Preclinical evaluation of the crude extract from the fruits of *Punica grantaum* L. (*Punicaceae*) for antimicrobial activity in *in vitro* and *ex vivo* experimental models: A comparative study

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Currently, natural products have been evaluated as sources of antimicrobial agents. Considering the increasing use of pomegranate, it has become important to establish a correlation between the phytochemicals and the antimicrobial properties of the crude extract used in Brazilian folklore medicine. The compositional analysis revealed the presence of classes of secondary metabolites of pharmaceutical interest, among them, tannins, flavonoids and phenolic compounds. The *in vitro* study was performed by broth micro-dilution susceptibility assay according to the protocols of the National Committee for Clinical Laboratory Standards and described the antibacterial and antifungal activities of the extract. The preeminent antimicrobial activities were recorded against *Staphylococcus aureus* and *Staphylococcus epidermidis*. In order to estimate the *ex vivo* antimicrobial activity of the extract, serum and granulomatous tissues were separately submitted to microbiological assay. The crude extract did not present any inhibition zone. Thus, it is possible to suggest that after the oral ingestion of the extract, bacteria present in the gastrointestinal tract of the animal, hydrolyze phytocompounds, which present antimicrobial potential in *in vitro* models but do not present sufficient serum and tissue concentrations to exert this activity in *ex vivo* and presumably in *in vivo* models.

**Key words:** *Punicaceae, Punica granatum* L., preclinical, antimicrobial, *in vitro*, *ex vivo*.

**INTRODUCTION**

*Punica granatum* L. (*Punicaceae*) is a large deciduous shrub or small tree which fruit (pomegranate), are common in the Mediterranean and has been therapeutically used as food in Brazil. The fruit is delimited by a leathery pericarp contained within are numerous arils, each as a single seed surrounded by a translucent juice-containing sac. Thin acrid-tasting membranes extend into the interior of the fruit from the pericarp, providing a lattice work for suspending the arils. The fruit itself gives rise to three parts: the seeds, about 3% of the weight of the fruit,
In Ayurvedic medicine, pomegranate has been used for the treatment of a variety of ailments including parasitic infections, diarrhea and ulcers (Jurenka, 2008). The root and stem barks are reported to have astringent effect (Alper and Acar, 2004) and antihelmintic activity (Gracious et al., 2001). The fruit rind is traditionally used in the treatment of a variety of ailments including parasitic infections, diarrhea and ulcers (Jurenka, 2008). The root is traditionally used to treat dysentery (Chopra et al., 1995). The flowers serve as a remedy for diabetes mellitus (Katz et al., 2007). The pharmacological activities of pomegranate include antioxidation (Dikmen et al., 2011), antihepatotoxicity (Kaur et al., 2006), antiplasmodial activity (Alper and Acar, 2004) and antihelmintic activity (Dell’Agli et al., 2009), anticancer (Albrecht et al., 2004) and antimicrobial properties (Reddy et al., 2007).

In the last few years, many important functions of fresh fruits and vegetables have been reported and they are now recognized as being good sources of natural antimicrobial agents (Berk and Tepe, 2013). Polyphenols have been acknowledged to have health beneficial effects, owing to derived products such as tannins, flavonoids and phenolic compounds. According to recent reports, pomegranate is rich in polyphenols, mainly ellagitannins and gallotannins, such as punicalin, punicalagin, pedunculagin, punigluconin, granatin B and tellimagrandin I (Noda et al., 2002). However, the correlation between the phytocompounds and the antimicrobial properties of the crude extract from the fruits of P. granatum L., which is therapeutically used in Brazilian folk medicine has not been investigated. Therefore, this preclinical evaluation aimed to clarify the antimicrobial activity of pomegranate through in vitro and ex vivo models to confirm its potential.

MATERIALS AND METHODS

Plant

Fresh ripe fruits of P. granatum L. were harvested from an orchard located in the municipality of Ribeirão Preto (São Paulo State, Brazil) in June 27th, 2012 and authenticated in the Department of Exact and Earth Sciences of Federal University of São Paulo.

Preparation of the crude extract

Fruits of P. granatum L. were previously washed in running water. Then, they were air dried at 50°C, crushed, milled in a knife mill to obtain 500 g and subsequently subjected to an extraction with water-ethanol solution (5.0 L, 70%) by maceration during seven days. The crude extract was filtered and concentrated on a rotary evaporator unit at 60°C under reduced pressure, furnishing 127 g (yield 25.4%) of the crude extract.

