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Evaluation of antioxidant and antimicrobial activities of pomegranate (*Punica granatum* Linn.) peel extracts

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Abstract

Pomegranate fruit (*Punicagranatum*) is an edible medicinal plants which is an important source of phytochemical compounds. The highest of phenolics and flavonoids content were found in the peel acetone (25.60±0.00 mg GAE/g DW) and 95% ethanol (33.44±0.05 mg RE/g DW) extracts, respectively. The highest antioxidant activities in the peel were shown in the 40% and 95% ethanol fractions (17.04±0.01 mg AAE/g DW) by DPPH method and in the acetone (25.60±0.00 mg AAE/g DW) by the FRAP method. For antimicrobial activity ofpeel extracts, all peel extracts exhibited broad spectrum activity against all microorganisms with the MICsranging 0.390-25.00 mg/ml by broth macrodilution method. The acetone fraction of peel displayed the highest antimicrobial activity. *Staphylococcus aureus*ATCC 1216 was the most sensitive strain to all extracts (MIC 0.390 mg/ml and MBC 1.562- 6.250 mg/ml). Thus, pomegranate peel could serve as a potential source of antioxidants and antimicrobial agents for use as active ingredient in pharmaceutical formulations, food and cosmetic products

Keywords: Pomegranate peel, Anitioxidant and Antimicrobial activity, MIC and MBC, Total phenolic and flavonoid content, *Punicagranatum*

บทคัดย่อ

ทับทิม (*Punicagranatum*) จัดเป็นพืชสมุนไพรกินได้ที่เป็นแหล่งของสารพฤกษเคมีที่สำคัญแหล่งหนึ่ง โดยปริมาณสารประกอบฟีนอลิคและฟลาโวนอยด์ทั้งหมดสูงสุดพบได้ในส่วนของสารสกัดเปลือกในอะซิโตน (25.60± 0.00 มิลลิกรัมเทียบเท่ากรดแกลลิกต่อกรัมน้ำหนักแห้ง) และ 95% เอทานอล (33.44±0.05 มิลลิกรัมเทียบเท่ารูทินต่อกรัมน้ำหนักแห้ง) ตามลำดับ ฤทธิ์ต้านอนุมูลอิสระสูงสุดโดยวิธี DPPH ของเปลือกทับทิมพบได้ในตัวทำละลาย 40% และ 95% ของเอทานอล (17.04±0.01 มิลลิกรัมเทียบเท่ากรดแอสคอร์บิกต่อกรัมน้ำหนักแห้ง) และโดยวิธี FRAP พบในส่วนของอะซิโตน (25.60±0.00 มิลลิกรัมเทียบเท่ากรดแอสคอร์บิกต่อกรัมน้ำหนักแห้ง) สำหรับฤทธิ์ยับยั้งแบคทีเรียก่อโรคของสารสกัดเปลือกทับทิมด้วยวิธีการเจือจางในอาหารเหลวแบบ macrodilution นั้น สารสกัดเปลือกทับทิมในตัวทำละลายทั้งหมดแสดงฤทธิ์กว้างในการยับยั้งเชื้อแบคทีเรียโรค โดยมีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อแบคทีเรียก่อโรคทดสอบได้ อยู่ในช่วง 0.390-25.00 มิลลิกรัมต่อมิลลิลิตรสารสกัดเปลือกด้วยอะซิโตนแสดงฤทธิ์ยับยั้งเชื้อแบคทีเรียก่อโรคได้สูงสุด และเชื้อ *Staphylococcus aureus* ATCC 1216 เป็นสายพันธุ์ที่มีความว่องไวต่อสารสกัดเปลือกทับทิบในตัวทำละลายทั้งหมด (MIC เท่ากับ 0.390 มิลลิกรัมต่อมิลลิลิตรและ MBC เท่ากับ 1.562-6.250 มิลลิกรัมต่อมิลลิลิตร) ดังนั้น เปลือกทับทิมจึงเป็นแหล่งที่มีศักยภาพของสารต้านอนุมูลอิสระและสารต้านจุลชีพเพื่อใช้เป็นส่วนผสมในเวชภัณฑ์ อาหารและผลิตภัณฑ์เครื่องสำอาง

