ANTIOXIDANT ACTIVITY OF PEPPERS (*Capsicum annuum* L.)
EXTRACTS AND CHARACTERIZATION OF THEIR PHENOLIC CONSTITUENTS

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SUMMARY

The aim of this study was to characterize the phenolic constituents and evaluate the antioxidant activity of five pepper (*Capsicum annuum* L.) cultivars harvested in the same season, geographic area and climatic conditions. Phenols, flavonoids and ascorbic acid of Anaheim, Bell, Caribe, Jalapeno and Serrano peppers were quantified, and antioxidant activity of their extracts were evaluated by the method of radical scavenging of DPPH• and ABTS•+. It was found that Serrano pepper had the highest ascorbic acid content, followed by Bell and Caribe, whereas the lowest values were found in Jalapeno and Anaheim. The highest contents of phenolic compounds were in Caribe and Bell peppers. The total flavonoid contents ranged from 25.38 ±3.44 (Anaheim) to 60.36 ±9.94 mg QE/100g fw (Caribe). The Bell and Caribe extracts showed the highest (p<0.05) stabilization of ABTS•+. The highest oxidation inhibition percentage for radical DPPH• was observed in Caribe extract, coinciding with the highest levels of gallic acid, chlorogenic acid, epicatechin, rutin, luteolin, resveratrol (r ≥0.85) and ascorbic acid. In conclusion, among the pepper cultivars studied, Caribe and Bell showed to have the best antioxidant properties and can be suggested as preferable for human consumption.

Introduction

Epidemiological studies have found an inverse correlation between consumption of fruits, vegetables and grains, and the risk of cardiovascular diseases and cancer (Ferrari and Torres, 2003; Ilow *et al*., 2008). This is attributed to the wide variety of nutrients and phytochemicals with beneficial biological effects to the human body. Among these effects is the ability to stabilize reactive oxygen species (ROS) that are commonly generated in normal metabolism as well as under various types of stress.

KEYWORDS / Antioxidant Activity / *Capsicum annuum* / Flavonoids / Pepper / Polyphenolic Compounds /
The ROS provoke damage to biomolecules such as proteins, RNA and DNA (Johnson and Loo, 2000). Hence the importance of the determination of the antioxidants present in different foods, and their possible role in human health.

Up to recent years, the protection against various diseases was attributed only to vitamin C (ascorbic acid), E (tocopherols) and carotenoids contained in vegetable foods. However, clinical and epide miological studies have demonstrated that other phytochemicals present in fruits and vegetables are implicated as antioxidants (Kaur and Kapoor, 2001).

At present, the most studied phytochemicals in plants are the phenolic compounds, because they have various functions in the human body, mainly as antioxidants (Kroon and Williamson, 2005). However, the composition and levels of individual phytochemicals with antioxidant potential present in vegetables do not necessarily reflect the total antioxidant capacity, which depends on the type and concentration of phytochemicals, as well as the synergistic or inhibitory interaction of molecules in the matrix. Therefore, it is important to study the phytochemicals present in vegetables of high consumption in some countries, such as peppers, in order to generate information about their potential health benefits. The fruits of pepper (Capsicum annum L.), are highly consumed in fresh form, processed or as spice in various dishes around the world. It has also been found that peppers are a good source of polyphenolic compounds with antioxidant properties (Howard et al., 2000; Marin et al., 2004).

The objective of this study was to identify and quantify the contents of phenolic acids, flavonoids and ascorbic acid present in the pericarp of five pepper (Capsicum annum L.) cultivars grown in Northwestern Mexico, and to evaluate their antioxidant activity.

Materials and Methods

Chemicals

The 6-hydroxy-2,5,7,8-tetraethyl-2-carboxylic acid (trolox), 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt radical (ABTS+), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), potassium persulfate, Folin-Ciocalteau reagent and standards (gallic acid, caffeic...
acid, catechin, chlorogenic acid, epicatechin, rutin, resveratrol, luteolin, and ascorbic acid) were obtained from Sigma-Aldrich Co. (Mexico). The solvents used were analytical and HPLC grade.

