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Research Article

In-Vitro Antidiabetic Activity of *Clinacanthus nutans* Extracts

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ABSTRACT
Diabetes mellitus is a prevalent disease which characterized by hyperglycemia. It is a condition in which blood glucose levels are elevated due to decrease in cellular glucose uptake and metabolism. The management of blood glucose level is critical in the treatment of this disease and this had been offered by α-amylase inhibitors. The present study was designed to determine the *in vitro* antidiabetic of *Clinacanthus nutans* extracts. The antidiabetic action was observed by the inhibition effects of the *C. nutans* extracts on α-amylase activity and glucose diffusion across the dialysis tube. It was found that the antidiabetic action of the *C. nutans* extracts was not related to glucose diffusion as they did not show any significant glucose entrapment ability. The ethanolic leaves extract of *C. nutans* exhibited the most significant inhibition effect on α-amylase activity (64.25%) compared to other extracts. However, its inhibitory activity was moderate when compared to the commercial drugs (captopril & acarbose). The ethanolic leaves extract (the best extract) was tested for the presence for flavonoids, saponins and tannin (most reported antidiabetic compounds). Its antidiabetic action might be due to the presence of flavonoids and tannins. However, the absence of saponins might be responsible for its moderate inhibition effect comparable to control. This study suggested the *in vivo* studies of this plant should be carried out to confirm its antidiabetic mechanism.

Keywords: *Clinacanthus nutans*, antidiabetic activity

INTRODUCTION

Diabetes is a clinical syndrome in which blood glucose are above ordinary level due to the deformities in insulin secretion, insulin activity or both. This disorder prevails worldwide with its occurrence increasing at an alarming rate globally. Basically, there are three principle types of diabetes, namely type 1 (juvenile diabetes), type 2 diabetes and gestational diabetes. The main cause of type 1 diabetes is known to be the failure of pancreas β cells to produce adequate insulin. Meanwhile, type 2 diabetes possesses the characteristic of insulin resistance which shows the inability of organism to react towards normal level of circulating insulin. Hormonal changes during pregnancy or insulin inadequacy are the primary causes of gestational diabetes. Failure of glucose in the blood to enter cells expands the glucose content in the blood which is known as hyperglycemia (high blood glucose). Specifically, type 2 diabetes mellitus (T2DM) is the most experienced type of diabetes, representing more than 80% of the aggregate instances of diabetes[1].

The use of plant derived products containing high concentrations of dietary fibre and complex polysaccharides have been proposed to be considered as prevention of hyperglycemia in diabetes[2]. Inclusion of viscous polysaccharides in the diet decreased postprandial blood glucose concentrations in subjects with type 2 diabetes. In particular, guar gum has decreased postprandial blood glucose concentrations in several experiments[3]. Besides, other therapeutic approach to reduce the hyperglycemia, particularly after a meal, is to retard the digestion and absorption of carbohydrates through the inhibition of carbohydrate hydrolyzing enzymes (such as α-amylase and/or α-glucosidases). Subsequently, these inhibitors could diminish the postprandial rise in blood glucose concentration. For example, acarbose (α-amylase inhibitor) could restrain the action of α-amylase enzyme leading to a slower rate in the breakdown of starch into glucose[4]. Thus, α- amylase inhibitors are the potential focuses in the development of lead compounds for managing the diabetic problem[5].

α-amylase is the enzyme in humans that responsible for the breakdown of starch to more simple sugars (dextrin, maltotriose, maltose and glucose). In spite of the fact that the action of the enzyme has not been directly involved in the etiology of diabetes, α-amylase inhibitors have long been thought to enhance glucose resistance in diabetic patients[6]. Extensive efforts have been made over the previous decades to discover a clinically effective α-amylase inhibitor with the point of getting better control of diabetes.

Clinacanthus nutans which is otherwise called Sabah snake grass having a place with the group of Acanthaceae[7]. This plant is a bit hedge that can be discovered all throughout South East Asia, fundamentally indigenous to Thailand, Indonesia and Malaysia. It has been utilized customarily as anti-venom, anti-inflammatory, analgesic, antidiabetic, anti-rheumatism, antiviral and antioxidant[8]. In addition, this plant has been traditionally used in

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Indonesia where its fresh leaves are boiled with hot water for obtaining the decoction for treating diabetes[7,9].

