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Exploring of *Coronilla varia* L. extracts as a source of high-value natural agents: Chemical profiles and biological connections

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ABSTRACT

Pharmacological studies have indicated that flavonoids are crucial compounds to eliminate drug resistance. In this report, the activity of ethyl acetate (EAE), methanol (ME) and water (WE) extracts of *Coronilla varia* L. on antioxidant and enzyme inhibitory activities, DNA protective effects, antiproliferative activities, apoptotic; autophagic and telomerase gene activity analysis in breast cancer cells and inhibitory effects on cell migration ability of malignant breast cancer cells were examined. In addition, HPLC-MS-MS was used for detection of chemical profiles of all extracts. Results showed that the highest concentration of the bioactive components was detected in EAE (50.86 mg GAE/g for phenolics and 25.66 mg RE/g for flavonoid). Also, EAE displayed significant antioxidant properties in radical scavenging and reducing power assays. Regarding enzyme inhibitory effects, EAE and ME were more active than WE. Some significant compounds such as, vitamin B5, riboflavin, citric acid, and isoflavonoid derivate – medicarpin, noscapine were detected only in WE. Apigenin was determined in all extracts. WE indicated the most shield effect on pDNA against oxidative damage. Half-maximal inhibitory concentrations of extracts on breast cancer cells were calculated with MTT cell viability test. *Bax* gene was up-regulated and anti-apoptotic gene known as *Bcl-2* was down-regulated on MDA-MB-231 cells after treated with WE. *TERT-1* gene was down-regulated after treated with EAE and ME for MDA-MB-231 and MCF-7 cells, respectively. Cell migration ability of both MDA-MB-231 and MCF-7 cells was prevented with EAE and ME. A more effective treatment strategy can be applied by combining these extracts with commercial chemotherapy drugs which cause apoptosis and cell migration inhibition *in vitro*.

1. Introduction

In Nagpur, known as a Sumerian clay plate and the oldest written evidence of 5000 years, it has been reported that pharmaceutical raw materials were produced from plants and used as a source of healing. This leaflet consists of 12 recipes containing more than 250 plants including alkaloids such as poppy, henbane and mandrake (Petrovska, 2012). Plant-derived chemicals are grouped based on their origin of biosynthesis. Utilization of these chemicals alone or mix with other therapeutic agents has played an important role in the development of cancer drugs including breast cancer (Elkady et al.,2012). Among the phytochemicals, flavonoids, which contain two benzene rings linked by a heterocyclic pyran ring, are the most well-known and studied in pharmacology (Otshudi et al., 2000).

Since the malignant tumor has taken over the human body with a complex development, different alternative ways have been considered for treatment. In addition, the recent applications indicated that the resistance mechanisms in cancer cells against anticancer drugs have created big trouble. For the last 50 years, researchers have demonstrated that how cancer cells growing in culture has become resistant to anticancer drugs through various mechanisms. The possible explanations for drug resistance mechanism in cancer cell lines can be an energy-dependent drug flow pump, alternatively known as *P*-glycoprotein (*P*-gp) or the multidrug carrier (Baguley, 2010). It has been shown that some flavonoids interact with ABC carriers such as *P*-gp, MRP1 and MRP2 and inhibit these carriers competitively. In the previous studies, some flavonoids such as genistein, naringenin, acacetin, kaempferol, quercetin, and xavopyridol were indicated to have reversible effects against BCRP (Breast Cancer Resistance Protein)

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mediated drug resistance (Castro and Altenberg, 1997; Katayama et al., 2007; Singh et al., 2000).

Coronilla varia (crown vetch), which belongs to the legume family, has dense stands with canopy shape up to 1 meter in height and the number of leaflets varies between 9 and 25. It grows in soils with an optimum pH range of 6.5–7 (Bajaj, 1990). Crown vetch was first used to control erosion in America. Nowadays, it has been widely distributed and has many medical usages for the treatment of prostate diseases, diuretic, and heart tonic (Dehpour et al., 2014). It is toxic to horses due to the presence of nitro glycosides. If consumed in large quantities, it can cause slow growth, stroke and even death. Crown vetch contains a toxic glycoside poison substance called "coronyline". It is therefore known to be one of the most toxic plants growing in the UK (Launert, 1981). In this study, it was aimed to examine chemical profile, the DNA protection properties against oxidative stress, enzyme inhibitory activities, antioxidant activities of *Coronilla varia* extracts. In addition, cytotoxic effects, and metastatic properties of plant extracts on MDA-MB-231 and MCF-7 were investigated.

2. Material and methods

2.1. Plant materials and extracts

Coronilla varia L. was collected at the Konya area in Turkey at the late spring of 2017. Identification and confirmation of plant material, as well as issuing of voucher specimen (GZ-17-1028, in Department of Biology, Selcuk University), was performed by botanist Dr. Murat Aydın Sanda from the Mus Alparslan University (Mus, Turkey). Details of plant material preparation were shown in supplementary materials.

To prepare extracts, maceration technique was selected for ethyl acetate (EAE) and methanol extracts (ME). For this purpose, powdered plant materials (10 gr) were mixed with these solvents at room temperature for 24 h. After, the solvents in all extracts were removed and converted to dry extract with a rotary evaporator under vacuum. For water extract (WE), 5 g of plants samples were measured and boiled in 100 mL water for 20 min and then lyophilized. All extracts were kept at +4 °C until they were used.

2.2. Total phenolic and flavonoid content

The ingredient of two major groups of phytochemicals, phenols-TPC and flavonoids-TFC, in obtained extracts was identified spectrophotometrically with appropriate Folin-Ciocalteu and aluminum chloride methods, respectively (Uysal et al., 2017). Expression of results was performed by equivalents of standards - gallic acid (in the case of TPC) and rutin (in the case of TFC).

2.3. HPLC-MS-MS analysis

To detect chemical profiles of the tested extracts, UHPLC system equipped with electrospray ionization source was used (Dionex Ultimate 3000RS and Orbitrap mass spectrometer, Thermo Scientific, USA). The Thermo Accucore C18 column (100 x 2.1 mm and 2.6 μm particle size) was utilized to separate compounds in the extracts. The obtained data were evaluated by Xcalibur 3.1 software (Thermo Scientific, USA) and TraceFinder 3.1 (Thermo Scientific, USA) and other analytical details were shown in our previous study (Zengin et al., 2018).

2.4. Identification of antioxidant and enzyme inhibitory effects

The inhibition ability of extracts was examined against biologically significant such enzymes as α-amylase, α-glucosidase, cholinesterases, and tyrosinase by *in vitro* assays (Uysal et al., 2017). Results were evaluated by official methods by utilizing with suitable standards (Uysal et al., 2017).

The antioxidant capacity of the extracts was also evaluated by different analysis including FRAP, CUPRAC, DPPH, ABTS, metal chelating and phosphomolybdenum. The details related to experiments and evaluation of results were performed based on our previous work (Uysal et al., 2017).

The significance of differences ($p < 0.05$) among the extracts was calculated using the parametric One-way ANOVA test together with Tukey's test. Venn diagram analysis of identified phytochemicals in the tested extracts was done with the online tool (http://jvenn.toulouse.inra.fr/app/example.html).

2.5. DNA protection analysis

The DNA protection ability of the extracts was indicated using pUC19 plasmid DNA (pDNA) which was isolated according to Manufacturer's kit protocol (Thermo scientific Genejet Miniprep Kit, USA). DNA damage was created by oxidative Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM $FeCl_3$) and the reaction tube was also included 5 and 10 mg/ml concentrations of extracts and pDNA (300 μg/μl). Samples were incubated for 30 min at 37 °C as described as in our earlier report (Yerlikaya et al., 2017). The DNA samples were separated with agarose gel (0.8%) and then were pictured under UV. DNA protection analysis were performed with three biological replications and DNA bands were examined with the gel image analysis device (Quantum, Vision-Capt., Vilber Lourmat SAS, France).

