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Research Article

CHEMICAL EVALUATION OF DIFFERENT PARTS OF *JUGLANS REGIA* L. AND SIMULTANEOUS DETERMINATION OF IMPORTANT POLYPHENOLS BY THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Juglans regia L. (Family - Juglandaceae), commonly known as Walnut, is a large deciduous tree. It contains several therapeutically active constituents, especially phenolic compounds. In the present communication a TLC method was established for the identification of five polyphenols in different parts of *J. regia* and their simultaneous quantification. The method was validated in terms of precision, repeatability, specificity, sensitivity, linearity and accuracy. The method permits reliable quantification and showed good resolution and separation from other constituents of extract on silica gel with toluene-ethyl acetate-formic acid (5:5:1 v/v/v) as mobile phase, and characteristic bands of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid were observed at R_f 0.34, 0.42, 0.56, 0.70 and 0.72 respectively. These polyphenols were estimated in terms of mg/g dry weight. The results indicated that caffeic acid was observed in all three parts although its quantity varied from part to part i.e. 0.558, 1.863 and 2.610 mg/g in leaf, stem bark and fruit respectively. However, gallic acid was estimated in stem bark (13.392 mg/g) and leaf (3.028 mg/g). On the contrary, (+) - catechin and (-) - epicatechin were present only in stem bark and syringic acid (0.511 mg/g) was observed only in leaves.

Keywords: *Juglans regia,* Polyphenols, TLC, Walnut.

INTRODUCTION

Juglans regia L. (Family - Juglandaceae), commonly known as walnut, is a large deciduous tree with a short trunk and broad crown, growing up to 40-60 m. It is native to the mountain ranges of Central Asia, Nepal, Tibet, northern India, Pakistan, Afghanistan, Turkmenistan and Iran to portions of Azerbaijan, Armenia, Georgia and eastern Turkey. All parts of the plant; stem, bark, leaves, fruits, seeds, seed oil are used in folk medicines to treat variety of health disorders including cancer[1]. Walnut leaves are alterative, anthelmintic, anti-inflammatory, astringent and depurative used for the treatment of constipation, chronic coughs, asthma, diarrhoea, dyspepsia, skin ailments[2, 3] and strumous sores[4]. Fruits are edible and used for frosted bite[5]; rheumatism[6], toothache[7, 8], fruit shell as tooth powder and sores of toes[9]. Stem bark is reported as anti-inflammatory, blood purifying, anticancer, depurative, diuretic

and laxative[4] and its decoction is useful in toothache[7].

Several polyphenols were reported from different parts of *Juglans regia*, like, gallic acid, protocatechuic acid, vanillic acid, (+)-catechin, (-)-epicatechin, myricetin from husks[10], p-coumaric, syringaldehyde from ripe fruits[11], 3-caffeoylquinic, 3-p-coumaroylquinic, 4-p-coumaroylquinic acids, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside, quercetin 3-pentoside, kaempferol 3-pentoside, quercetin, cyanadin, kaempferol, caffeic acid, p-coumaric acid, hyperin, quercitrin, kaempferol-3-arabinoside and quercetin-3-arabinoside from leaf[12, 13], and juglone, quercetin, myricetin, rutin, caffeic acid, and gallic acid from stem bark[14].

In the past two decades TLC / HPTLC is becoming a routine analytical technique because of advantages which include high sample throughput at low operating cost, easy sample preparation, short analysis time, and possibility of analysis of several samples at a time. A number of reports are available for the identification and estimation of polyphenolic compounds through this technique[14, 15-22]. Some reports on various analytical methods viz. spectrophotometry, column chromatography, HPLC, TLC/HPTLC, for the identification of phenolic compounds in various parts of *J. regia* are available[14, 23-27]. Literature survey revealed that no TLC/ HPTLC method has been reported for simultaneous determination of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid, and syringic acid in leaves, stem bark and fruits of *J. regia.* Hence, in this communication an attempt was made to evaluate aforesaid polyphenols and their quantitative estimation through TLC in different parts of *J. regia.*

MATERIALS AND METHODS

Plant material

Leaves, stem bark and fruits of *J. regia* were collected from Chaubatiya of Almora District during the months of March – April year 2014 and its identity was confirmed by matching with the samples in the Herbarium of the Institute. Samples were dried at 40°C in a hot-air oven. The samples were stored at 25°C in air-tight containers and powdered to 60 meshes when required.

