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Evaluating *Terminalia chebula* for Antibacterial and Anticancer Activity

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Abstract: Plants are inevitable source of drugs for the treatment of deadly diseases. The present study focuses on the use of ethanolic extract of the fruits of *Terminalia chebula* Retz as a remedy for bacterial infections and offer hope for cancer therapy. The plant extract was analyzed for the presence of phytochemicals. Crude ethanolic extract was screened for antibacterial activity using disc diffusion method and MIC. The anticancer activity was tested using MTT assay and DNA Fragmentation studies. Further investigations were aimed to obtain the best possible fractions suitable for antibacterial activity that might serve as lead molecules for the study of anticancer property as well. The results obtained showed good inhibition against the bacterial pathogens and significant antitumor effect against MCF-7 breast cancer cell lines. Therefore our analysis reveals the dual antagonist property of *T. chebula* that portray experimental evidence to the traditional claims reported by earlier researchers.

Keywords: *T. chebula*; antibacterial; anticancer; medicinal plants

I. INTRODUCTION

Health is complex and is posed with serious threats by social, behavioral and environmental factors. Women stand as testimony to the commonly affected population^[1]. It is a matter of concern to look into their well being as they play a prominent role in the society. The two most common problems that target women are urinary tract infections caused by pathogenic strains of bacteria and breast cancer. Microbes are ubiquitous in nature and are constantly evolving. The recent emergence of antibiotic resistant bacteria is an evolutionary lesson on microbial adaptation. Multidrug resistance (MDR) bacteria have created an alarming situation to look up for novel drugs with durable safety and efficacy standards^[2]. In the similar manner, cancer affects all living cells irrespective of age and gender. There is no available strategy for the prevention and cure of cancer. Now every researcher is focused on the use of herbal medicines to overcome the problem of antibiotic resistance and finding cures for the treatment of cancer in different parts of the world, with India in the forefront^[3]. According to WHO^[4] (2000), medicinal plants exert pharmacological action when administered.

So this work was aimed to unravel the therapeutic potential of medicinal plant *T. chebula* Retz.

II. MATERIAL AND METHODS

A. Collection and Preparation of Plant Extract

The fruits of *T. chebula* were collected from Pachamalai hills, Tiruchirappalli and authenticated by Rapinet herbarium, Department of Botany, St. Joseph College, Trichy. Ethanolic extract of the plant was prepared by Soxhlet extraction^[5]. The extract was screened for various phytochemicals^[6].

B. Separation of Compounds from Extract Using Column Chromatography

Different solvent mixtures based on polarity were used to elute the compounds present in crude ethanolic extract of fruit of *T. chebula*. Ratio of mixtures was maintained as per earlier reports. The method employed was the use of glass column packed with silica gel^[7].

C. Isolation And Identification Of Pathogens

The media used were purchased from Ponmani & Co., Trichy. The following media were used: Simmons Citrate agar, Nutrient Gelatin agar, Kovac's reagent, Mueller-Hinton agar, MR-VP broth, MacConkey agar, Nutrient broth/agar, Peptone broth. The antibiotics generally used for the treatment of UTIs were selected for the disc sensitivity method (Hi-media discs). The antibiotics are as follows: Ampicillin, Ciprofloxacin, Streptomycin, Tetracycline. Clinical isolates were obtained from local hospital.

The selected colonies based on the cultural, microscopic and microbiological examinations, were subjected to biochemical examination (oxidase test, catalase test, indole production test, methyl red test, voges-proskauer test, citrate utilization test, urease test) for confirmation of the pathogens.

D. Antimicrobial Sensitivity Testing

The susceptibility of the entire isolated organisms to selected antibiotics which were normally used to treat uropathogens was tested by disc diffusion assay. Sterile Mueller-Hinton agar plates were prepared and various antibiotic discs were selected. Identified pathogens were inoculated in peptone water tubes separately and incubated at 37°C for 1 hour. Using sterile cotton swabs for each test organism, incubated test organisms were inoculated on the surface of Mueller-Hinton agar plates three times, rotating the plate 60° after each streaking. Finally the swab was run around the edge of the agar. The cultures were allowed to dry on the plate for 5-10 minutes at room temperature. Various antibiotic discs were placed on the surface of the agar medium by gently pressing using a sterile forceps on the top of the discs (for better contact and effective diffusion of the antibiotics into the medium). The plates were incubated in an inverted position for 16-18 hours at 37°C.

