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Pharmacological Evaluation of *Najas gracillima* from the Bay of Bengal Coastal Regions: *In Vitro* and *In Silico* Studies

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Abstract

Najas gracillima is a green seaweed commonly found in North America and East Asia. This study explored its phytochemical composition and assessed its antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, along with α-amylase inhibition and cytotoxicity evaluations via the brine shrimp lethality bioassay (BSLB) and the HeLa cancer cell line. The acetone extract of *Najas gracillima* (ANG) showed a total phenolic content of 14.26 ± 1.86 mg GAE/g extract and a total flavonoid content of 10.27 ± 2.24 mg QUE/g extract. ANG demonstrated strong antioxidant activity (IC_{50} for DPPH: 14.57 ± 0.93 µg/mL; IC_{50} for ABTS: 32.17 ± 1.35 µg/mL) and moderate α-amylase inhibition (IC_{50}: 54.97 ± 3.36 µg/mL). While it showed mild cytotoxicity in the BSLB assay (LC_{50}: 673.08 µg/mL), no cytotoxic effects were observed against HeLa cells. Molecular docking analysis revealed that the identified compounds exhibited strong binding affinities to relevant drug target proteins associated with the observed biological activities. These findings suggest that *N. gracillima* may serve as a valuable source of bioactive compounds with potential applications in managing oxidative stress and diabetes.

Keywords: *Najas gracillima,* seaweed, macroalgae, marine organisms, antioxidant, antidiabetic.

1. Introduction

Seaweed, or macroscopic marine algae, is widely known for its high nutritional value as well as biologically active substances [1]. Marine algae have been used for dietary and therapeutic purposes since ancient times. Alginic acid is the oldest therapeutic component extracted from brown algae [2].

They are home to an exceptional variety of marine organisms, including seaweeds, which present them as a promising but largely untapped resource for the search for novel

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therapeutically relevant substances [3]. Seaweeds are categorized into three distinct types depending on their pigments: brown algae, red algae and green algae [4]. Green algae are characterized by their green pigmentation due to high chlorophyll content and unique biochemical compounds, including biologically active polyphenols, polysaccharides, and terpenoids, which possess antimicrobial, antioxidant, and other beneficial properties [5].
Diabetes mellitus (DM) is a disorder with various etiologies characterized by an imbalance in the metabolism of fat, protein, and carbohydrates, which impacts the action or secretion of insulin [6]. Free radicals have been linked to various kinds of ailments, including diabetes, cancer, atherosclerosis, and liver cirrhosis. Substances that can scavenge free radicals have the potential to improve various disease processes significantly. Hence, antioxidants play a vital role in protecting the human body from the harmful effects of reactive oxygen species [7]. The term "cervical cancer" indicates a tumor of a malignant nature that develops from cells that originate in the cervix uteri [8]. The compounds used in chemotherapy are ineffective for people with terminal cancer or metastases because the substances employed in it are only beneficial when administered to patients with small tumors identified at an early stage [9]. So, it is extremely crucial to explore novel anticancer agents and treatment approaches.

With over 12,000 known species, seaweeds comprise a diverse group of organisms that are found in most coastal regions worldwide. Research evidence showed that seaweed's bioactive compounds have enormous potential for use in pharmaceuticals and other biological fields [5], [10]. *Najas gracillima*, also known as slender water-nymph, is an annual, submerged, and green aquatic seaweed that originated from Asia. It is indigenous to North America and eastern Asia. Specifically, this species' current range in North America is distinct in many aspects. According to ecological categorization, this species requires cool northern environments with soft, clean, and unpolluted water. However, no scientific study has described the phytochemical composition or therapeutic properties of this marine seaweed until now. To address this gap, we have investigated the antioxidant, antidiabetic, and cytotoxic properties of the acetone extract of *Najas gracillima* (ANG) through a series of *in vitro* and *in silico* studies.

2. Materials and methods

2.1. Chemicals

All analytical-grade chemicals used in the study were supplied by the Department of Pharmacy, University of Chittagong.

2.2. Collection and extraction

N. gracillima was collected from the seashore area of Kutubdia, Cox's Bazar, Bangladesh. Mr. Mohammad Forkanul Hamid, from the Department of Fisheries at the University of Chittagong, identified the seaweed under accession number CU/DP/2023/01. The seaweed was dried and then ground into a fine powder, which was subsequently mixed with acetone and left for 14 days in a glass container. The mixture was subsequently filtered using Whatman filter paper (size 1), and the solvent was evaporated using a rotary evaporator to obtain the acetone extract. The sample was maintained at 4°C prior to further use.

2.3. Ethical approval

The departmental ethical review committee of the University of Chittagong approved the following experiment under the consent number AERB-FBSCU-20250107-(2).

2.4. Quantitative phytochemical analysis

The total phenolic content of the ANG extracts was measured using the Folin-Ciocalteu method, as outlined by Mannoubi *et al.,* [11] and expressed in milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract). The total flavonoid content was also determined using the aluminum trichloride method described by Mannoubi *et al.,* [11] and expressed in milligrams of quercetin equivalent per gram of extract (mg QUE/g extract).

2.5. Antioxidant activity

The antioxidant potential of ANG extracts was evaluated using two different methods: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity assays. These experiments were conducted according to the protocol described by Mannoubi *et al*.,[11]. In both experiments, ascorbic acid was used as a reference drug.

