Assesment of free radical scavenging activity and inhibitory properties of aqueous extract of *Cymodocea serrulata* segrass towards α- glucosidase and α –amylase enzyme

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Abstract

Aim: The study reports the *in vitro* free radical scavenging and α-amylase and α-glucosidase inhibition activities of aqueous extract of seagrass *Cymodocea serrulata* from Gulf of Mannar, Southeast Asia.

Methods: The free radical scavenging activity was evaluated by DPPH, Superoxide, Nitric oxide and Hydroxyl radical scavenging assays and α-amylase, α-glucosidase enzyme inhibitory activity. The *C. serrulata* showed effective DPPH radical scavenging activity of 69.45 µg/mL, Superoxide radical scavenging activity of 91.08.5 ± 0.02%, Nitric oxide radical scavenging activity of 86.89 ± 0.03% and Hydroxyl radical scavenging activity of 81.09 ± 0.3 % at a concentration of 5 µg/mL respectively. It also showed significant inhibitory activity against α-amylase with an IC_{50} value of 3.0 ± 0.04 (µg/ml) and the α-glucosidase inhibitory activity with an IC_{50} value of 3.7 ± 1.01 (µg/ml) at concentrations of 1-5 µg/mL.

Conclusion: *Cymodocea serrulata* was found to be a potent free radical scavenger and inhibitor of α-amylase and α-glucosidase enzymes.

Key words: Seagrass, *Cymodocea serrulata,* Free radical scavenging activity, α-glucosidase, α amylase inhibitory activity.

INTRODUCTION

Metabolic disorder of diabetes mellitus is characterized by increased blood glucose levels with instability in carbohydrate, protein and fat metabolism. [1] One of the novelty based therapeutic approach for treating diabetes is to decrease postprandial hyperglycemia. This can be achieved by delaying the glucose absorption through the inhibition of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase in the digestive tract. Liberation of glucose molecules from dietary complex carbohydrates which can be retard by α-glucosidase inhibitors and delay glucose absorption resulting in reduced postprandial plasma glucose levels and suppress postprandial hyperglycemia. [2]

Oxidative stress has been shown to have a significant effect in the causation of diabetes as well as diabetes related complications. [3] Hyperglycemia defining established diabetes can induce oxidative stress by various mechanisms. [4] The α-glucosidase enzymes are responsible for breakdown of carbohydrates to absorbable monosaccharide, α-glucosidase enzymes delay the absorption of ingested carbohydrates, reducing the postprandial glucose and insulin peaks. [5] Our previous study also showed that synthesized silver nanoparticles of seagrass *Enhalus acoroides* are known to contain α-glucosidase inhibitors. [6]

Majority of currently available drugs have been derived directly or indirectly from marine plants which are an excellent source of bioactive compounds. [7] Seagrass *Cymodocea serrulata* have long been used in traditional remedial purposes like fever and skin diseases including muscle pains, wounds, and stomach problems etc. [8] Marine plants have been reported for antibacterial, [9, 10] antifungal, [11] antiviral, [12] antiprotozoal [13] and antiinflammatory activities. [14] According to report, seagrasses are rich sources of antioxidant compounds. [15] They stand for

most significant and extremely productive ecosystems of the oceans, which offers a unique environment for the aquatic organisms and help in the stabilisation of sediments. [16] According to report, green plants contain rich sources of α-glucosidase and α-amylase enzyme inhibitors. [17] Eventhough, free radical scavenging and *in vitro* α-glucosidase and α-amylase inhibitory activities of *C. serrulata* are not well documented so far. The present study has revealed the *in vitro* free radical scavenging and α- glucosidase and α- amylase inhibitory activities of *C. serrulata*.

METHODS

Chemicals

All chemicals, reagents and solvents used in this study were of analytical grade and were purchased from Sigma-Aldrich (USA).

