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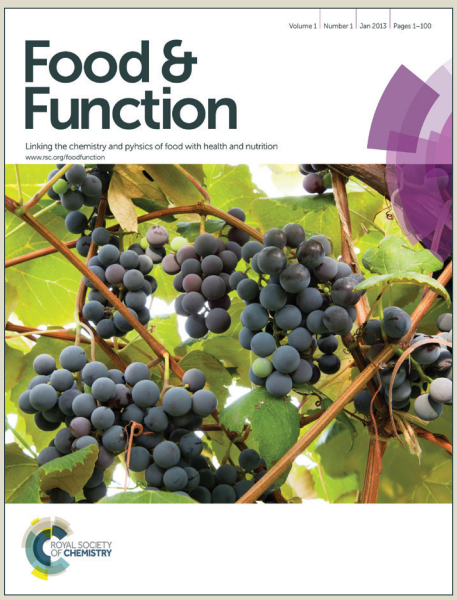

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ARTICLE

Chemical characterization of a red raspberry fruit extract and evaluation of its pharmacological effects in experimental models of acute inflammation and collagen-induced arthritis

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Berries are an important dietary source of fibres, vitamins, minerals and some biologically active non-nutrients. A red raspberry fruit extract was characterized in terms of phenolic content and the anti-inflammatory properties and protective effects were evaluated in two experimental models of inflammation. The antioxidant potential of the extract, the cellular antioxidant activity and the effects over neutrophils' oxidative burst, were also studied to provide a mechanistic insight for the anti-inflammatory effects observed. The extract was administered in a dose of 15 mg/kg, i.p. and significantly inhibited paw oedema formation in the rat. The same dose was administered via i.p. and p.o. route in the collagen-induced arthritis model in the rat. The extract has pharmacological activity and was able to significantly reduce the development of clinical signs of arthritis and markedly reduce the degree of bone resorption, soft tissue swelling and osteophyte formation, preventing articular destruction in treated animals.

Introduction

Rheumatoid arthritis as evolved from a syndrome of unknown cause to one for which distinct subsets of disease are emerging. Also, the growing knowledge of risk factors calls for preventive strategies.[1] This disease has become a prototype for the development of knowledge regarding new treatments targeting different molecular pathways. Rheumatoid arthritis is currently regarded as a modern-day medical dilemma, whereby early treatment can prevent disability in many patients but the most effective new drugs can be too expensive to administer to all people who might benefit.[1] This condition is known to be a chronic, systemic autoimmune disease[2], characterized by inflammation of the synovia, synovial hyperplasia with increased cell density, and infiltration of inflammatory cells leading to pannus formation and irreversible cartilage and bone destruction.[3] Collagen-induced arthritis (CIA) is an experimental model of arthritis that is initiated by the administration of type II collagen (CII), a component of the extracellular matrix of articular cartilage located in diarthrodial joints.[4] Trentham *et al* [5] first described this animal model of arthritis induction in rats immunized with CII. This model is considered of special interest in part because rats with CIA, like some (not all) of rheumatoid arthritis patients, expressed elevated titers of CII antibodies. The articular cartilage is composed mainly of type II collagen (CII; 60% of dry weight) and proteoglycan (10% of dry weight). Together, they are responsible for the biomechanical properties of the cartilage and confer tensile strength and load bearing capacity respectively.[6,7] In arthritis, the destruction of the cartilage is associated to a reduced synthesis of the matrix components by articular chondrocytes and an enhanced breakdown of the matrix by proteolytic enzymes, mainly the matrix metalloproteases.[3,8] The similarities between the joint pathology (including erosion) in CIA and rheumatoid arthritis have been the main driver for the use of this model in drug development when targeting rheumatoid arthritis.[4] The red raspberry (*Rubus idaeus* L.) fruit is known for containing several macro and micronutrients of pharmacological interest, including fibers, ascorbic acid and non-nutrients as bioactive phenolic compounds.[9] These compounds are mainly ellagitannins and anthocyanins, along with ellagic acid glycosides and flavonol conjugates.[10,11,12] Although some studies have started to unveil the anti-inflammatory effects of red raspberry extracts *in vitro* and *in vivo*, these studies are mostly preliminary and require further elaboration so that a translational approach for clinical practice is a realistic goal if considering an adjunctive therapy. Here we investigated the effects of a characterized red raspberry extract on the arthritic chronic inflammatory response caused by the injection of CII in the rat. The amount of components in the extract with expected biological activity could reasonably be achieved in humans, with the consumption of a regular daily quantity of fresh fruits.

Results and discussion

In this study, raspberries from unknown cultivar produced in Portuguese territory were used to prepare the red raspberry fruit extract with methanol: acetone (50%, v/v) extractant solution. The tested dose of 15mg/kg of extract would translate to the equivalent human daily consumption of 150 g of fresh red raspberry fruits. Spectrophotometric analyses were performed to determine total phenolic compounds, total flavonoids, total anthocyanins, total hydrolysable tannins and total condensed tannins. **Table 1** shows the concentration of these phenolic compounds, expressed per 100 g of fresh fruit and per mL of extract of fresh red raspberries. The total phenols content in the fresh red raspberries fruits analysed is comparable to values reported in the literature for these small fruits: 114.5 to 203.1 mg/100 g FF [13]; 129.4 to 183.1 mg/100 g FF [14]; and 124 to 134 mg/100 FF [15], the latter's values concerning red raspberries grown in Portugal. The concentration of total flavonoids and anthocyanins is also within the range reported in the literature. Total flavonoids content of red raspberry cultivars produced in Turkey range from 25.3 to 41.1 mg of catechin equivalents/100g of fresh fruit .[16]

Table 1 – Phenolic composition of the fresh red raspberry fruit and extract (values are expressed as means of three replicates ± standard deviation)

Parameter	/100g of Fresh Red Raspberry Fruit	/mL of Fresh Red Raspberry Extract
Total phenolic content (mg of GAE)	171.03 ± 4.17	8.57 ± 0.21
Total flavonoids content (mg of CE)	24.45 ± 1.44	1,22 ± 0.07
Total hydrolysable tannin content (mg of TAE)	166.13 ± 0.19	8.31 ± 0.01
Total condensed tannin content (mg of CE)	17.71 ± 0.25	0.89 ± 0.10
Total anthocyanin content (mg of C3GE)	20.04 ± 2.76	1.00 ± 0.14

GAE – Galic acid equivalents; **CE** – Catechin equivalents; **C3GE** – Cyanide-3-glucoside equivalents; **TAE** – tannic acid equivalents.

