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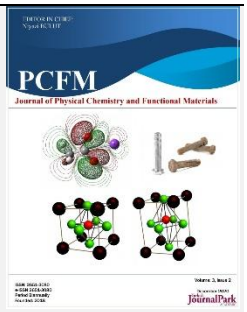

In vitro antiradical, cytotoxic, genotoxic and phytochemical properties of *Astragalus compactus* Lam. subsp. *compactus*

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ABSTRACT

In this study, it was aimed to determine the antiradical, cytotoxic, genotoxic and phytochemical compounds of ethanol, methanol and water extracts of *Astragalus compactus* Lam. subsp. *compactus* (guni) plant grown in Elazig. Antiradical activity of the plant was determined against ABTS, DPPH, and OH radicals, and the cytotoxic and genotoxic activities were determined against A2780 and MCF-7 cell lines with MTT and Comet assays. All extracts of *A. compactus* in all the radical scavenging tests showed lower antiradical activity than the standard antioxidant BHT. However, it was determined that this plant is rich in some important phytochemical compounds, such as phenolic, flavonoid, proanthocyanidin, and phytosterols. DNA damage studies of *A. compactus* extracts were carried out by the Comet assay and all the results showed that cell death occurred for the A2780 cell lines.

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1. INTRODUCTION

Plants have attracted people throughout history for both nutritional and therapeutic purposes. The plants are very rich in point of phenolic acids, phenols, flavonoids, tocopherols, carotenes, and important vitamins and these can provide protection against many diseases [1]. Living organisms provide protection against oxidative damage with the help of antioxidants such as α-tocopherol, β-carotene and ascorbic acid, which they receive with food, and enzymes such as glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) in the intracellular antioxidant defense system [2,3]. Many studies have shown that substances with antioxidant properties have important anticancer, antiviral, antimicrobial, antibacterial, anti-allergic, anti-mutagenic, anti-proliferative, anti-tumor, antiulcer and anti-metastasis features [4-6].

The oldest drugs used in the treatment of cancer are plants. They were first shown to be effective against lung, lymphoma and testicular cancer in scientific research conducted in the 1970s [7]. As a result of these studies, it was determined that plants regulate the patient's body balance and increase the resistance of patients against cancer-related damage to tissues. The anticancer properties of these plants are related to their phytochemical contents. Medicinal plants have been frequently used in cancer treatment since they are both cheap and easily accessible [8]. For this reason, scientists have investigated the natural

substances originated in the plant, their isolation, characterization, and determination of anticancer properties [9].

Astragalus L. genus is a perennial herbaceous plant within the Fabaceae family and is represented by approximately 3494 species on Earth [10]. Approximately 445 species of this genus grow in Turkey and among them 224 species are endemic. The gene center of *Astragalus* species, distributed in the Iran-Turan floristic region, is Eurasia, and Central and Western Asia are the distribution regions of this genus [11-13]. In Turkey, many *Astragalus* species are used among the people due to their liver protective, antioxidant, immune system regulating and antiviral properties. *Astragalus* species have also been used as a popular medicinal plant in European countries for many years [14]. The antiradical, antioxidant, antibacterial, antimicrobial, antitumor, anticancer and phytochemical properties of many *Astragalus* species have been determined [15-18]. *Astragalus compactus* subsp. *compactus* species are called "guni" in Turkish in Turkey [6]. Naghiloo et al. [19] determined only antiradical and phenolic content properties of the *Astragalus compactus* subsp. *compactus* taxon, and no anticancer, genotoxic and phytochemical contents were detected.

Due to their excellent properties mentioned-above, *Astragalus compactus* Lam. subsp. *compactus* species were used in this study. We investigated the *in vitro* antiradical, cytotoxic, genotoxic and phytochemical contents of ethanol, methanol and water extracts of these species.

2. MATERIAL AND METHOD

2.1. Collection Details of Plant Used in the Study

Astragalus compactus subsp. *compactus* (guni): **09.05.2020** Between Elazig and Keban, Around Altinkurek Village, Slopes. Altitude: 1460 meters. The voucher specimen number is Keser 002. This specimen was stored in the herbarium of Firat University, Faculty of Science, Department of Biology, Elazig/Türkiye.

