Original Research

Phytochemical Analysis and Antibacterial Activities of *Azadirachta indica* against Pathogens Isolated from Bovine Subclinical Mastitis

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Abstract

The phytochemical components of leaves of Azadirachta indica showed the presence of saponin, tannin and terpenoids and the absence of alkaloids, reducing sugars, glycosides and flavanoids in the methanolic extract and the presence of alkaloids, reducing sugars and terpenoids and the absence of saponin, tannins, glycosides and flavanoids in hydromethanolic extract. The test organisms were susceptible to concentrations higher than 50 mg / ml of the plant extracts. The MIC of methanolic extract of Azadirachta indica leaves was recorded to be 15.62 mg / ml for S. aureus and 31.25 mg / ml for both S. agalactiae and E. coli. The hydromethanolic extract of A. indica leaves showed MIC of 31.25 mg / ml for S. aureus, S. agalactiae and E. coli. The highest recovery rate was seen in a group, treated with intramammary infusion of enrofloxacin BID for a period of 3 days as compared to other treated group.

Key words: Antibacterial, *Azadirachta indica*, MIC, Phytochemical

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Introduction

Mastitis is the most prevalent production disease in dairy herds worldwide and under untreated conditions, it constitutes a serious problem with considerable economic consequences, mainly due to fall in milk production, decreased milk quality for dairy purposes and poor milk hygiene (Seegers *et al*., 2003). At least, 137 infectious causes of bovine mastitis are known to date and the commonest pathogens are *Staphylococcus aureus, Streptococcus agalactiae*, other *Streptococcus* species and Coliforms (Sumathi *et al*., 2008). Resistance of pathogens to common veterinary antibiotics hampers mastitis treatment and motivates the discovery of new antimicrobials. The antimicrobials obtained from plants are of much

therapeutic potential and are effective in treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Kokoska, 2002). Hydromethanolic extract of *Azadirachta indica* in mastitis have been reported to possess anti-inflammatory, antibacterial and immunomodulatory potential (De and Mukherjee, 2008). A few reports about the activity of this plant in cases of mastitis have been reported but its ameliorative effects in mastitis in order to replace conventional antibiotic therapy are yet to be explored.

Materials and Methods

Phytochemical Analysis

The methanolic and hydromethanolic extracts were analyzed, as per the methods suggested by Talukdar and Choudhary, 2010.

In Vitro Antibacterial Susceptibility Patterns

The antibacterial activity of all the extracts was screened by agar cup method as described by Cruickshank *et al.* (1975).

Minimum Inhibitory Concentrations (MIC) of the extracts having antibacterial activity was evaluated by tube dilution method as described by Robert and Scott (1966). Confirmation of MIC was done by sub culturing method as described by Rathchandi *et al*. (2001) with some modifications. Content from a tube was taken into inoculation loop in front of flame and streaked over a division of the nutrient agar petri plate (having 11 divisions) and repeated for all the 11 tubes over 11 divisions. The plate was incubated at 37^0C and after 18-24 hr these plates were examined for growth of bacteria in individual division of the plate. The lowest concentration of extract preventing the growth on the plate was taken as minimal inhibitory concentration (MIC) for that extract.

Group wise Treatment Protocol

Group I

Intramammary infusion with standard antibiotic therapy using Enrofloxacin (QuinIntas- Intas Pharmaceuticals Ltd., 10% injection) 150 mg diluted in 5 ml Phosphate Buffer Saline (PBS) in each infected quarters BID for a period of 3 days.

Group II

Intramammary infusion with methanolic extract (50 mg / ml) of *Azadirachta indica* dissolved in 5 ml PBS once daily for 7 days.

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Group III

Intramammary infusion with hydromethanolic extract (50mg/ml) of *Azadirachta indica* dissolved in 5 ml PBS once daily for 7 days.

Results and Discussion

The methanolic extract of *A. indica* was blackish in colour, sticky in nature and bitter in odour while the hydro-methanolic extract of *A. indica* was brownish in colour, less sticky than methanolic extract and bitter in odour. During present course of investigation the phytochemical analysis of both the extracts showed that the methanolic extract was positive for saponins, tannins, and terpenoids and negative for alkaloids, reducing sugars, glycosides and flavanoids (Table1) which was in close agreement to the findings of Dharajiya *et al.* (2012) and Vinoth (2012).

