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EVALUATION OF THE PHYTOCHEMICAL PROPERTIES AND DNA PROTECTIVE ACTIVITY OF *BOLANTHUS ORTEGIOIDES* (FISCH. and C.A. MEY.) MADHANI and RABELER

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ABSTRACT

The aim of this study was to elucidate the potential of the flora of Turkey by investigating the phytochemical properties of the endemic plant species *Bolanthus ortegioides* of the family Caryophyllaceae. The identity of the plant was validated through a phylogenetic approach with DNA barcoding using the internal transcribed spacer [ITS] region. Methanolic extract of *B. ortegioides* was examined by HPLC, and the total phenolic content was measured. In addition, pBluescript II SK(+) plasmid DNA cleavage was induced by ultraviolet (UV) photolysis of hydrogen peroxide (H_2O_2), and the activity of the methanolic extract of *B. ortegioides* against oxidative DNA damage was examined. The total phenolic content of the extract was 5.6 ± 0.25 µg (gallic acid equivalent [GAE])/mg. Of 15 phenolic compounds analysed by HPLC, vanillic acid was found to be a major compound. Chlorogenic, caffeic, epicatechin, p-coumaric, ferulic, rutin and chicoric acids were also detected. The results showed that *B. ortegioides* methanolic extract offered moderate protection against DNA damage.

Keywords: Phenolic content, methanolic extract, DNA cleavage, Caryophyllaceae, phytochemistry

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INTRODUCTION

Due to its diverse landscape and climate, Turkey has a very rich flora, with approximately 11,000 species, of which of 33% are endemic. There is great interest in traditional herbal medicines, and they provide a huge potential source for pharmaceutical studies (Kiliç and Bağci, 2013; Karakaya *et al*., 2020). The aim of this study was to elucidate the potential of the flora of Turkey by investigating the phytochemical properties of the endemic plant species *Bolanthus ortegioides* of the family Caryophyllaceae.

Caryophyllaceae Juss. is one of the largest dicotyledonous families worldwide, with about 90 genera and 2,400 species (Hamzaoğlu and Koç, 2019). The genus *Phryna* (Boiss.) Pax and Hoff. is a member of the Caryophyllaceae family and endemic in Turkey (Kiliç, 2013). *Phryna ortegioides* (Fisch. and C. A. Mey.) Pax and K. Hoffm. was the only known representative of the genus *Phryna* (Kiliç, 2013; Kiliç *et al*., 2013), until *P. hamzaoglui* Koç and Budak was discovered in Malatya, Turkey (Koç and Budak, 2018).

The analysis of molecular data has allowed researchers to solve unresolved biological questions (Baldwin, 1992). Based on molecular data, a previous study on the tribe Caryophylleae in the Caryophyllaceae family data revealed that the genus *Phryna* clearly belongs to the genus *Bolanthus* (Ser.) Rchb. As a result of this study, *Phryna* is now considered a synonym for *Bolanthus*, and therefore *P. ortegioides* is a synonym for *B. ortegioides* (Fisch. and C.A. Mey.) Madhani and Rabeler (Madhani *et al*., 2018). Other synonyms of *P. ortegioides* are *Tunica ortegioides* Fisch and Mey.; *Saponaria ortegioides* (Fisch and Mey.) Boiss. and Bal.; *Gypsophila ortegioides* (Fisch and Mey.) Boiss. (Kiliç, 2013; Kiliç *et al*., 2013); and *Phrynella ortegioides* (Fisch. and C.A. Mey.) Pax and K. Hoffm (Başköse and Dural, 2018).

Previous studies on the phytochemical properties of *B. ortegioides* concluded that this plant species is rich in triterpenoids and flavonoids (Horo *et al*., 2015; Geçibesler *et al*., 2017). Kiliç *et al*. (2013) found that the major components of the essential oil composition of *B. ortegioides* are germacrene D, borneol, bicyclogermacrene and pcymene. Other research on the chemical composition of lipophilic fractions of *B. ortegioides* reported significantmeaningful antioxidant activity (Geçibesler *et al*., 2016).

