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Quantification of phenolic and flavonoid contents and some biological activities of *Ornithogalum sigmoideum* Freyn & Sint

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ABSTRACT: *Ornithogalum* L. is a genus containing approximately 200 species that grow in warm areas of Europe, Asia and Africa. 54 of these species grow naturally in Turkey. They have been used for various medicinal purposes such as emetic and against abscess since ancient times and today consumed as food especially in the eastern region of the Black Sea. *Ornithogalum* species were found to be rich in cholestane glycosides, cholestane bidesmosides, cardenolide glycosides, flavonoid glycosides and saponins. According to the health-promoting effects of these compounds; we aimed to study, antioxidant, anti-inflammatory and cytotoxic effects of aerial parts and bulbs of *Ornithogalum sigmoideum* as well as bioactive compounds. The antioxidant activity was determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Anti-inflammatory activity of extracts was evaluated by the human red blood cell membrane stabilization method. Total phenolic and flavonoid contents were evaluated by Folin–Ciocalteu and aluminum chloride methods, respectively. The cytotoxic effects were evaluated on lung (A549), colorectal (HCT-116) and prostate (PC3) cancer cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Methanolic extract of aerial part of *O. sigmoideum* exhibited the greatest anti-inflammatory and antioxidant activity in all methods determined. Supporting these results, the highest amounts of total phenolic and flavonoid were obtained from this extract. MTT cell viability results showed that aerial part extract of *O. sigmoideum* was induced cell death more than the bulb extract in every-three cell line and the PC3 cells were more sensitive than the others were. However, the calculated IC_{50} values of all the extracts were too high for considering as cytotoxic.

KEYWORDS: Antioxidant ; anti-inflammatory ; cytotoxicity ; free radical ; *Ornithogalum sigmoideum.*

1. INTRODUCTION

Oxidative stress is defined as an imbalance between the antioxidant defence system and the generation of the production of reactive oxygen and nitrogen species which are called free radicals. Oxidative stress can lead and exacerbate inflammation and expression of genes involved in inflammatory signaling pathways. Thus, excessive production of free radicals can trigger various oxidation and inflammation-related diseases such as asthma, arthritis, autoimmune diseases, cancer, cardiovascular diseases, diabetes, metabolic and endocrine diseases, neurological disorders, pulmonary and renal diseases [1]. Antioxidants are endogen and exogen substances, which can neutralize and suppress free radicals and provide protection of organisms against the harmful effects of these unstable structures. Antioxidants may help prevent or delay cell damage caused by oxidation and inflammation. Moreover these molecules have disease-fighting potentials against several chronic and degenerative disorders including autoimmune, cardiovascular, dermatologic, neurodegenerative, ocular and respiratory system diseases and even aging and cancer [2-5]. Polyphenols and flavonoids, which are found in almost all fruits and vegetables, are natural antioxidant and anti-inflammatory compounds [6-8].

Ornithogalum L. is a genus that distributes in temperate regions of Europe, Asia and Africa. It comprises about 200 species and 54 of them naturally grow in Turkey [9-14]. The fresh plants (bulbs, leaves, stems) have

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been widely consumed as vegetables both raw and cooked and sold on the local markets for many years [14-18]. Some *Ornithogalum* species (sp.) have been reported to exhibit a wide range of biological activities such as anticancer, anti-inflammatory, antimicrobial, antioxidant and antitumor [9]. *Ornithogalum* sp. have long been used in the treatment of abscess and as emetic against poisoning since the time of Dioscorides. They are rich in cholestane glycosides, cholestane bidesmosides, cardenolide glycosides, flavonoid glycosides and saponins [14, 19-21]. A number of phenolic compounds have been reported in *Ornithogalum* sp. such as gallic acid, caffeic acid, protocatechuic acid, p-OH- benzoic acid, vanillic acid, ferulic acid and p-coumaric acid [9, 14].

