

Polyphenol-Rich Pomegranate Juice Reduces IgE Binding to Cashew Nut Allergens

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Running title: Pomegranate juice attenuates cashew allergens

ABSTRACT

BACKGROUND: Food allergy negatively impacts quality of life and can be life-threatening. Cashew nuts can cause severe reactions in very small amounts, and they are included in a group of foods most commonly responsible for causing food allergy. Polyphenols and polyphenol rich juices have been demonstrated to complex with peanut allergens. Here, the interaction between cashew nut allergens and polyphenol rich juices is evaluated biochemically and immunologically.

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RESULTS: Various juices, including pomegranate (POM), blueberry (BB), and concord grape (CG) juices, were evaluated for polyphenol content and formation of polyphenol-cashew allergen complexes. Among the various juices studied, POM juice showed a greater capacity to form complexes with cashew proteins. Dynamic light scattering (DLS) demonstrated a sharp increase in cashew protein extract particle size to around 3580 nm, and fewer cashew proteins were resolved by electrophoresis after treatment with POM juice. Immunoassays demonstrated reduced IgG and IgE binding to cashew allergens due to allergen precipitation by POM juice. These observations support the formation of complexes between polyphenol and cashew proteins that can prevent antibody recognition of cashew allergens through allergen precipitation.

CONCLUSION: POM juice treatment of cashew extract effectively reduces antibody binding through allergen precipitation, and these findings could be applied to the development of less allergenic cashew nut products and oral immunotherapy.

Keywords: cashew nut; allergen; allergy; polyphenol; pomegranate

INTRODUCTION

Approximately 5% of adults and 8% of children have been reported have food allergies.¹ The symptoms of food allergy generally occur within minutes to hours after intake of allergenic food.^{2,3} In severe cases, food allergy can be life-threatening and has been identified as the most major cause of anaphylaxis treated in emergency departments in a number of countries.^{1,4,5} Although more than 170 foods have been reported to be allergenic,² the eight most commonly reported foods causing allergy are milk, egg, fish, shellfish, peanut, tree nut, soy, and wheat.^{6,7}

Along with an increase in cashew nut consumption, there is an increasing number of cashew allergic reaction reports.⁸ Cashew nut allergens can be highly potent and cause severe reactions even in very small amounts.⁸⁻¹⁰ A clinical study by van der Valk et al.⁹ showed that 17% of study participants had anaphylactic reactions after ingesting just 1 mg of cashew nut protein. In addition, Davoren et al.¹⁰ reported that although more patients showed allergic reactions to peanut than cashew nut, the cashew nut allergy group had a greater chance of developing anaphylaxis, where 19 out of 22 patients showed positive reactions after ingestion of cashew nut.

The three cashew nut seed storage proteins thought to be responsible for causing allergic reactions are Ana o 1, Ana o 2, and Ana o 3.¹¹⁻¹⁴ Ana o 1 is a 7S vicilin-like protein of 50 kDa,¹² and Ana o 2 is a 11S legumin-like protein with two subunits linked by disulfide bonds, a basic subunit of 20 kDa and a larger acidic subunit of 33 kDa.^{11,13} Ana o 3, identified as 2S albumin of 13 kDa, is known to have at least three isoforms.^{11,14} These proteins have proven to be relatively resistant to food processing methods aimed at reducing their allergenic potency.¹⁵

Venkatachalam et al.¹⁶ systematically studied the effect of various physical and mechanical

processing methods, including autoclaving, blanching, microwave heating, roasting, γ -irradiation, and pH, on the stability of Ana o 1, Ana o 2, and Ana o 3. In most cases cashew nut allergens retained at least some immunoreactive after these treatments, showing high stability under various environments.¹⁵⁻¹⁷

There could be advantages to using natural food grade compounds to reduce or eliminate food allergen potency such as reduced costs, less equipment, and the obvious advantage of using natural additives. The application of phenolic acids and dietary polyphenols has been shown to effectively attenuate IgE binding and basophil degranulation to peanut allergens.¹⁸⁻²¹

Polyphenols are abundant in various fruits, vegetables, and beverages,^{22,23} and they offer several health benefits.²⁴ Polyphenols can reduce oxidative stress, and may prevent cancer or cardiovascular diseases.²⁴⁻²⁷ In addition, polyphenols may form soluble or insoluble complexes with protein through hydrophobic and hydrogen interactions.^{28,29} Soares et al.³⁰ reported that polyphenol associates with different proteins including bovine serum albumin and human α -amylase. The size and structure of polyphenol compounds significantly altered their binding affinity, with increased affinity as the molecular weight increased or in the presence of galloyl groups.³⁰ Tea polyphenols formed complexes with milk α - and β -caseins, and structural analysis demonstrated both hydrophobic and hydrophilic interactions.³¹ The idea was further extended to food allergens, where polyphenols interact with food allergens thereby reducing the allergenicity of allergenic foods. For example, Chung et al.¹⁸ reported the addition of phenolic acid to peanut extracts and liquid peanut butter reduced IgE binding. Similarly, tannic acid removed peanut allergens by forming polyphenol-protein complexes.¹⁹ Plundrich et al.^{20,21} studied the effect of various polyphenol-rich plant juices and extracts on peanut flour allergenicity, and their results

indicated that cranberry juice treated peanut flour samples had decreased allergic response in a mouse model.

