

Inhibition of Proliferation of Breast Cancer Cells MCF7 and MDA-MB-231 by Lipophilic Extracts of Papaya (*Carica papaya* L. var. Maradol) Fruit

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Abstract

Several epidemiological studies have suggested that carotenoids have antineoplastic activities. The objective of this study was to determine the antiproliferative effect of rich carotenoid lipophilic extracts of papaya fruit pulp (*Carica papaya* L., cv Maradol) in breast cancer cells, MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative), and in non-tumoral mammary epithelial cells MCF-12F. Antiproliferative effect was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and testing lipophilic extracts from different papaya fruit 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 in proliferation at 72 h with the RS4 papaya extract. Results suggested that lipophilic extracts had different action mechanisms on each type of cells and therefore, more studies were required to elucidate such mechanisms.

Keywords

Carica papaya, Antiproliferative Activity, Breast Cancer, Carotenoids

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1. Introduction

Cancer caused 8.2 million deaths in 2012, according to the Global Cancer Statistics, from which 521,000 were breast cancer related, and it is forecasted that by 2030 this number will increase to 22 million [1]. 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 [2].

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 [3]. In response to this, the US Department of Health and Human Services [4] has recommended 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 [5]–[7]. A high consumption of carotenoids could be associated with the reduction of the risk of breast cancer, because these types of compounds show several biological activities, from which the promotion of apoptosis in transformed cells stands out [8]. These antioxidants induce cell differentiation, repair damaged DNA, inhibit gene mutation, and activate tumor-suppressive genes [9]. Additionally, a panel of experts concluded that an inverse association of carotenoids with the risk of breast cancer is possible due to their antioxidant properties [10].

Mexico is the biggest exporter of papaya [11], a climacteric tropical fruit that has diverse bioactive components (BC) that give the fruit several antioxidant properties [12] [13]. This fruit is especially rich in carotenoids, such as lycopene, β -cryptoxanthine and β -carotene [12] [14]. Most of the research done in different cell lines have focused on the utilization of isolated doses of several BCs, and have not considered the importance of employing a mix of these, like those naturally occurring in the fruit, in order to determine the beneficial effect on health of a diet rich in fruits and vegetable. The objective of this study was to evaluate the antiproliferative effect of lipophilic extract (carotenoids) of “Maradol” papaya fruit on breast cancer cell MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) and non-tumoral breast epithelial cells MCF-12F for possible use as chemopreventive agent.

2. Materials and Methods

2.1. Plant Material

Fresh papaya fruit (*Carica papaya* L., cv. Maradol) was obtained from a local market in Hermosillo, Sonora, Mexico. Fruit were selected for uniformity in size, color, level of external ripeness, and were divided into 4 lots based on four ripeness stages: RS1 (fruit with 0% - 25% skin yellow color); RS2 > 25% to 50%; RS3 > 50% to 75%; and RS4 > 75% to 100%.

2.2. Papaya Extracts Preparation

Lipophilic extracts were prepared as described previously by Yahia *et al.* [15] and Gayosso García-Sancho *et al.* [16]. Papaya flesh dry sample (0.5 g) was briefly homogenized in 10 mL of hexane:dichloromethane (1:1, v/v), using an Ultra Turrax®T25 basic homogenizer (IKA Works, Wilmington, NC); then it was centrifuged at 9000 g for 10 minutes at 5°C. The 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 stirred at 100 rpm in a 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 Büchi low pressure evaporator. Samples were re-suspended in 2 mL acetone, filtered through nylon membrane of 0.45 μ m of pore size (Millipore Corp., Bedford, MA), and stored at -78°C until their utilization in cell culture.

2.3. 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, Queretaro, 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 containing 5% CO₂.

2.4. Determination of the Antiproliferative Activity

The antiproliferative activity of papaya extracts at four different stages of ripeness (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, as previously described [13], 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 shown in **Table 1**. 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 according to García-Solís *et al.* method [17], using concentrations of lipophilic extracts of 0.5%, 1% and 2% (v/v), then cells were incubated for 24, 48 and 72 h. As a positive control in each essay a treatment with 500 nM thapsigargin was included, which is considered to be a strong apoptotic inducer and inhibitor of cell proliferation [18]. Thapsigargin was dissolved in dimethyl sulfoxide (DMSO), which represents 0.1% (v/v) of the culture medium, and solvent controls were also included. MTT solution at 5 mg/ml was dissolved in 1 mL of phosphate-buffered saline (PBS); 20 µl of it 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 was 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.

2.5. Statistical Analysis

Comparisons of mean values of control and treatment cells were made using ANOVA, followed by Duncan's test, between control and each treatment group. 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). At least three replications for each sample were used to determine cell proliferation, and all experiments were performed at least in duplicate.

