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RESEARCH ARTICLE

In vitro antimicrobial efficacy of methanolic fruit extract of *Terminalia bellerica* against causative organism of Bovine Mastitis

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Abstract

The antimicrobial activity of methanolic extract of *Terminalia bellerica* (*T. bellerica*) dry fruit was tested by disc diffusion method, against 3 mastitis causing pathogens, two gram positive (*Staphylococcus aureus, Streptococcus agalactiae*) and one gram negative (*Escherichia coli*) strains of bacteria have been investigated. The antibiotic susceptibility test of the microorganisms to the methanolic extract of *T. bellerica* was compared with standards drug i.e. Ciprofloxacin. Methanolic extract of *Terminalia bellerica* against *S. aureus* was found to be highly susceptible forming highest zone of inhibition, suggesting that *T. bellerica* was strongly inhibitory towards this organism. Minimum inhibitory concentrations (MICs) of the extracts were also determined using broth dilution method. These results indicate that *T. bellerica* dry fruit possesses potential antimicrobial activity.

1. Introduction

To cure many diseases plants have been used as alternative treatments in developing countries. *Terminalia bellerica* Roxb, belonging to family Combrataceae, commonly known as Bahera in Hindi having wide array of bioactive principles which possess medicinal properties (Indian Herbal Pharmacopoeia, 2002). The tree is found in deciduous forests throughout the greater parts of India and also found in Myanmar, Sri Lanka, East and South East Asia. The dried fruit used for analgesic, astringent, brain tonic, expectorant and laxative purposes (Chaudhary, 2008).The fruit extracts of *T. bellerica* is having great hepatoprotective, antimicrobial and antihypertensive activity (Yu et al., 2002). Chemically, the fruit of *T. bellerica* reported the presence of β-sitosterol, gallic acid, ellagic acid, ethyl gallate, chebulagic acid, mannitol, glucose, galactose, rhamnose, and fructose.

"Mastitis" is the inflammation of mammary gland in dairy cows, accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissue (Samad, 2008). The mastitis pathogens commonly found in the udder (contagious pathogens) or the cow's surroundings (environmental pathogens), such as bedding, manure, soil, etc. The pathogens associated with contagious mastitis are Staphylococcus aureus (S. aureus) and Streptococcus agalactiae (*St. agalactiae*) (Hogan et al., 1989) and pathogens associated with environmental mastitis are Coliforms-particularly *Escherichia coli* (*E. coli*), *Enterobacter aerogenes, Klebsiella pneumonia* and *Serratia marcescens* and *Streptococcus uberis* (Monecke et al., 2007). Due to presence of synthetic antibiotics in dairy milk and adverse effects on population health and the agri-food chain (Taga et al., 2012). There is need to find sustainable alternatives for control of mastitis. So, the present study was done to analyze the antimicrobial activity of methanolic extracts of *Terminalia bellerica* against mastitis causing pathogens.

2. Materials and Methods

2.1 Collection of plant materials

T. bellerica was collected from West Singhbhum district of Jharkhand, with the help of traditional veterinary healers and experienced farmers in the field of traditional health care system were washed thoroughly

with water and dried at 40^0C and grounded into coarse powder for extract preparation. The sample was identified from Bareilly College, Bareilly.

2.2 Test Microorganisms

Microorganisms were isolated from mastitis milk sample as per the standard procedure (Griffin et al., 1977). Microorganisms were initially identified on the basis of colony morphology and odour on 5% blood agar as per Cruikshank (1962) and later by gram staining and growth on selective media, later identified by standard biochemical kits (HiStaph^{TM}, HiStrep^{TM} and Hi E. coli^{TM} identification kit HiMedia, Mumbai).

2.3 Preparation of methanolic extracts

The powdered material was macerated in methanol at the ratio of 50 gm of plants per 250 ml of methanol in a clean flat-bottomed glass container and percolated with methanol. The supernatants were collected after keeping the plant materials at 14 days in room temperature, and then filter the extract using whatmann filter paper no. 1. The extract was evaporated in hot air oven at 40^0C. The dried powder was weighed and reconstituted in sterile phosphate buffer saline (PBS, pH 7.4, 0.01M). The yield was 8.5 gm for methanolic extract. Finally the filtrate was filtered through membrane filter (pore size 0.45 µm) and stored in airtight vials at 4^0C for subsequent use. Extract was then used for further antimicrobial assay.

2.4 Phytochemical screening

For the presence of plant phytoconstituents such as Alkaloid, Flavonoid, Steroid, Saponin, Tannin and Glycosides of methanolic fruit extract of *T. bellirica*, qualitative assay, was carried out following standard procedure (Khandelwal, 2005).

