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In-Vitro Antidiabetic And Antioxidant Activity Of Bioactive Principle From *Sargassum Ilicifolium*.

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ABSTRACT

Oxidative stress performs a key position with the development of the two kinds of Diabetes mellitus. This condition arises when the production of oxidant exceeds the antioxidant activity in cells and plasma. This oxidant excess is due to two important mechanisms: Free radicals overproduction from the mitochondria and cellular NADPH oxidase activation via Angiotensin II receptors. Free radicals are chemicals species with its outer orbital having an unpaired electron. They include peroxides, superoxides and hydroxyl radical. In the body, they are capable of destroying the tissues due to the molecular reactivity of various macromolecules in the body, which leads to the development of insulin resistance. These effects can promote the evolution of complications of Diabetes mellitus. Seaweeds have been confirmed to be an outstanding resource of nutrients and secondary metabolites. In vitro studies of the effect of Phlorotannins from Sargassum ilicifolium on the biomarkers are surveyed. The present investigation suggests that the antidiabetic activity of Sargassum ilicifolium may be due to its hypoglycemic, antioxidant, and alpha-amylase inhibiting property. Thus, this seaweed may favor diabetic individuals. Further research is required to inquire into free radicals and its relationship with diabetes, and its complications, and to make lucid the mechanisms by which a rise in oxidative stress accelerates the evolution of diabetic complications, in an effort to increase the options for treatment.

Keywords: Free radicals, oxidative stress, Diabetes mellitus, Seaweeds.

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INTRODUCTION

The disturbances of carbohydrate, fats and protein metabolism leads to progressive disorder- **Diabetes mellitus**. It is characterized by chronic hyperglycemia, where the blood sugar levels are raised, either due to a deficit in insulin secretion or action (insulin resistance). Hence, a curative approach in regard to treating diabetes is to decrease high blood sugar post a meal, which can be achieved by the inhibiting α-glucosidase and α-amylase, which are the major digestive enzymes involved in the digestion of carbohydrates. α-amylase breaks down long chain carbohydrates like starch and disaccharides to glucose while α–glucosidase helps in intestinal absorption. These enzymes inhibitors are targeted for the development of the lead molecules for diabetes therapy.[**1]**

An imbalance between pro-oxidant and antioxidant homeostasis, results in **Oxidative stress**. The toxic reactive oxygen species (ROS) such as peroxyl radicals hydroxyl and superoxides, produced by them, attack the macromolecules like membrane lipids, proteins, and DNA, leading to many health problems such as cancer, diabetes mellitus, and age-related health deterioration condition. Antioxidants may have a positive impact on human health as they protect the human body against injury by ROS.[**2] Amongst the natural sources marine algae are considered to be an abundant source of antioxidants.** [**3]**

Seaweeds are plant-like organisms that mostly exist attached to rock or any other hard substratum along the coast. Secondary metabolites such as terpenoids, polysaccharides, polyphenols, sargachromenol, plastoquinones, steroids, glycerides glycerides etc., from the Sargassum species have several remedial activities, and are found throughout the tropical and subtropical regions. It has been considered as a therapeutic food of this century, as it contains many pharmacological properties. Further research is being carried out to find out more on its mechanisms.

Taxonomical classification

Division:	Phaeophyta
Order:	Fucales
Family:	Sargassaceae
Genus:	Sargassum
Species:	ilicifolium

Brown algae such as kelps and rockweeds or Sagassacean species and a few red algae contain phlorotannins, a type of tannins, proved for showing anti-diabetic, anti-cancer, anti-oxidative, antibacterial, radioprotective and anti-HIV properties. As they are called tannins, they have the ability to precipitate proteins. It has been observed that some phlorotannins (Fig.1) have the ability to oxidize and form covalent bonds with some proteins.[**4]**

After a meal, the glucose levels get elevated. Being an **alpha–amylase inhibitor**, it decreases the elevated glucose levels by decelerating the rate with which α–amylase converts polysaccharides to monosaccharide. Fast clearing rate of extracellular glucose from the blood important in diabetic people is prevented by low levels of insulin. Hence, in order to keep their glucose levels under control the diabetics are probable to have low alpha–amylase levels. So, α–amylase inhibitors have a vital role in controlling blood glucose levels.

