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ORIGINAL ARTICLE

Antioxidant and Antiglycation Effects of *Cucurbita argyrosperma* Seeds Polysaccharide

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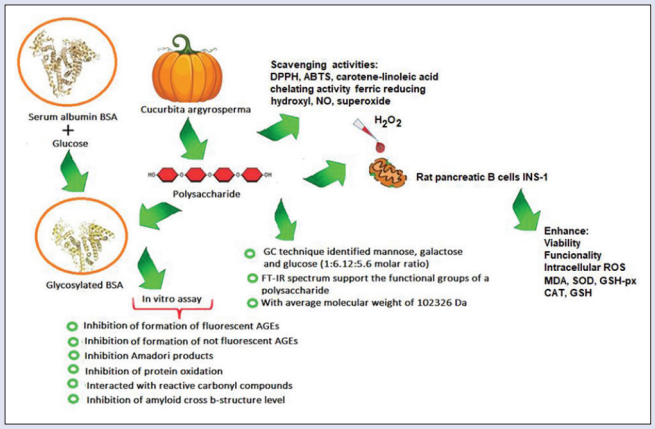
ABSTRACT

Background: Diabetes is related with oxidative stress caused by free radicals and glycation of the proteins. **Objectives:** This study aimed to isolate a polysaccharide (PLY) from *Cucurbita argyrosperma* seeds and determine their antioxidant and antiglycation effects. **Materials and Methods:** The PLY fraction was isolated by hot water extraction and purified by ion-exchange chromatography (A103S). La antioxidant capacities were evaluated by using lipid peroxidation, superoxide dismutase, glutathione peroxidase (GSH-Px), catalase, and malondialdehyde activities, and intracellular reactive oxygen species (ROS) with stimulation H_2O_2 in pancreatic β INS-1 cells. In addition, scavenging activities of PLY were estimated in 2,2 diphenyl-1-picrylhydrazyl radical, 2,2′ -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, β-carotene-linoleic acid, chelating activity, ferric reducing power, hydroxyl radical, nitric oxide radical, and superoxide anion radical assays. Therefore, the protective effect of PLY on reducing sugar-induced bovine serum albumin glycation was examined. **Results:** PLY consisted of mannose, galactose, and glucose in the molar ratio of 1.12:6.12:5.60 with an average molecular weight of 102326 Da. PLY had a pronounced radical scavenging potential as well as total antioxidant effect and prevented the cells ROS generation and increased the level of antioxidant enzymes. PLY inhibited the formation of carboxymethyllysine and fructosamine, prevented protein oxidation increasing the level of thiol, and decreasing protein carbonyl level, interacts with reactive dicarbonyl compounds (methylglyoxal), and decreased amyloid cross β-structure level. **Conclusion:** These results indicate that PLY enhanced antioxidant capacity and prevented oxidative stress and AGEs progression in its different stages. consequently, had promising effects on diabetes and associated disorders.

Key words: *Cucurbita argyrosperma,* glycation, oxidation, polysaccharide, protein oxidation

SUMMARY

- This study established that a polysaccharide isolated from *Cucurbita argyrosperma* seeds demonstrated a strong antiglycation ability *in vitro,* also shown promising inhibitory effects on glycation-induced protein cross-linking which occurred independently of the antioxidant properties exhibited. This investigation could lead to the possibility of using the plant extract or its purified active component for targeting diabetic complications. In addition, these results are important for identifying plants with potential to treat diabetic complications.



Abbreviations used: ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; AGEs: Advanced glycation end-products; BSA: Bovine serum albumin; CAT: Catalase; CML: Carboxymethyl-lysine; DMSO: Dimethyl sulfoxide; DPPH: 2,2 Diphenyl 1 picrylhydrazyl radical; FRAP: Ferric reducing power; FT-IR: Fourier-transform infrared spectroscopy; GSH-Px: Glutathione peroxidase; HPGPC: High-performance gel permeation chromatography; H_2O_2: Hydrogen peroxide; MDA: Malondialdehyde; MGO: Methylglyoxal; NBT: Nitroblue tetrazolium; PLY: Polysaccharide; ROS: Reactive oxygen species; SOD: Superoxide dismutase; T2DM: Type 2 diabetes mellitus; ATP: Adenosine triphosphate.

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INTRODUCTION

Diabetes mellitus is a disorder characterized by hyperglycemia (high blood sugar) and disturbances of protein, fat, and carbohydrate metabolism associated with various dysfunctions.[1] Diabetes type 2 (T2DM) is the metabolic disorder most prevalent and of greater incidence in worldwide. This is due among many factors to antioxidative enzyme deficiencies making the β-cells vulnerable against oxidative stress. Consequently, current hypoglycaemic drugs have limitations and side effects, furthermore a combination of antihyperglycaemic activity with the capacity to inhibit oxidative tissue damage would be ideal to control diabetes. The impaired antioxidant defense system and production of oxidative stress induced by chronicle dyslipidemia and hyperglycemia plays an important role in the etiology of type 2

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diabetes.[2] Hence, protection of the pancreatic β-cells is necessary for the prevention of diabetes.

Advanced glycation end-products (AGEs) are associated with chronic hyperglycemia leading to an alteration in the protein properties.[3] AGEs cause the generation of ROS to produce induction of cell apoptosis and DNA damage.[4] It has been reported that glycation of proteins is carried out in three stages early, intermediate, and late.[5] Consequently, T2DM subjects have a high accumulation of AGEs in the tissues, and for this reason, the excessive formation of AGEs results in complications of diabetes.[6]

Pumpkin (*C. argyrosperma*) is a plant native of Mesoamerica and is a member of the family of *Cucurbitaceae*, its seeds are used in folk medicine for the prevention and treatment of various human diseases, such as micturition disorders, diseases of the prostate, diabetes, and inflammation diseases.[7] It has been reported that many polysaccharides (PLYs) isolated from other Cucurbita fruits have a hypoglycemic effect by increasing plasma insulin in diabetic mice[8] as well as possess antioxidant,[9] inhibitory effect on AGEs formation and aldose reductase.[10] Therefore, in this study, we investigate the antioxidant and antiglycation effects of a PLY from *C. argyrosperma*.

