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Abstract

Folk medicine suggests that pomegranate (peels, seeds and leaves) has anti-inflammatory properties; however, the precise mechanisms by which this plant affects the inflammatory process remain unclear. Herein, we analyzed the anti-inflammatory properties of a hydroalcoholic extract prepared from pomegranate leaves using a rat model of lipopolysaccharide-induced acute peritonitis. Male Wistar rats were treated with either the hydroalcoholic extract, sodium diclofenac, or saline, and 1 h later received an intraperitoneal injection of lipopolysaccharides. Saline-injected animals (i.p.) were used as controls. Animals were culled 4 h after peritonitis induction, and peritoneal lavage and peripheral blood samples were collected. Serum and peritoneal lavage levels of TNF-α as well as TNF-α mRNA expression in peritoneal lavage leukocytes were quantified. Total and differential leukocyte populations were analyzed in peritoneal lavage samples. Lipopolysaccharide-induced increases of both TNF-α mRNA and protein levels were diminished by treatment with either pomegranate leaf hydroalcoholic extract (57% and 48% mean reduction, respectively) or sodium diclofenac (41% and 33% reduction, respectively). Additionally, the numbers of peritoneal leukocytes, especially neutrophils, were markedly reduced in hydroalcoholic extract-treated rats with acute peritonitis. These results demonstrate that pomegranate leaf extract may be used as an anti-inflammatory drug which suppresses the levels of TNF-α in acute inflammation.

Introduction

Inflammation is a protective biological response that involves blood vessels, immunological cells, and inflammatory mediators [1]. Among the most important inflammatory mediators, tumor necrosis factor-α (TNF-α) plays an important role in inflammation by regulating the release of other mediators. It is also involved in chemotaxis, especially of neutrophils, and induces the expression of a range of receptors that contribute to amplifying the inflammatory response. The excessive and/or chronic release of TNF-α has been implicated in a variety of pathological conditions such as rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, heart valve disease, and sepsis [2]. The treatment of inflammation in these conditions involves the use of anti-inflammatory drugs to minimize damage and improve patient’s quality of life. However, currently marketed anti-inflammatory drugs cause a range of adverse effects, including stomach upset and bleeding as well as serious cardiovascular events [3], in addition to the suppression of the immune system, especially when administered chronically [4]. As an alternative, anti-TNF-α drugs have been remarkably successful [5].

Natural compounds have been widely studied as potential anti-inflammatory drugs in the search of novel therapeutic options that may cause fewer adverse effects [6, 7]. In this context, pomegranate (Punica granatum L., Punicaceae) has been suggested to possess wound-healing, antimicrobial, and antioxidant properties [8-10]. However, its effects on inflammation are rather controversial. In a recent study, Al-Jarallah et al. [11] demonstrated that the administration of pomegranate extract reduces coronary artery atherosclerosis by reducing macrophage infiltration and the levels of monocyte chemotactic protein-1 in the myocardium. In contrast, when administered as an oral supplement pomegranate extract had no effects on pro-inflammatory cytokine production in hemodialysis patients [12]. Therefore, further
scientific studies are necessary to clarify the pharmacological properties of pomegranate extracts. Herein, we investigated the anti-inflammatory activity of a hydroalcoholic extract prepared from P. granatum leaves (HEP), especially in regards of TNF-α production and neutrophil influx, using a rat model of lipopolysaccharide (LPS)-induced acute peritonitis.

Results

The phytochemical screening of HEP showed the presence of coumarins, flavonoids (such as xanthones, flavone, flavonol, and flavanone), and phenolic acids. Other secondary metabolites classes examined were not detected in the HEP. HPLC analysis of the ethyl acetate fraction prepared from HEP showed peaks between 0 and 38 min, and most were well visualized at 325 nm (Fig. 1). The peaks were characterized as follows: peak 1 suggests the presence of 3,3′-di-O-methylellagic acid, peak 5 is related with the presence of kaempferol (3,5,7-trihydroxy-2-[4-hydroxyphenyl]-4H-chromen-4-one), and peak 7 suggests the presence of kaempferol 3-O-glycoside.

