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Evaluation of the Antimicrobial Activity of Aqueous Pomegranate (*Punica granatum* L.) Extract Against Shigella

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Abstract: Enteropathogen-caused diarrhea in Latin America is prevalent in children. The increasing number of reports on antibiotic resistance of Shigella in several parts of the world, recommend the search of alternative therapies. The use of natural inhibiting substances is an alternative treatment. The current study assessed *in vitro* antimicrobial activity of Aqueous Extracts (AE) from pomegranate peel (*Punica granatum* L.) against clinical isolates of Shigella using the following techniques; agar diffusion, Minimum Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC) and bactericidal activity (death curve). Phytochemical analysis revealed the presence of active inhibitors in the peel including phenolics and flavonoids. The AE from the pomegranate peel showed highest inhibition against *Shigella flexneri* with a MIC of 250 μg mL^{-1} and a MBC of 500 μg mL^{-1}. The death curve decreased 3 log units after 4 h indicating a bactericidal effect and photomicrographs showed the loss of cell wall integrity. AE activity was related to the higher content (46%) of total phenolic compounds.

Key words: Antimicrobial effect, pomegranate, Shigella, bacterial activity, antibiotic, Argentina

INTRODUCTION

Shigella is recognized by the World Health Organization as a major global public health risk. It is one of the principal causes of diarrhea in pediatric patients in developing countries and to a lesser degree in children in industrialized countries. The risk of contracting shigellosis is associated with deficiencies in environmental sanitation and personal hygiene (Folster *et al*., 2011; Niyogi, 2005; Penatti *et al*., 2007; Srinivasa *et al*., 2009).

Shigellosis, acute bacillary dysentery is manifested by the passage of loose stools mixed with blood or mucus and accompanied by fever, abdominal cramps and tenesmus. In most Latin American countries living conditions are crowded and sanitation is poor and diarrhea and dysentery caused by bacterial enteropathogens are among the main causes of morbidity and mortality. Shigellosis persists as an endemic disease being responsible for 8-12% of the diarrheal episodes and for 52% of the cases that require hospitalization. The highest rate of incidence of this disease is registered in children between 1 and 4 years of age (CDC, 2010; Pichel *et al*., 2007; WHO, 1996). Shigellosis is an important cause of diarrheal death. It has been reported that at least 140 million cases of shigellosis happen worldwide with 6,00,000 deaths annually and 60% of these fatalities occurs in children <5 years of age (Binsztein *et al*., 1999). It is one of the few enteric infections for which antimicrobials are prescribed with both clinical and epidemiological benefits but increasing resistance has been observed (Binsztein *et al*., 1999; Folster *et al*., 2011; Penatti *et al*., 2007).

In collaboration with WHONET-Argentina, the national network for surveillance of antimicrobial resistance, researchers organized a system for detection of local and regional outbreaks of *Shigella* sp. This included searching for clusters on the basis of genus, species and resistance phenotype and 19 statistical events were identified in a 12 months period. Of the six known outbreaks reported to the Ministry of Health, four closely coincided with SaTScan-detected events (Binsztein *et al*., 1999; Stelling *et al*., 2010). The increasing level of resistance of enteropathogenic bacteria to antimicrobial agents has become alarming and makes treatment of diseases caused by these pathogens more and more difficult. Antimicrobial susceptibility of isolates obtained from 4,364 children <5 years of age was

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assayed at the laboratory. All subjects suffered from acute diarrhea and resided in 7 different towns in Tucuman province. Resistance of diarrheagenic *E. coli* to ampicillin was 74.5% and to trimethoprim-sulfametoxazole 64.2% and resistance of *Shigella* sp. was 62 and 75.6%, respectively. However, resistance is not always evenly distributed and Argentina also presents geographic variations (Bennish *et al*., 1992; Binsztein *et al*., 1999; Stelling *et al*., 2010).

