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Full Length Research Paper

α-Amylase and α-glucosidase inhibitory effects of *Sclerocarya birrea* [(A. Rich.) Hochst.] subspecies *caffra* (Sond) Kokwaro (Anacardiaceae) stem-bark extracts

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Inhibition of intestinal α-amylase and α-glucosidase is an important strategy to control post-prandial hyperglycemia associated with type 2 diabetes mellitus. *In vitro* inhibitory effects of crude *Sclerocarya birrea* stem bark (SBSB) extracts against human urinary α-amylase and *Bacillus steatothermophilus* α-glucosidase were studied. Crude SBSB methanolic and acetone extracts inhibited human urinary α-amylase more potently than acarbose. Crude SBSB hexane extract was a strong inhibitor of α-glucosidase and weaker inhibitor of α-amylase. Furthermore, SBSB hexane extract significantly suppressed the rise in postprandial glucose level after oral administration of sucrose but failed to induce similar effects after oral administration of starch and glucose in both normal and diabetic rats. The results of this study suggest that the crude SBSB hexane extract may suppress the rise in postprandial hyperglycemia *in vivo* in part, through inhibition of alpha glucosidase.

Keywords: Diabetic rats, alloxan, postprandial hyperglycemia, diabetes mellitus.

INTRODUCTION

The stem bark, leaves and roots of *Sclerocarya birrea* (A. Rich) Hochst, subspecies *caffra* (Sond) Kokwaro, family Anarcardiaceae, are traditionally used in South Africa and some African countries to treat, manage and control a variety of human ailments, including diabetes mellitus (van Wyk et al., 2000). Numerous studies have established that *S. birrea* stem bark (SBSB) extracts possess among others activities, antibacterial (Eloff, 2001), antidiarrheal (Galvez et al., 1992), antihypertensive (Belemtougri et al., 2007) and antidiabetic (Ojewole, 2003; Dimo et al., 2007; Gondwe et al., 2008; van de Venter et al., 2008) properties.

With regard to the antidiabetic effects of SBSB, oral administration of moderate to high dose (100 to 800 mg/kg body weight) of an aqueous SBSB extract to streptozotocin-induced diabetic rats (STZ diabetic rats) significantly lowered their blood glucose level (Ojewole, 2003). In another similar study, oral administration of SBSB methanol/methylene chloride extract (300 mg/kg body weight) to STZ diabetic rats led to a significant reduction in blood glucose, increased plasma insulin levels, reduced plasma cholesterol, triglyceride and urea levels, and prevention of body weight loss in diabetic rats (Dimo et al., 2007). Recently, Gondwe et al. (2008) reported that SBSB ethanolic extract exhibited a dose-dependent reduction in blood glucose concentration without significantly affecting the blood insulin level of STZ diabetic rats. Furthermore, an *in vitro* cell culture-based assay carried out recently at the Nelson Mandela University, South Africa (van de Venter et al., 2008) reported that SBSB extract has the capacity to facilitate

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glucose utilization in Chang liver cell, C2C12 muscle cells and 3T3-L1 preadipocytes.

As far as the hypoglycemic mechanism of action of the SBSB extracts is concerned, results from research studies are inconclusive and contradictory. Ojewole (2003) and Dimo et al. (2007) have speculated, based on plasma insulin measurements, that the blood glucose lowering effects of SBSB extract could be associated with the stimulation of insulin secretion from the pancreatic beta cells. On the other hand, Gondwe et al. (2008) reported that *S. birrea* decreased the blood glucose level in STZ-induced diabetic rats without affecting the plasma insulin levels. Furthermore, van der Venter et al. (2008) suggested, on the basis of the observed marked increase in glucose utilization by cultured Chang liver cells and in C2C12 muscle cells supplemented with the SBSB extract, that the blood glucose lowering effects of the SBSB extract could be mediated via extra-pancreatic mechanisms. Thus, further studies are needed to clarify the blood glucose lowering mechanism of these extracts.

