

## INVESTIGATION ON THE MECHANISM OF ACTION OF THE LEAVES OF *TRIANTHEMA PORTULACASTRUM* ON HUMAN PATHOGENS

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### ABSTRACT

**Objective:** Many of the plants are used for medicinal purposes in human health needs. *Trianthema portulacastrum* has got many medicinal values and also a dietary plant, being used in Ayurveda. It exhibits nephroprotective, diuretic, anti-cancerous, antihelminthic, antipyretic, analgesic properties, anti-inflammatory activity, and also antibacterial activity. The objective of this study is to determine the mechanism of action of the leaves of *T. portulacastrum* on human pathogens.

**Methods:** The mechanism has been analyzed by membrane permeability, time-kill kinetics, hemolytic activity, DNA binding ability, post antibiotic effect, and bacterial membrane damage by scanning electron microscopy.

**Results:** This study reveal that the flavonoid and methanol fractions of *T. portulacastrum* have potent antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*. The leaves of *T. portulacastrum* has been proved as a safer drug indicating its noncytotoxic property and found to kill the bacterial cells by permeating the membrane and damage the DNA of the bacterial cells.

**Conclusion:** This study proved the mode of antibacterial action of *T. portulacastrum* on *S. aureus* and *K. pneumoniae* indicating its antibacterial efficacy on human pathogens.

**Keywords:** *Staphylococcus aureus*, Postantibiotic, *Klebsiella pneumoniae*, *Trianthema portulacastrum*.

### INTRODUCTION

Infectious diseases are the leading cause of morbidity and mortality worldwide, especially in developing countries. Infectious diseases are caused by microorganisms, such as bacteria, viruses, parasites, or fungi; the diseases that can be spread, directly or indirectly, from one person to another. The emerging of infectious diseases has been recognized as an important outcome of host-pathogen evolution leading to severe public health consequences [1].

Bacterial infectious diseases are serious health problems that have drawn the public in worldwide as a human threat, which extends to economic and social complications [2]. Antibiotic drugs are used to reduce the burden of common infectious diseases and become essential for many medical interventions [3].

In general, bacteria possess the genetic ability to acquire and to transmit resistance to therapeutic agents. Owing to the excessive use of antibiotics in human therapy, bacteria have developed several resistance mechanisms such as target site modification, expression of the efflux pumps and metabolic inactivation, contributing to the multidrug resistance bacteria [4].

Many of the plants are used today for medicinal purposes. Herbal medicine is mostly used to cure various diseases and physiological conditions by practiced traditional methods such as Ayurveda and Homeopathy. It is estimated that 40% of the world population depends directly on plant-based medicines. Drugs derived from plant sources play a significant role in the prevention and treatment of human diseases. Antimicrobial properties of medicinal plants are being increasingly reported [5].

Plants produce secondary metabolites as a response to adverse environmental conditions or developmental stages. The function or importance of these compounds is usually of an ecological nature as

they are used as defenses against predators, parasites, and diseases for interspecies competition, and to facilitate the reproductive processes (coloring agents, attractive smells, etc). From the medicinal point of view, the important secondary metabolites are alkaloids, flavonoids, tannins, terpenes, and phenolic compounds. These active constituents possess effective pharmacological activity [6].

Flavonoids are pigmented compounds having potent antimicrobial activity against a variety of bacterial and fungal pathogens and are mediated by their action on the microbial cell membranes. They may interact with membrane proteins present on bacterial cell wall leading to increased cell permeability and disruption against both Gram-positive and Gram-negative organisms [7].

One of the traditional medicinal plants is *Trianthema portulacastrum*, the family of Aizoaceae. It is the most common weed in the field crops. The plants have bitter, analgesic elements which cure Bronchitis and also used in inflammation and rheumatism. The leaves possess the diuretic property and also have been utilized for the treatment of jaundice. Likewise, various parts have also been reported as having antioxidant, analgesic, hepatoprotective, nephroprotective, antifertility, antihyperglycemic hypolipidemic, and anti-cancer properties [8].

The aim of this study is to determine the mechanism of action of the leaves of *T. portulacastrum* on bacteria by membrane permeability, bacterial time-kill kinetics, hemolytic activity, DNA binding ability, UV absorption method, post-antibiotic effect, and bacterial membrane damage by scanning electron microscopy (SEM) of *T. portulacastrum* leaves.

