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HR-LCMS Phytochemical Profiling and Evaluation of Anti-Diabetic Activity of Amphiroa Fragilissima by Key Enzymes Inhibition Assay

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KEYWORDS

Amphiroa Ffragilissima, α-glucosidase, α-amylase, Anti diabetic drugs, Seaweeds

ABBREVIATION HEEAF- Hydro ethanolic extract of Amphiroa Fragilissima, HR-LCMS- High resolution liquid chromatography-mass spectroscopy, AF- Amphiroa Fragilissima

ABSTRACT:

Introduction: Considering the seaweed extracts and their bioactive constituents which are able to inhibit enzymes involved in carbohydrate metabolism and reduce blood glucose during fasting, random and postprandial and same had been witnessed with *in-vivo* and *in-vitro* studies. In addition to their many industrial applications, seaweeds are well known for their ability to improve community nutritional status since they are richer sources antioxidants, macronutrients, and vitamins (B12, A, and K) than other plants. Seaweeds differ in their protein content depending on the species. It is found that the protein content of red and green seaweeds is higher than that of brown seaweeds. The red seaweeds like *Palmaria palmata* (dulse) and *Porphyra tenera* (nori) were observed that 36% and 49% of the dry matter consisted of macromolecule like protein respectively. Consequently, there is widespread use and significant exploitation of these two species. Recent investigations highlight novel prospects in the realm of medicine related to bioactive compounds derived from seaweeds. The potential bioactive substances of sea weeds such as alkaloids, flavonoids, polysaccharides, fatty acids, and polyphenols have antibacterial, antioxidant, anticancer, and immune-boosting qualities as functional foods.

Objectives: The current management of diabetes mellitus could be achieved with the help of synthetic hypoglycemic medications; however long-term use of these medications may lead to several side effects. So, there is a need for the natural compounds with anti-diabetic potential without any side effects. The current work intended to screen phytochemicals using High Resolution-Liquid Chromatography Mass Spectrometry and examine the *in-vitro* anti-diabetic properties for hydro-ethanolic extracts obtained from Amphiroa *Fragilissima* (HEEAF) by enzyme inhibition method.

Methods: The phytochemical analysis revealed the presence of alkaloids, flavonoids, proteins, free amino acids, gums, mucilage, carbohydrates, sterols, saponins, tannins, and polyphenols in HEEAF. The HR-LCMS study also proved the presence of various biologically active secondary metabolites like, Erucamide, 4-Hydroxybenzocyclobutene-1,2-Dione, Pentadecyl N-pentanoylalaninate, 7-Dehydrodesmosterol, Threo-Sphingosine, (-)-,Diethylene glycol n-butyl ether, Oleamide, sphinganine, Betaine, 2-Aminopalmitic acid, Stearoyl ethanolamide, Undecanamide, Coumarandione, Pentadecyl N-pentanoylalaninate, Octyl 5-O-pentanoyl-1-thiopentofuranoside, DL-Carnitine, Pentadecyl N-pentanoylalaninate, Embelin, Azelaic acid, 4-Caproylresorcinol, Pregna-4,6-diene-3,20-dione, (+)-[6]-Gingerol, Methyl (3E,6E)-3,6-dodecadienoate, BHQ, Brassylic acid and 4-Undecyl benzenesulfonic acid etc., in HEEAF. The anti-diabetic potential of HEEAF was investigated through the inhibitory action on α-amylase and α-glucosidase.

Results: The HEEAF showed significant increased inhibitory effects on α-amylase with IC_{50} value of 9.14 µg/ml against 3.12µg/ml for acarbose as the reference standard. Similarly, the significant inhibitory effect was showed on α- glucosidase with IC_{50} values of 65.34 µg/ml against 25.95µg/ml for voglibose as the standard drug. The *in-vitro* study revealed the possible mechanism of anti-

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diabetic activity of *Amphiroa Fragilissima* by enzyme inhibition method and can be carried forward for toxicity study as per OECD guidelines followed by *in-vivo* studies to prove its safety and efficacy.

