

Original Research Article

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## Assessment of Bactericidal and Phytochemical Properties of *Adhatoda vasica* Various Extracts against Gram Positive and Gram Negative Bacteria

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### ABSTRACT

#### Keywords

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Bacterial resistance against traditional antibiotics has posed a major problem nowadays and the phytoconstituents present in medicinal plants has the potential to combat bacterial infections. Leaves of *Adhathoda vasica* plant were screened for its medicinal property. Antibacterial activity was analyzed by preparing its extracts in different solvents like ethyl acetate, methanol and Distilled water (in increasing polarity). Results obtained were compared with commercial antibiotic Gentamicin (used as control) on gram positive and gram negative bacteria (*S. aureus*, *E. coli*, *Pseudomonas*, *Bacillus*, *S. typhi*). The results obtained showed effective results. Different extracts of leaves exhibited varied degree of inhibition against the selected microbes. The best result was observed in ethyl acetate extract (zone of inhibition against *E. coli* was 27mm). Phytochemical analysis also showed varied percentage of phytoconstituents responsible for the medicinal activity.

### Introduction

From prehistoric time, plants are used for their medicinal property. Various parts of the plant are used for the preparation of medicines for various ailments. Plants make many chemical compounds to protect themselves against fungi, bacteria or mammals, this act in same way on human body as allopathic drugs. These compounds present are estimated around about 12,000. Plants use as drugs has undergone tremendous increase as pathogens are developing resistance against the frequent use of drugs. New drugs with better effect are needed to provide good health facility. Another benefit

of these drugs are that they are safe and cost-effective, a gift for the non-industrialized and underdeveloped countries. Another reason for the development of these drugs is population rise, prohibitive cost of treatments, side-effects of other synthetic drugs. India is a large repository for herbal plants in world. About 8,000 herbals are codified in AYUSH systems in India. Worldwide, 80 percent of people rely on herbs, notified by WHO (world health organization). As per data three-quarter of population rely on plants and their extracts for health care ([https://en.m.wikipedia.org/wiki/medicinal\\_plants](https://en.m.wikipedia.org/wiki/medicinal_plants)). Commonly used

herbs in every household are aloe, tulsi, neem, turmeric and ginger for care of common ailments. Major diseases are also treated by the herbal plants. AYURVEDA and UNANI works on these medicinal plants help to provide their benefit to people (<https://nhp.gov.in> introduction and importance of medical plants and herbs). The active ingredients present in plant are many but mainly used and important are Alkaloids – bitter tasting chemicals. Glycosides – drug containing these are used to support the beating of the heart and act as diuretics; Polyphenols – drugs used to treat gynaecological problems; Terpenes – strongly aromatic and used by plant to repel herbivores.

Different herbs have different properties, used to care different ailments. Some are used to treat sores and boils while some have blood cleansing and antibiotic property. Fever and poisons cases are also cured. Astringent action, antacid property, cough care and many more action are served by the herbal plants. One of the herbal plants which are widely used is *Adhatoda vasica* leaves used to care ailments like asthma, tuber-culosis, piles, jaundice and many more. Commonly known Malabar nut or adulsa come from Acanthaceae family native to Asia. The plant ranges in Sri Lanka, India, Bangladesh, Pakistan, Indonesia and Malaysia. Leaves, bark, roots, flower of *Adhatoda* are full of medicinal property. Various extract of plants are used to care pulmonary, bronchial and asthmatic disorders. It is also used to speed childbirth (Kanthale *et al.*, 2014). The *adhatodha vasica* leaves extract were screened for their Phytochemical content. Quantitative test were used to detect the presence of alkaloids, tannins, flavonoids, saponins, phenolic acid. Presence of these phytochemicals in the medicinal plants indicates the presence of antibacterial properties against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*

and *S. typhi*. Aim of this study is to identify various medicinal properties of *Adhatodha vasica* plant. Leaf was the main part of the plant used for the observation of medicinal properties. Phytochemical analysis was also conducted and presence of these botanicals was determined in order to support the medicinal efficacy of the plant.

