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Review Article

Antioxidant and Antiproliferative Activity of Tropical Fruits Aqueous Extract

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Abstract

The antioxidant activity of aqueous extracts of five edible tropical fruits (*Spondias lutea, Hancornia speciosa, Spondias purpurea, Manilkara zapota* and *Averrhoa carambola)* was investigated using different methods. The amount of phenolic compounds was determined by the Folin-Ciocalteu reagent. Extracts showed neither reducing power nor iron chelation (between 0.01 and 2.0 mg/mL). *H. speciosa* exhibited the highest superoxide scavenging activity (80%, 0.5 mg/mL). However, at high concentrations (8.0 mg/mL) only *A. carambola*, *S. purpurea* and *S. lutea* scavenging 100% of radicals formed. *M. zapota* and *S. purpurea* had higher phenolic compound levels and greater OH radical scavenging activity (92%, 2 mg/mL). Antiproliferative activity was assessed with 3T3 fibroblasts and cervical tumor cells (HeLa). The most potent extract was *S. purpurea* (0.5 mg/mL), which inhibited HeLa cell proliferation by 52%. All extracts showed antioxidant and antiproliferative properties, characterizing them as functional foods.

INTRODUCTION

A functional food exhibits nutritional, metabolic, and therapeutic functions and has potential use in preventing and controlling certain diseases [1]. In addition to preventing cardiovascular diseases, these foods have anticarcinogenic and antioxidant properties.

According to the American Dietetic Association [2], fruits are one of the most basic forms of functional foods. In addition to their delicious flavor and aroma, they are important sources of vitamins, minerals and other essential antioxidants in human diet.

Due to the presence of antioxidants, fruit consumption has been associated with reduced risk of chronic diseases related to the oxidative stress induced by the production of free radicals in the human body [3]. However, although fruits are considered sources of natural antioxidants, their effectiveness depends on the chemical structure and concentration of these compounds. Total antioxidant content varies due to several factors such as plant variety, environment, climatic and soil conditions, genetic factors, degree of ripeness, storage and processing, among others [4].

Therefore, a fruit cannot be simply identified as functional food, without first knowing the antioxidant potential of the fruit in question.

Northeast Brazil shows a wide diversity of fruits, which have been widely consumed for many generations. However, little is known about their functional properties. In order to bridge this information gap, the present study aimed to determine the antioxidant and antiproliferative activity of the aqueous extract from five tropical fruit species consumed in Northeast Brazil.

MATERIALS AND METHODS

Materials

Iron sulfate, potassium ferricyanide, sulfuric acid and acetonitrile were obtained from Merck (Darmstadt, Germany). Nitro Blue Tetrazolium (NBT), ferrozine, $FeCl_2$, EDTA, methionine, riboflavin, gallic acid, trichloroacetic acid (TCA) and ammonium molybdate were purchased from Sigma-Aldrich Co. (St. Louis, USA). All other solvents and chemicals were of analytical grade.

Fruit samples

Fresh fruit samples with no apparent physical or microbial damage were collected separately. Samples included caja (*Spondias lutea*), mangaba (*Hancornia speciosa*), siriguela (*Spondias purpurea*), sapoti (*Manilkara zapota*), and carambola (*Averrhoa carambola*). All fruits were of eating quality, and were identically selected in terms of shape, size, color, and ripening stage.

Fruits were acquired in Pium-RN, Brazil, packed in plastic bags and taken to the laboratory (BIOPOL – Laboratory of Natural

Polymer Biotechnology) on the same day as collection, where they were washed under running water and stored at -76ºC.

Extraction

The clean fruits were seeded to obtain aqueous extracts and, in the case of the sapoti, the skin was removed. Pulps (400g) were then added to 800 mL of distilled water (1:2) and ground in a blender for 3 minutes at ambient temperature. Extracts were then centrifuged (8000 x g for 30 minutes at 40ºC) and filtered in a funnel with filter paper. After filtration, the aqueous extracts were lyophilized.

Determination of total phenolic content: Total phenolic content of tropical fruit aqueous extract was determined by the spectrophotometric method using the Folin – Ciocalteau reagent [5]. Results were expressed as µg of total phenolics in gallic acid equivalent (GAE) per mL of extract.

