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Antigenotoxic Effects of Piquiá (Caryocar villosum) in Multiple Rat Organs

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Abstract This study investigated the in vivo genotoxicity of piquiá pulp (Caryocar villosum) and its potential antigenotoxicity on doxorubicin (DXR)-induced DNA damage by comet assay and micronucleus test. In addition, the phytochemicals present in piquiá pulp were determined. Piquiá fruit pulp (75, 150 or 300 mg/kg b.w.) was administered by gavage to Wistar rats for 14 days, and the animals received an injection of saline or DXR (15 mg/kg b.w., i.p.) 24 h before they were euthanized. The phytochemical analysis revealed the presence of carotenoids; phenolic compounds, including flavonoids; tannins and α-tocopherol in piquiá pulp. No statistically significant differences were observed in the evaluated parameters, demonstrating the absence of cytotoxic and genotoxic effects of piquiá pulp at all tested doses. In liver, kidney, cardiac and bone marrow cells, piquiá significantly reduced the DNA damage induced by DXR. Our results showed that the lowest piquiá dose caused the largest decrease in DNA damage and the highest dose caused the smallest decrease, demonstrating an inverse dose–response of piquiá pulp. Furthermore, we observed a difference in the potential antigenotoxic effects in several tissues. In conclusion, our results demonstrated that piquiá pulp was not genotoxic and inhibited the genotoxicity induced by DXR, but some of the protective effects that were observed depended on the doses and experimental conditions. Therefore, further investigations are needed to clarify how piquiá pulp positively affects human health.

Keywords Polyphenols · Rats · Comet · Micronucleus · Antigenotoxic · Antimutagenicity

Abbreviations

ABTS 2,2,9-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
APCI Atmospheric pressure chemical ionization
BSA Bovine serum albumin
CE Catechin equivalent
DAD Diode array detector
% DNA Percentage of DNA
DXR Doxorubicin
ESI Electrospray ionization
GAE Gallic acid equivalent
MN Micronucleus
MNPCE Micronucleated erythrocyte polychromatic
MO Mineral oil group
MS Mass spectrometer
NCE Normochromatic erythrocyte
PCE Polychromatic erythrocyte
TAE Tannic acid equivalent
Introduction

Because fruit intake has an important role in promoting health, exploiting new and exotic fruits has garnered increased attention in recent years. *Caryocar villosum* (Aubl.) Pers. (local name: piquiá), of the Caryocaraceae family, is a large tree that grows in the virgin forests of the Amazon basin. Piquiá fruit pulp contains high levels of carotenoids, total phenolic compounds and flavonoids and exhibits free radical scavenger activity [1, 2]. Considering that piquiá pulp is part of the diet of the Amazon population, studying the pulp may provide important insight for commercializing this fruit for the food industry. We investigated the genotoxicity and antigenotoxicity of piquiá fruit pulp in the liver, kidney and heart cells of rats using the comet assay and the mutagenicity in bone marrow and peripheral blood cells by the micronucleus (MN) test. In addition, the phytochemicals present in piquiá pulp were determined. To the best of our knowledge, this is the first study that has investigated the potential antigenotoxic effects of piquiá. Assessing the genotoxic/antigenotoxic effects of this fruit pulp in an in vivo system is vital for establishing its safety and assessing potential hazards when combined with pharmacologic drugs, such as antitumor agents used in cancer therapy.

Materials and Methods

Samples and Chemical Agents

Piquiá fruits (*Caryocar villosum*) were acquired from Belém, Pará, Brazil. The yellow-colored pulp was separated from the seeds, weighed, ground and immediately lyophilized (Liobras Equipment, São Paulo, Brazil) and stored in the dark at −36 °C. Before it was administered to animals, the freeze-dried pulp was dissolved in mineral oil (25 % of the final solution) and then mixed in water. DXR (Rubidox®, CAS: 25316-40-9) was acquired from Laboratório Químico Farmacêutico Bergamo Ltda. (São Paulo, Brazil). All experiments were performed in minimal indirect light. All other reagents used were of analytical grade.