In the identification of plant samples, the works titled “Flora of Turkey and the East Aegean Islands” [20,21] and “List of Plants of Turkey (Vascular Plants)” [22] were used. The collected plant materials were dried in a laboratory environment, out of sunlight and at ambient temperature, and extraction processes were started without wasting time.

2.2. Extraction Process

The aerial parts of the *Astragalus compactus* subsp. *compactus* (guni) was first crushed in a blender and turned into powder for the extraction process. 20 grams of each plant sample was weighed, homogenized with 200 mL of solvent (ethanol, methanol and water) and centrifuged at 5000 rpm. At the end of the process, supernatants were taken and solvents were removed with appropriate procedures. The obtained extracts were stored in the deep freezer at -18 °C and dissolved at µg/mL concentration for analysis [23].

2.3. Determination of Antiradical Activity

The ABTS, OH and DPPH radical scavenging activities were determined by the methods of Re et al. [24], Halliwell et al. [25], and Brand-Williams et al. [26], respectively. All tests were repeated thrice and the average values were computed. The radical scavenging activity percentages (*RSA*(%)) were estimated by the following relation [27-29]:

$$RSA(\%) = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (1)$$

where A_0 is the control absorbance value and A_1 is the extract absorbance value.

2.4. Determination of Phytochemical Contents

The determination of total phenolic contents (gallic acid used as standard), total flavonoid contents (catechin used as standard), total proanthocyanidin content (catechin used as standard) were performed according to the methods of Slinkard and Singleton [30], Kim et al. [31] and Amaeze et al. [32], respectively. Fatty acids in the *A. compactus* aerial parts were analyzed according to Christie’s method [33] by Gas Chromatography (GC). The fatty acids analyses results were expressed as a percent of samples. The lipid-soluble vitamins and sterols were analyzed according to the method of Sánchez-Machado et al. [34] and Lopez-Cervantes et al. [35] by High Performance Liquid Chromatography (HPLC). The results of the analyses were expressed as µg/g.

2.5. Determination of Anticancer Properties

2.5.1. Cell Culture

The cell lines of MCF-7 human breast cancer and A2780 human ovarian cancer were used in the anticancer studies. These cells were retrieved from the American Type Culture Collection (ATCC).

2.5.2. MTT Test

The *A. compactus* extracts (water, methanol and ethanol) were studied for anticancer activity against the A2780 and MCF-7 cell lines. The viability of the cells was determined using 0.4% trypan blue. Effects of the % cell viability of extracts were evaluated by the MTT test [36,37].

2.6. Determination of the Genotoxicity with Comet Assay

Comet Assay, also known as single-cell gel electrophoresis, is one of the frequently used methods to detect genotoxic DNA damage in mammals [38]. The neutral comet assay technique described by Devlin et al. [39] was performed with some minor modifications. For this purpose, the ground slides were first coated with 0.65% high melting agarose (HMA) dissolved in PBS and left to dry in a microwave oven for 24 hours. Cultured MCF-7 and A2780 cells were incubated for 24 hours with the IC_{50} concentration of the extracts to be tested. At the end of

incubation, cells were mixed with low melting agarose (LMA) at 42 °C and spread onto HMA-coated ground slides, and then cover slipped very quickly. These slides were kept in the dark at +4 °C for 10-15 minutes until the agar turned into a solid state. Afterward, these slides were placed in freshly prepared cold lysis solution (prepared from the stock lysis solution by adding 1% Triton X-100 and 10% DMSO) consisting of a mixture of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH = 10.0) and kept in the dark at +4 °C for 1 hour.

2.6.1. Electrophoresis Process

Following lysis, the slides were placed in the same orientation in a horizontal electrophoresis tank (Bio-Rad, USA) filled with cold neutral electrophoresis buffer. The voltage and current of the tank were fixed at 25 V (0.83 V/cm) and 300 mA, respectively before the slides were placed, and the process was continued for 20 min. At the end of electrophoresis, the slides were subjected to neutralization with a neutralization buffer consisting of 0.4 M Tris, pH=7.5, 3 times for 5 min at 4 °C. After this process, the slides were stained with 50 µL ethidium bromide, coverslipped and kept in the dark at +4 °C for 20-30 min.

