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ARTICLE

Anti-inflammatory Effects of Persimmon (*Diospyros kaki* L.) in Experimental Rodent Rheumatoid Arthritis

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ABSTRACT

Persimmon (*Diospyros kaki* L.) fruits are used in traditional medicine largely due to their claimed beneficial effects on human health. The aim of this work was to evaluate the anti-inflammatory activity of a persimmon extract in rats with collagen-induced arthritis (CIA). CIA was induced in Wistar rats through an intradermal injection of an emulsion of bovine type II collagen (CII) in complete Freund's adjuvant (FCA). Macroscopic evidence of CIA first appeared as periarticular erythema and edema in the hind paws. The incidence of CIA was 100% by day 27 in the CII-challenged rats, and the severity of CIA progressed for 35 days. Radiographs revealed focal resorption of bone, with osteophyte formation in the tibiotarsal joint and soft tissue swelling. The histopathologic features included erosion of the cartilage at the joint margins. The persimmon extract showed an anti-inflammatory effect given the significant reduction in both the edema volume and radiological alterations attributed to CIA in the bone. We demonstrate that the administration of persimmon extract attenuates the degree of chronic inflammation and tissue damage characteristic of CIA in rats, most probably by the potent antioxidant characteristics of the extract.

KEYWORDS

antioxidant; collagen-induced arthritis; inflammation; persimmon

Introduction

Persimmon (*Diospyros kaki* L.) fruits have been used in traditional medicine for their positive effects on human health, mainly against hypertension, alterations of coagulation, pyrexia, and oxidative processes (Hibino et al. 2003; Gu et al. 2008). During the past years the popularity of persimmon has grown out of the traditional production areas (China, Japan, and Korea), becoming a promising crop in Brazil and in some Mediterranean countries such as Spain and Italy (Fao 2013). The fruits of the closely related *Diospyros lotus* L. are used as sedative, astringent, antiseptic, antidiabetic,

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antitumor, laxative, and febrifuge (Uddin et al. 2011). The fruits are also used for the treatment of diarrhea, dry cough, and hypertension (Rashed et al. 2012). Chemical investigation of the fruit constituents led to the identification of fatty acids, sugars, phenolic compounds, and nonvolatile acids (Loizzo et al. 2009; Rashed et al. 2012). The roots of *Diospyros lotus* L., traditionally used in several diseases, including pain syndromes and sleep disorders, were studied by Uddin et al. (2014), and it was concluded that the antinociceptive and anti-inflammatory effects of the roots of *D. lotus* appear to be partially attributed to the analgesic properties of some compounds, such as diospyrin and 8-hydroxyisodiospyrin, thus supporting the ethnopharmacological uses of *D. lotus* L. as antinociceptive, anti-inflammatory, and sedative (Uddin et al. 2014).

The nutritional status of rheumatoid arthritis (RA) patients is determined both by the intensity of the chronic inflammatory process observed in the course of the disease and by disease duration (Targonska-Stepniak and Majdan 2011). Widely available literature suggests that a high consumption of vegetables and fruits drives protective effects regarding the development of RA (Pattison et al. 2004). It is widely accepted that dietary antioxidants effectively suppress the release of inflammatory cytokines by significantly reducing reactive oxygen species (ROS), as demonstrated in several studies (Jung and Sung 2004; Kowalski et al. 2005; Surh et al. 2005). In fact, polyphenols may help to control oxidative stress and, consequently, the inflammatory response. Polyphenols, either isolated or as constituents of polyphenol-containing extracts, have been proven to act as antioxidants by protecting tissues against oxidative stress and associated pathophysiologic changes (Biesalski 2007). Recent studies have focused on the antioxidant activity of persimmon (*Diospyros kaki* L.) fruit, and the potent scavenging action against ROS was found to be mainly due to flavonoids, namely, flavan-3-ols with repeating units of catechin compounds (Lee et al. 2007).

Persimmons are rich in polyphenols, such as *p*-coumaric, catechin, epicatechin, epigallo catechin, condensed tannin (proanthocyanidins) (Giordani et al. 2011), and gallic acid (Gorinstein et al. 1994), and are known to contain proanthocyanidins (Halsam and Lilley 1988). Matsuo (1978) identified kaki-tannin in a Japanese persimmon and found that the tannin consisted of catechin, catechin-gallate, gallocatechin, and gallocatechin-gallate (Matsuo 1978). Other authors (Sattar et al. 1992) determined spectrophotometrically the total catechin content and total procyanidin content of persimmon. Catechins (flavan-3-ols) are polyphenolic compounds that may offer potential benefits to human health. They are related to various physiological functions, including a protective role against oxidative stress-related diseases, and antimutagenic and anticarcinogenic capacities (Suzuki et al. 2005). Catechins, as well as persimmon extracts, are known to induce apoptosis of Molt 4B cells (Achiwa et al. 1997). In more recent studies, persimmon procyanidins have been associated with various biological functions, including antioxidant (Jang et al. 2010; Tian et al. 2011; Tian et al. 2012), anti-inflammatory, antimicrobial (Borges-Argaez et al. 2007), hypolipidemic (Gorinstein et al. 1998; Kondo et al. 2004; Fukai et al. 2009; Zou et al. 2012), and antidiabetic activities (Lee et al. 2006) Persimmon pro-cyanidins have also been associated to a reduction of the risk of developing atherosclerosis (Park et al. 2008). Catechin, epicatechin, epigallocatechin, chlorogenic acid, caffeic acid and gallic acid (Chen et al. 2008), carotenoids (Daood et al. 1992), procyanidins (Suzuki et al. 2005; Gu et al. 2008), and ascorbic acid (Homnava

et al. 1990) are the main antioxidants in persimmon (Jung et al. 2005; Park et al. 2008). Persimmon is a widely consumed fruit, characterized by its high proanthocyanidin content (Uchida et al. 1990; Achiwa et al. 1997). Beyond the low molecular weight phenols in the edible part of persimmon, a total of 32 other compounds were detected recently, including gallic acid and its glycoside and acyl derivatives, glycosides of *p*-coumaric, vanillic and cinnamic acids, and different di-C-hexosides (Sentandreu et al. 2014). Gu et al (2008) described that proanthocyanidin from persimmon had more antioxidant activity than grape seed proanthocyanidin, suggesting that persimmon may be a source of therapeutically interesting polyphenolics (Gu et al. 2008). In fact, persimmon proanthocyanidin has also been associated with antioxidant activity, anti-inflammatory activity, and atherosclerosis prevention (Gorinstein et al. 2000).

