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RESEARCH ARTICLE

Development and Validation of a Rapid and Efficient Method for Simultaneous Determination of Scopolin and Scopoletin in *Convolvulus* Species by Ultra-high-performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: ***Background*:** *Convolvulus* species are extensively used in traditional medicines for the treatment of peptic ulcer diseases primarily associated with the presence of many bioactive compounds, such as coumarins.

***Methods*:** A validation and improvement of a rapid, sensitive and simple technique for bioactive compounds analysis of scopolin and scopoletin in *Convolvulus pilosellifolius (CP)* and *Convolvulus austroaegyptiacus (CA)* plant extracts using UHPLC-MS/MS were performed. Both plants extracts were subjected to high-resolution DART-ToF-MS technique for fast profiling of their constituents.

***Results*:** DART-ToF-MS spectra proved the presence of experimental mass at 193.05137 and 355.10371 *m/z* in the extract of *CA* plant and at 193.05084 and 355.10365 *m/z* in *CP* plant extract for scopolin and scopoletin compounds, respectively. The total chromatographic analysis time was less than 10 min with excellent resolution and negligible matrix effect. The validated parameter showed good linearity ($R^2 \geq 0.998$) over a wide concentration range of 0.05-10.0 μg mL^{-1} for both scopolin and scopoletin, detection limits of scopolin and scopoletin were 0.03 and 0.01 μg mL^{-1}, respectively. The method also showed good intraday and interday precisions (RSD<4.33%). The recovery percentage values were between 97.04 and 99.97% at three added concentration limits. The highest content of scopolin (3.485 mg g^{-1}) and scopoletin (0.795 mg g^{-1}) was detected in *CA* ethanolic extract. The studied plants were finally compared with all previous reports in terms of scopolin and scopoletin content.

***Conclusion*:** The results indicate that the suggested method can be used for regular analysis of active compounds in medicinal plant extracts.

ARTICLE HISTORY

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1. INTRODUCTION

Plant extracts are one of the most attractive sources for developing new drugs [1, 2]. Nowadays, the crude extracts from medical plants and their species are commonly used in the development and preparation of alternative traditional medicine and food additives [3-5].

Convolvulus austroaegyptiacus (*CA*) and *Convolvulus pilosellifolius* (*CP*) are plants belonging to *Convolvulus* genus. Both plants are usually used for the treatment of gastric ulcers in folk medicine. People in Saudi Arabia use the plant maceration for the treatment of ulcer [6]. Many compounds were identified from both plants such as phellandrenes, *p*-hydroxyphenylacetic acid, ferulic acid, scopolin, syringic acid, pinosylvin, taxifolin, vanillic acid, quercetin, scopoletin, myricetin, naringenin, and kaempferol. These two plants showed promising results for their anti-ulcer activity [7, 8].

Coumarins, which are widespread throughout various plant families, such as scopolin and scopoletin are a group of important natural compounds which have been found to have various biological activities such as; antifungal, anti-osteoporosis, anti-hypertension, anti-HIV, anti-arrhythmia, anti-tumor, antisepsis and antibacterial activities [9-13].

Interestingly, several analytical methods have been developed for the quantification analysis of coumarins content of different plants genus such as; LC-UV, GC-MS, and LC-MS [14-19]. Ultra-high performance liquid chromatography (UHPLC) has become an essential method for analyzing

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heterogeneous and biological compounds. An accurate determination of an analyte could be implemented using this method, by choosing suitable mobile, stationary phases and detector even without compounds being isolated in pure form. These techniques are more efficient, accurate, selective, reproducible, and sensitive than chemical and spectroscopic methods [20]. In order to increase the sensitivity to as low as pictograms per mass of sample or unit volume, HPLC coupled with a mass detector and many researchers used reverse phase HPLC coupled with mass spectrometry (HPLC-MS) to quantify coumarins in plants [21-24].

This work aims to improve an analytical process for instantaneous quantification of coumarin compounds taking into consideration many advantages such as: accurate quantitation, short analysis time, low volume injections and high sensitivity. In this study, UHPLC-MS/MS technique has been optimized and proved for quantification of active compounds concentrations such as scopolin and scopoletin in an ethanolic extract of both *CA* and *CP* plants. The amount of scopolin and scopoletin in both plants were finally compared with that of previously published reports.

2. MATERIALS AND METHODS

2.1. Plant Material and Reagents

Fresh aerial parts of both *CA* and *CP* were collected from the Al-Rawdha region, Saudi Arabia during summer 2017, identified by a taxonomist and compared with the published data [25]. All chemicals and reagents were of analytical grade or HPLC-grade.

