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Original Article

Isolation and characterization of Vasicine from *Adhatoda vasica* (Adusa)

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Abstract:
The research work is related to an improved process of isolation & characterization of Vasicine from *Adhatodha Vasica* (Sanskrit: Adusa). *Adhatodha vasica*, commonly known as Arusa, is a valued herb in Ayurvedic medicine. Roots, leaves and preparations of the plant are traditionally used as tonic, antiasthmatic, analgesic, anti-inflammatory and diuretic. *A. vasica* mainly contains Vasicine alkaoids including Vasicine which are specific to the Acantheceae family. Vasicinosides are biologically active secondary metabolites present in roots and leaves of A. vasica. In the present study, we have standardized the protocol for the isolation of Vasicine from the Adhatodha vasica punchang. Vasicine possess anti-inflammatory and anti-stress properties. This study contains newer and conventional method of isolation of Vasicine from *Adhatodha vasaka* as well Quantitative and qualitative techniques involved in purification of compound was followed throughout this research work. In this study, we have taken different trials based on hydro-alcoholic solvent composition. Based on different solvent extraction process pure 95% Vasicine was successfully isolated.

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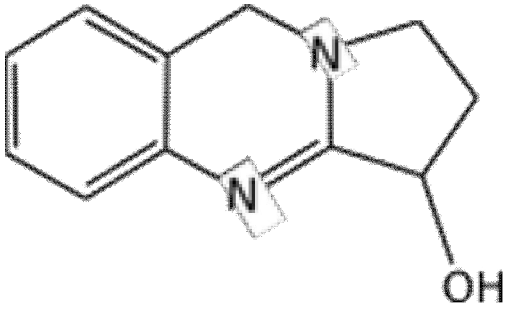
Introduction

Adhatoda vasica, generally known as Vasaka, is vital therapeutic plant that has been utilized as part of Ayurvedic & indigenous pharmaceutical for more than 3,000 years. In perspective of its changed restorative potential, it has additionally been subject of impressive present day logical consideration. Vasaka leaves are constituent of more than 200 definitions in Ayurveda, Siddha & Unani prescription, which are utilized as part of treatment of different physiological issue. Vasica shows up in WHO monographs on Selected Medicinal Plants & American Herbal Pharmacopeia monograph is additionally prospective.

In Ayurveda, Vasica is generally guaranteed to have powerful love potion, soothing, rejuvenate & life dragging out properties. It is additionally utilized as general vitality improving tonic known as Medharasayana, which implies that which advances learning & decent memory & in geriatric issues. Plant was generally used to advance energetic life, continuance, quality & wellbeing, sustaining time components of body & expanding creation of key liquids, muscle fat, blood, lymph, semen & cells. Comparability between these therapeutic properties & those of ginseng roots has prompted to Vasaka roots being called Malabar nut.

The flowers are either white or purple in color. Its trade name Vasaka is based on Sanskrit name [1]. Inflorescences in axillary spicate cymes, densely flowered; peduncles short; bracts broadly ovate, foliaceous. The leaves, flowers, fruit and roots are extensively used for treating cold cough, whooping cough, as sedative, expectorant, antispasmodic [2], chronic bronchitis [3] and asthma.



Vasicine Alkaloid

Figure 1: ***Adhatoda vasica*** **(Arusa)**

Common names – Arusa, Malabar nut

Family – Acanthaceae

Biological Source – It consists of fresh dried, leaves of *Adhatoda vasica.*

It grows in dry parts in sub-tropical regions. Rajasthan, Punjab, Haryana, Uttar Pradesh, Gujarat, Maharashtra & Madhya Pradesh.

Active constituents are Alkaloids: Vasicine, Vasicinone, Vasicinol, Vasicol etc.

Materials and Methods

(1) Plant material and Chemicals:

Roots and Leaves of *A. vasica* were collected directly from the forest of aravali Bharathari District Rajasthan. Materials were air dried at room temperature and powdered mechanically. The powdered of roots and leaves were used as the plant material for all analysis. Chemicals used in the process were listed in **table 1 and 2**.