In past phytochemical researches, a series of bioactive compounds were identified in *C. nutans* such as lupeol, β–sitosterol, stigmasterol, botulin, myricyl alcohol C-glycosyl flavones (vitexin, isovitexin, shaftoside, isomollupentin 7-O-β-glucopyranoside, orientin and isoorientin), sulphur-containing glucosides, cerebrosides mixer, a monoacylmonogalactosylglycerol, 13-hydroxy-(13-S)-phaeophytin b, pupurin-18-phytyl ester, phaeophorbide and chlorophyll derivatives. Botulin, lupeol, β-sitosterol, stigmasterol are usually found in the roots and leaves of *C. nutans* exceptional for flavonoid which is only present in its leaves[10].

This plant has been widely consumed traditionally by most of diabetes patients[11,12]. It is believed to contain antidiabetic components and this may merit further study. However, much uncertainty still exists about the potential of *C. nutans* as antidiabetic agent since its behaviour has not yet been scientifically investigated. Hence, in this study extracts from various parts of *C. nutans* have been examined for the α-amylase inhibitory action. Besides, the extracts were tested for their ability to hinder diffusion of glucose across a dialysis membrane for illustrating the glucose absorption through small intestine. Then, the best extract was subjected for the phytochemical screening and the identified bioactive compounds were used to explain the outcomes.

MATERIALS AND METHODS

Plant Material

C. nutans were obtained from farmers at Kg. Wang Tepus, Jitra, Kedah, Malaysia.

Chemicals and Reagents

A--amylase (BAN® 480 L, Science Technics, Malaysia) was produced from *Aspergillus oryzae* while starch was obtained from Merk, Malaysia. Other chemicals and reagents were of analytical grade and water used was glass distilled.

Plant Extraction

Three different parts (leaves, stem and mixture of leaves and stem) of *C. nutans* were cut and washed under tap water to remove all contaminants and oven (Binder, Malaysia) dried at 50 °C overnight. Then, they were ground to powder using mechanical grinder (RT-34 Grinder, Taiwan). The *C. nutans* powders were further extracted with two different solvents (ethanol and water) by using maceration method. They were all left to steep in covered containers for 24 h. Then, the resulting infusions were decanted and filtered. The ethanolic extracts were evaporated at 60 °C using rotatory evaporator (Buchi B-491 R-210, USA) while the aqueous extracts were freeze dried using VirTis Bench Top freeze dryer (SP Scientific Series, USA). Dried extracts were weighed and dissolved in 10% dimethylsulphoxide (DMSO) to yield a stock solution from which higher concentrations were prepared.

Glucose Diffusion Inhibitory Study

Glucose diffusion inhibitory study was carried out by using *in vitro* model consisted of a dialysis tube (6 cm×15 mm) (Spectra/Por®, MWCO:2000) into which 6 ml of *C. nutans* extracts and 2 ml of 0.15 M NaCl containing 1.65 mM D-glucose were added[13]. The dialysis tube was sealed at each end and placed in a centrifuge tube containing 45 mL 0.15 M NaCl. The tubes were shaken occasionally and incubated at 37 °C for 3 h. Concentration of glucose within the dialysis tubing was measured and control tests were conducted in the absence of *C. nutans* extracts. Glucose concentrations were analysed by DNS method. All tests were carried out in triplicate and the results were presented as means ± SEM.

