterial mechanisms.

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Key words: Agar well diffusion, Antibacterial, *Staphylococcus aureus*, *Escherichia coli*

Subclinical mastitis in dairy cows is a serious problem as it often remains undetected and is one of the main reasons for low output and subpar milk quality. In India, the monetary losses due to subclinical mastitis amounted to be INR 1390 per lactation (Sinha *et al*., 2014). The progressive and overuse of antibiotics in bovine mastitis is leading to antibiotic resistance and there is alarming rise in resistance against *S. aureus* strains to commonly used antibiotics (Yadav, 2018). Hence subclinical mastitis is a significant concern in the dairy industry and requires diligent management and preventive measures. In recent years, various strategies have been suggested to overcome the antibiotics resistance and researchers have used natural products as an alternative therapy. These agents can act alone or in combination with antibiotics to enhance the antibacterial activity against a wide range of bacteria (Sharma *et al*., 2021). Plant-based drugs offer cost-effective and less toxic treatment for infectious diseases with minimal side effects compared to synthetic antimicrobials (Sharma *et al*., *loc cit*.). *Vitexnegundo (V. negundo), Phyllanthusemblica (P. emblica)* and *Cymbopogoncitratus (C. citratus)* are the commonly used plants in ethnoveterinary medicine in the state of Himachal Pradesh (H.P.), India. However, studies on their antibacterial properties are limited. Therefore, the present study was designed with the objective to evaluate the antibacterial activity of *V. negundo, P. emblica* and *C. citratus* extracts against the common bacterial pathogens *i.e*., *S. aureus* and

E. coli, involved in bovine subclinical mastitis.

Materials and Methods

All the solvents used were of analytical grade. Absolute alcohol, Nutrient broth, Mueller Hinton agar (MHA), Nutrient agar (HiMedia®, Chennai, India) were used.

Preparation of extract

The leaves of *V. negundo* commonly known as Banna and *C. citrates* commonly known as Lemon grass were collected from different locations in and around Palampur (H.P.), India and shade dried at room temperature, whereas the *P. emblica* powder commonly known as Amla was collected from the local market. After drying, all these parts were powdered using a grinding machine. Aqueous and ethanolic extracts were prepared by maceration technique. In this process, the whole or coarsely powdered 100 grams of crude sample was soaked in one litre of ethanol solvent separately and allowed to stand at room temperature for at least 48-72 hours with frequent agitation until the soluble matter dissolves. After maceration, the extract was initially filtered using muslin cloth and then re-filtered again using a filter paper (Whatman filter paper No 1, Cat no.1001125). The filtrate obtained was evaporated in oven at a temperature of 40˚C. The residue obtained was kept at 4˚C in airtight bottles until use (Hamid and Janbaz, 2017**)**

Table I : Antibacterial activity of aqueous and ethanolic extract of *V. negundo, P. emblica* and *C. citrates* at various concentrations.

Extract	Concentration (mg/ml)	Zone of Inhibition (mm)	
		S. aureus	***E. coli***
Vitexnegundo (aqueous)	500	15.33±0.67	17.67±0.33
	250	13.67±0.33	15.67±0.33
	125	11.67±0.33	13.33±0.33
	62.5	< 10	< 10
Vitexnegundo (ethanolic)	500	13.67±0.33	14.67±0.33
	250	11.67 ±0.33	13.33±0.33
	125	10.67±0.33	-
	62.5	-	-
Cymbopogoncitratus (aqueous)	500	16.67±0.33	16.33±0.33
	250	15.33±0.33	14.67±0.33
	125	12.67±0.33	12.67±0.33
	62.5	-	-
Cymbopogoncitratus (ethanolic)	500	13.33±0.33	14.33±0.33
	250	10.67±0.33	12.67±0.33
	125	10.33±0.33	10.67±0.33
	62.5	-	-
Phyllanthusemblica (aqueous)	500	16.33±0.33	17.33±0.33
	250	14.67±0.33	15.33±0.33
	125	12.33±0.33	12.67±0.67
	62.5	< 10	< 10
Phyllanthusemblica (ethanolic)	500	17.67±0.33	15.67±0.33
	250	15.67±0.33	13.67±0.33
	125	14.33±0.33	12.67±0.33
	62.5	< 10	< 10
AMC		16	-
CPZ		-	20

