Extraction and thermal characterization of epicuticular wax from *Opuntia streptacantha* at different stages of maturation

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ABSTRACT

The content of epicuticular waxes on the surface of the cladodes of *Opuntia streptacantha* and their thermal characteristics based on the stage of maturation of the plant were determined. The moisture content of samples of cladodes from *O. streptacantha* at different stages of maturation was determined. The ultrastructure of the surface of the cladodes was determined in dehydrated samples employing scanning electron microscopy. The surface of the cladodes contains amorphous particles related to epicuticular waxes in all stages of maturation. The extraction of hydrophobic compounds was carried out through Soxhlet. The extracts obtained were thermally characterized utilizing DSC. Results showed no significant differences for the moisture content or hydrophobic compounds, regardless the stage of maturation. The melting temperature was different in young cladodes (1 and 2 years old) than old cladodes (3 and 4 years old); however, these temperatures are similar to those waxes with high commercial value, hereby we propose as a new alternative to commercial waxes for different technological applications.

Keywords: *Opuntia*, vegetable wax, DSC, Melting temperature, Microstructure.

INTRODUCTION

Species of the genus *Opuntia* is traditionally used in different ways, as food sources, medicines, cosmetics, and fodder; besides, as a building material and natural color source, depending on its biochemical characteristics (Stintzing and Carle, 2005). Studies conducted by Ramirez-Tobías *et al.* (2007) showed that the biochemical properties of cladodes of various species vary with the phenological stage of maturation. The concentration and composition of fiber, proteins, and carbohydrates of cladodes depend of its maturation stage; moreover, it has been reported that the lipid content is slightly higher in young cladodes (Nuñez-López *et al.*, 2013), confirming by Hernández-Urbiola *et al.* (2011) who showed that the lipid content in prickly pads flours decreased according to age.

One of the most important characteristics of the nopal is its high resistance to dehydration, due to the thickness of its cuticle. The cuticle consists mainly of two types of lipophilic polymers (cutine and epicuticular waxes), which are altered in their composition and ultrastructure by genetic, physiological, and environmental factors during their maturation (Tafolla-Arellano *et al.*, 2013).

Vegetable waxes have gained considerable attention in research, due to its natural origin, regulatory approval for use in food and commercial availability (Jana and Martini, 2016; Patel *et al.*, 2015; Blake *et al.*, 2014). Currently, the most commonly used waxes are candelilla, carnauba, rice, and sunflower wax where the main characteristics of these are their melting temperature and chemical composition. According to Ramírez-Gómez *et al*. (2016), candelilla wax has a melting point of 64.41 °C. In other study, Zheng *et al*. (2011) mentioned that the melting temperature of carnauba wax ranges between 80 and 88 °C. On the other hand, Dassanayake *et al*. (2011) reported melting temperatures of rice wax of around 78 and 81 °C. Blake *et al.* (2014) described that sunflower wax has a melting temperature between 74-77 °C. The chemical characteristics of vegetable waxes are determined by a heterogeneous mixture of long-chain esters, fatty acids, fatty alcohols, pentacyclic triterpenoids of alcohol and sterol esters (Blake *et al.*, 2014).

Several researchers have determined the microstructural characteristics of different vegetable waxes. Rykaczewski *et al.* (2016) observed the microstructure of epicuticular waxes in different *Opuntia* spp. at different phenological stages employing Environmental Scanning Electron Microscopy (ESEM). They determined that new cladodes have a complex three-dimensional nanostructure of epicuticular wax, while old cladodes have large cracks that break epicuticular wax on islands with a size of tens of micrometers. Barthlott *et al.* (1998) classified the microstructural characteristics of epicuticular waxes, where it catalogs different types of layered surfaces, softened layers and scabs; they also mentioned the presence of aggregates of crystals that form hollow cylinders surrounding the stomata, characteristic of succulent and xerophytic plants such as *Cactaceae*. On the other hand, Pérez-García and Sepúlveda-Sánchez (2011) determined that intercostal waxes in stomata protect the plant from dehydration. Therefore, the objective of this research were to quantify the content of epicuticular waxes on the surface of the cladodes of *Opuntia streptacantha* and define the thermal characteristics depending on the stage of maturation of the plant.

