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ARTICLE

Insulin Secretagogue Bioactivity of Finger Citron Fruit (*Citrus medica* L. var. *Sarcodactylis* Hort, Rutaceae)

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Finger citron [*Citrus medica* L. var. *Sarcodactylis* Hort, *Rutaceae*] (FC) fruits, widely cultivated in Japan, the southern provinces of China and Taiwan, are commonly used as functional vegetables and preserved as sweetmeats. Previously we identified the major compounds in essential oils (% in EO) of FC fruits to be *d*-limonene (51.24), γ-terpenene (33.71), α-pinene (3.40), and β-pinene (2.88). Documented evidence on its insulin secretion characteristics is still lacking. In parallel to compositional analysis, we performed in vivo the safety, hypoglycemic, and antidiabetic tests in Sprague-Dawley−SPF rats and Wistar DIO rats respectively. By kinetic analysis on the hypoglycemic patterns of the intraperitoneal glucose tolerance (IPGTT) and the insulin−glucose tolerance tests (IGTT), its insulin secretagogue effect was confirmed. In conclusion, FC fruits that concomitantly possess insulin secretagogue and slimming effects would be very beneficial to type 2 diabetes mellitus patients.

KEYWORDS: Insulin secretagogue; antiobese; type 2 diabetes mellitus; finger citron fruits

INTRODUCTION

Finger citron [*Citrus medica* L. var. *Sarcodactylis* Hort] (FC) belongs to the family Rutaceae (**Figure 1**). From the early 1700s until the late eighteenth century, FC was cultivated to merely serve the scenes and landscape in temples and magnificent gardens (*1*). Customarily, it is more popularly named "Finger citron", "Buddha hand citron", "Longevity orange", or "Five finger orange" in commercial vegetable markets (*2*). Since 1895 after the Japanese regime, FC fruits have been widely utilized as one kind of vegetable dishes or a thick vegetable soup in Taiwan. Presently, the cultivation has covered an area about 20000 acres from the north to the south in Taiwan, mostly emerging in Nan-Tou County in middle Taiwan. Because traditionally FC fruits have been considered to be beneficial to pancreas, liver and stomach functions, people like to take FC fruits as adjuvant herbal medicines to treat a diversity of chronic diseases like hypertension and respiratory tract infections (*3*). During harvesting season the overproduced FC fruits are often preserved as sweetmeats. Recently, in Southern China and Japan they are occasionally used as tonic ingredients to formulate crispy and cookies, and more frequently, they are used in combination with peaches to serve a premium fruit salad in many Japanese restaurants.

The volatile oil of fresh FC leaves consisted of 39 components (*4*). More recently, 6 new compounds were identified in FC leaves (*5*). In contrast, the essential oils of *Citrus medica* L. var. *ethrog* Engl. peels comprised only 34 components. Unlike those found in leaves, they revealed different bioactivity in many biochemical aspects. However, only until recently, twelve constituents were isolated from the dry and fresh FC fruits. Alternatively, a rather high level of polysaccharide content in FC fruits was also recognized. For several decades, FC fruits in fact have become popularly used as an adjuvant nutraceutical hypotensive as well as hypolipidemic foodstuffs in Japan, Southern China and Taiwan, yet literally its hypoglycemic bioactivity and related action mechanism have never been cited. In a preliminary survey, we found in FC fruits the presence of an extraordinarily huge amount of *d*-limonene, γ-terpenene and β-pinene. It is worth noting that these compounds had been reported to be beneficial to hyperlipidemia and obese (*6*, *7*). Since both obesity and dislipidemia are common features of insulin resistance, we carried out simultaneously an acute oral safety test with the Sprague-Dawley−SPF rats and an in vivo hypoglycemic study with the Wistar DIO rats, to investigate whether the whole FC fruits can be as effective and safe as these pure compounds.

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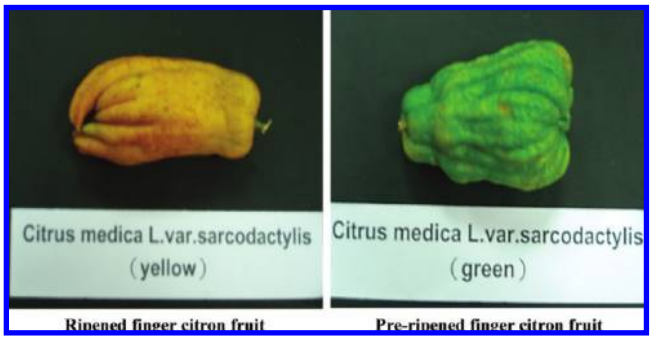

Figure 1. Appearance of finger citron [*Citrus medica* L. var. *Sarcodactylis* Hort] fruits. The yellow colored ripened finger citron fruits (left, RFC) and the green colored preripened finger citron fruits (right, PRFC).

MATERIALS AND METHODS

Source and Voucher of Finger Citron Fruits. Fruits of fingered citron (*Citrus medica* L. var. *Sarcodactylis* Hort, Rutaceae) were purchased from a local farm in central Taiwan and authenticated by the Taichung Research and Development Institute of Herbal Medicinal Plants in Taichung City, Taiwan.

Chemicals and Reagents. The authentic volatile compounds α-pinene and β-pinene etc. were purchased from Aldrich (U.K.). α-Terpinene and γ-terpinene and streptozotocin (STZ) were products of Sigma Chemical Co. (St. Louis, MO). *d*-Limonene and *cis*-β-ocimene etc. were provided by Merck Co. (Merck & Co., Inc., Whitehouse Station, NJ). All chemicals used were of reagent grade. The STZ solution was prepared by dissolving STZ in 0.1 M citric acid buffer (pH. 4.5).

Compositional Analysis of the Volatiles. FC fruits were ground and mashed with a minigrinder. One hundred grams of pulverized particles having size smaller than mesh #35 was collected and transferred into a 5 L steam-distillator, to which 1 L of purified water was added. The following procedures were conducted as previously reported starting from the procedure of steam-distillation (*8*). The yellowish essential oils were combined, filtered and stored at −20 °C for further analysis. The HP6890 series GC-system and the HP6890 series GC coupled with 5973 Network Mass Selective Detector were used for quantification of the volatiles in FC fruits. For determination of *d*-limonene a chiral column was used to replace the one regularly used. The operation and the quantification conditions for GC and GC/MS analysis were similar to those previously reported (*8*). For structural analysis of the volatiles, the database provided by Schönburg and Dielmann (*9*), Wiley MS Chemstation Libraries, NBS Computer Data Base, and the authentic patterns from the cited were referred.

