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Seasonal behavior of *Thalassia testudinum* (Hydrocharitaceae) metabolites

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Abstract: The marine angiosperm *Thalassia testudinum*, commonly known as turtle grass, is a dominant seagrass that grows in the Caribbean Sea shelf associated to *Syringodium filiforme*. The hydroalcoholic extract of *T. testudinum* is rich in polyphenols; the most abundant metabolite in this extract is thalassiolin B, a glycosilated flavonoid with skin damage repairing properties, and antioxidant capacity among others. The present study aimed at generating information about the seasonal behavior of secondary metabolites, as well as to study the antioxidant capacity of the *T. testudinum* leaves extract, collected monthly during 2012 from the Northeast coastline of Havana, Cuba. For this study, spectrophotometric methods were used to determine the concentrations of polyphenols, flavonoids, anthocyanins, soluble carbohydrates and proteins, chlorophylls *a* and *b*, and antioxidant activity of the extracts. In general, results demonstrated seasonal variations of the analyzed parameters. Extracts prepared from the vegetal material collected in October and November showed the highest values of polyphenols (58.81 ± 1.53 and 52.39 ± 0.63 mg/g bs, respectivally) and flavonoids (44.12 ± 1.30 and 51.30 ± 0.67 mg/g dw, respectively). On the contrary, the lowest values of polyphenols were found in extracts of leaves collected in July and August (15.51 ± 0.84 and 13.86 ± 0.48 mg/g, respectively). In accordance with these results, the lower value of Inhibitory Concentration (IC_{50}) was obtained to get a 50 % of maximal effect on free radical scavenging activity with the extracts prepared from leaves collected in October and November, and less significant IC_{50} was obtained from the extract prepared from leaves collected in August (5.63 mg/mL). A negative correlation (r= -0.694) was observed in this study between the content of polyphenols and the IC_{50} necessary to get the half of its antioxidant maximal effect. The high correspondence between the maximum values of polyphenols, flavonoids, carbohydrates and proteins in October and November, revealed a close relationship between these metabolites found in the extract of *T. testudinum*. Our hypothesis about the annual variation in the concentration of these metabolites was validated; and these results will support the correct harvesting of *T. testudinum* leaves for biotechnology and industrial purposes. Rev. Biol. Trop. 64 (4): 1527-1535. Epub 2016 December 01.

Key words: angiosperm, *Thalassia testudinum*, seasonal variation, polyphenols, antioxidant activity.

Marine angiosperm prairies represent one of the coastal tropical ecosystems of greater importance, because of their multiple ecological and economic benefits (Heck, Hays, & Orth, 2003), where fundamental biological processes also take place, such as reproduction, breeding and refuge of several species. These environments provide habitat and food to a wide diversity of marine organisms such as fishes, invertebrates, algae, plankton, bacteria and fungi, some of which are of commercial importance for fishing. Others are considered

in danger of extinction, such as the manatee, *Trichechus manatus* and the green turtle *Chelonia mydas* (Márquez & Jiménez, 2002).

Differing from other elements of marine biodiversity, marine meadows were little studied during the XIX century and most of the XX century. It was until the 1960's that research about their structure, physiology and functioning started, demostrating the importance of these valuable ecosystems and the need for their conservation.

Marine angiosperms are organisms that live in a complex habitat and are subject to regular extreme conditions (sudden changes in salinity, variation in irradiation, emersion periods due to tides effect, surf and predators); therefore, they have to adapt to new changing circumstances. These, cause the production of metabolites that could be of great usefulness for man, if they are isolated and used for therapeutical purposes (Srivastava, Saurav, Mohanasrinivasan, Kannabiran, & Singh, 2010). However, the biochemical composition of these plants has practically not been studied. In literature, there is a report by Dawes and Lawrence (1980), that refers to the content of lipids, structural proteins and soluble and insoluble carbohydrates in different parts of *T. testudinum,* and a recent study by Regalado et al. (2012) that reported the content of some polyphenols, flavonoids and other compounds, and determined the antioxidant capacity of an extract from this species. Thus, the objetive of this research was to report the seasonal behavior of secondary metabolites production, as well as of its antioxidants (from hydroalcoholic extract), in *T. testudinum,* collected monthly on the northeast coastline of Havana, Cuba, during 2012.

