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Plasma Glucose Lowering Effect of the Wild *Satureja khuzestanica* Jamzad Essential Oil in Diabetic Rats: Role of Decreased Gluconeogenesis

^{1,3}R. Shahsavari, ¹A. Ehsani-Zonouz, ²M. Houshmand, ¹A. Salehnia, ²G. Ahangari and ¹M. Firoozrai

¹Department of Biochemistry, School of Medicine and Cellular and Molecular Research Center, Iran University of Medical Sciences, Hemmat Highway, Tehran, Iran

²Department of Clinical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

³Razi Herbal Medicine Research Center, Lorestan University of Medical Sciences, Khoramabad, Iran

Abstract: This study was to evaluate the effect of the wild SKEO on activities and genes expression of hepatic Glycogen Phosphorylase (GP) and phosphoenolpyruvate carboxykinase (PEPCK) in normal and diabetic rats. The wild SKEO was orally administered at different doses (50 and 100 mg/kg/day) to normal as well as diabetic rats for 21 days. The levels of mRNA were determined using the quantitative real-time RT-PCR technique. The plasma glucose concentrations of diabetic rats receiving SKEO (100 mg kg⁻¹) compared with diabetic control were significantly decreased. Hepatic GP activity and its mRNA levels of diabetic rats treated with SKEO moderately increased. The activity of hepatic PEPCK and its mRNA levels were significantly decreased in normal rats treated with SKEO (100 mg kg⁻¹). The enhancement of PEPCK activity and its mRNA levels of diabetic treated rats with SKEO (100 mg kg⁻¹) was significantly decreased compared with diabetic control. In conclusion, an excessive inhibition of PEPCK in liver of diabetic rats treated with the wild SKEO may contribute to the plasma glucose lowering action of SKEO that seems to be in relation with antioxidant properties of SKEO.

Key words: *Satureja khuzestanica* Jamzad, medicinal plant, antidiabetic, gene expression, glycoregulatory enzyme, diabetic rats

INTRODUCTION

Satureja khuzestanica Jamzad is an endemic annual plant that is distributed in the southern of Lorestan Province (Jamzad, 1994). This plant has been employed as analgesic and antiseptic in the southern parts of Iran (Amanlou *et al.*, 2005). *Satureja khuzestanica* essential oil (SKEO) is reported to have a wide range of effects: it is anti-inflammatory and anti nociceptive (Amanlou *et al.*, 2005), antioxidant and antihyperlipidemic (Abdollahi *et al.*, 2003) and antimicrobial (Amanlou *et al.*, 2004). SKEO also is reported to have a beneficial effect on male rat fertility (Haeri *et al.*, 2006), on stimulatory of reproduction and on lowering the blood glucose levels in diabetic rats (Abdollahi *et al.*, 2003). It has been reported that SKEO prolongs the prothrombin time and activated partial thromboplastin time (Nazari *et al.*, 2005).

In earlier reports, the main components of the wild SKEO were carvacrol (93.9%), eugenol (1.0%), ρ -cymene (0.8%) and thymol (0.6%) (Farsam *et al.*, 2003). Carvacrol as the main component of the wild SKEO has been found to have significant antioxidant properties (Abdollahi *et al.*, 2003).

The liver plays an important role in maintaining blood glucose homeostasis by controlling hepatic glucose production via glycogenolysis and gluconeogenesis (Cherrington, 1999). Glycogen phosphorylase (GP) is a key regulatory enzyme that catalyzes the breakdown of glycogen to glucose-1-phosphate (Agius, 2007). Phosphoenolpyruvate Carboxykinase (PEPCK) catalyzes one of the rate-limiting steps of gluconeogenesis, the reaction of oxaloacetic acid to phosphoenolpyruvate (Aiston *et al.*, 2003). In earlier studies Streptozotocin (STZ)-induced diabetic rats exposed to SKEO have shown significantly decreased levels of plasma glucose (Abdollahi *et al.*, 2003). Normal treated rats with SKEO have also shown significantly decreased PEPCK activity and increased GP activity in the liver (Saadat *et al.*, 2004). However, it is unknown whether SKEO affects activities of these enzymes in STZ-induced diabetic rats. The present study was undertaken to evaluate the effect of the wild SKEO on activities and genes expression of hepatic GP and PEPCK in normal and STZ-induced diabetic rats. The plasma levels of glucose and insulin were also measured.

