

Integrating greener RP-HPLC method and chemical pattern recognition for the identification of *Acorus calamus* L. and its common adulterants in Indian markets

Mridul Kant Chaudhary¹, Ankita Misra¹, Adarsh Tiwari¹, Rupali Sharma¹, Sanjeev Kumar Lale², Nagayya Shiddamallayya³, Rabinarayan Acharya³, Sharad Srivastava^{1*}

¹Pharmacognosy Division, CSIR-National Botanical Research Institute, Lucknow, UP, 226001, India

²Central Ayurveda Research Institute, Jhansi, UP, 284003, India

³CCRAS, New Delhi, 110058, India

¹Author contributed equally

*Corresponding author: Dr Sharad Srivastava, E-mail: sharad_ks2003@yahoo.com.

Abstract

This work deals with the development of a greener RP-HPLC method and chemical pattern recognition for the identification of *Acorus calamus* L. collected from different natural sources and samples traded as '*Vacha*' in Indian herbal drug markets. The simultaneous quantification of α and β -asarone was done under 0.10% orthophosphoric acid (A) and acetonitrile (B) in isocratic manner (35:65 v/v) at 0.70 mL min⁻¹. The developed method was found greener on various metrics like AES, GAPI, NEMI, AGREE, AGREEprep than existing methods. The LC chromatogram showed 11 common peaks, containing β -asarone (peak 10) and α -asarone (peak 11) as major markers. The α and β -asarone were identified at R_t 10.69 ± 0.08 and 10 ± 0.06 minutes, and content varies from 0.02±0.001 to 0.71±0.07 and 2.76±0.03 to 39.26±0.14 µg/mg, respectively. Chemical pattern recognition was applied for quality evaluation of *Acorus* samples, and hierarchical cluster analysis bifurcates, 17 samples into two categories. Principal component analysis integrated with orthogonal partial least square discrimination showed that first two principal components efficiently reflects the similarity, and differences among the populations. Further, the component P7, P10 (β -asarone) and P11 (α -asarone) will serve as 'landmark component' for quality evaluation of natural and marketed samples. Results advocates that traded *Vacha* samples shares chemical similarity with authentic *Acorus* and the common adulterants (*Costus speciosus*, *Typha angustifolia*, *Alpinia galanga*) have entirely different profile. The integration of HPLC fingerprint and chemical pattern recognition will provide a scientific rationale for the identification of authentic raw drug and quality control of its adulterants/substitutes.

