The Journal of Phytopharmacology 2024; 13(1):4-11
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2024; 13(1): 4-11
January- February
Received: 11-01-2024
Accepted: 27-02-2024

doi: 10.31254/phyto.2024.13102

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Evaluation of secondary metabolites and chromatographic fingerprint profiling of stem bark of *Holarrhena antidysenterica* L.

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ABSTRACT

Holarrhena antidysenterica is an important medicinal plant in the Ayurvedic system of medicine. The present study aimed to screen secondary metabolites qualitatively and quantitively and to develop chemical fingerprint profiles of stem bark of the target species. The phytochemical screening findings revealed that the methanolic extract of stem bark contained alkaloids, flavonoids, phenols, steroids, tannins, cardiac glycosides, and terpenoids. HPTLC fingerprint profiles were developed which were specific to the mobile phase Toluene: Ethyl acetate: Diethylamine (6.5:2.5:1 v/v/v) and Rf values. The fingerprints are a valuable tool for quality assurance since these can be used as biochemical markers to differentiate between authentic drugs and adulterants.

Keywords: Phytochemical screening, Secondary metabolites, Conessine, Chemical fingerprints, *H. antidysenterica*.

INTRODUCTION

Holarrhena antidysenterica L. belongs to family Apocynaceae and commonly known as Kutaj and Indrajava [1-6]. It is found in tropical and subtropical regions of Asia, Africa, Burma, Sri Lanka, Pakistan and in Nepal also. It is distributed in spacious level in India and spread throughout up to an altitude of 4000 feet in deciduous forest in Himalayas and open wastelands [7-9]. It is an aromatic, woody, evergreen and deciduous small shrub or tree [7]. Different parts of this plant such as bark, root, stem and seeds are used as medicine in several traditional systems including Unani, Ayurveda, British Materica Medica [10] and are popular in Tibetian culture also [11]. In the traditional Ayurveda diagnosis system, it is one of the important medicinal plants with enormous value [12]. It equilibrates the kapha and pitta Doshas of the human body. This tree's seed are used for many Ayurvedic formulations to treat anti-diabetic [13], flatulence, jaundice, piles [14], cancer [15], chronic fever [16], worms [17], increase appetite [18] and toning up vaginal tissues after delivery [19]. The root & stem bark of the tree is an astringent, antidontalgic, anthelmintic, stomachic febrifuge, diuretic, antidropsical, colic, piles, amoebic dysentery, dyspepsia, and gastric disorders like diarrhea and indigestion, also used as a remedy in skin, chest affection and spleen related diseases [20]. The Bodo tribes of Assam also use this species as a medicinal tree [8] and the tribal people of Andhra Pradesh used stem bark for treating skin diseases [21]. The rural people of Himalaya use bark of this plant to diagnose dysentery. Leaves of the tree are used as febrifuge [22]. According to Tripura Unakoti community, mixture of seed powder and bark's decoction of this plant has preventive effect on diabetes [23].

The plant exhibits lots of pharmacological properties, such as anti-diabetic [24], anti-diarrheal [25], anti-inflammatry, anti-analgesic [26], anti-oxidants or free radicals reducing properties [27], anti- urolithic, anti-haemorrhoidal [28], diuretic, anti-amoebiasis [29], anti-hyperlipidemic [30], anti-microbial, anthelminthic [31], anti-mutagenic, anti-hypertensive [32], anti- malarial [33], inhibition of acetyl cholinesterase & stimulation of Central Nervous System (CNS) [34]. This plant has anti-bacterial activities against *Staphylococcus aureus, Escherichia coli, Klebsiella* species, *Proteus* species, *Pseudomonas* species, *Salmonella* species and *Vibrio cholera* also [35]. Leaves and bark of this plant displayed hepatoprotective activity and induced hepatic injury in rats by decreasing the levels of SGPT, SGOT, ALP enzymes and bilirubin respectively [36, 37].

The plant bark mainly contains around 25 types of alkaloids such as regholarrhenine-A, regholarrhenine-B, regholarrhenine-C, regholarrhenine-D, regholarrhenine-E and regholarrhenine-F, pubescine, norholadine, pubescimine, kurchinin, kurchinine, kurchinidine, holarrifine, holadiene, kurchilidine, kurchamide, kurcholessine, kurchessine, conessine, isoconessimine and the steroidal compounds holadyson and kurchinicin [35]. The alkaloidal conessine is the main chemical ingredient of the stem bark and is responsible for dysentery and helminthic diseases [19]. Lupeol and β-sitosterol have been found in

the unsaponifiable matter of bark [38]. The annual demand of the stem bark of this plant is 500-1000 MT.

