

SCREENING OF SEaweEDS COLLECTED FROM SOUTHEAST COASTAL AREA OF INDIA FOR α -AMYLASE INHIBITORY ACTIVITY, ANTIOXIDANT ACTIVITY AND BIOCOMPATIBILITY

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ABSTRACT

Objectives: Ten seaweeds namely, *Sargassum duplicatum*, *Sargassum wightii*, *Sargassum tenerrimum*, *Turbinoid conoids*, *Turbinoid ornate* and *Padina gymnospora* (brown seaweed), *Gracillaria gracilis*, *Chondrococcus hornemanni*, *Gracillaria edulis* (red seaweed) and *Caulerpa racemosa* (green seaweed) were harvested from the southeast coastal area of India and screened for α -amylase inhibitory activity, antioxidant activity and toxic effects.

Methods and Results: The collected seaweeds were subjected to solvent extraction using ethyl acetate, acetone and methanol. Among the ten seaweeds screened, the ethyl acetate extract of *G. gracilis*, *C. harnemanii* and *P. gymnospora* showed 81, 94% and 71% of α -amylase inhibitory activity respectively at the concentration of 300 μ g/ml. Whereas, seaweeds extracted using methanol showed moderate α -amylase inhibitory activity. All the three solvent extracts of *G. edulis* showed α -amylase inhibition. *C. harnemanii*, *G. gracilis* and *P. gymnospora* were subjected to further analysis due to their promising α -amylase inhibition. Antioxidant assays like DPPH, Deoxy ribose sugar damage assay were carried out, and *C. harnemanii* at the concentration of 300 μ g/ml, showed very good results against DPPH and deoxy ribose damage assay (78.02 and 85%). Haemolytic and cytotoxicity assays were performed in order to find the toxicity of the extracted compounds of *C. harnemanii*, *G. gracilis*, *P. gymnospora* and *C. harnemani*. No cytotoxicity was observed with the extracts of seaweeds except that of *G. gracilis*, which showed 4% toxicity as well as erythrocyte membrane damage.

Conclusion: From this study, it is shown that the ethyl acetate extract of *C. harnemanii* possesses potent α -amylase inhibitory activity. Further research is needed to identify the particular active principle responsible for the activity.

Keywords: α -amylase, Seaweeds, Toxicity, Solvents

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism resulting from absolute or relative lack of insulin secretion [1]. The prevalence of this disorder is on the rise and the global diabetic burden is expected to cross 440 million around the globe by the year 2030 [2]. Globally, among DM, type II DM (Non-insulin dependent DM) accounts for more than 90% of the cases [3]. In diabetes, the postprandial phase is characterized by a rapid and increased blood glucose levels, and the postprandial "hyperglycemic spikes" may be relevant to the pathophysiological conditions of type II DM [4]. Complications such as micro and macro vascular diseases associated with DM are proposed as independent risk factors for cardiovascular disease [5]. Intake of α -amylase inhibitors along with diet will help to regulate the postprandial hyperglycemic spikes. Therefore, the drugs against postprandial hyperglycemia are important in the management of pathophysiological conditions of DM.

The recent approach for controlling postprandial hyperglycemia is to inhibit the carbohydrate-hydrolyzing enzymes such as α -amylase and α -glucosidase in the digestive system [6]. Inhibitors of pancreatic α -amylase delay carbohydrate digestion and cause a reduction in the rate of glucose absorption thereby lowering the post-prandial serum glucose levels [7]. Some inhibitors like acarbose and miglitol, which are currently in clinical use, inhibit glycosidases such as α -glucosidase and α -amylase whereas others like voglibose inhibit α -glucosidase. However, many of these synthetic hypoglycemic agents have their own limitations. They are non-specific, produce serious side effects and fail to reduce the diabetic complications. The main side effects of these inhibitors are gastrointestinal disturbances viz., bloating, abdominal discomfort, diarrhoea and flatulence [8]. Natural products are given due importance in the treatment of DM owing to the negligible side effects, easy availability and affordability when compared to synthetic hypoglycaemic agents [9-10].

