4.0 RESULTS AND DISCUSSION

Infectious diseases are the leading cause of death worldwide. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens. Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents and resistance to old and newly produced drug. The increasing failure of antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Olaleye, 2015).

In recent years, there has been an increasing awareness about the importance of medicinal plants. Medicinal plants are still invaluable source of safe, less toxic, lower price, available and reliable natural resources of drugs all over the world (Kabbashi *et al.,* 2014*)*. The active drugs found in plants are secondary metabolites. It plays a vital role for their phytochemical constituents. Most of these molecules are secondary metabolites, of which atleast 12,000 have been isolated and characterized (Aktar *et al.,* 2011).

Drugs derived from medicinal plants are easily available, inexpensive, safe, and efficient. (Razmavar, 2014). One such plant that is used in traditional medicine is *Trianthema portulacastrum*. It has antibiotic, antifungal, antiseptic, analgesic, antipyretic, bronchitis, antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, antifertility, antihyperglycemic and hypolipidemic and anti-cancer activity. The leaves were used to cure jaundice, strangury and dropsy. The juice of the leaves dropped into the nostrils relives migraine (Lakshmi *et al.,* 2014).

Earlier studies in our laboratory had reported the antimicrobial activity of *Trianthema portulacastrum* of phytochemical fractions namely alkaloid, phenolics and flavonoids. These fractions were tested with the various bacterial species among which *Staphylococcus aureus* and *Klebsiella pneumoniae* were found to be the most sensitive organisms.

Hence, in this study we have analysed the mode of action of the leaves of *Trianthema portulacastrum* for its antimicrobial activity such as bacterial time kill kinetics, haemolytic activity, membrane permeability, UV absorbtion method and DNA binding affinity. The post antibiotic effect of both the methanolic extract and flavonoid fractions of *Trianthema portulacastrum* L. on the pathogens such as *Staphylococcus aureus*, a Gram positive bacteria and

Klebsiella pneumoniae, a Gram negative bacteria has been carried out. And, also the membrane damage on the pathogens was analyzed by Scanning Electron Microscopy.

4.1 PREPARATION OF METHANOLIC EXTRACT AND FLAVONOID FRACTIONS

The methanolic and flavonoid extract was prepared from 5g leaves of *Trianthema portulacastrum* L by the procedure given by Harborne method. The yield of methanolic extract and flavonoid fractions was found to be 27% and 25%respectively.

4.2 BACTERIAL TIME KILL KINETICS

Bacterial time kill kinetics was performed for methanolic extract and flavonoid fractions of *Trianthema portulacastrum* against *Staphylococcus aureus* and *Klebsiella pneumoniae*. The concentration of methanolic extract and flavonoid fractions were found to be 100 mg and125 mg respectively when they were tested for their antibacterial activity on selected pathogens. The results are presented in Figures 1 and 2.

The percentage of CFU at different time exposure was calculated by fixing the 0^{th}minute CFU as 100%. With the methanolic extract, the number of CFU of *Staphylococcus aureus* and *Klebsiella pneumoniae* were found to be decreased which has been shown in the Plates 2 and 3. At different time intervals, 30 minutes incubation was found to reduce the cells to 69% and 96%. Among different time 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 lead to the complete death of both the organisms. Hence methanolic extract showed more potent towards *Klebsiella pneumoniae* compared to *Staphylococcus aureus*.

When the bacteria were treated with the flavonoid fractions the number of CFU of *Staphylococcus aureus* and *Klebsiella pneumoniae* were found to be decreased. At different time intervals, 30 minutes incubation was found to reduce the cells to 98% and 73% (Plates 4 and 5). 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.

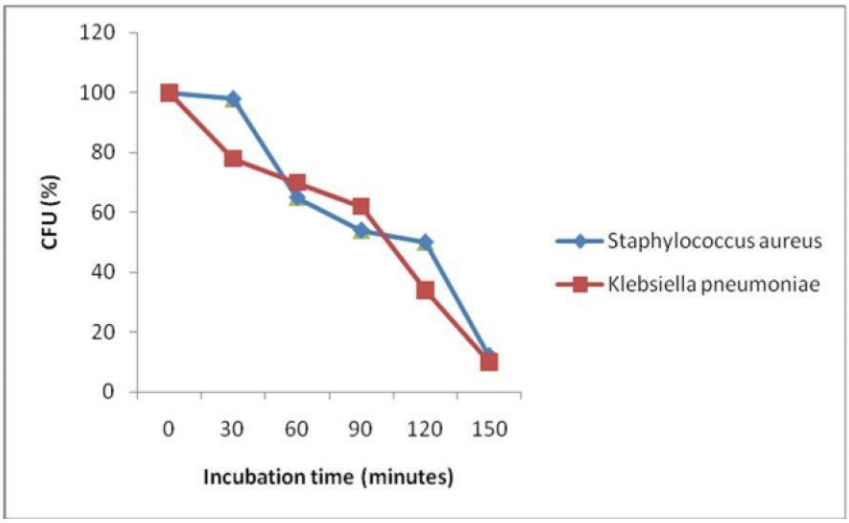


Figure 1: Bacterial Time Kill Kinetics of methanolic extracts of *Trianthema portulacastrum* against *Staphylococcus aureus* and *Klebsiella pneumoniae*

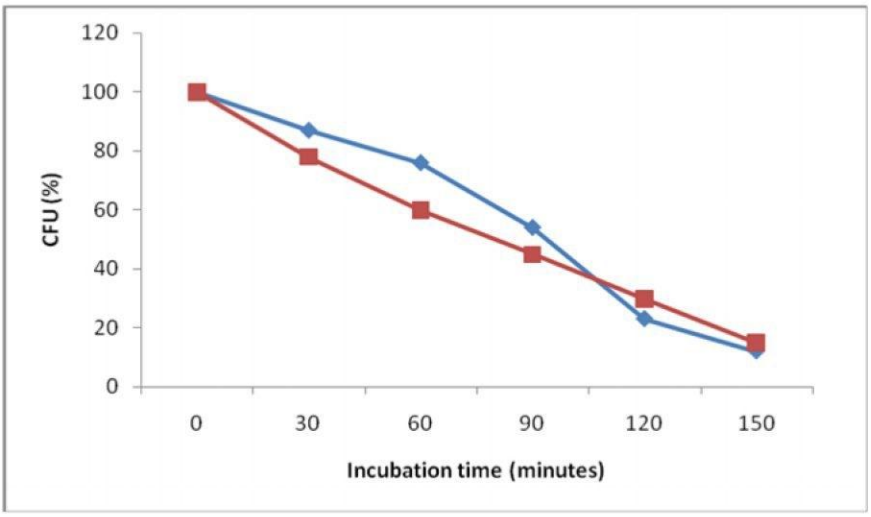
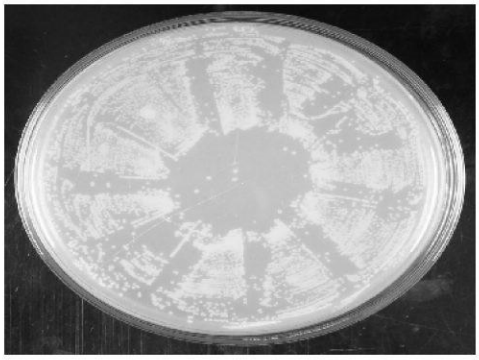


