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Effect of environmental factors on sanguinarine and berberine levels in root of *Chelidonium majus* by HPLC- PDA/MS method

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ABSTRACT

Chelidonium majus is a well-known medicinal plant of papaveraceae family which demonstrates numerous therapeutic effects due to the presence of isoquinolide alkaloids such as sanguinarine and berberine. In this study, after validation of an HPLC method, levels of these alkaloids in root samples of this plant collected from north of Iran were measured, and environmental factors affecting the amount of these compounds were also investigated. In this investigation, 9 groups of plants from different spots of north Iran were collected, and concentration levels of these alkaloids were measured applying a validated High Performance Liquid Chromatography- Photo diode Array/Mass Spectrometry (HPLC-PDA/MS) method. Then, the impact of geographical parameters encompassing altitude, average temperature, longitude and latitude coordinates on alkaloid contents was assessed. The validated method was selective, with good resolution, excellent linearity (r^2> 0.99), high accuracy, sensitivity and precision. Also, the results illustrated that there was a direct correlation between longitude and altitude with the amount of alkaloids in plant, which means the more the levels of these variables, the more the amount of mentioned alkaloids. In an opposite manner, levels of the alkaloids reversely correlated with latitude coordinates and temperature, in a way that decreasing these variables resulted in raising the amount of the alkaloids. The validated method is a simple, fast, accurate, precise and robust. Also the environmental factors can impact on alkaloid contents and higher levels of alkaloids were associated with lower temperatures, high longitude, less latitude, and more altitude from sea level.

Keywords: *Chelidonium majus*, sanguinarine, berberine, altitude, longitude, latitude, temperature

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INTRODUCTION

Chelidonium majus is anherbaceous perennial plant of papaveraceae family which contains a range of isoquinoline alkaloids e.g. sanguinarine and berberine. These alkaloids have shown anti-microbial [1-3], anti-tumur [4, 5], anti-malarial [6] and anti-inflammatory activities[7].

The content of secondary metabolites can be affected by different factors encompassing ecosystem, location, climate, environmental parameters, and physical or chemical stresses, etc[8-11]. As a matter of the fact, response of plants to these variables, depends on their ecophysiology and life history traits, and dramatically varies from one species to another. Ecosystem factors such as latitude/longitude coordination, altitude, terrain and average temperature put crucial impacts on the content of metabolites in plants. Several studies have noted that when the climate and ecosystem characteristics vary, the production of metabolites is also altered. Wallis et al.[8]reported that

variations in climate and terrain affected the biosynthesis ability of pine to produce mono, di and sesquiterpenes, flavonoids and lignins. Results of their study showed that regardless of ecosystem type, inhabitants of more northerly and westerly positions, and at lower elevations, were associated with higher levels of secondary metabolites. Oloumi et al. [11]indicated that phenolic compounds in roots of *Glycyrrhiza glabra* can be altered as a result of changing climate variables.

Determination of isoquinoline alkaloids is performed using different techniques, namely thin layer chromatography (TLC)[12], nuclear magnetic resonance (NMR) spectroscopy[13], gas chromatography (GC), gas chromatography coupled to mass spectrometry (GC-MS) [14], and liquid chromatography coupled to mass spectrometry (LC-MS) [15-20]. In 2006, Kursinszki separated isoquinoline alkaloids of *Chelidonium majus* using an RP-HPLC method [19].

In this assessment, we determined concentration levels of singunarine and berberine alkaloids in *Chelidonium majus* from Iran. The aim of our study was to develop a new LC-PDA/MS method for quantification of these alkaloids in Iranian plant and to probe the influence of geographical variables on the contents of sanguinarine and berberine in *Chelidonium majus*. Results of this study will lead us to new insights for potent sources of these alkaloids and their optimum growing sites.

EXPERIMENTAL SECTION

Materials

All HPLC solvents were purchased from Merck Company (Darmstadt, Germany). Sanguinarine and berberine were obtained from Sigma (St Louis, MO, USA). Deionized water produced by Milli-Q system was utilized all over the experiments (Bedford, MA, USA).