Antibacterial activity

Aqueous and ethanolic extracts of *V. negundo, P. emblica* and *C. citrates* were evaluated for their antibacterial activity against the common bacterial pathogens *S. aureus* and *E. coli* isolated from milk samples of cows affected with bovine subclinical mastitis. The antibacterial activity of herbal extracts was determined by agar well diffusion method (Perez *et al.*, 1990). Four equidistant wells of size 5 mm diameter were made in Mueller-Hinton Agar plates with the help of the sterilized gel puncher. The wells were sealed with hot melted agar. The bacterial isolate was suspended in the brain heart infusion broth and turbidity was adjusted 0.5 McFarland. Later, lawn culture of bacterial suspension was made on MHA agar plate with sterilized cotton swab. The extract was dissolved in dimethyl sulfoxide (DMSO) and different concentrations were made, out of which 50 µl were poured into different wells. Amoxyclav disc (30 ug) and Cefoperazone disc (75 ug) were kept as control against *S. aureus* and *E. coli* respectively. The plates were incubated at a temperature of 37°C for 24 hr. The procedure was done in triplicate form.

The data obtained in the research work undertaken were analysed and compared using standard formulas given for mean and standard error of mean in MS Excel sheet.

Results and Discussion

The results for the antibacterial activity of different extracts of *V. negundo, P. emblica* and *C. citrates* against gram-positive *S. aureus* and gram-negative *E. coli* are presented in Table I and illustrated in figure 1-14. The antibacterial potential of the extracts was found to be dose dependent. All the extracts showed varied inhibitory activity against strains of tested bacteria.

Results are presented as Mean±SE, SE-Standard error of mean. The sign (-) showed that the extracts used could not inhibit the growth of selected organisms *S. aureus* and *E. coli*.

V. negundo is one of the indigenous plant species employed in traditional methods to treat

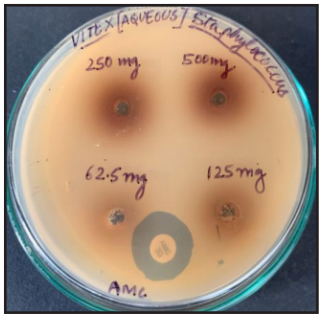

Fig. 1. Zone of inhibition of aqueous extract of *V. negundo* against *S. aureus*

Fig. 2. Zone of inhibition of aqueous extract of *V. negundo* against *E. coli*

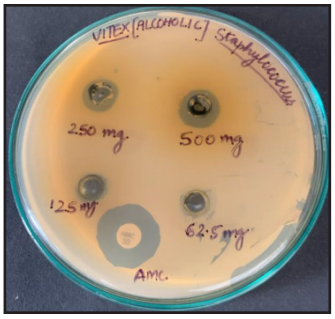

Fig. 3. Zone of inhibition of alcoholic extract of *V. negundo* against *S. aureus*

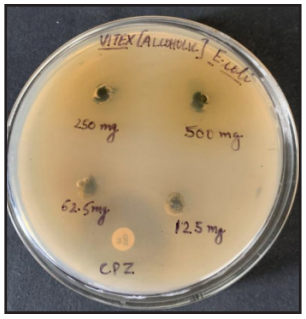

Fig. 4. Zone of inhibition of alcoholic extract of *V. negundo* against *E. coli*

mastitis and diarrhoea in animals (ICAR, 2006). In the present study aqueous extract from *V. negundo* leaves exhibited maximum zone of inhibition against *E. coli* (Fig. 14). Present results are in agreement with an earlier study in which alcoholic extract of *V. negundo* hindered the growth of *E. coli* with an inhibition zone of 13.10±0.76 mm (Dharajiya *et al*., 2015). Muhammad *et al.*, (2013) reported a zone of inhibition for aqueous extract of *V. negundo* leaves was 20.00±2.83 mm against *E. coli* which is in accordance with the present study which exhibited a zone of 17.67±0.33 mm. Present study was also in agreement with study conducted by Jahan *et al.*, (2007) who reported an inhibitory zone of12.0 mm aqueous extract at the concentration of 186 mg/ml against *S. aureus*. According to a study conducted by Tirumalasetty *et al.*, (2013) the antimicrobial effects of *V. negundo* leaf extract may be attributed to its bioactive compounds, such as alkaloids, tannins, flavonoids, glycosides, and saponins therefore validating the plant's traditional use in ethnomedicine. Devi *et al.*, (2008) tested the *Vitexnegundo* leaf extracts for antimicrobial efficacy on *S. aureus, E. coli and Klebsiellapneumoniae* and concluded that various extracts, including fresh and aqueous forms, exhibited notable antibacterial effects against all three bacteria even at different dilutions.