MATERIALS AND METHODS

Cladodes, determination of moisture and wax content

Cladodes of *O. streptacantha* were collected in different stages of maturation (1, 2, 3 and 4 years) from a nopal and maguey reserve area of the Ejido Palma Pegada, Salinas de Hidalgo, San Luis Potosí, Mexico, located at 22.435029 N and 101.4633.14 W. The collection was obtained between February and May 2018. The parenchyma, collenchyma and sclerenchyma were removed from the collected material, with the purpose to obtain the epidermis and periderms, where the cuticle is deposited. This cuticle was dehydrated in a convection oven (BINDER. FD53-UL, Germany) at 60 °C for 24 h to determine the percentage (wt/wt) of moisture in triplicate, according to the AOAC method 44.925.45. The waxy extract content was determined using the Soxhlet technique, using the AOAC 963.15 method, adding hexanes solvent (140 mL, Jalmek Scientific, Monterrey, Mexico) to 20 g of dehydrated sample.

Scanning Electron Microscopy

Dehydrated samples of the different stage of maturation of *O. streptacantha* were cut approximately 0.5×0.5 cm and placed on a circular copper slide (3 cm diameter), and were placed in a vacuum chamber (PLASMA CURRENT SPI Supplies SP1 Supplies División of STRUCTURE PROBE, Inc.). This chamber consisted in two modules, SPI-MODULE- Control y SPI-MODULE Sputter Coater, where a controlled negative atmosphere of 10^{-2} torr was generated using Argon. Samples were coated with a conductive metal (gold) for 4 minutes. Then the samples were placed in the scanning electron microscope (SEM; JEOL JSM-6390LV) to obtain images of the surface of cuticles at different magnifications through the Control User Interface software (Version 8.25 Copyright 2005, 2007 JEOL TECHNICS LTD).

Differential Scanning Calorimetry (DSC)

Approximately 4 mg of extract of each sample was placed in hermetic aluminum pans, then, the samples were introduced into the differential scanning calorimetry equipment (DSC, Model 2920; TA Instruments, New Castle, DE, USA). In order to determine the crystallization and melting profile, samples were heated at 130 °C for 10 min and then cooled from 130 °C to 0 °C at a rate of 10 °C/min, followed by second heating from 0 to 130 °C with a speed of 5 °C/min. The thermal parameters of crystallization temperature (T_{Cr}), melting temperature (T_F) and enthalpies of crystallization (ΔH_{Cr} and ΔH_F, respectively) were determined from the crystallization and melting profiles. The thermal parameters and the thermograms were analyzed using the Universal Analysis 2000 software (TA Instruments-Waters LLC, Version 4.5A). Two independent determinations were made (n= 2).

Statistical analysis

The phenological stage of cladodes was considered as a factor of variability, taking as an experimental unit a cladode. A variance analysis and Tukey's test of comparison of means ($p \leq 0.05$) for each evaluated trait were performed. The experimental design was completely randomized with three repetitions. The data was processed in the statistical package R-Studio Desktop version 1.2.5033 (2017).

RESULTS

Moisture and wax content

The moisture content present in cladodes at different phenological states, varying between 82 and 86 % (data not showed). The 1-year cladodes had the highest moisture content with 85.0±3.4 %, followed by 2, 3 and 4 years old (83.7±1.8 %, 82.6±0.5 % and 82.0±1.4 %, respectively). The results showed that the average moisture content decreases as their age increases. However, the statistical analysis for the percentage of humidity showed no significant differences related to the state of maturation of the cladodes (*p*=0.40). Astello-García *et al*. (2012) reported a moisture content between 80 and 95 % for *Opuntia ficus-indica,* suggesting that the chemical composition of commercial and wild species (e.g. moisture content) depends on the stage of maturation.

