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SEASONAL VARIATION IN PROPHYLACTIC SECONDARY METABOLITES OF *VARTHEMIA CANDICANS* IN TWO COASTAL HABITATS IN EGYPT

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Varthemia candicans (Delile) Boiss., naturally grows in the Northwestern costal region of Egypt. The present study was conducted to evaluate the diversity of *V. candicans* secondary metabolites in two habitats during different seasons. Results showed that flavonoids, alkaloids, saponins, and phenolic compounds content attenuated their maximum value during spring in sand dune habitat, however, it reached a peak during summer in Wadi Habbes habitat. HPLC analysis of phenolic compounds from aerial parts showed two common phenolic compounds in both habitats, eight unique phenolic compounds in the plant growing in sand dune, and four phenolic compounds characteristic to Wadi Habbes plants. The high antioxidant activity of the plant was attributed to its high phenolic compounds content.

Keywords: *Varthemia candicans*, phenolic compounds, alkaloids, medicinal plant, Egypt, HPLC

Nature has bestowed on people a very rich botanical wealth and a large number of naturally occurring plants in different parts of the world. Natural products from plant resources, including bio-molecules and secondary metabolites, usually exhibit some kind of biological activities in traditional and modern medicine. Due to their immense healing properties, plants are widely used in the human therapy, veterinary, agriculture, scientific research and in countless other areas (Kandukuri et al., 2009 and Varalakshmi et al., 2011).

The flora of Egypt comprises about 2165 species of plants distributed in different localities that vary in type of soil and prevailing climatic and other environmental conditions support a wide range of plant species. In addition, many plants have been successfully introduced and naturalized in Egypt (Boulos, 2005 and Haggag, 2009). Medicinal plants of Egypt represent an important source for investigations of antioxidants as medically important compounds. The medicinal value of these plants lies in some active chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, terpenoids and phenolic compounds (Edeoga et al., 2005).

The genus *Varthemia* is a member of Asteraceae, that encompasses about five species distributed throughout the Mediterranean region (Bremer, 1994). In Egypt, the genus *Varthemia* encompasses three species namely *V. candicans*, *V. montana* and *V. iphionoides* (Täckholm, 1974). *Varthemia candicans* is an aromatic perennial herb, that inhibits rocky places, semi-dryland and sand dunes (El-Kady, 1993).

Biochemical studies on the different species of the genus *Varthemia* led to the isolation of volatile constituents (Romero et al., 2003), squiterpenes (Sanchez et al., 2000 and Benito et al., 2002), monoterpenes (Ahmed and Jakupovic, 1990) and flavonoids (Rubio et al., 1995). To date, very few/or no phytochemical studies on *V. candicans* have been reported, although this genus was the subject of several chemical investigations. Some *Varthemia* species have antiprotozoal (Villaescusa et al., 2000), antimicrobial (Hammerschmidt et al., 1993), anti-inflammatory (Benito et al., 2002) and antidiabetic activities (Al-Howiriny et al., 2005).

The present study aimed to evaluate the antioxidative potential of *V. candicans* aerial parts inhibiting rocky places and loosely-attached sandy places in the Northwestern coastal region of Egypt during different growth seasons. Antioxidative potential in this study is represented in the pharmacologically active secondary metabolites including flavonoids, alkaloids, saponins and phenolic compounds.

MATERIALS AND METHODS

1. Collection of Plant Materials

The aerial parts of *V. candicans* were collected during 2011-2012 throughout the growth seasons (winter, spring, summer and autumn) from Wadi Habbes (master upstream rocky habitats), and oolitic sand dunes. The plant samples were washed with tap water thwice, followed by distilled water. The samples were then oven-dried at 60°C for five days. The dried samples were ground to fine powder, sieved through 0.2 mm sieve and stored in paper bags for further analysis.

