

## Antibiofilm effects of *Adhatoda Vasica* Nees. extracts in comparison with commercial vasicine on *Streptococcus Pneumoniae*

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### Abstract:

**Purpose:** Medicinal plants like *Adhatoda vasica* Nees. are used traditionally in Tripura to cure pneumonia, cough and cold. Pneumonia is a lung infection with cough, fever and hard time breathing. Pneumonia is mainly caused by bacteria like *Streptococcus pneumoniae*. Moreover, many of these pathogenic strains are showing acute resistant to many commercially available antibiotics due to the formation of biofilms. One of the main areas of modern day research in medical microbiology is to search for agents that can destroy microbial biofilms effectively. **Materials and Method:** The present study evaluates the antimicrobial activity of *Adhatoda vasica* Nees. extracts and commercially available vasicine against the biofilms of the bacteria - *Streptococcus pneumoniae* and their free-living forms. The extraction, identification and phytochemical screening of the leaf of *Adhatoda vasica* Nees. were done following the standard procedures. Thin layer chromatography had been done to show the presence of vasicine in the ethanol, methanol and water extracts. The antimicrobial activity of petroleum ether, ethanol, methanol and water extracts of *Adhatoda vasica* Nees. (20mg/ml) and commercially available vasicine (1mg/ml) against planktonic forms of bacteria were determined using the agar well diffusion method with tetracycline (20mg/ml) 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 micro titre plate method. The biofilm inhibition was observed both in compound microscope with crystal violet staining and in scanning electron microscope. The cell viability assay was assessed by Etbr/AO staining. **Results:** The results showed that the *A. vasica* Nees. leaf extract have remarkable zone of inhibition for the tested bacteria as compared to standard vasicine and tetracycline. However, the MIC values of *A. vasica* Nees. extracts (100 - 160 µg/ml) and vasicine (100-120 µg/ml) confirmed the high ability of these extracts for inhibition of planktonic bacteria. *A. vasica* Nees. extracts were efficient to inhibit biofilm and the concentration of each extract had a direct relation with the inhibitory effect. The extracts and standard vasicine showed a remarkable effect on cell viability of bacteria in biofilm which is also confirmed by SEM analysis. Finally, it can be suggested that the extracts of this plant and commercially vasicine be applied as antimicrobial agents against the pathogen, particularly in biofilm forms.

**Key Words:** *A. vasica* Nees, Vasicine, Biofilm, *Streptococcus pneumoniae*.

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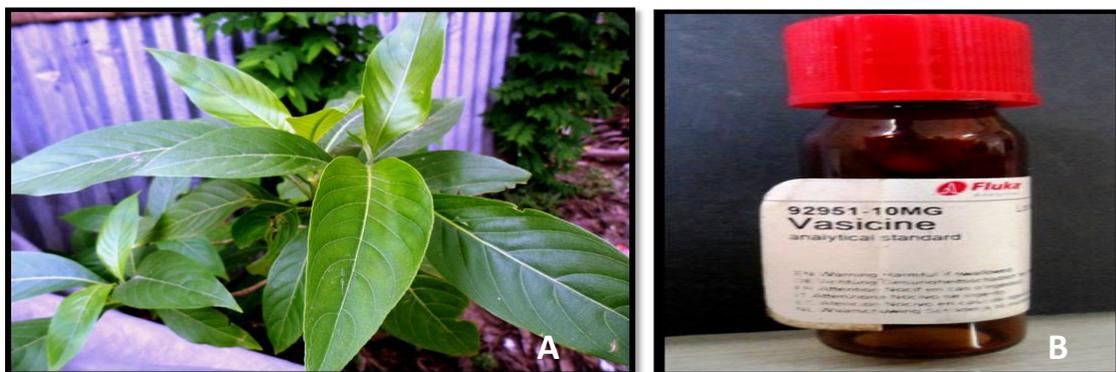
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### I. Introduction

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<sup>1</sup>. It is used in ayurvedic medicinal plant to treat cold, cough, asthma and tuberculosis<sup>2</sup>. The main action is expectorant and antispasmodic (bronchodilator)<sup>3</sup>. 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”<sup>4</sup>. 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<sup>5</sup>. The major alkaloids of the plant found to be biologically active are vasicine and vasicinone<sup>1</sup>.



**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<sup>6,7</sup>. The antimicrobial property of leaf extracts of *J. Adhatoda* (L.) in comparison with vasicine was shown by Rashmi Pa in 2012<sup>8</sup>. The leaves, flowers and roots of this plant used in herbal drugs against tubercular activities<sup>9</sup>, cancer<sup>10</sup> and possessed to have anti-helminthic properties<sup>11</sup>.

