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Evaluation of Antioxidant, Antimicrobial and Cytotoxicity of *Alcea kurdica* Alef

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Abstract

The purpose of this study is to evaluate the aqueous extract of *Alcea kurdica* Alef for antioxidant and antimicrobial activity as well as potential toxicity. Antioxidant activities were evaluated using 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging and Ferric Reducing Antioxidant Power (FRAP) reducing capacity assays as well as total phenolic compounds (TPC). Antimicrobial activity was assessed against some Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi using the agar dilution method. The plant extract was also assessed for *in-vitro* toxicity using the Promega Cell Titer 96 AQueous Non-Radioactive Cell Proliferation (MTS) assay. The aqueous extract of *A. kurdica* demonstrated potent free radical scavenging activity of 64% ± 1.64% and ferric reduction capacity of 2955.0 ± 0.04 mmol/g, as well as having 88.0 ± 0.002 mg gallic acid equivalents /gram plant extract. The aqueous extract of *A. kurdica* had modest antimicrobial activity against most tested microorganisms. Moreover, the plant extract did not have any toxic effects on human lung fibroblasts. Based on these findings, we conclude that *A. kurdica* may be utilized to prevent the growth of some microorganisms. The plant constituents behind these effects are the antioxidants and phenols. Further studies are needed to evaluate the therapeutic utility of *A. kurdica* extracts as antimicrobial agents.

Keywords: *Alcea kurdica* Alef, Antioxidant, Antimicrobial activity, Cytotoxicity.

1. Introduction

Recently, scientists have paid more attention to the role of natural antioxidants, mainly phenolic compounds, which may have more antioxidant activity than synthetic antioxidants (Velasco and Williams, 2011). Natural products, particularly those present in medicinal plants, have gained more interest as food ingredients because of their safety, accessibility, and positive impact on health (Ebrahimabadi *et al.*, 2010). A variety of natural antioxidant compound purified and derived from plant resources have been demonstrated to scavenge free radicals (Loo *et al.*, 2007). Epidemiological studies suggest that the consumption of plants can protect humans against oxidative damage by inhibiting or scavenging free radicals and reactive oxygen species (ROS) (Sun *et al.*, 2002; Materska and Perucka, 2005). Natural antioxidants exhibit a wide range of pharmacological activities, and have shown anticancer, anti-inflammatory, anti-aging, anti-ulcer, and antimicrobial properties (Mayne, 2003; Pinnell, 2003). For the past several decades, a variety of vegetables, crops, spices, and medicinal herbs have been analyzed in an effort to identify new and potentially useful antioxidants (Zheng and Wang, 2001). It has become evident that natural products may reduce oxidative stress through antioxidant action. For example, various phenolics and flavonoids, which are found naturally in fruit, vegetables, and some beverages, have been demonstrated to exert antioxidant effects through a number of different mechanisms (Nijveldt *et al.*, 2001).

Alcea kurdica, which belongs to the family Malvaceae, is a very polymorphic and widespread species found in east Iraq and west Iran. *Alcea* is an important source of mucilage and are widely distributed with about 70 species (Pakravan and Ghahreman, 2003). *Alcea* are usually found in the Penjween, Sharbazher area, and in Haji Omran and Garaguin in Kurdistan of Iraq. The *Alcea* is a delicate plant having sharp, pale yellow, dark reddish stems, and greyish green foliage. Traditionally *A. kurdica* have been widely used among the Kurdish population to treat a variety of diseases including tonsillitis, gastric ulcers, duodenal ulcers, pneumonia, urinary tract infections, and alopecia (Mati and de Boer, 2011). Based on literature survey, there are no ethnopharmacological studies on *A. kurdica* that originate from Kurdistan of

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Iraq. Based on the importance of *A. kurdica* in medicinal folk, this study was conducted to evaluate the antioxidant and antimicrobial activities of *A. kurdica* as well as potential toxicity against the human normal lung fibroblast cell line (Hs888Lu).

