Original Research Article

Total Phenolics, Flavonoids Contents, Antioxidant Activity and DNA Protective Effect of Lenten Rose (*Helleborus orientalis)*

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ABSTRACT

The aims of the present study were to evaluate the antioxidant activity and DNA Protective effect of *Helleborus orientalis* (HO) leaf extract against oxidative damage, and to determine the total phenolic and flavonoid contents of the plant species studied.

Methods: The total phenol content (TPC) of *H. orientalis* (*Ranunculaceae*) extract was determined using the Folin-Ciocalteu technique. The aluminum chloride colorimetric assay in the determination of The total flavonoid content (TFC) and was used, Analysis of Phenolic Acids was identified by High-Performance Liquid Chromatography (HPLC). Antioxidant activity was analyzed by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. Protective effect of *H. orientalis* leaf extract against to H_2O_2 was evaluated by using TAS, TOS methods and Comet assay.

Key findings: *H. orientalis* ethanol extracts contain high amounts of antioxidants. The HO leaf methanol extract (LME) decreased the DNA damage at all tested concentrations in a dose-dependent manner ($r=0.86$ $p<0.01$) against to H_2O_2.

Conclusions: The total phenol content in the extracts of different parts of the plant varied from 4.00 mg GAE/1 gr to 19.42 mg GAE/1 gr. The flowers had the highest phenol content (19.42 mg GAE/1 gr sample) and followed by the leaves (17.20 mg GAE/1 gr sample). The total flavonoid content in the extracts from different parts of the plant varied from 2.57 mg QE/1 gr to 11.88 mg QE/1 gr. The flowers had the highest flavonoid content (11.88 mg QE/1 gr sample) and followed by the leaves (10.21 mg QE/1 gr sample).

Antioxidant activity of fractions was explained as a percentage of DPPH radicals' scavenging and IC_{50} values (µg/ml). Leaf and flowers of HO are richer in antioxidant than its root and stem. As the concentration of leaf extracts used increased, the DNA

protective effect increased and it was statistically significant at overdoses of 2500 µg/mL. Total antioxidant status (TAS) levels were significantly (p <0.05) decreased in the H_2O_2 group (3.4±0.21) but *H. orientalis* was significantly (p<0.05) increased TAS levels in this group. When the concentration of leaf extracts used increased, the protective effect has also increased and statistically significant at overdoses of 2500 µg / mL (6.3±0.67). Total oxidant status (TOS) levels were significantly (p <0.05) increased in the H_2O_2 group (25.3±0.74) and *H. orientalis* was significantly (p<0.05) decreased TOS levels in groups.

Keywords: *Helleborus orientalis,* Phenolics, Flavonoid Contents, Antioxidant Activity, Protective Effect, Oxidative DNA Damage.

Comment [U1]: Keywords are usually arranged in alphabetical .

INTRODUCTION

There is little information on the therapeutic properties of *Helleborus* (family Ranunculaceae) species known as ornamental plants. For this reason, studies on the phytochemical structure and phytochemical properties of Helleborus species have been of interest in the last few years. There are studies including inherent extracts from these species which indicate that Helleborus plants are a helpful origin of chemical compounds with excellent therapeutic potency years (Maior and Dobrotă, 2013).

Some Helleborus extracts have immuno-stimulatory molecules and are used in anticancer treatment (Bussing and Schweizer, 1998). In vitro application of *H. niger* water extract resulted in a minor induction of sister chromatid exchanges in blood mononuclear cells from a healthy person. Additionally, Lindholm et al. (2002) noticed a powerful antitumor potential of some Helleborus species (*H. cyclophyllus* Boiss. *H. multifidus* and *H. hercegovinus*) (Lindholm et al., 2002). In cancer cells, the Reactive oxygen species (ROS) is maintained at higher levels than normal cells and mainly exerts its proliferative actions. When ROS levels are further increased by pro-oxidants so as to exceed a borderline level, the pro-apoptotic effects of ROS may exceed its proliferative effects and cytotoxic effects display in cancer cells, whereas the ROS levels in normal cells remain below the borderline level which is nontoxic to normal cells. Antioxidants treat cancers as cancer inhibiting agents (Jin et al., 2014).

Polyphenolic antioxidants are flavonoids and phenolic acid. These compounds most important natural bioactive secondary metabolites in plants (Ghasemzadeh and

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60 Ghasemzadeh, 2011). In addition, phenolic antioxidant compounds reduce the risk of
61 cancer by clearing free superoxide radicals. Phenols include both hydroxyl group and
62 aromatic ring. It has been reported that phenols are involved in the defense system against
63 microorganisms. Phenols directly cleans free radicals or act as electron donors against
64 hydrogen peroxide in reactions catalyzed by ascorbate peroxidase. Thus they reduce the
65 oxidative stress in the cell (Morina et al., 2008). In recent years it has been determined
66 that flavonol glycoside and phenolic glucoside derivatives isolated from some Helleborus
67 species and exhibit significant anti-proliferative and potent antioxidant activity (Braca A
68 et al., 2014; Prieto et al., 2006; Vitalini et al., 2011).

69 *Helleborus orientalis* is a perennial plant and is indigenous to Greece and Turkey
70 (Kazuki et al., 2003). Same antioxidant compounds have nowadays been isolated from
71 plants of this genus but information on the antioxidant activity of HO is limited. In order
72 to use this plant in modern medicine, it must be determined whether or not it causes
73 antioxidant effects in the cell. Comet assay is a technically easy, fast and highly sensitive
74 test to detects the small changes in the DNA structure (Liman, 2013). According to our
75 literature search, antioxidant effects of *H. orientalis* have not been examined yet by comet
76 assay.