Chemicals

All the solvents used in the experiments were of analytical grade and procured from Merck (Germany). Reference standards caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid, and syringic acid were procured from Sigma – Aldrich catalog numbers 03940590 (FLUKA), 43412 (FLUKA), 91215 (FLUKA), 60018 (FLUKA) and 63627 (FLUKA) respectively

Sample solution

Powdered (60 mesh) dried plant samples (10 g) were extracted with methanol (4 x 25 mL, three times each for 3 days). Extraction was checked by adding methanol (20 mL) to the exhausted part, filtering and evaporating to dryness. There was no residue available in evaporated sample; this was also confirmed with a simple TLC study. The extracts were combined (separately for each samples), filtered, evaporated to dryness using a rotary evaporator. Accurately weighed extract (10 mg) was dissolved in 1mL of methanol, to get 10 mg/mL of solution and filtered through a 0.45 µm filter membrane; the filtrate was used as sample solution for the quantification of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid.

Standard solution of polyphenol markers

All the marker compounds were completely soluble in methanol. Stock solutions (1 mg/mL) of standard polyphenols were prepared by dissolving 5 mg of each accurately weighed caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid, and syringic acid in 5mL of methanol separately. The stock solutions described were used for further dilutions for plotting the linear regression graph of the standard markers. The aliquots of 0.1- 0.5 mL of caffeic acid, gallic acid and syringic acid while 1-5 mL of (+)-catechin and (-)-epicatechin stock solutions were transferred to 10 ml volumetric flasks separately and the volume of each flask was adjusted to 10 ml with methanol to obtain desired concentration of standard phenolic markers i.e. 10, 20, 30, 40, 50 µg/mL solutions of caffeic acid, gallic acid, syringic acid and 100, 200, 300, 400, 500 µg/mL solutions of (+)-catechin and (-)-epicatechin.

Chromatography conditions

The ICH guidelines [28, 29] were followed for the validation of the analytical method developed for precision, accuracy and repeatability. TLC was performed on 20 x 10 cm TLC aluminium plates precoated with a 200 µm thick layer of silica gel 60F254 (SD Fine Chemicals Ltd, Mumbai, India, product no. 25447). Samples were applied using 100 µL Hamilton syringes with Linomat 5 applicator (Camag, Switzerland) under a flow of N2 gas. Linear ascending development was carried out with 20 mL toluene–ethyl acetate–formic acid (5:5:1 *v/v/v*) for co-chromatography with five polyphenol markers viz. caffeic acid, (+)-catechin, (-) -epicatechin, gallic acid and syringic acid. The plates were developed using a standardized solvent system in Camag glass twin trough chamber (20 x 10 cm); the experimental condition temperature 25 ± 2°C and relative humidity 40 %. The plates were developed to a distance of approximately 80 mm from the point of application. The chamber was previously saturated with mobile phase vapor for 30 min at room temperature (25 ± 2°C). After removal from the chamber, plates were completely dried using an air drier. The photographs were taken by CAMAG Reprostar 3 video documentation unit by illumination at UV 254 nm and 366 nm. Scanning was performed using a TLC Scanner 3 (Camag, Switzerland) at λ_{max} 300 nm in UV absorbance mode operated by winCATS software (version3.2.1).

Preparation of calibration curve of standard markers

10 ml of the standard solutions of each markers viz. caffeic, gallic, syringic acids (100-600 ng/spot) and (+)-catechin and (-)-epicatechin (1-6 µg/spot) were applied (band width: 6 mm, distance between the tracks: 10.5 mm, distance from the bottom edge: 10 mm, starting from the edge: 10 mm) in triplicate on separate TLC plates using automatic sample applicator. The plates were developed in a twin trough chamber (20 x 10 cm) to a distance of 8 cm using 20 ml of toluene–ethyl acetate–formic acid (5:5:1 *v/v/v*) solvent system at room temperature (25 ± 2°C) and relative humidity 40 %. After removal from the chamber, plates were completely dried in air at room temperature. Densitometric scanning was performed at 300 nm in absorbance mode using deuterium lamp. The area of resolved peaks was recorded. Calibration curve of (+)-catechin, caffeic acid, (-)-epicatechin, gallic acid and syringic acid were obtained by the winCATS software showing peak areas vs concentrations of respective standard markers applied.

