

RESEARCH ARTICLE

POLYPHENOLS/FLAVONOIDS ANALYSIS AND ANTIMICROBIAL ACTIVITY IN POMEGRANATE PEEL EXTRACTS

Omnia Hussein Abdu¹, Adel AM Saeed^{2,*}, and Taha Abubaker Fdhel³

¹Dept. of Pharmaceutical Chemistry, Faculty of Pharmacy - Aden, University of Aden, Aden, Yemen

²Dept. of Chemistry, Faculty of Science - Aden, University of Aden, Aden, Yemen

³Dept. of Chemistry, Faculty of Education - Aden, University of Aden, Aden, Yemen

*Corresponding author: Adel A. M. Saeed E-mail: adel_saeed73@yahoo.com

Received: 18 Jan 2020 / Accepted: 28 Jan 2020 / Published online: 07 Mar 2020

Abstract

The use of plants still plays a vital role to establish the basic health requirements in developing countries. The extracts of *P. granatum* and its compounds have shown antimicrobial activity. This study showed that the acetone (125.36 ± 0.01 and 70.5 ± 0.01) extract contained the highest amount of total polyphenols (TPPC) and total flavonoids content (TFC) respectively compared to methanol (105.6 ± 0.005 and 20.99 ± 0.004) extract while methanol extract content was higher than ethanol (94.14 ± 0.02 and 14.48 ± 0.005). The aqueous extracts content was the less one (83.17 ± 0.003 and 10.65 ± 0.003). When study the antimicrobial activity of four different prepared concentrations of four pomegranate peel extracts against microorganisms including two Gram-positive bacteria [*P. aeruginosa* and *S. aureus*], one Gram-negative bacteria [*A. baumannii*] and *Candida parapsilosis*. The result shown the antimicrobial activity of acetone extract on all microorganisms the highest value followed by methanol, ethanol, and aqueous extract activity. Besides, the activity of all extracts was good excepted aqueous extract has low antimicrobial activity against *S. aureus*.

Keywords: Pomegranate Peel Extracts, Polyphenols and Flavonoids Analysis, Antimicrobial Activity.

1. Introduction

Recently, there has been a rekindling interest in "rediscovering natural products" [1] due to a rapid increase in the rate of infections, antibiotic resistance in microorganisms and also due to side effects of synthetic antibiotics [2]. Plants are the gifts of nature used to cure number of human diseases. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with side effects and have an enormous therapeutic potential to heal many infectious diseases [3]. However, the herbal medications have been used for the relief of symptoms of the disease. Despite the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. Much interest, in plants, however, emanates from their long use in folk medicines as well as their prophylactic properties, especially in developing countries. [4]

Moreover, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security.

Pomegranate (*Punica granatum* L.) has attracted considerable attention for its health benefits in recent years. [5-7]

Polyphenols are secondary plant metabolites which are present in plants and more than 8000 polyphenols have been identified and traditionally use to heal both human and animals. [8]

Polyphenols have been classified into several types, namely, phenolic acids, flavonoids, stilbenes and lignans. Flavonoids (Fig. 1) chemically constitute the class of low-molecular weight phenolic compounds that are widely distributed amongst the kingdom Planta. [9]

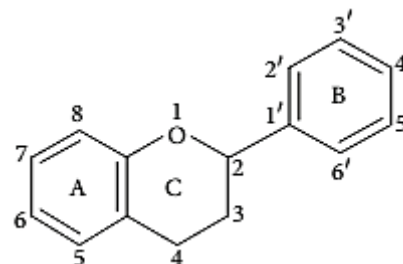


Figure 1: Basic skeleton of flavonoids

Polyphenolic and flavonoid content of peels of various pomegranate cultivars from different regions in the world have been evaluated. [10]

This work was done to evaluate polyphenols and flavonoids analyses and to determine antimicrobial activity of Yemeni pomegranate peel extracts.

2. Martials and Methods

2.1 Chemicals

The entire chemicals used in the present study were all of analytical grade.

2.2 Plant materials

Fresh pomegranate fruits cultivated in Saada Governorate were purchased from Aden's Market. The peels were removed manually from pomegranate fruits, washed in water then dried by keeping them in shadow at room temperature for three weeks.