α–glucosidase inhibitors are used by means of oral route, for treating Type II Diabetes mellitus. α–glucosidase inhibitors which are the competitive inhibitors of α–glucosidase enzyme, help in carbohydrate digestion. The α-glucosidases of the small intestine hydrolyze complex carbohydrates to simple sugars like glucose. α–glucosidase inhibitors act by inhibiting the breakdown of carbohydrates like starch. The speed of digestion of carbohydrates can be reduced by inhibiting these enzyme systems and less amounts of glucose is absorbed as the carbohydrates are not broken down into glucose. [**5]** In diabetic patients, the enzyme inhibitor drug therapy shows temporary effect by decreasing elevated blood glucose levels. Phlorotannins are α -amylase and α -glucosidase inhibitors, which exist naturally, and can be used as an effective therapy for treating post meal hyperglycaemia with lesser side effects. [**6]**

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This study involves screening of seaweeds for a number of biological activities, with potentially useful therapeutic property.

MATERIALS AND METHODS

Collection and Authentication of Plant material

Sargassum ilicifollium is brown algae, which was collected from Mandappam, Gulf of Mannar region, Rameshwaram, India. It was identified and authenticated by Dr. (late). V. Krishnamurthy, Director, Krishnamurthy Institute of Algalogy, Chennai.

Isolation and Purification of Phlorotannins

200mg of powdered algae was taken in a conical flask containing 100ml hexane and were defatted overnight. Hexane was removed and the algal matter was dried. 10ml of 7:3(acetone: water) was allowed to pass in the algae and the supernatant was filtered. Then the same ratio was continued with the algae for 3 days. The product formed from the filtrate was evaporated and dried. It was kept in a light resistant container and stored in the refrigerator until further use.

FT-IR Spectra of Phlorotannins

The phlorotannins of Sargassum ilicifolium was blended with 100gm of dried potassium bromide and were compressed to make a salt disc. The disc was then read spectrophotometrically. The frequencies of different components present in each sample were analyzed.

In-vitro Antioxidant activity by DPPH Scavenging Method:

Spectrophotometric analysis was used for measuring DPPH scavenging activity. Stock solution of DPPH (150µM) was prepared in ethanol[1]. 0.1 ml of various concentrations (31.25-2000 µg/0.1 ml) of phlorotannins and DPPH (1.9 ml) was added. The control was prepared similarly excluding test compound. In blank, ethanol replaced DPPH. BHA (31.25- 2000 µg/0.1 ml) was used as standard.The mixtures were kept in dark for 20 minutes, for the reaction to complete and the absorbance was read at 517 nm. The scavenging activity is inversely proportional to the absorbance of the reaction mixture.[**7]** The percentage inhibition of DPPH was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Average OD (Control)} - \text{Average OD (Sample)}}{\text{Average OD (Control)}} \times 100$$

In-vitro Antioxidant activity by Nitric Oxide Scavenging Method:

Nitric oxide free radical scavenging activity of the extract was determined.[**8]** 3 ml of the reaction mixture consisting 2 ml of 10 mM sodium nitroprusside in PBS and 1 ml of various concentrations (62.5-1000 µg/ml) of phlorotannins were incubated for 4 hours at 37˚C. A control was kept in the same manner. About 0.5 ml of Griess reagent was added after incubation. Ascorbic Acid (62.5- 1000 µg/ml) was used as the standard. At 546 nm the absorbance of the chromophore formed was read.The percentage inhibition of nitric oxide scavenging was calculated using the formula,

$$\% \text{ Inhibition} = \frac{\text{Average OD (Control)} - \text{Average OD (Sample)}}{\text{Average OD (Control)}} \times 100$$

Extraction of Alpha–Amylase enzyme

α–amylase enzyme extraction was done according to the method [**9]**, with slight modifications. About 500g of Malted wheat flour, previously freeze dried was mixed with 0.2% calcium acetate solution, and stirred

for two hours at room temperature. The suspension was centrifuged at 40^0C at 12,000 rpm for 10 minutes. A clear supernatant, brown in colour serves as the enzyme source and was stored at 2-3^0C.

