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ORIGINAL ARTICLE

Volatile and Phenolic Compounds in Freshwater Diatom *Nitzschia palea* as a Potential Oxidative Damage Protective and Anti-Inflammatory Source

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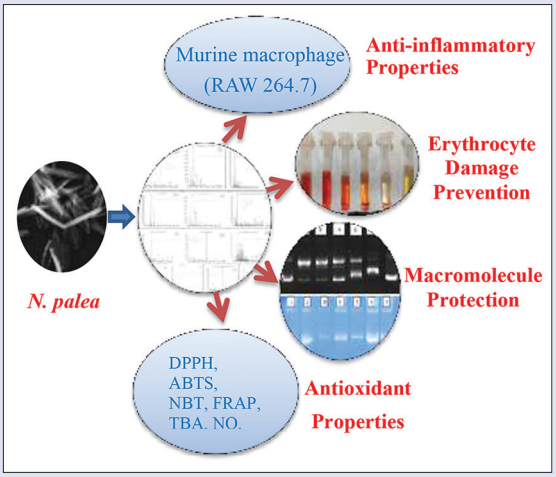
ABSTRACT

Background: *Nitzschia palea* is a freshwater diatom species (*Bacillariophyceae*), is easy to cultivate, and is a primary producer of organic matters in aquatic environments. **Materials and Methods:** In this study, proximate composition in axenic culture of *N. palea* was determined, phenolic content and volatile compounds were determined for methanol and ethyl acetate extracts. Both the extracts were evaluated for eight different *in vitro* antioxidant and free radical scavenging activities. Macromolecular damage prevention properties were determined by electrophoretic methods, and antihemolytic activity was validated by lactate dehydrogenase assay, atomic force microscope, and scanning electron microscope image analysis. **Results:** Both the extracts showed antioxidant, macromolecular damage prevention, and antihemolytic properties. Among the two extracts, ethyl acetate extract showed high activity compared to methanol extract. Based on this result, ethyl acetate extract was evaluated for *in vitro* anti-inflammatory properties using RAW 264.7 cells. The extract showed IC_{50} value of 50.73 µg/mL and inhibition of inflammatory cytokines such as nitric oxide, tumor necrosis factor-alpha, and prostaglandin E_2. The observed activity was correlated with identified important metabolites such as butyl isobutyl phthalate, pristane, and squalene of methanol extracts. Similar co-relation was observed for 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione, methyl palmitate, hentriacontane, and dibutyl phthalate were of ethyl acetate extract. **Conclusion:** The study concludes that *N. palea* has potentiality to isolate pharmacologically active metabolites using advanced chromatographic techniques, which can be useful in combating oxidative stress-related inflammatory diseases.

Key words: Antihemolytic, anti-inflammatory, antioxidant, diatom, metabolites, *Nitzschia palea*

SUMMARY

- Butyl isobutyl phthalate, pristane, and squalene were identified in methanolic extract, and hentriacontane, methyl palmitate, dibutyl phthalate, and several other compounds were identified in ethyl acetate extract of *Nitzschia palea*
- Proximate composition, elemental analysis by energy-dispersive X-ray spectroscopy, and phenolic profile revealed the presence of non-toxic elements
- Methanol and ethyl acetate extracts showed *in vitro* antioxidant, macromolecular, and erythrocyte damage protection
- Ethyl acetate extract showed potent *in vitro* anti-inflammatory properties.



Abbreviations used: GC-MS: Gas chromatography-mass spectrometry; LDH: Lactate dehydrogenase; AFM: Atomic force microscope; SEM: Scanning electron microscope; EDX: Energy-dispersive X-ray spectroscopy; NIST: National Institute of Standards and Technology; DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; FRAP: Ferric-reducing antioxidant power; NBT: Nitroblue tetrazolium; TBA: Thiobarbituric acid; AAPH: 2,2-azobis (2, amidinopropane) dihydrochloride; DNA: Deoxyribonucleic acid; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DMSO: Dimethyl sulfoxide; LPS: lipo-polysaccharide; EDTA: Ethylenediaminetetraacetic acid; BHT: Butylated hydroxytoluene; BSA: Bovine serum albumin; TNF-α: Tumor necrosis factor-alpha; PGE_2: Prostaglandin E_2; IC_{50}: 50% inhibition Concentration; DMEM: Dulbecco's Modified Eagle Medium.

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INTRODUCTION

Reactive oxygen species react with a large variety of cellular components, which leads to oxidative damage on lipids, proteins, and nucleic acids, which in turn triggers various chronic diseases and aging. Synthetic antioxidants are in practice to reduce oxidative damages in the human body. However, these were found to be liver damaging and carcinogenic in animal models.[1] Thus, it is important to replace these synthetic

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antioxidants with safe and natural antioxidants. When it comes to natural antioxidants, polyphenols have been reported in the human food which contribute to antioxidant activity with less side effects and exerting a positive health effect.[2] Most of the available natural antioxidants and health-promoting and disease-suppressing metabolites are derived from terrestrial plants and algal biomass, with microalgae serving as a reliable source.[3] The desired synthesis of antioxidants in microalgal cells can be controlled and easy purification methods can be developed. In addition to these advantages, they are free from herbicides and pesticides or any other toxic substances.[4]

Diatoms belonging to the class of *Bacillariophyceae* are micro algae constituting one of the most diverse groups of micro-organisms, which include conspicuous number of golden brown unicellular eukaryotic organisms having siliceous cell wall. Complete genome sequences were reported for *Thalassiosira pseudonana* (Centrales) and *Phaeodactylum tricornutum* (Pennales),[5,6] and it has proven that they have genes from the plant, bacteria, and animal kingdom, and this resulted in a unique metabolism to produce valuable metabolites.