Pure water was obtained through a Milli-Q system (Millipore, Bedford, MA, USA). The active compounds (scopolin and scopoletin) were purchased from Sigma-Aldrich (Darmstadt, Germany). Scopolin and scopoletin chemical structures are displayed in Fig. (**1**).

The stock solution of scopolin and scopoletin (100 µg mL^{-1}) was prepared individually by dissolving in HPLC-grade methanol and was kept for further dilution. Calibration curve for the working standard solutions was prepared by diluting the stock solutions to the concentration ranged from 0.07-10.0 µg mL^{-1} for scopolin and from 0.05-10.0 µg mL^{-1} for scopoletin (Fig. **2**). Before the injection process, both stock and standard solutions were prepared just before being analyzed.

2.2. Extraction Procedure and Sample Preparation

A 100 g of air-dried powder of both *CA* and *CP* were extracted with ethanol (300 mL × 3) in a Soxhlet apparatus [26]. The liquid ethanolic extract was dried using the vacuum at 35°C, yielding 6.5 and 5 g of the dry extract for *CA* and *CP*, respectively. Both extracts were stored in the refrigerator at 4°C until analysis.

Each extracted sample (20 mg) was dissolved in 25 mL methanol (HPLC-grade), followed by 10 minutes sonication of each sample. Finally, the volume of each sample was made up to 50 mL using methanol (HPLC-grade) and filtered over a 0.22 µm membrane filter.

2.3. Instrumentation

2.3.1. UHPLC Conditions

The chromatographic separation of coumarins was carried out on an EC Nucleodur C18 gravity polar Tec., Macherey-Nagel (Düren, Germany) with particle size 1.8 µm, 50 mm × 2 mm i.d., retained at temperature 30°C. All stand-

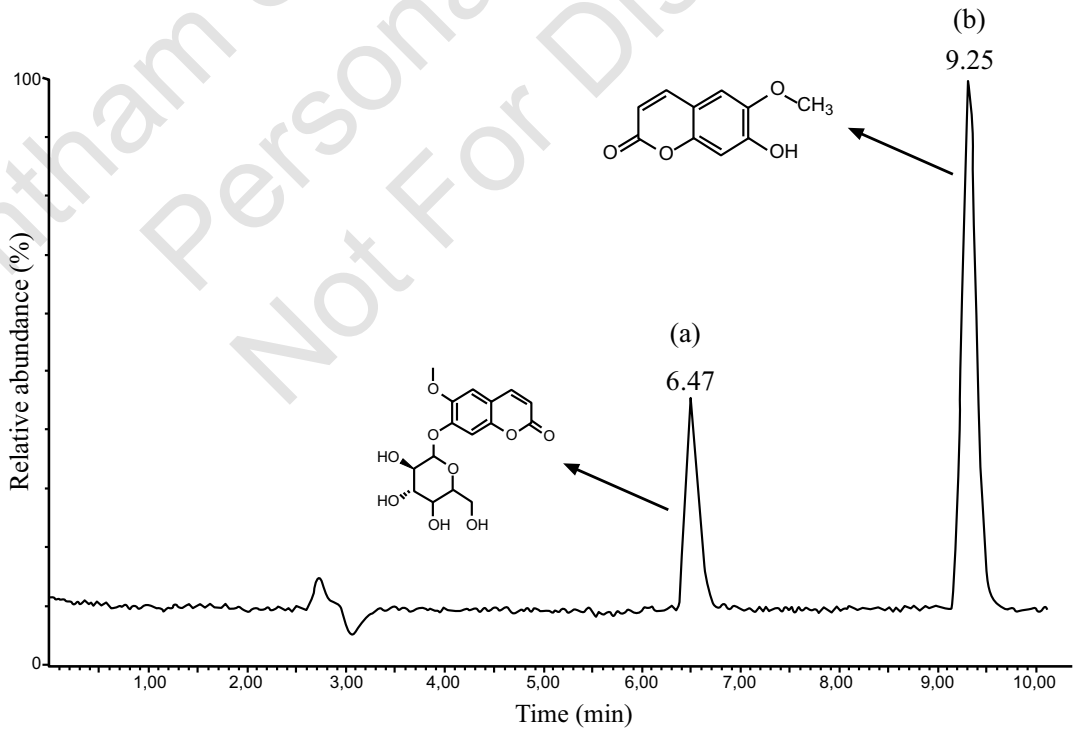

Fig. (1). UHPLC-MS/MS chromatogram of 1.0 µL injected (**a**) scopolin (1.0 µg mL^{-1}) and (**b**) scopoletin (1.0 µg mL^{-1}) standards in MRM mode.