Compositional analysis of the crude extract

The crude extract was preliminarily subjected to a qualitative analysis to detection of the secondary metabolities classes by chemical reactions characteristic for each substances (Delgado et al., 2013).

In vitro antimicrobial activity of the crude extract

Microbial strain

The crude extract was tested towards 5 reference bacteria, 2 Gram-positive strain: Staphylococcus aureus (ATCC 6538) and Staphylococcus epidermidis (ATCC 12228) and 3 Gram-negative strains: Pseudomonas aeruginosa (ATCC 15442), Klebsiella pneumoniae (ATCC 13883) and Escherichia coli (ATCC 10536) and 1 reference fungus, Candida albicans (ATCC 10231).

Experimental procedure

Broth micro-dilution susceptibility assay was used to determine the minimum inhibitory concentrations (MIC) according to the protocols of the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards 1997a, b). All tests were performed in Mueller-Hinton broth (Merck, Darmstadt, Germany) and overnight broth cultures of each microbial strain were prepared. Microorganism suspensions at a final concentration of 2 × 10^6 CFU/ml were added to microwell plates and the plates were incubated at 37°C for 24 h. The crude extract was dissolved in 1% dimethyl sulphoxide (DMSO) and serial doubling dilutions were prepared over the concentration range 1 to 100 µg/ml. As positive controls, two-fold serial dilutions ranging from 100 to 1.6 µg/ml of amikacin, amphotericin B and chloramphenicol were used. All determinations were performed in triplicate and repeated twice. The MIC value was defined as the lowest concentration of the crude extract at which the microorganism does not present visible growth. MICs ≤ 250 µg/ml are considered of interest for plant extracts (Lopes et al., 2013).

Ex vivo antimicrobial activity of the crude extract

Bacterial strain

A penicillin-sensible S. aureus strain (ATCC 25923) was used for the ex vivo test to determine MIC and minimum bactericidal concentration (MBC<sub>100</sub>) by using Mueller-Hinton broth (Merck, Darmstadt, Germany) and Salt Mannitol Agar (Merck, Darmstadt, Germany), respectively. The MBC<sub>100</sub> value was defined as the lowest concentration of the crude extract at which at least 99% of the microorganisms did not present visible growth. The same strain was used to carry out the regression line assay and the microbiological assay.

Drugs

Amoxicillin trihydrate was obtained from Sigma Chemical Co. (St. Louis, MO, USA.). Physiological saline solution (0.9% NaCl) was administered to the control animals.

Animals

Twenty-four adult male Wistar rats (Rattus norvegicus albinus), weighing 175 ± 25 g, were acquired from the biotery of ABC Medical School. The animals were housed in polyethylene cages (n = 6) in a climate-controlled environment (25 ± 4°C, 55 ± 5% humidity) with a 12 h (07:00 to 19:00) day length and had ad libitum access to food (Labina, Purina) and water. This project was performed in accordance with the guidelines of the Ethics in Research.
and the standards of use of laboratory animals in research of the Federal University of São Paulo (protocol #1356/12).

Granulomatous tissue model

All animals were anesthetized with a combination of ketamine 90 mg/kg/f.m. and xylazine 10 mg/kg/f.m and granulomatous tissue was induced as previously described (Matts-Filho et al., 2006). Briefly, four sterilized polyurethane sponge discs (density 35 kg/m³) were subcutaneously implanted in the back of all rats. These sponge discs (Proespuma Com. & Ind. Ltd., São Paulo, Brazil) were 12 mm in diameter and 5 mm in thickness, weighing 12.21 ± 0.73 mg.

Experimental groups

Seven days after the sponge introduction, all animals were assigned into three groups of eight animals each: amoxicillin (G1) 25 mg/kg per os, crude extract of P. granatum L. (G2) 300 mg/kg per os (Jain et al., 2013) and physiological saline (G3) 1.0 ml per os (0.9% NaCl). All drugs were administered in a single dose.

Surgical and sampling procedures

After 90 min of drug administration, blood samples were collected by cutting the carotid plexus of each animal under general anesthesia. Blood samples were centrifuged and 10 μl of serum was placed on three sterilized paper discs (6.25 mm) and dried at room temperature. Granulomatous tissues were delimitated and surgically removed. All discs and two granulomatous tissue samples of each animal were placed on Muller-Hinton agar plates inoculated with 10⁵ CFU/ml of S. aureus strain. After eighteen hours of incubation at 37°C, the inhibition zones were measured. Two granulomatous tissue samples of each animal were weighed and analyzed by a histological routine technique (HE). Granulomatous tissue weights, tissue and serum concentrations of group 1. Groups 2 and 3 did not present any inhibition zone considering both serum and tissue samples during the microbiological assay.