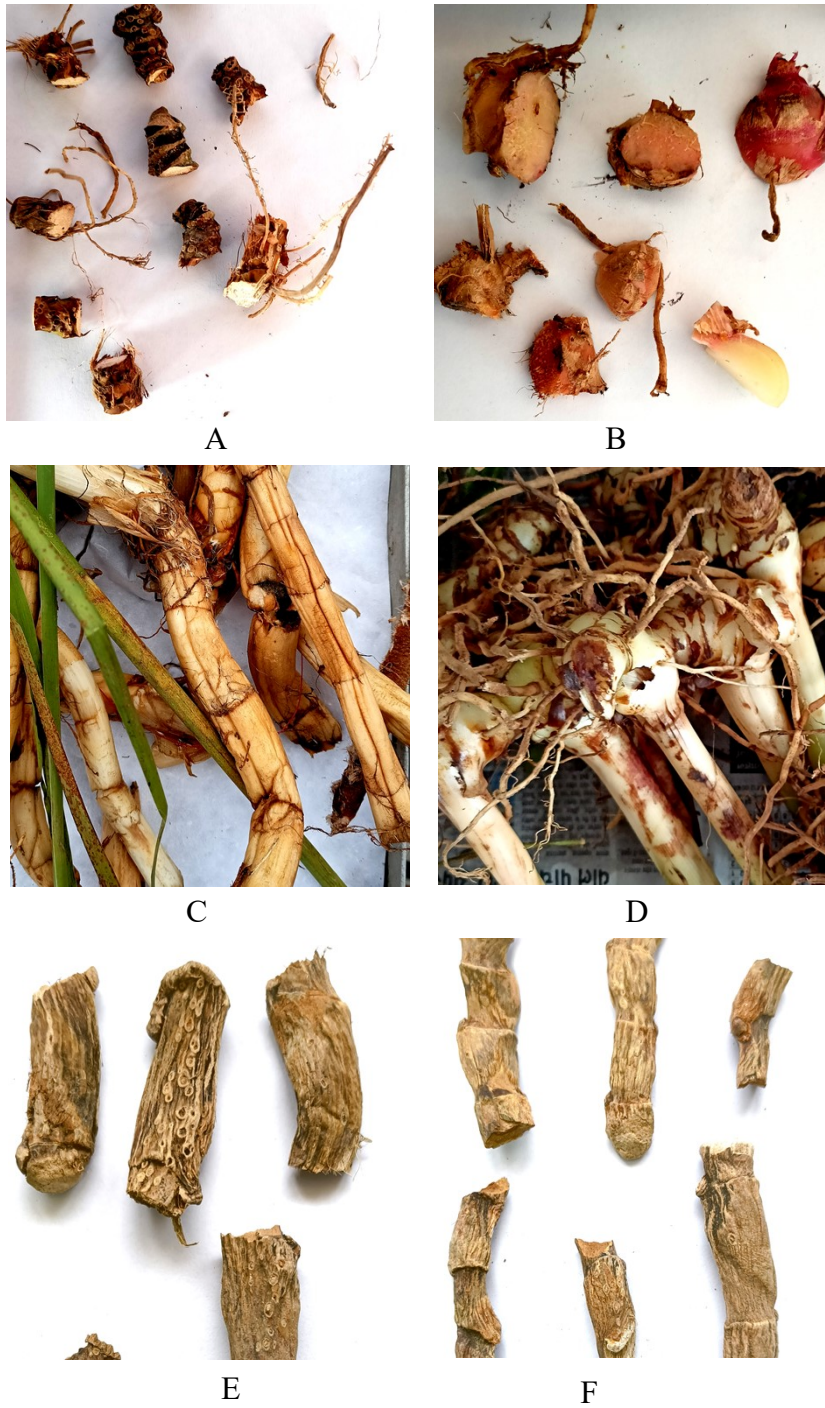


Fig. S1 Morphological appearance of *Acorus calamus* rhizome (A), *Costus speciosus* rhizome (B), *Typha angustifolia* rhizome (C), *Alpinia galanga* rhizome (D) and marketed *Vacha* samples (E&F).

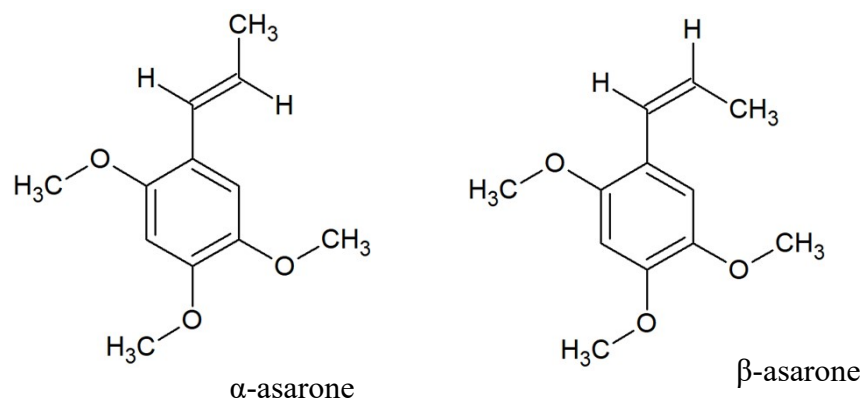


Fig. S2 Chemical structures of marker compounds; α -asarone and β -asarone.

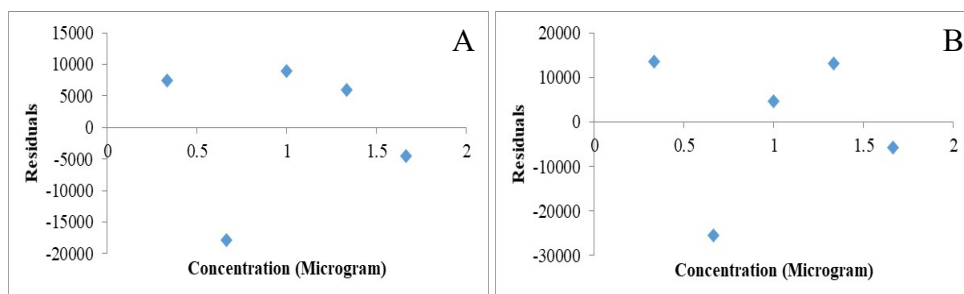


Fig. S3 Residual plot of the regression analysis for the calibration of standards α -asarone (A) and β -asarone (B).

Table S1 Recovery studies of secondary metabolites quantified through developed RP-HPLC method.

Chemical compound	Average recovery (%)	S.D*	RSD* (%)
α-asarone	97.12	1.76	1.75
β-asarone	100.28	2.41	2.48

* S.D: Standard deviation, RSD: Relative standard deviation, values are mean (n=3)

Table S2 System suitability parameters for proposed RP-HPLC method.

Parameters	Targeted metabolites	
	α-asarone	β-asarone
Resolution (R_s)	4.68	4.55
Capacity factor (K')	4.12	4.07
Selectivity (α)	2.96	2.81
Tailing factor	0.28	0.30
Relative peak area (% RSD)*	1.14	1.42
Relative retention time (% RSD)	0.17	0.16

* RSD: Relative standard deviation

Table S3 Number of chemical compound (peaks % area) present in *A. calamus* L. and its common adulterants, collected and marketed samples.