3. Results and Discussion

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-tumorigenic MCF-12F breast epithelial cells, as well as the effect on MCF-7 and MDA-MB-231 breast cancer cells is shown in **Figure 1**. In MCF-12F cells it was observed that papaya lipophilic extracts did not inhibit cell proliferation after 72h of treatment, which 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 was obtained; suggesting that carotenoids have a protective effect on normal cells by facilitating their growth [19]. This result suggests that MCF-12F cells could be a

Table 1. Contents of the main carotenoid compounds identified in the pulp of “Maradol” papaya.

| Ripeness Stage (RS) | Lycopene (mg/100 g DW) | β -Cryptoxanthin (mg/100g DW) | β -Carotene (mg/100 g 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 letter in the same column indicate significant differences ($P \leq 0.05$).

good model to explore the effect of papaya fruit carotenoids on initiating carcinogenesis of breast cancer.

We have reported on the physiological and biochemical changes that occur during the ripening of “Maradol” papaya [12]; but the effect of this process on the potential antiproliferative activity of the mix of carotenoid compounds in the fruit is not known; which is why we have decided to investigate the effect of carotenoids from lipophilic extracts of “Maradol” papaya, at four stages of ripeness on the proliferation of non-tumorigenic MCF-12F breast epithelial cells, as well as the effect on MCF-7 and MDA-MB-231 breast cancer cells. Cancer, one of the main causes of mortality, originates as the result of the interaction of various genetic, physical, chemical and biological factors that transform cells until they become malignant tumors [20]. Tumors are usually localized, but can disseminate to other organs, originating metastasis [1]. According to the Mexican Ministry of Health, breast cancer has become the main cause of death in Mexican women 25 years of age and older since 2006, replacing uterine cancer [21].

The effects of papaya extracts on MCF-7 cell line, which is the most utilized for studies of estrogen receptor-positive breast cancer [22], are shown in **Figure 2**. Cell proliferation levels were lower with RS3 and RS4 (67.3% and 66.8%, respectively), while proliferation has higher with RS1, and RS2 (77.3% and 75.5%, respectively), showing that lipophilic extracts of papaya reduced significantly the proliferation of MCF-7 cells, being generally more effective at 72 h of incubation. These results, which ranged from 22% and 33% in the inhibition of cell proliferation, could be related with the concentration of carotenoids contained in the extracts. In a preliminary study [12] we identified and quantified the three main carotenoids found in “Maradol” papaya at different stages of ripeness (**Table 1**), been lycopene, β -criptoxantine and β -carotene. This might indicate that bioactive

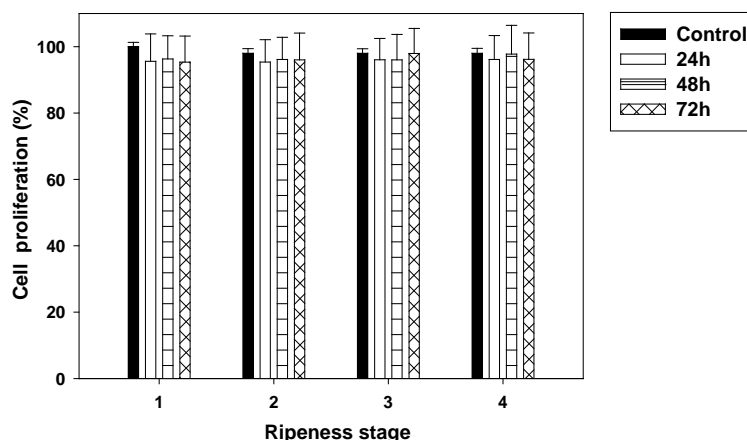


Figure 1. Effect of papaya extracts on cell proliferation in MCF-12F cells after 24, 48 and 72 h.

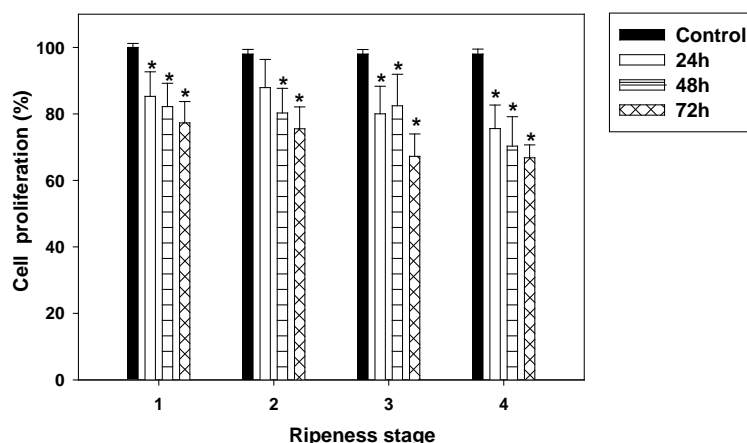


Figure 2. Effect of papaya extracts on cell proliferation in MCF-7 cells after 24, 48 and 72 h.

compounds of the extracts could act in a synergetic manner with other anticarcinogenic compounds, giving as a result an enhanced antiproliferative activity, hence, a greater health benefit [23]. Previous studies have shown the inhibition effect of carotenoids on this type of cancer cells: García Solís *et al.* [17] found that papaya extract had a significant inhibitory effect on proliferation of MCF-7 cells and Cui *et al.* [24] observed that β -Carotene induced apoptosis in MCF-7 cells.

