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Screening of Natural Bacterial Flora of Pomegranate Roots (*Punica granatum* L.) and their Antibiotic Activity in Taif, Saudi Arabia

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A B S T R A C T

Taif region is situated in the central foothills of the Sarawat mountains at an altitude of 2200 to 2500 m above the sea level. Special type of pomegranate is cultivated there and called taify pomegranate. Therefore, exploring the types of microorganisms tolerant to root exudates is important. The objective of this study was to identify bacteria, actinomycetes and potential bacterial pathogens of pomegranate roots and their antimicrobial susceptibility patterns as well as effect of fruit parts extract on isolated bacteria. A total of 102 isolate were isolated and identified by morphological, physiological, APi profiles and 16S-rRNA techniques. Out of 102 isolated bacteria, from free soil 38 (37.3%), rhizosphere 55(53.9%) and inside roots 9(8.8%). Isolated *Actinomycetes* represent 23 (22.5%), *Streptomyces*21 (20.6%) and *Sacchromyces cerevieace* 5(4.9%) of all isolated microorganisms. Isolates were identified as *B. subtilis, S. cerevieace, S. pneumoniae, S. pyogenes, K. pneumoniae, A. baumannii, P. aerogenosa, A. chroococum, H. seropedicae, A. lipoferum*and *E.aerogenes.* Susceptibility testing was done according to Clinical and Laboratory Standards Institute (CLSI) guideline. Tetracycline was effective against 75.5% of isolated pathogens. In general, Gram positive isolates were more susceptible to all antibiotic tested, whereas Gram negative isolates were less susceptible to all antibiotic tested. Antimicrobial activity of crude extracts of rind, fruit, and juice ofpomegranate fruitswere evaluated against 18Gram-positiveand 35 Gram-negative bacteria. Crude alcoholic extracts of rind, seeds extract (juice) and whole fruit were evaluated against isolated Gram-positive and Gram-negative by agar disc diffusion method. All fruit parts were active against all tested bacteria (*B. subtilis, E. aerogenes, K. pneumonia* and *P. aerogenosa*), and moreover, against N_2-Fixing bacteria (*A. chroococcu*m, *A.lipoferum* and *H.* seropedicae). Rind extract was less active against *P. aeruginosa*, juice against *E. aerogenes*, whole fruit extract against *B. subtilis*. In general, juice (seed extract) was relatively more active than extracts of other parts of the fruit. The descending order of activity of the extracted parts of the plant fruit was juice, rind and whole fruit, respectively. On the other hand, Gram- positive bacteria, *B. subtilis S. pneumoniae* and *A. chroococcum* were more sensitive than Gram-negative and their inhibition zones. Therefore, it may be concluded that fruits of pomegranate extracts have broad spectrum antimicrobial activity and their activity could be attributed, to a great extent, to their contents of alkaloids and flavonoids.

Introduction

Taif is a city in the Mecca Province of Saudi Arabia at an elevation of 1,879 m (6,165 ft) on the slopes of the Sarawat Mountains**.** Al Shafa is a small village situated high upon the Sarawat Mountains at an elevation of 2200 to 2500 meters above sea level, rich in agricultural products. Pomegranate (*Punica granatum* L.) is one of the important fruit crops in arid and semi-arid regions**.** Taif especially, in Shafa area produce pomegranate and grapes as remarkable fruits. Panichayupakaranant et al. (2010) reported that pomegranate (*Punica granatum* L.) is an ancient and important fruit crop of subtropical and tropical regions of the world. Although native of Iran and adjoining areas, pomegranate has been widely cultivated throughout India and Mediterranean regions of Asia, Africa and Europe. India is the largest pomegranate growing (1.3 lakh ha) and producing (11 lakh tons) country of the world followed by Iran with an area of 56,239 ha and production of 7.05 lakh tons. Pomegranate (*Punica granatum* L.), a species of Punicaceae, has recently become of great interest to the scientists who engage themselves in pharmaceutical, nutriological and pharmacological research, and new drug development, due to its distinctive multiple officinal parts and multiple bioactivities such as hypolipidemic, antioxidant, antiviral, anti-neoplastic, antibacterial, anti-diabetic, anti- diarrheal, and helminthic effects.