MATERIALS AND METHODS

Essential oil preparation: *Satureja khuzestanica* Jamzad, family of Lamiaceae, was collected from southern of Lorestan Province in Iran at flowering stage during September 2006. The plant has been identified by the Department of Botany of Research Institute of Forests and Rangelands in Tehran. A voucher specimen (No. 58416) has been deposited at the TARI herbarium. Aerial parts of the *Satureja khuzestanica* Jamzad were chopped, air-dried, powdered and then hydrodistilled using a Clevenger type apparatus for 5 h, giving yellow oil in a 2.8% yield. The oil was dried over anhydrous sodium sulfate and stored at 4°C (Sefidkon and Ahmadi, 2000).

Animals: Male albino rats of the wistar strain, with initial weights between 180 and 230 g, were made diabetic by STZ injection. Diabetes was induced by STZ (Sigma-Aldrich Inc., USA), which was a single administration intravenously in 0.1 mol L⁻¹ cold citrate buffer, pH 4.5, at a dose of 45 mg kg⁻¹ b.wt. After 96 h, whole blood glucose >18 mmol L⁻¹ and also exhibited glycosuria confirmed that these animals could be considered diabetic which used for further experimentation. The animals were randomly divided into six groups, consisting of six animals in each group as follows: group I: Normal control rats, group II: Diabetic control rats, group III and IV Normal rats that received the wild SKEO 50 and 100 mg/kg/day, respectively; group V and VI: Diabetic rats that received the wild SKEO 50 and 100 mg/kg/day, respectively, in aqueous solution orally for 21 days. At the end of the experimental period, in fed state, animals were sacrificed by decapitation between 8 and 10 a.m. The plasma was separated for glucose and insulin plasma levels determination. Liver was rapidly removed, sliced into very small pieces, washed with cold saline, frozen in liquid nitrogen and finally stored at -80°C until use.

Measurement of circulatory glucose and Insulin levels: Plasma glucose concentration was measured by the glucose oxidase method (Parsazmun Co., Tehran, Iran) and insulin was assayed using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden).

Measurement of PEPCK and GP assay: For both of the enzymes, portions of liver tissue were homogenized in 4 volumes of cold 20 mM Tris-HCl (pH 7.4) buffer, containing 1 mM EDTA for PEPCK and or containing 4 mM EDTA, 0.5 mM dithiothreitol for GP, using polytron homogenizer (Kinematica AG, Littau, Switzerland) for 45 sec pulsely. This crude homogenate was centrifuged for 60 min at 105,000 x g at 4°C, using Beckman ultracentrifuge type 90 Ti rotor (Palo Alto, CA, USA). The crude supernatant was used for enzyme assays. PEPCK activity was determined as earlier described by Petrescu *et al.* (1979). Glycogen phosphorylase activity was assayed spectrometrically using the crude supernatant by modification a previously described method (Aiston and Agius, 1999). AMP at 5 mM concentration was added instead of caffeine to the reaction mixture. Total protein concentration were determined by the method of Lowry *et al.* (1951). Enzyme activities are expressed as nmoles of substrate converted by 1 mg hepatic supernatant protein per minute.

RNA extraction and cDNA synthesis: Total RNA was extracted from each rat liver using high pure RNA tissue kit (Roche Diagnostics, Mannheim, Germany). The absorption of ratio 260/280 nm all of preparations ranged between 1.8 and 2.0 and RNA integrity was assessed by gel electrophoresis using an agarose-ethidium bromide gel. Total RNA (about 5 µg) was reverse transcribed using a transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Manneim, Germany) according to the manufacturer's instructions.

Real time RT-PCR: Real-time PCR was performed with a Lightcycler 2.0 system (Roche Applied science Indianapolis, USA) using lightcycler faststart DNA master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). The sequences of the specific primers for hepatic GP, PEPCK and β-actin (as an internal standard) gene expression are shown in Table 1. Samples were incubated in the Lightcycler apparatus for a predenaturation at 95°C for 10 min, followed by 45 PCR cycles. Each amplification cycle was 95°C for 10 sec, primer Tm (Table 1) for 15 sec and 72°C for 20 sec.

