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# ORIGINAL ARTICLE

# Brazilian berry extract (*Myrciaria jaboticaba*): A promising therapy to minimize prostatic inflammation and oxidative stress

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# Abstract

**Background:** Brazilian berry is a fruit popularly known as "Jaboticaba," rich in bioactive compounds with antioxidant and anti-inflammatory properties. Senescence and overweight are increasing worldwide and are considered risk factors to prostatic pathogenesis mainly due to oxidative and inflammatory processes induction. Thus, this study aimed to evaluate the effect of two increasing doses of the patented jaboticaba peel extract (PJE) on oxidative-stress and inflammation in the prostate of aging or high-fat-fed aging mice.

**Methods:** PJE and/or high-fat diet (HFD) treatments started with 11-month-old mice and lasted 60 days. The levels or the immunoexpression of different inflammatory (nuclear factor  $\kappa$ B [NF $\kappa$ B], CD3+, cyclooxygenase 2 [COX-2], toll-like receptor 4 [TLR4], phosphorylated signal transducers and activators of transcription 3 [pSTAT-3], tumor necrosis factor  $\alpha$  [TNF $\alpha$ ], interleukin 6 [IL-6], and IL-1 $\beta$ ) and oxidative-stress (catalase, superoxide dismutase 2 [SOD2], glutathione reductase [GSR], reduced glutathione, and glutathione peroxidase 3 [GPx3]) related molecules were analyzed by western-blotting, immunohistochemistry, and enzyme-linked immunosorbent assays.

**Results:** Both PJE doses reduced the levels of oxidative-stress-related molecules (GPx3, GSR, catalase), lipid peroxidation (4-hydroxynonenal), inflammatory mediators (COX-2, TNF- $\alpha$ , and pSTAT-3) and CD3+ T cells number, which were associated with the maintenance of the glandular morphological integrity in aging and HFD-fedaging mice. Nevertheless, only the high PJE dose reduced the NFxB and TLR4 levels in aging mice; and SOD2, IL-6, and IL-1 $\beta$  levels in HFD-aging mice. Aging itself promoted an oxidative inflammation in the prostate, interfering in the levels of the different oxidative-stress, lipid peroxidation, and inflammatory mediators evaluated, in association with high incidence of prostate epithelial and stromal damages. The HFD intake intensified aging alterations, showing an unfavorable prostatic micro-environment prone to oxidative and inflammatory damages.

**Conclusions:** PJE exerted a dose-dependent effect controlling inflammation and oxidative-stress in aging and HFD-fed aging mice prostate. This fact contributed to prostate microenvironment balance recovery, preserving the tissue architecture of this gland. Thus, the PJE emerges as a potential therapy to prevent inflammation and oxidative stress in the prostate.

#### KEYWORDS

aging, bioactive compounds, obesity, overweight, polyphenols

## 1 | INTRODUCTION

Aging is inherent process in human beings, being one of the major risk factors for prostate cancer.<sup>1,2</sup> The number of elderly people has increased in the world as well as the incidence of senescence consequences for human health.<sup>3,4</sup> Associated with this, a tendency to increase obesity during late life has been registered, making these important topics a concern.<sup>3</sup> Both aging and obesity seem to be directly involved in prostate pathogenesis, mainly by inducing oxidative stress, reactive oxygen species (ROS) accumulation, DNA damages, and inflammatory processes.<sup>5-9</sup> Richie et al<sup>7</sup> described alterations in glutathione cycle molecules during aging, and Colado-Velazquez et al<sup>10</sup> have observed that obesity modified total superoxide dismutase (SOD) and catalase activities in rodent prostate. These alterations predisposed the gland to an oncogenic phenotype by interfering with proliferative, inflammatory, and apoptotic pathways, which suggests they play an important role in prostate injury and malignance.7,9-11

The term oxidative inflammation has emerged to explain the cross talk between these processes and their influences in age- or obesity-related disorders.<sup>4,12</sup> The nuclear factor  $\kappa$ B (NF $\kappa$ B) has been highlighted as a key mediator of chronic oxidative inflammation in the prostate.<sup>6,8</sup> This molecule is involved in the expression of genes related to both oxidative and inflammatory pathways, stimulating the expression of downstream proinflammatory mediators such as interleukins (IL), toll like receptors (TLR), and cyclooxygenase 2 (COX-2).<sup>13-15</sup> Thus, controlling the NF $\kappa$ B action could be a relevant target to reduce inflammatory oxidative damages caused by aging or obesity in this gland.

In this context, antioxidant protection seems to be a promissory alternative to increase longevity and provide better life quality to the elderly. The Brazilian Berry (*Myrciaria jaboticaba* (Vell.) Berg) is a typical fruit from the country, popularly known as "Jaboticaba.<sup>#16</sup> Studies demonstrated that jaboticaba peel has bioactive compounds that exert in vivo and in vitro antioxidant effects, besides a positive effect on metabolic parameters altered by obesity.<sup>16-21</sup> A recent study by our research group, regarding the development of the patented jaboticaba peel extract (PJE), revealed it has a broader spectrum of bioactive compounds and a high in vitro antioxidant activity compared to other natural extracts previously reported.<sup>22</sup> Among the bioactive molecules found in the PJE, we highlight the presence of gallic acid, epicatechin, anthocyanins (cyanidine and delfinidine), kaempferol hexoside, chlorogenic acid, ellagic acid, quercetin, naringenim, rutin,