MATERIALS AND METHODS

General experimental

Fourier transform infrared spectroscopy (FT-IR) was determined on a Perkin-Elmer 1720 FT-IR in the range of 4000–500 cm^{-1}. Fluorescent AGEs were recorded in a spectrofluorometric detector (BIO-TEK, Synergy HT, USA). Solvents used as eluents were purchased from Fremont (California, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, USA).

Collection of the plant materials

Fresh *C. argyrosperma* was collected in Amecameca, Mexico State, and the plant was identified in the Herbarium of Escuela Nacional de Ciencias Biologicas-Instituto Politecnico Nacional. Samples of *C. argyrosperma* were stored under the voucher specimen number 16728.

EXTRACTION AND PURIFICATION OF POLYSACCHARIDE

Powdered air-dried seeds of *C. argyrosperma* (5 kg) were macerated with MeOH (20 L) for 5 days at room temperature and dried using a rotatory evaporator to yield a green gum (300 g), which was fractionated in water, for that purpose, the methanol extract was dissolved in water following of agitation at 50°C and allowed to stand for 30 min. The soluble extract was precipitated with absolute ethanol and was centrifugated at 4000 rpm for 15 min and then washed with ethanol, acetone, and ether, consecutively. The yield of the pure PLY was 1300 mg (0.026%). Protein was removed from crude PLY by ion-exchange chromatography (A103S) in a radial flow column (Superflow-250, Sepragen Co., Hayward, CA, USA), eluted with distilled water and a linear gradient of sodium chloride (0–0.4 mol/L) at room temperature with a flow rate of 30 mL/min. The carbohydrate content was monitored at 280 nm using phenol-sulfuric acid assay.[11]

Composition of polysaccharide

The monosaccharide content of PLY was evaluated using gas chromatography (GC, Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA). PLY (50 mg) was dissolved in 4 mL of trifluoroacetic acid (2 M; TFA) and was hydrolyzed in a sealed glass tube for 2 h at 110°C, the excess of TFA in the reaction was in the first instance eliminated by evaporation, and 4 mL of methanol was used to reach a dried product, this procedure was repeated two times. The neutral monosaccharides were reduced with $NaBH_4$ (2 mol/L; 3 mL) and acetylated with 4 mL of acetic anhydride at 100°C for 1 h. The alditol acetates were filtered through a nylon membrane (0.22 μm) for gas chromatography analysis and evaluated by GC Agilent-6890 GC (Waldbronn, Germany) equipped with an Agilent DB-1701 capillary column (30 m x 32 m, 0.25 μm) and a flame ionization detector. The temperatures of the detector and injector were 230°C and 250°C, respectively. The column temperature was maintained at 150°C for 3 min, increased to 230°C at a rate of 5°C/min, held for 30 min. Nitrogen was used as carrier gas at a flow rate of 1.3 mL/min. Ten standard monosaccharides were acetylated and assayed at the same conditions.[12]

Determination of the molecular weight of polysaccharide

The average molecular weight of PLY was evaluated by high-performance gel permeation chromatography (HPGPC) with a waters high-performance liquid chromatography (HPLC) system with ultrahydrogel, columns TM 120, and 1000 (7.8 mm × 300 mm) and a Waters 2410 RI detector. PLY (50 μL) was injected and eluted with a buffer 0.1 M $NaNO_3$ as a mobile phase, with a flow rate of 0.8 mL/min at 40°C.[13] The HPGPC system was recalibrated with various standard dextrans of different molecular weights (5–500 kDa).

Fourier-transform infrared spectroscopy spectral analysis of polysaccharide

Fourier-transform infrared (FT-IR) was used to determine the primary structure of the PLY. FT-IR spectrum of PLY was measurement with a wave range of 4000-400 cm^{-1}.[14]

Determination of protein content, analysis of total sugar content, and evaluation of uronic acid content

The Bradford assay was used to determine the protein content of PLY.[15] The sugar contents were evaluated by the phenol-sulfuric acid method[16] using D-glucose as standard. The uronic acid content was evaluated using the method of Blumenkrantz[17] with D-galacturonic acid as standard.

Assay antioxidant activity *in vitro*

The scavenging activities of PLY were evaluated at various concentrations of 20–200 μg/ml, namely 2,2 diphenyl 1 picrylhydrazyl radical (DPPH),[18] 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical,[18] β-carotene-linoleic acid,[19] chelating activity,[17] ferric reducing power (FRAP),[19] hydroxyl radical,[20] nitric oxide radical,[20] and superoxide anion radical[20] assays which were estimated.

Cell viability and functionality

Rat pancreatic β INS-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were preincubated with 1, 5, and 10 μg/mL of PLY for 2 h followed by the addition of 500 μM H_2O_2. Afterward, 24 h was added to each well 0.5 mg/mL of MTT (Sigma-Aldrich, St. Louis MI, USA) and incubated for 2 h at 37°C, and 200 μL dimethyl sulfoxide was aggregated to dissolve the formazan crystals formed within the cells. Color intensity was measured at 490 nm by a microplate reader (Thermo Fisher Scientific, FL, USA). The viability was expressed as the percentage in each treatment group with reference to that of the nontreated control. Cell integrity was measured by lactate dehydrogenase (LDH) assay as LDH released in the culture medium.

Briefly, after treatment, 0.2 mL culture médium of each group was evaluated for LDH activity using commercial kit (Cayman Chemical, MI, USA).

Evaluation of intracellular reactive oxygen species with stimulation H_2O_2

1×10^5 INS-1 cells per well were preincubated with PLY in different concentrations (1, 5, and 10 μg/mL) for 2 h. After incubation, the PLY-treated cells were treated with 500 μM H_2O_2 for 24 h, and the intracellular ROS level was evaluated by 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St Louis MI, USA) method.