2.6. Anticancer activity analysis

2.6.1. Materials for cell culture

Penicillin/Streptomycin, DMEM cell growth media, Fetal bovine serum (FBS), trypsin, MTT, ethanol, 2-prophanol and 100 × 17 mm cell culture corning plates were supplied from representative company of Sigma-Aldrich (Sigma-Aldrich, USA).

2.6.2. Preparation of plant extracts

EAE and ME were prepared in 0.1% DMSO and WE was dissolved in PBS. Homogenized extracts were put to 0.22 μm pore size filter to drain and they were kept at -20 °C until the experiment.

2.6.3. Cell culture maintenance

Basal-type MDA-MB-231 cells lines were cultured in DMEM medium including 10% FBS, 0.01 mg/mL human insulin, 1% non-essential amino acid and 0.1% penicillin/streptomycin. Hormone receptor-positive MCF-7 breast cancer cells were grown in DMEM including 10% FBS, 2mM L-glutamine, 0.1% penicillin/streptomycin at 37 °C in a 5% CO_2 humidified incubator. The time that cells upon reached to about 80 % density, they were passaged. For passage procedure, cancer cell lines were rinsed with PBS, and then separated from culture petri with 0.25% trypsin-EDTA solution for collection. After that, they were seeded to experiment plates.

2.7. Cell viability assay

Breast cancer cell lines (10,000) were seeded and let to attached on 96-well plates for 24 h. After cells were attained to the wanted level, they were exposed to 5 different doses (62,5; 125; 250; 500 and 1000 μg/ml) of all three extracts of *Coronilla varia* for 24, 48 and 72 h. Cytotoxicity test of extracts on breast cancer cell lines was carried out with the MTT test as mentioned in our previous study (Yerlikaya et al., 2017). The optical density of each sample was determined at 570 nm by using the microplate spectrophotometer (Multiskan Go, Thermo Scientific, USA). MTT-based cytotoxicity results were utilized for the determination of half-maximal inhibitory

concentration (IC_{50}) values of extracts on cells by log (inhibitor) vs. normalized response - variable slope analysis function with the aid of GraphPad Prism 7 software.

2.8. Expression profile of cell death and telomerase activity marker genes

After cells were reached to 10,000, they were treated with WE, EAE and ME at their IC_{50} values. RNA isolation was performed with RNA Purification Kit (Thermo Scientific, USA). After, total RNAs were treated with DNase and converted to complementary DNA by using BioRad cDNA synthesis kit. qRT-PCR analysis was carried out with Rotor Gene-Q (Qiagen, Germany). *Bax* forward (5'-CCCGAGGGTCTTTTTCCGAG-3') and reverse (5'-CCAGCCCATGATGGTTCTGAT-3'), *Bcl2* forward (5'-GGTGGGGTCATGTGTGTGG-3') and reverse (5'-CGGTTCAGGTACTCAGTCATCC-3'), *Beclin-1* forward (5'-GGCTGAGAGACTGGATCAGG-3') and reverse (5'-CTGCGTCTGGGCATAACG-3'), *LC3-II* forward (5'-GAGAAGCAGCTTCCTGTTCTGG-3') and reverse (5'-GTGTCCGTTCACCAACAGGAAG-3'), *TERT-1* forward (5'-GGATGAAGCGGAGTCTGGA-3') and reverse (5'-CGGAAGAGTGTCTGGAGCAA-3') and the reference gene human *GAPDH* forward (5'- AACATGTAAACCATGTAGTTGAGGT-3') and reverse (5- GGAAGGTGAAGGTCGGAGTC-3') primers were utilized for detection of gene expression levels. BioRad (USA) SYBR Green master mix was used for qRT-PCR analysis. Reaction amplification was achieved with the first denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing and extension at 57 °C for 30 s. ΔΔCt method was used for the relative quantification of gene expression (Pfaffl, 2001).

2.9. Wound healing assay

MDA-MB-231 and MCF-7 cell lines were maintained in growth medium in 6-well tissue culture plates. When they were reached to appropriate level, wound areas were injured with 200 μl sterile pipette tip in each petri dish. Cancer cells lines were treated with IC_{50} doses of extracts and control cells were exposed to 0.1% DMSO. All growth medium also included mitomycin *C* (10 μg/ml) to prevent cell proliferation. Images of wound parts and cell morphology were pictured under the inverted microscope with 4x objective (Düzgün et al., 2017).

3. Results and discussions

3.1. Chemical characterization

Total bioactive components (phenolics and flavonoids) were detected in the tested extracts. The results were shown in Table 1 which indicates the highest concentration of the components in EAE (50.86 mgGAE/g for phenolics and 25.66 mgRE/g for flavonoid). Among the tested extracts, WE contained the lowest level of these components. These findings are in good agreement with earlier reports which indicated a lower concentration of phenolics in the WE (Do et al., 2014; Yakoub et al., 2018).

Table 1
Total bioactive components of the tested extracts.

Extracts	Total phenolic con tent (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)
EAE	50.86 ± 4.35[a*]	25.66±0.13[a]
ME	29.83±0.60[b]	24.49±0.49[a]
WE	22.12±0.49[c]	6.88±0.70[b]

* Values expressed are means ± S.D. of three parallel measurements. GAE: Gallic acid equivalent; RE: Rutin equivalent; EAE: Ethyl acetate extract; ME: Methanol extract; WE: Water extract. Different letters indicate statistical differences in the extracts ($p < 0.05$).

In the last decade, phytochemical studies have been focused on determining chemical characterization of plant extracts and their biological activities. In this way, we may evaluate which compound/s can support the tested biological properties such as antioxidant, antimicrobial or anticancer. These pieces of information could provide a milestone for comprehensive approaches in further studies (Uthe et al., 2020).

Recent studies have been reported that spectrophotometric methods have several drawbacks (Granato et al., 2018; Margraf et al., 2015) and thus the obtained results may be not reflected to certain levels of phytochemicals. Hence, at least one chromatographic method is needed to understand certain phytochemical pictures in the natural extracts. Because of above-mentioned reasons, we also identified individual phytochemicals in the tested extracts by HPLC-MS.

The recognition of the detected compounds was determined according to their molecular mass measurements, isotopic pattern, their fragmentations, and then searched for identification in databases or compared with measurements of standards. For establishment of full composition, the screening of WE, EAE and ME was performed. A total of 64, 73 and 60 individual compounds were detected in ethyl acetate, methanol, and water extracts, respectively. In addition, 50 compounds of them were similar for all extracts (Supplementary Fig. S1). The results were listed in Table 2 and supplemental materials (Supplementary Table S1–S3). HPLC-MS-MS chromatograms and the associated analytical data of major compounds in *C. varia* showed that major constituents of flavonoids and polyphenolic compounds, besides such coumarins as scopoletin, scopoletin-7-O-glucoside, daphnoretin were determined in all extracts. Umbelliferone was also found only in ME. Those compounds were recently isolated from seeds of *C. varia* (Kovalev and Komissarenko, 1983). Chromatograms were indicated in supplementary materials (Supplementary Figs. S2–S4).

The presence of isoorientin-O-hexoside isomers, isoorientin-O-hexoside, isovitexin, kaempferol and astragalin were confirmed in earlier publications (Kovalev and Komissarenko, 1983), but the presence of other isoflavonoids in *C. varia* such as isoquercitrin, isorhamnetin-3-O-glucoside, isorhamnetin as well as uralenneoside were described firstly in this study. In contrast to other extracts, quercetin-3-O-glucuronide, quercetin-O-pentosyldeoxyhexosylhexoside, rutin and were only determined in ME. Isorhamnetin-O-hexosylhexoside, naringenin-6,8-di-C-glucoside, tetrahydroxy(iso)flavone-O-glucuronide were detected in ME and WE. Dimethoxy-tetrahydroxy(iso)flavone isomers, quercetin and quercetin-O-pentoside were found in both EAE and ME. The specific compounds such as vitamin B5, riboflavin, citric acid, and isoflavonoid derivate – medicarpin, noscapine were observed only in WE. The presence of alkaloid noscapine, typical for plants of the poppy family, was confirmed by standard measuring.

