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Sarojini A
Department of Fish Processing Technology, College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, Mangaluru, Karnataka, India

Gajendra
Department of Fish Processing Technology, College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, Mangaluru, Karnataka, India

Amitha
Department of Fish Processing Technology, College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, Mangaluru, Karnataka, India

Raju CV
Department of Fish Processing Technology, College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, Mangaluru, Karnataka, India

Corresponding Author:
Sarojini A
Department of Fish Processing Technology, College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, Mangaluru, Karnataka, India

Antibacterial and antioxidant properties of pomegranate (*Punica granatum*) peel extract

Sarojini A, Gajendra, Amitha and Raju CV

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Abstract
This study was carried out to investigate the potential use of pomegranate (*Punica granatum*) peel extract (PPE) as an effective antioxidant and antibacterial agent for food products. Total phenolic and flavonoid content of pomegranate peel extract was estimated. DPPH radical scavenging activity, metal chelating activity, ferric reducing power and antibacterial activity of pomegranate peel extract was compared with the synthetic antioxidant butylated hydroxyanisole (BHA). The result showed that, the total phenolic content present in the PPE was recorded in terms of Gallic acid equivalent (mg/g) and the value was 115.21 ± 1.32 mg GAE/g. Total flavonoid content of the sample was expressed as mg/g of quercetin equivalents. Total flavonoid content of the PPE was recorded as 18.11 ± 1.74 mg quercetin/g. The antioxidant activity of PPE was increased while the concentration of the PPE increased. Antibacterial activity was determined for PPE against five bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi* and *Pseudomonas fluroscens*) and *Staphylococcus aureus* had the higher zone of inhibition which was 40 ± 0.24 mm for the concentration of 10,000 ppm.

Keywords: Pomegranate peels extract, natural antioxidant, antibacterial activity, DPPH activity

Introduction
The oxidative decomposition of fats and oils in food products is the reason for off flavors and rancidity and it leads to poor nutritional quality and safety because of the formation of some potentially toxic compounds [1, 2, 3]. The increasing rate of oxidation can be retarded by the addition of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ). But, some of the commercial antioxidants, such as butylated hydroxytoluene (BHT), can promote DNA damage by binding to nucleic acids, so that, exert mutagenic, cancerous and cytotoxic effects [4].
Researchers found a perceptible progress in replacing these synthetic antioxidants with a number of plant origin natural antioxidant sources. The use of some synthetic antioxidants are restricted in many countries due to the undesirable effects on human health, leaving natural antioxidants as the only possible way to be utilized by the food industry [5, 6]. Processing waste products (e.g. fruit peels) of agricultural commodities could offer practical and economic sources of active antioxidants which could be an alternative for the synthetic ones [7, 8, 9]. Natural antioxidants can be acquired from agricultural byproducts such as rice bran [10], grape pomace [11], wheat bran [12], potato peel [13], apple peel [14] and pomegranate peel [15]. Phenolic compounds, including flavonoids, anthocyanins and tannins, are the main group of antioxidant phytochemicals with interesting properties and have deep value due to their biological and free radical scavenging activities [16, 17]. Natural antioxidants in food products could have greater application in increasing consumer acceptability and also improve the stability of the products [1, 8, 18, 19].
The pomegranate peel is considered as an agro-waste but it can be a potential source of antioxidants, phenols, flavanoids and also possesses antibacterial and antifungal activity [20]. Pomegranates have the highest concentration of punicalagin among the most commonly consumed fruits. Studies have shown that punicalagin has antioxidant, antifungal and antibacterial properties [21]. Pomegranate peels have been shown to possess higher antioxidant activity than edible portion (arils) owing to an abundance of flavonoids and tannins present [22]. Phytochemicals, derived from various plant sources have been shown to be a good alternative to synthetic chemical substances in preventing growth of several pathogenic bacteria [23]. Braga

et al. [24] observed that pomegranate extracts were able to inhibit not only the growth of *S. aureus* but also the production of enterotoxin. Hany *et al.* [25] found antimicrobial effects against *Bacillus subtilis*, *Staphylococcus* spp. and *Brucella* spp. The primary objective of this study was to investigate the potential use of pomegranate (*Punica granatum*) peel extract (PPE) as an effective antioxidant and antibacterial agent for food products.

Materials and Methods

Materials

Pomegranate (*Punicia granatum*) fruit peels were collected from a juice outlet at Mangalore, Karnataka for the preparation of pomegranate peel extract.

