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Antimicrobial, antioxidant, and antimutagenic activities of *Gladiolus illyricus*

[*Actividades antimicrobiana, antioxidante y antimutagénica de Gladiolus illyricus*]

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Abstract

Context: In the present study, the ethanolic extracts of the aerial parts and the rhizomes of *G. illyricus* were obtained.

Aims: To determine the antimicrobial, antioxidant, and antimutagenic properties of *G. illyricus* extracts.

Methods: The antimicrobial activity was studied with the disc diffusion method and the antioxidant capacity by inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the total antioxidant activity. The antimutagenic activity was investigated by Ames *Salmonella*/microsome mutagenic test. The bacterial mutant strains *Salmonella typhimurium* TA98 and TA100 were used to determine antigenotoxic potentials of the extracts.

Results: The ethanolic extracts of the aerial parts and the rhizomes showed moderate antimicrobial activity only on *Bacillus subtilis*. The IC_{50} value for DPPH radical of the aerial parts and rhizomes were 57.1 ± 1.3 and 48.1 ± 1.1 mg/mL, respectively. The total antioxidant activities of the aerial parts and the rhizomes of *G. illyricus* at 3.15 mg/mL concentration were 92.3 ± 1.4% and 91.5 ± 2.1%, respectively. These extracts showed antimutagenic effects at 0.5 and 5 mg/plate concentrations.

Conclusions: To our knowledge, this is the first study on antimicrobial, antioxidant, and antimutagenic activity of the ethanolic extracts of the aerial parts and the rhizomes from *G. illyricus.* Our results indicate that these extracts would exert several beneficial effects by virtue of their antioxidant and antimutagenic activities. These activities could be an important topic in the medical and cosmetic fields.

Keywords: Ames test; antimicrobial; antioxidant; *Gladiolus illyricus*.

Resumen

Contexto: En el presente estudio se obtuvieron extractos etanólicos de las partes aéreas y los rizomas de *G. illyricus* .

Objetivos: Determinar las propiedades antimicrobiana, antioxidante y antimutagénica de los extractos de *G. illyricus*.

Métodos: La actividad antimicrobiana fue estudiada mediante el método de difusión en disco y la capacidad antioxidante por la inhibición del radical 2,2-difenil-1-picrilhidracilo (DPPH) y la actividad antioxidante total. La actividad antimutagénica fue investigada mediante la prueba mutagénica de Ames Salmonella/microsoma. Para determinar el potencial antigenotóxico de los extractos se utilizaron las cepas mutantes de *Salmonella typhimurium* TA98 y TA100.

Resultados: Los extractos etanólicos de las partes aéreas y rizomas mostraron actividad antimicrobiana moderada solamente frente a *Bacillus subtilis*. Los valores de IC50 para el radical DPPH de las partes aéreas y rizomas fueron 57,1 ± 1,3 y 48,1 ± 1,1 mg/mL, respectivamente. La actividad antioxidante total de las partes aéreas y rizomas de *G. illyricus,* a concentración de 3,15 mg/mL, fueron 92,3 ± 1,4% y 91,5 ± 2,1%, respectivamente. Los extractos mostraron efectos antimutagénicos a concentraciones de 0,5 y 5 mg/plate.

Conclusiones: No se encontró en la literatura algún estudio sobre la actividad antimicrobiana, antioxidante o anti-mutagénica de los extractos etanólicos de las partes aéreas y los rizomas de *G. illyricus*. Estos resultados indican que estos extractos podrían ejercer efectos beneficiosos en virtud de su actividad antioxidante y anti-mutagénica. Estas actividades podrían ser un tema importante en los campos médicos y cosméticos.

Palabras Clave: Antimicrobiano; antioxidante; ensayo de Ames; *Gladiolus illyricus*.

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INTRODUCTION

Herbs have been used in many domains including medicine, nutrition, flavoring, beverages, dyeing, repellents, fragrances, cosmetics, smoking, and other industrial purposes (Dahanukar et al., 2000; Exarchou et al., 2002). A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials (Khalaf et al., 2008) and used for preventing and treatment of the infections, oxidation and mutations.

Antimicrobial resistance is now a major threat to public health, and controlling antimicrobial resistance is an international priority (Wise et al., 1998; WHO, 1999). This situation has forced scientists to search for new antimicrobial substances from various sources, such as medical plants (Şahin et al., 2003).

In recent years much attention has been devoted to the natural antioxidant and their association with health benefits (Arnous et al., 2001). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in scavenging and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994).

