ORIGINAL ARTICLE

Phytochemical study and bioefficacy of *Terminalia chebula* Retz. against some human pathogens

Geeta Singh, Padma Kumar, Alka Jindal
Department of Botany, Laboratory of Plant Tissue Culture and Secondary Metabolites, University of Rajasthan, Rajasthan, India

Objective: The antimicrobial activity of flavonoids of different parts (leaf, stem, stem bark, fruits) of *T. chebula* have been evaluated against seven bacterial (Gram +ve and Gram –ve) and three fungal strains. **Materials and Methods:** These flavonoids were screenedfor their antimicrobial activity by Disc Diffusion Assay. Minimum inhibitory concentration of the extracts was evaluated through micro broth dilution method, while minimum bactericidal/fungicidal concentration was determined by sub culturing the relevant samples. **Results:** In antimicrobial assay highest activity was observed by free flavanoid of fruits (IZ 30.83 mm, AI 1.39 + 0.024) against *Enterobacter aerogenes.* The range of minimum inhibitory concentration of tested extracts was 0.039-0.625 mg/ml while minimum bactericidal/fungicidal concentration ranged from 0.078-1.25 mg/ml. **Conclusion:** Result reveals that all the eight tested plant extracts inhibit the growth of selected pathogens, indicating broad spectrum bioactive nature of selected plant and hence can be exploited for future plant based antimicrobials.

Key words: *Terminalia chebula*, *Enterobacter aerogenes,* minimum inhibitory concentration, minimum bactericidal/fungicidal concentration, antibacterial activity, antifungal activity, total activity

INTRODUCTION

Medicinal plants are rich sources of developed secondary metabolites, which are potential remedies for different ailments. Extreme interest in plants with microbial activity has revived due to resistance, associated with antibiotics presently in use. The main advantage of natural agents is that they do not enhance the antibiotic resistance, a phenomenon commonly encountered with the long-term use of systematic antibiotics. The use of phytochemicals commonly called "biocides" is gradually gaining popularity.[1] There is growing interest in correlating phytochemical constituents of the plant with its pharmacological activity. It has been reported that the higher plants have shown to be a potential source for the new antimicrobial agents.[2]

Increasing use of natural medicines as a result of consumers seeking for complementary and/or alternatives to prescribed drugs, has provoked a great interest in research into medicinal plants. Some of these herbal products are commonly used to improve overall health, prevent and cure diseases.These are likely to act through a stimulation of receptor sites, immune system, inhibit pathogenesis of disease condition or have a lethal effect on pathogens. The discovery of bioactive compounds from plant origin offers an attractive approach to control infectious or non infectious diseases.

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Terminalia chebula Retz. (family Combretaceae) is a flowering evergreen tree attaining a height up to 30m, with widely spread branches and a brown rounded crown. It is native to India, Pakistan, Nepal, South West of China and Srilanka. *T. chebula* is called as 'King of Medicine' in Tibet and is always listed at the top of the list in Ayurvedic Materia Medica due to its extra ordinary power of healing.[3] *T. chebula* is basically an astringent, mild, safe purgative, stomatic and mild laxative. *T. chebula* contains tannins, chebulic acid, glycosides, sugar, triterpenoids, steroids and good quantity of phosphoric acid. This plant is commonly used to conquer over diseases like constipation, diarrhea, ulcer, gastroenteritis, asthma, cough, dyspepsia, hepatomegaly, renal calculi, urinary discharge, tumours, skin disease, anorexia.[4] It is also reported to posses antibacterial, antifungal, antiviral, carcinogenic, antioxidant, hypolipidemic, hepatoprotective, cardioprotective and antidiabetic activity.[5]

Antibacterial activities of *T. chebula* have been reported.[6,7] It is effective in inhibiting *Helicobactor pylori*, *Xanthomonas campestris*pv. *Citri* and *Salmonella*.[8-10] A water extract of *T. chebula* was found to have an antifungal activity.[11]

Address for correspondence: Dr. Geeta Singh, Laboratory of Plant Tissue Culture and Secondary Metabolites, Department of Botany, University of Rajasthan, Rajasthan, India. E-mail: geetsingh600@gmail.com
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Considering these reported medicinal values, the present work was carried out to examine the antimicrobial potential of flavonoids extract of *T. chebula* against some pathogenic microorganisms, those have not been screened earlier.

