Antioxidative and Neuroprotective Activities of Extracts from the Fruit Hull of Mangosteen (*Garcinia mangostana* Linn.)

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Key Words

Antioxidative activity · Neuroprotective activity ·
Garcinia mangostana Linn.

Abstract

Objective: The aim of this study was to investigate the antioxidative and neuroprotective activities of various extracts from the fruit hull of mangosteen (*Garcinia mangostana* Linn., GM). **Materials and Methods:** Four extracts: water, 50% ethanol, 95% ethanol and ethyl acetate, were used. The antioxidative activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl free-radical scavenging assay at extract concentrations of 1, 10, 50 and 100 µg/ml. Based on the free radical scavenging activity of the extracts, two (water and 50% ethanol) were selected for their protective activity in NG108-15 neuroblastoma cells against H₂O₂-induced oxidative stress and for cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Results:** All extracts exhibited antioxidative activity. The water and 50% ethanol extracts showed high free-radical scavenging activity with IC₅₀ values of 34.98 ± 2.24 and 30.76 ± 1.66 µg/ml, respectively. Both water and 50% ethanol extracts exhibited neuroprotective activity on NG108-15 cells. The highest activity was observed at the concen-

tration of 50 µg/ml for both the water and 50% ethanol extracts. For cytotoxicity test, none of the extracts was toxic to the cells except at the high concentration of 100 µg/ml. **Conclusions:** These results suggest that the water and 50% ethanol extracts from the fruit hull of GM may be potent neuroprotectants.

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Introduction

Free radicals have been found to be crucial because they can cause several severe diseases such as cancer, cardiovascular and cell degeneration [1–3]. This damage results from the imbalance between antioxidants and free radicals in the body [4]. Thus efforts are being expended in the search for substances that can prevent and inhibit the activity of free radicals. An important source of antioxidants is daily vegetables and fruits [1, 5, 6].

Oxidative stress-induced cell damage has been shown to be involved in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and stroke [7, 8]. The damage is mediated by reactive oxygen species (ROS), mainly superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). Accumulation of ROS in neuronal cells results in lipid peroxidation, protein and DNA damage,

and finally cell death [9]. Several studies revealed that ROS can be inhibited by antioxidants [10, 11].

The neuroprotective activity of various natural extracts has been reported in the literature [12–19]. The water extract of *Curcuma longa* reduces rat pheochromocytoma PC12 cell death induced by pyrogallol and H₂O₂ [12]. The water-soluble extracts of the seed of *Celastrus paniculatus* have neuroprotective effects against glutamate-induced toxicity in embryonic rat forebrain neuronal cells [13]. The ethanol extract of the seed of *Vitis vinifera* has neuroprotective effects on Mongolian gerbil forebrain [14].

Mangosteen is a tropical fruit available in south-east Asia. It has been used as traditional medicine to treat skin infections, wounds, and diarrhea. Several studies have revealed that xanthenes from the fruit hull exhibit antimicrobial [15] and anti-inflammatory effects [16]. The ethanol extract of *Garcinia mangostana* Linn. (GM) inhibits histamine release, prostaglandin E₂ synthesis [17], and HIV-1 protease [18]. The methanol extract of GM exhibits antiproliferative, apoptotic and antioxidative activities on SKBR3 human breast cancer cell line [19]. However, limited information is available on the neuroprotective activity of GM extracts. Previous reports revealed neuroprotective activities of antioxidative substances in various cell types. Curcumin exhibited dual action on H₂O₂-induced cell damage in NG108-15 cells [20]. The antioxidative activity of tert-butylhydroquinone showed protective activity against cell death in neuroblastoma SH-SY5Y cells [21]. Recently, Choto-san was shown to act as an antioxidant and neuroprotective agent against oxidative damage in NG108-15 cells [22]. In this study, we investigated the antioxidative and neuroprotective activity of the fruit hull extracts of GM against H₂O₂-induced oxidative damage in neuroblastoma NG108-15 cells and the toxicity of the extracts on these cells.

Materials and Methods

Materials

Mangosteens (*Garcinia mangostana* Linn.) were obtained in Prajeenburi Province, Thailand. NG108-15 cell line was kindly provided by Prof. Haruhiro Higashida, Department of Biophysical Genetics, Molecular Medicine and Biotransformatics, Kanazawa University Graduate School of Medicine, Japan. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co., USA. Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were from GIBCO-Invitrogen, USA. Dimethyl sulfoxide (DMSO) was from BDH Laboratories, UK. All other chemicals were of analytical grade.

