Determination of Salicin Content of Some Salix L. Species by HPLC Method

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In this paper, we find the salicin content of the nine species of Salix L from the province of Ankara, Turkey, namely Salix triandra, S. alba, S. excelsa, S. fragilis, S. babylonica, S. caprea, S. cinerea, S. pseudomedemii and S. amplexicaulis. A simple HPLC method was applied to the determination of Salicin of these nine species in barks and leaves of female and male. Chromatographic separation was carried out by a mobile phase consisting of bidistilled water, tetrahydrofuran and ortho-phosphoric acid (97.7: 1.8: 0.5) (v/v/v). The salicin amount of these samples was analyzed by measuring the peak area at the wavelength, 270 nm. A reversed phase phenyl column (250 x 4.6mm, 5μm) was used and flow rate was set to 1 ml/min. in an isocratic elution. The results provided by HPLC method was found in agreement with those indicated by European Pharmacopoeia. It was observed that S. babylonica female bark sample possess the highest salicin content (2.675), while S. caprea female bark (0.058) has the lowest salicin content as w/w (%).

Keywords: Salix, willow, salicin, HPLC method

Willow (*Salix* L.) species belong to the family Salicaceae including deciduous and dioecious trees and bushes inhabiting wetlands or humid areas and comprising 500 species all over the world [1]. 28 species of willows were recorded in Turkey, two taxa of them are endemic in Turkey [2,3,4]. In traditional medicine, the barks of willows are used for rheumatism, anti-pyretic, and pain reliever in Turkey [5]. Phenolic glycosides are commonly found as secondary metabolites of willows containing 1.5-11 % salicin and its derivatives [6, 7]. Within the Salix genus, these compounds have been used as taxonomic markers [6]. Willow bark is official in the European, German and British Herbal Pharmacopoeia [7, 9]. The Commission E monograph recommends liquid and solid preparations for internal use with an average daily dosage corresponding to 60-120 mg total salicin, which is equivalent to 6-12 g dried bark. Willow bark is used for diseases accompanied by fever, rheumatic ailments and headaches [7]. Daily consumption of Salicis cortex extract with 240 mg salicin per day affects platelet aggregation to a far lesser extent than acetylsalicylate. Both a lesser bleeding time and lower side drug effects were observed during treatment with willow bark extract [10]. A recent study proved that orally administered salicin produces antipyretic action without causing gastric injury [11]. Willow bark also has antioxidant activity mainly because of its phenolic compounds [12].

Although Turkey has a rich willow population with its 28 *Salix* species, there is no study about the salicin content of the barks and leaves of male and female specimens of willow species. This study fills this gap of information about this important medicinal plant growing in Ankara's region.

The proposed HPLC method is different from that proposed in [9]. Therefore, the method was optimized according to the experimental conditions in our laboratory. In this context, the proposed HPLC method was successfully applied to the determination of salicin content of the nine species of *Salix* L. in the province of Ankara, Turkey.

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Experimental part

Chemicals

Salicin (Merck-107665) used as the standard chemical was obtained from Merck Chemicals. Chromatographic grade-double distilled water, HPLC grade methanol (Merck 106018), HPLC grade tetrahydrofuran (Merck 108101) and HPLC grade *ortho*-phosphoric acid (Merck 100565) were used.

Plant Material

Plant materials were collected from various localities in the province of Ankara. During the collection of materials; bark and leaf samples for all species were taken considering both male and female specimens. Plant materials were dried in a cool and shad medium. Voucher specimens are prepared and stored at AEF (Ankara University, Faculty of Pharmacy Herbarium). Localities of the investigated plant samples are given in table 1.

Extraction

1.25 g of dried powdered material was macerated two times with 75 mL and finally 50 mL of methanol for 2.5 h at room temperature. The extracts were combined, then filtered and evaporated to dryness under a temperature not exceeding 40°C.

The residue was dissolved with 50 mL of HPLC grade Merck methanol. Solution was passed through a 0.45 μm filter and 20 μL extract was directly injected into the HPLC. The results were obtained as a mean value of three separate injections.