Analysis of Phytochemicals from Freeze-Dried Piquiá Pulp

Determination of total phenolic compounds, flavonoids, tannins and carotenoids

Phenolic compounds were extracted from 5 g of freeze-dried piquiá pulp with methanol:water (8:2, v/v) and quantified using the Folin-Ciocalteau colorimetric method [3]; the results are expressed as milligrams of gallic acid equivalent (GAE) per 100 g of freeze-dried pulp. The total flavonoid concentrations were quantified by a colorimetric assay described by Zhishen et al. [4], and the results are expressed as milligrams of catechin equivalent (CE) per 100 g of freeze-dried pulp. The tannins were quantified according to Brune et al. [5] (hydrolyzable tannins) and Waterman et al. [6] (condensed tannins), and the results are expressed as milligrams of tannic acid equivalent (TAE) per 100 g of freeze-dried pulp. The carotenoid concentrations were quantified according to De Rosso et al. [7] and the total carotenoid content was calculated using the specific extinction coefficient of zeaxanthin in ethanol $E_{1%}^1cm = 2540$ [8] and is expressed as milligrams of carotenoid per 100 g of freeze-dried pulp. All experiments were performed in triplicate, and the relative standard deviation was lower than 5 %.

HPLC Analysis

The carotenoids, phenolic compounds, ascorbic acid, tocopherols and tocotrienols from the freeze-dried piquiá pulp were analyzed in triplicate. The carotenoids, phenolic compounds and ascorbic acid were analyzed using a Shimadzu HPLC (Kyoto, Japan) connected in series to a diode array detector (DAD) (SPD-M20A) and a mass spectrometer (MS) from Bruker Daltonics (Esquire 4000 model, Bremen, Germany) with an ion-trap analyzer. An APCI (atmospheric pressure chemical ionization) interface was used in positive mode for carotenoids, and an ESI (electrospray ionization) source was used for phenolic compounds. Carotenoid analysis consisted of quantification and identification by HPLC-DAD-APCE-MS/MS as previously described by De Rosso et al. [7]. Phenolic compounds were analyzed using the same HPLC-DAD-MS described above. The phenolic compounds were separated on a C$_{18}$ Synergi Hydro column using a linear gradient of water:formic acid (99.5:0.5, v/v) and acetonitrile:formic acid (99.5:0.5, v/v) as the mobile phase. Absorption spectra were obtained between 200 and 600 nm, and the chromatograms were processed at 217 nm. The carotenoid and phenolic compound percentages obtained by HPLC-DAD, in triplicate, were calculated considering the areas of all major separated peaks. The ascorbic acid was extracted from 3 g of freeze-dried piquiá pulp with an aqueous solution of 1 % oxalic acid and quantified by HPLC-DAD after separation on a C$_{18}$ Shim-pack column with an isocratic mobile phase containing sulfuric acid. The tocopherol and tocotrienol contents were analyzed in accordance with the American Oil Chemists’ Society (AOCS, 2004) [9] using a normal-phase HPLC system (Perkin Elmer, Waltham, MA, USA). The oil from the freeze-dried piquiá pulp was extracted according to Bligh et al. [10] and then diluted in hexane before injection.

Animals and Treatments

Healthy, male Wistar rats, approximately 4–5 weeks old and weighing an average of 120 g, were obtained from...
Comet Assay of Liver, Kidney and Heart Cells