Extraction

The fruit bodies of mangosteen were cleaned to remove any residual compost. The hulls were separated and then dried. All dried hulls were ground and placed in 70°C distilled water at the ratio of hull powder:water of 1:4. The mixtures were boiled 4 times until no content of tannin was found by testing with 2% gelatin solution. The macerate filtrate was dried at 40–45°C in the hot air oven. The dry powder was macerated at room temperature for 7 days with distilled water, 50% ethanol, 95% ethanol or ethyl acetate. The extracts were filtered and evaporated to obtain the dry crude extracts. The obtained extracts were stored in a desiccator containing dry silica gel prior to use in each experiment.

Antioxidant Activity: DPPH Free Radical Scavenging Activity

The scavenging of DPPH free radicals was used for measuring the antioxidant activity of the extracts according to the method of Blois [23]. Briefly, stock solutions of the extracts were prepared by dissolving 0.1 g of dry extract in 50 ml 50% methanolic solution. The stock solution was diluted with 50% methanolic solution to obtain sample solutions at the concentrations of 1, 10, 50, and 100 µg/ml. The sample solutions were thoroughly mixed with freshly prepared 0.01% DPPH methanolic solutions at the ratio of 1:1, and kept for 30 min in the dark at room temperature. The amount of the reaction mixture was determined by UV-VIS spectrophotometer (U-2000, Hitachi, Japan) at 517 nm. The percentage of free radical scavenging activity was calculated as in the equation below:

DPPH inhibition (%) =

$$1 - \frac{\text{Absorbance in the presence of extract}}{\text{Absorbance in the absence of extract}} \times 100$$

The antioxidant activity of the extracts was expressed as IC₅₀, defined as the concentration of the extract required to inhibit DPPH radicals by 50%, using the exponential curve. Ascorbic acid was used as a standard antioxidant.

Neuroprotective Activity

Neuroprotectivity was evaluated in two separate protocols: neuropreventive and free radical scavenging activities using NG108-15 cells [24].

Cell Culture

NG108-15 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, hypoxanthine thymidine, aminopterin and minocycline solutions as a complete medium. The cells were cultured in 25-cm² flasks with 5 ml complete medium and incubated at 37°C with 5% CO₂. The cells were subcultured every 3–4 days. Cells from passages 4–5 were used for the experiments.

MTT Assay for Cell Viability and Cytotoxicity

The MTT assay was used to assess cell damage by the oxidants and cell viability protection by the extracts. A sample of 100 µl MTT solution (1 mg/ml in medium without FBS) was added to each well of the 96-well plates and then incubated for 3 h at 37°C. The medium was removed and 100 µl DMSO was then added to each well and vigorously mixed to dissolve the formazan crystals. The eluted samples were measured directly in a microplate reader at

550 nm (Microplate Manager, Bio-Rad Laboratories, Hercules, Calif., USA). The percentage cell viability was calculated according to the equation below:

$$\% \text{ of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

Experimental Design

NG108-15 cells were plated at a density of 2×10^3 cells/well in 96-well plates and incubated at 37°C with 5% CO₂ for 48 h. After 48-hour incubation, the cells were treated for the test experiments. To investigate the free radical scavenging or preventive activities of the extracts [25, 26], two different protocols were used.

Simultaneous Treatment. To assess the scavenging activities of the extracts, cells were simultaneously treated with the extracts and H₂O₂. The controls were optimal and normal cell cultures without addition of any extracts or oxidant agents, cells being exposed to oxidative damage without the presence of extracts. Cells were incubated at 37°C with 5% CO₂ for 2 h, washed twice with PBS, and fresh media were added to the wells. Cell viability was determined by MTT assay.

Preincubation Treatment. For the preventive effect of the extracts on cells, the NG108-15 cells were preincubated with various concentrations of the extracts and incubated at 37°C with 5% CO₂ for 2 h. After the removal of the media from the plates, cells were exposed to 200 µM H₂O₂. Cells were incubated at 37°C with 5% CO₂ for 2 h, washed twice with PBS, and fresh media were added to the wells. Cell viability was determined by MTT assay.

Cytotoxicity Assay. The cells were incubated with various concentrations of the extracts at 37°C with 5% CO₂ for 20 h. After 20-hour incubation, cell viability was determined using the MTT assay.

Statistical Analysis

Each experiment was performed at least 3 times. Sets of 7 or 8 wells for the MTT assay were used for each concentration of extracts. Results were expressed as means ± SE. Data were analyzed by one-way analysis of variance (ANOVA) followed by LSD post hoc test. A p value <0.05 was considered statistically significant.

