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EFFECT OF SOLVENT-TEMPERATURE EXTRACTION CONDITIONS ON THE INITIAL ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF MUITLE EXTRACTS AND THEIR DECAY UPON STORAGE AT DIFFERENT pH

EFECTO DE LAS CONDICIONES DE EXTRACCIÓN SOLVENTE-TEMPERATURA EN LA ACTIVIDAD ANTIOXIDANTE Y CONTENIDO DE FENOLES TOTALES EN EXTRACTOS DE MUITLE Y SU PÉRDIDA DURANTE EL ALMACENAMIENTO A DIFERENTES VALORES DE pH

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Abstract

The effect of solvent and extraction temperature on the initial antioxidant activity (AA) and total phenolic content (TPC) of Muitle (*Justicia spicigera*) extracts and their decay upon storage at different pH values was studied. Extraction with aqueous solvents, at either 25 or 60 °C, produced fresh extracts (pH 7.4) with higher initial TPC and AA. Extracts were adjusted to different pH´s displayed different TPC and AA decay rates upon storage. TPC had first order decay kinetics, characterized by a relatively quick constant (k_1) at relatively short storage times followed by a relatively slow constant (k_2) at relatively large storage times. ABTS and DPPH assays estimated different AA in the extracts, attributed to compatibility differences between the bioactive species and the free radicals.

Keywords: *Justicia spicigera*, total phenolic content, antioxidant activity, extraction conditions, decay kinetics.

Resumen

Se estudió el efecto del solvente y la temperatura de extracción en la actividad antioxidante inicial (AA) y el contenido de fenoles totales (TPC) en extractos de Muitle (*Justicia spicigera*) y su pérdida durante el almacenamiento a diferentes valores de pH. La extracción con solventes acuosos, a 25 o 60°C, produjeron extractos frescos (pH 7.4) con mayor TPC y AA iniciales. Los extractos fueron ajustados a diferentes valores de pH mostrando diferentes TPC y tasas de pérdida de AA durante el almacenamiento. TPC mostró una cinética de pérdida de primer orden caracterizada por una constante relativamente rápida (k_1) a tiempos relativamente cortos seguida de una constante relativamente lenta (k_2) a tiempos de almacenamiento largos. Pruebas de ABTS y DPPH estimaron diferentes AA en los extractos, atribuido a las diferencias de compatibilidad entre las especies bioactivas y los radicales libres.

Palabras clave: *Justicia spicigera*, contenido de fenoles totales, actividad antioxidante, condiciones de extracción, cinéticas de pérdida.

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1 Introduction

Aqueous extracts of aerial parts of Muitle (*Justicia spicigera* Schechtendal) have been used in traditional medicine in Mexico and elsewhere as antipyretic, blood depurative, anti-inflammatory, for treatment of diarrhea and diabetes, uterine cancer, amenorrhea, and have shown cytotoxic activities against human leukemic cells (Wong, 1976; Mahabir and Gulliford, 1997; Márquez *et al*., 1999; Sepúlveda Jiménez *et al*., 2009). Likewise, ethanolic extracts have demonstrated activity against Giardia duodenalis (Ponce-Macotela *et al*., 2001). The main compounds found in *J. spicigera* are kaempferitrin, O-sitosterol-3-β-glycoside, allantoin and cryptoxanthin, and are probably responsible for these activities (Euler and Alam, 1982; Domínguez *et al*., 1990). However, antioxidant activity studies of *J. spicigera* and other species from this genus are scarce. Sepúlveda-Jiménez *et al*. (2009) were the first in describing the antioxidant activity from *J. spicigera*, where the phenolic compounds and flavonoids contributed to this activity, suggesting *J. spicigera* as potential source of antioxidants for use against various free radical-related disorders.

The interest in phenolic compounds has been related primarily to their antioxidant activity; they also show important biological activity in vivo and may be beneficial in combating diseases related to excessive oxygen radical formation, where the antioxidant defense capacity of the human body is exceeded (Morelló *et al*., 2004; Mulero *et al*., 2010). However, these natural antioxidants are fairly unstable during harvesting, transportation, storage and processing (Reyes-Munguía *et al*., 2009). On the other hand, the initial phenolic content and antioxidant activity of the extracts depends on the solvent and extraction conditions used, mainly due to the complex interrelationship between the solvents and phenolics polarities and solubilities (Serrano-Maldonado *et al*., 2011; Ares *et al*., 2010). Also it is important to understand how storage time and pH of the phenolic compounds affects their stability before proceeding to use them in a commercial basis (Xu and Chang, 2007).