2.6. α-amylase inhibitory measurement

The enzyme (5 units/mL) was prepared in PBS buffer (20 mM, pH 6.7) containing 6.7 mM NaCl. Next, varying concentrations of the standard acarbose or the extract were added to the mixture, except for the blank. The mixture was incubated at 37°C for twenty minutes. Subsequently, a 5% (w/v) starch solution was incorporated, and the mixture was further incubated at 37°C for an additional 15 minutes. After incubation, the DNA reagent was added to the reaction mixture, which was then heated in a water bath at 100°C for 10 minutes [12]. The absorbance was measured at 540 nm. The formula for calculating the inhibition rate is as follows:

% inhibition = 100 − % reaction

Where % reaction is the result of (mean product in sample/mean product in control) × 100.

2.7. Cytotoxic activity

2.7.1. Brine shrimp toxicity bioassay (BSLB)

After 48 hours at room temperature (twenty to twenty-nine °C), the eggs of the brine shrimp species, *A. salina*, hatched in reconstituted seawater. Using a light source to draw them to one side of the vessel, the larvae (second instar) were gathered using a pipette. Using glass Pasteur pipettes and 250 ml glass flasks wrapped in parafilm, nauplii were separated from the parental stock cultures. Subsequently, 100 mL of test samples containing different concentrations of ANG extract (250, 500, and 1000 µg/mL) in 200 salinities were administered to 1000 individuals for each replicate and treatment group. Larvae mortality was measured after 24 hours, and the concentration needed to kill 50% (LC_{50}) of larvae was ascertained. Larvae of the positive control were cultured in 200 psu salinity.

2.7.2. Cytotoxicity test on HeLa cells

In brief, HeLa, a human cervical carcinoma cell line, was maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 1% penicillin-streptomycin (1:1), 0.2% gentamycin and 10% fetal bovine serum (FBS). Cells ($4.0*10^4$/200 µl) were seeded onto a 48-well plate and incubated at 37 °C with 5% CO_2. The next day, a 50µl sample (filtered) was added to each well. An inverted light microscope was used to evaluate cytotoxicity after a 24-hour incubation period, utilizing duplicate wells for each sample.

2.8. In silico investigation

2.8.1. Ligand preparation

Our previously reported GC-MS analysis of the ANG extract revealed fourteen small molecules [13]. For docking, these compounds were procured in 3D SDF format from the

PubChem database. If the 3D SDF format was unavailable, the 2D SDF format was downloaded and converted to 3D SDF with the Open Babel software. Before the docking simulation, all ligands were minimized and saved as pdbqt files using AutoDock Tools (version 1.5.6) [14].

2.8.2. Protein preparation

For the antioxidant and α-amylase inhibitory studies, human cytochrome P450 CYP2C9 (PDB ID: 1OG5) and human pancreatic α-amylase (PDB ID: 4W93) were selected. All protein structures were collected in PDB format from the RCSB Protein Data Bank (https://www.rcsb.org/structure). Using Discovery Studio 2020 [15], the protein structures were cleaned by deleting water molecules and other heteroatoms. Subsequently, the proteins underwent energy minimization using the steepest descent and conjugate gradient methods in Swiss-PDB Viewer (Version 4.1.0) [16]. The PDB files were then converted to pdbqt format with AutoDock Tools (version 1.5.6) and stored accordingly.

2.8.3. Molecular Docking Analysis

Molecular docking of the selected proteins with seaweed ligands was executed utilizing PyRx AutoDock Vina [17]. A semi-flexible docking approach was employed, with the proteins kept rigid while the ligands were flexible. AutoDock was used to specify parameters for the grid box, which was centered around the active site. Additionally, BIOVIA Discovery Studio Visualizer 2020 was utilized to visualize both two- and three-dimensional docking interactions.

2.8.4. Drug likeness and PASS prediction study

With the help of the SwissADME web server [18], the drug-likeness and oral bioavailability of compounds from *N. gracillima* were assessed based on Lipinski's Rule of Five (Ro5) criteria. The canonical SMILES of each compound were retrieved from the PubChem database and then input into the SwissADME server to obtain the associated parameters for each compound.

The biological activity prediction for *N. gracillima* compounds was determined using the PASS prediction tool. This tool assessed the simultaneous prediction of various biological activities using the chemical structure. It also calculated the estimated activity spectrum of compounds using probabilities of activity (Pa) and inactivity (Pi). Both probabilities, Pa and Pi, range from 0 to 1. However, values are presented as percentages of probability (%) [19].

2.9. Statistical Analysis

The data were presented as mean ± standard error of the mean (SEM). All statistical analyses were executed utilizing GraphPad Prism software (version 5.2).

3. Result

3.1. Total phenolic and flavonoid content

In this study, the TPC and TFC in the ANG extract were found to be 14.26 ± 1.86 mg GAE/g extract and 10.27 ± 2.24 mg QUE/g extract, respectively (Table 1).