In-vitro Anti-diabetic activity by Alpha–Amylase Inhibitory Assay:

α–amylase inhibitory activity was measured by the spectrophotometric method.[**9]** The assay mixture contained 200µL of 0.02M sodium phosphate buffer, 20µL of enzyme and 20µL of sample of phlorotannins in various concentrations (31.25-1000µg/0.1 ml) and then incubated for 10 minutes at room temperature. 200µL starch was added to all the tubes. In the same way, the control was prepared, without the test compound. Sodium phosphate buffer was used as blank. The reaction was terminated by the addition of 400µL of DNS reagent and boiled for 5 minutes. It was then cooled and diluted by adding 15 ml distilled water. Acarbose (31.25-1000µg/0.1 ml) was used as standard. The absorbance was read at 540 nm. The percentage inhibition of DPPH was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Average OD (Control)} - \text{Average OD (Sample)}}{\text{Average OD (Control)}} \times 100$$

Extraction of Alpha-Glucosidase enzyme

α–glucosidase enzyme extraction was done according to the method [**10]**, with slight modifications. 100g of freeze-dried barley was treated with 500mL of 0.02 M sodium phosphate buffer with pH 8.0, containing 0.002M of L-cysteine. The extract was centrifuged at 40C at 10,000 rpm for 5 minutes. A clear supernatant, brown in colour serves as the enzyme source and was stored at 2-3^0C.

In-vitro Anti-diabetic activity by Alpha–Glucosidase Inhibitory Assay:

Based on J.R. Stark et al with slight changes[**10]** The assay mixture consisted 0.5mL of sample in various concentrations (31.25- 1000µg/0.1 ml) of Sargassum ilicifolium with 0.5mL enzyme and 0.5mL substrate solution (3mg/mL Maltose in buffer). It was incubated at 37^0Cfor 60 minutes. 200µL of starch was added to all tubes. Control is prepared in the same manner without the test compound. Sodium acetate buffer was used as blank. Addition of 0.5mL sodium bicarbonate solution terminated the reaction. The absorbance was read at 420 nm. Acarbose (31.25-1000µg/0.1 ml) was used as standard.

Statistical Analysis:

The complete data was expressed as mean ± standard error of mean (S.E.M.).

RESULTS

FT-IR Study of Phlorotannins from Sargassum ilicifolium

The Fourier transform infrared spectroscopy identifies the functional group based on the peak value in the region of infrared radiation. The phlorotannins of Sargassum ilicifolium was passed into the FT-IR and based on the peak value the functional group of the components were separated.

The FT-IR spectra of Phlorotannins obtained from Sargassum ilicifolium showed a characteristic peak at 3714.23, 1164.79, 1519.6, 3031.55, 1643.05 cm^{-1} corresponding to OH stretching, C-O stretching, aromatic H stretching, aromatic C=C stretching respectively (Fig. 2).

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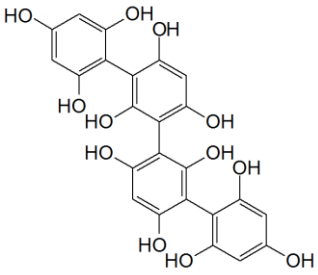

