

Inhibition of Proliferation of Colorectal Cancer Cells by Phenolic Extracts of Mandarin (*Citrus reticulata*) and Lime (*Citrus aurantifolia*) Fruit Waste

Francisco J Esparza-Martínez^{1,*}, Pablo García Solís², Rita Miranda-López³, Vicente Peña-Caballero¹

¹Departamento Ingeniería Agroindustrial, Universidad Guanajuato, campus Celaya-Salvatierra, Ing. Barros Sierra No. 201, Ejido de Santa María, Celaya Guanajuato, México

²Departamento de Medicina, Universidad Autónoma de Querétaro, Clavel No.200, Prados de La Capilla, Santiago de Querétaro, Querétaro. México

³Posgrado de Ingeniería Bioquímica, Tecnológico de Celaya, Celaya, Gto., México. Av. Tecnológico s/n, Celaya, Gto. México

*Corresponding author: fj.esparza@ugto.mx

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Abstract Several epidemiological studies have suggested that phenolics have antineoplastic activities. The objective of this study was to determine the antiproliferative effect of rich phenolics extracts of mandarin (*Citrus reticulata*) and lime (*Citrus aurantifolia*) fruit waste in colorectal cancer cells, RKO (carcinom) and HT-29 (adenocarcinom). Antiproliferative effect was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and testing phenolics extracts from different mandarin and lime fruit waste drying temperatures (fresh, 60, 90, 120°C), at different times (24, 48 and 72 h). Mandarin and lime phenolics extracts inhibit cell viability of colon cancer cell lines, HT-29 and RKO, at 24 h to 72 h. Results suggested that phenolics extracts had different action mechanisms on each type of cells and therefore, more studies were required to elucidate such mechanisms.

Keywords: antiproliferative activity, colorectal cancer, phenolic compounds

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1. Introduction

Cancer caused 8.8 million deaths in 2015, according to the Global Cancer Statistics, from which 774,000 were colorectal 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 has recommended to increase the consumption of fruits and vegetables from 5 to 13 portions a day [4]. The beneficial effect of diets rich in fruits and vegetables is attributed mainly to bioactive components (BC), such as carotenoids, phenolic compounds, flavonoids, vitamins C and E, that increases antioxidant, antimicrobial and antineoplastic capacities [5,6,7].

A high consumption of phenolics compounds could be associated with the reduction of the risk of 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 phenolics with the risk of colorectal cancer is possible due to their antioxidant properties [10].

Mandarin and lime are not climacteric tropical fruits that has diverse BC that give the fruit several antioxidant properties [11]. These fruits are especially rich in phenolics, such as hesperidin, naringin and naringenin [12,13]. Most of the research done in different cell lines have focused on the utilization of isolated doses of several BC and have not considered the importance of employing a mix of these, like those naturally occurring in the fruit, to determine the beneficial effect on health of a diet rich in fruits and vegetables. The objective of this study was to evaluate the antiproliferative effect of phenolics extracts of "Satsuma" mandarin and "Gallega" lime fruits on colorectal cancer cell lines HT-29 and RKO for possible use as chemo-preventive agent.

2. Materials and Methods

2.1. Plant Material

“Satsuma” (*C. reticulata* Blanco) mandarin fruits and “Gallega” (*C. aurantifolia* (Christm)) lime fruits from trees grown at El Marques, Queretaro, Mexico were harvested in November 2013 when commercially mature; they were sanitized (aqueous HCl, 0.05%, 1 min) before processing, to avoid contamination. Fruits were selected for their homogeneous size, free from defects and randomly divided in four batches, 150 fruit each. Mandarins and Limes from each batch were cut into two pieces and squeezed with a Hamilton Beach Electric juice squeezer (Hamilton, China) to produce juice and mandarin and lime waste (albedo, flavedo, residues of pulp and carpellary membranes), known hereafter as MW and LW, respectively. One batch of MW and LW was freeze-dried immediately after juice extraction (Fresh-MW and Fresh-LW); the other three batches were dried at 60, 90 and 120 °C (MW-60, LW-60, MW-90, LW-90, MW-120 and LW-120) in a tray dryer (UOP 8 Tray Dryer, Armfield, Ringwood, England) equipped with controls for temperature and airflow velocity. The dryer air velocity was of 1.5 m/s. Weight loss and airflow velocity were recorded during the drying process using a digital balance (Ohaus, Explorer, USA). Dehydration lasted until a moisture content of ~4.5% was achieved. The dried samples were ground and sieved through a 40-mesh sieve and stored at -20°C until analysis was performed.

