STUDIES ON CHROMATOGRAPHIC FINGER PRINT ANALYSIS AND ANTIBACTERIAL ACTIVITY OF *ADHATODA VASICA* LEAVES EXTRACTS

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Summary

Yields of the hot aqueous, methanolic and chloroform extracts of *Adhatoda vasica* leaves were found to be 10.36, 19.10 and 18.22 per cent, respectively. Qualitative phytochemical studies revealed the presence of alkaloids, flavonoids, saponins, sugars, tannins and glycosides in different extracts. Densitometric HPTLC analysis for finger-printing profile of the total hot chloroform and hot aqueous extracts of *Adhatoda vasica* revealed the presence of both the similar and different phytoconstituents in these extracts. Antimicrobial screening of hot aqueous, methanolic and chloroform extracts at 125, 250 and 500 mg/ml concentrations by disc diffusion assay method (25 µL/disc) against selected Gram positive (*Staphylococcus aureus* -MTCC 7405, *Bacillus* sp. -MTCC 4666) and Gram negative (*E. coli* – MTCC 1680, *Klebsiella* sp.- MTCC 4032) bacteria revealed that methanolic extract was moderately effective against *Staphylococcus aureus* and the zones of inhibition at 250 and 500 mg/ml concentrations were found to be 12.33±0.88 and 14.00±0.57 mm, respectively compared to the zone of inhibition of 19.33±0.57 mm of 0.02µg levofloxacin against *Staphylococcus aureus.* But hot methanolic extract almost lacked any such activity against rest of the three microbes. Hot chloroform and hot aqueous extracts were also found to be almost devoid of any antibacterial activity against these microbes. Therefore, results of the present study suggest that HPTLC and finger-printing profile should always be used for quality assurance of the plants-based formulations and *Adhatoda vasica* leaves do not possess any promising antibacterial activity worth exploiting in future drug-development programmes.

Keywords: *Adhatoda vasica,* HPTLC, Finger-printing, antibacterial activity, phytochemistry

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Introduction

Herbal drugs have been used by different civilizations in different parts of the world for centuries to treat different diseases in human beings and animals. Many of the traditional medicines are still included as part of the habitual treatment of various maladies (1). About 60 % of the total global population remains dependent on traditional medicines for their healthcare system (2). In India, thousands of plants are known to have medicinal values and use of their different parts to cure specific ailments has been in vogue since ancient times (3) because these are considered as potentially safe drugs. It is well known that some of the synthetic drugs have their origin from plant products (4) and recently, scientific interest in medicinal plants has burgeoned due to their better efficacy and ever increasing side effects of modern medicine.

Extensive and injudicious use of antibacterials over the last 50 years has lead to emergence of bacterial resistance and dissemination of resistance genes among pathogenic microorganisms. Therefore, to overcome the emerging menace, there seems an immediate need and also vast scope for antimicrobials of plant origin as screening of plant extracts and their products for antimicrobial activity has revealed that plants seem to be potential source of novel and future antibiotic prototypes (5, 6).

Adhatoda vasica is a well-known plant in Ayurvedic and Unani medicine primarily for respiratory disorders and there is an ancient Indian saying "No man suffering from phthisis need despair as long as the *Adhatoda* vasica plant exists" (7) and its medicinal properties are known in India and several other countries for thousands of years. On bibliographic survey of the total extracts of this plant, it was apparent that not much of information is available on fingerprinting and antimicrobial activity of the total extracts of leaves in different solvent systems. Therefore, in the present work we report the phytochemical and fingerprinting of phytoconstituents and antibacterial activity of Adhatoda leaves extracts.

Materials and Methods

Plant material and preparations of extracts

Dried leaves of *Adhatoda vasica* L. were purchased (Indian Drug Company, Delhi) and their identity was confirmed based on the taxonomy. Fifty gm of coarse powdered leaves (prepared in an electric blender) were packed in a thimble and placed in soxhlet appartus. Different solvents (water, methanol and chloroform) were used for extraction. Crude extracts obtained after hot percolation were concentrated to dryness with the help of rotatory evaporator under reduced pressure at $<40^{o}$C and the yields were determined. All the extracts were kept in air-tight containers and stored at 4 ^{o}C for further studies.

