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Antibacterial Effect of *Vernonia anthelmentica* (Kattu Seeragam) on Bovine Mastitis

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Abstract: Bovine mastitis is the common disease of mammary glands of dairy cows, caused by pathogenic bacteria, such as *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus* etc. The disease severely impacts the quality of milk and associated milk products. Three different bacterial species *Staphylococcus*, *Bacillus* and *Klebsiella* were isolated, identified and later treated with the seed extract of *Vernonia anthelmentica*. Well diffusion method was used to test the antibacterial effect of ethanol seed extract of *Vernonia anthelmentica*. Results showed that *Vernonia anthelmentica* was most effective against *Staphylococcus* compared to other two bacterial species. Phytochemical analysis of *Vernonia anthelmentica* revealed the presence of tannins, saponins, terpenoids etc. Present study suggests that seeds of *Vernonia anthelmentica* can be an effective antibacterial against bovine mastitis, which might be due to the presence of tannins, and terpenoids in the extract.

Keywords: Antibacterial agent, Bovine mastitis, *Staphylococcus*, *Bacillus*, *Klebsiella* Terpenoids. Tannins. *Vernonia anthelmentica*

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Introduction

India stands first in milk production in the world (Lahoti and Chole, 2009). Of the several diseases, which are rampant in cattle, bovine mastitis is one of the major disease which impacts the dairy industry in a big way. Bovine mastitis is the inflammation of the mammary glands in dairy cows caused by invasion and destruction of the milk producing tissue by pathogenic microorganisms (Schroder, 2009). Mastitis is caused as a result of complex interaction of infectious agents, environmental factors and

management practices, all together lower the quality of milk and milk products (Politis and Kawariharry, 1988). Bovine mastitis is not only caused by *Staphylococcus aureus*, but also by environmental pathogens such as *Streptococcus dysgalacti*, *Enterococcus faeciae*, *Escherichia coli*, *Klebsiella pneumonia* and *Bacillus* species. The most common treatment method to treat bovine mastitis is by the intra mammary infusion of antibiotics. However, in spite of introduction of various antibiotics to treat the disease, there are

alarming evidences of antimicrobial resistance being developed against these antibiotics. Recent studies by Ragunathan *et al.* (2018) suggests that various drugs such as penicillin, methicillin, genatmicin, erythromycin and tetracycline are the drugs of choice to treat bovine mastitis. Presently, vancomycin is the drug of choice for methicillin resistant *Streptococcus aureus* and there is an urgent need for developing a new natural antibiotic against bovine mastitis before it becomes vancomycin resistant. In this context there is need for exploring and developing new antibiotics against bovine mastitis to effectively combat the infection in a more natural way to replace chemical based antibiotics. There are several instances where a number of plant sources have been used to treat several pathogenic conditions including bovine mastitis (Patel *et al.*, 2013). In this context, *Vernonia anthelmintica* is one such plant reported to have several medicinal properties, including antimicrobial activity (Toyang and Verpoorte, 2013; Srivastava *et al.*, 2014).

The present study is an attempt to analyze and ascertain the antimicrobial activity of seed extract of *Vernonia anthelmintica* against bovine mastitis infection.

Materials and Methods

Seeds of *Vernonia anthelmintica* (Kattu seeragam) were collected from National Drug Store, Chennai. Three swabs of the cows udder was taken from three affected cattle. The swabs were collected from Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony Road, Chennai, Tamil Nadu, India.

Preparation of Sequential Extraction Using Selected Plant Materials:

Seeds of *Vernonia anthelmintica* (Kattu seeragam) was powdered using motor pestle and serial dilution of powdered sample was done in the ratio of 100:300 (300 ml of ethanol was added to 100 g of *Vernonia anthelmintica* seed powder and poured in to conical flask). The air-tight conical

flask was kept undisturbed for two days at room temperature and later contents of the flask were filtered using Whatmann filter paper No.1 and stored at room temperature for further use.

Isolation of Bacteria from Bovine Mastitis Sample:

Six petri plates of nutrient agar were prepared (250 ml of nutrient agar). The plates and nutrient agar were autoclaved after which the nutrient agar was cooled and poured into the petri plates. The plates were allowed to solidify. Followed by solidification, the bacterial loop were swabbed on the plates (two plates per loop). The plates were marked as affected 1, affected 2 and affected 3. The plates were incubated for 24 h at 37 C. After 24 h of incubation the bacterial colonies were formed on the plates. The bacterial colonies were sub-cultured to isolate the bacterial species.