2.2. Mandarin and Lime Extracts Preparation

The samples were extracted two times with 25 mL of aqueous methanol (80%) in an ultrasonic bath at room temperature for 60 min. After incubation, the samples were centrifuged at 5000 rpm for 10 min [14]. Supernatants (extracts) were combined and eventually the extracts were evaporated to remove methanol at 35°C in a Büchi low pressure evaporator (Rotavapor®-MODELO, Büchi Labortechnik AG, Flawil, Switzerland). Samples were, filtered through nylon membrane of 0.45 µm of pore size (Millipore Corp., Bedford, MA), and stored at -78°C until their utilization in cell culture.

2.3. Cell Culture

The tumoral colon epithelial cell lines RKO (ATCC No: CRL-2577™) and HT-29 (ATCC No: HTB-38™) were kindly supplied by Dr Teresa Garcia Gasca (Faculty of Natural Sciences Naturales, Autonomous University of Queretaro, Queretaro, Mexico). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (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 phenolics extracts of MW and LW at four different dryer temperatures in RKO and in HT-29 cells, was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, as previously described [15], with some modifications. Cells were seeded at a density of 10 x 10⁴ 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 treatments which correspond to MW-fresh, LW-Fresh, MW-60, LW-60, MW-90, LW-90, MW-120 and LW-120. Then, 200 µl of fresh medium were added with each phenolic extract at the following final concentrations of 1, 5 and 10% (v/v). 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 viability 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 viability. All experiments were performed at least in duplicate.

The kind and concentrations of phenolics in the extracts were determined previously according to method [16] and published previously by Esparza-Martínez [17].

2.5. Statistical Analysis

Comparisons of mean values of control and treatment cells were made using ANOVA, followed by Tukey Kramer test, between control and each treatment group. The correlations of the data are the result of the increase in the drying temperature, with the increase in the concentration of phenolic compounds and the decrease of cell viability. The statistical significance of difference ($P < 0.05$) for the treatment groups was determined relative to their respective control group, using the statistical software SPSS version 17.0 (SPSS Copyright © 1989–2009) and SAS version 8.0 (SAS Inst. Inc. Cary, NC, USA). At least three replications for each sample were used to determine cell viability, and all experiments were performed at least in duplicate.

3. Results and Discussion

3.1. Effect of Phenolic Extract of Waste Mandarin and Lime Fruits on the Cell Viability of Colon Cancer Cells RKO

The effect of phenolics extracts of MW dried at different temperatures on the cell viability of RKO cells, is

shown in Figure 1. MW extracts inhibited in a dose-response manner cell viability of RKO cells. After 24 h MW-120 had a greater effect on cell viability regarding treatments. Doses of 1, 5 and 10% of MW-120 inhibit cell viability by 27, 46 and 57%, respectively. While MW-60 had a lower effect on cell viability at doses of 1, 5 and 10%, cell viability was inhibited 21, 43 and 52%, respectively. In Figure 2 is shown the effects of phenolic extracts of LW dried at different temperatures on the cell viability of RKO cells. After 24 h treatment the dried sample at 120 °C has a greater effect on cell viability. Doses of 1, 5 and 10% of the dried sample at 120°C inhibit cell viability by 30, 49 and 56%, respectively. While samples dried at 60 °C has a minor effect on cell proliferation at doses of 1 and 10% inhibited cell viability by 18 and 44%, respectively. The effect of the lyophilized sample is similar to the dried sample at 60°C where the percentage inhibition of cell viability at the concentrations of 1, 5 and 10% of the extract is 35, 54 and 48%, respectively.

Finally, the solvent in which the extracts were also inhibited proliferation by 15%. In a study [18] provided a diet with hesperidin and found that at 500 ppm inhibits carcinogenesis up to 97% in rats injected with amoxymethane (is a carcinogenic chemical compound).

Nandakumar *et al.* [19], purified hesperidin orange peel and it showed that there was inhibition of cell proliferation of MCF-7 breast cancer, they used concentrations of 20, 40, 60, 80 and 100 mM of the purified hesperidin and

placed in interaction by dose-response and found they had a median lethal dose of 80 mM at 24 h and 60 mM for a period of 48 h.