Phytochemical screening

Qualitative phytochemical analysis of total extracts was carried out using standard procedures to detect flavonoids, alkaloids, triterpinoids, glycosides, steroids, saponins, fixed oils and fats, proteins, tannins, phenolic compounds (8).

HPTLC analysis

HPTLC analysis of *Adhatoda vasica* leaves extracts (chloroform and aqueous) was performed using Camag HPTLC system (Switzerland) comprising of Linomat 5 applicator, TLC scanner, twin trough developing chamber (20 x 10 cm), and Cats 4 software.

The chromatographic analysis was performed using aluminium sheets precoated with silica gel 60 F254 (10 x 10cm) as stationary phase. Aliquots of 4–8 µl of extracts (1 mg of extract

dissolved in 10 ml solvent) were spotted as sharp bands of 6 mm width using Linomat 5 applicator under nitrogen stream at spraying rate 6 s/ µl. A space of 8 mm was maintained between two bands. Mobile phase used for chloroform extract was Ethyl acetate: Chloroform: Petroleum ether in the ratio 2:2:5 v/v and for aqueous extract was n-butanol: water: acetic acid: formic acid in the ratio 10:5:5:2 v/v. The plates were developed using ascending technique to a distance of 7.5 mm. After development, the plates were dried using hot air dryer and densitometric measurements were performed at 254 and 366 nm in absorbance mode.

Antibacterial activity

Pure certified cultures of microbes, namely *Escherichia coli* (MTCC 1680), *Klebsiella pneumoniae* (MTCC 4032), *Bacillus sp.* (MTCC 4666) and *Streptococcus aureus* (MTCC 7405) were procured from IMTECH, Chandigarh and used for determining the antibacterial activity of test extracts. The growth media employed in the present study included Hi-Veg Nutrient Agar and Hi-Veg Nutrient broth (Hi-Media). The media were sterilized by autoclaving at 121 °C and 15 psi for 30 minutes.

Antibacterial activity was determined by disc diffusion method as described by Taylor *et al.* (9). The standard inoculum suspensions were swabbed over the surface of media. The oven-dried discs were impregnated with 25 µl of the leaf extracts and placed on the surface of medium. After an incubation period of 24 hour in BOD incubator, the diameters of inhibition zones around the discs was measured in mm (growth free zone). Levofloxacin (0.2 µg) was used as the positive control while methanol and chloroform as the negative controls.

Results and Discussion

Phytochemical screening

Percent yields of the *A. vasica* leaves extracts varied with the solvents used. It was least in hot aqueous medium (10.36) while highest in methanol (19.10) and chloroform (18.22); thus revealing that maximum phytochemicals are eluted in hot organic solvents compared to hot aqueous extract. Preliminary phytochemical analysis of the hot methanolic, aqueous and chloroform total extracts revealed the presence of alkaloids, saponins, sugars and glycosides in hot methanolic extract, tannins and sugars in hot aqueous extract while flavonoids, tannins and sugars in hot chloroform extract (Table I).

HPTLC analysis

TLC profile of chloroform and aqueous extracts of *Adhatoda vasica* leaves showed the presence of number of bands at 366 nm as shown in Fig 1 A and B, respectively, however, the separated bands in HPTLC plate of the same extracts were not very distinct at 254 nm. Densitometric measurements of these plates using Camag TLC scanner at 254 and 366 nm showed five distinct peaks in chloroform extract (Fig. 2,3) while five major and two minor peaks in aqueous extracts (Fig. 4,5) and the R_f values of different peaks observed in chloroform and aqueous extracts are summarized in Table II.

Table I: Chemical constituents present in different crude extracts of *Adhatoda vasica*

Type of extract	Chemical constituents							
	Alkaloids	**Flavonoids**	**Saponins**	**Sugars**	**Tannins**	**Glycosides**	**Fixed oils**	**Protein & A. Acids**
Hot Methanolic	+	-	+	+	-	+	-	-
Hot aqueous	-	-	-	+	+	-	-	-
Hot chloroform	-	+	-	+	+	-	-	-

Table II: R_f values of different peaks observed in chloroform and aqueous extracts of *Adhatoda vasica* leaves

Serial Number Resolved Bands	R_f value	
	Chloroform extract	Aqueous extract
1	0.18	0.14
2	0.27	0.22
3	0.33	0.31
4	0.63	0.37
5	0.73	0.43
6	-	0.78
7	-	0.81

Apparently, five distinct bands were observed in chloroform extract and seven bands in aqueous extracts. Figures 2 and 3 illustrate the HPTLC spectrum of chloroform and Fig. 4 and 5 show the HPTLC spectrum of aqueous extracts of leaves the test plant at 254 and 366 nm, respectively. Different peaks in the spectrum and their respective Rf values indicate the presence of different constituents in the extracts.