The worldwide spread of antibiotic-resistant pathogens has revived the search for antimicrobial compounds from natural sources including plants. Consequently, interest in the study of plants as a source of pharmacologically active compounds has increased. Besides, it is known that in some developing countries, plants are still the main medicinal source to treat infectious diseases.

Among the diverse plant compounds, polyphenols have received special attention due to their varied biological functions. Nevertheless, little is known about the antibacterial activity against enterobacteria which cause diarrhea and dysentery and the pharmacological effects of these plants. Hence, the necessity to study the properties of medicinal plants to validate their use in traditional medicine (Cowan, 1999). In previous studies we determined antimicrobial activity of Aqueous Extracts (AE) from popular shrubs and plants in the Northwest of Argentina (Agrimony, Cachiyuyo, Charrua (Aristolochia argentina), Duraznillo (Solanum glaucophyllum), Holy oak (Quercus ilex), pomegranate, guava (Psidium), Witch-hazel (Hamamelis), common fig, rose petals, oak, suico and blackberry against enteropathogens and it was demonstrated that the AE from Holy oak, pomegranate peel, hamamelis, rose petals and oak showed inhibition of Shigella strains. The AE from pomegranate peel showed the largest inhibition zone (unpublished data). Prashanth *et al*. (2001) studied *in vitro* antibacterial activity of several extracts and the MIC values (mg mL^{-1}) of the aqueous extract from *Punica granatum* L., for different pathogens were as follows: *Staphylococcus aureus* (25), *Escherichia coli* (50), *Klebsiella pneumonia* (not active), *Proteus vulgaris* (12), *Bacillus subtilis* (25) and *Salmonella typhi* (25).

Alanis *et al*. (2005) examined antibacterial properties of aqueous and methanolic extracts from 26 medicinal plants used in Mexico for treatment of gastrointestinal disorders. *Punica granatum* L. was assayed against *Shigella sonnei*, *Shigella flexneri* and *Salmonella* sp. A crude extract only inhibited 64% of the *Sh. sonnei* strains whereas the same extract with the same concentration of the active compound (8 mg mL^{-1}) inhibited 100% of the *Sh. flexneri* strains. Studies carried out in Uruguay, Paraguay and Argentina have shown that *Sh. flexneri* is the most frequent species isolated during diarrheal episodes, followed by *Sh. sonnei* (Lopez *et al*., 2000). Considering that in San Miguel de Tucumán, Argentina, Shigella is the second enterobacterium found in diarrheal episodes and that the use of medicinal herbs is very common in our population the aim of this study was to demonstrate antimicrobial activity of the AE from pomegranate peel against Shigella strains.

MATERIALS AND METHODS

Medicinal herbs: Information on medicinal uses of pomegranate was obtained through personal communication with traditional healers and from literature. Pomegranate peels were collected from herbalists' in San Miguel de Tucuman, Argentina.

Preparation of the Aqueous Extract (AE)

Decoction: Plant material (25 g) was boiled with 100 mL of water for 10 min. After cooling to 40-45°C, the AE liquid was filtered and subsequently lyophilized.

Standardization of the most active AE from pomegranate peel

Infusion: The 4 g of plant material were submerged in boiling water (100 mL) for 10 min to saturate the liquid with the active compounds present in the plant. After cooling to 40-45°C, the liquid was filtered and the AE lyophilized.

Decoction: The 4 g of material were boiled with 100 mL of water for 1, 5, 10, 15, 20, 25 and 30 min. After cooling to 40-45°C, the liquid was filtered and the AE lyophilized.

Micro-organisms: The following enteropathogens, previously isolated from patients admitted to hospitals in San Miguel de Tucuman were assayed for susceptibility to pomegranate AE: *Shigella flexneri* (n = 8), ampicillin-resistant *Sh. flexneri* (n = 1) and *Shigella sonnei* (n = 6). All strains had been characterized at the Laboratory of the Bacteriology Department of the Faculty of Biochemistry, Chemistry and Pharmacy, UNT, Tucuman.

