Full Length Research Paper

The effects of *Clausena anisata* (Wild) Hook leaf extracts on selected diabetic related metabolizing enzymes

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Accepted 14 March, 2012

*Clausena anisata* (Wild) Hook (Family Rutaceae) is an indigenous Southern African medicinal plant used to treat diabetes mellitus and other diseases. Although, the blood glucose lowering effect of the methanolic *C. anisata* leaf extract has been confirmed in diabetic rats, the mechanism(s) remains unknown. This study investigated the effects of crude extracts of *C. anisata* leaves on selected enzymes associated with carbohydrate metabolism, in order to determine the mechanism of action related to hypoglycaemic effects of *C. anisata*. Crude *C. anisata* leaf extracts were investigated for their inhibitory effects against human urinary α-amylase, α-glucosidase and glucose 6-phosphatase *in vitro* as well as rat α-amylase and α-glucosidase *in vivo*. Aqueous and methanolic extracts strongly inhibited (>80%) α-amylase and moderately inhibited (60 and 58%, respectively) rabbit hepatic glucose-6-phosphatase. These two extracts were less potent inhibitors of α-amylase than acarbose and significantly more potent inhibitors of G6Pase than sodium vanadate. Acetone and hexane extracts strongly inhibited (> 80%) *Bacillus stearothermophilus* α-glucosidase. Oral administration of acetone extract to fasted normal and streptozotocin-induced diabetic rats, 30 min before oral loading of both starch and maltose failed to prevent the rise in postprandial hyperglycemia in these animals. The *in vitro* inhibition of glucose-6-phosphatase by the aqueous and methanolic extracts of *C. anisata* needs to be confirmed *in vivo*. Results of this study suggest that the previously reported hypoglycemic activity of *C. anisata* extract could not be attributed to the inhibition of intestinal carbohydrate hydrolyzing enzymes.

**Key words:** *Clausena anisata*, α-amylase, α-glucosidase, glucose 6 phosphatase, postprandial hyperglycemia.

INTRODUCTION

Medicine in the 21st century has largely become a molecular science in which drug molecules are directed towards specific macromolecular targets whose bioactivity is pathogenic or at least associated with disease (Copeland et al., 2007). Among the biological macromolecules that can act as drug targets, enzymes hold a prominent role, because of their essential roles in metabolism and pathophysiology. Inhibition of a key enzyme involved in the pathogenesis of a specific disease may correct a disease-induced metabolic imbalance (Copeland et al., 2007; Rich, 2005). For these reasons, most of the pharmacological drugs used today are enzyme inhibitors. Indeed, a survey conducted, Hopkins and Groom (2002) found out that nearly half (47%) of the therapeutic drugs used in modern clinical practice are enzyme inhibitors.

In the context of type 2 diabetes mellitus, enzymes that have been targeted for therapeutic inhibition purposes include α-amylases (EC 3.2.1.1), α-glucosidases (EC
revitalization of damaged pancreatic beta cells; improvement of insulin sensitivity; mimicking the action of insulin and inhibition of carbohydrate metabolizing enzymes. To the best of our knowledge, leaf extracts of C. anisata have never been investigated for inhibitory effect against diabetic related carbohydrate metabolizing enzymes. Thus, the aim of the current study was to study the in vitro inhibitory effects of C. anisata leaf extracts on the activities of human urinary α-amylase, α-glucosidase and rat hepatic G6Pase and to confirm where possible the result of these in vitro studies in vivo using normal and STZ-induced diabetic rats.

MATERIALS AND METHODS

Reagents and chemicals

Organic solvents used for extraction of the plant material (hexane, acetone and methanol) were purchased from SAARCHEM (RSA). Bacillus stearothermophilus α-glucosidase glucose 6-phosphatase, potato starch, p-nitro phenyl α-D-glucopyranoside (pNPG), STZ, 3,5-dinitrosalicylic acid, sodium potassium tartrate and glucose-6-phosphate 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.