Ellagitannins are the dominant phytochemicals in ripe red raspberries and proanthocyanidins are minor polyphenolic compounds in those fruits.[17] Red raspberries analyzed in this study corroborate this data: total hydrolysable tannins content was 166.13 mg of tannic acid equivalents/100g of fresh fruit and, total condensed tannins was 17.71 ± 0:25 mg of catechin equivalents/100g of fresh fruit. Total anthocyanins content varied between 12.4 and 24.8 mg of cyanidin-3-glucoside equivalents/100g of fresh fruits produced in Turkey[30] from 19.0 to 51.0 mg of cyanidin-3-glucoside equivalents/100g of fresh fruit and from 16.0 to 78.0 mg of cyanidin-3-glucoside equivalents/100g of fresh fruit [18,19] for raspberry cultivars grown in Finland and in Italy respectively.

Anthocyanins are the major flavonoids of red raspberry. Anthocyanins (cyanidin-3-sophoroside, cyanidin-3-glucoside, cyanidin-3-sambubioside, pelargonidin-3-sophoroside, cyanidin-3-glucosyl-rutinoside, pelargonidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-xylosyl-rutinoside, pelargonidin-3-glucosyl-rutinoside, and pelargonidin-3-rutinoside), phenolic acids (gallic acid) and ellagitannins (ellagic acid) were identified and quantified at different wavelengths (520 nm, 258 nm, 280 nm and 378nm, respectively) after analysis by HPLC-DAD. The concentrations of these compounds in mg per 100g of fresh red raspberry fruit (FF) and in mg per mL of fresh red raspberry extract (FE) are shown in **Table 2**. Cyanidin-3-sophoroside and cyanidin-3-glucoside were the most abundant anthocyanins of the fresh red raspberries extract representing, respectively, 57% and 21% of the total anthocyanins (**Table 2**). The confirmation of the identification of anthocyanins was performed by LC-MS/MS (data not shown).

Table 2 – HPLC-DAD analysis: phenolic content of the fresh red raspberry fruit and extract (Values are expressed as means of three replicates ± standard deviation)

Phenolic compound	mg/100 g of Fresh Raspberry Fruit	mg/mL of Fresh Red Raspberry Extract
Gallic acid	2.40 ± 0.10	0.12 ± 0.005
Cyanidin-3-sophoroside	2.58 ± 0.08	0.32 ± 0.004
Cyanidin-3-glucoside	2.44 ± 0.05	0.12 ±0.003
Pelargonidin-3-sophoroside	1.00 ± 0.1	0.05 ± 0.005
Pelargonidin-3-glucoside	0,05 ± 0.01	0.003 ± 0.001
Cyanidin-3-rutinoside	1.12 ± 0.1	0.06 ± 0.005
Pelargonidin -3-glucosyl-rutinoside	0.06 ± 0.01	0.003 ± 0.001
Pelargonidin-3-rutinoside	0.15 ± 0.05	0.008 ± 0.003
Ellagic Acid	1.80 ± 0.01	0.09 ±0.001

Overall, the phenolic profile of the fresh red raspberries extract analyzed by HPLC-DAD is according to data available in the literature.[9,20,21] Samples of frozen raspberries used in the preparation of the extract had 16.77 ± 1,60 mg of ascorbic

acid/100g of fruit. Vitamin C was also present in the raspberry fruit extract despite the extraction procedure used in obtaining this extract. The concentration of ascorbic acid analyzed was 0.43 ± 0.052 mg of ascorbic acid/ ml of raspberry extract (1 mL of extract was obtained from 5 g of fruit so there was a loss about 50% ascorbic acid with the extraction process). Red raspberries fruits are known to be rich in vitamin C. According to published studies, the ascorbic acid content of fresh red raspberries ranged from 16.4 to 36.0 mg of ascorbic acid/100g of fresh raspberries [13,22], but the freezing process slightly affected the values of extracted ascorbic acid content, about 20-55% depending of the frozen storage time.[23]

On another note, there is a large amount of evidence that the production of reactive oxygen species, such as hydrogen peroxide, superoxide anion, and hydroxyl radicals, at the site of inflammation contributes to tissue damage. From the results presented so far, one can clearly identify red raspberry as a fruit abundant in natural antioxidants. Ellagitannins, anthocyanins, and vitamin C are the main individual antioxidant in raspberry.[17,21] According to Beekwilder *et al*.[17] these constituents were accounted for, respectively, 54%, 25% and 17% of the total antioxidant capacity of a ripe red raspberry (cv. 'Tulamine'). Borges *et al*.[20] published similar data. Several investigators have reported lists of values of antioxidant activity related to various foods, from different sources, using different methodologies. Wu *et al*.[24] reported values of the antioxidant activity evaluated by ORAC and HORAC assays of over 100 different kinds of foods, including fruits, vegetables, nuts, dried fruits, spices, cereals, infant, and other foods commonly consumed in the USA. Of all 35 fruits analyzed raspberry had the third highest value for ORAC (160 µmol of Trolox equivalents/100g of red raspberry fruit) and the seventh highest value for HORAC (4765 µmol of trolox equivalents/100g of red raspberry fruit). The antioxidant activity was evaluated by ORAC and HORAC assays. Results were, respectively, 1277 ± 38 µmol of Trolox equivalents/100g of fresh red raspberry fruit (63.9± 1.9 µmol of Trolox equivalents/mL of fresh red raspberry fruit extract), and 529 ± 33 µmol of Caffeic Acid equivalents/100g of fresh red raspberry fruit (26.5 ± 1.7 µmol of Caffeic Acid equivalents / mL of fresh red raspberry fruit extract). A cellular antioxidant activity (CAA) assay for quantifying the antioxidant activity of phytochemicals, food extracts, and dietary supplements is a more biologically relevant method than the chemistry antioxidant activity assays because it accounts for some aspects of uptake, metabolism, and location of antioxidant compounds within cells. Here a CAA of 78 µmol of Quercetin equivalents/ 100g of fresh red raspberry fruit (3.9 µmol of Quercetin equivalents/ mL of fresh red raspberry fruit) was determined. From a more mechanistic standpoint 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 red raspberry fruit extract (1.1-35 µg/mL) on human neutrophil oxidative burst an IC50 of 4.3 ± 1.1 µg/ml was determined (**Figure 1**). In both cases the extract demonstrated not only the ability to act as a potent intracellular antioxidant but also to interfere with neutrophils' function, inhibiting the release of deleterious reactive oxygen species that would amplify the inflammatory signals already triggered.

