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IN VITRO INHIBITORY POTENTIAL OF *Amaranthus viridis* AGAINST ALPHA-AMYLASE FOR DIABETES AND THE ANTIOXIDANT ACTIVITY

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Abstract

The occurrence of diabetes mellitus is ever-increasing worldwide, affecting the younger generation. In the current study, insulin and conventional medication have shown to cause adverse side effects. Hence, natural and safe approaches should be explored to prevent further progression of diabetes. Phenolic acid and flavonoid are natural phytochemicals that have antioxidant and anti-inflammatory properties that are associated with specific anti-diabetic agents. *Amaranthus viridis (A.viridis)* is a plant that has been utilized for medicinal purposes for its beneficial properties since prehistoric times. Thus, this study aims to investigate the antidiabetic effects of *A. viridis* leaf extract via *in vitro* and antioxidant assay. The *in vitro* assay of *A.viridis* was assessed by measuring its ability to inhibit diabetic linked enzyme α-amylase. The antioxidant properties were measured using a colorimetric assay. The α-amylase inhibition percentage was exhibited as dichloromethane>hexane> water>methanol. DCM has exhibited the lowest IC_{50} value of 18.08µg/ml. As for the antioxidant assay, total phenolic content showed methanol extract of *A.viridis* had the highest amount of phenolic acid, 68.54mg gallic acid(GAE)/g. Besides, total flavonoid content showed methanol extract of *A.viridis* had the highest amount of flavonoid content that is 185.4 mg quercetin equivalent (QE)/g. Furthermore, free radical scavenging activity showed the methanolic extract of *A.viridis* has the highest free radical scavenging potential. This study shows that *A.viridis* leaf extract exhibits significant a-amylase inhibitory activities and antioxidant properties that could be used to fight against the oxidative-stress related diseases.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder caused by an acquired or genetic deficiency in the secretion of insulin and affects the carbohydrates, protein, and fat metabolism. There are three different categories for diabetes mellitus which are gestational diabetes, Type 1 and Type 2 [1]. Type 2 diabetes mellitus (T2DM) is the commonly observed type in adults. It is estimated that 25% of the world population is affected by this disease. In 2019, the estimated number of individuals with diabetes worldwide is about 422 million [1]. An estimated 1.6 million deaths were directly caused by diabetes in 2016. The World Health Organization (WHO) has observed the prevalence and incidence rate for T2DM has increased tremendously over the last three decades [1].

The main symptom of diabetes mellitus is hyperglycemia that generates reactive oxygen species (ROS) that causes membrane damage and lipid peroxidation [2]. T2DM leads to abnormal blood glucose levels associated with impaired insulin signaling and insulin resistance. Eventually causing

β-cell dysfunction, an increase in oxidative stress, a change in lipid metabolism, and sub-clinical inflammation [2]. These and extra obscure mechanisms slowly lead to further complications, such as retinopathy, cardiovascular diseases, and neuropathy [3]. Hence, antioxidants play an important role in diabetes to protect the beta-cells from oxidation by inhibiting the peroxidation chain reaction.

Various approaches are being practiced for the management and prevention of T2DM such as control diet intake, exercising regularly, and the usage of different antidiabetic drugs [4]. Commercially there are many drugs available in the market for the management of this disease but most of the drugs come with some adverse side effects [3]. Therefore, attention is being focused on more efficient inhibitors for diabetes enzymes from natural sources to treat diabetes. In numerous metabolic processes involved in T2DM, flavonoids play crucial roles according to studies done *in vitro.*

Naturally occurring flavonoids have been broadly studied for α-amylase inhibitors over the last 10 years [5]. The α-amylase enzyme is produced in the pancreas and salivary gland [5,6], and complex carbohydrates are hydrolyzed to produce oligosaccharides and disaccharides which are further digested to monosaccharides [6,7]. Starch blockers are also called the α-amylase inhibitors which block the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose and other simple sugars [8]. Several flavonoid compounds have been tested on the inhibitory activity against α-amylase by Tadera et al. [9].

