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ANALYTICAL STUDIES OF (+)-CHELIDONINE, PROTOPINE, AND *l*-STYLOPINE IN *Chelidonium majus* GROWING IN GEORGIA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Chelidonium majus L. (greater celandine, swallowwort) is a medicinal plant and is the only species in the genus *Chelidonium* of the Papaveraceae family. *Ch. majus* contains various isoquinoline alkaloids: protoberberine and compounds with tertiary and quaternary benzo (c) phenanthridine structure [1–8]. The commercial drug (herb of *Chelidonium*) consists of dried aerial parts harvested during flowering time and is described in several European pharmacopoeias [9].

The major components of the drug are the alkaloids chelidonine, chelerithrine, sanguinarine, coptisine, protopine, desstylopine, and similar compounds. The *in vitro* cytotoxic activity of these compounds on human and animal tumor cell cultures considered with great interest because they could lead to promising cancer treatments [10–12].

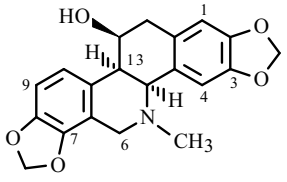
Earlier [13] it was shown that the aerial parts of *Ch. majus* growing in Georgia can serve as a good raw material for obtaining the equivalent crude drug and the purified components.

The present investigation reports the isolation of (+)-chelidonine, protopine, and *l*-stylopine from roots and shoots of *Ch. majus* and their determination by HPLC in the crude extract of the aerial and underground parts of *Ch. majus* collected during and after flowering time.

Plant Material. The plant material of *Ch. majus* was collected in April–May during the flowering phase and in June during seed ripening (in the experimental field of the Institute of Pharmacochemistry, Tbilisi, Georgia, 2008). A voucher specimen of the plant was deposited in the herbarium (# 281) of the Department of Pharmacobotany (head of the Department Sci. D. M. Churadze), Institute of Pharmacochemisty, Tbilisi, Georgia.

Sample Preparation. The dried powdered aerial parts (each sample 400 g) and roots (200 g) of *Ch. majus* were extracted in a Soxhlet apparatus with ethanol. The obtained extract was evaporated under reduced pressure. The residues were dissolved in 0.25 M sulfuric acid and filtered. The acidic layer was alkalized to pH 9 with ammonia (25%). The solution was extracted with chloroform, and the organic fraction was dried with anhydrous sodium sulfate and filtered; the chloroform fraction was concentrated under vacuum. Samples for HPLC analyses were taken from the obtained crude extracts.

Isolation of (+)-Chelidonine, *l*-Stylopine, and Protopine from the Crude Extract of *Ch. majus*. The crude alkaloid extract was isolated from *Ch. majus* (4.5 kg dried, powdered plant) by extraction in a percolator by the described method. The extract (10 g) was fractioned on a silica gel column (400 g). Elution was performed with chloroform followed by chloroform gradually enriched with methanol (98:2, 96:4, 92:8, 50:50). Elution was monitored by TLC.



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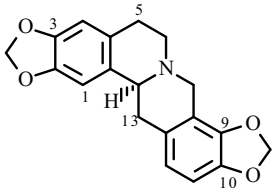

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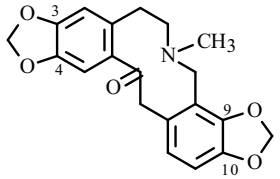

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TABLE 1. Data for Calibration Graphs

Alkaloid	Regression equation	Correlation coefficient rI	Linear range, mg/mL
(+)-Chelidonine (**1**)	y = 8342.01161x + 59.12244	0.99899	0.00898 – 1.19
l-Stylopine (**2**)	y = 8619.13252x + 72.87841	0.99955	0.00828 – 1.16
Protopine (**3**)	y = 7456.54270x – 143.83777	0.99819	0.00828 – 1.41