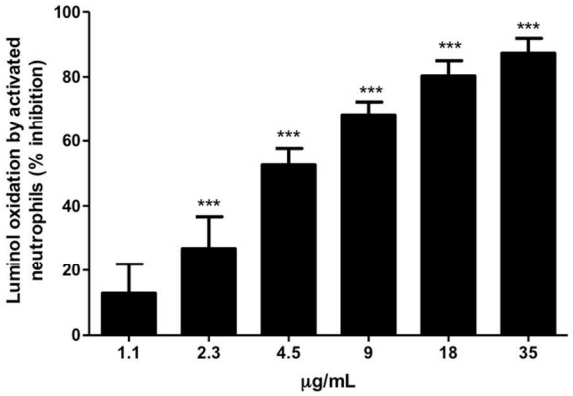

Figure 1. Inhibitory effect of raspberry (1.1-35 µg/mL) on human neutrophils' oxidative burst. Neutrophils' were stimulated with phorbol myristate acetate, as measured by luminol-amplified chemiluminescence. ***p<0.001 compared with the control assay (PMA alone). The values are given as the mean ± SEM (n≥4).

The carragenaan induced-inflammation of the hind paw has been used as a model to study anti-inflammatory activity of several antioxidants such as flavonoids and lycopene.[25,26] It has been reported that the early phase of carrageenan induced inflammation is related to the production of leukotrienes, histamine, platelet-activating factor and cyclooxygenase products, while the delayed phase is linked to neutrophil infiltration and the production of neutrophil-derived reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, as well as the release of other neutrophil-derived mediators.[25,27] The proposed mechanism of action for the extract would be able to explain the significant reduction of oedema formation (inflammatory response) observed 6 h after the administration of the phlogistic agent in this model. After 6h of λ□carrageenan administration, all treated groups showed statistically significant reduction of the paw oedema when compared with the carrageenan group (**Figure 2**). After 6 h, animals pre-treated with the extract showed no oedema development when compared to other groups and were comparable to normal rats. Animals treated with indomethacin and trolox showed a significant reduction of oedema development but nevertheless at a lower extent in comparison to the group administered with the red raspberry fruit extract.

The observed effect was of higher magnitude than the anti-inflammatory effect observed for a classic cyclooxygenase inhibitor such as indomethacin and a classic potent antioxidant such as trolox. It should also be considered that hydroperoxides are known signaling molecules. Redox active enzymes such as glutathione peroxidases, the thioredoxin system and/or peroxiredoxins thereof regulate the concentration and function. Expression of COX2 and iNOS is regulated by redox reactions, as is the whole inflammatory process. The Nrf2/Keap1 system is a key regulator of this system. In the carrageenan paw oedema model, treatment with the red raspberry extract started before carrageenan paw edema was induced, which might have activated this system.

This model is closely related to some of the alterations observed in the collagen-induced arthritis (CIA) in rats. CIA developed rapidly in rats immunized with CII. Periarticular erythema and

oedema were first observed in the hind paws between after day 21 post-challenge. A 100% incidence of CIA was observed by day 23 in all CII-immunized rats. No clinical signs of CIA were observed in rat fore paws during the 35-day evaluation period. Hind paw erythema and swelling increased in severity and time-dependent manner (data not shown) with a maximum arthritis index observed till day 35. Treatment with the extract both orally and intraperitoneally was able to significantly reduce oedema formation in the hind paws (**Figure 3**) when compared to untreated animals.

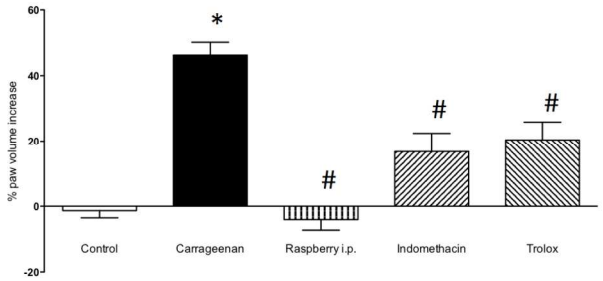

Figure 2. **Effects of red raspberry fruit extract on carrageenan-induced paw volume increase.** Effect of a single administration of red raspberry fruit extract (15 mg/kg, i.p.; *n*=5) on rat paw oedema development elicited by carrageenan 6 h after induction and comparison with the effect of indomethacin (10 mg/kg, i.p.; *n*=6) and trolox (30mg/kg, i.p.; *n*=6). The administration of the extract significantly inhibited rat paw oedema formation and the reduction was in significantly higher magnitude regarding the effects of trolox and indomethacin. The data are presented as mean ± SEM. *P<0.001 vs. control group; #P<0.001 vs. carrageenan group.

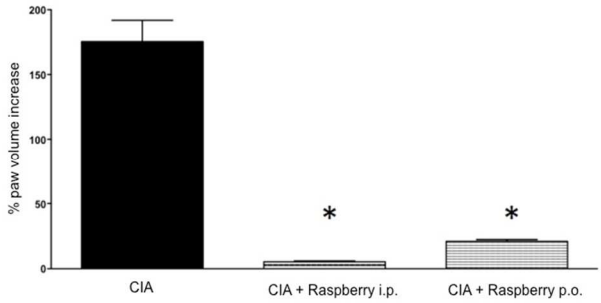

Figure 3. **Effects of red raspberry fruit extract on CIA associated paw volume increase on day 35.** Effect of treatment with red raspberry fruit extract (15 mg/kg, both i.p. and p.o.; *n*=5 per group) on rat paw oedema development associated to CIA (as seen on CIA group). Treatment with the extract significantly reduced paw oedema. Results are presented as mean ± SEM. *P<0.001 vs. CIA group.

On day 35, the histologic evaluation revealed that specimens from a non-treated control rat exhibit normal synovium, no cartilage damage and no marginal zone pannus or bone resorption. Samples from a vehicle-treated CIA rat exhibit severe synovitis, cartilage damage, marked infiltration and pannus, and bone resorption. Specimens obtained from animals treated *per os* and i.p. exhibit normal synovium, small cartilage damage, and reduced marginal zone pannus or bone resorption (**Figure 4A and 5**). When considering the radiographic examination of the hind paws 35 days after immunization with CII revealed bone matrix resorption and osteophyte formation at the joints (**Figure 4B and 6**). There was no evidence of CIA pathology in control (normal) rats. Radiographic examination confirmed that the red raspberry extract (administered by both routes) was able to markedly reduce the degree of bone resorption, soft tissue swelling and osteophyte formation, improving articular function in treated animals.