Ornithogalum sigmoideum Freyn&Sint. (*O. sigmoideum)*, whose local name is "Sakarca, Çökülce and Tükürük otu", spreads throughout The Black Sea region [10, 12]. Recent studies have been reported that different solvent extracts of *O. sigmoideum* showed moderate antioxidant activity according *in vitro* assays [14, 22].

This study aimed to evaluate cytotoxicity, antioxidant, anti-inflammatory effects and related total phenolic and flavonoid contents of aerial parts and bulbs of *O. sigmoideum* growing in Turkey. To the best of our knowledge, this is the first report on the anti-inflammatory and cytotoxic activity and total flavonoid content of *O. sigmoideum.*

2. RESULTS AND DISCUSSION

Many studies have been conducted on antioxidant properties and total amount of phenolic and flavonoid substances of *Ornithogalum* sp. [9, 14, 23-26]. It is known that polyphenolic substances and flavonoids have protective effects against various diseases such as cancers, cardiovascular diseases, osteoporosis and degenerative diseases, due to their antioxidant properties [27, 28]. However, there are only few reports on antioxidant activities of *O. sigmoideum.* The reports indicated that extracts possessed moderate antioxidant activities [14, 22].

In the present study; cytotoxicity, antioxidant and anti-inflammatory potentials of aerial parts and bulbs of *O. sigmoideum* were investigated. The half-maximal inhibitory concentrations (IC_{50}) of the extracts were calculated using calibration curves to evaluate these biological activities. Total phenolic and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents, respectively to demonstrate their phytochemical characteristics and activities. Our study gains importance as *O. sigmoideum* is consumed as a traditional food and medicine.

No study has been reported with total phenolic and flavonoid content, antioxidant, anti-inflammatory and cytotoxic activity data of *O. sigmoideum* together. Moreover, this is the first report to demonstrate anti-inflammatory and cytotoxicity activity as well as total flavonoid content of *O. sigmoideum.*

2.1. Antioxidant activity

ABTS and DPPH free radical scavenging assays are widely used to screen antioxidant capacities of plants, which measure the ability of a compound to scavenge ABTS and DPPH radicals respectively [29, 30]. The plant extracts possessed high and dose-dependent antioxidant capacities, in general.

ABTS free radical scavenging activities of extracts were shown in Table 1. Methanolic extracts of aerial parts of *O. sigmoideum* showed the highest ABTS free radical scavenging activity followed by aqueous extracts of aerial parts (IC_{50}=0.0449 and 0.0710 mg/ml, respectively). Aqueous extracts of bulbs of *O. sigmoideum* had the lowest ABTS free radical scavenging capacity with IC_{50} values of 0.2142 mg/ml. Our study is the first report on the ABTS radical scavenging activity of methanolic extracts of bulbs and aerial parts of *O. sigmoideum.* Similar to our results, one report suggested that hexanic, ethanolic and water extracts of *O. sigmoideum* exhibited moderate antioxidant activities through ABTS free radical scavenging and cupric reducing antioxidant capacity assays [22].

Table 2 revealed the total antioxidant activity, measured by DPPH free radical scavenging method. Methanolic extracts of aerial parts of *O. sigmoideum* exhibited the maximum inhibitor activity on DPPH free radical with IC_{50} values of 0.2172 mg/ml; whereas positive standard butylated hydroxytoluene (BHT) showed 0.0161 mg/ml. Similar to the results of ABTS assay, aqueous extracts of bulbs exhibited the lowest DPPH free radical inhibitory activity (IC_{50}=2.1864 mg/ml). Present study is the first report on the DPPH free radical scavenging activity of aqueous extracts of bulbs and aerial parts of *O. sigmoideum*. Another study was reported that methanolic extracts of *O. sigmoideum* aerial parts and bulbs showed antioxidant activities through ferric-reducing antioxidant power, ferrous ion-chelating, phosphomolybdenum-reducing antioxidant power, DPPH and superoxide radical scavenging assays and were rich in phenolic compounds [14].