Evaluation of lipid peroxidation, superoxide dismutase, glutathione peroxidase, catalase, and malondialdehyde activities

The supernatant from treated INS-1 cells was used for analysis of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and catalase (CAT) using commercial kits (Cayman Chemical, MI, USA) according to the manufacturer's protocols.

Evaluation of advanced glycation end-products *in vitro*

Ten mg/ml of BSA were co-incubated with glucose (250 mM/ml), phosphate buffer (pH 7.4), phosphate buffer, , 0.02% sodium azide, PLY (0.5, 1.0, 1.5, and 2.0 mg/ml), or aminoguanidine (AG, 1.0 mg/ml) used as standard for 4 weeks at 37°C. All the samples were dialyzed to eliminate unbound glucose against pH 7.4 phosphate buffer. Fluorescent AGEs were indicated at an excitation wavelength of 350 nm and emission wavelengths of 450 nm.[21]

Evaluation of non-fluorescent carboxymethyl-lysine

CML was analyzed using an enzyme-linked immunosorbent assay ELISA kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer × s protocols.

Formation of fructosamine adduct

Nitroblue tetrazolium (NBT) method was used for evaluating levels of fructosamine adduct.[22] A mixture, contained NBT reagent (2.0 ml, 0.3 mM), and 0.5 ml glycated sample in sodium carbonate buffer (100 mM, pH 10.3) was incubated for 15 min, at room temperature, then absorbance was determined at 530 nm using a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan) against a blank.

Determination of protein carbonyl content and thiol group estimation

The dicarbonyl content in glycated BSA was measured according to a previously published assay.[23] Briefly, 0.4 ml glycated sample was incubated with Girard-T stock solution (0.2 ml, 500 mM) and sodium formate (500 mM, 3.4 ml, pH 2.9) for 1 h at room temperature. The absorbance was determined at 294 nm against a blank without Girard-T stock solution and containing all the other reagents. Glyoxal was used as a standard to prepare the calibration curve treated in a similar assay. The thiol group in glycated albumin and native BSA were evaluated according to the DTNB method.[24] Glycated BSA (10 μL) was incubated for 15 min at room temperature with 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma-Aldrich, St Louis MI, USA) in 0.1 M PBS (pH 7.4). The absorbance was measured at 410 nm.

Formation of methylglyoxal-derived advanced glycation end-products

The BSA–MGO experiment was used for evaluating whether PLY acts in the middle stage of the formation of AGEs.[25] BSA solution (10 mg/mL) in 0.1 MPBS (125 mL, pH 7.4) was added at MGO (115 mL, 1 mM). After, PLY was added in concentrations of 0.5, 1.0, 1.5, 2.0 mg/mL to BSA–MGO reaction and incubated at 37°C for 7 days. Then, the fluorescence intensity was evaluated at the excitation wavelength of 370 nm and an emission wavelength of 420 nm.

Measurement of protein aggregation

Fifty microliters glycated sample was incubated with 50 μL of Congo red (100 μM; Sigma, St. Louis, MO, USA) in 10% ethanol/PBS for 20 min at 25°C. The absorbance was determined at 530 nm.[26] 100 μL of thioflavin T (64 μM) dissolved in 0.1 M of PBS (pH 7.4), were added to 10 μL glycated fructose samples and incubated at 25°C for 60 min. The degree of BSA glycation was measured by AGEs specific fluorescence (Ex 435 nm/Em 485 nm).

Statistical analysis

Results are shown as the mean ± standard error of the mean. The statistical significance of the data was determined by one-way ANOVA, followed by statistical significance differences by using Tukey's multiple comparison assays. The values of $P < 0.05$ were considered statistically significantly. All analyses were performed in SPSS statistical software (SPSS, Illinois, USA).

RESULTS

Characterization of the polysaccharide

Monosaccharide compositions of PLY were determined using the GC technique, the composition compared the retention time of standard

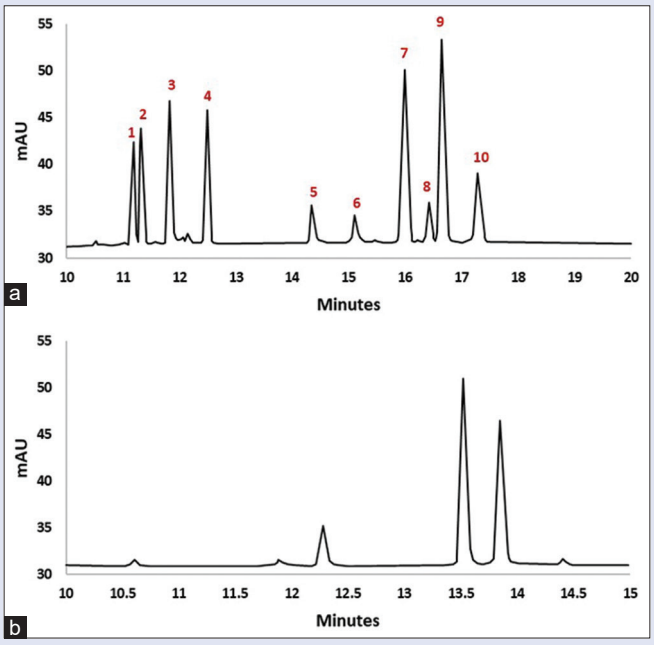

Figure 1: (a) Gas chromatogram: Rhamnose (1; RT = 11.1); Fucose (2; RT = 11.3); Arabinose (3; RT = 11.8); Xylose (4; RT = 11.8); Fructose (5; RT = 14.4); Ribose (6; RT = 15.4); Mannose (7; RT = 16.1); Galactose (8; RT = 16.5); Glucose (9; RT = 16.9); and Sorbose (10; RT = 17.4). (b) Monosaccharides contents in polysaccharide

monosaccharides under the same analytical conditions [Figure 1a] and was identified as mannose, galactose, and glucose in the molar ratio of 1:6.12:5.60 [Figure 1b]. Findings indicated that PLY is typically PLYs with the primary components mannose, galactose, and glucose, and this is consistent with the FT-IR spectroscopy data [Figure 2a]. FT-IR spectrum of PLY showed absorption peaks around 2923–2931 cm^{-1}, which were due to C – H stretching vibrations; the weak absorption peaks from 1400 to 1200 cm^{-1} were characteristic for PLYs.[27] Bands at 3265–3291 cm^{-1} were attributed to the hydroxyl group stretching vibrations. The intense absorption peaks at 1200–1021 cm^{-1} were assigned to C – O – C glycosidic stretching vibration. The peaks at 1121, 1026, and 1023 cm^{-1} indicated to the presence of a β-pyran linkage.[28] The strong signal at 917 cm^{-1} suggested the presence of β-glycosidic linkage,[28] supported by a weak band at 851 cm^{-1}. PLY exhibited signals at 1634, 1410 cm^{-1} and 1596 and 1410 cm^{-1} related to the absorption of the deprotonated carboxylic group (COO–).[29] The findings support that PLY has functional groups of a PLY.