2.3 Extracts preparation

A 200g-sample was pulverized and the powder material was packed in soxhlet apparatus subjected to continuous hot percolation for 6 h using 500 ml of a solvent (distilled water, methanol, ethanol, or acetone). Then, the solvent extract was concentrated under vacuum and dried at 50 °C [11] in Vaciotem-T oven (oven under pressure) to obtain 28.2g, 32.6g, 54.4g, and 62.3g for acetone, aqueous, ethanol, and methanol extracts respectively.

2.4 Microorganism

Three bacterial strains were procured from American Type Culture Collection (ATCC), The strains used were *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 27945 from High Authority for Drug Control. The fungal strain was isolated clinically from vaginal discharge and diagnostic in the Microbiology Department-Medical Faculty/University of Aden as *Candida parapsilosis*.

2.5 Qualitative and quantitative analysis

2.5.1 Determination of total polyphenol content (TPPC)

Total Polyphenol Content (TPPC) was estimated by the Folin-Ciocalteu Method (FCM) reported in [4]. Briefly, a 1 ml of the extract (1 mg/ml) was mixed with 9 ml of distilled water in a 25 ml volumetric flask. 2.5 ml of a 10 fold diluted Folin-Ciocalteu phenol reagent was added. After 5 min, 10 ml of 7.5% Na₂CO₃ solution was added to the mixture and made up to the mark with distilled water. The mixture was incubated in the dark for 90 mins. at room temperature. A set of standard solutions of gallic acid (40, 60, 80, 100, 120 and 140µg/ml) were prepared in the same manner as described for the extracts.

The absorbance of extract and the standard solution was read against the reagent blank (prepared in the same manner as extract where extract was replaced by distilled water) at 700 nm with a SCINCO PDA UV-Visible Spectrophotometer and LabPro® Plus software (Korea). The determination of TPPC in the extract was carried out in triplicate.

2.5.2 Determination of total flavonoids content (TFC)

Estimation of total flavonoid content was done by Aluminum Chloride Colorimetric Method (ACCM) as described in [12]. A 0.5ml of 1mg/ml of each extract/solvent stock solution was mixed with 0.1ml of 10% aluminum chloride and 0.1ml of potassium acetate (1M). In this mixture, 4.3ml of 80% solvent was added to make 5ml volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The sample blank was prepared similarly by replacing aluminum chloride with distilled water. The quercetin was used to make the calibration curve by preparation stock solution of 1mg/ml quercetin in solvent then diluted to different concentration (1.56, 3.125, 6.25, 12.5, 25, 50, 75, 100µg/ml). All prepared stock solutions were filtered through Whatman filter paper.

The absorbance of extract and standard solutions were read against the reagent blank at 430 nm using UV-Vis spectrophotometer. The total flavonoid content was determined from the calibration curve and expressed as microgram of quercetin equivalent per gram of the extracts. The determination of the total flavonoid in the extract was carried out in triplicate.

2.6 Antimicrobial analysis

2.6.1 Determination of inhibition zone

An Inhibition Zone Method (also known as an Agar-Well Diffusion Method) was employed [13] to determine the antimicrobial activity.

Several solutions of each extract/dis. water were prepared in different concentrations (*i.e.* 12.5, 25, 50, 100 mg/ml), and Mueller Hinton agar was prepared by dissolving 38g in one-liter solution. The agar plate was inoculated by spreading a volume of the microbial inoculum over the entire agar. Then, a hole with a diameter of 6 mm had punched aseptically with a sterile cork borer, and a volume (50µl) of the antimicrobial agent or extract solution at desired concentration was introduced into the well. Ciprofloxacin (antibiotic) was used as a positive control. The determination of inhibition zone of bacteria growth in the Mueller Hinton agar for extract was carried out in triplicate.

Modified Mueller Hinton agar was recommended for the diffusion of antifungal agents impregnated on paper disc through an agar gel as described in CLSI Approved Standard. For suitable fungal growth, Mueller Hinton Agar was supplemented with glucose to a final concentration of 2%. Addition of methylene blue to a final concentration of 5µg/ml was done to enhance zone edge definition. Then the same step was done as in the antibacterial assay. Use clotrimazole (antifungal drug) was used as a positive control. [14]

2.6.2 Determination of activity index

The activity index (Table1) of the crude plant extract was calculated as [11]:

$$\text{Activity Index (AI)} = \frac{\text{Zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic (drug)}} \quad (1)$$

2.7 Statistical analysis

Statistical analysis was carried out on the total polyphenol and flavonoid contents, antimicrobial activity. Three determinations of each of the measured compounds of pomegranate peel extracts were performed. Hence, the data were presented as means ($n=3$) \pm SD and a one way analysis of variance (ANOVA) at $P < 0.05$ was performed.

