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Phytochemical and Biological Investigation of *Nitraria retusa Asch*

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Dedicated to the soul of Prof. Abdullah El-Alali

ABSTRACT

Nitraria retusa Asch (Zygophyllaceae) of Jordanian origin has been investigated phytochemically and biologically. Three compounds, two new and one previously known, have been isolated and identified. The new compounds are 5,7-dihydroxy-3-deoxy vasicine (I) and 7-hydroxy-3-deoxy-1-vasiciene (II), while the known compound is *O*-acetyl nitraraine (I) but reported for the first time from this species. The relative levels of antioxidant activity and total phenolic content of the methanolic extract of *Nitraria retusa* and fraction B (the alkaloid rich fraction) were determined using the improved $ABTS^{\bullet+}$ method and the Folin-Ciocalteu colorimetric method, respectively. The total phenolic content of the methanolic extract and fraction B was found to be 234.66 and 114.53 mg GAE/g extract, respectively, while the total antioxidant capacity was found to be 429.3 and 809.9 μmol TE/g extract, respectively. The methanolic extract and fraction B were also tested for their general cytotoxicity using the brine shrimp lethality test (BSLT),but no activity was detected (LC_{50} >1000 ppm).
Keywords: *Nitraria retusa*, Al-Ghardaq, antioxidant activity, total phenolic content, Zygophyllaceae..

INTRODUCTION

Nitraria retusa Asch belongs to the family Zygophyllaceae.[1,2] The Zygophyllaceae (the bean caper or creosote bush) family contains 27 genera and 270 species of mostly shrubs, but also trees or annual or perennial herbs are included.[3] Species belonging to the genus *Nitraria* are widely distributed around the world; they are well adapted to arid climates, and some of them grow in the deserts and salt marshes of Central Asia (*Nitraria sibirica* Pall, *Nitraria schoberi* L., and *Nitraria komarovii*[4]), in the desert regions of South-East Europe (*Nitraria komarovii*, *Nitraria sibirica*), in the Middle East (*Nitraria schoberi*), in Australia (*Nitraria billardieri*), and in Africa (*Nitraria tridentata* or *Nitraria retusa*[3]).

The *Nitraria* genus is known to be rich in alkaloids, which are classically classified into three major groups (Figure 1): tripiperidine alkaloids (e.g., schoberine), indole alkaloids (e.g., nitrarine), and a group of spiro alkaloids which can be divided into two sub-groups, simple spiro alkaloids (e.g., sibirine) and complex spiro alkaloids (e.g., nitraramine, 1-pinitraramine).[5]

Of the 15 species of the genus *Nitraria*[6], two are reported to grow in Jordan. These are *Nitraria schoberi* and *Nitraria retusa* (or *Nitraria tridentata*)[1,2].

As part of our continuing endeavor to investigate the medicinal plants of Jordanian origin, a phytochemical investigation and biological evaluation of *Nitraria retusa* (Forssk.) Asch were pursued.

Nitraria retusa Asch is found flowering from April to May. It is known locally as Al-Ghardaq and described as a perennial, shrubby, spiny plant, 1-2 m long with many hanging stems, forming hemispherical growth. The leaves of the plant are fleshy, triangular, truncated to lobed, and greyish in colour. It has little white flowers (with five petals and 15 stamens) and fleshy red fruits with a diameter of 1 cm and three pyramidal chambers.[1,2]

Nitraria retusa is an important sand controller, and its leaves and twigs are occasionally grazed by sheep, goats and camels.[7,8] Its fleshy red fruits are eaten by human

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beings and birds, its wood is used as fuel by the local inhabitants[9], its bitter fleshy leaves are used as a poultice to reduce swellings, and its ashes are used to dry fluids secreted by infected wounds.[10,11]

From the leaves and young stems of *N. retusa*, Halem et al. isolated and fully characterized six flavonol glycosides: isorhamnetin 3-O-4-rham-galactosylrobinobioside, isorhamnetin 3-robinobioside, isorhamnetin 3-rutinoside, isorhamnetin 3-galactoside, isorhamnetin 3-glucoside, and free isorhamnetin.[12]

