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Chamaemelum nobile L. Aqueous Extract Represses Endogenous Glucose Production and Improves Insulin Sensitivity in Streptozotocin-induced Diabetic Mice

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Abstract: The present study was undertaken in order to evaluate the possible mechanisms of action involved in the hypoglycaemic activity of the aqueous extract of *Chamaemelum nobile* (CN) (20 mg kg^{-1}). This study was carried out in Multi Low-Dose Streptozotocin-induced (MLDS) diabetic mice. Hypoglycaemic effect of CN was studied after both single and repeated oral administration of CN aqueous extract (20 mg kg^{-1}). Endogenous glucose production was estimated using primed-continuous 3-^{3}H glucose infusion technique. While, euglycemic hyperinsulinemic glucose clamp technique was used to assess peripheral insulin sensitivity. Both single and repeated oral administration (s) of aqueous CN extract evoked a prominent hypoglycaemic activity in MLDS diabetic mice. In other hand, 3-^{3}H glucose infusion demonstrated that this hypoglycaemic activity was accompanied by a decrease in basal endogenous glucose production (EGP). EGP was lower in CS-treated group when compared to the control group, 15.5±0.5 vs 27.2±7.1 mg kg^{-1} min^{-1} ($p<0.001$) respectively. While, the metabolic clearance rate of glucose remains unchanged. In addition, we have demonstrated that CN treatment also improves insulin sensitivity in peripheral tissues, suggested by the observed higher levels of the glucose infusion rate. We can conclude that inhibition of basal endogenous glucose production and amelioration of insulin sensitivity in peripheral tissues account for the hypoglycaemic activity of aqueous CN extract in MLDS diabetic mice.

Key words: Endogenous glucose production, insulin sensitivity, streptozotocin, euglycemic hyperinsulinemic clamp, mice

INTRODUCTION

Diabetes mellitus is a complex metabolic disorder that is increasing tremendously all over the world. By 2030, the total number of people worldwide suffering from diabetes mellitus is projected to reach 366 millions[1]. Insulin resistance and excessive hepatic glucose production are two major pathophysiological abnormalities tightly associated with a cluster of diseases including diabetes mellitus, hypertension, dyslipidemia and central obesity[2-4]. Insulin resistance is defined as the inability of a known quantity of insulin to suppress hepatic glucose production and to promote glucose utilization in peripheral tissues, especially skeletal muscle and adipose tissue[5]. This insulin-resistant state seems to be due to defaults at insulin receptor and post-receptor levels[6-7]. Currently available therapeutic options for the treatment of diabetes mellitus include: dietary modification, the use of insulin or oral hypoglycaemic drugs (insulin secretagogues, insulin sensitizers and α-glucosidase inhibitors). Thiazolinediones, are the only available drugs that reduce insulin resistance in peripheral tissues by either mimicking or enhancing insulin action without affecting β-cells insulin secreting capacity[8]. Although these therapies can control many aspects of diabetes, numerous complications are common incidents of the disease and the mortality index due to this illness continue to increase.

Plants have been used to treat diabetes mellitus since the ancient times. *Chamaemelum nobile* (CN) (Asteraceae) locally known as "Babounge", a native shrub widely distributed throughout Morocco, is among the medicinal plants used in Moroccan folk medicine to treat a large variety of diseases including diabetes and hypertension[9]. Previously, we have reported that both single and oral repeated administration, for two weeks, of CN aerial parts aqueous extract exhibit a prominent hypoglycaemic activity in both normal and streptozotocin-induced diabetic rats[10]. The hypoglycaemic activity of CN has been attributed to the presence of Chamaemeloside, a HMG-containing

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flavonoid glucoside[11]. However, the precise cellular and biochemical mechanisms underlying this pharmacological activity remain to be elucidated.