Furthermore, is observed that the inhibition of cell proliferation of colon cancer RKO of different extracts bagasse lime in the time course of 48 and 72 h have a similar 24 h behavior, the difference is that the inhibition of proliferation reaches 56% in all treatments with 10% of the phenolic extract, to the extract with 5%, the inhibition of proliferation is 54% on average for all samples and finally to 1% of the extract, the inhibition of proliferation is 49% on average. Finally, the solvent in which the extracts were also inhibited proliferation by 15%. In a study [20], they found that hesperidin as a COX-2 and iNOS inhibitor, which might be related to the anti-tumorigenic efficacies.

Jaiprakash *et al.* [21] demonstrated that apoptosis is a major cause for inhibition of proliferation of pancreatic cancer cell line Pac-28 a flavanone (Limonin) in *Citrus aurantifolia* using a concentration of 6.25, 12.5, 25, 50, 100 and 200 mM extracts acetone, methanol and methanol-water (8: 2 v / v) and found that the best inhibition of proliferation of pancreatic cells was Pac-28 at 48 h of Dose-response interaction with a percentage of 25.1% acetone, 30.9% methanol and 60.5% with methanol-water to a concentration of 200 mM, found that improved regulation of P53 gene coding for the protein that regulates the cell cycle and induces apoptosis.

