

## Anti-diabetic effects of lemon balm (*Melissa officinalis*) essential oil on glucose- and lipid-regulating enzymes in type 2 diabetic mice

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The antioxidant activity of lemon balm (*Melissa officinalis*) essential oil (LBEO) on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and its hypoglycaemic effect in *db/db* mice were investigated. LBEO scavenged 97 % of DPPH radicals at a 270-fold dilution. Mice administered LBEO (0.015 mg/d) for 6 weeks showed significantly reduced blood glucose (65 %;  $P < 0.05$ ) and TAG concentrations, improved glucose tolerance, as assessed by an oral glucose tolerance test, and significantly higher serum insulin levels, compared with the control group. The hypoglycaemic mechanism of LBEO was further explored via gene and protein expression analyses using RT-PCR and Western blotting, respectively. Among all glucose metabolism-related genes studied, hepatic glucokinase and GLUT4, as well as adipocyte GLUT4, PPAR- $\gamma$ , PPAR- $\alpha$  and SREBP-1c expression, were significantly up-regulated, whereas glucose-6-phosphatase and phosphoenolpyruvate carboxykinase expression was down-regulated in the livers of the LBEO group. The results further suggest that LBEO administered at low concentrations is an efficient hypoglycaemic agent, probably due to enhanced glucose uptake and metabolism in the liver and adipose tissue and the inhibition of gluconeogenesis in the liver.

**Lemon balm (*Melissa officinalis*): Hyperglycaemic effects: Glucokinase: GLUT4: Sterol regulatory element-binding protein-1c**

Diabetes mellitus is a major public health problem that affects approximately 5 % of the world population<sup>(1)</sup>. Type 2 diabetes is the most common form, accounting for more than 90 % of patients, and is characterised by chronic hyperglycaemia resulting from abnormalities in glucose metabolism and insulin secretion and activity.

Many recent studies on the treatment of type 2 diabetes have focused on the potential use of plant constituents with hypoglycaemic and hypolipidaemic effects. Consequently, there has been a growing interest in herbal essential oils, due to their antioxidative and hypolipidaemic activities<sup>(2–5)</sup>. Several plant constituents have been implicated in insulin signalling pathways modulating glucose transport and glucose metabolism-related enzyme activation, and PPAR activation, all of which play roles in diabetes<sup>(6,7)</sup>. In particular, glucokinase (GCK) gene transcription is stimulated by insulin and increased GCK activity enhances glucose utilisation and uptake in the liver. There have been efforts over recent years to discover and develop GCK activators as a novel therapy for type 2 diabetes<sup>(8)</sup>. Glucose-6-phosphatase (G6Pase) is a key enzyme controlling hepatic gluconeogenesis and glucose output in liver<sup>(9,10)</sup> and is normally suppressed by insulin<sup>(11)</sup>. Reduced activity of two key gluconeogenic

enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase, decreases hepatic glucose production<sup>(9,10)</sup>.

In adipose and muscle tissues, insulin stimulates glucose uptake by rapidly recruiting GLUT4 from an intracellular compartment to the plasma membrane<sup>(12)</sup>. PPAR control the expression of many genes involved in glucose and lipid metabolism. In a previous study, a single molecule was found to act as a dual agonist for both PPAR- $\alpha$  and PPAR- $\gamma$ , producing simultaneous hypolipidaemic and hypoglycaemic effects, respectively<sup>(13)</sup>. Sterol regulatory element-binding protein (SREBP)-1c is primarily involved in the regulation of fatty acid biosynthesis<sup>(14)</sup>.

In addition to the gene expression changes in glucose metabolism and insulin signalling pathway, oxidative stress plays critical roles in insulin signalling and the aetiology of diabetic complications such as diabetic retinopathy, renal failure and atherosclerosis. Thus, appropriate intake of antioxidative nutrients is beneficial to prevent or ameliorate diabetic symptoms or the development of its complications.

Lemon balm (*Melissa officinalis*) is a well-known medicinal plant species used in perfumes, cosmetics, tea and food products in many countries, and it has been cited as a mild sedative, a spasmolytic and an antibacterial agent<sup>(15)</sup>. Lemon balm

**Abbreviations:** DPPH, 2,2-diphenyl-1-picrylhydrazyl; GCK, glucokinase; G6Pase, glucose-6-phosphatase; LBEO, lemon balm essential oil; PEPCK, phosphoenolpyruvate carboxykinase; SREBP, sterol regulatory element-binding protein.

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leaves contain many phytochemicals, including polyphenolic compounds, such as rosmarinic acid<sup>(16)</sup>, trimeric compounds<sup>(17)</sup> and some flavonoids<sup>(18)</sup>. Lemon balm tea contains 10 mg/l of essential oils and abundant citral<sup>(19)</sup>. Herbal essential oils generally contain a variety of volatile compounds, which may have medicinal properties, including hypolipidaemic and hypoglycaemic effects<sup>(2,4,5,14)</sup>. Some herbal essential oils also possess strong antioxidant activity due to their high contents of tocopherols and phenolic compounds<sup>(2,14,20–22)</sup>.

