Centro de Investigación en Alimentación y Desarrollo, A.C

"EVALUACIÓN DE LA CAPACIDAD ANTIOXIDANTE Y ANTIPROLIFERATIVA DE EXTRACTOS DE PAPAYA (*Carica Papaya* L.) var. MARADOL EN DIFERENTES ESTADOS DE MADUREZ"

POR

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CONTENIDO

Página

RESUMEN …….. 1

HIPÓTESIS……. 10

OBJETIVO GENERAL …….. 10

OBJETIVOS ESPECÍFICOS ……... 10

CAPÍTULO I ……..…. 12

Effect of Maturity Stage of Papaya Maradol on Physiological and Biochemical Parameters (2010). ***American Journal of Agricultural and Biological Sciences***. 5(2):199-208.

CAPÍTULO II ……..…. 23

Identification and Quantification of Phenols, Carotenoids and Vitamin C from Papaya (*Carica Papaya* L.) determined by HPLC-DAD-MS/MS-ESI (2011). ***Food Research International***. 44: 1284-1291.

CAPÍTULO III ……..…. 32

Contribution of Major Hydrophilic and Lipophilic Antioxidants from Papaya (*Carica papaya* L.) var. "Maradol" to Total Antioxidant Capacity. (Preparado: ***European Food Research and Technology***).

CAPÍTULO IV ……..… 60

Carotenoids: Antiproliferative Activity on Cell Lines. (Preparado: ***International Journal of Food Sciences and Nutrition***).

CAPÍTULO V ……..… 86

Inhibition of Cell Proliferation of Breast Cancer Cells MCF7 and MDA-MB-231 by Lipophilic Extracts of Papaya (*Carica Papaya* L.) var. "Maradol". (Preparado: ***International Journal of Food Sciences and Nutrition***).

CONCLUSIONES GENERALES ……..… 106

Producción Académica …….. ……. 109

SINOPSIS

Los nutrientes son componentes de los alimentos utilizados por el organismo y que hacen posible la vida, desempeñando funciones diferentes según su naturaleza. Los antioxidantes previenen la degeneración y muerte de las células que provocan los radicales libres retardando así los procesos de envejecimiento. El consumo de frutas y hortalizas en la dieta tiene un efecto benéfico para la salud y esto obliga al ser humano a buscar alimentos con propiedades antioxidantes para neutralizar el efecto no deseado de los radicales libres.

Existen numerosos estudios epidemiológicos que relacionan la ingesta de una dieta rica en frutas y hortalizas, con un menor riesgo de padecer enfermedades degenerativas, como son las enfermedades cardiovasculares y ciertos tipos de cáncer. Es por ello que el consumidor valora aquellos alimentos vegetales que además de proporcionarles los nutrientes indispensables para la vida, posean sustancias con un posible efecto protector, conocidas como compuestos fitoquímicos o bioactivos (carotenoides, compuestos fenólicos, vitaminas C, A, E, etc.). Estas sustancias químicas son constituyentes de alimentos de origen vegetal que proporcionan a los alimentos propiedades fisiológicas que van más allá de las nutricionales[1]. La mayoría de estas sustancias fitoquímicas se caracterizan por su capacidad antioxidante, que estando presentes a bajas concentraciones con respecto a un sustrato oxidable, retrasan o inhiben la oxidación de dicho sustrato. Sin embargo, el consumo de frutas y hortalizas que contienen estos compuestos es todavía bajo con respecto a las recomendaciones hechas por profesionales

de la salud[2]. De ahí la importancia nutricional que tiene un consumo adecuado de frutas y hortalizas, por su gran variedad y contenido de compuestos antioxidantes.

Dentro de los compuestos naturales de origen vegetal con actividad antioxidante se encuentran los compuestos fenólicos, quienes poseen el mayor espectro no solo en cuanto a su actividad antioxidante, sino también a su efecto bioactivo específico sobre determinadas patologías de carácter degenerativo en seres humanos[3]. Los compuestos fenólicos son un grupo de metabolitos aromáticos del metabolismo secundario de las plantas que comprenden alrededor de 8000 compuestos diferentes, pero todos poseen una estructura común con un anillo aromático, conteniendo al menos un grupo hidroxilo. El potencial antioxidante de los compuestos fenólicos se debe principalmente a sus propiedades redox, que les permite actuar como agentes reductores, donadores de iones hidrógeno, bloqueadores del oxígeno singlete y captadores de radicales hidroxilo[4].

Los carotenoides son compuestos lipofílicos de cuarenta átomos de carbono que pueden ser lineales o contener anillos en uno o ambos extremos y se clasifican en carotenos y xantofilas[5]. La estructura química de los carotenos comprende exclusivamente átomos de carbono e hidrógeno, mientras que en las xantofilas es posible distinguir diversos grupos oxigenados como hidroxilo, cetónicos, epóxidos, etc. En frutos tropicales como la papaya, el contenido de carotenoides se incrementa continuamente durante el proceso de madurez y son los causantes del típico color amarillo-naranja del fruto maduro[6]. El tipo y concentración de los carotenoides varía con el tipo y estado de madurez del fruto. Para el caso de papaya, los principales

carotenoides identificados en la pulpa son el licopeno, la β-criptoxantina y el β-caroteno. El rasgo estructural distintivo de los carotenoides es un sistema extenso de dobles enlaces conjugados, el cual consiste en alternar enlaces carbono-carbono (simples y dobles) y, que en general, se denomina cadena poliénica. Esta parte de la molécula conocida como el cromóforo, es responsable de la capacidad de los carotenoides de absorber luz en la región visible y en consecuencia su gran capacidad de coloración y acción antioxidante[6]. Por otro lado, se ha demostrado que los carotenoides exhiben actividades biológicas con impacto en las rutas de señalización celular, influenciando la expresión de genes o inhibiendo ciertas enzimas involucradas en el desarrollo de ciertos tipos de cáncer[7].

El estrés oxidativo inducido por los radicales libres puede causar daño al ADN, que al auto-repararse, da lugar a cambios como la mutación de una base, rompiendo de una a dos cadenas, produciendo entrecruzamiento de ADN y re-arreglo y rompimiento cromosomal. Sin embargo, el daño oxidativo inductor de cáncer, puede prevenirse o limitarse por la ingesta de antioxidantes provenientes de una dieta rica en frutas, vegetales y otras plantas alimenticias[8]. Existen varios estudios donde se ha observado el efecto de diferentes extractos vegetales en la capacidad antioxidante y antiproliferativa de diferentes tipos de células cancerosas. Los estudios *in vitro e in vivo* llevados a cabo hasta el momento, no han profundizado en el posible efecto benéfico que podrían tener los extractos de frutos de origen tropical como la papaya “Maradol”, por lo que sería interesante evaluar el efecto en líneas celulares de importancia para la salud humana.

La papaya es uno de los frutos tropicales de mayor consumo en nuestro país y México ocupa el primer lugar a nivel internacional en la exportación de este fruto[9]. Sin embargo, la información que existe sobre los cambios que ocurren durante la maduración en los niveles de compuestos fenólicos individuales, así como de carotenoides de papaya, es muy limitada. Por lo que el objetivo del presente estudio fue determinar los niveles y los cambios bioquímicos en el contenido de carotenoides y fenoles en diferentes estados de madurez de este fruto, así como la capacidad que poseen los extractos lipofílicos para inhibir la proliferación de líneas celulares cancerígenas.

En el **Capítulo I** se describe el efecto del estado de madurez de la papaya var. "Maradol" en los cambios fisiológicos, así como el contenido de diferentes compuestos bioactivos y su relación con la capacidad antioxidante. Para poder llevar a cabo las diferentes determinaciones, los frutos de papaya se seleccionaron de acuerdo a su nivel de madurez externa visual, dividiéndose en cuatro estados de madurez (EM): EM1 papaya amarilla 0-25%; EM2 >25 y 50%; EM3 >50 y 75% y EM4 >75 y 100%. La producción de CO_2 coincidió con un incremento en la producción de etileno durante la maduración del fruto, mientras que la firmeza tendió a disminuir, correlacionándose positivamente con la actividad de la enzima poligalacturonasa. En relación a los cambios en los parámetros de color L*, a*, b*, °Hue y Croma, se presentaron diferencias significativas ($p \leq 0.05$) entre los diferentes estados de madurez. Debido a que las frutas y vegetales contienen un gran número de compuestos esenciales que promueven la salud humana, se llevó a cabo la evaluación de la capacidad antioxidante de la papaya en los cuatro EM, utilizando las técnicas de DPPH, TEAC, ORAC y Folin-Ciocalteau. El

contenido de fenoles totales presentó una disminución en los niveles más altos de maduración; mientras que la mayor capacidad antioxidante se presentó en el EM1 con la técnica de DPPH y TEAC, atribuyéndose esto a los compuestos fenólicos y vitamina C. El mayor valor de capacidad antioxidante total (CAT) obtenido con la técnica de ORAC fue en el EM4, lo cual podría estar relacionado con los mayores niveles de carotenoides contenidos en estos frutos.

En el **Capítulo II** se muestran los resultados del análisis cualitativo y cuantitativo llevados a cabo por HPLC-MS de los principales compuestos fenólicos y carotenoides, así como de la vitamina C en pulpa y cáscara de papaya en los cuatro EM (EM1, EM2, EM3, EM4). Se analizaron extractos metanólicos hidrolizados en cáscara, observando que el contenido de fenoles tendió a disminuir con la madurez del fruto, logrando la identificación y cuantificación de: ácido ferúlico (277.49 a 186.63 mg/100gPS), *p*-coumárico (229.59 a 135.64 mg/100gPS) y cafeico (175.51 a 112.89 mg/100gPS); en pulpa sólo se detectaron trazas. Por otra parte, los principales carotenoides identificados en extractos lipofílicos saponificados de pulpa, aumentaron con la madurez, encontrándose niveles de licopeno de 0.36 a 3.40 mg/100gPS, β-criptoxantina (0.28 a 1.06 mg/100gPS) y β-caroteno (0.23 a 0.50 mg/100gPS). La vitamina C presentó un comportamiento similar con niveles que variaron de un 25.07 en EM1 a 58.59 mg/100gPS en el EM4. De acuerdo a estos resultados se infiere que el EM influye de manera significativa en el contenido de los compuestos bioactivos presentes en la papaya.

El **Capítulo III** presenta la contribución individual de los principales compuestos fenólicos en cáscara y carotenoides en pulpa a la CAT, en los diferentes EM de la papaya. Estas determinaciones se llevaron a cabo mediante las técnicas de DPPH y TEAC, utilizándose los estándares de los compuestos mayoritarios identificados: ácido ferúlico, cafeico y *p*-coumárico. Se encontró que el ácido cafeico fue el que contribuyó en mayor proporción a la CAT (14.98%), seguido del ácido ferúlico (6.22%) y *p*-coumárico (0.86%). Al combinar estos compuestos fenólicos para ver si existía un efecto sinérgico se observó que la combinación de los ácidos cafeico y ferúlico fueron los que presentaron la mayor sinergia, observándose la mayor CAT. Sin embargo, al combinar los tres ácidos fenólicos no se encontró un aumento significativo en la CAT ($p \leq 0.05$). Al evaluar la contribución individual de los carotenoides, se encontró que licopeno fue el que contribuyó en mayor proporción a la CAT (43.22%), seguido de la β-Criptoxantina (28.04%) y el β-Caroteno (11.60%). No se observó un efecto sinérgico al combinar licopeno con β-Caroteno. Por lo que es posible que la estructura química de estos compuestos, así como la capacidad para reaccionar con el radical, esté influyendo en los valores finales de la CAT.

El **Capítulo IV** presenta una revisión acerca de los resultados obtenidos en los diferentes estudios realizados *in vitro* e *in vivo* de la capacidad antiproliferativa que poseen los carotenoides. Estos compuestos exhiben actividades biológicas con impacto en la expresión de algunos genes, en la inhibición de enzimas específicas involucradas en el desarrollo de algunos tipos de cáncer y en las rutas de señalización celular[10]. Además, que pueden tener un efecto inhibitorio en la proliferación celular, previniendo

la detención de la fase G0/G1 del ciclo celular y disminuir la expresión de p53, p21 y ciclina D[11]. Sin embargo, también existen evidencias clínicas de que un consumo en concentraciones elevadas de β-caroteno, podría potenciar la proliferación celular, dando como resultado un aumento en el riesgo de tumores malignos[12].

Como última etapa de esta investigación, en el **Capítulo V** se presenta el efecto antiproliferativo de los extractos lipofílicos de carotenoides de pulpa de papaya en células de cáncer de pecho MCF-7 y MDA-MB-231, así como de células epiteliales no tumorales de mama. La determinación se llevó a cabo con la técnica del MTT (Bromuro de 3 (4,5 dimetil-2-tiazoil)-2,5-difeniltetrazólico), en los diferentes extractos lipofílicos (EM1, EM2, EM3, EM4) en tres tiempos diferentes (24, 48 y 72 h). Se observó que los extractos lipofílicos no inhibieron la proliferación celular de las células MCF-12F y MDA-MB-231. Sin embargo, las células MCF-7 mostraron una reducción significativa ($p \leq 0.05$) en la proliferación celular a las 72 h con el extracto EM4.

De acuerdo a los resultados obtenidos en el presente estudio, se puede concluir que los extractos de papaya var. "Maradol", posee diversos compuestos bioactivos, siendo los carotenoides los que aportan la mayor capacidad antioxidante, y que al parecer está relacionada con la disminución de la proliferación de células de cáncer de pecho MCF-7. Por lo que el consumo moderado de este fruto podría reducir los riesgos de presentar algunas enfermedades degenerativas como el cáncer. Sin embargo, a pesar de que existen evidencias de los beneficios potenciales de los compuestos fitoquímicos, se requiere de estudios más amplios para corroborar que realmente los compuestos

bioactivos presentes en papaya “Maradol”, tienen otros beneficios a la salud y así poder recomendar con mayor certeza, sobre las ventajas de su incremento en el consumo en la dieta saludables.

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HIPÓTESIS

Los carotenoides presentes en papaya (*Carica papaya* L) cv. "Maradol", en diferente estado de madurez, son los compuestos bioactivos que contribuyen en mayor proporción a la capacidad antioxidante total y antiproliferativa.

OBJETIVO GENERAL

Evaluar el efecto del estado de madurez de la papaya (*Carica papaya* L) cv. "Maradol" en el contenido de los principales carotenoides y su relación con la capacidad antioxidante y antiproliferativa.

OBJETIVOS ESPECÍFICOS

- ✓ Determinar los cambios fisiológicos que ocurren durante la maduración de la papaya y su relación con el contenido de compuestos bioactivos (vitamina C, carotenoides y fenoles).

- ✓ Evaluar la capacidad antioxidante de la papaya en cuatro estados de madurez, utilizando las técnicas de DPPH, TEAC, ORAC y Folin-Ciocalteau.

- ✓ Cuantificar la contribución individual de los principales fenoles y carotenoides presentes en los diferentes estados de madurez de la papaya, a la capacidad antioxidante total.

- ✓ Evaluar la capacidad antiproliferativa *in vitro* de los extractos de los principales carotenoides presentes en los diferentes estados de madurez de papaya.

CAPÍTULO I

Effect of Maturity Stage of Papaya Maradol on Physiological and Biochemical Parameters

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Effect of Maturity Stage of Papaya Maradol on Physiological and Biochemical Parameters

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Abstract: Problem statement: Nowadays, the worldwide increase in diseases has motivated consumers to increase the intake of fruits and vegetables, in response to various research reports indicating that fruits and vegetables can help prevent certain types of illnesses, due to their potentially high antioxidant properties. We evaluated the effect of the stage of ripeness of papaya fruit (*Carica papaya* L.) on the contents of bioactive components and their relation with antioxidant capacity. **Approach:** Whole papaya fruit were selected based on their visual ripeness, classifying them in four stages of ripeness (R1, R2, R3 and R4). Physiological and physical-chemical analysis performed included respiration, production of ethylene, firmness, pH, titratable acidity and total soluble solids, color (L*, a*, b*, °Hue, C); Polygalacturonase (PG) and Pectin Methyl Esterase (PME) activity, total phenolic content and antioxidant capacity (measured using DPPH, TEAC and ORAC assays). **Results:** The antioxidant capacity decreased approximately 27% in the RS4 when using DPPH and TEAC and increased when using ORAC (60.9%). PG activity increased from 8.14 (in RS1)-22.48 U gFW^{-1} (in RS4) as the stage of ripeness of papaya fruit increased. PME was affected in a similar manner with an activity of 0.5562 U gFW^{-1}, at the end of the ripening storage. A high correlation between PG activity and softening of ripen papayas was observed. **Conclusion/Recommendations:** It was observed that papaya fruit experienced changes in firmness, which is correlated with activity from two of the main enzymes: PG and PME and with the increase of respiration and production of ethylene. The various stages of ripeness showed very good antioxidant capacity, being higher in RS1, which is correlated with the higher content of phenolic contents found in this ripening stage.

Key words: *Carica papaya*, postharvest, antioxidants, phenols, antioxidant capacity

INTRODUCTION

In the last years, several experimental, clinical and epidemiologist studies have demonstrated that fruits and vegetables contain bioactive compounds with antioxidant and antimicrobial capacity, from different chemical classes such as phenolic compounds, carotenoids, vitamins Gonzalez-Aguilar *et al*. (2008). These were shown to help prevent cardiovascular diseases (Hu, 2003), atherosclerosis, decrease the risk of some types of cancers, among other health benefits (Yahia, 2009).

Papaya is one of the tropical fruits with important antioxidant properties and is also in great demand in international markets. In 2008, Mexico produced about 800,000 tons, of which, the Mexican states of Veracruz and Chiapas contributed with approximately 50% (SAGARPA, 2008). Antioxidant capacity of fruits and vegetables could be affected by a variety of factors, such as: cultivar, agronomic conditions, post-harvest

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manipulation and stage of ripeness (Kevers *et al*., 2007).

Information available on changes of individual phenols and carotenoids in papaya during ripening is limited; therefore methods for the determination of the Antioxidant Capacity (AOC) and for the evaluation of the evolution of papaya during ripening are needed. One of the most commonly used AOC techniques are DPPH (2, 2-diphenyl-1-picrylhydrazyl), TEAC (2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and Oxygen Radical Absorbing Capacity (ORAC) (Corral-Aguayo *et al*., 2008). The first two techniques develop discoloring reactions, which are proportional to the antioxidant capacity in the sample to reduce the radical and are measured using spectrophotometry. With ORAC, a fluorescent protein called fluorescein is used as an oxidable substrate and 2, 2'-azobis (2-amidinopropane) (AAPH) is used as a generator of peroxil radicals and AOC is quantified using a fluorometer. The AOC obtained by these methods are in function of the type and mixture of antioxidants. The objective of this work was to evaluate the effects of maturity stage on physiological and biochemical changes of "Maradol" papaya fruit associated with overall quality.

MATERIALS AND METHODS

Plant materials: Fresh papaya fruit (1-1.5 Kg) (*Carica papaya* L. cv. Maradol) was obtained from a commercial fruit distributor in Hermosillo, Sonora, Mexico and transported to the Fresh-cut Laboratory of the Centro de Investigación en Alimentación y Desarrollo, AC (CIAD). Fruit were selected according to their size, color and external ripeness. Afterwards fruit were sanitized with chlorinated water (200 ppm) for 3 min and were left to dry at room temperature for about 1 h. Fruit were selected subjectively according to surface color and divided in 4 groups of 15 fruit each, where four Ripeness Stages (RS) were established: RS1 represents papaya that is yellow 0-25%; RS2>25 and 50%; RS3>50 and 75% and RS4>75 and 100%.

Physiological and chemical analysis: Respiration and ethylene production rates were determined using three pieces of papaya fruit which were selected based on their RS. The 3 pieces of papaya were placed in sealed plastic containers for 2 h. Then, using a hypodermic needle, 1 mL from the headspace was extracted and then injected into a Varian Star 3400 CX gas chromatograph, equipped with the following: A Haysep N column of 200 mm in length and internal diameter of 3 mm; 80/100 μm size; a series of two detectors, one with Thermal Conductivity (TCD) for the quantification of CO_2 and the other Flame Ionization (FID) for the detection of ethylene and N_2 was utilized as a carrier gas. Temperature conditions were 50°C for the column, 70°C for the injector, 170°C for the TCD detector and 205°C for the FID detector. Concentrations of the standards were 5% O_2, 5% CO_2 and 1 ppm C_2H_4. To determine the concentration of each gas, the area under the curve was integrated and was compared with the areas of the known standards.

Papaya tissue firmness was measured by puncture method, using a Chatillon Penetrometer, Model DFM50 with an 8mm diameter flat-head stainless-steel cylindrical probe. Tissue's opposition force against the penetration was registered on 3 points in the equatorial region of the whole piece of fruit and results were reported in Newtons (N).

The pH, Titratable Acidity (TA) and Total Soluble Solids (TSS) determinations were done following the AOAC (1998) method, where 10 g of fruit were homogenized in 50 ml of distilled water; the mixture was filtered using organza fabric and 50 mL of the filtered mixture were taken to quantify pH and TA using a Mettler (Mod DL21) automatic Tritator. TA was expressed as a percentage of malic acid. TSS were measured directly from the filtered residue, using an Abbe digital refractometer and expressed in °Brix.