Safety and Slim Test. The safety tests were conducted according to the OECD Guideline 420, 2000 (*10*). In majority, the section of Acute Oral Toxicity-Fixed Dose Procedure (Revised draft 2000) was followed. To ensure the safety test, the instructions given in Federal Register 61 (*11*) were also adopted. The parameters to be taken included the survial rate, body weight, weights of liver, spleen and kidney, serum biochemical test, and tissue Hematoxylin and Eosin (HE) stains etc. The clinical serum biochemical tests included examination of serum aspartate aminotransferase (s-GOT), serum alanine aminotransferase (sGPT), and blood urea nitrogen (BUN).

This animal experimental project was approved by the Animal Model Experimental Ethics Committee of Hungkuang Univerity according to the 1972 Helsinki declaration. The whole protocol was performed according to OECD (*10*). Briefly, eighteen Sprague-Dawley−SPF rats, aged 5 weeks and weighing 140−190 g, were purchased from LOSC Biotech Co. (Taiwan) and used to carry out the acute toxicity test (File No. MZ-162 970312020 TA1). All other procedures in the guidance given were followed. From the eighth week on, after being fasted for 12 h, each rat was fed per os daily 1 mL of emulsion/100 gbw (equivalent to 2000 mg/kg) of the lyophilized pulverized samples of either the green colored preripened (PRFC) or the yellow colored ripened (RFC) fruits using a feeding needle. The whole experiment took a period of 7 days.

Herbal Nutraceutical Preparation for Safety Test. The FC fruit nutraceutics included two distinct categories: the PRFC and the RFC fruits (**Figure 1**). The lyophilized samples of whole PRFC and RFC fruits were pulverized. The powders were placed fresh in sterilized saline, homogenized and emulsified to attain the concentration as indicated. Each rat was fed 1 mL of emulsion/100 gbw (equivalent to 2000 mg/kg of the pulverized FC fruits).

Table 1. Ingredients of Experimental Animal Diets (%)[a]

	group	
ingredient	C	H
casein	20.0	20.0
sucrose	6	6
corn starch	51.8	37.8
corn oil	12.0	23.0
cholesterol		0.1
mineral premix[c]	4	4
vitamin premix[d]	1	1
choline	0.2	0.2
cellulose	5	5
total	100	100

[a] Based on AIN-76 formula (AIN, 1997). Amounts of corn oil were expressed in wt %. C: control diet containing corn oil 12%. H: high fat diet containing corn oil 23%. [c] Mineral premix: $CaHPO_4 \cdot 2H_2O$, NaCl, $K_3C_6H_5O_7$, K_2SO_4, MgO_3, MnO_3, Fe-citrate, $ZnCO_3$, $CuCO_3$, KI, $NaSeO_3$, $K_2SO_4 \cdot Cr_2(SO_4)_3 \cdot 24H_2O$. [d] Vitamin premix: Thiamine hydrochloride, pyridoxine hydrochloride, riboflavin, nicotinic acid, vitamin B_{12}, retinyl palmitate, vitamin D_3, vitamin E, vitamin K.

Serum Sampling and Analysis. The sera obtained were obtained and analyzed for parameters indicated according to the guidance given in OECD Guideline for the Testing of Chemicals #401: Acute Oral Toxicity Test, 1987; and Japanese Guidelines for Nonclinical Studies of Drugs Manual, 1995.

Histochemical Examination. At the end of the experiment, after the blood had been collected from the eyeball side, the animals were CO_2-anesthetized and sacrificed. The organs were dissected and rinsed. After being wiped with dry tissues, the organs were weighed and checked for gross apparent pathological changes. The dissected livers and kidneys were embedded into Tissue-Tek OCT (Sakura Co., Japan) and frozen at −20 °C in liquid nitrogen. The frozen specimens were microtomed (LEICA CM1900) to thin slices having thickness of 5 μm and then stained with Hematoxylin/Eosin (HE) stain. The finished slices were microphotographed with ProgC14 COOL CCD microscope.

Anti-Hyperglycemic Animal Experiment. *Animals.* The animal experimental project has been approved by the Animal Model Experimental Ethics Committee of Dong-Hwa Univerity according to the 1972 Helsinki declaration. These experiments were performed according to the "Guide for the Care and Use of Laboratory Animals". Briefly, ninety six Wistar DIO rats (weight 250 ± 20 g, age 1 month) were obtained from the Lux Biotech Co., Taiwan. The rats, eight in each cage, were acclimated and fed a basic chow consisting of 12% of fat for the first week before experimentation. The animal room was conditioned at a 12 h light/dark cycle and 72% relative humidity. All animals had free access to food and water.

Animal diets. Based on the formulation described in AIN-76 (*12*), the animal diets were prepared according to the formula listed in **Table 1**.

Preparation of Finger Citron Fruit Nutraceutics for Anti-Diabetes Mellitus Test. RFC or PRFC fruits were rinsed twice with sufficient double distilled water and sliced to separate the pulpiness from the epicarp. The two parts were lyophilized and pulverized respectively to avoid direct sunlight. For animal tests, 60 mg/kgbw were administered per os per day.

Type 2 DM Animal Model. A modified method of Zhang et al. (*13*) was followed to induce type 2 diabetes (T2DM) in rats (**Figure 2**). Briefly, ninety six rats were subgrouped into group 1 (24 rats to serve the normal group) and group 2 (72 rats) after being acclimated for one week. Group 1 was fed a regular diet, and group 2 was fed a high fat diet successively for another 5 weeks. After the average body weight attained 450 ± 15 g, group 2 was applied ip a dose of STZ 30 mg/kgbw (STZ treated group). To serve as the blank, group 1 received only the same amount of 0.1 M citric acid buffer (pH 4.5). STZ-treatment can induce partial destruction of β-cells in rats, resulting in insulin resistance-like symptoms, which pathologically

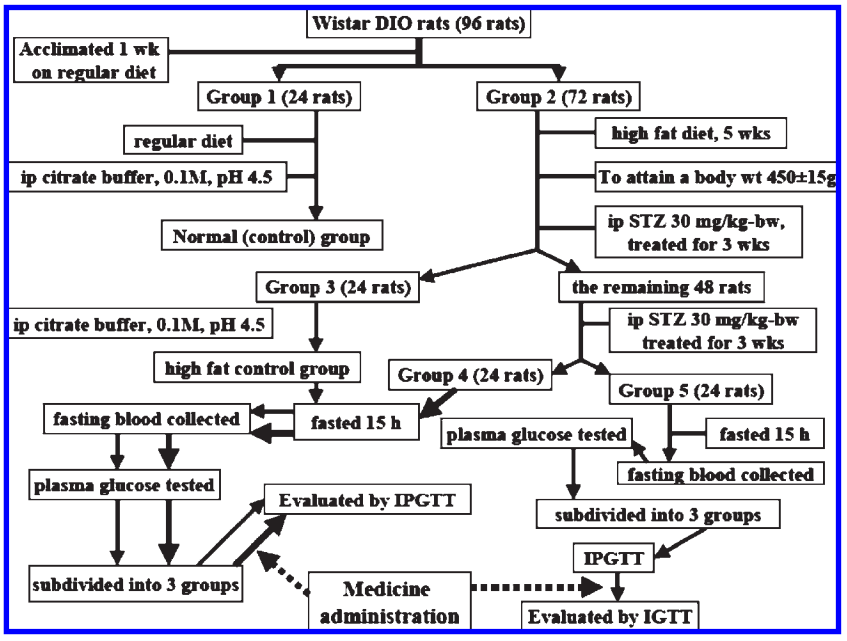

Figure 2. The flowchart for grouping and testing in animal model. (The major part was the protocol depicted from Zhang et al., 2003 (*13*)).

