Carnosic acid-rich rosemary (*Rosmarinus officinalis* L.) leaf extract limits weight gain and improves cholesterol levels and glycaemia in mice on a high-fat diet

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Abstract

Rosemary (*Rosmarinus officinalis* L.) extracts (RE) are natural antioxidants that are used in food, food supplements and cosmetic applications; exert anti-inflammatory and anti-hyperglycaemic effects; and promote weight loss, which can be exploited to develop new preventive strategies against metabolic disorders. Therefore, the aim of the present study was to evaluate the preventive effects of rosemary leaf extract that was standardised to 20% carnosic acid (RE) on weight gain, glucose levels and lipid homeostasis in mice that had begun a high-fat diet (HFD) as juveniles. The animals were given a low-fat diet, a HFD or a HFD that was supplemented with 500 mg RE/kg body weight per d (mpk). Physiological and biochemical parameters were monitored for 16 weeks. Body and epididymal fat weight in animals on the HFD that was supplemented with RE increased 69 and 79% less than those in the HFD group. Treatment with RE was associated with increased faecal fat excretion but not with decreased food intake. The extract also reduced fasting glycaemia and plasma cholesterol levels. In addition, we evaluated the inhibitory effects of RE *in vitro* on pancreatic lipase and PPAR-γ agonist activity; the *in vitro* findings correlated with our observations in the animal experiments. Thus, the present results suggest that RE that is rich in carnosic acid can be used as a preventive treatment against metabolic disorders, which merits further examination at physiological doses in randomised controlled trials.

Key words: *Rosmarinus officinalis* L.: Carnosic acid: Pancreatic lipase: PPAR-γ

The prevalence of metabolic disorders, such as obesity, hyperlipidaemia and hyperglycaemia, is rising dramatically in developing and industrialised nations. Obesity is reaching epidemic proportions worldwide[1] and is an established risk factor for various comorbidities, such as type 2 diabetes mellitus and CVD[2–4]. The development of obesity induces systemic oxidative stress[5] and affects the inflammatory state[6]. The constant increase in fat intake that is linked with sedentary lifestyles is the chief cause of this phenomenon[2].

Developing preventive and therapeutic solutions that impede the rise in metabolic disorders has become a primary goal in the past decade. In addition to pharmaceutical approaches, the use of natural products in physiological doses has been recognised as an effective regimen to improve several health conditions[7–9]. Plant-based treatments have been validated as strategies in the prevention of obesity and type 2 diabetes mellitus[10].

Rosemary (*Rosmarinus officinalis* L.) extracts are natural antioxidants that are used in food, food supplements and cosmetic applications[11–15]. Recently, rosemary extracts that have been standardised for carnosic acid and carnosol attained antioxidant status, garnering an additive E classification from the European Food Safety Authority, confirming its importance as a natural preservative in foods and beverages[13].

Carnosic acid-rich rosemary extract has been reported to have antioxidant activity *in vitro* by oxygen radical absorbance capacity and ferric-reducing/antioxidant power assays, and it inhibits the oxidation of Cu^{2+}-induced LDL *ex vivo*[15]. These antioxidant effects have been recapitulated *in vivo*. Consequently, carnosic acid-rich rosemary extract reduces oxidative stress in aged rats[16]. Carnosic acid has

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anti-inflammatory effects in cellular[17] and animal[18] models. Furthermore, carnosic acid has promising anti-obesity and anti-glycaemic effects.

In *in vitro* trials, carnosic acid inhibits pancreatic lipase[19], activates PPAR-γ[20] and prevents the differentiation of mouse pre-adipocytes into adipocytes[21] – all of which are important mechanisms in glucose and lipid homeostasis. The capacity of rosemary to regulate weight gain[22,23] and glycaemia[22–24] has been observed *in vivo*. Nevertheless, no randomised clinical trials have been reported using rosemary extracts to control obesity and hyperglycaemia, but this evidence encourages further study of carnosic acid-rich rosemary extracts to prevent the development of metabolic disorders.