(2) Isolation and Extraction:

Isolation was carried out by using newer and conventional method. 200 gms Vasaka punching (root and leave) powder in to 2 Liter Round bottom flask (R.B.F.). Then 750 ml petroleum ether was added to deffate the material. It was Refluxed at 60 ^{o}C for 90 min. after 90 min materials was filtrate and added further 800 ml of 75 % Methanol and reflux continue for four hours at 60 ^{o}C. After that, flask was allowed to stand for cool and filtered all solutions.

About 750 ml methanolic extract was collected, and transferred it into separation funnel. Now, extract with water (100 ml) followed by dichloromethane (3×200ml) and collect the lower layer separate out in to 1 litre beaker (total 600ml solution). Now about 8 to 10gms of activated charcoal were added into beaker and place beaker on water bath for 10 min.

Then, filter well by using Whattmann filter paper. Yellowish color solution obtains after filtration, which allows evaporating on water bath in evaporating dish. Allowed to Complete dry it on water bath and then cool the evaporating dish for 10 min at room temperature. A blackish colored semi-solid material was obtained at the bottom of the dish which was gummy in nature. Then, it was washed out with 70-80ml n-hexane and shake well, the dish, allowed to stand for 5 min, then decanted n-hexane in to other dish (because decanted hexane may contain powder.) 70ml n-hexane was added in to the dish which contained blackish material. Material was crushed by using mortar pastel to make dry powder. Yellowish-green powder obtains which was free flowing in nature. Obtained yellowish free flowing powder was subjected to thin layer chromatography (TLC) for Identification, and was subjected to High performance liquid chromatography (HPLC) for purification.

Methodology:

Pharmacognostical Evaluation

Data on outer morphology, life structures & micrometric information of composed plant material can be appropriately utilized as part of recognizable proof of plant material.

What's more, certain physico-synthetic parameters, for example, extractive esteem, fiery debris values & so on can give unpleasant thought regarding quality & virtue of unrefined medications.

Plant material

Fruits & stem roots of *Withania somnifera* & leaves of *Adhatoda vasica* were collected from Sariska, Bharahthari, District Alwar, and Rajasthan, India.

Identification

Plant materials were authenticated from Dr. Arvind S Dhabe Dr., Babasaheb Ambedkar Marathwada University, and Aurangabad. Collected plant material was dried in tray dryer at 55^{o}C for 24 h & powdered.

Preparation of herbarium

Herbarium was prepared per International curatorial practices. Collected specimens were placed in tray dryers carefully. Specimens were placed in plant press with uniform pressure. For 15 days, fresh dryers are used daily to remove moisture completely. After this, specimen was subjected for treatment with mercuric chloride in alcohol for about two minutes. Again, these specimens were dried. Specimens were spread on herbarium sheet of size 11.5X16 cm & stick with suitable adhesive. After identification, herbarium sheet was placed in species cover which was enclosed in genus cover. Specimen was given number by plant identifying authority is 0780 (reference number Bet/2010-11).

Preparation & storage

New products of soil barks were chosen for Microscopical review. Foods grown from ground barks were air dried & powdered by utilizing major metallic mortar & pestle took after by mechanical crushing. Powdered medications were then put away at room temperature & utilized for powder microscopy & extraction reason.

Morphological study:

Outer morphology was concentrated by strategies [4]. Simple microscope was used to carry out macroscopic study. Morphological parameters like shape, size, odour, colour & taste of fruits & stem bark were studied.

Microscopical analysis:

Fresh plant material was utilized to take free hand section of fruit as well as stem bark & it was then stained with several chemical staining agents according to optimized method. Disintegration of plant material was done by standard methods. In short scales were disintegrated by boiling in 5 % w/v aqu. Solution of NaOH for 5 min. After cooling it was washed with water, disintegrated parts were then reacted with 25% v/v aqu. Solution of chromic acid at room temperature for half hour. Pieces were cleaned by treating with solution of chloral hydrate & stained with mixture of toluidine blue & phloroglucinol-HCL (1:1) 5.

