J Plant Biotechnol (2015) 42:123-128
DOI:http://dx.doi.org/10.5010/JPB.2015.42.2.123
ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Production of Hypoallergenic Cocoa Beans by a Pregermination Treatment Method

Tri Handoyo · Akagawa Mitsugu · Kyung-Min Kim

Received: 15 May 2015 / Revised: 28 May 2015 / Accepted: 2 June 2015

Abstract Pregermination treatment of cocoa beans either with the testa, group PCB (+T), or without the testa, group PCB (-T), was studied here to determine whether this treatment (incubation up to 120 h at 25°C) has any effect on the levels of allergenic proteins or on chemical composition. Our proximate analysis included carbohydrates, proteins, and lipids. We used western blotting to measure changes in the amounts of allergenic proteins in the cocoa beans during the pregermination treatment. The protein and carbohydrate content of both groups (with or without the testa) decreased slightly during this treatment, whereas lipid content increased. Group PCB (-T) showed more rapid metabolic processes than did group PCB (+T) during the pregermination treatment. Western blot analysis showed that the cocoa beans contained an allergenic protein of ~28 kDa. Removal of the testa strongly reduced the amount of this allergenic protein after 72 h of the pregermination treatment. Generally, the pregermination treatment increased antioxidant activity in both groups. Significant differences in the antioxidant activity were observed between groups PCB (-T) and PCB (+T). Particularly, group PCB (-T) showed high antioxidant activity at 72 h of the pregermination treatment. Thus, the combination of cocoa beans without the testa and pregermination treatment (72 h) seems to be the optimal method for production of hypoallergenic cocoa beans rich in antioxidants for patients with allergic disorders.

Keywords allergy, allergenic protein, cocoa bean, hypoallergenic, pregermination

Tri Handoyo
Plant Physiology Laboratory, Faculty of Agriculture, Jember University, Jl. Kalimantan 37, Jember, Indonesia; Division of Plant Biosciences, School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, 80 Daehak-ro, Buk-Gu, Daegu 702-70, Korea

Akagawa Mitsugu
Department of Biological Chemistry, Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai 599-8531, Japan

K. M. Kim (✉)
Division of Plant Biosciences, School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, 80 Daehak-ro, Buk-Gu, Daegu 702-70, Korea
e-mail: kkm@knu.ac.kr

Introduction

Cocoa beans *(Theobroma cacao* L., family Sterculiaceae) are a commercially important raw material in the food industry and are consumed daily worldwide in the form of such products as cocoa powder, chocolate bars, and other cocoa derivatives (Belščak et al. 2009). Most countries have used chocolate as food or medicine, particularly the traditional populations of Central America and the Caribbean, who used cocoa beans for culinary and medicinal purposes (Grivetti et al. 2009). Cocoa beans are rich in phytochemicals and antioxidants. Several studies have shown that chocolate—when eaten in moderate amounts on a regular basis—can reduce blood pressure (Shrime et al. 2011), prevent heart diseases (Taubert et al. 2003), help prevent infectious and autoimmune diseases (Sanbongi et al. 1997), inhibit cancer growth (Demeule et al. 2002), and stress and defense (Kim et al. 2011; Kwon et al. 2012). Some people, however, are unable to consume cocoa bean products because they contain proteins that can cause allergic reactions (Abril-Gil et al. 2012). Worldwide, the number of children with food allergies is increasing, and allergy to chocolate is considered a serious health problem (Larche et al. 2006). The typical symptoms of chocolate allergy are generalized urticaria, atopic eczema/dermatitis syndrome, and in more severe cases, exercise-induced anaphylaxis (Perr 2004). Food technologies are developing rapidly, and the methods for production of hypoallergenic foods have improved. One of these methods involves pregermination treatment, which