Sample collection

Five commercially important pepper (Capsicum annuum L.) cultivars (SNITT, 2003): Anaheim (green pepper), Bell (bell pepper), Caribe (blond pepper), Jalapeno (typical) and Serrano (typical) were selected for this study and harvested in November 2008. Fruits of commercial size, green color and physiologically mature stage were harvested in Zamora, Sonora, Mexico (29º16'30.16''N, 110º53'13.93''O and 285masl). After harvest, fruits were transported to the Department of Scientific and Technological Research, Universidad de Sonora, Mexico. Peppers (5kg) without visible damage or physiological defects were selected, washed with distilled water, cut crosswise in halves or physiological defects were selected, washed with distilled water, cut crosswise in halves to remove seeds and the pericarp was cut in strips 5×0.5cm. The samples were stored up to one month at -20°C, until analysis.

Acetic acid determination

Ascorbic acid was determined as described by Doner and Hicks (1981). Fruit tissue (10g) was homogenized for 2min with 50ml of an aqueous solution containing 30g·l⁻¹ metaphosphoric acid and 80mL·l⁻¹ acetic acid. The homogenate was filtered and centrifuged for 15min at 10000rpm. The supernatant was filtered through filter paper (0.22μm). The ascorbic acid content was determined by means of a HPLC system (Varian 9012, Palo Alto, CA, USA) equipped with a UV-Vis detector (Varian 9050, Palo Alto, CA, USA), a water bondapack-NH₂ analytical column (3.9×300mm, 10μm), and a 10μl loop injector. The mobile phase was acetonitrile:KH₂PO₄ 0.05M (75:25 v/v) at a flow rate of 1.5ml·min⁻¹. Absorbance was read at 268nm. The ascorbic acid concentration was calculated using an external standard and expressed as mg ascorbic acid per 100g of fresh weight (fw; Robles-Sánchez et al., 2009). All tests were performed in triplicate.

Methanolic extracts

Ten grams of homogeneous samples from 5kg of each cultivar of fresh pepper were placed in conical tubes with 20ml of aqueous methanol (70:30, v/v) and homogenized in an Ultra-Turrax T 25 basic SI (IKA-Works Inc. Wilmington, NC, USA). The homogenate was then sonicated (Sonics 1510 R-DTH, Branson Ultrasonics Corporation, CT, USA) for 30min and centrifuged (16800g) (Centrifuge IEC CL3 IR, Thermo Electron Industries SAS, France) at 4°C for 15min. The supernatant was filtered thru Whatman Nº 2 paper. The methanolic extraction was done in darkness at room temperature (20 ±2°C) and was repeated twice to ensure maximum extraction of all the compounds. The extracts (0.25g·ml⁻¹) were frozen at -20°C for 48h until to analysis (Chitindingu et al., 2006).

Total phenols and flavonoids

The phenolic compounds in the extracts were spectrophotometrically determined at 750nm using the Folin-Ciocalteu 1N reagent and gallic acid as standard. The results are reported as mg of gallic acid equivalents (GAE) per 100g fresh sample (Molina-Quijada et al., 2010). Flavonoid content was determined by colorimetric assay (Molina-Quijada et al., 2010). In brief, 1ml of extract was mixed with 4ml of deionized distilled water and 0.3ml of 5% NaNO₂. After 5min, 0.3ml of 10% AlCl₃ was added, and after another minute, 2ml of 1M NaOH were added. The final volume was brought up to 10ml with deionized water, stirred, and lectures were taken at 515nm (UV-VIS spectrophotometer Cary 100, Varian Australia PTY LTD, Australia). Total flavonoids were expressed on a fresh weight (fw) basis as milligrams of quercetin equivalents per gram.

Phenolic compounds identification

The identification of phenolic compounds was performed according to the procedure described by Ramamurthy et al. (1992). Twenty μl of methanolic extract (0.25g fresh sample/ml of methanol 70%) were analyzed by liquid chromatography (HPLC ProStar 230, Varian, CA, USA), using a Supelcosil™ LC18 column (30×0.4cm×5μm particle size, Supelco, Bellefonte, PA, USA) and an ultraviolet detector (model 9050, Varian, CA, USA). The elution started with 90% solvent A (acetic acid 2%) and 10% solvent B (acetic acid/acetonitrile:water, 2:3:0.68, v/v/v) up to 100% solvent B at 30min, with a flow rate of 1.5ml·min⁻¹. The identification of individual phenolic compounds was performed comparing the retention times of standards and its absorption spectrum.

