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Multi-wavelength fusion spectral extraction-assisted HPLC for simultaneous quantification of multiple secondary metabolites in herbal matrices

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Abstract

The existing HPLC methods for the assaying of multiple markers in standardized herbal extracts use a single wavelength for quantification, which usually leads to lower assay than actual claims. It was because screening multiple phytochemical standards irrespective to their absorption maxima which drive decrease in peak are response which ultimately impact the assay. To overcome this issue, we have applied a methodology, called Mixed Standard Multi-Signal (MSMS) simultaneous detection, where multiple reference standards get extracted each with their respective λ_{max} and quantification will be done at the same λ_{max} in a single sequence data setup. This method was standardized using Single Signal Multi-Standards, Single Signal Single Standard, Multi-Signal Single Standard, for all individual reference standards, mixed reference standards, plant extracts, and Ayurvedic formulations. The results of our study demonstrate that higher assay reported total actives 5.04% by conventional detection and for current proposed method 13.81% with shorter span of analysis time and lesser solvent consumption. It helps to decide the actual standardization label claim on product which drives better commercial costings, and dosage regimen claims.

Keywords HPLC, DAD, MSMS, Simultaneous detection, Bioactives, Food, Herbal matrices

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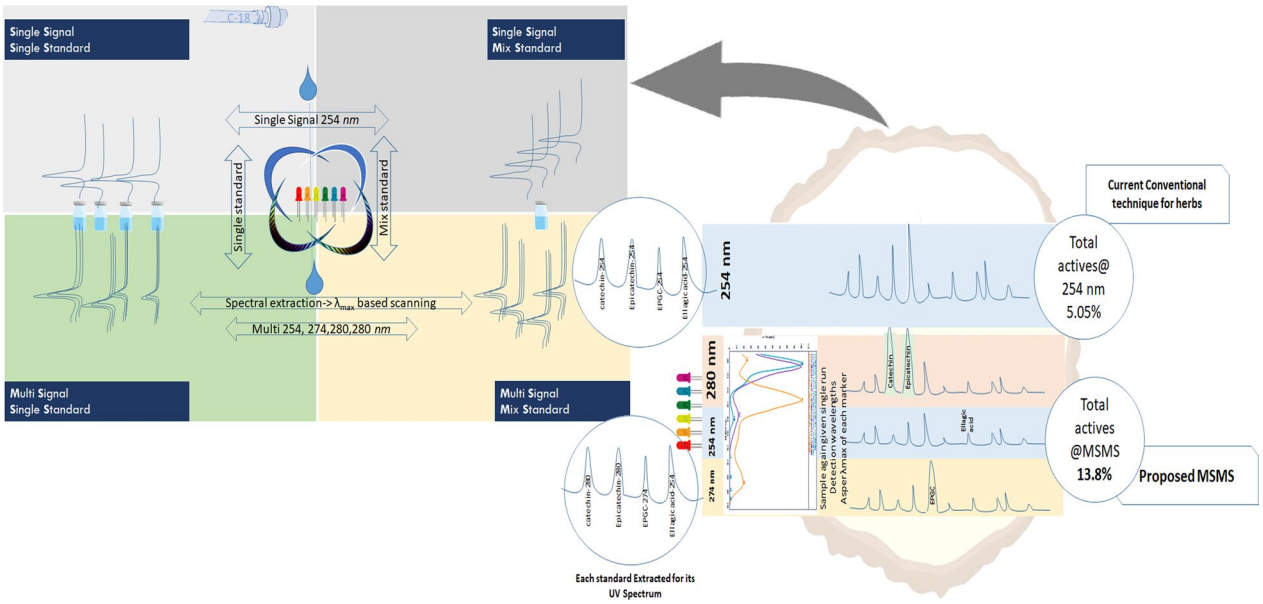
Graphical Abstract



Background

It was observed that many herbal extracts and nutraceuticals are getting standardized by HPLC with detection method using single wavelength to quantify the formulation's multiple phytochemicals. Phytopharmaceuticals a class of plant drugs introduced by Govt. of India states minimum 4 markers need to be quantified [1]. But the quantification process involves employing single wavelength to detect all 4 markers which inherently having different absorption maxima, and hence, it drives the lesser assay than actual presence which impact the dosage calculation for preclinical and clinical trials. Other methods Saroj Yadav et al. performed profiling of withanolides such as withanoside IV, V, VI, withanolide A, B, 12-deoxywithastromonolide profiling in *Withania somnifera* and commercially available brands at 227 nm [2]. Ganzera et al. quantified withaferin A withanolide D, at single detection wavelength 230 nm [3]. Aboli Girme et al. performed total withanolides assay which yielded 4% of the assay at 227 nm [4]. Ashley Dowell et al. performed total bacoside quantification in *Bacopa monnieri* 278 nm [5]. Gnanabhaskar et al. performed quantification of 4 phytochemicals glycyrrhizin, glabridin, formononetin, liquiritin quantified in glycyrrhiza glabra with single wavelength 254 nm [6].

The above-cited methods may usually yield lesser assay than actual due to applying single detection wavelength for quantification of multiple phytochemicals in herbal extracts. In this regard compound drugs and herbal matrices which have many chemicals constituents, their accurate quantification needs, multiple target molecule's multiple absorption maxima which gives multiple $\lambda_{max.}$. The sample matrix thereby re-analyzed with obtained $\lambda_{max.}$ as detection wavelength which leads to accurate assay with respective to the complete absorption maxima of each bioactive phytochemical in even in mixed condition. Wavelength screening generally employed in pharma sector for single API to get λ_{max} and afterward the further qualitative and quantitative studies will carry out on resulted λ_{max} for uni-constituent drug system.

