QUANTIFICATION OF SHIKIMIC ACID IN THE METHANOLIC EXTRACTS OF THREE *ALNUS* TAXONS GROWING IN TURKEY

Çiğdem ALTINYAY[1], Burçin ERGENE ÖZ[2*], Mehmet Levent ALTUN[2]

[1]The Ministry of Health of Turkey, Turkish Medicines and Medical Devices Agency, Ankara, Turkey
[2]Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06100 Tandoğan Ankara, TURKEY

Shikimic acid is a phenolic acid which is known to possess several activities and it takes attention as a leading compound to some synthetic medicinal substances as well. In this study, high performance liquid chromatographic analyses were carried out in order to determine shikimic acid contents of *Alnus glutinosa* subsp. *glutinosa, A. orientalis* var. *orientalis* and *A. orientalis* var. *pubescens*; acetonitrile and 0.2% *o*-phosphoric acid:water mixture with isocratic flow was used as mobile phase. The flow rate was 0.5 ml/min. The quantitative analysis of methanol extracts prepared from the leaves of *A. glutinosa* subsp. *glutinosa, A. orientalis* var. *orientalis* and *A. orientalis* var. *pubescens* revealed that shikimic acid amount was found to be 0.6491%, 0.4309% and 0.2452% respectively.

Key words: *Alnus*, Betulaceae, HPLC, Quantitative analysis, Shikimic acid.

Türkiye'de Yetişen Üç *Alnus* Taksonunun Metanollü Ekstrelerinde Şikimik Asit Miktar Tayini

Şikimik asit, çeşitli biyolojik aktivitelere sahip ve aynı zamanda, bazı sentetik maddeler için başlangıç materyali olarak dikkat çeken bir fenolik asittir. Bu çalışmada, *Alnus glutinosa* subsp. *glutinosa, A. orientalis* var. *orientalis* ve *A. orientalis* var. *pubescens* ile yüksek performanslı sıvı kromatografisi analizleri yapılmış; asetonitril ve %0.2 *o*-fosforik asit:su karışımı izokratik akış ile mobil faz olarak kullanılmıştır. Akış hızı 0.5 ml/dk olarak verilmiştir. *A. glutinosa* subsp. *glutinosa, A. orientalis* var. *orientalis* ve *A. orientalis* var. *pubescens* yapraklarından hazırlanan metanol ekstrelerinde şikimik asit miktarları sırasıyla, %0.6491, %0.4309 ve %0.2452 olarak tespit edilmiştir.

Anahtar kelimeler: *Alnus*, Betulaceae, YPSK, Kantitatif analiz, Şikimik asit.

***** Corresponding author: Tel: +90 312 2033039; Fax: +90 312 2131081.
***E-mail address*:** ergene@pharmacy.ankara.edu.tr

INTRODUCTION

The genus *Alnus* Miller, which belongs to Betulaceae family, is represented by approximately 35 species all around the world and grows especially in the hot regions of the Northern hemisphere (1). According to the records, two species (*Alnus glutinosa* (L.) Gaertner and *Alnus orientalis* Decne) and six taxons (*A. glutinosa* subsp. *glutinosa, A. glutinosa* subsp. *barbata* (C.A. Meyer) Yalt., *A. glutinosa* subsp. *antitaurica* Yalt., *A. glutinosa* subsp. *betuloides* Anşin, *A. orientalis* var. *orientalis, A. orientalis* var. *pubescens* Dippel) of this genus grow and known as Kızılağaç in Turkey (2,3).

This genus is traditionally used as antiperspirant, antipyretic, anticancer, antioxidant, antiinflammatory and for the treatment of dental abscess, rheumatism, hemorrhoid and some skin diseases such as chronic herpes, eczema and pruritus. In Turkey, the crushed leaves of *A. glutinosa* (L.) Gaertner is used for the medical care of incisions and wounds. Apart from the external use, the infusions prepared with the leaves are also used against rheumatism internally (4-7). The traditional use of *A. glutinosa* in Europa is reported as well. The dried barks of *A. glutinosa* are consumed as antipyretic and for the treatment of hemorrhoid and wounds in Portugal. The leaves, barks and roots of this species are used against cancer and especially inflammatory tumors (1). The phytochemical researches have revealed that *Alnus* species contain terpenoids, flavonoids, diarylheptanoids, phenolic compounds, steroids and tannins. Among all, diarylheptanoids are found to be the major substances (8).