The aim of this study was to investigate the phytochemical properties of *B. ortegioides* of the family Caryophyllaceae. With this aim in mind, we measured the total phenolic content of *B. ortegioides* methanolic extract using HPLC to identify phenolic compounds. We also tested the ability of the methanolic extract to confer

protection against oxidative stress and DNA damage. This is the first time that such data have been presented for the genus *Bolanthus* and species *B. ortegioides*.

The plant material used in the present study was collected by Prof. Ömer Kiliç in July 2018 and identified and registered as *P. ortegioides*. As a previous phylogenetic study revealed that *Phyrna* is now a synonym of *Bolanthus* (Madhani *et al*., 2018), and no published data argue against this classification, the plant material used in this study was treated as *B. ortegioides*. Precise identification of an organism that is used in a study is essential in all areas of biological sciences, as well as in biochemistry. Poor taxonomy can cause misleading and non-reputative results. In this study, we used an integrative approach incorporating morphological and molecular methods to identify the specimen. To validate the identification of the plant material, we amplified and sequenced the internal transcribed spacer (ITS) of nuclear ribosomal DNA, which is highly recommended as a core plant DNA barcode and is one of the most commonly used DNA markers in plant systematics (Cheng *et al*., 2015). In addition, we performed maximum likelihood (ML) analyses to reveal the status of *B. ortegioides* in the genus *Bolanthus* for the first time using the ITS sequence containing ITS 1, 5.8S rDNA gene and ITS 2 regions.

MATERIALS AND METHODS

Plant Samples: The plant material used in this study was collected by Prof. Ömer Kiliç in Sancak, Bingöl, Turkey, surroundings of Hasanova hamlet, steppe, stony slopes, 1,550–1,600 meters in July 2018. A voucher specimen was stored in Adiyaman University, Faculty of Pharmacy Herbarium (collection number: 4128). The morphological identification of the plant sample was performed according to volume 2 of the *Flora of Turkey* (Davis, 1966). An integrative taxonomic approach, as described in detail below, was followed to confirm the identity of the plant specimen.

Isolation of ITS Sequence and Phylogenetic Analysis: DNA was isolated from the herbarium material and grinded with liquid nitrogen (Cubero *et al*., 1999) according to the manufacturer's protocol using a DNeasy Plant Mini Kit (Qiagen). PCR was performed in a 25 µl reaction volume using approximately 20 ng of DNA, 1.5 units of Taq DNA polymerase (Fermentas), 10× buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP (800 µM), 5% of DMSO and 25 pmols of ITS4 and ITS5m primers (White *et al*., 1990; Sang *et al*., 1995), according to the following protocol: 5 minutes, 95°C initial denaturation; 35 cycles of 45 seconds, 94°C denaturation; 45 seconds, 50°C annealing; and 1 minute, 72°C extension, followed by a 5-minute final extension at 72°C. The PCR product was sequenced commercially by a biotechnology company (BMLabosis, Ankara, Turkey).

The sequence chromatograms were checked with the naked eye and aligned in BioEdit v7.0 (Hall, 1999). The homology and authenticity of the obtained sequence were checked via a search using the Basic Local Alignment Search Tool (BLAST) of the NCBI web server (Altschul *et al*., 1990). As the BLAST search found more than one closely related sequence belonging to different species of *Bolanthus*, further analyses were performed of ones with percent identity above 95%. KY468936.1 was not included, as it contains many ambiguous sites. The list of the species and their related sequences with GenBank numbers are given in Table 1. The multiple alignment of the sequences was conducted using the MAFFT online service (Katoh *et al*., 2019) with -L-INS-i parameters. Multiple alignments were checked and trimmed using Mesquite 3.7 (Maddison and Maddison, 2021). The final dataset containing 16 sequences 632 bp long (22 parsimony-informative, 34 singleton sites and 576 constant sites) was used for phylogenetic analyses. ML phylogenetic inference of the dataset was performed using the IQ-TREE web server (Trifinopoulus *et al*., 2016). A nucleotide substitution model was automatically calculated by ModelFinder software implemented in the web service (Kalyaanamoorthy *et al*., 2017). The best-fit model was TNe+G4 according to BIC, AIC and AICc. In addition, ultrafast bootstrap approximation with 1,000 replicates was used to test the branch support of the tree (Hoang *et al*., 2018). The constructed phylogenetic tree was rooted (midpoint rooting) and visualized using FigTree version 1.4.2 (Rambaut, 2010).