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, *Streptococcus pneumoniae* is the causative agent of bacterial pneumonia and the leading cause of infant mortality globally<sup>12</sup> due to acute resistance 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

- a. Preparation of standard vasicine:** Vasicine (10 mg) was procured from Sigma Aldrich. A solution of vasicine (5mg/ml) was prepared using methanol as solvent.
- b. 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.
- c. Culture and maintenance of test microorganisms for antimicrobial studies:** *Streptococcus pneumoniae* R6 strain was a kind gift from Dr. Arijit Bhattacharya, University of Ottawa, Canada. The strain was revived in Todd Hewith Broth (Hi-Media) and stored as 50% glycerol stock at -80° refrigerator.
- d. 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<sup>13</sup>.

- e. **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.

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

f. **Biochemical characterisation of crude extracts:**

1. **Detection of alkaloids:** The presence of alkaloids in the extract was determined by Mayer's Test, Dragendroff's Test and Wagner's Test <sup>14</sup>.
2. **Detection of carbohydrates:** The presence of carbohydrates was determined by Molisch's Test, Benedict's Test and Fehling's Test <sup>14</sup>.
3. **Detection of glycosides:** The presence of cardiac glycosides was determined by Legal's Test <sup>14</sup>.
4. **Detection of saponins:** The presence of saponins was determined by Froth Test and Foam Test <sup>14</sup>.
5. **Detection of phytosterols:** The presence of phytosterols was determined by Salkowski's Test <sup>14</sup>.
6. **Detection of phenols:** The presence of phenols was determined by Ferric Chloride Test <sup>14</sup>.
7. **Detection of tannins:** The presence of tannins was determined by Lead Acetate Test and Gelatin Tests <sup>14</sup>.
8. **Detection of flavonoids:** The presence of flavonoids was determined by Alkaline Reagent Test and Lead Acetate Tests <sup>14</sup>.
9. **Detection of proteins and amino acids:** The presence of protein and amino acids was determined by Xanthoproteic Test and Ninhydrin Test <sup>14</sup>.

- g. **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 <sup>15,16</sup>. The extracts were chromatographed on silica coated tlc plates (Sigma- Aldrich).

h. **Biological evaluation:**

i) **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<sup>17</sup>. Todd - Hewith agar was swabbed (sterile cotton swabs) with 24 hour old broth culture of *Streptococcus pneumoniae*. Wells were made in the 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.

ii) **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 *Streptococcus pneumoniae* R6 was grown for overnight on Todd Hewith broth (Hi-Media). 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. The broth culture was 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 the 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 *Streptococcus pneumoniae* R6 were taken as 0, 20,40, 60, 100, 120 µg/ml. 100 µl of the each culture was then inoculated onto solid medium and incubated for 36 h. The incubation temperature was 37°C, throughout the experiment. The MBC was defined as that concentration at which no viable bacteria was recovered <sup>18</sup>. For each phytochemical-treated system Dimethyl Sulphoxide (DMSO) was used as control.

iii) **Biofilm Formation Assay:**

The bacterial strain *Streptococcus pneumoniae* R6 was grown over-night on soyabean casein digest sheep blood agar plates (Hi-Media) at 37°C. Colonies of each strain were resuspended in Todd Hewith broth (Hi-Media) at  $od_{600}$  of 0.6. a 1:100 dilution of these suspensions were inoculated in fresh broth 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  $od_{620}$  of the resulting suspension was measured <sup>19</sup> using Synergy H1 hybrid reader.

**iv) Microscopic study of biofilm inhibition by the crude extract and compound:**

The bacterial strains *Streptococcus pneumoniae* R6 was grown over-night on Todd Hewith broth (Hi-Media) at 37°C. A 1:100 dilution of these suspensions were inoculated in fresh broth 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 Alrich) were added and incubated for 24 h at 37°C respectively. subsequently, the 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 <sup>20</sup>.

**v) Bacterial viability study of biofilm inhibition using EtBr/AO staining method:**

The bacterial strains *Streptococcus pneumoniae* R6 was grown over-night on Todd Hewith broth (Hi-Media) at 37°C. a 1:100 dilution of these suspensions were inoculated 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 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 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 <sup>21</sup>.

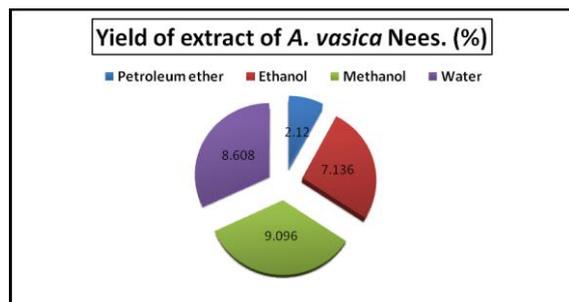
**vi) SEM Analysis**

To Evaluate The Action Of The Compounds On The Integrity Of Biofilm Of The Bacteria *S. Pneumoniae* R6, The Morphological Analysis Of The Bacteria Was Carried Out With The Antimicrobial Compounds With Treatment. The Bacterial Strain *S. Pneumoniae* R6 was Grown Over-Night On Todd Hewith 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<sup>23</sup>.

**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 *Streptococcus pneumoniae* R6 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</i> Nees. (%)
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.

