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ABTS radical scavenging and antimicrobial activities of flavonoid fractions from *Terminalia bellirica* fruit rinds

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ABSTRACT

Plants are widely used in traditional medicine from the beginning of mankind because of its significant healing power and least side effects. The extraction and isolation of biologically active components from plants for the designing and synthesis of novel drugs became an emerging research area for years. Plants possess a large spectra of secondary metabolites such as flavonoids, tannins, terpenoids, alkaloids, phenolic acids etc. they are the major contributors of pharmacological activities shown by plants. Present study aimed at the ABTS radical scavenging and antimicrobial testing of flavonoid fractions from the fruit rinds of *Terminalia bellirica*. The effect of flavonoids from diethyl ether and ethyl acetate extracts against six bacterial strains and two fungal strains are evaluated by the agar well diffusion method. The results revealed that these flavonoid containing fractions possess excellent ABTS radical scavenging activity with very low concentrations and considerable antimicrobial potential when compared with their respective standards.

Keywords: ABTS radical, antimicrobial, diethyl ether, ethyl acetate, flavonoid, *Terminalia bellirica*

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1. INTRODUCTION

Plants are the original sources of many biologically active compounds used by the pharmaceutical industries. The treatment using herbal plants is as old as mankind. The phenolics present in plants are mainly responsible for the therapeutic activities shown by them. They possess antioxidant, anticancer, anti-inflammatory, antimicrobial activities depending on the phytoconstituents present in them.[1].The extraction, isolation and identification of these biologically active compounds are necessary for active drug research. More ethnomedical information are used these days for the preparation of plant derived drugs.[2].WHO gives more attention on traditionally used medicinal plants because common men are still depending on them as their primary health care. Almost all the parts of plants possess some medicinal values such as leaves, bark, root. Fruits etc. Drugs obtained from plants are very much effective and possess least side effects when compared to synthetic drugs[3]

Plant secondary metabolites acting as the warriors of its defence mechanism. They include flavonoids, terpenoids, tannins, carotenoids, coumarins etc. Among these phytochemicals, flavonoids are wide spread in the plant kingdom and they can show an array of health benefits. Regular intake of flavonoids can prevent many degenerative diseases to a large extent.[4].The most described property of flavonoids is their antioxidant capacity. The hydroxyl groups present in them are mainly responsible for their free radical scavenging power.[5].A large variety of plant extracts are screened for antimicrobial studies also. Many folklore medicinal plants in the tribal area of Western ghats of India are subjected to antibacterial studies and many of them found to possess good activity against many microbial infections[6].

Antibiotics against different microorganisms are not much effective now because microorganisms became drug resistant due to the huge intake of them.[7]. The treatment of infectious diseases using medicinal plants is more effective and possess least side effects when compared to treatment with synthetic drugs.

Terminalia bellirica is a traditionally used medicinal plant from combrataceae family and its fruits are the major constituent of ayurvedic formulaton 'Triphala',a popular rejuvenating agent and is mainly prescribed for liver related diseases.[8]. Fruits of this plant mainly contains phenolic compounds such as gallic acid, ellagic acid and its

derivatives, esters, glycosides, aglycones etc. The fruits of this plant are used for long time to treat diseases like diarrhoea, cough, rheumatism, asthma etc.[9]. In the present study we are trying to evaluate the ABTS free radical scavenging and antimicrobial potential of flavonoids extracted from *Terminalia bellirica.*

2. MATERIALS AND METHODS

2.1 Sample Collection

The fruit rinds of *Terminalia bellirica* were purchased from the local market, payyannur, Kannur district, Kerala. The plant materials were identified by the taxonomist Dr. S. Sujanapal, KFRI, Thrissur. The fruit rind of the sample is thoroughly washed under running water and shade dried carefully. The well dried samples were grinded to powdered form and keep in air tight sample bottle.

2.2 Extraction

The powdered sample was weighed and then extracted with 80% methanol using Soxhlet extraction method. The extraction was continued until the solvent became colourless. The filtrate obtained was washed thoroughly with petroleum ether and then subsequently extracted with diethyl ether and ethyl acetate[10]. The petroleum ether fraction was discarded because it contains huge amount of fatty matters. Both diethyl ether (DT) and ethyl acetate (ET) fractions were collected and used for free and bound flavonoids respectively. The collected fractions were dried in a dessicator for future analysis.

2.3 Total phenolic and flavonoid content

The amount of total phenols present in the flavonoid fractions was determined by Folin-Ciocalteu's (FC) reagent using gallic acid as standard.[11]. The amount of flavonoids present in both extracts was determined with Aluminium chloride according to a previously reported method. Here Quercetin was used for calibration[12]

2.4 Qualitative test for flavonoids

The commonly used qualitative tests for the identification of flavonoids are Shinoda test, Lead acetate test and Alkaline reagent test [4].

