In vitro antioxidant and free radical scavenging activity of four *Alkanna* species growing in Iran

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ABSTRACT

Background: Medicinal plants are recognized as sources of natural antioxidants that can protect biological system from oxidative stress. **Objective:** In this study, the antioxidant activities of four species of the *Alkanna* genus (*Alkanna bracteosa, Alkanna frigida, Alkanna orientalis* and *Alkanna tricophila*) were evaluated. **Materials and Methods:** The dried roots of plant samples (100 g) were extracted separately by percolation method with MeOH/H_2O (80/20) and four fractions were provided respectively with hexane, chloroform, ethyl acetate, butanol and water. Antioxidant activities were investigated by using 1,1-diphenyl-2-picrylhydrazyl, ferric thiocyanate and thiobarbituric acid methods and compared with quercetin (as positive control). **Results:** The results indicated that the butanol fractions of four species had the highest antioxidant activity and radical scavenging activity compared with the other fractions studied ($P < 0.05$). The 50% effective concentration (half - effective doses) values of butanol fractions are less than quercetin and other fractions, so, these fractions showed potent antioxidant activity. This indicated that the active compounds in the root parts of *Alkanna* species dissolved in the butanol. All the fractions of four species of *Alkanna* had antioxidant activity, while, there were no significant differences ($P > 0.05$) between the total antioxidant activities of same fractions. The total antioxidant activity values of *Alkanna* fractions in a descending order are as follows: Butanol fraction > ethyl acetate fraction > total extract > hexane fraction > chloroform fraction > aqueous fraction. **Conclusion:** The antioxidant activities of butanol fractions of samples were higher than quercetin. This may be because most of the active compounds of *Alkanna* species dissolved in the butanol.

Key words: 1,1-diphenyl-2-picrylhydrazyl, *Alkanna*, antioxidant activity, ferric thiocyanate, thiobarbituric acid

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INTRODUCTION

Free radicals, like reactive oxygen species (ROS), reactive nitrogen species and reactive chlorine species, are normal by-products of metabolism, and they are introduced into the body from external sources of harmful chemicals in the environment, unhealthy foods, stress, certain drugs, cigarette smoke, etc., Increasing the intake of antioxidants can neutralize free radicals and protect the body from cell damage. In the body, oxidative stress results from an imbalance between the extent of ROS formation and the antioxidant defense mechanisms. Links between oxidative stress and adverse health effects have been suggested for several groups of diseases, including cardiovascular, respiratory and neurological diseases as well as the general aging process. Such adverse effects are mediated by free radical damage to lipids, proteins and DNA. Protection from damage occurs through the action of multiple antioxidants, some endogenously produced and some provided through dietary intake.[1,2] It is believed that medicinal plants are a potential source of antioxidants and ROS scavenger molecules.[3]

Alkanna is a genus of herbaceous plants including about 50 species of the family *Boraginacea*, originally from Europe, the Mediterranean and Western Asia. Alkannin, shikonin and their derivatives are a variety of enantiomeric hydroxylnaphthoquinone red pigments found among *Alkanna* species.[4] Alkannin and shikonin are potent pharmaceutical substances with a wide spectrum of

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biological properties, namely wound healing, antibacterial, anti-inflammatory and anticancer effects.[5-10] According to a previous study alkannin, shikonin and also extracts of *Alkanna tinctoria* roots exhibited extremely high radical scavenging activity.[11] Wound healing, antinociceptive and antiinflammatory activities of *Alkanna* genus have been pointed out in Iranian folk medicine to use this genus for wound bandage, cure inflammatory and pain-related ailments.[12] In a previous study, anti-inflammatory and antinociceptive effect of *Alkanna frigida* and *Alkanna orientalis* were determined.[12] Antiinflammatory and analgesic effects of *Alkanna bracteosa* and *Alkanna tricophila* were investigated.[13] Antimicrobial activity of sarothrin as a flavonoid from *A. orientalis* have been reported, later.[14] The aim of this study was to examine the possible antioxidant activity of four species of the genus *Alkanna* (*A. bracteosa* Boiss., *A. frigida* Boiss., *A. orientalis* (L.) Boiss. and *A. tricophila* Hub.-Mor).