Figure 1-4 indicates the zone of inhibition (mm) of aqueous and ethanolic extract of *V. negundo* against *S. aureus* and *E. coli* at 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml with a zone of AMC and CPZ, respectively against *S. aureus* and *E. coli.*

The present study revealed that the aqueous extract of *C. citratus* was found to be more effective against *S. aureus* (Fig. 13) in comparison to *E. coli* which is in accordance with the studies conducted by Naik *et al.*, (2010) and Nyamath and Karthikeyan (2018) which concluded that the *C. citratus* showed greater sensitivity to gram positive bacteria than gram negative bacteria. The study conducted by Choi *et al*., (2012) revealed that *C. citratus* essential oil exhibited antibacterial activity against both standard antibiotic resistant *S. aureus* strains

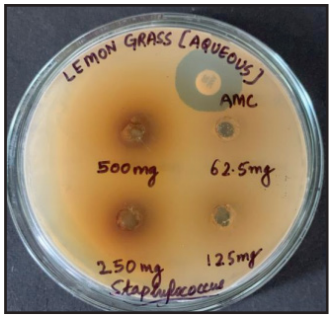

Fig. 5. Zone of inhibition of aqueous extract of *C. citrates* against *S. aureus*

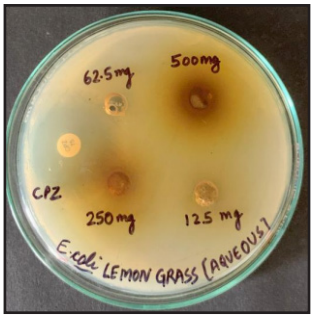

Fig. 6. Zone of inhibition of aqueous extract of *C.citratus* against *E. coli*

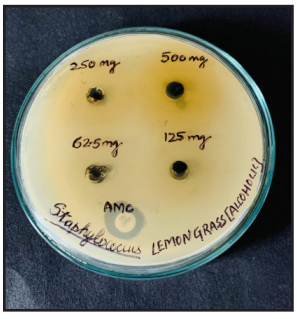

Fig. 7. Zone of inhibition of alcoholic extract of *C. citrates* against *S. aureus*

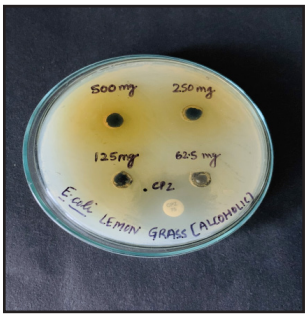

Fig. 8. Zone of inhibition of alcoholic extract of *C. citrates* against *E. coli*

and those isolated from a cow with chronic mastitis. The present research is in accordance with the study conducted by Jafari *et al*., (2012) which revealed the antibacterial property of methanolic extract of *C. citratus* and essential oil on *S. aureus, Bacillus cereus, E. coli and Pseudomonas aeruginosa* at various concentrations and noticed the effective inhibition against *S. aureus, B. cereus* and *E. coli*. Nyamath and Karthikeyan (*loc cit.*) also reported antibacterial activity of ethanolic extract of *C. citratus* with zone of inhibition of 12.50 mm. According to Gao *et al.,* (2020) citral, composed of neral and geranial, was the active component in lemongrass essential oil responsible for its antibacterial properties.

Figure 5-8 indicates the zone of inhibition (mm) of aqueous and ethanolic extract of *C. citrates* against *S. aureus* and *E. coli* at 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml with a zone of AMC and CPZ, respectively against *S. aureus* and *E. coli.*

In the present study, aqueous extract from *P. emblica* displayed a larger inhibition zone against *E. coli* compared to the ethanolic extract. The present study also revealed that both aqueous and ethanolic extract of *P. emblica* demonstrated the inhibition zone against *S. aureus,* with the latter showing slightly larger zone of inhibition, which is in accordance with the studies conducted by Saeed and Tariq (2007) and Javale and Sabnis (2010) on *P. emblica* aqueous extract against *S. aureus* which exhibited the zone of inhibition as 18.32±2.15 mm, 22.45±4.12 mm and 29.50±1.32 mm and 25.33±1.52 mm with the process of infusion and decoction, respectively. The current study further demonstrated that the *P. emblica* extract exhibited a higher effectiveness against gram-positive bacteria compared to gram-negative bacteria which aligns with the study conducted by Varghese *et al*., (2013) who concluded that the aqueous extract effectively hindered the growth of *S. aureus* to a greater extent than that of *E. coli*. Chaunde *et al.*,(2023) investigated the antibacterial potential of *P. emblica* in bovine sub-clinical mastitis and found amla effective against *S. aureus* and *E. coli* with inhibition zones of around 19.70 mm and 18.81 mm, respectively. According to a study conducted by Diwan *et al.,* (2018), phyllemblin was the main

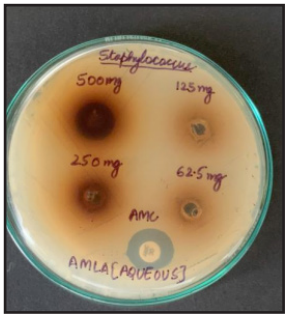

Fig. 9. Zone of inhibition of aqueous extract of *P. emblica* against *S. aureus*

