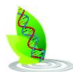

International Journal of Life Sciences Biotechnology and Pharma Research

ISSN 2250-3137 www.ijlbpr.com
Vol. 1, No. 4, October 2012
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Research Paper

ANTIBACTERIAL ACTIVITY OF *PUNICA GRANATUM* PEEL EXTRACTS AGAINST SHIGA TOXIN PRODUCING *E. COLI*

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Shiga toxin producing E. coli (STEC) is an emerging pathogen. These STEC strains are grouped as O157 and non O157 group. These non O157 include several serogroups. These strains also cause outbreaks similar to that of O157 E. coli. Antibiotic resistance is increasing among these STEC and usage of antibiotics for treatment of such strains result in induction of toxin production. Hence, phytochemicals can be used as an alternative to antibiotics. Punica granatum peels are rich in polyphenols, tannins and other secondary metabolites. The objective of this study is to analyse the bioactive phyto components of Punica granatum peel extracts against Shiga toxin producing E. coli. The methanol extract of P. granatum peel showed high antibacterial activity against STEC. HPLC analysis revealed Punicalagin (α) and (β). Ellagic acid was also present in the extract. GC-MS analysis revealed the presence of 36 compounds, some of them were not reported previously.

Keywords: Phyto chemical Analysis, GC-MS analysis, Ethyl acetate extract, *Punica granatum*, Shiga toxin producing *E. coli*

INTRODUCTION

Plants have potent biomedical and have components of phytomedicine. Medicinal plants are important source of antibacterial compounds. These plants contain secondary metabolites such as alkaloids, flavonoids, steroids, phenolics, terpenes, volatile oils etc. *Punica granatum* is a plant with a wide variety of activities. The whole plant has medicinal values and is investigated by various authors. Yoshikazu *et al*. (2001) investigated the inhibitory effect of several plant extracts on the production of verotoxin by enterohemorrhagic *Escherichia coli* O157 : H7 (EHEC). The extracts from four plant species, *Limonium californicum* (Boiss.) *A. Heller*, *Cupressus lustianica* Miller, *Salvia urica* Epling

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and *Jusiaea peruviana* L., were effective on the inhibition of verotoxin production. These findings suggested that the administration of any appropriate plant extract might prevent the production of verotoxin by EHEC in the human intestines. The objective of the present study was to analyze the peels of *Punica granatum* for its bioactive compounds against Shiga toxin producing *E. coli*.

MATERIALS AND METHODS

Preparation of Extract and Antibacterial Activity

Pomegranate fruits were collected from the local markets. Peels were removed and dried. The sample was spread out during daylight hours for 5 days until it dried to brittleness. Dried peels were powdered to get 60- mesh size using a mixer grinder. Thirty grams of powdered peel was extracted with different solvents like Petroleum ether, benzene, chloroform, ethanol and methanol with occasional shaking for 3 days at room temperature. The extracts were filtered, concentrated and dried at 50°C and the weight of each residue was recorded.

The prepared extracts were tested for antibacterial activity against shiga toxin producing *E.coli* (*E. coli* with stx2 genes, confirmed by PCR) by agar well diffusion method. 1 g of the prepared extract was dissolved in 1 ml of DMSO. 6mm wells were made on Mueller Hinton agar plates seeded with the test organisms and different concentrations of the extracts (10-100 mg) were loaded onto the wells. A gentamicin (G-30 mcg) disc was used as a standard antibiotic and DMSO was added in a separate well. The plates were incubated for 24 hours and after incubation the zone of inhibition was noted against each extract (Shiv Shanker Gautam *et al*., 2012).

Minimum Bactericidal Concentration

The MBC was performed to test the antimicrobial activity of active extract using tube dilution method. The MBC was defined as lowest concentration able to kill any microbe. Dilutions of the plant extract were prepared in sterile nutrient broth to get a final concentration of 2 mg, 4 mg, 8 mg, 16 mg, 32 mg and 64 mg/ml respectively (Mahfuzul Hoque *et al.,* 2007).

