

**ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF
TERMINALIA CRENULATA ROTH BARK**

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Summary

The development of bacterial resistance to presently available antibiotics has necessitated the search of new antibacterial agents. Plants and plant products are known to possess excellent antioxidant properties and play a significant role in preventing the conditions due to the excessive free radicals. The present study was aimed to evaluate the antimicrobial and antioxidant activities of the alcoholic and aqueous extracts from the bark of *Terminalia crenulata* Roth. Aqueous and alcoholic extracts prepared and were tested on Gram positive and Gram negative bacteria. Agar cup plate test was used to determine the sensitivity of the tested samples while the well micro-dilution was used to determine the minimum inhibitory concentration. The DPPH and Nitric oxide radical inhibiting activity were used to detect oxidative activity. The results of antimicrobial assays showed that all tested extract were active against all tested microbial species including gram positive and negative bacteria. The alcoholic extract showed direct antimicrobial activity against all tested microorganism with minimum inhibitory concentration ranging between 0.034 to 0.520 mg/ml, while aqueous extract showed 1.05 to 4.17 mg/ml, respectively.

The results of DPPH method showed 50% inhibition rate at the 116.62 µg/ml and 153.06 µg/ml with alcoholic and aqueous extract, respectively. Nitric oxide scavenging inhibition showed 50% inhibition rate at the 232.85 µg/ml and 474.80 µg/ml using alcoholic and aqueous extract, respectively. The overall results of this study indicates that the extract from roots have interesting antimicrobial and potential free radical scavenging activity for treatment of diseases.

Key words: Antimicrobial activity, DPPH method, Nitric oxide scavenging activity, *Terminalia crenulata* Roth.

Introduction

In developing countries, infectious diseases remain the main cause of the high mortality rates recorded; the majority of rural people has limited access to formal and adequate health services and thus heavily resources to traditional healers (1). Indigenous herbal remedies are widely used against many infectious diseases, but only few of them have been studied chemically and biologically in order to identify their active constituents (2). In modern medical practice, the alarming worldwide incidence of antibiotic resistance causes a need for new compounds that can act either by a direct antimicrobial activity or by inhibiting resistance mechanisms of microorganisms of medical importance. Medicinal plants represent a valuable source for this kind of compounds (3).

India, the richest floristic regions of the world, has a source of plants and their products, since antiquity. Man uses them as food and medicine as per his desires. Among the entire flora, estimated 2,50,000 higher plant species on earth, only 35,000 to 70,000 species (less than 1%) have been used for medicinal purpose (4). There are plenty of chances to find out a new compound derived from plant. The plant *Terminalia crenulata* Roth commonly known as 'Asaina' in Hindi, 'Black murdah' in English and 'Sadad' in Gujarati belongs to family Combretaceae.

The widespread pantropical Combretaceae comprises about 500 species and 20 genera out of which 11 occur in tropical Africa. The genera Combretum, Terminalia and Ptelopsis are known to include valuable medicinal plants, many of them used in African as well as Asian traditional medicine and some of them are used as anticancer. Species of Combretum and some species of *Terminalia* are known to contain powerful anticancer compounds. *Terminalia crenulata* Roth is a medium size to large, straight tree with 15-30 m in height growing with straight trunk, leaves sub opposite or upper most alternate, flower white or pale creamy-yellow and grayish black bark having longitudinal fissures and transverse cracks exfoliating in irregular flakes. It is distributed throughout India in semi-evergreen and mixed deciduous forest. It is used in diarrhoea, dysentery, astringent, cough, bronchitis, verminosis, leucorrhoea, gonorrhoea and burning sensation. Stem bark and leaves are mainly used as drug. The main constituent of the bark are tannins, polyphenolic compounds, triterpenes glycosides, flavonoides, reducing sugar and starch. The tannins from the bark possessed antimutagenic effect and anticancer activity. The genus *Terminalia* is chemically characterized by presence of tannins and related compound.

There is no record for pharmacognostical and preliminary phytochemical work on such a potential drug; hence, present work was under taken.

