J.Agric.Chem.and Biotechn., Mansoura Univ.Vol. 7 (12): 297 – 301, 2016

Antibacterial and Mutagenic Potency Screening of Different Pomegranate Cultivars Using rep-PCR

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ABSTRACT

Pomegranate fruit is a rich source of antimicrobial agents. Herein, the antibacterial potencies of four pomegranate cultivar peel extracts; Wonderful, H116, Manfalouty and Black, against five pathogenic bacteria *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli* have been evaluated with routine disc diffusion susceptibility testing. The gross effects of peel extracts on bacterial DNA have been assessed with the rep-PCR DNA fingerprinting technique. The results indicated that all cultivar peel extracts showed enhanced antibacterial activities against the tested strains compared to controls. The polymorphic DNA banding patterns of *E. coli* O157:H7, as a reference strain, treated with different cultivar peel extracts were used as a reference test to detect the mutagenic effect of peel extracts. The study indicated that pomegranate peel is a promising source as antibacterial agents or bacterial mutagens.

Keywords: Antibacterial potencies; rep-PCR; Pomegranate; peel extract

INTRODUCTION

Pomegranate, *Punica granatum* L., is an important fruit crop of tropical and subtropical regions of the world (Meerts *et al.,* 2009). The leaves, barks, peels and fruits of pomegranate have various medicinal properties. Therefore, the pomegranate has many therapeutic applications as antibacterial-**,** antifungal-**,** antiviral-**,** antioxidant-**,** antidiabetic-**,** anticarcinogenic-**,** antiparasitic- agent (Jurenka, 2008 ;Viuda-Martos *et al.,* 2010; Howell and D'Souza, 2013).

Pomegranate extracts, especially the peels, have a wide spectrum of activities on a number of important pathogenic as well as drug-resistant bacterial strains such as *Staphylococcus aureus* (Machado *et al*., 2003), *Salmonella typhimurium* (Tayel *et al*., 2012), *Escherichia coli* O157:H7 (Voravuthikunchai *et al*., 2004), *Pseudomonas aeruginosa* (Hayouni *et al*., 2011), *Listeria monocytogenes* and *Yersinia enterocolitica* (Al-Zoreky, 2009). Furthermore, it would be used as food preservation by preventing the actions of food spoilage bacteria (Howell and D'Souza, 2013).

The seeds and juice of pomegranate fruit only represent about 3 and 30 % of the fruit weight, respectively. The rest is peels, containing an interior network of membranes (Lansky and Newman, 2007). Among these parts, pomegranate peel extract has the highest antibacterial activity more than other fruit extracts (Howell and D'Souza, 2013).

Numerous chemical components have been detected in different parts of the pomegranate, hydrolyzable tannins (punicalins and punicalagins), punic acid, flavonols and anthocyanins that have the responsibility for pharmacological benefits of pomegranate. Among these components, the peel has high content of polyphenols which provide higher activity as antioxidant. The peel extract constituents would vary according to the tested cultivar of pomegranate (Duman *et al*., 2009).

Rep-PCR DNA finger-printing has been recognized as a general method that utilizes primers matching with short intergenic repeated sequences that have been found in bacterial genomes (Versalovic *et al*., 1991). These sequences called "Rep" sequence (repetitive extragenic palindromic sequence), highly conserved inverted repeats sequences and multiple copies (Versalovic *et al*., 1994). Rep-PCR technique is a widely applied for yield DNA fingerprints of different sources and generate very characteristic patterns. The rep-PCR is a high discriminatory power, reproducible, low cost, rapid and suitable for Gram-negative (G^-) and Gram-positive (G^+) bacteria (Versalovic *et al.,* 1994; Dombek *et al.,* 2000). Thus, rep-PCR method may be useful to fingerprint bacterial genomes.

This study was aimed to evaluate the antibacterial activities of four pomegranate (*Punica granatum* L.) cultivars against different pathogenic bacteria to determine the most important cultivar which has the highest antimicrobial potency. In addition, rep-PCR technique was used to investigate the possible mutagenic effect of peel extracts on bacterial DNA.

MATERIALS AND METHODS

Bacterial strains

Five different G+ and G− strains were used in the disc sensitivity assays; the G+ *Staphylococcus aureus* (ATCC 25923); *Bacillus cereus* (ATCC 33018) and the G− *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (O157:H7). These strains were kindly provided by Microbiology Department, Cairo University, Egypt. Cultures were maintained on LB agar slants kept at 4°C viable for routine use.