Conclusions: It has been concluded that, the important secondary metabolites had been identified in HEEAF by performing the preliminary phytochemical and HR-LCMS analyses. The investigation of inhibitory activities of *A. fragilissima* on α-amylase and α-glucosidase sheds information on the potential application of this seaweed in the management of diabetes. Subsequent isolation of the crude extracts will be needed to determine the primary compound accountable for its anti- diabetic effect. Further *in- vivo* research is required to confirm the pharmacological features of *Amphiroa fragilissima* as promising anti-diabetic drug. Therefore, HEEAF can be applied in the treatment and management of diabetes mellitus after performing toxicity studies as per OECD guidelines to establish their safety.

1. Introduction

Seaweeds are widely used in the biomedical, pharmaceutical, food and cosmetics industries. They are widely distributed from the tropics to the arctic regions as the primary producers in the environment. *Chlorophyta* (green algae), *Phaeophyta* (brown algae), *Rhodophyta* (red algae) and *Cyanophyta* (blue-green algae) are the four primary types of seaweeds. Seaweeds offer a valuable ecological and economic resource [1]. The Indian coast is inhabited by about 700 different types of marine algae in both deepwater and intertidal zones [2].

The substances that are derived from brown seaweeds and have been used for commercial purposes are mannitol, fucoidan, laminarin, iodine, and alginic acid. Agar-agar, agarose, and carrageenan and are widely employed in a variety of industries, including the pharmaceutical industries [2].

In addition to their many industrial applications, seaweeds are well known for their ability to improve community nutritional status since they are richer sources antioxidants, macronutrients, and vitamins (B12, A, and K) than other plants. Seaweeds differ in their protein content depending on the species. It is found that the protein content of red and green seaweeds is higher than that of brown seaweeds. The red seaweeds like *Palmaria palmata* (dulse) and *Porphyra tenera* (nori) were observed that 36% and 49% of the dry matter consisted of macromolecule like protein respectively. Consequently, there is widespread use and significant exploitation of these two species. Recent investigations highlight novel prospects in the realm of medicine related to bioactive compounds derived from seaweeds [2]. The potential bioactive substances of sea weeds such as alkaloids, flavonoids, polysaccharides, fatty acids, and polyphenols have antibacterial, antioxidant, anticancer, and immune-boosting qualities as functional foods [3,4]. Red seaweed belonging to the family Corallinaceae, *Amphiroa Fragilissima* (L.) Lamour is found in tropical and subtropical areas and lives in coral reefs. Because A. Fragilissima extracts contain bioactive ingredients such as alkaloids, flavonoids, saponins, and terpenoids and have antibacterial, antiviral, antioxidant, and anticancer properties [5,6]. The chromatographic techniques for characterizing plant metabolites have evolved in recent years, greatly aiding in the cataloging of many metabolites from pharmacologically significant plants [7].

2. Objectives

High morbidity and mortality rate of Diabetes mellitus, makes it as a global public health concern. Synthetic hypoglycemic medications can be used to tackle diabetes but long-term usage of synthetic medications may lead to several negative effects. As a result, there is a paradigm change in favor of using natural agents that may be anti-diabetic without any side effects. Because seaweed extracts and their bioactive constituents block enzymes that hydrolyze carbohydrates has been proved through *in-vitro* study and reduce random and postprandial blood glucose level therefore, they may have anti-diabetic effects [8]. By considering the above back ground, the current investigation was carried out with a preliminary qualitative photochemical analysis and the chemical constituents of HEEAF were cataloged using HR-LCMS analysis. Their pharmacological significance was then

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confirmed through *in-vitro* assays taking into account the extract has a potential to treat diabetes mellitus.

The current management of diabetes mellitus could be achieved with the help of synthetic hypoglycemic medications; however long-term use of these medications may lead to several side effects. So, there is a need for the natural compounds with anti-diabetic potential without any side effects. The current work intended to screen phytochemicals using High Resolution-Liquid Chromatography Mass Spectrometry and examine the *in-vitro* anti-diabetic properties for hydro-ethanolic extracts obtained from Amphiroa *Fragilissima* (HEEAF) by enzyme inhibition method.