Thus, the aims of this work were: (a) to evaluate the effect of the solvent and temperature extraction conditions on the initial total phenolic content and antioxidant activity of Muitle extracts; and (b) to determine the decay of these two parameters in extracts at different pH values stored for 20 days at 25 ^{a}C.

2 Materials and methods

2.1 Plant material and reagents

Leaves of Muitle (*J. spicigera* Schechtendal) from adult plants were collected in the month of June from Tepoztlan, Morelos, Mexico. Analytical reagents and standards used for the determination of total phenolic content (TPC), and antioxidant activity (AA) were: Folin-Ciocalteau reagent (FCR) and sodium azide (SA) both purchased from Hycel de Mexico, S.A. de C.V., Mexico, D.F., Mexico; 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) 2,2-diphenyl-1- picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (TROLOX), gallic acid standard (GAS), potassium persulfate (PPS), glycerol (G, Sigma Ultra, 99% GC) were obtained from Sigma-Aldrich Quimica, S.A. de C.V., Toluca, State of Mexico, Mexico; anhydrous sodium acetate (ASA), glacial acetic acid (GAA), hydrochloric acid (HCl), methanol (MeOH), and sodium hydroxide (NaOH) were acquired from J.T. Baker, S.A. de C.V. (Xalostoc, State of Mexico, Mexico); sodium carbonate (SC) was provided by Química Meyer (Mexico, D.F., Mexico); absolute ethanol (E_{100}) was procured from Tecsiquim S.A. de C.V. (Toluca, State of Mexico, Mexico); and propylenglycol (PG) was gotten from Polioles, S.A. de C.V. (Toluca, State of Mexico, Mexico). Deionized water (W) was used in all the experiments.

2.2 Preparation of Muitle extracts

Muitle stems and leaves were dried for 72 h at 38°C in an oven (SW-17TA, Blue-M, New Columbia, PA, USA). The dry vegetable material was finely milled and sieved using a Mesh 35, and 2.5 g of mill were extracted by maceration for 12 h with 100 g of different extraction solvents (two mono-component and three binary systems) at 25 and 60°C, respectively. These temperatures were chosen from preliminary experiments because high phenolic compounds extraction and a relatively low degradation caused by thermal effects were achieved. Codification of extraction conditions was done in base to the solvent´s abbreviation followed by the temperature used, as follows: Water (W25, W60), absolute ethanol ($E_{100}25$, $E_{100}60$), ethanol 500 g kg^{-1} ($E_{50}25$, $E_{50}60$), glycerol 50 g kg^{-1} ($G_5 25$, $G_5 60$), and propylenglycol 50 g kg^{-1} ($PG_5 25$, $PG_5 60$). The extracts were filtered using Whatman 4 filter paper. Fresh Muitle extracts presented an initial pH of 7.4, independently of the

extraction conditions used.

2.3 Characterization of Muitle extracts

Fresh Muitle extracts were diluted with the corresponding solvent system used in the extraction to 500 g kg^{-1}. The pH of the dilutions remained at 7.4. In order to obtain solutions at pH 3.5 and 5.5, the requisite amount of 1.0N HCl was added. All the extracts were stored for 20 days at 25 °C at the three pH values (3.5, 5.5, and 7.4), and their TPC and AA were determined up to 20 days. These pH values were chosen because they cover the pH range most used in the beverage, food and pharmaceuticals industries.

2.4 Total phenolic content (TPC) determination

TPC was measured using the method Yim *et al.* (2012), with slight modifications. In short, 0.02 mL of Muitle extract was mixed with 0.1 mL of FCR (500 g kg^{-1}). After 3 min, 0.3 mL of SC (200 g kg^{-1}) were added to the mixture and adjusted to 2 mL with deionized water. The mixture was allowed to stand in dark environment for 90 min. Absorbance was measured against the blank reagent at 725 nm using a Spectronic GENESYS 2 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). GAS was used for the calibration curve with a concentration range of 80-1000 mg kg^{-1} (R^2 = 0.99) and analyzed as above. Results were expressed as mg of Gallic Acid Equivalent (GAE) per kg (mg_{GAE} kg^{-1}).

