Research Paper

High-Performance Thin-Layer Chromatography Analysis for Class of Compounds in Some Crude Seed Extracts for Potential Male Contraceptive Activity

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The presence of various secondary metabolites in the crude plant extracts have always been a source of potential therapeutic activities in animals and humans. The varying phytochemical composition and concentrations are instrumental in deciding and shortlisting raw materials for extractions and isolation of active compounds. Therefore, preliminary phytochemical screening of the plant extracts along with their high-performance thin layer chromatography analysis for classes of compounds become prerequisite for any type of pharmacological study. Detailed profiling of secondary metabolites like cardiac glycosides, triterpenoids and steroids which are found to be instrumental in inducing contraception in male experimental model organisms will help us further understands the mechanism of natural male contraception. In this study, crude seed extracts of *Carica papaya* Linn., *Momordica charantia* Linn. and *Abrus precatorius* Linn. in suitable solvents were checked for the high-performance thin layer chromatography profiling of the above-mentioned metabolites. The results obtained were recorded and analysed for repeated authentication of plant parts and standardisation of extract preparation.

Key words: High-performance thin layer chromatography, cardiac glycosides, triterpenoids, steroids, male contraceptive

The need and interest in identification of the scientific class of compounds present in medicinally important plant materials is increasing worldwide, especially in developing countries where the use of herbal medicines is popular for their basic health needs. Various plant extracts are being used to cure and heal innumerable health disorders and injuries and this empirical knowledge comes from the plants defence system, which generates numerous compounds with diverse molecular structures, in far complex combinations to those derived from synthetic products, so the great interest in the identification and elucidation of new active principles. Preliminary phytochemical screening of the individual extracts of *Carica papaya* (*C. papaya*) Linn., *Momordica charantia* (*M. charantia*) Linn. and *Abrus precatorius* (*A. precatorius*) Linn., in various solvents like water, ethanol, chloroform, hexane, benzene and petroleum ether was carried out. It showed presence of various active phytochemicals like alkaloids, tannins, glycosides especially cardiac glycosides, diterpenes, triterpenoids and steroids. Out of all these phytochemicals found to be present in the different extracts, cardiac glycosides, triterpenoids and steroids are the class of compounds mainly responsible for the antifertility activity[1,2] (Table 1).

Cardiac glycosides are a class of organic compounds which are highly toxic and found in a number of plants, usually consisting of an aglycone (structurally related to steroid hormones) linked to one or more sugar molecules[3].

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Accepted 14 October 2024
Revised 11 March 2024
Received 03 July 2023
Indian J Pharm Sci 2024;86(5):1840-1849

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING FOR *C. papaya*, *M. charantia* AND *A. precatorius* EXTRACTS

S. No.	Test	*C. papaya*						*M. charantia*						*A. precatorius*					
		w.e.	e.e.	c.e.	p.e.	b.e.	h.e.	w.e.	e.e.	c.e.	p.e.	b.e.	h.e.	w.e.	e.e.	c.e.	p.e.	b.e.	h.e.
1	Test for alkaloids																		
	Dragendroff's reagent	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	+	+	-
	Wagner's reagent	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	+	+	-
	Mayer's reagent	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-	-
2	Test for proteins																		
	Ninhydrin test	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Millon's test	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-
	Biuret's test	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
3	Test for tannins																		
	Potassium dichromate ($K_2Cr_2O_7$) test	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-
	Ferric chloride ($FeCl_3$) test	+	-	-	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-
4	Test for sugars																		
	Benedict's test	+	+	+	-	-	-	+	+	+	-	-	-	+	+	-	-	-	-
	Fehling's test	+	+	+	-	-	-	+	+	+	-	-	-	+	+	-	-	-	-
	Mollish's test	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-	-
5	Test for steroids																		
	Salkowski's test	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	+	+	+
	Liebermann Burchard's test	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	+	+	+