### METHODS

#### Collection of plant

The leaves of *T. portulacastrum* (voucher no. 1629) were collected from the field of place in Usilampatti, Madurai district and the leaves were taken for further analysis.

### Preparation of flavonoid fractions

The leaves were taken and crushed using mortar and pestle. To the crushed sample, 20 ml of 80% ethanol was added. The conical flask was plugged and placed in a boiling water bath for 15 minutes with occasional shaking, and then the contents were centrifuged; supernatant thus collected was the phenolic extract. Approximately, half the volume of the phenolic fraction was transferred to a 50 ml separating funnel, and the sample was then extracted with petroleum ether (40-60°C). The aqueous layer thus obtained was the flavonoid extract.

### Preparation of methanolic extract

The leaves were collected, washed well under the running tap water and they were then air dried. The methanolic plant extract was prepared by taking fresh leaves and homogenized with 50 ml of methanol and was then left overnight in the shaker at room temperature and then centrifuged at 4000 rpm for 20 minutes. The supernatant containing the plant extract was then transferred to a pre-weighed beaker and the extract was concentrated by evaporating the solvent at 60°C. The crude extract was weighed and dissolved in a known volume of dimethyl sulfoxide, to obtain a final concentration of 20 mg/5 µl.

### Bacterial time-kill kinetics

Bacterial time-kill curves have been used to determine the kinetics of bacterial killing *in vitro* but not to determine whether an antibacterial agent is bacteriostatic. The time-kill kinetics antibacterial study of the leaf fractions of *T. portulacastrum* was carried out to assess the killing rate of the extract within a given contact time. The time-kill kinetics was performed by the appropriate concentration of methanolic extract and flavonoid fractions of *T. portulacastrum* against selected organisms. The test organisms used for the study were prepared by incubating the *Klebsiella pneumoniae* and *Staphylococcus aureus* in nutrient medium to get 1.0 McFarland standard (absorbance is 0.257) and to reach  $4 \times 10^7$  CFU/ml and the plates were incubated at 37°C for 16-24 hrs and the colonies were counted [9].

### Hemolytic activity

Hemolysis represents the disruption or lysis of the erythrocytes causing the release of hemoglobin. Hemolytic activity of the plant was measured at the amount of hemoglobin released by the lysis of goat blood cells (red blood cells [RBC]) obtained from slaughterhouse. The hemolytic activity of the methanolic extract and flavonoid fractions of *T. portulacastrum* was calculated by determining the hemoglobin release under 8% suspension of fresh goat erythrocytes at 414 nm [10].

### UV absorption method

The leakage of nucleic acids and proteins alters the membrane permeability of the bacterial cell membrane. The method was followed to determine the leakage of nucleic acids and proteins of methanolic extract and flavonoid fractions of *T. portulacastrum*, using  $UV_{260}$  and  $UV_{280\text{ nm}}$  spectrophotometrically. The bacterial cell suspension namely *S. aureus* and *K. pneumoniae* were treated with flavonoid and methanolic fractions and incubated at different period [11].

### Membrane permeability

To detect the leakage of reducing sugars and proteins through the membrane, tubes containing Mueller-Hinton (MH) broth with varying concentration of methanolic extract (100 mg) and flavonoid fractions (125 mg) were inoculated with pathogenic bacteria cells at final concentrations of  $10^8$  CFU/ml. The cultures were incubated at  $37 \pm 2^\circ\text{C}$  and shaken at 150 rpm for 18 hrs. The samples were centrifuged at 12,000 rpm, thereafter the supernatant was collected and frozen at  $-30^\circ\text{C}$ . Then, the supernatant was used for estimation of sugars and proteins [12].

### DNA binding assay

The DNA binding assay can assess the antimicrobial-DNA binding efficiency by noting the retardation rate of migration of DNA bands through agarose gels. The DNA binding abilities of the methanolic extract and flavonoid fractions of *T. portulacastrum* were examined

by analyzing the electrophoretic mobility of genomic DNA of bacteria on 1% agarose gel. The genomic DNA was isolated from the bacterial pathogens *S. aureus* and *K. pneumoniae*. The isolated genomic DNA was treated with flavonoid and methanol fractions and incubated for 1 hr followed by agarose gel electrophoresis.

### Post antibiotic effect

Post-antibiotic effect was performed *in vitro* as delayed bacterial growth after a short on off exposure to methanolic extract and flavonoid fractions of *T. portulacastrum* for 2 hrs. Then, the bacteria were pelleted, washed two times in 10 ml of phosphate-buffered saline and then resuspended in 4 ml MH broth. The tubes were placed back in the 37°C shaking incubator, and bacterial regrowth was measured at selected time points by plating 25 µl of samples of serial dilutions on agar plates and colonies were counted.