2.5 ABTS radical scavenging activity

The capacity of the extracts to scavenge $ABTS^{\cdot+}$ was based on that reported by Gong *et al.* (2012), with slight modifications. $ABTS^{\cdot+}$ was prepared by mixing an ABTS stock solution (7 mM) with 2.45 mM PPS. This mixture was allowed to stand for 12-16 h at room temperature in the dark. The $ABTS^{\cdot+}$ working solution was obtained by diluting the stock solution in MeOH to an absorbance of 0.7 ± 0.02 at 747 nm. Then 0.02 mL of the appropriately diluted sample was added to 3 mL of $ABTS^{\cdot+}$ working solution and mixed thoroughly. The reaction mixture was kept at room temperature in the dark for 1 h, and the absorbance was read at 747 nm during 20 days. A reagent control was measured in the same way. The data were reported as TROLOX equivalents (TEAC, mM). TEAC was calculated from the calibration curve using a range of 0.5-10 mM of TROLOX.

2.6 DPPH radical scavenging activity

Radical scavenging activity of the Muitle extracts was evaluated using $DPPH^{\cdot+}$ radicals as reported by Yim *et al.* (2012). Stock solution of DPPH (0.075 mM) in methanol was prepared daily. 3.8 mL of $DPPH^{\cdot+}$ working solution was mixed with 0.02 mL of Muitle extract. The mixture was shaken vigorously for 1 min and left to stand at room temperature in the dark for 30 min. Absorbance was measured against a blank reagent at 517 nm. Radical scavenging activity was determined by calculating the TEAC concentration using the TROLOX calibration curve mentioned above.

2.7 Extracts TPC decay kinetics

The Muitle extracts obtained with different solvents and adjusted to pH values of 3.5, 5.5, and 7.4 were monitored for TPC during storage at 25 °C, and the decay in TPC determined using a first order kinetic model with the following Eq. (1):

$$\ln[TPC]_t = \ln[TPC]_0 - kt \tag{1}$$

where $[TPC]_t$ is the TPC concentration (mg_{GAE} kg^{-1}) at time t (h), $[TPC]_0$ is the initial TPC concentration in Muitle extracts, and k is the decay rate constant (h^{-1}).

The variation in concentration with time provides a highly detailed description of how fast a reaction is occurring. In many circumstances, though, it is desirable to have a simple, approximate measure of the reaction rate, and the half-life ($t_{1/2}$) provides such a measure, and is given for a first order model (Walstra, 2002) as shown in Eq. (2).

$$t_{1/2} = \frac{\ln 2}{k} \tag{2}$$

$t_{1/2}$ is the time it takes for one-half of the original amount of material to react (assuming the compound in question is a limiting reactant) expressed in days. Intuitively, the faster the reaction proceeds the shorter $t_{1/2}$ will be.

2.8 Statistical analysis

Analyses were performed in triplicate for each sample for all the tests, and data is presented as means ± standard deviation (SD). Analysis of variance and comparison of treatment means (LSD 5% level) were performed using SPSS Statistics 17.0 (IBM Corporation, NY, USA).

3 Results and discussion

3.1 Effect of extraction solvent on initial TPC

The solvent system used affected the fresh Muitle extracts initial TPC as can be observed in Table 1. All the fresh extracts had a pH of 7.4, independently of the solvent used in the extraction with a solid content ranged from 1.5-1.6% (w/w). Initial TPC was higher than 700 mg_{GAE} kg^{-1} for most solvents, excepting E_{100}. Given the poor TPC yield by $E_{100}25$, this extract was not considered in the following assays. Non-significant highest initial TPC contents were displayed by $E_{50}25$, $E_{50}60$, PG_560 and G_525, with W showing significantly lower values than the former solvents, but significantly higher than the E_{100} solvent. These variations in the initial TPC can be attributed to the difference in polarity of the solvent systems used. The solvents with intermediate polarity, i.e., E_{50} [δ = 52.5], PG_5 [δ = 77.6], and G_5 [δ = 78.3], showed higher initial TPC than those with lower polarity (E_{100} [δ = 25]) or higher polarity (W [δ = 82]). It has been reported that Ginkgo leaves (*Ginkgo biloba* L.) and Henna leaves (*Lawsonia inermis*) extracts showed a significant difference in phenolic compounds content when extracted with solvents with different polarities (Ding, 1999; Uma *et al*., 2010). According to Li (1994), the solvents most used for extracting phenolic compounds (flavonoids) include water and aqueous methanol, ethanol, and acetone, without considering the possible toxicity associated with the residues of some of these solvents. While water is rather safe to use and is a good solvent for some flavonoid glycosides, its high polarity renders it a bad solvent for other flavonoids. Mixtures of alcohols and water are frequently used because they are more efficient in extracting phenolic compounds than mono-component solvent systems (Spigno and Faveri, 2007) probably because a more polar medium is created which facilitates the extraction of polyphenols. In this work the solvents used for the extraction of Muitle leaves was done based firstly on their safety and secondly on their ability to provide high TPC yields.