Phenolic acids are secondary metabolites which exhibit various biological activities and take attention especially with their antioxidant activity due to their potential for the protection from several diseases enhanced by oxidative stress (9). Shikimic acid (Figure 1) which is an important intermediate in the biosynthesis of lignin, aromatic amino acids (phenylalanine, tyrosine and triptophane), and most alkaloids of plants and microorganisms, is a phenolic acid and is reported to possess antiinflammatory, antipyretic, antioxidant, antibacterial and analgesic activities. It is a potential remedy for atherosclerosis with its activity to decrease blood viscosity and serum lipid levels such as LDL, total cholesterol, triglycerides and to increase HDL level. In vivo studies have revealed that shikimic acid is promising for the treatment of ulcerative colitis. Other results about the inhibitory effects of shikimic acid on platelet aggregation and blood coagulation are also remarkable. Apart from its biological activities, shikimic acid is used to synthetize an antiviral compound called oseltamivir which is administered to treat and prevent all the known strains of influenza virus (10-12). Shikimic acid was first isolated from the fruits of *Illicium religiosum* Sieb. by Eykman in 1885 (13) and literature researches have revealed that there are further phytochemical and ethnopharmacological studies about this compound. The study which analgesic, antipyretic and antiinflammatory activity of *Shinus polygamus* were evaluated showed that the most active fractions contain shikimic acid as well as β-sitosterol and quercetin (8). Shikimic acid was also isolated from the berries of *Juniperus oxycedrus* ssp. *oxycedrus* using bioactivity guided fractionation technique. In this study, it was shown that shikimic acid possessed hypoglycaemic activity (14). The quantification of shikimic acid was also evaluated in various studies. The HPLC analysis of *Prunus armeniaca* L. extract yielded the shikimic acid content as 23.78 mg/kg (15). According to the results of Chen *et al.* Shikimic acid content of Masson pine needle, Oriental arborvitae leafytwigs and star anise was found to be 5.71%, 8.95% and 1.74% respectively (16). The analysis conducted with the extracts prepared from the roots and aerial parts of *Eichhornia crassipes* (Mart.) Solms showed that the amount of shikimic acid in the aerial parts was 0.03-0.7%, while the amount was found to be 0.05-0.90% in roots (17).

In this study, quantitative analysis of *A. glutinos*a (L.) Gaertner subsp. *glutinosa, A. orientalis* Decne var. *orientalis* and *A. orientalis* Decne var. *pubescens* Dippel are conducted by the use of HPLC and shikimic acid contents of the leaves of these taxons are evaluated.

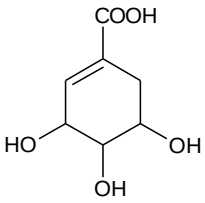

Figure 1. Shikimic acid

EXPERIMENTAL

Plant material

*A. glutinos*a subsp. *glutinosa*, *A. orientalis* var. *orientalis* and *A. orientalis* var. *pubescens* were collected from the localities which are listed in Table 1 and identified by Prof. Dr. Hayri Duman from Faculty of Science, Gazi University. The voucher specimens were deposited in the Herbarium of Ankara University, Faculty of Pharmacy (AEF).

Table 1. Plant material

Plant material	Locality	Herbarium no
A. glutinosa (L.) Gaertner subsp. *glutinosa*	Bolu-Sakarya, Beylice Çukurhan village, streamside, 261 m	AEF25991
A. orientalis Decne var. *orientalis*	Köyceğiz, Sandıras Mountain, Ağla, 766 m	AEF25990
A. orientalis Decne var. *pubescens* Dippel	Köyceğiz, lake side	AEF25989

Preparation of extracts

The leaves were dried and crushed. Powdered plant materials were weighed around 30 g and 500 mL of methanol was added for each. The mixtures were stirred at 550 rpm for 2 hours. At the end of this period, the extracts were filtered and the residues were macerated in the same conditions. The filtrates were combined and evaporated until dryness at 35-45°C.

