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DETERMINATION OF PROTOPINE IN *FUMARIA DENSIFLORA* DC. BY TLC-DENSITOMETRIC AND SPECTROPHOTOMETRIC METHOD

FUMARIA DENSIFLORA DC.' DA OPTİK DANSİTOMETRİK VE SPEKTROFOTOMETRİK YÖNTEMLE PROTOPİN MİKTAR TAYİNİ

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ABSTRACT

*The isoquinoline alkaloid protopine in Fumaria densiflora DC. (**Fumariaceae**) was quantitatively determined by TLC-densitometry and spectrophotometry. In TLC-densitometry two different solvent systems as toluene : chloroform : methanol : % 25 ammonium hydroxide (5:3:1:1) and cyclohexane : diethylamine (9:1) were used and protopine was detected as 0.351 and 0.352 % respectively. The content of protopine in Fumaria densiflora DC. was estimated as 0.366 % in spectrophotometric method. The quantitative data obtained by the use of two techniques were in good agreement.*

Key Words: *Fumaria densiflora DC., Fumariaceae, Protopine*

ÖZET

*Fumaria densiflora DC.' daki (**Fumariaceae**) izokinolin alkaloitlerinden olan protopinin % miktarı optik dansitometrik ve spektrofotometrik yöntemlerle saptanmıştır. Optik dansitometrik yöntemde toluen: kloroform: metanol : %25 amonyum hidroksit (5:3:1:1) ve siklohekzan: dietilamin (9:1) olmak üzere iki farklı solvan sistemi uygulanmış olup protopin miktarı % 0.351 ve % 0.352 olarak bulunmuştur. Spektrofotometrik yöntemde ise Fumaria densiflora DC.' nın protopin miktarı % 0.366 olarak saptanmıştır. Çalışmamız sonucunda bu iki yöntemde bulunan değerlerin birbiriyle uyumlu olduğu görülmüştür.*

Anahtar Kelimeler: *Fumaria densiflora DC., Fumariaceae, Protopin*

INTRODUCTION

Plants of the genus *Fumaria* have been used in traditional medicine as anti-hypertensives, diuretics, hepatoprotectants and laxatives (to treat gastrointestinal disorders), as well as in the treatment of some skin diseases (raches or conjunctivitis) (1,2). The biological activity of *Fumaria* is mostly associated with the presence of isoquinoline alkaloids in the plant. In the last few years, a large number of scientific reports have been described the properties of *Fumaria* (3,4). There are seventeen wild-growing species belonging to this genus (5,6).

Protopine is the most usual alkaloid found in *Fumaria* whatever type it is the active family of compound of the plant (7). It is an isoquinoline alkaloid with multiple pharmacological actions, such as inhibition of calcium influx through both voltage and receptor-operated channels (8) and inhibition of rabbit blood platelet aggregation (9). It also posesses anti-cholinergic and anti-histaminic (10) as well as anti-bacterial (11) activities. Structure of protopine is shown figure 1.

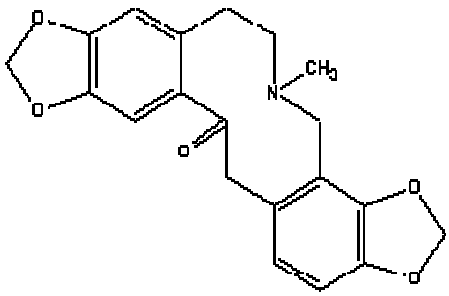

Figure 1. Protopine

The alkaloids isolated from the previous studies on *Fumaria* species are shown in table 1.