The ethanolic extract was prepared by immersing the dry powders in 80% ethanol for six hours, filtered through Whatman No. 1 filter paper. The residues were re-extracted again for more 2 times as previous and the clear supernatants were collected, combined and completed into definite volume.

2. Phytochemical Analysis

The obtained ethanolic extract (80%) was used for the quantification of secondary metabolites. Total flavonoids content was estimated by aluminum chloride colorimetric method according to Chang et al. (2002). The total alkaloids content was evaluated spectrophotometrically according to the method by Shamsa et al. (2008). Total saponin content was estimated quantitatively by the method described by Hiai et al. (1975). Total phenolic compounds content was estimated quantitatively using the method adopted by Jindal and Singh (1975).

3. HPLC Analysis of Phenolic Compounds

Phenolic compounds of plant sample were extracted according to the method outlined by Ben-Hammouda et al. (1995). The samples were filtered through a 0.22 µm filter membrane (Millipore, UK) prior to HPLC analysis. 50 µl of the sample (diluted accordingly) was injected onto hypersil C_{18} reversed-phase column (250 x 4.6 mm) (Dionex Ultimate 3000 UHPLC) connected with Diode array Detector (Chromeleon 7.1.1.1127).

A constant flow rate of 1.2 ml/min was used with two mobile phases: (A) 5% acetic acid in distilled water at pH (2.65) and (B) 0.5% acetic acid in 99.5% acetonitrile. The elution gradient was linear, starting with (A) and ending with (B) over 35 minutes. Phenolic compounds were detected by UV detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standers mixture chromatogram. The concentration of an individual compound was calculated on the basis of peak area measurements, and then converted to µg phenolic g^{-1} dry weight. All chemicals and solvents used were HPLC spectral grade (Sigma-Aldrich).

4. Evaluation of Total Antioxidant Activity

A procedure slightly modified from the method reported by Brand-Williams et al. (1995) and Bondet et al. (1997) was followed to evaluate the antioxidant capacity of the ethanolic extract using DPPH radical.

5. Statistical Analysis

Statistical analysis of the obtained results was carried out according to the technique adopted by Norusis (2007). Data were analyzed by one-way ANOVA with the Duncan's multiple range test (DMRT) to separate means with significance levels at $P \leq 0.05\%$. All statistical analyses were carried out using SPSS 19.0 software. The results are represented as the mean of three replicates ± standard deviation.

RESULTS AND DISCUSSION

1. Total Flavonoids Content

The total flavonoids content of *V. candicans* aerial parts collected from Wadi Habbes (WH) and Sand Dunes (SD) habitats at the different growth seasons is represented in fig. (1). Total flavonoids varied significantly with habitat, growth season and their interaction. Total flavonoids of *V. candicans* in WH increased from winter to autumn but this increase was slight in autumn. Flavonoids content in this habitat fluctuated between a maximum (8.2 mg g^{-1} d wt) at summer and a minimum (3.41 mg g^{-1} d wt) in winter. The flavonoids content of *V. candicans* in WH habitat was greater by 100.3%, 139.9% and 112.9% in spring, summer and autumn in comparison with winter, respectively. In the SD habitat, the total flavonoids content of *V. candicans* increased prominently from winter to spring and summer seasons, but the increase was attenuated by autumn season. Flavonoids content varied from its maximum (12.2 mg g^{-1} d wt) in the spring to its minimum (4.7 mg g^{-1} d wt) in the winter. The flavonoids content oscillation in SD habitat was 162.4, 149.6 and 7.3% higher in spring, summer and autumn, respectively as compared to that in winter.

