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Seasonal variations in phenolics, flavonoids and antioxidant capacity of some medicinal plants growing naturally in Wadi Halazien, Egypt

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Abstract
The environmental stresses such as high or cold temperatures, heat, UV irradiation and infection by pathogen stimulate the production of secondary metabolic products that have medicinal values and antioxidant properties such as phenolics and flavonoids. This study aimed to investigate the effect of seasonal variations of climatic conditions on phenolic and flavonoid compounds, as well as antioxidant capacity of three plant species belong to different families growing naturally in Wadi Halazein. The investigated plants were Seriphidium herba-alba, Teucrium polium and Peganum harmala. The results indicated that the total contents of phenolics and flavonoids was significantly affected by species and season and tended to increase during the climatic condition of summer season. The results of DPPH assay (radical scavenging) and FARP assay (ferric-reducing antioxidant power), indicated that under the climatic conditions of summer season, Serphedium herba alba showed the highest antioxidant capacity followed by T.polium and P.harmala. The ability of their ethanolic extracts to scavenge free radicals and chelate metals (ferric ion), indicated that phenolic and flavonoid compounds were the main compounds that contributed to antioxidant activity of the investigated plants. The HPLC analysis indicated that the highest values of medicinal compounds that have high antioxidant activity and high medicinal values, such as rutin (1.303 mg g^{-1}), quercetin (2.527 mg g^{-1}), apigenin 7-glucose (0.798 mg g^{-}1), kaempferol (1.644 mg g^{-1}) and apigenin (0.175 mg g^{-1}) were detected in the extract of Serphedium herba alba under the climatic conditions of summer season. Whereas, the highest content of naringin was detected in winter sample of Serphedium herba alba, while the high content of naringenin (4.012 mg g^{-1}) was detected in summer sample of P.harmala. This study supports using these plants as a source of natural antioxidant compounds especially Serphedium herba alba, which contains valuable antioxidant compounds that have anti-cancer and inflammatory activities.

Keywords*: Antioxidant activity, DPPH assay, FRAP, Serphedium herba alba*

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I. Introduction

Plants contain several secondary metabolic products that have medicinal values and antioxidant properties such as phenolics and flavonoids. The environmental stresses such as high or low temperatures, heat, UV irradiation and infection by pathogen stimulate their production [1]. The exposure of plants to multiple stresses such as heat and drought stresses with elevated level of CO_2, leads to production of ROS that affects the production of cell building materials, disturbance the photosynthetic and respiration processes and consequently contributes to cell death [2].

The selected plants belong to different families that inhabit the same habitats are exposed to the same environmental conditions to investigate the difference in physiological responses to the same stress conditions between different members represented different families. The investigated plants were *Seriphidium herba-alba, Teucrium polium and Peganum harmala*. *Seriphidium herba-alba* belongs to family Asteraceae. It is used widely in folk medicine for the treatment of diabetes mellitus, disorders as epilepsy, neurological, Alzheimer's disease and has antispasmodic and mild antibiotic activities as reported by [3],[4]. The second selected plant, *Teucrium polium* belongs to the family Lamiaceae. Species of the genus *Teucrium* are rich in essential oils, saponins, sterols, phenols, flavonoids, alkaloids [5] and fatty acids [6]. The third plant, *Peganum harmala* belongs to family Zygophyllaceae, is used as an analgesic and anti-histaminic [7], also had anti-inflammatory properties [8] antibacterial, antifungal and antiviral effects [9].

This study aimed to investigate the effect of seasonal variation of climatic conditions on phenolic and flavonoid compounds as well as antioxidant capacity of three plant species belong to different families.

II. Materials And Methods

1.Plant materials

The aerial parts of *Serphedium herba alba, Teucrium polilum and Peganum halmala* plants were collected in February and July from Wadi Halazein, Marsa Matrouh governorate. The samples of the three plants were identified in the Herbarium of the Desert Research Center.