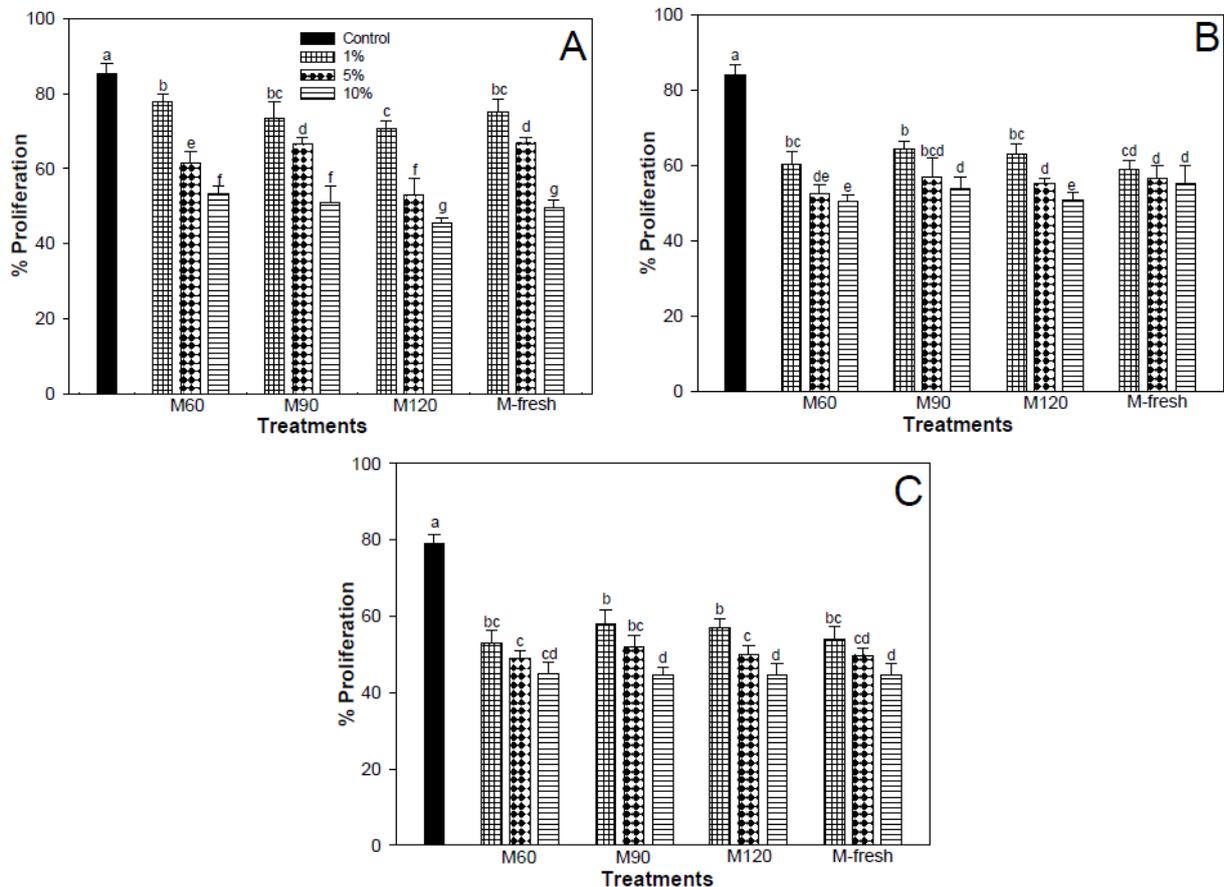


Figure 1. Dose-dependent inhibition of cell viability of RKO cells treated with phenolic extract of mandarin waste (MW for 24 (A), 48 (B) and 72 (C) h. MW was freeze-dried immediately after juice extraction (Fresh-MW); the other three treatments of MW were dried at 60, 90 and 120 °C (MW-60, MW-90, MW-120) in a tray dryer. The average and standard deviation (n = 3) is shown. Averages with different letters are significantly different at a level of significance of 5%

