

CHARACTERIZATION, *IN SILICO* AND *IN VITRO* DETERMINATION OF ANTIDIABETIC AND ANTI-INFLAMMATORY POTENTIAL OF ETHANOLIC EXTRACT OF *SARGASSUM WIGHTII*

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ABSTRACT

Objective: This study was intended to investigate and characterize the phytoconstituents of *Sargassum wightii*, evaluate its antidiabetic and anti-inflammatory potential under *in silico* and *in vitro* conditions.

Methods: The marine algae *S. wightii* was extracted with ethanol. The ethanolic extract was screened for various phytoconstituents, quantified for total flavonoid, and polyphenol content. Fourier transform infrared (FT-IR) and mass spectrometry were used for characterizing the ethanolic extract. The antidiabetic potential of the phytoconstituents was analyzed by molecular docking and enzyme inhibition assays. The anti-inflammatory potential was evaluated by albumin denaturation assay.

Results: The phytochemical screening revealed the presence of flavonoids, polyphenols, alkaloids, tannins, carbohydrates, proteins, oils, and fat present in the ethanol extract. The FT-IR analysis showed the presence of α, β unsaturated ketone, alcohol, and ether groups in the extract. MS analysis identified l - (+) - ascorbic acid 2, 6 dihexadecanoate and dotriacontyl isopropyl ether in the ethanolic extract. The molecular docking studies revealed that l - (+) - ascorbic acid 2, 6 dihexadecanoate interacted with both α -amylase and α -glucosidase with a consensus score of 5. The results of *in vitro* analysis showed that the ethanolic extract exhibited strong inhibitory activity on α -amylase (inhibitory concentration [IC₅₀] 8 mg/ml) and α -glucosidase (IC₅₀ 6 mg/ml), respectively. The ethanolic extract also inhibited the formation of advanced glycation end products (IC₅₀ 10 mg/ml). Further, the ethanolic extract inhibited the denaturation of albumin (IC₅₀ 2.5 mg/ml) thereby revealing the anti-inflammatory potential of *S. wightii*.

Conclusion: Hence, it can be concluded that *S. wightii* possesses antidiabetic and anti-inflammatory potential which may be due to the presence of l - (+) - ascorbic acid 2, 6 dihexadecanoate and flavonoids, polyphenols, alkaloids, tannins, carbohydrates, proteins, oils, and fat present in the extract.

Keywords: *Sargassum wightii*, α -amylase, α -glucosidase, Advanced glycation end products, Molecular docking.

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INTRODUCTION

Diabetes, chronic disorder of the carbohydrate metabolism is projected as the 7th major cause of death worldwide, accounting for 5.1 million deaths in 2013 [1]. Diabetes accounts for 12.3% (673 billion USD) of global health-care expenses which is expected to reach 802 billion USD by 2040 [2]. The complications of diabetes include blindness, heart attack, kidney failure, stroke, and leg amputation [3]. Management of hyperglycemia is the major challenge in the treatment of diabetes. Chronic hyperglycemia causes the nonenzymatic glycation of serum proteins, resulting in the formation of advanced glycation end products (AGE) [4]. Glycated albumin is responsible for complications such as diabetic retinopathy, diabetic nephropathy, atherosclerotic lesions, insulin insensitivity, oxidative stress, and inflammation [5,6].

Oral hypoglycemic drugs are widely employed in the management of diabetes. These hypoglycemic drugs target the two major enzymes, namely, pancreatic α -amylase and intestinal α -glucosidase to regulate the postprandial blood sugar level [7]. To overcome the adverse side effects of synthetic hypoglycemics, medicinal plants and herbs are explored to identify a suitable alternate in the management of diabetes [8,9]. Marine seaweeds are a prolific source of potential secondary metabolites which provide lead compounds in drug development. Seaweeds are widely utilized as food in coastal areas of Asian countries where the prevalence of diabetes is less than the western world. *Sargassum wightii* is a marine algae, which possesses antibacterial, anti-Alzheimer, antiasthmatic, and antitumor activities [10-12]. Although, previous studies have reported the hypoglycemic activity of *S. wightii* [13] the active ingredient and the mechanism of action are still not reported. Hence, this study is intended to isolate and characterize the active ingredient(s) of *S. wightii*, to

analyze its interaction with the critical enzymes (α -amylase and α -glucosidase) by molecular docking and to evaluate its antidiabetic potential under *in vitro* conditions.

