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Original Research Article

Hypoglycemic Activity of the Hexane Extract of Oriental Melon (*Cucumis melo L. var. makuwa* Makino) Seeds and Its Active Compounds

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Abstract - The aim of this work is to evaluate the potential of oriental melon (*Cucumis melo L. var. makuwa* Makino) seeds for the management of type 2 diabetes by controlling glucose absorption. The α-glucosidase and α-amylase inhibitory effects of the hexane extracts from oriental melon seeds were investigated. A bioassay-guided fractionation technique was used to elucidate the principal active components. The results show that the hexane extract from oriental melon seeds exhibited high inhibitory activities against α-glucosidase and α-amylase. The hexane extract was further fractionated into four sub-fractions. Among them, the sub-fraction F-1 exhibited the most potent anti-diabetic effect. The active components were isolated and identified by gas chromatography/mass spectroscopy (GC-MS). Free fatty acids showed significant hypoglycemic activity ($p < 0.001$) and fatty acid composition influenced enzyme inhibitory activities. These results suggest that oriental melon seeds could be used to prevent type 2 diabetes.

Key words - α-Amylase, α-Glucosidase, GC-MS, Oriental melon

Introduction

Type 2 diabetes mellitus, a metabolic disorder, is characterized by high blood glucose in terms of insulin resistance and relative insulin deficiency. During the disease state of diabetes mellitus, α-glucosidase and α-amylase can be detrimental because this biochemical defect causes blood glucose levels to be elevated. The inhibition of α-glucosidase and α-amylase can significantly reduce the post-prandial increase of blood glucose and therefore can be a good strategy in managing the blood glucose level in type 2 diabetes. This approach, retarding the absorption of glucose through the inhibition of the α-glucosidase and α-amylase, has been done by some researchers through clinical test (Rhabasa-Lhoret and Chiasson, 2004). Inhibition of these enzymes can delay carbohydrate digestion and consequently blunting the postprandial plasma glucose increasing (Kwon *et al*., 2006). Due to the relative high safety and low incidence of serious gastrointestinal side effects, the consumption of natural α-amylase and α-glucosidase inhibitors from food-grade plant sources offer an attractive strategy to control post-prandial hyperglycemia in contrast to traditional treatment with drugs (Ali *et al.,* 2006).

Oriental melon (*Cucumis melo L. var. makuwa*) are widely grown in Korea and favored by consumers, largely due to their high qualities and special flavors. Recently, the biological activities of oriental melon have been studied extensively. The anti-oxidant activity (Kim and Kang, 2010), quinone reductase inductive effect and cytostatic effect on cancer cellproliferation (Kim *et al*., 2009) of oriental melon extract were reported. Meanwhile, oriental melon seeds have attracted little attention because of the limited information as by-product. At recent, oriental melon seeds have been reported to possess various pharmacological activities, such as anti-inflammatory (Gill *et al*., 2011), anti-ulcer (Ismaila *et al*., 2010), and anti-cancer (Kim *et al*., 2012; Wang *et al*., 2007) activities. The extracts of oriental melon seeds, which may have better health-promoting effects, are of interest to us. Our previous study has shown that oriental melon seeds, an effective inhibitor of α-glucosidase and α-amylase, contain high levels of fatty acids (Chen and Kang, 2013). Therefore, the present study focused on characterizing the major active constituents of oriental melon seeds. In this work, the *in vitro* effects of the

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hexane extracts on inhibition of key enzymes relevant for hyperglycemia (α-glucosidase and α-amylase) were correlated to the chemical constituents as determined by gas chromatography-mass spectrometry (GC-MS). A bioassay-guided fractionation technique was used to elucidate the principal active components.

Materials and Methods

Plant materials

Oriental melon seeds were obtained from Chambio company located in Seongju-gun, South Korea. The seeds were deserted as agricultural waste. Nevertheless, care was taken that all of the samples analyzed were representative of the same initial batch, which was homogeneous. All extraction solvents used were of analytical grade.

