

## Determination of bioactive compounds of *Punicagranatum puniceae* and studying its antimicrobial activities against bacteria isolated from El-Manzala water treatment plant

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**Abstract:** *Punicagranatum puniceae* was found to be an effective antimicrobial agent especially in the field of water treatment as reviewed in literature. In this study, two organic solvent [Methanol:H<sub>2</sub>O; Methanol:Chloroform] extracts of *pomegranate peels* were screened for the potential antimicrobial activity through well agar diffusion method against 25 bacterial strains isolated from El-Manzala water treatment plant. All the extracts showed inhibitory activities for gram-positive and gram-negative bacteria. The Methanol:Chloroform extract was found to be effective against 23 bacterial strains and the methanol:H<sub>2</sub>O extract exhibited a higher inhibition activities against 24 bacterial strains. The phytochemical screening of extracts through GC-MS analysis answered for the major derivatives of *pomegranate peels* bioactive compounds.

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### 1. Introduction

*Punicagranatum puniceae*, commonly called pomegranate, recently described as nature's power fruit, is a plant used in folkloric medicine for the treatment of various diseases (Ajaikumar et al., 2005), widely cultivated in the Mediterranean region. Pomegranate has strong antioxidant and anti-inflammatory properties, recent studies have demonstrated its anti-cancer activity in several human cancers (Adhami and Mukhtar, 2007; Longtin, 2003). In addition, pomegranate peel extract with an abundance of flavonoids and tannins has been shown to have a high antioxidant activity (Abdel Moneim et al., 2011). Antimicrobial drug resistance in human bacterial pathogens is a worldwide issue and as a consequence, effective treatment and control of such organisms remain an important challenge. Bacterial resistance has appeared for every major class of antibiotic (Lambert, 2005). Since their introduction the emergence of resistant is evident, particularly for important pathogens such as *Escherichia coli*, *Salmonella spp.*, *Camphylobacter spp.*, *Enterococcus spp.* and *Staphylococcus spp.* Over the last decade research into the antimicrobial properties of traditional plant based medicines has been revisited (Melendez and Capriles, 2006; Navarro et al., 1996). Numerous plants have been screened for antimicrobial properties, for example Holecz et al. (2002) tested 13 plants used in Brazilian traditional medicine and they demonstrated activity against bacteria such as *Staphylococcus aureus* and *E. coli*. Melendez and Capriles (2006) tested 172 plant species used in Puerto

Rico and they demonstrated that 14 of these plants showed activity against bacteria including *S. aureus* and *E. coli*. Prashanth et al. (2001) tested a number of extracts of pomegranates against a range of bacteria (*S. aureus*, *E.coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Bacillus subtilis* and *Salmonella typhi*), and they found activity against all isolates. Braga et al. (2005) observed that pomegranate extracts were able to inhibit not only the growth of *S. aureus* but also the production of enterotoxin. They concluded that, the methanolic extract derived from 200 g of dried pomegranate produced bactericidal effects at 1%(v/v) over an extended incubation period 50 hours.

In the present study, the antimicrobial potency of chloroform, methanol extracts of pomegranate was investigated. Twenty five bacterial strains isolated from El-Manzala water treatment plant were used as test cultures. The antibacterial activity was determined by agar organisms well diffusion method. The preliminary phytochemical screening of plant extract was carried out to identify the derivatives in the extracts by using GC-MS technique.

**The aim of this work:** isolation of bacteria from El-Manzala water treatment plant, Purification and Identification of the isolated bacteria, Sensitivity testing for the isolated bacteria using pomegranate peel extract discs.

### 2. Materials and Methods

The plant extracts were prepared using the solvents water, methanol and chloroform. 10g of pomegranate peels were taken and homogenized with

100ml of the respective solvents. The crude preparation was left overnight in the shaker at room temperature and then centrifuged at 4000rpm for 20mins. The supernatant containing the plant extract was then transferred to a pre-weighed beaker and the extract was concentrated by evaporating the solvent at 60°C. The crude extract was weighed and dissolved in a known volume of dimethyl sulphoxide, to obtain a final concentration of 20mg / 5µl. Finally plants extracts were tested on bacterial isolates using Paper disc method.

## 2.2. Water samples

Samples of raw water before entering El-Manzala water treatment plant from different distances, as well as from treated water sample were collected in a clean, sterile glass bottles. The bottle is filled with raw water sample by placing it under water surface and closed immediately as soon as filled with adequate water sample. Then transferred to laboratory preserved in ice box as soon as possible.

