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FATTY ACIDS, PHENOLS CONTENT, AND ANTIOXIDANT ACTIVITY IN *Ibervillea sonorae* CALLUS CULTURES

CONTENIDO DE ACIDOS GRASOS, FENOLES, Y ACTIVIDAD ANTIOXIDANTE EN CULTIVOS DE CALLO DE *Ibervillea sonorae*

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Abstract

Ibervillea sonorae callus cultures were established in order to produce fatty acids (lauric, myristic, pentadecanoic, palmitic and stearic acids) and phenolic compounds. Highest callus induction (100%) was obtained in treatments containing 2.32 or 4.65 μM Kinetin (KIN) with 2.26 or 6.80 μM 2,4-Dichlorophenoxyacetic acid (2,4-D). Highest fatty acids (FA) production (48.57 mg g^{-1}), highest total phenol content (TPC; 57.1 mg gallic acid equivalents [GAE] g^{-1}) and highest antioxidant activity (EC_{50}; 573.3-855.7 μg mL^{-1}) was obtained from callus derived from 4.65 μM KIN with 6.80 μM 2,4-D. A direct relationship was observed between the callus growth index (GI), FA content, TPC and EC_{50}. FA content was significantly higher in callus (48.57 mg g^{-1}) than the reported for tuber roots from wild plant (0.25 mg g^{-1}). TPC and EC_{50} were higher in leaves (360.1 mg GAE g^{-1} and 66.0-75.5 μg mL^{-1}, respectively) than in callus.

Keywords: plant tissue culture, *Ibervillea sonorae*, fatty acids, phenols, antioxidant activity.

Resumen

Se establecieron cultivos de callo de explantes de hoja de *Ibervillea sonorae* para evaluar la producción de ácidos grasos (ácido láurico, mirístico, pentadecanoico, palmítico y esteárico) y compuestos fenólicos. La mayor inducción de callo (100%) se obtuvo en los tratamientos que contenían 2.32 o 4.65 μM de cinetina (KIN) en combinación con 2.26 o 6.80 μM de ácido 2,4-diclorofenoxiacético (2,4-D). El callo derivado del tratamiento 4.65 μM KIN con 6.80 μM 2,4-D mostró los valores más altos en términos de producción de ácidos grasos (FA; 48.57 mg g^{-1}), contenido de fenoles totales (TPC; 57.1 mg equivalentes de acido gálico [GAE] g^{-1}) y actividad antioxidante (EC_{50}; 573.3-855.7 μg mL^{-1}). Se determinó una relación directa entre el índice de crecimiento de callo (GI) y el contenido de los FA, TPC y EC_{50}. El contenido de FA en el callo fue significativamente mayor (48.57 mg g^{-1}) al reportado para las raíces del tubérculo de la planta silvestre (0.25 mg g^{-1}), mientras que TPC y EC_{50} fueron mayores en las hojas (360.1 mg GAE g^{-1} y 66.0-75.5 μg mL^{-1}, respectivamente) comparadas con el callo.

Palabras clave: cultivo de tejidos vegetales, *Ibervillea sonorae*, ácidos grasos, fenoles, actividad antioxidante.

