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Evaluation of antimicrobial properties of ethanolic extracts of stem of *Azadirachta indica* (Neem) and *Tinospora cordifolia* (Giloy) in subclinical mastitis affected cattle

Pooja Solanki, S Choudhary and Rajkumar Soni

Abstract
The present study was envisaged with the objective to determine antimicrobial activities of ethanolic extracts of medicinal plants including stem of *Azadirachta indica* and *Tinospora cordifolia* against organisms isolated from milk of subclinical mastitis affected cattle. Antimicrobial properties were determined *in vitro* by agar well diffusion-method against pathogenic bacteria *viz Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, E. coli, Bacillus cereus, Klebsiella pneumoniae and Pseudomonas aeruginosa*. The antibacterial activity measured as a zone of inhibition (in mm) for *Azadirachta indica* and *Tinospora cordifolia* extracts against pathogenic bacteria. *Azadirachta indica* plant ethanolic extracts were seen to possess comparatively higher antibacterial activity than *Tinospora cordifolia*. Studies have shown that both plants contain antibacterial properties and can be used in cases of subclinical mastitis.

Keywords: *Azadirachta indica*, bacteria, ethanolic extracts and *Tinospora cordifolia*

Introduction
Sub-clinical mastitis occurs worldwide, causing not only huge economic losses to milk production but also constant risk for other animals in the herd. In most dairy herds, subclinical mastitis causes greater losses than clinical mastitis (Bachaya *et al*., 2011) [3]. The most common bacteria found in isolation are *Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae* and *Escherichia coli* (Bramley *et al*., 1996) [5]. The increased use of antibiotics and lack of new drugs, vaccines and diagnostic aids have led to multidrug resistance as a worldwide issue. Because of this, the concept of using non-antibiotic strategies to treat mastitis is gaining attention.

The plants are the richest source of traditional medicine, modern medicine, nutritional supplement, folk medicine, pharmaceutical intermediate and chemical entity for synthetic drugs (Hammer *et al.*, 1999) [11]. Various medicinal plants have been recommended by the World Health Organization (WHO) since they are regarded as safe and effective alternatives to artificial drugs (Ahmad *et al.*, 2011) [1]. Additionally to their use as antibacterial, anti-inflammatory, and immune-modulating agents, medicinal plants can be used as an alternative therapeutic option against bovine mastitis. Phytochemical components in plants that possess a specific physiological and pharmacological effect in the body are the basis for the medicinal value of plants. The most important bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeoga *et al.*, 2005) [9].

Azadirachta indica (Neem) is one of the most important traditional medicinal plant, still considered as "Village dispensary" in India. The most important active constituent is azadirachtin and the others are nimbin, nimbolinin, nimbidin, nimbidol, gedunin, sodium nimbinate, salannin, and quercetin. Neem has antibacterial (Almas, 1999) [2], anti-inflammatory (Pillai and Santhakumari, 1981) and immunomodulatory properties (Upadhaya *et al.*, 1992).

Tinospora cordifolia which belongs to the Menispermaceae family is a large, deciduous, climbing shrub native to most of India. Several phytoconstituents isolated from *Tinospora cordifolia* belongs to different classes such as alkaloids, steroids, glycosides, diterpenoid lactones, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Meshram *et al.*, 2013). It is known to have anti-spasmodic, anti-inflammatory, antiallergic, anti-arthritic,

anti-periodic, anti-diabetic, anti-leprotic hepatoprotective, antioxidant and immune- modulatory properties (Singh *et al*., 2003) [24].

Materials and Methods

Bacterial isolates

Milk samples were collected from 100 apparently healthy cows from college dairy farm and private dairies in surrounding areas in Bikaner city of Rajasthan. Isolation and identification of bacteria in apparently normal milk was done as per Cowen and Steel (1975). Bacterial isolates were obtained through culture examination and series of biochemical tests (Himedia kit).

Collection of plant materials

In the present study, *Azadirachta indica* and *Tinospora cordifolia* were used. Stem of *Azadirachta indica* and *Tinospora cordifolia* were collected from nearby local area and identified by the Botanical Survey of India, Arid Zone Regional Centre, Jodhpur, Rajasthan. The plant materials were washed with water and dried in the oven at a temperature of 45-50ºC for 3-5 days till the weight became constant. All the dried plant materials were crushed to make powder and stored in clean and air tight container till further use. An ethanolic extract is prepared by a Soxhlet extraction method.

Preparation of stock inoculums

The antibacterial activity of ethanolic extracts of stem of *Azadirachta indica* and *Tinospora cordifolia* was screened by agar cup method as described by Cruickshank *et al*. (1975) [7].

