ANTIGLYCATION AND ANTIOXIDANT ACTIVITY OF POLYSACCHARIDES ISOLATED FROM FRUIT EXTRACT OF POMEGRANATE *(PUNICA GRANATUM)*

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Summary

Pomegranate *(Punica granatum)* has been used for centuries to confer health benefits in a number of inflammatory diseases. In present study, antioxidant, metal chelating and ferric ion reducing and antiglycation property was checked for the polysaccharide isolated from pomegranate fruit juice. At 20 µg/mL sugar concentration, it gave 87 % and 85 % inhibition in hydroxyl radical scavenging assay and Antiglycation assay respectively. It also had metal ion chelating and ferric ion reducing activity. It also inhibited fructosamine formation by 72% after 3 days of incubation. The above studies suggested that the inhibition of glycation exhibited by extract was not only due to its free radical scavenging property but also due to the modification in the amino or carbonyl groups in the Millard reaction, which resulted in the inhibition of fructosamine formation. On the SDS-PAGE profile, a band of glycated Bovine serum albumin (BSA) with a relative molecular weight larger than that of original BSA was observed. These results suggest that cross-linking between proteins or binding of carbohydrate to protein occurred during advanced glycation end products formation. So the sugars present in the extract may be donating the hydrogen atom to the free radical, and exhibiting the antioxidant activity. So polysaccharide isolated from pomegranate fruit juice can be used in preventing many diseases where free radical plays a vital role. As it has antiglycating activity, it can be used in delaying or preventing complications of diabetes and aging.

Keywords: Pomegranate, Antioxidant, Antiglycation, Advanced glycation end products, Diabetes and Free radicals

Abbreviations: BSA, Bovine serum albumin; kDa, Kilodalton; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamade gel electrophoresis; mL, milliliter; µM, micromolar; ROS, Reactive oxygen species; AGE, Advanced glycation end products; PFE, Pomegranate fruit extract;

Introduction

Reactive oxygen species (ROS) leads to many etiological and pathophysiological human diseases such as neurodegenerative disorders (e.g. Alzheimer disease, Parkinson disease, multiple sclerosis, Down's syndrome), Diabetes, Cardio vascular Diseases, Inflammation, Viral infections, Autoimmune pathologies, and Digestive system disorders such as Gastrointestinal inflammation and ulcer[1,2]. Free radicals are generated both by exogenous and endogenous sources. In Aerobic organisms, free-radicals are generated as a part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, by bacterial leucocytes or through xanthine oxidase activity. Exogenous sources are by atmospheric pollutants, radiations, transitional metal catalysts, drugs and cigarette smoke. Oxygen free radicals i.e. ROS can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and plays a role in the long-term complication of immune-related diseases[3,4].

Glycation is a non-enzymatic reaction between free amino groups of proteins and reducing sugars. This reaction is known as Maillard reaction. Glycation is closely associated with the pathogenesis of age- and diabetes-related complications like neuropathy, angiopathy and nephropathy[5]. This process represents a common posttranslational modification of proteins, which can impair their functions in living organisms. If the oxidative step is involved in glycation process, it is called as glycoxidation[3]. Free radicals, products of the autooxidation of the glycating sugar, and a heterogeneous group of substances called advanced glycation end products (AGEs) are formed in the course of glycoxidation[6, 7]. AGEs exert their actions by two different mechanisms, either by modifying structural intra- and extracellular proteins or by binding to their receptors that belong to the immunoglobulin family and are located in the plasma membranes of monocytes, macrophages, endothelial cells, and vascular smooth muscle cells. When AGEs bind to their receptors they initiate second messenger cascades. They also generate reactive oxygen species which modulate cellular function and can induce inflammatory processes[8].

Therefore, search for antiglycative and antioxidant agents from various sources is gaining lot of importance. There are several reports, which mentions about the identification of antiglycative and antioxidant agents from plant species. Chlorogenic acid, caffeic acid are the main substances responsible for the anti-glycation effect of maté tea (*Ilex paraguariensis*)[9]. Studies on guava leaf extracts show that they are potent antiglycation agents, which can be of great value in the preventive glycation-associated complications in diabetes[10]. There are several reports on plant polysaccharides being studied for antiglycation activity. Studies on polysaccharides of Longan fruit pericarp showed 60.4% antiglycation activity[11]. Water-soluble feruloyl oligosaccharides (FOs) from wheat bran, the ferulic acid esters of oligosaccharides, have been reported as natural antioxidants. Their inhibition of protein glycation in a bovine serum albumin (BSA)/glucose system is studied using fluorescence spectroscopy and SDS–PAGE[12]. An aqueous extract of polysaccharides from *Opuntia monacantha* cladodes suggested that it had good potential for inhibiting the formation of advanced glycation end products. Time- and dose-dependent effects were also observed for all *Opuntia* samples[13].