Determination of total antioxidant capacity (TAC)

The assay is based on the reduction of Mo (VI) to Mo (V) by the fruit extract and subsequent formation of a green phosphate/ Mo (V) complex at acid pH [5]. Tubes containing aqueous fruit extract and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate) were incubated at 95°C for 90 min.

After the mixture had cooled to room temperature, absorbance of each solution was measured at 695 nm against a blank. Antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

Hydroxyl radical scavenging activity assay

The scavenging activity of fruit extract against the hydroxyl radical was investigated using Fenton's reaction ($Fe^2 + H_2O_2$ $Fe^3 + OH^- + OH$). These results were expressed as inhibition rate. Hydroxyl radicals were generated using a modified method [6] in 3 mL sodium phosphate buffer (150 mM, pH 7.4), containing 10 mM $FeSO_4$. $7H_2O$, 10 mM EDTA, 2 mM sodium salicylate, 30% H_2O_2 (200 μL) and varying concentrations of aqueous fruit extracts. In the control group, sodium phosphate buffer replaced H_2O_2. Solutions were incubated at 37°C for 1 h, and the presence of the hydroxyl radical was detected by monitoring absorbance at 510 nm.

Superoxide radical scavenging activity assay

The assay was based on the capacity of aqueous fruit extract to inhibit photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system [5]. Each 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM) and 1 mL of sample solution. After blue form az an production, the increase in absorbance at 560 nm after 10 min illumination from a fluorescent lamp was monitored. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with the reaction mixture were kept in the dark and served as blanks.

Iron chelation

The ferrous ion chelating ability of samples was investigated according to earlier studies [5]. Briefly, the reaction mixture, containing samples of $FeCl_2$ (0.05 mL, 2 mM) and ferrozine (0.2 mL, 5 mM), was shaken well and incubated for 10 min at room temperature. Absorbance of the mixture was measured at 562 nm against a blank.

Reducing power

Reducing power of the samples was quantified as described further on Costa et al. [5]. Briefly, 4 mL of reaction mixture, containing different sample concentrations in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50°C for 20 min. The reaction was terminated by TCA solution (10% w/v). The solution was then mixed with distilled water and ferric chloride (0.1% w/v) solution and absorbance was measured at 700 nm. The results were expressed as a percentage of the activity shown by 0.2 mg/mL of vitamin C.

Cell proliferation studies

Cytotoxicity was determined by the MTT assay [7]. Cells were plated in 96-well plates at an initial density of 5 x 10^3 cells per well. After incubation for 24 h at 37ºC, cells were treated with concentrations of125, 250 and 500μg/mL and incubated for 48h. The MTT solution (5 mg/mL) was then added to each well and further incubated for 4 h at 37ºC. To solubilize the product of MTT reduction, 100μL of isopropanol containing 0.04 N HCl was added to each well and thoroughly mixed using a multichannel pipette. Optical density was read with an ELISA reader at 570 nm. The percent inhibition of cell proliferation was calculated as follows:

$$\%\ Inhibition = \frac{Abs\ 570\ nm\ control - Abs\ 570\ nm\ sample}{Abs\ 570\ nm\ control}\ x\ 100$$

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was conducted by one-way ANOVA using Graph Pad In Stat Software. Student-Newman-Keuls post-tests were performed for multiple group comparison. In all cases statistical significance was set at $p < 0.05$.

RESULTS

Total phenolic compounds

The amount of phenolic compounds in the aqueous extracts of the fruits under study is shown in Figure 1. Values ranged from 441.0 μg GAE/mL to 199.6 μg GAE/mL for *S. purpurea* and *S. lutea* respectively. Data analysis resulted in 2 groups, fruits with a large amount of phenolic compounds (*M. zapota, H. speciosa* and *S. purpurea*) and those with a small amount (*A. carambola* and *S. lutea*).

Antioxidant activity

Total antioxidant capacity (TAC): The TAC test evaluates the capacity of a sample to donate electrons into a slightly acidic environment, thereby neutralizing reactive species, such as oxygen reactive species. Results obtained in the present study are shown in Figure 2. We found no correlation between the amount of phenolic compounds and TAC values of the extracts.