Preparation of extract

Ten grams of powdered melon seeds extracted with 250 ㎖ hexane solvent at 150℃ for three hours and repeated twice. The extract was filtrated through Whatman No. 2 paper, then evaporated in vacuum at 45℃ by using a rotary evaporator (JP/N 1000S-W; Eyela, Japan) and water bath (Digital Water Bath SB-651; Rikakikai, Tokyo, Japan) to remove the hexane and stored at 2-4℃ until use.

Bioactivity guided fractionation of seeds extract

To identify bioactive molecules from the active extract of oriental melon seeds, bioactivity-guided isolation was proceeded (Fig. 1). The hexane extract was chromatographed over silica gel column (pore size: 35−75 ㎛ diameter) by gradient elution using n-hexane, diethyl ether, and acetic acid; flow rate was maintained at 5 ㎖/min throughout the experiment. In this step, with the gradation of n-hexane percentage, F-1, F-2, F-3 and F-4 fractions were obtained, respectively. Among them, F-1 was found to be the most potent inhibitor against α-amylase and α-glucosidase in the bioactivity evaluation. Hence, F-1 was further separated by flash column chromatography to generate F-1-1 fraction. Then, the biologically active fraction (F-1-1) (513.1 ㎎) was purified by preparative TLC (silica gel, n-hexane/diethyl ether/acetic acid, 100:30:3) to obtain OM-1 (79 ㎎) (Rf = 0.48) and OM-2 (4 ㎎) (Rf = 0.32). Fractions, OM-1 and OM-2 were identified based on their mass spectrometric fragmentation patterns. Each

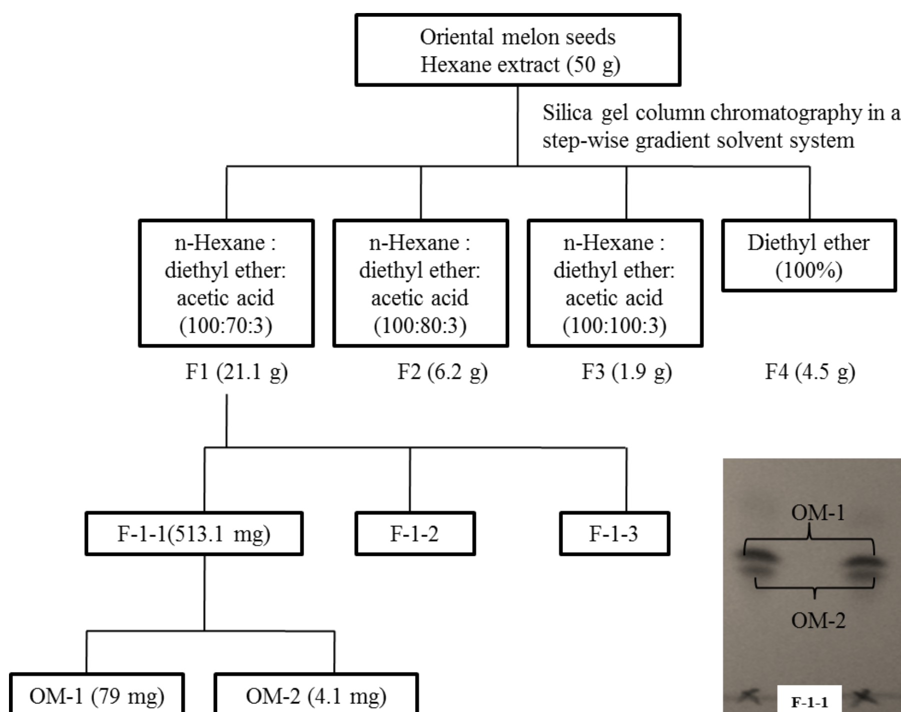

Fig. 1. A flow chart showing the fractionation procedures of oriental melon seeds.

fraction was dried using a rotary evaporator. All fractions were dried in vacuum and also reconstituted in DMSO at a concentration of 4 ㎎/㎖ for analysis.

