

## Extracts of *Adhatoda vasica* Nees. and vasicine inhibits biofilm formation of *Klebsiella pneumoniae*

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**Abstract:** *Adhatoda vasica* Nees. are used traditionally in Tripura to cure pneumonia, cough and cold. Pneumonia is mainly caused by bacteria like *Klebsiella pneumoniae*. Moreover, many of these pathogenic strains are showing acute resistance for many commercially available antibiotics due to its biofilm formation ability. The present study evaluates the antimicrobial activity of *Adhatoda vasica* Nees. extracts against the biofilms of *Klebsiella pneumoniae* and their free-living forms. After extraction and phytochemical screening of *Adhatoda vasica* Nees, the presence of vasicine in extracts was ascertained by thin layer chromatography. The antimicrobial activity for all the extracts and commercially available vasicine against planktonic forms of bacteria were determined using the agar well diffusion method with tetracycline as control. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values were evaluated by a macrobroth dilution technique. The anti-biofilm effects were assessed by microtitre plate method. The cell viability assay was assessed by EtBr/AO staining. Results shows that the *A. vasica* Nees. leaf extract have remarkable zone of inhibition for *Klebsiella pneumoniae* as compared to standard vasicine and tetracycline. Similarly, the MIC values confirmed its outstanding ability for inhibition of planktonic as well as biofilm form of the bacteria, and same is also been visualized and established further by cell viability studies and SEM analysis for the biofilm of *Klebsiella pneumoniae*.

**Key words:** *A. vasica* Nees, Vasicine, Biofilm, *Klebsiella pneumoniae*

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Date of Submission: 28-02-2018

Date of acceptance: 15-03-2018

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### I. Introduction

Tripura is a small hilly state of North-east India with a rich source of flora and fauna. The tropical climate of Tripura supports the growth of many medicinal plants and forest resources starting from the hills to the plains. Even today, most of the tribal population extract their livelihood from forests and depend on traditional herbal treatment practices. Many medicinal plants are used by the local practitioners to combat infectious disease like pneumonia. Herbal medicine is based on the premise that plants contain natural bioactive substances that can promote health and alleviate illness. *Adhatoda vasica* Nees. (Family- Acanthaceae) is a perennial, evergreen shrub found widely throughout the tropical regions of Southeast Asia. The name *J. adhatoda* (L.) Nees and *Adhatoda zeylanica* Medic are used synonymously. It is commonly known as Vasaka in Tripura. The plant is highly branched shrub (1.0 m to 2.5 mm height) with unpleasant smell and bitter taste. It has opposite ascending branches with white, pink or purple flowers (Dhankar et al., 2011). It is used in Ayurvedic medicinal plant to treat cold, cough, asthma and tuberculosis (Sharma et al., 1992). The main action is expectorant and antispasmodic (bronchodilator) (Karthikeyan et al., 2009). Moreover, the importance of Vasaka plant in the treatment of respiratory disorders can be understood from the ancient Indian saying, "No man suffering from phthisis need despair as long as the Vasaka plant exists" (Dymock et al., 1893). Thus the frequent use of *Adhatoda vasica* Nees. has resulted in its inclusion in the WHO manual "The Use of Traditional Medicine in Primary Health Care" which is intended for health workers in South-east Asia to keep them informed of the restorative utility of their surrounding flora (WHO fact sheets., 1990). The major alkaloids of the plant found to be biologically active are vasicine and vasicinone (Dhankar et al., 2011).



**Figure: 1. A.** *Adhatoda vasica* Nees. plant. **B.** Vasicine (Sigma)

The anti-inflammatory and antimicrobial properties of pyrroloquinazoline alkaloids from *Adhatoda vasica* Nees. have been reported by Singh, 2013 and Sheeba, 2012 (Singh et al., 2015; Sheeba et al., 2012). The antimicrobial property of leaf extracts of *J. adhatoda* (L.) in comparison with vasicine was shown by Rashmi Pa in 2012 (Rashmi et al., 2012). The leaves, flowers and roots of this plant used in herbal drugs against tubercular activities (Barry et al., 1955), cancer (Pandey., 2002) and possessed to have anti-helminthic properties (Ayyanar et al., 2008).

In many cases, pneumonia is treated at home. It often clears up in 2 to 3 weeks. But older adults, babies, and immune compromised patients may develop serious complications. It can be a community-associated pneumonia or healthcare-associated pneumonia. Pneumonia has bacterial, viral, fungal, and other primary causes. Amongst the pathogenic bacteria, *Klebsiella pneumoniae* is the causative agents of bacterial pneumonia and the leading cause of infant mortality globally (WHO fact sheets., 2013) due to acute resistant to many commercially available antibiotics and the formation of stringent biofilms. A number of intervention strategies are under-trial against infection associated pneumonic bacterial biofilms, most of the strategies are disadvantageous in terms of their impact on host systems. Application of phytochemicals independently or in combination with existing therapeutics might offer an important implication in this regard. However, it will be interesting to evaluate the antibacterial/ antibiofilm properties of *Adhatoda vasica* Nees. to validate scientifically its traditional usage against pneumonia.

## II. Materials And Methods

### 2.1 Preparation of standard vasicine

Vasicine (10 mg) was procured from Sigma Aldrich. A solution of vasicine (5mg/ml) was prepared using methanol as solvent.

### 2.2 Plant sample collection and identification

The fresh leaves of *Adhatoda vasica* Nees. were collected from Sepahijala Botanical Garden (23°39'52''N & 91°18'42''E). The plants are identified with the help of Department of Botany, Tripura University, India.

### 2.3 Culture and maintenance of test microorganisms for antimicrobial studies

Strain of *Klebsiella pneumoniae* MTCC 3384 was bought from IMTECH, Chandigarh. The strain was revived in Luria Bertanni broth (Hi- media) and stored as 50 % glycerol stock at -80°C refrigerator.

### 2.4 Processing of plant materials and extraction

Fresh leaves of plant *Adhatoda vasica* Nees. were collected and washed thoroughly 2-3 times with running water and once with sterile distilled water. Then the leaves were shade dried and placed in hot air oven at a temperature of 50°C for 4 - 5 days till the weight became constant. Plant materials were regularly examined to check any fungal growth or rotting. The dried leaves were powdered to obtain a very fine particle size using mechanical mixer grinder.