Results

Extraction Yield and Antioxidative Activity of the GM Extracts

The yield of the water, 50% ethanol, 95% ethanol and ethyl acetate extracts was 22.28, 2.01, 6.11 and 6.20%, respectively. The water, 50% ethanol, 95% ethanol, ethyl acetate extracts and ascorbic acid solution showed scavenging activity with IC₅₀ values of 34.98 ± 2.24, 30.76 ± 1.66, 58.46 ± 0.98, 77.84 ± 0.57 and 7.12 ± 0.36 µg/ml, respectively (table 1). Thus the water and 50% ethanol extracts had more effective scavenging activity than the extracts of 95% ethanol and ethyl acetate, but less than that of ascorbic acid solution, a standard antioxidant.

Table 1. Scavenging activity of DPPH radical of the water, 50% ethanol, 95% ethanol and ethyl acetate extracts

Extracts	IC ₅₀ , µg/ml
Water extract	34.98 ± 2.24
50% ethanol extract	30.76 ± 1.66
95% ethanol extract	58.46 ± 0.98
Ethyl acetate extract	77.84 ± 0.57
Ascorbic acid solution	7.12 ± 0.36

IC₅₀ values are the concentrations scavenging 50% DPPH radicals. Each value represents the mean ± SE of three different measurements.

Neuropreventive Activity

As determined by MTT assay, oxidative stress by H₂O₂ resulted in a decrease in cell viability by 62.44 ± 4.86% as compared with the control group (fig. 1). The water and 50% ethanol extracts showed the ability to inhibit cell death induced by H₂O₂. The activity of both extracts was found to be concentration-dependent at 1 and 50 µg/ml (fig. 1), however, a significant difference in neuroprotective activity was found only at the concentrations of 50 and 100 µg/ml. The 50% ethanol extract exhibited higher activity than the water extract with 98.27 ± 5.79% and 74.89 ± 7.56% cell viability, respectively. The neuroprotective activity of both extracts decreased at the concentration of 100 µg/ml.

Free Radical Scavenging Activity

Similar results were obtained in the neuroprotective activity test. A decrease in cell viability (52.34 ± 2.3%) compared with the control group was also observed after H₂O₂-induced cell death. GM extracts exhibited free radical scavenging activity. Dose-dependent free radical scavenging activity was observed at concentrations of 1–50 µg/ml for both extracts (fig. 2). However, significant differences in free radical scavenging activity were found at high concentrations of 50 and 100 µg/ml. The free radical scavenging activity of both extracts decreased at the concentration of 100 µg/ml.

Toxicity of GM Extracts on NG108-15 Cells

The GM extracts in the concentration range of 1–50 µg/ml had no cytotoxicity (fig. 3). However, in the presence of 100 µg/ml of 50% ethanol extract, the viabil-

Fig. 1. Neuropreventive activity of the water (□) and 50% ethanol (■) extracts in NG108-15 cells pretreated with GM extracts at various concentrations (1, 10, 50 and 100 µg/ml) for 2 h and exposed to H₂O₂-induced oxidative stress. Two hours later, cell viability was determined by MTT assay. Each value represents the mean ± SE of 3 wells. * p < 0.05; NS = not significant.

Fig. 2. Free radical scavenging activity of the water (□) and 50% ethanol (■) extracts. NG108-15 cells were simultaneously treated with various concentrations of the GM extracts (1, 10, 50 and 100 µg/ml) and H₂O₂. Two hours later, cell viability was determined by MTT assay. Each value represents the mean ± SE of 3 wells. * p < 0.05; NS = not significant.

Fig. 3. Effect of the water (□) and 50% ethanol (■) extracts on the viability of NG108-15 cells. Cells were incubated at 37°C with increasing concentrations of the GM extracts (1, 10, 50 and 100 g/ml) for 20 h. The toxicity of the GM extracts was determined by MTT assay. Each value represents the mean ± SE of 3 wells. * p < 0.05; NS = not significant.

ity of NG108-15 cells decreased to $39.45 \pm 6.93\%$ compared to water extract, indicating toxicity to the cells, whereas the water extract led to a slight decrease in cell viability.

Discussion

NG108-15 cells are a neuroblastoma-glioma hybrid cell line possessing both neuronal and glial properties; they have been widely used as a neuron model in electrophysiology and pharmacology research [27, 28]. Hydrogen peroxide was chosen to induce oxidative cell damage in the cultures because oxidative stress is believed to be an important mediator of neuronal cell death, and has been postulated to contribute to the pathogenesis of various neurodegenerative diseases [29]. Equally important, H_2O_2 is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals and is known to be toxic to many systems [30]. Among a great variety of reactive oxygen species, H_2O_2 plays a pivotal role because it is generated from nearly all sources of oxidative stress. Exogenous H_2O_2 can enter the cells and induce cytotoxicity due to its high membrane permeability [20, 26].