In this work and from the methanolic extract of the aerial parts of *N. retusa*, three compounds were isolated and characterized (Figure 2). Two of these are new: 5,7-dihydroxy-3-deoxy vasicine (I), and 7-hydroxy-3-deoxy-1-vasiciene (II); the other compound is known but new to the species: *O*-acetyl nitraraine (III). The structures of the compounds were elucidated by their spectral data (low resolution APCI-MS, high resolution ESI-MS, ^{1}H-NMR and 2D-COSY). Moreover, the relative levels of antioxidant activity and total phenolic content of the methanolic extract and fraction B (the alkaloid rich fraction) were determined using the improved $ABTS^{\bullet+}$ method and the Folin-Ciocalteu colorimetric method, respectively. The methanolic extract and fraction B were also tested for their general cytotoxicity using the brine shrimp lethality test (BSLT).

RESULTS AND DISCUSSION

Phytochemical Investigation of *N. retusa*

Methanolic extracts of the aerial parts of *N. retusa* were fractionated and purified as outlined in the experimental procedure. High concentrations of alkaloids were evident in the thin-layer chromatography (TLC) of fraction B as detected by distinctive green-brown spots after spraying with 5% phosphomolybdic acid in ethanol and the formation of a definite turbidity when treated with Wagner's and Mayer's reagents.

Compound I (1.26 mg, 0**.**00014% wt/wt of whole plant) was isolated as a yellowish powder. The atmospheric pressure chemical ionization (APCI) mass spectrum showed a parent molecular ion peak at *m/z* 205 $(M+H)^+$. The high resolution ESI-MS data (obsd. m/z 227.07910, calc. 227.07965 for $[M+ Na]^+$) revealed the molecular formula as $C_{11}H_{12}N_2O_2$, which corresponded to 7 degrees of unsaturation. The ^{1}H-NMR spectrum indicated the presence of a tetra substituted benzene ring and showed signals assignable to two aromatic protons at δ_H 6.92 (H-6) and 7.55 (H-8), and four methylene protons at δ_H 3.69 (H_2-1), 1.35 (H_2-2) and 2.35 (H_2-3). A broad doublet at 4.36 was assigned to the H-9 protons. Examination of the 2D-COSY data revealed correlations between H-1 and H-2 and between H-2 and H-3. The absence of a strong *J* coupling ^{1}H-^{1}H COSY correlation between the H-6 and H-8 protons of the phenyl ring provides proof of the existence of the two hydroxyl groups at the meta positions establishing the structure of I as 5,7-dihydroxy-3-deoxy vasicine, a new vasicine derivative, belonging to the quinazoline class of alkaloids.[13,14] The available spectral data were indicative that this compound is structurally related to vasicine which was isolated and identified from *Peganum harmala* and *Adhatoda vasica*.[15,16] Due to the low yield (0**.**00014% wt/wt), we were unable to run ^{13}C, HMBC or ROSEY.

Compound II (2.2 mg,0**.** 00024% wt/wt of whole plant) was obtained as a yellowish powder. The APCI mass spectrum showed a parent molecular ion peak at *m/z* 186.9 $(M+ H)^+$. The molecular formula was determined as $C_{11}H_{10}N_2O$ by HRESIMS data (obsd. m/z 186.07876, calc. 186.07931 for $[M]^+$), corresponding to 8 degrees of unsaturation. The spectral data of the compound indicated a structural similarity with the aforementioned alkaloid. The ^{1}H-NMR spectrum indicated the presence of a tri-substituted benzene ring. An AB pattern at 7.98 and 6.88 ppm (J = 8) was assigned to the aromatic protons H-5 and H-6, respectively. A singlet aromatic proton at 6.63 ppm was assigned to the H-8. A broad singlet at 3.93 was assigned to the H-9 protons, while the broad doublet at 5.72 and the broad peak at 5.35 ppm to the methine protons H-1 and H-2, respectively. The broad multiplet at 2.37 ppm was assigned to the H-3 protons. ^{1}H-^{1}H COSY revealed a correlation between H-1 and H-2 protons. A proof for the existence of one hydroxyl substituted

benzene ring was provided by the strong ^{1}H-^{1}H COSY correlation which was observed between H-5 and H-6 protons, establishing the structure of II as 7-hydroxy-3-deoxy-1-vasiciene.