2.6.2. Scoring

Scoring was performed using a Leica brand fluorescence microscope and Comet IV software. After the applications, cells were viewed under the microscope and the degree of DNA damage was assessed using the Comet IV software. By randomly counting at least 25 cells on each slide, the changes in the parameters of Tail Length, Tail Density, Olive Tail Moment, Head Diameter, and Head Density of the groups and the presence and rate of DNA damage were determined.

2.7. Statistical Analyses

Statistical evaluations of the results obtained from the analyses were made with the help of the SPSS Statistics 22.0 program. While the evaluation of antiradical tests was carried out with ANOVA and DMRT tests, the $p<0.05$ value was considered statistically significant. The results were presented as mean ± standard deviation. KOLMOGOROV SMIRNOV test was used in the evaluation of the results of anticancer analyses. While group comparisons were made with a one-way analysis of variance and variance homogeneities were made with the LEVENE test, it was observed that the variances were not homogeneous. Therefore, while multiple comparisons were made with the TAMHANE T2 test, the $p<0.05$ value was considered statistically significant. The results were presented as mean ± standard deviation.

3. RESULTS AND DISCUSSIONS

3.1. Antiradical Activity Results

Antiradical activity results of water, methanol and ethanol extracts of the aerial parts of *Astragalus compactus* subsp. *compactus* (guni) are presented in Table 1. According to these test results, the standard antioxidant BHT showed higher antiradical activity than all extracts in ABTS, DPPH and OH radical scavenging tests. For the ABTS radical scavenging test, the radical scavenging percentages are listed from largest to smallest as BHT (%90.33) > *A. compactus* Methanol (ACM) (%78.87) > *A. compactus* Ethanol (ACE) (%66.82) > *A. compactus* Water (ACS) (%65.96). For the OH radical scavenging test, the radical scavenging percentages are listed from largest to smallest as BHT (%89.28) > ACM (%84.12) > ACE (%71.96) > ACS (%59.23). For the DPPH radical scavenging test, the radical scavenging percentages are listed from the largest to the smallest as BHT (%89.98) > ACM (%66.99) > ACE (%57.42) > ACS (%37.48).

Table 1 Antiradical activities of *Astragalus compactus* subsp. *compactus* extracts (500 µg/mL)

Samples	$ABTS^{+\bullet}$ (%)	$OH^{\bullet}$ (%)	$DPPH^{\bullet}$ (%)
***A. compactus* Water**	65.96 ± 0.24^{c}	59.23 ± 0.51^{d}	37.48 ± 0.85^{d}
***A. compactus* Ethanol**	66.82 ± 0.17^{c}	71.96 ± 0.39^{c}	57.42 ± 0.93^{c}
***A. compactus* Methanol**	78.87 ± 0.35^{b}	84.12 ± 0.47^{b}	66.99 ± 0.29^{b}
BHT	90.33 ± 0.32^{a}	89.28 ± 0.19^{a}	89.98 ± 0.67^{a}

Groups with the same letter are statistically similar; $p<0.05$

When antiradical studies related to the taxon *Astragalus compactus* subsp. *compactus* were examined; only Naghiloo et al. [19] found that *A. compactus* extracts destroyed high levels of DPPH radicals; apart from this, it was understood that this species was not subject to any antiradical studies, but there were studies on the genus *Astragalus*. Keskin [12] determined that *A. diphtherites* stem and root methanol extracts scavenged 79.01% and 58.40% of DPPH radicals, 81.26% and 78.49% of OH radicals, respectively. In the same study, it was determined that *A. gymnalocepias* stem and root methanol extracts

scavenged 86.83% and 39.62% of DPPH radicals, 90.02% and 84.47% of OH radicals, respectively [12]. These results have higher values than the DPPH and OH radical scavenging activities of aboveground methanol extracts in our thesis. This may be because the relevant plants are different species and/or different plant parts were studied. In another study, Gharari et al. [15] observed that *A. alopecurus* essential oils scavenged 67% of DPPH radicals. Khan et al. [40] determined that *A. grahamianus* methanol extracts scavenged DPPH and ABTS radicals at very high rates.

