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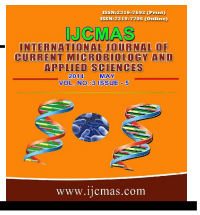

Original Research Article

Antimicrobial efficacy of *Terminalia bellerica* against virulence factors of respiratory pathogens

Shilpa Sabnis*

Flat-601, Rimple 4D, New Saraswati CHSL., Asha Nagar, Thakur Complex, Kandivli (E), Mumbai-400 101
Corresponding author

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A B S T R A C T

Medicinal plants have been used in treatment of various diseases from time immemorial. The increasing failure of chemotherapeutics, coupled with an increasing resistance towards antibiotics exhibited by various pathogenic microbial infectious agents has led to a search for alternative modes of treatment of these diseases. *Terminalia bellerica* commonly known 'Behada' or 'Belliric Myrobalan' has been routinely used in traditional medicinal for treatment of cough, fever etc. In the present study, an attempt was therefore made, to determine the antimicrobial activity of methanolic extract of *Terminalia bellerica* against two commonly encountered respiratory pathogens viz *Staphylococcus aureus and Klebsiella pneumoniae.* The preliminary antimicrobial analysis was carried out using the Agar ditch method which demonstrated the antimicrobial activity of *T. bellerica.* Methanolic extract of *Terminalia bellerica* was further found to inhibit the coagulase activity of *Staphylococcus aureus* as well as result in individual biochemical alterations. It was also found to bring about major alterations in the capsular morphology of *Klebsiella pneumoniae* after 24 h. and 48 h. of treatment respectively. The results obtained in the present study indicate that *Terminalia bellerica* extract possesses potential antimicrobial activity against most commonly encountered respiratory pathogens and can be used in treating diseases caused by these pathogens.

Introduction

According to Ayurveda, the origin of most diseases is found in either exogenous or endogenous dosha imbalance or in an inherent or acquired weakness of the tissues. The disease process is an actually thought to be reaction between the bodily humors (doshas) and tissues (dhatus) and is influenced by the environment.

Therefore, diagnosis in Ayurveda implies a time-to-time monitoring of the interaction between order (health) and disorder (disease) (Durappandiyan and Ayyamar 2006). As against this, in western medicine, diagnosis generally refers to the identification and treatment of an ailment once it is manifested.

Terminalia bellerica commonly known as 'behada' is one such medicinal plant routinely used in traditional medicine as a remedy from several ailments such as fever, cough, diarrhea, skin diseases, and oral thrush (Parekh and Chanda, 2007; Cowan, 1999). But, the major hindrance in the amalgamation of herbal medicines into modern medical practices is the lack of scientific and clinical data, and better understanding of efficacy and safety of the herbal products.

A comparative analysis of traditional medicines with modern drugs with regard to their efficacy of treatment has not been conducted for most of the herbal products. Integrating traditional medicine into the modern medicinal practices is the need of the hour. A systematic approach through experimental and clinical validation of efficacy is therefore required for any plant identified for its medicinal properties, as is done in modern medicine.

Therefore, in the present study an attempt was made to study the antimicrobial activity of *T. bellerica* against two commonly encountered respiratory pathogens viz. *S. aureus and K. pneumoniae* as well as to determine whether it has any effect on the factors responsible for virulence of these pathogens.

Materials and Methods

All media, chemicals (AR grade) and reagents were purchased from HiMedia ®, Laboraratories, Mumbai.

Preparation of methanolic extract of *Terminalia bellerica*

Ten grams of dried plant material was extracted with 100 ml of methanol in a conical flask and kept on a rotary shaker at 190-220 rpm for 24 h. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was collected and the solvent was evaporated to one-fifth of the original volume. It was stored at 4ºC in airtight bottles for further studies.

