

Co-sensitization to the fruit seeds and raw potato in children with cashew nut allergy

To the Editor,

Cashew nut, pistachio, and mango are the members of the *Anacardiaceae* botanical family and closely related to the *Rutaceae* family that citrus fruits belong.¹ In patients with cashew nut and pistachio allergy, anaphylactic reactions have been reported after chewing of the citrus and apple seeds.² However, there was no case with cashew nut and pistachio allergy who had allergy to quince in the literature. We have also observations that these patients had anaphylaxis after ingestion of pomegranate seed and raw potato. We aimed to document sensitization to raw/boiled potato, pulp of the mango around the seed, and the seeds of citrus fruits, apple, quince, and pomegranate via skin prick tests (SPT) in children with cashew nut allergy. Orange seed protein bands responsible for the allergy were analyzed via Western blot.

Forty-two children (patient group) and 12 age- and sex-matched controls were enrolled to the study. Further details are available in Supporting information section (Table S1). The median ages of the patients (male/female = 26/16) and the control group (male/female = 7/5) were 50.5 (29.0-76.3) and 67.0 (40.0-77.3) months, respectively. All the patients were allergic to cashew nut ($n = 42$) and 85.7% ($n = 36$) of them also sensitized to pistachio. The most common co-sensitization occurred to orange seed with a frequency of 54.7% and 58.3% of the children with cashew nut and pistachio allergy/sensitization, respectively (Table S2). Intriguingly, 35.7% of the patients with cashew nut and 38.8% of the children with pistachio allergy/sensitization had co-sensitization to raw potato, and the majority of them tolerated boiled potato. Co-sensitizations both with the apple and pomegranate seeds were identified in 13.8% and 11.9% of the patients with cashew nut and pistachio allergy/sensitization, respectively (Table S2). Additionally, sensitization to quince seed was detected in few patients (7.1% of cashew nut and 8.3% of pistachio sensitized children). Only, four children ($n = 4/42$; 9.5%) sensitized to mango pulp around the seed. In controls ($n = 12$), none had sensitization to cashew nut/pistachio and history of allergic reactions with the fruit seeds or raw potato.

Most of the patients could consume the fresh fruit juice; however, based on patients'/caregivers' declaration, they had never ingested the seeds previously. Allergic reactions to the fruits and raw potato occurred in seven patients and are summarized in Table 1 and Supporting information section.

The details of Western Blot analysis are given in Supporting information section. All the experiments were performed with a single patient serum who had anaphylaxis with orange seed and a nonatopic

patient serum. Protein bands were visible between 10 and 15 kDa, at 25 kDa, between 30 and 35 kDa, between 40 and 45 kDa, between 50 and 70 kDa, and between 80 and 115 kDa for the cashew nut (Figure 1A), and between 50 and 70 kDa and around 115 kDa for the orange seed (Figure 1B) in the patient. However, all protein bands of the orange seed became invisible after pre-incubation of the patient serum with the cashew nut extract (Figure 1B). Reduction in the intensity of the cashew nut protein bands was observed at 25 kDa, between 30 and 35 kDa, and between 40 and 45 kDa following the pre-incubation of patient serum with the orange seed extract (Figure 1A). To show that the cashew nut and orange seed allergen bands were not inhibited with unrelated allergens, inhibition assays were done with the cat and mite allergens ([®]ALK-Abelló) (Figure 1C). Furthermore, Coomassie blue staining was performed to visualize the protein content and quality of the cashew nut, orange seed, mite, and cat allergen extracts used in the assays (Figure 2).

Almost half of our patients co-sensitized to fruit seeds, particularly to the orange seed, as well-documented previously.¹ Most of the patients could consume fruit juices, but many had never consumed seeds of the fruits previously, according to the caregivers' declaration. However, one patient developed an allergic reaction with tangerine juice. The probable reason may be the seed of tangerine which might have mixed with the juice of the fruit. We also determined co-sensitization to the citrus seeds with pistachio in a relatively large pediatric population. However, studies showing the relationship between pistachio and fruit seeds only consist of case reports² rather than large case series/detailed studies.