Table 1: Total phenolic content, total flavonoid content, antioxidant and antidiabetic activity of the acetone extract of *Najas gracillima*

Extract/Standard	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QUE/g extract)	Antioxidant activity		Antidiabetic activity
			IC_{50}-DPPH (µg/ml)	IC_{50}-ABTS (µg/ml)	IC_{50}-α-amylase (µg/ml)
Acetone extract of *Najas gracillima*	14.26 ± 1.86	10.27 ± 2.24	14.57 ± 0.93	32.17 ± 1.35	54.97 ± 3.36
Ascorbic acid	-	-	13.72 ± 0.72	25.93 ± 1.76	-
Acarbose	-	-	-	-	45.75 ± 2.57

The data is represented as mean ± SEM. µg/ml = Microgram/milliliter

3.2. Antioxidant activity

With IC_{50} values of 14.57 ± 0.93 µg/mL for DPPH and 32.17 ± 1.35 µg/mL for ABTS, the ANG extract showed strong antioxidant activity. Meanwhile, the IC_{50} values for ascorbic acid, the standard reference, were 13.72 ± 0.72 µg/mL and 25.93 ± 1.76 µg/mL for DPPH and ABTS, respectively (Table 1). This indicates that the ANG extract has a free radical inhibition effect close to that of the reference standard. Figure 1 (A, B) illustrates the percentage of free radical inhibition by both ANG extracts and the standard ascorbic acid in DPPH and ABTS assays.

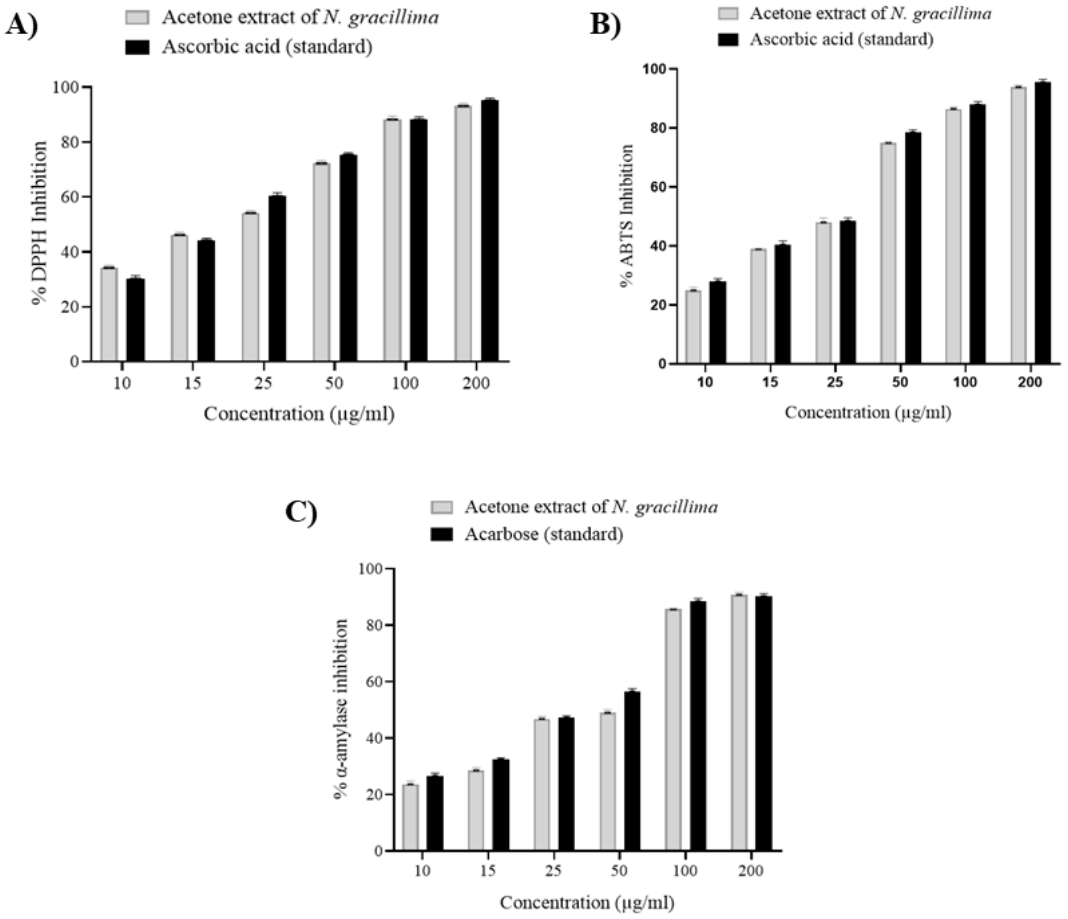

Figure 1: Percent inhibition graph of (A) DPPH, (B) ABTS, and (C) α-amylase by the acetone extract of *Najas gracillima* seaweed. All experiments were performed in triplicate, and data are expressed as mean ± SD.

α-amylase inhibitory activity

The ANG extract and acarbose standard both showed concentration-dependent inhibition of α-amylase, with IC_{50} values of 54.97 ± 3.36 µg/mL and 45.75 ± 1.52 µg/mL, respectively (Table 1 and Figure 1C). Given its IC_{50} value, the ANG extract demonstrated inhibitory activity comparable to that of the standard, indicating its strong α-amylase inhibitory effect.