and ascorbic acid.<sup>22,23</sup> The anti-inflammatory and antiobesity actions of the PJE have been previously described in aging or high-fat-fedaging mice. In these experimental models, this extract reduced the weight gain, improved insulin sensibility, prevented hepatic steatosis, besides reducing the hepatic levels of COX-2 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ).<sup>22</sup> Recently, Lamas et al<sup>24</sup> showed the PJE capacity to interfere in angiogenesis and hormonal pathways in the prostate, reducing the frequency of prostatic intraepithelial neoplasia (PIN) and well-differentiated adenocarcinoma foci in aging or high-fat-fed aging mice. Interestingly, in this study the PJE treatment also reduced the frequency of inflammatory infiltrates in the prostate, prompting us to investigate further the possible effect of this extract on inflammatory mediators.

Therefore, the aim of this study was to evaluate the PJE effect on inflammation and oxidative stress in the prostate of aging or high-fatfed aging mice. To accomplish this, we quantified the level of the endogenous antioxidant molecules and lipid peroxidation, besides the levels of NFxB and other inflammatory molecules related to its signaling pathway in the prostate of aging and high-fat-fed aging mice.

#### 2 | MATERIAL AND METHODS

### 2.1 | Patented jaboticaba peel extract

The protocol used to prepare the PJE was patented.<sup>25</sup> The PJE method of preparation consisted of the mixture of freeze-dried Myrciaria cauliflora (Vell. Berg) peel in an ethanol, followed by the solvent removal.<sup>22,25</sup> Previous studies by our research group, focusing on the development and characterization of the PJE, verified that PJE has a total phenolic content of 121.0 mg GAE/g, a total monomeric anthocyanins of 1381 mg cyd 3-glu 100 g<sup>-1</sup>, a total flavonoids content of 24.5 mg CAT g<sup>-1</sup>, 13.3 mg/g cyanidin-3-O-glucoside, 1.4 mg/g delphinidin-3-Oglucoside, 0.2 mg/g ellagic acid, 0.02 mg/g rutin, and 0.02 mg/g gallic acid.<sup>22-24</sup> In addition, the following bioactive compounds were also identified in PJE, considering the fragmentation pattern of the analytical standard: HHDP-galloylglucose, bis-HHDP-glucose (casuariin), bis-HHDP-glucose isomer (pedunculagin), HHDPgalloylglucose isomer, (-)-epicatechin, galloyl-bis-HHDPglucose (casuarinin), galloyl-bis-HHDPglucose (casuarictin), HHDP-digalloylglucose (tellimagrandin I), kaempferol hexoside, chlorogenic acid, HHDP-trigalloylglucose (tellimagrandin II), pentagalloyl hexose, myricetin-rhamnoside, quercetin-3-rhamnoside

(quercitrin), quercetin, and naringenin.<sup>22,24</sup> Furthermore, the PJE demonstrated a high in vitro antioxidant capacity, representing 4250  $\mu MTE^{-1}$  measured by ORAC and 6834.5  $\mu MTE^{-1}$  measured by ABTS.<sup>22</sup>

The experimental doses used in this study were based on previous studies by our group, which confirmed the safety of its administration to mice, considering the PJE dose-dependent effect in blood and liver parameters related to the metabolism of mice.<sup>22</sup> Also, the experimental doses administered herein presented smaller amounts of phenolic compounds, almost 10 times less than those usually used in the literature.<sup>16,20,22</sup> The lowest PJE dose (2.9 g/kg) used herein has 4.65 mg cyanidin-3-O-glucoside, 0.5 mg delphinidin-3-O-glucoside, 0.07 mg ellagic acid, 0.007 mg rutin, and 0.006 mg gallic acid; and the highest PJE dose (5.8 g/kg) used herein showed twice the content of phenolic compounds described above for the lowest dose.<sup>22,24</sup>

#### 2.2 | Animals and experimental procedures

A total of 70 male FVB mice were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science at the University of Campinas. All the experimental procedures were submitted to the Ethics Committee on Animal Use of the University of Campinas (Protocol: #3421-1). The animals were distributed into seven experimental groups (n = 10 mice/group) according to a specific experimental protocol (Figure 1). The mice were housed one per cage, under controlled lighting conditions (12 hours light-dark cycle), with food and drinking water ad libitum, during 60 days. The experimental period and the diets used in the present study were based on previous data by our group.<sup>22,24</sup>

At the end of the treatments, the animals were weighed on a semianalytical scale (Marte AS 5500; Marte, São Paulo, Brazil),

anesthetized with xylazine hydrochloride (5 mg/kg intramuscular [IM]; König, Sao Paulo, Brazil) and ketamine hydrochloride (60 mg/kg IM; Fort Dodge, Iowa; EUA). The euthanasia was performed by increasing the anesthetic level and ventral prostate samples were removed. Subsequently, blood samples were collected by left ventricle puncture in tubes containing EDTA. The data regarding the mouse weight gain and food intake was previous described in Lamas et al.<sup>22</sup>