TABLE 2. ^{13}C Chemical shifts of (+)-Chelidonine, Protopine, and *l*-Stylopine

C atom	(+)-Chelidonine	C atom	Protopine	C atom	*l*-Stylopine
1	107.4	1	108.17	1	105.53
1a	128.9	2	145.89	2	144.96
2	146.6	3	148.01	3	145.97
3	145.3	4	110.49	4	108.43
4	111.9	4a	136.18	4a	127.75
4a	125.8	5	31.81	5	29.58
6	53.56	6	57.82	6	51.29
6a	117.1	8	50.82	8	52.93
7	143.1	8a	117.91	8a	116.86
8	148.6	9	146.34	9	147.5
9	109.6	10	146.0	10	143.26
10	120.4	11	106.74	11	106.75
10a	131.1	12	125.08	12	121.05
11	72.4	12a	128.99	12a	128.54
12	39.7	13	46.50	13	36.49
13	42.6	14	–	14	59.76
14	62.9	14a	132.79	14a	130.70
N-CH_3	41.96	N-CH_3	41.47		
2,3-OCH_2O	101.1	2,3-OCH_2O	101.2	2,3-OCH_2O	100.79
7,8-OCH_2O	101.25	9,10-OCH_2O	100.86	9,10-OCH_2O	101.04

The obtained alkaloids were (+)-chelidonine (1.65 g), protopine (1.23 g), and *l*-stylopine (0.69 g).

The alkaloids were refined on a silica gel column (partial size 0.06–0.2 mm) and eluted with dichloroethane gradually enriched with methanol or chloroform gradually enriched with methanol.

The purity of the samples was verified on TLC plates (silica gel 60, Merck) with a mixture of solvents 1-propanol–water–formic acid (90:9:1) [8]. The separated spots were detected with Dragendorff reagent [14].

Their purities were: (+)-chelidonine – 100%, protopine – 97%, and *l*-stylopine – 99%.

Their identity was confirmed by ^1H and ^{13}C NMR correlation spectroscopy methods.

Materials. Acetonitrile and methanol were of HPLC purity. The solvents were purchased from VWR (Canada). The samples were isolated from the crude extract of celandine. The samples were dissolved in methanol to give a concentration of 1 mg/mL.

Apparatus. The qualitative and quantitative analysis of the alkaloids of celandine was carried out using a "Agilent-1100 series" (6JG46002) high-performance liquid chromatograph.

^1H and ^{13}C NMR spectra were obtained on a Avance 400 Bruker spectrometer (400.13 MHz for ^1H, 100.61 MHz for ^{13}C spectra) equipped with a 5 mm QNP-probe.

Optical rotation was measured using a Lipik-Rudiolf polarimeter at 20°C.

HPLC Conditions. A reversed-phase system consisting of silica gel column ODS (250 × 6.0 mm), partial size 10 μm, temperature 25°C, was used. A mixture of solvent A – water, pH 7.3 (buffered with Tris and HCl) and solvent B – acetonitrile (ACN) was used as a mobile phase. The flow rate was 1 mL/min, the injection volume was 10 μL, and UV absorbance detection was at 280 nm and run time 60 min.

Determination of Alkaloids by HPLC. The separation of protopine, (+)-chelidonine, and *l*-stylopine, using as the mobile phase a mixture of pH 7.3 water (with addition of Tris and HCl), and ACN, flow rate 1mL/min, UV detection at 280 nm, injection volume 10 mL, showed retention times for each alkaloid to be 17.254–17.264, 45.372–45.375, and 54.041–54.043 min, respectively

Since the purity of protopine was 97%, analytical studies of these alkaloids in the crude extracts of *Ch. majus* was not conducted using HPLC.

***Ch. majus* Harvested During Seed Ripening.** (+)-Chelidonine and *l*-stylopine were the major components of the crude drug from the aerial parts of celandine (collected during flowering time). Their content is about 6.4% and 3.1% of the total alkaloid fraction, respectively.

In comparison with those observed from the underground parts of the same plants harvested on the same date, *l*-stylopine was the main alkaloid in comparison with (+)-chelidonine, but protopine was not found.

***Ch. majus* Harvested After Seed Ripening.** (+)-Chelidonine was the minor component of the crude drug from the aerial parts, but *l*-stylopine was the major alkaloid in comparison with (+)-chelidonine. Their content is about 5% and 21.2% of the total alkaloid fraction, respectively.

However (+)-chelidonine was the main alkaloid in the underground parts of the same plant harvested at the same date. Its content is about 26% of the total alkaloid fraction.

Thus, the importance of seasonal changes in alkaloid contents in *Ch. majus* was established.

Moreover, this correlated with the high percentage of (+)-chelidonine and *l*-stylopine in the siliques and with the variation of the qualitative and quantitative composition of alkaloids from *Ch. majus* during its growth.

