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The effects of different inorganic salts, buffer systems, and desalting of Varthemia crude water extract on DPPH radical scavenging activity

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Abstract

The DPPH radical-scavenging activity of 25 inorganic salts, two buffer systems, and crude water extract of aerial parts of Varthemia (*Varthemia iphionoides*) before and after resins purification were investigated. Eight of the 25 inorganic salts tested quenched the DPPH radical colour. $Na_2S_2O_3$ and $FeCl_2$ showed markedly high DPPH colour-quenching activity, with inhibition of 65.3% and 47.7% respectively, at a concentration of 10 μg/ml. Four salts slightly increased the intensity of DPPH radical colour. The rest of tested salts, acetate buffer, and phosphate buffer at a concentration less than 0.1 mM did not affect DPPH radical colour. The DPPH radical-scavenging activity of BHT and catechol was considerably affected by the concentration of phosphate buffer (pH 7.0), and by acetate buffer (pH 5.0) at concentrations more than 0.01 mM in the case of BHT only. The DPPH radical-scavenging activity of a crude water extract of aerial parts of *Varthemia iphionoides* was much higher than that of an extract desalted by cation-exchange resin, indicating that iron ions apparently elevated the DPPH radical-scavenging activity of the extract. Therefore, desalting of plant extracts is important in order to obtain the true value of DPPH radical-scavenging activity.
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1. Introduction

Free radicals, which are generated in many bioorganic redox processes, may be involved in much of the oxidative damages to various components of the body (e.g., lipids, proteins and nucleic acids) and also in processes leading to mutations (Addis & Warner, 1991). Furthermore, radical reactions play a significant role in the development of chronic diseases such as cancer, hypertension, cardiac infarction, arteriosclerosis and others (Yen & Chen, 1995). In recent years, great interest has developed in the investigation of the potential of phenolic compounds, including catecholamine derivatives and plant flavonoids, as antioxidants, which play a crucial role in health maintenance and in the prevention of chronic and degenerative diseases. These compounds exhibit antiradical properties towards hydroxyl and peroxyl radicals, superoxide anions, and chelate transition metal ions, such as ferrous and ferric ions (Fernandez, Mira, Florencio, & Jennings, 2002; Morel, Lescoat, Cillard, & Cillard, 1994; Rice-Evans, Sampson, Bramley, & Holloway, 1997; Van Acker et al., 1996).

Several methods employing the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical have been developed for evaluating the radical-scavenging activity *in vitro*, because they are easy and convenient, and expensive reagents or sophisticated instrumentation are not required (Sanchez, Larrauri, & Saura, 1998). In these methods, the reduction of DPPH radical by radical scavengers is evaluated spectrophotometrically by monitoring the decrease in absorbance at 517 nm, as the DPPH radical is decolorised from deep violet to pale

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yellow. Although Blois reported in 1958 that inorganic ions interfere in the accurate determination of the DPPH radical-scavenging activity, none of these methods consider the effects of inorganic ions, either free or chelated. In a preliminary experiment, we have found that certain salts affected the DPPH radical-scavenging activity.

The aqueous extract of *Varthemia iphionoides* is commonly used in Jordanian folk-medicine for the treatment of diabetes mellitus and gastrointestinal disorders (Afifi, Saket, Jaghabir, & Al-Eisawi, 1997). Isolated flavonoids from this plant exhibited antifungal activity, antispasmodic effect on the smooth muscles of rabbits, and antiplatelet activity on human blood (Afifi & Aburaji, 2004; Afifi et al., 1991; Afifi, Al-khalil, & Aqel, 1990). The purpose of this study is to evaluate the effect of different inorganic salts, buffer systems, and desalting of a crude water extract of *V. iphionoides* on DPPH radical-scavenging activity.

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), catechol and 1,1-diphenyl-2-picrylhydazyl (DPPH) were purchased from Wako Chemical (Osaka, Japan). DOWEX 1-X8 and Amberlite-120 were obtained from Dow Chemical (Tokyo, Japan). Silica gel 60F-254 TLC from Merck, (Tokyo, Japan). Other chemicals were of analytical grade.