In literature, protein bands responsible for the citrus allergy have been identified as 10 kDa, between 15 kDa and 20 kDa, 20 kDa, and between 25 kDa and 37 kDa.³ Serum of our patient with allergic reactions to the orange seed revealed allergen bands between 50 and 70 kDa and at 115 kDa. However, these orange seed allergen bands disappeared in inhibition assay. Taken together, these results suggested the cross-reactivity between the cashew nut and orange seed and the primary sensitization to the cashew nut.

Of our patients, three developed allergic reactions to the pomegranate seeds, and two of them had anaphylaxis. In one of our patients with anaphylaxis to pomegranate seed, allergens have been studied in detail.⁴ Pathogenesis-related (PR) 4 protein other than the well-defined pomegranate allergen PR-14 protein has been documented in that case.⁴ In addition to PR proteins, seed storage proteins and pan

TABLE 1 Details of the patients with positive history upon the exposure to fruit seeds, fruit juices, and raw potato

Number of the patients with allergic reactions	Pomegranate (seed)	Raw potato	Orange (seed)	Tangerine (juice)
P2	Redness, itching around the mouth (tolerated pomegranate juice)	-	-	-
P3	Vomiting, difficulty in breathing (tolerated pomegranate juice)	-	-	-
P9	Generalized urticaria, pruritus, wheezing, and shortness of breath	-	-	-
P15	-	Lip angioedema (tolerated cooked potato)	-	-
P19	-	Lip angioedema (tolerated cooked potato)	-	Urticaria on face, itching around the mouth (no consumption of tangerine seed)
P23	-	Generalized urticaria (tolerated cooked potato)	-	-
P42	-	-	Lip angioedema, vomiting, coughing, urticaria on face	-