Three bands with the respective Rf values of 0.14, 0.22 and 0.78 or 0.81 in the aqueous extract of *Adhatoda vasica* leaves observed in the present study were very close to the Rf values of 0.12, 0.24 and 0.79 reported in HPTLC atlas of Ayurvedic single plant (*Adhatoda zeylanica*) drug mentioned in Ayurvedic Pharmacopoeia Volume III and IV, submitted to WHO by Central Council for Research in Ayurveda and Sidha, Department of AYUSH, Govt. of India (10). Thus suggesting that some of the phytoconstituents present in different species of the same genus may be similar while they also differ from one another as is evident from the present study. However, it is also true that the same Rf values always do not necessarily mean identical phytochemicals if the same chromatographic conditions are not used (11). Based on these observations, it may not be unreasonable to deduce that HPTLC and finger printing data should be used for quality-assurance of herbal drugs having the same medicinal plants or the same active phytoconstituents in medicinal plants-based formulations.

Further research work on separation of different fractions using column chromatography, determination of their other desirable pharmacological activities, HPTLC profile and phytochemistry of different fractions and HPLC and NMR studies for detection of active compounds is in progress.

Antimicrobial activity

Data on the antibacterial activities of different extracts (methanolic, chloroform and aqueous) of *Adhatoda vasica* leaves against *Staphylococcus aureus, Bacillus* sp. *E. coli* and *Klebsiella pneumoniae* are presented in Table 3. It is evident from the results of zone of inhibition that methanolic extract was moderately effective against *Staphylococcus aureus* (14.00±0.57 mm) and poorly against *Bacillus sp.* (8.67±0.88 mm) and *Klebsiella sp.* (8.33±0.33 mm). But aqueous and chloroform extracts of the test plant and also the negative controls (chloroform and methanol) failed to exhibit any antibacterial activity at all against all the four tested microbes as summarized in Table III.

Table III: Antibacterial activity of 25 µl disc load of different extracts of *Adhatoda vasica* against certain Gram-positive and gram-negative bacteria

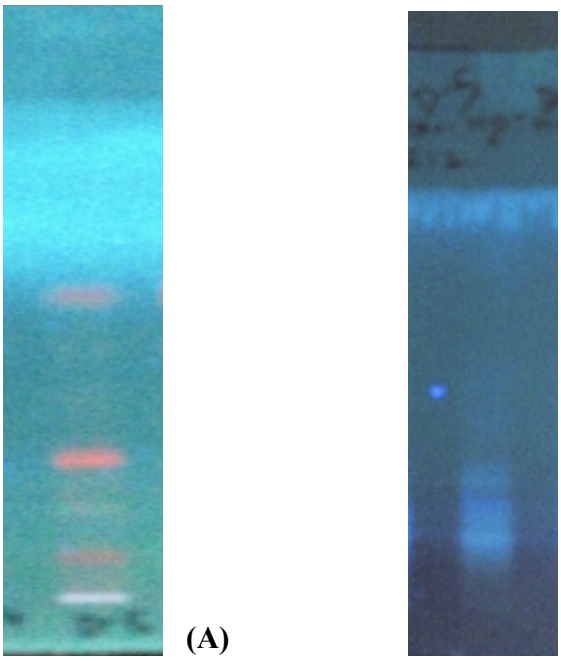
Test extracts/ drug/vehicle	Test organism(s)	Zone of inhibition (in mm) at different concentrations of extracts		
		125 mg/ ml	250 mg/ ml	500 mg/ ml
Hot aqueous	*E. coli*	-	-	-
	Klebsiella	-	-	-
	Staphylococcus	-	-	8.33±0.33
	Bacillus	-	-	-
Hot methanolic	*E. coli*	-	-	-
	Klebsiella	-	-	8.33±0.33
	Staphylococcus	-	12.33±0.88	14.00±0.57
	Bacillus	-	-	8.67±0.88
Hot chloroform	*E. coli*	-	-	-
	Klebsiella	-	-	-
	Staphylococcus	-	-	-
	Bacillus	-	-	-
Levofloxacin (0.02 µg)	*E. coli*	17.33±1.45		
	Klebsiella	14.67±0.88		
	Staphylococcus	19.33±0.57		
	Bacillus	14.66±1.20		

No zones of inhibition were observed in negative controls i.e. chloroform and methanol.