Cytotoxic activity

Brine shrimp toxicity assay

There was a notable correlation (R^2 = 0.7942) observed between the mortality rate of *A. salina* larvae and the ANG extract concentrations, implying that the brine shrimp mortality rose with higher extract concentrations (Figure 2). The ANG extract exhibited mild to moderate toxicity by Clarkson's toxicity index [20] against *A. salina* larvae with an LC_{50} value of 673.08 µg/ml (24 h). However, the LC_{50} of the Vincristine sulphate as a standard was 45.77 (19.75–106.06) µg/ml against the brine shrimp.

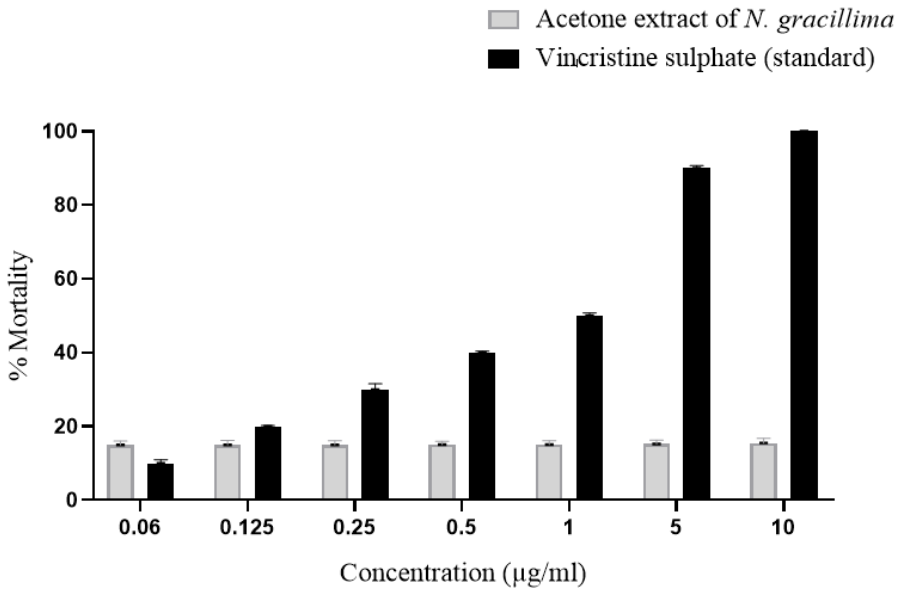

Figure 2: Lethality assay of the acetone extract of *Najas gracillima* seaweed against brine shrimp. All experiments were performed in triplicate, and data are expressed as mean ± SD.

Cytotoxic activity on HeLa cells

We observed that cell survival without the solvent was 100%, while survival with the solvent exceeded 95%. Similarly, cells treated with ANG extract also had a survival rate of over 95%, indicating that our extract exhibited no cytotoxic effects (Figure 3).