#### 2.3 | Western blot analysis

Five ventral prostate samples per group were frozen at -80°C and used in this analysis. The prostate samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer and the lysates were centrifuged at 14000 rpm 4°C for 20 minutes. Subsequently the supernatants were collected and the protein amount was quantified by the Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA). The same amount of protein (50 µg) per sample was separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gels and electroblotted on nitrocellulose membranes. The nonspecific sites were blocked with bovine serum albumin solution (1%-5%) for 1 hour at room temperature. Then, the membranes were overnight incubated at 4°C with the following primary antibodies for the detection of catalase (C0979; Sigma-Aldrich), glutathione peroxidase 3 (GPx3; ab-104448; Abcam), glutathione reductase (GSR; SAB100980; Sigma-Aldrich), SOD2 (HPA001814; Sigma-Aldrich), 4-hydroxynonenal (4HNE; ab 20953; Abcam), COX-2 (sc-376861; Santa Cruz Biotechnology), transcription factor xB (NFxB; ab 13594; Abcam), phosphorylated signal transducers and activators of transcription 3 (pSTAT-3; 3E2; Cell Signaling Technology), toll-like receptor 4 (TLR4; sc-16240; Santa Cruz Biotechnology), TNF- $\alpha$  (ab 8348; Abcam), and  $\beta$ -actin (sc-81178; Santa Cruz Biotechnology). The membranes were subsequently incubated for 2 hours with a specific horseradish peroxidase-conjugated



**FIGURE 1** Distribution of the FVB mice in the different experimental groups. Experimental groups (*n* = 10 *per* group): YG, AG, HfAG, JAG I, JAG II, HfJAG I, and HfJAG II. Diets: ST (Nuvital CR1, Colombo, Parana, Brazil/composition: 22 g % protein, 53 g % carbohydrate, 4.5 g % lipid, and 2.9 kcal/g); HF (composition: 20 g % protein, 50 g % carbohydrate, 21 g % lipid, and 4.5 kcal/g). The PJE and the vehicle (water) were administered daily by gavage. All the experimental treatments lasted 60 days. AG, aging group; HF, high-fat diet; HfAG, high-fat diet and aging group; HfJAG I, high-fat diet, PJE dose I and aging mice; HfJAG II, high-fat diet, PJE dose I and aging mice; JAG I, aging and PJE dose I group; JAG II, aging and PJE dose II group; PJE, patented jaboticaba extract; ST, standard diet; YG, young group [Color figure can be viewed at wileyonlinelibrary.com]

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secondary antibody: anti-mouse IgG (W4021; Promega Corporation), anti-rabbit IgG (W4018; Promega Corporation), or anti-goat IgG (14-14-06; KPL). Detection of the antigen-bound antibody was carried out for 5 minutes with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL), followed by image capture using the G-Box Chemi associated with the GeneSnap (Syngene, Cambridge, UK) image acquisition software. The band quantification was performed by densitometry using the Image J software. The results were expressed as means of the ratio with  $\beta$ -actin band intensity.

#### 2.4 | Total reduced glutathione (GSH) assay

The same lysates prepared for the Western blot analysis analysis were used in this assay. The total GSH content was evaluated according to the Ellman reaction using 5050-dithio-bis-2-nitrobenzoic acid, described by Anderson.<sup>26</sup> The intensity of the yellow color was read at 412 nm. The results were expressed as nmol/ $\mu$ g of protein.

# 2.5 | Morphology and immunohistochemistry analysis

Samples from the ventral prostate of five mice *per* group were fixed in Bouin for 24 hours. The prostate samples were rinsed with ethanol (70%), dehydrated, diaphanized, and embedded in plastic polymers (Paraplast Plus; St. Louis, MO; EUA). The samples were sectioned into  $5 \,\mu\text{m}$  thick slices using a micrometer (Hyrax M60; Zeiss, Germany), placed on silanized slides and stained with hematoxylin and eosin or submitted to immunohistochemistry.

The COX-2 and CD3 immunohistochemistry was done in prostate sections, based on the on the study of Kido et al.<sup>27</sup> COX-2 mouse monoclonal primary antibody (sc-376861; Santa Cruz Biotechnology) or CD3 rabbit monoclonal primary antibody (ab16669; Abcam) were used, followed by counter-staining with Harris' hematoxylin. This analysis was also performed in sections where the incubation step with the primary antibody was omitted (negative controls).

The slides staining with hematoxylin and eosin were scanned to provide a broader visualization of the morphological pattern observed in the different experimental groups, especially in the regions of COX-2 and CD3+ immunoreactivity. The prostatic features which were observed, such as the PIN, well-differentiated adenocarcinoma, and epithelium atrophy were based on previous studies from our group and others.<sup>24,27-29</sup>

To evaluate COX-2 immunoreactivity, 10 random images were captured (×40 magnification) *per* animal using a Nikon Eclipse E-400 microscope (Nikon, Tokyo, Japan) coupled to the NIS-Elements software. The COX-2 immunoreactivity was quantified, using a grid with 700 intersections over the captured imagens by means of the Image Pro Plus program (modified from Silva et al<sup>30</sup>). The intersections corresponding to the positive COX-2 immunoreactivity was counted and discriminated as epithelium or stroma COX-2 immunostaining. The total number of points, quantified in the epithelium or stroma, was divided by the total

number of intersections on the prostatic acini. The results were expressed as a percentage of prostate epithelium or stroma positive COX-2 immunostaining. The percentage of COX-2 total immunostaining was obtained by adding the percentage of COX-2 positively immunostained to the epithelium and stroma. Also, the COX-2 staining was scored and the frequency was graded on a 0 to 3 scale, according to the total percentage of positive staining areas: 0 (absence of immunostaining) 0%; 1 (weak immunostaining) 1% to 33%; 2 (moderate immunostaining) 34% to 66%; and 3 (intense immunostaining) more than 66%.<sup>31</sup> The percentage of total COX-2 positive immunostaining (0-100) was multiplied by the COX-2 staining score (0-3), resulting in an *H* score value adapted for cytoplasmic staining (0-300) (*H* score =  $3 \times \text{percentage}$  of total intense immunostaining +  $2 \times \text{percentage}$  of total moderate immunostaining) (modified from Beeghly-Fadiel et al.<sup>32</sup> Jalalabadi et al.<sup>33</sup> and Cass et al.<sup>34</sup>).