Figure 2: Bacterial Time Kill Kinetics of flavonoid fractions of *Trianthema portulacastrum* against *Staphylococcus aureus* and *Klebsiella pneumoniae*



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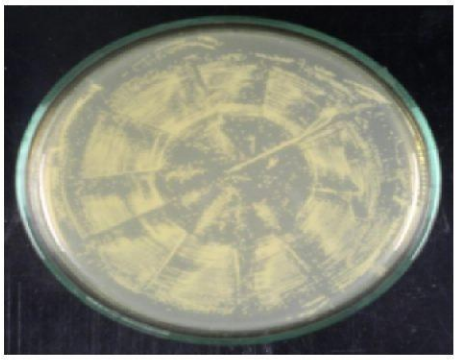

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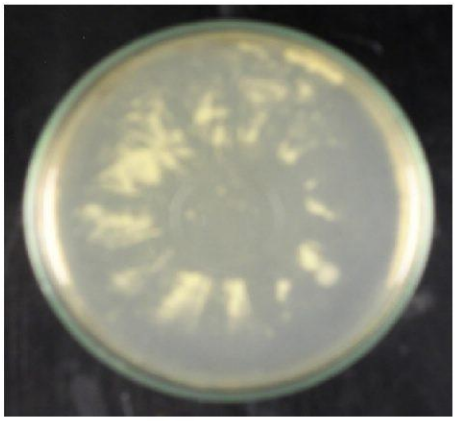

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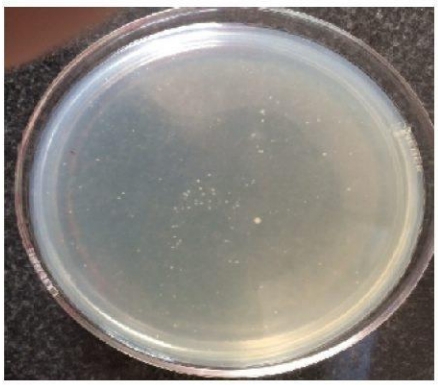

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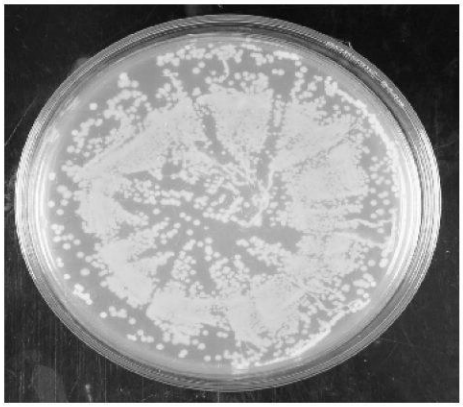
Plate 2: Influence of methanolic extract of *Trianthema portulacastrum* on *Staphylococcus aureus* at different time intervals



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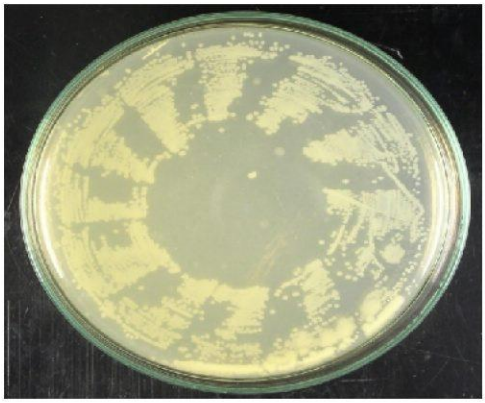

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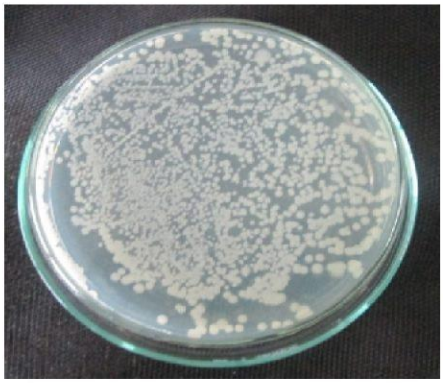

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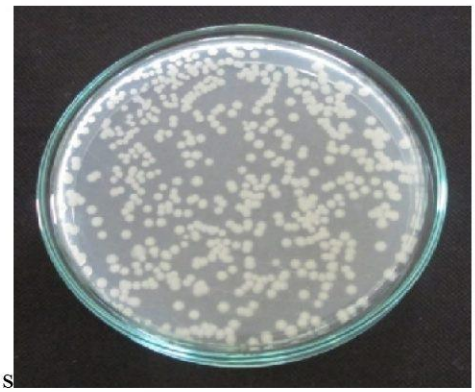

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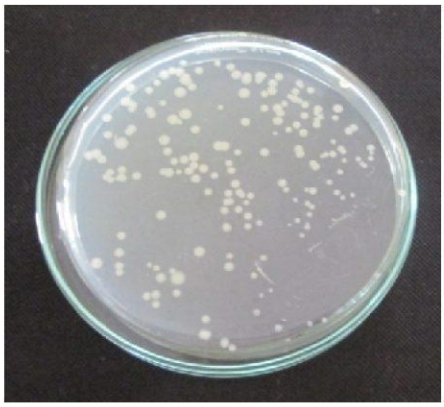

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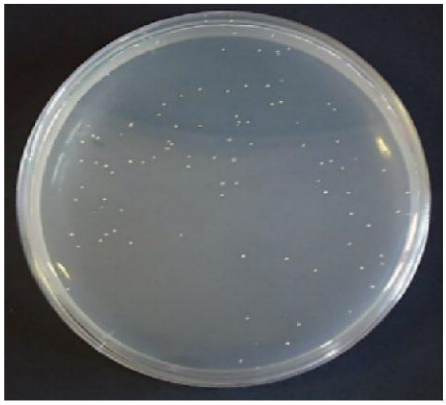

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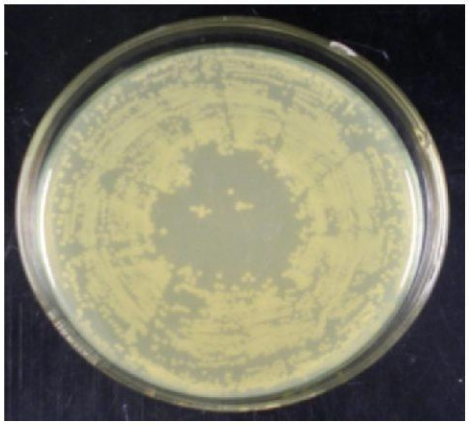
Plate 3: Influence of methanolic extract of *Trianthema portulacastrum* on *Klebsiella pneumoniae* at different time intervals