### SEM

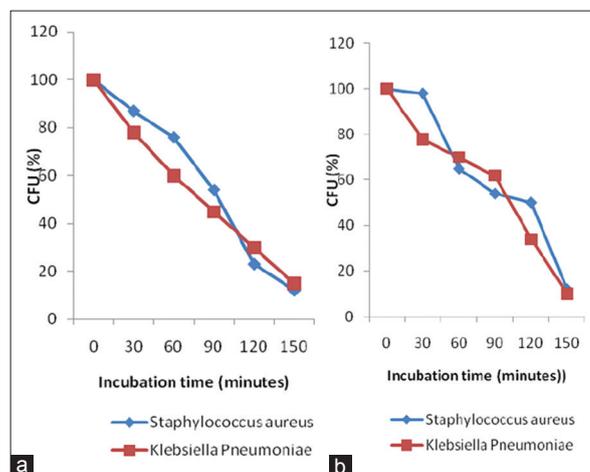
Bacteria were fixed with 2% glutaraldehyde, washed and resuspended in water, and then deposited onto silicon platelets as a 1 µl droplets. For samples on membrane filters, 10 µl of the treated cells suspension was placed onto a 0.45-µm pore-size membrane filter fixed for 1 hr with 2% glutaraldehyde, washed and post-fixed with 1% osmium tetroxide. The samples were dehydrated with graded ethanol and then air-dried. The cells were coated with 15 nm nano gold particles through automatic sputter coater. The samples were then viewed through SEM.

## RESULTS AND DISCUSSION

### Bacterial time kill kinetics

With the methanolic extract, the number of CFU of *S. aureus* and *K. pneumoniae* were found to be decreased (Fig. 1). At different time intervals, 30 minutes incubation was found to reduce the cells to 69% and 96%. Among different period of incubation, 90 and 150 minutes have been found to reduce the viable cells to 24% and 15%. Further increase in the incubation time leads to the complete death of both the organisms. Hence, methanolic extract showed more potent toward *K. pneumoniae* compared to *S. aureus*. When the bacteria were treated with the flavonoid fractions the number of CFU of *S. aureus* and *K. pneumoniae* were found to be decreased. At different time intervals, 30 minutes incubation was found to reduce the cells to 98% and 73%. Among various time intervals, 90 and 60 minutes have significantly found to reduce the viable cells to 60% and 54%, respectively. Further increase in the incubation time lead to the complete death of both of the selected pathogens.

In the results of time-kill kinetics, the flavonoid fractions of *T. portulacastrum* showed potent percentage reduction of viable cell



**Fig. 1: Bacterial time-kill kinetics (a) Flavonoid fractions of *Trianthema portulacastrum* against *Staphylococcus aureus* and *Klebsiella pneumoniae* (b) Methanol fractions of *T. portulacastrum* against *S. aureus* and *K. pneumoniae***

count and these fractions possess greater activity than methanol fractions.

Thus, flavonoid fractions were more effective against *S. aureus* than *K. pneumoniae*. Hence, flavonoid fractions were found to possess higher antibacterial activity than methanolic extract.

The reduction of initial viable cells with increasing concentration of the leaf extract led to microbial destruction in the ethanol leaf extract of *Pupalia lappacea* [13]. After 24 hrs of incubation, more than 5-log CFU/ml decreased and showed a reduction of 4-log CFU/ml of crude and methanolic extract of *Zingiber officinale* at a concentration of 128 µg/ml [14]. The time kill curves of α-terpineol, *Escherichia coli* had directly entered decline phase and were killed by α-terpineol at ×1 minimum inhibitory concentration (MIC) in 8 hrs and ×2 MIC in 4 hrs. Thus, the rate of killing was increased by increasing the concentration of α-terpineol [15].

#### Hemolysis assay

The methanolic extract and flavonoid fractions were found to lyse the RBC as 7% and 15%, respectively. Among these fractions, flavonoid fractions exhibited higher activity which might be due to the presence of all the phytochemical constituents. It clearly demonstrates that non-cytotoxic property of the leaves of *T. portulacastrum*. The results of hemolytic activity are presented in Table 1.