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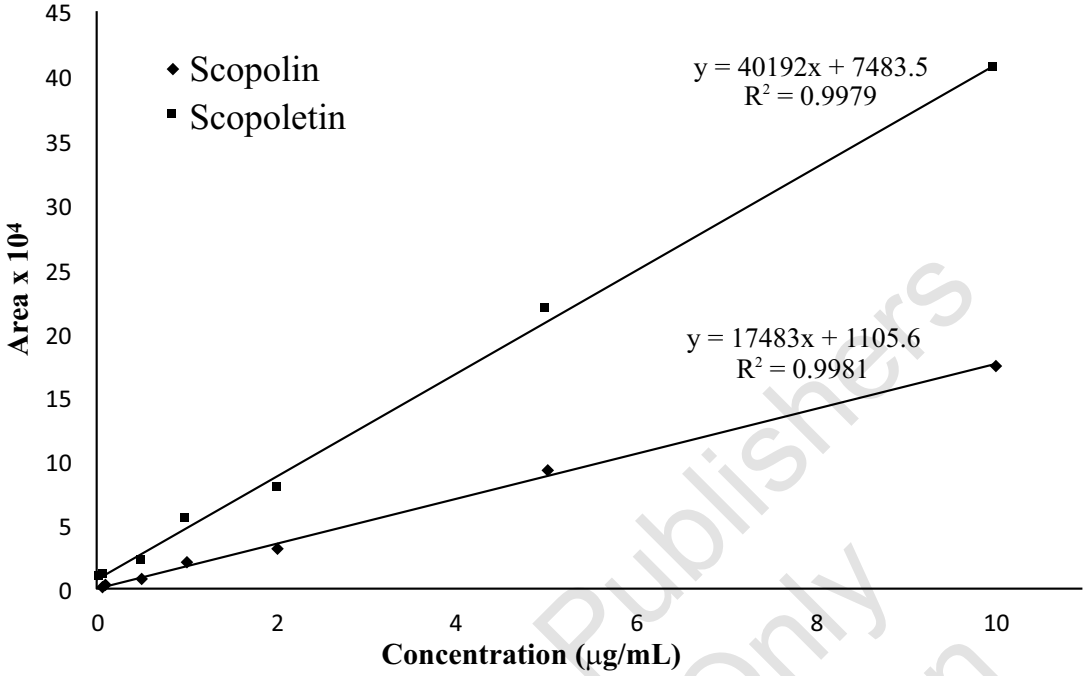

Fig. (2). Calibration curves of the standards scopolin and scopoletin.

ards and extract samples were subjected to Waters Acquity UPLC system (Waters, Manchester, UK) equipped with a column heater, an Acquity UPLC binary solvent manager and sample manager. In order to achieve the optimum chromatographic separation, a gradient mode was used by preparing a mobile phase mixture of methanol and water using 0.1% formic acid (30:70, v/v, after 10 min; 90:10, v/v), the flow rate was 0.2 mL min^{-1}. The injection volume was 1.0 μL.

2.3.2. Tandem Mass Spectrometry (MS/MS)

For detection of coumarin compounds, a triple-quadrupole mass spectrometer (Micromass Company Inc., Manchester, UK) was utilized with an Oerlikon rotary pump and Electrospray Ionization (ESI) source (SOGEVAC SV40 BI, Paris, France). Electrospray ionization with positive mode (ESI^+) was performed for MS/MS detection. Optimization monitoring conditions were applied for each compound. In order to achieve the collision energy and transition precursors ion-product for analysis of plant components, the Multiple Reactions Monitored (MRM) conditions were adjusted, whereas the formation of each precursor ion was accomplished by selection of optimized specific cone voltage (Table **1**). The optimum source conditions were as follows: source temperature 120°C, capillary voltage 3.2 kV, and desolvation temperature 300°C. The cone gas flows and desolvation were 60 and 600 L h^{-1}, respectively. High-purity nitrogen gas was used as nebulizing, cone gas and desolvation produced by a Peak Scientific NM30LA nitrogen generator (Inchinann, UK). For MS/MS measurements, a collision high-purity argon gas (99.99%) supplied by Speciality Gas Center (Jeddah, Saudi Arabia) was used and set a flow rate of 0.10 mL min^{-1}. Data acquisition was carried out by MassLynx V4.1 software (Micromass, Manchester, Lancashire, UK).

2.4. Validation Study

In order to validate the study and to ensure the method quality, various parameters were evaluated such as precision, linearity, limits of detection (LOD), recovery, and limits of quantification (LOQ). Seven standard calibration solutions (0.05, 0.10, 0.50, 1.0, 2.0, 5.0, 10.0 μg mL^{-1}) were injected in triplicate to estimate the linearity, LOD and LOQ of the method for scopolin and scopoletin compounds. The linear regression slope achieved using measured and added analyte concentrations was used to calculate the recoveries. Intraday precision was evaluated by spiked samples on the same day (five replicates), while interday precision was assessed over three consecutive days of the same samples (five replicates each day). LOD and LOQ were determined by spiked samples based on a signal to noise ratio of 10:1 for quantification limit and 3:1 for detection limit.

