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Research Article

In vitro Antimicrobial Activity Screening of *Punica Granatum* Extracts Against Human Pathogens

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Abstract

The demand for new antibiotics continues to grow due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. Currently, many scientists devoted in searching new biologically active antimicrobials which have possibly novel mechanisms of action, with no many side effects and wide therapeutic potentials. The study was done to evaluate the antimicrobial potentials of *Punica granatum* leaf extract against standard and drug resistant bacteria and *Cadidia albicans* using agar well diffusion assay and broth dilution methods. Active compounds of these plants which are responsible for the antimicrobial activities were extracted using four different solvents (acetone, methanol, chloroform and water). Vancomycin, methicillin, and amoxicillin were used as positive controls and pure water was as negative control in the well diffusion assay. Leaf crude extracts of the plant were showed various degrees of antimicrobial activities with the maximum mean inhibition zones reaches to 22.6 ± 5.50 mm in diameter with methanol leaf extracts against *methicillin* resistant *Staphylococcus aureus*. All the test organisms which were inhibited by all the extracts in agar well diffusion method also showed minimum inhibitory concentration values at concentration ranging from 6.25% to 50%. From all the test organisms used, *Punica granatum* relatively demonstrated better potential with minimum inhibitory concentration between 6.25% and 12% for all its extracts against on both standard and drug resistant *Streptococcus pneumonia*. Moreover, the most frequent minimum inhibitory concentration value among all extracts was 6.25%, followed 12.5%, 25% and 50%. Synergetic effects were obtained against *methicillin* resistant *Staphylococcus aureus* with the combined effects between chloroform extracts of *Punica granatum* with that of amoxicillin and vancomycin standard antibiotics. Results of the present investigation verified that the therapeutic potentials of *Punica granatum* leaf extracts to combat the raise of drug resistant bacteria and fungus hence could be proposed as an alternative approaches for the discovery of novel antimicrobial drugs.

Keywords: Crude extract; Synergetic effect; Drug resistant; Zone of inhibition

Introduction

Plants are used worldwide for the treatment of many diseases, and novel drugs continuously developed from plants. Globally the estimate of more than 20,000 species of plants used in traditional medicines, and all of these bioactive fractions used for development of new drugs [1]. In most developing countries, where coverage by health services is limited, it is to the traditional practitioner or to folk medicine that the majority of the population turns when sick. The treatment they receive is largely based on the use of medicinal plants [2]. Countries like China, Pakistan, India and Vietnam have identified potential usage of plant medicine and incorporated them into their overall health care system [3].

In view of the large number of the plant species potentially available for study, it is essential to have efficient systems of the methods to evaluate efficacy of medicinal plants as antimicrobial agent. The evaluation for antimicrobial agent of plant origin begins with thorough biological evaluation of plant extracts to ensure efficacy and safety followed by identification of active principles, dosage formulations, efficacy and pharmacokinetic profile of the new drug [4].

Biologists inspired on antimicrobial chemotherapy as soon as microorganisms were understood to be agents of infectious disease. In earlier times, plant products were sometimes used successfully in the treatment of disease, but neither doctors nor patients knew the basis for the action of these therapeutic agents. Many early medicines were used to cure protozoan diseases, rather than bacterial diseases. As early as 1619,

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it was known that malaria could be treated with the extract of cinchona bark (quinine) and that amoebic dysentery could be treated with ipecacuanha root (emetine) [5].

Antibiotics have long been considered the "magic bullet" that would end infectious disease. Although they have improved the health of countless numbers of humans and animals, many antibiotics have also been losing their effectiveness since the beginning of the antibiotic era. Bacteria have adapted defenses against these antibiotics and continue to develop new resistances, even as we develop new antibiotics. As more microbial species and strains become resistant, many diseases have become difficult to treat, a phenomenon frequently ascribed to both indiscriminate and inappropriate use of antibiotics in human medicine. Moreover, the use of antibiotics and antimicrobials in raising food animals has also contributed significantly to the pool of antibiotic resistant organisms globally and antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. There is no doubt that the use of antibiotics provides selective pressure that result in antibiotic resistant bacteria and resistance genes [6].

