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Quantification of Ellagic acid, Gallic acid and Picroside-I from *Phalatrikadi kvatha churna* by HPTLC

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Abstract

Objective: Phytochemical evaluation of an ayurvedic formulation *Phalatrikadi kvatha churna* and establishment of multiple marker based methods for its standardization using marker compounds/ biomarkers *viz.* picroside-I, gallic acid and ellagic acid. Materials and methods: Preliminary phytochemical analysis, development of fingerprint profiles and estimation of picroside - I, gallic acid and ellagic acid from the authentic sample and two market samples of the formulation. Results: Preliminary phytochemical screening showed the presence of phenols, tannins, flavonoids and sterols. The solvent systems developed for establishing fingerprint profiles and picroside-I, gallic acid, and ellagic acid gave good resolution of these compounds from the other components of the extract. The HPTLC methods developed for the estimation of marker compounds were validated in terms of instrumental precision, accuracy and repeatability. Picroside-I content of the authentic sample was found to be 0.16 %, whereas the two market samples contained very less picroside-I (~0.038%). The amount of gallic acid in the authentic sample was found to be 0.78%, while the other two market samples contained only about half the amount. The amount of ellagic acid in the authentic sample was found to be 0.12%, while the market samples contained 0.085% and 0.065% of free ellagic acid respectively. Conclusion: Qualitative and quantitative phytochemical evaluation of *Phalatrikadi kvatha churna* was carried out for multimarker based standardization using picroside-I, gallic acid, and ellagic acid by HPTLC. The TLC fingerprint profiles and TLC densitometric methods for the quantification of different markers can be used in the quality control of the formulation. The TLC densitometric methods developed can also be adopted for the quantification of these marker compounds in other preparations as well.

Key words: *Phalatrikadi kvatha churna,* Picroside-I, Gallic acid, Ellagic acid, HPTLC.

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1. Introduction

With growing world wide interest in plant based medicine in the last twenty years, herbal medicine has been enjoying renaissance throughout the developed world. The quality of herbal medicine i.e. the profile of the constituents in the final products has implications for efficacy and safety. Hence it is necessary to set certain quality control parameters for the formulations using modern analytical techniques. In this paper we report phytochemical evaluation of a reputed ayurvedic formulation *viz., Phalatrikadi kvatha churna.*

Phalatrikadi kvatha churna (PKC), widely used in ayurveda as antipyretic and antiemetic, contains *Picrorhiza kurroa, Triphala* and *Tricosanthes dioica* (Table 1) [1]. *P. kurroa* is well established in diseases of liver and spleen including jaundice and anaemia [2]. *Triphala,* three of the ingredients are fruit pulp of *Emblica officinalis, Terminalia bellerica, T. chebula,* contain large amount of tannins and the antioxidant activity of triphala is already established [3]. *Phalatrikadi kvatha churna* had shown beneficial results in the infective hepatitis with jaundice [4].

Phenolic compounds of natural origin, including gallic acid and ellagic acid have been shown to have significant biological activities including bactericidal activity, inhibition of HIV replication, free radical scavenging activity, inhibition of lipid peroxidation, cytotoxic activity, gastric protective action, anti-inflammatory activity [5]. Kutkin, an iridoid glycoside possesses abortifacient, analgesic, antidiarrheal, anti-inflammatory and hepatoprotective activity [6,7]. HPTLC method for the estimation of gallic acid, ellagic acid and picroside-I [8-10], HPLC method for ellagic acid estimation [11,12] and picroside-I estimation [13] were reported.

Phytochemical evaluation of PKC includes preliminary phytochemical testing, establishing TLC fingerprint profiles and developing HPTLC method for the estimation of three important marker compounds *viz.,* picroside-I estimation and for the simultaneous estimation of gallic acid and ellagic acid.

2. Materials and methods

2.1 Plant material and herbal formulation

Plant materials were collected and authenticated. Voucher specimens were preserved in our Pharmacognosy and Phytochemistry Department. The samples were powdered to 40 mesh and formulation was prepared as per the method prescribed in Ayurvedic formulary of India, with the help of an Ayurveda practitioner specialized in the preparation of classical formulations (Rasasastra) and classical text [1]. Two samples PKC were procured from the local market.

2.2 Chemicals

All chemicals used in the experiments were of analytical grade. Gallic acid was a gift sample from Tetrahedron Ltd., India. Ellagic acid was purchased from Natural Remedies Pvt. Ltd., Bangalore and kutkin (a mixture of picroside - I & II 64 : 36) was purchased from Regional Research Laboratory, Jammu, India.