Skin color was longitudinally determined on four points of each flat side of the fruit, using a Minolta CR-300 colorimeter. The L* value represents the luminosity of the fruit, where O = Black and 100 = White. The a* value ranges from the negative (green) to the positive (red) scale. The b* value could range from negative (blue) to positive (yellow). To know the real color changes of the fruit, a* and b* values were used to calculate the Hue angle (°Hue) and the Chroma (intensity), with the following equation:

$$°\text{Hue} = \text{arctg } b^*/a^*$$
$$C = [(a^*)^2 + (b^*)^2\ ]^{1/2}$$

Where:
°Hue = 0 represents a purple red
90° = Yellow
180° = Green-blue
270° = Blue

Enzyme assays: PG activity was determined following the method described by Gross (1982), with some modifications. The samples (10 g) were homogenized in an Ultra Turrax®T25 with 20 mL of 1% sodium bisulfite buffer and 6.0 pH. Next, they were filtered and a second wash of the residue was performed with

Am. J. Agri. & Biol. Sci., 5 (2): 199-208, 2010

20 mL of 1% sodium bisulfite, followed by a third wash with 15 mL of 1M NaCl. The extract's pH was adjusted to 6.0 and then the extract was stirred continuously for 3 hours in a Thermolyne Speci-Mix agitator, at 4°C. Afterward, it was filtered and was centrifuged at 9400 g at 4°C for 15 min. Enzyme solution (250 μL) was mixed well with substrate solution (2 mg polygalacturonic acid dissolved in 750 μL of sodium acetate buffer 37.5 μM, pH 4.4) and was incubated in water bath at 30°C for 2 h. Then the extract was centrifuged at 9400 g at 4°C for 15 min and 200 μL of the supernatant was taken and mixed with 1 mL of 0.1 M borate buffer at 9.0 pH with 200 μL cyanoacetamide (1%). Then the mixture was placed in a water bath at 100°C for 10 min and was left to cool down at room temperature. Absorbance was read using an UV-VIS VARIAN CARY 50 BIO spectrophotometer at 276 nm. Various levels of galacturonic acid solution were used to construct the standard curve (0-100 nmoles) for the PG activity assay. PG activity was expressed as Unit mg FW^{-1} and one activity unit was defined as the amount of enzyme that releases 1 nmol of reducing groups per 1 h. The assay was conducted three times for each RS.

To measure PME activity, a fruit sample (10 g) was homogenized with 25 mL of Tris-Cl 0.1 M buffer at pH 8.0, containing 0.3 M NaCl in an Ultra Turrax®T25 and placed in a Thermolyne Speci-Mix agitator at 4°C for 30 min, followed by centrifugation at 9400 g for 25 min at 4°C. The enzymatic extract was stored at -35°C until analysis and PME was determined following the method of Rouse and Atkins (1955), with some modifications. This method consists of the evaluation of the activity of the enzyme through titration, using as a substrate 25 mL of 1% pectin in 0.1N NaCl at 7.5pH, which was adjusted with 0.1N NaOH. The pectin was placed at water bath at 30°C for 10 min and 2 mL of the extract was added. Decrement of pH caused by the carboxylic groups, generated by the PME during the desertification of the pectin solution were kept constant at a 7.5 pH by titrating the solution with 0.049N NaOH for 10 min at room temperature (24°C). Titration was performed with an automatic Mettler DL21 titrator. Results were expressed as a unit of PME activity, which is defined as the amount that the enzyme required to hydrolyze 1 μmol of carboxyl groups, produced in 1 mL of pectin substrate per minute.

Biochemical evaluations: Papaya flesh sample (10 g) was homogenized in 20 mL of 80% methanol, using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC) at room temperature. The homogenate flesh was sonicated for 30 min in a Bransonic 2210 sonicator (Bransonic Ultrasonic Co., Danbury, CT) and later was centrifuged at 9400 g for 15 minutes at 4°C. The supernatant was collected and the precipitate was extracted again with 10 mL of 80% methanol, under the conditions previously described. The two supernatants were mixed, filtered using Whatman filter paper No.1 and evaporated in a rotary evaporator at 30°C. The concentrate was diluted with 6 mL of 80% methanol and stored at -35°C to be used in the determination of total phenols, DPPH, TEAC and ORAC. The extraction process was performed in triplicate per each RS.

Total phenols were determined according to Singleton and Rossi (1965), with some modifications. Sample of 50 μL were taken from a 2:8 dilution with 80% methanol) and 3 mL of HPLC-grade water and 250 μL of Folin-Ciocalteu 1N (1:1) reactive were added. After 5 min 750 μL of 20% Na_2CO_3 was added, followed by 950 μL of HPLC-grade water; shaken in a vortex and kept in the dark for 30 minutes. Absorbance was read using an UV-VIS VARIAN CARY 50 BIO spectrophotometer, at a wavelength of 765 nm. Results were expressed in mg of Gallic Acid Equivalents (GAE)/100 g of Fresh Weight (FW). Analyses were performed in triplicate per each RS.

DPPH was determined according to the Brand-Williams *et al.* (1995) technique, with some modifications. The stock solution was prepared by mixing 2.5 mg of DPPH radical with 100 mL of pure methanol. The solution was adjusted at an absorbance of 0.7±0.02 at 515 nm. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic) was used as a standard and 80% methanol was used as a blank, 3.9 mL of DPPH radical were placed in a test tube and 100 μL of the extract (2:8 dilution) were added. The mixture was shaken in a vortex and kept 30 min in the dark. Absorbance was then read in an UV-VIS VARIAN CARY 50 BIO spectrophotometer, at a wavelength of 515 nm. Results were expressed in EC_{50} (concentration of antioxidant required to reduce the absorbance of the radical by 50%) in gFW mL^{-1}. Analyses were performed in triplicate per each RS.

TEAC value was determined according to Miller *et al*. (1996) and Re *et al*. (1998). $ABTS^{\bullet+}$ cation was generated through the interaction of 19.2 mg of ABTS (2'2-azino-bis(3-ethylbenzotriazoline-6-sulfonic acid)), dissolved in 5 mL of HPLC-grade water and 88 μL of potassium persulfate ($K_2S_2O_8$) (0.0378 g mL^{-1}). It was incubated in the dark at room temperature for 16 h; then 1 mL of ABTS activated radical was taken and 88 mL of ethanol was added. The radical was adjusted at an absorbance of 0.7±0.02 at 734 nm. The reaction was initiated adding 2970 μL of $ABTS^{\bullet+}$ and

Am. J. Agri. & Biol. Sci., 5 (2): 199-208, 2010

30 μL of the extract or Trolox standard solution in methanol and. absorbance was monitored at 734 nm at 1 and 6 min. The percentage of inhibition was calculated and results were expressed as μmol of ET/100 gFW.

ORAC value was determined according to Robles-Sanchez *et al*. (2009). AAPH was used as peroxyl radical generator, fluorescein as fluorescent probe and Trolox as standard. The reaction mixture contained 100 μL of extracts, 1.65 mL of 75 mM phosphate buffer (pH 7), 150 μL of 0.8 M AAPH, 100 μL of 0.106 μM fluoresce in and phosphate buffer was used as a blank. Samples, phosphate buffer and fluoresce in were pre-incubated at 37°C for 15 min. AAPH was added to start the reaction and every 5 min the fluorescence was measured and recorded until the fluorescence of the last reading declined to less than 5%, respect to initial. The excitation and emission wavelength was set at 484 and 515 nm, respectively and each extract measurement was repeated 3 times. The values were calculated by using a regression equation between the Trolox concentration and the net area under the fluoresce in decay curve and were expressed as Trolox equivalents (μmol TE) per 100 gFW. The experiment was repeated at least 3 times during the 2009 season.

Statistical analysis: All determinations were conducted at least three times. Results were analyzed by multiple comparisons through A Variance Of Analysis (ANOVA) and the statistical significance through the Duncan's test. Differences in $p \leq 0.05$ were considered to be significant. The program Number Cruncher Statistical System version 6.0 software (NCSS, LLC) was used.

RESULTS

Respiration rate (CO_2 production) and ethylene production: As expected, the lowest respiration rate was observed in green fruit (RS1) followed by RS2, 3 and 4 (Table 1). These fruit had a respiration rate two-fold higher (15.4 mL CO_2/kg.h) than fruit from RS1 (7.8 mL CO_2/kg.h). The respiration rate coincided with the ethylene production which increased with the RS of fruit. The highest production of ethylene was observed in RS3 fruit (0.91 μL C_2H_4/kg.h), followed by RS4 (0.75 μLC_2H_4/kg.h) (Table 1).

Firmness: Firmness of papaya flesh tended to diminish with maturity stage and initial values of fruit (RS1), decreasing from 3.9-1.4 N (RS4) (Table 1). However, it is important to point out that although fruit in RS4 presented less firmness, its physical appearance and response to manual pressure was good according to the subjective evaluation. The last fruit was considered the most attractive and acceptable for the consumers.

Table 1: Production of CO_2 and C_2H_4 and changes in firmness in papaya (*Carica papaya*, L. cv. Maradol) in four stages of ripeness. Mean values in each column followed by a different letter at each ripeness stage are significantly different ($p<0.05$)

Ripeness stage	CO_2 (mL CO_2/kg.h)	C_2H_4 (μL C_2H4/kg.h)	Firmness (N)
1 (0-25% yellow)	7.94a	0.1988[a]	6.5a
2 (>25 and 50% yellow)	11.51b	0.5090b	3.9b
3 (>50 and 75% yellow)	14.80c	0.9108c	1.9c
4 (>75 and 100% yellow)	15.41c	0.7553c	1.4c

pH, TA and TSS: Maturity stage of papaya did not show a significant effect on pH values, which represents the presence of acidic groups, including organic acids, phenols and amino acids (Table 2). It was observed that in RS1, pH was 6.1 and increased to 6.4 in RS4 (Table 2). TSS varied from 5.4 in RS1-9.4-9.6 for RS3 and RS4, respectively. TA values did not show significant changes in the different maturity stages of papaya fruit.

Color: Hue angle (°Hue) represents changes in color of fruit, which ranges from 0 = Red, 90 = Yellow, 180 = Green-blue and 270° = Blue. °Hue value tended to change according to the RS, showing a minimum difference between the RS3 and 4 (Table 2); however, in general, there was a decrease in color (from 124-85). Chroma (C) levels describe the degree of saturation or intensity of color. Results obtained shows that papaya fruit increased their color intensity to similar levels in RS3 y 4, with values ranging from 29-61. These fruits were in good conditions, without apparent mechanical damages in their surface.

Enzyme assays: The highest PG activity was observed in fruit RS4 (22.48 U gFW^{-1}) and the lowest activity was in the RS1 (8.14 U gFW^{-1}) (Fig. 1A). This enzyme is commonly related with fruit softening and increases at the beginning of pectin's desesterification in ripened fruit. PME removes metoxyl groups from small ramifications of pectin or from partially esterified homogalacturonanes changing pectin's solubility, making it more sensitive to the attacks of other enzymes. PME activity usually initiates before that of PG. Figure 1B shows PME's activity in papaya flesh at various RS. PME activity increased gradually at RS1, RS2 and RS3; but decreased to 0.56 U gFW^{-1} in fruit of RS4.

Biochemical evaluations: Total phenolic contents of papaya fruit decreased with fruit ripening, with the highest values in RS1 (471.97 and 1.91 mgEAG/100 gFW) and the lowest in RS4 (358.67 and 0.88 mgEAG/100 gFW) in skin and flesh, respectively (Fig. 2). Commonly, phenols presented in higher concentrations in fruit skin than in flesh.

Am. J. Agri. & Biol. Sci., 5 (2): 199-208, 2010

Table 2: Changes in pH, AT, SST and color parameters (L*, a*, b*, °Hue and C) in papaya (*Carica papaya*, L. cv. Maradol) in four stages of ripeness. Mean values in each column followed by a different letter at each ripeness stage are significantly different ($p<0.05$)

Ripeness stage	pH	AT	SST (°Brix)	L*	a*	b*	°Hue	C
1 (0-25% yellow)	6.141a	0.074a	5.4a	40.01a	-16.20a	24.86a	124.69a	29.68a
2 (>25 and 50% yellow)	6.207a	0.062b	8.7b	53.30a	-10.98a	43.98a	105.00b	48.08b
3 (>50 and 75% yellow)	6.302a	0.056b	9.4b	60.73b	2.74b	62.79b	87.50c	60.50c
4 (>75 and 100% yellow)	6.401a	0.048b	9.6b	63.47b	4.86b	66.40b	85.81c	61.66c

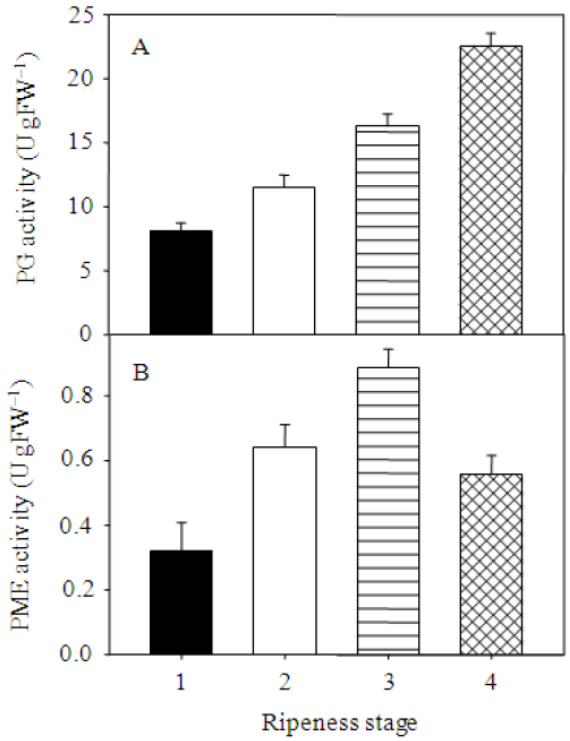

Fig. 1: Activities of polygalacturonase (A) and pectinmethylesterase (B) in papaya (*Carica papaya*, L. cv. Maradol) in four stages of ripeness. Data shows are means of at least three determinations and error bars indicate the standard deviation, expressed as U g FW^{-1}

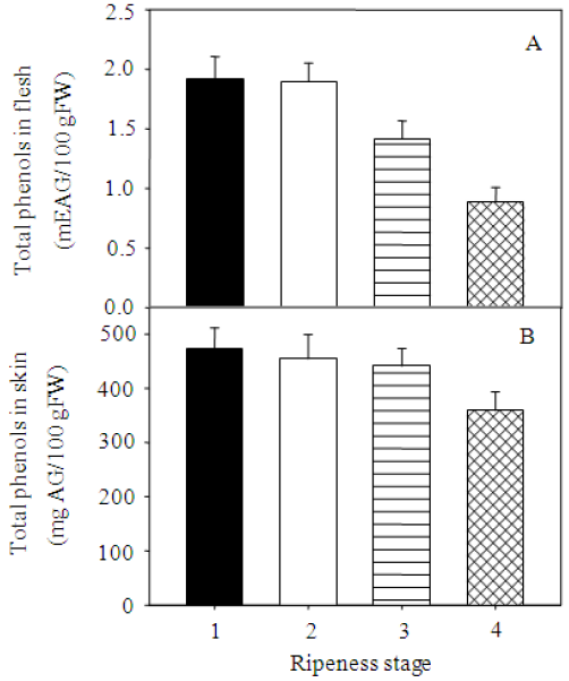

Fig. 2: Totals of phenol in papaya fresh (A) and skin (B) (*Carica papaya*, L. cv. Maradol) in four stages of ripeness. Data shows are means of at least three determinations and error bars indicate the standard deviation, expressed as mEAG/100 gFW

EC_{50} expresses the amount of antioxidants required to reduce by 50% the initial concentration of DPPH radical. Results obtained in the measurement of the AOC of papaya, expressed as EC_{50}, show that RS1 presented the highest antioxidant capacity (0.116 gFW mL^{-1}) in skin, while flesh registered 0.313 gFW mL^{-1}. The lowest AOC was recorded in RS4, with 0.1378 and 0.616 gFW mL^{-1} for skin and flesh, respectively (Fig. 3A).

The greatest AOC in papaya fruit was recorded at RS1 ripeness, in both the skin and flesh (593.77 and 160 µMET/100 gFW), while the lowest value was at RS4 (547.88 and 116.02 µMET/100 gFW) (Fig. 3B).

The antioxidant capacity assessed by ORAC increased as the fruit matured, showing that RS1 had 1065 µmTE/100 gFW, while RS4 had 1714 µmTE/100 gFW (Fig. 3C).

DISCUSSION

Respiration rate of "Maradol" papaya fruit was similar to that reported in papaya cv. Solo (Paull *et al*., 1997) with values ranging between 15-35 mL CO_2/kg.h at 20°C (Lam, 1990). Several studies on tropical fruit like mango and papaya observed that the higher the level of ripeness and/or storage temperature, the higher the respiration rate (Rivera-Lopez *et al*., 2005).

Am. J. Agri. & Biol. Sci., 5 (2): 199-208, 2010

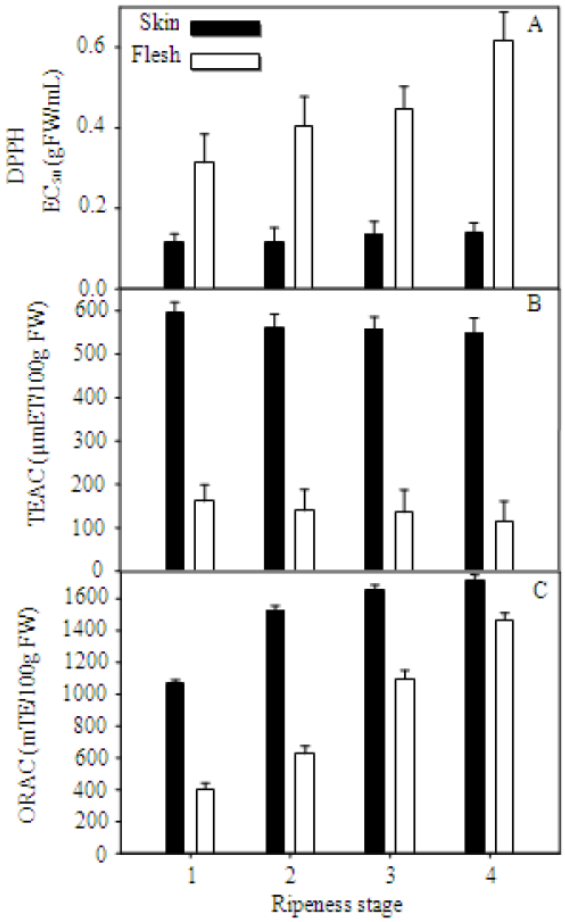

Fig. 3: Antioxidant capacity in papaya fresh and skin (*Carica papaya*, L. cv. Maradol) in four stages of ripeness through DPPH (A), TEAC (B) and ORAC (C). Data shows are means of at least three determinations and error bars indicate the standard deviation

The beginning of ripeness is usually accompanied by an increase of fruit respiration. Climacteric fruits such as papaya are characterized by an increment in their respiration and ethylene biosynthesis patterns during the ripeness process (Lelievre *et al*., 1997). In general, a series of events and reactions occur during the ripeness process, where molecules with high molecular weight (such as starch) are degraded, resulting in molecules with low molecular weight (such as sugars and organic acids, among others). Nevertheless, it is important to point out, that if fruit storage conditions are not appropriate, metabolism accelerates and could exhaust its energetic reserves, resulting in the loss of nutritional value of the fruit (Gonzalez-Aguilar *et al*., 2009).

Ethylene plays an important role in maturation and ripeness. When fruit initiates the ripeness process, small amounts of ethylene (0.1 ppm) can have a profound physiological effect on the fruit, because of its effect on the synthesis of enzymes responsible for physical, chemical and metabolic changes in plant tissue, which influences both firmness and taste of the fruit (Dunkley and Kerith, 1998). Mondal *et al*. (2008) observed that in guava in different RS, the production rate of ethylene increased reaching the climacteric point and then decreased, resulting in firmness loss, due to the activation of enzymes such as PG and PME.

One of the most important factors that affect firmness is the modification in cell walls of fruit and their degradation by pectolitic enzymes. Paull *et al.* (1999) observed an increase in the solubilization of pectin and hemicelluloses with a concomitant firmness loss during ripening of papaya which was enhanced with time and storage temperature. Similar patterns in firmness loss were observed recently in papaya cultivars with respect to our study (Nunes *et al*., 2006). Reduction of firmness observed in papaya correlated with the reduction of °Hue, but the change in color from green to yellow was not always present.

Texture changes have been related to the increase of cell-wall degrading enzymes, which are ethylene dependent. Other studies have reported that firmness loss is caused by the action of PME that remove methyl groups from esterifies galacturonic acids that increase with fruit ripening and enhance the accessibility of PG to its pectic substrate and β-galactosidase activity that increased during the last stages of ripeness (Karakurt and Huber, 2003). Lazan *et al*. (1989) and Chisari *et al*. (2009) observed that the loss of firmness appears first in the internal and later in the external mesocarp, attributing this pattern to the lack of synchronism in the degradation of pectin and hemicelluloses, suggesting that solubilization and depolymerization are two independent events in which PG is responsible for the solubilization of pectins, but not causal of fruit softening.

PG presented the greatest activity in papaya fruit flesh with a RS4 (22.48 U g FW^{-1}) and the lowest activity at a RS1 (8.14 U g FW^{-1}) (Fig. 1A). It has been reported that PG activity increases at the beginning of pectin's desesterification in ripe fruit and its activity has been correlated with softening of other fruit during ripening (Lohani *et al*., 2004).

PME removes metoxyl groups from small ramifications of pectin or from partially esterified homogalacturonanes. When separating methyl esters, PME not only provides a substrate for PG's action, but also modifies the pH from cell walls, promoting the

action from other enzymes (Chisari *et al*., 2009). Figure 1B shows PME's activity in papaya flesh at various RS, where this activity increased gradually at RS1, RS2 and RS3; but decreased to 0.56 U gFW^{-1} at RS4. A similar behavior has been observed in melons, grapefruits, peaches, kiwis, apples and papaya, where PME's activity *in vitro* decreased during ripening (Chisari *et al*., 2009).