has been studied as a means of increasing product quality. The pregermination treatment of seeds has been applied to various commercial crop species with the aim of increasing the concentration of bioactive compounds and reducing the level of allergenic proteins, e.g., in wheat (Yang et al. 2001), brown rice (Goufo and Trindade 2014), and buckwheat (Miyake et al. 2004). Similar efforts are now under way to improve cocoa beans for the use in production of cocoa powder, chocolate, and cocoa-related products because these phenolic compound-rich foods that are derived from the fermented, roasted, and milled seeds of *Theobroma cacao* L. (Arlorio et al. 2005) are now among the most widely consumed processed foods. The objective of our study was to evaluate a method that we call pregermination treatment with or without the testa, which may increase the quality of cocoa products. We used western blots to analyze the levels of possible allergenic proteins in the course of this treatment; additionally, we measured the amounts of proteins, lipids, carbohydrates, all phenolic compounds, and antioxidants.

Materials and methods

Preparation of pregerminated cocoa beans

The cocoa strain DR 38 was obtained from the Indonesia Cocoa and Coffee Research Institute, Jember, Indonesia. For each treatment, we used 5 kg of cocoa beans. We performed pregermination treatment of cocoa beans either with the testa, group PCB (+T), or without the testa, group PCB (-T). The beans were placed in an incubator at 25°C for various incubation periods (0, 24, 48, 72, 96, or 120 h).

Proximate analysis of the pregerminated cocoa beans

Carbohydrates, ash, proteins, and lipids were quantified using the methods approved by the Association of Official Analytical Chemists (Association of Official Analytical Chemists, 2002). Ash [grams of ash per 100 grams of dry matter (g ash/100 g DM)] was quantified after incubation of the samples at 550°C for 2 h. Protein content of the cocoa samples (g protein/100 g DM) was analyzed by the Micro Kjeldahl method (Association of Official Analytical Chemists, 2002). Lipids (g fat/100 g DM) were quantified using the Soxhlet extraction method: crude fat content was calculated from the weight loss after six-cycle extraction with N-hexane in a Soxhlet apparatus. Carbohydrate content was estimated by subtracting the total proportion (%) of crude proteins, lipids, crude fiber, moisture, and ash constituents in a sample from 100 (Oko et al. 2012), i.e., % carbohydrate = 100 - (% moisture + % crude fiber + % protein + % lipid + % ash).

Quantification of antioxidant activity and all phenolic compounds

Antioxidant activity was quantified by measurement of 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity (Brand-Williams et al. 1995). We extracted 1 g of the cocoa powder in methanol by shaking for 30 min. A 250-μL aliquot was then mixed with 5 mL of a 2,2-diphenyl-1-picrylhydrazyl solution (23.6 μg/mL in ethanol) and incubated for 24 h at room temperature in the dark to complete the reaction. Absorbance of each sample was measured at 515 nm. The antioxidant activity was calculated in 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents (mmol TE) per gram of grain.

A second aliquot (200 μL) of the methanol extract was added to 800 μL of 0.25N Folin−Ciocalteau reagent and 2 mL of 7.5% sodium carbonate in a test tube. The mixture was vigorously vortexed and diluted to 7 mL with deionized water. The reaction was allowed to proceed to completion for 2 h at room temperature in the dark; the mixture was centrifuged for 5 min at 10,000 rpm, and absorbance of the supernatant was measured at 765 nm on a Hitachi U-3900 spectrophotometer (Hitachi Co., Ltd., Japan). Methanol alone was used as a control. Gallic acid served as a standard, and the results were calculated in gallic-acid equivalents (milligrams per 100 g) of the sample. The reaction was conducted in triplicate, and the results were averaged (Katalinic et al. 2013).

Protein separation by conventional SDS-PAGE

This procedure was performed according to the method of Laemmli (Laemmli 1970). Samples were incubated with Laemmli sample buffer for 3 min at 100°C and then run on 12.5 % polyacrylamide slab gels at 25 V and 100 V for 1 and 4 h, respectively. After the electrophoresis, the gels were stained with Coomassie brilliant blue R-250 in 25% methanol with 10% acetic acid at room temperature for 90 min and were destained in 10% acetic acid without methanol or used for immunoblotting.