would be very similar to human T2DM. At three weeks after treatment with STZ, group 2 was further grouped into group 3 (24 rats) and the remaining group (48 rats). The group 3 rats received ip 1 mL/kgbw of citric acid buffer (0.1M, pH 4.5) to serve as the high-fat diet control (Fat group). The remaining 48 rats were similarly ip STZ-treated and subdivided into group 4 (24 rats) and group 5 (24 rats). Group 3 and group 4 were immediately fasted for 15 h to collect the fasting blood, and the fasting plasma glucose levels were taken. To further evaluate the anti-DM bioactivity of nutraceutics, the intraperitoneal glucose tolerance test (IPGTT) and the insulin−glucose tolerance test (IGTT) were performed (see below).

Intraperitoneal Glucose Tolerance Test (*IPGTT*). Group 4 was further divided into three subsubgroups, 8 rats in each and designated with groups 4-1, 4-2, and 4-3, respectively. The nutraceutical samples to be tested were applied immediately after collection of the fasting blood and before conducting IPGTT. All the subgroups 4 were ip injected with glucose 0.5 mg/kgbw. The subgroup 4-1 served as the IPGTT control. Subgroup 4-2 was fed per os 60 mg/kgbw pulpiness of lyophilized FC fruits (subgroup Pulpiness). Since the pulpiness part contained only a negligible amount of essential oils, the effect of essential oils in this regard can be overlooked. In contrast, the subgroup 4-3 was fed per os 60 mg/kgbw lyophilized FC epicarp (subgroup Epicarp). Both whole fruits contained 0.45 and 0.42% of essential oils respectively in PRFC and RFC (data not shown), while the majority of essential oil was contributed by the epicarp part, we estimated the epicarp diet would approximately reach a dose of 270 and 252 μg/kgbw, respectively.

These subgroups received ip glucose injection and nutraceutical administration. The blood samples were collected by vein puncture at the tail vein, and the plasma glucose levels at 30, 60, 90, and 120 min were determined. At this point, a level of 200 mg/dL threshold was set for screening type 2 DM rats. As declared by the World Health Organization (WHO), a fasting level of blood glucose in the range of 110 to 126 mg/dL can be diagnosed to be with impaired fasting glucose utilization. If at 120 min after glucose administration (ip 0.5 g/kgbw), the plasma glucose level still consistently persists in the range of 140 to 200 mg/dL, thereby impaired glucose tolerance is confirmed. By definition, a status of DM is confirmed when the fasting plasma glucose level exceeds 126 mg/dL and the IPGTT level at 120 min exceeds 200 mg/dL.

Insulin−Glucose Tolerance Test (*IGTT*). Similarly, on fasting for 15 h the fasting blood of group 5 was collected by vein puncture at the tail vein and thereby the fasting plasma glucose levels were determined. After blood collection, group 5 was immediately given FC fruit epicarp and/or pulpiness before conducting IPGTT and further divided into three subsubgroups and designated as subgroup 5-1, 5-2, and 5-3, respectively, each with 8 rats. After IPGTT was applied, all subgroups 5 (24 rats) were treated ip with insulin−glucose tolerance test (IGTT) using a mixture of glucose (0.5 g/kgbw) and insulin (25.2 USP U/mg, Sigma, St. Louis, MO) (0.2 U/kgbw). Subgroup 5-1 served as the control, subgroup 5-2 was fed per os 60 mg/kgbw of FC fruit pulpiness (subgroup Pulpiness), and subgroup 5-3 was fed 60 mg/kgbw of FC fruit epicarp (subgroup Epicarp), respectively. The blood samples were collected by vein puncture at the tail vein at intervals of 30, 60, 90, and 120 min, respectively. The plasma glucose levels were measured. According to Zhang et al. (*13*), in the IGTT, if the plasma glucose level at 120 min persists and still shows a level slightly higher than the fasting level irrespective of type 1 or type 2 DM animal models, the outcome would implicate clearly the status of insulin-resistance. Consequently, the insulin supplied exogenously would fail in blood glucose regulatory function. The rats with such a status are named as the "type 2 DM rats". Alternatively, under the circumstance that the plasma glucose level returns to normal glucose level within 60 to 90 min, and concomitantly if the plasma glucose level at 90 min is lower than the fasting values, the rats could be designated as "type 1 DM rats" which actually would possess a normal insulin response.

Statistical Analysis. The data obtained were processed with the statistical software SPSS (Version 10.0) to evaluate the significance in difference. A confidence level of $p < 0.05$ was considered to be significantly different between two data pairs to be compared.

RESULTS AND DISCUSSION

Compositional Profiles. The total essential oils were found to be 0.45 and 0.42% in PRFC and RFC, respectively (data not shown). Both PRFC and RFC fruits contained profound amount of monoterpenes but only very few amounts of sesquiterpenes, terpene aldehydes, and terpene alcohols (**Table 2**). Amazingly, in both fruits (RFC vs PRFC), exceptionally huge amounts of α-pinene (3.40 vs 2.92%), β-pinene (2.88 vs 2.45%), *d*-limonene (51.24 vs 57.63%), and γ-terpinene (33.71 vs 27.01%) occurred, which was not found in other citrus fruits. Moreover, the profiles were not associated with the ripening stage (**Table 2**). Consistent with the reported results (*3*), the ripened FC fruits contained a slightly higher concentration of α-pinene, β-pinene, α-phenllandrene, γ-terpinene, α-terpinolene, sesquiterpenes and terpene alcohols and lesser amounts of β-myrcene and *d*-limonene (**Table 2**).

Concomitantly, considering that some volatiles may be lost during lyophilization, we made a recheck on the amount of

d-limonene, β-pinene, and γ-terpinene after lyophilization and before feeding (unpublished). We found that although these constituents might vary in concentration from sample to sample, its mean values still retained at a level approximately within 5% accuracy. The reason might be due to the high pectin content in FC (about 40−48%, unpublished) that exerted adsorption and inclusion effects on these volatiles.