3.2 Effect of extraction temperature on initial TPC

The fresh Muitle extracts (pH 7.4) obtained with solvents W, E_{50} and PG_5 showed non-significant differences in initial TPC when extraction temperature was 25 or 60 °C, but a significant effect was observed on initial TPC when using E_{100} and G_5 solvents (Table 1). While initial TPC decreased for G_5 it increased for E_{100} as extraction temperature was raised from 25 to 60 °C. It has been reported that with certain materials, an increase in temperature promotes solvent extraction by enhancing both diffusion coefficients and the solubility of polyphenol content (Al-Farsi and Lee, 2008), and promotes the release of bound polyphenols by breaking the cellular constituents of plant cells (Wang *et al*., 2007). However, elevated temperatures may not be suitable for all kinds of phenolic compounds (Too *et al*., 2010), as they may degrade or coagulate with proteins (Spigno and Faveri, 2007). When the phenolic compounds are thermally stable, it may be inferred that the rate of extraction is higher than the rate of decomposition in the temperature range studied (Liyana-Pathirana and Shahidi, 2005).

Table 1. Initial TPC of Muitle extracts at different pH values

Muitle extract code		TPC $_{pH7.4}$ (mg_{GAE} kg^{-1})	TPC $_{pH5.5}$ (mg_{GAE} kg^{-1})	TPC $_{pH3.5}$ (mg_{GAE} kg^{-1})
W	25	917.09 ± 26.46 c,d	849.61 ± 19.54 c	865.73 ± 13.81 c
	60	872.77 ± 8.80 c	899.97 ± 21.32 c	885.87 ± 9.58c,d
E_{100}	25	190.98 ± 6.60 a	193.00 ± 6.98 a	196.36 ± 8.35 a
	60	624.02 ± 6.85 b	729.43 ± 25.64 b	608.59 ± 4.54 b
E_{50}	25	1018.80 ± 24.17 g	976.50 ± 28.63 e,f	970.46 ± 6.87 f
	60	977.51 ± 32.88 e,f,g	988.59 ± 18.71 e,f	969.79 ± 8.17 f
PG_5	25	959.05 ± 18.47 d,e,f	1018.13 ± 26.29 f	914.06 ± 15.98 d,e
	60	963.08 ± 26.76 d,e,f,g	849.61 ± 25.60 c,d	883.85 ± 14.23 c,d
G_5	25	989.93 ± 18.02 f,g	1006.72 ± 16.76 e,f	936.22 ± 16.87 e,f
	60	926.15 ± 14.52 c,d,e	952.33 ± 20.12 d,e	968.45 ± 13.81 f

Different lowercase letters in the same column represent data significantly different ($P \leq 0.05$).

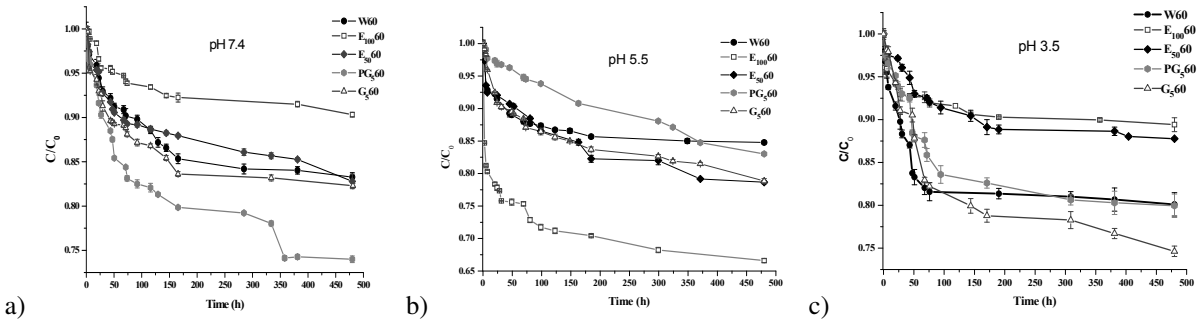

Fig. 1. Influence of solvent-time extraction conditions on the decay of total phenolic content of Muitle extracts adjusted to different pH values and stored at 25 C for 20 days.