Table 1. The alkaloids isolated from *Fumaria* species

SPECİES	NAME OF ALKALOIDS	NUMBER OF LITERATURE
Fumaria capreolata L.	Sanguinarine, protopine, cryptopine, β-allocryptopine, fumaritine, l-stylopine, coptisine, l-capnoidine, isoboldine, (+)-bicuculline, (+)-fumariline, (+)-parfumine, (-)-cheilanthifoline, (-)-scoulerine, adlumiceine, fumariline, fumarophycine, N-methylstylopine	12, 18, 21, 27
Fumaria officinalis L.	Sanguinarine, protopine, cryptopine fumariline, d-corydaline, l-sinactine, dl-bicuculline, fumarofine, l-scoulerine, fumaritine, corytuberine, parfumine, fumaricine, adlumidiceine, O-metylfumarofycine, stylopine, fumaroficine, palmatine, adlumiceine, N-methylstylopine	13, 19, 26, 27
Fumaria vaillantii Loisel.	Oxysanguinarine, (±)-8-acetonyldihydrosanguinarine, (±)-8-methoxydihydrosanguinarine, fumaramidine, dihydrosanguinarine, norsanguinarine, (+)-isocorydine, (-)-cordeline, (+)-juziphine, fumaricine, fumarophycine, O-methylfumarophycine	14, 27

Fumaria gaillardotii Boiss.	Protopine, fumaritine, fumaricine, l-stylopine, dl-bicuculline, N-methylhydrastine	15
Fumaria judaica Boiss.	Protopine, d-adlumidine, β-allocyrtopine, l-stylopine, fumaritine, coptisine, l-scoulerine	16
Fumaria macrocarpa Parlatore	Protopine, crytopine, fumariline, l-sinactine, α-adlumine, coptisine	17
Fumaria cilicica Hausskn.	Sanguinarine, protopine, cryptopine, d-corydaline, l-sinactine, dl-bicuculline, l-scoulerine	19
Fumaria densiflora DC.	Fumaramine, protopine, cryptopine, coptisine, palmatine, adlumidecine, sinactine, densiflorine, parfumine, parfumidine, fumaritine, cheilanthifoline, (±)-scoularine, isosalutaridine, (±)-aldumidine, N-methylhydrasteine, fumariline, (±)-bicuculline, β-allocrptopine, fumaricine, fumaritridine, fumarophycine, fumarofine, corytuberine, cis-N-methylstylopinium iodide, stylopine, fumaflorine, N-metylstylopine, dihydrosanguinarine	20, 23, 25, 26, 29
Fumaria bella P.D.	Isoboldine, sanguinarine, (+)-bicuculline, coptisine, protopine, (+)-fumariline, (+)-parfumine, (-)-cheilanthifoline, (-)-scoulerine, (-)-stylopine, (+)-adlumine, (+)-parfumine	21
Fumaria asepala Boiss.	Protopine, cryptopine, l-stylopine, l-scoulerine, d-bicuculline, sanguinarine	22
Fumaria parviflora Lam.	Protopine, parfumine, d-bicuculline, hydrastine, N-methlhydrastine, N-methylhydrasteine, microcarpine, sanguinarine, adlumiceine, coptisine, fumaritine, sinactine, N-methylstylopine	24, 27
Fumaria petteri Reichb. subsp. *thuretii*	Protopine, fumariline, fumaritine, fumaricine, l-sinactine, l-scoulerine, coptisine, l-capnoidine	24
Fumaria kralikii Jordan	Protopine, cryptopine, parfumine, l-scoulerine, d-bicuculline, d-adlumine, N-methylhydrasteine, sanguinarine	24
Fumaria schramii Pugsley	Fumaritine, corytuberine, parfumine, fumaricine, adlumidicein, adlumicein, N-metylstylopine, coptisine, O-metylfumaroficine, stylopine, fumaroficine, cryotopine, sinaktin, protopine, fumariline, palmatine	26
Fumaria agraria Lag.	Adlumiceine, coptisine, cryptopine, fumariline, fumaritine, fumarophycine, parfumine, N-methylstylopine, Domesticine, (+)-isoboldine, dihydrosanguinarine, noroxyhydrastinine, oxyydrastinine, (-)-stylopine, protopine, (-)-fumaritine-N-oxide, (+)-parfumine	27, 28
Fumaria muralis	Coptisine, cryptopine, fumariline, fumaritine, fumarophycine, O-methylfumarophycine, parfumine, N-methylstylopine	27
Fumaria spicata L.	Adlumiceine, coptisine, fumariline, fumarophycine, protopine, N-methylstylopine	27
Fumaria sepium Boiss.	Dihydrosanguinarine, noroxyhydrastinine, oxyydrastinine, coptisine, (-)-stylopine, protopine, densiflorine, (-)-fumaritine-N-oxide, (+)-parfumine	28
Fumaria indica Hasskn.	Fuyuziphine, (±)-α-hydrastine	30

