ORIGINAL ARTICLE

Evaluation of antimicrobial activity of alkaloids of *Terminalia chebula* Retz. against some multidrug-resistant microorganisms

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Alkaloids extracted from different parts (leaf, stem, stem bark, and fruits) of *Terminalia chebula* were screened for antimicrobial activity against nine bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Raoultella planticola*, *Enterobacter aerogens*, *Agrobacterium tumefaciens*, and *Klebsiella pneumoniae)* and two *fungi (Aspergillus flavus* and *Aspergillus niger)* and one yeast *(Candida albicans*). Minimum inhibitory concentration, Minimum bactericidal/fungicidal concentration, and Total activity of the extracts, against each sensitive test pathogen, were also evaluated. Alkaloids from all plant parts showed good antimicrobial activity against almost all the test microorganisms except *A. niger*, against which, none of the tested extracts showed activity. The largest zone of inhibition (IZ 20.75 mm) was observed against *P. aeruginosa*. The total activity of the leaf alkaloid was found to be the same and the highest (256.41ml/g) was against *E. aerogens* and *A. tumefaciens.*

Key words: Alkaloids, *T. chebula*, antimicrobial activity, minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration, *E. aerogens*

INTRODUCTION

Plants remain the most common source of antimicrobial agents. Their use as traditional health remedies is most popular in 80% of the world population in Asia, Latin America, and Africa, and is reported to have minimal side effects[1,2]. In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to the population. The rising incidence in multidrug resistance among pathogenic microbes has further necessitated the need to search for newer antibiotic sources. Plant materials remain an important source to combat serious diseases in the world. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in developing countries. The medicinal value of these plants lies in some chemically active substances that produce a definite physiological action on the human body. The most important among the bioactive constituents of plants are alkaloids, tannins, flavonoids, and steroids[3]. In recent years, infections have increased to a great extent and antibiotic resistance effects have become an ever-increasing therapeutic problem[4]. The natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms of action[5,6]. They are effective in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials[7]. Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine, and to reveal the active principle by isolation and characterisation of their constituents. Systematic screening of plant extracts may result in the discovery of novel active compounds[8].

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Terminila chebula Retz (*combretaeae*) is a medicinal plant widely distributed throughout India, Burma, and Srilanka. The dried ripe fruit of *T. chebula* also know as black myrobalan has widely been used in the treatment of asthma, sore throat, vomiting, hiccups, bleeding, piles, diarrhoea, gout, and heart and bladder diseases[9]. Black myrobalan is reported to have antioxidant and free radical scavenging activities[10].

T. chebula is reported to be an antimicrobial [11-15], hepato-protective[16,17], anti-inflammatory[18], immunomodulatory[19], an antioxidant[20-23] and adaptogenic.[24]

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MATERIALS AND METHODS

Preliminary Detection of Alkaloids

Each of the test samples was acidified by 5 ml of 2% HCl at 60°C for two hours, and later cooled and filtered. Formation of the white precipitate on the addition of the following reagents, individually to 2 ml of the above-mentioned solution, indicated the presence of alkaloids:

Wagner reagent: (Prepared by mixing 1.25 g I_2 and 2 g KI in 100 ml of distilled water).

Extraction of Alkaloids

Alkaloids were extracted from different parts of the selected plant by well-established methods after preliminary detection of alkaloids.[25] Different parts of *T. chebula* (stem bark, stem, leaf, and fruits) were taken for extraction. A finely powered sample (100 g) of the plant parts was extracted with 10% acetic acid in ethanol for four hours. The extracts were concentrated and were made alkaline with Ammonium Hydroxide (NH_4OH). The precipitate thus obtained was collected by centrifugation, washed with 1% NH_4OH, filtered, dried *in vacuo* and weighed. The extracts thus obtained were stored at 4°C in air tight glass vials for further use.

