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Comparison Of Phyto-Chemical Properties, Anti-Oxidant And Anti-Diabetic Activity Of Hydro-Ethanolic Extracts Obtained From Six Seaweed Species By In-Vitro Enzyme Inhibition Methods

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Abstract

The present study was conducted to compare the phytochemical properties of six different seaweeds like green algae (*Caulorpa racemosa* & *Ulva faciata),* red algae (*Amphiroa fragilissima* & *Porphira indica)* and brown algae (Sargassum *cristaefolium* & *Turbinaria ornata)* by using standard methods. The study revealed that, almost all six sea weeds (TO, AF, CV, SC, UF & PI) had flavonoids, poly phenols, carbohydrates, tannins, sterols, proteins & free amino acids. The alkaloids were present only in TO, AF, CV & PI and glycosides were present only in TO & UF. The anti-oxidant activity of HEEOSA was studied by *in-vitro* DPPH and nitric oxide scavenging assay methods. The anti-diabetic potential of HEEOSA was studied by using *in-vitro* enzyme inhibitory assays. The HEEOSA (TO, AF, CV, SC, UF & PI) showed significant dose dependent inhibitory effects on α-amylase with IC_{50} values of 14.25 µg/ml, 9.14µg/ml, 15.53 µg/ml, 18.4 µg/ml, 11.24 µg/ml & 13.05 µg/ml respectively and the reference standard acarbose was found to be3.12 µg/ml. The HEEOSA (TO, AF, CV, SC, UF and PI) and the standard drug voglibose showed significant dose dependent inhibitory effects against α-glucosidase withIC_{50}values of 82.36 µg/ml, 65.34 µg/ml, 95.42 µg/ml, 100.89 µg/ml, 71.146 µg/ml, 73.94 µg/ml & 25.95 µg/ml respectively. The inhibitory concentration (IC_{50}) of HEEOS against standard ascorbic acid by DPPH scavenging assay was found to be 4.78, 4.17, 4.86, 4.67, 4.79, 4.85 & 3.91µg/ml respectively. Similarly, inhibitory concentration (IC_{50}) of HEEOS and ascorbic acid against nitric oxide scavenging assay was found to be 4.69, 3.35, 4.85, 5.18, 4.47, 4.22 and 1.92 µg/ml for TO, AF, CV, SC, UF & PI respectively. Among the above six species of marine algae, *Amphiroa fragilissima* had maximum potential as anti-diabetic agent with IC_{50} value of 9.14 µg/ml and 65.34 µg/ml against α-amylase and α-glucosidase respectively, and also revealed its anti-oxidant potential with IC_{50} value of 3.36 µg/ml against 1.94 µg/ml for standard ascorbic acid by NOSA method and IC_{50} value of 4.17 µg/ml against the standard ascorbic acid (3.91µg/ml) by DPPH scavenging assay. Therefore, *Amphiroa fragilissima* could be selected for further studies like toxicity as per OECD guidelines and *in-vivo* studies by using animal models.

Keywords

Diabetes mellitus, Anti-oxidant, *Amphiroa fragilissima,* Voglibose and Acarbose.

Abbreviation

CV- *Caulorpa racemosa*, UF- *Ulva faciata*, AF-*Amphiroa fragilissima*, PI- *Porphira Indica*, SC- *Sargassum cristaefolium,* TO- *Turbinaria ornate.* HEEOSA- Hydro-ethanolic extract of six algae, DM- Diabetes mellitus, NOSA- Nitric oxide scavenging assay.

Introduction

The life style modifications like rapid urbanization, lack of physical activity, unhealthy diets etc., are the important causes for chronic metabolic disorder called as type II DM. American Diabetes Association; Diab Care (2013) states that, obesity is also an important cause for type II DM because 80 to 90% of diabetes patients are obese. The major outcome of diabetes mellitus is the persistent hyperglycemia which may be due to insufficient secretion or resistance of insulin. According to World Health Organization (2016) survey, the incidence of DM will be increased up to 592 million by the year 2035 and metabolic change of glucose tolerance will be increased up to 471 million, especially in South Asian countries like Sri Lanka (Jenum et al., 2012). The persistent elevated blood glucose level may lead to diabetes complications like hypertension, nephropathy, retinopathy, neuropathy and macro-vascular diseases which may lead to risk of morbidity and mortality which leads to reduction in life span of diabetic patients (Chawla et al., 2016). Polyphenols, tannins and saponins derived from plants or algae have inhibitory action on synergistic activity of both α- amylase and α-glucosidase and inhibit the digestion of starch (Nair et al., 2013). The inhibition of these enzymes may result in reduction of starch metabolism which further reduces postprandial blood glucose level. Ferna1ndez-Sanchez et.al revealed that, inflammation, anti-oxidative stress and obesity may lead diabetes mellitus (Ferná1ndez-Sánchez et al., 2011). Therefore, anti-diabetic drug should be able to reduce oxidative stress and obesity of the patients. The study reported by Wright. E et.al stated that, obesity and chronic inflammation in adipose tissues leads to molecular and cellular alterations of tissues (Wrigh et al., 2006). The oxidative stress is the major cause for the progress of chronic inflammatory conditions such as DM (Pal et al 2014). Therefore, the research is to focus on natural products to find a novel drug to prevent and treat oxidative stress, diabetes mellitus and their complications with minimum side effects. The seaweeds are the major sources for polyphenols, sterols, alkaloids, flavonoids, tannins, saponins, proteins, essential fatty acids, enzymes, vitamins, and carotenoids because of these components they could able to withstand even harsh environments as per De Souza et al., (2011).