Looking to the pharmacological importance of this plant, the current study intended to perform the preliminary phytochemical screening, evaluation of secondary metabolites and chromatographic fingerprint profiling of its commercially utilized stem bark.

MATERIAL AND METHODS

Chemicals and reagents

Chemicals, reagents, and solvents used in this investigation were all of analytical grade. Reference standard conessine, colchicine, quercetin, cholesterol, gallic acid, and tannic acid were purchased from Sigma-Aldrich, India.

Collection and processing of plant materials

Stem bark sample of *H. antidysenterica* was collected from the Barela range of West Mandla forest division. The herbarium of plant specimen was submitted in FE & CC division of ICFRE-TFRI, Jabalpur and identification No. 1700 was obtained. The bark sample was cleaned to remove the dust and other extraneous matter. Bark was dried in sunlight and pulverized into fine powder which was stored in airtight containers for further phytochemical analysis.

Qualitative phytochemical screening

1 gram of the powdered and dried stem bark sample was immersed in 25 ml of methanol for overnight. After that, the extract was filtered, and a preliminary phytochemical study was performed on the filtrate. The standard methods were followed for the initial phytochemical screening of the stem bark methanolic extract [39-42]. Observations of colors during the different phytochemical tests are given in Table 1.

Table 1: Preliminary phytochemical analysis of methanolic extract of stem bark

Phytochemicals	Tests	Observations
Alkaloids	Dragandroff's Test	Cremish/Brown/Red Orange precipitate
Flavonoids	Alkaline reagent test	Formation of intense yellow color which becomes colourless on addition of dilute acid
Phenols	$FeCl_3$ test	Formation of bluish black colour
Steroids	Liebermann-Burchard test	Presence of blue-green colour
Tannins	$FeCl_3$ test	Presence of blue-green precipitate
Cardiac glycosides	Keller-Kilianni test	Blue colour produced
Terpenoids	Salkowski test	Formation of reddish-brown coloration
Saponins	Frothing test	Formation of stable foam

Quantification of secondary metabolites

Estimation of Total Alkaloid Content (TAC)

20 ml of 80% methanol was used to extract 0.1g of the sample. After that extract was filtered, the filtrate was centrifuged for 10 minutes at 4000 rpm. A volume of 2 ml was made up by adding 80% methanol to 0.1 ml of the supernatant. 2 ml of extract, 2 ml of 0.025M $FeCl_3$, in 0.05M HCl, and 2ml of 0.05M phenanthroline in ethanol make up the reaction mixture. Kept the reaction mixture in hot water bath at 70±2ºC for 30 minutes. At 510 nm, the red-colored complex absorbance was measured against the blank reagent. TAC, which is expressed as mg of colchicine equivalent per gram of dry extract weight (mg CE/g dry extract wt), was calculated using the colchicine standard curve [43].

Estimation of Total Flavonoid Content (TFC)

12.5 ml of 95% methanol was added to 0.5 g of the sample, and it was let to stand overnight. After filtering the entire contents, 80% methanol was added to make a volume of 25 ml. 0.03 ml of extract was taken in a test tube with 0.1 M potassium acetate, 10% aluminum chloride, and 2.5 ml of distilled water. The test tube was then allowed to incubate for 30 minutes at room temperature. At 415 nm, the absorbance of reaction mixture was measured. Using the quercetin standard curve, TFC was calculated. The eventual result was measured as mg of quercetin equivalent per g of weight of dry extract (mg QE/g dry extract wt) [44].

Estimation of Total Phenol content (TPC)

TPC was estimated by the Folin-Ciocalteau method. To 0.5g of powdered sample, add 25 ml of 80% ethanol and kept the reaction mixture in rotary shaker for 1 hr. The mixture was filtered. 0.03 ml of the extract and 0.5 ml of the Folin-Ciocalteau were taken and distilled water was added to make the volume up to 3 ml then the mixture was allowed to incubate for 3 minutes. Following the incubation, 2 ml of 20% Na_2CO_3 was added, thoroughly mixed on a vortex mixer, and then allowed to incubate in hot water for an additional 1 minute. At 650 nm, the reaction mixture's absorbance was measured against a blank. The gallic acid standard curve was utilized in the measurement of TPC. Gallic acid equivalent in milligrams per gram of dry extract weight (mg GAE/g dry wt) was used to express the results [45].

