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RESEARCH ARTICLE

INHIBITORY EFFECTS OF AQUEOUS PERICARP EXTRACT OF *PUNICA GRANATUM* ON *IN VITRO* PROTEIN GLYCOXIDATION AND ADVANCED GLYCATION END PRODUCTS

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ABSTRACT

Objectives: In this study, we evaluated the antioxidant potential of aqueous pericarp extract of *Punica granatum* (*APPG*) using different model systems.

Methods: To establish the link between glycation and oxidation processes, we evaluated the extract for its *in vitro* radical scavenging against superoxide, hydroxyl, hydrogen peroxide, nitric oxide, ferric radical and $ABTS^{+}$ radical and antiglycation activities like the inhibitory activities on bovine serum albumin (BSA) and as well as protein oxidation markers including protein carbonyl formation (PCO).

Results: The results indicated that the APPG possesses the highest antioxidant activity against superoxide, hydroxyl, hydrogen peroxide, nitric oxide, ferric radical and $ABTS^{+}$ radical. *APPG* extract at different concentrations (10-250 µg/ml) has significantly quenched the fluorescence intensity of glycated BSA and the glycoxidation measured in terms of advanced glycation end products (AGEs). Furthermore, we demonstrated that the inhibitory effect of *APPG* extract in preventing oxidative protein damages including effect on PCO formation, which are believed to form under the glycoxidation processes.

Conclusion: These results clearly demonstrate that the *APPG*, owning to its antioxidant content, is capable of suppressing the formation of AGEs and protein oxidation *in vitro.*

INTRODUCTION

Free radicals are molecules containing one or more unpaired electrons in atomic or molecular orbitals. There is increasing evidence that abnormal production of free radicals *in vivo* increases oxidative stress on cellular structures and leads to oxidize nucleic acids, proteins, lipids or DNA [1].

Glycoxidation (Glycation) is a non-enzymatic reaction that occurs between a carbonyl group of a sugar and an amine group of a protein, which leads to formation of Schiff's base [2]. Subsequently, Schiff's base undergoes rearrangements to from amadori products, it undergoes oxidation to generate dicarbonyl compounds to form cross-linking fluorescent (e.g. pentosidine) and non-fluorescent adducts (e.g. N-(carboxymethyl) lysine [CML]) called as advanced glycation end products (AGEs) [3]. These AGEs causes change in molecular pathways that interrupt the pathogenesis of several important diseases, including carcinogenesis and cancer, cardiovascular diseases, neurodegenerative diseases and in the process of physiological ageing [4].

Nowadays, antiglycation agents (protein glycoxidation inhibitors) have attracted a lot of attention for alleviating diabetic complications [5]. A representative antiglycation drug is aminoguanidine (AG), acts by blocking the AGE formation by preventing the formation of AGEs from dicarbonyl precursors [4]. However, previous studies have shown that AGE has toxic effects in diabetic patients, such as flu-like symptoms, gastrointestinal problems and anemia [6]. Natural AGE inhibitors may have promising therapeutic potential in delaying the onset and prevention of aging and diabetic complications. Therefore, attention has also been focused on the antiglycation properties of phytochemicals present in fruits and vegetables [7,8]. *Punica granatum* commonly is commonly known as pomegranate. The pericarp of pomegranate is a rich source of tannins, flavanoids, polyphenols and some anthocyanins. The potential therapeutic properties of *Punica granatum* are treatment and prevention for diabetes [9], cancer [10,11] cardiovascular disease [12], dental problems [13], and erectile dysfunction [14], antioxidant [15] protection from ultraviolet (UV) radiation and antimicrobial activity[16]. Other potential applications include useful in infant brain ischemia, Alzheimer's disease[9], male infertility, arthritis [14], dermal wounds [17] and obesity [14]. Hence the present study focused on evaluation of pericarp extract of *Punica granatum* L. (Punicaceae), for its effects on protein glycoxidation by *in vitro* studies.

MATERIALS AND METHODS

Materials

Nitroblue tetrazolium (NBT), bovine serum albumin (BSA) were obtained from Merck. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4 dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO, USA). 2.4.6-Tri- (2 -pyridyl)-1, 3, 5-triazine (TPTZ), 2, 2 - Azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) and Trolox were obtained from Sigma Aldrich Chemical Co., Ltd. (England). All other reagents were of analytical reagent (AR) grade.