Mutations are the cause of inborn errors of metabolism, resulting in morbidity and mortality in living organisms. Besides inherited metabolic disorders, a spectrum of age-related human diseases, including cancer, are caused by mutations (Shon et al., 2004). A mutagen is considered an agent that is capable of destroying the integrity of the hereditary mechanism of a cell or organism. Any substance causing increased mutations can also increase the probability of cancer (Zaveri et al., 2011).

Natural antimutagenic compounds obtained from edible and medicinal plants are of particular importance in this regard because they produce no undesirable xenobiotic effects on living organisms that would offset any potential usefulness in cancer prevention in humans (Zahin et al., 2010).

The anticandidal activity of *Gladiolus gregasius* (Nguedia et al., 2004), antifungal activity of *Gladiolus dalenii* (Odhiambo et al., 2009), antimicrobial activity of *Gladiolus atroviolaceus* (Kahriman et al., 2012) and *Gladiolus psittacinus* (Munyemana et al., 2013) were investigated. However, as far as we know, no literature on the antimicrobial, antioxidant and antimutagenic effects of the ethanolic extract of the aerial parts and rhizomes of *G. illyricus* has been published. This is the first study using the ethanolic extract of *G. illyricus* to evaluate antimicrobial, antioxidant, and antimutagenic activities in order to enable their use in phytomedicine and other industries.

MATERIALS AND METHODS

Materials

1,1-diphenyl-2-picrylhydrazyl (DPPH), butylhydroxytoluene (BHT), ascorbic acid, β-carotene, linoleic acid, 4-nitro-o-phenylenediamine (4-NPD), sodium azide (NaN_3), and histidine/biotin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ethanol, chloroform, Mueller Hinton agar, Sabouraud dextrose agar, and Tween-40 (polyoxyethylene sorbitan monopalmitate) were obtained from Merck Co. (Darmstadt, Germany). Other chemicals were of reagent grade.

Plant material and preparation

The aerial parts and rhizomes of *G. illyricus* (Iridaceae) were collected from Mugla, Turkey. The leaves were air-dried at room temperature for seven days and were stored for later analysis. A voucher specimen (Herbarium No: 1602) has been taxonomically identified by Mehtap Donmez Sahin and deposited in the Herbarium of the University of Usak, Turkey.

The air dried and powdered plant samples were extracted with ethanol using the Soxhlet apparatus. Following evaporation, the extract was diluted in ethanol/water (1:1, v/v), and then kept in small sterile opac bottles under refrigerated conditions until used.

Microbial strains

Bacillus subtilis ATCC 6633 (ATCC, Rockville, USA), *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10239 were used as determined the antimicrobial activity.

Salmonella typhimurium TA98 and TA100 were used for the mutagenic and antimutagenic 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 (2000). Working cultures were prepared by inoculating nutrient broth with the frozen cultures, followed by an overnight incubation at 37°C with gentle agitation (Oh et al., 2008).

Antimicrobial activity

The antimicrobial activities of the extracts were assayed by the standard paper disc diffusion method (Collins et al., 1995; Murray et al., 1995). Twenty microlitres of each extract containing 3 mg crude extract were injected in discs of 6 mm in diameter. Mueller-Hinton agar and Sabouraud dextrose agar was used for the test bacteria and *C. albicans*, respectively. The media sterilised in a flask and cooled to 45-50°C were distributed to sterilised Petri dishes with a diameter of 9 cm (15 mL) after injecting cultures of bacteria and yeast (1 mL) and distributing the medium in Petri dishes homogeneously. Dishes injected with above-mentioned materials were located on a solid agar medium by pressing slightly. *P. aeruginosa* and the fungi were incubated at 30 ± 0.1°C for 18-24 h and 24-48 h, respectively. Other bacterial strains were incubated at 37 ± 0.1°C for 24-48 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested microorganisms. Ethanol was used as a negative control. Studies were performed in triplicate.

Antioxidant activity

Determination of DPPH radical scavenging activity

Antioxidant activity of the extracts were determined based on its ability to react with the stable DPPH free radical (Yamasaki et al., 1994). Fifty µL of the extract [1.25, 2.5, 5, 10, 50 and 75 mg/mL in ethanol/water (1:1, v/v)] was added to 5 mL DPPH solution (0.004%) in ethanol. After incubation at room temperature for 30 min, the absorbance of each solution was determined at 517 nm. Percentage of inhibition and the concentration of the sample required for 50% scavenging of the DPPH free radical (IC_{50}) were determined. BHT and ascorbic acid (1.25, 2.5, and 5 mg/mL concentrations) were used as a positive control. All determinations were performed in triplicate.

