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Original Article

Seasonal Variations in Carnosic Acid Content of Rosemary Correlates with Anthocyanins and Soluble Sugars

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Abstract

Seasonal variations may influence the quality and quantity of biologically active ingredients in medicinal plants. Rosemary (*Rosmarinus officinalis* L.) a member of the Lamiaceae family, contains valuable antioxidant, anticancer and antibacterial substances, including Carnosic acid (CA). Here, the fluctuations of important active compounds present in rosemary leaf extracts collected in Golestan, Iran were studied during the year of 2012-2013. Plant phenolics, flavonoids, ascorbates, anthocyanins and soluble sugars were analyzed spectrophotometrically, while CA content was measured by High Performance Liquid Chromatography (HPLC). The highest amounts of total flavonoids occurred in autumn; while CA, phenolics, ascorbic acids and soluble sugars were greatest in winter, probably due to regional high precipitation and subtle winters. Most of the above indicated active compounds were low in early summer. Furthermore, total anthocyanins and soluble sugars showed significant positive correlations with CA over the year. These data suggest that rosemary extracts from the collected leaves in winter contain greater amounts of biologically active compounds; and can be used for standardization of plant materials harvested throughout a year.

Keywords: Anthocyanin, Antioxidant activity, Carnosic acid, Rosemary, Soluble sugars

Introduction

Environmental conditions affect not only plant growth, but also the quality and quantity of secondary metabolites. Most medicinal plants show marked fluctuations in their active ingredients in different seasons; mainly due to variations in average seasonal temperature and precipitation. There are several assumptions concerning the best time and season for the collection of various parts of some medicinal plants; *e.g.* spring is suggested for the collection of bark and winter is suggested for the collection of essential oils [1]. Plants harvested during various seasons may contain completely different phytochemical properties [2], which may be of major importance from an agronomic, medicinal and economical point of view.

Carnosic acid (CA) is one of the most important plant substances, exclusively found in some Lamiaceae species such as rosemary (*Rosmarinus officinalis* L.) [3]. CA and carnosol (CAR), are suggested to contribute in more than 90% of the antioxidant activity of rosemary leaves. Dried rosemary leaves can contain between 0.1-10% carnosic acid, depending on the environmental conditions, plant variety and developmental stage [3, 4].

Important therapeutic applications of CA make it an attractive target for biological approaches aimed towards producing rosemary plants carrying elevated levels of CA, while at present, field-grown plants are the only source of CA. Furthermore, the contents of other antioxidant compounds such as phenolics [4], flavonoids [5], ascorbates [6], anthocyanins [7] and soluble sugars [8] can also be affected by seasonal variations. In this regard,

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several environmental factors such as light, temperature, humidity, drought, submergence and nutrient deprivation, which readily change during various seasons in temperate environments, can alter plant biochemistry, physiology and productivity leading to metabolite changes during plant growth and development [4].

In order to keep a high-quality standard, variations on composition and thus on antioxidant activity of plant extracts, caused by seasonality must be monitored throughout a year. There is no general rule for the best harvest time of a plant organ for a specific secondary metabolite. Although several studies reported the effects of different seasons on secondary metabolite production and accumulation in various plants [9-11], a complete figure that represents the best seasons for harvesting various medicinal plants bearing higher yields of secondary metabolites is still missing.

Here, for the first time, the effects of seasonal variation on CA content, as well as other compounds related to the rosemary antioxidant activity, such as phenolics, flavonoids, ascorbates, anthocyanins and soluble sugars and their correlations with CA content have been investigated in Golestan province, Iran. These results can be used for standardization of biologically active compounds present in rosemary, harvested in different seasons, and may recommend the best harvest time for this medicinally important plant species.

Material and Methods

Folin-Ciocalteu reagent was purchased from Merck (Germany), while all other chemicals and reagents were purchased from Sigma-Aldrich (Germany).