There are several polyphenol-rich beverages that are commonly consumed in the United States including pomegranate juice, red wine, concord grape juice, and blueberry juice, and these have higher polyphenol content compared to black cherry juice, açai juice, cranberry juice, orange juice, apple juice, and ice tea.³² Punicalagin (structure shown in Figure 1), a large polyphenol with a molecular weight greater than 1000, is the major polyphenol compound of pomegranate.^{33,34} CG juice and BB juice also contain a high amount of polyphenols including chlorogenic acid, peonidin-3-galactoside, and ellagic acid (Figure 1).^{32,33,35,36} The present study evaluated the ability of various polyphenol-rich juices to interact with cashew allergens.

EXPERIMENTAL

Materials

Cashew nuts were purchased from Nuts Online (Cranford, NJ, USA), pepsin from porcine gastric mucosa and Folin & Ciocalteu's phenol reagent was from Sigma-Aldrich (St. Louis, MO, USA), gallic acid and Clear Flat-Bottom Immuno Nonsterile 96-well plate was purchased from Thermo Fisher Scientific (Waltham, MA, USA), Mini-Protean TGX precast gels were from Bio-Rad Laboratories (Hercules, CA, USA), and LDS sample buffer (4×) was from Life Technologies (Carlsbad, CA, USA). POM juice used in this study was from POM Wonderful LLC (Los Angeles, CA, USA), CG juice was from Whole Foods Market Inc. (Austin, TX, USA), and BB juice was from R. W. Knudsen (Chico, CA, USA). Biotinylated anti-human IgE antibody and polyclonal rabbit anti-cashew antisera were purchased from Southern Biotech (Birmingham, AL,

USA) and Pierce Biotechnology (Rockford, IL, USA), respectively. Both IRDye 800CW Donkey Anti-Rabbit IgG and IRDye 800CW streptavidin were purchased from LI-COR (Lincoln, NE, USA). Human sera from cashew-allergic patients with a documented history of clinical cashew allergy were obtained from PlasmaLab International (Everett, WA, USA).

Cashew extract preparation

Defatted cashew flour was generated by defatting ground nuts with petroleum ether using a Soxhlet apparatus overnight. The defatted cashew flour was mixed with 100 mM sodium phosphate buffer (pH 6.8) for one hour, sonicated three times on ice for 15 s using a Sonic Dismembrator (Fisher Scientific Co., Orlando, FL, USA), and then centrifuged for 30 min at 12000 rpm at 4°C. The clarified extracts were collected, protein concentrations were determined by spectrophotometer at 280 nm (Pharmaspec UV-1700, Shimadzu, Kyoto, Japan), and samples were stored frozen at -20°C.

Determination of total phenolics

Total phenolics of POM juice/ BB juice/ CG juice were determined by colorimetric method using Folin-Ciocalteu reagent and expressed as mg gallic acid equivalents per liter.³⁷ To prepare samples, 70 µL of diluted juices were mixed with 350 µL 1:10 diluted Folin-Ciocalteu reagent (2N) for 2 min at room temperature, followed by adding 1050 µL of 7.5% Na₂CO₃. After incubation in darkness for 2 h, the absorbance of samples was measured at 765 nm using a UV-Visible spectrophotometer. To establish standard curve, gallic acid with concentrations ranging from 0 to 500 mg gallic acid/L was used.

Polyphenol assay for protein binding

Polyphenol interaction with cashew extract was evaluated by SDS-PAGE following steps from previous reports with minor modifications.^{18,19} To prepare polyphenol-cashew extract complexes, 3 μ L of (2 mg/mL) cashew extract was mixed with 27 μ L of POM juice/ BB juice/ CG juice of various concentrations at room temperature for 15 min. Then the mixture was centrifuged at 14,000 rpm for 5 min. Supernatant was collected and mixed with NuPAGE LDS sample buffer (4 $\times$) using a 1:4 (v/v) ratio prior to electrophoresis. SDS-PAGE was performed under non-reducing conditions without the addition of reducing agent into the sample buffer. After electrophoresis, gels were stained with SimplyBlue SafeStain and destained with water. The Odyssey CLx infrared imaging system (LI-COR, NE, USA) was used to capture and quantify gel images.

