Spatio-Temporal Variation of Terpenoids in Wild Plants of *Pentalinon andrieuxii*

Mickel R. Hiebert-Giesbrecht,[a] Fabiola Escalante-Erosa,[a] Karlina García-Sosa,[a] Gabriel R. Dzib,[b] Luz M. Calvo-Irabien,[b] and Luis M. Peña-Rodríguez*[a]

[a]Centro de Investigación Científica de Yucatán, Unidad de Biotecnología, Calle 43 No. 130, CP 97205, Colonia Chuburná de Hidalgo, Mérida, Yucatán, México, e-mail: lmanuel@cicy.mx
[b]Centro de Investigación Científica de Yucatán, Unidad de Recursos Naturales, Calle 43 No. 130, CP 97205, Colonia Chuburná de Hidalgo, Mérida, Yucatán, México

Pentalinon andrieuxii (MÜLL.ARG.) B.F.HANSEN & WUNDERLIN (Apocynaceae) is a vine native to the Yucatan peninsula, where it is widely used in Mayan traditional medicine to treat, among other ailments, the wounds caused by cutaneous leishmaniasis. Among the secondary metabolites isolated from *P. andrieuxii* are the triterpene betulinic acid and the chemically unusual tri-norsesquiterpene urechitol A; however, to date, there is no existing knowledge about the accumulation dynamics of the ubiquitous betulinic acid or the novel urechitol A in the plant. In this article, we report on the accumulation of both secondary metabolites in wild individuals of *P. andrieuxii*; our results show that while the content of betulinic acid in plant leaves bears no apparent relation to plant ontogeny, the content of urechitol A in root tissue is clearly related to plant development.

Keywords: *Pentalinon andrieuxii*, Apocynaceae, Secondary metabolite accumulation, Sesquiterpene, Triterpene, Urechitol A, Betulinic acid.

Introduction

Secondary metabolite accumulation dynamics in plant tissues is as diverse as secondary metabolism itself. The biosynthesis, transport, and accumulation or degradation of secondary metabolites is highly related to the immediacy for their need and the metabolic cost to produce them.[1] Because of this, studies on the production dynamics for secondary metabolites need to be considered individually, in each species, and for particular conditions.

Terpenes are the largest and most diverse group of secondary metabolites;[2] their role includes being structural constituents in cell membranes or active participants as mediators in ecological processes such as defense or pollinator attraction.[3] In spite of their diversity and function, the current understanding of terpenoid production dynamics in plant tissues is limited, with most studies focusing on economically important species, cultivated under controlled conditions.[4 – 6]

Pentalinon andrieuxii (MÜLL.ARG.) B.F.HANSEN & WUNDERLIN (Apocynaceae) is a perennial vine native to the Yucatan peninsula, which can grow up to the top of tree canopy and can be found from southern Florida to Nicaragua.[7] It is commonly used in Mayan traditional medicine to treat various ailments, specially the wounds derived from cutaneous leishmaniasis.[8] Although the phytochemical knowledge about *P. andrieuxii* has grown considerably in the past few years, with reports describing the production of sterols,[9] coumarins and pregnanes,[10] as well as triterpenoids, such as betulinic acid,[10][11] and the novel and chemically unusual urechitols,[12] the dynamics of the biosynthesis and accumulation of these secondary metabolites during plant development is still unknown. As part of a project directed towards establishing the biosynthetic origin of the novel campechane skeleton (**CS**) of the urechitols, we wish to report herein on the accumulation dynamics of two different terpenoids, the pentacyclic triterpene betulinic acid (**BA**) and the trinorsesquiterpenoid urechitol A (**UA**) (*Fig. 1*), during plant development.