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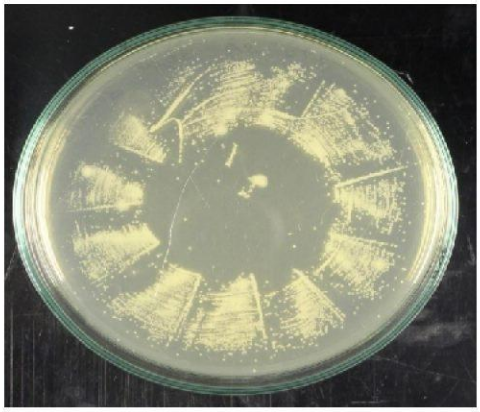

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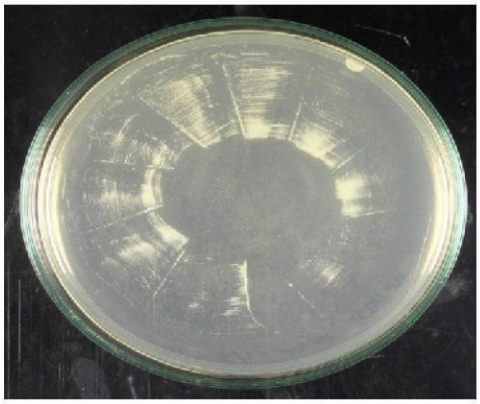

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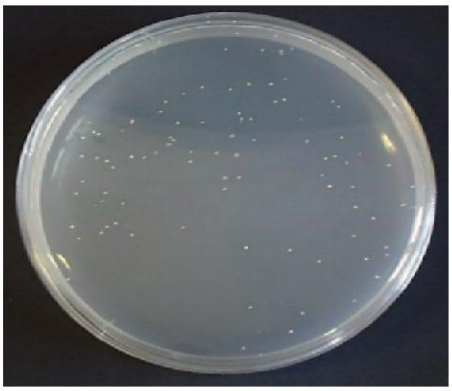

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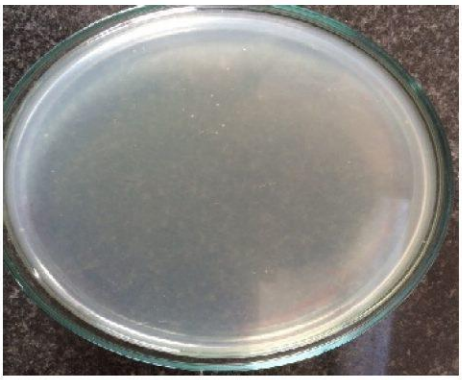

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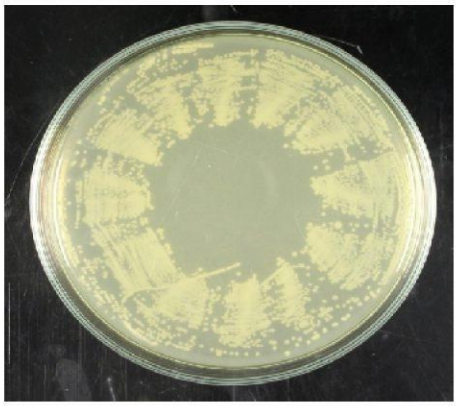
Plate 4: Influence of flavonoid fractions of *Trianthema portulacastrum* on *Staphylococcus aureus* at different time intervals



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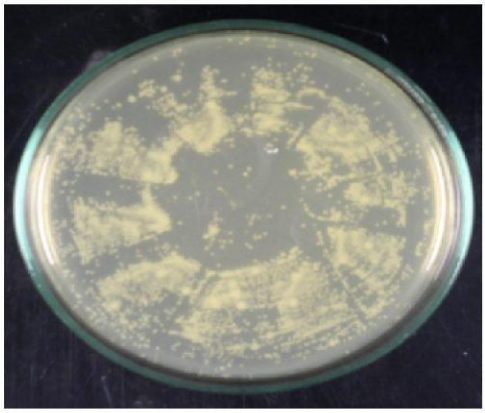

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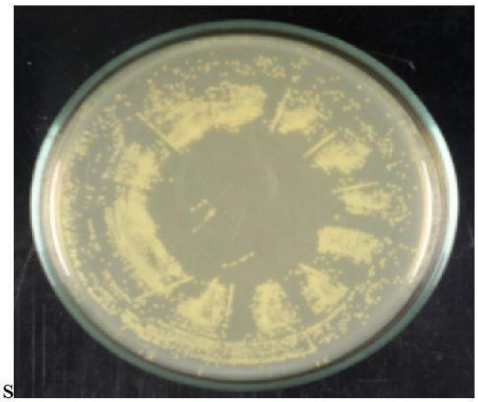

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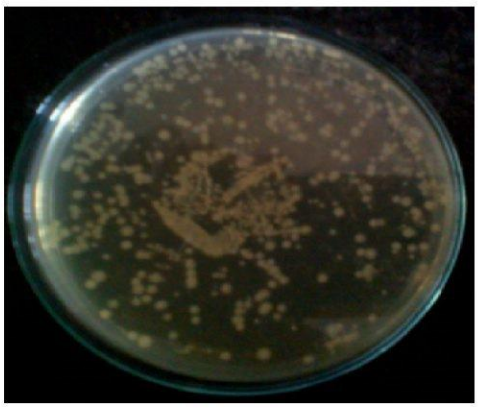

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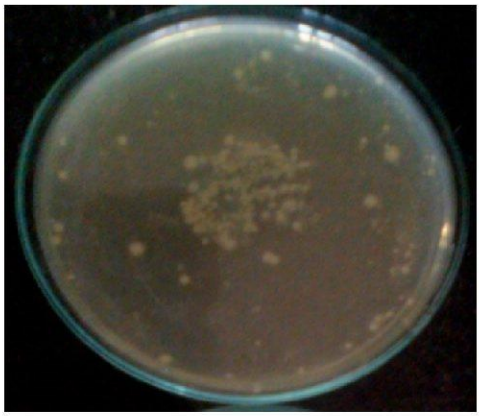

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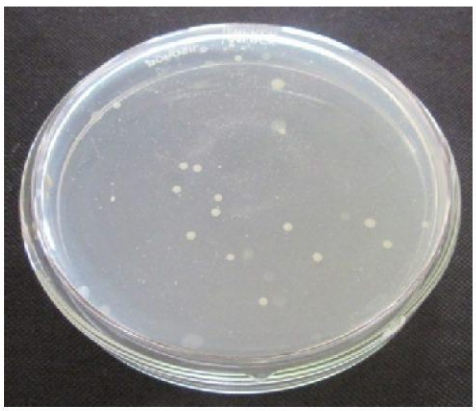

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Plate 5: Influence of flavonoid fractions of *Trianthema portulacastrum* on *Klebsiella pneumoniae* at different time intervals

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

There are several literatures available for the bacterial time kill kinetics of antimicrobial compounds. Agyare *et al.,* in 2015stated that the ethanol leaf extract of *Pupalia lappacea* exhibited antibacterial and antifungal activities where the results of bacterial time kill kinetics indicated the reduction of initial viable cells with increasing concentration of the leaf extract leading to microbial destruction.