3.2. Phytochemical Analysis Results

The amounts of phenolic compounds, flavonoids and proanthocyanidins in water, methanol and ethanol extracts of *Astragalus compactus* subsp. *compactus* aerial parts are presented in Table 2. According to these results, the total phenolic compound contents of the extracts were ranked as ACM (63.80 mg GAE/g extract) > ACS (52.51 mg GAE/g extract) > ACE (49.08 mg GAE/g extract); while total flavonoid contents were ACM (2821.33 µg CE/g extract) > ACE (2267.42 µg CE/g extract) > ACS (1895.64 µg CE/g extract); Total proanthocyanidin contents are listed as ACM (1221.89 µg CE/g extract) > ACE (1040.78 µg CE/g extract) > ACS (923.00 µg CE/g extract).

Table 2 Amounts of total proanthocyanidins, total flavonoids and total phenolic compounds of *Astragalus compactus* subsp. *compactus*

Samples	Total Flavonoid	Total Proanthocyanidin	Total Phenolic
***A. compactus* Water**	1895.64±3.87	923.00±2.00	52.51±1.02
***A. compactus* Ethanol**	2267.42±3.03	1040.78±1.96	49.08±0.77
***A. compactus* Methanol**	2821.33±2.29	1221.89±1.75	63.80±0.58

The amounts of flavonoids and proanthocyanidins are presented as µg catechin equivalent/g extract, and the amount of phenolic compounds are presented as mg gallic acid equivalent/g extract.

Fat-soluble vitamin, plant sterols and free fatty acid contents of the aerial parts of *Astragalus compactus* subsp. *compactus* are presented in Table 3. According to these results, *A. compactus* extracts contained 0.200 µg/g δ-tocopherol, 15.500 µg/g α-tocopherol, 0.133 µg/g vitamin D, 252.100 µg/g ergosterol, 2.467 µg/g stigmasterol, 20.533 µg/g β-sitosterol, 1.25% myristic acid (14:0), 23.69% palmitic acid (16:0), 7.62% palmitoleic acid (16:1), 12.87% stearic acid (18:0), 16.17% oleic acid (18:1), 10.37% linoleic acid (18:2), 28.03% linoleic acid (18:3), 37.81% It was determined that it contains saturated and 62.19% unsaturated fatty acids.

Table 3 Vitamin, phytosterol and free fatty acid contents *Astragalus compactus* subsp. *compactus*

Phytochemical Contents (µg/g)	***A. compactus* subsp. *compactus***
δ-tocopherol	0.200±0.06
α- tocopherol	15.500±0.56
Vitamin D	0.133±0.05
Ergosterol	252.100±1.27
Stigmasterol	2.467±0.25
β-Sitosterol	20.533±1.09
Fatty Acids (%)	
14:0	1.25±0.03
16:0	23.69±0.72
16:1	7.62±0.54
18:0	12.87±0.95
18:1	16.17±0.77
18:2	10.37±0.35

18:3	28.03±1.01
Saturated FA	37.81
Unsaturated FA	62.19

When phytochemical studies related to *A. compactus* subsp. *compactus* taxon were examined, it was understood that this species was not the subject of any study examining vitamins, sterols and free fatty acids; only Naghiloo et al. [19] determined that *A. compactus* extracts contained 5.18 µg GAE/mg total phenolic compounds. In studies related to *Astragalus* genus, Keskin [12] determined that *A. diphtherites* stem and root methanol extracts contained 76.1 mg GAE/g and 30.7 mg GAE/g total phenolic compounds, 39.31 mg QE/g and 2.31 mg QE/g total flavonoids, respectively. In the same study, it was determined that *A. gymnalocepias* stem and root methanol extracts contained 54.66 mg GAE/g and 17.66 mg GAE/g total phenolic compounds, 36.81 mg QE/g and 11.20 mg QE/g OH total flavonoids, respectively [12]. In another study, Gharari et al. [15] found that *A. alopecurus* essential oils contained 53.61 mg/g total phenolic compounds, 115.64 mg/g total flavonoids. Butkute et al. [41] found that *A. glycyphyllos* and *A. cicer* plants contained 17.1 mg GAE/g and 18.9 mg GAE/g total phenolic compounds, 12.8 mg RE/g and 3.42 mg RE/g total flavonoids, respectively. Ghaffari et al. [42] found that *A. creticus* methanol extracts contained 79.82 mg GAE/g total phenolics and 56.11 mg QE/g total flavonoids. All these studies and the results of this study showed that plants belonging to the *Astragalus* genus contain high amounts of phenolic compounds and flavonoids.

