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Full Length Research Paper

Investigation of antioxidant, cytotoxic and apoptotic activities of the extracts from tubers of *Asphodelus aestivus* Brot.

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In this study, potential antioxidant activities of crude extracts from *Asphodelus aestivus* Brot. (AA) tubers were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, and cytotoxic and apoptotic activity of the extracts on MCF-7 breast cancer cells were evaluated by trypan blue exclusion assay, comet assay and hoechst 33258/propidium iodide double staining. Crude diethyl ether and ethyl acetate extracts from AA have significant DPPH scavenging activity, except methanol and water (infusion and decoction) extracts. All of the extracts have cytotoxic activity on MCF-7 cells, time- and concentration-dependently. Although all of the extracts induced significantly deoxyribonucleic acid (DNA) damage, diethyl ether and ethyl acetate extracts induced apoptosis in the middle level and the others induced apoptosis in significant level. Results of this study suggest a dose-response relationship for all extract samples. The results demonstrates that antioxidant, cytotoxic and apoptotic properties of *A. aestivus* Brot. extracts show differentiation to extract type. Crude extracts were used in this study and we do not know which chemical(s) in the extracts exhibited cytotoxic, DNA damaging and apoptotic effects. Therefore, a further chemical identification of the crude extracts and further *in vitro* and *in vivo* studies about cytotoxic and apoptotic activities are required.

Key words: *Asphodelus aestivus* Brot., antioxidant activity, cytotoxic activity, apoptosis, comet assay, MCF-7 cells.

INTRODUCTION

Over the years, humans have relied on nature for their basic needs for the production of foodstuffs, shelters, clothing, means of transportation, fertilizers, flavors and fragrances and not least, medicines (Cragg and Newman, 2003). Medicinal plants continue to play an essential role in health care; it has been estimated by the World Health Organization (WHO) that approximately 80% of the world population rely mainly on traditional medicine for their primary health care (Farnsworth et al., 1985).

Although medicinal plants have been used for a long time, their chemical contents and pharmacological effects are not well understood in most cases. The most commonly found active constituents in medicinal plants include terpenes (sesquiterpenes, diterpenes, triterpenes), alkaloids, coumarins, lignans, quinines, flavonoids, tannins, stilbenes, curcuminoids, anthroquinons, polysaccharides etc. Some of them are alleged to have antioxidant activity (Larson, 1988; Ho et al., 1994; Ng et al., 2000; Cai et al., 2004; Asparganah and Ramezanloo, 2012). Antioxidant activity is a fundamental property important for human life. Many of the biological functions, including antimutagenicity, anticarcinogenicity, antiaging, among others, may originate from this property

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(Mclarity, 1997; Niki, 1997; Yang et al., 2001; Nasri et al., 2012; Alam et al., 2012).

In recent years, there has been a growing interest to isolate new compounds from plants which are effective for cancer treatment because cancer is an important disease and the second leading cause of death worldwide. Although over 60% of currently used anticancer agents are derived in one way or the other from natural sources (Cragg et al., 1997); numerous molecules in many medicinal plants still remain to be isolated or studied in detail (Mukherjee et al., 2001; Tsai, 2001; Lee, 1999). Many naturally occurring agents show chemopreventive and chemotherapeutic (anticancer) potential in a variety of bioassay systems and animal models (Galati and O'Brien, 2004).

Asphodelus aestivus Brot. (AA) common spring-flowering geophytes encountered on the Marmara, Aegean and Mediterranean coasts of Turkey, has been utilized traditionally for culinary and medicinal purposes. The leaves of AA are commonly consumed and cooked as a vegetable dish in Turkey. In traditional medicine, the tuber and roots of this plant are used against hemorrhoids, nephritis, burns and wounds (Baytop, 1999; Tuzlacı and Eryaşar-Aymaz, 2001). Phytochemical investigations suggested that *Asphodelus* species contain anthranoids, flavonoids and triterpenes (El-Fattah et al., 1997; Adinolfi et al., 1991; Van Wyk et al., 1995) and these chemicals have various pharmacological effects. For instance, *Asphodelus ramosus* has low anti-human immunodeficiency virus (HIV) effect (Bedoya et al., 2001) and *Asphodelus microcarpus* has low cytotoxic effect on rat pheochromocytoma (PC12) and human hepatoblastoma (HepG2) cells (Ljubuncic et al., 2005).

Although with the presence of information about phytochemical contents, anti-HIV effect and cytotoxic effect of various *Asphodelus* species, no data have been reported previously on the antioxidant, cytotoxic and apoptotic activities of *A. aestivus* Brot. Therefore, our study aimed at evaluation of potential antioxidant activity and cytotoxic and apoptotic activities of ethyl acetate, methanol and water (infusion and decoction) extracts from *A. aestivus* Brot. on MCF-7 breast cancer cells.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picryl-hydrazyl radical (DPPH), rutin, dimethylsulfoxide (DMSO), Dulbecco's modified eagle's medium (DMEM) , fetal bovine serum (FBS), trypan blue, triton X-100, low melting point agarose, normal melting agarose and ethidium bromide were all purchased from Sigma Aldrich Co. (St. Louis, USA).

