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NATURAL DRUGS

WATER-SOLUBLE COMPOUNDS OF LETTUCE INHIBIT DNA DAMAGE AND LIPID PEROXIDATION INDUCED BY GLUCOSE/SERUM DEPRIVATION IN N2A CELLS

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Abstract: Oxidative stress, increase of lipid peroxidation and resultant DNA damage are associated with pathophysiology of many human diseases such as acute and chronic CNS injuries and diseases, cancer, and also aging. This work was done to investigate whether water fraction from the hydroalcoholic extract of green leaf lettuce (*Lactuca sativa* L.) can protect N2a cells against glucose/serum deprivation (GSD)-induced lipid peroxidation and DNA fragmentation. The cells were cultivated for 12 h in GSD condition in the absence or presence of the lettuce fraction. The total antioxidant ability of the lettuce water fraction was determined using ferric reducing antioxidant power (FRAP) assay. The intracellular lipid peroxidation was evaluated by malondialdehyde (MDA) level. DNA damage was determined using single cell gel electrophoresis. Using FRAP assay, the antioxidant activity of lettuce water fraction was found to be 574 µmol/g, which is equivalent to 64.1 mg of pure ascorbic acid. Exposure of the cells to GSD condition led to a significant increase of MDA level and DNA fragmentation. Lettuce extract at 400 µg/mL could decrease the elevated intracellular lipid peroxidation and DNA damage. The present study demonstrates that lettuce exerts genoprotective effect through inhibition of oxidative stress.

Keywords: lettuce, water fraction, DNA damage, lipid peroxidation, antioxidant activity

It is well documented that oxidative stress, increase of lipid peroxidation and resultant DNA damage are major contributors to the pathophysiology of a variety of neurodegenerative disorders including ischemic stroke (1). By disruption of normal structure and function of cell and organelle membrane lipid bilayers, lipid peroxidation may alter membrane permeability, transport processes and ?uid plasticity and also result in the generation of several toxic aldehydes including malondialdehyde (MDA) and acrolein (2). These highly reactive electrophilic aldehydes react directly with DNA and proteins and these types of protein or DNA adducts, if not efficiently removed, deregulate cell homeostasis, which may finally lead to apoptosis (3, 4). It has been well documented that DNA damage is associated with many human diseases such as cancer, diabetes, neurological degeneration, and also aging (5–7). Therefore, to protect body cells against DNA damage, a balance must be preserved between reactive oxygen production and the antioxidant defense system (8). Consumption of natural antioxidants like vitamin A, ascorbic acid, vitamin E and plants flavonoids has positive effect in combating the oxidative stress (7).

Lettuce (*Lactuca sativa* L.), a vegetable of Compositae (Asteraceae) family, is an important leafy vegetable mainly consumed fresh in salad and a rich source of phytochemicals with antioxidant activity such as phenolic compounds, vitamins A, C and E, as well as minerals, which are essential for promoting health and preventing diseases (9–11). It has been reported to have some medicinal values including anti-inflammatory, analgesic and sedative-

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hypnotic activity (11–13). Deshmukh and co-workers showed that lettuce protect neurons against D-galactose-induced oxidative stress (14). Also, a recent study demonstrated that phenolic extract of romaine lettuce can inhibit H_2O_2-induced cytotoxicity *via* antioxidant activity (15). We previously found that ethyl acetate and water fractions of lettuce hydroalcoholic extract have cytoprotective properties against cell death induced by glucose and serum deprivation (16). The present work was carried out to investigate whether water fraction of lettuce hydroalcoholic extract has inhibitory effect on GSD-induced lipid peroxidation and DNA damage in N2a cells.

EXPERIMENTAL

Cell lines and chemicals

High glucose (4.5 g/L) Dulbecco's modified Eagles medium (DMEM), glucose-free DMEM and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Low melting point (LMP) and normal melting point (NMP) agarose were obtained from Fermentas (Glen Burnie, MD, USA). Ethylene diaminetetraacetic acid disodium salt (Na_2EDTA), Tris(hydroxymethyl)aminomethane, t-octylphenoxypolyethoxyethanol (Triton X-100), ethidium bromide, trichloroacetic acid, butylated hydroxytoluene, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) and thiobarbituric acid (TBA) were obtained from Merck (Darmstadt, Germany). Bicinchoninic acid (BCA) protein assay kit was purchased from Sigma (St. Louis, MO, USA).

