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Antibacterial and Synergistic Efficacy of *Punica granatum* Ethyl Acetate Extract Against Multidrug Resistant Bacterial Strains

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ABSTRACT

The study was aimed to study the efficacy of *P. granatum* ethyl acetate extract against multi drug resistant bacterial strains and to characterize the compounds at tributing synergistic and antibacterial potential by GC-MS analysis. Bacterial strains were screened for antibiotic resistance by the disk diffusion method. Antibacterial and synergistic activities of extract were explored by agar well diffusion method and microdilution method respectively. Synergy testing was carried out with antibiotics amikacin, ampicillin, ciprofloxacin, erythromycin, lincomycin, nitrofurantoin, tetracycline and trimethoprim. The extract was subjected to Gas chromatography and Mass spectroscopy (GC-MS) analysis to identify the bioactive compounds responsible for the studied activity. All the test bacterial strains were found to be multidrug resistant. Antibacterial studies showed that the ethyl acetate extract exhibited strong antibacterial activity with zone of inhibition and minimum inhibitory concentration ranged from 16-30 mm and 1.87 to 15 mg/ml respectively. The results of the synergistic studies revealed that good synergistic interaction was exhibited by the combination of the extract and antibiotic lincomycin and tetracycline. MICs of lincomycin and tetracycline were declined from 150 to 5µg/ml and 250 to 2.5µg/ml in combination with the peel extract. GC-MS revealed the presence of various medicinally significant phytochemicals like Citronellol, 5- Hydroxy methyl furfural and linoleic acid etc. Synergistic activity of *P. granatum* may be attributed to the different mode of action of the extract from the antibiotics. The findings provide substantial basis for future use of *P. granatum* ethyl acetate extract as potential antibacterial and antibiotic modulating agent.

Keywords: *Punica granatum*, Antibacterial, Antibiotic resistance,

Synergistic, GC-MS analysis.

INTRODUCTION

The universal escalation in the rate of microbial resistance to various antibiotics is a terrifying aspect; leading to reduction in the efficacy of the treatments. The discovery of antibiotics was considered an essential part in combating the infectious diseases caused by various microbial strains. However, the antibiotics achieved an amazing success in treating infectious disease but their success rate was limited by the emergence of antibiotic resistant strains, their toxic effects and narrow antimicrobial activity. Hence, the currently employed therapeutic approaches are not adequate enough to address these challenges. Under these circumstances, it becomes necessary to search for new antimicrobial agents or resistance modifiers.

Plants have always been exploited for their therapeutic and synergistic potential since the beginning of human civilization. This antimicrobial and synergistic potential of the plants can be attributed to the presence of various bioactive compounds present in plants. Numerous studies suggested that these herbal compounds in combination with antibiotics could offer novel effective therapies against bacterial infections through synergizing the antibiotics[1,2]. These natural compounds act by the disintegration of cytoplasmic membrane, destabilization of the proton motive force (PMF), electron flow, active transport and coagulation of the cell content [(3)]. These combinational studies offer an opportunity to sample a formerly unexplored area of bioactive chemical space and may lead to increased efficacy, reduced toxicity, prevention of the emergence of resistant microbes, and broader-spectrum of activity.

Punica granatum, commonly known as pomegranate or punica apple, belongs to the family Punicaceae. The plant has a strong ethno pharmacological background, used to treat disease like acidosis, dysentery, microbial infections, diarrhoea, helminthiasis, haemorrhage, and respiratory pathologies[4]. Its peel is used to treat infections found in human sexual organs as well as mastitis, acne, folliculitis, pile, allergic dermatitis, diarrhea and dysentery[5]. In addition, *P. granatum* is reported to have antioxidant, anti-atherosclerotic, anti-bacterial and antiviral properties[6,7]. However, to date, very few studies have been conducted on the antibacterial and synergistic activity of *P. granatum* peels. Therefore, the present study was carried out to further validate the *in vitro* antibacterial and synergistic potential of ethyl acetate extract of *P. granatum* peel against antibiotic resistant bacterial strains and to characterize the compounds possessing synergistic and antibacterial potential by GC-MS analysis.

MATERIALS AND METHODS

Microbial strains

Five Gram positive and three Gram negative bacterial strains, *Staphylococcus aureus* (MTCC 3160), *Staphylococcus epidermidis* (MTCC 3086), *Staphylococcus hominis* (MTCC 4435), *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 1885), *Klebsiella pneumonia* (MTCC 4030) and *Pseudomonas aeruginosa* (MTCC 7453) were used during the study. The bacteria were procured from Institute of Microbial Technology (IMTECH), Chandigarh.