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1 Introduction

Mexican folk medicine based on plant species has been extensively used for treating a large array of diseases. Such is the case of *Ibervillea sonorae* Greene (Cucurbitaceae), a climbing and native plant of north-western Mexico, commonly known as "wareque", "wereke" or "guareque". The leaves are used for treating skin ailments, stomach ulcers, and the tuber roots for counteracting diabetes mellitus (Hernández-Galicia *et al*., 2007). Pharmacological studies showed that the roots had significant hypoglycaemic activity on temporarily healthy and hyperglycaemic rabbits, and alloxan-diabetic mice. The fraction that showed higher hypoglycemic activity was that composed by a mixture of monoglycerids and fatty acids (Hernández-Galicia *et al*., 2007). On the other hand, there are no reports documenting which compounds are responsible for the curative effects of leaves. Nevertheless, it is known that closely related Cucurbitaceae species possess phenol compounds (Kolayli *et al*., 2010; Duke *et al*., 2001), so that it is likely that *I. sonorae* also bear phenolic compounds that display biological activity. Phenols are secondary metabolites produced in many plants as defence mechanism against environmental stress conditions such as attack by pathogens, insects or herbivores, wounding, ultraviolet light or/and heavy metal exposure, among others. Furthermore, phenols possess a great reactivity and show a wide variety of biological effects, including antibacterial, anti-inflammatory, antiallergic, hepato-protective, antithrombotic, antiviral, anticarcigenic and antioxidant activities (Soobrattee *et al*., 2005; Vermerris and Nicholson, 2006). It is known that free radicals are formed during the normal metabolism and under disease incurrence, causing a state of oxidative stress, thus damaging cells which can be scavenged through antioxidant activity of phenols (Vermerris and Nicholson, 2006; Soobrattee *et al*., 2005; Cowan, 1999). The high demand of *I. sonorae* has led to seek plant sources and appropriate technologies that assure their supply in high yields and constant composition. Plant tissue culture (PTC or *in vitro* culture) represents a viable technique for producing components with biological activity from vegetal material (Georgiev *et al*., 2009). The aim of this work was to: (a) determine if *I. sonorae* leaves contained phenolic compunds; (b) to establish *I. sonorae* culture conditions leading to the production of fatty acids and phenolic compounds, and (c) to compare the antioxidant activity of the phenolic compounds obtained from leaves and from callus.

2 Materials and methods

2.1 Plant material

I. sonorae young plants were provided by the Universidad Autonoma Metropolitana-Iztapalapa Campus (UAM-I) (Hernández-Galicia *et al*., 2007). On June 2010, the plants were fertilized and conditioned for 2 months in greenhouse (UAM-I). Immature leaves were removed from conditioned plants, and a part of them was used for the solvent extraction procedure described below, while the other part was used as source of explants to induce callus. The latter were washed with a 2% (v/v) soap solution for 10 min, rinsed and immersed for 30 s into a 70% (v/v) ethanol solution, followed by rinsing with distilled water. Afterwards they were immersed into 1.2% (v/v) sodium hipoclorite solution added with Tween-20 (three drops per 100 ml of solution), under low shaking conditions during 10 min, followed by aseptic rinsing with sterile distilled water (four times). Explants (5 x 5 mm segments) were put into the callus induction media culture. Each treatment for inducing callus consisted of 10 glass containers, every one containing three explants.

2.2 Establishment and propagation of callus cultures

The basal medium culture (BMC), which was used for all the treatments, consisted of Murashige and Skoog medium culture (MS, 1962) at half strength added with 3% (w/v) sucrose, 100 mg L^{-1} citric acid and 150 mg L^{-1} acid ascorbic. Phytagel at 0.2% (w/v) was used for solidifying the culture medium. For evaluating the callus response of immature leaves, the induction medium culture consisted of adding different combinations of plant growth regulators (PGR) to BMC: the cytokinin kinetin (KIN), and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) to medium culture. A factorial design (3×4; coded as KxDy) was employed. For KIN the applied concentration levels were 0.00 (K0), 2.32 (K1) and 4.65 (K2) μM, and for 2,4-D was 0.00 (D0), 2.25 (D1), 6.75 (D2) and 11.32 (D3) μM. All prepared media were adjusted to pH of 5.8, followed by the addition of 0.2% (w/v) phytagel, and sterilized at 121°C for 18 min. After 20 d of incubation, the frequency of callus inducement was assessed from the explants developing callus regarding the total number of treated explants. The treatments displaying the highest frequency of callus, which were easily disaggregated

and showed visually major growth in terms of biomass production, were chosen for being propagated in the respective media formulation (K1D1, K1D2, K2D1 and K2D2). Every 30 d, the selected calli (3.0 g fresh weight [FW]) were subcultured into new fresh media and incubated, with 8 subculture cycles being completed. Incubation conditions consisted of a photoperiod of 16 h (light)/8 h (dark) under white fluorescent light (50 μmol m^{-2} s^{-1}) at 26 ± 2°C, during 30 d. In the last two subcultures (cultured for 30 d), the biomass was harvested to separately use it to determine phenol and fatty acid contents, growth index and antioxidant activity. Due to no statistical differences were determined between all the results determined from those two subcultures, they are mentioned throughout the manuscript as an average. Once calli are obtained it is known that they can undergo somaclonal variation which can result in secondary metabolite variations among subculture cycles. So, a period of maintenance is recommended to follow in order to achieve stable cell lines, where stability can be inferred through obtaining results reproducible regarding assessed parameters from one subculture cycle to another (Bourgaud *et al*., 2001).