Results and Discussion

Developmental Stage Determination

Plant age estimation is among the most inaccessible parameters to establish plant development; consequently, the age of non-tree herbaceous plants has

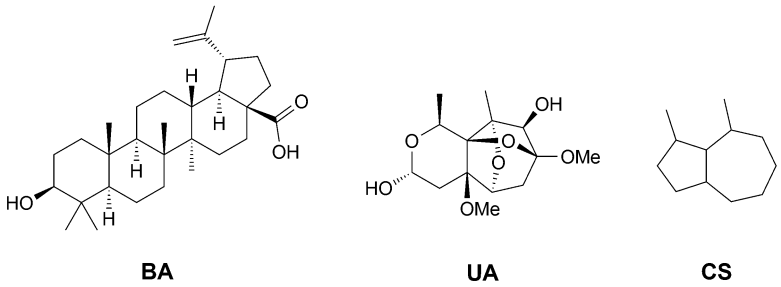

Figure 1. Chemical structures of betulinic acid (**BA**), urechitol A (**UA**), and campechane skeleton (**CS**).

been mostly ignored when studying wild populations.[13] Furthermore, most studies concerning herb age estimation focus on species growing in temperate climates,[14] which might be the reason why little is known about the life development history of tropical grown herbaceous perennials. Due to this lack of information, solid development indicators for wild *P. andrieuxii* plants had to be established for this investigation. Traditionally used parameters include morphological traits related to developmental stages (*e.g.*, seedling, sapling, juvenile, reproductive) or plant size (stem diameter and plant height).[15] A more recent approach for establishing the age of herbaceous perennial plants is herb-chronology,[16] which allows for a more precise description of plant growth. Herb-chronology relies in the appearance of growth rings formed in the stem or root tissue due to periodical and contrasting climatological conditions along development.[17] Although most of the work done using this approach has been applied to plants growing in temperate climates,[18][19] we have recently shown that herb-chronology is an adequate development marker for herbaceous plants growing in tropical climates.[20]

Four development indicators were used in this investigation: developmental stage (morphology), stem diameter at ground level (SDGL), plant height, and number of growth rings in root tissue (herb-chronology). Our data shows that each developmental stage, designated as young, intermediate, and adult, is clearly different from one another in morphological and quantitative terms (*Table 1*), although SDGL and the number of growth rings were found to have a more accurate prediction power for plant development than plant height. The limited use of plant height to predict plant development can be explained by the fact that, because *P. andrieuxii* is a vine, its height is highly influenced by the proximity and height of surrounding trees that serve as a support for this climbing growth habit.

Terpenoid Spatio-Temporal Distribution

Secondary metabolite location is mostly related to its role in plant tissues.[21] In some cases, the location of a secondary metabolite can be very specific, *e.g.*, artemisinin, a sesquiterpene used to treat malaria, accumulates only in glandular trichomes located on the leaves of *Artemisia annua*.[22] Alternatively, paclitaxel, a complex diterpenoid first isolated from the bark of *Taxus brevifolia* and mainly used for the treatment of cancer, has been found in many other members of the genus and in different plant organs such as needles, seeds, pulp, and stems.[23]

To determine the spatial distribution of **UA** and **BA** among different plant organs and developmental stages of *P. andrieuxii*, the content of both metabolites was monitored in the leaf, stem, and root extracts of plants from different developmental stages. Previous reports have shown a wide distribution of **BA** across many plant families[24] and in different plant organs;[25] conversely, **UA** has only been reported as a secondary metabolite isolated from the root extract of *P. andrieuxii*.[12] In this study, two very distinct dynamics in relation to metabolite accumulation were observed; **BA** was only detected in extracts from the

Table 1. Morphological and quantitative plant development parameters (mean and standard deviation)

Developmental stage	Habit	Plant height [m]	SDGL [mm]	Number of growth rings
Young	Self-supporting	0.26 ± 0.08	3.87 ± 1.36	2.88 ± 0.60
Intermediate	Beginning of climber	0.91 ± 0.34	8.01 ± 2.45	4.40 ± 0.52
Adult	Climber, flowering, fruits	6.13 ± 2.03	21.87 ± 4.13	6.38 ± 0.52

leaves of the plant, with mean contents ranging from 0.5 to 0.9 mg/g dry wt and no apparent relation to plant development (H(2) = 5.862, $P = 0.053$) (*Fig. 2a*). In contrast, **UA** was detected predominantly in the root extracts, with mean contents ranging from 0 to 0.5 mg/g, and showing a direct correlation with plant development (H(2) = 29.810, $P < 0.01$) (*Fig. 2b*).

