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Original Article

ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF FRUIT EXTRACTS OF *TERMINALIA BELLERICA*

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ABSTRACT

Objective: In current era, herbal products are measured to be the symbols of safety in comparison to the synthetic products that are regarded to be hazardous to human life and environment. Although herbs had been priced for their therapeutic importance, their phytochemical and pharmacological activities are conducted on different parts. With this, an attempt has been made to investigate the antimicrobial activity and phytochemical analysis of *Terminalia bellerica* fruits.

Methods: The antimicrobial activity was evaluated using agar well diffusion method against the bacterial (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Shigella flexneri,* and *Salmonella typhi*) and fungal (*Aspergillus niger, Mucor* species, *Aspergillus fumigatus, Rhizopus* species and *Aspergillus flavus*) isolates using aqueous, petroleum ether and chloroform extracts of *Terminalia bellerica* fruits. Phytochemical and FT-IR analysis was carried.

Results: It was observed that aqueous extract exhibited significant activity against the tested bacterial and fungal isolates, compared with chloroform and petroleum ether extract respectively. Phytochemical analysis of *Terminalia bellerica* extracts showed the presence of secondary metabolites like phenolics, alkaloids, flavonoids and tannins. The FT-IR analysis has revealed the presence of phenols, alcohol, amines and carboxylic acid as functional groups in *Terminalia bellerica.*

Conclusion: From this study, it can be concluded that *Terminalia bellerica* reveal antimicrobial activity against various human pathogenic bacteria.

Keywords: Antibacterial, Antifungal, *Terminalia bellerica,* phytochemical and FT – IR analysis.

INTRODUCTION

Nature is and will still serve as mans primary source for the cure of his ailments. However, the potential of higher plants as a source for new drugs is still largely unexplored [1]. The traditional system of using medicinal plants for curing many diseases dates back to the age of Rig Veda. Many microbial diseases can be cured by medicinal plants without any side effects and economical issues [2]. Multidrug resistance towards antibiotics and their related effects has an added effect to pursue the use of natural drugs [3]. Infection with various microorganisms is one of the leading causes for a number of diseases [4]. Infectious diseases are usually characterized by clear symptoms, so it is likely that traditional healers have been able to recognize such diseases and have developed effective therapies. In recent past, there has been tremendous increase in the use of plant based products in developing as well as developed countries resulting in an exponential growth of herbal products globally. A variety of phytochemicals are accumulated in plants accounting for their constitutive antimicrobial activities. World Health Organisation (WHO) noted that the majority of the world's population depends on traditional medicine for primary health care [5]. *Terminalia bellerica* (Combretaceae), a large deciduous tree found throughout India has enormous medicinal properties. The seed oil is used to cure skin diseases, premature graying of hair and can be applied on painful swollen parts. The fruits of bellerica can be used to treat cough, cold, hoarseness of voice, asthma, arrest bleeding, boost hair growth, impart black colour to hair, cure conjunctivitis, astringent and anti-diarrheal agent. Fruit extract of *T. bellerica* produced fall in blood pressure of rats at a concentration of 70 mg/kg body weight. The plant helps in loss of appetite, piles, lowering cholesterol, blood pressure, boosts immunity and prevents ageing. It also enhances the body resistance against diseases. It is used as traditional medicine to get remedies from all the above ailments by the local people of Coimbatore district [6].

Considering these facts, it is expected that the screening and scientific evaluation of the fruits of bellerica may provide novel antimicrobial compounds.

MATERIALS AND METHODS

All the chemicals and reagents used were from Hi- Media Pvt. Limited, Bombay, India. Glass wares used were from Borosil.

Collection of fruit

The fruits of *Terminalia bellerica* was collected during January-February 2013 in the areas in an around Coimbatore, Tamil Nadu. The fruit was authenticated and a voucher specimen was kept in the Department of Botany, Avinashilingam University for Women, Coimbatore, Tamil Nadu, India. The fruits were washed thoroughly under running tap water for 2 - 3 times to remove dirt and then shade dried at room temperature for a week. The dry fruits, devoid of seeds were ground into fine particles and kept in closed container before being stored at room temperature until further used.

Preparation of fruit extract

Ten grams of the ground sample of *Terminalia bellerica* was weighed and homogenized with 100 ml of petroleum ether, aqueous and chloroform separately. The crude preparation was left overnight in the shaker at room temperature and then centrifuged at 4000 rpm for 20 minutes. The supernatant containing the fruit extract was then transferred to a pre-weighed beaker and the extract was concentrated by evaporating the solvent at 60º C. For the preparation of aqueous extract, 10 g of the sample was added with 100 ml of distilled water and kept in a shaker at 90-120 rpm for 24 h at 30º C. The mixture was boiled at 60º C for 3 h and concentrated to one fourth of the original volume. The extracts were then concentrated to dryness under vacuum and reduced pressure using rotary evaporator. Then the crude extracts were dissolved in known volume of dimethyl sulphoxide (DMSO) to obtain a final concentration of 20mg / 5 µl. The aliquot was stored until it was used [7].