METHODS**Seaweed collection and extraction**

The seaweed *S. wightii* was collected from the Mandapam coastal region of Tamil Nadu, India. It was identified and authenticated by Dr. Saravanan, Scientist, CMFRI, Mandapam, Tamil Nadu, India. The collected seaweed was washed thoroughly to remove debris and shade dried, milled in an electric grinder. The dried algal powder (100 g) was extracted with ethanol (300 ml) for 72 hrs in an orbital shaker. The mixture was filtered, and the filtrate was evaporated in a rotary evaporator. The dried extract was used for further studies.

Phytochemical screening

The phytochemical composition of the ethanol extract was determined [14]. Quantification of flavonoids and polyphenols.

The total polyphenol content was determined using the Folin-Ciocalteu method [15]. In brief, different concentrations of the ethanol extract (100,200,300,400,500 μ g/ml) were mixed with 500 μ l of FCP and 150 μ l of sodium carbonate (2%) and incubated for 2 hrs in the dark. The absorbance of the samples was read at 760 nm in a Multimode plate reader (Perkin Elmer). A blank devoid of algal extract served as negative control and gallic acid served as positive control.

The flavonoid content was determined according to [16]. In brief, different concentrations of the ethanolic extract (100-500 μ g/ml) were

added with 100 μ l of aluminum chloride (10%) and 100 μ l of sodium acetate (1 M) and incubated for 30 minutes. The absorbance was read at 415 nm in a multimode plate reader (Perkin Elmer). A blank devoid of algal extract served as negative control. Quercetin was used as positive control.

Fourier transform infrared (FT-IR) and high-performance liquid chromatography (HPLC) analysis

The dried ethanolic extract (10 mg) of *S. wightii* was mixed with 10 mg of KBr and subjected to FT-IR analysis in Jasco FT-IR 4600 series. HPLC separation was carried out in Shimadzu HPLC 9A series (Japan) equipped with LC 20AD binary gradient pump, RF-fluorescence detector, and SPD- M20A diode array detector. The reverse phase separation was done in enable C18 G column of 250 \times 4.6 mm of particle size 5 μ m with a C18 guard column (3.9 \times 20 mm) of particle size 4 μ m maintained at 37°C with a flow rate of 1 ml/minute. The compounds in the ethanolic extract were separated using Acetonitrile and water in the ratio 30:70. The injection volume was 20 μ l, and the monitoring was done by the diode array detector set in the acquisition range of 200-700 nm. The total running time was set for 25 minutes. The retention time (RT) of the compounds was recorded [17].

Mass spectrometry analysis of *S. wightii*

The chemical composition and mass of compounds in the ethanolic extract was detected in the mass spectrometer of Shimadzu (GC-MS QP 2010 ULTRA) equipped with a direct injection port. The sample was injected directly and the emerging fragment ion pattern was collected. The ion source temperature was maintained at 200°C, and the interface was maintained at 250°C. The mass spectrometric voltage was set at 70eV. The National Institute of Standards and Technologies (NIST14) database was used to identify the separated peaks.

In silico analysis of *S. wightii* bioactives

Preparation of protein

The X-ray crystal structures of human α -amylase and α -glucosidase (PDB id: 5EOF and 2QLY) were retrieved from Protein Data Bank. The SYBL X1.3 Protein preparation program was used to prepare the proteins. The removal of small molecules, water, the addition of hydrogen, and assigning bond orders to the PDB-derived protein structure was carried out. The resulting protein structures were used for the *in silico* study.