Peak No.	Retention time (R _t)	Peak area (%)*																	
		AC-1	AC-2	AC-3	AC-4	AC-5	AC-6	AC-7	AC-8	AC-9	AC-10	AC-11	AC-12	AC-13	AC-14	CS-1	TA-1	AG-1	
1	2.22	3.88	9.27	1.94	0.65	1.39	9.64	1.53	13.2	3.29	3.15	3.94	3.52	2.58	1.78	ND	ND	ND	
		±	±	±	±	±	±	±	0±	±	±	±	±	±	±				
2	2.71	0.02	0.08	0.05	0.02	0.04	0.10	0.04	0.08	0.03	0.02	0.04	0.05	0.04	0.03				
		2.42	ND	1.34	0.51	0.69	3.22	0.72	4.21	2.18	0.68	0.84	0.92	0.59	0.52	ND	ND	3.3	
3	2.90	±		±	±	±	±	±	±	±	±	±	±	±	±			8±	
		0.05		0.03	0.02	0.05	0.08	0.02	0.04	0.02	0.04	0.05	0.04	0.05	0.04			0.0	
4	3.28	0.06	0.04	0.05	0.05	0.04	0.07	0.04	0.06	0.07	0.06	0.04	0.07	0.04	0.06	0.00	0.25	0.0	
		4.55	3.53	0.81	1.45	1.87	6.85	2.08	8.03	6.21	2.42	2.37	2.24	1.97	1.51	0.04	48.4	1.5	
5	3.42	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	6±	8±	
		0.03	0.06	0.01	0.02	0.03	0.05	0.04	0.05	0.03	0.03	0.07	0.06	0.04	0.04			4	
6	3.71	0.04	0.05	0.04	0.05	0.04	0.05	0.03	0.08	0.05	0.05	0.06	0.04	0.07	0.05				
		1.46	1.42	1.17	1.02	0.53	1.56	0.85	0.80	0.87	1.09	1.39	0.92	0.88	0.61	ND	11.7	5.4	
7	4.32	±	±	±	±	±	±	±	±	±	±	±	±	±	±			7±	
		0.02	0.05	0.04	0.06	0.02	0.04	0.03	0.05	0.03	0.04	0.04	0.05	0.06	0.06		0.11	0.0	
8	4.43	0.04	0.02	0.04	0.06	0.02	0.02	0.05	0.06	0.04	0.06	0.04	0.04	0.03	0.03	0.04			
		0.59	0.36	0.83	0.51	0.58	0.58	0.71	0.34	0.57	0.75	0.95	0.59	0.62	0.74	1.91	ND	ND	
8	4.43	±	±	±	±	±	±	±	±	±	±	±	±	±	±			6	
		0.32	0.49	0.28	0.24	0.17	0.30	0.26	0.42	0.27	0.14	0.22	0.23	0.17	0.16	ND	ND	ND	
		0.03	0.04	0.02	0.02	0.03	0.02	0.04	0.07	0.02	0.01	0.03	0.03	0.01	0.01				

9	5.13	2.09 ±	0.80 ±	1.16 ±	0.83 ±	1.52 ±	1.23 ±	1.14 ±	0.56 ±	1.10 ±	0.92 ±	1.12 ±	1.05 ±	1.12 ±	0.91 ±	ND	ND	ND
		0.07	0.05	0.06	0.04	0.07	0.05	0.04	0.04	0.04	0.05	0.04	0.04	0.03	0.04			
10	10.06 (β- asarone)	73.1 8 ±	75.3 8 ±	84.2 3 ±	88.9 2 ±	85.9 8 ±	67.1 1 ±	84.7 6 ±	49.0 9 ±	75.2 3 ±	81.9 7 ±	82.8 3 ±	83.1 3 ±	85.3 9 ±	86.0 1 ±	ND	ND	ND
		0.13	0.11	0.17	0.18	0.14	0.11	0.13	0.10	0.15	0.21	0.18	0.14	0.20	0.16			
11	11.77 (α- asarone)	3.40 ±	1.12 ±	1.15 ±	1.48 ±	2.03 ±	1.39 ±	2.33 ±	1.39 ±	2.65 ±	1.48 ±	1.91 ±	1.85 ±	1.62 ±	2.29 ±	ND	ND	ND
		0.06	0.05	0.02	0.08	0.07	0.04	0.03	0.04	0.08	0.06	0.05	0.04	0.05	0.04			

* values are ± SD (standard deviation), ND: Not detected.

Table S4 Assessment of greenness score of proposed and previously reported methods.

S. No.	References	Mobile phase composition	Run time (Min.)	Instrument	Analytical eco-scale
1	Mythili et al., 2013 ²⁰	0.1% TFA in water (A) and methanol (B)	10	HPLC	79
2	Nandakumar et al., 2012 ⁸	10mM ammonium acetate in water (A) and methanol (B)	2	HPLC-ESI-MS/MS	84*
3	Shailajan et al., 2015 ¹	Methanol (A) and Water (B)	10	RP-HPLC	82
4	Hermes et al., 2021 ⁹	Acetonitrile (A) and water (B) both contains 0.1% formic acid	12	LC-MS/MS	81
5	Hamil et al., 2016 ²¹	0.1% phosphoric acid (A), acetonitrile (B) and methanol (C)	30	HPLC	77
6	Proposed method	0.1% orthophosphoric acid in water (A) and acetonitrile (B)	20	RP-HPLC	83

* The greenness of method is high but only β -asarone was estimated.