Fig.1: Structure of Phlorotannins

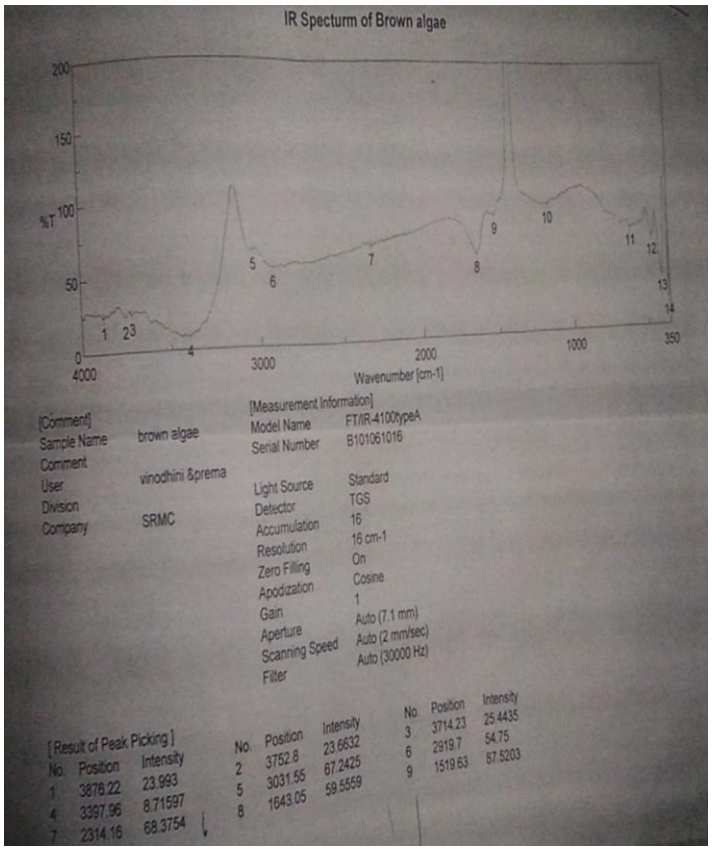

Fig 2: FT-IR spectra of Phlorotannins obtained from Sargassum ilicifolium

In-vitro Antioxidant activity by DPPH Scavenging Method:

DPPH assay is rapid, easy and cost effective method to measure an antioxidant effect, which involves the use of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), which is a free radical. DPPH is commonly used to test the ability of the compounds to act as scavengers of free radicals or hydrogen donors and it is based on the ability of DPPH to decolorize in the presence of an antioxidant. Phlorotannins from Sargassum ilicifolium scavenged DPPH free radical and showed a maximum percentage inhibition of 78.516±3.502% at 2000µg/0.1 ml concentration. DPPH scavenging activity of Sargassum ilicifolium is shown in Table 1 and represented graphically in Fig.3.

Table 1: DPPH Scavenging activity

Conc. (µg/0.1ml)	Percentage Inhibition (mean ± SEM)	
	Butylated Hydroxyanisole	Phlorotannins

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	(BHA)	(PT)
31.25	59.82±1.07	55.30±1.72
62.5	63.68±0.35	55.70±1.27
125	77.29±0.55	59.15±0.35
250	89.79±3.08	60.86±1.20
500	90.16±0.23	62.54±0.72
1000	93.52±0.25	62.99±1.58
2000	94.12±0.32	78.51±3.50

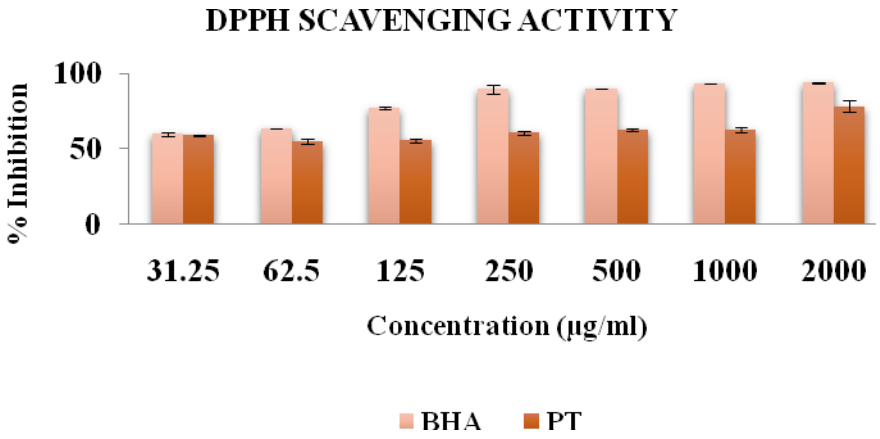

Fig 3: Graphical Representation of DPPH Scavenging activity

In-vitro Antioxidant activity by Nitric Oxide Scavenging Method:

Nitric oxide scavenging activity is based on the inhibition of nitric oxide radical generated from sodium nitroprusside, which interacts with oxygen to produce nitrate ions and measured colorimetrically by the use of Griess reagent. The phlorotannins confirmed the scavenging of nitric oxide radical, formed as a result of addition of sodium nitroprusside. NO scavenging activity was found to be increased with increase in concentration. It showed the maximum inhibition of 91.026±0.872% at 2000µg/ml. Nitric oxide scavenging activity of phlorotannins from Sargassum ilicifolium is shown in Table 2 and Fig. 4.