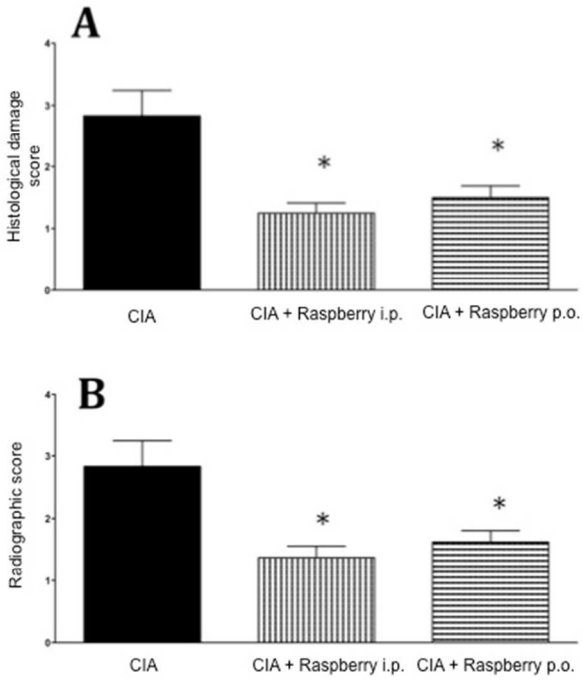

Figure 4. Effect of raspberry extract treatment (i.p. and p.o.) on the histologic damage score (A) and the radiographic score (B) in animals with collagen-induced arthritis (CIA). The values presented are the mean and standard error of the mean. *p<0,001 versus vehicle-treated (CIA).

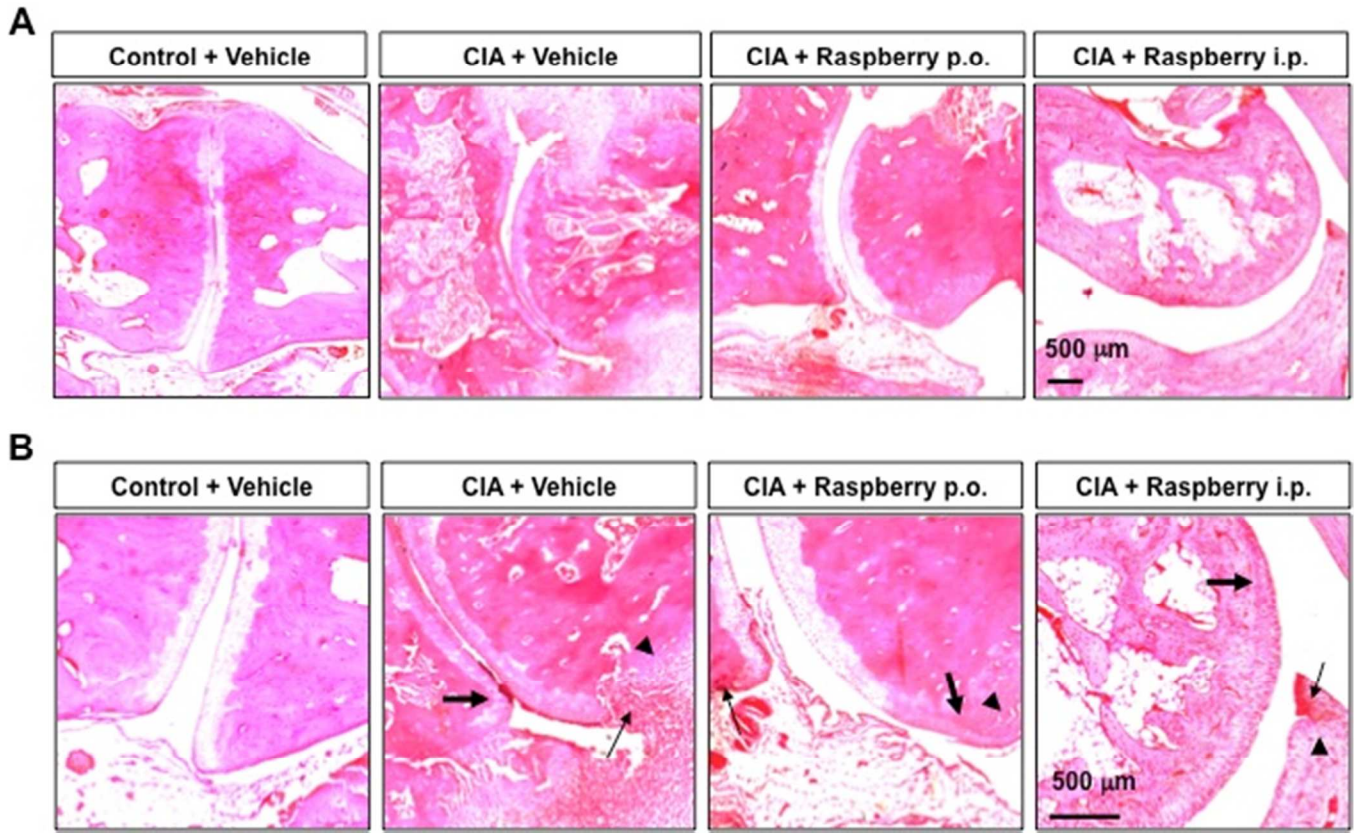

Figure 5. Histopathology findings (A) and close ups (B) regarding the effects of the red raspberry fruit extract in a rat CIA model. Rats were treated with the extract (15mg/kg, p.o or i.p., *n*=5 per group). Specimens from a non-treated control rat exhibit normal synovium, no cartilage damage and no marginal zone pannus or bone resorption. The specimen from a vehicle-treated CIA rat exhibits severe synovitis, cartilage damage (**large arrow**), and marked infiltration and pannus (**small arrow**) and bone resorption (**arrow head**). Specimens obtained from animals treated *per os* and i.p. exhibit normal synovium, small cartilage damage (**large arrow**), and reduced marginal zone pannus (**small arrow**) or bone resorption (**arrow head**). Scale bar equals 500 µm. Original magnification x100.