Although several reports have been published on herbal essential oils, there is no reported information, to our knowledge, regarding the molecular events involved in the glucose-regulating function of this oil, nor has there been any reported study on the hypoglycaemic effect of LBEO in relation to glucose metabolism in a type 2 diabetes model. Accordingly, we analysed the composition of LBEO and assessed its antioxidant effects. We then evaluated the glucose-lowering capacity of LBEO in a model of type 2 diabetes. To further understand the mechanism(s) involved in the beneficial effect(s) of LBEO in diabetes, the gene and protein expression profiles of regulatory enzymes involved in hepatic and adipocyte glucose uptake and hepatic gluconeogenesis were investigated. Furthermore, the influence of LBEO on PPAR- $\gamma$ , PPAR- $\alpha$  and SREBP-1c was examined in the liver and adipose tissue of type 2 diabetic mice.

## Materials and methods

### Chemicals

An enhanced chemiluminescence (ECL) plus detection system was obtained from GE Healthcare Life Sciences (Piscataway, NJ, USA). Anti-rabbit IgG and heavy and light (H&L) chain-specific peroxidase conjugate were purchased from Calbiochem (Darmstadt, Germany). Rabbit anti-mouse PPAR- $\gamma$ , rabbit anti-mouse GSK3 $\beta$ , mouse monoclonal anti- $\alpha$ -tubulin, and goat anti-mouse IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and rabbit anti-mouse GLUT4 was purchased from AbD Serotec (Oxford, UK). PowerScript RT was obtained from Clontech Laboratories (Palo Alto, CA, USA). The oligo(dT)<sub>15</sub> primer, random hexamers and GoTaq<sup>®</sup> Green Master Mix PCR kit were purchased from Promega (Madison, WI, USA). A mouse insulin ELISA kit was purchased from Shibayagi, Co., Ltd (Gunma, Japan). All other reagents used were purchased from Sigma Chemical (St Louis, MO, USA).

### Preparation of lemon balm essential oil

Lemon balm essential oil (LBEO) was prepared from lemon balm leaves harvested from the Arboretum of Korea University (Seoul, Korea) in June 2005. The leaves were stored in a plastic bag at  $-70^{\circ}\text{C}$  before analysis. A 20 g portion of leaves was ground using a commercial blender, followed by steam distillation and extraction with 500 ml distilled water and 30 ml diethyl ether for 2 h at atmospheric pressure. The extract was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> at atmospheric pressure and concentrated to 300  $\mu\text{l}$  using a gentle stream of N<sub>2</sub> gas. Extractions were performed in triplicate.

### Analysis of lemon balm essential oil by GC-MS

GC-MS analysis was conducted using a GC system (Agilent 6890 N; Agilent Technologies, Palo Alto, CA, USA) connected to a mass spectrometer (Quattro GC/MS/MS; Micromass, Manchester, UK). The GC was equipped with a capillary column (50 m length  $\times$  0.25 mm diameter  $\times$  0.2  $\mu\text{m}$  film thickness; AT-1701; Alltech, Lancaster, PA, USA). A 1  $\mu\text{l}$  sample of the extract was injected (splitless mode) into each column. The oven temperature was programmed to increase from 40 $^{\circ}\text{C}$ , with an initial holding time of 2 min, to 120 $^{\circ}\text{C}$  at 3 $^{\circ}\text{C}/\text{min}$ , and then finally to 200 $^{\circ}\text{C}$  at 5 $^{\circ}\text{C}/\text{min}$ . The flow rate of the He carrier gas was 1.0 ml/min. The injector and detector temperatures were held at 280 and 240 $^{\circ}\text{C}$ , respectively. Using perfluorotributyl amine, the parameters of the mass spectrometer were optimised for the best resolutions at 69  $m/z$ , 219  $m/z$ , 502  $m/z$  and 614  $m/z$ . Mass measurement was conducted using an electron ionisation (EI) positive ion source at 240 $^{\circ}\text{C}$  in the SCAN mode in the mass range of 33–350  $m/z$ .

### Identification and quantification

Total ion chromatograms of the samples were analysed using MassLynx 4.0 software (MassLynx 4.0 SCN 474; Micromass), and the compounds were positively identified using the Wiley mass spectral database (2002; John Wiley & Sons, New York, NY, USA).

### Antioxidant activity test

The effect of LBEO on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was estimated according to the method of Singh & Rajini<sup>(23)</sup>, with minor modifications. Samples of 900  $\mu\text{l}$  at various concentrations (10-fold to 47 829 690-fold dilutions) were mixed with 300  $\mu\text{l}$  of DPPH solution ( $1.5 \times 10^{-4} \text{ M}$ ) and then the tube was mixed by vortexing. The mixture was incubated at 37 $^{\circ}\text{C}$  for 30 min and the decrease in absorbance at 532 nm was measured. The antioxidant was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine product. The percentage inhibition of DPPH was calculated using the following equation:

$$\begin{aligned} \text{Radical-scavenging activity (\%)} \\ = ((A_{\text{sample}(517 \text{ nm})} / A_{\text{control}(517 \text{ nm})}) \times 100, \end{aligned}$$

where  $A_{\text{sample}(517 \text{ nm})}$  is the absorbance of the sample and  $A_{\text{control}(517 \text{ nm})}$  is the absorbance of the control at 517 nm.