2.2. Plant materials

Aerial parts of *V. iphionoides* were collected from the Alouk area near Amman, Jordan, in April 2004. Botanical identification of the collected material was done by Professor H. Takruri of the Faculty of Agriculture of the University of Jordan. A voucher specimen (vi-17-002) was deposited in the Faculty of Pharmacy, University of Jordan, Amman, Jordan.

The water extract was prepared as follows: the dried and ground aerial parts of the plant (250 g) was extracted with boiling distilled water (1500 ml) for 5 min with continuous stirring, and then left for 24 h at room temperature. The extract was filtered using Whatman No. 1 filter paper. The filtrate was evaporated to dryness under reduced pressure at 40 °C on a rotary evaporator (Eyela rotary evaporator NE-series, Tokyo, Japan). The dried extract was kept in a refrigerator for further analysis.

2.3. Assay of DPPH free radical scavenging activity

The DPPH radical-scavenging activity was assayed by the Blois method (1958). DPPH, BHT and catechol were dissolved separately in methanol to give concentrations of 6×10^{-5} M, 25 μg/ml, and 0.5 μg/ml, respectively. To 100 μl of a solution containing BHT, catechol or Varthemia water extract, 900 μl of methanol were added. The solution was mixed by vortex, followed by addition of 1000 μl of DPPH solution and mixed again by vortex for 1 min. After the solution was kept at room temperature for 30 min in the dark, its absorbance was measured at 517 nm (Hitachi U-3310 spectrophotometer, Tokyo, Japan), 100 μl of deionised water being used as the control. The DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = [(\text{absorbance of the control} - \text{absorbance of the sample}) / \text{absorbance of the control}] \times 100$$

2.3.1. Assay of DPPH radical colour quenching by inorganic salt solutions and buffers

The quenching of DPPH radical colour by inorganic salts and buffers was assayed at different concentration. Final concentrations of salts ranged from 1 to 400 μg/ml, and those of phosphate buffer (pH 7.0) and acetate buffer (pH 5.0) from 0.01 to 0.2 mM. To 100 μl of an inorganic salt solution or buffer, 900 μl of methanol were added. The solution was mixed by vortex, followed by addition of 1000 μl of DPPH solution and mixing again by vortex for 1 min. Decolorisation of DPPH radical was measured as described above. The quenching action of DPPH radical colour was calculated as follows:

$$\text{Quenching of DPPH radical colour (\%)} = [(\text{absorbance of the control} - \text{absorbance of the sample}) / \text{absorbance of the control}] \times 100$$

In order to observe, the quenching action of inorganic salts an appropriate volume of inorganic salt solution was spotted on a silica gel 60F-254 TLC plate. After the plate was sprayed with 0.1% DPPH methanolic solution, and allowed to stand at room temperature for 30 min, the colour change from purple to pale yellow was observed.

2.3.2. DPPH radical-scavenging activity of BHT in the presence of inorganic salts or buffers

A reaction mixture consisted of 100 μl of the inorganic salt solution or buffer, 100 μl of BHT solution (25 μg/ml), 800 μl of methanol, and 1000 μl of methanolic DPPH solution. Decolorisation of DPPH radical was measured as described above.

2.3.3. DPPH radical-scavenging activity of catechol in the presence of inorganic salts or buffers

A reaction mixture consisted of 100 μl of the inorganic salt solution or buffer, 20 μl of catechol solution (0.5 μg/ml), 880 μl of methanol, and 1000 μl of methanolic DPPH solution. Decolorisation of the DPPH radical was measured as described above.

2.4. Desalting of water extract of Varthemia

The dried aqueous-extract of Varthemia was dissolved in distilled water to give a concentration of 30.0 mg/ml. The solution (100 ml) was applied to a column of Amber-

lite IR120 H^+ (20 g), followed by elution with 300 ml of deionised water. The final washing was done with boiled, deionised water, until the effluent was shown to be pH neutral.