Table 1
LOCALITIES OF THE INVESTIGATED *SALIX* SPECIES

Species	Locality
Salix alba L	**B4 Ankara:** Beynam Atatürk Forest, Fındıcak Fountain, 1446m, 39°40.480N, 032°54. 595 E, 29.iv.2001, O. Arıhan, (AEF 22668)
Salix amplexicaulis Bory & Chaub	**B4 Ankara:** Beynam Atatürk Forest, within dense bush, 1300m, 39º 40.732 N, 32º 55.172 E, 26.iii.2002, O. Arıhan (AEF 22631)
Salix babylonica L.	**B4 Ankara:** Middle East technical University Campus, 960m, 15.viii.2001, O. Arıhan (AEF 22659)
Salix caprea L.	**A4 Ankara:** Kızılcahamam National Park, 1150m, 40º26.958 N, 32º36.959 E, 1152m, 31.3.2002, O. Arıhan, (AEF 22556)
Salix cinerea L.	**A4 Ankara:** Çubuk- Lake Karagöl, 1500m, 21.4.2002, A. Güvenç & O.Arıhan (AEF 22482)
Salix excelsa J.F. Gmelin	**B4 Ankara:** Lake Eymir, 900m, 28.viii.2001, O. Arıhan (AEF 22707)
Salix fragilis L.	**B4 Ankara:** Ankara-Bala roadside, entrance to the road to Beynam Atatürk Forest, 1250m, 29.iv.2001, O. Arıhan (AEF 22536)
Salix pseudomedemii E. Wolf.	**B4 Ankara:** Beynam Atatürk Forest, Fındıcak Fountain, 1446m, 39°40.480N, 032°54. 595 E, 4.iv.2001, O.Arıhan; (AEF 22725)
Salix triandra L.	**A4 Ankara:** Ankara Stream at Sincan – Fatih bridge, 800m, 13.iii.2002, O.Arıhan (AEF 22468)

Apparatus

The method was performed with a LC system consisting of a Hewlett Packard Series 1100 model. UV-VIS detector was set at 270 nm and peak areas were integrated automatically by computer using Agilent software programme. Separation was carried out using a reverse phase phenyl column (250 x 4.6mm, 5µm) and flow rate was set to 1ml/min. in an isocratic elution.

All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

Standard Solutions

Stock solution (25 mg/25 mL) of salicin was prepared in the mobile phase consisting of bidistilled water, tetrahydrofuran and *ortho*-phosphoric acid (97.7: 1.8: 0.5) (v/v/v). Standard series in the concentration range of 100-1000 µ/mL were obtained from the stock solution. The mobile as a solvent was used for all HPLC experimental studies.

Chromatographic conditions

HPLC analysis was performed by isocratic elution with flow rate 1 ml/min which is a modification presented in European Pharmacopeia [9]. The mobile phase composition consists of bidistilled water, tetrahydrofuran and *ortho*-phosphoric acid (97.7: 1.8: 0.5) (v/v/v). All solvents were filtered through a 0.45 µm Millipore filter before being used and degassed in an ultrasonic bath. Volumes of 20 µL extracts prepared from each sample were injected into the column. Quantification was realized by measuring the peak area at the wavelength 270 nm.

Results and Discussion

The determination of salicin according to gender and organs of 9 willow trees was performed. In Turkey, *Salix* species are grouped under two main categories: subgenus *Salix* (*S. triandra, S. alba, S. excelsa, S. fragilis, S. babylonica*) and subgenus Vetrix (*S. caprea, S. cinerea, S. pseudomedemii, S. amplexicaulis*). The outline of the developed HPLC and its chromatographic conditions for the determination of salicin in the above samples was explained in the following sections.

Method development

In our study, several chromatographic conditions were tested for the separation and determination of salicin in samples. Good separation and determination of nine *Salix* species in barks and leaves of female and male were performed by using the mobile phase consisting of bidistilled water, tetrahydrofuran and *ortho*-phosphoric acid (97.7: 1.8: 0.5) (v/v/v) and phenyl column (250 x 4.6mm, 5µm), at flow rate of 1 mL/min. Chromatograms were plotted by UV-VIS detector at the wavelength of 270 nm. Detector responses were measured as peak areas. The injection volume was 20 µg/mL and triplicate injections were used for each sample. At the flow rate of 1 mL/min the retention time was 8.44 min for salicin and sample as shown in figure1A and B, respectively.