2. Materials and Methods

2.1. Plant Extract

The dried leaves of the *A. kurdica* plant were purchased at the herbal market, Erbil, Iraq. The Voucher-ID and Vernacular name of *A. kurdica* were identified as Alef. EM2.1 Malvaceae Gule hero (Mati and de Boer, 2011). After identification, the plant leaves were ground into powder using an electrical blender then extracted using water solvent at a ratio of 1:20. The mixture was heated and stirred on a hotplate for 3 hrs at 65° C followed by cooling and filtration using Whatman No. 1 filter paper and a filter funnel. The mixture was evaporated under reduced pressure in Eyela™ rotary evaporator (Sigma-Aldrich, USA) and subjected to lyophilisation by freeze-drying (Labconco, Kansas, USA) to produce a powdered form of the extract. The extract was stored at -20° C for later use.

2.2. Antioxidant Experiments

The antioxidant activity of the aqueous extract was determined using the 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical assay which is based on the transfer of electrons between the DPPH reagent and the plant extracts. The method described by (Loo *et al*., 2007; Erel, 2004; Gorinstein *et al.*, 2003) was utilized with minor modifications. The DPPH value was expressed as percentage scavenging of the DPPH based on the following equation: DPPH (%) = (Absorbance of blank – Absorbance of sample) x 100 / Absorbance of sample. Each test was performed three times in triplicate and the results were expressed as mean percentage. Ferric Reducing Antioxidant Power (FRAP) assay was performed according to a previously described method (Erel, 2004). FRAP values were expressed as mmol of ferric reducing activity of the plant extract per gram of dry weight and Ferrous sulfate heptahydrate (FeSO4x7H2O) was used as a standard, based on the following equation: FRAP value of sample in mmol/L = (change in absorbance of sample from 0 to 4 min / change in absorbance of standard from 0 to 4 min) x FRAP value of standard.

2.3. Total Phenolic Compounds (TPC)

The total phenolic compounds (TPC) of aqueous plant extracts were determined by the Folin-Ciocalteu method (Miliauskas *et al.*, 2004), using gallic acid as a standard. Total phenolic content of the samples was determined, and the amounts of phenolic compounds in plant extracts were expressed in mg/g of extract and gallic acid equivalents (GAE), respectively. Each test was carried out three times in triplicates. Values were expressed as means.

2.4. Antimicrobial Activity Experiment

The ability to inhibit the growth of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi was observed using an overlay method (Williams *et al*., 1993). Antibacterial screening utilized the agar dilution method as previously described (Afolayan and Meyer, 1997). The dried plant extracts were dissolved in methanol to a final concentration of 50 mg/mL and sterilized by filtration through 0.45 µm Millipore filters. The activity of this solution was compared with different standard antimicrobial agents. Discs of streptomycin (S, 10 µg) and tetracyciline (TE, 30 µg) were used as positive controls for bacteria, neomycin (N, 30 µg) and nystatin (NY, 100 µg) were used as positive controls for fungi. Sterilized paper discs without the extract or antimicrobial agents were used as negative controls for all microorganisms. Final dilutions of the extract (ranging between 0.1 and 10 mg/mL) were prepared in molten Müeller Hinton agar medium (Lab M., Bury, Lancashire, UK) maintained in a water bath at 50° C. The organisms were streaked in radial patterns on the agar surface. Plates were incubated under aerobic conditions at 37° C for 24 hrs for the bacteria or 28° C for 48 hrs for the fungi. The organisms used were: *Bacillus subtitles*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter aerogenes*, *Candida albicans*, *Salmonella enterica*, *Fusarium oxysporum*, *Cladosporium macrocarpum*, and *Fusarium solani*. The bacteria were slanted on nutrient agar (Merck, Darmstadt, Germany), the yeast was slanted on Sabaroud's agar medium (Lab M., Bury, Lancashire, UK), and the fungi were slanted on potato dextrose agar medium (Lab M Limited, Bury, Lancashire, UK). Each test was conducted in triplicate. The lack of visible growth on the agar plates was used to indicate the inhibitory activity of the extracts.

