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Quantification of Some Phenolic Compounds in *Consolida thirkeana* (Boiss.) Bornm. by HPLC and Validation of Method

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ABSTRACT
Consolida species have traditional uses in the treatment of various diseases, especially skin diseases. There is also traditional use of some *Consolida* species in Turkey. Phenolic compounds have significant pharmacological effects, therefore it is important to know their amount in plants. *Consolida thirkeana* is endemic to Turkey and known as "boz mahmuz" and no study had conducted in terms of phenolic compounds. Therefore, some phenolic amount, which has been done for the first time for *C. thikeana*, was analyzed. In this study, *C. thirkeana* was analyzed quantitatively for caffeic acid, chlorogenic acid, hyperosit, and rutin by using HPLC and the method was validated (linearity, precision, accuracy, recovery, limits of detection (LOD), and limits of quantification (LOQ)). While chlorogenic acid (0.098%), caffeic acid (0.107%), rutin (0.078%), and hyperoside (0.134%) were detected in the aerial part, only rutin (0.007%) was detected in the root. As a result of this study, this endemic species was evaluated in terms of some phenolic compounds. It is thought that phenolic compounds can be determined on other *Consolida* species with this method.

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Consolida thirkeana (Boiss.) Bornm.'daki Bazı Fenolik Bileşiklerin YPSK ile Miktarı Tayini ve Yöntemin Validasyonu

ÖZET
Consolida türleri başta deri hastalıkları olmak üzere çeşitli hastalıkların tedavisinde geleneksel kullanıma sahiptir. Türkiye'de de bazı *Consolida* türlerinin geleneksel kullanımı mevcuttur. Fenolik bileşiklerin önemli farmakolojik etkileri bulunmaktadır, bu nedenle bitkilerde miktarlarının bilinmesi önemli bir yere sahiptir. *Consolida thirkeana*, Türkiye'de "boz mahmuz" olarak bilinen endemik bir türdür ve fenolik bileşikler açısından herhangi bir çalışmaya rastlanmamıştır. Bu nedenle *C. thikeana* için bazı fenolik bileşiklerin miktar tayini yapılmıştır. Bu çalışmada, *C. thirkeana*, YPSK kullanılarak kafeik asit, klorojenik asit, hiperosit ve rutin açısından kantitatif olarak analiz edilmiş ve yöntemin validasyonu (doğrusallık, kesinlik, doğruluk, geri kazanım, tespit limitleri ve ölçüm limitleri) gerçekleştirilmiştir. Toprak üstü kısımlarında, klorojenik asit (%0,098), kafeik asit (%0,107), rutin (%0,078) ve hiperosit (%0,134) tespit edilirken, toprak altı kısmı sadece rutin (%0,007) tespit edilmiştir. Bu çalışma sonucunda bu endemik tür bazı fenolik bileşikler açısından değerlendirilmiştir. Bu yöntem kullanılarak diğer *Consolida* türleri üzerinde fenolik bileşiklerin tayininin yapılabileceği düşünülmektedir.

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INTRODUCTION

Consolida (DC.) S.F. Gray is a genus with approximately 59 species found in Ranunculaceae family, and distributed in Europe, northern Africa, and western Asia. Anatolia, which is an important distribution area for *Consolida*, is also considered as the center of diversity in the Mediterranean (Munz, 1967; Ertuğrul et al., 2010; Jabbour and Renner, 2011; Yin et al., 2020). The general classification of *Consolida* was previously included in *Delphinium* L., then recognized as a separate genus (Suzgec et al., 2009; Ertuğrul et al., 2010; Jabbour & Renner, 2011; Pakravan et al., 2018; Yin et al., 2020). *Consolida thirkeana* (Boiss.) Bornm. is Turkish endemic species and called "boz mahmuz" (Güner et al., 2012; Hürkul, 2021; Tok & Yayla, 2022). *Delphinium thirkeanum* Boiss. is accepted as a synonym (Güner et al., 2012; Hürkul, 2021). Laciniate linear leaves, pale lilac flowers, and sessile follicles are characteristic of *C. thirkeana* (Güner et al., 2012; Hürkul, 2021).