Table 1. List of the species used in the phylogenetic analysis, together with their GenBank numbers.

Taxon name	GenBank Accession Number
Bolanthus ortegioides	OK500009
Bolanthus ortegioides	KX834008.1
Bolanthus sp. IB-2017	KY468936.1
Bolanthus confertifolia	KX834007.1
Bolanthus frankenioides var. *frankenioides*	KY406149.1
Bolanthus frankenioides var. *fasciculatus*	KY406148.1
Bolanthus huber-morathii	KX834006.1
Bolanthus minuartioides	KX834005.1
Bolanthus cherlerioides	KY406155.1
Bolanthus huber-morathii	KY406154.1
Bolanthus sp. IB-2016	KY406153.1
Bolanthus thymoides	KY406152.1
Bolanthus minuartioides	KY406151.1
Bolanthus spergulifolius	KY406150.1

Bolanthus stenopetalus	KY406147.1
Bolanthus mevlanae	KY406145.1
Bolanthus cherlerioides	MF401128.1
Bolanthus turcicus	KY406146.1

Plant Extract Preparation: The aerial parts at flowering stage of air-dried *B. ortegioides* (1,820 g) were dried in the shade, ground to a fine powder and macerated twice in succession with methanol at room temperature. After the filtrates were combined, methanol was removed using a rotary evaporator at 40°C. HPLC grade (100%) methanol (20-fold) was used for extraction at room temperature for 3 days for proper percolation and extraction. On the fourth day, the extract was filtered by filter papers (Whatman No. 1) The extract was then evaporated to dryness using a rotary evaporator and freeze dried. The plant extract was stored in a refrigerator (4°C) until use.

Quantitative Analysis of Phenolic Compounds by HPLC and Estimation of the Total Phenolic Content: The total phenolic content of the methanolic extract was estimated using Folin–Ciocalteu reagent according to a modified method described in previous studies (Singleton *et al*., 1999; Ekin *et al*., 2017). The experiment was conducted in triplicate, and the mean and standard deviation were calculated using Microsoft® Excel® software. For quantitative analysis of the extract, the Nexera-i LC-2040C 3D Plus HPLC system (Shimadzu) in the University of Health Sciences was used. A DAD (Diode array detector was used as the detector in the analysis process, and scanning was performed at 254 nm. Phenylhexyl 4.6 × 150 mm, 3 µm (UP) (InterSustain; GL Sciences, Japan) reversed phase filler column was used for separation of the phenolic components. The pump flow program with gradient elution was applied with 0.1% formic acid/deionized water (solvent A) and acetonitrile (solvent B) (HPLC grade, Merck) as the mobile phase. Throughout the analysis, the flow rate of the mobile phase was adjusted to 1 ml/minute. In total, 10 µl of sample and 10 µl of standard were injected into the device. The column temperature was set as 30°C. In total, 15 phenolic compounds were screened. The wavelength of maximum absorbance of each phenolic compound was determined, and all compounds were scanned at these maximum wavelengths. Standard graphics were drawn. Further extracts were prepared at a concentration of 1 mg/ml and delivered to the device.