PLY exhibits content of uronic acid (3.05%) which is supported to the absorption signal at 1634 cm^{-1} for a carbonyl group and showed a high content of carbohydrates (84.23%) and a low protein content was observed (0.019%). The molecular weights of PLY were evaluated using a linear relationship between the retention time and the logarithm of the molecular weight in the range of 5-500 kDa employing Dextran standards of different molecular weights [Figure 2b]. HPLC chromatographic results indicated that the molecular weight of PLY was >50 kDa, with an average molecular weight of 102326 Da. The PLY showed no absorption at 260 or 280 nm. The lack of nucleic acids and protein in PLY was demonstrated in the Bradford assay. Compound had a poor spectrum, and the results are not presented.

Antioxidant activity *in vitro*

The antioxidant effect of the polysaccharide is shown in table 1 where its effects are compared with the following standards quercetin, ascorbic acid, EDTA, curcumin, trolox, BHA, and gallic acid using DPPH radical, reducing power, chelating activity, β-carotene-linoleic, hydroxyl, superoxide, NO, and ABTS assays. Results clearly indicate that PLY has a noticeable capacity of scavenging toward free radical test compared to standards [Table 1].

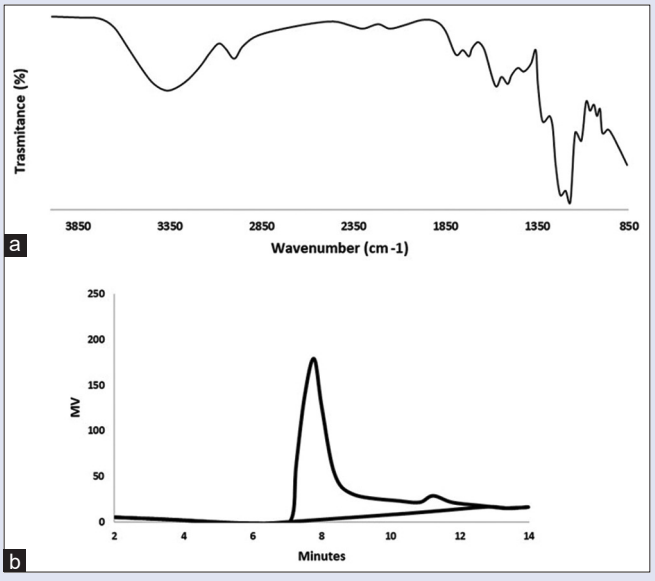

Figure 2: (a) FT-IR spectrum of polysaccharide; (b) Molecular weight of polysaccharide determined by HPGPC

Effect of polysaccharide on cell viability of H_2O_2-treated INS-1 cells

Exposition of INS-1 cells to H_2O_2 (500 μM) for 24 h derived in a significant cytotoxicity which was measured using MTT assay and LDH leakage test. Cell death was differently observed from H_2O_2 (500 μM) compared to control cells. The maximum inhibition in cell viability was observed of 49.1% at the highest concentration of 500 μM H_2O_2 ($P < 0.05$ vs. control) [Figure 3a]. Then, to evaluate the protective effect of PLY against H_2O_2-induced pancreatic cells damage, INS-1 cells were pretreated with 1, 5, and 10 g/mL of PLY during 2 h continued by treatment in the absence or presence of H_2O_2 for 24 h. Protective effect of PLY was observed of 68.5% at the dose of 1 μg/mL and 98% at 10 μg/mL of pretreatment.

Measurement of lactate dehydrogenase, ATP, reactive oxygen species, and malondialdehyde expression levels of H_2O_2-treated INS-1 cells

After we evaluated the cytotoxicity of PLY, LDH enzyme activity was performed to verify the membrane damage. INS-1 cells exposed to H_2O_2 (500 μM) for 24 h were measured the LDH activity. As shown in Figure 3b, an increase in LDH release of 33.3% was observed compared to control cells. However, PLY pretreatment reduced LDH release 1.75 fold. Pretreating the INS-1 cells with PLY significantly inhibits the increase of LDH levels in a dose-dependent manner. A decrease in Adenosine triphosphate (ATP) level was observed under oxidative conditions.