Although contents of **BA** in the leaves showed a weak correlation with plant height (linear $r^2 = 0.25$; *Pearson*'s $\rho = 0.534$, $P < 0.05$) (*Fig. 3a*), no statistically significant correlation was observed between the **BA** content in the leaves and the other development parameters (*Fig. 3b,c*). It is important to mention that while **BA** has been detected in the leaves from different plant species,[26] it has also been found in other organs, such as bark[27] and fruits.[28] The many biological activities reported for **BA**, and its common occurrence in exposed organs such as leaves, bark or fruits,[29] suggests a defensive role for this triterpenoid. Our data appear to confirm the ecological importance of **BA** by playing an important defensive role in the leaf tissue during the entire development of *P. andrieuxii*. It is also reasonable to understand that **BA** accumulation in the leaf tissue is not required, since the leaves are constantly being exchanged during plant development.

Unlike **BA**, the results of this investigation showed that **UA** content in the roots increases with each developmental stage (*Fig. 2b*). This was clearly established by the significant correlations observed between **UA** content and plant height (*Spearman*'s $\rho = 0.826$, $P < 0.01$) (*Fig. 3d*), between **UA** content and SDGL (*Spearman*'s $\rho = 0.823$, $P < 0.01$) (*Fig. 3e*), and between **UA** content and the number of growth rings (*Spearman*'s $\rho = 0.788$, $P < 0.01$) (*Fig. 3f*). These correlating results between **UA** content and the different plant development parameters further confirms the importance of herb-chronology, and the number of growth rings in the root tissue, as a solid indicator for establishing the age of herbaceous perennial plants growing in tropical climates.[20] The detection of **UA** mainly in the root extracts of the plant coincides with the original report of its being isolated from the root extract of *P. andrieuxii*;[12] although the biological meaning for the accumulation of this metabolite in the root tissue of the plant remains unknown, it has been widely reported that plant secondary metabolites accumulate during plant development for different reasons, *e.g.*, to serve as defense agents against herbivores or pathogens, or in response to abiotic stress or as signals of ecological relevance.[3]

Additionally, to date no biological activity has been described for **UA**; however, the novelty of the **CS** (*Fig. 1*) has stimulated an interest for understanding its biosynthetic origin. Initial attempts to incorporate labeled precursors into the sesquiterpenoid carbon have resulted in the isolation of labeled terpenoids[30] but not of labeled **UA**, emphasizing the importance of learning more about the production dynamics of **UA** during plant development. The results of this investigation showed that, while **UA** could not be detected in root tissue during the first stage of plant development (*Fig. 2a*), its content increased steadily after the second stage; this suggests that there might be a distinct event during the transition from first to second stage that is likely to trigger the biosynthesis of **UA**. Since flowering of the plant occurs during the third

