JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 2879-2885

Antibiofilm, Mutagenic and Antimutagenic Activity of *Allium sphaerocephalon L*

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(Received: 10 May 2014; accepted: 27 June 2014)

The ethanolic extracts (aerial parts and bulbs) of the *Allium sphaerocephalon*, has medicinal and food properties; these extracts were isolated, and its antibiofilm, mutagenic and antimutagenic activities were investigated. Minimum inhibitory concentrations (MICs) was calculated by broth microdilution method in microtiter plates against two Gram-positive (*S.aureus* and *B.subtilis*), two Gram- negative bacteria (*E.coli* and *P.aeruginosa*) and *C.albicans*. Antibiofilm effect of extracts was evaluated by microtiter plate assay. The mutagenic and antimutagenic activities were investigated by Ames Salmonella/microsome mutagenicity test. The ethanol extracts of *Allium sphaerocephalon* were effective against all the strains tested, with minimum inhibition concentrations ranging between 3.125 and 50 mg/ml. MIC results showed that the aerial parts extracts had the strongest growth inhibition effect against *C.albicans*. The aerial parts extracts also were able to inhibit 54.18%, 24.06% and 10.27% of *E.coli* biofilm structure in concentrations MIC, MIC/2 and MIC/4, respectively.The ethanolic extracts can be considered genotoxically safe, because it does not have any mutagenic effect at the tested concentrations. As a result, the ethanolic extracts of the *Allium sphaerocephalon* exhibited antimutagenic effect at 2.5 mg/plate concentration. Although the antioxidant capacity and inhibitory effects of *Allium sphaerocephalon* extracts/essential oils on planktonic bacteria have been investigated in a few studies, the antibiofilm activity and antimutagenic capacity of *Allium sphaerocephalon* ethanol extracts has not been reported to date. The aim of the present study was to investigate antibiofilm, mutagenic and antimutagenic activity of ethanol extracts of *Allium sphaerocephalon*.

Key words :*Allium sphaerocephalon*, Antimicrobial activity, Antibiofilm activity, mutagenic activity, antimutagenic activity.

Allium plants and their extracts contain different chemical compounds; an abundance of bioactive constituents namely organo-sulfur compounds, volatile sulfur compounds and proteins. Prostoglandins, fructan, vitamins, polyphenols, fatty acids and essential oils have also been identified[1]. Recent studies have confirmed the antibacterial, antifungal, antioxidant, anti-inflammatory and cytotoxic properties of Allium species[2-9].

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Cysteine sulphoxides possibly play a critical role in determining the characteristic smell and taste of these plants[10-11].Cysteine sulphoxides are physiologically active and are used as antibiotic and antitumor agents, especially in the context of stomach cancer treatment[11-12].Investigations of various wild species of the genus Allium have shown that some contains higher amounts of the cysteine sulphoxides than the cultivated species, and thus they may have considerable potential as spice, vegetable, and medicinal plants[13-14].*Allium sphaerocephalon* (round-headed leek) is a herbaceous, perennial plant with large, globe-shaped flower heads that inhabits insolated rocky slopes, sandy ground, vineyards and dry shrubby

habitats[15]. This ornamental plant cultivated in many European countries for medicinal and food purposes[16-18].Some cysteine sulfoxides, including alliin, methiin and isoalliin, have been reported as being present in *A. sphaerocephalon*[19].A new compound, a bisdesmosidic furanostanol saponin, isolated from the *A. sphaerocephalon* bulbs by Mimaki *et al*., 1996[20]. It was also reported that shyobunol, β-caryophyllene, α-cadinol, 3,5-diethy l-1,2,4-trithiolane and α-cadinene were main constituents of *A. sphaerocephalon* essential oil[21].Two previous studies have described the antimicrobial activity of *A. sphaerocephalon*[19-21].One of these studies, a fresh leaf extracts of *A. sphaerocephalon* have shown a weak antimicrobial activity against five bacteria and two fungal strains[19].Unlike the extracts of the *A. sphaerocephalon*, its essential oil has been reported to possess excellent antimicrobial activity[21].This is the first study using the ethanolic extracts of the *A. sphaerocephalon* to evaluate anti-biofilm, mutagenic and anti-mutagenic activities in order to enable their use in phytomedicine.