Fresh mature leaves of *Rosmarinus officinalis* L. collected from Golestan University campus, Gorgan, Golestan, Iran (54°25'E; 36°50'N) during 2012-2013 were air dried in the shade and ground to a fine powder using a cutting mill.

HPLC Analysis

Samples containing 500 mg of dry leaf powder in acetone (3 ml) were homogenized while being cooled on ice and the mixture was centrifuged (1500 g for 10 min) at 4 °C. The supernatant was then transferred to a test-tube and the residue was further extracted for the second time. The extracts were combined and centrifuged (1500 g for 10 min) at 4 °C, and 25 µl of the supernatant was injected into the HPLC column.

For detection of CA a reversed-phase HPLC method, using a perfect silica-based 300 ODS C_{18} column (4.60 mm x 150 mm, 5 µm) was employed at 25±0.5 °C. The separation was isocratically undertaken with a solvent consisting of 0.1% (v/v) aqueous phosphoric acid-acetonitrile at a flow rate of 1 ml/min. The HPLC run of the CA standard was performed under identical conditions. To quantify the CA present in each extract, the individual peak areas obtained with HPLC chromatograms at 228 nm were compared with areas obtained from the standard solutions.

Plant Extraction and Antioxidant activity measurements

One gram of each dry leaf sample was extracted in 25 ml of ethanol for 24 hours at room temperature, the residue was further extracted in the same manner and then filtered through Whatman No. 3 paper and concentrated by rotary evaporator at < 40 °C.

FRAP (Ferric reducing antioxidant power) assay was performed based on formation of the blue complex [Fe2+/TPTZ (2, 4, 6-tripyridyl-s-triazine)], as described by Benzie and Strain [12]. Formation of the blue complex was measured spectrophotometrically (Shimadzu UV-1800, Japan) at 593 nm and expressed as mmol Fe^{2+} gr^{-1} DW, using a standard curve of ferrous sulfate.

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity based on chemical reduction of the free radical produced from DPPH in the presence of hydrogen-donating antioxidants, was basically carried out, as described by Cuendet *et al*. [13]. The absorbance was spectrophotometrically measured at 517 nm (Shimadzu UV-1800, Japan) after 30 minutes. Percentage inhibition was calculated as described by Mohamad *et al*. [14]. The results were expressed as ascorbic acid equivalent antioxidant activity (AEAC) per DW (gr).

Total phenolic content of the extracts was measured using Folin–Ciocalteu reagent, as described by Meda *et al*. [15] and results were expressed as milligram gallic acid equivalent (GAE) per DW (gr). Total flavonoid of the extracts was determined using aluminum chloride colorimetric method, as described by Chang, *et al*. [16] and the data were expressed as milligram quercetin equivalents (QE gr^{-1} DW). Ascorbic acid

(AA) content was determined by 2, 6-dichlorophenolindophenol-dye method, as described by De Pinto *et al*. [17] and expressed as mg AA gr^{-1} DW. Total anthocyanin was measured according to Mita *et al*. [18]. The content of soluble sugars was assayed using phenol-sulfuric acid method [19] and the results were reported in terms of mg gr^{-1} DW.

Statistical Analysis

All experiments were carried out in triplicate and the results are presented as mean±standard error (SE). The analysis of variance (ANOVA) and Duncan tests were used for statistical analysis and values of $p \leq 0.05$ were considered as significant indicators.

Results

Seasonal variations in temperature and rain fall

Yearly temperature and rain fall fluctuations during the time course of experiment (2012-2013) were obtained from Iran Meteorological Organization website (www.irimo.ir). Accordingly, maximum and minimum temperatures were recorded in August (with an average of 29.2 ºC) and January (with an average of 7.9 ºC), respectively. The maximum and minimum rain falls were recorded in July (with an average of 110.9 mm) and August (with an average of 4 mm), respectively (Fig. 1).

