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Flavonoids and Functional Properties of Germinated Citron (*Citrus junos* Sieb. ex TANAKA) Shoots

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Abstract The main objective of this study was to investigate possible application of citron (*Citrus junos* Sieb. ex TANAKA) seeds, which are massively produced as by-products during citron tea process, into functional food materials. First of all, citron seeds were germinated and produced citron shoots were examined for their functional properties. When contents of flavonoids in citron seeds and their germinated shoots were compared, naringenin, neohesperitin, and hesperitin were remarkably increased in shoots after germination while naringin and didymin were decreased. Concentrations of limonin and nomilin were decreased by germination otherwise their unidentified derivatives were newly formed. A methanol extract of citron shoot had lower IC_{50} values [0.13 and 0.07 mg/mL for 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and ABTS, respectively] than citron seed extract in radical scavenging activities. Addition of 500 mg/mL of citron shoot extract suppressed fat accumulation in 3T3-L1 adipocytes by 36.9%. Oral administration of olive oil along with citron shoot extract (33 mg/kg body weight) to Sprague Dawley rats effectively inhibited absorption of lipid into a body by decreasing blood triglyceride levels from 105.1 to 74.9 mg/dL 2 hr after olive oil administration. According to these results, citron shoot extract as a rich source of flavonoids can be utilized for functional food ingredients with effective antioxidant and anti-adipogenic properties.

Keywords: citron seed, germination, citron shoot, antioxidant, anti-adipogenic

Introduction

Citron (*Citrus junos* Sieb. ex TANAKA) is a citrus fruit originating in East Asia. It was originated in China and introduced in Korea and Japan during the Tang dynasty. Citron is famous for its peculiar tart flavor that has attracted consumer's taste. When mature citron is processed for tea or other beverage products in Korea, massive amounts of seeds are collected and discarded. Therefore, it is worth to investigate functional potential of citron seeds for the purpose of adding values on by-products.

Seeds have a high demand for energy during early stage of germination, i.e., for repair and activation of metabolic machinery. Germination is the practice of soaking and leaving seeds until they germinate and begin to sprout. This practice is reported to be associated with improvements in the nutritional value of seeds by increasing nutrients such as free amino acids, carbohydrates, and phytochemicals (1-6). Seeds of fruits are generally known to have abundant antioxidant compounds to protect themselves from hazardous attacks from outside. Contents of polyphenols and their metabolites in buckwheat seeds were greatly increased during germination, which resulting in enhanced anti-adipogenic activities (7). Therefore, as a rich source of phytochemicals (8), contents of these phytochemicals in citron seeds also can be increased by germination. Since there are few reports on germinating citron seeds, it might be interesting to investigate changes in phytochemicals during germination and possible application of germinated citron products as functional food resources.

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Materials and Methods

Materials Mature citron was harvested on November, 2007, in Gochang, Jeollabuk-do, Korea. After citron was processed for tea, wasted citron seeds were collected and immediately delivered to our laboratory. Citron seeds were washed thoroughly under running tap water, dried at 50°C for 12 hr and stored at room temperature before further experiments. Equivalent amounts of citron seeds were divided either for extraction or germination.

Extraction For preparing citron seed extract, seed coats of dried citron seeds were removed by a rice huller (Model BCH 220; Daehwa Co., Daejeon, Korea). Then, dehulled citron seeds were pulverized and defatted with hexane for 24 hr followed by extracting with 20 volumes of methanol at 70°C for 3 hr. The methanol extract was concentrated under reduced pressure (34-36 kPa) using a rotary evaporator and stored at a deep-freezer.

Germination For germination, citron seed coats were carefully removed by hands and germinated for 3 weeks at 25°C in a dark room with spraying water for 1-2 min every 3 hr. After 3 weeks of germination, citron shoots (average 15 cm long) were separated from their seeds and roots, and dried at 50°C for 12 hr. Methanol extracts of citron shoots and seeds/roots were prepared by the procedure that was applied to methanol extraction of citron seeds.