Aqueous extract of leaves of *Jasminum auriculatum* showed higher activity at higher doses (30 mg/ml) as compared to control [16]. The results showed very low hemolytic effect of aqueous extract of *Aerva lanata* stem against normal human erythrocytes [17]. The methanol extract of *Maytenus royleanus* leaves and its various fractions against normal human erythrocytes showed that the ethyl acetate fraction exhibited minimum effect, whereas aqueous fractions showed the highest activity [18].

**Table 1: Hemolytic activity of flavonoid and methanol fractions of *T. portulacastrum***

Sample	Concentration (mg)	Percentage of hemolysis
Methanol fractions	100	15
Flavanoid fractions	125	7

*T. portulacastrum*: *Trianthema portulacastrum*

**Table 2: Determination of leakage of nucleic acids and proteins at UV260 and UV280 nm from *S. aureus***

Samples	Wavelength (nm)	Absorbance				
		0 minute	15 minutes	30 minutes	45 minutes	60 minutes
Control	260	0.004	0.004	0.004	0.004	0.004
	280	0.006	0.006	0.006	0.006	0.006
Methanolic extract	260	0.009	0.012	0.016	0.021	0.027
	280	0.032	0.045	0.048	0.067	0.078
Flavonoid fractions	260	0.014	0.025	0.028	0.032	0.034
	280	0.046	0.096	0.121	0.132	0.148

*S. aureus*: *Staphylococcus aureus*

**Table 3: Determination of leakage of nucleic acids and proteins at UV260 and UV280 nm from *K. pneumoniae***

Samples	Wavelength (nm)	Absorbance				
		0 minute	15 minutes	30 minutes	45 minutes	60 minutes
Control	260	0.003	0.003	0.003	0.003	0.003
	280	0.004	0.004	0.004	0.004	0.004
Methanolic fractions	260	0.012	0.032	0.046	0.049	0.052
	280	0.036	0.042	0.080	0.096	0.110
Flavonoid fractions	260	0.026	0.032	0.045	0.076	0.095
	280	0.067	0.086	0.098	0.108	0.127

*K. pneumoniae*: *Klebsiella pneumoniae*

#### UV absorption method

The values for absorbance at 260 nm and 280 nm for supernatants of *S. aureus* and *K. pneumoniae* treated in methanolic extract and flavonoid fractions of *T. portulacastrum* are shown in Tables 2 and 3. At regular time intervals of 15 minutes, the reading was taken up to 60 minutes.

The results of this study reveal that the higher leakage (release) of absorbing material was recorded from *S. aureus* while lowest leakage record was from *K. pneumoniae*, which seemed to be one of the modes of bactericidal actions. Both at 260 and 280 nm, *S. aureus* showed maximum leakage of protein and nucleic acid from the bacterial cell membrane when compared to *K. pneumoniae* at different time interval.

The treatment of *S. aureus* cells with spice extracts and essential oils induced the leakage of absorbing material over a period of 90 minutes [19]. The membrane leakage of cellular constituents having absorption at 260 nm and 280 nm was observed over a period of 90 minutes in the presence of spice extracts and essential oils against *S. aureus* [20].

#### Membrane permeability

Presence of reducing sugar and protein were estimated in the methanolic extract and flavonoid fractions of *T. portulacastrum* treated on the bacterial broth cultures and the results predicted the leakage of cell membrane of pathogenic bacteria (*S. aureus* and *K. pneumoniae*). The OD value obtained by estimation of reducing sugar and protein was referred with the standard graphs. Initially, the presence of reducing sugar protein was absent in the control indicating no leakage in cell membrane, while the leakage of membrane was noticed by estimating the reducing sugar and protein. The results illustrated that the flavonoid and methanol fractions of *T. portulacastrum* by serving as an inhibitors of cell membrane function, which contributes to their antibacterial potential. The results are shown in Fig. 2a and b.

Concentration of the reducing sugar and protein estimated were higher in glycosides and flavonoids compounds of *Caesalpinia Coriaria* treated with *S. aureus* cells [21]. The bacterial cells (*Escherichia fergusonii* and *Streptococcus mutans*) were treated with silver nanoparticle alone or in combination with antibiotics for 12 hrs and the amount of protein released in the suspension of the treated cells was significantly higher than the control groups or silver nanoparticles alone group [22].