3. RESULTS AND DISCUSSION

3.1. Optimization of Chromatographic Separation Conditions

First, the presence of some naturally occurring coumarin compounds in *CP* and *CA* plants extract has been screened by Direct Analysis in Real Time (DART) ion source coupled with Time of Flight-Mass Spectrometry (ToF-MS) high-resolution technique [8]. While coumarin, umbelliferone, aesculetin, and others have not been detected, DART-ToF-MS spectra proved the presence of experimental mass at 193.05137 and 355.10371 *m/z* in the extract of *CA* plant and at 193.05084 and 355.10365 *m/z* in *CP* plant extract, with unsaturated degree of 7.5 and 6.5 for scopolin and scopoletin, respectively.

Analysis of scopolin and scopoletin in the two plants using reversed-phase liquid chromatography with an improve-

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ment of a high throughput method is of high knowledge. The most important benefits of the UHPLC systems, where column dimensions are shorter and narrower (with a particle size less than 2.0 µm), are the ability to achieve fast and high resolutions at relatively low flow rates. A flow rate of 0.2 mL min^{-1} was found to be optimal for the analysis of scopolin and scopoletin, making an analysis time of <10 min achievable. Fig. (**1**) shows the typical chromatogram of scopolin and scopoletin (1.0 µg mL^{-1}) standards in MRM mode gained with the best working settings. Gradient elution with a various mixture of solvents was assessed. A mixture of water and methanol with 0.1% formic acid, (70:30, v/v, after 10 min; 10:90, v/v) at a flow rate of 0.2 mL min^{-1} was used as eluents. Moreover, the column was re-equilibrated under the final mobile phase composition, i.e., water/methanol 10:90, v/v, for one minute after each injection to wash the possible retained compounds from the plant samples, which are not detectable using the applied MS conditions. Several columns (C8, C18 and amide) for the chromatographic separation of scopolin and scopoletin were also tested. C18 column was the best column used for the separation of scopolin and scopoletin with better peaks symmetry and lower separation time. Over the whole studied conditions, the tailing factors for scopolin and scopoletin were less than 1.31 using C18 column, and located between 1.27-1.63 for both compounds separated by C8 and amide columns. Methanol solvent was injected as a quality control to minimize the carryover after each sample injection. Although the possibility of finding residues in the injector that may be responsible for the carryover peak through the experiments is minimum, the injector keeps washing after each sample injection by weak and strong solvents by auto-sampler of the UHPLC system.

3.2. Optimization of ESI-MS/MS Conditions

The optimization of ESI-MS/MS conditions was confirmed by infusing standards of 5 µg g^{-1} of scopolin and scopoletin in both positive and negative ionization modes in order to give the maximum analyte response and dissolve efficiently the organic/aqueous mobile phase. The optimization was performed in positive ionization mode where the negative mode has no, or relatively small analyte signals appeared under different ion source parameters. Therefore, the positive ionization mode was chosen for mass spectrometric detection and a highly abundant analyte signals appeared. The narrow chromatographic peaks (5 s width) required a fast scanning analyzer such as the triple quadrupole used in this work in order to define the peaks with enough points. Several ESI-MS conditions effects has been studied such as; cone voltage (5-90 V), desolvation gas (400-800 L h^{-1}), capillary voltage (2.0-4.5 kV), desolvation temperature (200-450°C) and source temperature (100-150°C). For both analytes, the maximum analyte response was achieved with 3.2 kV capillary voltage, 20 V of cone voltage for scopolin and 24 V for scopoletin, 300°C of desolvation temperature, 120°C of source temperature and 600 L h^{-1} of desolvation gas flow rate.

The studies indicate that the effect of the ESI-MS parameters above higher values was unimportant. Full scan mass spectra were recorded to select the most abundant *m/z* value. To estimate the performance of cone voltage and ionization values, the relative intensity for the most abundant *m/z* value was selected. The most abundant precursors were *m/z* 193 (scopoletin) and 355 (scopolin) which is displayed in Fig. (**3A**). Multiple reaction monitoring for a transition per compound was acquired, which is used for quantification analysis (Fig. **3B**). Under the operated MS/MS detection parameters, the proposed method displays high selectivity for determination of scopolin and scopoletin in plants extracts. The selectivity was achieved by the selection of unique MRM transitions for each compound, *i.e.*, 355.30 → 178.00 for scopolin and 193.28 → 132.90 for scopoletin, alongside with a complete matching of the peaks in both standard solutions and real samples. The optimized MS parameters (cone voltages, product and precursor ions, and collision energies) are summarized in Table **1**.

