High-Content Analysis of Chelidonine and Berberine from Iranian \textit{Chelidonium majus} L. Ecotypes in Different Ontogenetical Stages Using Various Methods of Extraction

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ABSTRACT

\textit{Chelidonium majus} is a perennial plant of the Papaveraceae family. This plant has been known as a rich source of isoquinoline alkaloids, chelidonine, and berberine, which are pharmaceutically important for their anti-cancerous activities. In the current study, four extraction techniques were compared in terms of their yield potential for chelidonine and berberine. Afterwards, High Performance Liquid Chromatography (HPLC) with a photodiode array type of UV/VIS detector was used for the detection of chelidonine and berberine from leaves and roots of five ecotypes of \textit{C. majus} during various ontogenetical stages. Based on our results, ultrasonic procedures and refluxing were the best techniques for extraction of these alkaloids. HPLC results inferred that chelidonine and berberine content of ecotypes belonging to the Northern provinces of Iran, i.e. Mazandaran (IBRCP1006619) and Gorgan (IBRCP1006625), were higher than the other ecotypes. Generally, the roots of the \textit{C. majus} were the most suitable organ for extraction of chelidonine at the generative stage, while at the vegetative stage, leaves are the most suitable organ for extraction of berberine.

Keywords: Extraction techniques, Generative stage, Isoquinoline alkaloids, Vegetative stage.

INTRODUCTION

\textit{Chelidonium majus} L. is a perennial plant from the family of Papaveraceae. It is distributed in Europe and Western Asia and also an introduced species in Northern America. Commonly, this plant is known as celandine, greater celandine, celandine poppy, elon-wort, felon-wort, rock poppy, swallowwort, and tetter-wort. The plant is highly toxic due to presence of various secondary metabolites, but has been adequately used in traditional medicines (Monavari et al., 2012). Pharmacological properties of \textit{C. majus} include anti-viral (Gerencer et al., 2006), anti-bacterial (Miao et al., 2011), anti-fungal (Hou et al., 2013), anti-protozoal (Kim et al., 2012), radioprotective (Song et al., 2003), anti-inflammatory (Park et al., 2011), anti-Alzheimer (Cahlikova et al., 2010), anticancer (Moussa et al., 2007), hepatoprotective (Biswas et al., 2008), and natriuretic and anti-diuretic (Koriem et al., 2013). An array of

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secondary metabolites present in C. majus is responsible for its therapeutic properties. One of the most common secondary metabolites in C. majus is alkaloids. Various alkaloids metabolized by C. majus are chelidonine, berberine, sanguinarine, coptisine, chelerythrine, protopine, etc. (Gilca et al., 2010). In addition to alkaloids, other secondary metabolites identified from this plant include flavonoids, saponins, vitamins (e.g. vitamins A and C), mineral elements, sterols, acids and their derivatives (Kopytko et al., 2005).

Two most pharmacologically important alkaloids identified from C. majus are chelidonine and berberine. Chelidonine is a benzophenanthridine alkaloid. This tertiary alkaloid (C_{20}H_{19}NO_{5}, molecular weight: 353.38 g mL^{-1}) has an anticancer effect (El-Readi et al., 2013). On the other hand, berberine (C_{20}H_{18}NO_{4}, molecular weight: 336.37 g mL^{-1}) is an isoquinoline alkaloid having anti-cancer activity (Kemeny-Beke et al., 2006; Mahata et al., 2011). Several studies have been conducted on the quantification of chelidonine and berberine from C. majus extracts (Kulp et al., 2011; Citoglu et al., 2009; Gu et al., 2010).

The concentration of alkaloids in plants is usually low, ranging between few percent (about 10 to 25%) and heterogeneously distributed within the plant tissue. Hence, adequate extraction methods are important for better recovery of alkaloids from a plant sources. Several studies have been published on the extraction of isoquinoline alkaloids, chelidonine and berberine from C. majus. Among the published protocol four methods of extraction were applied for C. majus in order to compare extraction procedures and identify the best method which shows the highest rate of chelidonine and berberine that save greater amount of anti-cancer agents in this plant. The salient features of these protocols are higher yield of alkaloids due to the used solvents that interact with the polar groups of alkaloids, faster extraction time, and lower operation costs.