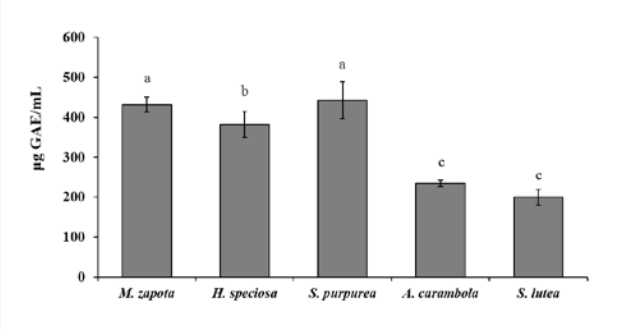

Figure 1 Total phenolic compounds of tropical fruit aqueous extract, expressed in gallic acid equivalents (GAE) μg/ mL. Different letters indicate a significant difference between concentrations of individual fruit aqueous extract using one-way ANOVA followed by the Student-Newman-Keuls test ($p < 0.05$).

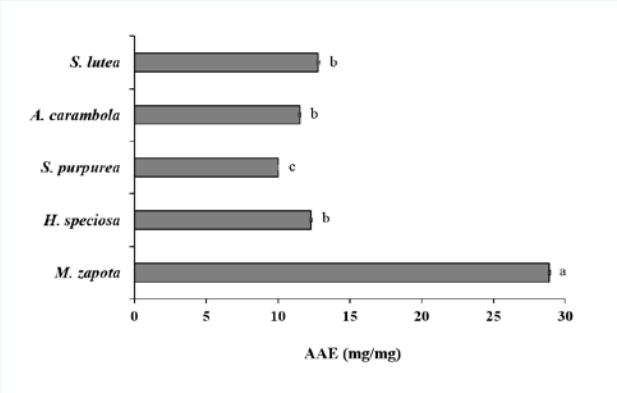

Figure 2 Total antioxidant capacity (TAC) of tropical fruit aqueous extract, expressed in ascorbic acid equivalents (AAE) mg/mg. Different letters indicate a significant difference between concentrations of individual fruit aqueous extract using one-way ANOVA followed by the Student-Newman-Keuls test ($p < 0.05$).

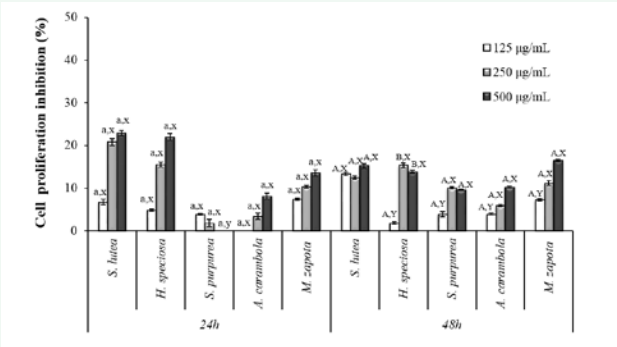

Figure 3 Antiproliferative activity of tropical fruit aqueous extracts with 3t3 cells. Data are expressed as mean ± standard deviation. a/A,B indicate a significant difference ($p< 0.05$) between different concentrations of the same fruit.x,y/X,Y indicate a significant difference ($p< 0.05$) between similar concentrations of different fruits. Statistical analysis was conducted separately at 24 and 48 hours using one-way ANOVA followed by the Student-Newman-Keuls test ($p < 0.05$).

Of the five species analyzed, *M. zapota* exhibited double the TAC value observed in the other fruit extracts.

Reducing power capacity: Reducing power is linked to electron transfer ability; thus, reducing activities were usually related to the development of reductones. Reductones terminate free radical chain reactions by donating a hydrogen atom. In most cases, irrespective of the stage in the oxidative chain in which antioxidant action is assessed, predominance of non-enzymatic antioxidative activity is mediated by redox reactions [5]. Thus, several studies have reported that antioxidant activity was concomitant with reducing power. However, our fruit extracts did not show any reducing property (from 0.01 to 2.0 mg/mL).