Kloepper 2003 and Bakker et al. 2007 reported that among PGPRs are representatives of the following genera: *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Azospirllium* and *Thiobacillus*. Some of these genera such as *Azoarcus* spp., *Herbaspirillum* and *Burkholderia* include endophytic species.

The pomegranate tree, which is said to have flourished in the Garden of Eden, has also been extensively used as a folk medicine in many cultures, documented at least as far back as the Egyptian Papyrus of Ebers, 50 B.C. (Wren, 1988). The most famous usage worldwide has been as a vermifugal or taenicidal agent (Zhicen, 1987; Kapoor, 1990), i.e., a killer and expeller of intestinal worms. According to one account, the alkaloids contained in the root, tree bark, and to a lesser extent, fruit rind, cause the "tapeworm to relax its grip on the wall of the intestine" thus allowing the weakened parasites to be easily expelled by a second herbal drug, one which is cathartic (Wren, 1988). The second major property of pomegranate hulls exploited in folk medicine is their strong astringency, making them a popular remedy throughout the world, in the form of an aqueous decoction (i.e., boiling the hulls in water for 10-40 minutes), for dysentery and diarrhea, and also for stomatitis (Boukef 1982; Caceres., 1987; Nagaraju and Rao, 1990). The decoction can be drunk, used as a mouthwash, douche or enema. Eating pomegranate regularly ensures optimal dental health as it destroys the bacteria responsible for causing formation of plaque.

Other ethnomedical explorations have documented pomegranate hull and/or root extract usage both orally and intravaginally to prevent fertility (Gujral *et al*.1960,Jochle, 1971) and abortion (Ramirez,1988) and to ameliorate assorted gynecological problems (Singh, 1980; Goh,1984). Other traditional uses of these materials have included treatments for snakebite (Jain and Puri 1984), diabetes (Singh 1986), burns (Siang 1983) and leprosy (Singh 1980). The fresh

fruit itself has been used as a refrigerant to lower fever (Arseculeratne 1985).

Panichayupakaranant et al. (2010) studied antibacterial, anti-inflammatory and anti-allergic activities of standardised pomegranate rind extract (SPRE) containing 13% w/w ellagic acid *in vitro*. The antibacterial activity of SPRE was determined using the disc diffusion and broth microdilution methods. SPRE exhibited a potent bacteriostatic effect against *Propionibacterium acnes*, a Gram-positive anaerobe, with a MIC of 15.6 lg/ml, and Gram positive facultative anaerobic bacteria, *Staphylococcus aureus* and *Staphylococcus epidermidis*, with MICs of 7.8–15.6 lg/ml. Anti-inflammatory activity of SPRE was evaluated by measuring the inhibition of nitric oxide (NO) production by murine macrophage-like RAW264.7 cells. SPRE exhibited a potent NO inhibitory effect, with an IC50 of 10.7 lg/ml. Evaluation of the anti-allergic activity showed that SPRE inhibited the release of b-hexosaminidase from antigen-stimulated rat basophilic leukemia (RBL-2H3) cells with an IC50 of 20.9 lg/ml. In addition, SPRE exhibited only moderate cytotoxicity on human keratinocyte cells, with CC50 of 33.6 lg/ml. These findings support the potential use of SPRE as a nutraceutical for antibacterial, anti-inflammatory and anti-allergic proposes.

The historical method for identification of new isolated microbes is dependent on the comparison of an accurate morphologic and phenotypic description of type strains or typical strains with the accurate morphologic and phenotypic description of the isolate to be identified. Microbiologists authoring standard references such as Bergey's Manual of Systematic Bacteriology or the Manual of Clinical Microbiology or compiling results from well-characterized strains such as those found at the Centers for Disease Control and Prevention or the American Type Culture Collection (ATCC) would publish tables summarizing the characteristics of each species of bacteria (Murray et al. 1999). Microbiologists would try to match the results for their unknown bacterial strain with a group in these tables. Not infrequently, there would be no perfect match and a judgment would have to be made about the most probable identification. Although various schema and computer programs were devised to help in these judgments, identification could vary among laboratories (Sussman et al. 1986). In the 1980s, a new standard for identifying bacteria began to be developed. In the laboratories of Woese and others, it was shown that phylogenetic relationships of bacteria, and, indeed, all life-forms, could be determined by comparing a stable part of the genetic code (Woese 1985).

Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S (also called the small subunit), and the 23S rRNA and the spaces between these genes. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S-rRNA gene (Garrity et al. 2001, Tortoli, 2003). The 16S- rRNA gene is also designated 16S-rDNA, and the terms have been used interchangeably: current ASM policy is that "16S-rRNA gene" be used. The 16S- rRNA gene can be compared not only among all bacteria but also with the 16S-rRNA gene of archeobacteria and the 18S rRNA gene of eucaryotes.

The use of PCR, over the last years, rapid purification, and automated DNA sequencing has significantly reduced the time to yield a high-quality sequence. The use of 16S- rRNA gene sequencing to study the relatedness of prokaryotic species is well

established and has led to increased availability of 16S- rRNA databases. The convergence of these technical and computational advances has also enhanced the application of 16S- rRNA gene sequence analysis to bacterial identification (Rantakokko-Jalava *et al.*, 2000). Recently, it was reported that subtle sequence differences in the 16S rRNA gene could be used for bacteria identification (Sacchi *et al.*, 2002) and for subtyping and identifying bacterial clones (Nilsson et al., 2003). Molecular techniques are used as identification tools for specific strains, genotyping and confirmation of the results. PCR and sequence analysis provide further information on the phylogenetic characteristics of the strains identified Harwood et al. (2009). Direct PCR (DPCR) provides an improved method for the detection and quantification of bacteria in samples (Fode-Vaughan et al. 2001). This is a more rapid and simple approach because the untreated environmental sample is used directly as a template in PCR, eliminating the steps of cell recovery or DNA extraction.

The information about prevalence and types of bacteria and *Actinomycetes* of pomegranate roots in Taif is scarce. To our knowledge, no research has been conducted on prevalence, isolation and identification of bacteria and potential bacterial pathogens and its distribution in pomegranate plant as well as their antibiotic susceptibility pattern. Therefore, the objective of this study was to isolate and identify potential bacterial isolates using morphological, biochemical, physiological, API test and 16S-rRNA techniques. In addition to, its distribution in pomegranate root area and their antibiotic susceptibility pattern. Also, we report the antimicrobial activity of pomegranate fruit extracts against different types of isolated bacteria from roots and soil surrounding the plant roots.

Materials and Methods

Sampling

Soil samples and pomegranate roots were collected from Al Shafa area and the university campus in Taif. Specimens of roots and soil were periodically collected. Standard microbiological techniques were used in isolation and identification of microorganisms (Holt et al., 1994).

Media

Different media were used for enumeration, isolation and identification.e.g. Plate count (Diffico, 1990), MacConky agar media, nutrient agar (Diffico, 1990), Jensen's media (Jensen et al. 2005), modified Tarozzi agar (Linzenmeier, 1958)and *S. griseus* agar media (Shirato and Motoyama, 1966).N-free media for N_2- fixing bacteria, Sabarroid media for fungi and MacConky media for pathogens.

Enumeration of Microorganism

Samples were collected from Taif regions. Samples were enumerated within 24 h by making tenfold serial dilution of samples using physiological saline. From the diluted sample, using a dropper pipette, 1 ml of each dilution was dropped onto Petri dish then the plate count agar for bacteria, and Jensen's agar medium, Tarozzi agar, *S. griseus* medium for *Actinomycetes* and *Streptomyces* were poured (Gerdhardt *et al.* 1984). Triplicates of plates were used for each dilution. Aliquots of freshly sieved soil were accurately weighed and then dried at 105°C for 3 days. The samples soil or grinded roots were then reweighed after they were first allowed to cool to room temperature in a desiccator. The factor for conversion of fresh weight to dry weight of soil were calculated, and all results were expressed

per gram (dry weight) of soil, rhizosphere soil and/or dry gram roots.

Isolation of Microorganisms

Different selective media were used as mentioned above in order to isolate potent *Actinomycetes* and/or *Streptomyces* for antibiotic production and different types of bacteria from the collected samples. Characterization of isolated microorganisms based on the morphological, physiological, and the biochemical characteristics presented in Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). Samples were processed within 24 h by making tenfold serial dilution of samples using physiological saline. From the diluted sample, using a dropper pipette, 1 ml of each dilution was dropped onto Petri dish then the nitrogen- free media for N_2- fixing bacteria, MacConky media for pathogens and Jensen media, nitrate-sucrose agar, for *Actinomycetes* were poured (Gerdhardt *et al*. 1984). Duplicates of plates were used for each dilution. Plates were incubated for 24 – 48 h at 30^{o}C for bacteria and 10-15 for *Actinomycetes* in an incubator. Each inoculum of microorganism developed into a discreet colony. All plates yielding 30 - 300 colonies were counted. The number of viable microorganisms in the sample were calculated from the number of colonies formed, the volume of inoculum used by dropper pipette and the dilution factor expressed in colony forming unit (CFU) (Krieg, 1984).