6	Test for flavonoids																			
	Lead acetate ($Pb(CH_3COO)_2$) test	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	
7	Test for glycosides																			
	Sodium hydroxide (NaOH) reagent	-	-	+	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	
	Keller-Kiliani test	-	-	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	
8	Test for quinones																			
	Concentrated Sulphuric acid (H_2SO_4)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	
9	Test for terpenoids																			
	Tin and thionyl chloride	-	-	-	+	+	-	-	-	-	+	+	-	-	-	+	-	-	-	
10	Test for diterpenes																			
	Copper acetate test	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-	-	+	
11	Test for saponins																			
	Foam test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
12	Test for triterpenoids																			
	Salkowski's test	-	-	+	+	+	+	-	+	+	+	+	+	-	+	-	-	+	-	
	Liebermann–Burchard test	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	+	+	+	
13	Test for lipids																			
	Alcoholic Potassium hydroxide (KOH) test	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	
14	Test for coumarins																			
	10 % Ammonium hydroxide(NH_4OH)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Note: (+): Present; (-): Absent; w.e.: Aqueous extract; e.e.: Ethanolic extract; c.e.: Chloroform extract; p.e.: Petroleum ether extract; b.e: Benzene extract and h.e: Hexane extract

These compounds are said to increase the output force of the heart and increase its rate of contractions by acting on the cellular sodium-potassium Adenosine Triphosphatase (ATPase) pump. They are steroidal in nature and are considered important drugs for treatment of heart failure and cardiac rhythm disorders. They also have potential therapeutic use for treating cancer owing to their specific cytotoxicity against cancer cells[4]. Digoxin, a cardiac glycoside isolated from *Digitalis* sp. was found to cause significant reduction in sperm motility without reducing sperm count as the drug does not affect spermatogenesis and cause damage to the testicular cells[2]. Triterpenoids are cyclised from oxidised squalene precursors by oxidosqualene cyclases, with more than 100 different cyclical triterpene scaffolds[5]. Triterpenoids have recently emerged as a unique metabolite with multi-functional anti-cancer activities as demonstrated by promising research studies along with a low toxicity profile. They are used for various medical purposes like for anti-inflammatory, analgesic, antipyretic, hepatoprotective, cardiotonic, sedative and tonic effects[6]. Many classes of triterpenoids lower cholesterol levels by inhibiting cholesterol synthesis[1]. Thus, it has the potential to regulate the production of hormones that involve cholesterol as a starting molecule. Triterpene, oleanolic acid, isolated from *Eugenia jambolana*, was found to exhibit anti-fertility effect on male albino rats without any toxic side effects[7]. Triterpenoids of *Nerium oleander* were also found to show anti-fertility effect in male albino rats by affecting spermatogenesis[8]. Steroids have the fundamental structure of four carbon rings called the steroid nucleus. The addition of different chemical groups at different positions on the backbone leads to the formation of many different types of steroid compounds including sex hormones progesterone and testosterone, the anti-inflammatory steroids like corticosteroids, cardiac steroids like digoxin and digitoxin etc. Plant steroids synthesised by cyclisation of 2,3-epoxysqualene into cycloartenol are further metabolised owing to the enzymatic conversion to produce biologically active steroids. Natural steroids act as regulators of lipid metabolism and influence the production and regulation of the sex hormones in the organism's body[1]. These plant steroids are found to possess many interesting medicinal and pharmaceutical activities like anti-tumor, immunosuppressive, hepatoprotective, anti-bacterial, sex hormones, anthelminthic, cytotoxic and cardiotonic activity[9-11].

Therefore, further studies and confirmation of the presence of those compounds with the help of High-Performance Thin Layer Chromatography (HPTLC) was carried out to prove their presence in the prepared crude drugs which will be administered to the male Wistar, albino rats in pharmacological evaluation.