Western blot analysis

The immunoblotting was optimized by means of preliminary experiments. After the electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Germany) at 2 mA/cm^2 for 60 min

using a Bio-Rad Trans-Blot Semi-Dry Electroblot System (Bio-Rad Laboratories, Hercules, CA) and a Tris-glycine transfer buffer system (25 mM Tris, 192 mM glycine, 0.1% SDS, and 10% methanol). The membrane was washed three times with Tris-buffered saline (25 mM Tris-HCl pH 7.4, 137 mM NaCl, and 2.68 mM KCl) containing 0.05% Tween 20 (TBS-T) and was then blocked with 1% polyvinylpyrolidone in TBS-T for 4 h at room temperature. After three washes with TBS-T, the membrane was incubated overnight with serum samples from seven patients. The serum samples were diluted 1:50 with TBS-T at room temperature. The membranes were washed three times with TBS-T and then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody (American Qualex, San Clemente, CA) at the dilution of 1:2,000 for 2 h at room temperature. The blots were then washed three times in TBS-T before visualization. Antibody complexes captured by the immobilized target protein were enhanced using 4-chloronaphthol staining and a 1% hydrogen peroxide detection system (Sigma-Aldrich, St. Louis, MO); the reaction of the peroxide radical with 4-chloronaphthol produces dark purple staining, which was measured using a Scanner CanoCanon LiDE 110 (Akagawa et al. 2007, Handoyo et al. 2008).

Statistical Analysis

The results were processed in the SPSS software (version 11.0 for Windows; SPSS, Chicago, IL). The mean and standard deviation were used to analyze the results. Differences were considered significant at $P < 0.05$, unless otherwise specified. All samples were tested in the experiments in triplicate.

Results and discussion

Changes in protein allergen levels during the pregermination treatment

Crude protein extracts of cocoa beans were immunoblotted with human serum samples. We detected a single band with a molecular weight of 28 kDa (Fig. 1). The serum samples were obtained from seven patients with various allergies, including urticaria, eczema, atopic dermatitis, itching, and a respiratory disease (Akagawa et al. 2007). The amount of the allergenic protein decreased in groups PCB (+T) and PCB (-T) during the pregermination treatment. The presence of the cocoa bean testa affected the response to the pregermination treatment because the level of the allergenic protein fell more rapidly in the PCB (-T) group than in the PCB (+T) group. Proteases are known to be responsible for the breakdown of allergenic proteins into small peptides and amino acids (Miyake et al. 2004).

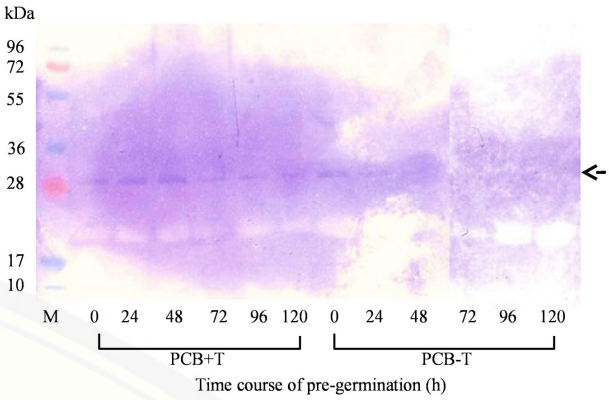

Fig. 1 Time-course western blotting of an allergenic protein during the pregermination treatment of cocoa beans with the testa [group PCB (+T)] or without the testa [group PCB (-T)]. M: molecular weight markers

Effects of the pregermination treatment on proteins, carbohydrates, lipids, and ash