Plant and extractions

A. aestivus Brot. were collected in March, 2006 from the West region of Adnan Menderes University Central Campus, Aydın, Turkey, in the Eastern Mediterranean region. The botanical identification was made by Dr. Ozkan Eren. A voucher specimen was deposited in the herbarium (AYDN 878) of the Faculty of Arts and Science, University of Adnan Menderes. Dried, ground tubers of *A. aestivus* (AA) (95 g) were extracted with a solvent series of increasing polarity (diethyl ether, petroleum ether, ethyl acetate, methanol and water). About 950 ml of solvent were added to 95 g plant material. After finishing the first soxhlet extraction (at 40°C for approximately 12 h, until the solvent became colorless) with diethyl ether and filtration, the plant material was dried and subjected sequentially to the second extraction with petroleum ether, the third extraction with ethyl acetate, and fourth extraction with methanol (Goffin et al., 2003; Lee et al., 2004; Miliauskas et al., 2004; Avcı et al., 2006). The extracts were evaporated and yielded 0.328, 0.796 and 15.178 g dried mass for diethyl ether, ethyl acetate and methanol extractions, respectively. Extracts from petroleum ether extraction was not possible.

After the methanol extraction, plant material was dried and subjected to the water (infusion) extraction. For water (infusion) extraction, 950 ml distilled water at 80°C was added to plant material for 10 min and extract was filtered. For second water extraction (decoction), 950 ml distilled water was added to 95 g dried plant material and boiled for 10 min and extract was filtered (Ljubuncic et al., 2005). Filtered extracts were lyophilized and yielded 2.069 and 2.491 g dried mass, respectively. Extracts were sealed in glass bottles and stored at -20°C until use.

Antioxidant activity

DPPH radical scavenging activity of the AA extracts

1,1-diphenyl-2-picryl-hydrazyl (DPPH) molecule that contains a stable free radical has been widely used to evaluate the radical scavenging ability of antioxidants. When DPPH reacts with an antioxidant compound that can donate hydrogen, it is reduced (Zhao et al., 2006). The free radical scavenging activity of diethyl ether, ethyl acetate, methanol and water (infusion and decoction) extracts from AA was tested by their ability to bleach the stable DPPH radical (Brand-Williams et al.,1995). One milliliter of 0.1 mM DPPH methanol solution was added to 3 ml of various concentrations (10, 25, 50, 75,100, 150, 200 and 300 µg/ml) of extracts in methanol. The mixture was shaken vigorously and left at room temperature. After 30 min, the absorbance of mixture was measured at λ = 517 nm. Tests were carried out in triplicate. Rutin (50 and 100 µg/ml), a citrus flavonoid glycoside, was used as a standard. Shimadzu PharmaSpec UV-1700 UV-Visible spectrophotometer was used. DPPH radical scavenging activity of extracts was calculated using the following equation:

Scavenging capacity (%) = 100 - [(Absorbance (Ab) of sample - Ab of blank) × 100 / Ab of control]

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

Cytotoxicity assay

Cell culture and treatments

MCF-7 breast cancer cell line was used in this study. Cells were grown and maintained in humidified incubator at 37°C and in a 5% CO_2 atmosphere. DMEM supplemented with 10% FBS, 1 mM glutamine, 1% non essential amino-acids, 100 units/ml of penicillin and 100 mg/ml of streptomycin was used as the culture medium for MCF-7 cell culture. Prior to the assay, concentrated stock solutions of extracts were prepared in DMSO and stock solutions were rediluted to the required concentrations using DMEM media. The maximum percentage of DMSO present in the wells was 0.1% (v/v),

a concentration that did not affect growth of cells. This datum was incorporated as a control element in all experiments. Cells were seeded at density of 5 × 10^4 cells/well into a sterile 24-well plate and allowed to adhere overnight. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with different concentrations (10, 25, 50, 75,100, 150, 200 and 300 µg/ml) of ethyl acetate, methanol and water (infusion and decoction) extracts from *A. aestivus* and incubated for 24 and 72 h under the same conditions. In order to avoid the effect of DMSO on cell proliferation and apoptosis, solvent controls were treated with similar concentrations of DMSO as used for sample preparation (in general, 0.1% DMSO). A control of growth medium was also run in parallel for each time period in cell line.

Cell viability assay

For the determination of cytotoxic activity of extracts on MCF-7 cells, viable cell numbers were determined using the trypan blue staining after the treatment (Son et al., 2003; Lee et al., 2005). In brief, a 0.4% solution of trypan blue was mixed to 50 µl of each cellular suspension during 5 min, spread onto a microscope slide and covered with a coverslip. Non-viable cells appear blue-stained. At least 300 cells were counted per concentration. Standard curves were prepared and 50% cytotoxic concentrations (CC_{50}), concentrations of extracts that caused 50% decrease in cell viability, were derived.