The alkaloids determinated from the previous studies on *Fumaria* species are shown in table 2.

Table 2. The alkaloids determinated from *Fumaria* species

SPECIES	**NAME AND QUANTITY OF ALKALOIDS**	**METHOD OF DETERMINATION**	**NUMBER OF LITERATURE**
Fumaria gaillardotii Boiss.	Protopine (% 0.11), fumaritine (% 0.05), fumaricine (%0.09), l-stylopine (% 0.09), dl-bicuculline (% 0.09), N-methylhydrastine (% 0.11)	HPLC	15
Fumaria judaica Boiss.	Protopine (% 0.18), d-adlumidine (% 0.17), β-allocyrtopine (% 0.11), l-stylopine (% 0.11), fumaritine (% 0.17), coptisine(% 0.04), l-scoulerine (% 0.03)	HPLC	16
Fumaria macrocarpa Parl.	Protopine (% 0.16), crytopine (% 0.11), fumariline (% 0.11), l-sinactine (% 0.13), α-adlumine (% 0.13), coptisine (% 0.07)	HPLC	17
Fumaria capreolata L.	Protopine (% 0.11), cryptopine (% 0.09), β-allocryptopine (% 0.09), fumaritine (% 0.10), l-stylopine (% 0.09), coptisine (% 0.05), l-capnoidine (% 0.12), sanguinarine (% 0.05)	HPLC	18
Fumaria officinalis L.	Sanguinarine (% 0.05), protopine (% 0.17), cryptopine (% 0.31), fumariline (% 0.14), d-corydaline (% 0.09), l-sinactine (% 0.05), dl-bicuculline (% 0.10), fumarofine (% 0.19), l-scoulerine (% 0.10)	HPLC	19
Fumaria cilicica Hausskn.	Sanguinarine (% 0.09), protopine (% 0.19), cryptopine (% 0.11), d-corydaline (% 0.18), l-sinactine (% 0.09), dl-bicuculline (% 0.15), l-scoulerine (% 0.06)	HPLC	19
Fumaria asepala Boiss.	Protopine (% 0.16), cryptopine (% 0.08), l-stylopine (% 0.15), l-scoulerine (% 0.05), d-bicuculline (% 0.12), sanguinarine (% 0.04)	HPLC	22
Fumaria capreolata L.	Protopine (% 0.12-0.11), fumarofine (% 0.10-0.09), cryptopine (% 0.09-0.09)	d.p.p. (differential pulse polarography)-HPLC	23
Fumaria asepela Boiss.	Protopine (% 0.18-0.17), cryptopine (% 0.13-0.12)	d.p.p. - HPLC	23
Fumaria gaillardotii Boiss.	Protopine (% 0.12-0.11)	d.p.p. - HPLC	23
Fumaria kraliki Jordan.	Protopine (% 0.40-0.38), parfumine (% 0.13-0.12), cryptopine (% 0.14-0.11)	d.d.p.-HPLC	23
Fumaria macrocarpa Parl.	Protopine (% 0.18-0.16), fumariline (% 0.12-0.11), cryptopine (% 0.11-0.11)	d.d.p. - HPLC	23
Fumaria judaica Boiss.	Protopine (% 0.19-0.18), β-allocryptopine (% 0.13-0.11)	d.d.p. - HPLC	23
Fumaria vaillantii Loisl.	Protopine (% 0.30-0.27), n-methylhydrastine (% 0.17-0.15), parfumine (% 0.19-0.16)	d.d.p. - HPLC	23
Fumaria microcarpa Boiss.	Protopine (% 0.33-0.31), n-methylhydrastine (% 0.25-0.23), β-allocryptopine (% 0.37-0.36)	d.d.p. - HPLC	23