Consolida species have been used for hundreds of years in the treatment of various diseases such as traumatic injury, rheumatism, sciatica, stomach-ache, intestinal worms, insomnia, lack of appetite, scabies, and other skin diseases. In Turkey, *Consolida* species are also used for body lice (Ulubelen et al., 1996; Baytop, 1999; Bitiş et al., 2006; Kostic et al., 2013; Yin et al., 2020). In the studies on the chemical components of *Consolida* species, 143 different compounds (alkaloids, flavonoids, and phenolic compounds) have been isolated. It has many biological activities because of the compounds it contains (Şener et al., 2007; Suzgec et al., 2009; Mericli et al., 2012; Rocchetti et al., 2020). It is known that flavonoid glycosides isolated from *Consolida* species have cytotoxic, anti-tyrosinase, anti-leishmaniasis, and anti-trypanosomatid activities (Díaz et al., 2008; Marin et al., 2009; Marín et al., 2017; Zengin et al., 2019).

Bioactive compounds such as flavonoids, and phenolic compounds are secondary metabolites produced under stress conditions. These phytochemical contents of the plants may change according to various stress conditions, climatic conditions, harvesting time of the plants and parts of the plant (Van Vuuren et al., 2007; Çiçek Polat et al., 2019; Kubes et al., 2018; Ouerfelli et al., 2021). One of the most important groups of bioactive compounds is phenolic compounds. These compounds have important pharmacological effects. Studies have found that phenolic compounds, such as chlorogenic acid, caffeic acid, rutin, and hyperoside, have significant antioxidant, anti-inflammatory, anticancer, and antimicrobial effects (Magnani et al., 2014; Gullón et al., 2017; Raza et al., 2017; Naveed et al., 2018; Birková et al., 2020; Bender & Atalay, 2021; Satari et al., 2021; Wang et al., 2021; Şeker Karatoprak et al., 2022).

High performance liquid chromatography (HPLC) is one of the most useful and easy methods used for the analysis of active compounds from plant samples. In HPLC analyses, it is important to find the appropriate solvent system for chromatographic separation of analytes. Therefore, validation of the method used for analysis is also important (Mendoza et al., 2011; Çiçek Polat & Coskun, 2016; Kendir et al., 2021). In the aim of this study, using high performance liquid chromatography, methanolic extracts of aerial part, and root of *C. thirkeana* were analysed quantitatively for caffeic acid, chlorogenic acid, hyperoside, and rutin. The reason why these four phenolic compounds were chosen for quantification was that they have proven important biological activities. Separate extracts were prepared to determine the compound profile in the aerial part and root. The linearity, precision, accuracy, recovery, limits of detection (LOD), and limits of quantification (LOQ) of the method were displayed thus demonstrating validation procedure.

MATERIALS and METHODS

Plant material

Specimens of *C. thirkeana* were collected at Ayaş, Ankara, Turkey, during the flowering period (Date:12.07.2020). The voucher sample was deposited in Herbarium of Ankara University, Faculty of Pharmacy (No: AEF 30483). (AEF: Ankara Üniversitesi Eczacılık Fakültesi Herbaryumu). Other samples collected from the same habitat on the same day were reserved for extract preparation.

Sample preparation

The aerial parts were separated from roots, and they were separately air dried in the shade. Methanol was used in the extraction process. Dried aerial parts and roots were separately powdered and extracted with methanol (Sigma-Aldrich) (24 h). Finally, the samples were extracted using an ultrasonic bath (25°C, 60 min.) (ISOLAB 621.05.010). After filtered, the extracts were concentrated with an evaporator (Heidolph WB2000) (Acıkara et al. 2019). Dry extract was dissolved in methanol (4 mg mL^{-1}). The samples prepared for analysis were stored in the refrigerator (+4°C) during the analysis.

High performance liquid chromatography (HPLC) analysis

For HPLC analysis, a liquid chromatographic system device (Agilent 1100 Series) (automatic injector, pump, thermostated column, and DAD) was used. Phenolic compounds (caffeic acid, chlorogenic acid, hyperoside, and rutin) were quantified in HPLC using a Waters Spherisorb C18 column (25 cm × 4.6 mm, 5μm) maintained at 40 °C. The mobile phase consisted of 0.01% formic acid (A) and acetonitrile (B) delivered at a flow rate of 1 mL/min. Detection of all samples was performed at a wavelength of 254 nm.