Overall, the protein, carbohydrate, lipid, and ash levels depended on the duration of the pregermination incubation and on whether the testa was removed. With regard to the testa treatment, we observed similar responses for beans with and without testa during the pregermination treatment period (Fig. 2). Protein content decreased in both groups PCB (-T) and PCB (+T) during the incubation from hour 24 to hour 120 (Fig. 2A). The amount of protein in the PCB (-T) group was slightly lower than that in the PCB (+T) group. The reduction in protein content likely resulted from hydrolysis of proteins by proteases during the incubation (Miyake et al. 2004). The amount of carbohydrates in beans of the PCB (+T) group decreased gradually during the incubation from hour 24 to hour 120 (Fig. 2B). A different pattern was observed in beans of the PCB (-T) group: carbohydrate content initially rose between hours 24 and 48 but then decreased. The PCB (+T) group showed lower water absorption than did the PCB (-T) group; this effect may have influenced the solubility of carbohydrates and the activity of hydrolytic enzymes.

During pregermination, cocoa beans require energy, which comes from the breakdown of carbohydrates (Kuo et al. 1988, Peterbauer et al. 2001). In the present study, lipid levels rose in cocoa beans in both groups with incubation time (Fig. 2C), although these levels increased more rapidly in the PCB (-T) group. Lipids are essential components of cell membranes. The increase in the amount of lipids with incubation time