In the present study, we aimed to determine the preventive effects of a rosemary extract that was standardised to contain 20 % carnosic acid (RE) on weight gain, glycaemia levels and lipid homeostasis in mice that were started on a high-fat diet (HFD) as juveniles. The animals were given a low-fat diet (LFD), a HFD or a HFD with 500 mg RE/kg body weight per d (mpk) (HFD-RE). Physiological and biochemical parameters were measured throughout the 16 weeks of treatment, and the effects on pancreatic lipase and PPAR-γ agonist activity *in vitro* were examined.

Experimental methods

Rosemary leaf extract

RE was prepared as described by Ibarra *et al.*[15].

Animals and diet

Male C57BL/6J mice, aged 4 weeks, were purchased from Elevage Janvier (CERJ, Le Genest Saint Isle, France). All mice were housed in a cage on a 12 h light–12 h dark cycle in a temperature-controlled environment during a 2-week acclimatisation, with *ad libitum* access to water and a control standard diet – an energy-balanced diet. After acclimatisation, the mice were randomised by body weight into three groups of eight animals. Each group was fed an experimental diet (Research Diets, Inc., New Brunswick, NJ, USA) for 16 weeks, as described in Table 1 (LFD, HFD and HFD-RE). Body weight was measured twice per week, and food intake was recorded once per week. All procedures were performed as per French guidelines for the care and use of experimental animals.

Blood biochemistry

Blood was collected from the retro-orbital sinus into EDTA-coated tubes under isoflurane anaesthesia after overnight fasting. Samples were collected at the beginning of the study (day 0) and after 16 weeks on the experimental diets. Blood samples were centrifuged at 4000 rpm for 15 min at 4°C to recover the plasma.

Biochemical levels were measured using commercial kits. Total cholesterol, TAG, glucose (kits CH3810, TR3823 and GL3815; Randox Laboratories Limited, Newbury, Berkshire, UK) and NEFA (kit 434-91717; Wako Pure Chemical Industries Limited, Osaka, Japan) were measured by spectroscopy. Insulin (kit INSKR020; Crystal Chem, Inc., Downers Grove, IL, USA) was measured by ELISA.

Faecal lipid measurements

Faeces were collected at weeks 0, 8 and 16, frozen at −80°C and pulverised. For each condition, faeces from eight mice, harvested during a 24 h period, were pooled. Total lipids were extracted from 100 mg of dried faeces as described[25]. Total lipid levels from several independent extractions were estimated by traditional gravimetric analysis: 500 µl of total lipids in chloroform were dried by evaporation and weighed.

The amount of faecal fat energy that was excreted, expressed in kJ/animal per d, was calculated in the lyophilised total fat that was excreted and collected throughout the experiment, assuming that 1 g lipid equals 37·7 kJ.

Pancreatic lipase activity assay

Human pancreatic lipase was purchased from Lee Biosolutions, Inc. (St Louis, MO, USA). Orlistat (tetrahydrolipstatin, a pancreatic lipase inhibitor) was purchased from Sigma Chemical Company (St Louis, MO, USA). Other chemicals were of reagent grade. The pancreatic lipase was diluted in dimethyl sulfoxide to obtain a final activity of $0·1 \times 10^6$ U/l. Orlistat was tested at two concentrations in dimethyl sulfoxide.

Lipase activity was measured using the ENZYLINETM Lipase Colour Assay kit (Biomérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Briefly, pancreatic lipase, substrate and the test sample were mixed gently, and incubated for 5 min at 37°C. Activator reagent was added, and the mixtures were incubated again for 6 min at 37°C. The recorded rate of increase in absorbance at 550 nm, due to the formation of quinone diimine dye, reflected pancreatic lipase activity.

PPAR-γ assay

PPAR-γ activation was measured in a cell-based luciferase assay. COS-7 cells (African Green Monkey SV40-transformed kidney fibroblast cell line), cultured in Dulbecco's modified Eagle's medium that was supplemented with 10 % fetal calf serum, were transiently transfected with a fusion protein GAL4/PPAR-γ and a DNA construct that harboured the gene reporter. The plasmid pGal5-TK-pGL3 was obtained by inserting five copies of the Gal4 (a yeast transcription factor) DNA-binding site upstream of the thymidine kinase promoter in pTK-pGL3.

The plasmid pGal4-human PPAR-γ was constructed by PCR amplification of the human PPAR-γ DEF domain (nuclear receptor Hinge region (D) + ligand binding domain (E) + C-terminal domain (F)) (aa 318–505). The resulting amplicons were cloned into pBD-Gal4 (Stratagene, La Jolla, CA, USA), and the chimera was subsequently subcloned into pCDNA3.