Assay of DNA Protective Activity of *B. ortegioides* Extract: To perform an in vitro assay of the DNA protective activity of *B. ortegioides* extract, the pBluescript II SK(+) (Stratagene) plasmid was used. For plasmid isolation, glycerol stock of *Escherichia coli* Dh5α cells carrying the pBluescript II SK(+) (Stratagene) plasmid was used to inoculate fresh LB medium containing ampicillin (100 µg/ml) and cultured at 37°C overnight with shaking at 220 rpm. The cells were harvested by centrifugation. Plasmid DNA was isolated using a plasmid DNA isolation kit (K0502; Thermo Fisher Scientific) according to the manufacturer's recommendations and stored at 4°C until used.

The DNA protective activity of the methanolic extract of *B. ortegioides* was tested by hydrogen peroxide (H_2O_2) photolysis by ultraviolet (UV) radiation, as previously described (Berk *et al*., 2011), with slight modifications. First, 5 µl of plasmid DNA (approximately 25 ng/µl), 5 µl of 3% H_2O_2 and 5 µl of plant extract (150 mg/ml, dissolved in ddH_2O) were mixed in a tube and then placed directly on the surface of a UV transilluminator (300 nm) for 5 minutes at room temperature. A negative control containing only plasmid DNA was not exposed to UV/H_2O_2 photolysis. The reaction mixtures were mixed with 3 µl of 6 × loading dye (Thermo Scientific), loaded on a 0.8% agarose gel and supplemented with 5 µl of 10 mg/ml of ethidium bromide. Electrophoresis was carried out at 90 V for 1 hour. The Quantum ST5 Gel Documentation system was used to visualize the gel.

RESULTS AND DISCUSSION

Identity of the Specimen: The plant specimens were collected and identified by Prof. Ömer Kiliç as *P. ortegioides* (now *B. ortegioides*, please see the Introduction) using classical morphological methods. Then, DNA was isolated from the specimen, and the ITS region was amplified and sequenced to confirm the identity of the plant. A 636 bp-long ITS sequence was obtained and submitted to GenBank (OK500009). The sequence was initially identified by a BLAST search. The search identified 16 closely related sequences that belong to the genus *Bolanthus* spp., with maximum scores ranging between 1,048 and 1,100 and percent identity between 95.92% and 98.26%. As the BLAST is a simple distance-based algorithm intended to find similarities between sequences, ML analysis was conducted to determine the relationship between 15 BLAST hits. There were 16 initial hits, but one sequence was discarded from the analysis because it had too many ambiguities. Most of these were found in a previous study on the phylogeny of *Bolanthus* (Koç *et al*., 2019). However, this study did not include *B. ortegioides*, as this species was in the genus *Phyrna* at the time the study was conducted. The ML tree derived from the dataset showed strong support (98%) for the relationship between *B. ortegioides* (KX834008.1) and the specimen used in this study, thereby confirming the identity of the specimen.(Fig. 1).

Phytochemical Analysis: Phenolics in plants have been reported to have numerous biological activities, one of which is antioxidant activity (Ekin *et al*., 2017). In this study, we examined the total phenolic content of *B. ortegioides* methanolic extract using the Folin–Ciocalteau

method and determined and expressed this in milligrams of GAE. The total phenolic content in the extract was 5.6 ± 0.25 µg (GAE)/mg (Fig. 2). In a previous study on *B. spergulifolius*, the total phenolic content in aqueous, methanolic and ethyl acetate extracts was 49.3, 46.91 and 23.2 mg GAE/g of dry weight, respectively (Derici *et al*., 2021). Similar total phenolic contents have been found in plants closely related to *Bolanthus*, such as *Saponaria officinalis* (6.57 µg GAE/mg) (Şengül *et al*., 2011), *Gypsophila pilulifera* (6.5 µg GAE/mg extract) (Özbek Yazici and Özmen, 2017) and *G. arrostii*, *G. pilulifera* and *G. simonii* (0.26, 0.54 and 15.15 µg GAE/mg extract, respectively) (Arslan and Çelik, 2013). When compared to the literature, the phenolic content of *B. ortegioides* in the present study seems average among closely related taxa and the genus *Bolanthus*.