Estimation of Total Steroid Content (TSC)

2 gm of powdered sample in chloroform solvent was kept overnight in a conical flask. The mixture was filtered and the filtrate was evaporated on rotary evaporator up to dryness and further dissolved in 25 ml of chloroform solvent and this extract was used for the analysis.

Reagents-

1. Bloor's reagent: It was prepared by mixing 3 volumes of ethanol and 1 volume of diethyl ether
2. Acetic anhydride-Sulphuric acid reagent: Prepared fresh reagent by mixing 20ml acetic anhydride with 1 ml conc. Sulphuric acid
3. Chloroform solvent
4. Cholesterol standard (1mg/ ml): Prepared standard solution by dissolving 1 mg of cholesterol in 1 ml of chloroform and mixed well

Procedure-

5 ml of Bloor's reagent was taken in a conical centrifuge tube, added 1 ml sample, and mixed vigorously for 1 minute. The tube was left in a slanting position for 30 minutes to settle the precipitate. The supernatant was decanted and evaporated to dryness at rotary evaporator. The residue was dissolved in 5 ml of chloroform in a test tube and to this added 2 ml mixture of acetic anhydride - sulphuric

acid and kept in dark for 15 minutes for the development of color. Mixture of 5 ml chloroform and 2 ml of acetic anhydride - sulphuric acid was used as blank. Calibration curve was prepared by using different concentrations (100 – 500 µL) of standard solution. The emerald green color formed was measured at 680 nm with the blank reagent [46].

Estimation of Total Tannin Content (TTC)

A 0.5 g sample was immersed in 75 ml of distilled water and left to incubate for 30 minutes at 80±2^{0}C in a hot water bath. After letting the mixture cool, it was filtered, and distilled water was added to get the volume up to 100 ml. 1 ml of Folin-Denis reagent, 2 ml of Na2CO, and 0.03 ml of the filtrate were gathered, and the volume was made up to 3 ml using distilled water. The absorbance at 700 nm was measured using the reaction mixture in comparison to a blank. The results were expressed as mg of tannic acid equivalent per gram of dry extract weight (mg TAE/g dry wt). TTC was calculated using the tannic acid standard curve [47].

Estimation of Total Cardiac glycosides Content (TCgC)

Cardiac glycosides were quantified by following the method described by [48]. A 5 g sample was taken and added to 25 ml of distilled water. This was mixed with 10 g of H_2SO_4 (pre-diluted with 10 ml H_2O). After that, it refluxed for 6-8 hours. cooled and extracted with chloroform (2 x 25ml). After that, distilled water was used to wash the chloroform layer until it was completely clean. Transferred to a pre-weighed beaker then dried in an oven to get constant weight. Using the following formula, the amount of cardiac glycosides was determined:

$$\text{Percentage of Cardiac glycoside} = \frac{(B - A) \text{ X } 100 \text{ X } 2}{\text{Weight of sample}}$$

where,
B = Weight of beaker with residue
A = Weight of empty beakers

Estimation of Total Terpenoid Content (TTrC)

1g of precisely weighed sample was soaked in ethanol for twenty-four hours and then filtered. Three times, 10 ml of petroleum ether was used to separate the extract. After the petroleum ether extract had fully evaporated in a hot water bath, it was taken and weighed again in a glass beaker that had been previously pre-weighed [49].

Petroleum ether yield percentage calculated by the formula:

$$\text{Petroleum ether yield \%} = \frac{w_i - w_f}{w_i} \times 100$$

Where, w_i = Weight of sample
w_a = Weight of empty glass beaker weight
w_b = Weight of glass beaker with evaporated extract
$w_f = w_b - w_a$

Development of chromatographic fingerprints using high performance thin layer chromatography (HPTLC)

Sample preparation

1 g of crude powder of sample A (stem bark of *H. antidysenterica*) and Sample B (Adulterant *Wrightia tinctoria*) was dissolved in 10 ml of methanol separately. Both the samples were vortexed and sonicated for 30 mins. The supernatants were used as working solutions for the generation of HPTLC fingerprints.