3. Methods

3.1 Chemicals

All chemicals used were the analytical grade purchased from Merck, Sigma Aldrich, India.

3.2 Collection of algae

Fresh and healthy *A. Fragilissima* algae had been collected from the Rameswaram coastal area in Tamil Nadu, and their authenticity was confirmed by the Department of Botany at Thiru. A. Govindasamy Gvt. Arts college in Thindivanam (TAGGACB/19-A/2022). The algae were shade- dried until constant weight, pulverized, and stored in an airtight container at ambient for further study.

3.3 Preparation of plant extract

Approximately 50 grams of powdered algae were extracted using a Soxhlet apparatus with 500mL of petroleum ether for 8 to 10 hours at 60 to 70°C. Then extracted with a 500mL of 90% ethanol for three days at 60 to 70°C for 8 to 10 hours per day. The ethanol was eliminated by concentrating the extract using a rotary evaporator at optimum pressure. The concentrated extracts were kept in a refrigerator (8 to 10°C) until further *in-vitro* and *in-vivo* analysis could be performed.

3.4 Preliminary phytochemical screening

A preliminary phytochemical screening was conducted on the HEEAF according to the standard methods.

3.4.1 Test for alkaloids

The HEEAF was dissolved in 5ml of $CHCl_3$ and filtered. Add two to five drops of 1M H_2SO_4 to the above solution and it was shaken well to produce two layers. The upper layer of acidic solution was tested with Dragendroff, Wagner and Meyer reagents and observed for appearance of colors or the precipitates. The purple color with Dragendroff reagent, brown color with Wagner reagent and white milky impetuous with Meyer reagents [9] were produced respectively which confirmed the presence of alkaloids in HEEAF.

3.4.2 Identification of flavonoids

The flavonoid content of HEEAF was assessed using the methodology of the previous study. The HEEAF was heated with 10 drops of HCl, 10 mL of distilled water and added a piece of magnesium. The appearance of a reddish-brown tint indicated the presence of flavonoids [10].

3.4.3 The methods for carbohydrate analysis

3.4.3.1 Anthrone analysis

A little HEEAF was dissolved in distilled water, shaken well and filtered. Added 1 mL of anthrone reagent to this filtrate and stirred. The formation of green or blue color signified the presence of carbohydrates.

3.4.3.2 Benedict's test

The HEEAF was shaken vigorously with 10 mL of water and filtered. Added 3mL of Benedict's reagent to this filter and then immersed it in a bath for about 10 mts. The appearance of red coloration suggested the presence of reducing sugar.

3.4.3.3 Fehling's test

The HEEAF was shaken vigorously with distilled water and filtered added 1mL of Fehling's A and B and heated on a water bath. The appearance of brick red color suggested existence of reducing sugar.

3.4.3.4 Molisch's test

The HEEAF in 10 mL of water was shaken vigorously and filtered. Added a few ml of Molisch's reagent to the filtrate and added conc. H_2SO_4 along the sides of the tube without shaking the test tube. The presence of carbohydrate was indicated by the formation of purple color ring between two junctions of the liquids [11].

3.4.4 Test for glycosides

The HEEAF was mixed with 10 mL of water, shaken well and filtered. After adding 1 mL of Molisch's reagent to the filtrate, a few drops of concentrated H_2SO_4 were applied along the sides of the test tube. The presences of glycosides were indicated by the formation of purple ring between two junctions [11].

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3.4.5 Test for tri terpenoids/steroids

The qualitative analysis of the crude HEEAF for tri terpenoid/ steroid content had been performed by adding few mL of 50% ethanol, boiled for 3 to 5 minutes, cooled and filtrate was dissolved in 1mL of diethyl ether. After that, the ether portion was separated &added 10 ml of $CHCl_3$ was added and mixed it, subsequently 0.5 g of anhydrous sodium sulfate was added. The resulting filtrate was separated into two separate test tubes and utilized in the subsequent methods.

3.4.5.1 Liebermann-Burchard's reaction

Taken equal volume of acetic anhydride in test tube I and it was gently shaken. A few drops of conc.$H_2SO_4$was added to the tube's side. The exitance of sterols and tri terpenes were indicated by the production of brownish-red ring between the polar and non-polar layers and a green color tint at the partition layer.