Organic acids provide most of the hydrogen ion sand normally decrease with ripening, producing an increase in pH. Studies performed by Sanudo Barajas *et al*. (2008) obtained a 5.3 pH in green "Maradol" papaya, which shows that pH tends to change, depending on the variety and the degree of ripeness of the fruit. The pH, TA and TSS results obtained in our study were similar to those obtained by others (Corral-Aguayo *et al*., 2008) in whole papaya, TSS tended to increase, while TA tended to decrease with fruit maturation. Storing fruit at low or high temperatures can affect negatively the TSS, as a result of acceleration of ripening (Nunes *et al*., 2006).

The highest color changes are observed when fruit ripe and, in general, the loss of chlorophyll makes yellow and red tones more evident, where carotenoids and other pigments are responsible for these colors. The L* value represents the luminosity or brightness and it was observed that papaya skin presented significant statistical differences ($p \leq 0.05$) between the RS1 and RS4 levels, with a tendency to increase, which corresponds to the change of values from 0 to 100 (from dark to light). Nevertheless, this change was minimum in RS3 and RS4 (Table 2). A similar pattern was observed in tomatoes (Marquez and Cortes, 2007) and apples (Rizzolo *et al*., 2006), where luminosity was higher in ripened fruit. Parameter a* shows important changes in the various RS, ranging from negative (green) to positive (red) values, which indicates the loss of chlorophyll and the biosynthesis of carotenoids in the fruit (Yahia and Ornelas-Paz, 2009). With respect to parameter b*, this was used to measure the changes in the fruit from blue to yellow colors and it is possible to observe that in RS1 and 4 exists a considerable change in color, but from RS3-4 there was no significant difference. The result of the increase on the values of parameters a*, b* and L* was similar to the results obtained in studies performed by Ornelas-Paz *et al*. (2008), where L* is correlated with the carotenoid content in the mesocarp of "Manila" mango.

°Hue value tended to change according to the RS, showing a minimum difference between the RS3 and RS4 (Table 2); however, in general, there was a decrease in color (from 124-85). Ornelas-Paz *et al*. (2008) correlated the reduction of Hue values with the contents of the major carotenoids present in mango. Chroma levels describe the degree of saturation or intensity of color. Results obtained show that papaya fruit increased their color intensity from 29-61 in RS3 and RS4.

Fruits and vegetables contain a great number of essential components, which promote health in humans, because of their beneficial effect against certain diseases, especially several cancer pathologies, acting as antivirals, anti-inflammatories and stimulators of immune response; some of these compounds include phenols (Yahia, 2009). Total phenolic contents of papaya in different RS decreased with fruit ripening, the highest values were recorded in RS1 (471.97 and 1.91 mgEAG/100 gFW) and the lowest in RS4 (358.67 and 0.88 mgEAG/100 gFW) in skin and flesh, respectively (Fig. 2). This pattern was similar to that observed in nectarines, peaches and plums, where phenolic contents were 2-6 times greater in the skin than in the flesh (Gil *et al*., 2002).

Mahattanatawee *et al*. (2006) analyzed different tropical fruits and found that ripened papayas (cv. Red Lady) contained the lowest amount of phenolic compounds, which coincides with our study performed recently with eight horticultural crops (Corral-Aguayo *et al*., 2008). Phenolic compounds can act as antioxidants and their activity is determined according with the chemical structure that posses. The reason of the difference between the results obtained in different tropical fruits might be because this capacity increases according to the number of hydroxyls present in fruit and their concentration has been correlated with the antioxidant ability of different types of fruit (Wang *et al*., 2008).

Corral-Aguayo *et al*. (2008) measured the AOC of "Maradol" papaya, obtaining higher values than those obtained in the present study. It is important to point out that this study used methanol as a solvent, therefore results represent the AOC of hydrophylic compounds (like ascorbic acid and phenolic compounds) as well as of those compounds that are not soluble in water, therefore, perhaps the values were lower because they did not react significantly with DPPH radical. On the other hand, Mahattanatawee *et al*. (2006) observed that in green papaya (cv. Red Lady) AOC is higher than in ripe papaya of the same variety, which is in agreement with our results. Studies performed by Gancel *et al*. (2008) in naranjilla indicated that the AOC measured as ED50 (dilution required to reduce by 50% the initial concentration of DPPH radical) was higher in flesh than in skin, which contrasts with the results that we obtained in papaya, where the highest AOC was observed in the skin. The reason for this could be the

interaction of phenolic compounds, organic acids and sugars, as well as the variety and atmospheric conditions, which results in greater AOC. Li-Chen *et al*. (2006) concluded that the AOC obtained in the skin of red pitayas could be the result of the high content and type of phenols (betalains) present in the fruit, because an increment of hydroxyl groups in their molecular structure is related with the increase of AOC.

It is recommended to use different techniques for the AOC evaluation of fruits and vegetables, because of the nature of the different types of compounds present in them and to obtain more reliable results. $ABTS^{\bullet+}$ or TEAC (Trolox equivalent AOC) methodology is widely used for compounds with hydrophilic or lipophylic nature. Also, the $ABTS^{\bullet+}$ radical has been used to confirm results obtained with DPPH, because both possess similar antioxidant mechanisms. It has been reported that phenolic compounds or ascorbic acids react vigorously with ABTS, while lipophylic compounds make them weaker (Perez-Jimenez *et al*., 2008).

The highest AOC in "Maradol" papaya was recorded at RS1 ripeness, in both the skin and flesh (593.77 and 160 μMET/100 gFW), while the lowest value was at RS4 (547.88 and 116.02 μMET/100 gpf) (Fig. 3B). Lako *et al*. (2007) observed that AOC was higher in papaya cv. *Annona* muricata, compared with other types of fruits, attributing these values to the considerable amount of flavonoids present (9 mg/100/g), although generally the content of these compounds is low in fruits.

The AOC assessed by ORAC increased as the fruit matured, showing that RS1 had 1065 μmTE/100 gFW, while RS4 had 1714 μmTE/100 gFW (Fig. 3C). Similar behavior in "Ataulfo" mangoes showed that antioxidant capacity increased during storage at 5 days (Robles-Sanchez *et al*., 2009), which may reflect the contribution of carotenoids (Yahia and Ornelas-Paz, 2009). However, the study performed by Tabart *et al*. (2009) in different fruit and vegetable juices concluded that used methods (ABTS, DPPH and ORAC) could provide widely different results, because some measure lipophylic, hydrophyilic compounds and others do not consider physiological cell conditions.

CONCLUSION

"Maradol" papaya experienced changes in firmness, which is correlated with activity from two of the main enzymes: PG and PME and with the increase of respiration and production of ethylene. Antioxidant capacity, measured using DPPH, ABTS and ORAC techniques, was higher in RS1, which is correlated with higher content of phenolic contents.

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CAPÍTULO II

Identification and Quantification of Phenols, Carotenoids, and Vitamin C from Papaya (*Carica papaya* L., cv. Maradol) Fruit Determined by HPLC-DAD-MS/MS-ESI.

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Identification and quantification of phenols, carotenoids, and vitamin C from papaya (*Carica papaya* L., cv. Maradol) fruit determined by HPLC-DAD-MS/MS-ESI

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ABSTRACT

Recent studies have demonstrated that vitamin C, phenols, and carotenoids are bioactive compounds that protect the body from oxidative stress, reducing the risk of cardiovascular diseases and some types of cancer. Qualitative and quantitative analysis of the major phytochemicals found in papaya fruit flesh and skin (*Carica papaya* L., cv Maradol) was conducted in four stages of ripeness, using high-performance liquid chromatography mass spectrometry. Phenolic compounds identified in the fruit skin tended to decrease with ripening. The compounds identified were ferulic acid (277.49 to 186.63 mg/100 gDW), *p*-coumaric acid (229.59 to 135.64 mg/100 gDW), and caffeic acid (175.51 to 112.89 mg/100 gDW). The following carotenoids, along with vitamin C, increased in flesh with ripening; lycopene (0.36 to 3.40 mg/100 gDW), β-criptoxanthin (0.28 to 1.06 mg/100 gDW), β-carotene (0.23 to 0.50 mg/100 gDW), and vitamin C (25.07 to 58.59 mg/100 gDW). These results indicate that stage of ripeness significantly influences the contents of bioactive compounds in papaya fruit.

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1. Introduction

In recent years, consumption of fruits and vegetables has increased considerably because of their benefits for good health (Yahia, 2010). Many biochemical and epidemiological studies have demonstrated that fruits and vegetables contribute to the reduction of several diseases, including cardiovascular, neurological, and carcinogenic illnesses (Stanner, Hughes, Kelly, & Buttriss, 2004). These benefits have been attributed, at least in part, to the amount of antioxidant compounds present in these foods, which reduce the oxidative stress produced by free radicals, and in consequence, cellular damage (Dosil-Díaz, Ruano-Ravina, Gestal-Otero, & Barros-Dios, 2008). Some of the most important antioxidant compounds present in fruits and vegetables include polyphenols, carotenoids, and vitamin C (Yahia, 2010).

Phenolic compounds are aromatic metabolites of plants secondary metabolism that have a common structure with an aromatic ring with at least one hydroxyl group, which provides the ability to neutralize reactive species, helping the body to protect itself from oxidative stress (Wojdyło, Oszmianski, & Laskowski, 2009). Additionally, phenols contribute to fruits' color and taste and have been described as possessing anticarcinogenic and antimutagenic activity (Al-Duais, 2009; Gorinstein et al., 2009). Various studies have shown that phenolic compounds have high antioxidant potential, resulting in a beneficial effect to human health (Vijaya Kumar Reddy, Sreeramulu, & Raghunath, 2010).

Carotenoids are lipophilic compounds formed by 8 isoprenoid units. They play a very important role in human health and nutrition, recognized as strong antioxidants due to their ability to trap singlet oxygen and eliminate the peroxyl radical (Al-Duais, 2009). Some carotenoids have pro-vitamin A activity (β-carotene, α-carotene, γ-carotene, β-cryptoxanthin) and reduce the risk of cancer and coronary vein disease (Yahia & Ornelas-Paz, 2010). Various *in vitro* and *in vivo* studies have shown that carotenoids prevent cardiovascular diseases and impact cell signaling pathways (Stahl & Sies, 2005), in addition to providing protection against some types of cancer (Yuan, Stram, Arakawa, Lee, & Yu, 2003).

Based on their structure, carotenoids are divided into two groups: carotenes (hydrocarbonated link) and xantophylls—with at least one oxygen molecule. The presence of double-conjugated links gives carotenoids the ability to act like photoprotectors (Tanaka, Sasaki, & Ohmiya, 2008), protecting membrane lipid peroxidation quenching reactive oxygen species (Rivera-Pastrana, Yahia, & Gonzalez-Aguilar, 2010). It has been reported that lycopene protects cells from reactive oxygen species (ROS) by stimulating the production of cellular enzymes such as superoxide dismutase and glutathione S-transferase (Goo et al., 2007).

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In several fruits such as papaya, the content of carotenoids increases with ripening (Wall, 2006). Lycopene, β-cryptoxanthin, and β-carotene are the main carotenoids that have been identified in papaya (Marelli de Souza, Silva Ferreira, Paes Chaves, & Lopes Teixeira, 2008). Vitamin C (ascorbic acid) is a hydrophilic vitamin present in fruit. It plays an important role because it is required for several metabolic processes like development of tissues and production of hormones (Puente, Pinto-Muñoz, Castro, & Cortés, 2010) and is also considered a powerful antioxidant reducing oxidative stress (Guoong, Mingjun, Fengwang, & Dong, 2009). In addition, it has been observed that vitamin C can act sinergically with other vitamins and play an important role to regenerate vitamin E. Recently, we reported the physiological and biochemical changes that occur during ripening of "Maradol" papaya and its antioxidant status (Gayosso-García Sancho, Yahia, Martínez-Téllez, & González-Aguilar, 2010). However, the identification and quantification of the most important phytochemicals responsible for the antioxidant activity were not reported. Therefore, the objective of this study was to determine the changes in vitamin C and identify and quantify the main phenols and carotenoids during ripening of "Maradol" papaya fruit (*Carica papaya* L.).

2. Materials and methods

2.1. Chemicals and solvents

Formic acid, acetronitrile, acetone, n-Hexane, dichloromethane, methanol, $Na_2S_2O_3$, and anhydrous granular sodium sulfate were purchased from J. T. Baker (Baker Mallinckrodt, Mexico). Diethyl ether, methyl tert-butyl ether (MTBE), lycopene (purity ≥ 90%), β-carotene (purity = 95%) from carrots were from Sigma-Aldrich (St. Louis, MO). Solvents used for chromatography were HPLC grade. Water was bidistillated, and HPLC grade water was obtained by a Milli-Q plus water purification system (Millipore Corp., Bedford, MA).

2.2. Plant material

Papaya fruits (*C. papaya* L, cv. Maradol) were obtained from a local market in Hermosillo, Sonora, Mexico. Fruits were selected for uniform size, color, level of external ripeness, and divided in four ripeness stages: RS1 represents papaya with yellow area on 0–25% of the skin; RS2 (25–50%); RS3 (50–75%), and RS4 (75–100%); we followed the criteria used previously by Fonseca, Rocha-Leal, Cenci, Cecon, and Bressan-Smith (2003) and Santamaría-Basulto et al. (2009). After selection, fruits were divided in lots of 12 fruit each, and flesh and skin were randomly sampled for chemical analysis of vitamin C, phenols and carotenoids. Samples were freeze-dried and stored at −70 °C until analysis.

2.3. Total carotenoids extraction

Total carotenoids (TC) were determined according to Yahia, Soto-Zamora, Brecht, and Gardea (2007) and Ornelas-Paz, Yahia, and Gardea (2008). Freeze-dried papaya tissue (0.5 g) was homogenized in 10 mL of hexane: dichloromethane (1:1, v/v), using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC) and centrifuged at 9000g for 10 min at 5 °C. Organic phase was separated, and procedure was repeated three times. For alkaline hydrolysis 10 mL of methanolic KOH 40% (1:1, v/v) was added to extracts for 1 h at 50 °C in a stirring bath set at 100 rpm. After saponification, 10 mL of 10% sodium sulfate was added for phase separation and the extracts were left for 1 h in the dark at room temperature. TC quantification was measured on top-phase aliquots in a Beckman DU-65 spectrophotometer at 450 and 470 nm. A calibration curve was performed using β-carotene in hexane as the standard and hexane as the blank. Extracts were evaporated in a Rotovapor® (Büchi Labortechnik AG, Flawil, Switzerland) at 30 °C in a Buchi low-pressure evaporator. Samples were resuspended in 2 mL acetone and filtered through nylon membrane of 0.45 μm of pore size (Millipore Corp., Bedford, MA) and directly injected into the HPLC mass spectrometry system.

2.4. High-performance liquid chromatography (HPLC)

Samples (30 μL) containing carotenoids were automatically injected into an HP 1100 series HPLC system (Hewlett-Packard, GmbH, Germany) equipped with a diode array detector. Absorption spectra for the main peaks were recorded at 430, 450, and 470 nm. The HPLC system was equipped with a C30 reversed-phase column (4.6 mm × 150 mm) with a spherical particle size of 3 μm (YMC Inc., Wilmington, NC), which was kept at 15 °C. The mobile phase was composed of methanol (A) and methyl tert-butyl ether (B), and the elution gradient was 0 to 100 % (B) in 55 min at a flow rate of 1 mL/min and 15 °C.

2.5. High-performance liquid chromatography-ApCI-mass spectrometry (HPLC-ApCI-MS) analysis

Mass spectra of the main carotenoids were obtained using the chromatographic system described above connected to a HP6210 model time-of-flight (TOF) mass spectrometer (Agilent Technologies Inc., Palo Alto, CA) equipped with an atmospheric pressure chemical ionization (ApCI) interface and MassHunter manager software (Version A.02.01). The ApCI–MS system was operated in the positive ion mode. High-purity nitrogen (99.999%) was used as nebulizing (20 psig) and drying gas at a flow rate of 5.0 L/min. Other ApCI–MS parameters were as follows: drying gas temperature 350 °C and the corona, capillary, fragmentor, and skimmer voltages were 4 μA, 4 kV, 200 V, and 60 V, respectively. Carotenoids were identified by comparing their retention time and UV–vis data with those obtained with reference standards as well as co-chromatography with added standards and using their mass spectra (*m/z* 100–800). Quantitative data for all-*trans*carotenoids were obtained by calibration curves of known standards. Contents of lycopene and β-carotene were obtained from the calibration curves of pure standards (0.001–0.1 g/L), and their correlation coefficients (r^2) were 0.9997 and 0.9996, respectively. Quantification of β-cryptoxanthin was performed using the calibration curve of β-carotene, because of its similarity with the spectrum.

2.6. Total phenols and antioxidant capacity

Total phenols (TP) and antioxidant capacity (AOC) of methanol extracts were measured as TEAC (trolox equivalent antioxidant capacity) and ORAC (oxygen radical absorbing capacity) (Gayosso-García Sancho et al., 2010).

2.7. L-Ascorbic acid (AA) and isoascorbic acid (IAA)

AA and IAA were determined according to Corral-Aguayo, Yahia, Carrillo-Lopez, and Gonzalez-Aguilar (2008). Samples of 0.5 g of freeze-dried powder were homogenized in 10 mL of extraction solution [0.1 M citric acid and 0.05% ethylenediaminetetraacetic acid (EDTA) at pH 2.35–2.40] using an IKA T25 basic homogenizer (IKA Works, Willmington, NC). Then, the homogenate was centrifuged at 15,000*g* for 10 min at 2 °C. The supernatant was separated and filtered through Sep-Pak C18 Vac 3 mL cartridge (Waters Co., Milford, CT). The first 5 mL was discarded, and the next 3 mL was analyzed. The cartridge was previously conditioned with 10 mL of ethanol and then with 10 mL of HPLC grade water. The residual water was expelled with air. A total of 3 mL of the sample was collected, and 1 mL (0.832 mg/mL) of 1,2-phenylendiamine prepared in methanol/water (5:95, v/v) was added. The samples were incubated for 37 min in the dark and filtered through a 0.45 μm nylon membrane. Aliquots of

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40 μL were injected in a HP 1100 Series HPLC (Hewlett-Packard/Agilent Technologies Co., Palo Alto, CA) with 10 μm Bondapak C18 column (3.9 mm × 300 mm), Sentry, 10 μm Bondapak C18 (3.9 mm × 20 mm) guard column, and diode array detector. The mobile phase consisted of 5 mM hexadecyltrimethylammonium bromide (cetrimide) and 50 mM KH_2PO_4 in methanol/water (1:99, v/v) at pH 4.6, and flow rate was 1.5 mL/min. L-Ascorbic acid was monitored at 254 nm, and isoascorbic acid was monitored at 261 nm. Calibration curves were prepared from known standards and used for quantification. The concentration range and the correlation coefficients (r^2) for the calibration curves were between 0 and 0.8 mg/mL and 0.9998 for ascorbic acid and between 0 and 0.8 mg/mL and 0.9995 for isoascorbic acid.

2.8. Separation and identification of phenolic acids

2.8.1. Preparation of extracts

Preparation and identification of phenolic acids were determined according to a modified method described by Ferreres et al. (2008). Papaya skin dry samples (0.5 g) were homogenized in 20 mL of 80% methanol, using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC), sonicated for 30 min at 30 °C in a 2510 model ultrasonic bath (Branson, Whetersfield, CT), centrifuged at 12,000*g* for 15 min at 5 °C in a Hermle centrifuge model Z323 K (Labortechnik Technologies, Germany), and then filtered through number 1 Whatman paper. For alkaline hydrolysis 10 mL of 4 M NaOH was added to phenolic extracts and left for 4 h in the dark at room temperature. After incubation, extracts were acidified to pH 2.0 with 4 M HCl, then, acidified solutions were extracted twice with 20 mL ethyl acetate. Extracts were evaporated in a Rotovapor® (Büchi Labortechnik AG, Flawil, Switzerland) at 35 °C in a Buchi low-pressure evaporator. Skin samples were resuspended in 10 mL of 80% methanol and filtered through nylon membrane of 0.45 μm of pore size (Millipore Corp., Bedford, MA) and directly analyzed by HPLC/UV-DAD/ESI-MS system.

2.8.2. High-performance liquid chromatography (HPLC)

Samples containing phenols were injected automatically into an HP 1100 series HPLC system (Hewlett-Packard, GmbH, Germany) equipped with a diode array detector (DAD). Absorption spectra for the main peaks were recorded at 280 and 320 nm. The HPLC system was equipped with a Xterra RP_{18} reverse phase column (4.6 mm × 250 mm) with a spherical particle size of 5 μm, which was kept at 25 °C. The mobile phase was composed of 1% formic acid (A) and acetonitrile (B), and the elution gradient was 2 to 100% (B) in 40 min at a flow rate of 0.5 mL/min and 25 °C. The injection volume was 20 μL.

2.8.3. High-performance liquid chromatography- mass spectrometry (HPLC-ESI-MS) analysis

Mass spectra of the main phenolic acids were obtained using the chromatographic system described above but with a 6210 model time-of-flight (TOF) mass spectrometer (Agilent Technologies Inc., Palo Alto, CA) equipped with an electrospray ionization (ESI) source operating at the negative ionization mode and MassHunter manager software (Version A.02.01). High-purity nitrogen (99.999%) was used as nebulizing (45 psig) and drying gas (11.0 L/min); gas and vaporizer temperature was 350 °C; and the corona, capillary, and fragmentor voltages were 4 μA, 4 kV, and 220, respectively. Phenolics were identified by comparing their retention time and UV–vis data with those obtained with reference standards as well as co-chromatography with added standards and using their mass spectra (*m/z* 100–650). Quantitative data for phenolics were obtained by calibration curves constructed with known standards.