Sample collection from plant natural ecosystem

Samples were randomly gatheredfrom natural ecosystems of *Chelidonium majus* in Northern Province of Iran, Mazandaranat may, 2014 and exact geographical location of the collection stations were determined and recorded using GPS (Table 1).The collected plants were confirmed by the Department of Botany, Shahid Beheshti University, Tehran, Iran and determined that they have similar morphology with the same age.

Table 1: collection sites of *Chelidonium majus*

Sample name	Height (m)	Latitude coordinates(N)	Longitude coordinates (E)	Average temperature (◦C)
R1	34	36 53' 11"	50 01' 06"	19.9
R2	36	37 12' 10"	50 55' 30"	21.6
R3	62	37 02' 21"	51 08' 05"	22.3
R4	240	36 46' 31"	51 37' 35"	22.0
R5	340	36 14' 26"	52 06' 14"	17.8
R6	591	36 29' 28"	52 14' 32"	19.0
R7	1359	36 25' 51"	52 37' 19"	23.0
R8	1462	35 81' 34"	54 33' 26"	16.8
R9	1901	35 52' 37"	58 07' 20"	16.4

Extraction procedure

50 ml hydrochloric acid (12 M) solution in methanol (0.5:100 v/v) was added to 1.0 gr of the dried and powdered root of the plant and the tube was placed in ultrasonic bath for 1 hour at 50°C. Then, sample was centrifuged at 6000 rpm for 10 min, and supernatant was collected.

As a clean-up step, chloroform was subsequently added to the supernatant so as to remove non-polar compounds. Finally, aqueous phase was alkaloids and purified alkaloids sediment was dissolved in 1 ml of methanol and was injected to high- performance liquid chromatography (HPLC).

High performance liquid chromatography- mass spectrometry (HPLC-MS)

HPLC system (Kenauer, Germany) equipped with K-1001 pump (Knauer, Germany), K-2008 PDA detector (Kenauer, Germany) and 20 µl loop was utilized. C_{18}Eurospher column (250×4.6 mm, 5 µm) was used. The mobile phase consisted of two different solutions, including solvent A, water (0.1% formic acid), and solvent B, acetonitrile. Separations were effected by a gradient elution program as follows: The initial mobile phase composition was 80% A, followed by a linear gradient to 10% A in 40 min and then from40–45 min were constant. The post-running time was 5 min. Flow rate of the mobile phase was 0.5 ml/min, and UV-VIS detector monitored the samples at 270 nm. Thermofisher Scientific LCQ ion trap mass spectrometer (Bremen, Germany) with mass

range of 100-4000 m/z was applied. Positive mode of Electrospray-Mass Spectrometry (ESI-MS) under capillary voltage of +2.0 KV and skimmer cone voltage of -20 V was applied.

Validation of the method

The HPLC method was validated in terms of precision, accuracy, and linearity according to ICH guidelines [21]. The accuracy of the assay method was evaluated in triplicate. The precision of the intra- and inter-day was evaluated by the repeated injection. Robustness of the method was demonstrated by changing the flow rate and wavelength. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting serial dilutions of solutions of the standards with known concentrations. The Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the signal-to-noise ratio of more than 3 times for LOD and 10 times for LOQ, respectively.

Statistical analysis

The experiment was carried out using a randomized complete block design (RCBD) considering five replications for each sample. The data were statistically analyzed by Statistical Analysis System (SAS) software and are mean ± SD (vertical bars) of five replications.

RESULTS AND DISCUSSION

Development of HPLC-PDA/MS

After extraction of isoquinoline alkaloids, a suitable HPLC method was developed and validated for determination of sanguinarine and berberine in root extract of *chelidinum majus*. In this favor, different compositions of the mobile phases were probed. According to the preliminary results, the mobile phase of acetonitrile/water (0.1% formic acid) accomplished in a gradient condition which was able to separate the favored alkaloids (Fig. 1). Prior to the full implementation in quantitative determination of the mentioned compounds, this method was comprehensively validated for its accuracy, linearity, robustness, specificity, precision and intermediate precision under various modified conditions.