Punica granatum is a fruit-bearing deciduous shrub or small tree. It belongs to family Punicaceae and distributed throughout the tropics and sub tropics, historically it was believed to be a symbol of fertility. It could be shrub or tree and usually grows to < 5 m, but can occasionally reach 12 m in favorable conditions [7]. The leaves are shiny and about 7.6 cm long [8]. Different parts of the plant such as bark, leaves, immature fruits and fruit rind have medicinal significance [9]. *Punica granatum* has been extensively used as a traditional medicine in many countries for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies [10]. Additionally, this plant is reported to have excellent antibacterial, antifungal, antiprotozoal and antioxidant properties [11-13]. Numerous phytochemical constituents have been reported to be present in different parts of the pomegranate plant making it pharmacologically precious [14,15].

However, the antimicrobial compounds from plants may inhibit growth of microorganisms by different mechanisms than those presently used [16]. So, there is a need to develop plant based new antibiotics which are effective against resistant microorganisms. So that, this research was done to evaluate the potential of plant extracts on clinical isolates of drug resistant bacteria strains and fungus (*Candidia albicans*). Moreover, synergistic effects of crude extracts in association with pure prescribed antibiotics were investigated against all the test bacteria and fungus.

Material and Methods

Study area

The study was conducted at Jig-jiga University in the Department of Biology which is located 675km away from Addis Ababa in Eastern Ethiopia from September, 2014 to April, 2015 and the laboratory work has been accomplished at Addis Ababa University.

Collection of plant materials

Each plant species *Punica granatum* was collected from Bobas, where the plants are widely available, and identified further in herbarium at Addis Ababa University, Botanical Herbarium. The fresh leaves of each plant was washed three times with pure (sterilized) water, allowed it to air dry at room temperature under shade (exposure to sun light was avoided to prevent loss of active components). The dried leaves were powdered with the help of pestle and mortar and store in sterile bottle [17] for further analysis.

Extraction of plant leaves

In this study, 50g of shade-dried powder was loaded in 1000 ml flask and mixed with 250 ml of 95% methanol, acetone, chloroform and sterilized water solvents for extraction and then shacked on a rotary shaker for three days. The extracts was filtered by passing through Whatman's filter paper No:1 and centrifuged at 5000 rpm for 15min. The solvent extracts was concentrated under reduced pressure using Rotavapor and preserve at 4°C in air tight bottle for further investigation.

Test organisms

Standard and drug resistant pathogenic bacteria were used for determination of antimicrobial activities of different extracts. In brief, the bacterial strains which was used to assess the antimicrobial properties of extracts includes both standard and drug resistant clinical isolates of gram positive and gram negative strains*: Staphylococcus aureus (S. aureus)* (MTCC2001), *Streptococcus pneumonia (S. pneumoniae)* (MTCC5954), *Escherichia coli (E. coli)* (MTCC8650), *Shigella boydii (S. boydii)* (MTCC7704) and as well as *Candida albicans (C. albicans)*. They were obtained from Pasture Institute of Microbiology and were maintained on Nutrient agar (NA) and Sabouraud-dextrose agar (SDA) (for the fungal strain only) and sub cultured before use.

Preparation of inoculums

The microbial stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments was prepared by transferring a loopful of cells from the stock cultures to test tubes of Muller-Hinton broth (MHB) and was incubated without agitation for 24 hrs at 37°C. To 5 ml of saline solution, 100 µl of culture was inoculated and incubated till it reached the turbidity equal to 0.5 McFarland standard solutions which is equivalent to 10^6-10^8 CFU/ml [18].

Antimicrobial activity assay

The antimicrobial activity assay was done by agar well diffusion method. In short, antibacterial activities of the different extracts were determined by agar well diffusion assay on MHA medium. The MHA was melted and cooled to 48 - 50ºC and poured into sterile Petri dishes to give a solid plate. Then standardized inoculums of 100 µl (1.5×10^8 CFU/ml, 0.5 McFarland) was added aseptically and streaked on the agar plate surface. Wells were prepared in the seeded agar plates with sterile cork borer (6 mm diameter). The test compound or crude extract (100 µl) was carefully dispensed into the wells (6 mm). This was done in triplicate in parallel to different control antibiotics. Extracts were allowed to diffuse for about 2 hrs before incubation. Plates were incubated overnight at 37°C. After overnight incubation, the plates were observed for the zone of inhibition and the diameter of the inhibition zone was measured. The presence of a zone of inhibition around each well was indicative of antibacterial activity. The diameters of zone of inhibition produced by the

extract agents were compared with those produced by the commercial control antibiotics.