Table 1. Inhibitory effects of plant extracts on ABTS free radical.

Plant Name	IC_{50} (mg/ml)	
	Aqueous extract	Methanolic extract
Control	-	-
***Ornithogalum sigmoideum* (bulbs)**	0.2142±0.0216*	0.1560±0.0240*
***Ornithogalum sigmoideum* (aerial parts)**	0.0710±0.0008*	0.0449±0.0022*
Trolox	0.0107±0.0001*	

* p<0.05; Statistically significant compared to control. Data were expressed as mean ± SEM.

Table 2. Inhibitory effects of plant extracts on DPPH free radical.

Plant Name	IC_{50} (mg/ml)	
	Aqueous extract	Methanolic extract
Control	-	-
***Ornithogalum sigmoideum* (bulbs)**	2.1864±0.3464*	0.5766±0.0056*
***Ornithogalum sigmoideum* (aerial parts)**	0.2766±0.0162*	0.2172±0.0084*
BHT	0.0161±0.0002*	

* p<0.05; Statistically significant compared to control. Data were expressed as mean ± SEM.

2.2. Anti-inflammatory activity

All the extracts possessed recognizable HRBC membrane stabilization activity as shown in Table 3. The results revealed that the percentage protection of HRBC membrane was found to be increased with concentration (Figure 1).

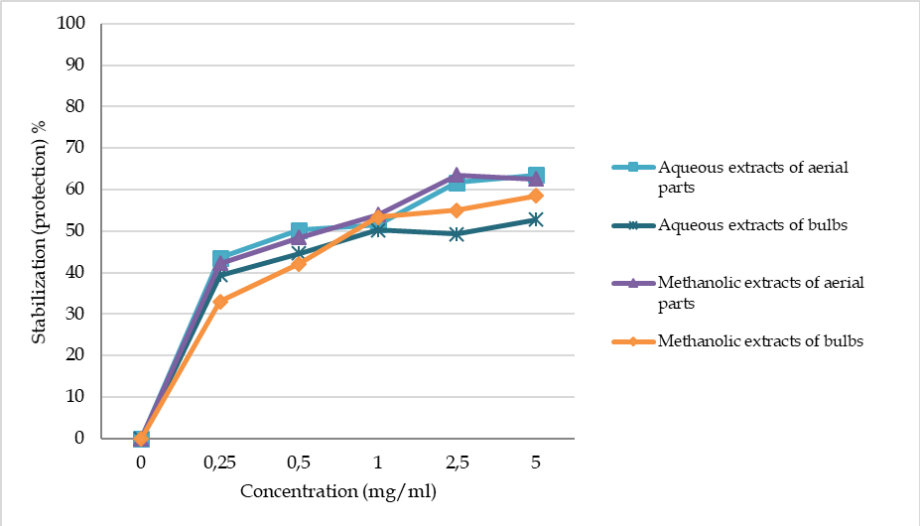

Figure 1. HRBC membrane stabilization (protection) activities of the extracts of *O. sigmoideum*.

In general, aerial parts exhibited higher anti-inflammatory activity than bulbs. Methanolic extracts of aerial parts of *O. sigmoideum* had the most potent protective effect on HRBC membrane with IC_{50} values of 0.7777 mg/ml. Based on the comparison of the IC_{50} values, the order of the HRBC membrane stabilization activity was found to be as follows: methanolic extracts of aerial parts>aqueous extracts of aerial parts>methanolic extracts of bulbs>aqueous extracts of bulbs (0.7777 mg/ml; 0.8575 mg/ml; 2.2333 mg/ml and 3.2726 mg/ml, respectively. The IC_{50} value of acetylsalicylic acid (ASA), which was used as a reference drug, was 0.2979 mg/ml. ASA exhibited higher HRBC membrane stabilizing activity than the most potent

extract. According to the results observed, the extracts can be considered to exhibit intermediate anti-inflammatory activity. Since the extracts are mixed structures, pure compounds can be found and isolate from these extracts which provide higher anti-inflammatory activity than ASA.