MATERIALS AND METHODS

Different parts of *T. chebula* (leaf, stem, bark, fruits) were collected from the University of Agriculture Sciences (Gandhi Krishi Vignyan Kendra, Bangalore.) and the specimen of the plant was identified at the department of Botany, University of Rajasthan. The sample specimen with No. RUBL20868 was submitted in the 'Herbarium' of Botany Department, University of Rajasthan.

Extraction Procedure

Plant parts were separately shade dried and finely powdered using a mixer. Free and bound flavonoids from root, stem, bark and fruits of *T. chebula* were Hundred grams each of finely powdered samples was Soxhlet extracted with 80% hot methanol (500 ml) on a water bath for 24 h and filtered.[12] Each filtrate was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, whereas ethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids, respectively. Ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2So_4 for 2 h and filtered. The filtrate was extracted in ethyl acetate and washed with distilled water to neutrality. Ethyl ether (free flavonoid) and ethyl acetate fractions (bound flavonoids) thus obtained were dried in vaccuo and weighed [Table 1]. The extracts were stored at 4°C and were re-suspended in their respective solvents to get 10 mg/ml concentration for antimicrobial assay.

Thin Layer Chromatography

Selected extract (bound flavonoid of fruit) which showed activity against all microorganisms tested was dissolved in ethyl acetate and applied on silica gel coated (0.2-0.3 mm) and activated glass plates (20×20 cm) in a oven at 100°C for 30 min. along with the standard reference compound of apigenin and kaempferol 1cm above the edge of the plates. These plates were developed in an organic solvent mixture of benzene, acetic acid and water (125:72:3), air dried and visualized under UV light.[13] Two spots (Rf 0.65, 0.31) were observed which were further confirmed by spraying the plates with 5% ethanolic ferric chloride solution [Table 2 and Figure 1]. A few other solvent system (n-butanol, acetic acid and water, 4:1:5; n-butanol, water 1:1; n-butanol, acetic acid and water 6:1:2) were used but in the present investigation the solvent system of benzene, acetic acid and water (125:72:3) gave excellent results. Rf value 0.65 obtained indicate the presence of apigenin in the bound flavonoids of fruit extract subjected to TLC.

Figure 1: Thin layer chromatography. E – Extract; A – Apigenin; K – Kaempferol

Table 1: Antimicrobial activity of free flavonoids of *T. chebula* by disc diffusion assay

Test microorganism	Extract							
	Leaf		Stem		Stem bark		Fruits	
	IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI
E. coli	-	-	-	-	11.66	0.448±0.034	-	-
P. aeruginosa	10.5	0.525±0.025	8.25	0.413±0.013	-	-	-	-
P. mirabilis	9.5	0.038±0.012	-	-	10.5	0.420±0.012	14.5	0.580±0.012
R. planticola	9.8	0.328±0.015	-	-	13	0.433±0.019	20.83	0.694±0.015
E. aerogens	12.5	0.568±0.013	10.5	0.477±0.013	10.75	0.489±0.012	30.83	1.397±0.020
B. subtilis	16	0.888±0.032	8.5	0.472±0.016	22	1.217±0.032	19.5	1.080±0.017
S. aureus	11.5	0.547±0.014	7.75	0.369±0.012	12.66	0.603±0.069	10.5	0.500±0.012
A. niger	-	-	-	-	-	-	-	-
A. flavus	13.66	0.911±0.198	-	-	-	-	14.5	0.966±0.019
C. albicans	19	1.353±0.084	11.25	0.803±0.125	13.5	0.964±0.021	26.5	1.892±0.250

IZ – Inhibition zone in mm (mean value; include 6 mm diameter of disc); AI – Activity index (IZ developed by extract/IZ developed by standard); ± – SEM; (-) – No activity; Extracts assayed in triplicate; IZ of standard drug streptomycin against *E. coli* (26 mm) *P. aeruginosa* (20 mm), *P. mirabilis* (25 mm), *R. planticola* (30 mm), *E. aerogens* (22 mm), *B. subtilis* (18 mm) *S. aureus* (21 mm), IZ of standard drug Itraconazol against *A. niger* (10 mm) & *A. flavus* (15 mm). IZ of standard drug Clotrimazole against *C. albicans* (14 mm)

