Electronic Journal of Biotechnology 40 (2019) 1–9

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

Chlorella sorokiniana and *Chlorella minutissima* exhibit antioxidant potentials, inhibit cholinesterases and modulate disaggregation of β-amyloid fibrils

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ARTICLE INFO

Article history:
Received 12 May 2018
Accepted 22 March 2019
Available online 1 April 2019

Keywords:
Algal biomass
Alzheimer's disease
β-Amyloid protein
Chlorella minutissima
Chlorella sorokiniana
Cholinesterases
Dichloromethane extract
Ethanol extract
FTIR
Gas chromatography
Hexane extract

ABSTRACT

Background: Microalgae are aquatic chlorophyll-containing organisms comprising unicellular microscopic forms, and their biomasses are potential sources of bioactive compounds, biofuels and food-based products. However, the neuroprotective effects of microalgal biomass have not been fully explored. In this study, biomass from two *Chlorella* species was characterized, and their antioxidant, anticholinesterase and anti-amyloidogenic activities were investigated.

Results: GC–MS analysis of the extracts revealed the presence of some phenols, sterols, steroids, fatty acids and terpenes. Ethanol extract of *Chlorella sorokiniana* (14.21 mg GAE/g) and dichloromethane extract of *Chlorella minutissima* (20.65 mg QE/g) had the highest total phenol and flavonoid contents, respectively. All the extracts scavenged 2,2-diphenyl-1-picrylhydrazyl, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) and hydroxyl radicals. The highest metal chelating activity of the extracts was observed in the ethanol extracts of *C. minutissima* (102.60 μg/mL) and *C. sorokiniana* (107.84 μg/mL). Furthermore, the cholinesterase inhibitory activities of the extracts showed that ethanol extract of *C. sorokiniana* (13.34 μg/mL) exhibited the highest acetylcholinesterase inhibitory activity, while dichloromethane extract of *C. minutissima* (11.78 μg/mL) showed the highest butyrylcholinesterase inhibitory activity. Incubation of the β-amyloid protein increased the aggregation of amyloid fibrils after 96 h. However, ethanol extract of *C. sorokiniana* and *C. minutissima* inhibited further aggregation of $A\beta_{1-42}$ and caused disaggregation of matured protein fibrils compared to the control. This study reveals the modulatory effects of *C. sorokiniana* and *C. minutissima* extracts on some mediators of Alzheimer's disease and provides insights into their potential benefits as functional food, nutraceutics or therapeutic agent for the management of this neurodegenerative disease.

How to cite: Olasehinde T, Odjadjare EC, Mabinya LV, et al. Chlorella sorokiniana and Chlorella minutissima exhibit antioxidant potentials, inhibit cholinesterases and modulate disaggregation of β-amyloid fibrils. Electron J Biotechnol 2019;40. https://doi.org/10.1016/j.ejbt.2019.03.008

1. Introduction

Recently, studies on the use of microalgal biomass as nutritional supplements, nutraceuticals and pharmaceuticals have gained much interest. Microalgae have a great biotechnological attribute that is similar to microbial cells, as they grow very fast in culture medium and are capable of producing several secondary metabolites [1]. The exposure of different species of microalgae to abiotic stress induces the secretion of diverse secondary metabolites. Hence, microalgal biomass is a reservoir of biologically active compounds such as fatty acids, phycobilins, carotenoids, sterols, polyphenols, polysaccharides and vitamins [2,3]. The use of microalgal biomass in the production of nutraceuticals, pharmaceuticals, animal and fish feed is also growing much interest [4]. Biomass derived from some microalgae has been used as dietary supplements for the treatment of hyperlipidemia, hypertension and diabetes [5,6]. The use of microalgae for the prevention of cardiovascular disease, stroke, [2], oxidative damage, inflammation and microbial infection [7] has been reported. Despite the extensive research on the biological activities of several species of microalgae, there are few studies on their neuroprotective effects. Hence, the neuroprotective effects of

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2019.03.008

microalgae against some neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) have attracted considerable attention.

Several factors such as oxidative stress-induced neuronal damage, loss of acetylcholine in cholinergic neurons and deposition of β-amyloid plaques in brain cells have been linked to the development of AD and PD [8,9,10]. Free radicals contribute to inflammatory processes, which may induce neuronal damage and memory dysfunction [11]. Accumulation of some metals such as iron and aluminum can initiate processes involving the generation of highly reactive radicals and production of β-amyloid fibrils in neuronal cells [12]. Furthermore, a low level of acetylcholine is associated with memory deficit in patients with AD owing to high cholinesterase activity. Cholinesterase activity regulates the concentration of acetylcholine, a neurotransmitter involved in the transmission of nerve impulses within the neurons [13]. Moreover, dysregulation in cholinesterase activities has been linked to the formation of β-amyloid plaques in brain cells [14]. The assembly and aggregation of β-amyloid fibrils is one of the major hallmarks of AD [15]. Hence, the prevention of continuous aggregation and the initiation of disaggregation of β-amyloid protein are important therapeutic strategies for the management of AD.

There has been extensive exploration for natural products from algae with antioxidant, cholinesterase inhibitory activity and anti-amyloidogenic properties that can be used as therapeutic interventions for the management of AD. Hexane and methanol extracts of some microalgae (*Rhodomonas salina*, *Nannochloropsis oculata*, *Tetraselmis chuii and Chlorella minutissima*) have been shown to possess cholinesterase inhibitory activities [16]. Furthermore, lipid extract of *Chlorella sorokiniana* containing polyunsaturated fatty acids also improved cognitive function in rats by elevation of noradrenaline and serotonin [17]. In this paper, we report for the first time the radical scavenging activity, metal chelating capacity, cholinesterase inhibitory effects and anti-amyloidogenic activity of *C. sorokiniana* and *C. minutissima*.

2. Materials and methods

2.1. Sample collection

Samples were aseptically collected into a clean 10-L plastic container from freshwater reservoirs located in Durban (29.645719 S, 31.122473 E and 29.678156 S, 31.032978 E), KwaZulu-Natal, South Africa. The samples were processed within 24 h of collection for enrichment, isolation and purification of microalgal species.