According to the wax content analysis, cladodes contain 2.5 to 3.2 % (wt/wt), having the highest wax content at 2 years (i.e., 3.2±0.6 %); 3-year cladodes recorded the lowest wax content (i.e., 2.3±0.8 %) (Fig. 1). Valdez-Cepeda *et al.* (2008) reported an increase in cactus fat as the cladode´s age increases, from the fat content data of 20 nopal varieties; however, the analyses showed no significant differences for the age of cladodes (*p*=0.77). Rojas-Molina *et al*. (2013) reported near 4 % yield of candelilla wax. On the other hand, De Freitas *et al.* (2019) mentioned that the yield of carnauba wax varied from 5.4 to 7.2 %, depending on the drying conditions. Therefore, it is considered that the content of waxes of the *O. streptacantha* according to the stage of maturity is low, according to the report for other species that produce wax.

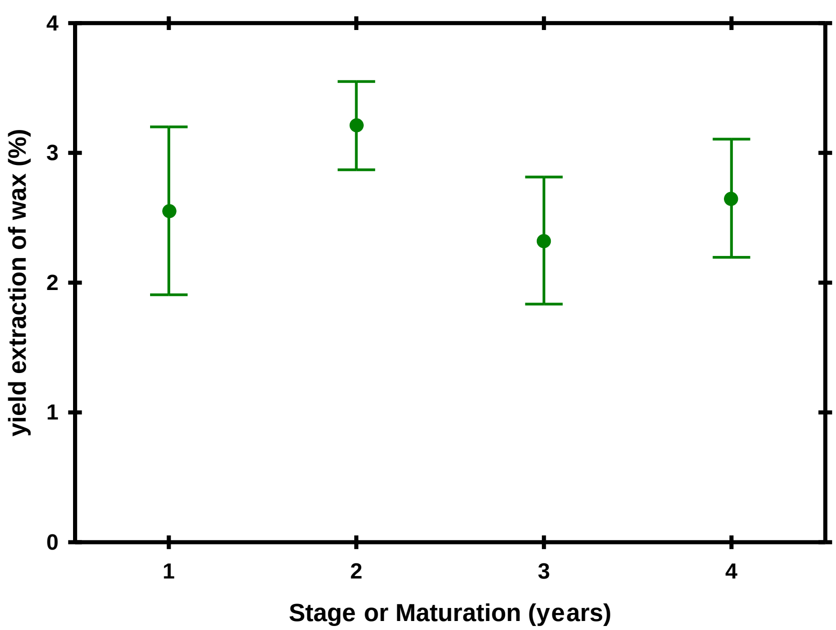

Figure 1. Hydrophobic compounds content on the surface of cladodes of *O. streptacantha* at different stages of maturation.

Scanning Electron Microscopy

The microscopic structures of the samples of O. *streptacantha* at different stages of maturation are shown in Figure 2. The four samples [i.e. 2A (1 year), 2B (2 years), 2C (3 years) and 2D (4 years)] showed a flat surface, with cracks and stomatic structures. Also, the images showed bright, amorphous, spherical particles and irregular platelet shape just as reported by Rykaczewski *et al.* (2016). These particles were observed more abundantly in Figure 2B (i.e., 2 years of maturation) compared to the other three images (2A, 2C, and 2D). Pérez-García and Sepulveda-Sánchez (2011) described the presence of wax crystals, platelet-like and with irregular shapes on the surface of palm leaves (*Sabal yapa* Wright ex Becc). In *O. ficus-indica* cultivars, showed translucent plaques of different shapes and sizes covering the epidermal cells of the cuticle, which are considered to be epicuticular waxes (Salem-Fnayou *et al.*, 2014). Therefore, in this study, the particles observed on the surface of the cladodes of *O. streptacantha* correspond to epicuticular waxes.

