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The Antimicrobial Effects of the Fruit Extracts of *Punica granatum, Actinidia deliciosa* and *Citrus maxima* on Some Human Pathogenic Microorganisms

Abboud El kichaoi[1], Mahmoud El-Hindi[2], Farida Mosleh[3] & Tarek Elbashiti[4]

Abstract

Objective: To evaluate *in vitro* the antimicrobial activity of ethanol, methanol and water extracts of *Punica granatum* (Pomegranate) "pulp", *Actinidia deliciosa* (Kiwifruit) "peel" and *Citrus maxima* (Pomelo) "pulp" against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. Methods: The extracts were prepared using soxhlet apparatus for 8 hours. Evaluation of antimicrobial activity was carried out using disc diffusion method and microdilution method (MIC) at different concentrations (100-0.195mg/ml) for *S. aureus, E. coli* and *P. aeruginosa*, and (200-0.39mg/ml) for *C. albicans*. Results: The results of this study showed that the water extract of pomegranate pulp "fresh and dried" showed maximum zone of inhibition against *S. aureus* and *E. coli* and the lowest minimum inhibitory concentration against all tested microorganisms. The ethanol extract of pomegranate pulp "fresh and dried" showed maximum zone of inhibition against *P. aeruginosa* and *C. albicans*. Conclusion: These results was revealed the importance of tested extracts in control of some human pathogenic microorganisms.If these results are confirmed by in vivo studies this make the tested extracts especially pomegranate fruit promising plants in medical treatments.

Keywords: Antimicrobial activity, Fruit extracts, Microdilution method, Disc-diffusion method, MIC.

[1] Microbiology Lab, Department of Biotechnology, Islamic University-Gaza, Gaza Strip, Palestine. Email: akichaoi@iugaza.edu.ps.
[2] Microbiology Lab, Department of Biotechnology, Islamic University-Gaza, Gaza Strip, Palestine. Email:mwhhindi@iugaza.edu.ps.
[3] Microbiology Lab, Department of Biotechnology, Islamic University-Gaza, Gaza Strip, Palestine. Email: fmosleh@iugaza.edu.ps.
[4] P.O. Box. 108, Rimal. Microbiology Lab, Department of Biotechnology, Islamic University-Gaza, Gaza Strip, Palestine. Email: tbashiti@iugaza.edu.ps. (Corresponding author)

1. Introduction

Medicinal plants are considered renewable natural resources and have an important and useful role in the protection of human health **(Mannargudi, 2013)**. Medicinal plants also are considered an important source because they contain plentiful amounts of antimicrobial agents **(Bobbarala *et al*., 2009).** Interest has increased in recent years in medical plants and herbs to use as a source of bioactive substances in control of human diseases resulted from microorganisms. Many studies have addressed the impact of plant extracts on the growth of microorganisms and thus the possibility of their use in the treatment of some diseases resulting from various microbial infections **(Nascimento *et al*., 2000; Mohana *et al*., 2008; Gurjar *et al*., 2012 & Djeussi *et al*., 2013).** Some Palestinian plants possess great ability against human bacterial **(Adwan & Mhanna, 2008)**. Bacteria, fungi and other microorganisms cause many health problems for humans and also for plants and animals, leading to loss in the agricultural and livestock production. In health care systems all over the world bacterial infections are one of the major issues due to serious problems that resulted from these infections **(Abeysinghe & Weeraddana, 2011).**

The presence of resistant strains of bacteria attributed to the extensive use of antibiotics **(Adwan & Mhanna, 2008)**. Plants play an important role in human health as a natural source of antioxidants, there are numerous medicinal plants described of common antioxidant plants **(Koleva *et al*., 2002; Gupta & Sharma, 2006; Saeed *et al*., 2012; & Nishaa *et al*., 2012).** In nature there are a wide variety of antioxidants which are different in their composition, physical and chemical properties **(Koleva *et al*., 2002).** Antioxidant is any substance prevents oxidation of cell content like proteins, carbohydrates and DNA at low concentrations **(Gupta & Sharma, 2006).** Based on a study of *N. Jaradat,* 2005 found one hundred and eighty four different plant species were used in folk medicine in Palestine for hypoglycemic, hypotensive and diuretic effects **(Jaradat, 2005).**

In Palestine and other different countries, several studies have been conducted in order to study the effect of plant extract against pathogenic microorganisms, which revealed the importance of these plants and thus the possibility of their use in the treatment of some diseases. Studies are still list to explore the most promising methods to extract these active compounds in plant and incorporated in the production of medicines and drugs.