We next investigated the anti-inflammatory effects of HEP in rats with acute peritonitis. LPS-induced increases of TNF-α mRNA expression were down-regulated by the administration of HEP (Fig. 2A). The administration of HEP significantly reduced the levels of serum and peritoneal TNF-α protein, whereas HEP-treated rats with acute peritonitis presented with lower levels of serum and peritoneal TNF-α in comparison with vehicle-treated rats.

The influence of HEP administration on leukocyte recruitment, especially neutrophils, was evaluated (Fig. 3). As expected, rats with LPS-induced acute peritonitis exhibited higher numbers of peritoneal leukocytes (Fig. 3A), especially neutrophils (Fig. 3B), when compared with the control group. Interestingly, the oral administration of HEP inhibited neutrophil recruitment from the blood into the peritoneal cavity. HEP-treated rats with acute peritonitis had lower numbers of neutrophils in their peritoneal cavity than those treated with vehicle. Likewise, treatment with sodium diclofenac inhibited neutrophil recruitment to the peritoneum in rats with acute peritonitis.

Discussion

Over the last decade, different studies have reported the presence of different secondary metabolites in diverse anatomical compartments of pomegranate [8]. This study demonstrated that HEP contains flavonoid compounds. Interestingly, flavonoids have a wide range of biological and pharmacological activities, including antiinflammatory, antioxidant, antimicrobial (antibacterial, antifungal and antiviral), anticancer, and antidiarrheal activities, in addition to anti-inflammatory effects [8, 13]. Some flavonoids, such as kaempferol, luteolin, apigenin, and quercetin, were previously suggested as anti-inflammatory agents for their ability to modulate immune cells and inhibit the production of proinflammatory cytokines [14, 15]. In the current study, the main bioactive compound was kaempferol, strongly suggesting an anti-inflammatory property for pomegranate HEP.

Although there are reports showing that pomegranate peel and seed extracts present with anti-inflammatory actions, very little is known of the effects pomegranate leaf extracts may have. In this context, the anti-inflammatory effect of pomegranate leaves...
was described in a mouse model of asthma [16]. However, the mechanisms by which pomegranate leaves affected the inflammatory process were not clear. Here, by using a rat acute peritonitis model, we demonstrated that pre-treatment with HEP produced from pomegranate leaves reduced TNF-α mRNA and protein levels in LPS-injected rats. Interestingly, anti-TNF-α drugs are alternatives to existing anti-inflammatory drugs because they are more effective and cause fewer side effects. Therefore, medications prepared from medicinal plants such as pomegranate may be an important source of anti-TNF-α molecules. Previous studies have described the anti-TNF-α activity of pomegranate whereas the anti-inflammatory and analgesic effects of standardized pomegranate rind extract were achieved by modulating the pro-inflammatory cytokines interleukin (IL)-β and TNF-α [17]. Large amounts of TNF-α are primarily produced and released by macrophages in response to LPS and other bacterial products [18]. TNF-α has numerous physiological effects, including on the hypothalamus (causing fever and anorexia). This inflammatory mediator can also stimulate the production of acute phase agents in the liver (IL-6, fibrinogen, and C-reactive protein) and macrophage-mediated phagocytosis. TNF-α is a potent chemoattractant for neutrophils by promoting the expression of adhesion molecules on endothelial cells, thus contributing to neutrophil migration. In the present study, we demonstrated that pomegranate HEP affected neutrophil migration to the peritoneal cavity by decreasing TNF-α levels. These results are in agreement with a previous study which showed that pomegranate rind extract diminishes myeloperoxidase activity (a marker for polymorphonuclear leukocyte infiltration) in a mouse model of croton oil-induced ear edema [17], suggesting this extract may represent a promising phytomedicine for the treatment of inflammatory diseases. In addition, Bachoul et al. [19] revealed that an aqueous extract of *P. granatum* peel inhibits neutrophil-mediated myeloperoxidase activity and attenuates LPS-induced lung inflammation in mice. Herein, we show that the most abundant bioactive molecule in the HEP is kaempferol. The inhibitory effects of this compound on TNF-α production have been previously described. Indeed, Luo et al. [20] showed that the oral treatment with kaempferol reduces the levels of TNF-α, in addition to IL-6, in type 2 diabetic rats. Kaempferol effects on cytokine production may be due to its ability to interfere with mRNA expression. Kim et al. [21] reported that kaempferol decreases NF-κB (p65 and p50) and AP-1 (c-Jun and c-Fos) levels in the nucleus and their transcriptional activity.