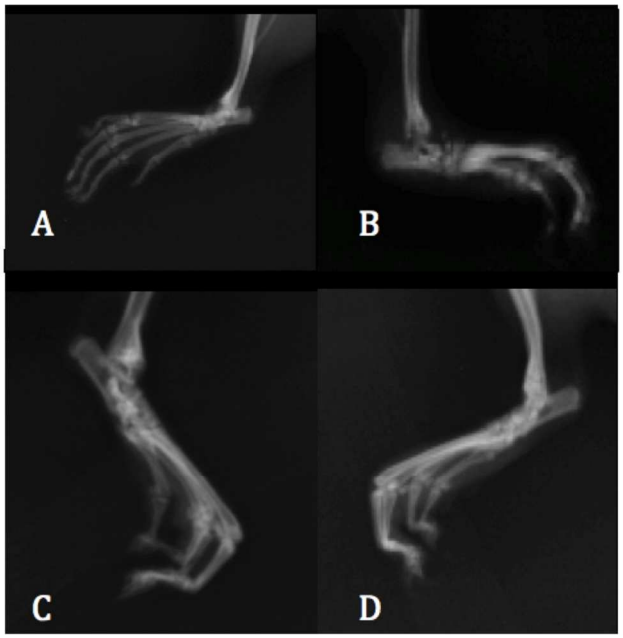

Figure 6. Radiographic progression of CIA in the tibiotarsal joints. (A) There is no evidence of pathological alterations in the tibiotarsal joints of control (normal) animals. **(B)** The hind paws of rats with CIA at day 35 demonstrated bone resorption and quite significant joint erosion. Treatment with the red raspberry fruit extract (15mg/kg) administered both *per os* **(C)** and i.p. **(D)** significantly suppressed the joint pathology and the soft tissue oedema in the hind paw. The X-ray images are representative of at least 3 experiments performed on different experimental days.

Our results show that macroscopic evidence of CIA first appeared as periarticular erythema and oedema of the hind paws. The incidence of CIA was 100% by day 23 in the challenged 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. Treatment of rats with the extract (15 mg/kg/day) both *per os* and intraperitoneally starting at the onset of arthritis (day 23) delayed the development of the clinical signs on days 24–35 and improved the histologic and radiographic scores of the knee joint and hind paw. The inflammatory signalling net is significantly attenuated with the proposed treatment, and there is even no significant production or release of pro-inflammatory cytokines

that would induce COX-2 expression (**Figure 7**) and there is also an attenuation in the production of nitric oxide, has concluded by the reduction of iNOS expression in samples collected from animals treated with the red raspberry extract (**Figure 8**). In addition, 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, namely, it is a well known fact that neutrophil-derived hypochlorous acid plays an important role in cartilage destruction during rheumatoid arthritis[28]. Considering the previously discussed inhibition of neutrophils oxidative burst (enhanced by the red raspberry extract; **Figure 1**), this effect also supports the observed effects under this experimental conditions, by modulating the release of pro-inflammatory neutrophil-derived products.

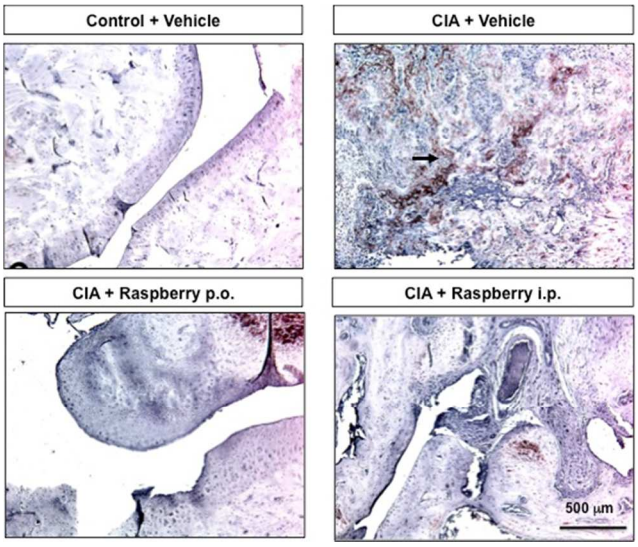

Figure 7. Red raspberry extract administration reduces COX2 activation in the CIA model. Rats were treated with the extract (15mg/kg, p.o or i.p., *n*=5 per group) and samples immunostained for COX2. Specimens from a non-treated rat exhibit almost no expression of COX2 while vehicle-treated arthritic mice exhibits massive production of COX2, namely near the infiltrates (brown staining - **large arrow**). Specimens from animals treated with the red raspberry fruit extract (15mg/kg) administered both *per os* and i.p. exhibit a reduced infiltration and COX2 staining (original magnification x100). Scale bar equals 500 µm.

Also, serum biochemical markers analysis to detect liver (AST and ALT), kidney (creatinine and urea) and neuromuscular (CK) injury/dysfunction, in animals subjected to a repeated administration of the extract as seen during the collagen induced arthritis experimental protocol, demonstrated the clear safety profile of this extract with no significant effects identified on these biochemical markers of injury in all experimental groups. In addition to the work of Jean-Gilles and collaborators[27], we definitely demonstrate here complete and consistent data collection regarding the effects of a red raspberry chronically administered in inflammation and rheumatoid arthritis. Our work hereby presented pursuits the previously published attempt to raise awareness for the pharmacological actions of this extract. Jean-Gilles and collaborators[27] looked at different endpoints and more into the *in vitro* actions of a similar, but not identical, extract over cartilage protection and used a similar animal model. We collected different data, providing a mechanistic insight for the pharmacodynamic actions of this extract, including immunohistochemistry characterization and effect of the extract on pro-inflammatory proteins expression and also on oxidative neutrophil burst. Our works also provides evidence that lower doses of the extract reduce inflammation (assessed by the carrageenan paw oedema model) and has a significant effect in the progression of arthritis. Contrariwise to the doses described previously in the literature[27], the effects observed in the current work can be translated to practice or to a pharmaceutical dosage, when formulated as a supplement, considering the average consumption of raspberry in the population.

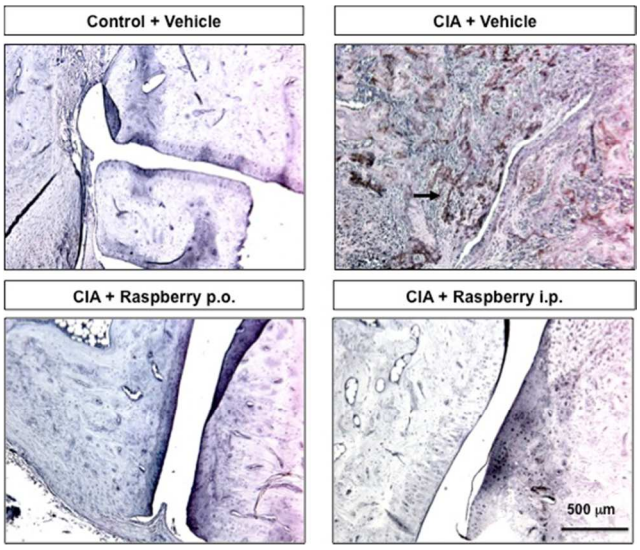

Figure 8. Red raspberry extract administration reduces iNOS activation in the CIA model. Rats were treated with the extract (15mg/kg, p.o or i.p., *n*=5 per group) and samples immunostained for iNOS. Specimens from a non-treated rat exhibit almost no expression of iNOS while vehicle-treated arthritic mice exhibits massive production of iNOS, namely near the infiltrates (brown staining - **large arrow**). Specimens from animals treated with the red raspberry fruit extract (15mg/kg) administered both *per os* and i.p. exhibit a reduced infiltration and iNOS staining (original magnification x100). Scale bar equals 500 µm.