Sample collection

C. serrulata (R. Brown) Ascherson & Magnus, a seagrass was collected Thondi (Lat. 9450 N and Lang 7930 E) is situated Gulf of Mannar, Tamilnadu, South India coastal region during the month of December 2019. It was identified and authenticated by a taxonomist. The young leaves having maximum size only were collected at 3–5 m depth using the SCUBA diving equipment model (SCUBA EA 2/07). All leaves were covered with a dense growth of Epiphytic diatoms. They were removed by scarping the individual leaves in 1 cm areas with the tip region in spatula and washed with distilled water. After cleaning, the leaves were dried in shade at room temperature for one week. The dried leaves were pulverized in to fine powder and subjected to extraction procedures.

Extraction phase

30 g of *C. serrulata* powder was boiled in 100 ml of distilled water for 5 min. Then the boiled content was filtered by Whatman No. 1 filter paper and the obtained filtrate was kept at 4ºC as an aqueous extract. Further the extract was subjected to free radical scavenging and α-glucosidase and α-amylase enzyme inhibitory assays. All these assays were conducted in triplicate manner.

DPPH free Radical Scavenging Assay

This assay was performed according to the standard method with slight modification. [18] 50 mL of extract was mixed with 50 µL of DPPH solution (0.2 mg/mL) and 100 µL of 0.1 M acetate buffer (pH 5.5). The reaction mixture was incubated for 20 min in dark at room temperature. The absorbance was read at 517 nm using a microplate reader. Ascorbic acid was used as standard reference and methanol was used as negative control. The IC_{50} value was calculated as the concentration required to reach a 50% reduction of DPPH.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined by the method of Halliwell et al., 1987. [19] Hydroxyl radical was generated by the Fe^{3}+-ascorbate-EDTA-H_2O_2 system (Fenton reaction). The assay is based on the quantification of the 2-deoxy-D-ribose degradation product, which forms a pink chromogen upon heating with TBA at low pH. The reaction mixture contained 0.8 mL of phosphate buffer solution (50 mmol L−1, pH 7.4), 0.2 mL of extractives/standard at different concentration (1–5 µg/mL), 0.2 mL of EDTA (1.04 mmol L−1), 0.2 mL of $FeCl_3$ (1 mmol L−1) and 0.2 mL of 2-deoxy-D-ribose (28 mmol L−1). The mixtures were kept in a water bath at 37°C and the reaction was started by adding 0.2 mL of ascorbic acid,

AA (2 mmol L−1) and 0.2 mL of H_2O_2 (10 mmol L−1). After incubation at 37°C for 1 h, 1.5 mL of cold thiobarbituric acid (TBA) (10 g L−1) was added to the reaction mixture followed by 1.5 mL of HCl (25 %). The mixture was heated at 100°C for 15 min and then cooled down with running tab water. The absorbance was read at 532 nm using spectrophotometer. The hydroxyl radical scavenging capacity was evaluated with the inhibition of percentage of 2-deoxy-D-ribose oxidation on hydroxyl radicals. The percentage of hydroxyl radical scavenging activity was calculated according to the following formula:

% hydroxyl radical scavenging activity = [A0−(A1−A2] × 100/A0

where A0 is the absorbance of the control without a sample. A1 is the absorbance after adding the sample and 2-deoxy-D-ribose. A2 is the absorbance of the sample without 2-deoxy-D-ribose. The % of inhibition was plotted against concentration, and from the graph IC_{50} value was calculated. The experiment was repeated thrice at each concentration**.**

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured by the method of Robak et al., 1988. [20] Superoxide radicals were generated in a PMS-NADH system by oxidation of NADH and assayed through reduction of NBT(Nitro blue tetrazolium). In this experiment, the superoxide radicals were generated in 3 mL of sodium phosphate buffer (100 mM, pH 7.4) containing 1 mL of NBT (150 μM) solution, 1 mL of NADH (468 μM) solution, and different concentrations of the *C. serrulata* extract (1–5 μg/mL) in water. The reaction started by adding 1 mL of PMS solution (60 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the

absorbance was measured against the blank. Ascorbic acid was used as the positive control. The decrease in the extent of NBT reduction, measured by the absorbance of the reaction mixture, correlates with the superoxide radical scavenging activity of the *C. serrulata* extract. The superoxide radical scavenging actiivty was calculated using the following formula:

Superoxide radical scavenging activity (%) = [(A0−A1)/A0 × 100]

where A0 is the absorbance of the control and A1 is the absorbance of sample or the standard.