The dried powder of leaves (125g) was soaked successively in petroleum ether, ethanol, methanol and water in the sample: solvent ratio of 1: 10 according to their polarity index. The mixture was subjected to intermittent shaking for 7 days. The extracts were filtered through Whatman filter paper no.1 and concentrated at 50° C using rotary evaporator (Ika, Japan). The concentrated extracts were stored in air tight container at 4° C refrigerator. For further experiments the extracts were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 1mg/ml (Malik et al., 2013).

## 2.5 Determination of percentage yield

The yield percentage of each crude extract was determined by percentage of weight of the dissolved extract after evaporation divided by the initial weight of the extract before addition of solvent.

$$\text{Yield percentage} = \frac{\text{Weight of the dissolved extract after evaporation}}{\text{Initial weight of the extract before addition of solvent}} \times 100$$

## 2.6 Biochemical characterisation of crude extracts

### 2.6.1 Detection of alkaloids

The presence of alkaloids in the extract was determined by Mayer's test, Dragendroff's test and Wagner's test (Joseph et al., 2013).

### 2.6.2 Detection of carbohydrates

The presence of carbohydrates was determined by Molisch's test, Benedict's test and Fehling's test (Joseph et al., 2013).

### 2.6.3 Detection of glycosides

The presence of cardiac glycosides was determined by Legal's test (Joseph et al., 2013).

### 2.6.4 Detection of saponins

The presence of saponins was determined by froth test and foam test (Joseph et al., 2013).

### 2.6.5 Detection of phytosterols

The presence of phytosterols was determined by Salkowski's test (Joseph et al., 2013).

### 2.6.6 Detection of phenols

The presence of phenols was determined by Ferric chloride test (Joseph et al., 2013).

### 2.6.7 Detection of tannins

The presence of tannins was determined by Lead acetate test and Gelatin tests (Joseph et al., 2013).

### 2.6.8 Detection of flavonoids

The presence of flavonoids was determined by alkaline reagent test and Lead acetate tests (Joseph et al., 2013).

### 2.6.9 Detection of proteins and amino acids

The presence of protein and amino acids was determined by Xanthoproteic test and Ninhydrin test. (Joseph et al., 2013)

## 2.7 Thin layer chromatography with commercial vasicine:

Thin layer chromatography has been done with the extracts and commercially available vasicine (Sigma-Aldrich) as standard to compare and see the presence of vasicine in the extracts. The petroleum ether extract, ethanolic extract, methanolic extract, water extract of leaves of *Adhatoda vasica* Nees. and standard vasicine were dissolved in chloroform and the mobile phase contained chloroform, methanol and ethyl acetate in the ratio 6:2:1 (Srivastava et al., 2001; Soni et al., 2008). The extracts were chromatographed on silica coated TLC plates (Sigma- Aldrich).

## 2.8 Biological evaluation:

### 2.8.1 Determination of antimicrobial activity

Agar well-diffusion method was done to determine the antimicrobial activity of the different extracts of *A. vasica* Nees leaves and commercially available vasicine (Sigma) using tetracycline as control (Sen et al., 2012). Luria Bertanni (LB) agar were swabbed (sterile cotton swabs) with 24 hour old - broth culture of *Klebsiella pneumoniae*. Wells were made in each of these plates using sterile cork borer. Stock solution of the plant extract was prepared at a concentration of 20 mg/ml viz. Petroleum Ether, Ethanol, Methanol, and Water. Vasicine (1mg/ml) and tetracycline (20mg/ml) were also prepared. About 100 µl of the plant extracts, vasicine and tetracycline were added into the wells and allowed to diffuse at room temperature for 2hrs. The plates were incubated at 37°C for 24 hours.

### 2.8.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assay was performed. The test strain *Klebsiella pneumoniae* MTCC 3384 were grown for overnight on Luria Bertanni Broth (Hi-media) respectively. 10 µl of the seed culture was inoculated into 5 ml of fresh respective broths in test tubes and incubated with shaking for 12 h. These broth cultures were diluted 1:500 in fresh broths. 20 µl of the diluted culture containing  $1.2 \times 10^5$  to  $2 \times 10^5$  CFU of each test strain was inoculated into 2 ml of broth containing the vasicine (Sigma Aldrich) and the extracts and incubated with shaking for 24 h. The concentration of the extracts for *Klebsiella pneumoniae* MTCC 3384 the extracts were taken in concentration of 0, 40, 80,120,160, 200 µg/ml. 100 µl of the each culture was then inoculated onto solid medium and incubated for 36

h. The incubation temperature will be 37°C, throughout the experiment. The MBC will be defined as that concentration at which no viable bacteria will be recovered (Provenzano et al., 2000). For each phytochemical-treated system Dimethyl sulphoxide (DMSO) was used as control.

### 2.8.3 Biofilm formation assay

The bacterial strains *Klebsiella pneumoniae* MTCC 3384 was grown over-night on Luria Bertanni agar plates at 37°C supplemented with 1 % dextrose. Colonies of each strain were resuspended in Luria Bertanni Broth (Hi-media) at OD<sub>600</sub> of 0.6. A 1:100 dilution of these suspensions were inoculated in fresh broth containing 1 % dextrose in 96-well polystyrene cell culture plates (Tarsons) for 3 hrs and then after 3 hrs extracts and vasicine (Sigma Aldrich) were added in the concentration of 0xMIC, 0.25xMIC, 0.50xMIC, 0.75xMIC and 1xMIC µg/ml and incubated for 24 h at 37°C. Subsequently, the respective media were removed carefully from the wells and the wells were rinsed twice with sterile distilled water. Then sterile distilled water was removed carefully from the wells and dried for 15 mins. Then the wells were filled with 1 % crystal violet stain ((Hi-media). After 5 min, the stain was removed from the wells and subsequently rinsed twice with sterile distilled water. The biofilm-associated crystal violet was resuspended with 99 % ethanol and the OD<sub>620</sub> of the resulting suspension was measured (Christensen et al., 1985) using Synergy H1 Hybrid reader.

### 2.8.4 Microscopic study of biofilm inhibition by the crude extract and compound

The bacterial strain *Klebsiella pneumoniae* MTCC 3384 was grown over-night on Luria Bertanni Broth (Hi-media) supplemented with 1 % dextrose respectively at 37°C. A 1:100 dilution of these suspensions were inoculated in fresh broth containing 1 % dextrose in test tubes (Borosil) of capacity 50 ml supplemented with glass coverslips (Borosil) for 3 hrs and then after 3 hrs extracts and vasicine (Sigma Aldrich) were added and incubated for 24 h at 37°C respectively. Subsequently, the respective media were removed carefully from the tubes and the tubes were rinsed twice with sterile distilled water. Then sterile distilled water was removed carefully from the tubes and dried for 15 mins. Then the tubes were filled with 5 ml of 1 % crystal violet stain ((Hi-media). Then the cover slips with biofilm associated crystal violet were taken out and examined under the compound microscope (Magnus) and photographed with digital camera (Allegruci et al., 2006).