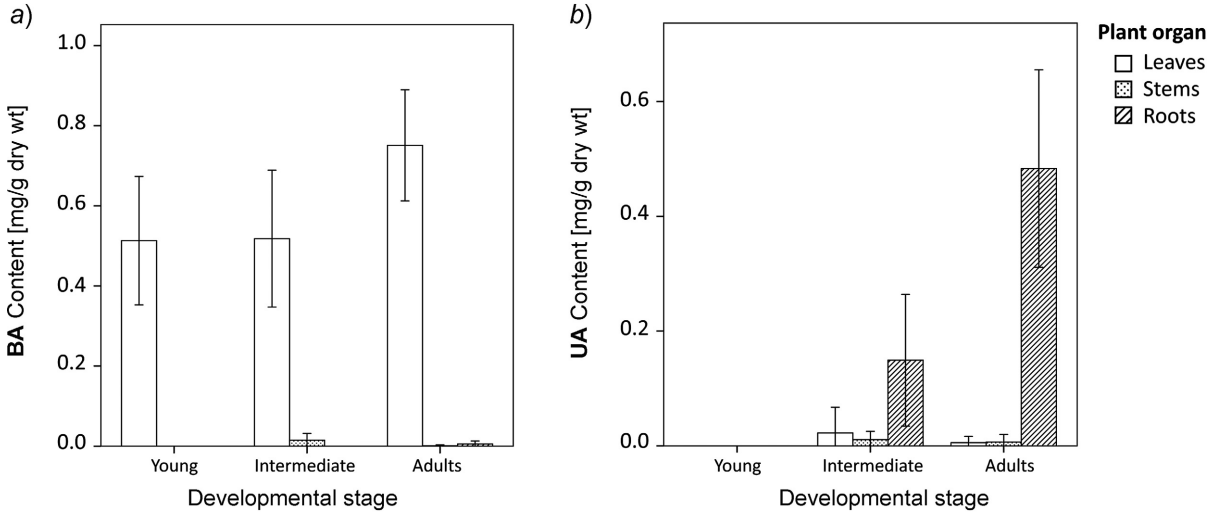

Figure 2. Mean **BA** *a*) and **UA** *b*) content in different plant organs and in different development stages in *Pentalinon andrieuxii*. *Significant at $P < 0.01$.

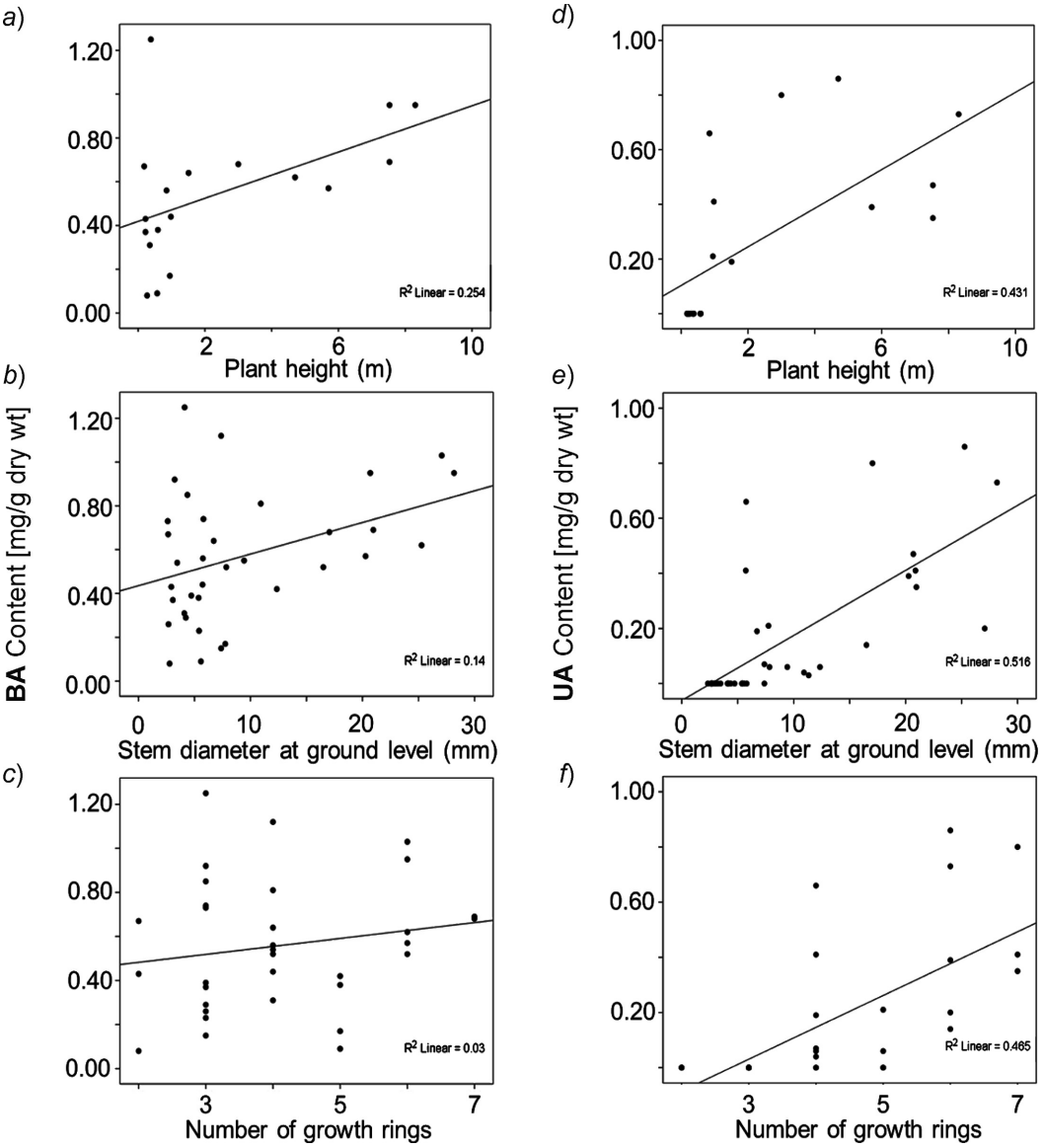

Figure 3. **BA** Leaf and **UA** root content related to SDGL (*a* and *d*), plant height (*b* and *e*), and number of growth rings (*c* and *f*).