Metal chelating activity: Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions. It is known to generate ROS by Fenton and Haber-Weiss reactions. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and consequent oxidative damage. The iron-chelating capacity test gauges the ability of antioxidants to compete with ferrozine in chelating ferrous ions. Activity is measured as the decrease in absorbance of the red Fe^{2+} ferrozine complex.

In this assay fruit extracts (from 0.01mg to 2.0 mg/mL) did not interfere with the formation of the ferrous complex using the ferrozine reagent, suggesting they had no chelating activity.

Superoxide radical scavenging: The effect of different extract concentrations on superoxide anions are shown in Table 1. All extracts assessed are capable of sequestering the superoxide radical, and three extracts (*S. purpurea, S. lutea, A. carambola*) were capable of scavenging 100% of radicals formed. Extract of *M. zapota* was the least potent, unable to sequester more than 60% of radicals formed. A low concentration (0.5 mg/mL) of *H. speciosa* showed the greatest superoxide scavenging capacity (~80%), a similar value to that observed with gallic acid (positive control) at the same concentration.

Hydroxyl radical scavenging: Table 1 show that all extracts exhibited dose-dependent hydroxyl radical scavenging activity (from 0.1 to 2.0 mg/mL). Extracts of *M. zapota* and *S. purpurea* displayed the highest hydroxyl radical scavenging percentage, around 92% (2.0 mg/mL). Interestingly, these two fruits had the largest amount of phenolic compounds (Figure 1). Another remarkable finding is that three extracts (*M. Zapota, S. purpurea,* and *S. lutea*) at a concentration of 0.1 mg/mL were capable of scavenging 50% of hydroxyl radicals at a lower concentration than gallic acid (positive control).

Cell proliferation studies

The antiproliferative activity of tropical fruit aqueous extracts was determined with two cell lines: HeLa (human cervical cancer cells) and 3T3 (rat fibroblast cells) for 24h and 48h using the MTT colorimetric method. 3T3 cells are commonly used to assess compound toxicity against normal cells. Our data showed that the extracts had low toxicity against these cells and interfered very little in 3T3 cell proliferation. Proliferation inhibition ranged from 0 to 20% in the first 24 hours, at the maximum concentration used. However, this did not increase after 48 hours of experiment (Figure 3).

The effect of extracts against Hela tumor cells was also evaluated. Results are shown in Figure 4. Under conditions tested, *H. speciosa* extract showed no antiproliferative activity (around 10% at the highest concentrations). The other extracts displayed antiproliferative activity, highlighted by extracts of *Spondias purpurea* and *Manilkara zapota* (500 μg/mL, 48h), which inhibited around 58 and 40% of cell proliferation, respectively.

No positive correlation also was found between the amount of phenolic compounds in the extracts and their antiproliferative activity. Although extracts of *Spondias purpurea* and *Manilkara zapota* showed higher amounts of phenolic compounds, they also exhibited greater antiproliferative activity.

DISCUSSION

The amount of phenolic compounds observed in the present study was lower than that found in some varieties of grapefruit juices, which showed around 535.0 μg GAE/mL. On the other hand, *M. zapota, H. speciosa* and *S. purpurea* exhibited higher values than those recorded in aqueous extracts of fruits consumed worldwide, such as apples (339.0 μg GAE/mL) and pineapples (358.0 μg GAE/mL) [8]. This demonstrates the great potential of these fruits as functional foods, given that plant phenolic compounds of are considered the main antioxidant compounds of fruits [9]. Furthermore, the food industry has shown considerable interest in plant extracts rich in phenolic compounds, since they retard degradation of various nutrients found in industrialized foods, thereby increasing their quality and nutritional value [10].

A comparison of the results obtained from TAC to the values of other investigators was quite limited, since this is the first literature study to assess the TAC of *H. speciosa*, *S. purpurea*, *A. carambola* and *S. lutea*. An exception was *M. zapota.* The approximate value of 30.0 AAE for aqueous extract of *M. zapota* found in the present study was similar to that described in an earlier study assessing the same extract [11]. On the other hand, extract of *M. zapota* from Singapore exhibited higher values than ours, around 101.4 AAE [12]. This difference could be due to natural variations, especially when cultivation conditions and stages at harvest are difficult to determine.