Bacterial cultures namely *Staphylococcus aureus* (NCIM 2079), *Escherichia coli* (NCIM 2688), *Bacillus subtilis* (NCIM 2063), *Salmonella typhi* (NCIM 2501) and *Pseudomonas fluroscens* (NCIM 2099) were procured from National chemical laboratory, Pune.

Methods

Preparation of pomegranate peel extract

The pomegranate peels were brought to the laboratory in ice box and washed thoroughly to remove dirt if any. Peels were then cut into small pieces and dried in oven at 60 °C for 12-48 h. After drying, the peels were ground in the kitchen blender to make the fine power to pass through 1mm sieve. The extraction procedure was carried out according to the methods as described by Iqbal *et al*. [26] with slight modifications. About 25g of pomegranate peel powder and 150 mL of ethanol were mixed well. The mixture was then subjected to shaking for 12h at the speed of 190 rpm in an ambient temperature. The mixture was filtered and residue was re extracted with same solvent. The filtrates of the mixture were placed under a hood in the rotary evaporator to remove the residual ethanol under vacuum at 40°C. The extract was collected and stored at -20°C in a sample container for further analysis.

Determination of total flavonoid and phenolic contents

Total phenolic contents

Total phenolic content was determined by Folin Ciocalteu reagent method described by McDonald *et al.* [27]. About 10 mg of PPE was dissolved in a 25 mL of 10% ethanol. A diluted extract of PPE (0.5mL) was mixed with 5mL of Folin Ciocalteu reagent (1:10 diluted with distilled water) and 4mL of 1M aqueous sodium carbonate. The mixture was allowed to stand for 15 min at room temperature and then the absorbance was measured at 765nm. The estimation of phenolic compounds in the extract was carried out in triplicate. The standard curve was prepared from Gallic acid at the concentration range of 20 to 100 µg/mL. Total phenolic values were expressed in terms of Gallic acid equivalent (mg/g) which is a common reference compound.

Total flavonoid content

Total flavonoid content was determined by aluminum chloride colorimetric method described by Chang *et al*. [28]. About 100 mg of PPE was taken and dissolved in 50 mL of 10% ethanol. From the dissolved sample solution (0.5 mL) was mixed 1.5mL of methanol, 0.1mL of 10% aluminum chloride, 0.1mL of 1M potassium acetate and 2.8mL of distilled water. It remained at room temperature for 30 min after that the absorbance of the reaction mixture was measured at 415nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 10 to 100 µg/mL. The total flavonoid content of the sample was expressed as mg/g of quercetin equivalents.

Antioxidant activity analysis of pomegranate peel extract

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH free radical scavenging activity of PPE was determined by DPPH assay according to the method described by Sanchez-Moreno *et al.* [29] with little modifications. The 1.5 mL of PPE at different concentrations was added with 1.5 mL of freshly prepared 0.1 mM DPPH solution. The control was prepared as the same without any extract. These solutions were thoroughly mixed at high speed by vortex mixer. The tubes were kept in the dark for 30 min at room temperature and then the absorbance was measured at 517 nm. Radical scavenging activity of PPE was compared with the BHA at different concentration. Radical scavenging activity was estimated as the inhibition percentage and was calculated using the following formula,

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \text{ X } 100$$

Metal chelating activity

Determination of metal chelating activity of extracts was done using the method of Dinis *et al.* [30]. The different concentration of extracts (1mL) was added to 2.7 mL distilled water and a solution of 0.1 ml of 2 mM ferrous chloride. This was followed by the addition of 0.2 ml of 5 mM ferrozine solution, which was left to react at room temperature for 10 mins. The absorbance was measured at 562 nm. A blank was run in the same way by using distilled water instead of extract. For the comparison of metal chelating activity of PPE, EDTA was used as reference. The percentage of metal chelating activity was calculated using the formula,

$$\text{Metal chelating activity (\%)} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \text{ X } 100$$

Ferric reducing antioxidant power (FRAP)

The reducing power capacity assessment was determined using the modified method of Yen and Chen [31]. The different concentration of extracts (1 mL) was mixed with 2.5 mL of phosphate buffer (200 mM and pH 6.6) and 2.5 ml of potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 mins. Then 2.5 ml of trichloroacetic acid (10 %) was added and centrifuged for 10 mins at 5000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank. The reducing power increases with the increase of absorbance. The total reducing power ability of PPE at different concentration was compared with BHA as a positive control.