Table 1: Phytochemical analysis of methanolic and hydromethanolic extract of *Azadirachta indica*

Phytochemical	Methanolic Extract	Hydromethanolic Extract
Alkaloids	-ve	+ve
Reducing sugars	-ve	+ve
Saponins	+ve	-ve
Tannins	+ve	-ve
Terpenoids	+ve	+ve
Glycosides	-ve	-ve
Flavanoids	-ve	-ve

Hydromethanolic extract was found positive for alkaloids, reducing sugars and terpenoids and negative for tannins, saponins, glycosides and flavonoids, which is in corroboration with the findings of De and Mukherjee (2008) who reported that the hydromethanolic extract of *A. indica* revealed the presence of triterpene and carbohydrate. It has been recorded that triterpene possesses anti-inflammatory and antimicrobial effects (Hunter *et al.*, 1997).

Based on the zones of inhibition of growth, the methanolic and hydro-methanolic crude extracts of leaves of *Azadirachta indica* against common mastitis pathogens viz., *Staphylococcus aureus, Streptococcus agalactiae* and *E. coli* showed that at the concentration of 100 mg / ml (as per standard method), both the herbal extracts (100 µl) individually exhibited the antibacterial activity against the pathogens. The zone of inhibition of methanolic extract of *A. indica* was 17.33±0.54 mm, 14.50±0.92 mm and 13.16±0.65 mm and that of hydromethanolic extract of *A. indica* was 14.66±0.73 mm, 12.83±0.77 mm and 11.33±0.96 mm for *S. aureus, Streptococcus agalactiae* and *E. coli*, respectively (Table 2). The activity of methanolic extract of *A. indica* against *S. aureus* and *E. coli* was higher than that of hydromethanolic extract of *A. indica.* Inhibitory zones formed by both the extracts of *A. indica* were different for different bacteria and the value was highest (17.33±0.54 mm) against *S. aureus* and lowest (11.33±0.96mm) for *E. coli*.

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Table 2: Comparative *in vitro* antibacterial activity of methanolic and hydromethanolic extract of *Azadirachta indica* to enrofloxacin against different bacterial isolates

Drug/Extract	Zone of Inhibition (mm)		
	Staphylococcus aureus	*Streptococcus agalactiae*	*E. coli*
Methanolic extract	17.33±0.54	14.50±0.92	13.16±0.65
Hydromethanolic extract	14.66±0.73	12.83±0.77	11.33±0.96
Enrofloxacin	27.33±0.46	25.83±0.77	21.83±0.52

On comparing both the extracts, the methanolic extract was found to be more effective than the hydromethanolic extract of *A. indica* (Table 1). The zone of inhibition of methanolic extract of *A. indica* at 100 and 200 mg/ml was 10 mm, 9 mm, 8 mm and 12 mm, 10 mm, 11 mm respectively, for *Staphylococcus aureus, Streptococcus* sp. and *E. coli* (Doss *et al*., 2012). The methanolic extract of *A. indica* showed variated zone of inhibition at 0.5%, 1%, 1.5% and 2% concentration which were 2 cm, 2.3 cm, 2.1 cm, 2 cm and 2.1 cm, 2.3 cm, 2.4 cm and 2 cm respectively against *E. coli* and *S. aureus* (Mishra, 2013). During the course of present investigation, enrofloxacin was found to be the best antibiotic against all tested bacteria. The inhibitory zones formed by enrofloxacin, methanolic extract of *A. indica* and hydromethanolic extract of *A. indica* were 27.33±0.46 mm, 25.83±0.77 mm, 21.83±0.52 mm; 17.33±0.54 mm,14.50±0.92mm 13.16±0.65 mm and 14.66±0.73 mm, 12.83±0.77 mm, 11.33±0.96 mm for *Staphylococcus aureus, Streptococcus agalactiae and E. coli*, respectively.