Identification and Characterization of Microorganism

The morphological characteristics of the isolates were identified by gram stain and biochemical reactions. The biochemical reactions include glucose fermentation, oxidase test, catalase production reaction; cell motility and reaction in tryptose soya broth were performed. According to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and the APi Kit profiling (Api, bioMerieux, France, 2009) isolates were identified and named based on morphological, physiological and biochemical characteristics. The genus-specific 16S- rRNA gene analysis were used to identify the isolates at the molecular level for further confirmation.

Culture Condition and Growth

Inocula were pregrown in 10 ml LB broth medium for 12 h. Cells were grown aerobically in 50 ml Erlenmeyer flasks. Flasks were filled to no more than 20 % capacity. All isolates were growing at 30^{o} C in an incubator shaker at 150 rpm. The absorbency of the culture was measured with a spectrophotometer at 660 nm. Cultures were usually harvested at absorbency 0.660. Cells were harvested by centrifugation for 5 min at 3,000 x g at room temperature (Krieg, 1984).

Antimicrobial Susceptibility

All isolates belonging to various genera were tested. Antimicrobial susceptibility testing was performed using the disc diffusion method on Mueller-Hinton agar plates for all bacteria and pathogens. The plates were incubated aerobically at 30°C for 18-24 hours for bacteria. The diameters of the zones of inhibition were measured with a ruler and compared with a zone-interpretation chart (NCCLS, 1999). Escherichia coli were used as the control. The antibiotics tested on each disc were Ampicillin 25 µg, Nalidixic Acid 30 µg, Ceftriaxone 30 µg, Augmentin (20/10 µg), Tetracycline (62.2%), Piperacillin 100 µg, Ciprofloxacin 30 µg, Gentamicin 10 µg and Amikacin 30 µg. Results of disk diffusion

method were interpreted in accordance to the Clinical and Laboratory Standards Institute (CLSI, 2009). *Streptomyces* and *Actinomycetes* were tested for production of antibiotics (data not shown).

Disc Diffusion Method

Antibacterial activities of the crude extracts were tested by using agar disc diffusion method as described by Kirby-Bauer with modification (Bauer et al. 1966).

16S-rRNAgeneAnalysis

Standard DNA methods (DNA isolation (using GenElute Bacterial Genomic DNA Kit, Mini-(SIGMA), agarose gel electrophoresis of DNA, restriction enzyme digestion, will be used as described by Ausubel *et al*. (1989) and Maniatis *et al*. (1982). To identify the isolates at the molecular level, the genus specific 16S-rRNA gene analysis using PCR were carried out. The forward primers and reverse primer were designed according to the convention of the Oligonucleotide Probe Database (OPD). The PCR were performed in a 50µl reaction volume containing 1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 2mM $MgCl_2$, 0.01% (w/v) gelatine), 250µM each of dGTP, dATP, dCTP and dTTP (dNTPs), 2.5 units of Taq DNA polymerases, 100 pmol of each primer and the DNA template, which released from the bacterial cells by boiling in a water bath for 5 min to lyses the cells and then the tubes were spun briefly to collect the condensate (Ausubel *et al*., 1989). PCR reaction conditions were: 94ºC for 3 min; 35 cycles of denaturation at 94ºC; annealing at 52ºC; and extension at 72ºC for 2 min each, followed by 7 min extension at 72ºC. Sequencing of the 1000 bp PCR product were carried out with the automated DNA sequencer (ABI, Gene line DNA sequencing, New York). The deduced sequence was aligned with the DNA data base available at the gene bank using the BLAST of the (http# www.ncbi.nlm.nih.gov/Blast/cgi).

Sequence Similarities and Phylogenetic Analysis

The BLAST program (www.ncbi.nlm.nih.gov/blst) were employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (Hall, 1999).