After transfection, the COS-7 cells were incubated for 24 h with RE to assess its capacity to activate PPAR-γ. Dimethyl sulfoxide was used as the reference control, and rosiglitazone was used as a positive control. The activation of PPAR-γ by RE

Table 1. Composition of the experimental low-fat diet (LFD), high-fat diet (HFD) or a HFD plus rosemary extract (RE) and macronutrient content (%)

	HFD				LFD				HFD-RE 0·5 % (500 mpk)			
	g	kJ	g/kg	kJ/kg	g	kJ	g/kg	kJ/kg	g	kJ	g/kg	kJ/kg
Compound									4·31	0	5·00	0
Casein 80 mesh	200	3349·4	233	3902·1	200	3349·4	190	3173·6	200	3349·4	231·89	3902·1
L-Cysteine	3	50·2	3	58·6	3	50·2	3	46·1	3	50·2	3·48	58·6
Maize starch	72·8	1219·2	85	1419·3	315	5275·4	299	4999·0	72·8	1219·2	84·41	1419·3
Maltodextrin 10	100	1674·7	117	1951·0	35	586·2	33	556·8	100	1674·7	115·95	1951·0
Sucrose	172·8	2893·9	201	3370·4	350	5861·5	332	5555·9	172·8	2893·9	200·36	3370·4
Cellulose BW200	50	0	58	0	50	0	47	0	50	0	57·97	0
Soyabean oil	25	942·0	29	1096·9	25	942·0	24	891·8	25	942·0	28·99	1096·9
Lard	177·5	6688·4	207	7795·8	20	753·6	19	715·9	177·5	6688·4	205·81	7795·8
Mineral mix	10	0	12	0	10	0	9	0	10	0	11·59	0
Calcium phosphate	13	0	15	0	13	0	12	0	13	0	15·07	0
Calcium carbonate	5·5	0	6	0	5·5	0	5	0	5·5	0	6·38	0
Potassium citrate	16·5	0	19	0	16·5	0	16	0	16·5	0	19·13	0
Vitamin mix	10	167·5	12	196·8	10	167·5	9	159·1	10	167·5	11·59	196·8
Choline bitartrate	2	0	2	0	2	0	2	0	2	0	2·32	0
Dye	0·05	0	0	0	0·05	0	0	0	0·05	0	0·06	0
Total	858·2	16985·4	1000·0	19791·0	1055·1	16985·8	1000·0	16098·2	862·5	16985·4	1000·0	19791·0
Protein (%)	23·66	20·02			19·24	20·01			23·66	20·02		
Carbohydrate (%)	40·27	34·08			66·35	69·02			40·27	34·08		
Fat (%)	23·60	44·92			4·27	9·98			23·60	44·92		

mpk, mg rosemary extract/kg body weight per d.
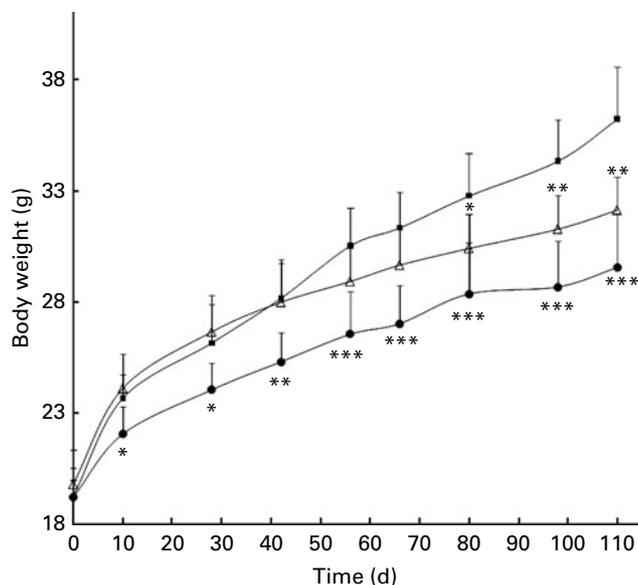
Fig. 1. Effects of rosemary extract (RE) standardised to 20 % carnosic acid on body weight after 16 weeks. Low-fat diet (■), high-fat diet (HFD, □) and HFD supplemented with RE (HFD-RE, ▣) at a concentration equivalent to 500 mg RE/kg body weight per d. Values are means, with standard deviations represented by vertical bars. Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

induced the expression of luciferase and a consequent increase in luminescence.