Powder plant materials of fruit & stem bark were separately reacted with phloroglucinol-HCL (1:1) solution, iodine & acetic acid solution to find out occurrence of lignified fibres, starch grains & Calcium oxalate crystals respectively. Series of digital images were taken by using Motic Digital microscope having 1/3'' CCD camera imaging accessory & with help of Motic Images 2000 (1.3 version) analysis software.

Fluorescence analysis

When fluorescence microscopic study is done for any herbal drug by using fluorescence microscope, it provides fluorescence emitted from tissues of herbal drugs under illumination. Herbal tissues contain many secondary metabolites or chemical structures & they have ability to emit light of specific wavelength.

Procedure: powdered samples were used for fluorescence analysis. On glass slide dry powdered sample of fruit & stem bark were separately taken & reacted with numerous drops of specified chemical reagents & within minute it was observed under UV lamp.

Physiochemical constants: [4, 6]

Ash Value

An ash value indicates contents of foreign matter in natural drug. By determining ash value traces of organic matter were removed which normally interfere with analytical determination. Generally, on incineration ash usually contains phosphates & carbonates along with calcium, potassium, sodium & magnesium in silicates format.

Determination of total ash

Accurately weighed amount (3gm) of medication was taken in silica pot & touched off at temperature not surpassing 450^{o}C. powdered drug was then spread in oven at bottom of tarred crucible. Then crucible containing drug was kept in muffle furnace & temperature was increased until drug becomes free from carbon. Crucible was removed from muffle furnace & kept in dedicator after cooling & note down weight. To get constant weight same procedure was repeated & calculated % of total ash with reference to air dried drug.

Determination of acid insoluble ash

Ash obtained in total ash determination was boiled with 25ml dilute HCL for 5 minutes & then filtered by using ash less filter paper. Insoluble ash on filter paper was further washed with hot water & transferred into tarred silica crucible. It was then lighted & weighed. Method was rehashed until two steady weights are gotten. % yield of corrosive insoluble fiery remains was then ascertained.

Determination of water soluble ash

Ash obtained from total ash determination was boiled with 25ml of water for 5 minutes & faltered through ash less filter paper.

Insoluble matter on fiery debris less channel paper was washed with boiling point water & touched off for 15 minutes at temperature not surpassing 4500c. Note down weight of insoluble matter after start & it was subtracted from weight of cinder to get genuine weight of water soluble ash. % yield was calculated with reference to air dried drug.

A. Elemental analysis of Ash:

Powdered drug material of fruit & stem bark were separately taken in muffle furnace & incinerated to get ash. It was then reacted with HCL (50%) for half hour & filtered to get filtrate, which is then utilized for elemental analysis by specific test. [4]

B. Test for Calcium

a. Test solution when treated with ammonium carbonate solution produces white precipitate, after boiling & cooling in ammonium sulphide solution it remains insoluble.

b. Test solution was reacted with potassium chromate gives yellow colour crystalline precipitate.

C. Test for Iron

a. Dilute HCL and potassium permanganate when mixed with test solution gives faint pink coloration.

b. Dilute HCL and solution of ammonium thiocyanate when mixed with test solution shows blood red coloration.

D. Test for Magnesium

a. Test arrangement in wake of overflowing with arrangement of ammonium carbonate gives white precipitation.

b. Test arrangement when blended with blend of weakens smelling salts & sodium phosphate arrangement gives white crystalline hasten.

E. Test for Potassium:

Test solution with perchloric acid gives white precipitation.

F. Test for sulphate:

a. Test arrangement & few drops of 5% $BaCl_2$ arrangement gives white crystalline BaSO4 hasten.

b. White lead acetic acid derivation reagent produces white hasten with test arrangement.

G. Test for phosphate:

Test arrangement arranged in HNO_3 when warmed with few drops of ammonium molybdate arrangement has not indicated yellow crystalline encourage.

H. Test for chloride:

a. Test arrangement arranged in HNO_3 when blended with few drops of 10% $AgNO_3$ Produces white accelerate.

b. Test arrangement with lead acetic acid derivation arrangement gives white hasten dissolvable in heated water.