คำสำคัญ: เปลือกทับทิม ฤทธิ์ต้านอนุมูลอิสระและยับยั้งเชื้อแบคทีเรีย MIC และ MBC ปริมาณสารฟีนอลิกและฟลาโวนอยด์ทั้งหมด ทับทิม

1. Introduction

Pomegranate (*Punica granatum*) is an important fruit which originated in the Middle East, eastward to Asia (China and India). It has been extensively used as a traditional medicine in many countries for the treatment of different types (1). In addition, this plant is reported to have excellent antibacterial (2-4), antifungal (3-5), and antioxidant activities due to an excellent source of phytochemical compounds such as polyphenolic compounds (6-7) include falvonoids, anthocyanins, condensed and hydrolysable tannin (8). The aims of many researches are to assess the neutraceutical qualities of pomegranate which are focused only on the fruit juice and fruit pulp from edible parts (9, 10) due to 50% of pomegramate which are peel will become an agricultural industrial wastes after making refreshing drink, aroma or wine (11-12). In fact, the peel of pomegranate fruit consists of wide varieties of phytochemical compounds such as gallotinnins, ellagic acid, gallagic acid, punicalins, punicalagins and ellagitannins (8, 13-14) and also has antimicrobial activity against various microorganisms such as bacteria, fungi, yeasts and viruses (15-19). Considering that the peel of pomegranate which is inedible part of fruit, can be used in pharmaceutical and other commercial products for developing the new value-added products from fruit wastes.Thus, the aims of the present study were to evaluate antioxidant and antimicrobial activities and to quantify the phenolic and flavonoid compounds in (40 and 95%) ethanol and acetone extract from pomegranate peel.

2. Materials and Methods

2.1 Preparation of Plant Extracts

The pomegranate (*Punica granatum*) fruits were collected from Phranakhon Sri Ayutthaya Province, in the central region of Thailand. Fresh pomegranate fruits were peeled manually and collected peels were then rinsed with tap water and distilled water, respectively. Then, peel was dried in hot air oven at 60°C for 24 hours. Dried peel was then taken for grinding by blender and powdered form of plant sample was soaked in 95% ethanol, 40% ethanol (rice whisky)or acetone in a ratio of 1:8 in the dark, at room temperature for 3 days separately. The mixtures were filtered through a sterile filter cloth and a sterile filter paper (Whatman No. 1), respectively. The filtrates obtained were subsequently concentrated to a small volume under vacuum on a rotary evaporator (Laborata 4000; Heidolph Instruments GmbH & Co. KG, Germany). All the concentrated extracts were stored at -20°C under dark condition prior to analysis. The final weight of the crude extracts were weighted and calculated for the %yield per original materials.

2.2 Determination of Total Phenolic content

Total phenolics of peel extracts were determined using the method of Singleton and Rossi(20).100µl portions of diluted extracts were introduced into test tubes followed by addition of 750µl of freshFolin-Ciocalteu reagent (1:10). After standing at room temperature for 5 min, 750µl of 6% (w/v) sodium carbonate (Na_2CO_3) was added and allowed to completely reacted for 90 min at the room temperature in the dark condition. And then, the absorbance ofthe reaction mixtures were recorded at 725 nm against blank. Total phenolics were calculated from standard gallic acid solutions (0.02 - 0.1 mg/ml) used under the same conditions, and concentrations were expressed as mg gallic acid equivalent per gramdry weight (mg GAE/g DW).

2.3 Total Flavonoids of Extracts

The content of flavonoids in the peel extracts were determined by aluminium chloride colorimetric assay (21). Briefly, 200ml of sample extracts and 2.3 ml 30% of methanol were added in tested tubes. Then, the mixture was added 100 µl of 0.5 M $NaNO_2$ and 100 µl 0.3 M $AlCl_3$, respectively. Next, the sample solution was thoroughly mixed with vortex and kept in the dark. And then, the absorbance was measured 5 min later at 506 nm. Total flavonoid were

calculated from standard rutin solution (0.01-0.05 mg/ml), and concentrations was expressed as mg of rutin equivalent per gram dry weight (mg RE/g DW).