Multi-wavelength screening concept was already in force but a different aspect and orientation is and not for quantification purpose, reported which is different from current MSMS concept. Xie et al. developed method for screening illegal adulterated antidiabetics in bitter melon, and ginseng soft gels were developed by multi-fingerprinting profiling analysis, their motive to detect quantify adulterants apart from herbal matrix [7]. Zhu et al. screened 19 batches of yankening tablet qualitatively and quantitatively by applying five random wavelength fusion fingerprint method, however they quantify 3 reference standards with single wavelength [8]. Gutiérrez et al performed almost same methodology for *Valeriana officinalis* and *Turnera diffusa* with 226, 254, 280 and 326 nm but to select respective wavelength is not happened upon pre-defined phytochemical λ_{max} and screening performed

just asses to number of compounds present, irrespective of quantification of mixed phytochemicals with respect to their λ_{max} [9, 9, 10, 10].

Xiang et al. Ri-Chuan Xio et al. applied multiple wavelengths randomly to screen the matrix and marker qualitatively by measuring the peak intensities [11, 12]. Nurgün Küçükboyacı et al. applied 4 wavelengths randomly for qualitative and quantitative screening and linearity calibration applied at single wavelength [13]. Yujing Zhang et al. applied five different wavelengths (203 nm, 220 nm, 250 nm, 280 nm and 344 nm) on the basis of class of secondary metabolites flavonoids, triterpene saponins, alkaloids and sodium benzoate. Linearity calibration mode was employed at different wavelengths but in different individual injections with different data sets which takes huge time and solvent consumption. The contrary to the above-cited publication current multiple wavelengths is selected based on target analyte absorption maxima-spectral extraction, thereby processing the linearity calibration data in single sequence of mixed standards which drives faster, accurate results.

Methods

Materials

All the reference standard/reference standards are purchased from commercial sources such as catechin (98%, BLD Pharma), epicatechin (97%, TCI), epigallocatechin (> 97%, TCI) and ellagic acid (98% TCI), and HPLC grade solvents such as orthophosphoric acid (OPA) buffer, acetonitrile, water and isopropyl alcohol are purchased from Qualigens chemicals. *Acacia catechu* hardwood is collected from Tapovan nursery section in Gwalior, MP, India. Three Ayurveda medications, Khadiradivati, Khadirarishta and Lavangadivati, are obtained from the pharmacy at RARI, Gwalior (MP).

Instrumentation

The Agilent Infinity 1260 series HPLC instrument is equipped with quat pump, auto sampler, C18 column (Agilent Eclipse XBD-18, 5 µ, 4.6 mm × 250 mm) and DAD WR detector, and Open Lab CDS software is used to conduct the chromatographic analysis. Gradient elution gradient flow program (Table 1) was applied for each type of chromatography method with Peak width >0.1 min (2 s response time) (2.5 Hz), Slit: 2 nm, Injection volume: 10 µL and run time of 35 min. For different methods, different detection channels/wavelengths are applied as required which are provided in result and discussion section (Table 2).

Table 1 Gradient elution and gradient flow program

Time (min)	0.1% OPA buffer (%)	Acetonitrile (%)	Flow rate (ml/min)
0	100	0	1.0
1	97	3	1.0
10	95	5	1.0
12	85	15	1.5
16	75	25	1.5
20	55	45	1.0
24	40	60	1.0
24.5	20	80	1.0
28	0	100	1.0
28.5	97	3	1.0
35	97	3	1.0

Sample preparation

Preparation of individual standards:

All reference standards, i.e., catechin, epicatechin, epigallocatechin, ellagic acid, are weighed 10 mg in 10 ml volumetric flask separately and dissolved in 10 ml methanol using ultra-sonicator to obtain 1 mg/ml (1000 ppm or 1000 mcg/ml) solutions and diluted this stock solution to 200 ppm. Diluted solution was filtered through 0.22 µ membrane filter before using for HPLC analysis.

Preparation of mix standard:

Each reference standards, i.e., catechin, epicatechin, epigallocatechin, ellagic acid, are weighed 10 mg in a single 10 ml volumetric flask and dissolved in 10 ml methanol using ultra-sonicator to obtain 1 mg/ml (1000 ppm or 1000 mcg/ml) solutions and diluted this stock solution to 200 ppm. Diluted solution was filtered through 0.22 µ membrane filter before using for HPLC analysis.

Acacia catechu (heartwood), Khadiradivati and Lavangadivati extraction:

The dried powdered 10 g of *Acacia catechu* (heartwood), Khadiradivati and Lavangadivati powder was extracted with 200 ml of 70% hydromethanolic solvent by using Soxhlet extraction for 3 h. The extract was evaporated to dryness under reduced pressure. The obtained extract was collected, dried and weighed; 1.2565 g of residue was obtained from extract which is stored separately at 4 °C for further studies.

Preparation of *Acacia catechu* (heartwood) extract sample:

Acacia catechu heartwood extract was weighed 100 mg in 10 ml volumetric flask and dissolved in 10ml methanol using ultra-sonicator to obtain 1mg/ml (10000 ppm or 1000 mcg/ml) solutions. This solution was filtered through 0.22µ membrane filter before using for HPLC analysis.

Preparation of formulation sample:

Table 2 Different calibration levels for compounds with particular detection signal

Vial	Mode	Compounds	Signal-nm	Conc. unit	L-1	L-2	L-3	L-4	L-5
Single vial	MSMS	(+)-Catechin	280	ppm	8	16	24	32	40
		(−)-Epicatechin	280	ppm	8	16	24	32	40
		Epigallocatechin	274	ppm	8	16	24	32	40
		Ellagic acid	254	ppm	8	16	24	32	40

Khadiradivati (13.2) and Lavangadivati (16.0 mg) was weighted in 10 ml volumetric flask separately and dissolved in 10 ml methanol using ultra-sonicator to obtain 1.32 mg/ml (1320 ppm or 1320 mcg/ml) solution and 1.6 mg/ml (1600 ppm or 1600 mcg/ml) solutions. This solution was filtered through 0.22 µ membrane filter before using for HPLC analysis.