3.4.5.2 Salwoski's test

Two to three drops of conc. H_2SO_4 was added to the mixer II which was added to create a lower layer. The presence of a steroidal ring was implied by the production of reddish-brown color during the intermediate stage [10].

3.4.6 Test for saponins

The qualitative analysis of the crude HEEAF for saponin content had been performed by dissolving HEEAF in distilled water which was heated and filtered. Added few mL of distilled water to the filtrate and was continuously trembled for almost five minutes. The presence of saponins was suggested by the formation of bubbles (froth) that persisted with bonding [12].

3.4.7 Test for tannins

The tannin content of the crude HEEAF was measured by Mehdinezhad's approach. The HEEAF was added with 15 to 20 mL of water, boiled on a water bath for 5 minutes and filtered. A few mL of filtrate was diluted by adding 5 to 10 mL of distilled water and mixed with two to three drops of 10% $FeCl_3$. The sudden formation of a brownish-green or bluish-black color indicated the exitance of tannins [12].

3.4.8 Test for phenolic compounds

Added an equal volume of HEEAF with 5 mL of distilled water and tested with gelatin and lead acetate solution as well. The formation of creamy white precipitate with both lead acetate and gelatin solution confirmed the presence of phenolic compounds [11].

3.4.9 Test for proteins and free amino acids

3.4.9.1 Millon's method

I mL of Millon's reagent was mixed with 1 mL of HEEAF to produce a cherry red color which confirmed the existence of free amino acids [11].

3.4.9.2 Ninhydrin test

Added 2 mL of HEEAF and equal volume of Ninhydrin reagent to produce a violet color which suggested the existence of free amino acids [11].

3.4.9.3 Biuret test

The HEEAF was added with 1 mL of 10% NaOH and 1 mL of 1% copper sulfate. The appearance of purple color suggested the presence of proteins [11].

3.4.10 Test for gums and mucilage

1mL of HEEAF was combined with 25 mL of 95% alcohol, shaken gently, filtered and the residue was allowed to air dry, its swelling ability was assessed. It indicated that mucilage and gums were present [11].

3.5 HR-LCMS Analytical method of HEEAF

The HR-LCMS analytical method was performed with the HEEAF. The HR-LCMS analysis was conducted at the IIT Bombay, Pawai, Mumbai, Sophisticated Analytical Instrument Facility (SAIF). Using Agilent HR-LCMS model G6550A with 0.01% mass resolution, chemical fingerprints of a chosen algal extract were created.

The C18 column (100 x 1.0 mm, particle size 1.8 µm; waters) was utilized for the chromatographic separations; and flow rate: 100 µl/minute, flush out factor: 5µl and injection volume: 8µl were employed for HR-LCMS analytical method. Methanol: acetonitrile (95:5) served as mobile phase A, whereas 10mM ammonium acetate in water served as mobile phase B. Thirty-five minutes was the run time. The HR-LCMS was kept running at 250^0C and 13 psi per minute. The MS acquisition method, the scanning rate of each spectrum per second was around the range of 50 (M/Z) to 1000 dalton (M/Z).

3.6 Anti-diabetic activity

3.6.1 *In-vitro α*-amylase assay

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The conventional approach was followed with slight modifications to assess HEEAF's α-amylase inhibitory activity. The acarbose was used as the reference standard and 100µl of α-amylase solution (0.1 mg/ml) was combined along with different concentrations of test samples ie., (10, 20, 40, 80, 160, and 320 µg/ml). The control was prepared without a test sample and reference standard and all were incubated at 37°C for 15 minutes. After adding 100 µL of starch solution to start the reaction and incubating at 37°C for 60 to 70 minutes. The test tubes were added with 10µl of 1M HCl to arrest the reaction and the 100 µL of iodine reagent was mixed. The optical density was assessed at 565 nm by UV spectrophotometer. The formula for measuring α-amylase inhibitory activity was as follows:

% Inhibition = [(Abs(test)–Abs.(control)]/ Abs(test)x 100

The extract concentrations that resulted in a 50% inhibition of enzyme activity (IC_{50} values) were plotted graphically.