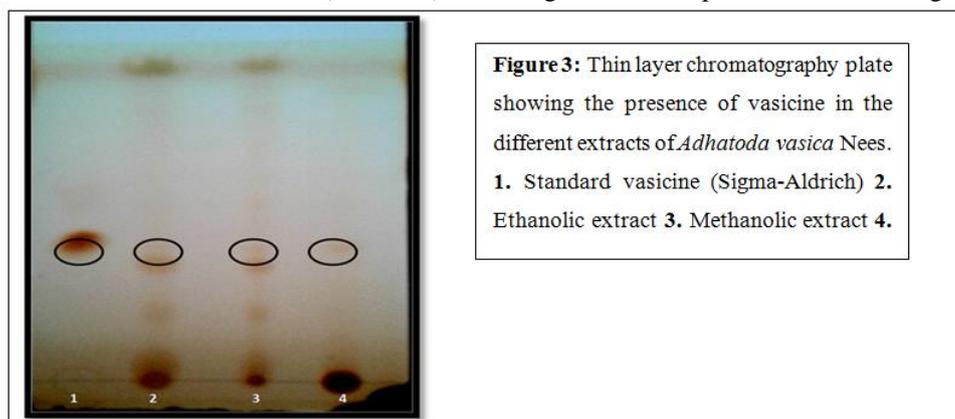
**Figure 2:** Showing yield percentage of the different extracts of *Adhatoda vasica* Nees.

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	Adhatoda Vasica Nees.			
		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





Sl .No	Treatment	<i>S. pneumoniae</i>
1	Tetracycline	17 ± 1.154701
2	Petroleum ether extract	9.33 ± 1.452966
3	Ethanol extract	10.67 ± 1.763834
4	Methanolic extract	12.67 ± 0.666667
5	Water extract	14.33 ± 0.881917
6	Vasicine	12.33 ± 0.881917

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

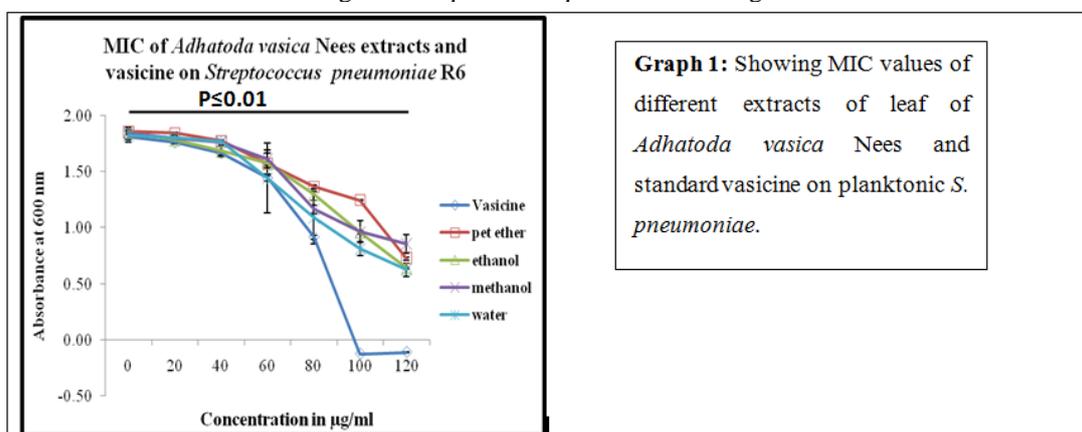
**Table 3:** Showing average zone of inhibition of the different extracts of *Adhatoda vasica* Leaves, tetracycline as control and commercial vasicine against *S. pneumoniae*

The Minimum Inhibitory Concentration (MIC) And Minimum Bactericidal Concentration (MBC) was determined for all the extracts of *Adhatoda vasica* Leaves and vasicine respectively. For *Streptococcus pneumoniae* R6, the MIC values of vasicine, petroleum ether extract, ethanol extract, methanol extract and water extract were 100 µg/ml, 120 µg/ml, 100 µg/ml, 100 µg/ml and 100 µg/ml respectively and the MBC values of vasicine, petroleum ether extract, ethanol extract, methanol extract and water extract were 120 µg/ml, 200 µg/ml, 160 µg/ml, 160 µg/ml and 160 µg/ml respectively. the values were tested statistically by Pearson correlation using SPSS.16 software. All the values of extracts were found significant as compared with vasicine at 0.01 level of significance having P values ≤ 0.01.

