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Original Article

IN VITRO AND *IN VIVO* ANTIHYPERGLYCEMIC EFFECT OF ACTIVE FRACTION OF *CLEOME RUTIDOSPERMA* DC

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ABSTRACT

Objective: The present study was aimed to investigate the possible mechanism of action of the active sub fraction of *C. rutidosperma*. This was done by *in vivo* and *in vitro* methods.

Methods: Aqueous extract of *C. rutidosperma* was sequentially fractionated and re-fractionated to yield the active sub fraction (based on *in vivo* evaluation). In the *in vivo* study, the active sub fraction was administered to streptozotocin-induced diabetic mice at graded doses in different prandial states; while for the *in vitro* method, assessment of the possible effects of the active sub fraction of the plant on glucose absorption by excised ileum as well as its effect on α-glucosidase, α-amylase and glucose 6 phosphatase activities was evaluated.

Results: There was significant reduction of blood glucose level by the active sub fraction at different prandial states. The active sub fraction produced comparable hypoglycemic effects as glibenclamide. Likewise, at the doses of 125 and 62.5 mg/kg b. w., it significantly ($p<0.05$) reduced postprandial hyperglycemia after administration. Glucose tolerance was significantly ($p<0.05$) improved in the presence of the active sub fraction to varying degrees. The active sub fraction of *C. rutidosperma* did not demonstrate inhibitory effects on glucose movement into external solution across the rats excised ileum except at 50g/L and 25g/L where a significant ($p<0.05$) inhibition of glucose movement was observed. Alpha-amylase inhibitory activity of the active sub fraction was found to be 22. 24% at the lowest concentration of 2.50 mg/ml while the highest concentration of 20.00 mg/ml gave 46.66% inhibition. On the other hand, acarbose (standard) showed inhibition of 16.00 % and 34.43% for its lowest and highest concentrations for α-amylase respectively. Similarly, the α-glucosidase inhibitory activity results were: 16.42% and 44.56% for *C. rutidosperma* and acarbose respectively for the lowest concentration of 2.50 mg/ml, while the highest concentration of 20mg/ml had 64.22% and 87.09% for *C. rutidosperma* and acarbose respectively. Also, the active sub fraction of the plant showed significant inhibitory activity against glucose 6-phosphatase in a dose dependent fashion.

Conclusions: The results revealed that the possible antihyperglycemic mechanism of action of the plant may be by reduction of postprandial hyperglycemia and improvement of glucose tolerance activity ; Inhibition of α-amylase and α-glucosidase enzymes in small intestine and consequently slowing down the absorption of carbohydrates from the gut.. Consequently, *C. rutidosperma* may be used as a dietary adjunct to counter hyperglycemia and it has the potential to be developed as new oral antidiabetic agent for the treatment of diabetes mellitus.

Keywords: Alpha amylase, Alpha glucosidase, *C. rutidosperma*, Glucose 6-phosphatase, Glucose Tolerance, Hypoglycemia, Postprandial – antihyperglycemia.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic disorder of carbohydrate, fat and protein metabolism that results from defects in both insulin secretion and/or insulin action and leads to hyperglycemia, lipoprotein abnormalities, raised basal metabolic rate, defect in enzymes and high oxidative stress induced damage to pancreatic beta cells [1]. The disease is associated with reduced quality of life and increased risk factors for morbidity and mortality. The long term hyperglycemia is an important factor in the development and progression of micro- and macrovascular complication, which include cerebrovascular diseases [2, 3], neuropathy, nephropathy and cardiovascular degeneration [4]. Several distinct types of diabetes mellitus exist and are caused by a complex interaction of genetics and environmental factors [5].

From ancient period, people have been using medicinal plants for the treatment of diabetes and WHO estimates that 80% of the World's population presently uses herbal medicine for primary health care [6]. Antidiabetic plants have the ability to restore the function of damaged pancreatic tissue by increasing the insulin or inhibiting the intestinal absorption of glucose [7].

