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ORIGINAL ARTICLE

Identification and Characterization of Polyphenols and Volatile Terpenoid Compounds in Different Extracts of Garden Sage (*Salvia officinalis* L.)

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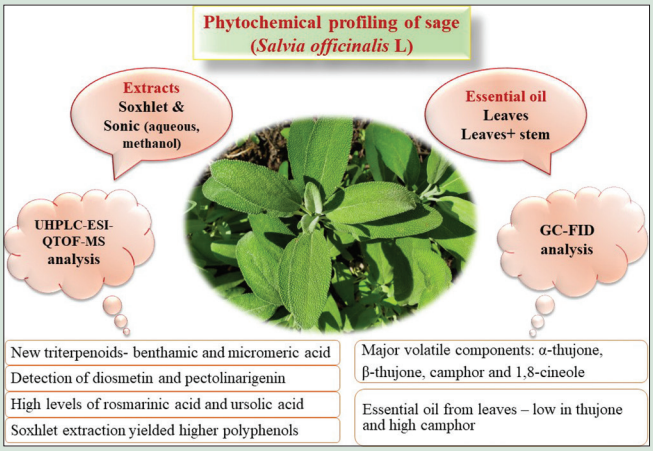
ABSTRACT

Introduction: Garden sage (*Salvia officinalis* L.) is an important medicinal and aromatic herb, used in various food, pharmaceuticals and cosmetic industries for its potential antioxidant properties. Leaves are the source of essential oils and polyphenols, used as a raw material in food and pharmaceutical industries. **Materials and Methods:** The study aimed to develop a complete phytochemical profile of *S. officinalis* leaves through liquid chromatography coupled with quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS) and gas chromatography with a flame ionization detector (GC-FID). Soxhlet and sonicated extract were analyzed using UHPLC, data-independent acquisition in negative electrospray ionization mode. Essential oil profiling of leaves in comparison with leaves+ stem was carried out using GC-FID. **Results:** Among the extraction methods, Soxhlet extraction yielded significantly high levels of caffeic acid (92.45 ± 1.92 µg/g), rosmarinic acid (18821.33 ± 150.20 µg/g), luteolin-7-glucoside (635.13 ± 11.20 µg/g), carnosic acid (27.48 ± 2.37 µg/g), carnosol (1347.67 ± 30.04 µg/g), and ursolic acid (14938.67 ± 82.20 µg/g). Among the 43 identified phenolic compounds, two flavonoids, diosmetin and pectolinarigenin and two triterpenoids benthamic and micromeric acids have been first time detected in *S. officinalis* leaves. The results of essential oil analysis indicated the presence of α-thujone (34.43- 38.93 %), β-thujone (6.03-7.58 %), camphor (15.77-18.12 %), 1,8-cineole (5.45-6.21 %), α-humulene (5.20 %), and camphene (4.29-5.10 %) as major volatile terpenoid components in *S. officinalis*. **Conclusion:** Soxhlet extraction found to be the best method for polyphenol extraction and the essential oil extracted only from leaves best suitable for therapeutic purposes due to less α-thujone and β-thujone content.

Key words: Garden sage, gas chromatography with a flame ionization detector, polyphenols, sonication, Soxhlet extraction, ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry

SUMMARY

- UHPLC-ESI-QTOF-MS enabled identification of more than 40 phenolic compounds in sage
- Methanolic extraction of sage yielded high concentration of polyphenols
- Soxhlet extracted samples contained more polyphenols than sonicated samples
- GC-FID analysis of oil resulted in high levels of α-thujone, β-thujone, camphor and 1,8-cineole
- Quality of essential oil from sage leaves better than leaves+ stem oil.



Abbreviations Used: UHPLC-ESI-QTOF-MS: Ultra-High-Performance Liquid Chromatography coupled with Electrospray Ionization, Quadrupole Time-of-Flight Mass Spectrometry; LC-MS: Liquid chromatography-mass spectrometry; SX: Soxhlet extraction (SX); SW: Sonic extraction in water; SM: Sonic extraction in Methanol; NIST: National Institutes of Standards and Technology; AOI: All-in-One; DIA: Data-independent acquisitions; SWATH: Sequential window acquisition of all theoretical fragment-ion spectra; GC-FID: Gas chromatography with a flame ionization detector

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INTRODUCTION

Salvia officinalis L., popularly known as garden sage or common sage, is a perennial aromatic herb belonging to the family *Lamiaceae*. It is recognized as a culinary herb in the western world, used in poultry stuffing, flavoring of meat, sausages, and fish. The herb is cultivated for the essential oils present in the leaves and stem, used in perfumes, cosmetics, and pharmaceuticals.[1,2] The herb is used medicinally to improve cognition and to reduce high blood pressure, excessive sweating, nervous disorders, depression, cerebral ischemia, and pharyngitis and also used as an antiseptic.[3,4] *S. officinalis* leaves are a rich source of polyphenolic compounds with more than 50 identified polyphenols, comprising an

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array of phenolic acids and flavonoids. These phenolic compounds include caffeic acid, rosmarinic acid, salvianolic acids, sagecoumarin, sagerinic acid, and ferulic acid, and the prime flavonoids comprise luteolin, apigenin, hispidulin, kaempferol, and quercetin.[5,6] The majority of the phenolic acids in *Salvia* species are derivatives of caffeic acid, which occurs mainly in dimeric form as rosmarinic acid.[7] As a natural source of flavonoids and polyphenolic compounds, *S. officinalis* possesses potent antioxidant, radical-scavenging, and antibacterial activities.[1] On the other hand, leaves are the source of renewable biomass that could be effectively converted to value-added chemicals, spice, medicine, insecticide, and essential oil.[1,8] Hence, the experiment was planned to study the complete biological efficiency and phytochemical profiling of *S. officinalis* herb chromatographically.

