Supporting information

Enhanced antioxidant effects of naringenin nanoparticles synthesized using high-energy ball milling method

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1. Materials and Methods

1.1. Chemicals, Materials and Reagents

Naringenin (MW =272.25, purity 98%) was purchased from TCI chemicals (INDIA) Pvt. Ltd. and methanol and MQ water. Primary antibody for cleaved caspase-3 (PA516335, Invitrogen), secondary antibody such as anti-rabbit IgG-Alexa Fluor 488 (Cat no. A-11008) and Annexin V-FITC PI Apoptosis Detection kit were purchased from Invitrogen, India. MTT (with 98% purity), fetal bovine serum (FBS), trypsin (with 98% purity), PBS, glycerol (with 98.2% purity), Triton X-100 (99%), and paraformaldehyde (with 99% purity) were obtained from Hi-Media Labs. Dihydroethidium (DHE; Sigma-Aldrich, D7008), and 2,7-dichlorofluorescin Diacetate (DCFDA; Sigma-Aldrich, D6883). Heat-inactivated fetal bovine serum (Gibco, 10270-106), penicillin–streptomycin (Cyagen, T150506G001), 0.25% trypsin EDTA (Gibco, 25200072), Dulbecco's Modified Eagle Medium without glucose (Himedia, AT186), low-glucose Dulbecco's Modified Eagle Medium (Himedia, AT006), Fluoroshield Mounting Medium with DAPI (Abcam, ab104139).

1.2. Methods

1.2.1. Synthesis of milled naringenin using high energy ball milling

Size reduction of naringenin by planetary ball mill technique included milling medium, balls and material. The balls which could be of different diameter occupy 30 -50% of the mill volume and its size depends on the feed and mill size. The large balls tend to break down the coarse naringenin and the smaller balls help to form fine product by reducing void spaces between the balls. Initially, milling of naringenin is carried out at different milling times these are 120 min, 240 min, 480 min, 600 min, and 720 min. After the milling of naringenin all processed powders are characterized by different characterization techniques. Primary characterization of naringenin included SEM by which we can easily examined that size of naringenin particles are reduced considerably manner. After SEM second most important characterization is XRD by which we concluded that milling process only affects the size of particle rather than other reaction parameters of naringenin. After the basic characterizations spectroscopy analysis of naringenin samples with different milling time to understand the solubility enhancement of naringenin after size reduction.

1.2.2. Scanning Electron Microscopy

For SEM, crude naringenin as well as different samples of milled naringenin were dispersed in double filtered ultrapure type-1 (Milli-Q) water. A drop of the samples was casted onto a clean silicon wafer and dried overnight. Gold coating was performed inside an auto-fine coater (JEOL JEC-3000FC; Tokyo, Japan) at 30 Pa's. The morphology of the crude naringenin as well as different samples of milled naringenin was then explored using a JEOL JSM-7600F scanning electron microscope Tokyo, Japan. The accelerating voltage was kept around 5.0−10.0 kV.

1.2.3. X-ray Diffraction Study

For X-ray diffraction (XRD), crude naringenin as well as different samples of milled naringenin in powdered form were analyzed by placing the on a zero background plate and measuring the wide-angle X-ray diffraction pattern using D-8 Advanced Eco diffractometer (Bruker, Germany) using nickel filtered Cu Kα radiation (λ = 1.54060 Å) with a scanning range 2θ = 5°−90° at room temperature.

1.2.4. UV-Vis Spectroscopy of the crude naringenin as well as milled naringenin samples

The UV-Vis analysis of the crude naringenin as well as different samples of milled naringenin were carried out using UV-Visible Spectrophotometer (Shimadzu, Japan). The UV-Vis Spectrum was scanned for all the samples in the range 200-800 nm. The peaks were recorded and analyzed by comparing the peak shift, peak broadening and absorbance of the spectrum. The value of absorbance for naringenin is taken at λ max and in case of naringenin the value of λmax in the wave-length range of 287- 300 nm. Solubility enhancement study of naringenin is carried out by UV-Visible spectroscopy. All UV-Visible spectra of naringenin recorded for the same dilution of naringenin with different parameters. The value of absorbance is calculated by standard curve of naringenin for different processed samples.