3.3 Effect of pH on TPC during storage

The Muitle extracts were adjusted to different pH values which caused slight variations in the initial TPC content (Table 1). The extracts at different pH values (3.5, 5.5 and 7.4) were stored at 25 °C and monitored for TPC for 20 days. In order to establish a comparative basis of the relative TPC degradation of the extracts, TPC values were normalized by plotting the ratio of TPC at time = t and t = 0 versus storage time. Fig. 1 shows the degradation profiles of Muitle extracts obtained at 60°C with storage time at different pH values. A similar behavior was found for the extracts obtained at 25°C (data not shown). The extraction conditions and pH affected TPC degradation. TPC degradation from lowest to highest was at pH 3.5: $E_{100}60 < E_{50}60 < E_{50}25 <$ W25 $<$ W60 $< PG_560 < PG_525 < G_525 < G_560$; at pH 5.5: $E_{50}25 <$ W60 $< PG_560 <$ W25 $< G_560 < E_{50}60 < PG_525 < E_{100}60$; and at pH 7.4: $E_{100}60 < E_{50}25 <$ W60 $< E_{50}60 < G_560 <$ W25 $< PG_525 < PG_560 < G_525$, respectively.

The rate at which TPC-time experimental data decreased were adjusted to several models (data not shown), but the model that best fitted the experimental data (R2 from 0.95 to 0.99) in all cases was a first-order model (Eq. 1). The experimental data fitting was made in two consecutive single processes with different kinetic rates: (1) An initial stage characterized by a steep gradient induced by the high initial TPC loss, probably promoted by reactive oxygen species (ROS), such as light and oxygen, that react with the phenolic compounds in a relatively short storage time (from t = 0 to $t = t_1$, with t_1 varying between 31.3 to 80.4 h), and (2) a second stage occurring at longer storage time (from $t = t_1$ to t = 480 h), where the TPC-time gradient is drastically diminished, probably because most of the phenolic compounds in the extracts have already reacted with ROS, resulting in a slowing down in the rate of loss. A representative graph depicting both the steep and the relatively flat gradient stages for PG_525 at pH 3.5 is presented in Fig. 2. The rate constants corresponding to the steep (k_1) and relatively flat (k_2) gradients of TPC-time decay are given in Table 2. The variability in the rate constants k_1 and k_2 for the Muitle extracts obtained with different extraction conditions and pH does not allow to establish a generalized behavior in the degradation pattern of TPC during storage time, other than the loss of TPC is considerable. Half-life time (t1/2) was calculated using k_1 values, because is in the first gradient when a greater loss in TPC was found.

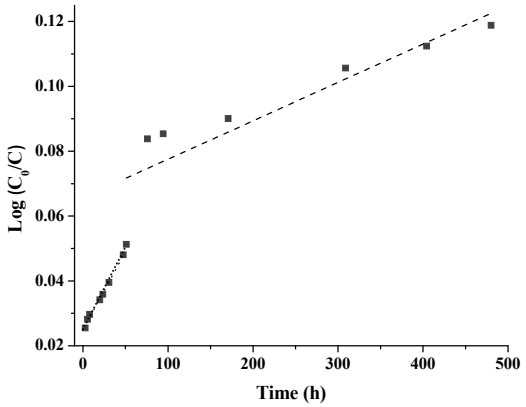

Fig. 2. Total phenolic content first order decay kinetics for extract PG525 at pH 3.5: (■), experimental data; (....), fitted data for the steep gradient (k_1); and (—), fitted data for the relatively flat gradient (k_2).

Table 2. Rate constants corresponding to the steep (k_1) and relatively flat (k_2) gradients of TPC-time decay of Muitle extracts when fitting the experimental data to a first-order kinetic model, and $t_{1/2}$ values for the steep gradient.