Immunoblotting

Samples resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 30 min at room temperature in phosphate buffered saline with Tween-20 (PBST) with 2% (w/v) nonfat dry milk. The membrane was then incubated with 1:1000 PBST diluted rabbit anti-cashew antibody for 1 h at room temperature. The membrane was washed three times for 5 min in PBST, followed by addition of anti-rabbit IRdye-800CW (1:10000 in PBST). After 30 min, the membrane was washed as above and subject to imaging with an Odyssey CLx infrared imaging system. The data were expressed as a percentage of IgG antibody binding using the following formula: (1) For whole cashew extract: IR800 value of each lane/IR800 value of lane of cashew extract without treatment; (2) For Ana o

3: IR800 value of Ana o 3 band/IR800 value of Ana o 3 band without treatment. The data were plotted as the average of three experiments with standard deviation as error bars.

Dynamic light scattering analysis

The average particle diameter of cashew extracts before and after POM juice treatment was measured with a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK).³⁸ The system was equipped with 90° detection optics with respect to the laser beam. To prepare POM juice treated samples, 540 µL of POM juice was mixed with 60 µL of cashew extract and incubated at room temperature for 15 min. Then the mixture was transferred to a polystyrene cuvette for analysis. Before each test, samples were equilibrated for 120 s at 25°C. Each sample was analyzed in triplicate with eleven runs of 10 s.

***In vitro* simulated gastric fluid (SGF) assay**

The stability of cashew extract after POM juice treatment was evaluated using a simulated gastric pepsin digestion essentially as described in Mattison et al.¹¹ 36 µL of cashew extract (2 mg/mL) was added to 324 µL of POM juice. After incubation for 15 min, the mixture was subject to centrifugation at 14000 rpm for 5 min. The polyphenol and cashew extract complexes were collected in centrifuge tubes for pepsin digestion, followed by adding simulated gastric fluid (SGF, 35 mM NaCl, 84 mM HCl, pH 1.2). As a control, 20 µL of the mixture was removed and incubated without pepsin digestion. Pepsin (4.8 µL of 1 mg/mL, 4000 U/mg) was added into the tube, giving a final concentration of 0.02 mg/mL. The tube was kept on ice throughout pepsin digestion. Samples (20 µL) of the digestive mixture were removed at 0, 5, 10, 20, 30, 40, 50, and 60 min into new tubes, which were centrifuged immediately at 14000 rpm for 5 min at

4°C. The supernatant was then collected and analyzed by both electrophoresis under non-reducing conditions and immunoblotting as described above.

Competitive enzyme-linked immunosorbent assay (ELISA)

Human sera IgE and rabbit IgG binding to POM juice treated cashew extract was evaluated by competitive ELISA as described by Mattison et al.³⁹ with minor modifications. Microtiter plates were coated with 50 µL of cashew extract in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) overnight at 4°C. Plates were blocked with 1% BSA (w/v) in PBST at room temperature for 30 min. POM juice with gallic acid equivalent concentrations ranging from 0 to 636 mg/mL were mixed with 9 µL of cashew extract, and the mixture was incubated at room temperature for 15 min, allowing for interaction between polyphenol from POM juice and cashew extract.¹⁹ The mixture was then centrifuged at 14000 rpm for 5 min, and 60 µL of supernatant was collected.¹⁹ Pooled human sera from cashew allergic patients (90 µL) was added into each tube and incubated at 37°C for 1 h, enabling the interaction between IgE antibody and cashew allergens. The mixture (50 µL) was transferred to the microtiter plate wells and incubated at 37°C for 1 h. The reaction solution was removed and wells were washed with 100 µL of PBST three times before adding 50 µL of biotinylated anti-IgE (1:1000 in PBST) to each well. After incubation at 37°C for 1 h, plate wells were washed as above and 50 µL of streptavidin-labeled IRdye-800CW (1:5000 in PBST) was added. After incubation for 30 min, plate wells were washed three times with PBST and imaged with Odyssey CLx infrared imaging system. For competitive ELISA with rabbit IgG, diluted rabbit anti-cashew sera (1:182 in PBST) was added to the POM juice treated cashew extract. After incubation for 1 h, 50 µL of the mixture was transferred to the blocked microtiter plate and incubated at 37°C for 1 h. The plate was then washed three times,

and 50 μ L of anti-rabbit antibody labeled with IRdye-800CW (1:1000 in PBST) was added into each well. The plate was incubated at 37°C for 30 min and washed and imaged with Odyssey CLx infrared imaging system. The data were plotted as the average of three experiments with standard deviation as error bars and were expressed as percent of IgE and IgG binding using the following formula: IR800 value of each sample/IR800 value of uninhibited sample $\times$ 100. The uninhibited sample was prepared by incubating cashew extract with POM juice with a concentration of 636 mg/mL. At this concentration, polyphenol from POM juice was predetermined to precipitate all assayable cashew allergen and served as the uninhibited standard.