## **Materials and Methods**

### **Plant material and extraction**

The dried mature leaves of *Adhatodha vasica* were collected from local area in Bhaniyawala Dehradun, India. Washed with distilled water and the leaves were separated and kept in a clean shaded place for 9-10 days, grounded to a powder and weight the whole powder. Cold Maceration method is used to prepare extracts. 250 ml of organic solvents ethyl acetate, methanol and water is taken and 25 gm of leaf and flower powder is soaked in it. Extracts obtained are made solvent free and concentrated by rotary evaporator and kept at 4°C in airtight bottle until further use (Akhter *et al.*, 2014).

### **Chemicals and Reagents**

Chemicals and reagents used are-for the study are- Ethyl acetate, Methanol, Conc. Ammonium hydroxide, 20% Acetic acid, Folin-Ciocalteu reagent, Sodium carbonate (20%), Dil.

Folin-phenol reagent (1:1 ratio with water), Gallic acid, Sodium nitrate(5%), Aluminum chloride(10%), Sodium hydroxide(4%), Polyvinyl pyrrolidone, Vanillin reagent(800gm of vanillin in 10ml of 99.5% ethanol), Sulphuric acid(72%), 2,6-dichloroindophenol sodium salt hydrate, Metaphosphoric acid(1%), L- Ascorbic acid and Distilled water. These reagent and chemical are used in a pure state (Table 1).

## **Microorganisms**

Five microorganisms representing Gram-positive and Gram-negative bacteria were used. The two gram-positive bacteria were *Staphylococcus aureus* and *Bacillus subtilis* and the three gram-negative bacteria were *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*.

## **Antimicrobial activity**

### **Agar well diffusion method**

Antibacterial activities of all the extracts (ethyl acetate, methanol, aqueous) of *A. vasica* were determined by agar well diffusion method (Chauhan, Neha *et al.*, 2012). In this method DMSO (dimethylsulphoxide) was dissolved in the extract to obtain 0.5mg/100µl and 1mg/100 µl concentrations. Commercial antibiotic (Gentamicin) and DMSO was taken as positive and negative control respectively. The test was done in triplicates and the final results obtained were presented as the mean zone of inhibition (Sawhney *et al.*, 2011)

### **Broth dilution MIC tests (NCCLS, 1993)**

This test was done to check the zone of inhibition which was minimum. This process is named as macro broth dilution assay. Muller- Hinton broth diluents was taken and 2-fold serial dilutions of all extract were prepared in the well on the basis of result obtained from agar well diffusion method.

Gentamicin and DMSO are positive and negative control respectively. 20 µl of test culture at concentration ( $5 \times 10^5$  cfu/ml) which is standard was inoculated and plates were incubated for 24 h at 37°C plate with minimum growth was taken and concentration is noted as minimum inhibitory concentration. Another value minimum bacterial count was calculated by spreading 20µl of MIC test

broth on a new plate incubating for 18- 24 h at 37°C. Dilution of plate showing no single bacterial growth was taken as MBC concentration.

Triplicates were used to perform test and mean MIC and MBC value were calculated and noted (Chauhan neha *et al.*, 2012)

## **Phytochemical analysis**

Phytochemical analysis was done in accordance to (Sharma *et al.*, 2014).

### **Test for glycosides**

Take 1ml of plant extract and add few drops of sulphuric acids and the mixture was allowed to stand for some time, formation of Reddish precipitate that means Presence of glycosides was confirmed.

### **Test for carbohydrates (Molisch's test)**

Take 1ml of extract and add 2ml of Molisch's Reagent. Now to this mixture, 2ml conc. sulphuric acid was added along the sides of the test tube. Presence of carbohydrates was confirmed by formation of reddish violet ring.

### **Test for flavonoids (Aqueous test)**

Take 1ml of plant extract, 1ml of aqueous NaOH was added. Presence of flavonoids was confirmed by yellow colour formation.

### **Test for saponins (Aqueous test)**

Take 1ml of extract, 5ml water was added and shake well in test tube shaker. Presence of saponins was confirmed by Lather formation.

### **Test for tannins (Ferric chloride test)**

Take 1ml of plant extract, 1ml of ferric chloride was added. Presence of tannins was confirmed by the formation of greenish black colour.