3.3. Method Validation

Assessing the linearity of the proposed technique was accomplished by investigation of the detection signals as a function of analyte concentration using the regression line by the method of least squares. Determination of scopolin and scopoletin in plant extracts by UHPLC-MS/MS analysis indicates that the calibration curves were linear over the range of 0.07-10.0 and 0.05-10.0 µgmL^{-1} for scopolin and scopoletin, respectively. The correlation coefficient (R^2) obtained was ≥0.998. The typical linear equations obtained for scopolin: $y = 17483\ x + 1105.6$ with $R^2 = 0.9981$, and for scopoletin: $y = 40192\ x + 7483.5$ with $R^2 = 0.9979$; where x the concentration of each compound in µg L^{-1} and y being the peak area. Calibration curves of scopolin and scopoletin are shown in Fig. (**2**).

The LOQ and LOD were evaluated by analyzing a blank sample (Milli-Q water) spiked with scopoletin and scopolin for three times at low concentration levels. The LOD and LOQ were calculated using the statistical Mass Lynx V4.1 software. The LOD values were 0.03 and 0.01 µg mL^{-1} while

Table 1. Retention time and data acquisition parameters of MRM transitions for each analyte used in UHPLC-MS/MS.

Analyte	Retention Time (min)	Precursor Ion $[M+H]^+$ *(m/z)*	Transition Product Ion *(m/z)*	Cone Voltage (V)	Collision Energy (eV)
Scopolin	6.47	355.30	178.00	20	18
Scopoletin	9.25	193.28	132.90	24	23

Ionization mode: ESI^+.
Dwell time was 0.1 s in all cases.

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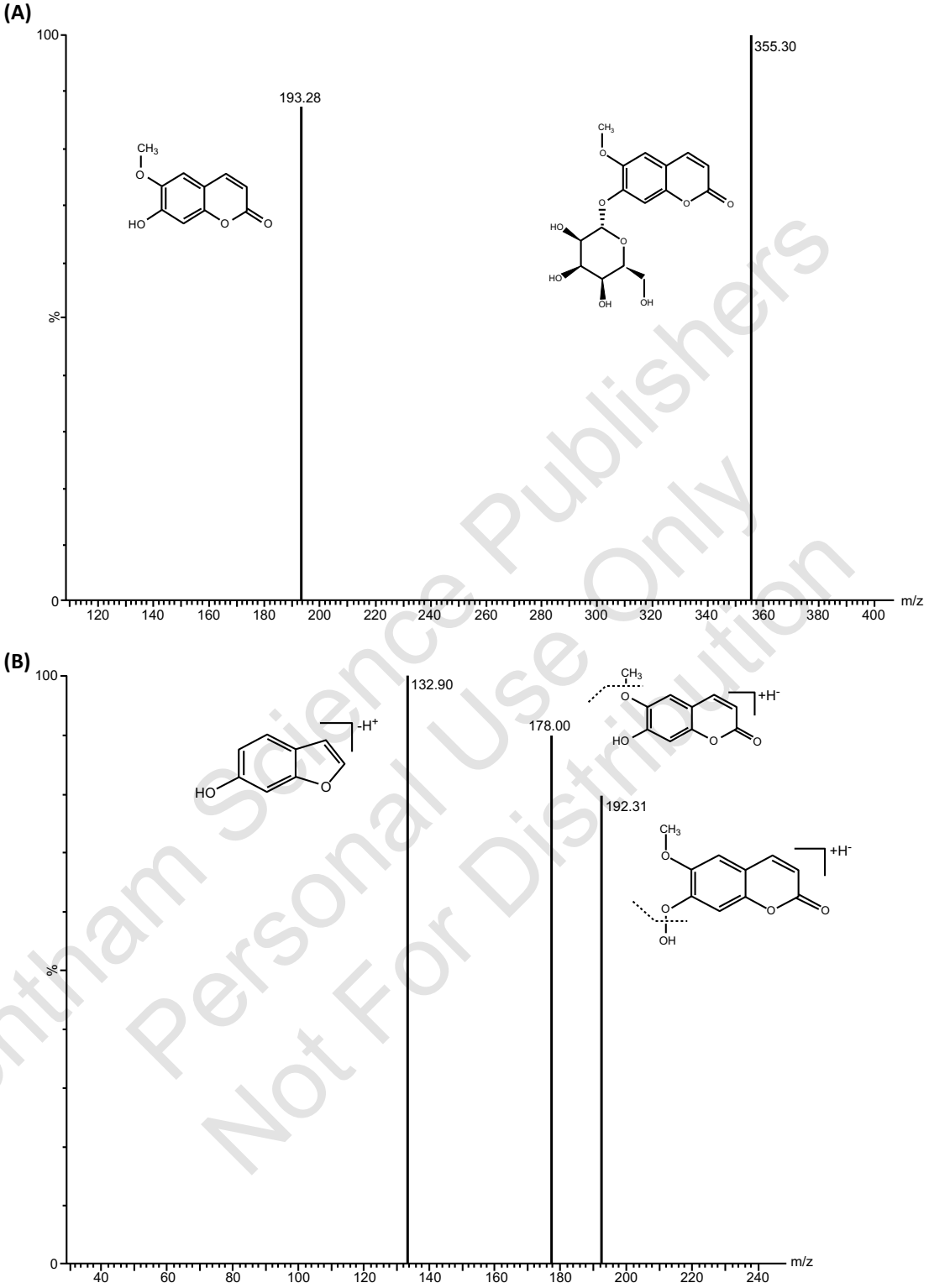