Marine seaweeds produce a diverse array of compounds that function as chemical defence systems facilitating their survival in extremely

competitive environments [11]. Research on the natural products and chemical defences of seaweeds over the past 40 years has resulted in the isolation of over 15,000 novel compounds, many of which have been shown to exhibit bioactive properties [12]. Seaweeds from the three groupings traditionally known as Chlorophyta (green seaweed), Rhodophyta (red seaweed) and Phaeophyta (brown seaweed) produce compounds with varying bioactivities [13]. In light of their reported broad-spectrum biological activities, seaweeds have been suggested as a promising source of bioactive substances that might have pharmaceutical applications [14].

The objective of this study was to screen α -amylase inhibitory activity of 10 different seaweeds collected from the southern coastal area of India and to analyze the antioxidant activity and toxic effects of the selected seaweeds.

MATERIALS AND METHODS

Chemicals

Porcine pancreatic α -amylase (EC 3.2.1.1), Dinitro salicylic acid and the other fine chemicals were purchased from Sigma Chemical Co. USA.

Algal samples

Six brown seaweeds (*Sargassum duplicatum*, *Sargassum wightii*, *Sargassum tenerrimum*, *Turbinoid conoids*, *Turbinoid ornate* and *Padina gymnospora*) three red seaweeds (*Gracillaria gracilis*, *Chondrococcus hornemanni* and *Gracillaria edulis*) and one green alga (*Caulerpa racemosa*) were collected from the southeast coastal area of India (Puducherry, Rameswaram, Tuticorin, Vizag and Kanyakumari) in August and September, 2012. Fresh seaweeds were individually washed in deionized water and air-dried in shade at room temperature. Dried samples were individually cut into small pieces (2 to 3 cm), homogenized, sieved using a 500 μ m sieve, and stored at -40°C until use.

Sample preparation

Shade dried seaweed materials were ground into powder. The extract was prepared by successive maceration of the powder (10 g) with different solvents like ethyl acetate, methanol and acetone (100 ml) at

room temperature for two days in an orbital shaker at 150 rpm. The final extract obtained was filtered by Whatman® No: 1 filter paper and the filtrate was lyophilised to obtain the powdered extract, which was used for further assays.

Determination of α -amylase inhibitor activity

About 500 μ l of each seaweed extracts and 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α -amylase solution (0.5 mg/ml) were incubated at 25 °C for 10 min. After pre-incubation, 500 μ l of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube. The reaction mixtures were incubated at 25° C for 10 min. After incubation, the reaction was stopped by the addition of 1.0 ml of dinitrosalicylic acid. The test tubes were incubated in a boiling water bath for 5 minutes and then cooled down to room temperature. The reaction mixture was diluted by the addition of 10 ml distilled water. The absorbance was than measured at 540 nm in an UV-Visible Spectrophotometer (UV-1100, Shanghai MAPADA Instruments Co., Ltd., China). The percentage of enzyme inhibition was calculated as:

$$\% \text{ of inhibition} = \{[A_{540} \text{ control} - A_{540} \text{ extract}] / A_{540} \text{ control}\} \times 100$$

Evaluation of antioxidant activity

DPPH free radical scavenging assay

The radical scavenging activity of the selected seaweeds was determined [15]. About, 1ml of 0.3mM alcoholic solution of DPPH was added to the solvent extracts of test samples at varying concentrations (100, 200 and 300 μ g/ml, respectively). After incubation of samples in dark for 30 minutes, the optical density was measured at 518 nm using methanol as blank and synthetic antioxidant Butylated Hydroxy Toluene (BHT) as positive control.

The radical scavenging activity (AA) was determined as:

$$AA\% = 100 - \{[(\text{Abs. sample} - \text{Abs. empty sample}) / \text{Abs. control}] \times 100\}$$

Determination of DNA sugar damage inhibition by Deoxyribose Assay

Freshly prepared solutions were used for this assay. The reaction mixture contained 0.5 ml of 2-deoxy-2-ribose, 0.5 ml of Phosphate buffer, 1 mL of the seaweed extracts at varying concentrations (in buffer), 2 mL of 4.8mM ferrous ammonium sulphate. After an incubation period of 1 hr at 37 °C, the extent of deoxyribose degradation was measured by the TBA (Thiobarbituric acid) reaction. 1.0 mL of TBA (1% in 50 mM NaOH) and 1.0 mL of TCA (Trichloro acetic acid) (2.8%) were added to the reaction mixture and the tubes were heated at 100 °C for 15 min. After cooling, the absorbance was read at 532 nm against a blank (containing only buffer and deoxyribose). The absorbance (A₁) read at the end of the experiment was used for the calculation of the percentage inhibition of deoxyribose degradation by the seaweed extracts. The percentage of DNA sugar damage inhibition was calculated as:

$$\% \text{ of Inhibition} = \{[A_0 - A_1] / A_0\} \times 100$$

A₀ = the absorbance of the control sample (containing no seaweed extracts) and A₁= the absorbance of the seaweed extracts.

