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Combined activity of Gracilaria edulis and Ulva reticulata on Diabetes associated Atherosclerosis: An In-vitro study

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ABSTRACT

Background: Diabetes-associated atherosclerosis is a complex metabolic condition driven by chronic hyperglycemia, oxidative stress, and inflammation. This study explores the therapeutic potential of hydroalcoholic extracts from two marine macroalgae, Gracilaria edulis (GE) and Ulva reticulata (UR), individually and in combination, focusing on their anti-diabetic and anti-inflammatory activities.

Methods: Extracts were prepared via Soxhlet extraction and analyzed for phytochemical composition and heavy metal content. Anti-diabetic properties were assessed through alpha-amylase, alpha-glucosidase, sucrase inhibition, hemoglobin glycosylation, and yeast glucose uptake assays. Anti-inflammatory effects were examined using HRBC membrane stabilization, nitric oxide scavenging, protein denaturation, and protease inhibition assays. The extracts were tested alone and in various GE:UR ratios (1:1, 1:2, 2:1) to evaluate synergistic effects.

Results: Phytochemical screening confirmed the presence of alkaloids, flavonoids, phenols, tannins, steroids, and terpenoids, with no detectable heavy metals. The 1:1 GE:UR combination exhibited the highest alpha-amylase (97.33%) and alpha-glucosidase (87.79%) inhibition. It also demonstrated strong inhibition of hemoglobin glycosylation (86.40%) and sucrase activity (82.42%). Glucose uptake was significantly enhanced (81.68%) in yeast cells. Anti-inflammatory assays showed notable results for the 1:1 combination, with high membrane stabilization (81.50%), nitric oxide scavenging (88.88%), protease inhibition (83.38%), and protein denaturation inhibition (87.89%).

Conclusion: The combined extracts of Gracilaria edulis and Ulva reticulata, especially in a 1:1 ratio, exhibit potent synergistic effects against key markers of diabetes-associated atherosclerosis. These findings support their potential development as safe, natural adjunct therapies for metabolic disorders. Further in vivo and clinical investigations are warranted.

Keywords: *Gracilaria edulis, Ulva reticulata, diabetes, atherosclerosis, enzyme inhibition, glucose uptake, anti-inflammatory, marine bioactives...*

1. INTRODUCTION

Gracilaria edulis and *Ulva reticulata* are two prominent species of marine macroalgae, gaining attention in recent years for their diverse bioactive compounds and their therapeutic potential.[1] The rising prevalence of chronic diseases such as diabetes-associated atherosclerosis calls for innovative approaches to mitigate these conditions. Research into natural and sustainable bioresources like marine algae offers promising avenues for developing novel therapeutic agents.[2]*Gracilaria edulis*, a red macroalga belonging to the family Gracilariaceae, is widely distributed in tropical and subtropical marine environments. It is primarily known for its role in the production of agar, a valuable polysaccharide widely used in the food, pharmaceutical, and biotechnology industries [3]. Apart from its industrial applications, Gracilaria edulis is rich in bioactive compounds, including sulfated polysaccharides, flavonoids, and polyphenols, which exhibit antioxidant, anti-inflammatory, and anti-diabetic properties[4–6].Studies have shown that the sulfated polysaccharides from *Gracilaria edulis* possess

significant hypoglycemic and hypolipidemic effects, making it a candidate for managing metabolic disorders[7].Its potential to modulate oxidative stress and inflammation further supports its application in conditions like atherosclerosis, where these mechanisms play a critical role in disease progression.

Ulva reticulata, commonly known as green seaweed, is a member of the Ulvaceae family and is widely distributed along coastal regions. It is rich in bioactive compounds, including ulvans, polyphenols, and sterols, which contribute to its pharmacological properties[6,8,9]. Ulvans, the sulfated polysaccharides extracted from *Ulva reticulata*, have been extensively studied for their anti-inflammatory, antioxidant, and cholesterol-lowering effects [10]These properties are particularly relevant in addressing diabetes-associated complications, such as atherosclerosis. The antioxidant activity of *Ulva reticulata* is attributed to its ability to scavenge free radicals and reduce oxidative stress, a key factor in the progression of atherosclerosis. Moreover, its role in modulating lipid profiles and preventing endothelial dysfunction underscores its therapeutic potential [11–13]

Diabetes-associated atherosclerosis is a leading cause of morbidity and mortality worldwide. Atherosclerosis, a chronic inflammatory condition characterized by the buildup of plaques in arterial walls, is accelerated in individuals with diabetes due to hyperglycemia, oxidative stress, and chronic low-grade inflammation[14]. The interplay between these factors contributes to endothelial dysfunction, vascular inflammation, and lipid metabolism dysregulation, resulting in an increased risk of cardiovascular events. Hyperglycemia induces the production of advanced glycation end products (AGEs), which activate pro-inflammatory pathways and exacerbate vascular damage (Singh et al., 2014). Additionally, oxidative stress in diabetic individuals leads to the oxidation of low-density lipoprotein (LDL), a critical step in atherogenesis. Conventional treatments for diabetes-associated atherosclerosis, including statins and anti-inflammatory drugs, are often associated with side effects and limited efficacy in addressing the underlying causes of the condition.[15–17]

Recent studies have employed advanced analytical techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) to elucidate the complex phytochemical composition of *Gracilaria edulis* and *Ulva reticulata*, providing deeper insights into their therapeutic potentials.

In the case of *Gracilaria edulis*, a study conducted by Kandasamy et al. (2021) utilized both LC-MS and GC-MS analyses to profile its metabolites. The research identified several bioactive compounds, including amino acids, fatty acids, triterpenoids, carotenoids, flavonoids, and phenolic compounds. These findings support the seaweed's traditional use in managing metabolic disorders and highlight its potential as a source of novel therapeutic agents. [18]

Similarly, *Ulva reticulata* has been subjected to GC-MS analysis to determine its bioactive constituents. A study by Swapna et al. (2020) revealed the presence of compounds such as dibutyl phthalate, n-hexadecanoic acid, and 1,2-benzenedicarboxylic acid in the methanolic extract of Ulva reticulata. These compounds are known for their antimicrobial, antioxidant, and anti-inflammatory properties, which may contribute to the therapeutic effects of the seaweed. [19,20]

The integration of these advanced analytical techniques has significantly enhanced our understanding of the complex phytochemical makeup of these marine macroalgae. By identifying and characterizing the bioactive compounds present in Gracilaria edulis and Ulva reticulata, researchers can better elucidate the mechanisms underlying their therapeutic effects. This knowledge paves the way for the development of algae-based nutraceuticals and pharmaceuticals aimed at managing chronic diseases such as diabetes-associated atherosclerosis.

The use of marine algae such as *Gracilaria edulis* and Ulva reticulata in therapeutic interventions offers a sustainable and natural alternative for managing diabetes-associated atherosclerosis. The combined activity of these two macroalgae could provide a synergistic effect, leveraging their unique bioactive compounds to target multiple pathways involved in disease progression. For instance, the antioxidant properties of both species can mitigate oxidative stress, while their anti-inflammatory and lipid-modulating effects can address key factors contributing to atherosclerosis. This research aims to explore the combined effects of Gracilaria edulis and Ulva reticulata on diabetes-associated atherosclerosis, focusing on their ability to reduce oxidative stress, inflammation, and lipid abnormalities. The study also seeks to elucidate the molecular mechanisms underlying their therapeutic effects, contributing to the growing body of evidence supporting the use of marine algae in managing chronic diseases. By identifying the bioactive compounds responsible for these effects and evaluating their efficacy in preclinical models, this work paves the way for the development of algae-based nutraceuticals and pharmaceuticals. Additionally, the study aligns with global efforts to promote sustainable and eco-friendly approaches to healthcare, leveraging the rich biodiversity of marine ecosystems.

2. MATERIALS AND METHODS:

Collection and Preparation of seaweed extract

About 5 Kg of two different seaweeds were collected from Mandapam Coast, Rameshwaram, Tamil Nadu, India. The seaweed samples were washed thoroughly in running tap water thrice, followed by distilled water. The washed seaweed samples were shade-dried and powdered. About 500-gram shade-dried material was ground in powder using a laboratory blender and sieved to obtain 60-70 mesh powdered seaweed.

