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Phenolic Content and Antioxidant Capacity of Infusions of *Vitis tiliifolia* (Humb & Bonpl. Ex Schult.) Leaves

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ABSTRACT

The antioxidant capacity and polyphenol contents in leaves of *Vitis tiliifolia* are unknown. Leaves from four accessions of *Vitis tiliifolia* grown *ex situ* in the collection of the Universidad Autónoma Chapingo, Veracruz - Mexico, were collected in Autumn (2015) and Spring (2016), dehydrated and used to make water infusions at 80°C for 5 minutes. The four accessions were propagated from wild grapevines which grow in Huatusco, Atlahuilco, Cosautlan and Ixtaczoquitlan. The aqueous infusions were analyzed to evaluate the antioxidant capacity by application of 2,2-DiPhenyl-1-PicrilHydrazyl (DPPH), and the total phenolic compounds (total reducing power) were determined spectrophotometrically by the Folin-Ciocalteu method. The polyphenols identification and quantification were determined using an ultrahigh resolution liquid chromatograph. Fourteen compounds, including *trans*-resveratrol, quercetin and rutin were identified. The infusions obtained from leaves of Huatusco and Ixtaczoquitlan accessions had the highest contents of total phenols. The infusions from leaves of Cosautlan and Ixtaczoquitlan accessions showed the highest antioxidant activities. The results indicate that the V*itis tiliifolia* leaves in infusions are a rich source of bioactive compounds. This is the first time that the phenolics content and the antioxidant capacity of leaf infusions of *Vitis tiliiflolia* leaves are reported. As a large variability was found in the compounds of the different accessions, a selection of the genotypes with the most suitable composition of the leaves for their use in infusions and subsequent cultivation could represent a way for the valorization of *Vitis tiliifolia* and to diversify the agricultural productions in tropical areas.

Keywords: Grapevine leaves, Mesoamerican *Vitis*, Polyphenols, *Trans*-resveratrol.

INTRODUCTION

Wild and cultivated *Vitis* vine leaves are used as food and also as therapeutic remedies in different countries (Tobar-Reyes *et al.*, 2009, 2010). The wild grapevines (*Vitis* spp.) in Europe (De Andrés *et al.*, 2012) and America (Cruz-Castillo *et al.*, 2009) are not cultivated, and sometimes they are overexploited *in situ* for the beneficial properties of their fruits and leaves in the human health.

Grapevine leaves are of great interest in phytotherapy as they exhibit antioxidant, anticancer, antispasmodic and antibacterial

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properties (Pignatelli *et al*., 2006). They are also used for the symptomatic treatment of mild to moderate chronic venous insufficiency in humans (Ortiz, 2004). Their properties are mainly due to their content of phenolic compounds that protect against various diseases because they eliminate free radicals (Yilmaz and Toledo, 2004). Among phenols, an important group of compounds in *Vitis* leaves is represented by flavonoids, which prevent platelets aggregation and induce muscle relaxation (Nijveltd, 2001), and together with proteoglycans, exert an inhibitory effect on allergic symptoms (Theoharides and Bielory, 2004). The stilbenoid, resveratrol, which is present in extracts of seeds, fruit and leaves of grapevine, may increase brain capacity and longevity (Barnes and Prasain, 2005). In plants, resveratrol accumulates in response to infections by fungi, UV radiation, external chemicals, and, in general, stress-causing factors (Jeandet *et al*., 2002). Then, as leaves of grapevines contain such useful compounds (Tobar-Reyes *et al*., 2009; Bárcena, 2014), the leaves of *Vitis tiliifolia*, native of the tropics in America (Tröndle *et al*., 2010), could represent an opportunity for the development of medicinal and nutritious infusions. In this context, the purpose of this work was to identify and quantify the total phenolic compounds, and the antioxidant capacity of leaf aqueous infusion extracts of the grapevine *Vitis tiliifolia*.