Toxicity test

Haemolytic assay

To evaluate the biocompatibility of seaweeds, hemolysis test was done [16]. The different solvents extracts of *C. harnemanii*, *G. gracilis* and *P. gymnospora* at the concentrations of 100, 200 and 300 μ g/ml were mixed with 100 μ l of erythrocyte stock dispersion and incubated at 37° C in an orbital shaker at 100 rpm. After incubation, the samples were centrifuged at 1500 rpm for 3 min. The absorbance of the supernatant was measured at 398nm. Saline solution (27%) was used as the negative control (nc, 0% lysis) and distilled water was used as the positive control (pc, 100% lysis). The hemolysis rate (HR) was calculated by the following equation:

$$HR = (Dt - Dnc \times 100\%) / (Dpc - Dnc)$$

Where, Dt, Dnc and Dpc were the absorbance of the tested sample, the negative control and the positive control, respectively.

Cytotoxicity test

Brine shrimp lethality assay

The toxic effect of the seaweeds extracts were determined by brine shrimp lethality assay [17]. The powdered extracts of *C. harnemanii*, *G. gracilis* and *P. gymnospora* at the concentrations of 100, 200 and 300 μ g/ml were dissolved in 0.01 mL of DMSO and mixed with 5 mL of sea water (pH= 8.8 and salinity = 28 %) containing ten *Artemia* sp. A control DMSO was also maintained. The vials were kept under illumination. Survivors were counted after 24 hrs and the percentage of mortality was calculated for test samples and control (DMSO and saline water).

Statistical Analysis

All the assays (α -amylase activity, toxicity test and antioxidant activity) were performed in triplicate and mean, SD values were calculated by SPSS® (v16) software for windows.

RESULTS

Inhibition assay for α -amylase activity

Seaweeds extracted using three different solvents were assessed for inhibition of α -amylase *in vitro*. In the above study, ethyl acetate extracts of *C. Recimosa*, *G. gracilis*, *C. harnemanii* and *P. gymnospora* showed potent α -amylase inhibitory activity. The crude ethyl acetate extract of *C. Recimosa*, *G. gracilis*, *C. harnemanii* and *P. gymnospora* at the concentration of 300 μ g/ml exhibited α -amylase inhibitory activity at 73, 81 and 94% (Table 1). Among the three seaweeds *C. harnemanii* showed the maximum inhibition (94%). The methanol extracts of *T. ornate* did not show any α -amylase inhibitory activity (Table 1). *Chondrococcus harnemanii*, *G. gracilis* and *P. gymnospora* were found to possess very good α -amylase inhibition. Only in *G. edulis* all the three solvent extracts demonstrated α -amylase inhibitory effect. Due to the very good inhibitory activity exhibited by the ethyl acetate extracts of *C. harnemanii*, *G. gracilis* and *P. gymnospora*, these extracts alone were subjected to further analysis.