2.9. Statistical analysis

The statistical significance of differences in phenolic compounds, carotenoids, and vitamin C concentrations in papaya of different ripeness stages was analyzed through an analysis of variance (ANOVA) and the multiple comparisons of means through the Duncan's test. Statistical differences were considered to be significant ($p \leq 0.05$) using the statistical software SAS version 8.0 (SAS Inst. Inc. Cary, NC, USA).

3. Results and discussion

The content of total carotenoids in papaya pulp (*C. papaya*, cv. Maradol) at different ripeness stages (RS) expressed as mg/100 gFW increased with the level of ripeness of the fruit. The highest values were found in fruit of RS4 (3.27 mg/100 gFW), while the lowest value was for RS1 (0.92 mg/100 gFW) (Fig. 1). During ripening, chlorophyll began to degrade, coinciding with carotenoid synthesis and resulting in a significant increase of yellow–orange color. Some tropical fruits such as mangos have a similar behavior as papaya, where color conferred by carotenoids plays an important role in the fruit acceptability by consumers (Yahia & Ornelas-Paz, 2010). The carotenoid content of "Maradol" papayas obtained in the present study were 2-fold higher than those reported for "Formosa" and "Sunrise" papaya (Mélo, de Lima Arroxelas Galvão, & Maciel Sucupira, 2006). These differences could be attributed to agricultural practices, sunlight exposure, production area, stage of ripeness, postharvest handling, and methodology used for analysis (Andersson, Olsson, Johansson, & Rumpunen, 2009; De Rosso & Mercadante, 2005; Ornelas-Paz et al., 2008).

The principal carotenoids in saponified extracts of papaya in different stages of ripeness were identified by comparison of mass spectrometry (MS) fragmentation pattern, retention times and UV–visible maximum absorption and mass spectra profile (*m/z*) of standard compounds, and they were lycopene, β-carotene, and β-cryptoxanthin (Table 1). A similar profile coincides with previous studies done in papaya (Chandrika, Jansz, Wickramasinghe, & Warnasuriya, 2003; Rivera-Pastrana et al., 2010; Wall, 2006). Chromatographic profiles of the main carotenoids present in saponified papaya extracts, which were obtained by HPLC-DAD, are shown in Fig. 2 and correspond to β-cryptoxanthin with a predominant ion $[M+H]^+$ at 553 and yielded ion fragments at *m/z* 409, 576, 653; β-carotene $[M+H]^+$ at 537 and yielded ion fragments at *m/z* 409, 539, 543, 662, and lycopene ($[M+H]^+$ at 537 and yielded ion fragments at *m/z* 530, 576, 669. Previous studies demonstrated the presence of carotenoids as esters in different fruits

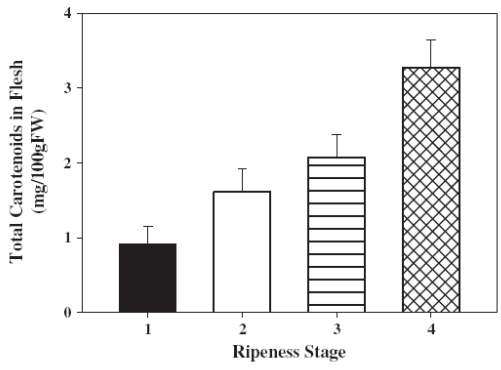

Fig. 1. Total carotenoids in papaya flesh (*Carica papaya* L. cv. Maradol) in four stages of ripeness. 1, 0–25% yellow; 2, >25 and 50% yellow; 3, >50 and 75% yellow; and 4, >75 and 100% yellow. Data shown are means of at least three determinations and vertical bars represent standard deviation expressed as mg/100 gFW.

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Table 1
Tentative identification of principal carotenoids by HPLC-ESI-MS in papaya flesh (*Carica papaya* L. cv. Maradol).

Compound name	RT (min)	$[M+H]^+$(Frag. *m/z*)
β-Cryptoxanthin	14.130	553 (409, 576, 653)
β-Carotene	16.112	537 (409, 539, 543, 662)
Lycopene	25.134	537 (530, 576, 669)

(Ornelas-Paz et al., 2008). Papaya extracts were saponified for the hydrolysis of esters and to remove chlorophyll and non-desired fatty acids that could interfere in the analysis. Our results coincided with those of Rivera-Pastrana et al. (2010), who reported a similar profile of carotenoids in "Maradol" papaya obtained from the same production area that the fruits of this study. Marelli de Souza et al. (2008) observed that the major carotenoids found in papaya were lycopene, β-cryptoxanthin, and β-carotene, with lycopene representing 65% of the total. Andersson et al. (2009) observed that the content of esterified carotenoids in cherries increased during ripening, which allows esterified carotenoids to integrate more quickly to the membranes, increasing the color of the fruit and its accumulation in chromoplasts (Yahia & Ornelas-Paz, 2010).

The concentrations of the main saponified and non-saponified carotenoids found in papaya pulp expressed in mg/100 gDW are shown in Fig. 3. The content of lycopene increased 10 times during ripening, showing that RS1 had 0.35 mg/gDW while RS4 had 3.5 mg/100 gDW (Fig. 3A) in the saponified samples. However, this increase was lower in β-cryptoxanthin and increased from 0.29 mg/100 gDW (in RS1) to 1.06 mg/100 gDW (in RS4) and was the second most abundant carotenoid in papaya, followed by β-carotene, which increased from 0.24 mg/100 gDW (in RS1) to 0.5 mg/100 gDW (in RS4). Significant differences ($P<0.05$) between the three carotenoids present in papaya were found between RS1 and RS4. A study performed by Kimura, Rodriguez-Amaya, and Yokoyama (1991) in "Common", "Solo", "Formosa" and "Tailandia" papaya, reported the effect of agricultural practice on the content of the three main carotenoids of papaya fruit. They found that lycopene increased 2-fold while β-carotene increased 2- to 5-fold with respect to that observed in the present study. Our results coincide with those obtained by Rivera-Pastrana et al. (2010) for lycopene, Corral-Aguayo et al. (2008) for β-carotene and those of Wall (2006), who performed studies on the contents of β-cryptoxanthin papaya produced under different farming systems, pointing out that the differences could be attributed to the type of farming and the location. The positive health benefits of lycopene contained in different fruits and vegetables have been widely reported, including reduction of cardiovascular problems (Singh & Goyal, 2008). Therefore, the possible benefits of papaya consumption fruit could be compared with those reported in other vegetables rich in lycopene such as tomato. However, the concentration of other phytochemicals present in these products that contribute to health needs to be considered.

Phenolic compounds were also identified in saponified and non-saponified extracts of papaya skin by HPLC-ESI-MS. The major phenolic compounds identified in saponified extracts were hydroxycinnamic acid sugar derivatives, while the non-saponified extracts showed only traces of these compounds in an acylated form (data not shown). Caffeic acid was identified tentatively as a [M–H]-deprotonated molecule (*m/z* 179), with loss of the CO_2 group in the form of negative ion, with an UV spectrum (λmax = 280, 320 nm) in a retention time (RT) of 16.8 min (Table 2). *p*-Coumaric acid was tentatively identified as a [M–H]-deprotonated molecule (*m/z* 163) in a RT of 19.04 min and yielded ion fragments at *m/z* 119 and 153. Ferulic acid was tentatively identified according to its UV spectrum (λmax = 280, 320 nm) as a [M–H]-deprotonated molecule (*m/z* 193), with a RT of 22.4 min, and yielded ion fragments at *m/z* 117, 134, 149, and 179. Profile of phenolic compounds but not concentration coincides with the first report on the identification of phenolics in "Maradol" papaya skin made by Rivera-Pastrana et al. (2010). Phenolic compounds have been reported to have antiradical, antimutagenic, and anticarcinogen properties and protect plants from UV radiation (Cantin, Moreno, & Gogorcena, 2009;

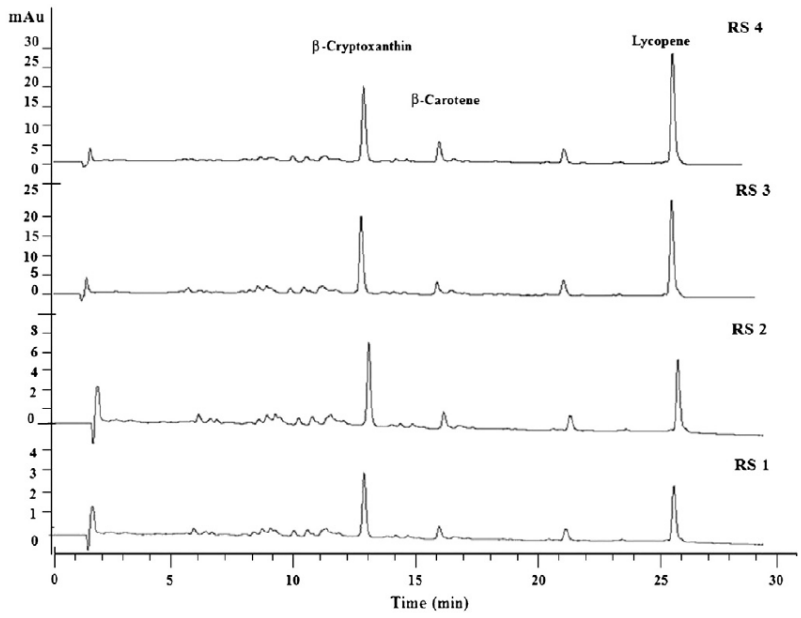

Fig. 2. HPLC carotenoids chromatograms at 450 nm in papaya flesh (*Carica papaya* L. cv. Maradol) in four stages of ripeness. RS1 (0–25% yellow), RS2 (>25 and 50% yellow), RS3 (>50 and 75% yellow), and RS4 (>75 and 100% yellow).

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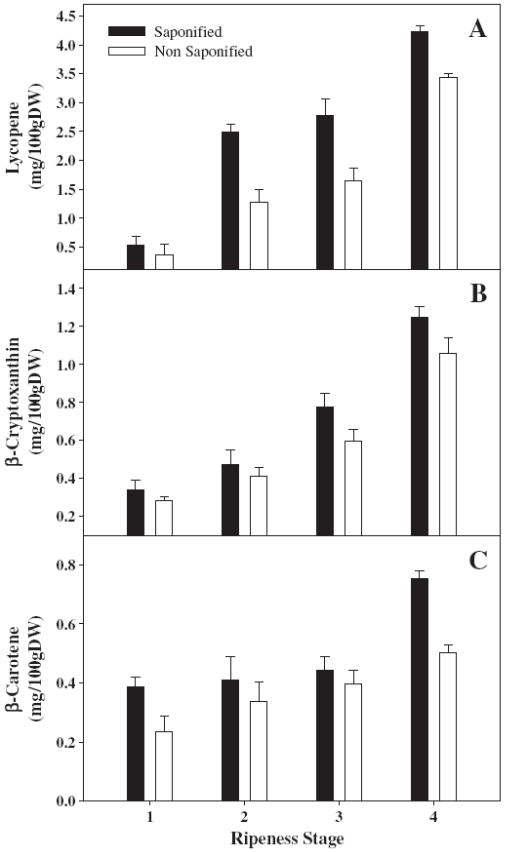
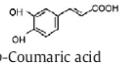
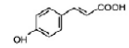
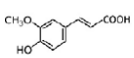


Fig. 3. Carotenoids concentrations in papaya flesh (*Carica papaya* L. cv. Maradol) saponified and non-saponified by HPLC in four stages of ripeness: Lycopene (A), β-Cryptoxanthin (B), and β-Carotene (C). Data shown are means of at least three determinations and vertical bars represent standard deviation expressed as mg/100 gDW.

Hounsome, Hounsome, Tomos, & Edwards-Jones, 2008). Ferulic acid synthesis occurs from phenylalanine via the shikimate (Barone, Calabrese, & Mancuso, 2009). Several studies have indicated anticarcinogenic mechanisms of phenolic compounds. Kawabata et al. (2000) found that ferulic acid had anticarcinogenic effect on colon cancer in rats, and this was correlated with the ability to scavenge free radicals and stimulate the cytoprotective effect of various enzymes. Caffeic acid

Table 2
Identification of principal phenolic acids by HPLC-ESI-MS in papaya skin (*Carica papaya* L. cv. Maradol).

Compound name	RT (min)	$[M-H]^-$ (Frag. *m/z*)
Caffeic acid	16.808	179 (135)
		
p-Coumaric acid	19.037	163 (119, 153)
		
Ferulic acid	22.413	193 (134, 117, 149, 179)
		

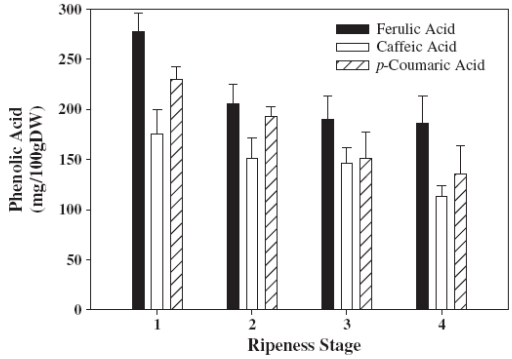


Fig. 4. Principal phenolic acids in papaya skin (*Carica papaya* L. cv. Maradol) in four stages of ripeness. Data shown are means of at least three determinations in HPLC-DAD at 320 nm and vertical bars represent standard deviation expressed as mg/100 gDW.

found in many fruits, vegetables, and coffee is commonly found as esterified form with quinic acid, also known as chlorogenic acid (Clifford, 1999). Da Cunha et al. (2004) observed that caffeic acid and its derivatives exert anti-inflammatory activity both *in vitro* and *in vivo*, and this activity was due in part to the removal of nitric oxide (NO) and its ability to modulate the expression of iNOS (inducible nitric oxide synthase). *p*-Coumaric acid is an intermediate in the synthesis of phenylpropanoids and has been shown to have antioxidant properties, lowers cholesterol, and provides a defense mechanism against atherosclerosis. Zang et al. (2000) found that *p*-coumaric acid oral administration (317 mg/day for 30 days) inhibited the oxidation of LDL, reduced serum cholesterol levels and did not affect HDL levels, and contributed considerably to the antioxidant capacity, which is directly related to the removal of ROS.

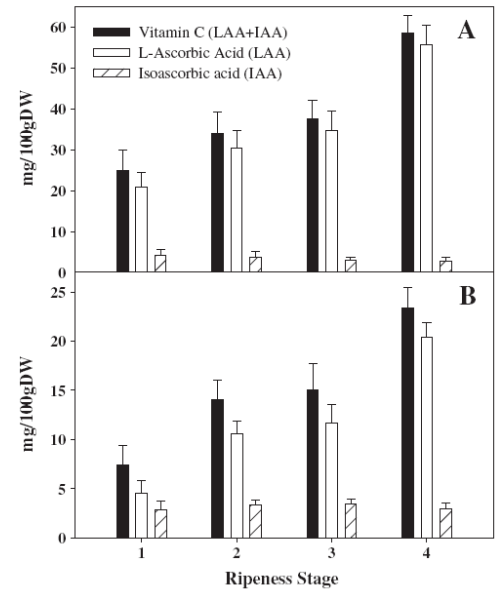


Fig. 5. Vitamin C (L-ascorbic acid+ Isoascorbic acid) in papaya flesh (A) and skin (B) (*Carica papaya* L. cv. Maradol) in four stages of ripeness. 1, 0–25% yellow; 2, >25 and 50% yellow; 3, >50 and 75% yellow; and 4, >75 and 100% yellow. Data shown are means of at least three determinations and vertical bars represent standard deviation expressed as mg/100 gDW.

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The major phenolic acids quantified by HPLC-DAD in papaya skin were ferulic, *p*-coumaric, and caffeic acids. The contents of phenolic acids decreased concomitantly with fruit ripening (Fig. 4). Ferulic acid had a concentration of 277.49 mg/100 gDW in RS1, decreased to 186.63 mg/100 gDW in RS4; *p*-coumaric acid showed a concentration of 229.58 mg/100 gDW in RS1 and 135.64 mg/100 gDW in RS4, while the concentration of caffeic acid was 175.50 mg/100 gDW in RS1 and 112.8892 mg/100 gDW in RS4. Studies performed in other fruits have determined that hydroxycinnamic acids are generally more abundant than hydroxybenzoic acids, and fruit skin normally has higher concentration of phenolic compounds than that of the pulp (Castillo-Muñoz, Fernández-González, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2009; Gancel, Alter, Dhuique-Mayer, Rualesand, & Vaillant, 2008; Rivera-Pastrana et al., 2010). It has been observed that phenolic compounds decreased during ripening (El Gharras, 2009). The health benefits of phenol compounds are associated with their role in the prevention of several disorders, related to the damaging effect of oxygen free radicals and ROS, (Valko et al., 2007). Liu (2004) found that ferulic acid is covalently conjugated to polysaccharides present in the cell wall, lignin, glycoproteins, and insoluble carbohydrate biopolymers. Among the antioxidant and anti-inflammatory properties that have ferulic acid, it has been observed that it has positive effects on Alzheimer's disease. Yan et al. (2001) observed that mice with this disease treated with a diet rich in ferulic acid decreased the activity of choline acetyltransferase, mainly due to the electron donation of its 3-methoxy and 4-hydroxyl groups on the benzene ring (Itagaki et al., 2009). Several epidemiological studies suggest an inverse relationship between consumption of foods rich in phenolic acids and the incidence of various diseases. Chung, Moon, Chang et al. (2004) found that caffeic acid and caffeic acid phenethyl ester supply significantly reduced liver metastasis, confirming the anti-tumor and anti-metastatic effects of these phenols.

Vitamin C, measured as L-ascorbic and isoascorbic acid and determined by HPLC at 261 nm, both in the skin and pulp was higher in pulp than in the skin, and the greater the stage of ripeness, the greater the vitamin C contents (Fig. 5). Significant differences ($P<0.05$) were found in vitamin C between the different RS. The largest amount of vitamin C in the pulp was 58.6 mg/100 gDW (in RS4), and the minimum was 25.1 mg/100 gDW (in RS1). However, skin had lower vitamin C values with 7.4 mg/100 gDW (in RS1) and 23.4 mg/100 gDW (in RS4). These results obtained in the pulp coincide with those obtained by Wall (2006) in eight varieties of papaya, where an average of 51.2 mg/100 g was reported. Corral-Aguayo et al. (2008) obtained 56.2 mg/100 g while Marelli de Souza et al. (2008) reported an average of 75.9 mg/100 g in three papaya cultivars. The content of vitamin C could vary, mainly because of the

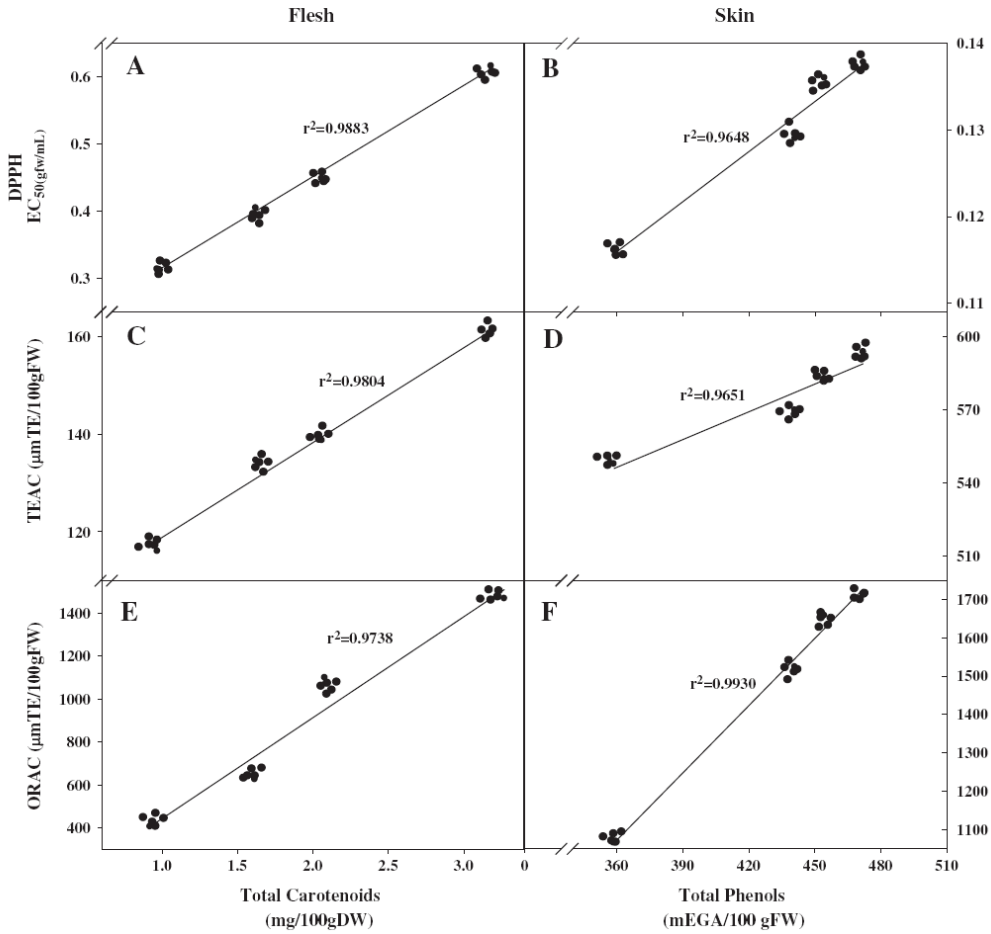

Fig. 6. Correlation of total carotenoids data in flesh and antioxidant capacity by (A) DPPH, (C) TEAC, and (E) ORAC assay. Correlation of total phenols data in skin and antioxidant capacity by (A) DPPH, (B) TEAC, and (C) ORAC assay.

