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ANTIBACTERIAL PROPERTY OF POMEGRANATE ARIL AND PEEL EXTRACTS FROM THREE DIFFERENT SOLVENTS

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ABSTRACT

Pomegranate is an ancient fruit that has been traditionally used in the management of diarrhoea, bronchitis and as a vermifuge. The pharmacological property of the fruit has been attributed to its antioxidant polyphenols. The emergence of resistance and scarcity of effective antibiotics have prompted research into the antimicrobial potential of polyphenols from fruits and vegetables including pomegranate. This study investigated the antibacterial potential of the fruit peel and aril extracts against some common tropical dermatological pathogens. The method used was 2,2-diphenyl-1-picrylhydrazyl (DPPH) to determine the radical scavenging activity (RSA) of the extracts. RSA was expressed as %inhibition of DPPH radical. Paper disc diffusion assay was used in determining the antibacterial potential of the fruit extracts with penicillin discs used as standard of comparison. Microdilution assay was used to determine the MIC and MBC of the fruit extracts. For the arils, the ethyl acetate extract demonstrated the highest RSA (87.5 %) and lowest IC_{50} value (10 µg/ml) compared to ethanol (%inhibition, 79 % and IC_{50}, 320 µg/ml) and hexane (%inhibition, 82.5 % and IC_{50}, 125 µg/ml) extracts. The peel extracts were similar in RSA (ethanol, 88.5 %, ethyl acetate 86.69 % and hexane 87.2 %) and IC_{50} (5 µg/ml for all solvent extracts). For antibacterial assay, all the extracts showed appreciable activity indices on *Staphylococcus epidermidis, Staphylococcus aureus*, *Streptoococcus pyogenes* and *Pseudomonas aeruginosa*. However, peel extracts demonstrated superior antibacterial effect against the pathogens compared to the aril extracts. Pomegranate fruit extracts have shown high antioxidant and antibacterial activity in this study and thus, could serve as active ingredients or adjuvants in the formulation of dermatological antimicrobial products.

KEYWORDS: **Antioxidants; Antibacterial; Pomegranate; Pathogens; Dermatological.**

INTRODUCTION

The developing countries account for about 80 % of the world population and rely on traditional medicine to alleviate several ailments that afflict them [1]. Diseases from pathogenic (disease-causing) microorganisms are the major causes of mortality and morbidity among these populations [2]. This is more so, because infectious diseases are easily transmitted from one person to another. The last century experienced a tremendous rise in the development of synthetic and semi-synthetic antibiotics [3] which has otherwise suffered a decline in the last three decades. Unfortunately, there is also an increasing emergence of new strains of pathogens and development of resistance to existing antibiotics thereby posing challenges to healthcare delivery [4,5]. However, some studies have pointed to the important role of plants in the discovery of new antimicrobials to curtail the spread of communicable

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diseases, menace of resistance and worrisome side effects of conventional antibiotics [6,7].

Most microorganisms are not harmful and can be found living in different parts of the body as normal flora [2]. Nonetheless, certain circumstances afford them the opportunity to be virulent and cause an array of topical, dermatological, systemic and food-borne diseases. Some of such opportunistic bacteria include *Escherichia coli*, *Staphylococcus spp.*, P*seudomonas spp*., *Streptococcus spp*., and some *Bacillus spp*.

Fruits and vegetables have for time immemorial served as source of food to man [8]. The health benefits associated with these types of plants have stimulated research into their phytochemical composition and pharmacological activity against several ailments [9]. Polyphenols are the chief constituents in fruits such as pomegranates and other berries [10]. The potential of polyphenols as antimicrobial has been documented [11,12]. Polyphenols are said to be produced by plants as chemical defences to resist infections from bacteria, fungi and nematode as well as attack by herbivores [13].

Pomegranate (*Punica granatum* L.) is a short tree that is widely cultivated across the globe. The fruit is berry-like, composed of an outer peel and inner juice laden ruby arils. Polyphenols constitute the major phytochemical component of the fruit [14]. The antioxidant property of pomegranate phenolic compounds has attracted scientific interest in recent times. They have been studied for their potential role to prolong the shelf life of products and for other medicinal and cosmetic applications [15,16]. The major groups of polyphenols present in pomegranate are the flavonoids and tannins and are responsible for most of the fruit's biological activity [17,18]. The folklore use of the fruit in treating diarrhoea, respiratory diseases and helminthiasis has inspired researchers to consider it for potential antimicrobial activity [19,20].