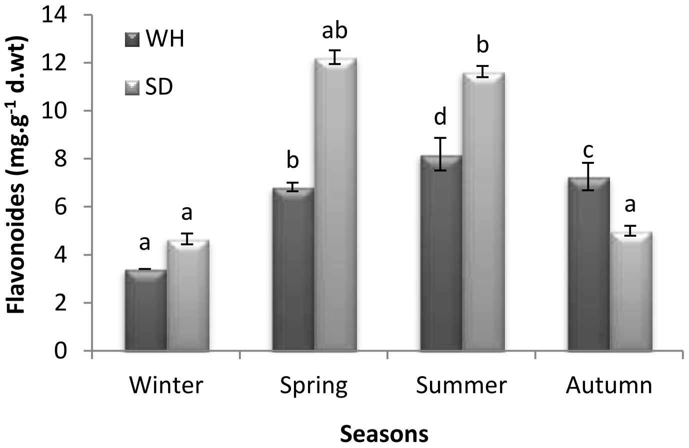

Fig. (1). Variation in total flavonoids content of *V. candicans* aerial parts as a function of growth habitat (WH and SD) and growth season.

The two columns with the same letter are non-significant, while columns with different letters are statistically significant.

Plants contain different polyphenolic compounds, e.g. flavonoids, phenolic acids, coumarins and anthocyanins and their content and composition is strongly influenced by different factors (Weidner and Paprocka, 1996). Polyphenols are involved in the defense mechanism of plants and their levels are enhanced by both biotic and abiotic stress (Dudjak et al., 2004). Flavonoids are important dietary compounds, having a capacity to inhibit DNA damage and lipid peroxidation and quench oxygen radicals (Kinoshita et al., 2005). They also improve high blood pressure, anticancer, antimicrobial, antioxidant, antibiotic and antiallergy actions (Kinoshita et al., 2005). Flavonoids are able to function as chelators for metals, depending on the molecular structure (Michalak, 2006 and Korkina, 2007).

2. Total Alkaloids Content

Fig. (2) represents the change in the total alkaloids content in the aerial parts of *V. candicans* growing in WH and SD habitats. With respect to WH habitat, the total alkaloids content increased gradually from winter till summer season then suddenly decreased in autumn season. The highest content (17.7 mg g^{-1} d wt) was detected in summer season and the lowest content (7.7 mg g^{-1} d wt) was observed in winter season. In SD habitat the total alkaloids content of the *V. candicans* aerial parts was duplicated from winter to spring season then decrease gradually till autumn season. The highest alkaloids content (18.3 mg g^{-1} d wt) was detected in the spring season, and the lowest alkaloids content (9.2 mg g^{-1} dwt) was in autumn season.

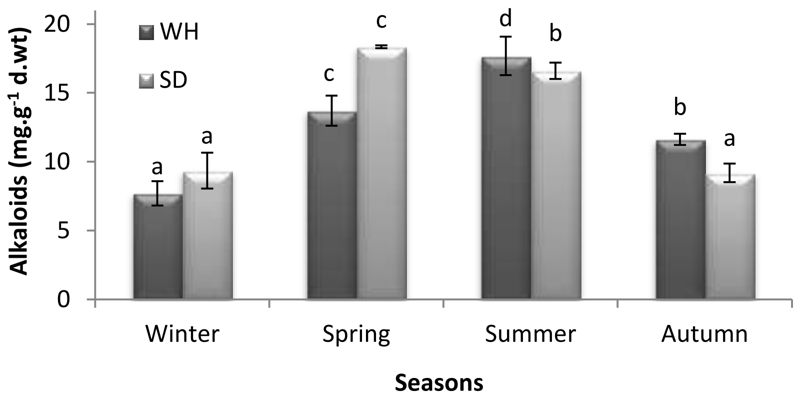

Fig. (2). Variation in total alkaloids content of *V. candicans* aerial parts as a function of growth habitat (WH and SD) and growth season.
The two columns with the same letter are non-significant, while columns with different letters are statistically significant.

