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Extraction, Characterization and Antibacterial activity of phytoconstituents from the leaves of *Adhatoda vasica*

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ABSTRACT
Medicinal plants play significant role in human health. Using synthetic pigments for the purpose of coloring food, clothes, fruit juices, paints are accepted worldwide previously, but due to hazardous impact of synthetic colors on environment and also on human health made to go for alternative sources of the pigments which are safe to use. The main objective of this research work was to extract the natural pigment which have valuable preference that will increase the supply of pigment from natural sources while minimizing environmental and health risks. The leaves of Adhatoda vasica was extracted for chlorophyll pigments from natural ecological source. Extraction of pigment was performed to obtain the crude extract. The crude extract were purified by flash column chromatography using different solvent as mobile phase. The preliminary phytochemical results demonstrated the presence of active substances including phenol, carbohydrate, glycosides, phytosterols, tannins, flavanoids, alkaloids, terpenoids and saponins. HPTLC fingerprinting was perfomed to check the presence of phytoconstituents in selected plant sample. Fourier transform infrared spectroscopy used to analyze the functional group of a band at 2989 cm^{-1}, 2995 cm-1, 2994 cm^{-1}, 2988 $cm^{-1}$and 2909 cm^{-1} associated with the stretching of C-H bonds. The pigment showed varying degree of inhibition on the test organisms(Bacillus cereus, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa). The maximum antibacterial activity of methanol extract of Adhatoda vasica was found at 18 mm against Bacillus cereus. The results showed that selected plant extract like Adhatoda vasica could revealed positive outcomes for phytochemical presence and antibacterial activity.
Key words: *Natural pigment, Flash column chromatography, FTIR spectroscopy, HPTLC fingerprinting analysis, Antibacterial activity*

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INTRODUCTION

Plants are vital to the survival of life on the planet. The manufacturing of natural colourants is expanding all around the world. Plant pigments are distinct compounds that give plants their distinctive colour by absorbing various wavelength of light. Chlorophyll is green photosynthesis pigment that contains a long hydrophobic phytol chain[1]. Extraction is required for the preparation of specific pigments as well as for gaining natural colorant extracts. *Adhatoda vasica* contains a number of bioactive compounds that can reveal health-promoting effects, including flavonoids, polyphenols, saponins and nitrate[2]. Phytochemical screening revealed the presence of tannins, saponins, flavonoid, alkaloid and phenolic component in plant. A comprehensive assortment of phytoconstituents in different extracts through HPTLC fingerprinting profiles displayed the existence of chlorophyll containing compounds. Infrared spectroscopy is a technique based on the vibrations of the atoms of a molecule and it is used to identify the functional properties of selected plant material. [3]. The antibacterial activities of *Adhatoda vasica* extract was investigated in vitro against various pathogen such as *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus* and *Escherichia coli*[4].

MATERIAL AND METHODS

The plant material for the proposed study such as *Adhatoda vasica* was collected from serenity botanical garden. The Processing of plant material such as washing, drying, grinding and storage was carried out at laboratory. Then physicochemical parameters like odor, taste, color, moisture content, total ash value and extraction yield were also observed in *Adhatoda vasica.* Determination of moisture content[5] and total ash value was calculated to check the purity of sample.
Extraction Process

The dehydrated plant material was grind using mortar and pestle to obtain fine powder and then it was passed through 1 mm sieve. To obtain crude extracts, 10 gram fine powders of both plant materials were soaked in 100 ml of various solvents such as methanol, acetone, chloroform, dichloromethane, ethanol and water separately for 5 to 7 days [6]. The plant material was filtered and the remaining solid was extracted to remove all the remaining liquid. The obtained liquid was purified by filtration. The solvent was extracted using rotatory vaccum evaporator under reduced pressure. The dried extract was placed in an airtight containerandkeptat4°Cuntilfurtherexamination[7]. The yields of weighted extracts were kept in small bottles at refrigerator (4 °C). Yield percentages were calculated using the following formula:

$$\text{Extract yield } \% = \text{weight of dried extract /weight of dried leave} \times 100$$