Determination of antibacterial activity of pomegranate peel extracts against food borne pathogens

In this study, five bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi* and *Pseudomonas fluroscens*) and agar well diffusion method [20] were used to determine the antibacterial activity of the pomegranate peel extracts. The concentration of the PPE used was 8000-10000 ppm. Alcohol was used as the positive control. Bacterial strains were first grown on Muller Hinton

agar medium for 18 to 24 h at 37°C. Muller Hinton agar was prepared in an Erlenmeyer flask and sterilized in an autoclave at 121°C for 15 min. Petri dishes with 20 ml of Muller Hinton agar were prepared, and the wells (5.0 mm in diameter) were cut from the agar under sterile conditions and then the plates were inoculated with 0.1 mL of the culture suspension (1 %, containing 10^6–10^7 cfu / mL) by spread plate technique. Then 10 µl of extracts were added into the wells of agar plates directly and same volume of alcohol was used as control. The bacterial cultures were then incubated at 37°C for 22-24 h. At the end of the incubation period, inhibition zones formed on the medium and the diameter of the inhibition zone was measured and recorded as mean diameter (mm). The measurements were done basically from the edge of the zone to the edge of the well. All experiments were carried out in triplicate.

Statistical analysis

Experiments and analyses were conducted in triplicate. The data presented as mean ± standard deviation.

Results and discussion

Total phenolic content of pomegranate peel extract (PPE)

Major phenolic compounds present in the plant kingdom are flavonoids, tannins and phenolic acids said by Rababah *et al.* [32]. These phenolic contents in plants are associated with their antioxidant activities, probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [33]. Pomegranate is a rich source of polyphenols. The polyphenols shows various biological activities, it helps in eliminating free radicals and retard oxidation [34]. The total phenolic content present in the PPE was estimated as 115.21 ± 1.32 mg of Gallic acid equivalents per gram (mg GAE/g). The result was compared with the findings of Saad *et al.* [35] and it was in agreement with their findings (100.4–181.0 mg GAE/g). The value found was less than the value 141.6 ± 3.42 (mg GAE/g) reported by Osman *et al.* [15]. The antioxidant activity of phenolics was mainly due to their redox properties that make them act as hydrogen donors, reducing agents, singlet oxygen quenchers and also may have a metallic chelating potential. In addition, synergism between the antioxidants in the extract makes the antioxidant activity depends on the concentration, structure and the interaction between the antioxidants [36]. The fruit peel contain major amount of phenolic compounds like punicalagins, gallic acids, ellagic acids and anthocyanins [37]. The variation in the phenolic content might be due to extraction procedure followed and condition of the pomegranate peel used because the phenolic content are highly affected by environmental conditions [38].

Total flavonoid content of pomegranate peel extract (PPE)

Flavonoids are known to be highly effective antioxidants by scavenging oxygen radicals. Moreover, the protective effects of flavonoids in biological systems are attributed to their capacity to scavenge free radicals, chelates metal catalysis, activate antioxidant enzymes, reduce alpha tocopherol radicals and inhibit oxidation [39]. Plumb *et al.* [40] and Lansky and Newman [41] reported that, various flavonoids like catechin, epicatechin, epigallocatechin-3-gallate, flavan-3-ol, kaempferol, kaempferolerol-3-O-glucoside, kaempferol-3-O-rhamnoglycoside, luteolin, luteolin 7-O-glucoside, Naringin, pelargonidin, prodelphindin, quercetin and rutin have been found in peel extracts of pomegranate which shows antibacterial, antiviral, antioxidant, anti-inflammatory and antineoplastic bioactivities.

Total flavonoid content of the sample was expressed as mg/g of quercetin equivalents and the total flavonoid content recorded in the present study was 18.11 ± 1.74 mg/g. The result was slightly higher than the findings of Jinnawat *et al.* [42] where the flavonoid content recorded was 16.66 ± 0.47 mg/g. Sweetie *et al.* [23] found that, the flavonoid content of PPE was 7.57 mg/g. Li *et al.* [43] reported that, flavonoids account for only a small part of total phenolics present in the peel extract of pomegranate.

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity of pomegranate peel extract (PPE)

The DPPH free radical scavenging activity has been commonly used to detect antiradical activity of various samples, due to its reactivity to lower concentrations of active compounds from natural sources. DPPH is a purple-coloured stable free radical with an absorption band at 517 nm. It is reduced to 2, 2-diphenyl-1-picrylhydrazine (yellow coloured) by accepting an electron or hydrogen radical from an antioxidant. The stable radical, DPPH, have a maximum absorbance at 517 nm and could readily undergo scavenging by antioxidants. Higher free radical scavenging activities of samples indicated by lower absorbance at 517 nm [3]. Pomegranate peel extract was analyzed for its DPPH free radical scavenging activity at different concentration and the result was given in Fig 1.