MATERIALS AND METHODS

Plant materials and extraction

The roots of *A. bracteosa* (6702-TEH), *A. frigida* (6701-TEH), *A. orientalis* (6700-TEH) and *A. tricophila* (6698-TEH) were collected from the province of Mazandaran, Iran, in July 2012 and were dried in the shade. The voucher specimens have been deposited in the herbarium of faculty of pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

The dried roots of plant samples (100 g) were extracted separately by percolation method with MeOH/H_2O (80/20) 3 times at room temperature, and the fractions were provided respectively with hexane, chloroform, ethyl acetate, butanol and water. All these fractions were evaporated by rotary evaporator and stored in the refrigerator to investigate the antioxidant activity. All samples were tested at several concentration in ethanol (0.1, 0.25, 0.5 and 1 mg/mL) for antioxidant activity.

Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin and linoleic acid were purchased from Sigma chemical co. Ltd, USA. Ferrous chloride ($FeCl_2$), thiobarbituric acids (TBA), trichloroacetic acid (TCA), ammonium thiocyanate were purchased from Merck, Germany. All other chemicals and reagents were of analytical grade.

1,1-diphenyl-2-picrylhydrazylradical scavenging test

The free radical scavenging activity was measured by DPPH using the method of Blois.[15] 0.3 ml of 0.1 mM DPPH solution was added to 2.7 ml absolute ethanol and 20 μl of different concentrations of samples. After 15 min, the absorbance was measured at λma × 517 nm. Decreasing the absorbance of DPPH solution indicates an increase in DPPH radical scavenging activity.

0.3 ml of DPPH solution and 2.7 ml of absolute ethanol was used as control. Quercetin was used as positive control (0.1, 0.25, 0.5 and 1 mg/mL). The experiment was repeated 3 times. Percentage of radical scavenging activity of samples was calculated by using the equation: Inhibition% = ([A0-As]/A0) *100 that A0 is the absorbance of the control and As is the absorbance of the sample. The radical scavenging activity was expressed as 50% effective concentration (EC_{50}) (the concentration of the sample [mg/mL], required for a 50% decrease in DPPH concentration) calculated by linear regression analysis (the inhibition% was graphed against the concentrations).

Ferric thiocyanate method

Ferric thiocyanate (FTC) method was applied to evaluate the amount of peroxide at the initial stage of lipid peroxidation. The peroxide reacted with $FeCl_2$ to form a reddish ferric chloride pigment.

The concentration of peroxide decreases as the antioxidant activity increases. 2 ml of sample or quercetin (as standard), 2.05 ml of linoleic acid (2.57%) in absolute ethanol, 4 ml of 0.05 M phosphate buffer (PH 7.0) and 1.95 ml of water was mixed and placed in an oven at 40°C in the dark. 9.7 ml of ethanol (75%) and 0.1 ml of ammonium thiocyanate (30%) was added to 0.1 ml of the reaction mixture. Exactly 3 min after the addition of 0.1 ml of $FeCl_2$ (0.02 M) in 3.5% hydrochloric acid to the mixture, the absorbance was measured at 500 nm every 24 h until the absorbance of the control reached maximum. The control was subjected to the same procedures as the samples, except that for the control, only the solvent (absolute ethanol) was added.[16] Antioxidant activity was measured based on the absorbance on the final day. The percentage of antioxidant activity was calculated by following formulae:

Percentage of antioxidant activity = ([Absorbance of control – Absorbance of test]/Absorbance of control) ×100

The antioxidant activity was expressed as EC_{50} (the concentration of the sample (mg/mL), required for a 50% decrease in peroxide concentration) calculated by linear regression analysis.

Thiobarbituric acid method

The method of Endrini *et al*.[17] was used to determine the TBA values of the samples. The formation of malonaldehyde is the basis for the TBA method that used for investigation the extent of lipid peroxidation. At low

pH and temperature at 100°C, malonaldehyde binds TBA to form a red complex that can be measured at 532 nm. The increased amount of the red complex correlates with the oxidative rancidity of the lipid. Two ml TCA (20%) and 2 ml TBA aqueous solution were mixed to 1 ml of sample solution prepared as in the FTC procedure and incubated in a similar manner. The mixture was placed in boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and the absorbance of the supernatant was determined at λma × 532 nm. Antioxidant activity was measured based on the absorbance on the final day. Percentage of antioxidant activity was calculated using the formulae that were used to calculate the antioxidant activity in the FTC method. The antioxidant activity was expressed as EC_{50} calculated by linear regression analysis.

Statistical analysis

All determinations were carried out in triplicates. Analysis of variance was performed by analysis of variance procedures, and $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

In this study, the antioxidant and radical scavenging activities of four species of the genus *Alkanna* (*A. bracteosa, A. frigida, A. orientalis* and *A. tricophila*) were determined by using DPPH, FTC and TBA methods.