The crude extract of selected plant material was purified by flash column chromatography. It was performed by the use of silica gel particles (60-120 mesh) using n-hexane and acetone solvent system. Pressurized gas (10psi to 15 psi) was used to drive the solvent through the column of stationary phase. Several thin layer chromatographies (TLC) using different mixture of hexane: acetone were done to determine the ideal parameters for flash chromatography. The best separation obtained with the use of hexane: acetone (2:1 v/v). To examine the numerous chemical groups found in extracts, qualitative preliminary phytochemical experiments were performed [8]. Secondary metabolites in *Adhatoda vasica* leaf extracts included alkaloids, flavonoids, saponins, polyphenols, tannins, terpenoids and glycosides [9].
HPTLC studies were carriedout using the standard method described by Wagner *et al*. 10ulofsample were loaded in silica gel TLC plate. The samples loaded plate was kept in TLC twin trough developing chamber (after being saturated with solvent vapour) with respective mobile phases, namely toluene- acetone-formic acid (4.5 : 4.5 : 1) for flavonoids and Ethyl acetate-methanol-water (10:1.35:1) for alkaloid[10]**.** The plate was developed up to 90mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photodocumentation chamber and the images were captured under visible light, UV 254 nm and UV 366 nm. The peak table, peak display and peak densitogram were noted [11]**.** Plant pigments were analyzed by FT-IR for the detection of functional groups (Hancock & Fabbri, 2011).
The test organisms such as *Bacillus cereus* (MTCC 736), *Staphylococcus aureus* (MTCC 2408), *Escherichia coli* (MTCC1650), *Pseudomonas aeruginosa* (MTCC 424) were used for the analysis of antibacterial activity. After 24 hours of incubation, zone of inhibition was measured. The diameter of zone of inhibition can be measured in millimeters. Negative controls were plates with dimethyl sulfoxide reagent and positive control were plates with chloramphenicol antibiotic solution [12].

RESULTS AND DISCUSSION

The several physicochemical features of plant powder was noted. The following table-1 shows the observed outcome:

Table 1: Moisture content and ash value of *Adhatoda vasica*

Plant	Moisture content (%)	Ash value (%)	Acid insoluble ash	Water soluble ash
Adhatoda vasica	3.95 %	15.05 %	0.95 %	6.7%

Table 2 displays the extraction yields as well as the physical properties of plant extracts. Extraction yields achieved in Methanol, Acetone, Chloroform, Petroleum ether solvent systems of 21.65 %, 14.03 %, 12.57 % and 11.09 % respectively. The extraction yield of chloroform extract of *Adhatoda vasica* was the lowest at 11.09 %. Methanol extract had the highest extraction yield of 21.65 %. Physicochemical properties of Plant extract such as colour and feeling of touch were also observed.

Table 2. Physical characteristics and % yield of Extract: *Adhatoda vasica*

Plant	Solvent	Colour of Extract	Sense of Touch	% Yield
Adhatoda vasica	Methanol	Green	Sticky	21.65 %
	Acetone	Green	Sticky	14.03 %
	Chloroform	Green	Sticky	12.57 %
	Petroleum ether	Light green	Sticky	11.09 %

The presence of significant phenolic compounds, saponins, tannin,flavonoid and other substances were identified in the plant sample by phytochemical assay.

Table 3. Preliminary tests for *Adhatoda vasica* extract.

Phytoconstituents		AVA	AVM	AVP	AVC
Alkaloid	Mayer's test	++	++	-	++
	Wagner's test	++	+	+	++
	Hager's test	+++	++	++	-
Carbohydrates	Molisch's test	++	++	+	+
	Fehling's test	+++	+	-	++
	Benedict's test	+	++	+	-
Saponins	Foam test	++	++	+	+
Proteins and anino acids	Millon's test	+	+	-	-
	Ninhydrin test	+	+	-	-
Phytosteroids and terpenoid	Libermann-Burchard's test	++	-	+	+
Fixed oil and fats	Spot test	+	+	+	+
Phenolic and flavonoids/Tannin	Ferric chloride test	++	++	++	+
	Gelatin test	+	++	+	+
	Lead acetate test	++	+	+	+
Diterpenes	Copper acetate test	-	++	-	++

+ indicates positive, ++ indicates moderate positive, +++ indicates Highly positive, - Indicates Absence

Alkaloid profile

A variety of extracts like methanol extract, acetone extract, chloroform extract and petroleum ether extract of *Adhatoda vasica* were used for HPTLC alkaloids profile that represented the presence of bands with Rf values ranged from 0.04 to 0.95, 0.04 to 0.9, 0.04 to 0.96 and 0.04 to 0.95respectively.