Pomegranate Fruits

The pomegranate fruits were collected from a taify farm at Taif. Different parts of the fruits was used rind, juice, seeds (white and red) and whole fruit extracted with methanol and aqueous extract.

Methods of Extraction

The fresh fruits was cleaned, freeze-dried and grounded into fine powder using an electric blender. The powder was dried in an oven at 40°C for 24 h, then the fine powder was sieved through 24-mesh. The fine powdered sample (10g) was extracted with 250 ml 80% methanol in water at room temperature (25°C) for 24 h in a shaking water bath. The extract was filtered by a Millipore filter with a 0.45µm nylon membrane under vacuum at 25°C. The samples wasstored at 4°C until use. For aqueous extract the fine powdered sample (10g) was extracted with 100ml of distilled water.

Data Analysis

All determinations were carried out in triplicates and the statistical analyses were carried out using SPSS 13.0.

Results and Discussion

Prevalence of Microorganisms in Pomegranate Roots

Total bacterial count and actinomycetes were estimated in soil, rhizosphere soil, as well as roots of pomegranate (Table 1). *Actinomycetes* and *Streptomyces* enumerated on 3 different media shown in Table (1). Moisture contents were ranged from 78.8-86.2% in all collected samples. The total bacterial count in soil, rhizosphere soil and roots of pomegranate was $1.7x10^5$ - $23.6x10^5$, $2.3x10^6$- $13.0x10^6$, and $1.7x10^3$- $28.3x10^3$, respectively. The total bacterial count was higher in rhizosphere soil than free soil as well as inside roots of pomegranate. This could be explained by abundance of plant root exudates, nutrients attracted by plant roots from surrounding soil and microorganisms exudates. The R/S ratio was between 2.3 - 5.51 in all analyzed samples. The presence and numbers of *Actinomycetes* and *Streptomyces* were various among samples of soil, rhizosphere soil and/or plant roots (Table 1). In general, numbers were higher in rhizosphere soil than free soil or inside plant roots. No growth was observed on *S. griseus* medium or Tarozzimedium for one free soil sample (2) or inside root sample (3).

Some microorganisms are able to colonize soil surrounding plant roots, the rhizosphere, making them come under the influence of plant roots (Hiltner 1904, Kennedy 2005). Rhizobacteria are rhizosphere competent bacteria able to multiply and colonize plant roots at all stages of plant growth, in the presence of a competing microflora (Antoun and Kloepper 2001) where they are in contact with other microorganisms. This could be explained by the interactions between plants and microorganisms and can be classified as pathogenic, saprophytic, and beneficial (Lynch1990). Beneficial interactions involve plant growth promoting rhizobacteria (PGPR), this refers to a group of soil and rhizosphere free-living bacteria colonizing roots in a competitive environment and exerting a beneficial effect on plant growth (Kloepper 2003, Bakker et al. 2007). However, numerous researchers considered rhizobacteria as endophytic bacteria in symbiotic association with plants (Bakker et al. 2007) e.g. Rhizobia with legumes and the actinomycetes with Frankia. Kloepper 2003 and Bakker *et al*. (2007) reported that among PGPRs are representatives of the following genera: *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Azospirllium*and *Thiobacillus*. Some of these genera such as *Azoarcus* spp., *Herbaspirillum*, and *Burkholderia* include endophytic species. However, *Pseudomonas* and *Bacillus* species constitute, together with *Streptomyces* species, the most bacteria often found in the rhizosphere of many plants. The soil microflora largely depends on the type of soil, temperature, moisture, plant growth, nutrients, pH, and many other factors which may vary between locations but also within a single plot and over very small distances (OECD, 2007).

Morphological, and Biochemical Characterization of Isolate

Isolates from free soil, rhizosphere soil and/or roots of pomegranate were isolated by enrichment culture technique and deposited in our microbial bank at Taif University, Saudi Arabia in our laboratory. The isolates were identified on the basis of their cultural, physiological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (9^{th} edition)

(Holt et al. 1994) and Api kit profiles (ApiBioMerieuxsa, 2009). About 102 isolates were collected in pure culture, 58 isolates were bacteria and 44 were *Actinomycetes* and *Streptomyces*. Phenotypic examination of the recovered isolates revealed that they belong to the genera of *Bacillus, Sacchromyces, Azotobacter, Azospirllum, Enterobacter, Herbaspirllum, Staphlococcus, Acintobacter, Streptococcus, Klebseilla, Streptomyces Actinomyces*and *Pseudomonas* (Table 2). About 37.3, 53.9 and 8.8% of isolated strains were isolated from free soil, rhizosphere and inside roots, respectively. All selected strains showed optimal growth at 30^{o}C but grows in different media. Strains were local isolates isolated by enrichment culture technique and deposited in our microbial bank at Taif University, Saudi Arabia in our laboratory.