2.2. Algal strain isolation and cultivation

Water samples from the freshwater reservoir mentioned above were enriched using BG-11 medium (Sigma-Aldrich, Germany). Trace metals for BG-11 were prepared using standard protocols [18]. The sample (15 mL) was inoculated into a 2 L Erlenmeyer flask containing 235 mL of the sterile enrichment broth containing 50 mg/mL of erythromycin. The flask was plugged with cotton wool and placed under UV illumination (54.36 μmol/m^2 s^{-1}) with continuous shaking at 180 rpm, 30°C for 14 to 21 d, with a 12:12-h light:dark cycles until visible cells were observed. After incubation, the mixed culture was serially diluted and used to inoculate appropriate growth media solidified with 1.5% bacteriological agar using the spread plate technique. The plates were further incubated under the same conditions previously described. Isolated microalgal colonies were further inoculated into fresh media broth. Samples were cultivated for 12 weeks of continuous batch photoautotrophic growth to accumulate enough biomass [19]. Microalgal cells were collected at the late exponential phase, centrifuged at 4000 × *g* and lyophilized using a freeze-dryer (CHRIST Alpha 1–2 LD plus, Germany). The yield of the dried biomass was approximately 1.2 g for each experiment. The dried biomasses were kept in glass vials and stored at 8°C for subsequent analysis.

2.3. Molecular identification of microalgal isolates

The microalgal species isolated were identified by 18S rDNA gene sequencing using primers and conditions reported by Moro et al. [20]. DNA extraction was performed using the Quick-DNA Fungal/Bacterial Miniprep Kit as indicated in the manufacturer's instructions (Zymo Research Corp., USA). The reaction mixture used for polymerase chain reaction (PCR) consisted of a 25-μL reaction mixture consisting of 1× buffer, 1-mM $MgCl_2$, 200-μM dNTPs, 0.4 μM of each primers and 2 U of Super-Therm Taq polymerase (Southern Cross Biotech., Cape Town, South Africa). The PCR program consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min, with an additional 10-min cycle at 72°C. The amplified DNA (5 μL) was electrophoresed in a 1.5% agarose gel at 60 V for 60 min. Thereafter, the gel was stained in 1% ethidium bromide for 10 min and visualized under UV light (Syngene, UK). The amplified products were sequenced (Inqaba Biotec, South Africa) and edited using Chromas Ver. 2.2.4 (Technelysium Pty Ltd., Brisbane, Australia). The sequences were then compared against those available in GenBank database using the basic local alignment search tool (BLAST) to identify the organism. The organisms were identified as *C. sorokiniana* and *C. minutissima*.

2.4. Extract preparation

For extract preparation, 40 mL of hexane was added to the dried biomass (1 g) in an amber bottle, which was placed on a shaker for 48 h. The hexane extract was filtered, and the process was repeated twice. The extracts were pooled together and evaporated under vacuum pressure. The extraction process was repeated for dichloromethane and ethanol. The extracts were resuspended in ethanol and used for subsequent analysis.

2.5. Total phenol content assay

Folin–Ciocalteu reagent (10%, 2.5 mL) and sodium carbonate (7.5%, 2.0 mL) were added to different dilutions of the extracts. The solutions were incubated on a water bath at 45°C for 40 min. After the incubation period, the absorbance of the mixture was measured at 765 nm using a V1200 spectrophotometer (Shanghai Mapada Instruments, China) [21]. The total phenol content of the extracts was calculated and expressed as gallic acid equivalent.

2.6. Total flavonoid content assay

The extracts (0.5 mL) were mixed with methanol (what %) (500 μL), 10% aluminum chloride (50 μL), potassium acetate (50 μL, 1 M) and water (1.4 mL). The solution was allowed to stand at room temperature for 30 min, after which the absorbance was measured at 415 nm [22]. Total flavonoid content of the extracts was calculated and expressed as quercetin equivalent.

2.7. Fourier transmission infrared spectroscopy analysis

The functional groups of the extracts were determined by scanning using an FTIR spectrophotometer (Spectrum Two, PerkinElmer, USA) at room temperature (25–28°C) at 370–4000 cm^{-1} spectral range. The functional groups were determined by comparing the peak frequencies to the IR spectroscopy correlation table [23].

2.8. Gas chromatography–mass spectrometry (GC–MS) analysis

The chemical constituents of the extracts of *C. sorokiniana* and *C. minutissima* were identified by GC–MS (Agilent 6890 series, China) equipped with a fused silica capillary column (30 m × 0.25 mm × 0.25 μm, coated by DB-5), with the EI operating at

70 eV. The injector temperature was set at 250°C. The oven temperature was programmed to hold at 40°C for 1 min, thereafter, with an increase of 3°C/min interval to 280°C. Helium was used as the carrier gas (1 mL/min). The compounds were identified by comparing the mass spectra with those in the National Institute of Standards and Technology (NIST) library [16].

2.9. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The extracts (52.5–200 μg/mL, 150 μL) were mixed with 150 μL of 0.1-mM DPPH radicals (previously dissolved in methanol). The control contained only the DPPH solution without the test samples [24]. The solution was placed in the dark, and the absorbance was measured after 30 min. The radical scavenging activity of the extracts was calculated as percentage of the control.

2.10. $ABTS^{\bullet+}$ (2,2-azinobis 3-ethylbenzothiazoline-6-sulfonate radical) assay

A modified method of Re et al. [25] was used to determine the total radical scavenging activity of the extract. ABTS (7 mM) and $K_2S_2O_8$ (a final concentration of 2.45 mM) were mixed together and placed in the dark for 16 h to generate ABTS radical. Then, the absorbance of the solution was measured at 734 nm and subsequently adjusted to 0.700 with ethanol. The extracts (13–53.3 μg/mL, 150 μL) were added to 150 μL $ABTS^{\bullet+}$ solution. The control experiment contained ABTS radical solution without the extracts. After 15 min, the absorbance was measured with a microtiter plate reader at 734 nm. The antioxidant capacity of the extracts was determined based on the decolorization of the ABTS solution. The radical scavenging activity was calculated as the percentage of the control.

2.11. Hydroxyl radical scavenging assay

The extracts (160–640 μg/mL) were added to a tube containing deoxyribose (20 mM, 120 μL), phosphate buffer (0.1 M, 400 μL), hydrogen peroxide (20 mM, 40 μL), and $FeSO_4$ (500 μM, 40 μL). The volume of the solution was made up to 800 μL with distilled water, and the solution was incubated at 37°C for 30 min. Five hundred microlitres of trichloroacetic acid (2.8%) and 400 μL of thiobarbituric acid (0.6%) were added to the solution. The tubes were placed in a boiling water bath (100°C) for 20 min for color development [26]. The absorbance of the solution was measured at 532 nm. The hydroxyl radical scavenging activity of the extracts was calculated using the formula described in Equation 1:

$$(\%)\ \text{Inhibition} = (\text{Abs}_{\text{con}} - \text{Abs}_{\text{sam}})/\text{Abs}_{\text{con}}{}^{*}\ 100 \qquad \text{[Equation 1]}$$

where Abs_{con} is the absorbance of the control experiment without the extracts, and Abs_{sam} is the absorbance of the test solution containing the extract.

