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Stimulation of Hepatic Glycogenolysis and Inhibition of Gluconeogenesis are the Mechanisms of Antidiabetic Effect of *Centaurea bruguierana* ssp. *belangerana*

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ABSTRACT

The aqueous, dichloromethane, ethyl acetate and methanol extracts of dried aerial fruiting parts of *Centaurea bruguierana* ssp. *belangerana* were investigated for hypoglycemic mechanism in diabetic rats. Diabetes was induced by intravenous administration of streptozotocin-alloxan. The methanol and ethyl acetate extracts were administered in a single effective dose of 200 mg kg⁻¹ and dichloromethane and aqueous extracts were administered in a single effective dose of 400 mg kg⁻¹. Blood glucose was determined every 1 h until 3 h post administration of the extracts. In the second experiment, the liver was surgically removed 3 h post treatment of diabetic rats with various extracts, homogenized and used for measurement of key enzymes of glycogenolysis (glycogen phosphorylase, GP) and gluconeogenesis (phosphoenolpyruvate carboxykinase, PEPCK). Treatment by dichloromethane, ethyl acetate, methanol and aqueous extracts and the glibenclamide, reduced blood glucose to 41.7, 55.0, 45.7, 29.5 and 34.5%, respectively. The aqueous extract showed the best effect in reduction of hepatic PEPCK activity (84.0%) and increased hepatic GP activity (134.5%), while glibenclamide showed 62.5 and 133.0% activity, respectively. None of the extracts affected blood insulin. Presence of sugar in dried aqueous extract could suppress the hypoglycemic effect during the first hour of the experiment. After 1 h, the hepatic mechanism overwhelmed and thus lowering effect in blood glucose appeared. The conclusion is that *C. bruguierana* ssp. *belangerana* is able to lower blood glucose via stimulation of hepatic glycogenolysis and inhibition of gluconeogenesis.

Key words: *Centaurea bruguierana* ssp. *belangerana*, diabetes, glucose, glycogenolysis, glycogen phosphorylase, gluconeogenesis, phosphoenolpyruvate carboxykinase

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and alterations in carbohydrate, lipid and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and/or insulin action. It is estimated that more than 220 million people worldwide have diabetes and this number is likely to more than double by 2030 (WHO, 2011). The high prevalence of diabetes as well as its long-term complications has led to an ongoing search for hypoglycemic agents from natural sources and some good sources have been introduced (Hasani-Ranjbar *et al.*, 2008, 2009a, 2011) in the recent years.

The genus *Centaurea* L. (Asteraceae, tribe Cardueae, subtribe Centaureinae) comprises 600 species widely distributed from Asia, Europe and tropical Africa to North America as aggressively invading weeds (Celik *et al.*, 2006). This genus consists of 88 species in the Flora Iranica. *Centaurea bruguierana* (DC.) Hand.-Mzt. ssp. *belangerana* (DC.) Bornm. (CBB) (sect. Tetramorphaea), a 5-50 cm annual herb with purple spiny flowers, is distributed in Iran, Transcaucasia, Afghanistan, Pakistan and Central Asia (Rechinger, 1980). During our expeditions in Iran, we found that the decoction of dried aerial fruiting parts of this plant, namely Baad-Avard in Bushehr province, south of Iran, is traditionally used as a hypoglycemic remedy in diabetes. Many species of the genus *Centaurea* have long been used in traditional medicine to cure various ailments, e.g., diabetes, diarrhea, rheumatism, malaria and used against coughs, as liver-strengthening, itch-eliminating and ophthalmic remedies (Flamini *et al.*, 2002; Shoeb *et al.*, 2006). Also a variety of secondary metabolites have been reported from different species of this genus including sesquiterpene lactones (Celik *et al.*, 2006; Marco *et al.*, 2005; Robles *et al.*, 1997; Yesilada *et al.*, 2004), flavonoids (Flamini *et al.*, 2002; Shoeb *et al.*, 2006, 2005; Akkal *et al.*, 2003; Flamini *et al.*, 2001), lignans (Celik *et al.*, 2006; Shoeb *et al.*, 2006; Masso, 1980) and alkaloids (Shoeb *et al.*, 2005, 2006).