In this study, the antibacterial property of the fruit extracts on some opportunistic dermatological pathogens would be evaluated.

MATERIALS AND METHOD

Extraction

The method used by Rohin et al. [21] as adopted with slight modification, 100 g of peel (P) and arils (A) were weighed and homogenised with a blender (Waring Commercial Laboratory Blender, USA) and labelled P and A respectively. 100 g each of P and A were then separately extracted by maceration in a 500 mL conical flask with 500 mL ethanol and kept in the dark for five days with vigorous agitation at intervals. The mixture was then filtered through Whatman no 41 (pore size 20-25 µ) filter paper under gravity. The filtrate was collected and concentrated under reduced pressure at 40 ºC with rotary evaporator.

The residue was air dried overnight before it was extracted with hexane following the above procedure. The procedure was repeated with ethyl acetate (EA) and finally with ethanol for a second time. The concentrated extracts were then finally allowed to evaporate to dryness in the oven and stored at -20 ºC until they were ready for use.

Free Radical Scavenging Activity

The free radical scavenging activity of the sample extracts were measured using the 96-well plate method [22, 23] with slight modification. Quercetin was used as standard. A total of 1 mg of extracts and quercetin were dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 1 mg/ml. Then using a 96-well micro plate, serial dilutions of the extracts and standards were made with 60 µl of DMSO. The volumes of all the samples were then adjusted to 100 µl by adding 40 µl of DMSO. 200 µl of 1 µM DPPH (2,2-diphenyl-1-picrylhydrazyl) was then added to all the sample concentrations including the blank. The micro plate was then placed in the dark and allowed to stand for 30 mins before absorbance was measured at 517 nm using Enzyme-Linked Immunosorbent Assay (ELISA) plate reader. The results were expressed as percentage of reduction of the initial DPPH absorption by test samples and used to calculate the half maximal inhibitory concentration (IC_{50}).

Antibacterial Assay

Test microorganisms

The antibacterial activity of the extracts from the peel and flesh of pomegranate obtained using ethanol, hexane and ethyl acetate were tested on the following bacterial strains; *Staphylococcus epidermidis* (ATCC 14990), *Staphylococcus aureus* (ATCC 9144), *Streptoococcus pyogenes* (ATCC 19615) and *Pseudomonas aeruginosa* (ATCC 14149). All bacteria strains were purchased from the American Type Culture Collection (ATCC), Manassas, USA. All strains were carefully identified and grown using standard microbial methods. The strains were first sub-cultured in Buffered Peptone

Water (BPW) and incubated at a temperature of 37 ºC for 18 hours.

Disc Diffusion Assay

The antibacterial activity of the sample extracts on the above bacteria strains was determined using Agar Paper Disc Diffusion Assay method as described by the National Committee of Clinical Laboratory Standards [24]. The microorganisms were prepared from an overnight broth culture and diluted using normal saline to give an inoculum size of about 10^8 cfu/mL. The bacterial suspension density of the culture was standardised turbidometrically to 500,000 – 1,000,000 colony forming unit (cfu) per millilitre (mL) at a wavelength of 600 nm. A total of 100 µL of suspension containing 10^8 cfu/mL of bacteria were spread on nutrient agar using sterile cotton bud.

Then 100 mg/mL concentrations of the extracts were prepared and filtered using 0.45 µm Millipore filters. Sterile discs were impregnated with 10 µL of extract solution and placed on the inoculated agar. Negative controls were prepared using DMSO, while penicillin G (10 µg/disc) was used as positive control to determine the sensitivity of each bacteria strain tested. The inoculated plates were incubated at a temperature of 37 ºC for 24 hours. Antibacterial activity was evaluated by measuring the zones of inhibition against the test organisms. The diameters of the inhibition zones were measured in millimetres (mm) by vernier callipers. All tests were repeated five times to minimize test error. An inhibition zone of 14 mm or greater (including diameter of the disc) was considered as having high antibacterial activity [25].