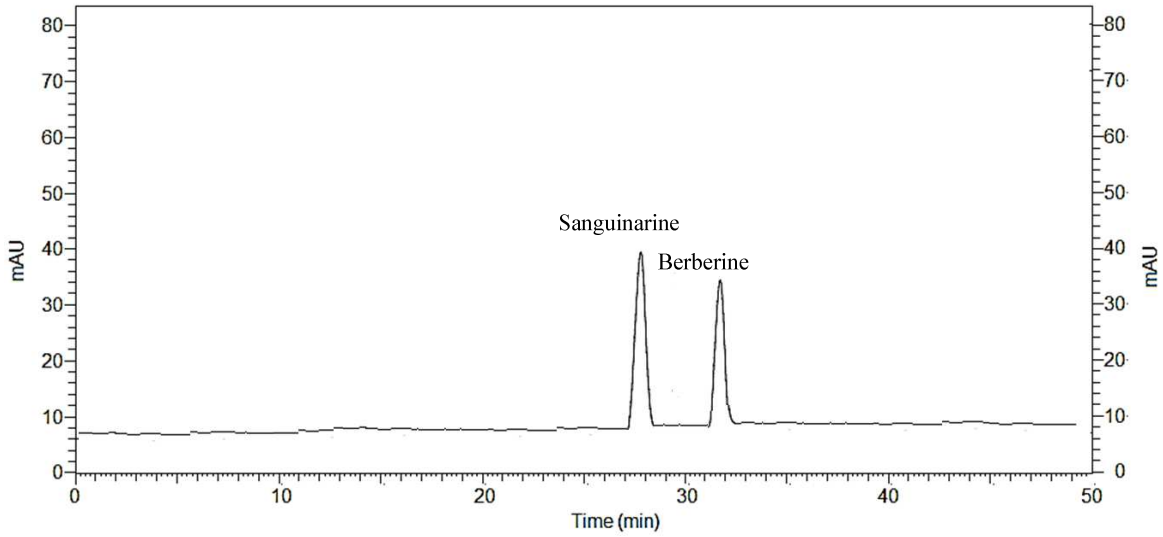


Fig. 1. Typical HPLC chromatograms of mixed standards using gradient method, Conditions: C18 Eurospher column (250×4.6 mm, 5 µm); PDA detection at 270 nm; mobile phase, (A) acetonitrile (B) 0.1 % formic acid; flow rate, 0.5 mL/min.

Six concentration levels were prepared and subjected to HPLC, and the corresponding peak areas were utilized so as to draw the calibration curves. Excellent linearity in a range of 0.10-30.0 µg/ml was achieved and the regression equations were y = 180212x-18.1816 with a correlation coefficient of 0.9969, and y= 100522x+5417.7 with a correlation coefficient of 0.9944 for sanguinarine and berberine, respectively. Limit of detection (LOD) and limit of quantification (LOQ) indicate the sensitivity of method and were low (Table 2). Calculated %RSD for peak areas related to triplicate injections of the standards was found to be less than 1.5%.These results indicate that the proposed HPLC method is sufficiently sensitive for the determination and quantitation of sanguinarine and berberine in *chelidinum majus* at low concentrations.

Table 2. Linear regression data, LOD and LOQ of sanguinarine and berberine

Analytes	Regression equations	R	Linear renge (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)

sanguinarine	y = 180212x-18.1816	0.9969	0.1-30	0.04	0.14
berberine	y= 100522x+5417.7	0.9935	0.1-30	0.06	0.20

Y=Ax+B, y is peak area; x is concentration of the alkaloids (µg/mL); r is the correlation coefficient of the equation.