E. Minimum Inhibitory Concentration

The minimum inhibitory concentration assay was determined by microtitre plate method^[7]

F. MTT Assay

MCF-7 cells were plated by adding 5×10^4 cells/mL suspension to each well of a 96-well tissue culture plate. The plate was incubated for a sufficient time to assure attachment and 40% to 60% confluency. The media was aspirated off and replaced with fresh media containing different concentrations of test extract. The last row was left as an untreated control. The plates were incubated at 37 °C, 5% CO₂, for 24, 48 and 72 hours, respectively. After incubation, the media was aspirated off and replaced with fresh media. Then, MTT solution was added in every well and incubated for 4 to 6 hours at 37 °C with 5% CO₂. After that, MTT-containing medium was removed gently and replaced with DMSO (200 µL per well) to mix the formazan crystals until dissolved. The plates were read on microtiter plate reader at 570 nm^[8,9].

G. DNA Fragmentation

Cells cultured in a 6-well plate at a density of 3×10^5 cells/2 ml and incubated in a CO₂ incubator overnight at 37°C for 24 hours. The spent medium was aspirated and cells treated with required concentration of experimental compounds and controls in 2 ml of culture medium and incubated the cells for 24 hours. The medium was removed from all the wells and given a PBS wash. PBS was removed and trypsin-EDTA solution was added and incubated at 37°C for 3-4 minutes. 2 ml culture medium was added and cells harvested. The tubes were centrifuged, washed with PBS and decanted. The pellet was resuspended in buffer and vortexed. Lysis buffer and Proteinase K were added and incubated at 65°C for 15mins in dry bath. Isopropanol was added and the mixture was then loaded to the spin column provided and centrifuged at 10,000rpm for 3minutes. The supernatant was discarded. 500µl of wash buffer was added to the spin column and centrifuged at 10,000rpm for 3minutes. 100µl of elution buffer was added to the column and centrifuged at 10,000rpm for 5minute. Finally elute thus obtained contained the DNA. The DNA was checked for fragmentation on Agarose gel electrophoresis^[10].

III. RESULTS

A. Collection And Preparation of Plant Extract

The fruits of the plant were identified as *Terminalia chebula Retz* by Dr. S. John Britto, the Director of RAPINAT Herbarium and Center for Molecular Systematics, St. Joseph's College, Trichy, Tamilnadu, India. A voucher specimen number HDP001 was obtained. Standardized common name in English is Chebulic myrobalan. The fruits of *T. chebula Retz* were powdered using a blender. It was made into coarse powder. The coarse powder (500 gm) of the sample was extracted with different solvents like 95% Ethanol by continuous hot percolation using soxhlet apparatus. After the completion of extraction, the extract was filtered and the solvent was removed by distillation under reduced pressure. The plant extract was screened to qualitatively analyze the presence of medically important secondary metabolites as already reported^[11].

B. Separation Of Compounds From Extract Using Column Chromatography

Based on the separation procedure, a total of 15 fractions were eluted. Among the eluted fractions, 12th, 13th, 14th fractions yielded brown coloured sediments that appeared to be crystalline in nature. Hence fractions 12, 13 and 14 (Fig1) were chosen for further analysis.

Fig1: Fractions obtained from column



C. Isolation and Identification of Uropathogens

The predominant uropathogens were isolated from urine samples of UTI patients and were identified as Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa and Staphylococcus aureus based on biochemical tests. Most of the bacterial isolates confirmed by various biochemical tests revealed to be multidrug resistant. The antibacterial activity of crude extract has been already reported^[11].