Plant materials and preparation

The ripened pomegranates (*Punica granatum*) were obtained from local market. The peels were manually separated and shade dried. The peels were powdered in a grinder to get 40-mesh size powder. The moisture content of peel powder was found to be 12%. The powder was suspended in 2% gum acacia and used in the experimental studies.

Superoxide radical scavenging activity

The assay was based on the capacity of the aqueous extract to inhibit formazan formation by scavenging the superoxide radicals generated in a riboflavin-light- NBT system [18]. The reaction mixture contained 58 mM phosphate buffer, pH 7.6, 20μM riboflavin, 6mM EDTA, and 50μM NBT, final volume made up to 3 ml, added in that sequence. Reaction was started by illuminating 40 volts. The reaction mixture with different concentrations of APPG for 15 minutes. Immediately after illumination, the absorbance was measured at 560 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes, with reaction mixture, were kept in the dark and served as blanks. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_O - A_{1\,x}}{A_O} \quad 100$$

Where A0 was the absorbance of the control, and A1 was the absorbance of the aqueous extract/standard.

Hydroxyl radical scavenging activity

Scavenging activity of hydroxyl radical was measured by the method of Halliwell *et al*., 1985 [19]. Hydroxyl radicals were generated by a Fenton reaction ($Fe3^{+}$-ascorbate-EDTA-H_2O_2 system), and the scavenging capacity of the extract and standard towards the hydroxyl radicals was measured by using deoxyribose degradation method. The reaction mixture contained 2-deoxy-2-ribose (2.8 mM), phosphate buffer (0.1 mM, pH 7.4), ferric chloride (20 μM), EDTA (100 μM), hydrogen peroxide (500 μM), ascorbic acid (100 μM) and various concentrations of the test sample in a final volume of 1 ml. The mixture was incubated for 1 h at 37 °C. After the incubation an aliquot of the reaction mixture (0.8 ml) was added to 2.8% TCA solution (1.5 ml), followed by TBA solution (1% in 50 mM sodium hydroxide, 1 ml) and sodium dodecyl sulphate (0.2ml). The mixture was then heated (20 min at 90 °C) to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All experiments were performed in triplicates. The percentage of inhibition was expressed, according to the following equation:

$$\% \text{ Inhibition} = \frac{A_O - A_1}{A_O} \times 100$$

Where A_0 was the absorbance of the control without a sample, A_1 is the absorbance in the presence of the sample.

Hydrogen peroxide radical scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of [20]. The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2. A solution of 43mM H_2O_2 was prepared in 0.1Mphosphate buffer (pH 7.4). APPG at concentration in 3.4mL phosphate buffer was added to 0.6mL of H_2O_2 solution (43mM) and absorbance of the reaction mixture was recorded at 230 nm. A blank solution contained the sodium phosphate buffer without H_2O_2.

DPPH radical scavenging activity

The potential AA of extracts, was determined on the basis of the scavenging activity of the stable 1,1 -diphenyl-2-picrylhydrazyl (DPPH) free radical. Aliquots of 1ml of a methanolic solution containing each pure compound were added to 3ml of 0.004% MeOH solution of DPPH. Absorbance at 517 nm, against a blank of methanol without DPPH, was determined after 30 min (UV, Perkin-Elmer-Lambda 11 spectrophotometer) and the percent inhibition activity was calculated [21]. IC50 values denote the concentration of sample required to scavenge 50% DPPH free radicals. All tests were run in triplicate and averaged.

Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [22]. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and IBPG and the reference compound in different concentrations (20, 40, 60, 80 and 100 μg) were incubated at 25°C for 150 min. After incubation 1.5ml of the Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride in 2% H3PO4) was added. The absorbance of the chromophore formed was measured at 546nm. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test samples.

Reducing power

The reducing power of the extract was determined according to the method of Oyaizu, 1986 [23]. Various concentrations of the extracts (mg/ml) in distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% of potassium ferricyanide water solution (2.5 ml, K3[Fe(CN)6]). The mixture was incubated at 50 8C for 20 min. Aliquots of trichloracetic acid (2.5 ml, 10% aqueous solution) were added to the mixture which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared FeCl3 solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. In this method, the higher the absorbance, the higher the reducing power.