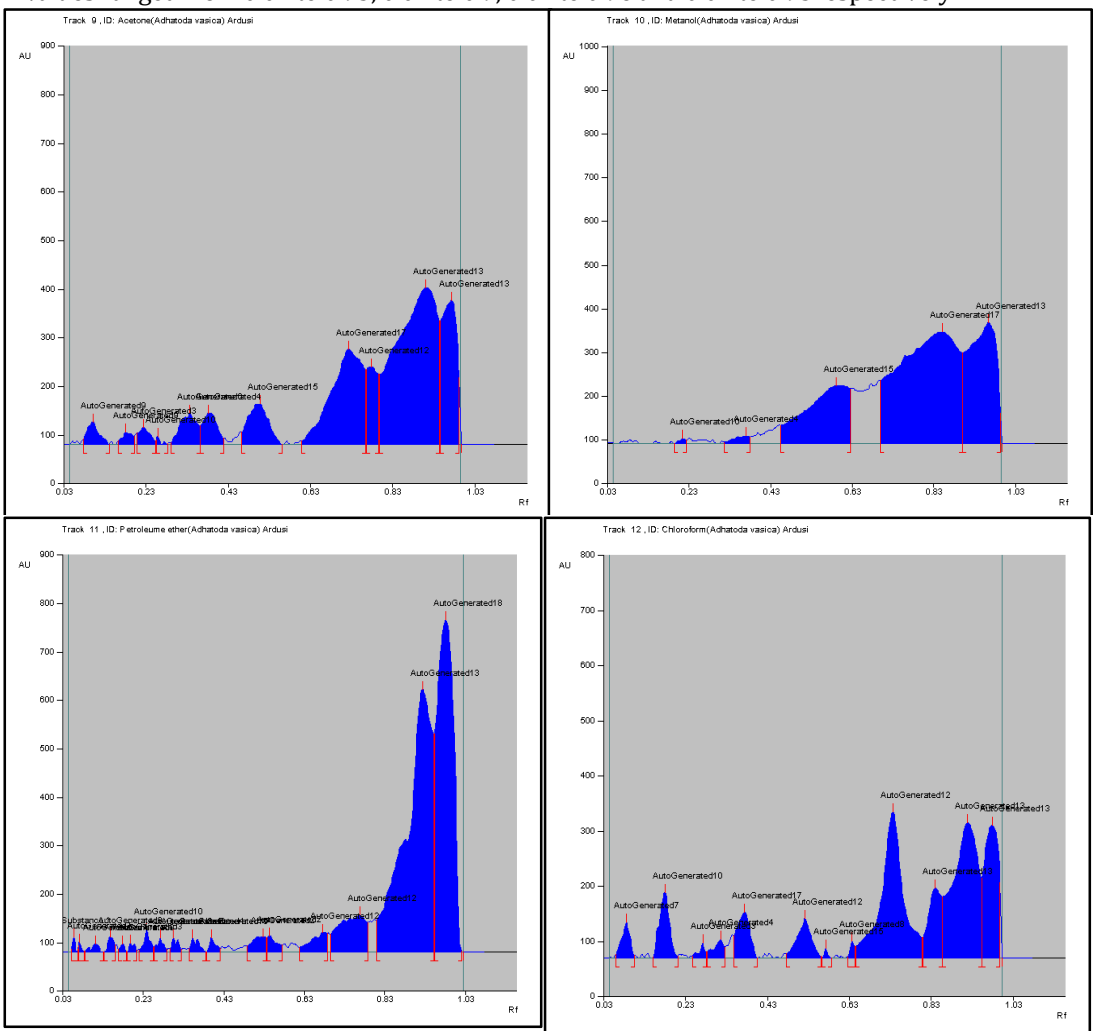

Figure 1: HPTLC peak densitogram of alkaloid profile (at 254 nm) of *Adhatoda vasica* plant pigment; AVM- methanol extract of *Adhatoda vasica* , AVA- acetone extract of *Adhatoda vasica* , AVC- chloroform extract of *Adhatoda vasica* , AVP- petroleum ether extract of *Adhatoda vasica*

Table 4. Peak table with retention factor (Rf) values of alkaloid compounds of *Adhatoda vasica* extract (AVA- Acetone extract of *Adhatoda vasica*, AVM- Methanol extract of *Adhatoda vasica*, AVP- Petroleum ether extract of *Adhatoda vasica*, AVC- Chloroform extract of *Adhatoda vasica*)

AVA		AVM		AVP		AVC	
Rf value	**Assigned substance**	**Rf value**	**Assigned substance**	**Rf value**	**Assigned substance**	**Rf value**	**Assigned substance**
0.22	Colchicine	0.22	Colchicine	0.09	Nicotine	0.22	Colchicine
				0.22	Colchicine		
0.23		0.23		0.31	Strychnine	0.41	Chelidonine
				0.41	Chelidonine		

HPTLC can be used as a phytochemical marker and is more effective in the field of plant taxonomy for secondary metabolite identification. The HPTLC results determined the presence of different types of alkaloids bands and validated different Rf values ranged from 0.04 to 0.96 (Table 4). The alkaloid band with Rf value 0.22 confirmed the presence of Colchicine, 0.31 confirmed the Strychnine, 0.09 confirmed the nicotine and 0.41 confirmed the Chelidonine in the AVA, AVM, AVP and AVC extract respectively.