Fig. (3). (A) UHPLC-MS/MS mass spectra of precursor ion of scopolin (*m/z* 355.30) and scopoletin (*m/z* 193.28) in MRM mode for *CA* and *CP* (B) UHPLC-MS/MS spectra of product ion of scopolin (*m/z* 355.30) and scopoletin (*m/z* 193.28) in MRM mode for *CA* and *CP*.

the LOQ values were 0.09 and 0.04 μg mL^{-1} for scopolin and scopoletin, respectively, which sufficiently fulfilled the quantification of scopoletin and scopolin in plant extracts by the UHPLC-MS/MS method. The obtained results are listed in Table **2**.

Interday and intraday determination of scopolin and scopoletin were used to evaluate the precision of the proposed method. The interday was measured by injection of the spiked sample (0.5 μg g^{-1}) along three consecutive days for five times, while the intraday was calculated from five

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replicate injections of the aforesaid spiked sample on the same day. These results were obtained by calculating the relative standard deviation (RSD%) of the acquired concentration values. The intraday RSD values for scopoletin and scopolin in *CA* plant extract were obtained as 1.35 and 3.50% and in *CP* plant extract 0.46 and 4.33%, respectively. The interday RSD values for scopoletin and scopolin in both *CA* and *CP* were less than 4.22. Intraday and interday precision values are listed in Table **2**. The results of the intraday and interday precision data shown good reproducibility for the UHPLC-MS/MS analysis of scopoletin and scopolin in both *CA* and *CP* plant extracts.

3.4. Plants Extract Analysis

Scopolin and scopoletin were unambiguously identified and quantified in the two plant extracts using developed and validated UHPLC-MS/MS methods by comparison of their retention times with those of standard compounds and also by mass spectra. Fig. (**4**) illustrates the UHPLC-MS/MS chromatograms of scopolin and scopoletin in *CA* and *CP* extracts. As there is no obvious matrix peak was eluted in the retention time of the analyzed compounds, the chromatograms revealed good resolution and did not exhibit any interference. Both of the corresponding compound and standard solution showed similar mass spectra with the same *m/z* value (Fig. **3A** and **B**). The highest content of scopolin (3.49 mg g^{-1}) and scopoletin (0.795 mg g^{-1}) was detected in ethanolic extracts of *CA* followed by *CP* (scopolin: 2.98 mg g^{-1} and scopoletin: 0.195 mg g^{-1}). According to our results, scopolin was found in a greater amount than scopoletin in both plants under investigation. On the other hand, no noticeable deterioration of separation efficiency, or reduction in the ionization efficiency or detection of sensitivity caused by matrix effect was observed for more than 120 injections of plant samples using the proposed UHPLC-MS/MS method for determining scopolin and scopoletin. This could be explained by using a short separation column under gradient elution and small injection volumes; however, the column was washed with 100% methanol once after several injections.

The recoveries of both scopoletin and scopolin were evaluated by spiking the two standards in three different concentration levels (low medium and high) to the plant extracts in order to validate the found results in the plant extracts. The recovery percentage values were obtained at 97.04-99.97% and presented in Table **3**. The recovery levels

Table 2. Validated parameters of UHPLC-MS/MS method.

Analyte	LOD[a] (µg mL^{-1})	LOQ[b] (µg mL^{-1})	Linearity (R^2)	Intraday (RSD%)[c]		Interday (RSD%)[c]	
				CA	*CP*	*CA*	*CP*
Scopolin	0.03	0.09	0.9981	3.50	4.33	3.44	4.22
Scopoletin	0.01	0.04	0.9979	1.35	0.46	1.33	0.54

[a] Limit of detection was estimated at a signal-to-noise ratio of 3.
[b] Limit of quantification was estimated at a signal-to-noise ratio of 10.
[c] Relative standard deviation (n=5).