The strains were identified by standard biochemical assaying for clinical diagnosis according to the Manual of Clinical Microbiology (Murray *et al*., 1999) and phenotype assaying according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). All organisms were maintained at -70°C in Hrain-heart Infusion (BHI medium) containing 25% (v/v) glycerol. Before testing, isolates were subcultured in BHI broth.

Antibacterial assaying

Agar well diffusion method: Agar plates (10 cm in diameter) were prepared using sterile Mueller Hinton Agar (MHA) (Britania). Inoculum size was 10^8 cells mL^{-1} as per Mc Farland standard and diluted in Mueller Hinton Broth (MHB) in order to obtain an adequate inoculum in each case. The number of cells in MHB was estimated by serial dilution (CLSI, 2006). Bacterial strains of standardized cultures (10^8 cfu mL^{-1}) were evenly distributed onto the surface of the agar plates using sterile cotton swabs. Wells with a diameter of 5 mm were punched in the agar with a sterile cork borer and 50 μL of the AE were poured into each well. Plates were incubated at 37°C for 24 h.

After incubation the plates were examined for inhibition of bacterial growth indicated by a clear zone around the wells. The size of the inhibition halos was measured and antibacterial activity expressed as the average diameter (mm) of the inhibition zone. Absence of an inhibition halo was interpreted as no activity. Each extract was assayed in triplicate and each experiment was repeated twice.

Determination of water-soluble compounds by lyphilization: About 50 mL of 4% pomegranate peel after decoction for 10 min yielded 10 mg of soluble compounds per mL of AE.

Quantitative determination of antimicrobial activity

Serial agar macrodilution method: Serial dilutions (final volume 1 mL) of AE were performed with MHB. After addition of 9 mL of MHA, the mixture was poured into petri dishes.

After solidification, plates were sown with 2 μL of each bacterial cell suspension (5×10^5 cfu mL^{-1}) and aerobically incubated at 37°C for 24 h. A growth control of each strain was included. The MIC was defined as the lowest AE dilution at which no growth was observed after incubation.

Broth microdilution method: Extract dilutions ranging from 0.5-0.02 were assayed against a final inoculum concentration of 5×10^5 cfu mL^{-1}. The inoculated test tubes were incubated at 37°C during 24 h. Presence of turbidity on the tube bottom indicated bacterial growth. MICs were determined as the first tube in which no turbidity was observed.

Determination of bactericidal activity of the AE from pomegranate peel (MBC): Cell death of the strains assayed was confirmed by reinoculating agar plates with 10 μL of each culture medium from the first tubes without turbidity used for the broth microdilution assay. The Minimal Bactericidal Concentration (MBC) was determined after 24 h of incubation at 37°C.

The MBC was defined as the lowest AE dilution that at least killed 99.9% of the bacteria. The broth macrodilution method was used to determine the MIC and MBC values of the extracts against the organisms assayed as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006).

Time-kill curve studies of *Shigella flexneri*: Bactericidal activity was determined through the time-kill curve method recommended by the CLSI. A standardized suspension of *Sh. flexneri* (5×10^5 cfu mL^{-1}) was added to MHB supplemented with the AE from pomegranate L to give a final concentration of twice the MIC. The mixtures were then incubated at 37°C for 12 h. Samples were taken after 0, 2, 4 and 6 h and serially diluted. Each dilution was plated out (0.1 mL) in duplicate. Total bacterial cell counts (cfu mL^{-1}) were determined after incubation at 35°C for 18 h. Bactericidal activity was defined as a 99.9% (≥3 log units) reduction in the total cell count of the original inoculum (CLSI, 2006).

