Chapter 14

Antioxidant and cytotoxic activities of Fukugiside- The major biflavonoid from *Garcinia travancorica* Bedd.

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Abstract

Garcinia species are well known as source of complex molecules with diverse biological activities, especially antioxidant and anticancer activities. The present chapter elaborates the *in vitro* antioxidant activity of *Garcinia travancoria* extract and isolated compounds. The biflavonoid fukugiside has been identified as the active compound with significant free radical scavenging activities in DPPH (IC_{50}: 8.34 µg/mL), superoxide(IC_{50}: 6.95 µg/mL), and reducing power assays. Cytotoxicity studies of the biflavonoid fukugiside revealed a dose dependent cancer cell growth inhibition in A431 and HeLa cells. The antiproliferative effect appears to be due to the ability of fukugiside to induce S-phase arrest and apoptotic cell death. In HeLa cells, fukugiside reduced the expression of MAPKp38 by 26.1% compared to untreated control.

Keywords: *Garcinia travancoria,* Biflavonoid, Fukugiside, Antioxidant, Cytotoxicity

Introduction

Cancer, the uncontrolled division of abnormal cells in the body, still remains a threat to humankind. Surgery, chemotherapy, and radiation are the widely practised treatment methods to combat cancer (Tannock, 1998). Besides being expensive, most chemotherapeutic and radiation treatments suffer from adverse side effects. The situation warrants effective therapeutic approaches, and encourages researchers to depend more on medicinal plants that produce new and novel chemotherapeutics (Sheldon *et al.*, 1997; Reed and Pellecchia, 2005). Over 60% of the clinically used anticancer drugs are of natural origin and most of them are derived from higher plants. Vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan, and irinotecan are examples for plant derived chemotherapeutics approved for use in cancer therapy (Lee, 1999).

Oxidative stress is perhaps a major cause for several diseases including cancer, and the chemical components of medicinal plants possessing antioxidant properties can protect the human body from oxidative stress and associated diseases (Guo *et al*., 2011, Nema *et al*., 2013). Phenolic compounds belonging to xanthones, biflavonoids and phloroglucnols present in *Garcinia* species were reported as potential antioxidant compounds (Merza *et al*., 2004; Rukachaisirikul *et al*., 2006; Jantan *et al*., 2012; Taher *et al*., 2012; Osorio *et al*., 2013; Jamila *et al*, 2014).

A number of extracts and isolated compounds from *Garcinia* species were reported to exhibit remarkable cytotoxic activity against different cancer cell lines. Polyisoprenylated benzophenones are perhaps the most promising group of secondary

metabolites in *Garcinia* species attributed with anticancer properties. The anticancer benzophenone garcinol induces apoptosis through the activation of caspases (Pan *et al*., 2001). Gambogic acid, the active component in gamboge, has potent cytotoxic activities against human hepatoma, gastric carcinoma, and lung cancer (Guo *et al*., 2004; Wang *et al*., 2008; Wu *et al*., 2004). Guttiferones, another group of polyisoprenylated benzophenones isolated from *Garcinia* species exhibited strong cytotoxic activity against different human cancer cell lines (Nguyen *et al*., 2011). Xanthones are another group of secondary metabolites from *Garcinia* species attributed with anticancer properties. Penangianaxanthone, cudratricusxanthone H, macluraxanthone C, and gerontoxanthone C from *G. penangiana* exhibited strong cytotoxic activity against three cell lines, MCF-7, NCI-H460) and DU-145 (Jabit *et al*., 2007). The xanthones bannaxanthone D, garcinone E and γ-mangostin inhibit cancer cell growth and promote cancer cell death in HeLa cells and the activity was more potent than clinically used anticancer drugs, camptothecin and etoposide (Han *et al*., 2008). Yahyaxanthone form *G. rigida* showed *in vitro* cytotoxic activity to L1210 murine leukemia cell lines (Elya *et al*., 2008). α-Mangostin, γ-mangostin, and 8-deoxy gartanin exerted strong growth inhibition in human melanoma SK-MEL-28 cell line (Wang *et al*., 2011). Gaudichaudione H, a xanthone from *G. oligantha* has potent apoptosis-inducing effect and cell growth inhibition effect on HeLa-C3 cells (Gao *et al*., 2012). 1,4,5,6-Tetrahydroxy-7,8-di(3-methylbut-2-enyl)xanthone, globuxanthone and garciniaxanthone E exhibited moderate activities against human leukaemic HL-60 cell line *in vitro* (Niu *et al*., 2012). Cowanin and fuscaxanthone B from *G. schomburgkiana* exhibited remarkable cytotoxicity towards HeLa cells (Vo *et al*., 2012). Xanthones from *G. cantleyana* such as 7-hydroxyforbesione, cantleyanone B, cantleyanone C, and deoxygaudichaudione A exhibited strong activity against the cell-lines, MDA-MB-231, MCF-7, CaOV-3, and HeLa cells (Shadid *et al*., 2007).