One known alkaloid, but new to the species, was also isolated from fraction B and its chemical structure was identified by ^{1}H-NMR, APCI mass spectrum analysis and comparison to literature data. The spectral data for compound III (2.59 mg, 0.00003% wt/wt of whole plant) were found to be in full agreement with those reported in the literature for *O*-acetylnitraraine.[17]

Biological Evaluation of *N. retusa*

The methanolic extract and fraction B of *N. retusa* were tested for their antioxidant activity and total phenolic content using the improved $ABTS^{\bullet+}$ method and the Folin-Ciocalteu colorimetric method, respectively, and for their general cytotoxicity using the BSLT.

Determination of Total Antioxidant Activity

The improved $ABTS^{\bullet+}$ method as described by Re et al. was used to determine the antioxidant capacity in this work.[18] The concentration-response curve for $ABTS^{\bullet+}$ as a function of five separately prepared stock solutions of Trolox standard (0.25, 0.5, 1, 1.5, and 2 mM) are shown in Figure 3A in terms of Trolox equivalent antioxidant capacity (TEAC, μmol Trolox equivalents per g plant extract). The results obtained were shown in the Table 1. *Nitraria retusa* methanolic extracts and fraction B showed high antioxidant activity with 429.3 and 809.9 μmol TE/g extract, respectively.

Determination of Total Phenolic Content

Total phenolic content were estimated using the Folin-Ciocalteu colorimetric method based on the procedure of Singleton and Rossi, using gallic acid as a standard phenolic compound.[19] A linear calibration curve of gallic acid in the range 20-500 μg/mL with r^2 value of 0.9992 was constructed (Figure 3B). The total phenolic content is expressed as gallic acid equivalents (GAE) in milligrams per gram extract. The results obtained were shown in Table 1. *N. retusa* methanolic extracts and fraction B showed a high total phenolic content of 234.66 and 114.53 mg GAE/g extract, respectively.

As noticed above, fraction B showed higher antioxidant activity (809.9 μmol TE/g extract) than the methanolic extract (429.3 μmol TE/g extract), while the methanolic extract showed a higher total phenolic content (234.66 mg GAE/g extract) than fraction B (114.53 mg GAE/g extract). These facts indicate clearly that non-phenolic chemotypes with strong antioxidant activity may be uncovered from fraction B where a lower phenolic content was associated with high-antioxidant activity.

Brine Shrimp Lethality Test (BSLT)

The crude methanolic extract and fraction B of *N. retusa* were tested for their general cytotoxicity using the BSLT.[20,21] Neither the crude methanolic extract nor fraction B showed any activity with LC_{50} values > 1000 ppm.

General Experimental

NMR experiments were performed in $CDCl_3$ with TMS as an internal standard; gs-COSY and ^{1}H NMR spectra were acquired using a Bruker 400 MHz NMR spectrometer (Fällanden, Switzerland). Low-resolution APCIMS were determined on an Agilent® (Palo Alto, CA, USA) ion-trap LC/MS system. High-resolution mass spectra (HRMS) were recorded by ESIMS on an Apex IV 7 Tesla Fourier-transform ion cyclotron resonance mass spectrometer (Bruker Daltonics). HPLC was performed on a Lachrom® MERCK-HITACHI (Tokyo, Japan), equipped with quaternary gradient L-7150 pump, L-7455 Diode-Array Detector, L-7200 auto-sampler, and D-7000 Interface. The preparative HPLC column was a Hibar® MERCK, pre-packed column RT 250-25, Lichrosorb® RP-18 (7 μm). The PTLC was carried out on 20×20 cm plates with silica gel F_{254} (Merck KGaA, Germany). Column chromatography was carried out using silica gel 60 (0.06-0.2 mm; 70-230 mesh) and the TLC utilized silica gel 60 with gypsum and pigment addition for UV visualization (both from Scharlau Chemie S.A., Barcelona, Spain). The TLC spots were visualized by UV (VILBER LOURMAT, 4 W-254 nm Tube) or made

visible by spraying the developed plates with 5% phosphomolybdic acid in EtOH.

The total antioxidant capacity assay was performed on a MultiSpec-1501, SHIMADZU® photodiode diode array spectrophotometer (Kyoto, Japan), fitted with Julabo F40, Ultratemp 2000 temperature control. The total phenolic content assay was carried out using a Spectronic 601 spectrophotometer (Milton Roy Company, USA). The incubator B 28 #04-68155 was obtained from Binder GmbH (Tuttlingen, Germany).