α-Amylase Inhibitory Assay

DNS Assay

A total of 250 μL of extract (0.1–10 mg/mL) and 250 μL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (0.5 mg/mL) were mixed in 10 mL test tube. This solution was preincubated at 25 °C for 10 min, after which 250 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added and further incubated at 25 °C for 10 min. The reaction had been terminated by adding 500 μL of DNS reagent and incubated in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using spectrophotometer (Helios Zeta, UK). A control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activities were calculated as percentage inhibition as per Eq. 1[14]. Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

% Inhibition = [(Abs_control-Abs_extract)/Abs_control] x 100% (Eq. 1)

Starch Iodine Assay

Approximately 1 mL of plant extract of different concentration (0.1–10 mg/mL) was taken in test tubes. A volume of 20 µL of α-amylase was added to each test tube and incubated for 10 min at 37 °C. After the incubation, 200 µL of 1% starch solution was added to each test tube and the mixture was further incubated for 1 h at 37 °C. Then, 8 mL of distilled water was added. Absorbance of the mixture was taken at 565 nm. Each experiment was done in triplicate. The α-amylase inhibitory activities were calculated as percentage inhibition as per Eq. 2[14]. Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

% Inhibition = [(Abs_extract-Abs_control)/Abs_extractl] x 100% (Eq. 2)

Quantitative Phytochemical Screening

Test for Flavonoids[15]

Alkaline reagent test: The stock solution (1 mL) was taken in a test tube and few drop of NaOH solution were added. An intense yellow color appeared in the test tube and it became colorless when a few drop of dilute hydrochloric acid added to the reaction mixture.

Ethyl acetate test: The test solution (1 mL) was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. Development of yellow coloration was an indication of the presence of flavonoids.

Test for Saponins[16]

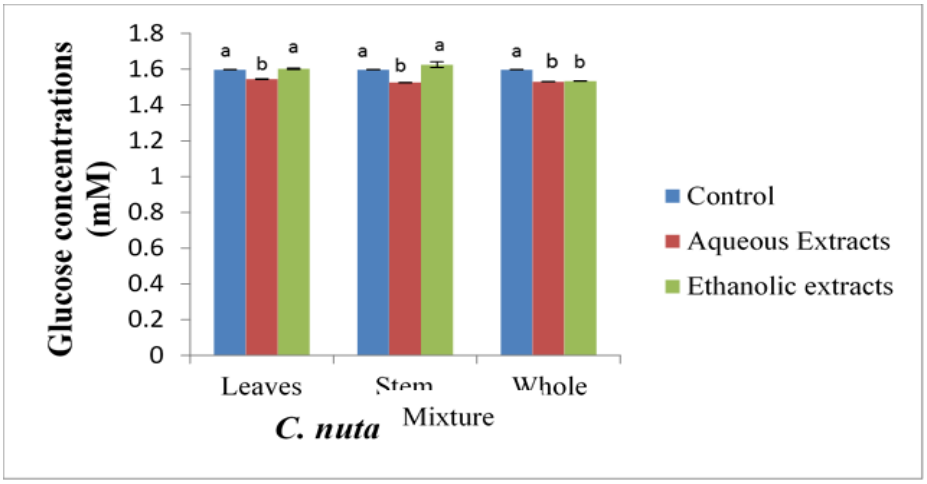

Figure 1: Effect of different extracts of *C. nutans* (1.25 mg/mL) on glucose level in external solution. Values were expressed as mean ± SEM (n=3). Different letters within each bar were significantly different (P<0.05) using Tukey's Honest Significant Different test.

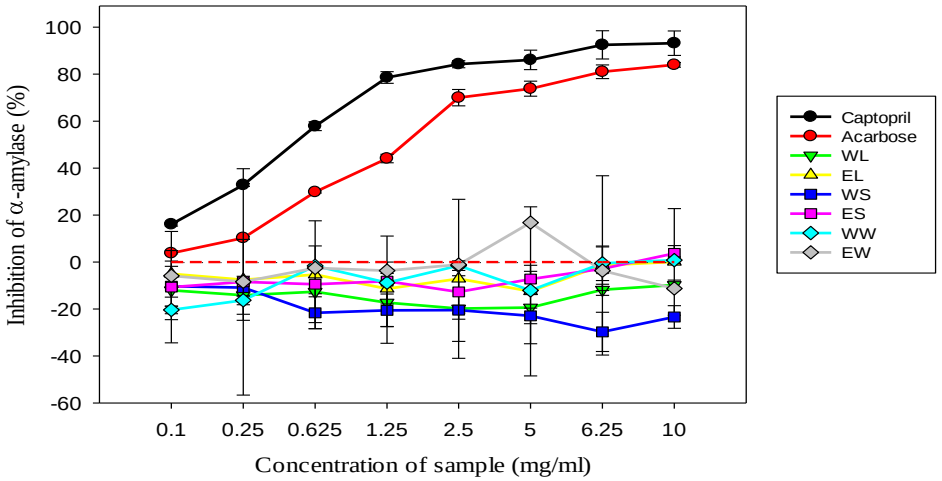

Figure 2: Effects of ethanolic and aqueous extracts of *C. nutans* at varies concentrations on the α-amylase activity as compared to controls (Captopril & Acarbose). The values are expressed as means ± SEM (n = 3).