Fumaria parviflora Lam.	Protopine (% 0.45-0.41), n-methylhydrastine (% 0.10-0.09), parfumine (% 0.06-0.07)	d.d.p. - HPLC	23
Fumaria cilicica Boiss.	Protopine (% 0.21-0.19), β-allocryptopine (% 0.30-0.30), cryptopine (% 0.12-0.11)	d.d.p. - HPLC	23
Fumaria petteri subsp. *thuretii*	Protopine (% 0.47-0.43), fumariline (% 0.25-0.22)	d.d.p. - HPLC	23
Fumaria officinalis L.	Protopine (% 0.19-0.17), fumariline (% 0.15-0.14), β-allocryptopine (% 0.20-0.19), cryptopine (% 0.33-0.31)	d.d.p. - HPLC	23
Fumaria densiflora DC.	Protopine (% 0.29-0.27), fumariline (% 0.21-0.20), β-allocrptopine (% 0.32-0.30)	d.p.p-HPLC	23
Fumaria parviflora Lam.	Protopine (% 0.42), parfumine (% 0.07), d-bicuculline (% 0.06), hydrastine (% 0.16), N-methylhydrastine (% 0.09), N-methylhydrasteine (% 0.05), microcarpine (% 0.03), sanguinarine (% 0.10)	HPLC	24
Fumaria petteri Reichb. subsp. *thuretii* Boiss.	Protopine (% 0.43), fumariline (% 0.22), fumaritine (% 0.08), fumaricine (% 0.21), l-sinactine (% 0.21), scoulerine (% 0.11), coptisine (% 0.08) , l-capnoidine (% 0.12)	HPLC	24
Fumaria kralikii Jordan	Protopine (% 0.38), cryptopine (% 0.11), parfumine (% 0.12), l-scoulerine 0.08), d-bicuculline (% 0.35), d-adlumine (% 0.27), N-methylhydrasteine (% 0.11), sanguinarine (% 0.08)	HPLC	24

There are some studies on the activity of some *Fumaria* species which are growing in Turkey:

Abbasoğlu et al. were isolated many isoquinoline alkaloids from *Fumaria* species growing in Turkey and screened antimicrobial activity of these alkaloids (31).

Şener et al. were screened antibacterial, antifungal, antiinflammatory, analgesic, anti-platelet and antihypertensive activities of the extracts from *Fumaria vaillantii*. Bioassay directed fractionation using different pharmacological tests with the extracts from *Fumaria vaillantii* has led to the determination of different bioactive compounds (32).

Aktay et al. were studied the hepatoprotective effects of the extracts of *Fumaria asepalea and Fumaria vailantii*. They were studied hepatoprotective effects of this plants' ethanolic extracts in rats. The exract of *Fumaria vailantii* prevented the elevation of plasma and hepatic malondialdehyde formation in active liver injury (33).

Şener and Orhan were measured acetylcholinesterase inhibitory activity of 10 *Fumaria* species (*Fumaria asepala, F. capreolata, F. cilicica, F. densiflora, F. judaica, F. kralikii, F. macrocarpa, F. parviflora, F. petteri* subsp. *thuretii* and *F. vailantii*) by a modified spectrophotometric method developed by Ellman. All of the extracts displayed high inhibitory activity (34).