### Animals and feeding protocol

Male C57BL/KsJ-*db/db* (*db/db*) mice were obtained from Orient Bio (Gyeonggi-Do, Korea). Animal rooms were maintained at 21 $^{\circ}\text{C}$  under humidity-controlled conditions and a 12 h light–12 h dark cycle. At 15 weeks of age, mice were fed normal chow or chow with *M. officinalis* essential oil (0.0125 mg LBEO/d) for 6 weeks. After feeding, the mice were fasted overnight (16–19 h), and blood samples were collected in purple top tubes containing EDTA once every 3 weeks. Plasma samples were obtained from blood by

centrifugation (10 000 rpm, 10 min). Glucose, total cholesterol, TAG and HDL-cholesterol levels were determined by enzymic methods (Asan Pharmaceuticals, Hwasung, South Korea). At the end of 6 weeks, an oral glucose tolerance test was performed and the mice were killed to obtain several organs. Organs were snap-frozen in liquid N<sub>2</sub> and stored at -80°C for total RNA and protein extraction. All experimental procedures involving animals were approved by the Korea University Institutional Animal Care and Use Committee.

#### Oral glucose tolerance tests

An oral glucose tolerance test was performed after 6 weeks following an overnight fast (16 h). The mice were administered glucose orally at 0.25 g/kg body weight. The blood glucose concentration was determined in tail blood samples taken 0, 15, 30, 60, 90 and 120 min after glucose administration using a glucometer (MyCare GAM-2200; Green Cross, Yongin, Korea).

#### Serum insulin levels

Blood was collected in tubes without heparin or EDTA and centrifuged (10 000 rpm; 10 min). The serum insulin level was determined using a mouse insulin ELISA kit (Shibayagi, Co., Ltd, Gunma, Japan); this reagent kit is for the quantification of insulin by a sandwich-technique enzyme immunoassay.

#### Isolation of total RNA and RT-PCR

Total RNA was extracted from liver or adipose tissue using a Sigma TRI reagent kit, according to the manufacturer's protocol, and was then dissolved in diethylpyrocarbonate-treated water. For cDNA synthesis, total RNA (2 µg) was reverse transcribed using PowerScript RT (Clontech, Mountain View, CA, USA), according to the protocol supplied, using a combination of oligo(dT)<sub>15</sub> primer and random hexamers. PCR was performed using the GoTaq<sup>®</sup> Green Master Mix PCR kit (Promega) in a 20 µl reaction mixture containing 1 µl of the RT reaction mixture and 0.5 µl of each primer (forward and reverse, 15 µM). PCR primers were designed using published nucleotide sequences for GCK<sup>(6)</sup>, G6Pase<sup>(6)</sup>, PEPCK<sup>(6)</sup>, GLUT2<sup>(24)</sup>, PPAR-α<sup>(25)</sup>, PPAR-γ<sup>(25)</sup>, GLUT4<sup>(26)</sup>, SREBP-1c<sup>(24,27)</sup> and β-actin<sup>(27)</sup>.

The following *in vivo* primers were used: for GCK, forward 5'-TTC ACC TTC TCC CCT GTA AGG C-3' and reverse 5'-TAC CAG CTT GAG CAG CAC AAG TCG-3'; for G6Pase, forward 5'-AAG ACT CCC AGG ACT GGT TCA TCC-3' and reverse 5'-TAG CAG GTA GAA TCC AAG CGC G-3'; for PEPCK, forward 5'-TGC TGA TCC TGG GCA TAA CTA ACC-3' and reverse 5'-TGG GTA CTC CTT CTG GAG ATT CCC-3'; for PPAR-α, forward 5'-CCT CAG GGT ACC ACT ACG GAG T-3' and reverse 5'-GCC GAA TAG TTC GCC GAA-3'; for PPAR-γ, forward 5'-TAG GTG TGA TCT TAA CTG TCG-3' and reverse 5'-GCA TGG TGT AGA TGA TCT CA-3'; for GLUT2, forward 5'-GGC TAA TTT CAG GAC TGG TT-3' and reverse 5'-TTT CTT TGC CCT GAC TGC CT-3'; for GLUT4, forward 5'-CCT GCC CGA AAG AGT CTA AAG C-3' and

reverse 5'-ACT AAG AGC ACC GAG ACC AAC G-3'; and for SREBP-1c, forward 5'-GGA GCC ATG GAT TGC ACA TT-3' and reverse 5'-GGC CCG GGA AGT CAC TGT-3'. The β-actin transcript (forward 5'-TGC TGT CCC TGT ATG CCT CT-3' and reverse 5'-AGG TCT TTA CGG ATG TCA ACG-3') was used as an internal control.

PCR using the GCK primer was performed with an initial cycle of 4 min at 94°C, followed by twenty-two cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, and a final extension for 5 min at 72°C. PCR using the G6Pase, PEPCK, PPAR-α, PPAR-γ, GLUT2, GLUT4, SREBP-1c and β-actin primers was performed similarly, with the exception of the annealing temperature (G6Pase, 57°C; PEPCK, 57°C; PPAR-α, 50°C; PPAR-γ, 52°C; GLUT2, 47°C; GLUT4, 54°C; SREBP-1c, 54°C; β-actin, 50°C) and the number of cycles (G6Pase, twenty cycles; PEPCK, nineteen cycles; PPAR-α, twenty-three cycles; PPAR-γ, twenty-six cycles; GLUT2, twenty-eight cycles; GLUT4, twenty-six cycles; SREBP-1c, twenty-five cycles; β-actin, twenty-two cycles). The β-actin transcripts were used as internal controls.