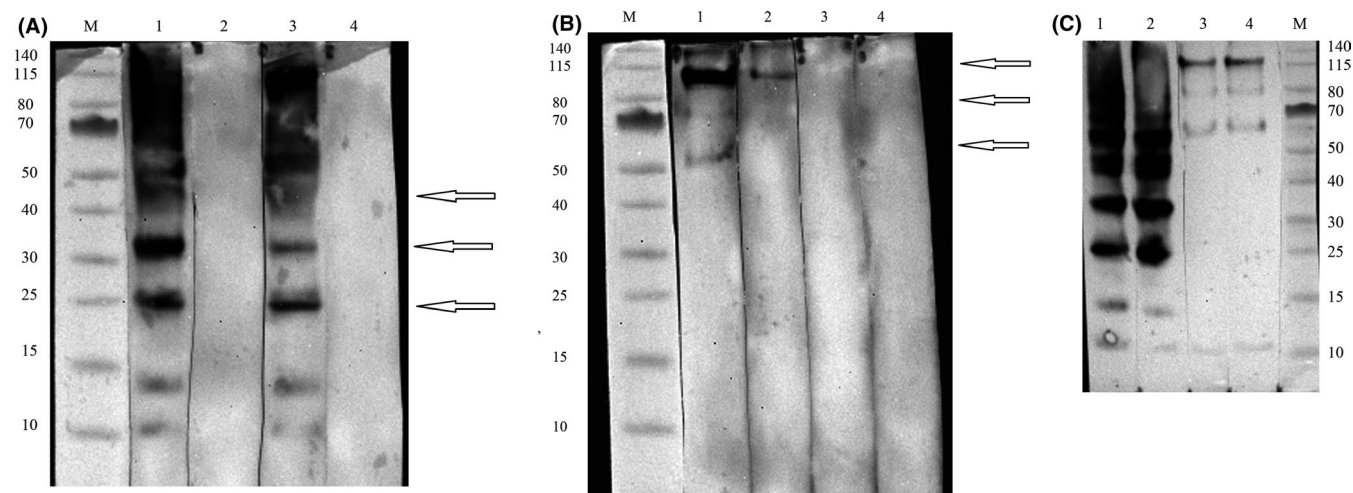


FIGURE 1 Western blot and inhibition assays of the cashew nut and orange seed allergens. (All the experiments were performed with serum of a single patient who had anaphylaxis with the orange seed.) A, Inhibition assay for the cashew nut allergens. Cashew nut allergen extract separated on gel and transferred to the membrane. Serum treatment for each lane was as follows: M: prestained page ruler, lane 1: patient serum, lane 2: patient serum pre-incubated with the cashew nut extract, lane 3: patient serum pre-incubated with the orange seed extract, and lane 4: serum of nonatopic individual. Protein bands of the cashew nut were between 10 and 15 kDa, at 25 kDa, between 30 and 35 kDa, between 40 and 45 kDa, between 50 and 70 kDa, and between 80 and 115 kDa. Bands at 25 kDa, between 30 and 35 kDa, and between 40 and 45 kDa were diminished in intensity following the pre-incubation of serum with the orange seed allergen extract. Arrow indicates the reduction in intensity of the band pattern of the cashew nut by orange seed extract. B, Inhibition assay for the orange seed allergens. Orange seed allergen extract separated on gel and transferred to the membrane. Serum treatment for each lane was as follows: M: prestained page ruler, lane 1: patient serum, lane 2: patient serum pre-incubated with the orange seed extract, lane 3: patient serum pre-incubated with the cashew extract, and lane 4: serum of nonatopic individual. Orange seed allergen bands were determined as between 50 and 70 kDa and around 115 kDa in the patient. However, these bands were disappeared after incubation with the cashew nut allergen extract. Arrows show the inhibition of orange seed allergens by the cashew nut extract. C, Inhibition assay for the cashew and orange seed allergen extracts with nonrelated allergen extracts. Lane 1: patient serum incubated with the mite allergen extract (cashew nut on membrane), lane 2: patient serum incubated with the cat allergen extract (cashew nut on membrane), lane 3: patient serum incubated with the mite allergen extract (orange seed extract on membrane), lane 4: patient serum incubated with the cat allergen extract (orange seed extract on membrane), and M: prestained page ruler

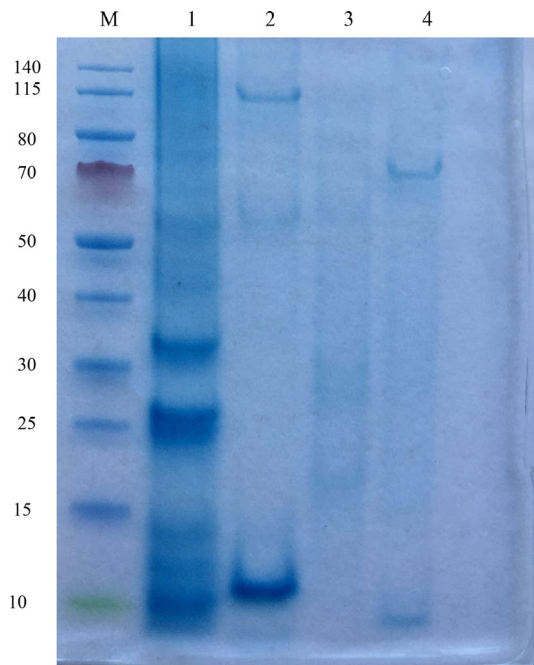


FIGURE 2 Allergen extracts separated on SDS-PAGE and stained with Coomassie blue. M: prestained page ruler, lane 1: cashew nut extract, lane 2: orange seed extract, lane 3: mite allergen extract ([®]ALK-Abelló), and lane 4: cat allergen extract ([®]ALK-Abelló)

allergens have been accused in co-allergy/co-sensitization to other foods in patients with tree nut allergy.⁵ These common allergens may be the reason of co-allergy/co-sensitization found between the pomegranate and cashew nut/pistachio in our study.