Many secondary metabolites play an important function as chemical defense compounds against herbivores, microbes or competing plants (Wink, 1997). One of the largest groups of chemical arsenals produced by plants is the alkaloids. Many of these alkaloids metabolic by-products are derived from amino acids and include an enormous number of nitrogenous compounds. Some plant alkaloids intensely affect herbivores and are involved in a variety of natural plant-insect interactions (Brown and Trigo, 1995). Alkaloids have important eco-chemical functions in the defense of the plant against pathogenic organisms and herbivores or, as in the case of pyrrolizidine alkaloids, as pro-toxins for insects, which further modify the alkaloids and then incorporate them into their own defense secretions (Hartmann, 1991). The function of alkaloids in plants is still largely obscure, although individual substances have been reported to be involved as growth regulators or insect repellents or attractants (Hegnauer, 1967). Some organisms use alkaloids for protection as Ornate Moth, pyrrolizidine alkaloids render these larvae and adult moths unpalatable to many of their natural enemies like coccinelid beetles, green lacewings, insectivorous hemiptera and insectivorous bats (Conner, 2009).

3. Saponin Content

The saponin content of *V. candicans* aerial parts in WH and SD habitats showed considerable variations in response to the change in the habitat and growth seasons (Fig. 3). The saponin content in *V. candicans* aerial parts in WH habitat increased six-folds from winter to summer season then markedly decreased at autumn season to about three-folds than summer season value. The highest saponin content (32.30 mg g^{-1} d wt) was recorded in summer season. While, the lowest saponin content (5.39 mg g^{-1} d wt) was recorded in winter season.

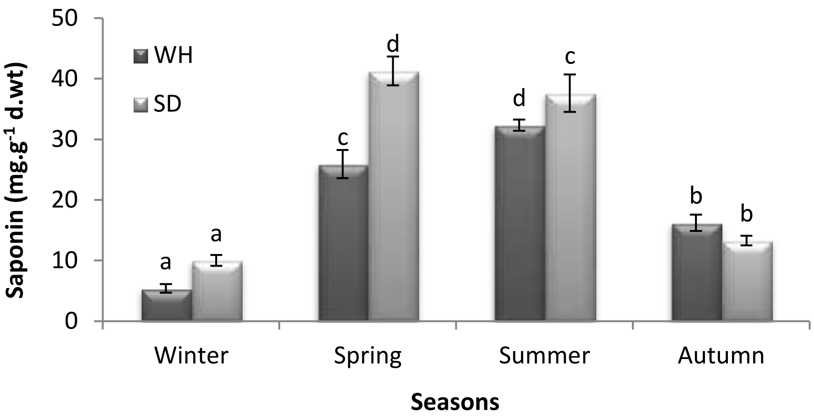

Fig. (3). Variation in total saponins content of *V. candicans* aerial parts as a function of growth habitat (WH and SD) and growth season.
The two columns with the same letter are non-significant, while columns with different letters are statistically significant.

In SD habitat, saponin content of *V. candicans* aerial parts increased at spring season about four-folds than winter season value, then saponin content decreased gradually till autumn season. The saponin content was 41.28 mg g^{-1} d wt in spring season as a maximum and was 10.0 mg g^{-1} d wt in winter season as a minimum value.

Saponins are naturally occurring surface-active glycosides. They are mainly produced by plants, but also by lower marine animals and some bacteria (Yoshiki et al., 1998). A number of factors, such as physiological age, environmental and agronomic factors, have been shown to affect the saponin content of plants (Yoshiki et al., 1998). Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack. Saponins may be considered a part of plant's defense systems, and as such have been included in a large group of protective molecules found in plants named 'phytoanticipins' or 'phytoprotectants' (Morrissey and Osbourn, 1999). It has also been suggested that saponins could be a source of monosaccharides (Barr et al., 1998). Saponins are valuable compounds to humans due to their use in pharmacy, industry, cosmetics, agriculture and the food market (Fenwick and Oakenfull, 2006 and Sun et al., 2009). Saponins have various effects that are attributed to a diverse range of properties, some of which induce both beneficial and detrimental effects on human health such as pesticidal, insecticidal and molluscicidal activity, allelopathic action, antinutritional effects, sweetness and bitterness (Hostettmann and Marston, 1995). Also saponins are useful in the human diet for controlling cholesterol, where it forms strong insoluble complexes with cholesterol resulting in lowering cholesterol activity in humans. Low saponin content of the diet may be partly responsible for the high incidences of heart disease in Western countries (Oakenfull and Sidhu, 1990). Saponins if repeatedly included in the diet may help the body protect itself from cancer.