2.Ecological study

2.1. Climatic data

The climate data consist of average temperature and the rate of rainfall of the studied habitat, provided by Applied Agricultural Meteorological Laboratory.

2.2. Soil analysis

Soil textures were analyzed according to Gee and Bauder [10] using the international pipette. Soil EC and soil reaction (pH) in soil water suspension (1:2.5) and the concentrations of sodium and potassium were determined according to Page [11], and the concentration of Cl was determined according to Jackson [12]. The concentrations of magnesium, calcium, bicarbonate ions (HCO_3) and carbonate (CO_3) were determined according to the method of Rowell [13].

3.Phytochemical study

3.1Determination of total phenolic content

The content of total phenolics in plant samples was determined according to Attard [14] using the Folin–Ciocalteu method. Samples were prepared at a concentration of 20 mg/mL in EtOH 80%, and a known volume (10 µL) was mixed with 100 µL of Folin-Ciocalteu reagent (Diluted 1: 10) in a 96-well microplate. Then, 80 µL of 4 N Na2CO3 was added and kept in dark for 20 minutes. The absorbance of blue colour was read at 630 nm. The content of total phenolics was calculated from the standard curve equation ($y = 4.1282x - 0.0894$ ($R^2 = 0.9925$)), where y is the absorbance at 630 nm and x is the concentration of gallic acid in mg/L. The results were expressed as milligrams gallic acid equivalent per gram dry weight used in extraction (mg GAE/g DW).

3.2. Identification of phenolic compounds by HPLC

Phenolic compounds were determined according to the method of Goupy et al. [15]. Five grams of sample were mixed with methanol and centrifuged for 10 min at 1000rpm, the supernatant was filtered through a 0.2 µm Millipore membrane filter. In a vial ,1-3ml were injected into HPLC Hewllet Packard (series 1050) equipped with solvent degasser, auto sampling injector, ultraviolet detector set at 280 nm and quaternary HP pump series (1100). For separation of phenolic compounds, packed column Hypesil BDS-C18, 4.0 x 250 mm was used at 35°C. Methanol and acetonitrile were used as mobile phases at flow rate of 1 ml/min. Standers of phenolic acid were dissolved in mobile phase and injected into HPLC. The concentration of phenolic compounds was calculated by data analysis of Hewllet Packard software, Germany, according to retention time and peak area.

3.3. Determination of total flavonoids

The total flavonoids content was determined using the aluminum chloride method as described by Kiranmai et al. [16] with minor modifications to be carried out in microplates. Briefly, 15 µL of sample/standard was placed in a 96-well microplate, then,175 µL of methanol was added followed by 30µL of 1.25 % $AlCl_3$. Finally, 30 µL of 0.125 M $C_2H_3NaO_2$ was added and incubated for 5 min. The content of total flavonoids was calculated from the standard curve equation ($y = 0.0034x + 0.1396$ ($R^2 = 0.9988$)), where y is the absorbance at 420 nm and x is the concentration of quercetin in mg/L. The results were expressed as milligrams quercetin equivalent per gram dry weight used in extraction (mg QE/g DW).

3.4. Identification of flavonoid compounds by HPLC

Flavonoid compounds were determined by HPLC according to the method of Mattila et al. [17]. Five grams of sample were mixed with methanol and centrifuged at 10000 rpm for 10 min and the supernatant was filtered through a 0.2µm Millipore membrane filter then 1-3 ml was collected in a vial for injection into HPLC Agilent 1200 series equipped with auto sampling injector, solvent degasser, ultraviolet (UV) detector set at 254 nm and quarter HP pump (series 1050). The column type was ODS column with a dimension of 5µm x4mm. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at a flow rate of 1 ml/min,

the column temperature was maintained at 35°C. Flavonoid standards from sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used to calculation of phenolic compounds concentration by the data analysis of HEWLLET packed software.