The quantification of CD3+ T cells were performed by the counting of CD3+ T cells in 10 imagens with ×100 magnification *per* animal, captured by a Nikon Eclipse E-400 microscope (Nikon, Tokyo, Japan) coupled to the NIS-Elements software. The number expressed from the CD3+ T cells was displayed as the mean value and standard deviation *per* experimental group (modified from Carvalho et al<sup>35</sup>).

## 2.6 | Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged at 3000 rpm, 4°C, for 10 minutes. The plasma obtained was used to determine IL-1 $\beta$  and IL-6 concentration using commercial kit reagents (Novex, Invitrogen). The absorbance of the samples were read using the Multi-Mode Microplate Reader Model Synergy H1M (Bio-Tek Instruments) with specific wavelength for each molecule as described in the respective protocol of each assay.

## 2.7 | Statistical analyses

The statistical analyses of the immunohistochemistry quantifications, Western blot analysis, total GSH assay, and ELISA data were carried out by analysis of variance (one-way analysis of variance) followed by Tukey's multiple range posttest or Student's *t* test when appropriate. A significance limit of *P* < .05 was considered. All these data were expressed as means ± standard deviation.<sup>36</sup> Pearson's correlation test was performed to obtain correlation values (*r*) between the number of CD3+ T cells and the total frequency of COX-2 immunostaining.

## 3 | RESULTS

# 3.1 | PJE reduced the oxidative stress by improving the prostatic antioxidant response in aging and high-fat diet (HFD)-fed aging mice

The AG group showed reduced GSH and GPx3 levels, and increased GSR, catalase, and SOD2 levels compared to the YG group. The GSR

and catalase levels were higher in the HfAG group than in the AG group. Both PJE doses increased the GPx3 level as well as reduced the GSR, catalase and SOD2 levels in JAGI and JAGII groups compared to the AG group. Nevertheless, only the highest PJE dose, administered in the JAGII group, improved the GSH level in relation to the AG group. The HfJAGI and HfJAGII groups displayed high GPx3, as well as low GSR and catalase protein levels, compared to the HfAG group. The dose-dependent effect of PJE was evidenced since only the high PJE dose intake, in HfJAGII group, led to increased GSH and reduced SOD2 levels compared to the HfAG group. Moreover, increased GPx3 and reduced catalase levels were observed in the HfJAGII group in relation to the HfJAGI group (Figure 2A-F).

# 3.2 | PJE led to a decrease of lipid peroxidation in the prostate

The lipid peroxidation evaluated through 4HNE level increased in the AG group compared to the YG group. The HfAG group showed a higher 4HNE level than the AG group. Both PJE doses reduced the lipid peroxidation in JAGI and JAGII groups compared to the AG group. Also, the HfJAGI and HfJAGII groups presented a reduction of the 4HNE level in relation to the HfAG group (Figure 2G,H).

# 3.3 | PJE treatment downregulated prostatic proinflammatory hallmarks dose-dependently

The AG group showed higher protein levels of COX-2, NF $\kappa$ B, TLR4, TNF- $\alpha$ , and pSTAT-3 than the YG group. The levels of these proinflammatory mediators increased even more in the HfAG group in relation to the AG group. Both PJE doses reduced the COX-2, TNF- $\alpha$ , and pSTAT-3 levels in JAGI and JAGII groups compared to the AG group. The effect of PJE treatment in aging mice was dose-dependent as evidenced by the fact that only the JAGII group showed a decrease in NF $\kappa$ B and TLR4 levels compared to the AG group, as well as resulted in even lower pSTAT-3 levels compared to the JAGI group. The HfJAGI and HfJAGII groups displayed a decrease in COX-2, NF $\kappa$ B, TLR4, TNF- $\alpha$ , and pSTAT-3 levels in comparison with the HfAG group. Moreover, the HfJAGII group showed even lower TNF- $\alpha$  levels compared to the HfJAGI group (Figure 3A-F).

# 3.4 | PJE treatment reduced the COX-2 immunoexpression in association with lower CD3+ T cells number

The positive COX-2 immunostaining and CD3+ T cells were observed in the prostate from the different experimental groups, varying in terms of immunostaining frequency and number, respectively. Pearson's coefficient showed a high and positive correlation (r = .9872; -The Prostate -WILEY

P < .0001) between the total COX-2 immunostaining frequency and the number of CD3+ T cells.

The YG group demonstrated a weak COX-2 epithelial immunostaining mainly in regions characterized by a simple folded epithelium with columnar cells and basal nuclei. The stroma presented fibromuscular features distributed concentrically around the acini and weak positive immunostaining for COX-2 in the YG group (Figure 4A-C and Table 1). Few CD3+ T cells were observed in the stroma, close to the acini fibromuscular layer (Figure 4D and Table 1).