Characterization of bioactive constituents

Thin-layer chromatography (TLC)

TLC was carried out on Merck plates (Darmstadt, Germany) percolated silica gel F254 (precoated Kiesel gel 60 F254, Merck, No. 5735, Darmstadt, Germany) and Kiesel gel 60 (70~230 mesh, Merck, No. 7734, Darmstadt, Germany) was used for silica gel column chromatography. Meanwhile, the solvent system was composed of n-hexane, diethyl ether, and acetic acid. The compounds were detected by iodine.

Gas chromatography/mass spectrometry (GC-MS)

GC-MS analysis was carried out using a Varian Star 3400 CX gas chromatograph equipped with a Varian Saturn 2000 ion trap MS detector, mounting 2 split/splitless 1078 universal capillary injectors (Varian, Palo Alto, CA, USA) equipped with a 30 m 0.25 ㎜ CPSil5 CB Low bleed/MS capillary column, 0.25 m film phase (Supelco, Bellefonte, PA, USA). The helium carrier head column pressure was 1.0 ㎖/min. Temperature programming was from 50℃ (hold 5 min) to 300℃ at 6℃/min; the transfer line and ion trap were at 250℃. Injector temperature was set at 250℃ to avoid decomposition. MS analyses were made in the electron impact (EI+) mode at 70 eV, the mass range was from m/z 40 to 400 and the chromatogram acquired in total ion current (TIC). Fractions, OM-1 and OM-2 were directly converted to trimethylsilyl (TMS) derivatives for GC-MS analysis.

α-Amylase inhibition assay

The α-amylase inhibition assay was performed using the chromomeric method adopted from Sigma-Aldrich. Forty ㎕ of sample and 200 ㎕ of *Aspergillusoryzae* α-amylase (EC 3.2.1.1) (4 U/㎖ prepared in ice cold distilled water) were mixed at 25℃ for 5 min. The reaction was started by the addition of soluble potato starch solution (0.5% w/v in 20 mM phosphate buffer, pH 6.9). After incubation for 3 min, DNS color reagent was added. The test tubes were placed in a water bath (85-90℃) for 10 min to develop color. Fifty microliters of the reacted mixtures were diluted with 175 ㎕ of distilled water in a 96-well microplate. The generation of maltose was quantified by the reduction of 3, 5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. This reaction (corresponding to color change from orange yellow to red) was detectable at 540 ㎚.

α-Glucosidase inhibition assay

This assay was performed according to our previous study (Chen and Kang, 2013). In brief, samples were diluted with 100 mM-phosphate buffer (pH 6.9) and incubated with α-glucosidase solution (0.76 U/㎖ in same buffer) at 25℃ for 5 min. Fifty ㎕ of 5 mM, p-nitrophenyl-α-D-glucopyranoside (prepared in same buffer) was added to each well in a time intervals after preincubation. In both cases before and after incubation, absorbance was recorded at 405 ㎚ and compared to a control.

Bioactivity analysis by correlating compounds

The pure standards and compounds were tested in the α-glucosidase and α-amylase inhibitory assay to explore structure-activity relationships. All of standards used in present study including α-linoleic acid (C16:2), oleic acid (cis-C18:1), palmitic acid (C16:0), and stearic acid (C18:0) were purchased from Sigma-Aldrich, Korea.

Statistics Analysis

Statistical analysis was performed by Statistical Analysis System (SAS, Cary, NC). Data were subjected to the analysis of variance (ANOVA), followed by mean comparisons by Ducan's multiple range test at $p < 0.05$. Results of three replicate readings were pooled and expressed as mean ± standard deviation.