The alkaline single-cell comet assay (pH>13) was performed according to Singh et al. [12]. Briefly, 0.4 g of each organ was sliced in 2 ml of chilled Hank’s solution. The trypan dye exclusion method was used to determine cell viability immediately before the comet assay; viability was above 75 % for all treatments. Cellular suspensions (80 μl) of each organ were mixed with 240 μl of a low-melting-point agarose (0.5 %) and spread onto microscope slides pre-coated with normal-melting-point agarose (1.5 %). The cells were immersed in a freshly prepared lysis solution consisting of 2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid, 10 % dimethylsulfoxide, 1 % Triton X-100 and 10 mM Tris at pH 10 for 22 h at 4 °C. After lysis, electrophoresis was performed in alkaline solution (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min with an electric field strength of 1 V/cm (25 V and 300 mA). Slides were subsequently immersed in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 5 min. Slides were dried at ambient temperature, fixed in ethanol for 2 min, stained with 30 μl of ethidium bromide (20 μl/ml) and analyzed immediately. Image analysis was performed at 400x magnification using a fluorescence microscope (Zeiss) with a 510–560 nm filter and a 590 nm barrier. One hundred nucleoids per animal (50 nucleoids per slide) were photographed using an Axion camera (Zeiss) and the AxioVision 3.1 program (Zeiss) and then analyzed using TriTek CometScore™ Freeware v1.5 software.

Micronucleus Test of Bone Marrow and Peripheral Blood Cells

The micronucleus test was performed on bone marrow and peripheral blood cells according to the protocols described by Schmid [14] and Holden et al. [15], respectively. The bone marrow cells were collected in fetal bovine serum (Invitrogen) and centrifuged, and the pellets were resuspended in 0.3 ml of supernatant for slide preparation. The slides were fixed in methanol for 10 min and then stained with Giemsa for 10 min (Sigma–Aldrich, CAS 51811-82-6). A small drop of peripheral blood (5 μl) was collected to prepare blood smears. The slides were fixed in absolute methanol for 10 min and stained with Acidine Orange (Sigma–Aldrich, CAS 10127-02-3). Coded slides were scored under 1,000× magnification using a fluorescence microscope (Zeiss, AxioStarPlus®) equipped with an excitation filter of 488 nm. Two thousand polychromatic erythrocytes (PCEs) were examined in the bone marrow [16] and 1,000 PCEs were analyzed in the peripheral blood [17] from each animal, and the number of micronucleated PCEs (MN-PCEs) was recorded. In the bone marrow, the percent of PCEs among 500 erythrocytes was determined in the same sample to evaluate the cytotoxic effect of any treatment. The percent reduction in the frequency of the micronucleated cells was calculated according to Waters et al. [13], as described previously.

Statistical Analysis

All data are expressed as the mean ± standard deviation (n=6/group). The normality of variable distributions was evaluated by the Kolmogorov–Smirnov test. The data were evaluated with a one way-ANOVA and Dunnnett’s test, using the GraphPad Prism 2.01 software program. A value of p<0.05 was considered statistically significant for all of the parameters evaluated.

Results

HPLC-DAD chromatograms illustrate the carotenoids (Fig. 1a) and phenolic compounds (Fig. 1b) detected in the freeze-dried piquiá pulp. The total contents of these compounds and the percentages of their major constituents are
shown in Table 1. The piquiá fruit pulp contained a large amount of total phenolic compounds and total carotenoids (236.2 mg GAE/100 g and 6.9 mg zeaxanthin equivalent/100 g, respectively), and tocopherols were also observed. Of the carotenoids, phenolic compounds and tocopherols found, antheraxanthin, gallic acid and α-tocopherol were the most abundant, respectively. Condensed tannins were not detected in piquiá pulp, as precipitate did not form after the addition of bovine serum albumin (BSA).

Table 2 presents data from a comet assay analysis of liver, kidney and heart cells. DNA damage did not increase in cells from rats treated orally with piquiá pulp, regardless of the piquiá dose administered ($p > 0.05$), demonstrating the absence of genotoxic effects at the indicated doses of piquiá pulp. As expected, animals treated with DXR exhibited a higher % DNA in the tail compared with the MO group. The data in Table 2 also show that treatment with piquiá pulp reduced the DNA damage induced by DXR in the liver, kidney and heart compared with the MO + DXR group. A difference in antigenotoxic effects was observed in several tissues. In liver cells, treatment with 75 mg/kg b.w. piquiá significantly reduced ($p < 0.05$) the % DNA in the tail, whereas in kidney cells, doses of 75 and 150 mg/kg b.w. significantly reduced ($p < 0.05$) the DNA damage induced by DXR. The dose of 300 mg/kg b.w. of piquiá pulp significantly decreased the % DNA in the tail in heart cells.