2.3 TLC Conditions

Plate: Precoated silica gel 60 F_{254} TLC plate (E. Merck) (0.2 mm thickness)

Spotter: CAMAG Linomat IV Automatic Sample Spotter

Developing chamber: CAMAG glass twin trough chamber (20 x 10 cm)

Scanner: CAMAG TLC Scanner 3 and CATS 4 software

Experimental conditions: Temperature 25±2°C, relative humidity 40 %

2.4 Phytochemical evaluation of the methanolic extract of Phalatrikadi kvatha churna

2.4.1 Stock solution of Phalatrikadi kvatha churna

Phalatrikadi kvatha churna (1.5 gm) was extracted with methanol (4x25 ml) and filtered, and the solvent was evaporated to dryness under reduced pressure, and it was subjected to preliminary phytochemical analysis for major chemical major groups [14,15].

2.4.2 TLC fingerprint profile of methanolic extract of Phalatrikadi kvatha churna

2.4.2.1 TLC fingerprint profile of methanolic extract with gallic acid and ellagic acid standards

TLC fingerprint profiles of the methanolic extract of different samples of *Phalatrikadi kvatha churna* were established with three chemical/ biological markers *viz.,* kutkin, gallic acid and ellagic acid (Table 2, 3).

15 µl stock solution of methanolic extract of *Phalatrikadi kvatha churna* was applied on precoated TLC plate with 5 µl each of standard solutions of gallic acid and ellagic acid. The plate was developed in a solvent system of toluene : ethyl acetate : formic acid : methanol (3:3:0.8:0.4 v/v) in a twin trough chamber up to a distance of 8 cm. After development, the plate was dried in air and scanned at 280 nm. The resolved bands were evaluated for their spectral details, and the relative concentrations were determined by densitometry. R_F, λ_{max} and shoulder inflections of the resolved bands were noted.

2.4.2.2 TLC fingerprint profile of methanolic extract with kutkin standard

15 µl stock solution of methanolic extract of *Phalatrikadi kvatha churna* and 5 µl of standard solution (100 µg/ml) of kutkin were applied on precoated TLC plate. The plate was developed in a solvent system of ethyl acetate-formic acid-methanol (6:0.6:0.4 v/v) in a twin trough chamber up to a distance of 8 cm. After development, the plate was dried in air and scanned at 280 nm. The resolved bands were evaluated for their spectral details, and the relative concentrations were determined by densitometry. R_F, λ_{max} and shoulder inflections of the resolved bands were recorded.

2.5 Quantification of marker compounds

For the quantification of gallic acid the method we reported earlier [8] was adopted. Solvent systems were optimized for good resolution of marker compounds in the samples. Since gallic acid and ellagic acid resolved in the same solvent system, they both were quantified simultaneously.

2.5.1 Preparation of sample solution

2.5.1.1 Sample solution for simultaneous quantification of gallic acid and ellagic acid

500 mg quantity of different drug samples extracted with methanol (4x25 ml) under reflux for 10 min each time. Extracts were filtered, pooled and concentrated to 25 ml.

2.5.1.2 Sample solution for quantification of Picroside-I

One gm each of the formulations were extracted with ethyl acetate (4x25 ml), extracts were filtered, pooled, evaporated to dryness and redissolved in 25 ml of methanol.

2.5.2 Preparation of standard solution

2.5.2.1 Standard solution of ellagic acid

A stock solution of ellagic acid (100 mg/ml) was prepared by dissolving 10 mg of accurately weighed ellagic acid in methanol and making up the volume to 100 ml with methanol. From this stock solution standard solutions of 15 to 35 mg/ml were prepared by transferring different aliquots (7.5 to 17.5 ml) of stock solution to 50 ml volumetric flasks and adjusting the volume with methanol.

2.5.2.2 Standard solution of kutkin

A stock solution of kutkin was prepared by dissolving 5 mg of accurately weighed kutkin in methanol and making up the volume to 25 ml with methanol. From this stock solution standard solutions of 20 to 100 µg/ml were prepared by transferring aliquots (1 to 5 ml) of stock solution to 10 ml volumetric flasks and adjusting the volume to10 ml with methanol.

2.5.3 Calibration curve

2.5.3.1 Calibration curve for ellagic acid

10 µl each of the standard solutions (150 to 350 ng per respective spot) were applied in triplicate on TLC plate. The plate was developed in a solvent system of toluene : ethyl acetate : formic acid : methanol (3:3:0.8:0.4 v/v) up to a distance of 8 cm. After development, the plates were dried in air and scanned at 280 nm. The peak areas were recorded. Calibration curve of ellagic acid was prepared by plotting peak areas *vs* concentration of ellagic acid applied.