Phytochemical screening: The AE compounds from pomegranate peel were separated by Thin Layer Chromatography (TLC) (Kieselgel 60 F254; 0.2 mm, Merck) with the following solvents; ethyl acetate; formic acid; glacial acetic acid; water (100:11:11:27), chloroform; methanol (9:1), chloroform; ethyl acetate (9:1), benzene; dioxane; glacial acetic acid (9:2.5:0.4) and toluene; chloroform and acetone (4.5:2.5:3.5). The separated compounds were visualized under ultraviolet light (254 and 360 nm; UV 5L-58 Mineralight Lamp) and sprayed with 1% ferric trichloride, natural product reagent (1% methanolic 2-aminoethyl diphenylborate) (Wagner *et al*., 1984) or aluminum chloride for phenolic compounds, methanolic potassium hydroxide for coumarins (Harborne, 1998), Dragendorff's reagent for alkaloids and anisaldehyde/sulfuric acid for steroids and terpenes (Krebs *et al*., 1969).

Determination of total phenolic compounds and flavonoid contents: Total phenol contents were determined using the Folin-Cicolteu method (Singleton *et al*., 1999) and flavonoids were estimated with the method by Popova *et al*. (2005). Concentrations were determined spectrophotometrically at 420 nm. Quercetin was used as standard.

Electron microscopy: Transmission Electron Microscopy (TEM) was carried out at the LAMENOA electron microscopy center (UNT) to observe the effects of the AE from pomegranate peel on *Sh. flexneri*. A standardized suspension of *Sh. flexneri* (5×10^5 cfu mL^{-1}) was prepared and added to MHB with pomegranate peel AE at a final concentration of 2 times the MIC. This mixture was incubated at 37°C during 12 h. Aliquots were taken after 0, 6 and 12 h, centrifuged at 12,000 rpm and the supernatant was discarded. Then a conserver was added to the pellets which were kept until further processing. Controls of the AE and bacterial inoculum were also prepared.

RESULTS AND DISCUSSION

AE from pomegranate peel after 30 min of decoction presented the largest inhibition zone: A 22 mm (Table 1).

Table 1: Inhibitory activity after different treatments of the Aqueous Extract (AE) from pomegranate peel against *Shigella flexneri*

AE treatments	Time	Inhibition halo (mm)
Infusion (min)	10	16
Decoction (min)	1	18
	5	19
	10	20
	15	20
	20	21
	25	22
	30	22

Minimum Inhibitory Concentration (MIC): From the 8 *Sh. flexneri* strains assayed, 4 showed a MIC of 0.05 mg mL^{-1} on solid medium and 4 strains including an ampicillin-resistant strain showed a MIC of 0.2 mg mL^{-1}, containing 0.5 and 2 mg of the soluble compound per mL of AE, respectively (Table 2).

MIC values for liquid and solid medium were similar for the 8 *Sh. flexneri* strains assayed which indicates a correlation between the MIC of solid and liquid medium. From the 4 strains that presented a MIC of 0.05 mg mL^{-1}, 3 showed a MBC of 0.1 mg mL^{-1} and one strain 0.11 mg mL^{-1}, containing 1 and 1.1 mg of the soluble compound per mL of AE, respectively (Table 3). From the 4 strains that had a MIC of 0.2 mg mL^{-1}, 3 showed a MBC of 0.33 mg mL^{-1} and one 0.25 mg mL^{-1} with 3 and 2.5 mg of the soluble compound per mL of AE, respectively (Table 4). In the death curve (Fig. 1), it can be observed that after 4 h the number of viable cells (cfu mL^{-1}) decreased 4 log units. This indicates that AE from pomegranate peel presented bactericidal activity against *Sh. flexneri*.

Phytochemical analysis and efficiency of soluble active compounds: The 10 mg of soluble compounds per mL of the AE were obtained from 50 mL of pomegranate peel at 4% after decoction for 10 min. Quantification of the soluble compounds revealed that AE from pomegranate peel consisted of 46% of total phenolic compounds and 13% of total flavonoids which corresponded to 28% of the total phenolic compounds. Phytochemical analysis of the

Table 2: MIC values of AE from pomegranate peel against *Shigella flexneri* on solid and in liquid medium. Values are given as mg of soluble compound per mL of AE

