

RESEARCH ARTICLE

Induction of Human Hepatocellular Carcinoma HepG2 Cell Apoptosis by Naringin

Ratana Banjerdpongchai*, Benjawan Wudtiwai, Patompong Khaw-on

Abstract

Naringin, a bioflavonoid found in *Citrus* seeds, inhibits proliferation of cancer cells. The objectives of this study were to investigate the mode and mechanism(s) of hepatocellular carcinoma HepG2 cell death induced by naringin. The cytotoxicity of naringin towards HepG2 cells proved dose-dependent, measured by MTT assay. Naringin-treated HepG2 cells underwent apoptosis also in a concentration related manner, determined by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) employing flow cytometry. Mitochondrial transmembrane potential (MTP) measured using 3,3'-dihexyloxycarbocyanine iodide (DiOC₂) and flow cytometer was reduced concentration-dependently, which indicated influence on the mitochondrial signaling pathway. Caspase-3, -8 and -9 activities were enhanced as evidenced by colorimetric detection of para-nitroaniline tagged with a substrate for each caspase. Thus, the extrinsic and intrinsic pathways were linked in human naringin-treated HepG2 cell apoptosis. The expression levels of pro-apoptotic Bax and Bak proteins were increased whereas that of the anti-apoptotic Bcl-xL protein was decreased, confirming the involvement of the mitochondrial pathway by immunoblotting. There was an increased expression of truncated Bid (tBid), which indicated caspase-8 proteolysis activity in Bid cleavage as its substrate in the extrinsic pathway. In conclusion, naringin induces human hepatocellular carcinoma HepG2 cell apoptosis via mitochondria-mediated activation of caspase-9 and caspase-8-mediated proteolysis of Bid. Naringin anticancer activity warrants further investigation for application in medical treatment.

Keywords: Naringin - hepatocellular carcinoma cells - HepG2 cells - apoptosis - caspase-9 - caspase-8

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Introduction

Naringin contains anti-inflammatory activity and induces oxidative stress via reactive oxygen species (ROS) such as superoxide anion radical generation (Lewinska et al., 2015). It shows cytotoxicity against various kinds of human cancer cell lines such as stomach (KATOIII and MKN-7), breast (MCF-7, MDA-MB-231), liver (HepG2, Hep3B and Huh7), cervical (HeLa), colon (Caco-2) and pancreatic (PK-1) cancer as well as leukemic (HL-60, U937, NALM-6 and Jurkat T) cells (Kanno et al., 2005). It also inhibits human cancer cell proliferation, including prostate cancer DU145 cells, cervical cancer HeLa and SiHa cells, chondrosarcoma cells, triple-negative breast cancer MDA-MB-231 cells and colon cancer Colo 320 cells, both *in vitro* and *in vivo* (Ugocsai et al., 2005; Vanamala et al., 2006; Li et al., 2013; Ramesh and Alshatwi, 2013; Tan et al., 2014). The cell growth inhibitory signaling in cancer cells of flavonoid glycosides, including naringin, diosmin and hesperidin, is via oxidative stress, stimulation of DNA double strand breaks (DSBs), DNA hypomethylation, micronuclei formation and apoptotic activation (Lewinska et al., 2015).

Naringin induces HeLa cell apoptosis through caspase-3 expression, nuclear factor-kappaB, cyclooxygenase-2 (COX-2) and caspase-1 (Zeng et al., 2014). At noncytotoxic concentrations, naringin suppresses chondrosarcoma cell migration and invasion by attenuation of vascular cell adhesion molecule-1 (VCAM-1) expression. Since VCAM-1 is linked to metastasis function, naringin down-regulates the synthesis of VCAM-1 (Tan et al., 2014). Naringin induces MDA-MB-231 cell apoptosis through G1 cell cycle arrest, a decrease of p21 due to the expression of inhibitor of apoptotic proteins (IAPs). It also induces MDA-MB-231 cell apoptosis via beta-catenin pathway (Li et al., 2013).