The results of absorbance at 500 nm of four species of the genus *Alkanna* (*A. bracteosa, A. frigida, A. orientalis* and *A. tricophila*) determined with the FTC method on the final day are shown in Figure 1. The results of total antioxidant activity of samples were shown in Table 1 in which the EC_{50} values of butanol fractions (EC_{50} = 0.17-0.20 mg/mL) are less than quercetin (as a positive control) (EC_{50} = 0.30 mg/mL) and other fractions. Hence, the butanol fractions of *Alkanna* species showed a higher total antioxidant activity compared to quercetin. There were significant differences ($P < 0.05$) between the total antioxidant activity of butanol fractions of four *Alkanna* compared with other fractions and quercetin. Results also indicated that all the fractions of four species of *Alkanna* showed antioxidant activity while, there were no significant differences ($P > 0.05$) between the total antioxidant activity of same fractions of four *Alkanna*.

The total antioxidant activity values of *Alkanna* fractions in descending order are as follows: Butanol fraction > ethyl acetate fraction > total extract > hexane fraction > chloroform fraction > aqueous fraction. The butanol fractions exert greater antioxidant activity compared to the other fractions. This indicated that the most of the active compounds in the root parts of *Alkanna* species dissolved in the butanol. The result obtained also showed that ethyl acetate fractions and total extracts of *A. bracteosa, A. frigida, A. orientalis* and *A. tricophila*

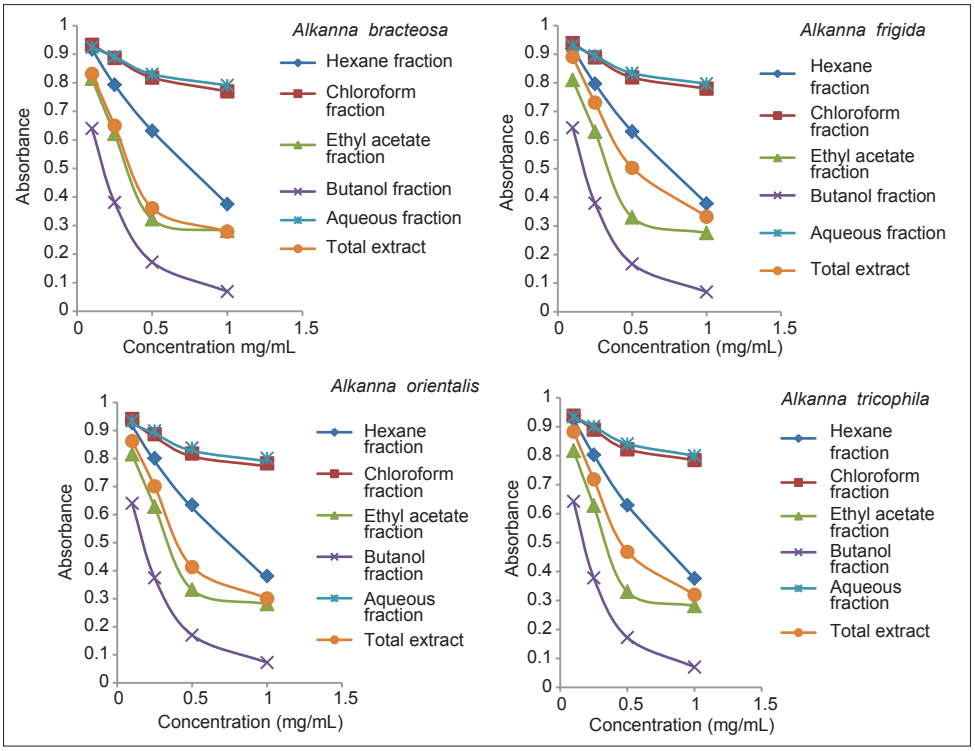

Figure 1: Antioxidant activity of four species of the genus *Alkanna (Alkanna bracteosa, Alkanna frigida, Alkanna orientalis* and *Alkanna tricophila*) determined with the ferric thiocyanate method on the final day

Table 1: Antioxidant activities of four species of the genus *Alkanna (A. bracteosa, A. frigida, A. orientalis* and *A. tricophila*) and quercetin (positive control) as expressed by half-effective doses (EC_{50}) (mg/ml)