HPLC analysis

Agilent Technologies LC 1200 series chromatograph was used for the analysis. The measurement was carried out at the wavelength of 210 nm using diode array dedector (G1215 DAD). The integration and processing of the chromatograms were provided by Agilent Software. The seperation of the samples was performed using ACE 5 C18 (150 mm×4.6 mm; 5 µm) column and 0.02% *o*-phosphoric acid in water as mobile phase with the isocratic flow rate of 0.5 mL/min. The injection volume was 10 µL and each analyse was proceeded 20 minutes. The method used for the analysis was modified from the method of Chen *et al.* (16).

Preparation of HPLC samples. The extracts were weighed and dissolved in methanol to obtain the concentrations of 2 mg/mL. The solutions were filtered through syringe filters with pore size of 0.45µ. Triplicate injections of 10 µL were performed.

Preparation of standard solutions and calibration. Shikimic acid which was isolated from the leaves of *A. glutinosa* subsp. *glutinosa* (not published) was dissolved in methanol to obtain standard solutions at the concentrations of 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 0.6 mg/mL, 0.7 mg/mL, 0.8 mg/mL and 1 mg/mL. Triplicate injections of 10 µL were performed for each concentration. The calibraiton curve was obtained by plotting the areas of each solution against concentration.

Validation procedure

Limit of detection (LOD) and limit of quantification (LOQ) values were determined at signal to noise ratio of 3 and 10 respectively. The solutions at LOD and LOQ concentrations of shikimic acid were prepared and experimentally verified by 9 injections.

RESULTS

In current study, quantitative analysis of shikimic acid in three *Alnus* species namely *A. glutinos*a subsp. *glutinosa, A. orientalis* var. *orientalis* and *A. orientalis* var. *pubescens*, growing in Turkey were performed by using HPLC technique. The data obtained were evaluated according to the retention time and UV spectrum of the standard and quantification was carried out with regard to the calibration equation obtained from the peak areas of the standard solutions at different concentrations. In order to acquire the calibration equation, 10 different concentrations of standard were injected as triplicate and the average of the peak areas were plotted against concentrations. The calibration possessed good linearity (Table 2). The chromatograms were obtained at the wavelength of 210 nm and the retention time of shikimic acid was found to be 2.36 min. The chromatograms of shikimic acid and the methanol extract of *A. glutinosa* subsp. *glutinosa* were given in Figure 2 and Figure 3 respectively. LOD and LOQ values were calculated due to the signal to noise ratio of 3 and 10 respectively and verified by 9 injections for each. The values determined as LOD and LOQ were also shown in Table 2.

Table 2. Linearity values of shikimic acid solutions

Standard	Calibration equation	r^2	RSD%	LOQ (µg/mL)	LOD (µg/mL)
Shikimic acid	y=15400x+1267	0.9900	1.032	8.000	2.700

x=concentration, y=area of the standard peak
RSD%: the percentage of relative standard deviation calculated as:
RSD%= (Standard Deviation/Average) X 100