Pomegranate ***(****Punica granatum,* Lythraceae***)*** has been used for centuries to confer health benefits in a number of inflammatory diseases. More recently standardized extracts of pomegranate fruit extract (PFE) have been shown to possess anti-inflammatory and cartilage sparing effects *in vitro*[14]. Published studies have shown that constituents of PFE inhibit the proliferation of human cancer cells and also modulate inflammatory subcellular signaling pathways and apoptosis when directly added to the culture medium[15]. Several groups have reported that consumption of pomegranate may have cholesterol lowering and cardiovascular and other chronic diseases preventing effects *in vivo*[16]. Pomegranate seeds are rich in sugars, polyunsaturated (n-3) fatty acids, vitamins, polysaccharides, polyphenols, and minerals and have high antioxidant activity. Studies have also shown that the antioxidant capacity of pomegranate juice is three times that of the popular antioxidant-containing beverages such as red wine and green tea, presumably due to the presence of hydrolyzable tannins in the rind, along with anthocyanins and ellagic acid derivatives[17].

In this regard, there are no reports on the antiglycation and antioxidant properties of polysaccharide fractions from pomegranate fruit juice. Therefore, the present investigation is focused on polysaccharide fractions with antiglycative and antioxidant properties.

Materials And Method

Ethanol, CBB-G_{250}, phenol, sulphuric acid, folin-ciocalteau reagent, sodium azide, nitro blue tetrazolium, sodium dodecyl sulphate, ferrous sulphate, TEMED and *L*-ascorbate, were purchased from Sisco Research Laboratory (SRL), India. All other chemicals and reagents used were of analytical grade and were purchased from SRL and Himedia, India.

Preparation of Pomegranate fruit extract

The pomegranate fruits were purchased from local market of Mysore, Karnataka. Fruit juice of pomegranate was prepared by squeezing and filtered to remove the fruit debris. The extract was kept at 4^0C overnight to precipitate sugars in the extract. Extract was centrifuged at 10,000 rpm at 4^0C for 20 min, and then the pellet was redissolved in minimum amount of distilled water, filtered through 0.22 micron filter and stored at -20^0 C till further use. The extract obtained was called as Pomegranate fruit extract (PFE). Protein[18] and Sugar[19] estimation was done for the Pomegranate fruit extracts.

Hydroxyl radical scavenging activity by *2*-Deoxy D-ribose assay

2-Deoxy D-ribose assay is done to determine the hydroxyl radical scavenging activity of pomegranate fruit juice extract and polysaccharide fraction in an aqueous medium[20]. The reaction mixture containing $FeCl_3$ (100µM), ascorbate (100µM), EDTA (104µM), H_2O_2 (1mM), 2-deoxy-D-ribose (2.8mM) were mixed with 20µg of extract in 20mM potassium phosphate buffer, pH 7.4 and incubated for 1hr at 37^0C. A similar assay was done with other known antioxidants such as ascorbic acid (Vit-C) at 400µM concentration serving as positive controls. The reaction mixture was heated at 95^0C in boiling water bath for 15min following the addition of 1mL of TBA (0.5%). Finally the reaction mixture was cooled in ice and optical density was measured at 535nm. The assay was carried out with appropriate blanks and controls. Antioxidant activity was expressed as percent inhibition of hydroxyl radical formation.

Ferric ion reducing power of PFE[21]

100µl of 4mM potassium ferricyanide solution was mixed with 200µL of 20mM phosphate buffer pH 6.5 in the presence or absence of pomegranate fruit juice extract and polysaccharide fraction. A similar assay was done with Ascorbic acid at 40µM concentration. The contents were incubated at 50^0C for 20min. 200µL of 10% TCA was added to the reaction mixture and centrifuged at 5000rpm for 10min at room temperature. The resulting supernatant was taken and mixed with 100µL of 2mM ferric chloride solution and final volume was made up to 1mL with distilled water and then incubated at 37^0C for 10min. The absorbance was recorded at 700nm. Absorbance increases with increase in reducing power.

