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Neuroprotective Effects of *Nannochloropsis oculata* Against AAPH-induced Oxidative DNA Damage in HT22 Cells

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Abstract: Water extracts of *Nannochloropsis oculata* (WNO) were investigated to determine whether they exhibited neuroprotective effects against oxidative-stress-induced cell damage by 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) in murine hippocampus neuronal (HT22) cells. We observed that WNO exhibited scavenging effects on hydroxyl and alkyl radicals as well as on intracellular Reactive Oxygen Species (ROS). As a consequence, WNO reduced AAPH-induced cell death, the fraction of sub-G₁ cells and DNA damage in HT22 cells. The results of this study demonstrate that WNO could be used as an alternative to more toxic synthetic antioxidants as an additive in food, pharmaceutical and cosmetic preparations.

Key words: Neuroprotection, water extract, *Nannochloropsis oculata*, oxidative stress, HT22 cells

INTRODUCTION

Oxidative stress is the accumulation of Reactive Oxygen Species (ROS) caused by increases in ROS production or suppression of ROS destruction. Neurons in the central nervous system are vulnerable to oxidative stress which plays a pivotal role in ischemia-associated neurodegeneration, Alzheimer's diseases, Parkinson's disease, stroke, head trauma and other neurodegenerative diseases (Coyle and Puttfarcken, 1993; Satoh *et al.*, 2006; Satoh and Lipton, 2007). The accumulation of ROS in neurons results in lipid peroxidation, protein oxidation, DNA damage and ultimately cell death (Satoh *et al.*, 2006; Satoh and Lipton, 2007). Therefore, the removal of excess ROS or the suppression of ROS generation by antioxidant activity may prove effective in preventing oxidative cell death. Antioxidants are thus one of the targets of drug development for neuroprotection (Keating, 2008; Satoh *et al.*, 1998; Satoh and Lipton, 2007). Recently, researchers have made considerable efforts to detect natural substances with neuroprotective potential, including a wide array of antioxidants that can scavenge free radicals and protect cells against oxidative damage (Craggs and Kalaria, 2010; Kelsey *et al.*, 2010).

2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) generates two potent ROS capable of inducing lipid peroxidation: alkoxy radical (RO[•]) and peroxy radical (ROO[•]) (Joshi *et al.*, 2006). These radicals are similar to those that are physiologically active and thus might initiate a cascade of intracellular toxic events leading to oxidation, lipid peroxidation, DNA damage and subsequent cell death (Joshi *et al.*, 2006; Yang *et al.*, 2011). We therefore used AAPH to establish a cell culture model of oxidative stress for studying neurotoxicity and neuroprotection in HT22 cells.

Finding new and safe antioxidants from natural sources, such as plant materials, is of current interest worldwide (Pratt, 1992). Such products could be used to prevent oxidative deterioration of food and to minimize oxidative damage in living cells (Pratt, 1992). Recent studies have reported that microalgae are a useful source of pharmacologically active material (Spolaore *et al.*, 2006), including antioxidant compounds. Marine microalgae have been mainly produced and sold as health food due to their high content of proteins, lipids, vitamins and other nutrients. Further understanding of the properties of proteins found in microalgae hydrolysates

would be useful for determining their usefulness as additives for food and nutritional supplements.

Nannochloropsis oculata is a unicellular marine microalga that is an important food source and additive used in the commercial rearing of many aquatic organisms. Live food organisms, such as rotifers, feed on microalgae and in turn, are used to rear the larvae of marine finfish (Durmaz, 2007). *N. oculata* is used as a food source in the aquaculture industry in Korea due to its high chlorophyll a content and diverse pigment composition (Sidharthan *et al.*, 2002). *N. oculata* is also being studied for its high fat and protein content (Renaud *et al.*, 1991). The high protein content of *N. oculata* makes it of interest in our search for novel antioxidants.