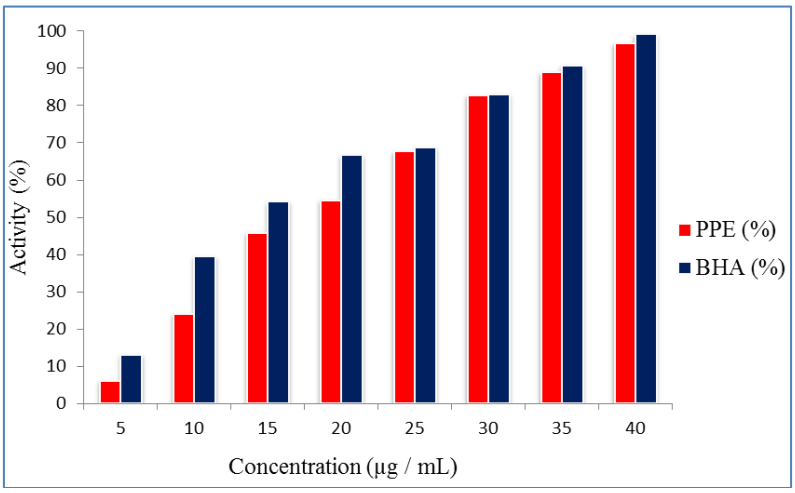

Fig. 1: DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity of pomegranate peel extract (PPE)

In the present study, the DPPH free radical scavenging activity was increased while the concentration of the PPE increased and the same trend was reported by Mutahar *et al.* [3]. The DPPH free radical scavenging activity was 6.14 ± 2.37 % at the concentration of 5 µg/mL and it increased to 54.61 ± 1.83 % at the concentration of 20 µg/mL. The percentage of DPPH free radical scavenging activity reached to 96.76 ± 0.87 % when the concentration of PPE increased to 40 µg/mL. The percentage of DPPH free radical scavenging activity of synthetic antioxidant BHA was 13.05 ± 2.19 % at 5 µg/mL concentrations and then it reached to 99.13 ± 0.12 % at 40 µg/mL concentrations. PPE effectively scavenged DPPH radicals and this was due that PPE possessed proton-donating ability and in association with a number of hydroxyl groups in the phenolic and flavonoid structures to stabilize free radicals [44]. The number of phenolic residues and hydroxyl groups present in the extract substantially affect the DPPH free radical scavenging activity [3].

Metal chelating activity of pomegranate peel extracts (PPE)

Pomegranate peel extract was tested for its metal binding capacity and that was assessed by its ability to compete with ferrozine for binding of ferrous ion and it was compared with EDTA. The result was given in Fig 2. The percentage of metal chelating activity of PPE was initially very low (1.82 ± 0.01 %) at the concentration of 100 µg/mL when compared to EDTA (71.32 ± 0.13 %) at the same concentration. The metal chelating activity increased to 35.53 ± 0.05 % for PPE at the concentration of 1000 µg/mL when the activity was 83.34 ± 0.37 % for EDTA at the same concentration. Polyphenols are chelators of metals and can inhibit Fenton and Haber–Weiss reactions, which generate hydroxyl radicals [23]. Metal chelation contributes important antioxidative effects by restricting metal-catalyzed oxidation reactive oxygen species (singlet oxygen) [45]. Sun *et al.* [46] reported that polyphenol extracts from pulp and peels of fruit revealed some metal chelating capability and they mentioned to the catechol group in B-ring of these polyphenol molecules. The finding was in agreement with Mladinka *et al.* [47]. Gulcin *et al.* [45] outlined that tannins (also referred as tannic acid, due to similar chemical structure) had a marked capacity for iron binding, suggesting that its main action as peroxidation protector may be related to its iron binding capacity.