Experimental methods

MSMS method standardized by using three modes of analytical strategies. Single Signal Single Standard (SSSS) individual detection, 4 individual phytochemical reference standards given 4 single injections separately and detected under single wavelength of 254 nm. Single-Signal-Mixed-Standard (SSMS) simultaneous detection, 4 reference standards mixed together and analyzed under single wavelength 254nm. SSSS, SSMS purpose of doing is to compare the peak area response of standards is same even in mixed condition. Multi-Signal Single Standard (MSSS) individual detection, 4 individual standards injected separately but the detection wavelengths are according to its λ_{max}. These λ_{max} were obtained after spectral extraction for each standard (Fig. 1). The acquisition sequence setup is mentioned in Additional file 1: Table S2. The linearity response and quantification parameters of all individual reference standards are catechin, epicatechin, ellagic acid, epigallocatechin mixed reference standards and *Acacia catechu* (heartwood) extract Khadiradivati and Lavangadivati (Additional file 1: Table S3). Comparative study of all methods is provided for better understanding of the advancements of new developed analysis method mentioned below

Single Signal Single Standard (SSSS) individual detection:

In this method, for a particular concentration (200 ppm) of each reference standards, HPLC was performed at 254 nm wavelength separately and area under the curve for each marker

Single Signal Mix Standard (SSMS) simultaneous detection:

In this method, for a mix solution of Reference standard, where each marker has particular concentration (200 ppm), HPLC was performed at 254 nm wavelength

Multi-Signal Single Standard (MSSS) individual detection:

In this method, for a particular concentration (200 ppm) of each reference standards, HPLC were performed at different wavelengths, i.e., 254 nm, 274 nm, 280 nm and 280 nm, separately.

Multi-Signal Mix Standard (MSMS) simultaneous detection:

In this method, for a mix solution of reference standard, each marker concentration (200 ppm) was observed under detection wavelength as mentioned for MSSS method.

Method validation

MSMS method are validated for specificity, linearity, precision the calibration curve of all 4 standards in mixed condition. Four standards are individually prepared on 5-level (L-1 to L-5) calibration with 100–500 ppm. When 4 different standards are mixed together, the diluted final concentration actually goes to 8–40 ppm. Then, these individual standards are mixed based on their linearity concentration 8, 16, 24, 32, 40 ppm level into one vial, and sequence set has created for calibration including plant material and three Ayurveda formulations. Different levels of calibration for different compounds in mixed condition processed with different wavelengths are provided in Table 3 with particular detection signal. Quantification was processed by Open lab CDS 3.5.0, and 3D UV quantitative default method with linear curve fitting mode of processing was employed.

Results

SSSS individual detection method (As raw method):

Chromatogram obtained from SSSS individual detection is shown in Fig. 2. This chromatogram results in an intense peak for ellagic acid at retention time (RT) of 14.999 min, a medium broad peak for epigallocatechin at RT of 11.567 min, low intense peaks for catechin at RT of 8.257 min, epicatechin at RT of 10.869 min. and a poor intense peak is observed at RT of 11.145 min. for epigallocatechin. The particular peak area for individual peaks is given in Table 4.

MSSS individual detection method (As standard method):

Table 3 Detection modes with their vial series and detection wavelength

Vial count	Detection mode	Compound `	Signal-SSSS and MSSS-nm	Signal-MSSS and MSMS-nm
1	SSSS and MSSS	(+)-Catechin	254	280
2	SSSS and MSSS	(−)-Epicatechin	254	280
3	SSSS and MSSS	Epigallocatechin	254	274
4	SSSS and MSSS	Ellagic acid	254	254
5	MSMS and SSMS	(+)-Catechin, (−)-Epicatechin, Epigallocatechin Ellagic acid	254	254, 274, 280, 280