## 2.3. Bacterial isolation, purification and identification

It was performed by inoculating 1.0 ml of primary effluent of each water samples and streaking on the Nutrient, MacConkey and Blood agar medium. Bacterial isolates were purified using streak method on nutrient, MacConkey and Blood agar medium. Colonies of different morphological forms were picked up and re streaked on the surface agar of plates containing the same isolation medium. After incubation for 24 hours, separate colonies of distinct shape and color were picked up and re streaked separately several consecutive times on to the surface of agar plates containing the isolation media to assure purity. Purity was checked up microscopically and morphologically using Gram stain. Pure isolates only were sub cultured on slants of the isolation medium and kept for further investigation. The purified colonies were prepared to be used for a complete biochemical identification using a VITEK 2 Compact system for bacterial identification.

## 2.4. Preparation and extraction of plant material

The plant extract of pomegranate peels was prepared using the solvents water, methanol and chloroform. 10g of the samples were taken and homogenized with 100 ml of the respective solvents. The crude preparation was left overnight in the shaker at room temperature and then centrifuged at 4000 rpm for 20 mins. The supernatant containing the plant extract was then transferred to a pre weighed beaker and the extract was concentrated by evaporating the solvent at 60°C. The crude extract was weighed, concentrated and dissolved in a known volume of dimethyl sulphoxide (DMSO), finally plants extract was tested on bacterial isolates using agar well diffusion method.

## 2.5. Agar well diffusion method

Analytical paper discs (6.0 mm in diameter) were saturated with the plant extract of pomegranate peels and aseptically placed on the surface of the inoculated plates seeded with different bacterial isolate separately.

## 2.6. GC/MS analysis

The qualitative and quantitative compositions of the plant extract were studied by Gas Chromatography and Mass Spectroscopy GC-MS analysis. A Perkin-Elmer gas chromatograph (model 8700), equipped with a flame ionization detector (FID) and HP-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm) was used for the chemical analysis of the essential oils. Injector and detector temperatures were set at 220 and 290°C, respectively. The column oven temperature was programmed from 80°C to 220°C at the rate of 4°C / min; initial and final temperatures were held for 3 and 10 minutes, respectively. Helium was used as a carrier gas with a flow of 1.5 ml /min. A sample of 1.0 µL was injected, using split mode (split ratio, 1:100). A built-in data-handling program provided by the manufacturer of the gas chromatograph (Perkin-Elmer, Norwalk, CT, USA) was used for quantification purposes.

### 2.6.1. Identification of compounds

Identification of compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library data (GC-MS) system (wiley 229) and confirmed by comparison of their retention indices with authentic compounds or with the compounds reported in literature. The relative percentage of each compound obtained according to it's under peak area in GC chromatogram, without the use of correction factors.

## 3. Results and Discussion

Isolation of bacterial isolates was performed by inoculation of water samples on nutrient agar, MacConkey's and blood agar plates, respectively. Results summarized in tables (1, 2, 3 and 4) indicates that total of 63 bacterial isolates could be isolated from 4 different locations at four consecutive times (Spring, Summer, Autumn and Winter seasons).

All bacterial isolates isolated during spring, summer, autumn and winter seasons were identified on a VITEK® 2 compact system for bacterial identification. The VITEK® 2 compact system is a fully automated system that performs bacterial identification by biochemical analysis using colorimetry. The VITEK® 2 compact system is highly automated and allows for the rapid, accurate identification of some bacterial strains in as little as two hours. The system's database is capable of identifying a variety of microorganisms.

Table 1. Bacterial isolates from El-Manzala water treatment plant at various locations growing on different media during spring season.

Location	Media	Result	Code of isolate	No of isolates
Intake	Nutrient agar	+	54	3
	MacCkonkey's agar	+	55	
	Blood agar	+	56	
Outtake	Nutrient agar	+	52	2
	MacCkonkey's agar	-	-	
	Blood agar	+	53	
5 km before Intake	Nutrient agar	+	57	3
	MacCkonkey's agar	+	58	
	Blood agar	+	59	
7 km before Intake	Nutrient agar	+	60	5
		+	61	
	MacCkonkey's agar	+	62	
	Blood agar	+	63	
<b>Total no. of isolates</b>				<b>13</b>

(+) = Positive for bacterial isolation, (-) = Negative for bacterial isolation, (Code of isolate) = the number for each positive bacterial isolate

Table 2. Bacterial isolates from El-Manzala water treatment plant at various locations growing on different media during summer season.

