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Almond Skin Polyphenol Extracts Stimulate the Activation of Diacylglycerol Kinase alpha via a 67 kDa Laminin Receptor

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ABSTRACT

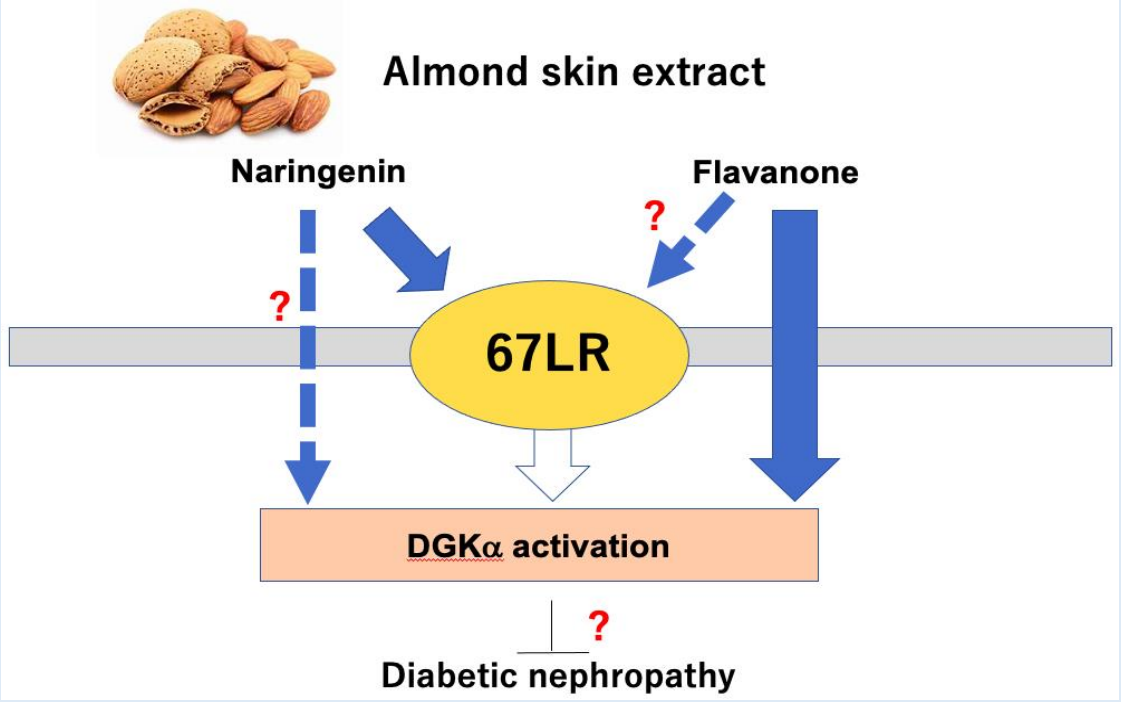
Background: Almond skins are the byproduct of the almond industry that are rich in dietary fibers and polyphenols. Recently, there has been an increasing interest in utilizing the phenolic compounds from almond skins for their functional benefits. Galloylated catechins activate diacylglycerol kinase α (DGKα) which is involved in the amelioration of diabetic nephropathy. Therefore, in this study, we investigated whether almond skin polyphenol extracts can also induce the activation of DGKα.

Methods: Phenolic contents in the almond skin polyphenol extracts were identified by liquid chromatography (LC)-time of flight mass spectrometry (TOFMS). Using confocal microscopy, the translocation of green fluorescent protein (GFP)-DGKα to the cell membrane was observed upon stimulation with almond skin polyphenol extracts. To check the involvement of 67 kDa laminin receptor (67LR), pre-treatment of anti-67LR antibody was used.

Results: We identified that naringenin and flavanone (2,3-dihydroflavone), which are among the phenolic contents in the almond skin polyphenol extracts identified, can also induce the activation of DGKα. In addition, we also investigated whether the pathway involves the same receptor as that of epigallocatechin-3-gallate (EGCg); the 67LR. Naringenin stimulated through the 67LR, while flavanone mainly used the 67LR-independent pathways.