Figure 01: Gracilaria edulis

Figure 02: Ulva reticulata

The powdered sample from each of the seaweed was subjected to Soxhlet extraction (Borosil, Mumbai, India) using different solvents such as Petroleum ether, Chloroform, Ethyl acetate, Ethanol, and Hydro-Alcoholic (Ethanol-Water 70:30). The powdered samples were made in a 100 g thimble using handmade filter paper. The plant sample-filled thimble was carefully placed inside the extractor chamber and poured with the respective solvent (1 L). The reservoir round bottom flask was heated to 78°C in a heating mantle. At least 15 refluxes were run for each sample to get good-quality seaweed extract. The resultant extract was condensed using a rotary evaporator (Buchi, Bangalore, India) under reduced temperature in vacuum conditions. The resultant precipitant was collected in a glass container for further analysis and stored at –20°C.

PHYTOCHEMICAL SCREENING

For saponins, a stable foam is formed when a sample is shaken vigorously with distilled water. Glycoside is detected through Liebermann's test, where a color change from violet to blue to green indicates the presence of a steroidal nucleus. Coumarin is detected by adding alcoholic sodium hydroxide to the solution, resulting in a yellow color. Alkaloids are detected through Mayer and Wagner's reagent, which results in turbidity. Flavonoids are detected through the Ferric chloride test, where a green-blue or violet colouration indicates the presence of a phenolic hydroxyl group. Tannins are detected through the addition of FeCl3 solution, resulting in a green precipitate. Terpenoids are detected through the Salkowski Test, where a greyish color develops when dissolved in chloroform and concentrated sulphuric acid. Steroids are detected by a red color in the lower chloroform layer when dissolved in chloroform and concentrated sulphuric acid. Anthraquinones are detected through Borntrager's reaction, where a powdered plant extract is heated in a steam bath, filtered, and cooled. A bright pink coloration is observed in the upper aqueous layer, indicating the presence of anthraquinones. Control tests are conducted by adding 10 mL of 10% ammonia solution in 5ml chloroform.

HEAVY METAL ANALYSIS

The sample *Gracilaria edulis* and *Ulva reticulata* was collected, washed thoroughly with distilled water to remove debris and salts, and air-dried at room temperature. The dried sample was powdered using a mechanical grinder and sieved to obtain a uniform particle size.0.5 g of the powdered sample was accurately weighed and transferred to a Teflon digestion vessel.10 mL of concentrated nitric acid (HNO_3) was added to the sample. The mixture was subjected to microwave digestion under controlled temperature and pressure conditions until complete dissolution of the sample was achieved. The digested sample was diluted to 50 mL with deionized water and filtered through Whatman No. 42 filter paper to remove any particulate matter. Heavy metals were quantified using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) following AOAC Official Methods. The instrument was calibrated using certified reference standards for each heavy metal, and the detection limit (DL) was set to 0.1 mg/kg.

ANTI-DIABETIC ASSAYS

Alpha-Amylase Inhibitory Assay: The assay was carried out using a modified procedure of McCue and Shetty [17]. A total of 250 µL of sample (6.25–20 µg/mL) was placed in a tube and 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (0.5 mg/mL) was added. This solution was pre-incubated at 25 °C for 10 min, after which 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and then further incubated at 25 °C for 10 min. The reaction was terminated by adding 500 µL of dinitrosalicylic acid (DNSA) reagent. The tubes were then incubated in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using spectrophotometer. A control was prepared using the same procedure replacing the sample with distilled water. Acarbose standard was also run in the same manner. The -amylase inhibitory activity was calculated as percentage inhibition:

$$\text{Inhibition (\%)} = \frac{\text{Abs 540 (control)} - \text{Abs 540 (sample)}}{\text{Abs 540(control)}} \times 100$$

Concentrations of sample resulting in 50% inhibition of enzyme activity (IC50) were determined graphically.

Alpha glucosidase inhibitory activity: The method of Watanabe et al. (1997) was used. Yeast α-glucosidase (0.7 U) dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/l bovine serum albumin, and 0.2 g/l NaN3, and 5 mM p-nitrophenyl- α-D-glucopyranoside in the same buffer (pH 7.0) were used as an enzyme and a substrate solution, respectively. The enzyme solution (1000 µl) and 100 µl of the test sample at various concentrations (100 to 500 µg/mL) were mixed, and absorbance at 405 nm was measured using a spectrophotometer (UV1800 Shimadzu, Japan). After incubation for 5 min, 50 µl of the substrate solution was added and incubated for an additional 5 min. The increase in absorbance from time zero was measured, and inhibitory activity was calculated as a percentage of the blank control. Acarbose standard was also run in the same manner. The % inhibition was calculated using the following formula

$$\text{Inhibition (\%)} = \frac{\text{Abs 405 (control)} - \text{Abs 405 (extract)}}{\text{Abs 405(control)}} \times 100$$

Nonenzymatic glycosylation of haemoglobin assay: The extracts were prepared in 0.01 M of phosphate buffer (pH 7.4) at a concentration of 1 mg/ mL. Precisely, 1 mL of 0.06% haemoglobin solution, 5 µL of 0.02% gentamycin, 1 mL sample solution, and 1 mL of 2% glucose solution was mixed. All of the reagent solutions were also prepared in the buffer. The mixture was incubated for 72 h at 37 °C in a dark environment. Thereafter, the degree of glycosylation was measured at 443 nm using UV-Vis spectrophotometry. Tocopherol was used as a standard drug. Determination of the percentage inhibition was calculated using the formula in Eq.

$$\text{Inhibition (\%)} = \frac{\text{Abs 443 (control)} - \text{Abs 443 (sample)}}{\text{Abs 443 (control)}} \times 100$$

Sucrase inhibition assay: A method to assay the effect of samples on sucrase enzyme activity [Honda and Hara 1993]. Enzyme solutions (10 µL) were incubated together with buffered solubilized sample (25–200 µg/ml in maleate buffer with pH 6.0) for 10 min at 37°C, while the volume was completed to 200 µL with maleate buffer (pH 6.0) in case of control, then the reaction was initiated by the addition of 100 µL of sucrose solution (60 mM). About 30 min later, the reaction was stopped by the addition of 200 µL of 3, 5-dinitrosalysilic acid reagent. The mixture was incubated in a boiling water bath for 5 min. Acarbose standard was also run in the same manner. The absorbance of each reaction was read at 540 nm. The percentages of inhibitory activities were calculated using the following formula:

Abs 540 (control) – Abs 540 (sample)

$$\text{Inhibition (\%)} = \frac{\text{Abs 540 (sample)}}{\text{Abs 540 (control)}} \times 100$$

Abs control represents the absorbance of the control reaction (containing all reagents except the tested sample), whereas the Abs sample is the absorbance of the tested sample. An untreated enzyme solution was used as control. All experiments were carried out 3 times.

Yeast Cell Glucose uptake assay: This assay was performed according to the well-defined method of Cirillo. Commercial baker's yeast was dissolved in distilled water to prepare 1% suspension. The suspension was kept overnight at room temperature (25∘C). On the next days, yeast cells suspension was centrifuged at 4200rpm (Microfuge 16 Centrifuge, FX241.5P Rotor, 50/60Hz and 220–240V) for 5 minutes. The process was repeated by the addition of distilled water to the pallet until a clear supernatant was obtained. Exactly 10 parts of the clear supernatant fluids were mixed with 90 parts of distilled water to get a 10%v/v suspension of the yeast cells.

Extracts were mixed with dimethyl sulfoxide (DMSO) till dissolution. The mixture was then supplemented with various concentrations (5, 10, and 25Mm) of1mLofglucose solution and incubated for 10min at 37∘C. To initiate the reaction, 100μL of yeast suspension was poured in the mixture of glucose and extract, vortexed, and incubated for another 60 minutes at 37∘C. After incubation, the tubes were centrifuged for 5 minutes at 3800rpm and glucose was estimated by using a spectrophotometer (UV 5100B) at 520nm. Absorbance for the respective control was also recorded on the same wavelength.

The percent increase in uptake was calculated by the formula: %increase in glucose uptake = (Abs. of control − Abs. of sample) (1) ×100, Abs. of control

where control is the solution having all reagents except the test sample. Metronidazole was used as a standard drug.

ANTI-INFLAMMATORY ASSAYS:

HRB membrane stabilization test: HRB membrane stabilization test was performed by the following described method proposed by Sadique et al. (1989). Fresh whole human blood (10ml) was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with an equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline. The reaction mixture 2ml consists of 1 ml of test sample solution and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula

Percentage Inhibition = (A of Control – A of Sample)/A of Control x100

Nitric oxide scavenging activity: Nitric oxide generated from sodium nitroprusside in an aqueous solution at physiological pH was measured by the Griess reaction (Marcocci et al., 1994). The reaction mixture (3ml) containing sodium nitroprusside (10mm) in phosphate buffer saline and the test extract (10, 25, 50 and 100µg/ml) was incubated at 25oC for 150min, after incubation 1.5ml of the reaction mixture was removed and 1.5ml of the Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% Napthylethyline diamine hydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Percent inhibition of nitric oxide scavenging was calculated using the formula.