Phosphomolybdinum method

The antioxidant activity of *APPG* extract was evaluated by the phosphomolybdenum method of Prieto, 1999 [24]. An aliquot of 0.1 ml of sample solution (equivalent to 100 lg) was combined with 1 ml of reagent solution (0.6M sulfuric acid, 28 mM

sodium phosphate, and 4 mM ammonium molybdate). In the case of the blank, 0.1 ml of methanol was used in place of sample. The tubes were capped and incubated in water bath at 95 ^{0}C for 90 min. After the samples were cooled to RT, the absorbance of the aqueous solution of each was measured at 695 nm.

Fe^{+2}Chelating activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al.,* 1994 [25]. To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of FeCl2 (2 mM) was added. After 30 sec, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as Chelating rate = (A0- A1)/A0×100, where A0 was the absorbance of the control (blank, without extract) and A1 was the absorbance in the presence of the extract.

Ferric reducing ability power

The FRAP method measures the absorption change that appears when the TPTZ (2,4,6 -tri pyridyl-*s*-triazine)-Fe^{3+} complex is reduced to the TPTZ-Fe^{2+} form in the presence of antioxidants [26]. An intense blue colour, with absorption maximum at 595 nm, develops Briefly, the FRAP reagent contained 2.5 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20 mM FeCl3 and 25 ml of 0.3 M acetate buffer, pH 3.6, was freshly prepared. The extracts were dissolved in ethanol at a concentration of 1 mg/ml. An aliquot of 0.2 ml of solution was mixed with 1.8 ml of FRAP reagent and the absorption of the reaction mixture was measured at 595 nm. Ethanolic solutions of known Fe (II) concentration, in the range of 50-1000 µM (FeSO4), were used for obtaining the calibration curve. The FRAP value represents the ratio between the slope of the linear plot for reducing Fe^{3+}-TPTZ reagent by plant extract compared to the slope of the plot for $FeSO_4$.

$ABTS^+$Assay

ABTS assay was based on the method of Re *et al* 1996 with slight modifications. ABTS radical cation ($ABTS^+$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS+ solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. After addition of 25µl of sample or Trolox standard to 2 ml of diluted $ABTS^+$ solution, absorbance at 734 nm was measured at exactly 6 min. The decrease in absorption at 734 nm was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of ABTS+ solution at different concentrations of Trolox. Appropriate blank measurements were carried out and the values recorded. Results were expressed as Trolox equivalent antioxidant capacity (TEAC) [27].

Estimation of protein carbonyl content

The effects of *APPG* extract on oxidative modification of BSA during glycoxidation process were carried out according to method described previously [28]. For determination of protein carbonyl content in the samples, 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the samples (1 mg). Samples were incubated for 30 min at RT. Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 3000g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrochloride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH. The data were expressed as nmol/mg protein.

In Vitro protein glycoxidation method

This assay was adopted from the literature used as an *in vitro* model for comparing the anti-glycation activities of various bean extracts. In brief, 5 g BSA and 14.4 g D-glucose were dissolved in phosphate buffer (1.5 M, pH 7.4) to obtain the control solution with 50 mg/mL BSA and 0.8 M D-glucose. 2 mL of the control solution was incubated at 37^{0}C for 21 days in the presence or absence of 1 mL of plant extracts in phosphate buffer (1.5 M, pH 7.4) (the final concentration of plant extract in the 3 mL test solution was 500 ppm). The test solution also contained 0.2 g/L NaN_3 to assure an aseptic condition. After 7 days of incubation, fluorescent intensity (excitation, 330 nm; emission, 410 nm) was measured for the test solutions [29]. Percent inhibition of AGE formation by each extract or compound was calculated using the following equation, % inhibition = [1 (fluorescence of the solution with inhibitors/Fluorescence of the solution without inhibitors)] ×100%.

RESULTS

Tables 1 Effect of Ascorbic acid and APPG on IC_{50} values of different *in vitro* models. Each value represents the Mean±SEM values. N=3.