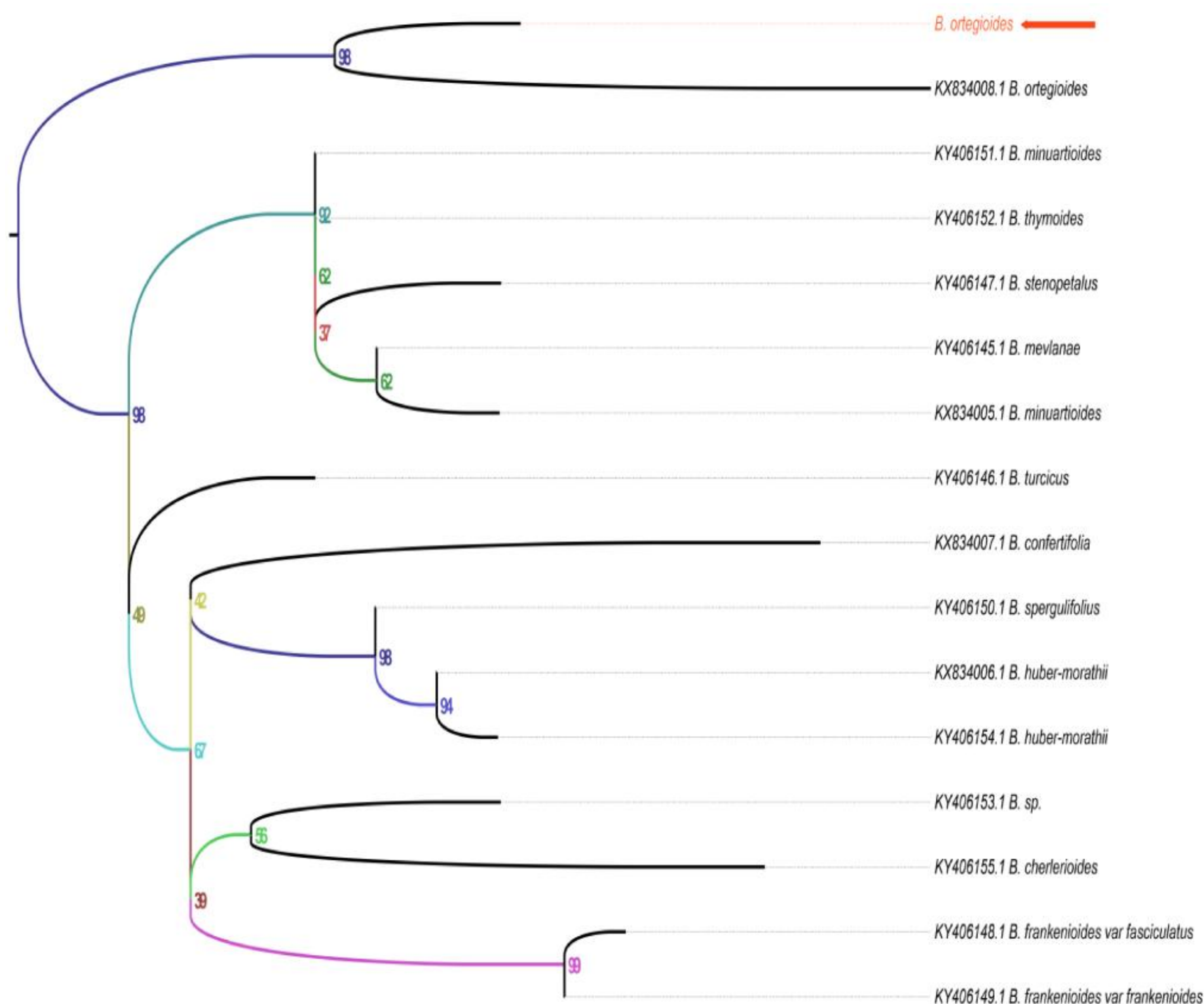

Figure 1. ML tree of 16 *Bolanthus* spp. constructed by IQ-TREE with ultrafast bootstrap support, showing the node values. The red arrow indicates the specimen used in the present study.