A.viridis locally known as "bayam pasir" in Malaysia is a diverse species in the botanical family [10]. The plant is often used as a source of medication and food where it is eaten as a vegetable in many parts of the world. The phytochemical investigation, hypocholesterolemia and anti-atherosclerotic effects of *A.viridis* leaf extract in hypercholesterolemia-induced rabbits have been studied [11], which suggests the presence of phenolic and flavonoid content, indicating the usage of *A.viridis* as an antioxidant agent [11]**.** Inhibiting diabetic enzymes such as α-amylase can be a key approach in the management of blood glucose levels in T2DM patients. Therefore, the objective of the current study is to investigate the antidiabetic effects of *A.viridis* leaf extract on the α-amylase enzyme in an *in vitro* assay.

MATERIALS AND METHODS

Preparation of Sample

Five kilogram (kg) of *A.viridis* sample was obtained from a local farmer. The plant sample was washed and was air-dried, and the stem was discarded. The sample was then ground using pestle and mortar.

Sequential Extraction of *A.viridis* Leaf

The grounded sample was placed in five different conical flasks and subjected to hexane following the 1:2 ratio and left to macerate on a shaker for 48 h. After 48 h, the extracts were filtered using a sieve and funnel. The filtration was repeated twice. The hexane extracts were then concentrated using a rotary evaporator and the concentrated extract was then freeze-dried to obtain the powder. The powder was labeled and stored in falcon tubes at 4ºC. The residue was immediately transferred into conical flasks where it is subjected to the next solvent which is dichloromethane (DCM) at a 2:1 ratio and the procedure was repeated. The steps above were repeated using methanol and water as the solvent. The powders were labeled and stored at 4 °C in a dark place.

Alpha-Amylase Inhibition Activity

The α-amylase enzyme was performed according to the method described by Manikandan [12]. The enzyme solution was prepared by mixing 2.75 µg of α-amylase in 100 ml of distilled water. A colorimetric reagent is prepared by mixing sodium potassium tartrate solution and 3, 5 dinitro salicylic acid solution 96 mM. The reaction mixture contained 1 mL of *A.viridis* of 10–100 µg/mL concentration and 1 mL of α-amylase solution. Both the reaction mixture and control were preincubated for 30 min and then 1 mL of starch solution was added to the reaction and incubated at 37°C for 10 min. The reaction mixture was stopped by the addition of 1 mL of DNS solution and the mixture was left to boil for 5 min. The negative control was prepared without sample and α-amylase enzyme solution and acarbose was used as the positive control. The absorbance was measured at 540 nm.

Determination of Enzyme Inhibition (%)

The α-amylase inhibitory activity was expressed as the inhibition percentage and was calculated using the equation given below:

[(Absorbance of control – Absorbance of inhibitor) /Δ Absorbance control] × 100.

The % α-amylase inhibition was plotted against the extract concentrations and the IC_{50} values were obtained.

Total Phenolic Content

Total phenolic content was estimated by Folin Ciocalteu's method [36]. 1 ml aliquots and standard gallic acid (20, 40, 60, 80, 100 µg/ml) were positioned into the test tubes and 5 ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent was mixed and shaken. After 5 min, 1.5 ml of 20 % sodium carbonate was added and volume made up to 10 ml with distilled water. It was allowed to incubate for 2 h at room

temperature. The intense blue color was developed. After incubation, absorbance was measured at 750 nm. The extracts were performed in triplicates. The blank was performed using reagent blank with solvent. Gallic acid was used as a standard. The calibration curve was plotted using standard gallic acid.