2.3 Extraction of phenols and fatty acids

Calli (30-d-old) fresh biomass samples (2 g) were lyophilized and weighted, and the growth index (GI) determined as reported by Nezbedova *et al*. (1999). Dried biomass (100 mg dry weight [DW]) was put with boiling solvent (50 mL) for 1 h, filtered and concentrated (10 mL). Methanol (MeOH) was used for extracting the phenolic compounds, while hexane was for extracting the fatty acids. Leaves (from wild plants acclimatized to greenhouse) were subjected to the same treatment than the calli for phenolic compounds extraction. All measurements were done in triplicate.

2.4 Derivatization and quantification of fatty acids

Hexane concentrated extracts were derivatized for producing fatty acid methyl esters (FAMEs) according to the procedure of Shehata *et al*. (1979). Briefly, each concentrated sample (0.5 mL) was esterified with boiling H_2SO_4-MeOH (1:2; 3mL) for 3 h then it was cooled and transferred to a separation funnel. Each sample was rinsed three times with distilled water (1.5 mL), and finally hexane (2 mL) was added. The organic phase was collected and dried with anhydride sodium sulphate. Fatty acids standards (Lauric acid, C12; Myristic acid, C14; Pentadecanoic acid, C15; Palmitic acid, C16; and Stearic acid, C18; Sigma-Aldrich Co., USA) were treated in the same way for developing their respective methyl esters. Stock standard FAME solutions (1.0 mg mL^{-1} for C12, and 10 mg mL^{-1} for the other FAMEs) were prepared. Further dilutions of each stock solution (2.5, 5.0, 7.5 and 10 mg mL^{-1}) were made in order to obtain the calibration curve of every FAME (coefficient of determination [r^2] = 0.9892, 0.9896, 0.9902, 0.9923, 0.9964 and 0.9977 for methyl esters of C15, C14, C18, C16 and C12, respectively). The quantitative analysis for FAMEs was carried out in an Autosystem XL gases chromatography with flame ionization detector (GC-FID) system (Perkin Elmer, USA), equipped with a capillary column (HP-5 bonded and cross-linked 5% siloxane, 30 m × 0.32 mm I.D., 0.025 μm film thickness; Agilent Technologies, USA). The temperature of the injection port was set at 300 °C, and for the detector was set at 300°C. While the oven temperature was programmed to initiate at 180 °C for 2 min, then the temperature was raised to 300 °C (at a rate of 5 °C min^{-1}) and held for 15 min. Carrier gas (Helium) flow rate was set at 1.0 ml min^{-1}, the injection volume was 1 μL in the split mode. Standards and samples were run in the same way. TotalChrom chromatography software (Perkin Elmer, USA) was used to acquire data from the detector. Each sample was injected almost three times ($n \geq 3$). For detecting, identifying and quantifying each FAME in samples, peak areas at the corresponding standard retention time were used from the calibration curve.

2.5 Determination of total phenol contents and $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical quenching

The calli that showed higher FAMEs content (K1D2, K2D2) were chosen for carrying out the total phenol and antioxidant measurements. Total phenol content (TPC) was determined from MeOH extracts by applying the Folin-Ciocalteau method (Aslan *et al*., 2007) and gallic acid (GA) was used to elaborate a calibration curve (1-25 mg mL^{-1}; r^2 = 0.9972); the results were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE g^{-1}). On the other hand, MeOH extracts were used to assess radical scavenging, so 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma-Aldrich Co., USA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich Co., USA) radicals were tested. The $ABTS^{\bullet+}$ assay