Ferrous ion chelating ability[22]

Ferrous ion chelating activity was measured for the fruit extract. The reaction solution contained ferrous chloride (200µM) and potassium ferricyanide (400µM) with or without out pomegranate fruit juice extract and polysaccharide fraction. A similar assay was done with Ethylene Diamine Tetra Acetic acid (EDTA) at 40µM concentration. The components in the reaction mixture were added in final volume of 1 mL distilled water and mixed. The reaction mixture was incubated at 20^0C for 10min. Formation of the potassium hexacyanoferrate complex was measured at 700nm. The assay was carried out at 20^0C to prevent Fe^{2+} oxidation. Lower absorbance indicated higher iron chelating capacity.

In vitro non enzymatic glycation of bovine serum albumin[23]

Bovine serum albumin (BSA, 20 mg/mL) was incubated in glucose (500 mM) and sodium azide (0.02%) with or without $CuSO_4$ (100µM) in 0.2 M phosphate buffer (pH 7.4). The test compound was added to the reaction mixture, and the reaction mixture was incubated for 3 days at 37^0C. After incubating, the fluorescent reaction products were assayed in a fluorescence spectrophotometer with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Results were expressed as percentage inhibition of formation of the glycated protein.

Spectrophotmetric analysis of fructosamine[24]

The procedure of fructosamine assay followed the method of Baker, et al 1994 with slight modifications. The reaction mixture which contained 0.2 mL glycated material and 0.8 mL nitro blue tetrozolium (NBT) reagent (300µM) in sodium carbonate buffer (100 mM, pH 10.35) was incubated at ambient temperature for 15 min, and the absorbance was read at 530 nm against a blank.

SDS-PAGE

Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of antiglycative protein was carried out in slab gels according to the method of LaemmLi[25]. BSA, glycated material with or without pomegranate fruit juice extract and polysaccharide fraction and molecular weight marker mix (15µg) were loaded onto the gel and the electrophoresis was carried out at 80V for 5% stacking gel and 40V for 12.5% resolving gel. After electrophoresis the gel was stained with 0.05% (w/v) Coomassie blue R_{250} dye in acetic acid:methanol:water (60:30:10, v/v) for 8 hrs. The gel was destained repeatedly in the same solution without the dye. The gel was stored in 7% acetic acid.

Statistical analysis

Statistical analysis was done using students *t*-test. All the values represent mean of triplicates and are expressed as Mean±SD. $p<0.05$ was considered as significant

Results and Discussion

Isolation of Pomegranate fruit extracts (PFE)

The pomegranate fruit juice was rich in of carbohydrates with negligible amount of proteins in it.

Evaluation of hydroxyl radical scavenging potential of PFE

PFE showed an inhibition of formation of hydroxyl radicals by 87% at 20 µg/mL concentration. Ascorbic acid (Vit C) at 400µM was able to inhibit hydroxyl radical by 89%. Test for antioxidant ability, PFE is a potential free radical scavenger than vitamin C **(Table 1)**. The antioxidant property of PFE could be due to the supply of hydrogen by PFE, which combined with radicals and thus forming a stable radical to terminate the radical chain reaction by acting as a chain break antioxidant. The other possibility was that PFE could combine with the radical ions that were necessary for the radical chain reaction; then the reaction was terminated. The exact mechanism of action, however, is still unknown.

Measurement of reducing power and chelation property

Because of the effectiveness of PFE on hydroxyl radical scavenging, it was further tested to find out its efficacy for reducing activity and chelation properties. PFE (25 µg/mL) showed 84% reducing power in comparison to ascorbic acid (88%) at 40µM concentration. Earlier authors have mentioned a direct correlation between antioxidant activities and reducing power of certain plant extracts[26]. The results obtained in the present investigation showed that the reducing power of PFE was likely to have contributed towards observed antioxidant effect. The ferrous ion–chelating effect was studied showed PFE (25 µg/mL) showed 88% reducing power in comparison to EDTA (40µM - 92%).

