Molecular profiling of *Punica granatum* rind: Phenolic composition with emphasis on Tannins and Catechins and *in-vitro* antimicrobial efficacy and interaction of Rind extracts with and without antibiotics against drug resistant bacteria

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Abstract- The rind (peel) of *Punica granatum* (pomegranate) is a rich source of bioactive phytochemicals, including phenolics, flavonoids, and tannins, which contribute to their strong antioxidant, antimicrobial, and anticancer properties. Today new sources of antimicrobial compounds need to be identified and improved strategy should be developed to combat multidrug resistance problem in pathogenic bacteria. Plant extract and phytochemicals demonstrating anti-microbial action needs to be exploited for their synergistic action between extracts and with antibiotics to exploit it in modern chemotherapy and combinational therapy. In the present study ethanol extract of *Punica granatum* rind was screened for their antimicrobial efficacy against a wide variety of drug resistant bacteria and yeast. The extracts showed promising action against one or more drug resistant bacteria as well as against *Candida albicans*. The extract of *Punica granatum* rind exhibited synergy with antibiotics, tetracycline, chloramphenicol, ampicillin and gentamicin against methicillin resistant *S. aureus* which has indicated their potential to be exploited in combination drug therapy after careful evaluation *in vivo* model. Phytochemical investigations included qualitative detection of phytochemicals including phenols and tannins, flavonoids, Total phenolic and tannin and catechin content was determined quantitatively. Ethanolic pomegranate rind extracts showed highest amount of phenolic and flavonoid content. The presence of catechin in pomegranate rind was determined by HPTLC. The extracts of pomegranate rind was found effective in inhibiting the growth of a number of bacteria.

Key words: Antimicrobial activity, MDR bacteria, MIC, synergistic activity antibiotics

Introduction

Pomegranate is an ancient fruit native to Persia which has been cultivated in the Mediterranean region through years (Viuda Martos 2010; Silvaj et al,2013). This fruit, which belongs to the family of Punicaceae, is botanically named *Punica granatum* (Chandra et al 2010). Pomegranate trees are considered as shrubs or small trees which grow 5 to 10 meters high (Silvaj et al,2013). Its flowers are red and can occur either as single blossoms or clusters of several blossoms (Baliga et al 2013 & Stover, 2007). Pomegranate is rich in bioactive

molecules, it has shown myriad medicinal properties due to its high phenolic content (Viuda Martos 2010; Akhtar et al,2015). Pomegranate, as a fruit rich in antioxidants, can intensively and positively contribute in humans' health. Pomegranates strong historical, cultural and religious significances, besides the researches determining the phytochemicals present in pomegranate triggered analysing and evaluating pomegranate rind.

The use of herbal and other natural substances is part of the fabric of traditional medicine in different part of the world. Medicinal plants have been found good source of therapeutic and novel compounds. Targeted screening of a large diversity of medicinal plants is expected to yield novel biological activities including problematic group of multidrug resistant bacterial pathogens.

Bacteria which have evolved numerous defences against antimicrobial agents and drug resistant pathogens are on the rise and such bacteria have become a global health problem. Nearly twenty years ago over 90% *S. aureus* strains were reported β-lactamase positive. Strains of β-lacatam resistant *Staphylococcus aureus* including MRSA now pose a serious problem to hospitalized patients and their care providers (Liu, et al., 2000). The production of β-lactamase is recognized as one of the main mechanisms of bacterial-resistance to β-lactamase antibiotics. Numerous compounds have been included in the list of β-lactamase inhibitors and some of these have shown potential clinical usefulness based on their synergistic-effects when they are combined with β-lactamase-labile antibiotics. Many β-lactamase were found to be resistant to β-lactamase inhibitors. Similarly multidrug resistant problem is common in members of family, Entero-bacteriaceae specially *E.coli*, *Salmonella*, *Shigella* and several other humans and animal pathogen like *Haemophilus influenza*, *Campyl-obacter*, *Pseudomonas* aeruginosa, *Mycobacterium tuberculosis* both in developing and developed countries (Eldelstein *et al.*, 2001; Tonkic *et al.*, 2005;).