Minimum Inhibitory Concentration Assay (MIC)

The antibacterial activity of the fruit aril and peel extracts obtained using the solvents ethanol, ethyl acetate and hexane was determined using microdilution method with slight modifications [26,27]. Initial concentrations of 100 mg/mL of the extracts were prepared by dissolving extracts in DMSO. 100 µL of extracts solution were then added to wells in triplicate and serially diluted with nutrient broth until seventh dilution. 10 µL of working bacteria culture as earlier prepared were then placed in each of the wells except blank containing only nutrient broth. A final volume of 200 µL in each well was obtained by making up volume with 90 µL of nutrient broth to give 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 mg/mL concentrations down the wells. Sterility controls were prepared for each extract concentration including broth control containing corresponding extract concentration without bacteria culture. Plates were then incubated in a shaker incubator (Stuart, UK) at 120 rpm, 37 °C for 24 h. The absorbance of the wells was read at 570 nm using micro titre plate reader (Bio-rad, US) after incubation. Serial dilutions of DMSO were also included to check for false antibacterial activity.

Minimum Bactericidal Concentration Assay (MBC)

Minimum bactericidal concentration of the fruit aril and peel extracts obtained using the solvents ethanol, ethyl acetate and hexane was determined using the method of Smith and Spencer with little modifications [28, 29]).

From the MIC assay described above, a loopful of bacteria from two consecutive wells from test extracts MIC wells were cultured in nutrient agar using streak plate method. The plates were then incubated at 37 °C for 24 hours. MBC were determined by the minimum concentration that allowed less than 1% of bacterial growth.

Statistical analysis

Data were expressed as the Mean ±Standard Error of the Mean (SEM). Data were analyzed statistically using one-way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons between the control and treated groups. Values of $P \leq 0.05$ were considered significant.

RESULTS

Figure 1 shows the radical scavenging activity of ethanol, ethyl acetate and hexane extracts from Pomegranate Aril. Figure 2 shows the radical scavenging activity of ethanol, ethyl acetate and hexane extracts from Pomegranate Peels.
Results of antimicrobial evaluation are depicted in Tables 1 and 2.

DISCUSSION

One of the mechanisms in which antioxidants exert their effect is by scavenging free radicals [30]. Fresh Pomegranate peel and arils were separately extracted successively with ethanol, ethyl acetate and hexane.
The DPPH is a stable free radical that is soluble in methanol and ethanol giving a yellow colouration. On reaction with compounds having radical scavenging potential, they reduce the compound by donating

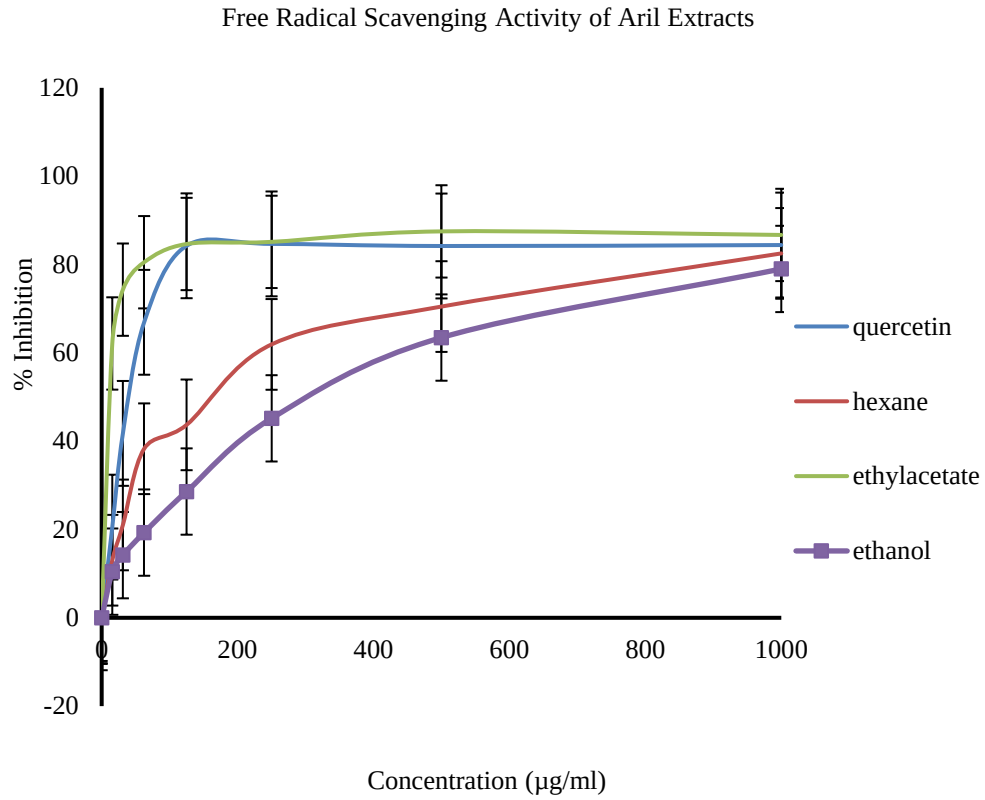

Figure 1: Radical scavenging activity of ethanol, ethyl acetate and hexane extracts from Pomegranate Aril.