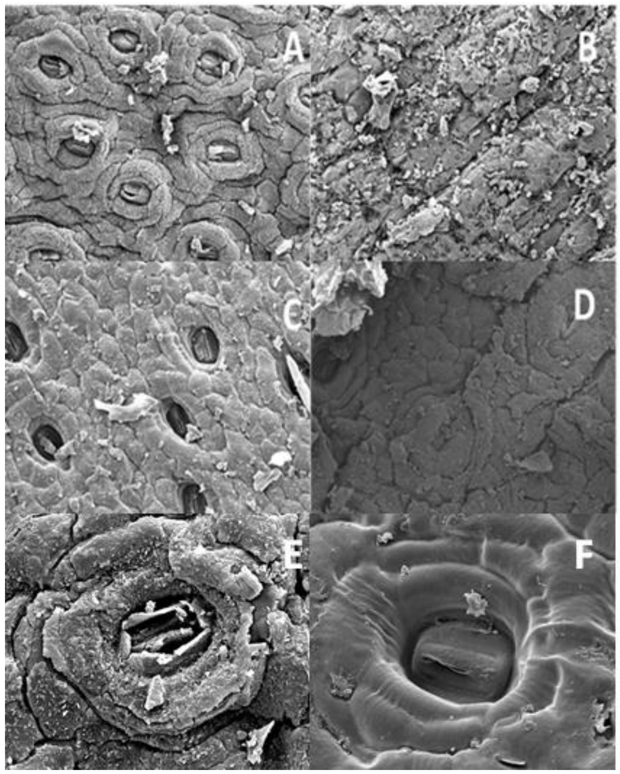

Figure 2. Microstructure of *O. streptacantha* at different stages of maturation. One year (A), 2 years (B), 3 years (C) and 4 years (D). Hydrophobic compounds present in stomata of 2-years *O. streptacantha* (E) and stomata of 2-year *O. streptacantha* with wax extraction (F). Magnification 250x.

The 2-year *O. streptacantha* microphotographs are shown in Figures 2E and 2F (without and with wax extraction, respectively). The presence of amorphous and bright particles around the stoma and the presence of irregular intercostal platelets in the same were observed in Figure 2E, similar to that reported by Salem-Fnayou *et al.* (2014), who demonstrated the presence of epicuticular waxes around the stoma in *O. ficus-indica*. Besides, Pérez-García and Sepulveda-Sánchez (2011) observed another type of wax, describing it as a thick, smooth, and waxy intercostal layer in the palm stomata. Barthlott *et al.* (1998) described crystalloids or their aggregations forming hollow cylinders surrounding stomata. Stomatic chimneys can derive from layers and scabs that are characteristic of succulent and xerophytic plants.

A stomatic structure of *O. streptacantha* is shown in Figure 2F after having undergone a solvent extraction process (i.e. Soxhlet), where a smooth surface is observed with elevated surrounding epidermal cells, without the presence of waxy scab structures or with the presence of a thin layer of waxes of only a few layers of molecules. This finding proves that the bright particles and intercostal platelets are removed by the solvent, so it is suggested that the extracts through liquid-solid extraction (i.e. soxhlet) are mostly epicuticular waxes.

Thermal analysis

The crystallization profile determined by DSC for all cladode extracts at different phenological stages reflected only one exotherm (data not shown), which represents the interval of temperatures required for crystallization of the compounds. The thermal parameters obtained from the crystallization profiles are shown in Table 1. Cladodes of 1-year had the highest crystallization temperature (i.e. 75.70±0.01 °C), followed by cladodes of 2-years with 75.59±0.18 °C, 4-years with 74±0.06 ºC and 3-years with the lowest crystallization temperature (i.e. 73.46±0.15 ºC). These results showed statistical differences (p<0.05), but cladodes of 1 and 2 years were not different. However, the crystallization temperatures of the waxes of the cladodes of 3 and 4-years showed differences.

Table 1. Thermal parameters of hydrophobic compounds of *O. streptacantha* at different stages of maturation. Crystallization temperature (Tcr), Melting Temperature Peak1 (T_F), Melting Temperature Peak 2 (T_{F2}), Crystallization Enthalpy (ΔH_{cr}) and Melting Enthalpy (ΔH_F).