2.4 DPPH- Scavenging Activity of Extracts

The DPPH radical-scavenging activity of sample extracts was determined through free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (22). Briefly, 900 µl of 0.1 mM DPPH in methanolic solution was mixed with 100 µl of samples in methanol. The mixture was left to stand in the dark, for 15 min at room temperature. Meanwhile, ascorbic acid (0.01-0.05 mg/ml) was used as reference under the same conditions. An equal amount of DPPH (900 µl) and methanol (100 µl) served as control.The decrease in DPPH absorbance (Abs) was measured at 517 nm. The DPPH radical-scavenging activity was calculated using the following formula:

$$\text{DPPH Scavenging effect (\%)} = [Abs_{control} - Abs_{sample} / Abs_{control}] \times 100$$

2.5 Ferric reducing/antioxidant power assay (FRAP)

The total reducing capacity was determined by using FRAP assay. FRAP assay was performed according to the method of Benzie and Strain (21) with some modification. The stock solution of FRAP reagent was initially prepared consisting of 300 mM acetate buffer (pH 6.3), 20 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) solution in 40 mMHCl and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was warm at 37°C in oven prior to use. Briefly, 300µl of peel extract was allowed to react with 2.7 ml of the FRAP reagent in test tubes for 30 min in the dark condition. Readings of the colored product were then taken at 593 nm by using spectrophotometers. The total reducing capacity by FRAP assay were calculated from ascorbic acid (0.01-0.05 mg/ml)standard, and concentrations was expressed as mg of ascorbic acid equivalent (AAE) per gram dry weight (mg AAE/ g DW).

2.6 Sources and maintenance of organisms

Six pathogenic strains were obtained from the laboratory of the Department of Biotechnology, King Mongkut's University of Technology North Bangkok, Thailand. The bacterial strains in the present study were Gram-positivebacteria including *Staphylococcus aureus*ATCC 1216 and *Bacillus cereus* DMST 5040 and Gram-Negative including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 1331. All bacterial strains were cultured on brain heart infusion (BHI, Difco) agar medium at 37°C for 24 hours.The culture was maintained by transfer to fresh medium at every 7 day interval.

2.7 Minimum Inhibitory Concentration (MIC) by broth macrodilution method

The estimation of MIC values of the extracts were carried out by two-fold serial dilution method. The inoculum of bacteriawas prepared by diluting the overnight bacteria culture in brain heart infusion (BHI) broth to a level of 10^8-10^9 cfu/ml (OD_{600} = 0.5). The extracts were prepared the initial concentrations by dissolving in 10% DMSO and then serial two-fold dilutions were made in a concentration range from 50 to 0.195 mg/ml with BHI in tested tubes. Then, 0.5 ml of the inoculums of tested strains was added in each tube, mixed on a vortex for 20 s and incubated at 37°C for 24 hours. Control samples were performed without adding the plant extract. The MIC was taken as the lowest concentration that inhibited the growth of the test microorganism after 24 hours of incubation (23).

2.8 Determination of the Minimum Bactericidal Concentration (MBC)

The tubes showed no apparent growth of microorganism in the MIC assay was subcultured on a BHI agar (Difco) plates by making streak the surface of agar, and incubated at 37°C for 24 hours. Least concentration of extracts showing no visible growth on new agar medium was taken as MBC.

2.9 Statistical Analysis

All the analyses were performed in triplicate. Results were expressed as means±standard deviation. Analysis of variance procedure (ANOVA) was performed with the statistical program MS Excel (Microsoft Office 2010 Professional) to analyze whether there was significant difference between each extract. Results were significant when $P < 0.05$.