Statistical analyses

Data shown in Figure 2, 3, and 6 represent mean values of three determinations with standard deviation used as error bars. The curve fitting of ELISA data was performed with Origin software (OriginLab Corporation, Northampton, USA).

RESULTS

Phenolic contents within POM juice, BB juice, and CG juice were determined by the Folin-Ciocalteu method and were expressed as gallic acid equivalents based on a gallic acid standard curve shown in Figure 2A.³⁷ As shown in Figure 2B, POM juice contained a higher phenolic concentration of 2725 mg/L compared with BB juice and CG juice, which had concentrations of 1729 mg/L and 1826 mg/L, respectively. These results are in line with previously reported data,³² in which POM juice had higher phenolic content than BB juice and CG juice. It is worth

noting that variety factors, including cultivar, growing location, growing temperature, level of carbon dioxide, and soil conditions, can affect the phenolic content within the fruit.^{40,41}

The effect of POM, BB, and CG juice on cashew allergens was evaluated with non-reducing SDS-PAGE using cashew extract samples after treatment with different concentrations of POM, BB, and CG juice. Fewer proteins were resolved after treatment (Figure 3A), suggesting interaction and formation of precipitates between polyphenol and cashew protein. The POM and CG juices formed precipitates through interaction with Ana o 1, Ana o 2, and Ana o 3. In contrast, although Ana o 1 and Ana o 2 were precipitated, a large amount of Ana o 3 remained after treatment with BB juice (Figure 3A).

The juice treated cashew extracts were evaluated by immunoblotting using rabbit anti-cashew antibodies. Significantly less IgG binding to cashew allergens was observed (Figure 3B) following the treatment, consistent with results obtained from SDS-PAGE analysis. As shown in Figure 3C, quantification of rabbit IgG binding in each lane was significantly reduced to less than 5%. The variation in protein precipitation between the juices and cashew nut allergens was apparent. Ana o 3 binding was reduced to 3% and 4% binding after treating with POM juice and CG juice, respectively (Figure 3D). However, around 30% of binding remained after treatment with BB juice, consistent with the higher Ana o 3 level observed in SDS-PAGE (Figure 3A). The data suggested that POM, BB, and CG juice effectively form complexes between polyphenol and cashew extract. Overall, POM juice demonstrated a greater capacity to form complexes with cashew proteins and reduce IgG binding through allergen precipitation.

The polyphenol-cashew nut extract complexes were evaluated by DLS measurement to assess particle size.³⁸ As shown in Figure 4, the untreated cashew extract has an average particle size of approximately 12 nm, with some aggregates of average size greater than 100 nm. However, the average size of POM juice treated sample shifted to approximately 3580 nm, with no peak corresponding to non-aggregated protein detected, indicating the formation of large matrices between polyphenol from POM juice and cashew extract.

The stability of the POM juice-cashew extract complexes to protease was evaluated *in vitro* using pepsin in an acidic environment (35 mM NaCl, 84 mM HCl, pH 1.2).^{11,20,21} The POM juice treated cashew extract was rapidly digested to fragments of smaller molecular weight (Figure 5A), and within 5 minutes the majority of Ana o 1 (50 kDa) and Ana o 2 were degraded. The Ana o 3 band, migrating just above the 10 kDa marker, remained undigested during the assay, consistent with previous studies.^{11,42} The signal intensity of a diffuse band migrating below the 10 kDa marker remained similar through the assay, indicating that they were not able to be digested further.¹¹ Immunoblotting with rabbit anti-cashew antibody was used to evaluate the immunoreactivity of cashew extract samples after digestion. As shown in Figure 5B, IgG binding to higher molecular weight proteins was significantly reduced after digestion, whereas Ana o 3 remained immunoreactive throughout the digestion process.¹¹ Binding to the diffuse band migrating under the 10 kDa marker was not observed. In general, POM juice treated cashew extracts were rapidly digested, and the digestion products were less immunoreactive. The results are consistent with data reported by Plundrich et al,²⁰ in which peanut protein-cranberry or green tea polyphenol complexes were rapidly hydrolyzed and had reduced antibody binding.

IgG binding to POM juice treated cashew extract was evaluated by competitive ELISA. As shown in Figure 6, POM juice treatment demonstrated a concentration dependent effect. The POM juice inhibited IgG binding through allergen precipitation with an IC₅₀ value of 119 mg/L. A similar experiment was performed with human sera (Figure 6B). A similar result with an IC₅₀ of 33 mg/L was observed, further demonstrating that the allergenic capacity of cashew extract was successfully reduced by precipitating cashew allergens with polyphenol-rich POM juice.