During our bibliographical survey, we found several papers on hypoglycemic activity of *Centaurea* species. Aqueous extracts (20 g kg⁻¹) of aerial parts of *Centaurea aspera*, *Centaurea calcitrapa*, *Centaurea melitensis* and *Centaurea solstitialis* elicited hypoglycemic effects in normal mice (Masso, 1980). Infusion of an active dose of 5 g kg⁻¹ of leaves and flowers of *Centaurea corubionensis* lowered blood glucose by 16-19% and increased circulating insulin by 27-50% in normoglycemic and glucose-induced hyperglycemic rats, but had no effect on alloxan-induced diabetic animals (Chucua *et al.*, 1988). Villar and Paya (1985) also reported the antidiabetic activity of *Centaurea seridis* var. *maritima*. However, *C. damascene* did not show any hypoglycemic activity in alloxan-induced diabetes in rats treated with 0.5, 1 or 2 g kg⁻¹ of the lyophilized extract (Ali *et al.*, 1997).

To our knowledge, only two studies have been conducted on the isolation of chemical constituents of *C. bruguierana* in literature comprising two sesquiterpene lactones, cnicin (a germacranolide), dehydromelitensin-8-acetate (an elemanolide) (Rustaiyan *et al.*, 1982) and flavonoids (Harras *et al.*, 1994). By now, two papers on antiplasmodial and anti-peptic ulcer effects of this species are available to date (Khanavi *et al.*, 2011a, b). Regarding flavonoids and other antioxidant components in this herb, the beneficial antidiabetic effect (Rahimi *et al.*, 2005) especially through liver metabolism (Hoseini *et al.*, 2006) is expected. As part of our ongoing screening of native Iranian plants for anti-diabetic activity, in this study we describe for the first time the mechanisms by which CBB controls Streptozotocin (STZ)/alloxan-induced diabetes.

MATERIALS AND METHODS

Plant materials: The aerial fruiting parts of CBB were collected from Borazjan, Bushehr Province, Southern Iran in July 2008 and identified by Prof. Gholamreza Amin, Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences (TUMS), where voucher specimen is deposited (6683-TEH).

Extraction: Dried aerial fruiting samples (200 g in two series for aqueous and organic solvents extraction, respectively) were extracted with distilled water (3×48 h, 600 mL each), Dichloromethane (DCM), Ethyl acetate (EtOAc) and Methanol (MeOH) (3×48 h, 600 mL each) in a percolator at room temperature. The combined extracts were concentrated to dryness under reduced pressure at 40°C to give aqueous extract (27.8 g), DCM extract (2.3 g), EtOAc extract (0.4 g) and MeOH extract (17.3 g). Freeze-dryer was used for final dryness of the aqueous extract. Analytical grade solvents were purchased from Merck (Darmstadt, Germany).

Animals: Wistar male rats (200-250 g) from animal house of TUMS were acclimatized to animal room conditions (25°C in a 12 h light-dark cycle) for 3 days and maintained to standard pellet diet and water *ad libitum*. The food was withdrawn 24 h before the experiment, but water was accessible. To avoid coprophagy, the rats were fasted in wire-bottomed cages. For each group, six rats were used. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals as well as the rules of Pharmaceutical Sciences Research Center ethics committee.

Preparation of test samples for bioassay: Single effective doses of MeOH and EtOAc extracts (200 mg kg⁻¹) and aqueous and DCM extracts (400 mg kg⁻¹) were obtained according to the results from a pilot study. Extracts were dissolved in distilled water and administered orally (test groups). Sodium Carboxymethyl Cellulose (Na-CMC) was used for suspending of DCM and EtOAc extracts in distilled water (0.5%). The control group animals received the same experimental handling as those of the test groups except that the extract treatment was replaced by administration of appropriate volumes of the dosing vehicle. Glibenclamide was dissolved in distilled water and used orally (5 mg kg⁻¹) as anti-diabetic drug (reference group). One group of animals was left as normal non-diabetic (sham group).