CA Variations During the Year

Foliar CA content of rosemary plants growing in Golestan, Iran was evaluated by HPLC (Fig. 2A). The presence of important compounds in each extract was identified based on the retention time, observed at 5.3 min. Results revealed that the average CA content increased slowly during the spring, with an average concentration of 57.8 mg gr^{-1} DW, and continued to increase in summer, autumn and winter with an average concentration of 67.5, 77.5 and 85.1 mg gr^{-1} DW, respectively. The highest and lowest amounts of CA were observed in January and April, respectively (Fig. 2B).

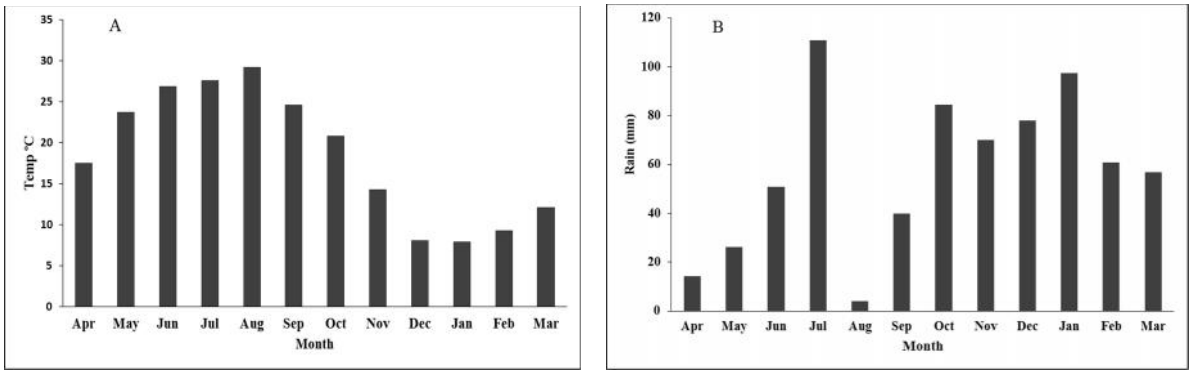

Fig. 1 Average temperature (A) and rain fall (B) during various months of the year.

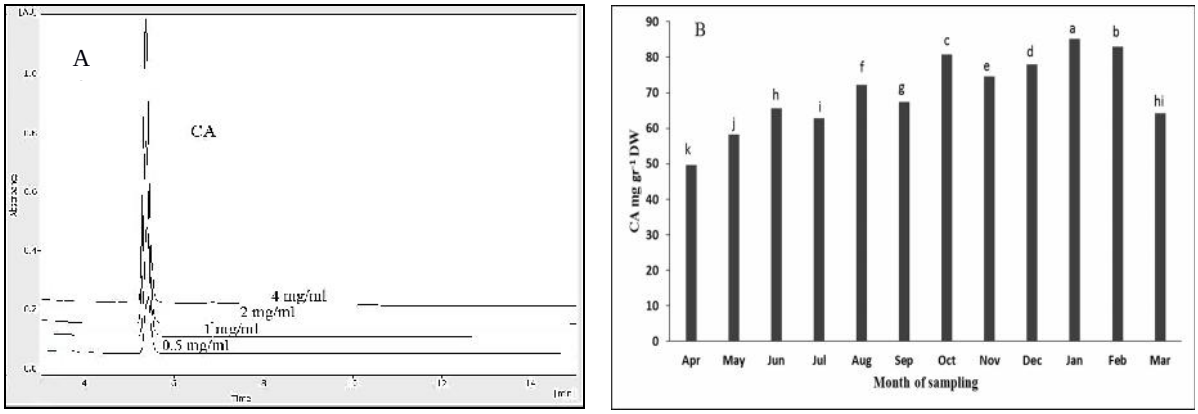

Fig. **2** HPLC chromatographs of different CA standard concentrations (A). Foliar content values of CA (mg gr^{-1} DW) during a year in Golestan, Iran (B).

Table 1 Correlation coefficients between CA, temperature, rainfall and other important rosemary active compounds.