HPLC grade MeOH and EtOH were obtained from Scharlau Chemie S.A. (Barcelona, Spain) and Fisher Scientific UK limited (Loughborough, Leicestershire UK), respectively. ABTS® was obtained from AppliChem GmbH (Ottoweg, Darmstadt, Germany), and potassium persulfate and Trolox® from Acros Organics (New Jersey, USA). Folin-Ciocalteu reagent (2N) was from Scharlau Chemie S.A. (Barcelona, Spain). Gallic acid monohydrate and sodium carbonate were from Janssen Chemica (Geel, Belgium) and Frutarom Ltd. (Berkhamsted, UK), respectively. Petroleum ether and dichloromethane were obtained from Scharlau Chemie S.A. (Clwyd, UK) and Gainland Chemical Company (Clwyd, UK), respectively. HPLC grade chloroform, analytical grade methanol, and HPLC grade acetonitrile were from TEDIA Company, USA. Dodeca-molybdophosphoric acid AR was obtained from S D Fine-Chem Ltd. Instant Ocean®, Synthetic Sea Salt, and Brine shrimp eggs (100% Artemia cysts) were obtained from Aquarium Systems. USA and Ocean Star® International (O.S.I.) Inc., respectively.

Plant Material

The leaves and stems of *Nitraria retusa* Asch (Zygophyllaceae) were collected during the flowering stage in April-May 2007 in the eastern part of Jordan in Al-Azraq and the southern part of Jordan in the Dead Sea. Eng. Mohammad Ghraibah, field taxonomist, Faculty of Agriculture, Jordan University of Science and Technology identified the collected materials. A voucher specimen (PHC# 113) was deposited at the herbarium museum of the Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan. The plant raw material was cleaned up and air-dried at room temperature. After drying, exact weight was recorded; where it was found to be 9.1 kg. Plant material was then grounded to powder using a laboratory mill, RetschMühle (RETSCH GmbH, Haan, Germany), stored at room temperature (22-23 °C), and protected from light until required for analysis.

Extraction and Fractionation

Extracts were prepared using cold maceration and the Soxhlet apparatus. In the cold maceration, extracts were generated via infusion by soaking each 300 g of the plant materials in 2 L methanol at room temperature for 24 h with intermittent shaking, followed by filtration to separate the marc; this process was repeated three times for 3 total days of extraction. The filtrates were combined and dried under reduced pressure to yield the MeOH fraction. In the second method, plant material was extracted with MeOH in a Soxhlet apparatus for 7 h for exhaustive extraction. The methanolic extract was then collected and concentrated, almost to dryness (MeOH fraction) under vacuum at 45°C using a rotary evaporator (RE 200, Bibby Steriline Ltd., Stone, UK). Methanolic fractions were then combined and used for further purification and fractionation.

The MeOH-extract was dissolved in 5% acetic acid, and then extracted with petroleum ether (Fraction A), after which the aqueous acid residue was made alkaline (pH 10) with 12.5% NH_4OH followed by extractions three times with CH_2Cl_2 (Fraction B) (4.53 g).

The TLC was carried out for fraction B using $CHCl_3$/MeOH [9:1] or $CHCl_3$/acetone/triethylamine [12:6:2] as a developing system. The spots were then visualized by spraying the plate with a 5% phosphomolybdic acid solution and then heating to 100 ºC for a few minutes, where yellowish and green-brown spots were observed. Methanolic extracts and Fractions A and B were tested for the presence of alkaloids using Wagner's and Mayer's reagents.

Depending on the TLC and the results of the

qualitative tests of alkaloids, conventional gravity-driven, open-column chromatography of fraction B (3.94 g) was carried out for fractionation and isolation of alkaloids. Gradient elution was used, starting with 50% hexane/dichloromethane (500 mL), then 100% dichloromethane (5 L). After that, the column was eluted with a mixture of methanol/dichloromethane of increasing polarity using the following system: 0.5% (7 L), 1% (15 L), 1.4% (24 L), 1.8% (12 L), 2% (8 L), 2.2% (8 L), 2.6% (7 L), and 3% (6 L). The eluted fractions were followed by TLC using $CHCl_3$/MeOH [9:1] or $CHCl_3$/acetone/triethylamine [12:6:2] and similar fractions were combined into pools.