Determination of total polyphenol contents Contents of total polyphenols in citron seeds, germinated citron shoots and seeds/roots were determined by the methods of Singleton *et al*. (9) with minor modification. Samples (0.125 mL) were added with distilled water (0.5 mL) and Folin-Ciocalteu's reagent (0.125 mL), and the mixture was incubated for 6 min at room temperature. Then, 1.25 mL of 7% sodium carbonate and 3.0 mL of distilled water were added and incubated for another 90 min. The absorbance at 760 nm was read and converted to total polyphenol contents according to the calibration curve of standard rutin (Sigma-Aldrich, St. Louis, MO, USA).

High performance liquid chromatography (HPLC) analysis of flavonoids and limonoids HPLC analysis of flavonoids and limonoids in citron seed and citron shoot extracts was performed using a JASCO PU-990 equipped with pump/851-AS, sampler/807-IT, integrator/LG-1580-04, gradient/DG 1580-54, and degasser (Jasco, Tokyo, Japan). Freeze-dried methanol extracts of citron seed and citron shoot were dissolved in water (5 mg/mL), membrane filtered (0.45-mm) and injected at ambient temperature into Eclipse XDB-C18 (4.6×250 mm, Agilent Technologies, Palo Alto, CA, USA). The mobile phases for flavonoids analysis were: (A) 50 mM phosphoric acid and (B) methanol with a flow rate of 0.7 mL/min. Gradient elution was applied from 98% (A) as follows: 0-10 min, 98-95% (A); 10-50 min, 95-65% (A); 50-60 min, 65-60% (A); 60-70 min, 60-40% (A); 70-90 min, 40% (A). Otherwise, the isocratic mobile phases (acetonitrile:methanol:water, 10:41:49) were used for limonoids analysis with a flow rate of 1.0 mL/min. Flavonoids and limonoids were detected at 280 and 210 nm, respectively. Four citrus flavanones (naringin, neohesperidin, naringenin, and hesperitin) and 2 limonoids (limonin and nomilin) were identified and confirmed by comparing their retention time on liquid chromatography (LC) with their standards (Sigma-Aldrich) and spiking them into the extracts. Standard didymin was purchased from Extrasynthese (Genay, France). Their contents in extracts were calculated based on the calibration curve on each standard.

Antioxidant activities The free radical scavenging activity of citron seed and citron shoot extracts were measured by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method proposed by Brand-Williams *et al*. (10). $ABTS^{+\bullet}$ radical scavenging assay was also carried out by the method of Roberta *et al.* (11). β-Carotene bleaching assay was carried out according to the method of Jayaprakasha *et al*. (12) with slight modification.

Inhibition of intercellular triglyceride (TG) formation in adipocytes 3T3-L1 cells were maintained in Dulbeco's modified Eagle's medium (DMEM) and supplement with 100 mg/mL of calf bovine serum at 37°C in 5% CO_2 cell incubator. Preadipocyte 3T3-L1 cells were grown in 6-well plates until cells reached confluence. The adipogenic differentiation was induced by addition of 10 μg/mL insulin, 0.5 mM 3-isobuthyl-L-methylxanthine (IBMX), and 0.25 μM dexamethasone (DEX) with 100 mg/mL of fetal bovine serum (FBS) in DMEM. To examine influence of citron seeds and citron shoots extracts on suppressing TG formation in adipocytes, both extracts were added to differentiation medium at 500 μg/mL each. After 48 hr, the medium was removed, and DMEM containing 100 mg/mL of FBS was replaced. The cells were further maintained for 6 days with changing the medium every 2 days. The degree of TG formation in the adipocytes was measured by Oil Red O staining followed by extracting dyeing agents with iso-propanol (13). The intensity of fat staining was measured by absorbance at 520 nm.