Finger Citron Fruits Were Safe for Uses. The acute toxicity test using 2000 mg/kg (or 1 mL/100 gbw) per os of FC fruits was shown to be totally nontoxic in nature despite its ripening stage (data not shown, but refer to **Table 3**, **Figures 3** and **4**). Some previous reports had demonstrated that *d*-limonene tends to increase the incidence of renal tubular tumors in male rats and mice in both genders, but results are still rather controversial (*6*). As popularly accepted, juniper fruits are very common health foods. In juniper fruits, the γ-terpinene content can reach a level as high as 600 mg/kg, while the toxic LD50 (rat) threshold of γ-terpinene had been shown to be 3650 mg/kg per os, or the acute dermal toxicity is roughly >5000 mg/kg by dermal transfusion (*14*).

Table 2. Dependency of Compositional Variation of the Volatiles on the Ripening Stage of Finger Citron Fruits[a]

				composition (%)	
compound	CAS No.	formula	MW	RFC	PRFC
		Monoterpenes			
α-thujene	002867-05-2	$C_{10}H_{16}$	136	1.29 ± 0.24	1.20 ± 0.03
α-pinene	000080-56-8	$C_{10}H_{16}$	136	3.40 ± 0.27	2.92 ± 0.22
camphene	000079-92-5	$C_{10}H_{16}$	136	0.03 ± 0.00	0.02 ± 0.00
β-pinene	000127-91-3	$C_{10}H_{16}$	136	2.88 ± 0.32	2.48 ± 0.33
β-myrcene	000123-35-3	$C_{10}H_{16}$	136	1.64 ± 0.03	1.76 ± 0.04
α-phellandrene	000099-83-2	$C_{10}H_{16}$	136	0.10 ± 0.02	−
α-terpinene	000099-86-5	$C_{10}H_{16}$	136	−	1.28 ± 0.03
d-limonene	005989-54-8	$C_{10}H_{16}$	136	51.24 ± 3.44	57.63 ± 4.33
β-ocimene	003779-61-1	$C_{10}H_{16}$	136	0.23 ± 0.03	0.93 ± 0.06
γ-terpinene	000099-85-4	$C_{10}H_{16}$	136	33.71 ± 2.44	27.01 ± 2.30
α-terpinolene	000585-62-9	$C_{10}H_{16}$	136	1.54 ± 0.06	1.25 ± 0.03
		Sesquiterpenes			
copaene	003856-25-5	$C_{15}H_{24}$	204	0.02 ± 0.00	−
β-elemene	000515-13-9	$C_{15}H_{24}$	204	0.05 ± 0.01	−
caryophyllene	000087-44-5	$C_{15}H_{24}$	204	0.06 ± 0.01	−
α-bergamotene	017699-05-7	$C_{15}H_{24}$	204	0.07 ± 0.01	−
α-humulene	006753-98-6	$C_{15}H_{24}$	204	−	−
germacrene-D	023986-74-5	$C_{15}H_{24}$	204	0.19 ± 0.03	0.15 ± 003
germacrene-B	015423-57-1	$C_{15}H_{24}$	204	−	+
bicyclogermacrene	100762-46-7	$C_{15}H_{24}$	204	0.06 ± 0.01	−
β-bisabolone	000495-61-4	$C_{15}H_{24}$	204	0.11 ± 0.02	0.03 ± 0.00
		Terpene Aldehydes			
neral	000106-26-3	$C_{10}H_{16}O$	152	0.46 ± 0.07	0.45 ± 0.06
		Terpene Alcohols			
terpinen-4-ol	000562-74-3	$C_{10}H_{18}O$	154	0.51 ± 0.03	0.34 ± 0.04
alpha-terpineol	000098-55-5	$C_{10}H_{18}O$	154	0.58 ± 0.05	0.48 ± 0.05
geraniol	000106-24-1	$C_{10}H_{18}O$	154	0.58 ± 0.06	0.55 ± 0.05

[a] RFC: yellow colored ripened finger citron fruits. PRFC: green colored preripened finger citron fruits. The total essential oils were found to be 0.45 and 0.42% in PRFC and RFC, respectively. "+" indicates to contain a level <0.01%, while "−" indicates "undetected".

Amazing Slimming Effect of FC Fruits Was Found. More attractively, the slimming effect of γ-terpinene in juniper and cumin has been well documented (*14*). Amazingly in our experiment, the average body weight decreased by −57.6% and −45.8% respectively by RFC and PRFC, an implication in the potent slimming effect of FC fruits (**Table 3**). Although the body weight prior to feeding was lower in groups PRFC and RFC, but the daily food intake ranged from 22.9 to 23.3 g /rat in all groups (not shown). Speculatively, the slimming effect must have resided on the compositional effect, being indpendent of the maturation stage of FC fruits. Similarly, the spleen weight and the corresponding ratio of spleen/body weight were significantly reduced by PRFC ($p < 0.05$) and RFC ($p < 0.01$) (**Table 3**). In addition, the serum analysis showed that there was no difference in sGOT, sGPT and BUN levels prior to and post consumption of PRFC fruits. In contrast, significant changes were found in the RFC administered group ($p < 0.05$) (**Table 3**). Furthermore, macroscopic examination also revealed that organs including livers, kidneys and spleens were all normal in appearance (data not shown). Microscopically, the histochemical examination by HE stains of livers and kidneys also exhibited normal status (**Figure 3** and **Figure 4**).

Clinically, it is all too understandable that for the diabetologists insulin resistance and hyperinsulinemia are in the center of reasoning of metabolic syndromes, whereas lipidologists, endocrinologists and metabolic researchers favored quite rightly the name obesity (*15*). Thus in some aspect the synergism of the slimming effect and the hypoglycemic effect in turn would improve better prognosis in treating DM. The reason why the ripened but not the preripened FC fruits possessed simultaneously such pronounced effects is still unclear. Obviously, it could be a consequence of insulin secretion enhanced by the FC fruits.

In addition, γ-terpinene is a potent ACE inhibitor, an aldose-reductase inhibitor, an antifeedant and a strong antioxidant. In Indian Herbs-Ancient Remedies, γ-terpinene has been used as the major component for weight loss, currently also having been approved by many clinical physicians (*7*).