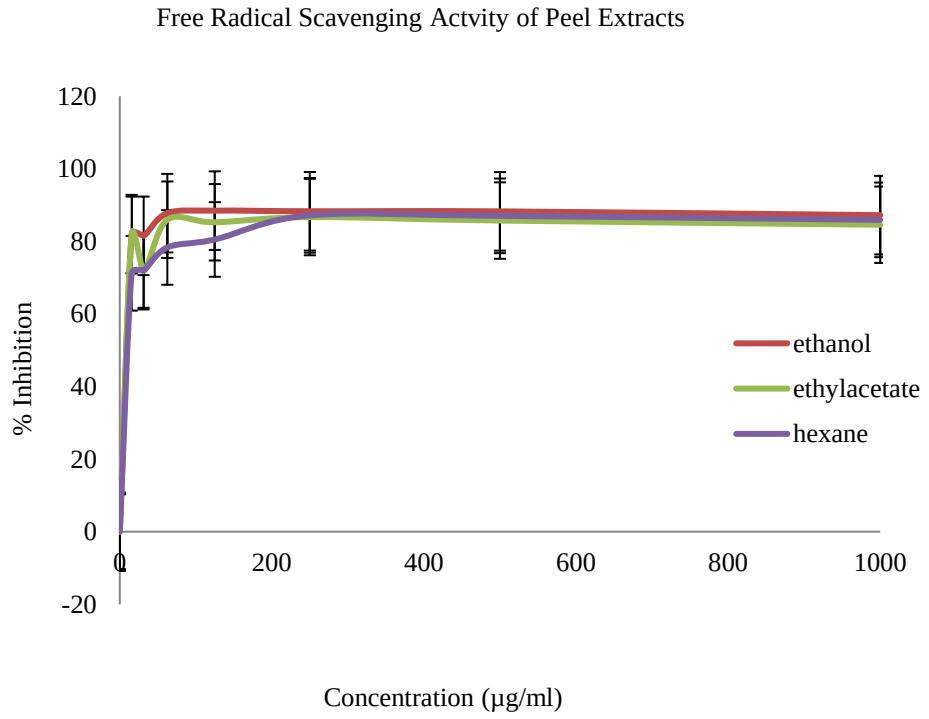

Figure 2: Radical scavenging activity of ethanol, ethyl acetate and hexane extracts from Pomegranate peels.

Table 1: Zones of inhibition of sample extracts against some bacteria strains.