Persimmon is also highly concentrated in sugars (about 12.5 g/100 g FW), with fructose, glucose, and sucrose being the major components, and in total ascorbic acid: 100–150 g of fresh persimmon clearly fulfills the recommended daily amount of vitamin C. The main carotenoid components are β-cryptoxantin (193 µg/100 g FW), β,β-carotene (113 µg/100 g FW), and β,ε-carotene (30 µg/100 g FW) (Giordani et al. 2011).

The chemical composition of the fruits, together with evidence generated in vivo and in vitro, suggests a relevant role for persimmon in the protection against ROS and, possibly, in the prevention of some human diseases (Giordani et al. 2011), such as RA. Animal models of autoimmune arthritis have proven to be valuable research tools for the study of pathogenic mechanisms of this disease as well as for testing new therapies. The collagen-induced arthritis (CIA) rat model is the most commonly used autoimmune model of rheumatoid arthritis, sharing several pathological features with RA. Autoimmune arthritis is induced in this model by immunization with an emulsion of complete Freund's adjuvant and type II collagen (CII), a major protein in cartilage—the target tissue of RA (Brand et al. 2007).

Because persimmon is a highly bioactive fruit, due to its high content of polyphenols and other constituents, we aimed to evaluate the anti-inflammatory activity of a fully characterized persimmon extract using an animal model of chronic inflammation such as the CIA model.

Materials and methods

Materials

Gallic acid (98%), sulfuric acid (95%–97%), and luminol were purchased from Fluka (Germany). Sodium hydroxide (98%), calcium chloride dihydrate, magnesium sulfate, and sodium hydrogencarbonate were purchased from Merck (Germany). Chloride acid, absolute ethanol (99.9%), methanol (99.9%), hydrochloric acid, glacial acetic acid (99%), and sodium acetate anhydrous (99%) were purchased from Carlo Erba Reagents (Italy). Phosphoric acid p.a. (85%) and ascorbic acid were purchased from Panreac Química (Spain). Acetonitrile HPLC gradient grade was purchased from VWR (Leuven, Belgium). Milli-Q water (18.2 MΩ cm) was obtained in Millipore – Direct Q3 UV System equipment (France). Potassium chloride was obtained from Pronalab (Portugal), sodium chloride and sodium salt of 2,6-

dichlorophenol indophenols (90%) from Riedel-de Haën (Germany). All other reagents were purchased from Sigma-Aldrich (USA).

Methods

Sample preparation of the persimmon (Diospyros kaki L.) fruit extract

Persimmon (*Diospyros kaki* L.) from an unknown cultivar produced within the Portuguese territory was used. The persimmon fruit extract was prepared according to Wu and Hwang (Wu and Hwang 2002) and Jang et al. (Jang et al. 2010) with some modifications. In summary, 20 ± 0.1 g of lyophilized persimmon fruit was extracted using acetone:water (80:20, v/v) solution and mechanically homogenized during 10 minutes, at room temperature and protected from light. The sample was centrifuged at 9,000 rpm for 10 minutes at room temperature. The supernatant was separated, and the extraction procedure was repeated three more times, using the same extracting solution. The supernatants were kept together and filtered. The sample was evaporated until almost dryness. Finally, the extract was made up with Milli-Q water to at least 0.5 g persimmon mL^{-1} to comply with oral gavage administration proposed in our study. The extract obtained was subdivided into aliquots and stored in falcon tubes at $-20\,^{\circ}C$, prior to analysis.

Determination of the total phenolic content

To determine the total phenolic content, the method of Stamatakis et al. (Stamatakis et al. 2009) was modified as per Figueira et al. (Figueira et al. 2014). Results were expressed as milligrams of gallic acid equivalents (mg GAE) per 100 g of fresh fruit and per ml of the extract. Analyses were performed in triplicate.

Determination of the total flavonoid content

Total flavonoid content was determined by the method of Zhishen, Mengcheng and Jianming, described by Cam and Hisil (Cam and Hisil 2010). Results were expressed in milligrams of catechin equivalents (mg CE) per 100 g of fresh fruit. Catechin was used as external standard in a concentration range from 20 to 100 mg/L. Analyses were performed in triplicate.

Determination of the total procyanidins content

This procedure was performed according to Swain and Hills (Swain and Hills 1959). Results were expressed as milligrams of catechin equivalents (mg CE) per 100 g of fresh fruit. Analyses were performed in triplicate.

Determination of the ascorbic acid content

The sample content of ascorbic acid was determined using a spectrometric method, as described in the Portuguese standard NP-303032 with some modifications (Figueira

et al. 2014). The ascorbic acid content of the sample was expressed in milligrams of ascorbic acid per 100 g persimmon. Samples were analyzed in triplicate.

Antioxidant activity

Oxygen radical absorbance capacity (ORAC). ORAC assay was carried out by the method of Huang et al. (Huang et al. 2002) modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Feliciano et al. (Feliciano et al. 2009). All data were expressed as micromoles of trolox equivalent antioxidant capacity (TEAC) per 100 g of fresh persimmon weight.

Hydroxyl radical adverting capacity (HORAC). The HORAC assay was based on a previously reported method (Ou et al. 2002), modified for the FL800 microplate fluorescence reader as described by Serra et al. (Serra et al. 2010). Data were expressed as millimoles or micromoles of caffeic acid equivalent antioxidant capacity (CAEAC) per 100 g of fresh persimmon weight. Results are a mean of six replicates.