Regression line

Amoxicillin and crude extract suspensions of 0.03, 0.05, 0.07, 0.10, 0.30, 0.50, 0.70, 1.0, 3.0, 5.0, 7.0 and 10 μg/ml were made by using drug-free serum of rats and 10 μl were placed onto dry paper-filter discs (6.25 mm). Three discs of each concentration were placed on the Mueller-Hinton agar, previously inoculated with 10⁶ CFU/ml of S. aureus strain. The resulting inhibition zones were measured (mm) after eighteen hours of incubation at 37°C. These zones and the drugs concentrations were used to obtain the regression line (software Excel XP® for Windows®).

RESULTS

Compositional analysis of the crude extract

Through the compositional analysis, it was possible to identify major classes of secondary metabolites of pharmacological interest present in the crude extract from the fruits of P. granatum L. exhibited in Table 1.

In vitro antimicrobial activity of the crude extract

The in vitro antimicrobial activity of the crude extract from the fruits of P. granatum L. (Punicaceae) against different microorganisms is displayed in Table 2.

Ex vivo antimicrobial activity of the crude extract

MIC and MBC₁₀₀ of amoxicillin against S. aureus (ATCC 25923) were, respectively 0.2 and 1.5 μg/ml. The limits of detection of the regression curve were 0.03 μg/ml (12 mm of inhibition zone diameter) and 10 μg/ml (31 mm of inhibition zone diameter). The relation between the diameter of inhibition zone (DIZ - in mm) and the concentration of amoxicillin (CA - in μg/ml) was DIZ = (3.23 × Ln (CA)) + 24.16, which showed a coefficient of regression (R) of 0.9851. This relation was used to estimate tissue and serum concentrations, considering the mean of tissue weights of each animal. The wet weight (mg) values (mean ± standard error of the mean) of the granulomatous tissue samples were 31.18 (± 1.98), 32.16 (± 2.16) and 31.88 (± 2.31), respectively for groups 1, 2 and 3. No statistically significant differences were observed among groups (p > 0.05) regarding the wet weight values. After seven days and ninety minutes, a delimitated fibrous capsule involving the sponge was observed in all samples. Fibroblasts, mesenchymal cells and new capillary formation were verified in large scale. Infectious exudates were not observed in any of the granulomatous tissues. Table 3 exhibits amoxicillin serum and tissue concentrations of group 1. Groups 2 and 3 did not present any inhibition zone considering both serum and tissue samples during the microbiological assay.

DISCUSSION

A compositional analysis has been accomplished to verify the presence of major classes of secondary metabolites of pharmaceutical interest in the crude extract from the fruits of P. granatum L. and then these phytochemical results were attached to its preclinical antimicrobial evaluation. In vitro tests performed with extracts rich in tannins or pure tannins, and pomegranate is a rich source of tannins (Afaq et al., 2005), have identified several biological activities, among them, bactericide and fungicide (Chung et al., 1998). From the pericarp of the fruit, rich in tannins, it has been described the isolation of granatins A and B, punicalagin and punicalin, which must be primarily responsible for the antimicrobial effect (Catao et al., 2006).

Flavonoids and phenolic compounds, also present in the crude extract are essential for growth and reproduction of vegetables and are produced as a plant's response to damage caused by microorganisms. Their benefits are usually connected to two properties: inhibition
Table 1. Compositional analysis of the crude extract.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Cardiotonic heterosydes</td>
<td>-</td>
</tr>
<tr>
<td>Fixed oils and fats</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>+</td>
</tr>
<tr>
<td>Proteins and amino acids</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. *In vitro* antimicrobial activity of *Punica granatum* L. (*Punicaceae*) species.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Concentration of extracts (µg/ml)</th>
<th>Control</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4000</td>
<td>2000</td>
<td>1000</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3. *Ex vivo* antimicrobial activity of *Punica granatum* L.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Serum (µg/ml)</th>
<th>Tissue (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>2.23</td>
<td>3.89</td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Physiological saline</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

of certain enzymes and antioxidant activity (Cotelle, 2001). Therefore, they can protect other components of the vegetables, such as carotenoids and vitamin C, digestive enzymes and the intestinal epithelial cells of oxidation of free radicals produced in the stomach (McDougall et al., 2005). Thus, tannins, flavonoids and phenolic compounds must act synergistically to constitute the antimicrobial potential of pomegranate.