pH	3.5 k_1 (h^{-1})	$t_{1/2}$ (days)	5.5 k_1 (h^{-1})	$t_{1/2}$ (days)	7.4 k_1 (h^{-1})	$t_{1/2}$ (days)
W25	7.23E-04	39.94	1.20E-03	24.07	5.48E-04	52.70
W60	1.29E-03	22.39	2.16E-03	13.37	4.71E-04	61.32
$E_{100}60$	2.71E-04	106.57	3.66E-03	7.89	4.49E-04	64.32
$E_{50}25$	7.48E-04	38.61	4.77E-03	6.05	5.04E-04	57.30
$E_{50}60$	3.78E-04	76.41	1.67E-03	17.29	7.08E-04	40.79
PG_525	4.98E-04	57.99	4.29E-03	6.73	5.06E-04	57.08
PG_560	8.77E-04	32.93	1.55E-04	186.33	9.44E-04	30.59
G_525	1.12E-03	25.79	7.38E-04	39.13	6.56E-04	44.03
G_560	1.04E-03	27.77	8.46E-04	34.14	6.98E-04	41.38

pH	3.5 k_2 (h^{-1})	R^2	5.5 k_2 (h^{-1})	R^2	7.4 k_2 (h^{-1})	R^2
W25	6.20E-05	0.96	4.88E-04	0.97	1.10E-04	0.99
W60	2.10E-05	0.98	2.07E-04	0.97	1.02E-04	0.99
$E_{100}60$	2.90E-05	0.96	4.77E-04	0.97	4.10E-05	0.97
$E_{50}25$	7.20E-05	0.97	1.96E-04	0.98	7.40E-06	0.96
$E_{50}60$	4.70E-05	0.97	5.57E-04	0.96	6.50E-05	0.98
PG_525	8.90E-05	0.99	3.09E-04	0.98	7.04E-05	0.95
PG_560	6.60E-05	0.96	1.55E-04	0.99	1.18E-04	0.97
G_525	6.50E-05	0.94	8.80E-05	0.98	1.72E-04	0.98
G_560	1.03E-04	0.98	1.01E-04	0.98	9.20E-05	0.94

Table 2 shows the $t_{1/2}$ for the extracts at different pH values. Since $t_{1/2}$ is a function of the initial TPC value, the TPC at $t_{1/2}$ may be higher for a given extract despite having a shorter $t_{1/2}$. This effect can be clearly discerned from our experimental data. While the highest half-life time (186.33 days) was displayed by $PG_5$60 at pH 5.5, followed by E_{100}60 (106.57 days) and E_{50}60 (76.41 days), the latter two at pH 3.5, their TPC concentrations at $t_{1/2}$ were of 424.81, 304.30, and 484.90 mg_{GAE} kg^{-1}, respectively; whereas, the lowest half-life times were exhibited by E_{50}25 (6.05 days), $PG_5$25 (6.73 days) and E_{100}60 (7.89 days), all at pH 5.5, and their TPC concentrations at $t_{1/2}$ were of 488.25, 509.07, and 364.72 mg_{GAE} kg^{-1}, respectively.

3.4 *Effect of extraction solvent on initial antioxidant activity*

It has been reported that solvent clearly influences the antioxidant capacity assay, but not all in the same way. The two most used assays for determining antioxidant activity of phenolic compounds are ABTS and DPPH. In the case of ABTS, the more polar the solvent is, the greater the ABTS value (Pérez-Jiménez and Saura-Calixto, 2006), while in the case of DPPH, the use of solvents with high polarity, seem to give low values for the extent of reduction (Molyneux, 2004).

Antioxidant activity was expressed as mM of Trolox equivalents (TEAC) as a more meaningful and descriptive expression than assays that express antioxidant activity as the percentage decrease in absorbance. Solvents used for polyphenol extraction had significant effects on ABTS, where the blue color due to the formation of ABTS free radicals ($ABTS^{\cdot+}$) is sensitive to the presence of antioxidants. Discoloration following sample addition indicates that ABTS radicals were quenched or reduced by the antioxidants. The order from higher to lower initial antioxidant activity determined by ABTS in the fresh Muitle extracts at pH 7.4 was: W60 $\geq$ $PG_5$25, $G_5$60, $PG_5$60, $G_5$25, E_{50}25 $\geq$ W25 > E_{50}60 > E_{100}60 (Table 3).

In the case of DPPH assay, which is a very fast method to evaluate the AA, it is possible to determine the antiradical power of an antioxidant by measuring the decrease in absorbance of an alcoholic solution of DPPH at 515 nm in the presence of a hydrogen donating antioxidant. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant through

donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 515 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (Juntachote and Berghofer, 2005; Turkmen *et al*., 2006). The scavenging activity in fresh Muitle extracts from higher to lower was as follows: $G_5$60 ≥ W60 ≥ $PG_5$25, E_{50}25, E_{50}60 > $PG_5$60, $G_5$25,W25 > E_{100}60 (Table 3).