Collectively, our data show that HEP from pomegranate leaves are able to suppress TNF-α production in acute inflammation, especially in conditions in which exacerbated levels of TNF-α are deleterious. However, future studies using other fractions of HEP and performing dose-response experiments are necessary to confirm its anti-inflammatory properties.

**Materials and Methods**

**Chemicals**

LPS (*Escherichia coli*, serotype 111:B4), phosphate buffered saline, sodium diclofenac (purity ≥ 98.5%), ketamine and xylazine were obtained from Sigma-Aldrich. SuperScript II reverse transcriptase, TaqMan GenEx Assay (RN01525859_g1 or RN01789812_g1) and TaqMan Universal PCR Master Mix were from Thermo Fisher Scientific and Rat TNF-α immunoassay kit was obtained from R&D System Inc. DNase and RNeasy Mini Kits were obtained from Qiagen. All other chemicals were of analytical grade.

**Plant material**

Fresh pomegranate (*P. granatum*) leaves were collected at the Atico Seabra Herbarium of the Federal University of Maranhão, Sao Luis, Maranhao, Brazil in March 2012. Prof. Therezinha Rego, PhD, Botanical Department of the Federal University of Maranhão, identified the plants, and a voucher specimen was deposited in the collection at the Herbarium (voucher number 01 002).

**Preparation of hydroalcoholic extract from *Punica granatum* leaves and reference drug**

Fresh leaves of the plant were air dried at 40 °C and then ground into a powder. The powder was macerated with a 70% alcohol solution in water at room temperature for 7 d under occasional agitation. The HEP was obtained by concentration using rotary evaporation, dried, and then lyophilized material was stored at −80 °C until use. The HEP was administered at a 250 mg/kg dose after being suspended in vehicle (saline, 0.9% NaCl suspension in distilled water). Sodium diclofenac (10 mg/kg; orally) in vehicle (saline) was used as a reference drug. Test drugs were orally administered in an equivalent volume of 10 mL/kg. The dose of 250 mg/kg was chosen based on pilot studies in which only the 250 mg/kg dose showed significant results in regards of total and differential cell counts (data not shown).

**Phytochemical analysis of the hydroalcoholic extract**

A phytochemical analysis of the HEP was evaluated by analysis of color intensity and/or precipitate formation as previously described [22] for the detection of various classes, such as anthocyanins, anthocyanidins, flavonoids, flavonols, xanthones, chalcones, leucoanthocyanidins, flavanones, catechins, tannins hydrolysable, tannins condensable, phenols, steroids, triterpenes and alkaloids.