Many studies have proved that pomegranate posses different therapeutic properties. It has been used in traditional medicine to treat diarrhea and internal parasites (**Mansour *et al*., 2013).** Also it rich in antioxidant tannins and flavonoids (**Chidambara *et al*., 2002).** A number of studies on pomelo extract have reported that it has antioxidant properties.

The flesh of pomelo has been used in the past as an appetizer and antitoxic **(He *et al*., 2012 & Caengprasath *et al*., 2013).** Results and experiments revealed that Kiwifruit a rich source of antioxidants. Also it is considered a good source of other nutrients such as potassium, and dietary fiber **(Singletary, 2012).** These properties found in these fruits are encouraging to carried out this study and made them promising plants in medical treatments.

2. Materials and methods

2.1. Chemicals

Mueller Hinton agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Dextrose Agar (SDA), RPMI 1640 Media, 80%Methanol, 80%Ethanol, Distilled water (D.W) and Dimethyl Sulphoxide (DMSO).

Chemicals	Manufacturer	Country
Mueller Hinton agar	Liofilchem	Italy
Mueller Hinton Broth	Liofilchem	Italy
Sabouraud Dextrose Agar	HIMEDIA	INDIA
RPMI 1640 Media	Sigma	Germany
Dimethyl Sulphoxide (DMSO).	Applichem	Germany
Methanol, Ethanol, Distilled water	CHEM Limited	India

2.2. Plant collection

The fruits used in this study (*Punica granatum*, *Actinidia deliciosa* and *Citrus maxima*) were collected from different areas in Gaza strip.

2.3. Extraction

The used parts from these fruits were pulp from (Pomegranate and Pomelo) and peel from (Kiwi fruit). Pulp and peel were separated from fruits and used as fresh and also air dried for 3 days. Then the fresh and dried parts cut into small pieces and 30 grams of these pieces extracted in a Soxhlet extractor by using 300 ml of 80%ethanol, 80%methanol, and water for 8 hours. The resulting extracts were evaporated using oven temperature 37°C for 3 days. Then all extracts were dissolved in DMSO. One gram of each extract was dissolved in 5 ml of DMSO. Thus 200 mg / ml of stock was obtained as a standard concentration of extracts. Then extracts were sterilized using 0.22 µm membrane filters and all samples were maintained at -4°C until the usage time **(Abeysinghe & Weeraddana, 2011).**

2.4. Antimicrobial susceptibility test

2.4.1. Microorganisms

The microorganisms which have been used in this study are the bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) and the fungi (*Candida albicans*) which were isolated from clinical samples delivered from El-Shifa Hospital. *S. aureus*, *E.coli* and *C. albicans* isolates were identified in microbiology laboratories of Islamic university-Gaza, by Stafsystem 18 R Kit for *S. aureus,* Enterosystem 18R Kit for *E. coli* and by culturing for *C. albicans* on Sabouraud's dextrose agar (SDA).

2.4.2. Antimicrobial test

2.4.2.1. Agar disc-diffusion method

Agar disc-diffusion method was followed to determine the antimicrobial activity. A suspension of inoculum of pathogenic microorganisms was introduced to MHA and SDA (cooled to 40-45°C) swirl gently to mix well. After solidification, sterile filter paper discs approximately 6mm in diameter were impregnated with stock extracts of concentration (200 mg/ml) and placed on the surface agar plate. Incubation period of 24h at 37°C for bacteria, and (24-48)h at 37°C for fungi. The antimicrobial activity was evaluated by measuring zones of inhibition of microbial growth surrounding the plant extracts **(Sharma, 2011).**

2.4.2.1. Minimum Inhibitory concentration

ANTIBACTERIAL ASSAY: The activity of extracts against microorganism was also determined by the broth macrodilution method (96- well plates).Extracts were diluted a number of times through a sterile diluent (MHB) after were diluted the obtained concentration ranges were from (100 to 0.1953) mg/ml. Then added 10 µl of inocula of overnight growth microorganisms to each well except a positive control.