For quantification, calibration curves were made for each of the identified phenolic compounds. Gallic acid, caffeic acid, catechin, and epicatechin were quantified at 280nm (wavelength at which maximum absorbance is present); resveratrol and chlorogenic acid at 320nm; and quercetin-3-rutinoside, known as rutin (flavonol), and luteolin (flavone) at 360nm.

Evaluation of antioxidant activity

For the measurement of the antioxidant activity of the pepper extracts, two methods were used. The first one evaluates the trolox equivalent antioxidant capacity (TEAC) and is based on the reduction of green/blue coloration produced by reaction of ABTS⁺ with the antioxidant present in the sample. A volume of 0.1ml of each methanolic extract (0.25g·ml⁻¹) was mixed with 3.9ml of the radical solution (3.84mg·ml⁻¹) and the absorbance was read at 754nm (UV-VIS spectrophotometer Cary 100, Varian Australia PTY Ltd., Australia) after 1min of reaction at room temperature, using ethanol as a control. The absorbance differential (Absi-Absof) was converted to inhibition percentage and antioxidant activity was calculated in μmoles equivalent trolox (ET) per g of fresh sample, using a calibration curve of trolox (an analogue of vitamin E water soluble) from 0.00 to 0.99nmol·ml⁻¹ (Kuskoiski et al., 2005).

The second method, the reduction of DPPH in presence of antioxidants, is detected as a change of color (from purple to yellow) in the solution. A volume of 3.9ml of DPPH solution (0.25mg·ml⁻¹ methanol) with 0.1ml of each of the dilutions of methanolic extracts (0.00 to 0.25mg·ml⁻¹) were mixed. The reaction was carried out for 30min and the absorbance was measured at 515nm in the UV-VIS spectrophotometer. The changes in absorbance at the beginning and the end of the reaction were transformed into percentage of inhibition (Materska and Perucka, 2005).

Statistical analysis

All determinations were carried out in triplicate and data were subjected to analysis of variance (ANOVA). Statistical analyses were performed using SigmaStat version 3.5 (Point Richmond, CA, USA). Significant differences among means were determined by the Tukey test; p values <0.05 were considered statistically significant.

Results and Discussion

Ascorbic acid

Ascorbic acid contents of the various peppers ranged
from 121.14 to 251.60mg/100g fw (Table I). These results confirm that the consumption of 100g of fresh peppers provides the recommended daily administration of ascorbic acid (100-200mg; Lee and Kader, 2000). Serrano pepper presented higher ascorbic acid content than Bell and Caribe, whereas the lowest values were found in Jalapeno and Anaheim peppers. Zhuang et al. (2012) reported that the ascorbic acid content of peppers is mainly dependent on the cultivars. Peppers are considered an important source of ascorbic acid (170-280mg/100g); therefore, they have been attributed health benefits (Kumar and Tata, 2009). However, it seems that other compounds with high antioxidant potential such as polyphenols may also contribute in this context.

**Total phenols**

In general, high levels of total phenols were found in all the pepper extracts studied. Caribe pepper presented the highest value and Jalapeno pepper the lowest (Table I). Vinson et al. (1998) and Sun et al. (2007) reported lower levels of total phenols in Bell peppers than those found in the present study. Contrarily, Helmja et al. (2007) reported a higher content of these compounds in pungent pepper (480mg/100g fw). Similarly, Kevser et al. (2007) reported higher levels of total phenols in red, yellow and green peppers (296, 284 and 215mg/100g, respectively), even higher than those found in spinach, broccoli, cucumbers and carrots. It is well known that content of phytochemicals, including phenolic compounds present in vegetables, is affected by the species and type of pepper, agronomic conditions, maturity (Righetto et al., 2005), postharvest handling and pre and postharvest treatments applied to the fruit (Howard et al., 2000). A close correlation between total phenols and antioxidant activity (r= 0.914 for DPPH; and r= 0.837for ABTS⁺) was found.