### 2.8.5 Bacterial viability study of biofilm inhibition using EtBr/AO staining method

The bacterial strain *Klebsiella pneumoniae* MTCC 3384 was grown over-night on Luria Bertanni Broth (Hi-media) at 37°C. A 1:100 dilution of these suspensions were inoculated in fresh broth containing 1 % dextrose in glass petridishes (Borosil) supplemented with glass coverslips (Borosil) in presence and absence of the extracts and vasicine (Sigma Aldrich) and incubated for 24 h at 37°C respectively. Subsequently, the respective media were removed carefully from the plates and the plates were rinsed twice with sterile distilled water. Then sterile distilled water was removed carefully from the plates and dried for 15 mins. Then the cover slips with biofilm were taken out and 100 µl of Ethidium bromide/ Acridine orange (1:1) solution was added to the coverslips and observed under fluorescent microscope (Shin et al., 2015).

### 2.8.6 SEM analysis

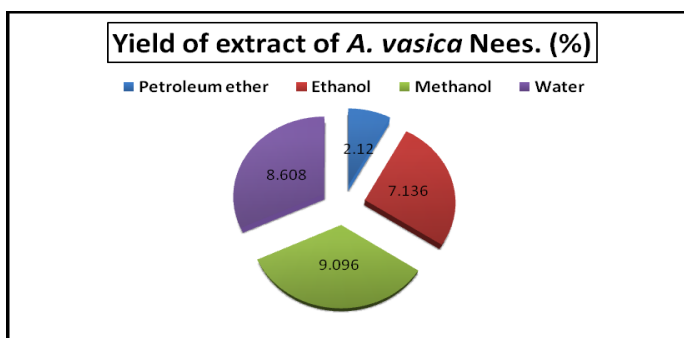
To evaluate the action of the compounds on the integrity of biofilm of the bacteria *K. pneumoniae* MTCC 3384, the morphological analysis of the bacteria was carried out with the antimicrobial compounds with treatment. The bacterial strain *Klebsiella pneumoniae* MTCC 3384 was grown over-night on Luria Bertanni Broth (Hi-media) at 37°C. A 1:100 dilution of these suspensions were inoculated in fresh broth containing 1 % dextrose in glass petridishes (Borosil) supplemented with glass coverslips (Borosil) in presence and absence of the extracts and vasicine (Sigma Aldrich) and incubated for 24 h at 37°C respectively. Subsequently, the respective media were removed carefully from the plates and the plates were rinsed twice with sterile distilled water. Then sterile distilled water was removed carefully from the plates and dried for 15 mins. The biofilms were fixed with 2.5% gluteraldehyde solution. After fixation for a minimum of 1 hr, samples are washed twice. The biofilms were then fixed by immersion in 1% osmium tetroxide in PBS for 30 min then, washed with the same buffer and dehydrated in a graded alcohol series of 50%, 70%, 95% and 100% ethanol consecutively for 5 min each. Then the cover slips were mounted on aluminum stubs by using double-sided carbon tape and coated with gold for scanning electron microscope (JEOL JSM –6390LV) in 15 kV accelerating voltage according to the method described by Kalchayanand, Dunne, and Ray (Kalchayanand et al., 2004).

## III. Results

In this investigation, we were mainly focussing on the effect of different extracts of fresh leaves of *Adhatoda vasica* Nees. on biofilm formation of *Klebsiella pneumoniae* MTCC 3384 in comparison to the commercially available vasicine (Sigma- Aldrich). So the fresh leaves were collected, identified and washed properly with tap water and then sterile distilled water. And then it was subjected to extraction in petroleum

ether, ethanol, methanol and water. After extraction, the percentage yield of the extracts was calculated and is shown in Table 1.

Solvent used	<i>A. vasica Nees.</i> (%)
Petroleum ether	2.12
Ethanol	7.136
Methanol	9.096
Water	8.608



**Table 1:** Showing yield percentage of the different extracts of *Adhatoda vasica Nees.*

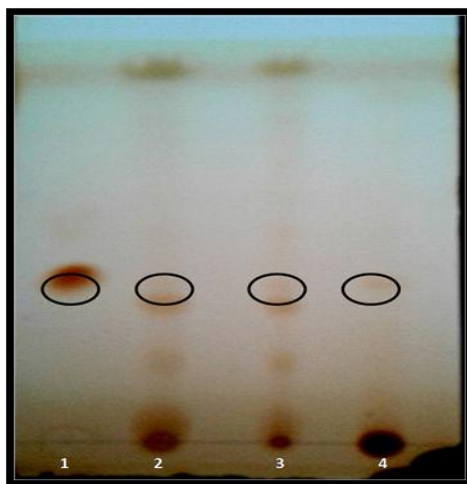
**Figure 2:** Showing yield percentage of the different extracts of *Adhatoda vasica Nees.*

The different biochemical tests of all the extracts of leaf of *A. vasica Nees.* were performed using the standard protocol to analyse the presence of phytochemicals in the different extracts. The results suggested that most of the phytochemicals were present in methanolic extract of the leaf of the plant. Total carbohydrates were found to be present in all the extracts but reducing sugar was present in petroleum ether and ethanolic extracts as compared to methanolic and water extracts. In case of alkaloids, it was seen that alkaloids were absent in only pet ether extract as compared to others. Cardiac glycosides were found to be present in only methanolic extract, saponins were absent in only ethanolic extract, phytosterols were present in all the extracts except water extract, tannins were present in all the extracts except ethanolic extract, whereas flavonoids were found to be present in all the extracts and proteins and amino acids were found to be present in only petroleum ether extracts. The results were shown in Table 2.