3. Results and Discussions

Most plants are able to produce a diverse range of bioactive molecules which become a rich source of different types of medicines. An interesting feature of plants in the present is focused on phytochemical compounds aspotential sources of functional substances such as antioxidant and antimicrobial substances. Pomegranate peel was extracted with a different polarity of solvents included 40% ethanol (rice whisky), 95% ethanol and acetone. Among the solvents, 95% ethanol afforded the most concentrated extract with the highest yield (48.88%). Whereas 40% ethanol and acetone gave maximum yield of 42.00% and 30.55%, respectively, and no significant differences were apparent between% yield of 2 types of ethanol. Thus, 40% ethanol (rice whisky) is one option that will be used to represent the ethanol solvent for extraction with lower cost.

3.1 Total phenolic and flavonoid content

Results for the total phenolic and flavonoid content of different extracts of pomegranate peel are given in Table 1. The highest total phenolic content was observed in acetone extract (25.60±0.00mg GAE/g DW) followed by 95% ethanol (12.80±0.00 mg GAE/g DW) and 40% ethanol (8.48±0.01 mg GAE/g DW), respectively, whereas the highest flavonoid content were showed in 95% ethanol extract (33.44±0.05 mg RE/g DW) followed by 40% ethanol (21.500.01 mg RE/g DW) and acetone (18.94±0.03 mg RE/g DW), respectively.

From the results shown in Figure 1, the total phenolic contents of ethanol fraction was significantly different from that of acetone fraction ($P < 0.05$) but no significant difference was found for 95% and 40% ethanol fractions. However,the phenolic content of 95% *ethanol* extract in this study was lower than that of 95% ethanol extract in India (18 mg GAE/g DW) (24). Additionally, this work showed that less polar solvent (acetone) could extract high amount of total phenolic compounds andless amount of total flavonoids. Yasoubi*et al*. (6) have reported

Table 1. Total phenolic and flavonoid content and antioxidant activities of Pomegranate (*Punica granatum*) peel extracts

Test	Antioxidant Activity		
	40% Ethanol extract (rice whisky)	95% Ethanol extract	Acetone extract
Total Phenolic Content (mg GAE/g DW; %)	8.48±0.01^{c}	12.80±0.00^{b}	25.60±0.00^{a}
Total flavonoids Content (Aluminum chloride method) (mg RE/g DW; %)	21.50±0.01^{b}	33.44±0.05^{a}	18.94±0.03^{c}
Total Antioxidant Capacity			
DPPH radical scavenging activity assay(mg AAE/g DW; %)	17.04±0.01^{b}	17.04±0.01^{b}	6.40±0.00^{a}
Ion Reducing Antioxidant Power (FRAP) (mg AAE/g DW; %)	12.80±0.00^{c}	21.28±0.01^{b}	25.60±0.00^{a}

GAE = Gallic Acid Equivalent; RE = Rutin Equivalent; AAE = Ascorbic acid Equivalent

^{abc} means in the same row with different superscript were significantly different ($P < 0.05$) by one way ANOVA and paired T-test