MATERIALS AND METHODS

Collection and post-collection treatment: *Thalassia testudinum* Banks ex König leaves were collected monthly during 2012 at Guanabo Beach (23º10' N - 82º07'01" W), Havana, Cuba. The species was authenticated by Dr. B. Martínez-Daranas (Center for Marine Research, Havana) and located in the collection of the National Aquarium of Cuba, with number IDO 039.

Freshly harvested monthly samples of *T. testudinum* leaves were washed with potable and distilled water to remove sand, epiphytes and salts, drained and then dried in an oven to constant weight at a temperature of 50 °C.

Extraction: A sample of 200 g of dried and ground *T. testudinum* leaves (6 mm of particle size) were continuously macerated with 2000 mL of ethanol: H_2O (1:1 vol/vol) over a period of seven days at room temperature in close flask. Extracts were filtered by filter paper Whatman No. 1 (180 mm of diameter) and concentrated using rotatory evaporator BUCHI to dryness under reduced pressure and low temperature (45 °C).

Soluble proteins determination: This determination was conducted using the method described by Bradford (1976), with Bovine Serum Albumine (BSA) as the reference standard (1 mg/mL). Results were expressed in milligram of BSA equivalents per gram of plant dry extract.

Total soluble carbohydrates determination: The soluble carbohydrate content was assayed by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Concentrations in the samples were measured by spectrophotometric absorbance to 487 nm using distilled water as blank and D (+) galactose as standard. Soluble carbohydrate content was expressed as in milligrams of D (+) galactose equivalents per gram of plant dry extract.

Chlorophylls *a* and *b* estimation: Chlorophyll *a* and *b* contents were determined by a modification of the spectrophotometric method proposed in the Handbook of Food Analytical Chemistry (Waterhouse, 2005). The modification made consisted in the determination of chlorophylls concentration in the dry extracts dissolved with acetone.

Total phenolic determination: The total polyphenol concentration was determined using Folin-Ciocalteu method (British Pharmacopeia, 2007). The concentrations in the samples were measured by spectrophotometric absorbance to 760 nm using ethanol: water as blank and pyrogallol as standard. Results were expressed as milligram of pyrogallol equivalents per gram of dry extract.

Flavonoids estimation: The aluminium chloride colorimetric method was modified from the procedure reported by Woisky and Saltino (1998). The modification consisted in the use of the sample resolved in distilled water. Concentrations in the samples were measured by spectrophotometric absorbance to 415 nm using distilled water as blank and quercetin as standard to prepare the calibration curve. The flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry extract.

Anthocyanins estimation: The determination was conducted by the method described by Fuleki and Francis (1968). The concentrations in the samples were measured by spectrophotometric absorbance to 520 nm using methanol acidified with 1 % HCl as blank and considering the coefficient of molar extinction of anthocyanins diglycosides as $E^{1\%}_{1cm}$ (37 700 L/cm.mol). The total anthocyanins content was expressed as mg of malvidine diglycosides equivalents per gram of dry extract content.

Antioxidant activity: The method used was a modification of Tabart, Kevers, Pincemail, Defraigne and Dommes (2009), consisting in the use of ethanol: water as diluent of the samples. Results were expressed as the average of inhibitory concentration of the extracts able to reduce the DPPH radical to one half of its initial value.

All analyses were performed in triplicate. Data were analyzed by Duncan's multiple range test at $p < 0.05$ (SPSS 10.0.1) for the identification of statistical seasonal differences during the study period. The Pearson correlation coefficient (r) was also computed between metabolites content and IC_{50} DPPH reduced.

RESULTS

The behavior of the soluble protein content extracted from *T. testudinum* (Fig. 1A) revealed significant differences among the sampling months ($p < 0.05$). The highest values were found in extracts from the leaves collected in October (41.22 ± 0.9 mg/g d.m) and November (39.43 ± 1.0 mg/g dm) ($p < 0.05$). The lowest values were detected in extracts from leaves collected in January 2012 (3.58 ± 0.05 mg/g dw), July (3.05 ± 0.41 mg/g dw) and August of the same year (13.93 ± 1.44 mg/g dw) ($p > 0.05$).