In contrast, both the AG and HfAG groups showed intense COX-2 immunolabeling in both the prostate epithelium and stroma. The increased COX-2 immunoexpression was associated with PIN regions and with well-differentiated adenocarcinoma foci. The PIN regions showed intense cell proliferation and stratification, as well as nuclear atypia. The well-differentiated adenocarcinoma foci identified were characterized by the basement membrane rupture and stromal invasion by epithelial cells. COX-2 immunostaining was also verified in proliferative inflammatory atrophy regions, characterized by reduced epithelial cell cytoplasm, and inflammatory infiltrates distributed throughout the glandular stroma. The glandular stroma was hypertrophic and hyperplastic in these groups, presenting a thick fibromuscular layer around the acini, which was intensely COX-2 immunostained (Figures 4E-G and 4I-K; Table 1) In addition, both the AG and HfAG groups showed a higher number of CD3+ T cells, than the YG group. These cells were organized in periacinar clusters (Figure 4H,L and Table 1).

Both groups treated with the PJE low dose (JAGI and HfJAGI), showed a moderate COX-2 immunolabeling, and the groups treated with the PJE high dose (JAGII and HfJAGII) showed a weak COX-2 immunolabeling. Low COX-2 immunostaining was predominant in the healthy morphological prostate regions, similar to the YG group, especially in JAGII and HfJAGII groups. (Figure 4M-O,Q-S,U-W,Y-AA and Table 1) The CD3+ T cell number diminished in the different PJE treated groups. In the JAGI and HfJAGI groups, the CD3+ T cells were organized in small clusters close to the prostate acini. In the JAGII and HfJAGII groups, the CD3+ T cells were predominantly isolated in the prostatic stroma. (Figure 4P,T,Y,BB and Table 1) A dose-dependent effect of the PJE was verified, due to the fact that the HfJAGII group demonstrated a lower COX-2 immunostaining frequency and a smaller number of CD3+ T cells than the HfAGI group (Table 1).

# 3.5 | PJE led to decreased IL-6 and IL-1 $\beta$ plasma levels

The AG group showed high plasma levels of IL-6 and IL-1 $\beta$  compared to the YG group. The level of these molecules in the HfAG group was as high as in the AG group. Both PJE doses reduced the plasma levels of these ILs in JAGI and JAGII groups in relation to the AG group. The dose-dependent effect of PJE was verified in the HfJAGII group, since only the treatment with the PJE high dose reduced the IL-1 $\beta$  and IL-6 plasma level compared to the AG group. Moreover, the IL-6



**FIGURE 2** Evaluation of oxidative stress and lipid peroxidation biomarkers in the prostate ventral lobe. A, Determination of the GSH total level. B, Relative frequency of GPx3. C, Relative frequency of GSR. D, Relative frequency of catalase. E, Relative frequency of SOD2. F, Representation of GPx3, GSR, catalase, and SOD2 Western-Blotting analysis. G, Relative frequency of 4-HNE. H, Representation of 4-HNE Western-Blotting analysis. Significant differences: <sup>a</sup>relative to YG group; <sup>b</sup>relative to AG group; <sup>c</sup>relative to HfAG group; <sup>e</sup>relative to HfJAGI group. Considering: \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001. *n* = 5 mice *per* group. 4-HNE, 4-hydroxynonenal; GSH, reduced glutathione; GSR, glutathione reductase; GPx3, glutathione peroxidase 3; SOD2, superoxide dismutase 2



**FIGURE 3** Western-blotting analysis of COX2, NF $\kappa$ B, TLR4, TNF- $\alpha$ , and pSTAT3 in homogenates of the prostate ventral lobe from mice of all experimental groups. Significant differences: <sup>a</sup>relative to YG group; <sup>b</sup>relative to AG group; <sup>c</sup>relative to HfAG group; <sup>d</sup>relative to JAGI group; <sup>e</sup>relative to HfJAGI group. Considering: \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001. *n* = 5 mice *per* group. COX2, cyclooxygenase-2; NF $\kappa$ B, nuclear transcription factor  $\kappa$ B; pSTAT3, phosphorylated signal transducer and activator of transcription 3; TLR4, toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ 

plasma level was lower in the HfAGII group than in the HfJAGI group (Figure 5A,B).

## 4 | DISCUSSION

Oxidative stress is an essential factor for prostate cancer and premalignant lesion development, showing a direct association with the inflammatory process.<sup>7,8,37</sup> The present results indicated a dosedependent positive effect of PJE treatment, diminishing a broad spectrum of inflammatory and oxidative molecules, contributing to prostate homeostasis and the morphological maintenance, which was altered by aging and/or HFD intake. There are no studies in scientific literature regarding the antioxidant effect of jaboticaba on the prostate microenvironment. However, Batista et al<sup>38</sup> showed that the ingestion of HFD supplemented with jaboticaba peel for 10 weeks reduced lipid peroxidation and SOD activity, besides increasing GPx activity in the mouse liver. In addition, Lenquiste et al<sup>16</sup> observed the reestablishment of catalase and GSH hepatic levels after jaboticaba peel tea ingestion by rats which were fed with HFD for 12 weeks. Our findings showed a systemic effectiveness of PJE, interfering in antioxidant molecules and in lipid peroxidation levels in the prostate of high-fat-fed aging mice, even after a low PJE dose and a reduced period of treatment. Taking into consideration, particularly, the HFD-fed aging mice, the high PJE dose showed a strong



