Research Square

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Integrated green ultrasonic extraction with UHPLC/DAD profiling for quantitative analysis, chemometric classification, and geographical standardization of phenolic acids, flavonoids, and PMFs in myrrh resins

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Research Article

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Abstract

Background

Myrrh resin contains phenolic acids, flavonoids, and polymethoxyflavones (PMFs); a standardization method for the simultaneous extraction and quantification of such phytochemicals is yet to be established. This study, for the first time, developed a green ultrasonic dismembrator-based extraction (UAG) along with a validated UHPLC-DAD protocol to quantify the major markers of myrrh in seven distinct geographical origins. **Methodology**: green solvents of water (H_2O), acetone (AC), ethanol (EtOH), and their 1:1 combination were evaluated to extract myrrh phytochemicals, using UAG. To quantify the markers: gallic acid (GA), p-coumaric acid (pCOU), quercetin (QT), naringenin (NG), citropten (CT), nobiletin (NB), and tangeretin (TG), an in-house UHPLC-DAD method was developed and validated.

Results

The analytical method exhibited linearity (r^2=0.99976–0.99990), low asymmetry (1.03–1.17), and LOD/LOQ ranges of 11.69–98.47 ppm and 35.43–298.40 ppm, respectively. The UAG showed more extract yield (17.06mg) for H_2O: EtOH, whereas AC: EtOH (1:1 v/v) yielded the highest total phytochemical content (90.78ppm). The extract yield across the origins was 277.59mg (SA1) to 366.04mg (IN1). About phenolic compounds, GA was observed in Indian origin only (16.67ppm), whereas pCOU varied widely (6.93–52.76 ppm), with Oman (O1) showing the highest yield. The flavonoid QT ranged from 22.02ppm (IN1) to 59.64ppm (SM1), whereas NG was observed only in O1, O2, and IN1. For PMFs, a restricted distribution was noticed: CT (6.72-10.02ppm) in SA2 and SM1; NB (4.45-9.27ppm) in O2 and SA2; TG was undetected. The statistical model of principal component analysis explained a cumulative variance of 69.39%, with QT and pCOU as the major discriminants.

Conclusion

The integrated UAG-UHPLC workflow provides a rapid, green, and robust platform for quantifying major markers in myrrh samples, to be utilized for standardization, targeted therapeutic formulation, and future metabolomic and bioactivity-driven research.

1. INTRODUCTION

The resin from myrrh (*Commiphora myrrha*) is widely recognized for its medicinal and traditional uses against inflammation, as well as antimicrobial, antioxidant, and wound-healing properties. These therapeutic effects are attributed to a diverse array of secondary metabolites, including phenolic acids, flavonoids, and polymethoxyflavones (PMFs). Yet again, the detailed quantitative phytochemical profile of myrrh remains inadequately characterized, particularly for the PMFs and flavonoids, which are gaining widespread recognition for their potential pharmacological role. Numerous studies have been reported previously; however, the focus of such research was either on the volatile oil profile using GCMS or on the crude phenolic content using colorimetric assays [1, 2], resulting in a significant gap for the targeted quantification of individual bioactive compounds.

LCMS/MS metabolomics has been recently investigated for myrrh; these investigations have predominantly been qualitative or semi-quantitative, providing a mere identification for the tentative compounds rather than a validated quantification. For instance, a metabolomic survey of *C. myrrha* previously identified several phenolic acids and flavonoids with a lack of quantification or validation of a reliable analytical workflow. [3] Consequently, the actual concentrations of marker compounds in myrrh resin, such as gallic acid (GA), p-coumaric acid (p-COU), quercetin (QT), naringenin (NG), citropten (CT), nobiletin (NB), and tangeretin (TG), remain undetermined. Therefore, a validated UHPLC-based quantification of phenolic acids, flavonoids, or PMFs in myrrh has not been reported to date, representing a significant analytical gap that impedes both pharmacological interpretation and standardization efforts. Further to the analytical gap, a concurrent need for the green extraction of these phytochemicals does exist.

The utilization of green extraction technologies, particularly ultrasonic-assisted extraction, is getting wider attention as an effective method for the recovery of phenolic acids and flavonoids from plant matrices. The proven efficiency of ultrasound extraction owes to its capacity to enhance mass transfer through acoustic cavitation. [4, 5] Several studies revealed the successful extraction of phytochemicals from medicinal plants: a markedly improved phenolic and flavonoid yield was observed for *C. asiatica* using ultrasound [6], superior recovery for phenolic and flavonoid compared to the traditional extraction systems, as reported in different plants [7], and the extraction of bio-compounds with high yield from *C. gileadensis* leaves. [8] Nevertheless, an optimized study employing an ultrasonic dismembrator for the extraction of phenolic, flavonoids, and PMFs from myrrh resins is yet to be reported. Likewise, a systematic comparison of the efficiency of various green solvents, in the presence of cavitation and acoustic effects, to maximize the recovery of phenolic, flavonoids, and PMFs from the myrrh resin is not reported. An additional gap concerns the lack of reports for validated UHPLC-DAD methods capable of simultaneously quantifying the structurally diverse phytochemicals present in myrrh. The analytical tool of UHPLC-DAD is well-known for its high resolution, fast analysis, and multi-wavelength detection capabilities; however, the existing analytical reports for myrrh have not employed validated chromatographic methods for targeted

simultaneous quantification of the major marker in resin. There is a single report in the existing literature addressing the phytochemicals of myrrh resins, detailing the identification of gallic acid, quercetin, and para-coumaric acid, but not their quantification. [3] A gap still exists for comprehensive data on the retention time reproducibility, limits of detection, limits of quantification, accuracy, precision, and linearity of the phenolics, flavonoids, and PMFs in myrrh. This lack of validated analytical tools has impeded efforts to standardize myrrh extracts and to facilitate the comparison of phytochemical profiles across different studies.

Furthermore, the reported extraction and analytical methods are insufficient to be employed for an in-depth chemometric analysis of myrrh samples from different geographical origins. The myrrh resin is sourced from diverse regions, including Yemen, Somalia, Oman, India, and Saudi Arabia. [9] There is a need to develop an optimized extraction and analytical tool with the potential to assess the influence of geographical origin on the quantitative distribution and variability of phenolic acids, flavonoids, or PMFs. [10, 11] The lack of geographical standardization is noteworthy to be explored, considering that environmental and genetic factors are recognized to strongly modulate phenolic biosynthesis in resin-producing species. The effect of the aforementioned factors has been reported to influence the phytochemical composition in various medicinal plants. [7, 10, 11] In addition, the current study will apply chemometric analysis using the statistical models of principal component analysis (PCA), K-means clustering, and paired samples t-test to differentiate myrrh chemotypes and to identify origin-specific phytochemical markers.

The present study aims to address the gaps by developing an integrated green ultrasonic extraction with UHPLC-DAD analysis for the simultaneous quantitative profiling of the key markers of GA, QT, pCOU, NG, CT, NB, and TG in myrrh resin from different geographical origins. Herein, six green solvent systems are optimized, followed by an in-house validation of the UHPLC-DAD method according to ICH guidelines, and the application of chemometric tools to compare samples from seven geographical origins. This research framework is the first of its kind for a comprehensive, validated, and origin-resolved phytochemical characterization of myrrh resin. The identification of origin-specific chemotypes offers novel insights into the chemical diversity of myrrh and establishes a foundation for future quality control, authentication, and bioactivity-driven research.