According to the WHO, there were 150 million diabetic patients worldwide by the year 2000, with a projection of 221 million people in 2010 and 300 million in 2025. The number of people with diabetes in urban areas is 172 million while 119 million live in rural areas. By 2030, the difference is expected to widen with 314 million people living in urban areas and 143 million in rural areas [8].

Traditional plant remedies have been used for centuries in the treatment of diabetes, but only a few have been scientifically evaluated [9]. The search for new agents with lower cost and better efficiency has therefore become a matter of major priority. The great number of plants used to manage diabetic patients in Africa might provide a useful source for the discovery of new compounds that can be used as pharmaceutical entities or simple dietary adjuncts to existing therapies [10]. Consequently, the current focus for appropriate anti-diabetic agents is herbal medicine. There is, however, a need for more in-depth investigation to confirm and advocate the benefits of these plants over existing therapies, including elucidation of their mechanism(s) of action and therapeutic effects, as the anti-diabetic evidence is often anecdotal [11].

Cleome rutidosperma DC is a low-growing herb, up to 70 cm tall, found in waste grounds and grassy places with trifoliate leaves and small, violet-blue flowers, which turn pink as they age. The plant is native to West Africa, from Guinea to Nigeria, Zaire and Angola. It has become naturalized in various parts of tropical America as well as Southeast Asia [12]. *Cleome rutidosperma* has been well studied by different researchers. The analgesic, antipyretic, anti-inflammatory, locomotory, antimicrobial, diuretic, laxative antioxidant, and antiplasmodial activities of the plant have already been reported [13-19]. *Cleome rutidosperma* is traditionally used in the treatment of paralysis, epilepsy, convulsions, spasm, earache, pain and skin disease [20].

Cleome rutidosperma has been shown to possess antidiabetic activities [21]. The antidiabetic activity of this plant has also been demonstrated in our previous studies [22]. However, mechanisms of action whereby this plant exert its blood glucose lowering effects on tissue or organs is unknown. Therefore, the present study was aimed at investigating the possible mechanism of action of the plant through *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Reagents and chemicals

All reagents and chemicals used were of analytical grade purchased from Sigma Chemical Company, St. Louis, Missouri, USA.

Plant materials

The plant (*Cleome rutidosperma*) was collected from Abraka in Delta State, Nigeria. It was identified at the Herbaruim Unit, Department of Plant Science, University of Benin, Benin City, Edo State-Nigeria, where a voucher specimen was deposited with number: UBHc0148.

Experimental animals

Male Wistar Albino rats weighing 120-200g and mice weighing 25-35g were obtained from National Veterinary and Research Institute (NVRI) Vom, Jos, Plateau State, Nigeria. The animals were allowed 3 weeks of acclimatization before commencement of experiment. They were fed on standard laboratory diet (Vital Feed Nig. Ltd, Jos Nigeria) and water *ad libitum* throughout the experiment. The animals were used according to the Guidelines of National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and in accordance with the principles of Good Laboratory Procedure (GLP) [23].

Treatment and extraction of plant samples

The plant sample was washed with distilled water and air-dried at room temperature, cut into small pieces and pulverized into fine powder using pestle and mortar. It was first extracted with petroleum ether and then distilled water by cold maceration method. Fifty grams(50g) powder of the plant was soaked in 200 ml of distilled water in airtight conical flask with daily shaking for three days at room temperature and was first filtered through double layered muslin cloth and then filtered through Whatman No 1filter paper. The filtrate was collected and concentrated at 50^{0}C using water bath and the dried extract was stored in the refrigerator at 4^{o}C till further use.