After 24 h, the cells were collected, and the luciferase assay was performed according to the manufacturer's instructions (SteadyGlow; Promega, Charbonnières, France). Luminescence was measured on a Tecan Ultra spectrophotometer (Tecan, Männedorf, Switzerland). All experiments were performed in quadruplicate. Relative luciferase activity of a sample was calculated as the ratio of mean luciferase activity in the test cells to that in the control cells, and PPAR-γ ligand-binding activity was expressed as the ratio of relative luciferase activity to that of the reference control.

Analysis of results

The animals were randomised based on total body weight by principal component analysis (GENFIT, Loos, France), resulting in groups of animals between which no statistical difference was observed for any parameter.

The data from the *in vivo* and *in vitro* studies are expressed as means and standard deviations. One-way ANOVA (one-way Bonferroni) and Student's *t* test were performed to compare groups using Sigma Plot 11.0 (2008) (Systat Software, Inc., Chicago, IL, USA). Statistical significance was considered at $P < 0.05$.

In the *in vivo* study, gains in the HFD-RE group are expressed as a percentage compared with those in the HFD and LFD control groups, which is calculated as:

$$Parameter\,(\%) = ((HFD - HFD{-}RE)/(HFD - LFD)) \times 100.$$

In the *in vitro* studies, changes are expressed relative to their respective controls.

Results

Effect of rosemary extract on body and organ weight and food intake in mice fed a high-fat diet

Body weight between the HFD and LFD groups began to differ significantly after the first week of treatment. In HFD-RE animals, body weight differed significantly after day 80 (Fig. 1) compared with that in HFD mice and weight gain peaked at 69 % ($P < 0.01$) at the end of the study (Table 2). This effect was associated with a 79 % ($P < 0.001$) less of an increase in epididymal fat mass; liver weight was unaffected by the treatment. No significant changes in food or energy intake were observed between the groups (Table 2).

Effects of rosemary extract on serum biochemical parameters

At 16 weeks of treatment, total fasting glycaemia, total cholesterol and NEFA levels rose significantly in the HFD group compared with the LFD animals. HFD-RE mice experienced 72 % ($P < 0.01$) less increase in plasma glucose levels and 68 % ($P < 0.001$) less an a rise in total cholesterol compared with HFD mice. No significant effects were observed in NEFA or TAG levels in HFD-RE mice compared with the HFD group (Table 3).

Fasting insulinaemia was also monitored; insulin levels remained low during the entire experiment, and no significant differences were observed between the groups (data not shown).

Table 2. Effects of chronic administration of rosemary extract (RE) standardised to 20 % carnosic acid on nutritional and weight parameters in mice fed a low-fat diet (LFD), a high-fat diet (HFD) or a HFD plus RE after 16 weeks

(Mean values and standard deviations)

Animal group	Food intake (g/animal per d)		Energy intake (kJ/animal per d)		Weight gain (g)		Liver weight (g)		Epididymal fat weight (g)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LFD	3·40	0·28	61·7	5·3	10·33***	2·18	0·99	0·10	0·45***	0·26
HFD	3·09	0·19	67·5	2·2	17·01	1·94	1·12	0·13	1·52	0·48
HFD-RE	3·01	0·10	62·8	1·3	12·37**	1·59	1·14	0·15	0·67***	0·15

Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): ** $P < 0.01$ and *** $P < 0.001$.