Simultaneous quantification of five polyphenols in the samples

20µl of sample solutions of methanolic extracts of leaves, stem bark and fruits of *J. regia* were applied in triplicate along with the standard phenolic markers on TLC plate. The plates were developed and scanned as mentioned above. The peak areas of the corresponding peaks of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid, and syringic acid were recorded and the amount of aforesaid five polyphenols was calculated using the calibration curve.

Repeatability for instrumental precision

Instrumental precision was checked by repeated scanning (n= 6) of the same spot of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid. The analysis was performed using standard solutions of 300 ng per band of caffeic acid, gallic acid, syringic acid, and 3000 ng band of (+)-catechin and (-)-epicatechin and to measure peak areas and expressed as relative standard deviation (% R.S.D.).

Inter-day and intra-day variation

The inter-day and intra-day variation for the determination of five polyphenols were carried out at three different concentration levels. The method was studied by analyzing aliquots of standard solution containing 200, 300, 400 ng/spot of gallic acid, caffeic acid, syringic acid and 2000, 3000, 4000 ng/spot of (+)-catechin and (-)-epicatechin on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % R.S.D.

Limit of detection and limit of quantitation

Sensitivity of the instrument was observed in terms of limit of detection (LOD) and limit of quantitation (LOQ). Based on the calibration curve the LOD and LOQ were estimated. LOD and LOQ were experimentally verified

by applying different concentrations of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid along with methanol as blank and determined on the basis of signal to noise ratio. LOD was considered as 3.3σ/S and LOQ as 10σ/S where σ corresponds to the average standard deviation of the y-intercepts of regression lines and S corresponds to the average slope of the regression lines.

Recovery

Recovery as accuracy was performed by the method of standard addition. The pre-analyzed samples were spiked with extra 50%, 100% and 150% of the standard caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid and the mixtures were re-analyzed by the proposed method. The experiment was conducted three times. This was done to check for the recovery of five polyphenols at different levels in the extracts. The percent recoveries and the average percent recoveries were calculated.

Specificity

Specificity of the method was ascertained by analyzing and comparing R_f values and the spectra of spot in the samples with that of standard polyphenols and by spiking standard caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid with the extract samples. The peak purity of all the phenolic markers was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

RESULTS AND DISCUSSION

TLC fingerprint and co-chromatography

Chromatographic fingerprinting is most popular technique and being used extensively in the recent years for quality assessment of herbal drugs, identification of adulterants/substitutes and batch to batch consistency of the products[21-23, 30-33]. In pharmacopoeias also the Rf values of general TLC profiles have been included as minimum requirement of quality standards of single herbal drugs and compound formulations[33]. Further, the developed fingerprint profiles of chemical components can not only be used for detection of markers of interest but also for the ratio of all the detectable constituents as well. However, TLC procedure was optimized with a view to quantify the polyphenols in herbal extract. Different compositions of acetic acid, acetone, *n*-butanol, chloroform, ethyl acetate, ethyl formate, formic acid, *n*-hexane, methanol, toluene, and water were tested as mobile phase for TLC analysis in order to obtain high resolution and reproducible peaks (Table 1). The mobile phase was tested using toluene - ethyl acetate - formic acid (5: 5: 1 *v/v/v*) to obtain high resolution and reproducible peaks. This mobile phase was also found to be the best for simultaneous determination of five polyphenols in different parts of *J. regia*. The mobile phase gave well-defined peaks at Rf = 0.34, 0.42, 0.56, 0.70, 0.72 for (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid respectively (Fig. 1). The spots were more pronounced when the chamber was saturated with mobile phase for 30 min at room temperature.

TLC fingerprint profile showed qualitative and quantitative variations of the chemical constituents (Fig. 1, Table 2). The band of caffeic acid at R_f 0.70 was observed in all the parts i.e. leaves, stem bark and fruits. Similarly, three additional other constituents at R_f 0.19 and 0.23 under UV 254 nm and UV 366 nm respectively were observed in all three parts of this plant. Likewise, a common black band at R_f 0.10 was observed in leaves and stem bark extracts under both wavelengths. However, additional black bands at R_f 0.23 under UV 254 nm and at R_f 0.84 under UV 366 nm were also present both the aforesaid parts. On the contrary, the band of gallic acid was observed at R_f 0.56 in stem bark and fruits and syringic acid at R_f 0.72 only in leaves. Besides, other characteristic identifying bands at R_f 0.15 (blue), 0.28 (dark blue), and 0.93 (pink) under UV 366 nm were also observed in leaves. Similarly, stem bark also showed characteristic identifying bands of (-)-epicatechin, (+)-catechin at R_f 0.34 and 0.42 respectively along with four additional bands at Rf 0.19 (blue), 0.66 (fluorescent blue), 0.78 (black) and 0.90 (dark blue) under UV 366 nm**.**