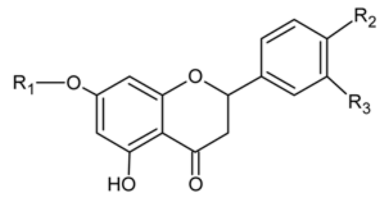
Anti-lipid absorption activity (animal study) All the following animal experiments were performed under the guidelines of the Laboratory Animal Experiment Committee of Korea Food Research Institute. Eight weeks old male Sprague Dawley rats (Hallym Experimental Animals, Gyeonggi, Korea) were divided into 2 groups of 5 rats each and orally administrated with 1.0 mL of distilled water (DW, control) or 10 mg/mL of citron shoot extract right after administrating 0.5 mL of olive oil. Blood samples were taken from an eye ground vein at 0, 1, 2, 3, and 4 hr after administration of oil using a heparanized capillary tube. The plasma was obtained by centrifuging blood samples at 1,200×g for 15 min. Plasma triglyceride level was measured enzymetically with a commercial assay kit (AM 157S-K; Asan Pharm. Co., Hwasung, Gyeonggi, Korea).

Statistical analysis The Statistical Analysis System (SAS) software ver. 6.11 was used to perform data analysis. All analyses were determined by Duncan's multiple range test at $p<0.05$.

Results and Discussion

Contents of phytochemicals in citron shoot Concentrations of total polyphenolic compounds in citron seeds before germination were 3.30 mg rutin equivalents/g dry matter. After 3 weeks germination, these amounts were increased to 6.63 mg (1.65 mg in shoots and 4.98 mg in seeds/root). Although more polyphenolic compounds were still in a germinated seed/root part than in a shoot, seed/root was too bitter to be used for functional food materials. Therefore, it was decided to focus more on investigating differences in properties of citron seeds and their germinated citron shoots in this study.

Composition of polyphenols in citron seeds and germinated citron shoots were compared after they were extracted with methanol. Contents of total polyphenolic compounds in methanol extract of citron seeds and citron shoots were 34.89 and 61.15 mg rutin equivalents/g dry extract, respectively. As shown in Fig. 1, compositions of polyphenolic compounds between 2 extracts were different. Major flavonoids in extracts were identified by comparing retention times of their standards followed by re-confirmation through spiking them into extracts. Didymin (III) was the most abundant citrus flavanones in citron seeds. Naringin (I), neohesperidin (II), naringenin (IV), and hesperitin (V) were other major flavanones in citron seeds. In citron shoot, however, there were abrupt increases in contents of naringenin, neohesperidin, didymin, and hesperitin whereas naringin was almost disappeared (Table 1). It is generally accepted that germination caused changes in both content and composition of flavonoids. Such changes are considered to be necessary in proper growth of



	Citrus flavanone	R_1	R_2	R_3
I	Naringin	Neohesperidose	OH	H
II	Neohesperidin	Neohesperidose	OCH_3	OH
III	Didymin	Rutinose	OCH_3	H
IV	Naringenin	H	OH	H
V	Hesperetin	H	OCH_3	OH

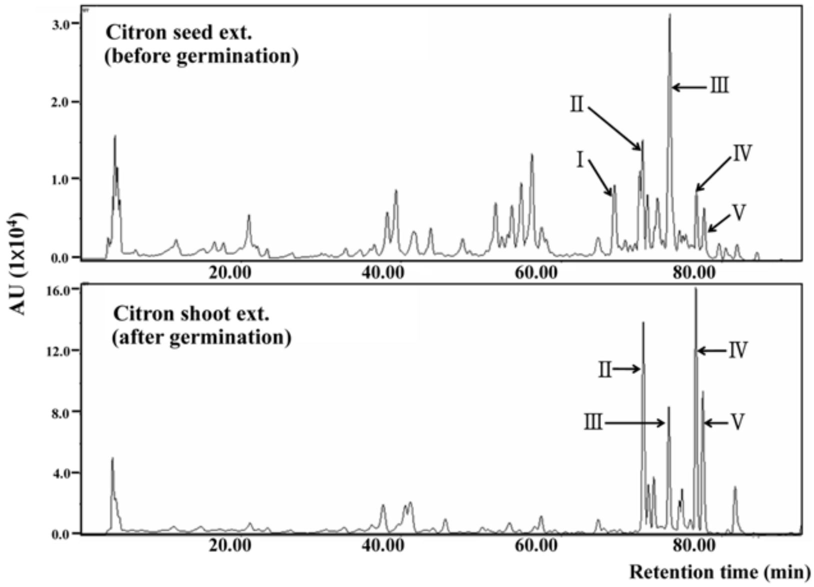


Fig. 1. Chemical structures of major citrus flavanones (upper) and HPLC chromatograms of polyphenolic compounds in methanol extracts of citron seeds and shoots (bottom).

