WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 7, 1038-1048. Research Article ISSN 2277–7105

AN INVITRO STUDIES OF CARALLUMA ATTENUATE PHYTOCHEMICALS AS INHIBITORY OF KEY ENZYMES RELEVANT FOR DIABETIC

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Article Received on
25 April 2017,

Revised on 15 May 2017,
Accepted on 05 June 2017

DOI: 10.20959/wjpr20177-8743

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ABSTRACT

Medicinal and natural herbal plant products are traditionally used from long time in many countries for the treatment of diabetes mellitus. The present study was designed to testify the potential anti-diabetic activity of flavonoid fraction of *Caralluma attenuata* by using *in-vitro* enzyme assay. The results of the invitro α-amylase and α-glucosidase inhibition test displayed that *Caralluma attenuata* flavonoid fraction had a similar inhibitory effect with that of the standard drug acarbose. Different concentration of flavonoid fraction of *Caralluma attenuata* showed a remarkable inhibitory activity, compared with the normal control group. To evaluation of invitro antidiabetic activities *Caralluma attenuate.*

KEYWORDS: *Caralluma attenuata*, Glucose, yeast cells, α-amylase and α-glycosidase.

INTRODUCTION

Medicinal plants have been part of the great healing traditions around the world going back thousands of years. The worldwide incidence of diabetes has risen in the past two decades. Many indigenous drugs have been used by the practitioners of the Ayurvedic system for the treatment of diabetes mellitus in India (Shirwaikar *et al*., 2005). Finding healing power in plants is an ancient idea (Middha *et al*., 2009). Biological actions of the plant products used as alternative medicines to treat diabetes are in relevance to their chemical composition.

Herbal products or plant products are rich in flavonoids, phenolic compounds, coumarins, terpenoids and other constituents which help to reduce blood glucose levels (Jung *et al*., 2006). Several species of herbal drugs with potential antidiabetic activity have been described in the scientific literature. Herbal drugs are prescribed due to their good effectiveness, fewer side effects in clinical experience and relatively low costs (Ren *et al*., 1997). Medicinal and natural herbal plant products are traditionally used from long time in many countries for the treatment of diabetes mellitus.

Bio-flavonoids are well-known for their multi-directional biological activities including anti-diabetic efficacy (Brahmachari, 2009). Type II diabetes mellitus is a heterogeneous disorder due to a combination of inherited and acquired factors that adversely affect glucose metabolism. It is thought that these factors lead to diabetes mainly by affecting β-cell function and tissue insulin sensitivity. This may occur because release is increased, because uptake is reduced, or due to a combination of factors such as increased release with a lesser increase in uptake (Gerich, 2000). In the normal individual, the concentration of glucose in blood is maintained at about 90 mg/dL of plasma. However, fasting blood glucose in diabetics may be 300-400 mg/dL and may even reach 1000 mg/dL (Johnson, 1998).

Type II diabetes is associated with insulin resistance initially and later, as the function of the β-cell decreases, insulin deficiency (Cerasi, 2000). Type II diabetes is characterized both by abnormalities of insulin secretion progressively leading to secretion failure as well as insulin resistance of all major target tissues (Haring 1999). Although insulin resistance is important in the early stages of type II diabetes, the failure in adequate β-cell compensation leads to the progression to the diabetic state. Compensation for insulin resistance is through increased secretion per β-cell or by an increase in β-cell mass through neogenesis or replication of the existing β-cells (Withers *et al*., 1998). Beta-cell mass is normally tightly maintained through a balance of β-cell birth (β-cell replication and islet neogenesis) and β-cell death through apoptosis. Most of the increase in β-cell mass with insulin resistance is probably due to increased β-cell number, but β-cell hypertrophy may also contribute (Weir and Bonner-Weir, 2004).