Evaluation of anti-glycation activity

The inhibition study for the production of AGEs was carried out for PFE. Pomegranate fruit juice and PFE were able to inhibit the production AGEs by 85% in comparison to Vit C (87%) at much lower dose **(Table 1)**. PFE acted as a glycation inhibitor because of its free radical scavenging property. The effectiveness of PFE in inhibiting hydroxyl radical formation and AGEs formation can speak about its potential uses for diabetic patients.

Table –1 Hydroxyl radical scavenging and Anti-glycation activity of polysaccharide isolated from of Pomegranate fruit extract

Treatment	Concentration	% Inhibition of hydroxyl radical formation	% Inhibition of protein glycation
PFE	20(µg/mL)	87 ± 3.10	85 ± 4.88
Asc	400 (µM)	89 ± 3.72	87 ± 4.56

Hydroxy radical scavenging activity and anti-glycation activity was done keeping positive and negative controls. Results are expressed as means ± SD of triplicates from three independent experiments. **PFE** = Pomegranate fruite extract, **Asc** = Ascorbic acid (Vitamin C)

The effect of PFE on the formation of fructosamine was studied by monitoring the reduction of NBT from the 1st day of incubation to the 3rd day. PFE inhibited fructosamine formation by 72% after 3 days of incubation. The possible explanation for the less formation of fructosamine in PFE treated sample was that PFE might have the ability to modify the amino or carbonyl groups in the Millard reaction that resulted in the inhibition of fructosamine formation.

The above two studies suggested that the inhibition of glycation exhibited by PFE was not only due to its free radical scavenging property but also due to the modification in the amino or carbonyl groups in the Millard reaction, which resulted in the inhibition of fructosamine formation.

On the SDS-PAGE profile, a band of glycated BSA with a relative molecular weight (Mw = 75 kDa) larger than that of original BSA was observed **(Figure 1)**. These results suggest that cross-linking between proteins or binding of carbohydrate to protein occurred during AGEs formation.

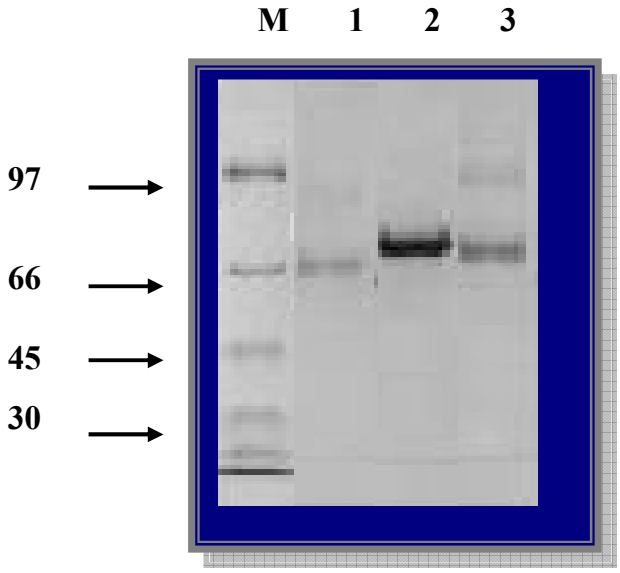

Figure –1 12.5 % SDS – PAGE profile of Glycated protein.

Lane M Molecular weight marker
Lane 1 BSA *(20 mg/mL)*
Lane 2 Glycated material *(BSA, 20 mg/mL) was incubated in glucose (500 mM) and sodium azide (0.02%) with or without $CuSO_4$ (100µM) in 0.2 M phosphate buffer (pH 7.4)*
Lane 3 Glycated material + SAMPLE *(As in 2 + with PFE – 35mg/mL)*

Conclusion

The results of the present work indicated that polysaccharides isolated from Pomegranate fruit juice possessed marked antioxidant, reducing power, metal ion chelation and anti-glycation properties. As it has antiglycating activity, it can be used in delaying or preventing complications of diabetes and aging. However, the *in vivo* antioxidant activity and the mechanism of action need to be further studied.