Fractionation of aqueous extracts

The crude aqueous extract of the plant was fractionated by initially dissolving 110 g of it in 500 mL of distilled water, mixed in a beaker and then filtered using No.1 Whatman filter paper. The filtrate was thereafter transferred into a 1 L separating funnel and extracted with 5 × 250 mL n-butanol. The combined n-butanol fraction was concentrated using water bath at 50 ^{0}C. The aqueous layer was sequentially extracted with 3 × 250 mL chloroform, 3 × 250 mL ethyl acetate and 3 × 250 mL methanol. The combined chloroform, ethyl acetate and methanol fractions were separately concentrated using water bath at 50 ^{0}C. Finally, the leftover aqueous fraction was also concentrated using water bath at 50 ^{0}C.

The methanol fraction was found to be most active fraction based on *in vivo* studies [24]. This fraction was further re-fractionated using silica gel-column chromatography (Qualikems, 60- 120 mesh). Four (4 g) grams of the methanol fraction was pre-adsorbed onto silica gel adsorbent (60–120 mesh) by firstly solubilizing it in little quantity of methanol, followed by addition of the silica gel (3 g) then mixing. The dried fraction-adsorbent mixture was then evenly loaded onto the top of the already packed column. The column was first eluted with 2 × 300 mL 100% chloroform followed successively by 2 × 300 mL chloroform- methanol in graded ratios (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and finally (0:10). Sub-fractions were collected and concentrated as described above.

The most active sub-fraction from *in vivo* studies [24] was: chloroform: methanol (5: 5). This active sub-fraction was used for the following assays:

Effects of plant sub fraction on glucose movement

A simple model system as described by Gallagher, *et. al.*, [25] was used to evaluate effects of plant sub fraction on glucose movement *in vitro*. The model for the present study involved the use of an excised ileum (6 cm) into which 2 ml of 0.15 M NaCl containing 0.22 mM D-glucose was added. The ileum was sealed at each end and placed in a 50 ml tube containing 45 ml of 0.15 M NaCl. The tube was placed on an orbital shaker (Balart Products, USA) and kept at 37^{0}C. The movement of glucose into the external solution was monitored at set time intervals. In the first series of experiments, the effects of 50 g/l plant sub fraction on glucose diffusion were compared to control tests conducted in the absence of plant sub fraction. At the end of the experimental period, the concentrations of glucose within the tubes were measured. A second experimental series investigated the concentration-dependent effects (6.25, 12.5, 25 and 50 g/l) of the plant sub fraction. All tests were carried out in triplicate. Glucose concentrations were measured using the glucose oxidase method of analysis.

Hypoglycemic and postprandial antihyperglycemic test

In the hypoglycemic test, mice were fasted 12 h prior to test whereas in the postprandial antihyperglycemic test, fed mice were fasted 1 h prior to test. After fasting period, the mice were given orally either plant sub fraction or Glibenclamide using a cannula. Blood was collected from the tail vein just before (0 h) and 2, 4 and 6 h after administration of sub fraction or Glibenclamide for the measurement of blood glucose concentration, which was done using Acute Check Active glucometer kit [26,27]. Five mice were used in each group.

Oral Glucose Tolerance Test (OGTT)

For OGTT evaluation, the mice were fasted for 12 h and blood was taken 30 min before administration of sub fraction of *C. rutidosperma* or Glibenclamide. Thirty minutes later, the mice from all groups were given glucose (1.5 g/kg) orally. Blood was collected from the tail vein just prior to glucose administration (0 min), and then 30, 60, 120 and 180 min after glucose loading for estimation of blood glucose using the glucometer. Also, five mice were used in each group.

Glucose 6-phosphatase inhibitory activity

Effect of the plant sub fraction on glucose-6-phosphatase activity was performed according to the method described by Baginsky *et. al.*, [28], using glucose-6-phosphatase from rabbit liver (Sigma, G5758). Briefly, 0.5 ml (25 units) of glucose 6-phosphatase (E. C. 3.2.3.2) was pre-incubated with 0.25 ml of plant sub fraction for 15 minutes at 37^{0}C. The reaction was then started by addition of 0.25 ml of 0.1 M glucose -6-phosphate in citrate buffer, pH 6.5. The reaction mixture was then incubated at 37^{o}C for 20 minutes. At the end of the incubation period, 2.0 ml of 2%(w/v) ascorbic acid and 10%(w/v)/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 compound whose absorbance was measured at 660 nm. A control was prepared using the same procedure except that the extract was replaced with distilled water and the glucose-6-phosphatase inhibitory activity was expressed as percentage inhibition. The glucose-6-phosphatase inhibitory activity was calculated using the equation:

% Inhibition = {(Ac – Ae)/Ac} 100

Where; Ac and Ae are the absorbances of the reaction mixture with distilled water (control) and the one with extract, respectively.