	FRAP	DPPH	Ascorbic Acid	Phenol	Flavonoids	Anthocyanins	Soluble Sugars	Temp	Rain fall
CA	0.221	0.084	-0.201	0.221	0.035	0.703**	0.374*	-0.546**	0.510**

** Significant at p< 0.01; * Significant at p<0.05

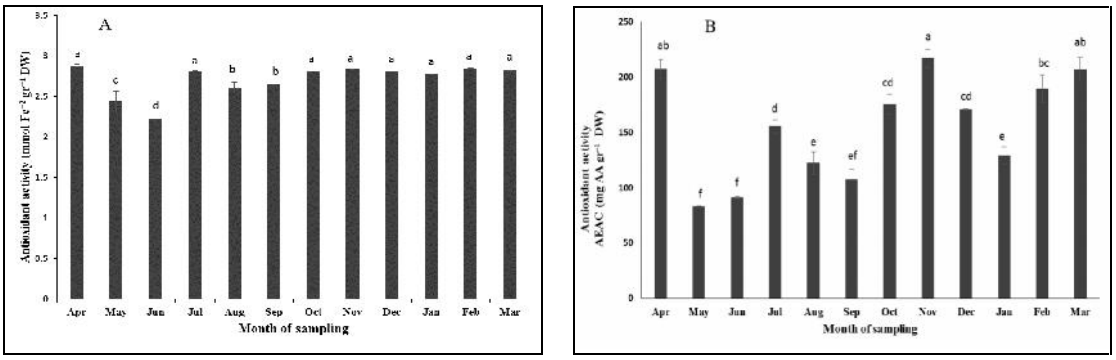

Fig. 3 Antioxidant activities detected in rosemary leaf extracts determined with the FRAP method (A) and DPPH method (B) during a year. Bars indicate Standard Error. Different letters indicate significant differences revealed by DUNCAN test (P<0.05).

Antioxidant Activity of Rosemary Leaf Extracts

Based on FRAP results, the maximum value of antioxidant capacity was 2.88 mmol Fe^{+2} gr^{-1} DW, detected in April; however, based on DPPH results, the maximum value of antioxidant capacity was 217.2 mg AA gr^{-1} DW, detected in November. These values were significantly higher than those detected during spring and summer. In both methods, leaf extracts from May and June exhibited the minimum antioxidant activity (Fig. 3). In addition, extracts collected in autumn and winter were rich in antioxidant constituents and demonstrated good antioxidant activity measured by both FRAP and DPPH assays.

Total Phenolic Content

The variation in total phenolic content of rosemary leaf extracts during a year and its correlation with CA content were analyzed (Table 1). Total phenolic content during different seasons varied by about 82.9% and ranged from 3.97 in June to 36.79 mg GAE gr^{-1} DW in March. In general, leaf extracts collected in autumn (with an average of 28.3 mg GAE g^{-1} DW) and winter (with an average of 28.59 mg GAE g^{-1} DW) contained more phenolics, as compared to samples collected during spring or summer with an average of 12.33 mg GAE gr^{-1} DW (Fig. 4).

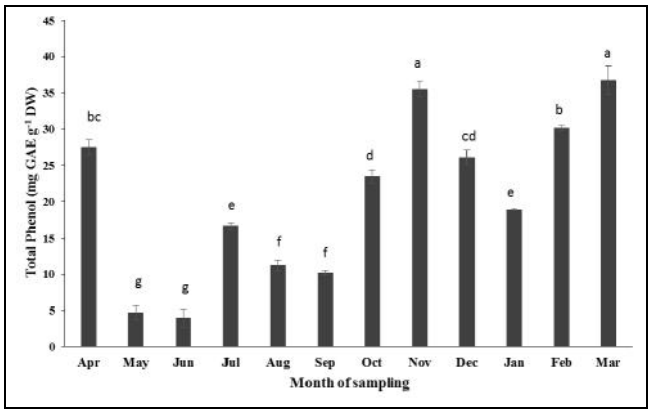

Fig. 4 Total phenolic content of rosemary leaf extracts under various months of the year. Bars indicate Standard Error. Different letters indicate significant differences (P<0.05).