Preliminary antimicrobial sensitivity of methanolic extract of *T bellerica* by Agar Ditch Plate Method

Sterile Nutrient Agar plates were prepared using 20 ml of sterile, cooled, and molten Nutrient Agar. A ditch of 8 cm × 1.5 cm size was cut out in the plate with the help of sterile scalpel to which 5 ml of sterile, cooled, and molten nutrient agar containing different concentrations (viz 5%, 10%, 15%, 20% v/v)of *T. bellerica* extract were added. A control plate containing Nutrient Agar without any extract was also included. The two test cultures viz. *S. aureus* and *K. pneumoniae* were streaked at the right angles to the ditch and parallel to each other. The plates were then incubated at 37ºC for 24 h. and the results were noted in terms of presence / absence of growth of the test cultures.

Effect of the methanolic extract on the biochemical characteristics

Various biochemical tests characteristic for *S. aureus* and *K. pneumoniae* (Bergey's manual, 1957) were carried out in presence and absence of methanolic extract of *T. bellerica*.

Effect of methanolic extract of *T. bellerica* on coagulase enzyme of *S. aureus*:

S aureus was grown in Walbum's media to enhance the production of coagulase. After 18 h. of incubation, this broth was used as

the inoculum. Different concentrations of methanolic extract of *T.bellerica* in the range 2% to 10% were prepared. Fresh human plasma was diluted 1:10 using sterile saline. To 0.5 ml of diluted plasma, 0.1 ml of the above inoculum was added. 1 ml of various concentrations of the methanolic extract of *T.bellerica* were then added to the tubes and incubated at 37ºC for 30 minutes to an hour. Positive and negative controls were also maintained. The plasma clot obtained was centrifuged and washed 3 times to remove any plasma proteins. The enzyme activity was estimated in terms of protein content of the plasma clot using Folin Lowry method (Lowry *et al.*, 1951).

Effect of methanolic extract on capsule of *K. pneumoniae*

Saline suspension of 18 h. old *K. pneumoniae* with 0.5 ml of methanolic extract of *T bellerica* were taken and incubated at 37ºC for 24 h. and 48 h. respectively. A control suspension without the methanolic extract was also included. These were then observed for changes in the capsule morphology on comparison with control using Manewal's capsule staining method (Cappuccino and Sherman, 1999).

Results and Discussion

Preliminary screening using 5% (v/v) to 20% (v/v) of methanolic extract of *T. bellerica* for its antimicrobial properties towards the respiratory pathogens, *Staphylococcus aureus, Klebsiella pneumoniae* indicated that the methanolic extract was indeed inhibitory to all the three respiratory pathogens. *S aureus* showed more inhibition in comparison with *K pneumoniae* which was only moderately inhibited by *T. bellerica* (Table 1). Earlier reports have always maintained that plant extracts are more active against Gram positive bacteria than Gram negative bacteria and the result observed in the study are found to be as per those reported earlier (Fyhrquist *et al*, 2004). Furthermore, the presence of capsular polysaccharide surrounding of *K pneumoniae* might have interfered in the antimicrobial activity of *T. bellerica* extract against it.

As *S. aureus* was found to be inhibited by methanolic extract of *T. bellerica*, further study was mainly aimed at determining whether it also caused any observable alterations in its morphology or biochemical characteristics. *S. aureus* did not show any morphological variation on treatment with methanolic extract of *T. bellerica* but, it did show some characteristics changes which were evident in the biochemical tests. The extract affected the catalase produced which was evident in terms of lesser amount of effervescence produced by *S. aureus* on incubation with the *T. bellerica* extract. This extract also interfered in the ability of *S. aureus* to reduce nitrate to nitrite as was evident in the change in the colour observed on testing with Griessillosvay's Reagent. The extract of *T. bellerica* also affected the fermentation pattern of *S. aureus*. It was observed that *S. aureus* was not able to ferment Glucose and Mannitol to the same extent on treatment with *T. bellerica* as it would do under normal circumstances (Table No. 2). This might be due to the reason that the active component of *T. bellerica* might have interfered with the enzymes involved in those respective pathways. All these result are in accordance with those reported for *T. chebula* (Elizabeth, 2005). Since both plants belong to the same family and fruits taste the same and