Plant material and preparation of extracts

Leaves of C. anisata were collected from the Lowveld National Botanical Garden in Nelspruit, Mpumalanga province (South Africa) where plant species are identified by a name tag. The leaves were air-dried at room temperature and homogenised into a fine powder using a coffee grinder. Crude C. anisata leaf (CCAL) hexane, acetone, methanol and aqueous extracts were prepared by a sequential extraction of 25 g of the dried powder with 250 ml of respective solvent (100% v/v) for 24 h. Hexane, acetone and methanolic extracts were evaporated to dryness in a rotary evaporator, whereas the aqueous extracts were lyophilized. 100 mg dry weight of each crude extract were further reconstituted with 10 ml of distilled water to provide (10 mg/ml) crude extracts which were used for in vitro enzyme inhibitory studies.

Study animals and induction of diabetes

Male Wistar rats weighing 220 to 280 g were obtained from the animal unit facility of the University of Cape Town, South Africa. The animals were kept in individual cages in an environmentally controlled room with a 12 h light/12 h dark cycle. The animals had free access to water and standard rat diet. The study was approved by institutional animal ethical committee. Diabetes mellitus was induced in 12 h fasted animals by intraperitoneal injection of STZ dissolved in sterile normal saline at a dose of 60 mg/kg body weight. Diabetes was confirmed in STZ-treated rats by measuring fasting blood glucose levels 72 h after STZ treatment. Rats with marked hyperglycemia (blood glucose level above 11.0 mM) were selected and used in the study. The study was approved by the University of Limpopo’s Animal Research Ethics Committee (AREC).
Alpha amylase inhibition

CCAL 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 CCAL extracts (10 mg/ml). Reaction was started by addition of 50 µl potato starch substrate (0.5%) dissolved in 20 mM phosphate buffer, pH 6.9. The reaction mixture was then incubated for a further 20 min at 37°C and the catalytic reaction terminated by addition of 2.0 ml of dinitrosalicylic acid reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The reaction mixture was then heated for 15 min at 100°C and α-amylase activity determined by measuring the absorbance at 540 nm. Each test was performed three times and the mean absorption was used to calculate percentage α-amylase inhibition extracts. Percentage α-amylase inhibition was calculated according to the following formula:

\[ \text{Alpha amylase inhibition} (\%) = \left( \frac{A_{\text{540 control}} - A_{\text{540 sample}}}{A_{\text{540 control}}} \right) \times 100 \]

The potency of crude CCAL extracts as inhibitors of human urinary α-amylase was 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).

Alpha glucosidase inhibition

The inhibitory effect of CCAL extracts on α-glucosidase activity was determined according to the chromogenic method described by Kim et al. (2005) using α-glucosidase from B. stearothermophilus. Briefly, 5 units of α-glucosidase were pre-incubated with 20 µg/ml of the different CCAL extracts for 15 min. pNPG (3 mM) as a substrate dissolved in 20 mM phosphate buffer, pH 6.9 was then 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 Na₂CO₃. 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:

\[ \text{Alpha glucosidase inhibition} (\%) = \left( \frac{A_{\text{405 control}} - A_{\text{405 sample}}}{A_{\text{405 control}}} \right) \times 100 \]

The potency of CCAL extracts as inhibitors of B. stearothermophilus α-glucosidase was assessed in terms of their IC\(_{50}\) values according to the method described by Cheng and Prusoff (1973).

Glucose 6-phosphatase inhibitory activity

The effects of CCAL extracts on G6Pase activity were investigated according to the method described by Baginski et al. (1974) with some modifications using G6Pase from a rabbit liver (Sigma, G5758). Briefly, 0.25 µl (25 units) of G6Pase (E.C. 3.2.3.2) was pre-incubated with 0.25 ml of CCAL extracts (water, methanol acetone and hexane) for 15 min at 37°C. The reaction was then started by addition of 0.25 µl of 0.1 M glucose -6-phosphate in citrate buffer, pH 6.5. The reaction mixture was then incubated at 37°C for 20 min. At the end of the incubation period 2.0 µl of 2/10% (w/v) ascorbic acid/trichloroacetic acid solution was added to stop the reaction. The inorganic phosphate liberated from the substrate by the enzyme was reacted with 1% ammonium molybdate solution to produce a blue-colored chromogen whose absorbance was measured at 660 nm. Percentage G6Pase inhibition was calculated according to the following formula:

\[ \text{Glucose 6 phosphate inhibition} (\%) = \left( \frac{A_{\text{405 control}} - A_{\text{405 sample}}}{A_{\text{405 control}}} \right) \times 100 \]

The potency of CCAL extracts as inhibitors of G6Pase was assessed in terms of their IC\(_{50}\) values according to the method described by Cheng and Prusoff (1973).