Cell culture

Human colon carcinoma Caco-2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and were routinely grown in RPMI 1640 medium supplemented with 10% of FBS and 2 mM of L-glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37 °C with a 5% CO_2 under a humidified atmosphere. The cellular antioxidant activity (CAA) assay was performed according to Wang and Joseph (Wang and Joseph 1999), modified by Serra et al. (Serra et al. 2010). The CAA of extracts was quantified according to Wolfe and Liu (Wolfe and Liu 2007). The EC_{50} values were stated as mean ± SD for triplicate sets of data obtained from the same experiment. EC_{50} values were converted to CAA values, expressed as micromoles of quercetin per gram of persimmon, using the mean EC_{50} value for quercetin from three independent experiments.

Animal care and maintenance for the in vivo experiments

Experiments were conducted as per the adopted EC regulations regarding animal welfare, and the studies follow the ARRIVE Guidelines for Reporting Animal Research, summarized at http://www.nc3rs.org.uk. All studies were carried out using male Wistar rats 5 weeks of age weighing 100–150 g (Harlan Iberica, Barcelona, Spain). All animals received a standard diet and water ad libitum.

Carrageenan-induced paw edema

Animals were randomly allocated into the following groups as described: (1) control group: animals were subjected to subplantar injection into the left hind paw of 0.1 mL sterile saline and administered with saline (1 mL kg^{-1}, intraperitoneal [i.p.]) ($n = 6$); (2) carrageenan group: animals were subjected to paw edema induction and administered

with saline (1 mL kg^{-1}, i.p.) ($n = 6$); (3) persimmon p.o. group: animals were subjected to paw edema induction and pretreated with the persimmon fruit extract (15 mg crude extract per kg, oral [p.o.]) 30 minutes before λ-carrageenan injection ($n = 6$); (4) indomethacin group: animals wee subjected to paw edema induction and pretreated with indomethacin (10 mg kg^{-1}, i.p.) 30 minutes before λ-carrageenan injection ($n = 6$); (5) trolox group: animals were subjected to paw edema induction and pretreated with trolox (30 mg kg^{-1}, i.p.) 30 minutes before λ-carrageenan injection ($n = 6$); (6) tempol group: animals were subjected to paw edema induction and pretreated with tempol (30 mg kg^{-1}, i.p.) 30 minutes before λ-carrageenan injection ($n = 6$). As previously described (Bignotto et al. 2009), paw volume was measured by means of a volume displacement method using a plethysmometer (Digital Plethysmometer LE7500; Letica Scientific Instruments, Letica, Spain) immediately after the injection of carrageenan (V_0 or basal volume) and 6 hours later (V_{6h}). The increase in paw volume was taken as the edema volume.

Collagen-induced arthritis in Wistar rats

CIA was induced as previously described (Szabo et al. 1998; Cuzzocrea et al. 2000; Figueira et al. 2014). On day 1, all rats were administered intradermally at the base of the tail with 100 µL of the emulsion (containing 100 µg of CII). On day 21, the second injection of CII in FCA was administered. In treated groups, animals ($n = 5$ per group) were treated with the persimmon fruit extract (15 mg crude extract per kg per day, p.o.) every 24 hours starting on day 23. Rats were evaluated daily for arthritis (Bignotto et al. 2009; Figueira et al. 2014; Figueira et al. 2016). Clinical severity was also determined by quantitating the change in the paw volume, as measured by plethysmometry on day 35. At the end of the experimental protocol, blood samples were collected into a BD Vacutainer SST II Advance gel and clot activator tube [3 × 5 mL (BD Diagnostics – Preanalytical Systems, Oxford, UK)] and were centrifuged (3,000 rpm for 10 min at room temperature) to separate serum, to be analyzed using a Cobas Analyzer Roche Automated system.

Histologic (light microscopy) assessment of arthritis. Histologic assessment of arthritis was performed according to Figueira et al. (Figueira et al. 2016). An investigator who was blinded to the treatment regimen performed the histologic examination, scoring the morphologic features on a scale of 0–3, where 0 = no damage, 1 = edema, 2 = inflammatory cells, and 3 = bone resorption.

Immunohistochemistry for iNOS and COX-2. After sacrifice, animals were perfused with 0.1 M phosphate buffer saline (PBS) (pH 7.4) followed by the same buffer containing 4% paraformaldehyde (PFA). Fixed tissues were postfixed in 4% PFA in PBS for 72 hours at room temperature (RT), decalcified, dehydrated through a graded ethanol series, and embedded in paraffin. Histopathologic features (inflammation, pannus formation, cartilage damage, and bone resorption) in knee joint specimens were observed following hematoxylin and eosin staining, and immunostaining was performed according to Figueira et al. (Figueira et al. 2016).

Radiographic analysis. After sacrifice, radiography of normal and arthritic rat hind paws was performed according to Silva et al. (Silva et al. 2015). An investigator who

Table 1. Phenolic composition and ascorbic acid content of the fresh persimmon fruit and extract.

Parameter	Per 100 g of fresh persimmon fruit	Per mL of the fresh persimmon extract	Literature data (mg/100g FW)
Total phenolic content (mg of GAE)	975 ± 6.5	5.36 ± 0.03	916.8 (Denev and Yordanov 2013)
Total flavonoid content (mg of CE)	225 ± 7.9	1.24 ± 0.000577 (0.00)	4.2 (Denev and Yordanov 2013)
Total procyanidins (mg of CE)	744 ± 8.6	4.09 ± 0.04	540.2 (Denev and Yordanov 2013)
Ascorbic acid content (mg AA)	18	0.09 ± 0.01	47 ± 39 (Giordani et al. 2011)

Values are expressed as means of three replicates ± standard deviation. GAE, gallic acid equivalents; CE, catechin equivalents; AA, ascorbic acid.

was blinded to the treatment regimen performed the radiographic scoring, using a scale of 0–3, where 0 = no bone damage, 1 = tissue swelling and edema, 2 = joint erosion, and 3 = bone erosion and osteophyte formation.