Contact and severe allergic reactions to raw potato have been described, and these subjects have tolerated cooked potato similar to our patients.⁶ The tolerance was attributed to heat-labile proteins that lose allergenic properties following the cooking process.⁶ Further, anaphylaxis with pectin in the patients with cashew nut/pistachio allergy/sensitization has been reported.⁷ Pectin is a complex set of polysaccharides that is present in many fruits (apple, quince, and citrus fruits) and vegetables (potato).⁸ The cross-reactivity between cashew nut/pistachio and pectin is thought to be due to a new, undefined carbohydrate allergen.⁸

Co-sensitization has been documented between the cashew nut and apple seed,² as we determined. Moreover, since it is widely consumed in our country and belongs to the same botanical family (*Rosaceae*) as apple, we performed SPT with quince seed and determined co-sensitization, even so the ratio was quite low. IgE antibodies to LTPs found in the foods of the *Rosaceae* family have been shown to react to foods in the *Anacardiaceae* family.⁹ Additionally, co-sensitization to apple and quince in our patients may be due to pectin which is also present in these fruits.

Although mango also belongs to the *Anacardiaceae* botanical family, we determined low co-sensitization ratios for mango contrary to the previous data.^{5,10} In the study,¹⁰ 41.3% of the patients co-sensitized to mango, but none of them had OFC-proven allergy. However, we did not perform OFC with mango to demonstrate the exact mango allergy in our patients, since mango is not a widely consumed food in Turkey.

This study demonstrated the co-sensitization in children with cashew nut/pistachio allergy/sensitization not only with the citrus seeds, but also with pomegranate, apple, and quince seeds, and with the raw potato in a relatively large pediatric population. Contrary to many reports, only a small number of patients had co-sensitization to mango. In Western blot analysis, we showed that the patient with cashew nut allergy who had anaphylaxis to orange seed had actually cashew nut allergy and only cross-reacted with the orange seed allergens.

CONFLICT OF INTEREST

All co-authors confirm agreement with the final statement. All authors declare that there is no conflict of interest. All authors declare that this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

AUTHOR CONTRIBUTIONS

Pinar Gur Cetinkaya, collected data, participated in review of files, data generation, entry and analysis, and prepared the manuscript. Esra Birben, made Western Blot analysis. Melike Kahveci, collected data. Aysegul Akarsu, collected data. Ozge Soyer, collected data and review the manuscript. Umit Murat Sahiner, collected data and reviewed the manuscript. Bulent Enis Sekerel, developed the design of the study, contributed the collection of data, evaluated the results, and wrote the manuscript with PGC.

ETHICAL APPROVAL

All listed authors have seen and approved the final version of the manuscript. The study protocol has been approved by the local ethical committee, and the parents/guardians gave written informed consent (Hacettepe University, GO 15/649-07).

Pinar Gur Cetinkaya¹
Esra Birben²
Melike Kahveci¹
Aysegul Akarsu¹
Ozge Soyer¹
Umit Murat Sahiner¹
Bulent Enis Sekerel¹

¹Division of Pediatric Allergy and Asthma Unit, Department of Pediatrics, Faculty of Medicine, Hacettepe University, Ankara, Turkey

²Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey

Correspondence

Bulent Enis Sekerel, Department of Pediatric Allergy, Hacettepe University School of Medicine, 06100 Ankara, Turkey.

Email: b_sekerel@yahoo.com

Pinar Gur Cetinkaya and Esra Birben Contributed equally.