Correlations						
		Vasicine	Petroleum Ether	Ethanol	Methanol	Water
Vasicine	Pearson Correlation	1	0.918**	0.975**	0.974**	0.972**
	Sig. (2-Tailed)		0.004	0.000	0.000	0.000
Petroleum Ether	Pearson Correlation	0.918**	1	0.981**	0.941**	0.959**
	Sig. (2-Tailed)	0.004		0.000	0.002	0.001
Ethanol	Pearson Correlation	0.975**	0.981**	1	0.975**	0.980**
	Sig. (2-Tailed)	0.000	0.000		0.000	0.000
Methanol	Pearson Correlation	0.974**	0.941**	0.975**	1	0.992**
	Sig. (2-Tailed)	0.000	0.002	0.000		0.000
Water	Pearson Correlation	0.972**	0.959**	0.980**	0.992**	1
	Sig. (2-Tailed)	0.000	0.001	0.000	0.000	

\*\* . Correlation Is Significant At The 0.01 Level (2-Tailed).  
N = 7

**Table 4:** Showing Pearson Correlation between the different extracts of *Adhatoda vasica* Nees and standard vasicine against *Streptococcus pneumoniae* using SPSS 16.

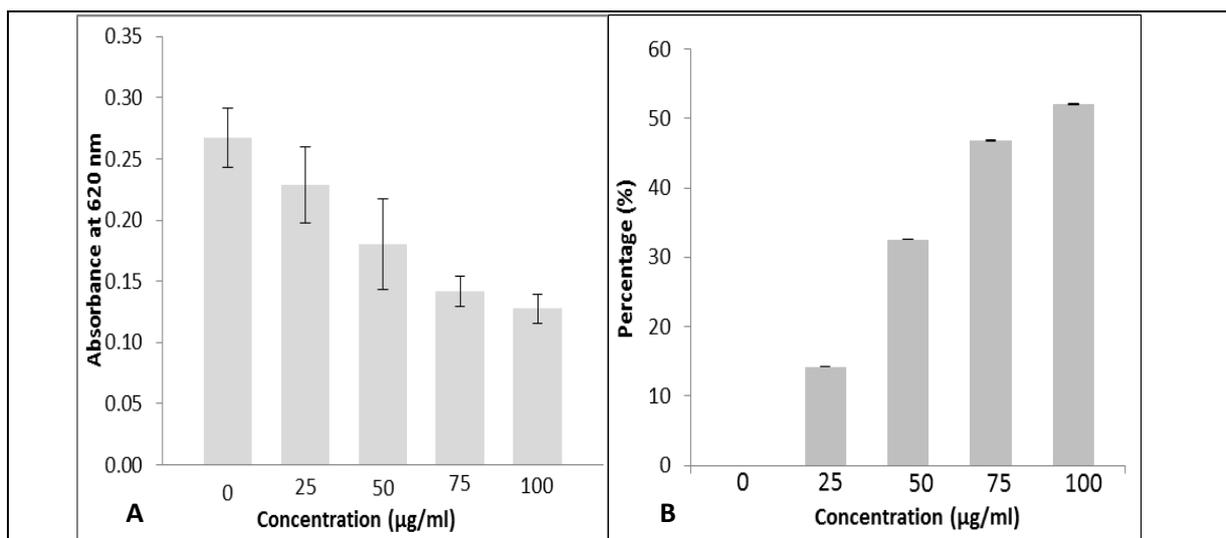
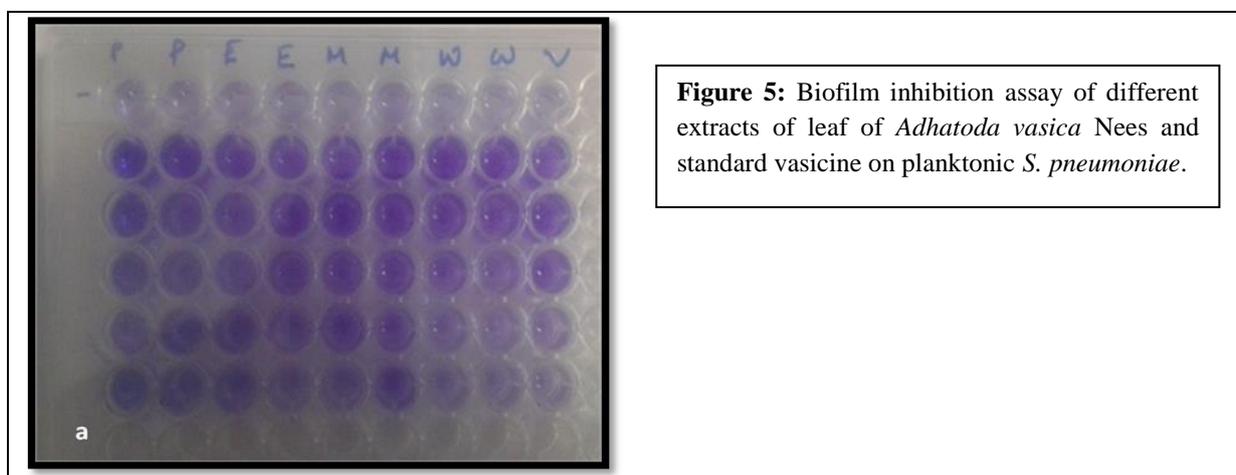