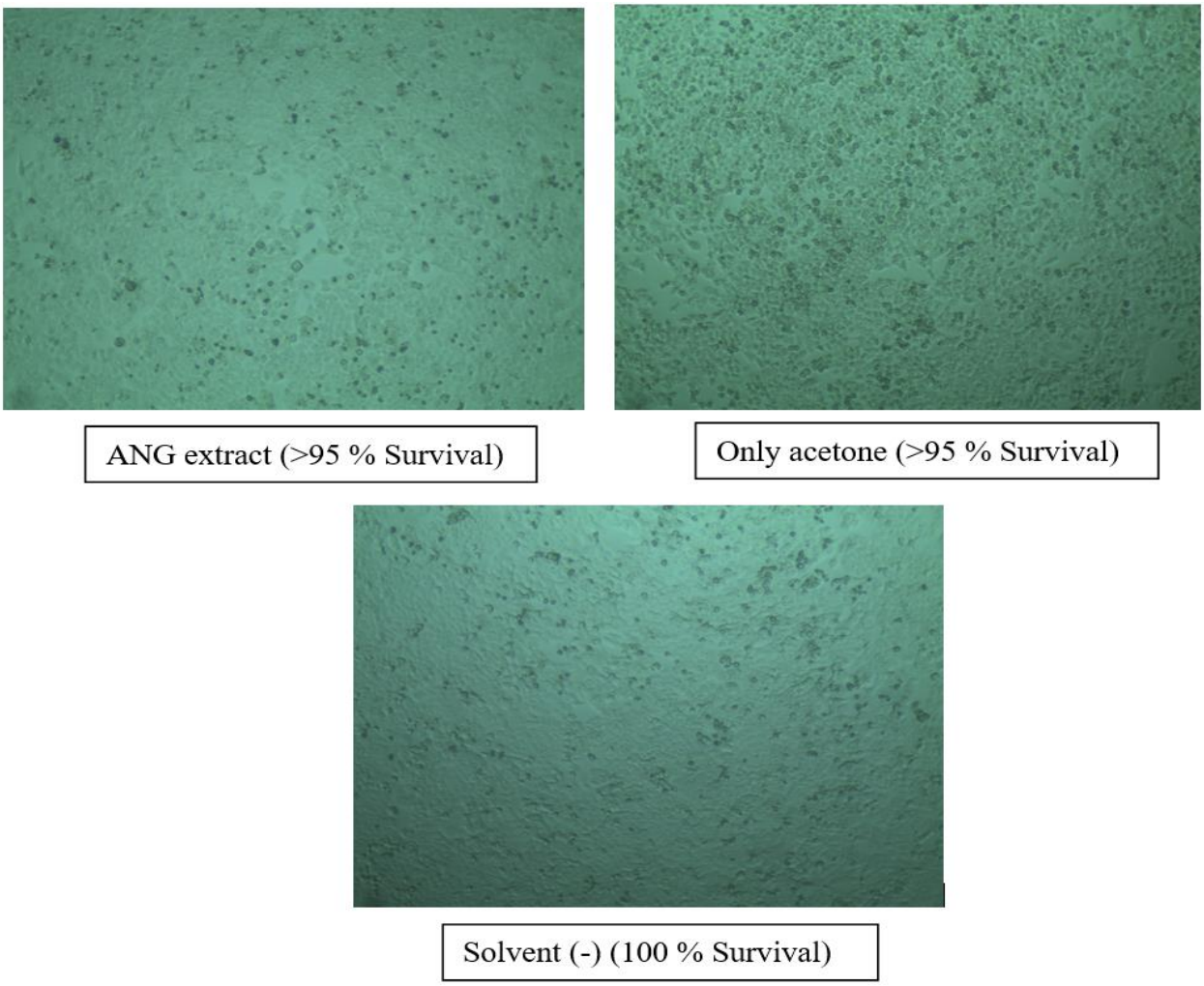

Figure 3: Cytotoxic activities of the acetone extract of *Najas gracillima* seaweed against the HeLa cancer cell line.

Molecular docking

Docking study for antioxidant activity

Human cytochrome P450 CYP2C9 (PDB ID: 1OG5) protein was used in this study to perform the antioxidant docking analysis. The compounds identified from the ANG extract showed binding affinity scores against this protein varying from -9.4 to -5.1 kcal/mol. The compound Gamma-sitosterol showed the highest binding affinity, with a score of -9.4 kcal/mol, which is greater than the standard ascorbic acid (-5.3 kcal/mol). It formed a total of eight hydrophobic interactions with six amino acids, namely- Ala-103, Ala-106, Val-113, Phe-114, Val-237, and Leu-366 (Figure 4A). Gamma-sitosterol (-9.4 kcal/mol), 3-oxo-5α-cholan-24-oic Acid (-9.2 kcal/mol), stigmasterol (-9 kcal/mol), 17-methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one (-8.8 kcal/mol), and Phytol (-6.3 kcal/mol) are the top five compounds based on the docking score (Table 2).

Table 2: Docking scores of the top docked compounds identified from the seaweed, *Najas gracillima,* with the human cytochrome P450 CYP2C9 (PDB ID: 1OG5).

Compound name	**Binding affinity (kcal/mol)**	**Hydrogen Bond Interactions**				**Hydrophobic Bond Interactions**			
		Conventional Hydrogen Bond		**Carbon Hydrogen Bond**		**Alkyl**		**Pi-Alkyl**	
		Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)
Gamma-sitosterol	-9.4					Ala-103	4.78688	Phe-114	4.83103
						Ala-103	4.54679	Phe-114	5.47385
						Ala-106	4.10618	Phe-114	5.11474
						Val-113	5.467		
						Leu-366	5.34872		
						Val-237	4.25857		
3-Oxo-5alpha-cholan-24-oic Acid	-9.2	Ile-205	2.49981	Ile-205	2.69809	Ala-103	3.7184	Phe-114	4.58724
						Leu-366	4.60923		
						Leu-366	4.26712		
						Pro-367	4.7032		
						Ala-477	4.06926		
						Leu-362	5.46432		
Stigmasterol	-9	Asn-217	2.27357			Ala-103	5.22432		
						Ala-103	4.9514		
						Ala-103	3.71394		
						Leu-208	5.25171		
						Leu-208	4.02438		
						Leu-208	4.39118		
						Ala-297	3.93467		
						Leu-102	5.37869		
						Val-113	3.5207		
						Val-237	4.41922		
						Met-240	5.39263		
						Val-292	4.09716		
17-Methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one	-8.8					Ala-103	4.32021	Phe-100	4.55806
						Val-113	5.36238	Phe-114	4.76909
						Val-113	3.54327	Phe-114	5.48274
						Leu-208	5.00023	Phe-114	5.42665
						Leu-208	5.12667	Ala-103	4.00755
						Ala-297	4.38799	Leu-366	5.23024
						Pro-367	4.41052	Pro-367	5.42068
Ascorbic acid	-5.3	Arg-97	2.24589	Phe-428	2.50641				
		Leu-366	3.03481	Ser-429	2.70241				
				Arg-433	2.47221				