Total Flavonoid Content

Total flavonoid content was measured with the aluminum chloride colorimetric assay [36]. One ml of aliquots and 1ml standard rutin solution (200, 400, 600, 800, 1000 µg/ml) was positioned into test tubes and 4ml of distilled water and 0.3 ml of 5 % sodium nitrite solution was added into each. After 5 min, 0.3 ml of 10 % aluminum chloride was added. On the 6th min, 2 ml of 1 M sodium hydroxide was added. Finally, the volume was made up to 10 ml with distilled water and mix well. Orange yellowish color was developed. The absorbance was measured at 510 nm. The blank was performed using distilled water. Rutin was used as a standard. The samples were performed in triplicates. The calibration curve was plotted using standard rutin.

Free Radical Scavenging Activity

A solution of DPPH was freshly prepared by dissolving 6 mg DPPH in 50 mL methanol. The dilution of the extract was prepared into 2x and 4x dilution. 2x dilution was prepared by adding 2ml 1x extract and 2ml of distilled water whereby 4x dilution prepared by adding 2ml of 2x dilution extract and 2ml of distilled water. Hence, 36 test tubes needed for all extract samples. The different extract samples were filled with 1ml of extract and 2ml of DPPH reagent. The same goes for 2x and 4x dilution. The test tube was then incubated in the dark for 20 min at room temperature. The decrease in absorbance was measured at 517 nm using a spectrophotometer. The percentage inhibition of radicals was calculated using the following formula:

$$\% \text{ inhibition} = \frac{(Acontrol - Asample) \times 100}{Acontrol}$$

Acontrol is the absorbance of DPPH solution without extract and Asample is the absorbance of the sample with DPPH solution. The half-maximal inhibitory concentration (IC_{50}) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50%. All tests were performed at least in triplicate, and graphs were plotted using the average of three determinations.

RESULTS AND DISCUSSION

Extraction of *A.viridis*

The objective of this study was to investigate the antidiabetic potential of *A.viridis* and the potential of various extracts of *A.viridis* leaf in inhibiting the α-amylase enzyme. The *A.viridis* leaves were extracted with increasing polarity of different solvents such as hexane, DCM, methanol, and distilled water. Different solvents were used in the extraction process to compare which solvent inhibits α-amylase activity the best. These different solvents have different polarities that affect the solubilities of the bioactive compounds in the leaf extract which produces the different inhibition percentages for different extracts [16].

α-Amylase Inhibition Percentage of the 4 Extracts in Various Concentrations (µg/ml)

The inhibition percentage exhibited by each extract is presented in Table 1. All the four extracts (hexane, DCM, methanol, and water) showed significant α-amylase inhibition at some tested concentrations. The standard positive control acarbose showed (83.84%) inhibition at 100 µg/ml concentration. The highest inhibition activity (at concentration: 10µg/ml) was observed in the DCM extract of *A.viridis* (73.85%) followed by hexane extract (67.69%) inhibition, then water extract (66.92%) inhibition while methanol extract showed the lowest inhibitory activity (64.87%).

Table 1: α-Amylase inhibition activity of the 4 extracts tested

Concentration (µg/ml)	Acarbose	Water	Hexane	DCM	Methanol
10	80.51±0.8882 ^{a}	66.92±0 ^{c}	67.69±0 ^{c}	73.85±0 ^{b}	64.87±0.4441 ^{d}
20	78.72±0.4441 ^{a}	54.62±0 ^{d}	39.23±0 ^{e}	65.38±0 ^{b}	57.95±0.4441 ^{c}
30	78.46±0 ^{a}	34.62±0 ^{d}	35.38±0 ^{d}	59.49±1.175 ^{b}	54.87±0.4441 ^{c}
40	73.33±1.175 ^{a}	31.54±0 ^{d}	26.92±4.35E-15 ^{e}	57.95±0.8882 ^{b}	54.10±0.8882 ^{c}
50	70.77±0 ^{a}	27.69±0 ^{a}	40.26±0.4441 ^{a}	43.59±0.4441 ^{a}	51.79±0.8882 ^{a}
60	72.31±0 ^{a}	40.77±0 ^{d}	29.23±4.35E-15 ^{e}	54.87±0.4441 ^{b}	43.85±8.7E-15 ^{c}
70	79.23±0 ^{a}	42.31±0 ^{e}	51.54±0 ^{c}	56.92±0 ^{b}	42.82±0.4441 ^{d}
80	81.79±0.4441 ^{a}	50.51±0.8882 ^{d}	55.38±0 ^{c}	63.85±8.7E-15 ^{b}	41.03±0.8882 ^{e}
90	83.33±1.175 ^{a}	61.79±0.4441 ^{c}	57.69±0 ^{d}	66.67±0.8882 ^{b}	40.51±1.601 ^{e}
100	83.85±0.7692 ^{a}	61.79±1.175 ^{d}	64.87±0.4441 ^{c}	68.97±0.4441 ^{b}	36.92±1.538 ^{e}