Table 3. Protective effects of plant extracts on HRBC membrane.

Plant Name	IC_{50} (mg/ml)	
	Aqueous extract	Methanolic extract
Control	-	-
***Ornithogalum sigmoideum* (bulbs)**	3.2726±0.2250*	2.2333±0.1036*
***Ornithogalum sigmoideum* (aerial parts)**	0.8575±0.0670*	0.7777±0.1233*
ASA	0.2979±0.0025*	

* $p<0.05$; Statistically significant compared to control. Data were expressed as mean ± SEM.

2.3. Total phenolic and flavonoid content

Phenols and flavonoids were maximally present in aerial parts and methanolic extracts. The total phenolic contents of the examined plant extracts were calculated from a calibration curve using the Folin-Ciocalteu's method, and the results were expressed as mg gallic acid equivalent (GAE) per g extract (r^2 = 0.9908) (Table 4). Methanolic extract of aerial parts and bulbs of *O. sigmoideum* had the greatest phenolic contents (388.2105 and 378.7368 mg GAE/g crude extract) respectively, while the lowest content was recorded for aqueous extracts of bulbs (243.1228 mg GAE/g crude extract).

Table 4. Total phenolic content of plant extracts

Plant Name	mg GAE/g extract	
	Aqueous extract	Methanolic extract
***Ornithogalum sigmoideum* (bulbs)**	243.1228±2.4749	378.7368±2.4119
***Ornithogalum sigmoideum* (aerial parts)**	344.5263±3.8795	388.2105±1.0526

Data were expressed as mean ± SEM.

The total flavonoid contents in the examined plant extracts using aluminium chloride colourimetric method were expressed in terms of quercetin equivalent (QE) (r^2= 0.9902) and the results were expressed in Table 5. Among all, the highest amount of flavonoids was found in methanolic extracts of aerial parts of *O. sigmoideum* (107.0417 mg QE/g crude extract) followed by aqueous extracts of aerial parts (79.7500 mg QE/g crude extract). The lowest amount of flavonoids was found in aqueous extract of *O. sigmoideum* bulbs (57.6667 mg QE/g crude extract).

Table 5. Total flavonoid content of plant extracts

Plant Name	mg QE/g extract	
	Aqueous extract	Methanolic extract
***Ornithogalum sigmoideum* (bulbs)**	57.6667±1.5404	68.9167±2.3199
***Ornithogalum sigmoideum* (aerial parts)**	79.7500±2.7847	107.0417±0.6250

Data were expressed as mean ± SEM.

2.4. MTT cell viability assay

The cytotoxic effects of methanolic extracts of bulbs and aerial parts of *O. sigmoideum* were investigated using MTT cell viability assay. The results were presented in Figure 2 and 3. Both bulb and aerial part extracts induced cell death in A549, HCT-116 and PC3 cells concentration-dependent manner. The cell viabilities were ranged from 88±5.2% to 24±5.8%, 98±5.0 to 40±4.5% and 74±9.0% to 6±1.4% in A549, HCT-116 and PC3 cells,

respectively at 100-1000 µg/ml (Figure 2). In the aerial part extract-treated A549, HCT-116 and PC3 cells, for 25-1000 µg/ml concentrations, the cells viabilities were calculated as 72±5.7% to 4±0.6%, 96±3.0% to 4±0.4% and 76±9.0% to 7±0.3%, respectively (Figure 3). According to the results, IC_{50} concentrations of bulb extracts (586.80±33.3; 846.71±45.8; 274.62±33.6) were higher than aerial part extracts (184.62±12.7; 187.79±17.3; 81.24±9.0) in A549, HCT-116, PC3 cells, respectively. According to the obtained results, the extracts cannot be considered as cytotoxic based on the criteria of the American National Cancer Institute (NCI). These values were farther than the criteria of a crude extract to evaluate as cytotoxic (IC_{50}<30 µg/ml), which was established by the NCI [31].