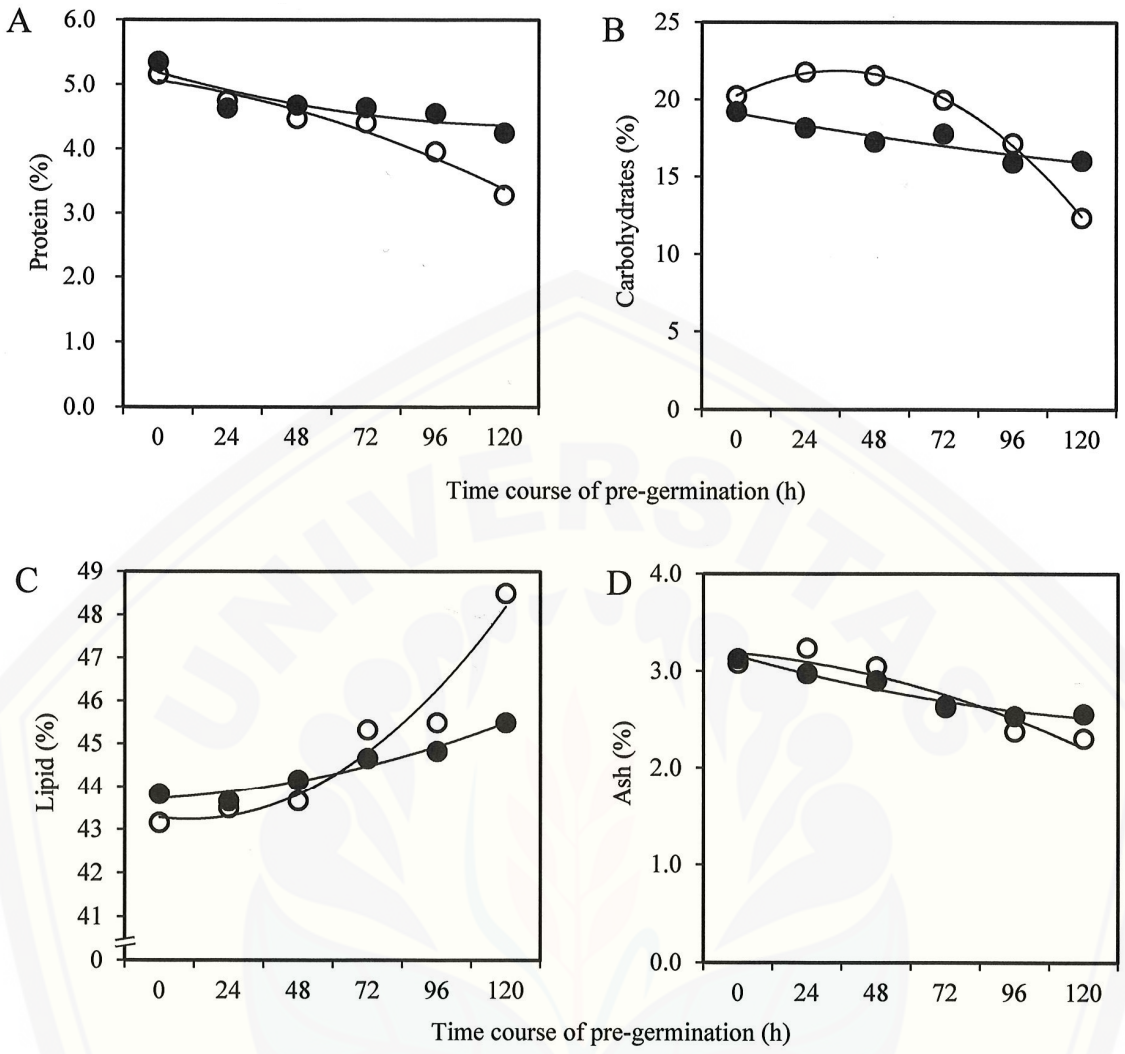

Fig. 2 Changes in the amount of (A) total soluble proteins, (B) carbohydrates, (C) lipids, and (D) ash in the cocoa beans subjected to pregermination incubation (25°C) for different periods. White circles: pregermination treatment of the cocoa beans without the testa [group PCB (-T)], black circles: pregermination treatment of the cocoa beans with the testa [group PCB (+T)]

indicated that the enzymes hydrolyzing carbohydrates and proteins contributed to the release of lipids from the membranes in the endosperm of the cocoa beans. The increase of lipid content showed the amount of lipids that were loosely bound in the complexes with other molecules. Lipids were easy to separate from the beans to obtain a premium cocoa powder with lipid content approximately 22-24% (International Cocoa Organization standard). Lipids, especially the unsaturated fat, are subject to oxidation and shorten shelf-life of food products (Borchers et al. 2000, Afoakwa et al. 2007). Ash content fell to a similar extent in groups PCB (-T) and PCB (+T) in the course of the incubation (Fig. 2D). The average ash content of the two types of cocoa powder was not different. Ash represents mineral content of foods, e.g., potassium, magnesium, copper, and iron (Afoakwa et al. 2007). Figure 3 shows changes in the amount of total soluble proteins, carbohydrates, lipids, and ash in the cocoa beans subjected to the pregermination treatment for different periods.

All phenolic compounds and antioxidant activity

The total amount of phenolic compounds in the cocoa beans of groups PCB (-T) and PCB (+T) fell with the incubation time (Fig. 3). The lowest total amount of phenolic compounds was present in the beans of the PCB (-T) group at all time points; after a 120-h incubation, PCB (-T) beans showed the average concentration of phenolic compounds of 1.20 μg/mg (Fig. 3A). The lower levels of phenolic compounds in the PCB (-T) group are presumably due to the activity of polyphenol oxidation enzymes. The total amount of phenolic compounds declined rapidly after incubation for 24-h. Imbibition of water was faster into the cocoa seeds without the testa than into