stage of development, and **UA** biosynthesis takes place before that, it is possible to speculate that the event triggering **UA** biosynthesis might be more related to ontogeny than phenology. Interestingly enough, the main ontogenic change occurring during the first to second development stage transition in *P. andrieuxii* is its going from an erect to a climber plant.[20] Future studies aimed at establishing the biosynthetic origin and biological role of **UA** should focus on this transitional period of development.

Experimental Section

Plant Material

Thirty-seven plant samples were collected at the end of both rainy (19) (December 2013) and dry (18) (May 2014) seasons in the state of Campeche, México (19°46′0.89′′N; 90°80′0.156′′W). A voucher specimen was deposited with the Herbarium of 'Unidad de Recursos Naturales' CICY, under the collection code Calvo, Dzib, Hiebert 334. Plants were grouped according to three developmental stages, with clearly distinct morphological and quantitative characteristics (*Table 1*). The height and SDGL were measured for each individual before collection. Each plant was kept separately, taken to the laboratory, washed to eliminate soil, and separated into leaves, stems, and roots (dry season root samples were also divided into phloem and xylem); after this, the plant material was air-dried for 48 – 72 h. Each dry sample was ground to a fine powder using a mill equipped with a 1-mm mesh, and stored at −20 °C until extraction.

Extraction Procedure

Each sample (*ca.* 1 g) was extracted with MeOH (100 ml) under sonication (20 min, 40 °C) and then filtered (*Whatman No. 1*). After washing the filter paper with 10 ml of MeOH, the solvent was evaporated under reduced pressure and the resulting extract was sonicated (20 min) with CH_2Cl_2 (100 ml). The resulting CH_2Cl_2-soluble fraction was stored at −20 °C until GC and HPLC analysis.

Plant Development Parameters

Developmental Stages. Young, intermediate, and adult. Young plants where characterized by an erect stem, height below 50 cm, slow growth, and with upper (younger) leaves bigger in size than lower (older) ones (inverted triangle shape).

Intermediate plants were starting vegetative growth, with a fast growing vine appearing in the apical end, with younger leaves smaller than older ones (triangle shape), and a height range of 60 – 190 cm.

Adult plants were taller than two meters and with visible signs of flowering and/or fruit production.

Stem Diameter at Ground Level. Stem diameter was measured using a *Digimatic CD-6 Caliper* (*Mitutoyo Corp.*).

Plant Height. Plant height was measured using a measuring tape for young and intermediate individuals and a standardized pole for adults.

Herb-Chronology Analysis. Top segments (*ca.* 5 – 10 cm) of each root sample were separated, labeled, and stored in an aq. EtOH soln. (75% *v/v*) until analysis. Sections of 45 – 50 μm of each specimen were obtained using a *Leica RM2125RT* microtome.

Tissue Staining and Photograph Protocol

Cross sections of each sample were separately stained using fluoroglucinol/HCl (*Wiesner* reaction) and toluidine blue (Grahan, 1984). Stained cross sections were photographed using a *Zeiss Stemi 2000-C Stereo-Microscope* and a *Moticam 2000* digital camera. Images where analyzed using the Motic Images Plus 2.0 software (*Motic China Group Co. Inc.*).

Growth Ring Analysis. The identification of growth rings was done following two main criteria: *i*) appearance of larger vessels and *ii*) radial distribution of the vessels.