Conclusion: These findings were additional function of almond skin polyphenol extracts and may implicate the benefits of the intake of nuts in daily diets. Polyphenols can be extracted from almond skins as an inexpensive source. The various health benefits of polyphenols can be applied to functional foods and supplements.

Keywords: flavonoid, lipid kinase, imaging, catechin, vitamin E, diabetic nephropathy



INTRODUCTION

Diabetic nephropathy is one of the major vascular complications that affects approximately 35% of patients with diabetes mellitus and is the leading cause of end-stage kidney disease [1]. The pathogenesis of the disease involves complex interplay between both genetic and environmental factors, leading to the stimulation of inflammatory responses and abnormal renal functions [2]. Hyperglycemia has been extensively studied as the major cause of glomerular hyperfiltration, and one of the underlying mechanisms is the protein Kinase C (PKC)-dependent pathways. PKC has diverse functions ranging from controlling the polyol pathway to the production of advanced glycation end products. It is activated by diacylglycerol (DAG) which increases during hyperglycemia through many pathways such as, de novo synthesis and conversion from phosphatidic acid (PA). The excess activation of PKC by DAG may enhance the activity of angiotensin II and the production of extracellular matrix (ECM), contributing towards diabetic nephropathy [3]. Recently, the amelioration of diabetic nephropathy has been focusing on reducing the activity of PKC through inhibition [4] or reducing the amount of its activator, DAG, by DAG kinase (DGK) [5].

DGK phosphorylates DAG forming PA and thus, attenuates the activity of PKC [6]. Upon activation, DGK translocates from the cytoplasm to the plasma membrane. Among the 5 subtypes of mammalian DGK

expressed in the glomeruli, DGKα has been shown to be stimulated by d-α-tocopherol [7] and the oral administration of d-α-tocopherol significantly reduced the loss of podocytes, which make slits for renal filtration [8]. The chroman ring of d-α-tocopherol is required for the activation and translocation of DGKα to the plasma membrane [9]. Subsequently, galloylated catechins have also been confirmed to activate DGKα, especially epigallocatechin-3-gallate (EGCg) which has been shown to induce the activation through the cell surface receptor 67-kDa laminin receptor (67LR) [10]. 67LR is the receptor for EGCg, the predominant green tea polyphenol, and is responsible for the anticancer activities of EGCg such as, cell growth inhibition and apoptosis [11]. The requirement for 67LR by galloylated catechins to activate DGKα may be the underlying mechanism for the biological effects of galloylated catechins. However, our understanding of the interactions between DGKα and 67LR is limited.

Almonds are rich in monounsaturated fats, proteins, and dietary fibers. Recently, along with almonds' highly nutritious profile, almond skin polyphenols have been extensively characterized as the source of antioxidants and bioactives. The polyphenols identified in almonds include proanthocyanidins, hydrolysable tannins and flavonoids [12]. Blanched almond skins are the by-product of the food processing industry which have a potential to be used as a functional ingredient, due to their high dietary fibers and polyphenol composition. A growing number of evidence supports the health benefits of almond consumption in improving metabolic syndrome [13]. Almond consumption has been shown to improve postprandial glycemia and hormonal control of patients with type 2 diabetes [14]. However, less is known about the molecular mechanism underlying these health benefits. Therefore, in this study, we investigated whether almond skin polyphenols stimulate the activation of DGKα via a 67LR.

MATERIALS AND METHODS

2.1 Almond skin polyphenol extraction: Almond skin polyphenol crude (ASP-A), 30% (ASP-B) and 50% (ASP-C) extracts were obtained following the same extraction but different purification procedures. Blanched almond skin (provided by Ezaki Glico Co., Ltd., Osaka, Japan) was dried at 60°C until the moisture was < 5%. Extraction was conducted by refluxing 1:10 (w/v) skin in 70% (v/v) aqueous ethanol for 1 hr at 80°C. The extract was filtrated through No. 5C filter paper (Advantech MFS, CA, USA) to remove the skin. For ASP-A, ethanol was evaporated, and the extract was passed through Whatman Grade 2 qualitative filter paper (GE Healthcare, NJ, USA) in the presence of 20% Radiolite 300 diatomite filter aid (Showa Chemical Industry, Tokyo, Japan).