	MIC (mg mL^{-1})												
Strain	5	3	2.5	2	1.7	1.4	1.3	1.1	1	0.5	0.3	0.25	0.2
Sh. flexneri A	-	-	-	-	-	-	-	-	-	-	+	+	+
Sh. flexneri F	-	-	-	-	-	-	-	-	-	-	+	+	+
Sh. flexneri G	-	-	-	-	-	-	-	-	-	-	+	+	+
Sh. flexneri H	-	-	-	-	-	-	-	-	-	-	+	+	+
Sh. flexneri B	-	-	-	-	+	+	+	+	+	+	+	+	+
Sh. flexneri C	-	-	-	-	+	+	+	+	+	+	+	+	+
Sh. flexneri D	-	-	-	-	+	+	+	+	+	+	+	+	+
Sh. flexneri E	-	-	-	-	+	+	+	+	+	+	+	+	+

Table 3: MBC values of AE from pomegranate peel against *Shigella* sp. Values are given as mg of soluble compound per mL of AE

	MBC (mg mL^{-1})												
Strain	5	3	2.5	2	1.7	1.4	1.3	1.1	1	0.5	0.3	0.25	0.2
Sh. flexneri A	-	-	-	-	-	-	-	-	-	+	+	+	+
Sh. flexneri F	-	-	-	-	-	-	-	-	-	+	+	+	+
Sh. flexneri G	-	-	-	-	-	-	-	-	-	+	+	+	+
Sh. flexneri H	-	-	-	-	-	-	-	-	-	+	+	+	+
Sh. flexneri B	-	-	-	-	-	-	-	-	+	+	+	+	+
Sh. flexneri C	-	-	+	+	+	+	+	+	+	+	+	+	+
Sh. flexneri D	-	-	-	+	+	+	+	+	+	+	+	+	+
Sh. flexneri E	-	-	+	+	+	+	+	+	+	+	+	+	+
Sh. flexneri E	-	-	+	+	+	+	+	+	+	+	+	+	+

Table 4: Phytochemical assaying of the Aqueous Extract (AE) from pomegranate peel after 10 min of decoction for active compounds. Coumarines and alkaloids were not detected

Parameter	Phenolic compounds (mg mL^{-1})	Total flavonoids (mg mL^{-1})	Dry weight (mg mL^{-1})
AE (Decoction 10 min)	4.60	1.30	10

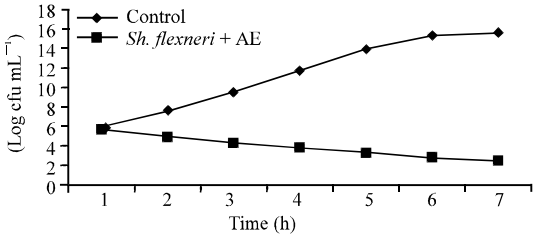

Fig. 1: Bactericidal effect of pomegranate peel on *Shigella flexneri* (death curve)

AE revealed the presence of phenolic compounds mainly flavonoids in the chromatographic systems assayed. Coumarines and alkaloids were not detected with the methods described in materials and methods (Table 4).

Electron microscopy: Electron microscopic examination of *Sh. flexneri* cells after treatment with AE from pomegranate peel revealed a pyriform shape with mammillated aspect (and numerous protuberances) of the bacterial cell after loss of the cell wall integrity. Development of a vesicle and the loss of cell material are clearly visible (Fig. 2 and 3).

Previous studies have demonstrated antibacterial activity of different plant extracts against a broad range of micro-organisms. Several researchers have confirmed the antibacterial potential of *Punica granatum* L. and hence its use in popular medicine. In traditional medicine in different countries, pomegranate has been used to treat dysentery, microbial infections, diarrhea, helminthiasis and respiratory pathologies (Arseculeratne *et al*., 1985; Misas *et al*., 1979; Jurenka, 2008).