Studies dealing with the mode of action of potential hypoglycaemic plants will confer scientific and systematic approach for the use of these plants as hypoglycaemic agents. The present study was undertaken in order to investigate the hypoglycaemic activity of the oral administration of the aqueous extract of CN aerial part and to investigate the possible beneficial effect the intravenous infusion of CN extract on some pathophysiological abnormalities associated with diabetes mellitus in multi-low streptozotocin-induced (MLDS) diabetic mice, an animal model of human type 1 diabetes[12]. In order to accomplish this goal, the effect of CN intravenous administration on basal glucose production was studied using primed-continuous 3-^{3}H glucose infusion[13] while insulin sensitivity was evaluated using euglycemic hyperglycaemic glucose clamp technique, the gold standard for assessing insulin resistance and insulin sensitivity[14].

MATERIALS AND METHODS

Plant: Plants material of *chamaemelum nobile* (Asteraceae) were collected from the Tafilalet region (semi-arid area) of Morocco in May-June 2004 and air-dried at 40°C. The plant was previously identified and authenticated by Pr. M. Rejdali (Agronomy and Veterinary Institute, Rabat) and a voucher specimen (ME 35)[9] was deposited at the herbarium of the Faculty of Sciences and Techniques Errachidia.

Preparation of the aqueous extract: Plant material was prepared according to the traditional method used in Morocco (decoction): 1 g of powdered fruits mixed with 100 mL distilled water were boiled for 10 min and then cooled for 15 min. Thereafter, the aqueous extract was filtered using a Millipore filter (Millipore 0.2 mm, St Quentin en Yvelines, France) to remove particulate matter. The filtrate was then freeze-dried and the desired dose (mg of lyophilized aqueous extract of CN aerial parts per kg body weight) was then prepared and reconstituted in 1.5 mL of distilled water. The extracts obtained were then given orally to different groups of mice at a dose of 20 mg kg^{-1} body weight. The dose of 20 mg kg^{-1} was used according to the Moroccan traditional phytotherapy.

Experimental animals: All experiments were performed in eleven-week-old male C57BL/6J mice (Janvier, Le Genest Saint Isle, France) weighing 30±5 g. Mice were housed in a controlled environment (inverted 12-h daylight cycle, lights off at 10:00 A.M.) with free access to food and water in groups of five mice per cage at 22°C. All animal experimental procedures have been approved by the local ethical committee of the Rangueil hospital (Toulouse, France).

Induction of diabetes: Diabetes was induced in adult fasted male C57BL/6J mice by repeated intraperitoneal injection of streptozotocin (80 mg kg^{-1}) for four consecutive days. Streptozotocin (Sigma, St Louis, Mo, USA) was dissolved in 0.1 M fresh cold citrate buffer at pH 4.5 before use. One week after the start of injections, only mice with blood glucose levels higher than 16 mM were considered as diabetic and then included in this study.

Acute and chronic oral treatment: Normal and diabetic mice was randomly assigned to two groups (n = 6 in each group). The control group received distilled water and the treated group received aqueous extract of CS at a dose of 20 mg kg^{-1} B.W. The CN aerial parts aqueous extract or vehicle were administered orally by gastric intubation using a syringe once daily at 10 h a.m. The hypoglycaemic effect was evaluated in fasted mice 1, 2, 4 and 6 hours after a single oral administration and after 4, 7 and 15 days of once daily repeated oral administration.

Surgery: In another set of experiment and in order to perform intravenous perfusions, a catheter was implanted under anaesthesia into the femoral vein of male C57BL/6J mice. The other extremity of the catheter was tunnelled under the skin of the back, exteriorized and secured in place at the back of the neck. The mice were then allowed to recover from the surgery in individual cages.

Endogenous glucose production: Diabetic mice were divided randomly to two groups, control group received continuous perfusion of physiological saline solution (NaCl 0.9%) and experimental group received continuous perfusion of CN aqueous extract (20 mg kg^{-1}) at a rate of 2 μL min^{-1} kg^{-1} body weight. The mice were fasted for six hours prior to the infusions. They were connected to the infusion apparatus two hours prior to the start of the infusions with free access to water. In order to determine the rate of endogenous glucose production in mice at post-absorptive state, 3-^{3}H-glucose (Perkin Elmer, Boston, MA) was infused continuously at a dose of 30 μCi kg^{-1} min^{-1} to ensure a detectable plasma D-(3H)^3-glucose enrichment. Plasma glucose and 3-^{3}H-

glucose were determined in 5 μL of blood sampled from the tip of the tail vein each 20 min following the 3-^{3}H-glucose or saline infusion.