All data are presented represents the mean ± standard deviation from triplicate measurements. One-way ANOVA was performed followed by Tukey's test for multiple comparisons. Values sharing the same superscript letters are not significantly different from each other ($p < 0.05$).

Inhibition Percentage of α-amylase on *A.viridis* Leaf Extract

Figure 1 (Fig.1) shows the results of the inhibitory activity of *A.viridis* on α -amylase. Based on the graph observed in the figure above, acarbose the positive control has exhibited a consistent and highest inhibition percentage. Among the four extracts, hexane, DCM, and water has a decrease and increase in trendline as the concentration increases. While the inhibition percentage for methanol decreases as the concentration increases.

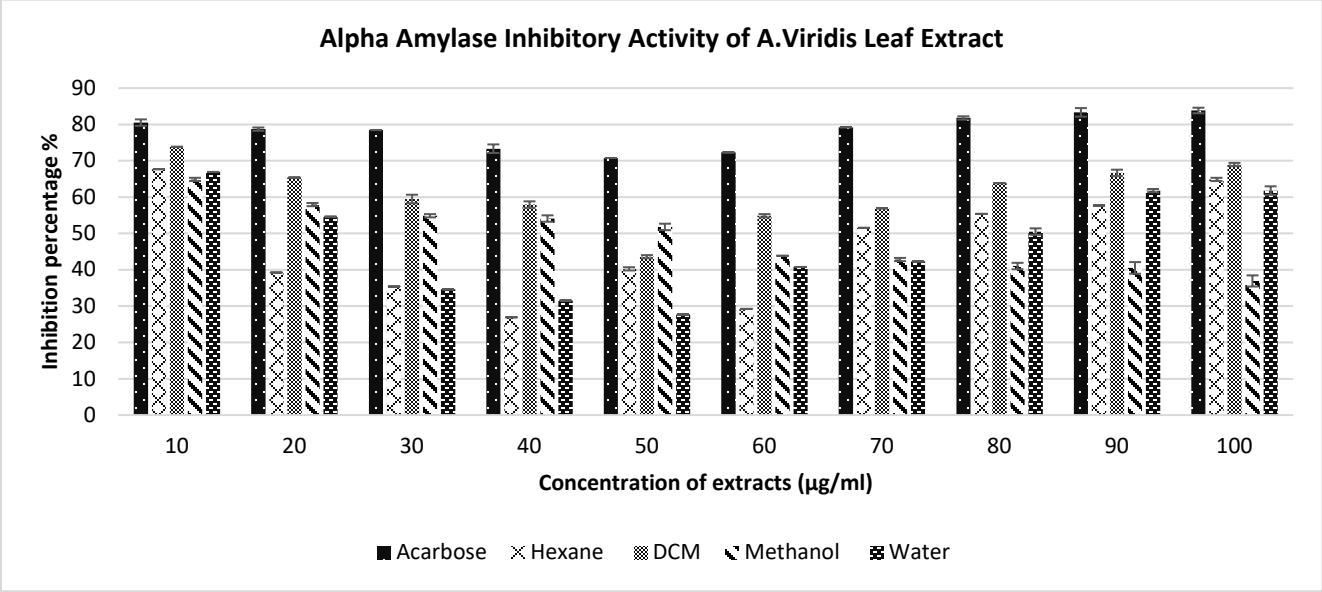

Figure 1: Percentage of α-amylase inhibition of different extracts with different concentrations (µg/ml)**.** All data are presented represents the mean ± standard deviation from triplicate measurements.