The currently T2DM has been treated with oral hypoglycemic agents like sulphonyl urea, gliptins, biguanides, insulin and its analogues etc., but the evidences revealed that health of patients may be affected because of the side effects and toxicity. Therefore, there is a need to identify a novel and natural remedy as an alternative with less or no side effects.

Materials and methods

Chemical Reagents

All chemicals used with analytical grade are purchased from Merck, Sigma Aldrich, India.

Collection of Plant Material

A fresh algae *Caulorpa racemosa, Ulva faciata, Amphiroa fragilissima, Porphira Indica, Sargassum cristaefolium* & *Turbinaria ornate* were collected from the Rameswaram coastal area in Tamilnadu and they were authenticated by Thiru. A. Govindasamy Government Arts college, Department of Botany at Thindivanam (TAGGACB/19-A/2022). The algae were shade-dried until constant weight was obtained, pulverized and stored in an airtight container for the further studies to be proceeded.

Preparation of plant Extract

Approximately 50 grams of all six powdered algae were extracted using a Soxhlet apparatus with 500 mL of petroleum ether for 8 to 10 hours at 60 to 70^0C. Further, extracted with 500 mL of 90% ethanol for three days at 60 to 70^0C for 8 to 10 hours per day. The ethanol was removed by using a rotary evaporator at low pressure to concentrate the extracts. The concentrated extracts should be at 8 to 10°C until further studies could be performed like *in-vitro, in-vivo* and toxicity.

Preliminary Phyto-chemical screening

A preliminary phytochemical screening was conducted with HEEOSA algae species by the below stated

standard methods.

Test for Alkaloids

The method described by Harborne, (1996) and Danapalanet al., (2013) were used to identify the alkaloid content of hydro-ethanolic extract of all six algae. Then the extracts were dissolved in 5ml of $CHCl_3$ and filtered separately and added two to five drops of 1M H_2SO_4 to all the filtrates and shaken to create two layers. The filtrates of upper acidic layers were dissolved in the Dragendroff reagent, Wagner reagent and Meyer reagent separately and observed for any color changes or the formation of precipitates. Alkaloids are suggested to be present by the appearance of purple colour in Dragendroff reagent, brown colour in Wagner reagent and white milky colour in Meyer reagent respectively.

Test for Flavonoids

The presence of flavonoids in crude hydro-ethanolic extracts of all six algae species were assessed by using the methodology described by Harborne Danapalanet al., (2013). Each extract of algae boiled in 10mL of distilled water along with 5–10 drops of HCl and a small piece of magnesium. The mixtures were kept to boil for five minutes. The appearance of reddish-brown tint suggested the presence of flavonoids.

Test for Carbohydrates

Anthrone test

1mL of HEEOSA and 10mL of distilled water were mixed, shaken well and filtered. Added 1mL of anthrone reagent to all the filtrates and stirred. The production of greenish blue color signified the carbohydrate presence (Harborne J B, 1996).

Benedict's test

1mL of HEEOSA and 10 mL of water were mixed, shaken vigorously and filtered. Added 3mL of Benedict's reagent to all the filtrates and immersed in a boiling water bath for about five minutes. Harborne J B (1996) states that, the production of red coloration suggested the presence of reducing sugar.

Fehling's test

1mL of HEEOSA were mixed with 10mL of water separately, shaken vigorously and filtered. Added 1mL each of Fehling's A and B to all the filtrates and were heated in a boiling water bath for five minutes. The formation of red coloration suggested the presence of reducing sugar (Harborne J B, 1996).

Molisch's test

1mL of HEEOSA were dissolved with 10 mL of water separately shaken vigorously and filtered. After adding a few drops of Molisch reagent to all the filtrates, conc. H_2SO_4 was added through the sides of test tubes. The presence of carbohydrates was indicated by the formation of violet color ring at junctions of two liquids according to Harborne J B (1996).

Test for Glycosides

1mL of HEEOSA were mixed with 10mL of water separately, shaken well and filtered. Added 1mL of Molisch's reagent to all the filtrate and a few ml of conc. H_2SO_4 was added at the sides of test tubes. The presence of glycosides was confirmed by the formation of violet color ring at junction of two solutions as per Harborne J B (1996).

Test for Tri Terpenoid/ Steroid

The qualitative analysis of HEEOSA for tri terpenoid/ steroid content had been performed according to Danapalan et.al (2013). The HEEOSA were taken separately and added 5mL of 50% ethanol. The mixtures were boiled in a boiling water bath for 5 to 10 minutes, cooled and filtered. After being dried in a dish evaporator, the filtrates were dissolved in 1mL of diethyl ether and stirred for five minutes. The ethyl ether portion was decanted and added 10 ml of chloroform and stirred for approximately five minutes and subsequently added 0.5g of

anhydrous sodium sulfate, gently shaken and filtered. The resulting filtrate was separated into two test tubes 1 & 2 and used for the subsequent tests:

Liebermann-Burchard's reaction

The equal volume of above filtrate and acetic anhydride was taken in test tube no: 1 and was shaken and added 1mL of conc. H_2SO_4 at the sides of tube. The presence of sterols and triterpenes were indicated by the formation of a brownish-red ring between the two liquids and a greenish tint was produced in partition layer as per Danapalan et.al (2013).

Salkowski's test

Two to three drops of pure sulfuric acid were added into test tube 2 to create a lower layer. The presence of a steroidal ring was implied by the formation of reddish-brown color during the intermediate stage as per Danapalan et.al (2013).

Test for Saponins

The qualitative analysis of HEEOSA content had been performed separately for saponins. 1mL of distilled water was added into all the extracts separately which were subsequently heated and filtered. Added 0.5mL of distilled water to all the filtrate and vigorously trembled for almost five minutes. The presence of saponins was suggested by the formation of bubbles (froth) that persisted with bonding Harborne J B (1996).