77 The aims of this study are to evaluate the antioxidant activity using different methods
78 and to determine the protective effect of *H. orientalis* extract against oxidative DNA
79 damage by employing a Comet assay.

80

81 **MATERIALS AND METHODS**

82 **Reagents and Plant material**

83 *Helleborus orientalis* (*Ranunculaceae*) were collected from Piraziz, Giresun, Turkey
84 (Latitude 40.924° - Longitude 38.128° - Height 290 m) in April 2017. The plant was
85 identified by Professor Mustafa Kargıoğlu, from the Department of Biology, Faculty of
86 Science and Literatures, Afyon Kocatepe University. An authenticated voucher specimen
87 (AKU9324) was deposited in the Herbarium of Faculty of Science and Literatures Afyon

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88 Kocatepe University. Its leaves and branches were dried at 27±2 °C in a dark room for
89 two weeks. Dried materials were milled (80–100 mesh) before extraction.

90 All the chemicals (Sigma-Aldrich) used in this study were filtered through a 0.45
91 Millipore membrane filter.

92 **Ultrasound-assisted extraction**

93 Extraction was performed utilizing Wise bath brand ultrasonic bath with 50 kHz
94 frequency. 1 g dried part of plant material powdered was extracted with 30 mL of a
95 methanol solution (70% methanol in distilled H_2O) in an ultrasonic bath at 27 °C
96 temperature for 30 min. It was watched out that the solvent and water level of flask in the
97 ultrasonic bath remained the same. After fixing the ultrasonic bath temperature and time
98 value, extraction proces was started. When the extraction was complete, the mixture was
99 first filtered with Whatman filter paper and then filtered with a 0.45 micron membrane
100 filter.

101 **Determination of total phenolic content (TPC)**

102 The Folin-Ciocalteu technique protocol was done according Kähkönen et al.
103 (1999). Absorbance was measured at 765 nm by a UV-Vis spectrophotometer and
104 compared to a Gallic acid (GA) calibration curve. The amount of the absorbance
105 measured in the extract was calculated from the equation of the standard curve prepared
106 with GA. TPC in the extract is expressed in terms of "mg GA/g sample".

107 **Determination of total flavonoid content (TFC)**

108 The TFC of the extract was determined by the aluminum chloride colorimetric
109 assay (Chang et al., 2002). The absorbance of the samples was read at 510 nm. The same
110 processes were used for quercetin used as standard and flavonoid contents of the samples
111 were calculated as equivalent to quercetin (mg QE / g sample).

112 **Analysis of Phenolic Acids by High-Performance Liquid Chromatography (HPLC)**

113 Determination of phenolic acids in extract was carried out with HPLC system
114 (Agilent 1260 series). Instrument control and data analysis were performed using Agilent

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115 HPLC Chemstation 10.1 edition through Windows 2000. Zorbax Extend-C18 (5 µm, 4.6
116 mm -150 mm, Agilent) column was used. The flow rate of the mobile phase was adjusted
117 at 0.5 mL/min. Mobile phase A was 0.02% Trifluoroacetic acid (TFA) solution in water,
118 and phase B was 0.02% TFA solution in methanol. The gradient conditions were as
119 follows: 0-5 min, 25% B; 5-10 min, 25-30% B; 10-16 min, 30-45% B; 16-18 min, 45%
120 B; 18-25 min, 45-80% B; 25-30 min, 80% B; 30-40 min, 80-25% B (The column
121 temperature: 25 °C, injection volume: 10 µL). As standard Vanillic acid (254), gallium
122 and syringic acid (275, 305), and rosmarinic acid (320 nm) were used (Wen et al., 2005).

Comment [U2]: Is it true ??? or should be 80 – 100 % ???

123 **Fourier Transform Infrared Spectrophotometer (FTIR)**

124 FTIR Analysis has been applied to determine functional groups present in the
125 methanol extracts of H. orientalis. About **1 mg** of dried material was analyzed by **FT IR**
126 (spectra frequency zones 3500-500 cm- 1) (Perkin Elmer Lambda 35)

Comment [U3]: Do ypu mean FTIR ?

127 **LC-Mass Analyze**

128 LC-Mass Analysis was applied to determine unknown substances in the structure
129 of *H. orientalis*. The dried ground samples were weighed 1 g and extracted in an
130 ultrasonic bath with 30 mL of 70% methanol. The analysis was performed by using the
131 6460 mass detector with the C18 column at 325 ° C of the ionization temperature using
132 the Jetstream ionization technique. The mobile phase A was 20 % acetonitrile and mobile
133 phase B was 80% distilled water containing 0.1% formic acid. The analysis was
134 performed with Triple Quadrupole LC-MS-MS device. Separation process was
135 performed with a 1200 model UPLC device. The injection volume was 10 µL.

136 **Antioxidant activity by DPPH test assay**

137 The DPPH assay protocol was carried out according to the method of Thaipong et
138 al. (2006). The absorbance of the samples (Shimadzu UV Mini 1240) was read at 515
139 nm. Antioxidant activity is expressed as % inhibition of DPPH.