2. METHODOLOGY

2.1 Standards, solvents, and instrumentation

The standard drugs for the study were obtained from Sigma-Aldrich, St Louis, MO, U.S.A. [gallic acid: GA, quercetin: QT, para-coumaric acid: pCOU] and UFC Biotechnology, Amherst, NY, U.S.A [naringenin: NG, tangeretin: TG, citropten: CT, nobiletin: NB]. The solvents, both analytical and HPLC-grade, were purchased from Merck Darmstadt, Germany [acetone: AC, acetonitrile: ACN, ethanol: EtOH, methanol: MeOH]. Water [H_2O] was purified in-house using a Millipore, USA, distillation system. The samples were extracted with an ultrasonic dismembrator using high-frequency waves [20kHz] generated via a probe and transducer [Fisher Scientific, Elma Hans Schmid Bauer GMBH, Singen, Germany]. The phenolic acids [GA, pCOU], flavonoids [QT, NG], and polymethoxyflavones [CT, NB, TG] were quantified using a UHPLC equipped with a diode-array detector (DAD) (Thermo Scientific™ Vanquish™, Thermo Scientific, Germany).

2.2 Procurement of samples and processing

The study collected seven myrrh samples (*Commiphora myrrha*) from different origins in Yemen (YN1), India (IN1), Somalia (SM1), and two each from Saudi Arabia [Taif (SA1) and Makkah (SA2)], and Oman [O1 and O2]. All the samples were ensured to be in a tightly closed container during transport, accessing them to the laboratory in their original condition without the loss of any quality.

Before starting the extraction, the myrrh samples were milled into powder and sieved through a stainless-steel mesh with 1.0mm openings [BS 410-1, Endecotts Ltd., London, England] to obtain a uniform powder with a consistent texture. Until the extraction process, the powder samples were stored in a tight container in a dry place to avoid moisture.

2.3 UHPLC/DAD method development and validation (MDMV)

2.3.1 Preparation of standard and stock solutions

The standard drugs (> 98%) GA, pCOU, QT, NG, CT, NB, and TG were dissolved in HPLC-grade EtOH: ACN (1:1 v/v) to prepare stock solutions of 1mg/mL. These stock solutions were diluted at seven different concentration points, in the linearity ranges of 0.5-250ppm [0.5, 2.5, 5, 25, 50, 100, and 250ppm] for GA and pCOU, and 1-500ppm [1, 5, 10, 50, 100, 200, and 500ppm] for QT, NG, CT, NB, and TG. The diluted linearity range points for all the standard drugs were mixed in the required volume from the stock solutions to prepare a mixed standard for the seven analytes with a final volume of 1mL. All the samples were diluted in the mobile phase (HPLC-grade ACN: H_2O, 1:1 v/v) and syringe-filtered (0.2µm). The samples were stored in a refrigerator at 4°C till further analysis. The calibration curve was developed using the prepared seven concentration points for the mixed standards, following UHPLC/DAD analysis.

2.3.2 Method development for myrrh phytochemicals

For the development of a chromatographic method, organic mobile phase (B) using acetonitrile, ethanol, and methanol, along with the aqueous mobile phase (A) water, was evaluated in both gradient (5–95%) and isocratic (10–80%) elution for B. Several columns with varying dimensions were tested for better peak separation using different modifiers of formic acid (0.01–0.1%) and ammonium acetate (1-10mM). The injection volume for the samples was optimized over the range of 1–10 µL, whereas the flow rate of the mobile phase was tested over the range of 0.5-1.2mL. The temperature for the column was set at 40°C, whereas the detection was performed using a MaxPlot 3D field (190-600nm) in the DAD, along with selective wavelengths (λ) of 260, 280, 300, and 320nm.

2.3.3 Method validation for myrrh phytochemicals

The developed analytical method was validated in terms of accuracy, limit of detection (LOD) and quantification (LOQ), linearity, determination coefficient (r^2), peak asymmetry, resolution, and tailing factor, as per the ICH (International Council for Harmonization) guidelines. [12]. For the R^2 value of the individual analyte, standard errors were calculated using the regression analysis, and the regression equation was generated. The slope and offset value, resolution, asymmetry, and tailing factors were acquired from the built-in software of Chromeleon (version 7.2.2.6890). To determine the accuracy, LOD, and LOQ for each analyte, the following equations were used.

Accuracy of the method = analyte concentration (recovered)/analyte concentration (initial) x 100

LOD of the method = 3.3 X intercept (SD)/regression equation (slope)

LOQ of the method = 10 X intercept (SD)/regression equation (slope)

2.4 Ultrasonic-assisted green extraction (UAG)

2.4.1 UAG method development (MD)

The extraction parameters were optimized for myrrh phytochemical extraction using ultrasonic dismembrator-based green extraction (UAG) using the solvents of ethanol (EtOH), acetone (AC), water (H_2O), and their combinations. To start the extraction, six samples for the powdered myrrh resin were prepared and extracted in the green solvents of H_2O (100%), AC (100%), EtOH (100%), H_2O: AC (1:1), H_2O: EtOH (1:1), and AC: EtOH (1:1). The USG parameters were set according to the previous method, with slight modification [13]: frequency (20kHz), amplitude (40%), pulse (10 on/5s off), extraction time (5min), temperature (ambient, 35°C), particle size < 1.0 mm, sample amount (100mg), and solvent volume (10mL). The extracted samples were cellulose-filtered (20mm), evaporated, and reconstituted in an HPLC-grade solvent, syringe-filtered (0.2µm), and diluted appropriately to get a final concentration of 1ppm for UHPLC/DAD analysis of myrrh phenolic, flavonoids, and PMFs.

2.4.2 UAG method validation (MV)

The UAG-MD was validated on a pilot scale using the optimized extraction parameters from method development. The solvent with the highest yield for the phytochemical was used to extract a larger amount of the myrrh powder using a larger volume of solvent. Briefly: 1000mg of the myrrh powder was extracted with 100mL of the AC: EtOH (1:1 v/v) using the UAG parameters for extraction. The extracted sample was filtered, dried, and reconstituted for UHPLC/DAD analysis, like the UAG-MD.

2.5 Applicability of the method in geographical origins

The applicability of the developed and validated method for UAG-UHPLC/DAD was determined using seven different samples of myrrh resins from various geographical origins. The samples were extracted according to the protocol followed in section 2.4.2 and analyzed via UHPLC/DAD for origin-driven variation in myrrh phenolic, flavonoids, and PMFs across the seven accessions.

2.6 Statistical analysis

The data were categorized into dependent (DV) and independent variables (IV) and coded in SPSS (Statistical Package for the Social Sciences: version 27.0). The appropriate statistical models were applied to mark the significant correlation and differences among the myrrh origins vs extract and phytochemical yields (P = 0.05: CI = 95%).