Diabetes induced by STZ-alloxan: Acute diabetes was induced in rats with a single intravenous administration of STZ-alloxan at a dose of 40 mg kg⁻¹ of each in 0.05 M citrate buffer (pH 4.5). Three days after injection of STZ-alloxan, diabetes was confirmed if a 90-100% increase in fasting blood glucose level of controls (70-80 mg dL⁻¹) or higher was observed in treated animals, meaning a range of 135 dL⁻¹ or higher (Black *et al.*, 1980).

Evaluation of hypoglycemic activity: The first experiment was done to evaluate blood glucose changes in diabetic control and extract-treated animals. Blood glucose measurement was performed by a glucometer from the mouse tail vein at 15 min and 1, 2 and 3 h after administration of the extracts to determine the peak level of glucose changes. In the second experiment that was designed to examine hepatic changes of key enzyme in glucose metabolism, diabetic animals were anesthetized 3 h post administration of the extracts and the liver was removed by transverse

abdominal incision and perfused with cold 0.9% normal saline and kept frozen at -70°C until homogenized. Blood samples were taken under anesthesia by cardiac puncture for further analyses of insulin concentration.

Hepatic cells PEPCK assay: The isolated liver was cut with scissors and homogenized at 4°C for 2 min with 3 volumes of cold 0.1 M mixture of phosphate buffer, pH 7.4, containing a 1 M mixture of KH_2PO_4 and K_2HPO_4 . The homogenate was centrifuged for 30 min at 14000 g at 0°C and the supernatant was used for the enzyme activity assay. Enzymatic activity was assayed at 25°C in the reverse direction (carboxylation of phosphoenolpyruvate to form oxaloacetic acid) in the presence of NADH in a coupled reaction catalyzed by glucose-6-phosphate dehydrogenase as described previously (Saadat *et al.*, 2004). The activity of PEPCK is expressed as unit per gram of liver protein.

Hepatic cells GP assay: The liver homogenate was centrifuged at 14000 g for 30 min and the supernatant was used for the enzyme activity assay. Liver homogenate was centrifuged at 14000 g for 30 min and the supernatant was used for the enzyme activity assay. Enzymatic activity was assayed in the direction of glycogen breakdown by measuring the reduction of NADP in a coupled reaction catalyzed by glucose-6-phosphate dehydrogenase. GP activity is expressed as unit per gram of liver protein (Saadat *et al.*, 2004).

Hepatic cells protein assay: The liver homogenate was centrifuged at 14000 g for 30 min and the supernatant was used for determination of protein concentration by use of Bradford reagent and bovine plasma albumin as standard.

Insulin assay: Rat ELISA insulin kit was purchased from DRG International Inc. (USA) and used for determination of blood insulin.

Glucose assay: Glucose levels were measured by a glucometer from Roche Accu-Chek Active (Germany).

Statistical analysis: The results were analyzed for statistical significance by one-way ANOVA and Tukey *post hoc* multi-comparison tests. All data were expressed as Mean \pm SEM of six animals in each group. Differences between groups with p-values less than 0.05 were considered statistically significant.

RESULTS

Blood glucose: Blood glucose was measured at 15 min, 1, 2 and 3 h after administration of the extracts at doses of 200 mg kg^{-1} for MeOH and EtOAc extracts and of 400 mg kg^{-1} for DCM and aqueous extracts to diabetic rats. The results showed that decrease in blood glucose concentration were 41.7, 55.0, 45.7 and 29.5% of the control for DCM, EtOAc, MeOH and aqueous extracts and 34.5% of the control for glibenclamide as reference drug at 3 h after oral administration. Glucose concentration started to decrease at 15 min and became steady at 3 h post administration of the extracts except than aqueous extract (Fig. 1).

GP activity: Administration of DCM, EtOAc, MeOH and aqueous extracts at defined doses increased GP activity in hepatic cells by 119.0, 121.0, 51.5 and 134.5% of the control and 133% for the glibenclamide at $p < 0.05$ (Fig. 2).

PEPCK activity: DCM, EtOAc, MeOH and aqueous extracts diminished PEPCK activity in hepatic cells by 69.5, 46.2, 73.9 and 84.0%, respectively while glibenclamide decreased the activity by 62.5%. All results were significantly different from control at $p < 0.05$ (Fig. 3).