2.5 Screening of antibacterial activity

The antibacterial assay of methanolic fruit extract of *T. bellerica* was performed by agar disc diffusion method (Baur et al., 1966). The pathogenic organisms, which were grown on 5% blood agar, were taken (3-4 colonies) and it was dipped in Nutrient broth. The organism was thoroughly mixed in solution; thereafter the turbidity of the solution was matched with the 0.5 McFarland's standard 1.5×10^8 cfu/ml. A sterile swab was dipped in this solution was smeared over Mueller-Hinton (MH) agar plate and were dried for 15 minutes. The antibiogram of the herbs were done by the disc diffusion methods (NCCLS, 1997). All the organisms (*S. aureus*, *st. agalactiae* and *E. coli*) isolated from bovine mastitis were taken at concentration of 1000 µl of 10^8 cells/ml and spread over Mueller-Hinton media plates. Sterile filter paper discs were impregnated in 25 µl of the prepared herbal extract (8000 µg/ml, 6000 µg/ml, 4000 µg/ml and 2000 µg/ml) for *Terminalia bellerica*. The inoculated plates were placed at 4^0C for 2 hours and thereafter the plates were incubated at 37^0C for 24 hours. The diameter of the zone of inhibition was measured in millimeters. Standard antibiotic discs of Ciprofloxacin (5 µg/disc) were used as positive control. All the tests were done in duplicate to minimize the test error. The extract/fractions that showed antimicrobial activity were later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial sample using suitable method.

2.6 Determination of Minimum Inhibitory Concentration (MIC)

Tube dilution method was done to determine the Minimum inhibitory concentration (MIC) of the extracts. A series of two fold dilution of each extract ranging from 100 mg/ml to 0.78 mg/ml was made in Muller Hinton broth as specified by National Committee for Clinical Laboratory Standards (NCCLS, 1998). In order to determine the MIC values, the extract or fractions were diluted in a simple dilution manner to make concentrations in the range of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 mg/ml. Then 1000 µl of standard inoculum of the bacterial strains matched to 0.5 Mc Farland's standards were seeded into each dilution. Two control tubes were maintained for each test batch. These included antibiotic control (tube containing extract and growth media without inoculum) and organism control (tube containing the growth medium and the inoculum).The tubes were then incubated at 37°C for 24 hours and checked for turbidity. MIC was determined as the highest dilution (that is, lowest concentration) of the extract that showed no visible growth.

3. Results and Discussion

The present study on methanolic fruit extracts of *T. bellerica* was strongly inhibitory to *S. aureus*, forming largest zone of inhibition (Table 2), i.e 20 mm and MIC values was 3.12 mg/ml (Table 3) with respect to *St. agalactiae* and *E. coli*, suggesting that *T. bellerica* was most effective against *S. aureus* (Elizabeth, 2005). Presence of different phytochemicals has been found to possess a wide range of activities, which may help in protection against infectious diseases (Gurib-Fakim, 2006). Methanolic fruit extract of *T. bellerica* showed the presence of

alkaloid, tannins and flavonoids. Presence of alkaloid present in the fruit extract might have inhibited the microorganism by impairing the enzymes involved in energy production, interferring the integrity of cell membrane and structural component synthesis. The phytochemical tannins in the fruit extract of *T. bellerica* might have prevented the development of microorganisms by precipitating the microbial protein (Hung and Chung, 2003). In many cases, antimicrobial effects of various plant extracts have been attributed to their flavonoid contents (Cafarchia et al., 1999).

Table 1: Phytochemical analysis of methanolic fruit extract of *T. bellerica*

Alkaloids	Flavanoids	Glycosides	Saponins	Steroids	Tannins
+	+	-	-	-	+

Table 2: Antimicrobial activity of *T. bellerica* showing zone of inhibition against microorganisms

S. No.	Solvent	Concentration	*Staphylococcus aureus*	*Streptococcus agalactiae*	*E. coli*
1	Methanol	2 mg	14 mm	8 mm	13 mm
		4 mg	15 mm	10 mm	15 mm
		6 mg	18 mm	11 mm	16 mm
		8 mg	20 mm	12 mm	17 mm
2	Ciprofloxacin	5 µg	25 mm	26 mm	21 mm

Table 3: Minimum inhibitory concentration (MIC) of Methanolic fruit extract of *T. bellerica*

Organisms	**MIC of Methanolic fruit extract of *T. bellerica***
S. aureus	3.12 mg/ml
St. agalactiae	12.5 mg/ml
E. coli	6.25 mg/ml

4. Conclusion

The present investigation was undertaken to evaluate the antibacterial property of methanolic fruit extracts *T. bellerica* and its antibacterial activity was checked by disc diffusion method against clinical isolates of *S. aureus*, *St. agalactiae* and *E. coli.* Of all the clinical isolates *S. aureus* exhibited maximum zone of inhibition 20 mm, followed by *E. coli* and *St. agalactiae*. Phytochemical investigation reported the presence of alkaloids, flavonoids and tannins. The results of the study support *T. bellerica* fruit extracts possess compounds with antimicrobial properties that can be used in novel drugs for the treatment of microbial diseases. Hence this plant can be further subjected to isolation of the therapeutic antimicrobials and further pharmacological evaluation.

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