**FIGURE 4** Photomicrographs of the prostate ventral lobe stained with HE, immunostained for COX2 or immunostained for CD3. Bar = 50 µm. Thick arrow, COX-2 or CD3+ immunostaining. The COX-2 and CD3+ immunostaining were quantified and/or classified according to the Table 1. *n* = 5 mice *per* group. At, epithelial atrophy; COX2, cyclooxygenase-2; Ep, epithelium; FL, fibromuscular layer; HE, hematoxylin and eosin; IN, inflammatory infiltrate; L, lumen; PIN, prostatic intraepithelial neoplasia; St, stroma; WDA, well-differentiated adenocarcinoma [Color figure can be viewed at wileyonlinelibrary.com]

#### TABLE 1 COX-2 and CD3 positive immunoreactivity in the prostate from different experimental groups

	COX-2					CD3+
Experimental groups	Epithelium immunoreactivity, %	Stroma immunoreactivity, %	Total immunoreactivity, %	Score scale (0-3)	H score	Number of CD3+ T Cell
YG	21.9 ± 2.7	6.8 ± 1.8	28.7 ± 0.9	1	28.7	1 ± 0.7
AG	54.3 ± 14.3 <sup>a,**</sup>	28.9 ± 2.6 <sup>a,***</sup>	83.2 ± 13.5 <sup>a,***</sup>	3	249.9	11 ± 1.4 <sup>a,***</sup>
HfAG	$62.5 \pm 4.3^{a,**}$	$29.1 \pm 4.1^{a,***}$	91.6 ± 7.0 <sup>a,***</sup>	3	274.8	$13 \pm 2.1^{a,***}$
JAG I	$28.6 \pm 2.3^{b,*}$	11.5 ± 3.6 <sup>b,***</sup>	40.1 ± 5.9 <sup>b,**</sup>	2	82.2	4 ± 1.4 <sup>b,***</sup>
JAG II	$20.3 \pm 2.6^{b,**}$	$9.4 \pm 2.4^{b,***}$	29.7 ± 3.4 <sup>b,***</sup>	1	29.7	3 ± 1.4 <sup>b,***</sup>
HfJAG I	37.1 ± 5.0 <sup>c,***</sup>	$14.2 \pm 1.0^{c,***}$	51.3 ± 6.0 <sup>c,***</sup>	2	102.6	7 ± 2.1 <sup>c,***</sup>
HfJAG II	22.6 ± 3.7 <sup>c,e,***</sup>	$9.4 \pm 4.3^{c,***}$	$32.0 \pm 0.7^{c,e,***}$	1	32.0	2 ± 1.2 <sup>c,e,***</sup>

*Note:* Score distribution according to predominant immunoreactivity range: 0 (0%), 1 (<33%), 2 (33%-66%), and 3 (>66%). The results of COX-2 epithelium, stroma and total immunoreactivity (%) and the CD3+ T cell number is displayed as mean values  $\pm$  standard deviation. *n* = 5 mice *per* group. Significant differences: <sup>a</sup>relative to YG group, <sup>b</sup>relative to AG group, <sup>c</sup>relative to HfAG group, <sup>e</sup>relative to HfJAGI group. Considering: \**p* < .05, \*\**p* < .01, and \*\*\**p* < .001.

interference, not only increasing GSH and GPx3 but also decreasing CAT and SOD2.

The amount of total phenolic and flavonoids, as well as the in vitro antioxidant activity of the PJE showed higher values than other jaboticaba extracts, utilizing different solvents for these the extraction and that of other purple fruits extracts.<sup>16,38-41</sup> Nogueira-Lima et al<sup>21</sup> suggested that ethanol-water solutions could be a good alternative for a solvent, considering it increases the phenolic compound solubility and the solute adsorption. In addition, the jaboticaba peel presents a great amount of flavonoids, and other phenolic compounds conjugated with glucosides or with a hydroxyl group, assisting in extraction using hydrophilic solvents.<sup>21-23,42</sup> Thus, the methodology applied in the PJE preparation provides high accessibility of phenolic compounds from the food matrix, leading to an extract with preserved amount and variety of bioactive compounds, which could have contributed, to the broad antioxidant and anti-inflammatory action.

Our results also confirmed that aging led to an increase in oxidative stress and consequently to high lipid peroxidation in the prostate, which further increased after the HFD intake. Prostate cancer cell cultures, treated with saturated fatty acid, showed increased catalase activity and lipid peroxidation levels, agreeing with the results observed herein during aging and HFD intake.<sup>43</sup> Catalase high levels are generally accompanied by high SOD levels, especially the manganese dependent SOD (MnSOD).<sup>44</sup> The MnSOD is the primary endogenous mitochondrial enzyme, encoded by SOD2, mainly related to upper levels of superoxide anions.<sup>45,46</sup> Studies regarding different SOD2 genotype have elucidated the fact that the Ala/Ala SOD2 genotype has considerable MnSOD activity, which is associated with reduced antioxidant status, increased DNA damage and the risk of aggressive prostate cancer.<sup>45,46</sup> A recent clinical trial on the effect of muscadine grape skin extract, which is rich in polyphenols, in men with biochemically recurrent prostate cancer, showed the supplementation increased the PSADT in Ala/Ala SOD2