The aim of this study was to compare four different optimized methods for extraction of chelidonine and berberine from C. majus, for selecting the most efficient extraction for recovering these alkaloids. In addition, we aimed to apply HPLC to quantify the chelidonine and berberine content of five Iranian C. majus ecotypes, using different plant tissues at various ontogenetical stages. Another objective was to determine the richest source of these alkaloids among the selected ecotypes, to be used for future studies.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Standards for the alkaloids, chelidonine (CAS No. 476-32-4; Lot No.BCBN1934V) and berberine (CAS No. 633-65-8; Lot No. SLBG1303V) were purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol and acetonitrile (HPLC grade), n-Hexane, formic acid, hydrochloric acid, chloroform, ammonia (25%), citric acid, and ammonium formate in all experiments were purchased from Merck (Darmstadt, Germany). Deionized water was obtained and purified using a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

**Preparation of Standard Solutions**

A stock solution containing 1 mg mL^{-1} of each analyte was prepared in methanol (Merck, Germany) and stored at 4°C. The working standard solutions were made by dilution of the stock solution in methanol, and then filtered by 0.2-µm membrane. Working solutions were stored at 4°C before injection into the HPLC.

**Plant Material**

Seeds of C. majus were collected from five different regions of the northern Iran (Table 1). The collections were submitted to the herbarium of the Iranian Biological Resources
Chelidone and Berberine from Iranian Chelidonium

Table 1. Regions where seeds of C. majus were collected.

<table>
<thead>
<tr>
<th>Accession no</th>
<th>Regions</th>
<th>Height (m)</th>
<th>Average temperature (°C)</th>
<th>Longitude coordinates (E)</th>
<th>Latitude coordinates (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBRCP1006619</td>
<td>Mazandaran; Mahmudabad-Amol; Mazandaran; Firoozkooh-Sari</td>
<td>1</td>
<td>27</td>
<td>52 17’ 0.9”</td>
<td>36 35’ 15.1 ”</td>
</tr>
<tr>
<td>IBRCP1006622</td>
<td>Mazandaran</td>
<td>1901</td>
<td>22</td>
<td>52 57’ 19.9”</td>
<td>35 52’ 37.2 ”</td>
</tr>
<tr>
<td>IBRCP1006623</td>
<td></td>
<td>340</td>
<td>25</td>
<td>52 54’ 32.9”</td>
<td>36 14’ 26.9 ”</td>
</tr>
<tr>
<td>IBRCP1006625</td>
<td>Gorgan</td>
<td>605</td>
<td>25</td>
<td>54 28’ 52.9”</td>
<td>36 45’ 23.4 ”</td>
</tr>
<tr>
<td>IBRCP1006626</td>
<td>Gorgan</td>
<td>34</td>
<td>29</td>
<td>54 7’ 20.6”</td>
<td>36 46’ 31.2 ”</td>
</tr>
</tbody>
</table>

Center (IBRC), Alborz, Iran, under voucher number e.g., IBRCP1006619, IBRCP1006622, IBRCP1006623, IBRCP1006625, IBRCP1006626 that all ecotypes have similar morphology with the same age.

The seeds were cultivated in the greenhouse facilities of IBRC from November 2015 till March 2016. The temperature at the facility was maintained between 20-25°C and plants were irrigated regularly.

The aerial parts and the roots for the different ecotypes of C. majus were collected randomly at two different stages, i.e. vegetative and generative. The plant materials were cleaned with the deionized water and dried in the oven at 40°C, then, powdered (20 mesh) for further experiments.

**Extraction Methods**

In this study, based on the previous researches (Lee et al., 2005; Artamonova and Kurkin, 2008; TalebiKouyakhi et al., 2008; Jahansooz et al., 2008; Baghalian et al., 2008; Baghalian et al., 2010; Citoglu et al., 2009; Sarkozi et al., 2006; Gu et al., 2010), the yield potential of four extraction methods for chelidonine and berberine were compared. These methods included: (1) Reflux with methanol (Ghanavi et al., 2013); (2) Ultrasonic procedure with water-methanol–HCl (Zhou et al., 2012); (3) Ultrasonic procedure with methanol–HCl (Kursinszki et al., 2006), and (4) Shaking incubator with citric acid (Borghini et al., 2015).

**High Performance Liquid Chromatography (HPLC) Analysis**

HPLC analysis was carried out using Agilent 1200 series (Walbronn, Germany). HPLC system consisted of a G1312B binary pump, a G1376A capillary pump, G1330B FC/ALS, G1379B Degasser, and G1377Amicrowips. Chemstation software was used for data acquisition, processing and reporting. Compounds were separated on a 5 μm ZORBAX Eclipse XDDBC18 reversed-phase column (150 mm×4.6 mm id; Agilent Technology, Germany). The column temperature was 30°C and the injection volume was 20 μL (in triplicate).