140 **Protective effect of leaf extract against to H_2O_2 by Comet assay**

141 The alkaline comet assay was done according to Singh et al. (1998) Negative and
142 positive controls (1xPBS and H_2O_2) were also included, and exposure time is in parallel with

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143 HO leaf extract dissolved in 1XPBS (500, 1000, 2500, 5000 and 10.000 µg/mL) for 0.5h at 37
144 °C. While supernatant was used for The oxidative stress index (OSI) determination, the pellet
145 was used for Comet assay. Electrophoresis was then carred out for 20 min at 25 V (1 V cm^{-1})
146 at 4 ^oC. Following electrophoresis, slides washed with neutralization buffer (0.4 M Tris, pH =
147 7.5) three times. The slides were covered with coverslip after staining with 60 µl of 20 µg/ml
148 ethidium bromide. Totally 300 comets per concentration were analyzed using a fluorescence
149 microscope (BAB-TAM-F, Turkey). Scores of slides were classified according to Koçyiğit et
150 al (2005). The mean ± standard deviations of the obtained data were calculated. . Significance
151 levels in different treatment groups were analyzed using Duncan multiple range tests (SPSS
152 23.0 version).

153 **Measurement of total oxidant status and oxidative stress index**

154 When Comet assay DNA damage study was carried out, the last supernatants were
155 taken into Eppendorf tubes and stored at -20 ˚C for used in TOS and TAS studies. The total
156 oxidant (530 nm) and oxidant (660 nm) level was estimated utilizing a completely automated
157 colorimetric technique created by Erel. TOS (530 nm) and TAS (660 nm) levels were
158 evaluated as spectrophotometric (Elisa Thermo Scientific) using Rel Test Kit RL0024 and
159 RL0017(Erel, 2004; Erel, 2005). Stress index was counted up according to the following
160 formula; TOS: (ΔAbsSample)/ (ΔbsStandard) X Conc. of standard TAS: ((ΔAbs H_2O) -
161 (ΔAbs Sample)) / ((ΔAbsH_2O) - (ΔAbs Standart)).

162 The oxidative stress index (OSI) of samples were determined with the ratio of TOS to
163 TAS. Standard deviations of the data obtained from the test samples were calculated and
164 analyzed using Duncan post-hoc one-way variance analysis (ANOVA).

165 **RESULTS**

166 **Phenolic contents**

167 TPC in plant extracts of *Helleborus orientalis* were given in Table 1. TPC in plant
168 extracts ranged from 19.42 to 4.39 mg GAE/1 g sample. The highest concentration of
169 TPC was measured at FME. TPC in LME was determined as 17.20 mg GAE/1 g sample.
170 A graph of the absorbance values versus the Gallic acid concentration was drawn and a
171 curve of Gallic acid calibration was generated. The calibration curve for Gallic acid was

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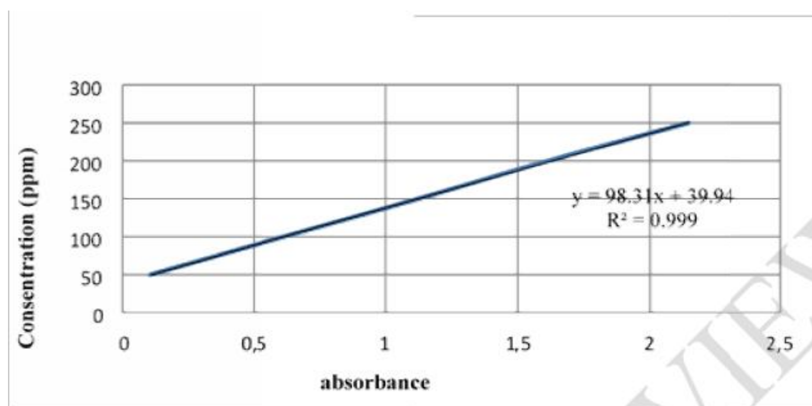
172 given in figure 1. Linearity was defined as (y=98.316 x+39.945) and regression
173 coefficient was defined as (R^2=0.9990).



174

175 **Fig1.** TPC linearity graph

176 **Table 1** The total phenolic contents of various plant extracts parts of *Helleborus*
177 *orientalis*

The methanol extract of *Helleborus orientalis*	mg GAE/1 gr of sample
Flower (FME)	19.42
Leaf (LME)	17.20
Body (BME)	6.86
Root (RME)	4.39

178

179 **Flavonoid contents**

180 The TFC in the extracts of different parts of the plant were given in Table 2 as
181 quercetin equivalent (QE). The highest amount of flavonoid content was found in FME
182 (11.880 mg QE/g) followed by LME (10.212 mg QE/g) BME (3.116 mg QE/g) and RME
183 (2.567 mg QE/g). A graph of the absorbance values versus the quercetin concentration
184 was drawn and a curve of quercetin calibration was generated. The calibration curve for
185 quercetin was given in Figure 2. Linearity was defined as (y=2438x+0.218) and
186 regression coefficient was defined as (R^2=0.9998).

Comment [U4]: what does this sign mean?

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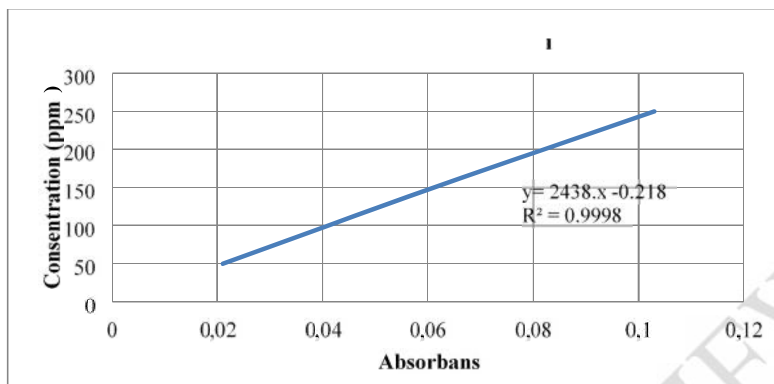

188

189 **Fig. 2.** TFC Linearity Graph

190 **Table 2** The total flavonoid contents of aerial parts of *Helleborus orientalis*

Comment [U5]: why use the term aerial parts? Does this plant have roots exposed to air?