**Total flavonoids**

The total flavonoids content (Table I) was significantly higher (p<0.05) in the Caribe pepper (60.36 ±9.95mg QE/100g fw), followed by Bell (38.64 ±0.37mg QE/100g fw). No significant differences (p<0.05) were observed among Anaheim, Serrano and Jalapeno peppers. According to the botanical classification scale of Peterson and Dwyer (1998) for flavonoid concentration, peppers were classified as having high flavonoid content. Howard et al. (2000) reported flavonoid contents from 17.17 to 85.49mg QE/100g fw in similar pepper varieties (Capsicum annuum L.). They observed a content of total flavonoids of 31.71mg QE/100mg fw for Bell pepper, similar to those found in this study. Sun et al. (2007) reported total flavonoids from not detectable to 80mg QE/100mg fw in Bell peppers of different colors. Marinaova et al. (2005) found a range of flavonoids between 13.7 (red pepper) and 27.4 (green pepper) mgQE/100g fw. These values contrast with those reported by Materska and Perucka (2005), who observed greater contents of flavonoids in red peppers than in green ones. These variations in flavonoid and phenol contents have been associated to pepper maturity, type of cultivar and growing conditions (Howard et al., 2000). The strongest correlation was between total flavonoids and antioxidant activity (DPPH; r= 0.885). There were also a significant positive but weaker correlation between total flavonoids and antioxidant activity (ABTS⁺, r= 0.629).

**Identification of phenolic compounds**

The main phenolic compounds identified in the five pepper cultivars were gallic acid, caffeic acid and chlorogenic acid, among phenolic acids; catechin, epicatechin, rutin and luteolin among flavonoids; and the stilbene, resveratrol (Table I). Gallic acid was the main phenolic compound found in all the pepper cultivars studied, with higher levels (p<0.05) than the other compounds. Caribe and Serrano peppers showed the highest levels (p<0.05) of gallic acid, followed by Bell, Anaheim and Jalapeno peppers.

Marin et al. (2004) reported similar levels of caffeic acid (1.37mg/100g fw) than those found in this study (1.10 ±0.50mg/100g fw) for Bell pepper. Estrada et al. (2000) found that phenolic acids content decreases with fruit ripening. It is possible that during this process, these compounds may participate in various metabolic reactions to form new compounds or retard oxidation reactions, thereby preventing the deterioration of the fruit. It is possible that the low presence of these compounds in advanced stages of fruit ripening makes it more vulnerable to oxidation reactions and could be one of the causes of high susceptibility to microbial attack and deterioration (González-Aguilar et al., 2010).

The major components in the flavonoid fraction were catechin, epicatechin and luteolin, which presented the highest levels (p<0.05) in the extracts from Bell and Caribe peppers (Table I). Materska and Perucka (2005) found that luteolin levels ranged from 2.11 to 4.99mg/100g fw in four types of pepper with different maturity stages. Marin et al. (2004) reported similar levels of luteolin (4.22mg/100g fw) than those observed in this study for green Bell pepper. However, Kevser et al. (2007) found quercetin (56mg/100g fw) and kaempferol (26mg/100g fw) as the major flavonoids in green peppers stored for 34 days at 4°C. Probably these differences could be attributed to the type of pepper and conditions to which the fruits were exposed.

The Bell and Caribe extracts presented the highest resveratrol content (p<0.05), while in Jalapeno extract this product was not detected. These values of resveratrol may be responsible of high antioxidant capacity of Bell and Caribe peppers. Fabris et al. (2008) found an antioxidant activity of resveratrol similar to butylhydroxytoluene and α-tocopherol. This com-