Experimental

Materials, solvents and reagents. Gallic acid (98%), sulphuric acid (95–97%) and Luminol were purchased from Fluka (Seelze, Germany). Sodium hydroxide (98%), calcium chloride dihydrate, magnesium sulfate and sodium hydrogencarbonate were purchased from Merck (Darmstadt, Germany). Chloride acid (0.1M), absolute ethanol (99.9%), methanol (99.9%), hydrochloric acid, glacial acetic acid, 99%, and sodium acetate anhydrous 99%, were purchased from Carlo Erba Reagents (Rodano, Italy). Cyanidin-3-O-glucoside was purchased from Extrasynthese (Lyon Nord, France). Tannic acid was purchased from BDH Laboratory reagents (Poole, England). Phosphoric acid p.a (85%) and ascorbic acid were purchased from Panreac Química (Barcelona, Spain). Acetonitrile HPLC gradient grade was purchased from VWR® (Leuven, Belgium). Milli-Q® water (18.2 MΩ.cm) obtained in a Millipore – Direct Q3 UV System equipment (Molsheim, France). Potassium chloride was obtained

from Pronalab (Abrunheira, Portugal), sodium chloride and Sodium salt of 2,6-dichlorophenol indophenols, 90%, from Riedel-de Haën (Hanover, Germany). All other reagents were purchased from Sigma-Aldrich (St. Louis, USA).

Preparation of the red raspberry (*Rubus idaeus* L.) fruit extract. For the present study, red raspberries from unknown cultivar produced in Portuguese territory were used. The red raspberry fruit extract was prepared according to the method described by Mullen *et al.*[11] with some modifications. Briefly, approximately 40 g of red raspberries were macerated with 90 mL of methanol: acetone (50%, v/v) solution (extractant solution), in an ice-cold pestle and mortar. After 10 min, the homogenate was centrifuged at 7000 rpm for 20 min at 4 °C, and the supernatant was removed. The extraction was repeated using 70 mL of extractant solution and the combined supernatants were mixed, filtered and evaporated at 35 °C until almost to dryness. Finally, the extract was made up with Milli-Q® water to at least 5g raspberries/mL, to comply with intraperitoneal and oral gavage administration. The extract obtained was subdivided into aliquots and stored in falcon tubes at -20 °C, prior to analysis.

Determination of total phenolic content. This determination was performed according to Stamatakis *G et al.*[29] with some modifications. Briefly, 0.1 mL of Folin-Ciocalteu reagent was added to 3.5 mL of the diluted extract (1:4000). After 3min, 0.4 mL of sodium carbonate solution (35% w/v) was added and a colorimetric reaction occurred. The previous mixture remained at rest one hour and after this period absorbance of the blue coloured solution was measured at 725 nm. Gallic acid was used as a standard and total phenolic content was calculated using a calibration curve (1–6 mg/L). The results were expressed as milligrams of gallic acid equivalents (mg GAE) per 100 g of fresh fruit and per mL of extract. Samples were analysed in triplicate.

Determination of total flavonoid content. Total flavonoid content was determined in the diluted extract (1:250) as described by Çam M *et al*.[30] Catechin was used as a standard and to calculate flavonoids content a calibration curve ranging from 20–100 mg/L was prepared. Absorbance of the pink coloured solutions was measured at 510 nm and results were expressed in mg of catechin equivalents (mg CE) per 100 g of fresh fruit and per mL of extract. Samples were analysed in triplicate.

Determination of total anthocyanins content. Total anthocyanins content was determined in the diluted extract (1:20) as described previously.[29] Cyanidin-3-*O*-glucoside was used as external standard and total anthocyanin content was calculated using a calibration curve (10 – 150 mg/L). Final absorbance was determined by the difference between the measured reference absorbance and the measured sample absorbance. For each anthocyanin, a correction factor of molecular weight (CFMW), was used, as described by Chandra *et al.*[31] The results were expressed as milligrams of cyanidin-3-*O*-glucoside equivalents (mg CGE) per 100 g of fresh fruit and per mL of extract. Samples were analysed in triplicate.

Determination of total anthocyanins content. The method described by Çam M *et al.*[30] Tannic acid was used as a standard and total hydrolysable tannins were determined using a calibration curve ranging from 300 to 1700 mg/L and per mL of extract. The results were expressed as milligrams of tannic acid equivalents (mg TAE) per 100g of fresh fruit. Samples were analysed in triplicate.

Determination of total hydrolysable condensed tannins content. The method described by Çam M *et al.*[30] Tannic acid was used as a standard and total hydrolysable tannins were determined using a calibration curve ranging from 300 to 1700 mg/L and per mL of extract. The results were expressed as milligrams of tannic acid equivalents (mg TAE) per 100 g of fresh fruit. Samples were analysed in triplicate.

Determination of total condensed tannins content. Determined in the diluted extract (1:10) using the vanillin assay according to method described by Çam M *et al.*[30] with modifications. Catechin was used as a standard. The calibration curve ranged between 16.7 to 166.5 mg/L. The results were expressed as milligrams of catechin equivalents (mg CE) per 100 g of fresh fruit and per mL of extract. Samples were analysed in triplicate.

Determination of ascorbic acid content. The ascorbic acid content in the sample was determined using a spectrometric method, as described in the Portuguese standard NP-3030[32] with some modifications. Briefly, 2 mL of the extract (10g of fresh fruit) were homogenized in a 100 mL flask with a 3% solution of metaphosphoric acid. 3 mL of this sample solution, 3 mL of a buffer solution of sodium acetate and glacial acetic acid and 1 mL of the dye solution, 2,6-dichlorophenol indophenol, were added to a 50 mL centrifuge tube. 10 mL of xylene were mixed to the previous and stirred vigorously for 6 seconds. The mixture was centrifuged at 3000 g for 3 minutes and the absorbance of the organic supernatant was read at 500 nm in a UV-Vis spectrophotometer, using xylene as a reference. The dye's volume consumed in this reaction was obtained from the calibration curve absorbance vs. volume of the dye solution. The calibration curve ranging from 150-1000 mg of ascorbic acid/L, was prepared following the procedure described above. The ascorbic acid content of the sample was expressed in mg of ascorbic acid per 100 g raspberries. Samples were analysed in triplicate.