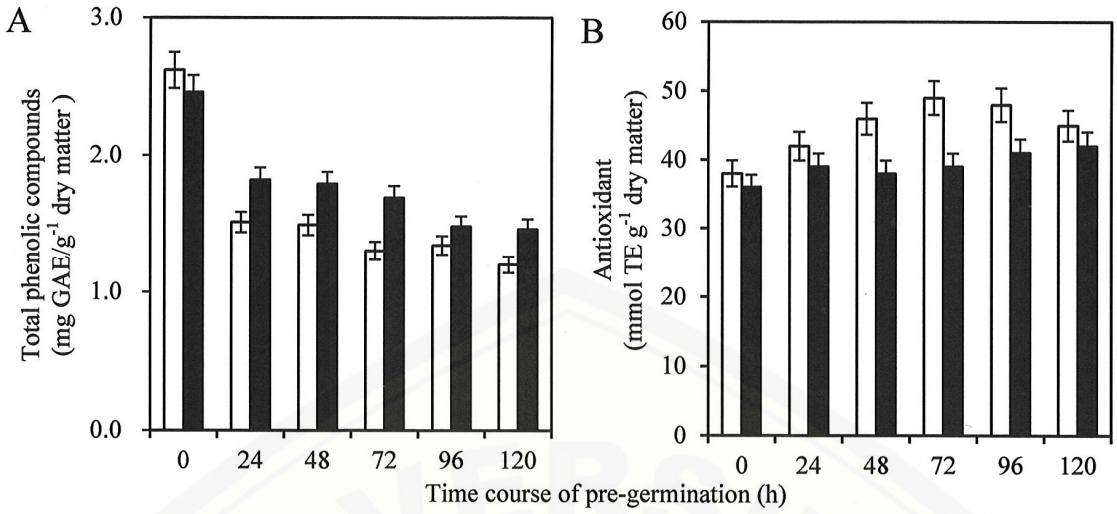

Fig. 3 Changes in the total amount of (A) phenolic compounds and (B) antioxidants in cocoa beans in the course of the pregermination treatment. White bars: pregermination treatment of the cocoa beans without the testa [group PCB (-T)], black bars: pregermination treatment of the cocoa beans with the testa [group PCB (+T)]

those with the testa; this result suggests that hydrolytic enzymes hydrolyze large molecules (proteins, lipids, and carbohydrates) into smaller parts, allowing polyphenol oxidizing enzymes to work faster. Antioxidant activity significantly increased in the course of the pregermination treatment in both groups (Fig. 3B). The PCB (-T) group showed a higher antioxidant activity than did the PCB (+T) group. For example, at hour 72 of the incubation, PCB (-T) beans showed a higher antioxidant activity (by approximately 49%). Generally, incubation at 25°C increased antioxidant activity in the cocoa beans with and without testae.

On the basis of these results, pregermination incubation seems to be an efficient method for increasing the quality of cocoa beans. Generally, the cocoa beans with and without the testa contained similar concentrations of proteins, carbohydrates, lipids, and ash at all incubation time points; however, the PCB (-T) group showed a more rapid reduction in protein allergens after 72-h incubation. Judging by the present results, we can conclude that a combination of incubation at 25°C and removal of testae may be used to increase the quality of pregermination cocoa beans as a source of chocolate products. Thus, modification of the processing of beans for cocoa manufacture may facilitate production of hypoallergenic cocoa bean products containing valuable nutrients and accelerate the development of hypoallergenic chocolate for human consumption.

Acknowledgments

This research was financially supported by the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia (2013). We are grateful to Mega Kartika Sari and Kiki Ulfaniah for their excellent assistance and kindness.

References

Abril-Gil M, Massot-Cladera M, Perez-Cano FJ, Castellote C, Franch A, Castell M (2012) A diet enriched with cocoa prevents IgE synthesis in a rat allergy model. Pharmacological Research 65:603-608

Afoakwa EO, Paterson A, Fowler M (2007) Factors influencing rheological and textural qualities in chocolate - a review. Trends in Food Science and Technology 18:290-298

Akagawa M, Handoyo T, Ishii T, Kumazawa S, Morita N, Suyama K (2007) Proteomic analysis of wheat flour allergens. Journal Agricultural and Food Chemistry 22:6863-6870

Arlorio M, Coïsson JD, Travaglia F, Varsaldi F, Miglio G, Lombardi G (2005) Antioxidant and biological activity of phenolic pigments from Theobroma cacao hulls extracted with supercritical CO_2. Food Research International 38: 1009-1014

Association of Official Analytical Chemists (2002) Official methods of analysis of AOAC International. Arlington: AOAC International