Preparation of standard solution

To compare the presence of conessine content in methanolic extracts of the sample and adulterant, a solution of 0.1 mg/ml conessine in methanol was prepared.

HPTLC fingerprint analysis

With the help of a 100µL Hamilton syringe and LinomatV applicator attached to the CAMAG HPTLC system, which was programmed through Win CATS software, 5 µL of each solution i.e. sample, adulterant, and standard were applied in the form of bands of width 8 mm using a 100µL CAMAG syringe on a 10 x 10 cm aluminum packed TLC plate that had been prewashed with methanol and coated with a 0.2 mm layer of silica gel60F 254 (E. Merck Ltd., Darmstadt, Germany). 10 ml of Toluene: Ethyl acetate: Diethylamine (6.5: 2.5: 1 v/v/v) in a CAMAG twin-through glass chamber (10 cm x 10 cm) saturated with mobile phase and sealed with a stainless-steel lid was used to develop the samples loaded TLC plate by the ascending process. The developed TLC plate was dried with hot air to remove the solvent and derivatized with Dragendorff's reagent. The plate image was taken in visual light (Rwhite). A tungsten light source was used to perform densitometric scanning at λmax = 520 nm using a CAMAG TLC Scanner4 equipped with WinCATS software. Number of bands and their Rf values, color, intensity, and HPTLC chromatograms were recorded.

Statistical Analysis

The experiments were performed in triplicate and the data was expressed as Mean ± SD.

RESULT AND DISCUSSION

A method of phytochemical screening is an essential phase in characterizing bioactive ingredients found in different plant species since it provides insight into the potential therapeutic properties of these substances. The preliminary phytochemical analysis detected the presence of secondary metabolites such as alkaloids, flavonoids, phenols, steroids, tannins, cardiac glycosides, and terpenoids in the methanolic extract while saponins were not detected (Table 2). The color observations of different phytochemical classes are shown in Fig. 1. The preliminary qualitative phytochemical analysis provides a brief overview of the secondary metabolites reported in the samples which have wide medicinal applications and are responsible for the therapeutic efficacy of the medicinal plants [50].

The calibration curves for the quantification of alkaloids, flavonoids, phenols, steroids, and tannins are given in Fig. 2-6 respectively. The secondary metabolite concentrations measured in stem bark sample of *H. antidysenterica* are presented in Table 3. The results showed the maximum TTC (70.90±0.46 mg TAE/g dry wt.) and minimum TCgC (0.028±0.01%) in stem bark sample.

Plants rely on secondary metabolites to survive harsh conditions. Pathogens such as bacteria, fungi, and viruses are highly susceptible to them. The secondary metabolites of each plant are unique in their types and functions. Modern pharmaceuticals and therapies are formulated with secondary metabolites such as alkaloids, flavonoids,

Table 2: Phytochemical screening of stem bark samples of *H. antidysenterica*

Phytochemicals	Methanolic extract
Alkaloids	+++
Flavonoids	++
Phenols	+++
Steroids	++
Tannins	+
Cardiac glycosides	++
Terpenoids	++
Saponins	-

(+) = present in low, (++) = present in moderate, (+++) = present in more quantity, (-) = not detected