Table 1: Sequences of primers for GP, PEPCK and β-actin gene expression

Gene name	Genbank accession	Primer sequences	Amplicon size (bp)	Temperature (°C)
GP	NM-022268	Forward: 5'-CCCGAGCACCCAATGACTTTAACC -3' Reverse: 5'- GCGAGTGC GGATGTGTGTCA -3'	298	66
PEPCK	NM-198780	Forward: 5'-GTCACCATCACTTCCTGGAAGA -3' Reverse: 5'-GGTGCAGAATCGCGAGTTG-3'	84	55
β-actin	NM-031144	Forward: 5'-ATGGATGACGATATCGCTGC-3' Reverse: 5'-CTTCTGACCCATACCCACCA-3'	150	57

Table 2: Effect of oral administration of the wild SKEO on the plasma levels of glucose and insulin for 21 days

Groups	Plasma glucose (mM)	Plasma insulin ($\mu\text{g L}^{-1}$)
Normal control	8.29 \pm 0.99	0.99 \pm 0.460
Normal+50 mg kg ⁻¹ wild SKEO	7.72 \pm 0.33	0.67 \pm 0.160
Normal+100 mg kg ⁻¹ wild SKEO	7.18 \pm 0.51	0.63 \pm 0.150
Diabetic control	22.31 \pm 2.5*	0.22 \pm 0.047*
Diabetic+50 mg kg ⁻¹ wild SKEO	20.22 \pm 0.95	0.24 \pm 0.055*
Diabetic+100 mg kg ⁻¹ wild SKEO	15.59 \pm 3.3***	0.33 \pm 0.075*

Values (Means \pm SEM) were obtained from each group of six rats. *p<0.001 compare to normal control and **p<0.001 compare to diabetic control group

The third segment consisted of a melting curve analysis and finally, a cooling program cooled the reaction mixture to 40°C. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each PCR. Product length and PCR specificity were checked further by 2% agarose gel electrophoresis and ethidium bromide staining. The relative quantity was calculated from a standard curve for each gene generated from diluted normal cDNA samples. Relative expression mRNA levels were normalized against β -actin.

Statistical analysis: To detect changes in variable between control groups and treated groups, we performed a one-way ANOVA followed by post-hoc multiple comparisons by Tukey's test. Bivariable correlations between enzyme activities and their mRNA levels in experimental groups were performed using Pearson correlation (r). A p \leq 0.05 was considered as statically significant.

RESULTS AND DISCUSSION

Effect of the wild SKEO on glucose and insulin plasma levels: The plasma glucose concentrations of STZ-induced diabetic rats were significantly increased compared with normal control rats while the levels of insulin decreased significantly (p<0.001). The plasma glucose concentrations of STZ-diabetic rats receiving SKEO (100 mg kg⁻¹) compared with diabetic control rats were significantly decreased (p<0.001). Although the plasma glucose concentration in diabetic rats treated with SKEO (100 mg kg⁻¹) was still markedly higher than normal control rats (p<0.001). The plasma insulin also was not significantly modified in diabetic rats treated with SKEO compared with diabetic control rats (p>0.05). Similarly, the plasma levels of glucose and insulin of normal rats treated with SKEO were not significantly altered compared with the normal control rats (p>0.05). Hence, plasma glucose lowering action of the

wild SKEO was only observed in STZ-diabetic rats as a hyperglycemia state (Table 2).

Effect of the wild SKEO on hepatic PEPCK activity and its mRNA levels: Activity of the hepatic PEPCK and its levels of mRNA in STZ-induced diabetic rats were significantly increased compared with normal control rats (p<0.001) (Fig. 1a, 2a). It has been suggested that increased gluconeogenesis is a main source of increased hepatic glucose production in diabetes (Agius, 2007).