Preparation of water fraction from the hydroalcoholic extract

The fresh green leaf lettuce (*Lactuca sativa* L.) collected from Neyshabure (North-East area of Iran) was identified by the herbarium of School of Pharmacy (Mashhad University of Medical Sciences, Iran) and a voucher specimen (12596) has been deposited in this institute. Fifty grams of the aerial parts were dried, powdered and subjected to the extraction for 24 h with 70% ethanol using Soxhlet apparatus. The extract was then dried on a water bath and the residue was suspended in 400 mL of distilled water. Then, it was partitioned with ethyl acetate (6 × 300 mL). The ethyl acetate-soluble fraction was separated and the remained solution further partitioned with *n*-butanol (6 × 300 mL). The superior *n*-butanol layer was also removed and the lower water-soluble layer was considered as water fraction (WF) (17–19). The resulting fraction was dried on a water bath (the yield was 19.9%) and working solutions were made up in culture media.

Cell culture and treatment

The N2a (mouse neuroblastoma cell line) cells were cultivated in high-glucose DMEM supplemented with 10% FBS. The cells at sub-confluent stage were harvested using trypsin and seeded in 96-well plate. Twenty four hours later, the standard medium was replaced by glucose- and serum-free DMEM (GSD) to induce DNA damage. The cells were further incubated in this condition for 12 h at 37°C and 5% CO_2. In treatment groups, the cells were preincubated with WF (50 and 400 µg/mL) for 2 h and then incubated simultaneously for another 12 h in GSD condition. These doses were chosen based on IC_{50} (concentration of 50% inhibition) calculated from earlier experiments (16). Blank and solvent controls were treated identically.

Ferric/reducing antioxidant power (FRAP) assay

The total antioxidant ability of the WF was determined using ferric reducing antioxidant power (FRAP) assay based on the method of Benzie and Strain (20) with some modifications (21). Briefly, to 1 mL WF in 0.1 M phosphate buffer (pH 7.3), 3 mL of freshly prepared and prewarmed (37°C) FRAP reagent (consisting of 300 mM acetate buffer pH = 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \times 6H_2O$ in the ratio of 10 : 1 : 1, v/v/v) was added and the reaction mixture was incubated at 37°C for 10 min. The ferric reducing ability of WF (the absorbance of the blue colored complex of Fe^{II}-TPTZ) was read against the blank (3 mL FRAP reagent + 1 mL phosphate buffer 0.1 M, pH 7.3) at 593 nm. Standard solutions of Fe^{II} in the range of 100 to 1000 µM were prepared from ferrous sulfate ($FeSO_4 \times 7H_2O$) in distilled water. The data were expressed as µmol ferric ions reduced to ferrous form per gram of the extract (FRAP value). Ascorbic acid was used as a reference. The antioxidant assay was repeated three times (22).

Lipid peroxidation assay

The end product of lipid peroxidation is malondialdehyde (MDA), which reacts with TBA to form a pink-colored complex with a peak absorbance at 530 nm (23). To perform the assay, the treated cells were scraped into trichloroacetic acid (2.5%, 1 mL) and centrifuged at 13000 × g at 4°C for 2 min. The lysate supernatant (500 µL) was removed and added to trichloroacetic acid (15%, 400 µL) and TBA 0.67%/butylated hydroxytoluene 0.01% (800 µL). BHT was added to prevent sample oxidation during processing. This mixture was vortexed, boiled for 20 min and then the reaction was stopped by cooling in an ice water bath. After cen-

trifugation at 2500 rpm for 10 min at 4°C, the fluorescence intensity of supernatant was read in excitation/emission of 530/550 nm (24). The MDA amounts were expressed as nmol/mg protein. Protein content was determined using BCA kit.