In recent years, there is more focus on secondary metabolites of diatoms. Kuczynska *et al*.[7] reviewed the current knowledge on diatom metabolites which include β-carotene, xanthophyll, diatoxanthin, diadinoxanthin, violaxanthin, antheraxanthin, and zeaxanthin complemented by some new insights regarding biological role and biosynthetic pathways. In addition to this, literature reveals leukemia cell death-inducing substances from the genera *Melosira, Amphora, Phaeodactylum,* and *Nitzschia*.[8] Because of this, the researcher's attention lies in the use of diatom species as a potential biomass because it can be easily cultivated in controlled conditions, handled as conventional laboratory micro-organisms, and genetically modified without any risk of dissemination. The present study describes the determination of proximate composition of *N. palea,* a *Bacillariophyceae,* and identification of its bioactive metabolites by gas chromatography-mass spectrometry (GC-MS). The study extended to evaluate antioxidant, macromolecular damage prevention, antihemolytic, and anti-inflammatory properties of methanol and ethyl acetate extracts.

MATERIALS AND METHODS

Chemicals – 2,2-azobis (2, amidinopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), nitroblue tetrazolium (NBT), bovine serum albumin (BSA), and butylated hydroxyanisole (BHA) were purchased from Sigma, Bangalore, Karnataka, India. Lactate dehydrogenase (LDH) estimation kit (11407002) was procured from Agappe, Bangalore, Karnataka, India. Tumor necrosis factor-alpha (TNF-α) (BMS607-3TWO), prostaglandin E_2 (PGE_2) (KHL1701) assay kits, and pRSET-A plasmid DNA were purchased from Invitrogen, Thermo Fisher Scientific, Bangalore, Karnataka, India. Murine macrophage cells (RAW 264.7) were procured from American Type Culture Collection, Chromachemie Laboratory Pvt. Ltd. Bangalore, Karnataka, India. All other chemicals used were of analytical grade procured from SRL, Bangalore, Karnataka, India.

Freshwater diatom species *N. palea* was isolated from blooming at Balmuri falls, river Cauvery, Karnataka, India, and cultured in newly designed photo bioreactor with optimized culture conditions (unpublished data). Biomass was obtained by mass culturing in 2000-mL Erlenmeyer flask containing 1000 mL of f/2 medium, at 23°C ± 2°C, 8:16 h light/dark cycle for 10 days. At the end of culture duration, the biomass was scraped to collect surface-attached cells, centrifuged at 5000 rpm, 4°C for 10 min, and pellets were washed thrice with 0.5-M ammonium formate to remove impurities and transferred in a rotary evaporator. Dried biomass was powdered in a pestle and mortar and stored at −20°C. Dried powder (20 mg) was extracted thrice with 30-mL ethyl acetate for 4 h at room temperature, the content was centrifuged at 5000 rpm for 10 min at 4°C, and the supernatant was recovered. The biomass (20 mg) was subsequently extracted twice with methanol (30 mL) for 4 h at room temperature; the supernatant was pooled and used for the determination of bioactivities.

Proximate compositions

Proximate chemical composition for axenic culture of *N. palea* was determined according to AOAC method,[9] and the elemental analysis was performed by energy-dispersive X-ray spectroscopy (EDX).

Total phenol and flavonoid contents

The total phenolic contents of both the extracts were determined by Folin–Ciocalteu method in comparison with standard gallic acid equivalent (GAE), and the flavonoid content was determined in comparison with standard catechin equivalent (CE).[2]

Gas chromatography-mass spectrometry analysis of ethyl acetate and methanol extracts

The bioactive compounds present in ethyl acetate and methanol extract of *N. palea* were determined by GC-MS analysis (Agilent GC-MS, 7890) with HP-5 column Rtx-5 ms (30 m × 0.25 mm × 0.25 μm). The carrier gas used was helium, and the flow rate of the carrier gas was 0.8 mL/min. Injection volume used was 1 μL, and sample concentration was maintained as 1 mg/mL. Each peak in the chromatogram was identified based on the retention index and also by comparing the fragmentation pattern of the compounds with the mass spectra in the National Institute of Standards and Technology (NIST) database. Chemical structures of the compounds were drawn using Chemdraw Ultra-8.0 software.

In vitro antioxidant and free radical scavenging activities

The radical scavenging activity of the extracts was studied using DPPH free radical assay, ferrous ion chelating, nitric oxide scavenging activity, ABTS radical scavenging activity, and ferric-reducing antioxidant power (FRAP).[2] Deoxyribose assay was carried out according to the method proposed by Halliwell *et al*.[10] Superoxide radical scavenging activity of the extracts (NBT method) was determined.[11]

Protective effect on the oxidation of biomolecules *in vitro*

Antilipid peroxidation activity (thiobarbituric acid [TBA] method) was carried out as described by Kumar *et al*.[12] AAPH-induced plasmid DNA strand break assay and protein oxidation protection assay by electrophoresis methods were carried out as described in our previous study.[13]

Hemolysis assay

AAPH-induced erythrocyte damage prevention efficacy of extracts was analyzed and expressed in IC_{50} values, and this was further validated by LDH assay.[14] Atomic force microscope (AFM) (Park NX 10, Italy) and scanning electron microscope (SEM) (ZEISS, Bangalore, Karnataka, India) image analyses were carried out to validate the result as per our previous study.[13] The morphological surface and dimensions of erythrocytes in terms of roughness, thickness, and waviness were analyzed using NOVA 1.1.0.1921 software.

In vitro anti-inflammatory properties

Cell viability using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

RAW 264.7 cells were cultured in DMEM medium supplemented with 10% inactivated fetal bovine serum, penicillin (100 IU/mL), and

streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with cell-dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in phosphate-buffered saline [PBS]). The above cultured cells (1×10^5) were seeded in a 96-well plate and incubated for 24 h at 37°C, in 5% CO_2 incubator. After 24 h, the monolayer was washed with medium, and 100 µL of different test concentrations of test drugs was added to the partial monolayer in 96-well plates. The plates were incubated at 37°C for 24 h in 5% CO_2 atmosphere. After incubation, the test solutions in the wells were discarded, and 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed, 100 µL of dimethyl sulfoxide was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured at 590 nm using a microplate reader Thermo Scientific™ Varioskan™ Flash Multimode Reader, Bangalore, Karnataka, India. The percentage growth inhibition was calculated, and results were expressed as IC_{50} values from the dose–response curves.[15]

Determination of nitric oxide production

RAW 264.7 cells (1×10^5 cells/mL) were seeded to a 96-well plate and after 24 h, the cells were preincubated with the extract (0–320 µg/mL) at 37°C for 1 h. Further, incubation was continued for another 24 h with lipo-polysaccharide (LPS) (1 µg/mL) at 37°C. After the incubation, nitric oxide (NO) accumulation was measured.[15] Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid), incubated at room temperature for 10 min, and the optical density was measured at 540 nm using fresh culture medium as a negative control.