3.6.2 *In-vitro* α-glucosidase inhibition assay

The effect of HEEAF on α-glucosidase activity was ascertained using the procedure outlined by Apostolidis et al [13]. 100mM phosphate buffer with a pH of 6.8 was used to prepare the substrate solution, p-nitrophenyl glucopyranoside (pNPG). Distinct conc. of HEEAF (10, 20, 40, 80, 160, and 320 µg/ml) were pre-incubated with 200µL of α-glucosidase for 10 minutes. To initiate the reaction, 400µl of a substrate (5mM pNPG) was mixed in 100mM 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. The yellow-colored reaction mixture was detected at 405nm using UV-VIS spectrophotometer. Voglibose is an approved drug and served as the positive control. Negative control was the buffer that lacked the test and reference standards. The α-glucosidase's inhibitory activity has been estimated with the help of below mentioned equation:

% Inhibition = [(Abs (blank (or) control) - Abs (Sample)/Abs(blank] x 100

The extract concentrations that resulted in a 50% inhibition of enzyme activity (IC_{50} values) were plotted graphically.

3.7 Statistical Analysis

All the experiments were conducted in triplicates and a statistically significant difference was estimated with $p \leq 0.05$ using IBM SPSS (vers.20) software. The results were appeared as mean ± SD.

4 Results

The hydro-ethanolic extract of the selected seaweed was prepared, stored and the preliminary phytochemical analysis was conducted based on the standard procedures.

The phytochemical analysis outcome is shown in Table 1. Alkaloids, flavonoids, carbohydrates, sterols, saponins, tannins, polyphenols, proteins, free amino acids, gums, and mucilage were found in HEEAF according to our study.

Table 1: Preliminary phytochemical screening of the HEEAF

Constituents	Tests	Inference
	Mayer's method	++
Alkaloids	Dragandorff's metn	++
	Hager's reagent	+
Flavonoids	Shinods test	++
	Molisch's analysis	++
Carbohydrates	Iodine	++
	Benedict's analysis	+
	Borntrager's test	--
Glycosides	Modified Borndrager's analysis	--

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	Keller Killiani test	--
	Raymond test	-
Sterols	Salkowski analysis	+++
	LibermanBurchard's analysis	++
Saponin	-	+++
Tannin	$FeCl_3$ test	++
	Gold Beater's skin test	++
Polyphenols	-	+ +
	Million's method	+ +
Proteins and amino acids	Biuret method	+ + +
	Ninhydrin reagent method	+ +
Gums	-	+

Note: (+++- immediate result was observed, ++- time duration was 3 to 5mts, +- time duration was above 5 mts and (-)- negative result)

The chemical composition of the crude HEEAF was characterized in the current investigation by using HR-LCMS. Figure 1 displays the chromatogram of HEEAF. Table 2 presents the phytochemical identification data from HR-LCMS. It summarized the tentative compounds that were identified from the extracts along with their molecular mass, retention time, suggested metabolite name, and molecular formula etc. A total of 29 compounds were eluted and identified.