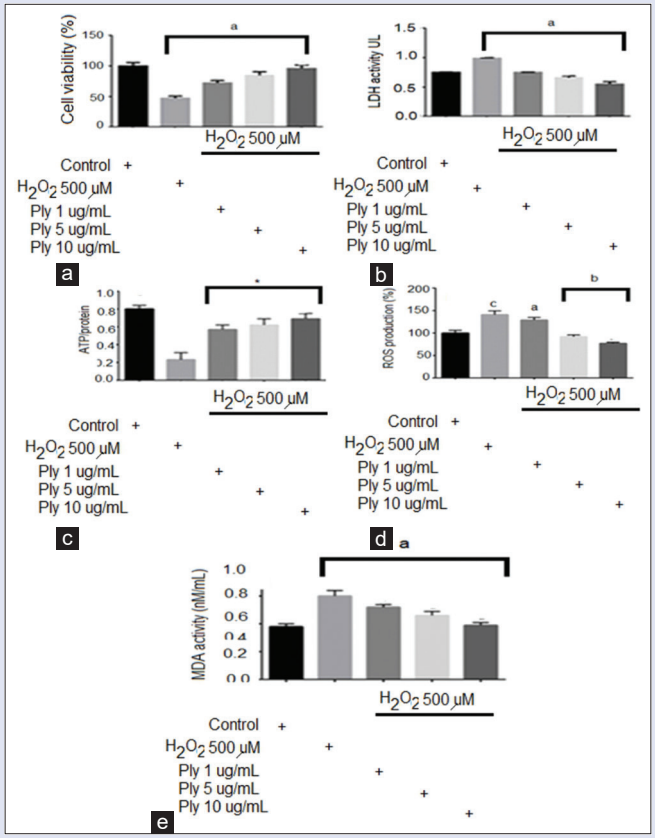

Figure 3: INS-1 cells stimulated H_2O_2; (a) cell viability; (b) LDH activity; (c) ATP concentration; (d) Reactive oxygen species production measured using DCFH-DA assay; (e) malondialdehyde activity; *$P < 0.05$ vs. H_2O_2 treated alone group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared to the control

Table 1: Antioxidant activity of polysaccharide 1 in the performed assays

Sample	EC_{50} (μg/mL)							
	DPPH radical	β-carotene-linoleic	Chelating activity	FRAP	Hydroxyl	Superoxide	NO	ABTS
1	4.6±1.02	14.4±3.08	43.1±1.50	8.1±2.38	25.7±3.11	34.9±4.21	74.3±5.45	5.7±1.37
Standard	EC_{50} (μg/mL)							
	DPPH radical	β-carotene-linoleic	Chelating activity	FRAP	Hydroxyl	Superoxide	NO	ABTS
Quercetin	2.39±0.01	10.1±1.35	221.9±12.64	2.1±0.19	-	42.6±5.71	-	-
Ascorbic acid	4.5±0.19	849.5±23.19	2.7±24.19	7.5±2.34	-	-	-	-
EDTA	-	-	5.7±1.07	-	-	-	-	-
Gallic acid	-	-	-	-	16.7±4.21	7.9±1.78		-
Curcumin	-	-	-	90.23±12.28	-	-	91.9±23.16	-
Trolox	4.2±0.91	-	27.3±6.12	-	35.5±3.5	-	-	9.0±2.05
BHA	0.43±0.002	15.1±2.24	282±15.4	7.68±2.60	26.85±7.3	-	-	7.2±2.32

EDTA: Ethylenediaminetetraacetic acid; FRAP: Ferric reducing power; DPPH: 2,2 Diphenyl 1 picrylhydrazyl radical; ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, BHA: Butylated hydroxyanisole, NO: Nitric oxide

Mitochondria-targeted antioxidants as PLY re-establish mitochondrial function and avoid the loss of intracellular ATP [Figure 3c]. Taking together, these findings indicate that mitochondria-targeted antioxidants can be important therapies in enhancing insulin secretion and protecting pancreatic islets in type 2 diabetes. The ability of H_2O_2 to produce intracellular ROS was determined using a nonfluorescent precursor of DCF (DCFH2-DA) and used as a test for oxidative stress, as shown in Figure 3d, when the INS-1 cells were exposed to 500 μM of H_2O_2, increased significantly the generation of ROS in 41%, compared with the control group. Instead pretreatment with PLY inhibited DCF fluorescence intensity, which is an indicator of ROS accumulation in a concentration-dependent manner in INS-1 cells compared to control. Pretreatment with 10 μg/mL of PLY decreased 1.83 fold ($P < 0.01$) in ROS accumulation compared with the levels in the control resulted in a high protection of H_2O_2 induced cell injury. The MDA level indicates the degree of lipid oxidation associated with pancreas damage in diabetes. The MDA level was increased in INS-1 cells exposed to high levels of H_2O_2 (500 μM) compared with the control group. PLY could significantly decrease the MDA content in islet β (INS-1) cells with concentration-dependent manner [Figure 3e], leading to a decrease in pancreatic damage caused byoxidative stress.

Measurement of superoxide dismutase, catalase glutathione peroxidase, and glutathione expression levels of H_2O_2-treated INS-1 cells

The improvement of PLY on neutralizing ROS-mediated oxidative stress was evaluated in levels of antioxidant enzymes such as SOD, CAT, and GSH-Px. Activity of antioxidant enzymes decreased in the presence of H_2O_2 for the ROS generation [$P < 0.05$; Figure 4a-c] compared with the control model. PLY pretreatment at dose of 10 μg/mL increased expression of SOD, CAT, and GSH-Px keeping the antioxidant enzyme activities. In this study, we used H_2O_2 toxicity in INS-1 cells as model to measure the antioxidant defense system in INS-1 cells treated with PLY previous to exposure to H_2O_2. PLY decreases cytotoxicity generated from H_2O_2, and significantly inhibits ROS generation increasing levels of CAT, SOD, and GSH-Px stimulated cellular antioxidant defense system, which maintaining an intracellular redox balance and acts as free radical scavenging. These results suggest that PLY could diminish H_2O_2-induced intracellular oxidant stress, either by protecting the severe depletion of antioxidant enzymes or by the direct scavenging of ROS.

Inhibitory effect of polysaccharide on advanced glycation end-products formation

Figure 5a shows the effects of PLY at concentrations of 0.5, 1.0, 1.5, and 2.0 mg/mL on AGEs formation through 4 weeks of incubation. The