3. RESULTS

3.1 UHPLCDAD MDMV

The UHPLC/DAD method successfully separated the targeted analytes in a runtime of 15 minutes (including equilibration for the method), where the retention times (min) of 2.523 (GA), 3.087 (pCOU), 3.547 (QT), 4.347 (NG), 7.17 (CT), 8.397 (NB), and 11.367 (TG) were observed for the seven analytes. The chromatographic conditions optimized were an isocratic elution for the mobile phase consisting of 55% ACN with 0.1% FA (B) and 45% H2O with 0.1% FA (A), injection volume of 5 µL, flow rate of 0.5mL/min, and column oven at 40°C, using the C18 column from Restek with dimensions of 150 × 4.6mm; 3µm.

The validation for the method under the ICH guidelines exhibited the ranges of 94.68–131.60% for accuracy, 1.56–2.50% for relative SD, 11.69-98.47ppm for LODs, and 35.43-298.40ppm for LOQs. The R^2 values for the analyte were 0.99976–0.9999 in the linearity range of 0.5–500 for the seven analytes. The peaks showed acceptable asymmetry (1.03–1.17), resolution (> 3.0) approaching 14.74, and tailing factor (< 1.2) values with theoretical plates > 10000. The detailed data for the UHPLC/DAD MDMV data for the individual analyte, along with the regression equations, are provided in Table 1. The chromatogram with RT for the seven analytes is shown in Fig. 1, whereas the CC with regression equations is provided in Fig. 2.

Table 1
The parameters for the development and validation of the UHPLC-DAD analytical method for myrrh phytochemicals

UHPLC MDMV parameters							
Parameters	**GA**	**pCOU**	**QT**	**NG**	**CT**	**NB**	**TG**
Mobile phase	55% ACN (0.1% FA): 45% H_2O (0.1% FA)						
Retention time (min)	2.523	3.087	3.547	4.347	7.17	8.397	11.367
Flow rate (mL/min)	0.5						
Elution mode	Binary mixture of organic and aqueous mobile phases with an isocratic elution						
Column oven temperature	40°C						
Column dimensions	Restek RP Ultra C18 (150X4.6mm; 3µm)						
Injection volume	5µL						
Peak resolution	5.02	3.46	5.24	14.74	5.15	10.01	0
Tailing factor	< 1.2						
Peak asymmetry	1.17	1.11	1.13	1.09	1.03	1.05	1.03
Theoretical plates	≥ 10000						
LODs (ppm)	26.15	11.69	30.42	31.68	25.35	98.47	36.08
LOQs (ppm)	79.24	35.43	92.19	96.00	76.83	298.40	109.32
Accuracy (%)	95.26–110.80	95.88–118.0	96.22-114.98	95.80-108.66	96.01–121.20	94.68–131.60	95.07–126.80
Rel. SD (%)	2.50	1.86	1.70	1.56	1.73	2.50	2.24
Linearity range (ppm)	0.5–250			1-500			
r^2-value	0.99976	0.99987	0.99989	0.9999	0.99988	0.99976	0.99981
Regression equation	offset: -0.041 & slope: 0.241	offset: -0.098 & slope: 0.399	offset: -0.141 & slope: 0.278	offset: 0.034 & slope: 0.251	offset: -0.048 & slope: 0.346	offset: -0.148 & slope: 0.125	offset: -0.294 & slope: 0.308
λ-nm	Chromatogram extraction; 300nm (UV range 190-800nm)						

3.2 UAG-MD

3.2.1 Extract yields

The method development showed a mean ± SD of 11.77 ± 3.50mg (*N = 6*) in the range (minimum-maximum) of 6.82mg (H_2O) to 17.06mg (H_2O: EtOH 1:1 v/v). The descending order for the extract yield (mg/100mg) exhibited was H_2O: EtOH (17.06)> H_2O: AC (13.94)> EtOH (11.73) > AC: EtOH (11.1) > AC (9.98)> H_2O (6.82). The data is provided in Table 2.

Table 2
Development of an ultrasonic extraction method for the optimization of solvent with higher yield for myrrh phytochemicals

S#	Solvent system (mL)	USE parameters	Solid: solvent ratio	Extract yield (mg)	GA (ppm)	pCOU (ppm)	QT (ppm)	NG (ppm)	CT (ppm)	NB (ppm)	TG (ppm)	Cumulative yield/ solvent
1	H_2O (100%)	Amplitude, 40%: pulse, 10/5s: time, 5min	100mg (dried powdered resin) in 10mL solvent	6.82	2.10	4.53	5.91	4.52	0.15	3.94	0.00	*21.15*
2	AC (100%)			9.98	12.42	13.54	9.01	0.23	5.01	19.15	0.00	*59.36*
3	EtOH (100%)			11.73	1.29	7.92	8.52	10.83	10.83	1.76	0.00	*41.15*
4	H_2O: AC (1:1 v/v)			13.94	3.69	4.72	11.10	15.89	0.20	16.97	0.00	*52.57*
5	H_2O: EtOH (1:1 v/v)			17.06	2.01	4.24	7.87	3.40	0.30	1.82	0.00	*19.64*
6	AC: EtOH (1:1 v/v)			11.1	5.12	22.97	20.46	10.13	4.88	27.22	0.00	*90.78*
Individual phytochemical yield					*26.63*	*57.92*	*62.87*	*45.00*	*21.37*	*70.86*	*0.00*	**284.65**

3.2.2 Yield for individual phytochemicals

The individual yield for the phytochemical across the six solvent systems showed a mean ± SD of 47.44 ± 20.07ppm, with more yield for NB (70.86ppm), followed by QT (62.87ppm), pCOU (57.92ppm), NG (45.0ppm), GA (26.63ppm), and CT (21.37ppm). On an individual basis, a large amount was observed for NB (27.22ppm) and pCOU (22.97ppm). The descending order across the myrrh phytochemicals was (ppm): NB (27.22)> pCOU (22.97) > QT (20.46) > GA (12.42) > NG = CT (10.83) > TG (0.00). TG was not observed in any of the solvents used for extraction.

The descending orders constructed were phenolic acids: pCOU > GA, flavonoids: QT > NG, and PMFs: NB > CT> TG. The phytochemical yield for the myrrh phytochemical is provided in Table 2.

3.2.3 Yield for the phytochemicals/solvent

The phytochemical yield in an individual solvent resulted in a sum with mean ± SD of 284.65 and 47.44 ± 26.65ppm in the range of 19.64ppm (H_2O: EtOH 1:1 v/v) to 90.78ppm (AC: EtOH 1:1 v/v).

The cumulative yield for the seven phytochemicals/individual solvent exhibited a higher amount for AC: EtOH (90.78ppm), followed by AC (59.36ppm), H_2O: AC (52.57ppm), EtOH (41.15ppm), H_2O (21.15ppm), and H_2O: EtOH (19.64ppm). The data for the individual and cumulative yield are provided in Table 2.

3.3 UAG-MV

3.3.1 Extract yields

The method validation resulted in an average yield of 327.71mg (*N = 7*; mg/g) with a sum ± SD of 2293.90 ± 28.55mg, in the range of (minimum-maximum) 277.59–366.04mg in AC: EtOH (1:1 v/v) solvent system. The details regarding the extract yields are shown in Table 3.