Hasan *et al.,* in 2015 reported that the killing kinetic assay of crude and methanolic extract of *Zingiber officinale* at a concentration of 128µg/ml showed an antibacterial activity against *Streptococcus mutans*. The results showed more than 5-log CFU/ml decrease after 24 hour of incubation and showed reduction of 4-log CFU/ml.

A study by Ikhane *et al., in* 2014 has stated that the methanol extract of *Paullinia pinnata* exhibited bactericidal property against *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 4 hour and 8 hour in various ranges of 800mg/ml and 400mg/ml respectively.

In another study with the time kill curves of α-terpineol, *Escherichia coli* had directly entered decline phase and were killed by α-terpineol at 1x MIC in 8 hour and 2x MIC in 4 hour. Thus the rate of killing was increased by increasing the concentration of α-terpineol (Li *et al.,* 2014).

Rajamuthiah *et al.,* in 2015 has studied the activity of niclosamide and oxyclozanide at a concentration of 4xMIC against *Methicillin-resistant Staphylococcus aureus (MW2) w*here oxyclozanide was able to kill MRSA completely during a 4 hour of exposure period and niclosamide only caused a six fold drop in Colony Forming Units (CFU) indicating that niclosamide is bacteriostatic whereas oxyclozanide is bactericidal against *staphylococcus aureus.*

Our study has revealed that the flavonoid fractions of leaves of *Trianthema portulacastrum* has more potential than the methanolic extract.

4.3 HEMOLYSIS ACTIVITY

Haemoglobin is a component of the RBC which forms usually at the later stages of erythropoiesis (RBCs formation). Hemolytic activity is a requirement to be tested because the plant possessing potent antimicrobial activity may not be useful in pharmacological preparations, if they possess hemolytic effect.

Hemolytic activity was performed for the methanolic extract and flavonoid fractions of *Trianthema portulacastrum* with the concentration of 125mg and 100mg respectively against goat red blood cells. The total hemolysis (%) was obtained using 0.1% Triton X-100.The results of hemolytic activity are presented in Table 1.

The results of the assay described that the methanolic extract and flavonoid fractions were found to lyse the red blood cells to 7% and 15% respectively. Among these fractions, flavonoid fractions exhibited lower hemolytic activity, indicating its significant role in drug formulation.

Table 1: Hemolytic activity of methanolic extract and flavonoid fractions of *Trianthema portulacastrum*

Sample	Concentration (mg)	Percentage of Hemolysis (%)
Methanolic extract	100	15
Flavonoid fractions	125	7

Many literatures are available regarding the Hemolytic activities

Aktar *et al* in 2011 stated that the hemolytic activity of crude methanol extract and its aqueous soluble fractions of both bark and seed of *Entada phaseoloides (L)* at 2.0mg/ml

significantly protected the lysis of mice RBC membrane induced by hypotonic solution as compared to the standard acetyl salicylic acid (0.10 mg/ml). Both these extracts exhibited 78.89% and 65.52% inhibition of hemolysis of red blood cells as compared to 84.44% standard acetyl salicylic acid.

In a study by Gupta and Caphalkar in 2015 on hemolytic activity of aqueous extract of leaves of *Jasminum auriculatum,* the resultsshowed higher activity at higher doses (30 mg/ml) as compared to control.

Another study by Kumar e*t al.,* in 2013 reported the very low hemolytic effect of aqueous extract of *Aerva lanata* stem against normal human erythrocytes suggesting the less/no toxicity of the plant on human erythrocytes.

A study on the *in vitro* hemolytic activity of 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 highest activity (Shabbir e*t al.,* 2013).

The results of hemolytic activity of methanolic extract and flavonoid fractions clearly demonstrates the non-cytotoxic property of the leaves of *Trianthema portulacastrum*.

4.4 UV ABSORPTION METHOD

Nucleic acids and proteins absorb UV light at a wavelength of 260nm and 280nm respectively. The presence of these materials in a bacterial suspension may be used as an indicator of damage of the cell membrane which in turn caused leakage of the intracellular materials into the surrounding and finally it might cause cell death.

The leakage of proteins and nucleic acids for methanolic extract and flavonoid fractions were checked using Spectrophotometer at 260nm and 280nm and was monitored over a period of 60 minutes. At regular interval time period 0,15,30,45 and 60 minutes the reading has been taken. The absorbance of 260nm (nucleic acid materials) and 280nm (proteins) was increased. The results suggest that phytochemical fractions alter the bacterial cell membrane resulting in the leakage of the nucleic acids and proteins.

The results are presented in Table 2 and 3. With the increase in time, there was a maximum leakage of proteins and nucleic acids from the microorganisms treated flavonoid fractions than with methanolic extract. Among the two microorganisms, *Staphylococcus aureus* was found to be more susceptible than *Klebsiella pneumoniae.*

Several literatures are available for the leakage of nucleic acid and protein due to the action of plant extracts. Karsha and Lakshmi (2010) revealed that the treatment of *Staphylococcus aureus* cells with spice extracts and essential oils induce the leakage of absorbing material over a period of 90 minutes. They suggested that the membrane permeability resulted with the leakage of the UV_{260} and UV_{280} absorbing material which might cause cell death.

In a study by Vani and Lakshmi in 2014, the membrane leakage of cellular constituents having absorption at 260nm and 280nm were observed over a period of 90 minutes in the presence of spice extracts and essential oils against *Staphylococcus aureus.* This revealed that the *Syzygium aromaticum* and *Cinnamomum zeylanicum* induced leakage rapidly than *Piper nigrum.*

Table 2: Determination of leakage of nucleic acids and proteins at UV_{260} and UV_{280} nm from *Staphylococcus aureus*

	Wavelength (nm)	Absorbance				
		0 mins	15 mins	30 mins	45 mins	60 mins
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
Flavanoid fractions	260	0.014	0.025	0.028	0.032	0.034
	280	0.046	0.096	0.121	0.132	0.148

Table 3: Determination of leakage of nucleic acids and proteins at UV_{260} and UV_{280} nm from *Klebsiella pneumoniae*

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

4.5 MEMBRANE PERMEABILITY ASSAY

Cell membrane acts as a barrier for transporting molecules in and out of the cell. A damage to the membrane leads to the leakage of intracellular substances which is essential for their cell survival.