Plants and the treatment of Diabetes Mellitus

The World Health Organisation (WHO) defines traditional medicine as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques applied singularly or in combination to treat, diagnose

and prevent illness or maintain well-being. In 2002 WHO launched its first comprehensive traditional medicine strategy to assist efforts to promote affordable, effective and safe use of traditional medicine and complimentary alternative medicine. The potential anti-hyperglycaemic activity might be due to the large presence of saponins in *Caralluma attenuata* (Su *et al*., 2009). Saponin from *T. terrestris* reported to possess hypoglycemic properties and produced protective effect in streptozotocin -induced diabetic rats by inhibiting oxidative stress (Amin *et al*., 2006).

MATERIALS AND METHODS

Collection and preparation of plant extracts

Stem of *Caralluma attenuata* were obtained from Sri Sairam Siddha Medical College and Research Centre, Saileo Nagar, West Tambaram, Chennai-44. The plants authenticated identification done by Dr. S. Sankaranarayanan, Asst. professor, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai-600 106 Tamil Nadu, India.

Phytochemical analysis of *Caralluma attenuata*

Flavonoid extraction from *Caralluma attenuata*

The herb (10 g) was extracted exhaustively with 70% aqueous methanol, combining maceration (24 h) with subsequent extraction at 60°C. The aqueous methanol extracts were evaporated in vacuum to a thick residue and left for 10 -12 h at 5–10°C. The dark green resinous solid was separated by filtration, treated with hot water, cooled, and filtered. The purified aqueous solution was extracted successively with EtOAc, and *n*-BuOH (Kovalev, 2009).

Glucose uptake in Yeast cells

The commercial baker's yeast in distilled water was subjected to repeated centrifugation (3,000×g, 5 min) until clear supernatant fluids were obtained and a 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of plant extracts (5-20µg/mL) were added to 1mL of glucose solution (5, 10, 15 and 25 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100µL of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 × g, 5 min) and amount of glucose was estimated in the supernatant (Cirillo, 1961). Metronidazole was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula.

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

α- amylase inhibition activity of flavonoid rich fraction from *Caralluma attenuata*

Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitory activity was based on the starch iodine method that was originally developed by (Hamdan and Fatimai 2010) and later employed by others for determination of amylase activity in plant extracts with some modifications. In alpha amylase inhibition method 1ml substrate- potato starch (1% w/v), 1 ml of methanol extract and ethyl acetate of different concentration such as 5, 10, 15 and 20 µg/ml, 1ml of alpha amylase enzyme (1% w/v) and 2ml of acetate buffer (0.1 M, 7.2 pH) was added.

$$\text{Inhibition of alpha- Amylase (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

α-glucosidase Inhibitory activity of flavonoid rich fraction from *Caralluma attenuata*

The α-glucosidase inhibitory activity was assessed by the standard method (Dong *et al*., 2012), with slight modifications. Briefly, a volume of 60µl of sample solution and 50 µl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plates at 37 ºC for 20 min. After pre-incubation, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 ºC for another 20 min. Then the reaction was stopped by adding 160 µl of 0.2 M NaCO3 into each well, and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had 60 µl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows.

$$\text{Inhibition Percentage-} \quad \frac{A_0 - A_1}{A_0} \textbf{X100}$$

Where, ACO is absorbance of the control and at is absorbance of the sample the concentration of inhibitors required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

RESULTS AND DISCUSSION

Many research and investigations of oral anti-hyperglycaemic agents of natural plant origin were used in traditional medicine have been studied and many of them have been found to posess the positive activity (Kirti *et al.,* 2008). Diabetes mellitus is a syndrome of imbalenced carbohydrate, fat and protein metabolism, caused due to either lack of insulin secretion or decreased sensitivity of the tissues to insulin, and is characterized by chronic hyperglycaemia (Setter *et al*., 2000; Shirwaikar *et al*., 2005).