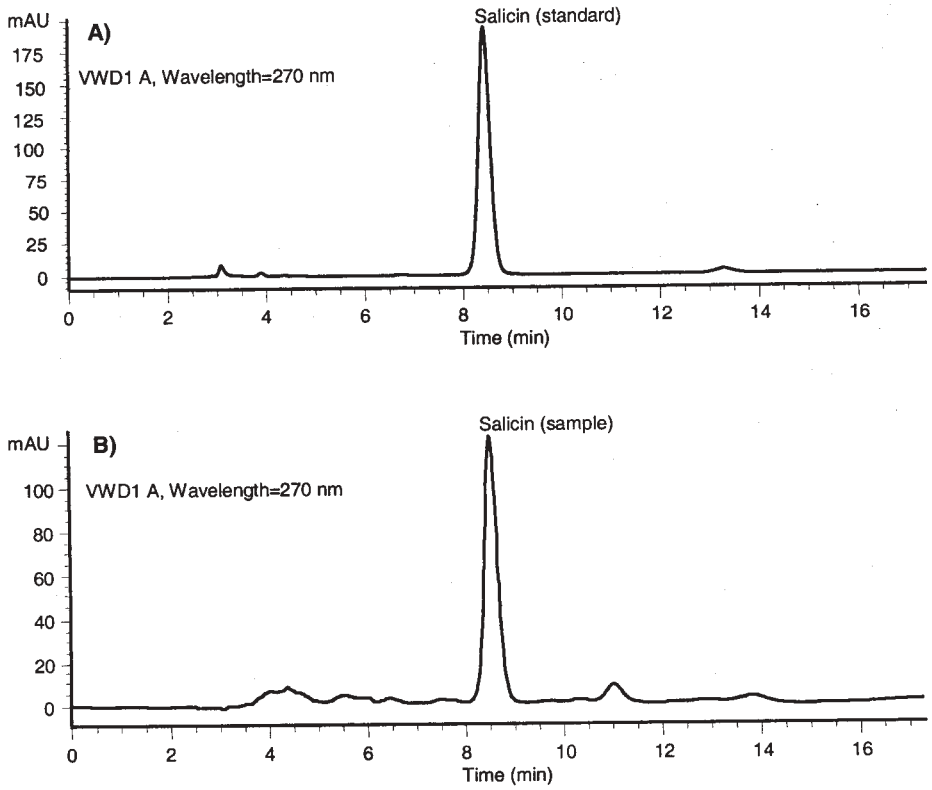

Fig. 1. Chromatograms of A) 100 mg/mL standard and B) sample (Salix babylonica female bark)

Table 2
LINEAR REGRESSION ANALYSIS AND ITS STATISTICAL RESULTS

m	n	r	SE(m)	SE(n)	SE(r)	LOD µg/mL	LOQ µg/mL
3.3842	42.1705	1.0000	0.0136	6.9037	9.6843	7.68	25.60

m = Slope of linear regression equation
n = Intercept of linear regression equation
r = Correlation coefficient of linear regression line
SE(m) = Standard error of slope
SE (n) = Standard error of intercept
SE(r) = Standard error of correlation coefficient
LOD = Limit of detection
LOQ = Limit of quantitation

Standard series in the concentration range of 100-1000 µg/mL salicin was prepared in the mobile phase. Calibration graph was obtained by using the relationship between concentration and peak areas. Linear regression analysis and its statistical results were summarized in table 2.

Method validation

In optimized chromatographic conditions, the validation of HPLC procedure was carried out by using the monograph of the International Conference on Harmonization (ICH) [17]. The linearity of HPLC detector response for the determination of salicin at 270 nm was observed in the working concentration range of 100-1000 µg/mL at the five different concentration levels. Analysis of each concentration was repeated three times. Each concentration was repeated three times and a pice of information on the variation in the peak area between samples having the same concentration was obtained. The linearity of the calibration functions of salicin at the working wavelength was confirmed by looking at the high value of the correlation coefficient (table 2). For this HPLC method, a linear regression function is represented in table 2.

The precision of HPLC procedure was tested by five replicate determinations at different concentration levels. Relative standard deviation was found to be 1.36 % as it is shown in table 3. The accuracy of the proposed HPLC method was tested by analyzing synthetic samples at different concentration levels. The mean recovery value was found 100.4 % (table 3).