The plant selected for study was based on its availability and its various therapeutic activities in various ailments mentioned in Ayurveda. In the present work, we have reported for the first time results of the combined investigations on *in-vitro* antibacterial and antioxidant activities of the extracts of *Terminalia crenulata* Roth (TCR).

Materials and Methods

Plant Material

The proposed material of *Terminalia crenulata* Roth bark was procured from South Gujarat Region, with the help of local

tribal and field botanist. The species for the proposed study was identified *Terminalia crenulata* Roth bark, by Dr. Minoo H. Parabha, Botanist, Department of Bioscience, Veer Narmad South Gujarat University, Surat, Gujarat, India, where the voucher specimen no. was VCJ/03/25032005.

Preparation of Extracts

Preparation of the extract of TCR powdered bark is done using alcohol and distilled water. The shade dried coarse powder of the bark (500 gm) was packed well in soxhlet apparatus and was subjected to continuous hot extraction with 90% alcohol until the completion of the extraction. The extract was filtered while hot and the resultant extract was distilled in vacuum under reduced pressure in order to remove the solvent completely. It is dried and kept in a desiccator till experimentation. Similarly, aqueous extract was prepared. Obtained extract was weighed practical yield and percentage yield was calculated in terms of air-dried powdered crude material (5, 6, 7).

Antimicrobial Activity

Microbial strains - The microorganisms used in the antimicrobial tests were Gram-positive (*Staphylococcus aureus* ATCC 25925, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* ATCC 12228 and *Micrococcus luteus* ATCC 10240) and Gram-negative (*Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 51812 and *Shigella dysenteriae* ATCC 25931) organisms have been selected. All strains were obtained from C. G. Bhakta institute of Biotechnology, Tarsadi, Bardoli, Gujarat, India.

Culture media - Nutrient agar (NA) (Himedia Laboratories Ltd., Mumbai, India) medium and Nutrient broth medium were used for the growth of bacteria. Suspend the ingredients in 1000 ml distilled water and boil to dissolve it completely. Adjust the pH of media 7.4 ± 0.2 (at 25°C) and sterilize it by autoclaving at 15 lbs pressure (121°C) for 15 min. The solution of the test extracts was prepared at the concentration of 50 mg/ml by dissolving in respective solvent in stopper specific gravity bottle & stored in refrigerator. The solution was removed from the refrigerator one hour prior to use & was allowed to warm up to room temperature.

Chemicals - Gentamycin (Alkem Laboratories Ltd., Mumbai, India) was used as reference antibiotic (RA) against bacteria and dimethylsulphoxide (DMSO) (Himedia, India) was used as solvent for tested samples. Other chemicals were used of analytical grade of S. D. Fine Chemicals, Mumbai, India.

Sensitivity test: Cup Plate Method (Zone of Inhibition) - This method depends on the diffusion of the various extracts from a cavity through the solidified agar layer of Petri dish, to an extent such that growth of the added micro-organism is prevented entirely in circular area or zone around the cavity containing the extracts. 0.2 ml of each of the seeded broth containing 10^6 – 10^7 cfu/ml test organism was inoculated on the two plates of solidified agar & spread it uniformly. Sterilize the cup-borer of 10 mm diameter by dipping it in alcohol followed by flaming it and make four wells, one in each quadrant, at equal distance in nutrient agar plate previously seeded with culture. Add 0.2 ml of reference antibiotic Gentamycin (50 μ g/ml) in to the two wells, in third well kept control solvent – DMSO and in fourth well test extracts solution was added. The plates were kept in refrigerator at 4 - 5⁰C for 30 min after addition to allow diffusion of the solution into the medium & then incubated the plate in upright position at 37⁰C \pm 1⁰C for 24 hrs. After the incubation period the diameter of the zone of inhibition in mm obtained around the well was measured. Interpret the result of extracts sensitivity as sensitive, moderate sensitive or resistant on the basis of mean zone diameter in mm. The MIC was defined as the lowest concentration of plant extract able to inhibit microorganism growth (8).