Assessment of tumor necrosis factor-alpha and prostaglandin E_2 production

LPS-induced RAW 264.7 cells treated with extracts were cultured in the same manner as described for the assessment of NO production. The concentrations of TNF-α and PGE_2 in the culture supernatants were measured by enzyme-linked immunosorbent assay kits in accordance with the manufacturer's protocols.

Statistical analysis

All the tests were performed in triplicate, standard deviation was expressed, and the results were analyzed by one-way ANOVA. Statistical significance was set at $P < 0.05$, and comparisons were made against the control and reference controls. The percentage of free radical scavenging activity of the compounds was calculated using formula 1, and results were expressed as IC_{50} value in µg/mL using standard antioxidant. FRAP value was calculated by formula 2, and results were expressed as mM/mg.

$$\text{Percentage inhibition (\%)} = \frac{(\text{Ab of controle} - \text{Ab of sample})}{\text{Ab of controle}} \times 100 \quad (1)$$

$$\text{FRAP value} = \frac{\text{Ab of sample} \times \text{FRAP value of standard}}{\text{Ab of standard}}$$

RESULTS

Proximate compositions

Diatom species show variations in their proportions of biochemical content such as protein, carbohydrate, lipid, and microelements. Approximate composition of *N. pale* dried biomass was found to be 34.76% ± 3.27% total protein (*N*X 6.25), 17.9% ± 2.75% total lipid (W/W), 16.95% ± 1.91% total carbohydrate in phenol sulfuric acid method, 27.20% ± 2.59% ash content, and 2.97% ± 0.54% moisture content. Elemental compositions were determined by EDX and expressed in percentage weight of the total elements. Among the major elements, in *N. pale,* oxygen concentration was found to be high (46.31 ± 0.62), followed by nitrogen (27.36 ± 0.35) and carbon (17.62 ± 0.35). Because it is a diatom species in which cell wall is made up of silica, silicate was also found as a major component (5.89 ± 0.07). Further, among the microelements identified, iron content was found to be high (0.81 ± 0.11) followed by calcium (0.69 ± 0.05), phosphorus (0.6 ± 0.06), magnesium (0.48 ± 0.04), aluminum (0.13 ± 0.02), and sulfur (0.11 ± 0.02).

Total phenol and flavonoid contents

The total phenolic content of methanol and ethyl acetate extracts of *N. palea* was found to be 19.85 ± 5.9 and 29.72 ± 7.38 mg/g (GAE) of extracts, respectively. Flavonoid content of methanol and ethyl acetate extracts was found to be 7.4 ± 14.98 and 12.37 ± 42.87 mg/g (CE), respectively.

Gas chromatography-mass spectrometry analysis of ethyl acetate and methanol extracts

The volatile organic compounds of methanol and ethyl acetate extracts of *N. palea* were analyzed by GC-MS [Supplementary Figures S1-2 for chromatograms]. The identities of expected compounds were confirmed by mass fragmentation analysis as shown in Figure 1a for methanol and Figure 1b for ethyl acetate extract. A total of 12 compounds were identified; among them, three compounds were present in methanol extract and nine compounds were identified in ethyl acetate extract [Supplementary Table 1 for compounds' details].

In vitro antioxidant and free radical scavenging activities

2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay

A dose-dependent increased quenching of free radical was observed in DPPH assay. Ethyl acetate extract demonstrated high activity with the lowest IC_{50} value (206.77 ± 11.63 µg/mL) followed by methanol extract (272.49 ± 9.53 µg/mL) [Table 1]. The data obtained clearly indicated that the DPPH scavenging ability of the ethyl acetate extract may be attributed to its potent hydrogen-donating ability.

Ferrous ion chelating assay

The results of ferrous ion chelating property of ethyl acetate and methanol extracts with standard EDTA equivalent showed IC_{50} values of 232.74 ± 8.24 and 259.68 ± 9.44 µg/mL, respectively [Table 1].

Table 1: Estimation of antioxidant and free radical scavenging activity of methanol and ethyl acetate extracts

Sample	DPPH*	Ferrous ion chelating$^{\Delta}$	NO scavenging$^{\epsilon}$	ABTS$^{£}$	D-ribose$^{\epsilon}$	NBT*	Lipid peroxidation$^{£}$	FRAP value$^{\epsilon}$
Methanol	272.49±9.53	259.68±9.44	-	56.29±14.8	362.76±42.74	-	647.59±34.01	33.78±0.6
Ethyl acetate	206.77±11.63	232.74±8.24	492.98±8.16	24.06±1.2	311.63±4.73	37.97±6.2	402.9±31.48	2.13±0.42
Standard	254.7±8.53*	113.25±5.1$^{\Delta}$	425.89±5.38$^{\epsilon}$	14.93±0.79$^{£}$	161.06±1.23$^{\epsilon}$	69.04±3.36*	290.09±5.76$^{£}$	6.19±0.30$^{\epsilon}$

IC_{50} values were represented with standard error mean of triplicate readings and standard antioxidants used were *BHT, $^{\Delta}$EDTA, $^{\epsilon}$L-Ascorbic acid and $^{£}$BHA. BHT: Butylated hydroxytoluene; EDTA: Ethylene diamine tetra acetic acid; BHA: Butylated hydroxyanisole; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; NBT: Nitro blue tetrazolium; FRAP: Ferric reducing antioxidant power; NO: Nitric oxide