Location	Media	Result	Code of isolate	No of isolates
outtake	Nutrient agar	+	23	3
	MacCkonkey's agar	+	24	
	Blood agar	+	25	
intake	Nutrient agar	+	26	4
		+	27	
	MacCkonkey's agar	+	28	
	Blood agar	+	29	
5km before Intake	Nutrient agar	+	30	4
		+	31	
	MacCkonkey's agar	+	32	
	Blood agar	+	33	
7 km before Intake	Nutrient agar	+	34	4
		+	35	
	MacCkonkey's agar	+	36	
	Blood agar	+	37	
<b>Total No. of isolates</b>				<b>15</b>

(+) = Positive for bacterial isolation, (-) = Negative for bacterial isolation, (Code of isolate) = the number for each positive bacterial isolate

Table 3. Bacterial isolates from El-Manzala water treatment plant at various locations growing on different media during autumn season.

Location	Media	Result	Code of isolate	No of isolates
Intake	Nutrient agar	+	39	4
	MacCkonkey's agar	+	40	
		+	41	
	Blood agar	+	42	
Outtake	Nutrient agar	+	38	1
	MacCkonkey's agar	-	-	
	Blood agar	-	-	
5km before Intake	Nutrient agar	+	43	4
		+	44	
	MacCkonkey's agar	+	45	
	Blood agar	+	46	
7 km before Intake	Nutrient agar	+	47	5
		+	48	
	MacCkonkey's agar	+	49	
		+	50	
	Blood agar	+	51	
<b>Total No. of isolates</b>				<b>14</b>

(+) = Positive for bacterial isolation, (-) = Negative for bacterial isolation, (Code of isolate) = the number for each positive bacterial isolate

Table 4. Bacterial isolates from El-Manzala water treatment plant at various locations growing on different media during winter season.

Location	Media	Result	Code of isolate	No of isolates
Intake	Nutrient agar	+	54	3
	MacCkonkey's agar	+	55	
	Blood agar	+	56	
Outtake	Nutrient agar	+	52	2
	MacCkonkey's agar	-	-	
	Blood agar	+	53	
5 km before Intake	Nutrient agar	+	57	3
	MacCkonkey's agar	+	58	
	Blood agar	+	59	
7 km before Intake	Nutrient agar	+	60	5
		+	61	
	MacCkonkey's agar	+	62	
		+	63	
	Blood agar	+	64	
<b>Total No. of isolates</b>				13

(+) = Positive for bacterial isolation, (-) = Negative for bacterial isolation, (Code of isolate) = the number for each positive bacterial isolate

Table 5. Identification of bacterial isolates isolated from El-Manzala water treatment plant during four seasons of the year.

No.	Suggested name	Organism code number on different media
1	<i>Micrococcus lutes</i>	33-53-55
2	<i>Edwardsiella ictalluri</i>	1-31-43
3	<i>Staphylococcus haemolyticus</i>	2-6-8-57
4	<i>Shigelladysenteriae</i>	3-4-62
5	<i>Klebsiella oxytoca</i>	5
6	<i>Bacillus megaterium</i>	7-9-18-23
7	<i>Bacillus firmus</i>	58
8	<i>Staphylococcus sciuri</i>	42-47-50
9	<i>Staphylococcus gallinarum</i>	20-26-29
10	<i>Staphylococcus Capitis</i>	49-52-61
11	<i>Bacillus pumilus</i>	15-35-54
12	<i>Bacillus coagulans</i>	19-22-25
13	<i>Staphylococcus Vitulens</i>	59
14	<i>Salmonella pullorum</i>	36
15	<i>Citrobacterfreundii</i>	46-51-56
16	<i>Shigella flexneri</i>	48-60
17	<i>Bacillus brevis</i>	32-34
18	<i>Bacillus mycoides</i>	11-12-16-63
19	<i>Enterobacter agglomerans</i>	28-39-44
20	<i>Proteus rettgeri</i>	21-38-40
21	<i>Providencia rettgeri</i>	27-30
22	<i>Enterobacter aewrogenes</i>	10-13
23	<i>Escherichia coli</i>	41-45
24	<i>Sphingomonas paucimobilis</i>	14-17
25	<i>Bacillus cereus</i>	24-37

All the total identified bacterial isolates 63 isolate belong to only 25 bacterial species as follow: *Bacillus cereus*; *B. brevis*; *B. coagulans*; *B. firmus*; *B. megaterium*; *B. mycoides*; *B. pumilus*; *Staphylococcus gallinarum*; *Staphylococcus haemolyticus*; *Staphylococcus capitis*; *Staphylococcus vitulins*; *Staphylococcus sciuri*; *Enterobacter agglomerans*; *Enterobacter aewrogenes*; *Klebsiella oxytoca*; *Edwardsiella ictalluri*; *Citrobacter freundii*; *Salmonella pullorum*; *Shigella dysenteriae*; *Shigella flexneri*; *Providencia rettgeri*; *Proteus rettgeri*; *Micrococcus lutes*; *Sphingomonas paucimobilis*; and *Escherichia coli* as shown in table (5).