16S rRNA Gene and Genetic Identification of Isolates

Sequencing of 16S- rRNA gene as a PCR based technique was used to identify the selected bacterial isolates. According to the alignment at the National Center for Biotechnology Information (NCBI), the sequences of studied isolates in (Table 2) were identified as *Bacillus subtilis, Sacchromyces cerevieace, Azospirllum lipeforum, Azotobacterchroococcum, Herbaspirllum seropedicae, Entrobacter aerogenes, S. pneumoniae, Acinetobacter calcoaceticus, S. pyogenes, K. pneumonia, Streptomyces griseus, Actinomyces octodloyts, P. aerogenosa* (DeSantis *et al*, 2006, El- Tarras *et al*. 2012, Shahaby *et al*. 2012, Shahaby 2014, Shahaby *et al*. 2015).

Antibiotic Resistance Profile of Bacterial Isolate

This study showed that antimicrobial susceptibility of isolated bacteria was: Ampicillin (37.7%), Nalidixic Acid (62.3%), Ceftriaxone (73.6%), Augmentin (64.2%), Tetracycline (75.5%), Gentamycin (75.5%), Ciprofloxacin (66.04%), Piperacillin (26.4%), and Amikacin (54.7%), (Table 3). Microorganisms are normally present in soil and more around plant roots. Some bacteria are pathogens and can live and survive inside roots of plants. The use of antibiotics for treatment of severe infections in human is routine in clinics and hospitals practice resulting in increased drug resistance. In this study, among the commonly used topical antibiotics 73.6 and 62.3% of all strains were piperacillin and ampicillin resistant, respectivily. *S. pyogones* isolate was sensitive to all antibiotics except for ciprofloxacin and piperacillin. While all *S. pneumonia* isolates were sensitive tonalidixic acid and ceftriaxone (Cef). About 73.6 % and 69.3% of all strains were resistant to both piperacillin and ampicillin, respectively. Moreover, 60.0% of all strains of *E. aerogenes* were nalidixic acid and piperacillin resistant. All *P. aerogenosa* isolates were resistant to ampicillin, augmentin and amikacin antibiotics. In addition to, 75.0% of *K. pneumonia* isolates were resistant to ampicilin, augmentin and amikacin. The N_2 – fixing *A. chroococcum* isolates were susceptible to tetracycline, while 71.4% of isolates were resistant to ceftriaxone and ciprofloxacin. The resistance of *A. lipeforum* isolates was between 10- 80 % for all antibiotics. While *Herbaspirllum* spp.resistance to antibiotics was 11.1-66.7%. *Acinetobacter baumannii* isolates were susceptible to both nalidixic acid and augmentin however, 50% of isolates were resistant to other antibiotics tested.

These results are in agreement with the study conducted by Caldwell *et al*. (1992), Joseph, (2009), Anagaw *et al*. (2011), Shahaby *et al*. (2015) and Tewelde *et al*. (2013). The increased susceptibility of some

isolates e. g. *S. pneumonia*, *S. pyogones*, *K. pneumonia*, *P. aerogenosa* and *A. baumanni* might be due to root exudates of pomegranate plant. Also, the reason for increased resistance for some antibiotics may be earlier exposure of the isolates to these drugs. In addition to, these drugs are very common due to low cost and often purchased without prescription in different areas (Jorgensen *et al*. 2015).