MATERIALS AND METHODS

Plant Material and Treatments

Mature leaves of four *Vitis tiliifolia* (Humb & Bonpl. Ex Schult.) accessions, 6-year-old, grown *ex situ* in the collection of the Universidad Autónoma Chapingo, at Huatusco, Veracruz, Mexico (19° 08′ 48" N 96° 57′ 00" W, 1,344 m), were randomly harvested from the whole vines at about 80 cm from the soil. The leaf samples were collected after fruit harvest in November 2015 (Autumn) and after flowering in June 2016 (Spring). These accessions were under the same type of soil and climatic conditions. They were from grapevines that grew wild in the counties of Huatusco, Ixtaczoquitlan, Cosautlan, and Atlahuilco in the state of Veracruz, Mexico (Cruz-Castillo *et al*., 2009).

The leaves were dried at 43°C to avoid leaves damage during six hours in an oven (Dehydrator Weston®). For the chemical extraction, 0.1 g of dehydrated powdered leaves were added to 20 mL of distilled water at 80°C for 5 minutes. Samples of the infusions were then stored at -20°C until analysis.

Total Phenolic Content

Total phenolic compounds (total reducing power) were determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton *et al.*, 1965), using gallic acid as calibration standard in concentrations from 0.5 to 4.5 mg mL^{-1}. Folin-Ciocalteu reagent was freshly prepared (1:10 v/v). In each microplate well, an aliquot of the infusions (25 µL), water (125 µL), Folin-Ciocalteu reagent (20 µL) and 20% Na_2CO_3 (30 µL) were added. After incubation for 30 min at room temperature, absorbance was measured at 750 nm in a microplate reader (Synergy 2 Microplate reader, Biotek International, software Gen5) versus a blank. The blank consisted of 25 µL of distilled water instead of the sample. A Gallic Acid standard curve with a linear range (2.5-29.0 µg GA mL^{-1}) was prepared from a freshly made 0.5 mg mL^{-1} gallic acid stock solution. The standard curve was linear (y= 40.267+0.0412 and R^2= 0.9824). Results were expressed as mg Gallic Acid Equivalents per gram of dry leaves (mg GAE g^{-1} DW). All samples were analyzed in triplicate.

Antioxidant Capacity

The antioxidant capacity application of 2,2-DiPhenyl-1-PicrilHydrazyl (DPPH) was

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carried out with leaves of *Vitis tiliifolia* in an aqueous extract. Three extracts were obtained from dry leaves of each of the four accessions. Each extract was evaluated four times for each antioxidant assay. The IC_{50} values were calculated from a graph of inhibition rate. DPPH free radical activity was evaluated according to Brand-Williams (1995). A 1 mM DPPH stock solution with 80% methanol was prepared dissolving DPPH. Trolox calibration curve was prepared with 80% methanol using concentrations from 3.99 to 39.95 µM. Tests were done to determine which extracts degraded approximately 50% of DPPH.

Blank was prepared in eight wells with 250 µL of methanol 80%. For each accession, 12 repetitions with extract aliquots of 200 µL and 50 µL of DPPH standard solution in methanol were added to wells of a 96 wells microplate. After incubation for 30 min at room temperature, the absorbance of each solution was read at 515 nm using a microplate reader (Biotek modelo Synergy 2) to evaluate the total removal capacity of the DPPH radical (%). Results were expressed as µmol Trolox Equivalents per gram of dry sample (TE) g^{-1} DW. The IC_{50} value was calculated from the curve of percentage of degraded DPPH against sample concentration. All leaves extracts were analyzed in triplicate.

Polyphenols Identification and Quantification

Phenolic compounds were extracted from 0.8 g of dehydrated leaves of each of the different accessions with 160 mL of water at 80°C, and allowed to cool for subsequent filtering. Afterwards, samples were stored at –80°C until analysis.

The detection and quantification of phenolic compounds were performed with a 1,290 infinity Agilent ultrahigh resolution liquid chromatograph coupled to a 6,460 Agilent triple quadrupole mass spectrometer, on a ZORBAX RRHD Eclipse Plus C18 reverse-phase column (2.1×150 mm, 1.8 µm; Agilent Technologies Inc., Santa Clara, CA, USA) thermostated at 40°C.