Table 3
Validation of the ultrasonic extraction method in myrrh samples from different origins with descriptive statistics

S#	Origin	USE parameters	Solid: solvent ratio	Extract yield (mg)	GA (ppm)	pCOU (ppm)	QT (ppm)	NG (ppm)	CT (ppm)	NB (ppm)	TG (ppm)	Cumulative yield/ solvent
1	**Oman 1 (O1)**	Amplitude, 40%: pulse, 10/5s: time, 5min	1000mg (dried powdered resin) in 100mL AC: EtOH (1:1 v/v)	331.87	0	52.76	25.38	4.74	0	0	0	*82.87*
2	**Oman 2 (O2)**			319.37	0	26.7	29.06	8.64	0	4.45	0	*68.84*
3	**Taif, KSA (SA1)**			277.59	0	32.19	28.56	0	0	0	0	*60.75*
4	**Makkah, KSA (SA2)**			310.87	0	46.86	36.74	0	10	9.27	0	*102.89*
5	**Yemen (YN1)**			345.22	0	28.74	31.28	0	0	0	0	*60.01*
6	**Somalia, Africa (SM1)**			342.98	0	9.651	59.64	0	6.72	0	0	*76.01*
7	**India (IN1)**			366.04	16.7	6.931	22.02	0.16	0	0	0	*45.78*
Individual yield for phytochemical				*327.71*	*16.7*	*203.8*	*233*	*13.5*	*16.7*	*13.7*	*0*	**497.16**

Descriptive statistics

Variable	Minimum	Maximum	Sum	Mean	Std. Deviation
Extract yield	277.59	366.04	2293.90	327.71	28.55
GA	0.00	16.70	16.70	2.38	6.30
pCOU	6.90	52.80	203.80	29.12	17.13
QT	22.00	59.60	232.70	33.24	12.52
NG	0.00	8.60	13.50	1.93	3.44
CT	0.00	10.00	16.70	2.39	4.20
NB	0.00	9.30	13.70	1.96	3.63

3.3.2 Yield for individual phytochemicals

The yield for GA revealed a sum of 16.70ppm with a mean ± SD of 2.38 ± 6.30ppm in the range of 0.0-16.70ppm. The sum (ppm) with mean ± SD (ppm) and ranges (ppm) for pCOU, QT, NG, CT, and NB observed were: [(203.80 and 29.12 ± 17.13; 6.90–52.80), (232.70 and 33.24 ± 12.52; 22.0-59.60), (13.50 and 1.93 ± 3.44; 0.0-8.60), (16.70 and 2.39 ± 4.20; 0.0–10.0), and (13.70 and 1.96 ± 3.63; 0.0-9.30)], respectively.

On an individual basis, QT was observed at higher levels (59.64 ppm), followed by pCOU (52.76 ppm), GA (16.7 ppm), CT (10 ppm), NB (9.27 ppm), and NG (8.64 ppm). TG was not observed in any of the tested myrrh resin origins, whereas GA was observed only for the Indian origin.

About phenolic acids, pCOU > GA, flavonoids QT > NG, and PMFs CT > NB. The data regarding individual yield for myrrh phytochemicals across the seven different origins are provided in Table 3.

3.3.3 Yield for the phytochemicals/solvent

The cumulative sum for the phytochemicals across the green solvents was 4797.16 ± 71.02ppm in AC: EtOH (1:1 v/v). A larger phytochemical yield of 102.89ppb was observed for SA2 (Makkah origin), followed by O1 (82.87ppb), SM1 (76.01ppb), O2 (68.84ppb), SA1 (60.75ppb), YNI (60.01ppb), and IN1 (45.78ppb). The data is shown in Table 3.

3.4 Extract and phytochemical yield for the origins

3.4.1 Extract yields

The optimized AC: EtOH (1:1) solvent system exhibited the highest extract yield of 366.04mg/g for the Indian sample (IN1), followed by Yemen (YN1; 345.22mg/g), and Somalia (SM1; 342.98mg/g), whereas the sample with the lowest yield was Taif, Saudi Arabia (SA1; 277.59mg/g). The details are shown in Table 3.

3.4.2 Yield for the phytochemicals

3.4.2.1 Phenolic acids

For the phenolic acids, GA was observed in the Indian sample (16.67ppm) only, while all other origins showed non-detectable levels. All the samples showed the presence of pCOU in the range of 6.93ppm (IN1) – 52.76ppm (O1). The highest pCOU concentration was seen in the O1 origin (Oman; 52.76ppm), whereas the lowest yield was observed in the IN1 (Indian sample; 6.93ppm), as shown in Table 3.

3.4.2.2 Flavonoids

Among the flavonoids, the Somalian origin (SM1) exhibited the highest QT content (59.64ppm), whereas the Indian (IN1) origin showed the lowest amount (22.02ppm).

The NG flavonoid was detected in O1 (4.74ppm), O2 (8.64ppm), and IN1 (0.16ppm) origins only. The origins of SA1, SA2, YN1, and SM1 were seen with a lack of detectable NG amount. The data is provided in Table 3.

3.4.2.3 Polymethoxyflavones (PMFs)

For the PMFs, TG was not detected in any of the origins, whereas NB was observed in Makkah (SA2; 9.27ppm) and Oman (O2; 4.45ppm) origins and CT in Makkah (SA2; 10.02ppm) and Somalian (SM1; 6.72ppm) origins. The details regarding PMFs are given in Table 3.

3.5 Statistical models

3.5.1 Paired samples t-test

The paired samples t-test for the extracts yield vs myrrh phytochemicals showed significant differences ($P < 0.05$). The mean with CI (confidence intervals) values observed were: GA [$M = 325.32 \pm 25.34$, 95%CI: 301.89–348.76, $t = 33.97$, $P < 0.001$], pCOU [$M = 298.59 \pm 40.06$, 95%CI: 261.54–335.64, $t = 19.72$, $P < 0.001$), QT [$M = 294.47 \pm 30.55$, 95%CI: 266.22–322.72, $t = 25.51$, $P < 0.001$], NG [$M = 325.77 \pm 29.03$, 95%CI: 298.93–352.62, $t = 29.70$, $P < 0.001$], CT [$M = 325.31 \pm 29.24$, 95%CI: 298.27–352.35, $t = 29.44$, $P < 0.001$], and NB [$M = 325.75 \pm 29.88$, 95%CI: 298.11–353.38, $t = 28.84$, $P < 0.001$).

The paired samples t-test for the origins vs extract and phytochemical yields revealed significant differences for pCOU [$M = 25.12 \pm 18.88$, 95%CI: 7.66–42.58, $t = 3.52$, $P = 0.013$] and QT [$M = 29.24 \pm 11.98$, 95%CI: 18.16–40.31, $t = 6.46$, $P < 0.001$]. The origin vs GA, NG, CT, and NB were seen with a lack of any significant differences. The data for the paired samples t-test is shown in Table 4.