Statistics

Statistical treatments were done using GraphPad Prism (version 5.0; GraphPad Software). Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison between groups was estimated using the one-way analysis of variance (ANOVA), followed by the Bonferroni's post hoc test. In all cases, p values lower than 0.05 were considered statistically significant. For the analysis of CIA results, the unpaired Student's t test was used.

Results

Total phenolic content

In this study, persimmon (*Diospyros kaki* L.) was extracted using an acetone:water (80:20, v/v) solution. This solvent was chosen because it provided the best results when we compared different extracting solutions (data not shown). The final extract was prepared according to the amount required for the animal assay. Results of the characterization of the extract expressed per 100 g of fresh fruit and per mL of the extract of fresh persimmon fruit are presented in Table 1.

Antioxidant activity

Antioxidant activity was evaluated by ORAC and HORAC assays. Results were, respectively, 1,517 ± 146 µmol trolox equivalents per 100 g of fresh persimmon fruit (7.581 ± 0.733 µmol trolox equivalents per mL of fresh persimmon fruit extract), and 984 ± 67 µmol caffeic acid equivalents per 100 g of fresh persimmon fruit (4.919 ± 0.334 µmol caffeic acid equivalents per mL of fresh persimmon fruit extract). Antioxidant intracellular activity was evaluated by CAA assay, and the result was 32.8 µmol of quercetin equivalents per 100 g of fresh persimmon fruit (0.1641 µmol of quercetin equivalents per mL of persimmon fruit extract). Considering the inhibitory effect of the fresh persimmon fruit extract (1.1–17.5 µg mL^{-1}) on human neutrophil oxidative burst, an IC50 of 7.5 ± 1.0 µg mL^{-1} was determined (Figure 1).

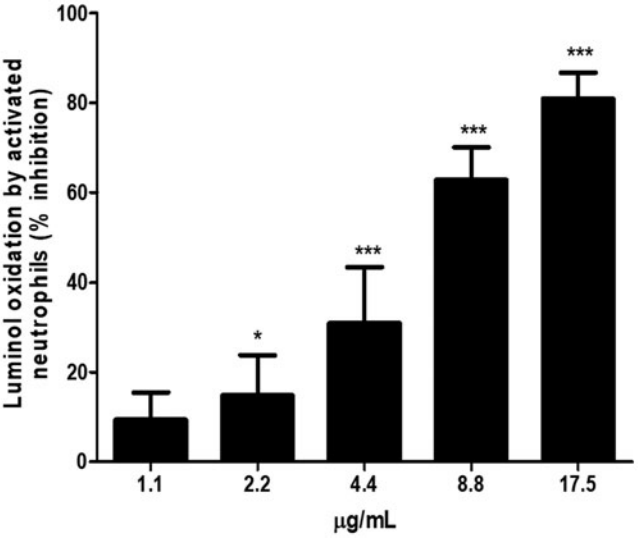

Figure 1. Inhibitory effect of persimmon on human neutrophils' oxidative burst. Persimmon extract (1.1–17.5 µg mL^{-1}) exhibited inhibitory effect on human neutrophils' oxidative burst stimulated with phorbol myristate acetate (PMA), as measured by luminol-amplified chemiluminescence. $^{*}p < 0.05$; $^{***}p < 0.001$ compared with the control assay (PMA alone). Values are expressed as mean ± SEM ($n \geq 4$). CIA, collagen-induced arthritis.

Acute inflammation model induced by carrageenan

After 6 hours of λ-carrageenan administration, all treated groups showed statistically significant reduction of the paw edema when compared with the carrageenan group (Figure 2). After 6 hours, animals administered with the extract showed less edema development when compared to the carrageenan group. Animals treated with indomethacin, trolox, and tempol showed a significant reduction of edema development. This model is closely related to some of the alterations observed in the collagen-induced arthritis in rats.

Collagen-induced arthritis

CIA developed rapidly in rats immunized with CII. Periarticular erythema and edema were first observed between the hind paws 21 days post-challenge. An incidence of 100% of CIA was observed by day 23 in all CII-immunized rats. No clinical signs of CIA were observed in rats' forepaws during the 35-day evaluation period. Hind paw erythema and swelling increased in severity and in a time-dependent manner (data not shown) with a maximum arthritis index observed till day 35. Treatment with the extract could significantly reduce edema formation in the hind paws (Figure 3) when compared to untreated animals. On day 35, samples from a vehicle-treated CIA rat exhibited severe synovitis, cartilage damage, marked infiltration and pannus, and bone resorption when compared to normal observations in control animals (not subjected to CIA induction). Specimens obtained from animals treated with the extract exhibited normal synovium, small cartilage damage, and reduced marginal zone pannus or bone resorption (Figure 4). Radiographic examination of the hind paws 35 days after immunization

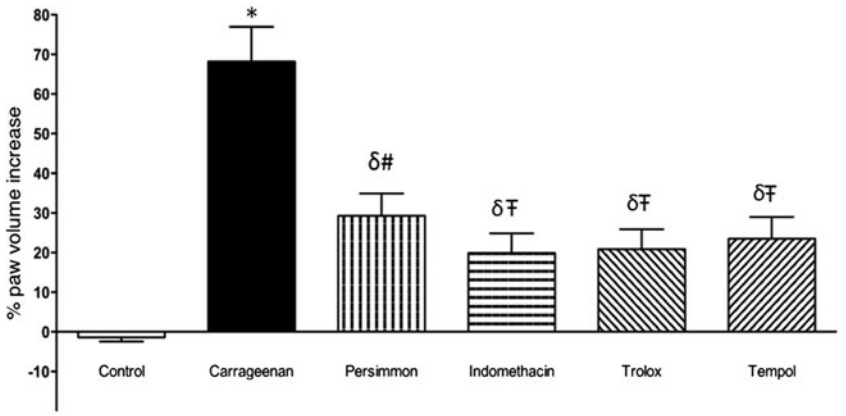

Figure 2. Effects of the persimmon fruit extract on carrageenan-induced paw volume increase. The effect of a single administration of the persimmon fruit extract (15 mg kg^{-1}, p.o.; $n = 6$) on rat paw edema development elicited by carrageenan 6 h after induction and comparison with the effect of indomethacin (10 mg kg^{-1}, i.p.; $n = 6$), trolox (30 mg kg − 1, i.p.; $n = 6$), and tempol (30 mg kg^{-1}, i.p.; $n = 6$). The administration of the extract significantly inhibited rat paw edema formation, and the reduction was in similar magnitude regarding the effects of trolox, indomethacin, and tempol. The data are presented as mean ± SEM. $^{*}p < 0.001$ versus control group; $^{\delta}p < 0.05$ versus control; $^{\#}p < 0.01$ versus carrageenan; $^{\mp}p < 0.001$ versus carrageenan.