Belščak A, Komes D, Horžić D, Ganić KK (2009) Comparative study of commercially available cocoa products in terms of their bioactive composition. Food Research International 42: 707-716

Borchers AT, Keen CL, Hannum SM, Gershwin ME (2004) Cocoa and Chocolate: composition, bioavailability, and health implications. Journal of Medicinal Food 3:77-103

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of free radical method to evaluate antioxidant activity. LWT Food Science and Technology 28:25-30

Demeule M, Michaud-Levesque J, Annabi B, Gingras D, Boivin D, Jodoin J, Lamy S, Bertrand Y, Béliveau R (2002) Green tea catechins as novel antitumor and antiangiogenic compounds. Current Medicinal Chemistry Anti-Cancer Agents 2:441−463

Goufo P, Trindade H (2014) Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, c-oryzanol, and phytic acid. Food Science and Nutrition 2:75-104

Grivetti LE, Shapiro HY (2009) Chocolate: History, culture, and heritage. (eds) J Wiley & Sons, Hoboken, NJ, pp. 3-15

Handoyo T, Akagawa M, Morita M, Maeda T, Mitsunaga T (2008) Hypoallergenic characteristics of wheat flour produced by stepwise polishing. International Journal of Food Properties 1:243-252

Katalinic V, Mozina SS, Generalic I, Skroza D, Ljubenkov I, Klancnik A (2013) Phenolic profile, antioxidant capacity, and antimicrobial activity of leaf extracts from Six Vitis vinifera L. varieties. International Journal of Food Properties 16:45-60

Kim SG, Wang Y, Huh HH, Kim YC, Choi IS, Agrawal GK, Rakwal R, Kang KY, Kim ST (2011) Proteomics of ionic stresses in rice: An overview. J Plant Biotechnol 38:130-136

Kuo TM, VanMiddlesworth JF, Wolf WJ (1988) Content of raffinose oligosaccharides and sucrose in various plant seeds. Journal Agricultural and Food Chemistry 36:32-36

Kwon YS, Jeong MJ, Cha J, Jeong SW, Park SC, Shin SC, Chung WS, Bae H, Bae DW (2012) Comparative proteomic analysis of plant responses to sound waves in *Arabidopsis*. J Plant Biotechnol 39:261-272

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Larche M, Akdis CA, Valenta R (2006) Immunological mechanisms of allergen-specific immunotherapy. Nature Review Immunology 6:761-771

Miyake K, Morita R, Handoyo T (2004) Characterization of graded buckwheat flours and some properties of germinated 'Mancan' buckwheat grains. Fagopyrum 21:91-97

Oko AO, Ubi BE, Efisue AA, Dambaba N (2012) Comparative analysis of the chemical nutrient composition of selected local and newly introduced rice varieties grown in ebonyi state of nigeria. International Journal of Agriculture and Forestry 2:16-23

Perr HA (2004) Novel foods to treat food allergy and gastro-intestinal infection. Current Gastroenterology Reports 6:254-260

Peterbauer TR, Richter A (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Seed Science Research 11:185-197

Sanbongi C, Suzuki N, Sakane T (1997) Polyphenols in chocolate, which have antioxidant activity, modulate immune functions in humans in vitro. Cell Immune 177:129-136

Shrime MG, Bauer SR, Mcdonald AC, Chowdhury NH, Coltart CEM, Ding EL (2011) Flavonoid-rich cocoa consumption affects multiple cardiovascular risk factors in a meta-analysis of short-term studies. Journal of Nutrition 141:1982-1988

Taubert D, Berkels R, Roesen R, Klaus W (2003) Chocolate and blood pressure in elderly individuals with isolated systolic hypertension. Journal of The American Medical Association 290:1029-1030

Yang F, Basu TK, Ooraikul B (2001) Studies on germination conditions and antioxidant contents of wheat grain. International Journal of Food Sciences and Nutrition 52:319-330