The frequency of MNPCE in bone marrow and peripheral blood cells and the PCE/NCE ratio are presented in Table 3. The number of micronuclei did not increase after treatment with 75, 150 or 300 mg/kg b.w. of piquiá pulp in either tissue, demonstrating that piquiá has no effects on these mutagenic endpoints at these doses. In bone marrow cells, the piquiá treatment alone decreased MNPCE frequency when compared to the MO group; however, this difference was not significant ($p > 0.05$). The administration of DXR resulted in a significant increase ($p < 0.05$) in micronuclei compared to the MO group. Simultaneous treatment with 75, 150 or 300 mg/kg b.w. of piquiá pulp + DXR resulted in a significant reduction of 50.0 %, 38.5 % or 39.3 %, respectively, in MNPCE frequency in bone marrow cells compared to the MO + DXR group. In peripheral blood cells, piquiá doses of 75, 150 or 300 mg/kg b.w. + DXR reduced the DNA damage by 36.3 %, 29.0 % or 17.2 %, respectively, compared with the MO + DXR group. However, these reductions were not significant. Bone marrow cytotoxicity was evaluated by quantifying the PCE/NCE ratio in 500 erythrocytes. The results revealed that piquiá pulp or DXR treatment did not decrease the PCE/NCE ratio compared with the MO group (Table 3).
Discussion and Conclusion

While most genotoxic studies have used fruit extracts or isolated compounds, we used whole fruit pulp in this study. Considering that humans consume fruit pulps rather than their extracts or isolated compounds, studies using pulp more accurately mimic the potential effects of the compounds in the fruits after ingestion compared with studies that use extracts. According to Zeiger [18], before a substance is claimed to be antigenotoxic, it should be evaluated for genotoxicity and rigorously tested with appropriate protocols. In the present study, none of the evaluated doses of piquiá pulp was genotoxic when analyzed by the micronucleus test and the comet assay. Concurrent negative control animals, treated with the solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should always be included in genotoxicity assays [19]. We included both a negative control group, in which the animals received water by gavage, and a MO group, treated in the same way as the piquiá and/or DXR groups. There were no significant differences between negative control and MO groups; therefore, only data from the MO groups are shown in the tables.

We selected DXR because it is an effective genotoxic agent in vivo and in vitro, and the administration of a single dose in rodents has been shown to induce genotoxicity in various tissues [20]. DXR increased DNA damage in both the micronucleus test and the comet assay. DXR induces genotoxicity and cardiotoxicity in normal cells via oxidative damage, which is caused by generation of free radicals [21]. Combinations of DXR and dietary antioxidants are currently being evaluated as therapies to inhibit the generation of free radicals and the development of DXR cardiotoxicity [22]. Given these characteristics and that piquiá pulp is rich in polyphenols and antioxidants, we chose DXR as a positive control in the micronucleus test and comet assay, where the heart was the organ in which adverse effects of DXR were assessed.

Studies in the literature have indicated that dietary antioxidants are more effective when applied before the genotoxic agent is administered [20]. The protective effect of piquiá pulp was observed in the micronucleus test in bone marrow cells at all evaluated doses. In peripheral blood cells, although no significant difference was observed, treatment with piquiá pulp decreased the MNPCE frequency. The comet assay revealed that piquiá pulp treatments decreased the DNA damage induced by DXR, and the greatest reductions were found in the liver and heart. The antigenotoxic potentials of fruits were also evaluated in other studies [23, 24]. Although the exact mechanism of the inhibition of DXR-induced genotoxicity by piquiá pulp has not been thoroughly elucidated, the antioxidant compounds may explain the protective effects of piquiá pulp verified in this study. A phytochemical analysis of piquiá pulp revealed that phenolic compounds and carotenoids are its main components. In addition, piquiá exhibited the most total phenolic compounds, flavonoids and antioxidant capacity (ABTS assay) compared with 18 other tropical fruits in a screen by Barreto et al. [1]. In the present study, among all of the compounds identified, the carotenoids antheraxanthin and zeaxanthin, as well as the phenolic compounds ellagic acid, rhamnoside and gallic acid, were the major compounds found in piquiá pulp.