3.5. DPPH radical scavenging capacity

The antioxidant activity of the extract was measured as described by Gebhard [18] using the 1, 1'–diphenyl– 2–picrylhydrazyl (DPPH) free radical scavenging capacity. Briefly, 0.1 mL aliquot of properly diluted plant extract in different concentrations was mixed with 3 ml of methanol solution of 0.004 % DPPH. Mixture was then vortexed for 1 min at 800 rpm and kept in the dark for 30 min at room temperature. Absorbance of samples was measured at 517 nm. The radical scavenging activity of the samples (antioxidant activity) was expressed as percent inhibition of DPPH0 radical as following:

% Inhibition = [(A control –A sample)/A control)] X 100

Results were expressed as µg sample/mL in the form of IC_{50}, determined by linear regression of IC and extract concentration at 50% inhibition.

3.6. Ferric-reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) was determined by the spectrophotometric method previously described by Benzie and Strain [19]. Three stock solutions were prepared: a 300 mM acetate buffer (3.1 g sodium acetate and 16 mL glacial acetic acid), pH 3.6, 10 mM TPTZ (2,4,6- tri (2- pyridyl) - 1,3,5- triazine) solution in 40 mM HCl, and 20 mM ferric chloride solution. Working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3.6H_2O$solution. This working solution was then warmed to 37C. A 75 mL aliquot of properly diluted plant extract in different concentrations was mixed with 1.425 mL of working solution. Absorbance was read at 593 nm 30 min after mixing in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe (III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous-TPTZ (Fe (II)-TPTZ) complex which absorbs strongly at 593 nm. The higher the FRP value, the greater was the reducing power, thus the greater the antioxidant activity

% Reducing power = [(A sample - A control) /A control] X 100

Where A sample is the absorbance of the sample in the presence of the extract and A control is the absorbance of the control. The result was expressed as µg sample/mL in the form of IC_{50}, determined by linear regression of IC and extract concentration at 50% reduction. The result was expressed as IC_{50} which corresponds to the concentration of the extract necessary to reduce 50% of ferric ferrous complex.

3.7. Statistical analysis

The data were subjected to two-way analysis of variance (ANOVA) and Duncan's multiple-range test ($P \leq 0.05$) using the statistical program, CoStat Version 6.311(CoHort soft-ware, Berkeley, CA 94701) according to Steel et al. [20].

III. Results And Discussion

1. Description of the study area and climatic data

Wadi Halazien is a rocky wadi located at the Northwest coast of Matrouh Governorate, Egypt at the distance about 40 km west of Matruh city at latitudes of 31° 25' 21" N and longitudes of 26° 51' 43" E. The Northern coast region of Egypt extends around 1000 km along the Mediterranean Sea and 30 km inland. This region is characterized by an arid Mediterranean climate that has a limited rainfall [21] The medicinal characteristics of plants may be affected by the fluctuation of environmental conditions between extremely low in winter season and high temperatures in summer season. Where the annual rainfall in Egypt shows a maximum rate over the Mediterranean coast with a rapid decrease toward the south. Average annual rainfall was 150 mm/year. The dry period extended to four months (from June to September). Most precipitation falls in January, with an average of 36 mm. The highest average temperature of 25.3 °C was recorded in August, as is considered the is the warmest month. The average high temperature during winter fluctuated between 18-20 °C, the average temperature is 13.1 °C in January.