Some antidiabetic agents such as acarbose, exert their blood glucose lowering effects through the inhibition of gastrointestinal carbohydrate hydrolyzing enzymes such as the salivary and pancreatic α-amylase, as well as the intestinal brush border α-glucosidase enzymes (Inzucchi, 2002; Cheng and Fantus, 2005). Inhibition of these enzymes may delay the digestion and absorption of carbohydrates, and consequently suppress the rise in postprandial blood glucose level after a mixed carbohydrate diet (Gin and Rigalleau, 2000; Bhandari et al., 2008). Acarbose, a microbial pseudo-tetrasaccharide, is an inhibitor of both α-amylase and α-glucosisidase (Asano, 2009), and is widely used clinically as an oral hypoglycaemic agent, in combinations with other antidiabetic agents, to control postprandial hyperglycemia (PPHG) (Van de Laar et al., 2005; Fujisawa et al., 2005). However, the use of acarbose as an oral hypoglycaemic agent is reported to be associated with gastrointestinal side effects such as abdominal discomfort, flatulence (gas), and diarrhoea (Lebovitz, 1997; Kwon et al., 2008), which are allegedly caused by the excessive inhibition of pancreatic α-amylase by acarbose (Bischoff et al., 1985; Horii et al., 1987). In contrast to acarbose, plant derived α-amylase and α-glucosidase inhibitors are reported to have lower inhibitory effect against α-amylase activity and stronger inhibitory activity against α-glucosidase (Kwon et al., 2008), an indication that plant extracts and their constituents may be effective therapeutic agents for the management and control of postprandial hyperglycemia with less side effects than acarbose.

At least to the knowledge of the authors, there are no previous reports on both the α-amylase and α-glucosidase inhibitory effects of crude SBSB extracts. The aim of the current study was to investigate the *in vitro* inhibitory effects of SBSB extracts in relation to acarbose, against the activities of human urinary α-amylase and *B. stearothermophilus* α-glucosidase, and to confirm appreciable *in vitro* enzyme inhibitory activities *in vivo* using normal and alloxan-induced diabetic rats.

MATERIALS AND METHODS

Chemicals and reagents

Organic solvents used for extraction of the plant material (hexane, acetone, and methanol) were all purchased from SARCHEM. *B. stearothermophillus* α-glucosidase (EC 3.2.1.20), potato starch, p-nitro phenyl α-D-glucopyranoside (PNPG), alloxan monohydrate, 3, 5-dinitrosalicylic acid and sodium potassium tartrate were all purchased from Sigma-Aldrich (St. Louis, MO. USA). Human urinary amylase (6342 units) was obtained from the urine of a patient with acute pancreatitis (National Health Laboratory Services, Dr George Mukhari Hospital, RSA). Acarbose (Glucobay 50 N1; Bayer Vital, Leverkusen, Germany) was obtained from a local pharmacy. All other chemicals used were of analytical grade.

Plant material and preparation of extracts

Stem barks of *S. birrea* were collected from an area around University of Limpopo (Medunsa Campus), South Africa, in July 2007 and authenticated by the South African National Biodiversity Institute (SANBI). Fresh stem barks of *S. birrea* were rinsed with tap water, air-dried at room temperature, cut into small pieces and ground into a fine powder using pestle and mortar. Hexane, acetone, methanol and aqueous SBSB extracts were prepared by sequential extraction of 25 g of the dried powder with 250 ml of the respective solvent (100% v/v) for 24 h. The filtered hexane, acetone and methanolic extracts were evaporated to dryness in a rotary evaporator, whereas the aqueous extracts were lyophilized. Dried extracts were reconstituted to 10 mg/ml in distilled water.

Study animals and induction of diabetes

Male albino Wistar rats weighing 220 to 280 g were obtained from the animal unit of the University of Cape Town, South Africa. The animals were kept in individual cages with a 12 h light / 12 h dark cycle and controlled conditions of temperature and humidity. The animals had free access to water and standard rat diet. The study was approved by the animal ethics committee of the University of Limpopo (AEC 09/06).

Diabetes mellitus was induced in 12 h fasted animals by intraperitoneal injection of alloxan monohydrate dissolved in sterile normal saline at a dose of 140 mg/kg body weight. Since alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were intraperitoneally treated with 20% glucose solution 6 h after alloxan treatment. The rats received 5% glucose solution in bottles for 24 h to prevent hypoglycaemia (Dhandapani et al., 2002). Diabetes was confirmed in alloxan-treated rats by measuring fasting blood glucose levels 72 h after alloxan treatment. Rats with fasting blood glucose level of 11.1 mmol/L (200 mg/dL) were selected and used in the study.