The gradient elution program was arranged with two eluents: eluent A, pure water–formic acid (99.9: 0.1, v /v); eluent B, acetonitrile–formic acid (99.9: 0.1, v /v). The flow rate was set at 0.1 mL min^{-1} and the injection volume at 5 µL. The gradient conditions of the mobile phase were: 0 minute 1% B, 0.1–40 minutes linear gradient 1–40% B, 40.1–42 minutes linear gradient 40–90% B, 42.1–44 minutes isocratic 90% B, 44.1-46 minutes linear gradient 90-1% B and 46.1-47 minutes 1% B isocratic (total run time 47 minutes). Mass spectrophotometry conditions were: Gas temperature of 300°C, with flow of 5 L min^{-1}, nebulization of 45 psi, capillary voltage of 3,500V and nozzles voltage of 500V. Fragment voltage was 100V, and throttle cellular voltage was 7V.

A dynamic Multiple Reaction Monitoring (dMRM) procedure was followed as reported by Durand-Hulak *et al*. (2015) on an Agilent 6460 134 Triplequadropole (QqQ) mass spectrometer. Quantification was performed using a calibration curve for each one of the 31 compounds analyzed with a concentration range from 0.03 to 30M. The determination coefficients were 0.99 or higher. Each polyphenol compound was reported as µg g^{-1} of sample (dry weight). Each assay was performed in triplicate and data were analyzed using Masshunter software version B.06.00 (Agilent).

Data Analysis

A completely randomized statistical design for the antioxidant capacity and the identified compound was used. ANOVA, Tukey's test ($P \leq 0.05$) for mean separation, and standard deviations of the means were performed with SAS (Statistical Analysis System, version 8.1).

RESULTS AND DISCUSSION

Total Phenols (Total Reducing Power)

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In general, the highest phenolic contents were found in Spring (June), after blooming, for all the accessions, with the exception of the Cosautlan one, which presented similar values in June and November. Leaves of the accession from Ixtaczoquitlan showed the highest value (31.6 mg GAE g^{-1} DW) (Table 1), followed by leaves of the accession from Huatusco (18.6 mg GAE g^{-1} DW) (Table 1).

Water extraction of compounds with reducing properties, among which are the phenolics from plants tissues, is efficient and recoveries achieve values from 75 to 90 % (Realini *et al*., 1981). The phenol contents (reducing power) of the aqueous infusions of the four accessions studied were higher than those reported for leaves of several other medicinal plants (Avonti *et al*., 2014; Pakade *et al*., 2013)**.** This indicates that leaves of *Vitis tiliifolia* have the potential for preparation of infusions for human consumption.

Antioxidant Activity

The DPPH antioxidant capacity was higher in the leaves of the accessions from Huatusco and Ixtaczoquitlan when the leaves were collected in Spring (June) and Autumn (November) (Table 1). In general, the antioxidant activity values shown in the present study were superior to those reported by Jiao *et al*. (2013) with a *Forsythia suspensa* leaf infusion. Regarding the mean Inhibition Concentration (IC_{50}), the leaf extracts of *Vitis tiliifolia* had an antioxidant capacity value between 21 and 26 μg mL^{-1}. Values lower than 30 μg mL^{-1} indicate a high antioxidant potential (Ramos *et al*. 2003; Zhu *et al*., 2011).

Identification and Quantification of Polyphenols

In the aqueous extractions, 14 different phenols were identified and quantified (Table 2). Leaves from Huatusco showed the highest values ($P \leq 0.05$) of catechin, quercetin glucoside, quercetin galactose, and epicatechin. Leaves from Cosautlan had the highest values ($P \leq 0.05$) for rutin and trans-resveratrol (Table 2). The Ixtaczoquitlan accession had the highest value of chlorogenic acid (Table 2), which is the principal phenol of the coffee beverage (Farah *et al*., 2005; Yuki *et al*., 2011). This accession also had high values of gallic acid (Table 2).

The content of resveratrol of the accession from Cosautlan was 8.3 times higher than that obtained by Tobar-Reyes (2010) with *Vitis* spp leaves. Leaves of the accession from Cosautlan presented a rutin content of 1,008 mg 100 g^{-1} DW that is about three times higher than that found in the skin of the fruit of *Vitis tiliifolia* (Jiménez *et al*., 2018) and more than six times higher than that found in the fruit skin of 10 *Vitis vinifera* cultivars (Lacopini *et al*., 2008). The recorded high values of rutin in the leaves of *Vitis tiifolia* could be due to the existence of high levels of quercetins (Table 2) that promote rutin compounds (Jeong *et al*., 2008).