Chromatographic Quantification

Quantification of Urechitol A by GC-FID. Quantitative analyses were carried out using a *Varian 430-GC* (*Varian*, Palo Alto, Ca., USA) gas chromatograph equipped with a *Varian PAL* autosampler (*Varian*, Palo Alto, Ca., USA). Separations were carried out using an *Ultra 1* column (25 m × 0.32 mm i.d. × 0.52 μm film thickness; stationary phase 100% dimethylpolysiloxane polymer; *Agilent Technologies*, Santa Clara, CA, USA). The GC conditions were set as follows: injector, splitless; injection volume: 1.0 μl; carrier gas: N_2; flow rate: 1.0 ml/min; split: off. Oven temperature program for roots and stems: 150 °C for 3 min, heating at a 9.3 °C/min rate to 260°, held for 5 min; for leaves: 150 °C for 3 min, heating at a 9.3 °C/min rate to 260°, held for 15 min. Injector and flame-ionization detection (FID) temps. were set at 250 and 280 °C, resp. FID conditions: sampling frequency: 12.5 Hz; data processed using the Galaxie software (*Varian*, Palo Alto, Ca., USA). A calibration curve was prepared using pure **UA** isolated from a root extract of *P. andrieuxii*. The standard solns. were injected in duplicate and the curve was constructed using five concentration levels (0.01 – 0.1 mg/ml). The resulting curve was linear in the concentration interval used (r^2 = 0.996).

Quantification of Betulinic Acid by HPLC-UV. Analyses were performed using a *Waters* HPLC chromatograph, equipped with a *Waters 600* binary pump, *Waters 717* plus autosampler, *Waters 2487* dual-wavelength ultraviolet detector (UV), and Empower Pro© software (2002, *Waters*, Milford, MA, USA). The anal. method (modified from *de Oliveira et al.*[31]) was developed using a *Waters Spherisorb® ODS2 RP-18* column (4.6 × 250 mm, 5 μm) (*Waters*, Milford, MA, USA). The mobile phase consisted of 90% MeCN (solvent *A*) and 10% H_2O (solvent *B*). The flow rate was set to 1.0 ml/min, the injection volume to 20 μl, column temp. was maintained at r.t., and the detection was carried out at λ 210 nm. The conditions used were adequate for a baseline separation of betulinic acid. A calibration curve was prepared with a commercial sample of **BA** (*Sigma–Aldrich*). The standard solns. were injected in duplicate and the curve was constructed using six concentration levels (0.01 – 0.5 mg/ml). The resulting curve was linear in the concentration interval used (r^2 = 0.990).

Statistical Analysis

Statistical analysis was carried out using the SPSS statistics software version 17.0.1. Data were analyzed in

order to determine the significance of differences in urechitol A and betulinic acid content between development stages and plant organs. Means were compared using the nonparametric *Kruskal–Wallis* test with a significance threshold of $P \leq 0.05$. A linear correlation analysis, using the *Spearman* coefficient, was performed in order to establish the relation between metabolite content and developmental stage indicators.

Acknowledgements

The authors wish to thank *CONACYT-México* for the financial support through research grant CB2013/223404; *M. R. H. G.* wishes to thank *CONACYT* for scholarship No. 366295.