Extract with media was used as a positive control and inoculum with media was used as a negative control. The test plates were incubated at 37°C for 24 h. After 18 h 50 µl of a 0.01% solution of 2, 3, 5 triphenyl tetrazolium chloride (TTC) as indicator was added to the wells and the plate was incubated for another hour. Since the colorless tetrazolium salt is reduced to red colored product by biological active bacteria, the inhibition of growth can be detected when the solution in the well remains clear after incubation with TTC **(Abu-Shanab *et al*., 2004).**

ANTIFUNGAL ASSAY: Before starting the antifungal assay, *C. albicans* was cultured on SDA media and incubated for 1-2 days at 37°C. Microbroth dilution assay for *C. albicans* carried out by adding 100 µl *C. albicans* suspension to each well except a positive control and the plate was incubated at 37°C for 24h to form the biofilm. After 24h the media was removed and washed three times with phosphate buffer saline then extracts were diluted a number of times through a sterile diluent (RPMI 1640 media) and incubated for 48h at 37°C **(Pierce *et al*., 2010).** MIC was determined at the lowest concentration that inhibited visible fungal growth.

3. Results

3.1. Bioactivity of fruit extract by disc diffusion method

The results in Table 1 showed the antimicrobial activity by disc diffusion method against tested microorganisms. For *S. aureus*, the water extract of pomegranate pulp " fresh and dried " showed the highest effect with a 23 mm zone of inhibition followed by ethanolic extract of pomegranate pulp "fresh" and "dried" with a 15mm and 10mm zone of inhibition, respectively. Kiwipeel "dried" and "fresh" extracted by ethanol and methanol showed moderate activity.

No antimicrobial activity was observed by pomelo "pulp" extracted by ethanol, methanol and water. For *E. coli*, the water extract of pomegranate pulp "dried" was showed the highest effect with a 20 mm zone of inhibition followed by pomegranate pulp "fresh" with a 17mm zone of inhibition. The ethanolic extract of pomegranate pulp "fresh" and "dried" also showed a good activity with a zone of inhibition (14mm).No antimicrobial activity was observed by pomelo "pulp" extracted by water at concentration of 200mg /ml. For *P. aeruginosa*, the ethanolic extract of pomegranate pulp "dried" showed the highest effect towards *P. aeruginosa* (with a 25mm zone of inhibition) followed by ethanolic extract of pomegranate pulp "fresh" with a 22mm zone of inhibition.

The water extract of pomegranate pulp "fresh" and "dried" showed good activity with a 18mm and 16mm zone of inhibition, respectively. For *C. albicans*, the ethanol extract of pomegranate pulp "fresh" showed the highest effect towards *C. albicans* with a 17mm zone of inhibition followed by ethanolic extract of pomegranate pulp "dried" with a 12mm zone of inhibition.The Kiwi peel "dried" and "fresh" extracted by ethanol, methanol and water showed little activity. No antimicrobial activity was observed by pomelo "pulp" extracted by ethanol, methanol and water and pomegranate pulp extracted by water and methanol.