Test for Tannins

The tannin presence of HEEOSA was measured by using Mehdinezhad's approach. The HEEOSA were boiled in a water bath for five minutes with 20 mL of distilled water and filtered. After diluting the cold filtrate (1mL) with water, added two to three drops of 10% ferric chloride, the establishment of impulsive and any color transformation was observed. The sudden formation of a brownish green color stated the presence of tannins as per Danapalan et.al (2013).

Test for Phenolic compounds

Added an equal volume of water to about 5 mL of HEEOSA separately and added gelatin with sodium chloride to produce a white precipitate. Similarly, a white precipitate was produced with lead acetate mixture which confirmed the presence of phenolic compounds according to Danapalan et.al (2013).

Test for free amino acids and proteins

Ninhydrin test

Danapalan et.al (2013) suggested that, the production of violet color when added HEEOSA with 1 mL of Ninhydrin reagent confirmed the occurrence of free amino acids.

Millon's test

Gently shake 1 mL of the Millon's reagent with 1 mL of HEEOSA separately. The presence of free amino acid was detected by the appearance of the cherry red color as per Danapalan et.al (2013).

Biuret test

1 mL of HEEOSA were taken separately in test tubes, mixed with 1 mL of 10%NaOH and 1 mL of 1% copper sulfate. The formation of purple color suggested the presence of proteins (Danapalan et.al (2013).

Test for Gums and Mucilage

Take 1mL of HEEOSA in separate test tubes, added 25 mL of 95% alcohol, shaken, filtered and residue was allowed to air dry. Danapalan et.al (2013) stated that, an assessment of swelling property confirmed the presence of gums and mucilage.

***IN-VITRO* ANTI-OXIIDANT ACTIVITY OF HEEOSA**

DPPH Assay

The anti-oxidant activity of HEEOSA was determined based on the method stated by Mansour Sobehet.al (2018). The hydrogen donating or electron donating ability of HEEOSA were determined by using

2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) in methanol based on the production of purple-color. The anti-oxidant effect of HEEOSA was estimated by using UV spectrophotometer. The 1mg/ml concentration of HEEOSA and 0.1mM DPPH were prepared. The different concentrations of HEEOSA (12.5 µL- 200 µL) were mixed with 4% DPPH (0.1 mm) solution and were placed in the dark for about half an hour of incubation at room temperature. The absorbance was measured at 517 nm and the inhibition percentage was calculated by using the following formula.

$$\% \text{ of Inhibition} = Abs_{(control)} - Abs_{(sample)} \times 100 / Abs_{(control)}$$

Nitric oxide scavenging assay

NOSA method of HEEOSA was determined by using the method reported in V.Kumar Priyanka (2020). The various concentrations like 2, 4, 6, 8 and 10µg/ml of HEEOSA and a standard ascorbic acid were taken in marked test tubes containing phosphate buffer saline (1ml). The volume of control was made up to 3ml with phosphate buffer saline and added 2 ml of 10 mM sodium nitroprusside into all the tubes except the control. Nitric oxide radicals were generated during the incubation period of 2 1/2 hours and added 1 ml of 0.33% sulphanilamide into 0.5ml of the solution taken from both samples and standard tubes, added 1ml of 0.1%N-(1-Naphthyl) ethylene diamine and incubate for 30 min at room temperature. The absorbance was measured at 516 nm and % of scavenging activity was found by using the below mentioned formula.

$$\% \text{ inhibition of nitric oxide radical activity} = Abs_{(control)} - Abs_{(sample)} \times 100 / Abs_{(control)}$$

Anti-diabetic activity

***In-vitro* α-Amylase inhibition assay**

The studies were conducted with hydro-ethanolic extracts of all the six algae species to find out their α-amylase inhibitory activity as per the reported studies by Domettila (2013) & Unnikrishnan (2015). Added 100 µl of α-amylase solution (0.1 mg/ml) with double folded dilutions (10, 20, 40, 80, 160 and 320 µg/ml) of standard drug acarbose and test solutions separately. The control was prepared without the sample and reference, and all the resulting solutions were pre-incubated at 37°C for 15 minutes. Added 100µL of starch solution into the above resulting solutions to start the reaction and incubated at 37°C for 60 minutes. The test tubes were added with 10µl of 1M HCl to arrest the reaction and then 100µL of iodine reagent was added. The UV absorbance was assessed at 565nm for all six samples and standard. The formula for measuring α-amylase inhibitory activity was as follows as per Nickavar B et al., (2011) & Teixera VL (2007).

$$\% \text{ Inhibition} = [(Abs_{test} - Abs_{control}) / Abs_{test}] \times 100$$

The extract concentration that resulted in 50% inhibition of enzyme activity (IC_{50} values) was plotted graphically (fig: 5).

***In-vitro* α-Glucosidase inhibition assay**

The inhibitory effects of HEEOSA on α- glucosidase inhibition was ascertained using the procedure outlined by Kumar P S et al (2013) and Sanger G et.al (2017). 100mM phosphate buffer with pH of 6.8 was used to prepare the substrate solution ie., p-nitrophenyl glucopyranoside (pNPG). Distinct concentrations of HEEOSA (10, 20, 40, 80, 160, and 320 µg/ml) and the standard drug voglibose were pre-incubated with 200µL of α-glucosidase for about 10 – 15 minutes. The reaction was initiated with 400µl of a substrate (5mM pNPG) solublised in 100 mM phosphate buffer (pH 6.8). After 20 minutes of incubation at 37°C, 1ml of 0.1M Na_2CO_3 was added to the reaction mixture to end the process and the blank buffer was prepared without the test and standards. The yellow-colored 4-nitrophenol was produced from pNPG and detected at 405 nm by using UV-VIS spectrophotometer. The

percentage of α-glucosidase inhibition had been estimated by the above-mentioned equation:

% Inhibition activity = Abs $_{(control)}$ – Abs $_{(sample)}$ / Abs $_{(control)}$ x 100

The extract concentration that resulted in 50% inhibition of enzyme activity (IC_{50} values) was plotted graphically (fig: 6).