Alpha-amylase inhibitory assay

This assay was carried out using a modified procedure of McCue *et. al.*, [29]. A total of 250 µL of sub fraction (2.5 – 20 mg/ml) was placed into tubes and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing porcine α-amylase solution (0.5 mg/ml) was added. The contents of the tubes were pre-incubated at 25^{0}C for 10 min, after which 250 µL of 1 % starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added. The reaction mixtures were

incubated at 25^{0}C for 10 min. The reaction was terminated by adding 1 ml of dinitrosalicylic acid (DNS) reagent and further incubated in boiling water for 5 min and cooled to room temperature. The content of each test tube was diluted with 5 ml distilled water and the absorbance measured at 540 nm in a spectrophotometer. A control was prepared using the same procedure except that the extract was replaced with distilled water. Acarbose was used as reference. The α-amylase inhibitory activity was calculated using the equation:

% Inhibition = {(Ac – Ae)/Ac} 100

Where; Ac and Ae are the absorbances of the reaction mixture with distilled water (control) and the one with extract, respectively.

Alpha-glucosidase inhibitory assay

The effect of the plant extracts on α-glucosidase activity was determined according to the method described by Kim *et. al.*, [30]; using α-glucosidase from *Bacillus stearothermophilus*. The substrate solution p-nitropheynyl glucopyranoside (pNPG) (3.0 mM) was prepared in 20 mM phosphate buffer, pH 6.9. Alpha-glucosidase (100 µL of 1.0 U/ml) was pre-incubated with 50 µL of the different concentrations of the sub fraction for 10 mins. Then 50 µL of 3.0 mM pNPG (as a substrate) dissolved in 20 mM phosphate buffer (pH 6.9) was added to start the reaction. The reaction mixture was incubated at 37^{0}C for 20 mins and stopped by adding 2 ml of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the yellow colored para-nitrophenol released from pNPG at 405 nm. Acarbose was used as reference. Also, a control was prepared using the same procedure except that the extract was replaced with distilled water.

The results (% Inhibition) are expressed as percentage of the blank (control) as in the equation below:

% Inhibition = {(Ac – Ae)/Ac} 100.

Where; Ac and Ae are the absorbance of the reaction mixture with distilled water (control) and the one with extract, respectively.

Statistical analysis

The results are presented as mean ± SD and were analysed using ANOVA followed by Tukey kramer multiple comparison test in Graphpad Prism, version 6.0 0 (Graph Pad Software, San Diego, CA, USA). and values of P< 0.05 were considered significant.

RESULTS

The hypoglycemic effects of the sub fraction of *C. rutidosperma* was evaluated and the results (fig. 1) indicate that the sub fractions of the plant have hypoglycemic effect in a dose dependent manner.

The effects of sub-fraction of *C. rutidosperma* and glibenclamide on postprandial glucose concentration in normal mice are shown in fig. 2. The results showed that the sub fraction of the plant at the doses of 125 and 62.5 mg/kg b. w., significantly ($p<0.05$) reduced postprandial hyperglycemia after administration. Also, administration of the sub fraction to normal mice in the glucose tolerance test (fig. 3) brought about significant decrease in blood glucose level 180 min after administration.

Shown in fig. 4, are the effects of sub-fraction of *C. rutidosperma* on glucose uptake. The results indicate that only the dose of 50g/L of the sub fraction could significantly decrease glucose movement in this assay.

