Research Article

High-performance thin-layer chromatography fingerprinting analysis of bioactive compounds in hydro-alcoholic extracts of polyherbal formulation

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ABSTRACT

Objective: The objective of the study was to explore the fingerprinting profile of bioactive compounds present in hydro-alcoholic extract of polyherbal formulation (HAF). **Materials and Methods:** High-performance thin layer chromatography (HPTLC) fingerprinting of HAF was performed and calculate Rf values at wavelength 580 nm. HAF consists of a hydro-alcoholic extract of 11 medicinal plants which have protective activity against cellular damage related activity. HPTLC method was performed to evaluate the presence of polyphenolics employing mobile phase n-hexane:ethyl acetate:formic acid (4:5:1, v/v/v). **Results:** Peaks were symmetrical in nature and no tailing was observed when plates were scanned at wavelength 580 nm. The Rf values of HAF at sample volume 25 µl (70 mg in 7 ml methanol + HCl) seen maximum in nos. with corresponding to percentage of peak area were observed to be 0.035 (1.23%), 0.113 (8.25%), 0.189 (7.57%), 0.254 (3.72%), 0.291 (5.37%), 0.381 (10.52%), 0.437 (11.18%), 0.547 (4.49%), 0.597 (5.99%), 0.64 (9.83%), 0.69 (27.35%), and 0.881 (3.22%). HPTLC was performed to confirm the quantitative presence of polyphenolics in extracts of HAF. **Conclusions:** The present standardization provides a specific and accurate tool to develop a confirmatory fingerprinting profile (HPTLC) of biomarkers present in HAFs for many curative activities.

KEY WORDS: Biomarker, Peak, Polyherbal formulation, Polyphenolics, Quantification

INTRODUCTION

Anti-oxidants are involved against the cell damage due to reactive oxygen species results cancer, liver, kidney, diabetes, and aging. The important preventive agents used against oxidation are polyphenolics flavonoids, phenolic acids and tannins[1-3] Vitamin E,[4] and Vitamin C.[5] The present investigation was directed to polyherbal formulations (HAFs) which incorporate an exceptional blends of herbs that have been utilized for a considerable decades to eradicate problems related to kidney disfunctioning. HAF is a polyherbal mix containing eleven herbs frequently prescribed by ayurvedic practitioners for protective effects against cellular oxidative damage. These are *Bergenia ciliata, Pedalium murex, Tribulus terrestris, Tinospora cordifolia, Sphaeranthus indicus, Saccharum officinarum, Saccharum spontaneum, Saccharum munja, Desmostachya bipinnata, Imperata,* and *Piper longum* (Pl). Pl has natural property to increase the bio-availability of any herbal formulation; it is likely to known as bio-enhancer.[6] Standardization promises constant composition of all herbals including analytical operations for identification, markers, and assay of active principles. Thin-layer chromatography (TLC) and high-performance TLC (HPTLC) are routinely used as valuable tools for qualitative determination of small amounts of impurities. Different researchers have proposed that HPTLC method enables high-quality resolution and can be used for quantization of biomarkers.[7-10] The present work confirms such findings like identification of biomarkers present in HAFs will be useful as protective or curative activity against oxidation due to the presence of polyphenolic compounds that are utilized for standardization purpose.[11-13]

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Website: jprsolutions.info	ISSN: 0975-7619

MATERIALS AND METHODS

Chemicals

Analytical grades of chemicals Alcohol, n-Hexane, ethyl acetate, formic acid, and anisaldehyde purchased and used for this study.

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Received on: 24-02-2017; Revised on: 26-03-2018; Accepted on: 28-04-2018

Apparatus

Server vision CATS-Server-PH, version 2.5.18053.1 (CAMAG, Switzerland), chromatographic plate (100x100mm), glass twin trough chamber 20 × 10 CAMAG, TLC scanner 4 linked to win cats software (CAMAG), 0.2 mm thickness pre-coated with silica gel 60 F254 (Merck) were used in this study. The experiment was carried out under the conditions with the temperature of (25 ± 2) °C and relative humidity of 40%.