Total flavonoid content of stem extract of *Caralluma attenuata*

In this context, the preliminary experiments revealed that 80% methanol was the best solvent for the extraction of flavonoids from *Caralluma attenuata* at 60 °C for 60 min since it afforded a maximum yield of flavonoids. The yields stem of *Caralluma attenuata* extracts ranged from 43% (w/w). Therefore, the total phenolic contents were reported as rutin equivalents (Table-1).

Table 1: Total flavonoid content *Caralluma attenuate* stem extract.

Sample	Yield of extract (g/100 g of defatted Content)	Total flavonoid content (mg rutin equivalents per gram flavonoid rich fraction)
Flavonoid exctract of *Caralluma attenuata*	42.1±1.7[a]	127.2±1.3[b]

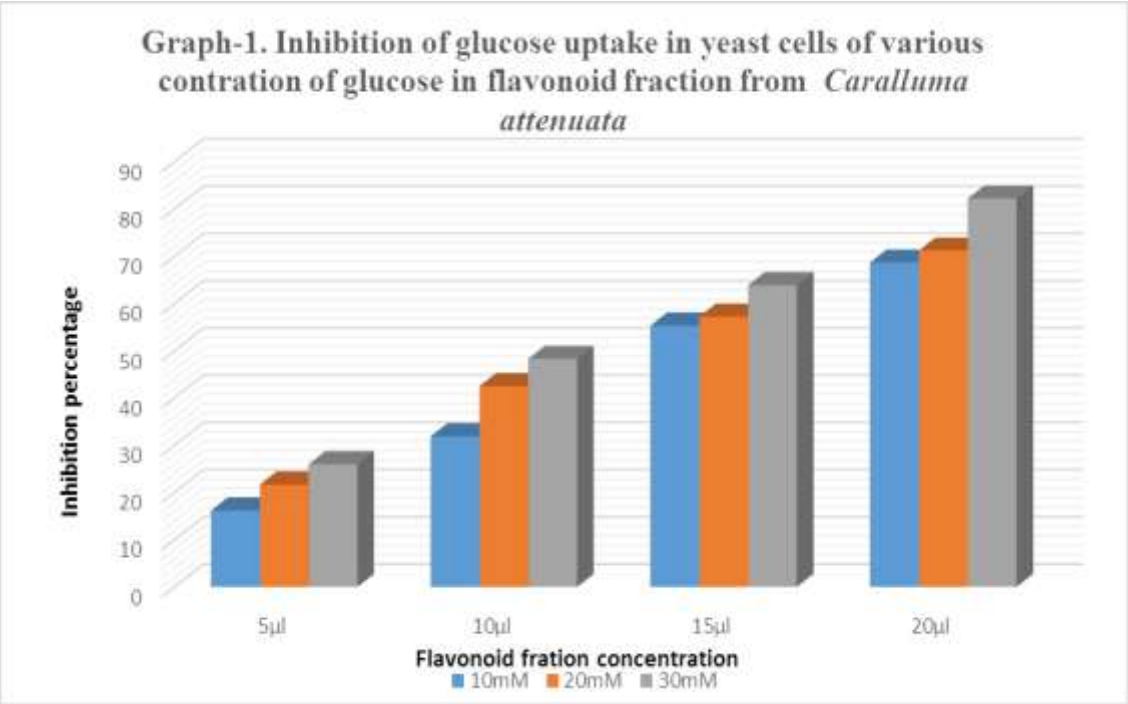
[a]Data are expressed as mean ± standard deviation (n = 3) on a fresh weight basis.

[b]Means in each column sharing the same letter are not significantly (P = 0.05) different from other.