Finger Citron Fruits Revealed Promising Hypoglycemic Bioactivity. The initial fasting plasma glucose level for normal rats ranged within 69.3−75.3 mg/dL (**Figure 5A**), whereas that of the fat group ranged within 73.2−75.5 mg/dL (**Figure 5B**); for the T2DM group, it ranged from 130.6 to 140.4 mg/dL (**Figure 5C**), and that for the IGTT group ranged within 100−106 mg/dL (**Figure 5D**). In a word, the normal fasting plasma glucose level may range from 69.3 to 75.5 mg/dL whether normal or fat, compared to the normal levels of 80 to 100 mg/dL in human. Amazingly, a significant difference was found between the normal−pulpiness and the normal−IPGTT or the normal−epicarp groups ($p < 0.05$) (**Figure 5A**), while the four groups,

Table 3. Variation of Body and Spleen Weights as Well as the Serum Biochemical Parameters[a]

	body weight (g)			spleen		serum biochemical parameter		
group	prior to feeding	prior to authenization	body wt gain	wt (g)	% ratio (wt/bw)	sGOT	sGPT	BUN
control	189.8 ± 7.6 a	235.5 ± 8.6 a	45.7 ± 3.0 a	0.5 ± 0.0 a	0.21 ± 0.01	13 ± 4 a	33 ± 3 a	7.9 ± 1.3 a
PRFC[b]	168.4 ± 3.6 a	193.2 ± 4.8 b	24.8 ± 4.5 b	0.4 ± 0.1 b	0.21 ± 0.05	11 ± 3 a	33 ± 5 a	7.6 ± 1.4 a
RFC[c]	162.9 ± 2.9 a	182.3 ± 7.5 b	19.4 ± 7.2 c*	0.3 ± 0.1 c	0.17 ± 0.03 b*	10 ± 2 b	36 ± 4 b	9.7 ± 1.2 b

[a] Data stated as mean ± SD ($n = 5$) from triplicate experiments. Data in the same column with different letters are significantly different from each other; $p \leq 0.05$ or (*) $p < 0.01$. [b] PRFC: prepared from the green colored preripened FC fruit powder. [c] RFC: prepared from the yellow colored ripened FC fruit powder.

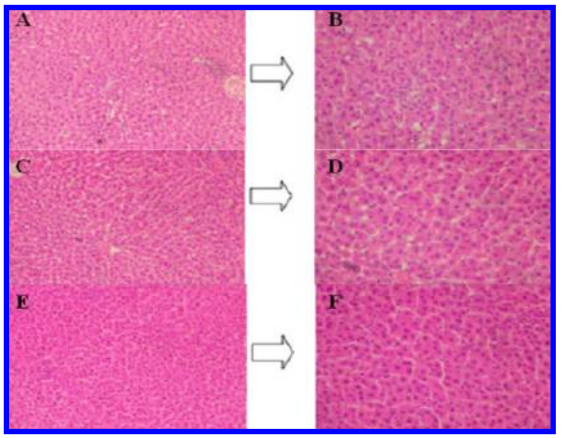

Figure 3. Histological examination of experimental rat livers by HE stain. Control: panels **A** and **B**. Group RFC: panels **C** and **D**. Group RFC: panels **E** and **F** (magnification: left, ×100; right, ×200).

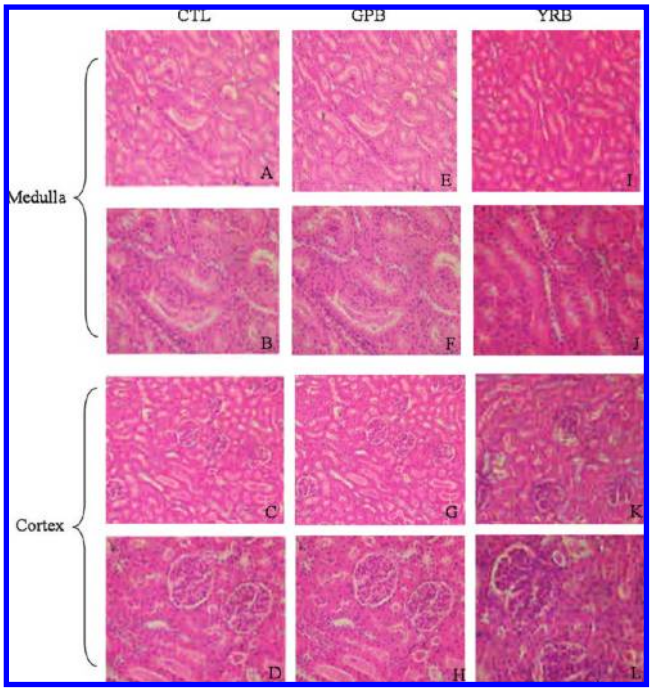

Figure 4. Histological examination of different experimental rat kidney tissues by HE stain. Upper 2 rows: medulla; magnification, **A**, **E**, **I**, ×100; **B**, **F**, **J**, ×200. Lower 2 rows: cortex; magnification, **C**, **G**, **K**, ×100; **D**, **H**, **L**, ×200. CTL: control. GPB or PRFC: green preripened finger citron fruits. YRB or RFC: yellow ripened finger citron fruit.

i.e. the normal, the fat, the T2DM, and the IGTT groups, when further subjected to IPGTT, and IPGTT−IGTT in parallel to the administration of FC fruits, revealed distinctly different results. In both the normal−pulpiness and normal−epicarp groups, the plasma glucose levels rose first to a peak at 30 min and then declined, while the IPGTT−control exhibited its peak value at 60 min (**Figure 5A**). And only the epicarp ingesting group returned to normal serum glucose level after 120 min. Serving as the control, the normal−IPGTT group was seen still retaining at 82 mg/dL even after 120 min post IPGTT, although slightly higher than the normal range (**Figure 5A**). The reason why the normal and the fat rats showed a transient rise in glucose level at 30 min after administration of FC preparations (**Figures 5A−5C**) is still unclear, while the reason that FC epicarp revealed better hypoglycemic effect than the FC pulpiness underlies the relevant bioactivity of essential oils in epicarp (**Figure 5A**), because as mentioned, the FC pulpiness contained only trace or negligible amount of essential oils. Differently, both FC pulpiness and epicarp suppressed the serum glucose in the fat group to a level approaching the normal range within 120 min (**Figure 5B**), indicating the higher susceptibility of the fat than the normal rats to FC nutraceutics regarding the antihyperglycemic bioactivity (**Figures 5A** and **5B**), which possibly could result from the interaction between different constituents present in different FC parts with different glucose metabolizing markers like adiponectin and leptin in the fat subjects. Supposedly, the fat rats are more susceptible to FC constituents leading to increased insulin secretion at a rate much faster than normal. In this regard, a similar trend but different extent was found for group T2DM (**Figure 5C**). In the T2DM−IPGTT group, the plasma glucose level that once reached a peak of 228 mg/dL at 30 min was respectively suppressed down from 213 and 216 mg/dL at 60 min to 155 and 162 mg/dL at 120 min respectively post FC administration. However, both FC preparations failed to restore the IPGTT induced hyperglycemic status to normal glucose levels even after a time relapse of 120 min. As frequently cited, the T2DM−IPGTT profile was more relapsed and flattened in the duration of 30 to 120 min, thus likely to retain at 210 mg/dL after 120 min in T2DM−IPGTT group. By contrasting to the other two abruptly declining profiles, an implication in a status of "typical insulin resistant type 2 DM" as defined by Zhang et al. (*13*) was observed (**Figure 5C**). At this point, a more improved hypoglycemic effect was achieved by insulin externally supplied (**Figure 5D**). At 30 min post IPGTT, the control IGTT−IPGTT group showed a peak plasma glucose level of 206 mg/dL, and the other two groups exhibited a plasma glucose level of 157 and 166 mg/dL, respectively, which readily declined to 173, 107, and 120 mg/dL, respectively, for the IGTT−IPGTT control, the FC−pulpiness and the FC−epicarp groups. By definition (*13*), these rats were assigned "type 2 DM rats" because the final glucose level at 120 min (173 mg/dL) was still much higher than its original fasting levels (100−106 mg/dL) (**Figure 5D**). Otherwise to compare the glucose degradation, the T2DM group showed a suppressed plasma glucose level from 210 to 155 and 162 mg/dL by ingestion of FC preparations. The corresponding percent efficiencies were 24.76 and 27.10% (**Figure 5C**). In contrast, the combined use of exogenous insulin with FC supplements elevated the percent efficiency to 38.20 and 30.60%, respectively (**Figure 5D**). Such a phenomenon could be attributed to either insufficient endogenous insulin supply (type 1 DM) or inherent insulin resistance (type 2 DM).