Table.1 Total Bacterial Count,*Actinomycetesand Streptomyceson* Various Media in Soil, Rhizosphereand Roots of Pomegranateplant

Sample+	Total count					
	Bacteria	*Actimycetes*				
	Moisture %		R/S ratio	Jensen's Medium*	*S. griseus* Medium**	Tarozzi Medium***
Soil	78.8	$23.6x10^5$	5.51	$1.6x10^4$	$0.8x10^4$	$2.0x10^4$
Rhizosphere	78.8	$13.0x10^6$		$0.3x10^5$	$1.1x10^4$	$0.\ 1x10^5$
Root		$28.3x10^3$		$1.6x10^3$	$0.28x10^5$	$2.83x10^3$
Soil	86.2	$9.31x10^5$	2.5	$12.93x10^3$	-	$11.38x10^3$
Rhizosphere	86.2	$2.3x10^6$		$11.1x10^2$	$9.3x10^4$	$3.1x10^3$
Root		$1.7x10^3$		$25.1x10^4$	$12.1x10^3$	$18.1x10^2$
Soil	82.5	$1.7x10^5$	2.3	$5.3x10^3$	$1.6x10^4$	$1.1x10^2$
Rhizosphere	82.5	$3.9x10^6$		$0.5x10^4$	$0.3x10^2$	3.1x10
Root		$2.8x10^6$		$1.4x10^4$	-	-

+, Each sample is an average of 5 mixed samples

-, No growth; *, Jensen *eta.l* (2005); **, Shirato and Motoyama (1966); ***, Linzenmeier (1958)

Table.2 Prevalence of Bacterial Isolates in Free Soil, Rhizosphere and Roots of Pomegranate

Isolates	Soil	Rhizosphere	Roots	Total
Bacillus subtilis	2	4	1	7
Sacchromycescerevieace	2	3	-	5
Azospirllumlipeforum	3	5	2	10
Azotobacterchroococcum	3	4	-	7
Entrobacteraerogenes	1	3	1	5
Streptococcus spp.	-	1	-	1
*Herbaspirllum*spp	3	4	2	9
*Actinomyces*spp	10	12	1	23
Streptomyces spp	11	10	-	21
K. pneumoniae	1	2	1	4
*Acinetobacter*spp.	-	2	-	2
S. pneumoniae	1	2	-	3
P. aerogenosa	1	3	1	5
Total	38	55	9	102

Table.3 Selected Antibiotics Sensitivity Pattern of Selected Isolated Microorganisms

Antibiotic* / Isolates	Number of strains sensitive to antibiotics (%)									
	No	Amp	Nal	Cef	Aug	Tet	Gen	Cip	Pip	Ami
G^+ bacteria										
B. subtilis	7	4 57.1**	5 71.4	6 85.7	4 57.1	4 57.1	4 57.1	6 85.7	3 42.9	5 71.4
A. chroococcm	7	3 42.9	6 85.7	2 28.6	5 71.4	7 100	5 71.4	2 28.6	nd	4 57.1
S. pneumoniae	3	2 66.7	3 100	3 100	2 66.7	2 66.7	1 33.3	2 66.7	1 33.3	2 66.7
S. pyogones	1 100	1 100	1 100	1 100	1 100	1 100	1 100	0	0	1 100
Total G^+	18	10 55.6	15 83.3	12 66.7	12 66.7	14 77.8	11 61.1	10 55.5	4 22.2	12 66.7
G^-bacteria										
A. lipeforum	10	2 20.0	5 50.0	9 90.0	8 80.0	8 80.0	9 90.0	9 90.0	nd	6 60.0
E. aerogenes	5	3 60.0	2 40.0	4 80.0	4 80.0	4 80.0	4 80.0	nd	2 40.0	3 60.0
H. seropedicae	9	3 33.3	4 44.4	8 88.9	7 77.8	6 66.7	5 55.6	7 77.8	5 55.6	6 66.7
K. pneumoniae	4	1 25.0	3 75.0	4 100	1 25.0	4 100	3 75.0	4 100	3 75.0	1 25.0
P. aerogenosa	5	0	2 40.0	1 20.0	0	3 60.0	5 100	5 100	nd	0
A. baumannii	2	1 50.0	2 100	1 50.0	2 100	1 50.0	1 50.0	nd	nd	1 50
Total G^-	35	10 28.6	18 51.4	27 77.1	22 62.9	26 74.3	27 77.1	25 71.4	10 28.6	17 48.6
Overall total	53	20 37.7	33 62.3	39 73.6	34 64.2	40 75.5	38 71.7	35 66.04	14 26.4	29 54.7

*Ampicillin (Amp), Nalidixic Acid (Nal), Ceftriaxone (Cef), Augmentin (Aug), Tetracycline (Tet), Gentamycin (Gen), Ciprofloxacin (Cip), Piperacillin (Pip), Amikacin (Ami), Not done (nd). **, % sensitive strains