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type of fruit cultivation, type of soil, weather, and level of fruit ripeness (Lee & Kader, 2000). Vitamin C or ascorbic acid is a powerful hydrosoluble antioxidant that protects the body against oxidative stress, because of its ability to trap hydroxyl and superoxide radicals. In addition, vitamin C is also involved in the synthesis of collagen (Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2008) and regenerates α-tocopherol by reducing α-tocopheroxyl radical (Niki, Noguchi, Tsuchihashi, & Gotoh, 1995). L-Ascorbic acid functions as a protective antioxidant for reactions that require reduced iron (Fe^{2+}) or copper (Cu^{1+}) metalloenzymes (Vasdev, Ford, Parai, Longerich, & Gadag, 2001), and a regular daily intake of 250–500 mg reduces oxidative damage by removing free radicals (Tariq, 2007).

An analysis was done on the correlation between the techniques to measure AOC and antioxidant compounds, where DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay showed an elevated correlation with carotenoids in the pulp ($R^2 = 0.9883$) (Fig. 6A), with phenolic compounds in the skin ($R^2 = 0.9648$) (Fig. 6B), and with ascorbic acid in the pulp ($R^2 = 0.9951$) (Fig. 7A). Similar results have been obtained in different types of fruits (Kevers et al., 2007). TEAC (trolox equivalent antioxidant capacity) assay showed a high correlation between carotenoids in the pulp ($R^2 = 0.9804$) (Fig. 6C), phenols in the skin ($R^2 = 0.9651$) (Fig. 6D), and ascorbic acid in the pulp ($R^2 = 0.9598$) (Fig. 7B). On the other hand, ORAC (Oxygen Radical Absorbing Capacity) assay also showed high correlation with carotenoids ($R^2 = 0.9738$) (Fig. 6E), phenols ($R^2 = 0.9930$) (Fig. 6F), and ascorbic acid ($R^2 = 0.9804$) (Fig. 7C). These results coincide with those obtained by Corral-Aguayo et al. (2008) and Guorong et al. (2009) but differ from the results obtained by others (Mahattanatawee et al., 2006), where the correlation of ascorbic acid with ORAC was minimum ($R^2 = 0.23$). These differences are perhaps due to the types of fruit cultivation and procedures used in the extraction of the sample (Cantin et al., 2009; Corral-Aguayo et al., 2008).

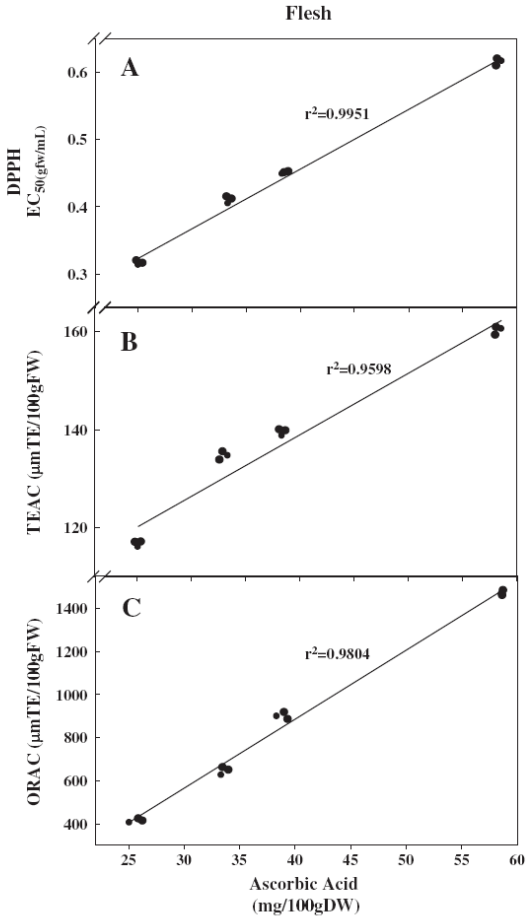

Fig. 7. Correlation of ascorbic acid data in flesh and antioxidant capacity by (A) DPPH, (B) TEAC, and (C) ORAC assay.

4. Conclusions

Results indicate that ferulic, *p*-coumaric, and caffeic acids are the most abundant acids in papaya skin. The most abundant carotenoids in pulp are lycopene, β-cryptoxanthin, and β-carotene. Vitamin C contents were higher in the pulp than in the skin, showing an increase of 233.7% in RS4 with respect to RS1. On the other hand, concentrations of phenolic compounds, carotenoids, and vitamin C were highly correlated with the antioxidant capacity measured by DPPH, TEAC, and ORAC. Results indicate that the consumption of ripened papaya is better due their higher concentrations of bioactive compounds, which can contribute to improve human health. As far as we know, this is the first report on "Maradol" papaya that evaluates the effect of RS on the changes of the most important phytochemical compounds. This information can be useful in determining the possible role of the identified compounds that can participate in the prevention of different health disorders. Further studies are needed to evaluate the biodisponibility of phytochemicals present in papaya, after being consumed fresh or processed.

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CAPÍTULO III

Contribution of major hydrophilic and lipophilic antioxidants from papaya (*Carica papaya* L.) var. "Maradol" to total antioxidant capacity.

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Contribution of major hydrophilic and lipophilic antioxidants from papaya (Carica Papaya L.) var. "Maradol" to total antioxidant capacity.

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Abstract Several studies have shown that phenol and carotenoid compounds protect the body against oxidative stress, reducing the risk of cardiovascular diseases and some types of cancers. The objective of this research was the determination of the following in papaya var. "Maradol": the individual contribution of the main phenolic compounds from the papaya's skin; the individual contribution of carotenoids from the pulp; the total antioxidant capacity at four ripening stages; and the individual and combined radical scavenging ability through essays DPPH (radical 2, 2-diphenyl-1-picryhydrazyl) and TEAC (radical 2, 20-azino-bis (3-ethylbenzothiazoline)-6 sulphonic acid). Phenolic acids' standards for this study were ferulic (FA), caffeic (CA) and *p*-coumaric (*p*CA) acids, where CA is the acid that contributes in greater proportion to the TAC (14.98%), followed by FA (6.22%) and *p*CA (0.86%). The phenol that showed the best DPPH• and ABTS•+ radical scavenging ability was CA, with 89.47% and 92.98%, respectively. The combination of CA and FA resulted in a significant synergy in the antioxidant capacity (94.92%), while the combination of the three phenols did not show a synergetic effect. Carotenoid standards were lycopene (Lyc), β-cryptoxanthin (BCr) and β-carotene (BC). Standard Lyc contributed in greatest extent to the TAC (11.9-43.22%), followed by BCr (10.95-28.04%) and BC (9.38-11.60%). Also, Lyc showed the best DPPH• and ABTS•+ radical scavenging ability with 62.12% and 94.26%; the combination of Lyc and BC did not show a synergetic effect (93.46%). Results showed that antiradical ability depends on the structure of the compound and its concentration.

Keywords Antioxidant activity · Carotenoids · Phenolics ·Radical scavenging activity · TEAC · DPPH

Introduction

There is plenty of epidemiological evidence demonstrating the association between a diet rich in fresh fruits and vegetables and the reduction of the risk of certain types of cancer and cardiovascular diseases [1]. Free radicals and other reactive species (RS) are constantly generated in vivo, playing a very important role in the age and patogenesis of a number of degenerative diseases due to their ability to alter several biomolecules (lipids, carbohydrates, proteins, nucleic acids), changing their structure and function [2]. Fruits and vegetables have valuable antioxidant compounds; the main protective action of these compounds is attributed to enzymes such as superoxide dismutase, catalasa and glutathione peroxidase, as well as to non-enzymatic antioxidants such as ascorbic acid, α-tocopherol, β-carotene, phenols, carotenoids and others [3].

Phenol compounds are commonly found in many fruits and vegetables, and it has been determined that they play a role in the defense mechanism against oxidative stress caused by reactive oxygen species (ROS) and free radicals. Additionally, phenols show multiple biological activities (antiproliferative, antiinflammatory, antimutagenic and antibacterial) [4]. Their antiradical activity is based in the structural relation between the different parts of their chemical structure [5]. Carotenoids are liposoluble antioxidants; they quench singlet oxygen by a physical mechanism, in which the excess energy of singlet oxygen is transferred to another carotenoid's electron-rich structure [6].

Total antioxidant capacity of fruits and vegetables could be attributed to different mechanisms and the combination of these could create synergetic [7], antagonistic or additive [8] effects. Several methods have been used to evaluate the antioxidant capacity of natural compounds in several foods; the two most common methods used are DPPH and ABTS, where the 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) and the 2, 20-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS•+) as generators of free radicals are used, respectively [9]. The mechanisms from both methods are similar, although essay DPPH has as a limitation the interference of color and the solubility of the sample; the reaction is dependent of the ability of the sample to scavenge free radicals, which is registered quickly by the change of color from purple to yellow, due to the capacity of donation of hydrogen [10]. On the other hand, ABTS radical has the advantage of being more versatile on polar samples and in the minimization of its interference on the spectrum when is being used at a maximum absorption of 760 nm [11]. The objective of this study was the evaluation of both the individual and combined contribution of the main phenol and carotenoid compounds that exist in papaya var "Maradol" at different ripeness stages, and their total antioxidant capacity.

Materials and methods

Standards and reagents

n-Hexane, dichloromethane, ethanol, methanol, K2S2O8, $Na_2S_2O_3$ were purchased from J. T. Baker (Baker Mallinckrodt, Mexico). Caffeic acid, ferulic acid, p-coumaric

acid, lycopene (purity≥90%), β-carotene (purity=95%) from carrots, 2,2-Diphenyl-1-picrylhydrazyl (DPPH·), **2**,2_-azinobis(3-ethylbenzo-thiazoline-6-sulfonate) $ABTS^{\cdot+}$, Trolox (6-hydroxy- 2,5,7,8-tetramethylchromane-2-carboxylic acid) all from Sigma Chemical Co (St Louis MO, USA).

Plant material

Papaya fruits (C. papaya L, cv. "Maradol") were obtained from a local market in Hermosillo, Sonora, Mexico. Fruits were selected for uniform size, color, level of external ripeness, and divided in four ripeness stages: RS1 represents papaya with yellow area on 0–25% of the skin; RS2>25 and 50%; RS3>50 and 75% and RS4>75 and 100%. After selection, fruits were divided in lots of 12 fruit each, and flesh and skin were randomly sampled and then, were freeze-dried and stored at −70 °C until analysis.

Sample preparation

Stock standard solutions (1 mmol/l) in methanol and acetone were prepared for each RS and appropriate dilutions were done for each type of measurement with the specific solvent of each method.

Extraction of hydrophilic fractions

Preparation of phenolic acids were determined according to a modified method described by Gayosso et al [12]. Papaya skin dry samples (0.5 g) were homogenized in 20mL of 80% methanol, using an Ultra Turrax®T25 basic homogenizer (IKA Works,

Willmington, NC), sonicated for 30 min at 30 °C in a 2510 model ultrasonic bath (Branson, Whetersfield, CT), centrifuged at 12,000g for 15 min at 5 °C in a Hermle centrifuge model Z323 K (Labortechnik Technologies, Germany), and then filtered through number 1Whatman paper. For alkaline hydrolysis 10 mL of 4 M NaOH was added to phenolic extracts and left for 4 h in the dark at roomtemperature. After incubation, extracts were acidified to pH 2.0 with 4 M HCl, then, acidified solutions were extracted twice with 20 mL ethyl acetate. Extracts were evaporated in a Rotovapor® (Büchi Labortechnik AG, Flawil, Switzerland) at 35 °C in a Buchi low-pressure evaporator. Skin samples were resuspended in 10 mL of 80% methanol and stored at -78°C to be used in the determination of DPPH and TEAC.

Extraction of lipophilic fractions

Carotenoids were determined according to Gayosso et al [12] and Ornelas-Paz et al [13] and Freeze-dried papaya tissue (0.5 g) was homogenized in 10 mL of hexane: dichloromethane (1:1, v/v), using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC) and centrifuged at 9000g for 10 min at 5 °C. Organic phase was separated, and procedure was repeated three times. For alkaline hydrolysis 10 mL of methanolic KOH 40% (1:1, v/v) was added to extracts for 1 h at 50 °C in a stirring bath set at 100 rpm. After saponification, 10 mL of 10% sodium sulfate was added for phase separation and the extracts were left for 1 h in the dark at room temperature. TC quantification was measured on top-phase aliquots in a Beckman DU-65 spectrophotometer at 450 and 470 nm. Extracts were evaporated in a Rotovapor®

(Büchi Labortechnik AG, Flawil, Switzerland) at 30 °C in a Buchi low-pressure evaporator. Flesh samples were resuspended in 2 mL acetone and stored at -78°C to be used in the determination of DPPH and TEAC.

Antioxidant capacity assay using DPPH radical

DPPH was determined according to the Brand-Williams et al [14] technique, with minor modifications for hydrophilic and lipophilic fractions and individual pure phenolics and carotenoids. The stock solution was prepared by mixing 2.5 mg of DPPH• radical with 100 mL of pure methanol. The solution was adjusted at an absorbance of 0.7±0.02 at 515 nm. Trolox (6-hydroxy-2, 5, 7, 8- tetramethylchromane-2-carboxylic) was used as a standard and 80% methanol was used as a blank, 3.9 mL of DPPH• radical were placed in a test tube and 100 μL of the extract were added. The mixture was shaken in a vortex and kept 30 min in the dark. The absorbance was read at 515 nm in an UV-VIS VARIAN CARY 50 BIO spectrophotometer. Antioxidant capacity of pure phenolics, carotenoids and each RS was expressed as Radical Scavenging Ability (%). Analyses were performed with a minimum of 6 replications per each RS and each standard.

Antioxidant capacity assay using ABTS radical

ABTS radical-scavenging activity for hydrophilic and lipophilic fractions and individual pure phenolics and carotenoids was determined according to Miller and Rice-Evans [15] and Re et al [16]. ABTS•+ cation was generated through the interaction of 19.2 mg of

ABTS (2'2-azino-bis(3-ethylbenzotriazoline-6-sulfonic acid)), dissolved in 5 mL of HPLC-grade water and 88 μL of potassium persulfate (K2S2O8) (0.0378 g mL^{-1}). The solution was held at room temperature in the dark for 16 h,then 1 mL of ABTS activated radical was taken and 88 mL of ethanol was added. The radical was adjusted at an absorbance of 0.7±0.02 at 734 nm. The reaction was initiated adding 2970 μL of ABTS•+ and 30 μL of the extract. The absorbance at 734 nm was at 1 and 6 min and antioxidant capacity of pure phenolics, carotenoids and each RS was expressed as Radical Scavenging Ability (%).Analyses were performed with a minimum of 6 replications per each RS and each standard.

Statistical analysis

The statistical significance of differences was analyzed through an analysis of variance (ANOVA) and the multiple comparisons of means through the Duncan's test. Statistical differences were considered to be significant ($p \leq 0.05$) using the statistical software SAS version 8.0 (SAS Inst. Inc.Cary, NC, USA).

Results and discussion

Percentage of individual contribution of phenolic compounds from papaya skin to TAC

In previous studies [17], once the total antioxidant capacity (TAC) was determined, we focused on the identification and quantification of the main phenol compounds that are

present at the highest concentrations on the hydrophilic portions of papaya skin [12], those being caffeic, ferulic and *p*-coumaric acids. Their concentration tended to decrease as the process of ripening of the fruit progressed (Table I); only traces were found in the pulp. These results coincide with previous reports from Rivera-Pastrana et al. (2010) in papaya fruit. Research done in other fruits have found that hydroxicinnamic acids are generally more abundant than hydroxibenzoic acids, and that skins have greater concentration of phenol compounds than pulp [18, 19], that their concentration decreased during ripening stages [20], and that their concentration is influenced by geography, variety and ripeness stages [21].

Later, an evaluation was performed of the individual contribution to TAC of phenol compounds of papaya skin in the hydrophilic extract. Figure 1 shows the percentage of individual contribution of phenol compounds; this calculation was done based on the TAC, which represents 100%, and using standards of these phenol acids according to the content found on the fruit at each of the ripening stages. It is possible to note that as RS advanced, the contribution of caffeic and ferulic acids decreased from 14.98 to 8.09% and from 6.92 to 6.22%, respectively; in the meanwhile, *p*-coumaric acid increased from 0.86 to 0.94. The rest of the 100% could be vitamin C, sugars and other phenols. Even when the concentration of caffeic acid is lower than of other acids, it is evident that the percentage of contribution to TAC is greater, because its activity depends mainly on its structure (dissociation energy, resonance, and steric hindrance derived from the substitution of hydrogen in the aromatic ring) and on its concentration in the food matrix [9]. A study performed by Jaikang et al. [22] concluded that the antioxidant activity of

caffeic acid occurs because of its possible relationship with the two hydroxyl groups that are present in the benzenic ring. In the case of ferulic acid [23] the study mentions that the presence of the metoxi group decreases the antioxidant capacity, concluding that ferulic acid is less effective than caffeic acid.

Percentage of carotenoids' individual contribution to TAC in papaya pulp

Just as in phenolic compounds, once TAC was determined, we focused on the identification and quantification of the main carotenoids that are present in highest concentrations of the lipophilic portion of the extract of papaya pulp [12]. Table 2 shows the highest concentration of carotenoids in papaya pulp: lycopene, β-carotene and β -cryptoxanthin; their concentration was inclined to increase with the process of ripening of the fruit. These results coincide with previous reports from Rivera-Pastrana et al. [24] and Wall [25] in papaya fruit.

Then an evaluation of carotenoid's individual contribution to TAC was performed in papaya pulp in lipophilic extract. Fig. 2 shows the percentage of carotenoids' individual contribution; this calculation was done based on the TAC, which is 100%, and using the standards for these carotenoids, according to the contents found in the fruit at each RS.

It was observed that RS advanced, generally the contribution of carotenoids increased, providing a total contribution of 32.23%, 41. 29%, 51.52% and 82.86% at RS1, 2, 3 and 4, respectively. The rest could be other carotenoid compounds and lipoproteins. The distinctive structural characteristic of carotenoids is formed by an extensive system of double conjugated links, which consist in the alternation of simple

and double carbon- carbon links that get stabilized by a resonance called polyenic chain. This part of the molecule, known as chromophore, is responsible for carotenoids' capacity for light absorption in the visible region, and consequently their great capacity for coloration and as antioxidants, giving them the ability to eliminate singlet oxygen and kidnap other reactive species of oxygen [6].

A study performed by Jimenez-Escrig et al. [26] showed that antioxidant capacity increased by the length of the system of the conjugated double links, and that it could be reduced with the addition of terminal rings (xanthophylls). On the other hand, studies done by Mortensen and Skisbsted [27] showed that the presence of an hydroxyl group in the terminal ring, such as in the case of β-cryptoxanthin, increased the antioxidant capacity, but at the same time this one is lower than the one provided by lycopene.

Evaluation of the effect of individual and combined phenol compounds in antioxidant capacity (% radical scavenging ability)

Once the percentage of contribution to TAC on behalf of phenol compounds was determined, the next step was to determine their individual and combined antioxidant capacity, using DPPH• and ABTS•+ radicals. The DPPH assay is based in the measurement of antioxidants' ability to lower radical DPPH• (2, 2-diphenyl-1-picrylhydrazyl). The test is quick and simple, and only a UV-vis spectrophototometer is needed to perform it, being the reason for its widespread use for the analysis of antioxidants [28].

For the evaluation of the percentage of individual radical scavenging ability, phenol acid standards were used in the quantified concentrations at different RS. Fig. 2 shows DPPH• scavenging ability of these compounds. Significant differences were observed in the scavenging ability of phenol acids: this ability decreased in the order CA (89.47%)>FA (62.27%)>pCA (43.43%). Results clearly show the importance of the effects of phenol structure, because antioxidant activity of hydroxycinnamic acids depends on the number of hydroxyl groups on the molecule, in addition of the effect given by the steric hindrance of their carboxyl group [29]. In a study performed to determine the antioxidant capacity of caffeic acid, it was observed that the capacity depended on the two hydroxyl groups that exist in the acid, and that every caffeic molecule could trap two peroxyl radicals [30].

Additionally, the effect of the combination of two or more phenols in the antioxidant capacity was evaluated, as shown on Fig. 3. The combination of CA and FA increased the antioxidant capacity (93.09%), while the combination of CA and pCA reduced the capacity (78.17%). The combination of FA and pCA resulted in the reduction of antioxidant capacity (57.71%), in comparison with the individual effect of FA. When combining the three phenol acids, radical scavenging ability was of 84.81% being a lower percentage compared to the obtained individually from the CA. A synergetic effect is produced when two or more antioxidants are present in a system, resulting in a total superior effect, which could be estimated by simple addition of their individual actions [31]. In general, results show that the combination of CA and FA creates a

significant synergy in the antioxidant capacity with respect to the individual use of these compounds, while the effect is lower with the combination of the three phenol acids.

Because the application of at least two essays is recommended to obtain more accurate information about the antioxidant capacity of a compound, an essay of TEAC was performed, which is based in the ability of antioxidants in the inhibition of radical cation ABTS•+ absorbance, through the donation of an electron or an H• reacting in aqueous and organic solvents [32]. Fig. 3 shows the results obtained for phenol acids, which were similar and slightly higher to the results obtained with DPPH essay. Several epidemiological studies suggest an inverse relation between the consumption of foods rich in phenol acids and the occurrence of a variety of diseases. Kang et al. [33] found that CA inhibited the activity of Fyn kinase, which belongs to the family of non-receptive proteins of tyrosine kinase, suppressing skin carcinogenesis, which suggests a chemo-preventive effect of this type of cancer. In a study performed by Chung et al. [34], liver metastasis was reduced significantly, confirming the anti-tumoral and anti-metastatic effects of CA and caffeic acid phenethylester (CAPE). With respect to the antioxidant and anti-inflammatory capacity of ferulic acid, it has been observed that this acid has positive effects against Alzheimer when mice were treated with FA, reducing the activity of the coline acetyltransferase [35]. In general, synergetic or antagonic effects of the compounds will depend on their structure, reactive mechanism and nature of the radical.