α -Amylase inhibition

SBSB extracts were screened for α-amylase inhibitory activity according to the method described by Ali et al. (2006) with slight modifications. Briefly, 50 µl aliquots of human urinary α- amylase (5 U/ml) were pre-incubated for 20 min with 50 µl aliquots of SBSB extracts (10 mg/ml). The reaction was started by the addition of 50 µl potato starch (0.5%) dissolved in 20 mM phosphate buffer at pH 6.9. The reaction mixture was incubated for a further 20 min at 37°C and the catalytic reaction terminated by addition of 2.0 ml of

DNS reagent (1% 3, 5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The reaction mixture was heated for 15 min at 100°C. Amylase activity was determined by measuring the absorbance at 540 nm. Each test was performed three times and the mean absorption was used to calculate percentage of amylase inhibition. Percentage α-amylase inhibition was calculated according to the following formula;

$$\text{Alpha amylase inhibition (\%)} = \frac{A_{540}\ \text{control} - A_{540}\ \text{sample}}{A_{540}\ \text{control}} \times 100$$

α-Glucosidase inhibition

The inhibitory effect of SBSB extracts on α-glucosidase activity was determined according to the chromogenic method described by Kim et al. (2005), using α-glucosidase from *B. stearothermophillus*. Briefly, 5 units of α-glucosidase were pre-incubated with 20 μg/ml of the different SBSB extracts for 15 min. Para-nitrophenyl-glucopyranoside (PNPG) (3 mM) dissolved in 20 mM phosphate buffer, pH 6.9 was added to start the reaction. The reaction mixture was further incubated at 37°C for 20 min and stopped by addition of 2 ml of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the yellow colored p-nitrophenol released from PNPG at 400 nm. Each test was performed three times and the mean absorption was used to calculate percentage α-glucosidase inhibition. Percentage α-glucosidase inhibition was calculated according to the following formula;

$$\text{Alpha glucosidase inhibition (\%)} = \frac{A_{400}\ \text{control} - A_{400}\ \text{sample}}{A_{400}\ \text{control}} \times 100$$

Determination of IC_{50} values of active SBSB extracts

The potency of crude SBSB extracts as inhibitors of catalytic activities of both human urinary α-amylase and *B. stearothermophillus* α-glucosidase was also assessed in terms of their IC_{50} values (inhibitor concentration that reduces enzyme activity by 50%) according to the method described by Cheng and Prusoff (1973). Briefly, aliquots of α-amylase and α-glucosidase enzymes were pre-incubated with increasing concentrations of SBSB extracts and acarbose (0.2 to 20 mg/ml). Catalytic reactions were started, terminated and enzyme activities determined as aforementioned. Dose-response curves of percentage inhibition versus plant extract concentration were plotted and the IC_{50} values of SBSB extracts and acarbose were estimated by interpolation.

Kinetics of inhibition against α-amylase and α-glucosidase

Inhibition modes of the crude SBSB extracts against α-amylase and α-glucosidase were determined according to the method described by Kim et al. (2005). Briefly, fixed amounts of both α-amylase and α-glucosidase were incubated with increasing concentrations of their substrates (starch and PNPG, respectively) at 37°C for 20 min, in the absence or presence of SBSB extracts (5 mg/ml). Reactions were terminated and absorption measurements carried out as aforementioned. Amounts of products liberated (reducing sugars as maltose and p-nitrophenol, respectively) were determined from corresponding standard curves and converted to reaction rates according to the following formula;

$$\text{Reaction rate (v) (mg.ml}^{-1}\text{.s}^{-1}\text{)} = \frac{\text{Amount of product liberated (mg.ml}^{-1}\text{)}}{1200\ \text{(s)}}$$

Inhibition types were then determined by Lineweaver–Burk plot ($1/v$ versus $1/[S]$) where [S] analysis of data is according to Michaelis–Menten kinetics (Lineweaver and Burk, 1934).