Microbial strains

The bacterial (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Shigella flexneri,* and *Salmonella typhi*) and fungal

(*Aspergillus niger, Mucor* species, *Aspergillus fumigatus, Rhizopus* species and *Aspergillus flavus*) isolates used in the present study were the clinical isolates obtained from P.S.G. Hospitals, Coimbatore, Tamil Nadu, India.

Culture media and inoculums preparation

Muller Hinton agar media / broth (Himedia, Mumbai, India) were used as the media for the culturing of the bacterial strains. Loop full of all the bacterial cultures were inoculated in the Muller Hinton broth and incubated at 37ºC for 24 hrs. Rose Bengal Chloramphenical agar/ broth (Himedia, Mumbai, India) were used as the media for the culturing of fungal strains. Loop full of all the fungal cultures were inoculated in the Rose Bengal Chloramphenical broth and incubated at room temperature for 72 hrs.

Antimicrobial assay

Well diffusion method

The agar well diffusion method was employed for the determination of antimicrobial activity of the extracts [8]. To brief, five wells were made in Muller Hinton agar plates and Rose Bengal Chloramphenical agar plates respectively using sterile cork borer (5 mm diameter). 50 µl of bacterial and fungal inoculum were swabbed on the above plates with sterile swabs separately. 20 µl of each extract, control (DMSO) and standard antibiotics (4 mg of Chloramphenical for bacteria and nystatin for fungi) were filled in the respective wells with the help of micropipette separately. The plates were then incubated at 37ºC for 24 hours for bacteria and at room temperature (25 - 30ºC) for five days for fungal isolates. The samples were tested in triplicates and the diameter for the zone of inhibition was measured as millimeter (mm) and the results were expressed as mean ± standard deviation.

Phytochemical screening of the extracts

The extracts obtained from the fruits of *Terminalia bellerica* were qualitatively tested to identify the presence of phytochemicals such as alkaloids, phenols, amino acids, flavonoids, saponins, tannins, quinones, carbohydrates, glycosides, steroids and terpenoids according to the method proposed by [10].

FT – IR analysis

FT-IR (Fourier Transform Infrared) is a tool used for identifying the types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond which can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. For the FT-IR study dried powder of aqueous extract (10 mg) of *Terminalia bellerica* fruits was taken in a mortar and pestle and ground with 2.5 mg of dry potassium bromide (KBr). The powder so obtained was filled in a 2 mm internal diameter micro-cup and loaded onto FT- IR set at 26°C ± 1°C. The samples were scanned using infrared in the range of 4000–400 cm^{-1} using Fourier Transform Infrared Spectrometer (Shimadzu, IR Affinity 1, Japan). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample [11].

Microdilution method

The minimum inhibitory concentration (MIC) was determined by micro dilution method using serially diluted *Terminalia bellerica* extracts according to the NCCLS protocol [9]**.** The aqueous extract were diluted to get series of concentrations from 100mg/ml to 1.56mg/ml in in sterile Muller Hinton broth using 96 - well plates. The microorganism suspension of 50µl was added to the broth dilutions and was incubated for 18 hours at 37ºC. MIC of each extract was taken as the lowest concentration that did not give any visible bacterial growth.

RESULTS

The results obtained are summarized in Table 1 indicating the growth inhibition produced by fruit extract of *Terminalia bellerica* towards bacterial and fungal isolates. The experimental results obtained from the present study illustrates that the aqueous extract was found to be more effective to control the bacterial and fungal growth when compared with chloroform and petroleum ether extracts respectively. All the bacterial and fungal isolates tested showed significant activity against the aqueous extract and the zone of inhibition ranged from 15 -23 mm. The chloroform extract of the fruits of *Terminalia bellerica* showed moderate zone of inhibition against the tested bacterial and fungal isolates (9-15 mm). The petroleum ether extracts of the fruits of *Terminalia bellerica* exhibited less zone of inhibition (8 -13 mm) against the tested microorganisms. The extracts exhibited significant zone of inhibition when compared with the tested standard antibiotics (Chloramphenicol and nystatin) and no zone of inhibition was observed in negative control (DMSO). The highest zone of inhibition was found against *Klebsiella pneumoniae* (23 mm) and *Aspergillus fumigatus* (22 mm) with aqueous extract and least inhibition against *Salmonella typhi* (8 mm) and *Aspergillus niger* (9 mm) with petroleum ether extract.