Hypoglycemic Effect Could Be Attributed to Its Huge Contents of *d*-Limonene and *β*-Pinene. Both the FC epicarp and pulpiness all showed a transient serum glucose elevation effect around 30−60 min after administration (**Figures 5A−5D**). Such a transient early stage plasma glucose elevation as well as the unique end-stage plasma glucose-elevation effects (**Figure 5A**) indeed had attracted our great interest for further investigation. However, both FC preparations eventually showed very effective hypoglycemic activity at 120 min after administration (**Figures 5B−5D**). We ascribed the hypoglycemic bioactivity of FC to its huge *d*-limonene and *β*-pinene contents. As widely cited, *d*-limonene has been popularly applied as a nutraceutic hypoglycemic agent (*6*). Eddouks, Lemhadri and Michel (*16*) reported the active constituent in caraway, *d*-limonene, plays an important role in hypoglycemic bioactivity. Previously, *d*-limonene, safrole and *β*-pinene were shown to possess significant hypoglycemic effect in the STZ-diabetic rats (*17*). Alternatively, the insulin emulsions prepared with soybean oil, triolein or trilinolein slightly but significantly decreased the serum glucose levels compared to the

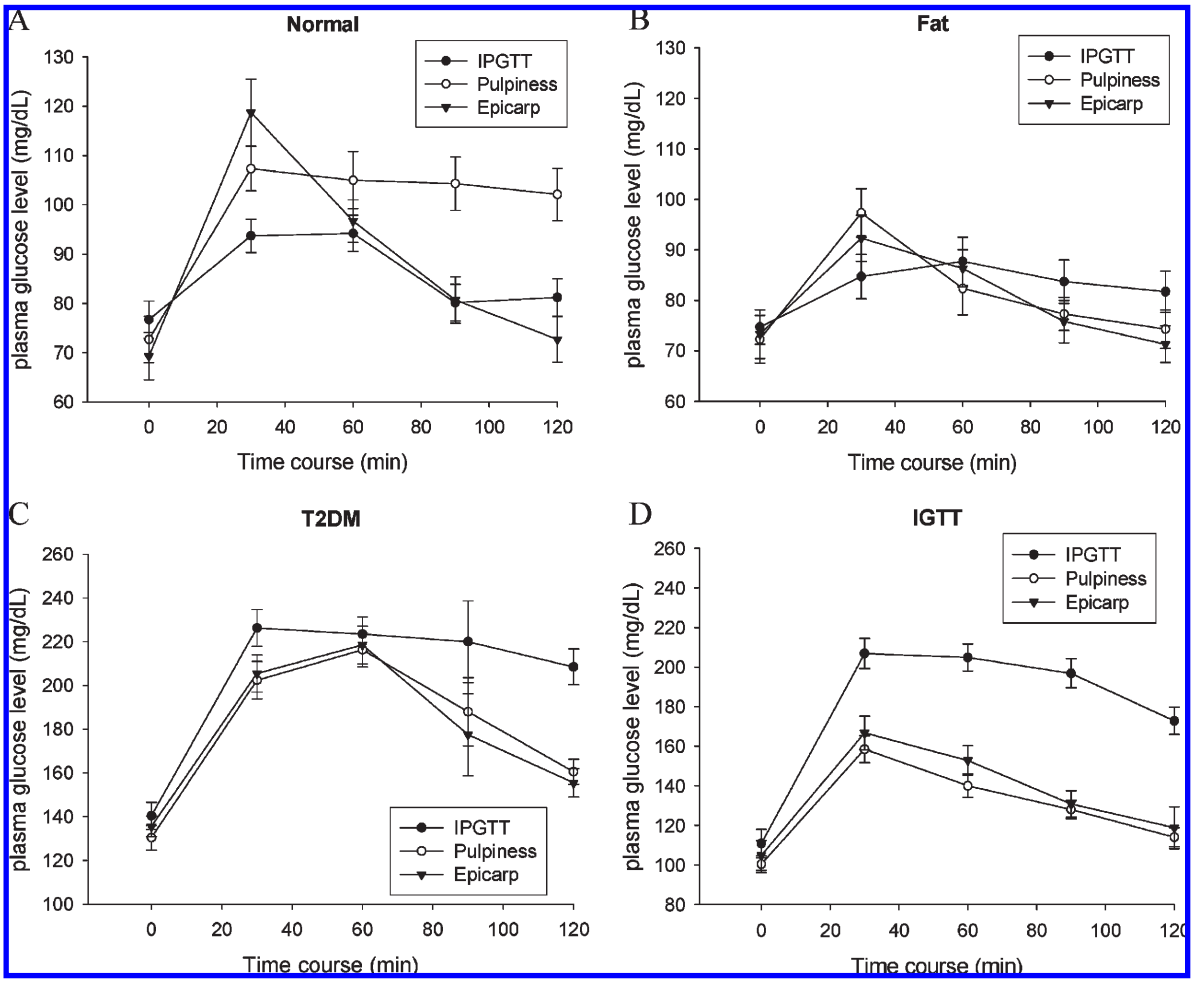

Figure 5. (**A**) Plasma glucose levels in the normal rat group after treatment with IPGTT, pulpiness and epicarp of the RFC fruits. Initially, all three subgroups received IPGTT, among which IPGTT without FC administered served as the control of the three. (**B**) Plasma glucose levels of the high fat-fed rats after treatment with IPGTT, pulpiness and epicarp of the RFC fruits. Initially, all three subgroups received IPGTT, among which IPGTT without FC administered served as the control of the three. (**C**) Plasma glucose levels of the T2DM rat group after treatment with IPGTT, pulpiness and epicarp of the RFC fruits. Initially, all three subgroups received IPGTT, among which IPGTT without FC administered served as the control of the three. (**D**) Plasma glucose levels of the STZ−insulin/glucose (IGTT) rat group treated with IPGTT, pulpiness and epicarp of the RFC fruits. Initially, all three subgroups received IPGTT, among which IPGTT without FC administered served as the control of the three.

nonemulsified insulin solution. By addition of 3% *d*-limonene or 3% menthol to the triolein emulsion, the hypoglycemic effect of insulin was significantly promoted in ileum but not in colon (*18*), implicating that the enhancement of hypoglycemic effect by *d*-limonene could be attributed in part to the insulin reclaim through a recyling process called "the reabsorption−reutilization mechanism".