Method validation

The method was validated (ICH 2005; Çiçek Polat &

Coskun, 2016). Each compound's stock reference solutions (caffeic acid, chlorogenic acid, hyperoside, and rutin) were made by dissolving 1 mg in 2 mL methanol (500 μg mL^{-1}). For the calibration curve, different concentrations of reference solution were injected in triplicate. Carrying on intra-day and inter-day variation, the precision of method was carried out, and differences were expressed by relative standard deviation (RSD). LOD is signal/noise value is 3:1, while LOQ is signal/noise value is 10:1. For LOD and LOQ, 10 injections of standards were made and averaged. For the recovery assay, 3 different known concentrations of standards were spiked into the sample solution. The mixtures were examined using the same method that was used to analyse the samples for standards.

Statistical analysis

All analyses were executed in triplicates and the mean values were calculated. All the data presented as the mean ± standard deviation (SD), relative standard deviation (RSD), linear regression analysis and calculations were performed using Microsoft Excel program.

RESULTS and DISCUSSION

In this study, aerial part, and root of *C. thirkeana* were analyzed quantitatively for caffeic acid, chlorogenic acid, hyperoside, and rutin by using HPLC. Methanol was used in the extraction process (Acıkara et al., 2019; Okur et al., 2020; Ayla et al., 2019). Yields of aerial part and root extracts are 15.85% and 5.84%, respectively. While chlorogenic acid (0.098%), caffeic acid (0.107%), rutin (0.078%) and hyperoside (0.134%) were detected in the aerial part, only rutin (0.007%) was detected in the root (Table 1, Figure 1, Figure 2).

A liquid chromatographic system device was used for HPLC analysis, and the method was validated. Within the ranges of 5 to 100 μg mL^{-1}, 5 to 100 μg mL^{-1}, 10 to 100 μg mL^{-1}, and 5 to 100 μg mL^{-1}, the calibration plots for caffeic acid, chlorogenic acid, hyperoside, and rutin were linear. The LOD and LOQ values for these phenolics were determined (Table 2).

Table 1. Contents of chlorogenic acid, caffeic acid, rutin and hyperoside in *C. thirkeana* methanol extracts (n=3).
Çizelge 1. C. thirkeana methanol ekstrelerinde kolorojenik asit, kafeik asit, rutin ve hiperosit içerikleri (n=3).

	Caffeic acid (% ± SD*)	Chlorogenic acid (% ± SD*)	Hyperoside (% ± SD*)	Rutin (% ± SD*)
Aerial part	0.107 ± 0.002	0.098 ± 0.001	0.134 ± 0.001	0.078 ± 0.003
Root	ND**	ND**	ND**	0.007 ± 0.001

*SD: Standard Deviation; **ND: Not Detected

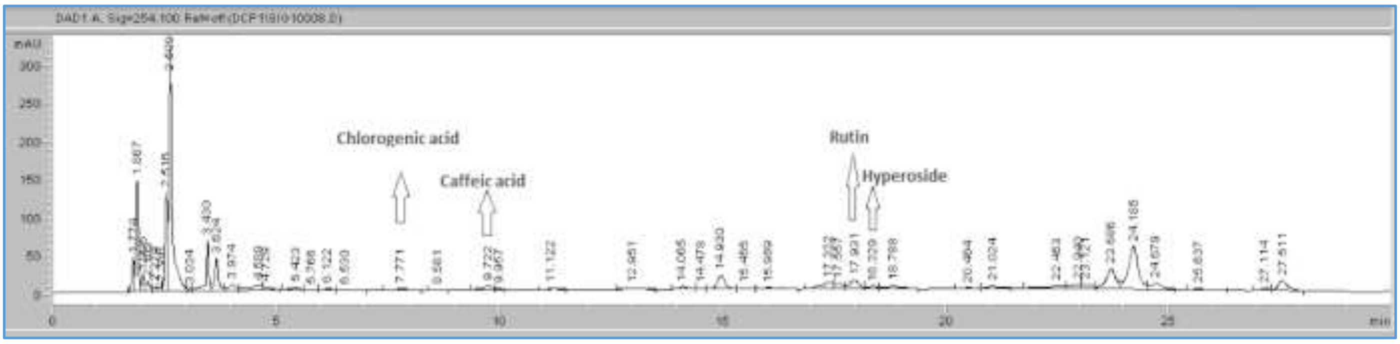

Figure 1. HPLC chromatogram of aerial part (*C. thirkeana*)
Şekil 1. Toprak üstü kısmının YPSK kromatogramı (C. thirkeana)