**HPLC analysis**

For HPLC analysis, an ethyl acetate fraction was prepared from HEP as previously described [23] and HPLC analysis was performed using a Prominence Shimadzu High Performance Liquid Chromatograph (Shimadzu) equipped with an injector with a 25 μL loop and a UV detector and a Kinetex C-18 column (250 × 4.6 mm, 5 μm; Phenomenex). The compounds of pomegranate extract were separated at room temperature using a gradient elution program at a flow rate of 0.6 mL/min. The mobile phases consisted of Milli-Q water containing 2% acetic acid (A) and methanol (B). The following linear gradient was applied: 0–2 min, 5% B; 2–10 min, 5–25% B; 10–20 min, 25–40%, 20–30 min, 40–50%, 30–40 min, 50–60% B; 40–50 min, 60–70% B, 50–80 min, and 70–100% B. The injection volume into the HPLC system was 25 μL, and DAD detection was performed at 200–500 nm. Before injection into the HPLC system, the extract was dissolved in the same solvent used for extraction (HPLC grade) to obtain a final concentration of about 5 mg/mL and then filtered through a 0.22-μm nylon syringe filter obtained from All sharp. The presence of the compounds was determined using controls obtained from Sigma-Aldrich and based on literature about the phytochemical profile of pomegranate [16].
Animals
Forty-two male Wistar rats with a weight between 180–220 g were used. All animals were obtained from the Animal Facility of the CEUAMA University, Sao Luis, Brazil. They were housed under controlled light (12 h light/12 h dark; lights on at 8 AM) and temperature conditions (23 ± 1°C) with access to water and food ad libitum. All experiments were performed in accordance with the welfare of experimental animals and all experimental protocols were approved by the Ethics Committee of the CEUAMA University (August 09, 2012; #242/2012).

Lipopolysaccharide-induced peritonitis and treatment
To assess the effects of HEP in the inflammation process, 36 animals were randomly divided into 6 groups (6 animals/group). The rats were orally pre-treated with HEP (250 mg/kg), sodium diclofenac (10 mg/kg), or saline (0.9%; 10 mL/kg), and then peritonitis was induced by a single injection (i.p.) of LPS (500 µg/kg, E. coli, serotype 111:B4). Saline-injected animals (0.9%, 10 mL/kg, i.p.) were used as controls. Animals were killed at 4 h after LPS injection following anesthesia with ketamine: xylazine, 120 mg/kg/10 mg/kg, i.p.) and peripheral blood samples were collected by cardiac puncture and the peritoneal lavage was harvested for analysis by injection of 10 mL of phosphate buffered saline on ice containing 0.5% sodium citrate. The abdomens were gently massaged and the blood-free cell suspension was carefully aspirated with a syringe. Abdominal washings were placed into plastic tubes and total cell counts were performed immediately using a Neubauer chamber.

Total and differential leukocyte counts
The total and differential leukocyte population was analyzed immediately in peritoneal lavage samples. The total leukocyte count was evaluated using a Neubauer chamber by using a light microscope after May-Grünwald Giemsa (MGG) type staining. A minimum of 200 leukocytes were counted for differential leukocyte determination.

TNF-α gene expression analysis
TNF-α mRNA expression in peritoneal lavage leukocytes was evaluated from total RNA extracted from samples by an RNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Total RNA was treated with DNase (Qiagen) and then reverse-transcribed with 200 U of SuperScript II reverse transcriptase (Thermo Fisher Scientific). RNA levels were determined by qPCR using a hydrolysis detection system (Thermo Fisher Scientific). The qPCR assays were carried out in 96-well plates using an ABI Prism 7500 SDS (Thermo Fisher Scientific) as previously described [24]. Briefly, qPCR were assayed in 25 µL reaction mixture containing of 2 µL of cDNA, 1 × TaqMan GenEx Assay (RN01 525859_g1 or RN01 789812_g1) and 1 × TaqMan Universal PCR Master Mix diluted in nuclease-free water. Ubiquitin mRNA was used as endogenous reference gene. Relative expression was calculated using the 2–ΔΔCt method.

Determination of TNF-α protein levels
TNF-α protein levels in serum and peritoneal lavage samples were analysed by enzyme-linked immunosorbent assay using a rat TNF-α immunoassay kit (R&D Systems Inc.) according to the manufacturer’s instructions. The optical density of samples was determined at 450 nm in a microplate reader and TNF-α levels were determined from the standard curve. Results were expressed as an absolute concentration (pg/mL).

Statistical analysis
The data obtained were analyzed using GraphPad software v. 6.0 and expressed as the mean ± standard deviation (S.D.). Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. A significant difference was considered when p-values < 0.05.

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Conflict of interest
The authors declare no conflict of interest.

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