Method	Ascorbic acid (µg/ml)	APPG (µg/ml)
Superoxide radical	04.27±0.31	05.52±0.06
Hydroxyl radical	29.71±0.11	31.40±0.08
Hydrogen peroxide radical	05.08±0.03	03.21±0.00
DPPH radical	53.92±5.22	42.27±0.02
Nitric oxide radical	16.93±0.07	13.63±0.07
Reducing power	05.52±0.01	07.47±4.35
Phosphomolybdinum method	03.19±0.03	03.90±0.07

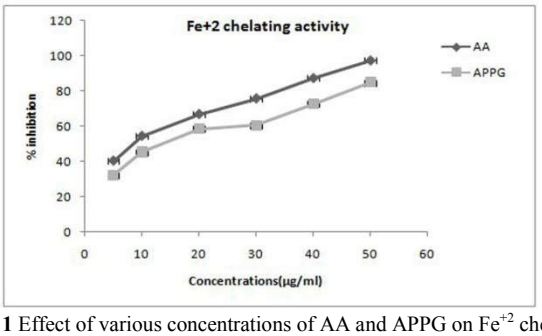

Figure 1 Effect of various concentrations of AA and APPG on Fe^{+2} chelating activity, Values represented as mean±SEM, n=3.

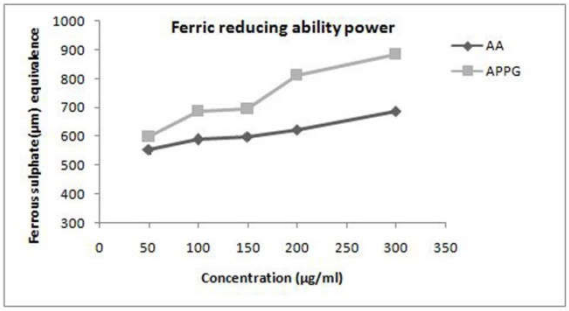

Figure 2 Effect of various concentrations of AA and APPG on ferric reducing ability power. Values represented as mean±SEM, n=3.

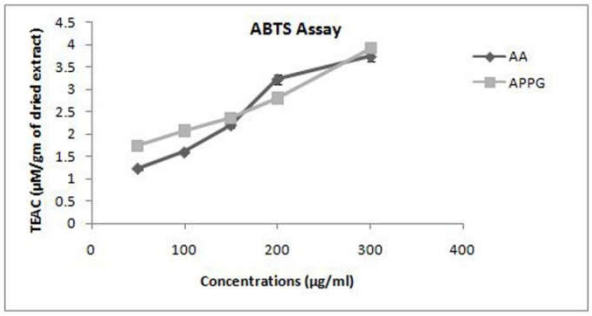

Figure 3 Effect of various concentrations of AA and APPG on ABTS^{+} Assay. Values represented as mean±SEM, n=3.

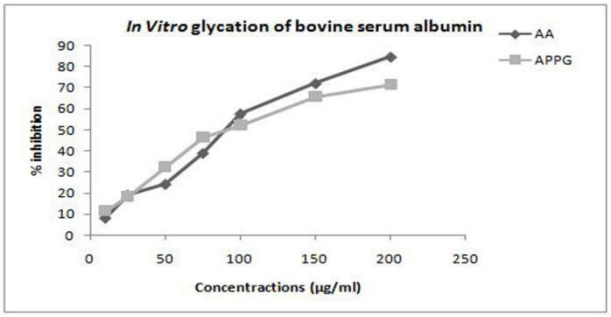

Figure 4 Effect of various concentrations of AA and APPG on in vitro glycation of bovine serum albumin. Values represented as mean±SEM, n=3.

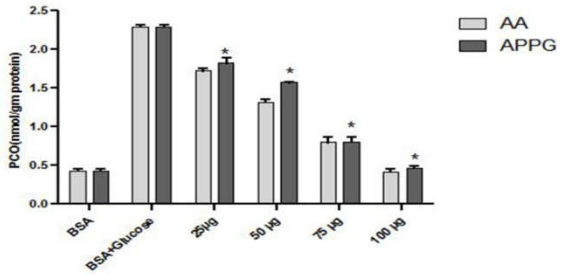

Figure 5 Effect of various concentrations of AA and APPG on protein carbonyl content

The APPG shown significant reduction in PCO content compared to that of BSA+ glucose treatment. P<0.0001significance followed by two way ANOVA followed by Bonferroni'spost test when compared with BSA+ glucose group.