Glucose uptake in Yeast cells of flavonoid fraction of *Caralluma attenuata*

The rate of glucose transport across cell membrane in yeast cells system is presented in "Graph-1". The amount of glucose remaining in the medium after a specific time serves as an indicator of the glucose uptake by the yeast cells. The rate of uptake of glucose into yeast cells was linear in all the 3 glucose concentrations. The flavonoid fraction of *Caralluma attenuata* exhibited significantly higher activity than at all concentrations. However the

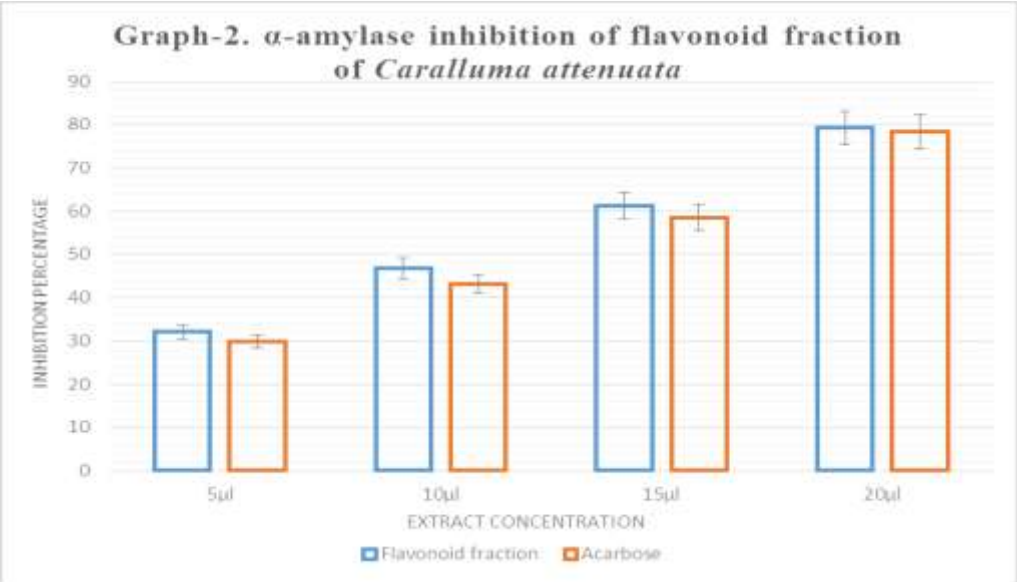
highest uptake of glucose was seen in 20mM Glucose concentration. The result showed the lower uptake of glucose by the yeast cells which conformed the highest activity.



"Graph-1". Glucose uptake in Yeast cells of flavonoid fraction of *Caralluma attenuate*

α-amylase inhibition of flavonoid fraction of *Caralluma attenuata*

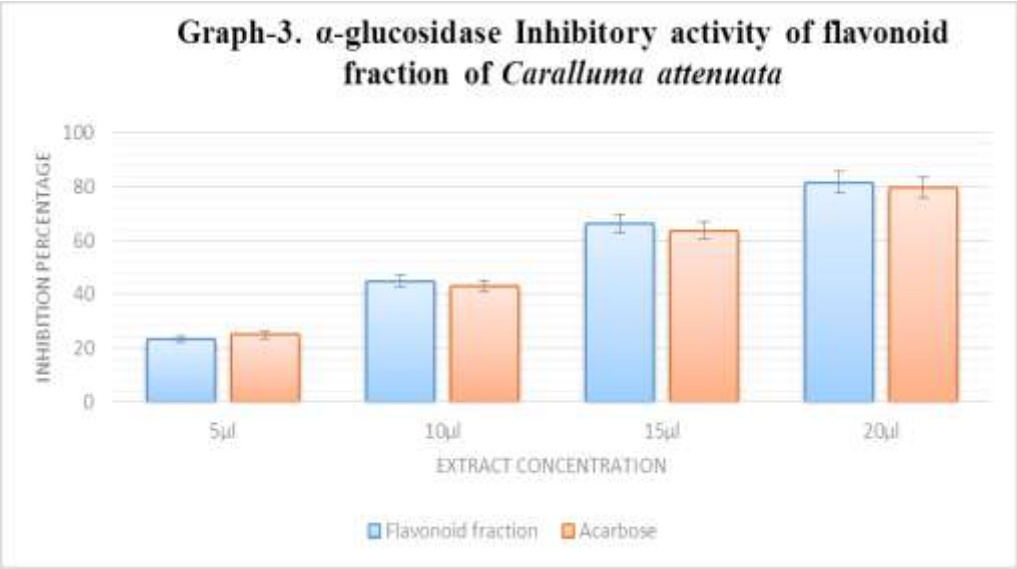
Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitors bind to alpha- bond of polysaccharide and prevent break down of polysaccharide in to mono and disaccharide. In present experimental study was observed that flavonoid fraction of *Caralluma attenuata* demonstrated significant Alpha amylase inhibition activity as compared to standard drug acarbose (Graph-2).