1.2.5. Effect of milling time on solubility of naringenin

In this spectroscopy analysis of different samples of naringenin milled at different time points were analyzed using UV-Vis spectroscopy. The UV-Visible spectrum of crude, 120 min, 240 min, 480 min, 600 min, 720 min processed were scanned and solubility value is calculated from the λmax value of absorbance.

1.2.6. Effect of milling time along with sonication on solubility of naringenin

The UV-Visible spectrum was scanned for all the crude as well as milled naringenin samples in the range of 200-800 nm. Before taking the value of absorbance at λmax all the samples are probe sonicated for 2 min at the amplitude of 20.

1.2.7. Effect of temperature on solubility of naringenin

Different samples of naringenin milled at different time points were analyzed at different temperatures using UV-Vis spectroscopy. Before taking the value of absorbance at λmax all samples were treated at different temperature condition with stirring for 10 min. As we know that as we increasing temperature solubility of flavonoids increases. In this study temperature conditions are 27°C, 50°C, 75°C, and 100°C.

1.2.8. Standard curve of Naringenin

1 mg naringenin was weighed accurately and dissolved in 1ml methanol to make the final concentration 1mg/ml. From the stock concentration, working concentration of 100µg/ml was prepared. 6 different dilutions were prepared from the working concentration i.e. 10, 20, 30, 40, 50, 60µl/ml with methanol and were scanned by UV-Visible spectrophotometer (Shimadzu, Japan) in the range of 200nm- 800nm to observe the linearity in the peak with increase in concentration of the drug. The values were recorded at peak maxima and a standard curve was prepared for the estimation of unknown concentration of the drug.

1.2.9. Dissolution study by UV-Visible spectroscopy

Sample used for the dissolution study were: pure naringenin (2 mg/ml), 120 min processed naringenin (2 mg/ml), 480 min processed naringenin (2 mg/ml), 600 min processed naringenin (2 mg/ml), 720 min processed naringenin (2 mg/ml). The dissolution media 100 ml were solutions at pH value 8.0 (PBS buffer) maintained at 37 ± 0.5 °C and stirred at 200 rpm. Dialysis membrane (obtained from Himedia (LA 392)) was used. Aliquots of the dissolution medium (1 ml) were withdrawn at suitable time intervals. These time intervals are 0 min, 15 min, 30 min, 1 hour, 2 hours, 4hours, 8hours, 12 hours, and 24 hours. The withdrawn samples were replaced by equal volumes of the fresh dissolution medium maintained at the same temperature (according to

the dissolution test (32). The samples were assayed by UV-Visible spectroscopy at the wavelength range of 287-300 nm. Each data point is the average of three determinations.

1.2.10. *In-vitro* biocompatibility assay

The cytocompatibility of milled naringenin samples and crude naringenin against SH-SY5Y human neuroblastoma cells were assessed using the MTT assay. Cells were seeded at a density of 1 × 103 cells/well in 96-well culture plate. Cells were treated with crude naringenin and milled naringenin at different concentrations for 24 h. To determine the *in-vitro* biocompatibility, we have chosen only milled naringenin sample which processed upto 720 min time point (N720). Then we washed the SH-SY5Y cells three times with PBS and incubated for further 24 h. Cell viability of metabolically active SH-SY5Y cells was evaluated for crude naringenin and milled naringenin by means of the EZcount MTT Cell Assay Kit (Himedia) according to manufacturer's protocol. MTT solution was added and incubated for 3−4 h and then formazan crystals were solubilized in 100 μL of solubilization buffer. Absorbance was taken at 590 and 630 nm using ELISA plate reader to calculate percent cell viability.

1.2.11. DCFDA assay for detection of ROS generation

The SH-SY5Y human neuroblastoma cells were treated with hydrogen peroxide (H_2O_2) and different concentrations of crude naringenin (15 μM and 20 μM) as well as different concentrations of milled naringenin (1 μM, 2 μM and 5 μM) and subsequently cells were treated with 2',7'-dichlorofluorescein (DCFH). In response to intracellular reactive oxygen species (ROS), DCFH converts to fluorescent product (DCF). After treatment cells were trypsinized, collected and washed using serum-free media. Cells were then treated with DCFH for 10 minutes in dark. Fluorescent product (DCF) was detected using fluorescence microscopy.