DISCUSSION

The reactive oxygen species are generated in the early and advanced glycation processes. The glycation phenomenon corresponds to the non enzymatic and non-oxidative covalent attachment of glucose molecule to protein. This process classically proceeds through early and advanced stages. In the early stage, reducing sugars such as glucose react with the amino groups of lysine side chains and the terminal amino group of proteins to form unstable Schiff bases and, through rearrangement, Amadori products. Then, this product undergoes slow and complex series of chemical reactions to form advanced glycation end products (AGEs) [30].

Antioxidants works as in various pathways by scavenging the radicals and decomposing peroxides by binding to the metal ions and some of them producing synergistic action. These free radicals act by damaging proteins, DNA, and other small molecules. These antioxidants work by three different mechanisms as antioxygen radicals, reducing substances and antiradicals. They work as metal chelators by chain breaking and reducing the chain initiation. Therefore antioxidant activity can be evaluated by different *in vitro* methods for different mechanisms.

In the present study, we evaluated the antiglycation properties of *Punica granatum* pericarp extract against both aqueous, lipid peroxyl free radicals and non enzymatic glycation and oxidation of BSA.

To measure the scavenging capability of AA and APPG by superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide and alkylperoxyl (ABTs^{+}) has been developed. The reducing capability of AA and APPG was measured by reducing power assay, phosphomolybdenum assay and ferric reducing ability assay. Estimation of metal chelating property of AA and APPG by Fe^{+2} chelating activity and the estimation of protein damage is by protein carbonyl content and by *in vitro* glycation of bovine serum albumin were carried out.

The APPG was found to be a moderate scavenger of superoxide radical generated in riboflavin-NBT-light system *in vitro* [31]. The ascorbic acid and APPG inhibited the formation of the blue formazan and the % inhibition was proportional to the concentration with an IC50 value of 4.27 µg/ml, 5.52 µg/ml respectively as shown in table 1. These results indicated that the tested extract had a notable effect on scavenging of superoxide when compared with ascorbic acid, which was used as positive control. Cells contain superoxide dismutase (SOD) as a cellular antioxidant enzyme, which removes this ubiquitous superoxide metabolic product by converting it into hydrogen peroxide and oxygen and this hydrogen peroxide radical readily decomposed into hydroxyl radical in the presence of Catalase in biological systems [32]. The APPG shown better scavenging activity in scavenging hydroxyl radical and hydrogen peroxide radical and the IC 50 values are 31.40µg/ml and 3.21 µg/ml respectively compared to standard ascorbic acid 29.27µg/ml and 5.18 µg/ml respectively (table 1). Nitric oxide is a free radical in terms of its unpaired electron. It reacts with O_2^- in termination reactions in the mitochondrial matrix, yielding peroxynitrite ($ONOO^-$).These oxyradical and peroxynitrite induce oxidative damage to mitochondrial DNA damage and protein inactivation and ATP synthesis [33]. The AA and APPG had shown better scavenging activity in scavenging nitric oxide free radical with IC 50 values 16.93 µg/ml and 13.63 µg/ml respectively.

The DPPH method based on the reduction of methanolic DPPH solution in the presence of hydrogen donating antioxidant due to the formation of the non radical form DPPH-H by the reaction. The APPG can able to reduce the stable radical DPPH to the yellow coloured diphenyl picrylhydrazine. Table 1 shows highest DPPH radical scavenging effect (IC50 value by 42.27 µg/ml) of APPG than standard ascorbic acid (53.92 µg/ml). In the phosphomolybdinum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of green Mo (V) complexes [34]. The AA and APPG showed concentration dependent %inhibition of Mo (V) with IC50 values of 3.19 µg/ml and 3.90µg/ml respectively.

For the measurements of the reductive ability, transformation of Fe3+ to Fe2+ in the presence of APPG has been investigated using the method of Oyaizu (1986) [23]. Earlier authors observed a direct correlation between antioxidant activities and reducing power of certain plant extracts [35]. The