Preparation of ligand

The 3D structures of l - (+) - ascorbic acid 2, 6 dihexadecanoate and dotriacontyl isopropyl ether were retrieved from pubchem (PubchemCID: 54686917 and 91692940) and used for the interactive analysis.

Molecular docking

The SYBL X1.3 dock suite program was utilized for analyzing the interaction of l - (+) - ascorbic acid 2, 6 dihexadecanoate and dotriacontyl isopropyl ether with α -amylase and α -glucosidase. The induced fit docking protocol of SYBL was used to evaluate the interaction between the ligands and the proteins. The best interaction was identified based on the total score and consensus score (C score) generated by the SYBL software.

Determination of *in vitro* antidiabetic activity

Inhibition of α -amylase activity

The inhibition of α -amylase by the ethanolic extract of *S. wightii* was evaluated as described previously [18]. In brief, 500 μ l of the extract (2-8 mg/ml) was incubated with 500 μ l of α -amylase (0.5 mg/ml in 0.02 mM phosphate buffer, pH6.9) for 10 minutes at 25°C. Following this 500 μ l of starch solution (1% in 0.02 mM phosphate buffer, pH6.9) was added and incubated for 10 minutes at 25°C. After incubation, the reaction was terminated by the addition of 1 ml of Dinitrosalicylic acid and the tubes were placed in a boiling water bath for 5 minutes. The contents were cooled and diluted by adding 5 ml of distilled water. The absorbance was read at 540 nm. A control with all the reaction contents,

but lacking the extract was prepared which represented 100% enzyme activity. The assay was done in triplicate, and the values represent mean \pm standard deviation (SD).

Inhibition of α -glucosidase activity

The efficiency of the algal extract to inhibit α -glucosidase was evaluated [19]. In brief, different concentrations (2-10 mg/ml) of the algal extract were incubated with 1 ml of maltose (2% solution in 0.2 M Tris buffer) for 5 minutes at 37°C. 1 ml of α -glucosidase (1 U/ml) was added to initiate the reaction. The reaction mixture was incubated for 40 minutes at 35°C. After incubation, 2 ml of 6N HCl was added to the reaction mixture and the absorbance was read at 540 nm. A control with all the reaction contents, but lacking the extract was prepared which represented 100% enzyme activity. The assay was done in triplicate and the values represent mean \pm SD.

Assessment of antiglycation activity

The antiglycation activity of the algal extract was evaluated based on previous studies [20] with some modification. In brief, different concentration (4-16 mg/ml) of the algal extract was incubated with 400 μ l of glucose solution (5 mM) and 500 μ l of BSA (1 mg/ml) for 24 hrs at 60°C in an incubator. The reaction was terminated by the addition of 10 μ l TCA (100% w/v), and the reaction mixture was incubated at 4°C for 10 minutes. Following incubation, the contents were centrifuged at 13,000 rpm for 5 minutes and the precipitate obtained was dissolved in alkaline PBS (pH 10) and the glycated BSA was quantified by measuring the absorbance at 440 nm. A negative control with 500 μ l of BSA replacing the algal extract and a blank with all the reaction contents but lacking the extract was prepared. The assay was done in triplicate and the values represent mean \pm SD.

Anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory efficiency of the algal extract was analyzed by the inhibition of albumin denaturation based on previous protocol [21] with slight modification. Different concentration (500 μ g-2 mg/ml) of the algal extract was incubated with 500 μ l of BSA (1%) for 20 minutes at 37°C followed by heating at 51°C for 20 minutes. The reaction contents were cooled and the turbidity developed was measured spectrophotometrically at 660 nm. A control with all the reaction contents, but lacking the extract was prepared which represented 100% enzyme activity. The assay was done in triplicate, and the values represent mean \pm SD.

RESULTS

For the current study, the ethanolic extract of *S. wightii* was characterized, and the antidiabetic and anti-inflammatory activity of *S. wightii* was evaluated by *in vitro* studies. The extraction of the algae by ethanol yielded 300 mg of dried extract. The extract was further analyzed. The results of phytochemical analysis (Table 1) of the ethanol extract indicated the presence of flavonoids, polyphenols, alkaloids, tannins, carbohydrates, proteins, oils, and fat.