The methanol extract of *Helleborus orientalis*	mg QE/ 1 g. of sample
Flower (FME)	11.880
Leaf (LME)	10.212
Body (BME)	3.116
Root (RME)	2.567

191

192 **Phenolic Acid Contents of *Helleborus orientalis***

193 The presence of Gallic acid, protoacetic acid, vanillic acid, caffeic acid, syringic
194 acid, coumaric acid and rosmarinic acid in phenolic acids was investigated by HPLC
195 method in the parts of *H. orientalis*. 26.52 ppm of Gallic acid in the flowers, 7.48 ppm of
196 rosmarinic acid in the body, 20 ppm of caffeic acid in the leaves and 19 ppm of
197 rosmarinic acid and 20 ppm of Gallic acid in the roots of *H. orientalis* plant were
198 determined by HPLC

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200 **FT-IR Spectrum analysis**

201 The FTIR spectrum of *H. orientalis* extracts is given in Fig 3 to 6. The data on the
202 peak values and the probable functional groups were presented in Tables 3.

203 **Table 3**

204 FTIR spectral peak values and functional groups obtained for the *H. orientalis* extract

Simple	Frequency (cm^{-1})	Functional group	Possible compound
Stem	3304.0	N-H bending	Amine and Amide Alkaloids
	2930	C-H group	Phenolic Aromatic Compounds
	1723.4	C=O carbonyl group	Saponins
	1592	C=C group	Phenolic Aromatic Compounds
	1035.9/1252.6	C-O-C stretching	Glycosides
Root	3307.1	N-H bending	Amine And Amide Alkaloids
	2855/2921.9 and 3012.8	C-H group	Phenolic Aromatic Compounds
	1707.3	C=O carbonyl group	Saponins
	1035.9/1156.3/1196.4 and 1274	C-O-C stretching	Glycosides
Flower	3248.3	N-H bending	Amine And Amide Alkaloids
	2847/2919.2	C-H group	Phenolic Aromatic Compounds
	1394.3	CH_3	Alkaloids
	1308/1035.9	C-O-C stretching	Glycosides
Leaf	3288.4	N-H bending	Amine and Amide Alkaloids
	2857.7/2921.9	C-H group	Phenolic Aromatic Compounds
	1512	C=C group	Phenolic Aromatic Compounds
	1038.5/1260.6	C-O-C stretching	Glycosides

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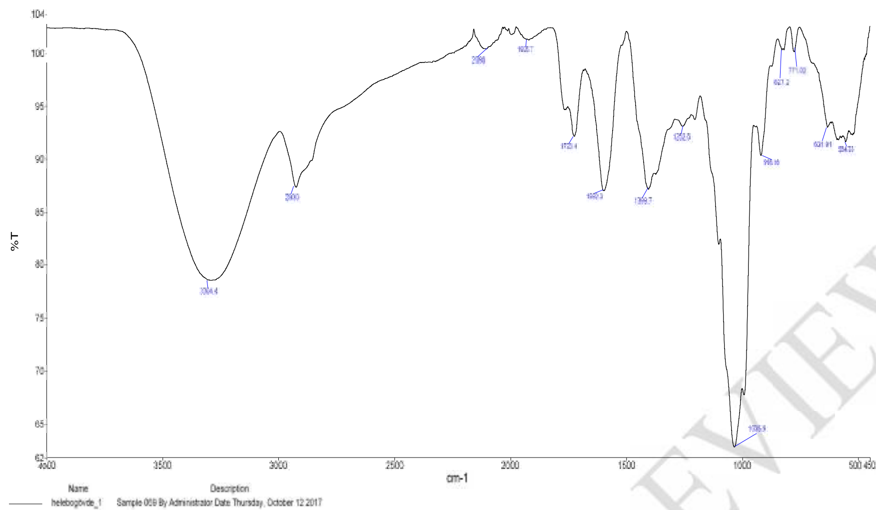

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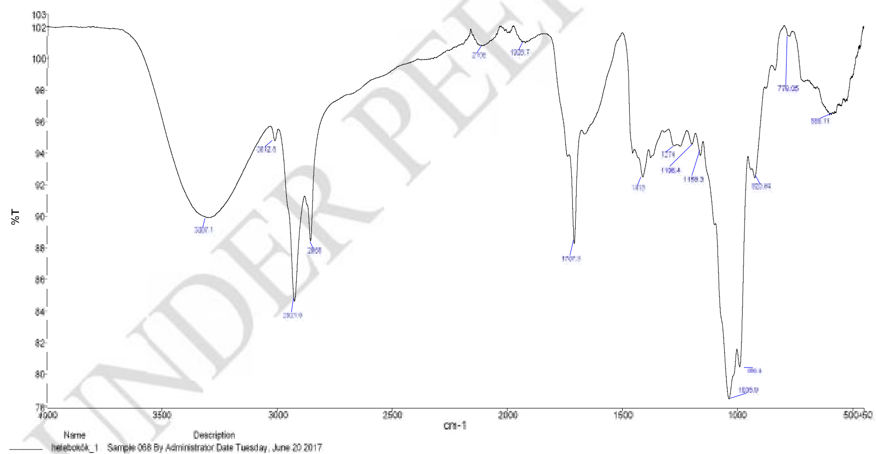
210 **Fig. 3.** FT-IR analysis of *H. orientalis* stem