Our findings about antibacterial activity of extracts of *Adhatoda* are in agreement with the observations of Dey *et al.* (12) as they also failed to observe any antibacterial activity of the aqueous extract against *Staphylococcus, Bacillus* and *Pseudomonas* while methanolic extract exhibited low activity against *E. coli, Klebsiella and Vibrio cholera.* But these are in contravention of the established antimicrobial activity against *Stahylococcus*, *Bacillus*, *Proteus* and *Candida* (13) and strong antimycobacterial activity of the natural alkaloids isolated from *Adhatoda* leaves (14). Therefore, from the results of present study and also reported by others, it can be reasonably inferred that *Adhatoda* total extracts do not seem to possess strong

antibacterial activity worth exploiting in drug-development programme. However on fractionation and purification, certain active principle(s) may be obtained with promising antimicrobial activity and provide some lead molecule(s) for future antibacterial drugs. Therefore, further studies are needed to isolate and elucidate the structure of active compounds present in different extracts of this plant.

Fig. 1: HPTLC plate showing different bands of phytoconstituents present in crude chloroform (A) and aqueous extracts (B) of *Adhatoda vasica* leaves at 366 nm.



(A) **(B)**

Fig. 2: HPTLC spectrum of hot chloroform extract of *Adhatoda vasica* leaves at 254 nm (in blue) and the red line depicts the base line.

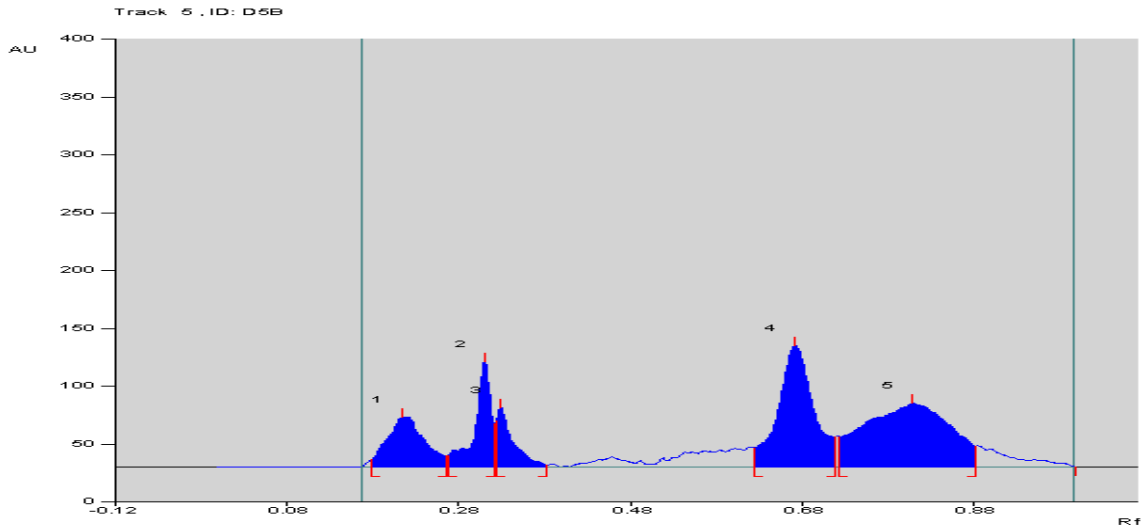
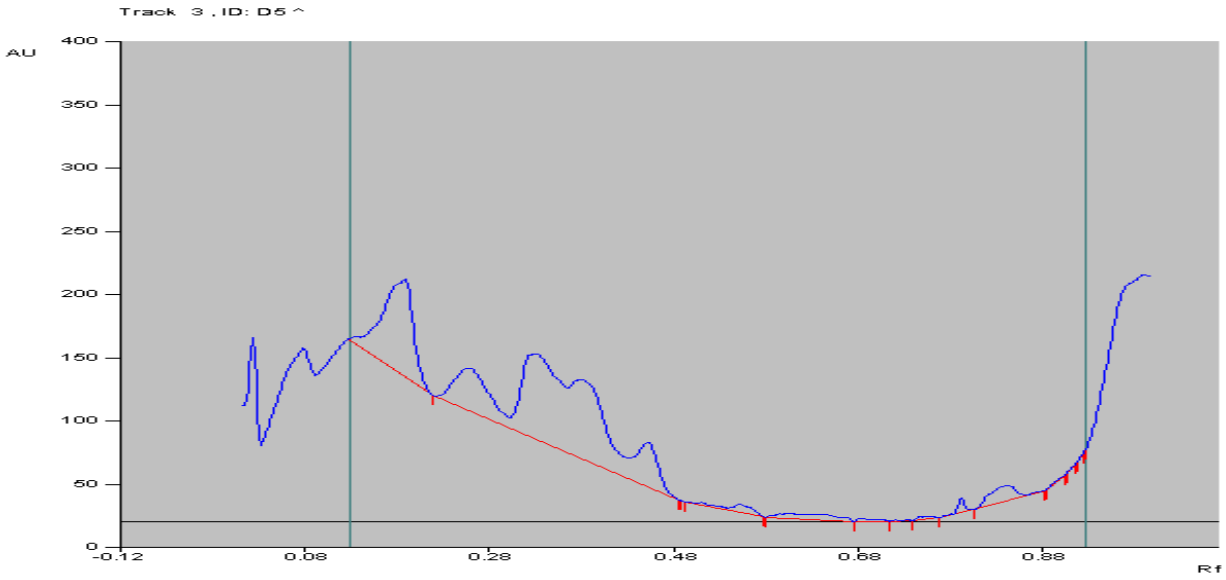