Euglycemic-hyperinsulinemic glucose clamp: The animals were allowed to recover from surgery for four days. Insulin sensitivity was assessed by using the euglycemic-hyperinsulinemic clamp as described previously[15]. Briefly, 6 h-fasted mice were divided randomly in two groups (n = 6 for each group). The control group was continuously infused with insulin at a rate of 4 mU kg^{-1} min^{-1} (physiological) and saline solution (2 μL min^{-1}) for three hours. In addition to insulin infusion, the experimental group was infused with CN aqueous extract (20 mg kg^{-1}) at a rate of 2 μL min^{-1}. Throughout the infusion, blood glucose level was assessed from blood samples (5 μL) collected from the tip of the tail vein when needed using a blood glucose meter (Roche Diagnostic, Meylan, France). Euglycemia was maintained at 5 mM by periodically adjusting a variable infusion of 16.5% glucose. Plasma glucose concentrations were determined in 5 μL of blood sampled from the tip of the tail vein every 10 min during the last hour of the infusion.

Calculations: 3-^{3}H-glucose enrichment was determined from the total blood after deproteinization which was performed as follows: 5 μL of venous blood were mixed with 250 μL of 0.3 M $Ba(OH)_2$ was added to precipitate the proteins and blood cells. The $Zn(OH)_2$ precipitate formed was spun down. An aliquot of the supernatant was evaporated to dryness to determine the radioactivity corresponding to 3-^{3}H-glucose. Another aliquot was directly mixed with the scintillation buffer to determine the radioactivity corresponding to 3-^{3}H-glucose. Thereafter, the difference between the first and the second aliquot corresponds to ^{3}H produced. In the third aliquot of the same supernatant, glucose concentration was assessed by the glucose oxidase method (Trinder, St Louis, Mo). Calculations were made with parameters collected during the last 60 min of the infusions when a steady-state 3-^{3}H-glucose enrichment was obtained. Glucose turnover was calculated by dividing the 3-^{3}H-glucose infusion rate by the plasma glucose specific activity. A steady specific activity with variations smaller than 15% was obtained during the last hour of the infusion. Mice showing larger variations in the specific activity were excluded from the study.

The total amount of the infused glucose was considered to be taken up by the body tissues and, under these steady-state conditions of euglycemia and hyperinsulinemia, glucose input was equal to glucose utilization. The glucose infusion rate (GIR) was calculated every 10 min during the clamp study. Metabolic clearance rate of glucose (MCR, mg kg^{-1} min^{-1}) was then obtained from GIR divided by the corresponding blood glucose concentration.

Statistical analysis: All the data reported are expressed as mean±SEM. Statistical analysis was performed using the Student's *t*-test. The values were considered to be significantly different when the P-value was less than 0.05 compared to baseline or control values.

RESULTS

Blood glucose levels, single oral administration: Figure 1 depicts the blood lowering effect of a single oral administration of the aqueous extract of CS fruits (20 mg kg^{-1}) in MLDS diabetic mice. CN treatment was accompanied with an important decrease of blood glucose levels which dropped from 19.41±2.02 to 13.57±1.54 and 9.10±1.41 mM two ($p< 0.01$) and six ($p<0.01$) hours after treatment respectively (Fig. 1).

Blood glucose levels; repeated oral administration: The effect of once daily repeated administration of aqueous CN extract (20 mg kg^{-1}) in MLDS diabetic mice was shown in Fig. 2. In diabetic mice treated with CN extract, blood glucose levels were decreased significantly by 39%. It dropped from 19.41±1.61-11.42±0.29 mM at the end of treatment ($p<0.01$) (Fig. 2).