A variety of biological activities such as antimicrobial, anti-inflammatory, antioxidative and antiproliferative activities have been revealed in the extracts of GM [16, 17, 20]. In this study, we determined the antioxidative activity of GM extracts by DPPH assay, which has been commonly used to evaluate the antioxidant activity of specific compounds or extracts [31, 32]. The assay provides information on the reactivity of test compounds with a stable free radical. The bleaching of DPPH absorption is representative of the capacity of test compounds to scavenge free radicals independently of any enzymatic activity. The antioxidative activities of the extracts of water and 50% ethanol were higher than those of 95% ethanol and ethyl acetate extracts; however, they were lower than that of ascorbic acid solution as a standard antioxidant (table 1). Different solvents could extract a different amount of active compound due to distinct affinity of the compounds and the solvents. The results implied that the most active compounds responsible for antioxidant activity are in the 50% ethanol extract. Our results clearly demonstrated that GM extracts were effective in scavenging DPPH radicals.

The ideal neuroprotective compound should not only exhibit free radical-scavenging activity but also modulate endogenous antioxidant defense systems. Activation of the function of endogenous antioxidant systems in tissue

is the characteristic of antioxidant compounds. Many plant extracts or antioxidants have been reported to exert an indirect scavenging effect on free radical-induced tissue injury by activating the function of endogenous antioxidant systems. Previous reports [25, 26] have revealed that Chito-san and *Ginkgo biloba* extracts showed neuroprotective activity by using preincubation and simultaneous treatment. On the other hand, pretreatment with curcumin was unable to protect the cells from H_2O_2 -induced oxidative damage, and instead caused a significant decrease in cell viability after H_2O_2 exposure [26]. Therefore, it is important to search for neuroprotective activity of GM extracts using two separate protocols: preventive and free radical scavenging activities. When the cells were treated with $200 \mu M$ of H_2O_2 , the results showed that H_2O_2 reduced the viability to 50–60% of control. H_2O_2 formed by two-electron reduction of O_2 is not a free radical but an oxidizing agent. In the presence of O_2 and transition metal ions, H_2O_2 can generate $OH\cdot$ via Fenton reaction. In addition, H_2O_2 can easily cross the cell membrane and exerts injurious effects on tissues through a number of different mechanisms, such as perturbing intracellular calcium homeostasis [33], decreasing intracellular ATP [34], inducing DNA damage [35], and inducing apoptosis [36]. Several previous studies have reported the neuroprotective effect of antioxidant and/or free radical-scavenging compounds against H_2O_2 -induced cell damage in various assay systems. Thus the antioxidants appear to be closely related to the neuroprotective effect.

When added simultaneously with H_2O_2 , GM extracts significantly protected NG108-15 cells against H_2O_2 -induced cell damage in a concentration-dependent manner at 1–50 $\mu g/ml$, but not for 100 $\mu g/ml$, compared to treatment with H_2O_2 alone (fig. 1). It is possible that the neuroprotective effect of GM extracts against the H_2O_2 -induced decrease in cell viability may at least partly result from its antioxidant and free radical scavenging properties. Pretreatment with GM extracts was also able to protect NG108-15 cells from H_2O_2 -induced oxidative damage, and instead caused a significant increase in cell viability after H_2O_2 exposure in a concentration-dependent manner at 1–50 $\mu g/ml$, but not at 100 $\mu g/ml$, compared to treatment with H_2O_2 alone (fig. 2). These results suggest that pretreatment of the cells with GM extracts could prevent cell damage by H_2O_2 . Exposure to GM extract alone at the highest concentration (100 $\mu g/ml$) did alter cell viability over the incubation period in comparison with non- H_2O_2 -treated control cells (fig. 3). This high concentration of extracts may cause significant toxicity in

cells under normal conditions resulting in a slight decrease in free radical scavenging activity and neuroprotective activity (fig. 1, 2). 50% ethanol extract showed more neuroprotective activity than the water extracts at all concentrations tested. This result was in accordance with the DPPH radical scavenging activity.

Summarizing our results, GM extracts have a potential to be neuroprotectant against oxidative stress-induced cell damage in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke. Although correlations between in vitro and in vivo results of GM extract have not yet been investigated, a good correlation has been reported in Alzheimer's disease [37], headache [38] and dementia [39] for some plants such as Choto-san, which has shown in vitro neuroprotective and free radical scavenging activity in NG108-15 cells.

Conclusions

This study demonstrates that the water and 50% ethanol extracts exhibited antioxidative and neuroprotective activities in neuropreventive and free radical activities in an in vitro model. The extracts demonstrated no cytotoxicity except at the highest concentration of the extracts (100 µg/ml). This implies that GM extracts have a potential for neuroprotection in oxidative stress-induced cell damage in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke. However, in vivo studies for clinical data should be carried out.

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