Naringenin, an aglycone form of naringin (structure as shown in Figure 1A), inhibits cancer cell growth, induces cell cycle arrest and apoptosis in HepG2 cells via p53, increased Bax/Bcl-2 ratio, cytochrome c release and activation of caspase-3 (Arul and Subramanian, 2013). The cytotoxic effect of naringin on HepG2 cells exists (Banjerdpongchai et al., 2016), but the mode of cell death and its mechanism remain unclear. Our results firstly demonstrated that naringin inhibited HepG2 cell growth through apoptotic cell death, increased caspases-9,

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-8 and -3 activities, increased pro-apoptotic Bcl-2 protein expression and mitochondrial transmembrane potential reduction.

Materials and Methods

Chemicals and reagents

Naringin (5,7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one) (chemical structure shown in Figure 1A), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), dimethyl sulfoxide (DMSO), dihexyloxacarbocyanine iodide (DiOC₆) and propidium iodide (PI) were obtained from Sigma-Aldrich, (St. Louis, MO, USA). Caspase-3, -8 and -9 activity determination kits, Dulbecco's modified Eagle medium (DMEM), penicillin G sodium, streptomycin and fetal bovine serum were supplied by GibcoBRL, Invitrogen Life Science Technologies, Thermo Fisher Scientific Inc., (Waltham, MA, USA). Annexin-V-Fluos kit and complete mini-protease inhibitor cocktail were obtained from Roche, (Indianapolis, IN, USA). Rabbit polyclonal antibodies to Bcl-xL, Bak, Bid, and mouse monoclonal antibody to Bax and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Abcam, (Cambridge, UK). Super Signal West Pico Chemiluminescent Substrate was obtained from Thermo Fisher Scientific Inc., (Waltham, MA, USA).

Cell culture

Human hepatocellular carcinoma HepG2 cell line was obtained from Dr. Prachya Kongtawelert. HepG2 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum, 25 mM sodium bicarbonate, 20 mM HEPES, 100 µg/ml streptomycin / 100 U/ml penicillin G sodium at 37°C and 5% CO₂.

Cytotoxicity assay

After HepG2 cells were treated with naringin (in dimethyl sulfoxide as a vehicle) at various concentrations for 24 h. MTT solution (dissolved in DMSO less than 0.1%) was added at the final dose of 100 µg/ml and incubated for 4 h at 37°C and 5% CO₂. The medium was removed and the violet crystals were dissolved in DMSO for 30 min. The absorbance was determined at 540 nm and reference wavelength at 630 nm by using spectrophotometer (BioTek, Winooski, VT, USA). The naringin at 10, 20 and 50% inhibitory concentrations (IC₁₀, IC₂₀ and IC₅₀) on HepG2 cell growth were determined and used for further experiments (Banjerdpongchai et al., 2014).

Determination of cell death mode

Hepatocellular carcinoma HepG2 cells were treated with various concentrations of naringin for 24 h and then stained with annexin-V-fluorescein isothiocyanate (FITC) and PI for 15 min. The cells were centrifuged at 200 x g for 5 min and then washed with phosphate-buffered saline (PBS). After the cells were resuspended in PBS, they were processed by using flow cytometry technique and analyzed with the Cell Quest software program of flow

cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) ((Banjerdpongchai et al., 2016),).

Mitochondrial transmembrane potential (MTP) assay

HepG2 cells were incubated with naringin at various concentrations for 24 h. Then the naringin-treated cells were incubated with DiOC₆ at 40 nM (the final concentration) for 15 min and investigated for the reduction of MTP by using flow cytometry technique (Banjerdpongchai and Wilairat, 2005).