4. Total Phenolics Content

The amount of total phenolics varied significantly in *V. candicans* aerial parts by the variation in the growth season and interaction of seasons with habitat but not significantly varied by the variation in habitation (Fig. 4). Total phenolics content of *V. candicans* in WH slightly varied from those of SD habitat during the different growth seasons. In WH habitat, total phenolics increased contentiously from winter till summer then decreased in autumn season. However, the furthermost content of phenolics of plant aerial parts in WH (30.6 mg g^{-1} d wt) was recorded in summer season, at the same time as the least phenolic content (25.7 mg g^{-1} d wt) was recorded in the winter season.

In the SD habitat the plant phenolic content increased from winter till spring then decreased through summer and autumn seasons. The highest phenolic content was 31.8 mg g^{-1} d wt during spring and the lowest content was 27.9 mg g^{-1} d wt during autumn season.

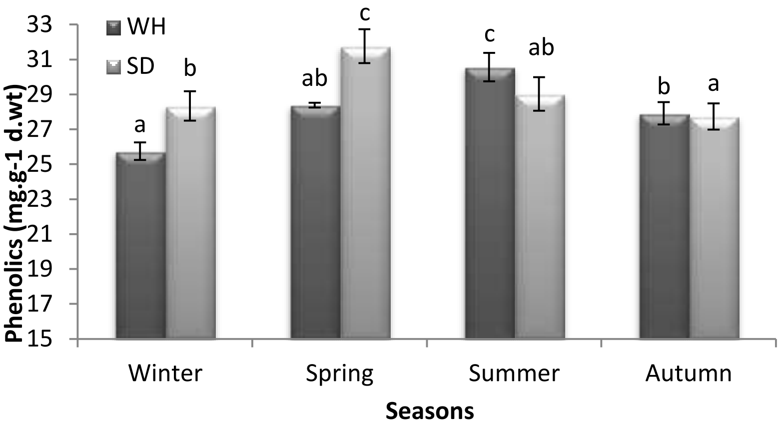

Fig. (4). Variation in total phenolics content of *V. candicans* aerial parts as a function of growth habitat (WH and SD) and growth season.

The two columns with the same letter are non-significant, while columns with different letters are statistically significant.

5. HPLC Identification of Phenolic Compounds

Quantitative and qualitative estimation of the phenolic compounds of *V. candicans* aerial parts in WH and SD habitats was achieved using HPLC. Compounds were separated, identified using authentic compounds and their concentration was determined (Table 1). The separated and identified phenolic compounds in *V. candicans* were fourteen, named, tannic acid, gallic acid, pyrocatechol, phananthrene, nicotinic acid, resorcinol, OH-benzoic acid, chlorogenic acid, phenol, vanillin, *ρ*-coumaric acid, ferulic acid, salicylic acid, and rutin.

The analysis data showed that, only two phenolic compounds, (phananthrene and nicotinic acid) were detected in *V. candicans* in both habitats (WH and SD). Phananthrene content was 7.26% in SD and 8.29% in WH. However, nicotinic acid content was 1.61% in SD and 4.33% in WH habitat, indicating higher percentage of the two phenolic compounds in *V. candicans* of WH habitat. In addition, *V. candicans* growing in this habitat was characterized by the presence of four distinctive phenolic compounds. These phenolic compounds in descending order according to their percentage are tannic acid (71.55%), resorcinol (7.51%), pyrocatechol (5.17%) and gallic acid (3.19%).