Total Flavonoids Content

As shown in Fig. 5, results clearly indicate that seasonal variation affected total flavonoids of rosemary samples. The highest and lowest concentrations of flavonoids were recorded in samples collected from autumn (37.42 mg QE gr^{-1} DW) and summer (24.64 mg QE gr^{-1} DW), respectively (Fig. 5).

Ascorbic Acid Content

The highest and lowest concentrations of ascorbic acid were recorded in samples collected during winter (0.11 mg gr^{-1} DW) and summer (0.09 mg gr^{-1} DW), respectively, while the average AA content of rosemary leaf extracts in various seasons remained similar (Fig. 6).

Total Anthocyanins Content

Results of this study showed that leaf extracts collected during spring (2.14 mg gr^{-1} DW) bear low content of total anthocyanins in comparison to the other seasons. Results also revealed that total anthocyanins increased abruptly in mid-summer to 3.5 mg gr¯1 DW (August) and remained fairly stable until the end of winter (3.67 mg g^{-1}DW). A strong correlation was observed between total anthocyanin and CA contents of the extracts within the time course of the experiment (Table 1 and Fig. 7).

Soluble Sugars

Results showed that soluble sugars increased during spring, slightly decreased during summer, and began to increase until the end of winter to a maximum of 69.85 mg gr^{-1} DW and then dropped abruptly by the beginning of spring to a minimum of 38.13 mg g^{-1} DW.

A significant positive correlation was observed between soluble sugar and CA contents (Table 1 and Fig. 7).

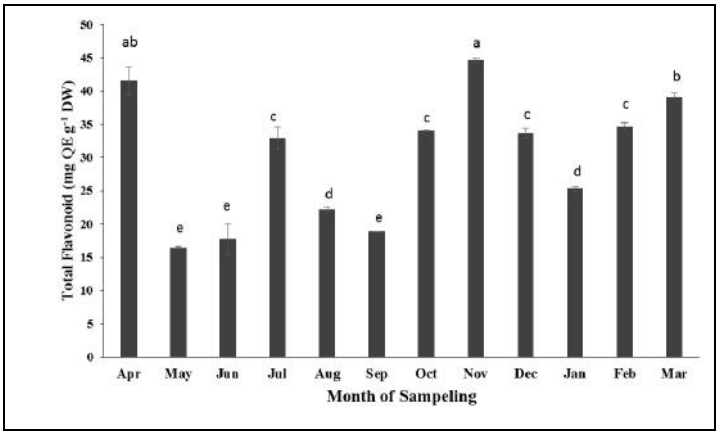

Fig. 5 Total flavonoids content of rosemary leaf extract in various months of the year (Mean±SE). Different letters indicate significant differences (P<0.05).

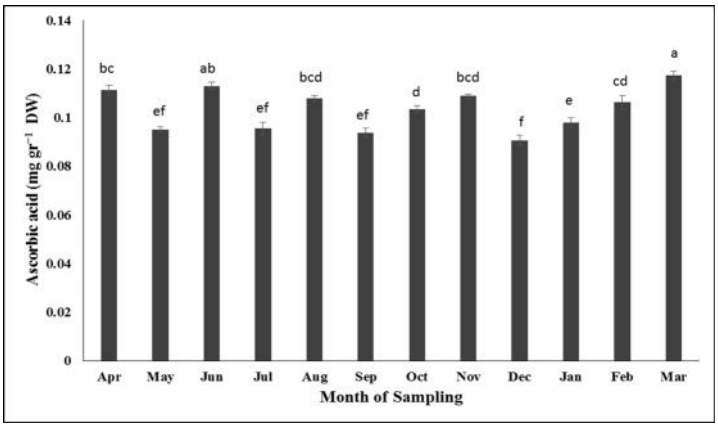

Fig. 6 Ascorbic acid content of rosemary leaf extract in various months of the year (Mean ± SE). Different letters indicate significant differences (P < 0.05).

