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High potential of agro-industrial by-products of pomegranate (*Punica granatum* L.) as the powerful antifungal and antioxidant substances

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ABSTRACT

Microorganisms such as fungi are one of the most important factors that cause oxidative processes during postharvest stage and consequently deterioration of agriculture products would not be unexpected. On the other hand, high antioxidant properties of industrial by-products of pomegranate propose them as powerful antioxidant and antifungal substances. So to investigate the antioxidant and antifungal properties of pomegranate, two independent factorial experiments based on randomized design with 5 replications were conducted. In the first experiment the effect of 3 different parts of pomegranate (peel, seed and leaf) and 2 different kinds of extracts (aqueous and methanolic) with 4 concentrations (0, 500, 1000 and 1500 ppm) were investigated on 3 postharvest fungi (*Penicillium italicum*, *Rhizopus stolonifer* and *Botrytis cinerea*). In the second experiment antioxidant capacity and phenolic content were measured for two different extracts from different parts. Based on the results the methanolic extract showed the highest inhibitory effects on the mycelia growth (IMG) and spore germination (ISG) with 49.82 and 41.25% respectively. On the other hand, peel and seed extracts had more inhibitory effect (IMG and ISG) than leaf extract. The phenolic content of peel extract were also measured 2.8 fold higher than pomegranate leaf extract and antioxidant capacity of peel, seed and leaf extracts of pomegranate were 55.3%, 35.7% and 16.4% respectively. Therefore, it seems that the high percentage of phenolic content in the peel and seed of pomegranate could cause the high antifungal and antioxidant activity of their extracts.

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1. Introduction

Pomegranate (*Punica granatum* L.) is a deciduous shrub that is native to Iran (Sarkhosh et al., 2007). By producing around 670,000 tons annually, Iran is the largest producer of this edible fruit (Anonymous, 2005). Because of the high antimicrobial activity against many pathogens, pomegranate has been widely used for the treatment of different types of human disease.

On the other hand, during the industrial processing of pomegranate, large volumes of industrial wastes are produced, which have a wide range of nutritional values. Therefore, in the recent years, the attention has been focused on the industrial by-products of pomegranate that have a high potential of antioxidant and antifungal properties (Orzuaa et al., 2009). Furthermore, the presence of abundant effective compounds has been reported in many parts of this plant such as leaves, barks, roots, peels, juice and seeds that cause high antimicrobial and antioxidant activity of them (Seeram et al., 2006).

Antioxidants have an important role in body's health. Recently, a series of studies had demonstrated that there is a positive correlation between prevention of many cancers and the amounts of absorption and consumption of vegetables and fruits that contain natural antioxidants (Casanova et al., 2008).

Ellagic acid is one of the most important compounds in peel of pomegranate. The phenolic structure of this compound causes its drastic antioxidant activity. Although pomegranate is used as an edible fruit, in recent years, medicinal properties of this fruit have been noticed by many investigators (Sarkhosh et al., 2007). Phenolic compounds especially punicalagin extracted from the peels of pomegranate have antimicrobial activity against *Candida albicans* (Burapadaja and Bunchoo, 1995). According to the type of tested microorganism, the reports about antifungal properties of the pomegranate peel extract are various. For example, this extract can prevent the growth of *Penicillum citrinum* and *Aspergillus ochraceus* for 8 and 3 days respectively. In some cases in China the peel extract of pomegranate is used as a fungicide (Seeram et al., 2006).

Nowadays, many people in Asia and Africa who are under the poverty line have to tolerate the malefic effects of fungi (Majumder et al., 1997). On the other hand, for controlling fungi, the most popular way is using synthetic chemicals and fungicides. But their

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high accumulation capacity in bodies of consumers, long durability period and many environmental disadvantages of them lead to hesitation for using of them (Barnard et al., 1997). Chang et al. (2008) mentioned that application of synthetic chemicals leads to creation of resistant strains. So during two recent decades, applications of some natural compounds such as essential oils and extracts for biological control of pathogens have been noticed.

Despite several previous studies on the antimicrobial properties of pomegranate against many human's disease, to date there is no any research about its antifungal activity against postharvest fungi, while it has been determined that the huge amounts of deterioration of agricultural products occur during storage because of oxidative processes and microorganisms attack (Casanova et al., 2008). So, the aim of the present study is to investigate the antifungal properties of aqueous and methanolic extracts of industrial wastes of pomegranate and to determine the phenolic content and antioxidant activity of this extracts for introducing them as a safe products.

2. Material and methods

2.1. Preparation of plant materials and fungi isolates

Pomegranate cultivar "shishe kab" that is cultured in Iran as a commercial cultivar, was selected for this investigation. Fresh peels, leaves and seeds of this cultivar were collected from Ferdows, Khorasan, Iran. The parts were dried in the shade and afterward powdered. Three common plant pathogenic fungi (*Penicillium italicum*, *Botrytis cinerea* and *Rhizopus stolonifer*) were selected from Center of Pomegranate Research of Ferdowsi University of Mashhad, Iran in 2009.