was made according Re *et al*. (1999) with brief modifications. The $ABTS^{\bullet+}$ radical cation was produced through the reaction of 7 mM ABTS with 2.45 mM potassium persulfate for 16 h under darkness and room temperature. The resulting solution was diluted with MeOH to achieve a value of absorbance of 0.7 ± 0.05 at wavelength of 734 nm. While $DPPH^{\bullet+}$ assay was conducted according to Sánchez-Moreno *et al*. (1998), with some modifications. A 0.1 mM $DPPH^{\bullet+}$ solution was prepared with MeOH. For both assays a volume of 3000 μL of radical solution (diluted $ABTS^{\bullet+}$ or $DPHH^{\bullet+}$) was mixed with 500 μL of sample (extracts from callus and leaves), and after 15 min under darkness and room temperature incubation the absorbance was recorded at 734 nm for $ABTS^{\bullet+}$ assay and 515 nm for $DPPH^{\bullet+}$ assay. Since EC_{50} is defined as the concentration of extract needed to decrease at 50 % the initial radical, different concentrations of extracts from calli or leaves were tested for each assay (0-3 mg of extract mL^{-1}). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich Co., USA) was used as standard, so different solutions were prepared to build a calibration curve (20-150 μM, $r^2 = 0.9903$). The results of radical quenching to achieve the EC_{50} were expressed as microgram of dry extract per ml of extract ($\mu g\ mL^{-1}$). Both assays were made three times for all samples ($n = 3$).

2.6 Statistics

All the results were statistically analyzed with NCSS software by performing One-way analysis of variance and the multiple comparison test of Tukey-Kramer. The significance level for all the statistical tests was established at 5 %. All the experiments were done by triplicate.

3 Results and discussion

3.1 Callus induction and calli development lines

The callus induction from explants of *I. sonorae* depended of the PGR treatments used. Callus response did not occur in explants grown under the control treatment (without PGR, K0D0) and those treatments consisting only of KIN in different concentrations (K1D0, K2D0). All the treatments made with 2,4-D alone or combined with KIN produced callus induction after seven days of incubation. Most of the induced calli were friable and white-yellowish in color after 7 days of incubation changing to a brown-yellowish color and slightly compact appearance after 20 days of incubation. The higher frequency response of callus induction (100%) was registered for treatments K1D1, K1D2, K2D1, K2D2, followed a significantly lower frequency response (75%) by treatments K0D1, K0D2, K0D3, K1D, and K2D3 (Fig. 1).

The callus induction in *in vitro* cultures is generally documented to be dependent on the addition of exogenous auxin and cytokinin, being critical the ratio between them to promote callus. An intermediate range of auxin and cytokinin is best for promoting calli formation, whilst a high ratio of auxin to cytokinin promotes root formation and a high ratio of cytokinin to auxin gives rise to shoots (Kakani *et al*., 2009). In plants, the auxin-cytokinin interaction determines the direction of cellular metabolism because their action regulates the expression of genes related to growth and development. Moreover, the action of both PGR is influenced by internal factors inherent to species and explants such as a differential sensitivity of receptor or transporter proteins and endogenous PGR levels (Mockaitis and Estelle, 2008; Sakakibara, 2006). Several PTC reports related to the Cucurbitaceae family, to which *I. sonorae* belongs, mainly deal with somatic embryogenesis (Elmeer and Hennerty, 2008).

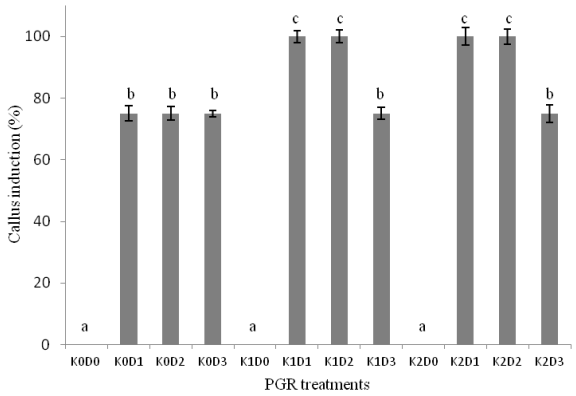

Fig. 1. Callus frequency response induced from immature leaves of *I. sonorae* under different concentrations of KIN with 2,4-D after 20 d of culture. The code treatments (KxDy) corresponded to levels of KIN: 0.00(K0), 2.32(K1) and 4.65(K2) μM with 2,4-D: 0.00(D0), 2.26(D1), 6.80(D2) and 11.32(D3) μM. Means (± SD) followed by the same lower case letter are non-significantly different at the 5% level.

However, few research works have been carried out with the aim of establishing PTC in cucurbit species showing medicinal properties, exceptions being those reported for *Ecballium elaterium* (Toker *et al*., 2003), *Cucurbita andreana*, and *Cucurbita andreana/Cucurbita maxima* hybrid (Halaweish and Tallamy, 1998). In the latter work best callus initiation was achieved with 9.0 μM 2,4-D and 4.65 μM KIN, result very similar to that found in this work with treatment K2D2.

