ORIGINAL ARTICLE

Bioprospecting of *Lacmellea standleyi* fruits (lechemiel)

Bioprospección de frutos de *Lacmellea standleyi* (lechemiel)

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ABSTRACT

Introduction: the idea to explore at least some of the regions diversity such as the Colombian Orinoco through bioprospecting study of *Lacmellea standleyi* (Woodson) Monach. arises as a consequence of ignorance of much of the floristic richness in Colombia and the potential relevance of much of this in the nutrition, health and industry.
Objective: to evaluate the antioxidant potential, nutritional and phenolic content, antimicrobial activity, and safety degree of aqueous, ethanol, and ethyl acetate extracts of *Lacmellea standleyi* fruits in three different ripening stages.
Methods: the nutritional value was evaluated using standardized methods to full fruit in its three ripening stages. Each of the extracts was chemically characterized by spectrophotometric assays. Antimicrobial activity was measured by the size of inhibition against strains of *Staphylococcus aureus*, *Escherichia coli*, and *Candida parpsilosis*; the acute toxicity of the fruits was measured through in vitro tests using *Artemia salina* as experimental model.
Results: the results show that green fruits are suppliers of antioxidant compounds. Higher levels of nutrients are found in the intermediate state and mature fruit has attractive organoleptic properties and a relatively high nutrient content.
Conclusions: the antioxidant capacity of *Lacmellea standleyi* fruits was evident in the three ripening stages, giving the plant a promising future in the pharmaceutical industry, standing out in this field the fruits in the green stage. Furthermore, the results suggest the application of the intermediate and mature fruits in the finished products development. The safety observed in the plant material warrants its use in human consumption.

Key words: *Lacmellea standleyi*, nutritional content, bioactive metabolites, antioxidant activity.

RESUMEN

Introducción: del desconocimiento de gran parte de la riqueza florística en Colombia y de la importancia que podría tener gran parte de esta en la nutrición, salud e industria, nace la idea de explorar, al menos en parte, la diversidad de regiones como la Orinoquía colombiana a través del estudio de bioprospección de los frutos de *Lacmellea standleyi* (Woodson) Monach.
Objetivo: evaluar el potencial antioxidante, el contenido fenólico y nutricional, la actividad antimicrobiana y el grado de inocuidad de los extractos acuoso, etanólico y de acetato de etilo, de los frutos de *Lacmellea standleyi* en 3 estadios diferentes de maduración.
Métodos: el valor nutricional se evaluó a través de métodos estandarizados, al fruto completo en sus 3 estadios de maduración; cada uno de los extractos se caracterizó químicamente a través de ensayos espectrofotométricos. La actividad antimicrobiana se midió mediante el tamaño del halo de inhibición frente a cepas de *Staphylococcus aureus*, *Escherichia coli* y *Candida parpsilosis*; la toxicidad aguda de los frutos se calculó mediante pruebas *in vitro*, usando como modelo experimental nauplios de *Artemia salina*.
Resultados: se pudo evidenciar que los frutos verdes aportan compuestos antioxidantes. En el estado intermedio se encuentran los niveles más altos de nutrientes y el fruto maduro ostenta atractivas propiedades organolépticas y un contenido relativamente alto de nutrientes.
Conclusiones: la capacidad antioxidante de los frutos de *Lacmellea standleyi* resultó evidente en los 3 estadios de maduración. Esto otorga al vegetal un futuro promisorio en la industria farmacológica, sobresaliendo en este campo los frutos en el estadio verde. Además, los resultados permiten sugerir la aplicación de los frutos en estado intermedio y maduro en la elaboración de productos alimenticios terminados. La inocuidad observada en el material vegetal garantizaría su uso en el consumo humano.

Palabras clave: *Lacmellea standleyi*, contenido nutricional, metabolitos bioactivos, actividad antioxidante.

INTRODUCTION

All the existing forms of life in the planet can be grouped under the term "biological diversity". Man has found multiple values of all the components of biodiversity and has taken advantage of them for his well-being.

From the colonial times many different countries, including the Spaniards, Portuguese, English, and Dutch, took diverse cultivations and other necessary plants from one continent to another. This was the beginning of the exploitation of the biodiversity with commercial aims. Nevertheless, the indiscriminate use of the resources and the urgent necessity to maintain the natural balance carried out the Conference on Environment and Development (Earth Summit) of 1992, which regulated at international level, and in a superb form, everything related to the conservation and the sustainable use of biodiversity. The systematic search with

commercial aims, of the genetic, biological and chemical elements of biodiversity is the fundamental intention of bioprospecting.

The Convention of the Biological Diversity (June of 1992), conscious of the intrinsic value of the biological diversity and ecological, genetic, social, economic, scientific, educative, cultural, recreational and aesthetic values of the biodiversity and also, recognizing its importance for the evolution and the maintenance of the necessary systems for the life of the biosphere, regulated the bioprospecting activities and assigned a formal protocol of participation in the benefits obtained from bioprospecting activities, in "co-participation access-benefit", according to the genetic resources and the traditional knowledge between the parts.