Kinetic Analysis Revealed FC Fruits Likely To Be Insulin Secretagogue Rather than Insulin Sensitizing. Whether FC fruits act as an insulin sensitizer or an insulin secretagogue, we performed kinetic analysis on the glucose uptake rates obtained from groups T2DM (**Figure 5C**) and IGTT (**Figure 5D**).

Assuming the glucose uptake rate is positively proportional to the in situ available plasma glucose and insulin concentration, we have

$$-\mathrm{d}G/\mathrm{d}t = k[G][I_{\mathrm{in}}] \quad (1)$$

where G is the plasma glucose level in mg/dL; I_{in} is the inherent plasma insulin level in U/mL; k is second order rate coefficient; and t is the reaction time.

In an infinitesimally short period of reaction time, the inherent insulin concentration can be assumed to be constant, hence eq 1 is transformed into

$$-\mathrm{d}G/\mathrm{d}t = k'[G] \quad (2)$$

in which k' is termed the pseudo-first-order rate coefficient, and

$$k' = k[I_{\mathrm{in}}] \quad (3)$$

Rearrangement of eq 2 gives

$$\mathrm{d}G/[G] = -k'\,\mathrm{d}t \quad (4)$$

where k' is the pseudo-first-order rate coefficient with respect to G, which on integration gives

$$\int_{G_0}^{G} \mathrm{d}\ln G = -k' \int_0^t \mathrm{d}t \quad (5)$$

or

$$\ln G/G_0 = -k't \quad (6)$$

Hence the in vivo glucose uptake obeys an exponential relationship regarding the glucose uptake time t.

Table 4. Kinetic Analysis of Glucose Degradation Rates Affected by Different Finger Citron Preparations in the Presence and the Absence of Insulin[a]

group	peak plasma glucose level (mg/dL)	onset time (min)	plasma glucose level at 120 min (mg/dL)	glucose degradation rate coefficient		
T2DM				k' [b]		
IPGTT	228 ± 9	60	210 ± 7	8.77×10^{-4}		100.0%
pulpiness	216 ± 6	60	162 ± 8	4.48×10^{-3}		511.0%
epicarp	213 ± 5	60	155 ± 7	4.70×10^{-3}		536.0%
IGTT				k'' [c]	k''' [d]	
IPGTT	206 ± 6	30	173 ± 7	1.78×10^{-3}	8.90×10^{-3}	100.0%
pulpiness	166 ± 7	30	120 ± 7	3.08×10^{-3}	1.54×10^{-2}	173.0%
epicarp	157 ± 6	30	107 ± 7	5.41×10^{-3}	2.71×10^{-2}	305.0%

[a] The amount of insulin used was ip 0.2 U/kgbw according to Sigma Co. (25.2 USP U/mg (Sigma, MO). Since both whole fruits contained 0.45 and 0.42% of essential oils respectively in PRFC and RFC (data not shown), and the pulpiness part contained only a negligible amount of essential oils, the effect of essential oils in this regard can be overlooked in groups fed pulpiness. In contrast, in the subgroups fed per os 60 mg/kgbw lyophilized FC epicarp (subgroup Epicarp) we estimated the amounts of essential oils fed were approximately 270 and 252 μg/kgbw, respectively from the PRFC and RFC epicarp. [b] The values of k' calculated according to eq 2. The dimension is min^{-1}. [c] The values of k'' calculated according to eq 10. The dimension is U/kg-min. [d] The values of k''' calculated according to eq 12. The dimension is min^{-1}:

Otherwise, in the presence of exogenously added insulin, which was 0.2 U/kgbw in this experiment, eq 2 is retransformed into

$$-dG/dt = k'[G][I_{ex}] \quad (7)$$

or

$$-dG/dt = k'[G][0.2\ U/kg] \quad (8)$$

$$-[dG/dt]/[G] = k'[0.2\ U/kg] \quad (9)$$

or

$$-[dG/dt]/[G] = k'' \quad (10)$$

where

$$k'' = k'[0.2\ U/kg] \quad (11)$$

Equation 10 will be called herein the noninsulin weighted rate coefficient. Further weighting for insulin of eq 9 leads to

$$k''' = [dG/dt]/[G][0.2\ U/kg] \quad (12)$$

Equation 12 will be called herein the insulin weighted glucose uptake rate coefficient.

The kinetic data obtained from **Figures 5A−5D** are listed in **Table 4**. The interpretation about the kinetic analytical results is as follows.

Without the external insulin supply (i.e., the T2DM group as shown in **Figure 5C**), the glucose uptake rate coefficients obtained by calculation from eq 2 were found to be 8.77×10^{-4}, 4.48×10^{-3}, and 4.70×10^{-3} min^{-1} for the IPGTT control, the pulpiness and the epicarp subgroups, respectively (values of k' in **Table 4**). When taking the dose of insulin into consideration, the corresponding data in the IGTT experiment (**Figure 5D**) were 1.78×10^{-3}, 3.08×10^{-3}, and 5.41×10^{-3} U/kg-min, respectively, when calculated from eq 10 (values of k'' in **Table 4**). After having been weighted with the exogenously added insulin (calculated by eq 12), the corresponding rate coefficients changed to 8.90×10^{-3}, 1.54×10^{-2}, and 2.71×10^{-2} min^{-1}, respectively (**Figure 5D**, values of k''' in **Table 4**). Thus with the help of the exogenous insulin, the glucose uptake rate was seen to have been increased from 8.77×10^{-4}, 4.48×10^{-3}, and 4.70×10^{-3} min^{-1} to 1.78×10^{-3}, 3.08×10^{-3}, and 5.41×10^{-3} U/kg-min. Or for better comparison in the same dimension, these same kinetic data were increased to 8.90×10^{-3}, 1.54×10^{-2}, and 2.71×10^{-2} min^{-1}, respectively. That was approximately improved by 10-fold between the two IPGTT groups, 3.44-fold between the two pulpiness groups, and 5.77-fold between the two epicarp groups (**Table 4**). Moreover, within the same T2DM group, it was found that the pulpiness and epicarp subgroups all showed over 5-fold hypoglycemic bioactivity over that of IPGTT control, indicating that the entire part of FC fruits was effectively hypoglycemic. To compare, a much lesser extent was found in the IGTT group, and the pulpiness and the epicarp groups were seen to have increased by only 1.73- and 3.05-fold, respectively (**Table 4**), an implication in the insulin secretagogue and non-insulin sensitizing bioactivity of FC fruits. More interestingly, the exogenous insulin apparently was seen to have suppressed more the bioactivity of pulpiness, indicating the pulpiness may have a stronger insulin secretagogue effect. To summarize, the insulin secretagogue bioactivity could be effectively blocked by the coexisting exogenous insulin. We propose that the FC preparations could act either as an activator for de novo insulin biosynthesis or a secretagogue for insulin release, which is subjected to the feedback antagonistic control by exogenous insulin. As a consequence, the glucose uptake rates were reduced.