#### Western blotting

Liver and adipose tissue were homogenised in a buffer containing 10 mM-2-amino-2-hydroxymethyl-propane-1,3-diol-HCl (pH 7.4), 0.1 M-EDTA, 10 mM-NaCl, 0.5 % Triton X-100, and one protease inhibitor cocktail tablet, at 4°C. The homogenates were then centrifuged (14 000 rpm, 10 min, 4°C). The protein concentration was determined using a Bio-Rad protein kit with bovine serum albumin (Sigma, St Louis, MO, USA) as the standard. Equal amounts of protein were boiled in sample buffer (with 5 % β-mercaptoethanol) for 5 min. The proteins were separated via 10 % SDS-PAGE and transferred to a nitrocellulose membrane (0.45 µm Protran Nitrocellulose Transfer Membrane; Schleicher & Schuell BioScience, Dassel, Germany). The membranes were then incubated with an anti-mouse GCK (rabbit polyclonal IgG), anti-mouse PPAR-γ (rabbit polyclonal IgG), or anti-mouse GLUT4 (rabbit polyclonal IgG) antibody, and monoclonal anti-α-tubulin (mouse Ig) antibody (1/700). After washing several times with PBS-0.1 % Tween 20, the membrane was incubated with 1/2500 anti-rabbit IgG or anti-mouse IgG with H&L chain-specific (goat) peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using an ECL kit (GE Healthcare Life Sciences, Piscataway, NJ, USA), according to the manufacturer's protocol, and exposed to high-performance chemiluminescence film for 10 s. Protein immunoblots were scanned with a 690 Bio-Rad densitometer using the Multi-Analyst program (Bio-Rad, Hercules, CA, USA) and quantified using SigmaGel software (Jandel Scientific, San Rafael, CA, USA).

#### Statistical analyses

Data from three independent experiments were expressed as mean values and standard deviations. One-way ANOVA followed by Tukey's test was used to compare the results from different treatments. Student's *t* test was used for comparisons between groups. Data were deemed to be statistically significantly different at *P* < 0.05.

## Results

### Composition of lemon balm essential oil

There were forty constituents identified in the essential oil of the leaves of lemon balm (*M. officinalis*) that accounted for 99.7% of the total oil components, as revealed by the GC-MS analysis (Table 1). Monoterpene hydrocarbons, including  $\beta$ -pinene (0.3%), oxygenated monoterpenes, including 2,3-dehydro-1,8-cineole (0.1%), linalool (0.8%), myrtenol (0.1%), (*Z*)-carveol (0.1%), geranial (65.4%), neral (24.7%), geranylacetate (7.4%) and sesquiterpene hydrocarbons, including caryophyllene (0.8%) and farnesene (0.1%), were found to be the major volatile compounds.

**Table 1.** Composition of the essential oil of lemon balm (*Melissa officinalis*)

No.	Compounds	Peak area (%)
Monoterpene hydrocarbons		
1	Sabinene	t
2	$\beta$ -Pinene	0.32
3	Limonene	t
4	Phellandrene	t
Oxygenated monoterpenes		
5	2,3-dehydro-1,8-cineole	0.05
6	Carvacrol	t
7	Epoxylinolool	t
8	$\alpha$ -Terpineol	t
9	Linalool	0.75
10	Borneol	t
11	Neroloxide	t
12	Citronellal	t
13	Myrtenol	0.08
14	Terpinene-4-ol	t
15	( <i>Z</i> )-Carveol	0.08
16	Menthen-2,3-diol	t
17	Neral	24.65
18	Anethole	t
19	Geranial	65.42
20	Citronellyl acetate	t
21	Geranylacetate	7.38
22	Damascenone	t
23	Eugenol	t
24	Dihydrocarveol	t
Sesquiterpene hydrocarbons		
25	Bicyclgermacrene	t
26	Caryophyllene	0.77
27	Germacrene-D	t
28	Farnesene	0.05
29	$\gamma$ -Elemene	t
30	Cadinene	t
Carbonyl compounds		
31	Nonanal	t
32	( <i>Z</i> )-Jasmone	t
33	$\beta$ -Ionone	t
Alcohols		
34	Benzylalcohol	t
35	Fenchylalcohol	t
36	Chavicol	t
Others		
37	Dodecane	t
38	Iso-geranol	t
39	Bourbonene	t
40	1-Dodecen-3-yne	0.09
Total area of identified peaks (%)		99.64

t, Trace amount (less than 0.05% of total peak area).

### Antioxidant activity of lemon balm essential oil

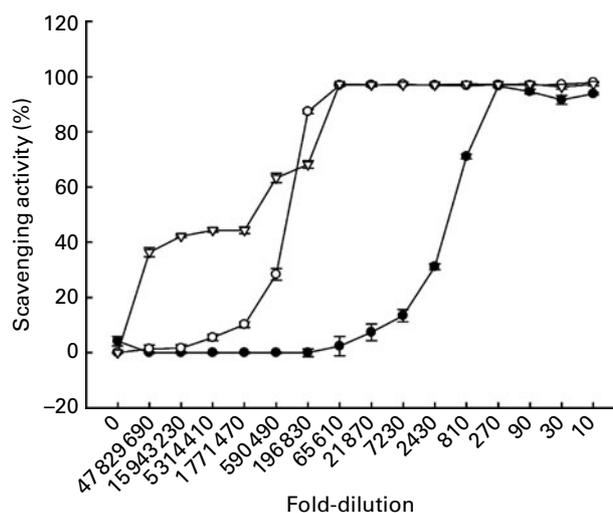
The effect of LBEO on DPPH radical-scavenging activity was tested (Fig. 1). DPPH reactivity is commonly used to determine the free radical-scavenging ability of antioxidative phytochemicals. In Fig. 1, the DPPH radical-scavenging activities of LBEO at various concentrations are compared with those of ascorbic acid and vitamin E. The DPPH radical-scavenging activity of LBEO increased significantly from the 196 830-fold dilution to the 270-fold dilution in a dose-dependent manner. A similar effect was found with ascorbic acid and vitamin E at high concentrations (270-fold to 10-fold dilutions), although these two antioxidants showed high antioxidant activities even at much higher dilutions.