Collection and Preparation and Extraction of Herbal Samples

All the medicinal plant materials were collected from different geographical areas of districts Fatehpur, Deoria and Agra, Uttar Pradesh, India. All the medicinal plants were authenticated from National Institute of Science Communication and Information Research (NISCAIR), New Delhi, India, under supervision of scientist Dr. Sunita Garg with different authentication number [Table1].

Plant Extraction

The different plant parts were dried for 2 weeks under shade, then at room temperature and were subjected to size reduction with a crusher and then passed through sieve no.40 to get uniform powder. Around 250 g of powdered plant material were subjected to extraction with a solvent such as petroleum ether (for the purpose of defatting) and alcohol (60%). The hydro-alcoholic (40:60) extracts were subjected for maceration process of cold extraction. Each extract was then distilled to dryness under reduced pressure using a rotatory evaporator to yield the respective dried extracts.[14-16]

Herbal Formulations

The ethanolic (60%) extracts of individual plant (hydro-alcoholic parts of powder extracts) were blends to develop HAF described in Table 1.[17,18]

HPTLC Analysis

Analysis was performed on 100 cm × 100 mm HPTLC silica gel 60 F 254 plates with the fluorescent indicator. Before starting the analysis, HPTLC plates were cleaned by pre-development with methanol by ascending method. HPTLC plate was immersed in a CAMAG glass chamber (20 cm × 10 cm), containing 30 mL methanol with dosage speed 150 nl/s and pre dosage volume 0.20 µl (HPLC grade) used as solvent system using valid diagnostics. The chamber was covered with glass lid and left till the development of the plate to the top with methanol. After complete development, the plate was removed from TLC glass chamber and dried in an oven at 105°C for 5 min. Six spots of HAF were applied (in the form of band) spots were shown in the form of bands on the same plate by means of a CAMAG Linomat 5 (automated spray-on applicator) equipped with a microsyringe and operated with the settings band length 6 mm, distance between band 15 mm, distance from the plate side edge 15 mm, and distance from the bottom of the plate 15 mm.[11,19,20]

Preparation of Sample

70 mg sample of hydro-alcoholic HAF was dissolve in 7 ml of methanol and also in same volume of a mixture of methanol and HCl and 15 µl, 20 and 25 µl volume of sample taken from the sample for the HPTLC analysis.

Preparation of Visualizing Agent

Place 170 ml methanol in 200 ml glass bottle and cool it down in water ice cube bath. To the ice-cold methanol add slowly at carefully 20 ml of catic acid and 10 ml of sulfuric acid and mix well allow the mixture to cool to room temperature, then add 1 ml of anisaldehyde.[21]

TLC Development and Scanning of HAF

The plate was developed by immersing sample HPTLC plate in a CAMAG glass chamber (20 cm × 10 cm) containing the solvent system cyclohexane:ethylacetate:formic acid (4:5:1 v/v/v). After complete development, the plate was allowed to dry by keeping in fume cupboard for 10 min and then kept in a hot air oven for 5 min at 105°C. The plate was scanned in the densitometer by linear scanning speed 20 mm/s, slit 6 mm × 0.45 mm (micro) at 580 nm

Table 1: Composition of HAF

Name of drug	Authentication No.	Part used	Quantity
HAF			
Bergenia ciliata	NISCAIR/RHMD/CONSULT/2016/2976-03-5	Roots	2 Part
Pedalium murex	NISCAIR/RHMD/CONSULT/2016/2976-03-1	Fruits	2 Part
Tribulus terrestris	NISCAIR/RHMD/CONSULT/2017/3050-77-6	Fruits	2 Part
Sphaeranthus indicus	NISCAIR/RHMD/CONSULT/2016/2976-03-4	Flowers	2 Part
Tinospora cordifolia	NISCAIR/RHMD/CONSULT/2016/2976-03-2	Stem	2 Part
Saccharum officinarum	NISCAIR/RHMD/CONSULT/2017/3050-77-1	Roots	2 Part
Saccharum spontaneum	NISCAIR/RHMD/CONSULT/2017/3050-77-5	Roots	2 Part
Saccharum munja	NISCAIR/RHMD/CONSULT/2017/3050-77-4	Roots	2 Part
Desmostachya bipinnata	NISCAIR/RHMD/CONSULT/2017/3050-77-2	Roots	2 Part
Imperata cylindrica	NISCAIR/RHMD/CONSULT/2017/3050-77-3	Roots	2 Part
Piper longum	NISCAIR/RHMD/CONSULT/2016/2976-03-3	Fruits	1 Part