Determination of minimum inhibitory concentration (MIC) and minimum microbial concentration (MMC) - The stock solution of different test extracts were prepared in concentrations of 50 mg/ml in respective solvent & used for study. Similarly the reference antibiotic concentration of 50 μ g/ml was prepared. Prepare 8 ml of broth containing 50 mg/ml concentration of extract for the first tube in row. Mix the content of universal bottle, using a pipette and transfer to the

first tube in row. Add 4 ml of broth to the remaining 4 ml of the universal bottle, mix and transfer 2 ml to the second tube in row, prepare dilutions up to the 10th tubes. Place 2 ml of extract free broth in the last tube. All tubes were placed in incubator at 37°C ± 1°C for 24 h. Inoculate tube containing 2 ml broth with the organism and keep at 4°C in a refrigerator overnight, to be used as standard for the determination of complete inhibition. The lowest concentration of test extract and reference antibiotic which caused apparently a complete inhibition of growth of organism was taken as minimum inhibitory concentration (MIC). The assay was repeated thrice (9, 10, 11, 12).

For the determination of MMC, a portion of a liquid (5µl) from each well that showed minimum zone was again plated on NA media and incubated at 37°C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC.

Antioxidant study

DPPH Scavenging Activity - The test sample (3ml extract solution in water) was mixed with 1ml (0.1 mM solution of DPPH in methanol) and different concentrations (40-200 µg/ml) were prepared. After 30 min at room temperature, the absorbance values were measured at 517nm and converted into percentage of antioxidant activity. Ascorbic acid was used as a standard control. Each assay was repeated thrice and the recorded as mean of the triplicate experiments, were graphically illustrated. Capability to scavenge DPPH radical was calculated by using following equation (13, 14, 15, 16).

$$\% \text{ Scavenging Effect} = [1 - \text{Abs. (s)}/\text{Abs. (c)}] \times 100 \quad [1]$$

Where, Abs. (s) = Absorbance of sample, Abs. (c) = Absorbance of control

Nitric Oxide Radical Inhibiting Activity - Nitric oxide scavenging activity was determined according to the method reported by Garrat. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide, which interact with oxygen to produce nitrite ions, which can be determined by the use of Griess Illosvoy reaction. Mix 2 ml (10

mM Sodium nitroprusside) in 0.5ml phosphate buffer saline (pH 7.4) with 0.5ml of extract at various concentrations (100-600 µg/ml) and was incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1ml sulphanic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1ml naphthylethylenediamine dihydrochloride (NEDA) (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance of the chromophore formed during diazotization of nitrite with sulphanic acid subsequent coupling with NEDA was read at 540nm. Ascorbic acid was used as a positive control. Experiment was done in triplicate. Capability to scavenge the nitric oxide radical was calculated by using following equation (17, 18, 19).

$$\% \text{ Inhibition} = \left[\frac{1 - \text{Absorbance of sample}}{\text{Absorbance of control}} \right] \times 100$$

Results and Discussion

Results of Antibacterial activity - The results of antibacterial sensitivity test of the plant extract are shown in (Table 1). The most active extract was alcohol extract (inhibition zone diameter 30.50 ± 0.289) followed by the aqueous extract (26.83 ± 0.441). These results indicated that most of the active constituents (responsible for exerting antibacterial action) in these plants are expected to be soluble in polar solvent. It authenticates that the entire tested microorganism are susceptible to aqueous extract and degree of susceptibility is given below in the decreasing order: *S. aureus* > *Bacillus Subtilis* > *Staphylococcus epidermis* > *Shigella dysenterie* > *Escherichia coli* > *Micrococcus luteus* > *Enetrobacter aerogens* > *Salmonella typhi*. On the other hand, the susceptibility to aqueous extract is in the order: *Salmonella typhi* > *Enetrobacter aerogens* > *Staphylococcus aureus* > *Bacillus subtilis* > *Shigella dysenterie*.

Table 1. Antibacterial activity of the alcoholic and aqueous extracts isolated from the bark of TCR and reference antibiotics determined by the cup plate method.