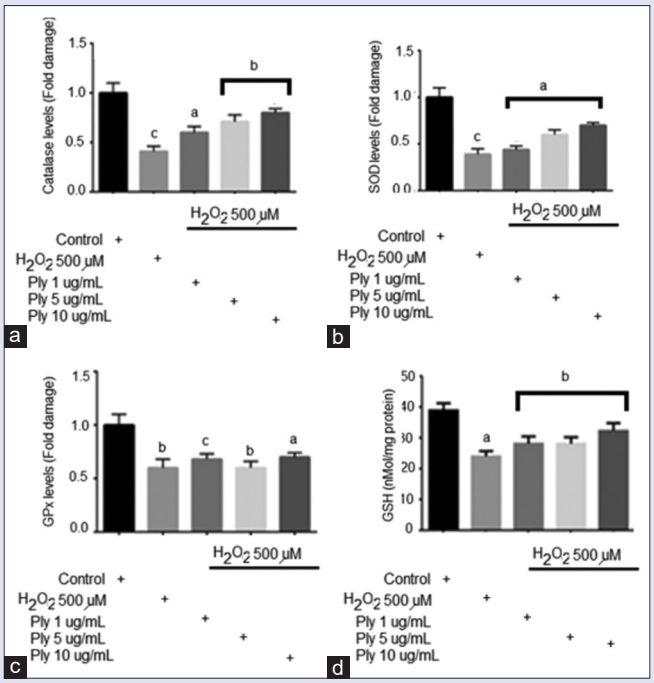

Figure 4: (a) Catalase levels; (b) Superoxide dismutase levels; (c) GPx levels; (d) GSH levels. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ compared to the control

fluorescent intensity of AGEs formation in the BSA/glucose systems markedly increased 3.6 fold 4 weeks of incubation compared to BSA, suggesting the progressive formation of AGEs. There was a significant ($P < 0.05$) reduction in AGEs formation compared to the control without treating with inhibition of 74.75 %, in the presence of 2 mg/ml of PLY This value is the percentage of inhibition obtained with the AG as a positive control (74.27%).

Inhibitory effect of polysaccharide on fructosamine formation

Fructosamines are early glycation products, as shown in Figure 5b; PLY significantly ($P < 0.05$) suppressed fructosamines formation with the range from 52.68% to 73.1% in BSA/glucose. The inhibitory effect of PLY was found to be less efficient than AG when it is used as a positive control, which had a reduction of 80.6%.

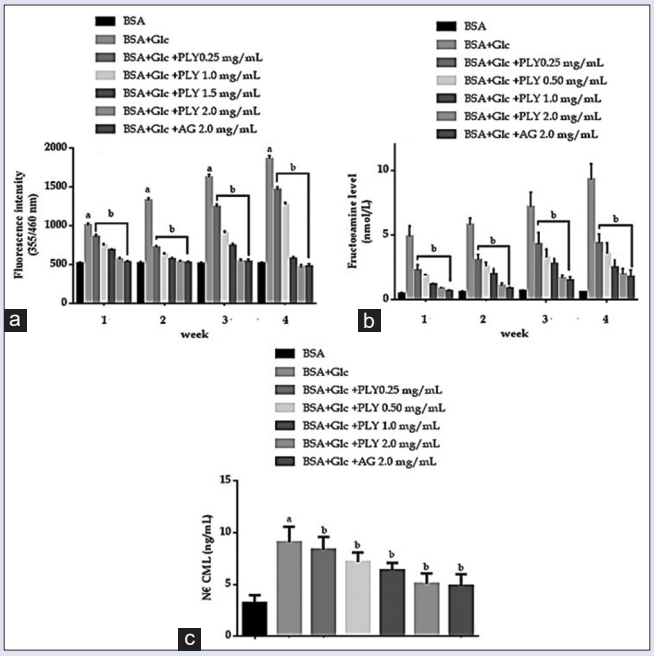

Figure 5: The effects of polysaccharide (0.5, 1.0, 1.5 and 2 mg/mL) and aminoguanidine (2.0 mg/mL) on: (a) fluorescent advanced glycation end-products formation; (b) Level of fructosamine; (c) formation of Nε-(carboxymethyl) lysine in the bovine serum albumin/Glc (glucose) system after 4 weeks of the experiment. Each value represents the mean ± standard deviation ($n = 3$). $^{a}P < 0.05$ compared to bovine serum albumin, $^{b}P < 0.05$ compared to bovine serum albumin + Glc

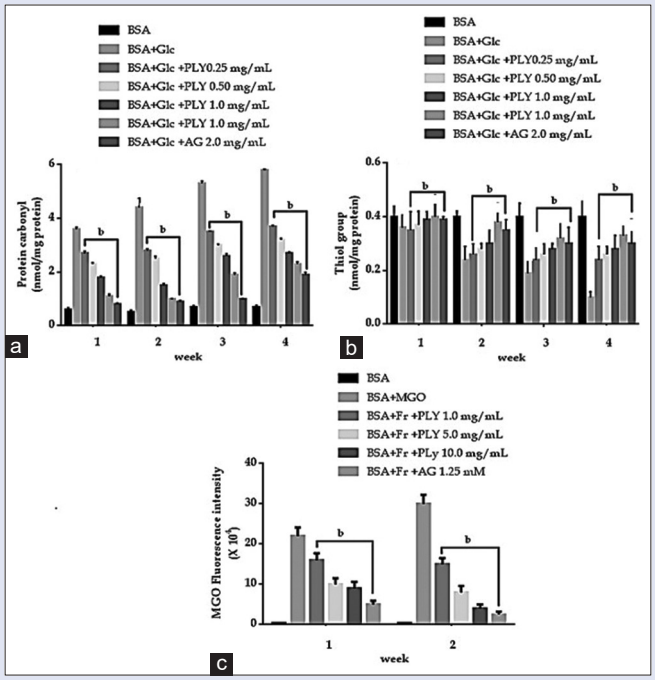

Figure 6: The effect of polysaccharide on glycation-induced protein oxidation. (a) protein carbonyl formation; (b) on thiol group content; (c) methylglyoxal content of fructose-modified bovine serum albumin; each value represents the mean ± standard deviation ($n = 3$). $^{a}P < 0.05$ compared to bovine serum albumin, $^{b}P < 0.05$ compared to bovine serum albumin + Fr

Measurement of Nε-carboxymethyl-lysine inhibitory effect of polysaccharide

Nε-CML is the most abundant product of glycation reaction, and its level was determined at week 4 of incubation. Nε-CML increased in BSA/glucose [2.8 fold; Figure 5c]. However, PLY at 2.0 mg/mL inhibited Nε-CML formation in 43.9% in BSA/glucose, nevertheless AG decreased Nε-CML in 46.1%formation in BSA/glucose.