2.5.3.2 Calibration curve for Picroside-I (using kutkin, a mixture of Picroside-I & II)

10 µl each of the standard solutions (200 to 1400 ng per respective spot) were applied in triplicate on a TLC plate. The plate was developed in a solvent system of ethyl acetate : formic acid: methanol (6:0.6:0.4 v/v) in a chamber up to a distance of 8 cm. After development, the plates were dried in air and scanned at 280 nm. The peak areas were recorded. Calibration curve of Picroside-I was prepared by plotting peak areas *vs* concentration of kutkin applied.

2.6 Estimation of marker compounds

2.6.1 Simultaneous estimation of gallic acid and ellagic acid

10 µl each of sample solutions were applied in triplicate on a TLC plate. The plate was developed and scanned at 280 nm. The peak areas and absorption spectra were recorded. The purity of gallic acid, and ellagic acid bands in the sample extracts was checked by recording the absorption spectra at start, middle and end position of the bands. The amount of gallic acid and ellagic acid in different samples was calculated using the respective calibration curve.

Table 1
Composition of *Phalatrikadi kvatha churna.*

Sl No.	List of the ingredients	Botanical name	Parts used
1	Haritaki	*Terminalia chebula*	fruit pulp
2	Bibhitaka	*Terminalia bellerica*	fruit pulp
3	Amlaki	*Embelica officinalis*	fruit pulp
4	Patola	*Tricosanthes dioica*	whole plant
5	Tikta	*Picrorhiza kurroa*	rhizome

2.6.2 Estimation of picroside-I

10 µl each of sample solutions were applied in triplicate on a TLC plate. The plate was developed and scanned as mentioned in section 2.5.3.2.

The peak areas and absorption spectra were recorded. The purity of picroside-I band in the sample extracts was checked by recording the absorption spectra at start, middle and end position of the band. The amount of picroside-I in different samples was calculated using the calibration curve.

2.7 Method validation

The methods were validated for precision, repeatability and accuracy. Instrumental precision was checked by repeated scanning of the same spot of gallic acid (450 ng), ellagic acid (160 ng) and picroside-I (640 ng) seven times and was expressed as coefficient of variance (% CV). The repeatability of the method was affirmed by analyzing 450 ng/spot of standard solution of gallic acid, 160 ng/spot of standard solution of ellagic acid and

Table 2.
TLC profile of methanolic extract of *Phalatrikadi kvatha churna* with gallic acid and ellagic acid standard solution scanned at 254 nm.

PS			MS1			MS2		
R_f	λ_{max}	Relative %	R_f	λ_{max}	Relative %	R_f	λ_{max}	Relative %
0.08	288	5.46	0.07	—	31.56	0.08	317	20.37
—	—	—	—	—	—	0.14	—	3.19
0.19	312	7.33	0.18	316	14.47	0.16	320	4.70
0.27	285	13.87	0.28	308	5.59	0.28	292	4.04
—	—	—	0.36	360	3.14	0.35	364	1.32
0.51	277	11.78	0.53	277	9.82	0.52	275	7.75
	(Ellagic acid)							
0.68	280	31.15	0.68	280	17.82	0.67	281	15.79
	(Gallic acid)							
0.79	328	3.34	—	—	—	0.78	324	5.73
0.86	265,299	24.36	0.86	259	11.61	0.86	260	36.09
0.94	279	2.44	—	—	—	—	—	—

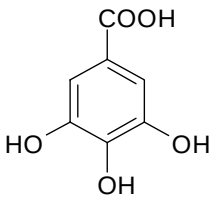
Table 3.
TLC profile of methanolic extract of *Phalatrikadi kvatha churna* with kutkin standard solution scanned at 254 nm.

PS			MS1			MS2		
R_f	λ_{max}	Relative %	R_f	λ_{max}	Relative %	R_f	λ_{max}	Relative %
0.08	292	15.08	0.07	—	43.62	0.08	315	53.57
0.14	302	9.45	0.11	312	4.83	—	—	—
0.24	304	1.18	0.19	309	6.52	0.29	316	1.40
0.34	—	0.70	0.26	310	3.21	—		—
0.41	297	4.56	0.40	314	2.90	0.38	312	4.86
0.59	290	25.76	0.59	291	2.45	0.58	292	2.39
	(Picroside-I)							
0.74	287	5.90	0.72	308	2.29	—	—	—
0.82	291	5.92	0.80	306	4.38	—	—	—
0.90	281	23.48	0.90	289	29.80	0.90	279	37.78
0.96	296	7.97	—	—	—	—	—	—

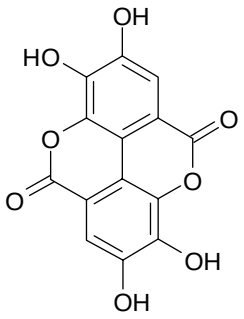
640 ng/spot of picroside-I after application on the TLC plate (n=5) and was expressed as % CV. (Table 4). Accuracy of the method was tested by performing recovery studies at two levels. The percent recovery as well as average percent recovery were calculated.