Microorganisms	Aqueous Extract	Alcoholic Extract	RA*
<i>Staphylococcus aureus</i> ATCC 25925	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	+	+	+
<i>Staphylococcus epidermis</i> ATCC 12228	-	+	+
<i>Micrococcus luteus</i> ATCC 10240	-	+	+
<i>Enterobacter aerogens</i> ATCC 13048	+	+	+
<i>Escherichia coli</i> ATCC 25922	+	+	+
<i>Salmonella typhi</i> ATCC 51812	+	+	+
<i>Shigella dysenterie</i> ATCC 25931	+	+	+

(-): Not active, (+): Active

*RA = Reference antibiotic – Gentamycin (50 µg/ml),

Alcohol extract is effective against all tested microorganism is having comparable result with Gentamycin against *S. dysnteries* and *S. typhi*. On the other hand, aqueous extract is susceptible to all the strains except *Escherichia coli*, *Staphylococcus epidermis* and *Micrococcus luteus*.

Table 2. Minimum inhibitory concentration (mg/ml) and Minimum microbial concentration (mg/ml) of aqueous and alcoholic extract from the bark of TCR and reference antibiotic ($\mu\text{g/ml}$) (Gentamycin).

Microorganisms	Minimum inhibition concentration			Minimum microbial concentration		
	Aqueous extract	Alcoholic extract	RA*	Aqueous extract	Alcoholic extract	RA*
<i>Staphylococcus aureus</i> ATCC 25925	2.08	1.05	9.55	4.16	2.10	9.55
<i>Bacillus Subtilis</i> ATCC 6633	2.10	0.130	4.65	4.20	0.263	9.55
<i>Staphylococcus epidermis</i> ATCC 12228	-	0.130	4.66	-	0.263	9.55
<i>Micrococcus luteus</i> ATCC 10240	-	0.263	9.45	-	1.05	9.55
<i>Enetrobacter aerogens</i> ATCC 13048	1.05	0.520	2.45	2.20	0.520	8.34
<i>Escherichia coli</i> ATCC 25922	-	0.263	4.91	1.05	0.520	8.34
<i>Salmonella typhi</i> ATCC 51812	1.05	0.520	155.31	2.10	1.10	4.16
<i>Shigella dysenteriae</i> ATCC 25931	0.520	0.130	80.25	1.05	0.520	2.10

(-): not tested because sample was not active by diffusion.

*RA = reference antibiotic (Gentamycin)

The results of MIC and MBC are shown in Table 2. It was observed from the result that the alcohol extract is most active extract with less MIC (0.034 – 0.52 mg/ml). Aqueous extract has high MIC then alcohol aqueous extract (1.05 – 4.17 mg/ml). All the strains were more susceptible to alcohol extract, our result indicate the presence of chemical compound in both extract with antibacterial activity against all strains comparable to Gentamycin (20, 21, 22, 23).

Results of *in - vitro* antioxidant activity - In today's environment, hyper physiological burden of free radical causes imbalance in homeostatic phenomenon between oxidants and antioxidants in the body. The imbalance leads to oxidative stress that is being suggested as the root cause of aging and various human diseases like arteriosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's and Parkinsonism. Thus free radical scavenging is very essential for preventing organ injury associated with shock, inflammation & ischemia or reperfusion. Therefore research in recent past have accumulated enormous evidence advocating enrichment of body system with antioxidants to correct vitiated homeostasis and prevent onset as well as treat the disease caused due to free radical and related oxidative stress. Stress, smoking, drugs & diet generates excessive free radicals in human body. Plants & plant products are known to possess excellent antioxidant properties & play a significant role in preventing the conditions due to the excessive free radicals. The results of absorbance and % inhibition showed decrease in the concentration of DPPH radical due to the scavenging ability of extract and standard ascorbic acid, as a reference standard (24, 25, 26, 27).