For ASP-B and ASP-C, the extract was passed through Whatman Grade 2 qualitative filter paper with 1% Radiolite 300 diatomite filter aid. Then, ethanol was removed, and the filtrate was centrifuged at 17,000 × g for 15 min at 4°C to remove insoluble particles. Column separation was performed using Diaion HP20 column (Mitsubishi Chemical, Tokyo, Japan). The column was washed with 10% (v/v) aqueous ethanol and eluted with 30% and 50% (v/v) aqueous ethanol to obtain ASP-B and ASP-C, respectively. The extracts were freeze-dried for storage.

2.2 Total phenolic content quantification: The total phenolic content was determined based on Folin-Ciocalteu method [15]. Stock solutions of almond skin polyphenol extracts were prepared at 10 mg/ml in methanol and diluted with Milli-Q water. 100 µl sample was mixed with 500 µl 10% Folin-Ciocalteu reagent (Nacalai tesque, Kyoto, Japan) for 3 min, then 400 µl 7.5% (w/v) aqueous sodium carbonate was added. The mixture was incubated in the dark for 1 hr, and absorbance was measured at 765 nm. The total phenolic contents were calculated in terms of Gallic Acid Equivalents (GAE).

2.3 LC-TOFMS analysis: Approximately 5 mg of almond skin polyphenol extract was mixed with 300 µl 1% (v/v) formic acid in acetonitrile containing internal standard solution (H3304-1002, Human Metabolome Technologies Inc., Tsuruoka, Japan). The mixture was homogenized three times at 1,500 rpm for 120 s using a tissue homogenizer (Micro Smash MS100R, Tomy Digital Biology, Tokyo, Japan). The mixture was homogenized again after adding 100 µl Milli-Q water, followed by centrifugation at 2,300 × g for 5 min at 4°C. The supernatant was filtrated through 3-kDa cut-off filter (Nanocep 3K Omega, Pall Corporation, Michigan, USA) to remove proteins, and further filtrated by using Hybrid SPE phospholipid 55261-U (Supelco, PA, USA) to remove phospholipids. The filtrate was desiccated and then dissolved in 100 µl 50% (v/v) aqueous isopropanol prior to analysis. LC-TOFMS was carried out through a facility service (Human Metabolome Technologies Inc., Tsuruoka, Japan).

2.4 GFP-DGKα translocation assay: Smooth muscle cells, DDT1-MF2, were cultured in high glucose DMEM (Wako, Osaka, Japan) supplemented with 2 mM L-glutamine, 5% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). At sub-confluence, cells were sub-cultured to 35 mm poly-lysine coated glass bottom dish (Matsunami, Osaka, Japan) at 1.0 x 10^5 cells/dish and incubated for 24 hr at 37°C. Then, lipofection was conducted following the protocol of FuGENE HD transfection reagent (Promega, WI, USA) with the plasmid encoding GFP-DGKα [9]. Stock solutions of almond skin polyphenol extracts, Epicatechin gallate (ECg) (Wako, Osaka, Japan), EGCg (LKT laboratories Inc., MN, USA), naringenin and flavanone (Sigma Aldrich, MO, USA) were prepared in methanol and diluted with Ringer's solution composed of Krebs-Ringer phosphate buffer (120 mM NaCl, 4.8 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4, 16.5 mM NaH2PO4/Na2HPO4, pH 7.4, and 5 mM β-D-glucose). Mixture sample contained ECg, flavanone and naringenin in the ratio of 16:740:8.1, respectively. 67LR blocking was performed prior to sample treatment where cells were treated with 20 µg/ml anti-67LR antibody (clone name MLuC5) (GeneTex, CA, USA) for 2 hr at 37°C. Transfected cells were treated with various concentrations of samples for 5 min at 37°C and fixed with 4% PFA. Images were taken with confocal fluorescence microscope (FV-1000, Olympus, PA, USA). Translocation rate was calculated as the average percentage of the number of cells where GFP-DGKα translocation was observed to the total number of GFP-DGKα expressing cells.