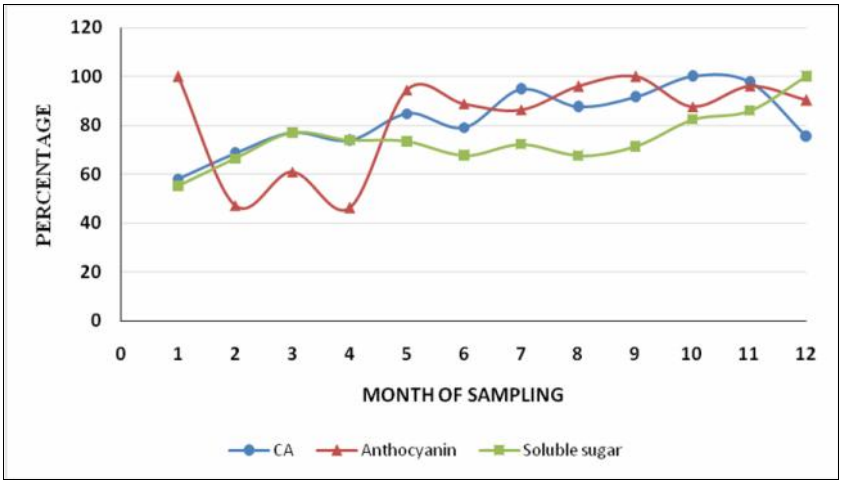

Fig. 7 Correlation between the CA content, total anthocyanins and soluble sugars of leaves extract during a year.

Discussion

In our experiments, variation analysis of CA content of rosemary during 12 months of a year revealed that the average concentration of CA increased by 41% during spring to autumn and reached to its maximum level in January. These data suggest that when, due to optimum temperature/precipitation and increased day length in spring, the plant vegetative growth increases, the leaf CA content also increases in the plant, however, during summer, due to reduced precipitation and increased temperatures, when the plants enter its reproductive stage, CA levels drops. Interestingly, CA increased during autumn and winter in Golestan, Iran, probably due to increased rainfall and subtle winters in this region. In agreement with this hypothesis, Luis and Johnson [9] reported that CA content in rosemary plants grown in Southern UK decreased by 50% during the summer (7 mg gr^{-1}FW in August), matching to the highest temperatures and lowest rainfall, and showed a recovery during September, October, and November, reaching maximum levels in December. Hidalgo *et al* [20] reported that in rosemary plants collected from C´ordoba, Spain, CA content increased slowly during the spring, peaked in the summer (46.1 mg g^{-1} DW on July), and dropped to the end of September, after which it continued to fall more slowly to a minimum of 21.4 mg g^{-1} DW by mid-February.

Leaf extracts prepared in various months of the year showed different levels of compounds important for antioxidant properties of the plant. Similarly, Lemos *et al* [10] showed that extracts from rosemary leaves collected in summer had better antioxidant activities, due to high levels of camphor and CA. Higher antioxidant activity in *Belli sperennis* L. flowers was observed in samples collectedfrom spring to autumn [21]. On the other hand, in *Ocimum basilicum* L. leaf and *Nothapodyte snimmoniana* (J.Graham) Mabb. bark extracts collected in winter exhibited the best antioxidant activity [22, 23]. Therefore, with regard to the accumulation of antioxidants, different plant species exhibit various responses, when exposed to seasonal variations. In agreement with this hypothesis, it is suggested that the antioxidant activity of plant extracts is correlated with their phenolic contents, which can be increased during plant responses to environmental stresses [12,24-26]. However, the best environmental condition for the accumulation of various active compounds varies between plant species. For instance, the shoots of *Mentha longifolia* (L.) Huds., contained more polyphenols and flavonoids in winter [27,28] while, in *Glycyrrhiza glabra* L. the major constituents liquiritin and glycyrrhizin were the highest in February and glabridin, glabrene and phenolics were the highest in November [11]. On the other hand, phenolic compounds are also reported to increase during summer high temperatures [29,30] or during spring in rosette leaves of *Chelidonium majus* L. [31]. Altogether, these data suggest that plant developmental stages and environmental conditions play crucial roles in production and accumulation of phenolics and flavonoid compounds in medicinal plants.