Carotenoids regulate cell growth, inhibit the growth of malignant cells, and promote apoptosis in transformed cells [24]. Additionally, some individual carotenoids, such as β -carotene and lycopene, have inhibited the growth of MCF-7 cells *in vitro* [25]. The antiproliferative effect of aqueous extracts of 14 plant foods on the MCF-7 breast cancer cell line was evaluated [17], and only papaya extract, rich in β -carotene (dose of 0.85 mg/mL) after 72 h of treatment, significantly decreased cell proliferation. On the other hand, a study performed in a group of women who followed a diet rich in carotenoids, observed that the survival probability increased during the first stages of breast cancer [26]. Other studies have shown the inhibiting effect of various compounds on MCF-7 cancer cells, especially during the early stages of carcinogenesis, through antioxidant and anti-inflammatory routes; the induction of apoptosis; the halting of cell cycle in early tumors, and in tumors with greater development; and the potential blocking of progression and metastasis, through the expression of genes that suppress tumors [19]. Though, carotenoids present in fruits and vegetables show several beneficial properties in humans (especially lycopene and β -carotene), such as antioxidant activity, increased intercellular communications, modulation of insulin-like growth factor-1 (IGF1), inhibition of cell proliferation, and enhancement of immune system function [27].

We have also explored the effect papaya extracts in the proliferation of estrogen receptor-negative breast cancer cell line MDA-MB-231 (Figure 3). MDA-MB-231 cells did not show any significant inhibitory effect in their proliferation in frank contrast with the effect on MCF-7 cells. Moreover, a slight non-significant cell proliferation increment was observed with extract RS1 after 48 and 72 h of treatment and this behavior changed in RS2 to RS4 extracts MDA-MB-231 cells with negative response to estrogens are considered a more de-differentiated form than MCF-7 cells, therefore they are considered more aggressive [28], and this could explain why their inhibition was not significant. Our results coincided with those obtained by other researchers [29], where the use of retinoids and β -carotene had no inhibitory effect on this type of cells. In a study performed with anoikis-resistant MDA-MB-231 cells [30], it was found that this type of cells can turn refractory to several chemotherapeutic agents, due to a gene overexpression related to nuclear factor kappa B ($NF-\kappa B$), which is one of the vital transcriptional cell factors, giving as a result greater aggressive behavior and greater cell resistance. Nevertheless, a possible way to reduce cell proliferation MDA-MB-231 could be the use of a mix of carotenoids, which have biological mechanisms that still have not been completely elucidated; then it would be necessary to focus on determining their molecular mechanism on this type of cell lines.

4. Conclusion

Papaya (*Carica papaya* L. var Maradol) is a tropical fruit that has significant antioxidative properties. Our re-

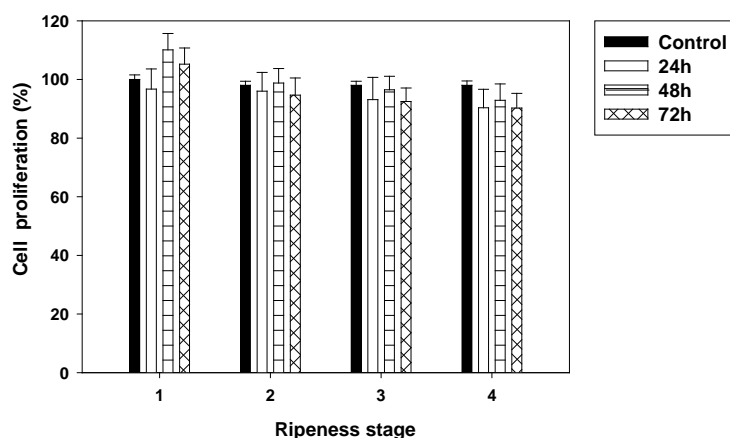


Figure 3. Effect of papaya extracts on cell proliferation in MDA-MB-231 cells after 24, 48 and 72 h.

sults showed that carotenoids present in ripened papaya could 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 completely elucidated, it is necessary to continue with this type of research to be able to determine the mechanism by which these types of compounds are able to provide a beneficial effect to human health.

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