I. Test for carbonate:

a. Test arrangement with weaken corrosive does not freed carbon dioxide.

b. Test arrangement with mercuric chloride arrangement does not create earthy red hasten.

c. Test arrangement with magnesium sulfate, white encourage was not framed.

J. Test for nitrate:

Test arrangement when warmed with H_2SO_4 and copper, no red exhaust where freed.

Moisture Content [4]

% w/w loss in weight resulting due to loss of water & volatile matter is called as Loss on drying. Drying can be done under definite conditions.

Accurately weighed amount (2gm) of each fruit & stem bark were separately taken on separate watch glass & kept for drying in hot air oven at 105^{o}c until two constant weights are achieved. Difference in weight represents moisture content of drug. Then % yield was determined & noted.

Extractive value [4, 6]

As per Indian Pharmacopoeial methods extractive values like water soluble extractive & alcohol soluble extractive values were determined.

A. Alcohol soluble extractive:

In closed apparatus air dried powder drug of fruit & stem bark were separately macerated with 100 ml of 90%v/v ethanol for one day. On later day, it was quickly filtered to avoid alcohol loss. In shallow evaporating dish with flat bottom precisely 25 ml of filtrate was taken & evaporated to dryness at about 105^{0}C & weighed.

B. Water soluble extractive

In similar way, water soluble extractive value was also determined. In short, in closed apparatus air dried powder drugs were macerated with 100 lm of water for one day with repeatedly shaking up to first 6 hours & allowed to keep as such without shaking for remaining 18 hours. On later day, it was quickly filtered to avoid alcohol loss. In shallow evaporating dish with flat bottom precisely 25 ml of filtrate was taken & evaporated to dryness at about 105^{o}C & weighed.

Extraction Methodology:

A. Choice of solvent for extraction:

Choice of solvent depends on nature of plant material & components to be isolated. Dried materials were usually powdered before extraction, whereas fresh plant (leaves, etc.) can be homogenized or macerated with solvent such as alcohol. Latter is also particularly useful for stabilizing fresh leaves by dropping them into boiling solvent [7].

Effective distinguishing proof of restoratively dynamic mixes from plant material is fundamentally reliant on dissolvable sorts utilized as part of extraction system., great dissolvable for plant extractions incorporates properties, for example, simplicity of vanishing, low poisonous quality, at low warmth, additive activity, advancement of quick physiologic assimilation of concentrate, failure to bring about concentrate to complex or separate. There are many variables that influences decision of dissolvable are amount of phytochemicals to be extricated, assorted qualities of various mixes removed, rate of extraction, simplicity of ensuing treatment of concentrates, differences of inhibitory mixes separated, poisonous quality of dissolvable in bioassay procedure, potential wellbeing peril of concentrates (Eloff JN, 1998). Decision of dissolvable is affected by what is proposed with concentrate. Since finished result will contain hints of remaining dissolvable, dissolvable ought to be non-dangerous & ought not to meddle with bioassay. Decision will likewise rely on upon focused on mixes to be separated [8].

Description various solvents used for extraction purpose are mentioned below.

1. **Water**: For extraction of plant material, water is universal solvent especially with antimicrobial activity. Primarily water is used by traditional healers but organic solvents have also used for plant extract & found to more consistent antimicrobial activity than water. Water soluble flavonoids do not have antimicrobial effects & water soluble compounds are important as antioxidant effect.
2. **Acetone**: Acetone is used to dissolve many hydrophilic & lipophilic compounds. It is miscible with water, having volatility & has low toxicity to bioassays used. This solvent is considered as very useful solvent for especially for antimicrobial study where more phenolic components are required in extract. In study, it was reported that extraction of tannins & other phenolic compounds was found to be better in aqu. Acetone than aqu. Methanolic extract [9].