The efficacy of methanolic and hydromethanolic the extract was 63.41%, 56.13%, 60.28% and 53.64%, 49.67%, 51.90% for *Staphylococcus aureus, Streptococcus agalactiae* and *E. coli*, respectively in comparison to enrofloxacin. Shakuntala *et al*. (2003) found highest sensitivity of enrofloxacin and ciprofloxacin while highest resistance of streptomycin and ampicillin against common isolates of microbes associated with mastitis in Meghalya. Sharma (2000) reported highest *in vitro* efficacy of enrofloxacin (93.07%) followed by cloxacillin (64.61%) against microbes associated with genesis of mastitis in Himachal Pradesh. He opined that the sensitivity pattern would vary depending on the intensive use of antibiotics in particular region for therapeutic uses in livestock. The MIC of methanolic extract of *Azadirachta indica* leaves was recorded to be 15.62 mg/ml for *S. aureus* and 31.25mg/ml for both *Streptococcus agalactiae* and *E. coli*. The hydromethanolic extract of *Azadirachta indica* leaves showed MIC of 31.25 mg/ml for *S. aureus, Streptococcus agalactiae* and *E. coli.* Abalaka *et al*. (2012) recorded the MIC and minimum bactericidal concentration (MBC) of ethanolic leaf extract as 5 mg/ml and 50 mg/ml respectively for *S. aureus* and *E. coli*. The MIC of methanolic extract of *Azadirachta indica* was 0.2 and 2 mg/ml against *Staphylococcus aureus* and *E. coli* respectively (Doss *et al*., 2012).

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In the present study, out of 112 crossbred cows studied, 63 were positive for subclinical mastitis. Among a total of 428 quarters, 142 quarters were found positive for subclinical mastitis. The overall and quarter wise prevalence of subclinical mastitis was computed to be 56.25% and 33.17% respectively (Table 3) which was in agreement to the findings of Gupta (2010) who found an overall prevalence of subclinical mastitis as 57.72% and quarter wise prevalence as 32.98% and Kumar (2010) who recorded an overall prevalence of subclinical mastitis as 54.09 % and quarter wise prevalence as 31.02 %.

Table 3: Overall and quarter wise prevalence of subclinical mastitis affected cows

Observations	No. of Animals	Total No. of functional quarters examined	Quarter Wise Prevalence			
			LF	RF	LH	RH
Cows examined	112	428*	109	111	103	105
SCM positive	63	142	26	25	45	46
Prevalence (%)	56.25	33.17	23.85	22.52	43.68	43.80

Out of total 142 positive samples, 95 (66.90%) samples were found positive for Staphylococcus spp., 33 (23.23%) for Streptococcus spp., 04 (02.81%) for *E. coli* and 10 (07.04%) for mixed infection (Table 4), which is in close agreement to the findings of Gupta (2010), that out of a total 156 positive samples, 67.95% were positive for *Staphylococcus* spp., 23.72% for *Streptococcus* spp., and 05.77% for mixed infection and Kumar (2010) who reported that out of total 148 quarters, 104 (70.27%) were infected with *Staphylococcus* spp., followed by 30 (20.27%) *Streptococcus* spp., 05 (03.37%) with *E. coli* and 07 (04.72%) for mixed infection.

Table 4: Bacterial prevalence in subclinical mastitis affected cows

Bacteria	Quarter Affected				Total
	RF	LF	RH	LH	
Staphylococcus spp.	19 (20%)	18 (18.94%)	30 (31.57%)	28 (29.47%)	95 (66.90%)
Streptococcus spp.	4 (12.12%)	6 (18.18%)	11 (33.33%)	12 (36.36%)	33 (23.23%)
E. coli	1 (25%)	0 (0%)	2 (50%)	1 (25%)	4 (2.81%)
Mixed infection	1 (10%)	2 (20%)	3 (30%)	4 (40%)	10 (7.04)

Enrofloxacin was found to be the most effective antibiotic against SCM in the present study. Sharma (2000) also reported highest in vitro efficacy of enrofloxacin (93.07%) followed by cloxacillin (64.61%) against microbes associated with mastitis in Himachal Pradesh. The highest recovery rate of 83.33% was seen in Group I (intramammary enrofloxacin treated) followed by Group II (intramammary methanolic extract treated) and Group III (intramammary hydromethanolic extract treated) with recovery rates as 66.66% and 50.00% (Table 5). No adverse reaction was observed with the intramammary administration of extracts during this study.

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Table 5: Group wise recovery rate

Group	Drug/Extract	Dose and Duration of Treatment	No. of Animals Treated	No. of Animals Recovered	Per cent Recovery
I	QuinIntas (Enrofloxacin-100mg/ml)	150mg bid for 3 days	6	5	83.33%
II	Methanolic extract of *A. indica*	250mg bid for 3 days	6	4	66.66%
III	Hydromethanolic extract of *A. indica*	250mg bid for 3 days	6	3	50.00%

Summary and Conclusion

Azadirachta indica leaf extract has antibacterial activity against mastitis pathogens. It is expected that using natural products as therapeutic agents will probably not elicit resistance in microorganisms. This can explain the rationale for the use of the plant in treating infections in traditional medicine. The plant could be a veritable and cheaper substitute for conventional drugs since the plant is easily obtainable and the extract can easily be made via a simple process of maceration or infusion. It is essential that research should continue to isolate and purify the active components of this natural herb and use in experimental animals. On comparing the three treatment groups, intramammary infusion of enrofloxacin for 3 days BID (group I) was graded as superior treatment for subclinical mastitic cows followed by intramammary infusion of methanolic extract for 7 days once daily (group II) and intramammary infusion of hydromethanolic extract for 7 days once daily (group III).