Table 4
paired t-test for extract yield vs myrrh phytochemicals, and origin of the myrrh sample vs extract yield and phytochemicals at *P* = 0.05 and CI = 95%

Paired differences for extract yield vs myrrh phytochemicals							
Pairs of variables		**Mean**	**SD**	**CI (95%)**		**t**	**P**
				Lower	**Upper**		
Pair 1	**Extract yield-GA**	325.32	25.34	301.89	348.76	33.97	**< 0.001**
Pair 2	**Extract yield-pCOU**	298.59	40.06	261.54	335.64	19.72	**< 0.001**
Pair 3	**Extract yield-QT**	294.47	30.55	266.22	322.72	25.51	**< 0.001**
Pair 4	**Extract yield-NG**	325.77	29.03	298.93	352.62	29.70	**< 0.001**
Pair 5	**Extract yield-CT**	325.31	29.24	298.27	352.35	29.44	**< 0.001**
Pair 6	**Extract yield - NB**	325.75	29.88	298.11	353.38	28.84	**< 0.001**
Paired differences for origin vs extract yield and myrrh phytochemicals							
		Mean	**SD**	**CI (95%)**		**t**	**P**
				Lower	**Upper**		
Pair 1	**Extract yield - origin**	323.71	27.34	298.42	348.99	31.33	**< 0.001**
Pair 2	**GA-origin**	-1.62	5.26	-6.49	3.25	-0.81	0.45
Pair 3	**pCOU-origin**	25.12	18.88	7.66	42.58	3.52	**0.013**
Pair 4	**QT-origin**	29.24	11.98	18.16	40.31	6.46	**< 0.001**
Pair 5	**NG-origin**	-2.07	5.18	-6.86	2.72	-1.06	0.33
Pair 6	**CT-origin**	-1.61	4.22	-5.51	2.29	-1.01	0.35
Pair 7	**NB-origin**	-2.04	4.56	-6.26	2.18	-1.18	0.28

3.5.2 K-mean cluster analysis

The statistical model for K-mean clustering suggested four clusters with samples: cluster 1 (1), cluster 2 (1), cluster 3 (4), and cluster 4 (1). Cluster 1 consists of one sample (SM1) with the highest amount of QT and a moderate amount of CT and extract yield. Cluster 2 consists of IN1, exhibiting the highest amount of GA and extract yield. The significant cluster was 4, where the highest amount of pCOU, CT, and NB were observed in SA2 (Saudi origin sample). The remaining samples exhibiting lower amounts of extract and phytochemicals yield were loaded in cluster 3. The data for K-mean is provided in Table 5 and Fig. 3.

Table 5
K-mean cluster analysis with PCA for the extract yield and origins vs myrrh phytochemicals based on *P* and F values

K-mean analysis					
Variables		**F-value**	**P-value**	**Cluster**	**samples**
Z-score: GA		0.02	0.74	1	1
Z-score: pCOU		3.08	0.19	2	1
Z-score: QT		51.91	**0.004**	3	4
Z-score: NG		0.35	0.80	4	1
Z-score: CT		0.07	0.82	**Total**	**7**
Z-score: NB		4.31	0.13		
Z-score: Extract yield		0.90	0.53		
PCA					
Variables	**PC1**	**PC2**	**KMO and Bartlett's Test**		
GA	-0.75	-0.12	Kaiser-Meyer-Olkin Measure of Sampling Adequacy		0.13
pCOU	0.82	-0.32	Bartlett's Test of Sphericity	X^2-*value*	28.25
QT	0.08	0.86		df	15
NG	0.38	-0.64		Sig.	0.02
CT	0.44	0.85			
NB	0.73	0.24			
Individual %variance	35.41	33.98			
Cumulative %variance	35.40	69.39			

3.5.3 Principal component analysis (PCA)

The PCA produced two components with a cumulative variability of 69.39% and individual variabilities of 35.41% (PC1) and 33.98% (PC2). The component with major variability (PC1) was dominated by pCOU and NB, whereas PC2 was loaded with QT and CT. GA, with a negative correlation, was loaded in PC1. The KMO and Bartlett's test of sphericity showed a high X^2-value (28.25) at $P = 0.02$, suggesting the adequacy of the PCA. The data for PCA are shown in Table 5 and Fig. 4.

4. DISCUSSION

Current research work, for the first time, highlights the presence and concentration of phenolic acids (GA, pCOU), flavonoids (QT, NG), and polymethoxyflavones (PMFs) (CT, NB, TG) in myrrh resins. This study provides a comprehensive evaluation of the ultrasound dismembrator-based green extraction (UAG), followed by an in-house developed and validated UHPLC-DAD analytical method for the quantification of GA, pCOU, QT, NG, NB, CT, and TG. The research framework follows a stepwise approach to develop a green extraction method (UAG-MD) using the green solvents of acetone (AC), ethanol (EtOH), water (H_2O), and their combinations. The UAG-MD was validated on a pilot scale using seven different origins of myrrh samples. The chemometric analysis was used to extract data related to the variability of myrrh phytochemicals, correlations, and significant differences among the geographical origins of myrrh.

To determine the myrrh phytochemicals, an in-house analytical method was developed and validated. The UHPLC-DAD method was able to determine the seven analytes in a single run with a shorter runtime of 15 minutes. The accuracy and linearity of the method, including peak asymmetry, resolution, and tailing factors, reflect its robustness.

To optimize the solvent selection process, an extraction method was developed (UAG-MD) using six different green solvent systems. The UAG-MD revealed significant differences in the extraction efficiency of the tested solvents. Though the highest crude extract yield was observed for H_2O: EtOH (1:1 v/v) solvent system, more phytochemical yield was observed for AC: EtOH (

1:1 v/v solvent system). This difference highlights the familiar distinction between extract quantity and extract quality. Generally, the water-rich solvents dissolve gums, sugars, and water-soluble resin components, thus increasing the crude yield. [14] On the contrary, a mix of organic

solvent systems widens the polarity range, improving the solubility for both phenolic acids and moderately hydrophobic flavonoids and PMFs. [15] This finding is in concordance with the previous reports, where a maximized recovery of phenolics and flavonoids was observed with the use of EtOH and AC during ultrasonic extraction. [6] The strong performance of the AC: EtOH (1:1 v/v) system is attributed to acetone's ability to accept hydrogen bonds and ethanol's polarity to improve the penetration of the matrix. [16, 17] Additionally, the cavitation produced by the ultrasonic energy breaks the matrix structures, further enhancing the diffusibility and solubilization phenomenon for the solvents. [17] The use of AC: EtOH for the extraction of myrrh phytochemicals is reported herein for the first time and may be relevant for industrial extraction and standardization.

To validate the applicability of the developed extraction (UAG-MD) and analytical method, the AC: EtOH system was used to extract myrrh phytochemicals from seven different origins (UAG-MV). The geographical origin exhibited widespread variation in terms of the extract yield and phytochemical composition. These differences reflect variations in the phytochemical composition due to environmental stressors (soil pH, temperature, altitude, salinity, irrigation, UV exposure, etc.) and genetic differences among myrrh species. [10, 11] A similar origin-based variability has been reported previously, where a significant difference in extraction efficiency and phenolic content was observed depending on the plant part and environmental conditions. [8, 18, 19] The resinous plants such as myrrh exhibit sensitivity to climatic factors, including drought, heat, and mineral content. These factors significantly modulate the biosynthesis of secondary metabolites and resin constituents. The elevated extractability observed in samples from India and Yemen could be attributed to either enhanced resin plasticity or a higher concentration of ethanol-soluble components. [10, 20] Conversely, the reduced yield from Taif (SA1) might suggest the presence of a denser, more polymerized resin matrix that exhibits diminished responsiveness to ultrasound cavitation. These geographically based factors need to be studied further in-depth.