The HPLC analysis of the phenolic compounds revealed the presence of chlorogenic, vanillic, caffeic, epicatechin, p-coumaric, ferulic, rutin and chicoric acids in the methanolic extract of *B. ortegioides* (Fig. 3). Among these phenolic compounds, vanillic acid was found in the highest amount, and chicoric acid was found in the lowest amount. The HPLC analysis did not detect the presence of gallic acid, 4-hydroxybenzoic acid, salicylic acid, apigenin-7-glucoside, cinnamic acid, quercetin or naringenin in the methanolic extract of *B. ortegioides*. The phenolic contents of *B. ortegioides* and their quantities are shown in Table 2.

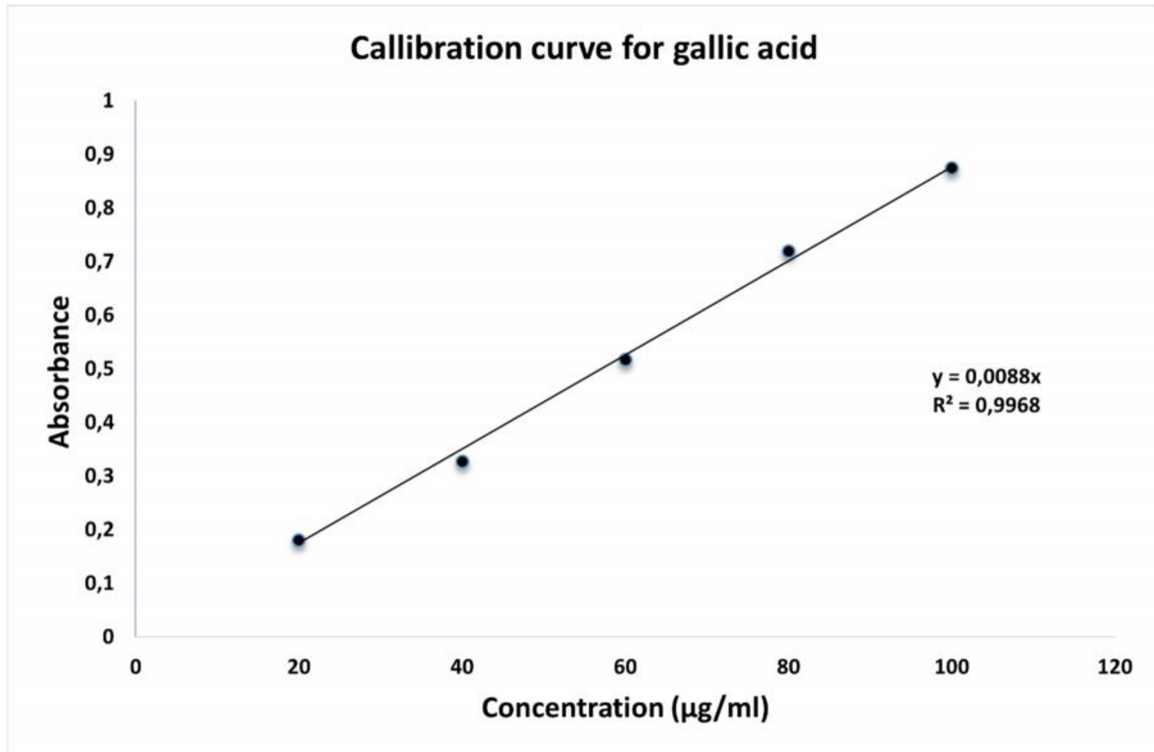

Figure 2. Standard curve of gallic acid for total phenolic content.