Table 1: Preliminary analysis of phytochemicals from *Sargassum wightii* ethanol extract

Phytochemicals	Ethanol extract
Tannins	++
Flavonoids	++
Terpenoids	-
Saponins	+++
Glycosides	+++
Carbohydrates	+++
Fats and oils	++
Resins	++
Phytosterols	++
Protein	+

+: Presence, ++: Moderate, +++: High, -: Absence

The quantification of secondary metabolites namely flavonoids and polyphenols showed that *S. wightii* is a good source of these metabolites. The polyphenol content was found to vary in the range of 21-55.2 µg and the flavonoid content was between 40 and 133 µg in 100-500 mg of the algal extracts. The strong peaks observed in FT-IR analysis revealed the presence of C-O stretching of alcohol (3346 cm⁻¹), C-O stretching of acid (2979 cm⁻¹), C-O stretching of ether (1251 cm⁻¹), and α, β unsaturated ketone (1639 cm⁻¹). The HPLC chromatogram showed the presence of two prominent compounds with retention time 20.75 and 22.739 minutes (Fig. 1).

The mass spectrometry analysis (Fig. 2) of the ethanol extract confirmed the presence of these two compounds and based on the ion chromatogram pattern and NIST14 library search these compounds

were identified as dotriacontyl isopropyl ether and ascorbic acid 2, 6 dihexadecanoate with M.wt. 508 and 652, respectively.

The interaction of these compounds with α-amylase and α-glucosidase was analyzed *in silico* using the SYBL X 1.3 docking software. The results of the interaction (Fig. 3) showed that l - (+) - ascorbic acid 2, 6 dihexadecanoate interacted with both α-amylase and α-glucosidase with a total score of 3.216 and 10.87, respectively, whereas the other compounds (dotriacontyl isopropyl ether) did not show any interaction with both the enzymes analyzed.

The antidiabetic activity of the ethanolic extract was determined by its potential in inhibiting the enzymes α-amylase and α-glucosidase, which are responsible for the postprandial hyperglycemia in diabetes. The