Table.4 Effect of Different Pomegranate Extracts on the Selected Isolated Bacterial Cultures in Solid Medium

Diameter of inhibitionzoneinmm(DIZ)			
Bacteria Tested	Rind	Seeds Extract (Juice)	Whole fruit
B. subtilis	20	18	11
Entrobacteraerogenes	19	16	18
K. pneumonia	21	24	26
S. pneumoniae	20	17	19
A. chroococcum	18	19	12
A. lipoferum	21	20	18
P. aerogenosa	17	22	15
Herbaspirllumseropedicae	18	19	20

Effect of Pomegranate Fruit Extracts

Phytochemical constituents of medicinal plants are secondary metabolites that may act as antimicrobial agents (Marjorie 1999). In this study extracts of *pomegranate* fruits were evaluated for their antimicrobial activities. Crude alcoholic extracts of rind, seeds extract (juice) and whole fruit were evaluated against isolated Gram-positive and Gram-negative by agar disc diffusion method. As shown in Table 4, while, fruit parts were active against all tested isolated bacteria (*B. subtilis*, *E. aerogenes*, *K. pneumonia* and *P. aerogenosa*), and moreover, against N_2-Fixing bacteria (*A. chroococcum*, *A.lipoferum* and *H. seropedicae*). Rind extract was less active against*P. aerogenosa* (17mm), juice against *E. aerogenes* (16mm), whole fruit extract against *B. subtilis*(11mm).The inhibition zone diameters of alcoholic extracts of different parts of pomegranate fruit against the eight tested bacteria ranged between 11 and 26mm (Table 4). In general, juice (seed extract) alcoholic extract was relatively more active than extracts of other parts of the fruit. The descending order of activity of the extracted parts of the plant fruit was jucie, rind and whole fruit, respectively (Table 4). On the other hand, Gram- positive bacteria, *B. subtilis S. pneumoniae* and *A. chroococcum* were more sensitive than Gram-negative and their inhibition zones ranged between 11 and 19 mm. The higher susceptibility of the tested Gram-positive bacteria than Gram-negative bacteria to pomegranate fruit extracts is consistent with previous studies on the antibacterial activity of natural products (Smith-Palmer et al. 1998, Ceylan, Fung 2004, Lopez *et al*. 2005, Panichayupakaranant *et al*. 2010, Howell1 and D'Souza, 2013). This may explained by fact that Gram-negative bacteria possess an outer membrane which acts as a barrier which prevents or decreases the penetration of numerous antimicrobials (Nikaido1996, Gao *et al* 1999, Duffy and Power 2001). Because Gram-positive bacteria lack the outer membrane, and possess only a cytoplasmic membrane, it is more vulnerable to damaging molecules and this leads to the leakage of their cytoplasm contents(KalembaandKunicka2003).

P. aeruginosa and *E. aerogenes* were found to be more sensitive to different extracts of pomegranate fruit compared to the other tested Gram-negative bacteria. This is rather interesting because both organisms are known to be less susceptible to antimicrobials including antibiotics, preservatives, antiseptics and disinfectants (Russell 1991, McDonnell and Russell 1999). This resistance is attributed to the less permeable outer membrane of *P. aeruginosa* (McDonnell and Russell 1999).*K. pneumonia* was the least sensitive Gram-negative bacterium. It was reported previously that this bacterium have a lower susceptibility to the antibacterial effect of *Nigella sativa* extracts (Burkill 1985). This lower sensitivity may be attributed to the surrounding capsule which is made of acidic polysaccharides (Domenico *et al*. 1994). The large partially negatively charged macromolecular structure of the capsule was reported to decrease the uptake of antimicrobials like peptides (Campos et al. 2004). This study showed that the capsule might also decrease the uptake of the antibacterial phytoconstituents of *C. erectus* extracts presumably, as a result of their repulsion or attraction to its charged polysaccharide.

In conclusion, Extracts of different parts of pomegranate fruit investigated in this study possessed broad-spectrum antimicrobial activity against Gram-positive, and Gram-negative. The broad-spectrum antibacterial activity of the fruit extracts, confirms its use

as a health remedy in folklore medicine. Therefore, it may be concluded that fruits of pomegranate extracts have broad spectrum antimicrobial activity and their activity could be attributed, to a great extent, to their contents of alkaloids and flavonoids.

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