Table 2: Nitric oxide scavenging activity

Conc. (µg/0.1ml)	Percentage Inhibition (Mean ± SEM)	
	Ascorbic Acid	Phlorotannins (PT)
31.25	63.18±5.40	55.68±0.93
62.5	74.63±2.71	62.39±3.38
125	82.48±1.62	63.92±6.12
250	91.13±0.43	82.37±1.55
500	93.13±0.08	86.49±0.72
1000	95.48±0.19	91.02±0.87

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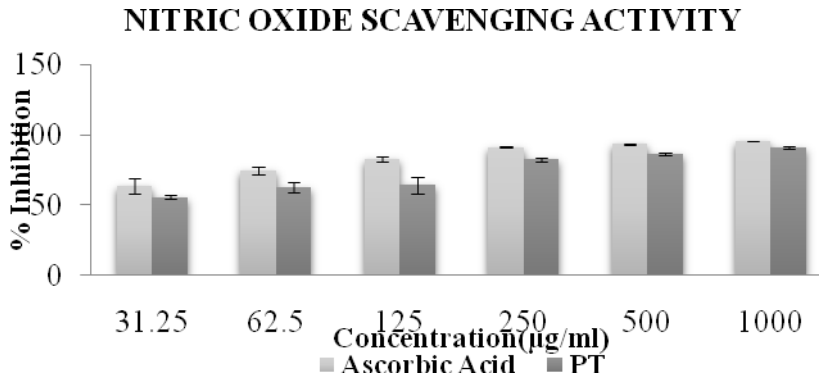

Fig 4: Graphical Representation of Nitric oxide scavenging activity

In-vitro Anti-diabetic activity by Alpha–Amylase Inhibitory Assay:

α-amylase inhibitory activity is based on the inhibition of enzyme-substrate complex by addition of phlorotannins to the mixture. α-amylase inhibitory activity was found to be increased with increase in concentration. It showed the maximum inhibition of 90.703±0.455% at 2000µg/ml. α-amylase inhibitory activity of phlorotannins from Sargassum ilicifolium is shown in Table 3 and represented graphically (Fig. 5).

Table 3: Alpha-amylase inhibitory activity

Conc. (µg/0.1ml)	Percentage Inhibition (mean ± SEM)	
	Acarbose	Phlorotannins (PT)
31.25	52.29±0.27	44.53±0.37
62.5	67.32±2.26	63.11±0.49
125	78.96±0.88	77.94±0.32
250	87.80±0.82	84.89±1.02
500	90.17±0.28	87.22±0.42
1000	91.59±0.41	90.70±0.45

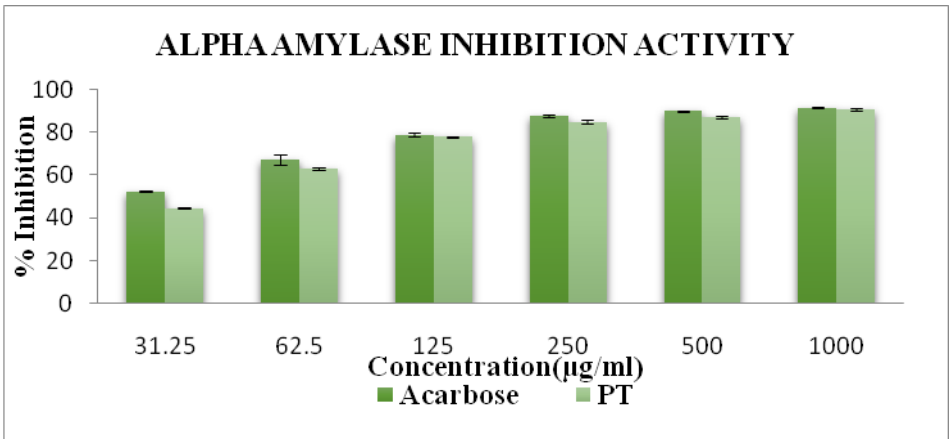

Fig 5: Graphical Representation of Alpha-amylase inhibitory activity

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In-vitro Anti-diabetic activity by Alpha–Glucosidase Inhibitory Assay:

α-glucosidase inhibitory activity is based on the inhibition of enzyme-substrate complex by addition of phlorotannins to the mixture. α-glucosidase inhibitory activity was found to be increased with increase in concentration. It showed the maximum inhibition of 90.9±0.288% at 2000µg/ml. Alpha-glucosidase inhibitory activity of phlorotannins from Sargassum ilicifolium is shown in table 4 and represented graphically (Fig. 6).