Sample	Conc. (mg/ml)	*S. epidermidis*	*S. aureus*	*S. pyogenes*	*P. aeruginosa*
Ethanolic aril extract (FA)	100.00	22.0(0.88)	21.0(0.85)	22.5(0.78)	23.0(0.68)
	50.00	17.0(0.68)	17.5(0.70)	19.5(0.67)	18.5(0.54)
	25.00	15.5(0.62)	15.5(0.62)	18.0(0.62)	15.5(0.46)
	12.50	14.5(0.58)	12.0(0.48)	13.0(0.45)	13.5(0.40)
	6.250	10.5(0.42)	10.0(0.40)	11.5(0.40)	11.0(0.32)
	3.123	8.5(0.34)	8.0(0.32)	10.0(0.35)	10.0(0.29)
Ethyl acetate aril extract (FE)	100.00	20.0(0.8)	22.5(0.9)	18.5(0.64)	22.0(0.65)
	50.00	18.5(0.74)	19.0(0.76)	17.0(0.59)	19.0(0.56)
	25.00	16.0(0.64)	17.5(0.70)	14.5(0.5)	17.0(0.50)
	12.50	13.0(0.52)	15.0(0.60)	12.5(0.43)	14.5(0.43)
	6.25	9.0(0.36)	11.0(0.55)	11.0(0.38)	11.5(0.34)
	3.13	7.5(0.30)	10.0(0.40)	10.0(0.35)	9.5(0.28)
Hexane aril extract (FH)	100.00	18.5(0.74)	20.0(0.80)	24.0(0.83)	23.0(0.68)
	50.00	16.0(0.64)	17.0(0.68)	21.5(0.74)	19.0(0.56)
	25.00	13.5(0.54)	14.5(0.58)	18.5(0.64)	14.0(0.41)
	12.50	11.0(0.55)	12.0(0.48)	15.0(0.52)	12.0(0.35)
	6.25	9.5(0.38)	10.0(0.40)	12.5(0.43)	10.0(0.29)
	3.13	7.0(0.35)	9.0(0.36)	11.0(0.38)	10.0(0.29)
Ethanolic peel extract (PA)	100.00	21.0(0.85)	20.0(0.80)	20.5(0.71)	25.5(0.75)
	50.00	18.5(0.74)	20.0(0.80)	19.5(0.67)	22.5(0.66)
	25.00	16.0(0.64)	18.0(0.72)	17.0(0.59)	20.0(0.59)
	12.50	13.5(0.65)	16.0(0.64)	15.0(0.52)	17.0(0.50)
	6.25	12.0(0.48)	12.0(0.48)	12.5(0.43)	14.0(0.41)
	3.13	9.5(0.38)	8.0(0.32)	11.0(0.38)	12.0(0.35)
Ethyl acetate peel extract (PE)	100.00	23.5(0.94)	21.0(0.85)	23.5(0.81)	27.0(0.79)
	50.00	19(0.76)	19.0(0.76)	20.0(0.69)	20.0(0.59)
	25.00	16.5(0.66)	18.0(0.72)	19.0(0.66)	17.5(0.51)
	12.50	13(0.52)	15.0(0.60)	16.5(0.57)	15.5(0.46)
	6.25	12(0.48)	12.0(0.48)	13.5(0.47)	13.0(0.38)
	3.13	10.5(0.42)	10.0(0.40)	11.5(0.40)	13.0(0.38)
Hexane peel extract (PH)	100.00	23.0(0.92)	25.0(1.00)	23(0.79)	27.5(0.81)
	50.00	18.0(0.72)	18.5(0.74)	21(0.72)	19.5(0.57)
	25.00	15.0(0.60)	16.5(0.66)	18.0(0.62)	18.0(0.53)
	12.50	12.5(0.50)	13.5(0.54)	14.5(0.50)	13.0(0.38)
	6.25	11.0(0.55)	10.0(0.40)	12.5(0.43)	10.5(0.31)
	3.13	9.0(0.36)	8.0(0.32)	10.5(0.36)	9.5(0.28)
Penicillin G (control)	10 IU	25.0	25.0	29.0	34.0

*Values in parenthesis indicate activity indices of samples against respective bacteria

Table 2: The minimum inhibitory concentrations and minimum bactericidal concentrations of Pomegranate fruit peel and Aril extracts.

Sample	*S. aureus*		*S. epidermidis*		*Strep. pyogenes*		*Ps. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ethanol Aril extract	0.781*	0.781	0.781	0.781	3.125	3.125	1.563	3.125
EA Aril extract	3.125	12.250	0.781	0.781	12.250	12.250	0.781	1.563
Hexane Aril extract	1.563	1.563	0.781	0.781	12.250	12.250	0.781	1.563
Ethanol peel extract	0.781	0.781	1.563	1.563	3.125	3.125	0.781	1.563
EA peel extract	0.781	0.781	0.781	1.563	3.125	12.250	0.781	1.563
Hexane peel extract	0.781	0.781	0.781	0.781	1.563	1.563	1.563	1.563

MBC= minimum bactericidal concentration, MIC= minimum inhibitory concentration
*Concentrations expressed as mg/mL.

hydrogen thereby converting it to the light yellow compound, α,α-diphenyl-*β*-picryl hydrazine [31].

All the extracts demonstrated high free radical scavenging effect at the concentrations tested. The peel extracts were observed to show greater inhibition as compared to the arils. The percentage inhibition for the peel extracts were 88.5, 86.69 and 87.2 % for ethanol, ethyl acetate and hexane respectively, whereas the percentage inhibition for the aril extracts were 79, 87.5 and 82.50 % for ethanol, ethyl acetate and hexane respectively. The IC_{50} for the three peel extracts extrapolated from the graph was 5 µg/L while the IC_{50} for the aril extracts were 320, 10 and 125 µg/mL for ethanol, ethyl acetate and hexane, respectively.