Results and Discussion

The hexane extracts of oriental melon seeds showed the inhibitory effects against α-glucosidase and α-amylase (Chen and Kang, 2013). In order to elucidate active principles of oriental melon seeds, bioassay-guided fractionation was performed. Four fractions were prepared. The fractions showed enzyme inhibitory activities dose dependently. Activity data of the fractions were expressed as percent inhibitory

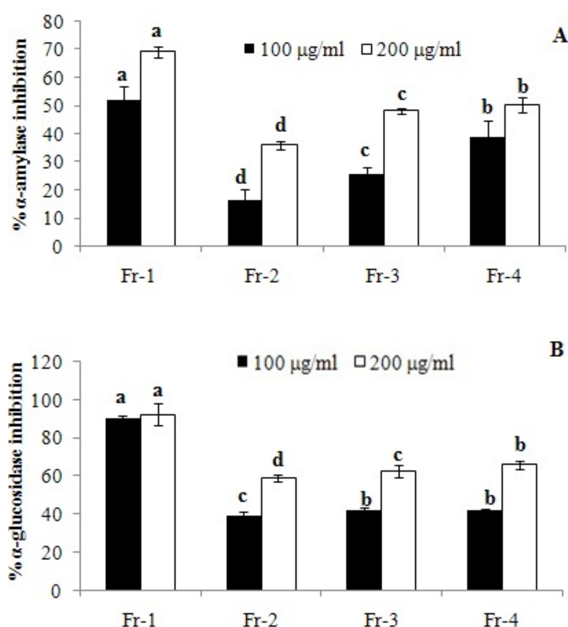

Fig. 2. α-Amylase (A) and α-glucosidase (B) inhibition by sub-fractions of the hexane extract from oriental melon seeds at concentrations of 100 ㎍/㎖ and 200 ㎍/㎖. Data represent the mean of three independent experiments, each performed in duplicated samples.

Table 1. Inhibition of α-amylase and α-glucosidase by the fractions and compounds

Fraction/ compound	α-Amylase inhibition	α-Glucosidase inhibition
	IC_{50} (㎍/㎖)[z]	IC_{50} (㎍/㎖)[z]
F-1-1	96 ± 2^{d}	50 ± 2^{c}
F-1-2	200 ± 10^{a}	110 ± 6^{a}
F-1-3	175 ± 5^{b}	100 ± 8^{a}
OM-1	48 ± 1^{e}	25 ± 0.5^{d}
OM-2	147 ± 3^{c}	83 ± 2^{b}
Linoleic acid	80 ± 4 (μM)	120 ± 8 (μM)
Oleic acid	92 ± 3 (μM)	160 ± 4 (μM)
Palimitic acid	118 ± 13 (μM)	189 ± 1 (μM)
Stearic acid	116 ± 7 (μM)	201 ± 6 (μM)
Acarbose	20 ± 0.9 (μM)	22 ± 1 (μM)

[z]IC_{50} values means standard deviation were obtained from dose effect curves by linear regression performed in at least three independent experiments (each in duplicated samples). Different letters in the same column indicate a significant difference by Ducan's multiple ranges at $p < 0.05$.

effect on either α-amylase or α-glucosidase (Fig. 2). Among four fractions, F-1 showed high enzyme inhibitory activities reached to 69.1% and 92.2% at the concentration of 200 ㎍/㎖, on α-amylase and α-glucosidase, respectively. Hence, F-1 was selected for further study and separated by flash column chromatography to generate F-1-1, F-1-2, and F-1-3 fraction. As shown in Table 1, F-1-1 had much stronger inhibitory effect on α-glucosidase and α-amylase than F-1-2 and F-1-3. IC_{50} (50% inhibition dose) values of F-1-1 were 96 and 50 ㎍/㎖ for α-amylase and α-glucosidase, respectively. Therefore, the biologically active fraction (F-1-1) was further separated by preparative TLC to obtain OM-1 and OM-2. OM-1 showed inhibitory effect on α-glucosidase as strong as acarbose.