Plant materials

The pomegranate fruits (Wonderful; W, H116; H, Manfalouty; M, and Black; B cultivars) were collected from the field station of Horticultural Research Institute, ARC, Giza, Egypt. Fruits were washed well with running water, dried on filter papers, aseptically washed and manually peeled. The collected peels were cut into small pieces, dried in oven at 40 °C for 48h, then grinded in liquid nitrogen and kept in at −20°C. For extraction, 25 grams of each peel sample were soaked in 100 ml of 2.5 % dimethyl sulfoxide (DMSO) and 80 % methanol kept in shaking water bath for 24 h at room temperature. Extracts were filtered, concentrated by continuous heating at 40°C and stored at 4°C until use.

Antibacterial bioassay of peel extracts

Antibacterial activities of the peel extracts were evaluated with agar well diffusion method (NCCLS, 2009). One hundred ml of Mueller-Hinton broth

(Laboratorios Conda SA, Madrid, Spain) were inoculated and incubated with each bacterial strain at 37 °C for overnight. All strain cultures were suspended and diluted to 10^6 CFU/ml (0.5 McFarland). Two hundreds µl of the culture suspension of each test strain were added to 20 ml of Mueller-Hinton solidified medium, cooled at 40°C, mixed well and poured into Petri plates. Wells of 9 mm diameter were cut in agar. Wells were filled with 15 mg/ml of different peel extracts. DMSO (2.5%) or methanol (80%) was used as a negative control. Streptomycin (1.2 µg/ml) was used as a positive control to detect the sensitivity of each bacterial strain and to compare the relative percent of antibacterial activity (Gaber *et al.*, 2015). The plates were incubated at 37°C for 24 h and the diameters of inhibition zones (IZ) of tested strains were measured in mm to evaluate the antibacterial activities.

rep-PCR DNA fingerprinting

rep-PCR DNA fingerprinting method was utilized to evaluate the destined mutagenic effects of peels on bacterial genetic materials. The effect of peel extracts on bacterial genetic material was determined by comparing DNA profiles of *E. coli* (as a reference) treated with H116 peel extract with untreated naïve controls. Genomic DNA from *E. coli* pure culture was extracted using genomic DNA purification kit® (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Rep-PCR fingerprinting was performed as described by Kon *et al.* (2009). Ten oligonucleotide primers were used to amplify multi-size amplicons of bacterial genomic DNA. The primer sequences (Macrogen, S. Korea) are listed in Table 1. The rep-PCR amplification program was adapted from Gaber *et al.* (2015).

Table 1. The sequences of rep-PCR primers

Primer Name	Sequence	Annealing temp. (°C)	References
Rep-2A	5'-AACGACTTATCAGGCCTAC-3'	52	Çepni and Gürel (2012)
Rep-2C	5'-GGCGGCTTATCGGGCCTAC-3'		Çepni and Gürel (2012)
Rep-2D	5'-CCCGCCTTATCCGGCCTAC-3'		Çepni and Gürel (2012)
Rep 12	5'-AGAGAGAGAGAGAGAGC-3'		Gaber *et al.* (2015)
Rep 18	5'-ACACACACACACACACG-3'		Gaber *et al.* (2015)
Rep 19	5'-AGAGAGAGAGAGAGAGTT-3'		Gaber *et al.* (2015)
BOX A1R	5'-CTACGGCAAGGCGACGCTGACG-3'		Çepni and Gürel (2012)
BOX B1	5'-TTCGTCAGTTCTATCTACAACC-3'		Genersch and Otten (2003)
BOX C1	5'-TGCGGCTAGCTTCCTAGTTTGC-3'		Genersch and Otten (2003)
$(GTG)_5$	5'-GTGGTGGTGGTGGTG-3'		Gevers *et al.* (2001)

Statistical analyses

All experiments were carried out in triplicates (n=3). All statistical analyses were performed using SPSS 13.0 software (IBM SPSS Statistics for Windows—IBM Corp., Armonk, NY, USA).

RESULTS

Antibacterial bioassay of peel extracts

The potency of peel extracts against the tested pathogenic bacteria was assessed according to clear inhibition zone. The increasing of IZ by comparing positive and negative control reflected a high peel extract antibacterial activity and vice versa. Eight extracts, four DMSO- and four methanol-based, of four pomegranate cultivar peels were used against five bacterial strains.

Table 2 shows the antibacterial activity of 2.5% DMSO extracts. DMSO extract of W peel showed the maximal inhibitory activity against the G^+ *S. typhimurium*; Likewise, H showed a maximal activity on G^- *P. aeruginosa* and *E. coli*. However, it displayed the least inhibitory effect on *B. cereus*. Extract of W and M showed low inhibitory effect for *P. aeruginosa* (Fig. 1).