About phenolic compounds, the study highlighted distinct differences in the phytochemical profiles when comparing samples across various origins. The phenolic GA was exclusively detected in the Indian sample, suggesting either a unique chemotype specific to this origin or the influence of environmental factors promoting GA biosynthesis. As is known, phenolic acids are products of the phenylpropanoid pathway, and their accumulation is known to be highly sensitive to factors such as environmental stress, nutrient availability, and genetic predispositions. The restricted presence of GA in IN1 could therefore indicate localized activation of shikimate-derived pathways or differential expression of key enzymes, including shikimate dehydrogenase and gallic acid synthase. [10, 20, 21] In contrast, pCOU was detected in all origins, though its concentration varied considerably, with the highest levels observed in the Oman sample. This observation is consistent with prior research indicating that pCOU functions as a central intermediate in the phenylpropanoid pathway, and its accumulation is significantly modulated by environmental stressors such as UV exposure and pathogen pressure. [7, 21] The significant origin effect observed for pCOU with its widespread distribution reinforces its potential utility as a chemotaxonomic marker for myrrh standardization and quality variation.

The distribution of the flavonoid exhibited distinct geographical patterns. The QT flavonoid was the highest in Somalia and lowest in India-origin samples, suggesting that African myrrh populations may experience environmental conditions, such as high UV radiation or drought, that stimulate flavonol biosynthesis. [22] Usually, flavonoids are recognized as stress-responsive metabolites, and similar origin-dependent QT variation has been reported in UAE studies of other medicinal plants. [6, 20, 21] The compound NG, a key flavanone precursor, was observed only in O1, O2, and IN1, indicating differential metabolic flux through the conversion of flavanone to flavonol/PMF pathway among origins. This restricted distribution suggests that some plants and their species may rapidly convert NG into downstream flavonoids, while others accumulate it due to bottlenecks in enzymatic conversion. [22, 23]

The PMF profile exhibited a restricted distribution across the samples studied, where CT and NB were detected in only a few origins, and TG was absent from all samples. While PMFs are primarily characteristic of Citrus species [24], their low-level detection in myrrh samples may suggest the presence of structurally related methylated flavonoid derivatives or trace compounds with similar fragmentation patterns. However, due to their limited abundance and the absence of confirmatory enzymatic or biosynthetic evidence, these findings require cautious interpretation and cannot be directly attributed to specific O-methyltransferase activity. [25, 26] The elevated PMF content observed in the Makkah (SA2) sample is notable, given that NB is recognized for its potent anti-inflammatory and anticancer activities. The absence of TG may signify either its biosynthetic absence or concentrations below the method’s limit of detection. To date, none of the studies reported any targeted quantification of CT, NB, and TG in myrrh samples, rendering this dataset a novel contribution to myrrh phytochemistry. Further investigations using high-resolution mass spectrometry may be employed to authenticate the presence of PMFs in the myrrh sample.

The data were analyzed using statistical models for chemometric analysis to elucidate the factors influencing chemical variation. The paired t-tests revealed that the geographical origin significantly affected the concentrations of pCOU and QT across the origins, but not GA, NG, CT, or NB. These findings suggest that phenolic acids and flavonoids are more susceptible to environmental modulation when compared to PMFs. The statistical model of K-means clustering identified QT as the most prominent discriminant variable, a finding consistent with its broad dynamic range observed across different origins. Though the QT amount was highest in SMI and GA in IN1, the sample from Saudi Arabia

(SA2) exhibited the highest amounts for pCOU, CT, and NB. The PCA revealed two principal components: PC1, primarily influenced by pCOU and NB, and PC2, primarily influenced by QT and CT. This dual-axis structure implies that phenolic acids/PMFs and flavonoids constitute two semi-

independent metabolic domains within the chemical composition of myrrh resin. Such comparative chemometric patterns have been reported in previous investigations, supporting the data observed herein. The details regarding the distribution of phenolics, flavonoids, and PMFs in these seven different origins are presented in Fig. 5. The outcome for statistical models signifies that the presence of four markers (pCOU, QT, CT, and NB) may be utilized to classify the geographical origins of myrrh, thereby providing a foundational basis for subsequent authentication and quality control frameworks. The data are supported by the previous study showing that myrrh resins contain gallic acid, para-coumaric acid, and quercetin as the phenolic and flavonoid compounds in the myrrh resin. [3]

The integration of UAG and UHPLCDAD in this study indicates that the optimized AC: EtOH (1:1 v/v) extraction system carries the potential to be scaled for the industrial production of standardized myrrh extracts, facilitating the enrichment of specific bioactive classes. The successful origin-dependent phytochemical variation may aid the selection of raw materials optimized for specific applications, such as those requiring antioxidant, anti-inflammatory, or PMF-rich extracts. To broaden the scope of the phytochemical investigation for myrrh, UHPLCMS/MS investigation, for both the targeted and untargeted metabolomics, may be undertaken to validate the identified chemotaxonomic markers. Furthermore, correlating these phytochemical profiles with biological activities such as anti-inflammatory assays, antimicrobial testing, and cytotoxicity studies may help establish their functional relevance and therapeutic potential. For the observed chemotypic variation, the integration of transcriptomic or genomic analyses may be employed to elucidate the genetic basis of phytochemical variation, providing deeper insight into the biosynthetic pathways that contribute to myrrh's chemical diversity.

The study successfully developed and validated a framework for a more comprehensive phytochemical understanding of myrrh resin via integration of ultrasonic-dismembrator-based green extraction, analytical method validation, and chemometric interpretation. This research establishes a methodological and conceptual foundation for the subsequent research, industrial standardization, and therapeutic investigation of myrrh resin.

Conclusion

This study developed a rapid, green, and analytically robust method for profiling phenolic acids, flavonoids, and polymethoxyflavones in myrrh resin. The optimized AC: EtOH (1:1 v/v) ultrasonic extraction, coupled with a validated UHPLCDAD method, enabled the simultaneous quantification of seven marker compounds. The outcomes of the study revealed significant chemotypic variations attributable to geographical origin. The specific patterns associated with geographical origin were identified, including the exclusive presence of GA in Indian myrrh, high levels of QT in Somali resin, and enriched PMFs in samples from Makkah. The outcomes from this study suggest that biological and environmental factors contribute to the observed chemical diversity in myrrh. The findings herein establish a basis for developing standardized extraction protocols, authenticating the geographical origin of myrrh, and facilitating the targeted formulation of myrrh-based therapeutic agents. The future directions for myrrh research should integrate metabolomics, bioactivity assays, and sustainable sourcing strategies to further elucidate the pharmacological and commercial utility of myrrh resin.

Declarations

Conflict of interests

There is no conflict of interest applicable in this study.

Consent to publish

The author agrees with the current format for the manuscript to be published.

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Availability of data

Any data generated in this study is presented in the respective tables and figures.

Authors contribution

RA (conceptualization and study design; literature search; ultrasonic extraction and analytical method validation for UHPLC-DAD; chemometric analysis using SPSS; write-up and final revision of the manuscript).