**FIGURE 5** Enzyme-linked immunosorbent assay. A, Determination of IL-6 plasmatic concentration in the experimental groups. B, Determination of IL-1 $\beta$  plasmatic concentration in the experimental groups. Significant differences: <sup>a</sup>relative to YG group, <sup>b</sup>relative to AG group; <sup>c</sup>relative to HfAG group; <sup>d</sup>relative to JAGI group; <sup>e</sup>relative to HfJAGI group. Considering: \*P < .05, \*\*P < .01, and \*\*\*P < .001. *n* = 5 mice *per* group

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genotype patients and reduced their basal oxidative state, suggesting effective therapy.<sup>47</sup> It is known polyphenols such as epicatechin, can inhibit the NADPH-oxidase enzyme, resulting in superoxide production reduction and oxidative stress decrease.<sup>48</sup> Also, the anthocyanin, present in a great majority in the PJE, has a phenolic structure with hydroxyl radical in specific locations which mediates and improves the scavenging of superoxide and singlet oxygen, inhibiting the per-oxide formation and the free radical activity of these molecules.<sup>49,50</sup> Based on these data, we could suggest that the reduced SOD2, observed herein after the PJE treatment, could indicate that one of the PJE action pathways was the reduction of the production of super-oxide anions in the prostate of aging or high-fat-fed aging mice. The low catalase levels after PJE treatment were expected as their action and tissue levels are associated with SOD level.

In contrast to the results herein, reduction in the total SOD and catalase activities was described in the prostate of obese rats after sucrose intake.<sup>10</sup> Nevertheless, the percentage of sucrose in the diet was high, providing a higher daily caloric amount than in the present study.<sup>10</sup> Pires et al<sup>51</sup> suggested that the increase or maintenance of the antioxidant enzyme activity is a process that occurs before its inhibition. as an attempt to reach the oxidative balance, varying according to the exposure time or type of factor/substance used to induce oxidative stress.<sup>51</sup> Thus, the use of more aggressive methods to trigger oxidative stress could lead to the inhibition of the endogenous antioxidant machinery, showing lower levels of the antioxidant molecules. Interestingly, Colado-Velazquez et al<sup>10</sup> verified the recovery of SOD and CAT activities and reduced prostate epithelium and stromal proliferation in aging animals that ingested a high-sucrose diet and a palm tree leaf extract, which is rich in bioactive compounds. Their results suggested that the antioxidant action of the bioactive compounds is not restrict to HFD intake. In fact, the ingestion of both sucrose and fat, or even aging, are associated with important hormonal alterations in the body, especially related to alterations in the testosterone levels, which is an important stimulator of ROS production, oxidative stress and prostatic damages.<sup>10</sup> Previous studies by our research group showed the PJE supplementation reduced the androgen and estrogen receptor levels, mainly through downregulation of aromatase, in the prostate of high-fat-fed aging mice. Thus, the PJE capacity to interfere in hormonal mediators could indicate a significant involvement in the modulation of the endogenous antioxidant machinery and in the oxidative stress reduction observed herein.

Moreover, we showed in the study herein that aging, associated or not with HFD intake, interfered with the glutathione antioxidant cycle. Sekine et al<sup>52</sup> verified that HFD intake induced GPx3 low levels in mouse prostate, which was especially related to the local increase of ROS levels and the amplification of oxidative-related cell/tissue damage. The relatively low GSH level is considered a consequence of its oxidation, suggesting oxidative stress onset, whereas high GSR levels during oxidative stress could be interpreted as a response to maintain the upper levels of the antioxidant glutathione, indicating an attempt to neutralize this stress.<sup>53</sup> Recently, Ishola et al<sup>54</sup> verified that the *Moringa oleifera* leaf extract, which is rich in quercetin and other flavonoids that are also present in the PJE, reduced the GSH and lipid peroxidation level in rats with benign prostatic hyperplasia, preserving the integrity of prostatic cells. The study of flavonoids showed they could modulate the g-glutamylcysteine synthetase and glutamate cysteine ligase enzymes which are directed related to the GSH synthesis.<sup>51,55,56</sup> These data contributed to our understanding that the improvement of GSH and GPx3 levels after the PJE treatment suggested the glutathione cycle was the main endogenous antioxidant tool stimulated in the prostate to promote hydrogen peroxide neutralization. The low GSR levels after the PJE treatment could be related to an attempt to reduce GSH and to recovery the natural oxidative levels in this gland. The PJE reducing lipid peroxidation in the prostate reinforced this premise since it represents an endpoint of oxidative injury.

Another novelty of our findings is that we indicated, for the first time in scientific literature that PJE reduced the chronic inflammatory process observed in the prostate of aging or HFD-fed aging mice, by means of reducing the levels of essentials inflammatory mediators. Previous studies have already demonstrated that the PJE or lyophilized jaboticaba peel reduced the IL-6, IL-1 $\beta$ , COX-2, and TNF- $\alpha$  levels in the liver of HFD-fed mice, showing that they had a relevant response reducing hepatic pathogenesis.<sup>20,22</sup> By and large, some authors believe that the antioxidant activity of bioactive compounds that lead to the reduction of oxidative stress, is one important action mechanisms in the downregulation of inflammatory mediators levels.57,58 Reinforcing this fact, Henning et al<sup>59</sup> verified that the oxidative stress reduction, due to the polyphenol epigallocatechin ingestion, which was also identified in the PJE used herein, was associated with a decrease in the inflammatory process; angiogenesis; and tumor growth in a xenograft prostate cancer mouse model.