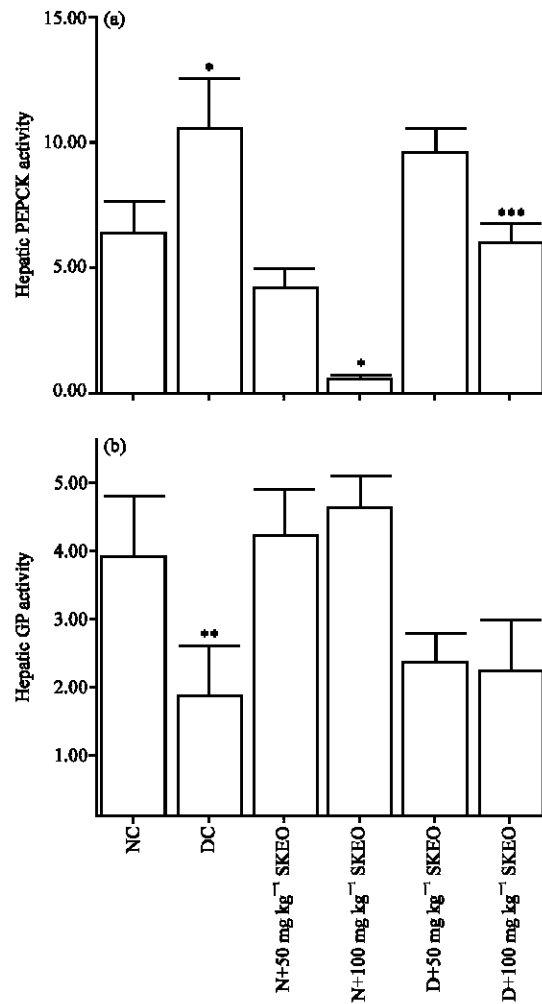


Fig. 1: Effect of oral administration of the wild SKEO on hepatic activity of the, (a) PEPCK and (b) GP for 21 days. NC: Normal Control; DC: Diabetic Control; N: Normal; D: Diabetic. Values (Mean \pm SEM) were obtained each group of six rats. *p<0.001 and **p<0.05 compare to normal control and ***p<0.001 compare to diabetic control. Enzyme activity is expressed as nmol/mg protein/min

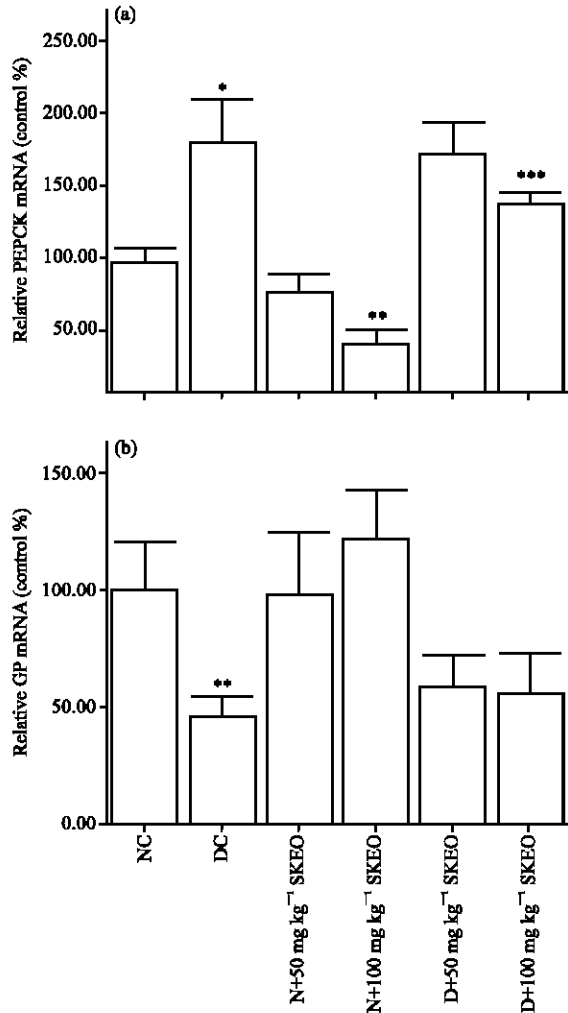


Fig. 2: Effect of oral administration of the wild SKEO on hepatic mRNA levels of the, (a) PEPCK and (b) GP for 21 days. NC: Normal Control; DC: Diabetic Control; N: Normal; D: Diabetic. Values (Mean±SEM) were obtained from each group of six rats. *p<0.001 and **p<0.05 compare to normal control and ***p<0.05 compare to diabetic control

Hepatic PEPCK activity and its mRNA levels in normal and diabetic rats treated with SKEO (100 mg kg⁻¹) were significantly reduced compared with normal control and diabetic control rats, respectively (p<0.05). Diabetic and normal rats treated with SKEO (50 mg kg⁻¹) in hepatic PEPCK activity were not significantly reduced compared with diabetic control and normal control groups of rats, respectively (p>0.05). The earlier reports indicated that hyperglycemia results in generation of reactive oxygen species (ROS) ultimately leading to increased oxidative stress (Ceriello, 2000). This elevation of oxidative stress