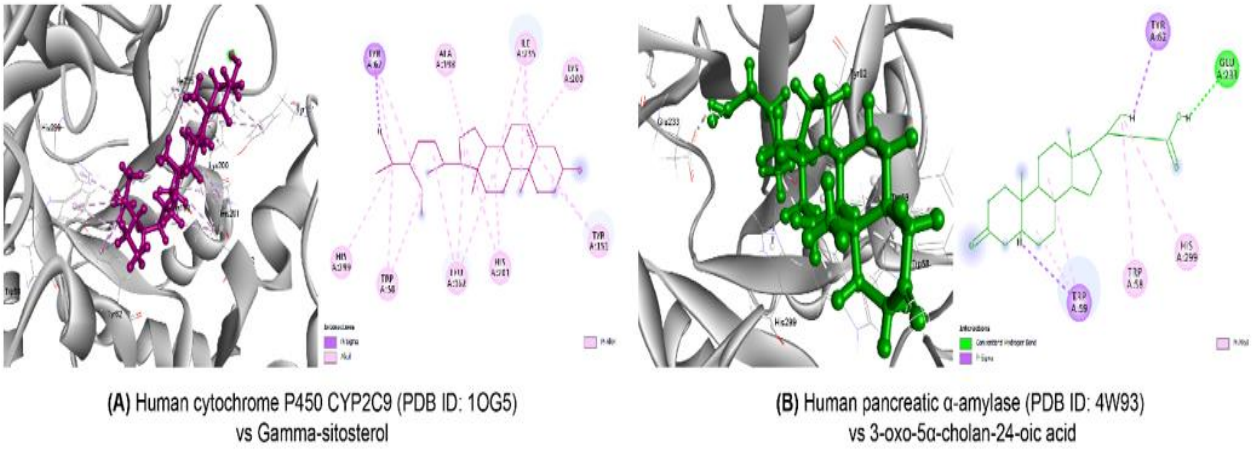

Figure 4: Molecular docking interactions of the top docked compound with each selected drug target protein

Docking study for antidiabetic activity

In this study, human pancreatic α-amylase (PDB ID: 4W93) was utilized to assess the α-amylase inhibitory potential of compounds identified from *N. gracillima* seaweed. The docking scores of these compounds ranged from -9.4 to -4.9 kcal/mol, indicating their binding affinity to α-amylase. Among these compounds, four showed higher binding affinities compared to the standard inhibitor acarbose (-7.1 kcal/mol): 3-oxo-5α-cholan-24-oic acid (-9.4 kcal/mol), gamma-sitosterol (-9.3 kcal/mol), 17-methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one (-9 kcal/mol), and stigmasterol (-8.9 kcal/mol) (Table 3). The top-ranked compound, 3-oxo-5α-cholan-24-oic acid (-9.4 kcal/mol), formed one conventional hydrogen bond with Glu-233 and established seven hydrophobic interactions with four amino acids: Tyr-62, Trp-58, Trp-59, and His-299 (Figure 4B)

Table 3: Docking scores of the top docked compounds identified from the seaweed, *Najas gracillima,* with the human pancreatic α-amylase (PDB ID: 4W93).

Compound name	Binding affinity (kcal/mol)	Hydrogen Bond Interactions				Hydrophobic Bond Interactions							
		Conventional Hydrogen Bond		Carbon Hydrogen Bond		Alkyl		Pi-Alkyl		Pi-Sigma		Pi-Pi stacked	
		Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)	Amino Acid Residue	Distance (Å)
3-Oxo-5alpha-cholan-24-oic Acid	-9.4	Glu-233	2.16059					Trp-58	5.31477	Trp-59	2.52957		
								Trp-59	4.39254	Tyr-62	2.72156		
								Trp-59	4.13972				
								Trp-59	5.28604				
								His-299	5.14247				
Gamma-sitosterol	-9.3					Leu-162	5.08678	Trp-58	5.15662	Tyr-62	2.6287		
						Leu-162	4.85158	Trp-58	4.76602				
						Leu-162	5.44245	Tyr-62	4.38363				
						Ala-198	4.08015	Tyr-62	4.56921				
						Lys-200	4.94858	Tyr-151	5.49045				
						Ile-235	5.4974	Tyr-151	4.32443				
						Ile-235	3.95264	His-201	5.21108				
						Ile-235	3.9773	His-201	5.10186				
								His-299	4.7535				
17-Methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one	-9			Asp-356	2.96274	Leu-165	4.78498	Trp-58	5.39237			Trp-59	3.71754
								Trp-59	4.30946			Trp-59	4.75304
								Trp-59	4.51045				
								Trp-59	4.72108				
								Trp-59	5.36831				
								Tyr-62	4.43148				
Stigmasterol	-8.9	Ile-235	2.37913	Ile-235	3.00151	Leu-162	4.80427	Trp-59	4.36255				
						Leu-162	5.23187	Trp-59	4.96819				
						Lys-200	5.03817	Trp-59	4.97828				
						Ile-235	4.44073	Trp-59	4.92243				
						Ile-235	4.00401	Tyr-62	3.99432				
								Tyr-151	5.46009				
								Tyr-151	5.28679				
								His-201	4.44604				
Acarbose	-7.1	Trp-59	2.21531	His-101	2.37053								
		Asp-356	2.13828	Trp-357	2.92123								
		Asp-197	2.37506	Glu-233	2.26821								
		His-201	2.68547	Glu-233	2.7871								
				Asp-356	2.83246								
				Asp-197	2.92187								
				Asp-300	2.53447								

Drug likeness and PASS prediction studies

Lipinski's rule of five was employed to assess the drug-likeness properties of compounds identified from *N. gracillima* seaweed. Ro5 is a theoretical as well as computational procedure for estimating the drug-likeness potential of small molecules and determining

whether a compound with specific therapeutic effects has characteristics that indicate it is orally bioavailable. The Ro5 suggests that poor oral bioavailability can occur when: (i) Log P > 5, (ii) hydrogen bond donors > 5, (iii) hydrogen bond acceptors > 10, as well as (iv) the molecular weight > 500 [21]. Based on our investigation, all compounds conform to the Lipinski rule of five and exhibit drug-like characteristics (Table 4).