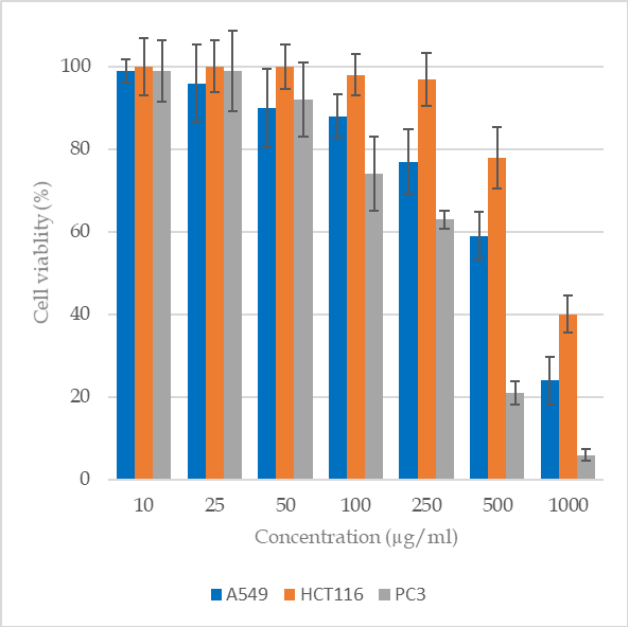

Figure 2. MTT results showed the cytotoxic effect of methanolic extracts of bulbs of *O. sigmoideum* in A549, HCT-116 cells and PC3 cells. The cells were treated with different concentrations (10-1000 µg/ml) of the extracts for 24 h. Results are given as median of cell viability (%). The error bar represents ± SEM.

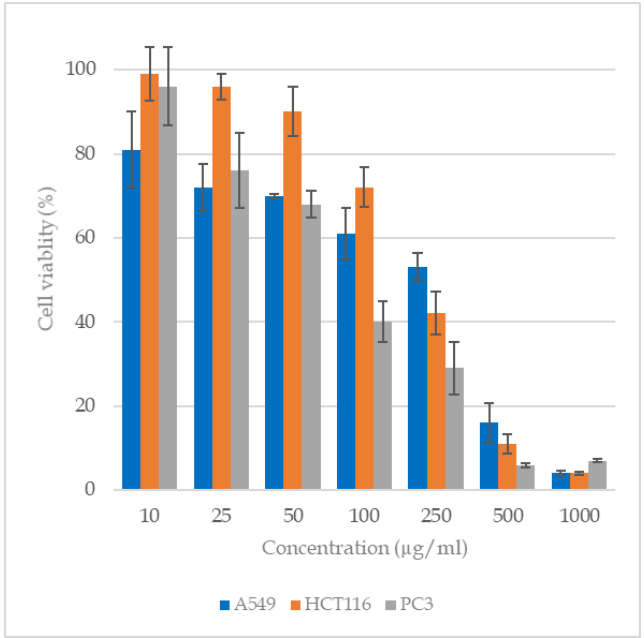

Figure 3. MTT results showed the cytotoxic effect of methanolic extracts of aerial parts of *O. sigmoideum* in A549, HCT-116 cells and PC3 cells. The cells were treated with different concentrations (10-1000 µg/ml) of the extracts for 24 h. Results are given as median of cell viability (%). The error bar represents ± SEM.

3. CONCLUSION

The current study reveals useful information about antioxidant, anti-inflammatory and cytotoxic properties and phenolic and flavonoid contents of different parts of *O. sigmoideum*. It was found that all the extracts possessed concentration-dependent inhibition of ABTS and DPPH free radicals. HRBC membrane stabilization activity of these extracts were increased in a concentration dependent manner as well. In general, aerial parts and methanolic extracts exhibited higher biological activities and also these are rich in phenolic and flavonoid compounds. These potentials of the extracts may be due to the presence of various phenolic and flavonoid compounds. The results also suggest that the methanolic extracts of aerial parts of *O. sigmoideum* was the best antioxidant and anti-inflammatory extract and the richest regarding to phenolic and flavonoids among all. The concentrations of cell death observed were too high to indicate the extracts as cytotoxic. As a consequence, *O. sigmoideum* might be consumed safely as food and a promising source of potential antioxidant and anti-inflammatory compounds.