Statistical Analysis

IBM SPSS Statistics version 20 was used to collect data from any type of file. The collected data were used mainly to create tabulated reports, charts, plots of distributions, descriptive statistics and statistical analyses of complex data. All the experiments were preceded in triplicates and the significant difference was considered with $p \leq 0.05$ as per statistical analysis by using IBM SPSS (version 20) software. Results were represented as mean ± SD.

Results

The HEEOSA were analyzed for preliminary phyto-chemicals based on standard protocols. The phytochemical analysis outcome is shown in Tab: 1. Alkaloids, flavonoids, carbohydrates, sterols, saponins, tannins, polyphenols, proteins, free amino acids, glycosides, gums and mucilage were found in hydro - ethanolic extracts of algae.

Table 1: Preliminary Phytochemical screening of HEEOSA species

Constituents	Tests	T. ornate	Amphiroafra gilissima	Caulorpa racemosa	Sargassum cristaefolium	Ulva Faciata	Porphira Indica
Alkaloids	Mayer's reagent	+	+	+	_	_	+
	Dragandroff's reagent	+	+	+	_	_	+
	Hager's reagent	+	+	+	_	_	+
Flavonoids	Shinoda's	+	+	+	+	+	+
Carbohydrates	Molisch's	+	+	+	+	+	+
	Iodine	+	+	+	+	+	+
	Benedict's	+	+	+	+	+	+
Glycosides	Borntrager's	+	_	_	_	+	_
	Modified Borndrager's	+	_	_	_	+	_
	Keller Killiani	+	_	_	_	+	_
	Raymond	+				+	_
Sterols	Salkowski	+	+	+	+	+	+

	LibermanBurchard's	+	+	+	+	+	+
Saponins	Froth	+	+	+	_	+	_
Tannins	Ferric chloride	+	+	+	+	+	+
	Gold Beater's skin test	+	+	+	+	+	+
Polyphenols	-	+	+	+	+	+	+
Proteins and freeamino acids	Millon's	+	+	+	+	+	+
	Biuret	+	+	+	+	+	+
	Ninhydrin	+	+	+	+	+	+
Gums and Mucilage	-	+	+	+	+	_	_

Note: (+) present and (-) absent

Anti-oxidant activity

Anti-oxidant activity of HEEOSA by *In- vitro* assay method

The determination of anti-oxidant activity of HEEOSA was carried out by DPPH scavenging assay and Nitric oxide scavenging assay.

Table: 2: Determination of DPPH scavenging activity of HEEOSA

S. NO	**Conc. of ascorbic acid& HEEOSA**	**% Inhibition of ascorbic acid(µg/ml)**	**% Inhibition of TO (µg/ml)**	**% Inhibition of AF (µg/ml)**	**% Inhibition ofCV (µg/ml)**	**% Inhibition ofSC (µg/ml)**	**% Inhibition of UF (µg/ml)**	**% Inhibition of PI (µg/ml)**
1	2	34.49±0.12	29.14±0.06	31.59±0.09	28.97±0.05	29.76±0.08	27.83±0.18	28.56±0.28
2	4	51.19±0.27	47.51±0.41	49.98±0.17	46.88±0.34	48.61±0.15	45.32±0.27	45.87±0.08
3	6	67.5±0.3	58.45±0.25	65.96±0.81	57.54±0.45	57.78±0.17	60.21±0.36	58.65±0.45
4	8	80.50±0.07	70.44±0.37	78.43±0.09	69.87±0.63	71.98±0.54	73.52±0.43	70.68±0.05
5	10	97.16±0.61	86.72±0.71	94.76±0.51	85.87±0.05	88.8±0.64	88.56±0.31	87.10±0.17
6	IC_{50}	3.91	4.78	4.17	4.86	4.67	4.79	4.85