The 2012 yearly profile of non-structural carbohydrates (Fig. 1B) appears to be very similar to that obtained for *T. testudinum* soluble proteins: Maximum values in extracts from leaves collected in October (266.88 ± 0.9 mg/g dw) and November (227.80 ± 1.2 mg/g dw) ($p < 0.05$). Similarly, the lower values were found in the extracts corresponding to July (67.87 ± 2.3 mg/g dw) and August (78.56 ± 1.13 mg/g dw) ($p < 0.05$), respectively. A positive correlation was found between the content of carbohydrates and the content of non-structural soluble proteins ($r= 0.705$; $p < 0.01$), that showed the physiological relation existing between these components.

The concentrations of chlorophyl *a* and *b* extracted from *T. testudinum* showed statistically significant differences among the months of study ($p < 0.05$). The maxima of both chlorophyls coincided in the extracts from leaves collected in May, June, October and November; while the lowest concentrations of both chlorophyls *a* and *b* corresponded to extracts of January, July and December 2012. Specifically for chlorophyl *a*, the lowest value was from the leaves collected in January 2012 (0.26 ± 0.03 µg/g dw), while chlorophyl *b* showed its lowest value in leaves collected in July (0.43 ± 0.06 µg/g dw) (Fig. 1C and Fig. 1D).

A positive correlation between the contents of chlorophyl *a*, chlorophyl *b* and soluble

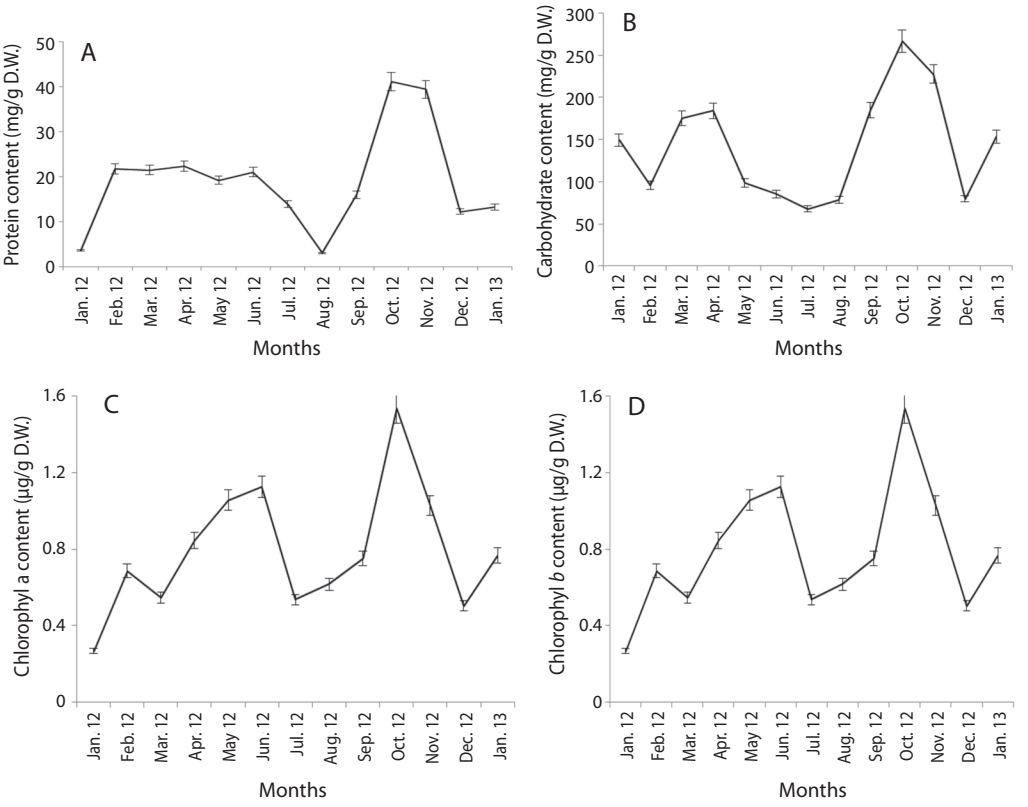

Fig. 1. Seasonal behavior of primary metabolites of *Thalassia testudinum*. A: soluble protein content; B: non-structural carbohydrates content; C: chlorophyl *a* content; D: chlorophyl *b* content.