Table 4: Lipinski rule of five parameters of compounds from *N. gracillima* seaweed

Compound Name	Molecular weight	Hydrogen bond donor	Hydrogen bond acceptor	Log P	Lipinski's rule of five
Hentriacontane	436.8	0	0	11.9	Yes
Hexadecanoic acid, 2-hydroxy-1-	272.42	2	3	4.34	Yes
Gamma-sitosterol	414.7	1	1	7.19	Yes
Cyclohexanamine, N-3-butenyl-N-methyl-	167.29	0	1	2.84	Yes
Cyclopentane, 2-(1-hydroxy-2-propyl)-1,3-dimethyl-	156.26	1	1	2.47	Yes
Stigmasterol	412.7	1	1	6.97	Yes
(Z)-9-Octadecenamide	281.5	1	1	5.32	Yes
3-Oxo-5α-cholan-24-oic Acid	374.6	1	3	4.85	Yes
11,14-Eicosadienoic acid, methyl ester	322.5	0	2	6.44	Yes
11,14-Octadecadienoic acid, methyl ester	294.5	0	2	5.7	Yes
10,13-Dimethyltetradecanoic acid	256.42	1	2	4.95	Yes
n-Propyl 11-eicosenoate	352.6	0	2	7.41	Yes
Phytol	296.5	1	1	6.22	Yes
17-Methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one	308.4	0	2	4.19	Yes

Using the PASS online tool, compounds from *N. gracillima* seaweed were assessed for antioxidant, antidiabetic, and antibacterial properties, and the potent compounds demonstrated a Pa value that exceeded the Pi value (Table 5).

Table 5: PASS prediction value of compounds from *N. gracillima* seaweed for antioxidant and antidiabetic activities

Compound Name	Antioxidant activity		Antidiabetic activity	
	Pa	Pi	Pa	Pi
Hentriacontane	0.170	0.079	0.228	0.010
Hexadecanoic acid, 2-hydroxy-1-	0.230	0.042	0.521	0.020
Gamma-sitosterol	0.178	0.072	-	-
Cyclohexanamine, N-3-butenyl-N-methyl-	-	-	-	-
Cyclopentane, 2-(1-hydroxy-2-propyl)-1,3-dimethyl-	0.141	0.114	0.137	0.076
Stigmasterol	0.215	0.048	-	-
(Z)-9-Octadecenamide	0.167	0.082	0.215	0.043
3-Oxo-5α-cholan-24-oic Acid	0.158	0.093	0.126	0.105
11,14-Eicosadienoic acid, methyl ester	0.296	0.024	0.400	0.013
11,14-Octadecadienoic acid, methyl ester	0.296	0.024	0.400	0.013
10,13-Dimethyltetradecanoic acid	0.371	0.015	0.353	0.019
n-Propyl 11-eicosenoate	0.269	0.030	0.342	0.022
Phytol	0.475	0.008	-	-
17-Methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one	0.226	0.044	0.193	0.137

4. Discussion

Seaweeds are among the most valuable marine plant resources currently utilized due to their well-documented health benefits. They are considered valuable sources of structurally distinct bioactive chemical substances, offering the potential to discover new functional foods or therapeutic agents [22]. This study aimed to investigate the phytochemical constituents and evaluate the pharmacological activities, specifically the antioxidant, antidiabetic, and cytotoxic effects, of the acetone extract of *Najas gracillima*.

Phenolic compounds serve as vital secondary metabolic products in plant metabolism that promote growth, stress resistance, antioxidant activity, and pigment development. Plant extracts contain phenolic and flavonoid chemical substances, which exert antibacterial, free radical-scavenging, and antioxidant activities that improve human health [23]. Due to these significant biological activities, determining the total phenolic and flavonoid content is essential [12]. Our result revealed that a significant amount of TPC (14.26 ± 1.86 mg GAE/g extract) and TFC (10.27 ± 2.24 mg QUE/g extract) were found in the ANG extract.

The presence of phenolic and flavonoid compounds in most plant samples is attributed to their antioxidant properties. The amount, location, planar structure, and presence of C2-C3 double bonds, among other hydroxyl substituents, impact the antioxidant properties of flavonoids for metal chelation, scavenging of free radicals, and inhibition of enzymes that produce free radicals [12]. DPPH is widely used to assess the scavenging effects of antioxidant standards and test samples, as it is one of the stable and nitrogen-centered free radicals that decrease in the presence of free radical scavengers. When hydrogen is accepted from a suitable donor, it causes a change in the DPPH solution, changing from its usual dark purple hue to a yellow diphenylpicryl hydrazine. Again, the ABTS has a stable radical cation in its free state. When an antioxidant is added to a solution containing this radical, the radical is reduced, and this reduction is dependent on the test compound's antioxidant activity [24]. Our results demonstrated that the ANG extract exhibited significant inhibition of DPPH and ABTS, with IC_{50} values of 14.57 ± 0.93 and 32.17 ± 1.35 µg/mL, respectively. Moreover, the reference standard ascorbic acid IC_{50} values against DPPH and ABTS were 13.72 ± 0.72 µg/mL and 25.93 ± 1.76 µg/mL, respectively. The ANG extract showed a more or less similar IC_{50} value compared to the reference standard ascorbic acid, indicating its potent antioxidant activity. Moreover, Cytochrome P450 is a diverse family of enzymes responsible for catalyzing oxidation-reduction reactions. CYP2C9 accounts for approximately 20% of all CYP450 enzymes in the liver and also plays an important part in metabolizing around 15% of drugs and xenobiotics. Additionally, the overexpression of CYP2C9 can trigger oxidative stress, potentially contributing to the progression of various diseases [25]. In a molecular docking investigation, the compounds identified from the ANG extract exhibited binding affinity scores against this protein ranging from -9.4 to -5.1 kcal/mol, with gamma-sitosterol displaying the highest affinity (-9.4 kcal/mol), significantly surpassing the standard ascorbic acid (-5.3 kcal/mol). Thus, this *in silico* investigation reinforces the antioxidant properties of ANG extract demonstrated *in vitro*.