The world health organization (WHO) reported that 80% of the world's population rely chiefly on traditional medicines and major part of the traditional therapies involve the use of plant extracts or their active constituents (WHO, 1993).

Many plant metabolites are now used globally as drugs. It has been estimated that 14-28% of higher plant species are used medicinally, that only 15% of all angiosperms have been investigated chemically and that 74% of pharma-cologically active plants derived components were discovered after following upon ethanobotanical use of plants (Eloff, 1998). The plants are valuable in the three basic ways:
(1) they are used as source of direct therapeutic agent.
(2) As a source of new bioactive metabolites including antimicrobial, antihelminthic and antiprotozoan etc.
(3) they serve as raw material base for elaboration of more complex semi-synthetic chemical compounds.

According to a report published in the 'Journal of the American medical

association', more than 630 million visit are made to alternative practitioners each year in the U.S. also more than 15 million adults take herbal remedies while taking other medication (Hoffman, 2004). Concerted efforts have been made all over the world to explore the various biological and specific pharmacological activities and their active compounds all over the world. However, targeted screening with improved strategy to evaluate the efficacy of various potential plants against problematic multi drug resistant bacteria is in the stage of infancy.

It is expected that plant extract showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens. However very little information is available on such activity of plant extract (Lee *et al.*, 1998). In the recent years, plants have been screened against multidrug resistant bacteria including *Staphylococcus aureus*, *Salmonella paratyphi*, *Escherichia coli*, *Shigella dysentriae* and *Candida albicans*. The selection of medicinal plant was based on their traditional uses in India and reported antimicrobial activity of many medicinal plants (Chopra *et al.,* 1992; Ahmad *et al.,* 1998; Mehmood *et al.,* 1999).

The recent development in the phytopharmacology is development of multicombinational drug against multidrug resistant bacteria. This has been possible due to interaction among plant extracts (Phytocompounds) and with other chemotherapeutic agents that may be synergistic or additive in their interaction. The development of these drugs has grown a new future in the area of phytopharmacology and medical practices.

At present multi drug therapy or combinational antibiotic therapy is in use. However its efficacy may be severely hindered against several MDR bacteria. Therefore, there is an increased request to develop novel drugs against multi drug resistant bacteria. One possible approach is to screen unexplored Indian medicinal bioactive plant extracts for their potential to be used against multi drug resistant bacteria.

Considering the vast potential of Indian medicinal plants as an anti-infective agent, we have selected *Punica granatum* on the basis of their traditional uses, ethanopharmocoloical data and local availability. The present study has been planned to identify the active phytochemicals tannins and catechins and antimicrobial activity against drug resistant microbial pathogens and to assess synergy with antibiotics *in vitro.*

Material and methods

Plants material- Fresh pomegranate fruit was bought from the local market and washed thoroughly using water. The rind of the pomegranates was separated, washed with distilled water and dried. The dried rind was powdered using commercial grinder. Samples were kept in sealed plastic bags and stored at low temperature in dark, until use.

The authentic plant material was obtained from the Himalaya WellnessCompany, Dehradun, India. The identification of the samples was further confirmed by the plant taxonomist in the Department of Pharmacognosy, HWC Dehradun. The voucher specimen has been deposited in the Department of Pharmacognosy, HWC, Dehradun shown in Table-2.

Drug resistant and sensitive bacterial strains used in the screening programme- The Standard strains were obtained from different National and International Culture Collection Centers/ Collection of individual scientist and clinical isolates were collected from Department of Microbiology, HWC Dehradun Uttarakhand. Multidrug resistant bacteria include the strains of *Staphylococci* including methicillin resistant *Staphylococcus aureus* (MRSA), *Psuedomonas aeruginosa* and R-plasmid harbouring strains of *E. coli.* MRSA and some other Gram positive and Gram negative bacteria were also used in our laboratory. The details of the test strains and their relevant characteristics are mentioned in Table-1.