Single cell gel electrophoresis (comet) assay

The alkaline SCGE (comet) assay was conducted based on the method described previously (25, 26). After 12 h incubation in glucose- and FBS-free medium, the cells were harvested for alkaline single cell gel electrophoresis (comet) assay. In brief, 10 µL of cell suspension was mixed with 90 µL LMP agarose and the mixture was layered over a microscope slide precoated with a layer of 100 µL NMP agarose. The slides were then covered with a cover slip and placed on ice to allow agarose to cool down and solidify. Finally, another layer of LMP agarose was added on top. The slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, 1% Triton X-100, pH 10) and kept at 0°C overnight. After that, the slides were placed on a horizontal gel electrophoresis platform containing a prechilled alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 40 min. They were then electrophoresed (25 V, 300 mA) at 0°C for 30 min. Finally, the slides were rinsed gently three times with 400 mM trizma solution (adjusted to pH 7.5 by HCl) to neutralize the excess alkali, stained with 50 µL of 20 µg/mL ethidium bromide, and covered with a cover slip. While undamaged cells resemble an intact nucleus without a tail, damaged cells have the appearance of a comet. For analysis, 150 nuclei

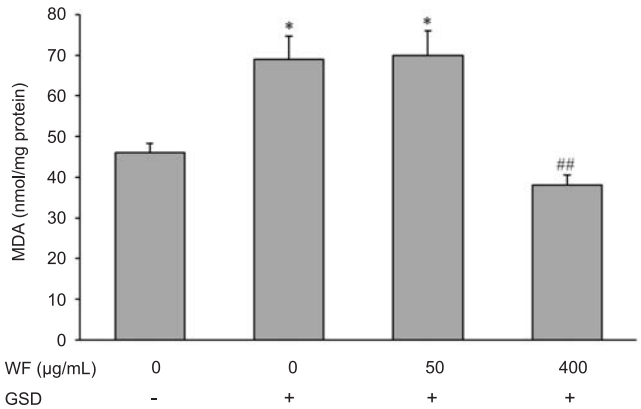

Figure 1. Effect of water fraction (WF) of lettuce on lipid peroxidation induced by glucose/serum deprivation (GSD) in N2a cells. The lipid peroxidation level was evaluated by measuring the concentration of malondialdehyde (MDA). Data are the mean ± SEM of two independent experiments performed in triplicate; *$p < 0.05$ *vs.* untreated cells cultured in normal condition; ##$p < 0.01$ *vs.* untreated cells cultured in GSD condition

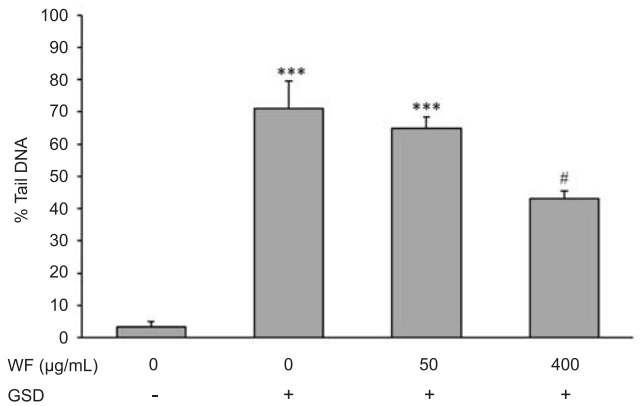

Figure 2. Effect of water fraction (WF) of lettuce on DNA damage in N2a cells cultured in the glucose/serum deprivation (GSD) condition. The percent of DNA in the comet tail (% tail DNA), which is an estimation of DNA damage, was determined using comet assay. Data are mean ± SEM of two independent experiments performed in triplicate; ***$p < 0.001$ *vs.* untreated cells cultured in GSD condition; #$p < 0.05$ *vs.* untreated cells cultured in GSD condition

were randomly selected from three replicated slides and examined using fluorescence microscope equipped with an excitation filter of 520–550 nm and a barrier filter of 580 nm. The percent of DNA in the comet tail (% tail DNA), which is an estimation of DNA damage, was analyzed using the computerized image analysis software (CASP software).

Statistical analysis

The results were presented as the mean ± SEM. Comparison between groups was made by one way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Differences were considered significant when p values were less than 0.05.

RESULTS

Total antioxidant activity

Using the FRAP assay, the antioxidant activity of lettuce water fraction was found to be 574 µmol/g of dry water fraction of lettuce leaves, while the antioxidant potential of pure vitamin C was 8960 µmol/g.