Test dilution of herbal extract

The different dilutions of herbal extracts were prepared and tested. Different dilutions were prepared by dissolving prepared extracts of *Azadirachta indica* and *Tinospora cordifolia* in triple glass distil water by serial dilution method to yield different concentration *viz.* 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml.

Results and discussion

Evaluation of *In vitro* antibacterial activity of ethanolic extract of *Azadirachta indica*

The antibacterial activity of ethanolic extract of *Azadirachta indica* in terms of the zone of inhibition against subclinical mastitis bacteria is depicted in Table 1.

The average zone of inhibition against *Staphylococcus aureus* was 19.83 ± 0.008 mm, 16.55 ± 0.024 mm, 12.35 ± 0.011 mm, 9.15 ± 0.021 mm and 6.82 ± 0.021 at concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml, respectively. The average zone of inhibition against *Staphylococcus epidermidis* was 17.85 ± 0.017 mm, 14.65 ± 0.026 mm, 10.74 ± 0.011 mm and 8.23 ± 0.014 at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus agalactiae* was 16.95 ± 0.027 mm, 13.21 ± 0.008, 10.36 ± 0.008 mm and 7.26 ± 0.015 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus dysgalactiae* was 16.27 ± 0.021 mm, 12.54 ± 0.024 and 9.61 ± 0.008 mm at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Streptococcus uberis* was 16.55 ± 0.026 mm, 12.32 ± 0.012 mm and 8.41 ± 0.008 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *E. coli* was 14.57 ± 0.030 mm, 11.51 ± 0.017 mm and 7.61 ± 0.008 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Bacillus cereus* was 13.96 ± 0.017 mm, 9.05 ± 0.029 mm and 7.35 ± 0.014 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Klebsiella pneumoniae* was 15.45 ± 0.008 mm, 10.34 ± 0.015 mm and 7.84 ± 0.012 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Pseudomonas aeruginosa* was 14.31 ± 0.032 mm, 9.56 ± 0.018 mm and 7.15 ± 0.020 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively.

Table 1: Mean ± SE values of zones of inhibition (mm) of ethanolic extract of *Azadirachta indica* against subclinical mastitis pathogens at different concentration

S. No.	Bacterial isolates	Zone of inhibition (mm)				
		500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml
1.	*Staphylococcus aureus*	19.83±0.008	16.55±0.024	12.35±0.011	9.15±0.021	6.82±0.021
2.	*Staphylococcus epidermidis*	17.85±0.017	14.65±0.026	10.74±0.011	8.23±0.014	-
3.	*Streptococcus agalactiae*	16.95±0.027	13.21±0.008	10.36±0.008	7.26±0.015	-
4.	*Streptococcus dysgalactiae*	16.27±0.021	12.54±0.024	9.61±0.008	-	-
5.	*Streptococcus uberis*	16.55±0.026	12.32±0.012	8.41±0.008	-	-
6.	*E. coli*	14.57±0.030	11.51±0.017	7.61±0.008	-	-
7.	*Bacillus cereus*	13.96±0.017	9.05±0.029	7.35±0.014	-	-
8.	*Klebsiella pneumoniae*	15.45±0.008	10.34±0.015	7.84±0.012	-	-
9.	*Pseudomonas aeruginosa*	14.31±0.032	9.56±0.018	7.15±0.020	-	-

Results of present study were the same as results obtained in study carried out by Francine *et al*. (2015) [10] who recorded that ethanol extracts were found more effective than aqueous extracts. In present study, ethanolic extract of *Azadirachta indica* was found to be most effective against genus *Staphylococcus* specially *Staphylococcus aureus*. Sarmiento *et al*. (2011) [23] and Rathod *et al*. (2010) [22] also found that ethanolic extract of *Azadirachta indica* exhibited maximum effect against *Staphylococcus aureus*. Pandey *et al*. (2014) [20] evaluated that the ethanolic extract of *Azadirachta indica* showed significant antibacterial activity against *E. coli* and *Staphylococcus aureus*, though it inhibited the growth of *Staphlococcus aureus* more effectively as compared to *E. coli.* Mamman *et al*. (2013) [14] studied that stem-bark extracts of *Azadirachta indica* were more effective than the leave extract in terms of antibacterial activity. Biswas *et al*. (2002) [4] reported that stem bark is bitter than the leaves, it contains more of nimbidin which to be the main active anti-bacterial ingredient of *Azadirachta indica*. Tirumalasetty *et al*. (2014) [25] observed that the ethanolic leaf extracts of neem showed inhibition zone of 16 mm on *E. coli*, 15 mm on *Klebsiella*, 14 mm on *Pseudomonas aeruginosa* and 20 mm on

Staphylococcus aureus. However, there are some other studies that reported results contradictory to the present study. Francine *et al*. (2015) [10] reported that there was no antibacterial activity of ethanol and aqueous extracts of *Azadirachta indica* against *E. coli*. The possible differences in results may be due to the differences in methods of extractions and concentrations used in each study (Kumar *et al*., 1997) [13].