Phenolic profile

Adhatoda vasica were used for HPTLC phenolic profile that represented the presence of bands with Rf values ranged from 0.09 to 0.91, 0.04 to 0.97, 0.07 to 0.75, 0.05 to 0.95 respectively.

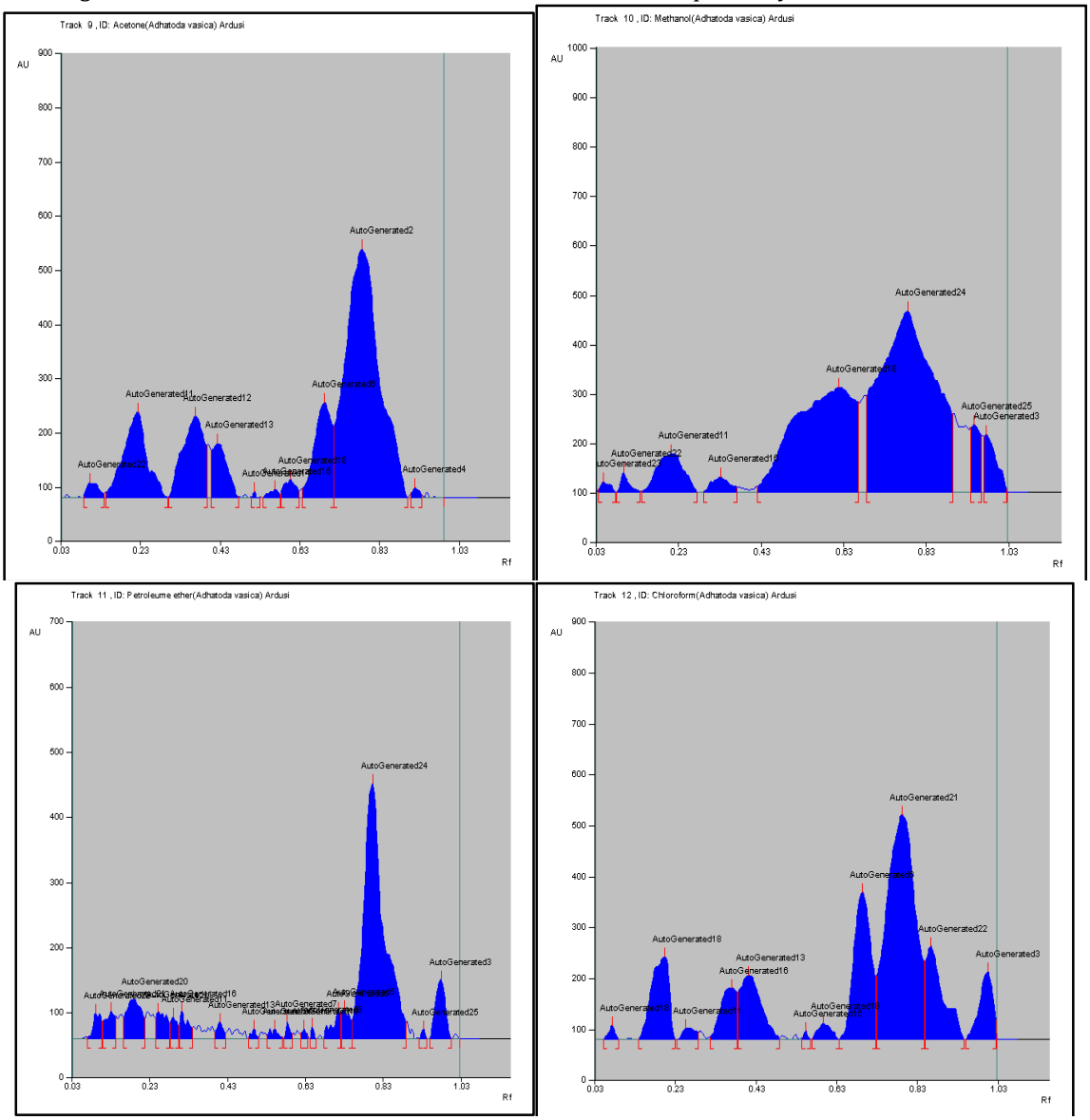

Figure 2: HPTLC peak densitogram of phenolic profile (at 254 nm) of *Adhatoda vasica* plant pigment; AVM- methanol extract of *Adhatoda vasica* , AVA- acetone extract of *Adhatoda vasica* , AVC- chloroform extract of *Adhatoda vasica* , AVP- petroleum ether extract of *Adhatoda vasica*