D. Antimicrobial Sensitivity Testing

All the fractions contributed to development of zones of inhibition against the 5 isolates tested. Of these, 13th and 14th fraction produced inhibition zones in all the isolates. Also, E. coli and Klebsiella were found to be sensitive to all the fractions. The antibacterial activity of the selected fractions against the uropathogens was assessed by disc diffusion assay (Table 1).

Among the fractions tested, fraction 14 demonstrated the maximum zone size of 15 mm. The size of the zones of inhibition ranged between 8±1.03 to 15±0.92 mm for E. coli, 6±0.62 to 10±1.11 mm for Klebsiella sp., 7±0.67 to 8±1.24 mm for Proteus sp., 18±1.27 to 10±0.35 mm for Staphylococcus sp. Pseudomonas sp., was found to be resistant to few fractions but they produced zones of inhibition of size 8±1.28 and 9±0.71 mm for 13th & 14th fraction respectively.

Table:1Antibiogram patterns of the isolated uropathogens

S.No.	Fractions	Zones of inhibition of Clinical isolates (diameter in mm)				
		E. coli	Proteus vulgaris	Klebsiella pneumoniae	Pseudomonas aeruginosa	Staphylococcus aureus
1.	Fraction 11	15±0.92	7±1.14	8±1.04	Resistant	18±1.27
2.	Fraction 12	13±1.21	8±0.75	6±0.62	Resistant	15±1.62
3.	Fraction 13	10±0.83	7±0.67	10±1.11	8±1.28	12±0.91
4.	Fraction 14	11±2.33	8±1.24	8±1.75	9±0.71	10±0.35
5.	Fraction 15	9±0.71	Resistant	6±1.33	Resistant	10±0.84

Data obtained are expressed as Mean±SD of triplicates.

Table: 2Antibiogram patterns of positive and negative control

S.No.	Control	Zones of inhibition of Clinical isolates (diameter in mm)					
		E. coli	Proteus vulgaris	Klebsiella pneumoniae	Pseudomonas aeruginosa	Staphylococcus aureus	
1.	Reference drugs	Amp	Resistant	Resistant	Resistant	Resistant	Resistant
		Cipro	Resistant	Resistant	Resistant	Resistant	Resistant
		Strep	18±7.6	22±2.5	14±5.8	20±1.1	22±2.3
		Tet	15±5.1	19±3.2	17±3.2	17±4	21±3.6
2.	DMSO	Resistant	Resistant	Resistant	Resistant	Resistant	

Data obtained are expressed as Mean±SD of triplicates

E. Estimation of Minimum Inhibitory Concentration

The MIC values of fractions obtained from ethanolic extract were found to be in the range of 50-200µg/ml for the isolated uropathogens.

Table: 3 Minimum inhibitory concentration doses of isolated uropathogens (µg/ml)

S.No	Fractions	E. coli	Proteus vulgaris	Klebsiella pneumoniae	Pseudomonas aeruginosa	Staphylococcus aureus
1.	Fraction 11	150	100	150	200	150
2.	Fraction 12	150	150	100	150	150
3.	Fraction 13	100	150	150	100	100
4.	Fraction 14	50	100	50	150	150
5.	Fraction 15	150	200	150	150	150

F. MTT Assay

Screening of ethanolic extract of *T. chebula* demonstrated remarkable anticancer activity against MCF-7 cells. The pattern of cell inhibition was found to be concentration dependent. The maximum concentration used in the study was 450 µg/ml and the minimum was 25 µg/ml. The invitro dosage and time-dependent effects (24 hrs, 48 hrs, 72 hrs) of extract against MCF -7 cells have been reported by us earlier where the IC₅₀ value of crude extract was found to be 334.54 µg/ml^[12].