Samples	Antioxidant reaction		
	DPPH	FTC	TBA
A. bracteosa			
Hexane fraction	0.67±0.07	0.70±0.01	0.64±0.10
Chloroform fraction	1.96±0.01	2.32±0.31	3.19±0.54
Ethyl acetate fraction	0.30±0.09	0.32±0.02	0.28±0.12
Butanol fraction	0.17±0.08	0.16±0.07	0.11±0.03
Aqueous fraction	2.07±0.53	2.75±0.64	4.63±0.74
Total extract	0.41±0.01	0.35±0.02	0.34±0.05
A. frigida			
Hexane fraction	0.70±0.01	0.70±0.07	0.64±0.08
Chloroform fraction	2.14±0.51	2.42±0.56	3.31±0.65
Ethyl acetate fraction	0.33±0.01	0.32±0.02	0.28±0.04
Butanol fraction	0.20±0.01	0.16±0.06	0.11±0.02
Aqueous fraction	2.23±0.51	2.80±0.64	4.79±0.89
Total extract	0.48±0.03	0.46±0.07	0.38±0.09
A. orientalis			
Hexane fraction	0.69±0.07	0.70±0.01	0.64±0.03
Chloroform fraction	1.95±0.51	2.44±0.74	3.22±0.50
Ethyl acetate fraction	0.31±0.08	0.32±0.05	0.28±0.07
Butanol fraction	0.17±0.03	0.15±0.05	0.10±0.01
Aqueous fraction	2.30±0.87	2.80±0.84	4.38±0.93
Total extract	0.46±0.04	0.38±0.08	0.37±0.07
A. tricophila			
Hexane fraction	0.69±0.05	0.70±0.07	0.64±0.04
Chloroform fraction	1.92±0.50	2.62±0.43	3.19±0.47
Ethyl acetate fraction	0.32±0.18	0.32±0.08	0.28±0.07
Butanol fraction	0.18±0.03	0.15±0.03	0.10±0.09
Aqueous fraction	2.13±0.57	2.77±0.76	4.45±0.78
Total extract	0.48±0.10	0.42±0.07	0.35±0.04
Quercetin positive control	0.30±0.02	0.30±0.01	0.30±0.04

DPPH: 1,1-diphenyl-2-picrylhydrazyl, FTC: Ferric thiocyanate, TBA: Thiobarbituric acid, *A. bracteosa: Alkanna bracteosa, A. frigida: Alkanna frigida, A. orientalis: Alkanna orientalis, A. tricophila: Alkanna tricophila*, EC_{50}: 50% effective concentration

had no significant differences ($P > 0.05$) compared to quercetin. So, ethyl acetate fractions and total extracts showed high antioxidant activity by using DPPH, FTC and TBA methods. On the other hand, aqueous fractions did not appear to have potential as antioxidant agents (EC_{50} = 4.38-4.79 mg/mL). Radical scavenging activity of *A. orientalis* methanolic extract was investigated, previously and EC_{50} was 0.7 mg/mL[18] that in this study, EC_{50} of *A. orientalis* total extract in DPPH assay was 0.46 mg/mL.

In the body, antioxidants act as free radical scavengers and thus protect cells from being exposed to free radicals and further cellular damage. This is the mechanism by which they protect the human body from several diseases attributed to the reactions of radicals. Numerous substances have been suggested to act as antioxidants in this genus. Various phenolic antioxidants such as flavonoids, tannins, hydroxynaphthoquinones and coumarins have been shown to scavenge radicals.[17-20] In previous studies, some flavonoids were identified from *A. orientalis*[21] and pyrrolizidine alkaloids were reported from *A. tinctoria*.[22] The different compounds can react with active oxygen radicals such as hydroxyl radicals,[23] superoxide anion radicals[24] and lipid peroxy radicals.[25,26] In addition, hydroxynaphthoquinones such as alkannin, shikonin and their derivatives have been introduced as the main constituents of the genus *Alkanna* in several studies.[4,9,11,27,28] In the previous studies, alkannin and shikonin exhibited high radical scavenging activity[11,27] while, there are not evidences that alkannin and shikonin were isolated from butanol fraction. So, hydroxynaphthoquinones and other compounds such as flavonoids can be responsible for the antioxidant activity of genus *Alkanna*.

CONCLUSION

The results of the present study indicated that the different species of *Alkanna* collected from north of Iran showed same antioxidant activity and the butanol fractions of four species had the highest antioxidant activities compared to the other fractions studied ($P < 0.05$). The antioxidant activities of butanol fractions of samples were higher than quercetin. This may be because most of the active compounds of *Alkanna* species dissolved in the butanol.

Furthermore, ethyl acetate fractions and total extracts of *A. bracteosa, A. frigida, A. orientalis* and *A. tricophila* showed potent antioxidant activity equal to that of quercetin.

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