The accuracy of the method was determined using recovery test. After the addition of accurate amount of each standard at three levels (10, 20 and 30%) to the extract, we analyzed it by the proposed HPLC method. The recoveries were calculated and are reported in Table 3. The recoveries obtained were close to 100% in almost all cases and this method can be considered accurate. To evaluate the precision of the method we repeated it by assaying 5 replicate injections of standards at the same concentration, during the same day and 5 continuous days. The intra-day precision was <0.7%, and inter-day precision was <1.1% for the alkaloid standards (Table 3). Since the results were within the acceptable range confirm the accuracy and precision of the method.

Robustness was evaluated to ensure that the HPLC method is insensitive to small changes in the experimental conditions. In order to assess the robustness of the method, we modified several parameters, such as flow rate from 0.5 to 0.6 mL/min, and wavelength from 270 nm to 280, and no significant changes were observed in the resolution or response of the standard peaks. The results indicated good linearity, sensitivity, accuracy, precision, specificity, and robustness of this method to be suitable for the analysis of alkaloids in *Chelidinum majus*.

Table 3. Recovery, intra- and inter-day precision of HPLC assay of alkaloids in *Chelidinum majus*

Analytes	Recovery (%)	RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)
sanguinarine	95.21	<1.2	<0.6	<0.9
berberine	94.77	<1.0	<0.7	<1.1

In order to exhibit the ability of the method for the analysis of the root extract of *Chelidonium majus*, crude extract was subjected to HPLC under optimum chromatographic separation conditions (Fig. 2). Moreover, for identification of the related peaks of the sanguinarine and berberine, crude extract was spiked with standard solutions, and the corresponding mass spectra were acquired (Fig.3).

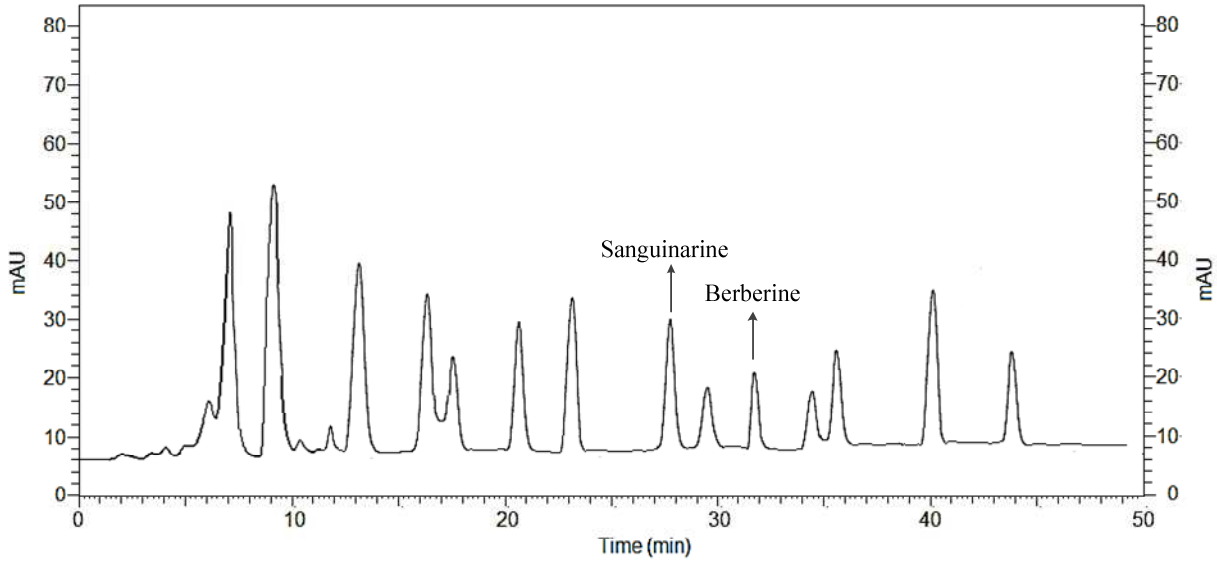


Fig.2. HPLC chromatogram of extract using gradient method, Conditions: C18 Eurospher column (250×4.6 mm, 5 µm); PDA detection at 270 nm; mobile phase, (A) acetonitrile (B) 0.1 % formic acid; flow rate, 0.5 mL/min

Quantification of sanguinarine and berberine were performed using calibration curves. Results displayed that sanguinarine occurs in much more amounts than berberine within root samples of *Chelidonium majus*(Table 4). Quantification of the two alkaloids in collected samples revealed that concentration levels of sangunarine and Berberine varied from 0.1091 to 0.0187, and 0.0059 to 0.0146 mg/g of plants dry weight, respectively.