Polyphenols, such as gallic acid, quercetin and kaempferol present in the leaves of *V. tiliifolia* were higher than those measured in muscadine (*Vitis rotundifolia* Mixch) leaves (Pastrana *et al*., 2003). In ten cultivars of white and purple grapes, gallic acid was in the range of 7.6-9.5 μg 100 g^{-1} FW. These values are lower than those found in the Ixtaczoquitlan accession (12.5 μg g^{-1} DW). Also, the kaempferol was lower than that observed in the Atlahuilco accession. Likewise, the quercetin content was lower than that observed in the accession from Huatusco. Thus, the presence of these compounds in our water extracts indicate the potential of *Vitis tiliifolia* oven dried leaves for the elaboration of infusions rich in phenols.

The estimated mean intake of *trans*-resveratrol for humans is 933 μg d^{-1} (Zamora *et al*., 2008). According to our results, drinking a cup of infusion of oven dried *Vitis tiliifolia* leaves (0.8 g) would contribute

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Table 1. Total phenols, antioxidant capacity, and IC_{50} by DPPH of aqueous infusions of oven dried leaves from four accessions of *Vitis tiliifolia* leaves collected in Spring (June-J) and in Autumn (November-N).[a]

Accession (Date)	Total phenols (mg GAE g^{-1} DW)	DPPH (µ mol TE g^{-1})	IC_{50} (µmol L^{-1})
Atlahuilco (J)	11.0 ± 0.3 d	251.2 ± 18 b	21.53
Atlahuilco (N)	5.3 ± 0.0 e	149.7 ± 16 d	21.53
Cosautlan (J)	8.9 ± 0.3 d	118.3 ± 18 e	25.76
Cosautlán (N)	9.4 ± 0.4 d	205.8 ± 10 c	24.05
Huatusco (J)	18.6 ± 0.2 b	315.5 ± 14 a	21.29
Huatusco (N)	14.1 ± 0.3 c	142.6 ± 16 d	23.03
Ixtaczoquitlan (J)	31.6 ± 0.4 a	271.8 ± 15 ab	21.88
Ixtaczoquitlan (N)	13.9 ± 0.2 c	152.1 ± 11 d	20.53

[a] Letters identify differences of interactions between accession and date of sampling by Tukey (P≤ 0.05).

Table 2. Content of phenolic compounds of aqueous infusions of oven dried leaves from four accessions of *Vitis tiliifolia* collected in Spring (June).[a]

Phenolic compounds (µg g^{-1} DW)	Atlahuilco	Cosautlan	Huatusco	Ixtaczoquitlan
Gallic acid	***	***	1.25 ±0.2 b	12.46 ± 0.6 a
Catechin	31.3 ± 10 c	49.5 ±12 c	1533.1 ± 18 a	193.5 ± 15 b
Vanillic acid	79.4 ± 24 a	75.6 ± 26 a	86.4 ± 20 a	114.4 ± 30 a
Chlorogenic acid	****	***	238.74 ± 70 b	961.20 ±89 a
Caffeic acid	25.5 ± 5 b	88.2 ±9 a	33.3 ± 10 b	2.1 ± 6 c
Epicatechin	85.2 ± 15 b	132.4 ± 22 a	91.4 ± 30 ab	37.2 ± 12 c
Vanillin	16.8 ± 3 b	20.2 ± 3 b	17.1 ± 5 b	41.1 ±8 a
Coumaric acid	***	5.2* ±2	***	***
Routine	302.3 ± 52 c	10008.5 ±74 a	2045.6 ± 100 b	246.7 ± 89 c
Quercetin galactose	1527.2 ± 444 a	1551.6 ±487 a	2221.0 ± 482 a	134.7 ± 100 b
Quercetin glucoside	9282.6 ±1272 b	8052.4 ±921 b	14979.6 ±2000 a	751.1 ±200 c
Kaempferol-3-o-glucoside	105.8 ±20 a	95.1 ±20a	82.3 ± 37 a	***
Trans-resveratrol	***	160.3 ± 5 a	74.9 ± 5 b	6.4 ± 3 b
Quercetin	39.7 ± 12 b	50.6 ±11 b	101.1 ±20 a	***

[a] Means with the same letter within each row do not differ statistically (Tukey, P≤ 0.05).
(***) Values under quantification limit.

about 17.1% of the daily intake of this metabolite. The infusion of *Vitis tiliifolia* leaves represents a therapeutic and nutritional alternative for families that have access to wild grape populations in their localities. However, further studies are needed on possible side effects because an over dosage would cause nephrotoxicity (Cottar, 2010). This is the first time that the antioxidant capacity, the content, and identification of phenols in leaves of *Vitis tiliifolia* in infusion are reported.