Chemicals and Antibiotics- All the antibiotic discs were purchased from Hi-Media Lab Pvt Ltd, Mumbai, India. The indicator dye p-iodonitro tetrazolium violet were purchased from Sigma Chemical Co., USA. MMS and Sodium azide were purchased from Sisco Reseach Laboratory, India. All the other media/chemicals used were of analytical grade.

Bacterial cultures- Bacterial isolates were obtained from different sources (Table-1) and were subjected to antibiotic sensitivity by disc diffusion, method (Bauer et al., 1966).

ß-lactamase production- The method described earlier (Ahmad et al., 2008) was used for detection of proudtcion of ß-lactamase.

Culture Media and Inoculum preparation- Nutrient broth/ Agar and Muller–Hinton broth/ agar (Hi-Media Pvt. Ltd., Mumbai, India) were used to grow the test bacteria at appropriate temperature 30-37 ^{0}C for 18hrs and then appropriately diluted in sterile 0.8% saline solution to obtain a cell suspension of 10^5 –10^6 CFU/ml.

Preparation of plant extracts and its fractionation- Plant extracts were prepared as described earlier (Ahmad and Beg 2001) with a little modification. 800 gram of dry, plant powder was soaked in 2.5 liter of 70% ethanol, for 8–10 days and stirred after every 10 hr using a sterilised glass rod. At the end of extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). This alcoholic filtrate was concentrated under vacuum on rotary evaporator at 40 °C and then stored at 4 °C for further use. The crude extract was prepared by dissolving known amount of the dry extract in DMSO, to have a stock solution of 100 mg/ml concentration.

Extraction for HPTLC: Accurately weigh 1 g of dried pomegranate rind powder and extract using 10 mL of analytical-grade methanol. Sonicate for 15-20 minutes.

HPTLC method development of pomegranate peels – Used TLC silica gel G 60 F254 (10 × 10) plates are used with solvent system **chloroform: ethyl acetate: formic acid (4:3:3 v/v/v) as a solvent system.** The sample was spotted on precoated TLC plates using Linomat 5 applicator, the ascending mode is being used for the development of thin-layer chromatography, TLC plates were developed from solvent front position 85.00 mm. The plates were air-dried and then scanned at 254 nm and 366 nm. (Pooja Gadkari and Sanjay Daharwal, 2022).

Antimicrobial assay- The agar well diffusion method (Perez et al. 1990) as adopted earlier (Ahmad and Beg 2001) was used. 0.1 ml of diluted inoculum (10^5 CFU/ml) of test organism was spread on Muller-Hinton agar plates. Wells of 8 mm

diameter were punched into the agar medium and filled with 100µl of plant extract of 10mg/ml concentration and solvent blank (DMSO) separately. The plates were incubated at 37 °C, over night. The antibiotic (chloramphenicol) at 100µg/ml conc. was used in the test system as positive control. Zone of inhibition of bacterial growth around each well was measured in mm.

Minimum inhibitory concentration of plant extracts- Minimum inhibitory concentration of plant extracts against test bacterial strains was determined by tube broth dilution method, using specific dye (p-iodonitro tetrazolium violet) as an indicator of growth (Eloff 1998). 2 ml of the plant extract was mixed with 2 ml of Muller-Hinton broth (Hi-Media Ltd., Mumbai, India) and serially diluted into the next tube and so on. 2 ml of an actively growing culture of different test strains was added before incubating for over night, at 37 °C. After examining turbidity visually, 0.8 ml of 0.02 mg/ml indicator dye (p-iodonitro tetrazolium violet) was added to each tube and incubated at 37 °C. The tubes were examined for the colour development, after 30 min. Absence of growth was also confirmed by spreading 0.1 ml of broth from such test tube on normal nutrient agar plate.

Synergistic interaction of plant extracts with antibiotics- Synergistic interaction between antibiotics, like ampicillin, tetracycline and chloramphenicol with crude plant extracts was studied by agar well diffusion method. For determining the synergistic effects of plant extract with antibiotic, the wells were punched at a predetermined distance so that their inhibitory circles touch each other only tangentially without influencing each other as recommended by Ahmad et al. (2008). The wells were inoculated with plant extract and antibiotic separately. Plates were then incubated at 37 °C, for 18 hours. Enlargement of inhibition zones indicates a positive interaction (synergism).