Starch tolerance test

24 rats (12 normal and 12 alloxan-induced diabetic rats) were divided into four groups consisting of six rats (n = 6) in each group: Group I, normal experimental rats; Group II, normal control rats; Group III, diabetic experimental rats; Group IV, diabetic control rats. After an overnight fast (18 h), Group I and III rats were given SBSB acetone extract (300 mg/kg body mass) by means of an intragastric tube. Group II and IV received distilled water at the same time. Twenty minutes after administration of plant extract, all rats were given orally, potato starch (3 g/kg body mass). Post-prandial blood glucose levels were measured before (0 min) and at 30, 60, 90, 120, and 150 min after oral administration of potato starch using Glucometer 4 Ames (Bayer Diagnostics). Post-prandial blood glucose curves of experimental rats were plotted and compared with those of control rats.

Sucrose tolerance test

Four days after performing the starch tolerance test, sucrose tolerance tests were performed in the same group of rats used for the starch tolerance test. The procedure for performing the sucrose tolerance test was similar to the one used in the starch tolerance tests except that instead of starch, sucrose (5 g/kg body mass) was orally administrated to all groups of rats, 20 min after administration of the plant extract.

Statistical analysis

Data expressed as mean ± SD were analyzed using the Sigma Stat statistical program (version 8.0). Comparisons were made between normal and alloxan-induced diabetic rats as well as between treated and untreated alloxan-induced diabetic rats by means of unpaired Student's t-test and their significance were established by ANOVA. Differences of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

There is a growing interest among researchers to discover new and effective α-glucosidase inhibitors with minimal side effects, from medicinal plants with known and scientifically proven antidiabetic properties (Grover et al., 2002; Onal et al., 2005; Bnouham et al., 2006; Bhat et al., 2008). As part of this research niche area, we investigated the inhibitory effects of SBSB extracts and acarbose against human urinary amylase and *B. steatothermophilus* α-glucosidase. Crude SBSB methanolic, acetone and hexane extracts had appreciable *in vitro* inhibitory activity (> 50 %) against α-amylase. Both the crude SBSB methanolic and acetone extracts inhibited amylase significantly more than acarbose ($p < 0.05$) and the hexane fraction (Figure 1). Crude SBSB acetone and hexane extracts had a high inhibitory potency (>80%) against α-glucosidase. This level of inhibition was comparable to the effects of