The crude extract from the fruits of *P. granatum* L. (*Punicaceae*) was evaluated for *in vitro* antimicrobial activity against six different microorganisms. The minimum inhibitory concentration (MIC) is determined by inoculating the organism into a series of test wells that contain a standard amount of broth and serial dilutions of the antimicrobial agent being tested. Following a period of incubation, the wells are examined for growth. The MIC number is the lowest concentration of drug that inhibits growth of the pathogen. Usually, successful treatment of infection is achieved by merely inhibiting multiplication of the microorganism and relying on a healthy immune system. Thus, the assay evinced broad spectrum antimicrobial property; contents of the ethanolic extract were sufficient to inhibit the growth of all the tested microorganisms. Among the selected microorganism cultures, the preeminent antimicrobial activity was recorded against *S. aureus* and *S. epidermidis*, which presented significant MIC values (Lopes et al., 2013).

All the tested strains evidenced sensibility towards the crude extract, causing damage to the morphology, as irregularities on its superficies and cell wall. Moreover, microorganisms which are responsible for diarrhea and typhoid diseases (*S. aureus* and *E. coli*), wound infection (*S. aureus, E. coli* and *P. aeruginosa*), respiratory disorders (*S. aureus, K. pneumoniae* and *P. aeruginosa*) and skin infections (*S. aureus, S. epidermidis* and *C. albicans*) were inhibited considerably, which in turn, prove the local effect of the extract. These results are in accordance with previous studies that investigated the *in*
vitro antimicrobial activity of *P. granatum* L. (Moorthy et al., 2013).

On the other hand, the *ex vivo* assay permitted to confirm *S. aureus* susceptibility through MIC and MBC100 values of amoxicillin (Koneman et al., 1997). As observed in previous studies, the microbiological method was accurate enough to measure drugs concentrations (Mattos-Filho et al., 2006). This method has the same precision as high performance liquid chromatography (HPLC) assay and it has been widely used for determining this type of test samples concentrations (Charles and Chulavnatol, 1993). The period of seven days used for the development of granulomatous tissue in the present study is adequate. Different periods (7, 14, 21 and 28 days) were observed for development of granulomatous tissues in rats and it has been concluded that the period did not interfere with the pharmacokinetics of the drugs (Groppo et al., 2004).

While acoxolin has an intestinal absorption through passive diffusion (Sugawara et al., 1989) and through oligopeptide transporter system present mainly in kidney and intestine (Moore et al., 2000), there are no data regarding the bioavailability of any pomegranate extracts. The oral ingestion of the crude extract of *P. granatum* L. did not present any inhibition zone during the *ex vivo* microbiological assay, since pomegranate phytocompounds must be metabolized during digestion, suggesting that the bioactive compounds that should have provided an *ex vivo* activity may not be the same as those identified in the extract (Johannsmeier and Harris, 2011). Thus, it shall be explained by the action of enzymes, such as tannase, an extracellular enzyme produced by bacteria in the presence of tannic acid, which hydrolyzes esters and side connections of tannins (Battestin et al., 2004). Based on these facts, it is suggested that the crude extract from the fruits of *P. granatum* L. (G2) did not evidence *ex vivo* antimicrobial activity, since after the oral ingestion of the extract, bacteria present in the gastrointestinal tract of the animal, hydrolyze phytocompounds, which present antimicrobial potential in *in vitro* models, but do not present sufficient serum and tissue concentrations to exert this activity in *ex vivo* and presumably in *in vivo* models. Thus, the compositional analysis evidenced secondary metabolites with known antimicrobial action. The *in vitro* results obtained, in turn, were promising, because the tested strains demonstrated sensibility towards the crude extract, causing damage to the morphology, as irregularities on its superficies and cell wall. However, pomegranate phytocompounds did not present sufficient serum and tissue concentrations to exert this activity in the *ex vivo* model proposed. Presently, the crude extract from the fruits of *P. granatum* L., considered a promising source for alternative treatment requires *in vivo* and clinical studies to confirm its antimicrobial capacity, promoting scientists to continue studies on safety, efficiency and standard quality issues for *P. granatum* L. (*Punicaceae*).

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REFERENCES