Table 4: Alpha-glucosidase inhibitory activity

Conc. (µg/0.1ml)	Percentage Inhibition (Mean ± SEM)	
	Acarbose	Phlorotannins (PT)
31.25	58.44±0.27	42.87±0.44
62.5	67.45±0.86	53.72±0.35
125	73.38±0.43	63.41±0.24
250	80.41±0.47	79.39±0.32
500	91.80±0.37	86.97±0.41
1000	93.38±0.40	90.90±0.28

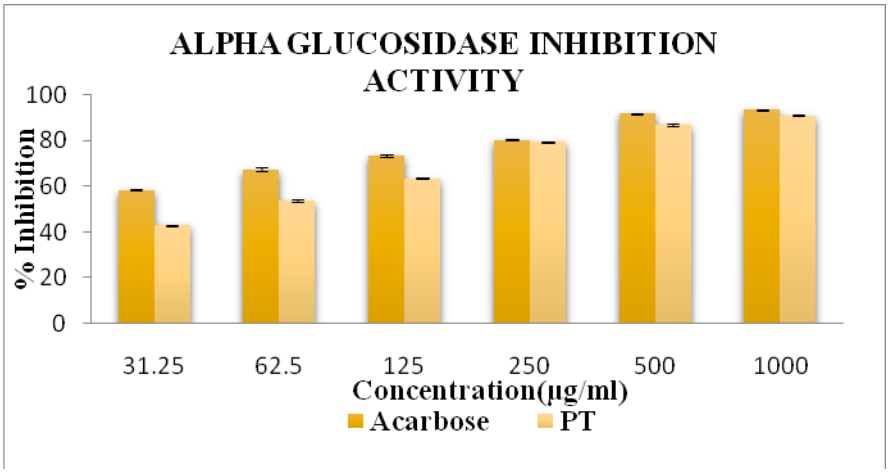

Fig 6: Graphical Representation of Alpha-glucosidase inhibitory activity

DISCUSSION

Phlorotannins from the seaweed extract demonstrates potent antioxidant activity in different in vitro assays. The DPPH radical scavenging showed a maximum percentage inhibition of 78.516±3.502% at 2000µg/0.1 ml concentration which was compared with butylated hydroxyl anisole (BHA) as a standard, having the maximum percentage inhibition of 94.12±0.32 (Table 1). In addition to this, phlorotannins also possess potent nitric oxide scavenging activity with maximum inhibition of 91.026±0.872% at 2000µg/ml, compared with Ascorbic acid as the standard which had the maximum percentage inhibition of 95.486±0.195 (Table 2). The result of this study shows that phlorotannins from the Sargassum ilicifolium can be used easily as an available source of natural antioxidants. The in vitro anti-diabetic studies demonstrated that phlorotannins had α-amylase and α-glucosidase inhibitory activity. It showed the maximum inhibition of 90.703±0.455% at 2000µg/ml and 90.9±0.288% at 2000µg/ml for α-amylase and α-glucosidase inhibitory activities respectively. Acarbose was used as the standard with the maximum inhibition of 91.593±0.415 and 93.383±0.405 µg/ml for α-amylase and α-glucosidase inhibitory activities respectively.[**11]**

Several important secondary metabolites that include alkaloids, phenols, flavanoids, saponins have been identified as the potential source of seaweeds. These secondary metabolites participate extensively in their defense against diseases, microorganisms and stress. They have been extensively studied for their importance in providing good health and defense against diseases and thus seaweed could definitely behave as potential hypoglycaemic and anti-diabetic agent. Hence, preliminary FT-IR served as an initial step in identifying the functional group of the active components. Phlorotannins from Sargassum ilicifolium showed considerable anti-diabetic and anti-oxidant activity.[**12]**

CONCLUSION

Our preliminary pharmacological studies on phlorotannins from Sargassum ilicifolium provide as a part of scientific support in traditional medicine, particularly in diabetes. However, further pharmacological investigations are required to understand its principal mode of action on the antidiabetic activity.

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