"Graph-2" α-amylase inhibition of flavonoid fraction of *Caralluma attenuate.*

α-glucosidase Inhibitory activity of flavonoid fraction of *Caralluma attenuata*

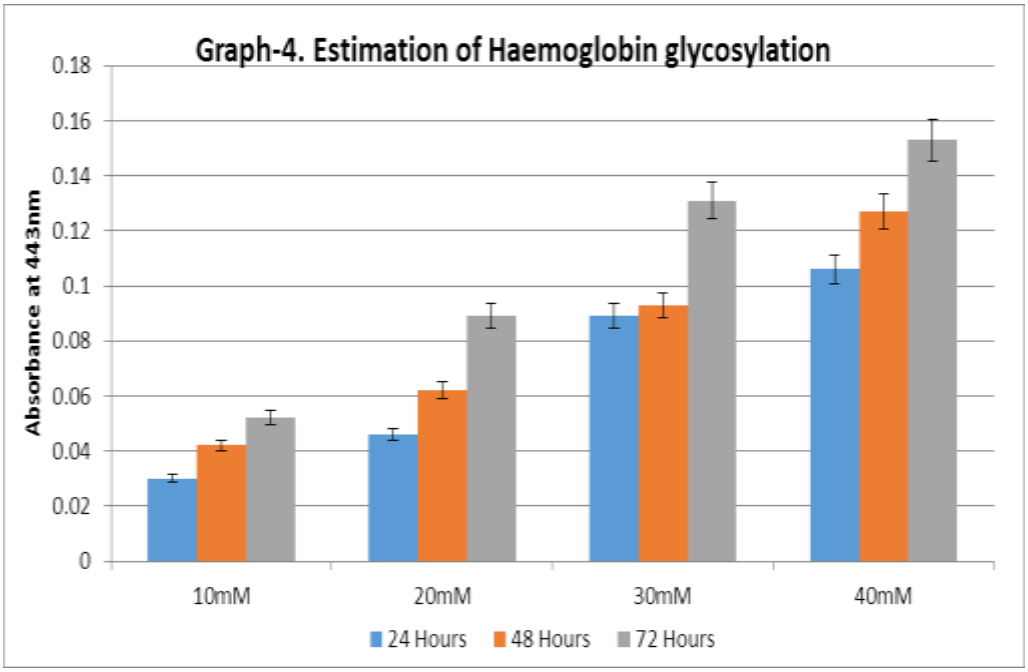
The results of *in-vitro* α-glucosidase inhibitory study are showed in "Graph-3". The flavonoid fraction of *Caralluma attenuata* showed a concentration-dependent inhibition of enzyme. The highest concentration of 20 µl/ml tested showed a maximum inhibition of nearly 86.71% flavonoid fraction of *Caralluma attenuata* seems to be less potent in α-glucosidase inhibitory potential compared to Acarbose. It may be that α-glucosidase is more sensitive towards Acarbose with the concentration required for 50% inhibition (IC_{50}) found to be 2.09 mg/ml.