3. Results and discussion

3.1 Determination of total polyphenol content (TPPC) and total flavonoid content (TFC)

To perform the calculation of total polyphenol content and total flavonoid content in the four extracts a standard calibration curve was obtained from a series of different Gallic acid (GA) and Quercetin (Q) concentrations respectively. Figures 2 and 3 are λ_{\max} curve using UV-Vis spectrophotometer for Gallic acid and Quercetin respectively while relevant calibration curves for the same substances are shown in Figs 4 and 5.

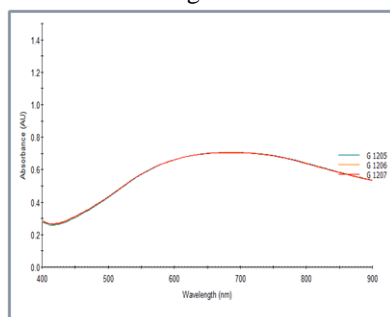


Figure 2: λ_{\max} curve for gallic acid using UV-Vis Spectrophotometer

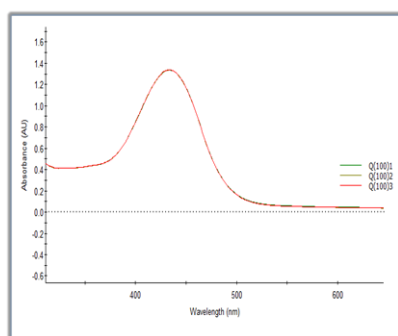


Figure 3: λ_{\max} curve for Quercetin using UV-Vis Spectrophotometer

The total phenolic content and total flavonoid content were determined from the calibration curve and expressed as milligram of gallic acid equivalent (GAE) and quercetin equivalent (QE) respectively per gram of the extracts by using equation below:

$$C = \frac{cv}{DW} \quad (2)$$

where, C= gallic acid equivalent or Quercetin equivalent in mg/g; c= concentration of gallic acid or quercetin established from calibration curve in mg/ml; v= volume of extract in ml in the experiment, and DW = the dry weight

of the plant extract in g. The results were represented in Table 1. The acetone extract contents had the highest amount of TPPC and TFC compared to methanol extract while methanol extract content was higher than ethanol and aqueous extracts. The aqueous extract content was the lowest amount of TPPC and TFC. It is well known that, the concentration of phenolic content usually is higher than the concentration of flavonoid in most cases (see Table2).

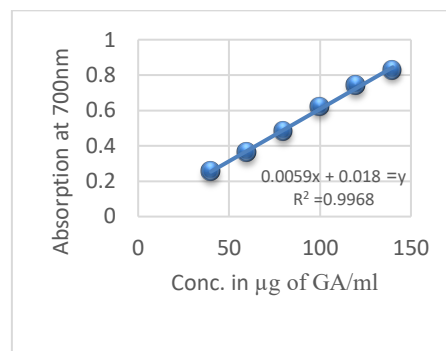


Figure 4: A calibration curve of Gallic acid

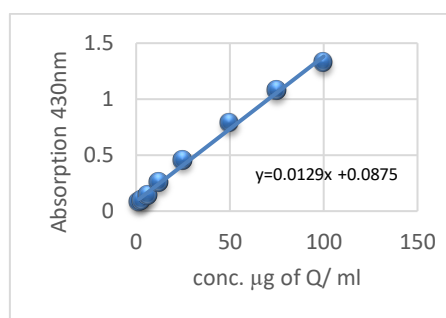


Figure 5: A calibration curve of Quercetin

The order of the solvent extracts was: acetonic > methanolic > ethanolic > aqueous

Table 1: The mean values of total polyphenols, flavonoids for applied solvents extract of pomegranate peel.

Extract Experiment	Aqueous extract	Ethanolic extract	Methanolic extract	Acetonic extract
TPPC(mg/g)				
*(Total polyphenol content)	83.17 \pm 0.003	94.14 \pm 0.02	105.6 \pm 0.005	130.36 \pm 0.01
TFC(mg/g)**				
(Total flavonoid content)	10.65 \pm 0.003	14.48 \pm 0.005	20.99 \pm 0.004	70.5 \pm 0.01

Each value in the table is represented as mean \pm SD ($n = 3$).