Niric acid radical scavenging activity

This was adopted by the method of Panda et al., 2009. [21] The extracts were prepared from a crude extract stock of 10 mg/ mL in water. Extracts and standard ascorbic acid were then serially diluted with distilled water to make concentrations from 1−5 µg/ mL. They were stored at 4°C. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid freshly before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the extracts and incubated at 25°C for 180 mins. The extract was mixed with an equal volume of freshly prepared Griess reagent. Negative control was maintained. The colour tubes contained ethanol extracts at the same concentrations with no sodium nitroprusside. A volume of 150 µL of the reaction mixture was transferred to 96-microtiter well plate. The absorbance was read at 546 nm using a Hitachi UV-Vis microplate reader. Ascorbic acid was used as the positive control. The percentage inhibition of the extract

and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the extract and ascorbic acid were calculated using the following formula:

Nitric acid radical scavenging activity (%) = [(A0−A1)/A0 × 100]

where = absorbance of control sample and = absorbance in the presence of the samples of extracts or standards.

α-glucosidase enzyme inhibition assay

The α-glucosidase inhibition property was determined using the modified method of Matsui et.al., 1996. [22] The α-glucosidase reaction mixture contained 2.9 mM P-nitrophenyl-α-glucopyranoside (pNPG), 25 µL of *C. serrulata* extract (1-5 µg/mL conc.) in DMSO and 0.6 U/ml baker's yeast α-glucosidase in sodium phosphate buffer, pH 6.9. Control tubes contained only DMSO, enzyme and substrate while in positive controls acarbose replaced by the seagrass extract. Mixtures without enzyme, seagrass extract and acarbose served as blanks. The reaction mixtures were incubated at 25ºC for 5 min, after which the reaction was stopped by boiling for 2 min. Absorbance of the resulting p-nitro phenol (pNP) was read at 405 nm using spectrophotometer and was considered directly proportional to the activity of the enzyme.

Glucosidase activity was determined as percentage of control as follows:

$$\% \text{ Activity of sample} = A_{405} \text{ of sample} - A_{405} \text{ of controls} / A_{405} \text{ control} \times 100$$

The activity in controls (with α- glucosidase but without inhibitor) was considered to be 100%. Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50} values) were determined graphically.

α-amylase enzyme inhibition assay

The α-amylase activity was determined by the method of Hansawasdi et.al., 2000. [23] Starch azure (2 mg) was suspended in each of the tubes containing 200 µL of 0.5 M Tris-HCl buffer (pH 6.9) and 0.01 M $CaCl_2$. The tubes containing substrate solution was boiled for 5 min and was then incubated at 37ºC for 5 min. *C. serrulata* extract (200 µL) was taken in each tube containing different concentrations (1-5 µg/mL) of DMSO. Porcine pancreatic amylase (PPA) was dissolved in Tris-HCl buffer to form a concentration of 2 units /mL and 100 µL of this enzyme solution was added to each of the above mentioned tubes. The reaction was carried out at 37ºC for 10 min and was stopped by adding 500 µL of 50% acetic acid in each tube. The reaction mixture was centrifuged at 3000 rpm for 5 min at 4ºC. The absorbance of the resulting supernatant was read at 595 nm using a spectrophotometer. The α-amylase inhibitory activity was calculated as follows:

$$[(A_c+) – (A_c-)] – [(A_s-A_b)] / [(A_c+) – (A_c-)] \times 100$$

Where A_c+, A_c-, A_s and A_b are defined as the absorbance of 100% enzyme activity (only solvent with enzyme), 0% enzyme activity (only solvent without enzyme activity), a test sample (with enzyme) and a blank (a test sample without enzyme), respectively.