Also, several studies demonstrated that NFkB activation could either stimulate or be stimulated by ROS in obese animals, resulting in oxidative inflammation.<sup>5,10,13,37</sup> The NF<sub>K</sub>B acts as a key molecule considering the maintenance of inflammatory state, stimulating downstream inflammatory mediators, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , besides interfering in genes related to cell proliferation, survival, and invasion.<sup>13</sup> Liu et al<sup>14</sup> observed that COX-2 expression, regulated by NF<sub>x</sub>B binding, promoted a chronic inflammation via the TLR4-derived signaling pathway in prostate cancer cell culture treated with saturated fatty acids.<sup>6,14,60,61</sup> In addition, the NF<sub>k</sub>B and STAT-3 signaling crosstalk, keeps the NFxB permanently activated, sustaining prostatic chronic inflammation, the lymphocyte accumulation; and the neoplastic lesion development after HFD intake by adult mice.<sup>6,13,62</sup> Recently, Ignacio et al<sup>63</sup> proposed that reduced NF $\kappa$ B levels by muscadine grape skin extract supplementation were associated with cell-cycle arrest, reducing the tumor proliferative characteristics in a xenograft prostate cancer. According to Davalli et al<sup>64</sup> green teaderived polyphenols reduced NF<sub>K</sub>B binding to DNA, decreasing the expression of its active subunit, as well as the activation of downstream molecules and proliferative processes in prostatic cancer cell culture. Moreover, the study of renal ischemia and melanoma models clarified that interfering in the TLR4/NFxB pathway is an important strategy to reduce tissue damages, thereby reducing IL-1β, IL-6, and TNF-α serum levels.<sup>57,65</sup>

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A positive correlation of CD3 + T lymphocytes cell and COX-2 expression have been described in canine breast cancer, mainly due to prostaglandins secretion by lymphocytes.<sup>35</sup> According to these authors, increased CD3+ T cell/COX-2 were correlated with increased tumor aggressiveness, high rates of tumor inflammation and necrosis, as well as to metastasis development. Yang et al<sup>66</sup> suggested the number of CD3+ T cells and other lymphocytes could be used to evaluate the prostate cancer progress and prognosis, considering a lower expression of CD3+ T cells at the end of radio-therapy treatment of men with prostate cancer. The present results showed the decrease in CD3+ T lymphocytes after PJE treatment was also positively correlated with low COX-2 immunoexpression. These results suggest that the CD3+ T cell decrease could have contributed, at least in part, to reduce the inflammatory signaling mediator level.

Generally speaking, we believe the antioxidant properties of the PJE reduced the oxidative stress in the prostate of aging or high-fatfed aging mice, negatively stimulated the intraprostatic inflammation in different signaling pathways, which was proportional to the oxidative damage. In addition, the PJE capacity to reduce NF $\kappa$ B represents a promissory step to minimize the generalized chronic prostatic oxidative inflammation due to the fact that NF $\kappa$ B can regulate the level of downstream inflammatory mediators and interfere in different inflammatory pathways in the prostate. Thus, we encourage the development of future studies focusing on specific PJE action mechanisms involved with NF $\kappa$ B to clarify this perspective.

The study of polyphenols absorption, excretion, and tissue accumulation could contribute toward the understanding of their therapeutic effect.<sup>67</sup> The anthocyanins, an important class of polyphenols present in the PJE, are rapidly absorbed in the small intestine, due to the presence of the  $\beta$ -glucosidase enzyme in the microbiota.<sup>67,68</sup> Also, they are easily degraded, reaching a maximal concentration in feces 24 hours postconsumption.<sup>67,69,70</sup> When anthocyanins reach the microbiota, occur their biotransformation into metabolites, which are essential for its absorption and biological activity.<sup>67</sup> Felgines et al<sup>71</sup> verified that the anthocyanins from blackberry, in the form of native anthocyanins or as anthocyanin metabolites (cyaniding monoglucuronide, methylated forms of cyanidin 3-glucoside and cyanidin monoglucuronide, and aglycones) can be found in the plasma and in the prostate, in greater amounts than in testis, heart and adipose tissue. Also, one of the ellagic acid metabolites, urolithin-A, was identified in the mouse prostate after Pomegranate extract supplementation.<sup>72</sup> It is unclear how these natural polyphenols or metabolites concentrate in the prostate, however these data suggests this event could be related to their positive action in this gland.

## 5 | CONCLUSION

This study brings significant implications about the jaboticaba (Brazilian berry) extract effect on prostatic oxidative inflammation. The antioxidant and anti-inflammatory actions of this extract represents a new promissory adjutant approach to reestablish the prostatic microenvironment balance altered by aging associated or not with HFD intake, suggesting the maintenance of a normal crosstalk between the prostatic epithelium and stroma. The jaboticaba extract antioxidant and anti-inflammatory effects were dosedependent; especially considering the tissue responses in a drastically damaged prostatic microenvironment after the HFD intake, suggesting its effectiveness could be related to the tissue injury level.

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#### CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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