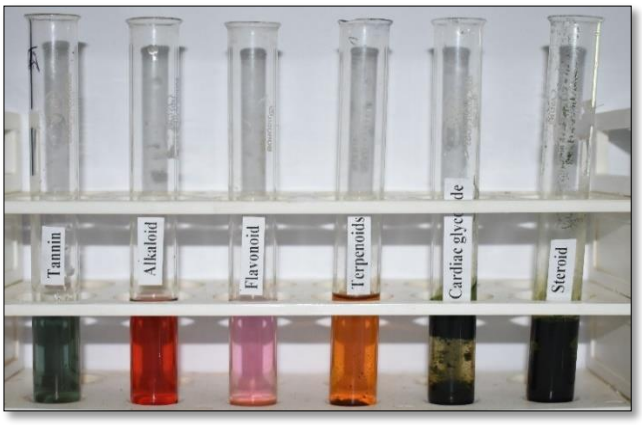

Figure 1: Color developed in preliminary phytochemical tests of different groups

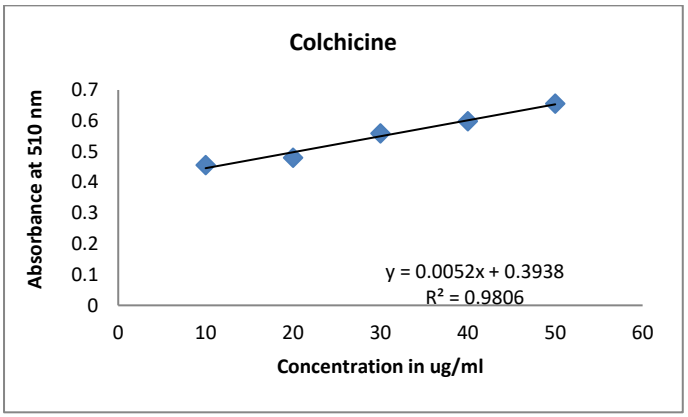

Figure 2: Calibration curve of Colchicine

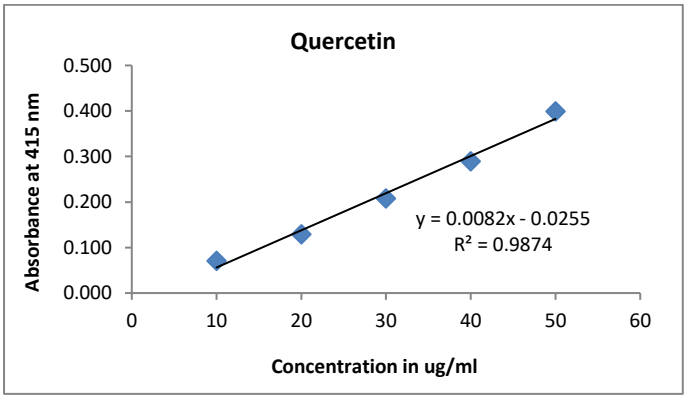

Figure 3: Calibration curve of Quercetin

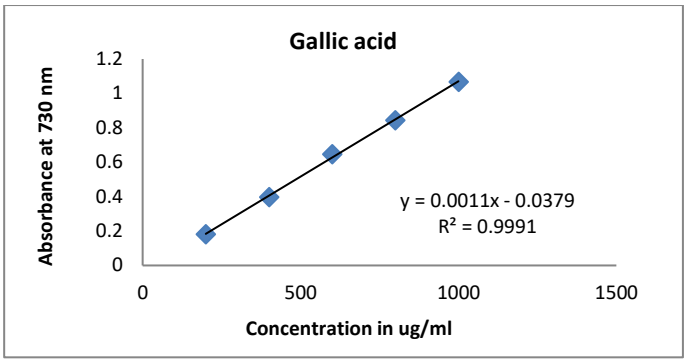

Figure 4: Calibration curve of gallic acid

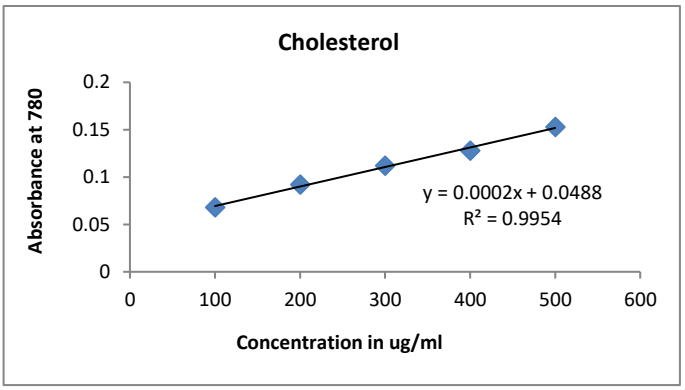

Figure 5: Calibration curve of cholesterol

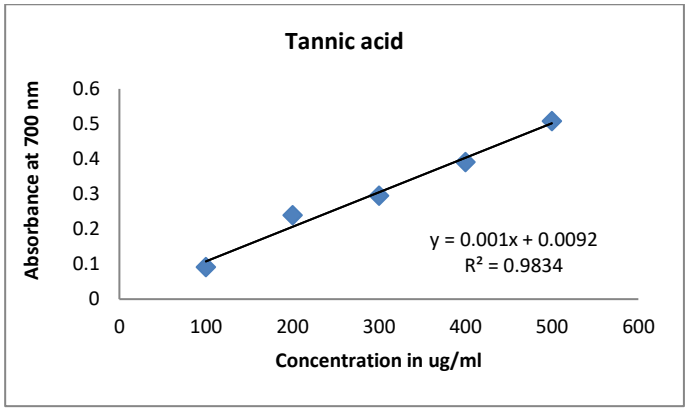

Figure 6: Calibration curve of tannic acid

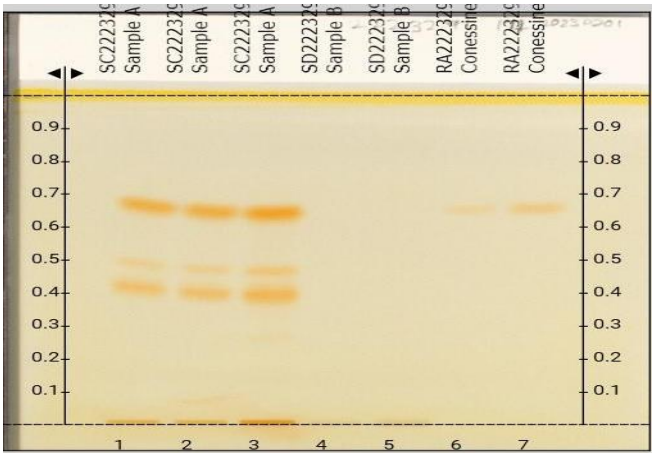

Figure 7: HPTLC fingerprint profiles of Sample A, Sample B and Conessine

tannins, terpenoids, phenols, steroids, and saponins. Alkaloids are well known for their therapeutic uses as cardio protection, anesthetics, and anti-inflammatory drugs. Various well-known alkaloids like nicotine, ephedrine, strychnine, morphine, quinine etc are extensively used in medicinal contexts [51]. Flavonoids have a variety of health rewards such as anti-viral, anti-cancer, anti-inflammatory, anti-oxidant, cardio-protective, and neuroprotective activities [52]. Cellular defense mechanisms against cancer and atherogenesis have been reported in relation to phenolic substances. Additionally, phenols and phenolic compounds have considerable antibacterial activity [39]. In terms of the possible applications of medications and nutraceuticals, tannins are important. By blocking important enzymes involved in microbial metabolism, tannins inhibit the growth of bacteria and