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Study of antibacterial and antioxidant properties of pomegranate peel weak acid extracts

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ABSTRACT

In order to investigate whether the weak acid solution is an effectively pomegranate peel polyphenols extract agent or not, this work took 5% aqueous acetic acid as weak acid extraction agent, water and 50% aqueous ethanol extract as contrast agent to extract pomegranate peel polyphenols, the total phenol content, total oxidation resistance, free radical scavenging ability, superoxide anion radical scavenging capacity of extracting solution and extract antibacterial ability as evaluation criterion to study the extracting effect of 5% acetic acid aqueous solution. It showed that pomegranate peel polyphenols extract by 5% acetic acid aqueous solution has higher total phenol content, antioxidant capacity, free radicals scavenging ability, superoxide anion radical scavenging capacity, and antibacterial ability against E. coli, yeast and mold. As a result, 5% acetic acid aqueous solution may be a more appropriate extract agent for pomegranate peel polyphenols.

KEYWORDS

Pomegranate peel polyphenols;
Acetic acid;
Free radicals;
Antioxidation;
Bacteriostasis.

INTRODUCTION

Pomegranate peel is rich in polyphenols, including tannins, flavonoids and organic compounds including ellagic tannin, gallic tannins, ellagic acid, gallic acid, catechin, anthocyanin, chlorogenic acid, ferulic acid, quercetin and other compounds[1]. In recent years, studies have shown that pomegranate peel polyphenols have antioxidant, antibacterial, antidiarrheal, anti-tumor and other functions, and thus shown great application value in food, medicine, daily-use chemical and other fields[2-8].

The tradtional extraction solvents for pomegranate peel polyphenols are water, methanol, ethanol, acetone, chloroform and ethyl acetate. It was reported that the composition of pomegranate peel polyphenols extracting by different solvents is not the same, and extraction with polar solvents has greater antioxidant capability compared to non-polar one, and a proper polarity of solvent could yield a higher antioxidant activity than other solvents[9-14]. The antioxidant capability is also affected by other factors, including solid-liquid ratio, temperature, particle size of pomegranate peel and extract methods. Small-sized pomegranate peel particles could yield higher antioxidant activity, and the total phenolic content and antioxidant activity of pomegranate peel polyphenols increase as an inverse function of peel particle size[14]. Qu et al. showed that the yield and content

of antioxidants increased with reduced particle size, increased water/sample ratio and temperature, but antioxidant activity was low when extraction temperature was high. A recommended extraction condition was peel particle size of 0.2mm, water/peel ratio of 50/1 (w/w), temperature of 25 °C, and extraction time of 2 min, which gave the high antioxidant yield (11.5%) and content (22.9%), and DPPH scavenging activity of 6.2mg/g[15]. Some assistant method can also increase the antioxidant capability of pomegranate peel polyphenols, such as pressurised water, microwave-assistant, ultrasonic-assisted, Etc. Up to 262.7mg/g of hydrolyzable tannic acid equivalents and 116.6 mg/g of punicalagin, the secondary component of pomegranate peel polyphenols could be extracted with pressurized (102.1 atm) de-ionized water at 40 °C by Cam et al[16]. Reza et al. optimized the ultrasonic assisted extraction process of pomegranate peel polyphenols using surface response methodology, and they recommended an optimal condition for industrial applications, 70% ethanol-water mixture as solvent, temperature of 60 °C and extraction time of 30 minutes[17].

Some researchers studied the antimicrobial and antioxidant potential of the active ingredients of pomegranate peel extracts, exploring their prevention and treatment role in atherogenic, gastro-mucosal injuries, ethanol- and acetone-induced ulceration and diabetic oxidative damage[18-21]. Many studies have confirmed pomegranate peel as a remarkable source of antioxidants[22,23]. The study made by Keita Saito[24] about anti-oxidative function of thousands of natural plant showed that the antioxidant activity of pomegranate peel extract ranked fourth, and it is verified by experiment that its antioxidant activity is related to its polyphenol content. Some researches focused on the anti-tumor potential of pomegranate peel polyphenols[7,8,25]. The antimicrobial mechanisms of pomegranate peel polyphenols based on their ability to precipitate membrane protein and inhibit enzymes such as glycosyltransferases[26]. Pomegranate peel polyphenols have been widely exploited against Staphylococcus aureus and hemorrhagic Escherichia coli[26-28], Al-Zoreky proved the inhibitory effect against Listeria monocytogenes, Staphylococcus aureus, Escherichiacoli and Yersinia enterocolitica of an 80% methanolic extract of pomegranate peel by in vivo and in situ experiment[29]. Negi et al. studied the bacteriostasis action of Granatum acetone, methanol and water extract on Bacillus cereus, Bacillus coagulens, Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa. Activity of acetone extract is the strongest and MIC concentration is 150-200ppm. Activity of aqueous extract is the worst and MIC concentration is 400-700 ppm[13, 30].