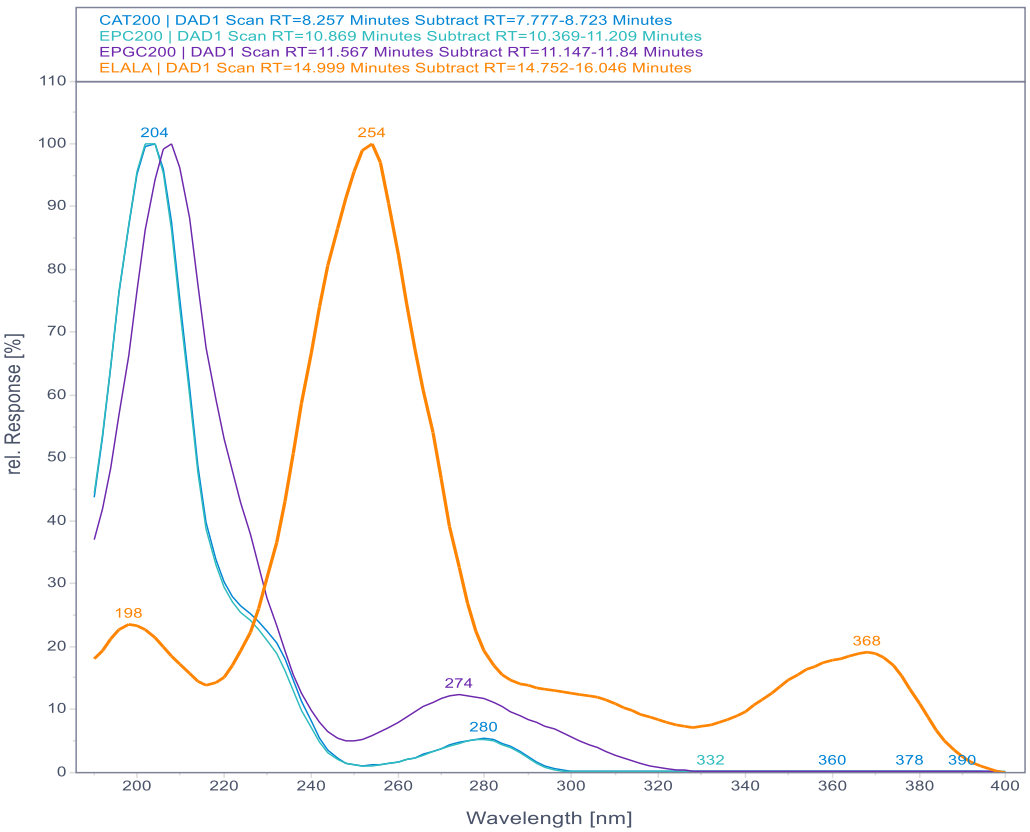


Fig. 1 UV absorption spectrum overlay stack of individual reference standards extracted from HPLC–DAD system. Catechin: 280 nm, epi catechin: 280 nm, epigallocatechin: 274 nm, ellagic acid: 254 nm

Chromatogram obtained from MSSS individual detection is shown in Fig. 3. This chromatogram results in an intense peak with highest peak area for ellagic acid at retention time (RT) of 14.999 min at 254 nm signal. Another instance peaks a medium broad peak for epigallocatechin at RT of 11.567 min, low intense peaks for catechin at RT of 8.257 min, epicatechin at RT of 10.869 min and a poor intense peak is observed at RT of 11.145 min. for epigallocatechin. The particular peak area for individual peaks is given in Table 5.

SSMS simultaneous detection method (Currently used in industries):

Chromatogram obtained from SSMS simultaneous detection is shown in Fig. 4. This chromatogram results in an intense peak for ellagic acid at retention time (RT) of 14.999 min., a medium broad peak for epigallocatechin at RT of 11.567 min., low intense peaks for catechin at RT of 8.257 min., epicatechin at RT of 10.869 min. and a poor intense peak is observed at RT of 11.145 min. for epigallocatechin. The particular peak area for individual

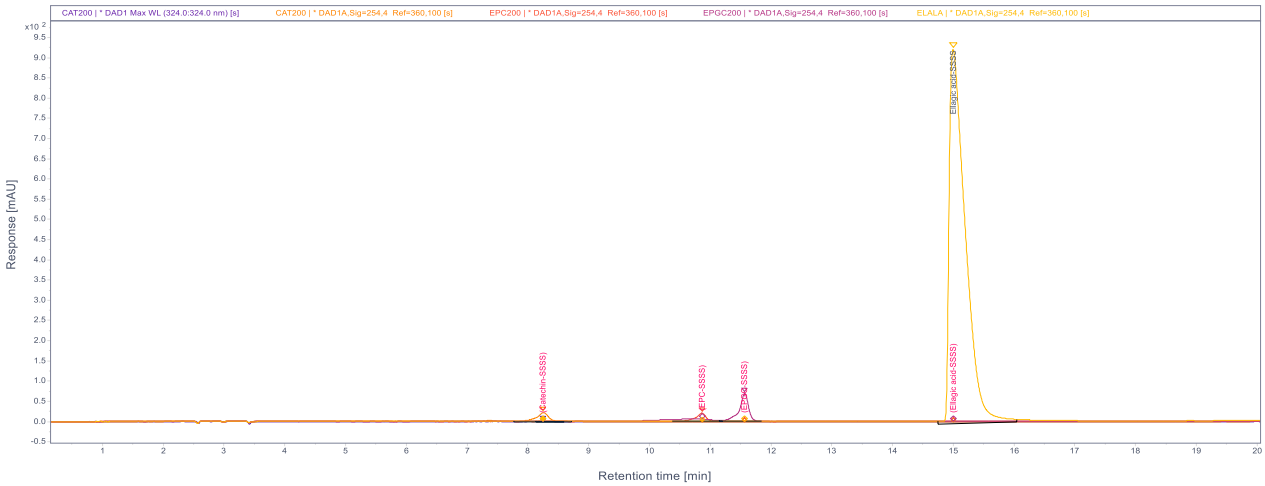


Fig. 2 Chromatogram showing peak detection for each individual marker at 254 nm

Table 4 Screening of individual reference standards by SSSS individual detection method

Standard	Conc. (ppm)	Signal (nm)	RT (min.)	Peak area (mau)
Catechin	200	254	8.257	316.48
Epicatechin	200	254	10.869	257.99
Epigallocatechin	200	254	11.567	750.21
Ellagic acid	200	254	14.999	16,996.16

peaks is provided in Table 6. It is observed that SSSS individual detection method and SSMS simultaneous detection method result in comparable area for each particular peaks.

MSMS simultaneous detection method (New proposed method):

Chromatogram obtained from MSMS simultaneous detection is shown in Fig. 5. This chromatogram results in an intense peak with highest peak area for ellagic acid at retention time (RT) of 14.999 min. at 254 nm signal. Another instance peaks a medium broad peak for epigallocatechin at RT of 11.567 min, low intense peaks for catechin at RT of 8.257 min., epicatechin at RT of 10.869 min. and a poor intense peak is observed at RT of 11.145 min. for epigallocatechin. The particular peak area for individual peaks is provided in Table 7. It is observed that MSSS individual detection method and MSMS simultaneous detection method result in comparable area for each particular peaks.