Preparative Thin Layer Chromatography

Preparative TLC of the bound flavonoids from fruit of *T. chebula* was carried out on silica gel coated and activated (0.4-0.5mm thick) glass plates in the selected solvent (benzene, acetic acid and water). Spot of Rf value 0.65 was marked in each plate and was collected and eluted with ethyl acetate. Elutes were pooled, completely dried and re-chromatographed to test the purity of the isolated compound.

Infra Red Spectral Study of Eluted Compound

The isolated compound was crystallized, weighed and subjected to melting point and infra red spectral studies on Perkins Elmer model 555 spectrophotometer in KBr pellets. Apigenin (Rf 0.65; UV fluorescent-blue; ammonia-bright yellowish green; $FeCl_3$–brownish; m.p. 340°) was identified in the fruit of plant [Figure 2].

Selected Test Microorganisms

Pathogenic microorganisms selected for study include seven bacteria, viz., *Escherichia coli* (MTCC no. 46), Pseudomonas *aeruginosa* (MTCC 1934), Proteus *mirabilis* (MTCC 3310), *Raoultella planticola* (MTCC 2271), *Enterobacter aerogenes* (MTCC 2822), *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 3160) and three fungal strains, viz., *Candida. albicans* (MTCC 183), *Aspergillus flavus* (MTCC 277), Aspergillus niger (MTCC 282). Selected microorganisms were procured from IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on "Muller- Hinton Agar Medium" (Beef extract 2.0 g; Peptone 17.5 g; Starch 1.5 g; Agar 17.0 g; in 1000 ml of distilled water; Final pH 7.4±0.2) at 37±2°C while fungal strains were grown on "Sabouraud Dextrose Agar Medium" (Peptone 10 g; Dextrose 20 g; Agar 20 g in 1000 ml of distilled water; pH adjusted to 6.8-7.0 at 27±2°C).

Antimicrobial Screening of Extracts

Disc diffusion assay (DDA) was performed for antimicrobial assay.[14,15] MH agar (for bacteria) and SD agar (for fungi) base plates were seeded with the standard inoculum size of bacteria, yeast and fungi (1×10^8 CFU/ml for bacteria, 1×10^7 CFU/mlfor yeast and 1×10^6 CFU/ml for dermatophytic fungi). Sterile filter paper discs (6 mm in diameter) were impregnated with 100 µl of each of the extract (10 mg/ml concentration) to give a final concentration of 1 mg/disc, left to dry in vaccuo to remove residual solvent, which might interfere with the determination. Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate along with standard drugs streptomycin (1 mg/disc) for bacteria, itraconazol (1 mg/ml) for *A. niger* and *A. flavus* and Clotrimazole (1 mg/ml) for *C. albicans* respectively. The plates were kept at 4°C for 1h for diffusion of extract, thereafter were incubated at 37±2°C for 24 h; 27±2°C for 48 h and 27±2°C for 5-7 days for bacteria, yeast and fungus, respectively. Zone of inhibition (IZ) or depressed growth of microorganisms was measured and the 'Activity Index' (AI) for each extract was calculated.

Table 2: Rf values of spots of flavonoid extract obtained in thin layer chromatography (solvent system benzene:acetic acid:water 125:72:3)

Flavonoid extract	Rf
Spot 1	0.31
Spot 2	0.65
Standard apigenin	0.65
Standard kaempferol	0.86

Rf of spot no. 2 matches with standard apigenin

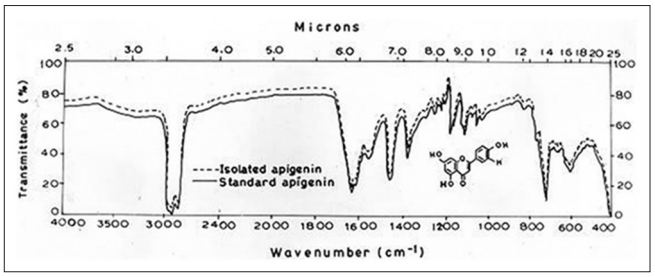

Figure 2: Infra red of Apigenin

Minimum Inhibitory Concentration and Minimum Bactericidal/Fungicidal Concentration