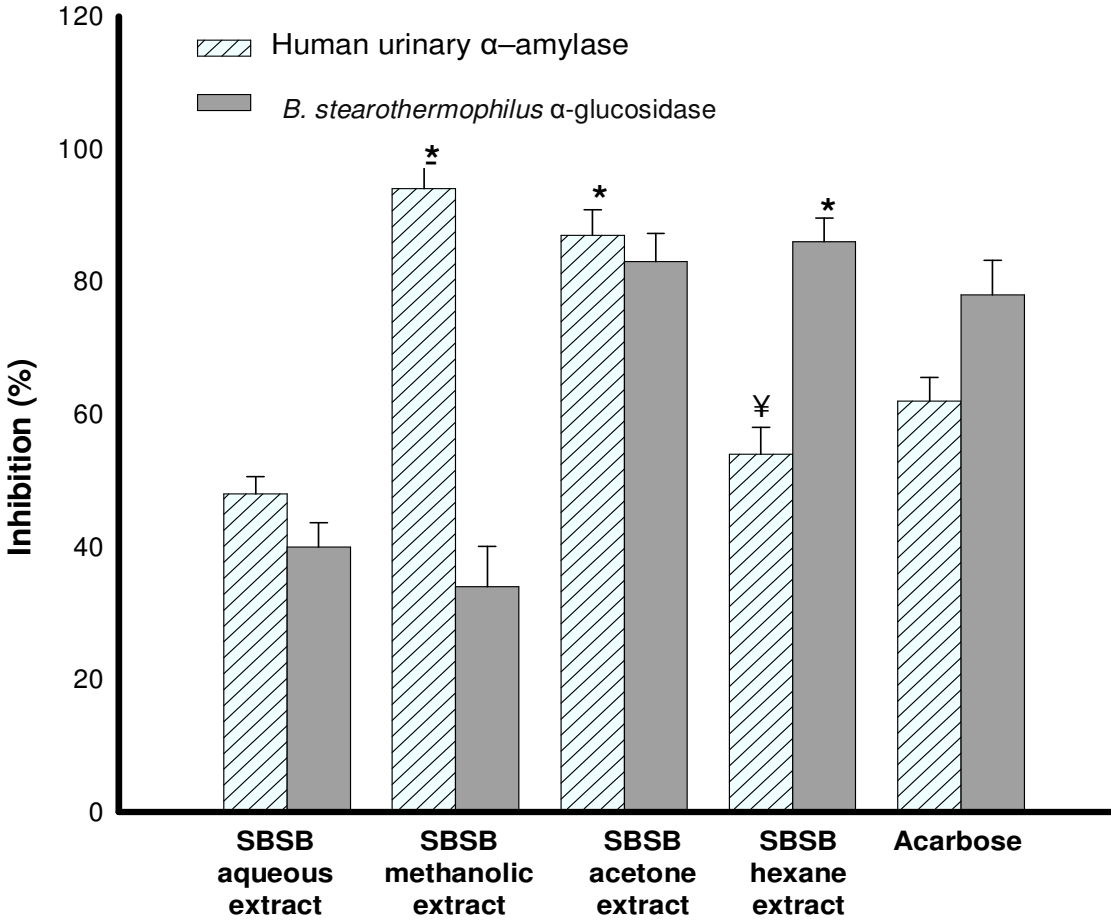

Figure 1. *In vitro* inhibition of human urinary α-amylase and *B. stearothermophilus* α-glucosidase activities by crude SBSB extracts and acarbose.

acarbose (Figure 1). Crude SBSB methanolic extract inhibited α-glucosidase significantly less ($p < 0.05$) than acarbose. The aqueous extract was a less potent inhibitor of both enzymes. These observations suggest that unlike the use of acarbose and crude SBSB methanolic and acetone extracts, the use of crude SBSB hexane extract as an oral hypoglycaemic agent might be associated with less gastrointestinal side effects.

The IC_{50} values for the SBSB methanolic, acetone and hexane extracts, as well as acarbose, against α-amylase and α-glucosidase activities were determined from corresponding dose-response curves. The methanolic and acetone extracts were more potent inhibitors of amylase (IC_{50} values of 7.8 and 8.4 mg/ml, respectively) (Table 1). The acetone and hexane extract had good α-glucosidase inhibitory activity with IC_{50} values of 0.029 and 0.019 mg/ml, respectively. These values were comparable to the IC_{50} value resulting from acarbose (IC_{50} = 0.026 mg/ml).

Established inhibitors of α-amylase and α-glucosidase enzymes include: Pseudosacharides such as acarbose, miglitol and vogiblose (Asano, 2009), and phenolic phytochemicals (Kim et al., 2000; McDougall et al., 2005; Tadera et al., 2006; Matsui et al., 2007). Whereas most phenolic phytochemicals are reported to inhibit α-glycosidases enzymes in a non-compititive manner, pseudosaccharides, by virtue of being substrate analogs, inhibit α-glucosidases in a competitive manner. The mode of inhibition of the crude SBSB methanolic and hexane extracts against both α-amylase and *α*-glucosidase was confirmed by Lineweaver–Burk plots (Figure 2A and B). Crude SBSB methanolic extract competitively inhibited α-amylase, whereas the hexane extract non-competitively inhibited α-glucosidase. These observations suggest that amylase is inhibited mostly by polar SBSB metabolites (probably pseudosaccharides) and α-glucosidase is inhibited mostly by non-polar SBSB metabolites. Further studies are needed to identify the nature and identity of α-amylase and α-glucosidase inhibitory metabolites in these SBSB extracts.

In vitro enzyme inhibitory effects are not always reproducible *in vivo*. It was thus necessary to confirm the observed *in vitro* results in *vivo*. This was done by investigating the effect of plant extract on PPHG in

Table 1. The IC_{50} values (mg/ml) of various SBSB extracts compared with IC_{50} values of acarbose against α-amylase and α-glucosidase.