4. MATERIALS AND METHODS

4.1. Chemicals

ASA, aluminium chloride, BHT, Folin&Ciocalteu's phenol reagent, gallic acid, methanol, potassium persulfate, sodium carbonate, sodium chloride, sodium nitrite, trolox, quercetin, ABTS, DPPH, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). Dulbecco's modified eagle medium (DMEM) in high glucose (Wisent, Canada), MTT (Serva, Germany), trypsin/EDTA (Multicell, USA), streptomycin/penicillin (Wisent, Canada) were purchased from commercial sources.

4.2. Plant collection and identification

O. sigmoideum, which is known as 'sakarca' by local people, was obtained from a Perşembe district bazaar in the city of Ordu-Turkey in May 2018. Each sample along with the aerial part and bulbs of the plant was authenticated by Dr. Derya Çiçek Polat from Ankara University.

4.3. Preparation of extracts

After samples (aerial parts and bulbs) were dried separately, they were powdered. The powdered samples were separately extracted with a magnetic stirrer methanol for methanolic extracts and distilled water for aqueous extracts (50g sample, 300ml×3). After being filtered, methanol extract was concentrated with a rotary evaporator and aqua extract was frozen at -20°C, then lyophilized. The methanol extracts yield of aerial parts and bulbs was calculated as 11.29% and 10.52% (w/w) respectively. The aqueous extracts yield of aerial parts and bulbs was calculated as 15.12% and 13.81% (w/w) respectively.

4.4. Antioxidant activity

4.4.1. DPPH free radical scavenging activity

The DPPH free radical scavenging activities of the extracts were determined by their ability to bleach the stable radical DPPH with slight modifications of the method of Blois [32]. The reaction mixture contained different concentrations of the extracts and DPPH in methanol. Absorbance was measured after 30 minutes incubation at 517 nm using a microplate reader. All the experiments were done in triplicate. BHT was used as a reference compound. The DPPH scavenging activity was calculated as the percentage of the radical reduction using the formula below.

$$\text{Inhibition (\%)} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control})] \times 100 \quad \text{[Eq. 1]}$$

IC_{50} values were determined from a calibration curve for each extract and the results were expressed as mean± SEM.

4.4.2. ABTS free radical scavenging activity

The method of Re et al. [33] was used to determine ABTS free radical cation decolourization activity of the extracts with minor modifications. ABTS was produced by reacting 7 mM ABTS aqueous solution with 2.45 mM potassium persulfate. The reaction mixture was allowed to stand in the dark at room temperature for 12-16 hours. Intensely-coloured ABTS radical cation was diluted with ethanol to give an absorbance value

of 0.700±0.020 at 734 nm (pH=7.4). The absorbance of the reaction mixture, containing different concentrations of plant extracts and ABTS solution, was measured 6 minutes after addition to the wells at 734 nm using a microplate reader and the percentage of inhibition was calculated. All the experiments were done in triplicate. Trolox, the water-soluble α-tocopherol analogue, was used as a reference compound. . The ABTS scavenging activity was calculated as the percentage of the radical reduction using the formula below.

Inhibition (%) = [(Absorbance of control- Absorbance of sample)/ Absorbance of control)] x 100 [Eq. 2]

IC_{50} values were determined from a calibration curve for each extract and the results were expressed as mean ± SEM.