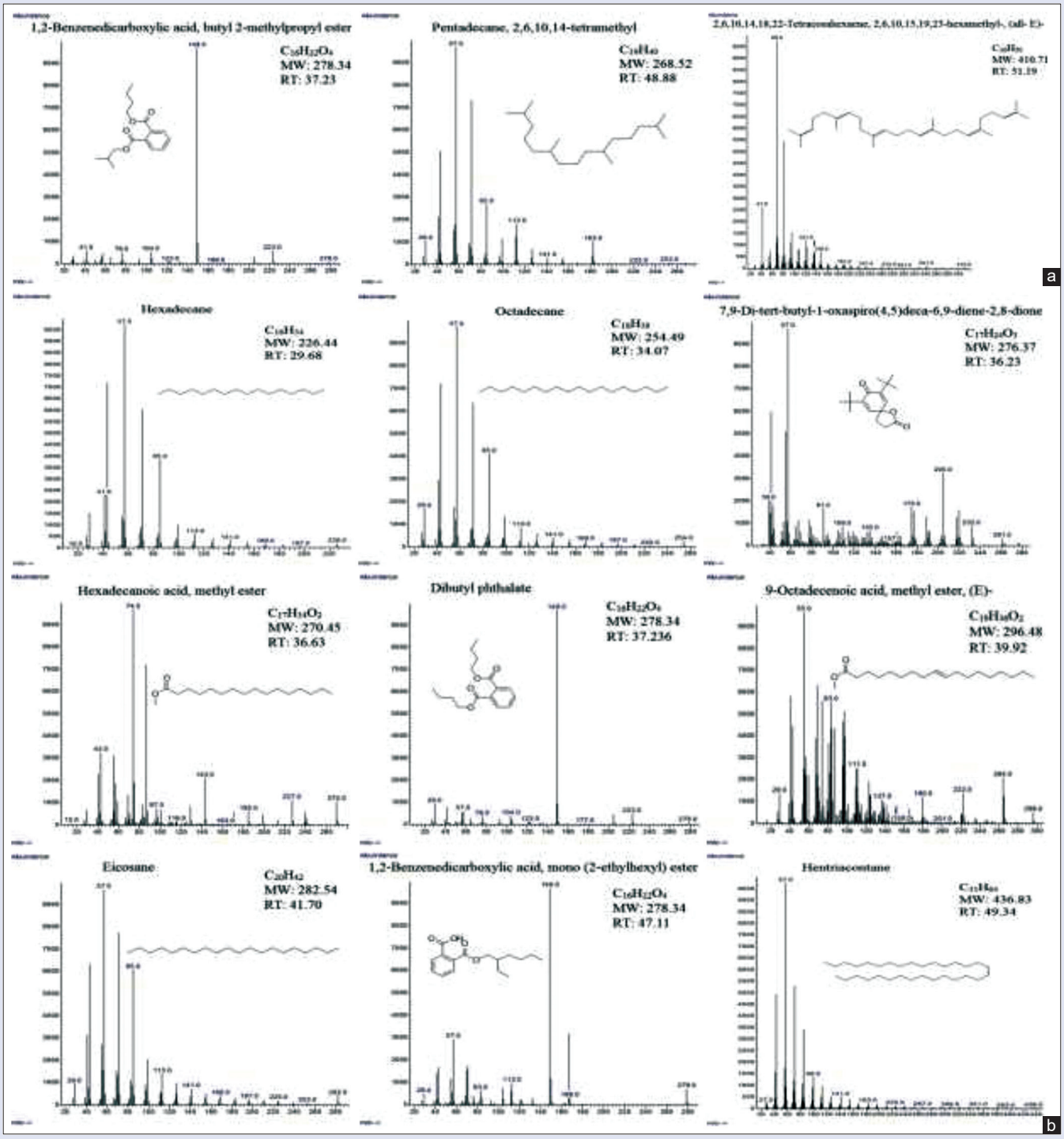

Figure 1: Bioactive compounds identified in methanol (a) and ethyl acetate extracts (b) of *Nitzschia palea* by GC-MS with NIST database. GC-MS: Gas chromatography-mass spectrometry; NIST: National Institute of Standards and Technology

Nitric oxide scavenging activity

The result of NO scavenging assay showed remarkable result only in ethyl acetate extract and not in methanol extract. Ethyl acetate extract showed IC_{50} value of 492.98 ± 8.16 μg/mL [Table 1], equivalent to standard L-ascorbic acid.

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid radical scavenging activity

ABTS assay result showed that ethyl acetate extract was found to be a standard BHA equivalent with IC_{50} value of 24.06 ± 1.2 μg/mL and methanol extract showed IC_{50} value of 56.29 ± 14.8 μg/mL [Table 1].

Deoxyribose assay

The results of deoxyribose assay showed that ethyl acetate extract has the activity equivalent to standard L-ascorbic acid with IC_{50} of 311.63 ± 4.73. However, methanol extract showed comparatively less activity with IC_{50} of 362.76 ± 42.74 µg/mL [Table 1].

Superoxide radical scavenging activity (nitroblue tetrazolium method)

Ethyl acetate extract showed inhibition of blue-colored NBT formation, and the decrease of absorbance indicates the consumption of superoxide anion. This was calculated as percent reduction of superoxide anion scavenging capacity, and the result showed IC_{50} value of 37.97 ± 6.2 µg/mL in comparison with butylated hydroxytoluene as standard [Table 1].

Antilipid peroxidation activity

Lipid peroxidation assay showed that both ethyl acetate and methanol extracts inhibited lipid peroxidation in a dose-dependent manner with IC_{50} values of 402.9 ± 31.48 and 647.59 ± 34.01 µg/mL, respectively [Table 1].

Ferric-reducing antioxidant power assay

The IC_{50} values of ferric-reducing prevention capacity of ethyl acetate and methanol extracts were found to be 2.13 ± 0.42 and 33.78 ± 0.6 µg/mL, respectively, which indicates the potential antioxidant activity of ethyl acetate extract compared to methanol extract [Table 1].