2.5 Statistical analysis: All values are expressed as mean (SD). Statistical analysis was performed using R 3.6.1 (R Core Team, 2019). The data were analyzed using one-way analysis of variance (ANOVA) with a post-hoc Tukey HSD test. * and **means $p < 0.05$ and ** $p < 0.01$, respectively.

RESULTS AND DISCUSSION

3.1 Effects of almond skin polyphenol extracts on DGKα localization: The extract contained mainly polyphenols and the effects of almond polyphenols on DGKα activation were investigated through the translocation of GFP-DGKα to the plasma membrane. After stimulation with different almond skin polyphenol extracts, GFP-DGKα was observed to aggregate as spots on the cell membrane, like the pattern of EGCg (Fig. 1A). At 50 µg/ml, both ASP-B and ASP-C showed higher translocation rates than the EGCg control (Fig. 1B). Fig. 2 shows the total phenolic contents of respective almond skin polyphenol extracts, indicating that the total phenolic contents of almond skin polyphenol extract remarkably increased after purification. Corresponding to the amount of total phenolic contents, ASP-A induced the lowest translocation rate compared to the other fractions.

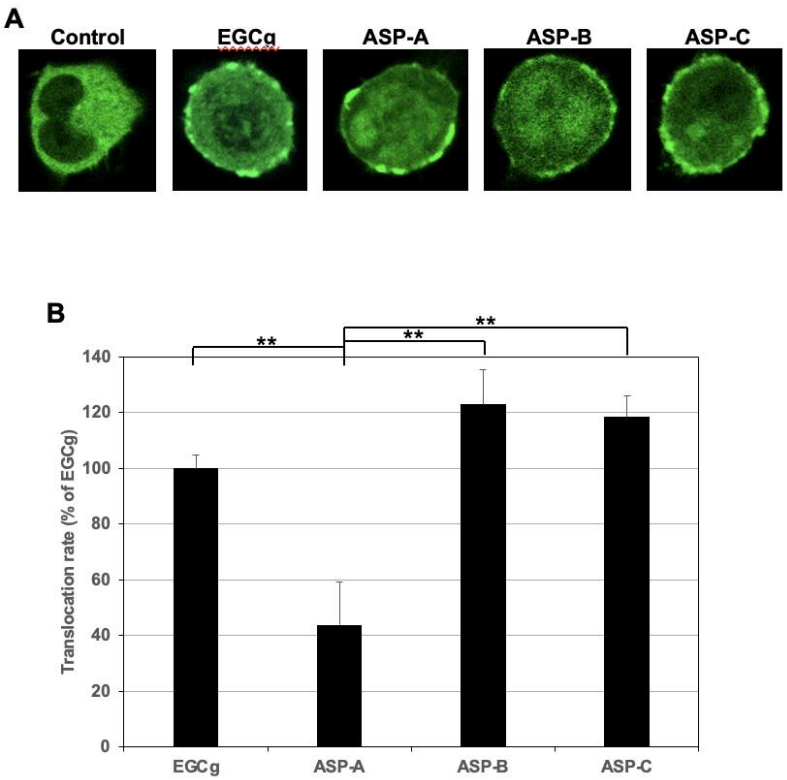


Fig 1. Effects of almond skin polyphenol extracts on GFP-DGKα localization. (A) Typical images of GFP-DGKα after stimulation with almond skin polyphenol extracts. (B) Comparison of the translocation rates of GFP-DGKα induced by different almond skin polyphenol extracts, compared to EGCg positive control, at 50 μg/ml. (** $p < 0.01$)

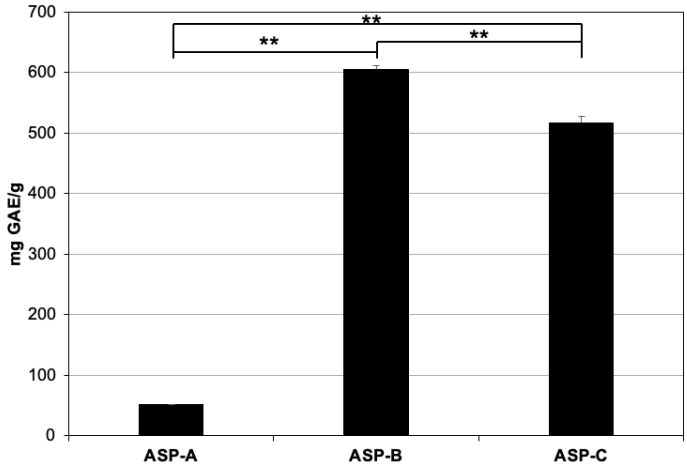


Fig 2. Total phenolic contents of almond skin polyphenol extracts, expressed as gallic acid equivalents (GAE) per g extract (dry weight). (** $p < 0.01$)