*TPPC expressed as mg GAE/g DW

TFC expressed as mg Q/g DW

As reported in [15] the TPPC in the peel of Mumbai, Indian pomegranate in methanol extract was higher than that of aqueous extract.

Barzegar with his co-workers [16] stated that the number of phenolic compounds in Yazd (Iranian) pomegranate peel acetone extracts were the highest followed by methanol, ethanol, and water. This resembles our recent results.

Elfalleh and his co-workers [17] reported that the content of TPPC in the peel of Gabès pomegranate (Tunisian pomegranate) 53.65 ± 4.13 for aqueous extract, and 85.60 ± 4.87 for methanol extract and this was resembled to the results in this study where methanol content of TPPC was higher than aqueous extract but the content of TPPC in Yemeni pomegranate was higher than that in Gabe's pomegranate. Also TFC in the peel of Gabe's pomegranate (21.03 ± 1.62) for aqueous extract and (51.52 ± 8.14) for methanol extract were nearly same as in this work. But the TFC in Yemeni pomegranate was less than that in Gabe's pomegranate.

As mentioned in [18] the result of TPPC for methanol extract (103.2 ± 2.77) was approximately equal to the TPPC in this study but the TFC in this study was much lesser than in [18]. Farag et al. [19] reported that the content of TPPC in the juice of peel of El-Menia pomegranate, Egyptian pomegranate was (58.63 ± 0.129). This result was less than the TPPC that obtained by extraction by any of four solvents (acetone, methanol, ethanol, and water). While the TFC in the juice of peel 47.32 ± 0.032 was higher than TFC

in methanol, ethanol and aqueous extract but fewer than TFC in acetone extract.

3.2 Antimicrobial assay

The antimicrobial activity of the above-mentioned extracts was separately determined using a modified agar-well diffusion method (Table 2). In general, the bioactivity of peel extracts on the microorganisms tested had high total flavonols, phenolics, anthocyanins, and organic acids. Similarly, confirmed that phenolics were the most important compounds against bacteria, among that gallic acid was identified as the most active compound for inhibition of bacteria tested. The inhibitory effect of phenolic compounds could be explained by adsorption to cell membranes, interaction with enzymes, substrate and metal ion deprivation. [20]

The antimicrobial activity of four different prepared concentrations of four pomegranate peel extracts against microorganisms including two Gram-positive bacteria (*P. aeruginosa* and *S. aureus*) and one Gram-negative bacteria (*A. baumannii*) was recorded after 24hr of incubation. The result was analyzed statistically and compared with the antimicrobial activity of the control (Ciprofloxacin (Anti-bacterial drug) and Clotrimazole (Anti -fungal drug)).

Table 2: Antimicrobial activity of pomegranate extract against microorganisms tested by agar well diffusion (inhibition zone) method

No.	Microorganisms	Inhibition zone in mm					Mean ±SD	Standard antibiotic 100mg/ml	Zone (mm)	Activity index
		Concentration in mg/ml								
		12.5	25	50	100					
Antimicrobial Activity of Aqueous Pomegranatre Peel Extract										
1	<i>S. aureus</i>	no	10	11	13	8.5 ± 5	Ciprofloxacin	40	0.212	
2	<i>P. aeruginosa</i>	18	20	22	24	21± 2.2	Ciprofloxacin	40	0.525	
3	<i>A. buamannii</i>	22	26	27	28	25.75 ± 2.3	Ciprofloxacin	40	0.644	
4	<i>Candida parapsilosis</i>	12	13	14.5	16	13.9 ± 1.5	Clotrimazole	30	0.463	
Antimicrobial Activity of Ethanol Pomegranatre Peel Extract										
5	<i>S. aureus</i>	12	14	19	21	16.5 ± 3.6	Ciprofloxacin	40	0.412	
6	<i>P. aeruginosa</i>	18	20	22	24	21 ± 2.2	Ciprofloxacin	40	0.525	
7	<i>A. buamannii</i>	21	24	24	26	23.75 ± 1.8	Ciprofloxacin	40	0.644	
8	<i>Candida parapsilosis</i>	14	14	16	17	15.25 ± 1.3	Clotrimazole	30	0.508	
Antimicrobial Activity of Methanol Pomegranatre Peel Extract										
9	<i>S. aureus</i>	13	15	20	22	17.5 ± 3.6	Ciprofloxacin	40	0.438	
10	<i>P. aeruginosa</i>	20	22	24	26	23 ± 2.2	Ciprofloxacin	40	0.575	
11	<i>A. buamannii</i>	22	26	27	28	25 ± 1.6	Ciprofloxacin	40	0.625	
12	<i>Candida parapsilosis</i>	14	15	16	17	15.5 ± 2.2	Clotrimazole	30	0.517	
Antimicrobial Activity of Acetone Pomegranatre Peel Extract										
13	<i>S. aureus</i>	22	24	26	28	25 ± 2.2	Ciprofloxacin	40	0.625	
14	<i>P. aeruginosa</i>	22	24	26	28	25 ± 2.2	Ciprofloxacin	40	0.625	
15	<i>A. buamannii</i>	26	26	26	29	26.75 ± 1.3	Ciprofloxacin	40	0.669	
16	<i>Candida parapsilosis</i>	17	18	19	20	18.5 ± 1.1	Clotrimazole	30	0.617	