### TABLE I

<table>
<thead>
<tr>
<th>Peppers</th>
<th>Anaheim</th>
<th>Bell</th>
<th>Caribe</th>
<th>Jalapeno</th>
<th>Serrano</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolics (GAE)</td>
<td>97.99</td>
<td>103.26</td>
<td>154.30</td>
<td>59.34</td>
<td>94.85</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>69.30</td>
<td>81.80</td>
<td>101.30</td>
<td>49.10</td>
<td>94.60</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>2.20</td>
<td>1.10</td>
<td>1.00</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.97</td>
<td>0.49</td>
<td>1.79</td>
<td>0.20</td>
<td>0.70</td>
</tr>
<tr>
<td>Flavonoids (QE)</td>
<td>25.38</td>
<td>38.64</td>
<td>60.36</td>
<td>28.77</td>
<td>32.73</td>
</tr>
<tr>
<td>Catechin</td>
<td>3.68</td>
<td>1.85</td>
<td>3.47</td>
<td>0.11</td>
<td>1.03</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>7.35</td>
<td>3.70</td>
<td>7.93</td>
<td>0.10</td>
<td>1.17</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.38</td>
<td>1.90</td>
<td>7.90</td>
<td>0.20</td>
<td>1.98</td>
</tr>
<tr>
<td>Luteolin</td>
<td>0.35</td>
<td>4.75</td>
<td>5.09</td>
<td>0.20</td>
<td>0.57</td>
</tr>
<tr>
<td>Stilbene</td>
<td>0.35</td>
<td>4.75</td>
<td>5.09</td>
<td>0.20</td>
<td>0.57</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.38</td>
<td>1.22</td>
<td>1.45</td>
<td>Nd</td>
<td>0.80</td>
</tr>
<tr>
<td>Ascorbic acid (AAE)</td>
<td>121.14</td>
<td>220.42</td>
<td>210.81</td>
<td>156.36</td>
<td>251.60</td>
</tr>
</tbody>
</table>

Values in each column with different letters (a-e) show a significant difference (p<0.05). GAE: gallic acid equivalents; QE: quercetin equivalents; AAE: ascorbic acid equivalents. Nd: not detected.
B Bell and Caribe extracts showed a higher antioxidant activity (34.44 ±0.43 and 33.60 ±1.35 μM ET/g fw, respectively) than that observed in Anaheim, Serrano and Jalapeno extracts (p<0.05; Figure 1). Sun and Tanumihardjo (2007) reported a lower antioxidant capacity (about 8.40 μM ET/g fw) for Bell extract than that found in this study. Navarro et al. (2006) conducted a study on antioxidant activity under salt stress at different stages of Bell pepper ripening; they concluded that a moderate salinity causes a significant increase in the antioxidant activity (ABTS⁺ radical scavenging). The caffeic acid, chlorogenic acid, catechin, epicatechin, rutin and resveratrol contents correlated positively with the antioxidant activity by ABTS⁺ and DPPH⁺ tests. The highest positive correlations among phenolic contents and antioxidant activities were found for catechin (r = 0.96), epicatechin (r = 0.94), and rutin (r = 0.89) with the DPPH⁺ radical; and resveratrol (r = 0.88) with the ABTS⁺ radical. Commonly, a good correlation between the values of specific groups of antioxidants and the antioxidant activity could determine the real contribution that is in function of the type and concentration of the individual antioxidant compounds presented in the sample (Jacobo-Velázquez and Cisneros-Zevallos, 2009).

The antioxidative activity of phenols is related to the number and position of the hydroxyl groups present in their chemical structure and its relative concentration in the matrix (Rice-Evans et al., 1996). The concept of specific antioxidant capacity (ratio of antioxidant capacity/total soluble phenols) has been considered as a new parameter to understand the activity of a mixture of phenols leading to the neutralization of free radicals; due to the synergistic, additive or antagonistic behavior of phenolic compounds to inhibit reactive oxygen species (Jacobo-Velázquez and Cisneros-Zevallos, 2009). Further studies should be conducted on this concept.

**Conclusions**

According to the results obtained, the pepper cultivars studied have levels of phenolic constituents that contribute to a high antioxidant activity and may be considered as a good source of natural antioxidants. Caribe and Bell peppers had the highest antioxidant capacity, which correlated with the highest levels of total phenols and flavonoids. The highest positive correlations of phenolic contents and antioxidant activities were for catechin, epicatechin, rutin and resveratrol. However, other compounds (e.g. ascorbic acid) present in the peppers could contribute to the antioxidant activity and therefore should be considered in order to understand how the individual contribution of each group of phytochemicals to the total antioxidant activity. The information presented in this study can help promote the consumption of peppers in fresh form. To complement this work, studies of the effect of different technological processes on the phytochemical content of peppers extracts are in process.

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