Inhibitor	IC_{50} (mg/ml)	
	α-Amylase	α-Glucosidase
SBSB methanolic extract	7.8 ± 1.01*	0.032 ± 0.08
SBSB acetone extract	8.4 ± 0.8*	0.029 ± 0.10
SBSB hexane extract	21.6 ± 1.22	0.019 ± 0.06
Acarbose	16.5 ± 1.12	0.026 ± 0.04

SBSB, *Slerocarya birrea* stem bark.

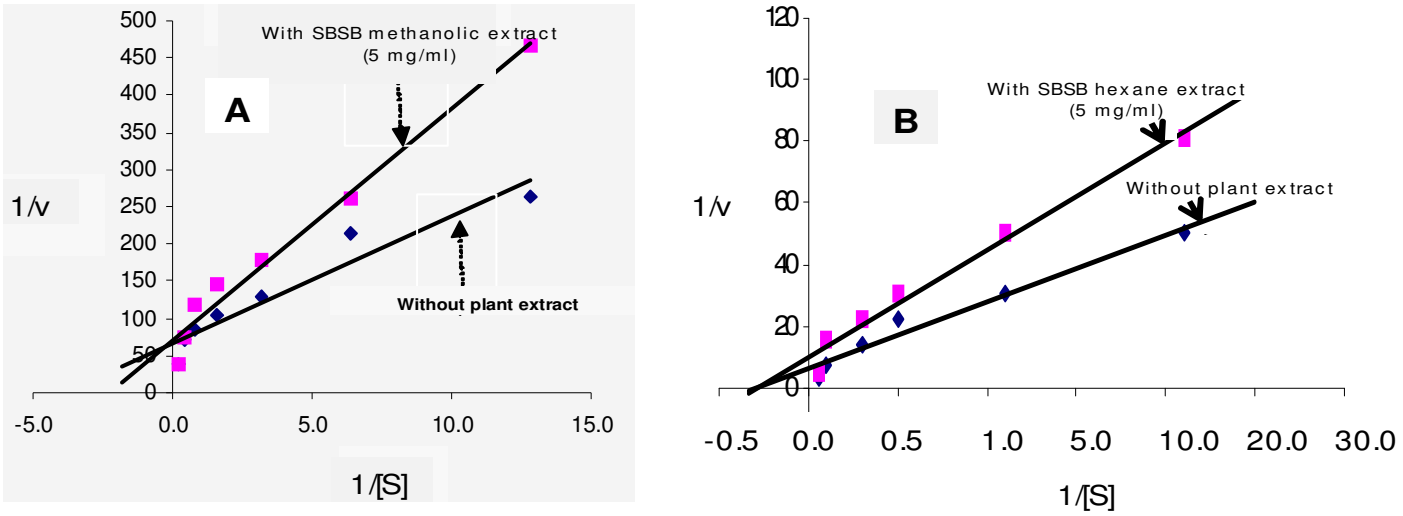

Figure 2. Lineweaver-Burk plots for the investigation of mode of inhibition of (A) human urinary amylase activity by crude SBSB methanolic extract and (B) *B. steatothermophilus* α-glucosidase by crude SBSB hexane extract.

normal and diabetic rats challenged with oral administration of potato starch and sucrose. Oral administration of plant extract to rats fed sucrose significantly suppressed the rise in PPHG in both normal and diabetic rats, whereas administration of extract to rats did not affect the rise in PPHG

Oral administration of acarbose (50 mg/kg body weight) to 18 h fasted normal and diabetic rats 20 min before oral administration starch (3 g/kg body weight), as expected, significantly suppressed the rise in PPHG at 30 and 60 min in these rats (Figure 3C and D). However, oral administration of crude SBSB hexane extracts (300 mg/kg body weight) 20 min before starch loading failed to lower PPHG in both normal and diabetic rats. Both acarbose (50 mg/kg body weight) and the crude SBSB hexane extract (300 mg/kg body weight), when administered 20 min before an oral load of sucrose significantly suppressed the rise in PPHG in normal and diabetic rats. There was however, no significant difference between the effect of acarbose and SBSB hexane extract on PPHG in both normal and diabetic rats (Figure 4E and F). Our study suggests that the reported hypoglycaemic effects of extracts of *S. birrea* in diabetic rats (Ojewole, 2003; Dimo et al., 2007; Gondwe et al., 2008) may in part, be mediated through the inhibition of α-glucosidase activity.