Table 5. Peak table with retention factor (Rf) values of phenolic compounds of *Adhatoda vasica* extract (AVA- acetone extract of *Adhatoda vasica*, AVM- methanol extract of *Adhatoda vasica*, AVP- petroleum ether extract of *Adhatoda vasica*, AVC- chloroform extract of *Adhatoda vasica*)

AVA		AVM		AVP		AVC	
RF value	**Assigned substance**	**RF value**	**Assigned substance**	**RF value**	**Assigned substance**	**RF value**	**Assigned substance**
	Phenolic 8		Catechin	0.43	Phenolic 5	0.05	Phenolic 2
0.72		0.28		0.49	Phenolic 6	0.26	Phenolic 4
				0.72	Phenolic 8	0.49	Phenolic 6
0.73		0.29		0.75	Quercetin		

The HPTLC results determined the presence of different types of phenolics bands and validated different Rf values ranged from 0.09 to 0.95 (Table 5). The phenolic band with *Rf* value 0.75 confirmed the presence of quercetin and 0.28 confirmed the catechin in the AVM and AVP extract.

Flavonoid profile

Adhatoda vasica were used for HPTLC Phenolic profile that represented the presence of bands with Rf valuesrangedfrom0.08 to 0.92, 0.12 to 0.91, 0.13 to 0.91, 0.12 to 0.92respectively.