Note: Values are means of triplicates

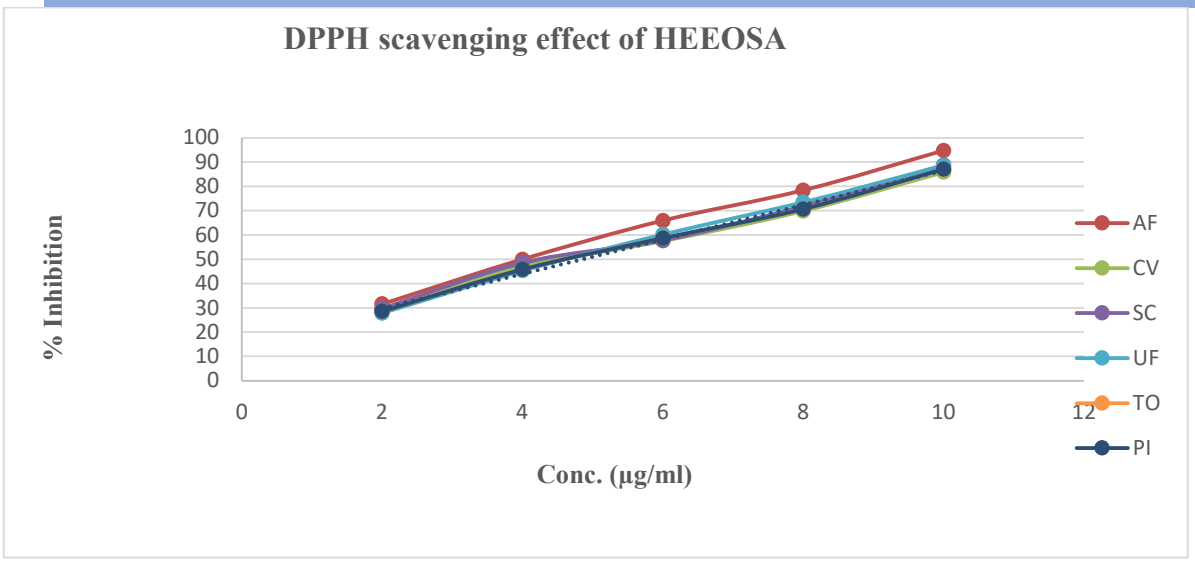

Fig 1: DPPH scavenging effect of HEEOSA

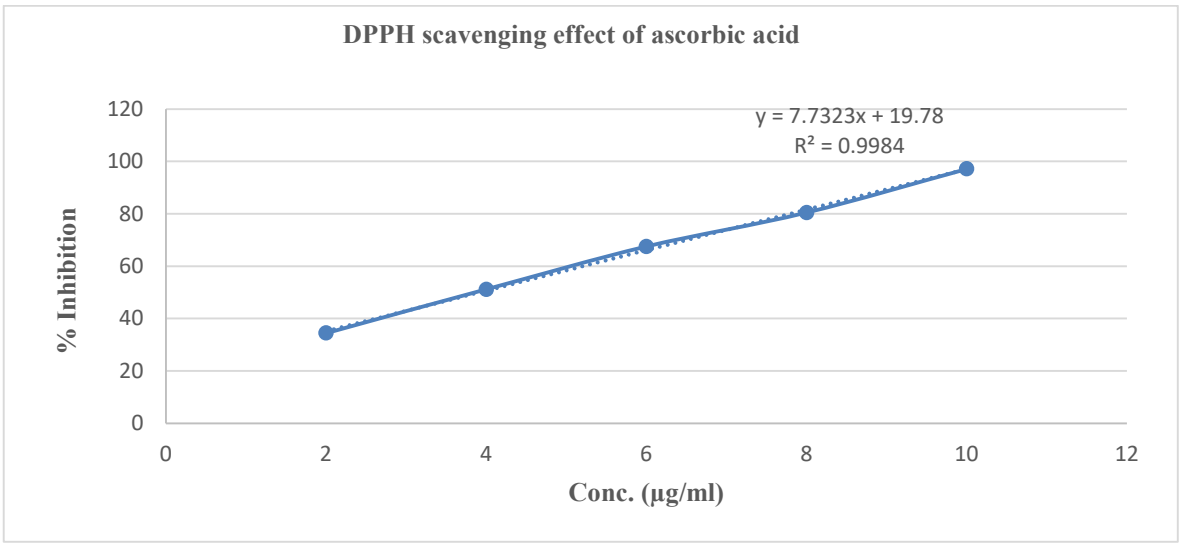

Fig 2: DPPH scavenging effect of ascorbic acid

The inhibitory concentration (IC_{50}) of HEEOSA against DPPH scavenging effects were found to be 4.78, 4.17, 4.86, 4.67, 4.79, 4.85 & 3.91µg/ml for TO, AF, CV, SC, UF, PI and standard ascorbic acid respectively.

Table 3: Determination of nitric oxide scavenging activity of HEEOSA

S. NO	Conc.of Ascorbic Acid & HEEOSA	% Inhibition of ascorbic acid (micro gm/ml)	% Inhibition of TO (micro gm/ml)	% Inhibition of AF (micro gm/ml)	% Inhibition of CV (micro gm/ml)	% Inhibition of SC (micro gm/ml)	% Inhibition of UF (micro gm/ml)	% Inhibition of PI (micro gm/ml)
1	2	50.65±0.09	38.52±0.04	40.69±0.80	37.63±0.32	35.26±0.45	38.95±0.81	39.51±0.43
2	4	60.26±0.51	50.65±0.64	53.21±0.08	48.36±0.47	45.38±0.18	50.24±0.07	51.25±0.25
3	6	78.32±0.05	55.36±0.34	68.65±0.74	56.23±0.54	55.64±0.45	56.58±0.12	57.69±0.34
4	8	88.05±0.81	60.58±0.09	77.65±0.09	61.25±0.06	62.58±0.08	62.54±0.51	64.21±0.05
5	10	98.35±0.58	66.54±0.72	85.52±0.68	67.35±0.42	68.24±0.54	70.25±0.34	72.65±0.09
6	IC_{50}	1.92	4.69	3.35	4.85	5.18	4.47	4.22