"Graph-3". α-glucosidase Inhibitory activity of flavonoid fraction of *Caralluma attenuate.*

Estimation of haemoglobin glycosylation

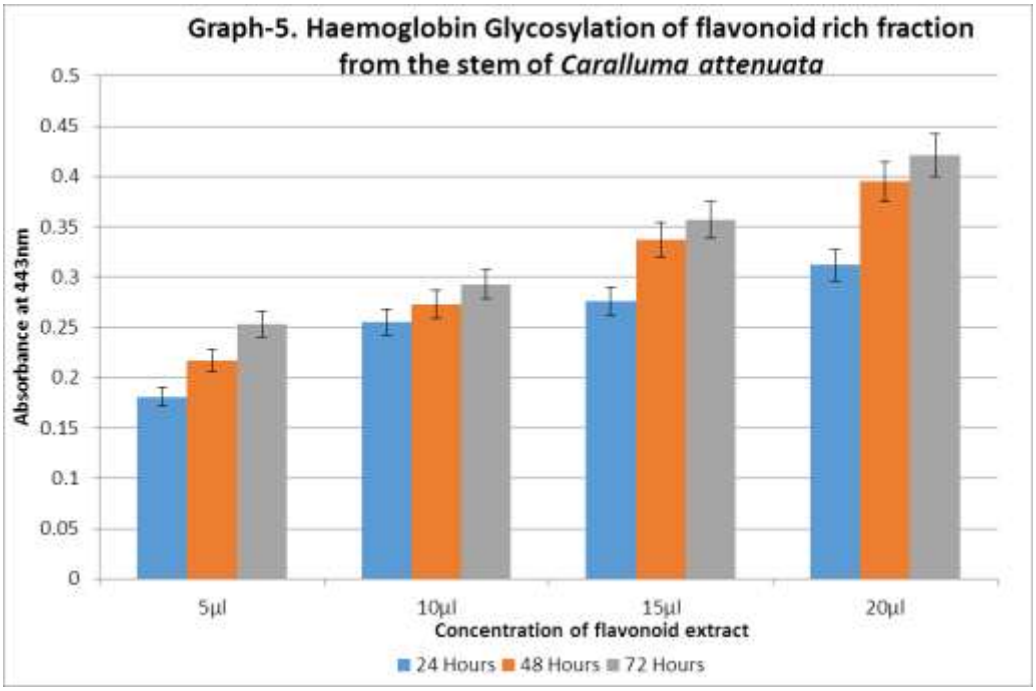
Increased meditation of glucose in the blood leads to its binding to hemoglobin which may result in the formation of the reactive oxygen species. Plant extracts play an important role the inhibition of the glycosylation end products. An increase in the glycosylation was observed on incubation of hemoglobin with the increasing concentration of the glucose (5mM, 10mM, 15mM and 20mM) over a period of 72hrs (Graph-4).



"Graph-4". Estimation of Haemoglobin glycosylation of *Caralluma attenuate.*

Haemoglobin Glycosylation of flavonoid rich fraction from the stem of *Caralluma attenuate.*

The flavonoid rich fraction of *Caralluma attenuata* exhibits potent anti-diabetic activity. The percentage inhibition of glycosylation was found to be dose dependent. As the concentration of drug increases formation of glucose-hemoglobin complex decreases and free haemoglobin increases, which show the inhibition of glycosylated haemoglobin. The present experimental study was observed that synergic effect of *Caralluma attenuata* flavonoid extract demonstrated significant activity (Graph-5).



"Graph-3". Haemoglobin glycosylation of flavonoid rich fraction from *Caralluma attenuate.*

CONCLUSION

In conclusion, the potential anti-diabetic effect of flavonoid rich fraction of *Caralluma attenuata* was well established and the possible underlying mechanisms of this effect may be related with that flavonoid rich fraction from the *Caralluma attenuata* could inhibit α-amylase and α-glucosidase activity to decrease the absorption of carbohydrates from food; and also, flavonoid rich fraction from the *Caralluma attenuata* could directly stimulate insulin secretion and preserve the function of β-cell to ameliorate glucose metabolism. So, further investigations are deserved to elucidate specific components and their mechanisms of flavonoid rich fraction from the *Caralluma attenuata* for its anti-diabetic effect.

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