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Figures

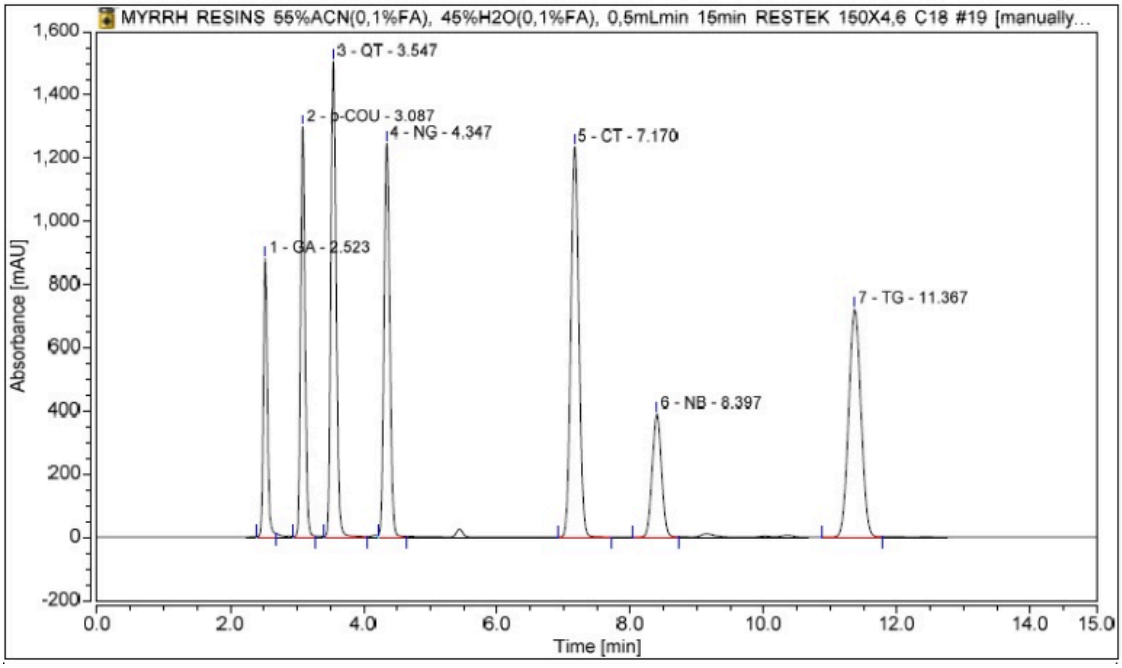


Figure 1

A representative chromatogram for myrrh phytochemicals with retention time (min) and absorbance (mAU) within the runtime of 15 minutes

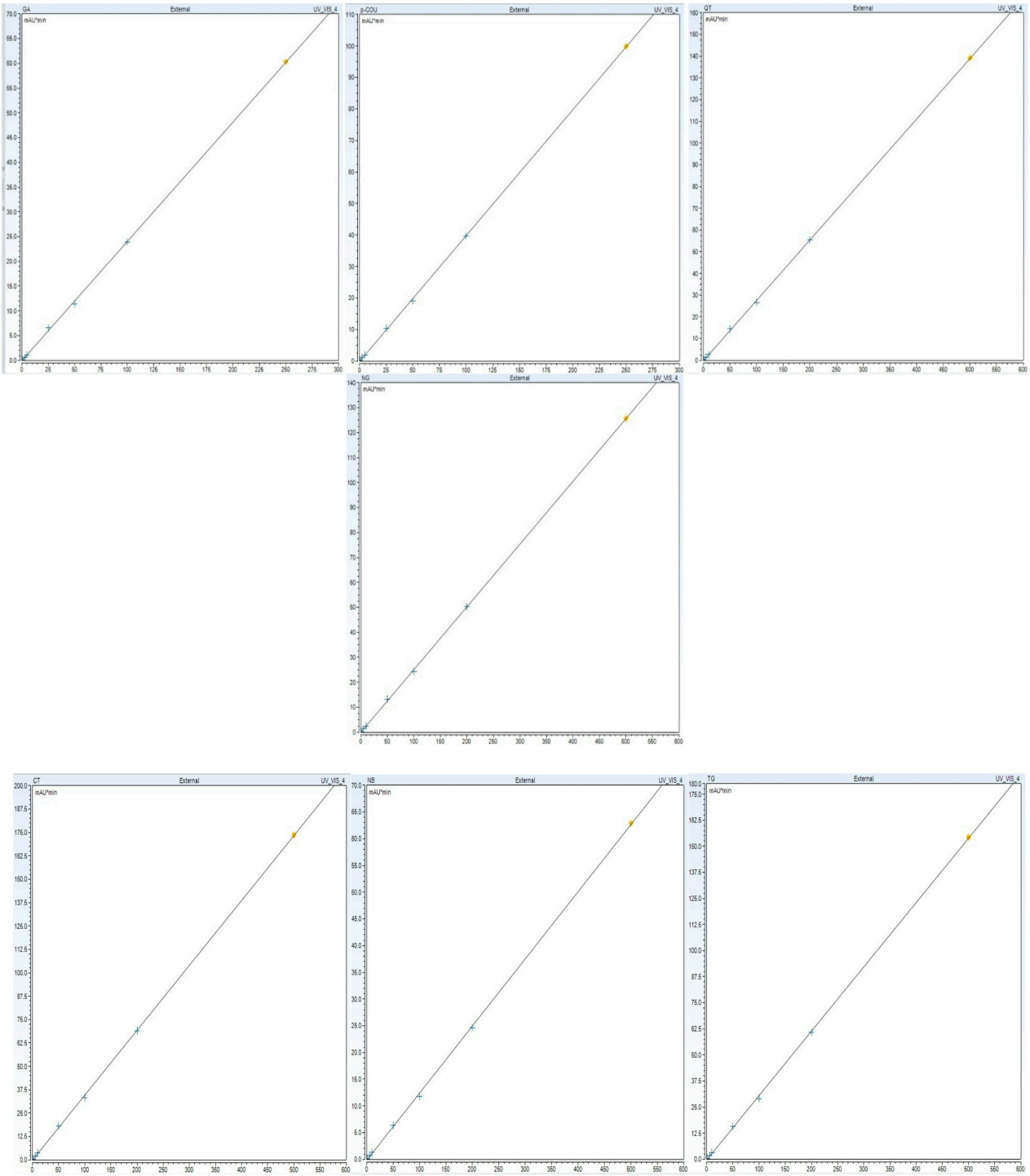


Figure 2

calibration curve diagrams for the standard drugs of GA, pCOU, QT, NG, CT, NB, and TG, respectively