Comprehensive previous researches, extraction solvent of pomegranate peel polyphenols is usually water, different concentrations of alcohol, ether, acetone solution or ester solvent. Previous studies showed that pomegranate peel polyphenols is weak acid substance. According to the principle of similar miscibility, Pomegranate peel polyphenols should have better solubility in acid solvent. This article took 5% acetic acid aqueous solution as extraction agent, water and 50% ethanol-water solution as comparative extracting agent to investigate the effect of 5% aqueous acetic acid solution on the extraction of pomegranate rind polyphenols, and carried out preliminary experiments and antioxidant antibacterial test with three kinds of extractant, and its aim is to provide certain ideas and scientific basis for the development and application of pomegranate peel polyphenols resources.

EXPERIMENTAL

Material and methods

Anhui Huai pomegranate peel (direct peel, dried) Distilled water, laboratory made. Ethanol (AR), glacial acetic acid (AR), Na_2CO_3 (AR), concentrated sulfuric acid (98%), hydrochloric acid (37%), ammonium molybdate (AR), trisodium phosphate (AR), vitamin C (AR), pyrogallol (AR) gallic acid standard, potatoes (PDA) medium (BR) and yeast extract peptone dextrose agar (YPD) medium (BR) were purchased from Sinopharm Chemical reagent Co., Ltd.; Tris (hydroxymethyl) amino methane, 2,2-diphenyl-2-picrylhydraz-yl(DPPH), Folin-phenol (Folin-ciocalteu) reagents, LB medium: sigma.

General procedure

Extraction of the pomegranate peel polyphenols

The pomegranate peel is dried to constant weight in an oven in 40°C, crashed in high speed pulverizer, and passed through a 60-mesh sieve, then bagged to

standby application. Weigh accurately three pieces of 20.00g of pomegranate peel powder, according to liquid ratio of m: V = 1:10, then add in 200mL of water, 50% ethanol, 5% aqueous acetic acid, respectively, reflux and extract for 1.5 h for three times, filter and merger filtrate, namely corresponding pomegranate peel polyphenols extract of three kinds of extraction agent. The extract solutions were used directly for the test total of phenolic content and antioxidant properties. Pomegranate peel polyphenols extract in the same volume was concentrated under reduced pressure to a paste, dried in vacuum oven to obtain pomegranate peel water extract, 50% ethanol extract and 5% acetic acid extract for antimicrobial properties evaluation.

Detection method

Determination of total phenolic content of pomegranate peel polyphenols extracts

Determination of total polyphenol content uses Forint-phenol colorimetric method (Folin-Ciocalteu method)[3]. Use Gallic acid as the standard. Forint - phenol reagent is diluted for 2 times before using. Determination of standard curve: Weigh accurately 44.3mg of gallic acid standard substance dried in vacuum oven to constant weight. Dissolved in water and diluted to 100 ml. This was allocated to the concentration of 8.86 ìg / ml, 17.72 ìg / ml, 35.44 ìg / ml, 70.88 ìg / ml, 88.60 ìg / ml of solution. Respectively take 1ml of the different concentration of solution add to 10ml of colorimetric tube, and then add 1ml of deionized water in turn, 0.5 ml of the forint - phenol solution diluted for 2 times, 1.5ml of 26.7% Na_2CO_3 solution, and use water volume to 10ml, response for 2h at room temperature at last. Taken solution without standard liquid as blank control, scan on the ultraviolet spectrophotometer (island ferry UV - 7550) to get the peak value of maximum absorption peak at 760nm. Perform regression of Concentration by the absorbance and standard curve is obtained. Determination of polyphenols content in pomegranate peel extract: Absorb 1.0ml of sample 1, sample 2 and sample 3, and respectively dilute with an equal amount of distilled water to the concentration below 0.08 mg/ml. Take 1 ml of diluted sample liquid add to 10 ml of colorimetric tube, add 1 ml of deionized water, 0.5 ml of the forint - phenol solution diluted 2 times, 1.5 ml of 26.7% sodium carbonate solution in turn, use water volume to 10ml, response for 2h at room temperature, determine its absorbance at 760nm. Measured absorbancy is substituted into the standard curve, and total polyphenol content in the sample are obtained.