Table 3. Effects of chronic administration of rosemary extract (RE) standardised to 20 % carnosic acid on plasma lipid and glucose levels in mice fed a low-fat diet (LFD), a high-fat diet (HFD) or a HFD plus RE after 16 weeks

(Mean values and standard deviations)

Animal group	Total cholesterol (mg/l)		TAG (mg/l)		NEFA (mmol/l)		Glucose (mg/l)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LFD	1076·6***	48	891·4*	125·9	1·46	2·2	1286***	182·5
HFD	1475·8	136·3	1297·3	374·6	1·45	1·1	1978·1	492·6
HFD-RE	1205***	154·6	1021·1	256·8	1·47	2·8	1480**	113·3

Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Effect of rosemary extract on faecal fat excretion

HFD animals had higher faecal fat excretion values (7·63 (SD 1·27) mg/100 mg) compared with LFD mice (2·74 (SD 0·10) mg/100 mg, $P < 0.001$) after 16 weeks. Moreover, RE-treated mice experienced a significant 1·2-fold increase ($P < 0.01$) in total faecal content compared with HFD animals (Fig. 2).

Throughout the experiment, there was no significant difference in fat energy intake between the HFD and HFD-RE groups. However, faecal fat energy excretion rose 1·3-fold ($P < 0.05$) in RE-treated mice (1·13 (SD 0·16) kJ/animal per d) v. the HFD group (0·87 (SD 0·15) kJ/animal per d). Faecal fat energy excretion differed between the LFD group (0·32 (SD 0·01) kJ/animal per d, $P < 0.001$) and HFD mice (Fig. 3).

In vitro analysis of the mechanism of rosemary extract

As shown in Fig. 4, 100 μg/ml RE ($P < 0.001$) inhibited pancreatic lipase activity by 70 % compared with Orlistat. RE also activated PPAR-γ by 1·66-fold ($P < 0.001$) in a dose-dependent manner compared with the blank control at 30 μg/ml (Fig. 5), whereas activation in the positive control, rosiglitazone, was 4·35-fold ($P < 0.001$) that in the blank

control at 10 nm. Therefore, 30 μg/ml RE are able to activate PPAR-γ by 19·70 % compared with the positive control.

Discussion

The production of RE, estimated to exceed 100 tonnes annually, has risen considerably in recent years due to its widespread use in food, beverage, flavour, food supplements and cosmetic applications[11–15]. Consequently, technologies to develop standardised extracts from rosemary have evolved tremendously with regard to quality and reproducibility. Recently, we have demonstrated the importance of standardisation in determining the biological activity of plant extracts. Furthermore, depending on the content, we have observed that the antioxidant activities of various preparations of RE vary[15].

Rosemary exerts various biological activities with which preventive nutritional strategies against metabolic disorders, such as obesity, dyslipidaemia and diabetes, can be developed[19–23]. Nevertheless, individual studies have examined specific extracts, the chemical description of which is not always available. Thus, it can be difficult to extrapolate the health benefits of a unique RE to another solely on the basis of published data.
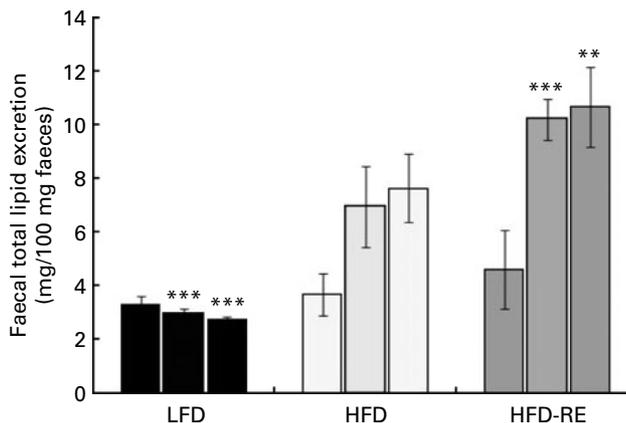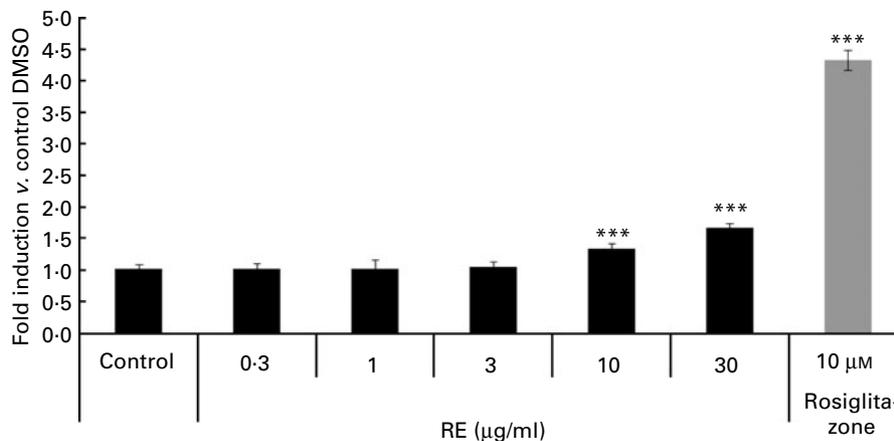