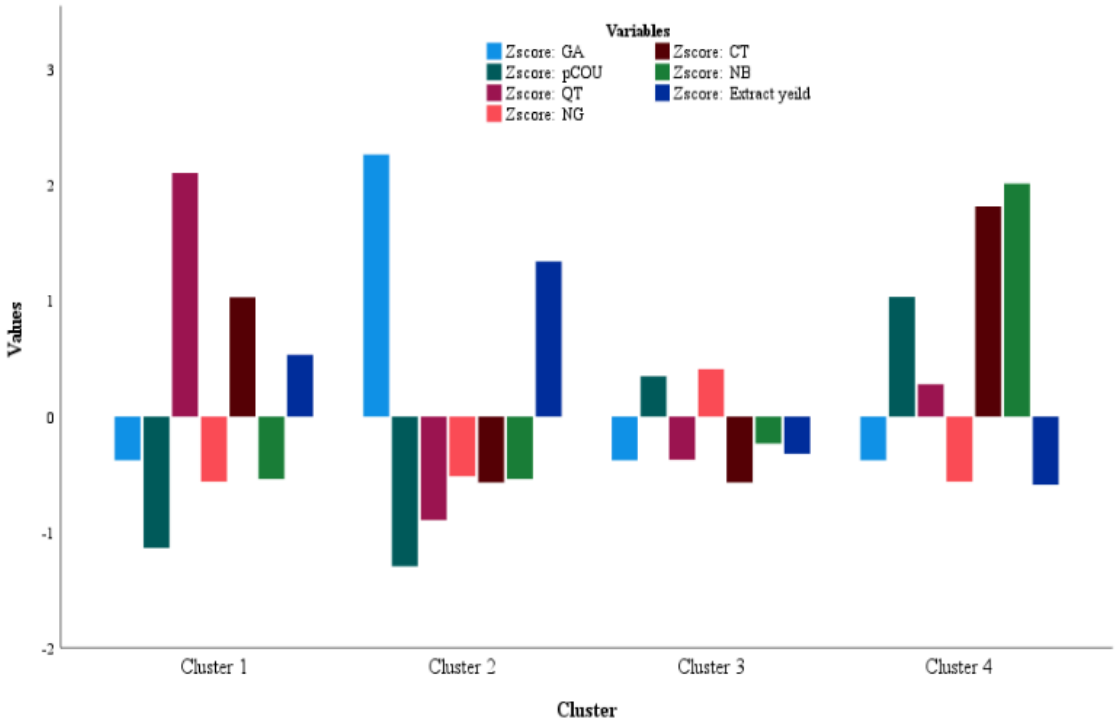


Figure 3

clusters representing the distribution of variables in K-mean analysis

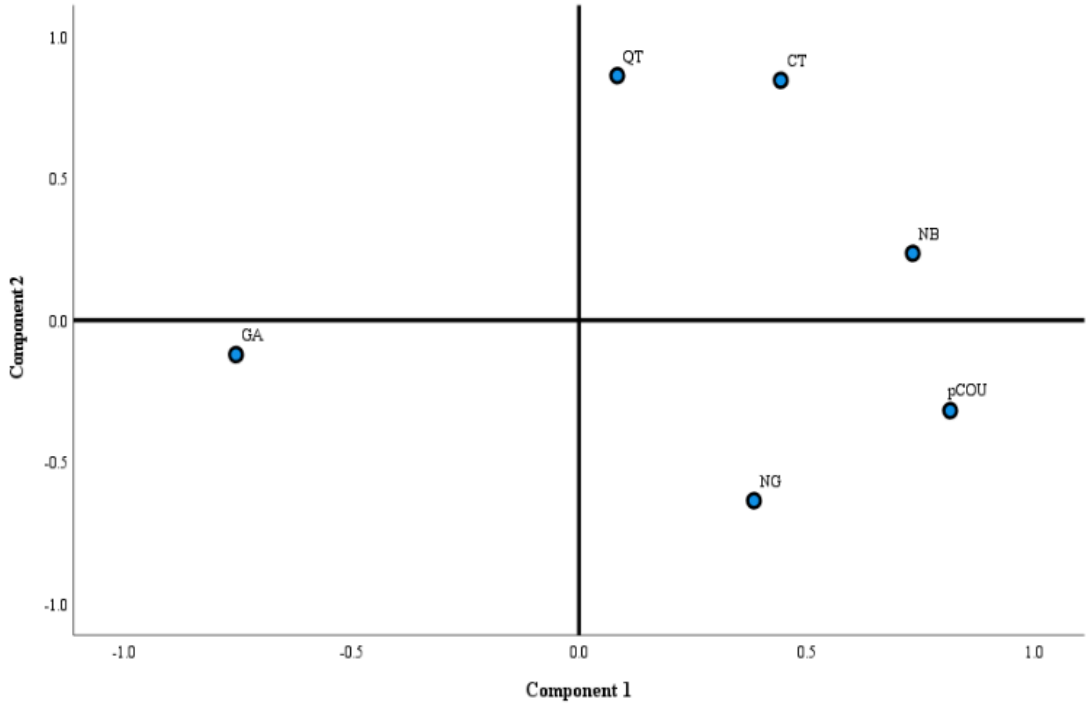


Figure 4

distribution of the variables in different components observed in PCA

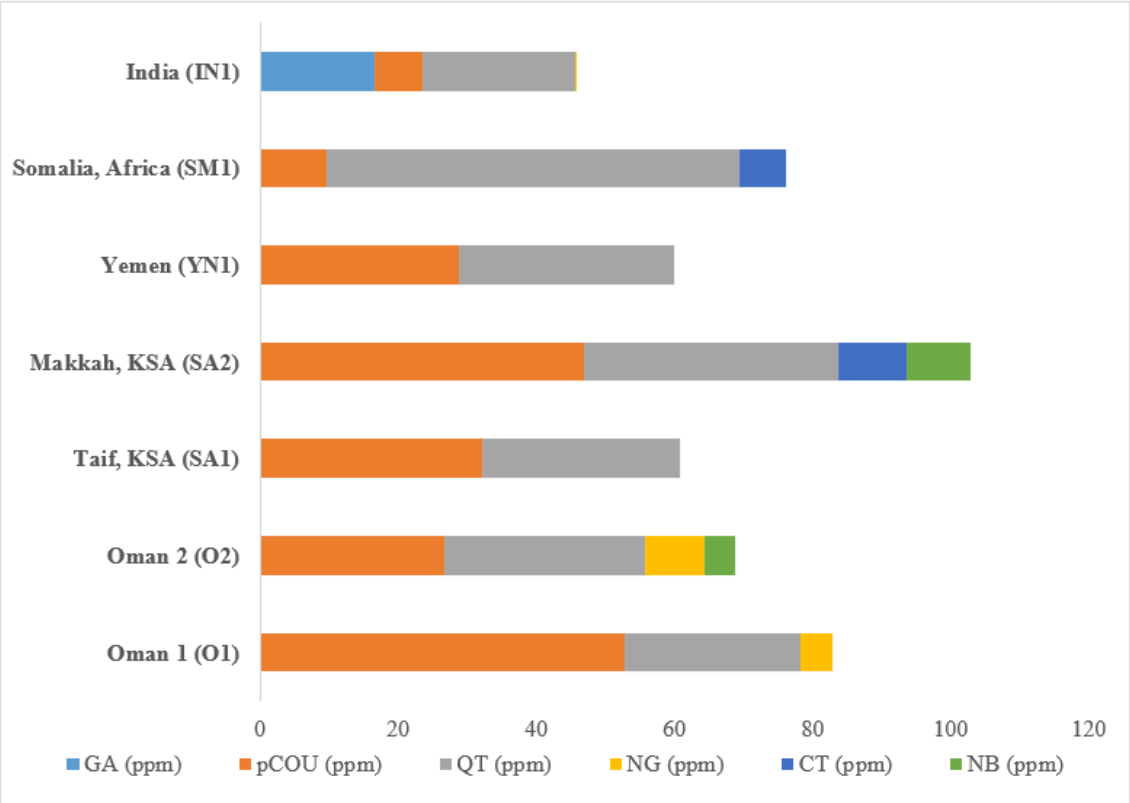


Figure 5

Distribution of phenolic acids, flavonoids, and polymethoxyflavones in seven different origins of the myrrh samples

Supplementary Files

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- Graphicalabstract.png