3. Results and discussion

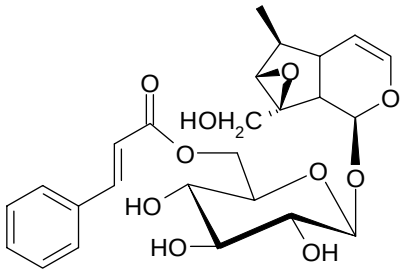
Preliminary phytochemical analysis of the polyherbal formulation PKC showed the presence of phenolics, tannins, steroids/ terpenoids.



Gallic acid



Ellagic acid



Picroside - I

Table 4.
Method validation parameters for the estimation of markers.

Parameters	Gallic acid	Ellagic acid	Picroside-I
Instrumental Precision (% cv)(n=7)	0.083	0.78	0.81
Repeatability	1.07	1.50	1.03
Limit of detection	50 ng	50 ng	65 ng
Limit of quantification	150 ng	150 ng	250 ng
Specificity	Specific	Specific	Specific
Linearity (Correlation coefficient)	0.997	0.988	0.996
Range (ng/spot)	150-750	150-350	256-896

Table 5.
Amount of various markers estimated *Phalatrikadi churna* (% w/w)*.

Markers	PS	MS1	MS2
Gallic acid	0.780 ± 0.059	0.368 ± 0.0084	0.348 ± 0.0023
Ellagic acid	0.121 ± 0.0056	0.085 ± 0.0049	0.065 ± 0.049
Picroside-I	0.165 ± 0.001	0.037 ± 0.007	0.039 ± 0.018

* Mean ± S.D (n=3)

Further TLC fingerprint profiles of the formulation were established and co-chromatography using important biomarkers of the ingredients like gallic acid, ellagic acid and kutkin was carried out. For establishing fingerprint profiles, the methanolic extract of the formulation was resolved using suitable solvent systems. Chromatogram and absorption spectra of the resolved bands were recorded. R_f, λ_{max}, shoulder inflections if any, relative percentage and colour of the resolved bands were noted for the prepared sample (Table 2, 3).

The solvent systems were optimized to achieve good resolution of each of the marker compounds in the sample extracts. Identity of the different markers was confirmed by comparing the absorption spectra of the marker compounds in the sample extracts and the respective reference standards. Purity of band of each marker in the sample track was confirmed by overlaying the absorption spectra recorded at start, middle and end position of the band (Fig. 3a, 3b & 3c).

Kutkin obtained was a mixture of picroside I and II, in a ratio of 64 : 36. In the sample extracts picroside-I was found to be present. A band in the sample extract resolved at the same R_f as picroside-II was found to be of a different compound, as revealed by the comparison of the absorption spectra.

Efforts were made to simplify the sample preparation step and resolve more than one

Table 6.
Recovery study of marker compounds by HPTLC method.

Amount of Marker compound present (µg)	Amount of marker added (µg)	Amount of marker found (µg)*	Percent recovery*	Average percent recovery
Gallic acid				
950	475	1419 ± 0.098	99.58 ± 0.019	99.73
950	950	1897 ± 0.002	99.89 ± 0.053	
Ellagic acid				
980	490	1475 ± 0.009	100.34 ± 0.032	99.91
980	980	1950 ± 0.004	99.48 ± 0.048	
Picroside-I				
825	414	1237.12 ± 0.86	99.84 ± 0.80	99.79
825	820	1640.31 ± 0.12	99.74 ± 0.59	

* Mean ± S.D (n=3)

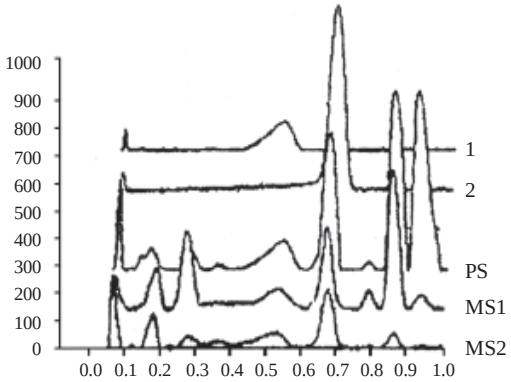


Fig. 1
TLC densitometric chromatogram of sample solutions of *Phalatrikadi kvatha churna* with gallic acid and ellagic acid standard solution
1 - Ellagic acid; 2 - Gallic acid; PS - Sample solution of prepared sample; MS1 - Sample solution of market sample 1; MS2 - Sample solution of market sample 2