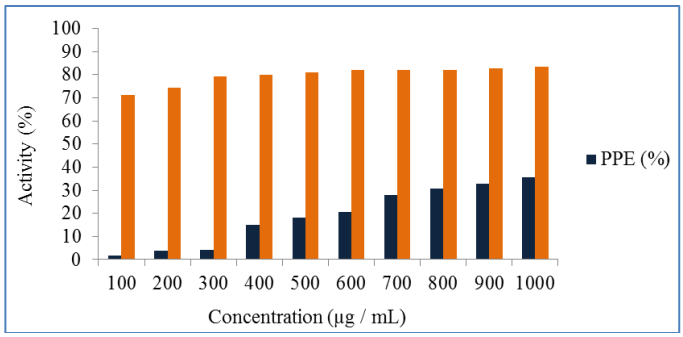

Fig. 2: Metal chelating activity of pomegranate peel extracts (PPE)

Ferric reducing powers (FRP) of pomegranate peel extract (PPE)

The reducing power of a composite is associated to its electron transfer capacity and may serve as a notable indicator of its potential antioxidant activity [48]. Pomegranate peel extract was analyzed for its ferric reducing power and the result was presented in Fig 3. The result showed that the reducing power increased with the increase in the concentration of PPE. In the reducing power assay, the presence of antioxidants in the extracts would result in the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. Increasing absorbance at 700 nm indicates an increase in reductive ability. In this present study, the reducing power of pomegranate peel extracts also increased with an increase in their concentration. The reducing power indicated presence of reductones which have the ability to break free radical chains by donating hydrogen atoms and thus converting them to a more stable non-reactive species [3]. Negi *et al.* [1] also found a significant reducing power of pomegranate peel extract.

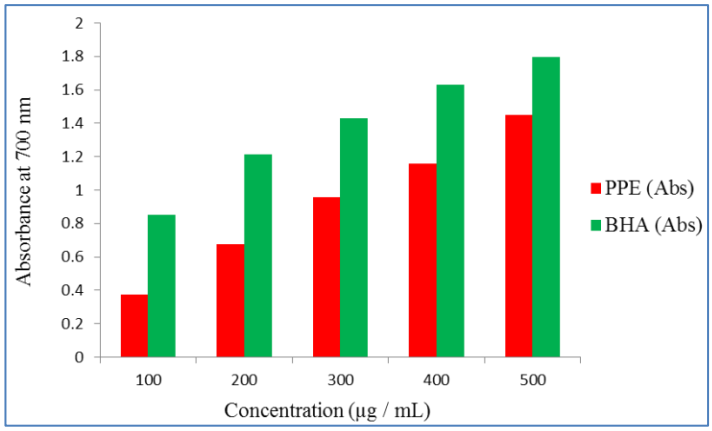

Fig 3: Ferric reducing power (FRP) of pomegranate peel extract (PPE)

Antibacterial activity of pomegranate peel extract (PPE)

The phenolic acids and tannin – rich ellagitannins of *Punica granatum* have antibacterial activity [49]. Determination of

antibacterial activity of pomegranate peel extract (PPE) was done for five bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi* and *Pseudomonas fluroscens*) and the result was presented in Table 1. *Staphylococcus aureus* shown the highest zone of inhibition when compared to all the five bacterial strains tested. The antibacterial activity of peels of *Punica granatum* may be an indicative of the presence of metabolic toxins or broad spectrum antimicrobial compounds that act against both gram positive and gram negative bacteria [20]. The maximum antibacterial activity of extracts against different bacterial strains was found in the order *S. aureus* > *B. subtilis* > *S. typhi* > *E. coli*. Melendez and Capriles [50] determined the antibacterial properties of a number of tropical plants using the disc diffusion method against *E. coli* and *S. aureus*. They found that pomegranate extract produced inhibition zone sizes of 11 and 20 mm, for *E. coli* and *S. aureus* respectively. Negi and Jayaprakasha [1] reported that PPE was less effective against *E. coli*. Research showed low concentration of *P. granatum* extract led to delay in *S. aureus* growth, while in a higher concentration of *P. granatum* extract, growth of *S. aureus* was eliminated [24].

Table 1: Antibacterial activity of pomegranate peel extract (PPE)

Species	Zone of inhibition
B. subtilis	26±0.31mm @ 10,000 ppm
S. aureus	40±0.24mm @ 10,000 ppm
S. typhi	16±0.45mm @ 10,000 ppm
E. coli	12±0.21mm @ 10,000 ppm

Conclusion

Lipid oxidation is one of the major problems in fish food products. In this study, pomegranate peel extract (PPE) at the higher concentration showed the potential antioxidant effect equal to the synthetic antioxidant BHA and showed good antibacterial activity. This study proved that the pomegranate peel extract (PPE) which obtained from the pomegranate juice fabric as a byproduct can be used as an effective natural antioxidant in food products.

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