Table 1. Contents of major citrus flavanones in methanol extracts of citron seeds (before germination) and citron shoots (after germination) (mg/g d.w.)

Flavonoids / Extracts	Naringin	Neohesperidin	Didymin	Naringenin	Hesperetin
Citron seeds	2.45	1.77	7.45	0.72	0.658
Citron shoots	-	15.63	12.24	10.51	20.01

plants through enhanced protection from an outside.

Limonoids, as another important phytochemicals, belong to a group of chemically related triterpenoids found in Rutaceae and Meliaceae families. Limonoids are highly oxygenated with fewer hydroxyl groups than polyphenols. Limonoids have attracted attention due to their insect antifeedant, growth regulating activities, and anti-carcinogenic activities (14,15). Limonoids in citron seeds were mostly composed of limonin (2.6 mg/g d.w.) and nomilin (1.5 mg/g d.w.) (Fig. 2). As germination proceeded, however, their concentrations were decreased whereas their unidentified derivatives (UI) were newly formed in shoots.

Antioxidant activities of citron shoot extract Epidemiological surveys have shown an inverse relationship between the intake of fruit and the incidence of coronary heart disease and some types of cancer (16,17). Antioxidants are also well-recognized for their potential role in reducing incidence of such diseases (18-21). Therefore, antioxidants in fruits are assumed to play major roles in such health beneficial effects. Citron seeds take 14-16% of total citron fresh weight. Grapefruit seeds that closely resemble citron seeds are famous for their excellent antioxidant capabilities (22,23). When citron seeds were fractionated with different solvents, those fractions also showed efficient antioxidant activities (8).

Antioxidant activities of citron seed and citron shoot extracts were compared. Methanol extract of citron shoots was more efficient in DPPH radical scavenging activity through all concentrations than methanol extract of citron