Desta (1995) affirmed that pomegranate is used in Ethiopia as a taeniacide drug because it presents relatively low toxicity and pomegranate fruits are not toxic. Toxicity of *Punica granatum* L. has not been intensively studied. Amorin (1995) did not observe any toxic effects in rats treated with aqueous extracts from pomegranate similar to those used in popular medicine.

In Mexico, Navarro *et al*. (1996) studied the antibacterial activity of methanolic extracts of 12 plants including pomegranate peel. The extracts were assayed against *S. aureus*, *E. coli*, *P. aeruginosa* and *Candida albicans*. The researchers revealed a MIC of 0.62 mg mL^{-1} for *S. aureus* and 10 mg mL^{-1} for *E. coli* and *P. aeruginosa*.

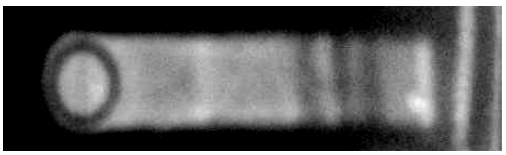

Fig. 2: TLC of the Aqueous Extract (AE) from pomegranate peel using a chloroform: methanol mixture (9:1) as solvent

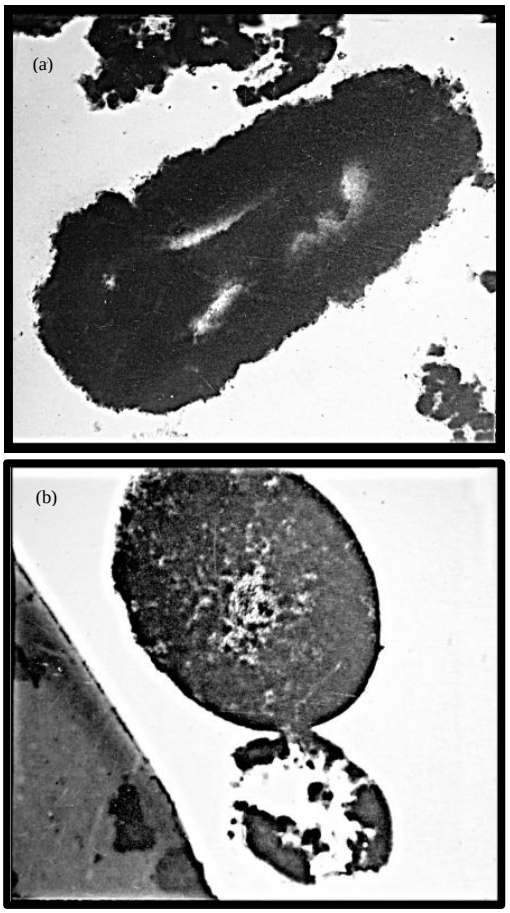

Fig. 3: a) A pyriform shape with mammillated aspect (and numerous protuberances) of the bacterial cell can be observed after loss of the cell wall integrity. b) Development of a vesicle and the loss of cell material are clearly visible

In Arabia, Al-Zoreky (2009) assessed the antimicrobial activity of a methanolic extract from pomegranate peel against *L. monocytogenes*, *S. aureus*, *S. enteritidis*, *E. coli* and *Y. enterocolitica*. They found that the extract presented antibacterial effect against *S. enteritidis* and *L. monocytogenes* with a MIC of 4 mg mL^{-1} for *S. enteritidis* and a reduction of >1 log