G. DNA Fragmentation

DNA from MCF -7 cells treated with standard drug, crude ethanolic extract (Sample A), untreated control cells (UT) were electrophoresed along with 1 kb DNA ladder (L) to observe the “ladder” pattern of band formation as reported in case of apoptosis. DNA ladder served as reference. Untreated cells showed a single band confirming DNA to be intact without undergoing any serious damage. Standard drug (STD) treated cells exhibited “ladder pattern” due to DNA damage mediated by apoptosis. The crude ethanolic extract (S-A) treated cells showed similar stacking of DNA resulting in ladder-like pattern confirming the mechanism of action of cell death mediated by apoptosis as observed in Fig2.

The cells were incubated with an IC₅₀ concentration of extract of *T. chebula* for 24 hrs. The treated cells collected by trypsinization were observed for DNA ladder formation. Therefore, the cytotoxic effect of *T. chebula* extract was mediated through induction of apoptosis.

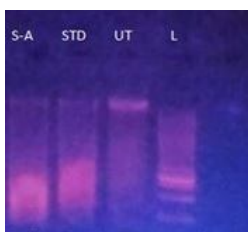


Fig:2 DNA band formation observed in agarose gel

IV. DISCUSSION

The ethanolic extract of *T. chebula* Retz was found to contain different types of phytochemicals like alkaloid, flavonoid, phenolic compounds and tannins which was also in accordance with other reports^[13,14,15].

Urinary tract infection (UTI) one of the most common bacterial infections prevalent in the society targeting humans especially women is a matter of serious concern. Among the different extracts (aqueous, acetone and ethanol) investigated to support the use of traditional medicinal plants, against a few multi – drug resistant pathogens of urinary tract infections such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* by disc – diffusion method the results showed that ethanolic extract of most of the plants such as *Terminalia chebula* exhibited strong antibacterial activity against pathogens causing complicated UTI. This support the use of folklore associated medicinal plants in the treatment of bacterial infections practiced by the tribals of central India inhabiting the Mahakoshal region^[16].

The anti-proliferative effect of *T. chebula* was assessed based on different concentration and limited the study to its IC₅₀ in MCF-7 cell line. IC₅₀ value is the effective dose required to inhibit the proliferative response by 50%. Treatment with the extract obtained

from *T. chebula* Retz showed significant cytotoxic effect at different concentration when compared to control. Cytotoxic effect was found to be dose- dependent. Antiproliferative effect was compared with standard reference drug Tamoxifen.

The U937 cells treated with ethanolic extract of fruits of *T. chebula* with varying concentrations showed significant reduction in cell viability as assessed by MTT assay. Similar cytotoxic effects of the extract of *T. chebula* containing gallic acid and chebulagic acid have been reported^[10].

DNA ladder assay is simple, sensitive, cost effective and rapid for estimating apoptosis in single cells. Apoptosis was visualized as a ladder pattern of 180-300bp by standard agarose gel electrophoresis. Defects in apoptotic pathways play an important role in the development and progression of cancer. Modulation of the apoptotic signaling pathways towards normality by means of drugs or treatment strategies have the ability to eliminate cancer cells^[17]. During apoptosis, genomic DNA is chopped into ~180bp or its multiples at certain regions (internucleosomal regions), hence appears as a ladder when electrophoresed. This is a characteristic feature that serves as a hallmark of apoptosis and is not present in necrosis^[18]. DNA fragments are more reliable biochemical marker for apoptosis. The conventional gel electrophoresis is used to separate low molecule weight DNA that occur as “ladder” pattern of discontinuous DNA fragments that stands as a benchmark of apoptosis. This pattern serves as a marker of apoptotic mode of cell death. DNA fragmentation into oligonucleosomal ladders is seen in early events of apoptosis in the range of 20-300kb^[19]. Thus the various experimental evidences pointed above justify the medicinal properties of *T. chebula* and its use as a drug by the tribal's. Further investigations are under progress.

V.CONCLUSION

Our findings have provided strong experimental evidences to the nature of the chosen plant *T. chebula* Retz. The medicinal property of the plant has yielded good zones of inhibition against the tested pathogens. The antiproliferative nature was also determined successfully. This clearly depicts the possibilities of creating new avenues for the identification and validation of safe lead molecules that could act as drug candidates.

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