Determination of MIC and MBC/MFC

The Minimum Inhibitory Concentration (MIC) of *Punica granatum* leave extracts were determined according to methods described by [19]. Each extracts were diluted to concentrations ranging from 6.25% to 50%. To each dilution of leave extract, nutrient broth tubes were seeded with 100 µl of the pathogenic standard and resistant clinical bacterial inoculums, and clinical isolate of the fungus. Negative control tubes with no bacterial and fungal inoculation was simultaneously maintained. Tubes were incubated aerobically at 37°C for 24 h. The lowest concentration of the extract that produced no visible growth (turbidity) was recorded as the MIC. Dilutions showing no visible growth for the MIC were sub cultured onto a fresh MHA plate and incubated at 37°C for 24 h for the determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC). In brief, dilution showing no visible growth in the determination of MIC was streaked (sub cultured) on to MHA and incubated for 24 h for the bacteria and the fungus. The least concentration of the extract with no visible growth after incubation was taken as the MBC and MFC.

Synergetic effect determination of crude plant extracts and pure antibiotics

The crude extracts and standard antibiotics were diluted to (10% chloroform extract, 10%, amoxicillin, 10% vancomycin) as well as combination of extracts with clinically prescribed drugs were diluted to (10% extract plus 10% (amoxicillin, vancomycin) to evaluate whether synergetic effect was there or not. Then 100 µl of each crude extract was added into the wells of 6 mm to examine inhibition zone differences after incubation [20].

Data Analysis

The data was analyzed using SPSS version 21.0. Means and standard deviations of the triplicate analysis were calculated using analysis of variance (ANOVA) to determine the significance differences between the means using Duncan's Multiple range test ($p \leq 0.05$) when the F-test demonstrated significance. The statistically significant difference was determined as $p \leq 0.05$.

Results

Extracts yielded

The plants were extracted using four different solvents to increase the possibility of getting the bioactive components, thus their leaf were treated with each solvent chosen and the crude extracts yielded are calculated in percentage. From 50g of *Punica granatum* leaf powder, 23%, 24%, 14% and 21% of crude antibiotics were collected for diffusion and dilution analysis with acetone, methanol, and chloroform and water solvents, respectively.

Determination of antimicrobial activity of plant extracts

The extracts were tested for the antimicrobial activity against the selected bacterial and fungal strains using agar well diffusion assay and broth dilution methods.

Agar well diffusion method

All extracts of *Punica granatum* against *S. aureus* (MTCC2001) showed various degrees of antibacterial activity. Both methanol (22.3 ± 1.15) and chloroform (19.6 ± 2.88) crude extracts were resulted significantly ($p \leq 0.05$) wider clear zones than the disc antibiotics of methicillin (12.3 ± 2.30) and amoxicillin (13 ± 1.00). On the other hand, only methanol (22.3 ± 1.15) extracts were showed better activity than to that of water (15.0 ± 2.64) among all sample extracts against on the same strain.

Inhibition zones of methanol (22.6 ± 5.50) and chloroform (20.3 ± 7.57) extracts were significantly ($p \leq 0.05$) greater than the all commercial antibiotics vancomycin (15 ± .00), methicillin (0 ± .00) and amoxicillin (11.6 ± 2.8) towards MRSA. Similarly, acetone (18.0 ± 5.0) and water (12.0 ± 3.60) inhibition zones were statistically greater than methicillin and amoxicillin positive controls on MR*SA*. Moreover, there was no statistically significant ($p>0.05$) inhibition difference observed between the three solvent crude extracts, although greater the water extracts (Table 1).

Among all the crude antibiotics of the same plant tested against *S. pneumonia* (MTCC5954), acetone (21.6 ± 1.52) extracts were showed significantly greater clear zones than the controls vancomycin (16.6 ± 2.51), methicillin (10.3 ± 1.52), amoxicillin (15.6 ± 1.15), and to other solvent extracts methanol (12.3 ± 0.57), water (16.0 ± .00) and chloroform (14.3 ± 0.57). Alternatively, methanol, water and chloroform extracts were showed considerable antimicrobial effect with no significant difference between each other (Table 2).