Age (years)	T_{cr} (ºC)	T_F (ºC)	T_{F2} (ºC)	ΔH_{cr} (J/g)	ΔH_F (J/g)
1	75.70±0.01 a*	73.37±0.04 a	80.04±0.13 a	170.35±0.04 a	177.25±0.54 a
2	75.59±0.18 a	73.59±0.20 a	79.65±0.07 a	173.80±2.69 a	182.15±0.50 a
3	73.46±0.16 c	71.10±2.28 a	77.30±0.42 b	126.35±2.33 c	139.60±1.84 c
4	74.00±0.06 b	73.32±0.13 a	77.86±0.31 b	137.42±3.32 b	152.45±5.44 b
SEM	0.08746	0.8097	0.1935	1.7361	0.6289
p-value	0.0001	0.2442	0.0014	0.0001	0.0003

* Average values followed by the same letter were not significantly different (Tukey p≤0.05), SEM= Standard Error of the Mean.

Figure 3 shows the melting profiles of the hydrophobic compounds of cladodes at different phenological stages. The endotherms evidence the melting of the extracts and the temperatures at which the hydrophobic compounds change of phase. All samples of *O. streptacantha* at different phenological stages showed two endotherms. The first of them is the main hydrophobic compounds (i.e. waxes) and are shown as the highest signal in the heat flow. The second peak of the endotherm is presumably the fusion of components of greater molecular weight. Zheng *et al.* (2011) concluded that the variation of melting points of different natural waxes is due to the composition and molecular weight of samples.

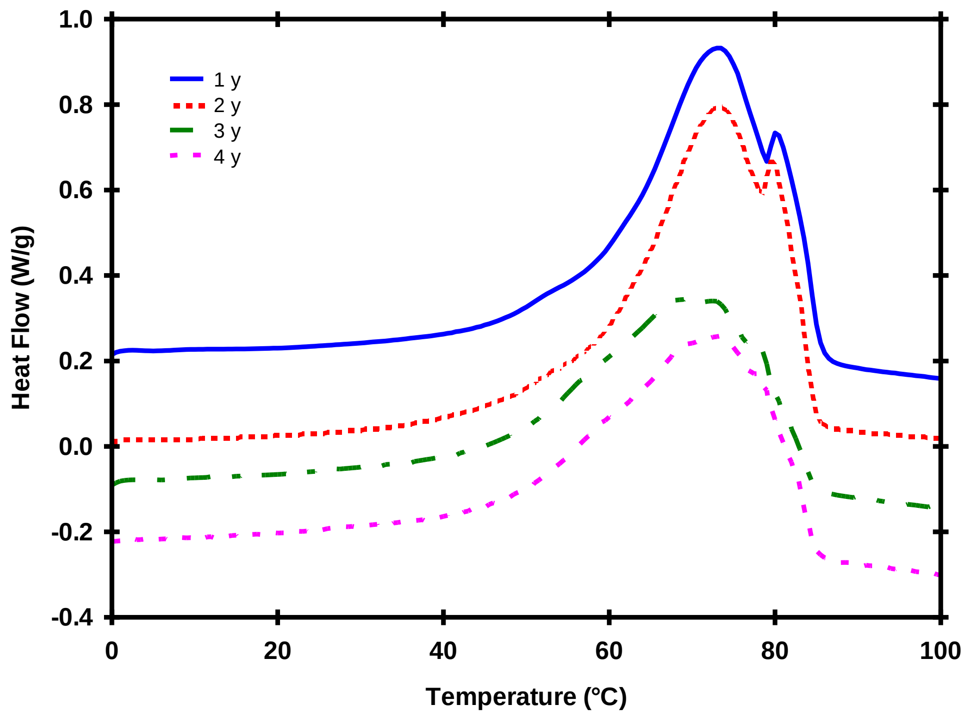

Figure 3. Melting thermograms of hydrophobics compounds of *O. streptacantha* at different stages of maturation.