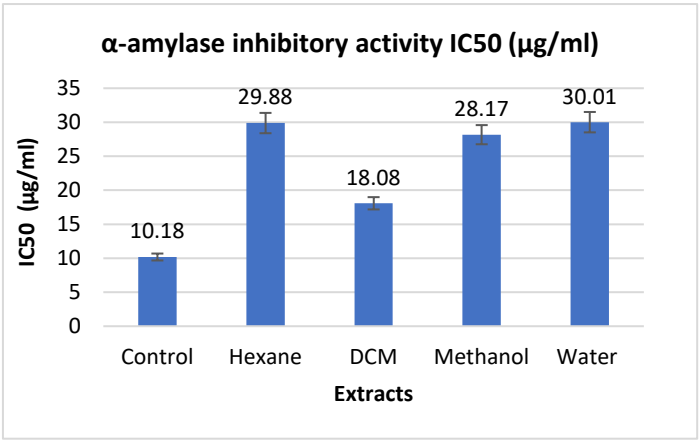

Figure 2: IC_{50} (µg/ml) of α-amylase inhibitory activity of *A.viridis* leaf extracts

IC_{50} (µg/ml) of α-amylase Inhibitory Activity

The inhibition percentage of α-amylase against the extract concentrations was performed and the IC_{50} values were calculated. The DCM crude extract exhibited the lowest IC_{50} of 18.08µg/ml and the IC_{50} values of methanol, hexane, and water extracts were 28.17, 29.88, and 30.01µg/mL respectively. Meanwhile, the standard positive control acarbose exhibited an IC_{50} value of 10.18µg/ml.

As the results observed in Table 1, acarbose was used as the positive control which inhibited the α-amylase activity highest at 100µg/ml with 83.85±0.7692% inhibition. Among the different extracts, DCM showed the highest inhibition percentage on *α*- amylase (73.85±0) followed by hexane (67.69±0), water (66.92±0) and methanol (64.87±0.4441) with the least inhibition percentage at 10µg/ml. In this study, the DCM extract of *A.viridis* leaf showed significant scavenging capability compared with the positive control acarbose. The assay revealed that the extracts contain α-amylase inhibitory compounds. This study suggests that DCM is the best extraction solvent for *A.viridis*. Studies have shown DCM solvent has the capability to have higher inhibiting potential than the polar solvents [17].

In Figure 2 (Fig.2), DCM has exhibited a consistent inhibition percentage which shows significant inhibitory activity at all the concentrations tested. Hexane and water have a decreasing (at concentration: 30µg/ml) and increasing (at concentration: 70µg/ml) trend as the concentration increases. Pipetting errors during the assay mixture may have led to this trend [18,19]. The inhibition percentage decreases with the increase in extract concentration for methanol (Fig.2). This could be due to the active compounds with a mixture of numerous interactions in the extract that

displays high activity at very low concentrations [18]. It is possible that with the increase in the concentration of a plant extract, some components of it become less soluble and so the trend goes against expectancy [18].

The half-maximal inhibitory concentration (IC_{50}) measures the effectiveness of *A.viridis* leaf extract in inhibiting α-amylase activity [18]. The IC_{50} values (μg/mL) of *A. viridis* extracts revealed good inhibitory potential and radical scavenging properties. Based on the results observed in Fig.2, the IC_{50} value of DCM extracts are almost similar to acarbose a commonly recommended anti-diabetic drug [15]. The *A. viridis* leaf extract for DCM exhibited the lowest IC_{50} value compared to the other solvents. This shows that DCM has a higher potency in inhibiting α-amylase compared to hexane, methanol, and water.