Fig. 2. Effects of rosemary extract (RE) standardised to 20 % carnosic acid on total faecal lipid content at weeks 0, 8 and 16. Low-fat diet (LFD), high-fat diet (HFD) and HFD supplemented with RE (HFD-RE) at a concentration equivalent to 500 mg RE/kg body weight per d. Values are means, with standard deviations represented by vertical bars of pooled data (n 6; except for LFD, n 3). Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): ** $P < 0.01$, *** $P < 0.001$.
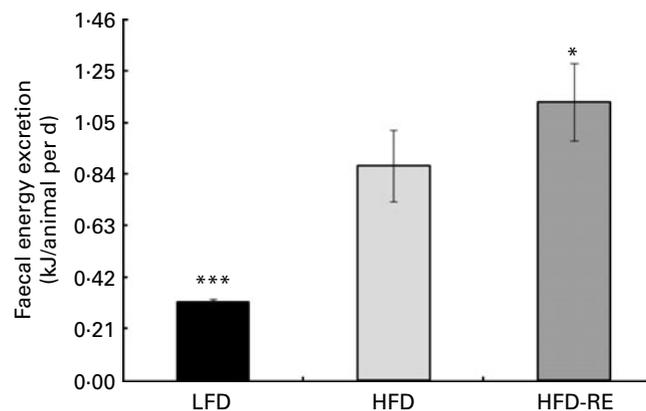
Fig. 3. Effects of rosemary extract (RE) standardised to 20 % carnosic acid on total faecal energy excretion over 16 weeks. Low-fat diet (LFD), high-fat diet (HFD) and HFD supplemented with RE (HFD-RE) at a concentration equivalent to 500 mg RE/kg body weight per d. Values are means, with standard deviations represented by vertical bars of pooled data (n 6; except for LFD, n 3). Mean values were significantly different from HFD control (Student's t test): * $P < 0.05$, *** $P < 0.001$.
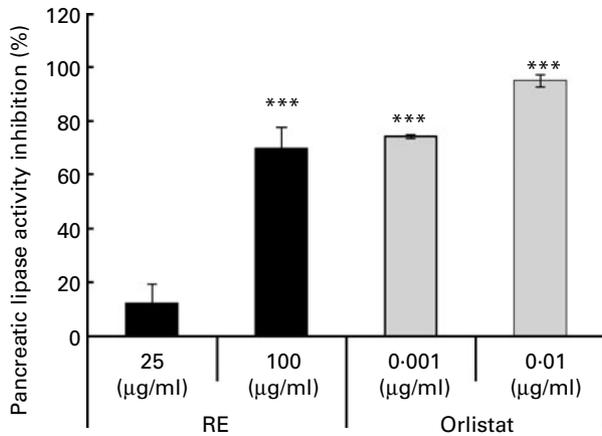
Fig. 4. Effects of rosemary extract (RE) standardised to 20 % carnosic acid on pancreatic lipase inhibition in vitro. Values are means, with standard deviations represented by vertical bars expressed as a percentage of inhibition of three independent experiments. Mean values were significantly different from the blank control (Student's t test): *** $P < 0.001$.

In a previous study, we identified RE as the most potent antioxidative extract in an ex vivo LDL oxidation model[15], prompting us to determine whether it affects other metabolic parameters in a HFD mouse model. In the present study, the administration of a 44·92 % fat diet to 6-week-old C57BL/6J mice for 16 weeks resulted in significant increases in body weight, epididymal fat mass, glycaemia and cholesterol, compared with a LFD, confirming previous reports[26,27].