The differences in AA thrown by the ABTS and DPPH assays may be due to the nature of the solvent and the radicals, as the type of solvent and polarity may affect the single electron transfer and the hydrogen atom transfer, which are the main aspects in the measurements of AA. Also the presence of non-antioxidant compounds in extracts could affect the results (Pérez-Jiménez and Saura-Calixto, 2006).

In practical terms, our results indicate that the Muitle extracts contain diverse phenolic compounds whose extraction is directly related to the compatibility of the compounds with the solvent system, and the antioxidant activity is a result of the reaction of the sum of the individual components of the substrate with the free radical molecule used for the assay, be it ABTS or DPPH. Thus, on the end, AA will depend on the type of phenolic compounds extracted by a given extraction system and on the nature of the scavenging molecule (Molyneux, 2004).

3.5 *Effect of extraction temperature on initial antioxidant activity*

The extraction temperature had a significant effect on AA, determined by ABTS and DPPH, when extraction was performed with W and E_{50}. With W, an increase in extraction temperature produced an increase in AA, but the opposite effect occurred with E_{50}. Extraction temperature variation did not affect significantly AA with the rest of the solvents, excepting $G_5$25, which displayed an increase in AA (determined by DPPH) as temperature increased (Table 3). These changes could be due to the equilibrium principle, in which higher temperatures could increase the extraction rate and reach maximum phenolic compounds content recovery and therefore higher antioxidant compounds content. However, elevated temperatures may not be suitable for all kinds of phenolic compounds. Liyana-Pathirana and Shahidi (2005) reported that only samples with higher proportions of thermally stable polyphenols are more appropriate to extract under elevated temperatures. Thus, from our results it may be inferred that most of the phenolic compounds extracted from *J. spicigera* are thermo-stable.

3.6 *Effect of extract pH on antioxidant activity during storage*

Changing the pH of the fresh Muitle extracts from 7.4 to 3.5 and 5.5 affected differently the initial AA, depending on the solvent system and on the assay used to determine AA (Table 3). Because of the lowest TPC yield displayed by E_{100}25, this extract either was not considered in AA assays. With ABTS, lowest initial AA occurred with E_{100}60 regardless of the pH of the extract, whereas at pH 5.5 highest initial AA corresponded to $PG_5$25, and at pH 3.5 to $PG_5$60, respectively. With DPPH, also lowest AA happened with E_{100}60 regardless of the pH of the extract, at pH 5.5 highest initial AA corresponded to G_5, and at pH 3.5 to $G_5$25 and E_{50}60, respectively. The results could be attributed to the different conformations adopted by the different phenolic compounds as result of the different degree of dissociation of OH groups induced by the changes in pH, affecting the number of active ionized groups partaking in the reaction with the free radical molecules of $ABTS^{\cdot+}$ and $DPPH^{\cdot+}$, and causing changes in AA (Akrem *et al*., 2007; Juntachote and Berghofer, 2005).

Table 4 shows the loss in AA exhibited by the Muitle extracts after 20 days of storage. With the ABTS assay, the highest loss was displayed by E_{100}60 at all pH's tested. Non-significant lowest AA loss was exhibited at pH 7.4 by W25 and $G_5$60, at pH 5.5 by W25, $G_5$60, and E_{50}60, and at pH 3.5 by E_{50}25, E_{50}60, $PG_5$25 and $G_5$60. With the DPPH assay the lowest AA loss occurred for the ethanolic extracts (E_{100} and E_{50}), while the highest loss happened for W60, followed by W25, all these results regardless of pH value. The differences in the results obtained using the ABTS and DPPH assays are most likely due to the compatibility of the extracts with free radicals of different chemical nature. Both methods are complementary and allow for a more thorough understanding of the factors affecting the stability and functionality of the Muitle extracts.

Table 3. Initial antioxidant activity in Muitle extracts determined by ABTS and DPPH assays.