Phytochemicals	Tests	<i>Adhatoda vasica Nees.</i>			
		Petroleum ether	Ethanol	Methanol	Water
Carbohydrates	Molisch's	+	+	+	+
	Benedict's	+	+	-	-
	Fehling's	+	+	-	-
Alkaloids	Mayer's	-	+	+	+
	Dragendroff's	-	+	+	+
	Wagner's	-	-	+	+
Glycosides	Legal's	-	-	+	-
Saponins	Froth	+	-	+	+
	Foam	+	-	+	+
Phytosterols	Salkowski's	+	+	+	-
Phenols	Ferric chloride	-	+	+	+
Tannins	Lead acetate	+	-	+	+
	Gelatin	+	-	+	+
Flavonoids	Alkaline reagent	+	+	+	+
	Lead acetate	+	+	+	+
Proteins and aminoacids	Xanthoproteic	+	-	-	-
	Ninhydrin	+	-	-	-

**Table 2:** Phytochemical screening of the different extracts of *Adhatoda vasica Nees.*

Thin layer chromatography was carried out to verify the presence of vasicine in the extracts. The result showed that the Rf value of the spot obtained in the case of ethanolic, methanolic and water extracts were same as the spot obtained in standard vasicine (Rf ~ 0.70). The image of the TLC plate was shown in figure no.2



**Figure 3:** Thin layer chromatography plate showing the presence of vasicine in the different extracts of *Adhatoda vasica* Nees. 1. Standard vasicine (Sigma-Aldrich) 2. Ethanolic extract 3. Methanolic extract 4. Water extract.



Sl .no	Treatment	Zone of inhibition (mm)
1	Tetracycline	25 ± 1.154701
2	Petroleum ether extract	19.67 ± 0.666667
3	Ethanolic extract	21.67 ± 1.452966
4	Methanolic extract	25.33 ± 0.881917
5	Water extract	22.91 ± 0.948004
6	Vasicine	25.17 ± 0.669162

**Figure 4:** Antimicrobial activity of different extracts of *Adhatoda vasica* Nees. and standard vasicine in comparison with tetracycline as control against *Klebsiella pneumoniae*.

**Table 3:** Showing average zone of inhibition of the different extracts of *Adhatoda vasica* leaves, tetracycline as control and commercial vasicine against *K. pneumoniae*

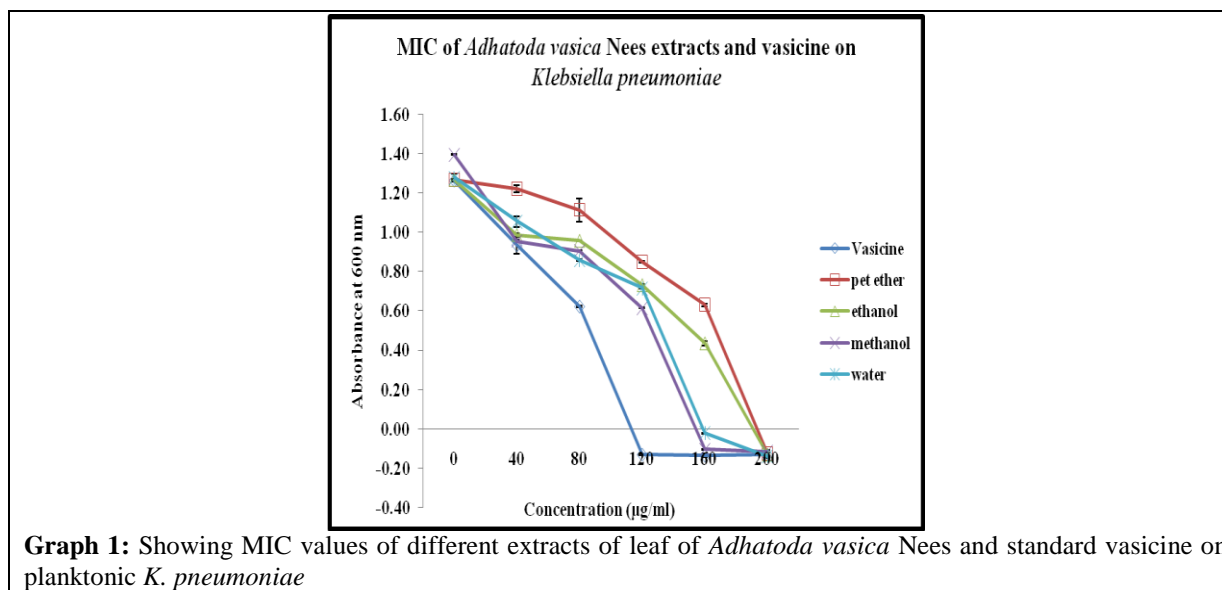
The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined for all the extracts of *Adhatoda vasica* leaves and vasicine respectively. For *Klebsiella pneumoniae* MTCC 3384, the MIC values of vasicine, petroleum ether extract, ethanol extract, methanol extract and water extract were 120 µg/ml, 160 µg/ml, 160 µg/ml, 160 µg/ml and 160 µg/ml respectively and the MBC values of vasicine, petroleum ether extract, ethanol extract, methanol extract and water extract were 160 µg/ml, 200 µg/ml, 200 µg/ml, 200 µg/ml and 200 µg/ml respectively. The values were tested statistically by Pearson Correlation using SPSS.16 Software. The values of some extracts were found significant (\*) as compared to the vasicine at 0.05 level of significance having p values ≤ 0.05 and some were found highly significant (\*\*) at 0.01 level of significance with p values ≤ 0.01.

Correlations						
		Vasicine	Petroleum ether	Ethanol	Methanol	Water
Vasicine	Pearson Correlation	1	0.769	0.822*	0.892*	0.860*
	Sig. (2-tailed)		0.074	0.045	0.017	0.028
Petroleum ether	Pearson Correlation	0.769	1	0.984**	0.873*	0.909*
	Sig. (2-tailed)	0.074		0.000	0.023	0.012
Ethanol	Pearson Correlation	0.822*	0.984**	1	0.933**	0.948**
	Sig. (2-tailed)	0.045	0.000		0.007	0.004

*Extracts of Adhatoda vasica Nees. and vasicine inhibits biofilm formation of Klebsiella pneumoniae*