The conventional technique, Soxhlet extraction (SX) with methanol, has been a widely accepted method to extract the maximum bioactive compounds in herbs.[9] Although it is an efficient method for small-scale laboratory extraction, it is time-consuming and not suitable for heat-labile compounds, besides the residual effect of organic solvents. On the other hand, ultrasound extraction for a short period (1–2 h) at low frequencies (20 kHz) increases the yield of alkaloids in herbal extracts, which significantly reduces extraction time and solvent consumption.[10,11] Hence, in the present study, *S. officinalis* leaves were extracted with sonication using water and methanol as solvents, besides SX with methanol. There are few reports on the identification and quantification of phenolic compounds in *S. officinalis* through liquid chromatography-mass spectrometry (LC-MS), with a limitation of distinguishing the compounds only with unit mass resolution.[5,6] Therefore, the study was conducted to identify and quantify the polyphenols in *S. officinalis* leaves extracted through Soxhlet apparatus and sonication, by ultra-high-pressure LC, coupled with electrospray ionization quadrupole time-of-flight MS (UHPLC-ESI-QTOF-MS). The UHPLC gives clearly resolved peaks in less time with higher selectivity and sensitivity, and simultaneously, QTOF-MS identifies multiple targeted and untargeted constituents of the sample based on their exact mass (*m*/z) and fragmentation pattern with high m/z resolution; this technology can even distinguish isobaric compounds by exact mass with different elemental positions.[12,13] On the other hand, gas chromatography with a flame ionization detector (GC-FID) is useful in the analysis of volatile components of the essential oils at trace levels with high sensitivity, stability, and high linear dynamic range.[14,15] Thus, the study also included a GC-FID evaluation of the volatile fraction of essential oil extracted from *S. officinalis* leaves and leaves + stem, mainly to enhance the total biomass content and oil recovery per unit area.

MATERIALS AND METHODS

Plant material

S. officinalis herb was grown organically at the Regenerative Organic Farm, the Maharishi University of Management, Fairfield, Iowa, USA, located at 41°01' Northern latitude, 91°96' Western longitude with an altitude of 238 m above mean sea level. Seeds were started in the greenhouse, and the 6-week-old seedlings were transplanted into the field at 45 cm × 30 cm distance during the 1st week of May. Leaves were harvested in the 2nd week of August, air-dried for 10 days, and powdered for aqueous and methanolic extractions. Leaves along with stem portion leaving 20 cm from the ground were harvested and cut into 2" pieces and air-dried for 15 days for essential oil extraction.

Chemicals

LCMS grade acetonitrile was purchased from Honeywell, Burdick and Jackson, USA, and LCMS grade methanol was purchased from Honeywell, Riedel-de-Haen, USA. LCMS grade formic acid and glacial acetic acid were procured from Merck, Germany. Caffeic acid, rosmarinic acid, carnosic acid, ursolic acid, and luteolin-7-glucoside were purchased from Toronto Research Chemicals, Canada. Carnosol and ^{13}C-caffeic acid were purchased from Cayman Chemical, USA. Ultrapure water produced using a Milli-Q; A10 water purification system (Millipore Sigma, USA) was used throughout the experiment.

Preparation of samples for liquid chromatography

Three extracts were used for LC-MS analysis, Soxhlet methanol extract, and sonic water and methanol extracts.

Soxhlet extraction

10 g of *S. officinalis* leaf powder was extracted in 250 ml LC-MS grade methanol using the Soxhlet apparatus and at 50°C for 6 h or until we get the clear solvent in the extraction chamber. The extraction was vacuum evaporated and the volume was made up to 100 ml, and the extract was then filtered using a 0.2-µ Nalgene filter unit from Thermo Fisher Scientific Inc.

Sonic (ultrasound) extraction with water or methanol

10 g of leaf powder was extracted in 100 ml of distilled water or analytical grade methanol for 2 h with a frequency of 40 kHz in Bransonic-52 ultrasonic bath unit from Branson, USA. The extract was filtered using a 0.2-µ Nalgene filter and stored.

All extractions were made in triplicate and stored at −20°C until chromatographic analysis.

Identification and quantification of polyphenols by ultra-high-performance liquid chromatography coupled with electrospray ionization with quadrupole time-of-flight mass spectrometry

The *S. officinalis* leaf extracts were analyzed using LC coupled with electrospray negative ionization QTOF-MS (UHPLC-ESI-QTOF-MS). The analysis was carried out by reverse-phase UHPLC using a Shimadzu Nexera UHPLC system (Kyoto, Japan) that was directly connected to a QTOF Triple TOF 5600 mass spectrometer (AB SCIEX, Concord, Canada) in direct injection mode. The autosampler (Shimadzu SIL30AC, Kyoto, Japan) was operated in full injection mode filling a 50 µl loop with 10 µl analyte for optimal sample delivery reproducibility. Briefly, after injection, sample mixtures were transferred onto the analytical C_{18} HPLC column (C-18 Kinetex XB, 1 mm ID × 5 cm, 2.6 µm particle size, 100 Å pore size, Phenomenex, CA, USA) and eluted at a flow rate of 250 µl/min. Pumps (Shimadzu LC30AD, Kyoto, Japan) were operated in the following multi-step linear gradient with different proportions of mobile phase B: 0 min, 10% B; 10 min, 90% B; 12.5 min, 90% B; 15 min, 10% B; and 20 min, 10% B, with a total runtime of 20 min, including mobile phase equilibration. Mobile phases A and B were 0.1% of acetic acid made in Milli-Q water and acetonitrile, respectively. Column oven (Shimadzu CTO30A, Kyoto, Japan) was set to 40°C.

Data-independent acquisitions, MS/MS^{ALL} with SWATH® acquisitions

Mass spectra and tandem mass spectra data were recorded in ESI "negative ion" and "high sensitivity" mode, with a resolution of ~35,000 full-width half-maximum on the Triple TOF 5600. The ion spray needle voltage was at −4500 V with drying gas temperature of 600°C; ion source Gas 1 (nebulizer) and Gas 2 (heater) values were 50 psi each; curtain gas was 35 psi. The collision-energy values for TOF MS were 5 eV and for MS/MS experiments was 25 eV, with a spread of 15eV. The sample ions were fragmented using collision gas and nitrogen, and

the mass range for QTOF-MS was fixed to ± 1 m/z. In the SWATH-MS2 acquisition, a variable SWATH window was used to cover the mass range of m/z 50–1000 in 16 segments (15 ms × 48.5 ms), yielding a cycle time of 0.8268 s, which includes one 50 ms MS1 scan. SWATH-MS2 generates multiple MS/MS spectra, a combination of all the analytes in the selected Q1 m/z window. During the execution of the LC method, the mass spectrometer was externally calibrated using a known mixture of masses from Sciex (P/N 4460134, AB SCIEX, Concord, Canada). The mixture was injected at the beginning of each run, and all the spectra were calibrated before compound identification.