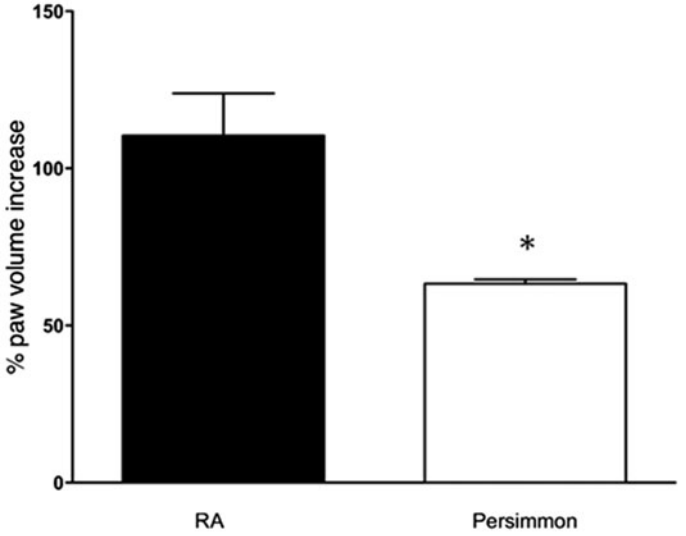

Figure 3. Effects of the persimmon fruit extract on CIA-associated paw volume increase on day 35. The effect of treatment with the persimmon fruit extract (15 mg kg − 1 p.o.; $n = 12$ per group) on rat paw edema development associated with CIA (as seen in the RA group; $n = 12$). Treatment with the extract significantly reduced paw edema. Results are presented as mean ± SEM. $^{*}p < 0.001$ versus CIA group. CIA, collagen-induced arthritis; RA, rheumatoid arthritis.

with CII revealed bone matrix resorption and osteophyte formation at the joints (Figure 5). There was no evidence of CIA pathology in control rats. Radiographic examination confirmed that persimmon extract could reduce the degree of bone resorption, soft tissue swelling, and osteophyte formation, improving articular function in treated

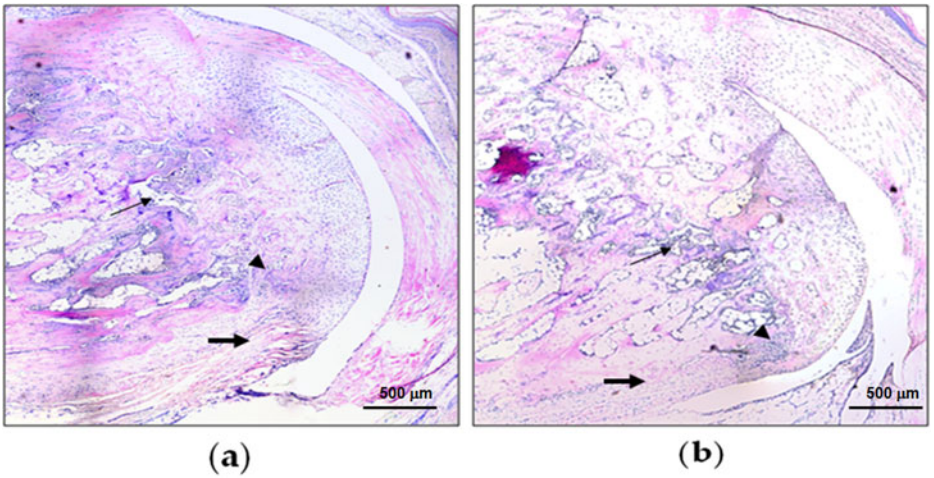

Figure 4. Histopathologic findings in rats from arthritis model treated with persimmon extract. Rats were treated with 15 mg/Kg/day of persimmon extract by gavage. (a) Specimens from a vehicle-treated arthritic rat exhibit severe synovitis, cartilage damage (large arrow), and marked infiltration and pannus (small arrow) and bone reabsorption (arrow head). (b) Specimens from a rat treated with persimmon extract exhibit less cartilage damage (large arrow) but maintain the infiltration (small arrow) and bone reabsorption (arrow head). (Original magnification ×100).

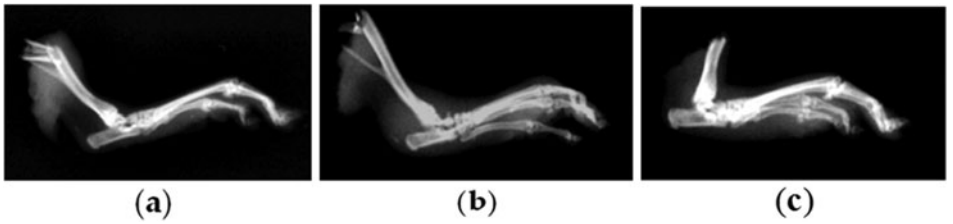

Figure 5. Radiographic progression of CIA in the tibiotarsal joints. (a) There is no evidence of pathological alterations in the tibiotarsal joints of control (normal) animals. (b) The hind paws of rats with CIA at day 35 demonstrated bone resorption and significant joint erosion. (c) Treatment with the persimmon fruit extract (15 mg kg − 1) administered per os significantly suppressed the joint pathology and the soft tissue edema in the hind paw. The X-ray images are representative of at least three experiments performed on different experimental days. CIA, collagen-induced arthritis.

animals. Investigators who were blinded to the treatment regimen performed the histologic and radiographic examinations, and scoring of the lesion features showed a beneficial effect of the extract administration on both parameters (Figure 6). Animals subjected to CIA exhibited a marked induction of COX-2 and iNOS expression, namely, near the infiltrates. There was no significant alteration on with COX-2 (Figure 7) or iNOS (Figure 8) expression in samples collected from animals treated with the persimmon extract.