3.2. Effects of antioxidant and enzyme inhibitory

The measurement of antioxidant capacity of the plant extracts could provide the first insights about their potential uses. However, one uniform assay for the full antioxidant picture has not been reported yet. At this point, different chemical assays, such as free radical quenching, reducing power or metal chelating, are recommended for highlighting antioxidant properties of natural extracts. For this reason, the antioxidant capacity was tested by several chemical assays in the present study and the results were illustrated in Table 3. We observed different results in the free radical scavenging assays. Although the methanol extract exhibited the strongest DPPH quenching ability, the lowest ABTS scavenging ability was found in same extracts. The differences may be linked with the nature of radicals and similar results were indicated by earlier studies (Floegel et al., 2011). In an earlier study, Sientzoff et al. (2015) were investigated the antioxidant properties of *Securigera varia* (*Coronilla varia*)

Table 2
HPLC-MS-MS analysis of the tested extracts.

No	Name	Formula	Rt	$[M + H]^+$	$[M - H]^-$	EA	MeOH	Water	Ref
1	Citric acid	C6H8O7	1.56		191.01918	-	-	+	
2	Pantothenic acid	C9H17NO5	5.18	220.11850		-	-	+	
3	Uralenneoside	C12H14O8	10.03		285.06105	+	+	+	
4	Dihydrokaempferol-6-C-glucoside	C21H22O11	14.11		449.10839	+	+	+	
5	Scopoletin-7-O-glucoside	C16H18O9	14.29	355.10291		+	+	+	
6	Naringenin-C-hexoside-O-hexoside	C27H32O15	15.33		595.16630	+	+	+	
7	Coumaroylquinic acid isomer 1	C16H18O8	16.78		337.09235	+	+	+	
8	Naringenin-6,8-di-C-glucoside	C27H32O15	16.87		595.16630	-	+	-	
9	Tetrahydroxy(iso)flavanone-C-hexoside	C21H22O11	17.19	451.12404		+	-	-	
10	Tetrahydroxy(iso)flavanone-C-hexoside isomer 1	C21H22O11	17.21	451.12404		-	+	-	
11	Apigenin-di-C-hexoside	C27H30O15	17.88	595.16630		+	+	+	
12	Tetrahydroxy(iso)flavanone-C-hexoside isomer 2	C21H22O11	18.04	451.12404		-	+	-	
13	7-Hydroxycoumarin (Umbelliferone)	C9H6O3	17.61	163.03952		-	+	-	(Sherwood et al.,1973)
14	Isoorientin-O-hexoside isomer 1	C27H30O16	18.14		609.14556	+	+	+	(Kovalev and Komissarenko, 1983)
15	Riboflavin	C17H20N4O6	18.57	377.14611		-	-	+	
16	Scopoletin (7-Hydroxy-6-methoxycoumarin)	C10H8O4	18.45	193.05009		+	+	+	(Sherwood et al., 1973)
17	Isoorientin-O-hexoside isomer 2	C27H30O16	18.50		609.14556	+	+	+	(Kovalev and Komissarenko, 1983)
18	Vicenin-2 (Apigenin-6,8-di-C-glucoside)	C27H30O15	18.91		593.15065	+	+	+	
19	Coumaroylquinic acid isomer 2	C16H18O8	19.08		337.09235	+	+	+	
20	Naringenin-C-hexoside	C21H22O10	19.27	435.12913		+	+	-	
21	Naringenin-C-hexoside isomer 1	C21H22O10	19.28	435.12913		-	-	+	
22	Methoxy-trihydroxy(iso)flavanone-C-hexoside isomer 1	C22H24O11	19.60	465.13969		+	+	+	
23	Naringenin-C-hexoside isomer 2	C21H22O10	19.85	435.12913		-	-	+	
24	Methoxy-trihydroxy(iso)flavanone-C-hexoside isomer 2	C22H24O11	20.15	465.13969		+	+	+	
25S	Noscapine (Narcotine)	C22H23NO7	20.61	414.15528		-	-	+	
26	Isoorientin (Homoorientin, Luteolin-6-C-glucoside)	C21H20O11	20.66	449.10839		+	+	+	(Kovalev and Komissarenko, 1983)
27	Myricetin-3′-O-glucoside (Cannabiscitrin)	C21H20O13	20.87		479.08257	+	+	-	
28	Isorhamnetin-O-hexosylhexoside	C28H32O17	20.90		639.15613	-	+	+	
29	Quercetin-O-pentosyldeoxyhexosylhexoside	C32H38O20	21.51		741.18782	-	+	-	
30	Dihydrokaempferol (Aromadendrin)	C15H12O6	21.83		287.05557	+	+	-	
31	Isovitexin (Homovitexin, Apigenin-6-C-glucoside)	C21H20O10	22.20	433.11348		+	+	+	(Kovalev and Komissarenko, 1983)
32	Tetrahydroxy(iso)flavone-O-glucuronide	C21H18O12	22.25		461.07201	-	+	+	
33	Hyperoside (Hyperin, Quercetin-3-O-galactoside)	C21H20O12	22.62		463.08765	+	+	+	
34	Methoxy-trihydroxy(iso)flavone-C-hexoside	C22H22O11	22.64	463.12404		+	+	+	
35	Quercetin-3-O-glucuronide	C21H18O13	22.66		477.06692	-	+	-	
36	Isoquercitrin (Hirsutrin, Quercetin-3-O-glucoside)	C21H20O12	22.86		463.08765	+	+	+	
37	Rutin (Quercetin-3-O-rutinoside)	C27H30O16	22.94	611.16122		-	+	-	
38	Methoxy-pentahydroxy(iso)flavone-O-hexoside	C22H22O13	23.17		493.09822	+	+	-	
39	Quercetin-O-pentoside	C20H18O11	23.43		433.07709	+	-	-	
40	Quercetin-O-pentoside isomer 1	C20H18O11	23.44		433.07709	-	+	-	
41	Quercetin-O-hexoside	C21H20O12	23.75		463.08765	+	+	+	
42	Kaempferol-O-hexoside	C21H20O11	24.07		447.09274	+	+	+	
43S	Myricetin (3,3′,4′,5,5′,7-Hexahydroxyflavone)	C15H10O8	24.13		317.02974	+	+	-	
44	Quercetin-O-pentoside isomer 2	C20H18O11	24.18		433.07709	-	+	-	
45	Kaempferitrin (Kaempferol-3,7-di-O-rhamnoside)	C27H30O14	24.26		577.15573	+	+	+	
46	Astragalin (Kaempferol-3-O-glucoside)	C21H20O11	24.62		447.09274	+	+	+	(Bodalski and Rzadkowska-Bodalska, 1966)
47	Kaempferol-O-pentoside isomer 1	C20H18O10	24.81		417.08218	+	+	+	
48S	Isorhamnetin-3-O-glucoside	C22H22O12	24.87		477.10330	+	+	+	
49	Dimethoxy-tetrahydroxy(iso)flavone-O-hexoside	C23H24O13	24.94		507.11387	+	+	-	
50	Kaempferol-O-pentoside isomer 2	C20H18O10	25.16		417.08218	+	+	+	
51	Kaempferol-O-malonylhexoside	C24H22O14	25.55		533.09314	+	+	+	
52	Kaempferol-O-3-Hydroxy-3-methylglutaroylhexoside	C27H28O15	25.78		591.13500	+	+	+	
53	Methoxy-trihydroxy(iso)flavone-O-glucuronide	C22H20O12	25.81		475.08766	+	+	-	
54	Tetrahydroxy(iso)flavone-O-acetylhexoside	C23H22O12	26.47		489.10331	+	+	+	
55	Daphnoretin	C19H12O7	26.69		351.05048	+	+	+	(Sherwood et al., 1973)
56	Dihydroxy(iso)flavone	C15H10O4	26.74		253.05009	+	+	+	
57S	Quercetin (3,3′,4′,5,7-Pentahydroxyflavone)	C15H10O7	26.90		301.03483	+	+	-	
58S	Naringenin (4′,5,7-Trihydroxyflavanone)	C15H12O5	27.14		271.06065	+	+	+	
59	Dimethoxy-tetrahydroxy(iso)flavone	C17H14O8	27.77		345.06105	-	-	+	
60	Dimethoxy-tetrahydroxy(iso)flavone isomer 1	C17H14O8	27.74		345.06105	+	+	-	
61	Sebacic acid	C10H18O4	27.91		201.11268	+	+	+	(Bodalski and Rzadkowska-Bodalska, 1966)
62S	Kaempferol (3,4′,5,7-Tetrahydroxyflavone)	C15H10O6	29.24		285.03991	+	+	+	(Sherwood et al., 1973)
63	Methoxy-trihydroxy(iso)flavone isomer 1	C16H12O6	29.34		299.05556	+	+	+	
64	Cirsiliol (6,7-Dimethoxy-3′,4′,5-trihydroxyflavone)	C17H14O7	29.41		329.06613	+	+	+	
65S	Apigenin (4′,5,7-Trihydroxyflavone)	C15H10O5	29.59		269.04500	+	+	+	
66	Methoxy-trihydroxy(iso)flavone isomer 2	C16H12O6	29.68		299.05556	+	+	+	
67	Trihydroxy-trimethoxy(iso)flavone isomer 1	C18H16O8	29.71		359.07669	+	+	+	
68S	Isorhamnetin (3′-Methoxy-3,4′,5,7-tetrahydroxyflavone)	C16H12O7	29.74		315.05048	+	+	-	
69	Pilosin (4′,6-Dimethoxy-5,7,8-trihydroxyflavone)	C17H14O7	29.75		329.06613	+	+	+	