DISCUSSION

Among the three studied polyphenol-rich juices, POM juice demonstrated a greater capacity to form complexes with cashew proteins. The structure and concentration of polyphenol within the three different fruit juices is varied, and is likely responsible for the different capability of fruit juices to interact with cashew extract.^{18,19,30,37} Overall, Ana o 3 was harder to precipitate than Ana o 1 and Ana o 2. This is possibly due to the smaller size of Ana o 3, which has fewer active sites available for the interaction with polyphenol molecules, resulting in less protein-polyphenol cross-linking. Therefore, the precipitation of Ana o 3 by polyphenol is less effective than Ana o 1 and Ana o 2. In addition, structural differences between Ana o 3 and Ana o 1 or Ana o 2 could be another reason explaining the different binding capability. Previously, Soares et al.³⁰ studied the binding affinities of BSA and α -amylase with (+)-catechin, suggesting the important role of the three-dimensional structure of the proteins for the interactions.

With POM juice treatment, we observed reduced IgG and IgE binding to cashew nut allergens through allergen precipitation. In addition, it was reported that the interaction of polyphenol

with protein could directly modulate the process of allergic sensitization.^{19,43} Moreover, it was shown that polyphenol directly interferes with T-helper 2 activation, which inhibits allergy development.^{44,45} Other than possible effects on allergy alleviation, another health benefit from polyphenol is their endogenous antioxidant ability, which limits the extent of cellular injury from free radicals during the allergic reaction.^{43,44,46} With these health benefits, one of the potential applications of POM juice would be used as food additive for the development of less allergenic cashew products.¹⁹

POM juice treated cashew extract also holds the potential to be used for oral immunotherapy and provide protection against cashew sensitization. This assumption is supported by a previous study, in which peanut allergic mice were challenged with polyphenol treated peanut extract and the complexes are proved to be hypoallergenic.²¹ This implies the possible application of polyphenol-allergen complexes towards the development of oral immunotherapy. Further investigations such as *in vivo* or clinical studies are required to confirm the application.

CONCLUSION

To our knowledge, this is the first study of interaction between dietary polyphenols and cashew extract proteins. Polyphenols contained within POM juice formed complexes with cashew allergens and reduced IgG and IgE binding by precipitating allergens. The results are expected to assist in the development of cashew nut products with reduced immunoreactivity, and hold the potential to be used for oral immunotherapy.

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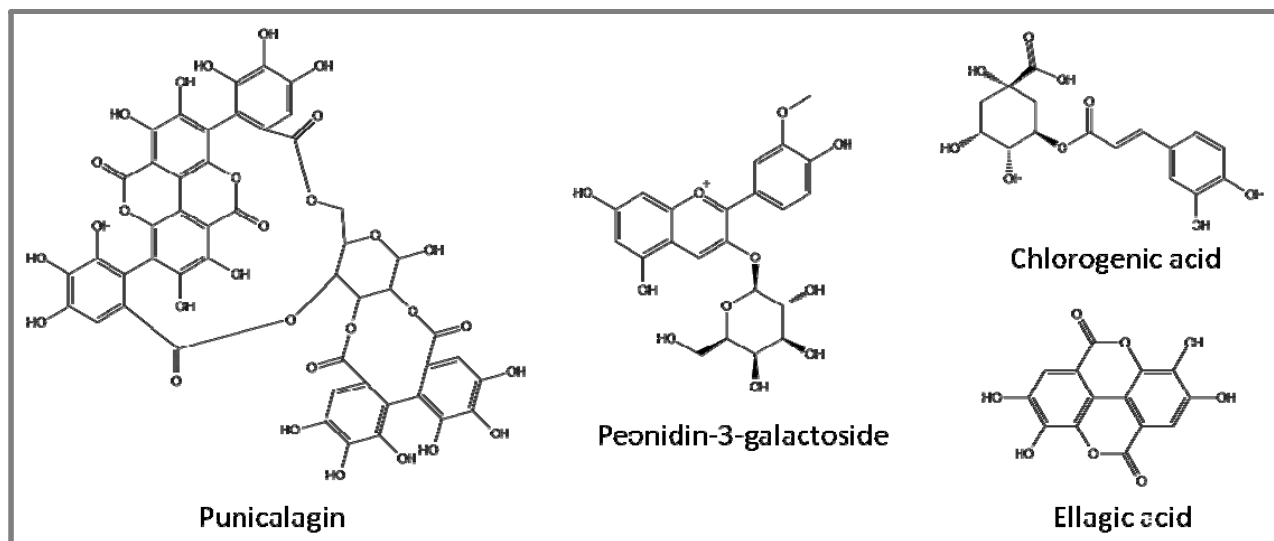


Figure 1. Structure of punicalagin, peonidin-3-galactoside, ellagic acid, and chlorogenic acid.