Table 3. Recovery study of scopolin and scopoletin added to plant extract samples.

Plant	Analyte	Added (µg g^{-1})	Found (µg g^{-1}) ± SD[a]	Recovery (%)
CA	Scopolin	1.0	3.97 ± 0.01	97.72
		5.0	5.95 ± 0.05	99.40
		10.0	8.43 ± 0.05	99.39
	Scopoletin	1.0	1.29 ± 0.01	99.87
		5.0	3.29 ± 0.03	99.97
		10.0	5.76 ± 0.05	98.42
CP	Scopolin	1.0	3.46 ± 0.05	99.57
		5.0	5.45 ± 0.04	99.54
		10.0	7.95 ± 0.02	98.65
	Scopoletin	1.0	0.69 ± 0.01	97.04
		5.0	2.69 ± 0.02	97.70
		10.0	5.18 ± 0.03	99.78

SD = standard deviation.
[a] Mean of three measurements.

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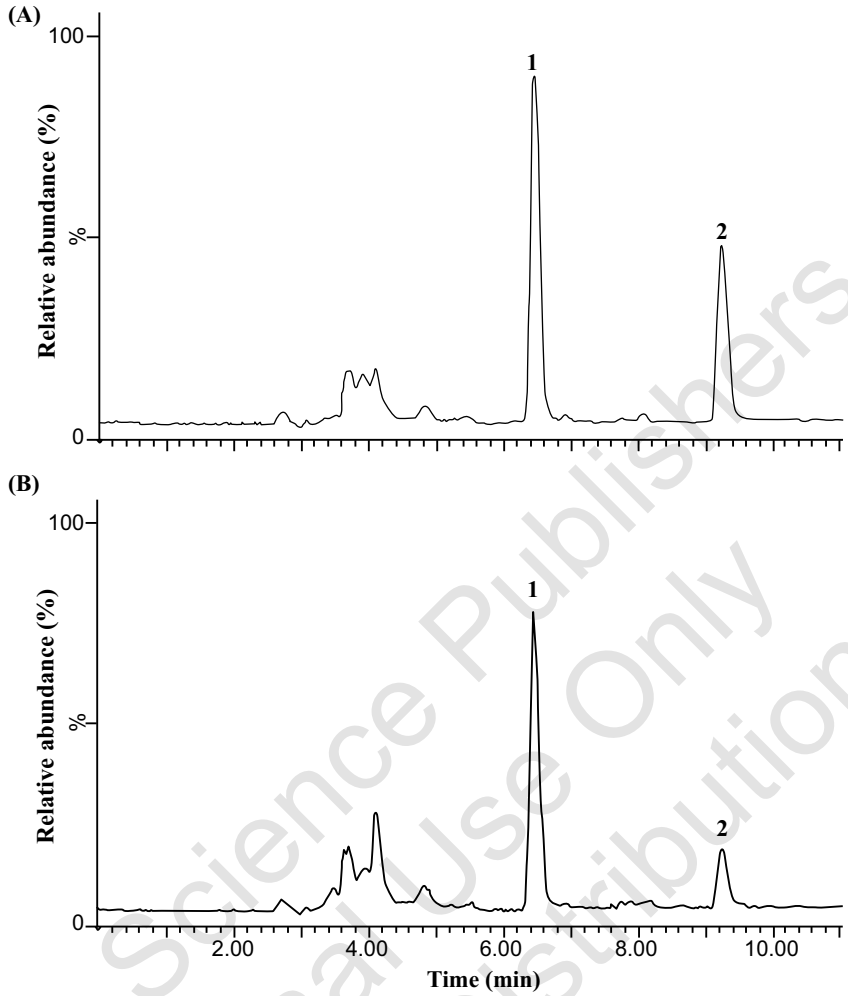

Fig. (4). UHPLC-MS/MS chromatogram of studied plant extracts under optimum conditions (**A**) *CA* (**B**) *CP*, 1 = scopolin, 2 = scopoletin.

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were acceptable for both analytes. In addition, good repeatability of the recovery test (RSD < 4.33%, n = 3) in all spiked levels was achieved.