Table 4. Amount of sanguinarine and berberine in root samples of *Chelidonium majus*

Root Samples	Concentration of sanguinarine (mg/g dw)	Concentration of berberine (mg/g dw)
R1	0.0229	0.0079
R2	0.0204	0.0059
R3	0.0213	0.0066
R4	0.0300	0.0078
R5	0.0510	0.0112
R6	0.0340	0.0094

R7	0.0404	0.0117
R8	0.0800	0.0146
R9	0.1091	0.0156

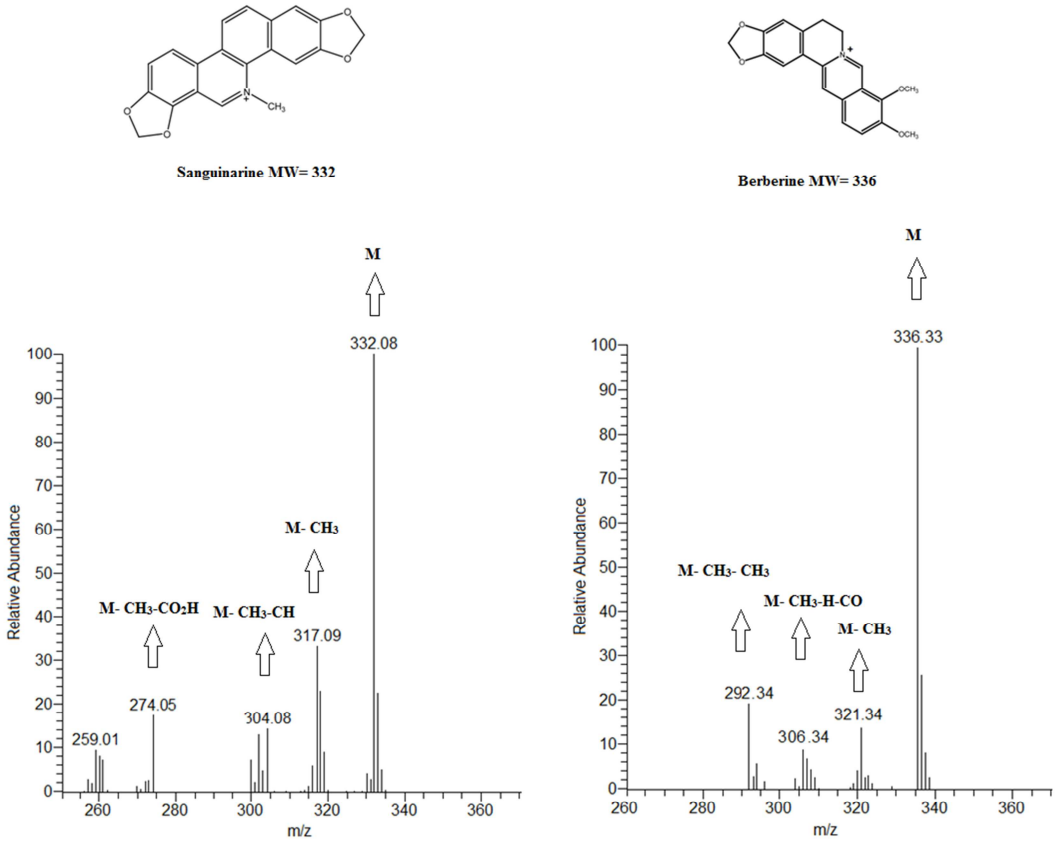


Fig. 3. full ESI mass spectrum of A) sanguinarine and B) berberine

Correlation of sanguinarine and berberine with environmental factors

After quantification of sanguinarine and berberine in root extracts, dependence of the content of these compounds on environmental variables including altitude, latitude and longitude coordinates and average temperature were evaluated. As it is obvious fromFig.4a, quantification of both alkaloids exhibited a direct relation between alkaloid content and the altitude of the plant collection site. According to the results, as the altitude(height) of the plant ecosystem increased, content of both alkaloids raised in the root of plant. On the other hand, a distinct behavior appeared with the impact of longitude and latitude on the contents of alkaloids in the plants (Fig.4b and 4c), in a way that increasing the longitude coordinates caused an upturn in contents of both alkaloids, while a rise in the latitude coordinates declined their contents. Moreover, there is a reverse relation between the contents of the alkaloids and temperature of the plant growth site (Fig.4d). As the average temperature of the plant growing site rises, a downwards trend is observed for alkaloid contents in root samples. This implies that temperature has a negative effect on the content of alkaloids in this plant. Therefore, results indicate that inhabitants in more northerly and less westerly positions, and at higher elevations, were accompanied by higher alkaloid levels. As it is proved by the previous studies as well, variations in environmental variables such as latitude and climate resulted in enhancement of biosynthesis of the secondary metabolites or storage of