The leakage of intracellular components from the bacterial cell membrane leads to the release of protein and nucleic acid from the membrane which is analysed by UV absorption method. In order to quantify the amount of protein and sugar released from the bacterial cell envelope and to compare the membrane permeability potential of the phytochemical fractions, this assay was carried out. The results are shown in Figures 3 and 4.

The culture of *Staphylococcus aureus* and *Klebsiella pneumoniae* were incubated in Mueller Hinton broth with appropriate concentration of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* for overnight in a shaking incubator at 37°C. Then the

overnight samples were centrifuged and the supernatant were used to estimate the leakage of cellular substances such as sugar and protein.

Membrane permeability was performed by treating the culture of *Staphylococcus aureus* and *Klebsiella pneumoniae* with the methanolic extract and flavonoid fractions of *Trianthema portulacastrum*. The supernatant obtained, was used to estimate the leakage of sugar. The level of reducing sugar was increased in both methanolic extract and flavonoid fractions which indicated the leakage of cell membrane permeability. The level of reducing sugar was found to be greater than the control value. Hence, both the culture of *Staphylococcus aureus* and *Klebsiella pneumoniae* were more susceptible to the methanolic extract and flavonoid fractions damaging the membrane, leading to leakage of reducing sugar (cellular substances).

Similarly, the supernatant obtained from the treated culture of *Staphylococcus aureus* and *Klebsiella pneumoniae* with the methanolic extract and flavonoid fractions of *Trianthema portulacastrum* were taken for the estimation of leaked protein. The level of protein increased in both these fractions showed the leakage of cell membrane integrity. The level of protein was found to be higher than the control value. Hence, both the organisms are susceptible to methanolic extract and flavonoid fractions which results in the leakage of protein.

This explains that the flavonoid fractions of the *Trianthema portulacastrum* has the ability to the damage bacterial cell membrane. Among the two tested pathogens, *Staphylococcus aureus* was found to be susceptible when compared to *Klebsiella pneumoniae.*

There are several literatures available to support this study are as follows

Anandhi *et al.,* in 2014 stated that the phytocompounds were treated on the bacterial culture such as *E.coli, Staphylococcus aureus and Klebsiella pneumoniae* and the results predicted the leakage of the cell membrane of pathogens. Thus, the concentration of the reducing sugar and protein estimated were higher in glycosides and flavonoids compounds of *Caesalpinia Coriaria* treated with *Staphylococcus aureus* cells

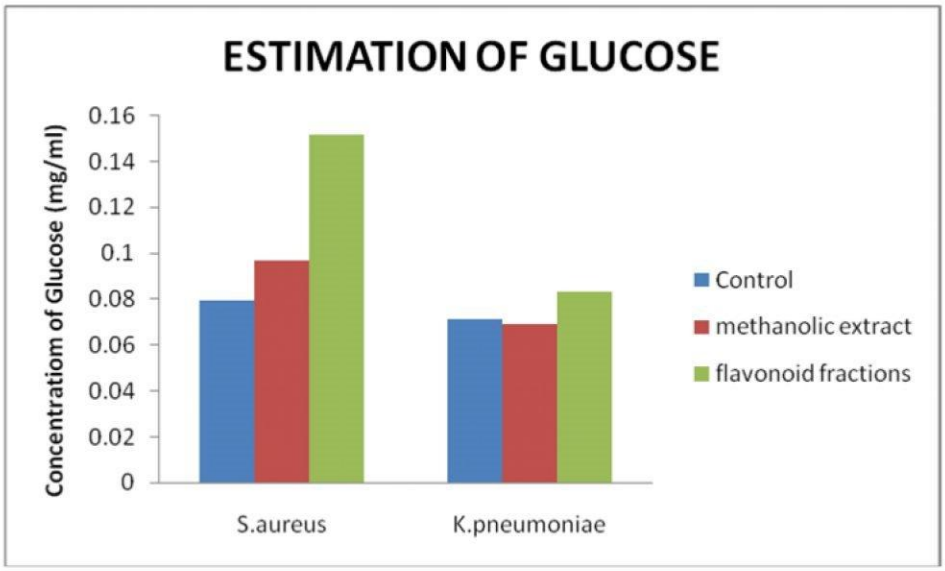


Figure 3: The leakage of reducing sugar by membrane permeability of methanolic extract and flavonoid fractions of *Trianthema portulacastrum*

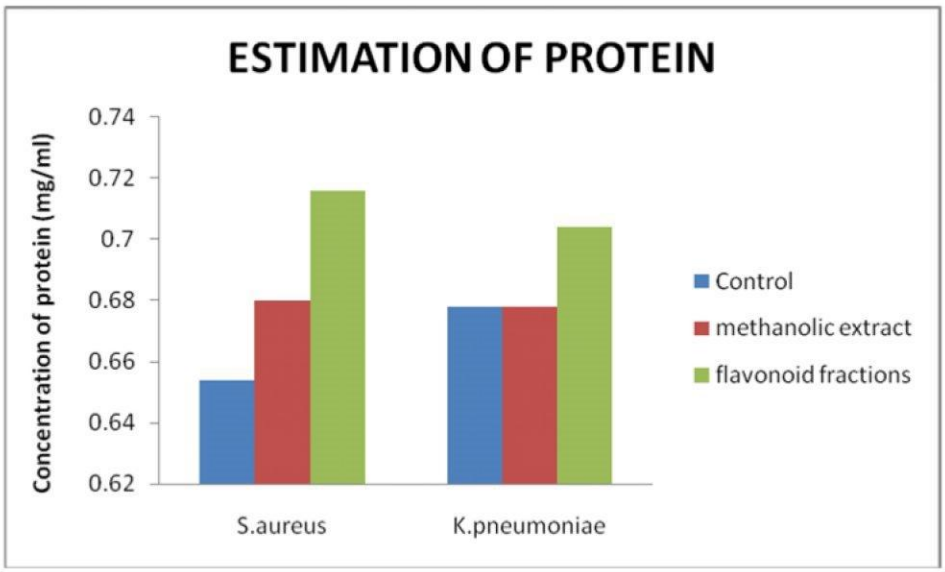


Figure 4: The leakage of protein by membrane permeability of methanolic extract and flavonoid fractions of *Trianthema portulacastrum*

In this study by Gurunathan in 2014, the bacterial cells (*Escherichia fergusonii*and *Streptococcus mutans*) were treated with silver nanoparticle alone or in combination with antibiotics for 12 hour 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. These results revealed that the higher amount of protein was leaked through Gram –negative bacterial membranes as compared to Gram- positive bacterial membrane.

The results of membrane permeability assay clearly demonstrate that the methanolic extract and flavonoid fraction of *Trianthema portulacastrum* causes an extensive damage to the membrane leading to its antibacterial potential.