***Ch. majus* Harvested During Flowering Period.** Apart from identification of (+)-chelidonine, *l*-stylopine, and protopine, several other small peaks may also be due to the presence of alkaloids, but in the absence of appropriate standards, their identification was not possible.

The linear regression of individual (+)-chelidonine, *l*-stylopine, and protopine solutions confer between 0.00898 – 1.19 mg/mL, 0.00828–1.16 mg/mL, 0.00828–1.41 mg/mL, respectively. Aside from the amount of each alkaloid injected and the intensity of the absorption at 280 nm, other data for calibration graphs are given in Table 1.

Chemicals. During the preliminary separation of the crude alkaloids of *Ch. majus* through a silica gel column (see experiment), the fraction obtained by elution with chloroform yielded (+)-chelidonine (**1**), and that by elution with mixtures of chloroform gradually enriched with methanol yielded *L*-stylopine (**2**), and protopine (**3**).

(+)-Chelidonine, $C_{20}H_{19}NO_5$, mp 136–137°C (EtOH); $[\alpha]_D$ +118° (*c* 0.5, EtOH). UV (λ_{max}, nm, lg ε): 205 (4.85), 238 (3.96), 289 (3.91). ^{1}H NMR ($CDCl_3$, δ, ppm, J/Hz): 2.3 (3H, s, NCH_3), 2.98 (1H, tr, H-13), 3.09, 3.23 (2H, dd, J = 18, H-12), 3.43, 4.09 (2H, dd, J = 15.8, H-6), 3.58 (1H, br.s, H-14), 4.24 (1H, br.s, H-11), 5.93, 5.94 (2H, dd, J = 1.4, J = 1.4, 7,8-OCH_2O), 5.96, 6.00 (2H, dd, J = 1.26, J = 1.4, 2,3-OCH_2O), 6.65 (2H, s, H-1), 6.67 (2H, s, H-4), 6.75, 6.78 (2H, dd, J = 7, J = 7, H-9, 10). ^{13}C NMR ($CDCl_3$, δ): see Table 2.

Protopine, $C_{20}H_{19}NO_5$, mp 207–208°C (MeOH); $[\alpha]_D$ 0° (*c* 0.5; MeOH). UV (λ_{max}, nm, lg ε): 205 (4.99) 238 (4.00), 289 (4.00). ^{1}H NMR (MeOD, δ, ppm): 1.94 (3H, s, N-CH_3), 6.9 (1H, s, H-1), 6.65 (1H, s, H-4), 6.7 (2H, s, H-11, 12), 5.92 (2H, s, 2,3-OCH_2O), 5.98 (2H, s, 9,10-OCH_2O), 2.4–2.7 (4H, m), 2.9–3.7 (4H, m). ^{13}C NMR ($CDCl_3$, δ): see Table 2.

***l*-Stylopine**, $C_{19}H_{17}NO_4$, mp 204–205°C; $[\alpha]_D$ –315° (*c* 0.5; $CHCl_3$). ^{1}H NMR (MeOD, δ, ppm, J/Hz): 6.72 (1H, s, H-1), 6.67 (1H, d, J = 8.0, H-11), 6.62 (1H, d, J = 8, H-12), 6.58 (1H, s, H-4), 5.95 (1H, d, J = 1.5, OCH_2O), 5.91 (2H, s, OCH_2O), 5.96 (1H, d, J = 1.5, OCH_2O), 4.08 (1H, d, J = 15, H-8b), 3.55 (1H, dd, J = 11, 3.4, H-14), 3.54 (1H, d, J = 15, H-8a), 3.12 (2H, m, H-5b/H-6b), 2.8–3.21 (2H, qd, J = 16, 11.5; 3.7, H-13), 2.63 (2H, m, H-6a/H-5a). ^{13}C NMR ($CDCl_3$, δ): see Table 2.

The physical and ^{1}H, ^{13}C NMR spectral data, including DQF-COSY, HMBC, and HSQC spectra, obtained for the structural formula of (+)-chelidonine, protopine, and *l*-stylopine are in agreement with literature data [1, 5, 14].

The increased interest shown for the alkaloids of celandine stem from the potential use of their derivatives in medicine. The method described here may be applied in further studies directed towards the isolation and identification of alkaloids of biological and chemical interest.

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