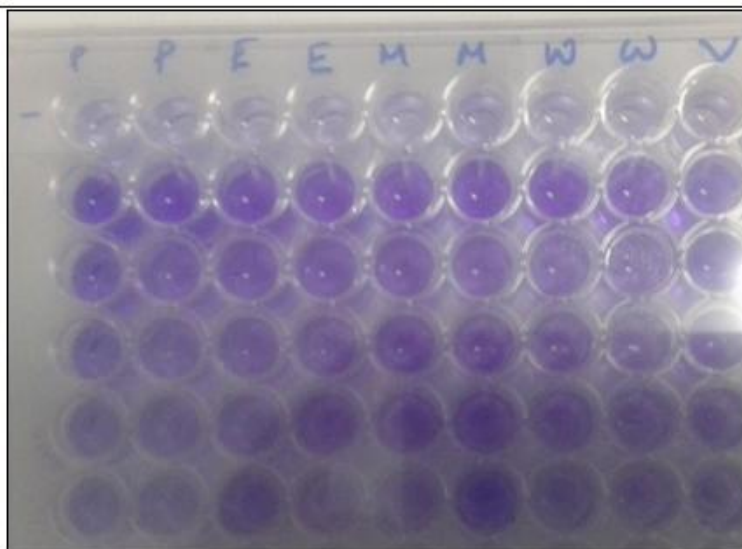
Methanol	Pearson Correlation	0.892*	0.873*	0.933**	1	0.989**
	Sig. (2-tailed)	0.017	0.023	0.007		0.000
Water	Pearson Correlation	0.860*	0.909*	0.948**	0.989**	1
	Sig. (2-tailed)	0.028	0.012	0.004	0.000	
*. Correlation is significant at the 0.05 level (2-tailed).						
**. Correlation is significant at the 0.01 level (2-tailed).						
N= 6						

**Table 5:** Showing Pearson correlation between the different extracts of *Adhatoda vasica* Nees and standard vasicine against *Klebsiella pneumoniae* using SPSS 16.

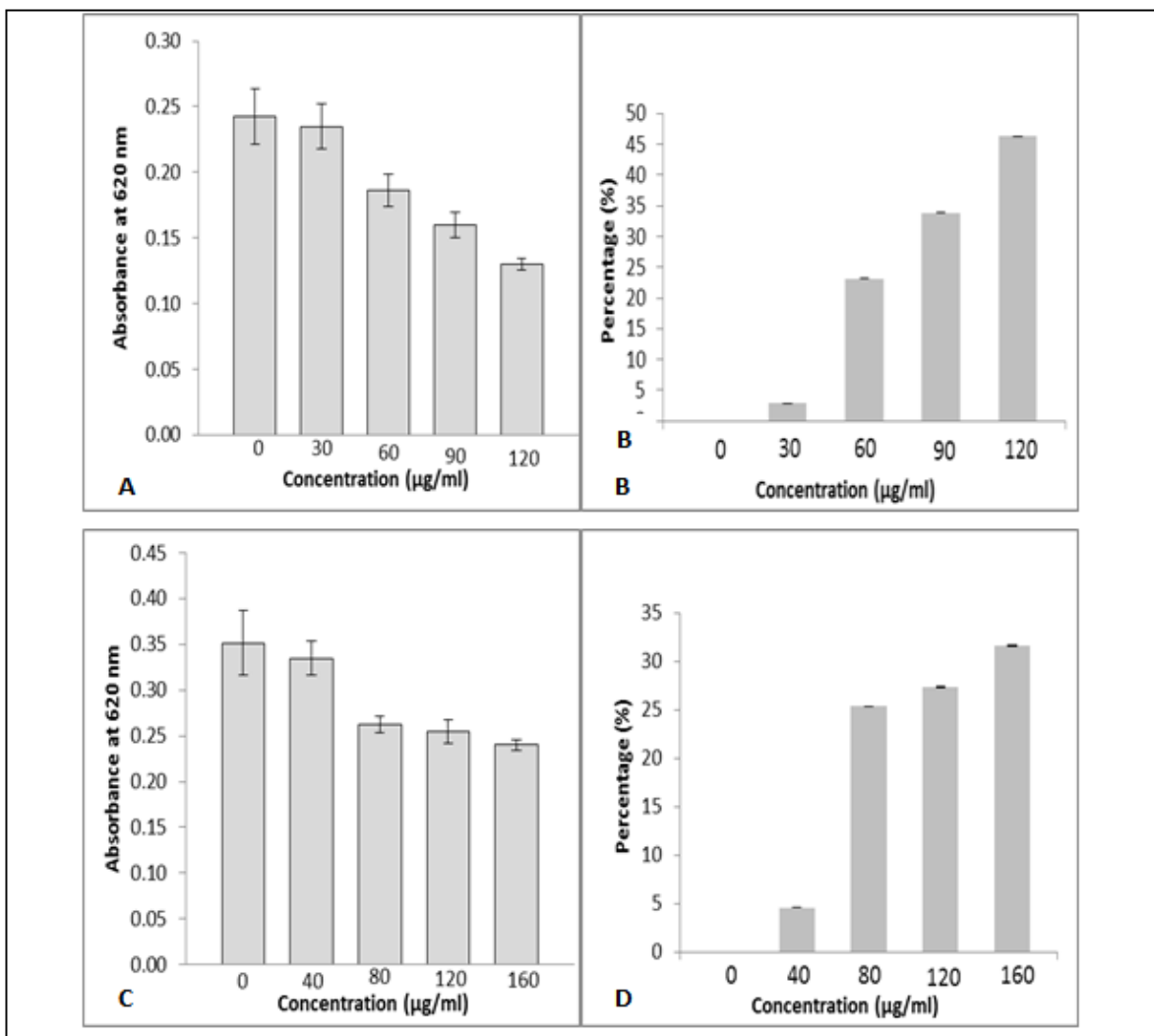


The biofilm formation assay with crystal violet stain was studied for all the extracts of *Adhatoda vasica* Nees. leaves and vasicine (Figure 5). It was found that all the extracts and vasicine have a significant effect on the biofilm formation of the organism. The biofilm inhibitory studies have been conducted for commercial vasicine and different extracts (petroleum ether, ethanol, methanol, water) of *A. vasica* Nees. The commercial vasicine was taken as a control and the study was conducted for different concentration of 0, 30 60, 90 and 120 µg/ml and the proportional decline of values are 0, 2.89, 23.14, 33.88 and 46.28 % respectively. The different concentrations of petroleum ether extract (0, 40, 80, 120 and 160 µg/ml) shows remarkable percentage biofilm inhibition and the values are 0, 4.56, 25.36, 27.35 and 31.62 % respectively. Similar studies were carried out with ethanol, methanol and water extract for the same concentration. The values for ethanol extract are 0, 29.87, 40.27, 50 and 53.36 %, methanol extract are 0, 27.21, 39.8, 43.2 and 47.28 % and for water extract are 0, 35.26, 46.8, 55.13 and 57.37 % respectively which shows notable biofilm intervention of the organism (Graph 3)