Statistical analysis

The statistical analysis was performed using one way analysis of variance (ANOVA). Results are expressed as mean ± SD and n = 3.

RESULTS

DPPH free Radical Scavenging Assay

DPPH free radical scavenging activity, expressed as IC_{50} values of *C. serrulata* aqueous extract was shown in Figure 1. *C. serrulata* exhibited a significant dose-dependent inhibition of DPPH activity with an IC_{50} value of 3.7 ± 0.09 (µg/ml). This value was comparable with that of the reference standard, ascorbic acid's IC_{50} value of 2.9 ± 2.21 (µg/ml), revealing the antioxidant activity of *C. serrulata*.

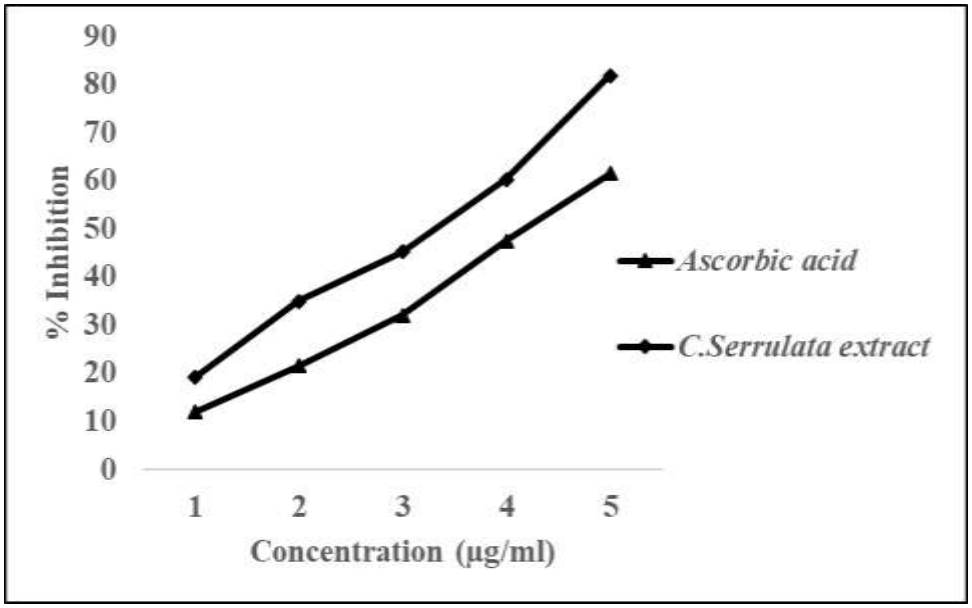

Figure 1. DPPH radical scavenging activity of *C.Serrulata* extract

Hydroxyl radical scavenging activity

The results of the extract and standard were 81.09 ± 0.3 % µg/mL and 87.12 ± 0.1 % µg/ mL, respectively. The IC_{50} value of the extract was less than that of the standard ascorbic acid (Table 1). Standard and the extracts exhibited a dose dependent inhibition of the hydroxyl radicals.