On the other hand, *V. candicans* growing in SD habitat showed anotherpattern of phenolic compounds content, where it is characterized by the presence of ten distinctive phenolic compounds not found in the plants growing in WH habitat. The percentage of those phenolics components in those habitats varied from 19.39% (*ρ*-coumaric acid) to 3.48% (phenol).

Table (1). The percentage of detected phenolic compounds in *V. candicans* aerial parts in Wadi Habbes (WH) and Sand Dunes (SD) habitats by HPLC.

Peak	RT (min.)	Compound	Habitat %	
			WH	**SD**
1	1.417	Tannic acid	71.55	ND
2	1.780	Gallic acid	3.19	ND
3	1.967	Pyrocatechol	5.17	ND
4	2.730	Phananthrene	8.29	7.26
5	3.067	Nicotinic acid	4.33	1.61
6	5.033	Resorcinol	7.51	ND
7	9.820	OH-benzoic acid	ND	7.76
8	10.077	Chlorogenic acid	ND	6.45
9	11.067	Phenol	ND	3.48
10	14.133	Vanillin	ND	8.69
11	16.160	ρ-coumaric acid	ND	19.39
12	16.973	Ferulic acid	ND	10.43
13	17.367	Salicylic acid	ND	7.73
14	18.213	Rutin	ND	10.33

RT = Retention time ND = Not Detected

Phenolic compounds give the plant an important value as they exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Middleton et al., 2000; Puupponen-Pimia et al., 2001; Manach et al., 2005). Also, phenolic compounds could be a major determinant of antioxidant potentials of foods and could therefore be a natural source of antioxidants. Some phenolic compounds recorded in the study plant have medical importance such as:

1. Gallic acid has high antioxidant capacity, significantly higher than any other antioxidant and about five times higher than Vitamin C in juice of apple (Sakagami and Satoh, 1997).

2. Ferulic acid and gallic acid inhibit the mycelial growth of *Fusarium* spp. by 85.1%, where it can decrease the damping off (Ahtiainen et al., 2003).

3. Tannic acid was used as a treatment for many toxic substances, such as strychnine, mushroom, and ptomaine poisonings (Sturmer, 1999).

4. Nicotinic acid has been used for over 50 years to increase levels of high density lipoproteins (HDL) in the blood and has been found to decrease the risk of cardiovascular events modestly in a number of controlled human trials (Bruckert et al., 2010).

5. Salicylic acid is known for its ability to ease aches and pains and reduce fevers (Madan and Levitt, 2014).

6. *p*-Coumaric acid has antioxidant properties and is believed to reduce the risk of stomach cancer (Ferguson et al., 2005) by reducing the formation of carcinogenic nitrosamines (Kikugawa et al., 1983). Also, p-coumeric acid has been found to help honey bees to detoxify certain pesticides (Mao et al., 2013).

7. Rutin inhibits platelet aggregation (Navarro-Nunez et al., 2008) as well as it decreases capillary permeability, making the blood thinner and improving circulation. Recent studies confirm rutin could help in preventing blood clots, so could be used to treat patients at risk of heart attacks and strokes (Reporter, 2012).

6. Antioxidant Scavenging Activity

The percentages of antioxidant scavenging activity of *V. candicans* aerial parts in WH and SD habitats at the various growth seasons are represented in fig. (5). The antioxidant activity of the ethanolic extract in WH habitat showed slight fluctuation, where it decreased from winter to spring season, then showed an increase during summer season then, re-decreased at autumn season to its lowest value. The antioxidant scavenging activity of *V. candicans* aerial parts mostly remained constant all over the plant growth seasons throughout this habitat, it showed a maximum 87.97% activity in the summer season and a minimum 81.391% one in the autumn season.