Quantitative analysis was performed by diluting the extracted samples with 0.1% formic acid (1/10–1/10,000) to quantify the samples within the linearity range of standard calibration curve, avoiding MS signal saturation. Subsequently, the diluted samples were centrifuged at 14,000 rpm for 15 min, and further, the supernatant added with 25 µl of the internal standard mixture (^{13}C caffeic acid/diclofenac/chloramphenicol). All samples were extracted and analyzed in triplicates. The standard calibration curves (6–250 ng/ml) for caffeic acid (y = 0.00485x + 0.00658; R^2 = 0.9983), rosmarinic acid (y = 0.03863x + 0.06813; R^2 = 9984), carnosol (y = 0.05899x − 0.08327; R^2 = 9924), and luteolin-7-O glucoside (y = 0.03269x + 0.05975; R^2 = 9975) were constructed for quantification of those compounds. The calibration curve for carnosic acid (y = 5.26301e-5x^2 + 0.00373x + 0.12615; R^2 = 9971) and ursolic acid (y = 0.01283x + 0.25563; R^2 = 9944) were created at higher concentration (24–1000 ng/ml) as the MS signal fails to record sensitivity at lower levels. All samples were extracted and analyzed in triplicates. Unknown polyphenolic compounds and flavonoids were identified based on their accurate mass (*m*/z) and molecular (*m*/z) ion fragmentation using Peak View Software (ver. 2.2, AB SCIEX, Concord, Canada), Master View, Library View (AB SCIEX, Concord, Canada), National Institutes of Standards and Technology (NIST), and AOI database.

Essential oil extraction

200 g of air-dried *S. officinalis* herb was steam distilled for 120 min at 100°C with a small-scale Clevenger-type apparatus; however, maximum oil recovery was within 90 min, and after that, there was no oil yield. Two different kinds of essential oils were extracted: (1) *S. officinalis* leaves along with the stem and (2) *S. officinalis* leaves alone, discarding the stem portion. Steam distilled oil samples were dried on anhydrous sodium sulfate salt and filtered through Whatman grade 5 cellulose filter paper. Filtered oil was stored in amber color glass bottles in a cool, dark place before chemical analysis. All the extractions were carried out in triplicates. Further, the essential oil samples were diluted with *n*-hexane (Millipore-Merck KGaA, Germany) before chromatographic analysis.

Essential oil analysis of *Salvia officinalis*

Essential oil samples of *S. officinalis* were analyzed using GC-FID from Agilent Technology Hewlett Packard 6890 series (USA) connected to DB-5 column coated with 5% phenyl methyl siloxane (HP-5) having 30 m × 0.25 mm × 0.25 µm dimension used for the analysis. The injection volume of 0.2 µl diluted essential oil was injected into the capillary GC column. The FID and the injector were maintained at 325°C and 250°C, respectively. Hydrogen was used as the carrier gas, the flow rate through the column was 23.9 ml/min, and the split ratio was set to 20:1. The column oven temperature was maintained at 56°C initially and then raised to 250°C at the rate of 3.1°C/min. The run time for each sample was 75 min. Identification of volatile compounds was based on the internal laboratory database developed using authentic compounds as well as pure essential oil samples and Adams data library as a retention time and retention index reference for the DB-5 column using Agilent Chem Station software.[16]

Statistical analysis

The results of polyphenol quantification were expressed as mean ± standard deviation; the data were analyzed statistically using single-factor ANOVA in MS Excel software. The critical difference at 5% level of significance or Tukey's honestly significant difference test (at $P < 0.05$) was used to compare the significant difference between the treatments.[17]

RESULTS AND DISCUSSION

Quantitative analysis of *Salvia officinalis* leaf extracts

Phenolic compounds such as caffeic acid, carnosic acid, ursolic acid, rosmarinic acid, carnosol, and luteolin-7-O glucoside were quantified using calibration curves for each of their respective reference standards. Concentrations of phenolic compounds (µg/g) in different extracts of *S. officinalis* analyzed through UHPLC-ESI-QTOF-MS are presented in Table 1. All three extraction methods, Soxhlet, sonic extraction with water, and methanol, recorded substantial concentrations of phenolic compounds in *S. officinalis*. Among the extraction methods, SX yielded significantly higher concentrations of caffeic acid (92.45 ± 1.92 µg/g), rosmarinic acid (18821.33 ± 150.20 µg/g), luteolin-7-glucoside (635.13 ± 11.20 µg/g), carnosic acid (27.48 ± 2.37 µg/g), carnosol (1347.67 ± 30.04 µg/g), and ursolic acid (14938.67 ± 82.20 µg/g). Hot continuous extraction or SX combined with methanol solvent might enhance the solubility of polyphenols, flavonoids, anthocyanins, and other bioactive compounds present in herbs, maximizing the extraction of phenolic compounds.[18] There were only a few studies reported regarding polyphenol analysis and quantification in *S. officinalis*.[19-22] Hamrouni-Sellami *et al*. studied the phenolic contents in methanol extracts of *S. officinalis* grown in Tunisia through reverse-phase HPLC and reported much less concentration of phenolic acids, caffeic acid (44.37 µg/g), rosmarinic acid (110.6 µg/g), luteolin 8.11 µg/g), and carnosol (2.07 µg/g) as compared to the present study.[23]

Ultrasound extraction in methanol exhibited significantly higher concentrations of carnosic acid, ursolic acid, rosmarinic acid, carnosol, and luteolin-7-O glucoside than the aqueous sonicated samples. Ultrasound is known to disrupt plant cell walls, thereby facilitating the release of extractable compounds and enhancing mass transport of solvent from the continuous phase into plant cells, and this effect boosts recovery of polyphenols, especially when optimal solvent, such as methanol, is used.[11,24] *S. officinalis* leaf extracted by sonication using water as solvent yielded the lowest phenolic acid and flavonoid content, compared to sonication and SX with methanol. Phenolic compounds are known to form complex molecules that are insoluble in an aqueous base.[25] Ultrasonically assisted solvent extraction reported to be more efficient, and ultrasonic extraction in water-based media was compared with herbal decoction process.[11] Hence, in the current study, the efficacy of methanol sonic extraction was more than the aqueous extraction. In the present investigation, the caffeic acid concentration in sonicated methanol (SM) and aqueous extracts (SW) was similar, which likely reflects the solubility of caffeic acid in both water and methanol. Lower rosmarinic acid levels in aqueous *S. officinalis* extracts were reported by Kontogianni *et al*. while working with different solvents for the herb extraction process.[26] The significantly higher concentrations of rosmarinic and ursolic acid [10.8–18.8 mg/g and 1.1–14.9 mg/g, respectively, Table 1], measured in the methanolic extracts prepared in this study, demonstrate that *S. officinalis* is a high-quality source of these compounds, which are useful for treatment of an array of diseases, including gastrointestinal inflammation, colitis, colon cancer, and nervous system inflammation.[27]