Orhan et al. were studied antiviral and antimicrobial profiles of isoquinoline alkaloids from *Fumaria* species growing in Turkey. The chloroform:methanol (1:1) extracts of a number of the plant species belonging to *Fumaria vaillantii* Loiss., *F. capreolata* L., *F. kralikii* Jordan., *F. asepela* Boiss., *F. densiflora* DC., *F. flabellata* L., *F. petteri* Reichb. subsp. *thuretii* (Boiss.) Pugsley, *F. macrocarpa* Boiss., *F. cilicica* Hauskkn., *F. parviflora* Lam., *F. judaica* Boiss. were screened for their anticholinesterase activity on acetylcholinesterase and butyrylcholinesterase enzymes by in vitro Ellman method. All of the *Fumaria* extracts displayed highly potent inhibition against both of the enzymes at 1 mg/ml concentration compared to the standart (35).

Various techniques ranging from straightforward TLC to complex immunoassays have been used for the determination of isoquinoline alkaloids in plants extracts; however, only a limited number of alkaloids determined with these methods (26).

In this study, TLC-densitometric and spectrophotometric methods have been used to determine the protopine content of *Fumaria densiflora* DC. The results obtained from the two analytical methods have been compared.

MATERIALS AND METHODS

Plant Material

The *F. densiflora* DC. was collected from West Anatolia in İzmir-Kemalpaşa, in April 1999, and identified by M.Ali Önür. A voucher specimen (No:1239) is deposited in the herbarium of the Department Pharmacognosy of the Faculty of Pharmacy in Ege University, in Izmir.

Extraction of alkaloid

Aerial parts of plants (25 g) were dried, powdered and extracted with ethanol in a Soxhlet apparatus until Mayer's test was negative, and then the evaporated in vacuo. The ethanolic residue was taken up in % 1 hydrochloric acid (50 ml), filtered and the aqueous acid solution brought to pH 9-9.5 with % 25 ammonium hydroxide and extracted with chloroform (5 x 150 ml). The extracts were dried over anhidr sodium sulphate and the solvent evaporated to afford a crude extract of alkaloids.

Reagents and solvents

Protopine (Merck 7989) was used as a standart. All of the analytical solvents and reagents were purchased from Merck.

Sample solutions

Sample solutions were prepared by dissolving in chloroform : methanol (8:2) 40 mg (TLC densitometric assay and spectrophotometric assay) of accurately weighed extract in a 10 ml volumetric flask.

TLC-densitometric assay

A Shimadzu high speed TLC-Scanner CS-920 was used with the following settings: beam size 0.4x0.4 mm, X=24, Y=10, L=3; AZS off, wavelenght 290 nm. Silica gel 60 F_{254} (20x20 cm, 0.25 mm, Merck 5715) plates were used. The mobile phases were 1° toluene : chloroform : methanol : %25 ammonium hydroxide (5:3:1:1) and 2° cyclohexane : diethylamine (9:1). Samples were applied with Hamilton syringes (15 mm from the bottom edge of the plate). The mobile phase 1° was allowed to run a distance of 15 cm, mobile phase 2° was allowed to run a distance of first 10 cm and second 15 cm in a satured tank. Protopine solutions (2, 4, 6 and 8 µl) were applied on a TLC plate and developed under the above mentioned conditions. The developed plates were initially air-dried. The resolved compounds were quantitated on the high-speed TLC scanner at 290 nm using a D_2 lamp. The calibration curves (Figure 2 and 3) showed a linear relationship between the concentrations and areas on TLC plates. Aliquots (20, 25 and 30 µl) of sample solutions were spotted on each TLC plate, and after the development, the areas of the spot on the plate were integrated by TLC densitometry. For every sample the procedure was repeated three times.