In order to simultaneously determine chelidonine and berberine, a gradient program with two solvent systems including A, water (0.1% formic acid), and B, acetonitrile, was applied. Separation was performed with 20% B at the beginning, which gradually changed to 90% B within 40 minutes, remaining 5 minutes at this point for re-equilibration. The flow rate of the mobile phase was 0.5 mL min⁻¹. The photodiode detector was scanned from 210 to 400 at 2-nm intervals. The chromatographic profile was recorded at 280 nm. Retention times and UV–visible
absorption spectra of the samples were compared with the standards.

**Statistical Analysis**

All tests were performed in triplicate using factorial experimental design. Data were expressed as mean±SD values, while significance (P values less than 0.05 to 0.01) of the differences between the mean values was determined by Duncan’s multiple range test using Statistical Analysis System Ver. 9.1 (SAS) software.

**RESULTS AND DISCUSSION**

**Calibration Curves**

Calibration curves for chelidonine were drawn at 2,500, 5,000, 10,000, 25,000 ng mL$^{-1}$, whereas in case of berberine it was 60, 125, 250, 500, 1,000, 2,500, 5,000 ng mL$^{-1}$. Linear regression and linear range of each alkaloid are shown in Table 2. All calibration curves showed the best-fitting linear regression ($r \geq 0.999$) within tested ranges. Twenty µL of each standard solution in triplicate was injected on to the HPLC column and absorbance was recorded at 280 nm. The concentrations of chelidonine and berberine in samples were calculated from their peak areas by use of the calibration curve.

**HPLC Analysis**

HPLC is commonly used for separation of metabolites from *C. majus* extract using both normal and Reverse Phase (RP) columns (Suchomelova et al., 2007; Taborska et al., 1994; Bugatti et al., 1987; Zuo et al., 2008).

Application of HPLC for separation of chelidonine and berberine requires some modifications to obtain fast and efficient protocol for high recovery within a short run time (less than 20 min). In the present study, due to the effect of polar functional groups and chromatographic behavior of chelidonine and berberine induced by non-polar HPLCC18 column; acetonitrile-formic acid was selected as the most convenient elution mobile phase following Ghanavi et al. (2015).

Typical Chromatogram for chelidonine and berberine mixed standards (5,000 ng mL$^{-1}$) are shown in Figure 1. For each sample, analysis was carried out in triplicate. Chromatograms obtained for chelidonine and berberine extracts from leaves and roots of five ecotypes of *C. majus* during vegetative and generative stages are shown in Figures 2-5. As depicted, two alkaloids separated within 12 minutes and data represent the means±standard deviation of three independent experiments (Table 4). The ANOVA of the different tissues and growth stages data of *C. majus* in five ecotypes is shown in Table 5.

**Extraction Methods**

Selection of extraction procedures and solvents depends on several factors, which includes the physicochemical nature of secondary metabolite, nature of plant material (fresh parts, dried parts) and their particle size (Yadav et al., 2003). As the

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**Table 2. Linear regression data of chelidonine and berberine.$^{a}$**

<table>
<thead>
<tr>
<th>Linear range (ng mL$^{-1}$)</th>
<th>$r$</th>
<th>Regression equations</th>
<th>Alkaloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500-25000</td>
<td>0.999</td>
<td>$Y= 35.80x-17.22$</td>
<td>Chelidonine</td>
</tr>
<tr>
<td>60-5000</td>
<td>0.999</td>
<td>$Y= 0.125x+1.53$</td>
<td>Berberine</td>
</tr>
</tbody>
</table>

$^{a}$ $Y=Ax+B$, $Y$ is peak area; $x$ is concentration of the alkaloids (ng mL$^{-1}$), $r$ is the correlation coefficient of the equation.
Chelidonine and Berberine from Iranian Chelidonium

Figure 1. Chromatogram of Chelidonine (CHE) and Berberine (BER) mixed standards.

Figure 2. Separated chelidonine and berberine chromatograms from roots of five Iranian C. majus ecotypes at vegetative stage.

Figure 3. Separated chelidonine and berberine chromatograms from roots of five Iranian C. majus ecotypes at generative stage.

Figure 4. Separated chelidonine and berberine chromatograms from leaves of five Iranian C. majus ecotypes at vegetative stage.

chemical property of a solvent is directly correlated to the final yield, solvents such as methanol and its mixtures (methanol-water, acidic methanol with HCl) along with acetic acid and other solvents were selected on the basis of polarity of alkaloids (Madziga et al., 2010; Yalavarthi and Thiruvengadarajan, 2013).
Our results showed that ultrasonic and reflux methods were the most efficient techniques for extracting chelidonine and berberine, respectively. Concentration of chelidonine and berberine obtained using different extraction methods are presented in Table 3.