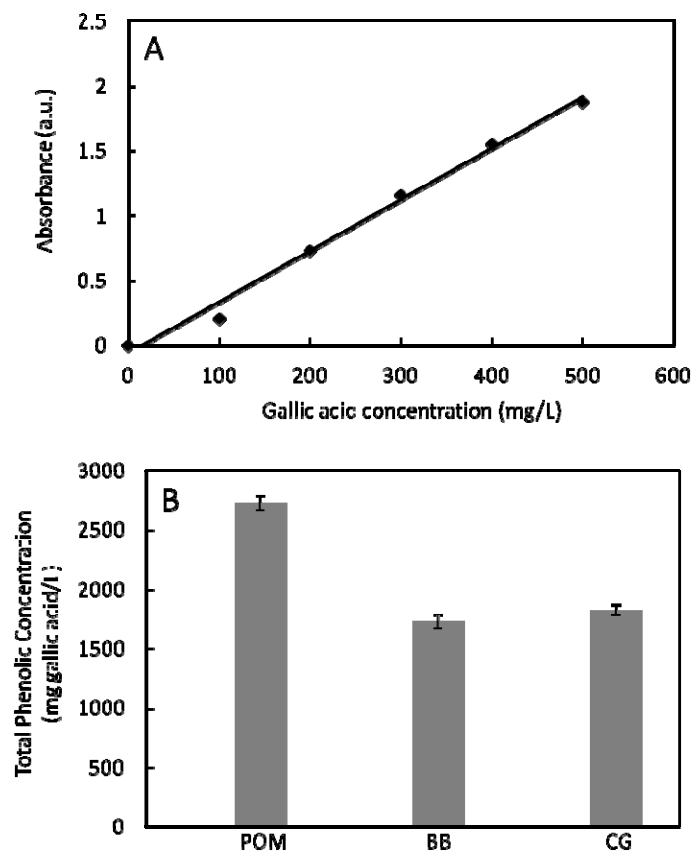


Figure 2. Folin-Ciocalteu method was used to quantify phenolic content within the samples. (A) The standard curve of gallic acid with concentrations ranging from 0 mg/L to 500 mg/L for determination of phenolic content (gallic acid equivalents). (B) Phenolic contents of POM/BB/CG juices were calculated with three independent replicates (POM: 2725 mg/L; BB: 1729 mg/L; CG: 1826 mg/L). Data are represented as mean values of three determinations with standard deviation used as error bars.