(*continued*)

Table 2 (*Continued*)

No	Name	Formula	Rt	$[M + H]^+$	$[M - H]^-$	EA	MeOH	Water	Ref
70	Dimethoxy-tetrahydroxy(iso)flavone isomer 2	C17H14O8	29.89		345.06105	+	+	-	
71	Liquiritigenin (4′,7-Dihydroxyflavanone)	C15H12O4	29.93		255.06573	+	+	+	
72	Hydroxy(iso)flavone	C15H10O3	30.69	239.07082		+	+	+	
73	Hydroxy-methoxy(iso)flavone	C16H12O4	30.76	269.08139		+	+	+	
74	Medicarpin (3-Hydroxy-9-methoxypterocarpan)	C16H14O4	30.94	271.09704		+	+	+	
75	Trihydroxy-trimethoxy(iso)flavone isomer 2	C18H16O8	31.22		359.07669	+	+	+	
76	Pinocembrin (5,7-Dihydroxyflavanone)	C15H12O4	32.15		255.06573	+	+	+	
77	Dihydroxy-methoxy(iso)flavone	C16H12O5	32.36		283.06065	+	+	+	
78	Dihydroxy-trimethoxy(iso)flavone	C18H16O7	33.05	345.09743		+	+	+	
79	Dihydroxy-tetramethoxy(iso)flavone	C19H18O8	33.17		373.09235	+	+	+	
80S	Chrysin (5,7-Dihydroxyflavone)	C15H10O4	33.20	255.06573		+	+	+	
81	Dodecanedioic acid	C12H22O4	33.26		229.14399	+	+	+	
82	Dimethoxy-hydroxy(iso)flavone	C17H14O5	35.22	299.09195		+	+	-	

Rt – retention time, min
Fr. – fragment
S – compound confirmed by standard

extracts and fractions. IC_{50} value for methanol extract was reported as 92.6 μg /ml in DPPH scavenging assay.

CUPRAC and FRAP assays were used to evaluate reducing power, which reflects to electron-donating ability of the tested samples. The abilities in CUPRAC assay can be ranked as ethyl acetate>methanol>-water. However, the FRAP abilities of the extracts were very close. As reported in Table 3, the ME exhibited the best metal chelating ability with 12.16 mg EDTAE/g, followed by WE and EAE. Regarding phosphomolybdenum assay, the highest and lowest ability were observed by methanol and water, respectively. Generally, the obtained antioxidant results were same trends of total phenolic and flavonoids content in the tested extracts. This approach is also in agreement with previous studies, in which a positive correlation between total bioactive content and antioxidant properties were reported (Devi et al., 2019; El Atki et al., 2019).

In recent years, enzymes have been gaining great interest as drug targets and thus they are main players for designing novel products to manage several diseases in pharmaceutical area. As an example, amylase is one of key enzymes in carbohydrate catabolism and thus the inhibition of this enzyme could manage the level of blood glucose in the patients with diabetes. Also, the inhibition of cholinesterase may be useful to increase the cognitive functions in the patients with Alzheimer's disease. That is way, several drugs have been produced as enzyme inhibitors, and however, they can cause unpleasant side effects. Thus, novel inhibitors from natural sources are required to replace the synthetic ones (Mzoughi et al., 2019; Rauf and Jehan, 2017).

In the current work, we investigated the inhibitory effects against tyrosinase, amylase and cholinesterases (AChE and BChE) and the results were illustrated in Table 4. The strongest anti-cholinesterases ability was found in EAE, while the WE was not active on these enzymes. Amylase and glucoside inhibitory effects orders were same: ethyl acetate>methanol>water. The best tyrosinase inhibitory effect was observed in methanol extract and other extracts exhibited quite similar effect. Herein, the enzyme inhibitory properties of the EAE and ME may be ascribed to the availability of phenolics. This phenomenon was also described by several researchers, who found a positive correlation between total bioactive components and enzyme inhibitory properties (Kocak et al., 2016; Muddathir et al., 2017; Reza et al., 2018). To date, no data regarding enzyme inhibitory effects of *C. varia* was observed and this current work could shed light to the potential of this plant in the literature.

3.3. DNA protective effect

Reactive oxygen species (ROS), reactive nitrogen species (RNS) as well as environmental toxic agents, including heavy metals from the Fenton reaction, cause DNA damage and results in mutations. In this study, we firstly targeted to determine DNA protective ability of *C. varia* extracts against mostly deleterious Fenton reaction. As it can be seen from Fig. 1, the ME and WE protected the plasmid DNA against the Fenton mixture and formed distinct DNA bands. Accordingly, the WE showed the highest protective activity with a protective effect of

Table 3
Antioxidant activities of the tested extracts.

Samples	DPPH (mg TE/g extract)	ABTS (mg TE/g extract)	CUPRAC (mg TE/g extract)	FRAP (mg TE/g extract)	Phosphomolybdenum (mmol TE/g)	Metal chelating ability (mg EDTAE/g)
EAE	$33.81\pm0.78^{b*}$	323.99 ± 1.77^{a}	106.10 ± 3.86^{a}	55.67 ± 1.86^{a}	1.02 ± 0.06^{a}	4.16 ± 0.12^{c}
ME	38.28 ± 0.76^{a}	190.71 ± 5.29^{c}	93.46 ± 1.30^{b}	53.84 ± 0.88^{a}	1.17 ± 0.07^{a}	12.16 ± 0.06^{a}
WE	29.63 ± 1.22^{c}	219.77 ± 4.17^{b}	60.74 ± 1.35^{c}	54.18 ± 0.56^{a}	0.82 ± 0.04^{b}	9.59 ± 0.49^{b}

* Values expressed are means ± S.D. of three parallel measurements. TE: Trolox equivalent; EDTAE: EDTA equivalent; EAE: Ethyl acetate extract; ME: Methanol extract; WE: Water extract. Different letters indicate statistical differences in the extracts ($p < 0.0$

Table 4
Enzyme inhibitory properties of the tested extracts.