proteins was observed. Chlorophyl *b* was significantly correlated with the content of proteins ($r = 0.768$; $p < 0.01$), and with chlorophyl *a* ($r = 0.800$; $p < 0.01$). On the other hand, chlorophyl *a*/chlorophyl *b* ratio showed a significant correlation with the content of chlorophyl *a* ($r = 0.524$ and $p < 0.01$).

A stadistically significant seasonal variation was evident in all 2012 in the content of polyphenols ($p < 0.05$). This secondary metabolite showed its maximum values in the leaves collected in October (58.81 ± 1.53 mg/g dw) and November (52.39 ± 0.63 mg/g dw); while the lower values corresponded to leaves collected in January (7.19 ± 0.13 mg/g dw), May (15.51 ± 0.84 mg/g dw) and August (13.86 ± 0.48 mg/g dw) (Fig. 2A).

Flavonoid concentration values showed a similar pattern as polyphenols, which were subject to a significant variation among the months of the year ($p < 0.05$) and showed their maximum values in leaves collected in the months of October (44.12 ± 1.30 mg/g dw) and November (51.30 ± 0.67 mg/g dw). The lowest values corresponded to leaves collected in January 2012 (9.47 ± 0.29 mg/g dw), May (11.06 ± 0.52 mg/g dw) and August of the same year (10.82 ± 0.53 mg/g dw) (Fig. 2B).

The monthly behavior of antocyanins in extracts *of T. testudinum* is shown in figure 2C. Statistically significant differences among the studied months were evident ($p < 0.05$). As observed in the content of polyphenols and flavonoids, the extracts of greater antocyanins content corresponded to the leaves collected in October (5.99 ± 0.21 mg/g dw) and November (6.40 ± 0.06 mg/g dw) ($p < 0.05$). The lowest value was observed in leaves collected