### **Test for alkaloids (Dragondroff's reagent)**

Take 1ml of plant extract add 5-6 drops of dragondroff's reagent. Presence of alkaloids was confirmed by the formation of creamish/brownish-red/ orange precipitate.

### **Quantitative and qualitative analysis (Sharma *et al.*, 2014)**

Phytochemicals- Phenolics, Saponins, Flavonoids and Ascorbic acid Tannins and Alkaloids provide antibacterial properties to the medicinal plant against *S. aureus*, *S. typhi*, *E. coli*, *Bacillus* and *Pseudomonas*. Phenolics, Saponins, Flavonoids and Ascorbics were detected by Quantitative tests and Tannins and Alkaloids by Qualitative tests.

### **Test for total phenolic content**

Sidduraju and Becker method was used to estimate total phenolic content of *A. vasica* by using folin ciocalteace reagent. 1ml solution of 20 microgram leaf extract were prepared with distilled water.

Then in this two reagents were added- 500µl diluted folins- phenol reagent (1:1ratio with water) and 2.5ml of Na<sub>2</sub>CO<sub>3</sub> (sodium carbonate) 20%. Then it was shaken well and incubated for 40 min. in dark condition, for the development of colour. After it, the absorbance was measured at 725nm. Gallic acid calibration curve was constructed and linearity was obtained in the range of 10-50 µgm/l. Then standard curve was used to obtain the total phenolic content in the plant

extract which was expressed as mg. of Gallic acid equivalent (GAE/g extract).

### **Test for total saponin content**

It was estimated by the method described by Makkarte *et al.*, based on vallin-sulphuric acid colorimetric reaction with some modifications. About 50µl pf plant extract was added in 250µl of vanillin reagent (800mg of vanillin in 10ml of 99.5% ethanol) and was mixed.

Then 2.5ml of 72% sulphuric acid was added and it was mixed well. This solution was kept in a water bath at 60°C for 10min. after 10min. Ice cold water was used to cool it and the absorbance was measured at 544nm. The values obtained were expressed as diosgenin equivalent (mg DE/g extract) which was derived from a standard curve.

### **Test for total flavonoids content**

The total flavonoids content was estimated using the procedure described by Zhishen *et al.*, A total of 1ml of plant extract were diluted with 200µl of distilled water separately followed by the addition of 150µl of sodium nitrite (5%) solution.

This mixture was incubated for 5min. and then 150µl of aluminium chloride (10%) solution was added and allowed to stand for 6min. Then 2ml of sodium hydroxide (4%) solution was added and made up to 5ml with distilled water.

The mixture was shaken well and left it for 15min at room temp. The absorbance was measured at 510nm. Appearance of pink colour showed the presence of flavonoids content. The total flavonoids content was expressed as rutin equivalent mgRE/g extract on a dry weight basis using the standard curve.

## Results and Discussion

### Antibacterial activity

Various extracts of *A. vasica* leaves showed potent antimicrobial activity against the pathogens causing infections in humans. The E1 extract was found to be the most effective in suppressing the growth of selected pathogens as the values of ZOI were higher. S1 was the highest susceptible pathogen as the ZOI was 27mm (1mg/100µl) and least susceptible was S5 as ZOI was 20mm (1mg/100µl). S5 at 1mg/100µl (ZOI values) showed maximum susceptibility against E2 extract and S2 at 1mg/100µl (ZOI values) was least susceptible. E3 extract effectively inhibited S2 at 1mg/100µl (ZOI values) and least inhibition was shown against S4 (ZOI values). The data obtained from antimicrobial activity of E1, E2 and E3 extract against pathogens were tabulated in tables 3, 4 and 5. The extracts that showed high efficacy against selected pathogens were subjected to minimum inhibitory concentration (MIC) assay by two-fold serial dilution method (2:2) (9, 10) (Table 2).

### Phytochemical analysis of *Adhatodha vasica* leaves extracts

#### Qualitative analysis

Phytochemical analysis of *Adhatodha vasica* leaves in ethyl acetate extract exhibit the presence of Saponins, carbohydrates, flavonoids, tannins and alkaloids. Glycosides are absent in both the extracts. Methanolic extract also exhibit the presence of saponins, carbohydrates, flavonoids, tannins and alkaloids. Glycosides are absent in both the extracts. Aqueous extract exhibit the presence of saponins, carbohydrates, flavonoids, tannins and alkaloids. Glycosides are absent in both the extracts. Results are presented in table 6 (Fig. 2).