Blood insulin: Treatment by the extracts did not change blood insulin level in diabetic rats when compared to controls ($p > 0.05$) while glibenclamide significantly increased insulin by 2.5 times of control (Fig. 4).

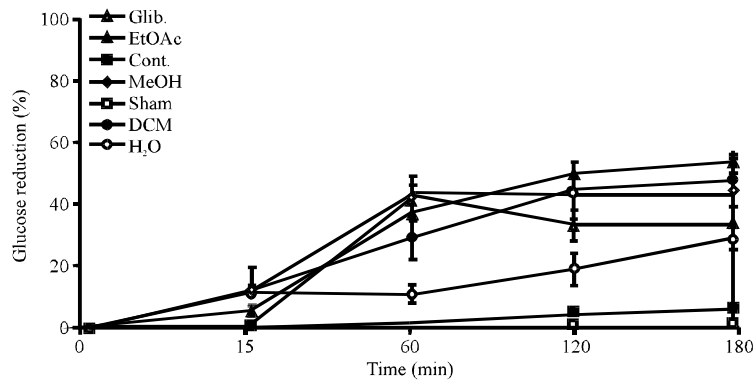


Fig. 1: Effects of different extracts of *C. bruguierana* ssp. *belangerana* on blood glucose reduction. Data are Mean±SEM of six animals per group and reported as percentage of reduction of control. All data are significantly different from control at $p < 0.05$ at 1st, 2nd and 3rd h post administration of the extracts (The aqueous extract showed significant difference from control at $p < 0.05$ only at 3rd h)

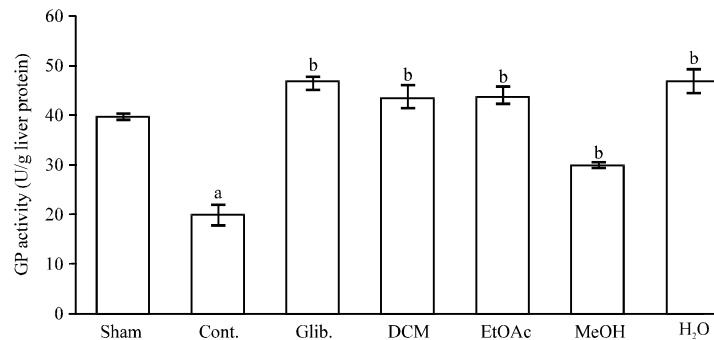


Fig. 2: Effects of different extracts of *C. bruguierana* ssp. *belangerana* on hepatic GP activity in diabetic rats. Data are Mean±SEM of six animals per group. ^{a,b}Significantly different from sham and control at $p < 0.05$, respectively

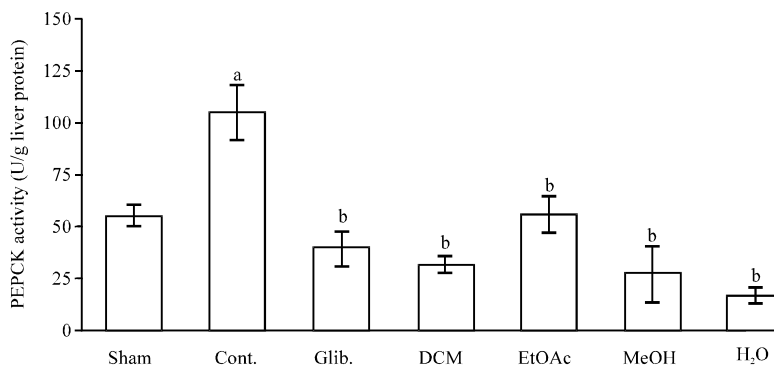


Fig. 3: Effects of different extracts of *C. bruguierana* ssp. *belangerana* on hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity. Data are Mean±SEM of six animals per group. ^{a,b}Significantly different from sham and control at p<0.05, respectively