Determination of DNA damaging effects of the AA extracts on MCF-7 cells by alkaline comet assay

The comet assay is a very sensitive method for measuring DNA strand breaks, which has the additional unusual feature that analysis is performed on individual cells. The assay has been of value in fundamental investigations of cellular responses to DNA damage and in *in vitro* and animal studies of genotoxicity (Collins et al., 1997). Currently, comet assay is often used since it is fast, convenient and of easy application (Singh et al., 1988; Hartmann et al., 2003). DNA damaging effect of the AA extracts was determined by alkaline comet assay (Singh et al., 1988). For the comet assay, 100, 150, 200 and 300 µg/ml extract concentrations which have high cytotoxic effect, were used. Cells were seeded at density of 5 × 10^4 cells/well into a sterile 24-well plate and allowed to adhere overnight. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with 100, 150, 20 and 300 µg/ml concentrations of AA diethyl ether, ethyl acetate, methanol and water (infusion and decoction) extracts, and incubated for 72 h under the same conditions. Then, cells were trypsinised and centrifuged.

After centrifugation, cells were suspended in 150 µl of molten 0.5% low melting point agarose (LMPA) in PBS without calcium and magnesium. Then, 150 µl aliquots of the cell suspension were rapidly spread on three slides pre-coated with 85 µl of 1% normal melting agarose (NMA) and cover-slipped (24 × 50 mm). After the agarose was allowed to solidify for 5 min at 0°C, the cover slips were gently removed and a third layer of 75 µl LMPA was added. The slides were then placed in a tank filled with lysis solution (2.5 M NaCl, 0.1 M EDTA, pH 10, 10% DMSO and 1% Triton X-100 both freshly added) at 4°C. After 1h, the slides were removed from the lysis solution and incubated in fresh electrophoresis buffer (0.3 M NaOH and 1 M EDTA, pH > 13) for 20 min at room temperature for unwinding of DNA. Electrophoresis was then carried out at room temperature in the same electrophoresis buffer for 30 min at 0.7 V/cm and 300 mA. After electrophoresis, the slides were gently washed twice for 5 min in fresh neutralization buffer (0.4 M Tris-HCL, pH 7.59), followed by dehydration in absolute methanol.

The slides were stained with 75 µl 1× ethidium bromide and cover slipped. For each treatment concentration, 100 randomly selected cells from each of three slides (300 cells per concentration) were evaluated for DNA damage visually using a ×40 objective on a fluorescent microscope (Olympus BX 51). The DNA damage was quantified by visual classification of cells into five categories of "comets" corresponding to the tail length (Anderson et al., 1994): (i) undamaged: Type 0; (ii) low-level damage: Type I; (iii) medium-level damage: Type II; (iv) high-level damage: Type III and; (v) complete damage: Type IV. The extent of DNA damage was expressed as the mean percentage of cells with low, medium, high and complete damaged DNA, which was calculated as the sum of cells with damage from Types I, II, II and IV. From the arbitrary values assigned to the different categories (from Type 0 = 0 and Type 4 = 4), a genetic damage index (GDI) was calculated for each concentration level (Pitarque et al., 1999).

Hoechst 33258 and propidium iodide (HOPI) double staining for apoptosis determination

Apoptotic effect of the extracts (at 100, 150, 200 and 300 µg/ml concentrations) was determined by Hoechst 33258 and propidium iodide staining, which allows to distinguish between apoptosis, and necrosis was performed according to the method described by Grusch et al. (2002). MCF-7 cells (5 × 10^4 per ml) were seeded in 24-well plate and treated with 100, 150, 200 and 300 µg/ml concentrations of the extracts for 72 h. Hoechst 33258 (HO) and propidium iodide (PI) were added directly to the culture medium to final concentrations of 5 and 2 mg/ml, respectively. After an incubation period of 1 h at 37°C, the cells were examined under an Olympus BX 51 fluorescence microscope equipped with appropriate filters for Hoechst 33258 and PI. This method allows for a distinction between early apoptosis, late apoptosis and necrosis. Cells were counted under the microscope and the number of apoptotic cells was given as a percentage.

Statistical analysis

All experiments were performed in triplicate and analyzed by One Way analysis of variance (ANOVA) (SPSS 11.5 program). Statistically significant difference was considered at the level of $p < 0.05$.

RESULTS

DPPH radical scavenging activity of the AA extracts

DPPH radical scavenging activities of the extracts were presented in Table 1 as scavenging activity (%) and IC_{50} values. There were eight concentrations (10, 25, 50, 75, 100, 150, 200 and 300 µg/ml) in each of the extracts. Results of DPPH assay suggested that scavenging activity percentage of the AA extracts showed variation at different concentrations. Although diethyl ether and ethyl acetate extracts have significant scavenging activity, methanol and water (infusion and decoction) extracts have very low scavenging activity on DPPH radical. Therefore, IC_{50} values of methanol and water extracts could not be determined. Furthermore, diethyl ether extract was found to be more effective (IC_{50} value: 22.46 ± 0.01 µg/ml) than ethyl acetate extract with regard to DPPH radical scavenging ability (IC50 value is 188.90±0.03 µg/ml) (Table 1). However, IC50 values of

Table 1. DPPH radical scavenging activity of the *A. aestivus* Brot. tuber extracts.