Morphological Characterization of Bacteria:

A clean slide was taken and the smear was prepared by using bacteria taken from the colony and mixed with a drop of distilled water. The slide was heat fixed. The slide was flooded with crystal violet and allowed to stand for 2 min then washed with distilled water. Later, Gram's iodine was added and left for 2 min. The slide was washed with ethanol and then with distilled water in a slanting position. Finally a few drops of Safranin was added. After 2 min, the slide was washed with distilled water and air dried. The air dried slide was observed under the microscope for characterization of bacteria, and this was followed by biochemical tests, for confirmation of bacterial species.

Indole Test (Tryptophanase Hydrolysis):

This test is performed to determine the ability of an organism to produce indole from the amino acids tryptophan using the enzyme tryptophanase. The isolate were incubated in the broth at optimum temperature for 24-48 h. Kovac's reagent (10-12 drops) was added to broth and observed for the formation of colored layer on the

surface of the medium.

Methyl Red Test (MR Test):

This test was performed to determine the ability of an organism to produce mixed acid end products from glucose fermentation. The isolates were inoculated into MR-VP broth and incubated for 3-5 days at optimum temperature. Three to four drops of methyl red reagent was added to interpret the MR-positive and MR-negative isolates, based on red or yellow colour development.

Voges-Proskauer Test (VP Test):

This test was executed to determine the ability of an organism to produce acetoin; 2, 3 butane diol and ethanol. The isolates were inoculated into MR-VP broth and incubated for 3-5 days at optimum temperature. 1 ml of culture was then pipette out and to it Barritt's solution A (alpha-naphthol) and Barritt's solution B (KOH) were added in equal proportions. The solution was agitated vigorously and incubated for 15 to 20 min for the formation of red colour.

Citrate Utilization Test:

This test was performed to determine whether the organism is capable of using citrate as the sole source of carbon with production of the enzyme citritase. The isolates were streaked onto Simmons citrate agar slants and incubated at optimum temperature for 24-48 h and observed for any color change.

Triple Sugar Iron Test (TSI Test):

The ability of an isolate to produce H₂S (Hydrogen sulfide) was carried out by stabbing the isolate into TSI (triple sugar iron) medium and incubated. Gaps, cracks or bubbles in the agar medium indicate the gas production.

Phytochemical Analysis:

Qualitative Phytochemical Analysis was performed to test the presence of carbohydrates, tannins, saponins, flavinoids, alkaloids, quinones,

glycosides, Cardiac Glycosides, Terpenoids, Triterpenoids, Phenols, Coumarins, Steroids and Phytosteroids, Philobatannins and Anthraquinones by using standard protocols (Harborne, 1973; Smolenski *et al.*, 1974; Sofoworam 1993; Kolawole *et al.*, 2006; Ayoola *et al.*, 2008; Suresh Kumar *et al.*, 2009; Jana and Shekhawat, 2010; Manas *et al.*, 2010).

Antibacterial Activity Using Agar Well Diffusion Method:

The samples were screened for antibacterial activity against bacterial strains (affected 1, affected 2, affected 3) using agar well diffusion method. (Malibari, 1991; Zhou *et al.*, 2006; Gong *et al.*, 2009; Zhang *et al.*, 2009). Nutrient Agar (NA) plates were inoculated with test organisms. The plates were evenly spread out. Then wells were prepared in the plates with a cork borer. Each well was loaded with 20, 40, 60, 80 µl of corresponding concentration of sample and 10 mg of Tetracycline dissolved in 1 ml of 10% DMSO was used as a positive control. The plates were incubated for 24 h at 37 C. The development of inhibition zone around the well was measured (diameter) and recorded.

Results

Morphological Characterization of Bacteria:

The bacterial isolates were subjected to Gram staining to identify the morphological characteristics of the bacteria (Table 1) and subjected to biochemical test for confirmation of the bacteria. From morphological and biochemical study, the bacteria isolates such as affected 1, affected 2 and affected 3 was confirmed as *Klebsiella* sp., *Bacillus* sp. and *Staphylococcus* sp., respectively (Fig. 1).

Qualitative Phytochemical Analysis of Vernonia anthelmentica (Kattu Seeragam):

The results of qualitative phytochemical analysis of *Vernonia anthelmentica* seed extract showed the presence of carbohydrates, tannins, saponins,

Table 1: Biochemical test for characterization of bacterial species

Biochemcial Test	Positive			Negative		
	Isolate 1	Isolate 2	Isolate 3	Isolate 1	Isolate 2	Isolate 3
Indole test	+	+				-
Methyl red test	+	+	+			
Voges-Proskaver test	+	+	+			
Citrate utilization test			+	-	-	
Triple sugar test			+	-	-	

Bacterial Isolate 1

Bacterial Isolate 2

Bacterial Isolate 3

Fig. 1: Bacterial isolates

quinones, terpenoids, phenols, steroids, philobattannins and absence of flavonoids, glycosides, Cardiac glycosides, coumarins and anthraquinones (Table 2).