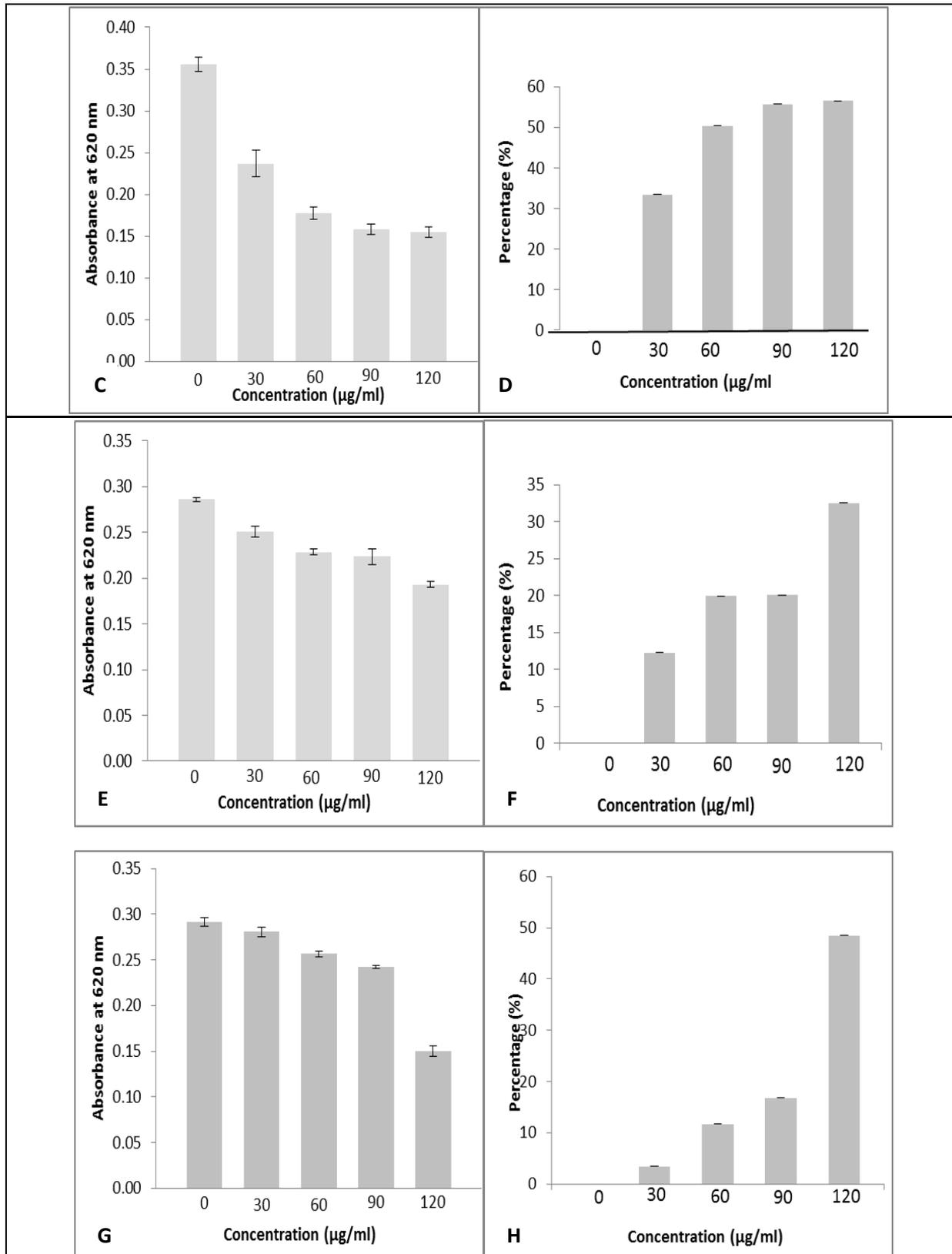
*Antibiofilm effects of Adhatoda vasica Nees. extracts in comparison with commercial vasicine*