Fig. 3: HPTLC Spectrum hot chloroform extract of *Adhatoda vasica* leaves at 366 nm (in blue) and the red line depicts the base line.



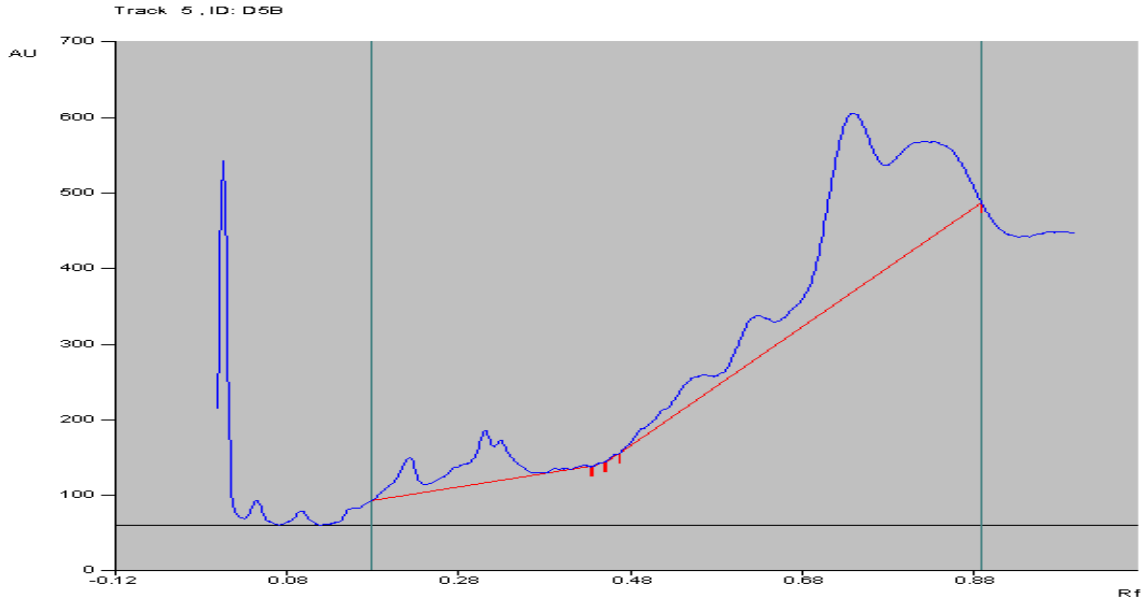


Fig. 4: HPTLC spectrum of hot aqueous extract of Adhatoda vasica leaves at 254 nm (in blue) and the red line depicts the base line.

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