Selected Test Microorganisms

Pathogenic microorganisms selected for the study include nine bacteria, namely, *E. coli* (MTCC 46), *P. aeruginosa* (MTCC 1934), *P. mirabilis* (MTCC 3310), *S. aureus* (MTCC 3160), *B. subtilis* (MTCC 121), *R. planticola* (MTCC 2271), *E. aerogens*(MTCC 2822), *A. tumefaciens (MTCC 431), K. pneumoniae (MTCC 4030)*, two fungal strains, namely, *A flavus (MTCC 277), A. niger (MTCC 282), and one yeast C. albicans* (MTCC 183). The selected microorganisms were procured from IMTECH, Chandigarh, India. The 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 kept 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 Extract

Disc diffusion assay (DDA) was performed for antimicrobial screening.[26,27] MH agar (for bacteria) and SD agar (for fungi) base plates were seeded with the standard size of bacterial, yeast, and fungal inoculums (1×10^8 CFU/ml for bacteria, 1×10^7 CFU/ml for yeast, and 1×10^6 CFU/ml for fungi). Sterile filter paper discs (6 mm in diameter) were impregnated with 100 μl each of the extract (10 mg/ml concentration) to give a final concentration of 1 mg/disc and left to dry in vacuo, to remove the residual solvent, which might interfere with the determination. The extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate along with streptomycin (1 mg/disc) for bacteria, itraconazol (1mg/ml) for *A. niger* and *A. flavus*, and clotrimazole (1 mg/disc) for *C. albicans*, respectively. The plates were kept at 4°C for one hour for diffusion of the extract, and thereafter, they were incubated at 37±2°C for 24 hours; 27±2°C for 48 hours, and at 27±2°C for five to seven days for bacteria, yeast, and fungus, respectively. The zone of inhibition (IZ) or depressed growth of microorganisms was measured and the Activity Index (AI) for each extract was calculated.

$$AI = \frac{\text{Inhibition Zone of the sample}}{\text{Inhibition Zone of the standard}}$$

Minimum Inhibitory Concentration and Minimum Bactericidal/Fungicidal Concentration

Minimum inhibitory concentration (MIC) was determined for the plant extract showing antimicrobial activity against test pathogens in a disc diffusion assay. The broth microdilution method was followed for determination of the MIC values.[28] Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make a 10 mg/ml final concentration, and this was added to the broth media in 96-wells of microtitre plates, using a two-fold serial dilution. Thereafter, a 100 μl inoculum of standard size was added to each well. Bacterial and fungal suspensions were used as negative controls, while broth containing the standard drug was used as a positive control. The microtitre plates were incubated at 37±2°C for 24 hours for bacteria, 27±2°C for 48 hours for yeast, and 27±2°C for five to seven days for fungi. Each extract was assayed in duplicate and each time two sets of microtitre plates were prepared; one was kept for incubation, while another set was kept at 4°C for comparing the turbidity in the wells of the microtitre plate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtitre plate that showed no turbidity after incubation. The turbidity in the wells in the microtitre plate was interpreted as a visible growth of microorganisms. The minimum bacterial/fungicidal concentration (MBC/MFC) was determined by sub-culturing 50 μl from each well showing no apparent growth. The least concentration of extract showing no visible growth on sub-culturing was taken as MBC/MFC.

Total Activity

Total activity (TA) is the volume at which the test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g of plant material by the MIC of the same extract or an isolated compound, and is expressed in ml/g.[29] In mathematical terms it can be expressed as

$$TA = \frac{\text{Amount extracted from 1 g plant material}}{\text{MIC of the extract}}$$

RESULTS AND DISCUSSION

The alkaloid content estimated in each gram of plant material was recorded [Table 1, Figure 1]. The leaves of the plant were recorded to have maximum alkaloid content (10 mg/gdw) followed by fruits (6.5 mg/gdw), stem bark (5 mg/gdw), and stem (2.5 mg/gdw). Alkaloid extracts were then screened against the selected test pathogens [Table 2]; [Figures 2-5]. Most of the extracts showed antibacterial and/or antifungal potential against the selected pathogens under study.