The Amerindian ethnic communities have elaborated a very particular knowledge transmitted from generation to generation, about how to exploit the natural resources from which they count on to obtain their daily sustenance. An example of this is the Guahibos and the Sikuanis, settlers of the Silvan zones of the Orinoquía Region of Colombia, who take advantage of the abundant latex from the "Lechemiel" tree (*Lacmellea standleyi*, Apocynaceae) as a substitute for the animal milk and consume the fruits of the plant as a snack due to their pleasant flavor. The peasant population of the region also uses these fruits for the creation of by-products like wines, jellies and ice cream.

The Apocynaceae family has awakened the interest of the investigators to test a variety of bioactivities in equal number of species. These works have resulted in obtaining compounds of pharmacological interest. Example of it are the called "vinca alkaloids", vincristine, and vinblastine isolated from *Catharantus roseus¸* which have found application in the chemotherapy of breast, liver and lung cancer, leukemia and lymphomas.[1] Another example constitutes *Rauwolfia serpentine*, a plant from which the indole alkaloids ajmalicine and reserpine have been isolated, recognized for their antiarrhythmic and hypotensile properties.[2] Other studies on species of Apocynaceae reveal the potentiality of this family in the nutritional,[3-5] pharmacological[6,7] and bioactivity field.[8,9]

For a long time it is has been known that some types of plants have certain analogy in the structures of the isolated secondary metabolites, basically if the species are related through genetic material in such form that it can be expected vegetal of the same family to have biosynthetic tools that lead to chemical compounds with similar bioactivities. Despite the bibliography of interest in this work, we don't see many investigations related to *L. standleyi;* a promissory future could be expected for it if it is considered the vegetal family to which it belongs and the antecedents mentioned in the previous paragraph.

Lacmellea standleyi (Woodson) Monach. is a native tree of the tropical rain forests of South America, where it grows approximately to a 355 altitude (meters above sea level) and to 26 °C aprox. *Lechemiel* undergoes fundamental changes of coloration and consistency as a result of its process of maturation, going from a fleshy fruit in form of a berry, rounded (2-2,5 centimeters of diameter), persistent chalice, hard consistency and abundant content of chlorophyll and carotenoid, that gives it a green coloration, to a product of dark yellow color, with soft pulp of sweet flavor, abundant hyaline mucilage that covers the (brown) round seed, of 1cm of diameter and not attached to the pulp (Fig. 1). In Colombia, the fruit is only known by indigenous and peasants in the Orinoquía region and; although it is considered important in the nutritional diet of these settlers, its commercialization is little to none.

Fig. 1. Lechemiel fruits (*Lacmellea standleyi*) in three stages of ripening.

To determine the biological properties and/or the chemical composition of a specific nutritional material gives intrinsic value to the product, orients towards the search of bioprospecting elements and, finally, helps in the awareness of the biological diversity of a region or nation.

The contribution of this work was to determine the nutritional value, the chemical nature of the main nuclei of secondary metabolites, the antioxidant potentiality against radicals DPPH and ABTS, total phenolic content, the total soluble antioxidant capacity and the acute toxicity of the harvested fruits of *Lacmellea standleyi* in three stages of ripening (green, intermediate and mature), aiming to have scientific bases to be able to argue the traditional knowledge of the settlers of the Colombian Orinoquía, and to offer possibilities to use the fruit in the nutraceutical or pharmaceutical industry.

METHODS

Vegetal material

Lacmellea standleyi (Woodson) Monach. (Apocycaceae) fruits refer code N°. COL. 527660 assigned by the *Herbario Nacional Colombiano* (Bogotá, Colombia), were collected in the Colombian east in their natural habitat; berries of different wild trees, from the tropical rain forest (latitude 35 °0, 26 °C, 355 m.a.s.l).

A systematic ethnographic method was used to collect information and to select the fruits for the analysis, including interviews with peasants of the region, observation and photographs. The fruits of "Lechemiel" were transported to the laboratory, classified according to their color as green, intermediate and mature consistency and frozen to assure the stability of the nutritious substances in the samples stored before the analysis.

Chemical reagents

Folin-Ciocalteau, ammonium persulfate and radicals DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)-6-sulfonic) of Sigma-Aldrich S.A. (U.S.A.) were used. The remaining reagents were analytic grade (Merck USA).

Preparation of the extracts

From the dry and crushed vegetal material (sieve 2 mm), a macerated ethanolic was prepared with ethyl acetate of the fruits in each stage of maturation (1:10 vegetal/material solvent). With equal proportions, an aqueous extract was elaborated by decoction. Each extract was put under physical chemistry.

Chemical analysis

In each plant extract the presence of some nuclei of secondary metabolites was determined, such as: flavonoids, coumarins, saponins, quinones, tannins, free alpha-amino group, cardiac glycosides, terpenes and steroids, terpenic lactones, saponins, alkaloids and phenylpropanoids, according to the individual qualitative tests for each chemical group[10] and the classic methodology of fractioning by solvent polarity.