Measurement of caspases-3, -8 and -9 activities

After HepG2 cells were treated with naringin at various ICs for 24 h, the cells then were trypsinized. The cells were lysed and the cell lysate with equal amount of proteins was incubated with the caspase-specific tetrapeptide substrates linked to *p*-nitroaniline. The peptides were DEVD-, IETD- and LEHD-*p*-NA for caspase-3, -8 and -9 assay, respectively. The lysate was incubated with each substrate for 1 h and the absorbance was determined by using microplate reader (BioTek, Winooski, VT, USA) (Banjerdpongchai et al., 2013).

Bcl-2 family protein expression levels in naringin-treated HepG2 cells by immunoblotting

After HepG2 cells were incubated with naringin at various doses for 24 h, the cells were washed once in ice-cold PBS. Then the naringin-treated cells were incubated at 4°C for 10 min with ice-cold cell lysis buffer (70 mM KCl, 250 mM sucrose, 0.25% Triton X-100 in PBS containing complete mini-protease inhibitor cocktail). The cell lysate was centrifuged at 20,000 x g for 20 min; the supernatant with the equal amount of protein, 50 µg, were separated by 15% SDS-PAGE; and the separated bands of proteins were transferred onto nitrocellulose membrane. After treatment with 5% non-fat milk in PBS containing 0.2% Tween-20, the membrane was incubated with rabbit polyclonal antibodies to Bak, Bid and Bcl-xL and mouse monoclonal antibody to Bax, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were developed and visualized on X-ray film with Super Signal West Pico Chemiluminescent Substrate. The band density was determined by using densitometer compared to the control protein actin and analyzed by image software.

Statistical analysis

The data were shown as mean ± S.D. performed in triplicate and three times independently. One-way ANOVA was employed for statistical difference in multiple groups, whereas two groups of data were compared by Student's *t*-test with *p* value of <0.05 by using SPSS program version 22.0.

Results

Cytotoxicity of naringin on HepG2 cells

Naringin has inhibitory concentration as follows, 10% (IC₁₀) at 39.67±16.80 µM, 20% (IC₂₀) at 73.1±19.0 µM and 50% (IC₅₀) at 172±10.39 µM, respectively, from MTT assay. Naringenin is less toxic than naringin with

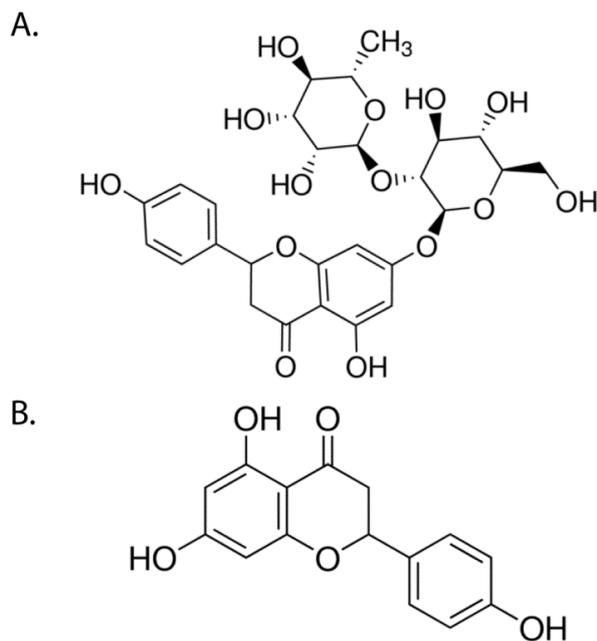


Figure 1. Chemical Structures of Naringin (A) and Naringenin (B)

IC₅₀ concentration more than 200 μ M ((Banjerdpongchai et al., 2016),).

Naringin-induced HepG2 cell apoptosis

Cancer cells undergo programmed cell death by various stimuli such as chemotherapeutic drugs, natural products or radiation through various mechanisms, viz., apoptosis, autophagic cell death and necroptosis (Lu and Walsh, 2012; Safa, 2013; Su et al., 2015). The cytotoxic effect of naringin was due to apoptosis as evidenced by staining with annexin-V-FITC/PI and employing flow cytometry technique. Percent early apoptotic cells increased in a dose response manner (Figure 2). The annexin-V positive and PI negative cell population in the right lower quadrant of the dot plot increased dose-dependently and these cells represented the early apoptotic cells (Figure 2A). Bar graph shows the increase of the early apoptotic cells significantly compared to without treatment or negative control (Figure 2B).