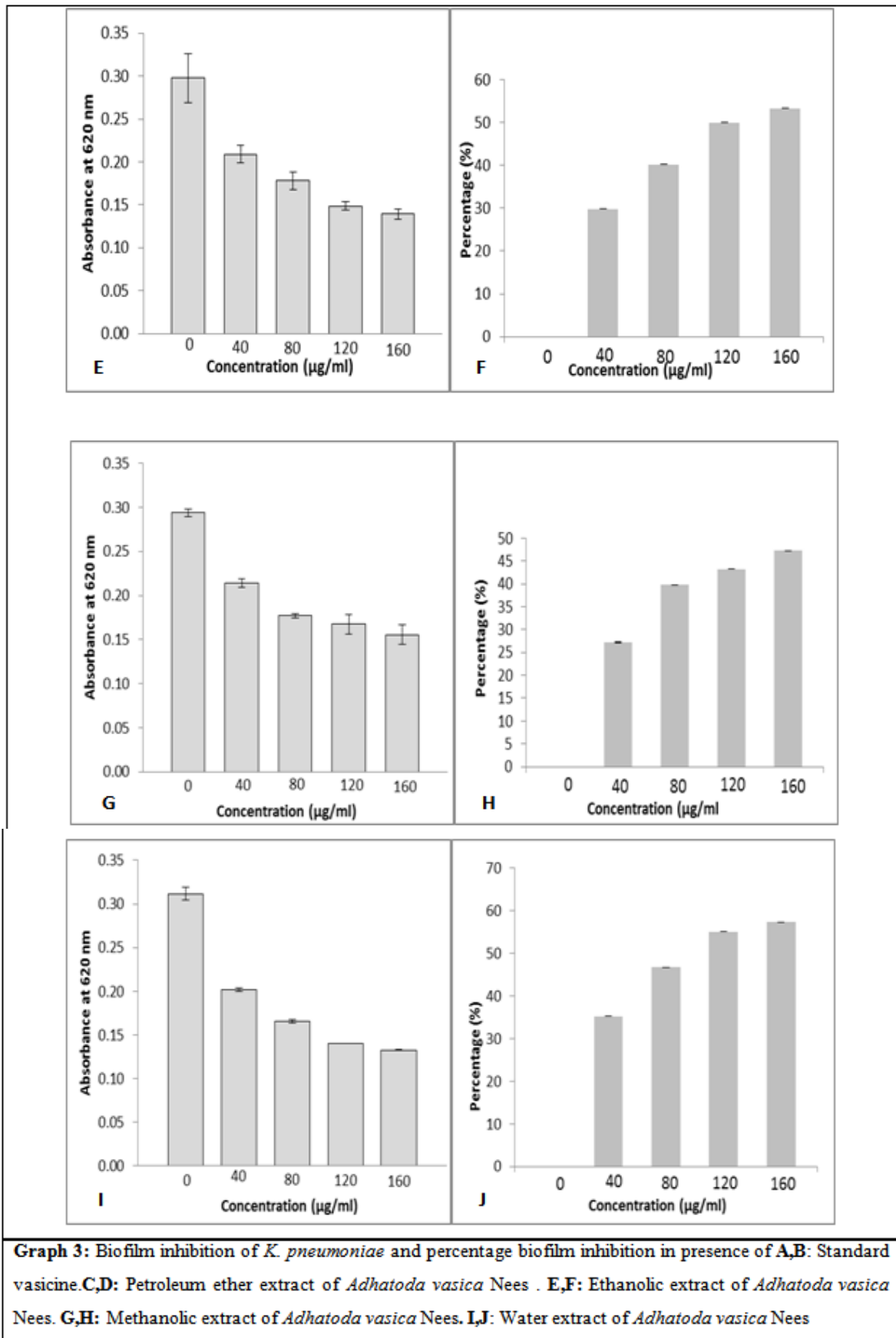
The microscopic examination of the biofilm adherence was done with organisms adhered on sterile glass coverslips incubated in presence and absence of the treatment with commercial vasicine and different *A. vasica* extracts (petroleum ether, ethanol, methanol and water). Then the coverslips were recovered and stained with 1 % crystal violet stain which shows considerable inhibition of streptococcal biofilm adherence by the different extracts of *Adhatoda vasica* Nees and commercial vasicine (Figure 6). Similar biofilm intervention was seen in case of cell viability study with EtBr/ AO staining. The green cells take up acridine orange as they are live cells but in case of dead cells ethidium bromide can enter into the cell and stain it red. So live cells and dead cells can be differentiated easily (Figure 7). Similarly, biofilm inhibition was seen in case of SEM analysis of the biofilm in presence of extracts of *Adhatoda vasica* Nees, vasicine and control antibiotic tetracycline (Figure 8)