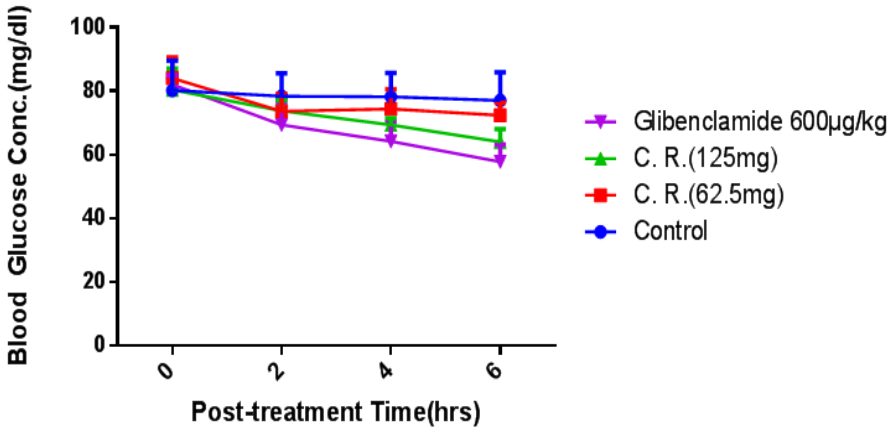

Fig. 1: Effects of sub-fraction of *C. rutidosperma* (C. R) and Glibenclamide on blood glucose level in normal mice

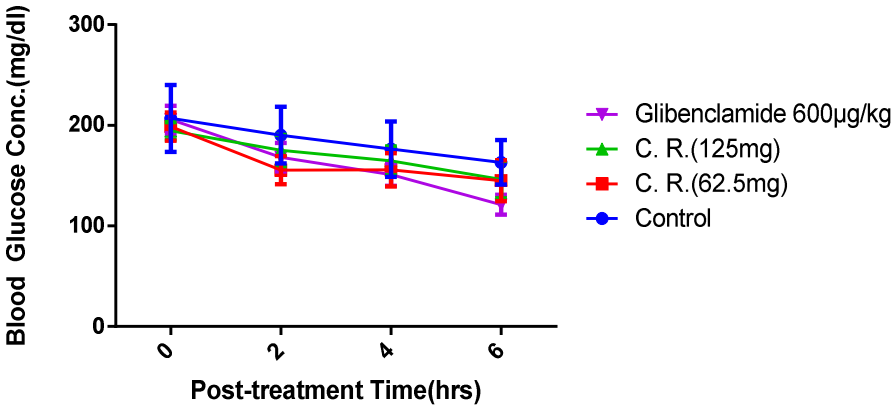

Fig. 2: Effects of sub-fraction of *C. rutidosperma* (C. R.) and Glibenclamide on postprandial glucose concentration in normal mice

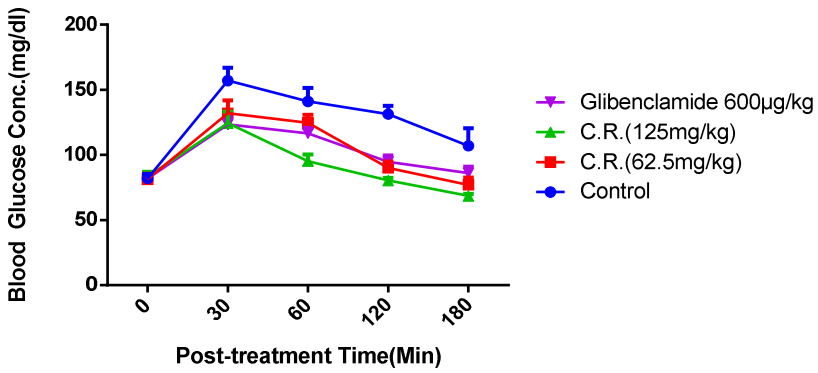

Fig. 3: Effects of sub-fraction of *C. rutidosperma* (C. R.) and Glibenclamide on glucose tolerance in normal mice