MATERIALS AND METHODS

Collection and processing of plant material:

The plant materials were collected from local markets and gardens and those materials were authenticated from Blatter Herbarium, St. Xavier's College, Mumbai. The specimen of *C. papaya* L. matches with the specimen number KGM-1079 of Kalpit Mhatre. The specimen of *M. charantia* L. matches with the specimen number 3800 of S.M. Almeida. The specimen of *A. precatorius* L. matches with the specimen number S.H.813 of P.S. Herbert. For phytochemical screening, 4 g of powdered drug was macerated with individual solvents like distilled water, ethanol, benzene, hexane, chloroform and petroleum ether in closed flasks for 24 h with frequent shaking. Those were then filtered using Whatman's filter paper. All the extracts obtained were tested using standard methodology for the metabolites using respective reagents and treatments[12-19]. 1 g of the dried powder of seeds of *C. papaya* was extracted in 50 ml of chloroform, 1 g of the dried *M. charantia* seed powder in 50 ml of benzene and 1g of the seed powder of *A. precatorius* was extracted in 50 ml of ethanol by cold maceration for 24 h and then subjected to rotary evaporator for total removal of solvent. An oily thick brown paste was obtained from *C. papaya*, sticky semi solid dark brown paste was obtained from *M. charantia* and a blackish brown sticky powder was obtained from *A. precatorius* which was used for further analysis.

Preliminary phytochemical screening:

Test for tannins: To test for the presence of tannins, 3 ml of extract is combined with different reagents. Adding 3 ml of $Pb(CH_3COO)_2$ forms a white precipitate, indicating tannins; adding 3 ml of 5 % $FeCl_3$ solution results in a blue-black coloration, indicating tannins; and adding 3 ml of $K_2Cr_2O_7$ causes a dark coloration, confirming the presence of tannins.

Test for proteins: To test for the presence of proteins, 3 ml of extract is combined with different reagents. In the biuret test, adding 1 ml of 4 % NaOH and 1 ml of 1 % Copper sulfate ($CuSO_4$) results in violet or pink coloration, indicating proteins. In Millon's test, adding 3 ml of Millon's reagent forms a white precipitate that

turns brick red upon heating, indicating proteins. In the sulphosalicylic acid test, adding 1 ml of sulphosalicylic acid to 2 ml of extract causes cloudiness, confirming proteins. In the ninhydrin test, adding 1 ml of ninhydrin reagent to 2 ml of extract produces a deep blue or purple coloration, indicating proteins.

Test for reducing sugars: To test for the presence of reducing sugars, 2 ml of extract is combined with different reagents. In Fehling's test, adding a mixture of 1 ml each of Fehling's A and Fehling's B reagents and heating in a boiling water bath for 10 min results in a yellow coloration, indicating reducing sugars. In Benedict's test, adding 2 ml of Benedict's solution to the extract and heating in a boiling water bath for 10 min produces yellow, green, or red coloration, confirming the presence of reducing sugars.

Test for carbohydrates: In Molisch's test for carbohydrates, 2 ml of extract is mixed with 2 drops of Molisch's reagent, followed by a few drops of concentrated Sulfuric acid (H_2SO_4). The formation of a violet ring at the interface indicates the presence of carbohydrates.

Test for glucosides: To test for the presence of glucosides, 2 ml of extract is mixed with 2 ml of 1 % aqueous picric acid solution and left aside for 15 min, followed by the addition of 1 ml of aqueous Sodium carbonate (Na_2CO_3). A red coloration indicates the presence of glucosides.

Test for flavonoids: To test for the presence of flavonoids, 2 ml of extract is combined with different reagents. In the $FeCl_3$ test, adding neutral $FeCl_3$ produces a green coloration, indicating flavonoids. In the Shinoda test, adding 1 ml of Hydrochloric acid (HCl) and a pinch of finely chopped magnesium ribbon to the extract results in a pink, orange, or red coloration, confirming the presence of flavonoids.

Test for triterpenes: To test for the presence of triterpenes, 2 ml of extract is combined with different reagents. In Salkowski's test, adding concentrated H_2SO_4 results in a yellow coloration in the lower layer after standing, indicating triterpenes. In the Liebermann–Burchard test, adding a few drops of acetic acid and 1 ml of concentrated H_2SO_4 produces a deep red coloration at the junction of two solvents, confirming triterpenes. In Tschugajen's test, mixing 2 ml of extract with 5 ml of acetyl chloride and a pinch of Zinc chloride ($ZnCl_2$) then warming in a hot water bath, results in eosin red coloration, indicating triterpenes.

Test for terpenoids: To test for the presence of terpenoids, 2 ml of extract is mixed with a pinch of tin and 1 ml of Thionyl chloride ($SOCl_2$) solution. The appearance of violet coloration indicates the presence of terpenoids.