Our data revealed that total anthocyanin content increased in mid-summer. It may suggest that accumulation of anthocyanins in rosemary can be activated by high temperatures and/or low

precipitation. Plants in sub-optimal water conditions not only experience dehydration stress but increased risk of oxidative stress, which can cause membrane damage unless attenuated by antioxidants [32]. Given the antioxidative properties of anthocyanins, their increase during summer could be related to the protection of sensitive structures, such as cell membranes [33] or for protection of chlorophyll from degradation while increasing the percentage of bound-water within the cells. Combined with other anti-stress activities attributed to anthocyanins (including their solar shield and antioxidative capacities), this phenomenon may allow rosemary leaves to better tolerate dry seasons. In addition, the influence of other seasonal factors such as light intensity and temperature on anthocyanin contents should also be considered. Lu and Yang [34] showed that, elevated levels of light and temperature stimulate anthocyanin biosynthetic genes in *Solanum pinnatisectum* Dunal. Although protection against solar radiations may not be a universal feature in all anthocyanogenic leaf tissues, this property might prohibit the possibility of photo-oxidative stress, especially during high light periods.

Results presented in this study showed a strong correlation between total anthocyanins and CA content of the extracts (Fig. 7). *Rosmarinus* L. species normally grow in high light conditions and their photosynthetic tissues suffer from photo-oxidation [35]. Exposure to high light conditions often results in increased production of ROS in plants. Triantaphylides *et al*. [36] showed that oxidative damage motivated by high light involves singlet oxygen ($^1O_2$) as the major ROS induced by the excess energy in photosynthetic membranes. Plants have evolved several isoprenoids involved in mechanisms to dissipate this excess energy. Anthocyanins can efficiently protect photosynthetic lipids from photo-oxidation as they are capable of ROS scavenging. During this process, they particularly prohibit $^1O_2$ oxidation reactions [37] and dissipate the excess energy as heat [38]. Lipophilic rosemary extracts containing an array of compounds, including CA perform scavenging activities against ROS, notably $^1O_2$ [39]. A similar role can be postulated for soluble sugars, which fulfill various functional roles in plant metabolism. They might either directly detoxify ROS in chloroplasts and vacuoles or indirectly stimulate antioxidative defense systems [40]. Higher photosynthetic activity induces both the generation of ROS and massive accumulation of soluble sugars. It has been suggested that sugars themselves might be effective candidates for the oxidative burst in tissues exposed to a wide range of environmental stresses [40]. It has been suggested that plant phenolic contents increased under water stress by hydrolyzing soluble sugars [41]. Therefore, it can be hypothesized that an interaction of anthocyanins, soluble sugars and phenolic compounds, such as CA may act as a part of an integrated redox system, quenching ROS and contributing to stress tolerance, especially in tissues with high anthocyanins and soluble sugars.

Conclusions

The influence of environmental growing conditions can modulate the contents of CA and thus the antioxidant potential of rosemary leaf extracts. Since the time of harvest may be of major importance from an agronomic, medicinal and economic point of view; variations in the CA content in leaves of rosemary plants have been studied in Northern Iran. Total anthocyanins and soluble sugar content were found as the main components, which correlated with CA content in the time course of this study. These results suggest that climate condition can modulate the contents of CA in rosemary leaves by different mechanisms. Therefore, rosemary leaves harvested during various seasons contain different CA content and different phytochemical properties, which can be used in human health care.

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