Reduction of mitochondrial transmembrane potential (MTP) in naringin-treated HepG2 cells

Naringin induced the loss of MTP, which was evidenced by the lower fluorochrome DiOC₆ fluorescence intensity

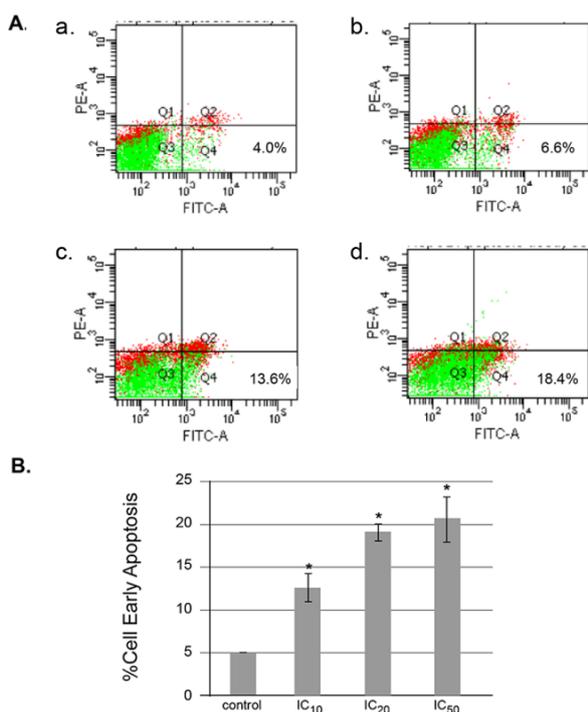


Figure 2. Apoptosis Induction of HepG2 Cells by Naringin. HepG2 cells were treated with naringin at various concentrations for 24 h and stained with annexin-V-FITC/PI and determined by flow cytometry as described in Materials and methods. Dot plot analyses (a) of naringin-treated HepG2 cells are (a) control (without treatment) and various concentrations of naringin at (b) IC₁₀, (c) IC₂₀ and (d) IC₅₀ in μ M unit. Bar graphs (b) of mean percentage and standard deviation (mean \pm S.D.) of early apoptotic cells (annexin-V-FITC positive and PI negative cells) in each concentration are in triplets and from three independent experiments. The significance of statistical value compared to control (without treatment) is marked with asterisk, $p < 0.05$