Test for steroids: To test for the presence of steroids, 2 ml of extract is combined with different reagents. In Salkowski's test, adding concentrated H_2SO_4 results in the lower layer turning yellow after standing, indicating steroids. In the Liebermann-Burchard's test, adding a few drops of Acetic acid (CH_3COOH) and 1 ml of concentrated H_2SO_4 produces a deep red coloration at the junction of the two solvents, confirming the presence of steroids.

Test for diterpenes: To test for the presence of diterpenes, 2 ml of extract is mixed with 2 ml of copper acetate solution. The formation of a green coloration indicates the presence of diterpenes.

Test for glycosides: To test for the presence of glycosides, 2 ml of extract is combined with different reagents. In the NaOH test, adding 1 ml of water and 1 ml of NaOH solution results in a yellow coloration, indicating glycosides. In Keller-Killiani's test, adding 1 ml of water, 1 ml of glacial acetic acid, 1 ml of $FeCl_3$ solution, and 0.5 ml of concentrated H_2SO_4 produces a brown ring at the junction of the two solvents, confirming the presence of glycosides.

Test for anthraquinones: To test for the presence of anthraquinones, 1 g of powdered drug is boiled in dilute H_2SO_4 for 3 min and then filtered. The filtrate is divided into three parts, and each part is treated with 2 ml of chloroform, ether, and benzene, respectively. After the organic layer is separated, ammonia is slowly added. The appearance of a pink to red color in the ammoniacal layer indicates the presence of anthraquinones.

Test for quinones: To test for the presence of quinones, 2 ml of extract is mixed with 1 ml of concentrated H_2SO_4. The appearance of a yellow coloration indicates the presence of quinones.

Test for alkaloids: To test for the presence of alkaloids, 2 ml of extract is mixed with 1 ml of Dragendroff's reagent. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

Wagner's test: To test for the presence of alkaloids, 2 ml of extract is mixed with 1 ml of Wagner's reagent. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

Mayer's test: To test for the presence of alkaloids, 2 ml of extract is mixed with 1 ml of Mayer's reagent. The

formation of a creamy white precipitate indicates the presence of alkaloids.

Test for phenols: To test for the presence of phenols, 2 ml of extract is mixed with 1 ml of $FeCl_3$ solution. The appearance of a blue or green coloration indicates the presence of phenols.

Test for saponins: To test for the presence of saponins, 2 ml of extract is mixed with 1 ml of water. The formation of foam that persists for more than 10 min after vigorous shaking indicates the presence of saponins.

Test for lactones: To test for the presence of lactones, 2 ml of extract is mixed with a few drops of 10 % NaOH and 1 ml of 0.3 % nitroprusside sodium reagent. The appearance of a dark red coloration indicates the presence of lactones.

Baljet test: To test for the presence of lactones, 2 ml of extract is mixed with 2 ml of methanolic sodium picrate solution and 1 ml of NaOH. The formation of a light wine-red coloration indicates the presence of lactones.

Test for lipids: To test for the presence of lipids, 2 ml of extract is mixed with 2 ml of alcoholic KOH. The formation of soap indicates the presence of lipids.

Test for coumarins: To test for the presence of coumarins, 2 ml of extract is mixed with 10 % NH_4OH solution. The appearance of intense fluorescence indicates the presence of coumarins (Table 1).

Sample preparation for HPTLC analysis:

After total removal of solvent, Chloroform extract of *C. papaya*, benzene extract of *M. charantia* and ethanolic extract of *A. precatorius* were weighed and individual loading samples were prepared by addition of a diluent i.e., chloroform:methanol (1:1) in the concentration of 10 mg/ml. For the prepared combination, the loading samples of the individual extracts were combined and were used as a loading sample for our combination.