Effect of lettuce on lipid peroxidation

Exposure of N2a cells to glucose- and serum-free condition resulted in a significant ($p < 0.05$) increase of MDA level (69.0 ± 5.7 nmol/mg protein) as compared to control cells cultured in standard condition (46.0 ± 2.4 nmol/mg protein). The content of MDA was significantly ($p < 0.01$) decreased in the cells treated with 400 µg/mL (38.0 ± 2.5 nmol/mg protein) of the WF (Fig. 1). The lettuce in low concentration (50 µg/mL) failed to change MDA concentration (70.0 ± 6.0 nmol/mg protein).

Effect of lettuce on oxidative DNA damage

The cells cultured in standard condition showed only minor DNA fragmentation (3.4 ± 1.5%) as estimated by measurement of percent of DNA in the comet tail (% tail DNA). However, as shown in Figure 2, glucose and serum deprivation significantly increased the percent of DNA fragmentation (71.0 ± 8.5%, $p < 0.001$). A significant decrease in DNA damage was seen following treatment with 400 µg/mL (43 ± 2.5%, $p < 0.05$) of the WF. Again, the lettuce fraction was ineffective on DNA damage at concentration of 50 µg/mL.

DISCUSSION AND CONCLUSION

In the present study, we showed that lettuce has an overall protective effect against glucose/serum deprivation-induced lipid peroxidation and DNA fragmentation. Deprivation of cells from glucose and serum is a reliable *in vitro* model for induction of cytotoxicity and evaluation of cytoprotective activity of natural materials (27). It is well documented that lipid peroxidation induced by oxidative stress plays an important role in DNA damage (6). Since the GSD-induced overproduction of MDA was markedly inhibited by WF of lettuce, it is reasonable to assume that protective effect of this fraction is mediated, at least partially, thorough its antioxidant properties. This is in agreement with previous studies, which revealed that lettuce is a good source of antioxidants such as quercetin, caffeic acid, tocopherol, ascorbic acid, carotenoids and phenolic compounds (3, 8). Using FRAP assay, it is estimated that 1 g WF contains an amount of antioxidant power similar to that found in 64.1 mg of pure ascorbic acid (vitamin C), highlighting the enormous antioxidant potential of lettuce. Due to several reasons, one may consider the FRAP assay as suitable method for assessment of total antioxidants in plants. In the FRAP assay, pretreatment is not required, stoichiometric factors are constant and linearity is maintained over a wide range. Moreover, the FRAP assay, unlike other assays, directly measures antioxidants or reductants in a sample. The other assays indirectly measure the inhibition of reactive species (or free radicals) generated in the reaction mixture and therefore, the results also strongly depend on the type of reactive species used (28). Our result is in agreement with that of Tiveron et al. (29) who showed lettuce present high antioxidant activity not only in the FRAP assay (447.1 µmol Fe^{II}/g), but also in the DPPH (2,2-diphenyl-1-picrylhydrazyl radical) (77.2 µmol Trolox/g) and the α-carotene bleaching (90%) methods. The antioxidant activity of lettuce correlated very well to the total phenolic contents, mainly flavonoids (quercetin glycosides, luteolin) and phenolic acids (caffeoyl and p-coumaroyl esters) (29, 30). Heimler et al. (31) also found the considerable polyphenol content and antiradical activity in *Lactuca sativa*.

Antioxidant agents have differing solubilities: water-soluble ascorbic acid, phenolic compounds, glutathione and urate, lipid-soluble tocopherols and carotenoids and intermediate-soluble hydroxycinnamic acids and flavonoids (32, 33). Since we used water-soluble fraction of lettuce, it seems that water-soluble antioxidants such as ascorbic acid and phenolic compounds are mainly involved in the cytoprotective activity of lettuce. Beside, in our preliminary works, it was found that ethyl acetate fraction (intermediary water-soluble antioxidants) of lettuce can also induce cytoprotective activity (unpublished data). Therefore, the exact nature of compounds

responsible for the protective action of lettuce still remained to be elucidated.

Recently, Okada and Okada (34) reported that aqueous extract of lettuce seeds offer protection against amyloid α (Aα)-mediated oxidative stress and cell death using hippocampus neurons, probably through high phenolic content and radical scavenging activity. Neuroprotective effects of phenolic extract of romaine lettuce and its pure caffeic acid derivatives against oxidative stress caused by hydrogen peroxide were also reported in PC12 cells (15).

In summary, our study revealed that water-soluble fraction of lettuce exerts protective effect against glucose/serum deprivation-induced DNA damage. This effect is mediated through inhibition of oxidative stress.

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