Preliminary *in vitro* screening of the antimicrobial activity of the plant extracts from *T. chebula* was studied against some microorganisms, using the 'Disc Diffusion Assay'. The result indicated that gram-negative bacteria *E. aerogens, A. tumefaciens, and K. pneumoniae* were the most sensitive strains among the tested pathogens. The strongest inhibitionzone was recorded for the alkaloids from fruits (IZ 17.5, AI 0.795±0.013) followed by alkaloids from the stem bark (IZ 19, AI 0.678±0.036; IZ 14.75, AI 0.867±0.015), respectively. The alkaloids of *T. chebula* showed no activity against *A. niger.* Alkaloids from the fruits of *T. chebula* showed the highest activity (IZ 20.75, AI 1.038±0.038) against *P. aeroginosa*, with modest activity against *A. flavus* (IZ 8.5, AI 0.567±0.034). The stem alkaloid showed activity against all the pathogenic microorganisms tested except *A. niger*. The leaf alkaloid

Table 1: Alkaloid content of different parts of *T. chebula*

Plant part	Quantity of the extract mg / gdw
Leaf	10.0
Stem	2.5
Stem bark	5.0
Fruits	6.5

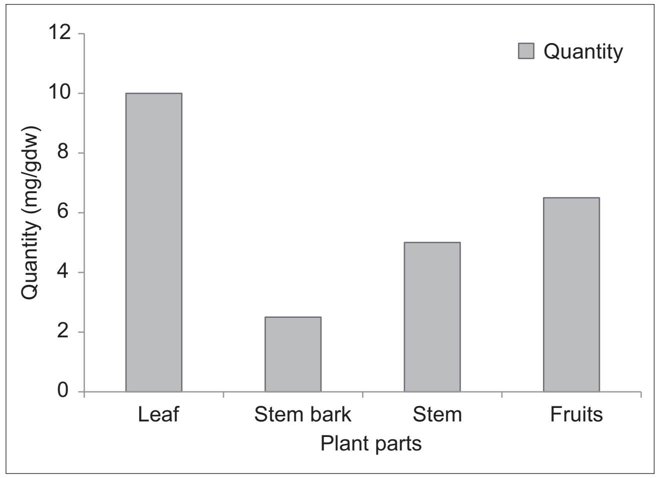

Figure 1: Alkaloid content of different parts of *T. chebula*

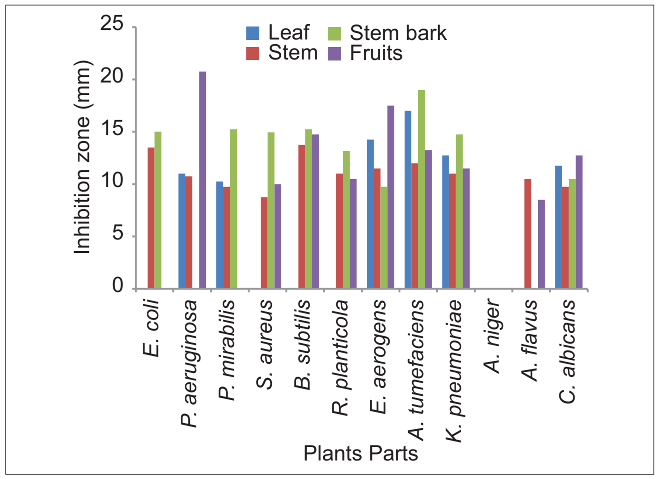

Figure 2: Antimicrobial activity of *T. chebula*

Table 2: Antimicrobial activity of Alkaloids of *T. chebula* by disc diffusion assay

Test microorganisms	Plant parts							
	Leaf		Stem		Stem bark		Fruits	
	IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI
E. coli	–	–	13.50	0.519±0.058	15.00	0.577±0.039	16.25	0.625±0.010
P. aeruginosa	11.00	0.550±0.014	10.75	0.538±0.013	–	–	20.75	1.038±0.038
P. mirabilis	10.25	0.410±0.010	9.75	0.390±0.010	15.25	0.610±0.010	–	–
S. aureus	–	–	8.75	0.416±0.012	14.95	0.714±0.024	10.00	0.476±0.024
B. subtilis	–	–	13.75	0.764±0.042	15.25	0.847±0.042	14.75	0.819±0.042
R. planticola	–	–	11.00	0.367±0.017	13.16	0.439±0.039	10.50	0.350±0.034
E. aerogens	14.25	0.648±0.012	11.5	0.523±0.023	9.75	0.443±0.012	17.50	0.795±0.013
A. tumefaciens	17.00	0.607±0.072	12.00	0.428±0.036	19.00	0.678±0.036	13.25	0.473±0.009
K. pneumoniae	12.75	0.750±0.015	11.00	0.647±0.017	14.75	0.867±0.015	11.50	0.676±0.029
A. niger	–	–	–	–	–	–	–	–
A. flavus	–	–	10.50	0.700±0.034	–	–	8.50	0.567±0.034
C. albicans	11.75	0.839±0.018	9.75	0.696±0.018	10.50	0.750±0.020	12.75	0.910±0.018