Previous studies have shown that various organic solvent extracts of *N. oculata* possessed antioxidant activities or exerted protective effects against H₂O₂-induced cell death (Custodio *et al.*, 2012; Liao *et al.*, 2011). To make use of these extracts as food additives, food grade extraction of *N. oculata* is required, eliminating the use of harmful chemicals or organic solvents in the extraction medium. Plant extracts made with water would have obvious advantages in relation to certification and safety for use in foods (Moller *et al.*, 1999). We therefore used water as the solvent to extract hydrophilic antioxidants present in the microalgae. As it pertains to our interest in evaluating the antioxidant activities of *N. oculata*, we have focused on identifying neuroprotective activities of the water extract.

MATERIALS AND METHODS

***N. oculata* cultures:** The microalga *N. oculata* was originally obtained from the National Research Institute of Aquaculture Fisheries Research Agency (Kanagawa, Japan) and screened for growth and biomass production at the Jeju Center of Korea Basic Science Institute, Korea. The *N. oculata* cells were grown in f/2-Si medium in artificial sea water which has the following composition (per liter): NaNO₃ (75 mg), NaH₂PO₄·H₂O (5 mg), FeCl₃·6H₂O (3.15 mg), Na₂EDTA·2H₂O (4.36 mg), MnCl₂·4H₂O (0.18 mg), CoCl₂·6H₂O (0.01 mg), CuSO₄·5H₂O (9.8 µg), Na₂MoO₄·2H₂O (6.3 µg), ZnSO₄·7H₂O (22 µg), B₁₂ (0.5 µg), biotin (0.5 µg) and thiamine HCl (0.1 mg) (Kim *et al.*, 2011). All solutions were autoclaved at 121°C for 20 min. The vitamin solution was sterilized using a 0.22 µ filter.

A seed culture of *N. oculata* (approximately 1×10⁵ cells mL⁻¹) was used to inoculate 4 L of f/2-Si medium in an Erlenmeyer flask. The culture was kept illuminated with fluorescent lamps (Philips TLM 40W/54RS) at an irradiance level of 50 µmol m⁻² sec⁻¹

using a 12/12 (light:dark) photoperiod cycle. Microalgal cultures were aerated continuously by stirring air and were kept at 26±1°C with 60% of humidity under continually controlled conditions.

Proximate composition of *N. oculata*: After freeze-drying, the proximate composition of *N. oculata* was determined. The crude lipid, ash, carbohydrate and protein contents were determined using the AOAC methods.

Amino acid composition analysis: Two milligrams of freeze-dried *N. oculata* was hydrolyzed with 6.0 N HCl in a sealed vacuum ampoule at 110°C for 24 h for amino acid composition analysis. The HCl was removed from the hydrolyzed sample on a rotary evaporator and the hydrolysate re-dissolved in 2 mL aquadest. Amino acids were separated by ion exchange chromatography and identified by reaction with ninhydrin and photometric detection at 570 nm (440 nm for proline) using an automatic amino acid analyzer (ARACUS, Membrapure; Germany). An amino acid standard solution (100 nM mL⁻¹) was used to calibrate the analyzer and calculate the amount of each amino acid in the sample (Kim *et al.*, 2008).

Preparation of water extracts (WNO): Microalgal cells were collected by centrifugation (4,500 rpm, 4°C, 30 min) and washed twice with deionized water. Microalgal pellet was stored in a freezer at -70°C for 24 h. The sample was then freeze-dried at -50°C at 5 m torr. The freeze-dried sample was ground to a fine powder. For the water extraction, the powdered sample (5 g) was extracted with 500 mL distilled water at 25°C for 24 h. The water extract was collected by filtering and was then concentrated (Heo *et al.*, 2012).

Cell culture: The HT22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, penicillin (100 U mL⁻¹) and streptomycin (100 µg mL⁻¹), cultures were maintained at 37°C in a 5% CO₂ incubator (Heo *et al.*, 2012).