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212 **Fig. 4.** FT-IR analysis of *H. orientalis* root

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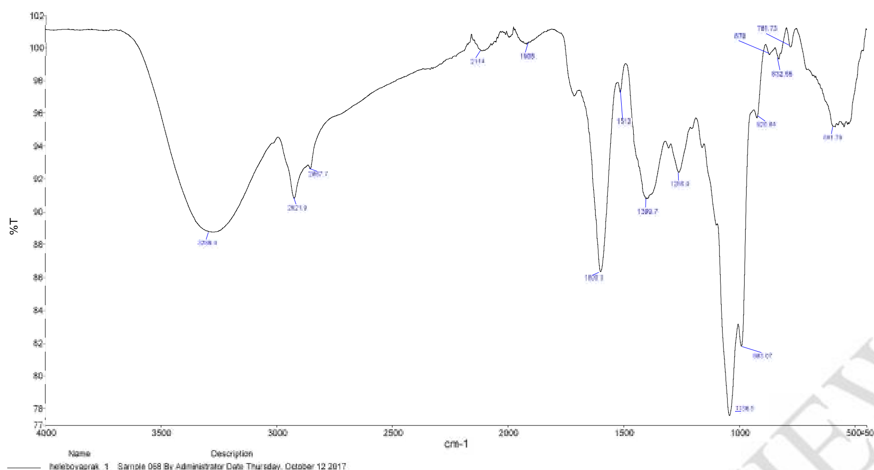

Fig. 5. FT-IR analysis of *H. orientalis* leaf

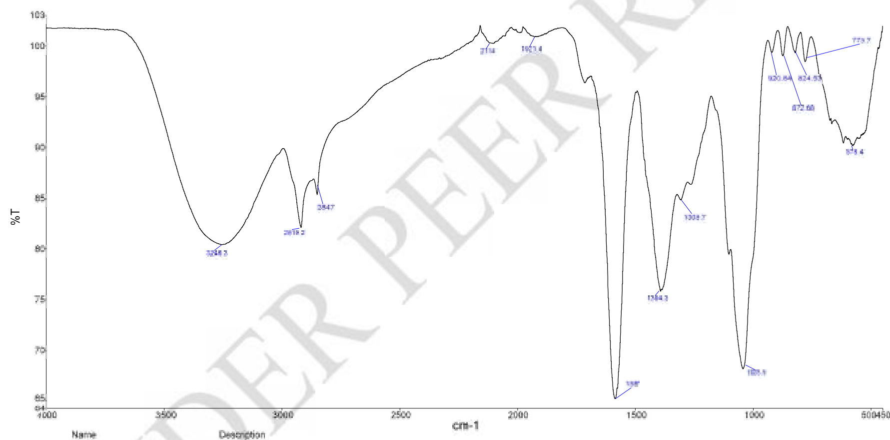

Fig. 6. FT-IR analysis of *H. orientalis* flower

LC-MS results

In LC-MS analysis, glycosides equivalent to the molecular weights of cardioactive glycosides were determined. The hellebrin, hellebrigenin in the leaves of the plant and hellebrigenin in the body, hellebrigenin in the root and the presence of helleborine were determined. In the leaves of the plant hydroxycinnamic acids and other

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222 polar compounds containing caffeic acid, flavonol glycosides containing quercetin have
223 been identified. In the roots of the plant, quercetin containing flavonol glycosides was
224 found. The presence of quercetin-containing flavonol glycosides was determined in the
225 body of the plant. Flowers of the plant have hydroxycinnamic acids and other polar
226 compounds containing caffeic acid and flavonol glycosides quercetin were found.

227 **Antioxidant activity by DPPH Assay**

228 From the plot plotted against the % inhibition value calculated against different
229 concentrations of antioxidants, linear regression resulted in Antioxidant concentrations
230 which cause 50% inhibition was calculated by linear regression. The results are expressed
231 as EC50 (µl) in table 4.

232 **Table 4** EC50(µl) values of aerial parts of *Helleborus orientalis*

Comment [U6]: why use the term aerial parts? Does this plant have roots exposed to air?

The methanol extract of *Helleborus orientalis*	EC50 (µl/ml)
Flower (FME)	18.00
Leaf (LME)	13.00
Body(BME)	28.00
Root (RME)	40.00

233

234 **TAS and TOS levels**

235 There were significant increases of TOS levels whereas decreases TAS in the H_2O_2
236 group compared to the control group (Table 5). Additionally, treatment leaf extract decreased
237 to their increased oxidant levels in the H_2O_2 group compared to the control group ($p<0.05$).
238 These results supported that the administration of leaf extract decreased Oxidative Stress
239 Index (OSI) compared to the H_2O_2 group ($P < 0.05$).

240 **Table 5** TAS, TOS levels and OSI index in groups.