These findings suggest that inhibiting α-amylase activity through the *A.viridis* leaf extract delays the starch hydrolysis which is one of the ways the plants exhibit the hypoglycemic effect [14,23]. Blocking carbohydrate hydrolyzing enzymes such as α-amylase can control the blood glucose level in diabetic patients [20]. Modulation of α-amylase activity of compounds in these extracts can lead to reducing blood glucose levels because the presence of an α-amylase inhibitor will inhibit the normal starch conversion pathway [21].

Total Flavonoid and Phenolic Content

The total phenolic content for aqueous, hexane, DCM, and methanol extracts were estimated by Folin Ciocalteu's method using gallic acid as standard [27]. This method estimates the capability to react with oxidizing agents of phenolic compounds. According to Fig.3, total phenolic content in 4 different extracts of *A.viridis* ranged from 19.47 to 68.54mg gallic acid equivalent (GAE)/g. The methanol extract of *A.viridis* had the highest amount of phenolic acid, 68.54mg while hexane extract of *A.viridis* had the lowest that is 19.47 mg gallic acid equivalent (GAE)/g.

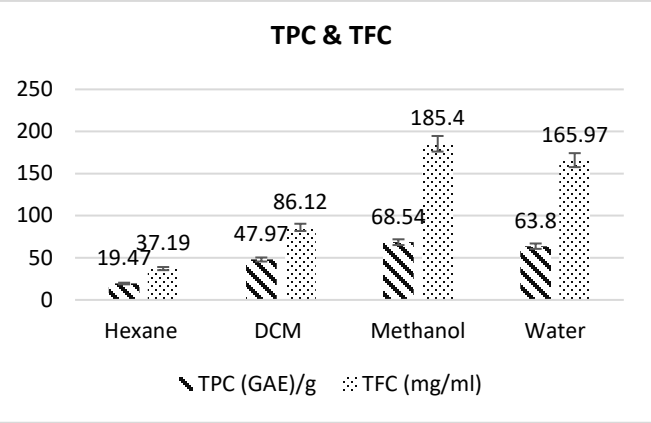

Figure 3: Total phenolic and total flavonoid content of different extracts

The total phenolic contents in plant extracts of *A.viridis* is based on the type of extract. The different solvent used in extraction has a different polarity. The high solubility of phenols in polar solvents provides a high concentration of these compounds in the extracts obtained using polar solvents for the extraction [31]. It may also be due to the complex formation of certain phenolic compounds in the extract that are most soluble in methanol [32]. The phenolic compounds in the methanol extraction possess more phenol groups in the hexane extract. Based on the results of TPC, methanol has the highest phenolic content.

The total flavonoid content for aqueous, hexane, DCM, and methanol extracts were measured with the aluminum chloride colorimetric assay using rutin as standard. Fig.3 shows the flavonoid content of the 4 different extracts of *A.viridis.* According to the result, total flavonoid content in 4 different extracts of *A.viridis* ranged from 37.19 to 185.4 mg rutin curve equivalent, mg RU/g.

It was observed that the results of solvents on TFC are similar to TPC. The highest TFC was obtained in methanol extract of *A.viridis* which is 185.4 mg while hexane extract had the lowest amount of flavonoid content, 37.19 mg RU/g. A similar trend was observed in the amount of TPC.

Phenolic and flavonoid content has a positive relation with antioxidant activity as phenolic acid has the reducing capacity which aids in preventing harmful effects of oxidative processes on macromolecules [30]. Therefore, a high amount of phenolic content in methanol extraction suggests that *A.viridis* has the potential to prevent reactive oxygen species from disrupting the cell function and insulin sensitivity which ultimately aids in glucose tolerance in diabetic patients [31].

