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In Vitro α-Amylase/α-Glucosidase Inhibitory Activities and *In Vivo* Improving Glucose Tolerance and Hypoglycemic Effect of *Ceratonia siliqua* Leaves Aqueous Extract

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Abstract

Ceratonia siliqua (Fabaceae) is a plant used by traditional medicine as a remedy for the diabetes treatment. This study aims to evaluate the hypoglycaemic, anti-hyperglycemic and the anti-diabetic activities of an aqueous extract of carob leaves (CSLAE). The experiments were carried out on normal and diabetic rats model. These latter revealed that oral administration of the extract at the dose of 500 mg kg^{-1} resulted a considerable decrease in blood glucose ($P < 0.05$) after three hours of treatment. Administration of CSLAE at graded doses (100, 250 and 500 mg/kg^{-1} *b.w*) significantly produced an anti-hyperglycemic effect after glucose administration at the dose of 2 g kg^{-1}. These results also showed that oral administration of the same dose decreased blood glucose levels by 72.72% ($p < 0.05$) in diabetic rats, 15 days after treatment. Furthermore, CSLAE exhibited potent inhibitory activities against α-glucosidase and α-amylase. Thanks to its richness bioactive compounds, the extract showed its complexation with glucose *in vitro*. These results suggested that food preparations obtained with *C. siliqua* leaves might contribute to modulate the digestion of carbohydrates in humans.

Keywords: *Ceratonia siliqua Leaves; Anti-Hyperglycemic Effect; Blood Glucose; α-Glucosidase; α-Amylase; Rat*

Introduction

For a long time, the diabetes has been considered as pathology of the rich countries, but currently, it has been shown that this pathophysiology exists also in the developed countries [1]. The diabetes mellitus type 1 resulting from selective and autoimmune destruction of pancreatic β cells. While, the diabetes mellitus type 2 is distinguished by insulin resistance or lack of secretion of this hormone. There are also other specific types, such as gestational diabetes and impaired glucose homeostasis [2].

Glucosidases are a category of digestive enzymes which reduce the dietary carbohydrates into simple sugars that are quickly absorbed by the small intestine. Glucosidase inhibitors like acarbose diminish the rate of carbohydrate digestion and delay the carbohydrate absorption from the digestive tract. Therefore, they have a potential to prevent the development of type 2 diabetes mellitus by lowering the post-prandial glucose levels [3].

Anti-hyperglycaemic herbal medicine is currently enjoying remarkable progress due to the discovery of a huge number of plant extracts antidiabetic. The use of plants is a usual practice in the world. Today more than 800 plants have been identified and studied as a potential treatment for type II diabetes. The essential active ingredients isolated and recognized as antidiabetic are the secondary metabolites (flavonoids, mucilages, glycans, triterpenoid, alkaloids, saponosides and tannins) [4].

The carob tree or *Ceratonia siliqua* is an evergreen sclerophyllous tree, which can reach 7 to 20m in height and a circumference at the base of the trunk of 2 to 3m. It is evergreen, drought tolerant but not very cold tolerant. The leaves are oval in shape, dark green and shiny on top and reddish beneath and have a petiole 10 - 20 cm long. They are alternately pinnate and have 2 to 5 pairs of leathery leaflets, oval and entire, slightly indented at the apex and paripinnate [5]. Leaves are used in folk medicine as antidiarrheal, diuretic pharmaceutical and cosmetic agents and chemical investigation involved the presence of tannins and polyphenols [6].

Numerous investigations have shown also that the use of leaves associated with polyethylene glycol (PEG) improves the digestibility and nutritional quality of the tannins contained in the leaves [7].

In this study, our purpose is to evaluate the effects of aqueous extract of carob leaves on glucose level and hyperglycemia in rats and on both α-amylase and α-glucosidase activities *in vitro*.