Hydroxyl radical scavenging activity: Hydroxyl radicals were generated using the Fenton reaction and reacted rapidly with nitron spin trap DMPO; the resultant DMPO-OH adduct was detectable with an ESR spectrometer (Rosen and Rauckman, 1984). The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) containing 0.2 mL DMPO (0.3 M), 0.2 mL FeSO₄ (10 mM) and 0.2 mL H₂O₂ (10 mM) using a JES-FA electron spin resonance spectrometer (JEOL, Tokyo, Japan) set to the following conditions: central

field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 ; and temperature, 298 K (Ahn *et al.*, 2007).

Alkyl radical scavenging activity: Alkyl radicals were generated by AAPH. The phosphate buffered (pH 7.4) reaction mixtures contained 10 mM AAPH, 10 mM 14-POBN and indicated concentrations of test samples. Reaction mixtures were incubated at 37°C in a water bath for 30 min (Hiramoto *et al.*, 1993) and then transferred to a 100 μ L Teflon capillary tube. The spin adduct was recorded using a JESFA ESR spectrometer. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5 and temperature, 298 K.

Intracellular reactive oxygen species (ROS) scavenging activity: To detect intracellular ROS, HT22 cells were seeded in 96-well plates at a concentration of 1×10^5 cells mL^{-1} . After 16 h, the cells were treated with different concentrations of WNO (12.5, 25, 50 and 100 μM) and incubated at 37°C in a humidified atmosphere. After 1 h, AAPH was added at a concentration of 10 mM and the cells were incubated for an additional 1 h at 37°C. Finally, 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA; 5 $\mu\text{g mL}^{-1}$) was introduced to the cells and 2',7'-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a PerkinElmer LS-5B spectrofluorometer (Norwalk, CT, USA) (Kim *et al.*, 2006).

Cytotoxicity assay: Cell viability was evaluated by a colorimetric MTT assay. HT22 cells were seeded in a 96-well plate at a concentration of 1×10^5 cells mL^{-1} . After 16 h, the cell were pretreated for 1 h with WNO at different concentration (25, 50, 100 and 200 $\mu\text{g mL}^{-1}$), then 10 μL AAPH 10 mM was added to the cell culture medium and incubated for 24 h at 37°C. MTT stock solution (50 μL ; 2 mg mL^{-1}) was then added to each well to obtain a total reaction volume of volume of 250 μL . After 4 h of incubation, the plates were centrifuged for 10 min at 2,000 rpm and the supernatant was aspirated. The Formazan crystals in each well were dissolved in DMSO. The amount of purple Formazan was determined by measuring the absorbance at 540 nm (Kim *et al.*, 2006).

Determination of DNA damage (Comet assay): A comet assay was performed to determine the degree of oxidative DNA damage (Singh *et al.*, 1995). The cells were seeded in 96-well plates at a concentration of 1×10^5 cells mL^{-1} . After 16 h, the cells were pretreated for 1 h with WNO at

different concentrations (100 and 200 $\mu\text{g mL}^{-1}$). AAPH (10 μL , 10 mM) was then added to the cell culture medium. Cells were incubated for 1 h at 37°C, harvested and washed twice with PBS. The harvested cells were mixed with 100 μL of 0.7% low melting point agarose and added to 1.0% normal melting point agarose-coated slides. After cooling at 4°C for 10 min, the slides were covered with another 100 μL of 0.7% LMPA and allowed to solidify at 4°C for 40 min. Slides were then immersed in lysis solution (2.5 M NaCl, 100 μM EDTA, 10 mM tris, 1% sodium lauroylsarcosine and 1% Triton X-100) for 1 h at 4°C. The DNA was unwound, followed by electrophoresis at 25 V/300 mA for 20 min. The slides were then neutralized by soaking in 0.4 M Tris buffer (pH 7.5) for 10 min 2 times and dehydrated with 70% ethanol. The percent fluorescence in the DNA tail of each cell (tail intensity, TI; 50 cells from each of 2 replicate slides) on the ethidium-bromide-stained slides was measured by image analysis (Kinetic Imaging, Komet 5.0, UK) using a fluorescence microscope (Leica DMLB, Germany).