Samples	AChE inhibition (mg GALAE/g extract)	BChE inhibition (mg GALAE/g extract)	Tyrosinase inhibition (mg KAE/g extract)	α-amylase inhibition (mmol ACAE/g extract)	α-glucosidase inhibition (mmol ACAE/g extract)
EAE	1.39 ± 0.09^{a}	0.40 ± 0.08^{b}	24.98 ± 2.47^{a}	0.59 ± 0.03^{a}	20.02 ± 0.26^{a}
ME	1.27 ± 0.14^{a}	0.63 ± 0.13^{a}	28.91 ± 2.91^{a}	0.50 ± 0.01^{b}	4.95 ± 0.19^{b}
WE	na	na	24.24 ± 2.22^{a}	0.09 ± 0.01^{c}	2.33 ± 0.26^{c}

*Values expressed are means ± S.D. of three parallel measurements. GALAE: Galatamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent; na: not active; EAE: Ethyl acetate extract; ME: Methanol extract; WE: Water extract. Different letters indicate statistical differences in the extracts ($p < 0.05$).

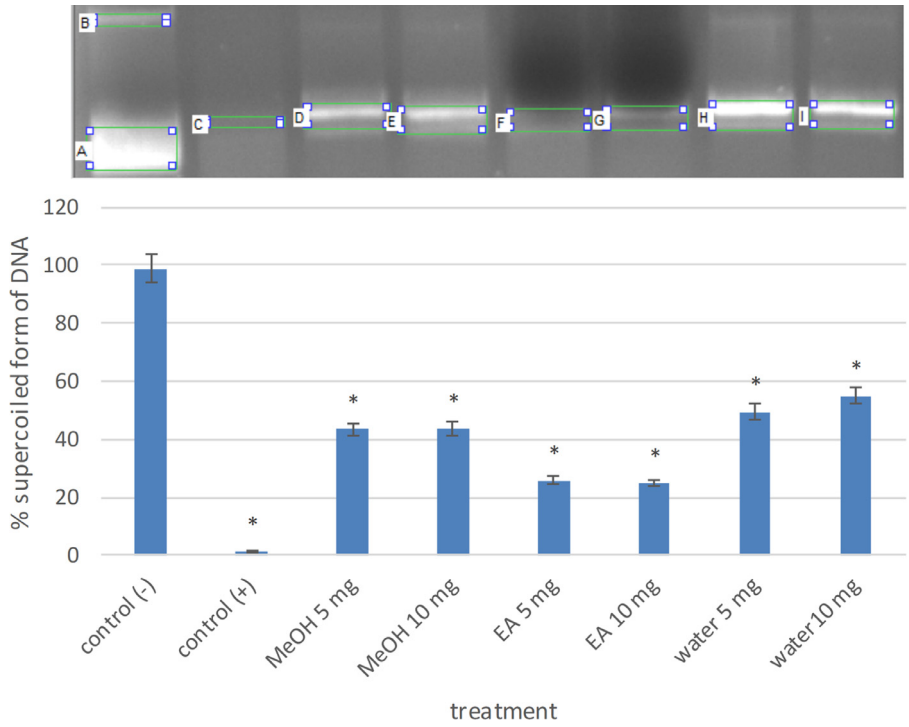

Fig.1. pUC19 plasmid DNA bands and % of protected supercoiled DNA after treated with EAE, ME and WE of *Coronilla varia*. **A, B**; (−) control, **C**; (+) control, **D, E**; 5 and 10 mg/ml ME, **F, G**; 5 and 10 mg/ml EAE, **H, I;** 5 and 10 mg/ml WE. * illustrates $p < 0.05$.

more than 50%. Afterward, ME indicated a DNA protection activity of about 40%. There was little protection activity for EAE compared to positive control. DNA protection activities of extracts can be explained by phenolic compounds found in *C. varia L.* aerial parts. Apigenin was determined in all extracts. Then, quercetin derivatives, 7-hydroxycoumarin (umbelliferone), tetrahydroxy(iso)flavanone-C-hexoside isomer 2 and rutin (quercetin-3-O-rutinoside) were also present in ME. Citric acid, pantothenic acid, riboflavin, naringenin-C-hexoside isomer 2 and noscapine (narcotine) were detected only in WE. This also accords with our earlier observations, which showed that metabolic compounds were mainly related to antioxidant activity. As mentioned in our previous studies, quercetin and its derivatives possess the good capacity for free radical scavenger, antioxidant and have the ability of DNA protection against Fenton reaction (Yerlikaya et al., 2019,2017). Apigenin is rich in vegetables, fruits, beverages, parsley, grapes, apples, chamomile tea and wine and chemically known as 4′,5,7-trihydroxyfavone. According to recent study, apigenin was considered as a class II drug of Biopharmaceutical Classification System. Physiologically, it functions as anti-cancer, antioxidant, antiviral, antibacterial, anti-inflammatory and blood pressure reduction (Yan et al., 2017). Apigenin is a good cancer chemo-preventive agent and showed cytostatic and anti-angiogenic effect *in vitro* (Horvathova et al., 2004). It was indicated in a study that rutin and quercetin prevented the formation of superoxide and hydroxyl radical in the Fenton reaction (Horvathova et al., 2004). In one study, it was evaluated that antioxidant properties of umbelliferone, knowns as 7-hydroxycoumarin and as a result of this, flavonoid decreased lipid peroxidation activity in STZ-diabetic rats (Ramesh and Pugalendi, 2006). Riboflavin is a water-soluble vitamin (so for this reason present only water extract). It was showed that deficiency of riboflavin leaded to oxidative damage in the gills of fish (Chen et al., 2015). Other crucial compound known as pantothenic acid or pantothenate is an important vitamin which provides biosynthesis of coenzyme A (CoA) in mammalian cells. CoA is a cofactor enzyme for oxidation of fatty acids, carbohydrates, pyruvate, lactate, ketone bodies and amino acids (Tahiliani and Beinlich, 1991). Some vitamins (including vitamin C, E, β-carotene) are known with their antioxidant capacity. Also, pantothenate inhibited deoxyribose oxidation in a study (Hu et al., 1995). Our results seem to be consistent with other research which revealed that phenolic compounds caused DNA protection activity because of having high antioxidant capacity like as *C. varia* extracts.

3.4. Anticancer effect

Cell viability analysis of extracts on MDA-MB-231 and MCF-7 breast cancer cells was evaluated by MTT-based cytotoxicity test. Cells were exposed to various doses of all extracts for 24, 48 and 72 h. As a result, the cell viability of both cells was diminished with time and dose-dependent manner (Fig. 2). The most effective extracts on cancer cells were determined as EAE and then ME. In addition, WE was more effective in MDA-MB-231 cells than MCF-7 cells. According to the cell viability test, IC_{50} values of extracts on the cells were also calculated. For MCF-7 cells, the lowest IC_{50} values of MeOH and EA were calculated as 817 μg/ml and 371 μg/ml, respectively (Supplemental Figure 5). No IC_{50} value was calculated for WE because the cell viability of MCF-7 cells was higher than 50% after treated with WE. For MDA-MB-231 cells, the lowest IC_{50} values of ME, EAE and WE were determined as 597,7 μg/ml, 386,4 μg/ml and 824,1 μg/ml, respectively (Supplemental Fig. 6). The IC_{50} value of substances is an important concentration for brightening biological activities inside the cell signaling pathway. For determination of cell death mechanism at mRNA transcript level in MDA-MB-231 and MCF-7 cell lines, IC_{50} values of all extracts were used. *Bax* and *Bcl-2* genes were selected to evaluate apoptotic death mechanism. Based on our findings, *Bax* (pro-apoptotic gene) was up-regulated and *Bcl-2* (anti-apoptotic gene) was down-regulated after treatment of WE in MDA-MB-231 cells (Fig. 3). These results showed that apoptotic cell death mechanism could initiate inside MDA-MB-231 cell due to the application of WE. Interestingly, WE mainly contains noscapine (narcotine) different from other extracts. Noscapine has several medical applications, including anti-stroke, anti-cancer and cough suppressant. It is a