Validation of developed TLC method

The TLC densitometric method was validated in terms of instrument precision, linearity, recovery, LOD and LOQ (Tables 3-5). The developed TLC method for estimation of (-)-epicatechin, (+)-catechin,

gallic acid, caffeic acid, and syringic acid showed linearity and a good correlation coefficient in concentration range of 1000-5000 ng spot^{-1} for (+)-catechin and (-)-epicatechin and 100-500 ng spot^{-1} for gallic acid, caffeic acid, and syringic acid with respect to the peak area (Table 3). The precision study was comprised of repeatability, inter-day and intra-day variations. The developed method was found to be precise with % R.S.D. 1.48, 1.20, 1.25, 0.88 and 0.66 for (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid respectively. The % R.S.D. of intra-day variation ranges from 0.936-1.112, 0.899-1.381, 1.392-1.745, 1.275-1.461 and 1.122-1.783 for (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid respectively. However, % R.S.D. for inter-day variation was in the range of 1.526-1.874, 1.268-1.779, 1.491-1.906, 1.557-1.691 and 1.464-1.934 for (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid respectively (Table 4). The aforesaid results indicate that the proposed method was precise and reproducible. The average percentage recoveries at three different levels of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid were found to be 99.35%, 99.66%, 99.23%, 99.63% and 99.45%, respectively (Table 5). The purity of the marker components in the sample extracts were also confirmed by spiking and comparing absorption spectra at three different levels, viz. peak start, peak apex and peak end positions of the spot.

TLC densitometric quantification of polyphenols

There is no method for the simultaneous detection and quantification of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid in different parts of *J. regia.* Hence, we developed a precise, validated and simple TLC method for qualitative and quantitative determination of aforesaid five phenolic compounds in leaves, stem bark and fruits of this important plant of commerce. The amounts of caffeic acid, (+)-catechin, (-)-epicatechin, gallic acid and syringic acid in the samples were calculated by the densitometric scanning and using the software and the identity of all the aforesaid marker bands from the sample extracts were confirmed by spiking and overlaying absorption spectra obtained from standard solutions of respective phenolic compounds with the samples. For the quantitative determination of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid, the analysis of methanolic extract of different parts of *J. regia* were repeated three times. The average content of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid are given in Table 6. It is clear that these polyphenols vary from sample to sample in their presence and quantity. It is notable that the caffeic acid was present in all three parts although its quantity varies from part to part. On the contrary, syringic acid was present only in the leaves and gallic acid was absent only in the leaves. However, (-)-epicatechin and (+)-catechin were present only in stem bark.

Table 1: Different compositions of the mobile phase for simultaneous analysis of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid from different parts of *J. regia* through TLC

S. No.	Solvents	Ratios
1.	Ethyl acetate : methanol : water	10 : 1 : 1
2.	*n*- Hexane : ethyl acetate : formic acid	2.9 : 1.9 : 0.1
3.	Chloroform : ethyl acetate : formic acid	7.5: 6 : 0.5
4.	*n* -Butanol : acetic acid : water	6 : 2 : 1
5.	Toluene : ethyl acetate : formic acid	5 : 5 : 1
6.	Toluene : ethyl acetate : formic acid	6 : 4 : 1
7.	Toluene : ethyl acetate : formic acid	4 : 6 : 1
8.	Toluene : ethyl formate : formic acid	6 : 4 :1
9.	Toluene : acetone : formic acid	9 : 9 : 2
10.	Ethyl acetate : formic acid : water	8.5 : 1 : 1.5

Table 2: R_f values of the components present in different parts of *J. regia*

R_f	Under UV 254 nm			Under UV 366 nm		
	Leaves	Stem bark	Fruits	Leaves	Stem bark	Fruits
0.10	+	+	-	Black	Black	-
0.15	+	-	-	Blue	-	-
0.19	+	+	+	-	Blue	-
0.23	+	+	-	Black	Black	Black
0.28	+	-	-	Dark blue	-	-
0.34	-	+	-	-	Black	-
0.42	-	+	-	-	Black	-
0.50	-	+	-	-	-	-
0.56	-	+	+	-	Dark Blue	Light Blue
0.61	-	+	-	-	-	-
0.66	-	+	-	-	Fl. Blue	-
0.70	+	+	+	Fl. Blue	Fl. Blue	Fl. Blue
0.72	-	-	-	Ink Blue	-	-
0.78	-	-	-	-	Black	-
0.84	-	+	-	Light Blue	Greenish Yellow	-
0.90	-	-	-	-	Dark Blue	-
0.93	-	-	-	Pink	-	-