The minimum inhibitory concentration (MIC) of the extracts to inhibit the microorganisms was determined using the microdilution method. Since the aqueous extract showed the maximum zone of inhibition, the MIC was determined only with this extract. Table 2 depicts the MIC values of the extract against the tested bacterial and fungal isolates.

The aqueous extract could inhibit the growth of *E. coli* and *A. fumigatus* at a minimum concentration of 6.25mg/ml when compared with other microbial isolates (Table 2). The MIC values for the standard antibiotics against the tested microbes were depicted in Table 2. The results further validate the activity of aqueous extracts against all the tested bacterial and fungal isolates.

The phytochemical analysis of the fruit extracts of *Terminalia bellerica* was tabulated in table 3. It revealed the presence of alkaloids, phenol, tannins and flavonoids.

Table 1: Antimicrobial activity of the fruit extracts of *Terminalia bellerica*

Microorganisms	Zone of inhibition in diameter (mm)				
	Petroleum ether	Chloroform	Aqueous	Positive control	Negative control
Escherichia coli	11.6±1.5	13.6±1.5	14.6±1.5	21.6±1.5	-
Pseudomonas aeruginosa	9.3±2.5	12.6±2.5	13.6±1.5	26±1.0	-
Klebsiella pneumoniae	12.6±1.5	14.3±2.0	22.6±2.5	18±2.5	-
Shigella flexneri	9.3±2.0	16	21.3±1.5	12±2.0	-
Salmonella typhi	8±2.0	8.6±1.5	10±1.5	10.6±1.5	-
Aspergillus niger	9±1.0	11.3±2	17.6±1.5	20±1.0	-
Mucor species	10.3±1.5	17	20.6±1.5	13.6±1.5	-
Aspergillus fumigatus	13.3±1.5	14.6±1.5	19.3±1.5	23.3±1.5	-
Rhizopus species	12±2.0	15±2.0	19.3±1.5	23.3±1.5	-
Aspergillus flavus	10.3±1.5	10.3±1.5	20.3±2.5	24±1.0	-

Positive control – Chloramphenicol (Bacteria), Nystatin (Fungi), Negative control – DMSO

Table 2: Minimum Inhibitory Concentration of aqueous extract of *Terminalia bellerica* against bacterial and fungal isolates

Bacterial isolates	Concentration (mg/ml)	Standard Antibiotics (mg/ml)
Escherichia coli	**6.25**	12.5
Pseudomonas aeruginosa	25	25
Klebsiella pneumoniae	50	100
Shigella flexneri	12.5	50
Salmonella typhi	100	12.5
Aspergillus niger	50	25
Mucor species	25	50
Aspergillus fumigatus	**6.25**	12.5
Rhizopus species	50	12.5
Aspergillus flavus	12.5	100

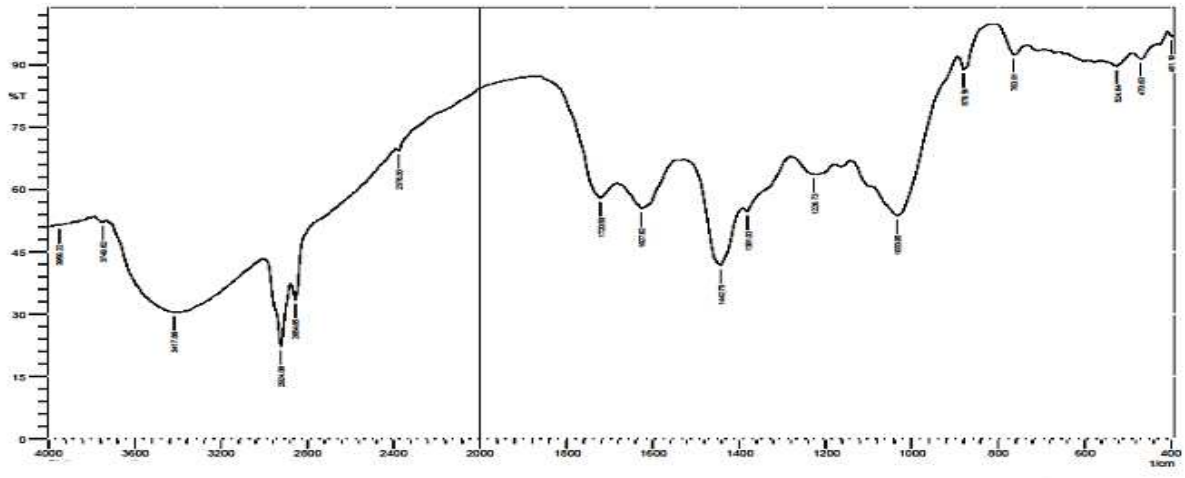
Standard Antibiotics – Chloramphenicol (Bacteria), Nystatin (Fungi)