Minimum inhibitory concentration (MIC) was determined for plant extract showing antimicrobial activity against test pathogens in disc diffusion assay. Broth micro dilution method was followed for determination of MIC values.[16] Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration and then was added to broth media of 96-wells of micro titer plates using two fold serial dilution. Thereafter 100 µl inoculum of standard size was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. The micro titer plates were incubated at 37±2°C for 24 h for bacteria, 27±2°C for 48 h for yeast and 27±2°C for 5-7 days for fungi. Each extract was assayed in duplicate and each time two sets of micro titer plates were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the wells of micro titer plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bacterial/fungicidal concentration (MBC/MFC) was determined by subculturing 50 µl from each well showing noapparent growth. Least concentration of extract showing no visible growth on subculturing was taken as MBC/MFC.

Total Activity

Total activity (TA) is the volume at which test extract can be diluted without loosing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g.[17]

RESULTS

Antimicrobial activity (assessed in terms of inhibition zone and activity index) of the plant extracts, tested against selected microorganisms were recorded [Tables 1 and 3; Figure 3]. Among Gram +ve bacteria, maximum activity was exhibited by free flavonoid extracts of stem bark against *B.subtilis* (IZ 22, AI 1.21 + 0.032) and *S. aureus* (IZ 12.66, AI 0.603±0.069). Among Gram −ve bacteria maximum activity was exhibited by free flavonoids of fruits against *E. aerogens* (IZ 30.83, AI 1.39±0.020. Free flavonoids (IZ 20.83, AI 0.694±0.015) of fruits demonstrated maximum inhibition against *R. planticola*. Against *P. mirabilis* bound flavonoids of fruits showed maximum activity (IZ 19, AI 0.760±0.061). Likewise maximum antifungal activities was against *A. flavus* (IZ 26, AI 1.73±0.039) was demonstrated by bound flavonoids of fruits. Free flavonoids of leaf showed significant inhibitory effect against *C. albicans* (IZ 19, AI 1.35±0.084).

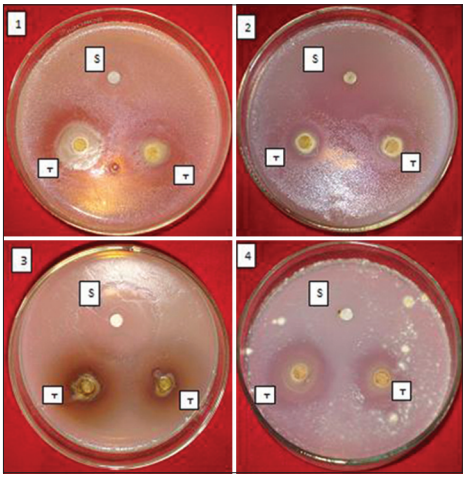

Figure 3: Inhibition Zone of extracts of selected plants against microorganisms S: Standard Disc; T: Test extracts Disc (1) *Terminalia chebula*/Fruit/E1/ *Candida albicans* (2) *Terminalia chebula*/Fruit/E1/ Raoultella planticola (3) *Terminalia chebula*/Fruit/E2/ *Enterobacter aerogens* (4) *Terminalia chebula*/Fruit /E2/ *Aspergillus flavus*

MIC and MBC/MFC values [Tables 4 and 5] were evaluated for those plant extracts which had shown activity in 'Disc Diffusion Assay'. In the present investigation lowest MIC values 0.039 mg/ml was recorded against *P. mirabilis, R. planticola, E. aerogens, B. subtilis, S. aureus, A. flavus* and *C. albicans*, whereas against *E. coli* and *P. aeruginosa* lowest MIC (0.156 and 0.312 mg/ml) was observed, indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found equal for three free flavonoids and five bound flavonoids of *T. chebula* indicating cidal nature of extracts.