About 237 fractions were collected from the chromatographic column, which were then grouped into 18 pools. The 18 pools were found to be rich in alkaloid content, so they were all subjected to further purification using a reversed-phase semi-preparative HPLC method (method A) and separated into 9 pools. These were then subjected to further purifications using a normal-phase preparative HPLC method (method B) for which the Lachrom® MERCK-HITACHI HPLC equipped with a quaternary gradient L-7150 pump connected to a manual injector, a L-7455 diode-array detector, a Hibar® MERCK, pre-packed column RT 250-25 and a Lichrosorb® RP-18 (7 µm) preparative HPLC column was used. The mobile phase was a gradient blend of 0.1% formic acid in water and acetonitrile (100:00 to 50:50 over 40 min), method A, and 9% methanol in chloroform and chloroform (05:95 to 90:10 over 48 min), method B. The flow rate used was 10 mL/min; the detector was set at 220 nm (method A) and at 250 nm (method B) and the injection volume was 2 mL.

The samples to be injected were dissolved in 2 mL mobile phase 1:1 ratio. The samples, in the range of 75-150 mg, were injected manually. Effluent solutions were collected according to the eluted peaks, as they appeared on the monitor, in 15-20 mL vials, and allowed to dry in the air. The dried residues were then dissolved in chloroform (HPLC grade, Scharlau) and checked by TLC to ensure purity using chloroform/methanol [91:9] (v:v) systems. Fractions obtained from semi-preparative HPLC were applied for further clean up by preparative thin-layer chromatography using dichloromethane/methanol, as a developing system of (9:1) ratio.

Structural Elucidation of Isolated Compounds

Chemical structures of isolated compounds have been elucidated using different spectroscopic and spectrometric techniques, principally, Mass Spectroscopy (APCI-MS), high resolution ESI-MS, One Dimension Nuclear Magnetic Resonances (1D-NMR), Proton Nuclear Magnetic Resonances (^{1}H-NMR) and 2D-NMR (COSY).

Biological Evaluation of *N. retusa*

The methanolic extract and fraction B of *N. retusa* were tested for their antioxidant activity and total phenolic content using the improved $ABTS^{\bullet+}$ method and the Folin-Ciocalteu colorimetric method, respectively, and for their general cytotoxicity using the BSLT.

Determination of Total Antioxidant Activity

The antioxidant capacity assay was carried out using the improved $ABTS^{\bullet+}$ method as described by Re et al.[18]

Determination of Total Phenolic Content

The methanolic extract and fraction B of *N. retusa* were tested for their total phenolic content. Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method based on the procedure of Singleton and Rossi (1965), using gallic acid as a standard phenolic compound.[19]

Brine Shrimp Lethality Test (BSLT)

BSLT was used to test the methanolic extract and fractions A and B for their general cytotoxicity. The BSLT was performed as described previously by Meyer et al and McLaughlin and Rogers using (-)-colchicine as a positive control.[20,21]

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Table 1: Antioxidant activity and total phenolic content results of *Nitraria retusa.*

Plant Fraction	Antioxidant activity (*μ*mol TE/g extract)	Total Phenolic Content (mg GAE/g extract)
Methanolic extract	429.3 ± 2.4	234.66
Fraction B	809.9 ± 10.6	114.53

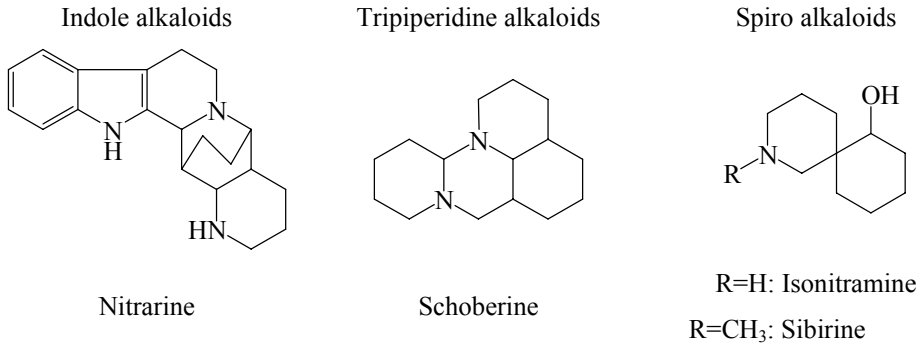
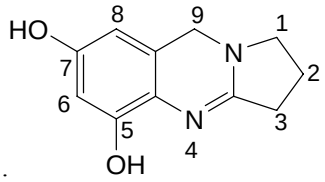
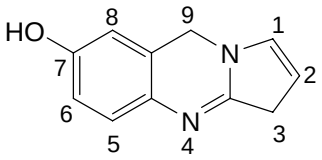