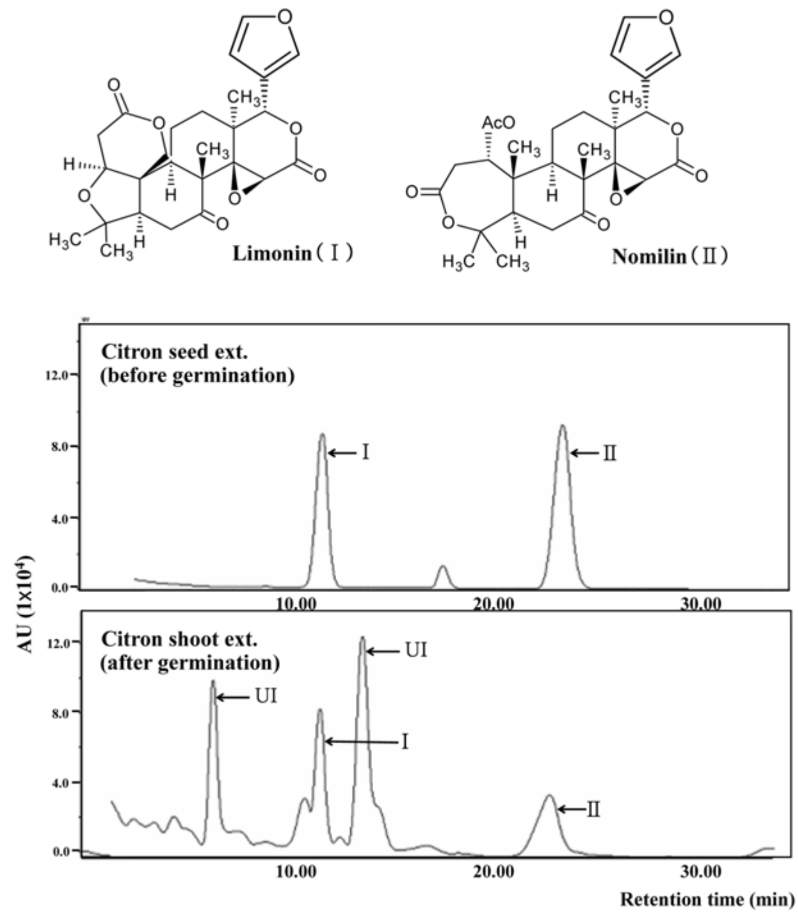


Fig. 2. Chemical structures of limonoids (upper) and HPLC chromatograms of limonoid compounds in methanol extracts of citron seeds and shoots (bottom).

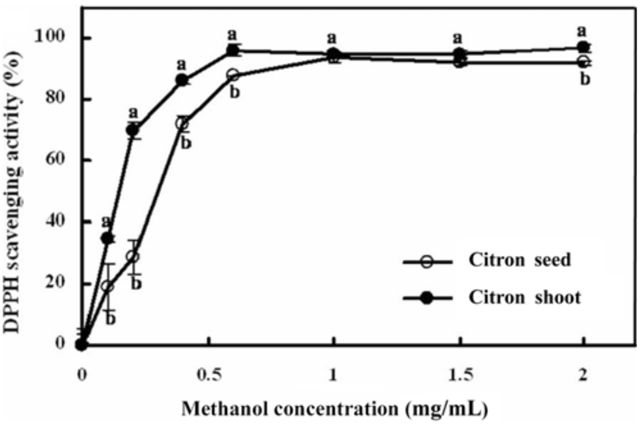


Fig. 3. DPPH radical scavenging activities of methanol extracts of citron seeds and citron shoots at different concentrations. Values are mean±SD of 6 experiments in each concentration. Different letters indicate significant difference at the level of $p<0.05$ between samples.

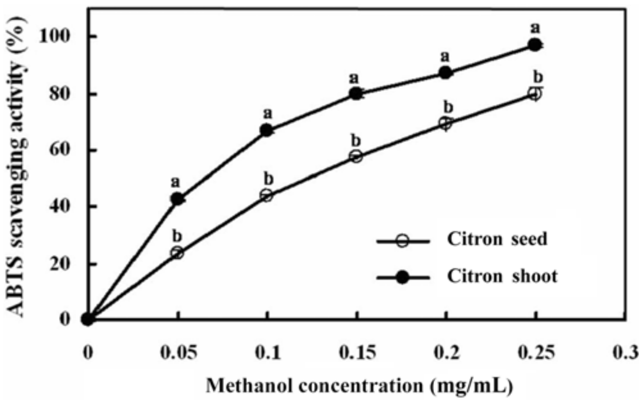


Fig. 4. ABTS cation radical scavenging activities of methanol extracts of citron seeds and shoots at different concentrations. Values are mean±SD of 6 experiments in each concentration. Different letters indicate significant difference at the level of $p<0.05$ between samples.

seeds (Fig. 3). The IC_{50}, which is the amount of antioxidants to decrease initial concentrations of DPPH radicals by 50%, of citron seed and citron shoot extracts were estimated to 0.29 and 0.13 mg/mL, respectively.

Similar results were observed in ABTS cation scavenging activities (Fig. 4). Addition of methanol extract of citron shoots with various concentrations was more efficient in scavenging ABTS cation radicals than citron seeds. Values of IC_{50} of citron seed and citron shoot extracts were estimated to 0.12 and 0.07 mg/mL, respectively. More efficient radical scavenging activities of citron shoot extract may come from their higher concentrations of antioxidant ingredients like flavonoid compounds. Previously, a