Cell-cycle analysis: Cell-cycle analysis was performed to determine the proportion of DNA damage sub-G₁ hypodiploid cells (Nicoletti *et al.*, 1991). HT22 cells were seeded in a 96-well plate at a concentration of 1×10^5 cells mL^{-1} . After 16 h, the cell were pretreated for 1 h with WNO at different concentration (100 and 200 $\mu\text{g mL}^{-1}$), then 10 μL AAPH 10 mM was added to the cell culture medium. After 12 h of incubation, the cells were harvested at the indicated time and fixed in 1 mL of 70% ethanol for 30 min at 4°C. They were then washed twice with PBS and incubated in the dark in 1 mL of PBS containing 100 μg Propidium Iodide (PI) and 100 μg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect of fucoxanthin on the cell cycle was determined by changes in the percentage of cells in each phase of the cell cycle and assessed with histograms generated by software programs CellQuest and ModFit (Wang *et al.*, 1999).

Statistical analysis: All data are presented as the Mean \pm SD of at least three replicates. Significant differences among the groups were determined using the unpaired Student's t-test. $p < 0.05$ was considered statistically significant (Kim *et al.*, 2010).

RESULTS AND DISCUSSION

Marine microalgae are sources of natural antioxidants and a variety of bioactive compounds with potential medicinal value have been isolated from these organisms

(Chisti, 2007; Kang *et al.*, 2012). Among the microalgae, *N. oculata* is an important source of antioxidants for human consumption (Custodio *et al.*, 2012; Liau *et al.*, 2011). However, it is unknown whether *N. oculata* exerts neuroprotection against AAPH-induced neuronal damage. We have thus evaluated the neuroprotective effects of WNO against oxidative stress in HT22 cells.

Microalgae vary in their proportions of protein (6-52%), carbohydrate (5-23%) and lipid (7-23%) (Brown and Jeffrey, 1995). We found that the approximate composition of freeze-dried *N. oculata* in mass culture is as follows: 41.6±1.9% protein, 19.4±2.1% lipid, 13.1±1.9% carbohydrate, 21.7±2.4% ash and 3.2±0.4% moisture, on a dry weight basis (Table 1). Proteins are composed of different amino acids and hence the nutritional quality of the protein is determined by the content, proportion and availability of its amino acids. The amino acid composition of *N. oculata* is illustrated in Table 2. Glutamic (11.9%) and aspartic (10.2%) acid were the most abundant amino acids, representing up to 22.1% of the total amino acids. According to Mabeau and Fleurence (1993), high levels of aspartic and glutamic acids are responsible for the special flavor and taste of seaweed. Leucine, proline and alanine are the second group of amino acids found in high levels in *N. oculata* (9.8, 9.3 and 8.1%, respectively). Many proteins from animal and plant sources have been shown to have antioxidant activities that protect against the peroxidation of lipids or fatty acids upon hydrolysis. Others actively scavenge active oxygen species (Okada and Okada, 1998).

We measured the ability of WNO to scavenge hydroxyl radicals, alkyl radicals and intracellular ROS (Fig. 1). Cells treated with 200 µg mL⁻¹ WNO demonstrated reduced levels of hydroxyl and alkyl radicals (40.9 and 79.6%, respectively) (Fig. 1a, b), indicating that the extract possesses radical scavenging activity; radical scavenging occurred in a dose-dependent manner. The intracellular ROS scavenging activity of WNO was 21.4% at 25 µg mL⁻¹, 33.5% at 50 µg mL⁻¹, 44.6% at 100 µg mL⁻¹ and 68.3% at 200 µg mL⁻¹ (Fig. 1c). This antioxidant activity in the hydrophilic extract could be due to the presence of proteins, as reported by other authors (Kang *et al.*, 2012; Kang *et al.*, 2011).