### Quantitative analysis

Phytochemical assessment of *A. vasica* plant leaves extracts was done using quantitative methods. The E1 extract showed the presence of phenol, saponins and flavanoids upon investigation (Table 7). Flavonoid was found to be the highest (0.106) followed by saponins (0.052) and phenol (0.023). The E2 extract possessed the highest amount of phenol (0.037) whereas saponins and flavonoids were found to be less in comparison (0.019). The E3 extract upon investigation revealed the presence of saponins (as 0.094) as highest followed by phenol (0.011) and flavonoids (0.009). These botanicals exhibit the potent inhibitory activity of leaves extracts against all the selected human pathogens.

### Antibacterial activity

*Adhatoda vasica* leaves extract were used to study for antibacterial properties of plant. *S. typhi* showed least influence against the all three extracts (E1, E2 & E3) of *A. vasica*.

E1 extract showed strong inhibitory activity against *E. coli* followed by *Bacillus*, but moderately inhibit the growth of *S. typhi* and *S. aureus* and was least effective against *Pseudomonas*. Maximum ZOI for E2 extract was shown by *S. typhi* followed by *S. aureus*, *E. coli*, *Pseudomonas* and *Bacillus*.

E3 extract effectively inhibit the growth of *Bacillus*, *S. aureus*, *Pseudomonas*, *E. coli* and *S. typhi*. In the study by Ramachandra *et al.*, 2013, done on antibacterial activity of extracts of *A. vasica*, methanolic extract was found to possess maximum antibacterial activity against *S. aureus* while in our study maximum inhibitory activity was recorded by E1 extract for *E. coli* (Tables 8–10 and Fig. 1).

**Table.1** The yield and physical properties of *A. vasica*

S.no	Solvent used	Yield (g/250ml)	Colour	State
1.	Ethyl acetate E1	11.016	Blackish green	Viscous
2.	Methanol E2	15.277	Blackish green	Viscous
3.	Water E3	16.755	Brownish	Solid

**Table.2** Antibacterial activity of Gentamicin

S.No	Microbial Culture	Concentration of the E1 extract	
		0.5mg/100µl	1mg/100µl
1.	S1	31	35
2.	S2	26	30
3.	S3	27	32
4.	S4	24	28
5.	S5	22	27

**Table.3** Antibacterial activity of *Adhatodha vasica* E1 extract

S.No	Microbial Culture	Concentration of the E1 extract	
		0.5mg/100µl	1mg/100µl
1.	S1	24	27
2.	S2	19	21
3.	S3	21	25
4.	S4	21	23
5.	S5	14	20

**Table.4** Antibacterial activity of *Adhatodha vasica* E2 extract

S.No	Microbial Culture	Concentration of the E2 extract	
		0.5mg/100µl	1mg/100µl
1.	S1	13	15
2.	S2	9	11
3.	S3	10	13
4.	S4	18	18
5.	S5	15	17

**Table.5** Antibacterial activity of *Adhatodha vasica* E3 extract

S.No	Microbial Culture	Concentration of the E3 extract	
		0.5mg/100µl	1mg/100µl
1.	S1	9	11
2.	S2	14	16
3.	S3	9	12
4.	S4	8	9
5.	S5	11	14

**Table.6** Phytochemical analysis of *Adhatodha vasica* leaves extracts

S. No.	Phytochemicals	Leaves		
		Ethanollic extract	Methanollic extract	Aqueous extract
1	Glycosides	-ve	-ve	-ve
2	Carbohydrates	+ve	+ve	+ve
3	Flavonoids	+ve	+ve	+ve
4	Saponins	+ve	+ve	+ve
5	Tannins	+ve	+ve	+ve
6	Alkaloids	+ve	+ve	+ve