Discussion

In this study, an extract of persimmon fruit (*Diospyros kaki* L.) was prepared using acetone:water (80:20, v/v) solution (the choice of solvent was based on previous studies

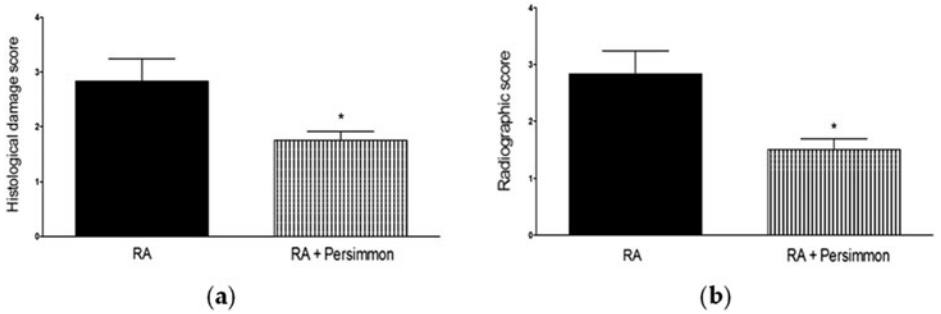

Figure 6. Effects of persimmon extract treatment (p.o.) on the (a) histologic damage score and the (b) radiographic score in animals with collagen-induced arthritis (RA). The results are expressed as mean ± SEM and were compared using an unpaired *t* test using a Graph Pad Prism Statistical Package (version 5.0). A *p* value less than 0.05 was considered to be statistically significant. $^{*}p < 0.001$ versus vehicle-treated (RA).

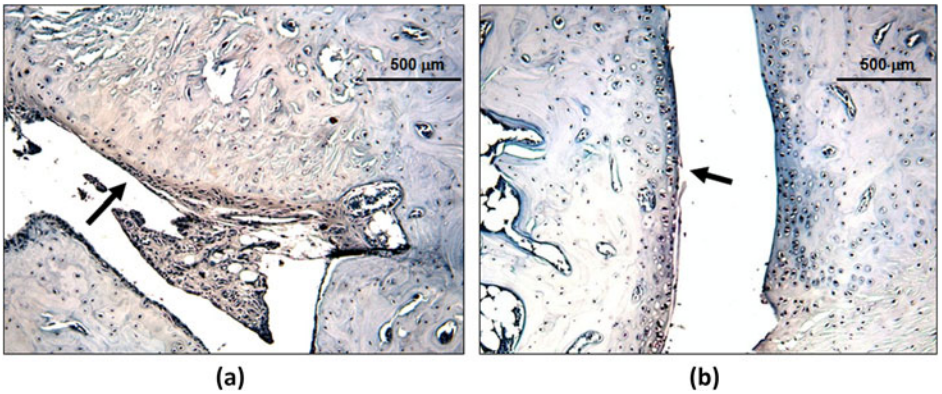

Figure 7. Immunostainning for COX-2 expression in arthritic joint samples. Specimens from a nontreated rat exhibit almost no expression of COX-2 while (a) vehicle-treated arthritic mice exhibit massive production of COX-2, namely, near the infiltrates (brown staining – large arrow). (b) Specimens from animals treated with the persimmon extract (15 mg kg^{-1}, $n = 5$ per group) administered per os did not significantly reduce COX-2 staining (original magnification ×100). Scale bar equals 500 µm. COX-2, cyclooxygenase-2.

that compared different extracting solutions). A characterization of the extract was performed and results, expressed per 100 g of fresh fruit and per milliliter of the extract of fresh persimmon fruit, are presented in Table 1.

In the literature, the data regarding total polyphenol content of persimmons is quite diverse. The total polyphenols are reported to be between 12.7 and 29.5 mg of GAE/100g of FW (Veberic et al. 2010). Other authors report soluble polyphenol concentrations to vary in the range of 1.3 mg to 1,550 mg/100g FW of total polyphenols, even for the same astringent cultivar Triumph (Gorinstein et al. 2001; Park et al. 2008). In fact, by considering the research of Jang et al. (2010), conducted on homogeneous samples of the nonastringent cultivar Fuyu, the lowest concentration of soluble polyphenols was 454 mg GAE/100 g FW when extracted with ethanol, about half the highest concentration determined with water extraction (860 mg GAE/100 g FW) (Jang et al. 2010). Per

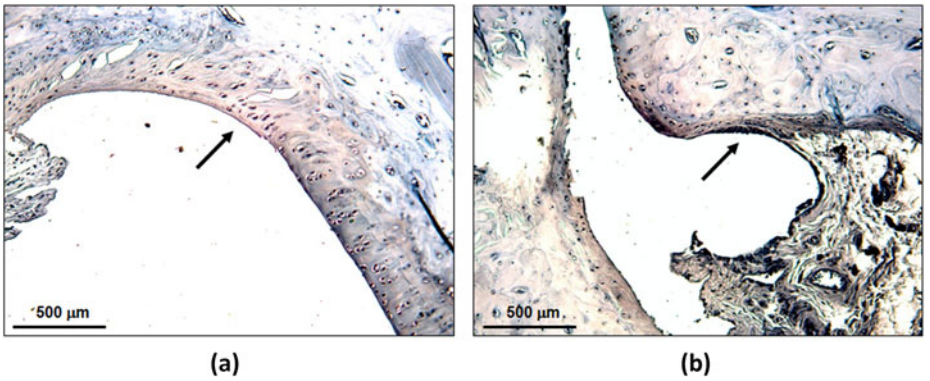

Figure 8. Immunostainning for iNOS expression in arthritic joint samples. Specimens from a nontreated rat exhibit almost no expression of iNOS while (a) vehicle-treated arthritic rats exhibit massive production of iNOS, namely, near the infiltrates (brown staining – large arrow). (b) Specimens from animals treated with the persimmon fruit extract (15 mg kg − 1) administered by gavage did not significantly reduce iNOS staining (original magnification × 100). Scale bar equals 500 µm. iNOS, inducible nitric oxide synthase.