In order to determine the nutritional value of the fruits of Lechemiel (humidity, ashes, ethereal extract, crude fiber, gross protein), standard methods were applied, including the spectroscopic methods for the quantification of the total and reducing sugars, vitamin C, vitamin A, and high-performance liquid chromatography (HPLC) to quantify vitamins E and K.[11] In the case of vitamin E, it was analyzed after making an alkaline saponification, later extraction of n-hexane and quantification by HPLC with a diode-array detector.[12] On the other hand, vitamin K was analyzed following the procedure described by Booth[13] and Peterson.[14]

Determination of total phenols

The total phenolic content of the extracts was quantified using the Folin-Ciocalteau reagent (FC).[15] A defined volume of the extract prepared at different concentrations was graduated with water (10 mL). A defined volume of this solution was mixed with FC reagent (2.5 mL) and with sodium carbonate (2 mL, 7.5 %); the mixture was incubated (20 min, 50 °C), cooled off completely and the absorbance was read to 750 nm against a target (solvent and reactive). The optical density of the samples was interpolated in a calibration curve prepared with gallic acid (0.5-40 µg/mL), obtained by linear regression. The phenolic content of the extracts is expressed as equivalent milligrams of gallic acid per gram of dry sample.

Antioxidant activity

ABTS Method

The method developed by *Re* and collaborators,[16] was applied with some modifications: the radical was obtained after the reaction of the ABTS (7 mM) with ammonium persulfate (4.9 mM), incubated to room temperature (± 25 ºC, 16 h) and in the dark. The formed radical was diluted with ethanol until obtaining a value of absorbance between 0.70 (± 0.1) to 740 nm (λmax). The previous solution (160 µL) was mixed with ethanol (3.04 mL) and the absorbance was read again. The sample was added within the cell (40 µL) reading after 1 minute and, in a continuous form, during 6 minutes. Ascorbic acid (A.A was used as pattern; 5-15 µg/mL). The results were expressed as the equivalent antioxidant potential of ascorbic acid (%), calculated by the equation:

$$\%PAEAA = AABTS\text{-}A6minAABTS \times 100$$

Where:

APEAA: Antioxidant potential equivalent to ascorbic acid, expressed as a percentage.
AABTS: ABTS absorbance before adding the sample.
A6min: Absorbance of the reaction mixture for 6 minutes

DPPH method

The interaction of the components of the samples with DPPH free radical was evaluated applying the method described by *Brand-Williams* et al.[17] An ethanolic solution of the radical was mixed with vegetal extract (1:3, sample/radical) in different concentrations (5-5 000 µg/mL). After incubating (20 min, room temperature and the dark), its absorbance was read, λ517nm, to determine later the average inhibiting concentration (CI_{50}). A gallic acid solution was used as pattern (A.G.: 5-20 µg/mL). The potentiality to stabilize the DPPH radical of samples and patterns of reference was directly proportional to the capacity to degrade of the violet color of the radical, which was determined by equation:

$$\left[\frac{\text{Abs.DPPH-Abs.muestra}}{\text{Abs.DPPH}}\right]\times 100$$

Total antioxidant hydrosoluble capacity (TAHC)

The method of phosphomolybdenum was applied in order to evaluate the TAC of the extracts.[18,19] Ascorbic acid (AA, 500 ppm) was used as pattern; the results were quantified according to the following equation:

$$\text{THAC}=\frac{A\lambda}{\varepsilon(213\mu M^{-1})}\times\frac{\text{Vmixture}}{\text{Vsample}}\times\frac{\text{Vextract}}{\text{Wsample}}$$

Where:

A: Absorbance at 695 nm.
λ*:* is the inverse of the extinction coefficient (213 μM^{-1}).
$V_{mixture}$: Volume of the mixture obtained in the test.
V_{Sample}: Sample volume used in the test.
$V_{extract}$: Volume of extract obtained by extraction method.
W_{sample}: Sample weight used to make the extract.

Acute toxicity of the extracts

The harmlessness of the fruits of Lechemiel was determined by bioassays, using as an experimental model the nauplii of *Artemia salina* in a wide range of concentrations (10-10 000 µg/mL) of aqueous, ethanolic extracts and ethyl acetate. Following the method described by *Mcgawa*,[20] with some modifications: The eggs of the microcrustacean, obtained in a commercial store, were set to hatch in artificial seawater (3.8 % of salinity, artificial light, 24 h). The nauplii thus obtained were placed (10 individuals) in standard size vials with artificial seawater (5 mL). Separately, 1 mL of sample was taken to dryness to eliminate the interference of the solvent, DMSO (3 drops) was added to the remainder, and a day later the mortality readings were performed. The results were analyzed with program EPA Probit Analysis

Program version 1.5 *United States Environmental Protection Agency* (U.S. EPA) to determine the values of DL and DL_{50}.