Determination of total antioxidant capacity of pomegranate peel polyphenols extracts

Total antioxidant capacities in pomegranate peel extracts were determined by the formation of phosphomolybdenum complex method. Phosphomolybdic reagent concentration is always 0.6mol/L of concentrated sulfuric acid, 28mmol/L of sodium phosphate and 4mmol/L of ammonium molybdate solution. In the 10ml of tube, sequentially add above-mentioned 4ml of phosphomolybdic reagent solution, 0.4ml sample solution, incubate 90min at 95°C of constant temperature in water bath, measure the absorbancy at 695nm wavelength on the UV spectrophotometer (Shimadzu UV-7550) for three parallel samples on each sample and take the average[31].

Free radical scavenging capacity of pomegranate peel polyphenols extract

Anion radical scavenging capacity in pomegranate peel polyphenols extract is determined by the DPPH method. Preparation of DPPH formulated solutions: Weigh 0.0128g of DPPH, dissolve it with absolute ethanlol in 50 ml of volumetric flask to volume, shake and store in a refrigerator as stock solution (6.5×10^{-4}mol/L). When using, diluted with ethanol to a concentration of 1.0×10^{-4}mol/L and preserved avoiding light. At the same time, three kinds of pomegranate peel extract were configured to the same concentration of solution as an alternate. Determination of DPPH· free radical scavenging ability: Samples indicated by A_i let stand for 30 min at room temperature, add in the cuvette, and measure the absorbance of the sample A_i, A_j, A_c at a wavelength of 517nm, the sequence of DPPH sample adding reaction liquid was shown in TABLE 1. Calculate DPPH scavenging ability of pomegranate peel polyphenol extracts extracted by different extraction agent according to equation (1).

$$SA(\%) = [1 - (A_i - A_j) / A_c] \times 100\% \quad (1)$$

A_c - Absorbancy of DPPH and solvent's mixture; A_i - Absorbancy of DPPH·and sample after reaction; A_j - Absorbancy of the sample and solvent's mixture

The above test was repeated for three times to get

TABLE 1 : The sequence of DPPH sample adding reaction liquid

	Reaction liquid 1	Reaction liquid 2
A_c	2 ml DPPH solution	2mL 50% aqueous ethanol
A_i	2 ml DPPH solution	2 ml sample solution
A_j	2 ml sample solution	2 ml 50% ethanol aqueous

average value of the clearance.

Superoxide anion (O_2-) scavenging ability of Pomegranate peel polyphenols extract

Free radical scavenging of Pomegranate peel extract was evaluated by pyrogallol auto-oxidation systems. Join 9ml of pH value of 8.2 of Tris-HCl buffer in 20ml of tubes and let it stand in constant temperature water bath at 25 °C for 20min, and inject 0.04ml of pyrogallol solution (45mmol/L pyrogallol dissolved in 10mmol/L HCL) pre-heated at 25 °C using a micro syringe, immediately mix and pour into 1cm of cuvette, place at room temperature for 5 min and measure absorbancy at 420nm, i.e.A_0, represents pyrogallol auto-oxidation rate. Previously added 0.1mL of three kinds of aqueous solution of pomegranate peel polyphenol extracts in the Tris-HCl buffer, and then determine pyrogallol oxidation rate according to the above method to obtain A_1, while doing a reagent blank A_2. According to formula (2), calculate inhibition ratio d (%):

$$d\ (\%) = [A_0 - (A_1 - A_2) / A_0] \times 100\% \quad (2)$$

Antibacterial properties of pomegranate peel polyphenol extract

In order to exclude the impact of extract solvents on the antimicrobial property of pomegranate peel polyphenols extract, the extraction of the same volume was concentrated under reduced pressure to a paste, dried in vacuum oven to obtain pomegranate peel water, 50% ethanol and 5% acetic acid extract powders for antimicrobial properties evaluation.

Strains of assessing antibacterial properties of pomegranate peel polyphenol extract were molds, yeasts and E.Coli. The inhibiting ability of pomegranate peel polyphenol extract to yeast and E. Coli were evaluated by punch inhibition zone method, while inhibiting ability to Mildew using inoculation RBI mothed.