reducing properties are generally associated with the presence of reductants [36,37], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [38]. The reducing power of APPG increased with increasing concentrations. However, the reducing power of ascorbic acid (5.52±0.01) was relatively more pronounced than that of APPG with IC50 value 7.42±4.35 µg/ml (table 1). In nature iron can be found as either ferrous (Fe^{2+}) or ferric (Fe^{3+}). If iron is free within the cell it can catalyze the conversion of hydrogen peroxide into free radical [39,40]. Minimizing these ions may afford protection against oxidative damage by inhibiting production of ROS. Reducing power is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action, can be strongly correlated with other antioxidant properties [41]. The data obtained from Fig. 1, reveals that APPG has an effective capacity for iron binding, suggesting that its action as an antioxidant may be related to its iron binding capacity. The AA and APPG showed IC_{50} values of 8.86±0.13 and 17.36±0.07 µg/ml respectively for Fe+2 chelating activity (fig 1). FRAP assay measures the reducing ability of antioxidant that react with ferric tripyridyltriazine (Fe3+-TPTZ) complex and produce a coloured ferrous tripyridyltriazine (Fe^{2+}- TPTZ) [26]. In FRAP assays the AA and APPG showed 687.75±0.65 and 886.05±0.65 of ferrous sulphate (mM) equivalence respectively. Using this assay, APPG shown to increasing FRAP value in dose dependent effects shown in Fig 2.

The scavenging activity of the APPG on the radical ABTS, generated by potassium per sulfate was compared with a standard amount of trolox [42] which was expressed as trolox equivalent antioxidant capacity (TEAC value). In general, medicinal plants may play an important role in chemical protection from oxidative damage by possessing endogenous antioxidants such as phenolic compounds. Hagerman *et al*., 1998 [41] have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{+}$). In Fig 3 the AA and APPG showed TEAC values of 3.74±0.12 and 3.92±0.04 mM/gm dried extract respectively in ABTS radical scavenging activity. The presence of tannins present in the APPG extract might be responsible for scavenging the ABTS radical.

The incubation of BSA with D-glucose for 21 days induced a loss of helical structure in native BSA by protein glycation [43]. Such glycation was found to be inhibited in the presence of APPG that found to induce an increase in the helicity of BSA (more negative ellipticity values). Therefore, APPG may stabilize the native protein structure. The IC_{50} values of inhibition of *in vitro* glycation of bovine serum albumin was found to be in a dose dependent manner with an IC 50 value of AA and APPG was found to be 102.7± 1.73 and 110.4±1.13µg/ml respectively (fig 4). The presence of phytochemicals present in APPG might be responsible for the stabilization of native structure of BSA and inhibition of glycation.

To further evaluate the inhibition of protein glycation by APPG, the protein carbonyl (PCO) content which is a byproduct of glycation of BSA was estimated [44]. The APPG and AA were found to decrease the formation of PCO in a dose dependent manner. As shown in fig 4, glycation elicited a significant increase of carbonylation and glycation of BSA in the presence of glucose compared to the control sample without reducing sugar. However a significant effect on the inhibition of protein oxidation due to glycation was exerted in a dose-dependent manner with increasing the concentrations of APPG as showed in fig 5. The AA and APPG shown to decrease the formation of PCO to 81.85% and 79.64% inhibition as shown in fig 5.

Many researchers reported that the phenols and flavanoids had antioxidant activity [46]. The active principles present in *Punica granatum* pericarp extract such as tannins like ellagic acid, gallic acid, corilagin, gallagyldilactone, granatin A, granatin B, pedunculagin, punicalagin, punicalin [47,48]; anthocynidines like delphinidine, cyanidine, pelargonidine [49] and flavonoids like catechin, epicatechin, flavan-3-ol, kaemniferol, luteolin, naringin, pelergonidin, prodelphinidin, quercitin and rutin [50,51,52] might be responsible for the *in vitro* protein glycoxidation.

CONCLUSIONS

It is concluded that, using different *in vitro* models for estimation the antioxidant potential of *Punica granatum* showed better effect in scavenging the different free radicals. In addition to antioxidant activity it also showed better activity against the formation of protein carbonyl content and protect the structural changes in BSA during glycation processes. All the activities might be due to the presence of high levels of tannins and polyphenols in aqueous pericarp extract of *Punica granatum*.