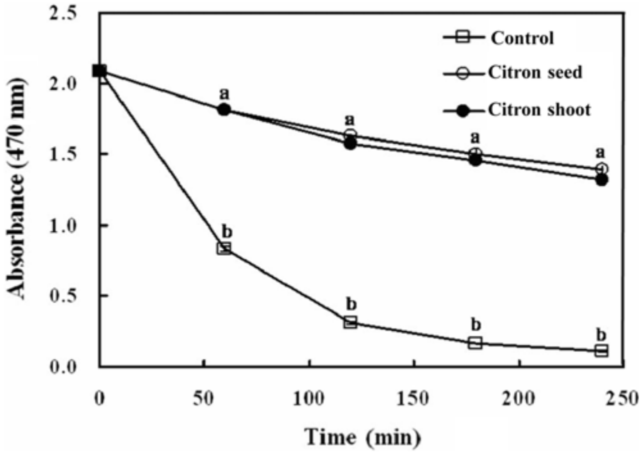


Fig. 5. Antioxidant activities of control, methanol extracts of citron seeds, and shoots at different concentrations in β-carotene-linoleate model system. Values are mean±SD of 6 experiments in each time period. Different letters indicate significant difference at the level of $p<0.05$ between samples.

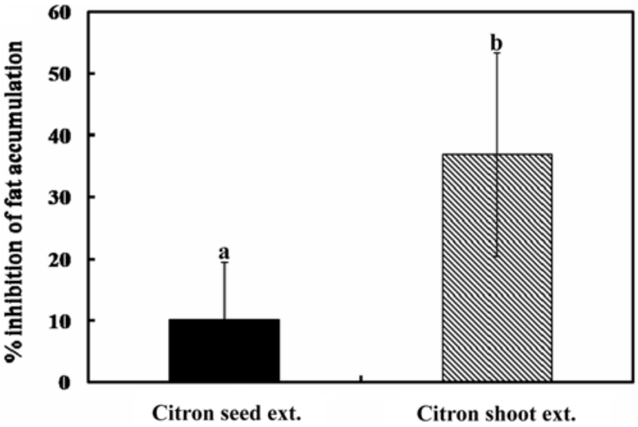


Fig. 6. Effects of additions of citron seed and citron shoot extracts on suppressing adipocyte differentiation in 3T3-L1 cells. Methanol extracts of citron seed and citron shoots were added at 0.5 mg/mL. Values are the mean±SE of 5 experiments in each group. Different letters indicate significant difference at the level of $p<0.05$ between samples.

positive relationship between radical scavenging activities and total polyphenol contents in various citron fractions was found (8). Also, different compositions in such flavonoids between citron seed and citron shoot extracts may cause differences in their radical scavenging activities. However, contents of limonin and nomilin as other key antioxidants in citron seeds were higher (27.44 and 15.60 mg/g dry extract, respectively) than those in citron shoot extract (9.62 and 4.21 mg/g dry extract, respectively) although concentrations of unidentified limonoids metabolites in citron shoots were not taken into consideration.

Effects of both citron seed and citron shoot extracts on β-carotene bleaching were investigated. β-Carotene bleaching assay measures the degree of denatured β-carotene molecules that are attacked by linoleic acid free radicals formed upon the abstraction of a hydrogen atom in their methylene groups by hydrogen peroxide. As β-carotene looses its double bonds upon the attack of linoleic acid free radical, its characteristic orange color disappears. In the absence of citron extracts (control), β-carotene underwent rapid discoloration (Fig. 5). Addition of citron seed and citron shoot extracts effectively suppressed bleaching of β-carotene up to 4 hr indicating that addition of citron seed and shoot extracts prevent β-carotene from oxidation by neutralizing the linoleate free radical and other free radicals formed in the system. Since structural features of limonin and nomilin probably contribute to their strong antioxidant activities in relatively hydrophobic environment, the citron seed extracts that contained higher amounts of these 2 limonoids seemed to have almost equal anti-β-carotene bleaching activities with citron shoot extract.