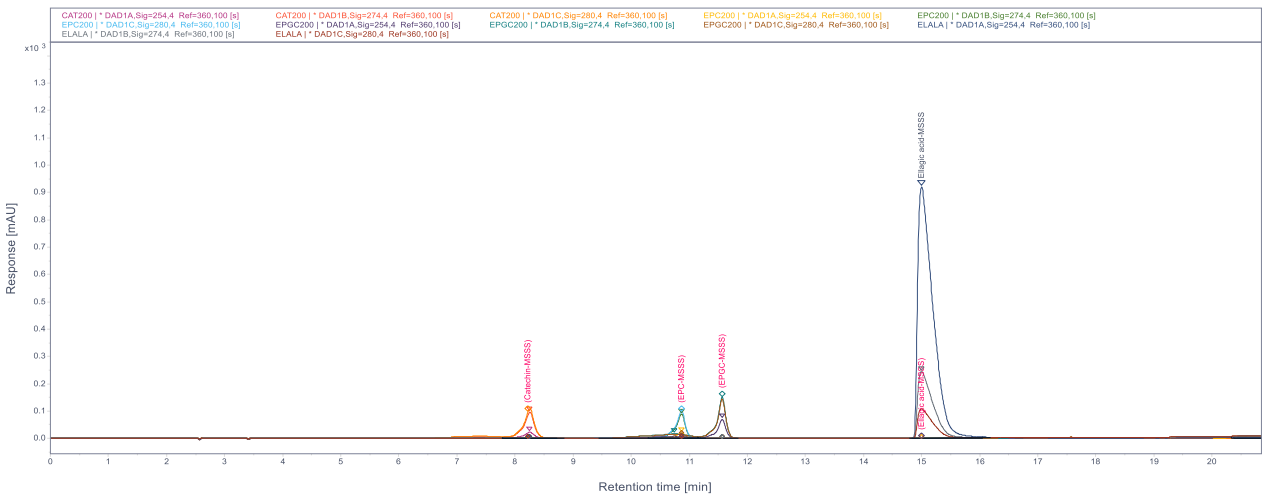


Fig. 3 Chromatogram showing peak detection for individual marker at different signals

Table 5 Screening of individual reference standards by MSSS individual detection method

Standard	Conc. (ppm)	Signal (nm)	RT (min.)	Peak area (mau)
Catechin	200	280	8.257	1488.11
Epicatechin	200	280	10.869	1211.53
Epigallocatechin	200	274	11.567	1627.87
Ellagic acid	200	254	14.999	16,996.16

Table 6 Screening of individual reference standards by SSMS individual detection method

Standard	Conc. (ppm)	Signal (nm)	RT (min.)	Peak area (mau)
Catechin	200	254	8.257	313.59
Epicatechin	200	254	10.869	268.72
Epigallocatechin	200	254	11.567	765.65
Ellagic acid	200	254	14.999	16,996.16

Method standardization and validation:

For standardization, we suppose the mixed standard solution as known concentration having solution and chromatographic results are compared with the area obtained from the all methods. Raw SSSS individual detection method results poor area under the curve for all reference standards except ellagic acid, but use of MSSS individual detection method provides maximum area under the curve for all reference standards at different wavelengths. This study shows that peak area of different reference standards is not providing the accurate results when they are analyzed by using any particular single wavelength. Results obtained from SSSS individual detection method and MSSS individual detection method show the difference in peak area of epigallocatechin is 2.1 times for catechin and epicatechin is 4.7 times. This difference in peak area of different reference standards at different wavelengths refers to the inaccurate quantification results. Currently used industrial method/SSMS individual detection method shows similar value as shown by SSSS individual detection method, which reports wrong quantification with standard MSSS individual detection method, while new proposed MSMS simultaneous detection method shows similar value as shown by MSSS individual detection method, which reports accurate quantification with standard MSSS individual detection method. A comparative area under the curve plot for all four methods is shown in Fig. 6. This indicates the newly proposed MSMS simultaneous detection method is able to quantify compound drugs/formulations and phytochemicals in shorter time and less run cycles during analysis with actual assay.

Table 7 Screening of individual reference standards by MSMS individual detection method

Standard	Conc. (ppm)	Signal (nm)	RT (min.)	Peak area (mau)
Catechin	200	280	8.257	1492.01
Epicatechin	200	280	10.869	1227.89
Epigallocatechin	200	274	11.567	1619.29
Ellagic acid	200	254	14.999	16,996.16