2.12. Metal chelating assay

One hundred fifty microlitres of freshly prepared $FeSO_4$ (500 μM) was introduced into a mixture of 168 μL of 0.1 M Tris–HCl (pH 7.4), sodium chloride (0.8% (*w*/*v*), 218 μL) and the extracts (32–160 μg/mL). A solution containing 0.25% of 1,10-phenanthroline (3 μL) was added to the mixture [27]. The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe^{2+} chelating ability was subsequently calculated using Equation 1.

2.13. Cholinesterase activity assay

The acetylcholinesterase (AChE) inhibitory activity of the extracts was evaluated as described by Perry et al. [28]. Forty microlitres of (0.28 U/mL) enzyme (AChE type V from electric eel; Sigma Aldrich), 140 μL of 3.3 mM 5,5-dithiobis-(2-nitrobenzoic) acid (prepared in 0.1 M phosphate-buffered solution, pH 7.0, containing 6 mM $NaHCO_3$), extracts (5.3–21.3 μg/mL) and 80 μL of phosphate buffer (pH 8.0) were added to each well of the microplate. The solution was incubated for 20 min at 25°C. Acetylthiocholine iodide (Sigma-Aldrich, Germany) (0.05 mM, 40 μL) was added to each well, and the absorbance was determined using a microtiter plate reader (Synergy MX Biotech) at 412 nm for 3 min immediately after the addition of the substrate. The same experiment was used to determine the butyrylcholinesterase (BChE from equine Serum, Sigma-Aldrich) activity of the extracts using butyrylcholine iodide. The enzyme inhibitory activities were expressed as percentage inhibition.

2.14. Inhibition of β-amyloid ($A\beta_{1-42}$) peptide aggregation

2.14.1. Preparation of peptides

β-Amyloid fragment 1–42 ($A\beta_{1-42}$) was dissolved in 200 μL of 50-mM sodium hydroxide. The solution was left for 3 min, after which 700 μL of deionized water was added to the same tube. The solution was made up to 1 mL with phosphate-buffered saline (PBS) to give a final concentration of 25 μM. The solution was sonicated for 3 min and centrifuged (Heraeus Multifuge 3SR+, Thermo Fisher Scientific, Germany) at 4000 × *g* for 20 min at 4°C [29]. Algal extracts (80 and 160 μg/mL) were added to the solution containing $A\beta_{1-40}$ (final concentration, 5 μM) and incubated at 37°C. Aliquots were taken at different time intervals (6, 24, 48, 72 and 96 h) for Thioflavin-T assay and electron microscopy study.

2.14.2. Thioflavin-T (ThT) assay

This assay was used to determine Aβ aggregation and disaggregation kinetics. One millimolar of thioflavin-T solution (prepared in glycine-NaOH, pH 8.5) was added to the aliquots taken at intervals ($A\beta_{1-42}$ with/without algal extracts) and vortexed for 10 s. Thioflavin fluorescence intensity signals were measured using a microtiter plate reader (Synergy Mx BioTek, USA) at excitation (450 nm) and emission (480 nm) wavelengths. The control experiment was without the extracts. ThT solution and phosphate-buffered saline, pH 6.6, was used as a blank. This was done at time t = 0, 24, 48, 72 and 96 h) [30].

2.14.3. Transmission electron microscopy

This analysis was used to determine the presence or absence of Aβ aggregates. Ten microliters (10 μL) of the $A\beta_{1-42}$ peptide incubated with/without the algal extracts was taken at intervals t = 24, 48, 72 and 96 h and placed on a carbon-coated copper grid. The grid was negatively stained with a drop of uranyl acetate solution (2%). After drying for 1 h, the grids were scanned under a transmission electron microscope (Carl Zeiss Libra 120 Plus [120 KV], Germany).

2.15. Statistical analysis

The experiments were carried out in triplicate, and results are expressed as mean ± standard error of mean (SEM). The data were subjected to analysis of variance (ANOVA) to determine differences between the extracts using GraphPad prism 6.0. Differences at $P < 0.05$ were considered to be significantly different.

3. Results and discussion

3.1. Phenolic content of algal extracts

Molecular identification of the algal isolates revealed the two algae to be *C. sorokiniana* and *C. minutissima*. The total phenol content assay revealed detectable levels of polyphenols in the microalgal extracts. The results given in Table 1 show that the polar extracts presented significantly ($P < 0.05$) higher phenolic content than less polar and

Table 1
Total phenol (mg/g) and flavonoid contents (mg/g) of hexane, dichloromethane and ethanol extracts of *C. sorokiniana* and *C. minutissima*.

Extract	Total Phenol (GAE mg/g)	Total flavonoid (QE mg/g)
C. sorokiniana		
Ethanol	14.21 ± 0.37^{a}	11.54 ± 0.32^{f}
Dichloromethane	10.13 ± 0.17^{b}	18.36 ± 0.52^{a}
Hexane	6.94 ± 0.36^{c}	14.36 ± 0.29^{d}
C. minutissima		
Ethanol	13.35 ± 0.39^{d}	10.83 ± 0.29b
Dichloromethane	7.70 ± 1.23^{e}	20.65 ± 0.82^{c}
Hexane	4.89 ± 0.47^{f}	10.21 ± 0.52^{b}

Values are presented as mean ± standard deviation of replicates (n = 3). Values with different superscript letters along the same column are significantly different ($P < 0.05$).

non-polar extracts. The highest total phenol content was exhibited by ethanol extracts of *C. sorokiniana* (14.21 mg GAE/g) and *C. minutissima* (13.35 mg GAE/g). The hexane extracts of both species (*C. sorokiniana* [6.94 mg GAE/g] and *C. minutissima* [4.98 mg GAE/g]) had the lowest total phenol contents (Table 1). However, dichloromethane extracts from *C. sorokiniana* (18.36 mg QE/g) and *C. minutissima* (20.65 mg QE/g) exhibited the highest total flavonoid contents compared to that of both ethanol and hexane extracts, respectively (Table 1).