Materials and Methods

Plant material

Ceratonia siliqua leaves were harvested from the region of Menzel-bourguiba (Northwestern of Tunisia) during the month of June 2014.

Leaves aqueous extract preparation

Carob leaves were dried in the oven at a temperature of 50 ± 5°C for 72 hours. Then, the dry matter was thoroughly milled in an electric mixer. Powder of leaves was dissolved in bi-distilled water (1/10; w/v) and incubated at room temperature for 24h in a shaking bath. The sample was filtered through a colander (0.5 mm mesh size). Finally, the lyophilized CSLAE (extraction yield = 10%) was immediately used for in our experiments.

Animals

The experiments were carried out on male Wistar rats, whose body weight varies between 180 and 210g. These animals were placed in polycarbonate cages at a rate of 6 per cage and maintained under standard animal facility conditions [Brightness (12h light/12h darkness), temperature (22 ± 2°C) and relative humidity (60 - 70%)]. They are fed a standard diet in the form of corks from the industrial society of Badr-Utique, Tunisia. Water and food are provided at will throughout the duration of treatment.

Treatment of rats

Evolution of blood glucose level in normal rats

Fasted rats were divided into four groups consisting of six animals in each group. The hypoglycemic effect of the aqueous extract of carob leaves was tested for 3 hours. The rats received different doses of the aqueous extract at the beginning of the experiment (0 minute). Blood samples are taken at the level of the tail vein for 3 hours (0 minute, 30 minutes, 60 minutes, 120 minute and 180 minutes). Glucose is measured using a glucometer (Accu-Chek, Roche, QC, Canada) [8].

Oral glucose tolerance test (TTOG)

Twenty-four rats were divided into four groups (six rats in each group) and were kept fasting overnight. 30 minutes before the administration of glucose (2 g kg^{-1} b.w.), the rats received three doses of the extract (100, 250 and 500 mg kg^{-1} b.w.) by oral route. Blood samples were collected from rats by tail vein puncture at regular times: 0 minute, 30 minutes, 60 minutes, 120 minutes and 180 minutes [8].

Antidiabetic effect of the aqueous extract

36 rats were divided in to six equal groups as follows:

(i) Control group: Rats of this group received by gastric gavage a normal saline (0.5 ml/100g body weight per rat).

(ii) Diabetic group: Rats were made diabetic by a single intraperitoneal injection of alloxane (150 mg kg^{-1}, body weight per rat).

(iii) Carob leaves supplement group: The diabetic rats were forcefully fed with aqueous extract at the dose of 100 mg kg^{-1} body weight per rat per day for 15 days by gavage.

(iv) Carob leaves supplement group: The diabetic rats were forcefully fed with aqueous extract at the dose of 250 mg kg^{-1} body weight per rat per day for 15 days by gavage administration.

(v) Carob leaves supplement group: The diabetic rats were forcefully fed with aqueous extract at the dose of 500 mg kg^{-1} body weight per rat per day for 15 days by gavage administration.

(vi) Reference molecule supplement group: The diabetic rats were forcefully fed with glibenclamide at the dose of (10 mg kg^{-1}) body weight per rat per day for 15 days by gavage administration.

In vitro glucose complexation: Glycosylation test

The purpose of this test is to estimate the ability of CSLAE containing flavonoids to complex free glucose *in vitro*, thus demonstrating its role as a glucophage. This trial will tell us about one of the hypoglycemic mechanisms. For this, 100µl of a glucose solution (1 g L^{-1}) is mixed with 100 µl of the extract at different concentrations: 10 mg, 20 mg and 40 mg mL^{-1}. The control consists of 100 µl of distilled water. The mixture thus obtained is incubated at 37°C for 15 minutes. Free glucose was determined according to the Bertrand method [9].