Note: Values are mean of 3 readings

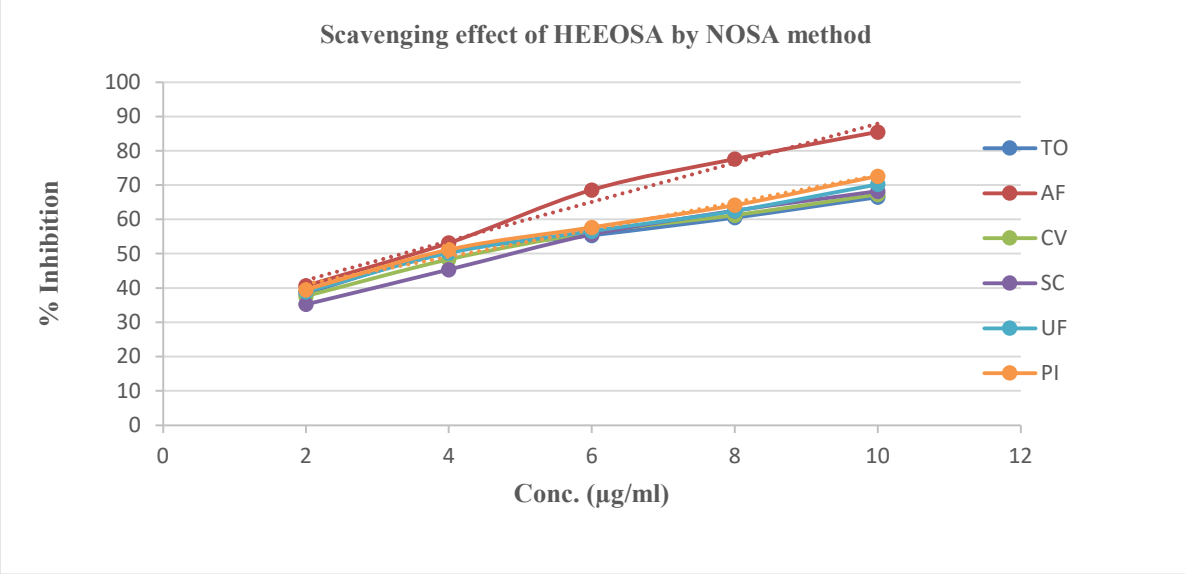

Fig 3: Nitric oxide scavenging effect of HEEOSA

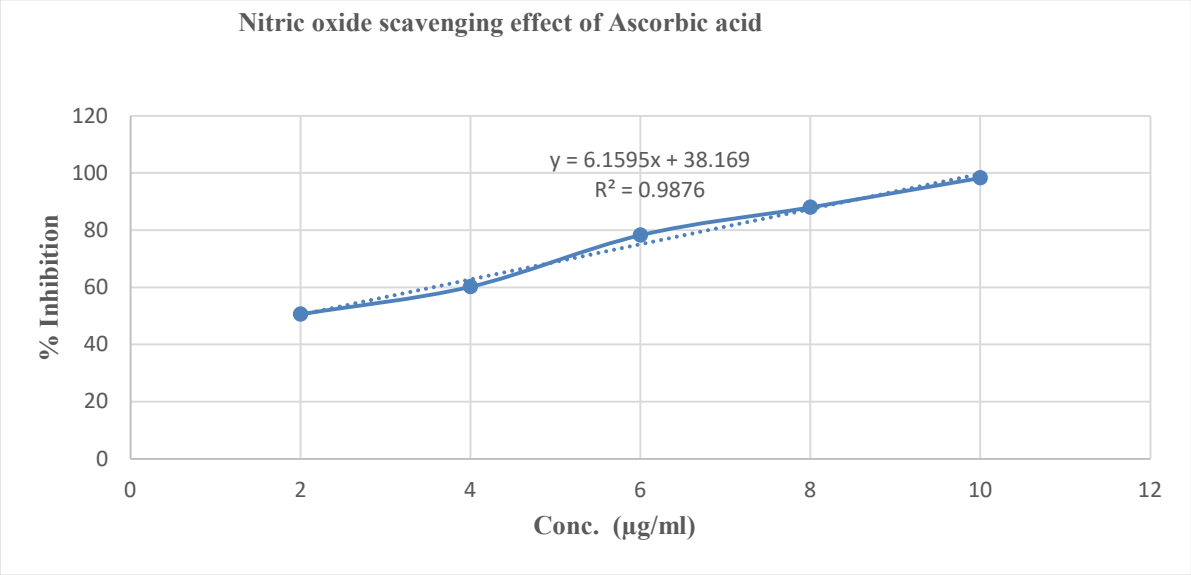

Fig 4: Nitric oxide scavenging effect of ascorbic acid

The inhibitory concentration (IC_{50}) of HEEOSA against NOSA was found to be 4.69, 3.35, 4.85, 5.18, 4.47, 4.22 and 1.92 µg/ml for TO, AF, CV, SC, UF, PI and standard ascorbic acid respectively.

Anti-diabetic activity

***In-vitro* anti-diabetic activity of HEEOSA**

The *in-vitro* inhibitory assays were performed on α-amylase and α-glucosidase with HEEOSA to study their anti-diabetic potential. All six algae extracts showed significant dose dependent inhibitory effect against α-amylase with IC_{50} value of the given samples (TO, AF, CV, SC, UF& PI) and acarbose were found to be 14.25 µg/mL, 9.14 µg/mL, 15.53 µg/ml, 18.4 µg/ml, 11.24 µg/ml, 13.05 µg/ml and 3.12 µg/mL respectively. Similarly, HEEOSA showed dose dependent significant inhibitory effects against α-glucosidase with IC_{50} value of samples (TO, AF, CV, SC, UF & PI) and reference standard voglibose were found to be 82.36 µg/mL, 65.34 µg/mL, 95.42 µg/mL, 100.89 µg/mL, 71.146 µg/mL, 73.94 µg/mL and 25.95 µg/mL respectively.