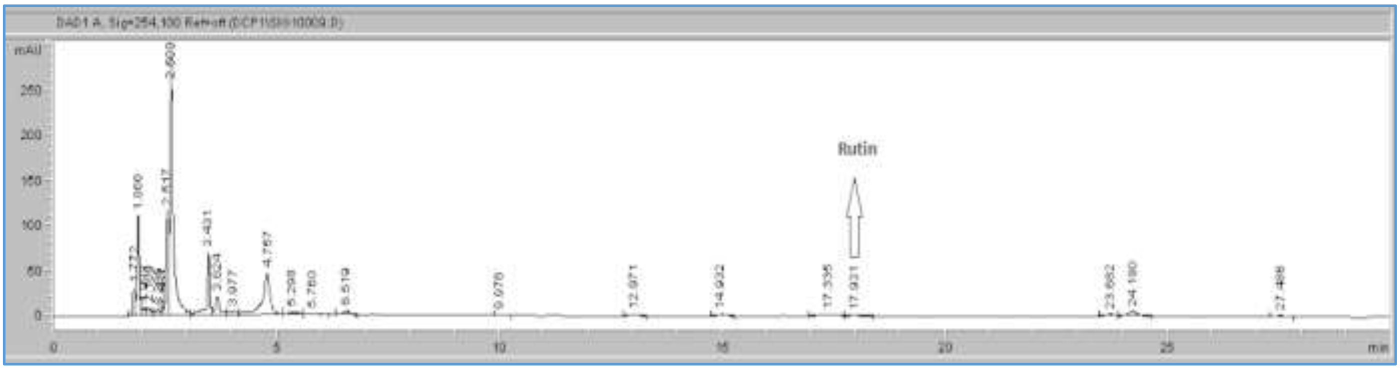

Figure 2. HPLC chromatogram of root (*C. thirkeana*)
Şekil 2. Toprak altı kısmının YPSK kromatogramı (C. thirkeana)

Intra-day and inter-day variations were used to determine the method's precision. The result showed that relative standard deviation (RSD) values were always less than 3% (Table 3).

For a recovery assay, 3 different known concentrations of standards were spiked into the sample solution. The mean extraction recovery of caffeic acid, chlorogenic acid, hyperoside, and rutin was in the range of 96.124-101.270%, 97.837-101.881%, 97.879-101.103, and 97.289-101.778, respectively (Table 4).

Table 2. Calibration values for standards.
Çizelge 2. Standartlar için validasyon değerleri

Standards	Calibration range (μg mL^{-1})	Linear Equation	Correlation factor (r^2±SD*)	RT** (min)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Caffeic acid	5-100	y=65.998x-244.18	0.985 ± 0.005	9.5	0.371	1.237
Chlorogenic acid	5-100	y=11.965x-9.2492	0.99 ± 0.008	7.6	0.777	2.590
Hyperoside	10-100	y=14.1x-36.65	0.996 ± 0.004	18.4	0.348	1.160
Rutin	5-100	y=23.625x+9.898	0.995 ± 0.006	17.9	0.036	0.123

*SD: Standard Deviation; **ND: Not Detected

Table 3. Intra-day and inter-day precision data of the method.
Çizelge 3. Metodun gün içi ve günler arası kesinlik verileri.

Standards	Amount (μg mL^{-1})	Intra-day precision (RSD*%)	Inter-day precision (RSD*%)
Caffeic acid	5	1.105	0.846
	10	0.709	0.342
	25	0.315	1.255
	50	2.486	1.133
	100	0.730	2.558
Chlorogenic acid	5	1.347	1.579
	10	1.291	2.784
	25	2.913	1.357
	50	1.060	0.580
	100	0.831	2.932
Hyperoside	10	2.744	2.607
	25	2.145	0.820
	50	2.859	0.711
	100	0.415	1.090
Rutin	5	2.735	0.502
	10	1.806	1.116
	25	0.595	0.361
	50	1.354	2.659
	100	1.423	1.717

*RSD: Relative Standard Deviation

Table 4. Recovery assay's statistical data of the method (n=3).
Çizelge 4. Methot geri kazanım testinin istatistiksel verileri (n=3).