The study was also showed that acetone (17.0 ± 6.24), water (15.6 ± 2.30) and chloroform (17.6 ± 0.57) extracts *Punica granatum* were measured a significantly ($p \leq 0.05$) greater inhibition zones than the parallel tested commercial antibiotics vancomycin (10.0 ± .00), methicillin (0 ± .00) and amoxicillin (0 ± .00) towards the drug resistant *S. pneumonia*, however no significant inhibition zones difference obtained between the solvent extract themselves. Comparably methanol (10.3 ± 0.57) extracts did not elicit statistically better activity than vancomycin (10.0 ± .00) control antibiotics on the same resistant pathogen.

Values are means of triplicate determination values within the same row and column followed by different superscripts are statistically different ($p \leq 0.05$) against each tested microorganisms

Acetone (18.6 ± 4.6), methanol (17.0 ± 1.00) and chloroform (15.0 ± .00) extracts of the same plant were recorded a significantly ($p \leq 0.05$) greater inhibition zone than vancomycin (12.3 ± 0.57), methicillin (8.6 ± 0.57) and amoxicillin (11.0 ± 1.00) against *E. coli* (MTCC8650), though no significant clear zone difference obtained among themselves. And there was no antibacterial activity showed with the water (0 ± .00) extracts.

All, except water, extracts of *Punica granatum* provoke a remarkable antibacterial activity and efficiency with inhibition zones of acetone (14.3 ± 2.51), methanol (12.0 ± 3.46) and chloroform (13.0 ± 1.00) extracts against drug resistant *E. coli* with no significant difference between one another. Nevertheless, all the controls and water extracts were exhibited no activity at all on the same test bacteria.

Test micro organisms	Solvents used for extraction	Mean inhibition zone (mm)				
		Crude extracts	Positive controls			Negative control
			V	Me	Am	Pure water
S. aureus (MTCC2001)	A	20.3 ± 5.50^{ab}	19.3 ± 2.51^{a}	12.3 ± 2.30^{b}	13 ± 1.00^{b}	0
	M	22.3 ± 1.15^{a}	19.3 ± 2.51^{a}	12.3 ± 2.30^{b}	13 ± 1.00^{b}	0
	W	15.0 ± 2.64^{b}	19.3 ± 2.51^{a}	12.3 ± 2.30^{b}	13 ± 1.00^{b}	0
	C	19.6 ± 2.88^{ab}	19.3 ± 2.51^{a}	12.3 ± 2.30^{c}	13 ± 1.00^{c}	0
MRSA	A	18.0 ± 5.0^{a}	15 ± .00^{ab}	0 ± .00^{c}	11.6 ± 2.8^{b}	0
	M	22.6 ± 5.50^{a}	15 ± .00^{b}	0 ± .00^{c}	11.6 ± 2.8^{b}	0
	W	12.0 ± 3.60^{b}	15 ± .00^{b}	0 ± .00^{a}	11.6 ± 2.8^{b}	0
	C	20.3 ± 7.57^{a}	15 ± .00^{b}	0 ± .00^{c}	11.6 ± 2.8^{b}	0
S. pneumonia (MTCC5954)	A	21.6 ± 1.52^{a}	16.6 ± 2.51^{b}	10.3 ± 1.52^{c}	15.6 ± 1.15^{b}	0
	M	12.3 ± 0.57^{b}	16.6 ± 2.51^{a}	10.3 ± 1.52^{b}	15.6 ± 1.15^{a}	0
	W	16.0 ± .00^{b}	16.6 ± 2.51^{b}	10.3 ± 1.52^{a}	15.6 ± 1.15^{b}	0
	C	14.3 ± 0.57^{b}	16.6 ± 2.51^{b}	10.3 ± 1.52^{a}	15.6 ± 1.15^{b}	0
S. pneumonia (drug resistance)	A	17.0 ± 6.24^{a}	10.0 ± .00^{b}	0 ± .00^{c}	0 ± .00^{c}	0
	M	10.3 ± 0.57^{b}	10.0 ± .00^{b}	0 ± .00^{a}	0 ± .00^{a}	0
	W	15.6 ± 2.30^{ab}	10.0 ± .00^{c}	0 ± .00^{d}	0 ± .00^{d}	0
	C	17.6 ± 0.57^{a}	10.0 ± .00^{b}	0 ± .00^{c}	0 ± .00^{c}	0

Where: mean ± Standard deviation in triplicate, V=Vancomycin, Me= Methicillin, Am= Amoxicillin, A= Acetone, M= Methanol, W= Water, C= Chloroform, *= statistically not significant b/n different solvent extracts (p≥ 0.05).