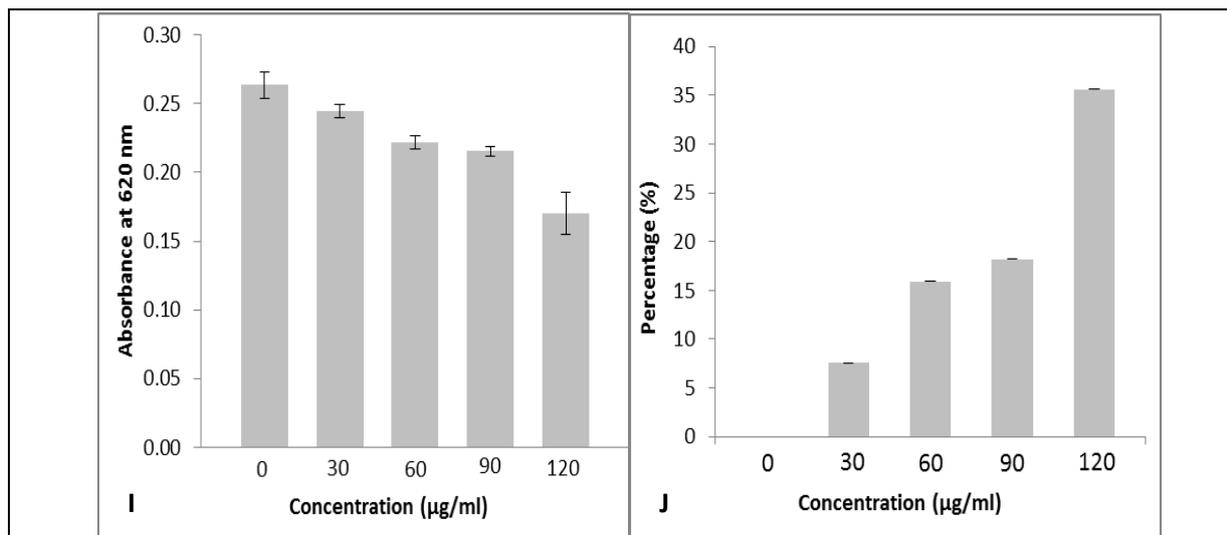
The biofilm formation assay with crystal violet stain was done for all the extracts of *Adhatoda vasica* Nees. Leaf and commercial vasicine. It was found that all the extracts and vasicine have a substantial effect on the biofilm formation of the organisms (Figure 5).

The streptococcal 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, 25, 50, 75 and 100 µg/ml and the proportional decline of values are 0, 14.23, 32.58, 46.82 and 52.06 % respectively. Similar study with ethanolic extract for the said concentration shows 0, 12.24, 19.93, 20.03 and 32.52 % inhibition, methanolic extract for the same concentration shows 0, 3.44, 11.68, 16.83 and 48.45 % inhibition and water extract shows 0, 7.58, 1.91, 18.18 and 35.61 % inhibition respectively. the petroleum ether extract shows a different inhibition pattern as compared to other extracts. the concentration as taken as 0, 30, 60, 90 and 120 µg/ml as per the MIC values and the percentage biofilm inhibition values are 0, 33.43, 50.28, 55.62 and 56.46 % respectively (Graph 2)

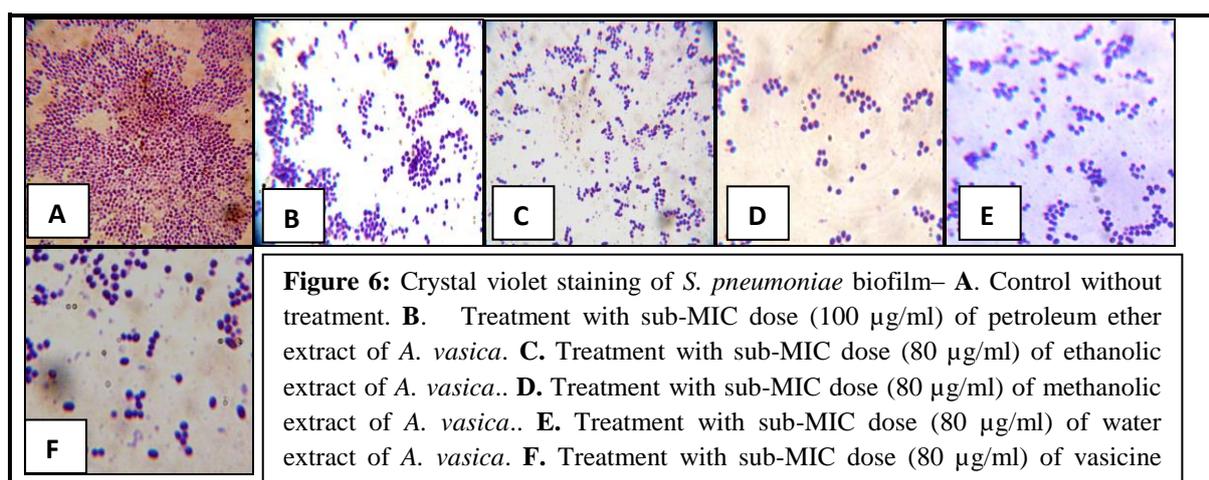
The microscopic examination of the biofilm intervention with crystal violet stain shows considerable inhibition of streptococcal biofilm by the different extracts of *Adhatoda vasica* Nees and commercial vasicine (Figure 6) and similar observation is also obtained from SEM analysis of the samples (Figure 8). Similar biofilm intervention pattern 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).