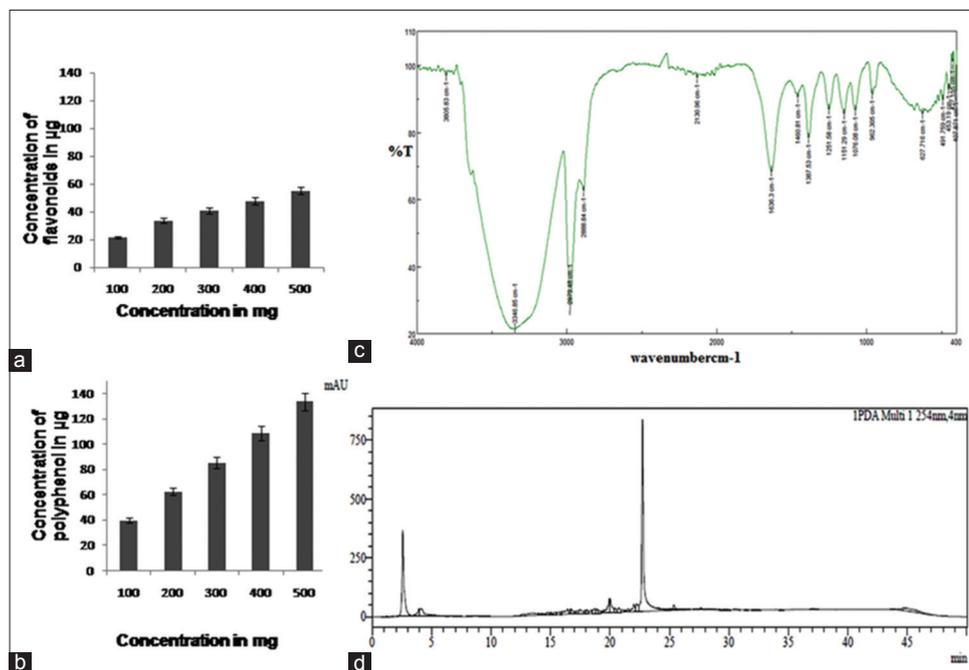


Fig. 1: Characterisation of ethanolic extract of *Sargassum wightii*. (a) Quantification of flavonoids in 100-500 mg of ethanol extract, (b) quantitative analysis of polyphenols in 100-500 mg of ethanol extract. The values represent mean±standard deviation, (c) Fourier transform infrared spectra of ethanol extract, (d) high-performance liquid chromatography chromatogram of ethanol extract

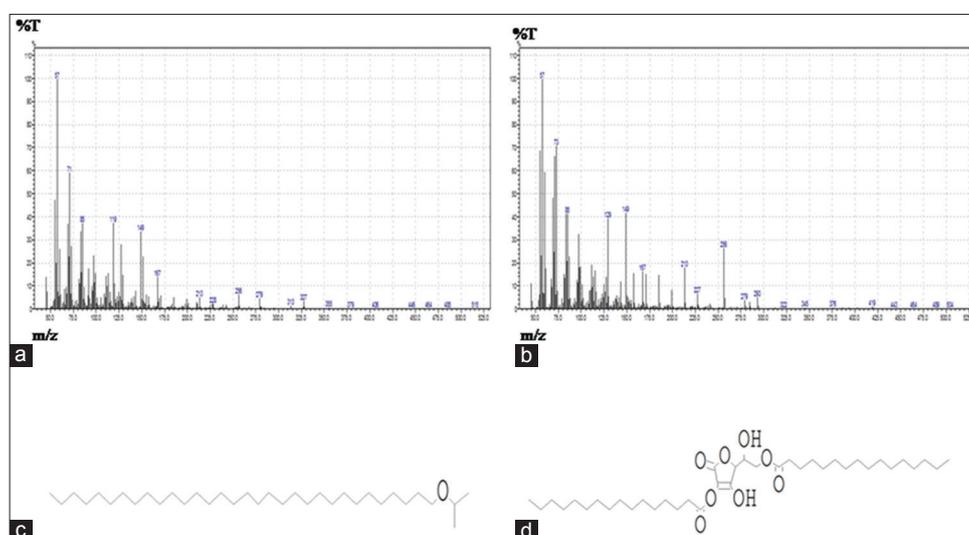


Fig. 2: Mass spectrometry analysis of ethanolic extract. (a) Ion chromatogram of dotriacontylisopropyl ether, (b) Ion chromatogram of ascorbic acid 2, 6 dihexadecanoate, (c) structure of dotriacontylisopropyl ether, (d) structure of ascorbic acid 2, 6 dihexadecanoate

ethanolic extract exhibited a dose-dependent inhibition of α -amylase ranging from 16.76% at 1 mg/ml to 50.53% at 8 mg/ml (Fig. 4a). The extract exhibited an inhibitory concentration (IC_{50}) value of 8 mg/ml. In case of α -glucosidase (Fig. 4b), the ethanolic extract exhibited significant inhibition with 90.1% at 10 mg/ml to 13.166% at 2 mg/ml. The IC_{50} was determined as 6 mg/ml. The protective effect of the ethanolic extract by inhibiting the formation of advanced glycation end products was analyzed. The extract exhibited good inhibition against the glycation of albumin. The inhibition of glycation (Fig. 4c) varied with increasing dosage. The inhibition of glycation was 28.23% at 4 mg/ml and increased to 50% at 10 mg/ml. The IC_{50} of the extract was 10 mg/ml. The anti-inflammatory activity of the ethanol extract was analyzed based the inhibition of albumin denaturation. The ethanolic extract exhibited 58.69% of inhibitory activity at 3 mg/ml. The IC_{50} value of the extract was observed at 2.5 mg/ml (Fig. 4d).