HAF: Polyherbal formulation

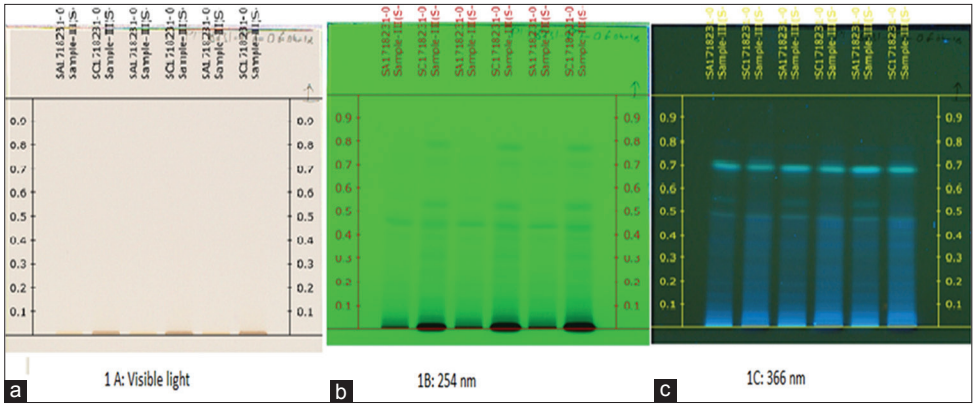

Figure 1: High-performance thin layer chromatography finger printing profile of polyherbal formulation, 1a: Before development under visible light, 1b: Before development at 254 nm, 1c: Before development under 366 nm, after development under 366 nm

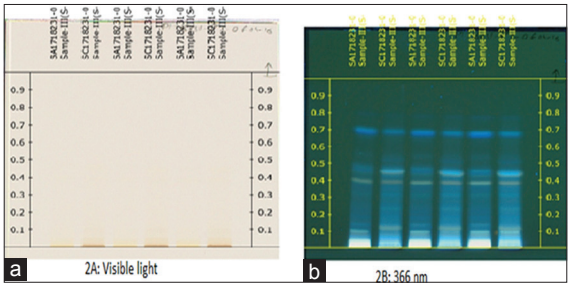

Figure 2: (a) After derivatization under 254 nm, (b) after derivatization under 366 nmm

using a TLC Scanner 4 CAMAG with a tungsten lamp source. In the HPTLC derivatization, plate gives data resolution at 25 µm/step, filter K400 and mercury lamp were used at wavelength 366 nm.[22-24]

Integration Parameters

In integration parameters, starts from bound observed between 0.00 and 1.00, smoothing seen in savitzky-golay of order 3 and window, baseline correction shows lower slop with noise 0.05 with peak detection gauss (legacy) with sensitivity 0.1, separation 1, and threshold 0.1 scan at wavelength 580 nm.

RESULTS AND DISCUSSION

For the chromatographic separation of phenolic compounds, HPTLC method reported by Morlock *et al*.,[25] was modified to optimize the resolution of the phenolic acids and flavonoids. In the current study, quantitative estimation of biologically active components was conducted in the HAFs using HPTLC. Phytoconstituent present in HAF; therefore, in the quantitative estimation represented in fingerprinting [Figures 1 and 2] for optimization of method, mobile phase composition was employed to achieve good separations.