RESULTS AND DISCUSSION

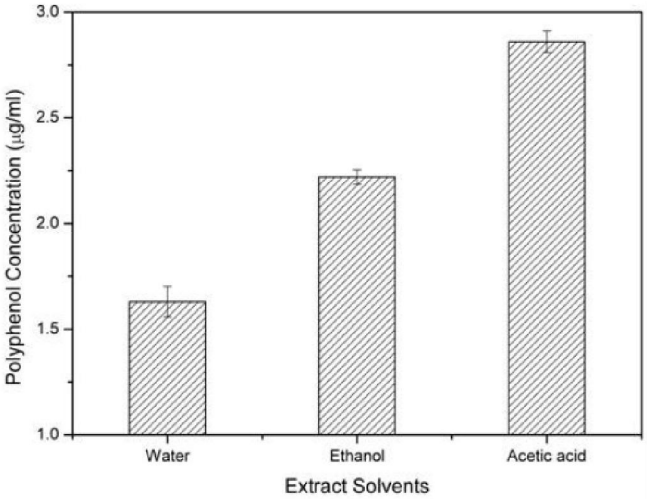

Figure 1 : Total phenol content of three pomegranate peel extraction solutions corresponding to different extraction agents

Contrast of three kinds of pomegranate peel polyphenol extracts

Figure 1 showed the total phenol content of three pomegranate peel extract solutions correspond to water, 50% aqueous ethanol and 5% aqueous acetic acid as extraction solvents, respectively.

As can be seen from Figure1, different extraction agents to extract pomegranate peel polyphenols were different. Total phenol content of pomegranate peel extract preparing with water as extracting agent is the lowest, with 5% acetic acid aqueous solution as extracting agent is the highest, with 50% ethanol aqueous solution as extracting agent falls in between. That is to say, adding organic solvent in the water can improve the efficiency of the extraction of pomegranate peel polyphenols, especially under acid condition, and 5% acetic acid aqueous solution is more effective for extraction of pomegranate peel polyphenols as extraction agent.

The reason may be that the pomegranate peel polyphenols are mainly ellagic tannin in which molecules contain several phenolic hydroxyl groups to make pomegranate peel polyphenols have strong and weak polar nature. This polarity allows pomegranate peel polyphenols more easily to dissolve in a polar solvent. Therefore, the efforts of 50% aqueous ethanol and 5% acetic acid solution are better than water's for extraction of polyphenols pomegranate peel. In addition, low-acid nature of pomegranate peel polyphenols is close to that

FULL PAPER

of acetic acid aqueous solution. According to the principle of similar miscibility, polyphenols granatum aqueous acetic acid at 5% greater than the solubility in water and 50% aqueous ethanol, Therefore, 5% aqueous acetic acid has higher extraction efficiency. Solubility of pomegranate peel polyphenols in 5% acetic acid aqueous solution is bigger than that in water and 50% ethanol water, so 5% acetic acid aqueous solution has higher extraction efficiency.

Figure 2 illustrated the different total anti-oxidant capacity of three pomegranate peel extracts solutions. As can be seen from the figure, compared with aqueous extract of pomegranate peel polyphenols, total antioxidant capacities of ethanol extract and acetate extract of pomegranate peel polyphenols improved significantly. The total antioxidant capacity of acetate extract of pomegranate peel polyphenols was slightly higher, closer to its ethanol extract.

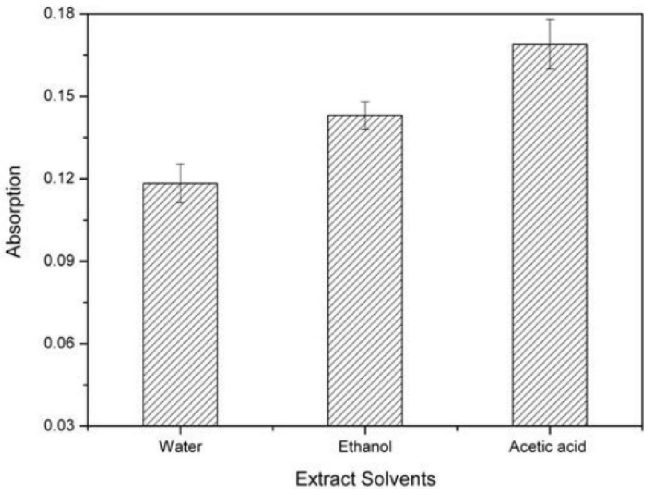

Figure 2 : Total anti-oxidant capacity of three pomegranate peel polyphenol extraction solutions