3.2 Identification of DGKα stimulating flavanones in almond skin polyphenol extracts: To identify the bioactive compounds that induced DGKα activation, ASP-B and ASP-C were subjected to polyphenol composition analysis by LC-TOFMS (Fig. 3). 34 phenolic compounds were identified; 26 of which were flavonoids. Interestingly, EGCg was not detected in the almond skin polyphenol extracts. ASP-B contained 15 flavonoids, while ASP-C contained 23 flavonoids. The structural basis behind the activation of DGKα by catechins requires both

the chroman ring and the gallate moieties [10]. However, DGKα activation was still observed for ASP-C which lacked galloylated catechins. Therefore, other active compounds should be present in ASP-C that induced the activation of DGKα. It has been reported that only the chroman ring was sufficient for the action of d-α-tocopherol [9]. There were 14 unmodified flavonoids identified in the almond skin polyphenol extracts; 4 of which belonged to the flavanone subgroup and only flavanone (2,3-dihydroflavone) and naringenin were detected in both extracts. ASP-C contained higher flavanone than ASP-B of $1.3x10^{-1}$ and $7.4x10^{-2}$ relative concentration units, respectively. Naringenin was also higher in ASP-C than ASP-B of $1.7x10^{-2}$ and $8.1x10^{-4}$ relative concentration units, respectively. Thus, we focused on the flavanone and the naringenin.