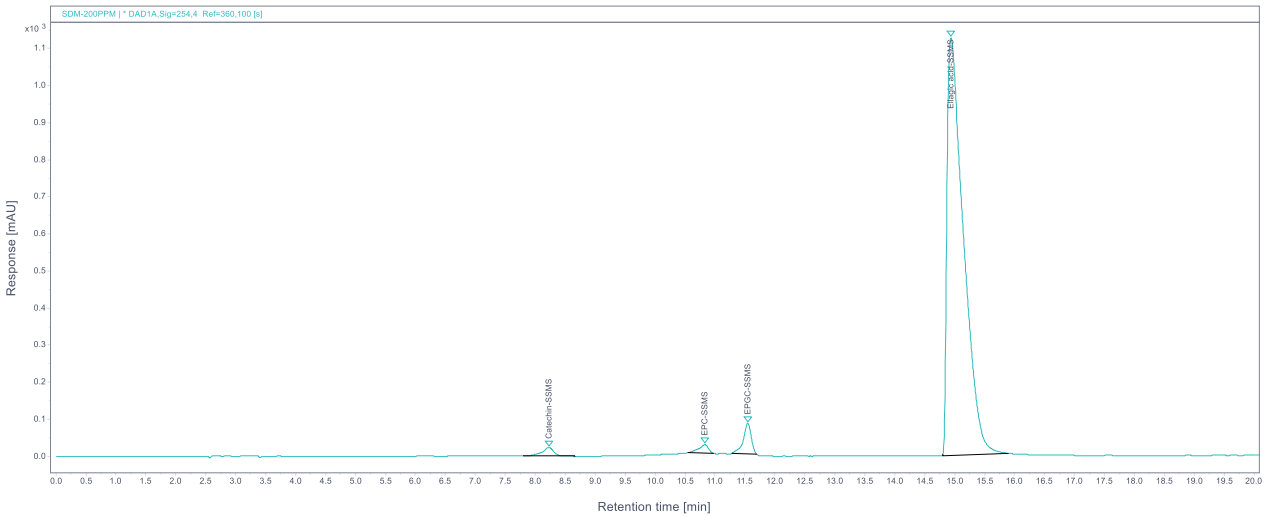


Fig. 4 Chromatogram showing peak detection for each mixed marker at 254 nm

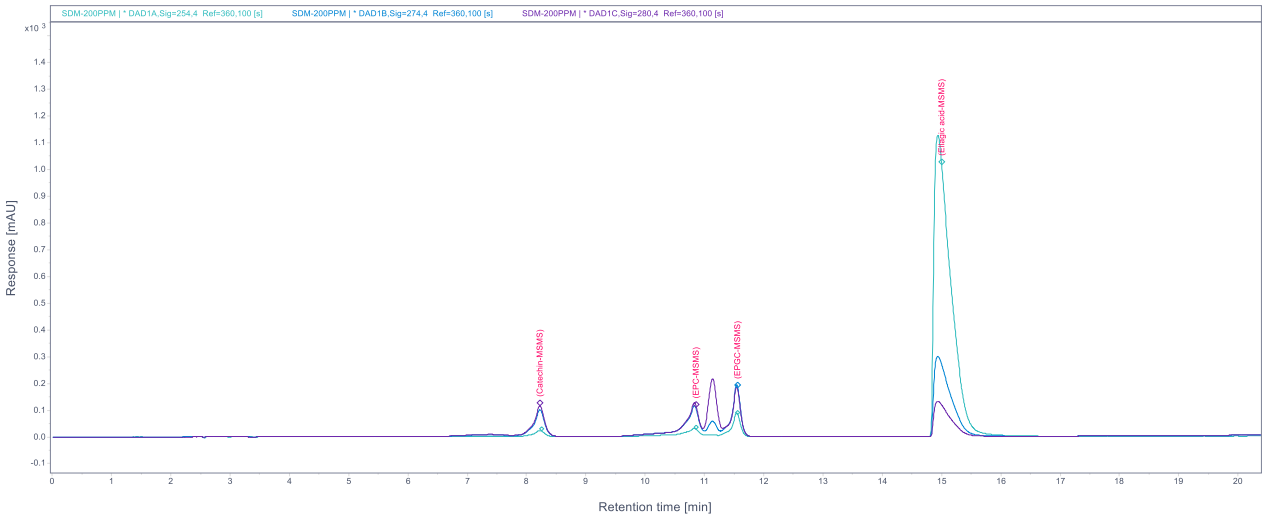


Fig. 5 Chromatogram showing peak detection for mixed marker at different signals

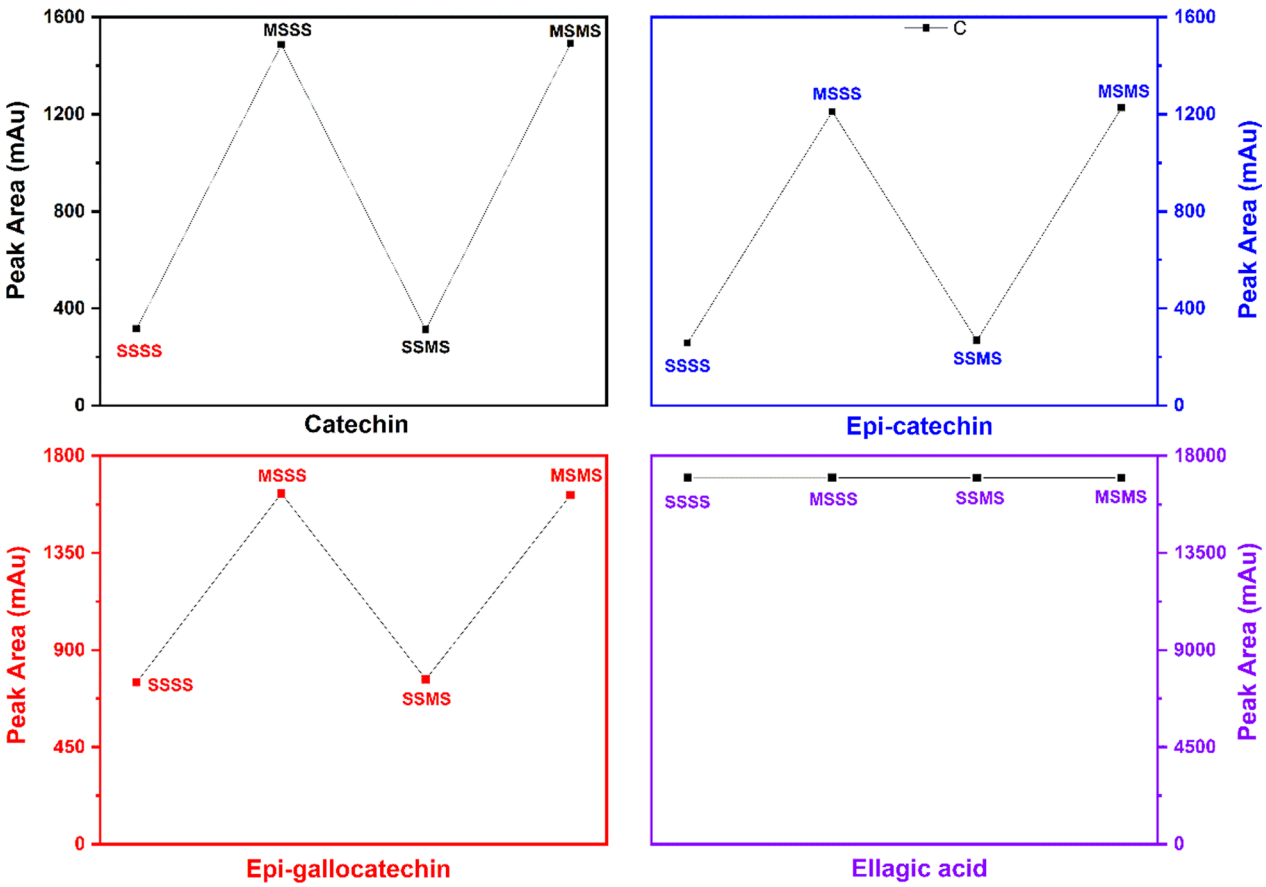


Fig. 6 Comparative quantitative analysis of reference standards by different detection methods

Comparative study of SSMS individual detection method and MSMS simultaneous detection method is carried out for single drug (*Acacia catechu*) and two different formulations (Khadiradi gutika and Lavangadivati) phytochemical analysis to find out the real deviation of real assay values of reference standards in particular plant material and formulation. Results observed for single plant drug are analyzed in two parameters such as peak area and percentage assay. In *Acacia catechu*, single plant material SSMS simultaneous detection method gives lesser peak areas for catechin, epicatechin and epigallocatechin with lower percentage assay, i.e., 4.96% of catechin, 0.03% epicatechin and 0.05% of epigallocatechin. Beside that MSMS simultaneous detection method gives accurate peak area and assay, i.e., 13.7% of catechin, 0.05% epicatechin and 0.06% of epigallocatechin which is comparable with the standard MSSS single detection method (Table 8).

The MSMS method is further validated by specificity, linearity, precision. For specificity, 4 standards are injected individually and in mixed condition to ensure there will not be any interferences in retention times of sample matrices, and linearity results also showing linear response with regression value 0.999, LOD and LOQ calculated as per ICH results are given in Table 9. Individual linear calibration plot is given in supporting information.

Table 8 Phytochemical analysis of single plant drug and formulations by different detection methods