Development of HPTLC chromatogram:

Fine particle size silica gel coated glass plate 5715 (20 cm×20 cm) or Thin-Layer Chromatography (TLC) silica gel 60 F_{254} aluminium sheets from MERCK™ were selected and were heated at 120° for 15-20 min for removal of adsorbed water if any. The samples were loaded onto the silica plates using CAMAG® automatic TLC sampler in varying concentrations. A CAMAG® Twin Trough Chamber (TTC) (20×20) cm was rinsed using methanol and dried completely. For efficient saturation of the chamber, a filter paper (Whatman No. 1) was kept inside the TTC, respective mobile phase was poured in and kept shut for 20 min, undisturbed. Complete saturation of the TTC was done and the loaded silica TLC plate was introduced into the TTC and the mobile phase was let to run up to 115 mm. After confirming the proper chromatogram development in a CAMAG® TLC visualizer, spraying of appropriate derivatizing agent was carried out using piezoelectric spray of sample level 4 in CAMAG® derivatizer. The derivatized silica TLC plate was scanned in 254 nm, 366 nm and white light using CAMAG® TLC scanner, which was attached to data recording software and Retention factor (R_f) values were recorded. The developed spots were viewed as peaks at wavelengths of selected Ultraviolet (UV) regions (fig. 1)[20].

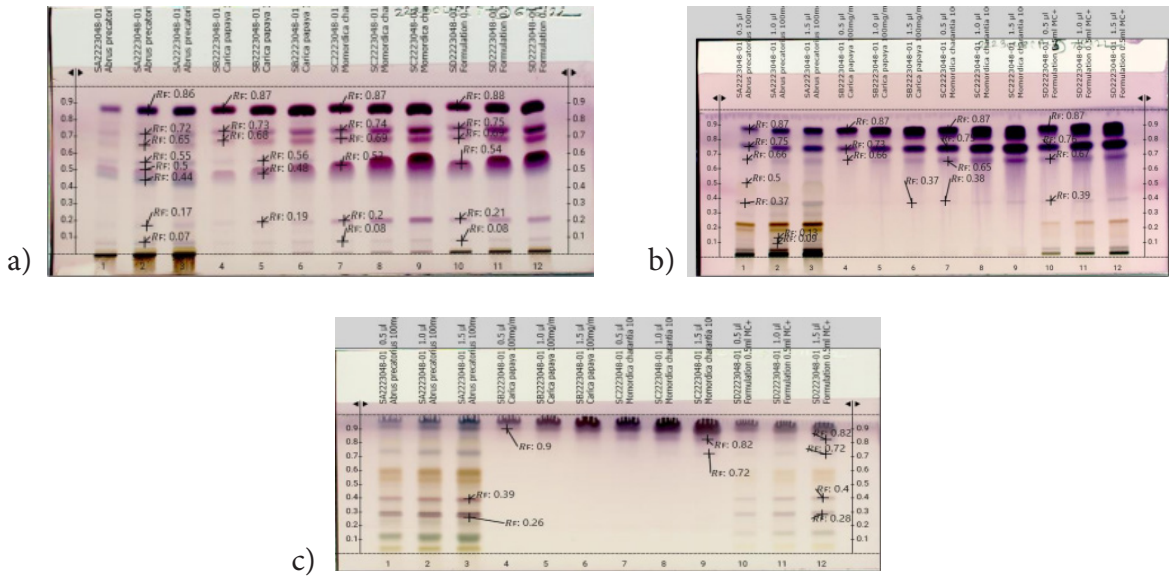

Fig. 1: HPTLC plate for, (a): Triterpenoids; (b): Cardiac glycosides and (c): Steroids