Kinetics of inhibition against α-amylase, α-glucosidase and G6Pase

Inhibition modes of the CCAL extracts against human urinary α-amylase, B. stearothermophilus α-glucosidase and G6Pase were determined according to the method described by Kim et al. (2005). Briefly, fixed amounts of both human urinary α-amylase and B. stearothermophilus 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 CCAL extracts (5 mg/ml).

Reactions were terminated and absorption measurements were carried out as described earlier. Amounts of products liberated were determined from the corresponding standard curves and converted to reaction rates according to the following formula.

\[ \text{Reaction rate} (y)(\text{mg} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}) = \frac{[\text{Product}] (\text{mg} \cdot \text{ml}^{-1})}{\text{Incubation time} (\text{s})} \]

Lineweaver–Burk plots were used to determine modes of inhibition as well as K\(_{m}\) and V\(_{max}\) values.

Starch tolerance test

Twenty four rats (12 normal and 12 STZ-induced diabetic rats) were divided into four groups of six rats (n = 6) each: Group I (normal experimental rats), Group II (normal control rats), Group III (diabetic experimental rats) and Group IV (diabetic control rats). After an overnight fast (18 h), Groups I and III rats were given CCAL acetone extract (300 mg/kg body mass) by means of an intragastric tube. Groups II and IV received distilled water (vehicle control) at the same time. 20 min after administration of the plant extract, all rats were given potato starch (3 g/kg body mass) orally. Postprandial blood glucose levels were then 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, Germany). Postprandial blood glucose curves of experimental rats were plotted and compared with those of control rats.

Maltose tolerance test

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

Statistical analysis

Data, expressed as mean ± standard deviation (SD) were analyzed
RESULTS

In vitro enzyme inhibitory activities

Water, methanol, acetone and hexane extracts of C. anisata obtained by sequential extraction were screened for enzyme inhibitory activities against human urinary α-amylase, B. stearothermophilus α-glucosidase and rabbit hepatic G6Pase (Figure 1).

The crude water, acetone and methanol extracts of CCAL strongly inhibited (> 80%) human urinary α-amylase. C. anisata leaf acetone and hexane extracts showed appreciable (> 80%) inhibitory activity against B. stearothermophilus α-glucosidase, whereas water and methanol showed moderate inhibitory activities (60 and 58%, respectively) against rabbit hepatic G6Pase.

IC₅₀ values of C. anisata extracts

IC₅₀ values of C. anisata water, methanolic, acetone and hexane extracts against human urinary α-amylase, B. stearothermophilus α-glucosidase and rabbit G6Pase were determined from dose-response curves and were compared with those of 0.5 mg/ml acarbose and 0.5 mg/ml sodium vanadate. Both water and methanol C. anisata leaf extracts appear to be significantly less potent inhibitors of human urinary α-amylase than acarbose (P < 0.001) (IC₅₀ values are significantly higher than that of acarbose) and significantly more potent inhibitors G6Pase than sodium vanadate (IC₅₀ values are lower than that of sodium vanadate). On the other hand, the acetone and hexane leaf extracts of C. anisata appear to be significantly less potent inhibitors of B. stearothermophilus α-glucosidase than acarbose (Table 1).

Mode of inhibition of active C. anisata extracts

Modes of inhibition of CCAL water extract against human urinary α-amylase and rabbit hepatic G6Pase as well as the mode of CCAL hexane extract against B. stearothermophilus α-glucosidase were determined by means of Lineweaver-Burk double reciprocal plot of 1/v versus 1/[S] (Figure 2).

Modes of inhibition of CCAL methanolic and aqueous against human urinary α-amylase and rabbit hepatic G6Pase were found to be reversible and non-competitive (Figure 2A and C, respectively). The mode of inhibition of B. stearothermophilus α-glucosidase by CCAL hexane extracts was found to be reversible and Competitive.
Table 1. IC50 values of CCAL extracts compared with those of acarbose and sodium vanadate.