Antimicrobial activity

The capacity of the extracts to inhibit the microbial development was determined according to the size of inhibition halo presented against strains of *Escherichia coli* (Gram -. ATCC25922), *Staphilococos aureus* (Gram +. ATCC29213) and the fungus *Candida parapsilosis* (ATCC22019) provided by the Clinic Laboratory of the Federico Lleras Acosta Hospital of Ibague. The inhibitory effect was calculated by the following equation:

$$\%Inhibition = Dhe - Dhnc \times Dhpc$$

Where:

Dhe: is the mean diameter of halos of inhibition for the extracts.
Dhnc: the mean diameter of inhibition zones for the negative control.
Dhpc: the mean diameter of inhibition zones for the positive control.

Statistical analysis

All the tests were performed in triplicate (n= 3). Each replica was analyzed individually and the data was reported as the average of three determinations (n= 3 × 3) ± standard deviation SD (n= 3 × 3). Through the statistical program INFOSTAT, version 2009. (Group InfoStat, FCA, National University of Cordova, Argentina), the data were put under a variance analysis, with interaction between solvent and stage, Duncan's multiple range test, homogeneity of variance and distribution of the remainders; the values of $p < 0.05$ were considered significant.

RESULTS

Nutritional content

Table 1 shows the results obtained in the nutritional analysis of the fruits of "Lechemiel" in three ripening stages.

Phytochemical screening

In all the extracts, the presence of phenolic type compounds (tannins, flavonoids, coumarins, and phenylpropanoids) was observed. In addition, terpenes and/or steroids were evident. Metabolites like flavonoids and tannins were revealed mainly in the ethanolic extracts, for the three stages of ripening, whereas the content of compounds of terpene and steroid nature were abundant in the green fruit. The presence of alkaloids was detected in the extracts of green and intermediate fruits, but not in the mature ones.

Table 1. Nutritional analysis of the fruits of "lechemiel" in three stages of ripening

Parameters		Stages of maturation											
		Green				Intermediate				Mature			
Humidity (%)		75.6 ± 0.031				61.8 ± 0.045				78.4 ± 0.037			
Fiber (%)		5.6 ± 0.72				6.0 ± 0.78				6.4 ± 0.90			
Protein (%)		5.6 ± 0.098				4.7 ± 0.28				4.2 ± 0.54			
Fat (%)		3.1 ± 0.70				0.8 ± 0.087				0.8 ± 0.029			
Ash (%)		3.8 ± 0.18				1.8 ± 0.37				4.5 ± 0.26			
Nitrogen-free extract		81.9 ± 0.073				86.7 ± 0.33				84.1 ± 0.67			
Total carbohydrate available (mgEG/gdvm)		300 ± 0.53				542 ± 0.58				565 ± 0.34			
Reducing carbohydrates (mgEG/gdvm)		249.8 ± 0.33				296 ± 0.76				105.6 ± 0.50			
Hexoses (mgEG/gdvm)		215.56 ± 0.068				256.12 ± 0.093				72.49 ± 0.091			
Pentoses (mgER/gdvm)		34.24 ± 0.45				42.88 ± 0.24				33.11 ± 0.63			
ºBRIX		-				-				25.1 ± 0.016			
Vitamin C (mg/100g)		0.18 ± 0.042				0.02 ± 0.059				0.03 ± 0.09			
Vitamin A (IU)		2261 ± 0.015				2603 ± 0.023				1007 ± 0.043			
Vitamin E (mg/100g)		1.02 ± 0.008				0.95 ± 0.007				1.0 ± 0.005			
Vitamin K (µg/100 g)		60 ± 0.002				46 ± 0.009				50 ± 0.023			
Major minerals (mg/kg)		Ca	K	Mg	Na	Ca	K	Mg	Na	Ca	K	Mg	Na
		0.02 ± 0.003	1.58 ± 0.005	0.06 ± 0.002	0.01 ± 0.002	0.08 ± 0.07	1.24 ± 0.03	0.03 ± 0.02	0.01 ± 0.05	0.09 ± 0.02	1.58 ± 0.1	0.12 ± 0.04	0.01 ± 0.002
Minor minerales (mg/kg)	P	1.04 ± 0.02				0.88 ± 0.03				0.56 ± 0.003			
	Fe	3.3 ± 0.007				1.75 ± 0.09				3.1 ± 0.04			
	Zn	1.65 ±0.1				1.60 ± 0.09				1.15 ± 0.02			
	Cu	0.25 ±0.03				0.21 ± 0.06				0.05 ± 0.01			
	Mn	4.6 ±0.09				4.25 ± 0.07				4.1 ±0.1			
	S	0.029 ± 0.004				0.043 ± 0005				0.019 ± 0.002			

The results are expressed as milligrams equivalent to glucose per gram of dry vegetal material (mgEG/gdvm) or as equivalent milligrams of ribose per gram of dry vegetal material (mgER/gdvm). The values correspond to the average of three determinations (n= 3 ± SD).

Determination of total phenolics

Table 2 shows that the total phenolic content in the extracts of the fruits of Lechemiel appears in greater proportion in the aqueous extract, obtaining values between 11,27 ± 0,80 for the green fruit, 1,83 ± 0,09 in intermediate stage and 1,6 ± 0,09 in the mature fruit.