possess quite a few similar phytochemical substances, this seems to be justified. Furthermore, the active *T. bellerica* were also found to after the coagulase active of *S. aureus*. Coagulase is a major virulence factor of *S. aureus* which converts host plasma fibrinogen to fibrin forming blood clots. In the human host, the action of coagulase produces clotting of the plasma in the immediate vicinity of the bacterium. The resulting increased effective diameter of the bacterium makes it difficult for the defense reactions of the host to deal with the infecting cell. In particular, the defensive mechanism of phagocytosis, where the bacterium is engulfed by a host cell and then dissolved, is rendered ineffective. This enables the bacterium to persist in the presence of a host immune response, which can lead to the establishment of an infection. Thus, coagulase can be described as one of the major virulence factors of *Staphylococcus aureus*. This enzyme was inhibited by the active component of *T. bellerica* which was confirmed when *S. aureus* grown in presence of *T. bellerica* extract was unable to form coagulation of normal human plasma indicating either inactivation of coagulase or absence of protein products. These results are also in accordance with those cited by Elizabeth (2005). Therefore, a further attempt was made to study extent of the effect of *T. bellerica* on the coagulase activity of S. aureus. Various concentrations of methanolic extract of *T. bellerica* ranging from 2% v/v to 10% v/v were used and the amount of enzyme activity was measured in terms of the amount of clot formed which was measured in terms of its protein content using Folin Lowry's method (Lowry *et al.*, 1951). With increasing concentration of methanolic extract of *T. bellerica*, the amount of clot formed was found to decrease. There was a reduction in enzyme activity from 41.33% to 94.7% (Table No. 3). *T. bellerica* could bring about almost 94.7% reduction in this activity suggesting the effectiveness of *T. bellerica* against these respiratory pathogens.

Methanolic extract of *Terminalia bellerica* at a concentration of 10% v/v was found to only slightly affect the growth of *K. pneumoniae*. Therefore further attempt was made to determine whether it caused any morphological or biochemical alterations in *K. pneumoniae* at that concentration. With this aim, morphological biochemical tests were performed (Cappuccino and Sherman, 1999). Without any treatment with *T. bellerica*, *K. pneumoniae* was found to show its characteristic morphology, Gram staining characteristics and pattern of biochemical tests. But, on treatment with 10% v/v *T. bellerica*, differences were observed. Though on Gram staining after treatment extract of *T. bellerica*, morphological alterations were evident, the capsule staining patterns using Schaeffer and Fulton's method showed changes after 24 h. and 48 h. of treatment with the extract. Within 24 hrs, the capsule started getting more and more diffuse (Fig.1, 2) while after 48 h, the capsule almost disappeared (Fig. 3) suggesting that a longer incubation with methanolic extract of *T. bellerica* or using a higher concentration of the extract might alter the sensitivity pattern of K. pneumoniae to *T. bellerica*. Furthermore, Cowan (1999) has cited that the activity of certain lipophilic flavanoids known to be synthesized by plants in response to microbial infection is due to their complex ability with extracellular and soluble proteins and with bacterial cell wall. The results of our study are in accordance with these reports as the methanolic extract of *T. bellerica* resulted in alterations in the capsular morphology.

Table.1 Antimicrobial sensitivity of *T. bellerica* by Agar Ditch Method

Organisms used	**Concentration of methanolic extract of *T. bellerica* (v/v)**			
	5%	**10%**	**15%**	**20%**
S aureus	+	+++	+++	+++
K pneumoniae	-	+	+	+

Key: +++: highly inhibitory, + : moderately inhibitory, - : no inhibition

Table.2 Biochemical characteristics for *S. aureus*

Biochemical Tests	**Results**	
	Without extract	**With extract**
Glucose : Ae	+	+/-
An	+	+/-
Mannitol : Ae	+	+/-
An	+	+/-
Gelatinase:	+	+
Nitrate Test:	+	-
Catalase:	+	+/-
Coagulase:	+	-
6.5% NaCl:	+	-