Reduction of glucose (%) = (control - sample/control) x 100

Determinations of α-amylase and α-glucosidase inhibitory activities by CSLAE

The inhibitory effect of CSLAE on α-amylase was analyzed as reported precedently by Yang and Kong (2016) with some modification [10]. Briefly, soluble starch solution (0.5%) was prepared by dissolving starch in phosphate buffer (pH 6.9, 20 mmol/L, containing 6.7 mM NaCl) and gelatinized at 90°C for 20 minutes, sample was dissolved in DMSO at 25 mg/mL as stock solution and diluted with phosphate buffer to different concentrations (0, 100, 300 and 500 µg/mL), and 500 µL sample solution and α-amylase (3.185 unit/mL) were incubated at 37°C for 15 minutes. After incubation, 500 µL soluble starch were added at 37°C for 5 minutes and the mixture was stopped with 1.0 mL dinitrosalicylic acid color reagent. After that, the mixture was boiled for 10 minutes and cooled to room temperature. The reaction mixture was then diluted 2 times with distilled water, and absorbance was measured at 540 nm using a spectrophotometer. The readings were compared with the control, which contained phosphate buffer instead of sample solution.

Moreover, the α-Glucosidase inhibitory activities were assessed according to the method illustrated by McCue., *et al*. (2005), with some modifications [11]. The enzyme solution contained 20 µl α-glucosidase (0.5 unit/ml) and 120 µl (0.1M) phosphate buffer (pH 6.9). p-Nitrophenyl-α-D-glucopyranoside (5 mM) in the same buffer (pH 6.9) was used as a substrate solution. 10µl of test samples, dissolved in DMSO at diverse concentrations, were mixed with enzyme solution and incubated for 15 minutes at 37°C. 20 µl of substrate solution were added and incubated for 15 minutes. The reaction was finished by adding 80 µl of 0.2M sodium carbonate solution. Absorbance of the wells was measured at 405 nm, while the reaction without CSLAE was used as control. Acarbose was used as positive control and each experiment was conducted in triplicate. The inhibitory activities were determined using following formula:

Inhibitory effects (%) = [(DOcontrol − DOsample) /DOcontrol] × 100.

Statistical analysis

The results were explored using the one-way analysis of variance (ANOVA) and Statview statistical software. The data were expressed as means ± standard error of the mean (S.E.M.). All statistical tests were two-tailed, and a p value of 0.05 or less was considered significant.

Results

Effect of CSLAE on changes in blood glucose levels in normal rats

Fasting blood glucose (FBG) levels were within the range of 97 - 105 mg dL-1 in all the groups at 0h. Table 1 shows the evolution of blood glucose (mg dL^{-1}) in normal rats after administration of different doses of CSLAE (100, 250 and 500 mg kg^{-1}) and during 3h. In this context, we recorded a progressive decrease of glucose level compared to the control rats.

T (min)	Control	CSLAE-100	CSLAE-250	CSLAE-500
0	100 ± 13	105 ± 5	97 ± 3	99 ± 7
60	97 ± 6	96 ± 3	78 ± 5[a]	78 ± 4[a]
120	101 ± 7	80 ± 2[a]	69 ± 2[a]	64 ± 2[a]
180	115 ± 2	78 ± 3	58 ± 7	48 ± 1

Table 1: *Influence of administration of Ceratonia siliqua leaves aqueous extract on the evolution of blood glucose levels in normal rats.*

The results are expressed as mean ± SEM (n = 6), a: p < 0.05, the difference is significant compared to the control rats.

Oral Glucose Tolerance Test (TTOG) after CSLAE administration

A state of hyperglycemia has been recorded following the administration of glucose solution (2 g kg^{-1} b.w.). However, the co-administration of the aqueous extract leads to a progressive diminution in the level of the blood glucose. The both doses (250 and 500 mg kg-1) of the extract can restore the normal value of glucose in rats (Figure 1).