Antioxidant activity

In an attempt to verify the relation between antioxidant activity and phenolic compounds, the total hydrosoluble antioxidant capacity (THAC) of *L. standleyi* fruits associated to the total phenolic content was determined in this work. The results are illustrated in figure 2.

Table 2. Performance bioactive compounds and phenolic content of lechemiel fruits under the action of three solvents of different polarity

Solvent	Stage of maduration	Extract performance (%)	Total phenolic content mgEAG/gdvm*
Water	Green	18.44 ± 0.1	11.27 ± 0.80
	Intermediate	25.53 ± 0.3	1.83 ± 0.09
	Mature	26.50 ± 0.5	1.6 ± 0.09
Ethanol	Green	3.06 ± 0.2	4.17 ± 0.98
	Intermediate	1.40 ± 0.1	0.95 ± 0.33
	Mature	0.49 ± 0.05	0.13 ± 0
Ethyl acetate	Green	27.47 ± 0.9	1.55 ± 0.11
	Intermediate	40.34 ± 0.2	0.59 ± 0.05
	Mature	35.30 ± 0.6	0.63 ± 0.02

* milligrams equivalent to gallic acid per gram of dry vegetal material (mgEG/gdvm). The values correspond to the average of three determinations (n= 3 ± SD).

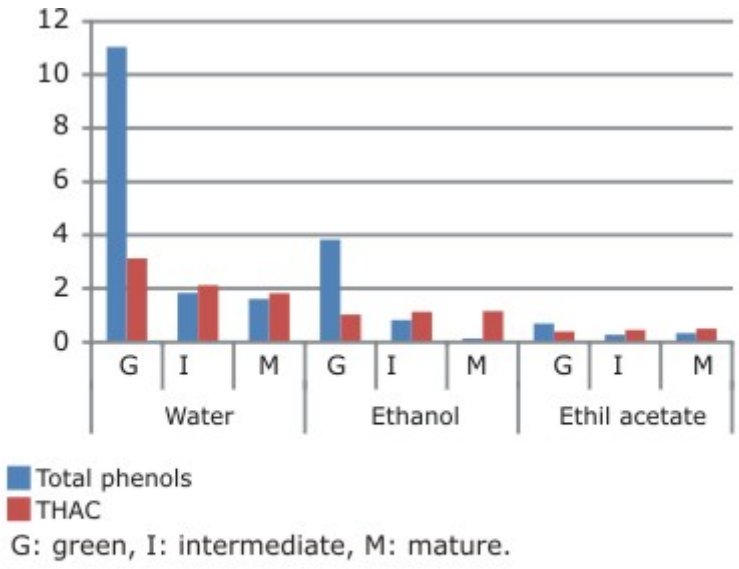

Fig. 2. Total hydrosoluble antioxidant capacity (THAC) *vs.* the total phenolic content of *Lacmellea standleyi*, expressed in miligram of acid per gram of dry vegetal material.

Based on the analysis of ripening stage, figure 3 illustrates the green fruit as the one that has greater THAC (3,138 ± 0,071; 2,122 ± 0,15; 1,835 ± 0,358 mgEAA/gmvs), whereas the mature fruit shows the lowest potential (1,835 ± 0,358; 1,154 ± 0,003; and 0,497 ± 0,031), when treating them with water, ethanol and ethyl acetate, respectively. In that order, the total phenolic content is displayed. Associated to the high polarity of these chemical components it is the ability of solvents to extract them from vegetal material: water>ethanol>ethyl acetate.

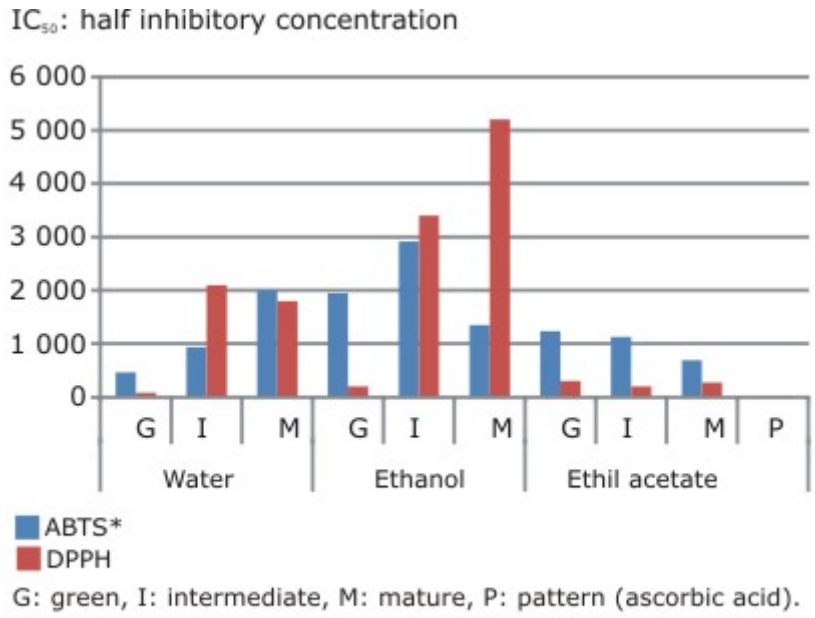

Fig. 3. Antiradical capacity against DPPH and ABTS of the fruits of Lechemiel in three stages of ripening.