Standards	Concentration in sample (μg mL^{-1})	Amount spiked (μg mL^{-1})	Mean amount found in mixture (μg mL^{-1})	Mean recovery (%)	RSD*
Caffeic acid	0.006	0.003	0.0045	96.124	0.204
		0.006	0.006	96.739	1.200
		0.012	0.009	101.270	0.331
Chlorogenic acid	0.003	0.0015	0.0025	101.881	1.570
		0.003	0.003	97.837	2.359
		0.006	0.0045	98.854	2.694
Hyperoside	0.005	0.0025	0.00225	98.937	1.620
		0.005	0.005	101.103	1.636
		0.01	0.0075	97.879	1.502
Rutin	0.003	0.0015	0.0025	101.778	2.726
		0.003	0.003	99.369	1.355
		0.006	0.0045	97.289	2.519

*SD: Standard Deviation, *RSD: Relative Standard Deviation

Some phytochemical studies have been carried out on *Consolida* genera and there are many alkaloids isolation studies on *Consolida* species grown in Turkey. In the study of Ulubelen et al. (1996), consolidine (a new norditerpenoid alkaloid), pubescenine, gigactonine, desolline and ajaconine alkaloids were isolated from the aerial parts of *C. oliveriana* (DC.) Schrödinger. Bitiş et al. (2006) isolated delphatine, delcaroline, browniine, which are very toxic alkaloids and hetisine, dehydronapelline, 12-epidehydronapelline alkaloids from aerial parts of *C. olopetala* (Boiss.) Hayek. Especially Mericli et al. have many isolation studies on *Consolida* species. Mericli et al. (1999) isolated hetisine, hetisinone and ajadelphinine alkaloids from aerial parts of *C. stenocarpa* (Davis & Hossain) Davis. Mericli et al. (2001) isolated delcosine, delsoline, gigactonine, lycoctonine, takaosamine, atisine and hetisinone diterpenoid alkaloids from aerial parts of *C. regalis* S.F.Gray subsp. paniculata (Host) Soo var. paniculata. Mericli et al. (2012) isolated methyllycaconitine and leucanthumsine alkaloids from aerial part of *C. thirkeana* (Boiss.) Bornm. and they also isolated browniine, gigactonine and neolinine alkaloids from aerial parts of *C. sulphurea* (Boiss. & Hausskn.) P.H. Davis.

In terms of phenolic compounds, 93 phenolic acids were detected in the study on some *Consolida* species (*C. glandulosa* (Boiss. & A. Huet) Bornm, *C. hellospontica* (Boiss.) Chater, *C. raveyi* (Boiss.) Schrödinger, *C. regalis* (Boiss.) Schrödinger, *C. staminosa* P.H. Davis & Sorger and *C. stenocarpa* (Davis & Hossain) Davis) which grown in Turkey. However, in this study, information about these species was not given separately (Rocchetti et al., 2020). *p* -hydroxybenzoic, caffeic, ferulic and *p* - coumaric acids have been detected in aerial parts of *C. armeniaca* (Stapf ex Huth)F.C.Schrad and protocatechuic, vanillic, cinnamic, chlorogenic, galllic, sinapic and benzoic acids, kaempferol, quercetin, and hyperoside have been detected in *C. orientalis* (J.Gay) Schrödinger (Yin et al., 2020). Phenolic compounds have also been isolated in other species belonging to the Ranunculacea family. *p* -Hydroxy benzoic, caffeic, *p* -coumaric, chlorogenic and trans-aconitic acids were isolated from *Delphinium formosum* Boiss.&A.Huet (Dürüst et al., 2001). Caffeic, ferulic, isoferulic, fukinolic, cimicifugic A, and cimicifugic B acids were isolated from *Actaea racemosa* L. (Li et al., 2003). Considering the studies conducted, data could not be reached in terms of quantification. In addition to the detection of bioactive compounds, the determination of their amounts is also important.

This study was conducted for the first time for *C. thirkeana*. According to the results, chlorogenic acid, caffeic acid, rutin, and hyperoside were detected in *C. thirkeana*. At the root, only rutin was found. When the amount of rutin was compared, it was found that it was higher in the aerial part. Studies on *Consolida* species have generally been studied with aerial parts and bioactive compounds have been isolated. This is due to the presence of more compounds in the aerial parts.

CONCLUSION

The method used in this study for the HPLC determination of chlorogenic acid, caffeic acid, rutin, and hyperoside in *C. thirkeana* extracts is rapid, simple, effective, and reliable. The linearity, intra-day and inter-day precision, recovery, LOD, and LOQ of the method were all validated. At the end of this study, *C. thirkeana* was evaluated in terms of these phenolic compounds. Elaborated studies are needed to determine the other major compounds and it is thought that with this method, phenolic compounds can be determined on other *Consolida* species as well. Thus, comparisons can be made between species in terms of the amount of phenolic compounds.

Author's Contributions

The contribution of authors is equal.

Statement of Conflictof Interest

Authors have declared no conflict of interest.

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