Evaluation of *In vitro* antibacterial activity of ethanolic extract of *Tinospora cordifolia*

The antibacterial activity of ethanolic extract of *Tinospora cordifolia* in term of the zone of inhibition against subclinical mastitis bacteria is depicted in Table 2.

The average zone of inhibition against *Staphylococcus aureus* was 18.91 ± 0.012 mm, 16.16 ± 0.027 mm, 11.83 ± 0.018 mm and 8.31 ± 0.008 mm at concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Staphylococcus epidermidis* was 17.34 ± 0.020 mm, 14.10 ± 0.041 mm, 10.41 ± 0.005 mm, 8.12 ± 0.017 at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus agalactiae* was 16.55 ± 0.026 mm, 12.67 ± 0.021, 10.07 ± 0.017 mm and 7.06 ± 0.026 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus dysgalactiae* was 16.36 ± 0.020 mm, 12.36 ± 0.008 and 7.62 ± 0.005 mm at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Streptococcus uberis* was 15.25 ± 0.020 mm, 11.15 ± 0.017 mm and 7.32 ± 0.021 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *E. coli* was 14.13 ± 0.050 mm, 9.92 ± 0.014 mm, 7.23 ± 0.014 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Bacillus cereus* was 13.22 ± 0.035 mm, 9.05 ± 0.026 mm and 7.12 ± 0.012 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Klebsiella pneumoniae* was 15.06 ± 0.026 mm, 10.12 ± 0.015 mm and 7.53 ± 0.018 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively. The average zone of inhibition against *Pseudomonas aeruginosa* was 13.89 ± 0.011 mm, 9.31 ± 0.008 mm and 7.03 ± 0.008 at the concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml, respectively.

Table 2: Mean ± SE values of zones of inhibition (mm) of ethanolic extract of *Tinospora cordifolia* against subclinical mastitis pathogens at different concentration

S. No.	Bacterial isolates	Zone of inhibition (mm)				
		500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml
1.	*Staphylococcus aureus*	18.91±0.012	16.16±0.027	11.83±0.018	8.31±0.008	-
2.	*Staphylococcus epidermidis*	17.34±0.020	14.10±0.041	10.41±0.005	8.12±0.017	-
3.	*Streptococcus agalactiae*	16.55±0.026	12.67±0.021	10.07±0.017	7.06±0.026	-
4.	*Streptococcus dysgalactiae*	16.36±0.020	12.36±0.008	7.62±0.005	-	-
5.	*Streptococcus uberis*	15.25±0.020	11.15±0.017	7.32±0.021	-	-
6.	*E. coli*	14.13±0.050	9.92±0.014	7.23±0.014	-	-
7.	*Bacillus cereus*	13.22±0.035	9.05±0.026	7.12±0.012	-	-
8.	*Klebsiella pneumoniae*	15.06±0.026	10.12±0.015	7.53±0.018	-	-
9.	*Pseudomonas aeruginosa*	13.89±0.011	9.31±0.008	7.03±0.008	-	-

In present study, ethanolic extract of *Tinospora cordifolia* showed antibacterial properties against all tested bacteria. The maximum zone of inhibition was found against *Staphylococcus aureus* and minimum against *Bacillus cereus*. Jeyachandran *et al*. (2003) [12] and Mishra *et al*. (2014) [17] also reported that ethanolic extracts of *Tinospora cordifolia* exhibited significant antibacterial activity against all tested bacteria *viz*. *Staphylococcus aureus*, *E. coli*, *Proteus vulgaris*, *Salmonella typhi and Pseudomonas aeruginosa* etc. Nagaprashanthi *et al*. (2012) [18] reported that the hydro alcoholic extract of *Tinospora cordifolia* creeped on *Azadirachta indica* Tree (TC1) exhibit effective antimicrobial activity against all the organisms, while the extract of *Tinospora cordifolia* (TC2) creeped on fencing exhibits inhibition zone on limited species such like *Staphylococcus aureus* (12 mm), *Klebsiella pneumonia* (10 mm), *Pseudomonas* spp. (8 mm), *Aspergillus niger* (6 mm), *Aspergillus fumigates* (8 mm) and *mucor* spp. (12 mm). The results of study suggested that *Tinospora cordifolia* creped on neem tree having the potential antimicrobial activity similar to *Azadirachta indica*.

Evaluation of *In vitro* antibacterial activity of combination of ethanolic extract of *Azardirachta indica* and *Tinospora cordifolia*

The antibacterial activity of ethanolic extract of *Azadirachta indica* and *Tinospora cordifolia* in term of the zone of inhibition against subclinical mastitis bacteria is depicted in Table 3.