3.3. Anticancer Analysis Results

The IC_{50} values of anticancer activity results of *A. compactus* subsp. *compactus* ethanol, water and methanol extracts on the A2780 and MCF-7 cancer cell lines are shown in Table 4 and Figure 1. *A. compactus* methanol extract (1.11 µg/mL) has better anticancer activity for the A2780 cell lines than all the other extracts; *A. compactus* methanol extract (24.71 µg/mL) has a better anticancer activity for the MCF-7 cell lines than all the other extracts. To the best of our knowledge, there is no report about anticancer properties in *A. compactus* subsp. *compactus*.

Although no anticancer studies were found on the *A. compactus* subsp. *compactus* taxon, some species of the *Astragalus* genus were determined to be the subject of many anticancer studies. For example, Ghaffari et al. [42] showed that *A. creticus* methanol extracts had low cytotoxic activity against HeLa (human cervical adenocarcinoma) and 3T3 (mouse fibroblast) cell lines. Yağlıoğlu et al. [43] determined that *A. leucothrix* secondary metabolites exhibited cytotoxic activity against HeLa and C6 (rat glioma) cells, while Mihaylova et al. [44] determined that saponin derivatives obtained from *A. glycyphyllos* extracts had almost perfect cytotoxic activity on bladder cancers (T-24 and CAL-29) and lymphoma cancers (MJ and HUT-78). In another study, Sheik et al. [45] stated that *A. membranaceus* plant has anticancer activity on breast, nasopharynx, larynx, lung, stomach, liver, colon, prostate, ovary, uterus, cervical cancers, leukemia and gynecological malignancies. The results of this study and the other studies mentioned above prove that *Astragalus* species have significant activity in terms of anticancer properties. Agzamova et al. [46] found that *A. lehmannianus* methanol extracts showed weak cytotoxic activity against PC-3 (prostate cancer) and HT-29 (colon cancer) cell lines.

Table 4 The IC_{50} values of endemic *A. compactus* aerial parts extracts against A2780 and MCF-7 cancer cell lines for the anticancer activity assay at 24 hours

Samples (µg/mL)	**A2780**	**MCF-7**
***A. compactus* Water**	1.48	367.50
***A. compactus* Ethanol**	4.57	81.59
***A. compactus* Methanol**	1.11	24.71