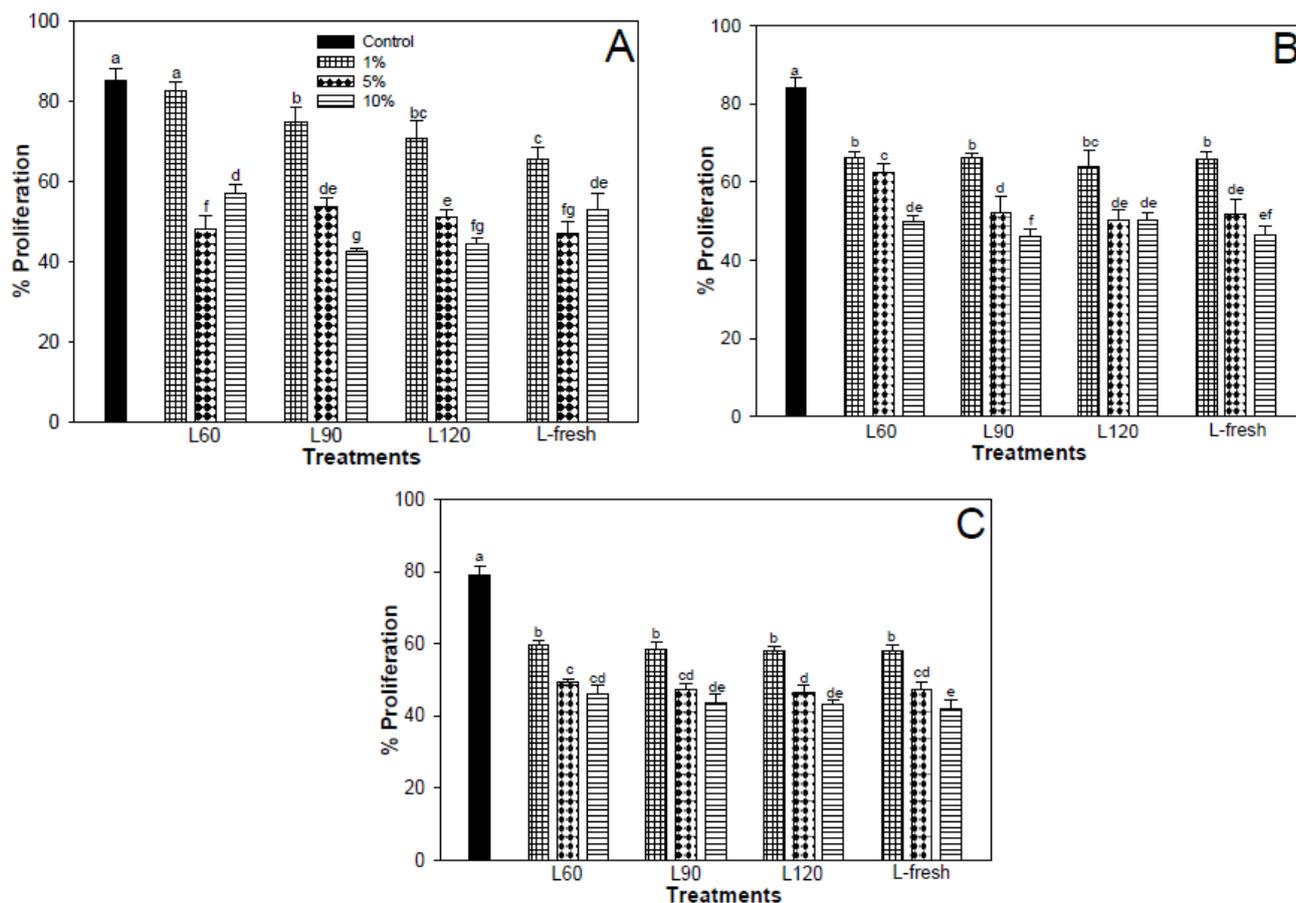


Figure 2. Dose-dependent inhibition of cell viability of RKO cells treated with phenolic extract of lime waste (LW) for 24 (A), 48 (B) and 72 (C) h. LW was freeze-dried immediately after juice extraction (Fresh-LW); the other three treatments of LW were dried at 60, 90 and 120 °C (LW-60, LW-90, LW-120) in a tray dryer. The average and standard deviation (n = 3) is shown. Averages with different letters are significantly different at a level of significance of 5%

3.2. Effect of Phenolic Extract of Waste Mandarin and Lime Fruits on the Proliferation of Colon Cancer Cells HT-29.

The effect of phenolics compounds from methanol extracts of “Satsuma” (*C. reticulata* Blanco) mandarin fruits, at four dried samples (fresh-MW, MW-60, MW-90, and MW-120), on the proliferation of tumorigenic HT-29 colon epithelial cells, is shown in Figure 3, a dose dependent effect and time course of inhibition of proliferation of colon cancer cells HT-29 methanol extracts of different waste tangerine observed. After 24 h treatment the dried sample at 120°C and lyophilized have a greater effect on proliferation compared to other treatments. Doses of 1, 5 and 10% of the dried sample at 120°C inhibit proliferation by 21, 29 and 58%, respectively, whereas the lyophilized sample inhibits proliferation by 20, 36 and 58%. While samples dried at 90°C have a minor effect on cell proliferation at doses of 1, 5 and 10% inhibit proliferation by 15, 19 and 48%, respectively. Furthermore it is observed that the inhibition of proliferation of colon cancer cells HT-29 extracts of different tangerine residues in the time course of 48 and 72 h have a similar behavior to 24 h, the difference is inhibition of proliferation reaches 68% in the treatments at

120°C and lyophilized to 10% of extract, whereas in the sample at 60°C with 10% of the extract inhibition of proliferation is 56% on average and finally the sample dried at 90°C is the one with less inhibition of proliferation with 1, 5 and 10% extract inhibition of proliferation is 29, 34 and 46% respectively. Finally, the solvent in which the extracts were also inhibited proliferation by 11%. In a study [22], They examined Examination of the expression of apoptosis-regulating genes indicated that hesperidin treatment decreased the expression of B-cell CLL/lymphoma 2 (BCL2) mRNA and increased the expression of BCL2-associated X protein (BAX). The expression and activity of the major apoptotic factor caspase3 (CASP3) was increased significantly with hesperidin treatment. Hesperidin down-regulated the protein expression of pro-CASP3, and up-regulated the level of active CASP3.

Ai-Yih *et al.* [23], used peel of *Citrus sulcata* where extracted naringin and hesperidin, identified inhibition of proliferation with a median lethal dose at a concentration of 250 µM, the extract was tested on a lung cancer cell line A549, they attribute to hesperidin and naringin positively regulate many genes involved in the cell cycle of A549 cell lung cancer and also down regulate mutated genes which cause irregularities in the cell cycle.