Determination of protein carbonyl content

Protein carbonyl content is considered as an oxidative modification of BSA and is an intermediate stage marker. The carbonyl content of glycated BSA/glucose (6.0 fold to 8.2 fold increase) was significantly ($P < 0.05$) increased during the 4 weeks of incubation. PLY can inhibit protein carbonyl content in the range from 36.20% to 60.34% in BSA/glucose [Figure 6a] compared to nonglycated BSA at 4 weeks of incubation, while that by AG showed inhibition of 67.2%.

Thiol groups measurement

Structural alteration of glycated BSA can be evaluated by depleting the thiol group. When BSA was incubated with glucose or fructose, the level of thiol groups significantly ($P < 0.05$) decreased through the 4 weeks of incubation. Inhibition of depleting thiol group was in the range of 24%–69.6% in BSA/glucose [Figure 6b], whereas AG prevented the depletion of protein thiol groups by 66.6%.

Effect of polysaccharide on bovine serum albumin/methylglyoxal

The fluorescence intensity of BSA/MGO incubated by 1 and 2 weeks increased by 55 fold and 75 fold, respectively, compared with BSA incubated by 1 and 2 weeks. When PLY (1–10 mg/mL) was incubated with BSA/MGO, the formation of MGO-derived significantly ($P < 0.05$) decreased. After 1 week of incubation, reduction ranged was 27.2% to 59.5% and 50% to 86.6% in week 2 [Figure 6c]. However, AG at week 2 suppressed almost completely the formation of MGO-derived.

Antiaggregatory effect of polysaccharide in Congo red and Thioflavin T assays

Glycation induces conformational changes of protein and increases the level of amyloid cross β-structure, having an essential role in protein aggregation. The ability of PLY to retard aggregation of BSA was evaluated using two amyloid markers such as Congo red and Thioflavin T assays.

As shown in Congo red assay [Figure 7a], we found a significant increase of 1.2 fold in amyloid cross-β structures at 4 weeks of incubation. However, BSA/Fr incubated with PLY (0.25–2.0 mg/mL) significantly ($P < 0.05$) attenuated the level of amyloid cross-β structure in a concentration-dependent manner in a range of 11.1% to 18%. Furthermore, co-treatment with AG (2 mg/mL) at week 4 of incubation showed a significant ($P < 0.05$) reduction in the level of amyloid cross-β structure (11.2%).

Thioflavin T assay shown in Figure 7b, the BSA/Fr group exhibited significantly ($P < 0.05$) more amyloid cross-βstructures than the nonglycated group. After 4 weeks of incubation with PLY the % inhibition of amyloid cross-β structures was 24.7%, 38.8%, 46.2%, and 57% at concentrations of 0.25 mg/mL, 0.50 mg/mL, 1.0 mg/mL, and 2.0 mg/mL, respectively. However, treatment with AG (2 mg/mL) exhibited a fluorescence intensity (55%) similar to PLY at the same concentration.

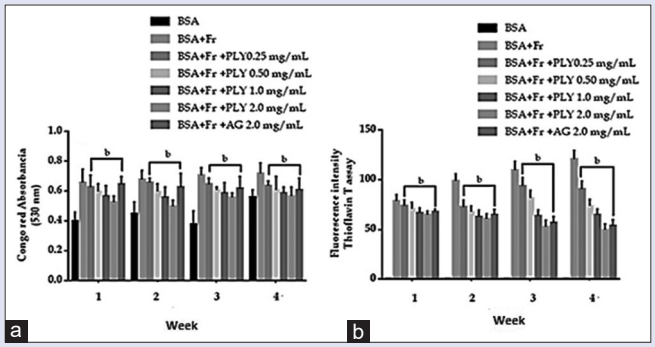

Figure 7: Inhibitory effect of polysaccharide on (a) amyloid cross-β structures by Congo red assay. (b) Amyloid cross-β structures by Thioflavin T assay. Results are showed as the mean ± standard deviation $^{a}P < 0.05$ when compared to bovine serum albumin; $^{b}P < 0.05$ when compared to bovine serum albumin/Fr at the same week of incubation

DISCUSSION

The PLY isolated has a molecular weight of 102326 Da. The PLY from *C. argyrosperma* consisted in mannose, galactose, and glucose monosaccharides by GC, indicated the presence of β-glycosidic bonds. However, it was proved that PLY had the potential radical scavenging ability to different radicals and decreasing AGEs formation.

Since is known that a single test to evaluate antioxidant effects of the compounds with different chemical and physical properties, modes of action, is not enough, thus is recommendable use several assays to evaluate plant extracts or compounds. Of these, the most commonly used for the evaluation of antioxidant activities including DPPH radical, ABTS•+ cation radical, chelating activity, β-carotene-linoleic, FRAP, hydroxyl, superoxide, and NO. PLY demonstrated good antiradical effect, like to ascorbic acid and trolox. The possible mechanism of PLY can be owing to electron donation capacity to the free radicals completing the radical chain reaction increasing the half-life of natural antioxidants. ABTS•+ cation radical assay shown that PLY has greater chelating ability toward ABTS•+ cation radical than BHA and trolox used as standard. Metal chelating ability is one antioxidant mechanism, which in biological systems catalyzing the oxidative changes of cellular content and is known that poorly liganded or free iron is the source of many diseases involving apoptosis and cell death. Thus, the metal chelating ability of the polysaccharide was studied. PLY has high metal chelating ability in comparison with quercetin and BHA. OH radical reacts with DNA, proteins, and amino acids, causing cell damage or death.[29] PLY showed a high *in vitro* antioxidant effect toward hydroxyl radical scavenging activity compared with the antioxidant gallic acid, trolox at tested concentrations. Nevertheless, PLY has activity similar to that of BHA. PLY has ability to counteract the formation of NO radicals. This compound executing major free radical scavenging activity against NO as suggested their EC_{50} values compared with curcumin used as standard. The transformation of SOD by oxygen-free radicals to H2O2 molecules is caused by an excessive generation of ROS producing lipid peroxidation.[30] PLY showed major activity to the gallic acid and quercetin. In the discoloration of β-carotene test, oxidation of linoleic acid generates hydroperoxyl radicals, conjugated dienes, and lipid hydroperoxides.[31] The addition of PLY difficulty the whitening preventing formation of linoleic hydroperoxyl radicals. PLY showing major activity to the ascorbic acid, and no differed significantly in activity with BHA. PLY demonstrated that reducing power ability could be due to reduction of Fe^{3+} ferricyanide complex to the ferrous form, by donation of a hydrogen atom breaking the free radical chain. PLY showed major activity to the curcumin and no differed significantly in activity with ascorbic acid and BHA.