To each of these dilutions, a loop full of STEC culture adjusted to 0.5 Mc Farland standard, was inoculated and all the tubes were incubated at 37°C for 24 hrs. After incubation, loopful from each tube was inoculated onto nutrient agar plates. The plate without growth was recorded as MBC.

Phytochemical Screening

Phytochemical screening for flavonoids, alkaloids, tannins, saponins and terpenoids were done following standard methods as described by Harborne 1998, Trease and Evans, 1989 and Sofowora, 1993).

Thin Layer Chromatography and TLC Bioautography Analysis

The methanol extracts were applied at 2.5 cm from the base of silica gel G TLC plate. The TLC plates were developed with ethyl acetate: methanol: water (81:11:8) and were run in duplicate. 5% ferric chloride reagent was sprayed and the chromatogram was observed. The second set of chromatogram, developed as described above in TLC plates which was not subjected to chemical treatment for observing spots was loaded with the inoculum of *E. coli* in molten Mueller Hinton Agar, over the TLC plates. After the solidification of the medium, the TLC plate was incubated overnight at 37°C.

Subsequently, bioautogram developed was sprayed with 1% aqueous solution of 2, 3, 5, triphenyl tetrazolium chloride (TTC) and incubated at 37^{0}C for 4 h. Inhibition zone indicated the presence of active compounds (Anjana *et al.,* 2010). Ellagic acid and the antibiotic rifampin were used as controls.

High Performance Liquid Chromatography

For injection in HPLC system, the methanol extract was used. The HPLC was performed on YMC C18 column, 5¼m, 250 × 4.6mm, using a gradient consisting of solvent A (water/formic acid, 90/10) and solvent B (methanol), at a flow rate of 0.7ml/minute (Prieto *et al.,* 2005).

Column Chromatography and Gas Chromatography- Mass Spectrum Analysis (GC-MS)

1 g of the crude methanol extract was subjected to column chromatography using silica gel (100-200 mesh) and eluted with ethyl acetate. This ethyl acetate fraction of the *Punica granatum* rind was taken for GC-MS analysis, since ethyl acetate could extract most of the bioactive compounds (Sangeetha and Vijayalakshmi, 2011).

The eluted ethyl acetate fraction was dried and dissolved in ethanol. GC-MS analysis of this extract was done using Agilent GC-MSD-5975 C (412 SLPL ES GC- MSD) system. Interpretation of mass spectrum GC-MS was done using the database of National Institute Standard and Technique (NIST 11.L).

RESULTS AND DISCUSSION

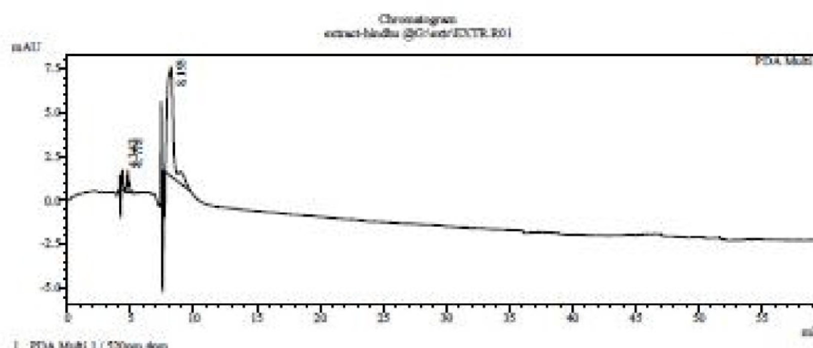
The weights of all the residues in Petroleum ether, benzene, chloroform, ethanol and methanol were 3.0%, 2.6%, 3.3%, 3.0% and 3.3% respectively. The antibacterial activities of different extracts were tabulated (Table 1 and Figure 1). The methanol extract of *Punica granatum* showed high antibacterial activity. Similar antibacterial activity was shown by several authors (Trivedi and Kazmi, 1979; Anesini and Perez, 1993; Voravuthikunchai *et al.,* 2004; Vishal Jain *et al.*, 2011).