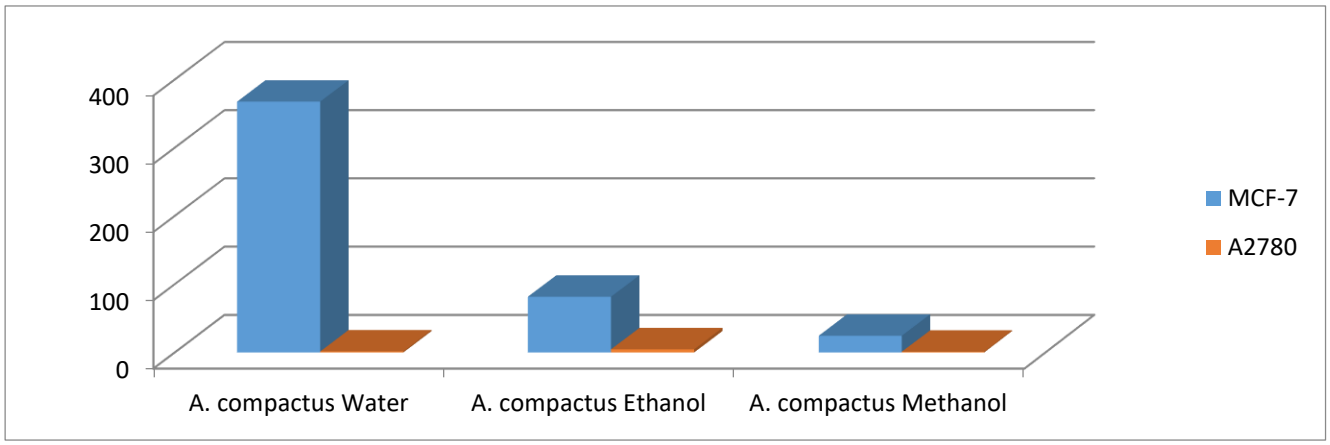

Figure 1 The IC_{50} values of *A. compactus* extracts against MCF-7 and A2780 cancer cell lines

3.4. Genotoxic Analysis Results

The DNA damage analysis results of ethanol, methanol and water extracts of *A. compactus* aerial parts in MCF-7 and A2780 cell lines according to IC_{50} values in effective doses are given in Table 5, Table 6 and Figure 2. As a result of the Comet experiments, it was determined that the plant extracts added to the culture medium were effective on the parameter values of the cells in the mentioned tables and that this situation was statistically significant ($p<0.05$). It was determined by Comet Assay that the decrease in cell viability after 24 hours of incubation was mainly due to the death of the cells due to DNA damage in the A2780 cell lines.

Table 5 DNA damage of *A. compactus* extracts on A2780 cell line at 24 hours

	Control	Solvent	ACW	ACE	ACM
Tail Length (TL)	4007.4±1552.64	3696.29±845.56	8409.09±16708.19*	3946.15±1072.65	3958.62±1552.3
Tail Intensity (TI)	104.25±63.22	80.39±31.38	410.45±1248.06*	107.23±40.43	91.98±58.42
Olive Tail Moment (OTM)	970.77±613.81	647.17±250.93	2957.46±9446.74*	901.34±473.41	965.86±907.44
Head Length (HL)	18992.59±4025.1	19659.25±3180.13	22200±4261.45	18600±3756.16	18413.79±3816.47
Head Intensity (HI)	1298.67±699.1	1451.53±494.27	1933.33±719.21	1361.55±563.04	1161.01±547.61

(Results are presented as Mean ± Standard Deviation. *$p<0.05$)

Table 6 DNA damage of *A. compactus* extracts on MCF-7 cell line at 24 hours

	Control	Solvent	ACW	ACE	ACM
Tail Length (TL)	3560±1284.81	3928.57±1553.21	nd	4866.66±2307.17	4738.46±2397.17
TailIntensity (TI)	406881.63±264873.38	534828.21±258781.27	nd	436061.96±320325.61	497564.53±275939.64
Olive Tail Moment (OTM)	391257.26±271381.57	434532.92±299851.59	nd	483969.96±321379.46	407408.88±292673.09

Head Length (HL)	15080±1996.27	15242.85±1533.19	nd	17362.96±2128.94*	15707.69±2198.34
Head Intensity (HI)	431583.56±205834.31	614303.85±335831.93	nd	267881.25±309435.23*	341653.57±342570.85

(Results are presented as Mean ± Standard Deviation. *$p<0.05$; nd: not detected)