3.2. FTIR characterization

The FTIR spectra shown in Fig. 1A and B were used to identify the functional groups of the phytochemicals present in *C. sorokiniana* and *C. minutissima*. The bands obtained from the FTIR analysis of both algal species were similar. The results confirmed the presence of the following functional groups O—H, N—H, —C═C, CH, —C═O, COO^{-}, P═O, C—O, and C—O—C in both algal species. The bands obtained in the FTIR spectral were compared with those given in previous reports. The results given in Fig. 1 revealed some bands at 3261 cm^{-1} and 3253 cm^{-1} for *C. sorokiniana* and *C. minutissima*, respectively. These bands are within 3700–3300 cm^{-1}, which is the characteristic region for hydroxyl groups (O—H) and stretching of primary amines (N—H). The O—H functional group represents the presence of polyphenols or polysaccharides [31]. Hydroxyl (O—H) groups are present in flavonoids and phenolic acids as well as other phytochemicals such as polysaccharides. Furthermore, the bands that appeared at 2899 and 2892 cm^{-1} for *C. sorokiniana* and *C. minutissima*, respectively, could be linked to the presence of lipids including fatty acids. D'Souza et al. [23] reported that the region between 1800 and 1500 cm^{-1} shows features of proteins. The bands identified at 1531 (*C. sorokiniana*) and 1515 cm^{-1} (*C. minutissima*) indicate that some proteins are present in the algal biomasses. The bands identified at 1219 and 1206 cm^{-1} could be features of phytochemicals with phosphodiesters. Phytochemicals with phosphate moieties or sugar phosphate esters are capable of chelating metals [31]. *C. sorokiniana* showed bands at 1170, 1112 and 1106 cm^{-1} in the spectral data, while *C. minutissima* showed a band at 1013 cm^{-1}, which were identified to be polysaccharides. Yee et al. [32] reported that bands between 1200 and 900 cm^{-1} are characterized by functional groups with C—O—, —C—C, C—O and C—O—P, which represent polysaccharides. Hence, our findings suggest that *C. sorokiniana* and *C. minutissima* may contain phenolic compounds, proteins, fatty acids and polysaccharides. Moreover, the growth condition used in the cultivation process in this study may influence the presence or absence of the chemical constituents of the algal strains. Previous reports have shown that different culture conditions may stimulate the production of some chemical compounds and contribute to the biological activities of the algal strains [33,34].

3.3. GC-MS analysis of algal extracts

The chemical constituents of the hexane, dichloromethane and ethanol extracts of *C. sorokiniana* and *C. minutissima* were analyzed and identified by gas chromatography–mass spectrometry. Table 2 and Table 3 revealed that phenols, fatty acids, sterols and terpenes are the main classes of compounds that are present in the extracts of *C. sorokiniana* and *C. minutissima*. Moreover, the abundance of these compounds varies as shown in the results. The percentage composition of fatty acids was high in *C. sorokiniana* extracts compared to that in *C. minutissima*. Some of these fatty acids include n-hexadecanoic acid, 11-hexadecenoic acid, 15-methyl-methyl ester, undecanoic acid, ethyl ester, 2,2-dimethylpropanoic acid and 2,6-di methylnon-1-en-3-yn-5-yl ester. Some fatty acids play important roles in neurological processes such as cognitive function, neuronal plasticity and cholinergic transmission. The presence of fatty acids in the algal extracts corroborates previous reports that indicate that microalgae are rich in fatty acids and serve as a source of nutritional supplements [16]. Furthermore, the GC–MS analysis also revealed the presence of some steroids such as estra-5(10)-en-3-one-17-ol, acetate, D-homoandrostane, pregna-5,8-diene-3-β,11α-diol-20-one diacetate (5α13α), 5α-androstan-17β-ol, 2β, 3β-epoxy-2-methyl- and androstan-3-one, (amino carbonyl) hydrazone, (5α). Steroids are secondary metabolites that are derived from cholesterol. They have diverse structures and biological activities. Sterols are subgroups of steroids and were also identified in *C. sorokiniana* and *C. minutissima* extracts. The sterols detected in the algal extracts include ergost-25-ene-3,5,6,12-tetrol, lanosterol and ergost-22-en-3-ol. Sterols have been reported to exhibit radical scavenging activity, anti-inflammatory activity and extenuate β-amyloid-induced neurotoxicity [16,35]. Terpenes such as neophytadiene, lup-20 (29)-en-28-ol (botulin), and phytol (diterpene alcohol) bacharrane were also identified in the extracts. Appreciable levels of phenols such

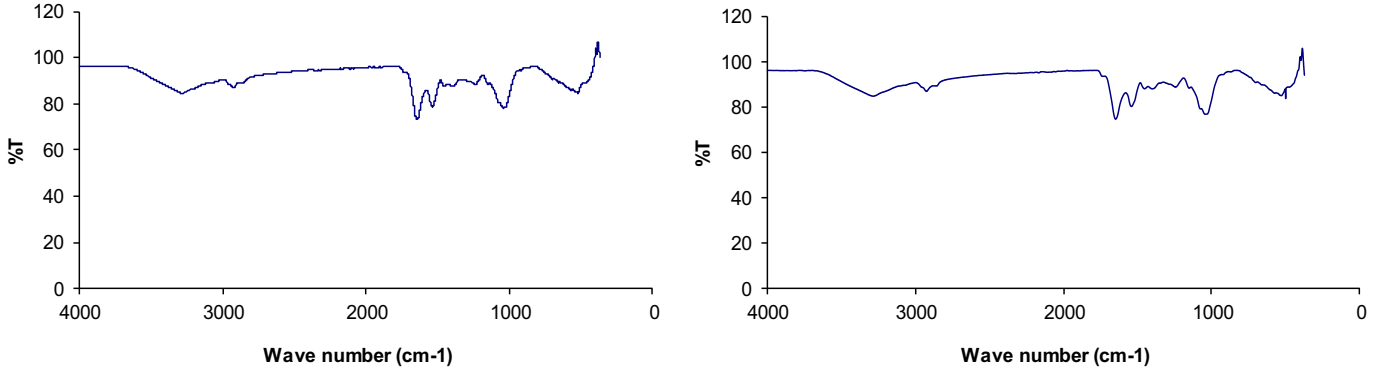

Fig. 1. FTIR analysis of *C. sorokiniana* (A) and *C. minutissima* (B) biomass.

Table 2
GCMS analysis of hexane, dichloromethane and ethanol extracts of *C. sorokiniana*.