Fl. Fluorescent

Table 3: Validation of TLC densitometry method for estimation of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid, and syringic acid

Property	(-)-Epicatechin	(+)-Catechin	Gallic acid	Caffeic acid	Syringic acid
R_f	0.34	0.42	0.56	0.70	0.72
Repeatability (n=6) %RSD	1.48	1.20	1.25	0.88	0.66
Limit of detection [ng/band]	499.67	808.91	78.63	25.55	24.53
Limit of quantitation [ng/band]	1514.16	2451.24	238.27	77.44	74.32
Specificity	Specific	Specific	Specific	Specific	Specific
Linearity [ng/band]	1000-5000	1000-5000	100-500	100-500	100-500
Linear Regression (r)	0.9963	0.9932	0.9983	0.9963	0.9971

Table 4: Intra-day and inter-day precision of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and syringic acid

Marker	Concentration (ng/spot)	Intra-day precision*	Inter-day precision*
(-)-Epicatechin	2000	0.936	1.874
	3000	1.112	1.526
	4000	1.032	1.667
(+)-Catechin	2000	1.369	1.716
	3000	0.899	1.268
	4000	1.381	1.779
Gallic acid	200	1.695	1.906
	300	1.392	1.491
	400	1.745	1.886
Caffeic acid	200	1.461	1.557
	300	1.275	1.578
	400	1.308	1.691
Syringic acid	200	1.122	1.464
	300	1.321	1.744
	400	1.783	1.934

* =% R.S.D; mean (n= 3).

Table 5: Recovery studies of polyphenols at 50%, 100% and 150% addition by the proposed TLC densitometric method

Marker	Amount of marker added (µg)	Amount of marker found (µg±SD)	Recovery* (%)	Average recovery (%)
(-)-Epicatechin	50	50.21±1.35	100.42	
	100	98.37±1.41	98.37	99.35
	150	148.89±0.97	99.26	
(+)-Catechin	50	49.87±1.63	99.74	
	100	98.56±0.89	98.56	99.66
	150	151.03±1.56	100.69	
Gallic acid	50	48.75±1.22	97.5	
	100	100.35±1.08	100.35	99.23
	150	149.78±1.74	99.85	
Caffeic acid	50	51.87±0.92	103.74	
	100	99.63±1.86	99.63	99.63
	150	147.78±1.48	98.52	
Syringic acid	50	49.35±0.88	98.7	
	100	99.21±0.91	99.21	99.45
	150	150.67±1.25	100.45	

* Mean± SD (n= 3).

Table 6: Percentage of polyphenols in the extract of three parts of *J. regia*

Polyphenols	Leaf	Stem bark	Fruit
(-)-Epicatechin	-	0.369±0.36	-
(+)-Catechin	-	0.162±0.78	-
Gallic acid	-	1.339±1.23	0.303±0.71
Caffeic acid	0.056±1.03	0.186±0.94	0.261±1.35
Syringic acid	0.511±1.47	-	-

Value-Mean± SD (n= 3).