**DNA binding assay**

To clarify the molecular mechanism of action, we examined the DNA binding ability of the test compounds. The genomic DNA was isolated from both *S. aureus* and *K. pneumoniae* and the purity was checked by UV absorption spectra at 260 nm/280 nm. The DNA binding ability of the methanolic extract and flavonoid fractions of *T. portulacastrum* was determined by the gel retardation method. The results of DNA binding assay for *S. aureus* are presented in Table 4 and Plate 1. The results of DNA binding assay for *K. pneumoniae* were shown in the Table 5 and Plate 2.

The results of electrophoretic mobility indicated that there is a significant damage in methanolic extract and flavonoid fractions treated DNA of *S. aureus*. The genomic DNA of *S. aureus* was found to be intact while there is a significant retardation on the agarose gel. These results are also confirmed by the intergral density values (IDV) obtained by the gel documentation system.

On comparison of the IDV of the *S. aureus* DNA the flavonoid fractions treated *S. aureus* DNA, we can confirm that there is a remarkable damage in the DNA treated with the flavonoid fractions, contributing to its DNA binding ability than the methanolic extract. Replacing one or more of the electron donating groups such as hydroxyl or furan ring results in an increase in the DNA cleavage affinity [23]. The cell-penetrating peptide

**Table 4: Effect of methanolic extract and flavonoid fractions of *T. portulacastrum* on genomic DNA of *S. aureus***

Sample	IDV of the bands
Genomic DNA of <i>S. aureus</i>	260000
Genomic DNA of <i>S. aureus</i> with methanolic extract	44254
Genomic DNA of <i>S. aureus</i> with flavonoid fractions	26372

*S. aureus*: *Staphylococcus aureus*, *T. portulacastrum*: *Trianthema portulacastrum*, IDV: Integral density values

**Table 5: Effect of methanolic extract and flavonoid fractions of *T. portulacastrum* on genomic DNA of *K. pneumoniae***

Sample	IDV of the bands
Genomic DNA of <i>K. pneumoniae</i>	1435523
Genomic DNA of <i>K. pneumoniae</i> with methanolic extract	10563
Genomic DNA of <i>K. pneumoniae</i> with flavonoid fractions	2378

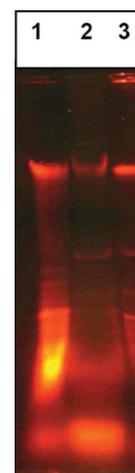
*T. portulacastrum*: *Trianthema portulacastrum*, *K. pneumoniae*: *Klebsiella pneumoniae*, IDV: Integral density values

analog, P7, exerts antimicrobial activity against *E. coli* ATCC25922 via penetrating cell membrane and targeting intracellular DNA [24].

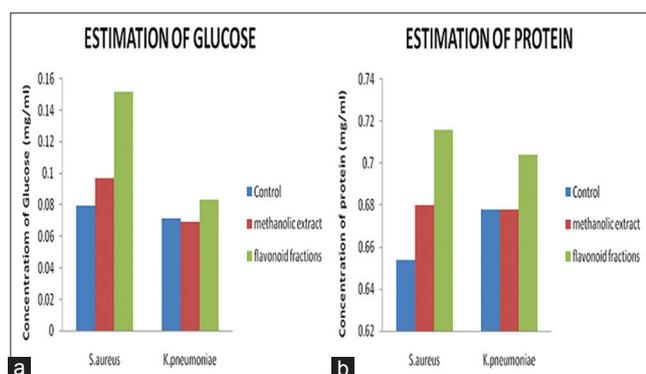
**Post antibiotic effect**

Post antibiotic effect was performed for methanolic extract and flavonoid fractions of *T. portulacastrum* against *S. aureus*. The results are depicted in Fig. 3. The concentration of flavonoid and methanol fractions was found to be 125 mg and 100 mg, respectively. At 2 hrs, the cells were found to be rapid bactericidal activities. The results of this assay indicated that both the methanolic extract and flavonoid fractions showed a good post antibiotic effect with the selected pathogens.

Effect of cefquinome against *Staphylococcus aureus* at different concentrations of  $\times 1$ ,  $\times 2$ ,  $\times 4$  MIC for up to 2 hrs [25]. The activity of anthracimycin against contemporary methicillin-resistant *S. aureus*. The compound exhibited minimal post antibiotic effects against USA300 methicillin-resistant *S. aureus* (MRSA), with regrowth rapidly after removal of the compound within 3 hrs at 5 times its MIC [26]. The marinopyrrole has a potent role which exhibited a concentration - dependent bactericidal activity against MRSA strains, a prolonged post antibiotic effect superior to that of vancomycin and linezolid, and are highly favorable [27].