Although the water does not allow obtaining greater bioactive compound concentrations over 20 % (18, 44 %), it does extract the greater amount of phytophenols (11,27 ± 0,80) and, consequently, this extract reveals the highest THC. It follows then, that the antioxidant compounds in green fruit are mostly of polar nature, fundamentally of phenolic type. The correlation between the two variables was expressed by the equation:

$$\text{Absorbance} = 0.1982[\text{THAC}] + 0.8067;\ R^2 = 0.6$$

Biological activity

The results obtained in the safety test against nauplii of *A. salina*, are expressed in terms of the average lethal dose (LD_{50} value). This statistic allows knowing the stimulus level that causes a respond in 50 % of the individuals of the population under study, being considered as an important parameter of characterization.

In the present investigation it was found that the concentrations at which half of the population of *A. salina* nauplii dies oscillates between 18 209.61 ± 400.06 and 27 230.60 ± 107.83 mg/L, for both aqueous and ethanolic extracts of the three ripening stages, respectively. With regard to, in the ethyl acetate extracts was not observed mortality in exposed organisms.

With respect to the obtained values when measuring the inhibiting capacity of microbial growth on bioactive compounds of Lechemiel, the data indicate that the percentage of inhibition of the extracts ranks between zero and 0.2 %, represented by the diameter of the inhibition halos.

DISCUSSION

Nutritional content

The fruits of Lechemiel are not different from the great majority of similar products considering the water level they have. While the green fruit stores water to accomplish the abundant number of physiological processes which it is put through, in the optimal state of maturation this is a result of the degradation of the peptic substances, because the product softens and stores more water.

It must be considered that the fruits are vegetal foods containing more that 60 % water that, besides genetic factors, varies in a wide range and it does not constitute a constant parameter given the influence of the environmental relative humidity, the state of maturity and time of harvesting, among others. In general, the humidity content in a food is, frequently, an index of stability of the product, since a relation exists, although imperfect, between the water content and its capacity of deterioration.

The values of ash, gross protein, ethereal extract and nitrogen-free extract (N.F.E.) show little correlation with the fruit ripening state; on the contrary, it is noticeable that the concentration of these parameters decays from the green stage to the mature one, or similar values between the intermediate fruit and the mature one are obtained. The exception constitutes the crude fiber contents, in which the increase of the measurement seems to depend on the degree of maturity. It could be inferred that this group of compounds (non-starch polysaccharides) undergoes a little transformation during the ripening process.[21]

Regarding the protein content, a decrease associated with the advance of the maturity degree is noticed. It should be noted that the progressive degradation of these nutrients into simpler ones, demands a great consumption of energy and water that make the chemical and biological value of protein in a nutritional product not constant, but dependent of a series of variables like the species, the age and the physiological state of the natural product.

On the other hand, the lipids found in the fruit pulp are the major energy source of the seed and a vital component for the embryo and seedling development; this would explain why the lipid material (ethereal extract) is markedly decreased as the ripening process of the *L. standleyi* fruits advances, insinuating a possible translocation effect of this group of compounds towards other parts of the plant, like for example the seeds.

Other nutrients show greater level in the intermediate state and an abrupt reduction when reaching the optimal maturation level. Such is the case of the reducing carbohydrates (hexoses and pentoses).

The explanation could find support in the continuous metabolic transformations that happen in the intermediate state and which is part of the mechanism to prepare for the seed maturation and development. We must not forget that carbohydrates,

specially the glucose, are molecules that initiate the primary and some secondary metabolic processes.[10]

The nitrogen-free extract is formed by monosaccharides, disaccharides, starch, pectins, resins, organic acids, tannins, pigments, water soluble vitamins, cellulose, hemicellulose, lignin and other nitrogen-free materials; becoming therefore a fraction of great nutritional importance. The portion of digestible carbohydrates results from the aggregation of fiber and digestible nitrogen-free extract. The values of these two nutrients (table 1) indicate that the fruits of *L. standleyi*, in spite of undergoing physiological changes, conserve their nutritional level, while maintaining an almost constant digestibility throughout the ripening process.

The high content of total carbohydrates available in the mature fruit suggests the presence, not only of the previously mentioned carbohydrates, but also of other sugars like saccharose, or the fructose, an aspect related to the considerably high value of suspended solids (25.1 °Brix). This characteristic also contributes to the pleasant sweet flavor of the fruit, which upholds the liking for its fresh consumption by the settlers of the Orinoquía region, especially from the children, who consider it a treat.