Among the various solvent systems tried the solvent system containing n-hexane:ethyl acetate:formic acid in the volume ratio of 4:5:1 resulted in good separation of the polyphenolics. TLC plates were observed under ultraviolet light for the presence of phytoconstituents like gallic acid, which were detected by prominent dark brown spots. The spots developed were dense, compact and typical peaks of different chemicals were obtained. Peaks were symmetrical in nature, and no tailing was observed when plates were scanned at wavelength 580 nm.

The Rf Values of HAF at volume 15, 20, and 25 µl were taken to perform shown in Figures 1 and 2 of chromatogram at 580 nm. It is evident from Table 2 that in 15 µl of HAF; there were 6 spots at the Rf values at maximum 0.23, 0.27, 0.41,0.52, 0.66, and 0.78 and at the end of Rf values 0.24, 0.29, 0.46, 0.544, 0.68, and 0.80 and percentage peaks were 3.46, 5.65, 27.60, 12.14, 28.60, and 22.57. 15 µl of HAF; there were 9 spots at maximum peak were 0.008, 0.142, 0.23, 0.26, 0.34, 0.40, 0.48, 0.58, and 0.75, at end peak Rf values were 0.01, 0.18, 0.25, 0.28, 0.36, 0.44, 0.53, 0.60, and 0.79. Percentage peak covered 1.36, 10.65, 10.20, 4.36, 6.23, 12.35, 15.98, 5.98, and 32.85. The HAF were prepared using volume 20 µl seen 7 spots, at start of the peak Rf values 0.19, 0.25, 0.37, 0.47, 0.59, 0.72, and 0.82 were shown and percentage area of peaks 3.39, 4.70, 20.0, 12.14, 28.69, 20.29, and 10.78 at wavelength 580 nm. HAF in solvent methanol with HCl, there were 10 spots seen with Rf values at start of the peak 0.00, 0.123, 0.192, 0.252, 0.299, 0.381, 0.44, 0.54, 0.64, and 0.70 with percentage area of the peaks 1.58, 9.14, 8.87, 4.27, 5.96, 12.01, 13.06, 4.84, 11.71, and 28.55. The Rf values and peaks (starting, maximum, and end) of HAF at volume 25 µl prepared and performed then peak area covered were noted.

The maximum Rf values of HAF using volume 25 µl were 0.035, 0.113, 0.189, 0.254, 0.291, 0.381, 0.437, 0.547, 0.597, 0.641, 0.695, and 0.881 and corresponding of percentage of peak area covered was 1.23, 8.25, 7.57, 3.72, 5.37, 10.52, 11.18, 4.49, 5.99,