MATERIALS AND METHODS

Plant Material

The whole plants of wild-growing *A. sphaerocephalon* were collected in July, 2012 from natural populations in the vicinity of Salkim (Kavaklidere, Mugla), Southwest Turkey. The plant sample was identified by Dr. Mehtap Dönmez SAHIN and the voucher specimen has been deposited in the Herbarium of Faculty of Education, University of Usak under acquisition number 1275. The plant samples were air-dried at room temperature for later analysis.

Preparation of the ethanolic extracts

The air-dried and powdered plant materials (aerial parts and bulbs) (30 g) were extracted with ethanol (Merck) (300 ml) using the Soxhlet apparatus. The extracts were evaporated and then kept in small sterile opac bottles under refrigerated conditions until used.

Antimicrobial activity

Microbial strains

The in vitro antimicrobial activity of the ethanol extracts of *A. sphaerocephalon* was tested against a panel of laboratory control strains from the American Type Culture Collection, the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, the Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10239. All micro-organisms were maintained at 20°C under appropriate conditions and regenerated twice before use.

Broth microdilution assay

The minimal inhibitory concentrations (MICs) of the ethanol extracts of *A. sphaerocephalon* were determined by a broth microdilution method in 96-well microtitre plates[22]. The test medium was Mueller-Hinton Broth (MHB) and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (100µL) were inoculated into the wells of 96-well microtitre plates (Nunc F96 MicroWell™ plates; Nunclon TM Δ, Denmark) in the presence of extracts with different final concentrations (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 mg/ml). Plates were prepared in triplicate, then placed in an incubator at 37°C for 24 h for bacteria or at 30°C for 48 h for *C.albicans*.The MIC was defined as the lowest concentration of extract at which no visible growth was observed.

Effect on biofilm formation

The effect of different concentrations of extracts (ranging from 1 to 0.125 MIC) on biofilm-forming ability was tested on polystyrene flat-bottomed microtitre plates as described by Merritt *et al*. (2005)[23]. Briefly, 1% of overnight cultures (OD adjusted to 0.4 at 600 nm) of test pathogens were added into 200 µl of fresh TSB medium and cultivated in the presence and absence of extracts without agitation for 48 h at 37 ºC. The wells containing only TSB served as control. After incubation, the wells were washed with water to remove planktonic bacteria. The remaining bacteria were subsequently stained with 0.1% crystal violet solution for 10 min at room temperature. Wells were washed once again to remove the crystal violet solution that is not specifically staining the adherent bacteria. Microplates inverted and vigorously tap on paper towels to remove any excess liquid and air dried. 200 µl of 95% ethanol and 33% glacial acetic acid (Sigma Chemical Co) poured in Gram-negative bacteria *C.albicans* wells and Gram-positive bacteria wells, respectively.

Biofilm stains solubilized at room temperature. The stained biofilms were resuspended in 200 µl phosphate buffer saline (PBS) and OD_{550} was measured by spectrophotometry using an microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland). Percentage of inhibition of the tested extracts was calculated using the formula [1-(OD_{550} sample/OD_{550} control)]x 100%. All tests were done as triplicates.

Mutagenic and antimutagenic activity

Bacterial strains

S. typhimurium TA98 and *S. typhimurium* TA100 were used for the mutagenity and antimutagenity tests. The strains were analyzed for their histidine requirement, biotin requirement, the combination of both, rfa mutation, excision repair capability, the presence of the plasmid pKM101, and spontaneous mutation rate according to Mortelmans and Zeiger[24]. Working cultures were prepared by inoculating nutrient broth with the frozen cultures, followed by an overnight incubation at 37°C with gentle agitation[25].

Viability assays and determination of test concentrations

Cytotoxic doses of the *A. sphaerocephalon* ethanolic extracts were determined by the method of Mortelmans and Zeiger(2000)[24]. The toxicity of ethanolic extracts toward *S. typhimurium* TA98 and TA100 was determined as described in detail in cited papers[26-27]. These tests confirmed that there was normal growth of the background lawn, spontaneous colony numbers within the regular range, and no significant reduction in cell survival. Thus,for the concentrations and conditions reported here, no toxicity or other adverse effects were observed.

Mutagenicity and antimutagenicity tests

In this study, the plate incorporation method was used to assess the results of mutagenicity and antimutagenicity assays[28]. The known mutagens 4-nitro-o-phenylenediamine (4-NPD) (3 µg/plate) for *S. typhimurium* TA98 and sodium azide (NaN_3) (8 µg/plate) for *S. typhimurium* TA100 were used as positive controls and ethanol was used as negative control.