unit in the case of *L. monocytogenes* in food during storage at 4°C. Nascimento *et al.* (2000) studied the antimicrobial effect of *Punica granatum* L. against *Staphylococcus aureus* ATCC 6538, *Salmonella cholerasuis* ATCC 10708, *P. aeruginosa* ATCC 15442, *Bacillus subtilis*, *K. pneumoniae*, *Shigella* sp., *Proteus* sp., *P. aeruginosa*, *Enterobacter aerogenes*, *Escherichia coli* and *S. aureus* (resistant to antibiotics) and they only found inhibition zones (≥7 mm) for *P. aeruginosa* ATCC 15442 and *Bacillus subtilis*. Holetz *et al.* (2002) studied the antibacterial activity of *Punica granatum* L. fruits and they found MIC values (μg mL^{-1}) of 62.5 for *S. aureus* ATCC 25923 and >1,000 for *B. subtilis* ATCC 6623, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442. Alanis *et al.* (2005) studied the antibacterial properties of aqueous and methanolic extracts from 26 medicinal plants used in Mexico for the treatment of gastrointestinal disorders. *Punica granatum* L., was assayed against *E. coli* ATCC 25922, *Sh. sonnei*, *Sh. flexneri* and *Salmonella* sp. They showed that only 64% of the *Sh. sonnei* strains was inhibited with an extract containing 8 mg mL^{-1} of active compound whereas this concentration inhibited 100% of the *Sh. flexneri* strains. These results agree with the findings.

Mathabe *et al.* (2006) examined antibacterial activity of methanolic, ethanolic, acetonic and aqueous extracts from the root of *Punica granatum* L. (100 mg mL^{-1}) against *S. aureus* ATCC 25923, *Salmonella typhi* ATCC 0232, *V. cholera*, *E.coli* ATCC 35218, *Sh. dysenteriae*, *Sh. flexneri*, *Sh. sonnei* and *Sh. boydii*. They determined inhibition zones of 12.7 and 30.7 mm for *Sh. sonnei* and *Sh. flexneri*, respectively and the MIC value of the aqueous extract was 0.156 mg mL^{-1} for both species. These studies show that aqueous and organic extracts from *Punica granatum* L. were equally active against the gram-negative bacteria *Sh. sonnei* and *Sh. boydii*. This contrasts with findings by other scientists who affirmed that aqueous extracts showed less activity against gram-negative bacteria than organic extracts (Lall and Meyer, 2000; Matu and van Staden, 2003; Shale *et al.*, 1999). Some scientists have attributed the antimicrobial activity to the presence of water-soluble tannins (Djipa *et al.*, 2000; Otshudi *et al.*, 2000). The present study found that the aqueous extract of pomegranate peel consisted of 46% of total phenolic compounds and 13% of total flavonoids that corresponded to 28% of the total phenolic compounds. Novel gallotannins and ellagitannins isolated from the bark of the fruit of *Punica granatum* L., are the main components responsible for the antimicrobial action of this species (Hussein *et al.*, 1997, Vidal *et al.*, 2003; Machado *et al.*, 2003). Hussein *et al.* (1997) and Naz *et al.* (2007) found that antimicrobial activity observed with species of the family Punicaceae would be due to presence of flavonoids and tannins. Some assays with flavonoids showed a reduction of 1,000 times or more in viable cell counts of MRSA-YK, *S. aureus* NCTC 6571 and MRSA-16 suggesting that flavonoids have bactericidal activity. However, it has been demonstrated recently that some flavonoids induced the formation of pseudomulticellular aggregates both in antibiotic-resistant and sensitive strains of *S. aureus* (Machado *et al.*, 2003). As a result, flavonoids may not kill bacterial cells but induce the formation of bacterial aggregates and thus reduce the number of cfu in cell counts. The results of electron microscopy in the current study may confirm the bactericidal effect of aqueous extract from *Punica granatum* L. initially demonstrated by the quantitative and qualitative microbiological assays with *Sh. flexneri*. The micrographs clearly demonstrate the effect on the cell wall of this micro-organism.

CONCLUSION

The current study determined that the aqueous extract from pomegranate peel (*Punica granatum* L.) which consists of 46% of phenolic compounds presented highest antimicrobial activity against *Shigella flexneri* with a MIC between 0.5 and 2 mg mL^{-1} and a MBC between 1 and 3 mg mL^{-1}. It also showed that the extract produced a decrease of 3 log units in bacterial growth after 4 h of incubation and electron microscopy results demonstrated the loss of cell wall material. These findings confirm bactericidal activity of the AE from pomegranate peel against *Sh. flexneri*.

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