Samples	Concentrations (µg/ml)	DPPH scavenging activity (%) ±SD	IC_{50} values (µg/ml) ± SD
Rutin	50	89.38±0.001	7.77±0.03
Diethyl ether	10	17.74±0.003	22.46±0.01
	25	55.81±*0.001	
	50	62.26±*0.006	
	75	29.35±0.001	
	100	32.26±0.000	
	150	28.72±0.001	
	200	31.23±0.007	
	300	43.55±0.010	
Ethyl acetate	10	13.87±0.006	188.90±0.03
	25	25.81±0.001	
	50	34.84±0.007	
	75	37.74±0.007	
	100	39.68±0.005	
	150	39.78±0.009	
	200	53.23±0.002*	
	300	54.32±0.003*	
Methanol	10	10.65±0.008	-
	25	9.35±0.000	
	50	10.97±0.002	
	75	11.61±0.001	
	100	17.10±0.008	
	150	14.11±0.002	
	200	22.26±0.001	
	300	25.94±0.013	
Infusion (Water)	10	8.50±0.003	-
	25	9.29±0.002	
	50	7.31±0.002	
	75	8.69±0.007	
	100	8.30±0.009	
	150	8.49±0.004	
	200	12.85±0.006	
	300	13.87±0.011	
Decoction (Water)	10	3.95±0.006	-
	25	5.53±0.008	
	50	5.34±0.009	
	75	7.71±0.009	
	100	8.10±0.008	
	150	9.52±0.008	
	200	11.07±0.006	
	300	13.28±0.009	

*$p < 0.05$.

diethyl ether and ethyl acetate extracts are significantly higher than that of rutin (7.77±0.03 µg/ml), therefore radical scavenging activities of these extracts are lower than that of rutin.

Cytotoxic activity of the AA extracts on MCF-7 cells

Table 2 present the cytotoxic activity of AA extracts tested on MCF-7 cells as cytotoxic activity (%) and CC_{50} values. Even so, methanol and water (infusion and decoction) extracts exhibited high cytotoxic activity on MCF-7 cells, time and concentration dependently ($p < 0.05$); diethyl ether and ethyl acetate extracts have no significant influence on viability of MCF-7 cells in the ranges of applied concentrations and treatment times, so that diethyl ether and ethyl acetate extracts exhibited no cytotoxic activity at 24 h. Therefore, CC_{50} values of the diethyl ether and ethyl acetate extracts at 24 h could not be determined. Cytotoxic activity of AA extracts on MCF-7 cells after 72 h increased in the order of decoction (water) > methanol > infusion (water) > diethyl ether > ethyl acetate. Of the five extracts tested, decoction extract exhibited the greatest potency against MCF-7 cells by eliciting CC_{50} at concentrations of 48.63 µg/ml at 72 h exposure time. Higher concentrations of infusion (water), methanol, diethyl ether and ethyl acetate extracts were required to elicit the same degree of cytotoxicity.

DNA damaging effects of the AA extracts in MCF-7 cells

The percentage of damaged nuclei, genetic damage index and arbitrary units as measured in the alkaline comet assay were presented in Table 3. As presented in the table, all of the AA extracts (except for 100 µg/ml ethyl acetate extract) were found to induce significant DNA damage after 72 h of treatment compared to control groups ($p < 0.05$) (Figure 1). Although ethyl acetate extract exhibited low damaging effect at 100 µg/ml concentration on DNA of MCF-7 cells, higher concentrations of this extract were found to be significantly effective. Among the extracts, only DNA damaging effect of diethyl ether extract in MCF-7 cells was found to be < 50%, but this extract induced also significantly DNA damage in comparison with control groups ($p < 0.05$). DNA damage percentage in MCF-7 cells increased after extract treatments concentration-dependently.

Genetic damage index and arbitrary unit values also increased in extract treatment groups dose-dependently compared to control groups and reached statistically significance, mainly by the increased percentage of Type II, III and IV damages in the extract treatment groups ($p < 0.05$). DNA damaging effect of AA extracts on MCF-7 cells after 72 h increased in the order of infusion (water) > decoction (water) > ethyl acetate > methanol > diethyl ether.

Apoptotic effects of the AA extracts on MCF-7 cells

The percentage of apoptotic cells detected by the Hoechst 33258/propidium iodide double staining method after treatment with AA extracts (100, 150, 200 and 300 µg/ml) for 72 h was evaluated, and the results are shown in Table 4 as percentage of apoptotic cells and percentage of necrotic cells. As indicated in the table, diethyl ether and ethyl acetate extracts induced apoptosis slightly (9.00 to 22.66% and 8.33 to 23.00%, respectively) in MCF-7 cells at all concentrations after 72 h, while the other extracts induced significantly high apoptosis after 72 h, concentration-dependently. Although diethyl ether and ethyl acetate extracts induced apoptosis in the middle level, these values were found to be statistically significant. Apoptotic effect of AA extracts on MCF-7 cells after 72 h increased in the order of decoction (water) > infusion (water) > methanol > diethyl ether > ethyl acetate. The extracts induced also necrosis in MCF-7 cells, and percentages of necrotic cells reached statistically significant level after some extract treatments (Table 4).