The WNO was further evaluated to determine whether it could protect against neuronal cell death induced by AAPH. MTT assays revealed dose-dependent excitotoxicity of AAPH (2.5-40 mM) on primary cultured hippocampal neurons (Fig. 2a). Stimulation with 10 mM AAPH resulted in a loss of cell viability of about

Table 1: Proximate compositions of the *N. oculata*

Contents	Concentration (%)
Crude protein	41.6±1.9
Crude lipid	19.4±2.1
Crude carbohydrate	13.1±1.9
Ash	21.7±2.4
Moisture	3.2±0.4
Total	100

Table 2: Amino acid composition of the *N. oculata*

Amino acid	Concentration (%)
Aspartic	10.2
Threonine	5.3
Serine	4.2
Glutamic	11.9
Glycine	5.9
Alanine	8.0
Cysteine	0.0
Valine	5.4
Methionine	0.9
Isoleucine	4.4
Leucine	9.8
Tyrosine	3.0
Phenyl-alanine	4.9
Histidine	3.9
Lysine	4.4
Ammonium	1.5
Arginine	7.0
Proline	9.3
Total	100.0

50%; we therefore, used 10 mM AAPH to induce cell insults in all subsequent experiments. AAPH treatment alone induced a reduction in cell viability to 41.2%. Inclusion of WNO in AAPH cell treatments (25, 50, 100 and 200 µg mL⁻¹) prevented AAPH-induced cell damage, restoring cell viability to levels of 43.5, 47.1, 53.7 and 64.1%, respectively (Fig. 2b). The activity of WNO, as observed in this study, implies that WNO could be used as a natural antioxidant to potentially reduce the levels of pro-oxidants such as free radical and ROS.

DNA damage is known to be one of the most sensitive biological markers for evaluating oxidative stress (Heo *et al.*, 2005; Kim *et al.*, 2006). The comet assay (single cell gel electrophoresis) is a rapid and sensitive fluorescence microscopic method for detection of primary DNA damage on the individual cell level. This technique is extensively used to evaluate the genotoxicity of test substances (Ahn *et al.*, 2007; Heo and Jeon, 2009). We thus used the comet assay to measure the protective effects of WNO on DNA damage in AAPH-treated cells. The exposure of cells to AAPH increased the cell tail length and the amount of DNA in the tails increased by 61.8%. Treatment of cells with 200 µg mL⁻¹ WNO resulted in reduction of the increase in tail DNA to 30.4% (Fig. 3a). We also examined photomicrographs of different DNA migration profiles after treatment with WNO (Fig. 3b). In