***Acacia catechu* Heartwood**										
Marker	Rt	SSMS			MSSS			MSMS		
		Signal nm	Area	Assay %	Signal nm	Area	Assay %	Signal nm	Area	Assay %
Catechin	8.2	254	2280	4.96	280	4023	13.8	280	4011	13.7
Epicatechin	10.8		14.7	0.03	280	22.0	0.04	280	25.0	0.05
Epigallocatechin	11.6		24.9	0.05	274	44	0.06	274	47.5	0.06
Ellagic acid	15.2		6420	0.0004	254	6420	0.0004	254	6420	0.0004
Cumulative assay		**5.0404**			**13.9**			**13.81**		
Khadiradi gutika										
Marker	Rt	SSMS			MSSS			MSMS		
		Signal nm	Area	Assay %	Signal nm	Area	Assay %	Signal nm	Area	Assay %
Catechin	8.2	254	86.0	3.9	280	441.3	4.1	280	448.1	4.2
Epicatechin	10.8		172.1	0.7	280	168.1	1.2	280	172.1	1.3
Epigallocatechin	11.6		6.7	0.1	274	17.0	0.24	274	17.2	0.25
Ellagic acid	15.2		368.6	0.23	254	368.6	0.23	254	368.6	0.23
Cumulative assay		**4.9**			**5.7**			**5.9**		
Lavangadivati										
Marker	Rt	SSMS			MSSS			MSMS		
		Signal nm	Area	Assay %	Signal nm	Area	Assay %	Signal nm	Area	Assay %
Catechin	8.2	254	176.7	6.6	280	980.6	7.4	280	989.7	7.6
Epicatechin	10.8		99.5	1.6	280	519.2	3.0	280	539.5	3.2
Epigallocatechin	11.5		15.6	0.2	274	42.3	0.4	274	41.9	0.4
Ellagic acid	14.9		908.7	0.4	254	908.7	0.4	254	908.7	0.4
Cumulative assay		**8.8**			**11.2**			**11.6**		

Table 9 Results of HPLC validation

Validation parameter	Catechin 280 nm	Epicatechin 280 nm	Epigallocatechin 274 nm	Ellagic acid 254 nm
Specificity-Rt	8.2	10.8	11.6	15.2
UV peak purity	99%	99%	99%	98%
Linearity				
Conc. Range	10–50 ppm			
R^2	0.99911	0.99915	0.99954	0.99965
LOD-ppm	1.58	2.01	0.93	1.48
LOQ-ppm	4.81	6.09	2.84	4.5
Precision				
Intraday area precision RSD	1.03%	0.69%	0.21%	0.49%
Area response factor Concentration/Avg. response	14.9	19.9	9	225.4

Intraday area precision evaluation leads to acceptable %RSD within acceptable limit of 2.5%. The developed method has shown acceptable performance in terms of sensitivity, specificity, linear response and without interfering sample matrix