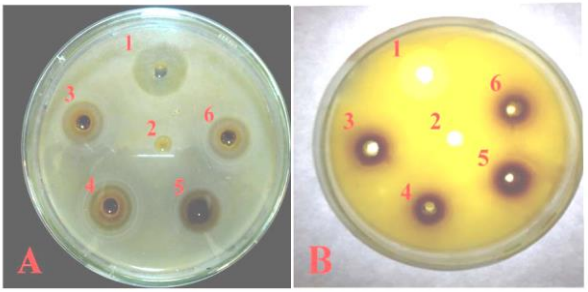

Figure 1. Inhibition zone of four pomegranate cultivar peel DMSO and methanol extracts against *Pseudomonas aeruginosa* strain. 1: Positive control; 2: Negative control; 3: Wonderful; 4: H116; 5: Black; 6: Manfalouty; A: DMSO extracts; B: methanol extracts.

Table 2. Antibacterial activity[±] of four pomegranate cultivar peel DMSO extracts against the tested bacterial strains

	Organisms	Positive control (Streptomycin)	[+]W	H	M	B	Negative control (2.5% DMSO)
G^+ strains	*S. aureus* ATCC 25923	27.6±1.20	40.6±0.88***	39.0±0.57**	40.6±0.84***	33.0±1.15*	9.6±0.33
	B. cereus ATCC 33018	34.3±0.82	33.6±0.33†	22.3±0.88†	31.3±0.33†	24.0±1.15†	9.0±0.00
G^- strains	S. typhimurium ATCC 14028	29.3±0.88	41.0±0.57***	29.6±1.20*	34.3±0.81**	34.6±0.75**	9.0±0.00
	P. aeruginosa ATCC9027	30.0±0.57	30.6±0.77†	41.3±0.85***	29.6±0.81†	33.6±0.66**	9.6±0.33
	E. coli O157:H7	13.3±0.25	34.6±0.33**	39.3±0.79***	33.0±1.51**	36.6±1.20***	9.6±0.31

[±]Measured as inhibition zone in mm diameter, taking the full-length diameter of zones.
[+]Different pomegranate cultivars W: Wonderful; H: H116; M: Manfalouty; B: Black.
Each value is the mean±S.E. of 3 different experiments. †*P*>0.05; **P*<0.05; ***P*<0.01; ****P*<0.001.

The antibacterial activities of peel methanolic extracts are shown in Table 3. The extracts of W, H and M had inhibited the growth of *S. aureus*, while, H and B extracts were more effective to inhibit *B. cereus.* On the other hand, M extracts showed the maximal inhibitory values against *E. coli*, *P. aeruginosa*, *S. typhimurium*. On contrast, B and H extracts displayed the minimal inhibition activity against *P. aeruginosa*.

rep-PCR DNA fingerprinting

The possible mutagenic effects of pomegranate peel extracts on bacterial genetic materials were evaluated by using rep-PCR DNA fingerprinting technique (Fig. 2). *E. coli* gDNA profiles were used as a marker of mutagenicity, post-treatment with H116 peel extracts (DMSO and methanolic fractions) and those untreated.

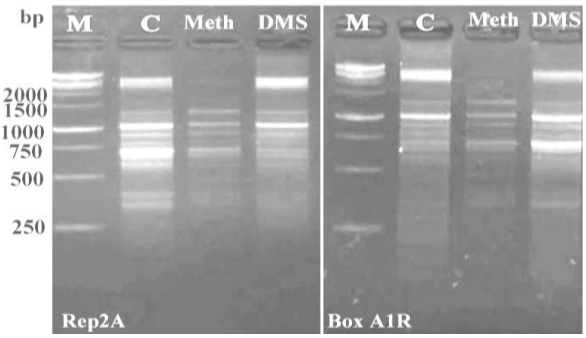

Figure 2. rep-PCR DNA fingerprinting of treated and untreated *E. coli* strains with Rep2A and BoxA1R primers. M: 100 bp DNA ladder, C (control): untreated *E. coli*, Meth: treated *E. coli* by 2 mg/ml of peel methanol extract, DMS: treated *E. coli* by 2 mg/ml of peel DMSO extract.