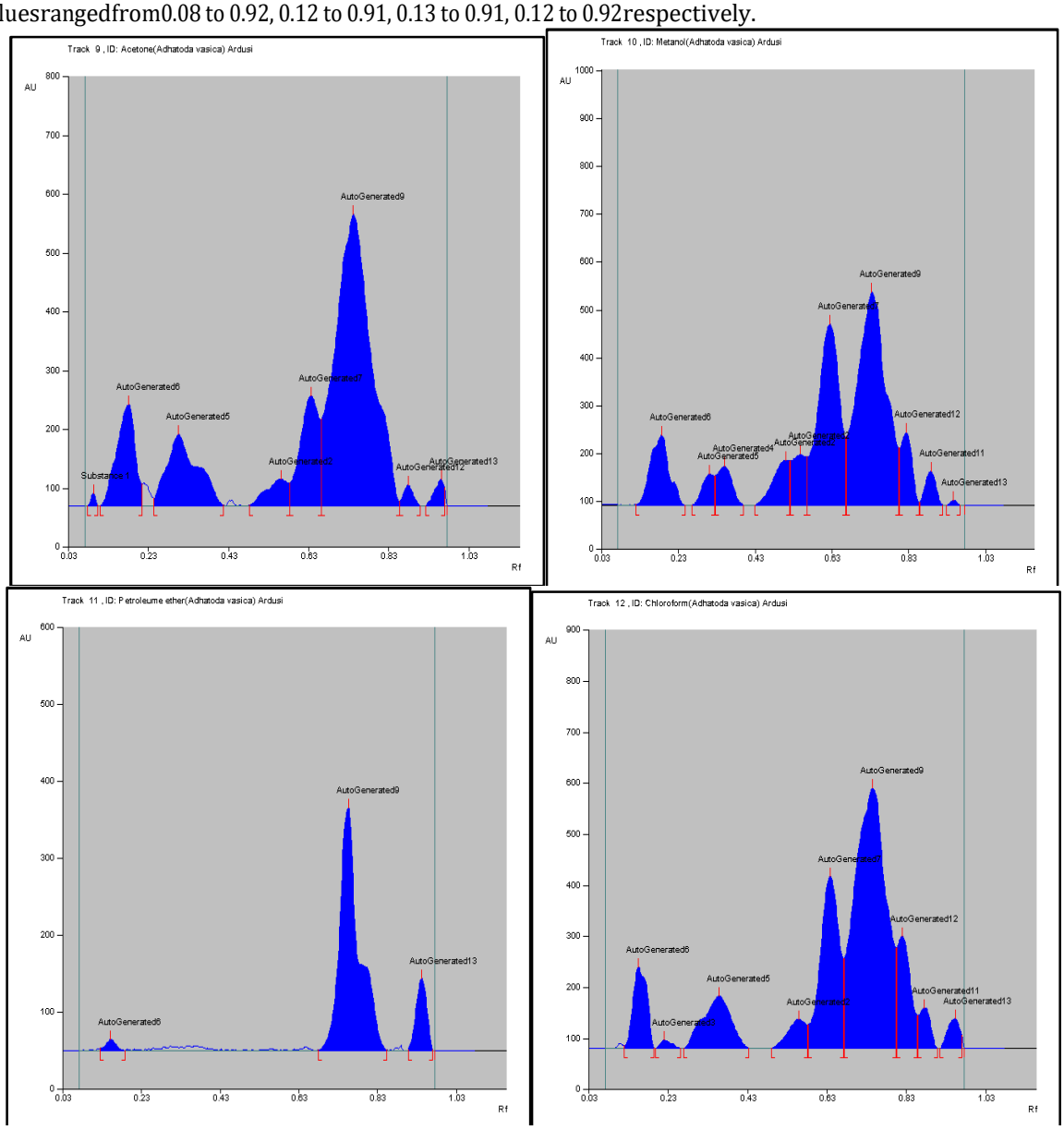

Figure 3: HPTLC peak densitogram of flavonoid profile (at 254 nm) of *Adhatoda vasica* plant pigment; AVM- methanol extract of *Adhatoda vasica* , AVA- acetone extract of *Adhatoda vasica* , AVC- chloroform extract of *Adhatoda vasica* , AVP- petroleum ether extract of *Adhatoda vasica*

Table 6. Peak table with retention factor (Rf) values of flavonoid compounds of *Adhatoda vasica* extract (AVA- acetone extract of *Adhatoda vasica*, AVM- methanol extract of *Adhatoda vasica*, AVP- petroleum ether extract of *Adhatoda vasica*, AVC- chloroform extract of *Adhatoda vasica*)

AVA		AVM		AVP		AVC	
RF value	**Assigned substance**	**RF value**	**Assigned substance**	**RF value**	**Assigned substance**	**RF value**	**Assigned substance**
0.18	Rutin	0.36	Flavonoid 4	0.68	Flavonoid 6	0.68	Flavonoid 6
0.68	Flavonoid 6						

The HPTLC results also determined the presence of different types of flavonoid bans and validated different Rf values renged from 0.08 to 0.92 (Table 6). The flavonoid band with Rf value 0.18 confirmed the presence of rutin in the AVA extract.