Percentage Inhibition = (A of Control – A of Sample) /A of Control× 100. A- absorbance.

Inhibition of albumin denaturation: Method of Mizushima et al was followed with minor modifications24. The reaction mixture was consisting of test extracts at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amount of 1N HCl. Diclophenac sodium was taken as standard drug. The samples were incubated at 37oC for 20 min and then heated at 57oC for 30 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

Percentage Inhibition = (A of Control – A of Sample)/A of Control x100

Proteinase inhibitory activity: Proteinase inhibitory activity was performed according to the modified method of Oyedepo et al. (1995). The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations. The mixture was incubated at 37oC for 5 min. The 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. Then 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage of proteinase inhibitory activity was calculated using the following formula.

Percentage Inhibition = (A of control – A of sample)/A of control x100.

3. RESULTS:

Phytochemical Analysis:

Table 1: Qualitative Phytochemical Screening Results

Constituents	***Gracilaria edulis* (G)**	***Ulva reticulata* (U)**
Alkaloids	+	+
Anthraquinones	–	–
Coumarins	–	–
Glycosides	–	–
Flavonoids	+	–
Phenol	+	+
Saponins	–	–
Steroids	+	+
Tannins	+	+
Terpenoids	+	+

Table 2: Quantitative Phytochemical Analysis Results

S.No.	**Assay**	**Unit***	***Gracilaria edulis* (G)**	***Ulva reticulata* (U)**
1	Total Alkaloid Content	mg Atropin equivalent/g DE	81.25	94.71
2	Total Flavonoid Content	mg Quercetin equivalent/DE	51.76	44.33
3	Total Phenol Content	mg Gallic acid equivalent/g DE	325.5	358.1
4	Total Terpenoid Content	mg Linalool equivalent/g DE	32.37	28.91

Qualitative Screening:

Both ***Gracilaria edulis* (G)** and ***Ulva reticulata* (U)** contain alkaloids, phenols, steroids, tannins, and terpenoids. Flavonoids are only present in ***Gracilaria edulis***. Anthraquinones, coumarins, glycosides, and saponins are absent in both samples.

Quantitative Analysis:

Total Alkaloid Content: Higher in Ulva reticulata (94.71 mg Atropin equivalent/g DE) than in *Gracilaria edulis* (81.25 mg). Total Flavonoid Content: Higher in *Gracilaria edulis* (51.76 mg Quercetin equivalent/DE) compared to Ulva reticulata (44.33 mg). Total Phenol Content: *Ulva reticulata* (358.1 mg Gallic acid equivalent/g DE) has a slightly higher content than *Gracilaria edulis* (325.5 mg). Total Terpenoid Content: Slightly higher in *Gracilaria edulis* (32.37 mg Linalool equivalent/g DE) than in Ulva reticulata (28.91 mg).

Heavy Metal Analysis:

Table 3: Heavy Metal Analysis

S.No.	**Parameter**	**Method**	**Unit**	**Result**
1	Lead (Pb)	AOAC 20th Edn. 2016, 99.11	mg/kg	BDL (Below Detection Limit: 0.1)
2	Arsenic (As)	AOAC 20th Edn. 2016, 986.15	mg/kg	BDL (Below Detection Limit: 0.1)
3	Cadmium (Cd)	AOAC 20th Edn. 2016, 999.11	mg/kg	BDL (Below Detection Limit: 0.1)

4	Mercury (Hg)	AOAC 20th Edn. 2016, 971.21	mg/kg	BDL (Below Detection Limit: 0.1)
5	Chromium (Cr)	AOAC 20th Edn. 2016, 974.27	mg/kg	BDL (Below Detection Limit: 0.1)

The analysis covers five heavy metals: Lead (Pb), Arsenic (As), Cadmium (Cd), Mercury (Hg), and Chromium (Cr). All metals were analyzed using the AOAC 20th Edition methods, which are widely recognized for accuracy in food and environmental testing. For all five parameters, the results were BDL (Below Detection Limit). The detection limit for each metal was specified as 0.1 mg/kg, meaning the concentration of these metals in the samples is below this threshold and hence considered negligible. The absence of detectable levels of these heavy metals indicates that the samples are safe and do not pose a toxicological risk related to these parameters.

Invitro Enzymatic Assay (Anti-diabetic)

Table 4: Alpha amylase inhibition activity of *Gracilaria edulis* and *Ulva reticulata* at Different Concentrations

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	Ulva reticulata	GU 1:1	GU 1:2	GU 2:1	STD
1.	0.2	41.30± 0.04	28.50± 0.06	55.66± 0.08	43.00± 0.07	47.77± 0.08	57.66± 0.08
2.	0.4	54.15± 0.05	39.71± 0.08	64.62± 0.09	53.96± 0.08	62.56± 0.09	66.62± 0.09
3.	0.6	61.79± 0.09	51.79± 0.09	71.22± 0.10	62.71± 0.11	69.47± 0.11	76.22± 0.10
4.	0.8	75.00± 0.11	62.64± 0.10	85.84± 0.12	73.96± 0.12	79.66± 0.12	87.84± 0.12
5.	1	83.01± 0.12	71.60± 0.12	97.33± 0.14	79.32± 0.12	84.67± 0.13	99.33± 0.14

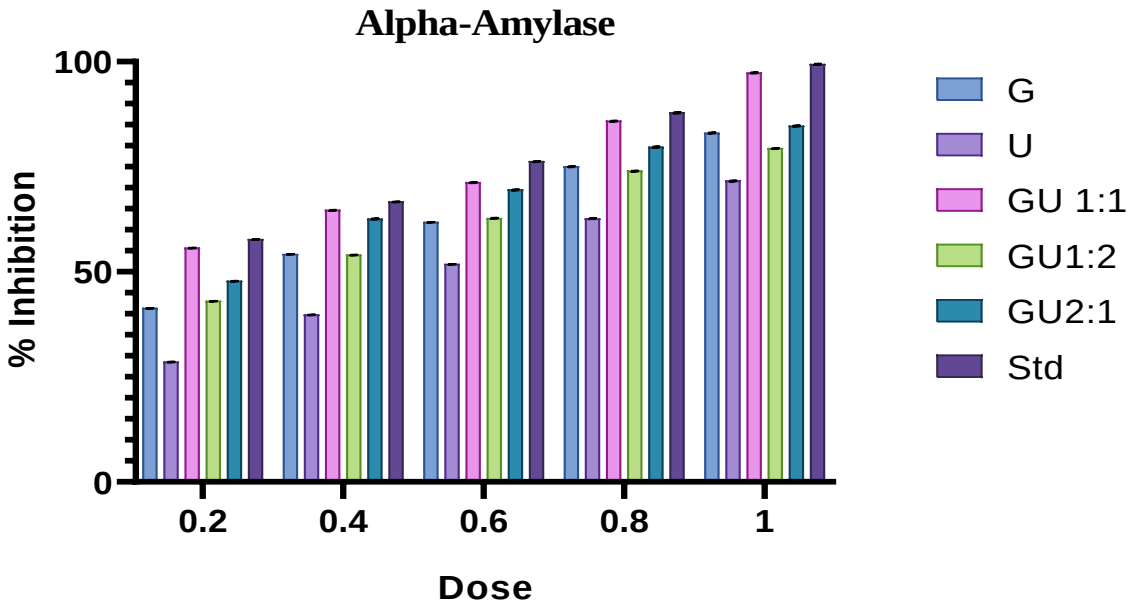

Figure 03: Illustrates the alpha-amylase inhibition (%) at different doses (0.2, 0.4, 0.6, 0.8, and 1) for various groups, including G, U, GU 1:1, GU 1:2, GU 2:1, and a standard (Std) reference. A dose-dependent increase in inhibition is observed across all groups, with GU 1:1 and the standard (Std) demonstrating the highest inhibitory effects, followed by GU 2:1 and

G, while U shows the lowest inhibition. Statistical analysis using two-way ANOVA followed by Tukey's multiple comparison test reveals significant differences among groups, with $p \leq 0.0001$ (****) for all comparisons: G vs. U = ****, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****. These results strongly indicate that GU 1:1 exhibits significantly higher inhibitory activity than the other test groups, suggesting its potential therapeutic application in metabolic disorders related to alpha-amylase inhibition.