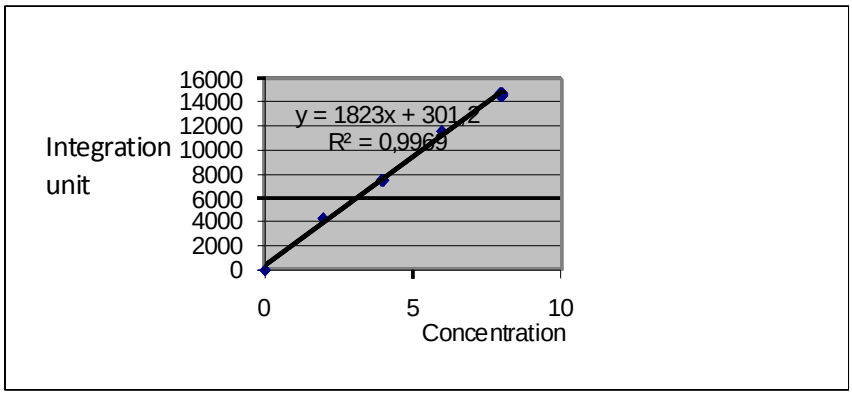

Figure 2. Calibration curve for the determination of protopine
by TLC-densitometry in toluene : chloroform : methanol : %25 ammonium hydroxide (5:3:1:1)

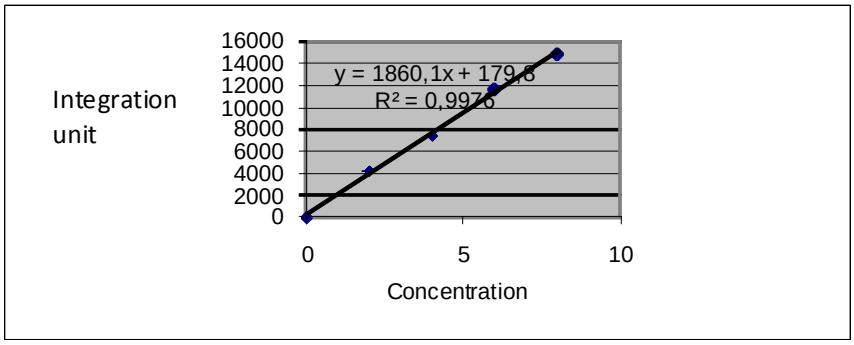

Figure 3. Calibration curve for the determination of protopine by TLC-densitometry in cyclohexane : diethylamine (9:1)

Spectrophotometric Assay

A Shimadzu UV-160A model spectrophotometer was used. Silica gel G (20 x 20 cm, 0.5 mm thick, Merck 5715) plates were used. The mobile phase was cyclohexane : diethylamine (9:1). Samples (40, 60, 80, 100, 120, 200 µl) were applied as bands (10 cm long) on silica gel G plates.

After development, the band of protopine was detected under 254 nm UV-light and scraped. The compound was eluted with chloroform : methanol (8:2) and the solution was evaporated to dryness. The residue was then dissolved in methanol, transferred to a volumetric flask and the volume was adjusted to 10 ml with methanol. The absorbance of this solution was measured at 290 nm against a blank using quartz. Then, the standard curve was drawn (Figure 4).