The content of vitamin A, (intermediate stage mainly, 2 603 IU), and the minerals within, for example: potassium, iron, zinc and manganese; all of them are connected to diverse metabolic functions of the organism such as the osmotic regulation, being part of enzymes or acting like coenzymes.[22] In general it is known that fruits are contributors to the fiber, carbohydrate (mainly sugars), mineral, and vitamin diet.

It is important to mention that Lechemiel fruits seem to adjust very well to the behavior of a climacterical fruit: [23] it is initially green and then it changes to red shades. The chlorophyll diminishes as maturation and lycopene increase. As ripening advances, hardness, soluble solids and the ascorbic acid also diminish, from which it is expected in addition a decrease of O_2, increase of CO_2, ethylene and the starch during the breathing.

Phytochemical screening

The alkaloids presence was detected in the extracts of green and intermediate fruits, but not in the mature ones. It is worth to point out that *L. standleyi* belongs to the Apocynaceae family, particularly rich in indole and bis-indole type alkaloids,[24] these have great interest in the scientific world and are widely used in the treatment of multiple diseases,[25,26] like anticancerigenic (vinblastine), hypertensive (yohimbine) and specifically the *Aspidosperma ramiflorum* species that presents indole alkaloids with activity against *Leishmania amazonensis*.[27]

On the other hand, most of the members of the Apocynaceae family are provided with laticifers formed by individual or branched cells that produce a milky, reddish (Aspidosperma) or transparent (*Thenardia*) latex, which contains glucosides and alkaloids that can be very toxic (*Asclepias linaria* Cav.). From a long time ago, certain species of the Apocynaceae s.l. family have been used as poison.[28] Nevertheless; others are nowadays used as ornamental plants or effective drug against leukemia (*Catharanthus roseus* (L.) G. Don) and even though the family is integrated by toxic plants, some have been included as seasoning for regional dishes.[29]

It is important to consider that the conditions of the tropic cause that this region displays, provides a high diversity of flora that conforms a potential material, little explored scientifically.

Determination of total phenolics

In general, it is observed that the content of these metabolites seems to depend more on the maturity stage of the fruit than on the solvent used to extract them in the quantification process; in all the cases the mature fruit has lower content.

It is important to mention that the enzymatic system of many fruits can use phenolic compounds as substratum. One of these enzymes is polyphenol oxidase (PPO), which has been related to the browning processes of the pulp.[30] The activity of this enzyme is increased significantly during ripening, which seems to explain the decrease in the concentration of total phenolics as the state of vegetative development of the fruit of Lechemiel advances (table 2).

In addition, it must be considered that PPO is an enzyme soluble in cytosol and related to membranes and that the evolution of the maturation goes accompanied by changes of permeability of the cellular membranes, from which it is deduced that the maturity of the fruit favors the enzyme-substratum interaction and, consequently, the oxidation of the phenolic components, to which carbon dioxide and ethylene production contributes in great way during ripening.[31,32] The fruits and vegetables are formed by sets of living cells that go through metabolic reactions even after having been removed from the plant that provides the water and the nutrients.

It is important to also note that all the solvents showed a greater extractor force when they were exposed against the intermediate fruit (table 2). Nevertheless, ethanol showed the lowest capacity to extract bioactive compounds (extract performance), including phytophenols.

Nevertheless, the highest phenolic compound levels appear in the green fruit, regardless of the solvent used. The highest values obtained with the ethyl acetate make to think about phytophenols moderately hydroxylated. Individual differences distinguish each group of phytophenols, depending on the variations in number and distribution of the hydroxyl groups, as well as the nature and extension of the alkyl groups and/or glycosylation.[16, 33]

Antioxidant activity

Different levels of concentration of the extracts of *L. standleyi* were used to determine the half maximal inhibitory concentration (IC_{50}) against DPPH (50-5000 µg/mL) and ABTS (300-3000 µg/mL); understanding this statistical parameter as a measurement of the capacity of the antioxidant, solubilized in reliable the respective solvent, to stabilize half of the initial concentration of the free radicals in them. Working with 50 % as pattern test avoids possible ambiguities of making measures in the ends and reduces the amount of required tests.[34]

Figure 3 was elaborated to illustrate and to compare antiradical antioxidant activity of the Lechemiel fruits in three stages of ripening, against DPPH and ABTS. It is clearly observed that the aqueous extract of the green fruit is the most active against both radicals, with a half maximal inhibitory concentration of 80 ± 0,34 (DPPH) and 465,98 ± 0,03 (ABTS). These values turned out to be the lowest among the extracts, although not comparable to the ones obtained with ascorbic acid (18, 95 ± 0, 17 and 13, 48 ± 0, 03, when acting against DPPH and ABTS, respectively).

Combining the information related to the type of solvent, the stage of maturity and the antioxidant activity that figure 2 illustrates, it would be possible to state that the antioxidant potential of Lechemiel would be associated not only to compounds of

phenolic nature but also to another type of chemical compounds of lower polarity and solubilized by ethyl acetate, for example vitamin E and carotenoid pigments (table 1). Among them it could be mentioned ß-carotene, main responsible for the yellow or orange color of some fruits. The antioxidant activity of this terpenoid has already been demonstrated.[35] Its chemical nature allows extracting it with solvents such as ethyl acetate instead of water or ethanol.