Extract treatments caused morphological changes in the nuclear chromatin of the MCF-7 cells (Figure 2). Although the cells in untreated group were stained with a less bright blue color, which was homogenous by HOPI staining; the apoptotic cells in extract treatment groups were stained much brighter than control cells and showed typical apoptotic features, such as cell shrinkage, nuclear fragmentation marginalization, chromatin condensation and apoptotic bodies. Necrotic cells appeared in violet-red fluorescence without chromatin condensation and apoptotic bodies. Especially apoptotic bodies, a specific and distinct feature of apoptotic cells were found at significant levels in the extract treated cells. These results suggested that AA extracts have apoptotic effect at various levels, depending on extract type on MCF-7 cells.

DISCUSSION

Over the ages, humans use plants for various basic needs such as foodstuffs, cloth, drug etc. Currently, medicinal plants are used intensively by people for treatment of various illnesses in many countries. Natural products have long been an important source of treatments of cancer. Recently, a growing interest is present to investigate the mechanism responsible for the anticancer effects of medicinal plants and plant-based drugs (Kültür, 2007; Leong et al., 2011). Many chemopreventive agents have been associated with antiproliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage or transformation into cancer cells (Khan et al., 2007).

Free radical scavenging is a generally accepted mechanism for antioxidants (Barbaste et al., 2002), and the preferred method for evaluation of the free radicals

Table 2. Cytotoxic activity of the *A. aestivus* Brot. tuber extracts on MCF-7 cells.

Sample	Concentrations (µg/ml)	Cytotoxic activity (%) ± SD at 24 h	CC_{50} value (µg/ml) ±SD at 24 h	Cytotoxic activity (%) ± SD at 72 h	CC_{50} value (µg/ml) ± SD at 72 h
Control	-	0.66±0.580	-	1.33±0.577	-
Solvent control (DMSO)	0.1%	1.00±1.000	-	1.66±0.580	-
Diethyl ether	10	12.33±0.577	> 300	21.67±0.600*	188.83±1.00
	25	15.33±0.577		24.33±0.577*	
	50	21.33±0.577*		35.00±0.000*	
	75	27.67±0.600*		39.67±0.600*	
	100	31.33±0.577*		42.33±0.577*	
	150	33.00±0.000*		46.67±0.600*	
	200	39.33±0.577*		50.67±0.600*	
	300	49.00±0.000*		58.00±1.000*	
Ethyl acetate	10	15.67±0.600	> 300	20.67±0.600	215.29±0.60
	25	19.33±.,577		26.33±0.577*	
	50	22.67±0.600*		31.00±0.000*	
	75	28.33±0.577*		38.67±0.600*	
	100	34.00±0.000*		43.67±0.600*	
	150	36.33±0.577*		45.67±0.600*	
	200	39.00±1.000*		48.67±0.600*	
	300	40.67±0.600*		53.00±0.000*	
Methanol	10	21.33±0.577*	94.84±0.57	25.33±0.577*	82.06±0.53
	25	25.67±0.600*		27.33±0.577*	
	50	29.00±1.000*		41.00±1.000*	
	75	33.33±0.577*		45.67±0.600*	
	100	54.33±0.577*		61.00±1.000*	
	150	58.67±0.600*		67.67±0.600*	
	200	61.67±0.600*		71.00±0.000*	
	300	69.33±0.577*		75.33±0.577*	
Infusion (Water)	10	30.33±0.577*	185.92±0.60	32.33±0.577*	89.68±0.57
	25	33.67±0.600*		35.33±0.577*	
	50	36.00±0.000*		39.33±0.577*	
	75	38.33±0.577*		46.33±0.577*	
	100	41.33±0.577*		52.67±0.600*	
	150	45.33±0.577*		57.00±0.000*	
	200	51.67±0.600*		63.67±0.600*	
	300	55.00±1.000*		70.67±0.600*	
Decoction (Water)	10	31.00±0.000*	112.26±0.58	41.00±0.000*	48.63±0.60
	25	35.33±0.577*		44.33±0.577*	
	50	39.67±0.600*		50.33±0.577*	
	75	42.33±0.577*		54.67±0.600*	
	100	47.67±0.600*		59.00±0.000*	
	150	57.33±0.577*		61.67±0.600*	
	200	63.33±0.577*		69.00±1.000*	
	300	66.67±0.600*		76.00±1.000*	

*$p < 0.05$

Table 3. Analysis of DNA damage as measured by comet assay in MCF-7 cells treated with *A. aestivus* Brot. tuber extracts.