### Effects of lemon balm essential oil on plasma glucose levels, oral glucose tolerance test, and serum insulin concentrations

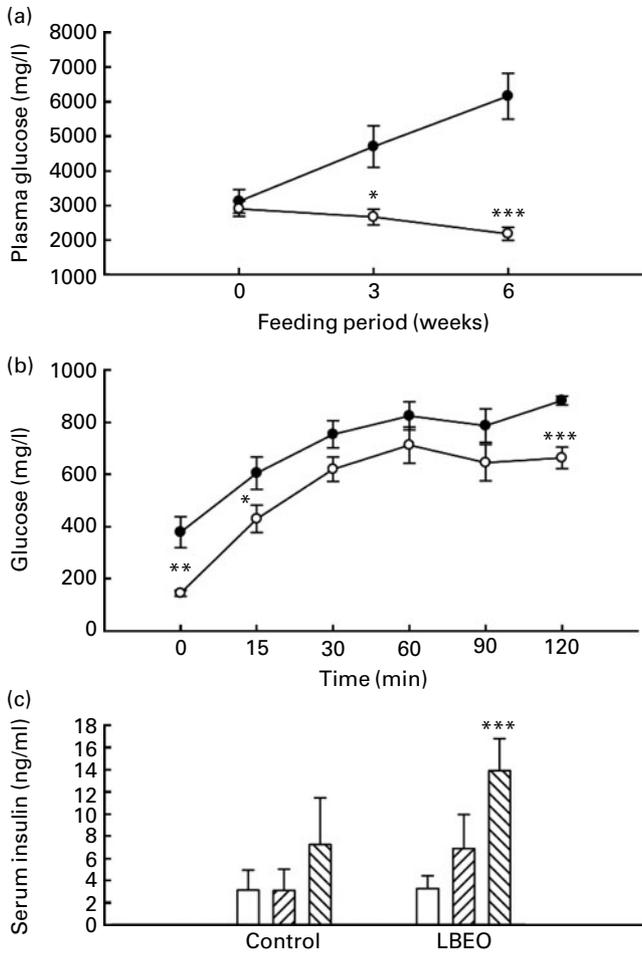
The baseline values (week 0) for plasma blood glucose were similar between groups, although levels in the LBEO group decreased significantly after 3 and 6 weeks of LBEO treatment, compared with the control group, and blood glucose levels decreased by up to 64.6% (Fig. 2(a)). The administration of LBEO also improved glucose tolerance in *db/db* mice (Fig. 2(b)). Blood glucose levels at 0, 15 and 120 min after glucose loading were significantly ( $P < 0.05$ ) lower in the LBEO group v. the control group (Fig. 2(b)). In contrast, serum insulin levels showed a significant increase ( $P < 0.05$ ) in the LBEO group, compared with the control group, at 3 and 6 weeks (Fig. 2(c)). Body weight (28.3 (SD 0.6) v. 28.2 (SD 0.5) g in control and LBEO mice, respectively) and total fat content (2.6 (SD 0.1) v. 2.5 (SD 0.1) g in control and LBEO, respectively) were not changed after 6 weeks of LBEO feeding.

### Effects of lemon balm essential oil on plasma lipids levels

Plasma TAG concentrations were significantly lower ( $P < 0.05$ ) in the LBEO group than in the control group

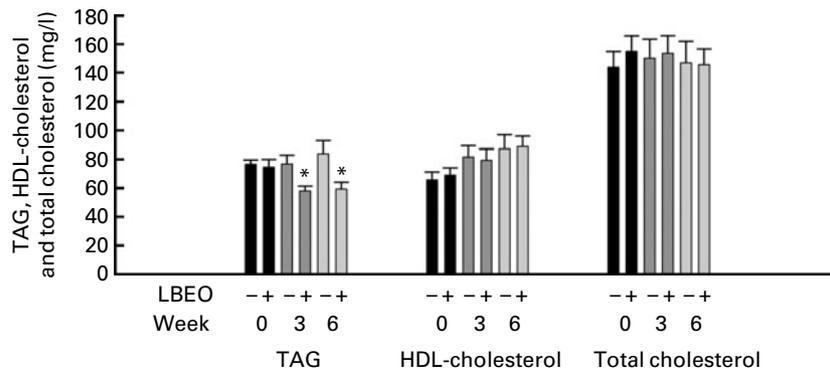


**Fig. 1.** Effects of lemon balm (*Melissa officinalis*) essential oil (—●—) compared with ascorbic acid (—○—) and vitamin E (—▽—) on 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity. Values are means ( $n = 4$ ), with standard deviations represented by vertical bars.