Cucumis melo L. var. makuwa Makino seeds contain substantial amounts of valuable fatty acids that can be recovered for possible applications in food. Our previous study reported that fatty acids were highly presented in oriental melon seeds, such as linoleic acid, oleic acid, and palmitic acid (Chen and Kang, 2013). In normal phase TLC, the stationary phase (silica gel) is polar and the mobile phase is non-polar (the used solvent system contains an amounts of solvents such as hexane). Free fatty acids can be easily separated from the residual lipids by means of TLC and subsequently determined by GC eventually in combination with MS. Phytochemical screening of the fraction F-1-1 showed presence of unsaturated fatty acids. TLC of F-1-1 using n-hexane/diethyl ether/acetic acid (100:30:3) as the mobile phase showed two major distinct spots (Rf = 0.48, Rf = 0.32) in the iodine chamber, indicating unsaturation. Previous study reported that the free fatty acids in plasma were checked by TLC with the n-hexane/diethyl ether/acetic acid as mobile phase (Ubhayasekera *et al*., 2013). Each fraction containing high fat concentrations cannot be analyzed directly by GC-MS. For this reason, they were hydrolyzed and then converted to TMS derivatives. The fractions of OM-1 and OM-2 were analyzed by GC–MS (Fig. 3 and Fig. 4) and the peaks were compared with those of reference compounds analyzed under the same conditions. Preliminary identifi cation of the compounds was carried out using the auto-library search function of the Mass Spectral Library. Fatty acids obtained from OM-1, and alkanes of OM-2 were summarized in Table 2. OM-1 contained high concentrations of fatty acids. Four distinct cationic adducts corresponding to the four tested fatty acids were detected at the expected m/z values.

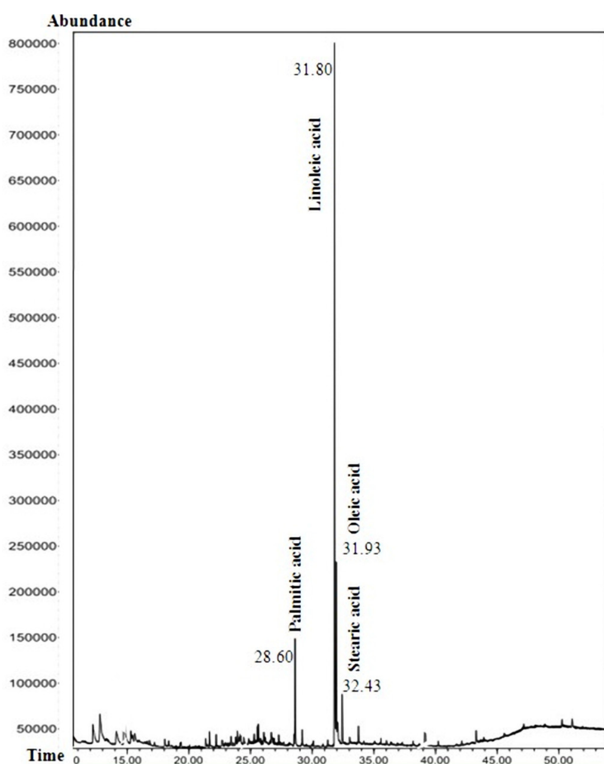

Fig. 3. Total ion chromatograms of fraction OM-1.

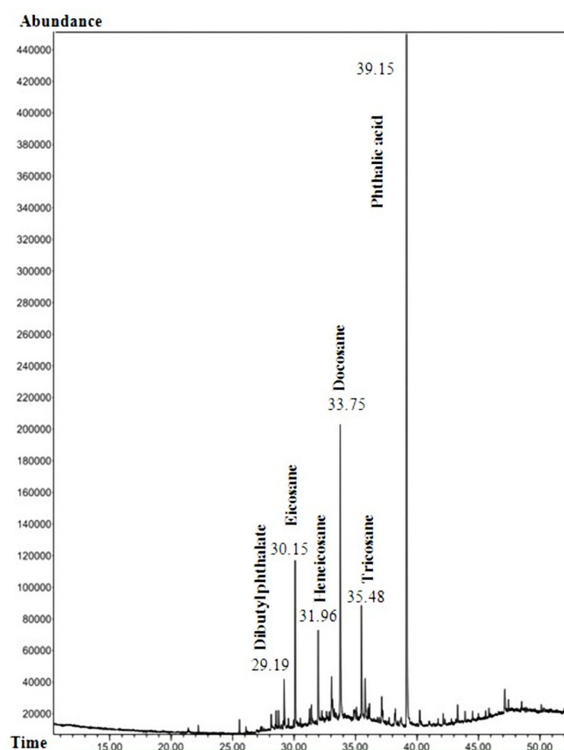

Fig. 4. Total ion chromatograms of fraction OM-2.