Qualitative analysis of *Salvia officinalis* leaf extracts

Most of the qualitative analysis of phenolic compounds in garden sage was reported either by HPLC or high performance liquid chromatography with diode array detector (HPLC-DAD) based on their retention time, ultraviolet-visible, and mass spectra.[19,23] The high resolution, accurate mass, UHPLC-ESI-QTOF-MS analysis used in this study facilitated even more in-depth identification and characterization of known and previously unknown compounds based on their molecular formulae, exact mass measurements, and MS/MS fragmentation patterns.[28] In the present study, negative ionization mode $[M-H]^-$ was used because it was reported to be more sensitive for analysis of phenolic acids and flavonoids as compared to positive ionization mode.[28-30] Several polyphenolic compounds present in *S. officinalis* leaf extracts have been identified using UHPLC-ESI-QTOF-MS under negative ESI conditions $[M-H]^-$ along with retention time, molecular weight, and mass (*m*/z) fragmentation pattern. These results are presented in Table 2. The compounds without reference standards were identified tentatively by comparing the mass spectra data, ion fragmentation, and molecular weight (*m*/z) with data available in the literature as well as mass spectral library obtained from the NIST.[19,31]

As shown in Table 2, 43 different phenolic compounds have been identified, including phenolic acids, flavonoids, and terpenoids in three extracts of *S. officinalis.* All three extracts contained various polyphenols, and among them, two flavonoid compounds, diosmetin ($[M-H]^-$ m/z 299.05) and pectolinarigenin ($[M-H]^-$ m/z 313.07), have been identified for the first time in *S. officinalis* leaf extracts that we have prepared. Diosmetin and pectolinarigenin, previously found in other herbs, are reported to have potent anti-inflammatory and anticancer properties.[32,33] The two new triterpenoids, benthamic acid ($[M-H]^-$ m/z 471.34) and micromeric acid ($[M-H]^-$ m/z 453.33), are also reported here for the first time to be detected by QTOF-MS in *S. officinalis* leaves.

Phenolic acids, namely caffeic acid ($[M-H]^-$ m/z 179.04), ferulic acid ($[M-H]^-$ m/z 193.05), rosmarinic acid ($[M-H]^-$ m/z 359.08), and sagerinic acid ($[M-H]^-$ m/z 719.16), were detected in all three *S. officinalis* extracts. Danshensu ($[M-H]^-$ at m/z 197.05) and scutellarin ($[M-H]^-$ at m/z 461.07) were identified only in sonic extracts with methanol. These identities were confirmed as danshensu by comparison with the m/z ion fragmentation pattern (135.0450, 123.0450, 72.9947, and 179.0349) in the NIST MS library and previous literature data.[22] Danshensu ($[M-H]^-$ at m/z 197.05), caffeic acid 3-glucoside ($[M-H]^-$ at m/z 341.09), ferulic acid ($[M-H]^-$ at m/z 193.95), rosmarinic acid ($[M-H]^-$ at m/z 359.08), and methyl rosmarinate ($[M-H]^-$ at m/z 373.09), all shared many of the same MS/MS (*m*/z) ion fragments (179.03), since they are all structurally related to caffeic acid. Many phenolic acids of *Salvia* species were previously reported to be caffeic acid derivatives, mostly formed by esterification of caffeic acid with danshensu.[34] The phenolic acids derived from caffeic acid were also extensively reported in polyphenol studies of *S. officinalis*.[5,22,35] Similarly, sagerinic acid ($[M-H]^-$ m/z 719.16) and salvianolic acid L ($[M-H]^-$ m/z 717.14) were also found to share similar (*m*/z) MS/MS ion fragments (359.07) as they derive from rosmarinic acid ($[M-H]^-$ m/z 359.08). Lu and Foo reported sagerinic acid and salvianolic acid to be dimers of rosmarinic acid with a potent phenolic antioxidant activity in *S. officinalis*.[36]

Methanol was found to be highly efficient in extracting the polyphenols, flavonoids, phenolic acids, and terpenoid compounds from *S. officinalis* leaves than aqueous extraction. The important flavonoids present in all the extracts of *S. officinalis* were gallocatechin ($[M-H]^-$ m/z 305.07), luteolin-7-O-glucoside ($[M-H]^-$ m/z 447.09), isorhamnetin-3-glucoside ($[M-H]^-$ m/z 477.10), hispidulin glucuronide ($[M-H]^-$ m/z 175.09), apigenin-7-glucuronide ($[M-H]^-$ m/z 445.08), and homoplantaginin ($[M-H]^-$ m/z 461.11). However, apigenin, diosmetin, and pectolinarigenin flavonoids were identified only in methanolic samples. Phenolic diterpenoids such as rosmanol, rosmadial, carnosol, and carnosic acids were also identified in all the *S. officinalis* extracts. However, the phenolic triterpenoid compounds such as asiatic acid, benthamic acid, micromeric acid, and ursolic acids were detected only in methanolic samples. A triterpenoid compound, betulinic acid

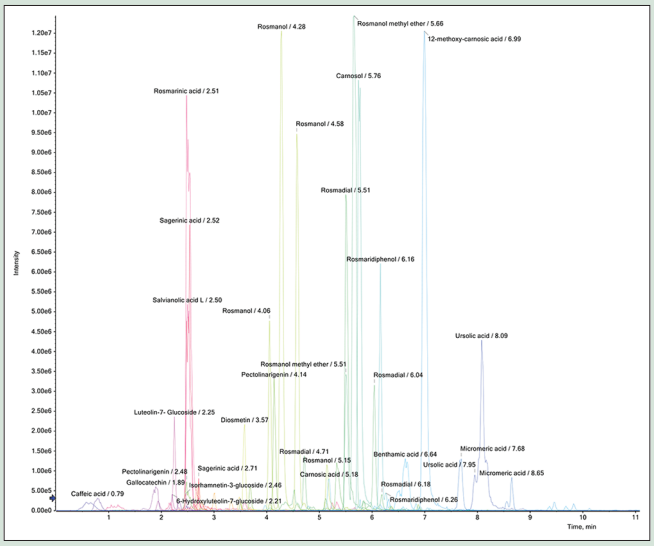

Figure 1: The relative abundance of phenolic compounds in sage leaves - Soxhlet extraction analyzed through ultra-high-performance liquid chromatography coupled electrospray ionization quadrupole time-of-flight mass spectrometry (intensity of phenolic compounds vs. elution time)