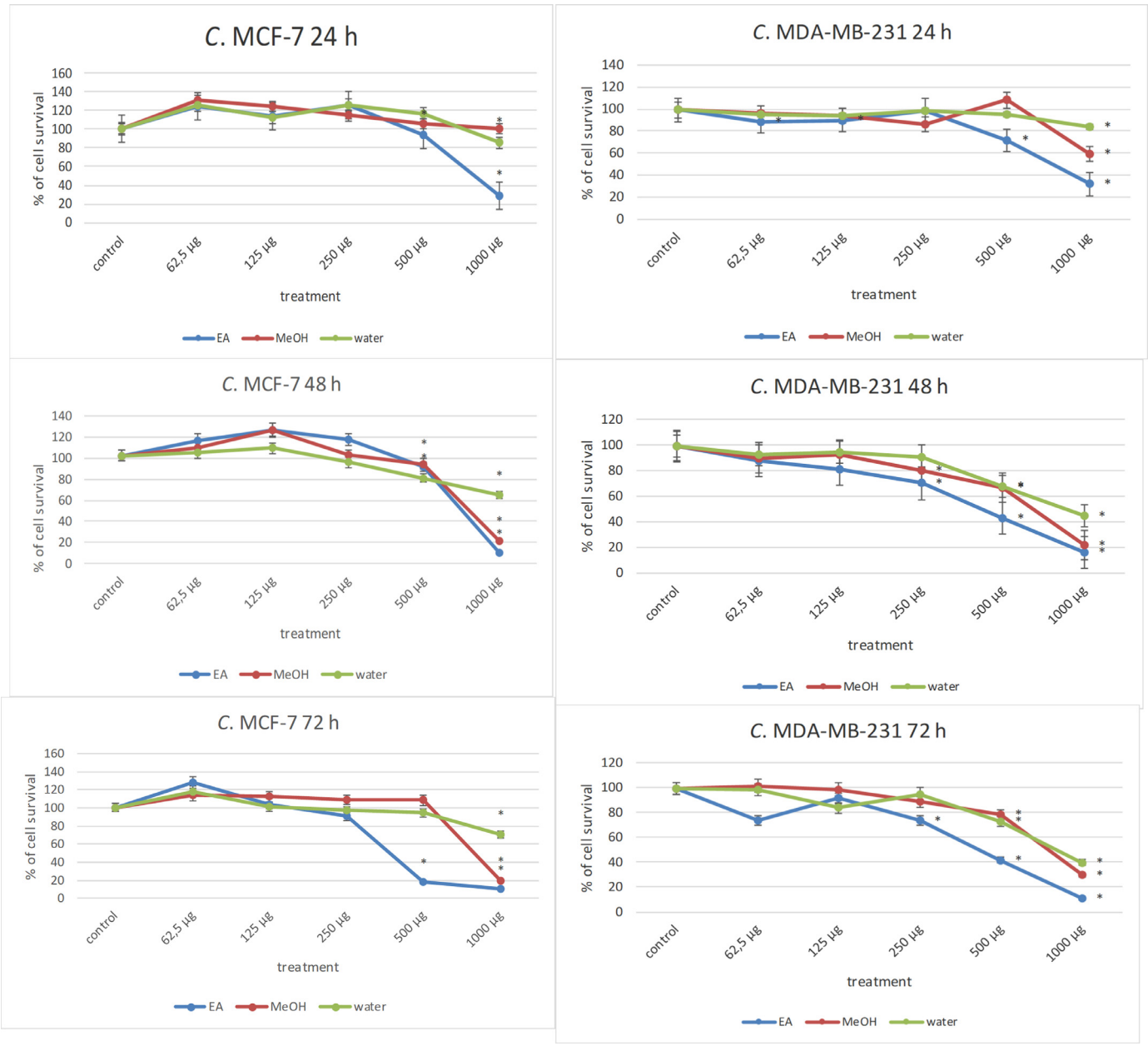

Fig. 2. % of MCF-7 and MDA-MB-231 breast cancer cells' viability after treatment of different concentrations of EAE, ME and WE with 24, 48 and 72 h. * shows $p < 0.05$.

safe drug used effectively in low toxicity. Several studies revealed that noscapine is able to arrest cells in the mitotic phase of growth (Mahmoudian and Rahimi-Moghaddam, 2009). Also, it caused apoptosis phenomena via binding to microtubule assembly and arrested cells in mitosis in solid breast tumor in mice (Aneja et al., 2006). In addition, noscapine could reverse the resistance mechanism of the drug and able to make more active the effect of vincristine and doxorubicin in OVCAR3 cell lines (Rahbar-Roshandel et al., 2008). Moreover, it was observed that noscapine increased regulation of *Bax*/*Bcl-2* ratio in K562 apoptosis-resistant cells (Mahmoudian and Rahimi-Moghaddam, 2009). These results are in line with those of early works which showed that noscapine caused apoptotic cell death in various cancer lines. In our study, *Bax* and *Bcl-2* genes were down-regulated after treated with EAE. In addition, after the application of ME in MDA-MB-231 cells *Bax* gene was down-regulated, *Bcl-2* gene was up-regulated. These findings clearly showed that the cell death mechanism of MDA-MB-231 cells was not apoptosis after treated with ME. On the other hand, after treatment of EAE, expression of *Bax* gene was raised and *Bcl-2* gene expression was reduced in MCF-7 cells (Fig. 4). Apigenin was detected in the tested extracts. In human cancers, it was showed that apigenin is a potent initiator of apoptosis via either the intrinsic or extrinsic pathway (Yan et al., 2017). In one study, it was shown that apigenin decreased the mitochondrial outer membrane potential and cytochrome c was released to cytosol and activated procaspase 9 and finally apoptotic cell death mechanism was taken place by the way of intrinsic pathway in human promyelocytic leukemia HL-60 cells (Wang et al.,1999). *Bax* and *Bcl-2* gene expression increased in MCF-7 cells after application of ME. It was considered that these unexpected results can be due to anti-synergistic effects of metabolic substances in ME. *Beclin-1* and *LC3-II* genes were also used for evaluation of autophagic mechanism. As a result, *Beclin-1* and *LC3-II* genes were up-regulated independently from *Bcl-2* gene in MCF-7 cells after treated with EAE. In contrast, expression of *Beclin-1* and *LC3-II* genes were reduced independently from *Bcl-2*