RESULTS AND DISCUSSION

As the seeds of *C. papaya*, *M. charantia* and *A. precatorius* are very promising in various pharmacological and clinical studies, a systematic analysis for determination of their standard pharmacognostic and phytochemical characters is of prime importance (Table 1). These characteristics are said to work through specific and non-specific mechanisms. However, extensive and in-depth research is required to evaluate the precise mechanism, active principles, and the safety profile of the plant as a remedy for different health conditions. The phytochemical constitution of the plant drugs is said to be responsible for therapeutic effects. These compounds are the primary and the secondary metabolites, resulting through the plant metabolism. Phytochemical constitution and their concentration differ not only from plant to plant, but also in different parts of the plant. So preliminary screening of phytochemicals in a given plant sample or powdered drug helps us get an idea of the various active constituents present in there and the possible therapeutic uses. Although many solvents were found to extract many of the phytoconstituents, the intensity of positive results and the ability to extract all the bio-active ones responsible for a particular activity varied (Table 2). Therefore, selection of solvent which shows the presence of target phytoconstituents like cardiac glycosides, triterpenoids and steroids along with higher percentages of extractive value is of prime importance. Complete removal of solvent from the prepared extractive is done to ensure the pharmacological activity is only a result of the phytoconstituents and not the solvent itself. Investigations like these also help us provide alternative sources of herbal drugs and thus reduce the commercial load on particular plant parts. Phytochemical analytical techniques like HPTLC are used for identifying a genuine drug amongst adulterated material and also confirming the presence of the pharmacologically active phytoconstituents. Animal trials carried out by various researchers on pharmacological activities of the selected seed samples led to shortlisting of solvent used for extraction procedure. The three classes of compounds; cardiac glycosides, triterpenoids and steroids are considered to be active in inducing contraceptive effects in male rats. These compounds directly target either level of cholesterol and other reproductive hormones and metabolites which are responsible for maintaining normal reproductive functions. Thus, the use of HPTLC analysis for identification of class of compounds serve as important tools in determining the identity and purity of these drugs and also for developing reference phytochemical standards (fig. 2-fig. 4).

TABLE 2: R_f VALUES OF TRITERPENOIDS, CARDIAC GLYCOSIDES AND STEROIDS IN EXTRACTS

S. No.	Name of the sample	R_f values	Colour	Intensity
Terpenoids				
1	*A. precatorius*	0.07	Pale violet	Low
		0.17	Pale violet	Low
		0.44	blue	Medium
		0.5	Pink violet	Medium
		0.55	Pale violet	Low
		0.65	Pale violet	Low
		0.72	violet	Medium
		0.86	purple	High
2	*C. papaya*	0.19	Pale violet	Low
		0.48	purple	High
		0.56	Pale violet	Low
		0.68	violet	Medium
		0.73	violet	Medium
		0.87	purple	High
3	*M. charantia*	0.08	Pale violet	Low
		0.2	Pink violet	Medium
		0.53	violet	Medium
		0.69	violet	Medium

		0.74	violet	Medium
		0.87	purple	High
Cardiac glycosides				
1	*A. precatorius*	0.09	Pale violet	Low
		0.13	Pale violet	Low
		0.37	Blue	Medium
		0.5	Pale green	Low
		0.66	Pale violet	Low
		0.75	purple	High
		0.87	purple	High
2	*C. papaya*	0.66	Pale violet	Low
		0.73	Purple	High
		0.87	purple	High
		0.37	Pale violet	Low
3	*M. charantia*	0.37	Pale violet	Low
		0.65	Purple violet	Medium
		0.75	purple	High
		0.87	purple	High
Steroids				
1	*A. precatorius*	0.26	Pink violet	Medium
		0.39	Pink violet	Medium
2	*C. papaya*	0.9	Violet	Low
3	*M. charantia*	0.72	Violet	Low
		0.82	Violet	Medium