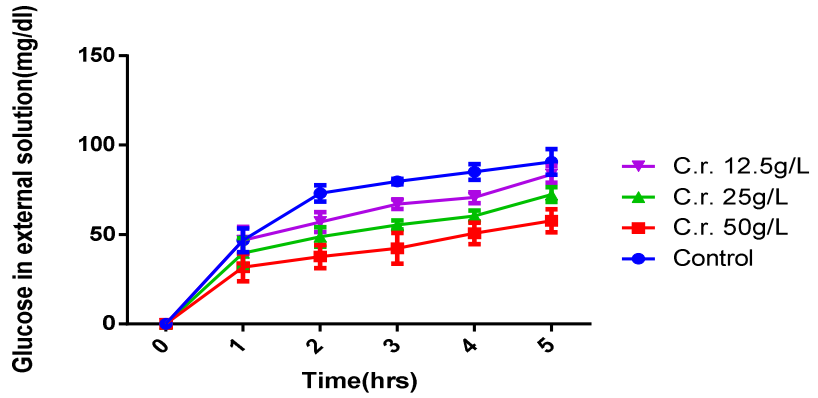

Fig. 4: Effects of sub-fraction of *C. rutidosperma* (C. r.) on glucose absorption by excised rat ileum

The percentage inhibition of α-amylase by sub-fraction of *C. rutidosperma* and acarbose is shown in fig. 5. There was significant inhibition ($p < 0.05$) displayed by sub fraction the plant in a dose dependent manner. The maximum inhibition of this enzyme by sub fraction of *C. rutidosperma* was 46.66%, while acarbose had 34.43% inhibition at the concentration of 20 mg/mL.

Also, the sub fraction of the plant demonstrated a dose dependent % inhibition of α-glucosidase as illustrated in fig. 6. At the highest concentration of the sub fraction and acarbose (20 mg/ml), the inhibitory activities were: 64.22% and 87.09% for *Cleome rutidosperma* and acarbose respectively.

Similarly, fig. 7 shows the percentage inhibition of glucose 6-phosphatase by sub-fraction of *C. rutidosperma*. From the result, it is seen that the plant sub fraction demonstrated good glucose 6-phosphatase inhibitory activities at the lowest concentration of 2.50 mg/ml and the highest concentration of 20 mg/ml. A dose dependent inhibition pattern of this enzyme was shown by the plant.

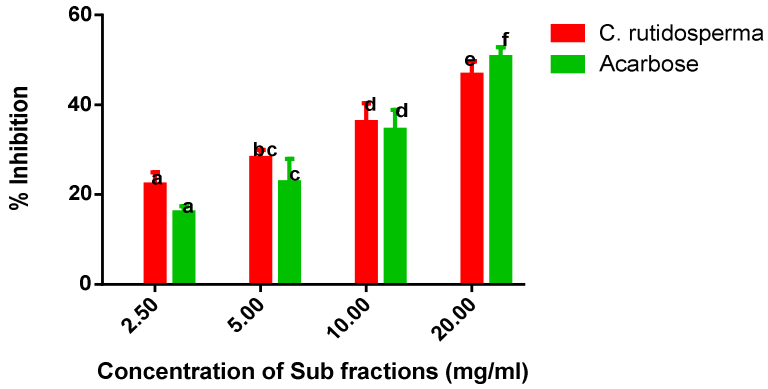

Fig. 5: Percentage Inhibition of α-amylase by Sub-fraction of *C. rutidosperma* and Acarbose.
***Bars with different letters differ significantly ($p<0.05$)**