Analysis of the red raspberry extract by HPLC-DAD. HPLC analysis was carried out using a HPLC system from Thermo Finnigan (Surveyor model) equipped with a diode-array detector (DAD) and an electrochemical detector (Dionex, ED40). Chromatographic separation was carried out on a Lichrocart RP-18 column (250 x 4 mm, 5 µm, Merck) at 35ºC. Diode array detector (DAD) was programmed for scanning between 192 and 798nm. The injection volume was 20 µL. For chromatographic separation, the mobile phase was pumped at a flow rate of 0.7 mL/min and consisted of Milli-Q® water: phosphoric acid (99.9:0.1,v/v), (Eluent A), and Milli-Q® water:acetonitrile:phosphoric acid (59.9:40.0:0.1, v / v / v), (Eluent B). The gradient was 0–15 min from 0% to 20% of eluent B; 10 min with 20% eluent B; 25–70 min, from 20% to 70% eluent B; 70–75 min, with 70% of eluent B; 75–85 min from 70% to 100% eluent B; 85–90min, with 100% eluent B; 90–95min from 100% to 0% of eluent B; and 95–100 min 100% of eluent A. The acquisition and treatment of data were performed using Chromquest Software version (Thermo Finnigan–Surveyor, San Jose, CA, USA). Identification of compounds was done by comparing retention time, spectra and spiking samples with known amounts of pure standards, whenever available.

Oxygen radical absorbance capacity (ORAC). The method of Huang *et al.*[33] modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Feliciano *et al.*[34] was used. This assay measured the ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from AAPH. All samples, including the blank and the controls, were analysed as six replicates. Final ORAC values were calculated by a regression equation between the trolox concentration and the net area under the FL decay curve. All data were expressed as micromoles of trolox equivalents antioxidant capacity (TEAC) per 100 g of fresh raspberry weight.

Hydroxyl radical adverting capacity (HORAC). The method developed by Ou *et al.*[35] modified for the FL800 microplate fluorescence reader as described by Serra *et al* [36] was used. Caffeic acid was used as a standard as it provides a wider linear range as compared to gallic acid. Data was expressed as mmol or µmol of caffeic acid equivalents antioxidant capacity (CAEAC) per 100 g of fresh raspberry weight. Results are a mean of six replicates.

Cellular antioxidant activity (CAA). Human colon carcinoma Caco2 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and were grown in RPMI 1640 medium supplemented with 10%FBS and 2mM of glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC with 5% CO_2 in a humidified atmosphere. For CAA assay, Caco2 cells were seeded at a density of 2×10^4 cell/well (determined by trypan blue exclusion assay) on 96-well plate in 100 µl of growth medium. The medium was changed every 48 hours and the experiments were performed when cells reach confluence (≈72 hours). After that, medium was removed and cells were washed twice with PBS. Triplicate wells were treated for 1 h with 100 µl of different concentrations of raspberry extracts plus 25 µM DCFH-DA diluted in PBS. Then, medium was removed and replaced by PBS containing 600 µM AAPH. The 96-well microplate was placed into a fluorescence reader (FL800, Bio-Tek Instruments, Winooski, VT, USA) at 37ºC. Emission at 530 ± 25 nm was measured after excitation at 485 ± 20 nm every 5 min for 1 h. Each plate included triplicate control wells (cells treated with DCFH-DA and oxidant, namely AAPH) and blank wells (cells treated with DCFH-DA without oxidant). Quercetin was used as a standard. Cellular antioxidant activity (CAA) of extracts was quantified according to Wolfe and Lui [37]. The EC_{50} values were stated as mean ± SD for triplicate sets of data obtained from the same experiment. EC_{50} were converted to CAA values, expressed as micromoles of quercetin per g of raspberry, using the mean EC_{50} value for quercetin from three independent experiments.

Evaluation of neutrophil oxidative burst. Isolation of human neutrophils was performed by gradient density, as previously reported by Freitas *et al.*[38] The obtained cell suspensions contained more than 99% of neutrophils and the control of their viability showed more than 95% of the cells excluding trypan blue solution 0.4%. Isolated neutrophils were kept in ice until use. Tris glucose (25 mM Tris, 1.26 mM $CaCl_2.2H_2O$, 5.37 mM KCl, 0.81 mM $MgSO_4$, 140 mM NaCl, and 5.55 mM D-Glucose) was the incubation media used. The chemiluminescent probe luminol has been thoroughly studied and used for monitoring the production of reactive species by neutrophils, namely the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$) and peroxynitrite anion ($ONOO^-$).[39] The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring ROS-induced oxidation of luminol, according to a previously described procedure.[40] The reaction mixtures contained neutrophils ($1x10^6$cells/ml) and the following reagents at the indicated final concentrations (in a final volume of 250 µl): tested compounds at various concentrations, luminol (500 µM) and phorbol myristate acetate (PMA; 160 nM). Cells were pre-incubated with luminol and the tested compounds for 5 minutes before the addition of PMA and the measurements were carried out at 37ºC, under continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve. This peak was observed at around 10 minutes. Effects are expressed as the percent inhibition of luminol oxidation. Each study corresponds to, at least, four individual experiments, performed in triplicate in each experiment. Neutrophils' viability was assessed by the trypan blue assay after exposure to the tested compounds. Cell viability was maintained over 98%, after 1 h of exposure to the different extracts, at the maximum concentration tested.

Animal care and maintenance for the in vivo experiments. Experiments were conducted according to the Home Office Guidance in the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London, UK and the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), as well as to the currently adopted EC regulations. Finally, the studies are in compliance with the ARRIVE Guidelines for Reporting Animal Research' summarised at www.nc3rs.org.uk. Hence, the Ethics Committee of the Research Institute endorsed the animal study protocol, considering also that authors Sepodes and Rocha, are licensed by the Portuguese General Directorate of Veterinary to coordinate and conduct independent animal research. All studies were carried out using male Wistar rats with 5 weeks of age weighing 100–150g (Harlan Iberica, Barcelona, Spain). All animals received a standard diet and water *ad libitum*.