References

[1] J. Gershenzon, *J. Chem. Ecol.* **1994**, *20*, 1281.
[2] P. M. Dewick, 'Medicinal Natural Products - A Biosynthetic Approach', John Wiley & Sons, Ltd., West Sussex, 2009.
[3] R. Croteau, T. M. Kutchan, N. G. Lewis, in 'Biochemistry & Molecular Biology of Plants', Eds. B. Buchanan, W. Gruissem and R. Jones, John Wiley & Sons, Ltd, West Sussex, 2000, p. 1250.
[4] R. Slimestad, M. J. Verheul, *J. Agric. Food Chem.* **2005**, *53*, 3114.
[5] S. Ceunen, J. M. C. Geuns, *Phytochemistry* **2013**, *89*, 32.
[6] P. D. Brown, J. G. Tokuhisa, M. Reichelt, J. Gershenzon, *Phytochemistry* **2003**, *62*, 471.
[7] J. F. Morales, *Darwiniana* **2009**, *66*, 217.
[8] M. T. Pulido, L. Serralta, 'Lista anotada de las plantas medicinales de uso actual en el Estado de Quintana Roo, México', Centro de Investigaciones de Quintana Roo, Quintana Roo, 1993.
[9] A. Yam-Puc, L. Chee-González, F. Escalante-Erosa, A. Arunachalampillai, O. F. Wendt, O. Sterner, G. Godoy-Hernández, L. M. Peña-Rodríguez, *Phytochem. Lett.* **2012**, *5*, 45.
[10] L. Pan, C. M. Lezama-Davila, A. P. Isaac-Marquez, E. P. Calomeni, J. R. Fuchs, A. R. Satoskar, A. D. Kinghorn, *Phytochemistry* **2012**, *82*, 128.
[11] D. B. Domínguez-Carmona, F. Escalante-Erosa, K. García-Sosa, G. Ruiz-Pinell, D. Gutierrez-Yapu, M. J. Chan-Bacab, A. Giménez-Turba, L. M. Peña-Rodríguez, *Phytomedicine* **2010**, *17*, 379.
[12] A. Yam-Puc, F. Escalante-Erosa, M. Pech-Lopez, M. J. Chan-Bacab, A. Arunachalampillai, O. F. Wendt, O. Sterner, L. M. Peña-Rodríguez, *J. Nat. Prod.* **2009**, *72*, 745.
[13] F. H. Schweingruber, P. Poschlod, *For. Snow Landsc. Res.* **2005**, *79*, 195.
[14] H. Dietz, F. H. Schweingruber, *Can. J. Bot.* **2002**, *649*, 642.
[15] M. Begon, M. Mortimer, D. Thompson, 'Population Ecology: A Unified Study of Animals and Plants', Blackwell Science, Oxford, 2002.
[16] H. Dietz, I. Ullmann, *Ann. Bot.* **1998**, *82*, 471.
[17] von Arx G., S. R. Archer, M. K. Hughes, *Ann. Bot.* **2012**, *109*, 1091.
[18] von Arx G., H. Dietz, *Plant Biol.* **2006**, *8*, 224.
[19] Y. Liu, Q. Zhang, *J. Integr. Plant Biol.* **2007**, *49*, 144.
[20] M. R. Hiebert Giesbrecht, M. Sc. Thesis, Centro de Investigación Científica de Yucatán at Mérida, 2015.
[21] A. Ramakrishna, G. A. Ravishankar, *Plant Signal. Behav.* **2011**, *6*, 1720.
[22] M. V. Duke, R. N. Paul, H. N. ElSohly, G. Sturtz, S. O. Duke, *Int. J. Plant Sci.* **1994**, *155*, 365.
[23] H. N. ElSohly, E. M. Croom, W. J. Kopycki, A. S. Joshi, M. A. ElSohly, J. D. McChesney, *J. Environ. Hortic.* **1997**, *15*, 200.
[24] S. Fulda, *Mol. Nutr. Food Res.* **2009**, *53*, 140.
[25] S. Jäger, H. Trojan, T. Kopp, M. N. Laszczyk, A. Scheffler, *Molecules* **2009**, *14*, 2016.
[26] N. Frighetto, R. M. Welendorf, A. M. Pereira da Silva, M. J. Nakamura, A. C. Siani, *Phytochem. Anal.* **2005**, *16*, 411.
[27] G. Zhao, W. Yan, D. Cao, *J. Pharm. Biomed. Anal.* **2007**, *43*, 959.
[28] S. Guo, J. Duan, Y. Tang, S. Su, E. Shang, S. Ni, D. Qian, *J. Pharm. Biomed. Anal.* **2009**, *49*, 1296.
[29] C. Chandramu, R. D. Manohar, D. G. L. Krupadanam, R. V. Dashavantha, *Phyther. Res.* **2003**, *17*, 129.
[30] L. M. Peña-Rodríguez, A. Yam-Puc, N. Knispel, N. Schramek, C. Huber, C. Graßberger, F. G. Ramírez-Torres, F. Escalante-Erosa, K. García-Sosa, M. R. Hiebert-Giesbrecht, M. J. Chan-Bacab, G. Godoy-Hernández, A. Bacher, W. Eisenreich, *J. Org. Chem.* **2014**, *79*, 2864.
[31] B. H. de Oliveira, C. A. M. Santos, A. P. D. M. Espíndola, *Phytochem. Anal.* **2002**, *13*, 95.

Received March 12, 2016
Accepted June 7, 2016