Samples	Concentrations (µg/ml)	Proportion of damaged nuclei (%)				% of damaged cells	Genetic damage index (GDI)	Arbitrary units (Au)
		Type I	Type II	Type III	Type IV			
Control	-	1.00	1.33	0.00	1.00	3.33	0.10	30
Solvent control (DMSO)	0.1%	1.33	0.33	1.67	2.00	5.33	0.15	46
Diethyl ether	100	5.33	4.67	5.00	8.00	23.00*	0.62*	185
	150	5.33	6.33	8.67	11.33	31.66*	0.89*	268
	200	7.33	7.67	11.00	16.33	42.33*	1.21*	363
	300	6.33	8.00	13.00	20.33	47.66*	1.43*	428
Ethyl acetate	100	4.67	3.67	3.33	4.33	16.00	0.39	118
	150	7.00	6.33	3.67	5.00	22.00*	0.51*	152
	200	3.67	8.33	16.33	24.33	52.66*	1.67*	500
	300	3.00	3.66	20.67	38.33	65.66*	2.26*	677
Methanol	100	8.00	8.67	6.00	9.33	28.00*	0.81*	242
	150	7.33	9.33	9.00	10.67	36.33*	0.96*	287
	200	9.33	10.00	11.00	17.33	47.66*	1.32*	395
	300	11.33	9.67	13.66	25.00	59.66*	1.72*	515
Infusion (Water)	100	8.67	9.00	11.00	15.33	44.00*	1.21*	363
	150	13.00	10.67	11.33	16.66	51.66*	1.35*	405
	200	15.67	11.67	12.66	17.33	57.33*	1.46*	439
	300	16.67	13.00	15.33	19.33	64.33*	1.66*	498
Decoction (Water)	100	8.33	9.33	11.33	14.67	43.66*	1.20*	359
	150	12.33	11.00	12.33	17.00	52.66*	1.39*	418
	200	15.00	11.00	12.33	18.00	56.33*	1.46*	438
	300	17.33	11.67	13.00	20.66	62.66*	1.62*	487

*$p < 0.05$. GDI: (Type 1 + 2. Type II + 3. Type III + 4. Type IV) / (Type 0 + Type I+ Type II+ Type III+ Type IV). Source: Pitarque et al. (1999).

scavenging activities is DPPH assay (Brand-Williams et al., 1995). DPPH is a stable free radical that has been widely used as a substrate to evaluate the antioxidant activity of various samples (Blois, 1958; Jung et al., 2003). The DPPH radical scavenging assay seems to be a rapid and accurate method for assessing the antioxidant activity of plant extracts. The results are highly reproducible and comparable to other free radical scavenging methods (Gil-Izquierdo et al., 2001). The DPPH radical scavenging activity of antioxidants is thought to be due to their

Table 4. Apoptotic effects of the *A. aestivus* Brot. tuber extracts on MCF-7 cells.

Samples	Concentrations (µg/ml)	Total cells	Apoptotic cells (%) ± SD	Necrotic cells (%) ± SD
Control	-	300	1.66±0.00	-
Solvent control (DMSO)	0.1%	300	2.00±0.01	-
Diethyl ether	100	300	9.00±0.54*	3.00±0.02
	150	300	14.67±0.57*	4,66±0.03
	200	300	17.33±0.60*	3.33±0.02
	300	300	22.66±0.60*	7.66±0.05*
Ethyl acetate	100	300	8.33±0.57*	4.00±0.02
	150	300	11.66±0.50*	5.33±0.03
	200	300	19.00±0.57*	7.00±0.06
	300	300	23.00±0.60*	9.00±0.06*
Methanol	100	300	18.33±0.57*	7.00±0.05
	150	300	25.66±0.60*	9.00±0.09*
	200	300	38.00±1.00*	8.00±0.06*
	300	300	42.33±1.50*	9,66±0.10*
Infusion (Water)	100	300	26.33±0.54*	6.33±0.05
	150	300	30.66±0.60*	7.66±0.06*
	200	300	37.33±1.50*	6.00±0.05
	300	300	45.00±1.00*	9.33±0.10*
Decoction (Water)	100	300	25.66±0.50*	5.00±0.03
	150	300	35.00±0.60*	7.00±0.06
	200	300	42.33±1.00*	8.66±0.09*
	300	300	49.66±1.50*	8.00±0.10*

*$p < 0.05$

hydrogen-donating ability.

The aim of this study was to evaluate the potential antioxidant activity and cytotoxic and apoptotic activities of different crude extracts of *A. aestivus* Brot. (AA) on MCF-7 breast cancer cell line. The different crude extracts (diethyl ether, ethyl acetate, methanol and water (infusion and decoction)) of AA were assessed for their DPPH scavenging ability. Their free radical scavenging activities were compared with the activity of well-known antioxidant and flavonoid rutin. The results of DPPH scavenging assay suggested that the ethyl acetate and methanol extracts have significant DPPH radical scavenging activity (IC_{50} values: 22.46 and 188.90 µg/ml, respectively) but diethyl ether and water (infusion and decoction) extracts have very low DPPH radical scavenging activity.

Although ethyl acetate and methanol extracts have significant DPPH radical scavenging activity, these activities seemed to be lower than that of flavonoid rutin (7.77 µg/ml) (Table 1). However, we could say that the extracts have hydrogen-donating ability and could serve as free radical inhibitors or scavengers, possibly acting as primary antioxidants. Among the diethyl ether, ethyl acetate, methanol and water (infusion and decoction) extracts, ethyl acetate extract is the most effective DPPH radical scavenger. Each herb contains generally different phenolic compounds, with different amount of antioxidant activity (Djeridane et al., 2006). The difference in antioxidant capacity of different extracts may be attributed to differences in their chemical composition such as ascorbic acid, limonoids, carotenoids, terpenoids and flavonoids. Furthermore, some of the extracts have hydrophilic and hydrophobic compounds and those samples may not work efficiently in some *in vitro* model systems (Jayaprakasha et al., 2008). Therefore, diethyl ether and water (infusion and decoction) extracts tested in this study were not efficient in DPPH assay system.