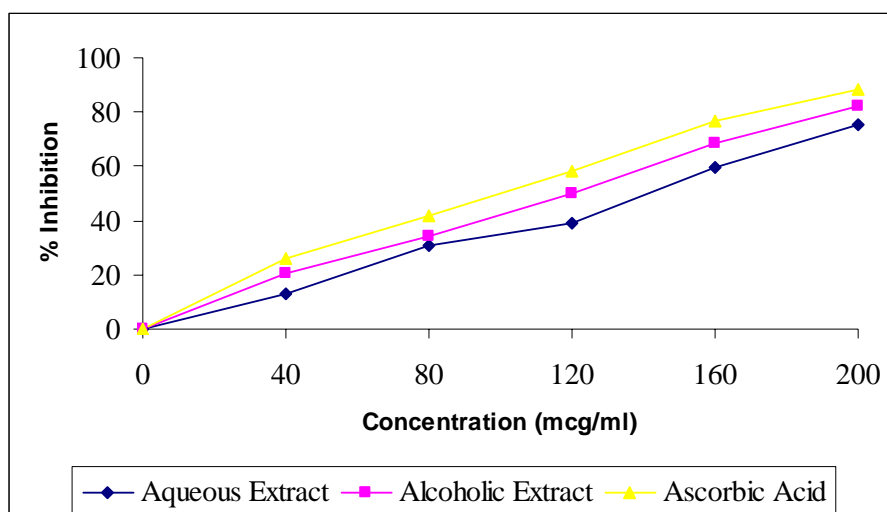


Fig.1. Graphical representations of *In vitro* Antioxidant activity of TCR bark extract by DPPH method.

Fig 1 shows *in vitro* antioxidant activity of TCR leaves extract by DPPH method. In DPPH method alcoholic extract of TCR bark presented more activity than aqueous extract. 200 $\mu\text{g/ml}$ of alcohol, aqueous extracts and ascorbic acid exhibits 82.5, 75.3 and 88.6 % inhibition and the EC_{50} (μg) – 116.62, 153.06 and 96.15 $\mu\text{g/ml}$, respectively (28, 29, 30, 31, 32).

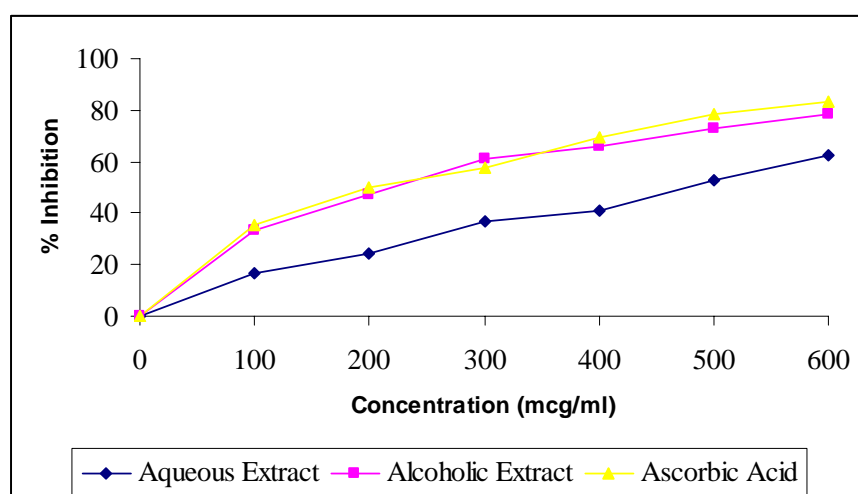


Fig.2. Graphical representations of *In vitro* Antioxidant activity of TCR bark extract by Nitric oxide method.

Fig 2 shows *in vitro* antioxidant activity of TCR leaves extract by Nitric oxide method. In Nitric oxide method alcohol extract of TCR bark presented more antioxidant activity than aqueous extract., Alcoholic extracts, Aqueous extract and Ascorbic acid exhibits 78.3%, 62.8% and 83.5%, inhibition and the EC₅₀ (µg) – 474.80, 232.85 and 200.76 µg/ml respectively.

The alcoholic extract exhibited more antioxidant activity with low EC₅₀ value in these two methods. The Phytochemical analysis indicated the presence of phenolic compounds and flavanoids in extract. Several such compounds were known to possess antioxidant activity. Hence, the observed activity may be due to the presence of any of these constituents.

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