Table (6) showed that 24 bacterial isolates including *Bacillus cereus*; *B. brevis*; *B. coagulans*; *B. firmus*; *B. megaterium*; *B. mycoides*; *B. pumilus*; *Staphylococcus gallinarum*; *Staphylococcus haemolyticus*; *Staphylococcus capitis*; *Staphylococcus vitulins*; *Staphylococcus sciuri*; *Enterobacter agglomerans*; *Enterobacter aewrogenes*; *Klebsiella oxytoca*; *Edwardsiella ictalluri*; *Citrobacter freundii*; *Salmonella pullorum*; *Shigella dysenteriae*; *Shigella flexneri*; *Providencia rettgeri*; *Proteus rettgeri*; *Sphingomonas paucimobilis*; and *Escherichia coli* are sensitive to Pomegranate peels extract when extracted with polar:polar solvent [Methanol:H<sub>2</sub>O]

except *Micrococcus lutes* which are resistant.

Twenty three bacterial isolates including *Bacillus cereus*; *B. brevis*; *B. coagulans*; *B. firmus*; *B. megaterium*; *B. mycoides*; *B. pumilus*; *Staphylococcus gallinarum*; *Staphylococcus haemolyticus*; *Staphylococcus capitis*; *Staphylococcus vitulins*; *Staphylococcus sciuri*; *Enterobacter agglomerans*; *Enterobacter aewrgenes*; *Klebsiella oxytoca*; *Edwardsiella ictalluri*; *Citerobacter freundii*; *Salmonella pullorum*; *Shigella flexneri*; *Providencia rettgeri*; *Proteus rettgeri*; *Sphingomonas paucimobilis*; and *Escherichia coli* are sensitive to *Pomegranate peels* extract when extracted with polar:non-polar solvent [Methanol:Chloroform] except *Shigella dysenteriae* and *Micrococcus lutes*.

Similar results were obtained by Dahham *et al.* (2010) when they studied the anti-bacterial and anti-fungal activities of pomegranate peel extract (rind), seed extract, juice and whole fruit on their selected bacteria and fungi. The peel extract has shown highest antimicrobial activity compared to other extracts. The highest antibacterial activity was recorded against *Staphylococcus aureus* in their study. On the other hand, in this study the highest antibacterial activity was recorded against *Enterobacter agglomerans* clear zone= 35 mm when the extraction was carried out by polar:polar solvent Methanol:H<sub>2</sub>O and *Providencia rettgeri* clear zone= 35 mm, *Bacillus pumilus* clear zone= 35 mm, *Edwardsiella ictalluri* clear zone= 35 mm when the extraction was carried out by polar:non-polar solvent Methanol:Chloroform.

In this study, the methanol extract of pomegranate peels showed high antibacterial activity compared to chloroform extract. Similar antibacterial activity was shown by several authors (Trivedi and Kazmi, 1979; Anesini and Perez, 1993; Voravuthikunchai *et al.*, 2004; Jain *et al.*, 2012).

Our results are identical to that observed by Sadeghian *et al.* (2011), the later reported that the aqueous and methanolic extracts of *pomegranate* fruit skin showed good antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, also the methanolic extract presented strong antifungal effect on *Candida albicans*. The methanolic extract was found to be more effective than aqueous one against all the tested microorganisms. They concluded that the extracts from pomegranate fruit skin possess strong antimicrobial activity against the tested microorganisms. Therefore this plant showed important source of new antimicrobial compounds to treat bacterial and fungal infections.

Satish *et al.* (2008); McCarrell *et al.* (2008) and Gould *et al.* (2009) recorded similar results using aqueous extract of *pomegranate*, also similar results were recorded by Voravuthikumchai *et al.* (2005) and

Bajracharya *et al.* (2008) using Alcoholic extract from *pomegranate*.