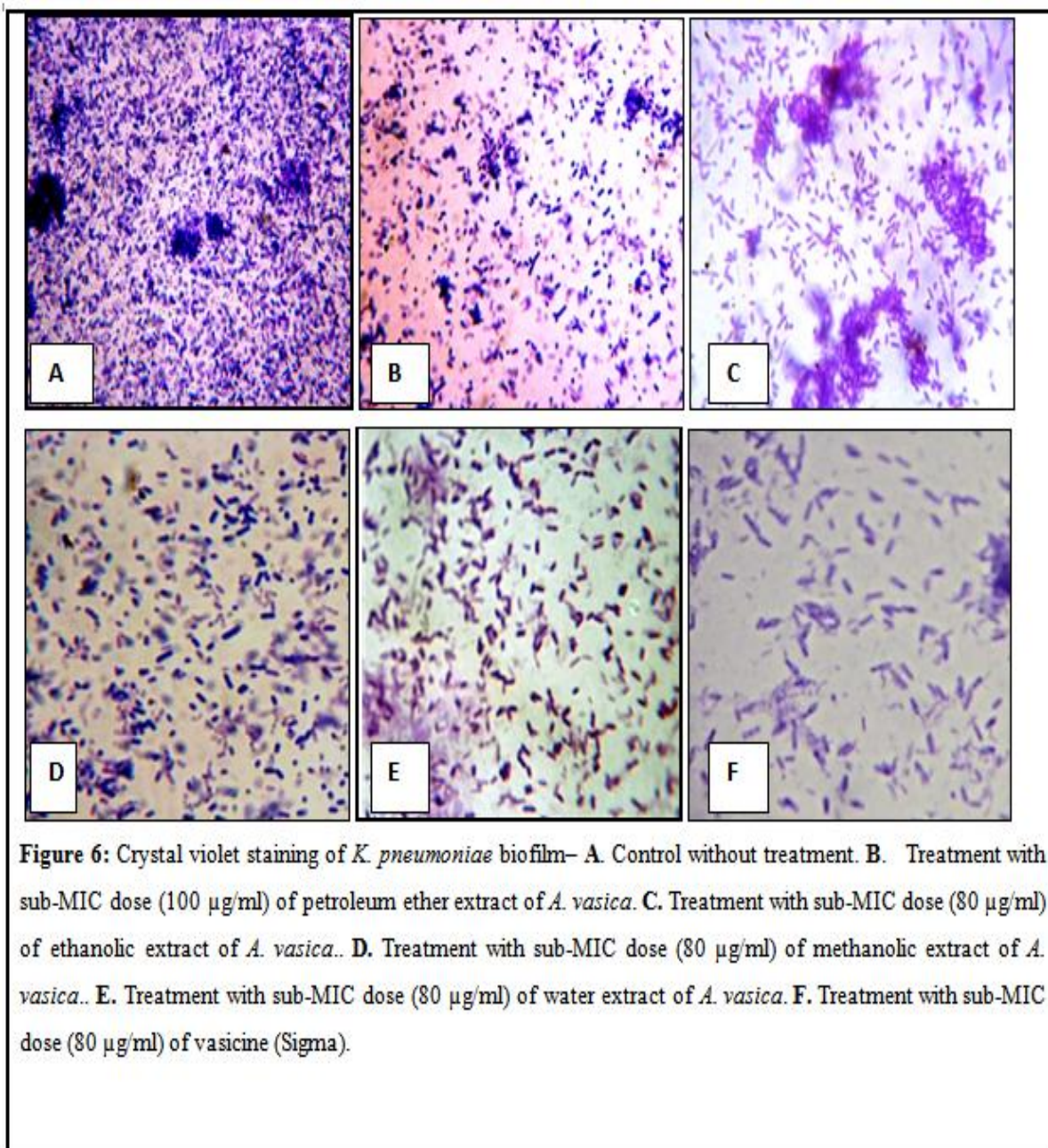


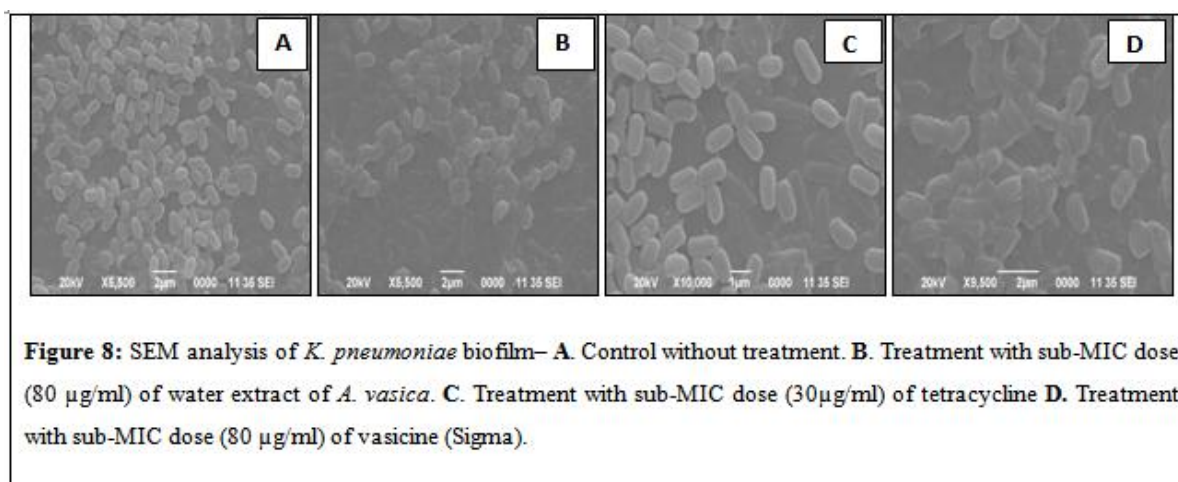
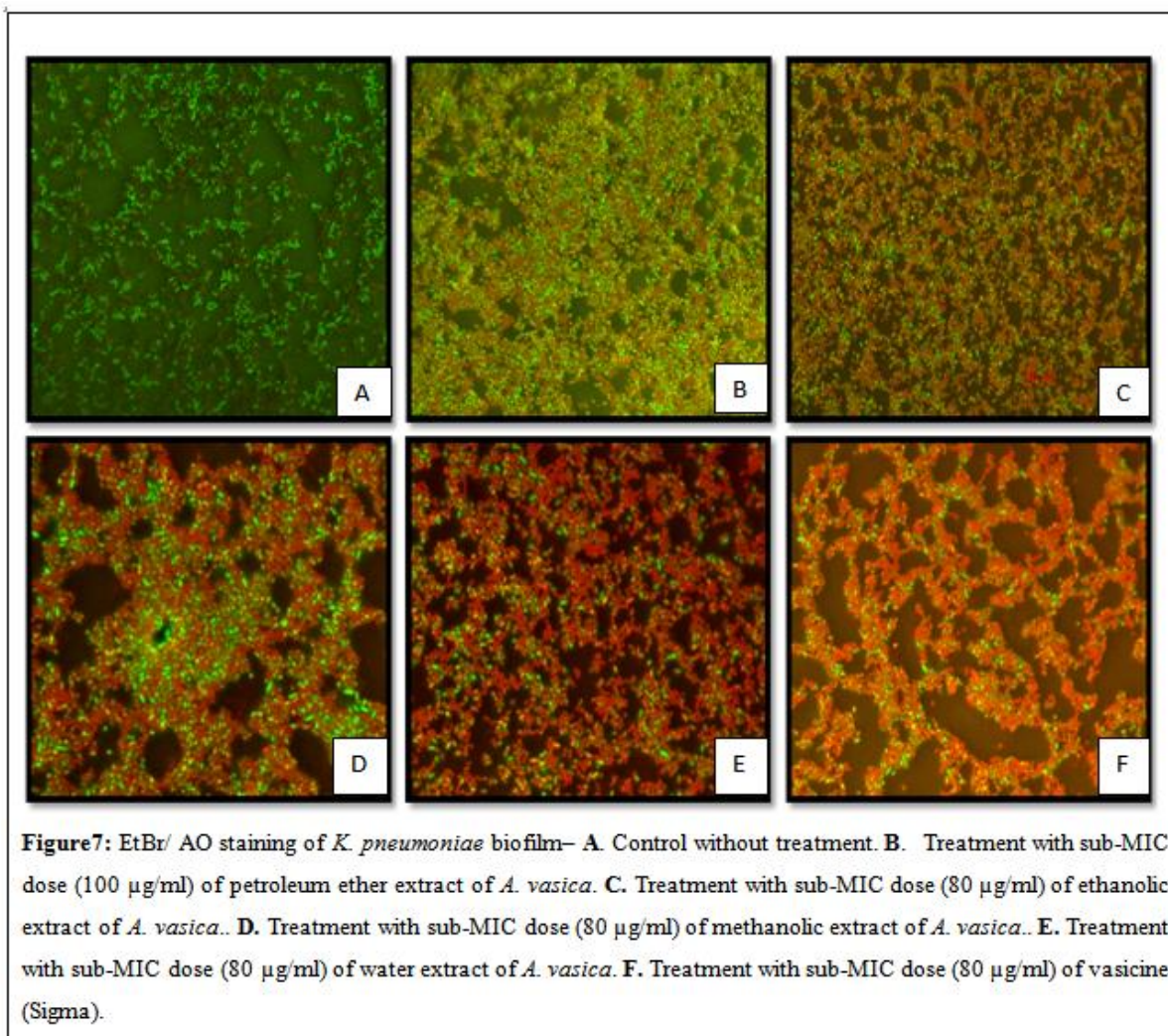
**Figure 5:** Biofilm inhibition assay of different extracts of leaf of *Adhatoda vasica* Nees and standard vasicine on planktonic *K. pneumoniae*