The melting temperature of the second endotherm of the 1 and 2-year cladodes sample reached values of 80.04±0.13 °C and 79.65±0.07 °C, respectively (Fig. 3). These temperatures were not different but different to 3 and 4-year-old samples. The 3 and 4-year-old cladodes reflected melting temperatures in the second endotherm of 77.30±0.42 °C and 77.86±0.31 °C, respectively, which were not different (Table 1). However, the results showed differences in the melting temperatures of the second exotherm between young cladodes (i.e., 1 and 2 years) and mature cladodes (i.e. 3 and 4 years). This indicates that there are probably hydrophobic compounds with higher melting temperature in the cuticle in younger cladodes than in older cladodes.

Finally, from the DSC data, the enthalpies of crystallization (ΔH_{cr}) and fusion (ΔH_F) of the samples at different phenological stages are shown (Table 1). In all cases, melting enthalpy is slightly greater than the crystallization enthalpy because the chemical components of the extracts probably underwent recrystallization during the cooling process (Zheng *et al.*, 2011). It is observed that the enthalpies of crystallization and melting in the phenological stages of 1 and 2-years were not different (Table 1). On the other hand, the enthalpies of crystallization and melting of the phenological stages of 3 and 4 years were different from each other and between the enthalpies of the young cladodes (i.e. 1 and 2 years) (Table 1). These results indicate that the cuticle of the young cladodes produces larger molecular structures, more abundant or probably more complex than the cuticle waxes of the cladodes of greater phenological development.

CONCLUSIONS

The cuticle of *O. streptacantha* used in this research contains significant concentrations of epicuticular waxes regardless of the stage of maturation of the cladodes. The analyzed extracts showed melting temperatures similar to waxes with high commercial value, holding evidence to be proposed as a new alternative to commercial waxes for different technological applications.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

Astello-García, M.G., Santos Díaz, M.D.S., Reyes-Agüero, A., Barba De La Rosa, A.P. 2012. *Opuntia* spp. as a source of bioactive compounds. *ACS Symposium Series* 1109: 101-111. https://doi.org/10.1021/bk-2012-1109.ch008.

Barthlott, W., Neinhuis, C., Cutler, D., Ditsch, F., Meusel, I., Theisen, I., Wilhelmi, H. 1998. Classification and terminology of plant epicuticular waxes. *Botanical Journal of the Linnean Society* 126(3): 237-260. https://doi.org/10.1006/bojl.1997.0137.

Blake, A.I., Co, E.D., Marangoni, A.G. 2014. Structure and physical properties of plant wax crystal networks and their relationship to oil binding capacity. *Journal of the American Oil Chemists' Society* 91(6): 885-903. https://doi.org/10.1007/s11746-014-2435-0.

Dassanayake, L.S.K., Kodali, D.R., Ueno, S. 2011. Formation of oleogels based on edible lipid materials. *Current Opinion in Colloid and Interface Science* 16(5): 432-439. https://doi.org/10.1016/j.cocis.2011.05.005.

de Freitas, C.A.S., de Sousa, P.H.M., Soares, D.J., da Silva, J.Y.G., Benjamin, S.R., Guedes, M.I.F. 2019. Carnauba wax uses in food - A review. *Food Chemistry* 291: 38-48. https://doi.org/10.1016/j.foodchem.2019.03.133.

Hernández-Urbiola, M.I., Pérez-Torrero, E., Rodríguez-García, M.E. 2011. Chemical analysis of nutritional content of prickly pads (*Opuntia ficus indica*) at varied ages in an organic harvest. *International Journal of Environmental Research and Public Health* 8(5): 1287-1295. https://doi.org/10.3390/ijerph8051287.

Jana, S., Martini, S. 2016. Physical characterization of crystalline networks formed by binary blends of waxes in soybean oil. *Food Research International* 89: 245-253. https://doi.org/10.1016/j.foodres.2016.08.003.