Discussion

Any formulation, food material or plant extractive materials have plenty of chemical ingredients. When these samples are analyzed under single wavelength, only a few compounds show their full absorption and results of maximum area under the curve are not getting measured. Hence, this quantification method needs modified methodology which is sustainable with HPLC-DAD instrument and provides more accurate result with saving time compared to the conventional method. In this regard, this work represents an efficient, sensitive, simple, rapid, cost-effective in terms of less injection count, less solvent consumption validate analytical method. Herein, four chemical reference standards, i.e., catechin, epicatechin, ellagic acid, epigallocatechin and epigallocatechin, are selected as reference materials; *Acacia catechu* (heartwood) extract is taken as plant material for phytochemical analysis sample and two Ayurvedic formulations, i.e., Khadiradivati and Lavangadivati, are taken as formulations. The selection of plant material is done based on the presence of all four reference standards [14] and selection of compound drug is done based on the presence of plant material in formulation [15, 16]. Method validation and standardization are done by using three different analytical strategies: SSSS individual detection, SSMS simultaneous detection and MSSS individual detection of individual reference standards, mix standard of reference standards, plant material and compound drug formulations. For multi-signal analysis 254 nm, 270 nm, 280 nm, wavelengths are used which are extracted from SSSS injections and the absorption maxima wavelengths for ellagic acid (254 nm), epigallocatechin (274 nm), epicatechin and catechin (280 nm) [17]. The reference absorption spectrum is also obtained and provided in Fig. 1 for individual marker in the HPLC-DAD system.

The standardization is done on the basis of SSSS and SSMS individual detection method, which is currently in industrial use and here taken as conventional method to compare. Moreover, standardization is done using comparative results obtained from each method compared to the SSSS individual method for known and unknown samples. All selected reference standards are individually (in SSSS) analyzed at single wavelength of 254 nm (Table 4) and the same are analyzed at different wavelengths (in MSSS) of 254 nm, 270 nm, 280 nm (Table 5) for accurate quantification of particular marker as per their absorption maxima with individual run. A mixture of marker solution is simultaneously (in SSMS) analyzed at single wavelength of 254 nm (Table 6), which is currently in industrial use. In order to check deviation from the accurate quantification, we performed MSSS individual detection method and a mixture of marker solution is MSMS simultaneous detection method (Table 7) at different wavelengths of 254 nm, 270 nm, 280 nm.

These results confirm that the currently used SSMS simultaneous detection method is not capable to detect real concentration of phytochemicals obtained from plant samples; on the other hand, MSMS simultaneous detection method is providing real concentration of each ingredient and useful in industrial applications. Moreover, the result obtained from the comparative analysis study of two different formulations, i.e., Khadiradi gutika and Lavangadivati (Table 8), also supports the merits of MSMS simultaneous detection method over

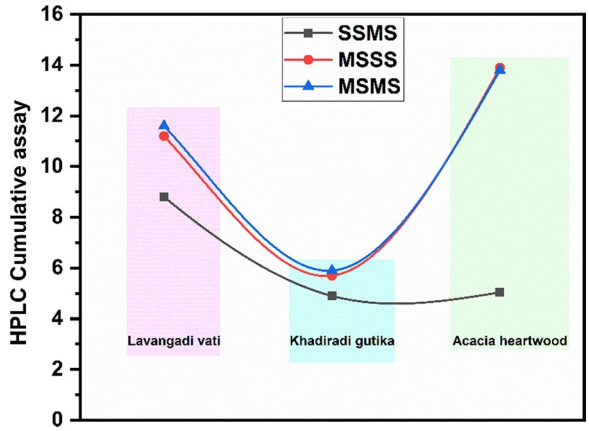


Fig. 7 Quantitative cumulative assay of single drug and formulations from different detection methods

SSMS simultaneous detection method. Cumulative assay obtained from different SSMS simultaneous detection methods also refers to large deviation from the standard SSMS single detection method; beside that MSMS simultaneous detection method reports almost similar cumulative assay that conforms the validity of new developed method (Fig. 7).

Conclusion

In this study, we successfully developed a new HPLC analytical method, i.e., MSMS simultaneous detection method, for the qualitative and quantitative analysis of finished products. It has several benefits over the SSMS simultaneous detection method, including that it can obtain real and accurate quantification of phytochemicals. It only requires a single sequence run of all reference standards in mixed condition, which saves experimental time, energy and cost. It provides actual results that are comparable to those obtained by the standard MSSS single detection methods or currently established methods. It allows for quick sample analysis, and this method is a valuable tool for industries and researchers who need to set real-parameter accurate assays of their products and conduct pharmacological studies to set the dose of particular single drugs/compounds/formulations. It should be practiced as regular protocol for all standardization of herbal extracts, nutraceuticals where multiple phytochemicals involved.

Abbreviations

HPLC-DAD	High-Performance Liquid Chromatography-Diode Array Detector
ppm	Parts per million
mg	Milligram
mcg	Microgram
ml	Milliliter
nm	Nanometer
LOD	Limit of detection
LOQ	Limit of quantification
RSD	Relative standard deviation
RT	Retention time
SSSS	Single Signal Single Standard
SSMS	Single Signal Multi-Standard
MSSS	Multi-Signal Single Standard
MSMS	Multi-Signal Multi-Standard

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43094-023-00555-4.

Additional file 1 contains system suitability test parameters, HPLC chromatogram of single drug (*Acacia catechu*), and compound formulations (Khadiradi gutica and Lavangadivati) with linearity fit model.

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Author contributions
Danaboina identified and executed the experimentation of chromatographic analysis, Tiwari analyzed and designed the standardization needs for the methodology, Kurra supported the bench work sample preparation of calibration solutions, Shreekala helped to choose the traditional formulations, and Meena supervised entire operation and guided at every step.

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Availability of data and materials
Data available on request from the authors.

Declarations

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Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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