These findings suggest that **GU 1:1 may be a promising candidate for managing metabolic disorders** involving alpha-amylase inhibition, potentially aiding in **postprandial glucose regulation**. Further studies, including **mechanistic evaluations and in vivo validation**, are necessary to explore its full therapeutic potential.

Table 5: Alpha glucosidase inhibition activity inhibition Based on Watanabe et al., 1997 Method

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	*Ulva reticulata*	GU 1:1	GU 1:2	GU 2:1	STD
1.	0.2	16.22± 0.06	12.05±0.02	37.27±0.00	23.93±0.01	27.80±0.00	49.27±0.00
2.	0.4	25.00± 0.09	20.18±0.02	57.18±0.03	46.65±0.02	49.04±0.03	67.18±0.03
3.	0.6	38.72±0.11	34.17±0.04	62.77±0.05	53.56±0.05	59.15±0.07	72.77±0.05
4.	0.8	45.90±0.14	41.15±0.07	79.95±0.07	69.41±0.07	73.40±0.08	89.95±0.07
5.	1	51.76±0.16	49.67±0.09	87.79±0.11	76.81±0.08	82.46±0.10	95.79±0.11

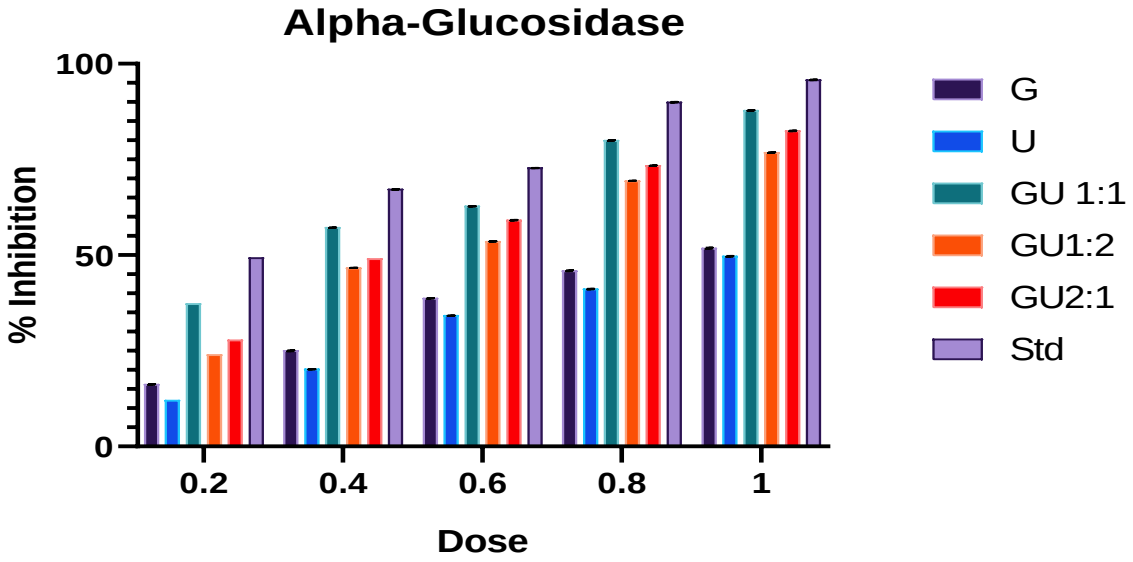

Figure 04: The bar graph represents the alpha-glucosidase inhibitory activity of different formulations (G, U, GU 1:1, GU 1:2, GU 2:1, and Std) at increasing doses (0.2, 0.4, 0.6, 0.8, and 1 mg/mL). The results indicate a dose-dependent increase in inhibition, with GU 1:1 and the standard (Std) showing the highest inhibitory effects. The statistical analysis using two-way ANOVA and Tukey's multiple comparison test revealed significant differences among the groups. The statistical significance between groups (Tukey's test, $p \leq 0.0001$ ()) was observed forG vs. U = ****, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****. These findings confirm that GU 1:1 exhibits the most potent inhibition of alpha-glucosidase activity, followed by GU 2:1 and GU 1:2, while G and U showed relatively lower inhibition. The standard (Std) displayed the highest inhibition, further validating the effectiveness of the tested formulations. The statistical analysis confirms that the differences between groups are highly significant ($p \leq 0.0001$, ****), suggesting a strong potential of GU

1:1 as an effective alpha-glucosidase inhibitor, which could be beneficial in managing postprandial hyperglycemia and diabetes-related metabolic disorders. Further in vivo and mechanistic studies are required to establish its full therapeutic potential.

The results confirm that **GU 1:1 exhibits the most potent inhibition of alpha-glucosidase activity**, followed by **GU 2:1 and GU 1:2**, while **G and U showed relatively lower inhibition**. The standard (Std) displayed the highest inhibition, further validating the effectiveness of the tested formulations. The statistical analysis confirms that the differences between groups are **highly significant ($p \leq 0.0001$, **),** suggesting a strong potential of **GU 1:1 as an effective alpha-glucosidase inhibitor**, which could be beneficial in **managing postprandial hyperglycemia and diabetes-related metabolic disorders**. Further in vivo and mechanistic studies are required to establish its full therapeutic potential

Table 6: Non enzymatic glycosylation of haemoglobin by Based on Parker et al., 1981 Method

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	***Ulva reticulata***	**GU 1:1**	**GU 1:2**	**GU 2:1**	**STD**
1	02.	37.39± 0.05	31.91± 0.01	44.85± 0.02	30.50± 0.01	38.75± 0.02	54.85± 0.02
2	0.4	40.73± 0.09	34.85± 0.02	57.08± 0.06	42.73± 0.04	47.39± 0.03	67.08± 0.06
3	0.6	51.57± 0.21	38.30± 0.04	60.32± 0.11	56.88± 0.06	59.92± 0.06	70.32± 0.11
4	0.8	54.20± 0.30	40.63± 0.06	72.15± 0.20	64.30± 0.09	69.49± 0.09	82.15± 0.20
5	1	58.97± 0.42	54.97± 0.09	86.40± 0.27	70.12± 0.14	79.24± 0.16	96.40± 0.27

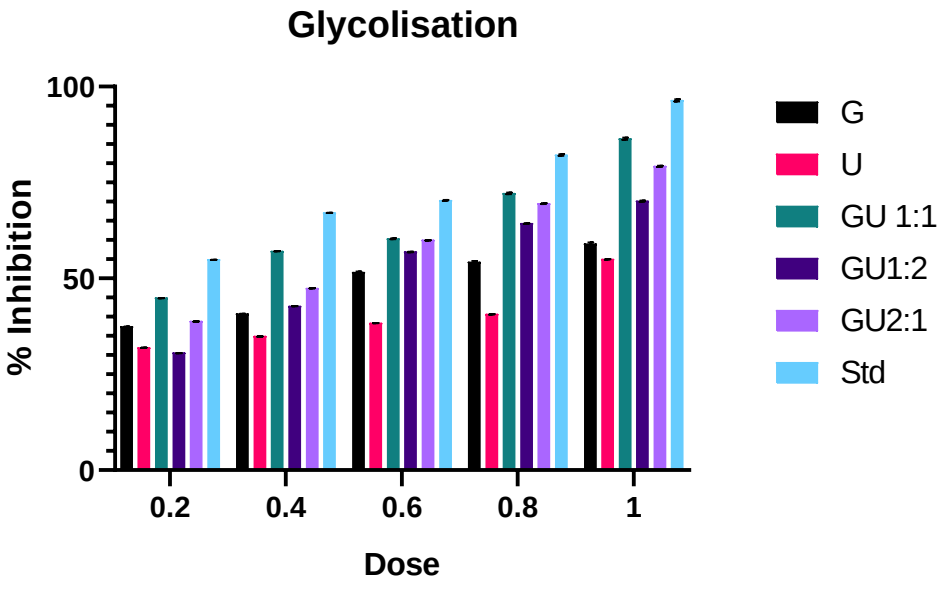

Figure 05: The bar graph illustrates the percentage inhibition of glycosylation by different formulations (G, U, GU 1:1, GU 1:2, GU 2:1, and Std) at increasing doses (0.2, 0.4, 0.6, 0.8, and 1 mg/mL). The results demonstrate a dose-dependent increase in inhibition across all groups, with GU 1:1 exhibiting the highest inhibition, followed by GU 2:1 and GU 1:2, whereas G and U showed relatively lower inhibition. The standard (Std) displayed the highest inhibitory activity, serving as a reference for comparison. Statistical analysis using two-way ANOVA followed by Tukey's multiple comparison test revealed highly significant differences ($p \leq 0.0001$) among all formulations. Specifically, significant differences were observed in comparisons betweenG vs. U = ****, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****,

U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****. These findings indicate that GU 1:1 is the most effective formulation in inhibiting glycosylation, showing statistically significant superiority over all other formulations. The standard exhibited the highest inhibition, serving as a benchmark for comparison. The significant differences across all groups suggest the potential of GU 1:1 as an effective inhibitor of glycosylation, which may have promising implications in managing diabetes-related complications. Further studies, including in vivo and mechanistic evaluations, are required to validate its therapeutic potential.