The average zone of inhibition against *Staphylococcus aureus* was 20.22 ± 0.014 mm, 17.13 ± 0.012 mm, 14.83 ± 0.018 mm, 11.72 ± 0.025 mm and 9.14 ± 0.078 at concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml, respectively. The average zone of inhibition against *Staphylococcus epidermidis* was 17.26 ± 0.098 mm, 16.77 ± 0.012 mm, 14.67 ± 0.008 mm, 11.25 ± 0.018 and 7.60 ± 0.012 at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 32.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus agalactiae* was 17.30 ± 0.005 mm, 16.21 ± 0.008, 13.79 ± 0.005 mm, 10.43 ± 0.024 mm and 7.21 ± 0.008 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus dysgalactiae* was 17.64 ± 0.014 mm, 15.83 ± 0.020 mm, 13.9 ± 0.011 mm and 10.16±0.014 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Streptococcus uberis* was 16.85 ± 0.015 mm, 15.73 ± 0.011 mm, 13.53 ± 0.017 mm and 9.64 ± 0.026 at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *E. coli* was 15.37 ± 0.304 mm, 12.34 ± 0.008 mm, 10.24 ± 0.014 mm and 9.12 ± 0.014 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against

Bacillus cereus was 14.23 ± 0.011 mm, 11.78 ± 0.008 mm, 9.42 ± 0.012 mm and 8.18 ± 0.008 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Klebsiella pneumoniae* was 15.84 ± 0.020 mm, 11.45 ± 0.008 mm, 9.25 ± 0.026 mm and 7.36 ± 0.015 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively. The average zone of inhibition against *Pseudomonas aeruginosa* was 14.74 ± 0.001 mm, 10.63 ± 0.011 mm, 9.23 ± 0.116 mm and 7.21 ± 0.012 mm at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml, respectively.

Table 3: Mean ± SE values of zones of inhibition (mm) of ethanolic extract of combination of *Azadirachta indica* and *Tinospora cordifolia* against subclinical mastitis pathogens at different concentration

S. No.	Bacterial isolates	Zone of inhibition (mm)				
		500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml
1.	*Staphylococcus aureus*	20.22±0.014	17.13±0.012	14.83±0.018	11.72±0.025	9.14±0.078
2.	*Staphylococcus epidermidis*	17.26±0.98	16.77±0.012	14.67±0.008	11.25±0.018	7.60±0.012
3.	*Streptococcus agalactiae*	17.3±0.005	16.21±0.008	13.79±0.005	10.43±0.024	7.21±0.008
4.	*Streptococcus dysgalactiae*	17.64±0.014	15.83±0.020	13.76±0.02	10.16±0.014	-
5.	*Streptococcus uberis*	16.85±0.015	15.73±0.011	13.53±0.017	9.64±0.026	-
6.	*E. coli*	15.37±0.304	12.34±0.008	10.24±0.014	9.12±0.014	-
7.	*Bacillus cereus*	14.23±0.011	11.78±0.008	9.42±0.012	8.18±0.008	-
8.	*Klebsiella pneumoniae*	15.84±0.020	11.45±0.008	9.25±0.026	7.36±0.015	-
9.	*Pseudomonas aeruginosa*	14.74±0.001	10.63±0.011	9.23±0.116	7.21±0.012	-

Acccording to the study, it was concluded that combination of ethanolic extracts of both *Azadirachta indica* and *Tinospora cordifolia* were effective against common subclinical mastitis organisms *viz*. *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, E. coli, Bacillus cereus, Klebsiella pneumonia and Pseudomonas aeruginosa*. Mantena *et al*. (2006) [15] reported that berberine is an active constituent extracted from the *Tinospora cordifolia,* shows various pharmacological action, whereas Debashri and Tamal, 2012 [8] recorded that azadirachtin is an active compound extracted from the *Azadirachta indica* tree which possesses antibacterial, antiviral, antifungal, and insecticidal activities. Patil *et al*. (2017) [21] studied that Methanolic and ethanolic extracts of *Tinospora cordifolia, Azadirachta indica* and *Ocimum santum* showed strong antibacterial effect against *Staphylococcus epidermidis, Staphylococcus aureus* and moderate antibacterial effect against *Escherichia coli, Proteus vulgaris, Enterobacter aerogenes, Salmonella typhi* and *Salmonella typhimurium* while mild effect on *Pseudomonas aeruginosa.*

Conclusions

Present study concluded that ethanolic extract of stem of *Azadirachta indica* and *Tinospora cordifolia* possessed potent anti bacterial activity and can be used in the treatment of subclinical mastitis in dairy cattle with highest efficacy of combination of ethanolic extracts of *Azadirachta indica* and *Tinospora cordifolia*.

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