Table 1: α -amylase inhibitory activity of crude extracts of seaweeds

Name	% of inhibition of methanol extracts			% of inhibition of acetone extracts			% of inhibition of ethyl acetate extracts		
	100 μ g	200 μ g	300 μ g	100 μ g	200 μ g	300 μ g	100 μ g	200 μ g	300 μ g
<i>T. conoids</i>	-	-	-	-	-	-	-	-	-
<i>C. recimosa</i>	27 \pm 0.03	11 \pm 1.0	35 \pm 0.02	-	-	-	40 \pm 0.7	60 \pm 0.4	68 \pm 0.5
<i>S. duplicatum</i>	53 \pm 0.05	60 \pm 0.8	65 \pm 0.06	-	-	-	45 \pm 0.6	65 \pm 0.7	68 \pm 0.7
<i>G. gracilis</i>	50 \pm 0.03	60 \pm 0.6	68 \pm 0.04	-	-	-	65 \pm 0.2	78 \pm 0.1	81 \pm 0.9
<i>G. Edulis</i>	25 \pm 0.8	42 \pm 0.5	57 \pm 0.9	29 \pm 0.3	50 \pm 0.6	53 \pm 0.1	48 \pm 0.04	50 \pm 0.5	60 \pm 1.2
<i>C. harnemanii</i>	44 \pm 0.3	54 \pm 0.3	61 \pm 1.0	-	-	-	78 \pm 0.6	92 \pm 0.3	94 \pm 0.08
<i>S. wightii</i>	21 \pm 0.08	40 \pm 0.3	53 \pm 0.6	-	-	-	-	-	-
<i>S. Tenerrimum</i>	30 \pm 0.03	52 \pm 0.3	65 \pm 0.2	-	-	-	-	4 \pm 0.2	12 \pm 0.1
<i>P. gymnospora</i>	44 \pm 0.9	52 \pm 0.6	58 \pm 0.06	-	-	-	44 \pm 0.2	63 \pm 0.3	71 \pm 0.6
<i>T. ornatab</i>	21 \pm 0.07	29 \pm 0.5	45 \pm 0.1	-	-	-	-	-	-

(-) no detectable α -amylase inhibitory activity

Evaluation of antioxidant activity

DPPH free radical scavenging assay

The ethyl acetate extracts of the three selected seaweeds showed free radical scavenging property at all the three concentrations studied. DPPH can be used in determining radical scavenging activity as it forms a stable molecule on accepting an electron or hydrogen atom. The results obtained are shown in Table 2. *Chondrococcus harnemanii* extracts showed satisfactory effect in inhibiting DPPH. The scavenging effects of the seaweed extracts (300µg/ml) on the DPPH radical was found to be increasing in the following order: *G. gracilis* (64%) < *P. gymnospora* (65%) and < *C. harnemanii* (79%). Among the various solvent extracts analyzed for DPPH scavenging activity, ethyl acetate extracts of seaweeds showed higher radical inhibition activity, which is comparable with that of standard BHT (85%) at 300 µg/ml.

Table 2: Evaluation of DPPH activity of the ethyl acetate extracts of the seaweeds

Name	DPPH (%)		
	100µg/ml	200 µg/ml	300µg/ml
BHT	65±0.5	76±0.8	85±0.2
<i>H. gracilis</i>	45 ± 0.7	48 ± 0.6	64 ± 0.3
<i>P. gymnospora</i>	53 ± 0.8	61± 0.3	65 ± 0.8
<i>C. harnemanii</i>	66 ± 0.3	73 ± 0.6	79 ± 0.05

DNA Sugar damage assay

The ability of the extracts to prevent the damage of ribose sugar moiety of the DNA was studied. The best inhibition of the ribose sugar damage is exhibited by the ethyl acetate extracts of *C. harnemani*, *G. gracilis* and *P. gymnospora* (300µg/ml) with a percentage inhibition of 88, 65 and 56.04 % respectively (Table 3).

Table 3: Evaluation of DNA Sugar damage activity of the ethyl acetate extracts of the seaweeds

Name	Deoxyribose (%)		
	100µg/ml	200 µg/ml	300µg/ml
<i>H. gracilis</i>	55 ± 0.06	66 ± 0.9	88 ± 0.7
<i>P. gymnospora</i>	40 ± 0.8	55 ± 0.4	65 ± 0.04
<i>C. harnemanii</i>	66 ± 0.2	77 ± 0.4	88 ± 1.0

Haemolytic assay

In this study, *G. gracilis* showed a minimal toxic effect at the concentration of 300µg/ml (Figure1), and the other two samples did not show erythrocyte membrane damage at the test concentrations.

Cytotoxicity Assay

The cytotoxicity was performed using the larvae *Artemia salina* (Brine shrimp). The percentage mortality for 24 hrs was calculated. Among the three seaweed samples, *C. harnemani* and *P. gymnospora* showed denial cytotoxicity (Table 4) and the maximum toxicity among the extracts tested was shown by *G. gracilis* (4% at 300 µg/ml).