Table 1: Concentrations of phenolic compounds (µg/g) in different extraction of *Salvia officinalis* analyzed through ultra-high-pressure liquid chromatography, electrospray ionization, coupled with quadrupole time-of-flight mass spectrometry

	Polyphenol content (µg/g) in sage					
	Caffeic acid	Rosmarinic acid	Luteolon-7-glucoside	Carnosic acid	Carnosol	Ursolic acid
Extract						
Soxhlet	92.45±1.92^b	18,821.33±150.20^c	635.13±11.20^c	27.48±2.37^c	1347.67±30.04^c	14,938.67±82.20^c
Sonication-water	73.24±1.62^a	67.41±2.03^a	53.25±3.66^a	1.79±0.38^a	6.10±0.60^a	0.72±0.04^a
Sonication-methanol	73.86±1.84^a	10,874.67±53.45^b	304.27±6.60^b	12.68±1.10^b	537.60±17.20^b	1098.67±16.23^b
Mean	79.85	9921.14	330.88	13.98	630.45	5346.02
F test	**	**	**	**	**	**
SEM±	1.04	53.15	4.50	0.88	11.54	27.93
CD at 5%	3.59	183.92	15.58	3.04	39.93	96.65

**Significant at 5% level, values followed by different letters indicate a significant difference between the treatments at $P<0.05$. SEM: Standard error of the mean; CD: Critical difference

Table 2: Polyphenolic compounds in different *Salvia officinalis* leaf extracts identified by liquid chromatography tandem-mass spectrometry (ultra-high-pressure liquid chromatography, electrospray ionization coupled with quadrupole time-of-flight mass spectrometry)

Compound	Formula	Mass $[M-H]^-$ (*m/z*)	RT (min)	MS2 (*m/z*) fragments	SX	SW	SM
Danshensu	$C_9H_{10}O_5$	197.05	0.50	135.0450, 123.0450, 72.9947, 179.0349	–	–	+
Caffeic acid 3-glucoside	$C_{15}H_{18}O_9$	341.09	0.60	179.0339, 221.0449, 135.0445, 251.0564, 281.0667	+	+	+
Caffeic acid	$C_9H_8O_4$	179.04	0.79	135.0445, 134.0370	+	+	+
Luteolin-7-O-rutinoside	$C_{27}H_{30}O_{15}$	593.15	1.47	285.0431	–	+	–
Ferulic acid	$C_{10}H_{10}O_4$	193.05	1.65	134.0372, 179.0273	+	+	+
Gallocatechin	$C_{15}H_{14}O_7$	305.07	1.85	225.1126, 96.9597	+	+	+
6-Hydroxyluteolin -7-O-glucuronide	$C_{21}H_{18}O_{13}$	477.07	2.03	301.0328	–	+	+
6-Hydroxyluteolin-7-glucoside	$C_{21}H_{20}O_{12}$	463.09	2.21	301.0338	+	–	+
Luteolin-7-glucoside	$C_{21}H_{20}O_{11}$	447.09	2.25	285.0403	+	+	+
Scutellarin	$C_{21}H_{18}O_{12}$	461.07	2.31	285.0371, 113.0241, 175.0215	–	–	+
Isorhamnetin-3-glucoside	$C_{22}H_{22}O_{12}$	477.10	2.46	315.0709	+	+	+
Apigenin-7-O-glucoside	$C_{21}H_{20}O_{10}$	431.10	2.49	269.0825, 355.0719	+	–	–
Salvianolic acid L	$C_{36}H_{30}O_{16}$	717.14	2.50	359.0745, 179.0332	+	–	–
Rosmarinic acid	$C_{18}H_{16}O_8$	359.08	2.51	197.0380, 161.0816, 179.0282	+	+	+
Sagerinic acid	$C_{36}H_{32}O_{16}$	719.16	2.52	359.0719	+	+	+
Hispidulin glucuronide	$C_{22}H_{20}O_{12}$	475.09	2.72	299.0533, 284.0312	+	+	+
Apigenin-7-glucunoride	$C_{21}H_{18}O_{11}$	445.08	2.61	269.0445	+	+	+
Homoplantaginin	$C_{22}H_{22}O_{11}$	461.11	2.68	283.0233, 298.0464	+	+	+
Trihydroxy flavone	$C_{15}H_{10}O_5$	269.05	2.88	117.0349	–	+	–
Hispidulin	$C_{16}H_{12}O_6$	299.06	2.96	284.0323	–	+	–
Methyl rosmarinate	$C_{19}H_{18}O_8$	373.09	3.02	135.0450, 175.0400, 179.0362, 197.0450	+	–	+
Apigenin	$C_{15}H_{10}O_5$	269.05	3.50	117.0345	+	–	+
Diosmetin	$C_{16}H_{12}O_6$	299.06	3.57	285.0334, 136.9871	+	–	+
2,3,4,4a, 10,10a-Hexahidro-5,6-dihydroxy-1, 1-dimethyl-7-(1-methylethyl)-9 (1H)-Phenantrenone	$C_{19}H_{26}O_3$	301.18	4.02	283.1696	+	–	–
Rosmanol	$C_{20}H_{26}O_5$	345.17	4.06	301.1779, 183.1668	+	–	+
3,7-Dihydroxy-3-4-dimethoxyflavone	$C_{17}H_{14}O_6$	313.07	4.15	298.0472, 283.0239, 255.0296	–	+	–
Pectolinarigenin	$C_{17}H_{14}O_6$	313.07	4.15	283.0249, 298.0496, 117.0347, 163.0038, 183.0451, 227.0354	+	–	+
Epirosmanol	$C_{20}H_{26}O_5$	345.17	4.28	283.1709	+	+	+
Genkwanin	$C_{16}H_{12}O_5$	283.06	4.54	268.0374, 117.0342, 240.0428	+	–	+
Epiisorosmanol	$C_{20}H_{26}O_5$	345.17	4.58	283.1673	+	+	+
5,6,7,10- tetrahydro-7-hydroxy rosmariquinone derivative	$C_{20}H_{26}O_5$	345.17	5.11	301.1797	+	–	+
Carnosic acid	$C_{20}H_{28}O_4$	331.19	5.18	287.2004	+	–	+
Asiatic acid	$C_{30}H_{48}O_5$	487.34	5.35	407.3317, 425.3443	+	–	+
Rosmadial	$C_{20}H_{24}O_5$	343.15	5.51	299.1650, 243.1026	+	+	+
Rosmanol methyl ether	$C_{21}H_{28}O_5$	359.18	5.67	284.1696, 283.1675, 300.1712, 329.1723	+	–	+
Carnosol	$C_{20}H_{26}O_4$	329.18	5.76	286.1850, 285. 1826	+	+	+
Rosmadial isomer	$C_{20}H_{24}O_5$	343.15	6.04	299.1596	+	+	+
Rosmaridiphenol	$C_{20}H_{28}O_3$	315.20	6.16	286.1871, 285.6091	+	–	+
Benthamic acid	$C_{30}H_{48}O_4$	471.35	6.64	-	+	–	+
12-methoxy-carnosic acid	$C_{21}H_{30}O_4$	345.21	6.99	301.2167, 287.1962	+	+	+
Micromeric acid	$C_{30}H_{46}O_3$	453.34	7.68	-	+	–	+
Betulinic acid	$C_{30}H_{48}O_3$	455.35	7.95	-	+	–	–
Ursolic acid	$C_{30}H_{48}O_3$	455.35	8.09	456.3635	+	–	+