The effluent was concentrated to dryness. A part of the effluent was used for determining DPPH radical-scavenging activity and the contents of iron and copper ions. The remainder was dissolved in deionised water, and applied to a column of DOWEX 1-X8 (CC grade) (20 g). The column was developed with deionised water (100 ml). The final washing was done with boiled deionised water, until the effluent was shown to be chlorine free and pH neutral. The effluent was concentrated to dryness and used for determining DPPH radical-scavenging activity and the contents of iron and copper ions.

2.5. Determination of iron and copper contents of Varthemia and water extracts

Dried aerial parts of Varthemia (0.5 g), 150 mg of the aqueous extract, 10 mg of the aqueous extract free from cations, and the aqueous extract free from salts were accurately weighed into a Teflon microwave vessel, and mixed with 7 ml of concentrated nitric acid. The samples were digested for 1 h in a microwave oven system (ETHOSD, Milestone, New York, USA) provided with a rotating 6-position carousel. After a 5 min rest under ventilation, the carousel was removed, and placed in cold water. The digest of Varthemia was diluted with deionised water to 25 ml. The digests of the others were diluted with deionised water to 10 ml. The final solutions were analysed using an inductively coupled plasma atomic emission spectrometer SPS 1700-ICP (Seiko Instruments, Tokyo, Japan).

3. Results and discussion

3.1. Quenching of DPPH radical colour by inorganic salt solutions and buffers

Of 25 inorganic salts tested, 5 inorganic salts quenched the DPPH radical colour in a dose-dependent manner (Table 1). $Na_2S_2O_3$ and $FeCl_2$ markedly quenched it by 65.3% and 47.7%, respectively, at a concentration of 10 μg/ml, and CuCl quenched it weakly, indicating that these salts probably donated one electron to the DPPH radical. No increase in quenching by $Na_2S_2O_3$ at concentrations over 5 μg/ml, and quenching by $Fe(NO_3)_3$ and $Cu(NO_3)_2$ could not be explained. I_2, KI, $K_2S_2O_5$ and $K_2Fe(CN)_6$

Table 1
DPPH radical quenching activity (%) of different inorganic salts and buffers

Inorganic salt	DPPH radical quenching activity (%)							
	1 μg/ml	5 μg/ml	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	250 μg/ml	400 μg/ml
$FeCl_2$	9.4 ± 1.9	25.6 ± 1.7	47.7 ± 5.8	74.5 ± 4.1	93.2 ± 3.3	CI[c]	CI	CI
$Fe(NO_3)_3$	4.7 ± 1.6	7.4 ± 1.9	14.2 ± 3.1	18.7 ± 2.7	21.4 ± 4.1	24.0 ± 6.3	28.1 ± 7.9	33.2 ± 5.3
CuCl	ND[b]	ND	2.7 ± 1.2	8.9 ± 2.3	14.8 ± 3.0	57.6 ± 7.3	CI	CI
$Cu(NO_3)_2$	ND	ND	5.4 ± 0.9	16.5 ± 2.1	22.8 ± 3.3	26.7 ± 4.2	28.2 ± 3.9	30.1 ± 5.3
$Na_2S_2O_3$	11.6 ± 0.8	59.9 ± 1.2	65.3 ± 1.8	65.6 ± 0.8	66.9 ± 0.0	66.9 ± 0.0	66.9 ± 0.0	66.9 ± 0.0
I_2	ND	8.3 ± 2.8	9.4 ± 2.6	13.0 ± 1.9	15.3 ± 2.7	19.4 ± 4.5	CC[d]	CC
KI	ND	1.4 ± 0.6	3.8 ± 0.8	9.0 ± 2.0	13.0 ± 2.8	17.6 ± 4.0	23.8 ± 2.1	27.3 ± 3.6
$K_2S_2O_5$	ND	1.2 ± 0.2	3.8 ± 0.8	7.9 ± 1.8	13.8 ± 2.4	16.0 ± 2.8	23.4 ± 3.2	26.2 ± 2.7
$K_3Fe(CN)_6$	–[a]	–	ND[b]	ND	CC	CC	CC	CC
K_2SO_3	–	–	ND	ND	−4.7	−10.7	−18.1	–
K_2SO_4	–	–	4.4	1.25	−6.5	−18.2	T[e]	T
K_2HPO_4	–	–	ND	ND	ND	−3.0	1.8	2.3
K_2CO_3	–	–	ND	ND	ND	ND	2.2	6.0
K_2CrO_4	–	–	ND	ND	ND	−3.0	CC	CC
$MgCl_2$	–	–	−5.0	−7.4	−8.8	−8.7	–	–
$CaCl_2$	–	–	ND	−2.0	−6.5	−8.0	–	–
$CaCO_3$	–	–	−2.7	−3.0	−2.5	2.4	–	–