ORCID

Pınar Gur Cetinkaya  <https://orcid.org/0000-0002-2138-802X>
Esra Birben  <https://orcid.org/0000-0003-1192-309X>
Melike Kahveci  <https://orcid.org/0000-0002-4459-4272>
Aysegul Akarsu  <https://orcid.org/0000-0002-1544-3408>
Ozge Soyer  <https://orcid.org/0000-0002-7444-251X>
Umit Murat Sahiner  <https://orcid.org/0000-0003-0088-913X>
Bulent Enis Sekerel  <https://orcid.org/0000-0001-7402-6850>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplementary Table 1. Demographic features of the study population

	Patients (n=42)	Controls (n=12)	<i>p value</i>
Gender (M) (n/%)	26 (61.9%)	7 (58.3%)	0.825
Current age of the children (months)*	50.5 (29.0-76.3)	67.0 (40.0-77.3)	0.303
sIgE ≥ 15 kU/L for the cashew nut (n/%; cashew nut sIgE was applied to 24 children)	9 (37.5%)	-	-
sIgE 0.35-15 kU/L for the cashew nut (n/%; cashew nut sIgE was applied to 24 children)	15 (62.5%)	-	-
sIgE ≥ 15 kU/L for the pistachio (n/%; pistachiosIgE was applied to 23 children)	10 (43.5%)	-	-
sIgE 0.35-15 kU/L for the pistachio (n/%; pistachio sIgE was applied to 23 children)	13 (56.5%)	-	-
Total IgE (k/UL)*	350 (124-1054)	133.5 (88.4-1182.5)	0.397
Eosinophil %*	3.3 (2.4-7.9)	2.9 (1.9-7.9)	0.673
Eosinophil count*	300 (200-700)	400 (150-550)	0.567
Atopic Dermatitis (n/%)	34 (81%)	8 (66.7%)	0.298
Allergic Rhinitis (n/%)	11 (26.2%)	3 (25%)	0.516
Asthma (n/%)	11 (26.2%)	3 (25%)	0.934
Concomitant food allergy other than the tree nuts/peanut (n/%)	32 (76.2%)	8 (66.7%)	0.511
Family history of atopy (n/%)	12 (28.6%)	3 (25%)	0.809
Anaphylaxis with the pistachio (n/%)	9 (21.4%)	-	-
Allergic reactions other than anaphylaxis with the pistachio (n/%)	6 (14.3%)	-	-
Anaphylaxis with the cashew nut (n/%)	4 (9.5%)	-	-
Allergic reactions other than the anaphylaxis with cashew nut (n/%)	6 (14.3%)	-	-

*; Median (Interquartile Range)

Supplementary Table 2. Co-sensitizations in children with cashew nut allergy/sensitization

	Ratio of sensitization (≥ 3 mm) in patients with positive cashew nut SPT (≥ 8 mm; n=42)	Ratio of sensitization (≥ 3 mm) in patients with positive pistachio SPT (≥ 3 mm; n=36)	Ratio of sensitization (≥ 3 mm) in control group (n=12)
Orange seed	23 (54.7%)	21 (58.3%)	1 (8.3%)
Lemon seed	20 (47.6%)	19 (52.7%)	1 (8.3%)
Grapefruit seed	20 (47.6%)	19 (52.7%)	0
Tangerine seed	19 (45.2%)	18 (50%)	0
Bitter Orange seed	15 (35.7%)	15 (41.6%)	0
Kumquat seed	18 (42.8%)	17 (47.2%)	0
Raw Potato	15 (35.7%)	14 (38.8%)	2 (16%)
Pomegranate seed	5 (11.9%)	5 (13.8%)	1 (8.3%)
Apple seed	5 (11.9%)	5 (13.8%)	0
Mango pulp around	4 (9.5%)	4 (11.1%)	0

(n=42) and control group (n=12)

the seed			
Quince seed	3 (7.1%)	3 (8.3%)	0
Boiled Potato	1 (2.3%)	1 (2.7%)	0

Supplementary Table 3. Correlation between the cashew nut/pistachio and fruit seeds/potato
SPT results