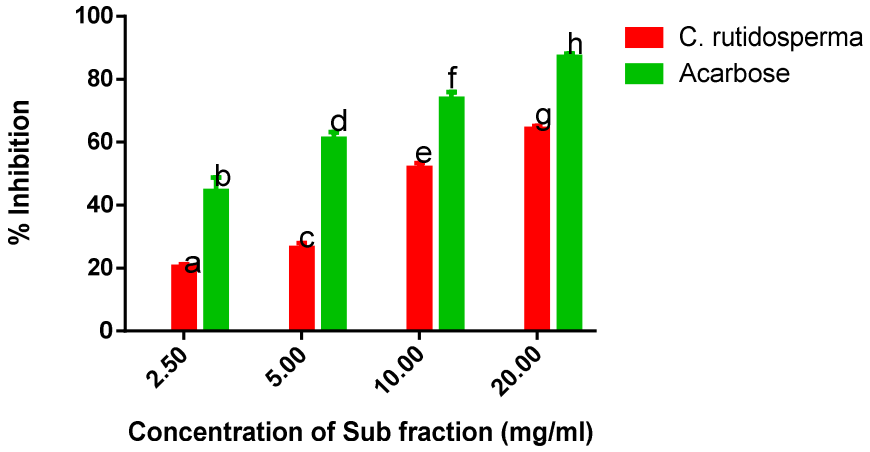

Fig. 6: Percentage inhibition of α-glucosidase by sub-fraction of *C. rutidosperma* and Acarbose.
***Bars with different letters differ significantly (p<0.05)**

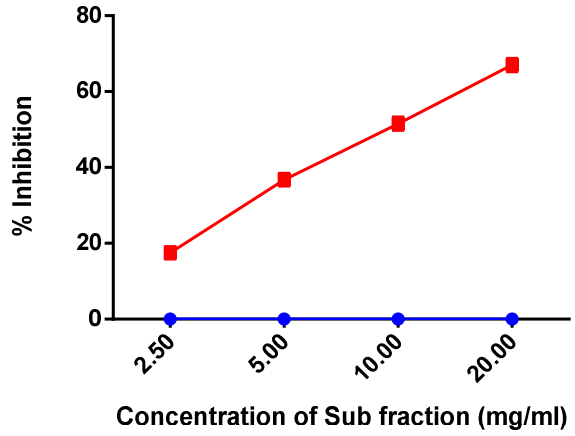

Fig. 7: Percentage Inhibition of Glucose 6-phosphatase by Sub-fraction of *C. rutidosperma*

DISCUSSION

The use of herbal medicine is a common practice in many countries, particularly in Asia [31] and Africa [32]. The currently available drug regimens for management of diabetes mellitus have certain drawbacks and therefore, there is a need to find safer and more effective antidiabetic drugs [33, 34].

In this study, the evaluation of antihyperglycemic effect of sub fractions of *C. rutidosperma* was evaluated in different prandial states. Hypoglycemic test in fasting state was done to evaluate whether the sub fraction have tendency to produce severe hypoglycemia. The test on postprandial antihyperglycemia was done to evaluate the potential of the sub fractions to challenge postprandial hyperglycemia whereas post – glucose – load antihyperglycemia test was done to evaluate the potential of sub fraction to improve glucose tolerance. Significant (p<0.05) reduction of blood glucose level by sub fraction of *C. rutidosperma* at different prandial states were observed from the results. The control of postprandial hyperglycemia is one of the beneficial therapy for management of type II diabetes mellitus [30] along with nutrition, oral hypoglycaemics and insulin therapies [35]. In addition, controlling postprandial hyperglycemia could also prevent the development of macro-and microvascular complications associated with diabetes [36]. The use of plants with postprandial antihyperglycemic property such as *Mucuna pruriens* [37] and *Cynara cardunculus* [38] as well as plants with potential of improving glucose tolerance such as *Helicteres ixora* [39] and *Tournefortia hartwegiana* [40] may benefit the diabetic patient in controlling postprandial hyperglycemia.