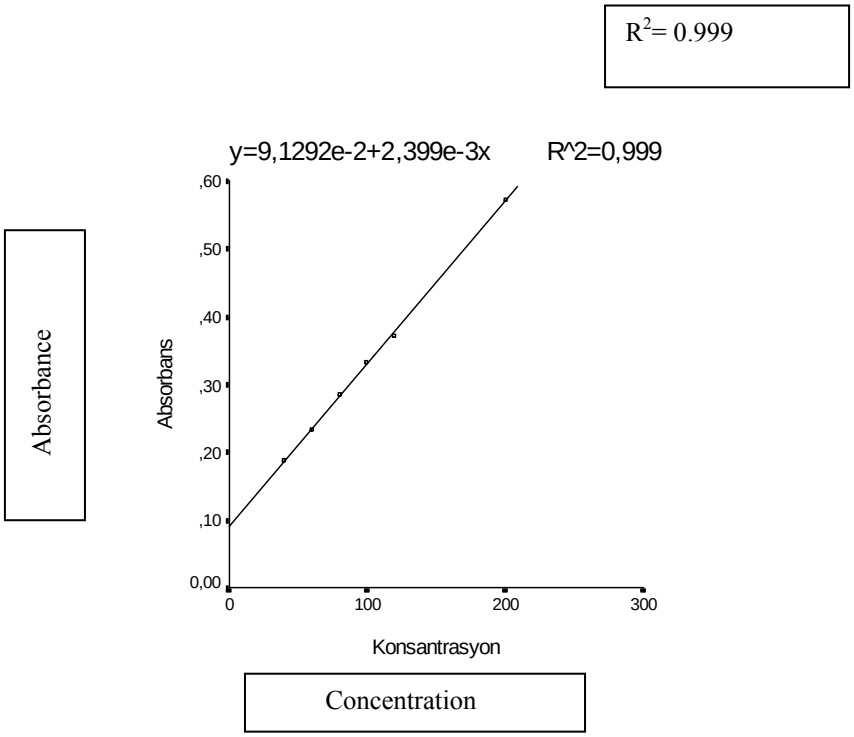

Figure 4. Calibration curve for the spectrophotometric determination of protopine

The above described procedure was followed by using 40 mg samples. The assay was repeated three times. The protopine content in extracts was calculated from the regression equation of the Standard curve.

Statistical analysis

Results obtained from the TLC-densitometric and spectrophotometric analyses were as mean ±S.E. and compared by paired *t*. Test ($p<0.05$)

RESULTS AND DISCUSSION

Protopine in Herba Fumariae densiflorae extracts was quantitatively determined by TLC-densitometry and spectrophotometry.

For testing the quantitative accuracy of the TLC-densitometric method, the analyses of the reference subtance and extracts were repeated on three different plates on 2 different solvents. The TLC-densitometric calibration curves (Figure 2 and Figure 3), used to calculate the protopine content in the extracts, was expressed by the following linear equations:

1- According to solvent system [Toluene : chloroform : methanol : %25 ammonium hydroxide (5:3:1:1)]:

$$y = 1823x + 301.2;\ R^2 = 0.9969$$

2- According to solvent system [Cyclohexane : diethylamine (9:1)]:

$$y = 1860.1x + 179.8;\ R^2 = 0.9976$$

Where y is the integration unit and x is the protopine concentration (µl/ml).

For the spectrophotometric assay of protopine, a calibration curve was prepared (Figure 4), expressed by the following linear equation:

$$y = 9.1292e^{-2} + 2.3099e^{-3x};\ R^2 = 0.999$$

The results of quantitative determinations of protopine in Herba Fumariae densiflorae by TLC-densitometry (in two different solvent system) and spectrophotometry are shown in Table 3. Comparison of the TLC-densitometric results with those obtained from spectrophotometric experiments showed no significant differences, confirming the reliability of the spectrophotometric data.

Table 3. The results of determination of % Protopine with TLC-densitometric and spectrophotometric method

Determination method	Solvent system	% Protopine
TLC-densitometry	Cyclohexane:diethylamine (9:1)	0.03511 ± 0.0012
TLC-densitometry	Toluene:chloroform:methanol: %25 ammonium hydroxide (5:3:1:1)	0.03521 ± 0.0007
Spectrophotometry	Cyclohexane:diethylamine (9:1)	0.3660 ± 0.0016

Temizer et al. determined protopine from some *Fumaria* species and they found the content of protopine in *Fumaria densiflora* DC. as 0.29 and 0.27 % by differential pulse polograph and HPLC respectively (23). Suau R. et al. reported the content of protopine in *Fumaria densiflora* DC. as 0.76 % by GC-MS analysis (29). Our results obtained by the two different methods were similar to Temizer' s study.

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