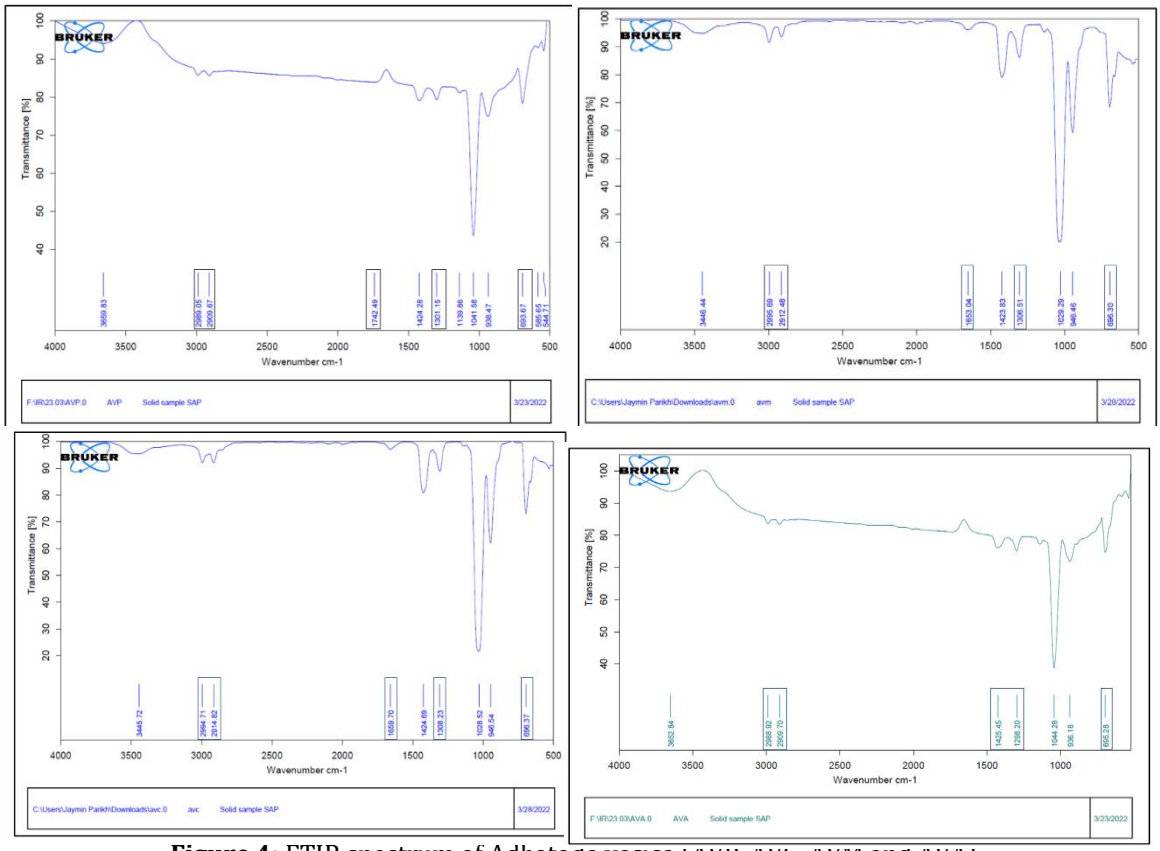

Figure 4: FTIR spectrum of Adhatoda vasica (AVP, AVC, AVM and AVA)

Table 7: FTIR spectrum range of *Adhatoda vasica* extract

Extract	Functional group	Frequency (Cm^{-1})
AVP	C-H alkane (stretching)	2989, 2909 (3000-2840)
	C=O stretching (Ester)	1742 (1750-1735)
	C-N stretching (aromatic amine)	1301 (1342-1266)
	C=C bending (alkene)	693 (730-665)
AVM	C-H alkane (stretching)	2995, 2912 (3000-2840)
	C=N stretching (imine / oxime)	1653 (1690-1640)
	C-N stretching (aromatic amine)	1306 (1342-1266)
	C=C bending (alkene)	696 (730-665)
AVC	C-H alkane (stretching)	2994, 2914 (3000-2840)
	C=N stretching (imine / oxime)	1659 (1690-1640)
	C-N stretching (aromatic amine)	1308 (1342-1266)
	C=C bending (alkene)	696 (730-665)
AVA	C-H alkane (stretching)	2988, 2909 (3000-2840)
	C-H bending (bending)	1425 (1450)
	C-N stretching (aromatic amine)	1298 (1342-1266)
	C=C bending (alkene)	695 (730-665)

The FTIR spectra obtained for the extract showed all characteristic bands of chlorophyll. The spectrum shows a band at 2989 Cm^{-1}, 2909 Cm^{-1}, 2995 Cm^{-1}, 2912 Cm^{-1}, 2994 Cm^{-1}, 2914 Cm^{-1}, 2988 Cm^{-1} and 2909 Cm^{-1} associated with the stretching of C-H bonds. At 1742 Cm^{-1} was observed a charactersticband of the C=O stretching vibration and at 1301 Cm^{-1} , 1306 Cm^{-1}, 1308 Cm^{-1} and 1298 Cm^{-1}were observed a characteristic band of the C-N stretching (aromatic amine group). A band at 695 Cm^{-1} and 696 Cm^{-1} corresponding to C=C bending (alkene group). A band at 1653 Cm^{-1} and 1659 Cm^{-1} corresponding to C=N of aeromatic ring stretching vibration.

The antibacterial activity of different extract of *Adhatoda vasica* was tested against bacterial pathogens such as *Bacillus cereus* (MTCC 736), *Staphylococcus aureus* (MTCC 2408), *Escherichia coli* (MTCC1650) and *Pseudomonas aeruginosa* (MTCC 424). The specific zone of inhibition against various types of pathogenic bacteria was shown in Table.

Table 8: Zone of inhibition of *Adhatoda vasica* extract against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*

Sample	Zone of inhibition (mm)																			
	Escherichia coli					*Pseudomonas aeruginosa*					*Staphylococcus aureus*					*Bacillus cereus*				
	2 mg	4 mg	6 mg	8 mg	10 mg	2mg	4 mg	6 mg	8 mg	10 mg	2 mg	4 mg	6 mg	8 mg	10 mg	2 mg	4 mg	6 mg	8 mg	10 mg
AVA	-	10	11	12	13	10	10	11	12	13	10	11	12	13	14	11	12	16	17	18
AVM	10	12	13	15	16	11	12	13	14	15	11	12	13	14	15	11	13	16	17	18
AVP	09	11	12	13	14	09	10	11	12	13	09	10	12	13	14	10	10	15	15	16
AVC	09	11	12	13	15	11	12	12	13	14	11	12	13	14	15	12	14	17	18	19
Positive control	14	16	18	19	21	15	17	18	19	21	15	16	18	19	21	16	18	21	23	24