2.2. Preparation of the extracts

In this research aqueous and methanolic extracts were used. Powdered plant materials (2 g) of peel, seed and leaves of pomegranate were macerated with 20 ml solvent (distilled water or methanol) for 24 h at room temperature. The macerated was first filtered through double layered muslin cloth and then spun at 3000 rpm for 15 min. The extracts were concentrated by using Rotary Evaporator at 40 °C and afterward dried in oven at 50 °C for 48 h. Finally, by using these dried extracts, the different concentrations of aqueous and methanolic extracts (500, 1000, 1500 ppm) were prepared (Fernandes et al., 1997).

2.3. Antifungal activity against the mycelia growth

After sterilization the PDA (potato dextrose agar) medium, it was cooled in a water bath to 40 °C. The aqueous and methanolic extracts were mixed with sterile molten PDA for obtaining the final concentrations (0, 500, 1000 and 1500 ppm). 15 ml of each medium (contains different concentrations of extracts) was poured into 90 mm Petri plates and then were inoculated with 5 mm plugs from 7-day-old cultures. Five replicates were used per treatment. Plates were incubated at 28 ± 1 °C in 12 h light/12 h dark cycle. Growth inhibition of treatment against control was calculated by using the following formula:

$$\text{Inhibitory of mycelia growth}\,(\%) = \left(\frac{a-b}{a}\right) \times 100$$

where a and b represented the average increase in mycelium growth of control and average increase in mycelium growth of treatment, respectively (Satish et al., 2007).

2.4. Antifungal activity against the spore germination

Spore suspension of plant pathogens were obtained from their respective 7-day-old cultures and mixed with sterile distilled water to obtain the homogenous spore suspension of 2×10^6 spore/ml. 500 μl of spore suspension of each fungi was added to Petri plates that contained different concentrations of extracts (0, 500, 1000 and 1500 ppm) and spread with sterile lob. After 20 h, spore germination was counted around a limited area (2 cm diameter) under the microscope (Bajpai et al., 2007).

$$\text{Inhibitory of spore germination}\,(\%) = \left(\frac{a-b}{a}\right) \times 100$$

where a and b represented the average of spore germination in control and treatments, respectively.

2.5. Total phenols and antioxidant activity evaluation

Determination of the total phenols was done by applying the Folin–ciocalteu regent using a UV–vis spectrophotometer at 660 nm (Singleton and Rossi, 1965).Gallic acid used as standard absorbance read. Results were expressed as milligram (mg) gallic acid equivalents per gram dry weight (Mousavinejad et al., 2009). DPPH method was used to measure the antioxidant activity of pomegranate extracts based on the evaluation of the free radical scavenging capacities of the extracts (Gil and Tomas, 2000). In this method 0.1 ml of methanolic extract of each plant sample was combined with DPPH (500 μmol in methanol). The mixture was strongly shaken and after 30 min the change in absorbance at 517 nm was measured. Finally, calculation of antioxidant activity was done by using the following formula:

$$\text{Antioxidant activity} = \left(1 - \frac{A_{\text{sample}}(517\,\text{nm})}{A_{\text{control}}(517\,\text{nm})}\right) \times 100$$

Antioxidant activity of extracts was compared with the activity of butylated hydroxyanisole (BHA) and L-ascorbic acid.

2.6. Experimental design and statistical analysis

In this study the effect of aqueous and methanolic extracts of 3 different parts of pomegranate (peel, seed and leaf) and with 4 concentrations (0, 500, 1000 and 1500 ppm) were investigated on 3 postharvest fungi (*P. italicum*, *R. stolonifer* and *B. cinerea*) in one factorial experiment and the antioxidant activity and phenolic content of these two extract methods from different parts of pomegranate (one concentration) were evaluated in another experiment. Both experiments conducted in five replications. The analysis of the data was conducted by Statistical Analysis System (SAS) software Version 9.1 and Mean values and standard error (SE) were calculated for all tests.

3. Results

Results showed that the effect of plant material type, extraction method, type of fungi and extract concentration on the inhibitory of mycelia growth (IMG) and inhibitory of spore germination (ISG) were significant (Table 1). Leaf extract of pomegranate had the least effect on ISG and there were no significant differences between seed and peel extract. Among the fungi, *P. italicum* has the least ISG (17.86%) compared with *R. stolonifer* (30.29%) and *B. cinerea* (30.92%) (Table 1). As shown in Fig. 1, the lowest amount of ISG was observed in the treatment of leaf extract for *P. italicum*.

The effect of methanolic extract on IMG was 90.8% higher than the aqueous extract (Table 1). In addition, there was no significant difference between *R. stolonifer* and *B. cinerea*. The percentage of

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