Evaluation of the individual and combined effect of carotenoids in antioxidant capacity (% radical scavenging ability)

Following the determination of carotenoids' percentage of contribution to TAC, we determined their individual and combined antioxidant capacity, using DPPH• and ABTS•+ radicals. During the individual evaluation of the percentage of radical scavenging ability, carotenoid standards were used in the quantified concentrations at different RS. Fig. 3 shows the DPPH• scavenging ability of these compounds; significant differences are observed in the antioxidant activity of lycopene (62.12%) and β-carotene (12.06%). It is well known that carotenoids possess strong differences in their redox potentials, due to their molecular structure.

A study performed to determine the antioxidant capacity of several carotenoids concluded that this capacity depends on the structure of each specific carotenoid, increasing in the following order: lycopene > β -cryptoxanthin > α-carotene > β-carotene > zeaxanthin > lutein [26]. Several in vitro studies have noted that lycopene is a powerful antioxidant, a quench singlet oxygen, and has the ability to scavenge free radicals [36]; in the meantime, β-carotene eliminates free radicals, neutralizes singlete oxygen and protects DNA from its mutagenic activity [37]; one mole of β-carotene can quench 250 to 1000 molecules of singlet oxygen [38] and may donate electrons instead of hydrogen atom to free radicals, and become β-carotene radical action [27].

During this study, we obtained values for the percentage of radical scavenging ability, because of carotenoids' double link structure, with spectrums that can be overlap on radical DPPH• at 515nm, resulting in the recording of lower values [39]. In contrast, the evaluation with essay TEAC recorded superior values in the antioxidant capacity. β-

carotene had a 30.04% radical scavenging ability, while lycopene was three times larger (94.26%) (Fig. 3). Lycopene's quenching ability has been related to the aperture of the b-ionone ring in its chain [40]. Carotenoids' antioxidant capacity will depend on the number of conjugated double links and on the presence of oxygenated functions in its molecule [41].

On the other hand, the combination of lycopene and β-carotene's scavenging ability was measured using radical DPPH• and ABTS•+. Fig. 3 shows the results obtained, where this combination did not increase antioxidant capacity (61.76%), compared to the individual results from lycopene using DPPH technique, and at the same time there were no significant differences in the individual and combined evaluation using the TEAC technique (93.46%). While comparing the molecular structures of lycopene and β-carotene, it has been confirmed that carotene's ability for scavenging radical ABTS increases with the extension of cromophore [15]. Other studies have observed that the mix of carotenoids has been more effective against liposome oxidation, that the use of individual carotenoids and that the combination with lycopene and lutein promote a synergetic effect, increasing the antioxidant activity of the mix, in comparison with a low synergetic effect obtained when using individually α-carotene, β-carotene, and other carotenoids [42].

Other studies have suggested that antioxidant activity is the result of the combination of each one of the components of the mix, and that a synergetic or antagonist effect can be generated, depending on the environment where the compounds are found [43]. Carotenoid compounds have important biological activities, such as antioxidant activity,

the stimulation of the intercellular communication, the control of cellular growth, the intercellular differentiation in growth control, cell differentiation (mutagenesis inhibition), and the modulation of immune response [6]. Results suggest that scavenging ability of the combination of carotenoids was higher than the one presented by β-carotene alone, which is in favor of the increase of antioxidant capacity of these types of compounds.

Conclusions

Our results show that the contribution of phenolic acids to the TAC depends mostly on their structure, that the combination of ferulic acid and caffeic acid recorded the best radical scavenging ability, while the combination of caffeic, ferulic and p-coumaric acid did not showed a synergetic effect that could contribute to increasing antioxidant capacity. In relation to the contribution of carotenoids to the TAC, lycopene was the compound that contributed the most, followed by β-criptoxantin, showing again that the structure of the molecule played an important role in the results obtained. Radical scavenging ability results showed that the interaction of lycopene with β-carotene did not have an antagonic effect, and although it is necessary to perform further studies to understand the mechanisms involved in the increase of the synergy of this type of compounds, this mix could be considered for the design of dietary supplements that could contribute in the improvement of human health.

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Figure legends

Fig. 1. Percentage of the contribution of total antioxidant capacity (TAC) of the main phenol acids in the skin of papaya var. "Maradol" at different RS.

Fig. 2. Percentage of the contribution to total antioxidant capacity (TAC) of the main carotenoids in papaya var. "Maradol" at different RS.

Fig. 3. Radical Scavenging Ability (%) of single phenolics, carotenoids and in combination measures by DPPH and TEAC methods (**FA:** ferulic acid; **CA:** caffeic acid; **pCA:** p-Coumaric acid; **BC:** β-Carotene; **L:** lycopene.

Table legends

Table 1. Contents of the main phenol compounds identified in the skin of papaya var. "Maradol". Values in the same column with different letters vary significantly ($p \leq 0.05$).

Table 2. Contents of the main carotenoids identified in the pulp of papaya var. "Maradol". Values in the same column with different letters vary significantly ($p \leq 0.05$).

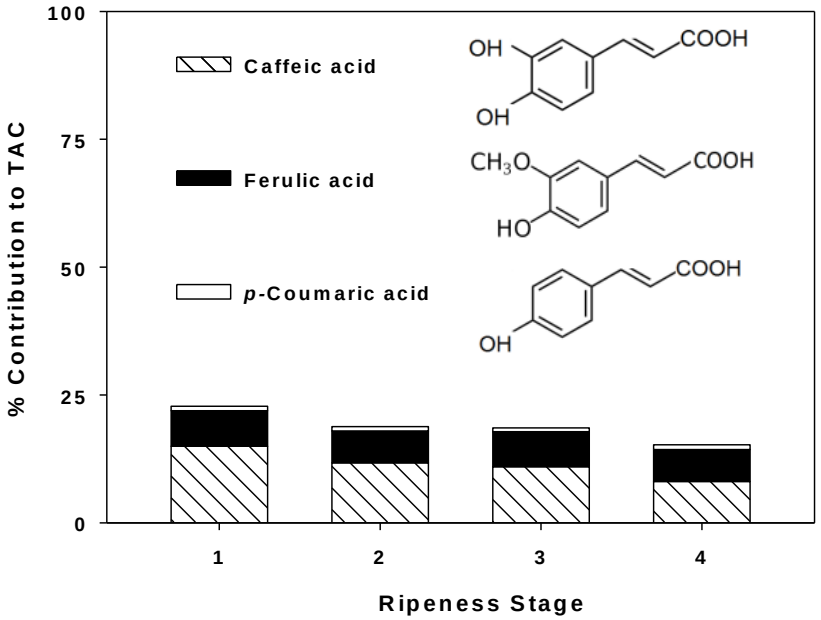
100
75
50
25
0
% Contribution to TAC
Caffeic acid
Ferulic acid
p-Coumaric acid
OH
OH
COOH
CH3O
HO
COOH
OH
COOH
1
2
3
4
Ripeness Stage

Fig. 1

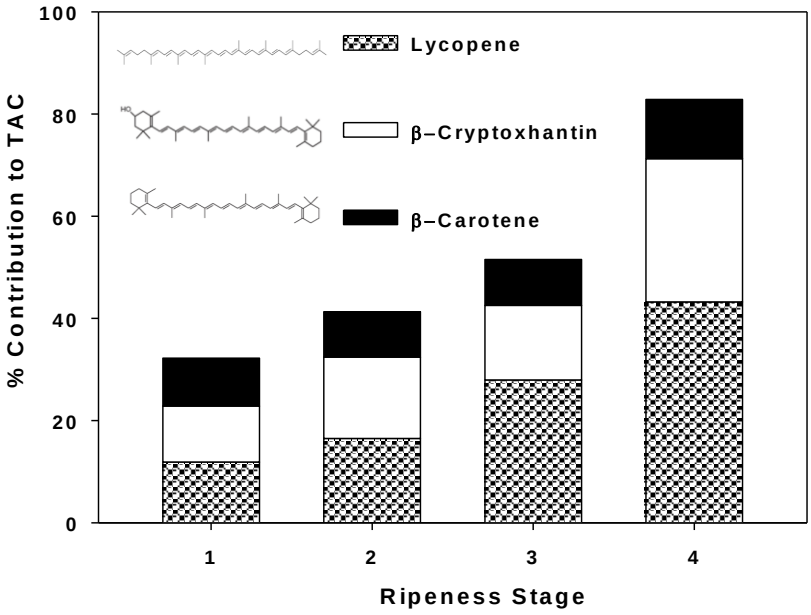
100
80
60
40
20
0
% Contribution to TAC
Lycopene
β–Cryptoxhantin
β–Carotene
1
2
3
4
Ripeness Stage

Fig. 2

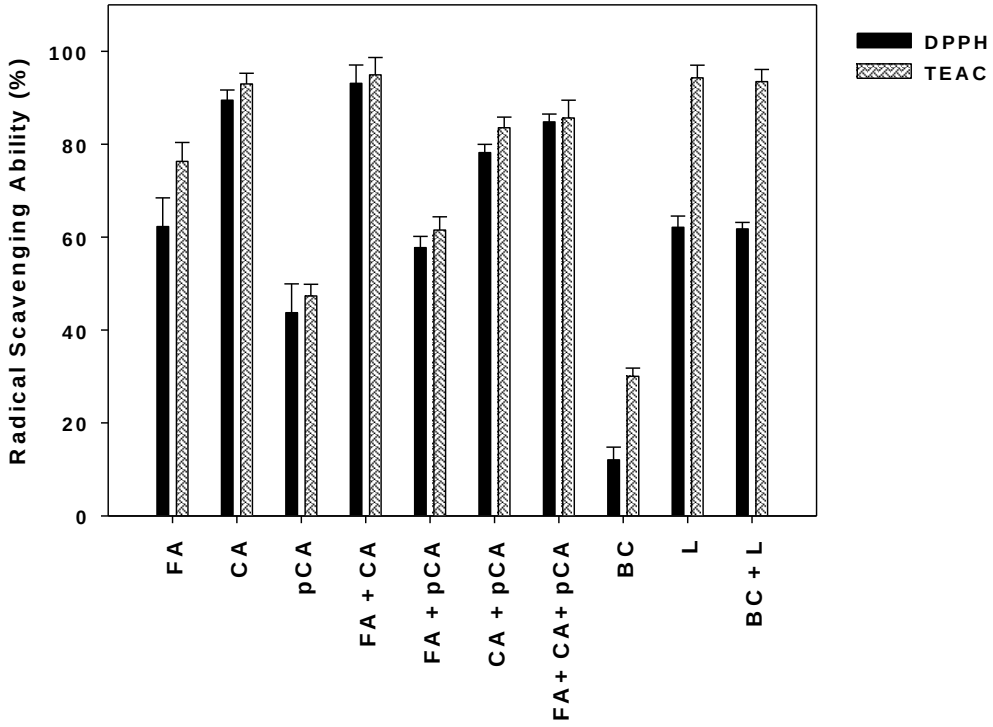
Radical Scavenging Ability (%)
0
20
40
60
80
100
DPPH
TEAC
FA
CA
pCA
FA + CA
FA + pCA
CA + pCA
FA+ CA+ pCA
BC
L
BC + L

Fig. 3

Table 1

Ripeness Stage	Caffeic Acid	Ferulic Acid	*p*-Coumaric Acid
	mg/100gDW		
1	175.50[a]	277.49[a]	229.58[a]
2	151.37[a]	205.19[b]	192.86[b]
3	146.21[a]	189.80[b]	151.11[c]
4	112.88[b]	186.63[b]	135.64[c]

Different letters in the same column indicate significant differences ($p \leq 0.05$)

Table 2

Ripeness Stage	Lycopene	β-Cryptoxanthin	β-Carotene
	mg/100gDW		
1	0.524[a]	0.331[a]	0.387[a]
2	2.48[b]	0.558[b]	0.409[a]
3	2.78[b]	0.794[c]	0.441[a]
4	4.23[c]	1.295[d]	0.752[b]

Different letters in the same column indicate significant differences ($p \leq 0.05$)

CAPÍTULO IV

Carotenoids: Antiproliferative Activity On Cell Lines

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Carotenoids: Antiproliferative Activity on Cell Lines

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Abstract

Nowadays, fruits and vegetables are recognized for their important components, and because their intake help in the prevention of a great number of illnesses, such as cardiovascular diseases and some types of cancer. Carotenoids are pigments found in most plants, prevent photo-oxidative damage, attract pollinators, and potentially may play a role as antioxidants, in both humans and foods. Carotenoids are the product of the isoprenoid biosynthetic route; they are lipophilic with forty atoms of carbon that could be totally linear or have rings in one or both extremes. In human blood, they have been identified mainly with lutein, zeaxanthin, β-cryptoxanthin, lycopene, β-carotene, and α-carotene. In several *in vitro* and *in vivo* studies it has been demonstrated that carotenoids exhibit biological activities with impact on: the expression of some genes; the inhibition of specific enzymes involved in the development of some types of cancer; and on the routes of cellular signalization. Carotenoids can have an inhibitory effect in cell proliferation, preventing the arrest in G0/G1 phase of cell cycle, and reducing the expression of p53, p21 and cyclin D. However, there is also evidence that the intake of β-carotene in high concentrations could enforce cell proliferation, resulting in the increase of the risk of malignant tumors. In this review, results obtained in the various *in vitro* and *in vivo* studies show the antiproliferative capacity of carotenoids.

Keywords: Carotenoids, Lycopene, β-carotene, β-cryptoxanthin, oxidative stress, proliferation *in vitro*, proliferation *in vivo*.

Introduction

Numerous epidemiological studies have associated a diet high in fruits and vegetables with the reduction of the risk of cardiovascular diseases, cancer, and diabetes (Riccioni et al. 2008). Fruits and vegetables are a source of phytochemicals that act as antioxidants and prevent or reduce antioxidative stress. Oxidative stress, generated by several reactive oxygen species (ROS), has been identified as the cause of several chronic diseases (Cerutti 1985). Carotenoids are important phytochemicals that have antioxidant action, and protect oxidative stress of cells through various mechanisms (Maiani et al. 2009). Some carotenoids, mainly β-carotene and β-cryptoxanthin, are precursors of vitamin A, which intervenes in important human physiological processes (Paiva and Rusell 1999).

Carotenoids are substances that possess antiproliferative activity (Larsson et al. 2010), and lycopene is the carotenoid that has the greatest antioxidant and antiproliferative capacity, due to its quenching ability of singlet oxygen (Omoni and Aluko 2005). β-carotene is considered a liposoluble antioxidant that can reduce the risk of heart attacks and increase the efficiency of the immune system (Bjelakovic et al. 2007). However, recent studies have observed that β-carotene not only is not able of reducing the risk of lung cancer, but can have the opposite effect of increasing it in people smoking (Lin et al. 2009). β-cryptoxanthin is a carotenoid that protects against cancer, cardiovascular diseases, and reduces macular degeneration of the eye (Lorenzo et al. 2009). The

purpose of this review is to reveal the results obtained in the measurement of carotenoids' antiproliferative effect on various cell lines.

Reactive Oxygen Species (ROS)

In the past few years, the number of cardiovascular and chronic diseases has increased, due mainly to the changes on people's lifestyles and nutrition. In response, several epidemiological studies have recommended the increase of the intake of foods rich in phytochemicals (carotenoids, phenols, vitamin C), which not only provide beneficial effects on human health, but also play a very important role in the prevention of this type of diseases (Ignarro et al. 2007; Vitale et al. 2010).

Reactive oxygen species (ROS) are highly reactive molecules, generated endogenically through metabolic processes and other factors, such as lifestyle, exercise, diet, and smoking (Story et al., 2010). When concentrations of ROS are found in levels higher than normal, oxidative stress is induced, which is associated with several chronic illnesses, such as cancer and cardiovascular diseases (Rao and Agarwal 1999).

Carotenoids

Carotenoids are natural pigments, synthesized by plants and microorganisms. They are liposoluble tetraterpenoids, and have an extensive system of doubled conjugated links, called polyene chain (Yahia y Ornelas-Paz 2010). About 600 carotenoids have been

identified: carotenes (hidrocarbonated carotenoids) and xanthophylls (carotenoids with at least one molecule of oxygen), partially responsible for yellow, orange, and red colors in fruits and vegetables (Perera & Mei 2007); from these, 20 have been identified in human blood and tissue, mainly lycopene, α-carotene, β-carotene, lutein y β-cryptoxanthin (Figure 1) (Gerster, 1997).

The polyene chain, named chromophore, is responsible for the absorption of light, and provides the ability of coloration. It is classified as cyclic or acyclic, and in ripened fruit, most of its components are found esterified with fatty acids. Carotenoids are a system of double conjugated links, with linear, rigid molecules, with *all-trans* configuration, while those that have *cis*-isomers shape have a lesser ability to crystallize or aggregate, making them faster to be solubilized, absorbed and transported than all-*trans* carotenoids (Yahia and Ornelas-Paz 2010).

Fruits and vegetables constitute the main sources of carotenoids in human diet (Johnson 2002), and their consumption has been correlated with the lesser incidence of chronic-degenerative illnesses, such as cardiovascular diseases and cancer. Based on this, the interest to further analyze the benefits of these compounds has increased (O'Sullivan et al. 2007). Several research studies have demonstrated the antiproliferative capacity of carotenoids in several cellular lines of cancer (Pastori et al. 1998; Karas et al. 2000; Boileau et al. 2003).

Antioxidant Activity

Carotenoids have a great potential to decrease free radicals, due to their antioxidant capacity, which is related to their system of double links (Birt 2009). One example is lycopene, the most powerful antioxidant to quenching singlet oxygen (Di Mascio et al. 1989). During physical stabilization, singlet oxygen transfers its excitation energy to the carotenoid, and later it releases it to the environment in the form of heat. This is the reason recent epidemiological studies have been focused on the modes of lycopene's action (El-Agamey et al. 2004).

Reactive oxygen and nitrogen species are capable of damaging several types of biomolecules (lipids, proteins, carbohydrates and DNA), causing degenerative diseases in humans, although it has been observed that carotenoids could be involved in the scavenging of this type of species (van den Berg et al. 2000).

Carotenoids are partially responsible for colors ranging from yellow to red. Some have provitamin A activity (β-carotene, α-carotene y β-cryptoxanthine), and also have a powerful antioxidant activity, while others like lycopene lack provitamin A activity, due to the absence of a beta ionone terminal ring (Rao and Rao 2007).

Lycopene is a liposoluble antioxidant, synthesized by many microorganisms and plants, but not by animals or humans (Paiva et al. 1999). It has two non-conjugated double links, making it very reactive to oxygen and free radicals, and able to exist in *cis* and *trans* isometric forms (Rao and Rao 2007). The double links' instability to light, heat and chemicals promotes isomerization (Henry et al. 2000). Lycopene's *all*-trans

isomer is commonly found in food (Nguyen and Schwartz 2001), while *cis* isomeres are found in human blood and serum (Boileau et al. 1999; van Breemen and Pajkovic 2008). *Cis* isomeres are more stable and have a greater antioxidant capacity than *all*-trans isomeres (Chasse et al. 2001).

Lycopene could reduce the risk of chronic illnesses, including cardiovascular diseases (CVD) and cancer caused by oxidative stress (Wertz et al. 2004). Lycopene's antioxidant activity exists because of its ability to trap peroxil radicals, and because of its singlet oxygen-quenching property (Shi 2000), which is twice as high as that of β-carotene and 10 times higher than that of α-tocopherol (Agarwal and Rao 2000).

Antiproliferative activity *in vitro*

Carotenoids present in fruits and vegetables show several beneficial properties in humans (especially lycopene y β-carotene), such as antioxidant activity, inhibition of cell cycle, induction of apoptosis, increased intercellular communications, modulation of insulin-like growth factor-1 (IGF1), inhibition of cell proliferation, modulation of carcinogenic metabolism, and enhancement of immune system function (Figure 2) (Wertz et al. 2004; Sharoni et al. 2004, Yahia and Ornelas-Paz 2010).

Obermuller-Jevic et al. (2003) observed that lycopene inhibited the growth of normal human prostate epithelial cells *in vitro*, through the arrest of the transition from G1 to S phases of cell cycle. This reduction depended on the dose utilized in cyclin D1, protein

involved in the facilitation of cell cycle's progression, and overexposed in several kinds of tumors (Moyano et al. 2004). In a study performed by Burgess et al. (2008), where seven human cell lines where used to measure the effect of lycopene, a reduction of the proliferation of liver adenocarcinoma cell line (Hep-G2) was observed, as well as noncancerous lung cell line IMR-90, although the proliferation of the following were not affected: A431 lines, skin carcinoma, DU-145, prostate carcinoma, HS-68, noncancerous skin, A549, lung carcinoma, and HS-578T, breast carcinoma.

Carotenoids have biological mechanisms that still have not completely been elucidated, so researchers have been focusing on determining their activity in several cell lines. Levy et al. (1995) researched the effect of lycopene in comparison to α and β-carotene, using endometrial (Ishikawa), mammary (MCF-7), and lung (NCI-H226) human cancer cells. Results showed that lycopene was more effective to inhibit cell growth than α- and β-carotene, which was by far a less effective inhibitor. Additionally, lycopene also suppressed IGF1 growth, which is the most important autocrine/paracrine regulator of growth of endometrial and mammary cancerous cells.