G. travancorica is a Western Ghats endemic tree species and the phytochemical studies of the plant showed the biflavonoid glycoside fukugiside as the major constituent (AnuAravind *et al*., 2016). The present chapter evaluates the antioxidant and cytotoxic activity of fukugiside isolated from *G. travancorica*.

1. Antioxidant activities of *G. travancorica* leaf methanol extract and isolated compounds

The isolated biflavonoids GB-1a, GB-1, GB-2 and morelloflavone-7''-O-β-D-glycoside (**Figure 1**), and leaf methanol extract (GTL) were studied for their antioxidant activities by various *in vitro* free radical scavenging assays**.** The activities were measured as percentage, calculated using the formula % scavenging = $[(A_{control}-A_{sample})/A_{control}]$ x 100 and reported as IC_{50} value; the concentration of sample required to scavenge 50% of radicals. Experiments were done in triplicate and the results were expressed as mean value with standard deviation.

The *in vitro* antioxidant activities of the extract and isolated biflavonoids against DPPH and superoxide radicals are shown in **Table 1**. High quantity of phenolics (435.53±23.85 mg/g extract) and flavonoids (143.4±11.60 mg/g of extract) present in the leaves showed a direct correlation with its antioxidant potential. The IC_{50} value of DPPH radical scavenging activity of morelloflavone-7''-O-β-D-glycoside was 8.34±2.12 µg/ml, comparable to that of standard ascorbic acid (3.2±0.50 µg/ml). In superoxide radical scavenging assay also, the compound showed comparable activity (IC_{50} 6.95±1.33 µg/ml), close to standard ascorbic acid (IC_{50} value of 5.8±0.25 µg/ml). In reducing power assay, the

activity of the compound was very close to that of standard ascorbic acid (**Figure 3**). Though the antioxidant activity of glycosylated flavonoids is usually weaker than the corresponding aglycones, bioavailability is generally enhanced by the presence of glucose moiety (Ratty and Das 1988). The potential antioxidant activity of morelloflavone-7''-O-β-D-glycoside can be attributed to 3", 4"- dihydroxy unit present in the B ring. The B ring hydroxyl configuration is the most significant determinant of scavenging activity of flavonoids (Bors *et al*, 1990).

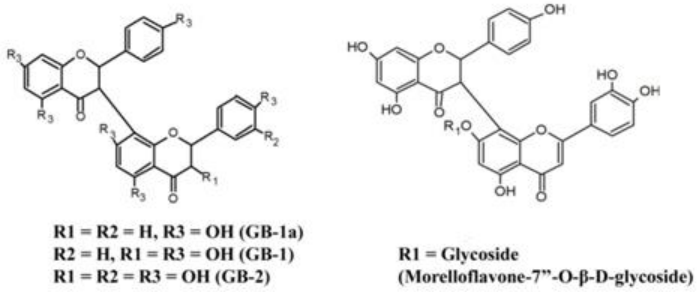

Figure 1. Structures of the biflavonoids GB-1a, GB-1, GB-2, and morelloflavone-7''-O-β-D-glycoside

Table 1. *In vitro* radical scavenging asays (DPPH and superoxide radical) of *G. travancorica* leaf methanol extract and isolated compounds

Extract/ compound	DPPH IC_{50} value (µg/mL)	Superoxide IC_{50} value (µg/mL)
G. trav. Lf MeOH extract	18.9±1.80	53.2±3.09
GB-1a	31.98±1.14	42.13±0.51
GB-1	22.31±2.33	37.52±2.10
GB-2	11.93±0.58	23.31±1.60
Morelloflavone-7''-O-β-D-glycoside	8.34±2.12	6.95±1.33
Ascorbic acid	3.2±0.50	5.8±0.25