<table>
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<tr>
<th>Inhibitor</th>
<th>IC50 (μg/ml) against</th>
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<tr>
<td></td>
<td>human urinary α-amylase</td>
</tr>
<tr>
<td>CCAL aqueous extract</td>
<td>1947 ± 50**</td>
</tr>
<tr>
<td>CCAL methanolic extract</td>
<td>2436 ± 62**</td>
</tr>
<tr>
<td>CCAL acetone extract</td>
<td>ND</td>
</tr>
<tr>
<td>CCAL hexane extract</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>84 ± 11</td>
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<tr>
<td>Sodium vanadate</td>
<td>ND</td>
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</tbody>
</table>

IC50 values of CCAL extracts, acarbose and sodium vanadate against enzyme activities were determined as described in the material and methods and IC50. Results are expressed as mean IC50 value ± SD, n = 3. Mean IC50 values statistically different compared with those of acarbose, ND: not done.

Figure 2. Lineweaver-Burk plots of (A) activity of human urinary α-amylase in the absence or presence of the CCAL methanolic extract, (B) activity of B. stearothermophilus α-glucosidase in the absence or presence of the CCAL hexane extract and (C) activity of rabbit hepatic G6Pase in the absence or presence of CCAL aqueous extract.

(Figure 2B).

Effects of C. anisata acetone extract on postprandial hyperglycemia

The effect of the CCAL acetone extract on PPGH was studied in both normal and STZ-induced diabetic rats by means of oral starch and maltose tolerance tests. C. anisata leaf acetone extract failed to bring about any significant changes in PPGH induced by oral administration of either starch or maltose in both normal and diabetic rats (Figure 3).

DISCUSSION

Plant extracts have long been used to treat diabetes in
various systems of medicine and are currently accepted as an alternative for diabetic therapy (Bnouham et al., 2006; Tanira, 1994; Bailey and Day, 1989; Jung et al., 2006; Dey et al., 2002). However, for many plant extracts, there is no clear understanding of the mechanism of their hypoglycaemic action (Bhandari et al., 2008; Tanira, 1994). In the current study, the effects of CCAL on the activities of human urinary α-amylase, B. stearothermophilus α-glucosidase and rabbit hepatic G6Pase were investigated in vitro.

C. anisata leaf aqueous, methanolic and acetone extracts led to over 80% inhibition of human urinary α-amylase. The inhibition of human urinary α-amylase by the CCAL hexane extract (56%) could be regarded as insignificant when compared with the effect of the other three polar extracts. These observations suggest that human urinary α-amylase and presumably human pancreatic α-amylase, are inhibited by more polar constituents of C. anisata leaves. This is in agreement with the results of related studies which reported α-amylase inhibitory activities in the more polar extracts of plant materials (Bhandari et al., 2008). Thus, the α-amylase inhibitory activity of CCAL extracts could be attributed to the presence of polyphenols, flavonoids and their glycosides which are known to be soluble in more polar solvents (Jung et al., 2006).

Figure 3. Effects of CCAL acetone extract on PPGH induced by oral administration of either maltose in normal rats (A) and in diabetic rats (B) or starch in normal rats (C) and diabetic rats (D).
On the other hand, *B. steartothermophilus* α-glucosidase was found to be strongly inhibited by both acetone and hexane extracts of *C. anisata* leaves. In this study, the aqueous and methanolic extracts produced relatively weak enzyme inhibitory activities (12 and 30%, respectively). This suggests that *B. steartothermophilus* α-glucosidase, at least in the present study was inhibited by relatively non-polar constituents of CCAL extracts. Based on the findings of other similar studies, candidate phytochemicals responsible for α-glucosidase inhibition, observed in the current study will include terpenoids and non-polar flavonoids (Andrade-Cetto et al., 2008; Hara and Honda, 1990; Ortiz-Andrade et al., 2007). Rabbit hepatic G6Pase was moderately inhibited in the current study by both aqueous and methanolic extracts of *C. anisata* (58 and 56%, respectively). Thus, like human urinary α-amylase, rat hepatic α-glucosidase is inhibited by relatively polar, hydrophilic constituents of *C. anisata* leaf extracts.