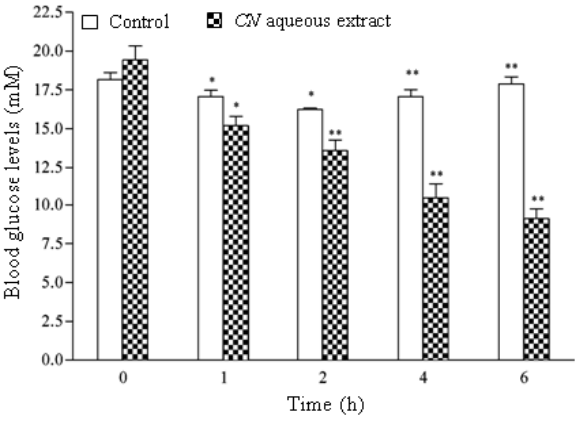

Fig. 1: Plasma glucose levels (mM) over 6 h after single oral administration of CN aqueous extract (20 mg kg^{-1}) in diabetic mice. Data are expressed as means ± SEM, n = 6 mice per group. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ when compared to baseline values (0 h)

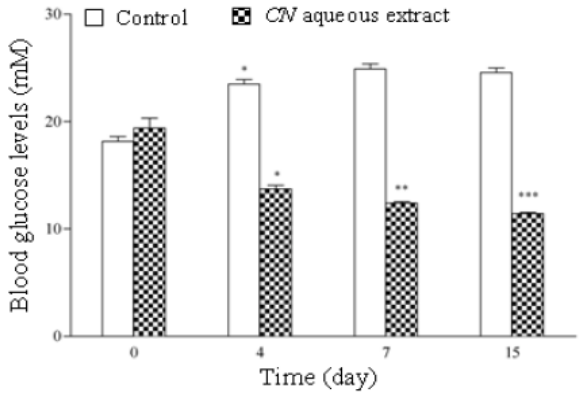

Fig. 2: Plasma glucose levels (mM) over once daily repeated oral administration of CN aqueous extract (20 mg kg^{-1}) for 15 days in diabetic mice. Data are expressed as means±SEM, n = 6 mice per group. *: p<0.05; **: p<0.01 when compared to baseline values (J0)

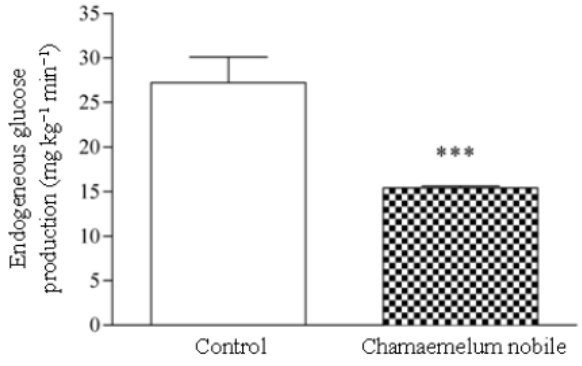

Fig. 3: Endogenous glucose production (mg kg^{-1} min^{-1}) after the CN aqueous extract infusion (20 mg kg^{-1}) in diabetic mice. Data are expressed as means±SEM, n = 6 mice per group. ***: p<0.001 when compared to control group

Endogenous glucose production: In MLDS mice, aqueous CN extract infusion at a dose of 20 mg kg^{-1} produced a strong hypoglycaemic effect. Blood glucose levels decrease from 18.83±0.44 to 5.44±0.37 mM three hours after CN infusion (p<0.001) (Table 1). Parallel to the potent decrease in blood glucose levels, CN infusion (20 mg kg^{-1}) produced a strong decrease in endogenous glucose production (EGP). At the end of the infusion period, EGP values were significantly lower in CS treated group when compared to the control one, 15.4±0.5 *vs* 27.2±7.1 mg kg^{-1} min^{-1} respectively (p<0.001) (Fig. 3).