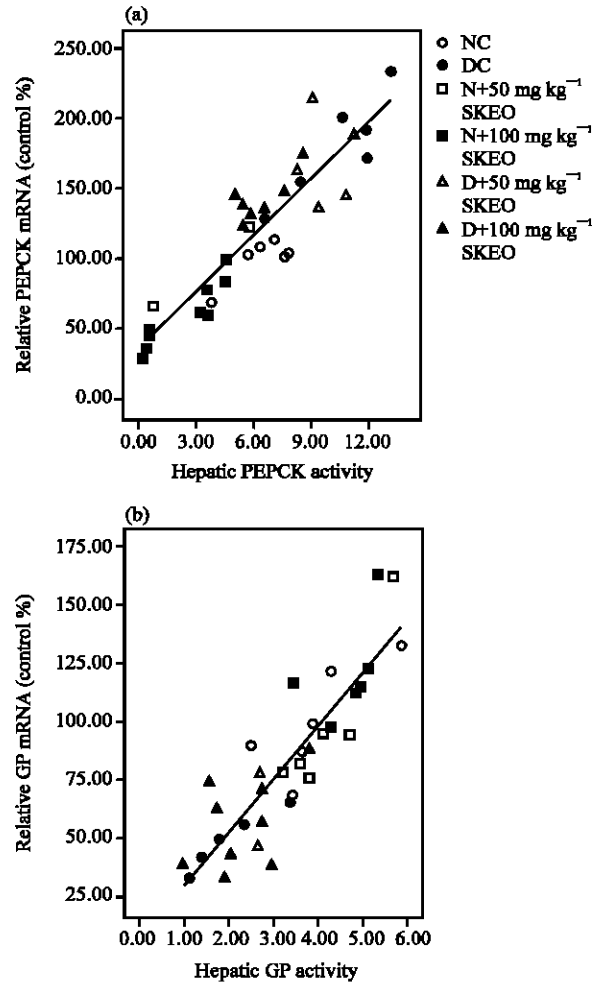


Fig. 3: Correlation between the enzyme mRNA levels and its activity of rat livers. (a) Correlations are significant at p<0.01 with r = 0.914 for PEPCK and (b) Correlations are significant at p<0.01 with r = 0.883 For GP

is involved in the up-regulation of gene expression of PEPCK via activating the stress-activated signaling pathways (Waltner-Law *et al.*, 2000). The high correlation between rates of activity and mRNA levels of PEPCK (Fig. 3a) supports a fundamental role for the wild SKEO in promoting inactivation of PEPCK synthesis through suppression of the factors involving the stress-activated signaling pathways that is mediated ROS. The main components of essential oil are rich in phenolic compounds (such as carvacrol, p-cymene and tymol) and phenylpropan compounds (such as eugenol) which were isolated as antioxidant components from the wild *Satureja kuzestanica* Jamzed (Farsam *et al.*, 2003). It has been reported by Kaneto *et al.* (1999) that the flavonoids

acts synergistically as antioxidant. In this context, several antidiabetic plants have been reported to have the ability of decreasing PEPCK activity through their antioxidant properties (Govorko *et al.*, 2007; Zang *et al.*, 2006).

Effect of the wild SKEO on hepatic GP activity and its mRNA levels: Activity of the hepatic GP and its levels of mRNA in STZ-induced diabetic rats were significantly decreased compared with normal control rats ($p < 0.05$). Diabetic rats treated with SKEO showed a 11 and 22% elevation in hepatic GP activity and its mRNA levels compared with diabetic control rats, respectively. Hepatic GP activity and its mRNA levels in normal rats treated with SKEO were associated with a mean of 21% elevation compared with normal group of rats (Fig. 1b, 2b). Although these differences are not significant ($p > 0.05$). As shown in Fig. 3a, the increase in GP mRNA levels in normal and diabetic treated rats liver occurred in parallel to the increase in GP activity ($r = 0.883$ at $p < 0.01$) (Fig. 3b). The levels of insulin negligibly increased in STZ-diabetic rats treated with SKEO compared with diabetic control group. Hence, the chronic effect of insulin on the activation of the GP activity that is mediated through the stabilization of its mRNA levels was observed (Venkata-Rao *et al.*, 1995).

Results demonstrate that a reduction in hepatic glucose output by significant decreased PEPCK levels seem to be slightly compensated by stimulation of moderate elevation in hepatic GP. In conclusion, results indicate that an excessive inhibition of PEPCK in liver of STZ-induced diabetic rats treated with SKEO may contribute to the plasma glucose lowering action of SKEO, through decreased gluconeogenesis, that seems to be in relation with antioxidant properties of SKEO.

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