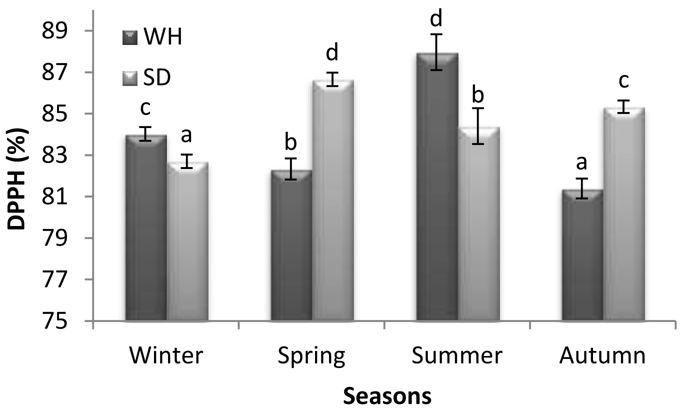

Fig. (5). Variation in antioxidant scavenging activity (%) of *V. candicans* aerial parts as a function of growth habitat (WH and SD) and growth season.

The two columns with the same letter are non-significant, while columns with different letters are statistically significant.

In the SD habitat, the percentage of antioxidant scavenging activity of *V. candicans* also fluctuated with an increasing trend from winter to autumn with a marked increase in spring season. It showed slight change between a maximum of 86.654% in the spring and a minimum of 82.707% in the winter season. The antioxidant scavenging activity is the most commonly used method for assessment of the antioxidant properties and strongly correlated with phenolic compounds (Maisuthisakul et al., 2007). Many authors (Sanita et al., 1998 and Dong et al., 2006) reported an increase in the total antioxidants scavenging activity in plants to cope with abiotic stress conditions. Adamo et al. (2004) have pointed out that some environmental conditions are capable of breaking the chemical bonds of polyphenols, thereby releasing soluble phenols of low molecular weights, leading to an increase of antioxidant capacity.

CONCLUSION

The coastal regions around the world contain a wide range of wild plants with obscure medicinal importance. Such plants contain great diversity of phytochemicals with a great potential in treating many chronic diseases. Thus further studies are still required to explore the phytochemically active ingredients of such plants at their natural habitats throughout the growth seasons to maximize the benefit from these natural resources. *V. candicans*, as a wild plant, showed high content of medicinally important phytochemicals in its aerial parts. The harvest time varies according to the season and habitat in order to obtain the uppermost quantity of such compounds. In the rocky places, the best time for harvest was during summer season, but in the sandy places it is suggested during the spring season.

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التغيرات الموسمية في محتوى الأيض الثانوي ذات الأثر الطبي في المجموع الخضري لنبات زعتر الحمار في منطقتين على ساحل مصر

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زعتر الحمار هو نبات بري ينمو طبيعيًا في الساحل الشمالي الغربي لجمهورية مصر العربية. ولقد أجريت هذه الدراسة لتقييم التنوع والإختلاف في محتوى مركبات الأيض الثانوي الموجودة بالمجموع الخضري لنبات زعتر الحمار في بيئتين متباينتين، خلال فصول السنة المختلفة. وأظهرت نتائج التحاليل أن محتوى الفلافونيدات والقلويدات والصابونينات والمركبات الفينولية سجلت أقصى قيمة لها خلال موسم الربيع في منطقة الكثبان الرملية، بينما أظهرت هذه المركبات أقصى قدر من قيمتها خلال موسم الصيف في وادي حابس ذو الطبيعة الصخرية. وأظهرت نتائج الفصل الكروماتوجرافي السائل ذو الضغط العالي للمركبات الفينولية لمستخلصات نبات زعتر الحمار وجود مشترك لإثنين من المركبات الفينولية في كلا البيئتين، وثمانية مركبات فينولية فريدة من نوعها في النباتات التي تنمو بمنطقة الكثبان الرملية، بالإضافة إلى أربعة مركبات فينولية أخرى مميزة لنباتات منطقة وادي حابس. ولقد خلصت الدراسة إلى أن النشاط المضاد للأكسدة في هذا النبات يعزى إلى محتواه العالي من المركبات الفينولية.