Nuñez-López, M.A., Paredes-López, O., Reynoso-Camacho, R. 2013. Functional and hypoglycemic properties of Nopal cladodes (O. ficus-indica) at different maturity stages using in vitro and in vivo tests. *Journal of Agricultural and Food Chemistry* 61(46): 10981-10986. https://doi.org/10.1021/jf403834x.

Patel, A. R., Babaahmadi, M., Lesaffer, A., Dewettinck, K. 2015. Rheological profiling of organogels prepared at critical gelling concentrations of natural waxes in a triacylglycerol solvent. *Journal of Agricultural and Food Chemistry* 63(19): 4862-4869. https://doi.org/10.1021/acs.jafc.5b01548.

Pérez-García, M., Sepúlveda-Sánchez, J.D. 2011. Micromorfología de ceras epicuticulares en hojas maduras de *Sabal yapa* Wright ex Becc. (Arecaceae). *Polibotánica* 32: 153-161.

Ramírez-Gómez, N.O., Acevedo, N.C., Toro-Vázquez, J.F., Ornelas-Paz, J.J., Dibildox-Alvarado, E., Pérez-Martínez, J.D. 2016. Phase behavior, structure and rheology of candelilla wax/fully hydrogenated soybean oil mixtures with and without vegetable oil. *Food Research International* 89: 828-837. https://doi.org/10.1016/j.foodres.2016.10.025.

Ramírez-Tobías, H.M., Reyes-Agüero, J.A., Pinos-Rodríguez, J.M., Aguirre-Rivera, J.R. 2007. Efecto de la especie y madurez sobre el contenido de nutrientes de cladodios de nopal. *Agrociencia* 41(6): 619-626.

Rojas-Molina, R., De Leon-Zapata, M.A., Sucedo-Pompa, S., Aguilar-Gonzalez, M.A., Aguilar, C.N. 2013. Chemical and structural characterization of candelilla (*Euphorbia antisyphilitica* Zucc.). *Journal of Medicinal Plants Research 7*(12): 702-705. https://doi.org/10.5897/JMPR11.321.

Rykaczewski, K., Jordan, J.S., Linder, R., Woods, E.T., Sun, X., Kemme, N., Manning, K.C. Cherry, B.R., Yarger, J.L., Majure, L.C. 2016. Microscale mechanism of age dependent wetting properties of prickly pear cacti (*Opuntia*). *Langmuir 32*(36): 9335-9341. https://doi.org/10.1021/acs.langmuir.6b02173.

Salem-Fnayou, B., A., Zemni, H., Nefzaoui, A., Ghorbel, A. 2014. Micromorphology of cactus-pear (*Opuntia ficus-indica* L. Mill.) cladodes based on scanning microscopies. *Micron* 56: 68-72. https://doi.org/10.1016/j.micron.2013.10.010.

Stintzing, F. C., Carle, R. 2005. Cactus stems (*Opuntia* spp.): A review on their chemistry, technology, and uses. *Molecular Nutrition and Food Research* 49(2): 175-194. https://doi.org/10.1002/mnfr.200400071.

Tafolla-Arellano, J.C., González-León, A., Tiznado-Hernández, M.E., García, L.Z., Báez-Sañudo, R. 2013. Composition, physiology and biosynthesis of plant cuticle. Composición, fisiología y biosíntesis de la cutícula en plantas. *Revista Fitotecnia Mexicana* 36(1): 3-12.

Valdez-Cepeda, R.D., Blanco-Mácias, F., Vázquez-Alvarado, R.E., Magallanes-Quintanar, R. 2008. Producción y usos del nopal para verdura. *Revista de Salud Pública y Nutrición.* Edición Especial No. 14: 1-19.

Zheng, H., Zhang, R., Zhang, H., Feng, Y., Li, K., & Zhang, W. 2011. Thermal analysis of four insect waxes based on differential scanning calorimetry (DSC). *Procedia Engineering* 18: 101-106. https://doi.org/10.1016/j.proeng.2011.11.016.