Table 3. Antibacterial activity[±] of four pomegranate cultivar peel methanolic extracts against the tested bacterial strains

	Organism	Positive control (Streptomycin)	[+]W	H	M	B	Negative control (80% methanol)
G^+ strains	*S. aureus* ATCC 25923	31.0±0.57	39.3±0.88***	39.6±0.33***	39.6±0.60***	33.6±0.33**	9.6±0.29
	B. cereus ATCC 33018	34.6±0.27	31.3±1.45†	34.3±0.71*	29.3±0.66†	34.6±0.21*	10.3±0.33
G^- strains	*S. typhimurium* ATCC 14028	29.6±0.32	39.6±0.33***	34.6±0.83**	39.3±0.61***	40.3±0.30***	9.0±0.09
	P. aeruginosa ATCC 9027	30.6±0.66	38.3±0.57***	24.6±0.49†	42.6±0.16***	24.3±0.73†	9.6±0.32
	E. coli O157:H7	11.3±0.88	34.0±0.57***	39.3±0.22***	40.0±0.57***	31.0±0.57***	9.3±0.30

[±]Measured as inhibition zone in mm diameter, taking the full-length diameter of zones.

[+]Different pomegranate cultivars W: Wonderful; H: H116; M: Manfalouty; B: Black.

Each value is the mean±S.E. of 3 different experiments. †P>0.05; *P<0.05; **P<0.01; ***P<0.001.

Table 4 displays the polymorphism between DNA profiles of *E. coli*, treated and untreated, using a ten primer set. By the comparison between the DNA profiles of untreated *E. coli* (control) with DNA profile of methanol-treated *E. coli* and DMSO-treated *E. coli*, the total number of bands was 85 bands; while the polymorphic numbers of bands was 43 and 12 bands, respectively.

The total percentage of polymorphism of all primers represented 46.07 and 11.9%. The polymorphism percentages ranged between 80% for Rep-19 primer to 20% for Box-B1 primer and 30.7% for Box-C1 primer to 0% for Rep-2D or Box-B1or $(GTG)_5$ primers, respectively. DNA profile of *E. coli* treated with DMSO extract yielded the highest number of bands compared to *E. coli* that treated by methanol extract in all rep-PCR profiles.

Moreover, DNA profiles of methanol-treated *E. coli* are distantly-related to the untreated *E. coli* (control), and DNA profiles of DMSO-treated. On contrary, DNA profiles of DMSO-treated *E. coli* are closely similar to DNA profile of untreated *E. coli* (control), i.e. no significant differences between them. Accordingly, it was clear that the methanol extract is more effective than the DMSO extract.

Table 4. The polymorphism between DNA profiles of *E. coli* (treated and untreated) using ten primers of rep-PCR.

Primer Name	DNA profiles of untreated *E. coli* (control) comparison with DNA profile of methanol-treated *E. coli*			DNA profiles of untreated *E. coli* (control) comparison with DNA profile of DMSO-treated *E. coli*		
	Total bands	Polymorphic bands	Polymorphism (%)	Total bands	Polymorphic bands	Polymorphism (%)
Rep-2A	11	5	45.4	11	1	9.09
Rep-2C	10	6	60	10	1	10
Rep-2D	6	2	33.3	6	0	0
Rep 12	8	4	50	8	1	12.5
Rep 18	6	2	33.3	6	1	16.6
Rep 19	10	8	80	10	3	30
BOX A1R	9	3	33.3	9	1	11.1
BOX B1	5	1	20	5	0	0
BOX C1	13	10	76.9	13	4	30.7
$(GTG)_5$	7	2	28.5	7	0	0
Total	85	43	---	85	12	---
Average	8.5	4.3	46.07	8.5	1.2	11.9

DISCUSSION

The various *in vitro* pharmacological properties of pomegranate natural phytochemicals were reported by Howell and D'Souza (2013). In this study, peel extracts of different cultivars showed enhanced antibacterial potencies as measured by the standard disc diffusion methods, taking into context the high resistance of G− strains over G+ ones. This ability to inhibit the growth of a wide-spectrum of bacterial strains was reported in various works (Al-Zoreky, 2009; Gaber *et al*., 2015). However, the mechanisms underlying the antimicrobial potential are not fully understood (Viuda-Martos *et al*., 2010). The peel forms about 50% of the fruit gross weight and represent a rich source of polyphenols, tannins (including ellagitannins and