The protective mechanisms of fruits and vegetables have been related to their antioxidant contents, such as carotenoids and a vast array of polyphenolic compounds [25], and it is likely that these compounds together provide better

### Table 2

% DNA in the tail (mean ± SD) from liver, kidney and heart cells of rats treated with different doses of piquiá pulp alone (75, 150 or 300 mg/kg b.w.) or in combination with doxorubicin (DXR, 15 mg/kg b.w.) in a subacute (14 days) treatments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% DNA in tail</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>8.4±3.6</td>
<td></td>
</tr>
<tr>
<td>Piquiá 75 mg/kg</td>
<td>8.5±1.3</td>
<td></td>
</tr>
<tr>
<td>Piquiá 150 mg/kg</td>
<td>7.5±2.5</td>
<td></td>
</tr>
<tr>
<td>Piquiá 300 mg/kg</td>
<td>8.9±1.6</td>
<td></td>
</tr>
<tr>
<td>MO + DXR</td>
<td>19.0±7.6a</td>
<td></td>
</tr>
<tr>
<td>Piquiá 75 + DXR</td>
<td>10.4±3.1b</td>
<td>81.1 %</td>
</tr>
<tr>
<td>Piquiá 150 + DXR</td>
<td>12.8±5.1</td>
<td>58.4 %</td>
</tr>
<tr>
<td>Piquiá 300 + DXR</td>
<td>15.1±3.8</td>
<td>36.7 %</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>6.5±1.6</td>
<td></td>
</tr>
<tr>
<td>Piquiá 75 mg/kg</td>
<td>8.7±2.3</td>
<td></td>
</tr>
<tr>
<td>Piquiá 150 mg/kg</td>
<td>9.2±3.2</td>
<td></td>
</tr>
<tr>
<td>Piquiá 300 mg/kg</td>
<td>10.3±3.1</td>
<td></td>
</tr>
<tr>
<td>MO + DXR</td>
<td>21.0±4.4a</td>
<td></td>
</tr>
<tr>
<td>Piquiá 75 + DXR</td>
<td>12.8±3.0b</td>
<td>56.5 %</td>
</tr>
<tr>
<td>Piquiá 150 + DXR</td>
<td>12.9±4.3b</td>
<td>55.8 %</td>
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<tr>
<td>Piquiá 300 + DXR</td>
<td>14.4±4.4a</td>
<td>45.5 %</td>
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<tr>
<td>Heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>8.7±2.6</td>
<td></td>
</tr>
<tr>
<td>Piquiá 75 mg/kg</td>
<td>8.8±3.3</td>
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<tr>
<td>Piquiá 150 mg/kg</td>
<td>9.9±1.5</td>
<td></td>
</tr>
<tr>
<td>Piquiá 300 mg/kg</td>
<td>9.8±3.9</td>
<td></td>
</tr>
<tr>
<td>MO + DXR</td>
<td>20.6±5.2a</td>
<td></td>
</tr>
<tr>
<td>Piquiá 75 + DXR</td>
<td>15.5±5.3</td>
<td>42.8 %</td>
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<tr>
<td>Piquiá 150 + DXR</td>
<td>16.1±5.0</td>
<td>37.8 %</td>
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<tr>
<td>Piquiá 300 + DXR</td>
<td>12.4±2.9b</td>
<td>68.9 %</td>
</tr>
</tbody>
</table>