3.2. Bioactivity of fruit extract by microdilution method

MIC values of all tested fruit extracts against tested microorganisms are summarized in Table 2. For *S. aureus*the MIC of the water extract of pomegranate pulp "fresh" and "dried", kiwi "fresh" and "dried" and pomelo "dried" showed very strong activity with the best MIC (3.125mg/ml). Followed by water extract of pomelo "fresh", ethanol and methanol extract of pomegranate pulp "fresh" and "dried" with MIC (6.25mg/ml).Also ethanol extract of kiwi peel “fresh and dried” showed MIC (6.25mg/ml). MIC of the ethanol and methanol extract of pomelo "fresh" and "dried" and methanol extract of kiwi peel "fresh" and "dried" showed moderate activity with MIC (50mg/ml). For *E. coli* the MIC of the water extract of pomegranate pulp "fresh" showed very strong activity with the best MIC (1.56mg/ml).Followed by water extract of pomegranate pulp "dried" and kiwi peel "fresh" and "dried" with MIC (3.125mg/ml).

MIC of the ethanol and methanol extract of pomegranate pulp and kiwi peel "fresh" and "dried" showed good activity with MIC (12.5mg/ml) and the MIC value of Pomelo pulp "fresh and dried" extracted by water also showed good activity with MIC (12.5mg/ml). The MIC value of ethanol and methanol extracts of pomelo pulp was (25mg/ml). For *P. aeruginosa* the MIC of water extract of pomegranate pulp "fresh" and "dried", kiwi peel "fresh" and "dried" and pomelo pulp "fresh" and "dried" showed the best activity with MIC (12.5mg/ml). Followed by ethanol and methanol extract of pomegranate pulp "fresh" and "dried" with MIC (25mg/ml). Also ethanol extract of kiwi peel "fresh" and "dried" and pomelo pulp "fresh" and "dried" showed MIC (25mg/ml). MIC of methanol extract of pomelo "fresh" and "dried" and kiwi peel "fresh" and "dried" showed moderate activity with MIC (50mg/ml). For *C. albicans* the MIC of the water extract of pomegranate pulp "fresh" and "dried" showed strong activity with MIC (6.25mg/ml).

Followed by ethanol and methanol extract of pomegranate pulp "fresh" and "dried" with MIC (12.5mg/ml).MIC of water, ethanol and methanol extracts of kiwi peel "fresh" and "dried" showed activity with MIC (25mg/ml). MIC of water extract of pomelo "fresh" and dried "showed moderate activity with MIC (50mg/ml). MIC of the ethanol and methanol extract of pomelo "fresh" and "dried" showed activity with MIC (100mg/ml).

Table1: Antimicrobial activity of fruit extracts by disc diffusion method.

Microorganisms		Dick diffusion method "mm"					
	Solvent	R1	R2	K1	K2	B1	B2
	E	15	10	8	6	-	-
S. aureus	M	9	9	7	-	-	-
	W	23	23	10	10	-	-
	E	14	14	9	6	7	7
E. coli	M	7	10	7	6	9	9
	W	17	20	8	8	-	-
	E	22	25	6	6	7	7
P. aeruginosa	M	13	11	6	6	7	8
	W	18	16	8	8	8	7
	E	17	12	7	7	-	-
C. albicans	M	-	-	9	9	-	-

R1: Pomegranate pulp fresh, R2: Pomegranate pulp dried, K1: Kiwi peel fresh, K2: Kiwi peel dried, B1: pomelo pulp fresh, B2: pomelo pulp dried, E: Ethanol, M: Methanol, W: Water.

Table2: Antimicrobial activity of fruit extracts by microdilution method

Microorganisms	Solvent	MIC "mg/ml" R1	R2	K1	K2	B1	B2
	E	6.25	6.25	6.25	6.25	12.5	25
S. aureus	M	6.25	6.25	50	50	50	50
	W	3.125	3.125	3.125	3.125	6.25	3.125
	E	12.5	12.5	12.5	12.5	25	25
E. coli	M	12.5	12.5	12.5	12.5	25	25
	W	1.56	3.125	3.125	3.125	12.5	12.5
	E	25	25	25	25	25	25
P. aeruginosa	M	25	25	50	50	50	50
	W	12.5	12.5	12.5	12.5	12.5	12.5
	E	12.5	12.5	25	25	100	100
C. albicans	M	12.5	12.5	25	25	100	100
	W	6.25	6.25	25	25	50	50

R1: Pomegranate pulp fresh, R2: Pomegranate pulp dried, K1:Kiwi peel fresh, K2: Kiwi peel dried**,** B1**:**pomelo pulp fresh, B2: pomelo pulp dried, E: Ethanol, M: Methanol, W: Water.