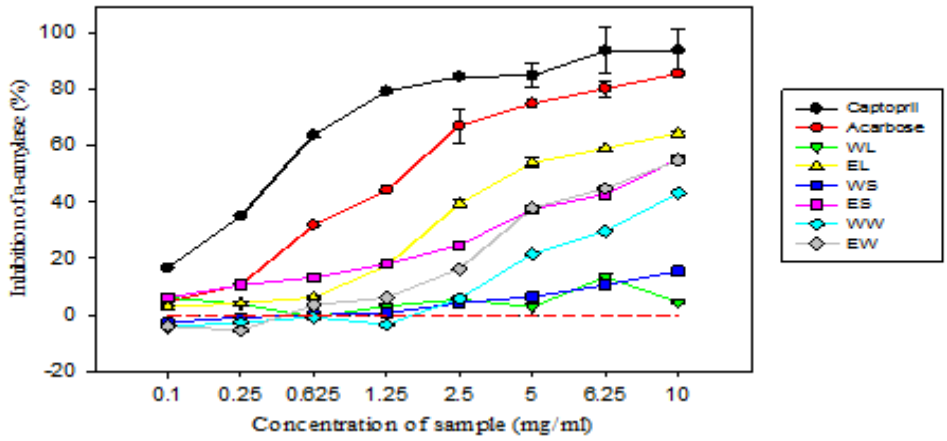

Figure 3: Effects of ethanolic and aqueous extracts of *C. nutans* on the α-amylase activity as compared to controls (Captopril & Acarbose). The values are expressed as mean ± SEM (n = 3).

Froth test: The stock solution (1 mL) was taken in a test tube and diluted with 20 mL of distilled water. It was shaken by hand for 15 min. A foam layer was obtained on the top of the test tube indicated the presence of saponins.
Foam test: Small amount of extract was shaken with little quantity of water. The foam produced persisted for ten minutes indicated the presence of saponins.

Test for Tannins[16]

Ferric chloride test: About 0.5 g of plant extract was boiled in 20 mL of water in a test tube and filtered. Few drops of 0.1% ferric chloride was added and observed for a brownish green or blue black coloration as an indication of tannins.
Acetic anhydride test: The stock solution (3 mL) was taken in a test tube and mixed with chloroform and acetic anhydride (1 mL). Finally, sulphuric acid (1 mL) was added carefully by the side of test tube to the solution. A green color was formed which showed the presence of tannins.

RESULTS AND DISCUSSION

Glucose Diffusion Activity

Glucose diffusion test was conducted to investigate the effect of ethanolic and aqueous extracts from various parts of *C. nutans* with respect to its glucose retardation activity across the dialysis tube (Fig. 1). It shows that all the aqueous extracts of *C. nutans* exhibited a significant inhibition effect compared to the control. In contrast, the ethanolic leaves and stem extracts did not show any entrapment ability in decreasing glucose movement into the external solution. However, the inhibition effect exhibited by the ethanolic mixture extract was significantly different compared to control.
The antidiabetic action of *C. nutans* extract in this study is not likely to be related to glucose diffusion. This result is in accord with study on the entrapment ability of aqueous and ethanolic extract of *Teucrium polium* on glucose diffusion activity[17]. On the other hand, the results were contradicted with studies conducted to identify antidiabetic mechanism of aqueous extract of *Nigella sativa*, *Eugenia jambolana*, *Andrographis paniculata* and *Gymnema sylvestre* by the same method[18]. The contradict finding shows that plants extracts may had different role on glucose diffusion. Hence, the antidiabetic action of *C. nutans* might be related to different mechanism such as inhibition of α-amylase activity instead of glucose diffusion.
In this study, the dialysis tubing technique was used as a simple model to evaluate the potential of plant extracts to retard the glucose diffusion in the intestinal tract. Movement in this system is assisted by the convective activity of intestinal contractions[13,19]. Due to the simplicity of the study, the antidiabetic mechanism was only tested by diffusion of glucose through a normal dialysis membrane whereas in the body, there are various transporters which work in synchronization with other molecules to transport glucose[20]. Further studies are needed to be conducted in order to confirm the *in vivo* action of *C. nutans* with respect to the glucose diffusion. Moreover, further studies are necessary to check the role of viscosity of both aqueous and ethanolic extracts of *C. nutans* on glucose diffusion activity since thicker fluids will have greater viscosity and the molecules move slower around each other. Thus, the diffusion rate will decrease with increase in viscosity.