Other studies have focused in determining the dose in which lycopene could be effective in several cell lines. Salman et al. (2007) examined the antiproliferative and apoptotic effect of lycopene in the following cell lines, at different concentrations: human colon carcinoma (HuCC), B chronic lymphocytic leukemia (EHEB), human erythroleukemia (K562) and Raji, a prototype of Burkitt lymphoma cell line. Results showed that lycopene could perform, only with a significant dose-dependent effect on

the proliferation capacity of K562, Raji and HuCC cell lines, so the conclusion was that the effect of lycopene depends on the dose and the type of cell line.

In a study performed by Kotake-Nara et al. (2001) to find out the chemioprotector effect of 15 carotenoids in 3 cell lines of human prostate cancer (PC-3, DU 145 and LNCaP), it was observed that phytofluene, β-carotene, lycopene, neoxanthin and fucoxanthin significantly reduced cell proliferation, while phytoene, canthaxanthin, β-cryptoxanthin and zeaxanthin did not affect the growth of the prostate cancer cells.

Experimental studies have also shown that lycopene inhibits cell proliferation of human colon cancer cells, by suppression of Akt, and increased the expression of p27Kip1 (Tang et al. 2008). Protein AKt plays a very important role in the routes of cell survivorship, inhibiting apoptotic signals; for this reason it is activated as a response to DNA damage and with a clear implication of cancer generation (Nicholson and Anderson 2002).

Several investigations have demonstrated the antiproliferative effect of carotenoids on MCF-7 and T47d mammary cancer cells, which, supplemented with lycopene, significantly reduced the IGF1 induction of cell signaling, and decreased the levels of cyclin D1, maintaining the levels of p27 in E-cdk2 cyclin complexes (Nahum et al. 2001). Protein p27 inhibits cell cycle through cyclins in phase G1, prior to the restriction point, so the loss or reduction of its expression relates to the formation of mammary tumors (Masciullo et al. 2000).

On the other hand, in most of the studies, lycopene has been associated with its ability to block cell cycle in G0/G1 phase in breast and prostate cancer cell lines (van Breemen et al. 2008). Park et al. (2005) reported that lycopene induced the arrest in G0/G1 phases, and blocked S phase, decreasing the proliferation of Hep3B human hepatoma cells. Nevertheless, it is important to point out that several research teams have obtained different results, indicating that lycopene, in physiological ranges, does not affect cell proliferation, suggesting the need to perform more careful studies (Burgess et al. 2008).

Antiproliferative effects of lycopene were reported by Fornelli et al. (2007) in human breast cancer cell line MCF-7, observing the induction of Gap Junction Intercellular Communication (GJIC), which was confirmed through the increase of connexin 43 expression. Gap junctions are water-filled pores connecting the cytosol of neighboring cells, which allow an exchange of low molecular weight compounds, and are made up of transmembrane proteins (connexins). These results coincided with those presented by Forbes et al. (2003), who suggested that the increase of connexin 43 could be transcedental to revert the malignant processes in carcinogenesis.

When evaluating the effects of lycopene in human cancer cells derived from the oral cavity (KB1), Livny et al. (2002) observed that lycopene strongly and dose dependently inhibited the proliferation of KB-1, and also upregulated the transcription and expression of the GJIC protein connexin 43. Other studies performed by Kim et al. (2002) to measure the effect of lycopene in the proliferation of human prostate cancer cells (LNCaP), observed that lycopene decreased their growth, having a dose-response effect.

Although *in vitro* and *in vivo* studies do suggest that lycopene reduces the risk of cancer due to its antioxidant properties, improvement of gap-junctional communication, upregulation of detoxification systems, modulation of signal transduction pathways, reduction of cell proliferation, and delay of cell cycle progression (Bhuvaneswari and Nagini, 2005). In a study performed by Chen et al. (2010) it was observed that lycopene inhibited the proliferation of vascular smooth muscle cells (VSMCs) from G1 into S phases, in dose-dependent concentrations.

Some publications have shown that carotenoids are able to modify the differentiation and progression of cell cycle. Murakoshi et al. (1989) observed that α-carotene had an inhibitory effect, 10 times more powerful than β-carotene in the proliferation of cell lines of human neuroblastom GOYO, in a dose-time dependent manner.

Pastori et al. (1998) studied the effect of lycopene in an individual form, as well as associated to α-tocoferol in two human prostate carcinoma cell lines (DU-145 and PC-3), and found that lycopene not only inhibited cell proliferation, but also that, together with α-tocoferol, lycopene had a strong inhibitory effect in both cell lines. Prakash et al. (2001) designed a study to determine the effects of β-carotene, lycopene and canthaxanthin in the proliferation of human breast cancer cells (MCF-7, Hs578T and MDA-MB-231). They observed that β-carotene significantly reduced the proliferation of MCF-7 and Hs578T cells; lycopene inhibited the growth of MCF-7 and MDA-MB-231 cells; and canthaxanthin did not inhibit the proliferation of the three cell lines utilized.

In a study performed by García-Solís et al. (2009) to evaluate the antiproliferative capacity of 14 extracts of nutritional plants in breast cancer cell MCF-7, it was observed that only papaya extract was able to reduce cell proliferation, suggesting that the combination of phytochemicals of this fruit determine its biological activity, and that lycopene is a powerful antioxidant that inhibits cell proliferation in breast cancer, through the reduction of cyclin D levels and the inhibition of the phosphorylation of Rb gene (Nahum et al. 2001).

Otsuki et al. (2010) examined the effect of the aqueous extracts of papaya leaves in the inhibition of the growth of cell lines of cervical carcinoma (Hela), breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), lung adenocarcinoma (PC14), pancreatic epithelioid carcinoma (Panc-1), and mesothelioma (H2452). It was observed that the extract inhibited cell proliferation in a dose-dependent manner.

In the study performed by Rahmat et al. (2002) to determine the anticarcinogenic potential of pure lycopene and lycopene extracted from cantaloupe and papaya juice in cell lines of human liver cancer (HepG2) and breast cancer (MDA-MB-231), it was observed that pure lycopene and papaya juice reduced cell proliferation of liver cancer cell line (HepG2), while cantaloupe juice had anticancerigen properties, specifically in breast cancer cell line (MDA-MB-231). In general, the juices showed to be more effective with lycopene extract to inhibit the proliferation of cancer cells.

β-carotene is an antioxidant that protects human bodies against the damage caused by free radicals, giving it anticarcinogenic properties. Because of this, Palozza et al. (2009)

analyzed the effects of β-carotene on the proliferation of human colon adenocarcinoma cells (HT-29), and observed that there was inhibition of cell growth, dose dependent, with a halt on the progression of cell cycle in G0/G1 and G2/M phases.

Antiproliferative activity *in vivo*

β-Cryptoxanthin is found in citrics and in animal products such as eggs and butter (Granado et al. 1996). It is an antioxidant that prevents damage caused to biomolecules by free radicals (lipids, proteins and nucleic acids); it has provitamin A activity; and with in vitro studies it has been determined that it plays an important role in the prevention of some cancers (Soprano et al. 2004; Dhuique-Mayer et al. 2005; Tanaka et al. 2000). Toniolo et al. (2001), investigated the effect of β-cryptoxanthin in the potential risk of mammary cancer, finding that its presence in serum was related to the reduction of breast cancer in a dose-dependent manner, while α-carotene and β-carotene had no effect. To determine the chemopreventive properties of β-Carotene, lycopene, β-cryptoxanthin, zeaxanthin and lutein, Mühlhöfer et al. (2003) performed biopsies of colorectal adenomas, and results obtained suggested that carotenoids could be used as biomarkers for the predisposition of colorectal cancer, and that carotenoid supplementation could be beneficial in the cases of colorectal adenoma.

Narisawa et al. (1999) studied the effect of β-cryptoxanthin in rats F344, and observed that those rats that were fed with a diet supplemented with 25ppm of β-cryptoxanthin, showed a significantly less incidence of colon cancer in comparison to the control group, hence suggesting that a diet with β-cryptoxanthin could reduce colon carcinogenesis.

Because various epidemiological studies have suggested that lycopene acts against certain types of cancer, Narisawa et al. (1998) researched the inhibition of the proliferation of colon carcinogenesis by lycopene and tomato juice in F344/NSIc rats, concluding that tomato juice Rich in lycopene may have a protective antiproliferative effect against colon cancer.

In a study performed by Kim et al. (2000) in B6C3F1 mice of both sexes, to see the effects of lycopene on liver, colon and kidney carcinomas, it was observed that there was no effect on colon and kidney tumors, but there was a reduction of hepatocelular carcinomas in male mice, but no in the females.

Several studies performed in animals have shown the effect of lycopene in the inhibition of the proliferation of several types of cells. To test the effect of lycopene in the proliferation of prostate cancer cells, Limpens et al. (2006) used a model of BALB/c nude mice. Results showed that lycopene reduced the growth of tumors when it was combined with α-tocopherol acetate.

When evaluating the effect of lycopene in prostate cancer cell growth (DU145) in BABLB/c nude mice, Tang et al. (2005) observed that lycopene reduced the growth of the tumors. Nagasawa et al. (1995) researched the effect of the chronic intake of lycopene on the growth of breast cancer in SHN virgin mice, and found that those mice fed with lycopene reduced the growth of breast cancer. Other Studies have suggested that supplementation of lycopene could reduce the growth of prostate cancer, but more studies are needed to confirm this affirmation (Haseen et al., 2009).

In vitro and in vivo models have shown that b-carotene has a powerful capacity to scavenging free radicals with beneficial effects on human health, such as cell proliferation inhibition (Patil et al. 2009). Several studies suggest that carotenoids could be powerful inhibitors of tumor growth. In studies with animal models, when carotenoids where applied, a reduction of carcinogenesis was observed, indicating that carotenoids are capable of inhibiting cell proliferation during the first stages of the tumor (Collins 2001). However, Wolf (2000) observed that when ferrets' diet had a high supplementation of β-carotene combined with the daily exposure to cigarette smoke, cell proliferation increased, and facilitating lung cancer. In contrast, Liu et al. (2004) observed that low doses of b-carotene supplemented to ferrets, had protective effects in animals' lungs.

In a study performed by Cheng et al. (2007) to study the effects of lycopene carotenoids, β-carotene y lutein in the proliferation of cell nuclear antigen (PCNA) in oral cancer induced in hamsters, it was observed that carotenoids inhibited cell proliferation, acting as suppressors of the carcinogenesis in the animals, through the inhibition of the PCNA expression.

Conclusions

Carotenoids are lipophilic compounds present in fruits and vegetables; they possess antioxidant and antiproliferative activity, being able to reduce the risk of certain kinds of cancer. Because of their presence in human blood and their antiproliferative properties,

the most widely analyzed carotenoids are: lycopene, β-carotene, lutein and β-cryptoxanthin. In various *in vitro* and *in vivo* studies, evidence has been found that lycopene is the carotenoid with the most anticarcinegic potential, due to its antioxidant properties that facilitate mechanisms such as modulation of gap junctional intercellular communication, immune systems, cell cycle regulation, apoptosis, cell differentiation, and antiproliferative activity. However, future research on fruits must focus in the elucidation of mechanisms in which carotenoids reduce cell proliferation in certain types of cancer.

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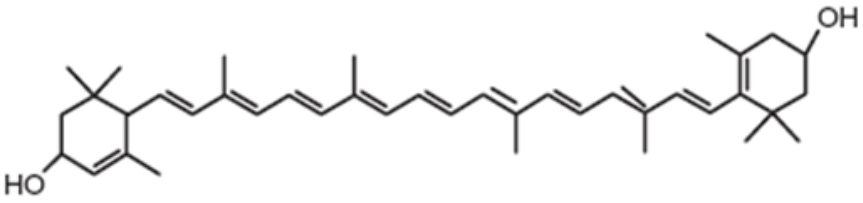
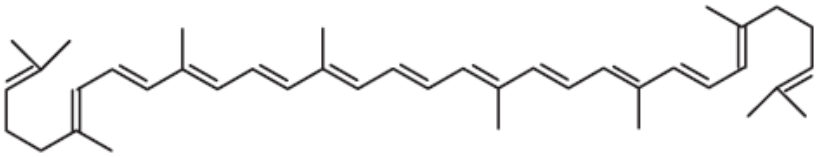


Lutein



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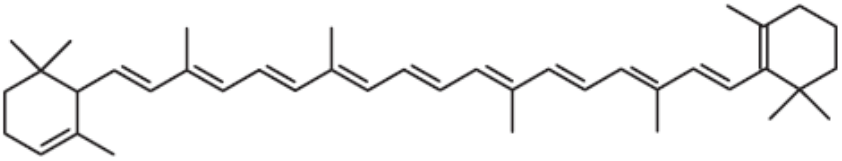
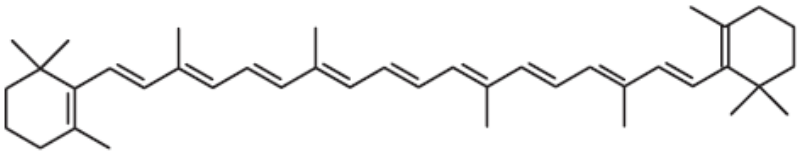
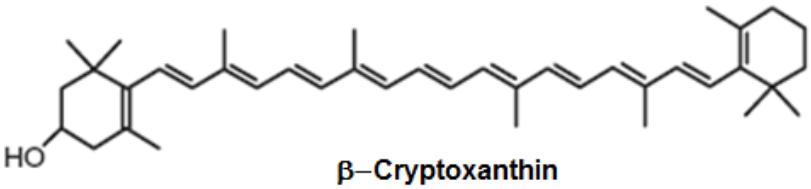


α– Carotene



β– Carotene



β–Cryptoxanthin

Figure 1 Examples of carotenes and xanthophylls

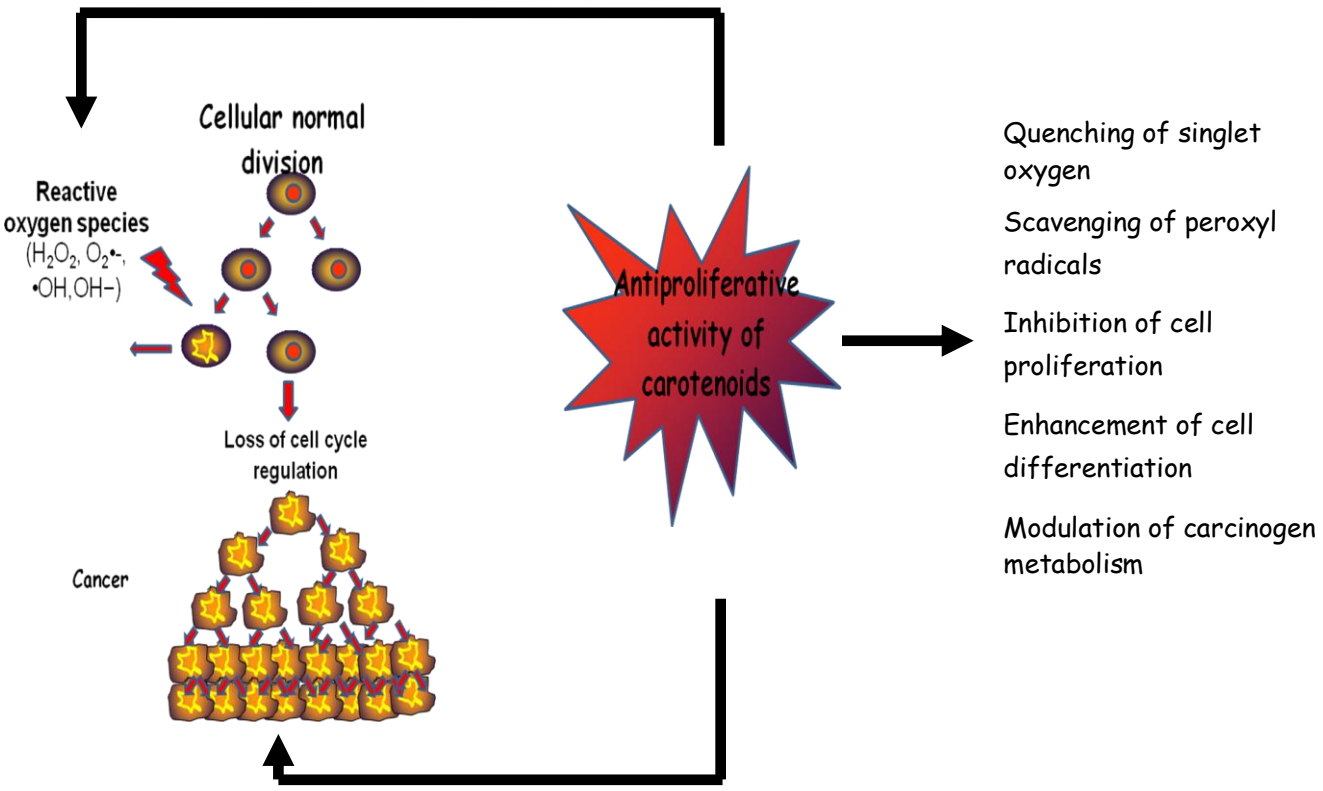


Figure 2 Several beneficial properties in humans of carotenoids

CAPÍTULO V

Inhibition of Cell Proliferation of Breast Cancer Cells MCF-7 and MDA-MB-231 by Lipophilic Extracts of Papaya (*Carica Papaya* L.) var. "Maradol".

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Inhibition of Cell Proliferation of Breast Cancer Cells MCF-7 and MDA-MB-231 by Lipophilic Extracts of Papaya (*Carica Papaya* L. var. "Maradol")

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Abstract

Several epidemiological studies have suggested that carotenoids have antineoplasic activities. The objective of this study was to determine the antiproliferative effect of rich carotenoid lipophilic extracts of papaya pulp (*Carica papaya* L., cv "Maradol") on breast cancer cells, MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) and on non-tumoral mammary epithelial cells MCF-12F. Antiproliferative effect was evaluated using the methylthiazolydiphenyl-tetrazolium bromide (MTT) assay and testing lipophilic extracts from different papaya ripening stages (RS1, RS2, RS3, RS4), at different times (24, 48 and 72 h). Papaya lipophilic extracts do not inhibit cell proliferation of MCF-12F and MDA-MB-231 cells. However, MCF-7 cells showed a significant reduction of proliferation at 72 h with the RS4 papaya extract. Results suggested that lipophilic extracts had different action mechanisms on each type of cell. More studies are needed to elucidate this mechanism.

Key words: *Carica papaya*, antiproliferative activity, breast cancer, carotenoids, papaya

Introduction

According to the Global Cancer Statistics cancer caused 7.6 million deaths in 2008, from which 460,000 were breast cancer related, and it is forecasted that by 2030 this number will increase to 11 million (World Health Organization 2011). Recent research studies

established that if oxidative stress (generated by an overproduction of free radicals) is excessive, and DNA repairing systems are surpassed, a mutagenesis and carcinogenesis could be promoted (Lee et al. 2004).

Several studies have suggested that the consumption of fruits and vegetables could reduce the risk of many chronic diseases, and have a protective effect against certain types of cancer (Yahia 2010). In response to this, the US Department of Health and Human Services (USDA/HHS 2010), is now recommending to increase the consumption of fruits and vegetables from 5 to 13 portions a day. The beneficial effect of diets rich in fruits and vegetables is attributed mainly to bioactive components (carotenoids, phenolic compounds, flavonoids, vitamins C and E) that provide antioxidant, antimicrobial and antiproliferative properties (Gonzalez-Aguilar et al. 2008; McEligot et al. 2005; Borek 2004). A high consumption of carotenoids could be associated with a reduction of the risk for breast cancer, because these types of compounds show several biological activities, from which the promotion of apoptosis in transformed cells stands out (Sumantran et al. 2000). These kinds of antioxidants induce cell differentiation, repair damaged DNA, inhibit gene mutation, and activate tumor-suppressive genes (Rock 2002). Additionally, a panel of experts concluded that an inverse association of carotenoids with the risk of breast cancer is possible, due to the antioxidant properties of carotenoids (Krinsky and Johnson 2005).

Cancer is one of the main causes of mortality. It originates as the result of the interaction of various genetic, physical, chemical and biological factors that transform

cells until they become malignant tumors (Musa-Veloso et al. 2009). Tumors are usually localized, but can disseminate to other organs, originating metastasis (World Health Organization 2011). According to the Mexican Ministry of Health, breast cancer, has become the main cause of death by cancer in Mexico in women 25 years and older since 2006, replacing uterine cancer (Knaul 2009). Mexico is the biggest exporter of papaya (SAGARPA 2010); a climacteric tropical fruit that has a combination of bioactive components (BC) that confer the fruit several antioxidant properties (Gayosso-García Sancho et al. 2010; Corral-Aguayo 2008). This fruit is especially rich in carotenoids, such as lycopene, β-cryptoxanthin and β-carotene (Marelli de Souza et al. 2008; Rivera-Pastrana et al. 2010; Gayosso-García Sancho et al. 2011). Most of the research performed in different cell lines have focused on the utilization of isolated doses of several BCs, and have not considered the importance of employing a mixture, such as the naturally found in fruit, in order to determine the beneficial effect on health of a diet rich in fruits and vegetable. The natural ripening process of fruits can affect their nutritional content in different manners. Recently we reported the physiological and biochemical changes that occur during the ripening of "Maradol" papaya (Gayosso-García Sancho et al. 2010); but the effect of this process in the potential antiproliferative activity of papaya extracts from different RS containing a phytochemical mixture rich in carotenoid compounds is unknown. Therefore, we considered of scientific importance to evaluate the effect of carotenoids from lipophilic extracts of "Maradol" papaya, at four stages of ripeness (RS1, RS2, RS3 and RS4), on the proliferation of non-tumorogenic MCF-12F breast epithelial cells, as well as the effect on MCF-7 (estrogen receptor

positive) and MDA-MB-231 (estrogen receptor negative) breast cancer cells and its possible use as chemopreventive agent.