The findings indicate that GU 1:1 is the most effective formulation in inhibiting glycosylation, showing statistically significant superiority over all other formulations. The standard (Std) demonstrated the highest inhibition, serving as a benchmark for comparison. The significant differences observed across all groups ($p \leq 0.0001$) highlight the potential of GU 1:1 as an effective inhibitor of glycosylation, which may have promising implications in the management of diabetes-related complications. Further studies, including in vivo and mechanistic evaluations, are required to validate its therapeutic potential.

Table 7: Sucrase inhibition activity, Based on Honda and Hara, 1993 Method

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	***Ulva reticulata***	**GU 1:1**	**GU 1:2**	**GU 2:1**	**STD**
1.	0.2	14.91± 0.05	12.08± 0.02	47.28± 0.08	32.74± 0.02	43.40± 0.03	47.28± 0.08
2.	0.4	24.18± 0.15	19.71± 0.07	55.88± 0.17	44.73± 0.05	57.56± 0.08	65.88± 0.17
3.	0.6	39.75± 0.21	30.68± 0.11	67.11± 0.18	57.94± 0.08	62.48± 0.13	77.11± 0.18
4.	0.8	48.73± 0.30	41.56± 0.15	78.26± 0.29	68.22± 0.08	74.84± 0.15	88.26± 0.29
5.	1	53.93± 0.36	48.81± 0.20	82.42± 0.34	77.07± 0.09	80.20± 0.21	92.42± 0.34

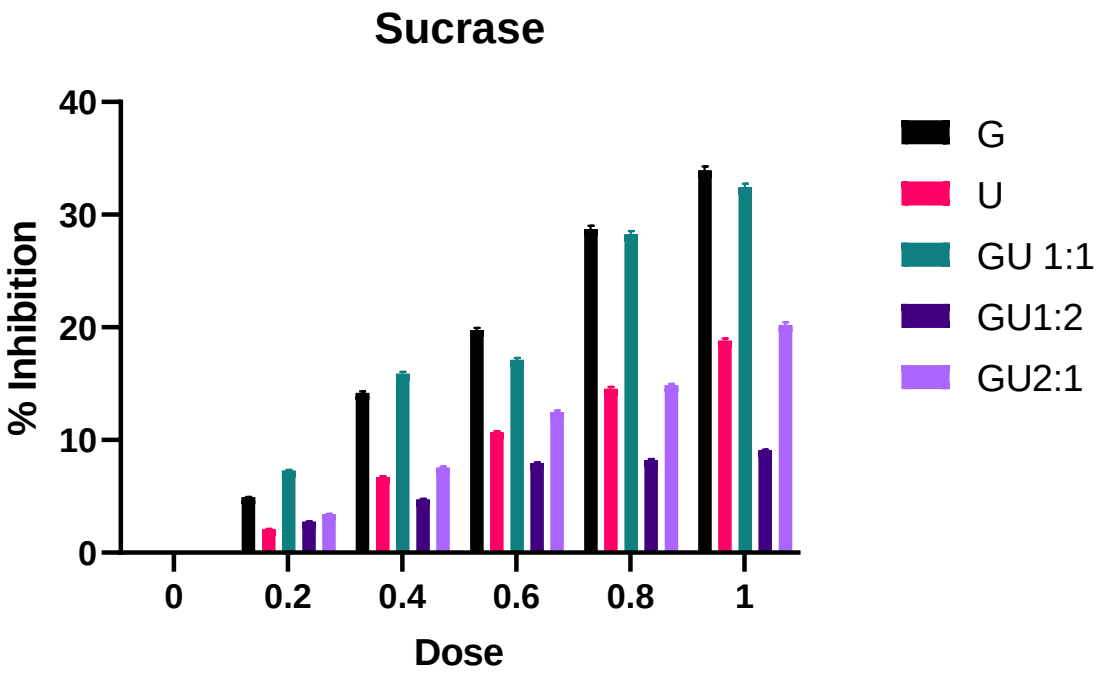

Figure 06: The bar graph represents the percentage inhibition of sucrase at different doses for various groups, with the x-axis showing increasing dose levels (0 to 1) and the y-axis displaying percentage inhibition (0% to 40%). A dose-dependent increase in sucrase inhibition is observed across all groups. G (black) exhibits the highest inhibition, reaching its peak at dose 1, while U (red) shows a similar trend but with lower inhibition levels. Among the combinations, GU 1:1 (teal) demonstrates significant inhibition, closely competing with G at higher doses. GU 2:1 (light purple) exhibits moderate inhibition, surpassing GU 1:2 (dark purple), which consistently shows the lowest inhibition among all groups. The standard (Std) shows the highest inhibition across all doses, establishing its efficacy as a reference compound. Specifically, significant differences were observed in comparisons between G vs. U = ****, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****.

From these results, a clear dose-dependent inhibition of sucrase across all groups, with G and the GU 1:1 combination exhibiting the highest inhibitory effects. The varying inhibition levels among different combinations indicate that the ratio of components plays a crucial role in determining effectiveness. GU 1:1 emerges as the most promising combination, closely matching G in its inhibitory potential, while GU 1:2 consistently shows the weakest activity. These findings suggest that specific formulations and concentrations can significantly impact enzyme inhibition, highlighting the potential of optimized combinations for enhanced therapeutic efficacy.

Table8: Yeast glucose uptake assay Based on Bhutkar and Bhise, 2013 Method

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	***Ulva reticulata***	**GU 1:1**	**GU 1:2**	**GU 2:1**	**STD**
1.	0.2	29.97± 0.11	24.17± 0.11	48.68± 0.11	39.20± 0.11	40.75± 0.11	58.68± 0.11
2.	0.4	33.33± 0.12	31.07± 0.12	57.21± 0.14	42.30± 0.12	43.59± 0.13	67.21± 0.14
3.	0.6	43.41± 0.17	36.43± 0.14	60.31± 0.16	57.73± 0.14	51.09± 0.15	70.31± 0.16
4.	0.8	48.58± 0.19	38.50± 0.14	76.25± 0.17	61.09± 0.15	64.96± 0.17	86.25± 0.17
5.	1	54.78± 0.21	41.60± 0.16	81.68± 0.20	73.93± 0.17	79.87± 0.19	91.68± 0.20

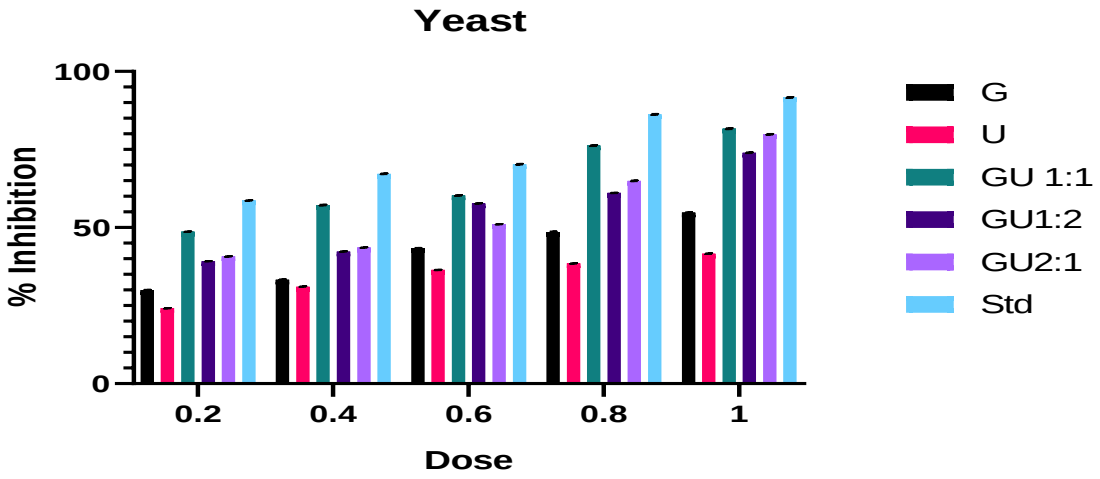

Figure 07: The bar graph represents the percentage inhibition of yeast α-glucosidase activity by different formulations (G, U, GU 1:1, GU 1:2, GU 2:1, and Std) at varying doses (0.2, 0.4, 0.6, 0.8, and 1 mg/mL). The data reveal a dose-dependent