Muitle extract code		$ABTS_{pH7.4}$ (mM TEAC)	$ABTS_{pH5.5}$ (mM TEAC)	$ABTS_{pH3.5}$ (mM TEAC)	$DPPH_{pH7.4}$ (mM TEAC)	$DPPH_{pH5.5}$ (mM TEAC)	$DPPH_{pH3.5}$ (mM TEAC)
W	25	6.29±0.12 c	5.80±0.13 c	5.76±0.28 b,c	4.45±0.06 b	5.72±0.05 d,e	4.82±0.13 c,d
	60	6.74±0.10 d	6.34±0.16 e	6.28±0.17 c,d	5.85±0.18 c,d	5.48±0.05 c,d	4.24±0.04 b
E_{100}	25	ND	ND	ND	ND	ND	ND
	60	4.33±0.04 a	3.01±0.15 a	2.77±0.19 a	3.52±0.30 a	2.90±0.07 a	3.53±0.11 a
E_{50}	25	6.42±0.06 c,d	4.83±0.10 b	5.32±0.64 b	5.63±0.15 c	3.23±0.09 b	5.03±0.08 d,e
	60	5.95±0.13 b	6.10±0.11 c,d,e	6.19±0.15 b,c,d	5.52±0.08 c	5.88±0.07 e	5.46±0.10 g
PG_5	25	6.61±0.18 c,d	7.35±0.19 f	5.74±0.16 b,c	5.74±0.29 c	6.35±0.13 f	5.20±0.16 e,f
	60	6.54±0.12 c,d	6.34±0.18 d,e	7.13±0.13 e	5.56±0.22 c	5.43±0.25 c	4.34±0.03 b
G_5	25	6.46±0.15 c,d	5.88±0.12 c,d	6.52±0.13 c,d	4.93±0.10 b	6.62±0.05 g	5.51±0.05 g
	60	6.55±0.11 c,d	6.24±0.16 d,e	6.26±0.49 c,d	6.24±0.22 d	6.71±0.11 g	4.58±0.03 c

Different lowercase letters in the same column represent data significantly different ($P \leq 0.05$); ND = not determined.

Table 4. Decay in antioxidant activity in Muitle extracts at different pH values after 20 days of storage.

Muitle extract code		$ABTS_{pH7.4}$ (%)	$ABTS_{pH5.5}$ (%)	$ABTS_{pH3.5}$ (%)	$DPPH_{pH7.4}$ (%)	$DPPH_{pH5.5}$ (%)	$DPPH_{pH3.5}$ (%)
W	25	42.75±1.90 a	42.88±2.06 a,b	57.22±4.18 b,c	68.31±2.56 e	66.97±1.60 e	69.60±2.89 e
	60	50.44±1.79 c	59.99±2.66 c	60.65±1.55 c,d	93.16±0.35 f	80.24±0.34 f	82.69±0.51 f
E_{100}	25	ND	ND	ND	ND	ND	ND
	60	87.53±1.20 e	87.69±3.22 d	93.48±3.17 e	23.49±8.22 a	16.68±3.14 a	23.75±2.07 a
E_{50}	25	54.51±1.40 d	44.41±1.55 b	51.98±6.03 a,b	33.87±9.61 c,d	21.35±3.68 b	28.02±2.29 b,c
	60	49.73±1.86 c	41.81±3.22 a,b	50.22±1.86 a	21.45±5.10 a	16.49±0.91 a	22.14±1.56 a
PG_5	25	46.42±2.45 b	58.50±2.92 c	51.37±2.03 a	32.29±3.64 b,c,d	22.34±1.85 b	28.34±3.02 c
	60	48.92±1.90 b,c	61.34±1.76 c	64.64±3.42 d	39.31±5.76 d	31.21±2.96 c	33.42±2.12 d
G_5	25	46.88±1.42 b	59.57±1.32 c	65.58±1.03 d	29.05±2.05 a,b,c	36.62±1.89 d	37.02±0.74 d
	60	42.61±2.21 a	40.16±2.83 a	48.26±6.07 a	24.16±7.19 a,b	24.22±0.72 b	24.11±5.17 a,b

Different lowercase letters in the same column represent data significantly different ($P \leq 0.05$); ND = not determined.

Conclusions

The solvent and temperature extraction conditions significantly affected the initial total phenolic content and the antioxidant activity of the Muitle extracts. Adjusting the pH also had a significant effect on the total phenolic contents and antioxidant activity of the extracts with storage time. The extracts obtained using solvents with higher polarity displayed more effective radical-scavenging activity than those obtained using less polar solvents. These results indicate that the selective extraction from botanicals sources, by appropriate solvents and suitable methods, is important for obtaining bioactive compounds with high phenolic contents and antioxidant activities. Thus, this work establishes the importance of employing adequate solvent and temperature extraction conditions for obtaining extracts from *Justicia spicigera* with high initial total phenolic contents and antioxidant activities, and for minimizing their degradation with storage time at specific pH's, and contributes for a better performance of these extracts in practical applications.

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