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), *S. aureus* (21 mm), *B. subtilis* (18 mm), *R. planticola* (30 mm), *E. aerogens* (22 mm), *A. tumefaciens (28 mm), and K. pneumoniae (17 mm). IZ of itraconazol against A. niger (10 mm) and A. flavus (15 mm).* IZ of clotrimazole against *C. albicans* (14 mm)

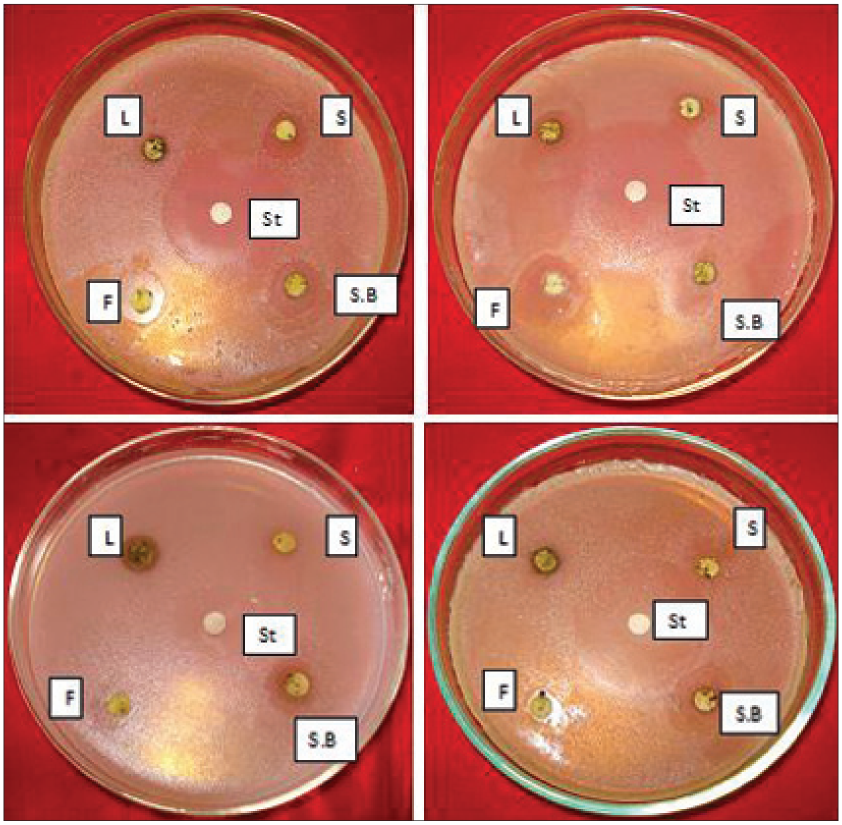

Figure 3: Inhibition zone of extracts of selected plants against microorganisms. St – Standard Disc; L – Leaf extracts Disc; S – Stem extracts disc; S.B. – Stem bark extracts disc; F – Fruits extracts disc. 1. Against *E. coli* 2. Against *P. aeruginosa* 3. Against *P. mirabilis* 4. Against *S. aureus*