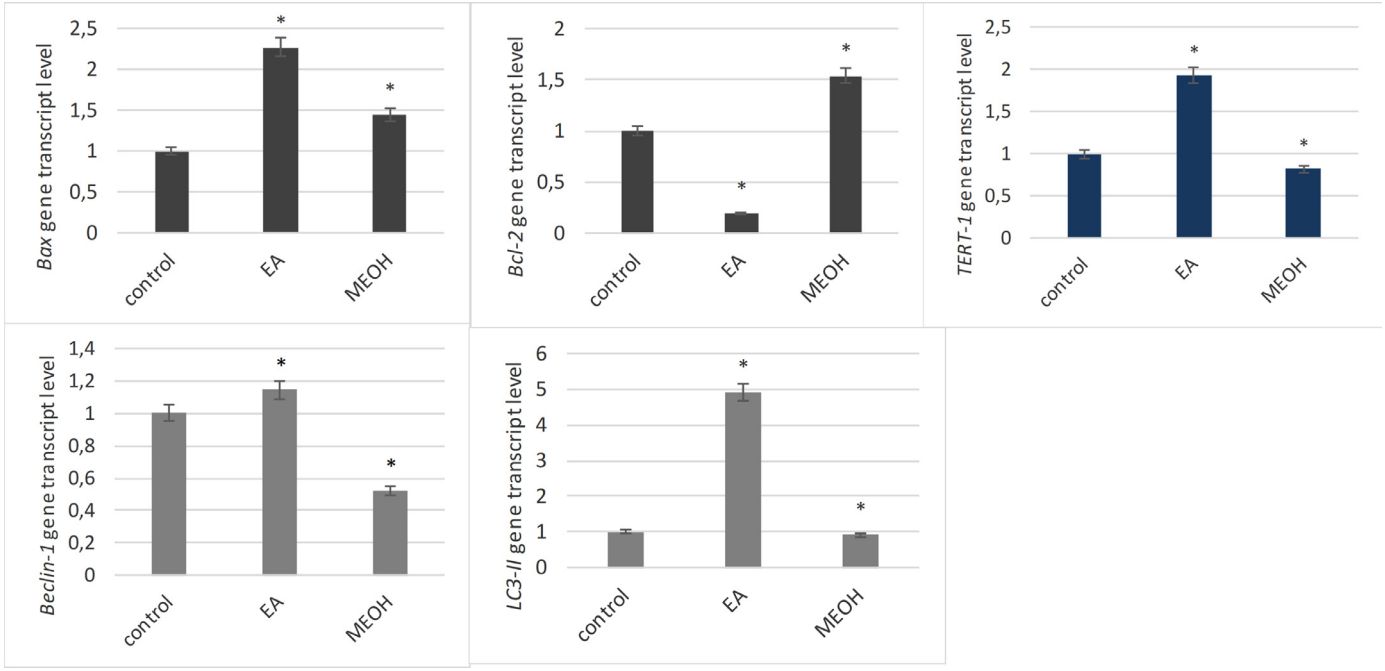

Fig. 3. Gene expression diagrams of MCF-7 cells after exposed to *C. varia* extracts. mRNA transcript levels were investigated by qRT-PCR. *GAPDH* was used as internal control gene. $^{*}p < 0.05$.

gene in MCF-7 cells after exposed to ME. In addition, expressions of *Beclin-1* and *LC3-II* genes were increased in MDA-MB-231 cells after treatment of EAE. However, *LC3-II* gene was up-regulated, and *Beclin-1* was down-regulated in MDA-MB-231 cells after application of ME. *Beclin-1* was down-regulated in MDA-MB-231 cells correlated with down-regulation of *Bcl-2* and there was no significant difference in *LC3-II* gene expression after treatment of WE. It was considered that these results can be due to crosstalk mechanism between apoptosis and autophagy marker genes.

In oncology research, it is very important to understand the molecular mechanism and telomere activity that regulates aging, since stopping division in cancer cells is one of the best treatment strategies. *TERT* (telomerase activity marker gene) expression has been shown to increase in approximately 4% of all cancers (ovarian cancer, lung adenocarcinoma, lung squamous cell carcinoma, esophageal carcinoma, and adrenocortical carcinoma) (Colebatch et al., 2019). *TERT-1* gene was examined for analyzing of telomerase activity in this study. *TERT-1* gene was down-regulated after treated with EAE

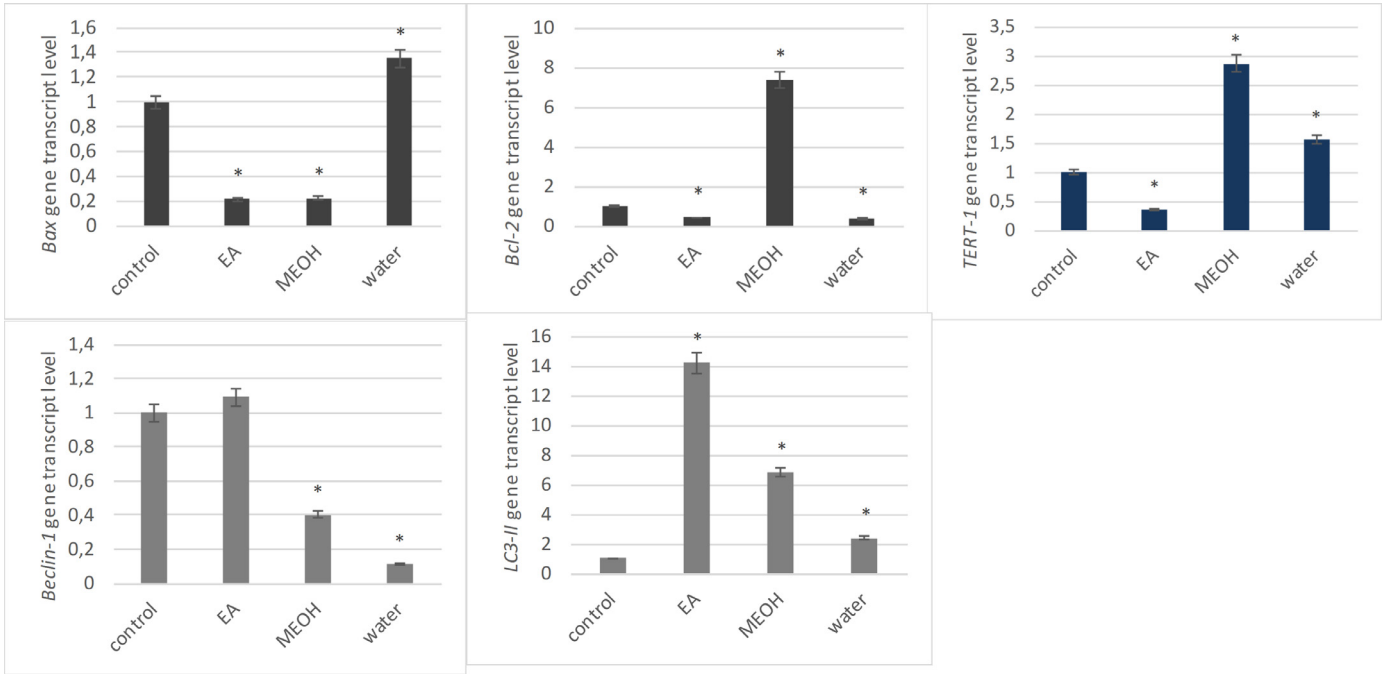

Fig. 4. Gene expression diagrams of MDA-MB-231 cells after exposed to *C. varia* extracts. mRNA transcript levels were investigated by qRT-PCR. *GAPDH* was used as internal control gene. $^{*}p < 0.05$.