Results are the average of three measurements ± S.D.
[a] Values were not measured.
[b] ND: Not detected.
[c] CI: Complete DPPH colour inhibition.
[d] CC: Changes in colour occurred.
[e] T: Turbidity occurred.

slightly quenched the DPPH colour at concentrations over 5 μg/ml. K_2HPO_4, KH_2PO_4, KCl, KF, KBr, KSCN, KNO_3, $CaCO_3$, K_2CO_3, K_2CrO_4, $MnCl_2$ and NaCl did not affect the DPPH radical colour at concentrations up to 400 μg/ml, but $MgCl_2$, $CaCl_2$, K_2SO_3 and K_2SO_4 seemed to increase the intensity of DPPH colour slightly. Furthermore, the effect of inorganic salts on DPPH colour was qualitatively confirmed by measuring the colour change from purple to pale yellow by eight inorganic salts at concentrations of 400 μg/ml (Fig. 1).

Acetate buffer did not quench DPPH radical colour at any of the concentrations examined (0.01–0.2 mM), while phosphate buffer enhanced the DPPH radical colour at concentrations above 0.05 mM.

3.2. DPPH radical-scavenging activity of BHT in the presence of inorganic salts or buffers

The radical-scavenging activity of BHT in the presence of different inorganic salts is summarised in Table 2. The DPPH radical-scavenging activity of BHT in the presence of salts is the sum of that of BHT and the DPPH radical colour-quenching activity of the salt.

Surprisingly, K_2HPO_4 and K_2CO_3 in the presence of BHT exhibited complete DPPH colour-quenching at concentrations of 10 and 50 μg/ml, respectively. These two salts (without BHT addition) showed no DPPH radical colour-quenching activity (Table 1), but the presence of these two salts with BHT in the reaction mixture increased the DPPH radical scavenging activity of BHT (Table 2). KH_2PO_4 at a concentration of 100 μg/ml exhibited DPPH radical colour-quenching activity similar to that of BHT standard at 25 μg/ml. The rest of the tested inorganic salts did not affect the DPPH scavenging activity of BHT. $FeCl_2$ at a concentration of 25 μg/ml with BHT standard showed complete DPPH colour-quenching.

Acetate buffer (pH 5.0) increased the DPPH radical scavenging activity of BHT at concentrations over 0.01 mM (data not shown). The phosphate buffers (pH 7.0) at concentration ranges of 0.005–0.20 mM with BHT standard, surprisingly exhibited complete DPPH colour quenching, even though it has been reported that the optical absorption of DPPH is stable, with respect to changes in pH from 5.0 to 6.5 (Blois, 1958).

It has been reported that, highly alkaline solutions affect DPPH inhibition (Blois, 1958). The effect of K_2HPO_4 and K_2CO_3 on DPPH inhibition appeared only when they were mixed with BHT, which may suggest that the existence of such salts in extracts possessing radical scavenger(s), may lead to inaccurate DPPH radical-scavenging activity measurement.