References

1. Abalaka, M., Oyewole, O A. and Kolawole, A R. 2012. Antibacterial Activities of *Azadirachta indica* against Some Bacterial Pathogens. *Adv. in Life Sci.* 2(2): 5-8.
2. Cruickshank, R., Duguid, J P., Marmion, B P. and Swain, R H A. 1975. Medicinal microbiology, 2nd edn, Vol. II. The English Language Book Society, Churchill, Livingston, Edinburgh, London and New York. 119p.
3. De, U K. and Mukherjee, R. 2008. Expression of cytokines and respiratory burst activity of milk cells in response to *Azadirachta indica* during bovine mastitis. *Trop. Anim. Health Pro*. 41: 21-25.
4. Doss, A., Mubarack, H M., Vijayasanthi, M. and Venkataswamy, R. 2012. *In-vitro* antibacterial activity of certain wild medicinal plants against bovine mastitis isolated contagious pathogens. *Asian J. of Pharm. and Clin. Res.* 5(12): 90-93.
5. Gupta, D. 2010. Evaluation of Immune-therapeutic and Antioxidative effect of some herbs in bovine subclinical mastitis. Thesis, Ph.D. G. B. Pant University of Agriculture and Technology, Pantnagar (India).
6. Hunter, M S., Rowald, E. and Durley, R C. 1997. Four new clerodane diterpenes from leaves of *Curcuma guianensis* which inhibit the interaction of leukocyte function antigen 1 with intracellular adhesion molecule. *J. Nat. Prod*. 60: 894-899.
7. Kokoska, L., Polesny, Z., Rada, V., Nepovim, A. and Vanek, T. 2002. Screening of some Siberian Medicinal Plants for antimicrobial activity. *J. Ethnopharmacol.* 82:51–53.
8. Kumar, S. 2010. Development of herbal remedies for management of subclinical mastitis in bovines. Thesis, M.V.Sc. G. B. Pant University of Agriculture and Technology, Pantnagar (India). 59 p.

DOI 10.5455/ijlr.20170920023525

9. Mishra, A., Mamta, Neema, Niketa, Poonam, Pranjul and Priyanka 2013. Antibacterial effects of crude extract of *Azadirachta indica* against *Escherichia coli* and S*taphylococcus aureus*. *Int. J. Sci. Env. Tech.* 2(5): 989 – 993.
10. Robert, B W. and Scott, E G. 1966. *Diagnostic Microbiology*. 2nd edn. the C. V. Mosby Company Ltd., Tokyo, Japan. pp 257-272.
11. Seegers, H., Fourichon, C. and Beaudeau, F. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Vet. Res.* 34: 475-491.
12. Shakuntala, I., Giri, S C., Yadav, B P S. and Kumar, A. 2003. Bacterial isolates from bovine mastitis and sensitivity pattern to different antibiotics. *Indian J. Dairy Sci.* 56(5): 341-342.
13. Sharma, A K. 2000. Studies on prevalence and therapeutics of mastitis in dairy animals. M.V.Sc. Thesis. H.P.K.V. Palampur (H.P.). 61p.
14. Sumathi, B R., Veeregowda, B M. and Gomes, A R. 2008. Prevalence and antibiogram profile of bacterial Isolates from clinical bovine mastitis. *Vet.World*. 1 (8): 237-238.
15. Talukdar, A D., Choudhary, M D., Chakraborty, M. and Dutta, B K. 2010. Phytochemical screening and TLC profiling of plant extracts *Cyathea gigantea* and *Cyathea brunoniana*. *J. Sci. Technol. Bio. Env. Sci.* 5(1):70-74.
16. Vinoth, B., Manivasagaperumal, R. and Rajaravindran, M. 2012. Phytochemical analysis and antibacterial activity of *Azadirachta indica* a juss. *Int. J. Res. Plant Sci.* 2(3): 50-55.

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