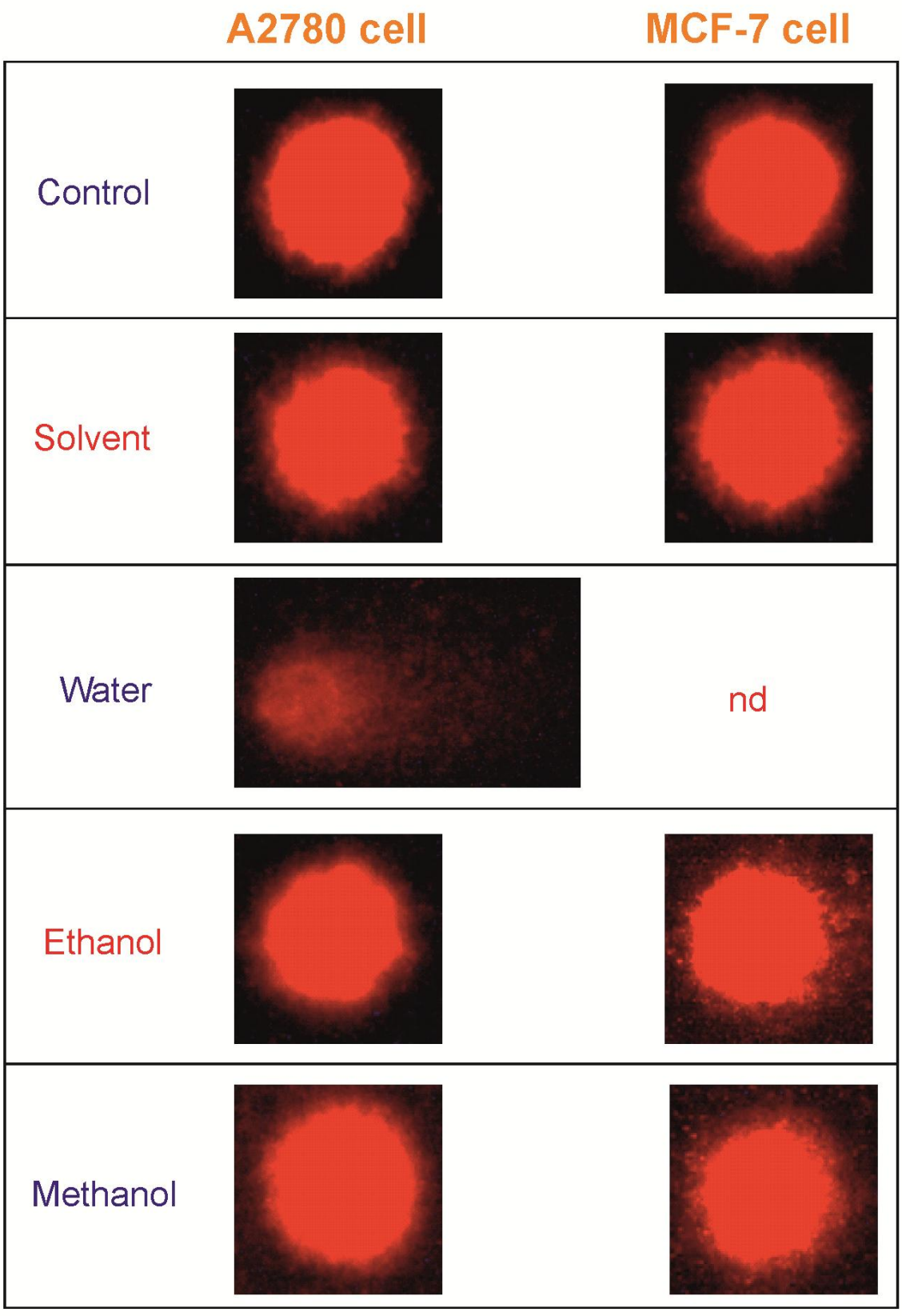

Figure 2 Images obtained from Comet Assay trials for *A. compactus* extracts against MCF-7 and A2780 cancer cell lines

4. CONCLUSIONS

According to the antiradical analysis results, it was determined that *A. compactus* subsp. *compactus* ethanol, methanol and water extracts showed lower activity than the standard antioxidant BHT in ABTS, DPPH and OH radical scavenging tests. According to phytochemical content analysis, it was observed that *A. compactus* subsp. *compactus* methanol extract contained higher amounts of total flavonoids, total phenolics and total proanthocyanidins compared to other extracts. It was found that the plant is rich in α-tocopherol, phytosterols and unsaturated fatty acids. According to the anticancer analysis results, it was understood that *Astragalus compactus* subsp. *compactus* (guni) plant has anticancer activity against human ovarian cancer (A2780) cell lines depending on the increasing dose. In addition, as a result of the genotoxic analyses performed, it was understood by the Comet test that the plant carries out its anticancer activities through DNA damage.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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