Figure 3 was comparison of free radical scavenging ability of three pomegranate peel polyphenols extraction solutions, correspond to water, 50% aqueous ethanol and 5% aqueous acetic acid. As can be seen from the figure, three kinds of extracts has certain scavenging effect on DPPH free radicals, pomegranate peel polyphenols extract with three kinds of extraction agent have close ability to remove free radicals, acetate extract is better than that of ethanol extract and water extract. It represents that radical scavenging ability of extracts is closely related to the content of polyphenols. This is similar to research results by Negi et al. on

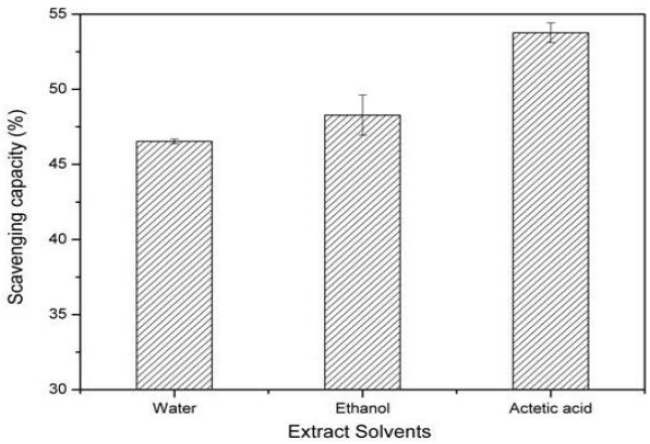

Figure 3 : Free radical scavenging ability of three pomegranate peel polyphenols extraction solutions

free radicals scavenging activity of pomegranate peel extract[10], and is in accordance with the determination results of previous total phenol content and total antioxidant capacity.

Tannins in pomegranate peel polyphenols have strong antioxidant effect, and can react with free radicals to play a role in scavenging free radicals. Because 5% of acetic acid aqueous solution had better efficiency on extraction of pomegranate peel polyphenols, the corresponding extraction solution had better ability of resisting oxidation and scavenging free radical, and 50% ethanol aqueous solution was second, with water the worst. Experimental total antioxidant activity and DPPH· free radical scavenging ability, however, had no a linear relationship with the results of the total phenol content, which might has something to do with different types and quantities of concomitant polyphenols and other physiologically active ingredients. Data showed that the larger ellagic tannins monomer molecular weight was, the more inclusive phenolic hydroxyl and HHDP groups were and the higher its antioxidant and free radical scavenging ability were[3].

Figure 4 showed the superoxide anion radical scavenging abilities of three pomegranate peel polyphenols extraction solutions. Different from assessment results of previous total antioxidant capacity and free radical scavenging ability, the order of three kinds of extraction agent corresponding pomegranate peel polyphenols extracts on scavenging superoxide anion is: acetate extract > aqueous extract > pomegranate peel polyphenols ethanol extract. This phenomenon of non-positively related to the sort of superoxide anion radical scavenging and that of extract total phenolic content is prob-

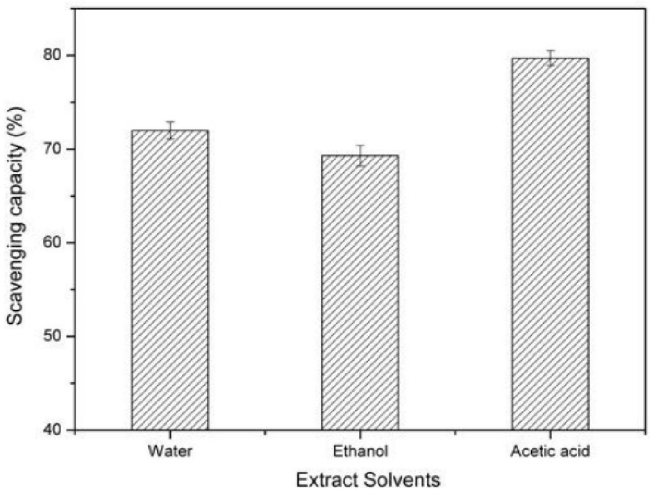

Figure 4 : Superoxide anion (O_2-) radical scavenging abilities of three pomegranate peel polyphenols extracts

ably caused by different of extracts' physiological active ingredients and kinds. The specific mechanism remains to be further in-depth research.