Table 4: The IC_{50} value determination for α- amylase inhibition assay

S.No	Conc (µg)	TO	AF	CV	SC	UF	PI	Acarbose
1	10	15.8±0.05	36.73±0.04	18.92±0.1	9.70±0.02	29.82±0.4	28.63±0.8	52.58±0.80
2	20	74.14±0.03	68.72±0.05	70.87±0.3	68.44±0.08	68.94±0.05	66.65±0.4	77.95±0.09
3	40	86.78±0.05	82.89±0.13	79.81±0.02	84.59±0.07	83.53±0.7	79.85±0.6	83.45±0.05
4	80	89.57±0.12	88.48±0.07	84.52±0.2	88.21±0.09	87.26±0.8	88.26±0.21	91.02±0.07

5	160	92.11±0.08	90.49±0.02	89.11±0.5	89.76±0.21	90.68±0.08	92.10±0.09	94.93±0.09
6	320	93.66±0.15	94.30±0.08	91.20±0.08	91.33±0.13	92.51±0.5	94.29±0.51	95.39±0.55

Note: Values are mean of triplicates

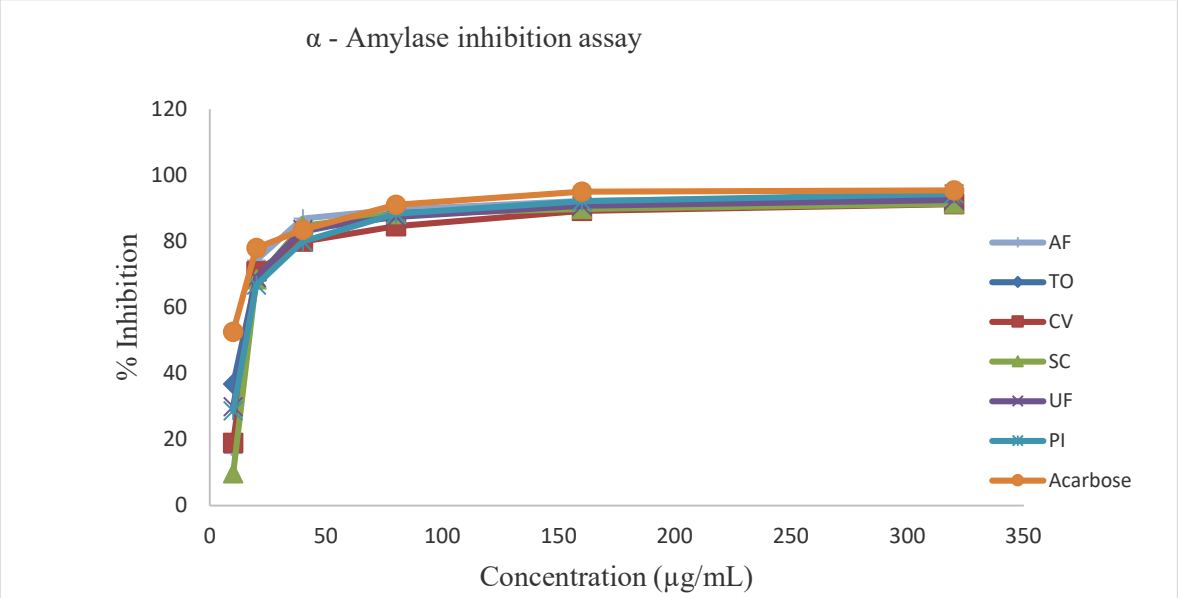

Figure 5: The IC_{50} value determination for α-amylase inhibition assay

Table 5: The IC_{50}value determination for α- glucosidase inhibition assay

S.No	**Conc** (µg)	**TO**	**AF**	**CV**	**SC**	**UF**	**PI**	**Voglibose**
1	10	2.33±0.09	7.22±0.2	5.52±0.05	4.04±0.05	4.04±0.08	9.10±0.4	23.96±0.54
2	20	15.53±0.5	24.41±0.56	12.29±0.08	15.70±0.52	19.12±0.58	19.12±0.58	46.44±0.65
3	40	32.66±0.23	37.84±0.05	11.95±0.36	30.73±0.48	39.27±0.24	35.85±0.9	61.80±0.3
4	80	49.40±0.56	57.7±0.58	47.97±0.46	49.68±0.23	59.93±0.26	56.51±0.7	80.59±0.78
5	160	66.98±0.09	71.31±0.23	58.45±0.58	58.45±0.0	66.42±0.08	68.69±0.08	88.84±0.9