In this study we use pomegranate peels instead of pomegranate leaf extracts so that our results are nearly comparable with Hegde *et al.* (2012), the later focuses on antimicrobial properties, phytochemical analysis and antioxidant potential of leaf extracts of pomegranate. The methanolic extract inhibited *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi* and *Proteus mirabilis*, whereas the chloroform and aqueous extracts exhibited moderate inhibitory effects against the test bacteria. Comparing to standard on the other hand, only methanolic extract demonstrated antifungal activity against *Aspergillusniger*, *Aspergillus flavus*, *Trichophyton rubrum*, *Candida albicans* and *Cryptococcus sp.* The phytochemical screening of the methanolic extract of the leaves revealed the presence of carbohydrates, reducing sugars, sterols, glycosides, phenolics, tannins, flavonoids, proteins and saponins, whereas, gums were not detected.

Analysis of pomegranate peels extract revealed that the presence of several identified compounds tabulated in table (7) and figure (1). In this study, analysis revealed the presence of hexadecanoic acid, methyl ester (13.3%), n-Hexadecanoic acid (9.61%), Malonic acid, ethyl 3-hexyl ester (4.50%), 1,2,3-Propanetriol, 1-acetate (4.51%), tert-Hexadecanethiol (3.16%), 5-Hydroxymethylfurfural (3.36%), 9,12-Octadecadienoic acid (Z,Z)-methyl ester (2.33%), Ethylene, 1,1-diphenyl- (2.32%), Cyclohexane, 1-(cyclohexylmethyl)-4-(1-methylethyl)- (1.5%), Octadecanoic acid (6.2%). Hexadecanoic acid, methyl ester (13.3%), n-Hexadecanoic acid (9.61%), 1,2,3-Propanetriol, 1-acetate (4.51%), Malonic acid, ethyl 3-hexyl ester (4.50%) and Octadecanoic acid (6.2%) were the compounds showing higher area%.

In our study hexadecanoic acid, methyl ester (13.3%) was the highest abundant compound as shown from both peak area in Table (7) and height of peak in Figure (3), followed by n-Hexadecanoic acid (9.61%). These results are contradicting with Lali *et al.* (2012) who showed that n-Hexadecanoic acid was the highest bioactive compound followed by gamma sitosterol as the second one, they also identified several compounds similar to that observed in our study such as hexadecanoic acid, methyl ester (13.3%), Malonic acid, ethyl 3-hexyl ester (4.50%), 1,2,3-Propanetriol, 1-acetate (4.51%), Hydroxymethylfurfural (3.36%), 9,12-Octadecadienoic acid (Z,Z)-methyl ester (2.33%), Ethylene, 1,1-diphenyl- (2.32%).

Further the presences of several compounds in pomegranate peels were previously reported by several authors. The compounds found in literature were Hydroxymethylfurfural (Keheyani and Giulianelli,



2006), Ethylene, 1,1-diphenyl- (2.32%) (Lali et al. 2012). In conclusion to that part pomegranate peels is a good source of antibacterial compound, different extraction methods and different solvents will elute different bioactive compounds. GC-MS analysis revealed the presence of 10 compounds.

#### 4. Conclusion

- This paper studied antimicrobial activities of the bioactive compounds of pomegranate peels against

bacteria isolated from El-Manzala water treatment plant.

- This work recorded that most of the isolated bacteria was sensitive to pomegranate peels according to antimicrobial sensitivity test applied in this work.
- This is good idea to apply natural products of some desert plants as antimicrobial agents in the future at large scale.

Table 6. Antimicrobial activity of tested pomegranate peels extract against bacteria isolated from El-Manzala water treatment plant.

Plant extract		Methanol:H <sub>2</sub> O extract (10:90 %)	Methanol:Chloroform extract (50:50 %)
		Inhibition Zone (mm)	
1	<i>Staph. Haemolyticus</i>	30	29
2	<i>EdwardsiellaIctalluri</i>	25	35
3	<i>Bacillus Mycoides</i>	25	25
4	<i>KlebsiellaOxytoca</i>	25	25
5	<i>CitrobacterFruendii</i>	25	20
6	<i>Bacillus Brevis</i>	15	16
7	<i>Bacillus Coagulanse</i>	15	25
8	<i>Protusrettgerri</i>	20	20
9	<i>Staphylococcus Sciuri</i>	16	29
10	<i>ShigellaFlexneri</i>	31	29
11	<i>Enterobacteraewrogene</i>	12	11
12	<i>ShigellaDysintrie</i>	20	R
13	<i>Staph. Gallinarum</i>	29	20
14	<i>Bacillus Cerus</i>	25	25
15	<i>Micrococcus Lutes</i>	R	R
16	<i>Bacillus Megterium</i>	30	25
17	<i>EnterobacterAgglomeranse</i>	35	33
18	<i>Salmonellapullorum</i>	16	19
19	<i>ProvidinicaRettegri</i>	25	35
20	<i>Bacillus Pumiles.</i>	25	35
21	<i>Staph. Capitis</i>	30	25
22	<i>SphingomonasPaucimobilis</i>	23	18
23	<i>Staph. Vitulinus</i>	25	20
24	<i>Bacillus Firmus</i>	11	9
25	<i>E.coli</i>	30	30