3.2 FAMEs contents in cellular lines

All of the *in vitro* line cultures (K1D1, K1D2, K2D1 and K2D2) produced C12, C14, C15, C16 and C18 FAMEs. Significant total FAMEs differences were observed among treatments, being from highest to lowest: K2D2> K1D2> K1D1> K2D1. Treatment K2D2 showed a significant higher content of C12, C14, C15, C16, and C18, than the rest of the treatments (0.39, 12.51, 9.13, 15.92 and 10.62 mg g^{-1}) (Table 1). Within K2D2, significant differences in the content of the individual fatty acids were observed, and were from higher to lower as follows:C16>C14>C18>C15>C12 (Table 1). Production of FAMEs seems to be closely related to GI. The higher GI was for a given treatment, the higher was its FAMEs contents (Table 1). All the cellular lines produced significantly higher FAMEs concentrations than that reported by Hernández-Galicia *et al*. (2007) for tuber roots (0.25 mg mg g^{-1}). These authors also found that these FAMEs possessed hyploglycemic activity, so that it may be inferred that our cellular lines may also display hypoglucemic activity. Furthermore, the five fatty acids (C12, C14, C15, C16 and C18) found in our calli have been associated with hypoglycemic activity (Hernández-Galicia *et al*., 2007). Other cucurbits used to counteract diabetes have shown their effectiveness by means of displaying hypoglycemic activity, attributed to sterols, flavonoids, terpens and steroids (Andrade-Cetto and Heinrich, 2005). However, among all the reported natural products responsible for antidiabetic properties from many plants, just few of them are related to lipids and fatty acids (Qi *et al*., 2010; Negri 2005), such in the case of oleanolic acid from *Ligustrum lucidum*, hypothesized as stimulator of insulin release (Gao *et al*., 2007).

Table 1. Fatty acid methyl esters (FAMEs) content from extracts of *I. sonorae* cellular lines.

Calli line	FAMEs content from *I. sonorae* calli samples (mg g^{-1}) Individual content					Total content	GI
	C12	C14	C15	C16	C18		
K1D1	$0.27\pm0.01^{a,A}$	$8.76\pm0.45^{a,C}$	$6.19\pm0.25^{b,B}$	$11.20\pm0.37^{a,D}$	$8.74\pm0.41^{a,C}$	35.16 ± 0.30^{b}	1.77 ± 0.01^{b}
K1D2	$0.28\pm0.01^{a,A}$	$8.98\pm0.18^{a,C}$	$7.23\pm0.29^{c,B}$	$9.95\pm0.62^{a,D}$	$10.82\pm0.30^{b,E}$	37.26 ± 0.26^{c}	2.03 ± 0.01^{c}
K2D1	$0.29\pm0.02^{a,A}$	$8.89\pm0.14^{a,C}$	$5.48\pm0.05^{a,B}$	$10.20\pm0.78^{a,D}$	$8.40\pm0.33^{a,C}$	33.26 ± 0.25^{a}	1.70 ± 0.01^{a}
K2D2	$0.39\pm0.02^{b,A}$	$12.51\pm0.41^{b,D}$	$9.13\pm0.56^{d,B}$	$15.92\pm0.18^{b,E}$	$10.62\pm0.30^{b,C}$	48.57 ± 0.29^{d}	2.61 ± 0.01^{d}
TR*						0.25*	

Within a column, mean (± SD) of each sample followed by the same lower case letter indicate non-significantly differences ($p > 0.05$). Within a row mean (± SD) of each sample followed by the same upper case letter indicate non-significantly differences ($p > 0.05$). All the data shown are the mean of three replications from the last two subcultures. *TR, tuber roots, data were taken from Hernández-Galicia *et al*. (2007).

Table 2. Total phenol content (TPC) and radical quenching (EC_{50}) from extracts of calli lines and leaves of *I. sonorae*.