Plant phenolic and flavonoids compounds such as quercetin, ferulic acid, anthocyanins, catechin, and resveratrol were shown in epidemiological studies to regulate glycemia via increased glucose uptake, insulin secretion, and inhibition of lipid peroxidation, alpha-glucosidase, and alpha-amylase [33,34]. Several flavonoid compounds were tested for their inhibitory activity against α-amylase and showed that the potency of inhibition is correlated with the number of hydroxyl groups on the B ring of the flavonoid scaffold [9]. Studies have demonstrated flavonols and flavones enzyme inhibitory capacity depend on hydrogen bonds between the hydroxyl groups of the polyphenol ligands and the catalytic residues of the binding site and formation of a conjugated α-system that stabilizes the interaction with the active site [35]. The recent findings show the structure-activity relationship of polyphenols inhibiting α-amylase has concluded that the hydroxylation galloylation of flavonoids, including catechins, improved the inhibitory effects against α-amylase [36].

Free Radical Scavenging Activity

The antioxidant activity of different extracts of *A.viridis* was determined using a methanol solution of DPPH reagent. A

freshly prepared DPPH solution exhibits a deep purple colour with an absorption band at 517 nm. DPPH radical is a stable organic free radical [38]. It loses this absorption when accepting an electron or a free radical species, which results in the purple colour to fade because antioxidant molecules eliminate DPPH free radicals and convert them into a colourless product [38]. It can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations.

The DPPH assay measured hydrogen atom (or one electron) donating activity and hence provided an evaluation of antioxidant activity due to free radical scavenging [39,40]. Free radicals are inevitably produced in biological systems and encountered exogenously which eventually leads to cause various disorders [40]. Antioxidants are the compounds capable to remove the free radicals by intervening in the free radical-mediated oxidative process. These antioxidants are mostly found naturally in many plants. Thus, it is important to study the antioxidant activity of the plant to protect against oxidative damage.

Table 2: Inhibition percentage of different extracts

Extracts	Inhibition percentage (%)		
	1x dilution	2x dilution	4x dilution
Hexane	2.50	43.59	58.02
DCM	1.80	58.92	69.24
Methanol	0.10	82.77	86.87
Water	13.73	67.74	80.46

The order of antioxidant activity in A.viridis leaf extract is as such methanol > water > DCM > hexane. The results in the free radical scavenging ability of the different extracts of leaves of *A.viridis* based on the inhibition percentage is presented in Table 2. It is evident from the figure, that the methanolic extract of *A.viridis* has the highest and the hexane extraction has the lowest free radical scavenging potential. Based on the results, it suggests that methanol extraction of *A.viridis* has a more potent antioxidant property of scavenging free radicals.

In this study, our findings showed a strong relationship between total phenolic and flavonoid content and antioxidant activity. This suggests that the antioxidant capacity of these extracts results from the contribution of the phenolic compounds present.

CONCLUSION

In conclusion, the antidiabetic properties observed in *A.viridis* is a potential therapeutic approach for the management of T2DM. These compounds are used in studies with the interest of their significant role in the treatment of free radical stress-related diseases and it showed that *A.viridis* have hypoglycemic effects. Studies have also shown that flavonoid has improved diabetic symptoms and liver injury in diabetic patients. Hence, the large presence of flavonoids in the leaves of *A.viridis* proves that it could be used as future medicine in preventing diabetes mellitus. The results from this study provide scientific support to the use of *A.viridis* for the treatment of diabetes, and the potential role of α-amylase inhibition with its antioxidant activity.

There are many literature studies and observations on α-amylase over the last decade. However, the mechanism of action should be further investigated because in an *in vitro* assay the xenobiotic metabolism cannot be investigated. Further studies on the toxicity investigations of the leaf extract should be conducted together with the isolation and identification of bioactive compounds in *A.viridis*. Not all individuals living with diabetes have access to obtain affordable treatment, including insulin. Therefore, encouraging the population for a healthy lifestyle with the consumption of greens is one cost-effective way to reduce diabetes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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