100 nuclei were analyzed per animal, with six animals per group. SD standard deviation. MO Mineral oil

a Significantly different from MO (p<0.05)
b Significantly different from MO + DXR (p<0.05)

ANOVA and Dunnett’s test were used to determine significance of differences
Table 3 Frequency (mean ± SD) of micronucleated polychromatic erythrocytes (MNPCE), percentage reduction and PCE/NCE ratio in bone marrow and peripheral blood cells of rats treated with different doses of piquiá pulp alone (75, 150 or 300 mg/kg b.w.) or in combination with doxorubicin (DXR, 15 mg/kg b.w.), in a subacute (14 days) treatment

| Treatments          | Bone marrow* | | Peripheral blood** | |
|---------------------|--------------|---------------------------|---------------------------|
|                     | MNPCE/1000   | Reduction                 | PCE/NCE                   | MNPCE/1000   | Reduction                 |
| MO                  | 4.2±0.9      | 0.8±0.3                   | 2.0±1.2                   |
| Piquiá 75 mg/kg     | 1.6±0.7      | 0.6±0.2                   | 1.8±1.6                   |
| Piquiá 150 mg/kg    | 2.1±0.8      | 0.7±0.2                   | 2.3±1.0                   |
| Piquiá 300 mg/kg    | 2.6±0.9      | 0.7±0.3                   | 2.6±0.8                   |
| MO + DXR            | 16.4±3.4\*   | 0.7±0.4                   | 13.0±4.4\*                |
| Piquiá 75 + DXR     | 10.3±1.7\*\(\) | 50.0 %                   | 9.0±2.9\*                | 36.3 %       |
| Piquiá 150 + DXR    | 11.7±2.6\*\(\) | 38.5 %                   | 9.8±5.2\*                | 29.0 %       |
| Piquiá 300 + DXR    | 11.6±2.2\*\(\) | 39.3 %                   | 11.1±3.9\*               | 17.2 %       |

*p<0.05. **Significantly different from MO (p<0.05). \(\) Significantly different from MO + DXR (p<0.05). ANOVA and Dunnett’s test were used to determine significance of differences.

protective effects than a single molecule does alone [26]. Scavenging of free radicals may be considered one of the most important antioxidant functions of phenolic compounds; moreover, these compounds also act as metal chelators, function as inhibitors or activators of a large variety of mammalian enzymes, and interfere with the pathways that regulate cell division and proliferation, detoxification, and inflammatory and immune response [27]. Both carotenoids and phenolic compounds exhibited protective effects on DNA, and several studies have evaluated their antigenotoxic and antimitogenic effects in vivo and in vitro [28, 29]. Whereas individual polyphenolic compounds have been extensively evaluated, studies of piquiá pulp and its biological properties have been lacking.

Our results showed that the lowest piquiá dose caused the largest decrease in DNA damage and the highest dose caused the smallest decrease, demonstrating an inverse dose–response of piquiá pulp. Furthermore, we observed a difference in potential antigenotoxic effects in several tissues. The assessment of dose–response relationships is complicated by the fact that many chemoprotective compounds act simultaneously at different protection levels [30], and it is worth noting that the functions of antioxidants are dependent on some factors and under certain conditions; for example, at high concentrations, phenols can initiate an autoxidation process and behave like pro-oxidants [27]. Other studies have also demonstrated an inverse dose-dependent relationship [26, 31]. As such, the lack of a dose–response relationship might be attributed to the activation of different mechanisms depending on the piquiá dose used.

In conclusion, under the conditions employed in this study, the results demonstrated that piquiá pulp was not genotoxic in rat bone marrow, peripheral blood, liver, kidney or heart cells as determined by the micronucleus test and comet assay. Piquiá also plays a role in inhibiting the genotoxicity induced by the antitumor agent DXR, but some of the protective effects observed depended on the doses and experimental conditions. Therefore, further investigations are needed to clarify how the properties of piquiá pulp can positively affect human health.

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