The superoxide radical is a highly toxic species produced by innumerable biological and photochemical reactions. It is produced in vivo and leads to the formation ofH_2O_2via a dismutation reaction, which is another oxidant agent very harmful to cells [13]. Similar superoxide scavenging values were found with extracts (from 0.5 to 1.0 mg/mL) of the strawberry tree (*Arbutus unedo L.*) [14]. On the other hand, values obtained with *H. speciosa* extract (0.5 mg/mL) were higher than those observed in extracts of *Choerospondias axillaris* [15] and *Psidium guajava* [16] which sequestered around 38 and 60% of superoxide radicals formed, respectively, at a concentration of 0.5 mg/mL.

The hydroxyl radical is the most reactive of oxygen reactive species, causing serious damage to biomolecules. Its elimination is highly desirable, since the elevated reactivity of this radical is associated to cell damage, leading to several diseases [15].

Hydroxide scavenging activity of extracts analyzed here was not as potent as that described for extract of the Indian gooseberry (*Phyllanthus emblica*), which scavenging around 80% of radicals formed, at a concentration of 25.0 μg/mL [17]. However, the activity observed here was much more potent than that reported for extracts of other fruits, such as bitter melon *(Momordica charantia),* known in Brazil as "melão de São Caetano". This fruit exhibited low hydroxyl radical scavenging capacity, since with 1.6 mg/mL the extract was capable of scavenging only 37.1% of hydroxyl radicals [18].

Extracts of red pitaya peel (*Hylocereus undatus*) and white pitaya peel (*H. costaricensis*), at a concentration of 2.0 mg/mL, showed scavenging activity of only 64.0 and 58.0%, respectively [19]. The scavenging activity of fruits studied here was also

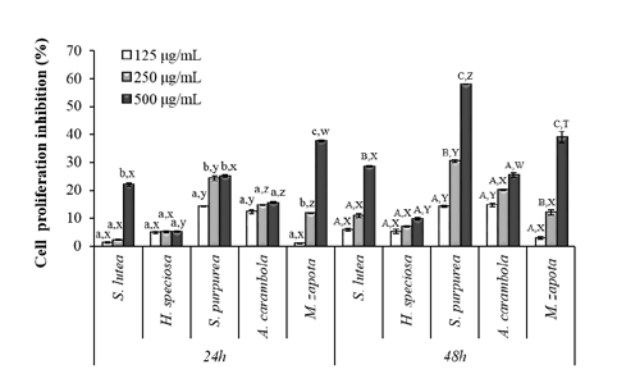

Figure 4 Antiproliferative activities of tropical fruit aqueous extracts with Hela cells. Data are expressed as mean ± standard deviation. a/A,B indicate a significant difference ($p< 0.05$) between different concentrations of the same fruit.x,y/X,Y indicate a significant difference ($p< 0.05$) between similar concentrations of different fruits. Statistical analysis was conducted separately at 24 and 48 hours using one-way ANOVA followed by the Student-Newman-Keuls test ($p < 0.05$).

Table 1: Hydroxyl and superoxide radical scavenging activity of tropical fruits.