Amount of extracts isolated from each gram of plant parts [Table 6] and Total activity (TA) was calculated and recorded [Table 7]. Total activity indicates the volume at which extract can be diluted with still retaining ability to kill microorganisms. Most of the extracts showed high values of TA against *P. mirabilis, E. aerogenes, A. flavus* and *C. albicans*, which proves the potential to inhibit the growth of the test microorganisms, even at low concentration. Maximum TA values calculated were 506.41, 160.25, 1012.82, 506.41, 1012.82, 1282.05 ml against *E. coli, P. aeruginosa, P. mirabilis, R. planticola, E. aerogens, B. subtilis, S. aureus, A, flavus* and *C. albicans*, respectively.

DISCUSSION

T. arjuna was found to have antibacterial activity.[18] Fruit

Table 3: Antimicrobial activity of bound flavonoids of *T.chebula* by disc diffusion assay

Test microorganism	Extract							
	Leaf		Stem		Stem bark		Fruits	
	IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI
E. coli	-	-	-	-	-	-	16	0.15±0.022
P. aeruginosa	-	-	8.5	0.425±0.014	-	-	-	-
P. mirabilis	-	-	-	-	16	0.640±0.023	19	0.760±0.061
R. planticola	13.5	0.450±0.010	-	-	9.5	0.316±0.010	19.83	0.661±0.015
E. aerogens	14.5	0.659±0.159	11	0.500±0.026	14.8	0.659±0.159	29	1.313±0.026
B. subtilis	11.5	0.638±0.016	16.3	0.907±0.049	17.5	0.972±0.049	14	0.777±0.032
S. aureus	9.8	0.468±0.021	8.5	0.404±0.014	11.16	0.531±0.029	13.83	0.658±0.029
A. niger	-	-	-	-	-	-	-	-
A. flavus	9.5	0.633±0.019	-	-	-	-	26	1.733±0.039
C. albicans	11.5	0.821±0.107	15.5	1.105±0.035	15	1.070±0.070	26	1.875±0.072

IZ – Inhibition zone in mm (mean value; include 6 mm diameter of disc); AI – Activity index (IZ developed by extract/IZ developed by standard); ± – SEM, (-) – No activity. Extracts assayed in triplicate, IZ of standard drug streptomycin against *E. coli* (26 mm), *P. aeruginosa* (20 mm), *P. mirabilis* (25 mm), *R. planticola* (30 mm), *E. aerogens* (22 mm), *B. subtilis* (18 mm) *S. aureus* (21 mm), IZ of standard drug Itraconazol against *A. niger* (10 mm) & *A. flavus* (15 mm). IZ of standard drug Clotrimazole against *C. albicans* (14 mm)

Table 4: Minimum inhibitory concentration and minimum bactericidal/fungicidal concentration values of free flavonoids of *T. chebula* against test pathogens

Test microorganism	Extract							
	Leaf		Stem		Stem Bark		Fruits	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MFC/MBC	MIC	MFC/MBC
E. coli	-	-	-	-	0.156	0.312	-	-
P. aeruginosa	0.312	0.625	0.625	1.25	-	-	-	-
P. mirabilis	0.312	0.312	-	-	0.312	1.25	0.156	0.312
R. planticola	0.312	0.625	-	-	0.156	0.312	0.039	0.078
E. aerogens	0.078	0.156	0.039	0.078	0.078	0.312	0.039	0.039
B. subtilis	0.078	0.312	0.312	0.625	0.078	0.039	0.078	0.156
S. aureus	0.156	0.312	0.625	1.25	0.078	0.312	0.312	0.625
A. niger	-	-	-	-	-	-	-	-
A. flavus	0.078	0.156	-	-	-	-	0.156	0.156
C. albicans	0.039	0.078	0.312	0.625	0.312	0.625	0.078	0.078

MIC – Minimum inhibitory concentration (mg/ml); MBC/MFC – Minimum bactericidal/fungicidal concentration (mg/ml)

Table 5: Minimum inhibitory concentration and minimum bactericidal/fungicidal concentration values of bound flavonoids of *T. chebula* against test pathogens