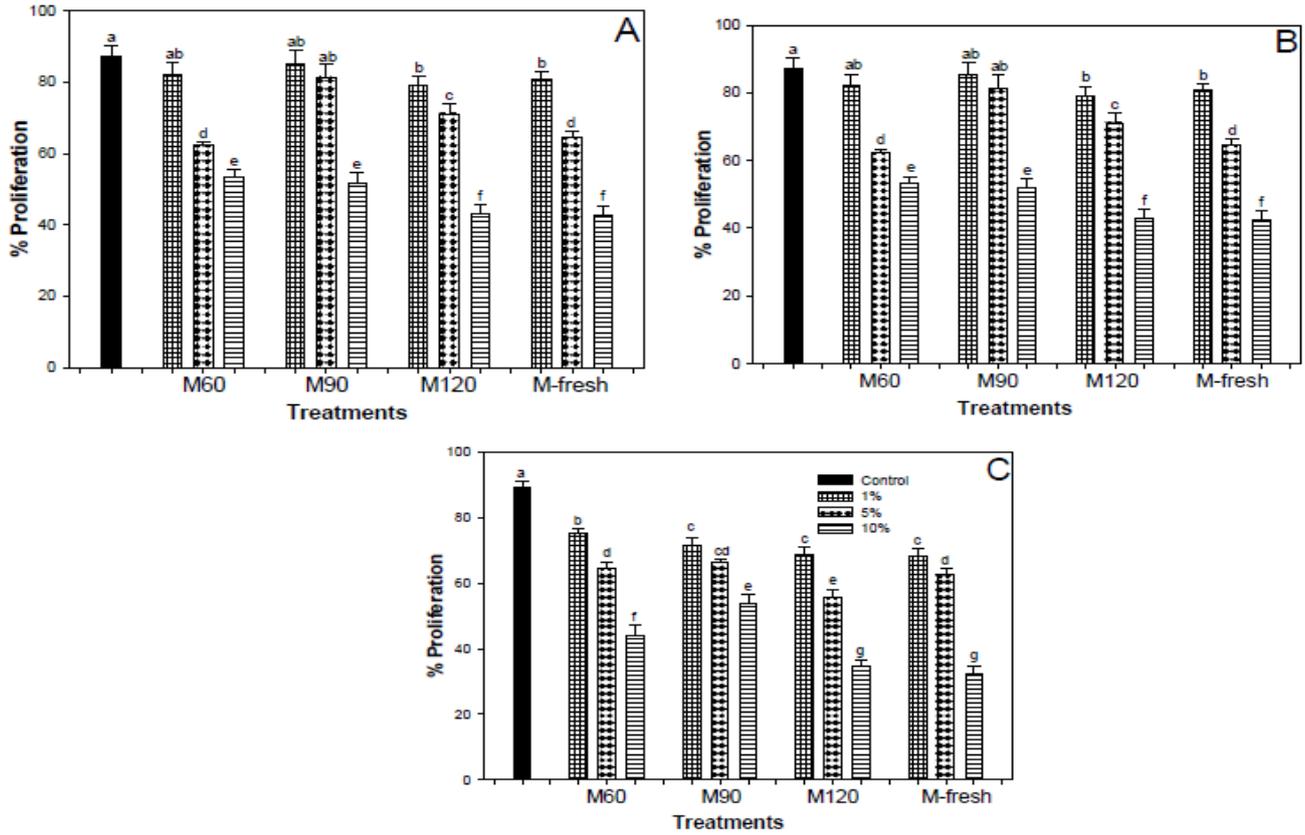


Figure 3. Dose-dependent inhibition of cell viability of HT-29 cells treated with phenolic extract of mandarin waste (MW) for 24 (A), 48 (B) and 72 (C) h. MW was freeze-dried immediately after juice extraction (Fresh-MW); the other three treatments of MW were dried at 60, 90 and 120 °C (MW-60, MW-90, MW-120) in a tray dryer. The average and standard deviation (n = 3) is shown. Averages with different letters are significantly different at a level of significance of 5%