R= no inhibition zone

Table 7. Showing Compounds, Retention Times and Area% from GC-MS analysis of tested *Pomegranatum* extract.

Peak number	Retention Time RT (min)	Area %	Chemical Name	Molecular weight (Mwt)
1	19.0	2.32	Ethylene, 1,1-diphenyl-	180
2	19.5	4.51	1,2,3-Propanetriol, 1-acetate	134
3	21.0	3.36	5-Hydroxymethylfurfural	134
4	26.5	2.33	9,12-Octadecadienoic acid (Z,Z)-methyl ester	280
5	28	4.50	Malonic acid, ethyl 3-hexyl ester	216
6	31.5	1.5	Cyclohexane, 1-(cyclohexylmethyl)-4-(1-methylethyl)-	237
7	<b>32.5</b>	<b>13.3</b>	<b>Hexadecanoic acid, methyl ester</b>	<b>270</b>
8	37	3.16	tert-Hexadecanethiol	258
9	<b>38</b>	<b>9.61</b>	<b>n-Hexadecanoic acid</b>	<b>256</b>
10	41.5	6.2	Octadecanoic acid	284

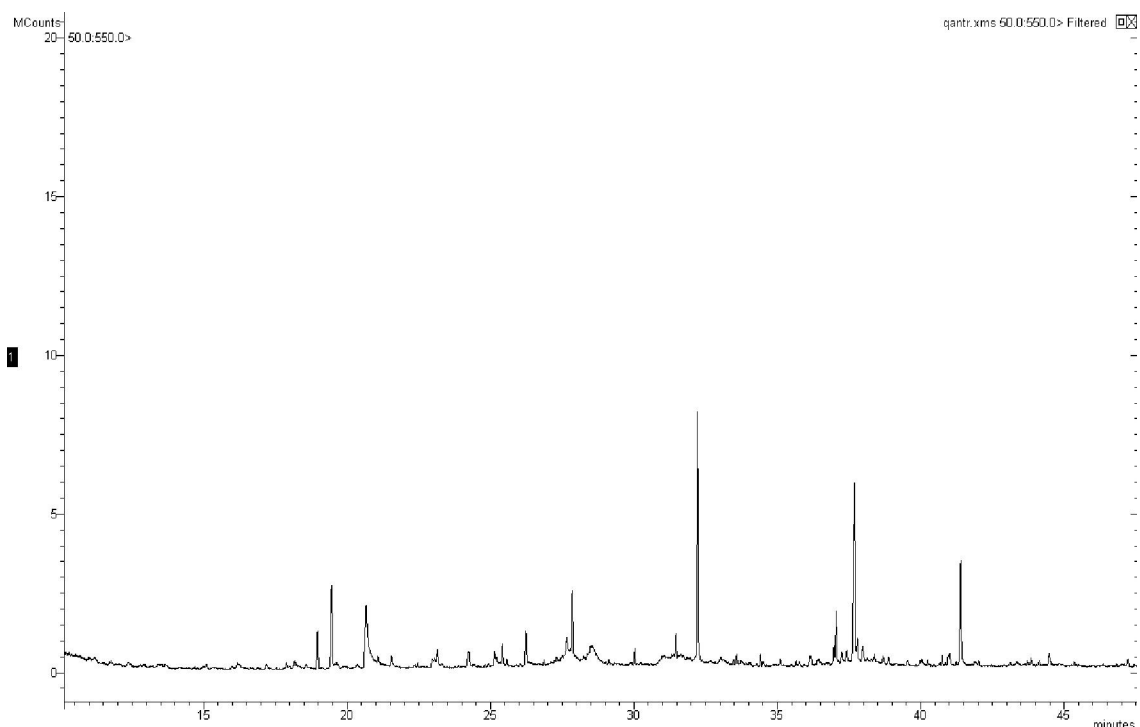


Figure 3. The chromatogram of all compounds produced from GCMS analysis of pomegranate peels extract.

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