α-Amylase Inhibition Assay

DNS Method

The DNS reagent assay is a common method to estimate the amount of reducing sugars in different types of samples. This method measured the amount of reducing sugar yielded after treatment of the test solution with α-amylase. It shows that captopril (93.21% ± 0.054) and acarbose (83.97% ± 0.008) exerted dose dependent inhibition effects against α-amylase activity. However, effect of all *C. nutans* extracts was uncertainty (Fig. 2). The unstable inhibition effects exerted by the *C. nutans* extracts were observed through the enhancement of colour intensities, which resulted with higher absorbance values compared to the control. Thus, the inhibition effects by *C. nutans* remain inconclusive. The results of this study were contrast with study conducted on *Nisamalaki churna* and *Morinda lucida* which shows significantly inhibited α-amylase activity as indicated in DNS assay[21,22].
The contradict results had raised important questions about the nature of DNS in detecting different reducing sugars, specifically on the factors which cause fluctuations in colour development. Several factors have been address by some researcher[23,24] and they concluded that
Different reducing sugars generally yield different colour intensities and this may affect the final colour measurement of the test solution and reagent.
Rochelle salt in the DNS reagent interferes with the protective action of sulphite which then results in unstable colour in the presence of phenol.
Decomposition reactions of sugar in alkaline solution competes for the availability of 3,5-dinitrosalicylic acid during hydrolysis.
The findings of the current work indicated that *C. nutans* extracts exerted uncertain inhibition effects when reacted with DNS reagent. The possible reaction mechanisms involved were not clearly understood and it was beyond of the scope of this study. Hence, a further study with more focus on the possible mechanisms involved by the *C. nutans* extracts when reacted with the DNS reagent is therefore suggested.

Starch Iodine Method

Despite the uncertain inhibition activity of the *C. nutans* extracts shows in DNS method, all the extracts show a dose-dependent inhibition effect against α-amylase activity by starch iodine method. This method measured the remaining of starch in the test solution after treated with α-amylase. However, the inhibition activity is not significant compared to the control drug i.e. captopril and acarbose (93.53% ± 0.075 and 85.34% ± 0.007 respectively). Among the extracts, the leaves ethanolic extract (10 mg/mL) shows an appreciable α-amylase inhibition effect (64.25% ± 0.006). The ethanolic stems and mixture of stems and leaves extracts of *C. nutans* moderately inhibited the α-amylase.
In overall, it can be concluded that the ethanolic extracts

Table 1: IC_{50} values for controls & *C. nutans* extracts.

Samples		IC_{50} (mg/ml)
Control	Captopril	0.435 ± 0.001^{a}
	Acarbose	1.572 ± 0.003^{b}
Ethanol	Leaves	4.283 ± 0.001^{c}
	Stem	8.432 ± 0.010^{d}
	Whole	8.192 ± 0.008^{e}
Aqueous	Leaves	nd
	Stem	nd
	Whole	nd

nd=not determine;
The values are expressed as means ± SEM with n=3. Means down vertical column not sharing common letter are significantly different (P <0.05).

Table 2: Phytochemical compositions of the best *C. nutans* extract (leaves ethanolic extract).