Radical scavenging activity of compounds in tested extracts was influenced also by the number and location of hydroxyl groups, glycolisation, and other substitutions.

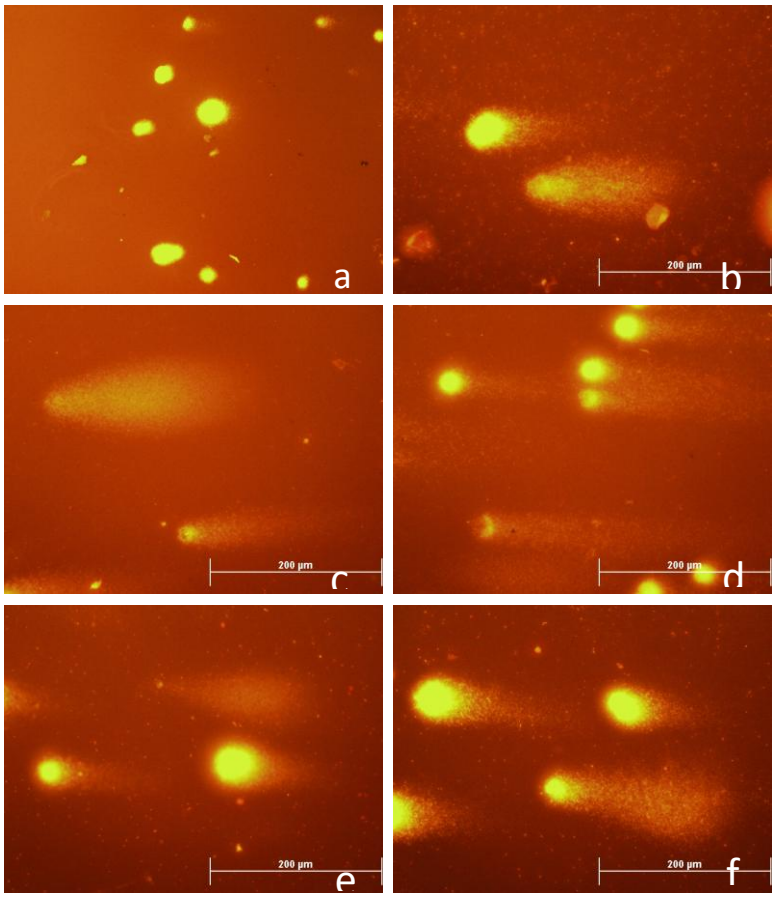

Figure 1. DNA damages after the *A. aestivus* extract treatments in the MCF-7 cells visualized with fluorescence microscopy. (a) control (undamaged); (b) 300 µg/ml diethyl ether extract treatment (Type I and Type IV); (c) 300 µg/ml ethyl acetate extract treatment (Type IV); (d) 300 µg/ml methanol extract treatment (Type III and Type IV); (e) 300 µg/ml infusion extract treatment (Type II and Type IV); (f) 300 µg/ml decoction extract treatment (Type II and Type III).

The methylation of hydroxyl groups can result in a much reduced effect on antioxidant activity (Rao et al., 2007). From chemical and kinetic viewpoints, it should be reasonable to assume that the free hydroxyl group at the C-3 position would enhance the free radical scavenging ability, since according to the reduction mechanism of free radicals, a scavenger should be capable of providing electrons or hydrogen or of receiving electron pairs, while the hydrogen bond between the 3-hydroxyl group and the 4-keto group would increase this ability (Rice-Evans et al., 1996; Decker, 1997).

Previous phytochemical investigations performed on *Asphodelus* species have resulted in the isolation of anthranoids, flavonoids and triterpenes (El-Fattah et al., 1997; Adinolfi et al., 1991; Van Wyk et al., 1995). Flavonoids have been shown to exhibit antioxidant and pro-oxidant activities, which often have been linked to their beneficial effects in cancer therapy (Birt et al., 2001). In recent years, a plant derived-bioactive substance that is capable of selectively arresting cell growth in tumor cells has received considerable attention in cancer chemopreventive approaches (Galati et al., 2000; Jang et al., 2005). On the other hand, flavonoids may not act as conventional hydrogen-donating antioxidants but may exert anticancer and apoptosis inducing properties in cells, through actions at protein kinase and lipid kinase signaling pathways (Hadi et al., 2000).

Recent studies suggested that *A. ramosus* has low anti-HIV effect (Bedoya et al., 2001) and *A. microcarpus* has low cytotoxic effect on rat pheochromocytoma (PC12) and human hepatoblastoma (HepG2) cells (Ljubuncic et al., 2005). In our study, methanol and water (infusion and decoction) extracts were shown to have strong cytotoxic activity against MCF-7, and diethyl ether and ethyl acetate extracts have no significant cytotoxic activity (Table 2). However, all of the extracts showed significant DNA damaging and apoptotic activity at all concentrations after 72 h (Tables 3 and 4).