Compounds	Relative abundance (%)		
	Hexane	DCM	Ethanol
1. Neophytadiene	–	0.64	–
2. 9,10-Anthracenedione, 1-amino-4-hydroxy-	–	0.33	–
3. Methyl dehydroabietate	–	0.78	–
4. Butylated Hydroxyl Toluene (BHT)	0.64	–	0.83
5. 1,4-Benzenediamine, N-(1,3-dimethylbutyl)-N′-phenyl-	2.55	–	–
6. 3-Buten-2-one, 4-(2,5,6,6-tetramethyl-1-cyclohexen-1-yl)-	–	–	0.19
7. 5-α-Androstan-17β-ol, 2beta.,3.β.-epoxy-2-methyl-	–	–	0.23
8. Isonipecotic acid, N-(3-phenylpropionyl)-, pentyl ester	–	1.17	–
9. Longifolene-(V4)	–	0.59	–
10. Androstan-3-one, (aminocarbonyl)hydrazone, (5.α.)-	–	4.29	–
11. Phenol, 2,4-bis(1-methyl-1-phenylethyl	–	5.41	–
12. Tacrine	–	2.01	–
13. Benzenemethanol, 4-(1,1-dimethylethy)-	–	–	0.71
14. Imidazolo[2,1-b]thiazole, 5-(3-indolyl)	–	–	1.10
15. Phenol, 2-[4-amino-6-(2-pyridin-4-ylvinyl)-[1,3,5]triazin-2-yl]-	–	–	0.78
16. Benzo[d]pyrazolo[3,4-b]azepin-3(2H)-one, 5,6-dihydro-5,5-dimethyl-	–	–	0.23
17. Phenol, 4,4′-(1,3,3-trimethyl-1-pr opene-1,3-diyl)bis-	–	–	6.49
18. Methyl dehydroabietate	–	–	0.89
19. 1H-Inden-5-ol, 2,3-dihydro-3-(4-hydroxyphenyl)-1,1,3-trimethyl-	–	–	0.63
20. [2-(5-Methoxy-2-methyl-1H-indol-3-yl)-ethyl]-(6-methyl-pyridin-2-ylethyl)-amine	–	–	0.41
21. 4-[3-Ethoxypropylamino]benzo-1,2,3 -triazine	–	1.35	1.10
22. Myristamide, N-ethyl-	–	–	1.60
23. Cyclopentane, 1-butyl-2-propyl-	0.24	2.87	–
24. 2-Myristynoyl-glycinamide	–	–	1.41
25. 1,2-Benzisothiazole, 3-(hexahydro-1H-azepin-1-yl)-, 1,1-dioxide	–	–	2.01
26. D-Homoandrostane, (5.α.,13.α)	–	2.00	–
27. Phenol, 4-[2-[4-(dimethylamino)phenyl]-5-phenyl-1H-imidazol-4-yl]-	–	2.56	–
28. Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-(1α,2β,5β)	0.31	–	–
29. 2-Pentadecanone, 6,10,14-trimethyl	0.40	–	–
30. 4-Chloro-6-methoxy-2-methylquinoli n-8-amine	–	–	1.61
31. Methyl (5-hydroxy-1H-benzimidazol-2-yl) carbamate	–	–	2.78
32. Ergost-22-en-3-ol, (3β,5α,22E,24R)-	–	1.56	–
33. 1,2-Benzenedicarboxylic acid, bis2-methylpropyl) ester	0.33	–	–
34. 7,9-Di-tert-butyl-1-oxaspiro eca-6,9-diene-2,8-dione	11.87	–	–
35. cis-10-Nonadecenoic acid	1.61	–	–
36. n-Hexadecanoic acid	11.24	0.52	–
37. 11-Hexadecenoic acid, 15-methyl-methyl ester	–	0.10	–
38. Hexanoic acid, 3,5,5-trimethyl- 2,7-dimethyloct-1-en-3-yn-5-yl ester	–	1.28	–
39. Aziridine, 1-(1,2,3,4-tetrahydro-2-naphthyl)-	–	0.12	–

–: Not determined.

as butylated hydroxyl toluene, butylated hydroxyl anisole, phenol and 2,4-bis(1-methyl-1-phenylethyl) were also identified in the extracts. Phytophenols are known for their radical scavenging and metal chelating activities [36,37].

3.4. Radical scavenging activities of microalgae extracts

The results of the DPPH radical scavenging activity of the extracts are shown in Table 4. All the extracts exhibited DPPH radical scavenging activity at different capacities. No significant difference was observed between the radical scavenging activities of ethanol (145.26 μg/mL) and dichloromethane (143.85 μg/mL) extracts of *C. minutissima*. Moreover, both extracts exhibited the highest radical scavenging activity compared to other extracts as shown by their IC_{50} values given in Table 4. Similar results were observed in the ethanol (146.06 μg/mL) and dichloromethane (146.05 μg/mL) extracts of *C. sorokiniana*. There was no significant difference in the radical scavenging activity of both extracts, but they showed slightly lower scavenging activity than *C. minutissima* extracts. Hexane extracts from *C. sorokiniana* and *C. minutissima* showed lower radical scavenging activity than other extracts. The highest radical scavenging activity of the hexane extracts were 41% (*C. sorokiniana*) and 49.3% (*C. minutissima*) at the highest concentration (210 μg/mL).

There was no significant difference in the ABTS radical scavenging activities of all the extracts as shown in Fig. 2. The ethanol, dichloromethane and hexane extracts of both algal species exhibited strong scavenging activities against ABTS radical with percentage scavenging activity of 85.35 ± 0.46, 84.45 ± 0.46 and 85.03 ± 0.64 (*C. sorokiniana*) and 84.24 ± 1.60, 84.45 ± 0.63 and 86.05 ± 0.47 (*C. minutissima*) at their highest concentration (53.33 μg/mL), respectively (Fig. 2).

Furthermore, the microalgae extracts scavenged OH radicals as shown by the IC_{50} values in Table 4. Hexane extracts of *C. sorokiniana* (352.93 μg/mL) and *C. minutissima* (344.35 μg/mL) exhibited the strongest scavenging activity against OH radicals, although the latter exhibited higher activity than the former. Ethanol and dichloromethane extracts of *C. minutissima* (464.11 μg/mL) had significantly higher scavenging activity than *C. sorokiniana* (482.63 μg/mL), respectively. The observed radical (DPPH, ABTS and OH) scavenging activities of the extracts depict their antioxidant capacity. These results suggest that *C. sorokiniana* and *C. minutissima* extracts contain antioxidants that are capable of donating electrons, mopping up free radicals and preventing radical-induced oxidative damage. The observed OH radical scavenging activity of the extracts also suggests their ability to prevent the production of highly reactive radicals and lipid peroxidation product (malondialdehyde). Antioxidants play a major role in preventing the initiation and progression of some neurodegenerative diseases [38]. Due to minimal levels of antioxidant defense and high polyunsaturated fatty acid content, brain cells are susceptible to attack by free radicals, which may lead to neuronal damage, loss of cholinergic transmission and cognitive dysfunction [39]. The radical scavenging activities of *C. minutissima* and *C. sorokiniana* extracts could be linked to the

Table 3
Chemical constituents of ethanol, dichloromethane and hexane extracts of *C. minutissima*.