the values reported in other studies, for this fruit the total phenolic content varies from 916.8 mg GAE/100g FW to 400.2 mg per 100 g FW, and this content was strongly affected by the cultivar and maturity stage (Denev and Yordanov 2013). Our results for total polyphenol content were 975 ± 6.5 mg of GAE/100g of FW (5.36 mg of GAE/mL) and are in agreement with the values reported in the literature by other authors (Jang et al. 2010; Denev and Yordanov 2013). The wide range of variability observed can be attributed to environmental effects, even though the different extraction methods applied and analytical protocols could also significantly influence the results (Giordani et al. 2011), as could the different persimmon cultivars analyzed and the stage of maturation.

In general, the polyphenol content (and particularly the proanthocyanidin content) is strongly affected by the cultivar and maturity stage. Proanthocyanins decreased proportionally during maturation from 540.2 CE/100g FW to 90.2 CE/100g FW (Denev and Yordanov 2013). In our study, proanthocyanidin content was 744 ± 8.6 CE/100g FW (4.09 mg of EC/mL).

Total flavonoids represent just a very small part of the total polyphenols in persimmon (Denev and Yordanov 2013), and in our extract are about 225 ± 7.9 CE/100 g FW (1.24 mgEC/mL). The concentration of ascorbic acid in the extract was 0.09 ± 0.01 mg of ascorbic acid per mL of the persimmon extract corresponding to 18 mg/100g FW. According to published data, ascorbic acid content of the persimmon fruit ranges from 3.5 mg/100 g FW in the astringent variety Costata to 146 mg/100 g FW in the nonastringent cultivar Hana Fuyu. Astringent and nonastringent genotypes have significantly different ascorbic acid concentrations, the mean values of which were 34 ± 22 and 71 ± 51 mg/100 g FW, respectively (Giordani et al. 2011). However, values ranging from 0.68 to 1.129 mg/100g in *Diospyros* cultivars grown in Portugal have been previously determined (Vinha et al. 2011). *Diospyros kaki* is highly concentrated in vitamins and a moderate source of ascorbic acid (Singh and Joshi 2011). Our results demonstrate that persimmon is a fruit with a large amount of phenolic antioxidants

compared to other fruits such as apples, which have a content of total phenolic compounds of 422.5 mg GAE/100g FW (Mikulic-Petkovsek et al. 2007) and 69.6–141.0 mg CE/100g FW (Gu et al. 2004) of total procyanidins, or red raspberry (*Rubus idaeus L.*), with 171 mg GAE/100g FW of total phenolic compounds, 24.45 mg CE/100g FW of total flavonoids content, and 17.71 mg CE/100g FW of total procyanidins content (Figueira et al. 2014).

In addition, our extract showed higher antioxidant activity compared to other extracts from several fruits, including apples (Serra et al. 2010) and strawberries (Kevers et al. 2007). In our study, the persimmon extract showed 1,517 ± 146 µmol TEAC/100g FW (7.581 ± 0.733 µmol TEAC/mL) by ORAC and 984 ± 67 µmol CAEAC/100g FW (4.919 ± 0.334 µmol CAEAC/mL) by HORAC. The studies with apples (Fuji, Starking, Reineta Parda, Gala Galaxy, and Golden) by Sera et al. (Serra et al. 2010) found 1,133 ± 70 µmol TEAC/100g and 612 ± 79 µmol CAEAC/100g, by ORAC and HORAC, respectively. Strawberry (Fragaria x. ananassa) exhibited 1,190 µmol TEAC/100g as determined by ORAC (Kevers et al. 2007).

According to several reports considering the antioxidant activity of persimmon (Jung et al. 2005; Chen et al. 2008; Park et al. 2008; Fukai et al. 2009; Jang et al. 2010; Gorinstein et al. 2011; Jang et al. 2011), the majority of the studies employed DPPH and ABTS assays, which are very rapid and easy to perform but have some serious limitations. For example, DPPH radical is resistant nitrogen radical in contrast to highly reactive physiologically relevant radicals. Often antioxidants, which react fast with peroxyl radicals, react very slowly with DPPH. There is also evidence that DPPH reacts reversibly with some polyphenols, resulting in altered radical-scavenging value (Huang et al. 2005). The methods chosen in this study embrace different aspects of the antioxidant action and give a comprehensive view on the antioxidant potential of the investigated extract. This is important because it is recommended to use more than one antioxidant assay for detailed understanding of the principles of antioxidant properties of antioxidants (Ciz et al. 2010).

Cellular antioxidant activity (CAA) assay is a more biologically relevant method than the antioxidant activity assays because it considers the uptake, metabolism, and location of antioxidant compounds within cells (Figueira et al. 2014). Values obtained for the extract used in this study were CAA of 32.8 µmol of quercetin equivalents per 100 g of fresh persimmon fruit (0.1641 µmol of quercetin equivalents per mL of fresh persimmon fruit extract).

From a mechanistic point of view, we aimed to determine not only the efficacy of exogenous antioxidant compounds from the extract within a cellular environment (evaluated by CAA), but also the ability to actively inhibit the oxidative burst response from neutrophils infiltrated in the injured tissue. Considering the inhibitory effect of the fresh persimmon fruit extract (1.1–17.5 µg mL^{-1}) on human neutrophil oxidative burst, an IC50 of 7.5 ± 1.0 µg mL^{-1} was determined (Figure 1). In both cases, the extract demonstrated not only the ability to act as an intracellular antioxidant but also the ability to interfere with neutrophils' function, inhibiting the release of deleterious reactive oxygen species that would amplify the inflammatory signals already triggered.