Table 1: Blood glucose levels (mM) and metabolic clearance rate of glucose (EGP) (mg kg^{-1} min^{-1}) after the CN aqueous extract infusion (20 mg kg^{-1}) in diabetic mice. Data are expressed as means±SEM For each group n = 6 mice

Experimental groups	Blood glucose levels (mM) 0h	3h	Metabolic clearance rate of glucose (mg kg^{-1} min^{-1})
Control	18.44±1.33	9.83±1.63	0.0270±0.007
CN treated	16.12±1.13	5.44±0.37***	0.0250±0.003^{NS}

***: p<0.001 when compared to baseline values (0 h). NS: No significant difference when compared to control group

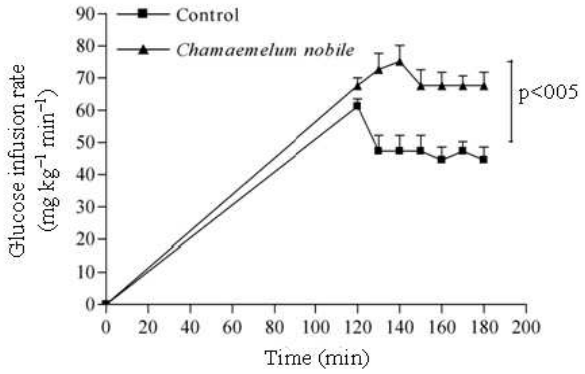

Fig. 4: Glucose infusion rate (mg kg^{-1} min^{-1}) after CN aqueous extract infusion (20 mg kg^{-1}) in diabetic mice. Data are expressed as means±SEM, n= 6 mice per group

Metabolic clearance rate of glucose: There are no significant differences in basal Metabolic Clearance Rate (MCR) of glucose between CN treated diabetic group and control group (Table 1).

Euglycemic hyperinsulinemic clamp: Figure 4 shows the time-course of Glucose Infusion Rate (GIR) in CS-treated and control diabetic groups. The total amount of the infused glucose was considered to be taken up by the body tissues and, under these steady-state conditions of euglycemia and hyperinsulinemia, glucose input was equal to glucose utilization. Because a plateau GIR was achieved during the last 30 min of the clamp procedure, GIR was used as an indicator of whole body glucose utilization and reflects the whole body insulin sensitivity. It was significantly higher in CN treated group then in control groups, 80.72±3.47 *vs* 67.68.55±10.06 mg kg^{-1} min^{-1} respectively (p<0.05) (Fig. 4).

DISCUSSION

The present study was designed to investigate the hypoglycaemic activity of the aqueous extract of

Chamaemelum nobile (CN) and to evaluate the possible beneficial effect of CN infusion on hepatic glucose production and whole body insulin sensitivity in multi-low dose streptozotocin-induced diabetic mice. Multiple injections of low doses of streptozotocin are known to affect β-cells, eliciting a subsequent immune destruction with macrophage and lymphocyte islet infiltration, leading to β-cell lysis[16]. This animal model mimics some basic aspects of recent-onset type 1 diabetes in human patients[12]. Our results clearly demonstrate that both single and repeated oral administration of aqueous CN extract were accompanied by an important decrease in blood glucose levels in MLDS diabetic mice. This finding is with concordance with our previous work which has demonstrated the same prominent hypoglycaemic activity in both streptozotocin-induced diabetic rats and normal rats[10]. Although, the cellular and biochemical mechanisms underlying this pharmacological activity remain unknown. In order to elucidate the possible mechanism of CN hypoglycaemic effect, we have analysed the effect of aqueous CN extract perfusion in MLDS diabetic mice. The effects of this perfusion on blood glucose levels, basal endogenous glucose production and insulin sensitivity were studied in conscious and unrestrained diabetic mice.

Our results clearly demonstrated that continuous infusion of CN extract for three hours lowered both blood glucose levels and endogenous glucose production, whereas the metabolic clearance rate of glucose remains unchanged. Previously, we have reported that inhibition of endogenous glucose production accounts for the hypoglycaemic activity of *Spergularia purpurea* aqueous extract in streptozotocin-induced mice[17]. The liver plays a strategic role in the control of glucose homeostasis. Hepatic glucose production is determined by the rate of hepatic glycogen breakdown, which is regulated by glucose-6 phosphatase and by the rate of hepatic gluconeogenesis, which is regulated by phosphoenolpyruvate carboxykinase. Excessive hepatic glucose production is characteristic of untreated or poorly controlled diabetes mellitus[18]. It has been suggested that insulinopenic state is associated with basal glucose overproduction and that increased gluconeogenesis is the main source of hepatic glucose overproduction[19-20]. It seems to be a result of the lack of insulin repressing activity on the two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase[21]. CN may exert its hypoglycaemic effect by repressing the activities of these key enzymes. Such metformin-like effects have been previously reported to be related to the hypoglycaemic activity of several medicinal plants and herbal preparations[22-26]. However, it is well known that depletion in hepatic and muscular glycogen content is one of main metabolic features of diabetes mellitus[27]. So, it is not excluded that CN may also prevent decrease in glycogen content especially via the activation of rate-limiting glycogenic enzyme, glycogen synthase leading to diminution in the total hepatic glucose output. A similar effect was reported by[23] to explain the hypoglycaemic of aqueous extract of *Pterocarpus marsupium* in diabetic rats.