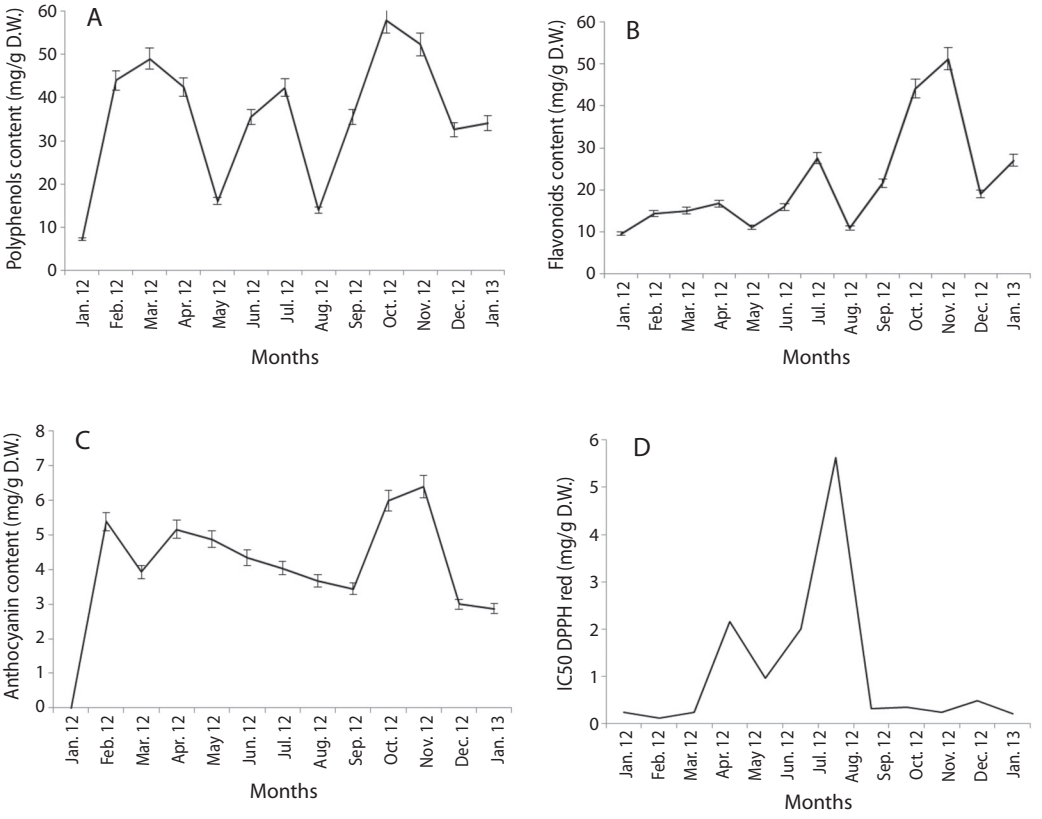

Fig. 2. Seasonal behavior of secondary metabolites of *Thalassia testudinum*. A: polyphenols content; B: flavonoids content; C: anthocyanins content; D: IC_{50} DPPH reduced.

in January 2012 (0.004 ± 0.36 mg/g dw), coinciding with the month of lowest content of polyphenols and flavonoids.

A significant correlation between the values of antocyanins and polyphenols was evident ($r= 0.699$; $p < 0.01$), and with flavonoids ($r= 0.540$; $p < 0.01$). On the other hand, we found a positive correlation between antocyanins and chlorophyl *a* ($r= 0.733$; $p < 0.01$) and of antocyanins with proteins ($r= 0.799$; $p < 0.01$).

The mean inhibitory concentrations of extracts of *T. testudinum* for achieving 50 % in the reduction of DPPH free radical (IC_{50}) are shown in figure 2D. The leaves harvested in different months showed significant differences among them ($p < 0.05$). The extract corresponding to leaves collected in March 2012 showed the lowest Inhibitory Concentration to reduce the free radicals to one half of their initial value. The leaves collected in January 2012 did not show antioxidant activity; this result also coincided with the lowest values of polyphenols, flavonoids and antocyanins found in this month.

The highest mean inhibitory concentration (IC_{50}) was observed in August with a value of 5.63 mg/mL. This result coincided with one of the lowest values of polyphenols, flavonoids and antocyanins found in all months of the study.

A negative correlation ($r= -0.694$, $p < 0.01$) was found between the concentration of polyphenols and the $IC_{50.}$ It means that lower sample concentrations are required for achieving 50 % of the reduction of DPPH radical, indicative of the antioxidant activity. On the other hand, another negative correlation

was found between the IC_{50} and the content of carbohydrates ($r = -0.528$; $p < 0.01$).

No correlation was found between the content of flavonoids and antocyanins with the mean inhibitory concentration necessary to reduce DPPH to one half of its initial value.

DISCUSSION

Seasonal changes in the concentration of major soluble components such as proteins and carbohydrates, showed a similar trend along the year, because both can be used for plant growth and development (Dawes & Lawrence, 1980). A similar behavior between soluble carbohydrates and proteins was found by Pradheeba, Dilipan, E., Nobi, E. P., Thangaradjou, T., & Sivakumar (2011) in other species of marine plants, for which they found a positive correlation between the content of carbohydrates and the soluble proteins. The fact that an increase in the concentrations of these metabolites was found in *T. testudinum* in October and November 2012 suggests that during the rainy period in Cuba (October and November), some favorable conditions arise; these can be: an increase of accumulated nutrients in the environment, adequate salinity and optimum temperatures that promote an increase in the photosyntetic rate favoring the plant development.

Generally, it was found that the values of chlorophyl *b* were greater than the values of chlorophyl *a* for almost all the study period. These results coincided with the report by Fillit (1995), where they showed that marine angiosperms are particularly rich in chlorophyl *b*.