Compounds	Relative abundance (%)		
	Hexane	DCM	Ethanol
1. Erythritol	1.98	–	–
2. 2,5-cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-hydroxy-4-methyl-	0.40	–	–
3. Butylated hydroxytoluene	0.99	0.09	0.18
4. 3-Tert-Butyl-4-hydroxyanisole	–	0.07	0.24
5. 2,4-Di-tert-butylphenol	–	–	0.16
6. 7,9-Di-tert-butyl-1-oxaspiro (4,5)d	–	–	8.23
7. N-Methyl-N-n-butyl-2-alpha-naphthylethenylamine	–	0.03	–
8. Neophytadiene	–	0.16	–
9. Psoralen, 3-(alpha,alpha-dimethylallyl)-	–	4.29	–
10. Phenol, 2-amino-4-tricyclo(3,7)]dec-1-yl-	–	0.06	–
11. 5β-Androstan-17β-ol, 2β,3β-epoxy-2-methyl	0.15	0.34	–
12. Resorcinol, 4-[(2-hydroxy-5-methyl-3-pyridyl)azo]	0.20	–	–
13. Imidazolo[2,1-b]thiazole, 5-(3-indolyl)	1.56	–	–
14. 4b,8-Dimethyl-2-isopropylphenanthrene, 4b,5,6,7,8,8a,9,10-octahydro-	0.61	–	–
15. 5-Nitro-3-phenyl-1H-indazole-	–	0.20	–
16. 1H-Inden-5-ol, 2,3-dihydro-3-(4-hyl)indan-6-ol	–	0.87	0.93
17.Phenol, 4,4′-(1,3,3-trimethyl-1-propene-1,3-diyl)bis-	3.60	0.54	–
18. 4-[3-Ethoxypropylamino]benzo-1,2,3-triazine	0.42	1.13	–
19. Lanosterol	–	2.14	–
20. 2,2-Dimethylpropanoic acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester	0.99	2.11	–
21. Pregna-5,8-diene-3β,11α-diol-20-one diacetate	–	1.75	–
22. Noraporphin-7-one, 4,5,6,6a-tetradehydro-10-ethoxy-1,2,9-trimethoxy-	1.84	–	–
23. Nor-17beta(H)-hopane	1.19	2.64	–
24. D-Homoandrostane, (5α,13α)-	–	2.05	–
25. Hexanoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	–	0.70	–
26. Bromo-4,5-dimethoxycinnamic acid	–	0.96	–
27. 2-Ethylacridine	–	0.28	–
28. Lup-20(29)-en-28-ol	0.46	–	–
29. 4-Chloro-6-methoxy-2-methylquinolin-8-amine	–	–	1.61

– Not determined.

presence of some phytophenols such as butylated hydroxyl toluene and butylated hydroxyl anisole and diterpenes (phytol). Betulin — a triterpene that was identified in *C. minutissima* extracts — may also contribute to its antioxidant activity. Previous report has established that betulin is a potent antioxidant [40].

3.5. Metal chelating activities

The microalgal extracts exhibited metal chelating activities at 32–160 μg/mL. According to the IC_{50} values in Table 4, ethanol extracts of *C. minutissima* (102.60 μg/mL) exhibited the highest metal chelating activity followed by ethanol extract of *C. sorokiniana* (107.84 μg/mL). Hexane extract of *C. sorokiniana* (Percentage chelating activity below 50%) exhibited the lowest chelating activity compared to the dichloromethane extracts. These results revealed that *C. minutissima* and *C. sorokiniana* extracts possess metal chelating capacities. Many antioxidants have been reported as potent metal chelators. Hence, the observed chelating activities could be associated with the phenolic compounds that were identified in the extracts. Fe^{2+} ions are capable of reacting with hydrogen peroxide or lipid hydro peroxides, which leads to the formation of highly reactive radicals that are initiators of lipid peroxidation [41]. The products of lipid peroxidation are capable of attacking cells, thereby causing cell injury and/or damage.

3.6. Cholinesterase inhibitory activities

The algal extracts inhibited AChE and BChE activities at different concentrations. The extract concentrations capable of causing 50% enzyme inhibition are shown in Table 4. Hexane extract of *C. minutissima* exhibited moderate (<50% inhibition) inhibitory effect on AChE, while all other extracts exhibited potent (>50% inhibition) inhibitory activity. Our findings revealed that ethanol extract of *C. sorokiniana* (11.94 μg/mL) exhibited the highest inhibitory effect on AChE activity, followed by ethanol extract from *C. minutissima* (13.34 μg/mL). These results contradict the report

Table 4
IC_{50} (μg/mL) of hexane, dichloromethane and ethanol extracts of *C. sorokiniana* and *C. minutissima*.

Extract	DPPH	OH	Fe^{2+}	AChE	BChE
C. sorokiniana					
Ethanol	146.06 ± 3.16^{b}	524.79 ± 10.37^{f}	107.84 ± 1.08^{c}	11.94 ± 0.11^{a}	12.40 ± 0.22^{f}
DCM	146.05 ± 3.17^{b}	508.34 ± 5.90^{a}	130.61 ± 0.45^{d}	16.20 ± 0.17^{b}	12.89 ± 0.12a
Hexane	–	352.93 ± 6.22^{c}	–	21.97 ± 0.96^{c}	–
C. minutissima					
Ethanol	145.26 ± 1.17^{e}	464.11 ± 2.85^{d}	102.60 ± 0.98^{b}	13.34 ± 0.11^{d}	14.68 ± 0.32^{d}
DCM	143.85 ± 1.57^{e}	482.63 ± 1.40^{b}	121.84 ± 0.87^{f}	15.23 ± 0.05^{e}	11.78 ± 0.10^{c}
Hexane	–	344.35 ± 2.06^{e}	115.75 ± 2.87^{b}	–	–

Values are presented as mean ± standard deviation of replicates (n = 3). Values with different superscript letters along the same column are significantly different ($P < 0.05$). –: Not determined.