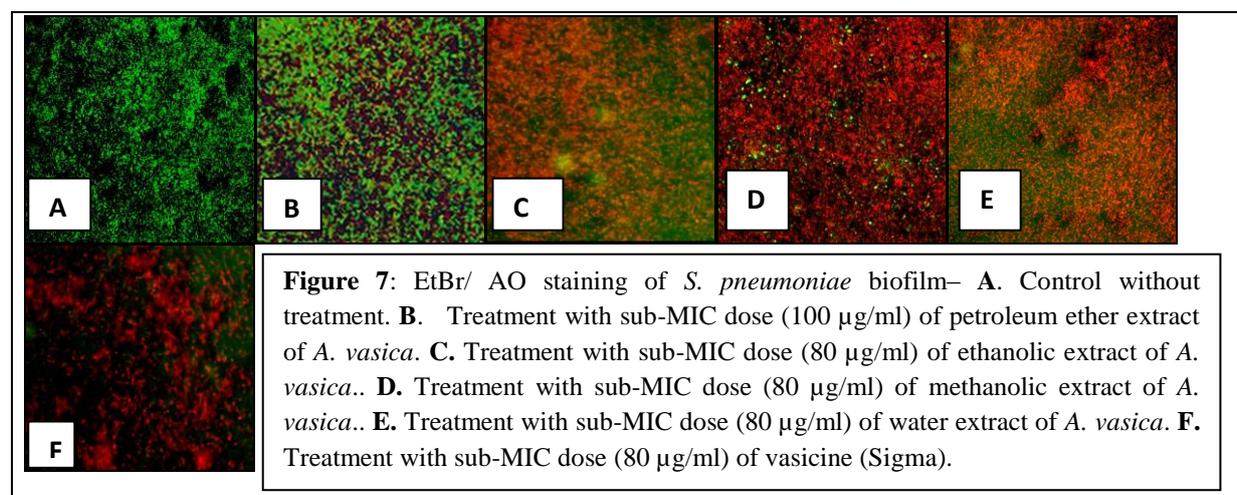




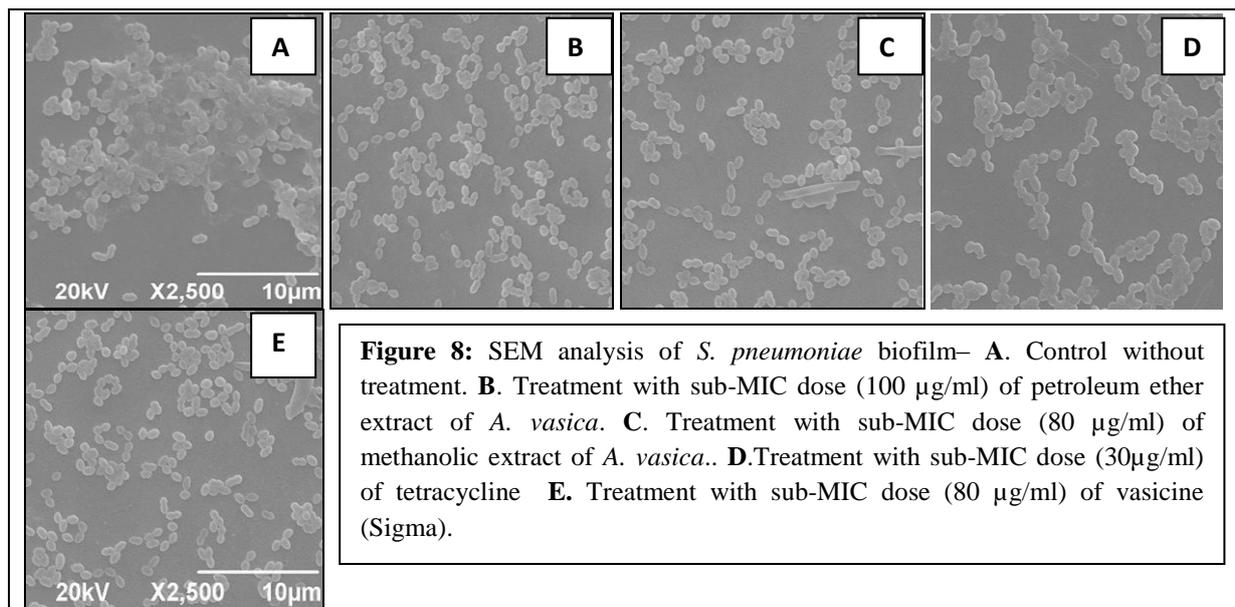
**Graph 2:** Biofilm inhibition of *S. pneumoniae* and percentage biofilm inhibition in presence of **A,B:** Standard vasicine. **C,D:** Petroleum ether extract of *Adhatoda vasica* Nees. **E,F:** Ethanolic extract of *Adhatoda vasica* Nees. **G,H:** Methanolic extract of *Adhatoda vasica* Nees. **I,J:** Water extract of *Adhatoda vasica* Nees



**Figure 6:** Crystal violet staining of *S. pneumoniae* biofilm– **A.** Control without treatment. **B.** Treatment with sub-MIC dose (100  $\mu\text{g/ml}$ ) of petroleum ether extract of *A. vasica*. **C.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of ethanolic extract of *A. vasica*.. **D.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of methanolic extract of *A. vasica*.. **E.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of water extract of *A. vasica*. **F.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of vasicine



**Figure 7:** EtBr/ AO staining of *S. pneumoniae* biofilm– **A.** Control without treatment. **B.** Treatment with sub-MIC dose (100  $\mu\text{g/ml}$ ) of petroleum ether extract of *A. vasica*. **C.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of ethanolic extract of *A. vasica*.. **D.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of methanolic extract of *A. vasica*.. **E.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of water extract of *A. vasica*. **F.** Treatment with sub-MIC dose (80  $\mu\text{g/ml}$ ) of vasicine (Sigma).