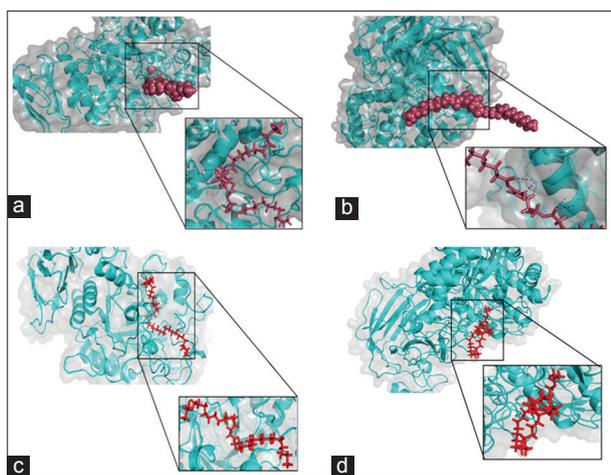


Fig. 3: Interaction studies of dotriacontylisopropyl ether and ascorbic acid 2, 6 dihexadecanoate with α amylase and α glucosidase. (a) Interaction of ascorbic acid 2, 6 dihexadecanoate with α amylase, (b) interaction of ascorbic acid 2, 6 dihexadecanoate with α glucosidase (c) interaction of dotriacontylisopropyl ether with α amylase, (d) interaction of dotriacontylisopropyl ether with α glucosidase

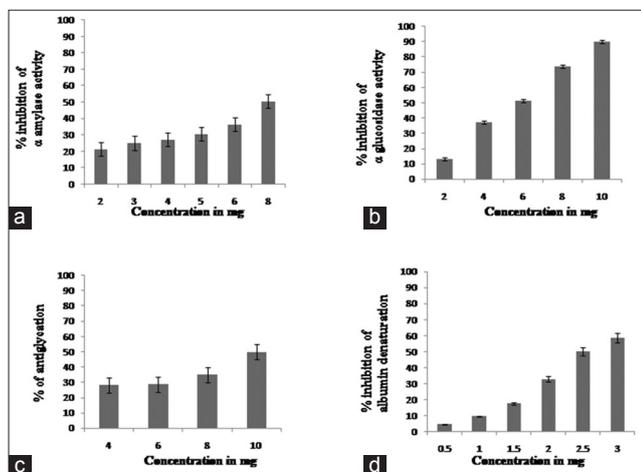


Fig. 4: Analysis of antidiabetic and anti-inflammatory potential of ethanolic extract. (a) Analysis of α amylase inhibitory activity of ethanolic extract in varying concentration (2-8 mg/ml), (b) analysis of α glucosidase inhibitory activity of ethanolic extract in varying concentration (2-10 mg/ml), (c) inhibition of protein glycation by ethanolic extract (4-10 mg/ml), (d) inhibition of albumin denaturation by ethanolic extract (1-3 mg/ml). Results expressed are mean \pm standard deviation

DISCUSSION

Diabetes is an endocrine disorder causing premature death globally. Hyperglycemia resulting from uncontrolled postprandial blood sugar is the major challenging factor in diabetes. Although considerable advancements have been made in the biomedical field, the management of blood glucose is quite challenging. The excess of blood sugar causes glycation of hemoglobin, resulting in the formation of reactive oxygen species (ROS) [22]. The generation of ROS leads to denaturation of proteins resulting in prolonged inflammation, delayed wound healing, micro, and macrovascular changes. One of the recent strategies adopted in the management of diabetes involves the inhibition of α -amylase and α -glucosidase thereby regulating the blood glucose release. This study is an attempt to find a suitable natural alternate source of marine origin for the management of diabetes.