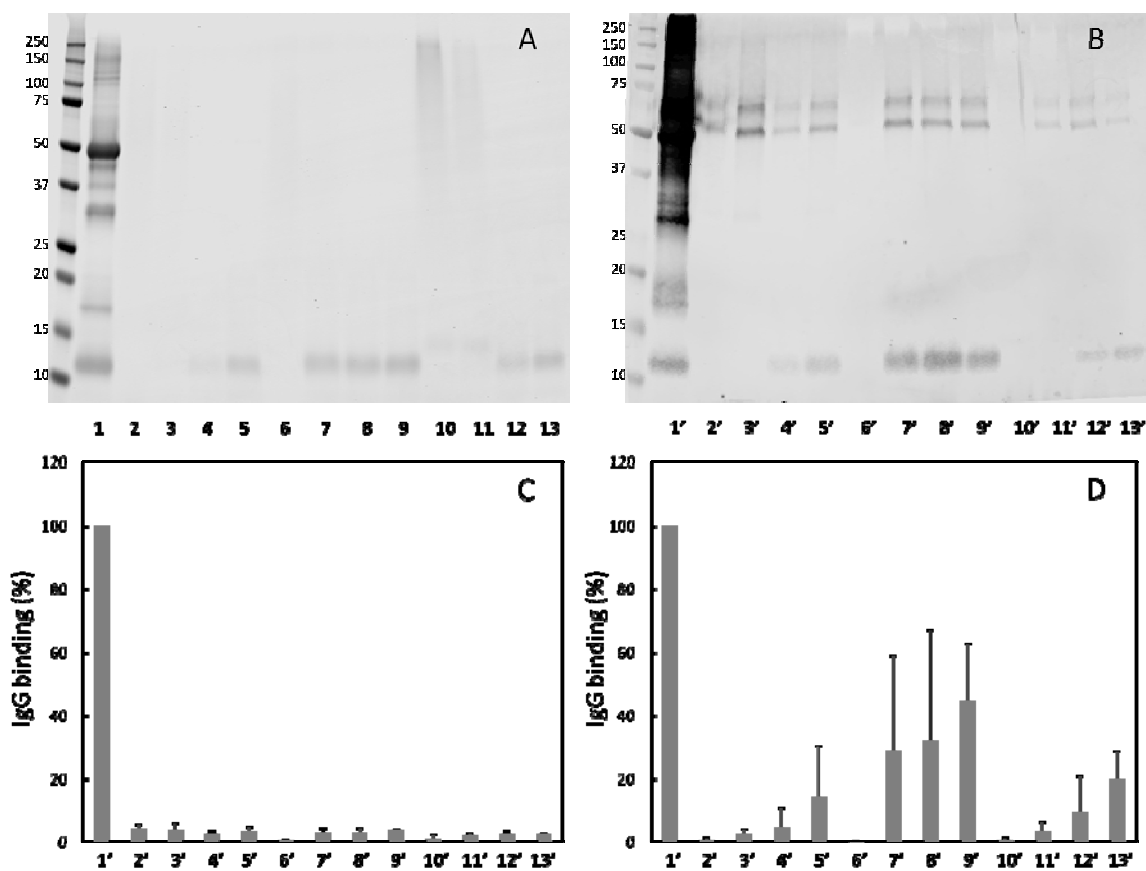


Figure 3. Polyphenol from POM, BB, CG juices interact with cashew extract. Cashew extract after treatment of POM, BB, and CG juices was analyzed by (A) non-reducing SDS-PAGE and (B) immunoblot with rabbit anti-cashew antibodies. Lanes: (1, 1') cashew extract as control; (2, 2') POM juice as control; cashew extract treated with POM juice (3, 3') 2725 mg/L (no dilution); (4, 4') 908 mg/L (three times dilution); and (5, 5') 303 mg/L (nine times dilution); (6, 6') BB juice as control; cashew extract treated with BB juice (7, 7') 1729 mg/L (no dilution); (8, 8') 576 mg/L (three times dilution); and (9, 9') 192 mg/L (nine times dilution); (10, 10') CG juice as control; cashew extract treat with CG juice (11, 11') 1826 mg/L (no dilution); (12, 12') 609 mg/L (three times dilution); and (13, 13') 203 mg/L (nine times dilution). Normalization of rabbit IgG binding to cashew allergens was confirmed by quantifying the 800 nm channel signal. Quantification of IgG antibody binding to cashew allergens in each lane was shown in (C); Quantification of IgG antibody binding to Ana o 3 in each lane was shown in (D). Data in (C) and (D) represent mean values of three independent determinations with standard deviation used as error bars.

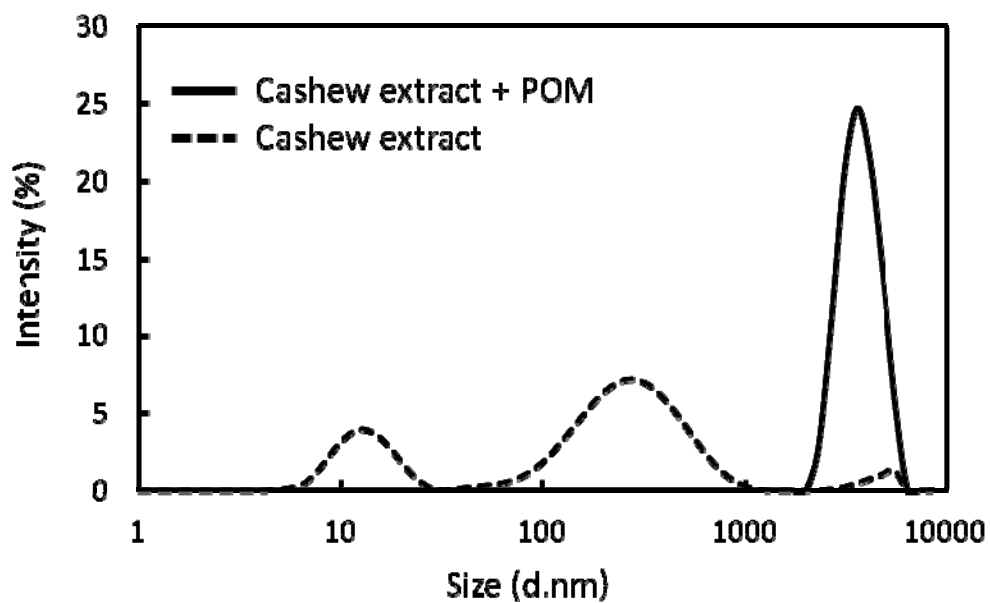


Figure 4. Dynamic light scattering analysis of particle size of cashew extract. Size distribution of cashew extract before (dashed line) and after (solid line) treating with POM juice. Results are shown as intensity on the Y-axis by average size in nanometers on the X-axis. The average size of cashew extract increased to around 3580 nm after POM juice treatment.