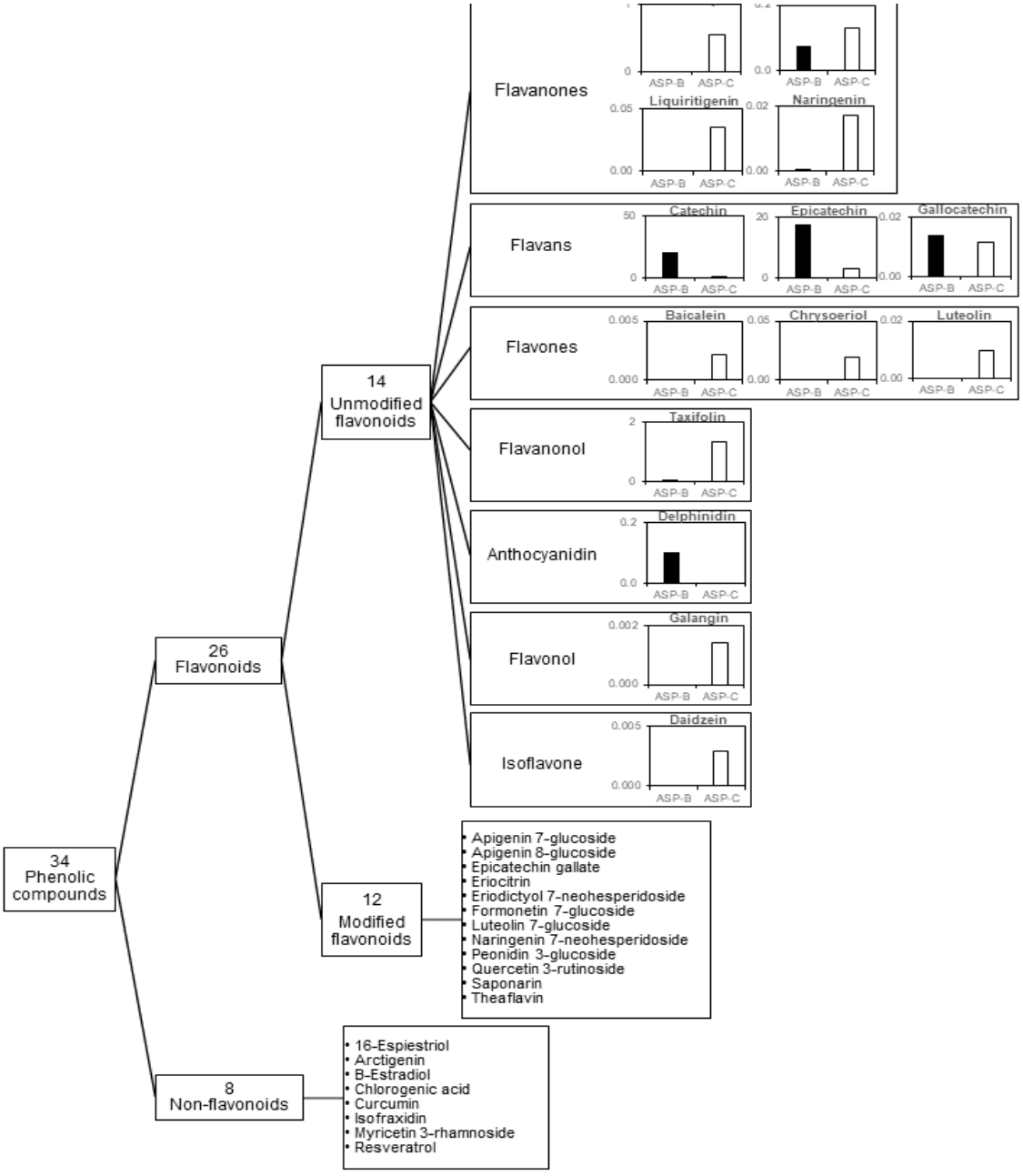


Fig 3. Classification of the phenolic compounds found in almond skin polyphenol extracts by LC-TOFMS. Comparisons of the relative concentrations of unmodified flavonoids in ASP-B (filled) and ASP-C (unfilled) are also shown

Upon stimulation with flavanone and naringenin, GFP-DGKα was observed to accumulate around the cell membrane (Fig. 4A). The dose-dependent effect of flavanone was comparable to that of ECg (Fig. 4Bi). On the other hand, naringenin showed a moderate effect of just approximately 26% translocation rate at 50 μg/ml. Although the results showed a variable effectiveness of different flavanones, they suggest that, at least, flavanone and naringenin are bioactive compounds contained in almond skins to activate DGK□. This was supported by the observation that the mixture sample, containing the same ratio of ECg: flavanone: naringenin as the ASP-B, was effective in inducing the translocation of DGKα (Fig. 4Bii).

Interestingly, the dose-dependent effect of the mixture sample was lower than that of ASP-B. The difference indicates the presence of other DGKα activating phenolic compounds in the almond skin polyphenol extracts, apart from ECg, flavanone and naringenin. Further experiments are required to analyze the phenolic contents of the almond skin polyphenol extracts to identify other DGKα activating compounds. In addition, we had better check if the other small components contained in the extract can activate DGKα or not.