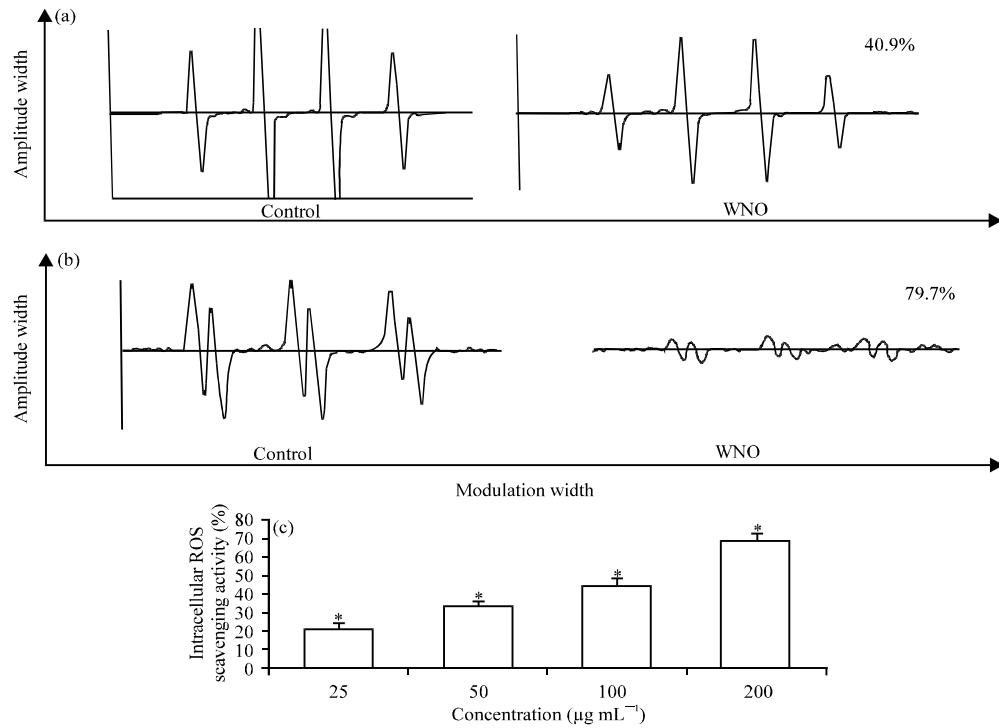


Fig. 1(a-c): Effects of WNO on scavenging radical and reactive oxygen species (ROS), (a) Hydroxyl, (b) Alkyl radicals were detected using ESR spectroscopy and (c) Intracellular ROS was detected by DCFH-DA method. *Significantly different from control at $p < 0.05$

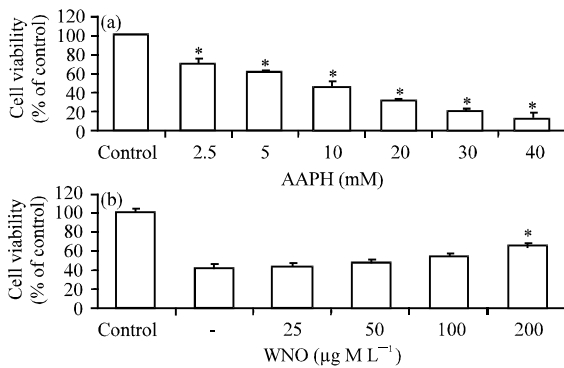


Fig. 2(a-b): Protective effects of WNO against AAPH-induced cell death. Cell viability was assessed 24 h after AAPH stimulation, (a) Dose-dependent cytotoxic effects of AAPH on cell viability using MTT assay and (b) Effects of WNO on the loss of HT22 cell viability after exposure to 10 µL of 10 mM AAPH as determined by MTT assay. *Significantly different from control at $p < 0.05$

the positive control (AAPH treatment only), DNA was severely and systemically damaged. The quantity of tail DNA was reduced by WNO.

In addition to our DNA content evaluations, the protective effects exerted by WNO were confirmed via, flow cytometry. DNA content analysis of AAPH-treated HT22 cells revealed an increase in the proportion of cells with sub-G₁ DNA content to 26.0% (Fig. 4). This result indicates that DNA damage was induced by AAPH. However, the addition of WNO (200 µg mL⁻¹) along with AAPH significantly reduced sub-G₁ DNA contents (11.7%), suggesting that WNO protected the cells against AAPH-induced DNA damage and that the cells were protected against injuries associated with oxidative stress. Our results show that WNO can be used as an effective natural antioxidant and may also prove useful in functional food and pharmaceutical field, owing to its protective effects against cell damage.

More in-depth research is needed to define the health benefits and clinical effects of *N. oculata*. However, these findings demonstrate the protective effects of *N. oculata* against oxidative stress in HT22 cells. This neuroprotection is mediated, at least in part, through free radical scavenging.

These results indicate that *N. oculata* could be a natural source of neuroprotective agents and could be applied to the functional food field. Further studies