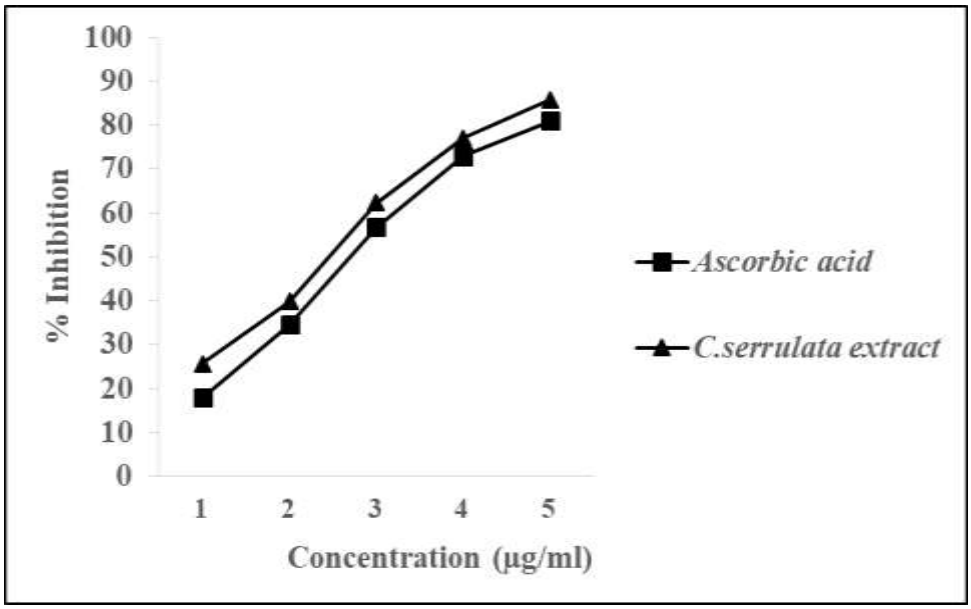

Figure 2. Hydroxyl radical scavenging activity of *C.Serrulata* extract

Table 1. IC_{50} values of free radical scavenging activity and alpha amylase and alpha glucosidase inhibitory activity

S.No	Assays	IC_{50} value	
		***C.serrulata* extract**	**Standard**
1.	DPPH Radical scavenging activity	3.7 ± 0.09 (µg/ml)	2.9 ± 2.21 (µg/ml)
2.	Hydroxyl Radical scavenging activity	2.8 ± 2.23 (µg/ml)	2.4 ± 1.12 (µg/ml)
3.	Superoxide Radical scavenging activity	2.7 ± 0.04 (µg/ml)	2.1 ± 0.98 (µg/ml)
4.	Nitric oxide Radical scavenging activity	3.1 ± 2.34 (µg/ml)	2.8 ± 2.12 (µg/ml)
5.	Alpha amylase Inhibitory activity	3.7 ± 1.01 (µg/ml)	3.4± 1.23 (µg/ml)
6.	Alpha glucosidase Inhibitory activity	3.0 ± 0.04 (µg/ml)	2.2 ± 0.14 (µg/ml)

Superoxide radical scavenging activity

The superoxide radical scavenging activity of *C. serrulata* extract was assayed by the PMS-NADH system is shown in Figure 3. The results of superoxide radical scavenging activity of *C. serrulata* extract was increased noticeably with an increase in concentrations with an IC_{50} value of 2.7 ± 0.04 (µg/ml) and with that of the reference standard, ascorbic acid's IC_{50} value of 2.1 ± 0.98 (µg/ml)