Table 1: Comparison of inhibition zone among crude extracts of *Punica granatum* using different solvents and also with commercial antibiotics against Gram positive bacteria and *C. albicans* (clinical isolate).

Test micro organisms	Solvents used for extraction	Mean inhibition zone (mm)				
		Crude extracts	Positive controls			Negative control
			V	Me	Am	Pure water
E. coli (MTCC8650)	A	18.6 ± 4.6^{a}	12.3 ± 0.57^{b}	8.6 ± 0.57^{b}	11.0 ± 1.00^{b}	0
	M	17.0 ± 1.00^{a}	12.3 ± 0.57^{b}	8.6 ± 0.57^{c}	11.0 ± 1.00^{b}	0
	W	0 ± .00^{b}	12.3 ± 0.57^{a}	8.6 ± 0.57^{c}	11.0 ± 1.00^{a}	0
	C	15.0 ± .00^{a}	12.3 ± 0.57^{b}	8.6 ± 0.57^{c}	11.0 ± 1.00^{bc}	0
E. coli (drug resistance)	A	14.3 ± 2.51^{a}	0 ± .00^{b}	0 ± .00^{b}	0 ± .00^{b}	0
	M	12.0 ± 3.46^{a}	0 ± .00^{b}	0 ± .00^{b}	0 ± .00^{b}	0
	W	0 ± .00^{b}	0 ± .00^{a}	0 ± .00^{a}	0 ± .00^{a}	0
	C	13.0 ± 1.00^{a}	0 ± .00^{b}	0 ± .00^{b}	0 ± .00^{b}	0
S. boydii (MTCC7704)	A	14.0 ± .00^{a}	11.3 ± 0.57^{b}	0 ± .00^{c}	10.3 ± 2.08^{b}	0
	M	13.0 ± 1.00^{a}	11.3 ± 0.57^{a}	0 ± .00^{c}	10.3 ± 2.08^{a}	0
	W	9.0 ± .00^{b}	11.3 ± 0.57^{a}	0 ± .00^{c}	10.3 ± 2.08^{ab}	0
	C	11.3 ± 3.21^{ab}	11.3 ± 0.57^{a}	0 ± .00^{c}	10.3 ± 2.08^{a}	0
C. albicans (clinical isolate)	A	15.3 ± 1.52^{a}	0 ± .00^{b}	0 ± .00^{b}	0 ± .00^{b}	0
	M	10.0 ± 1.00^{b}	0 ± .00^{a}	0 ± .00^{a}	0 ± .00^{a}	0
	W	11.3 ± 0.57^{b}	0 ± .00^{a}	0 ± .00^{a}	0 ± .00^{a}	0
	C	16.6 ± 1.52^{a}	0 ± .00^{b}	0 ± .00^{b}	0 ± .00^{b}	0

Where: mean ± Standard deviation in triplicate, V= Vancomycin, Me= Methicillin, Am= Amoxicillin, A= Acetone, M= Methanol, W= Water, C= Chloroform *= statistically not significant b/n different solvent extracts (p≥ 0.05).

Table 2: Comparison of inhibition zone among crude extracts of *Punica granatum* using different solvents and also with commercial antibiotics against Gram negative bacteria and *C. albicans* (clinical isolate).

Crude extracts of acetone (14.0 ± .00) Inhibition zone was significantly (p≤ 0.05) greater than vancomycin (11.3 ± 0.57), methicillin (0±.00) and amoxicillin **(**10.3 ± 2.08**)** against *S. boydii* (MTCC7704). Unlikely, methanol (13.0 ± 1.00) and chloroform (11.3 ± 3.21) extracts were showed no significant (p>0.05) clear zone difference with that of the controls vancomycin and methicillin. However, the water extracts of the same plant didn't induce significantly better potential over the controls used on the standard bacteria.

Punica granatum extracts of all crude antibiotics were also showed a various range of activities on *C. albicans*. Inhibition zones of acetone (15.3 ± 1.52) and chloroform (16.6 ± 1.52) were significantly (p≤ 0.05) greater than the other two methanol (10.0 ± 1.00) and water (11.3 ± 0.57) solvent extracts, but there was no antifungal activities were shown by the controls (Table 3).

Values are means of triplicate determination values within the same row and column followed by different superscripts are statistically different (p≤ 0.05) against each tested microorganisms.