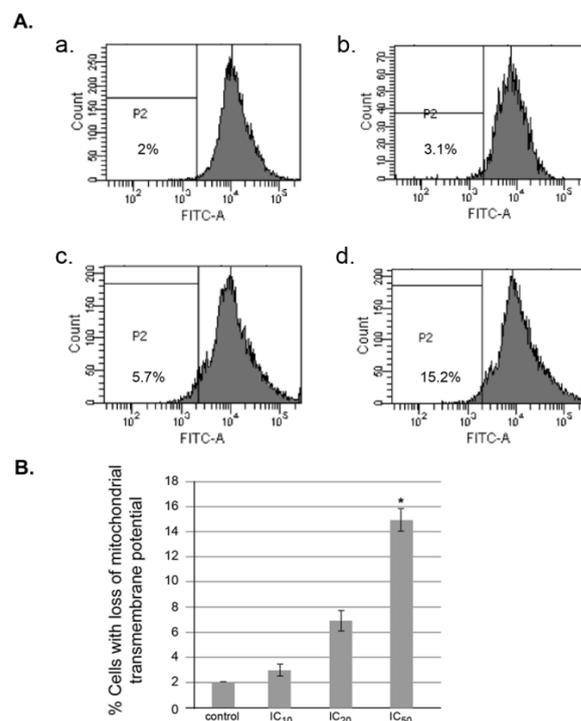


Figure 3. Disruption of Mitochondrial Transmembrane Potential (MTP) in Naringin-treated HepG2 Cells. The human Hepatocellular carcinoma HepG2 cells were treated with naringin at various concentrations for 24 h and the MTP was measured by staining the cells with DiOC₆ and employing flow cytometry. The relative fluorescence intensity was measured. The histograms (a) of naringin-treated cells are shown in (a) control (without treatment) and various concentrations of naringin at (b) IC₁₀, (c) IC₂₀ and (d) IC₅₀. Bar graphs (b) of HepG2 cells treated with naringin corresponding to (a) are shown as mean with the standard deviation (S.D.). The significance of statistical value compared to control (without treatment) is marked with asterisk, $p < 0.05$

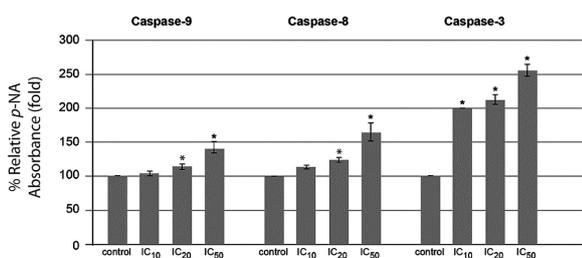


Figure 4. Caspase Activities of Naringin-treated HepG2 Cells. The caspase-9, -8 and -3 activities of HepG2 cells after treatment with naringin for 24 h were measured by using specific tetrapeptide substrates tagged with para-nitroaniline (*p*-nitroaniline) and the absorbance of cleaved *p*-NA was determined by spectrophotometer. The significance of statistical value compared to control (without treatment) is marked with asterisk, $p < 0.05$

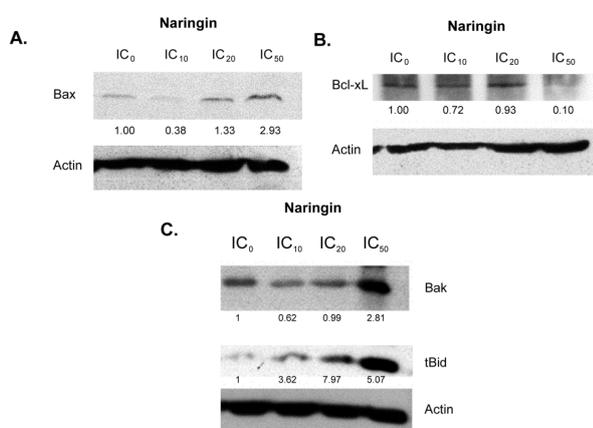


Figure 5. Apoptotic Protein Expression Levels of Bcl-2 family in Naringin-treated HepG2 Cells. The expression of pro-apoptotic Bax (a), anti-apoptotic Bcl-xL (b), and pro-apoptotic Bak and BH3-only Bid in truncated form (truncated Bid, tBid) (c) were demonstrated by immunoblotting. The number under the bands is (are) the fold(s) of band density compared to control as verified by using actin as a constitutive protein

in the mitochondria of HepG2 cells after treatment with naringin compared to control (without treatment) as shown in histogram (Figure 3A). The percentage of cells with MTP reduction increased in a concentration-dependent manner as shown in Figure 3B.

Enhancement of caspases-9, -8 and -3 activities in naringin-treated HepG2 cell apoptosis

Apoptosis is mediated via two main pathways, viz., intrinsic and extrinsic pathways as mentioned above. The caspases-9 and -8 activities are significantly activated in the mitochondrial (intrinsic) and death receptor (extrinsic) pathways, respectively. Caspase-3 is stimulated in the downstream cascade of both initiator caspase-9 and -8. Caspase-3 is an effector or executioner caspase of both pathways (Shalini et al., 2015).