The crude drug *kaki-yô*, prepared from the leaves of *Diospyros kaki* Thunberg (Ebenaceae), has been utilized as a hypotensive drug in Japanese traditional medicine

(Funayama and Hikino 1979). The effect of five flavonoid compounds isolated from the leaves of *Diospyros kaki* on the stimulus-induced superoxide generation and phosphorylation of tyrosine residues of protein in human neutrophils was investigated by Chen et al. (2002). The five compounds examined were kaempferol 3-*O*-*β*-d-galactopyranoside, kaempferol 3-*O*-*β*-d-glucopyranoside, isorhamnetin 3-*O*-*β*-d-glucopyranoside, quercetin 3-*O*-*β*-d-galactopyranoside, and quercetin 3-*O*-*β*-d-glucopyranosyl-(6→1)-α-l-rhamnopyranoside. Flavonoid compounds suppressed stimulus-induced superoxide generation and tyrosyl phosphorylation (Chen et al. 2002). Other authors have also discussed the effects of persimmon fruit or of the fruit extract on human neutrophils' oxidative burst, and although the results are similar, none of them based their conclusions on the same assay we have used in this study.

Carrageenan-induced inflammation of the hind paw in rodents has been used as a model to study the anti-inflammatory activity of several antioxidants such as flavonoids and lycopene (Bignotto et al. 2009; Figueira et al. 2014; Silva et al. 2015). In the early phase of carrageenan-induced inflammation, there occurs the release of leukotrienes, histamine, platelet-activating factor, and cyclooxygenase products, while in the delayed phase there is neutrophil infiltration and, consequently, the production of neutrophil-derived reactive oxygen species as well as the release of other neutrophil-derived mediators (Jean-Gilles et al. 2012). The proposed mechanism of action for the extract used in this study would explain the significant reduction of edema formation (inflammatory response) observed 6 hours after the administration of the carrageenan in this model. The effect observed was of similar magnitude in relation to the effect seen for a cyclooxygenase inhibitor (such as indomethacin) for a potent water-soluble antioxidant (such as trolox) and for a low-molecular-weight superoxide dismutase mimetic (such as tempol).

Other authors demonstrated that the anti-inflammatory effects of isolated diospyrin and 8-hydroxyisodiospyrin and of the extract (chloroform fraction) of roots from *Diospyrus lotus* were dose dependent, using diclofenac as control (Uddin et al. 2014). In the experimental setting of the study by Uddin et al. (Uddin et al. 2014), the maximum percent inhibition (58%–78.43%) of paw edema was observed after the third hour of carrageenan injection in chloroform fraction treated animals. Other studies investigated the protective effects of the aqueous extract of *Diospyros kaki* on mast cell–mediated allergic inflammation and determined its possible mechanisms of action by using in vitro and in vivo mast cell–based models; in these studies the extract was able to inhibit the release of histamine and *β*-hexosaminidase from mast cells by modulating cAMP and intracellular calcium levels (Kim et al. 2013). The extract decreased gene expression and the secretion of the proinflammatory cytokines, tumor necrosis factor (TNF)-α, and interleukin (IL)-1*β* by inhibiting nuclear factor-*κ*B (Kim et al. 2013), and these effects where comparable to those observed with disodium cromoglicate. These findings provide evidence that an aqueous extract of *Diospyros kaki* inhibits allergic inflammation and suggest the therapeutic application of aqueous extract of *Diospyros kaki* in allergic inflammatory disorders (Kim et al. 2013). However, anti-inflammatory effects of *Diospyros kaki* have not yet been elucidated in a chronic path such as rheumatoid arthritis model.

Our results show that macroscopic evidence of CIA first developed as periarticular erythema and edema of the hind paws. The incidence of CIA was 100% by day 23 in rats, and the severity of CIA progressed over a 35-day period. Radiographs revealed focal resorption

of bone, with osteophyte formation in the tibiotarsal joint and soft tissue swelling as expected. Treatment of rats with the extract (15 mg kg^{-1} per day) per os starting at the onset of arthritis (day 23) significantly delayed the development of the clinical signs on days 24–35 and improved the histological and radiographic scores of both the knee joint and hind paw. Results showed that, in this model of CIA and administration timeline, no alteration of COX-2 and iNOS expression was observed in the arthritic joint tissues (Figure 8). These results are in accordance with the absence of effect on the neutrophil infiltration as determined by our histological analysis, although histological markers of arthritic lesions were attenuated (less cartilage damage). Along with the observed effects of the extract on the in vitro reduction of oxidative burst of neutrophils (Figure 1), the beneficial effects of persimmon extract in this model might be related to an attenuation of neutrophil activation and consequent reduction of the release of proinflammatory neutrophil-derived products. This is also in accordance with the positive results observed in the paw edema studies, both representing inflammation models typically with high correlation to neutrophil activation (Rocha et al. 2015). Of all cells implicated in the pathology of rheumatoid arthritis, neutrophils possess the greatest cytotoxic potential, owing to their ability to release degradative enzymes and reactive oxygen species; it is a well-known fact that neutrophil-derived hypochlorous acid plays an important role in cartilage destruction during rheumatoid arthritis (Schiller et al. 1995). Consequently, inhibition of neutrophil activation will directly interfere with the pathologic mechanisms behind injury in RA.

Here we provide original data, proposing a rationale for the pharmacodynamic actions of this extract, including histological and immunohistochemistry characterization and evaluation of the effect of the extract on paw edema models (induced by CIA and carrageenan) and on oxidative neutrophil burst. These effects could be observed in practice considering the average consumption of persimmon in the population. A dose of 15 mg kg^{-1} of the extract would translate to the equivalent human daily consumption of 175 g of fresh persimmon fruit. This consumption of the fresh fruit or of an extract of the fruit might be a useful pharmacological tool in the management of chronic arthritic conditions associated with active inflammation, along with the identification of specific constituents of the extract that might be useful in the dietary supplementation of patients with RA.

Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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