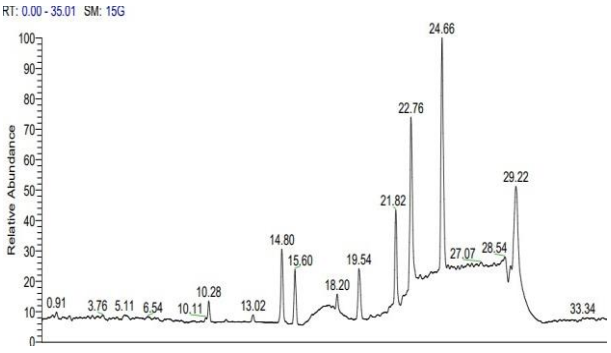

Fig: 1- The HR-LCMS chromatogram of HEEAF

Table 2: The phytochemical identification of HEEAFby HR-LCMS

S.No	Name of the Compounds	Formula	RT (min)	Mass
1.	4-Undecylbenzene sulfonic acid	$C_{17}H_{28}O_3S$	21.169	312.17625
2.	Embelin	$C_{17}H_{26}O_4$	15.26	294.18364
3.	Azelaic acid	$C_9H_{16}O_4$	10.716	188.10431
4.	Spherophorol	$C_{13}H_{20}O_2$	15.321	208.14593
5.	4-Caproylresorcinol	$C_{12}H_{16}O_3$	14.201	208.10957
6.	Pregna-4,6-diene-3,20-dione	$C_{21}H_{28}O_2$	19.972	312.20924
7.	(+)-[6]-Gingerol	$C_{17}H_{26}O_4$	14.743	294.18358
8.	Methyl (3E,6E)-3,6-dodecadienoate	$C_{13}H_{22}O_2$	14.607	210.16162
9.	BHQ	$C_{14}H_{22}O_2$	15.251	222.16167
10.	Brassylic acid	$C_{13}H_{24}O_4$	14.674	244.16749
11.	S-(tert-Butyl) (2-[(2Z)-5-hydroxy-2-	$C_{16}H_{26}O_3S$	24.15	298.16054

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	pentenyl]- 3-oxocyclopentyl) ethanethioate			
12.	Erucamide	$C_{22}H_{43}NO$	17.679	337.33382
13.	4-Hydroxybenzocyclobutene-1,2-Dione	$C_8H_4O_3$	19.548	148.01602
14.	Pentadecyl N-pentanoylalaninate	$C_{23}H_{45}NO_3$	25.079	383.33925
15.	7-Dehydrodesmosterol	$C_{27}H_{42}O$	25.129	382.32317
16.	THREO-SPHINGOSINE, (-)-	$C_{18}H_{37}NO_2$	21.616	299.28224
17.	Diethylene glycol n-butyl ether	$C_8H_{18}O_3$	10.526	162.12563
18.	Oleamide	$C_{18}H_{35}NO$	23.281	281.27165
19.	Sphinganine	$C_{18}H_{39}NO_2$	16.642	301.29787
20.	Betaine	$C_5H_{11}NO_2$	28.868	117.07915
21.	(4bR,12aS)-2-Butyl-12a-methyl-1,3-dioxo-dodecahydronaphtho[2,1-f]isoquinolin-8-yl acetate	$C_{24}H_{31}NO_4$	22.77	397.22475
22.	2-Aminopalmitic acid	$C_{16}H_{33}NO_2$	19.322	271.25096
23.	Stearoyl ethanolamide	$C_{20}H_{41}NO_2$	23.82	327.31331
24.	Dispiro[5.2.5.2] hexadecan-1-one	$C_{16}H_{26}O$	21.687	234.19832
25.	2,2,6,6-Tetramethyl-4-piperidinol	$C_9H_{19}NO$	14.73	157.14665
26.	7-Butyl-hydroxy-1-azaspiro[5.5] undecan-2- one	$C_{14}H_{25}NO_2$	15.066	239.18846
27.	Undecanamide	$C_{11}H_{23}NO$	15.633	185.1779
28.	Coumarandione	$C_8H_4O_3$	21.817	148.01602
29.	Pentadecyl N-pentanoylalaninate	$C_{23}H_{45}NO_3$	25.503	383.33928

Determination of IC_{50} values for α-amylase and α-glucosidase enzymes

The *in-vitro* inhibitory assay method was performed on α-amylase and α-glucosidase with HEEAF to investigate its anti-diabetic potential. The HEEAF showed significant increase in inhibitory effects on α-amylase having IC_{50} value of 9.14µg/ml and for reference standard acarbose was found to be 3.12 µg/ml. Similarly, HEEAF showed significant inhibitory effect onα-glucosidase having IC_{50} value of 65.34µg/ml and standard drug voglibose was found to be 25.95 µg/ml. The IC_{50} value determination presented in table 3 & 4 also in figure 2 & 3 for α-amylase and α-glucosidase respectively.