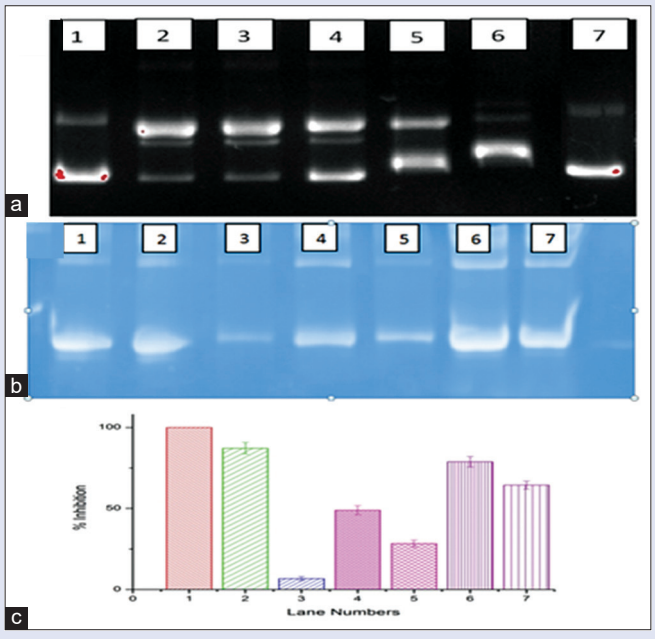

Figure 2: The macromolecule protection by electrophoretic method; (a) DNA; Lane 1 – reference control, 2 – negative control, 3 – methanol extract (10 µg), 4 – methanol extract (20 µg), 5 – ethyl acetate (10 µg), 6 – ethyl acetate (20 µg) and 7 – positive control. (b) Protein; Lane 1 – reference control, 2 – positive control 3 – negative control, 4 – methanol fraction (20 µg) 5 – methanol extract (10 µg), 6 – ethyl acetate (20 µg), 7 – ethyl acetate (10 µg). (c) Protein band density; the band density for protein oxidation inhibition (%) lane numbers is same as column number at X axis. Error bar represents mean ± standard deviation of triplicates, and test samples are significantly different from control at $P < 0.05$

Protective effect on oxidation of biomolecules *in vitro*

Plasmid deoxyribonucleic acid strand break assay

Ethyl acetate and methanol extracts were evaluated for their protective effect on AAPH-induced damage of DNA. The agarose gel (1%) pattern of DNA added with minimum concentrations (10 and 20 µg/mL) of extracts to show the visible differences in gel pattern is shown in Figure 2a. Reference control without AAPH (lane 1) showed a single band representing supercoiled circular DNA (ScDNA). Negative control (only AAPH, lane 2) showed two bands on agarose gel electrophoresis, wherein the faster moving band represents the ScDNA and the slower moving band corresponds to the open circular form (OcDNA). This indicated complete damage of ScDNA and it was converted to OcDNA. Whereas treatment with methanol extract in lane 3 (10 µg) and lane 4 (20 µg) and ethyl acetate extract in lane 5 (10 µg) and lane 6 (20 µg) and positive control added with standard quercetin (lane 7) provided significant protection to the ScDNA in a dose-dependent manner.

Protein oxidation protection assay

SDS-PAGE electrophoretic band pattern of BSA exposed to AAPH and added with minimum concentrations (10 and 20 µg/mL) of extracts to show the visible differences in gel pattern is presented in Figure 2b. The corresponding densitometric analysis of bands in terms of percentage of protein oxidation is presented graphically in Figure 2c. The protein BSA as such was considered the reference control (lane 1) which showed oxidative damage when incubated with AAPH (negative control, 400 mM for 2 h, lane 3). Standard quercetin (lane 2) was used as positive control, followed by methanol extract of 20 µg (lane 5) and 10 µg (lane 4) and ethyl acetate extract of 20 µg (lane 7) and 10 µg (lane 6). The prevention efficacy was analyzed by the density of bands and compared with both positive and negative controls, and the results were expressed in percent protein oxidation inhibition [Figure 2c].

Hemolysis assay

In hemolysis assay, the ethyl acetate and methanol extracts were evaluated for AAPH-induced antihemolytic properties. The ethyl acetate extract showed potent activity of 50% inhibition at 65.5 ± 2.37 µg/mL followed by methanol extract which showed 50% inhibition at 92.6 ± 9.29 µg/mL. Further, the experiment has been extended for morphological examination of erythrocytes by AFM and SEM image analyses. The AFM study revealed that the surface morphology of erythrocytes without extracts (negative control) showed higher value of average roughness (4.49 nm), lesser value of profile (height/thickness 2.04 nm), and multiple peaks in waviness [Figure 3A, a-d]. This higher value of roughness and lesser value of profile and multiple peaks in waviness indicate that the untreated erythrocytes were shrunken which leads to damage because of AAPH. Further, methanol extract-treated erythrocytes showed that the slightly lower roughness 3.63 nM and slightly higher profile of 2.75 nm [Figure 3B, a-c] with the waviness showed simple peak [Figure 3B, d]. When compared to negative control, this indicates that bioactive compounds in methanolic extracts protect the erythrocytes at lesser extent. Similarly, the ethyl acetate extract showed average roughness of 3.03 nM [Figure 3C, a-b] and profile of 4.75 nM [Figure 3C, c], with waviness of single peak [Figure 3C, d]. These results confirmed that the ethyl acetate extract has potent inhibition of AAPH-induced hemolysis when compared to methanol extract. Further, the above-prepared erythrocytes were observed by SEM which revealed a clear difference in their effects on Erythrocyte membrane morphology, i.e., erythrocytes treated with only AAPH (negative control) showed shrinkage [Figure 4a], and erythrocytes treated with methanol extract exhibit slight membrane protection from the AAPH damage [Figure 4b]