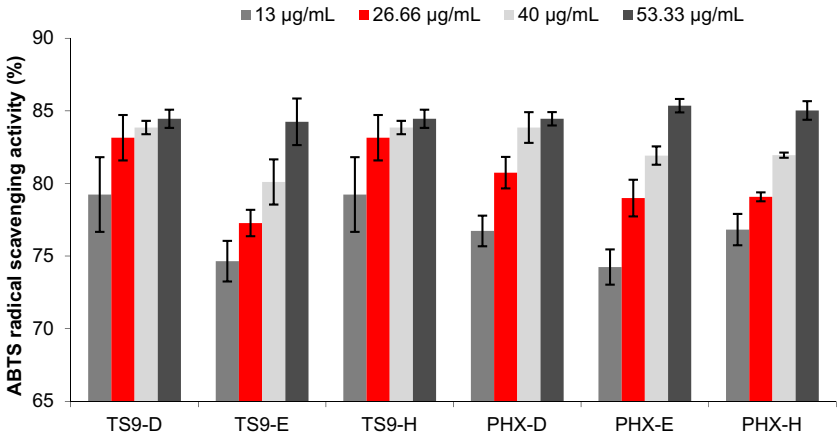

Fig. 2. ABTS radical scavenging activity of *C. minutissima* and *C. sorokiniana* extracts. Key: TS9-D, TS9-E and TS9-H – Dichloromethane, ethanol and hexane extracts of *C. minutissima*; PHX-D, PHX-E and PHX-H – Dichloromethane, ethanol and hexane extracts of *C. sorokiniana*, respectively.

of Pereira et al. [42], which revealed that methanol extracts of *Picochlorum* sp., *Nannochloropsis* sp. and *Desmochloris* sp. did not show potent inhibitory effect on AChE activity. The inhibition of AChE is a common therapeutic strategy for the management of AD. Reduction in AChE activity increases acetylcholine levels at the synaptic cleft in neurons, hence making it available for neurotransmission and thereby improving cognition and memory function [13]. These results suggest that *C. minutissima* and *C. sorokiniana* extracts contain potent inhibitors of AChE, which can be explored for the management of AD. Moreover, ethanol extract of *C. sorokiniana* exhibited the highest AChE inhibitory activity. The AChE inhibitory effect of these extracts may be linked to the presence of phytophenols, diterpenes and triterpenoids. Similarly, the extracts inhibited BChE activity at different concentrations. Hexane extracts of *C. minutissima* and *C. sorokiniana* exhibited moderate (<50% inhibition) inhibitory effect on BChE activity at the highest concentration (5.3–21.3 µg/mL) while other extracts exhibited potent (>50% inhibition) inhibitory activity (Table 4). Dichloromethane extract (11.78 µg/mL) of *C. minutissima* showed the highest BChE inhibitory activity, followed by ethanol (12.40 µg/mL) and dichloromethane (12.89 µg/mL) extracts of *C. sorokiniana*. Ethanol extract of *C. minutissima* (14.68 µg/mL) exhibited the least inhibitory effect as shown by the IC_{50} in Table 4. Our findings revealed that dichloromethane and ethanol extracts of *C. sorokiniana* and *C. minutissima* are more potent against BChE activity than *Botryococcus braunii* and *Nannochloropsis oculata* extracts as reported by Custodio et al. [16]. An impairment in the regulation of BChE activity leads to rapid degradation of acetylcholine and a decline in memory function [13,33]. Furthermore, increase in BChE activity has been linked to a progression in the pathogenesis of AD through the initiation of β-amyloid-induced neurotoxicity in the brain cells of AD patients [43]. Hence, the inhibitory effects of the extract may prevent dysregulation in BChE activity and progression of AD. Most drugs that ameliorate cholinergic dysfunction in AD patients target AChE activity. Hence, the exploration for bioactive compounds with a strong inhibitory effect on AChE and BChE has gained much interest. The observed dual inhibitory effects of *C. minutissima* and *C. sorokiniana* extracts on AChE and BChE could be linked to their bioactive constituents, which could be explored as potential cholinesterase inhibitors for the management of AD. Some of the compounds (phytol and betulin) present in the extracts are dual inhibitors of AChE and BChE [44,45]. Tacrine, a known cholinesterase inhibitor used for the management of AD, was also identified in dichloromethane extracts of *C. sorokiniana.*

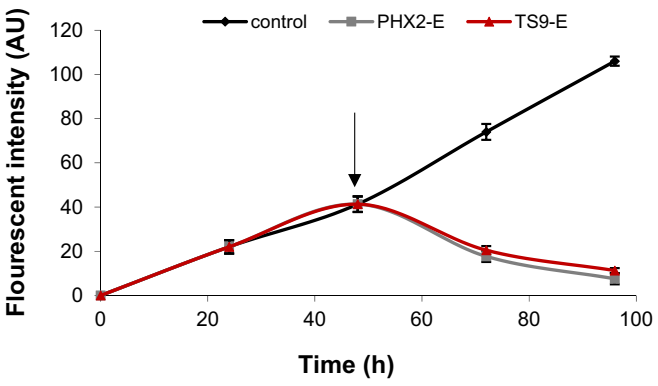

Fig. 3. Effect of ethanol extracts of *C. sorokiniana* on $A\beta_{1-42}$ fibrils as determined by thioflavin-T fluorescence assay. Key: Control – $A\beta_{1-42}$ alone; PHX-E – ethanol extract of *C. sorokiniana* (160 µg/mL); TS9-E – ethanol extract of *C. minutissima* (160 µg/mL). The arrow in the graph indicates the time at which the extract was added to the reaction mixture.

3.7. Effect of ethanol extract of C. sorokiniana and C. minutissima on $A\beta_{1-42}$ fibrils

Fig. 3 shows the results of the thioflavin-T assay, which involves the effect of ethanol extracts of *C. sorokiniana* and *C. minutissima* on $A\beta_{1-42}$ fibrils. $A\beta_{1-42}$ was incubated for 0–96 h at 37°C. The incubation of the protein triggered the formation and aggregation of amyloid fibrils. An increase in fluorescence intensity was observed with increase in incubation time, which indicates an increase in the formation of amyloid fibrils and aggregation of the protein. The ethanol extracts of *C. sorokiniana* and *C. minutissima* (160 µg/mL) were added separately to the reaction mixture after 48 h and then incubated at 37°C. The result revealed that *C. sorokiniana* and *C. minutissima* significantly reduced thioflavin-T fluorescence intensity with increase in incubation time (72 and 96 h). This suggests that the extracts inhibited the formation of $A\beta_{1-42}$ fibrils after 48 h. These results are further confirmed with the data obtained from the electron microscope study. The representative electron micrographs shown in Fig. 4 revealed that the formation of $A\beta_{1-42}$ fibrils increased with incubation time from 6 to 96 h as shown in Fig. 4A, B, C and F. Minimal levels of the fibrils were observed after 6 and 24 h with a low level of aggregation. Furthermore, matured $A\beta_{1-42}$ fibrils were observed after 72 and 96 h (Fig. 4C and F), which indicates further aggregation of the protein. However, *C. sorokiniana* and *C. minutissima* extracts inhibited fibril formation after 72 h with the reduction of the fibrils as shown in