Chlorophyls content in marine plants can be strongly influenced by the availability of light; when temperature increases, the photosyntetic speed increases markedly, although above certain values (30-35 °C), it decreases abruptly. In this study, a significant correlation between the monthly concentrations of chlorophyls *a* and *b,* and the carbohydrates concentration was evident. A reduction in photosyntesis, and hence in the content of carbohydrates in the colder months of the year in Cuba (such as December and January) was evident, as the enzymes involved in the photosyntesis process usually slow down their activity at lower and higher temperatures.

The increment of phenolic compounds in leaves of marine angiosperms is generally attributed to defense mechanisms in these plants, facing the growth of epiphytic algae, predators and pathogens (Subhashini, Dilipan, Thangaranhou, & Papenbrock, 2013). The allopathic effects of phenolic compounds in seagrass are well known according to what was pointed out by Dumay, Costa, Desjobert, and Pergent (2004); as a matter of fact, polyphenols inhibit growth and germination of the Magnoliophyta (Harrison, 1982), and interfere in their physiological functions like antioxidants (Osawa Ramarathnam, Kawakishi, & Namiki, 1991) and enzymatic inhibitors (Van Sumere, 1989). The action mechanism through which phenolic compounds protect lipids from the oxidative damage has been widely discussed in literature. It has been pointed out that polyphenols act by interrupting the chain propagation reactions of Lipid Peroxidation (LPO) (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Other studies refer to their property of capturing the peroxyl and alkyl radicals in the initiation and propagation phases of LPO (Liu et al., 2002; Sano, Yoshida, Degawa, Miyase, & Yoshino, 2003). Yamamoto, Moon, Tsushida, Nagao, and Terao (1999) reported that the orthodihydroxyl structure in the B ring of polyphenols is required to obtain a maximum activity entrapment of these lipidic radicals. Higuchi, Yonemitsu, Koreeda, & Tsunenari (2003) mentioned that the chelating activiy of iron ions is another possible inhibition mechanism of LPO. According to that, it is valid to think that one of the mechanisms through which the extract of *T. testudinum* inhibit LPO *in vitro*, could be related to its capacity of trapping free radicals.

The production and accumulation of polyphenolic compounds is taken as an indicator of physiological stress that can be induced by abiotic factors, such as the scarcity of nutrients (Chalker-Scott & Fuchigami, 1989), contamination by heavy metals (Ragan &

Glombitza, 1986) and the changes in temperature (Rivero et al., 2003).

The seasonal trend observed in polyphenol concentration of *T. testudinum* leaves showed significant variations with the months. The highest contents were found in October and November, and also in March and April; however, water temperature in these months was not the determinant abiotic factor of their variations. In October, when the rainy period starts in Cuba, epiphytic algal blooms may have been promoted, and also may have originated the synthesis of polyphenols in *T. testudinum*. Dumay et al. (2004) found high levels of polyphenols in the adult leaves of the species *Posidonia oceanica*, related to its interaction with the macrophyte *Caulerpa taxifolia*; besides he found an increase in leave length, due to a competition for light, and particularly, in the production of phenolic compounds, which showed their biosynthesis as a chemical defense.

The flavonoids concentration extracted from *T. testudinum* showed a significant annual variation, in correspondence with the maxima of polyphenols in October and November. Rowley et al. (2002) isolated three glycosilated flavonoids thalassiolin a, b and c, as active principles of *T. testudinum* with anti-HIV activity. On the other hand, Regalado et al. (2009) isolated thalassiolin b and showed its antioxidant and dermoregenerating activity.

The polyphenols concept includes any metabolite that, in its structure, has a benzenic ring with one or more hydroxylic substitutions; so that both, flavonoids and antocyanins, are within this great family of chemical compounds. In general, they have been classified by their functions as grabbers of free radicals (stabilizing the reactive species), citoprotectors and DNA damage inhibitors (Evans & Johnson, 2010), as well as their repellent activity to herbivorism of the marine pastures (Millan, 1984).