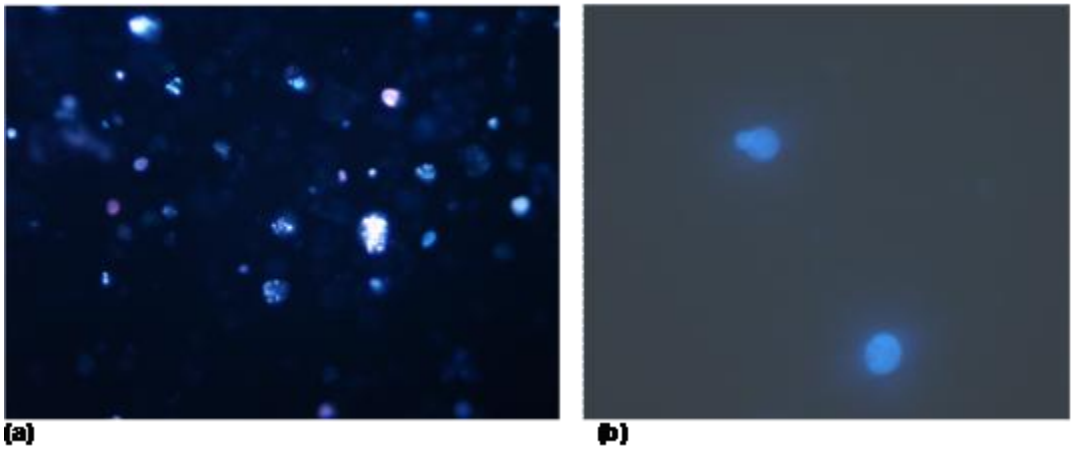

Figure 2. Apoptotic cells after the *A. aestivus* extract treatments in the MCF-7 cells visualized with fluoresce ence microscopy after HO/PI double staining. (a) control (normal) cells; (b) apoptotic cells indicated white arrow after than 300 µg/ml decoction extract treatment.

Targeting cell cycle and apoptotic pathways has emerged as an attractive approach for treatment of cancer. Apoptosis is important to eliminate undesired cells during the development and homeostasis of multicellular organisms. Normally, DNA damage increases the levels of p53 tumor suppressor protein, which transcriptionally activates the WAF1/CIP1/p21 gene whose protein product triggers cell cycle arrest to permit DNA repair (Elledge and Lee, 1995; Shao et al., 2006). However, if the repair is unsuccessful, cells commit apoptosis to safeguard the genome (Hautgraaf et al., 2006). As the HO/PI staining results showed, the extract treated MCF-7 cells clearly showed apoptotic morphology and the apoptotic cell rate significantly increased in a dose-dependent manner. At the same time, comet assay results also showed significant increase of DNA damage in extract treated MCF-7 cells, dose-dependently. Therefore, when the MCF-7 cells were treated with the high concentrations of the extracts (100, 150, 200 and 300 µg/ml), an irreparable DNA damage was possibly induced and apoptosis was initiated due to presence of phytochemicals, especially flavonoids in the extracts.

In a recent paper, Ueda et al. (2001) reported that a flavonoid, baicalin, induces apoptosis in Jurkat cells as pro-oxidant. Also, cytotoxic activity of extracts from this plant on MCF-7 cells in our study may be due to the presence of flavonoids in extracts. Also, the cause of DNA damage arising with extract treatments may be due to the presence of flavonoids that act as pro-oxidant, and they could induce apoptotic cell death, resulting in oxidetive DNA damage. It has been suggested that flavonoids can intercalate with the DNA molecule (Havsteen, 1983) and it has been shown that some flavonoids are mutagenic with pro-oxidant effects (De Carvalho et al., 2003). Flavonoids might therefore cause cytotoxic effect by inducing DNA damage, and in addition to flavonoids, possibly also other components in the crude extracts can have dual actions (Demma et al., 2009). Previous studies suggested that plant-derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells (Marklund et al., 1982) and antitumor activity of these antioxidants is either through induction of apoptosis (Ruby et al., 1995) or by inhibition of neovascularization (Ming et al., 1998).

Necrotic cells were also observed in this study. The treatment with extracts and percentage of the necrotic cells were found statistically significant at some extract concentrations (Table 4). It is important to distinguish tissue necrosis from molecularly defined necrotic cell death. Pathologic tissue necrosis can be the end product of apoptosis, autophagic, and/or necrotic cell death. In naturally occurring tumor tissue, necrosis occurs when the death rate exceeds the ability of cells to clear dying cells and is likely to be the sum of apoptotic, autophagic, and necrotic cell death processes (Amaravadi et al., 2007).

Findings of this study revealed that constituents in ethyl acetate and diethyl ether extracts of AA have antioxidant activity, but methanol and water extracts of AA have low antioxidant activity. However, all extracts from AA induced cytotoxicity significantly, as apoptotic cell death caused DNA damage in MCF-7 breast cancer cells.

Conclusion

This is the first study about antioxidant, cytotoxic and apoptotic effects of AA tuber extracts. Result of the present study demonstrates that antioxidant, cytotoxic and apoptotic properties of AA extracts show differentiation to extract type. Although antioxidant activities of methanol and water extracts are not significant, all of the

extracts have significant cytotoxic and apoptotic activity. Results of this study are important because these results can contribute to researches about finding of new compounds from plants, which can be used for breast cancer treatment, but further investigations are needed to identify the active components and establish the exact mechanism of action in each extracts in order to explain their therapeutic efficacy for breast cancer.

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