3.5. Comparison Study

One of the objectives of this research is to compare *CA* and *CP* plants with the other published research in terms of scopolin and scopoletin content. To the best of our knowledge, five studies using eight different plants have reported the amounts of scopolin and scopoletin previously. HPLC coupled to UV or MS were the most used analytical techniques for quantification of active compounds such as scopolin and scopoletin in the plants. In this study, the performance of UHPLC separation and MS/MS detection has been combined to get a fast, sensitive and selective analysis of scopolin and scopoletin with negligible matrix effect. Table **4** summarizes the contents of scopolin and scopoletin obtained from *CA* and *CP* plants in comparison with the previous literature reports [27-31]. With the exception of *Morinda tinctoria* plant (15.8 mg g^{-1} scopoletin) [31], *CA* and *CP* contain a relatively high amount of scopolin and scopoletin in comparison with the other studied plants. However, since the biological activity is directly related to the active compounds content, these data are very useful in terms of the feasibility of the isolation of scopolin and scopoletin compounds.

The developed method has been compared with other previous works for determination of scopolin and scopoletin in different plants. All reported works used C18 stationary phases at conventional scale chromatography, *i.e.*, 4.6 mm internal diameter columns packed with 5 µm stationary phase particle size and a mobile phase flow rate of 1 mL min^{-1}. In a validation point of view, the data show advantages of the proposed method in terms of sensitivity based on LOD values; 0.01 µg mL^{-1} in comparison with 0.39-7.9 µg mL^{-1} for the other methods. The second main advantage of the described method is the lower separation time for the targeted compounds; this is very clear in the run time values presented in Table **4**. However, its axiom that the short UHPLC columns (5 cm length, packed with sub-2 µm stationary phase particle size) exhibits higher efficiency and lower analysis time than the conventional HPLC columns (25 cm length, packed with 5 µm stationary phase particle size). The proposed method showed more benefits in terms samples injection volumes (1 µm in comparison with 10-20 µm) and mobile phase solvents consumption (five times lower).

Table 4. Comparison of the studied plants with other reported plants in terms of scopolin and scopoletin contents, percentage recovery values and other analytical parameters.

Plant	Analytical Method	Scopolin		Scopoletin		Separation Column	LOD*	Flow Rate (mL min^{-1})	Injection Volume (µL)	Run Time (min)	Refs.
		Recovery (%)	mg g^{-1}	Recovery (%)	mg g^{-1}						
Canscora decussate	HPTLC/UV	ND	ND	≥ 99.8	1.68×10^{-6}	--	31 ng $spot^{-1}$	--	--	--	[27]
Chimonanthus nitens	HPLC/MS	≥ 97.6	2.16	≥ 95.7	0.23	Ultimate XB-C18 (250×4.6 mm, 5 µm)	7.9 µg mL^{-1}	1.0	10	80	[28]
Chimonanthus salicifolius			1.43		0.15						
Chimonanthus zhejiangensis			0.26		0.05						
Chimonanthus grammatus			1.06		0.12						
Convolvulus pluricaulis	HPLC/UV	ND	ND	≥ 99.1	1.74	Phenomenex C18 (250×4.6 mm, 5 µm)	5 µg mL^{-1}	1.0	20	30	[29]
Aegle marmalos	HPLC/UV	ND	ND	≥ 96.25	0.14	Purospher Star RP-18 (250×4.6 mm, 5 µm)	0.39 µg mL^{-1}	1.0	20	15	[30]
Morinda tinctoria	HPLC/UV	ND	ND	ND	15.8	Luna C18 (250×4.6 mm, 5 µm)	ND	1.0	20	20	[31]
CA	UHPLC-MS/MS	≥ 97.72	3.49	≥ 98.42	0.795	EC Nucleodur C18 (50×2 mm, 1.8 µm)	0.01 µg mL^{-1}	0.2	1	10	This work
CP		≥ 98.65	2.98	≥ 97.04	0.195						

* LOD of scopoletin; ND: Not detected.

Reduction of solvents consumption is very important in the generation of lower amounts of waste and decrease the cost of analysis. On the other hand, the lower injection volumes becoming more significant if the extracted samples are limited. The total comparison data is summarized in Table **4**.

CONCLUSION

In this work, a method for the determination of scopolin and scopoletin in two *Convolvulus* plant extracts by UHPLC with tandem mass spectrometry detection has been developed. Many significant improvements in analytical speed, resolution and sensitivity have been offered by the developed UHPLC-MS/MS-based technique. A wide linearity range (0.05-10.0 µg mL^{-1}) as well as intra-day/inter-day precision (RSD<4.33%) and high percentage recovery (97.04-99.97%) were obtained for quantitative determination of the two active compounds. The results realized with this system as resolution and analysis time approve that they are enough to suggest it as a strong approach for the fast and dependable simultaneous analysis of scopolin and scopoletin in plant extracts. Based on these experiments, the proposed method shows the potential for routine determination of active compounds in medicinal plant extracts.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data sets used and/or analysed during this study are available from the corresponding author on request.

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CONFLICT OF INTEREST

The authors declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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