**Fig. 2.** Effects of lemon balm (*Melissa officinalis*) essential oil (LBEO;  $\square$ – $\square$ ) compared with control ( $\bullet$ – $\bullet$ ) on (a) plasma glucose, (b) glucose tolerance and (c) serum insulin levels at 0 weeks ( $\square$ ), 3 weeks ( $\square$ ) and 6 weeks ( $\square$ ). Values are means for six mice, with standard deviations represented by vertical bars. Mean value was significantly different from that of the control group: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

(a 29.2% decrease; Fig. 3). However, no significant difference in plasma total cholesterol or HDL-cholesterol was observed compared with the control group at 3 or 6 weeks (Fig. 3).



**Fig. 3.** Effects of lemon balm (*Melissa officinalis*) essential oil (LBEO) on plasma TAG, HDL-cholesterol and total cholesterol levels. Values are means for six mice, with standard deviations represented by vertical bars. \* Mean value was significantly different from that of the control group ( $P < 0.05$ ).

*Gene and protein expression of hepatic glucose-regulating enzymes*

A number of key hepatic glycolytic and gluconeogenic genes were assayed by RT-PCR and Western blotting. The LBEO supplementation resulted in a significant decrease in G6Pase and PEPCK mRNA levels compared with the controls (Fig. 4(a)), whereas the GCK mRNA and protein levels were increased significantly in response to LBEO administration in *dbldb* mice compared with controls (Fig. 4(b) and (c)).

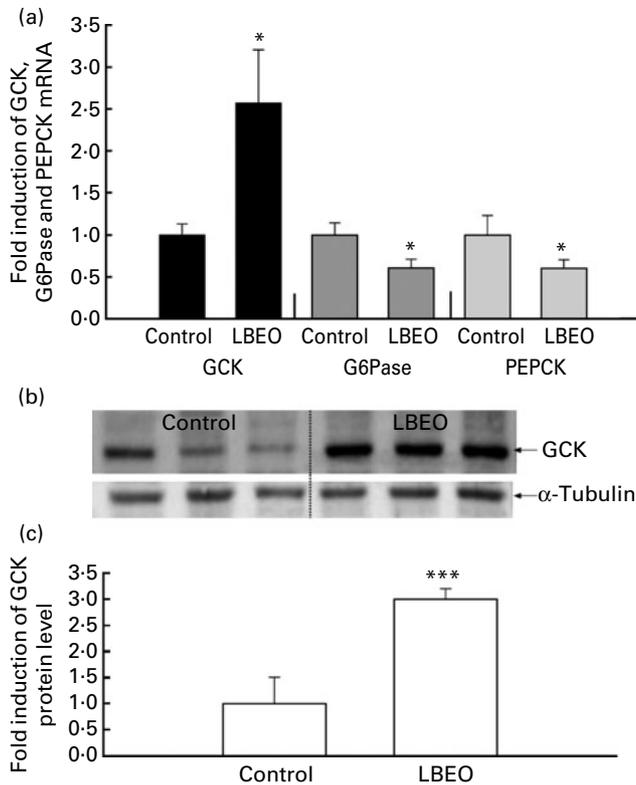
*Expression of glucose transporters, PPAR- $\gamma$ , PPAR- $\alpha$  and sterol regulatory element-binding protein-1c*

Changes in GLUT4, GLUT2, PPAR- $\gamma$ , PPAR- $\alpha$  and SREBP-1c expression were determined. The mRNA expression of hepatic GLUT4 and SREBP-1c and adipocyte GLUT4, PPAR- $\gamma$ , PPAR- $\alpha$  and SREBP-1c was significantly higher in the LBEO group than in the control group (Fig. 5(a)). Hepatic and adipocyte GLUT4 mRNA levels were 1.5- and 2.6-fold higher, respectively, and their corresponding protein levels were approximately 1.5- and 1.3-fold higher, respectively, in the LBEO group (Fig. 5(a) and (b)). Adipocyte PPAR- $\gamma$  protein expression increased significantly in the LBEO-fed group. However, no significant difference was observed in hepatic GLUT2, PPAR- $\gamma$  or PPAR- $\alpha$  mRNA transcription.

**Discussion**

In previous studies, we showed that Asian plantain (*Plantago asiatica*) and wormwood (*Artemisia princeps*) essential oils had strong antioxidant effects; they also showed hypocholesterolaemic effects through the suppression of 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase expression and the up-regulation of LDL receptor expression, *in vitro* and *in vivo* (2,14). This prompted us to examine other herbal essential oils for activities that may be helpful in preventing and treating various diseases. Thus, we assessed the hypoglycaemic effects of LBEO.