Table 3: Qualitative phytochemical analysis of fruit extract of *Terminalia bellerica*

Phytochemicals	*Terminalia bellerica* extracts		
	Petroleum ether	Chloroform	Aqueous
ALKALOIDS			
Dragendroff's Reagent	+	+	+
Hager's test	-	-	-
Wagner's Reagent	-	-	-
PHENOLS			
Ferric chloride test	+	+	+
Lead acetate test	+	+	+
AMINO ACID			
Ninhydrin test	-	-	-
FLAVONOIDS			
Schinoda's test	+	+	+
Lead acetate Test	+	+	+
SAPONINS			
Froth test	-	-	-
TANNINS			
Breamer's test	+	+	+
QUINONES			
Borntrager's test	-	-	-
CARBOHYDRATES			
Molish test	-	-	-
Fehling's test	-	-	-
GLYCOSIDES			
Legal's test	-	-	-
STERIODS/TERPENOIDS			
Libermann – Burchardt test	-	-	-

The FT –IR spectrum of the aqueous extract of the fruits of *Terminalia bellerica* in the range of 400 – 4000 cm^{-1} revealed the presence of many functional groups. It exhibits the peak at 3950, 3749, 3417, 2924, 2854, 2376, 1720, 1627, 1442, 1381, 1226 and 1033 cm^{-1} which indicates the presence of –OH, -COOH, -NH and C=O groups respectively (Fig. 1).

Fig. 1: FT –IR spectrum of the fruit extract of *Terminalia bellerica*



DISCUSSION

Infectious diseases have become the major cause and serious concern in public health issues. The occurrence of drug resistant strains with less susceptibility to antibiotics due to mutation is challenging amongst the researcher to invent newer drugs [12]. At this scenario, evaluation of antimicrobial substances from various sources of medicinal plants is considered to be a pivotal role. The demonstration of activity against the test bacteria provides scientific base for the local usage of this plant in the treatment of various ailments. The fact that the extracts were active against bacterial and fungal isolates tested may indicate a broad spectrum of activity. This observation is very significant because of the possibility of developing therapeutic substances that will be active against multidrug-resistant organisms. The results of the study supports the traditional application of the fruit extract of *Terminalia bellerica* and suggests the presence of compounds with antimicrobial properties that can be used as antimicrobial agents in novel drugs for the treatment of microbial diseases [13].

The aqueous extract of the fruits of *Terminalia bellerica* confirmed the antimicrobial effect on bacterial and fungal isolates, suggesting that the phytochemicals present in the extract may deactivate various cellular enzymes which play a vital role in metabolic pathways of these microorganisms. It has also been found that the phytochemicals may denature the proteins of the cells, which as a result impairs normal cellular process.

A variety of phytochemicals present in the plant extracts are non-nutrient compound possess biological activity that can be of valuable therapeutic index. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases [14]. The phytochemical screening of fruit extract of *Terminalia bellerica* showed the presence of alkaloid, phenol, tannins and flavonoids. The phytochemical alkaloid present in the fruit extract might have inhibited the microorganism by impairing the enzymes involved in energy production, interfering the integrity of cell membrane and structural component synthesis. The growth of the fungus might have been inhibited due to the presence of phenol which might have induced the swelling, plasma seeping and leakage, distortion, abnormal branching or fusion and wrinkling of hyphae. Presence of tannins in the fruit extract of *Terminalia bellerica* might have prevented the development of microorganisms by precipitating the microbial protein and making nutritional proteins unavailable for them [15]. It has also been reported that tannins have been found to form irreversible complexes with proline rich proteins resulting in the inhibition of cell protein synthesis [16]. The presence of characteristic functional groups may be responsible for the medicinal properties of *Terminalia bellerica* which contain high therapeutic content. Determination of respective antimicrobial potential and toxicological evaluation of these extracts with the view to formulate novel chemotherapeutic agents to be used in future is worth mentioning.

CONCLUSION

The results of the study support the traditional application of the fruit extracts which possess compounds with antimicrobial properties that can be used in novel drugs for the treatment of microbial diseases. Further pharmacological evaluations, toxicological studies and possible isolation of the therapeutic antimicrobial from this fruit are the future challenges.

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