3.3. DPPH radical-scavenging activity of catechol in the presence of inorganic salts or buffers

The radical-scavenging activity of catechol in the presence of different inorganic salts is summarised in Table 3. The DPPH radical-scavenging activity of catechol in the presence of salts is the sum of that of catechol and the DPPH radical colour-quenching activity of salt. K_2HPO_4 and K_2CO_3 at different concentrations caused changes in DPPH colour and interfered with readings, providing inaccurate results for the actual DPPH radical scavenging activity.

K_2SO_4 and phosphate buffers showed a decrease in DPPH radical scavenging activity at increasing concentra-

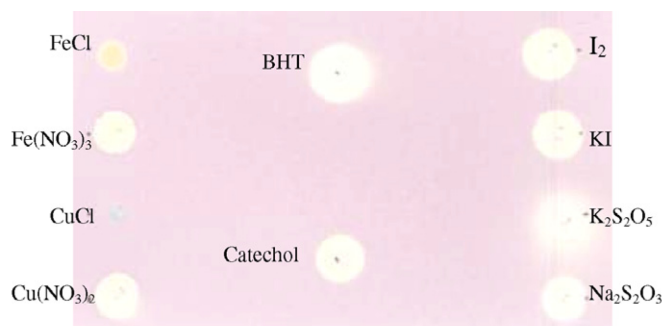

Fig. 1. The DPPH radical colour quenching by radically active inorganic salts, BHT and catechol.

Table 2
DPPH radical-scavenging activity of different inorganic salts with BHT at concentration of 25 μg/ml

Sample	DPPH radical-scavenging activity (%)						
	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml	300 μg/ml	400 μg/ml
BHT	–[a]	16.9 ± 1.0	–	–	–		–
BHT + KI	–	–	31.1 ± 1.9	38.5 ± 2.1	47.7 ± 3.2	52.1 ± 2.4	54.2 ± 3.7
BHT + K_2SO_4	–	–	20.4 ± 2.2	22.3 ± 1.9	26.6 ± 3.3	T[b]	T
BHT + K_2HPO_4	85 ± 0.7	90 ± 0.0	90 ± 0.0	CC[c]	CC	CC	CC
BHT + KH_2PO_4	24.1 ± 1.7	25 ± 2.6	28.2 ± 1.8	32.3 ± 2.2	37.7 ± 3.4	40.4 ± 3.9	40.4 ± 5.1
BHT + K_2CO_3	–	82 ± 0.0	82 ± 0.0	CC	CC	CC	CC

Results are the average of three measurements ± S.D.
[a] Values were not estimated.
[b] T: Turbidity occurred.
[c] CC: Changes in colour occurred.

Table 3
DPPH radical-scavenging activity of different inorganic salts with catechol at concentration of 0.5 μg/ml

Sample	DPPH radical-scavenging activity (%)									
	0.5 μg/ml	0.005 mM	0.01 mM	0.050 mM	0.10 mM	0.30 mM	0.6 0 mM	1.20 mM	1.8 mM	2.4 mM
Catechol (Cat.)	20.8	–[a]	–	–	–	–	–	–	–	–
Cat. + KI	–	–	–	–	–	23.7 ± 1.3	30.8 ± 2.1	34.6 ± 1.7	37.5 ± 2.6	41.6 ± 3.8
Cat. + K_2SO_4	–	–	–	–	–	20.4 ± 2.5	19.6 ± 4.0	7.2 ± 3.0	T[b]	T
Cat. + K_2HPO_4	–	–	–	CC[c]	34.8 ± 1.9	37.0 ± 2.1	CC	CC	CC	CC
Cat. + KH_2PO_4	–	–	–	17.2 ± 1.8	18.2 ± 2.2	18.7 ± 2.7	19.2 ± 3.1	19.7 ± 2.6	20.2 ± 1.9	21.4 ± 3.2
Cat. + K_2CO_3	–	–	–	–	37	CC	CC	CC	CC	CC
Cat. + Phosphate buffer	–	44.5 ± 1.9	38.6 ± 1.6	3.4 ± 0.9	2.6 ± 1.7	−17.9 T	T	T	–	–