increase in inhibitory activity across all formulations, with GU 1:1 showing the highest inhibition among the test groups, closely followed by GU 2:1 and GU 1:2. The standard (Std) exhibited the most potent inhibition, serving as a reference for comparison. Formulations G and U demonstrated the lowest inhibitory effects, suggesting their limited activity against yeast α-glucosidase. The standard (Std) displayed the highest inhibitory activity, serving as a reference for comparison. Statistical analysis using two-way ANOVA followed by Tukey's multiple comparison test revealed highly significant differences ($p \leq 0.0001$) among all formulations. Specifically, significant differences were observed in comparisons between G vs. U = ****, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****.

The findings indicate that GU 1:1 is the most effective formulation in inhibiting yeast α-glucosidase, with statistically significant inhibition compared to other formulations. The strong inhibitory effect suggests its potential application in managing postprandial hyperglycemia by targeting α-glucosidase activity. Further studies, including in vivo validation and mechanistic investigations, are warranted to establish its efficacy and therapeutic relevance in diabetes management.

Invitro Enzymatic Assay (Anti-inflammatory)

Table 9: Antioxidant Activity Based on HRB Membrane Stabilization Assay (Sadique et al., 1989)

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	*Ulva reticulata*	GU 1:1	GU 1:2	GU 2:1	STD
1.	0.2	18.16± 0.04	13.29± 0.04	40.88± 0.06	36.29± 0.03	31.63± 0.03	50.88± 0.06
2.	0.4	24.80± 0.08	20.03± 0.05	55.14± 0.09	40.88± 0.06	39.69± 0.05	65.14± 0.09
3.	0.6	39.90± 0.12	28.63± 0.08	67.21± 0.16	54.80± 0.08	43.78± 0.08	77.21± 0.16
4.	0.8	43.63± 0.19	36.84± 0.09	74.69± 0.20	68.03± 0.10	58.03± 0.10	84.69± 0.20
5.	1	59.63± 0.23	49.93± 0.18	81.50± 0.24	71.26± 0.12	67.62± 0.13	91.50± 0.24

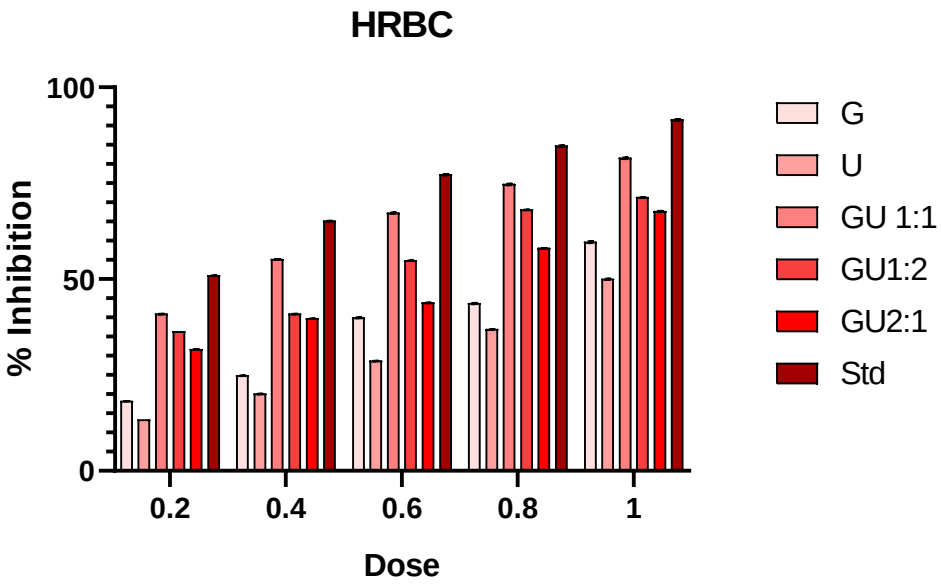

Figure 08: The bar graph illustrates the percentage inhibition of hemolysis in the Human Red Blood Cell (HRBC)

membrane stabilization assay at different doses (0.2–1 mg/mL) for various formulations (G, U, GU 1:1, GU 1:2, GU 2:1, and Std). The standard (Std) consistently exhibits the highest inhibition across all doses, confirming its superior membrane stabilization effect. Among the test groups, GU 2:1 and GU 1:1 show higher inhibition percentages, indicating their potential to protect HRBC membranes from damage. G also demonstrates moderate inhibition, while U and GU 1:2 exhibit comparatively lower activity. Specifically, significant differences were observed in comparisons between G vs. U = **, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****. Statistical analysis reveals a significant difference ($p < 0.05$) between GU 2:1 and other formulations (U, GU 1:2, and G), suggesting its enhanced membrane stabilization effect. GU 1:1 also shows significant inhibition compared to U ($p < 0.05$), while no significant difference is observed between GU 1:1 and G ($p > 0.05$). The standard exhibits significantly higher inhibition than all test groups ($p < 0.001$).**

The results suggest that GU 2:1 and GU 1:1 possess strong membrane stabilization properties, which may contribute to their anti-inflammatory potential. These formulations may serve as promising agents for preventing hemolysis-related inflammatory conditions, warranting further in vivo validation.

Table 10: Antioxidant Activity Based on NO Inhibition Assay (Marcocci et al., 1994)

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	***Ulva reticulata***	**GU 1:1**	**GU 1:2**	**GU 2:1**	**STD**
1.	0.2	19.00± 0.05	14.83± 0.01	42.17± 0.02	31.55± 0.01	42.28± 0.02	52.17± 0.02
2.	0.4	26.14± 0.09	22.48± 0.02	56.73± 0.06	44.45± 0.04	53.93± 0.03	66.73± 0.06
3.	0.6	31.74± 0.21	28.55± 0.04	61.39± 0.11	56.94± 0.06	66.42± 0.06	71.39± 0.11
4.	0.8	41.47± 0.30	36.73± 0.06	71.22± 0.20	69.52± 0.09	74.83± 0.09	81.22± 0.20
5.	1	53.89± 0.42	49.52± 0.09	88.88± 0.27	75.01± 0.14	80.7± 0.16	98.88± 0.27

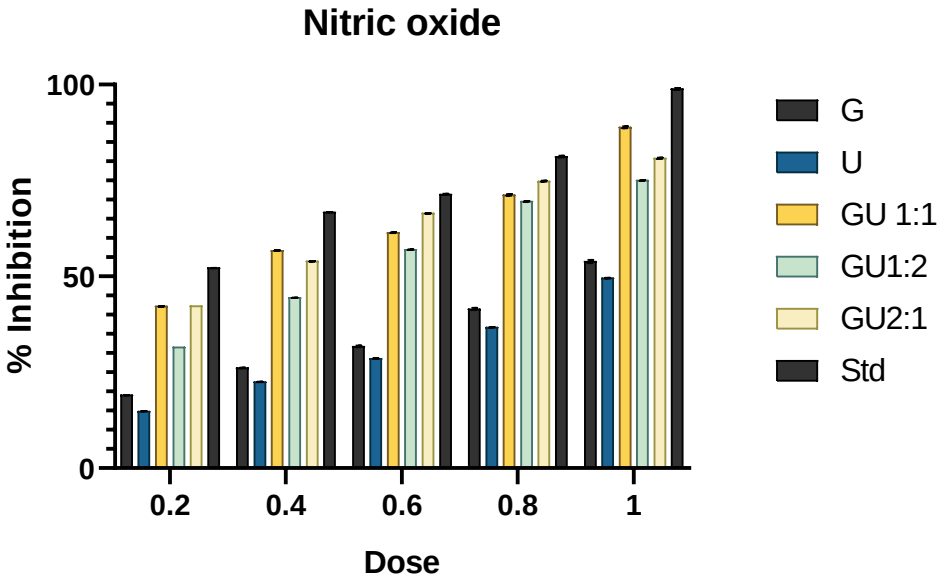

Figure 09: The bar graph represents the percentage inhibition of nitric oxide (NO) production at different doses (0.2–1 mg/mL) for various formulations (G, U, GU 1:1, GU 1:2, GU 2:1, and Std). The standard (Std) exhibits the highest inhibition at all doses, confirming its strong NO-scavenging activity. Among the test formulations, G and GU 1:1 demonstrate the most significant inhibition, particularly at higher doses, followed closely by GU 2:1 and GU 1:2. U exhibits the lowest inhibition, indicating its weaker NO-scavenging potential. Statistical analysis using two-way ANOVA followed by Tukey's multiple comparison test revealed highly significant differences ($p \leq 0.0001$) among all formulations. Specifically, significant differences were observed in comparisons between G vs. U = ****, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****. No significant difference ($p > 0.05$) is observed between GU 1:1 and G, suggesting comparable NO-inhibitory potential. The standard exhibits significantly higher inhibition than all test groups ($p < 0.001$).