4.6 DNA BINDING ASSAY

DNA is important to the replication of all living forms including bacteria. DNA damage interferes with DNA replication and transcription, and thereby induces mutations, chromosomal aberrations, cellular senescence, and apoptosis. These interactions are essential to cellular metabolism and the survival of all organisms.

In order to clarify the molecular mechanism of action, we examined the DNA binding ability of the test compounds. The genomic DNA was isolated from both *Staphylococcus aureus* and *Klebsiella pneumoniae* and the purity was checked by UV absorption spectra at 260nm/280nm. The concentration of methanolic extract and flavonoid fractions were taken as 100mg and 125mg respectively. The DNA binding ability of the methanolic extract and flavonoid fractions of *Trianthema portulacastrum* was determined by the gel retardation method. The DNA from *Staphylococcus aureus* and *Klebsiella pneumoniae* were treated with the methanolic extract and flavonoid fractions of *Trianthema portulacastrum* for one hour at room temperature followed by the agarose gel electrophoresis. The results of DNA binding assay for *Staphylococcus aureus* are presented in the Table 4 and plate 6. These results are also confirmed by the Integral Density Values (IDV) obtained using a digital gel documentation software. The results of DNA binding assay for *Klebsiella pneumoniae* were shown in the Table 5 and plate 7.

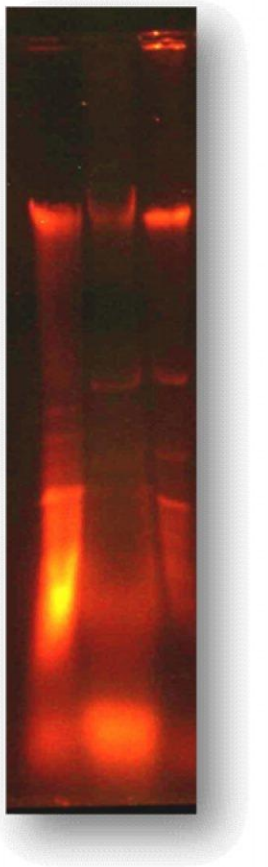
Table 4: Effect of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* on genomic DNA of *Staphylococcus aureus*

Sample	IDV of the bands
Genomic DNA of *Staphylococcus aureus*	260000
Genomic DNA of *Staphylococcus aureus* with methanolic extract	44254
Genomic DNA of *Staphylococcus aureus* with flavonoid fractions	26372

The results of electrophoretic mobility indicated that there is a significant damage in methanolic extract and flavonoid fractions treated DNA of *Staphylococcus aureus*. The genomic DNA of *Staphylococcus aureus was* found to be intact while there is a significant retardation on the agarose gel. Also methanolic extract and flavonoid fractions have the DNA binding ability which is indicated by the damaged DNA in the agarose gel. These results are also confirmed by the Intergral Density Values (IDV) obtained by the gel documentation system.

On comparison of the ID values of the *Staphylococcus aureus* DNA and the flavonoid fractions treated *Staphylococcus 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.

1 2 3



- Lane 1 : Genomic DNA of *Staphylococcus aureus*
- Lane 2 : Genomic DNA with methanolic extract
- Lane 3 : Genomic DNA with flavonoid fractions

Plate 6: Effect of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* on DNA of *Staphylococcus aureus*

Table 5: Effect of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* on genomic DNA of *Klebsiella pneumoniae*

Sample	IDV of the bands
Genomic DNA of *Klebsiella pneumoniae*	1435523
Genomic DNA of *Klebsiella pneumoniae* with methanolic extract	10563
Genomic DNA of *Klebsiella pneumoniae* with flavonoid fractions	2378

The ID values of the flavonoid fractions treated DNA is much lesser than the control genomic DNA. This indicates the intensity of the damage occurred on the genomic DNA by the antibacterial compounds.

From the results and the calculated IDV value, it was clear that the flavonoid fractions were found to have more DNA binding capacity than the methanolic extract.

There are several reports available to prove the DNA binding ability

It was observed that replacing one or more of the electron donating groups such as hydroxyl or furan ring results in an increase in the DNA cleavage affinity (Al-Omair *et al.,* 2015).

In a study by Park *et al.,* in 1998 reported that the DNA or RNA binding abilities of buforin II and magainin 2 were analyzed by DNA or RNA bands at various weight ratios of peptides on Agarose gel (1%). This result indicates that buforin II binds to DNA and RNA atleast over 20 times tightly than magainin 2.

Therefore DNA binding assay as indicated by the agarose gel and the IDV values confirmed that the leaves of *Trianthema portulacastrum* and flavonoid fractions have the good

DNA binding ability, for them to become a better antibacterial agents resulting in its antibacterial activity.

- Lane 1 : Genomic DNA of *Klebsiella pneumoniae*
- Lane 2 : Genomic DNA with methanol fractions
- Lane 3 : Genomic DNA with flavonoid fractions

Plate 7: Effect of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* on DNA of *Klebsiella pneumoniae*

4.7 POST ANTIBIOTIC EFFECT

Post antibiotic effect (PAE) is defined as persistent suppression of bacterial growth after a brief exposure (1 or 2h) of bacteria to an antibacterial agent even in the absence of host defence mechanism. In PAE, inhibition of bacterial growth is seen when either the antibacterial agent is no longer present in bacterial medium or if present; its concentration is well below the MIC (Sharma *et al.,* 2002).

Post antibiotic effect was performed for methanolic extract and flavonoid fractions of *Trianthema portulacastrum* against *Staphylococcus aureus.* The results are depicted in Figure 5, Plates 8 and 9. The concentration of flavonoid and methanol fractions was found to be 125mg and 100mg respectively. At 2 hour, the cells were found to be rapid bactericidal activities.