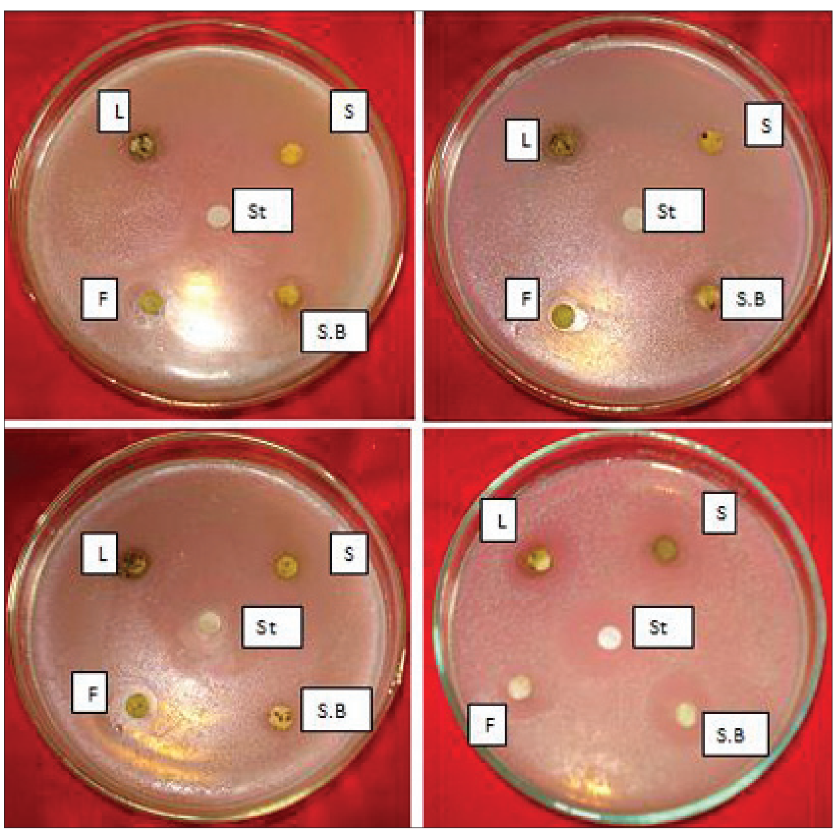

Figure 4: Inhibition zone of extracts of selected plants against microorganisms. St – Standard Disc; L – Leaf extracts Disc; S – Stem extracts disc; S.B. – Stem bark extracts disc; F – Fruits extracts disc. 1. Against *B. subtilis* 2. Against *R. planticola* 3. Against *E. aerogens* 4. Against *A. tumefaciens*

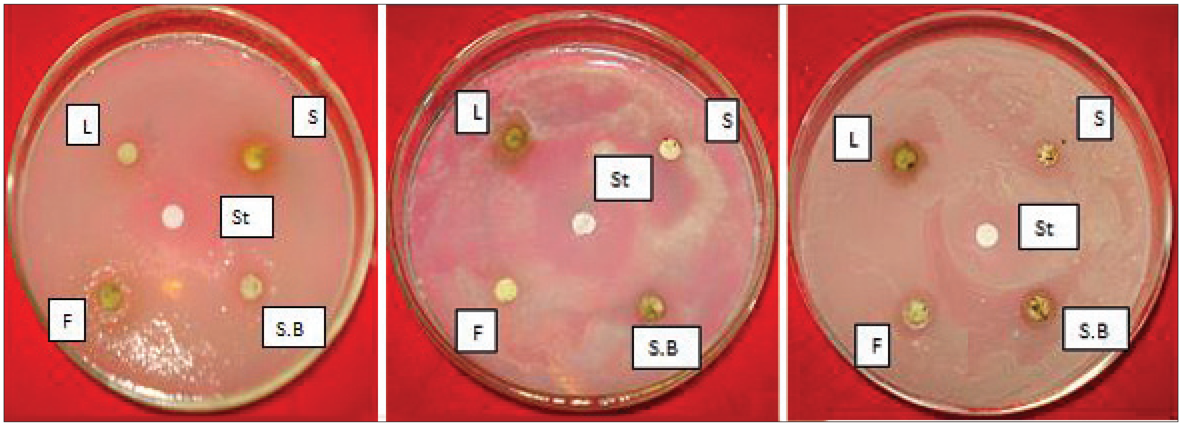

Figure 5: Inhibition zone of extracts of selected plants against microorganisms.St – Standard Disc; L – Leaf extracts Disc; S – Stem extracts disc; S.B. – Stem bark extracts disc; F – Fruits extracts disc. 1. Against *K. pneumoniae* 2. Against *A. flavus* 3. Against *C. albicans*