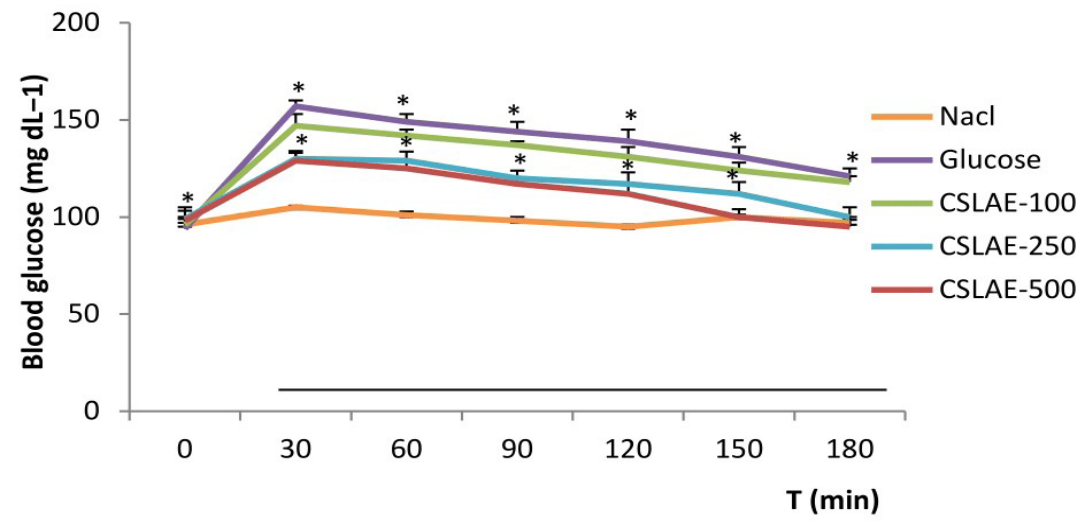

Figure 1: *OGTT after acute CSLAE oral treatment in rats. Rats were gavaged intragastrically with various doses of EAFC (100, 250 and 500 mgkg^{-1} body weight) and OGTT (glucose, 2 g kg^{-1} body weight). Significantly different from control (glucose), *: P < 0.05.*

Evaluating effect of CSLAE on glycemia after induction of diabetes with alloxan

The induction of experimental diabetes by alloxan in rats caused a considerable increase in the level of glucose in the blood. While, the treatment of these animals by CSLAE shows an antihyperglycemic effect. This activity is done in a dose-dependent manner.