The free radical scavenging activity among the extracts using the DPPH method were similar in IC_{50} values. Fawole *et al.* [21] reported a radical scavenging activity of 71.5-83.5 % at 1000 µg/mL similar to that of this study [12]. The peel extracts also demonstrated high RSA even at concentrations of 5 µg/mL, making it a very suitable source of antioxidant. Other literature have demonstrated pomegranate peel to be an excellent antioxidant superior to other fruits [32-34]. The peel was superior in antioxidant activity than the arils in agreement with another study [32]. There were scanty studies on the antioxidant activity of the aril extract as researchers prefer to use the juice for their studies. It was observed that free radical scavenging activity of the extracts was not solely due to the presence of polyphenols. This could be the case of the hexane extracts which had no polyphenol detected both had high RSA. The RSA reported could be attributed to other non-polyphenol antioxidant compounds.

The antibacterial activity of the fruit extracts was determined using paper disc agar diffusion assay using penicillin discs, 10 IU as standard (positive control). The size of the diameter of the region around the disc without bacterial growth was measured as zone of inhibition which is a measure of the antibacterial property of the tested extracts. The wider the diameter of the zone of inhibition, the greater the activity. The zones of inhibition of the extracts were then compared with the standard and calculated as activity index. Activity index greater than '1' means the extract has higher activity than the standard, while activity below '1' means activity is less than that of the standard. The zones of inhibition and activity indices of the extracts are shown in table 1 below.

Pomegranate has attracted a lot of interest in the last few decades due to its numerous potentials for the prevention and treatment of chronic diseases. The pharmacological property of the fruit has been attributed to the polyphenols contained in the peel and arils of the fruit. Polyphenols are natural plant antioxidant compounds with biological activity in animals including humans. Although the peel is inedible, it has been shown to contain more

polyphenols than the arils and consequently greater antioxidant activity. The emergence of resistance and dwindling rate of discovery of new antimicrobial agents has necessitated research into antibacterial potential of plant polyphenols.

In this study, three solvents were used to successively extract the extractive components of pomegranate peels and arils. Each solvent is expected to extract a set of unique compounds with varying pharmacological effect.

All the extracts tested demonstrated high antibacterial activity at high concentrations but little or no activity at concentrations lower than 250 µg. Thus, the antibacterial activities of the extracts are dose dependent consistent with the work of Sachin & Nitave [35]. The antibacterial activities of the extracts (22-27 mm) at 100 % concentration were similar to penicillin (25-30 mm), a broad-spectrum antibiotic. The extracts also had similar activities against both gram negative (*P. aeruginosa*) and gram positive (*S. pyogenes*, *S. aureus*, *S. epidermidis*) bacteria. Thus, pomegranate fruit extracts could possess broad spectrum antibacterial activity. This is in agreement with the works of Opara *et al.* [36] and Fawole *et al.* [27] but contrary to the work of Kanatt *et al.* [37], which reported no activity against Gram-negative bacterial by peel extracts [12,36,37]. In a similar study, pomegranate ethanolic peel extract was found to be effective against *S. aureus* and *P. aeruginosa,* with zones of inhibition (ZOI) of 25.5 mm [38].

To determine the bacteriostatic and bactericidal activities of the extracts, MIC and MBC methods were used respectively. An MIC and MBC value range of 0.78 to 12.25 mg/mL were recorded in this study. The extracts had similar MBC and MIC values. The values obtained for this study are similar (0.49-1.95 mg/mL) to those of Howell & Souza [39].

The antibacterial activity was also compared with DPPH radical scavenging activity. The antioxidant and antimicrobial activity of pomegranate peel extracts against *S. aureus* and *P. aeruginosa* have been studied and shown to be useful in prolonging the shelf life of chicken products by 2-3 weeks [35]. Even though differences can be observed in the antioxidant activity of the extracts, the antibacterial activity were quite similar. This is a little contrast to the study of Negi & Jayaprakasha [40], which showed that there was a positive correlation between antibacterial activity and antioxidant activity [40]. However, the methods of extraction and solvents used in the study are not same with this research. This could influence the variation in activities. The antibacterial activity of the fruit extracts is thought to be due to the precipitation of bacterial cell wall proteins by the polyphenol contents [12].

CONCLUSION

It can be concluded from this study that pomegranate peel and aril extracts possess dose dependent antibacterial activity against the dermatological pathogens tested (*Staphylococcus epidermidis, Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*). The activity indices of the extracts against the organisms tested were similar to that of penicillin. Additionally, the extracted have demonstrated appreciable antioxidant activities. Thus, the extracts could serve as active ingredients or adjuvants in the formulation of dermatological antimicrobial products.

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