Results are the average of three measurements ± S.D.
[a] Values were not estimated.
[b] T: Turbidity occurred.
[c] CC: Changes in colour occurred.

tions. An increase in other inorganic salts or acetate buffer (pH 5.0) concentrations did not cause significant differences to the DPPH radical scavenging activity of catechol. $FeCl_2$ at a concentration of 25 μg/ml with BHT standard showed complete DPPH colour-quenching.

3.4. DPPH inhibition of crude water and resins-purified extracts of Varthemia

The results described above showed that certain inorganic salts occurring in aqueous extracts of plants probably affected the DPPH radical-scavenging activity of the extracts. The activity of the water extract of Varthemia was examined before and after desalting. The DPPH-scavenging activity of the crude water extract of Varthemia after cationic-resin purification was lower than that of the crude water extract before resins purification (Table 5). This may be related to the higher amount of iron and other cations of lower valence state that existed in the crude water extract of Varthemia (Table 4). Iron and copper were shown to be examples of interfering inorganic ions, although the existence of other inorganic ions in lower valence states is possible.

Table 4
Iron and copper contents of *Varthemia iphionoides* and its water and resins-purified extracts

Sample	Iron (μg/g)	Copper (μg/g)
Varthemia	480.5 ± 7.0	7.86 ± 0.1
Crude water extract	5.2	ND[a]
Cationic effluent	1.0	ND
Cationic and anionic effluent	ND	ND

Results are the average of three measurements ± S.D.
[a] ND: Not detected.

The anionic effluent of the crude water extract exhibited the highest DPPH-scavenging activity out of all the extracts. This may be related to the actual DPPH radical-scavenging activity of radical scavenger(s) present in this extract, and the removal of all inorganic matter. These results were similar to a study carried out on spinach by Aehle et al. (2004), showed that the antioxidant efficiency of the spinach polyphenol extracts was significantly increased by the elimination of a major part of the non-phenolic components.

In many cases, when more than one method is used for the determination of radical-scavenging activities of crude extracts, e.g., the use of DPPH and ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), quite different results were obtained for the same extract. In many cases these differences are either due to the kinetics of radical scavenging reactions in the different systems or the ability of compounds to act as antioxidants in particular systems. However, the interference from inorganic ions is not usually considered.

Hence, for a comprehensive assessment of scavenging activities of plant extracts, cationic and anionic resin purification of the extract to eliminate free or chelated metal ions, that may alter the results, should be taken into consideration.

Table 5
DPPH radical-scavenging activity (%) of crude and resin purified extracts of *Vartheimia iphionoides*

Varthemia extracts	DPPH radical-scavenging activity (%)			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
Crude water extract	28.9 ± 2.5	52.1 ± 6.3	84.6 ± 1.7	CI[a]
Cationic-purified extract	20.0 ± 0.3	29.5 ± 2.5	53.9 ± 2.8	CI
Cationic–anionic purified extract	46.2 ± 2.2	72.8 ± 0.7	CI	CI

Results are the average of three measurements ± S.D.
[a] CI:Complete inhibition of DPPH colour.

4. Conclusions

DPPH is a good, rapid, easy and convenient method for the determination of antiradical activities for pure compounds or crude plant extracts, but the existence of inorganic ions, especially in lower valence states, may interfere and must be eliminated or determined separately. The use of buffers at certain concentrations also should be considered when this method is used. Good results may be obtained by using this method after the resins purification of crude extracts because most radically active compounds like flavonoids have the ability to chelate iron ions, which lead to inaccurate estimation of the actual DPPH radical scavenging activity. Acetate buffers can be used in any extracts, but phosphate buffers have limited use.

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