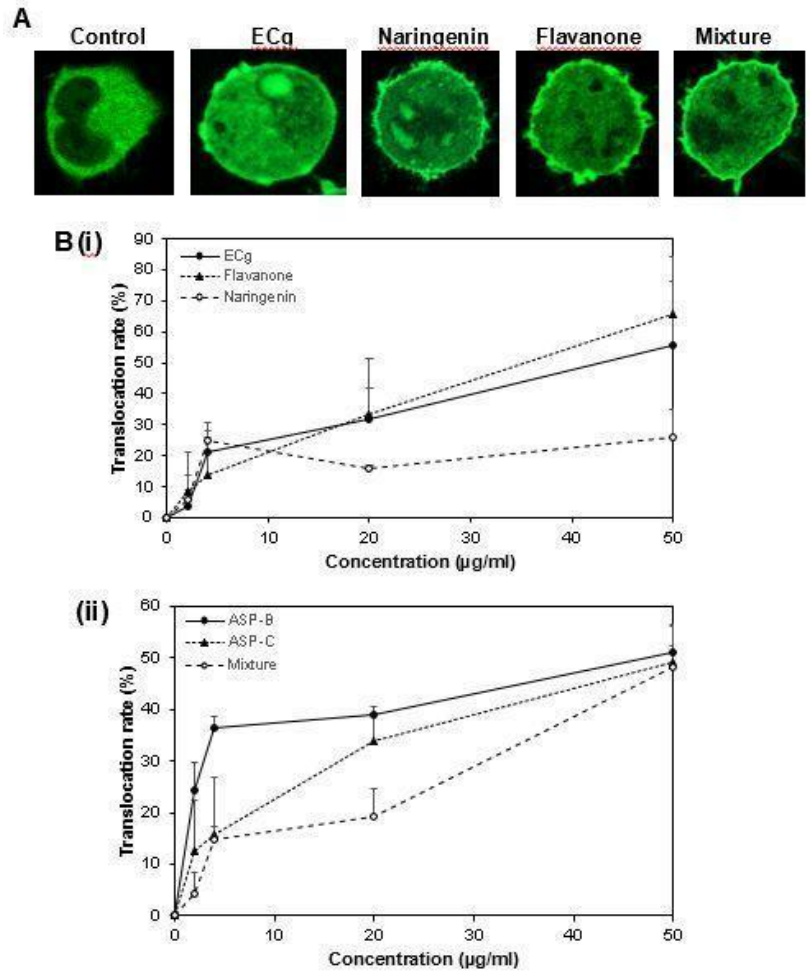


Fig 4. Effects of different phenolic compounds and almond skin polyphenol extracts on GFP-DGKα localization. (A) Typical images of GFP-DGKα after stimulated with different flavanones and a mixture sample, containing the same ratio of ECg:Naringenin:Flavanone as ASP-B. ECg was used as a positive control. (B) Dose-dependent effects on GFP-DGKα translocation induced by (i) flavanones and ECg; (ii) almond skin polyphenol extracts and a mixture sample.

3.3 Involvement of 67LR in the activation of DGKα: 67LR is involved in the activation of DGKα by both EGCg and α-tocopherol. To assess whether 67LR is also required for the activation of DGKα by flavanones, we investigated the effect of the anti-67LR antibody on the translocation. The extent of the anti-67LR antibody inhibition on GFP-DGKα activation varied depending on the extracts' phenolic compositions (Fig. 5). Blocking the 67LR with antibody significantly attenuated the translocation of GFP-DGKα induced by naringenin and ECg, by approximately 27% and 83%, respectively. This shows that naringenin DGKα activates via 67LR. On the other hand, the flavanone-induced translocation showed the tendency to be inhibited by the presence of anti-67LR antibody, yet the difference was not statistically significant. This highlights the presence of 67LR-dependent and 67LR-independent pathways utilized by different phenolic compounds in activating DGKα. Due to the lipophilic structures of the flavanones, they can readily enter the cell through passive diffusion and bind to cytoplasmic receptors to induce signal transductions. Flavanone has been shown to bind to peroxisome proliferator-activated receptor γ (PPARγ) [16]. Therefore, it may be possible that flavanones activate DGKα via an intracellular receptor, rather than binding to the cell surface 67LR. However, the different side chains of the flavanones influence their hydrophobicity and affect the reactivity and the mechanism of action. There is some evidence supporting the binding of flavanones to cell surface receptors, such as the human β2-adrenergic receptor [17] and FMS-like tyrosine kinase 3 (FLT3) [18]. Although evidence for naringenin binding to cell surface receptors is limited, the presence of hydroxyl side chains on naringenin reduces its lipophilicity, and thus it may be possible that naringenin preferentially binds to the cell surface 67LR. The low DGKα activation effect of naringenin may highlight the low affinity interaction with the 67LR. The binding of naringenin to 67LR will need to be further elucidated and whether the binding occurs at the same motif as EGCg [11].