Antibiotic discs

High potency discs of eight antibiotics, amikacin (30µg); ampicillin (10µg); ciprofloxacin (5µg); erythromycin (15µg); lincomycin (2µg); nitrofurantoin (300 µg); tetracycline (30 µg) and trimethoprim (5 µg) were purchased from Hi-media Pvt. Ltd., Bombay (India).

Collection of plant material

P. granatum fruits were purchased from the local market of Kurukshetra, India and their peels were removed. Specimens were identified and authenticated by, Department of Botany, Kurukshetra University, Kurukshetra. The peels were thoroughly washed with tap water followed by distilled water, dried under shade for two weeks and ground into fine powder. After sieving (80 mesh) they were transferred to airtight polyethylene zipper bags, labeled and stored till further use. Voucher specimen was deposited at the Wild Life Institute of India, Dehradun, under specimen number GS442 for future reference.

Preparation of plant extract and its fractionation

The powdered *P. granatum* fruit peels (100 g) were soaked in ethyl acetate in a clean and dry reagent bottle covered with a lid at 37°C for overnight. The extraction was done by hot continuous soxhlet extraction method. Resulting extract was evaporated and concentrated to dryness using the rotatory evaporator at 50°C and stored at -4°C till further uses.

In vitro studies

Antibiotic resistance screening test

Resistance of test strains to different antibiotics was determined by the disk diffusion method as described by the Clinical and Laboratory standard Institute (CLSI) standards[8]. The test strains were first enriched in Nutrient broth for 24 h at 37°C by picking-off technique[9]. Using sterile swab sticks, plates were seeded with 1ml of suspension of the test strains containing approximately 10^6 cells. Antibiotic discs were dispensed on the plates seeded with organisms. The plates were incubated at 37°C for 24h and antibiotic resistance was interpreted by inhibition zones.

Antibacterial susceptibility testing - agar diffusion method

Susceptibility of the antibiotic resistant strains to the ethyl acetate peel extract, antibiotics and to the combination of ethyl acetate extract and antibiotics was determined using the modified Kirby–Bauer diffusion technique[10]. The bacterial strains (10^6 CFU) were cultured on nutrient agar by using spread plate technique and wells of 8 mm diameter were made for loading the extract. Each of the bored wells was filled with 50 µl of ethyl acetate extract as a control or 50 µl of antibiotics as a positive control. To determine the synergistic effect, 50 µl of both ethyl acetate extract as well as antibiotic have been added into well. The plates were allowed to stand for 1h at room temperature for diffusion of the extract and antibiotics into agar and incubated at 37°C for 24h. The diameter of the zones of inhibition produced by the peel extract alone, antibiotic alone and their combinations were measured and interpreted using the CLSI zone diameter interpretative standards (2008).Synergistic effect was considered when combinations exhibited enlargement of combined inhibition zone size by 5 mm[11].

Minimum inhibitory concentration

MICs of antibiotics and ethyl acetate peel extract were determined by micro dilution technique using 96-well microtiter plates as described by the National Committee for Clinical Laboratories standards[12]. In case of synergistic interaction, the MIC of the antibiotics was determined by making serial dilution of the antibiotics. Ethyl

acetate extract solutions were separately added into the tubes in a final concentration of 30mg/ml and then bacterial inoculum size of 10^6 CFU/ml was added to each tube. Controls without ethyl acetate extract, without bacterial inoculum or with ethyl acetate extract only were included in the experiment. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism. Tubes were incubated at 37^0C for 24 h. The activity was measured as a function of turbidity at 660 nm. Lack of turbidity was further confirmed by pouring suspension aliquot of 0.1 ml into pre-sterilized Petri dishes with nutrient agar medium. The tests were conducted in triplicate.