SX: Soxhlet; SW: Sonication with water; SM: Sonication with methanol; +: Detected in the sample; –: Not detected in the sample; RT: Retention time

with a pseudomolecular weight $[M-H]^-$ at m/z 455.35, was detected only in Soxhlet-extracted samples of *S. officinalis*. Even though ursolic acid and betulinic acid have the same pseudomolecular weight ($[M-H]^-$ at m/z 455.35), the former was identified through the reference standard while the later molecule was confirmed by comparison to the NIST mass spectral library.

The relative abundance of identified phenolic compounds in SX, sonication with water, and methanol is presented in Figures 1-3, respectively, reflecting the relative efficiencies of these methods for recovery of various compounds from *S. officinalis*. In SX, besides rosmarinic acid, rosmanol, rosmanol methyl ether, rosmadial, 12-methoxy carnosic acid, sagerinic acid, and salvianolic acid L. were also found in abundance [Figure 1]. The high intensity of pentacyclic triterpenoids, ursolic acid, benthamic acid, and micromeric acid, was recorded in methanolic extracts of *S. officinalis*. Presence of more than one peak corresponding to the same molecular mass but different elution times was due to the presence of isomers, such as rosmanol, rosmadial, sagerinic acid, and pectolinarigenin. Rosmanol ($[M-H]^-$ m/z 345.17) recorded three peaks at 4.06, 4.28, and 4.58 min, representing rosmanol (MS^2 m/z fragments 301.1779, 183.1668), epirosmanol (MS^2 m/z fragment 283.17), and epiisorosmanol (MS^2 m/z fragment 283.17), respectively [Figures 1 and 3]. There were two clear peaks observed for the rosmadial molecule ($[M-H]^-$ m/z 343.15) at 5.51 min (MS^2 m/z 299.1650, 243.1026) and 6.04 min (MS^2 m/z 299.1596) with similar fragmentation patterns characteristic of this molecule. Similar results were obtained during the

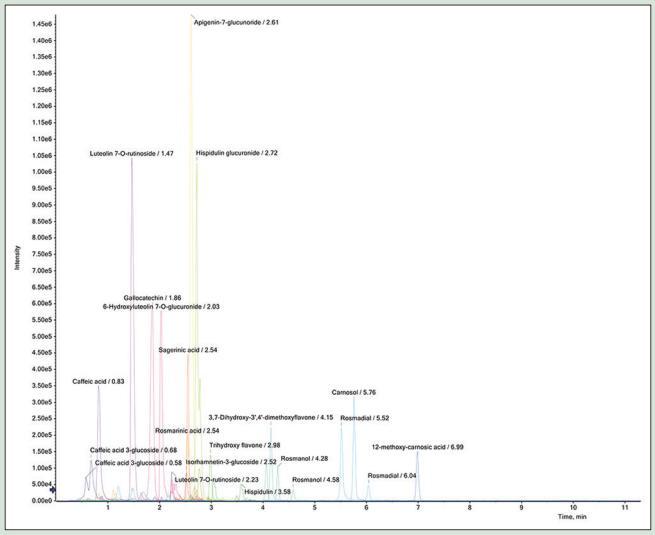

Figure 2: The intensity of phenolic compounds in sage leaves extracted by sonication with water, analyzed through ultra-high-performance liquid chromatography coupled electrospray ionization quadrupole time-of-flight mass spectrometry (intensity vs. elution time)

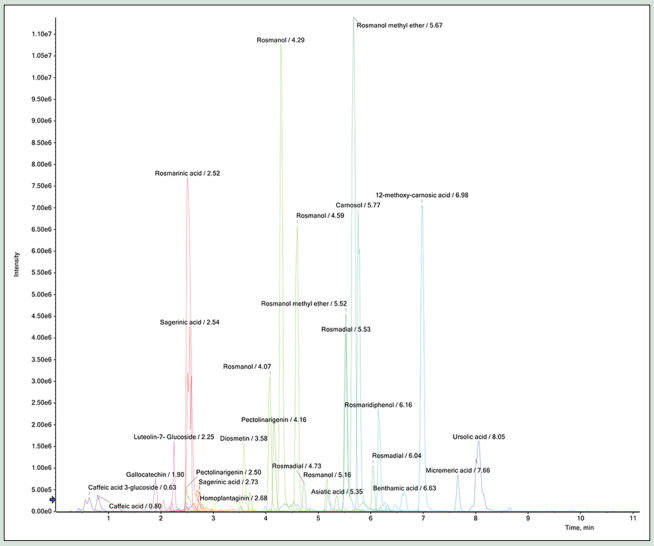

Figure 3: The concentration of phenolic compounds in sage leaves extracted by sonication with methanol, analyzed through ultra-high-performance liquid chromatography coupled electrospray ionization quadrupole time-of-flight mass spectrometry (intensity vs. elution time)

chromatographic determination of polyphenols in aqueous infusions of sage tea, where Zimmermann *et al*. observed 4–5 peaks of rosmanol and carnosol with similar m/z fragmentation patterns.[22]