2.4.1 Shinoda Test

The extracts were mixed with small pieces of Magnesium ribbon and Con: HCl was added drop wise into this mixture. Development of red colour after a few minutes indicates the presence of flavonoids.

2.4.2 Lead acetate Test

Lead acetate solution (10%) was added drop wise to the extracts, Formation of yellow colour or yellow creamy precipitate indicates the presence of flavonoids.

2.4.3 Alkaline reagent Test

To the extracts, NaOH solution was added. Intense yellow colour formed which loses the intensity on the addition of dilute acid indicates the presence of flavonoids**.**

2.5 Total anti-oxidant activity by phosphomolybdenum method.

The total antioxidant capacity of the extracts was evaluated by the phospho-molybdenum method.[13]. 1mg/mL of sample was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate).The tubes containing the reaction mixture were incubated at 95°C for 90 min. Absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer *(*UV Pharmaspec 1700*)* against blank after cooling to room temperature. Methanol (0.3 mL) in the place of extract was used as the blank. The total antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

2.6 ABTS free radical scavenging assay

The free radical scavenging activity of the flavonoid fractions was determined by ABTS radical cation decolorisation assay.[10]This radical cation was generated by the reaction with 7mM ABTS in water and 2.45mM potassium persulphate in 1:1 ratio and store in a dark room 12 hours before the assay started. The ABTS radical cation solution was diluted with methanol. The reaction was initiated by the addition of 1ml of diluted ABTS to100 µl of different concentration of sample and 100 µl of methanol as control. The absorbance was read at 734 nm and the percentage inhibition was calculated.The antioxidants present in the plants extracts inhibit the ABTS radical cation and as a result decolorisation occurs.

2.7 Antimicrobial screening studies

Antibacterial assay of extracts against six bacterial strains both gram negative and gram positive and antifungal assay against two fungal strains were done by agar well diffusion method.

2.7.1 Test Organisms

The bacterial strains used are *E.coli* (MTCC48),*Staphylococcus aureus* (ATCC 25923),*Kelbsiella pneumoniae* (ATCC 13883) ,*Streptococcus mutans* (MTCC 890), *Bacillus substilis*(MTCC121T),*Pseudomonas fluorescense*(MTTC664) and the fungal strains are *Aspergillus niger* (ATCC 16404*)* ,*Candida albicans*(ATCC 10231).The bacterial cultures were maintained in Muller Hinton Agar Medium and fungal strains wre seeded on potato dextrose agar medium, growth of culture adjusted according to McFards Standard, 0.5%. All the strains used were CLSI (formerly NCCLS-National Committee for Clinical Laboratory Standards, 1999) standard reference strains used for antimicrobial susceptibility tests.

2.7.2 Antibacterial assay.

Antibacterial assay was done by Agar well diffusion method. [14]. The antimicrobials present in the samples are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters. Petriplates containing 20ml Muller Hinton Agar Medium were seeded with bacterial culture of *E.coli, Klebsiella pneumoniae, Pseudomonas fluorescence, Bacillus substilis Streptococcus mutans* and *Staphylococcus aureus*. Wells of approximately 10mm was bored using a well cutter and different concentrations of flavonoids from both DT and ET fractions of Terminalia bellirica, such as 250µg/mL, 500µg/mL and 1000µg/mL were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. Streptomycin was used as a positive control and the solvent DMSO as negative control.

2.7.3 Antifungal assay

Antifungal assay was also done by Agar well diffusion method. Here the antifungals present in the samples were diffuse out and interact with microorganisms and the resulting zone of inhibition is measured in millimetres**.** Potato Dextrose agar plates were prepared and overnight grown species of fungus, *Aspergillus niger and Candida*

albicans were swabbed. Wells of approximately 10mm was bored using a well cutter. Different concentrations of both the flavonoid fractions from *Terminalia bellirica* such as 250µg/mL, 500µg/mL and 1000µg/mL were added. The zone of inhibition was measured after overnight incubation at room temperature and compared with that of standard antimycotic, Clotrimazole .Here DMSO was used as the negative control[15]

3. RESULTS AND DISCUSSION

3.1 Total Phenolic and Flavonoid Content

Total phenolic and flavonoid content of both extracts is shown in Table 1. The Phenolic compounds are one of the most effective antioxidative constituents of plants. Total phenolic content of diethyl ether and ethyl acetate extract was calculated from the standard curve of gallic acid, y= 0.022x+0.052, R^2= 0.994. Whereas the estimation of the total flavonoid content of the same was calculated from the standard graph of Quercetin Y= 0.008x-0.026, R^2= 0.994. Diethyl ether extract of the sample possess higher amount of total phenolics and flavonoids when compared to the ethyl acetate fraction.