	Cashew nut		Pistachio	
	rho	p value	rho	p value
Pistachio	0.600	<0.001	-	-
Cashew nut	-	-	0.600	<0.001
Kumquate seed	0.639	<0.001	0.471	0.002
Orange seed	0.585	<0.001	0.400	0.009
Lemon seed	0.557	<0.001	0.350	0.023
Grapefruit seed	0.534	<0.001	0.353	0.022
Tangerine seed	0.525	<0.001	0.338	0.028
Bitter Orange seed	0.484	0.001	0.508	0.001
Apple seed	0.406	0.008	0.238	0.129
Pomegranate seed	0.400	0.009	0.069	0.664
Mango pulp around the seed	0.127	0.425	0.135	0.392
Quince seed	0.023	0.883	-0.031	0.844
Raw Potato	-0.067	0.674	0.140	0.375
Boiled Potato	0.016	0.921	0.234	0.136

Methods

Patients

This prospective study was conducted at the Pediatric Allergy Department of Hacettepe University between July 1th, 2018 and August 30th, 2019. Forty-two children with cashew nut SPT wheal diameters of ≥ 8 mm indicating a clinical reactivity with $>95\%$ accuracy¹, and age- and sex-matched children with tree nut allergy other than the cashew nut and/or pistachio were enrolled to the study. Of these 42 children with cashew nut allergy/sensitization, 10 were reactive to the cashew nut (six of them had oral food challenge (OFC)-proven cashew nut allergy), 32 patients did not consume the cashew nut previously. The parents/patients were questioned whether the patients had ever consumed the juice or chewed the seeds of citrus fruits (orange, lemon, grapefruit, tangerine, bitter orange, and kumquat), apple, quince, pomegranate, and had ever eaten raw/boiled potato and pulp of the mango around the seed. Allergic reactions following the ingestion of citrus fruits (orange, lemon, grapefruit, tangerine, bitter orange, and kumquat), apple, quince, ~~mango~~, and pomegranate seeds, mango pulp around the seed, and raw potato were recorded.

Skin Prick Tests

Skin prick tests (SPT) were performed for the seeds of citrus fruits (orange, lemon, grapefruit, tangerine, bitter orange, and kumquat), pomegranate, apple, quince, for the mango pulp around the seed, and for the raw/boiled potato as a panel to the entire study population with positive (histamine) and negative (saline) controls. Allergen extracts were prepared for the cashew nut, pistachio, fruit seeds, and potato according to the protocol as described previously.² Unfortunately, obtaining a purified protein allergen extraction from the raw potato

was not possible. The patients had positive SPT results with prick-to-prick application of the raw potato; however, none of them had positivity with the raw potato allergen extract in skin prick testing. Therefore, we performed prick-to-prick test to the patients both with the raw and boiled potatoes. Positive SPT was accepted as a wheal diameter of 3 mm or larger. The mean SPT result was calculated by taking the average of two perpendicular diameters of the wheal after 15 minutes following the application of allergens to the skin. Total and specific IgE (sIgE) levels were measured via the Pharmacia CAP system (Immunocap-CAP; Pharmacia & Upjohn, Uppsala, Sweden); sIgE levels ≥ 0.35 kUA/L were accepted as positive for the cashew nut/pistachio.

Western Blot Analysis

Since protein extract of the raw potato could not be obtained, and one of the patients with pomegranate seed anaphylaxis has been previously studied in detail,³ Western blot analysis was performed only for the patient with anaphylaxis to orange seed and serum of a non-atopic healthy control was used as negative control for experiments. To show that the cashew nut and orange seed allergen bands were not inhibited with unrelated allergens, inhibition assays were done with the cat and mite allergens ([®]ALK-Abelló, Hørsholm, Denmark). Furthermore, Coomassie blue staining was performed to visualize the protein content and quality of the cashew nut, orange seed, mite, and cat allergen extracts used in the assays. To further characterize the orange seed allergen proteins in the patient who experienced anaphylaxis with the orange seed, we analyzed orange seed allergen extract that was prepared for the current study via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Since the amount of allergen protein in the orange seed extract that we prepared was low (5 microgram/microliter protein in cashew nut