	TAS (mmol Trolox Equiv./ L.)	TOS (mM H_2O_2 Equiv. / L.)	OSI
Control	7.3±0.02[a]	11.2±0.85[a]	3.21±1.2[a]
H_2O_2	3.4±0.21[b]	25.3±0.74[b]	8.14±3.12[b]

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500 µg/L + H_2O_2	3.2±0.14[b]	24.12±0.72[b]	8.10±3.11[b]
1000 µg/L + H_2O_2	3.1±0.11b[c]	23.17±0.69[b]	8.10±3.14[b]
2500 µg/L + H_2O_2	6.3±0.67[cd]	19.07±0.67[c]	4.25±2.14[c]
5.00µg/L + H_2O_2	6.1±0.64[de]	19.21±0.64[dc]	4.29±2.11[dc]
10.000µg/+ H_2O_2	6.2±0.61[e]	19.11±0.81[dc]	4.32±3.01[dc]

*Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation

Protective effect of leaf extract against to H_2O_2

The content of phenolic acid in the fruit fell in the first and second phase of the flowering phase while the phase increased in the fourth (Sahri et al., 2011). For this reason, leaf extracts were used in the Comet experiment. Protective effect of HO leaf extract against to H_2O_2 is given to Table 6. Exposure of HO leaf extract decreased the DNA damage at all concentrations in a dose-dependent manner ($r=0.86$ $p<0.01$) against to H_2O_2. While the highest DNA damage was observed the positive control (305±5.51), the lowest one observed in the negative control (6±1). The decreasing DNA damage showed statistically significant results ($p<0.05$) above the 2500 µg/mL of HO leaf extract.

Table 6 Protective effect of HO leaf extract against to H_2O_2

Treatment	DNA Damage (Arbitrary Unit ±SD)*
Control	6±1[a]
H_2O_2 200 µM	305.33±5.51[b]
500 µg/mL + H_2O_2	304.33±3.21[b]
1000 µg/mL + H_2O_2	302±5.57[bc]
2500 µg/mL + H_2O_2	296.33±2.52[cd]
5.000 µg/mL + H_2O_2	293.33±3.06[de]
10.000 µg/mL + H_2O_2	288.67±3.51[e]

* Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation

DISCUSSION

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259 Over the past few years, different Helleborus species have been the subject of
260 phytochemical investigations because of their potential to produce important secondary
261 metabolites. However, studies in the literature have been limited to a few studies with
262 *Helleborus orientalis* species. To the best of our knowledge, no data have been published
263 on total phenolic and flavonoid content *Helleborus orientalis* and relationship protective
264 effect of leaf extract against to H_2O_2 by Comet assay. Therefore the aim of our study was
265 to examine the possible antioxidant potential of *H. orientalis* Lam species with
266 distribution in Piraziz, Giresun, Turkey.

267 Kumar and Laltha show that (2014) ethanol extract of *H. niger* exhibits excellent
268 antioxidant activity in streptozotocin and nicotinamide-induced diabetic rats induced type 2
269 diabetes model. In our study, ethanol was used as the solvent in *H. orientalis* extraction
270 Roman et al (2010) results revealed that the concentrated *H. purpurascens* extracts have a
271 high antioxidant activity. Similar to the findings we have in our study, Paun et al (2014)
272 evaluated that *H. purpurascens* and *H. officinale* polyphenolic extracts and they show that the
273 plant flowers had the highest phenol content followed by the plant leaves. Cakara et al. (2011)
274 reported that the antioxidative properties of helleborus leaf extracts were caused by high
275 levels of phenolic and flavonoids.

276 In this study, the concentration of TPC in *H. orientalis* ethanol extracts ranged from
277 19.42 to 4.39 mg GAE/1 gr of the sample. The plant flowers had the highest phenol content
278 (19.42 mg GAE/1 gr) followed by the plant leaves (17.20 mg GAE/1 gr) (Table 2). The
279 highest amount of flavonoid content was found in FME (11.880 mg QE/g) followed by LME
280 (10.212 mg QE/g) (Table 4). The maximum total flavonoid content of *H. orientalis* methanol
281 extracts was found in flowers and leaves (10.212 mg QE / 1 g) in spectrophotometric
282 measurement (11.880 mg QE / 1 g).

283 The bands at 1700-1600 cm-1 in the FTIR spectrum are caused by the stretching of the
284 carbonyl groups C = O and C = C or also associated with phenolic molecules (Tahir et al.,
285 2017). It has been reported that the vibration in the spectral region of 1540 2121175 cm-1
286 may be from O-H, C-O, C-H, and C = C deformation [213], or flavonol and phenol [Tahir et
287 al., 2017; Masek et al., 2014; Nickless et al, 2014). Masek et al. reported that the vibrations in
288 the spectral region of 940-1175 cm^{-1} were due to C-OH groups, C-O in phenol, carbohydrate-
289 linked C-C and C-O (Nickless et al, 2014). In our study, C-H, C = O, C-O-C, C methanol OH,
290 C = C bond stress in root, stem, flower, and leaf methanol extracts. This shows that phenolic

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291 compounds are present in the methanol extracts of *H. orientalis.* In this study, the results of
292 HPLC analysis data, LC- Mass, FTIR spectral findings and spectrophotometric phenolic/
293 flavonoid substance support each other.

294 DPPH is considered to be an easy and fast method for the correct determination of
295 antioxidant activity (Katalinic et al, 2004). In this study, the sweeping activity of DPPH
296 radical is found in the highest flower extract. The sweeping activity of the lowest DPPH
297 radical was determined at the root. Plant extracts have higher antioxidant activity than many
298 antioxidant substances. (Table 6). This is probably related to depends on redox properties of
299 phenolic compounds (Adedapo et al., 2009; Katalinic et al, 2004; zheng and Wang, 2001).