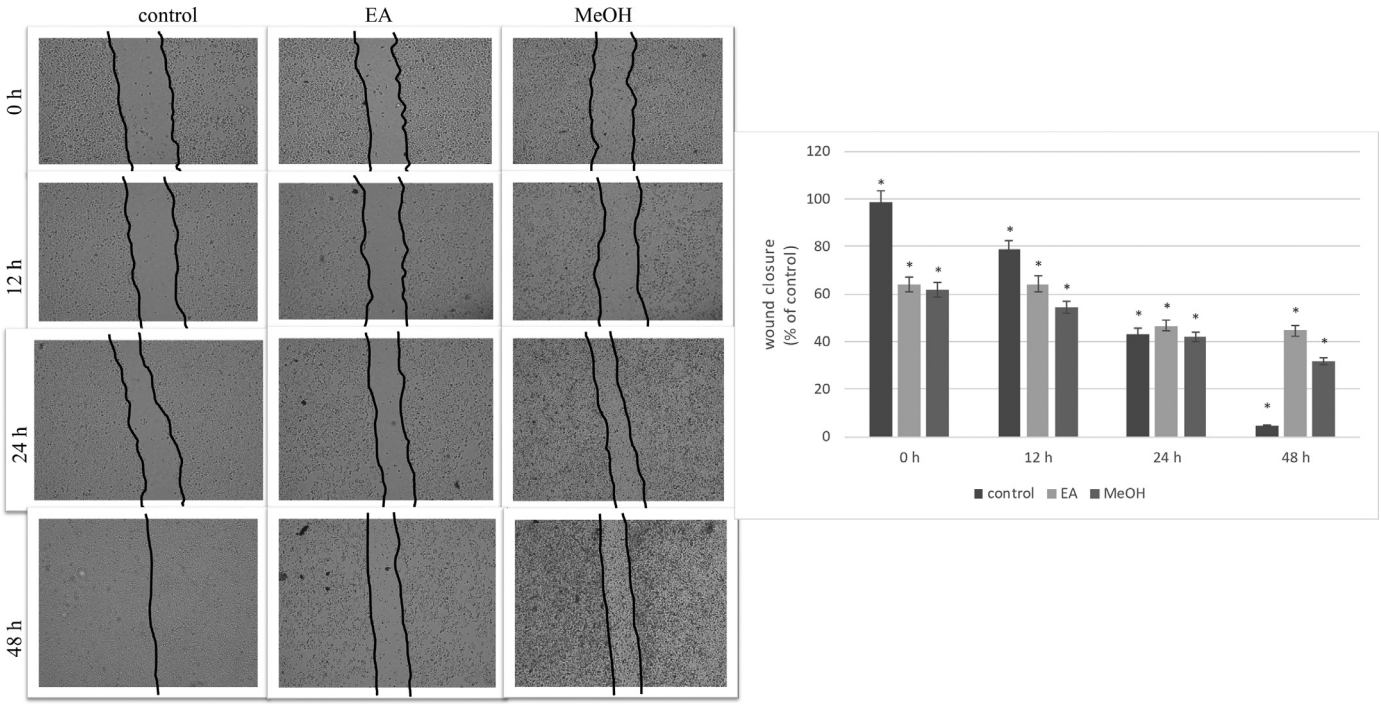

Fig. 5. Cell morphology images of scratched areas and % of cell movement ability of MCF-7 breast cancer cells after treated with *C. varia* extracts. All experimental growth media contained mitomycin C (10μg/ml) for blocking proliferative ability of cells. $^{*}p < 0.05$.

and ME for MDA-MB-231 and MCF-7 cells, respectively (Figs. 3 and 4). According to our results, ME includes rutin, so down-regulation of telomerase activity on MCF-7 breast cancer cells can be related to presence of rutin. Telomerase activity of cancer cells is related to the highly proliferative ability of malign cells. It was reported that rutin is able to inhibit cancer cell growth and angiogenesis in colorectal cell lines (Chen et al., 2013).

For evaluation of anti-metastatic activity of extracts on breast cancer cell lines, wound healing scratch method was utilized. Cells were exposed to IC_{50} values of extracts and wound closure was traced under the inverted microscope with time points in 0, 12, 24 and 48 h. As indicated in Figs. 5 and 6, cell migration ability of MDA-MB-231 and MCF-7 cells were suppressed after treatment of EAE and ME. In MDA-MB-231 cells, the wound was closed in 48 h after treated with WE. Different from the benign tumor, malignant tumors can be able to migrate and metastasize to other tissue. Metastatic activity is one of the major problems of chemotherapy agent due to progressive resistance mechanism against the drug. Experiments on *in vitro* cancer cells and *in vivo* animal models showed that apigenin is able to obstruct movement and invasion of cancer cell (Yan et al., 2017). In a

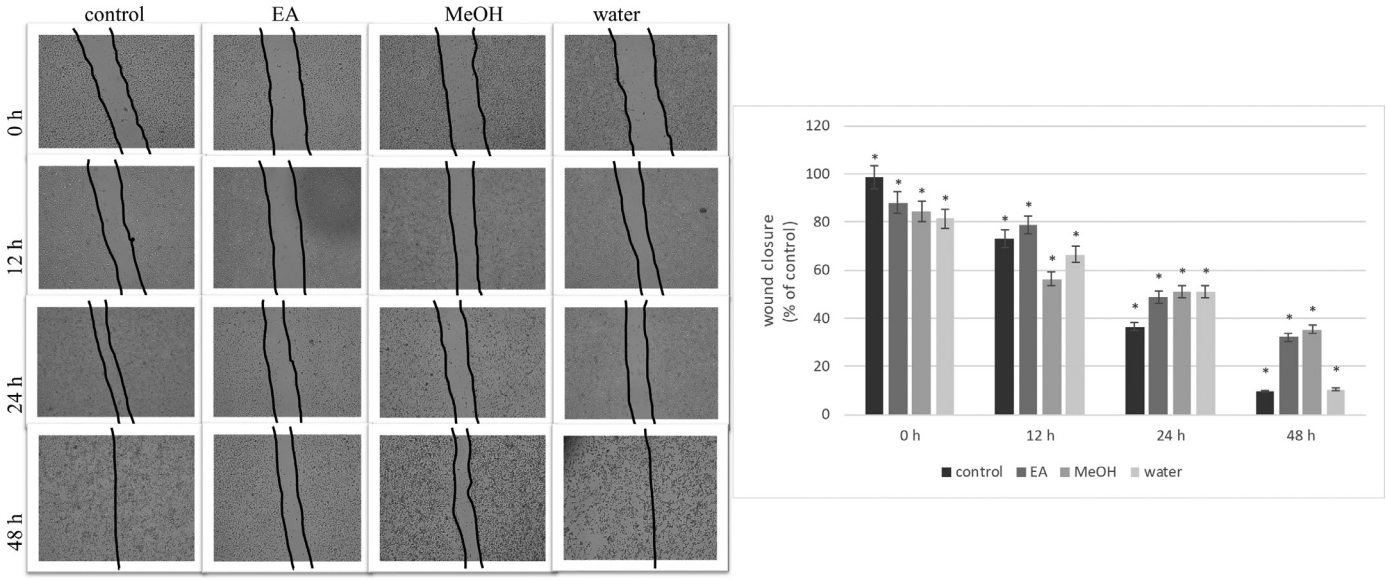

Fig. 6. Cell morphology images of scratched areas and % of cell movement ability of MDA-MB-231 breast cancer cells after treated with *C. varia* extracts. All experimental growth media contained mitomycin C (10μg/ml) for blocking proliferative ability of cells. $^{*}p < 0.05$.

study, apigenin prevented migration and cell invasion in a dose-dependent manner in prostate cancer DU145 cells (Zhu et al., 2015). Our findings are match those observed in earlier studies.

4. Conclusion

This study is the first report which demonstrates the mechanism of chemical profile, biological and pharmacological activities (DNA protection, enzyme inhibitory, antioxidant, and anti-cancer effects) of aerial parts of *C. varia* plants. It was thought that the DNA protection activity of C. varia for WE and ME may be due to presence of naringenin, apigenin, quercetin derivatives, riboflavin and pantothenic acid. The raise in *Bax/Bcl-2* ratio in MCF-7 cells treated with *C. varia* EAE was thought to be due to apoptotic cell death. It has been confirmed by comparison with other studies in the literature that this activity may be due to the availability of apigenin derivatives in the EAE. The increase in *Bax* / *Bcl-2* ratio in MDA-MB-231 cells treated with *C. varia* WE was thought to be associated with the noscapine (narcotin) substance present in the WE. EAE and ME of *C. varia* plant were found to suppress cell migration by inhibiting metastatic activity in MDA-MB-231 and MCF-7 breast cancer cells. Various studies have shown that between 30 and 50% of the population in industrialized countries utilize alternative medicine to avoid or cure various health-related complications. The modulating effects of polyphenols (flavonoid-derived compounds) on cell-cycle regulatory proteins have been demonstrated in several studies. Phytochemicals have angiogenic, anti-inflammatory, antioxidant, antiproliferative and pro-apoptotic effects by affecting various signaling pathways. In addition, the IC_{50} values (>200 μg/ml) for the tested extracts were higher than that of National Cancer Institute and only ethyl acetate extract (IC_{50}=386.4 μg/ml) exhibited weak cytotoxic activity. However, considering the side effects of synthetic drugs, the ethyl acetate extract might be useful for designing anticancer applications. Taken together, *C. varia* can be one valuable agent in natural therapy for designing novel pharmaceutical ingredients. Considering chemical profile of the tested extracts, we strongly suggested that the plant should be tested in terms of *in vitro* or *in vivo* toxicity profiles in further studies.

Declaration of Competing Interest

There is no conflict of interest

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2021.02.025.

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