Key: + : positive test; - : negative test; +/- : faintly positive

Table.3 Effect of *T. bellerica* on coagulase enzyme of *S.aureus*

Concentration of methanolic extract of *T. bellerica* (% v/v)	**Concentration of protein (mg/ml)**	**Percentage reduction in enzyme activity**
2	75	-
4	44	41.33
6	30	60
8	10	85.56
10	4	94.66

Table.4 Biochemical characteristics for *K. pneumoniae*

Biochemical Tests	Results without extract	Results with extract
Glucose: Ae	AG	AG
An	AG	A
Lactose: Ae	AG	AG
An	AG	AG
Maltose: Ae	A	(A)
An	A	A
Mannitol: Ae	AG	(A)
An	AG	AG
Indole:	-	-
Methyl Red:	+	-
Voges-Prauskeur:	-	-
Citrate:	++	(+)
TSI : butt	A	A
slant	A	A
H_2S	-	-
gas	G	NG
Capsule:	+	+/-

Key: AG: Acid and gas production A : Acid production
G : Gas formation (A): Weak acid production

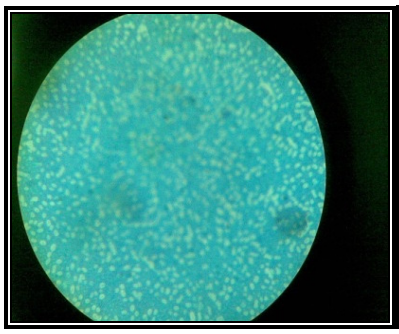

Fig 1: Capsule before addition of extract

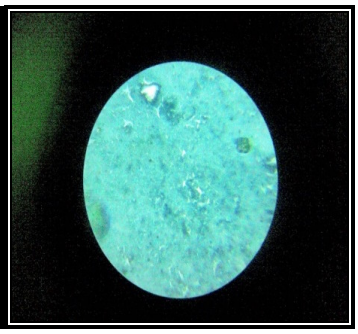

Fig. 2: Capsule as seen 24 h after addition of the extract

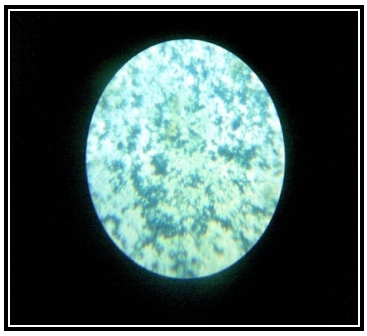

Fig 3: Capsule as seen 24 h after addition of the extract

K. pneumoniae is actually an organism possessing a polysaccharide capsule which confers upon its characteristic morphology and virulent properties as if prevents diffusion of antibiotic materials through it thus protecting the organism (Stanier, 1987). The above finding therefore suggests that *K. pneumoniae* which showed resistance to 10% v/v extract of *T. bellerica* (Table No.1 and 4) might show an altered sensitivity patterned with 10% v/v *T. bellerica* under circumstances mentioned above. Similarly, *K. pneumoniae* also showed some variations especially in the fermentation pattern of Glucose, Mannitol and Maltose after

treatment. With regards to glucose utilization, *K. pneumoniae* showed no gas production while maltose and mannitol were also not completely fermented thereby, producing lesser acid than under normal circumstances. Furthermore, under normal circumstances *K. pneumoniae* is able to utilize citrate as a sole source of carbon and energy for growth and ammonium salt as the sole source of nitrogen thereby changing the colour of bromothymol blue from green to blue. But, after treatment with *Terminalia bellerica* the process was found to be affected thus preventing the entire slant from turning green (Table No. 4).

In a nutshell, the results obtained from our investigations confirmed the therapeutic potency of methanolic extract of *Terminalia bellerica* against the virulence factors of respiratory tract pathogens.

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