Materials and Methods

Plant Material

Fresh papaya fruit (*Carica papaya* L, cv. "Maradol") was obtained from a local market in Hermosillo, Sonora, Mexico. Each fruit was selected for uniform size, color, level of external ripeness, and then the fruit was divided in 4 lots based on ripeness stages: RS1 (fruit with 0-25% yellow color); RS2 (>25 to 50% yellow color), RS3 (>50 to 75% yellow color), and RS4 (>75 to 100% yellow color), as previously described [12].

Papaya extracts preparation

Lipophilic extracts were prepared as described previously by Gayosso-García Sancho et al. (2010) and Yahia et al. (2007). Papaya flesh dry sample (0.5g) was homogenized in 10 mL of hexane:dichloromethane (1:1, v/v), using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC); then it was centrifuged at 9000 g for 10 minutes at 5°C. Organic phase was separated and the procedure was repeated three times. For alkaline hydrolysis, 10 mL of methanolic KOH 40% (1:1, v/v) were added to extracts for 1 hour, at 50°C, and at a 100 rpm stirring bath set. After saponification, 10 mL of 10% sodium sulfate were added for phase separation, and the extracts were left for 1 hour in the dark, at room temperature. Extracts were evaporated in a Rotovapor®

(Büchi Labortechnik AG, Flawil, Switzerland); at 30°C in a Buchi low pressure evaporator. Samples were re-suspended in 2 mL acetone, and filtered through nylon membrane of 0.45 µm of pore size (Millipore Corp., Bedford, MA). Samples were stored at -78°C until their utilization in the cell cultures.

Cell culture

The non-tumoral breast epithelial cell line MCF-12F and breast cancer cell lines MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) were kindly supplied by Dr C. Aceves (Instituto de Neurobiología, UNAM, Mexico). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (basal medium), and then were incubated at 37°C in a 95% humidified atmosphere of 5% CO_2.

Determination of the antiproliferative activity

The antiproliferative activity of papaya extracts at the four different RS in MCF-12F and in MCF-7 and MDA-MB 231 cells, was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, previously described by García-Solís et al. (2009), with some modifications. Cells were seeded at a density of 5000 cells/well, in 96-well flat-bottomed plates, in a final volume of 100 µl, and incubated for 24 hours

prior to the addition of lipophilic extracts of papaya. Then, 100 µl of fresh medium were added at carotenoid concentrations of 0.92, 1.61, 2.08 and 3.27 mg/mL, which correspond to RS1, RS2, RS3, and RS4, respectively. The kind and concentrations of carotenoids in each papaya extract are show in Table I. Acetone at non-toxic level (0.5%) was used in the medium as a control. The kind and concentrations of carotenoids in the extracts were determined previously by Gayosso-García Sancho et al. (2011). Cells were incubated for 24, 48 and 72 h. As a positive control, a treatment with 500 nM thapsigargin was included in each assay, which is considered to be a strong apoptotic inducer and inhibitor of cell proliferation (Jackisch et al. 2000). Thapsigargin was dissolved in dimethyl sulfoxide (DMSO), which represents 0.1% (v/v) of the culture medium, and a solvent controls were also included. MTT solution at 5 mg/ml was dissolved in 1 mL of phosphate-buffered saline (PBS) and 20 µl of this solution was added to each of the 96 wells at 24, 48 and 72 hours of incubation, at 37 °C for 1 hour. The solution in each well containing MTT, media and dead cells was removed by suction, and formazan crystals were dissolved with 100 µl DMSO in each well. DMSO was used as solvent control. The plates were then shaken and the absorbance was measured using a micro plate reader (Multiskan Ascent®, Thermo electron corporation) at 610 nm. Cell proliferation was determined using the average of absorbance units reading from the wells and expressed as percentage with respect to the control (untreated cells). At least three replications for each sample were used to determine cell proliferation. All experiments were performed at least in duplicate.

Statistical analysis

Comparisons of mean values of control and treated cells were made using ANOVA, followed by Duncan's test. The statistical significance of difference ($P < 0.05$) for the treatment groups was determined relative to their respective control group, using the statistical software SAS version 8.0 (SAS Inst. Inc. Cary, NC, USA).

Results and Discussion

Papaya lipophilic extracts RS1, RS2, RS3 and RS4 did not significantly inhibit cell proliferation of MCF-12F cells compared to control, on average only; there was a decrease of 7% after 24, 48 and 72 h of treatment (Figure 1). This suggests that the main component of extracts, carotenoids, did not affect cell growth in a normal breast epithelium. Other studies observed that when using β-carotene, a similar behavior were obtained; suggesting that carotenoids have a protective effect on normal cells by facilitating their growth (Thangaiyan and Anupam 2009). This result suggested that MCF-12F cells could be a good model to explore the effect of papaya carotenoids on initiation of carcinogenesis of breast cancer cells.

Figure 2 shows the effects of papaya extracts on MCF-7 cell line, which is the most utilized for studies of estrogen receptor-positive breast cancer (Simstein 2003). Lypophilic extracts of papaya reduced significantly the proliferation of MCF-7 cells, being generally more effective at 72h of incubation. The highest cell proliferation was observed when using RS1 and RS2 extracts (77.29 and 75.51%). However, the RS3 and RS4 extract reduced significantly the cell proliferation in 67.24% and 66.8%,

173 respectively. These results, which ranged from 22% and 33%, in the inhibition of cell
174 proliferation, could be related with the concentration of carotenoids contained in the
175 extracts. In a previous study (Gayosso-García Sancho et al. 2011) we identified and
176 quantified the three main carotenoids found in "Maradol" papaya at different stages of
177 ripeness (Table I): lycopene, β-cryptoxanthin and β-carotene. These carotenoids appear
178 to act synergically between them and with other anticarcinogenic compounds present in
179 the papaya extract, giving as a result an enhancement of the antiproliferative activity
180 (Yang and Liu 2009). We corroborated previous studies of the inhibiting effect of
181 carotenoids in this type of cancer cells (García-Solís et al. 2009).

182 Carotenoids regulate cell growth, inhibit the growth of malignant cells, and promote
183 apoptosis in transformed cells (Cui et al. 2007). Additionally, some individual
184 carotenoids, such as β-carotene and lycopene, inhibited the growth of MCF-7 cells in
185 vitro (Prakash et al. 2001). The antiproliferative effect of aqueous extracts of 14 plant
186 foods on the MCF-7 breast cancer cell line was evaluated by García-Solís et al. (2009),
187 and only papaya extract, rich in β-carotene (dose of 0.85 mg/mL) after 72 h of
188 treatment, significantly decreased cell proliferation. On the other hand, a study
189 performed in a group of women who followed a diet rich in carotenoids, observed that
190 the survival probability increased during the first stages of breast cancer (Rock et al.
191 2009). Other studies have shown the inhibiting effect of various compounds on MCF-7
192 cancer cells, especially during the early stages of carcinogenesis, through antioxidant
193 and antinflammatory routes; the induction of apoptosis; the halting of cell cycle in early
194 tumors, and in tumors with greater development; and the potential blocking of

195 progression and metastasis, through the expression of genes that suppress tumors
196 (Thangaiyan and Anupam 2009). Carotenoids present in fruits and vegetables show
197 several beneficial properties in humans (especially lycopene and β-carotene), such as
198 antioxidant activity, increased intercellular communications, modulation of insulin-like
199 growth factor-1 (IGF1), inhibition of cell proliferation, and enhancement of immune
200 system function (Yahia and Ornelas-Paz 2010).

201 We evaluate the effect papaya extracts in the proliferation of estrogen receptor–
202 negative breast cancer cell line MDA–MB–231 (Figure 3). MDA-MB-231 cells did not
203 show any significant inhibitory effect in their proliferation contrary to the effect on
204 MCF-7 cells. Moreover, a slight non-significant cell proliferation increment was
205 observed with extract RS1 after 48 and 72 h of treatment and this behavior changed in
206 RS2 to RS4 extracts MDA-MB-231 cells with negative response to estrogens considered
207 more de-differentiated form than MCF-7 cells, and considered more aggressive (Ross
208 and Perou 2001). This could explain why their inhibition was not significant. Our results
209 coincided with those obtained by other researchers (Prakash et al. 2000), where the use
210 of retinoids and β-carotene had no inhibitory effect on this type of cells. In a study
211 performed with anoikis-resistant MDA-MB-231 cells (Yu et al. 2009), it was found that
212 this type of cells can turn refractory to several chemotherapeutic agents, due to a gene
213 overexpression related to nuclear factor kappa B (*NF-κB*), which is one of the vital
214 transcriptional cell factors, giving as a result greater aggressive behavior and greater cell
215 resistance. Nevertheless, a possible way to reduce cell proliferation MDA-MB-231
216 could be the use of a mix of carotenoids, which have biological mechanisms that still

have not been completely elucidated. Therefore it would be necessary to focus in determining their molecular mechanism on this type of cell lines.

Conclusions

Papaya (*Carica papaya* L. cv, "Maradol") is a tropical fruit that has excellent antioxidative properties. Our results showed that carotenoids present in ripe papaya could partially contribute to the reduction of the proliferation of MCF-7 breast cancer cells. Although the relationship between the consumption of carotenoids and breast cancer is not yet completely clear, it is necessary to continue with this type of research to determine the mechanism by which these types of compounds are able to provide a beneficial effect to human health.

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319 **Figure legends:**

320 **Figure 1** Effect of papaya extracts on proliferation in MCF-12F cells after 24, 48 and 72
321 h. Vertical bars indicate means± standard deviation, n=6. No significant differences
322 were shown with respect to the control from 2 independent experiments and each
323 experiment was conducted in triplicate.

324 **Figure 2** Effect of papaya extracts on proliferation in MCF-7 cells after 24, 48 and 72 h.
325 Vertical bars indicate means± standard deviation, n=6. **P*-value <0.05 are significant
326 difference with respect to the control from 2 independent experiments and each
327 experiment was conducted in triplicate.

328 **Figure 3** Effect of papaya extracts on proliferation in MDA-MB-231 cells after 24, 48
329 and 72 h. Vertical bars indicate means± standard deviation, n=6. No significant
330 differences were shown with respect to the control from 2 independent experiments and
331 each experiment was conducted in triplicate.

332

333 **Table legends**

334 **Table I.** Contents of the main carotenoid compounds identified in the pulp of "Maradol"
335 papaya fruit.

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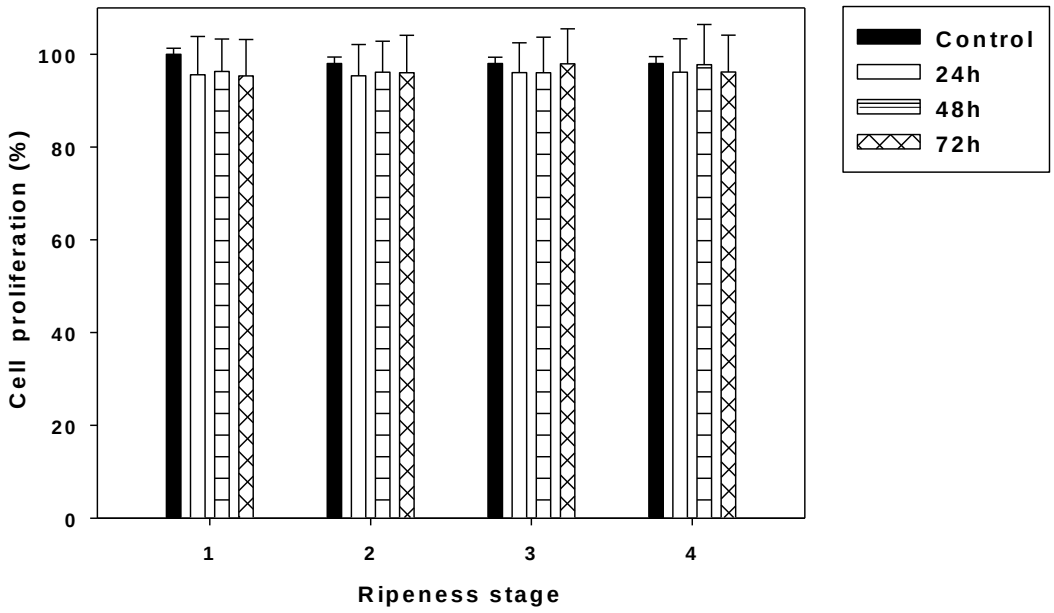
Control
24h
48h
72h
Cell proliferation (%)
0
20
40
60
80
100
1
2
3
4
Ripeness stage

Figure 1

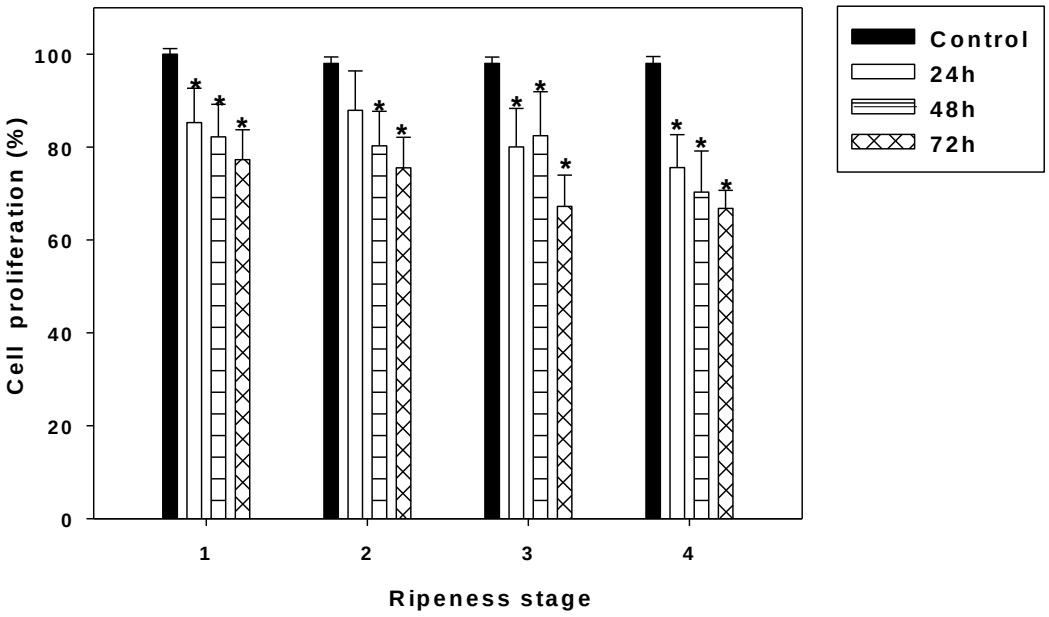
Control
24h
48h
72h
Cell proliferation (%)
0
20
40
60
80
100
1
2
3
4
Ripeness stage

Figure 2

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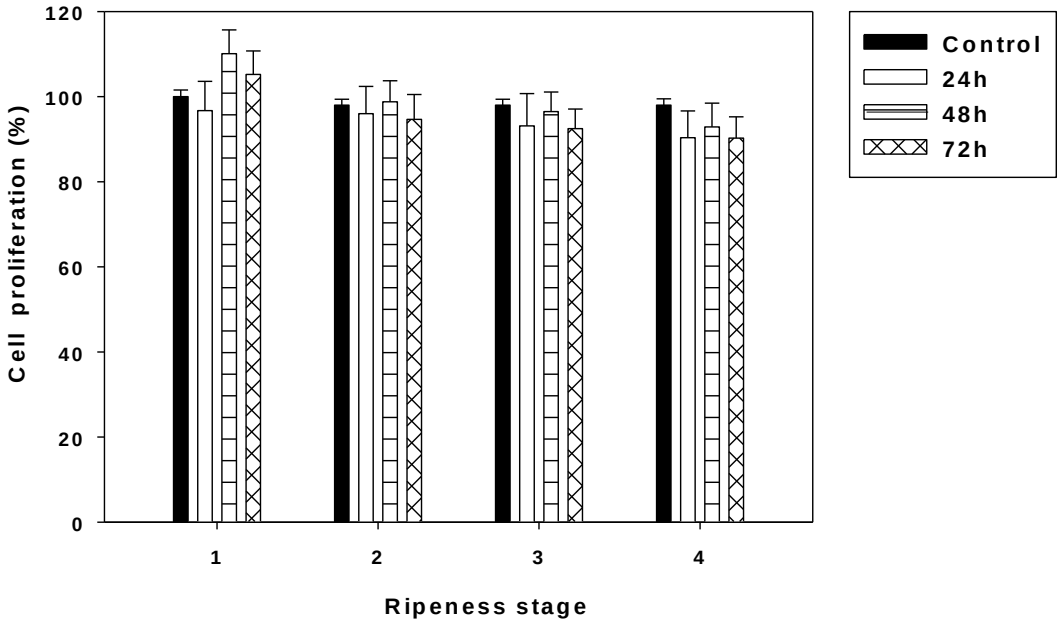

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374 Figure 3

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Table I

Ripeness Stage (RS)	Lycopene (mg/100g DW)	β-Cryptoxanthin (mg/100g DW)	β-Carotene (mg/100g DW)
1	0.524[a]	0.331[a]	0.387[a]
2	2.48[b]	0.558[b]	0.409[a]
3	2.78[b]	0.794[c]	0.441[a]
4	4.23[c]	1.295[d]	0.752[b]

Different letters in the same column indicate significant differences ($p \leq 0.05$)

CONCLUSIONES GENERALES

1. Los cambios en firmeza que ocurren durante la maduración de papaya, están directamente correlacionados con la actividad de poligalacturonasa (PG), pectinmetilesterasa (PME) y con el aumento en la producción de CO_2 y etileno.

2. Existe una correlación negativa entre la capacidad antioxidante del fruto medida por las técnicas de DPPH y TEAC con el contenido de fenoles; mientras que con la técnica de ORAC, ésta tendió a aumentar, correlacionando positivamente este comportamiento con el alto contenido de carotenoides y fenoles.

3. Los ácidos fenólicos mayoritarios en cáscara de papaya fueron: ferúlico, *p*-coumárico y cafeico, cuyas concentraciones disminuyeron durante el proceso de maduración. Los carotenoides más abundantes en pulpa fueron licopeno, β-criptoxantina y β-caroteno, observando que sus concentraciones se incrementaron con la maduración del fruto. El contenido de vitamina C fue mayor en pulpa que en cáscara, mostrando un incremento del 233.7% en el EM4, con respecto al EM1. Por otra parte, las concentraciones de los compuestos fenólicos, carotenoides y vitamina C, se correlacionaron con la capacidad antioxidante medida por DPPH, TEAC y ORAC. Los resultados indican que en estado maduro, las concentraciones de los compuestos bioactivos en general, son mayores, por lo cual es recomendable el consumo de papaya madura, ya que podría contribuir a mayores beneficios en la salud humana, debido a que reducen diversos procesos oxidativos en el organismo.

4. La contribución a la capacidad antioxidante total (CAT) de los ácidos fenólicos, está en función de su concentración y estructura química, ya que la posición y número de OH presentes en la molécula, son los responsables de su capacidad para estabilizar las especies reactivas de oxígeno. La combinación de ácido ferúlico con ácido cafeico fue la que presentó la mejor capacidad de reducción del radical, por su potencial redox menor y mayor capacidad para donar electrones. Sin embargo, la combinación de los ácidos cafeico, ferúlico y p-coumárico, no mostró un efecto sinérgico que contribuyera a aumentar la capacidad antioxidante, al parecer la combinación de estos compuestos produjo un efecto antagónico.

5. En cuanto a la contribución de los carotenoides a la CAT, el licopeno fue el compuesto que presentó la mayor contribución, seguido de la β-criptoxantina. De la misma forma, la estructura química de licopeno le confiere la propiedad de donar con mayor facilidad electrones para estabilizar los radicales libres. Su alto contenido en papaya "Maradol" es de gran importancia para el organismo ya que en forma combinada con β-caroteno, vitamina C y los compuestos fenólicos, pueden contribuir a mayores beneficios al consumidor. Con el fin de elucidar los mecanismos involucrados en el aumento de la CAT, es necesario llevar a cabo más estudios para conocer con mayor claridad la potenciación y sinergia entre este tipo de compuestos.

6. Las amplias propiedades antioxidantes y los altos niveles de carotenoides presentes en los frutos en el EM4 contribuyeron a la reducción de la proliferación de células de cáncer MCF-7.

Este trabajo de investigación mostró que la papaya Maradol posee compuestos bioactivos cuyo contenido varía de acuerdo a su estado de madurez. La pulpa del fruto es la que posee el mayor contenido de vitamina C y carotenoides, siendo éstos últimos los que contribuyeron en mayor proporción a la capacidad antioxidante. Por lo que la ingesta de papaya, combinada con otros fitoquímicos derivados de la dieta, podría contribuir a la prevención de diversas enfermedades y probablemente en la reducción del riesgo de cáncer de mama.

PRODUCTIVIDAD ACADÉMICA

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La Contribución del Consumo de Frutas y Hortalizas a la Salud Humana. Simposio del Laboratorio de Fitoquímicos y Nutrición de la Universidad Autónoma de Querétaro. 12 de junio del 2009.

Estancia de Investigación

Laboratorio de Fitoquímicos y Nutrición de la Facultad de Ciencias Naturales de la Universidad Autónoma de Querétaro. De junio a octubre del 2009 con el Dr. Elhadi Yahia K.

Laboratorio de Endocrinología y Nutrición de la Facultad de Medicina de la Universidad Autónoma de Querétaro. De noviembre 2009 a abril 2010 con el Dr. Pablo García Solís.