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References

1. Stadtman ER, Levin RL. Protein oxidation. *Ann. NY Acad. Sci.* 2000; 899:191-208.
2. Brownlee M. Glycation and diabetic complications. *Diabetes*.1994; 43: 836-41.
3. Wolff SP and Dean RT. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem. J.* 1987; 245: 243-250.
4. Swathi P and Eswar Kumar K. Advanced glycation end products: A review. *Journal of Global Trends in Pharmaceutical Sciences*, 2014; 5(2): 1729-37.
5. Matika Z, Radoff S, Rayfield EJ. Advanced glycosylation end products with diabetic nephropathy. *N Engl J Med*. 1991; 325:836-842.
6. Bolton Wk, Cattorn DC, Williams ME, Alder SG, Appeal GB. Randomized trial of advanced glycation end products in diabetic nephropathy. *Am J Nephrol*. 2004; 24:32-40.
7. Yokozawa T and Nakagawa T. Inhibitory effects of Luobuma tea and its components against glucose-mediated protein damage. *Food. Chem. Toxicol*. 2004; 42:975-981.
8. Eskander EF, Won Jun H. Hypoglycaemic and hyperinsulinemic effects of some Egyptian herbs used for the treatment of Diabetes mellitus (type II) in rats. *Egy. J. Pharm*. 1995; 36: 331-342.
9. Middha SK, Usha T, RaviKiran T. Influence of *Punica granatum* L. on region specific responses in rat brain during alloxan-induced diabetes. *Asian Pacific Journal of Tropical Biomedicine.* 2012; 2(2): 905-909.

10. Dikmen M, Ozturk N, Zturk YO. The antioxidant potency of *Punica granatum*L. fruit peel reduces cell proliferation and induces apoptosis on breast cancer. *Journal of Medicinal Food*. 2011;14(12):1638-1646.
11. Hong MY, Seeram NP, Heber D. Pomegranate polyphenols down-regulate expression of androgen-synthesizing genes in human prostate cancer cells overexpressing the androgen receptor. *Journal of Nutritional Biochemistry*. 2008; 19(12):848-855.
12. Jurenka J. Therapeutic applications of pomegranate (*Punica granatum*L.): a review. *AlternativeMedicine Review*. 2008; 13(2):128-144.
13. Viuda-Martos M, Fernandez-Lopez J, Perez JA. Pomegranate and its many functional components as related to human health: a review, Comprehensive Reviews in Food Science and Food Safety. 2010; 9(2): 635-654.
14. Kanatt SR, Chander R, Sharma A. Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. *International Journal of Food Science and Technology*. 2010; 45(2): 216-222.
15. Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chem*. 2006; 96:254-260.
16. Hayouni EA, Miled K, Boubaker S. Hydroalcoholic extract based-ointment from *Punica granatum* L. peels with enhanced *in vivo* healing potential on dermal wounds, Phytomedicine. 2011; 18(11): 976-984.
17. Murthy KN, Reddy VK, Veigas JM, Murthy UD. Study on wound healing activity of Punica granatum peel. *J Med Food*. 2004; 7:256-9.
18. Beauchamp C and Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem*. 1971; 44: 276-277.
19. Halliwell B and Gutteridge JMC. In Free radicals, ageing, and disease, free radicals in biology and medicine, Oxford: Clarendron Press. 1985; 2:279-315.
20. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea, Carcinogenesis. 1989; 1003-1008.
21. Braca A, Tommasi ND, Bari LD, Pizza C, Politi M, Morelli I. Antioxidant principles from Bauhinia terapotensis. *Journal of Natural Products*. 2001; 64: 892-895.
22. Ebrahimzadeh MA, Nabavi SF. *In vitro* antioxidant and free radical scavenging activity of Leonurus cardiaca subsp. Persicus, Grammosciadium platycarpum and Onosma demawendicum, *Afr J of Biotech*. 2010; 9(51): 8865-8871.
23. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition*. 1986; 44: 307-31.
24. Prieto P, Pineda M, Aguilar MM. Spectrophotometric quantitation of antioxidant capacity through the formation of a phoshomolybdenum complex: specific application to the determination of vitamin E. Anal. *Biochem*. 1999; 269: 337-341.
25. Dinis CP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys*. 1994; 315: 161-9.
26. Benzie IF, Strains JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Anal Biochem*. 1996; 239:70-6.
27. Re R Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol Med*. 1996;26: 1231-7.
28. Ardestani A and Yazdanparast R. Antioxidant and free radical scavenging potential of Achillea santolina extracts. *Food. Chem*. 2007; 104:21-29.
29. Wu CH and Yen GC. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *Journal of Agricultural and Food Chemistry*. 2005; 53: 3167-3173.
30. Pennathur S and Heinecke JW. Mechanisms for oxidative stress in diabetic cardiovascular disease. *Antioxid Redox Signal.* 2007; 9: 955-69.
31. Mossine VV, Linetsky M, Glinsky GV, Ortwerth BJ, Feather MS. Superoxide free radical generation by Amadori compounds: the role of acyclic forms and metal ions. *Chem. Res. Toxicol*. 1999; 12: 230-236.
32. Giorgio M, Trinei M, Migliaccio E, Pelicci PG. Hydrogen peroxide a metabolic byproduct or a common mediator of ageing signals. *Nat.Rev.Mol.Cell Bio.* 2007; 8(9): 722-8
33. Wink DA and Mitchell JB. Chemical biology of nitric oxide; Insights into regulatory, cytitoxic, cytoprotective mechanisms of nitric oxide. *J.Biol.Chem*. 1998; 273: 11044-11048.
34. Halliwell B, and Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol*. 1990; 186:1-85.
35. Uma Shankar S and Arun Kumar. *In vitro* antioxidant activity of *Rubus ellipticus* fruits. *J Adv Pharm Technol Res*. 2011; 2(1): 47-50.
36. Pin-Der Duh, X. Antioxidant activity of burdock (Arctium lappa Linne): its scavenging effect on free-radical and active oxygen. *Journal of the American Oil Chemists Society* 1998; 75: 455-461.
37. Gayathri GA, Gayathri Mahalingam and R. Nathiya. Quantitative Phytochemical Analysis, In vitro Reducing Power and Anti-oxidant Activity of Methanol Leaf Extract of Acanthus ilicifolius. *International Journal of Pharmacognosy and Phytochemical Research*. 2014; 7(1): 181-186.
38. Gordon MH. (1990). The mechanism of antioxidant action in vitro. In B. J. F. Hudson (Ed.), Food antioxidants (pp. 1-18). London: Elsevier Applied Science.
39. Andrews NC. Disorders of iron metabolism. *N.Engl.J.Med*. 1992; 341(26):1986-95
40. Conrad ME, Umbreit JN. Disorders of iron metabolism. *N.Engl.J.Med.* 2000; 342(17):1293-4
41. Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW. Higher molecular weight oplant polyphenolics(Tannins) as biological antioxidant. *J Agric Food Chem*. 1998;46: 1887-1892.
42. Robert RE, Pellegrini M, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical caution decolorization assay. *Free Radical Bio Med*. 1999;26: 1231-1237.