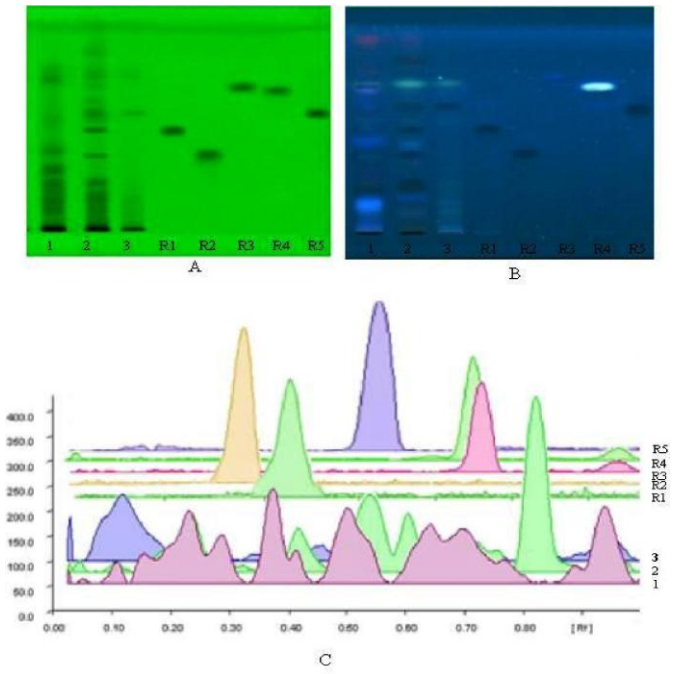

Fig. 1: TLC image and digital scanning profiles of different parts of *J. regia* and polyphenol markers. A, Visualization under UV 254 nm; B, Visualization under UV 366 nm; C, Densitometry scanning profile at 300 nm. 1-Leaves, 2-Stem bark, 3-Fruits, R1- (+)-Catechin, R2- (-)-Epicatechin, R3- Syringic acid, R4- Caffeic acid, R5- Gallic acid

CONCLUSIONS

A TLC densitometry method was developed to work out the different parts (leaves, stem bark and fruits) of *J. regia* and estimation of five polyphenolic. The method was found to be simple, precise, specific, sensitive, and accurate and can be used for the simultaneous detection of (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid, and syringic acid in different parts of *J. regia.* This analysis can be performed without any special sample pretreatment and sixteen samples can be analyzed on a single TLC plate. Secondly, this is an important edible and medicinal plant and also used for the treatment of various disorders in the indigenous systems of medicine. The establishment of this study can provide the quality control markers of this plant.

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REFERENCES

1. Bown D. Encyclopedia of Herbs and their uses. Darling Kinderley, London. 1995;145.
2. Launert E. Hamlyn Guide - Edible and Medicinal Plants of Britain and Northern Europe, The Hamlyn Publishing Group Ltd., London, UK. ISBN 0-600-37216-2. 1981.
3. Duke JA, Ayensu ES. Medicinal Plants of China. Reference Publications, Inc., Algonac, Michigan. ISBN 0-917256-20-4.1985.
4. Chopra RN, Nayar SL, Chopra IC. Glossary of Indian Medicinal Plants (Including the Supplement). Council of Scientific and Industrial Research, New Delhi, India. 1986;145-146.
5. Dar GH, Vir J, Kachroo P, Buth GM. Ethnobotany of Kashmir-I. Sind Valley. J Econ Tax Bot. 1984;5:668-675.
6. Hajra PK, Chakraborty PC. A survey of wild plants sold in the Lal market of Gangtok. Indian J Forestry. 1981;4:217-220.
7. Negi KS, Tiwari JK, Gaur RD. Economic importance of some common trees in Garhwal Himalaya: An ethnobotanical study. Indian J Forestry. 1985;8:276-289.
8. Gupta RK. Some unusual and interesting food plants of the Garhwal Himalaya. Agric. Trop Bot Appl. 1962;9(11-12):532-535.
9. Shah NC, Joshi MC. An ethnobotanical study of Kumaon region of India. Econ Bot. 1971;2:414-422.
10. Stampar F, Solar A, Hudina M, Veberic R, Colaric M. Traditional walnut liqueur – cocktail of phenolics. Food Chem. 2006;95(4):627-631.
11. Colaric M, Veberic R, Solar A, Hudina M, Stampar F. Phenolic acids, syringaldehyde, and juglone in fruit of different cultivars of *Juglans regia*. J Agri Food Chem. 2005;53(16):6390-6396.
12. Amaral JS, Seabra RM, Andrade PB, Valentao P, Pereira JA, Ferreres F. Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves. Food Chem. 2004;88(3):373-379.
13. Rastogi RP, & Mehrotra BN. Compendium of Indian Medicinal Plants, Vol. V, 1990-1994, Central Drug Research Institute, Lucknow, Publication and Information Directorate, New Delhi. 1998; 465.
14. Ghosh P, Katiyar A. Densitometric HPTLC analysis of juglone, quercetin, myricetin, rutin, caffeic acid, and gallic acid in *Juglans regia* L. J Planar Chromatogr Mod TLC. 2012;25(5): 420-425.
15. Waksmundzka-Hajnos M. Chromatographic separations of aromatic carboxylic acids. J Chromatogr-B. 1998;717:93-118.
16. Onyilagha J, Bala A, Hallett R, Gruber M, Soroka J, Westcott N. Leaf flavonoids of the cruciferous species, *Camelina sativa*, *Crambe* spp., *Thlaspi arvense* and several other genera of the family Brassicaceae. Biochem Systemat Ecol. 2003;31:1309-1322.
17. Simonovska B, Vovk I, Andrensek S, Valentova K, Ulrichova J. Investigation of phenolic acids in yacon (*Smallanthus sonchifolius*) leaves and tubers. J Chromatogr-A. 2003;1016:89-98.
18. Seemungal A, Petroczi A, Naughton D P. Application of thin-layer chromatography to rank the efficacies