In the evaluation of postprandial antihyperglycemia, the sub fraction at low and moderate dose of 125 mg/kg b. w. significantly reduced blood glucose after 4 and 6 h of administration (fig. 2). The postprandial antihyperglycemic effects of the plant was comparable to that of glibenclamide. In the oral glucose tolerance study, all doses of the sub fraction significantly attenuated glucose concentration when compared to the control group. At 30 min after glucose administration, blood glucose level increased rapidly from the fasting value to a peak and then subsequently decreased. The sub fraction exhibited remarkable blood glucose lowering effect at 90 min. This suggests that sub fraction of *C. rutidosperma* have the ability to improve glucose tolerance in normal mice. Moreover, these activities were even more effective than Glibenclamide as shown in fig. 3. This observation indicates that the glucose tolerance activity produced by the sub fraction was more potent than Glibenclamide.

Management of blood glucose level is a critical strategy in the control of diabetes and its complications [41]. Alpha-amylase and α-glucosidase inhibitors have been useful as oral hypoglycemic drugs for the control of hyperglycemia in diabetes mellitus patients. Inhibition of these enzymes delay carbohydrate digestion and prolong overall carbohydrates digestion time causing a reduction in the rate of glucose absorption and consequently reducing postprandial plasma glucose rise [42].

The moderate inhibition of α-amylase by the sub fractions of *C. rutidosperma* at the highest concentration of 20mg/ml indicates that the plant is a mild inhibitor of this enzyme, which is a desirable effect over synthetic drugs with very high inhibitory effects. This observation is in agreement with the report of Pinto *et. al.,* [43] that dietary management of hyperglycemia linked to diabetes can be targeted through foods or botanical supplements that have moderate α-amylase inhibition.

As for α-glucosidase inhibition, inhibition of the enzyme displayed by sub fraction of the plant at the highest concentration of 20mg/ml as shown in fig. 2, suggests that the sub fraction contain potent α-glucosidase inhibitor. This is a beneficial effect of the plant because any plant or drug which is a strong inhibitor of α-glucosidase could serve as effective therapy for postprandial hyperglycemia with minimal side effects [44].

Results of the present investigation indicated that *C. rutidosperma* exhibited appreciable inhibitory activity towards glucose-6-phosphatase activity in a dose dependent manner. This activity of

the plant may be an advantage over synthetic drugs that inhibit the enzyme, such as sodium orthovanadate and sodium tungstate with their associated toxicity [45,46]. To our knowledge, the present study demonstrates for the first time the potent inhibitory activities of this plant on these enzymes.

Hepatic glycogenolysis and gluconeogenesis are major causes of fasting hyperglycemia seen in both type 1 and type 2 diabetes mellitus [47]. Inhibition of enzymes involved in gluconeogenesis and/or glycogenolysis therefore constitutes an alternative approach to suppress hepatic glucose production and lower fasting plasma glucose [48]. Glucose-6-phosphatase (G6Pase) catalyses the final reaction in hepatic glucose production by both gluconeogenesis and glycogenolysis, and has been proposed as a potential target for antihyperglycaemic drugs for type-2 diabetes [49,48].

On the basis of the above results therefore, we suggest that the mechanisms by which the plant extracts and fractions mediate their antihyperglycemic activity may be: (a) Reduction of postprandial hyperglycemia and improvement of glucose tolerance activity (b) Inhibition of glucose 6-phosphatase thereby causing a reduction of hepatic gluconeogenesis and glycogenolysis and (c) Inhibition of α-amylase and α-glucosidase enzymes in small intestine and consequently slowing down the absorption of carbohydrates from the gut.

CONCLUSION

Active sub fraction of *C. rutidosperma* at the doses of 125 and 62.5 mg/kg b. w., significantly reduced postprandial hyperglycemia particularly after the 4 and 6 h of administration. Also, Glucose tolerance activity was significantly improved in the presence of sub fraction of the plant. Sub fraction of the plant demonstrated good α-glucosidase, α-amylase and glucose 6-phosphate inhibitory activities. Therefore, *C. rutidosperma* would be promising candidate for the treatment of diabetes.

CONFLICT OF INTERESTS

Declared None

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