The potency of a crude plant extract or a purified phytochemical as an inhibitor of a particular enzyme is often evaluated in terms of its IC₅₀ value when compared with that of a reference inhibitor of the same enzyme (Burlingham and Widlanski, 2003). In the current study, the IC₅₀ values of CCAL extracts as inhibitors of human urinary α-amylase and *B. steartothermophilus* α-glucosidase were determined and compared to those of acarbose, a known inhibitor of both enzymes. The aqueous and methanolic extracts of *C. anisata* leaves were found to be less potent inhibitors of human urinary α-amylase (IC₅₀ values of 1947 and 2436 μg/ml, respectively) than acarbose (IC₅₀ = 84 μg/ml). The CCAL acetone and hexane extracts were also found to be less potent inhibitors of *B. steartothermophilus* α-glucosidase (IC₅₀ values 1020 and 2068 μg/ml, respectively) than acarbose (IC₅₀ = 36 μg/ml). The IC₅₀ values of CCAL aqueous (494 μg/ml) and methanol (1012 μg/ml) extracts as inhibitors G6Pase were significantly lower than sodium vanadate (1651 μg/ml) (P < 0.05), an observation which suggests that these extracts are more potent inhibitors of G6Pase than sodium vanadate.

Inhibitors of enzymes obeying Michaelis-Menten kinetics are often characterized in terms of their effects on the kinetic constants, Kᵐ and Vₘₐₓ using either Lineweaver-Burk plots or Dixon secondary plots (Burlingham and Widlanski, 2003). In the current study, CCAL aqueous and methanolic extracts demonstrated non-competitive (Vₘₐₓ decreased whereas Kᵣ remained the same) mode of inhibition against both human urinary α-amylase and rat hepatic G6Pase. These observations suggest that the α-amylase and G6Pase inhibitory components of CCAL extracts do not resemble the normal substrates of the enzymes in structure (Smith et al., 2005; Burlingham and Widlanski, 2003). On the other hand, *C. anisata* acetone extract competitively inhibited *B. steartothermophilus* α-glucosidase. This observation suggests that α-glucosidase inhibitory components present in the CCAL acetone extracts could resemble the normal substrates of this enzyme in structure (Vanable and Aschenbrenner, 2007).

In vitro enzyme inhibitory activities are not always applicable in vivo (Yoon and Robyt, 2003; Yoon et al., 2004). Therefore, it is necessary to confirm the observed in vitro inhibitory effect CCAL extracts on α-amylase and α-glucosidase in vivo. Thus, postprandial blood glucose levels were determined in normal and STZ-induced diabetic rats after oral administration of both potato starch and maltose. Acetone was chosen as an extractant of choice for in vivo experiments, because it is known to extract both polar and non-polar compounds from plant materials (Jones and Kinghorn, 2005; Eloff, 1998). However, the acetone extract of *C. anisata* leaves failed to suppress the rise in postprandial glucose level after carbohydrate ingestion. This observation suggests that the in vitro inhibitory activities of both the aqueous and acetone extracts of *C. anisata* leaves are not applicable in vivo.

Conclusions

*C. anisata* leaf aqueous extract was a more potent inhibitor of rat hepatic G6Pase than sodium vanadate. Further studies are needed to characterize this extract and to isolate G6Pase inhibitory components. Although, CCAL acetone extracts strongly inhibited both human urinary α-amylase and *B. steartothermophilus* α-glucosidase in vitro, CCAL aqueous acetone extract failed to suppress PPGH in normal and STZ-induced diabetic rats. Thus, this study proves that the previously reported hypoglycaemic activity of CCAL extract (Jones and Kinghorn, 2005) could not be attributed to its inhibition of digestive α-glucosidase enzymes, and hence may not suppress postprandial glucose levels.

ACKNOWLEDGEMENTS

The authors are grateful for the financial assistance from the Department of Physiology, University of Limpopo and the National Research Foundation (NRF). LJ Shai is a recipient of an NRF grant under the Thutuka-REDIBA programme.

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