GCMS analysis

P. granatum ethyl acetate extract was subjected to GC-MS analysis to identify the phytochemicals responsible for the studied activity. The tested extract was analyzed by GC-MS using Shimadzu Mass Spectrometer-2010 series. 1 µl of sample was injected in GC-MS equipped with a split injector and a PE Auto system XL gas chromatograph interfaced with a Turbo-mass spectrometric mass selective detector system. The MS was operated in the EI mode (70 eV). Helium was employed as the carrier gas and its flow rate was adjusted to 1.2 ml/min. The analytical column connected to the system was an Rtx-5 capillary column (length-60m × 0.25mm i.d., 0.25 µm film thickness). The column head pressure was adjusted to 196.6 kPa. Column temperature programmed from 100˚C (2 min) to 200˚C at10˚C/min and from 200˚ to 300˚C at 15˚C/min withhold time 5 and 22 min respectively. A solvent delay of 6 min was selected. The injector temperature was set at 260°C. The GC-MS interface was maintained at 280°C. The MS was operated in the ACQ mode scanning from m/z 40 to 600.0. In the full scan mode, electron ionization (EI) mass spectra in the range of 40–600 (m/z) were recorded at electron energy of 70 eV. Compounds were identified by comparing mass spectra with library of the National Institute of Standard and Technology (NIST), USA/Wiley.

Statistics

All experimental results are expressed as mean ± standard deviation (SD) of three determinations. One-way analysis of variance was performed on the means to determine whether they differed significantly, with $P < 0.05$ being regarded as significant.

RESULTS AND DISCUSSION

Disc diffusion assay

Resistance profiling of the microbial strains was done by using eight different antibiotics belonging to different classes (Table 1). All eight bacterial strains were found to be multi drug resistant as they were showing resistance to 4 or more than 4 antibiotics. Results were interpreted according to the CLSI zone diameter interpretative standards, 2008. The tested strains exhibited 100% resistance to ampicillin, lincomycin, nitrofurantoin and tetracycline and 100% susceptibility to amikacin and ciprofloxacin antibiotics. This result is in accordance with the previous work of Waili *et al*., 2012[13] and Luna *et al*., 2007[14] as they also reported about prevalence of resistance to various antibiotics in *P. aeruginosa*, *S. aureus, E. coli* and majority of the *B. cereus* and all of the *B. thuringiensis* isolates respectively. Resistance to test antibiotics may be attributed to various efflux systems present in bacteria.

Antibacterial susceptibility testing - Agar diffusion method

The bacterial isolates exhibited a varied degree of susceptibility to the extract, antibiotics, and their combinations. The zone of inhibitions for *P. granatum* ethyl acetate peel extract ranged from 16 mm to 30 mm (Table 2). Similar reports on antibacterial

activity of *P. granatum* were also reported by Machado *et al*., 2003[15]. Zone of inhibition for the antibiotics ranged from 17mm to 30 mm while no antibacterial activity was shown by ampicillin, lincomycin and nitrofurantoin at the concentration of 10µg/ml. Antibacterial activity of antibiotics was tested at 10µg/ml as according to CLSI Performance Standard for Antimicrobial Susceptibility Testing, 2007, MIC breakpoint for most of the antibiotics is less than 10µg/ml. Maximum activity was shown by trimethoprim against *P. aeruginosa*. Combinational studies revealed that the combination of ethyl acetate extract and antibiotic lincomycin and tetracycline exhibited synergistic interaction in term of enlargement of combined zone of inhibition. Combination of extract and lincomycin showed synergism against *S. aureus*, *S. epidermidis*, *S. hominis, E. coli* and *P. aeruginosa* while tetracycline exhibited synergistic interactions against *S. aureus*, *S. epidermidis*, *S. hominis, B. subtilis, E. coli* and *P. aeruginosa.* Other combinations did not show any synergistic interaction. Synergistic interaction of the *Punica granatum* with tetracycline is also reported by Hussin and El-Sayed, 2011[16]. Different mode of action of the extract from the antibiotics may be an important factor in the enhanced bactericidal efficacy observed when used in combination.

Minimum inhibitory concentration (MIC)

MIC is considered as the gold standard for determining the susceptibility of organism to antimicrobials. MICs ranged from 1.87 to 15 mg/ml for ethyl acetate extract (Table2). The present findings revealed that different bacterial strains exhibited different levels of susceptibility to the extract. The differences in bacterial susceptibility to the extract may be due to the differences in cell wall composition or genetic content of their plasmids[17]. Lowest MIC was recorded for ethyl acetate extract against *B. cereus* and *P. aeruginosa*.