Among various treatment approaches to control hyperglycemia, the widely accepted method is fundamental enzyme inhibition. Alterations in the enzymes responsible for carbohydrate metabolism, specifically α-amylase, lead to elevated blood glucose levels in DM patients. At neutral to moderately acidic pH, α-amylase breaks down polysaccharides into glucose for optimum absorption in the gut. Regulating α-amylase levels can lower hyperglycemia and prevent consequences, including diabetic nephropathy, retinopathy, and neuropathy [26]. In this study, we have investigated the α-amylase inhibitory potential of ANG extract. Acarbose was utilized as the standard drug, an oligosaccharide that can postpone the absorption of carbohydrates and the release of glucose from starch [27]. Both the extract (IC_{50}: 54.97 ± 3.36 µg/mL) and standard acarbose (IC_{50}: 45.75 ± 2.57 µg/mL) showed concentration-

dependent α-amylase inhibitory activity. In a molecular docking study, compounds from *N. gracillima* showed binding affinity scores against pancreatic α-amylase ranging from -9.4 to -4.9 kcal/mol, with 3-oxo-5α-cholan-24-oic acid exhibiting the strongest affinity (-9.4 kcal/mol), notably exceeding the standard acarbose (-7.1 kcal/mol). This computational analysis further validates the α-amylase inhibitory potential of the ANG extract as observed *in vitro*.

BSLB is a very useful technique to determine cytotoxic activities by estimating the median lethal concentration (LC_{50}), which has been observed for several toxins and extracts. Using BSLB, many naturally extracted products with LC50 values less than 1000 µg/mL have been identified as containing physiologically active compounds [28]. Our extract showed an LC_{50} value of 673.08 µg/ml (24 h), indicating moderate cytotoxicity. However, ANG extract did not exhibit noticeable cytotoxicity against the HeLa cell line.

A key aspect of discovering new drugs is the use of computer-aided drug design (CADD) techniques [29]. Among these, molecular docking is commonly used to predict ligand-target interactions and to elucidate the therapeutic actions of bioactive compounds. To validate our experimental results, we employed molecular docking to predict the mode of action of the compounds identified in the ANG extract against key drug target proteins linked to oxidative stress, diabetes, and bacterial infections. Our investigations also revealed that all compounds identified from *N. gracillima* adhere to Lipinski's rules, suggesting their potential as effective drug candidates and indicating their safety for oral use. Additionally, the PASS prediction tool was utilized to assess varying levels of bioactivity and to clarify the multiple effects of these compounds. According to this tool, most compounds exhibited a higher Pa (predictive activity) than Pi (predictive inactivity) for antioxidant, antidiabetic, and antibacterial activities, highlighting their potential therapeutic value. In summary, the findings from both laboratory and computational analyses underscore the acetone extract of *N. gracillima* as a promising source of antioxidant and antidiabetic properties.

5. Concluding remarks and future perspective

The findings of this research investigation suggest that the acetone extract of *N. gracillima* seaweed can be a beneficial source of natural antioxidants and a promising candidate for moderate antidiabetic therapy. In addition, molecular docking simulations revealed that several bioactive potential compounds had a high binding affinity with particular proteins and, according to the Lipinski rule of five, demonstrated drug-like characteristics. Furthermore, the experimental results have been consistent with PASS predictions for seaweed components. In summary, research on *N. gracillima*'s potential for medicinal use has yielded encouraging results; however, several uncertainties should be considered for further studies. The results of this study showed that the *N. gracillima* acetone extract exhibits low cytotoxicity, strong antioxidant properties, and moderate antidiabetic properties. These conclusions, however, are based only on *in vitro* tests, which ignore the intricate physiological and metabolic processes that take place *in vivo*. The wider understanding of its therapeutic potential is limited by the limited cytotoxicity testing and the use of a single enzyme model for antidiabetic evaluation. Furthermore, it is challenging to assign the observed effects to specific constituents because the study used a crude extract without isolating particular bioactive compounds. Future research should focus on mechanistic cellular studies, broader enzyme inhibition assays, the isolation and characterisation of active compounds, and *in vivo* validation. Supporting *N. gracillima*'s therapeutic potential will also require assessing toxicity across several cell lines and creating efficient delivery methods.

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