In this study, the effect of the ethanol extract of *S. wightii* in inhibiting α -amylase and α -glucosidase, prevention of AGE formation and anti-inflammatory activity was evaluated under *in vitro* conditions. The phytochemical screening of the algal extract showed the presence of various phytoconstituents which are in agreement with earlier studies [23]. The quantification of flavonoids and polyphenols shows that *S. wightii* is rich in metabolites which are an important source of antioxidant molecules and are efficient free radical scavengers. Seaweeds are rich in antioxidants which may be attributed to their flavonoid and polyphenol content [24,25]. The FT-IR analysis confirmed the presence of various functional groups which is inconsistent with previous study [26]. The HPLC analysis showed the presence of two major compounds which were identified as dotriacontyl isopropyl ether and l-(+)-ascorbic acid 2, 6 dihexadecanoate by mass spectrometry. The identification of these compounds by MS analysis supports the FT-IR data which showed the presence of α, β unsaturated ketone (1639 cm^{-1}) group and hydroxy groups at 1639 and 3346 cm^{-1} which are present in ascorbic acid 2, 6 dihexadecanoate and the ether group at 1251 cm^{-1} present in Dotriacontyl isopropyl ether. Further Ascorbic acid 2, 6 dihexadecanoate is reported to confer wound healing property [27], antibacterial [28] and antitumor [29] properties. The characterization studies were followed by the *in silico* analysis where the interaction of ascorbic acid 2, 6 dihexadecanoate and dotriacontyl isopropyl ether with α -amylase and α -glucosidase was analyzed. The interaction study revealed that Ascorbic acid 2, 6 dihexadecanoate interacted with α -amylase and α -glucosidase efficiently with an SYBL C score of 4 and 5, respectively. The active site of α -amylase harbors the amino acids GLU 233, ASP 300 and ASP 197. These amino acids are essential for the breakdown of sugar residues by the enzyme. Ascorbic acid 2, 6 dihexadecanoate is found to interact with GLU 233 in the active site of α -amylase, thereby inhibiting the binding of sugar molecule to the enzyme. Thus, the interaction results put forth that the inhibition exerted by *S. wightii* on α -amylase is mediated through the binding of ascorbic acid 2, 6 dihexadecanoate to GLU 233 in the active site. Similarly ascorbic acid 2, 6 dihexadecanoate interacted with α -glucosidase with a SYBL C score of 5 through the residues ASN 621 and ARG 624. The docking study further revealed that dotriacontyl isopropyl ether present in the algal extract did not interact with both the enzymes analyzed. Thus, the interaction studies showed that the inhibition exhibited by the algal extract on α -amylase and α -glucosidase is mediated through ascorbic acid 2, 6 dihexadecanoate and not by dotriacontyl isopropyl ether.

The algal extract showed potent inhibition of α -amylase and α -glucosidase. Although previous studies have reported the inhibitory activity of methanol and ethyl acetate extracts of *S. wightii* against these enzymes, the compounds involved and the mechanism varies [30,31]. The inhibition of formation of AGE by the algal extract further supports its antidiabetic activity. The analysis of anti-inflammatory activity of *S. wightii* showed that the compounds of the algae offer protection against denaturation of proteins thus preventing inflammation of serum proteins. Denaturation of proteins causes a loss of their biological function and also leads to micro and macrovascular complications in diabetes. The inhibition of albumin denaturation suggests that can

limit the inflammatory response generated by proteases and enzymes in tissue inflammation [32]. Although similar studies [13,30] have reported the antidiabetic property of *S. wightii*, the potential bioactive compound and the mechanism of interaction were unrevealed. Thus, the current study has enabled in identifying the major bioactive compound and also the mechanism of its action in functioning as an antidiabetic compound. As the use of herbal plants in the management of diabetes is gaining momentum [33], the current study throws light on the antidiabetic potential of *S. wightii* which can be utilized efficiently in the treatment of diabetes. The *in vitro* analysis of antidiabetic and anti-inflammatory activity of *S. wightii* revealed that the algae not only possess potent inhibitors of α -amylase and α -glucosidase but also can offer protection from inflammatory response which alleviates the complications of diabetes.

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