The minimum bactericidal concentration of *Punica granatum* was 4.0mg/ml. The Minimum bactericidal concentration is low, thus showing good antibacterial activity against Shiga toxin producing *E. coli*. Phytochemical analysis of these extracts showed the presence of alkaloids, flavanoids and tannins. Voravuthikunchai *et al.*

Table 1: Antibacterial Activity of *Punica granatum* Against Shiga Toxin Producing *E.coli*

S. No	Extract	Zone diameter (mm)					
		Punica granatum (mg)					
		10	20	40	60	80	100
1.	Petroleum ether	–	–	–	8	10	12
2.	Benzene	–	–	–	–	–	10
3.	Chloroform	–	–	–	–	–	12
4.	Ethanol	8	11	12	13	14	15
5.	Methanol	14	16	16	17	19	22
6.	Gentamicin (30¼g)			16			
7.	DMSO			–			

Figure 1: HPLC Chromatogram of Methanol Extract



Peak Table @ Gr\extr\EXTR.R01

Ch1 520 nm 4 nm

Peak #	Ret Time	Area	Height	Area (%)	Height (%)
1	4.343	4122	1102	1.840	12.887
2	4.778	11634	1266	5.192	14.811
3	8.193	208333	6182	92.969	72.303
Total		224090	8551	10.000	100.000

(2005) studied the antibacterial activity of *Punica granatum* pericarp extracts against different strains of Enterohaemorrhagic *Escherichia coli* O157:H7 and Non O157:H7 strains. The ethanolic extract was found to be the most effective and the Minimum Inhibitory Concentration was 0.09 mg/ml.

Brown colour spots were observed when sprayed with 5% ferric chloride. Spots were observed at Rf values of 0.04 cm, 0.07 cm, 0.092 cm, 0.23 cm, 0.30 cm and 0.439 cms. Ellagic acid and rifampin showed Rf value of 0.07cms. Bioautography showed a zone

Table 2: Table Showing Compounds, Retention Times and Area% From GC-MS Analysis

Sl. No.	Peak Number	Retention time (min)	Area %	Compound	Molecular weight	Molecular formula	Abundance %
1.	1	3.508	0.86	Propanedioic acid	104.06	$CH_2(COOH)_2$	62.19
2.	2	4.539	1.59	2,4-Dihydroxypyridine	111.10	C_5H_5NO2	15.41
3.	3	5.396	0.69	N-Nitroso-2-methyloxazolidine	116.14	$C_4H_8N_2O_2$	49.11
4.	4	6.151	1.92	1-Propanol, 2-ethoxy-	104.15	$C_5H_{12}O_2$	50.31
5.	5	6.631	1.51	2,5-Furandicarboxaldehyde	124.0942	$C_6H_4O_3$	61.68
6.	6	6.936	1.99	Undecane	156.31	$CH_3(CH_2)_9CH_3$	83.67
7.	7	7.444	1.69	Acetamide, 2,2'-thiobis	59.07	CH_3CONH_2	61.83
8.	8	7.662	1.44	Malonic acid, ethyl 3-hexyl ester	216.2741	$C_{11}H_{20}O_4$	88.19