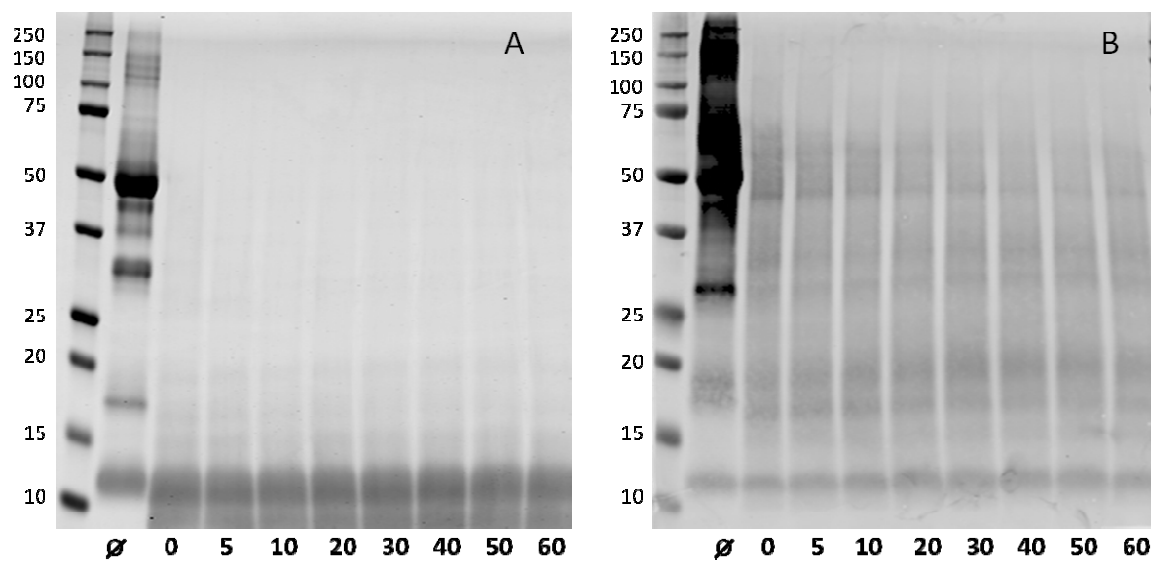


Figure 5. *In vitro* simulated gastric fluid digestion of POM polyphenol-cashew extract complexes. The digested samples were taken at time points 0, 5, 10, 20, 30, 40, 50, 60 min. Cashew extract without pepsin digestion was loaded as a control (Lane Ø). Samples were subject to both (A) SDS-PAGE analysis under non-reducing conditions and (B) immunoblotting using rabbit antibodies to cashew proteins.

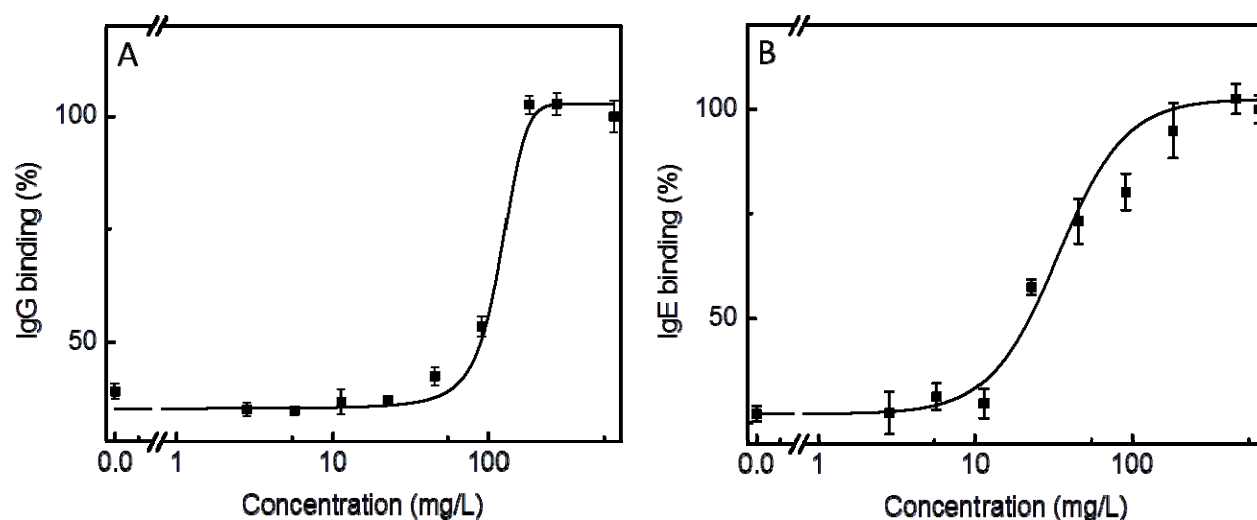


Figure 6. Treatment of cashew extract with POM juice reduced IgG and IgE binding to cashew nut allergens. (A) Diluted rabbit anti-cashew sera were used for competitive IgG binding; (B) pooled sera from cashew nut allergic patients were used for competitive IgE binding to cashew extract after treatment with POM juice. POM juice concentration (gallic acid equivalents) is indicated on the X-axis, and relative IgG and IgE binding in IR800 units is indicated on the Y-axis. The data plotted are representative of three repetitions with standard deviations as error bars