Table 1: Total phenolic and flavonoid content of diethyl ether and ethyl acetate extract

Sample	TPC (mg GAE/g)	TFC (mg QE/g)
Diethyl ether(DT)	171.21± 0.73	41.20± 0.79
Ethyl acetate(ET)	158.46± 0.27	32± 0.18

3.2 Qualitative tests for flavonoids

Both diethyl ether and ethyl acetate fractions showed strong positive results on all of the qualitative tests of flavonoids.

3.4 Total antioxidant activity

The total antioxidant activity of the extracts is shown in table 2.Both the extracts exhibits comparatively better antioxidant capacity. The assay is based on the reduction of Mo-VI to Mo-V by the extract and subsequent formation of a green phosphate Mo –V Complex at acid pH.[16] The Total antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Table 2: Total antioxidant activity of the extracts

SL.NO:	Sample	OD at 695nm	Concentration of antioxidants in µg/mg of sample
1	ET	0.869	108.63
2	DT	1.438	179.75

3.5 ABTS Radical scavenging assay

ABTS is a popular peroxidase substrate for the determination of antioxidant activity[17].Both the fractions possess very high ABTS radical scavenging activity with very low concentrations. The extent of decolorisaion of radical cation depends on the antioxidants present in the extracts.

Table 3: ABTS radical scavenging activity of extracts

Sample	Concentration (µg)	OD at 734 nm	% of inhibition
Control at 0 mins	-	0.672	
DT	2.5	0.250	62.79
	5	0.022	96.7
	7.5	0.007	98.95
	10	0.005	99.25
ET	2.5		53
	5	0.012	98.2
	7.5	0.005	99.25
	10	0.004	99.4

3.6 Antimicrobial assays

The ethyl acetate and diethyl ether fractions showed significant inhibition of both bacterial and fungal strains. The zone of inhibition shown by the extracts in the antbacterial and antifungal assays are given in the table 4&5 and the photographs of zone of inhibition of both samples and standards against the tested microorganisms are also given. The extracts were active against several tested microorganisms and can be used against the diseases caused by them. The inhibitory potential of the extracts in some of the strains are even comparable with the standards streptomycin and clotrimazole.

Table 4: Antibacterial activity of the flavonoid fractions

	Organism	Zone of Inhibition				
		+ve control	-ve control	T1	T2	T3
Sample DT	*E.coli*	30	-	12	16	20
	Pseudomonas fluorescence	20	-	-	9	10
	Bacillus substilis	27	-	12	14	16
	Klebsiella pneumoniae	19	-	10	11	15
	Staphylococcus aureus	29	-	13	16	17
	Streptococcus mutans	22	-	10	13	16
Sample ET	*E.coli*	30	-	20	24	26
	Pseudomonas fluorescence	20	-	-	10	12
	Bacillus substilis	27	-	14	16	18
	Klebsiella pneumoniae	19	-	12	13	17
	Staphylococcus aureus	29	-	14	16	18
	Streptococcus mutans	22	-	20	22	25

(T1,T2 and T3 represents concentrations of the sample at 200,500,1000 µg/ml respectively.

The concentration of the standard taken was 100µg/ml.)