Table 2 (Cont.)							
Sl. No.	Peak Number	Retention time (min)	Area %	Compound	Molecular weight	Molecular formula	Abundance %
9.	9	7.720	2.04	4H-Pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl	144.1253	C6H8O4	81.88
10.	10	8.577	3.38	Catechol	110.1	C6H4(OH)2.	26.56 g/mol
11.	11	9.041	4.18	5-Hydroxymethylfurfural	126.11	C6H6O3	67.18
12.	12	9.303	0.69	1,2,3-Propanetriol, 1-acetate	134.13	C5H10O4	32.33
13.	13	9.956	0.87	1,2-Ethanediol, monoacetate	104.1	C4H8O3	33.47
14.	14	10.058	5.30	3,4-Difluorobenzoic acid, 4-dodecyl ester	326.4212	C19H28F2O2	17.98
15.	15	10.334	0.98	Benzene, 2-methoxy-1,3,4-trimethyl	150.2176	C10H14O	94.43
16.	17	11.263	0.67	Sebacic acid, di(but-2-enyl) ester	310.4284	C18H30O4	77.70
17.	20	13.108	1.12	Benzoic acid, 4-ethoxy-,ethyl ester	194.23	C11H14O3	56.51
18.	21	13.456	1.11	Dodecanoic acid	200.32	C12H24O2	86.88
19.	23	15.693	0.76	Tetradecanoic acid	228.3709	C14H28O2	91.11
20.	24	16.259	3.11	2,3,5-Trimethyl-6-propylpyrazine	164.2474	C10H16N2	47.15
21.	26	17.436	2.60	Hexadecanoic acid,methyl ester	270.450	C17H34O2	71.52
22.	27	17.668	1.32	n-Hexadecanoic acid	256.42	C16H32O2	97.92
23.	28	17.770	13.23	n-Hexadecanoic acid	256.42	C16H32O2	97.92
24.	30	18.423	0.70	2,4,6-Triisopropylbenzoic acid	248.3606	C16H24O2	60.54
25.	31	19.062	1.61	9,12-Octadecadienoic acid (Z,Z)-methyl ester	280.4455	C18H32O2	99.88
26.	32	19.120	1.25	6-Octadecenoic acid, methyl ester	296.487	C19H36O2	83.94
27.	33	19.396	8.41	9,12-Octadecadienoic acid (Z,Z)-	280.445	C18H32O2	92.73
28.	37	23.927	0.68	Ethanamine, 2-phenoxy	137.17	C8H11NO	32.54
29.	38	24.015	2.02	3,7-Benzofurandiol, 2,3-dihydro—2,2-dimethyl	180.2	C10H12O3	91.03
30.	39	24.465	1.67	Ethylene, 1,1-diphenyl-	180.24	C14H12	60.89
31.	40	24.741	1.87	Hesperetin	302.27	C16H14O6	70.16
32.	41	24.930	1.17	Squalene	410.71	C30H50	58.59
33.	43	27.123	2.14	Vitamin E	430.70	C29H50O2	49.92
34.	44	28.052	2.00	Ergost-5-en-3-ol,(3.beta.)-	400.680	C28H48O	92.04
35.	45	28.357	4.64	Stigmasterol	412.69	C29H48O	76.72
36.	46	28.938	9.14	gamma.-Sitosterol	414.7067	C29H50O	70.43

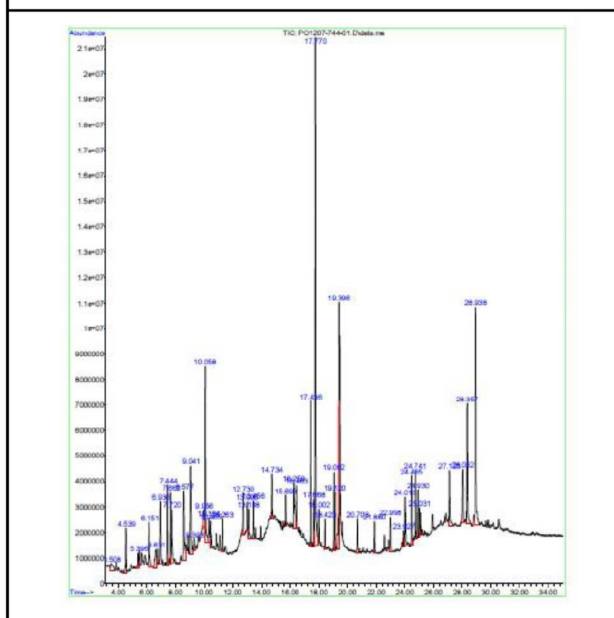
formation over the spots with Rf values of 0.07 and 0.439 cms.

The HPLC chromatogram showed 3 peaks with RT of 4.3, 4.7 and 8.1 min (Figure 1.) The first two peaks with RT 4.3 and 4.7 coincide with Punicalagin (±) and (β) respectively (Vishal Jain *et al*., 2011).