Table 3: MIC and MBC / MFC values of *T. chebula* against test pathogens

Test microorganisms	Plant parts							
	Leaf		Stem		Stem bark		Fruits	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	-	-	0.078	0.156	0.039	0.156	0.039	0.078
P. aeruginosa	0.078	0.156	0.156	0.312	-	-	0.039	0.039
P. mirabilis	0.078	0.312	0.312	0.625	0.078	0.156	-	-
S. aureus	-	-	0.625	1.25	0.078	0.078	0.156	0.312
B. subtilis	-	-	0.039	0.156	0.039	0.078	0.078	0.156
R. planticola	-	-	0.078	0.156	0.078	0.312	0.156	0.312
E. aerogens	0.039	0.078	0.156	0.312	0.078	0.156	0.039	0.078
A. tumefaciens	0.039	0.078	0.156	0.156	0.039	0.039	0.039	0.078
K. pneumoniae	0.078	0.078	0.078	0.312	0.039	0.078	0.156	0.312
A. niger	-	-	-	-	-	-	-	-
A. flavus	-	-	0.156	0.625	-	-	0.312	0.625
C. albicans	0.156	0.312	0.312	0.625	0.156	0.625	0.078	0.078

MIC – Minimum inhibitory concentration (mg / ml), MBC – Minimum bactericidal concentration (mg / ml)

Table 4: Total activity of alkaloids of *T. chebula*

Test microorganisms	Total activity			
	Plant parts			
	Leaf (ml/g)	Stem (ml/g)	Stem bark (ml/g)	Fruits (ml/g)
E. coli	–	32.05	128.20	166.66
P. aeruginosa	128.20	16.02	–	166.66
P. mirabilis	128.20	8.01	64.10	–
S. aureus	–	4	64.10	41.66
B. subtilis	–	64.10	128.20	83.33
R. planticola	–	32.05	64.10	41.66
E. aerogens	256.41	16.02	64.10	166.66
A. tumefaciens	256.41	16.02	128.20	166.66
K. pneumoniae	128.20	32.05	128.20	41.66
A. niger	–	–	–	–
A. flavus	–	16.02	–	20.83
C. albicans	64.10	8.01	32.05	83.33

showed lower activity against test organisms as compared to other extracts.

The MIC and MBC/MFC values [Table 3] were evaluated for those plant extracts showing activity in the diffusion assay. In the present investigation, the lowest MIC value of 0.039 mg/ml was recorded against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Enterobacter aerogens*, *Agrobacterium tumefaciens and Klebsiella pneumoniae*, whereas, MIC against *Proteus mirabilis*, *Staphylococcus aureus*, *Raoultella planticola* and *Candida albicans* was 0.078 mg/ml, indicating significant antimicrobial potential in the test extracts. The range of MIC and MBC/MFC of the extracts recorded was 0.039 – 0.625 mg/ml and 0.039 – 1.25mg/ml, respectively. The total activity [Table 4] was the highest for leaf alkaloid (256.41 ml/g) against *E. aerogens* and *A. tumefaciens*.

$$\text{Total activity} = \frac{\text{Extract per gram dried plant part}}{\text{MIC}}$$

In the current investigation *T. chebula* showed its antimicrobial potential against pathogens that are involved in a number of human diseases. Screening of the plant under investigation has not been worked out for alkaloids so far, mostly crude extracts have been reported earlier, but without evaluating MIC, MBC/MFC, or Total activity, which are indispensible to ascertain the antimicrobial potentiality in any of the screened compounds or extracts. Among all the pathogens the gram-negative bacteria *Enterobacter aerogens*, *Agrobacterium tumefaciens*, and *Klebsiella pneumoniae* have been found to be the most sensitive, and are known to be resistant to the action of most antimicrobial agents, including plant-based extracts.[30, 31] Gram-negative bacteria have an outer phospholipid membrane with structural lipopolysaccharide components, which make their cell wall impermeable to antimicrobial agents.[32] Further studies can be done to identify the specific chemical compound responsible for the antimicrobial activity.

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