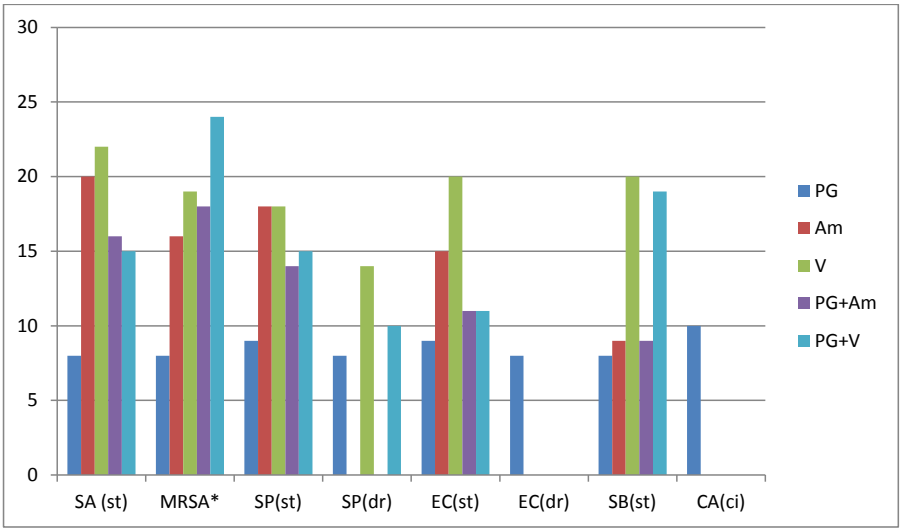
MIC and MBC determination

The crude antibiotics which were showed various range

Crude extracts		Tested microorganisms							
		SA (st)	MRSA	SP(st)	SP(dr)	EC(st)	EC(dr)	SB(st)	CA(ci)
MIC	A	6.25	6.25	6.25	6.25	6.25	12.5	12.5	25
	M	6.25	12.5	6.25	12.5	12.5	25	6.25	12.5
	W	6.25	25	6.25	12.5	no	No	12.5	25
	C	6.25	6.25	12.5	6.25	12.5	50	25	6.25
MBC/MFC	A	6.25	12.5	6.25	6.25	12.5	25	25	50
	M	12.5	25	12.5	25	12.5	25	12.5	25
	W	12.5	50	6.25	25	no	no	12.5	25
	C	6.25	6.25	25	12.5	25	50	25	12.5

Keys: SA (st) = *S. aureus* (MTCC2001), *MRSA= methicillin* resistant *S. aureus, SP*(st)= *S. pneumonia* (MTCC5954), *SP*(dr)= *S. pneumonia* (drug resistance), *EC*(st)= *E. coli* (MTCC8650), *EC*(dr)= *Escherichia coli* (drug resistance), *SB*(st)= *Shigella boydii* (MTCC7704), *CA*(ci)= *Candida albicans* (clinical isolate)

Table 3: MIC and MBC/MFC determination of different concentration of *Punica granatum* against different tested microorganism.



Keys: PG = 10% crude Chloroform extract of *Punica granatum*, Am= 10% Amoxicillin, V= 10% Vancomycin, PG+Am = 10% (*Punica granatum* extract + Amoxicillin), PG+V = 10% (*Punica granatum* extract + Vancomycin), *= bacteria showed synergetic effect
SA (st) = *S. aureus* (MTCC2001), *MRSA= methicillin* resistant *S. aureus, SP*(st)= *S. pneumonia* (MTCC5954), *SP*(dr)= *S. pneumonia* (drug resistance), *EC*(st)= *E. coli* (MTCC8650), *EC*(dr)= *Escherichia coli* (drug resistance), *SB*(st)= *Shigella boydii* (MTCC7704), *CA*(ci)= *Candida albicans* (clinical isolate)

Figure 1: Association effect between Chloroform extract of *Punica granatum* and pure antibiotics against bacteria and *C. albicans* (clinical isolate)

of MIC/MBC on the test bacteria, had also similarly perform a different degree of inhibitory and fungicidal efficacy with MIC/MFC ranges from 6.25% to 50% on *C. albicans*.

Looking at the lowest MIC value (6.25%), all the different extracts of *Punica granatum* showed a promising inhibitory effect on *S. aureus* (MTCC2001), while their bactericidal concentration was found to be 12.5% with methicillin and water extracts. Moreover, from all the test organisms used, *Punica granatum* relatively demonstrated better potential with MIC between 6.25% and 12% for all its extracts against on both standard and drug resistant *Streptococcus pneumonia*.