Treatment of mice with 500 mpk of RE reduced the gains in weight that were induced by the HFD without affecting food intake or fat energy intake. It also lowered epididymal fat tissue weight significantly compared with HFD mice. In addition, total faecal lipid content increased in HFD-RE mice compared with the HFD group, which correlates with the amount of total faecal fat energy that was excreted. Based on the present study and other reports[19,22], limiting lipid absorption in the intestine is a potential mechanism by which RE prevents weight gain.

This hypothesis is strongly supported by evidence of the in vitro inhibitory effect of RE on pancreatic lipase activity, a key enzyme in the digestion and absorption of fat. Moreover, similar effects have recently been reported with an ethanolic extract of rosemary that contains rosmarinic acid, carnosol and carnosic acid, wherein the treatment of 15-week-old diet-induced obesity mice with 200 mpk of extract limited the weight gain that was induced by a 50 d HFD and increased the lipid faecal content by 2·2-fold. Ninomiya et al.[19] observed that after 2 weeks of treatment with 20 mpk of carnosic acid alone, the weight of ddY (Deutschland, Derker, Yoker) mice fell by 7·6 % compared with the control group. Thus, in the present study, the effects of RE on faecal fat excretion and, consequently, faecal fat energy excretion partially explain the observed reductions in body weight.

In addition to its effects on physiological measures, RE significantly reduced elevated cholesterol levels that were induced by the HFD. Although these effects were not observed by Harach et al.[22] or Ninomiya et al.[19], they were observed in human subjects with Orlistat[28–30] and in animal models with other plant-based pancreatic lipase inhibitors[31,32]. Dietary cholesterol absorption has been proposed to be associated with fat digestion; Young & Hui[33] have shown that minimal TAG hydrolysis is sufficient to increase cholesterol transport significantly from lipid emulsions to intestinal cells. Consequently, pancreatic lipase inhibition has been proposed to be a target against which lipid malabsorption can be triggered to control TAG and cholesterol levels[33].

In the present study, fasting glycaemia was reduced in animals in the HFD-RE group compared with the HFD control group. Few studies have evaluated the effect of rosemary on diabetes. Anti-hyperglycaemic effects can be induced by an ethanolic RE in the alloxan diabetic rat model[34] and by a water RE in a mouse model[24], whereas no such effect has been observed in the diet-induced obesity mouse model with an extract that contains rosmarinic acid, carnosol and carnosic acid[22]. Based on these data, it appears that the

Fig. 5. Effects of rosemary extract (RE) standardised to 20 % carnosic acid on PPAR-γ activation in vitro. Values are means, with standard deviations represented by vertical bars expressed as a percentage of inhibition of three independent experiments. Mean values were significantly different from the blank control (Student's t test): *** $P < 0.001$. DMSO, dimethyl sulfoxide.

anti-hyperglycaemic activity and preventive effects of an extract against type 2 diabetes mellitus depend on its composition and the animal model in which it is tested.

Recently, it has been reported that the glucose-lowering effect of rosemary is attributed to PPAR-γ activation, in which carnosic acid and carnosol were proposed be the active compounds[20]. Therefore, we examined the in vitro effects of RE on PPAR-γ activation, hypothesising that the glucose-lowering effects are mediated through this mechanism.

In the present study on C57BL/6J mice, we used an effective dose of RE – 500 mpk – that contains 100 mpk of carnosic acid. The European Food Safety Authority Panel on Food Additives has estimated the dietary exposure for adults and pre-school children (aged 1·5–4·5 years) to carnosol plus carnosic acid to be 0·04 and 0·11 mpk, respectively[13]. Thus, considering the normal dietary exposure of carnosic acid, we used a pharmacological dose of RE.

In addition, the Panel also notes that the margin between the not observable adverse effect level of carnosol plus carnosic acid, as calculated in 90 d rat studies, is equivalent to 20–60 mpk, and the mean intake of carnosic acid-rich rosemary extracts is estimated to be 500–1500 mg/d in adults and 182–546 mg/d in pre-school children[13]. Therefore, future randomised clinical trials that aim to confirm the efficacy of RE in humans should consider these values to establish an effective and safe dose.

In conclusion, we have demonstrated that a carnosic-standardised RE limits weight gain and improves plasma lipid and glucose levels in a HFD mouse model. These data confirm its potential for use in preventive strategies against metabolic disorders and encourage the initiation of further studies to recapitulate the physiological activity of RE in human subjects.

Acknowledgements

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