Table 2. Retention times and mass spectrometric data for TMS derivatives of lipids by GC-MS

	Fatty acids or m/z values detected	Retention time (min)	Exact masses	$[M-H]^-$ ions detected	Relative peak area (%)
OM-1	Palmitic acid	28.61	256.42	256	8.9
	Linoleic acid	31.80	280.24	280	50.6
	Oleic acid	31.93	282.25	282	14.9
	Octadecanoic acid	32.43	284.48	284	4.4
OM-2	Dibutyl phthalate	29.19	278.34	278	3.9
	Eicosane	30.08	282.55	282	9.5
	Heneicosane	31.96	296.57	296	6.2
	Docosane	33.75	310.62	310	21.4
	Tricosane	35.48	324.62	324	7.7
	Phthalic acid	39.14	166.13	166	47.8

The dominant fatty acid components in the fraction OM-1 were linoleic acid (18:2 n-6c, 50.6% of total fatty acids), oleic acid (18:1 n-9c, 14.9%), palmitic acid (16:0, 8.9%), and stearic acid (18:0, 4.4%). Total content of unsaturated fatty acids (UFA) in OM-1 is 65%, while relative amount of saturated fatty acids (SFA) is 13%, respectively. The ratio of polyunsaturated fatty acid (PUFA) to SFA (palmitic and stearic acid) is 3.8, while 4.9 is the ratio of UFA to SFA, which is recommended to be a functional ratio to reduce cardiovascular disease risk (Sánchez-Muniz *et al*., 2003). In addition, it is well known that polyunsaturated fatty acid plays an important role in the structure and function of many membrane proteins (Donald, 2002). These results suggest that oriental melon seeds may serve as a potential dietary source of PUFA. As a result of GC-MS for OM-2, alkanes were observed (Fig. 4). Docosane, eicosane, tricosane, heneicosane,

and dibutyl phthalate were the main substances of OM-2 (Table 2).

Four fatty acids (linoleic acid, oleic acid, palmitic acid, and stearic acid) determined in OM-1 were responsible for its high enzyme inhibitory effects. Kusunoki *et al*. (2007) reported that positive correlation between palmitic acid and homeostasis model insulin resistance index in human. With regard to polyunsaturated fatty acid, linoleic acid (C18:2) and monounsaturated acid, oleic acid (C16:1), good inhibitory effects against α-amylase and α-glucosidase were also observed. It was reported that methyl palmitate, methyl linoleate and methyl linoleneate inhibited 73.4%, 66.5% and 68.5% on α-glucosidase activity at a concentration of 200 μmol/mL, respectively (Miyazawa *et al*., 2005). Previous study has reported that consumption of polyunsaturated fatty acids lead improvement of hyperlipidemia and prevention of arteriosclerosis (Hu *et al*., 2002).

The principal active components from oriental melon (*Cucumis melo L. var. makuwa* Makino) seeds were elucidated. Free fatty acids were isolated as potential hypoglycemic compounds of oriental melon seeds through activity-guided fractionation. Four fatty acids (linoleic acid, oleic acid, palmitic acid, and stearic acid) determined in OM-1 were responsible for high enzyme inhibitory effects of the hexane extract of oriental melon seeds. GC-MS has been demonstrated to be useful in the current work for identification of the active compounds showing α-glucosidase and α-amylase inhibitory activity. These results suggest that oriental melon seeds, which at present are considered as agricultural waste, could be used as an anti-diabetic agent for type 2 diabetes.

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