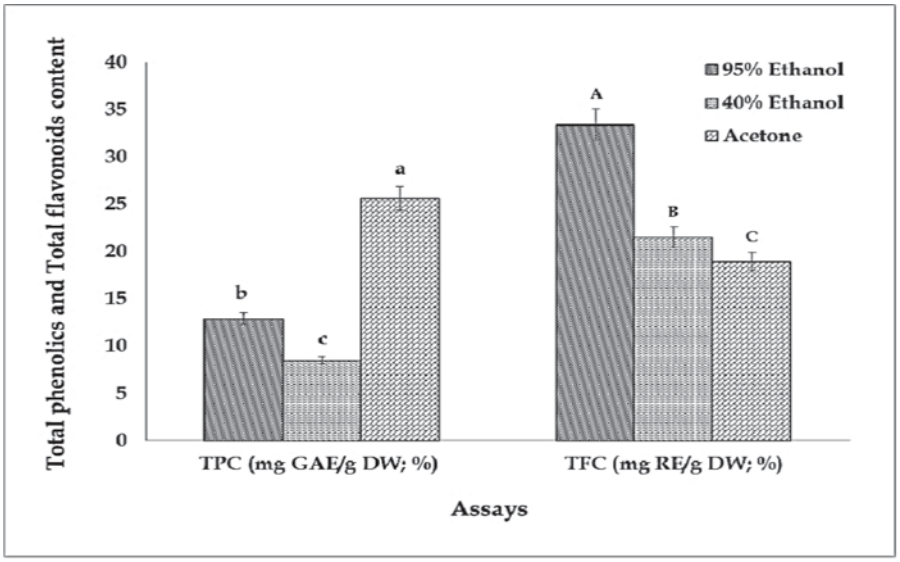

Figure 1. Total phenolics and flavonoids content of different extracts of pomegranate (*Punica granatum*) peel ($P < 0.05$; one way ANOWA and paired T-test)

that the acetone extract of pomegranate peel showed higher amount of phenolic compounds compared with those of the other solvents which was similar to the result of this work. The variability in total phenolics and flavonoids content among studies was dependent on solvents used for extraction, sources of samples and pomegranate varieties.

3.2 Antioxidant activities

The DPPH scavenging activity has been widely used to test the antiradical activity in different samples due to stable radical, sensitivity in low concentration and short-term detection. The decrease in absorbance of the DPPH$^{\cdot}$ radical caused by antioxidants indicates the scavenging capacities. As presented in Table 1 the investigated pomegranate peelextracts differed in the DPPH radical-scavenging activity and the total reducing capacity by FRAP. Ethanol (40 and 95%) exhibited the highest DPPH radical scavenging activity (17.04±0.01mg AAE/g DW) whereas acetone extract showed Ferric Reducing Antioxidant Power (FRAP) (25.60±0.00 mg AAE/g DW) (Figure 2). The relationship between phenolic content or flavonoid content and antioxidant activities (DPPH and FRAP) in different extracts of pomegranate peel were not, the highest antioxidant activities were not dependent on the total phenolics or flavonoid content.

3.3 Antimicrobial activities

Quantitative evaluation of antimicrobial activity of all extracts was carried out against 6 different Gram-negative and Gram-positive bacteria by broth macrodilution techniques. The MIC values of all extracts were presented in Table 2. In this study, the MIC and MBC values of peel extracts had range from 0.390 to 12.50 mg/ml and 1.562 to >50.00 mg/ml, respectively.The acetone extract of pomegranate peel was the most effective against all pathogenic strains at MIC values between 0.39-6.25 mg/ml on the tested strains. For the MIC value, 95% ethanol extract was more effective on *B. cereus* DMST 5040, *E. coli* ATCC 25922 and *K. pneumoniae* in comparing with those of 40% ethanol extracts ($P<0.05$), but the MBC values were not significantly different (Table 2). The highest antimicrobial activity of acetone extracts from pomegranate peel have been reported by Negi and Jayaprakasha (25) and the opposite was found by Nuamsetti *et al*. (26) who reported that acetone extract of peel was less active than ethanol and hot water extract against *S. aureus*, *E. coli* and *S. typhimurium*. For each tested bacterial strain, the

most sensitive bacterial strain was *S. aureus* ATCC 1216, whereas the most resistant bacterial strain was *K. pneumoniae*. Moreover, the Gram positive strains were more sensitive to peel extracts than Gram-negative strains. This might be due to the difference of the composition of their cell walls.