Phytochemical analysis of plant extracts- Major phytocompounds, in the crude extracts of plants, were detected by standard colour tests and thin layer chromatography, as described by(Ahmad and Beg 2001).

Results and Discussion

Antimicrobial activity of plant extracts against drug resistance pathogenic bacteria

Multiple drug resistance in pathogenic bacteria has emerged as important problem in many countries of the world. There are now increasing case reports documenting the development of clinical resistance to newer and broad spectrum antibacterial drugs like fluroquinolone (norfloxacin, Cipro-floxacin, oflaxacin etc.) in many pathogenic bacteria. In the present study, clinical isolates of *S. aureus P. aeruginosa, E. coli,* and *Candida albicans* were used. These microbial strains are found to be resistant to one or more antibiotics, showing the common occurrence of drug resistance. These finding are in agreement with the reports of previous workers as these strains have been previously tested for their sensitivity to antibiotics (Ahmad and Arina, 2001; Aqil et al., 2005, Aqil and Ahmad, 2007; Jafri et al., 2014). Further these test isolates of bacteria were also tested for the production of β-lactamases **(Table-1).**

In the present study, *Punica granatum*

was selected on the basis of their traditional uses in treatment of different disease in India and worldwide. Only alcoholic extracts of plant material have been used as the alcohol was found suitable solvent for the extraction of antimicrobially active constituents from plants (Eloff,1998; Ahmad et al.,1998). Antibacterial activity of crude extracts of *Punica granatum* against Gram positive bacteria (7 distinct isolates of *S. aureus*) and Gram- negative bacteria (*E.coli*, *P. aeruginosa*) and a yeast (*C. albicans*) is presented in **(Table-1)**. Activity of ethanolic crude extracts against Gram positive bacteria and Gram negative MDR bacteria and *Candida albicans* showed broad spectrum **(Table-3)**.

Table-1 Antibiotics resistant pattern and β-lactamase production by test strains

Name of bacteria	Strains code	β-lactamase hydrolyzing β-lactam antibiotics		Resistant pattern of used strains against antibiotics
		Ampicillin	Benzyl penicillin	
Staphylococcus aureus	SA-03	+	+	Cx, M, A. Pn, Cf, Do, Sm, Na
Staphylococcus aureus	SA-08	–	–	Cx, M, A, Pn, Cf, Sm,
Staphylococcus aureus	SA-11	+	+	Pn, Am, M, S, T, Do, Na, Cu,
Staphylococcus aureus	SA-21	+	+	Cx, M, A, Pn, Cf, Do, Sm,
Staphylococcus aureus	SA-22	+	+	Sensitive to all drugs
Staphylococcus aureus	SA-28	+	+	Pn, Am, Cx, Cf, M, Pc, Kt, T, S,
Staphylococcus aureus	SA-29	+	+	Cx, M, A, P,
E.coli	UP-2556	–	–	Pn, A, Cx, Do,
E.coli	EC-14	+	+	Pn, A, Cx, M, Ce, Cfx, Cep, Cu,
E.coli	EC-20	+	+	Pn, A, Cx, M, Ce, Cfx, Cu, Va, T, E,
P. aeruginosa	*P.aeruginosa*	NT	NT	A,C,T,Na, Co, Cx, Am, M

Pn, Penicillin, A, Ampicillin; Cx, Cloxacillin, Ce, Cephotaxime; Cu, Cefuroxime; Cfx, Cefixime, Cefpodoxime; M, Methicillin; Va, Vancomycin; Nf, Nitrofurantoin, Nx, Norfloxacin, Nv, Novobiocin Co, Co-trimoxazole; Na, Nalidixic acid; T, Tetracyline; C, Chloramphenicol; Do, Doxycycline; E, Erthromycin.