are an excellent source of antioxidants [53, 54]. Steroids play vital role in pharmacognosy and pharmaceutical sector which used in enhancement of Gluconeogenesis, enhanced lipolysis, anti-inflammatory and enhance catabolism also [55]. Cardiac glycosides are used in treating the heart failure, irregular heartbeats, and other heart related issues [56]. Terpenoids enhance the human health due to their anti-inflammatory, anti-fungal, analgesic, anti-bacterial, anti-hyperglycemic, anti-cancer, anti-parasitic and anti-viral activities. These have a wide range of physiologically relevant properties [57].

Plant-based drugs can be standardized effectively with chemical fingerprinting. Chemical fingerprinting can be used to identify species, strains, and geographical origins of plants [58]. HPTLC fingerprinting method is a better, linear, precise, and accurate way of identifying, authenticating, standardizing, and characterization medicinal plant species [59].

The recorded HPTLC fingerprint profiles of methanolic extract of stem bark of *H. antidysenterica* (sample A), adulterant (sample B) and conessine are represented in Fig. 7 which showed three major bands in sample A including one band of reference compound conessine while there was no band observed in sample B. Number of bands, Rf values, colour and intensity in HPTLC profiles are given in Table 4. The corresponding chromatograms of sample A and conessine standard obtained after densitometric scanning are depicted in Fig. 8. There was no chromatogram observed for sample B.