Results obtained in Table 2 illustrated the different effect of pomegranate peel four extracts on the five microorganisms, that means pomegranate peel extracts showed antibacterial activities against *S. aureus* and *P. aeruginosa*, *A. buamannii*, also showed antifungal activity against *Candida Parapsilosis*.

On the other hand, the activity index of all extracts with the antimicrobial activity of the control ciprofloxacin and clotrimazole displayed the highest value was especially for acetone extract.

100mg/ml of acetone, methanol, ethanol, and aqueous extracts of pomegranate peel showed inhibition zone of 28 mm, 22 mm, 21mm and 13mm respectively against *S. aureus* and noted that the aqueous extract had very low activity against *S. aureus*. When compared the activity of Acetone, methanol, ethanol, and aqueous extracts with the antibacterial activity of the control ciprofloxacin the activity index are 0.62, 0.44, 0.41 and 0.21 respectively. As mentioned in [21] the antimicrobial activity of acetone extract of pomegranate peel against *S. aureus* is higher than methanol followed by ethanol. Also [11] showed that the activity of methanol extract of peel against *S. aureus* was lower than in this study

Moreover, the antibacterial activity of all extracts on *P. aeruginosa* and *A. buamanni* were in the order acetone > methanol > aqueous > ethanol extracts.

The antifungal activity (Table 2) of all extracts on *C. parapsilosis* was in this order acetone > methanol = ethanol > aqueous.

From equation (1) and as it mentioned in Table 3 it can be concluded that in comparison with positive control drugs the inhibition effectiveness of extract on the microorganisms follows the order: *C. parapsilosis* > *S. aureus* > *P. aeruginosa* ≈ *A. buamanni*.

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مقالة بحثية

تحليل الفينولات المتعددة/الفلافونيدات والفاعلية المضادة للميكروبات في مستخلصات قشرة الرمان

أمينة حسين عبده¹، عادل أحمد محمد سعيد^{2*}، طه أبوبكر فضل³

¹ قسم الكيمياء الصيدلانية، كلية الصيدلة - عدن، جامعة عدن، عدن، اليمن

² قسم الكيمياء، كلية العلوم - عدن، جامعة عدن، عدن، اليمن

³ قسم الكيمياء، كلية التربية - عدن، جامعة عدن، عدن، اليمن

* الباحث الممثل: عادل أحمد محمد سعيد، البريد الإلكتروني: adel_saeed73@yahoo.com

استلم في: 18 يناير 2020 / قبل في: 28 يناير 2020 / نشر في: 07 مارس 2020

الملخص

لا يزال استخدام النباتات يلعب دوراً حيوياً مهماً لتحقيق المتطلب الصحي الأساسي لتطوير البلدان. أظهر مستخلصات قشور الرمان فاعلية مضادة للميكروبات. أعلى كمية من الفينولات والفلافونيدات تم استخلاصها باستخدام الاسيتون يليه المذيب الميثانول ثم الايثانول. أقل كمية كانت في المستخلص المائي. تم اختبار الفاعلية المضادة للميكروبات باستخدام هذه المستخلصات وأربع تراكيز مختلفة منها ضد الميكروبات الموجبة [*P. aeruginosa* and *S. aureus*] والسالبة [*A. baumannii*] وفطر *Candida parapsilosis*. ما عدا الاسيتون، وكانت فاعلية جيدة ما عدا المستخلص المائي الذي لم يظهر فاعلية تجاه بكتيريا *S. aureus*.

الكلمات الرئيسية: مستخلصات قشرة الرمان، تحليل الفينولات المتعددة والفلافونيدات، الفاعلية الميكروبية.

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