2.Soil properties

The physical and chemical properties of Wadi Halazein soils indicated that the soil texture was loamy sand in the first depth (0-20 cm) and loam in the second depth (20-40 cm). The percentages of sand, silt, and clay in the first depth were 80.63 %, 16.67 %, and 2.70 g%, respectively, while in the second depth, their percentages were 46.45 %, 41.30 %, and 12.25 %, respectively. The percentage of soil moisture content at first depth was 5.65 % in the winter season and 2.80% in the summer season and slightly increased to 6.25% and 3.56%, respectively. The value of pH was 8.44 in the first depth and 8.80 in the second depth. The values of

calcium magnesium and sodium were 4.85 $meql^{-1}$, 5.15 $meql^{-1}$and 21.84 $meql^{-1}$ in the first depth and 4.85 $meql^{-1}$, 5.15 $meql^{-1}$ and 21.84 $meql^{-1}$in the second depth. The concentration of potassium was 0.93 $meql^{-1}$ in the first depth and 0.39 $meql^{-1}$ in the second depth. The contents of bicarbonate were 1.94 and 1.60 $meql^{-1}$, while the percentage of total calcium carbonate ($CaCO_3$%) was 26.75% in the first depth and 27.50% in the second depth. The presence of high percentages of silt and clay particles makes carbonate more active, which may cause a decrease in the availability of phosphorus, manganese, copper and zinc [22].

2.Seasonal variation of total phenolic and flavonoid contents

As shown in Table (1), there was a significant difference in the contents of total phenolics and flavonoids between species or seasons. The concentration of total phenolics was significantly increased in summer in all the investigated plants, its values were 24.67 ± 1.28, 13.83 ± 0.28 and 8.24 ± 0.11 mg g^{-1} in *Serphedium herba alba* (*S.h.alba*), *Teucrium polilum* (*T.polium*) *and Peganum halmala* (*P.harmala*) in winter and increased to 30.72 ± 0.35, 17.05 ± 0.20 and 9.23 ± 0.26 mg g^{-1} in summer respectively. Similarly, the concentration of total flavonoids tended to increase under the climatic conditions of summer season in all the investigated plants, its values were 2.58 ± 0.2, 0.10 ± 0.03 and 0.30 ± 0.07 mg g^{-1} in winter and significantly increased to 5.20 ± 0.45, 0.34 ± 0.02 and 1.24 ± 0.06 mg g^{-1} *S.h.alba , T.polium and P.harmala* in summer season, respectively.

Table 1. Total phenolic and flavonoid contents of *S.h.alba, T.polium* and *P.harmala* and DPPH and FRAP values during winter and summer climatic conditions

Plant species	Seasons	Total phenolics (mg /g)	Total flavonoids (mg /g)	IC_{50} of DPPH (µg /mL)	IC_{50} of FRAP (µg /mL)
S.h.alba	Winter	24.67 ± 1.28^{b}	2.58 ± 0.2^{b}	232.05	133.77
	Summer	30.72 ± 0.35^{a}	5.20 ± 0.45a	191.95	27.97
T.polium	Winter	13.83 ± 0.28^{d}	0.10 ± 0.03^{e}	677.96	264.92
	Summer	17.05 ± 0.20^{c}	0.34 ± 0.02^{d}	589.36	184.21
P.harmala	Winter	8.24 ± 0.11^{f}	0.30 ± 0.07^{d}	816.15	337.68
	Summer	9.23 ± 0.26^{e}	1.24 ± 0.06^{c}	694.76	300.50

Values are expressed as mean ± SD (n = 3). In each column values followed by different letters are significantly different at $p < 0.05$

The obtained results indicated that *S.h.alba* showed the highest content of total phenolics and flavonoids compared with *T.polium and P.harmala* , which was compatible and supported with the results of DPPH and FRAP assays. Where the DPPH and FARP assay used as indicator to the antioxidant capacity of plant extracts based on the ability of scavenging of free radicals and reducing ferric ions to ferrous ions [23]. Therefore, the low value needed to scavenge of DPPH˙ and reduce ferric tripyridytriazine compex, indicated high antioxidant capacity of the used plant extract.

Regarding the results of DPPH and FARP assay (Table 2&3), the *S.h.alba* showed the highest antioxidant capacity under the climatic conditions of summer season followed by *T.polium* and *P.harmala.* The significant correlations between phenolic and flavonoid compounds and antioxidant activity, as well as chelating properties were reported by others [24], [25],[26],[27]. The ability of their extracts to scavenge free radicals and chelate metals (ferric ions) indicated that phenolic and flavonoid compounds were the main compounds that contributed to antioxidant activity of the investigated plant.