Total antioxidant activity by the β-carotene-linoleic acid method

The total antioxidant activity of the extracts were evaluated by the β-carotene-linoleic acid model (Jayaprakasha and Rao, 2000). β-carotene (0.5 mg) in chloroform (1 mL), 25 µL of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed together. The chloroform was completely evaporated using a vacuum evaporator and the resulting solution was diluted with 100 mL of oxygenated water. Aliquots (2.5 mL) of this mixture were transferred into different tubes containing 0.5 mL of samples at 3.15 mg/mL concentration in ethanol/water (1:1, v/v). The same procedure was repeated with the positive control BHT and ascorbic acid at 3.15 mg/mL, and a blank. The emulsion system was incubated for up to 2 h at 50°C. Measurement of absorbance was continued until the color of β-carotene disappeared in the control. After this incubation period, absorbance of the mixtures was measured at 490 nm. All determinations were performed in triplicate.

The bleaching rate (R) of β-carotene was calculated using the following formula. R=ln (a/b)/ t where, ln= natural log, a= absorbance at time 0, b= absorbance at time t (120 min). The

antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using the formula AA= $[(R_{Control}- R_{Sample})/R_{Control}]$ x 100. Antioxidative activity of the extract was compared with those of BHT and ascorbic acid at the same concentration.

Mutagenic and antimutagenic activity

Viability assays and determination of test concentrations

Cytotoxic dose of the extract was determined by the method of Mortelmans and Zeiger (2000). The toxicity of the extract toward *S. typhimurium* TA98 and TA100 was determined as described in detail elsewhere (Santana-Rios et al., 2001; Yu et al., 2001). 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 antimutagenic tests

In this study, the plate incorporation method was used to assess the results of mutagenic and antimutagenic assays (Maron and Ames, 1983). The known mutagens 4-NPD (3 µg/plate) for *S. typhimurium* TA98 and NaN_3 (8 µg/plate) for *S. typhimurium* TA100 were used as positive controls and ethanol/water (1:1, v/v) was used as a negative control in mutagenicity and antimutagenic tests.

In the mutagenicity test performed with TA98 and TA100 strains of *S. typhimurium*, 100 µL of the overnight bacterial culture, 100 µL test compounds at different concentrations (0.05, 0.5, and 5 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 antimutagenic test performed with the same strains, 100 µL of the overnight bacterial culture, 100 µL mutagen, 100 µL test compounds at different concentrations (0.05, 0.5, and 5 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 antimutagenic assays (Maron and Ames, 1983). 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 antimutagenic 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= $[(M-S_1)– (M-S_0)] \times 100$. Inhibition was defined as: < 25% as no antimutagenicity; 25-40% moderate antimutagenicity; and >40% of inhibition as strong antimutagenicity (Ikken et al., 1999; Negi et al., 2003; Evandri et al., 2005).

Statistical analysis

Experiments were performed in triplicate and results were recorded as mean ± SD. Data was entered into a Microsoft Excel database and analyzed using SPSS (version 20.0). $P<0.05$ was considered as statistically significant. Statistical analyses of the antioxidant activity were performed using the Kruskal-Wallis test.

RESULTS AND DISCUSSION

The antimicrobial activities of the extracts were determined by the disc diffusion method (data not shown). The results showed that the ethanolic extracts have only slight inhibition effect on *B. subtilis* ATCC 6633. The inhibition zone of the of the aerial parts and rhizomes on *B. subtilis* ATCC 6633 were 7 and 8 mm, respectively.

Free radical-scavenging capacity of the corresponding extracts, measured by DPPH assay, and the IC_{50} values of the extract, BHT, and

ascorbic acid are shown in Table 1. Lower IC_{50} value indicates higher antioxidant activity.

The results indicate that the radical-scavenging activity of BHT and ascorbic acid were higher than that of the extracts. Total antioxidant activities of the ethanolic extract of the aerial parts and the rhizomes of *G. illyricus*, according to β-carotene-linoleic acid method, are shown in Table 2 ($p<0.05$). The total antioxidant activity of the extracts was higher than that of ascorbic acid at the same concentration.

Table 1. Free radical scavenging capacities of the ethanolic extract of the aerial parts (GiAPe) and rhizomes (GiRe) of *G. illyricus* and reference compounds measured in DPPH assay.

Treatment	IC_{50} value (mg/mL)
GiAPe	57.1 ± 1.27^{d}
GiRe	48.1 ± 1.08^{c}
BHT	0.95 ± 0.01^{b}
Ascorbic acid	0.48 ± 0.02^{a}

The IC_{50} values were obtained by linear regression analysis. Different letters symbolize significant differences ($p < 0.05$) by mean of the nonparametric Kruskal-Wallis test. Data represent the mean ± SD of at least n = 3. BHT: butyl hydroxytoluene.