them, as a defense mechanism [8-11]. Almost always, increasing the altitude is associated with lower temperatures of the plant growing site. This may shed light on the approximately similar behavior of variations in alkaloid contents towards higher altitudes and lower temperatures, since they both have the same effect on alkaloid concentration levels. Bhatt et al. [22] observed an upwards trend for concentration levels of secondary metabolites, as the plant growing sites were located in western and northern spots of Himalayan Mountain. The same manner was concluded in our study, in a way that northern (upper longitude) and western (higher latitude) situations exhibited higher levels of alkaloids. Since the Alborz and Zagroos mountains are located in western and northern parts of Iran, it would be rational to observe lower altitudes associated with eastern and central flat lands, and hence reduced contents of the alkaloids in eastern /southern growing sites.

Berberine bridge enzyme (BBE) isolated from *Eschscholzia californica*, is a plant enzyme participating in alkaloid biosynthesis via catalyzing the challenging oxidative cyclization of (*S*)-reticuline to(*S*)-scoulerine (Scheme 1) [23]. This enzyme contributes to the production of benzophenanthridine alkaloids as a defense mechanism in response of plants to pathogens. In our study, a downwards trend for alkaloid contents appeared, as the temperature of the plant growing site increased. Effect of lower temperatures on key enzymes of the biosynthesis of isoquiniline alkaloids, probably BBE, may account for this. Lower temperature is considered as an environmental abiotic stress and can cause responses in plant in which alkaloids may significantly take part. Regarding the results of previous investigations as well as those of ours, we suggest that environmental variables can put substantial impact on alkaloid contents. Higher concentrations of sanguinarine and berberine in *Chelidonium majus* were associated with lower temperatures, high longitude, less latitude and more altitude from sea level.