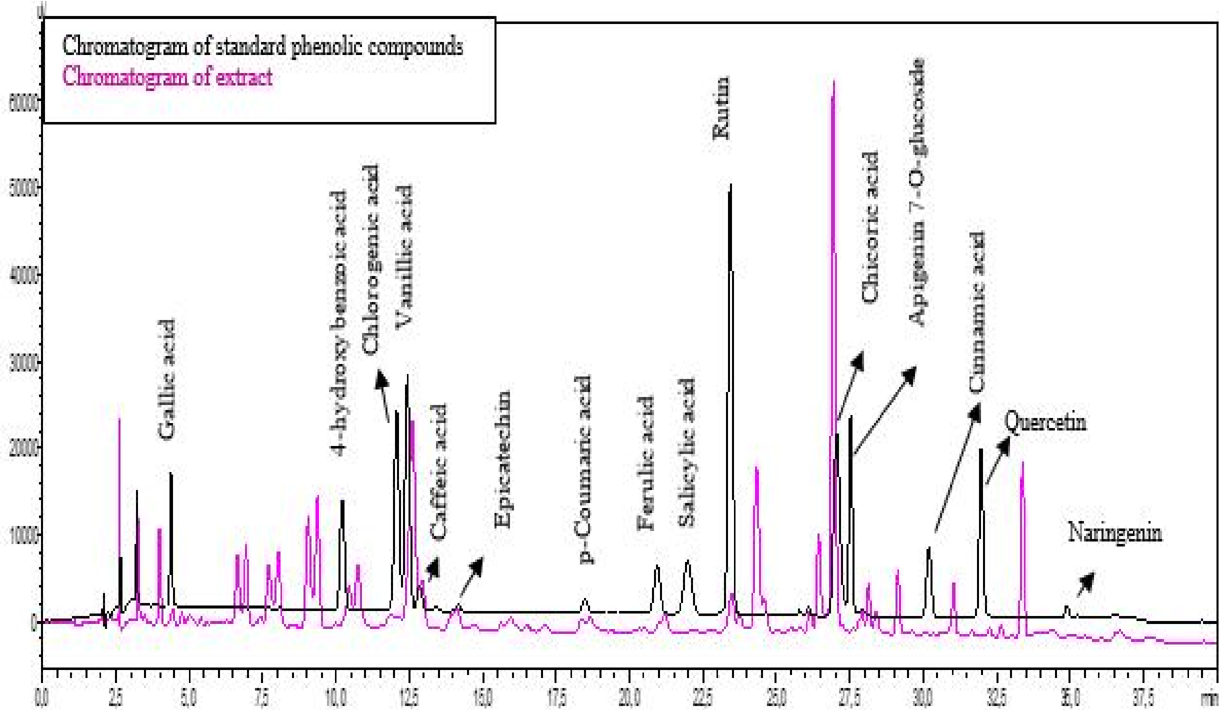

Figure 3. HPLC chromatogram of phenolic standards and extract at 280 nm, together with their retention times (minutes). The chromatogram of standard phenolic compounds is shown in black, and the chromatogram of the extract is shown in pink.

The present study provides the first data obtained from a HPLC analysis of phenolics in *B. ortegioides* methanolic extract. Some previous studies have described flavonoids (Geçibesler and Aydın, 2020), lipophilic fractions (Geçibesler *et al*., 2016), triterpene saponins (Horo *et al*., 2015) and essential oil

compositions (Kiliç *et al*., 2013) of *B. ortegioides.* It is emphasized that HPLC data of phenolics of the genus *Bolanthus* is not available in the current literature (Özbek Yazici and Özmen, 2017). In a HPLC analysis of the phenolic content of extracts from four *Gypsophila* L. taxa, which are closely related to *Bolanthus*, Altay *et al*. (2019) reported the presence of 3,4-dihydroxybenzoic acid, vanillin and p-coumaric acid.