The ethanolic extract of the aerial parts and the rhizomes, which were tested at three different concentrations, including 0.05, 0.5, and 5 mg/plate, did not exhibit any mutagenic effect in the mutagenicity assays performed with *S. typhimurium* TA98 and TA100 (data was not shown).

The possible antimutagenic potential of the extracts was examined against 4-NPD, and NaN_3 in *S. typhimurium* TA 98 and TA 100, respectively. The results were evaluated by using standard plate incorporation method and summarized in Table 3.

In the antimutagenic assays performed with TA98 and TA100 strains, the extract of the aerial parts and the rhizomes exhibited antimutagenic effects at 0.5 and 5 mg/plate concentrations ($p<0.05$). The strongest antimutagenic activity was observed at 5 mg/plate concentration of the aerial parts against *S. typhimurium* TA98 strain. As a result, only one concentration (0.05 mg/plate) of the extract of the aerial parts and the rhizomes did not exhibit any antimutagenic effect (inhibition < 25%) against *S. typhimurium* TA98 and TA100. The antimutagenic activity of the extract was determined as dose dependent.

Table 2. Antioxidant activity of the ethanolic extract of the aerial parts (GiAPe) and rhizomes (GiRe) of *G. illyricus* in *β*-carotene- linoleic acid test system.

Treatment	Antioxidant activity (%)
GiAPe	92.3 ± 1.4^{b}
GiRe	91.5 ± 2.1^{b}
BHT	95.7 ± 0.1^{a}
Ascorbic acid	63.7 ± 3.2^{c}

The concentration used in all extracts and reference compounds was 3.15 mg/mL. Different letters symbolize significant differences ($p < 0.05$) by mean of the nonparametric Kruskal-Wallis test. Data represent the mean ± SD of at least n = 3. BHT: butyl hydroxytoluene.

Toxic substances, whether synthetic or natural, may be mutagenic agents. Since cancer has become the number one cause of death, much attention has been focused on the chemoprevention of cancer, with minor success, while little attention has been paid to substances found in medicinal plants and herbal medicines that might serve as protection against the chemical mutagens that act as initiators in the carcinogenic process (Shon et al., 2004).

There is a wide range of prospective human health applications for plant species that possess antimutagenic properties. Various studies have shown that natural antioxidants can reduce or inhibit the mutagenic potential of mutagens and carcinogens (Negi et al., 2003; Zahin et al., 2010). Herbal remedies and phytotherapeutic drugs currently in use have also been shown to prevent free radicals from attacking DNA, indicating the potential, it has been suggested, to inhibit aging as well as cancer (Ghazali et al., 2011).

Table 3. The antimutagenic assay results of the ethanolic extract of the aerial parts (GiAPe) and rhizomes (GiRe) of *G. illyricus* for *S. typhimurium* TA98 and TA100 bacterial strains.

Treatment	Concentration (mg/plate)	Number of revertants			
		TA98		TA100	
		Mean ± SD	Inhibition (%)	Mean ± SD	Inhibition (%)
Ethanol	-	6.6 ± 3.4^{e}	-	40.5 ± 7.1^{e}	-
4-NPD	3	353.1 ± 32.4^{d}		-	-
NaN_3	8	-	-	427.7 ± 25.2^{d}	-
GiAPe	0.05	336.0 ± 32.9^{c}	4.9	342.0 ± 24.1^{c}	15.1
	0.5	215.5 ± 21.2^{b}	39.0	267.8 ± 26.0^{b}	33.6
	5	179.8 ± 27.0^{a}	49.1	236.0 ± 22.3^{b}	41.5
GiRe	0.05	287.7 ± 23.1^{c}	6.5	370.2 ± 25.8^{c}	13.5
	0.5	227.5 ± 13.5^{b}	26.0	286.2 ± 21.5^{b}	33.1
	5	139.3 ± 19.0^{a}	54.7	132.6 ± 25.6^{a}	69.0

Ethanol was used as negative control (without any mutagen), 4-nitro-o-phenylenediamine (4-NPD) and sodium azide (NaN_3) were used as positive controls for *S. typhimurium* TA98 and TA100 strains, respectively. A regression analysis was carried out in Microsoft Excel between percent inhibition of mutagenicity and log values of concentrations of the plant extract. Different letters symbolize significant differences ($p < 0.05$) by mean of the nonparametric Kruskal-Wallis test. Data represent the mean ± SD of at least n = 3.

CONCLUSIONS

The ethanolic extracts of the aerial parts and the rhizomes of *G. illyricus* are safe at the tested concentrations, exhibited important antimutagenic and antioxidant properties. The obtained results could form a good basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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