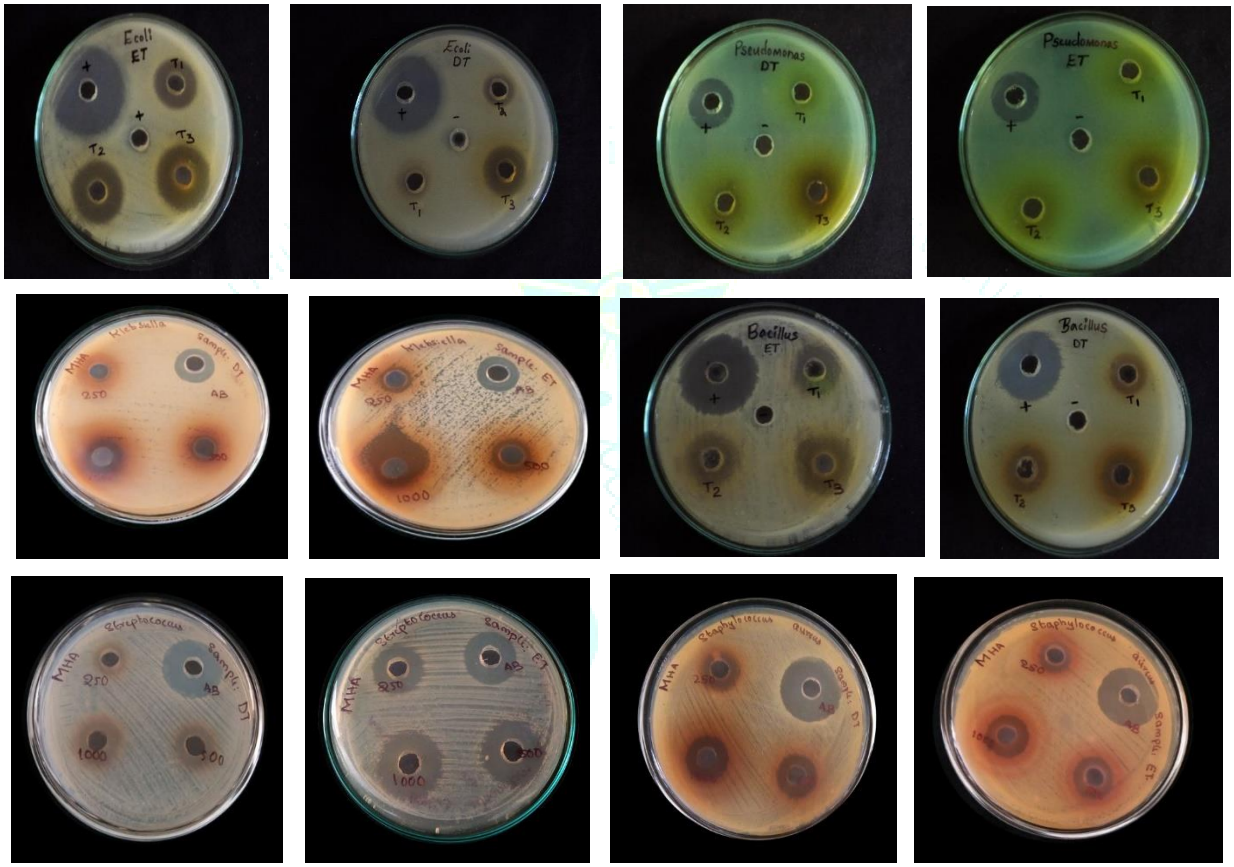

Fig 1:Images of zone of inhibition of both ET and DT in six different bacterial strains

Table 5:Antifungal activity of the flavonoid fractions

	Organism	Zone of inhibition				
		+ve control	-ve control	T1	T2	T3
Sample DT	*Aspergillus niger*	28	-	12	15	19
	Candida albicans	18	-	-	12	16
Sample ET	*Aspergillus niger*	28	-	14	17	21
	Candida albicans	18	-	10	12	17

(T1,T2 and T3 represents concentrations of the sample at 200,500,1000 µg/ml respectively.

The concentration of the standard taken was 100µg/ml.)

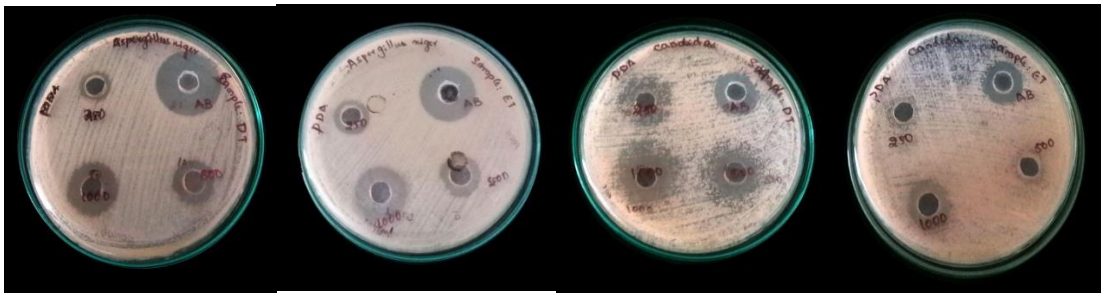

Fig 2: Images of zone of inhibition of both ET and DT in two different fungal strains.

CONCLUSION

Terminalia bellirica is a popular medicinal plant widely used in Ayurvedic medicinal systems.The present study aimed to evaluate the antioxidant,free radical scavenging and antimicrobial potential of its flavonoid fractions of fruit rinds of Terminalia bellirica.The extracts posses a very strong inhibition of ABTS radical and are active against a broad spectrum of microorganisms both bacterial and fungal.These activities are mainly attributed to the phenolics particularly flavonoids present in them.These flavonoid rich fractions posses strong dpph,hydroxyl,super oxide,nitric oxide radical scavenging activity,which was described in an earlier study.These fractions can be used in the treatment of oxidative damages and many infectious diseases caused by the tested microorganisms.The next step of this research will be the isolation of the active component from these fractions and the incorporation of this component in to the matrix of a core-shell nanparticle for more effective drug delivery.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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