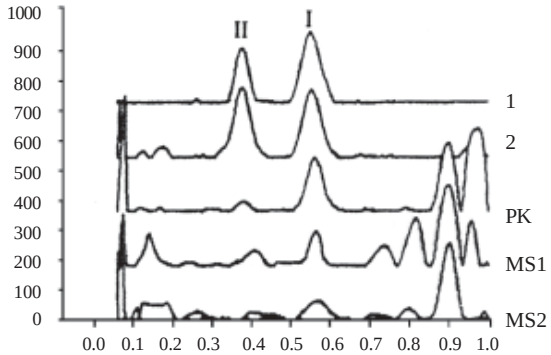


Fig. 2
TLC densitometric chromatogram of sample solutions of *Phalatrikadi kvatha churna* with Kutkin standard solution
1 - Kutkin standard solution; I - Picroside-I; II - Picroside-II; PK - Sample solution of *Picrorhiza kurroa* ; PS - Sample solution of prepared sample; MS1 - Sample solution of market sample 1; MS2 - Sample solution of market sample 2

marker in the same solvent system, which enabled simultaneous estimation of gallic acid and ellagic acid (Fig. 1). The amount of gallic acid in the authentic sample was 0.78%, whereas in market samples it was 0.35 - 0.37%. Ellagic acid was found to be 0.12% in the authentic sample, whereas it varied from 0.065 - 0.085 in the market samples. In authentic sample picroside-I was found to be 0.165%, but in market samples it was found to be in very less amount i.e. 0.037-0.039% (Fig. 2).

The average percentage recovery at two different levels of gallic acid and ellagic acid were found to be 99.73%, 99.91% respectively and average percent recovery of picroside–I was found to be 99.79%. (Table 6).

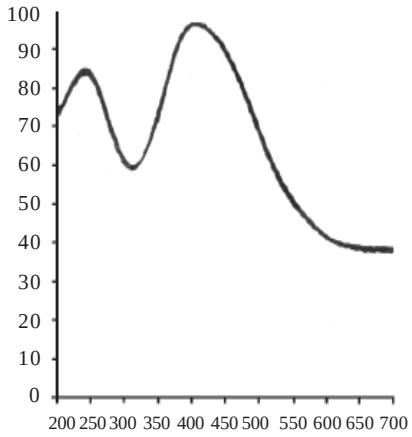


Fig. 3a

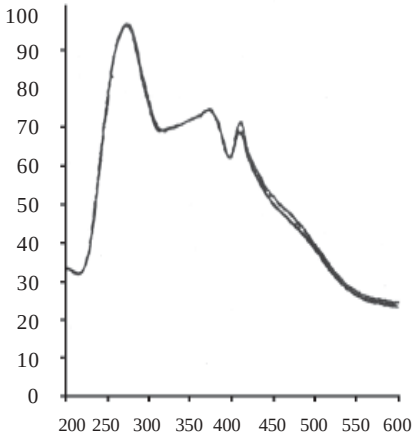


Fig. 3b

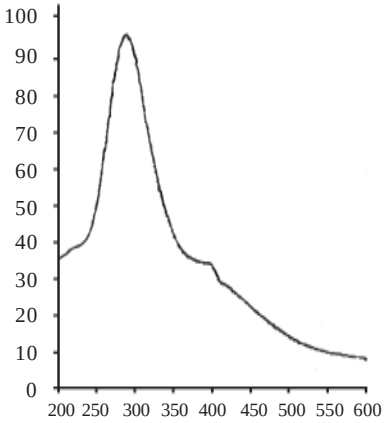


Fig 3c

Fig. 3.
Overlay UV absorption spectra of gallic acid (3a), ellagic acid (3b) and picroside–I (3c) in the sample extract of prepared sample (PS).

4. Conclusion

The methods developed for the extraction and TLC densitometric quantification of different markers *viz.*, gallic acid, ellagic acid and picroside-I from the PKC were simple with minimum sample clean-up requirement. The multiple-marker based approach described in the present work for the standardization of the polyherbal formulation aid in setting quality control parameters and can serve routine quality control purpose.

Multiple marker based phytochemical evaluation of the polyherbal formulation *Phalatrikadi kvatha churna* is an effort towards meeting quality standards for herbal preparations, especially in the present times when herbal preparations in general and Ayurveda in particular are gaining acceptance all over the world.

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