Table 2: Densiometric scan of HAF at wavelength 580 nm

Sample volume at 15 µl (70 mg HAF in 7 ml methanol)									
Peak	**Start**		**Max**			**End**		**Area**	
	Rf	**Height**	**Rf**	**Height**	**%**	**Rf**	**Height**	**Area**	**%**
1.	0.206	0.0012	0.230	0.0203	5.79	0.244	0.0100	0.00048	3.46
2.	0.258	0.0024	0.276	0.0405	11.53	0.295	0.0066	0.00078	5.65
3.	0.378	0.0195	0.415	0.0958	27.29	0.461	0.0170	0.00380	27.60
4.	0.494	0.0137	0.522	0.0464	13.22	0.544	0.0252	0.00167	12.14
5.	0.615	0.0213	0.662	0.0831	23.67	0.687	0.0433	0.00393	28.60
6.	0.745	0.0360	0.781	0.0650	18.50	0.806	0.0414	0.00310	22.57
Sample volume at 20 µl									
1	0.197	0.0001	0.229	0.0254	4.57	0.253	0.0044	0.00075	3.39
2	0.253	0.0043	0.270	0.0600	10.79	0.288	0.0109	0.00104	4.70
3	0.378	0.0257	0.402	0.1251	22.48	0.446	0.0247	0.00443	20.00
4	0.476	0.0172	0.509	0.0661	11.87	0.539	0.0315	0.00269	12.14
5	0.596	0.0325	0.647	0.1276	22.93	0.675	0.0604	0.00639	28.69
6	0.728	0.0395	0.766	0.0928	16.68	0.801	0.0358	0.00450	20.29
7	0.827	0.0455	0.855	0.0595	10.69	0.8720	0.0463	0.00239	10.78
Sample at 25 µl									
1	0.192	0.0000	0.226	0.0338	4.77	0.243	0.0176	0.0083	2.91
2	0.254	0.0092	0.270	0.0720	10.18	0.286	0.0152	0.00130	4.59
3	0.369	0.0374	0.395	0.1528	21.59	0.440	0.0236	0.00563	19.83
4	0.468	0.0150	0.504	0.0800	11.31	0.539	0.0322	0.00340	11.95
5	0.590	0.0269	0.641	0.1748	24.70	0.673	0.0692	0.00812	28.57
6	0.718	0.0379	0.756	0.1278	18.06	0.788	0.0364	0.00548	19.29
7	0.801	0.0343	0.848	0.0664	9.38	0.873	0.0406	0.00366	12.87
Sample at 15 µl (70 mg HAF in 7 ml methanol + HCL)									
1	0.000	0.0000	0.008	0.0308	5.03	0.019	0.0000	0.00031	1.39
2	0.123	0.0207	0.142	0.0912	14.89	0.181	0.0021	0.00232	10.65
3	0.191	0.0000	0.237	0.0732	11.96	0.253	0.0164	0.00226	10.20
4	0.253	0.0164	0.267	0.0548	8.96	0.289	0.0000	0.00097	4.36
5	0.305	0.305	0.0035	0.344	0.0366	5.98	0.363	0.0225	6.23
6	0.385	0.0265	0.408	0.0708	11.57	0.445	0.0150	0.00274	12.35
7	0.445	0.0150	0.482	0.0708	11.57	0.445	0.0150	0.00274	12.35
8	0.556	0.0136	0.582	0.0395	6.45	0.603	0.0243	0.00133	5.98
9	0.709	0.0479	0.755	0.1398	22.84	0.797	0.0329	0.00728	32.85
Sample at 20 µl									
1	0.000	0.0000	0.011	0.0403	5.13	0.023	0.0000	0.00044	1.58
2	0.123	0.0221	0.143	0.0964	12.26	0.183	0.0000	0.00257	9.14
3	0.192	0.0000	0.236	0.0846	10.75	0.252	0.0212	0.00250	8.87
4	0.252	0.0212	0.266	0.0668	8.49	0.288	0.0003	0.00120	4.27
5	0.229	0.0034	0.347	0.0462	5.87	0.360	0.0292	0.00168	5.96
6	0.381	0.0335	0.402	0.0856	10.88	0.439	0.0151	0.00338	12.01
7	0.440	0.0151	0.480	0.0802	10.20	0.523	0.0079	0.00368	13.06
8	0.548	0.0120	0.578	0.0443	5.63	0.595	0.0239	0.00136	4.84
9	0.640	0.0466	0.667	0.0704	8.95	0.696	0.0460	0.00330	11.71
10	0.700	0.0450	0.741	0.1717	21.84	0.786	0.0213	0.00804	28.55
Sample at 25 µl									
1	0.000	0.0000	0.014	0.0431	3.88	0.026	0.0000	0.00050	1.27
2	0.035	0.0000	0.053	0.0250	2.26	0.067	0.0146	0.00049	1.23
3	0.113	0.0193	0.143	0.1067	9.61	0.189	0.0000	0.00326	8.25
4	0.189	0.0000	0.238	0.0987	8.89	0.253	0.0290	0.00299	7.57
5	0.254	0.0290	0.266	0.0812	7.32	0.291	0.0000	0.00147	3.72
6	0.291	0.0000	0.343	0.0608	5.47	0.359	0.0416	0.00212	5.37
7	0.381	0.0477	0.400	0.1077	9.70	0.435	0.0167	0.00415	10.52
8	0.437	0.0166	0.482	0.0929	8.37	0.527	0.0077	0.00441	11.18
9	0.547	0.0175	0.577	0.0572	5.15	0.593	0.0302	0.00177	4.49
10	0.597	0.0284	0.624	0.0734	6.61	0.640	0.0584	0.00236	5.99
11	0.641	0.0580	0.665	0.0877	7.90	0.695	0.0467	0.00388	9.83
12	0.695	0.0467	0.740	0.2398	21.59	0.782	0.0191	0.01079	27.35
13	0.881	0.0256	0.899	0.0361	3.25	0.938	0.0014	0.00127	3.22