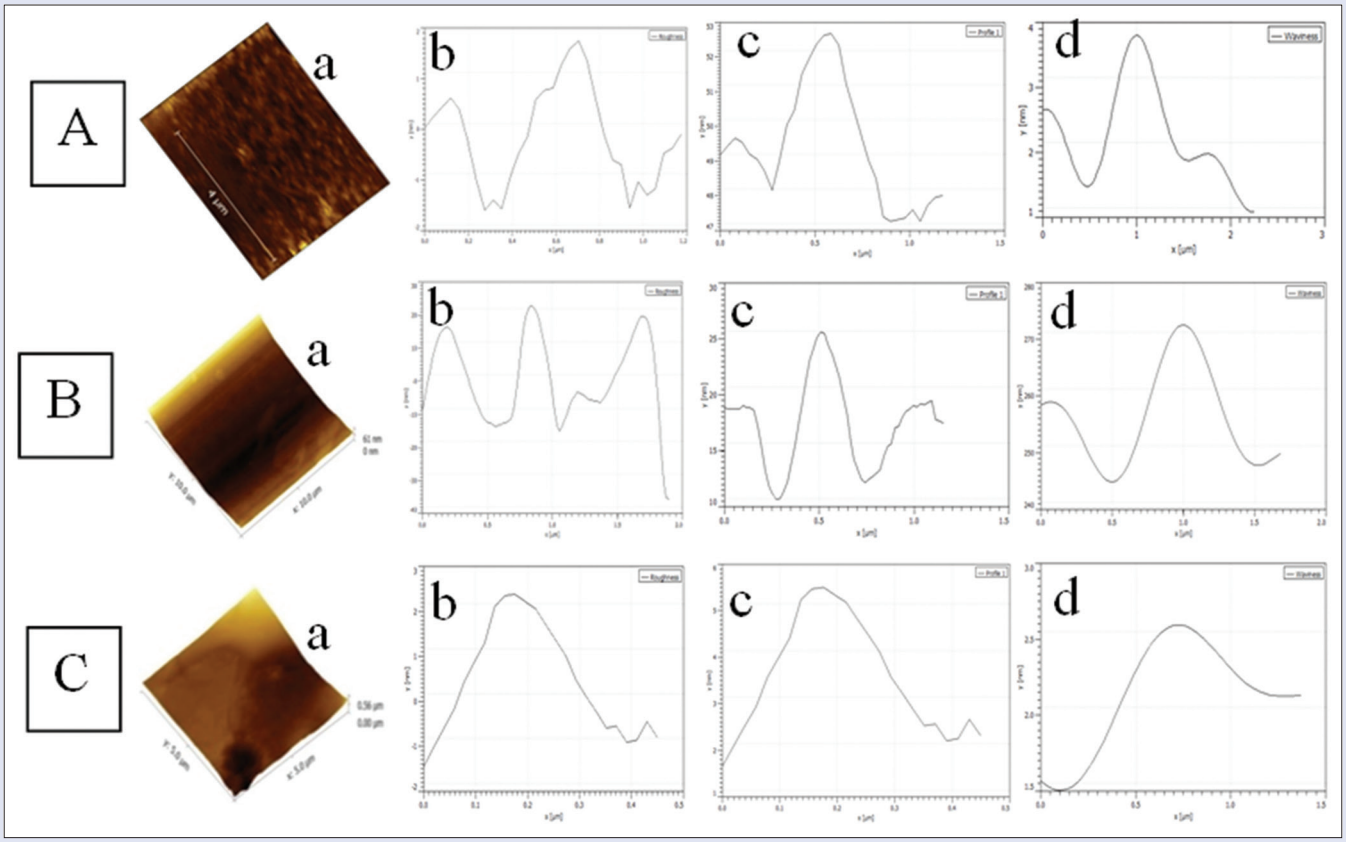

Figure 3: Morphological observation of erythrocytes by atomic force microscopic image analysis. (A) Erythrocytes treated with 100 mM AAPH for 2 h. (B) Erythrocytes treated with methanol extract (50 µg/mL) and AAPH for 2 h. (C) Erythrocytes treated with ethyl acetate extract (50 µg/mL) and AAPH for 2 h. (a-d) indicated image, average roughness, height/thickness called profile, and surface waviness of the erythrocytes, respectively. AAPH: 2,2-azobis (2, amidinopropane) dihydrochloride

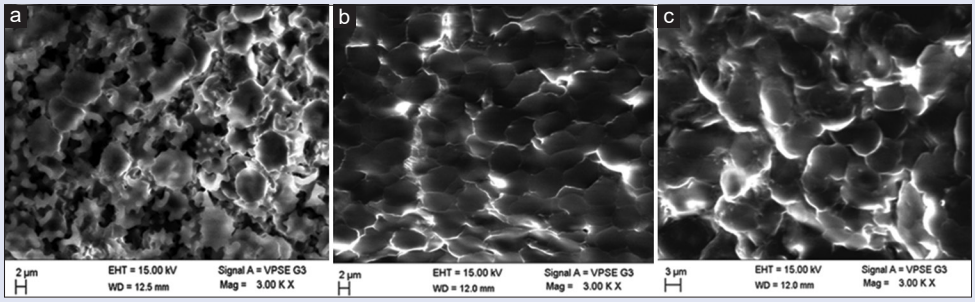

Figure 4: Morphological observation of erythrocytes by scanning electron microscope (a) Erythrocytes treated with 100 mM AAPH for 2 h (b) AAPH + methanol extract (50 µg/mL) treated for 2 h (c) AAPH + ethyl acetate extract (50 µg/mL) treated for 2 h. AAPH: 2,2-azobis (2, amidinopropane) dihydrochloride

compared to negative control. AAPH-induced erythrocytes treated with ethyl acetate resulted in an improved unaltered erythrocyte membrane compared to methanol extract [Figure 4c]. This antihemolytic nature of *N. palea* extracts is due to the presence of phenolic and volatile metabolites.

Lactate dehydrogenase assay for erythrocyte damage protection

LDH is an enzyme used as a marker for inflammatory reaction, as it is released outside the cells upon cell destruction. The result of LDH assay showed [Figure 5] high LDH-releasing activity with AAPH-induced erythrocytes (control), and the activity was dose dependently decreased with methanol and ethyl acetate extract pretreatment. Up to 32% of decrease in LDH activity was observed with 40 µg/mL of ethyl acetate extract [Figure 5].

In vitro anti-inflammatory properties

Cell viability in terms of growth inhibition of the extract was found to be dose dependent [Figure 6a-d], and the IC_{50} was calculated using dose–response curves that showed maximum dose of 50.73 µg/mL [Supplementary Material]. Ethyl acetate extract of *N. palea* showed reduced production of NO level against LPS-induced RAW 264.7 cells in a dose-dependent manner. In particular, by assuming that LPS induced 100% production of NO, the extract at different concentrations of 20 µg/mL, 40 µg/mL, and 60 µg/mL exhibited 91.38% ±6.24%, 78.42% ±5.79%, and 72.26% ±5.63% of NO production, respectively [Figure 6e]. Further, the ethyl acetate extract also showed inhibition of pro-inflammatory cytokines such as TNF-α [Figure 6f] and PGE_2 [Figure 6g]. Among

these, TNF-α was reduced to a higher extent when compared to PGE_2 and for both cytokines, maximum inhibition was observed at 60 µg/mL. The concentration of TNF-α and PGE_2 in ethyl acetate extract-treated RAW 264.7 cells was found to be 79.42 ± 4.18 and 478.6 ± 16.47 pg/mL, respectively [Figure 6f-g].