Table 2. Antioxidant activities of *S.h.alba, T.polium* and *P.harmala* extracts with different concentrations against DPPH

Plants	Seasons	Concentration of extract (µg/mL) DPPH%				
		100	200	400	800	1000
S.h.alba	Winter	31.22 ± 0.205^{b}	52. 22±0.173	67.87 ± 0.505^{b}	97.19 ± 0.40^{b}	98.19 ± 0.395^{a}
	Summer	37.10 ± 0.505^{a}	52.34±0.405	69.68 ± 0.295^{a}	98.15 ± 0.261^{a}	98.50 ± 0.309^{a}
T.polium	Winter	9.80 ± 0.288^{d}	16.44 ± 0.495^{d}	25.19 ± 0.46^{f}	61.84 ± 0.228^{d}	72.40 ± 0.505^{c}
	Summer	11.35 ± 0.352^{c}	19.61 ± 0.42^{b}	36.35 ± 0.262^{c}	66.51 ± 0.425^{c}	81.45 ± 0.17^{b}
P.harmala	Winter	7.04 ± 0.35^{e}	9.95 ± 0.62^{e}	26.70 ± 0.340^{e}	48.57 ± 0.615^{f}	61.09 ± 0.477^{e}
	Summer	10.11 ± 0.606^{d}	19.16 ± 0.311^{b}	33.33 ± 0.505^{d}	56.71 ± 0.33^{e}	68.67 ± 0.285^{d}

Values are expressed as mean ± SD (n = 3). In each column values followed by different letters are significantly different at $p < 0.05$

Table 3. Ferric-reducing antioxidant power of *S.h.alba, T.polium* and *P.harmala* extracts with different concentrations

Plants	Seasons	Concentration of extract (µg/mL) FRAP%			
		100	200	300	400
S.h.alba	Winter	42.61±0.395[b]	63.57±0.305[b]	80.76±0.605[b]	97.19±0.40[b]
	Summer	60.14±0.495[a]	71.48±0.495[a]	83.51±0.192[a]	98.95±0.62[a]
T.polium	Winter	23.71±0.52[d]	39.86±0.689[d]	53.95±0.45[d]	72.85±0.405[d]
	Summer	38.14±0.405[c]	47.77±0.47[c]	71.82±0.028[c]	87.97±0.15[c]
P.harmala	Winter	6.19±0.221[f]	23.02±0.03[f]	45.36±0.585[f]	60.48±0.345[f]
	Summer	18.9±0.355[e]	26.46±0.35[e]	47.08±0.35[e]	71.48±0.236[e]

Values are expressed as mean ± SD (n = 3). In each column values followed by different letters are significantly different at $p < 0.05$

The results of analysis of phenolics by HPLC (Table 4), indicated that most of phenolic compounds tended to accumulate during the climatic condition in summer season, except in *P.harmala* ,the content of pyrogallol, catechin caffeic acid, ellagic acid and coumarin was found to increase in high concentration during winter climatic conditions. Also, the concentrations of 3-0H tyrosol and catechol tended to increase in all the investigated plants in winter season. The results also indicated that *S.h.alba* contained the highest values of chlorogenic acid (2.532mg g^{-1}), caffeic acid (0.367mg g^{-1}), caffeine (1.091 mg g^{-1}) in response to summer climatic conditions and highest value of ellagic acid in winter season.