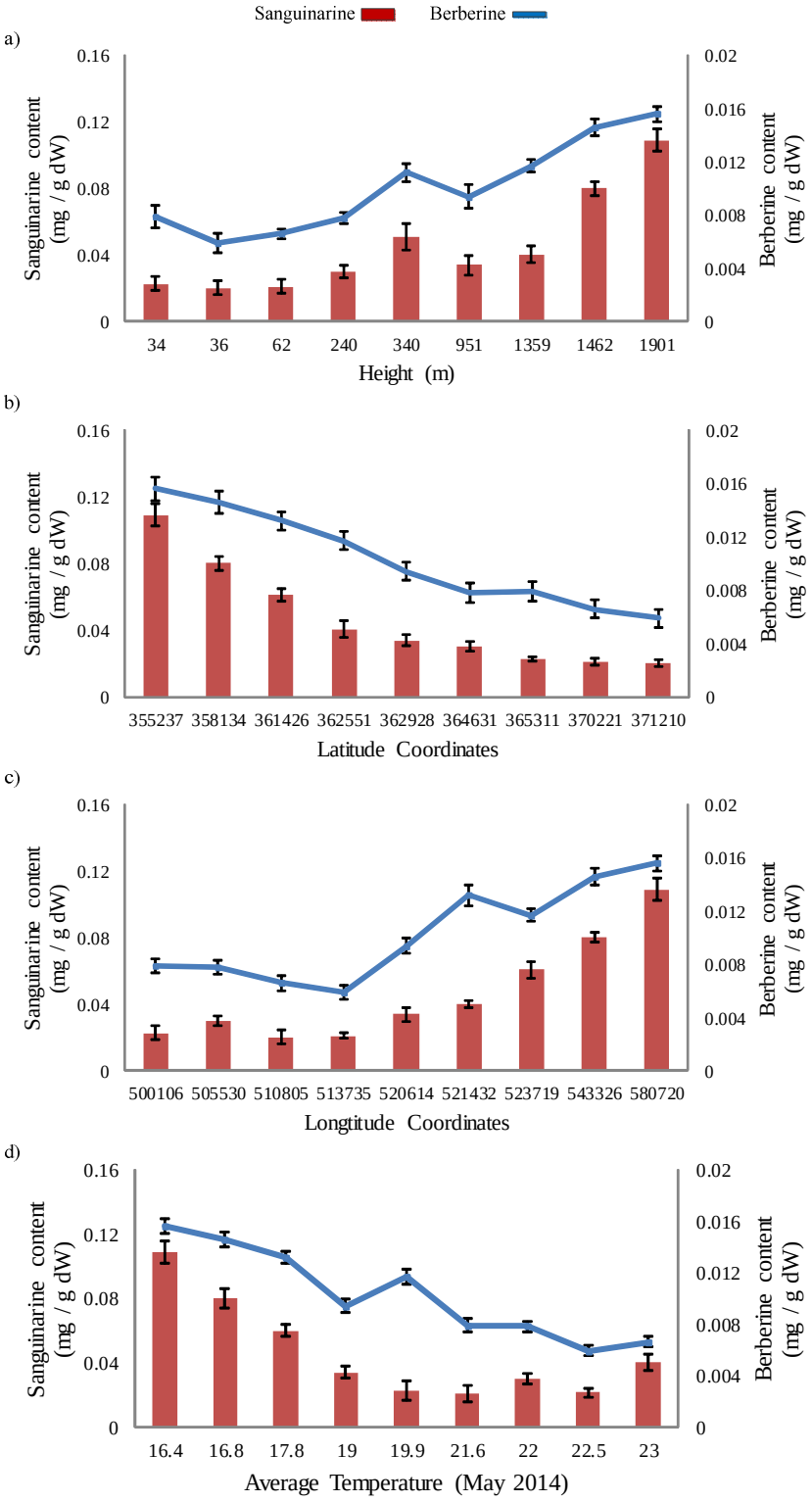
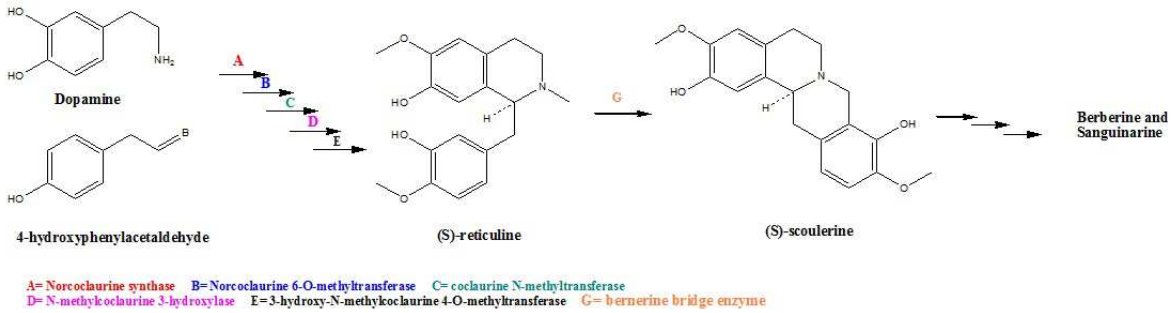