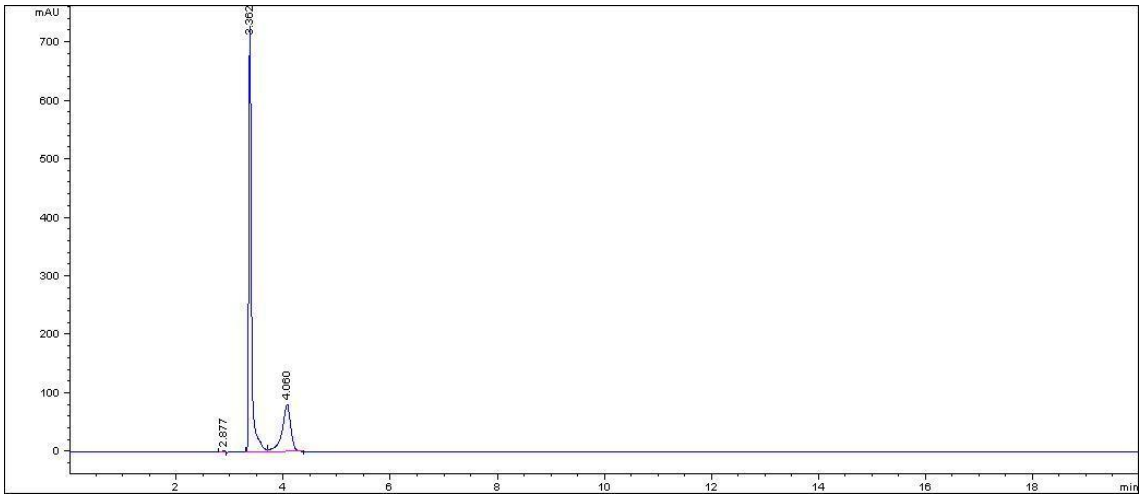

Figure 2. The HPLC chromatogram of shikimic acid

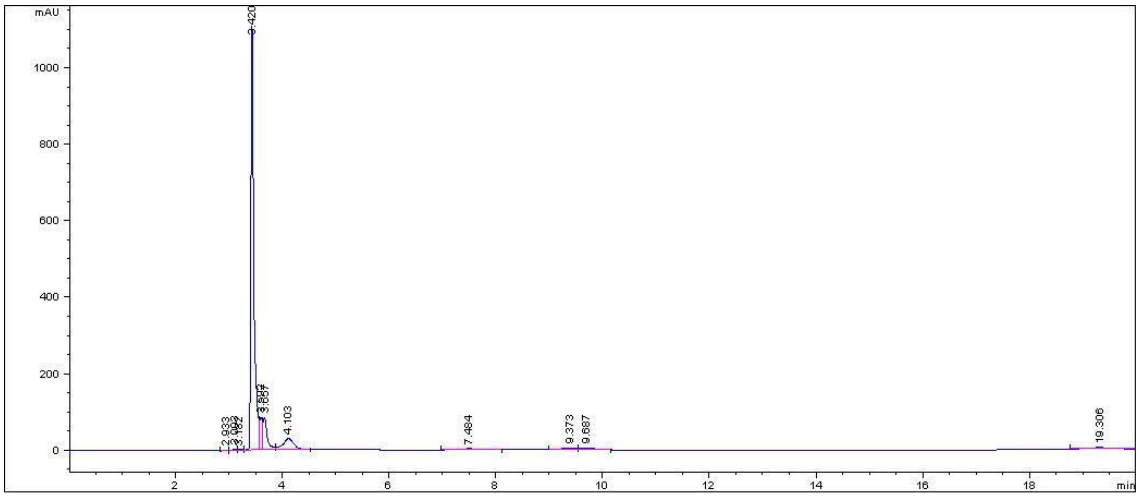

Figure 3. The HPLC chromatogram of *A. glutinosa* subsp. *glutinosa*

According to the calibration equation, shikimic acid contents of the methanol extracts of *Alnus* sp. leaves were determined. As shown in Table 3, *A. glutinosa* subsp. *glutinosa* was found to have the highest shikimic acid content with the value of 0.6491%.

Table 3. Amounts of shikimic acid in the leaves of *Alnus* sp.

Plant material	Shikimic acid amount (mg/mL)	Shikimic acid amount (w/w%)
A. glutinosa subsp. *glutinosa*	0.1969	0.6491
A. orientalis var. *orientalis*	0.0739	0.2452
A. orientalis var. *pubescens*	0.1300	0.4309

CONCLUSION

Shikimic acid is the crucial component of the shikimate pathway which leads to the biosynthesis of essential aromatic amino acids, lignin and several alkaloids in plants and microorganisms. It also plays a role as a precursor of cinnamic acids and flavonoids. Apart from this, shikimic acid is required for the assimilation of folic acid, alkaloids and vitamins. Therefore shikimic acid appears to present in the content of several plants and exhibits various biological activities. It also takes attention with its potential as a starting material for some synthetic compounds such as oseltamivir which is an antiviral drug against H5N1 influenza virus and (-)-zeylenone which exhibits antiviral, antibacterial and anticancer activities. Thus the natural sources which contain shikimic acid are important to fulfill the requirement of industry as well as biological activities of its own (9,10).