of five antioxidants in red wine. J Planar Chromatogr Mod TLC. 2011;24(4):320-324.
19. Pandey M M, Rastogi S, Rawat AKS. 2010. Optimization of a HPTLC method for the separation and identification of phenolics. J Planar Chromatogr Mod TLC. 2010;23(2):108-111.
20. Khatoon S, Singh N, Srivastava N, Rawat AKS, Mehrotra S. Chemical evaluation of seven *Terminalia* species and quantification of important polyphenols using HPTLC. J Planar Chromatogr Mod TLC. 2008;21(3):167-177.
21. Khatoon S, Singh H, Singh K, Goel AK. TLC evaluation and quantification of phenolic compounds in different parts of *Dendrophthoe falcata* (Linn. f.) Etting. J Planar Chromatogr Mod TLC. 2010;23(2):104-107.
22. Khatoon S, Singh H, Goel AK. Use of HPTLC to establish the chemotype of a parasitic plant, *Dendrophthoe falcata* (Linn. f.) Etting. (Loranthaceae), growing on different substrates. J Planar Chromatogr Mod TLC. 2011;24(1):60-65.
23. Zhang Z, Liao L, Moore J, Wua T, Wang Z. Antioxidant phenolic compounds from walnut kernels (Juglans regia L.). Food Chem. 2009;113(1):160-165.
24. Jakopic J, Veberic R, Stampar F. Extraction of phenolic compounds from green walnut fruits in different solvents. Acta Agric Slov. 2009;93(1):11-15.
25. Bujdoso G, Vegvari G, Hajnal V, Ficzek G, Toth M. Phenolic profile of the kernel of selected persian walnut (*Juglans regia* L.) cultivars. Not Bot Horti Agrobo. 2014;42(1):24-29.
26. Noumi E, Snoussi M, Trabelsi N, Ksouri R, Hamdaoui G, Bouslama L, Bakhrou A. Antioxidant activities and reversed phase-high performance liquid chromatography (RP-HPLC) identification of polyphenols in the ethyl acetate extract of Tunisian *Juglans regia* L. treated barks. J Med Pl Res. 2012;6(8):1468-1475.
27. Liu J, Meng M, Li C, Huang X, Di D. Simultaneous determination of three diarylheptanoids and alpha-tetralone derivative in the green walnut husks (*Juglans regia* L.) by high-performance liquid chromatography with photodiode array detector. J Chromatogr-A. 2008;1190(1-2):80-5.
28. International Conference on Harmonization (ICH) Guideline Q2B. Validation of Analytical procedures, Methodology (Step 5, Nov. 1996; CPMP/ICH/281/95ICH 1996). 2005.
29. International Conference on Harmonization (ICH) Guideline. Q1A (R2): Stability testing of New Drugs Substances and Products. International Conference on Harmonization/IFPMA: Geneva. 2003.
30. Khatoon S, Rai V, Rawat AKS, Mehrotra S. Comparative pharmacognostic studies of three *Phyllanthus* species. J Ethnopharmacol. 2006;104:79-86.
31. Singh N, Khatoon S, Srivastava N, Rawat AKS, Mehrotra S. Qualitative and quantitative standardization of *Myrica esculenta* Buch.-Ham. stem bark through HPTLC. J Planar Chromatogr Mod TLC. 2009;22 (4): 287-291.
32. Khatoon S, Singh H, Rathi A, Ojha SK, Rawat AKS. Standardization of an Ayurvedic formulation- Kalyanavleha and estimantion of curcumin using HPTLC. Indian J Tradit Know. 2014;13(3):535-542.
33. Khatoon S. Macro-microscopy and planar chromatography - Important tools for quality control and identification of adulterants/substitutes of Unani Drugs. Int J Adv Pharm Med Bioallied Sci. 2015;3(1):58-64.