Combination studies revealed that there is a decrease in the MIC in case of combination between ethyl acetate peel extract and antibiotics (lincomycin and tetracycline) against all the test strains. (Table3).MICs of lincomycin and tetracycline were declined from 150 to 5µg/ml (30 fold reduction) and 250 to 2.5µg/ml (100 fold reduction) in combination with ethyl acetate extract. Maximum reduction in MIC of lincomycin was observed against *E. coli* while in case of tetracycline it was observed against *P. aeruginosa*. Thus, bacterial strains even resistant to these antibiotics become sensitized in combination with ethyl acetate extract. This implies that that use of plant extract and antibiotics could inhibit the growth of antibiotic resistant bacteria better than the use of plant extract/antibiotics alone. The plant extract thus stand out as genuine source of potential resistance modifying agents. Our results are in agreement with various reports which showed that plant extracts increase the activity of various antibiotics with significant reduction in the MICs of the antibiotics[18].

GCMS analysis

GCMS of ethyl acetate extract was conducted to determine the nature of phytochemicals which were responsible for the studied activity. GC-MS chromatogram of ethyl acetate extract of *P. granatum* is shown in Figures 1and compounds are listed in Table 4.Citronellol, 5- Hydroxy methyl furfural, linoleic acid and myristic acid were most prevalent in *P. granatum* ethyl acetate extract. Citronellol is strongly effective against several species of microbes[19], so it may be responsible for antibacterial activity of plant extract. Other significant compounds revealed by GC-MS analysis were Cyclooctanediol, valeric acid and vitamin E; have substantial medicinal properties.

CONCLUSION

The ethyl acetate peel extract of *P. granatum* showed the potential synergy with antibiotics lincomycin and tetracycline and thus suggested that effective therapy may be achieved with these antibacterial combinations. The study also revealed the presence of various medicinally significant phytochemicals attributing antibacterial and synergistic potential to the *Punica granatum* extract. Thus the findings provide substantial basis for the future exploration of the phytochemicals present in *P. granatum* crude extract as potential antibacterial and antibiotic modulating agent. Further studies are needed on animal model to evaluate the bioactivity of the phytochemicals present in the plant.

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Conflict of interest

There is no conflict of interest.

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Table 1. Antibiogram of the Bacterial Strains

S. No.	Antibacterial Agent	Disc Content	Zone of Inhibition (mm)							
			S. A	S. E	S. H	B. C	B. S	E. C	K. P	P. A
1.	Amikacin	30 µg	18±1 (S)	17±0 (S)	17.33±0.57 (S)	17±1 (S)	18.33±0.57 (S)	18.33±0.57 (S)	17±1 (S)	17.66±0.57 (S)
2.	Ampicillin	10 µg	R	R	R	R	R	R	R	R
3.	Ciprofoxacin	5 µg	21.33±0.57 (S)	21±0 (S)	22.33±0.57 (S)	20±1 (S)	21±1 (S)	22.66±0.57 (S)	22.33±0.57 (S)	23±0 (S)
4.	Erythromycin	15 µg	20.33±0.57	20±1 (I)	18.66±0.57 (I)	R	R	15±1 (I)	23.66±0.57 (S)	25.33±0.57 (S)
5.	Lincomycin	2µg	R	R	R	R	R	R	R	R
6.	Nitrofurantoin	300 µg	R	R	R	R	R	R	R	R
7.	Tetracycline	30 µg	R	R	R	R	10 (R)	10 (R)	R	R
8.	Trimethoprim	5 µg	R	R	20±1 (S)	R	R	R	R	30±0 (S)

S.A: *S. aureus*, S.E: *S. epidermidis*, S.H: *S. hominis*, E.C: *E. coli,* K.P: *K. pneumonia*, B.C: *B. cereus*, B.S: *B. subtilis*, P.A: *P. aeruginosa*. R: resistant, I: intermediate, S: susceptible.