In the current study, the quantitative analysis of shikimic acid found in the methanolic extracts of the leaves of *Alnus* species gathered from Turkey were performed by using HPLC method. To our knowledge this is the first study that reports the shikimic acid contents of the leaf methanolic extracts of *Alnus* species growing in Turkey. The results showed that *Alnus* species could be considered as good shikimic acid sources to be used in shikimic acid isolation.

REFERENCES

1. Ludwiczuk A, Saha A, Kuzuhara T, Asakawa Y, Bioactivity guided isolation of anticancer constituents from leaves of *Alnus sieboldiana* (Betulaceae), Phytomedicine 18(6), 491-498, 2011.
2. Davis, PH, Flora of Turkey and the East Aegean Islands, Vol.7, Edinburg: Edinburg University Press, 1982.
3. Davis, PH, Flora of Turkey and the East Aegean Islands, Vol.11, Edinburg: Edinburg University Press, 2000.
4. Loi MC, Poli F, Sacchetti G, Selenu MB, Ballero M, Ethnopharmacology of Ogliastra (Villagrande Strisaili, Sardinia, Italy), Fitoterapia 75, 277-295, 2004.
5. Tuzlacı E, Şifa Niyetine Türkiye'nin Halk İlaçları, pp. 29, Alfa Basım Yayın ve Dağıtım Ltd. Şti., İstanbul, 2006.
6. Neves JM, Matos C, Moutınho C, Queıroz G, Gomes LR, Ethnopharmacological notes about ancient uses of medicinal plants in Tras-os-Montes (northern of Portugal), J Ethnopharmacol 124, 270-283, 2009.
7. Choi SE, Park KH, Jeong MS, Kim HH, Lee DI, Joo SS, Lee CS, Bang H, Choi YW, Lee M, Seo SJ, Lee MW, Effect of *Alnus japonica* extract on a model of atopic dermatitis in NC/Nga mice. J Ethnopharmacol, 136(3), 406-13, 2011.
8. Sati SC, Sati N, Sati OP, Bioactive constituents and medicinal importance of genus *Alnus*, Pharmacogn Rev 5(10), 174-183, 2011.
9. Robbins RJ, Phenolic acids in foods: An overview of analytical methodology, J Agric Food Chem 51, 2866-2887, 2003.
10. Erazo S, Delporte C, Negrete R, Garcia R, Zaldivar M, Iturra G, Caballero E, Lopez JL, Backhouse N, Constituents and biological activities of *Shinus polygamus*, J Ethnopharmacol 107(3), 395-400, 2006.
11. Bochkov D V, Sysolyatin SV, Kalashnikov AI, Surmacheva IA, Shikimic acid: review of its analytical, isolation, and purification techniques from plant and microbial sources, Journal of Chemical Biology, 5(1), 5-17, 2012.
12. Estevez AM, Estevez RJ, A short review on the medicinal chemistry of (-)-shikimic acid, Mini-Rev Med Chem 12, 1443-1454, 2012.
13. Bohm BA, Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid), Chemical Reviews 65, 435–466, 1965.
14. Orhan N, Aslan M, Pekcan M, Deliorman Orhan D, Bedir E, Ergun F, Identification of hypoglycaemic compouns from berries of *Juniperus oxycedrus* subsp. *oxycedrus* through bioactivity guided isolation technique, J Ethnopharmacol 139, 110-118, 2012.
15. Hasib A, Jaouad A, Mahrouz M, Khouili M, HPLC determination of organic acids in Moroccan apricot, Cienc Tecnol Aliment 3(4), 207-211, 2002.
16. Chen XY, Yuan L, Wei L, Wang M, Lei YX, Content analysis of shikimic acid in the masson pine needles and antiaggregating activity, IJAST 2(4), 110-114, 2014.
17. Cardoso SF, Lopes LMX, Nascimento IR, *Eichhornia crassipes*: an adventageous source of shikimic acid, Rev Bras Farmacogn 24, 439-442, 2014.