DISCUSSION

Diatoms produce massive blooms and are considered non-toxic exception of domoic acid in some species. Recently, Marella *et al.*[16] showed the usefulness of dual application of diatoms species for biodiesel production and the biomass for water treatment. Lebeau and Robert[17] reviewed the use of biomass of selected diatom species for various biotechnological applications such as aquaculture, food source, paintings, and intracellular metabolites for pharmaceutical and cosmetic applications. In the present study, for the first time, we report the identification of volatile metabolites in *N. palea* for screening their biological activities.

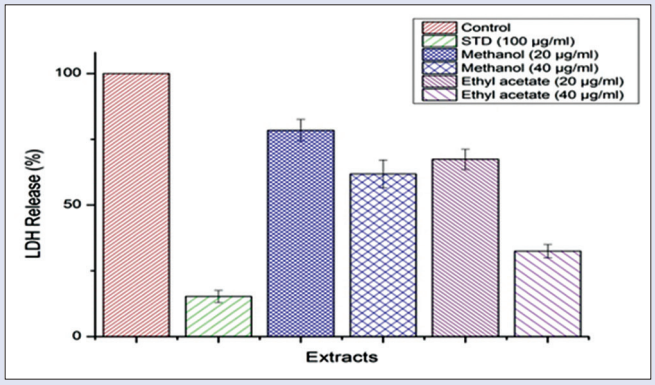

Figure 5: Lactate dehydrogenase assay by AAPH-induced erythrocytes treated with methanol and ethyl acetate extracts (20 and 40 µg/mL) for 2 h by considering the control as 100%; the amount of LDH release was calculated in percent. Error bar represents mean ± of triplicates, and test samples are significantly different from control at $P < 0.05$. AAPH: 2,2-azobis (2, amidinopropane) dihydrochloride; LDH: Lactate dehydrogenase

The proximate composition was determined for *N. palea* biomass, and the results represented the presence of higher amounts of protein, ash content, lipids, and carbohydrates accordingly. In literature, some of the other diatom species have also shown similar results, particularly the evaluation of effect of three color lights for culturing *Chaetoceros* sp.[18] All these variations of compositions are dependent on species-specific and physicochemical parameters used for culture. Elemental compositions determined by EDX analysis showed more oxygen concentration followed by nitrates, carbon, silicate, iron, calcium, magnesium, phosphorus, aluminum, and sulfur accordingly. This is in agreement with the findings of Orcutt and Patterson[19] who determined the elemental composition in 11 species which include four *Nitzschia sp.* However, elemental composition determination showed the absence of hazardous materials such as lead, mercury, and arsenic.

In microalgae, not only carotenoids are considered a source of antioxidants, but phenolic compounds also have a role in antioxidant activity.[19] In the analysis of total phenolic and flavonoid content of methanol and ethyl acetate extracts of *N. palea,* higher concentration of total phenolic and flavonoid content was observed in ethyl acetate extract compared to methanol extract. In recent years, determination of phenolic contents in diatom species to exhibit antioxidant properties has gained interest. The antioxidant potential of phenolic content was successfully demonstrated in *P. tricornutum*,[20] *Nitzschia laevis*,[4] and *Phaeodactylum* sp.[21]

The present study is a first attempt to analyze the volatile metabolites of diatom species especially in *N. palea* by GC-MS data matching with NIST library. The following identified metabolites of methanol extract were discussed based on their bioactive potentiality: 1,2-benzenedicarboxylic acid and butyl 2-methylpropyl. These compounds are responsible for the

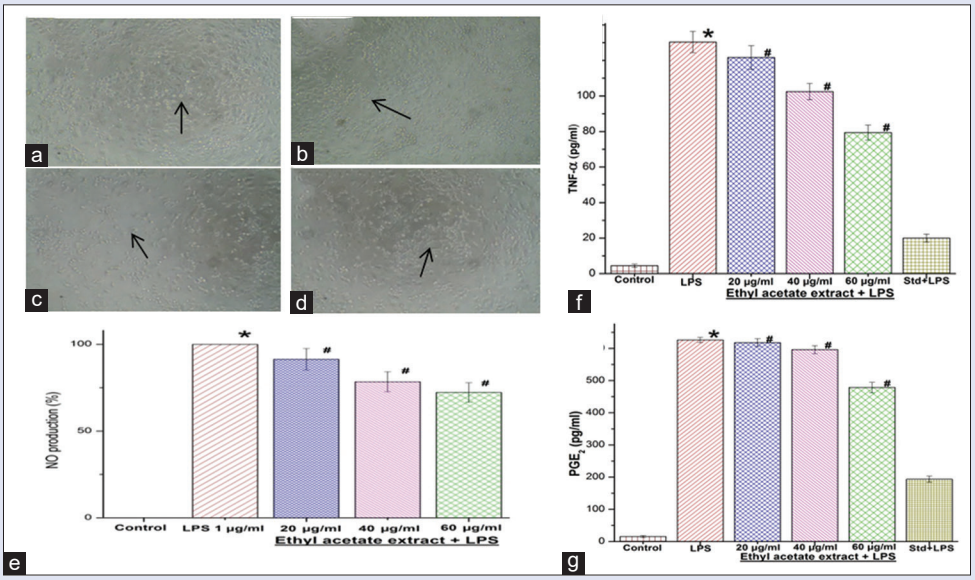

Figure 6: Cell viability and inhibition of inflammatory cytokine NO, TNF-α, and PGE_2 production on RAW 264.7 cells: Treated with ethyl acetate extract (a) 2.05 µg/mL, (b) 16.47 µg/mL, (c) 65.89 µg/mL and (d) Control (the arrows indicate cell dense zone). (e) Production of NO, (f) TNF-α, and (g) PGE_2 which were pretreated with ethyl acetate extract# (20, 40, and 60 µg/mL) for 1 h followed by stimulation with 1 µg/mL of LPS for 24 h. Error bar represents mean ± standard deviation of triplicates, and marked with # are significantly different from control* at $P < 0.05$. NO: Nitric oxide; TNF-α: Tumor necrosis factor-alpha; PGE_2: Prostaglandin E_2, LPS: lipo-polysaccharide