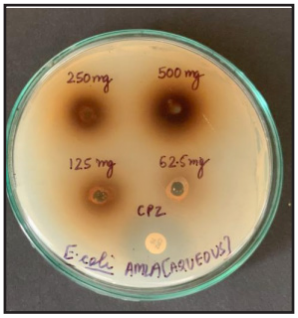

Fig. 10. Zone of inhibition of aqueous extract of *P. emblica* against *E. coli*

Fig. 11. Zone of inhibition of alcoholic extract of *P. emblica* against *S. aureus*

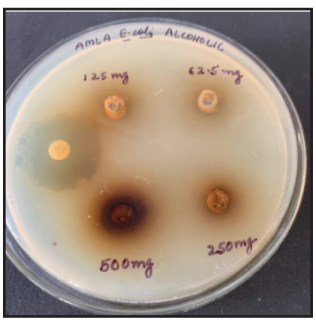

Fig. 12. Zone of inhibition of alcoholic extract of *P. emblica* against *E. coli*

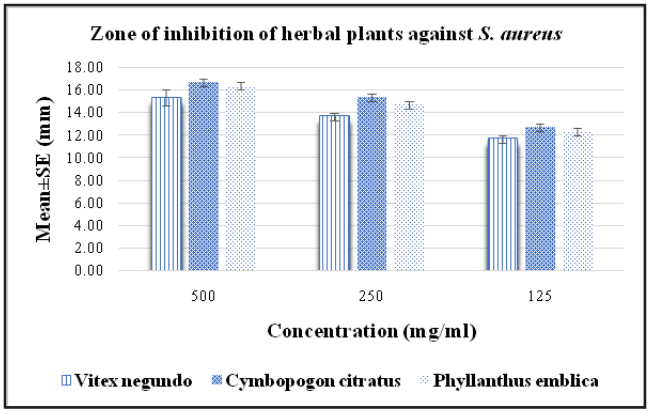

Fig. 13. Comparative Mean±SE of mean (in mm) of aqueous extract of *V. negundo, C. citrates* and *P. emblica* against *S. aureus* at different concentrations of 500mg/ml, 250mg/ml and 125mg/ml.

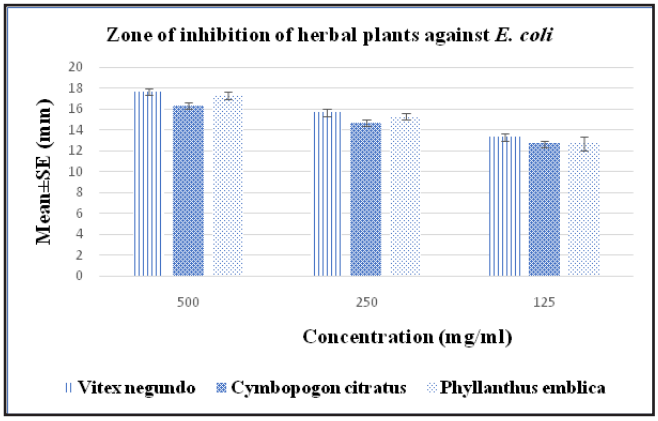

Fig. 14. Comparative Mean±SE of mean (in mm) of aqueous extract of *Vitexnegundo, Cymbopogoncitratus* and *Phyllanthusemblica* against *E. coli* at different concentrations of 500mg/ml, 250mg/ml and 125mg/ml.

active compound in *Emblica* responsible for its antibacterial properties.

In this study, the antibacterial properties for the combination of three extracts were not investigated. Moreover, as per authors knowledge, there is no study on the therapeutic efficacy of these herbal plants through intramammary route. Further studies could be planned to test the *in vivo* efficacy of these herbal plants against bovine subclinical mastitis.

Figure 9-12 indicates the zone of inhibition (mm) of aqueous and ethanolic extract of *P. emblica* against *S. aureus* and *E. coli* at 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml with a zone of AMC and CPZ, respectively against *S. aureus* and *E. coli.*

Conclusion

The results obtained from the *in-vitro* studies showed stronger antibacterial activity of all the extracts against the bacterial isolates from subclinical mastitis. Among the extracts maximum activity was shown by aqueous extract of *V. negundo* against gram negative bacteria whereas the aqueous extract of *C. citrates* exhibited the maximum activity against gram positive bacteria. Further studies focussing on isolation and characterization of bioactive compounds are needed to elucidate antibacterial mechanisms. The bioactive compounds once isolated can be tested clinically in cases of subclinical mastitis.

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