Using the euglycemic hyperinsulinemic glucose clamp technique we have confirmed that aqueous CN extract infusion improved the whole body insulin sensitivity in MLDS diabetic mice. Streptozotocin-induced diabetes leads to the early apparition of insulin resistance in hepatic and peripheral tissues[28-29]. During the insulin infusion rate (4 mU kg^{-1} min^{-1}) used in this study, insulin levels of about 100 μU mL^{-1} were achieved. At high physiological insulin levels, changes in GIR are thought to be caused mainly by changes in the insulin receptor binding. Although we did not measure the effect of insulin on hepatic glucose production (HGP), according to Tominaga et al., HGP can be completely suppressed under an insulin infusion rate of 3 mU kg^{-1} min^{-1}[30]. Therefore, the GIR is essentially synonymous with the rate of the total body glucose utilization[31]. Consequently, the results of the present study suggest that CN administration could improve insulin sensitivity in peripheral tissues. Many herbal preparations and medicinal plants have been reported to possess similar insulin sensitizing activities both in experimental study and clinical investigations, *Indigofera mysorensis*[32], *Gymnema sylvestre*[30], *Panax ginseng*[33-34], *Trigonella foenum-graecum*[35], *Sanguis draxonis*[36] and *Acanthopanax senticosus*[37], Gasha-jinki-gan[38-40] and dietary Guar gum[41]. Improvement of peripheral insulin sensitivity may be a consequence of the stimulation of insulin signalling pathway[39,42]. A similar mechanism has been reported to explain the insulin sensitizing activity of cinnamon and fenugreek extracts[35,39,43]. After three weeks of cinnamon treatment (300 mg kg^{-1}), the skeletal muscle insulin-stimulated IR-β and the IRS-1 tyrosine phosphorylation levels were 18 and 33% higher in treated rats[42]. However, the hypoglycaemic activity of fenugreek seed extract was mediated through the stimulation of an insulin signalling pathway especially in adipocytes and liver cells[35]. Stimulation of insulin signalling pathway in insulin sensitive tissues (liver, muscle and adipose tissues) promotes glucose

utilization and leads to decrease blood glucose levels. It is well known that the antidiabetic thiazolidinediones reduce the insulin resistance of peripheral tissues and that this insulin sensitizing property is mediated through a subfamily of nuclear receptors, peroxisome proliferator activated receptor gamma (PPAR-γ)[43]. PPAR-γ receptors are found in key target tissues for insulin action, such as adipose tissue, skeletal muscle and liver and evidence indicates that these receptors are important regulators of adipocyte differentiation, lipid homeostasis and insulin action[44]. The reported hypoglycaemic activity of CN extract could be mediated by a beneficial effect on PPAR-γ signalling network. A similar mechanism has been demonstrated in diabetic animals treated with *Punica granatum*[45] and *Pterocarpus marsupium*[46] extracts. However, the precise molecular mechanism underlying this insulin sensitizing property of CN aqueous extract needs further experimental investigations to be demonstrated.

In summary, this is the first report that CN treatment decreases endogenous glucose production and improves insulin sensitivity in peripheral tissues, two major pathophysiological abnormalities associated with diabetes mellitus, in multi-low dose streptozotocin-induced diabetic mice. Our results support overall *in vivo* anti-hyperglycaemic activity of *Chamaemelum nobile* extract.

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