All extracts of *Punica granatum* showed a strong inhibitory and bactericidal activity against *S. pneumonia* (MTCC5954) and drug resistant *S.pneumonia* with the MIC and MBC ranged from 6.25% to 12.5%, respectively. On the other hand, all the crude extracts of *Punica granatum* showed MIC ranged from the lowest concentration value 6.25% to 25%, but their MBC reaches to the highest dilution point (50%), against MR*SA*, *S. boydii* (MTCC7704) and *C. albicans*.

The minimum concentration that was enough to inhibit the growth of bacteria with MIC 6.25% was more effective against gram positive than gram negative with all different extractions of *Punica granatum.* There was no any inhibitory and bactericidal activity exhibited towards on both *E. coli* (MTCC8650) and *E.coli* (drug resistant) gram negative bacteria for water extracts of *Punica granatum*.

Evaluation of synergetic effect of plant extract and pure antibiotics

Evaluation of synergetic effect whether the plant crude extracts can exert synergism when tested in combination with the selected commercial antibiotics has been investigated in the study and it's presented below in the Figure 1.

MR*SA* was showed for synergetic effect for the combination of *Punica granatum* chloroform extract with both amoxicillin and vancomycin where their inhibition zone recorded 18mm and 24mm in diameter, respectively.

Column chromatography

One of the crude extracts (*Punica granatum* acetone) was subjected for purification the bioactive components by column

chromatography using silica gel (60-120 mesh). Apiece of cotton was placed at the bottom of the column. Elution was carried out using different solvents (petroleum ether, mixture of petroleum: acetone (ratio), acetone) as per method recommended by [21] with some modifications. Silica gel packed with solvents in the column.

The crude *Punica granatum* acetone extract was loaded at the top of the column and successive fractionated elutes were collected separately in different clean pre weighted test tubes. Eluents were evaporated at room temperature. The fractions were checked for their antibacterial activity against *S. aureus* and *E. coli* (drug resistance) by agar well diffusion method. The fractions with petroleum ether were active against *S. aureus*, fractions eluted by the mixture petroleum ether: acetone (1:6) and acetone showed antibacterial activity against both targeted bacteria. Therefore, this implicated the plant may contain different constituents of polar and non-polar compounds.

Discussion

The antimicrobial effects of *Punica granatum* were previously studied. Indeed, it is reported that leaves of *Punica granatum* are widely used as antimicrobial agents [22]. Moreover, in this study all the four different solvent extracts tested also showed antibacterial and antifungal activities. This may be due to the presence of some metabolic toxins or new bioactive compounds [23] and its antibacterial activity could indicate the presence of wide spectrum secondary metabolites than tannins. The study also showed that, all the extracts of the same plant possesses strong antibacterial activities against on both selected Gram-positive and Gram-negative bacteria, and similarly it was also reported [24,25] that it had the ability to inhibit the activity of drug resistant *S. aureus* and *E. coli*. However, it was in contradictory to the study [25] that the water extracts of the *Punica granatum* didn't show any antibacterial activity against the standard and drug resistant *E.coli* strain in our investigation.

Höfling, et al. [26] reported that methanol extracts of *Punica granatum* showed antifungal activity against *C. albicans*. These results are in accordance to results obtained in the present study for *C. albicans* wherein antifungal activity was observed for all the four different solvent extracts. Subsequently, a multidisciplinary approach shall be conducted to identify the novel active constitutes which are specifically responsible for the antifungal activity, then to contribute the best solution to the current productivity crisis facing the scientific community engaged in drug discovery and development.

Punica granatum chloroform extract were measured for combined effects against to all pathogens used in the sensitivity test and specifically showed synergistic effect against MRSA. Data from the literature as well as our results reveal the great potential of the plant for therapeutic treatment, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search for new compounds. Once extracted, and before being used in new therapeutic treatments, they should have their toxicity test *in vivo.*

In conclusion, the results certainly demonstrated that the therapeutic potentials of *Punica granatum* leaf extracts in the control of resistant bacteria and fungus; which are becoming a threat to human life, in all the techniques applied to check its efficiency. An *in vitro* investigation of each crude extracts has also exhibited an inhibitory effect towards in all targeted microorganisms.

Conflict of Interest

The authors declared that there is no conflict of interest.

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