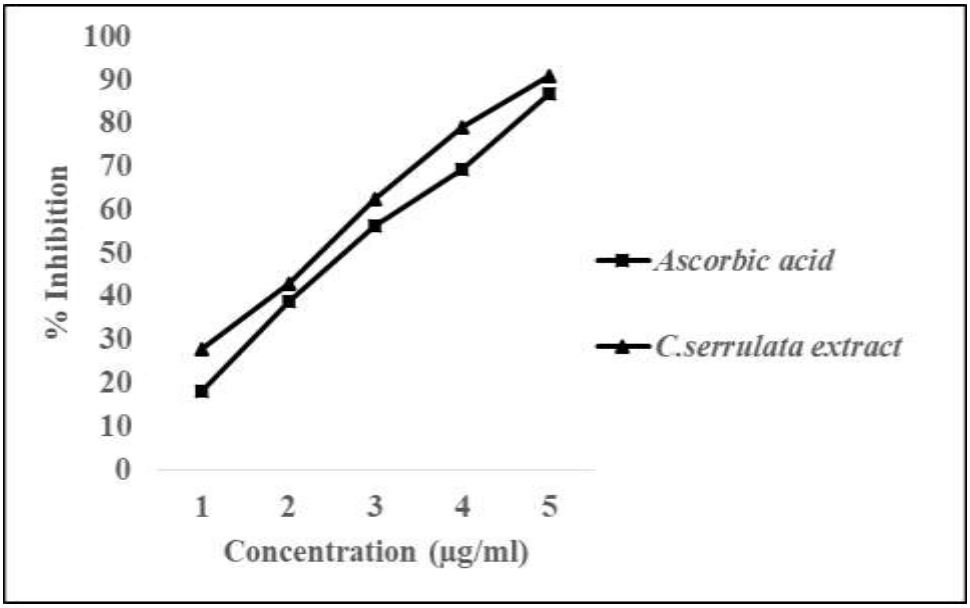

Figure 3. Superoxide radical scavenging activity of *C.Serrulata* extract

Nitric oxide radical scavenging activity

C. serrulata extract also caused a good dose-dependent inhibition of nitric oxide with an IC_{50} value of 3.1 ± 2.34 (µg/ml) is shown in Figure 4. Acorbic acid was used as a reference compound and it's IC_{50} value was 2.8 ± 2.12 (µg/ml). The IC_{50} value of the extract was less

than that of the standard. The extract effectively decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* at 546 nm.

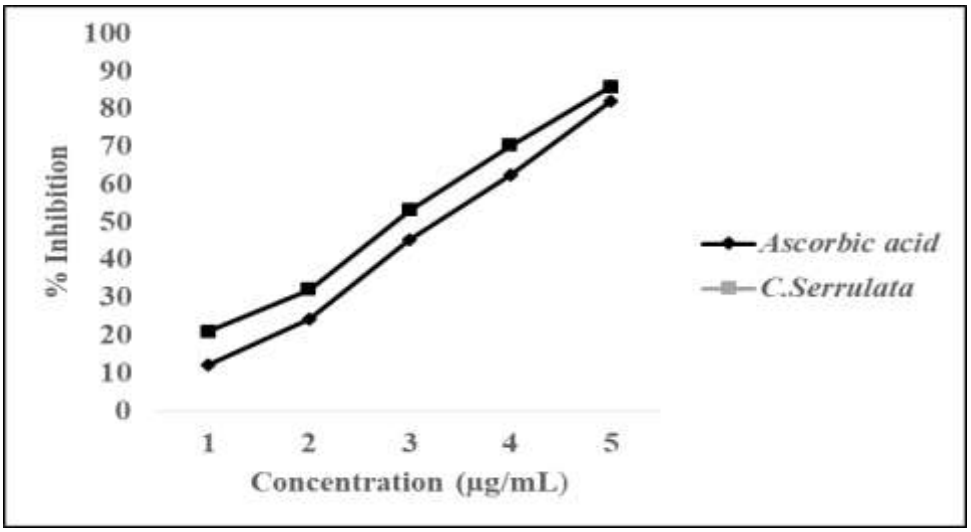

Figure 4. Nitric oxide radical scavenging activity of *C.Serrulata* extract

Inhibition of α-amylase activity

In this study, the aqueous extract of *C. serrulata* was found to possess favorable α-amylase i +nhibitory effects on starch break down in *in vitro*. The α-amylase inhibitor effectiveness of extracts were compared on the basis of their resulting IC_{50} values. *C. serrulata* inhibited the activity of α-amylase with an IC_{50} value of 3.7 ± 1.01 (µg/ml).

Acarbose, the positive control used in this study was inhibited α- amylase activity with an IC_{50} value of 3.4± 1.23 (µg/ml) (Figure 5).