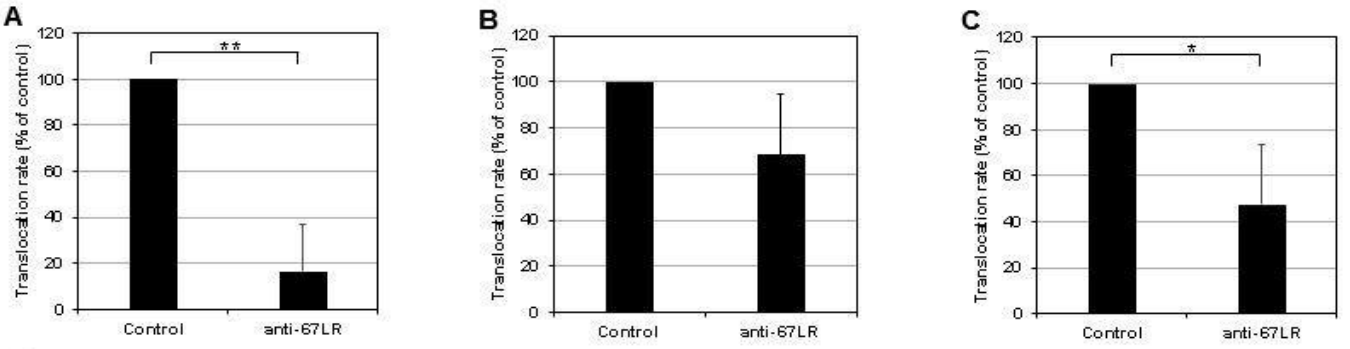


Fig5. Effects of pretreatment with the 67LR antibody on the translocation of GFP-DGKα upon stimulation with different compounds. (A) ECg; (B) Flavanone; (C) Naringenin; (* $p < 0.05$, ** $p < 0.01$)

The activation of DGKα may be related to alterations to the cell membrane through lipid raft modifications. D-α-tocopherol has been reported to associate with lipid rafts, modulating the membrane lipid composition and influencing signal transductions [19]. 67LR has also been identified to associate with lipid rafts which affected the activity of EGCg [20]. Previously, the activities of DGKα and DGKζ have been shown to be modulated by membrane properties and compositions [21]. Therefore, compounds that can alter the properties of the cell membrane are likely to affect the activation of DGKα.

Recently, focus has been on utilizing the beneficial

almond skin polyphenols for their antioxidant capacity [22], anti-inflammation [23] and anti-viral effects [24]. Here, we demonstrated that almond skin polyphenols stimulate the activation of DGKα via a 67LR. DGKα has also been reported to be involved in insulin secretion by pancreatic β cells [25]. 67LR has been identified as cell surface receptor, and there has been reported several signal pathways through this receptor. Thus, there are potentials in almond polyphenols in activating DGKα or signaling pathway through 67LR. For example, there is a potential in ameliorating diabetes through the synergistic effects of both improving the kidney function and regulating insulin secretion, although the effect will have to be further validated through clinical trials involving patients with diabetic nephropathy. The findings in this study provide additional functions of almond skin polyphenol extracts and may implicate the benefits of the intake of nuts in daily diets.

CONCLUSION

Almond skin polyphenol extracts induced the activation of DGKα. The effect was dependent on the phenolic acid compositions of the extracts. ASP-B contained fewer types of flavonoids than ASP-C, yet its effect was stronger. Among the phenolic compounds identified in the almond skin polyphenol extracts, flavanone and naringenin were also found to activate DGKα. The 67LR was required for the induction of DGKα activation by ECg, and naringenin. However, flavanone stimulated DGKα via a 67LR-independent pathway.

List of Abbreviations: ASP: Almond skin polyphenol crude; DAG: diacylglycerol; DGK: diacylglycerol kinase, ECg: epicatechin gallate; EGCg: epigallocatechin-3-gallate; ECM: extracellular matrix; FBS: fetal bovine serum; GAE: gallic acid equivalents; GFP: green fluorescent protein; LC: liquid chromatograph; MS : mass spectrometry; PKC: Protein Kinase C; 67LR: 67 kDa laminin receptor; PA: phosphatidic acid; PPARγ: peroxisome proliferator-activated receptor γ

Author Contributions: B. Chinthammit and S. Okamoto designed and conducted the experiments. B. Chinthammit interpreted the results and drafted the manuscript. Y. Shirai provided supervision throughout this work.

Conflicts of Interest: The authors declare no conflict of interest.

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