The chromatogram pattern of Soxhlet-extracted samples and SM samples were similar, and the relative abundance of phenolic compounds in both extracts mostly matches with each other, even though the intensity of phenolic compounds in SX was higher [Figures 1 and 3]. There was a similar number of phenolic compounds detected in methanol extracts of *S. officinalis* produced by both Soxhlet and ultrasound procedures. Exposure of samples to high temperatures for an extended period using an optimal solvent, such as methanol, in SX and the acoustic cavitation effect of ultrasound, both increase the permeability of cell walls and enhance the release of compounds in the extract.[10,37] However, it was clear that yields with SX were higher as compared to sonicated extracts. This might be due to increasing extraction efficiency with an increase in time and solvent volume.[38] Even though the SX yielded maximum polyphenols, the extraction process took a long time and consumed a large volume of methanol.

Analysis of *S. officinalis* sonication extract in water revealed high concentrations of conjugated polyphenols, including apigenin-7-glucuronide, hispidulin glucuronide, luteolin 7-O-rutinoside, 6-hydroxyleteolin 7-O-glucuronide, and sagerinic acid [Figure 2]. These were present at proportionately higher levels in the aqueous extracts, presumably because conjugation increases the water solubility of polyphenols. In general, the same phenolic compounds were present in the aqueous extracts as those found in methanol extracts; however, the relative amounts of the compounds were different, probably due to differences in solubility in water versus methanol. Further, in general, the levels of phenolic compounds were substantially lower in aqueous, as compared to methanol extracts. The higher solubility of complex phenolic compounds in organic solvents has been previously noted.[25,39,40]

High-resolution and accurate mass LC-MS/MS chromatograms contain comprehensive information of all molecules present in the sample that are amenable to the ionization technique and polarity used. We used mass spectral library searching used to identify non-targeted compounds based on their mass spectral fragmentation pattern. This strategy of identifying compounds based on their molecular fragmentation fingerprint is very useful and very powerful but is limited to the compounds present in the MS/MS libraries available. Using this methodology, we found that the three different *S. officinalis* extracts that we prepared contained a vast number of polyphenols, of which only a relative few are present in currently available mass spectral libraries and therefore capable of being named in the present study. Thus, there are many yet-unidentified phenolic compounds observed in the mass spectral datasets of the *S. officinalis* extracts we generated using the SWATH untargeted analytical protocol. Indeed, more compounds were detected but remain unidentified than were identified based on the existing mass spectral libraries. As additional mass spectral libraries become available, the datasets that we have generated and others can be analyzed further to identify and characterize many more additional polyphenols based on m/z fragmentation patterns.

Essential oil profiling through gas chromatography coupled with flame ionization detector

Steam distillation of *S. officinalis* leaves yielded 1.30% of essential oil and distillation of leaves along with stem yielded 1.25% of oil on a dry weight basis. The fresh-to-dry conversion ratio of the herb was 33%. As per the available literature, essential oil content in *S. officinalis* has been found to vary from 1.1% to 2.8% depending on the cultivar, soil, and the weather conditions during the crop growth.[41,42]

Analysis of volatile components present in the essential oil by GC-FID is presented in Table 3. The analysis resulted in the separation of 38 volatile terpenoid compounds accounting for 99.47% and 99.49% of the oil composition extracted from *S. officinalis* leaves + stem and leaves, respectively. Oxygenated monoterpene content was slightly higher (71.78%) in leaves + stem oil as compared to leaf oil (69.43%), especially monoterpene ketones. Essential oils with a high level of monoterpene ketones have been reported to exhibit strong antioxidant activity.[43] On the other hand, *S. officinalis* leaf oil was found to have more sesquiterpenoids and diterpenoid content, indicating the presence of higher molecular weight components that make the oil more stable with an enduring flavor. The mirror image of GC-FID analysis of

Table 3: Volatile terpenoid compounds in *Salvia officinalis* essential oil extracted from leaves + stem and leaves analyzed through gas chromatography with a flame ionization detector

Compound	RT	RI	Formula	Area %	
				Leaves + stem	Leaves
cis-Salvene	4.16	847	C_9H_{16}	0.62	0.73
Trans-salvene	4.35	858	C_9H_{16}	0.09	0.11
Tricyclene	5.76	921	$C_{10}H_{16}$	0.13	0.14
α-thujene	5.87	924	$C_{10}H_{16}$	0.31	0.25
α-pinene	6.07	932	$C_{10}H_{16}$	2.34	3.30
Camphene	6.48	946	$C_{10}H_{16}$	4.29	5.10
Sabinene	7.19	969	$C_{10}H_{16}$	0.36	0.19
β-Pinene	7.30	974	$C_{10}H_{16}$	2.55	1.91
Myrcene	7.71	988	$C_{10}H_{16}$	1.30	1.37
α-Phellandrene	8.15	1002	$C_{10}H_{16}$	0.05	0.06
α-Terpinene	8.56	1014	$C_{10}H_{16}$	0.15	0.20
p-Cymene	8.82	1020	$C_{10}H_{14}$	0.39	0.36
d-limonene	8.98	1024	$C_{10}H_{16}$	1.77	2.28
1,8 cineol	9.06	1026	$C_{10}H_{18}O$	5.45	6.21
cis-β ocimene	9.29	1044	$C_{10}H_{16}$	0.07	0.06
γ-Terpinene	10.04	1054	$C_{10}H_{16}$	0.38	0.44
cis-Sabinene hydrate	10.34	1065	$C_{10}H_{18}O$	0.20	0.14
Terpinolene	11.14	1086	$C_{10}H_{16}$	0.36	0.45
Linalool	11.65	1095	$C_{10}H_{18}O$	0.27	0.32
α-Thujone	11.87	1101	$C_{10}H_{16}O$	38.93	34.43
β-Thujone	12.23	1112	$C_{10}H_{16}O$	7.58	6.03
Trans-sabinol	13.16	1137	$C_{10}H_{16}O$	0.12	0.15
Camphor	13.31	1141	$C_{10}H_{16}O$	15.77	18.12
Neo-iso-3-thujanol	13.91	1147	$C_{10}H_{18}O$	0.15	0.06
Borneol	14.13	1165	$C_{10}H_{18}O$	1.76	2.13
terpin-4-ol	14.60	1174	$C_{10}H_{18}O$	0.28	0.26
α-Terpineol	15.14	1186	$C_{10}H_{18}O$	0.10	0.13
Myrtenol	15.37	1194	$C_{10}H_{16}O$	0.15	0.11
Bornyl acetate	18.98	1284	$C_{12}H_{20}O_2$	0.78	1.09
3-Thujanol acetate	19.27	1295	$C_{12}H_{20}O_2$	0.24	0.25
β-Caryophyllene	24.23	1417	$C_{15}H_{24}$	3.67	3.20
Aromadendrene	24.88	1439	$C_{15}H_{24}$	0.10	0.19
α-Humulene	25.53	1452	$C_{15}H_{24}$	5.21	5.20
Germacrene A	27.09	1508	$C_{15}H_{24}$	0.12	0.14
Caryophyllene oxide	30.24	1582	$C_{15}H_{24}O$	0.27	0.29
Viridiflorol	30.58	1592	$C_{15}H_{26}O$	2.17	2.42
Humulene epoxide II	31.18	1608	$C_{15}H_{24}O$	0.38	0.56
Manool oxide	45.32	1987	$C_{20}H_{34}O$	0.61	1.11
Identification of total components				99.47	99.49
Normonoterpenes				0.71	0.84
Monoterpenes				14.45	16.11
Oxygenated monoterpenes				71.78	69.43
Monoterpene alcohols				8.48	9.51
Monoterpene ketones				62.28	58.58
Monoterpene esters				1.02	1.34
Sesquiterpenes				11.92	12.00
Diterpenes				0.61	1.11