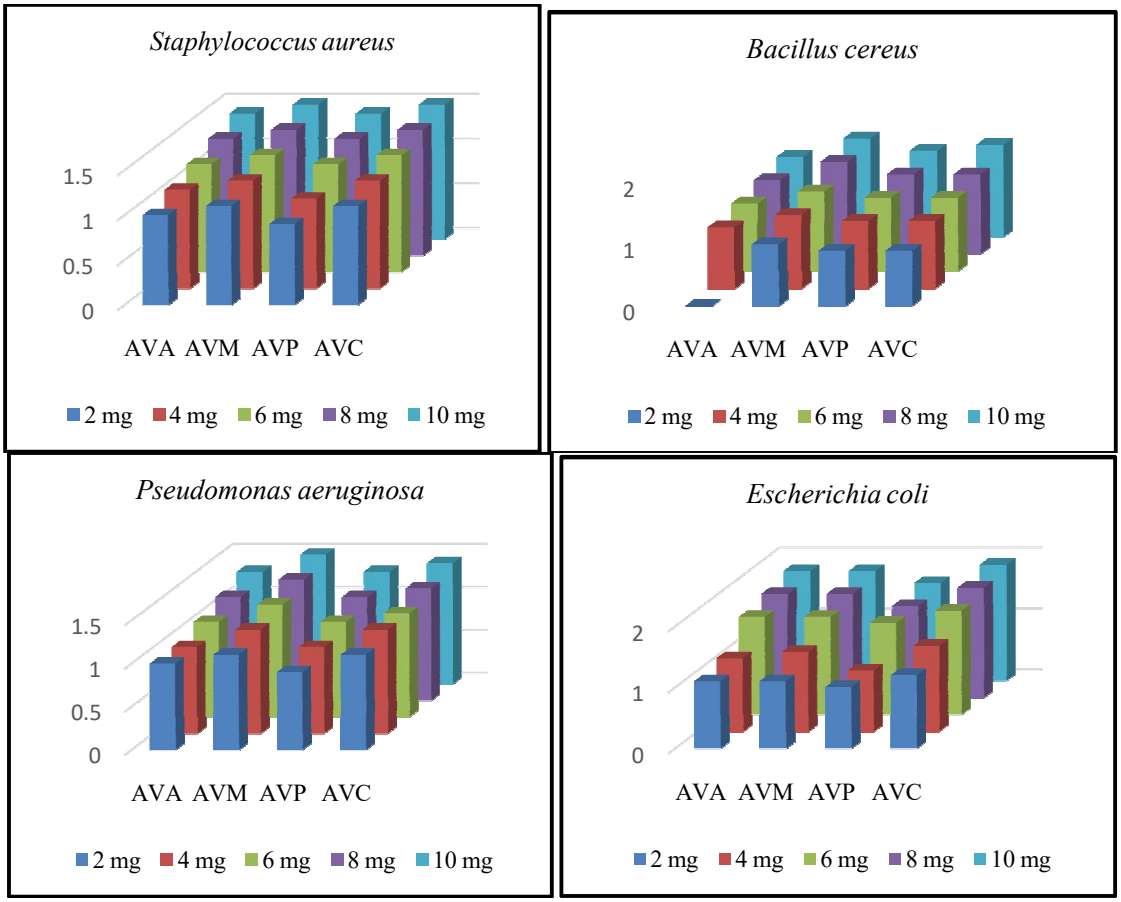

Figure 5: Zone of inhibition of methanol extract, acetone extract, chloroform extract and petroleum ether extract against *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa and Escherichia coli*

Among these, the methanol extract was found more effective against all selected bacteria. The maximum antibacterial activity of methanol extract of *Adhatoda vasica* was found at 18 mm against *Bacillus cereus* (MTCC 736) and minimum 9 mm against selected pathogen. Minimum antibacterial activity was observed in acetone extract and petroleum ether extract of *Adhatoda vasica*.This may be the indication of the broad spectrum of antibiotic compounds present in the pigment due to the use of different solvents.

CONCLUSION

The methanol extract of *Adhatoda vasica* was found to have the highest effective and was also found the maximum extraction yield being a polar solvent. According to the results of this study, it is indicated that the all the extracts derived from the *Adhatoda vasica* plants include numerous phytochemicals such as alkaloid, phenol, tannin, flavonoid, saponin and others. HPTLC fingerprinting confirmed the presence of some flavonoid and alkaloid compound in different extract of selected plants which might be responsible for stability properties of plant pigment. The plant pigment and isolated compounds exhibit higher antibacterial activity against gram-positive and gram negative bacteria so the plant pigment can be used as an easily accessible source of natural antibacterial agent.

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