Fig. 4. Correlation between amount of sanguinarin and berberine with A) altitude, B) Latitude, C) Longitude and D) Average temperature



Scheme 1. biosynthesis pathway of sanguinarine and berberine

CONCLUSION

This work proposes a validated method for quantification of sanguinarine and berberine in *Chelidonium majus*. This method is a simple, fast, accurate, precise and robust. Also we conclude that environmental variables can put substantial impact on alkaloid contents and higher concentrations of sanguinarine and berberine were associated with lower temperatures, high longitude, less latitude, and more altitude from sea level.

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REFERENCES

[1] Wongbutdee J. *Thai Pharm Health Sci J* **2008**;4(1):78-83.
[2] Dzink JL, Socransky SS. *Antimicrob Agent Chem* **1985**;27(4):663-665.
[3] Facchini PJ. *An Rev Plant Physiol Plant Mol Biol* **2001**;52:29–66.
[4] Sun Y, Xun K, Wang Y, Chen W. *Anti-Cancer Drug* **2009**;20(9):757–769.
[5] Simanek V. Benzophenanthridine alkaloids. In: Brossi A ed.The Alkaloids. New York, NY, Academic Press; **1985**;26:185–240.
[6] Syed Abd Aziz SS, Mat RopiMukhtar MR, Hadi HA, Abdullah NR, Awang K. *J Sains Mat* **2009**;1(2):80 -86.
[7] Lenfeld J, Kroutil M, Marsalek E, Slavik J, Preininger V, Simanek V. *Planta Med* **1981**;43(2):161–165.
[8] Singh SP,Parmar SS, Stenberg VI, Farnum SA. *J Hetero Chem* **1977**;15(4):541-544.
[9] Anna Petruczynik A, Waksmundzka-Hajnos M, Plech T, Tuzimski T, Hajnos ML, Józwiak G, Gadzikowska M, Rompała A. *J Chromatogr Sci* **2008**;46:291-297.
[10] Suau R, Cabezudo B, Valpuesta M, Posadas N, Diaz A, Torres G. *Phytochem Anal* **2005**;16:322–327.
[11] Luo XB, Chen B, Yao SZ. *Phytochem Anal* **2006**;17:431-438.
[12] Kursinszki L, Sarkozi A, Kery A, Szoke E. *Chromatogra* **2006**;63:S131-S135.
[13] Ma C, Fan M, Tang Y, Li Z, Sun Z, Ye G, Huang C. *Biomed Chromatogr* **2008**;22:835–850.
[14] YueGu Y, Qian D, Duan J, Wang Z, Guo J, Tang Y, Guo S. *J Sep Sci* **2010**;33:1004–1009.
[15] Sarkozi A, Janicsa G, Kursinszki L, Kery A. *Chromatogr* **2006**;63:S81-S86.
[16] Chang-Qun N, Li-Yi H. *J Chromatogr A* **1991**;542:193-199.
[17] Christopher M, Wallis CM, Huber DPW, Lewis KJ. *JChem Ecol* **2011**;37:607–621.
[18] Sergio Rasmann S, Agrawal AA. *Ecol Let* **2011**;14:476–483.
[19] Jaakola L, Hohtala A. *Plant Cell Envir* **2010**;33(8):1239-1247.
[20] Oloumi H, Hassibi N. *J Med Plant Res* **2011**;5(25):6011-6016.
[21] ICH Q2B: Validation of analytical procedures. London: methodology (CPMP/ ICH/281/ 95); **1996**.
[22] Harish C, Andola HC, Gaira KS, Rawal RS, Rawat MSM, Bhatt ID. *J Herb Spi Med Plant* **2011**;17:329–338.
[23] Glenn WS, Runguphan W, Oconnor SE. *Cur Opin in Biotech* **2013**;24(2):354–365.