Fruits	mg/mL	Inhibition (%)	
		Hydroxyl radical scavenging	Superoxide radical scavenging
Manilkara zapota	0.10	50.9 ± 2.6 ^{a}	nd
	0.25	52.8 ± 4.3 ^{a}	nd
	0.50	66.5 ± 2.0 ^{b}	41.6 ± 1.5 ^{a}
	1.00	87.4 ± 1.4 ^{c}	45.3 ± 3.5 ^{a}
	2.00	92.2 ± 0.5 ^{c}	45.2 ± 0.0 ^{a}
	4.00	nd	61.1 ± 3.3^{b}
	8.00	nd	62.0 ± 0.9^{b}
Hancornia speciosa	0.10	26.7 ± 4.7 ^{a}	nd
	0.25	50.3 ± 1.6^{b}	nd
	0.50	58.9 ± 0.2^{b}	80.1 ± 7.7 ^{a}
	1.00	73.2 ± 3.6^{c}	81.5 ± 10.6 ^{a}
	2.00	76.0 ± 1.2^{c}	86.3 ± 8.3^{b}
	4.00	nd	88.3 ± 5.0^{b}
	8.00	nd	89.9 ± 1.5^{b}
Spondias purpurea	0.10	58.3 ± 5.0 ^{a}	nd
	0.25	65.9 ± 0.4 ^{a}	nd
	0.50	79.5 ± 1.8 ^{b}	33.5 ± 3.9 ^{a}
	1.00	88.4 ± 2.2^{b}	44.8 ± 6.3 ^{b}
	2.00	92.6 ± 0.3^{b}	47.1 ± 0.5 ^{b}
	4.00	nd	81.6 ± 8.9^{c}
	8.00	nd	101.5 ± 2.0^{d}
Averrhoa carambola	0.10	17.9 ± 0.3 ^{a}	nd
	0.25	27.4 ± 1.7^{b}	nd
	0.50	49.6 ± 2.3^{c}	43.4 ± 4.5 ^{a}
	1.00	82.1 ± 2.2^{d}	49.8 ± 2.5 ^{a}
	2.00	92.0 ± 0.7^{e}	68.3 ± 3.8^{b}
	4.00	nd	92.5 ± 0.8^{c}
	8.00	nd	102.1 ± 0.7^{c}

Spondias lutea	0.10	63.1 ± 1.1 [a]	nd
	0.25	60.6 ± 2.3 [a]	nd
	0.50	74.1 ± 2.2[b]	36.3 ± 3.1 [a]
	1.00	76.3 ± 3.2[b]	46.9 ± 10.5 [a]
	2.00	79.9 ± 1.1[b]	56.4 ± 0.2 [a]
	4.00	nd	101.4 ± 8.1[b]
	8.00	nd	101.7 ± 3.4[b]
Gallic acid	0.10	40.5 ± 1.3 [a]	41.0 ± 3.1 [a]
	0.25	63.3 ± 2.1 [a]	73.1 ± 2.6 [a]
	0.50	92.0 ± 1.2[b]	81.1 ± 3.2[b]
	1.00	98.4 ± 2.3[b]	90.1 ± 3.5 [a]

Data are expressed as means ± standard deviation. Different letters indicate a significant difference between tropical fruits using one-way ANOVA followed by the Student-Newman-Keuls test ($p < 0.05$). nd – not determined.

greater than that observed for extracts from other sources such as the fungi *Agrocybe cylindracea* [20] and *Ganoderma tsugae* [21] and seaweeds [5] which, even at high concentrations (> 5.0 mg/mL), were unable to sequester more than 30% of hydroxyl radicals.

Results show that the fruit extracts investigated in the present study exhibited substantial hydroxyl radical sequestering capacity and could be used to help prevent or reduce damage provoked by oxidative stress.

Assessment of the antiproliferative activity of fruit extracts against tumor cells has been increasing in recent years. A study with extract of haritaki fruit (*Terminalia chebula*) and human prostate cells (PC-3) showed dose-dependent antiproliferative activity, reaching a maximum of around 0.4 mg/mL. Data also show a positive correlation between antiproliferative activity and phenolic compound content, which led the authors to suggest the latter are responsible for the antiproliferative activity of extracts [22]. Positive correlations were also observed between the amount of phenolic compounds and antiproliferative activity with extracts of rosehips and black chokeberries against colon cancer cells (HT-29) and breast cancer cells (MCF-7). The authors proposed that the antiproliferative activity of extracts is due to the presence of phenolic compounds [23].

Antiproliferative activity is likely dependent on different types of phenolic compounds, some being more toxic to cells than others. In the future, we intend to purify the different phenolic compounds present in fruit extracts studied here, assess them as *in vitro* and *in vivo* antiproliferative compounds, and identify their action mechanisms.

CONCLUSIONS

Data obtained demonstrated that tropical fruits, especially *Spondias purpurea* and *Manilkara zapota,* can inhibit oxidative damage by acting in both the initiation and termination phase of the oxidant agent formation process, as well as inhibiting tumor uterine cell proliferation, because the correlation between oxidative stress and cancer. These characteristics show their great potential for dietotherapeutic and nutritional applications.

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