   It was also found that both acetone & methanol is good solvent for saponins, which have antimicrobial activity [8].
3. **Alcohol**: Ethanoic extract was found to be more effective for polyphenols content as compared to aqu. Extract. It means they are more effective in seeds & cell walls degradation which possess unipolar character & responsible for release of polyphenols from cells. Diminish in action of aqu. Concentrate is because of nearness of chemical polyphenols oxidase which debase polyphenols from concentrate. While if there should be occurrence of methanol & ethanol it is latent. & water provides better medium for growth of microorganisms as compared to alcohol. Flavonoid is major group of compounds present in plants it was detected in higher concentrations with 70% ethanol due to its high polarity than pure ethanol. Polarity of ethanol can be increased by adding 30% of water in pure ethanol [10]. More ever, it was also found that ethanol easily penetrates cellular membrane for extraction of intracellular ingredients from plants materials [11]. Since all compounds identified from plants dynamic against microorganisms are sweet-smelling or soaked natural mixes, they are for most part gotten through underlying extraction by ethanol or methanol.

   Alcohol in many cases is good all-purpose solvent for preliminary extraction. Subsequently material can be macerated in blender & filtered, but this is only necessary if exhaustive extraction is being attempted. When isolating substances from green tissue, success of extraction with alcohol is directly related to extent chlorophyll is removed into solvent & when tissue debris, on repeated extraction, is completely free of green color. It can be assumed that all low molecular compounds have been extracted [12].

   Alcohol is general solvent for many plant constituents (accept most fixed oils) & as such many give problems in subsequent elimination of pigments, resins, etc. Water immiscible solvents are widely used besides light petroleum (essential & fixed oils, steroids) ether & $CHCl_3$ (alkaloids, quinines). Extraction of organic base generally requires basification of plant material if water immiscible dissolvable is to be utilized. For fragrant acids & phenols fermentation might be required.
4. **$CHCl_3$**: $CHCl_3$, hexane & methanol were used for successive extraction of terepenoids lactones with activity concentrating in $CHCl_3$ fraction. Even though tannins & terepenoids is found in aqu. Phase, but they are obtained by treatment with less polar solvents [13].
5. **Ether**: Commonly ether is used selectively for extraction of coumarins & fatty acids [13].

Table 1: Solvents used for extraction of active components [13]

Solvents	Active components
Water	Anthocyanins, Starches, Tannins, Saponins, Terepenoids, Polypeptides, Lectins
Ethanol	Tannins, Polyphenols, Polysaccharides, Flavonol, Sterols, Alkaloids, Terepenoids
Methanol	Anthocyanins, Terepenoids. Saponins, Tannins, Xanthoxylines, Totarol, Quassinoids, Lactones, Flavones, Phenones, Polyphenols
$CHCl_3$	Terepenoids, Flavonoids
Ether	Alkaloid, Terepenoids, Coumarins, Fatty acids
Acetone	Phenols, flavnols

Preparation of crude drug for extraction: [7]

Entire plant material was dried under shade & then coarsely powdered with help of mechanical grinder. Powder was passed through sieve No 40 & stored in air tight container for extraction.

Extraction procedure:

In Soxhlet apparatus about 50 g of powdered plant material was filled in cotton bag & consecutively extracted with help of different solvents. Different extracting solvents used were water, petroleum ether (60-80^{o}C), benzene, $CHCl_3$, ethyl acetate & methanol. Powdered material was getting dried before extracting with next solvent, to below 50^{o}C in oven. On these successive extracts, extractive values & preliminary phytochemical studies were performed.

Thin Layer chromatography [14-16]

Thin-layer chromatography was Carry out on a precoated silica gel 60F254 plate using *Vasicine* as a reference standard**. Mobile Phase:** Chloroform: methanol (9.0: 1.0), **Test solution:** To 3 g of the substance being examined, add 25 ml of *methanol*, heat on a water bath for 10-15 minutes, cool and filter, **Standard solution:** Dissolve 10 mg of *Vasicine* in 10 ml of methanol, **Procedure:** Apply 10 l each of the test and standard solutions on a TLC plate as bands of 10 mm. Develop the plate to a distance of 8 cm from the line of application. Dry the plate in air and examine under 254 nm. Spray the plate with a solution of *anisaldehyde sulphuric acid reagent* (figure 3.1) Heat the plate at 110 Celsius for about 5 minutes till the bands are clearly visible. Results are shown in table 3