Table-2 Antibacterial activity of plant extracts against Gram positive bacteria

S. No	Scientific Name (Family)	Percent Yield	Antimicrobial activity (Radius in mm) ± SD						
			SA-03	SA-08	SA-11	SA-21	SA-22	SA-28	SA-29
1.	*Punica granatum*	6.25	–	12.2 ± .25	9.33± 0.41	8.5±0 .24	18.2± 0.2	10.16± 0.2	11.33± 0.2

SD – Standard deviation

Table-3 Antibacterial activity of plant extracts against Gram negative bacteria

S. No	Scientific Name (Family)	Antimicrobial activity (Zone in mm) ± SD*							
		SM-06	SM-07	SM-08	UP 2566	EC-14	EC-20	P	CA
	Punica granatum	12.1± 0.6	15.2±0.4	9.2± 0.1	13.0 ±0.4	8.2± 0.2	8.2± 0.2	6.4±0.2	18.3± 0.1

***SD – Standard deviation**

Similar findings have been reported by Mehmood et al., 1999 and few other workers (Aqil and Ahmad, 2007). *Punica granatum* peel extracts were also evaluated for their potency in terms of Minimum Inhibitory concentration against a variety of MDR bacteria as shown in **(Table-4)**. MIC values of varied greatly from 0.41 mg/ml to 4.81 mg/ml against test bacteria. Variation in MIC values might be due to difference in cell well composition and intrinsic tolerance of the test isolates, nature and composition of phytoconstituents.

Table- 4 Activity profile of crude plant extracts of Punica granatum in terms of Minimum inhibitory concentration (MIC)

S. No	Plant Extract	Yield in mg/100 gm of dry powder	Minimum inhibitory concentration against test microorganisms (mg/ml)										
			SA						EC				
			SA-03	SA-08	SA-11	SA-21	SA-28	SA-29	EC-M	EC-14	EC-20	P	CA
1	*Punica granatum*	10.25	3.5	3.62	4.62	0.41	1.38	4.16	4.11	2.03	2.03	4.81	1.30

NT - Not tested, Organisms key : SA – *Staphylococcus aureus,* , EC - *E.coli,* P – *Pseudomonas aeruginosa,* CA- *Candida albicans* .

Phytochemcial analysis of *Punica granatum* extract was made for the presence of major phytocompounds like alkaloids, flavonoides phenols & tannins as depicted in **(Table-5)**. The presence of Catechin and epicatechin was detected in the crude extracts by HTLC as given in **(Plate- 1 and 2)**. The differences in phytocompounds might be responsible for varied activity & MIC values. These observations were supported by many workers (Nakamura et al., 2002; Shanab et al., 2004). Thus our antimicrobial screening results also justify the traditional uses of these plants in ailments and localized skin infections caused by *S.aureus, E.coli, P.aeruginosa,* and *Candida albicans*.

Table 5 Phytochemical analysis of active plant extracts for major bioactive compounds

S. no.	Plant name	Part used	Phytocompounds detected					
			Alkaloids	Flavonoids	Glycosides	Phenols	Tannins	
							Epi/ gallo	Condensed salts
1.	*Punica granatum*	Rind	+	+	–	+	+	–