A good agreement between the analysis results was observed for HPLC method. During the analysis procedure, the interference and the systematical error were not observed.

In accordance with ICH [17], the limit of detection (LOD) and the quantitation (LOQ) were calculated by using the standard deviation of the response and the slope of the linear regression line (table 2).

The calibration range, according to salicin concentration presented in *Salix* species was designated in the practical range to give an accurate, precise and linear response.

The matrix effect in samples, nine *Salix* species, was studied for the proposed HPLC method. We observed the matrix effect does not give any error for the determinations.

Table 3
RECOVERY DATA OBTAINED BY APPLYING HPLC METHOD TO SYNTHETIC SAMPLES

No:	Added µg/mL	Found µg/mL	Recovery (%)
1	100	99.03	99.0
2	200	205.11	102.6
3	300	299.64	99.9
4	400	396.24	99.1
5	500	505.19	101.0
6	1000	1009.79	101.0
		Mean	100.4
		RSD	1.36

RSD = Relative standard deviation

Table 4
EXPERIMENTAL RESULTS FOR SALICIN CONTENT IN THE BARKS AND LEAVES OF *SALIX* SPECIES BY HPLC METHOD

	Barks (Salicin %) mean ± SD		**Leaves** (Salicin %) mean ± SD	
Species	**Male**	**Female**	**Male**	**Female**
Subgenus Salix				
S. alba	-	0.881±0.0134	0.127±0.0205	0.319±0.0049
S. excelsa	0.415±0.0049	0.223±0.0092	0.328±0.0163	0.320±0.0042
S. fragilis	0.182±0.0078	0.256±0.0162	1.030±0.0064	1.340±0.0148
S. triandra	-	0.370±0.0014	0.070±0.0198	0.089±0.0007
S. babylonica	0.755±0.0085	2.675±0.0184	0.222±0.0014	0.734±0.0156
Subgenus Vetrix				
S. amplexicaulis	2.050±0.0064	1.754±0.0226	1.719±0.0064	0.852±0.0007
S. caprea	0.245±0.0099	0.058±0.0050	0.170±0.0049	0.115±0.0064
S. cinerea	0.569± 0.0028 (unknown gender)			
S. pseudomedemii	0.829±0.0057	-	1.286±0.0134	1.080±0.0007

Salicin analysis

The proposed HPLC were applied to the determination of salicin in the barks and leaves of male and female in nine *Salix* (*Salix triandra, S. alba, S. excelsa, S. fragilis, S. babylonica, S. caprea, S. cinerea, S. pseudomedemii* and *S. amplexicaulis*) . The assay results of nine *Salix* species are shown in table 4. Their percent mean and standard deviation values are summarized in the same table. Good agreement was observed for the proposed HPLC approach and literature methods.

It is known that male willows have lower salicin content than females [13, 14]. Our results show that salicin content of female barks and leaves of Subgenus *Salix* is higher than the males. The salicin content of male barks and leaves of Subgenus Vetrix is higher than the females. Therefore, among all species, the highest salicin content was found in the *S. babylonica* female bark (2.675 %) (see Fig. 1B) and the lowest was found at *S. caprea* female bark (0.058 %) (table 4).

There was no previous study for comparison of male and female *Salix cinerea* bark and leaf specimens however, the period for the collection of the specimens in this study was not suitable for gender identification. The salicin content was determined also for those samples (table 4). As it is well known, the salicin amount is related with harvest times, seasonal variations, locality, gender and age of the plant and the part of the bark harvested as well as species with diversity [15].

The results of our study and the findings in literature are fairly comparable with those presented in [16]. Especially for *Salix caprea,* the results are exactly the same. The comparisons are not possible for *S. cinerea, S. pseudomedemii* and *S. amplexicaulis* since no records were found in literature.

Conclusions

In this study, an HPLC method was developed and applied to the determination of salicin content of the nine species of *Salix* L. in Ankara region, Turkey. We observed that the proposed HPLC approach gave us successfully assay results for the analysis of salicin in all samples. The experimental results for *S amplexicaulis* was found in good aggrement with those prepsented by European Pharmacopoeia [9].

This paper is the first study of the analysis of salicin in willow species growing in Turkey done according to their gender and organs. The results of this current work form a basis for the future studies in this field.

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