4.5. Anti-inflammatory activity

HRBC membrane stabilizing activity of the extracts was determined as an indicator of anti-inflammatory activity [34, 35]. Fresh whole human blood was taken from healthy human volunteers who had not taken any steroidal, nonsteroidal anti-inflammatory or anticoagulant drugs for 4 weeks before the experiment. The tubes were centrifuged at 3000 rpm for 10 minutes, then the packed cells were washed with isosaline (0.85 %, pH 7.2) at least three times. 10% v/v suspension with isosaline was prepared. The reaction mixture consisted of test sample and 10% red blood cell suspension. All the centrifuge tubes containing reaction mixture were incubated at 56°C for 30 minutes. After the incubation, the tubes were cooled, and the reaction mixture was centrifuged at 2500 rpm for 5 minutes. The absorbance was measured at 560 nm using a microplate reader. All the experiments were done in triplicate. ASA was used as a reference drug. The percentage of hemolysis and protection were calculated according to the formula:

Hemolysis % = (Absorbance of sample/ Absorbance of control) ×100 [Eq. 3]

Protection (Stabilization) % = 100 – [(Absorbance of sample/ Absorbance of control) ×100] [Eq. 4]

IC_{50} values were determined from a calibration curve for each extract and the results were expressed as mean ± SEM.

4.6. Phytochemical screening

4.6.1. Determination of total phenolic content

The amount of total phenolic content in the extracts was determined with the Folin-Ciocalteu method with minor modifications [36]. Total phenolic content was determined from a standard curve of different concentrations of gallic acid. Total phenolic contents were expressed as mg GAE per g crude extract.

4.6.2. Determination of total flavonoid content

The amount of total flavonoid content in the extracts was determined by the aluminium chloride colourimetric method with minor modifications [37]. Total flavonoid content was determined from a standard curve of different concentrations of quercetin. Total flavonoid content was expressed as mg QE per g crude extract.

4.7. Cytotoxicity

4.7.1. Cell culture

Human colorectal (HCT-116) (ATCC®CCL-247TM), lung (A549) (ATCC®CCL-185TM) and prostate (PC3) (ATCC®CCL-1435TM) cancer cell lines were grown in high glucose DMEM supplemented with 10% fetal bovine serum, 1% penicillin (100 U/ml) and streptomycin (100 µg/ml), and maintained at 37°C in 5% CO_2 incubator. The passage process was performed twice a week and culture media was changed after every 2 days. The cells were treated with Trypsin-EDTA (0.25%) and sub-cultured when reached to 90% confluence into 6/96 well plates or 25 cm^2 flasks according to the assay.

4.7.2. MTT cell viability assay

Cells were seeded in 96-well plates quantities of $1x10^4$/well for 24 h. The stock solutions of 1000 mg/ml extracts were dissolved in methanol, after removing the culture medium, cells were treated with exposure medium containing the extracts (final concentrations: 10, 25, 50, 100, 250, 500, and 1000 µg/ml). DMSO 10% and methanol 0.4% was used as positive and solvent controls, respectively. Plates were incubated in 37 °C, humidified incubator with 5% CO_2 for 24 h. Afterwards, the medium was changed with 100 µl of serum-free medium containing 0.5 mg/ml MTT to each well and incubated at 37°C for 4 h. Thereafter, the MTT containing medium was gently removed. For dissolving formazan crystals 150 µl DMSO was added to each well and then shaken for 10 min. The absorbance was measured at 570 and 690 nm using a microplate reader [38]. The percentage of cell viability was calculated according to the formula:

Cell viability %= ((Absorbance of control-Absorbance of treat)/Absorbance of control) ×100 [Eq. 5]

4.8. Statistical analysis

IBM SPSS Statistics for Windows, Version 25.0. (Armonk, NY: IBM Corp.) was used for statistical analysis of results. Results were expressed as mean value ± standard error of the mean (SEM). All the assays were conducted at least in triplicates. The linear regression analysis was performed to find out the correlation coefficient. Statistical significance was evaluated employing one-way analysis of variance (ANOVA) and post hoc LSD test. A p-value of less than 0.05 was considered as statistically significant.

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