1.2.12. DHE assay for detection of cellular superoxide anion radical generation

The SH-SY5Y human neuroblastoma cells were seeded onto sterile coverslip. After 24 hours, cells were treated with PBS (control), hydrogen peroxide (H_2O_2) as well as different concentrations of crude naringenin (15 μM and 20 μM) as well as different concentrations of milled naringenin (1 μM, 2 μM and 5 μM). Subsequently, cells were then trypsinized, collected and washed using serum-free media and treated with 10μM dihydroethidium (DHE) (in FBS-free

media) and incubated in dark for half an hour at 37°C in CO_2 incubator. After incubation, cells were washed with PBS and imaged using a fluorescent microscope.

1.2.13. Immunofluorescence

To investigate the effects of different concentrations of milled naringenin (1 μM and 2 μM) against hydrogen peroxide (H_2O_2)-treated SH-SY5Y human neuroblastoma cells, we investigated a apoptosis marker i.e., cleaved caspase-3. Briefly, cells (1×10^4) were grown in a sterile 6-well plate onto poly-L-lysine-coated coverslips for 24 hours. Cells were treated with PBS (control), H_2O_2 and different concentrations of milled naringenin (1 μM and 2 μM). After 24 hours, cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min and then again washed three times with PBS. Subsequently, cellular permeabilization was done using0.1% Triton X-100 in PBS for 10 min and then incubated in blocking buffer (5% BSA in PBS + 0.1% Tween 20) for 30 min. After blocking, cells were treated with anti-cleaved caspase-3 primary antibody overnight at 4 °C, then washed with PBS and secondary antibody such as Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (for cleaved caspase-3) was added for 1 h at room temperature, washed with washing buffer, and stained with DAPI for nuclear staining. Cells were washed with PBS, mounted, and imaged using 40× objective of inverted fluorescent microscope (Zeiss Axio Vert.A1).

1.2.14. Evaluation of effects of H_2O_2 and milled naringenin on Apoptotic Cell Death by Annexin V PI Assay Using Flow Cytometry

To further assess the protective effects of milled naringenin against H_2O_2-induced apoptosis in SH-SY5Y cells, Annexin-V/propidium iodide (PI) assay using flow cytometry was performed. The cells were treated with PBS (control) and milled naringenin at the doses of 1 μM and 2 μM. Subseqestly, cells were washed with PBS and transferred to CellPro flow cytometry tubes. Annexin V-FITC (5μL) and PI (1 μL of 100 μg/mL) were added to the cell suspension, followed by incubation at room temperature in the dark for 30 min. After this step, 400 μL of binding buffer was added, and the samples were analyzed by a BD FACS Aria Fusion Flow cytometer (BD Biosciences). The results were then analyzed using CellQuest 3.0 software. The dots in the lower left quadrant, upper left quadrant, upper right quadrant, and lower right quadrant represents viable healthy cells (Annexin V−, PI−), necrosis undergoing cells (Annexin V−, PI+), cells in late apoptosis (Annexin V+, PI+), and cells in early apoptosis (Annexin V+, PI−),

respectively. The experiment was carried out in triplicate and data are expressed as mean ± SEM. Quantification was performed using GraphPad Prism software Version 5.0.

1.2.15. Statistical analysis

Results were expressed as mean ± Standard Error of Mean. All data were analyzed using analysis of variance (ANOVA) followed by Tukey's test. Values of $p<0.05$ were considered as significant. All the statistical analyses were performed using graph pad prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA).

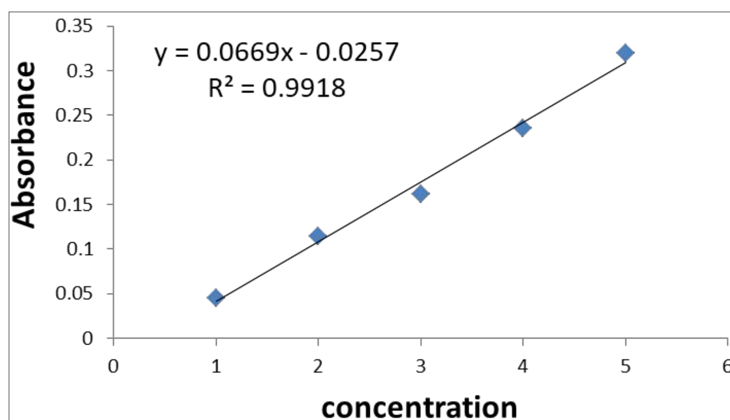

Figure S1: Standard curve drawn for various absorbance versus naringenin concentrations for assessment of its solubility and other properties