High performance liquid chromatography [17-21]

High performance liquid chromatography was performed during different trials for isolation methods. HPLC analysis was performed on a Shimadzu LC-20AD pump system equipped with a Shimadzu SPD-20AT UV- Visible detector with the detection wavelength set at 230 nm and 20 L Rheodyne injector loop. A column was a reversed-phase (Luna C18 4.6 mm x 260 mm – particle size 5) eluted at a rate of 1.0 mL/min with a solvent system {acetonitrile: 1% Glacial Acetic acid – 6:4 (V/V)}. Sample was prepared in the HPLC grade methanol. Results are shown in table 4

Table 2: List of Chemicals Used in TLC and HPLC analysis

S. No.	Name of Chemicals	Grade of chemicals
1	Chloroform	HPLC grade, Merck
2	Toluene	AR grade, Merck
3	Methanol	HPLC grade, Merck
4	Acetonitrile	HPLC grade, Merck
5	Formic acid	AR grade, Merck
6	Glacial acetic acid	AR grade, Merck
7	Acetone	HPLC grade, Merck
8	Ethyl acetate	AR grade, Merck
9	Water for HPLC	HPLC grade, Merck

Results and Discussions

Vasicine was successfully isolated from the plant material of *Adhatodha vasica.* Isolated Vasicine was found to be above 90 % pure by HPLC analysis. Isolated Vasicines how single spot at same Rf value corresponding to Standard Vasicine. Thin layer chromatography and High performance liquid chromatography was performed for identification of final compound.

Silica coated TLC is convenient and suitable for the analysis of Vasicinnosides including Vasicine. It is often used to monitor fractions or finally purified Vasicinnosides. Chloroform - methanol (95:5) is frequently used solvent system for aglycones and chloroform - methanol (90:10) for glycosides.

The obtained purified compound is expected to be Vasicine, so the mobile phase for TLC of sample and standards were changed as chloroform: methanol in the ratio 9:1 with same spraying reagent. The single compounds were analyzed in TLC with standards Vasicine. The standard Vasicine showed the same Rf value as 0.65. From this result, it can be stated that the purified compound is Vasicine.

Results are shown in **table 3**.

Table 3: Results of TLC study in Rf value

TLC FIGURE NUMBER		RF VALUE DETECTION AT		
		368nm	251nm	After spraying
Figure 1	RS	-	0.64	0.62
	T	-	0.28, 0.45, 0.55, 0.66,0.72	0.28, 0.45, 0.55,0.66, 0.72, 0.78, 0.84,
Figure 2	RS	0.65	0.65	0.65
	T	0.65	0.28, 0.45, 0.54, 0.58, 0.65,0.76, 0.81, 0.82,0.84	0.28, 0.45, 0.58, 0.65,0.76

The purified compound was further confirmed by High Pressure Liquid Chromatography analysis with standard Vasicine as reference compound. Both standard and purified compound obtain peak at same retention time (3.957 min) as shown in **figure 2 and 3** respectively. Results of HPLC analysis are shown in below **table 4**.

Table 4: Results of HPLC study

Sample Name	Fig. No.	Retention Time	Peak Area	Wt/ml (mg/ml)	Conc. (ppm)	% Purity
Vasicine	3.2	8132.155	3.957	1mg/10ml	100ppm	97.00%
Test	3.3	7753.429	3.957	1mg/10ml	100ppm	92.48%