In effect, the antioxidant included in the extracts obtained from the green fruit (aqueous, ethanolic, and ethyl acetate), as well as the ones coming from the intermediate and mature fruit (ethyl acetate), were those that showed greater ability to stabilize radical DPPH, if their action is compared against the ABTS. When trying to associate the total phenolic content recorded in table 1 with the antioxidant activity in each ripening stage that shows figure 2, little correlation between these chemical components is observed as well as the shown bioactivity.

The antioxidant contents in the aqueous extract from the green fruit and the three ethyl acetate extracts were the most active against the ABTS. It is important to mention that the antioxidant compounds in a food can be soluble, liposoluble, insoluble or connected to the cellular walls.[36] Then, using different solvents and measuring the efficiency in the bioactive compound extraction, important factors need to be considered at the time of evaluating the antioxidant activity of a vegetal product.

On the other hand, the method of the DPPH can be used for solid or liquid samples and it is not specific for a determined type of antioxidant. It is rather related to the antioxidant integral capacity of the sample. On the other hand the radical ABTS, test with greater efficiency the ability of flavonoids and phenolic compounds, considering its properties as proton or electron donors.[37]

Synthetic radical ABTS+ is used to test highly colored vegetal extracts, considering its maximum absorbency to 734 nm, where many of vegetal products do not absorb at that wavelength, therefore reducing the possibilities of interferences of absorbent compounds in the region of the visible one. In addition, it can be applied to an either hydrophilic or lipophilic system. It could then be thought that for the case of Lechemiel fruits the antiradical activity against the ABTS gives an idea closer to the potentiality of the fruit than when it is done against DPPH.

The green stage is the fruit with the highest total antioxidant capacity and higher soluble phenolic content presenting a direct correlation (r= 0,84), the same behavior can see with ABTS+ and DPPH antiradical assays. Then, a measurement of the antioxidant capacity by different methodologies helps to understand the functional properties of a food or natural product.

In folk medicine, many vegetables contain a wide variety of phenolic compounds. They act in the stabilization of free radicals and the inhibition of lipid peroxidation, especially flavonoids.[38] Also fruits and vegetables offer a surprising arsenal of functional substances, where components of phenolic nature stand out.[39]

Biological activity

Many species of the Apocynaceae family cause toxic reactions to those who consume them. Such is the case of the fruits of *Nerium oleander, Thevetia peruviana*[40] and *Marsdenia hilariana*.[41] Additionally, the validity of a nutritional product is not only in its nutritional content or its organoleptic properties but also in its biosecurity at the time of being used as food. Consequently, bioassays were made to allow seeing the

safety of the extracts of fruits of *L. standleyi*, trying to obtain information to ensure its applicability as consumable food and/or possible raw material in the nourishing or nutraceutical industry.

The *A. salina* nauplii are easily available commercially, they present a low cost and therefore this assay may be useful and practical to test large number of samples for preliminary toxicity screening.[42,43] Dose response experiments indicate that the aqueous and ethanolic extracts present a high LD_{50} dose, which allows infer that the fruits in all stages of ripening are harmless.

On the other hand, no mortality in exposed organisms was observed in the ethyl acetate extracts, which seems to be a consequence of the low capacity of the solvent to extract the great majority of bioactive metabolites, such as coumarins, cardiac glycosides and alkaloids, among others.[44,45]

With respect to antimicrobial activity measurement for most of the extracts this diameter turned out to be very small or absent, when compared to the positive controls. As well as for the safety test, the deficiency of antimicrobial activity responds to the absence of secondary metabolites[46] that provide the capacity to inhibit microbial growth to these fruits.

In conclusion, the results of this study show thus that fruits of *Lacmellea standleyi*, known in the Colombian region of Orinoquía as *Lechemiel*, can be used in any of their three stages of maturation like this: the green fruits as great contributors of antioxidant compounds, in the intermediate state of ripening are the highest levels of nutrients, and the mature fruit shows attractive organoleptic properties and a relatively high content of nutrients that make possible their application in the elaboration of finished food products. In addition, the safety of the vegetal material is a guarantee of its use for human consumption.

The use of solvents of different polarity allowed detecting the presence of different phytocompounds in the three stages of ripening of *L. standleyi* fruits of and its relation to the antioxidant activity. In this particular case, the water seems to be best the solvent when extracting compounds with antioxidant potential. On the other hand, the proposed stages of maturation as object of the study expose the way in which diverse compounds modify their amounts as the maturity advances, making evident the processes of translocation and compound degradation.

The antioxidant activity in *L. standleyi* seems not to be exclusive of compounds of phenolic nature, but of all the set of compounds that the vegetal material has. This way, the antioxidant capacity of *Lacmellea standleyi* fruits, was evident in the three stages of ripening, granting to the vegetable a promissory future in the pharmacologic industry, being the fruits in the green stage the most outstanding in this field.

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