Up to the present, investigators have conducted only a few studies exploring the potential benefits of essential oils as hypoglycemic agents and publications on the subject are still very scarce. Some essential oils may aggravate diabetes, e.g., rosemary essential oil showed hyperglycemic and insulin release inhibitory effects in diabetic rabbits (*19*). Broadhurst et al. (*20*) have emphasized that the lipophilic fraction of aromatic plants (i.e., essential oils) are not generally responsible for any antidiabetic activity. As a contrast, Talpur et al. (*21*) indicated that an oral administration of a combination of essential oils including cinnamon, cumin, fennel, oregano, myrtle, besides others, was able to enhance insulin sensitivity and to lower circulating glucose in type 2 diabetes (*22*). Which constituent was responsible for its slimming effect was not clear. The effects seen could have been due to other phytochemical components (flavonoids, coumarins, limonoids, etc.) besides *d*-limonene, β-pinene, and γ-terpinene. Obviously, more research is needed to elucidate their action mechanism of hypoglycemic activity.

LITERATURE CITED

(1) Brands, S. J. (comp.) (1989−2006). Systema Naturae 2000. *The Taxonomicon*. Universal Taxonomic Services: Amsterdam, The Netherlands. Accessed October 11, 2006.

(2) Mei, S. F.; Zhaou, H.; Liu, S. R.; Mei, Z.; Hsih, L. S.; Lu, G. C. Development of Bergamot resources and its utility post development. *Chin. Seed Ind.* **2006**, *10*, 68–69.

(3) Chen, Y. S.; Gong, Z. L. Review of research progress in *Citrus medica* polysaccharide. *Food Sci. Technol.* **2003**, *3*: DOI. cnki: ISSN: 1005-9989.0.2003-03-039.

(4) Chen, J. H.; Lin, Z. M.; Sheng, F. *The study of chemical compositions of top note of Bergamot*. **1989**, DOI: cnki: ISSN: 0479-8023.0.1989-02-011.

(5) Chang, Y.; Kong, L. Y. Studies on the constituents of *Citrus medica* L. var. *Sarcodactylis* Swingle. **2006**, *Chinese Modern Medicines 6*.
(6) Sun, J. d-Limonene: safety and clinical applications (Clinical report). *Altern. Med. Rev*. **2007**, *12* (3), 259–264.
(7) Medical professionals. India Herbs Affiliate Program: Yogic−Slim web site: http://www.india-herbs.com/affiliates.
(8) Cheng, M. C.; Lin, L. Y.; Yu, T. H.; Peng, R. Y. Hypolipidemic and antioxidant activity of Mountain celery (*Cryptotaenia japonica* Hassk) seed essential oils. *J. Agric. Food Chem*. **2008**, *56* (11), 3997–4003.
(9) Schonburg, G.; Dielmann, G. Identification by means of retention parameters. *J. Chromatogr. Sci*. **1973**, *11*, 51–159.
(10) OECD Guideline 420. Acute Oral Toxicity-Fixed Dose Procedure. Revised draft, **2000**.
(11) Food and Drug Administration (FDA). Single Dose Acute Toxicity Testing for Pharmaceuticals. *Fed. Regist*. **1996**, *61*, 43934–43935.
(12) American Institute of Nutrition (AIN). Report of the American Institute of Nutrition ad hoc Committee on Standards of Nutritional Studies. *J. Nutr*. **1977**, *107*, 1340–1348.
(13) Zhang, F.; Ye, C.; Li, G.; Ding, W.; Zhou, W.; Zhu, H.; Chen, G.; Luo, T.; Guang, M.; Liu, Y.; Zhang, D.; Zheng, S.; Yang, J.; Gu, Y.; Xie, X.; Luo, M. The rat model of type 2 diabetic mellitus and its glycometabolism characters. *Exp. Anim*. **2003**, *52*, 401–407.
(14) Millennium Specialty Chemicals Inc. *Material Safety Data Sheet*. Supercedes: April 18, 2002. Revision Date: October 21, 2002. Product code: 01F26A. (A Lyondell Company, Jacksonville, FL 32201.)
(15) Hanefeld, M.; Leonhardt, W. *The Metabolic Syndrome* (*Das metabolische Syndrom*). Gustav Fischer Verlag: Jena, Germany, 1997; ISBN 3-437-31096-8.
(16) Eddouks, M.; Lemhadri, A.; Michel, J. B. Caraway and caper: potential anti-hyperglycaemic plants in diabetic rats. *J. Ethnopharmacol*. **2004**, *94*, 143–148.
(17) Peungvicha, P.; Thirawarapan, S. S.; Temsiririrkkul, R.; Watanabe, H.; Kumar, Prasain, J.; Kadota, S. Hypoglycemic effect of the water extract of *Piper sarmentosum* in rats. *J. Ethnopharmacol*. **1998**, *60*, 27–32.
(18) Morishita, M.; Matsuzawa, A.; Takayama, K.; Isowa, K.; Nagai, T. Improving insulin enteral absorption using water-in-oil-in-water emulsion. *Int. J. Pharm*. **1998**, *172*, 189–198.
(19) Al-Hader, A.; Hasan, Z.; Agel, M. Hyperglycemic and insulin release inhibitory effects of *Rosmarinus officinalis*. *J. Ethnopharmacol*. **1994**, *43*, 217–221.
(20) Broadhurst, C.; Polansky, M.; Anderson, R. 2000. Insulin-like biological activity of culinary and medicinal plant aqueous extract in vitro. *J. Agric. Food Chem*. **2000**, *48*: 849−852.
(21) Talpur, N.; Echard, B.; Ingram, C.; Bagchi, D.; Preuss, H. Effects of a novel formulation of essential oils on glucose-insulin metabolism in diabetic and hypertensive rats: a pilot study. *Diabetes Obes. Metab*. **2005**, *7*, 193–199.
(22) Abdollahi, M.; Salehnia, A.; Mortazavi, S. Antioxidant, antidiabetic, antihyperlipidemic, reproduction stimulatory properties and safety of essential oil of *Satureja Khuzestanica* in rat in vivo: a toxicopharmacological study. *Med. Sci. Monit*. **2003**, *9*, 331–335.

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