DNA Protective Activity: Hydroxyl radicals can cause nicks in DNA, which can lead to carcinogenesis, mutagenesis and cytotoxicity (Saravanakumar *et al*., 2014). DNA scission has been shown to be an effective method to evaluate antioxidant activity against hydroxyl radicals in vitro (Chandrasekara and Shahidi, 2011). In this work, the ability of methanolic extract of *B. ortegioides* to protect against oxidative damage and DNA cleavage induced by UV photolysis of H_2O_2 using an in vitro method was evaluated. As shown in Figure 4, the protection conferred against DNA damage by *B. ortegioides* methanolic extract was moderate. In accordance with the findings of the present study, the results of previous studies showed that plant species belonging to the Caryophyllaceae family exhibit DNA protective activity, preventing DNA damage by hydroxyl radicals (Boukhira *et al*., 2015; Aliyazicioğlu *et al*., 2017; Özbek Yazici and Özmen, 2017). In the present study, vanillic acid was the major component of *B. ortegioides* methanolic extract, along with epicatechin and rutin, which were the other components found in high amounts. These phenolic compounds are known to prevent the production of oxidative and UV induced DNA damage (Porto *et al*., 2003; Kumar *et al*., 2011; Ojha, 2016). Therefore, these compounds may account for the protective activity of *B. ortegioides* methanolic extract against DNA damage in the present study.

This is the first study to shed light on the phenolic composition and content of *B. ortegoides* methanolic extract. The results of this study provide evidence for biological activities of *B. ortegoides* extract, such as protection against DNA damage. More in vitro and in vivo assays are essential to identify and analyse the active components of the extract to develop a new natural drug for use in the pharmaceutical field.

Table 2. Phenolic compounds of methanolic extract of *B. ortegioides.*

ID#	Name	Ret. time	Conc.	Unit	Channel	Peak#	Area	Height	Area%	S/N
1	Galllic acid	No peak detected	0,000	mg/L	Ch2 271 nm	--	0	0	0,000	--
2	4-hydroxybenzoic acid	No peak detected	0,000	mg/L	Ch1 254 nm	--	0	0	0,000	--
3	Chlorogenic acid	12,154	0,387	mg/L	Ch3 325 nm	1	12906	1455	100,000	0.56
4	Vanillic acid	12,608	8,479	mg/L	Ch4 260 nm	1	400127	29786	100,000	8.32
5	Caffeic acid	12,935	2,000	mg/L	Ch5 248 nm	1	38269	3847	100,000	0.86
6	Epicatesine	14,128	6,156	mg/L	Ch6 277 nm	1	44026	3685	100,000	1.15
7	p-coumaric acid	18,651	0,204	mg/L	Ch7 308 nm	1	16128	1131	100,000	0.35
8	Ferulic acid	21,219	0,432	mg/L	Ch8 322 nm	1	25924	2341	100,000	0.88
9	Salicylic acid	No peak detected	0,000	mg/L	Ch9 235 nm	--	0	0	0,000	--
10	Rutin	23,472	2,542	mg/L	Ch1 254 nm	1	51650	4143	100,000	1.01
11	Chicoric acid	27,271	0,152	mg/L	Ch10 327 nm	1	1689	282	100,000	0.12
12	Apigenin-7-glucoside	No peak detected	0,000	mg/L	Ch11 336 nm	--	0	0	0,000	--
13	Cinnamic acid	No peak detected	0,000	mg/L	Ch12 276 nm	--	0	0	0,000	--
14	Quercetin	No peak detected	0,000	mg/L	Ch1 254 nm	--	0	0	0,000	--
15	Naringenin	No peak detected	0,000	mg/L	Ch13 288 nm	--	0	0	0,000	--

Figure 4. DNA protective activity of *B. ortegioides* methanolic extract. 1: 5 µl of plasmid DNA as a control, 2: 5 µl of plasmid DNA + 5 µl of 3% H_2O_2 + 5 µl of dH_2O + UV (5 minutes), 3: 5 µl of plasmid DNA + 5 µl of 3% H_2O_2 +UV (5 minutes) + 5 µl of plant extract.

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Conflict of interest: No potential conflict of interest was reported by the authors.

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