Conclusion

In conclusion, the present study has demonstrated that *S. birrea* extract exerted both α-amylase and α-glucosidase inhibitory action *in vitro*, and significantly prevented a rise in postprandial blood glucose levels. The data from this preliminary investigation should provide a basis for further investigations where the active component that results in angiotensin converting enzyme (ACE) inhibition can be isolated. Strong inhibition of α-glucosidase and low inhibition of α-amylase could be potentially used as an effective complementary therapy for postprandial hyperglycemia with minimal side effects.

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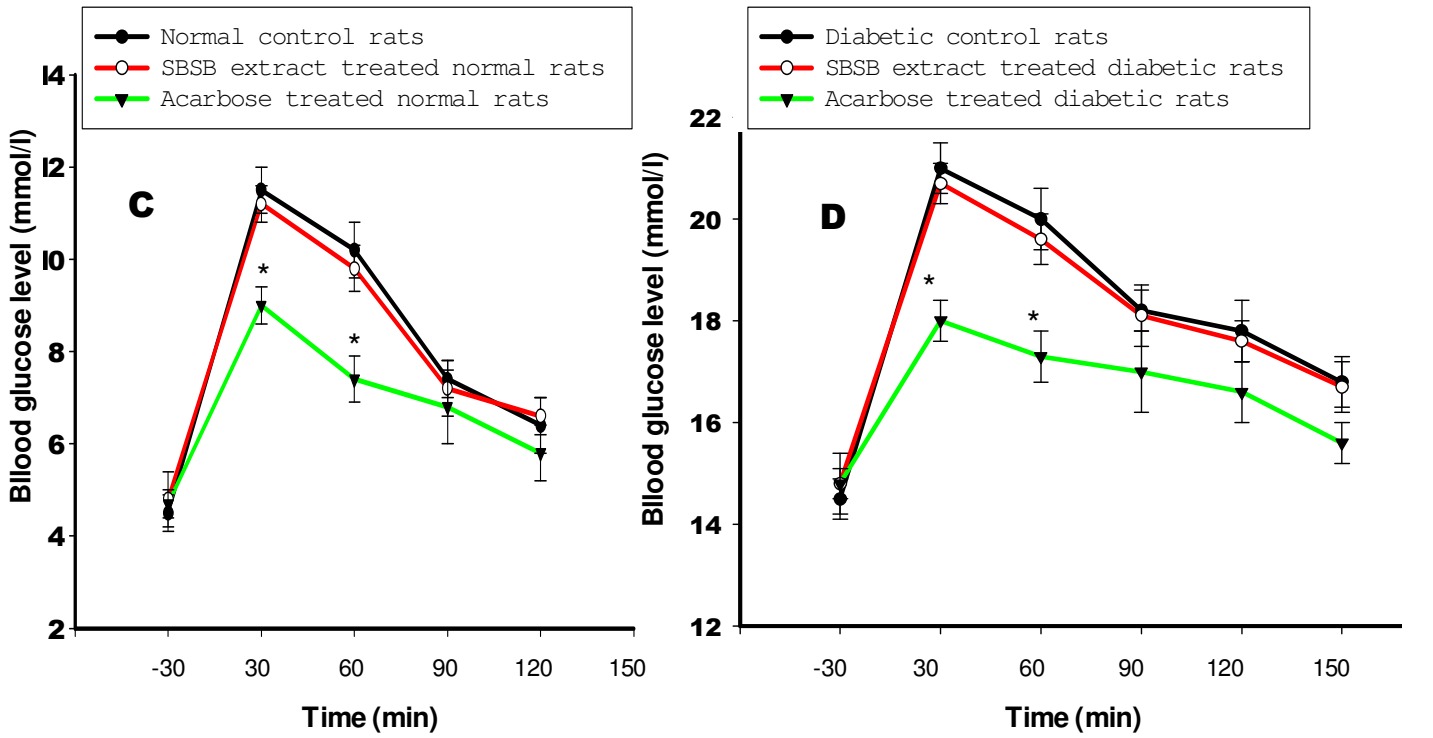

Figure 3. Blood glucose response during oral starch tolerance tests in **(C)** normal control rats, SBSB hexane extract (300 mg/kg body weight) treated normal rats and acarbose (50 mg/kg body weight) treated normal rats and in **(D)** diabetic rats, SBSB hexane extract (300 mg/kg body weight) treated diabetic rats and acarbose (50 mg/kg body weight) treated diabetic rats.