6	320	80.70±0.34	81.95±0.09	70.11±0.09	71.82±0.45	80.36±0.5	76.94±0.9	92.37±0.45

Note: Values are mean of triplicates

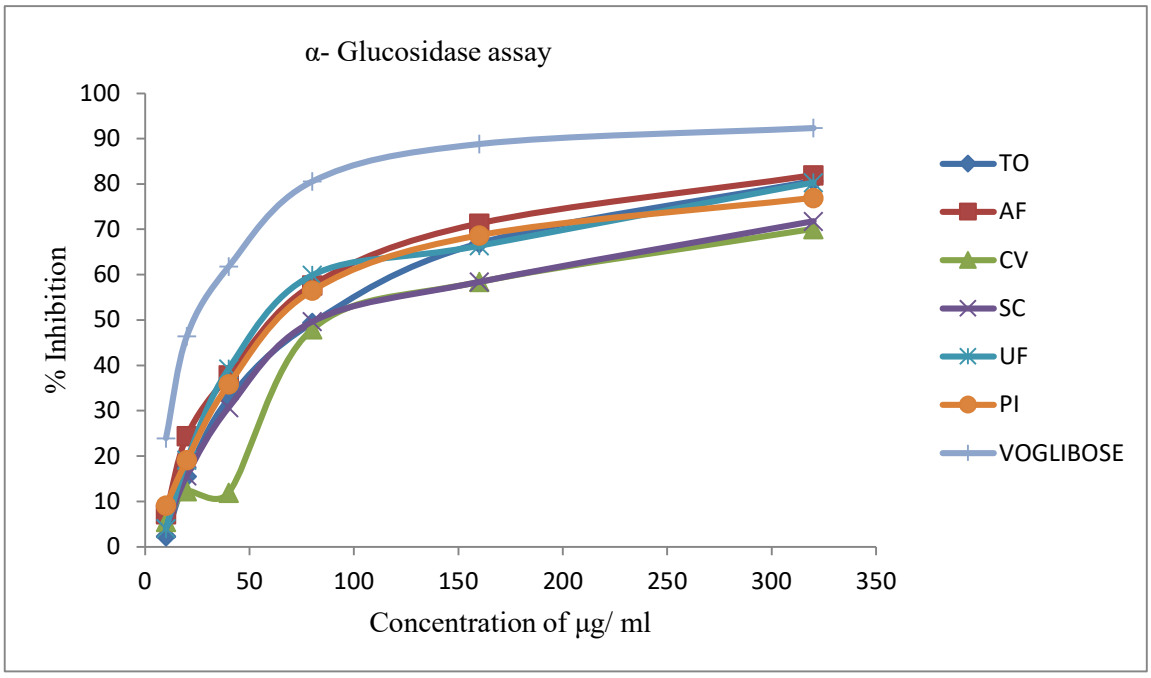

Fig 6: The IC_{50}value determination for α- glucosidase inhibition assay

Discussion

The important public issues towards human health all over the world are oxidative stress, diabetes, cancer, anti-biotic resistance etc., especially in developing and developed countries. The main cause of above-mentioned diseases are abnormal nutritional supplements, life style modification, lack of physical activity and also because of increased free radicals production or insufficiency of antioxidant defense mechanism as stated by Wild S (2004). Seaweeds are proven sources of naturally occurring bioactive molecules with various pharmacological activities as per Unnikrishnan P S (2015) & Mehdinezhad N (2016). Type II diabetes mellitus is a carbohydrate metabolic disorder caused by insufficient insulin secretion, insulin resistance or both and oxidative stress according to Samuel Tadesse (2016) & Zhang D (2008). The anti-diabetic drugs are not only to reduce the serum glucose by reducing intake through GI by inhibiting α-glucosidase or α-amylase or DPP- 4 and they should also able to suppress the oxidative stress by scavenging free radicals as per Ranilla L G (2010). The anti-oxidant activity of HEEOSA was studied by *in-vitro* DPPH scavenging and nitric oxide scavenging assay methods. The study suggested that, the inhibitory concentration (IC_{50}) of H EEOSA against DPPH scavenging effects were found to be 4.78, 4.17, 4.86, 4.67, 4.79, 4.85 & 3.91µg/ml for TO, AF, CV, SC, UF, PI and standard ascorbic acid respectively. The IC_{50} values of HEEOSA against nitric oxide scavenging assay were found to be 4.69, 3.35, 4.85, 5.18, 4.47, 4.22 and 1.92 µg/ml for TO, AF, CV, SC, UF, PI and standard ascorbic acid respectively. *Amphiroa fragilissima* had maximum anti-oxidant activity with IC_{50}value of 4.17µg/ml against 3.91µg/ml of standard ascorbic acid as per DPPH scavenging assay method and 3.35µg/ml (AF) & 1.92 µg/ml (std) as per nitric acid assay method and AF was found to be potential anti-oxidant.

Many bioactive compounds like phenols & derivatives, alkaloids, saponins, aldehydes, alcohols obtained from marine algae were proven that could be used to treat DM, oxidative stress, cancer, inflammation etc., according to

Sharifuddin Y (2015), Boopathy NS (2010), Kim KY (2009) & Sakthieaswari P (2016). The current study stated that, HEEOSA were investigated for phyto-chemical screening and suggested that, almost all six sea weeds were found to contain flavonoids, polyphenols, carbohydrates, tannins, sterols, proteins & free amino acids. The alkaloids were present only in TO, AF, CV & PI and glycosides were present only in TO & UF. The current study revealed that, TO, AF, CV, SC, UF & PI had significant dose dependent inhibitory effects on α-amylase with IC_{50} values of 14.25 µg/ml, 9.14µg/ml, 15.53 µg/ml, 18.4 µg/ml, 11.24 µg/ml & 13.05 µg/ml respectively and the reference standard acarbose was found to be 3.12 µg/ml. The same extracts of TO, AF, CV, SC, UF, PI and the standard drug voglibose showed significant dose dependent inhibitory effects against α-glucosidase with IC_{50}values of 82.36 µg/ml, 65.34 µg/ml, 95.42 µg/ml, 100.89 µg/ml, 71.146 µg/ml, 73.94 µg/ml& 25.95 µg/ml respectively. Among the six species of marine algae *Amphiroa fragilissima* had maximum potential as an anti-diabetic agent with IC_{50} value of 9.14µg/ml and 65.34 µg/ml against α-amylase and α-glucosidase respectively. Freitas A.M (2002) & Seung-Hong Lee [a] (20216) stated that, the demand for alternative therapy has been raised because of non-toxicity and less side effects of the products derived from plants especially for treating diabetes mellitus.

CONCLUSION

The current study had concluded that, all the six marine species were found to contain important bio-active substances like, alkaloids, flavonoids, carbohydrates, sterols, saponins, tannins and poly phenols. The study also summarized that; HEEOSA could be used to manage diabetes mellitus because of their inhibitory action on both α-amylase and α-glucosidase which may lead to reduce post-prandial hyperglycemia. Therefore, HEEOSA could be adopted to reduce the stress because of their anti-oxidant activity. Hence the seaweeds which were investigated may be the source for the development of an anti-oxidant and anti-diabetic drugs especially, *Amphiroa fragilissima* because of its high potential when compared to other species.

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