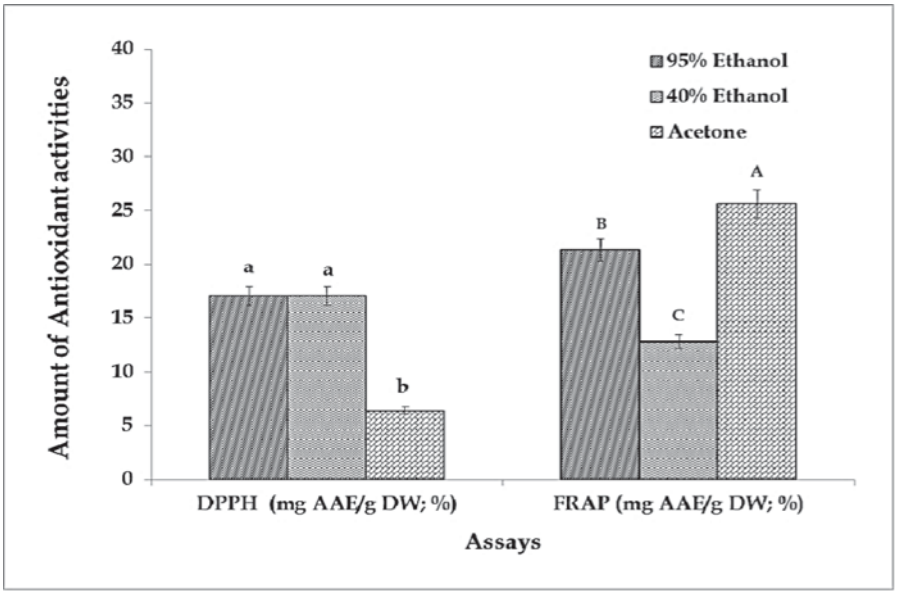

Figure 2. Antioxidant activity of different extracts of pomegranate (*Punicagranatum*) peel ($P < 0.05$; one way ANOWA and paired T-test)

Table 2. The MIC and MBC of pomegranate (*Punica granatum*) peel extracts against some pathogenic strains by broth macrodilution method

	Microorganisms	MIC and MBC (mg/ml)					
		40% ethanol extract (rice whisky)		95% ethanol extract		Acetone extract	
		MIC[1/]	MBC[2/]	MIC[1/]	MBC[2/]	MIC[1/]	MBC[2/]
Gram-positive	*B. cereus* DMST 5040	$1.562^{c,A}$	$25.00^{b,A}$	$0.781^{d,B}$	$12.50^{b,A}$	$0.781^{b,B}$	3.125^{d}
	S. aureus ATCC 1216	0.390^{d}	$6.25^{c,A}$	0.390^{e}	$6.25^{c,A}$	0.390^{c}	$1.562^{c,B}$
Gram-negative	*E. coli* ATCC 25922	$1.562^{c,A}$	$50.00^{a,A}$	$0.781^{d,B}$	$25.00^{a,B}$	$0.781^{b,B}$	$25.00^{a,A}$
	P. aeruginosa ATCC 27853	$1.562^{c,A}$	$25.00^{b,A}$	$1.562^{c,A}$	$25.00^{a,A}$	$0.390^{c,B}$	$3.125^{d,B}$
	S. typhimurium ATCC 13311	$3.125^{b,A}$	>50.00	$3.125^{b,A}$	>50.00	$0.781^{b,A}$	6.250^{c}
	K. pneumoniae	$25.00^{a,A}$	>50.00	$12.50^{a,B}$	>50.00	$6.250^{a,C}$	12.50^{b}

^{abcde} means in the same column with different superscript were significantly different ($P < 0.05$) by one way ANOVA and paired T-test

$^{1/ABC}$ means in the same column with different superscript were significantly different ($P < 0.05$) by one way ANOVA and paired T-test

$^{2/ABC}$ means in the same column with different superscript were significantly different ($P < 0.01$) by one way ANOVA and paired T-test

4. Conclusions

The natural bioactive compounds of plant extracts will help to develop new products such as antioxidant and antimicrobial agents. Our work clearly showed that pomegranate peel extracts possessed abundant phytochemicals containing phenolic and flavonoid compounds and exhibited the antioxidant activities by DPPH and FRAP assays. Additionally, their extracts exhibited excellent potential compounds as broad-spectrum antimicrobial agents. However, the solvent types and solvent ratios for extraction have significant effect on antioxidant and antimicrobial activities of plantsand also help cost reduction in operation such as 40% and 95% ethanol extract in this work showed no significant difference in the result. Thus, pomegranate peel is one of the agro-industry waste which could be used as preservative in the *value-added product*s of pharmaceutical and food applicationsin the future.

5. Acknowledgement

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