The results suggest that GU 1:1 and G possess strong NO-scavenging properties, which may contribute to their anti-inflammatory potential. Further in vivo validation is recommended to confirm their efficacy in mitigating oxidative stress and inflammation.

Table 11: Antioxidant Activity Based on Protease Inhibitor Assay (Oyedepo and Femurewa, 1995)

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	***Ulva reticulata***	**GU 1:1**	**GU 1:2**	**GU 2:1**	**STD**
1.	0.2	16.91± 0.02	14.74± 0.00	40.29± 0.01	31.47± 0.00	43.68± 0.00	50.29± 0.01
2.	0.4	24.26± 0.03	19.94± 0.00	54.26± 0.03	46.91± 0.02	57.35± 0.01	64.26± 0.03
3.	0.6	32.03± 0.05	26.62± 0.00	69.41± 0.04	50.59± 0.02	63.24± 0.01	79.41± 0.04
4.	0.8	38.24± 0.05	31.97± 0.01	77.50± 0.05	65.00± 0.03	72.06± 0.03	87.50± 0.05
5.	1	41.91± 0.06	38.18± 0.02	83.38±0..05	79.41± 0.04	81.68± 0.03	93.38± 0.05

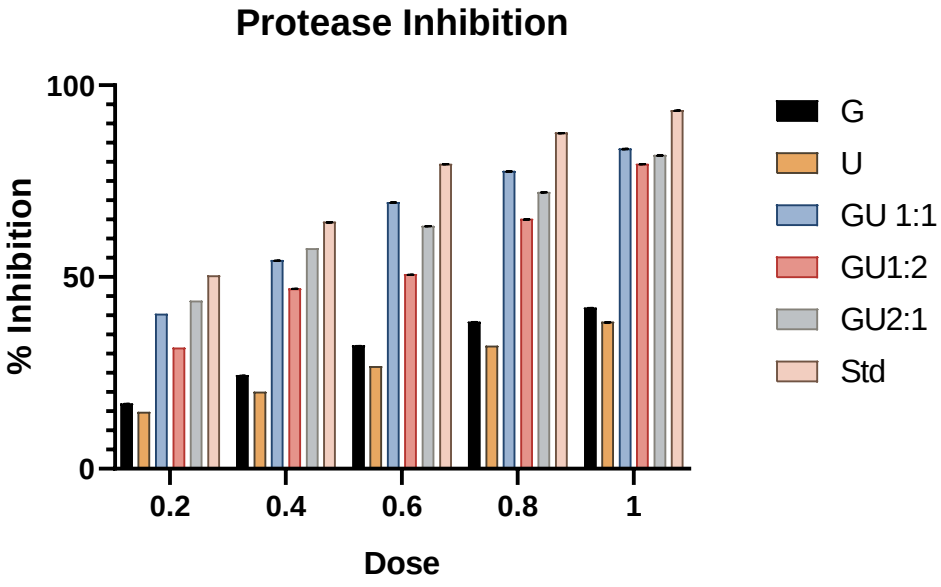

Figure 10: The protease inhibition assay evaluates the ability of different formulations to inhibit protease activity,

which is crucial in conditions involving excessive proteolysis, such as inflammation and tissue degradation. The bar graph reveals a dose-dependent increase in inhibition, indicating that higher concentrations of each formulation lead to greater protease inhibition. The standard (Std) shows the highest inhibition across all doses, establishing its efficacy as a reference compound. Specifically, significant differences were observed in comparisons between G vs. U = **, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****.**

From these results, it can be concluded that **GU 1:1 and GU 2:1 are the most potent protease inhibitors among the tested formulations**. Their performance suggests potential applications in **therapeutic interventions where protease inhibition is critical, such as in inflammatory disorders, wound healing, and tissue protection.** Further studies, including mechanistic investigations and in vivo evaluations, would be beneficial to confirm their effectiveness and explore their potential use in clinical settings.

Table 12: Antioxidant Activity Based on Protein Denaturation Inhibition Assay (Mizushima and Kobayashi, 1968)

S.No	Dose in mg/ml	Inhibition (%)					
		Gracilaria edulis	*Ulva reticulata*	GU 1:1	GU 1:2	GU 2:1	STD
1.	0.2	46.48± 0.03	32.39± 0.02	49.86± 0.01	22.68± 0.00	38.03± 0.02	56.86± 0.01
2.	0.4	56.34± 0.04	46.48± 0.03	56.76± 0.02	31.31± 0.01	47.89± 0.03	66.76± 0.02
3.	0.6	69.01± 0.05	56.34± 0.04	66.62± 0.03	43.94± 0.01	47.75± 0.04	76.62± 0.03
4.	0.8	71.83± 0.05	61.97± 0.04	72.25± 0.03	52.39± 0.02	57.75± 0.04	82.25± 0.03
5.	1	78.87± 0.06	66.20± 0.05	87.89± 0.03	60.85± 0.02	67.61± 0.04	97.89± 0.03

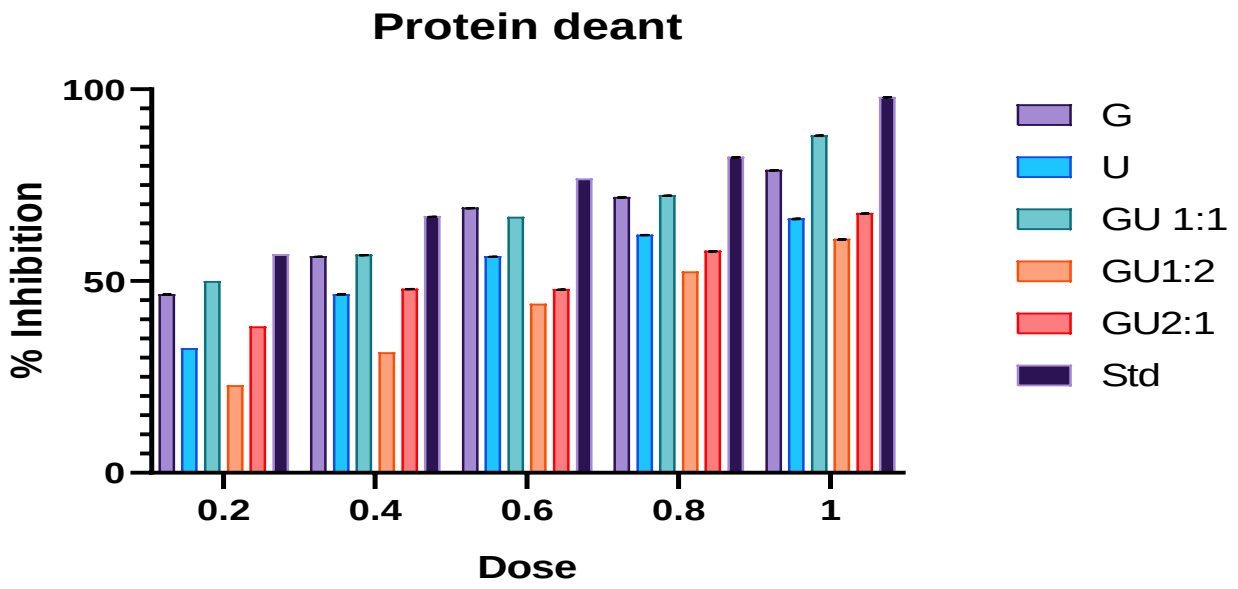

Figure 11: The bar graph illustrates the percentage inhibition of protein denaturation by different formulations (G, U, GU 1:1, GU 1:2, GU 2:1, and Std). The standard (Std) consistently exhibits the highest inhibition, establishing its strong anti-denaturation properties. Among the test formulations, GU 1:1 and G demonstrate the most potent