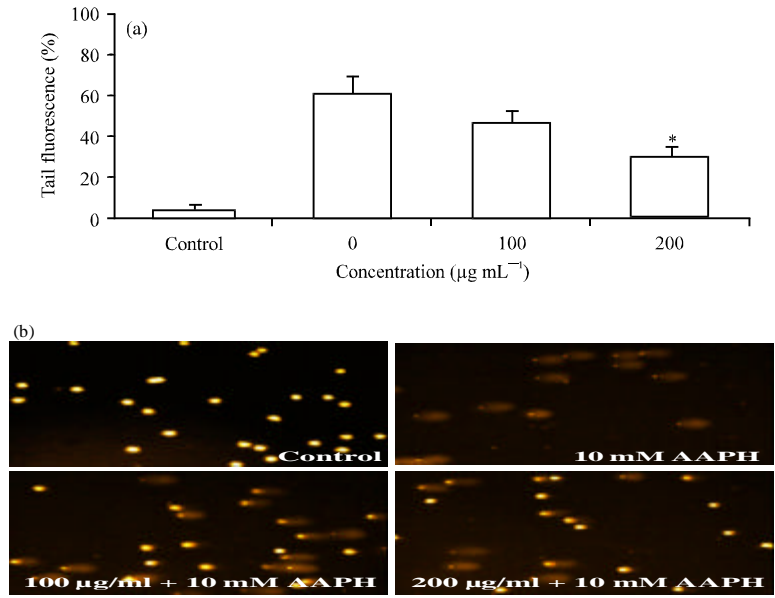


Fig. 3(a-b): Protective effects of different WNO concentrations against AAPH-induced DNA damage, effect on (a) DNA tail and (b) DNA migration. *Significantly different from control at p<0.05

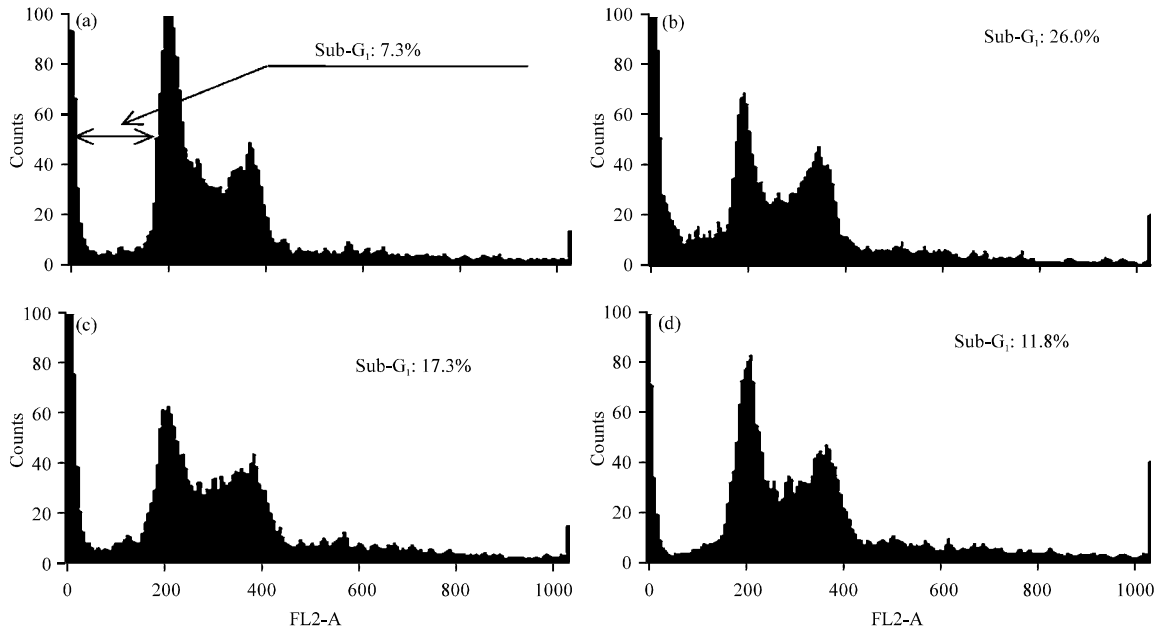


Fig. 4(a-d): Effects of WNO on cell cycle and DNA damage in HT22 cells of (a) Control, (b) 10 mM AAPH, (c) 100 µg mL⁻¹ WNO+10 mM AAPH and (d) 200 µg mL⁻¹ WNO+10 mM AAPH groups. The cells were stained with PI and analyzed via flow cytometry

are necessary to isolate the bioactive peptides from WNO, to elucidate the mechanism of their neuroprotective effects and to characterize their *in vivo* neuroprotection activity.

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