**Figure 8:** SEM analysis of *S. pneumoniae* biofilm– **A.** Control without treatment. **B.** Treatment with sub-MIC dose (100 µg/ml) of petroleum ether extract of *A. vasica*. **C.** Treatment with sub-MIC dose (80 µg/ml) of methanolic extract of *A. vasica*.. **D.**Treatment with sub-MIC dose (30µg/ml) of tetracycline **E.** Treatment with sub-MIC dose (80 µg/ml) of vasicine (Sigma).

#### IV. Discussion

Pneumonia causing and biofilm forming bacterial agent *Streptococcus pneumoniae* develop 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.<sup>22</sup>

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 not alkaloid, glycosidic and 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 compounds (Table 2). It is established that vasicine is an alkaloid. Hence it infers that the compound extracted in petroleum ether is not vasicine. to check the presence of vasicine in ethanol, methanol and water extract thin layer chromatography is performed. The similarity in Rf values in comparison to Rf of commercial vasicine 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 compared to commercial vasicine against streptococcus pneumoniae for the methanolic extract (figure 4). This might be due to the presence of other phytochemicals along with vasicine in the methanolic extract, which might be showing some synergistic effect. Next to methanol the water extract is showing good effects followed by ethanolic extract. However in the case of petroleum ether the activity is recorded to be minimum compared to all other extracts for the organism. This might be due to the absence of vasicine component in the extract (Figure 4)

The MIC values of vasicine, petroleum ether extract, ethanol extract, methanol extract and water extract were 100 µg/ml, 120 µg/ml, 100 µg/ml, 100 µg/ml and 100 µg/ml respectively against *Streptococcus pneumoniae* R6 (Graph 1). The MIC determined for the extracts (methanol, ethanol and water) show similar values as compared to commercial vasicine whereas the MIC value of petroleum ether was quite higher. It clearly indicates and signifies the presence of vasicine in methanol, ethanol and water extracts and its absence in petroleum ether. Similarly the MBC values also follow the same trend. The MBC for pure commercial vasicine 120 µg/ml which is less than methanol, ethanol and water extract. this might be attributed to the purity of commercial vasicine compound. The correlation analysis among the different extracts and commercial vasicine were found highly significant. This implies the similar trend of effect in all the extracts along with commercial vasicine statistically. Similar patterns of results are also being seen for the anti streptococcal activity by agar well diffusion method, MIC and MBC.

Further the compounds are being checked for its ability of biofilm intervention against *Streptococcus pneumoniae* R6. The percentage inhibition of biofilm at different concentration is depicted in Graph 2 for all the extracts and commercial vasicine where the percentage reduction was seen uniformly increasing as per the concentration in the commercial vasicine. Whereas, in extracts of ethanol, methanol and water the uniformity as per increase in concentration does not follows well. This might be due to impedance of other associated

phytocompounds along with vasicine in the extract. However, the trend of increase in rate of inhibition as per increase in concentration is observed for all the extracts. The maximum concentration considered for all the treatments are the MIC concentration itself and maximum inhibition is being observed in petroleum ether extract (56.46%) at its MIC concentration. Moreover, the 60 and 90 µg/ml concentration is also showing more than 50 % intervention for the biofilm formation. Therefore, it can be inferred that the vasicine is more effective in planktonic form but in biofilm intervention of streptococcus pneumoniae some unidentified non alkaloid fraction of *Adhatoda vasica* phytocompound which can be extracted only with petroleum ether seems to be more potential. Same trend of inhibition is also being visualised in microscopic observation where cell viability studies were carried with crystal violet staining (Figure 6), EtBr/AO staining (Figure 7) and SEM analysis (Figure 8). Therefore, it needs further detailed studies and fractional analysis of the individual phytocompounds extracted with petroleum ether which might be more promising than vasicine and may lead some potential pneumococcal drug for future.

## V. Conclusion

Present study concludes that the extracts of ethanol, methanol and water contain vasicine. The MIC And MBC values of *Adhatoda Vasica* extracts are found promising against *Streptococcus pneumoniae* in its planktonic form. Commercial vasicine and vasicine in ethanol, methanol and water extracts shows reasonably high effect in biofilm form. However there is an unidentified non alkaloid compound in petroleum ether extract of *A. vasica* showing even more efficiency towards streptococcal biofilm intervention than vasicine at low concentration of 60µg/ml and it needs further studies to know the compound in detail.

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