tannic acid (TA), and flavonoids (Machado *et al*., 2002). These phytochemicals are in part responsible for antimicrobial potencies of pomegranate. Adsorption of polyphenols on bacterial membranes cause membrane disintegration and disruption and flocculation of different cellular contents and consequently, interfere with membrane transport and enzymatic activities (Nychas *et al*., 2003; Tiwari *et al*., 2009). Also, tannins have bactericidal potential resulted from its ability to form stable complexes with proteins or metal ions, like Cu and Fe, or sugars leading to inhibition of enzymatic activities of the cell and disruption of cell wall permeability (Chung *et al.*, 1998; Aguilera-Carbo *et al.*, 2005). Worth mentioning, the high level of vitamin C on fruit peel fractions showed enhanced antibacterial activity against pathogenic bacteria (Opara *et al.*, 2009). It was suggested that antibacterial efficacy of pomegranate is dependent on cultivar types and geospatial factors. Hence, it was suggested that the difference in values of susceptibility testing may result from the differences in phytochemical constituents of the tested cultivars as reported by Duman *et al.* (2009).

In this study, rep-PCR technique was used to investigate the effect of peel extracts on bacterial gDNA. The rep-PCR technique could differentiate between the closely related bacterial strains. In general, the primers of rep-PCR displayed polymorphism between DNA profiles of *E. coli*, treated and untreated. The effect of phytochemicals of pomegranate peel extracts on bacterial DNA was studied scarcely. Maxwell (1993) referred to the antibacterial drugs applied to target the DNA gyrase and DNA topoisomerase by binding to B subunit of these enzymes; subsequently, ATP hydrolysis inhibition that required for ATP-dependent DNA supercoiling. Also, ellagitannins have the ability to inhibit DNA gyrase that affect DNA replication (Weidner-Wells *et al*., 1998). In addition, the transcriptional and translational steps are putatively affected by these tannins (Sakagami *et al*., 2001). Lim *et al.* (2013) concluded that TA widely affects expression profiles of many homeostases-regulatory genes of *Pseudomonas protegens* Pf-5 total transcriptome. Furthermore, phenolics affect the expression of antibiotic biosynthesis genes of *P. protegens* (de Werra *et al.* 2011).

The study has been proposed that the polymorphic banding patterns of tested bacterial strains are due to the effect of phytochemicals in pomegranate extracts in agreement with Gaber *et al*. (2015). Phytochemicals may play a role as bacterial mutagens which induce frame shift mutations. These mutations are resulted from deletion of at least one nucleotide, causing disappearance of some genetic material and hence, resulted in changing the primer matching sites. The deletion or frame shift mutations cause interruption in DNA and protein synthesis and affect gene over all expression.

CONCLUSION

The four tested pomegranate cultivar peels showed enhanced antibacterial activities against the tested pathogenic strains. The phytochemicals of peel extract are largely responsible for the antibacterial activity of pomegranate. Also, this phytochemicals may have effects on bacterial gDNA. Therefore, extraction, purification and production of phytochemicals of pomegranate are a must to develop different therapeutic purposes, the subject of future work.

Conflict of interest:

Authors have no conflict of interest.

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فحص كفاءة أصناف مختلفة من الرمان كمضادات ومطفرات للبكتريا بإستخدام rep-PCR

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يُعتبر الرمان مصدراً غنياً بالعوامل المضادة للميكروبات ، وفى هذه الدراسة وبإستخدام طريقة Disc diffusion تم تقييم كفاءة مستخلصات القشر من أربعة أصناف من الرمان (Wonderful , H116, Manfalouty, Black) كعوامل مضادة للبكتريا الممرضة وهى *Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium, Pseudomonas aeruginosa* and *Escherichia coli* . وكذلك تم تقييم التأثير الناتج عن المعاملة بمستخلصات القشر كتأثير مُطفر على المادة الوراثية لبكتريا *Escherichia coli* (كسلالة مرجعية) عن طريق الإختلافات الوراثية التى تنتج بإستخدام rep-PCR DNA fingerprinting technique. وقد أوضحت النتائج أن كل مستخلصات قشر الرمان سواء مستخلصات الـ Methanol أو مستخلصات الـ DMSO أظهرت نشاطاً وكفاءة ضد السلالات البكتيرية المستخدمة مقارنة بالكنترول. وعلى جانب أخر فقد تم الإستدلال علي التأثير المطفر لمستخلصات القشر عن طريق نسبة الـ polymorphism التى أظهرها تكنيك الـ rep-PCR فى المادة الوراثية للسلالة المُعاملة مقارنة بغير المُعاملة حيث تبين أن مستخلص الـ Methanol كان أكثر تأثيراً من مستخلص الـ DMSO على المادة الوراثية ، وبذلك فإن الدراسة تشير إلى أن قشر الرمان يعد مصدراً جيداً يمكن تطويره كمضادات للبكتريا ومُطفر للمادة الوراثية فى البكتريا