Table 3: The IC_{50} value determination of α-amylase inhibition assay

Conc. (µg)	AF	Acarbose
10	36.73± 0.05	52.58± 0.15
20	68.72± 0.22	77.95± 0.62
40	82.89± 0.52	83.41± 0.81
80	88.48± 0. 81	91.02± 0.08
160	90.49± 0.09	94.93± 0.71
320	94.30± 0.18	95.39± 0.08

All the values are mean of 3 findings

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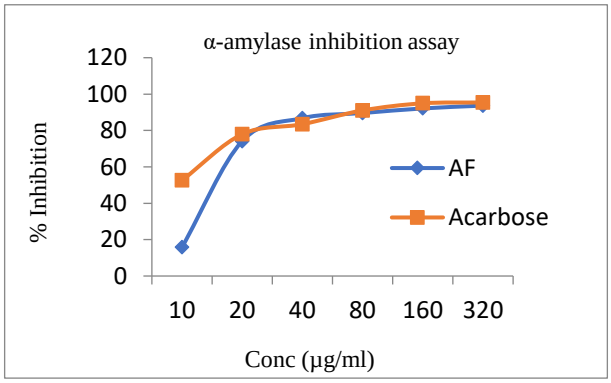

Fig 2: The IC_{50} value estimation of α-amylase inhibition assay

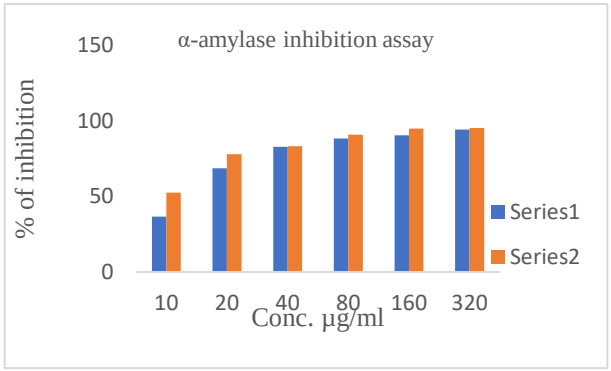

Fig 3: The IC_{50} value estimation of α-amylase inhibition assay

Table 4: The IC_{50} value estimation of α-glucosidase inhibition assay

Conc. (µg)	AF	Voglibose
10	7.22±0.09	23.96±0.09
20	24.41±0.92	46.44±0.11
40	37.84±0.81	61.80±0.25
80	57.71±0.05	80.59±0.38
160	71.31±0.42	88.84±0.28
320	81.95±0.35	92.37±0.06

All the values are mean of 3 findings

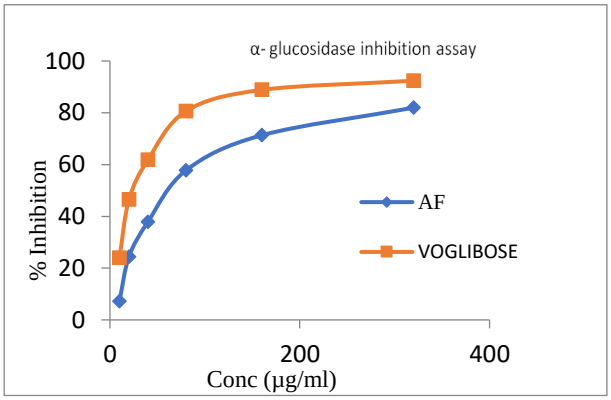

Fig 4: The IC_{50}value determination of α-glucosidase inhibition assay

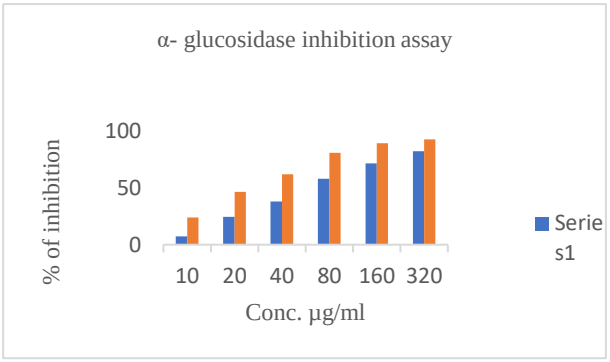

Fig 5: The IC_{50}value determination of α-glucosidase inhibition assay

5 Discussion

An abundance of structurally unique and physiologically active metabolites could be found in marine species. The pharmaceutical industry may find possible bioactive substances of interest in the secondary or primary metabolites produced by these algae [14].