high antimutagenic activity, anti-androgenic effect, and α-glucosidase inhibitory activity. They were identified in octopus[22] and *Laminaria japonica*.[23] Pentadecane, 2,6,10,14-tetramethyl (commonly called Pristane) – a natural saturated terpenoid alkane – is present in shark liver oil and stomach oil of birds. It induces plasma cell tumorigenesis and helps to study autoimmune diseases. 2,6,10,14,18,22-tetracosahexaene 2,6,10,15,19,23-hexamethyl-(all-E) – commonly called Squalene – was identified in many plant species, i.e., *Broussonetia luzonica*.[24] Similarly, in ethyl acetate extract, we identified ten volatile compounds; among them, only bioactive compounds were discussed. 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione was identified and the same has been identified from a plant *Oryza sativa*.[25] Narendhran *et al*.[26] identified the same compound in a bacterial species *Streptomyces cavouresis* for its antioxidant properties. Hexadecanoic acid methyl ester, commonly known as methyl palmitate, was identified in *Hibiscus sabdariffa*,[27] which has proven to depress phagocytic activity and immune response, prevent Kupffer cell activation for postoperative survival in liver transplanted rats, and exhibit anti-inflammatory and antifibrotic effects.[28-31] Dibutyl phthalate was produced from *Streptomyces albidoflavus*.[32] Identified Hentriacontane, generally present in leaf wax, and having anti-inflammatory mechanism, in addition to it Kim *et al*.[33] demonstrated the suppression of caspase-1 activation *in vivo* model from the plant *Oldenlandia diffusa*.

Based on the presence of the above bioactive compounds, we have subjected methanol and ethyl acetate extracts to eight antioxidant assays. The bleaching of DPPH is used as a representative of free radical or antioxidant scavenging activity by plant as well as microalgal extracts. This method is based on the principle that the amount of hydrogen-donating capacity of extract required to reduce radical form DPPH by 50% to form the non-radical form (DPPH-H). Iron is an essential element for life, and it can stimulate lipid peroxidation by Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. Therefore, the ability of extracts to chelate/bind metal ion was tested in the present study. The blue-green ABTS radical decoloration assay is widely used to assess the antioxidant activity of plant extracts. The reducing ability of extracts was analyzed by FRAP assay, and this activity depends on the ability of the extract to donate hydrogen atoms to inactivate the free radical formation. TBA reacts with malondialdehyde to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. The deoxyribose assay allows the determination of the rate constants for reaction between antioxidant molecules and hydroxyls. Extracts can inhibit the blue NBT formation and the decrease of absorbance at 560 nm with antioxidants, indicating the consumption of superoxide anion in the reaction mixture. Results of these assays revealed that the ethyl acetate extract has potent antioxidant properties in all the eight assays compared to methanol extract. Whereas methanol extracts showed less antioxidant activity in six assays except nitrate scavenging and NBT assays. This poor performance of the methanol extract is in agreement with the less number of volatile bioactive compounds present in it. The observed results are in accordance with a recent report by Kandikattu *et al*.[34] who demonstrated the biological activities of *Cyperus rotundus*.

In addition to antioxidant assays, the study was extended to analyze the diatom extract for prevention efficacy of macromolecular damage induced by oxidative stress and validated by electrophoretic methods. DNA damage has been observed in several stress conditions such as cancer, diabetes, and neuronal diseases.[35] Similarly, oxidative damaged proteins are highly resistant to degradation and accumulate as fragments to cause neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.[36] In the literature, several herbal extracts have been reported to protect the macromolecules such as DNA[12,37] and protein (BSA) from oxidative damage using electrophoretic methods.[12,36] Our findings demonstrated DNA and protein damage prevention efficacy of both methanol and ethyl acetate extracts. Ethyl acetate extract showed higher prevention when compared to methanol extract; this difference in result is in agreement with the results of phenolic, flavonoid content, and volatile compounds present in both extracts. Our results suggested that, biomass of *N. palea* as a source of natural antioxidants, in addition to carotenoids, volatile compounds, and phenolic contents, is also responsible for biological activities.

The membrane of erythrocytes has polyunsaturated fatty acids and proteins, cellular oxygen, and iron; hence, it is susceptible to oxidative damage. AAPH generates peroxyl radicals which can cause lipid peroxidation, protein damage, and finally lead to hemolysis to release hemoglobin. In the present study, the fact of the presence of metabolites having antioxidant and anti-inflammatory properties in both extracts of *N. palea* made us to evaluate hemolysis inhibition and anti-inflammatory properties. Preliminarily, we have determined the percent inhibition of erythrocyte induced by AAPH; results revealed that ethyl acetate extract has potent inhibitory activity compared to methanol extract. Further, the results were validated by AFM and SEM image analyses followed by LDH release assay which is strongly recommended to study the prevention of damage of erythrocytes. In the cell viability test, lesser inhibitory effect on RAW 264.7 cells was evidenced with inhibitory effect of crude extract on the secretion of NO, TNF-α, and PGE_2. As it is a crude extract, the results show quite lesser inhibition when compared to the standard drug indomethacin. This indicated that the presence of anti-inflammatory compounds in extract has played a major role in the prevention of inflammation, but it needs to isolate lead compounds to achieve best result similar to standard. Recent studies also identified anti-inflammatory components such as polysaccharides in a diatom species *P. tricornutum*.[15,38] Based on these findings, further studies are required for screening diatom species to develop methods to isolate bioactive compounds. Furthermore, enhancing the production of bioactive compounds by the induction of physicochemical stress is one of the approaches.

CONCLUSION

The bio active volatile and phenolic compounds such as squalene and pristane in methanol extract and methyl palmitate and hentriacontane in ethyl acetate extracts were identified in freshwater diatom species, *N. palea*. From the results, it can be concluded that *N. palea* can be a potential source of antioxidant, antihemolytic, and anti-inflammatory activities. There is a requirement of toxicity studies of extracts in detail and characterization of identified compounds to establish their therapeutic applications and for human consumption.

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Conflicts of interest

There are no conflicts of interest.

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