**Table.7** Quantitative analysis of *Adhatodha vasica* different extracts

Extracts	Phytochemicals		
	Phenol	Saponins	Flavonoids
E1 (µg/ml)	<b>0.023</b>	0.052	<b>0.106</b>
E2 (µg/ml)	<b>0.037</b>	0.019	<b>0.019</b>
E3 (µg/ml)	0.011	<b>0.094</b>	0.009

**Table.8** The MIC, MBC and MIC Index values of ethyl acetate extract Against different pathogens

Organism	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
S1	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
S2	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
S3	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
S4	0.5- 0.0156	0.0156	0.0312	0.0625	0.125	2	2
S5	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2

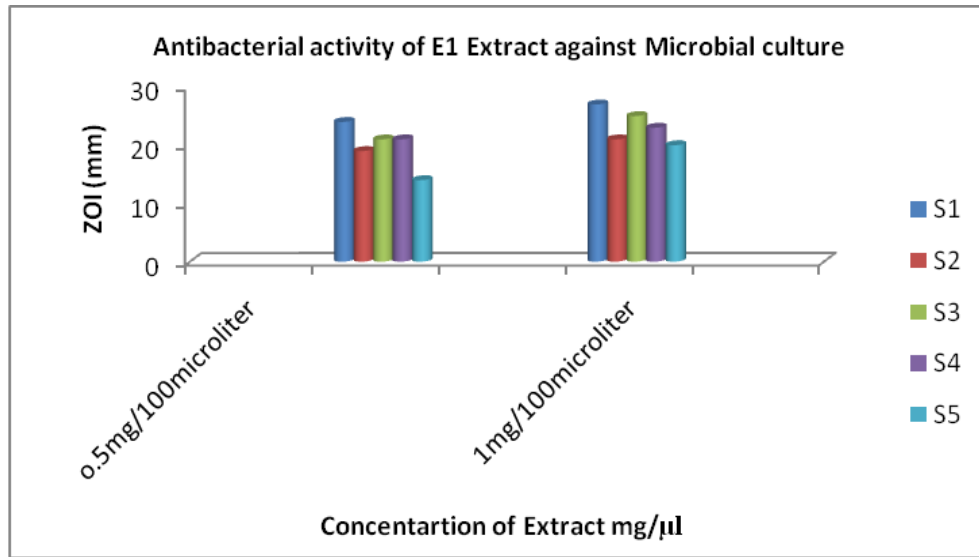
**Table.9** The MIC, MBC and MIC Index values of methanol extract against different pathogens

Organism	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
S1	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
S2	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S3	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S4	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S5	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2

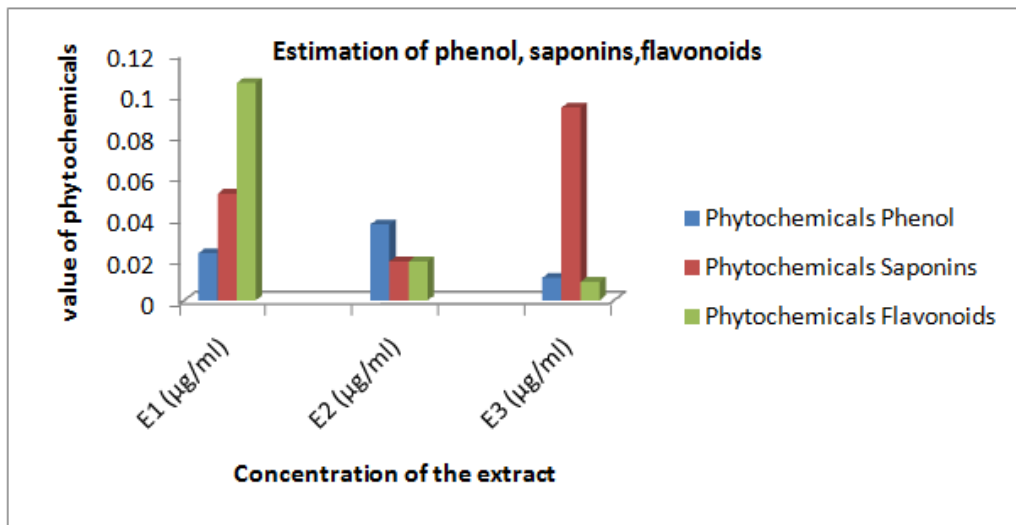
**Table.10** The MIC, MBC and MIC Index values of aqueous extract against different pathogens