To confirm the mitochondrial and death receptor signaling pathways of naringin-induced apoptosis in HepG2, the specific individual caspase substrate was used

and the product was measured for its absorbance by the calorimetric method of para-nitroaniline. All substrates were linked to the corresponding individual caspase-specific tetrapeptides (Banjerdpongchai et al., 2015b). In naringin-treated HepG2 cells, the caspases-9, -8 and -3 activities increased in a dose-dependent manner especially at IC₅₀ as shown in Figure 4.

Bcl-2 family protein expressions in naringin-induced HepG2 cell apoptosis

Bcl-2 family proteins are categorized into three main types: viz., pro-apoptotic (containing of multidomains, e.g., Bax and Bak); anti-apoptotic (with multidomain, such as Bcl-xL, Bcl-2 and Mcl-1); and pro-apoptotic proteins with BH3 domain-only (BH3-only, such as Bid, Bad, Bim, Noxa and Puma) (Vela and Marzo, 2015). The Bcl-2 family proteins play pivotal roles on apoptosis via mitochondria-mediated (intrinsic) pathway (Vela and Marzo, 2015). However, for Bid (BH3-only) is a molecule linked between extrinsic and intrinsic pathways. It is proteolyzed by caspase-8 from extrinsic pathway and subsequently stimulates mitochondrial pathway.

In naringin-induced HepG2 cell apoptosis, the expression levels of pro-apoptotic proteins (Bax and Bak) increased; whereas that of BH3-only proteins, Bid, increased in its cleaved form (truncated Bid or tBid). Bid is proteolyzed by caspase-8 in extrinsic receptor pathway and tBid activates other pro-apoptotic proteins in intrinsic pathway, indicating that naringin induced HepG2 apoptotic cell death via both intrinsic (Bax, Bak) and extrinsic (Bid) pathways. Whereas the expression of anti-apoptotic protein, i.e. Bcl-xL, decreased; which confirmed the involvement of intrinsic pathway (Figure 5).

Discussion

The active compounds extracted from *Citrus* seeds include the following flavonoids: nobiletin, hesperetin, hesperidin, naringenin, tangeretin and naringin. Naringin is also isolated from grapefruits and at its dose of 200 mg/kg suppresses murine aberrant crypt foci (Vanamala et al., 2006).

The structure of naringin, 4',5,7-trihydroxyflavanone 7-rhamnoglucoside, contains a disaccharide unit (C₂₇H₃₂O₁₄, MW 580.53) (as shown in Figure 1A) whereas the chemical structure of (±)-naringenin, (viz., (±)-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one or described as 4',5,7-trihydroxyflavanone (C₁₅H₁₂O₅, MW 272.25)), is aglycone (Figure 1B). Both are isoflavanone, but the flavanone glycosides will be metabolized by hydrolase enzymes, affecting its permeability, efficiency and activity on the target cells. Generally isoflavanone glycosides contain the sweet taste and are commercially favorable for the consumers.

From the present study, naringin, at IC₅₀ concentrations (172±10.39 μM), induced HepG2 cell early apoptosis 20.5±2.6% (Figure 2A), whereas naringenin (200 μM) induces early apoptosis 4.8% ((Banjerdpongchai et al., 2016)). It indicates that naringin has anticancer activity more than that of naringenin, as evidenced by the percentage cells with exposure of phosphatidylserine (PS)

to the outer layer of cell membrane.

Naringin induces human cancer cell apoptosis via various mechanisms and signalling pathways. In cervical cancer SiHa cells it induces apoptosis via death receptor and mitochondrial pathways with high p53, Bax, Fas and FADD (Ramesh and Alshatwi, 2013).

Naringenin is cytotoxic to leukemic cells more than other kinds of cancer and it induces apoptosis in both HL-60 leukemic and Caco-2 colon cancer cells. The effect of naringin in animals by oral administration shows less suppression on tumor growth than naringenin (Kanno et al., 2005).