The seaweed *A. fragilissima* was chosen for the current investigation and tested for phytochemical elements. Our findings indicated the existence of alkaloids, flavonoids, carbohydrates, sterols, polyphenols, proteins, free amino acids, gums, and mucilage in HEEAF. The same kind of outcomes was shown in earlier research [15, 16].

The result of the HR-LCMS analysis revealed that, almost 29 compounds were present in HEEAF. Embelin was one of the compounds in our investigation which turned up our study because it is a naturally occurring benzoquinone molecule with numerous biological features that are significant to treat or prevent cancer in humans [17].

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Azelaic acid was one of the substances in our investigation. Whole grains including wheat, rye, and barley contain azelaic acid which is a natural source of saturated dicarboxylic acid with nine carbon atoms. Both the European Medicines Agency and the US FDA have given the approval for azelaic acid as therapeutically applicable to treatment melasma, skin pigmentation, medium- to-severe vulgaris, and inflammatory acne [18]. The unexplored phytochemicals like Erucamide, Threo-Sphingosine, Oleamide, sphinganine, Betaine, Undecanamide, Coumarandione, Pentadecyl N-pentanoylalaninate,4- Caproylresorcinol, Pregna-4,6-diene-3,20-dioneand 4-Undecyl benzenesulfonic acid had been cataloged from HEEAF for their bioactive potentials to represent unique natural interventions. *Amphiroa* species extract were shown to have antibacterial, antiviral, antioxidant, and anticancer properties in a prior study. The same could be achieved with *A. fragilissima* because of its abundant primary and secondary metabolites [5].

Pancreatic breaks down starch, whereas intestine α-glucosidase absorbs glucose. These two enzymes work together to aid in digestion of carbohydrate. It plays an essential role to produce linear and branched malto-oligosaccharides and measures the extent of carbohydrate digestion by hydrolyzing inner α-1,4-glucosidic bonds. The α-glucosidase plays a crucial role in converting carbohydrates into glucose subsequently leads to postprandial hyperglycemia [19].

Seaweeds may help to manage diabetes mellitus because they inhibit the hydrolysis of carbohydrates which lowers post-prandial hyperglycemia. The current *in-vitro* study on α-amylase and α-glucosidase inhibition assay was performed with HEEAF to investigate its anti-diabetic potential. The study showed significant inhibitory effects against α-amylase with IC_{50} value of 9.14 µg/ml and 3.12 µg/ml for both HEEAF and the reference standard acarbose respectively. Similarly, there was a significant inhibitory effect onα-glucosidase having IC_{50} values of 65.34 µg/ml and 25.95 µg/ml for HEEAF and the standard drug voglibose respectively. The current investigation had promised a strong dose dependent inhibition of HEEAF on α-amylase and α- glucosidase.

Similarly, it had been reported that 2- bromophenols, 2,4,6-tribromophenol, and 2,4-dibromo phenol of *Grateloupia elliptica Holmes* had inhibitory action on α-glucosidase [21]. The same ingredients were present in HEEAF therefore, it may also have the same α-glucosidase inhibition.

It has been concluded that, the important secondary metabolites had been identified in HEEAF by performing the preliminary phytochemical and HR-LCMS analyses. The investigation of inhibitory activities of *A. fragilissima* on α-amylase and α-glucosidase sheds information on the potential application of this seaweed in the management of diabetes. Subsequent isolation of the crude extracts will be needed to determine the primary compound accountable for its anti- diabetic effect. Further *in- vivo* research is required to confirm the pharmacological features of *Amphiroa fragilissima* as promising anti-diabetic drug. Therefore, HEEAF can be applied in the treatment and management of diabetes mellitus after performing toxicity studies as per OECD guidelines to establish their safety.

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