Carrageenan-induced paw oedema. The carrageenan-induced paw oedema of the rat hind paw is a suitable model to study acute local inflammation and widely considered to be one of the most useful models in the evaluation of anti-inflammatory activity.[26] Paw oedema was induced by a single sub-plantar injection into the rat left hind paw of 0.1ml of a 1% λ-carrageenan sterile saline solution. Paw volume was measured by means of a volume displacement method using a plethysmometer (Digital Plethysmometer LE7500; Letica Scientific Instruments, Letica, Spain). Paw volume was measured immediately after the injection of carrageenan (V_0 or basal volume) and 6h later (V_{6h}). The increase in paw volume was taken as oedema volume. Animals were randomly allocated into the following groups as described: (*i*) Control group: animals were subjected to subplantar injection into the rat left hind paw of 0.1 mL sterile saline and administered with saline (1 ml/kg, i.p.) (*n*=6); (*ii*) Carrageenan group: animals subjected to paw oedema induction and administered with saline (1 ml/kg, i.p.) (*n*=6); (*iii*) Raspberry i.p. group: animals subjected to paw oedema induction and pre-treated with red raspberry fruit extract (15mg crude extract/kg, i.p.) 30 minutes before λ-carrageenan injection (*n*=5); (*iv*) Indomethacin group: animals subjected to paw oedema induction and pre-treated with indomethacin (10mg/kg, i.p.) 30 minutes before λ-carrageenan injection (*n*=6); (*v*) Trolox group: animals

subjected to paw oedema induction and pre-treated with trolox (30mg/kg p.o.) 30 minutes before λ-carrageenan injection (*n*=6).

Collagen-induced arthritis in Wistar rats. Bovine CII was dissolved in 0.01M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at -70°C until used. Complete Freund's adjuvant (FCA) was prepared by the addition of *Mycobacterium tuberculosis* H37Ra at a concentration of 2mg/ml. Before injection, CII was emulsified with an equal volume of FCA. CIA was induced as previously described.[4,41] On day 1, all rats were administered intradermally at the base of the tail with 100µl of the emulsion (containing 100µg of CII). On day 21, a second injection of CII in FCA was administered. In treated groups, animals (*n*=5 per group) were treated with red raspberry fruit extract (15 mg crude extract/kg/day intraperitoneally and *per os*) every 24 hours, starting on day 23. Rats were evaluated daily for arthritis.[26] 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 – Prearalytical Systems, Oxford, UK)] and were centrifuged (3000 rpm for 10min at room temperature) to separate serum, to be analysed by a Cobas Analyzer Roche® Automated system. Alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury) and aspartate aminotransferase (AST, a non-specific marker for hepatic injury) were measured to evaluate the liver function. Creatine Kinase (CK) was measured to evaluate the neuromuscular injury. Urea and creatinine were measured to evaluate the renal function.

Histologic (light microscopy) assessment of arthritis. On day 35, animals were sacrificed while under anaesthesia, and the paws and knee joints were removed and fixed in formalin for light microscopy examination. Knee joint tissue samples were obtained at the end of the experimental period and were fixated in a solution of paraformaldehyde in PBS (10% v/v), at room temperature. Before dehydration and inclusion in paraffin blocks, samples were decalcified with formic acid (10%) for a minimum of 8 days. Samples were dehydrated using ethanol gradients and included on paraffin blocks, following sectioning at 3µM. All sections were deparaffinized with xylol and hydrated in progressively less concentrated solutions of ethanol, ending with distilled water. Afterwards, they were stained with hematoxilin/eosin, dehydrated, diphanized and placed on a mount fluid. All sections were observed with an optical microscope with the 10x and 40x lens and photographed. For the light microscopy analysis, an optical microscope Zeiss, a photographic camera Leica DFC490, and an images capture software Leica IM50 were used. 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 post-fixed in 4% PFA in PBS for 72 h 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-eosin staining. For immunostaining, 6 mm sections were submitted to antigen retrieval in 20 mM citrate buffer with 1.5% H_2O_2 for 15 min at RT in the dark, incubated for 10 min in Tris/EDTA buffer at 84°C and blocked for 1 h at RT in 1% bovine serum albumin (BSA) in PBS. Primary antibodies, rabbit anti-COX-2 (Cell Signaling #4842, 1:100) and mouse anti-iNOS (BD Transduction Laboratories #610328, 1:100) were used in 0.5% BSA in PBS overnight at 4°C. After washing in PBS, sections were incubated for 1 h at RT with antibodies anti-rabbit and anti-mouse coupled with horseradish peroxidase (Santa Cruz Biotechnology, 1:5000) in 0.5% BSA in PBS, incubated for 10 min in SIGMAFAST™ DAB with Metal Enhancer (SIGMA) and mounted with EntellanÒ (Merck). Tissue sections were visualized in an Axioskop bigthfield microscope.

Radiographic analysis. Animals were anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneally) and placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiography of normal and arthritic rat hind paws was performed using an X-ray machine, with a 40-kW exposure for 0.01 seconds. An investigator who 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. Statistic treatments were done using GraphPad PrismTM (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 as statistically significant.

Conclusions

Our results are in line with the current understanding on the biological effects of polyphenols and other antioxidant compounds contained in red raspberry fruit and their relevance to human health. The effects observed that can be related with antioxidant activity of the extract can be due to the phenolic composition but also to the presence of ascorbic acid. However, the doses usually used to inhibit oxidative stress are 10 mg/Kg/day[42] and the animals in our study were administered with only 0.8 mg/Kg/day, a much lower dose. It's our opinion that even if ascorbic acid contributed to the antioxidant activity of the extract, the majority of the observed effect must be related to the phenolic content of the extract. Interestingly, the oral administration of the extract is associated to the same protective effects observed when a parenteral route is used and this is especially important when translating to the clinical use of this extract. Here we provide the mechanistic justification and reasoning for the potential of a diet supplemented with a standardized red raspberry fruit extract or similar quantities of the fresh fruit, to provide protection against the development and progression of rheumatoid arthritis, paving the way for further research in this area. In this type of study it's essential to appropriately translate the extract dosage from one animal species to another. When considering the translation from the

non-clinical setting to the human use, the tested dose of 15mg/kg of extract would translate to the equivalent human daily consumption of 150 g of fresh red raspberry fruits as per Reagan-Shaw *et al*.[43] No significant changes in serum markers of organ injuty/dysfunction were observed on day 35 in treated and untreated animals. Although several biological effects based on epidemiological studies can be scientifically explained, a better knowledge of some variables of the extract constituents' bioavailability (such as the kinetics of absorption, distribution and elimination) facilitates the design of future clinical studies. The role of the compounds described in this study in human health is still a fertile area of research. Based on our current scientific understanding, this product could offer a renewed opportunity for the prevention and/or adjunctive treatment of chronic inflammatory human diseases. This study demonstrates that a red raspberry fruit extract with pharmaceutical quality or fresh red raspberry fruits could potentially be used as a supplement or a dietary source of pharmacologically active compounds that may have an active role in delaying the progression of inflammatory articular injury associated to arthritic conditions.

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