HPTLC Chromatograms

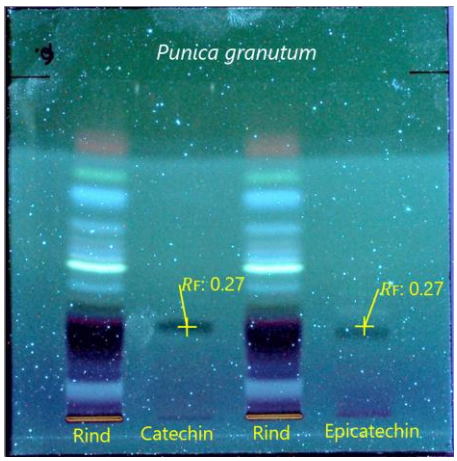

PLATE-1

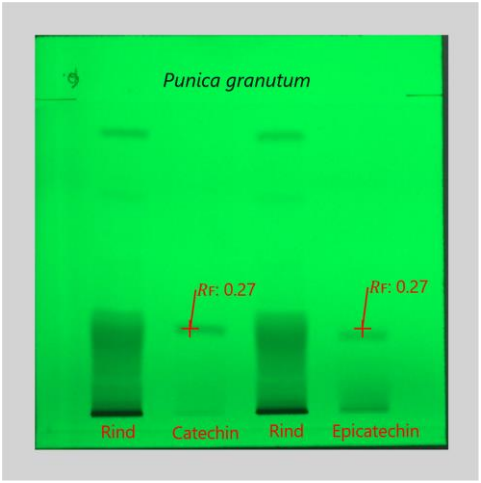

PLATE-2

Synergism in plant extracts- In the traditional system of medicine (Ayurveda and Unani-Tibbiya) formulation of herbal drugs are prepared as a mixture of many crude extracts in different preparations. It is commonly believed that various active phyto-constituents of plant extracts posses additive or synergistic activity.

Multiple antibiotic therapy is now considered an effective way to control infectious diseases caused by drug resistant bacteria. Phytocompounds which may have strong activity against antibiotic resistant bacteria is expected to give strong synergistic and additive effect with antibiotics. Considering this known fact we have tried to see the possible synergistic effect between plant extracts *Punica granatum* and antibiotics(Table-6). *The* extract showed synergistic interaction with tetracycline, chloramphenicol, ampicillin and gentamycin against mutlidrug resistant *S. aureus* (MRSA) strain. The above findings show that synergistic interactions are specific and the possible reason may be found in the interaction of different phyto-constituents with antibiotics. This result agrees with the observation of synergistic interactions of medicinal plants with chloramphenicol as reported by Lee (1998) and Aqil et al., (2005).

Table 6 Synergistic interaction between *P.granatum* extract and antibiotics

Strains Used	Plant extract (P)	r_P (in mm)	Antibiotic (A)	r_A (in mm)	Combined radius (r_P + r_A) in mm	Enlargement of Zone-size (in mm)	Synergism
SA-03	*P.granatum*	6.0 ± 0.4	C T Gm Am NA	16.8 ± 0.7 17.1 ± 0.8 14.0 ± 0.7 14.0 ± 0.2 –	25.1 ± 0.4 23.4 ± 0.5 24.5 ± 0.5 23.0 ± 0.6 –	3 – 4 3 –	+ – + + –
SA-08	*P.granatum*	7.0 ± 0.8	C T Gm Am	11.0 ± 5 12.0 ± 0.5 12.0 ± 0.5	23.1 ± 0.5 19.1 ± 0.3 22.1 ± 0.4	5 – 3 –	+ – + –

			Na Cf	10.1 ± 1.0 10.0 ± 0.3 16.2 ± 0.6	17.0 ± 0.5 17.8 ± 1.5 23.8 ± 0.6	– –	– –
SM-07	*P.granatum*	5.5 ± 0.2	C T Am Na Cf`	11.3± 0.17 5.1± 0.1 8.4 ± 0.2 – 8.2 ± 0.5	16.3 ± 0.4 10.0 ± 0.3 13.6 ± 0.3 – 13.4 ± 0.7	– – – – –	– – – – –
EC-20	*P.granatum*	8.3 ± 0.3	C T Gm Am Na Cf	14.3± 0.2 16.3 ± 0.1 15.2 ± 0.3 12.1 ± 0.1 11.4 ± 0.1 20.4 ± 0.1	22.6 ± 0.5 24.4 ± 0.2 27.3 ± 0.3 20.4 ± 0.2 19.6 ± 0.2 28.4 ± 0.2	– – – – – –	– – – – – –

Conclusion

This preliminary investigation indicated that plant extracts of *Punica granatum(peel)* showing broad spectrum antimicrobial activity and synergy could be further tested to determine the efficacy *in vivo* against MDR bacteria. Active fractions of various plants may also be exploited in preparation of herbal formulation of improved efficacy and quality.

Disclaimer Statement

Authors declare that no competing interest exists. The products used for this research are commonly used products in research. There is no conflict of interest between authors and producers of the product.

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