GC-MS ANALYSIS

Ethyl acetate fraction was checked for antibacterial activity by agar well diffusion method. A zone diameter of 17 mm was produced. On analysis by GC-MS, several compounds were identified and tabulated (Table 2). The chromatogram is shown in Figure 2. Thirty six compounds were identified in this study. Sangeetha and Vijayalakshmi (2011), had identified 18 different compounds from ethyl acetate fraction of Punica granatum rind and concluded that Pyrogallol was present in the highest concentration (41.88%) and it is one of the active principles in that extract. In this study, n-Hexadecanoic acid (13.23%), 9,12-Octadecadienoic acid (Z,Z)-(8.47%), 3,4-Difluorobenzoic acid, 4-dodecyl ester (5.30%), Stigmasterol (4.64%) and 5-Hydroxymethylfurfural (4.18%) were the compounds showing higher area%. Pyrogallol was absent in our results but 5-Hydroxymethylfurfural was the next abundant compound in their study. In our study also 5-Hydroxymethylfurfural was present as the fifth abundant compound.

Figure 2: Chromatogram of Ethyl Acetate Fraction of *Punica granatum* Peel Extracts



Further the presence of several compounds in Punica granatum were previously reported by several authors. The compounds found in literature were Di hydroxyl pyridine (Jennifer *et al*., 2009), N-Nitroso-2-methyl-oxazolidine (Sebastian and Hamide, 2008), 2,5-Furandicarboxaldehyde (Keheyan and Giulianelli, 2006), Undecane (Tang, 2004), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (Ashok Kumar and Vijayalakshmi, 2011; Rane Zab *et al*., 2012), Catechol (Onslow, 1920), Hesperetin (Swarnamoni Das and Sarajita Barman, 2012) and Squalene (Caligiani *et al*., 2010).

Two new beta-sitosterol esters have been isolated by Bagri *et al*., 2009, from the flowers of *Punica granatum* Linn. We report the presence of Stigmasterol in ethyl acetate extract of peels. GC-MS analysis of unsaponifiable fractions of Punica granatum seed oils by Augustina *et al*. (2010), revealed that β-sitosterol (up to 8069 mg/kg) and cycloartenol (5916–7766 mg/kg) were predominant in phytosterol and triterpene fractions, while β- and d-tocopherol were the most abundant vitamin E forms. But in our study, in the pomegranate peel ethyl acetate fractions, gamma.-Sitosterol were identified. Jiang *et al*. (2008) had reported the presence of Ergost-5-en-3-ol, (3.beta.), as one of the main compounds of bioactive n-butyl alcohol fraction of *P. granatum*.

CONCLUSION

Punica granatum peel is a good source of antibacterial compound against Shiga toxin producing *E. coli*. The methanol extract showed high antibacterial activity. HPLC analysis revealed 3 peaks and 2 peaks were identified as Punicalagin (±) and (β). TLC shows the presence of ellagic acid. Thus different extraction methods and different solvents will elute different bioactive compounds. GC-MS analysis revealed the presence of 36 compounds. n-Hexadecanoic acid (Manilal *et al*., 2009), 9,12-Octadecadienoic acid (Z,Z)- (Milosevic *et al*., 2010), 3,4-Difluorobenzoic acid, 4-dodecyl ester (Chue *et al*., 2011), Stigmasterol (Jesus Manriquez-Torres *et al*., 2007) and 5-Hydroxymethylfurfural (Muhammad Gulfraz *et al*., 2010). This study also detects new compounds which were not previously reported from Punica granatum. Those compounds are Propanedioic acid, Malonic acid, ethyl-3-hexyl ester, 1,2,3-propanetriol 1- acetate, ethenamine-2-phenoxy, 3,7-benzofurandiol, 2,3-dihydro-2,2-dimethyl, Ethylene, 1,1-diphenyl-, 1-propyl 2-ethoxy-, acetamide,2,2'-thiobis, 1,2-ethane diol monoacetate, benzene, 2-methoxy 1,3,4-trimethyl, sebacic acid, benzoic acid,4-ethoxy-ethyl ester, do decanoic acid, tetra decanoic acid, 2,3,5-trimethyl 6-propyl pyrazine, n-hexadecanoic acid,2,4,6-triisopropyl benzoic acid, 9,12-Octadecenoic acid and 9,12-Octadecadienoic acid (Z,Z-). Further the activity of each bioactive component should be investigated.

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