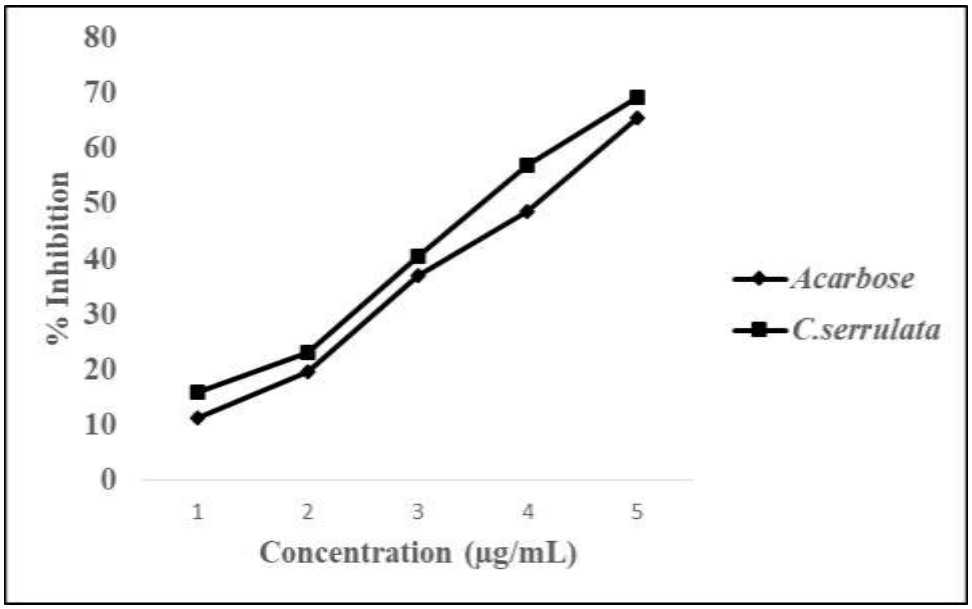

Figure 5. Alpha amylase inhibitory activity of *C.Serrulata* extract

Inhibition of α-glucosidase activity

The α-glucosidase inhibitor effectiveness of aqueous extract of the *C. serrulata* was compared on the basis of their resulting IC_{50} values. *C. serrulata* inhibited the activity of α-glucosidase with an IC_{50} value of 3.0 ± 0.04 (µg/ml). Acarbose, the positive control was inhibited the activity of α-glucosidase with an IC_{50} value of 2.2 ± 0.14 (µg/ml) was shown in Figure 6.