extract, 0.1 microgram/microliter protein in orange seed extract), 10 times higher orange seed extract than the cashew nut allergen was used for Western blot inhibition assays. To show the cashew nut and orange seed specific IgE cross reactive allergen bands in the patient serum, both allergen extracts were run on 4-12%Bis-Tris gel and transferred to the nitrocellulose membrane. The membrane was incubated with 1/5 diluted patient serum for overnight. After that, the membrane was incubated with horseradish peroxidase⁴for the conjugation of anti-IgE antibody and visualized by charge-coupled device (CCD) camera. To determine the cross-reactivity between the orange seed and cashew nut in the patient, Western blot inhibition assay was performed. In this method, serum was pre-incubated with 2 µl cashew nut protein extract or 20µl orange seed protein extract overnight at 4°C prior to incubation with the nitrocellulose membrane. If there was a reduction or marked decrease in the intensity of the band pattern, the cross-reactivity was considered to be present.

Statistical Analysis

Statistical analyses were performed using SPSS Version 22.0 statistical software package (IBM SPSS Statistics Chicago, Ill). The data were given as median and interquartile ranges (IQR) in not normally distributed variables. Statistical comparisons were performed using Mann-Whitney *U* test on ranks as appropriate. The statistical tests were 2-sided, and a *p-value* ≤ 0.05 was regarded as statistically significant. The Spearman correlation coefficient (*r*) was used to determine the strength of the relationship between mean SPT wheal diameters to each allergen.

Results

Clinical reactions

Three had allergic reactions with the pomegranate seed, two of which had anaphylaxis. Of the seven patients, one had urticaria on the face after the consumption of tangerine juice, but this patient had never eaten tangerine seed. Three patients had allergic reactions upon contact of the mouth with or consumption of the raw potato; however, they could consume boiled/fried/cooked potato, and prick-to-prick SPTs were negative with the boiled potato. One patient had lip angioedema, vomiting, coughing, and urticaria on the face after accidentally chewing of the orange seed (**Supplementary Table 3**). The rest of the study population had not previously consumed neither the raw potato nor the seeds of fruits. Additionally, we did not perform OFC tests to the patients with the seeds and raw potato for ethical reasons.

Correlation between cashew nut/pistachio and fruits/raw potato

The strongest correlation in the patients with cashew nut allergy/sensitization was identified with kumquat ($r=0.639$, $p<0.001$), orange ($r=0.585$, $p<0.001$), lemon ($r=0.557$, $p<0.001$), grapefruit ($r=0.534$, $p<0.001$), and tangerine seeds ($r=0.525$, $p<0.001$) (**Supplementary Table 3**). Similarly, the strongest correlations with pistachio were found with citrus seeds (**Supplementary Table 3**).

86 **SupplementaryTable 1.** Demographic features of the study population

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Supplementary Table 3.Correlation between the cashewnut/pistachio and fruit seeds/potato SPT results

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Lemon seed	0.557	<0.001	0.350	0.023
Grapefruit seed	0.534	<0.001	0.353	0.022
Tangerine seed	0.525	<0.001	0.338	0.028
Bitter Orange seed	0.484	0.001	0.508	0.001
Apple seed	0.406	0.008	0.238	0.129
Pomegranate seed	0.400	0.009	0.069	0.664
Mango pulp around the seed	0.127	0.425	0.135	0.392
Quince seed	0.023	0.883	-0.031	0.844
Raw Potato	-0.067	0.674	0.140	0.375
Boiled Potato	0.016	0.921	0.234	0.136

Supporting References

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3. Buyuktiryaki B, Bartolomé B, Sahiner UM, et al. Pomegranate allergy and pathogenesis-related protein 4. *Ann Allergy Asthma Immunol* 2013;111:231-232.
4. Klionsky DJ, Abdelmohsen K, Abe A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 2016;12:1-222.