2.5. MTS Assay

The cytotoxic activity of aqueous extract of *A. kurdica* was determined using Promega Cell Titer 96 AQueous Non-Radioactive Cell Proliferation (MTS) assay (Lestari et al., 2005). The MTS assay is a colorimetric test for the determination of cell viability in cytotoxicity assays. The assay utilized the human normal lung fibroblast cell line (Hs888Lu), purchased from American Type Culture Collection (ATCC, The Global Bioresource Centre, Manassas, VA, USA). Hs888Lu cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) with high glucose content, 1% non-essential amino acids (PAA Laboratory GmbH, Austria), 2% L-glutamine (200 mM) (Sigma, USA), 1% penicillin/streptomycin (100 x) (PAA Laboratories GmbH, Austria), 1% sodium pyruvate (1 mM) (Sigma-Aldrich, USA), and supplemented with 10% fetal bovine serum (FBS, PAA Laboratory GmbH, Austria). Cells (1 x 105 cells/mL) were seeded in a 96-well plate using 100 µL per well, and incubated at 37° C with 5% CO2 in a humidified atmosphere for 24 hrs before the addition of the plant extract. Dilutions of *A. kurdica* extract ranging in concentration from 125 µg/mL to 2000 µg/mL were added to the culture plate in triplicates, and then incubated for 24 hrs under the same conditions. Following the treatment, 20 µL of the MTS reagent (pre-warmed to 37° C) was added to each of the 96-wells and the plate was incubated at 37° C for 3 hrs. The absorbance was recorded using Glomax multi detection system (Promega, USA) at 492 nm. The experiment was conducted three times in

triplicates and the mean was calculated and expressed as percentage of the value observed with no plant extract treatment (control).

2.6. Statistical Analysis

The data were analyzed using One-way ANOVA test by Statistical Package for Social Sciences (SPSS) version 17.0 program. A *p* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Antioxidant Experiments

The DPPH free radical scavenging abilities of the positive control and plant extracts are expressed as a percentage of inhibition. Based on the values calculated from the linear standard curves (y = 2.002x; R2 = 0.9819) as in Table 1, the aqueous extract of *A. kurdica* showed high free radical scavenging activity towards DPPH with 69.4% ± 1.19% inhibition. The ferric reducing ability of A. kurdica was 258.33 ± 0.97 mmol/g (Table 1). The FRAP value was calculated from a standard calibration curve equation (y = 0.0011x; R2 = 0.9987) as in Table 1. The TPC of *A. kurdica* was 80 ± 0.98 mg gallic acid equivalents per g of extract (Table 1). The potent radical scavenging effect is positively associated with the high content of phenolic components consistent to what has been previously reported by Gorinstein and coworkers (Gorinstein *et al*., 2003; Qader *et al*., 2011). Furthermore, Scalzo and coworkers (Scalzo, 2005) and Giorgi and coworkers (Giorgi *et al.*, 2005) have demonstrated a correlation between antioxidant activity and TPC. Therefore, the antioxidant capacity of A. kurdica could be related to its phenolic content.

Table 1. The antioxidant activity and total phenolic compounds of aqueous extract of A. kurdica

Antioxidant assay	Gallic Acid	Vitamin C	Quercetin	*A. kurdica*	Standard Curve Equation
DPPH (%)	88.8 ± 0.41	87.5 ± 0.02	-	69.4± 1.19	y = 2.002x R^2 = 0.9819
FRAP (mmol.g^{-1})	1216.67 ± 1.02	432.67 ± 0.14	-	258.33 ± 0.97	y = 0.0011x R^2 = 0.9987
TPC (mg GA eq. g^{-1})	-	-	118± 0.37	80 ± 0.98	y = 0.9917x R^2 = 0.9984

DPPH % and FRAP (mmol.g^{-1}) values represent the mean ± SEM of triplicate experiments. "-": not used.

3.2. Antimicrobial Activity of A. kurdica Extract

The aqueous extracts of *A. kurdica* exhibited modest antimicobial activity against different microbial organisms (Table 2). The extract did not inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Salmonella enterica*, and *Fusarium solani*. On the other hand, it demonstrated modest antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cladosporium macrocarpum*, and *Fusarium oxysporium*. Among the tested microorganisms, the filamentous fungi *Cladosporium macrocarpum* and *Fusarium oxysporium*, were the most affected by the extract, with inhibition zones of 15 mm. Therefore, the *A. kurdica* plant is a potential source of novel antimicrobial compounds especially against some fungal and bacterial pathogens (Gram negative). The antimicrobial activity of this plant extract has been previously reviewed by Sharifi (2012). Generally, phenolic compounds have been illustrated to possess different bioactivities including free radical scavenging. Furthermore, the antioxidant activities of phenolics provide important protective mechanisms in a variety of disease conditions (Alshawsh *et al.*, 2012). Several studies have reported that phenolic compounds mediate the antimicrobial activities of various plant extracts (Dordevic *et al.*, 2007; Alshawsh *et al.*, 2012; Ebrahimabadi *et al.*, 2010). Hence, this study has evaluated *A. kurdica*'s antioxidant and phenolic contents as well as its antimicrobial activity.