In order to eliminate the effects of ethanol and acetic acid on microbial growth, the evaluation of anti-bacterial property respectively extracted 100ml from prophase preparation of three kinds of pomegranate peel polyphenols extracts at first, stressed concentration to the paste, and then be freeze-dried, and dissolved three kinds of the obtained pomegranate peel polyphenols extracts after drying in 100ml of distilled water, and used the redissolved pomegranate peel polyphenols solution for antibacterial performance test.

TABLE 2 and TABLE 3 were the comparison of antibacterial properties of three pomegranate peel polyphenols extracts. The inhibiting ability of pomegranate peel polyphenols extract to the growth of mold was evaluated by Inoculation RBI method. The smaller the circle of mold fungus growth, the better inhibiting ability it has. As can be seen from the data in the table, the circle of corresponding fungal strains to acetate extract of pomegranate peel polyphenols, having a diameter of only 2.0mm, indicating acetate extract had a good role in inhibiting the growth of mold. The circle of corresponding mould strains to acetate extract of pomegranate peel polyphenols had a diameter of 12.0mm, aqueous extract of 16.0mm. It basically did not show a significant antibacterial activity. This is somewhat different from the results of previous studies. Previous research results indicated that the inhibitory effect of pomegranate peel polyphenols extract on the growth of mold was very weak. However, 5% acetic acid extract of pomegranate peel polyphenols showed distinct features. It is a notable research point to further study biological component of acetate extract of pomegranate peel polyphenols. Inhibiting ability of pomegranate peel polyphenols extract to yeast and E.coli was evaluated by inhibition zone method. The larger bacteriostatic circle, the better antibacterial effect was. By the data in TABLE 2, the circle of corresponding yeast strains to acetic acid extract of pomegranate peel polyphenols had a diameter of 15.0mm, and diameter of E. coli bacteriostatic circle was 25.7mm. The circle of corresponding yeast strains to alcohol extract had a diameter of 10.7mm, and diameter of E. coli bacteriostatic circle was 20.3mm. The circle of corresponding yeast strains to aqueous extract had a diameter of 10.0mm, and diameter of E. coli bacteriostatic circle was 18.3mm. Analysis shows that the three kinds of extracts had a good inhibitory effect on E. coli and yeast, and pomegranate peel polyphenols extract was better than that of acetate ethanol extract which was better than that of water extract.

TABLE 2 : Inhibiting ability of pomegranate peel polyphenols extract to the mold growth

Pomegranate Peel Extracts	Mould bacteria circle size/mm
Aqueous extract	Three bacteria laps, 16.0
50% aqueous ethanol extract	Three bacteria laps, 16.0
5% aqueous acetic acid extract	Bacteria circle is small, 2.0

TABLE 3 : Inhibiting ability of pomegranate peel polyphenols extract to Yeast and E. Coli

Pomegranate Peel Extracts	Average size of the bacteriostatic circle /mm	
	Yeast	E.Coli
Aqueous extract	10.0	18.3
50% aqueous ethanol extract	10.7	20.3
5% aqueous acetic acid extract	15.0	25.7

On the whole, acetate extract of pomegranate peel polyphenols had a significantly inhibitory effect on yeast, E. coli and fungi, which was better than its ethanol extract and water extract. Therefore, 5% of acetic acid aqueous solution was a more ideal extracting agent in terms of antibacterial property.

CONCLUSIONS

FULL PAPER

This work is to investigate whether 5% aqueous acetic acid solution is effective to extract pomegranate peel polyphenols or not by taking water, 50% ethanol and 5% acetic acid solution as extraction agent to extract pomegranate peel polyphenols, and taking total phenolic content, total antioxidant properties, scavenging free radicals, superoxide anion radical scavenging ability and antibacterial ability as evaluation standards. The results showed that pomegranate peel polyphenols extract with 5% acetic acid aqueous solution as extraction agent preparation had a higher total phenol content. Whether antioxidant capacity, free radical scavenging ability, or superoxide anion radical scavenging capacity was more outstanding. It was particularly worth mentioning here that pomegranate peel polyphenols 5% acetic acid extract not only has a good antibacterial effect on E.coli and yeast, but also mold. This was a new finding which did not find in previous research results and deserves further research. As a result, extracting agent with 5% of acetic acid aqueous solution for pomegranate peel polyphenols was more appropriate.

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