Table 2. Zone of inhibitions produced by ethyl acetate extract (30mg/ml), antibiotics (10µg/ml) and their combinations *in vitro*

Micro-organism	Ethyl Acetate Extract (mg/ml)	AMK (AMK + EA)	AMP (AMP + EA)	CIP (CIP + EA)	ERY (ERY + EA)	LIN (LIN + EA)	NIT (NIT+ EA)	TET (TET + EA)	TMP (TMP + EA)
S. aureus	24.66±0.57	18±1 (24.33±0.57)	R (24.33±0.57)	21.33±0.57 (24.66±0.57)	R (24±1)	R (29.33±0.57)	R (24±0)	R (29.33±0.57)	R (24±1)
S. epidermidis	18±0	17±0 (17±1)	R (18.33±0.57)	21±0 (21±1)	20±1 (20±1)	R (31±0)	R (18±0)	R (23±0)	R (18±0)
S. hominis	22.66±0.57	17.33±0.57 (22±0)	R (22.33±0.57)	22.33±0.57 (22.33±0.57)	18.66±0.57 (22.33±0.57)	R (27.66±0.57)	R (22±1)	R (27.66±0.57)	20±1 (20.33±0.57)
B. cereus	24±0	17±1 (24±1)	R (24.33±0.57)	20±1 (24.66±0.57)	R (24±0)	R (24±0)	R (24±1)	R (24±1)	R (24.33±0.57)
B. subtilis	22±0	18.33±0.57 (22.66±0.57)	R (22.66±0.57)	21±1 (22±1)	R (22±0)	R (22±1)	R (22±1)	10±1 (22.66±0.57)	R (22.66±0.57)
E. coli	16±0	18.33±0.57 (18±1)	R (16±1)	22.66±0.57 (22±1)	15±1 (15±1)	R (27.66±0.57)	R (16±0)	10±1 (22.33±0.57)	R (16±0)
K. pneumonia	18±0	17±1 (18±0)	R (18±0)	22.33±0.57 (22.66±0.57)	23.66±0.57 (23±0)	R (18±0)	R (18±0)	R (18±0)	R (18±0)
P. aeruginosa	30.66±0.57	17.66±0.57 (30±0)	R (30±0)	23±0 (23±1)	25.33±0.57 (25±0)	R (35.66±0.57)	R (30.33±0.57)	R (35±1)	30±0 (30±1)

AMK: Amikacin, AMP: Ampicillin, CIP: Ciprofloxacin, ERY: Erythromycin, LIN: *Lincomycin,* NIT: Nitrofurantoin, TET: Tetracycline, TMP: Trimethoprim, EA: Ethyl Acetate extract, R: resistant, (): Synergistic activity.

Table 3. Minimum inhibitory concentration of antibiotics alone, plant extract alone and in combination

Microorganism	Ethyl Acetate Extract (mg/ml)	AMK (AMK + EA)	AMP (AMP + EA)	CIP (CIP + EA)	ERY (ERY + EA)	LIN (LIN + EA)	NIT (NIT+ EA)	TET (TET + EA)	TMP (TMP + EA)
S. aureus	3.75	10 (10)	100 (100)	2.5 (2.5)	50 (50)	200 (5)	200 (200)	200 (5)	100 (100)
S. epidermidis	3.75	5 (5)	150 (150)	1.25 (1.25)	50 (50)	150 (10)	50 (50)	150 (5)	100 (100)
S. hominis	3.75	10 (10)	100 (100)	5 (5)	100 (100)	150 (5)	50 (50)	150 (5)	5 (5)
B. cereus	1.87	5 (5)	150 (150)	5 (5)	150 (150)	100 (20)	100 (100)	100 (50)	20 (20)
B. subtilis	3.75	5 (5)	100 (100)	2.5 (2.5)	100 (100)	150 (50)	50 (50)	20 (10)	20 (20)
E. coli	15	5 (5)	150 (150)	5 (5)	50 (50)	150 (5)	100 (100)	20 (5)	150 (150)
K. pneumonia	3.75	10 (10)	100 (100)	5 (5)	150 (150)	150 (50)	100 (100)	150 (50)	50 (50)
P. aeruginosa	1.87	10 (10)	50 (50)	2.5 (2.5)	250 (250)	250 (10)	150 (150)	250 (2.5)	2.5 (2.5)

AMK: Amikacin, AMP: Ampicillin, CIP: Ciprofloxacin, ERY: Erythromycin, LIN: *Lincomycin,* NIT: Nitrofurantoin, TET: Tetracycline, TMP: Trimethoprim, EA: Ethyl Acetate extract, (): MIC decline in combination.