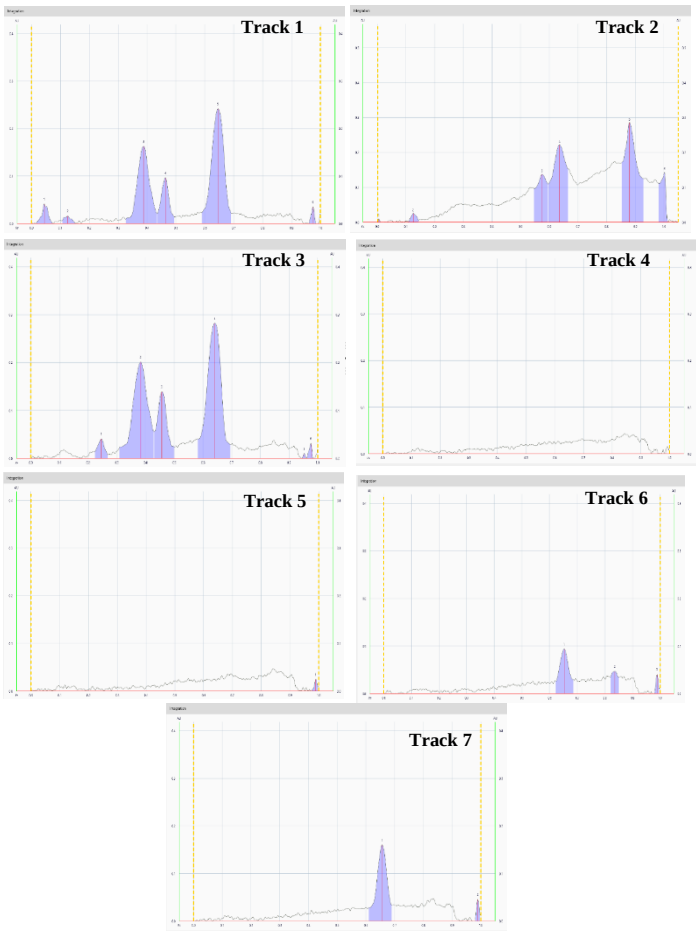

Figure 8: HPTLC chromatograms of sample A, sample B and conessine standard

Table 3: Quantification of secondary metabolites in stem bark of *H. antidysenterica*

Contents of secondary metabolites						
TAC (mg CE/g dry wt.)	**TFC (mg QE/g dry wt.)**	**TPC (mg GAE/g dry wt.)**	**TTC (mg TAE/g dry wt.)**	**TSC (mg Cyc/g dry wt.)**	**TCgC (%)**	**TTrC (%)**
22.87±0.34	3.46±0.07	51.00±0.58	70.90±0.46	0.80±0.02	0.028±0.01	3.43±0.11

Table 4: Number of bands, Rf values, colour and intensity in HPTLC profiles of samples

Wavelength	Track No.	No. of bands	Rf	Color	Intensity
After derivatization with Dragendorff's reagent					
Sample A					
Rwhite	1, 2 & 3	3	0.4	Orange	Medium
			0.47	Orange	Low
			0.65	Orange	High
Sample B					
Rwhite	4 & 5	0	-	-	-
Conessine					
Rwhite	6 & 7	1	0.65	Orange	High

These chemical fingerprint profiles can be useful for the authentication of stem bark samples of *H. antidysenterica* and distinguishing with adulterants. The developed chromatograms will be specific with standardized solvent system Toluene: Ethyl acetate: Diethylamine (6.5: 2.5: 1, v/v/v) and Rf values. Conessine, an important steroidal alkaloid found effective in various chronic diseases and has various biological activities such as Acetylcholinesterase, antibacterial, anticercarial, antidiarrheal, antifeedant, antimycobacterial, antiplasmodial, antitrypanosomal, cytotoxic, hypotensive, leishmanicidal, restoring antibiotic efficacy [60] and antimarial [61].

CONCLUSION

H. antidysenterica has been majorly used in ancient and modern pharmacological diagnosis. The present investigation described the variety of secondary metabolites that exist in the stem bark of this plant, and this study validates its therapeutic significance. The developed chemical fingerprint profiles can be stored as electronic fingerprints and utilized for quality standardization and authentication of stem bark of this valuable species.

Conflict of interest

The authors declare that they have no conflict of interest.

Financial Support

None declared.

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HOW TO CITE THIS ARTICLE
Pawar G, Parihar S, Saxena HO. Evaluation of secondary metabolites and chromatographic fingerprint profiling of stem bark of *Holarrhena antidysenterica* L. J Phytopharmacol 2024; 13(1):4-11. doi: 10.31254/phyto.2024.13102