Among the five Iranian *C. majus* ecotypes investigated in this study, the final yield of chelidonine and berberine substantially varied between different parts of the plant and growth stages (Table 4). Among ecotypes, we recovered the highest amount of chelidonine from IBRCP1006619 (Mahmudabad-Amol, Mazandaran), followed by IBRCP1006622 (Firoozkooh-Sari, Mazandaran) and IBRCP1006623 (Mazandaran). Ecotypes from Gorgan, *i.e.* IBRCP1006625 and IBRCP1006626 showed a great variation in concentration of chelidonine in terms of different plant tissues and different ontogenetical stages. Irrespective of the ecotypes, chelidonine concentrations in the roots increased with the transition of the plant from vegetative stage to generative stage. While this relationship was reverse in the case of the leaf tissues. For all ecotypes, chelidonine concentration in the roots were higher than that of the leaves. In terms of plant growth stages, the chelidonine concentration was relatively higher in the generative stage than in the vegetative and this is applicable to all ecotypes.

In terms of berberine content, ecotypes from Gorgan had significantly more of this alkaloid than Mazandaran. IBRCP1006625 (Gorgan) in vegetative stage produced high levels of berberine, followed by IBRCP1006623, IBRCP1006622 and IBRCP1006619 (Mazandaran), and lastly IBRCP1006626 (Table 4). The concentration of berberine was higher in the leaves than in the roots for all ecotypes. In term of growth stages, the vegetative stage had a relatively higher concentration of berberine than in the generative stage (Table 4). Berberine concentrations in all ecotypes decreased during the transition of the plant from vegetative to the generative stage.

By statistically comparing our data, it revealed that the ecotypes with the higher amount of berberine in leaves showed lower levels of chelidonine in the roots. Among the ecotypes from Mazandaran, IBRCP1006619 produced high levels of chelidonine (19.13±0.15 mg g⁻¹ of dry weight) at the generative stage in root and the ecotype of Gorgan, IBRCP1006625

### Table 3. Concentration of isolated chelidonine and berberine with different extraction methods.

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Chelidonine (mg g⁻¹ DW)</th>
<th>Berberine (µg mg⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refluxing with methanol</td>
<td>6.57 ± 0.11</td>
<td>0.90 ± 0.09</td>
</tr>
<tr>
<td>Ultrasonic with water–methanol–HCl</td>
<td>10.99 ± 0.14</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Ultrasonic with methanol–HCl</td>
<td>12.04 ± 0.01</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>Shaking incubator with Acidic condition on dried parts</td>
<td>6.85 ± 0.11</td>
<td>0.05 ±0.02</td>
</tr>
</tbody>
</table>
produced highest levels of berberine (3.98±0.02 µg mg\(^{-1}\) of dry weight) at vegetative stage in leaf. Hence, it is quite evident that the metabolisms for these alkaloids change between the developmental stages of the plant and between tissues.

In previous study, Ghanavi et al. (2013) quantified the isoquinoline alkaloids content of C. majus using refluxing technique. Their study showed berberine content of C. majus stem was 1.28 µg mg\(^{-1}\). Sarkozi et al. (2006) investigated the chelidonine content from the leaf and root in C. majus, and their result is consistent with our study. They observed a significant difference in chelidonine content in various organs of C. majus. The chelidonine concentration in roots and leaves was more than in other organs. They recovered 0.18 and 3.76 mg of chelidonine from dry weight of leaf and root, respectively. Gu et al. (2010) investigated the chelidonine content from the roots of C. majus and Berberine from Iranian Chelidonium