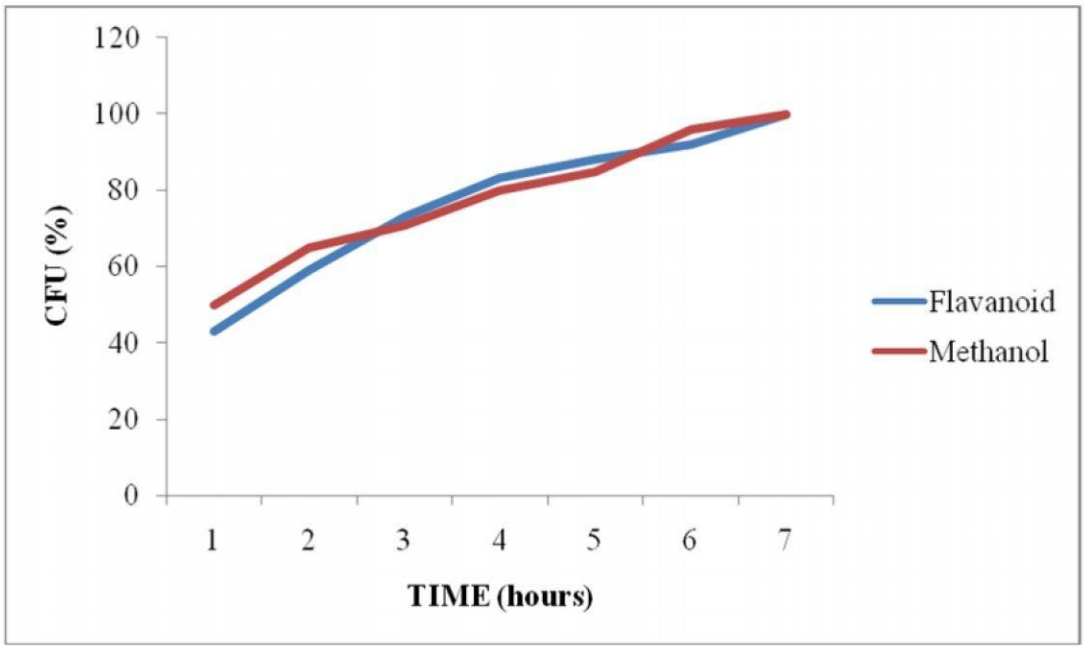
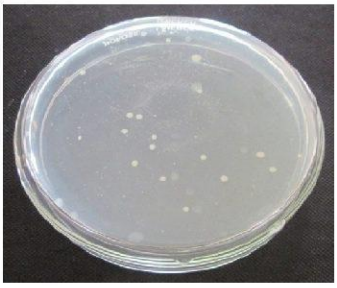
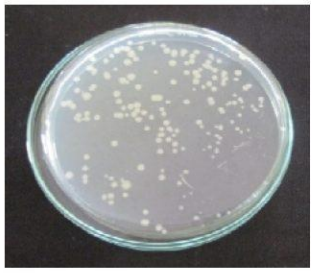


Figure 5: Post Antibiotic Effect Methanolic extract and Flavonoid fractions of *Trianthema portulacastrum* against *Staphylococcus aureus*



1 hour



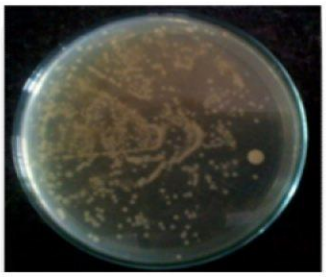
2 hours

3 hours

4 hours

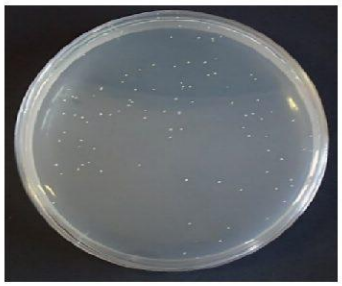
5 hour s

6 hours



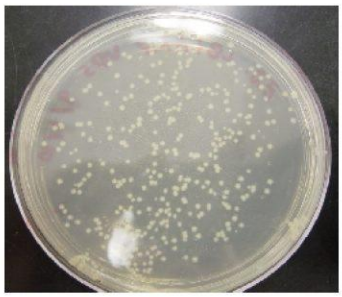
7 hours

Plate 8: Influence of methanolic extract of *Trianthema portulacastrum* on *Staphylococcus aureus* at different time intervals

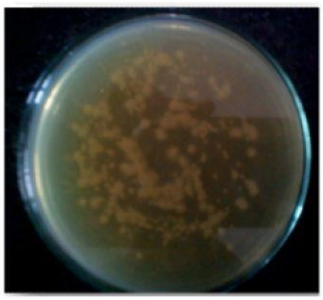


1 hour

2 hours



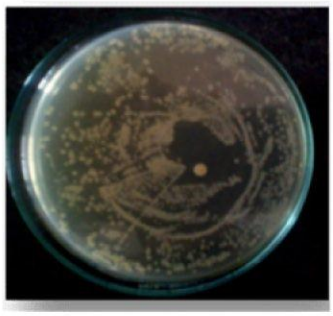
3 hour s



4 hours

5 hours

6 hours



7 hours

Plate 9: Influence of flavonoid fractions of *Trianthema portulacastrum* on *Staphylococcus aureus* at different time intervals

The post antibiotic effect was measured by incubating *Staphylococcus aureus* with methanolic extract and flavonoid fractions for 2 hours, thereafter the bacteria were washed to remove the fractions and the re-growth of the bacteria was analysed at every one hour. The results of this assay indicated that both the methanolic extract and flavonoid fractions showed a good post antibiotic effect with the selected pathogens.

There are several reports in the literature indicating the post antibiotic effects of antimicrobial compounds. In this study by Ahmad *et al.,* in 2015 had reported the post antibiotic effect of cefquinome against *Staphylococcus aureus* at different concentrations of 1X, 2X, 4X MIC for upto 2 hours.

Haste *et al.,* in 2011 revealed that 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.

Hensler e*t al., in* 2014 has studied the activity of anthracimycin against contemporary methicillin-resistant *Staphylococcus aureus*. The compound exhibited minimal post antibiotic effects against USA300 MRSA (methicillin-resistant *Staphylococcus aureus)*, with regrowth rapidly after removal of the compound within 3 hours at five times its MIC.

Another study by Li *et al.,* in 2001 carried out the post antibiotic effect of colistin and colistin methanesulfonate on three strains exhibited rapid bactericidal activity of 2 to 3 hours at 16 times of MIC after 15 minutes of exposure.

Both the methanolic extract and flavonoid fractions produced a significant post antibiotic effect of 2 hours against *Staphylococcus aureus*.

4.8 SCANNING ELECTRON MICROSCOPY (SEM)

Electron microscopy was used to investigate the mechanism of action of *Trianthema portulacastrum* fractions in pathogenic microorganisms. Scanning electron microscopy (SEM) was used to observe membrane damage and the morphological changes in bacteria *Staphylococcus aureus*.

Among the two tested pathogens, *Staphylococcus aureus* was found to be more susceptible when compared with *Klebsiella pneumoniae.* Hence *Staphylococcus aureus* was taken to analyse the surface morphology by Scanning Electron Microscopy.

The SEM images represent the methanolic extract and flavonoid fractions treated *Staphylococcus aureus* cells as shown in plate 9 and 10. 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.