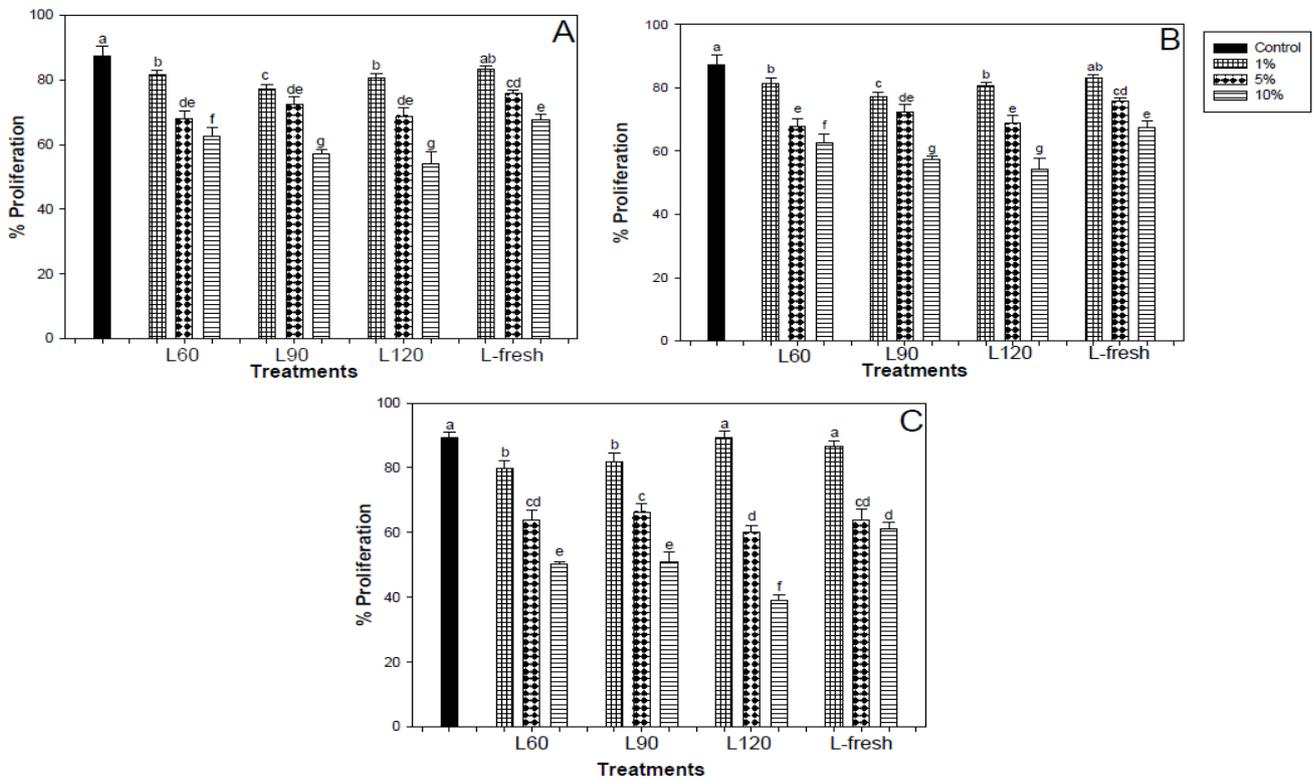


Figure 4. Dose-dependent inhibition of cell viability of HT-29 cells treated with phenolic extract of lime waste (LW) for 24 (A), 48 (B) and 72 (C) h. LW was freeze-dried immediately after juice extraction (Fresh-LW); the other three treatments of LW were dried at 60, 90 and 120 °C (LW-60, LW-90, LW-120) in a tray dryer. The average and standard deviation (n = 3) is shown. Averages with different letters are significantly different at a level of significance of 5%

The effect of phenolics compounds from methanol extracts of “Gallega” (*C. aurantifolia* (Christm)) lime fruits, at eight dried samples (fresh-LW, LW-60, LW-90, and LW-120), on the proliferation of tumorigenic HT-29 colon epithelial cells, is shown in Figure 4, a dose dependent effect and time course of inhibition of proliferation of colon cancer cells HT-29 methanol extracts of different waste lime is observed. After 24 hours of treatment the sample dried at 90 and 120°C has a greater effect on proliferation with respect to the other treatments. Doses of 1, 5 and 10% of the sample dried at 120°C and 90 inhibit proliferation by 23, 28, 47, 20, 32 and 46%, respectively. While samples dried at 60°C have a minor effect on cell proliferation at doses of 1 and 10% inhibit proliferation by 19 and 38%, respectively. The effect of the lyophilized sample is less than the dried sample at 60°C where the percentage inhibition of proliferation at the concentrations of 1, 5 and 10% of the extract is 17, 25 and 33%, respectively.

Furthermore, inhibition of cell proliferation of colon cancer HT-29 of the different extracts waste lime in the time course of 48 and 72 h have a similar 24 h behavior, the difference is that inhibition proliferation reaches 61% in the treatment at 120°C with 10% of the extract, 50% by the treatment at 90°C with 10% of the extract, finally lyophilized sample with 1, 5 and 10% extract the inhibition of proliferation is 14, 37 and 39% on average. Finally, the solvent in which the extracts were also inhibited proliferation by 11%. In another study [24], demonstrated that dietary hesperidin during either the initiation, post-initiation or entire period phase significantly inhibited DMH-induced colon carcinogenesis in rats.

Citrus fruit contains other possible chemopreventive agents. These include d-limonene [25], naringin, diosmin

[26], and limonin [27]. Some of these can modify activities and expression of the detoxifying enzymes and CYPs [28]. Thus, citrus fruit is one of the rich sources of cancer chemopreventive agents.