300 The presence of phenolic acids (ferulic acid, caffeic acid and chlorogenic acid) and
301 flavonoids (kaempferol and quercetin derivatives) in the methanolic extract of Helleborus
302 atrorubens leaves was shown. (Cakara et al., 2011; Shahri et al., 2011). Many studies
303 demonstrated that caffeic acid has anti-mutagenic and anti-carcinogenic properties. Chen et al
304 (1995) show that caffeic acid is decrease lipoperoxyl radicals by inhibiting the reaction of
305 lipid peroxidation chain.

306 In our study, 26.52 ppm of gallic acid in the flowers, 7.48 ppm of rosmarinic acid in
307 the body, 20 ppm of caffeic acid in the leaves and 19 ppm of rosmarinic acid and 20 ppm of
308 gallic acid in the roots of *H. orientalis* plant were determined by HPLC. Also, we couldn't
309 determine some peaks in HPLC because of didn't use the standards. Although all of the
310 obtained data show that *Helleborus orientalis* has got rich antioxidant content, no literature on
311 the Comet assay of *H. orientalis* plant extracts was found in the literature review. Although,
312 *Helleborus odorus* and *H. hercegovinus* extracts have strong antioxidant activity, *H.*
313 *multifidus* has stronger antiproliferative activity (Cakara et al.1995, Chen et al, 1995). The
314 leaves extracts have better antioxidant activity and secondary metabolites are present in
315 higher amounts than the root extracts. On the other hand, *H. hercegovinus* root extracts
316 exhibit high antitumoral activity. Moreover, Kumar and Lalitha findings directly, verified at
317 the first time that the ethanol extract of *H. niger* root exhibited perfect activities type 2
318 diabetic rats (Kumar and Lalitha., 2014).

319 Helleborus flower development was divided into six stages and the amount of
320 phenolic content varies according to the flowering period (Zheng and Wang, 2001). For this
321 reason, Leaf extract was used for determination of protective effect ın this study.

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322 Protective effect against oxidative stress of *H. orientalis* was determined using
323 TAS, TOS method and Comet assay. Exposure of HO leaf extract decreased the DNA
324 damage at all concentrations in a dose-dependent manner ($r=0.86$ $p<0.01$) against to
325 H_2O_2 ın the Comet essay. The decreasing DNA damage showed statistically significant
326 results ($p<0.05$) above the 2500 μg/mL of HO leaf extract. OSI value was found to
327 decrease significantly ($p<0.05$) at 2500 4.2g / L of HO leaf extract compared to positive
328 control (4.25 ± 2.14).

329 **CONCLUSIONS**

330 This is the first report on the activity of protective effect of leaf extract of *H. orientalis*
331 against H_2O_2. The study shows that *H. orientalis* leaf and flowers extracts have a high
332 antioxidant activity, thus it can be considered a good source for further medicinal
333 applications. The following investigations should be based on the purification and chemical
334 identification of *H. orientalis* extract.

335 **References**

336 Kumar, K.V., Lalitha, K.G., 2014. Antihyperglycemic, Antihyperlipidemic and Antioxidant
337 Activities of Ethanolic Extract of Helleborus niger Linn Roots in Streptozotocin-
338 Nicotinamide Induced Diabetic Rats. Journal of Pharmaceutical Research. 13(3), 92-
339 101.

340 Adedapo, A., 2009. Antioxidant properties of the methanol extracts of the leaves andstems of
341 Celtis. Africana. Records of Natural Products. 3: 23–31.

342 Apetrei, N.S., Lupu, A.-R., Calugaru, A., Kerek, F., Szegli, G., Cremer, L., 2011. The
343 antioxidant effects of some progressively purified fractions from Helleborus
344 purpurascens, Rom. Biotechnol. Lett. 16, 6673-6681.

345 Braca, A., Prıeto, J. M., De Tomması, N., Tome, F., And Morellı, I., 2004. Furostanol
346 Saponins and Quercetin Glycosides From The Leaves Of Helleborus Viridis L.
347 Phytochemistry. 65(21), 2921-2928.

348 Büssing, A., Schweizer, K., 1998. Effects of a phytopreparation from Helleborus nigeron
349 immunocompetent cells in vitr, J. Ethnopharmacol. 59, 139–146

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Comment [U9]: italic

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UNDER PEER REVIEW

350 Čakar, J., Parić, A., Vidic, D., Haverić, A., Haverić, S., Maksimović, M., 2011. Antioxidant
351 and antiproliferative activities of Helleborusodorus Waldst. & Kit, H. multifidus Vis.
352 and H. hercegovinus Martinis. Nat. Prod. Res. 25, 1969–1974.

353 Chang, C., Yang, M., Wen, J.H., 2002. Chern Estimation of total flavonoid contentin propolis
354 by two complementary colorimetric methods. J. Food Drug Anal. 10,178-18

355 Chen, Y.F., 1995. Anti-inflammatory and analgesic activities from roots of Angelica
356 pubescens. Planta Med. 61(1),2-8.

357 Erel O., 2005. A new automated colorimetric method for measuring total oxidant status. Clin
358 Biochem. 38, 1103-1111.

359 Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity
360 using a new generation, more stable ABTS radical cation. Clin Biochem. 37, 277-285.

361 Ghasemzadeh, A and Ghasemzadeh, N., 2011. Flavonoids and phenolic acids: Role and
362 biochemical activity in plants and human. Journal of Medicinal Plants Research. 5(31),
363 6697-6703.

364 Jin, S., Wu Y., Deng, S., Zhang, J., Chen, X.Q., 2014. Oxidants and Antioxidants in
365 Metabolic Syndrome and Cancer. Oxid Med Cell Longev. 2014:178962.