As shown in this study, the maximum contents of polyphenols, flavonoids and antocyanins in *T. testudinum* were found in October and November. Likewise, it was proven that in these months the antioxidant activity of these extracts increased, thus confirming the role played by these metabolites in the antiradical activity (Athiperumalsamy, Rajeswari, Poorna, Kumar, & Jesudass, 2010). In this study, a highly significant inverse correlation between the mean inhibitory concentration (IC_{50}) and the concentration of polyphenols was shown. Several authors have determined that there is a positive linear correlation between the content of total phenols and the reducing power, and they have demonstrated that the antiradical activity in plants is a consequence of the concentration of some of their secondary metabolites, such as polyphenols; this fact has been observed in different extracts of plants (Zheng & Wang, 2001; Karawita et al., 2005).

On the other hand, a high content of polyphenols is not always translated into a high reducing power, as this could be influenced by the presence of other antioxidant components (Wang, Zhang, Duan, & Li, 2009). In our study, something similar occurred in July, for which high values of polyphenols, flavonoids, antocyanins, carbohydrates and proteins were obtained; however, the antioxidant activity did not show interesting values.

The high correspondence that exists between the maximum values of polyphenols, flavonoids, carbohydrates and proteins in October and November revealed a close relationship among these metabolites. This was also shown by the highly significant correlations found among them, a fact that supports the hypotesis that the specimens collected in these dates were in favorable conditions for their growth and development.

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RESUMEN

Comportamiento estacional de los metabolitos de *Thalassia testudinum* (Hydrocharital: Hydrocharitaceae). La angiosperma marina *Thalassia testudinum*, comúnmente conocida como "hierba tortuga", es un pasto marino dominante que crece en el Mar Caribe asociada

a *Syringodium filiforme*. El extracto hidroalcohólico de *T. testudinum* es rico en polifenoles; el metabolito más abundante en este extracto es thalassiolina B, un flavonoide glicosilado con propiedades para la reparación de daños en la piel y la capacidad antioxidante, entre otros. El objetivo del presente estudio fue conocer el comportamiento estacional de los metabolitos secundarios, así como la capacidad antioxidante del extracto de hojas de *T. testudinum* recolectadas mensualmente durante el 2012, en la costa noreste de La Habana, Cuba. Para este estudio fueron empleados métodos espectrofotométricos para la determinación de la concentración de polifenoles, flavonoides, carbohidratos y proteínas solubles, clorofilas a y b y la actividad antioxidante del extracto. En sentido general, los resultados demostraron las variaciones estacionales de los parámetros analizados; los extractos preparados a partir de material vegetal recolectado en octubre y noviembre mostraron los mayores valores de polifenoles y flavonoides (44.12 ± 1.30 y 51.30 ± 0.67 mg/g bs respectivamente) y se encontraron los valores más bajos en los extractos de hojas recolectadas en julio y agosto. (15.51 ± 0.84 y 13.86 ± 0.48 mg/g respectivamente). De acuerdo con los resultados, se obtuvo el valor más bajo de la concentración inhibitoria (CI_{50}) necesaria para obtener un 50 % de efecto máximo en la actividad de captación de radicales libres con los extractos preparados a partir de las hojas recolectadas en octubre y noviembre y la CI_{50} menos significativa se obtuvo a partir del extracto preparado a partir de las hojas recolectadas en agosto (5.63 mg/ml). Se observó una correlación negativa (r= -0,694) entre el contenido de polifenoles y la CI_{50} necesaria para obtener la mitad del efecto antioxidante máximo. La alta correspondencia que existe entre los valores máximos de polifenoles, flavonoides, carbohidratos y proteínas en octubre y noviembre reveló una estrecha relación entre estos metabolitos, encontrados en el extracto de *T. testudinum*. La hipótesis de la variación anual de la concentración de estos metabolitos fue validada. Estos resultados se tendrán en cuenta con el fin de seleccionar el momento de recolecta de las hojas de *T. testudinum* para su uso con fines biotecnológicos e industriales.

Palabras clave: angiosperma, *Thalassia testudinum*, variación estacional, polifenoles, actividad antioxidante.

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