4. Discussion

The study of the relationship between plants and people (Ethnobotany) are employed in choosing the plants for the development of pharmaceutical industries **(Saranraj & Sivasakthi, 2014).** Plant kingdom is one of the important sources of the natural product due to both medicinal and economical values **(Kharjul *et al*., 2012).** The first step to development of pharmacological agents to treat diseases is the *in vitro* antibacterial activity assay. The search for new antimicrobial agents is of great concern today due to the increasing development of drug resistance to human pathogens and the appearance of unwanted effects of certain antifungal agents **(Höfling *et al*., 2010).**

This current study was aimed to detect the effectiveness of some fruit extract toward *S. aureus*" which have multi-resistance characteristic against antibiotics", *E. coli*, *P. aeruginosa* and *C. albicans* for producing new antimicrobial agent of great benefit to mankind. In this study different extracts of pomegranate pulp, kiwi peel and pomelo pulp were evaluated for exploration of their antimicrobial activity against pathogenic bacteria and fungi. Susceptibility of each fruit extracts were tested by serial microdilution method (MIC) and agar disc diffusion method.

All extracts showed a low value in MIC and these results obtained are in agreement with the previous study (*Adwan & Mhanna*), which explained this result that crude extracts have many different phytochemicals which might inhibit bacteria and fungi by different mechanisms **(Adwan & Mhanna**, **2008)**. Secondary metabolites in plant such as carotenoids, flavonoids, vitamin, alkaloids and pigments which have biological significance and may have some kind of resistance mechanisms e.g. enzymatic inactivation, target sites modifications and decrease intracellular drug accumulation **(Abeysinghe & Weeraddana, 2011).** Our experiments showed that all ethanol, methanol and water of pomegranate pulp "fresh and dried" were active against the tested microorganism.

The water extract provided more powerful antimicrobial activity as compared to organic extracts, and this may attributed to the water extract was found to be richer in polar phenols than ethanol and methanol **(Triantaphyllou *et al*., 2001)**, and this compound may be increase the antimicrobial activity for water extracts. The effect of ethanol and methanol extract of kiwi peel and pomelo pulp for antimicrobial activity showed some difference in the activity between different alcoholic extract may be due to the difference between extract compounds in this two extract. This result is similar to those of **(Sen & Batra, 2012)** in *Melia Azedarach l.* extracts by using Methanol, Ethanol, Petroleum ether and water, as solvents. In these experiments, pomelo pulp showed weak activity as compared to pomegranate pulp and Kiwi peel may be due to some plant extracts containing inhibitor substances for the growth of microorganisms and part of them may lose their inhibitory ability during extraction methods.

In this study, crude extracts appear to be more potent inhibitor against Gram-positive bacteria than Gram-negative bacteria which could be due to the difference in the structure of the bacterial cell wall.

Our results were consistent with previous *in vitro* study which reported the plant antibiotic substances effect appear to be an inhibitor to Gram-positive than Gram-negative bacteria **(Elbashiti *et al*., 2011).** In conclusion, this study revealed that the pomegranate pulp contain potential antimicrobial components that may be of great use for the development of pharmaceutical industries as a therapy against various diseases. The results of the study support the development of new antimicrobial drug from tested fruit extracts. *In vitro*, our results revealed that crude extracts proved their effectiveness with acceptable degree in control of growth some pathogenic microorganism however in vivo experiments are needed to confirm these results.

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Comments

This paper fall within general project that aims to solve some of environmental and health problems by reducing the use of chemical fertilizers, pesticides and drugs and replace them by natural material or organisms. This present study promote to return to nature by utilizing natural compounds from plant kingdom in control of some diseases resulted from pathogenic microorganisms and thus replace or minimize the use of synthetic compounds.

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