inhibition, closely approaching the standard at higher doses, whereas GU 2:1 and GU 1:2 show moderate effects. U exhibits the lowest inhibition, indicating weaker anti-denaturation potential. Specifically, significant differences were observed in comparisons between G vs. U = **, G vs. GU 1:1 = ****, G vs. GU 1:2 = ****, G vs. GU 2:1 = ****, G vs. Std = ****, U vs. GU 1:1 = ****, U vs. GU 1:2 = ****, U vs. GU 2:1 = ****, U vs. Std = ****, GU 1:1 vs. GU 1:2 = ****, GU 1:1 vs. GU 2:1 = ****, GU 1:1 vs. Std = ****, GU 1:2 vs. GU 2:1 = ****, GU 1:2 vs. Std = ****, and GU 2:1 vs. Std = ****. The standard is significantly more effective than all test groups ($p < 0.001$). The results indicate that GU 1:1 and G possess strong anti-inflammatory potential, warranting further in vivo investigations to validate their therapeutic relevance.**

The results suggest that GU 1:1 and G are the most effective among the tested formulations in inhibiting protein denaturation, indicating their strong anti-inflammatory potential. The statistical significance confirms that **GU 1:1 has a superior effect compared to U, GU 1:2, and GU 2:1 ($p < 0.05$), but is not significantly different from G.** Given the promising inhibition levels, further studies, including in vivo evaluations and mechanistic investigations, are necessary to validate their therapeutic relevance in inflammation-related conditions.

4. DISCUSSION

The present study systematically investigated the combined therapeutic efficacy of *Gracilaria edulis* (GE) and *Ulva reticulata* (UR) extracts in mitigating key factors associated with diabetes-associated atherosclerosis. The data generated provide clear evidence of the bioactive potential of these marine macroalgae, particularly in their antioxidant, anti-diabetic, and anti-inflammatory activities. The phytochemical analysis revealed the presence of important secondary metabolites, including alkaloids, flavonoids, phenols, terpenoids, steroids, and tannins in both algae. Notably, the total phenol content was higher in *Ulva reticulata*, whereas *Gracilaria edulis* exhibited greater flavonoid content, indicating a complementary phytochemical profile in the combination of both species.[21–23]

Heavy metal analysis confirmed that both seaweed samples were devoid of detectable levels of toxic metals such as lead, arsenic, cadmium, mercury, and chromium, reinforcing the safety of these marine resources for pharmacological applications.[20,24]

The enzymatic assays demonstrated that both *Gracilaria edulis* and *Ulva reticulata*, individually and in combination, possess significant inhibitory activity against alpha-amylase and alpha-glucosidase, two key enzymes implicated in the digestion of carbohydrates. These results are consistent with earlier reports of seaweed-derived polysaccharides and polyphenols acting as competitive inhibitors of digestive enzymes [25,26]. Among the different formulations tested, the combined extracts in a 1:1 ratio (GE:UR) displayed the highest alpha-amylase inhibition (97.33%) at 1 mg/mL concentration, followed closely by the 2:1 ratio (84.67%). Similarly, alpha-glucosidase inhibition was markedly enhanced in the combined extract formulations compared to individual extracts. These findings suggest that the synergistic interaction between bioactive compounds in the combined extracts results in greater enzyme inhibition, thereby potentially regulating postprandial hyperglycemia and delaying glucose absorption.

The non-enzymatic glycosylation of haemoglobin, a critical marker in diabetic complications, was also significantly inhibited by both individual and combined extracts. The highest inhibition (86.40%) was observed with GE:UR at 1 mg/mL, while the standard displayed promising inhibitory activity (96.40%). The reduction in glycation indicates the antioxidant potential of the seaweed extracts in preventing protein damage caused by hyperglycemia-induced oxidative stress.[25,27]

Furthermore, sucrase inhibition assays demonstrated a similar trend, with *Gracilaria edulis* exhibiting the strongest inhibitory effect, followed by the combined extract (1:1). These results align with the enzyme inhibition data, suggesting that the seaweed extracts effectively modulate carbohydrate metabolism by acting at multiple enzymatic targets.[28,29]

The glucose uptake assay using yeast cells revealed a concentration-dependent increase in glucose utilization by all extract formulations. The combination in a 1:1 ratio also demonstrated significant glucose uptake enhancement (81.68%) and G:U (2:1) combination revealed the percentage inhibition of 79.87%. The increased glucose uptake can be attributed to the presence of polyphenols and terpenoids in the extracts, which are known to stimulate glucose transport and utilization.[30,31]

In addition to their anti-diabetic potential, the seaweed extracts demonstrated noteworthy anti-inflammatory activity. The HRBC membrane stabilization assay indicated that the combined extract (1:1) offered maximum membrane protection (81.50%), comparable to *Gracilaria edulis* (59.63%). The nitric oxide scavenging activity further validated the anti-inflammatory potential of the extracts, with the 1:1 combined extract (88.88%). This indicates the ability of the extracts to attenuate oxidative stress-induced inflammatory responses, a major contributor to endothelial dysfunction and atherogenesis in diabetic patients.

Proteinase inhibitory activity and inhibition of protein denaturation assays also highlighted the therapeutic potential of the extracts. The combined extract (1:1) exhibited significant protease inhibition (83.38%) and effectively prevented protein denaturation (87.89%) at the highest concentration tested. The protective effects against proteolytic enzymes and protein denaturation suggest a broader anti-inflammatory role of these marine extracts.[32]

Taken together, the findings of this study emphasize the multifaceted bioactivity of *Gracilaria edulis* and *Ulva reticulata*. The combination of these algae exhibited synergistic effects in enzyme inhibition, glucose uptake, and anti-inflammatory assays, providing a promising natural strategy for the management of diabetes-associated atherosclerosis. The observed activities can be attributed to the rich content of polyphenols, flavonoids, alkaloids, and terpenoids in the extracts, which act through various mechanisms, including oxidative stress mitigation, inhibition of carbohydrate digestive enzymes, and membrane stabilization.[33]

The results of this in vitro study offer a scientific rationale for the use of these marine resources as functional food ingredients or nutraceuticals in metabolic disease management. However, further in vivo and clinical studies are warranted to validate these findings and to elucidate the precise molecular pathways involved. Additionally, the identification and isolation of specific bioactive compounds responsible for these therapeutic effects will be crucial for developing standardized formulations.

5. CONCLUSION

The present study underscores the therapeutic potential of marine macroalgae *Gracilaria edulis* and *Ulva reticulata* in combating diabetes-associated atherosclerosis. The phytochemical profiling revealed a rich presence of bioactive constituents such as phenols, flavonoids, alkaloids, terpenoids, and tannins, which likely contribute to the observed biological activities. Both individual and combined extracts demonstrated significant anti-diabetic potential through effective inhibition of key carbohydrate-digesting enzymes, enhancement of glucose uptake, and reduction in non-enzymatic glycosylation of haemoglobin. Additionally, the extracts exhibited remarkable anti-inflammatory properties by stabilizing cell membranes, scavenging nitric oxide radicals, inhibiting proteinase activity, and preventing protein denaturation.[34–36]

The combination of *Gracilaria edulis* and *Ulva reticulata*, particularly in the 1:1 and 2:1 ratios, displayed synergistic activity, outperforming the individual extracts in several assays. These findings suggest that the combined extracts can target multiple pathological pathways associated with diabetes and its vascular complications, thereby offering a comprehensive natural therapeutic strategy.[37]

Importantly, the absence of detectable heavy metals in the seaweed samples reinforces their safety profile and suitability for therapeutic use.[38] However, it is essential to further investigate these findings through detailed in vivo experiments and clinical trials to establish efficacy, bioavailability, and safety in human subjects. Additionally, isolation and characterization of the specific bioactive molecules responsible for these effects will contribute to the development of standardized, sustainable, and cost-effective nutraceuticals or adjunct therapies for metabolic disorders.

The study highlights the promise of marine algae as an untapped bioresource in addressing the growing burden of diabetes and its complications, supporting the integration of marine-based functional foods and phototherapeutics in modern healthcare systems.

Conflict of Interest: Nil

Author Contribution:

Devi. M- Conducted the research work

Geetha B- Checked the correctness of the paper

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