Table 4. Chemical composition of *P. granatum* ethyl acetate extract

Peak	Compound	R. time	Peak area (%)
1	Valeric acid, 2-acetyl-4-methyl-, ethyl ester	4.617	1.22
2	1,2-Cyclooctanediol	4.844	2.19
3	5-Hydrxoymethylfurfural	6.402	35.16
4	7-Hexadecenal	7.674	0.67
5	alpha.-Calacorene	8.138	0.07
6	Caprylonitrile	8.716	2.95
7	Hydroxyphenethyl alcohol	9.003	0.42
8	1-Octadecanol	10.251	1.43
9	Isoelemicin	10.738	0.09
10	Farnesene epoxide	10.931	0.19
11	Furan, 2-Octyl	11.434	1.15
12	Tricosanoic acid, methyl ester	11.817	0.66
13	3-Furoic acid, tert-butyldimethylsilyl ester	12.064	0.48
14	Myristic acid	12.374	0.99
15	Loliolide	12.777	0.33
16	Oleyl alcohol	13.015	1.39
17	Oleyl alcohol	13.269	0.65
18	Oleyl alcohol	13.462	1.14
19	cis-11-Eicosenoic acid, methyl ester	13.711	0.10
20	Myristic acid	14.638	15.83
21	Oleyl alcohol	15.245	0.46
22	Linoleic acid, methyl ester	15.567	1.91
23	Citronellol	15.840	8.74
24	Linoleic acid	16.215	10.02
25	9,12,15-Octadecatrien-1-ol	16.892	0.17
26	Dichloroacetic acid, tridec-2-ynyl ester	17.318	1.13
27	7-Hexadecyn-1-ol	17.693	0.34
28	Eicosapentaenoic acid	18.345	0.12
29	Phthalic acid, mono-(2-ethylhexyl) ester	19.681	0.33
30	Wogonin	21.444	1.81
31	N-Lauroyl-p-aminopheno	22.093	0.24
32	Spinacen	23.831	2.42
33	Geranylgeraniol	24.293	0.55
34	14-Beta-H-pregna	24.710	0.54
35	Geranylgeraniol	25.217	0.04
36	Geranylgeraniol	25.450	0.15
37	14B-Pregnane	25.770	0.09
38	Geranylgeraniol	26.089	0.13
39	Geranylgeraniol	26.171	0.17
40	Globulol	26.870	0.21
41	Vitamin E	27.151	2.15
42	Stigmasterol	28.058	0.30
43	Stigmast-5-en-3-ol	28.493	0.69

44	1, 40-Tetracontanediol	30.115	0.19
		100.00	

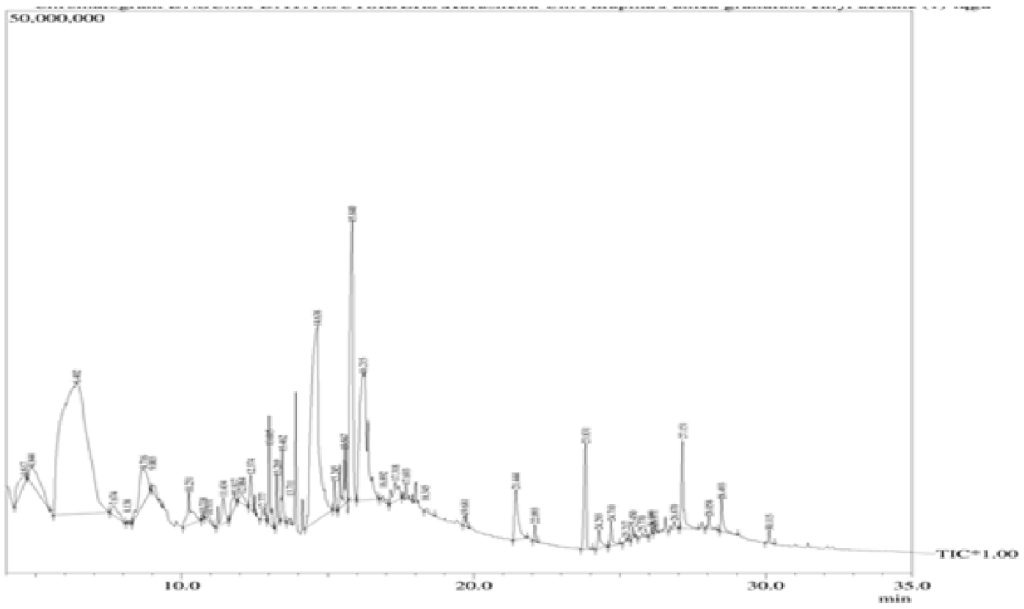

Figure 1. GC-MS chromatogram of *P. granatum* ethyl acetate extract