In the mutagenicity test performed with TA98 and TA100 strains of *S. typhimurium*, 100 µl of the overnight culture, 100 µl of test compounds at different concentrations (2.5, 1.25, and 0.625 mg/plate), and 500 µl phosphate buffer were added to 2 ml of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose plates. Histidine independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 or 72 h.

In the antimutagenicity test performed with the same strains, 100 µl of the overnight bacterial culture, 100 µl mutagen, 100 µl test compounds at different concentrations (2.5, 1.25, and 0.625 mg/plate), and 500 µl phosphate buffer were added to 2 ml of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose plates. Histidine independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 or 72 h.

The plate incorporation method was used to assess the results of mutagenicity and antimutagenicity assays[28]. For the mutagenicity assays, the mutagenic index was calculated for each concentration, which is the average number of revertants per plate divided by the average number of revertants per plate with the negative control. For the antimutagenicity assays, the inhibition of mutagenicity was calculated by using the following equation (M: number of revertants/plate induced by mutagen alone, S_0: number of spontaneous revertants, S_1: number of revertants/plate induced by the extract plus the mutagen): % Inhibition = $[(M\text{-}S_1)\text{ “ }(M\text{-}S_0)] \times 100$.

25-40% inhibition was defined as moderate antimutagenicity; 40% or more inhibition as strong antimutagenicity; and 25% or less inhibition as no antimutagenicity[29-31].

RESULTS AND DISCUSSION

The MICs of ethanolic extracts of *A. sphaerocephalon* aerial parts and bulbs was determined for Gram-positive and Gram-negative bacteria as well as for fungi using the standard broth microdilution susceptibility test. The MICs are given in Table 1. The highest activity was expressed by aerial parts extract, MIC for *C.albicans* was 3.125 mg/ml. Bulb extract of *A. sphaerocephalon* at a concentration of 25 mg/ml was also bacterio static for *C.albicans.* It was found that the growth of *B.subtilis* and *S.aureus* was inhibited by both (aerial parts and bulbs) extracts at the concentrations of 12.5-25.0 mg/ml. MIC

values obtained for Gram-negative *P.aeruginosa* and *E.coli* were 25.0-50.0 mg/ml (Table 1).

Biofilms are sources of diverse problems in various areas. In dairy industry, biofilms are often sources of biological contaminants and they also contribute to increased equipment corrosion rates[32]. In the public health sector, the colonization of medical surfaces, such as catheters and other indwelling devices, by biofilms, plays a decisive role in the problem of healthcare-associated infections[33]. These are the reaons why many research groups investigate potential strategies, which could be accessory or alternative to antibiotic therapy. Currently, natural plant compounds are on the focus of some biotechnological companies which are looking for the new antimicrobial and anti-biofilm drugs[34]. Employing a microtiter plate assay for biofilm study, the results revealed that the inhibitory effect of *A. sphaerocephalon* extracts on biofilm appeared to be dose-related (Table 1). Despite the differences of inhibitory effects among the strains, a general attenuated level of biofilm formation in the presence of MIC and subinhibitory concentrations of ethanol extracts of *A. sphaerocephalon* was observed. In the presence of aerial part extracts at

Table 1. Antimicrobial and antibiofilm activities of *A. sphaerocephalon* ethanol extracts

Microorganism	Extract	Planktonic	% inhibition on biofilms			
		MIC (mg/ml)	MIC	MIC/2	MIC/4	MIC/8
*B.subtilis*ATCC 6633	Aerial parts	12.5	35.7±0.74	22.32±4.65	-	-
	Bulbs	12.5	39.03±2.60	21.91±3.80	-	-
S.aureus ATCC 25923	Aerial parts	25	36.73±2.15	23.02±2.97	10.55±1.65	2.79±0.53
	Bulbs	25	44.12±2.20	17.52±1.06	10.52±0.70	-
*P.aeruginosa*ATCC 27853	Aerial parts	50	40±0.72	26.35±0.50	12.96±1.25	3.82±0.98
	Bulbs	25	15.12±0.81	-	-	-
E.coli ATCC 25922	Aerial parts	50	54.18±4.71	24.06±2.74	10.27±1.38	-
	Bulbs	25	29.76±2.82	17.53±2.27	7.01±1.50	-
C. albicans ATCC 10239	Aerial parts	3.12	16.83±1.44	4.91±0.35	-	-
	Bulbs	25	41.12±2.30	21.87±1.20	5.07±0.75	-