Anti-adipogenic activity of citron shoot extract To examine anti-adipocyte differentiating activities of citron seed and citron shoot extracts, 500 μg/mL of citron seed extract and citron shoot extract were separately added to 3T3-L1 postconfluent preadipocytes with hormone cocktail and incubated for 2 days. The cells were, then, maintained for another 6 days in the maintaining medium, and the extent of fat accumulation in cells were measured by Oil Red O staining. When degree of fat accumulation in citron seed or citron shoot extract added adipocytes were compared with fully developed adipocytes (control), it was clearly shown that addition of citron shoot extract suppressed accumulation of fat (36.9%) in 3T3-L1 cells more effectively than citron seed extract (10.1%) (Fig. 6). These results indicate that citron shoot extract suppresses either gene expressions of enzymes related to fat accumulation in 3T3-L1 or differentiation from pre-adipocytes into adipocytes.

Flavonoids could suppress adipogenesis by either inhibiting the signals that promote adipogenesis or decreasing adipose tissue mass. In intact rat adipocytes, quercetin inhibited insulin stimulated glucose transport, oxidation and its incorporation into lipids (24,25). Harmon and Harp (26) reported that genistein inhibited mitotic clonal expansion and induced lipolysis in adipocytes. We previously reported that germinated buckwheat of higher concentrations of flavonoids had better anti-adipogenic activities than buckwheat seeds (7). HSU and Yen (27) reported that addition of flavonoids such as naringin, naringenin, and hesperidin significantly suppressed formation of TG in adipocytes. Therefore, flavonoids in citron shoots may also play certain roles in suppressing intracellular TG formation in adipocytes.

Anti-lipid absorbing activity of citron shoot An animal study was conducted to investigate effects of oral administration of citron shoot extract on suppressing excessive lipid absorption into a body. Dietary lipid is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase that hydrolyzes lipid into fatty acid and 2-monoacylglycerol. Therefore, it is beneficial to find dietary compounds that can inhibit activity of pancreatic lipase for preventing excessive absorption of lipid and accumulation in a body. When SD rats were orally administered olive oil alone (control group), increase in blood TG reached to maximum (105.1 mg/dL) after 2 hr and maintained to 93.8 mg/dL after 4 hr

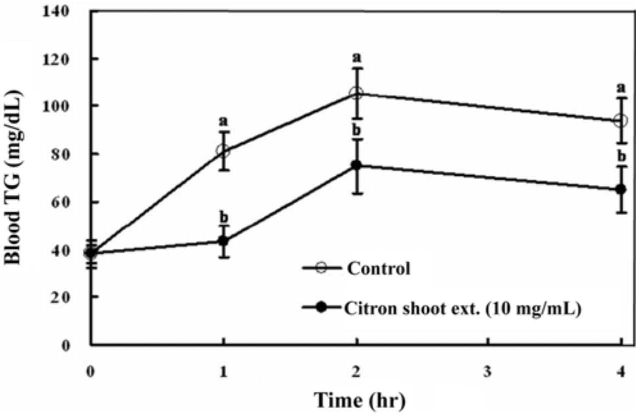


Fig. 7. Effects of oral administration of citron shoot extract on suppressing abrupt increase in blood triglyceride (TG) after olive oil administration. Ten rats were divided into control and citron shoot groups.

of olive oil administration (Fig. 7). Subsequent administration of citron shoot extract (10 mg), however, suppressed abrupt increase in blood TG levels to 74.9 mg/dL after 2 hr and 65.0 mg/dL after 4 hr indicating that citron shoot extract contain some ingredients inhibiting pancreatic lipase activity either by blocking active sites of the enzyme or surrounding substrate lipid in order to isolate substrate lipid from lipase's attack. A further study, however, is necessary to verify lipase inhibiting activities of citron shoot extract.

We investigated possible application of citron seeds, which are massively produced as wastes during citron tea processing, into value-added functional food materials. Germination of citron seeds was performed to achieve this purpose and the properties of germinated citron shoots were examined. Germinated citron shoot was a rich source of phytochemicals such as citrus flavanones and limonoids derivatives. Methanol extract of citron shoot exhibited excellent antioxidant and anti-lipidemic activities. According to these results, germination could be one good alternative to add values on wasted citron seeds.

Acknowledgments

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