366 Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, TS., 1999.
367 Heinonen M.et al. Antioxidant activity of plant extracts containing phenolic
368 compounds. J. Agric. Food Chem. 47, 3954–3962.

369 Katalinic, V., 2004. Antioxidant effectiveness of selected wines in comparison with(þ)-
370 catechin. Food Chemistry. 86:593–600.

371 Kazuki, W., Mimaki, Y., Sakagami, H and Sashida, Y., 2003. Bufadienolide and Spirostanol
372 Glycosides from the Rhizomes of Helleborus orientalis J. Nat. Prod. 66,236-241.

373 Koçyiğit, A., Keles, H., Selek, S., Guzel, S., Celik, H., Erel, O., 2005. Increased DNA
374 damage and oxidative stress in patients with cutaneous leishmaniasis. Mutat. Res. 585,
375 71–78.

Comment [U11]: italic

Comment [U12]: italic

UNDER PEER REVIEW

376 Liman, R., 2013. Genotoxic effects of Bismuth (III) oxide nanoparticles by Allium and Comet
377 assay Chemosphere. 93 (2), 269-273.

378 Lindholm, P., Gullbo, J., Claeson, P., Göransson, U., Johansson, S., Backlund, A., 2002.
379 Selective cytotoxicity evaluation in anticancer drug screening of fractionated plant
380 extracts. J. Biomol. Screen. 7, 333-340.

381 Maior, M.C., Dobrata, C., 2013. **Natural Natural** compounds discovered in Helleborus sp.
382 (Ranunculaceae) with important medical potentiali Article in Central European
383 Journal of Biology. 8(3), 272-285.

384 Masek, A., Chrzescijanska, E., Kosmalska, A., & Zaborski, M., 2014. Characteristics of
385 compounds in hops using cyclic voltammetry, UV–VIS, FTIR and GC–MS analysis",
386 Food Chemistry. 156, 353–361.

387 Morina, F., Jovanović, L., Kukavıca, B., Veljović-Jovanović, S., 2008. Peroxidase,
388 phenolics, and antioxidative capacity of common mullein (Verbascum thapsus
389 L.) grown in a zinc excess. .Archives in Biological Science Belgrade. 60(4),
390 687–695.

391 Muzashvili, T., Skhirtladze, A., Sulakvelidze, T., Benidze, M., Mshviladze, V., Legault, J.,
392 Pichette, A., Kemertelidze, E., 2006. Cytotoxic activity of Helleborus caucasicus A.
393 Br. Georg. Chem. J. 6, 684–685.

394 Nickless, E. M., Holroyd, S. E., Stephens, J. M., Gordon, K. C., & Wargent, J. J., 2014.
395 Analytical FT-Raman spectroscopy to chemotype Leptospermum scoparium and
396 generate predictive models for screening for dihydroxyacetone levels in floral nectar.
397 Journal of Raman Spectroscopy. 45(10), 890–894.

398 Paun, G., ,Litescu, S.C. , Neagu, E.,Tache A., Radu G.L., 2014. Evaluation of Geranium
399 spp.,J Enzyme Inhib Med Chem. 29(1), 28–34

400 Prıeto, J. M., Sıcılıano, T., And Braca, A., 2006. A New Acylated Quercetin Glycoside And
401 Other Secondary Metabolites From Helleborus Foetidus. Fitoterapia. 77(3), 203-207.

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UNDER PEER REVIEW

402 Roman, P.G., Neagu, E., Radu, G.L., 2010. Membrane processes for the purification and
403 concentration of Helleborus purpurascens extracts and evaluation of antioxidant
404 activity. Rev. Chim. (Bucharest). 61, 877-881.

405 Shahri, W., Tahir, I., Islam, S.T., Bhat, M.A., 2011, "Physiological and biochemical changes
406 associated with flower development and senescence in so far unexplored Helleborus
407 orientalis Lam. cv. Olympicus", Physiol Mol Biol Plants. 17(1), 33-9.

408 Shahri, W., Tahir, I., Islam, S.T., Bhat, M.A., 2011. Physiological and biochemical changes
409 associated with flower development and senescence in so far unexplored Helleborus
410 orientalis Lam. cv. Olympicus. Physiol Mol Biol Plants. 17(1), 33–39

411 Singh, N..P., Mc.Coy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for
412 quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175,184-
413 191.

414 Tahir, H., Xiaobo, Z., Zhihua L., Jiyong, S., Zhai, X., Wang, S , Mariod, A., 2017. Rapid
415 prediction of phenolic compounds and antioxidant activity of Sudanese honey using
416 Raman and Fourier transform infrared (FT-IR) spectroscopy. Food Chemistry. 226,
417 202–211.

418 Thaipong, K., Boonprako, U., Crosby, K., Cisneros-Zevallos, L., Hawkins Byrne, D., 2006.
419 Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant
420 activity from guava fruit extracts. J. Food Compos. Anal. 19, 669-675.

421 Vıtalını, S., Braca, A., and Fıco, G., 2011. Study On Secondary Metabolite Content Of
422 Helleborus Niger L. Leaves. Fitoterapia. 82(2), 152-154.

423 Wen, D., Li, C., Di, H., Liao, Y., Liu, H., Agric J., 2005, A universal HPLC method for the
424 determination of phenolic acids in compound herbal medicines. Food Chem. 53,
425 6624–6629.

426 Zheng, W and Wang, S.Y., 2001. Antioxidant activity and phenolic compounds in selected
427 herbs. Journal of Agricultural and Food Chemistry.49, 5165–5170.

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