The present results showed that the LBEO had strong antioxidant activity and contained large amounts of neral (24.7%) and geranial (65.4%) volatile oils. In the present study, LBEO administered orally (0.0125 mg/d) for 6 weeks did not appear to produce any toxicity in *dbldb* mice; in fact, the activities of plasma transaminases (aspartate aminotransferase and alanine



**Fig. 4.** Effects of lemon balm (*Melissa officinalis*) essential oil (LBEO) on hepatic glucokinase (GCK), glucose-6-phosphatase (G6Pase), and phosphoenolpyruvate carboxykinase (PEPCK) mRNA transcription (a) and hepatic GCK protein expression (b and c). The density of each band on the Western blot (protein) and RT-PCR gel (mRNA) was quantified using Sigma-Gel software (Jandel Scientific, San Rafael, CA, USA). The mRNA and protein levels in each sample were normalised against the quantity of 18s RNA or  $\alpha$ -tubulin. Values are means for six mice, with standard deviations represented by vertical bars. Mean value was significantly different from that of the control group: \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .

aminotransferase) decreased in response to LBEO supplementation (data not shown), indicating that LBEO did not induce liver damage at the dose used here. To date, twenty chemical components have been isolated from the leaves of lemon balm<sup>(19)</sup>. In the present study, the major aromatic components were neral (20.4%) and geranial (27.8%); thus, the present results regarding the composition and antioxidant activity of lemon balm leaf essential oil were consistent with previous reports<sup>(19,21)</sup>.

Since oxidative stress and reactive oxygen species could cause diabetes and its complications, appropriate intake of antioxidative nutrients, such as LBEO, may be beneficial to prevent or ameliorate diabetic symptoms or complications. There is evidence that reactive oxygen species in cells act as a double-edged sword in modulating insulin signalling<sup>(28,29)</sup>. Reactive oxygen species are generated in response to insulin and they are somewhat necessary to normal insulin activities, but, on the other hand, recent evidence suggests that reactive oxygen species are negative regulators of insulin signalling, rendering them putative mediators in the development of insulin resistance and obesity. Oxidative stress is also particularly important in the development of various diabetic complications such as diabetic retinopathy, renal failure and atherosclerosis. There are mainly four mechanisms regarding

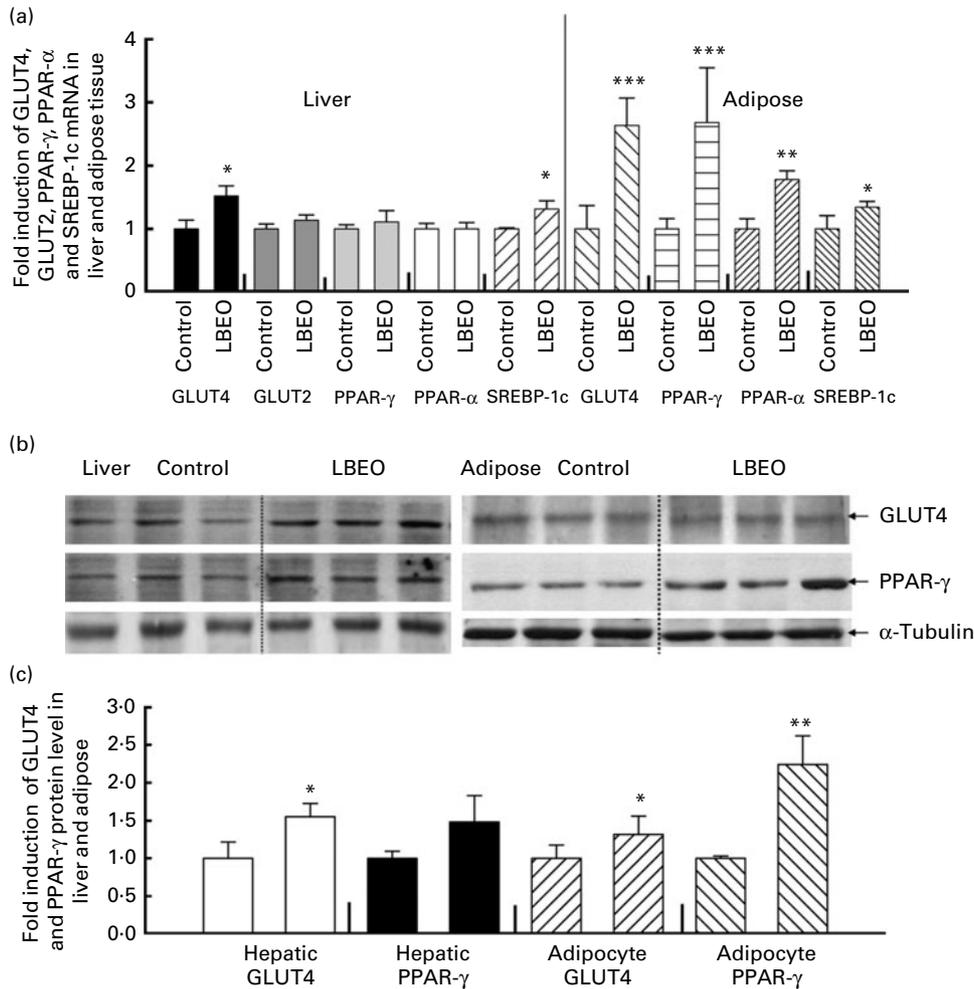
these<sup>(30)</sup>: first, activation of protein kinase C isoforms; second, increased hexosamine pathway flux; third, increased polyol pathway flux, which aldose reductase mediates conversion of glucose to sorbitol and excess sorbitol causes oxidative damage and activates stress genes; fourth, increased advanced glycation endproduct formation, which bind to specific cell surface receptors and lead to post-receptor signalling and further generation of reactive oxygen species. Thus, the antioxidant effect of LBEO will help to prevent diabetes and its complications.