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References

1. Repetto M, Llesuy S. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. Braz J Med Biol Res 2002; 35:523-534.
2. Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in food plants. Mutation Res 2003; 524:9-20.
3. Sabu M, Kuttan R. Antidiabetic activity of medicinal plants and its relationship with their antioxidant property. J Ethnopharmacol 2002; 81:155-160.
4. Boynes JW. Role of oxidative stress in the development of complication in diabetes. Diabetes 1991; 40:405-411.
5. Monnier VM,. Sell DR, Nagaraj RH, et al. Maillard reaction-mediated mole- cular damage to extracelluar matrix and other tissue proteins in diabetes, aging and uremia. Diabetes 1992; 41:36–41.
6. Hunt JV, Dean RT, Wolff SP. Hydroxyl radical production and autoxidative glycosylation, Biochem. J. 1988; 256:205– 212.
7. Ceriello A. Oxidative stress and diabetes-associated complications. Endocr Pract. 2006;12:60–62.
8. Luciano Viviani G, Puddu A, Sacchi G, et al. Glycated fetal calf serum affects the viability of an insulin-secreting cell line in vitro. Metabolism 2008; 57:163–169.
9. Deborah H. Markowicz Bastos, John Schulze, Marina F, Ferreira Souza, Gugliucci A. Caffeic and chlorogenic acids in Ilex paraguariensis extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. Fitoterapia 2009; 80:339-344
10. Ju-Wen Wu, Chiu-Lan Hsieh, Hsiao-Yun Wang, Hui-Yin Chen. Inhibitory effects of guava (*Psidium guajava L*.) leaf extracts and its active compounds on the glycation process of protein Food Chem, 2009; 113:78-84.
11. Bao Yang, Mouming Zhao, Yueming Jiang. Anti-glycated activity of polysaccharides of longan (*Dimocarpus longan Lour*.) fruit pericarp treated by ultrasonic wave Food Chem., 2009; 114:629-633.
12. Jing Wang, Baoguo Sun, Yanping Cao, Yuan Tian. Protein glycation inhibitory activity of wheat bran feruloyl oligosaccharides. Food Chem 2009; 112:350-353

13. Mouming Zhao, Ning Yang, Bao Yang, Yueming Jiang, Guihe Zhang. Structural characterization of water-soluble polysaccharides from Opuntia monacantha cladodes in relation to their anti-glycated activities. Food Chem 2007; 105:1480-1486
14. Ahmed S, Wang N, Hafeez BB, Cheruvu VK, Haqqi TM. Punica granatum L. extract inhibits IL-1beta-induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF-kappaB in human chondrocytes in vitro. J Nutr 2005; 135:2096–2102.
15. Seeram NP, Adams LS, Henning SM, et.al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 2005; 16:360–367.
16. Esmaillzadeh A, Tahbaz F, Gaieni I, Alavi-Majd H, Azadbakht L. Cholesterol-lowering effect of concentrated pomegranate juice consumption in type II diabetic patients with hyperlipidemia. Int J Vitam Nutr Res 2006; 76:147–151.
17. Gil MI, Tomas-Barberan FA, Hess-Pierce B, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem. 2000; 48:4581–4589.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. Biol Chem 1951; 193:265-273.
19. Dubois M, Giles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determin ation of sugars and related substances. Anal. Chem 1956; 28:350 356.
20. Halliwell B, Gutteridge JMC, A sample test tube assay for determination of rate constant for reaction of hydroxyl radical. Anal Biochem 1982; 126:131-138.
21. Shih Peng Wong, Lai Peng Leong, Jen Hoe William Koh, Antioxidant activities of aqueous extracts of selected plants. Food Chemistry 2006; 99:775-783
22. Ningappa M, Srinivas L, Purification and characterization of ~35kDa antioxidant protein from curry leaves (*Murraya koenigii L.)* Toxicology in Vitro, 2008; 22:699-709.
23. Yam Faguchi F, Ariga T, Yoshimura Y, Nakazawa H. Antioxidative and anti-glycation activity of garcinol from Garcinia indica fruit rind. J. Agric. Food Chem., 2000; 48:180–185.
24. Baker JR., Zyzak DV, Thorpe SR, Baynes JW. Chemistry of the fructosamine assay: D-glucosone is the product of oxidation of Amadori compounds during the fructosamine assay. Clinical Chemistry 1994; 40:1950–1955.
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 1970; 227:680-685.
26. Pin-Der-Duh X, Pin-Chan-Du X, Gow-Chin-Yen X, Action of methanolic extract of mung hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. Food Chem. Toxicol 1999; 37:1055–1061.