Table 4. Phenolic compounds of of *S.h.alba, T.polium* and *P.harmala* under winter and summer climatic conditions

Phenolic Compounds mg/g	*S.h.alba*		*T.polium*		*P.harmala*	
	Winter	Summer	Winter	Summer	Winter	Summer
Pyrogallol	0.042	---	0.026	0.002	18.11	0.021
Gallic acid	0.005	0.005	0.004	0.001	---	0.016
3-0H Tyrosol	0.011	0.008	0.004	0.004	0.017	0.012
Catechol	0.200	0.163	0.103	0.071	0.187	0.092
4-Aminobenzoic	0.010	0.032	0.007	0.005	0.021	0.009
Catechin	0.232	0.584	0.115	0.155	0.703	0.248
Chlorogenic acid	0.774	2.532	0.084	0.065	0.182	0.198
Benzoic acid	--	---	0.045	0.091	0.084	0.162
Caffeic acid	0.114	0.367	0.164	0.220	0.146	0.033
Vanillic acid	0.089	0.138	0.020	---	---	0.400
Caffeine	0.150	1.091	0.055	0.078	--	---
Ferulic acid	1.158	3.921	--	0.164	0.124	0.140
Ellagic acid	1.634	1.582	0.304	0.589	0.522	0.489
Coumarin	0.056	0.284	0.030	0.087	0.062	0.043

The results of analysis of flavonoids by HPLC (Table 5), indicated that the content of most flavonoids compounds increased under the climatic conditions of summer seasons. The highest values of medicinal compounds that have high antioxidant activity and high medicinal values, such as rutin (1.303 mg g^{-1}), quercetin (2.527 mg g^{-1}), apigenin 7-glucose (0.798 mg g-1), kaempferol (1.644 mg g^{-1}) and apigenin (0.175 mg g^{-1}) were detected in the extract of *S.h.alba* under the climatic conditions of summer season. Whereas the highest content of naringin was detected in winter sample of *S.h.alba* ,while high content of naringenin (4.012 mg g^{-1}) was detected in summer sample of *P.harmala*. Flavonoids are considered the most powerful antioxidants [28] induced by abiotic stress that act as potential inhibitors to lipid peroxidation [29] and lipoxygenase enzyme and have anti-cancer and inflammatory activity [30].

Table 5. Flavonoid compounds of of *S.h.alba, T.polium* and *P.harmala* under winter and summer climatic conditions

Flavonoids Compounds mg/g	*S.h.alba*		*T.polium*		*P.harmala*	
	Winter	Summer	Winter	Summer	Winter	Summer
Rutin	1.303	4.172	0.067	0.072	0.018	0.033
Naringin	4.641	1.285	0.543	0.883	0.097	0.062
Rosmarinic acid	----	0.159	0.033	0.068	0.006	0.016
Quercetin	0.851	4.277	0.044	0.084	0.022	0.107
Apigenin 7-glucose	0.211	0.798	0.061	0.082	0.072	0.154
Quercetin	1.926	2.527	0.056	0.079	--	0.115
Naringenin	---	0.222	0.015	0.027	0.702	4.012
Kaempferol 3-2-P-coumaroyl glucose	1.338	---	0.086	0.078	0.426	0.683
Kaempferol	0.432	1.644	0.049	0.019	0.021	0.032
Acacetin 7neo-rutinoside	---	---	--	---	---	0.264
Apigenin	0.086	0.175	0.083	0.047	0.021	0.022

IV. Conclusion

The results indicated that the investigated plants; *Seriphidium herba-alba*, *Teucrium polium* and *Peganum harmala* vary in response to the climatic fluctuation in Mediterranean region. The results indicated that most of phenolics and flavonoids compounds tended to accumulate during the climatic condition in summer season The methanolic extracts of the investigated plants showed have high antioxidant capacity, which indicate the of value of these plants as a source of natural antioxidant compounds. The results also proofed that the compositions and the yield of phenolic and flavonoid compounds were significantly affected by climatic conditions. Also, this study supports the use of these plants as a source of natural antioxidant compounds especially *Seriphidium herba-alba,* which contains valuable antioxidant compounds that have anti-cancer and inflammatory activities.

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