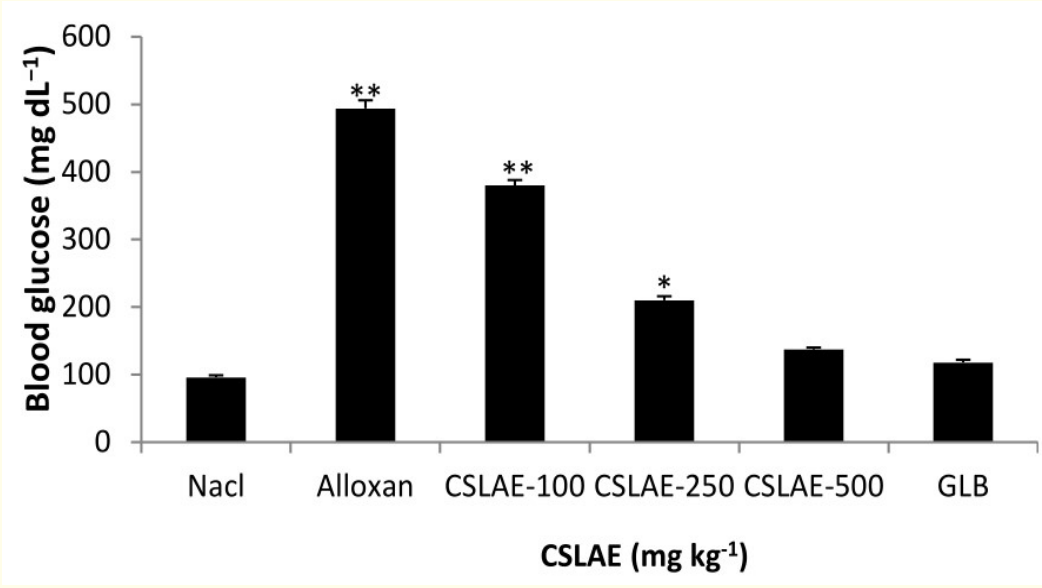

Figure 2: *Effect of the CSLAE at increasing doses (100, 250 and 500 mg kg^{-1}) on the glucose level after induction of diabetes with alloxane (150 mg kg^{-1}) in rats. Significantly different from control (Nacl), *: P < 0.05.*

CSLAE and Glucose Complexation *in Vitro*: Glycosylation Test

There is a significant drop in the initial glucose concentration in the tubes containing the aqueous extract of the carob tree leaves compared to the control tubes. Indeed, the percentage of glucose reduction may exceed a value of 50% at a dose of 40 mg mL^{-1}.

		% of glucose reduction
Glucose	1g L^{-1}	0
CSLAE	10 mg mL^{-1}	22.43
	20 mg mL^{-1}	39.55
	40 mg mL^{-1}	55.67
	80 mg mL^{-1}	67.23

Table 2: *Complexation of the Ceratonia siliqua leaves aqueous extracts (10, 20, 40 and 80 mg mL^{-1}) with glucose in vitro. The results are expressed as mean ± SEM (n = 3).*

Investigation of carbohydrate metabolism enzymes inhibitory activities by CSLAE

Ceratonia siliqua leaves aqueous extract at various doses (100, 200, 300, 400 and 500 µg mL^{-1}) exhibited a potent inhibition against α-amylase and α-glucosidase. Indeed, this reduction was realized in a dose-dependent manner.

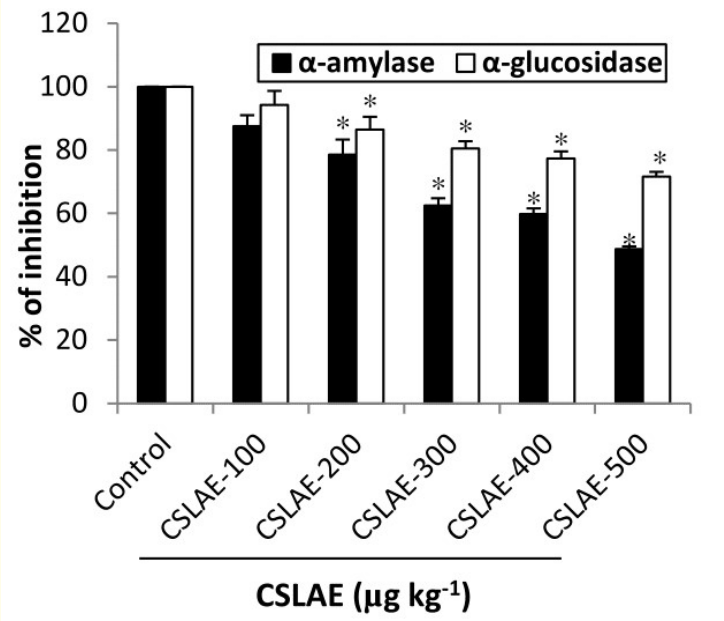

Figure 3: *Inhibitory effects of α-amylase and α-glucosidase were measured with different concentrations of CSLAE (100, 200, 300, 400 and 50 0µl kg-1). Significantly different from control, *: P < 0.05.*

Discussion

Diabetes is due to an excess of sugar in the blood. It is insulin that normally regulates blood sugar (sugar level). In case of diabetes, there is insulin deficiency or resistance to the action of this hormone. The body becomes unable to use glucose as a source of energy. This accumulates in the blood instead of being absorbed by the cells, thus causing chronic hyperglycaemia. The slowing down of intestinal glucose uptake by secondary metabolites is a promising new therapeutic strategy [12].