Antibacterial Activity Using Agar Well Diffusion Method:

The zone of inhibition against *Bacillus* sp. was recorded as 5, 5.5 and 6 (mm) for 20 µl concentration, 4.8, 6.9 and 7.1 (mm) for 40 µl concentration, 6.5, 7 and 8 (mm) for 60 µl concentration and 8.3, 9.5 and 10 (mm) for 80 µl concentration. The zone of inhibition against *Staphylococcus* sp. was recorded a 5.7, 7.8 and 7.9 (mm) for 20 µl concentration, 6, 7.1 and 8 (mm) for 40 µl concentration, 7.2, 6.5 and 9 (mm) for 60 µl concentration and 8.3, 6.9 and 9.2 (mm) for 80 µl concentration. The zone of inhibition against

Klebsiella sp. was recorded as 3, 4 and 5 (mm) for 20 µl concentration, 3.3, 5.3 and 6 (mm) for 40 µl concentration, 5.7, 6.5 and 7 (mm) for 60 µl concentration and 7, 8.4 and 9 (mm) for 80 µl concentration.

The result of antibacterial activity of *Vernonia anhelmentica* seed extract showed wider zone of inhibition against *Staphylococcus* sp. when compared with other two bacteria namely *Klebsiella* sp. and *Bacillus* sp. (Table 3; Fig. 2).

Discussion

The present study demonstrates that ethanol seed extract of *Vernonia anhelmintica* was found to be quite effective as an antibacterial agent against three different species of pathogenic bacteria causing bovine mastitis. Bacterial species,

Table 2: Phytochemical Screening of *Vernonia anthelmantica*

S.No	Test	Extract
1.	Carbohydrates	+
2.	Tannins	+
3.	Saponins	+
4.	Flavanoids	-
5.	Quinones	+
6.	Glycosides	-
7.	Cardiac glycosides	-
8.	Terpenoids	+
9.	Phenols	+
10.	Coumarins	-
11.	Steroids and Phytosteroids	+
12.	Philobatannins	+
13.	Anthraquinoinines	-

Fig. 2: Zone of Inhibition in *Vernonia anthelmantica* (Kattu Seeragam) against Bovine Mastitis

such as *Staphylococcus*, *Klebesella* and *Bacillus* sp. were isolated from the mastitis infection isolate and were identified as per the standard protocol. Antibacterial activity was analyzed using well diffusion method. The seed extract of

Vernonia antihelminica was found to be effective against all the three bacterial species, but particularly seems to be more effective against *Staphylococcus*. These /results are in accordance with the studies of Kalayou *et al.* (2012) and

Table 3: Zone of Inhibition of seed extract of *Vernonia anthelmintica*

	Extract Concentration of <i>Vernonia anthelmintica</i>			
<i>Klebsiella sp</i> (Bovine mastitis)	20 µl	40 µl	60 µl	80 µl
	3 mm	3.3 mm	5.7 mm	7 mm
	4 mm	5.3 mm	6.5 mm	8.4 mm
	5 mm	6 mm	7 mm	9 mm
<i>Bacillus sp</i> (Bovine mastitis)	20 µl	40 µl	60 µl	80 µl
	5 mm	4.8 mm	6.5 mm	8.3 mm
	5.5 mm	6.9 mm	7 mm	9.5 mm
	6 mm	7.1 mm	8 mm	10 mm
<i>Staphylococcus sp</i> (Bovine mastitis)	20 µl	40 µl	60 µl	80 µl
	5.7 mm	6 mm	7.2 mm	8.3 mm
	7.8 mm	7.1 mm	6.5 mm	6.9 mm
	7.9 mm	8 mm	9 mm	9.2 mm

Ravula *et al.* (2012) who have reported antibacterial and synergistic activity of *Vernonia* species. Phytochemical studies by authors like Ravula *et al.* (2012) have reported the presence of alkaloids, terpenoids, tannins, saponins, flavinoids, quinine etc. and suggested that the antibacterial and other pharmacological properties of *Vernonia anthelmintica* may be attributed to these phytochemical constituents. In accordance with these observations, our phytochemical analysis of seeds of *Vernonia* has also shown a similar

phytochemical composition i.e., tannins, saponins, terpenoids, phenols, steroids etc. Based upon these studies, it may be said that antibacterial activity against bovine mastitis infection in the present study may be attributed to these phytochemical compounds, in particular tannins and terpenoids as suggested by Shiney *et al.* (2013) and Kapoor *et al.* (1969). Results from the present study suggest that seed extract of *Vernonia* may be considered as a natural antibiotic against certain species of bacteria

causing bovine mastitis.

Conclusion

The results of present study indicates that *Vernonia anthelmentica* seed extract can be considered to treat bovine mastitis in a natural way and in future may be used as a most sought natural antibiotic to treat vancomycin resistant bacteria.

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