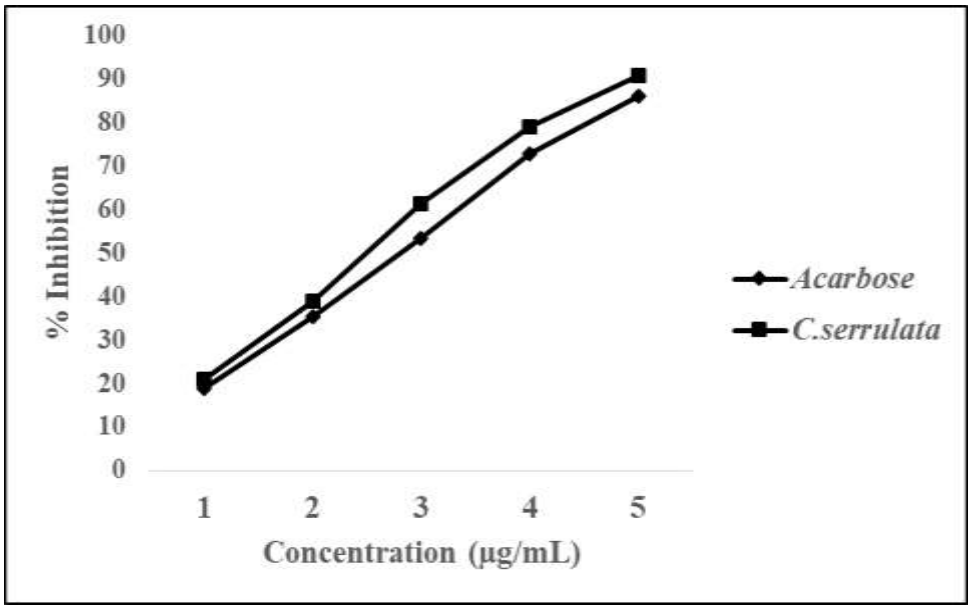

Figure 6. Alpha glucosidase inhibitory activity of *C.Serrulata* extract

DISCUSSION

Antioxidants are chemical constituents which prevent and stabilize the injury caused by free radicals by supplying electrons to these damaged cells. [24] Plants containing rich sources of antioxidant compounds. [25] In this present study, the antioxidant property of seagrass *C. serrulata* extract was measured by DPPH, Hydroxyl radical, Superoxide radical, Nitric oxide radical assays and ascorbic acid was used as standard. All the assays were showed promising results.

DPPH free radical scavenging assay is globally accepted method for screening antioxidant activity of plant extracts. Our results revealed that the *C. serrulata* extract showed a potent free radical scavenging activity when compared with ascorbic acid. According to Huang (2005) [26] *C. serrulata* extract might have the presence of high amount of phenolic compounds which reflected as greatest antioxidant activity and also scavenge the DPPH radicals by their hydrogen

donating ability. The results obtained in this study also showed that *C. serrulata* extract showed higher DPPH radical scavenging activity due to it's electron transfer ability.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage. [27] *C. serrulata* extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. The IC_{50} value indicates that the plant extract is a better hydroxyl radical scavenger than the standard ascorbic acid.

Superoxide anion is similarly very injurious to cellular constituents. [28] As shown in Figure 3, the superoxide radical scavenging activities of the *C. serrulata* and the ascorbic acid standard are increased markedly with increasing concentrations. The results suggest that the *C. serrulata* has a potent scavenger of superoxide radical than ascorbic acid. These results suggested that *C. serrulata* has a significant superoxide radical scavenging activity.

According to Tylor (1997), [29] It is well known that nitric oxide has an important role in various inflammatory processes where as Huie (1993) [30] also stated that the toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. *C. serrulata* inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The present study also showed that the extract has more potent nitric oxide scavenging activity when compared to ascorbic acid as standard.

An effective approach for the controlling of carbohydrate metabolic disorders, including diabetes mellitus type II is the inhibition of the α-glucosidase enzyme. In this present study, *C. serrulata*

seagrass from the coastal waters of Mandapam, southeast coast of India, were screened for their α-amylase and α-glucosidase inhibitory activity. Inhibitors of α-glucosidase and α-amylase enzyme delay the breaking down of carbohydrate in the small intestine and diminish the postprandial blood glucose excursion in a person suffering from diabetes. [31]

In this present study, seagrass *C. serrulata* was screened for it's α-amylase and α-glucosidase inhibitory activity. The aqueous extract of *C. serrulata* showed α-glucosidase and α-amylase enzyme inhibitory activity and was might be due to the presence of several phytochemicals such as flavonoids, saponins, and tannins where as in Kwon, 2007 [32] also stated that α-amylase and α-glucosidase inhibitors isolated from plants was purely due to the flavonoids presence which have features of inhibiting α-amylase and α-glucosidase activities.

Conclusions

It can be concluded that *in vitro* analysis of *C. serrulata* possessed significant antioxidant, α-amylase and α-glucosidase inhibitory activities.This is might be due to the synergistic effect of the phytochemical constituents of the seagrass. Hence, *C. serrulata* can be used as an alternative dietary supplement for the treatment of diabetes. This study needs further research for the effects on the hyperglycemic properties in diabetic rats and also identifies the hyperglycemic principle (s) to reveal their mode of action.

Conflict of interest

The authors report no conflicts of interest. The authors only are answerable for the content and inscription of the paper.

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