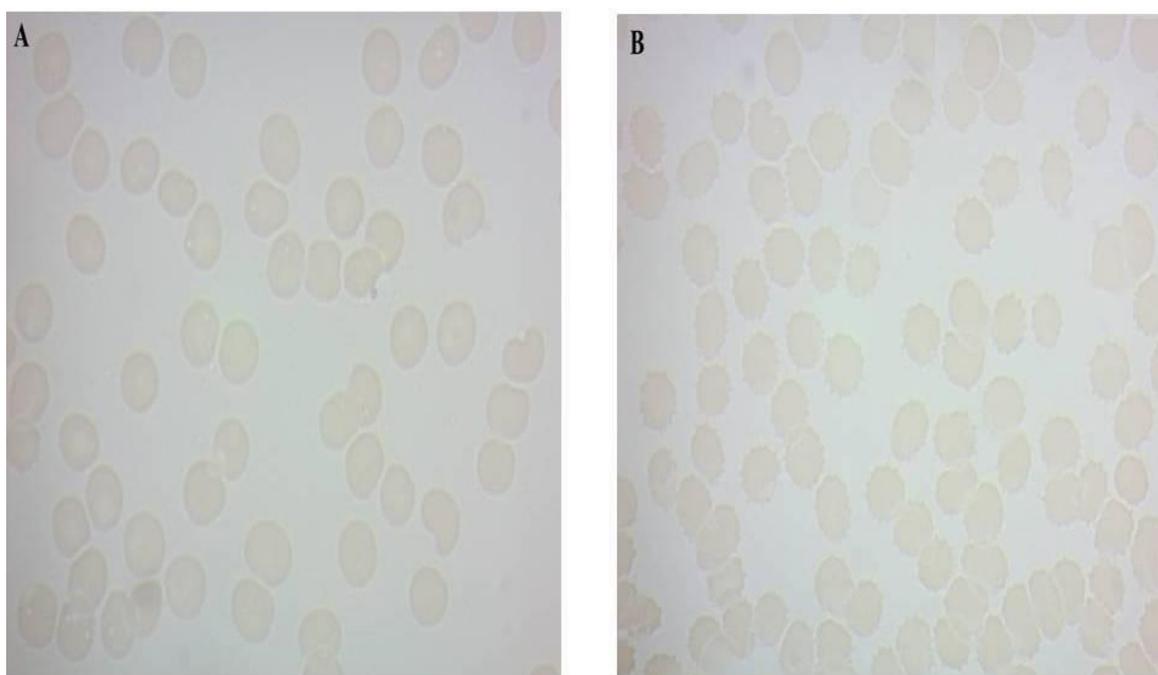


Fig. 1: (A) Control (B) Test microscopic image of erythrocyte membrane damage of *H. gracilis* at 300 µg concentration (at 100X).

Table 4: Cytotoxicity effect of the ethyl acetate extracts of seaweed samples

Name	Initial number of larve	% of mortality		
		Concentrations µg/ml		
		100	200	300
<i>H. gracilis</i>	10	-	2	4
<i>P. gymnospora</i>	10	-	-	-
<i>C. harnemanii</i>	10	-	-	-

DISCUSSION

The search for new pharmacologically active agents from natural sources can lead to the discovery of potent and specific inhibitors for α-amylase [9]. Pharmacological properties of α-glucosidase inhibitors

such as acarbose that can also inhibit pancreatic α-amylase revealed that the complications of DM such as onset of renal, retinal and neurological changes and the development of ischaemic myocardial lesions are prevented or delayed [18]. Long-term day-to-day management of diabetes, with acarbose is well tolerated and can

improve glycaemic control as monotherapy, as well as in combination therapy [19]. Acarbose is derived from *Actinoplanes* Sp. The major adverse effects of acarbose are abdominal distention, flatulence, meteorism and mild diarrhoea [20]. The search for safer, specific, and effective hypoglycemic agents has continued to be an important area of investigation. Natural extracts from readily available traditional medicinal plants offer a great potential for the discovery of new antidiabetic drugs [20]. In this study, antidiabetic activity of seaweed extracts was extensively studied in an attempt to screen for potent inhibitors of α -amylase with antioxidant and free radical scavenging activity.