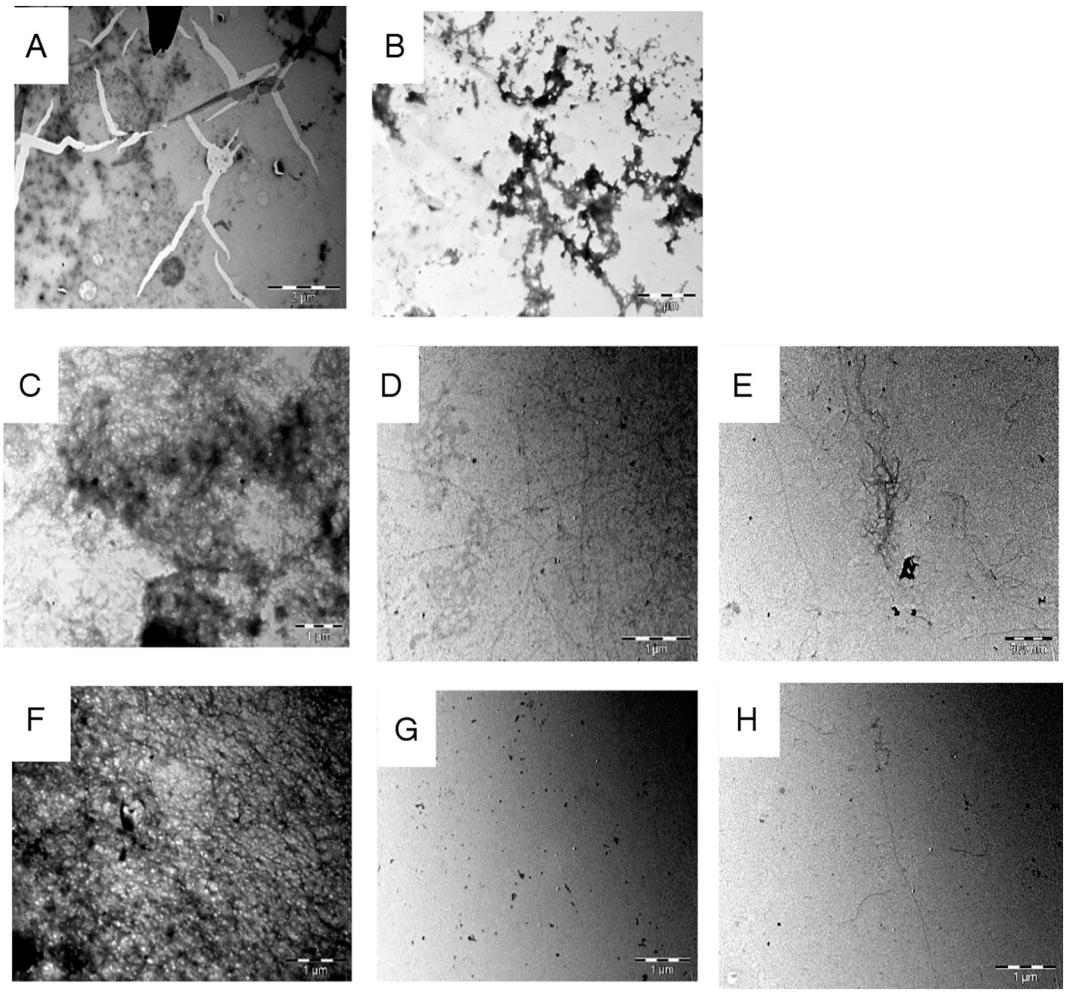

Fig. 4. Representative electron micrographs of the effects of ethanol extracts of *C. sorokiniana* and *C. minutissima* on preformed Aβ1–42 fibrils. (A) $A\beta_{1-42}$ alone after 6 h; (B) $A\beta_{1-42}$ alone after 24 h (C) $A\beta_{1-42}$ alone after 48 h. (D) $A\beta_{1-42}$ [incubated for 48 h] and 160 μg/mL of *C. sorokiniana* extract; (E) $A\beta_{1-42}$ [incubated for 48 h] and 160 μg/mL of *C. minutissima* extract. (F) $A\beta_{1-42}$ alone after 96 h; (G) $A\beta_{1-42}$ [incubated for 96 h] and 160 μg/mL of *C. sorokiniana* extract; (H) $A\beta_{1-42}$ [incubated for 96 h] and 160 μg/mL of *C. minutissima.*

Fig. 4D and E**,** respectively. The disruption of further aggregation of the fibrils was observed after 96 h as shown in Fig. 4G and H. The disappearance of matured fibrils (identified in Fig. 4F after 96 h) was also observed in Fig. 4G and H. This result shows that apart from inhibition of $A\beta_{1-42}$ fibril formation, ethanol extracts of *C. sorokiniana* and *C. minutissima* (160 μg/mL) also caused disaggregation of the fibrils, which was formed after 48 h (Fig. 4A–C) and can be observed in the clear disappearance of the fibrils in Fig. 4G and H. A similar result was obtained by Ramesh et al. [46], which revealed that aqueous extract of *Caesalpinia crista* inhibited and disaggregated $A\beta_{1-42}$ fibrils. Aggregation and deposition of β-amyloid fibrils in neurons are major events that contribute to the pathogenesis of AD [47]. Moreover, the accumulation of β-amyloid fibrils can trigger loss of cholinergic neurons and neurodegeneration in AD [14]. These findings revealed that *C. sorokiniana* and *C. minutissima* showed therapeutic potentials relevant for the management of AD. The mechanism of inhibition and disaggregation of the extracts is not well-understood. However, our findings suggest that fatty acids, sterols and phenolic compounds that are present in the extract may induce disaggregation of the fibrils. Previous reports have established that phenolic compounds [46], sterols [48] and polyunsaturated fatty acids such as docosahexaenoic acid and eicosapentaenoic exhibited anti-amyloidogenic activity [3]. The anti-amyloidogenic activity of *Nannochloropsis oceania* was attributed to omega-3 fatty acids [49] Hence, *C. sorokiniana* and *C. minutissima* could be potential sources of therapeutic agents with potent anti-amylodogenic activity.

4. Conclusion

The radical scavenging, metal chelating and cholinesterase inhibitory activities of hexane, dichloromethane and ethanol extracts of *C. sorokiniana* and *C. minutissima* suggest their potential application as antioxidants and cholinesterase inhibitors. The ability of the ethanol extracts of *C. sorokiniana* and *C. minutissima* to inhibit continuous protein aggregation and induce disaggregation of β-amyloid fibrils suggests their potentials as potent neuroprotective agents, although further work is still in progress to identify the chemical constituents of the extracts by liquid chromatography–mass spectrometry (LC–MS) and determine their neuroprotective effects in neuronal cells. This study revealed the potentials of *C. sorokiniana* and *C. minutissima* extracts to modulate important mediators that have been implicated in the pathophysiology of Alzheimer's disease.

Financial support

We thank the South African Medical Research Council, The World Academy of Science and the National Research Foundation of South Africa for financial support.

Conflict of interest

The authors declare no conflict of interest.

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