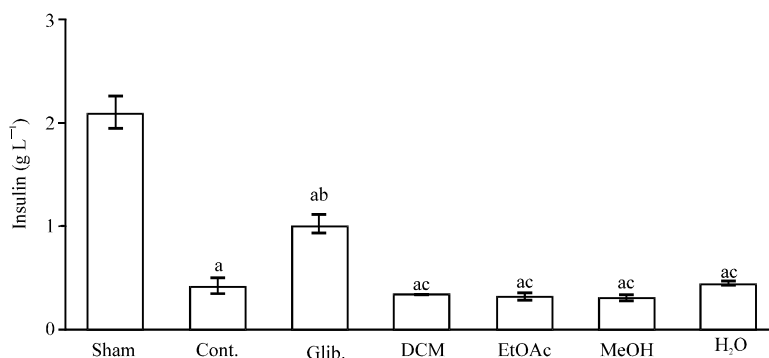


Fig. 4: Effects of different extracts of *C. bruguierana* ssp. *belangerana* on blood insulin. The results are Mean±SEM of six animals per group, ^{a,b,c}Significantly different from sham, control and glibenclamide at p<0.05, respectively

DISCUSSION

The dried aerial fruiting parts of *Centaurea bruguierana* ssp. *belangerana* (CBB) has been used as a decoction for hypoglycemic activity in diabetes in folk medicine of Borazjan, Bushehr Province in southern Iran. The present study was carried out to investigate the claimed ethnobotanical hypoglycemic activity of the CBB and possible mechanism in STZ/alloxan-induced diabetic rats. Our results confirmed that oral administration of the CBB various extracts could lower blood glucose in diabetic rats. However, the extracts did not show any statistically significant difference in their activity.

Treatment by the extracts, except than aqueous one significantly decreased blood glucose nearly equal to that of glibenclamide at 1 h post administration which increased during next 2 h, while glibenclamide effect started decreasing. The hypoglycemic effect reached steady state starting from the 2nd h and was likely to continue after 3rd h (Fig. 1). However, the hypoglycemic effect of the aqueous extract increased significantly after 1 h and continued increasing even after 3 h. The results obtained from hepatic enzymes assay elicited that the aqueous extract is most

potent reducer of hepatic PEPCK activity (84% Glib. 62.5%) and increaser of hepatic GP activity (134.5%, Glib. 133%) in comparison to controls, 3 h post administration. None of the extracts showed any effect on blood insulin. However, glibenclamide showed significant increase in insulin by 2.5 times of the control that is in agreement with previous knowledge (Serrano-Martin *et al.*, 2006).

According to our findings, CBB showed its hypoglycemic activity by increasing hepatic glycogenolysis via stimulating GP activity and decreasing hepatic gluconeogenesis with inhibition of PEPCK. We can assume that the hypoglycemic activity of CBB is similar to that of metformin an oral hypoglycemic drug used in type 2 diabetes, which reveals its hypoglycemic mechanism of action primarily by suppressing hepatic gluconeogenesis through activating AMP-activated protein kinase (AMPK) (Kajbaf *et al.*, 2007). Data obtained from hepatic enzymes assays showed that the aqueous extract was the most prominent compared to other groups. While the results from glucose reduction assay did not match the observed hepatic mechanism. According to Fig. 1, the glucose reduction percentage of all groups reached steady state after 2 h, while the aqueous extract showed the trend of increasing of hypoglycemic activity seeming to continue if tested even after 3 h. The reason for this observation could be the presence of sugar in dried aqueous extract which could suppress the hypoglycemic effect during the first hour of the experiment. After 1 h, the hepatic mechanism overwhelms and thus lowering effect in blood glucose appears.

Diabetes is the disease of the century and the current belief is that obesity (Hasani-Ranjbar *et al.*, 2009b) and pollution of the environment (Mostafalou and Abdollahi, 2012) progress incidence and complications of diabetes. The best scenario as hypothesized recently is to combine natural antioxidants (Hosseini and Abdollahi, 2012) to provide a strong antioxidant with strong antidiabetic effects like IMOD (Mohammadirad *et al.*, 2011) or to get other beneficial effects like anti-hyperlipidemia (Momtaz and Abdollahi, 2010) that are usually associated with diabetes. Since the results of this study interestingly confirmed the ethnobotanical use of CBB as a long-term treatment of diabetes, the chronic antidiabetic investigation of the aqueous extract or acute treatment with sugar-free aqueous extract and finally the isolation and structure elucidation of the active compounds would be of interest.

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