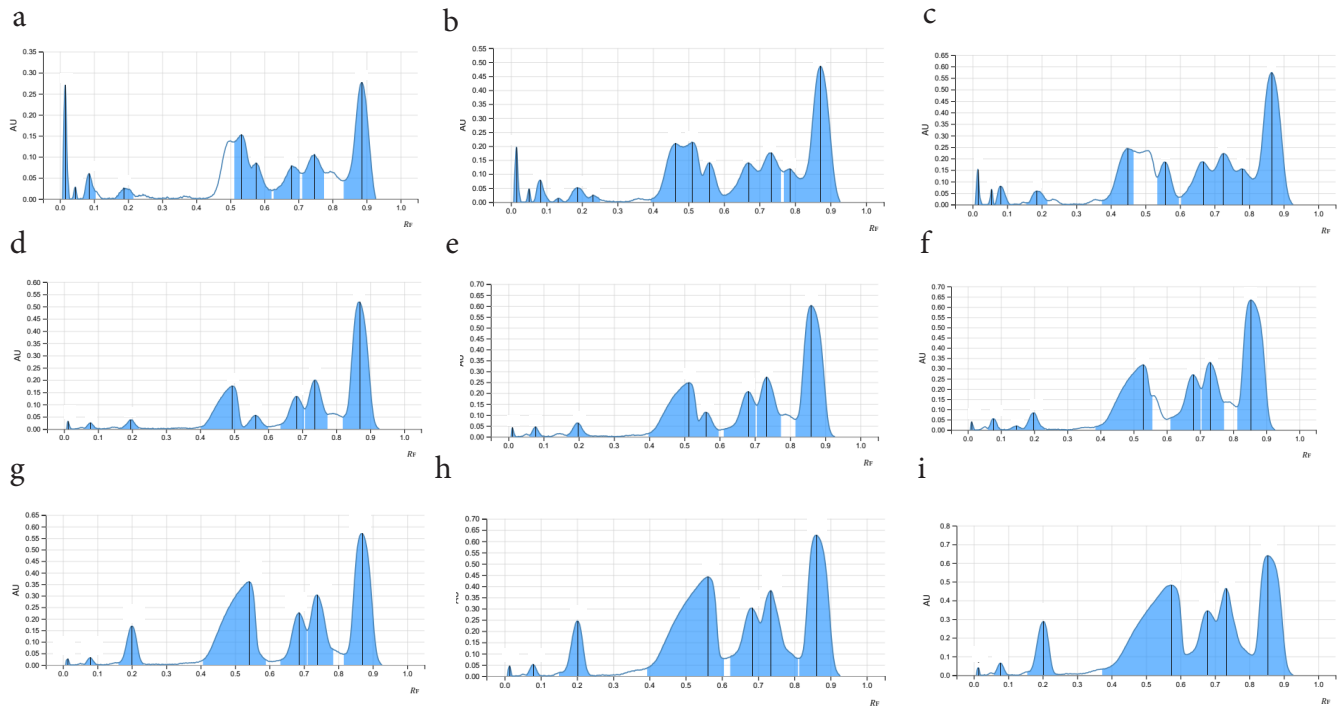

Fig. 2: HPTLC profiling of terpenoids
Note: X-axis: R_f and Y-axis: Peak area

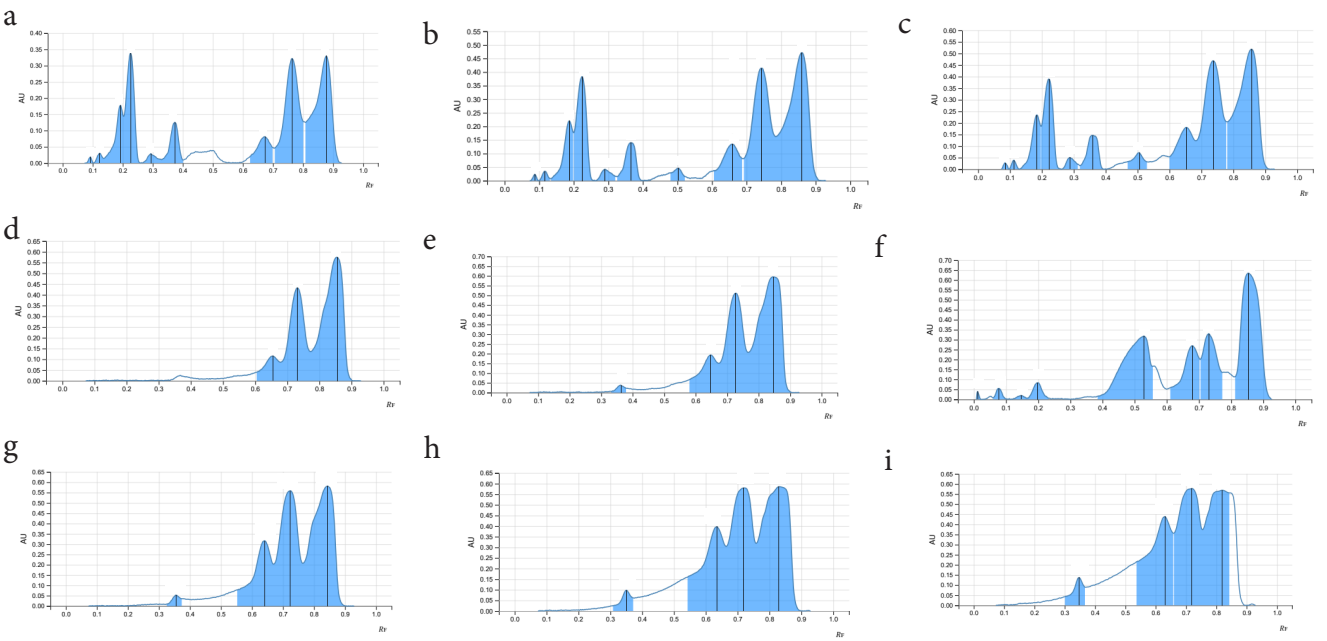

Fig. 3: HPTLC profiling for cardiac glycosides
Note: X-axis: R_f and Y-axis: Peak area

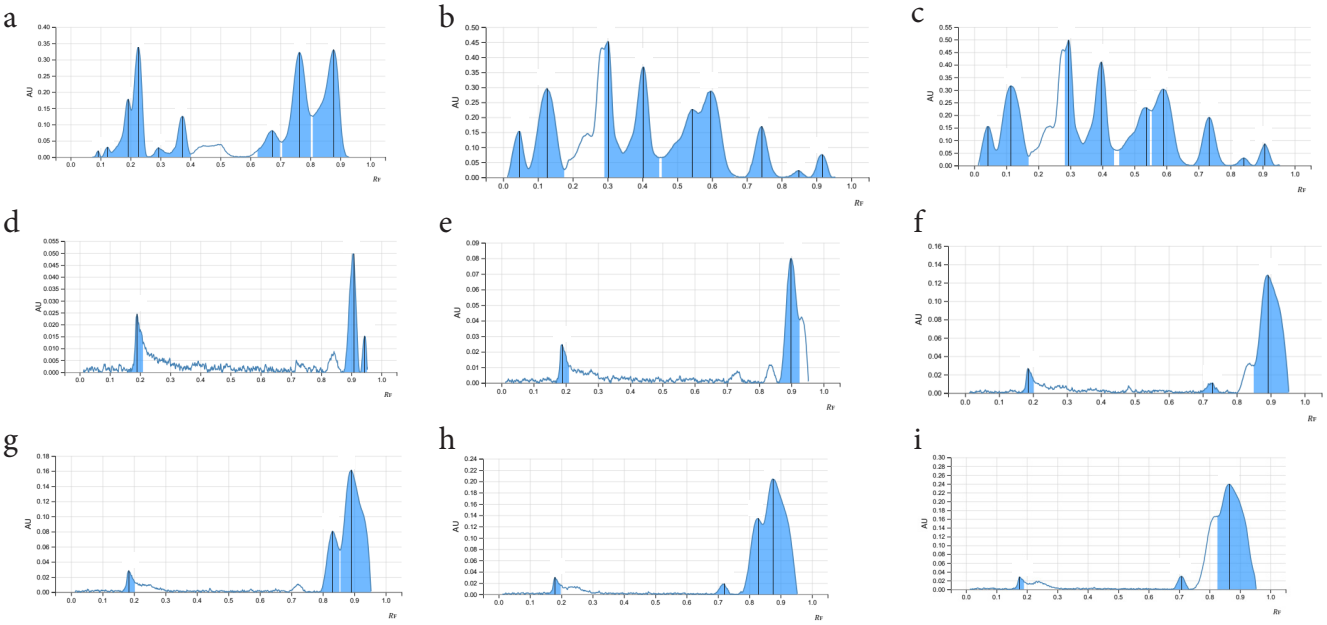

Fig. 4: HPTLC profiling for steroids
Note: X-axis: R_f and Y-axis: Peak area

Acknowledgement:

The authors would like to express their gratitude to the Blatter Herbarium, St. Xavier's College, Mumbai for authentication of the plant samples and also Anchrome Enterprise Ltd., Mulund for assisting in the HPTLC analysis of the sample extracts.

Conflict of interests:

The authors declared no conflict of interests.

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