In the present study, LBEO supplementation significantly reduced plasma glucose levels compared with the control group, and augmented glucose tolerance in a type 2 diabetic model. Serum insulin concentrations were increased significantly in the LBEO group compared with the control group. These results are consistent with those of previous reports describing the hypoglycaemic effects of citrus flavonoids and Du-zhong (*Eucommia ulmoides* Oliver) leaf water extract, and increased plasma insulin levels in response to treatment with Du-Zhong leaf water extract<sup>(6,7)</sup>.

LBEO treatment decreased glucose concentrations by stimulating GCK activity and inhibiting G6Pase activity in the livers of *db/db* mice. Hepatic GCK activity was increased significantly, whereas those of G6Pase and PEPCK were decreased significantly in the LBEO group compared with the control group. Key liver genes for carbohydrate and lipid homeostasis are regulated by insulin and glucose<sup>(31,32)</sup>. GCK catalyses the conversion of glucose into glucose-6-phosphate in the liver, thus playing a key role in the control of glucose homeostasis by supplying glucose-6-phosphate for glycogen storage, glycolysis or the pentose phosphate pathway. For these reasons, the GCK gene has been considered as a potential target for the pharmacological treatment of type 2 diabetes in recent years<sup>(33)</sup>. A major effect of insulin in the liver is the induction of GCK gene expression, which is a key step in the subsequent activation of hepatic glycolytic and lipogenic gene expression by glucose<sup>(34–36)</sup>. Many studies have also indicated that G6Pase and PEPCK activities are higher in type 2 diabetes mellitus<sup>(7,37,38)</sup>, whereas supplementation with several plant constituents significantly lowered G6Pase and PEPCK activities<sup>(6,7)</sup>. Additionally, an abnormal increase in hepatic glucose production is a major symptom of diabetes and contributes to fasting hyperglycaemia<sup>(39)</sup>, apparently as a consequence of increased G6Pase activity and decreased GCK activity. Among glucose-regulating genes, the enhanced expression of the hepatic PEPCK gene has been identified in most forms of diabetes, and contributes to increased hepatic glucose output<sup>(40)</sup>. Accordingly, LBEO treatment appears to improve glucose metabolism through an increase in GCK activity and a decrease in gluconeogenic enzyme activity (i.e. G6Pase and PEPCK).

SREBP-1c has been proposed as a major mediator of insulin action on GCK transcription<sup>(41,42)</sup>. In a previous study, GCK gene transcription was regulated by SREBP-1c and insulin in cultured rat hepatocytes, and SREBP-1c activation up-regulated insulin-sensitive GLUT4 expression in liver, muscle and adipocytes<sup>(43)</sup>. Insulin binding to the insulin receptor regulates glucose uptake into cells via GLUT4, indicating a major role for GLUT4 in glucose uptake and metabolism.

PPAR- $\gamma$  activation restores the glucose-sensing ability of  $\beta$ -cells through the increased expression of GLUT2 and



**Fig. 5.** Effects of lemon balm (*Melissa officinalis*) essential oil (LBEO) on hepatic GLUT4, GLUT2, PPAR-γ, PPAR-α and sterol regulatory element-binding protein (SREBP)-1c, and on adipocyte GLUT4, PPAR-γ, PPAR-α and SREBP-1c mRNA transcription (a), and GLUT4 and PPAR-γ protein levels in liver and adipose tissue (b and c) of *db/db* mice. The density of each band on the Western blot (protein) and RT-PCR gel (mRNA) was quantified using SigmaGel software (Jandel Scientific, San Rafael, CA, USA). The mRNA and protein levels in each sample were normalised against the quantity of 18s RNA or α-tubulin expression. Values are means for six mice, with standard deviations represented by vertical bars. Mean value was significantly different from that of the control group: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

GCK<sup>(44)</sup>. PPAR-γ activation increases the expression and translocation of GLUT1 and GLUT4 to the cell surface, thus increasing glucose uptake in adipocytes and muscle cells<sup>(45)</sup>, and reducing glucose plasma levels.

In the present study, serum insulin levels were significantly higher in LBEO-supplemented *db/db* mice compared with control mice. Insulin-regulated GLUT4 mRNA levels were also significantly higher in the LBEO group than in the control group. However, the molecular mechanism(s) by which insulin regulates GCK gene expression remain(s) controversial.

In the present study, the plasma TAG concentrations were significantly lowered in the LBEO group, which was observed in association with a simultaneous increase in SREBP-1c mRNA transcription and PPAR-γ protein expression in the liver and adipose tissue. SREBP-1c promotes the expression of genes involved in fatty acid synthesis. PPAR-γ activation induces the expression of genes controlling adipocyte fatty acid metabolism, including those that encode lipoprotein lipase and fatty acid transport proteins, thus leading to

lipolysis of plasma TAG, uptake of fatty acids and the storage of TAG in adipocytes.

In conclusion, the present data suggest that LBEO is an anti-hyperglycaemic agent that mediates its effects through the activation of GCK and the inhibition of G6Pase and PEPCK in the liver. Increased GLUT4, SREBP-1c, PPAR-γ and PPAR-α expression in the liver and adipose tissue may provide additional anti-diabetic benefits.

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M. J. C. was the main researcher in performing animal experiments, lipid and gene expression analyses and also wrote the manuscript. S.-Y. C. assisted in immunoblot

analysis. M. J. H. B. assisted in data interpretation and manuscript preparation. K. H. K. provided GC/MS analysis data. S.-J. L. was the principal investigator in the project.

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