**Plate 1: Effect of methanolic extract and flavonoid fractions of *T. portulacastrum* on DNA of *Staphylococcus aureus*, Lane 1: Genomic DNA of *S. aureus*, Lane 2: Genomic DNA with methanolic extract, Lane 3: Genomic DNA with flavonoid fractions**



**Fig. 2: (a) Leakage of reducing sugar by membrane permeability of flavonoid and methanol fractions of *Trianthema portulacastrum* (b) Leakage of reducing sugar by membrane permeability of flavonoid and methanol fractions of *T. portulacastrum***



**Plate 2: Effect of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* on DNA of *Klebsiella pneumoniae*, Lane 1: Genomic DNA of *K. pneumoniae*, Lane 2: Genomic DNA with methanol fractions, Lane 3: Genomic DNA with flavonoid fractions**

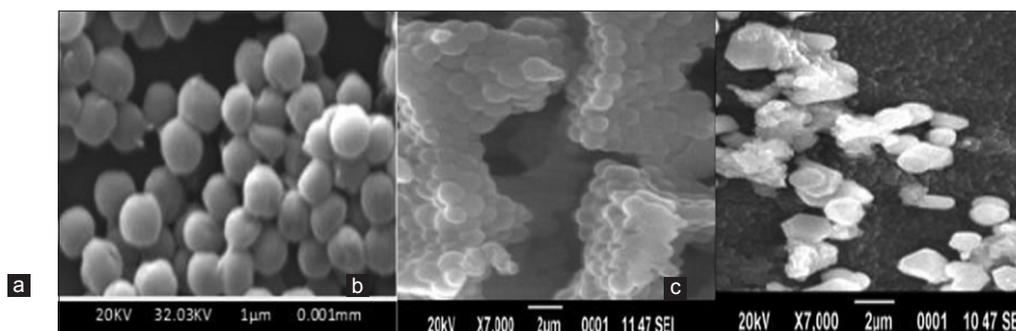


Plate 3: Scanning electron microscopic images of *Staphylococcus aureus* (a) Control cells (b) *S. aureus* treated with methanolic extract (c) *S. aureus* treated with flavonoid fractions

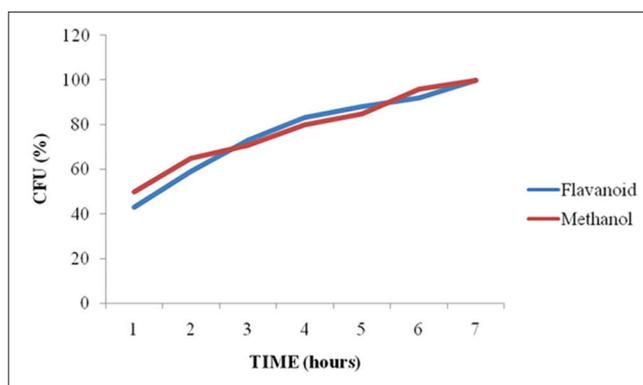


Fig. 3: Post antibiotic effect methanolic extract and flavonoid fractions of *Trianthema portulacastrum* against *Staphylococcus aureus*

#### SEM

The SEM images represent the methanolic extract and flavonoid fractions treated *S. aureus* cells as shown in Plate 3a-c. There is distortion in the shape of cells, with depressions on the surface as a result of exposure to both the fractions. The frequency of dead and damaged cells was found to be more in flavonoid fractions than in methanol fractions. It revealed deformed and destroyed cells with probable depletion of their intracellular cell content.

The membrane damage was clearly seen in *S. aureus* treated methanolic extract and flavonoid fractions of the leaves of *T. portulacastrum*.

Curcumin I treated *S. aureus* cells have indicated the distortion in the shape of the cells, with depression on the surface of the cells. After exposure of Catechin-Cu nanoparticles for 3 hrs, the quantity of *S. aureus* greatly decreased and cell walls became wrinkled and damaged. Similarly, in *E. coli* cells, the shape and size of cells also damaged dramatically and there were a lot of materials attached on the bacterial surface [28].

#### CONCLUSIONS

The results of this study proved the mode of antibacterial action of *T. portulacastrum* on *S. aureus* and *K. pneumoniae*. Therefore, the leaves of *T. portulacastrum* has been proved as a safer drug indicating its noncytotoxic property and found to kill the bacterial cells by permeating the membrane and damage the DNA of the bacterial cells.

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