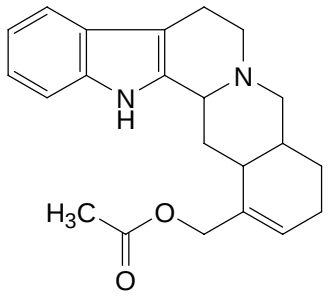
Figure 1: Major alkaloidal groups of the *Nitraria* genus.



5,7-dihydroxy-3-deoxy vasicine (I)



7-hydroxy-3-deoxy-1-vasiciene (II)



O-acetylnitraraine (III)

Figure 2: Chemical structures of isolated compounds.

A) B)

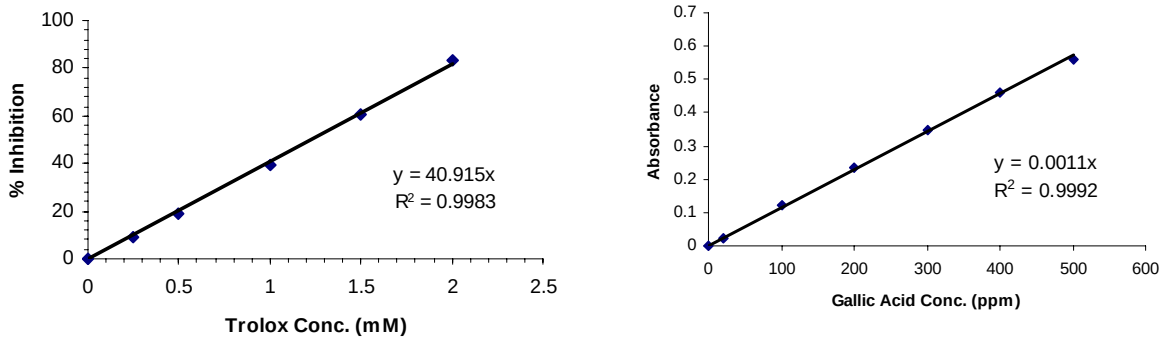


Figure 3: Concentration-response curve for the absorbance at (A) 734 nm for $ABTS^{\bullet+}$ as a function of a standard Trolox solution and at (B) 765 nm for a gallic acid standard.

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فصل المركبات الفعالة لنوع من نبات تابع لجنس الغرقد ودراسة فعاليته البيولوجية

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ملخص

تم في هذا البحث دراسة نوع من نبات تابع لجنس الغرقد Nitraria retusa، ينمو في الأردن، كيميائياً وبيولوجياً. تم فصل البنية الكيميائية وتحديدها في ثلاثة مركبات، اثنان منهما جديدان، والثالث معروف سابقاً، ولكن يفصل لأول مرة من هذا النبات. المركبان الجديدان، هما: 5,7-dihydroxy-3-deoxy vasicine (I), and 7-hydroxy-3-deoxy-1-vasiciene (II)، والمركب المعروف سابقاً، هو :(III) O-acetylnitraraine . تم تحديد محتوى المستخلص الكحولي الخام والجزء الغني بالقلويدات [الجزء(ب)]، من المواد الفينولية الكلية، وتقييم فعاليتها كمضادة للأكسدة، باستخدام، وعلى التوالي، طريقة Folin-Ciocalteu اللونية، وطريقة ABTS•+ المحسنة. أظهر المستخلص الكحولي الخام والجزء (ب) 234.66 و114.53 GAE لكل غرام، على التوالي، من المواد الفينولية. أما بالنسبة للفعالية كمضادة للأكسدة، فكانت 429.3 و809.9 مايكرومول TE لكل غرام، على التوالي. تم اختبار سمية المستخلص الكحولي الخام والجزء (ب) على يرقات القريدس، ولم تظهر أي منها فعالية.

الكلمات الدالة: Nitraria retusa، الغرقد، مضادات الأكسدة، المواد الفينولية الكلية، Zygophyllacea.

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