-: No inhibition

Table 2. Theantimutagenicity assay results of the ethanolicextracts of *A. sphaerocephalon* for*S. typhimurium*TA98 and TA 100 bacterial strains

Test items	Concentration (mg/plate)	Number of revertants			
		TA98		TA100	
		Mean±S.error	Inhibition %	Mean±S.error	Inhibition %
Negative control		7.4 ±2.3[a]		15.75 ±4.11	
4-NPD[b]	3	401.6±4.5		-	
NaN_3[b]	8	-		430 ±19.07	
Extract of aerial part	2.5	203.5±20.46	49.33	323.6±4.5	24.72
	1.25	262.3±15.5	34.68	376.6±13.57	12.4
	0.625	322.3±15.17	19.74	398±3.6	7.44
Extract of bulb	2.5	102±35.02	76.60	331.3±17.21	22.94
	1.25	263±43.91	34.52	378±16.7	12.09
	0.625	263.5±16.92	34.39	407±5.47	5.34

[a] Values expressed are means ± SD of three parellel measurements. The regression analysis was carried out in Microsoft Excel between percent inhibition of mutagenicity and log values of concentrations of the plant extracts.4-nitro-α-phenylene diamine

[b] 4-NPD and NaN_3 were used as positive controls for *S.typhimurium* TA98 and TA100 strains, respectively.

concentrations of MIC, MIC/2 and MIC/4, the mean biofilm formation values were equal to 40.0, 26.35 and 12.96% for *P.aeruginosa* and 54.18, 24.06 and 10.27 % for *E.coli*, respectively. In the presence of bulb extracts at concentrations of MIC and MIC/2, the mean biofilm formation values were equal to 39.03 and 21.91% for *B.subtilis* and 44.12 and 17.52 % for *S.aureus*. At a concentration of 3.125 mg/ml, aerial parts extracts of *A. sphaerocephalon* exhibited 16.83% inhibitory effect on *C.albicans* biofilm formation.The bulb extracts at concentrations between MIC-MIC /4 exhibited 5.07-41.12% inhibition on *C. albicans* biofilm formation.

Cancer is considered as one of the main causes of mortality throughout the industrial world in the present century. Scientists believe that damage to the genetic material, changes in DNA sequence and continuity, mutation in genes and other genetic changes in chromosomal structures play important roles in carcinogenesis[35]. The use of anti-mutagens and anti-carcinogens in everyday life is the most effective procedure for preventing human cancer and genetic disease[36].Natural antimutagens from edible and medicinal plants are of particular importance because they may be useful for human cancer prevention and have no undesirable xenobiotic effects on living organisms[37-38]. In recent years, plants have gained much attention due to their antimutagenic activities, which may inactivate or reverse the effects caused by some mutagens. Furthermore, studies have demonstrated that substrates with antioxidant properties are potential antimutagens or carcinogens[39]. The ethanolic extracts of the aerial part and bulb of *A. sphaerocephalon,* which was tested at three different concentrations, including 0.625, 1.25 and 2.5 mg/plate, did not exhibit any mutagenic effect in the mutagenicity assay performed with *S.typhimurium* TA98 and TA100 (data was not shown).The possible antimutagenic potential of the extract was examined against 4-NPD and NaN_3in *S. typhimurium* TA 98 and TA 100, respectively. The results were evaluated by using standard plate incorporation method and summarized in Table 2.

In the antimutagenicity assays performed with TA98 and TA100 strains, the extracts exhibited antimutagenic effects at 2.5, 1.25,and 0.625 mg/plate concentrations. The strongest antimutagenic activity was observed at 2.5 mg/plate concentration of bulb and aerial part extracts against *S.typhimurium* TA 98 strain. However, extracts of *A. sphaerocephalon* did not exhibit any antimutagenic effect against *S. typhimurium* TA 100. The data obtained on antimutagenic potential of bulb extract showed its moderate activity against TA 98 at 1.25 and 0.625 mg/plate extract concentrations. Similar antimutagenic activity against TA98 was determined at a concentration of 1.25 mg/plate of aerial part extract.

The results presented in this article indicate that the ethanolic extracts of *A. sphaerocephalon* are mostly non-mutagenic and antimutagenic. Also the results in this study demonstrated potent in vitro activity in inhibiting biofilm formations of *E.coli, P.aeruginosa, S.aureus, B.subtilis* and *C.albicans* by *A. sphaerocephalon* ethanol extracts.

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