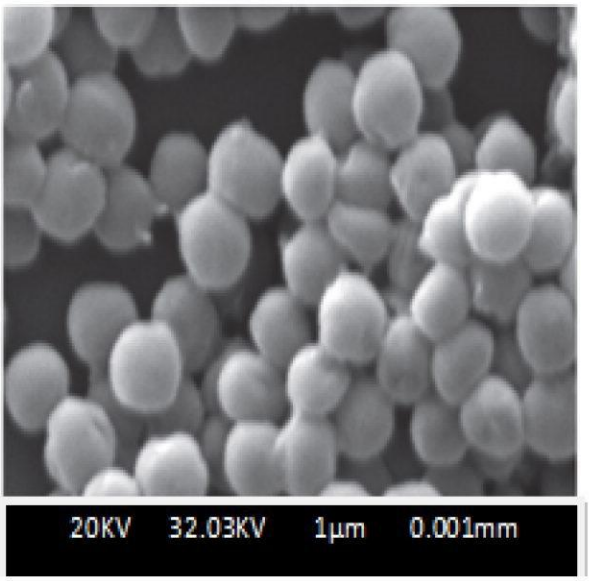
Using Scanning electron microscopy, the membrane damage was clearly seen in *Staphylococcus aureus* treated methanolic extract and flavonoid fractions of the leaves of *Trianthema portulacastrum*.

Several studies using Scanning electron microscopy has been carried out to analyse the morphology changes in bacterial cells.

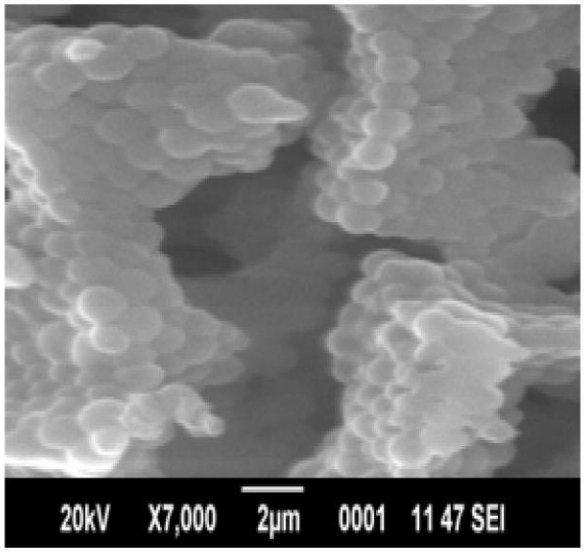
Tyagi *et al*., (2015) stated that the Scanning electron microscopy of 25µM, 50µM and 100µM curcumin I treated *Staphylococcus aureus* cells have indicated the distortion in the shape of the cells, with depression on the surface of the cells.

Erika *et al*., (2015) reported that the essential oil from *Origanum vulgaris* Linna and reference drugs such as chloramphenicol, triclosan and neomycin proved the membrane damage when treated with tested bacteria including *Micrococcus luteus, Proteus vulgaris, Staphylococcus aureus* and*Corynebacterium xerosis* where considerable morphological changes were observed when compared to the control group.

Li *et al.,* (2015) stated that the untreated *Escherichia coli* and *Staphylococcus aureus* were rod-shaped and round shaped, respectively, both with smooth and intact cell walls. After exposure of Catechin-Cu nanoparticles for 3h, the quantity of *Staphylococcus aureus* greatly decreased and cell walls became wrinkled and damaged. Similarly, in *Escherichia coli* cells, the shape and size of cells also damaged dramatically and there were a lot of materials attached on the bacterial surface.

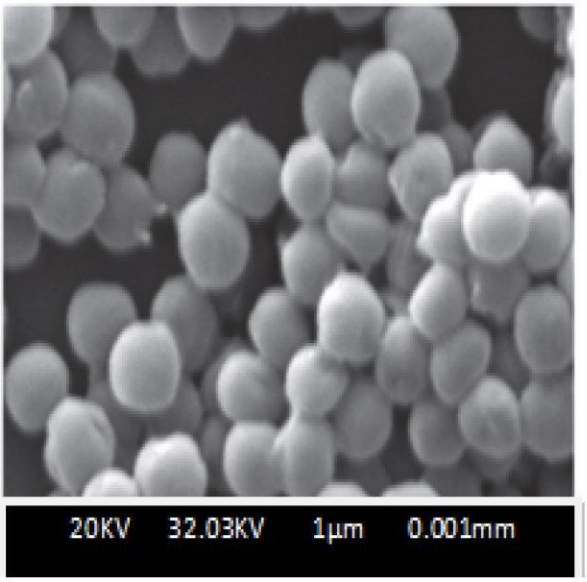


(A) *Staphylococcus aureus* cells

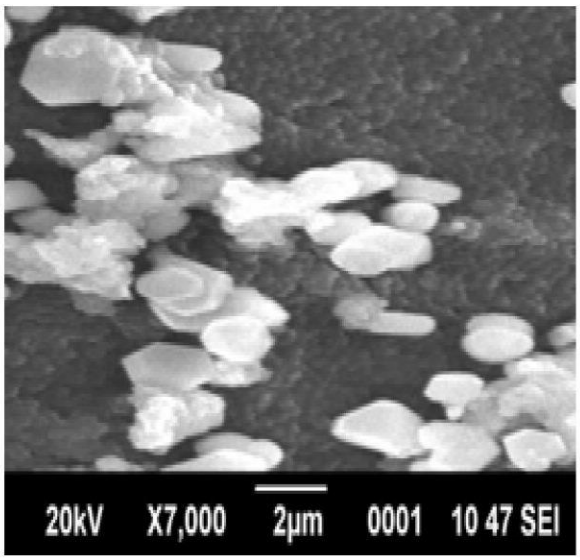


(B) Methanolic extract treated *Staphylococcus aureus*

Plate 10: Scanning electron microscopic images of *Staphylococcus aureus* with treated methanolic extract



(A) *Staphylococcus aureus* cells



(B) Flavonoid fractions treated *Staphylococcus aureus*

Plate 10: Scanning electron microscopic images of *Staphylococcus aureus* treated with flavonoid fractions

The results of scanning electron microscopy analysis clearly demonstrate that the methanolic extract and flavonoid fractions are able to damage the cell membrane of the studied bacteria.

From the above observations, it has been proved the mechanism of the action of methanolic extract and flavonoid fractions of *Trianthema portulacastrum* leaves against bacterial pathogens. The study on bacterial time kill kinetics gave a better knowledge about the bacteriostatic property of the phytochemical fractions. The hemolytic study of the phytochemical fractions concludes that flavonoid fractions does not cause the hemolysis. The determination of leakage of nucleic acids and proteins by UV absorption method has showed the maximum leakage towards the flavonoid fractions, ultimately resulting in the release of intracellular components of the bacterial cell membrane. The mode of action on membrane permeability assay explained that these phytochemicals fractions cause membrane damage leading to the leakage of protein and sugars. The DNA binding capacity of the phytochemical fractions shows that flavonoid fractions binds to the DNA of the microorganism and degrade them which finally caused their death. The Post antibiotic effect clearly proved that both the methanolic extract and flavonoid fractions produced a significant post antibiotic effect of 2 hours against *Staphylococcus aureus*. Scanning Electron Microscopy proved that the methanolic extract and flavonoid fractions are able to damage the cell membrane of the studied bacteria.