The exposure of INS-1 cells to a high H_2O_2 level (500 μM) for 24 h significantly ($P < 0.05$) reduced their viability, while PLY treatment suppresses cell death suggesting that PLY protected INS-1 cells against oxidative conditions. In this study, we demonstrated that PLY protects pancreatic β-cell under oxidative (H_2O_2) and glucotoxic conditions as results of a suppression in oxidative stress, oxidative injury, renovation of intracellular ATP and the conservation of the morphology, function and mitochondrial content. Hyperglycemia is main contributing factor to the development of type 2 diabetes favoring the production of mitochondrial ROS in pancreatic islets which is consequence of low expression of antioxidant enzymes in pancreatic cells[32] making them susceptible to impairment by mitochondrial ROS. An important cause of the formation of reactive aldehyde and lipid peroxidation (MDA) in pancreatic islets is mitochondrial ROS. Lipid peroxidation produces oxygen-free radical causing toxicity to membrane-bound enzymes and cellular organelles contributing to progressive degradation of pancreatic β-cell function. Thus, evaluation of lipid peroxidation can supply a good index of the functional impairment of INS-1 cells. Exposure to a high H_2O_2 concentration increased the content of lipid peroxides in pancreatic cells. PLY reduces lipid accumulation by preventing mitochondrial ROS in oxidative (H_2O_2) conditions.

Enzymes antioxidant in the human includes CAT SOD, GSH-Px, and GSH which are important defense enzyme that acts like biological scavenger of ROS in the organism. These enzymes prevent glucose toxicity, consequently they avoid diabetes impairment of pancreatic β cells. Findings indicate that PLY reduced the MDA level and increased the level of enzyme antioxidant in H_2O_2-treated INS-1 cells. Our results suggest that the mechanism by which PLY protects INS-1 cells from H_2O_2-induced cytotoxicity is decreasing lipid peroxide production and raising the activity of antioxidant enzymes.

The accumulation of the reaction products of protein glycation in living organisms leads to modifications of tissue proteins. Many studies have shown a significant role for glycation in the progress of normal aging and the pathogenesis of metabolic diseases. Glucose metabolites participate in the glycation reactions generating an Amadori product (fructosamine).[33] Therefore, the inhibition of fructosamine can be a therapeutic form to reduce vascular complications. The reaction of Amadori products with amino acids leads to a formation of irreversible AGEs. The findings indicated that PLY successfully inhibited the initial stage of glycation inhibiting conversion to AGEs.

The measured BSA/fructosamine levels are used as a biomarker of the accumulation of Amadori products in the early glycation. The results indicated that PLY inhibited the level of fructosamine, preventing conversion into Nε-CML produced from the oxidative breakdown of the Amadori product, which is an indicator in the formation of nonfluorescent AGEs.

The ROS is produced during glycoxidation and glycation, oxidizing the side chains of amino acid residues to generate carbonyl groups, reducing the oxidative defense of protein by decline thiol groups. A loss of free thiol groups by alteration of cysteine residues and the increase in carbonyl content was evaluated during the glycation process as a marker of protein oxidation. In our study, results indicate that glucose-induced glycation increased protein carbonyl level and decreased thiol group testifying to the increase in protein oxidation.[34] When PLY was incorporated in the same systems, it has shown remarkable potential in suppressing the protein carbonyl content and increased the protein thiol group.

MGO is a reactive carbonyl species capable of protein glycation leading to the formation of AGEs. In the intermediate stage, reactive carbonyl species, particularly methylglyoxal and 3-deoxyglucosone, are produced

by the degradation of Amadori products and autoxidation of glucose. The reaction with lysine residues and MGO is irreversible, forming MGO-derived lysine dimer. Besides, PLY reduced the formation of AGEs through a direct trapping MGO. PLY from *C. argyrosperma* seeds can form adducts with MGO when it is incubated.

The formation of protein aggregation can be affected by glycation, which is known to produce conformational modifications in proteins. These aggregates form an amyloid cross β-structure, which modifies protein stability and structure.[35] Accumulation of protein aggregation in the tissues causes the progression of pancreatic islet amyloidosis inducing β-cell damage and impairs insulin secretion and neurodegenerative disease. Findings establish that PLY can suppress the level of amyloid cross β-structure in albumin *in vitro*. In all assays conducted in this study was used, drug AG was employed which is a hydrazine compound, which traps intermediates at the initial glycation stages, avoiding AGEs formation.[36]

CONCLUSION

PLY exhibited strong antioxidant activities, which were comparable to the commercial antioxidant ascorbic acid, EDTA, trolox, and BHA. Findings indicate that PLY enhanced viability, reduced ROS production, increased level of CAT, SOD, GPx, and GSH level restoring mitochondrial function in H_2O_2-exposed INS-1 cell, in addition, can protect the initial and intermediate stages of glycation, leading to a reduction in the formation of AGEs in the late stage. The beneficial effect of PLY to reduce AGEs formation may be due to antiglycation mechanisms, such as blocking the formation of late-stage Amadori products. PLY acts as carbonyl trapping agent forming dicarbonyl adducts, decreasing the number of free carbonyl groups in the early stages of glycation and breaking the cross-linking structures in the formed AGEs. These results demonstrated different modulatory roles of PLY in the maintenance of β-cell physiology against ROS and formation of AGEs.

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Conflicts of interest

There are no conflicts of interest.

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