The study of the variation of the polyphenols content and total flavonoids in equivalents of gallic acid and quercetin showed a high level of polyphenols (62.50 ± 6.52 mg g-1 of DM) as well as a level of 21, 32 ± 2.22 mg g^{-1} of MS in total flavonoids within carob leaves. On the other hand, the study of the phenolic composition of carob leafs by HPLC showed the presence of kaempferol, tannic acid and catechin hydrate [13].

New prevention or treatment strategies for type 2 diabetes can be defined based on the inhibition of nutrient uptake. It is clear that new agents that can inhibit the intestinal absorption of glucose can have a significant impact on the efficacy of treatment of type 2 diabetes [14].

Our results show that increasing dose CSLAE results in a dose-dependent reduction in blood glucose levels. In this context, several studies have demonstrated the anti-hyperglycemic or antidiabetic effect of several medicinal plants [15].

Indeed, polyphenols have a regulating effect on the intestinal absorption of glucose [16]. Other polyphenols, such as those found in green tea (epigallo-catechin gallate (EGCG) and epicatechin gallate (ECG), decrease intestinal glucose transport probably through competitive inhibition of SGLT1 [17]. Flavonoids glycosides inhibit active glucose transport while aglycones inhibit glucose-assisted transport

in Caco-2 intestinal cells [18]. Other studies suggest that flavonoids inhibit the facilitated transport of glucose by the GLUT2 transporter located at the apical membrane of the enterocytes [14].

Kaempferol is a relatively abundant flavonol in the aqueous extract of carob leaves. In addition, kaempferol is isolated from the leaves of *Bauhinia forficata*, is able to decrease the increase of serum glucose and increase glucose uptake in rat muscle also more effective than insulin [19]. *In vitro* results demonstrated that kaempferol (10 μM) treatment promotes viability, inhibits cell apoptosis and reduces caspase-3 activity in β-cells and human islets chronically exposed to hyperglycemic disease [20].

The phytochemical exploration of the carob leaves indicated also that it may include rutin, interketones, and organic constituents, oils (volatile oils and fatty acids). The hypoglycemic effect of the aqueous extract may be attributed to its constituents such as rutin, saponins, and organic constituents [21].

The α-amylase and α-glucosidase inhibition actions are considered to be effective strategies for the control of diabetes by reducing the glucose absorption through the intestinal mucosa [22]. In this respect, Irondi., *et al*. (2017) reported that *Adansonia digitata* leaves extract, with high kaempferol levels, exhibited a strong inhibitory activity against α-amylase [23]. In addition, it has been reported that the catechin, is a potent α-amylase inhibitor. In recent years, there have been many reports about the α-glucosidase inhibitory activities of the simple polyphenols such as phenolic acids and flavonoids [24,25]. Although it has been reported that hydrolyzable tannins could inhibit the activity of α-glucosidase [26].

On another hand, *C. siliqua* germ may contain relatively high quantities of further glycosylated derivatives of (iso)schaftoside. Apigenin 6,8-C-di-glycosides have been elucidated as powerful α-glucosidase inhibitors [27].

Poovitha and Parani reported also that the protein extracts from the fruits of the two varieties of bitter gourd inhibited α-amylase and α-glucosidase *in vitro* and reduced the glucose level in blood at a comparable extent with acarbose when given intragastrally to Streptozotocin-induced experimental diabetic rats [28].

Conclusion

In conclusion, we consider that consumption of polyphenol-rich medicinal plants can be advantageously used in preventing and treating diabetes and may be useful for regulating carbohydrate metabolism and related disorders. The findings of the current study may also shed light on a way of generating a new class of amylase/glucosidase inhibitors that will discriminately inhibit the on-target enzymes with negligible undesired off-target side effects.

Declaration of Interest

Only the authors are responsible for the content of this paper.

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