Seaweeds have been identified as an under-exploited plant resource and a source of functional food [21]. They have also been identified as rich sources of structurally diverse bioactive compounds with great pharmaceutical and biomedical potential. In particular, the brown seaweeds have a variety of biologically active compounds, including pigments, fucoidans, phycocolloids, and phlorotannins [22]. Several studies are being done for the isolation of bioactive compounds from seaweeds. It has been pointed out that those compounds have variety of biological activities such as antioxidant, anticoagulant, antihypertension, antibacterial, and antitumor activities [22, 23].

Seaweeds are known to contain α -glucosidase and α -amylase inhibitors [24]. Red seaweeds of the family *Rhodomelaceae* contain bromophenols with α -glucosidase inhibitory activity; one of the family *Rhodomelaceae* bears a 3,4-dihydroxybenzyl skeleton [25, 26].

In this study, we investigated the α -amylase inhibitory effect of seaweeds collected from the southeast coastal area of India and elucidated the possible use of seaweed compounds as anti-hyperglycemic agent. The three different species of seaweeds (*C. harnemanii*, *G. gracilis* and *P. gymnospora*) were subjected to organic solvent extraction and ethyl acetate extract showed maximum α -amylase inhibitory activity with less toxic effect. The seaweeds selected for the study were evidenced for higher inhibitory activity against α -amylase than that of the commercial carbohydrate digestive enzyme inhibitor, acarbose [27]. Polyphenolic compounds such as flavonoids from terrestrial plants and phlorotannins from marine seaweeds are known to be associated with a variety of proteins to form complexes. Recent studies have demonstrated that the hydroxyl groups in polyphenolic compounds may therefore, have an important role in promoting inhibitory activity [28].

In the present study, *C. Hornemanni* and *G. Gracillis* (red seaweed) indicated the maximum α -amylase activity without toxic effects. Similarly, α -glucosidase and α -amylase inhibitor bromophenol, C_6H_5BrO , is produced by *Polyopes lancifolia* and *Grateloupia elliptica* (red seaweeds). It has been used in the therapy of type II DM to efficiently control the blood sugar with starch-containing diets. Many other species of seaweeds were reported to produce various other components, which inhibit α -glucosidase and α -amylase activity [29].

Polysaccharides and polyphenolic compounds enriched fractions from the brown seaweed *Ascophyllum nodosum* showed the maximum α -glucosidase inhibitory activity. Diphlorethohydroxycarmalol isolated from *Ishige okamurae* (brown seaweeds) was acting as a potent α -glucosidase and α -amylase inhibitor. It alleviates postprandial hyperglycemia in diabetic mice, which reflected to our sample *P. gymnospora* [29]. Sargaquinoic acid and sargahydroquinoic acid from *Sargassum yezoense*, brown seaweed stimulate adipocyte differentiation through PPAR α /gamma activation in 3T3-L1 cells [28]. Phenolic antioxidant-mediated α -glucosidase and α -amylase inhibitors have been isolated from the *Ascophyllum nodosum* [28].

CONCLUSION

This study is a preliminary report on anti-diabetic properties of seaweeds, six brown seaweeds (*Sargassum duplicatum*, *Sargassum wightii*, *Sargassum tenerrimum*, *Turbinoid conoids*, *Turbinoid ornate* and *Padina gymnospora*) three red seaweeds (*Gracillaria gracillis*, *Chondrococcus hornemanni* and *Gracillaria edulis*) and one green seaweeds (*Caulerpa racemosa*) harvested from the southeast coastal area of India. The seaweeds were extracted with ethyl acetate, acetone and methanol. The *in vitro* α -amylase inhibitory activity of ethyl acetate extracts of seaweeds showed very good results. Ethyl acetate extracts

of *G. gracilis*, *C. harnemanii* and *P. Gymnospora* inhibit α -amylase at 73, 81 and 94% respectively and *C. harnemanii* showed the maximum inhibition (94%) in 300 μ g/ml concentrations. The methanol extract of the seaweeds showed moderate results. The ethyl acetate extracts of *C. harnemanii*, *G. gracilis* and *P. gymnospora* were found to exhibit potent α -amylase inhibitory activity. All the three extracts of *G. edulis* exhibited α -amylase inhibitory activity. *C. harnemanii*, *H. gracilis* and *P. gymnospora* were subjected to antioxidant assays. In that, *C. harnemanii* showed promising DPPH and deoxyribose protecting activity. Detailed research is needed to identify the active principle responsible for antidiabetic activity of *C. harnemanii*.

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