Table 2. The antimicrobial activity of *A. kurdica* aqueous extract

Organisms	Bacteria						Fungi			
	Gram Positive		Gram Negative				Unice-llular	Filamentous		
	B.s	S.a	*E.a*	S. e	P.a	E.c	C.a	C.m	F.o	F.s
Inhibition Zone (mm)										
AKE	00	00	00	00	12	12	13	15	15	00
S 10µg	14	ND	ND	ND	ND	12	00	ND	ND	00
TE 30µg	18	ND	ND	ND	ND	23	00	ND	ND	00
N 30 µg	0	ND	ND	ND	ND	00	16	ND	ND	15
NY 100µg	0	ND	ND	ND	ND	00	00	ND	ND	15

AKE *A. kurdica* extract, *B.s*: *Bacillus subtilis*. *S.a*: *Staphylococcus aureus*. *E.a*: *Enterobacter aerogenes*. *S.e*: Salmonella enterica. P.a: *Pseudomonas aeruginosa*. *E.*c: *Escherichia coli)*. *C.a*: *Candida albicans*. *C.m*: Cladosporium macrocarpum. F.o: Fusarium oxysporum. and F.s: Fusarium solani. 00 = no inhibition zone. ND = not detected, S: streptomycin. TE: tetracycline (TE), N: neomycin, NY: nystatin.

3.3. MTS Assay

In this study, normal lung fibroblast cells (Hs888Lu) have been used for toxicity evaluation. The results of cytotoxic activity of aqueous extract of *A. kurdica* are summarized in Figure 1. Data were expressed as percentage of the value observed with no plant treatment (control). Cytotoxicity has been assessed using different concentrations of the extract. None of the extract concentrations had any cytotoxic effect as there were no significant differences ($P<0.05$) between the cytotoxicity of the plant extract concentrations and the control. Previous studies have used normal lung cells as a model to evaluate toxicity (Najim *et al.*, 2010). This is the first time that the cytotoxic activity of *A. Kurdica* has been assessed against the human normal lung fibroblast cell line Hs888Lu.

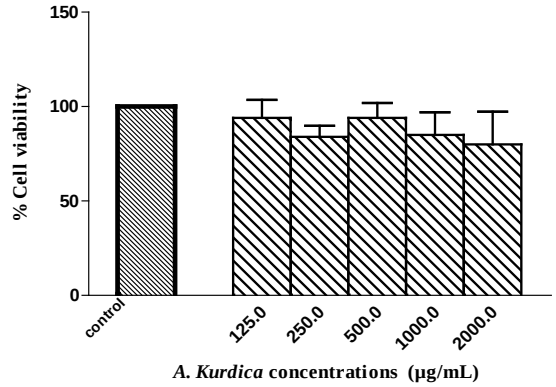

Figure 1. Cytotoxic activities of aqueous of *A. kurdica* in normal lung fibroblast Hs888Lu cell line at concentrations of 125-2000 µg/mL and 24 hrs exposure times. Each bar represents the mean ± SEM of triplicates. Control: no plant extract treatment.

4. Conclusion

In the present study, *A. kurdica* exhibited potent antioxidant activity which might be useful for the therapy or management of disorders involving ROS-mediated pathology. Further *A. kurdica* demonstrated interesting antimicrobial activity against growth of selected microorganisms. Notably, the plant extract did not have any cytotoxic effects against the normal lung fibroblast (Hs888Lu) cell line. Ultimately, this study confirmed that the aqueous extract of *A. kurdica* is able to scavenge free radicals and possessed modest antimicrobial activity.

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Conflict of Interest

The authors declare no conflicts of interest.

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