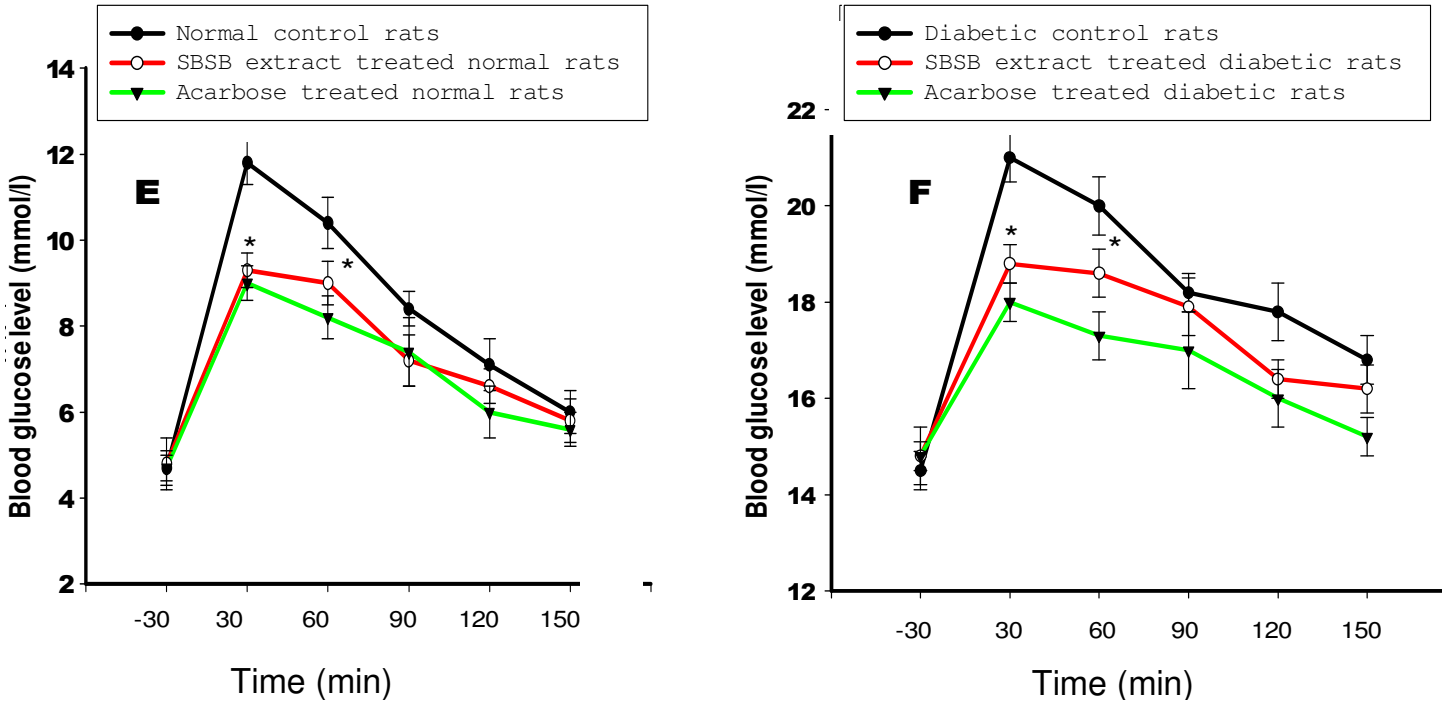

Figure 4. Blood glucose response during oral sucrose tolerance tests in **(E)** normal control rats, SBSB hexane extract (300 mg/kg body weight) treated normal rats and acarbose (50 mg/kg body weight) treated normal rats and in (**F**) diabetic control rats, SBSB hexane extract (300 mg/kg body weight) treated diabetic rats and acarbose (50 mg/kg body weight) treated diabetic rats.

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