CONCLUSIONS

The results of our experiments showed that oven dried leaves of *Vitis tiliifolia* have a great potential for being used for the preparation of aqueous infusions with antiradical activity and could be suggested for human consumption. This was due to the presence in the infusion of metabolites of

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nutritional and pharmacological importance, including *trans*-resveratrol in its free form, along with gallic acid, catechin, vanillic acid, chloragenic acid, caffeic acid, epicatechin, rutin, quercetin galacose, and quercetin glucoside. *Vitis tiliifolia* is spread in tropical areas in America as wild vines and the use of its leaves in infusion could have commercial potential for the elaboration of therapeutic drinks. As we found a large variability in the compounds released by the leaves of different accessions, a selection of the genotypes with the most suitable composition of the leaves for their use in infusions and subsequent cultivation could represent a way for the valorization of *Vitis tiliifolia* and diversification of agricultural production in tropical areas.

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محتوای فنولی و ظرفیت آنتی اکسیدانی عصاره آبی(اینفیوژن) برگ های *Vitis tiliifolia* (Humb & Bonpl. Ex Schult.)

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چکیده

ظرفیت آنتی اکسیدانی و محتوای فنولی برگ های *Vitis tiliifolia* دانسته نیست. در این پژوهش، در پاییز ۲۰۱۵ و بهار ۲۰۱۶، برگ های ۴ نمونه ثبت شده از *Vitis tiliifolia* که درمحل کلکسیون دانشگاه Universidad Autónoma Chapingo, Veracruz, در مکزیک پرورش یافته بودند، برداشته شد واز آنها برای تهیه عصاره آبی (اینفیوژن) در ۸۰ درجه سانتی گراد به مدت ۵ دقیقه استفاده شد. این ۴ نمونه ثبت شده از تاک های وحشی در مناطق Huatusco، Atlahuilco، Cosautlan، وIxtaczoquitlan تکثیر شده بود. عصاره های آبی به دست آمده مورد تجزیه قرار گرفت تا ظرفیت آنتی اکسیدانی آن ها با استفاده از 2,2-diphenyl-1-picrilhydrazyl (DPPH) ارزیابی شود و محتوای مواد فنول کل (توان احیاکنندگی کل) هم با اسپکتروفتومتربه روش Folin-Ciocalteu تعیین شد. شناسایی و کمّی کردن پلی فنول ها با کاربرد کروماتوگراف مایع با وضوح فوق العاده بالا انجام شد و ۱۴ ماده شامل *trans*-resveratrol، quercetinو rutin شناسایی شد. عصاره آبی به دست آمده از برگ نمونه های ثبت شده از مناطق Huatuscoو Ixtaczoquitlan بیشترین مقدار فنول کل را داشتند. عصاره های آبی به دست آمده از نمونه های ثبت شده Cosautlan و Ixtaczoquitlan بیشترین فعالیت آنتی اکسیدانی را نشان دادند. نتایج نشان داد که برگ های *Vitis tiliifolia*در فرایند عصاره گیری منبعی غنی از مواد زیست-فعال (Bioactive) است. این نخستین بار است که محتوای فنولی و ظرفیت آنتی اکسیدانی عصاره آبی برگ های *Vitis tiliifolia* گزارش می شود. از آنجا که مواد موجود در نمونه های ثبت شده این پژوهش تغییرات زیادی نشان میداد، انتخاب مجموعه ای از ژنوتیپ های دارای مناسبترین ترکیبات در برگ ها برای استفاده در عصاره آبی و متعاقب آن کاشت این محصولات می تواند راهی باشد برای ارزش گذاری *Vitis tiliifolia* و تنوع دادن به تولیدات کشاورزی در مناطق گرمسیر.

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