Organism	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
S1	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S2	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S3	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S4	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2
S5	0.5- 0.0156	0.0156	0.0312	0.25	0.5	2	2

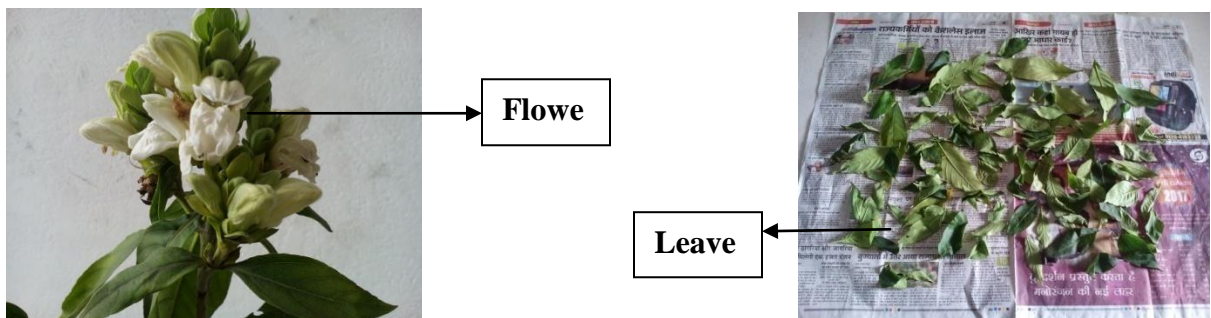
**Fig.1** The antibacterial activity of E1 extract of *A. vasica* against bacterial culture



**Fig.2** The presence of different phytochemicals in various extracts of *A. vasica*



**Plant material and extraction**





### MIC (Minimum Inhibitory Concentration)

MIC is the concentration of the extract at which there is maximum inhibitory activity against microorganisms. The extracts which showed maximum inhibition on microorganisms were subjected to minimum inhibitory concentration (MIC) assay by two-fold serial dilution method (2:2) (25:26). Lowest MIC value for E1 extract was 0.625mg/ml for *S. aureus*. E2 extract showed lowest MIC concentration for *E. coli* was 0.125mg/ml and the E3 extract showed lowest concentration of MIC for *E. coli*, *Bacillus*, *Pseudomonas* and *S. typhi* i.e. 0.125mg/ml. Elgal *et al.*, (2017) in their study done on *A. vasica*, showed that *E. coli* and *S. aureus* were highly susceptible against the ethanolic extract of the plant having MIC concentration of 3.125mg/ml followed by S3 (MIC=12.5mg/ml). While in our study the lowest MIC concentration was recorded against E1 extract for *S. aureus*.

### Phytochemical screening

The E1, E2 and E3 extracts obtained from the leaves of *A. vasica* were found to be strongly active against the selected human microbes. Microbes showed four type of bactericidal effects: 1. They inhibit cell wall synthesis 2. They stop microbial protein and nucleic acid synthesis 3. They disrupt microbial membrane structure and function 4. They block metabolic pathways through inhibition of key enzymes (Sawhney *et al.*, 2011). These pathogens cause various types of diseases in humans. In E1 extract Flavonoids content was found to be highest then saponins then phenolic. In E2 extract phenolic content was found to be highest then Flavonoids then saponins. In E3 extract saponins content was found to be highest then phenolic then Flavonoids of *A. vasica*. Presence of alkaloids, flavonoids, triterpenoids, tannins and glycosides was also confirmed by Ramachandran *et al.*, 2013.

In our study we have focused on searching the alternative means of therapy as herbal drug formulations for bacterial infections caused by Gram positive and Gram negative bacteria in humans. The phytochemicals present in herbal drugs possess the potentiality to cure various bacterial infections and have negligible or no side effects. Various extracts of leaves of *Adhatoda vasica* has exhibited effective antimicrobial activity against bacterial pathogens along with the significant phytochemicals that can further be explored to obtain and use its formulations as a cure for various ailments.

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