### Table 4

<table>
<thead>
<tr>
<th>Organ</th>
<th>Ecotype</th>
<th>Metabolite</th>
<th>Growth stages</th>
<th>Vegetative stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>IBRC P1006619</td>
<td>CHE</td>
<td>19.13±0.15</td>
<td>15.52±0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BER</td>
<td>0.29±0.01</td>
<td>0.53±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.45±0.28</td>
<td>12.87±0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.86±0.02</td>
<td>1.65±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13.26±0.15</td>
<td>12.5±0.30</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.28±0.02</td>
<td>0.42±0.01</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>12.21±0.09</td>
<td>11.93±0.24</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.10±0.02</td>
<td>0.21±0.01</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>10.93±0.15</td>
<td>9.15±0.05</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.37±0.01</td>
<td>0.54±0.08</td>
</tr>
<tr>
<td>Leaf</td>
<td>IBRC P1006619</td>
<td>CHE</td>
<td>6.52±0.24</td>
<td>5.08±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BER</td>
<td>0.43±0.02</td>
<td>1.65±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.99±0.23</td>
<td>6.33±0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.45±0.01</td>
<td>2.00±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14.45±0.22</td>
<td>8.17±0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.51±0.01</td>
<td>2.83±0.01</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>15.23±0.14</td>
<td>9.60±0.05</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.56±0.01</td>
<td>3.98±0.02</td>
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<tr>
<td></td>
<td></td>
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<td>15.58±0.11</td>
<td>10.96±0.14</td>
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<td></td>
<td></td>
<td></td>
<td>0.42±0.01</td>
<td>1.53±0.01</td>
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### Table 5

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>MS</th>
</tr>
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<tbody>
<tr>
<td>Tissue</td>
<td>1</td>
<td>117.91</td>
</tr>
<tr>
<td>Ecotype</td>
<td>4</td>
<td>2.88</td>
</tr>
<tr>
<td>Growth stage</td>
<td>1</td>
<td>150.07</td>
</tr>
<tr>
<td>Tissue×Ecotype</td>
<td>4</td>
<td>93.75</td>
</tr>
<tr>
<td>Tissue×Growth stage</td>
<td>1</td>
<td>36.49</td>
</tr>
<tr>
<td>Ecotype×Growth stage</td>
<td>4</td>
<td>0.47</td>
</tr>
<tr>
<td>Tissue×Ecotype×Growth stage</td>
<td>4</td>
<td>7.56</td>
</tr>
<tr>
<td>Error</td>
<td>38</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Coefficient of variation 1.52
majus collected from different regions in China and Mongolia by Ultra-Performance LC (UPLC) method with photodiode array detection. The maximum chelidonine content from these two regions were about 2.34±0.00 and 2.87±0.05 mg, respectively. They recovered a low berberine concentration from all samples (averaged 0.13 mg). In another study, the differences of chelidonine and berberine contents in C. majus extracts from 14 different geographic areas in China and showed that the ranges of chelidonine and berberine recovered were 514 to 1,012 mg kg\(^{-1}\) and 499 to 1,161 mg kg\(^{-1}\), respectively (Zhou et al., 2012).

In our study, we reconfirmed ultrasonic and reflux as better methods than shaking incubator for extraction of chelidonine and berberine from C. majus. The advantages of ultrasonication compared to the reflux and shaking incubator are requirement of shorter extraction time and temperature, conserving the chemical structure of the metabolites. In contrast, shaking needs longer extraction time and higher temperature giving less yield due to decomposition of the metabolites (Arulpriya et al., 2013). However, reflux is another efficient method that in some cases was superior than ultrasonic extraction (Lee et al., 2013), but simplicity of the apparatus, faster operational time, and ability to tandem process multiple samples (Porevsky et al., 2014; Manika et al., 2013) makes ultrasonication the preferred extraction method. For the first time, we recovered the highest amount of chelidonine and berberine using ultrasonic and reflux techniques coupled with developmental stages of the C. majus. We believe that the higher yield of the alkaloids in our study was outcome of the different ontogenetical stages of the plant we investigated.

CONCLUSIONS

In the present study, chelidonine and berberine contents from five ecotypes of C. majus were assessed from roots and leaves at different developmental stages using four different extraction methods. Since even slight increase in the amount of chelidonine and berberine is more important than optimizing a new extraction method; in this study, the yield potential of four already published methods were compared to reach the highest amount of these two alkaloids. Based on our results, the root is the most suitable organ for extraction of chelidonine at the generative stage. In the case of berberine, leaves at the generative stage are the most suitable organ for extraction. Generally, the ecotypes from Mazandaran (IBRCP1006619) and Gorgan (IBRCP1006625) showed a higher concentration of chelidonine and berberine, respectively. According to the results, we determined the richest source of these alkaloids among the selected ecotypes, which can be used for RNA-sequencing to discover a potential connection between gene expression and content variation for chelidonine and berberine, using differential regulatory mechanisms underlying secondary metabolic pathways in two different tissues of C. majus.

ACKNOWLEDGEMENTS

The authors would like to acknowledge University of Tabriz and Alborz University of Medical Sciences for financial support of this research.

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Chelidonine and Berberine from Iranian Chelidonium———JAST