Test microorganism	Extract							
	Leaf		Stem		Stem bark		Fruits	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MFC/MBC	MIC	MFC/MBC
E. coli	-	-	-	-	-	-	0.078	0.156
P. aeruginosa	-	-	0.312	1.25	-	-	-	-
P. mirabilis	-	-	-	-	0.078	0.312	0.039	0.078
R. planticola	0.078	0.156	-	-	0.312	1.25	0.078	0.156
E. aerogens	0.078	0.312	0.156	0.312	0.039	0.078	0.039	0.039
B. subtilis	0.156	0.312	0.039	0.078	0.078	0.078	0.078	0.156
S. aureus	0.312	0.625	0.625	1.25	0.312	0.625	0.156	0.312
A. niger	-	-	-	-	-	-	-	-
A. flavus	0.156	0.312	-	-	-	-	0.039	0.039
C. albicans	0.312	0.312	0.078	0.078	0.039	0.078	0.078	0.312

MIC – Minimum inhibitory concentration (mg/ml); MBC/MFC – Minimum bactericidal/fungicidal concentration (mg/ml)

Table 6: Flavonoids content of different parts of *Terminalia chebula*

Plant part	Extracts	Quantity of the extract mg/gdw
Leaf	E_1	50
	E_2	9.5
Stem	E_1	5
	E_2	3.5
Stem bark	E_1	14
	E_2	5.5
Fruits	E_1	6.8
	E_2	39.5

E_1 – Free flavonoids; E_2 – Bound flavonoids

ethanol extract of *T. chebula* Retz. exhibited antibacterial activity against *S. aureus* (MRSA) and the compounds responsible for this activity were gallic acid and its ethyl ester.[19] The clinical pathogen *E. coli* showed a MIC value of 6.25 mg/ml which is higher than the present study (0.078 mg/ml). Terpenoides from *T. avicennioides* showed antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa*.[20] Results of the present study reveal that all plant extracts tested, inhibited the growth of selected bacteria and fungi, indicating broad spectrum bioactive nature of selected plant. Most of the extracts of *T. chebula* were found to be potent inhibitor for tested organisms except *E. coli*, against which only two extracts of the plant (free flavonoids of stem bark and bound flavonoids of fruits) showed activity. Excellent activity was shown by free and bound flavonoids of *T. chebula* with low MIC and MBC/MFC values. Higher values of MFC/MBC as compare to MIC values indicate the bacteriostatic/ fungistatic nature of the extracts. Free flavonoids of leaf, fruits were found to be bactericidal against *P. mirabilis* and *E. aerogens*, whereas bound flavonoids of stem bark and fruits were found to be bactericidal against *B. subtilis* and *E. aerogenes*. On the other hand free flavonoids of fruits were recorded to be a fungicidal against *A. flavus*, bound flavonoids of leaf, stem were recorded fungicidal against *C. albicans* and bound flavonoids against *A. flavus*.

In the present scenario when existing antibiotics are gradually becoming ineffective against pathogenic microorganisms, such studies should highly be encouraged, so that new and alternative sources for future antibiotics

Table 7: Total activity flavonoids of *T. chebula* against test pathogens

Test microorganism	Extract							
	Leaf		Stem		Stem bark		Fruits	
	E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2
E. coli	-	-	-	-	89.74	-	-	506.41
P. aeruginosa	160.25	-	8	11.21	-	-	-	-
P. mirabilis	160.25	-	-	-	44.87	70.51	43.58	1012.82
R. planticola	160.25	121.79	-	-	89.74	17.62	174.35	506.41
E. aerogens	641.02	121.79	128.20	22.43	179.48	141.02	174.35	1010.82
B. subtilis	641.02	60.89	16.02	89.74	179.48	70.51	87.17	506.41
S. aureus	320.51	30.44	8	4.12	179.48	17.62	21.79	253.20
A. niger	-	-	-	-	-	-	-	-
A. flavus	641.02	60.89	-	-	-	-	43.58	1012.82
C. albicans	1282.05	30.44	16.02	44.87	17.62	141.02	87.17	506.41

Total activity – Extract per gram dried plant part; MIC

may be explored well in advance. Results of the study reveal that *T. chebula* can be potential candidate to be explored for future.

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