Phytochemical	Test	Ethanolic Leaves Extract
Saponins	Foam test	-
	Froth test	-
Tannins	Ferric chloride test	+
	Acetic anhydride test	-
Flavonoids	Alkaline reagent test	+
	Ethyl acetate test	+

'+' = present '- '= absent

from different parts of *C. nutans* exerted better inhibition effects compared to the aqueous extracts. These results matched with study on the inhibition of α-amylase by leaf extracts of *Picralima nitida*. Nevertheless, the shown inhibition effects were moderate as compared to the control[22]. This research reveals that the ethanolic extracts of *C. nutans* have the potential to be evaluated as antidiabetic regimen. However, its inhibition mode was not clearly understood, but there are some suggestions that the plant phenol (flavonols) might cause conformational changes in the structure. Hence, further studies for evaluating its antidiabetic potential and determination of its actual mode of inhibition must be conducted. Besides, studies on the isolation and characterization of the active compound responsible for this inhibitory effect are recommended.

IC_{50} Analysis

The *IC_{50}* analysis is commonly used for quantifying an inhibitor's effect. It can be defined as the concentration of an inhibitor required to reduce the rate of an enzymatic reaction by 50%. In this study, the ethanolic leaves extract showed an appreciable *IC_{50}* value compared to the ethanolic stems and mixture extracts. However, its *IC_{50}* value was higher as compared to captopril and acarbose (Table 1). Acarbose and captopril had been proven to possess high efficacy in inhibiting α-amylase activity[25,26]. Hence, it was concluded that the ethanolic leaves extract is more effective in inhibiting α-amylase activity compared to other extracts, but less effective when compared to control.

Quantitative Phytochemical Screening

The best extract of *C. nutans* (the ethanolic leaves extract) was tested for flavonoids, tannins and saponins. These bioactive compounds were selected as most of the antidiabetic compounds fall under these categories. Table 2 shows that the ethanolic extract of *C. nutans* leaves contain flavonoids, while the presence of tannin was ascertained and saponins were absence. The presence of the compounds might be responsible for the antidiabetic activity of the extract.

The phenolic fractions of plants have long been recognized to inhibit carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase in mammals[27,28]. Condensed tannin was believed to exhibit antidiabetic activity through inhibition towards carbohydrates-hydrolyzing enzymes[29] and it was reported that tannin could delay the glucose absorption in human intestine[30]. This was proven by study conducted to determine *in vitro* antidiabetic of soursop leaves brew through α-glucosidase inhibition found that inhibition activity by soursop leaves brew was related to the presence of tannin[31]. However, this study showed that the presence of tannins in the *C. nutans* extracts was ascertained. Thus, more tests on tannins should be conducted for more relevant results.

Saponin have been suggested as amylase inhibitor[32] and the ability of saponins to reduce elevated plasma blood glucose makes saponins as an excellent candidate in the treatment of diabetes mellitus[33]. However, in this study, saponins have been found to be absence and this might be a possible explanation for the medium antidiabetic action exhibited by the *C. nutans* extracts since saponin had been clarified as an antidiabetic compound in most of reported antidiabetic studies[33-36].

CONCLUSION

Herbal remedies are considered convenient for management of diabetes due to their traditional acceptability and availability, low cost, and lesser side effects. The therapeutic approaches had focused on decreasing the postprandial hyperglycemia which can be achieved through the inhibition α-amylase. This investigation concluded that both ethanolic and aqueous extracts of C. *nutans* do not retard the glucose diffusion activity. This study has identified that the ethanolic extracts of *C. nutans* exhibited better inhibition effects compared to the aqueous extracts. The leaves ethanolic extract displayed the most significant inhibition effect as compared to other extracts. This might be due to the presence of flavonoids. However, the exhibited inhibitory action was lower compared to the both tested commercial drugs (captopril & acarbose). The absence of saponins were might be responsible for its moderate α-amylase inhibition activity. This study suggested that the possible antidiabetic mechanism by this plant was not related with glucose diffusion. However, it was found that the ethanolic extracts of *C. nutans* have the potential to be evaluated as antidiabetic regimen. Hence, further studies prior to evaluate their antidiabetic potential must be conducted.

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