Sample	TPC (mg GAE g^{-1})	EC_{50} (μg mL^{-1}) Assay	
		$DPPH^{\bullet+}$	$ABTS^{\bullet+}$
Leaves	360.1 ± 18.0^{c}	75.5 ± 5.0^{a}	66.0 ± 2.6^{a}
K1D2	21.2 ± 1.0^{a}	2798.4 ± 133.6^{c}	1973.1 ± 106.8^{c}
K2D2	57.1 ± 3.3^{b}	855.7 ± 25.0^{b}	573.3 ± 15.0^{b}

Within a column mean±SD (of three replications from the last two subcultures) with the same lower case letter are non- significantly different ($p > 0.05$).

3.3 TPC and antioxidant activity in calli and leaves

Significant higher TPC and antioxidant activity were found in K2D2 callus (57.1 mg GAE g^{-1} and 573.3-855.7 μg mL^{-1}, respectively) than in K1D2 callus (21.2 mg GAE g^{-1} and 1973.1-2798.4 μg mL^{-1}, respectively) (Table 2). As in the case of FAMEs production, TPC and antioxidant activity seem to be closely related to GI (Tables 1 and 2). Comparison of TPC and antioxidant activity between K2D2 callus and leaves show that the latter possessed significantly higher values for both parameters (360.1 mg GAE g^{-1} and 66.0-75.5 μg mL^{-1}, respectively) (Table 2). Chew *et al*. (2011) categorized TPC into 4 classes based on mg GAE $100g^{-1}$ as follows: i) high for values > 5000, ii) medium high ranging between 3000-5000, iii) medium low ranging between 1000-3000, and iv) low for values < 1000. So, by this standard, the leaves showed medium high TPC, whereas both calli showed low TPC (Table 2). Likewise, Kuete and Efferth (2010) ranked the efficiency of radical scavenging at 50% of initial amount, where lower radical quenching expressed as μg mL^{-1} means a higher antioxidant activity, as follows: i) high for values < 50, ii) moderate ranging between 50-100, and iii) low for values> 100. Thus, leaves had a moderate radical scavenging capacity based on the values obtained for the $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ assays, while both calli possessed a low radical scavenging capacity (Table 2).

Several curcubit species have been reported as possessing phenolic compounds and antioxidant activity, but no reports exist regarding these two parameters for *I. sonorae*. The results found in this work indicate that TPC in leaves was significantly higher, and for K2D2 callus non-significantly different, than TPC (ranging from 49.7-157.6 mg GAE g^{-1}) reported for several cucurbit species (Local Food-Nutraceuticals Consortium, 2005; Wu and Ng, 2008). On the other hand, the antioxidant activity found in leaves was significantly higher, but for both calli it was significantly lower, than values reported for some cucurbit species (129.94-500.00 μg mL^{-1}, Local Food-Nutraceuticals Consortium, 2005; Wu and Ng, 2008). Nevertheless, the antioxidant activity found in the leaves of *I. sonorae* was significantly higher than that reported for vitamin E by Wu and Ng (IC_{50} = 172.21 μg mL^{-1}, 2008). These results tend to explain the popular belief attached to *I. sonorae* leaves for remeding or counteracting diseases associated with oxidative stress. Likewise, fruits of capulin (*Prunus serotina*) which are widely used in Mexico to treat various diseases, showed significant antioxidant and antimicrobial activities and had high content of anthocyanin phenols (Jiménez *et al*., 2011). Molecular studies have revealed that phenolics can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. These processes mark the point of no return in the cell death process, although the exact mechanism involved is not fully elucidated. Phenolic compounds may prevent oxidative stress and through their antioxidant activity exert protective effect by selectively inhibiting or stimulating key protein in the cell signaling cascades (Soobrattee *et al*., 2005).

Conclusion

This work establishes that *I. sonorae* leaves possess high amount of phenolic compounds and antioxidant activity that are higher or equivalent to those found in other cucurbit species. Also an explanation is provided as to why the leaves are used in folk medicine for treating diverse illnesses related to oxidative stress. Likewise, it was shown that it is possible to establish callus cellular lines for producing fatty acids and phenolic compounds with antioxidant activity. Fatty acids content in callus was higher than in the reported for tuber roots, but the opposite was found for the phenolic compounds content. Nevertheless, this work provides the stepping stone for enhancing the production of fatty acids and phenolic compounds from *I. sonorae* through the use of plant tissue culture techniques.

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Nomenclature

EC_{50}	concentration of extract needed to decrease at 50% the initial radical, μg mL^{-1}.
GI	growth index.
TPC	total phenol content per gram of dry extract, mg GAE g^{-1}.

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