3.3. Correlations between of the Phenolic Compounds in Lime and Mandarin Waste and Different Concentrations of Extracts in the Colon Cancer Cells

In the Table 2 shown some correlations of the most representative flavonoids of mandarin and lime extracts, exist a correlation of the 0.84 and 0.86 between the hesperidin and the 10% of the extracts at 24 and 48 h of interaction doses depend with the extracts and the colon cancer cells. Tanaka [29], observed a decrease in the proliferation of HT-29 colon cancer cells in rats when they used mandarin juice with high hesperidin content, Sakata [30], founded that hesperidin decreases the expression of cyclooxygenase-2 (COX-2), nitric oxide synthase (iNOS) and prostaglandin E₂ (PGE₂), but mainly hesperidin inhibits COX-2 and iNOS that are related to anti-tumorigenic efficacy.

Also, in Table 2 it can be observed that naringin and naringenin have positive correlations between 0.82 to 0.91, which means that the higher concentration of phenolic extracts during the curse of time, exist a decrease in the proliferation of colon cancer cells. Moon [31], they found that hesperidin, naringin and naringenin influence the metabolism of cytochrome P450 and specifically in the complexes CYP1A1, CYP1A2 and CYP3A4 respectively, the decrease in the activity of these enzymatic complexes causes a decrease in carcinogenesis

Table 1. Contents of the main phenolics of mandarin and lime waste

Treatment	Eriocitrin (mg/g DW)	Hesperidin (mg/g DW)	Naringin (mg/g DW)	Naringenin (mg/g DW)
Fresh MW ^a	197.24 ± 6.07 a	182.52 ± 4.98 b	54.69 ± 1.44 c	6.45 ± 0.13 d
Fresh LW ^a	97.24 ± 3.71 d	181.32 ± 5.83 b	74.94 ± 1.88 a	15.52 ± 0.89 a
MW-60 ^b	187.12 ± 1.09 b	202.53 ± 14.91 a	75.41 ± 3.33 a	4.48 ± 0.19 e
LW-60 ^b	47.33 ± 2.17 f	142.87 ± 8.64 d	55.39 ± 2.18 c	8.28 ± 0.34 c
MW-90 ^b	141.38 ± 10.89 c	208.31 ± 12.73 a	76.85 ± 4.91 a	8.17 ± 0.41 c
LW-90 ^b	142.24 ± 7.91 c	188.53 ± 9.87 ab	48.95 ± 2.77 d	11.16 ± 0.27 b
MW-120 ^b	152.13 ± 8.97 c	161.72 ± 9.67 c	53.72 ± 2.89 cd	4.54 ± 0.28 e
LW-120 ^b	75.32 ± 2.57 e	139.23 ± 5.17 e	63.29 ± 3.59 b	14.47 ± 0.85 a

^amandarin and lime waste; ^bmandarin and lime waste dried at 60, 90 and 120°C.

Means in the same column with a common letter are not significantly different ($p < 0.05$, Tukey).

Table 2. Correlations between phenolic compounds and the decrease of cell proliferation

	Hesperidin	Naringin	Naringenin
MW & LW 5A			0.82*
MW & LW 10A	0.84*	0.90*	
MW & LW 10B	0.86*	0.91*	0.83*

MW & LW 5A, indicated that the extract of mandarin waste (MW) and lime waste ((LW) at 5% concentration (5) has been in contact with de cells for 24 h (A)

MW & LW 10A, indicated that the extract at 10% concentration has been in contact with de cells for 24 h

MW & LW 10B, indicated that the extract at 10% concentration has been in contact with de cells for 48 h

*Correlations with a level of significance, $\alpha = 0.05$.

4. Conclusion

Mandarin (*C. reticulata* Blanco) and lime (*C. aurantifolia*) are tropical fruits that have antioxidant properties. Our results showed that phenolic extracts of MW and LW inhibited cell proliferation in a dose dose-depended manner of colorectal cancer cell lines, HT-29 and RKO. Inhibition of cell proliferation of cancer cells had strong correlation with specific phenolic compounds present in extracts of fruit waste such as hesperidin, naringin and naringenin.

Statement of Competing Interests

We affirm that the manuscript has not been previously published, is not currently submitted for review to any other journal and will not be submitted elsewhere before a decision is made.

There is no conflict of interest for the authors listed above.

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