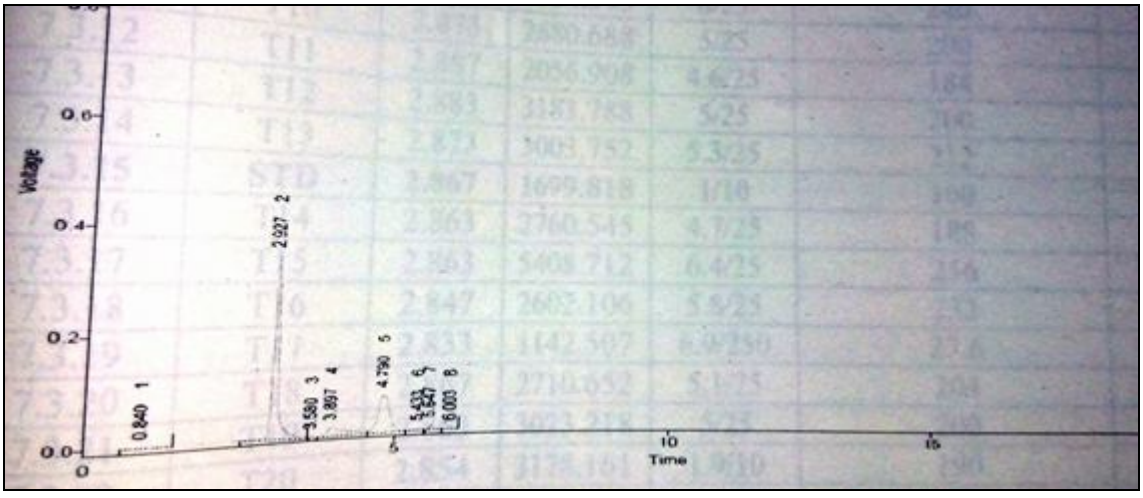


Figure 2: HPLC chromatogram of test Vasicine

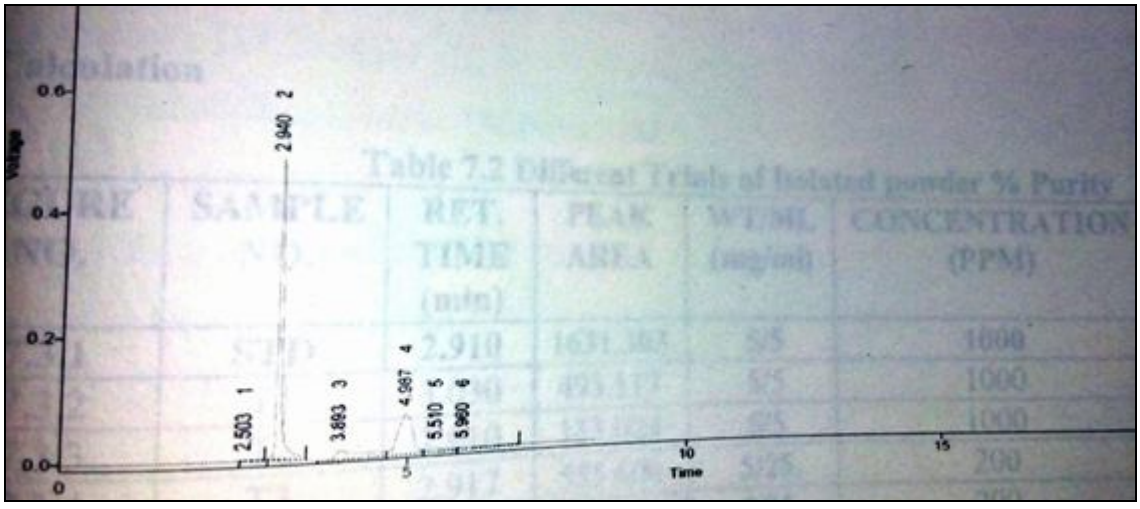


Figure 3: HPLC chromatogram of Sample Standard Vasicine (97%)

Conclusion

From the present study, it can be concluded that the *Adhatodha vasica* that contain Vasicine compound were eluted using simple techniques with less cost effect and they are quantified with the HPLC techniques. So, the obtained Vasicine compound will be used as the marker for analyzing the unknown compounds. 500 micro gram Vasicine compound obtains approximately from the 200 grams of the dried roots of *Adhatodha vasica.*

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Conflict of Interest: There is no conflict of interest

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