43. Hye Young K and Kyong K. Protein Glycation Inhibitory and Antioxidative Activities of Some Plant Extracts in Vitro. *Agric*. *Food Chem* 2003; 51 (6): 1586-1591.
44. Rosa Martha PG, Sandra L D, Ivan C R, Adriana Maria N G. Anti-glycation Effect of Spices and Chilies Uses in Traditional Mexican Cuisine. *Journal of Natural Products,* 2010;3: 95-102.
45. Hanasaki Y, Ogawar S, Fukui S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radical Biology and Medicine*. 1994;16: 845-850.
46. Tanaka T, Nonaka GI, Nishioka I. Tannins and related compounds. C. Reaction of dehydrohexahydroxydiphenic acid esters with bases, and its application to the structure determination of pomegranate tannins, granatins a and b. Chemical and Pharmaceutical Bulletin. 1990;38: 9424-9428.
47. Satomi H, Umemura K, Ueno A, Hatano T, Okuda T, Noro T. Carbonic anhydrase inhibitors from the pericarps of *Punica granatum* L. *Biological and Pharmaceutical Bulletin.* 1993; 16: 787-790.
48. Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem*. 2000; 48:4581-9.
49. Noda Y, Kaneyuka T, Mori A, Packer L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. *Journal of Agricultural and Food Chemistry*. 2002; 50:166-171
50. Artik N. Determination of phenolic compounds in pomegranate juice by using HPLC. *Fruit Processing*. 1998; 8: 492-499.
51. De pascual TS, Santosm BC, Rivas GSL. Quantitative analysis of flavin-3-ols in Spanish food stuffs and beverages. *Journal of Agricultural and food chemistry.* 2000; 48: 5331-5337.
52. Van Elswijk DA, Schobel UP, lansky EP, Irth H, Vander greef J. Rapid dereplication of estrogenic compounds in pomegranate (*Punica granatum*) using on line biochemical detection coupled to mass spectroscopy, 2004; 65: 233-241.

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