#### IV. Discussion

Pneumonia causing and biofilm forming bacterial agents like *Klebsiella pneumoniae* develops characteristics that offer tough antibiotic resistance due to EPS formation. The traditional use of *Adhatoda vasica* Nees. is very common in Tripura for herbal healing of pneumonia. It has been reported that the active major alkaloid extracted from *Adhatoda vasica* Nees. are vasicine and vasicinone. Along with this other phytochemicals are adhatodine, vasicolinone, vasicoline, anisotine, isoniazid, ethambutol, pyrazinamide etc (Jha et al., 2012).

The petroleum ether, ethanol, methanol, water extracts of *Adhatoda vasica* Nees and its phytochemical screening for carbohydrates, alkaloids, glycosides, saponins, phytosterols, phenols tannins, flavonoids, protein and amino acids indicates that the phytochemicals extracted in petroleum ether is non alkaloid, non glycosidic and non phenolic compound. The compound extracted in ethanol is non glycosidic, non saponin, non protein and amino acid. Whereas the compound in methanolic and water extract is a non protein and amino acid compound (Table 1). As there is no alkaloid compound in petroleum ether extract, it infers that the compound extracted is not a vasicine. To check the presence of vasicine in ethanol, methanol, and water thin layer chromatography is performed. The similarity in Rf value indicated the presence of vasicine in all of them (Figure 3).

The antimicrobial activity of the extracts of *Adhatoda vasica* Nees are being compared in three replicates taking tetracycline as control and vasicine as standard. The result depicted higher activity in *Klebsiella pneumoniae* for the methanolic extract. However the activity against *Klebsiella pneumoniae* is recorded to be higher than the standard vasicine and control tetracycline (Figure 4). This might be due to the presence of other alkaloids along with vasicine in the methanolic extract showing combine effect. Next to methanol the commercial vasicine is showing good effects followed by water, ethanolic and petroleum ether extract of *Adhatoda vasica* Nees.

The MIC values of vasicine, petroleum ether extract, ethanol extract, methanol extract and water extract were 120 µg/ml, 160 µg/ml, 160 µg/ml, 160 µg/ml and 160 µg/ml respectively against *Klebsiella pneumoniae* MTCC 3384 (Graph-1). The MIC determined for the extract shows similar concentration for petroleum ether, methanol, ethanol and water extract whereas the concentration for vasicine (commercial) was lower. The MBC values also follow the same trend. However, the MBC values for pure commercial vasicine is 160µg/ml which is less than petroleum ether, methanol, ethanol and water extract. It might be attributed to the purity and efficacy of vasicine in pure form towards microorganisms. The correlation analysis among the different extracts and commercial vasicine were found highly significant (Table-2). This implies the similar trend of effect for all extracts and the commercial vasicine statistically. Same patterns of results are also being seen for the antimicrobial activity by agar well diffusion method.

Further the compounds are being checked for its ability of *Klebsiella pneumoniae* biofilm intervention. The percentage inhibition in biofilm formation at different concentration for all the extracts and commercial vasicine is depicted in graph 2. The percentage reduction in biofilm formation was observed to be uniformly increasing as per the concentration in commercial vasicine and petroleum ether, ethanol, methanol and water extract. So, it implies that, the trend of increase in rate of inhibition is directly proportional to the increase in concentration of the extracts. Same trend is also been observed in microscopical studies using Crystal violet staining (Figure-6) and EtBr/ AO staining (Figure-7) However, the maximum inhibition in biofilm formation was observed in water extract of *Adhatoda vasica* followed by ethanol and methanol. The percentage inhibition of *Klebsiella pneumoniae* biofilm with water extract 11% higher than the commercially available vasicine (positive control) which can be attributed to some additional active fraction of compound along with vasicine showing synergistic effect. However, the petroleum ether showed minimum inhibition. Similarly it was confirmed by SEM analysis (Figure -8) that water extract of *Adhatoda vasica* Nees has the maximum effect. It infers that unknown active fraction of the compound is commendably water-soluble and has remarkable effect on gram negative *Klebsiella pneumoniae*. Further fractional analysis of water extract and detail studies of the active fraction in the compound may lead to some promising antibiofilm drugs for gram negative pneumonia causing *Klebsiella pneumoniae*.

## V. Conclusion

The study concluded that the water extracts of *A. vasica* is more active than commercially available vasicine towards gram negative *Klebsiella pneumoniae*. The MIC and MBC values of water and ethanol extracts are found promising against *Klebsiella pneumoniae* in its planktonic form as well as in the biofilm forms. However, it indicates that, there is an unidentified active compound in water and ethanol extract of *A. vasica* showing more efficiency towards gram negative *Klebsiella pneumoniae* biofilm intervention than vasicine even at below MIC concentration, and it needs further studies to know the compound in detail.

## Acknowledgement

We would like to thank the **Department of Science and Technology** for funding the project under **Women scientist -A programme**. We would also like to thank to the Department of Microbiology and State Biotech Hub, Tripura University for giving us lab facilities. We would like to thank Department of Microbiology, Lady Brabourne College, Kolkata for their lab facilities.

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Dr. Bipin Kumar Sharma "Extracts of *Adhatoda vasica* Nees. and vasicine inhibits biofilm formation of *Klebsiella pneumoniae*." *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)* 13.2 (2018): 37-49.