Most flavonoids, including naringin, occur as O-glycosides with the most common subunit as glucose. Other mono- or polysaccharides includes glucorhamnose, galactose, arabinose and rhamnose. The beta-linkage of these sugars resists pancreatic hydrolase; hence, the microorganisms in the intestine are responsible for its hydrolysis reaction. The hydrolases in cytosol are also able to deglycosylate flavonoids to allow the site(s) for phase II enzyme conjugation reactions (Heim et al., 2002).

Cecal bacteria in herbivores enzymatically degrade flavonoid polymers and cleave monomeric flavonoids into monophenolic acids. All phenolic acids have lower antioxidant activity than the parent flavonoids, whereas hydroxycinnamates are more active than other derivatives, e.g., hydroxyphenylacetates and hydroxybenzoates (Rice-Evans et al., 1996). The antioxidant activity of phenolic acids correlates to the specific number and plane of hydroxyl groups (Rice-Evans and Miller, 1996).

Factors influencing the biological effects of naringin include structure, absorption, pharmacokinetics, pharmacodynamics, biotransformation and relative activities of metabolites. The structure of flavonoids define their activity so called "structure-activity relationships, SAR" (Cao et al., 1997).

Cytochrome P450 enzymes, e.g., CYP1A isozymes, modifies flavonoids' structure by hydroxylation reaction. Flavonoids inhibit various P450 isoforms, such as CYP1A (Doostdar et al., 2000). Phase II conjugation in the liver and enterocytes consists of glucuronidation, sulfation and methylation reactions. Small amount of free flavonoid aglycones is generated. Reduction capacity of the A-ring is more than that of B-ring and conjugation at the 3'- or 4'-position increases the reduction potential (Spencer et al., 1999).

Proteins in the food, intestine and blood circulation milieu potentially mask the bioactivity of polyhydroxyl flavonoids. Naringin is indirectly modulated by enzymatic reactions, metal (cofactor) chelating, and directly by radical scavenging, which affects its bioavailability and antioxidant efficacy in human-beings (Arts et al., 2002).

Intriguingly some flavonoid glycosides are rapidly up-taken into the gastrointestinal epithelial cells and more available for the organisms compared to aglycone form, due to their interaction and transported by glucose transporters at the intestinal epithelium (Gee et al., 1998).

The anti-proliferative activity of naringin in gastric adenocarcinoma AGS cells is via autophagic cell death with the cytoplasmic vacuoles and autophagosomes. Its signaling mediates through downregulation of PI3K/Akt/

mTOR pathway, upregulation of p21, activation of Beclin 1 and LC3B, and phosphorylation of mitogen activated protein kinases (MAPKs) (Raha et al., 2015).

Naringin completely inhibits apoptotic cell death and blocks DNA damage induced by cytosine arabinoside Ara-C treatment. It inhibits hydrogen peroxide-induced murine leukemia P388 cytotoxicity and apoptosis and reduces Ara-C-induced oxidative stress. Naringin increases antioxidant enzyme activities, i.e., catalase and glutathione peroxidase, leading to apoptosis inhibition (Kanno et al., 2003; Kanno et al., 2004).

Naringin is a hepatoprotective compound due to its suppressive effect on protein phosphatase enzymes of algal toxins, i.e., okadaic acid and microcystin-LR. These two toxins elicit the hepatocyte apoptotic cell death, which is prevented by naringin (Larsen et al., 2002).

Taken together, naringin has both the cytotoxic and protective effect on the liver. From the results, it induced human hepatocellular carcinoma HepG2 cell apoptosis via caspases-9, -8 and -3 activities and the increase of truncated Bid (BH3-only protein) levels. The protein expression levels of Bax and Bak were increased and Bcl-xL level decreased. Therefore, naringin with potential of anti-hepatocellular carcinoma action in vitro may be developed for further clinical treatment. Further toxicity test in both normal animals and human-beings is required.

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