RT: Retention time; RI: Retention indices for DB-5 column

S. officinalis essential oil from leaves + stem and only from leaves with the relative abundance of volatile compounds against retention time is depicted in Figure 4. Both sage leaves + stem and leaf essential oil were characterized by high α-thujone (34.43%–38.93%), β-thujone (6.03%–7.58%), camphor (15.77%–18.12%), 1,8-cineole (5.45%–6.21%), α-humulene (5.20%), and camphene (4.29%–5.10%). A similar composition with elevated thujone levels was reported in sage grown in Poland and Brazil.[8,42,44] However, some essential oil of sage from Egypt and Tunisia also recorded higher camphor content (23%–26%), almost equal to α-thujone content.[45,46]

Even though the oil content from leaves and leaves + stem was almost same, the oil yield per unit area was more in leaves + stem. However, as compared to *S. officinalis* leaves + stem oil, leaf oil was found to be safer and of finer quality as the later contained less thujone. α-thujone reported to be toxic on the brain, liver, and kidney cells and might cause convulsions by consumption of sage essential oils rich in thujone content.[47,48] The comparatively high concentration of toxic thujones seems to be characteristic of sage leaves cultivated in different locations as well.[49,50] Hence, the leaf oil could be used for therapeutic purpose whereas leaves + stem oil might be used as an effective insecticide. Studies have also shown that therapeutic properties sage depends on camphor, 1,8-cineole, α-thujone, and β-thujone content. The essential oil of *S. officinalis* analyzed in the present study also recorded high camphor and 1,8-cineole, which are known to enhance radical scavenging activities of essential.[51] The essential oil profile of *S. officinalis* defined by the ISO 9909 was as follows; α-thujone (18%–43%), β-thujone (3%–8.5%),

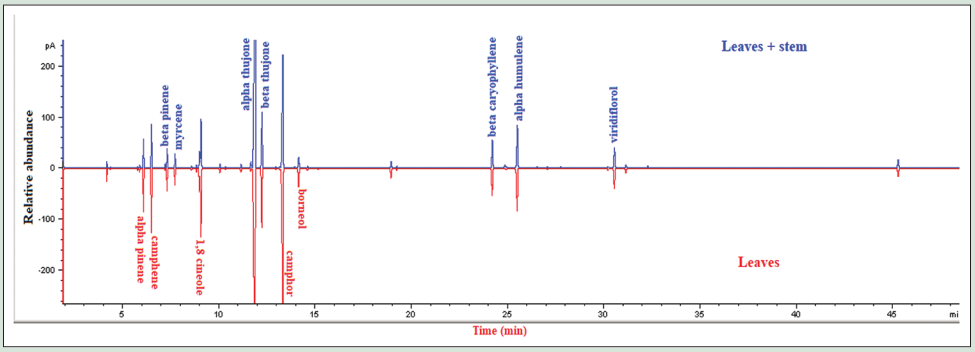

Figure 4: The analysis of *Salvia officinalis* essential oil from leaves + stem and only from leaves, the mirror image of gas chromatography with a flame ionization detector analysis with the relative abundance of volatile compounds against retention time

camphor (4.5%–24.5%), 1,8-cineole (5.5%–13%), α-humulene (0%–12%), camphene (1.5%–7%), and α-pinene (1%–6.5%).[52] The chromatographic analyses of our extracts were consistent with these values, indicating that oils produced using the variety of *S. officinalis* and the procedures which we used would be suitable for international trade.

CONCLUSION

The Soxhlet method was more efficient in extracting phenolic compounds as compared to sonic extraction, and of the three methods compared, SX can be considered the best extraction method for the preparation of phenolic extracts from *S. officinalis*. However, sonic extraction of *S. officinalis* in methanol was comparable to SX concerning intensity and diversity of phenolic acids and flavonoids. Hence, sonic extraction at low temperatures with significantly less time and solvent consumption was found to be suitable for large-scale preparation of phenolic compounds. UHPLC-ESI-QTOF-MS methodology for the analysis proved to be very efficient in the identification and characterization of targeted and untargeted phenolic compounds present in the *S. officinalis* extract. However, there is substantial scope to investigate more deeply a large number of yet-to-be-unidentified phenolic compounds present in *S. officinalis*. The lower temperature, more gentle sonic extraction may be found advantageous for the efficient recovery of a larger number of novel compounds. Essential oil profiling through GC-FID in the present study revealed the presence of 38 different terpenoid compounds. It would be fruitful to characterize *S. officinalis* essential oil by mass chromatographic techniques in the future.

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Conflicts of interest

There are no conflicts of interest.

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