9.83, 27.35, and 3.22, respectively. All the Rf values were displayed in Table 2 and percentage peak shown in Figure 3.

CONCLUSION

For the quality assurance of ayurvedic products, the standardization by HPTLC for qualitative identification of active compounds present in the HAF is very important. The present investigation revealed the presents of bioactive compounds, namely phenols, flavonoids, tannins, terpenoids, and steroids in HAF. HPTLC of HAF demonstrated the presence of several polyphenolic compounds corresponding to the plant extract and its HAF. Regarding the role of phenolic compounds, HAF could be an appropriate

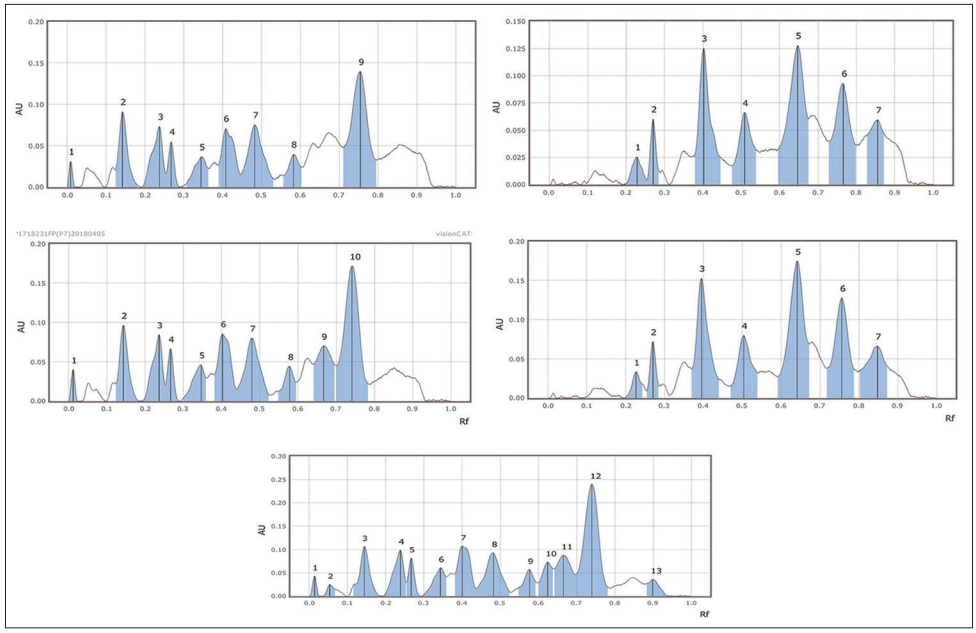

Figure 3: Fingerprinting of polyherbal formulation at wavelength 580 nm

option for traditional uses of Indian medicinal plants and prepared HAF to consider against disease caused by cellular damage. HPTLC fingerprinting profile of HAF has disclosed many active constituents at different characteristic peaks, and Rf values were observed. The result of HPTLC will be helpful to ensure the quality, safety, sustaining development, and reproducibility of HAF.

ACKNOWLEDGMENT

Authors are highly obliged to Chairman Mr. P.K. Gupta, Vice-Chairman Mr. Y.K. Gupta and Dean Dr. Kashmira J. Gohil. Anand College of Pharmacy, Agra (Sharda Group of Institutions) and Anchrom Lab, Mumbai, for providing laboratory support.

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Source of support: Nil; Conflict of interest: None Declared