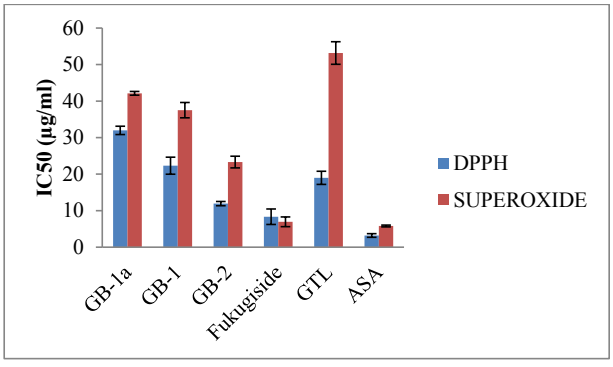

Figure 2. IC_{50} values of DPPH and superoxide radicals scavenging assay (GB-1a, GB-1, GB-2, Fukugiside, **GTL**- *G. travancorica* leaf methanol extract, **ASA**- standard ascorbic acid)

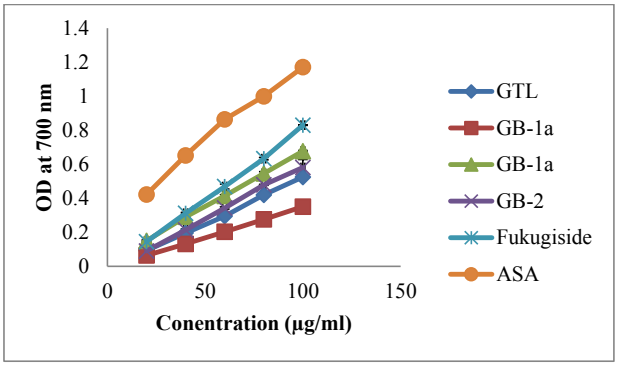

Figure 3. Reducing power assay (GB-1a, GB-1, GB-2, Fukugiside, **GTL**- *G. travancorica* leaf methanol extract, **ASA**- standard ascorbic acid)

2. Growth inhibitory effect of Fukugiside on cancer cell lines A431, HeLa, HT29 and normal cell line WRL68 cells

MTT assay was performed by seeding ~5000 cells per well in a 96 well plate and treating them under sub confluent conditions, with different concentrations of fukugiside such as 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 150 µg/mL respectively. The experiment was performed in batches with respect to the incubation time as 48 hrs. MTT assay is widely used in the *in vitro* evaluation of the biosafety of plant extracts and compounds. This colorimetric assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, coloured formazan product which is measured spectrophotometrically at 570 nm. Reduction of the dye MTT occurs only in metabolically active cells and the level of activity is a measure of the viability of the cells.

The study done on A431 and HeLa cells showed that fukugiside exhibited a concentration dependent cytotoxicity to both the cell lines. The cells were incubated with varying doses of fukugiside (1µg, 10 µg, 50 µg and 100 µg and 150 µg) and MTT assay was performed. Fukugiside inhibited the proliferation of human epidermal cancer cell line A431 and cervical cancer cell line HeLa in a dose dependent manner. Fukugiside exhibited significant cell death in A431 cell line with LD_{50} value of 150 µg/mL. Severe morphological changes were observed in HeLa cells treated with fukugiside under phase contrast microscope. Comparatively higher activity was exhibited by fukugiside against HeLa cells with LD_{50} value of 82.80 µg/mL compared with untreated control (**Figure 4**). The study done on normal liver cell line WRL68 and colorectal cancer cell line HT-29 cells treated with varying doses of fukugiside (1µg, 10 µg, 50 µg and 100 µg and 150 µg) did not exhibit any toxicity to the cells. From the results indicate that the compound exhibited toxicity to cancer cell lines A431 and HeLa in a dose dependent manner and no toxicity was observed against normal cell line WRL68.

Acridine orange/ethidium bromide (AO/EB) staining is used to visualize nuclear morphology and apoptotic body formation that are characteristic of apoptosis. Acridine orange is an important dye that will stain both live and dead cells, whereas ethidium bromide stain only those cells that have lost their membrane integrity (Jayadev *et al*., 2004).

Table 2. Cell viability in Fukugiside treated A431 and HeLa cells by MTT assay

Test material		% Cell viability	
		A431	HeLa
Control (0.01% DMSO)		100	100
Fukugiside (µg/mL)	1	110±3.6	85.85±0.19
	10	125±4.3	64.86±3.79
	50	86±3.4	56.50±2.46
	100	64±3.6	43.55±0.52
	150	49±3.4	39.88±.67

Values are mean±SD of three separate determinations. Cells were incubated at 37° C for 48 hrs in DMEM media in CO_2 incubator

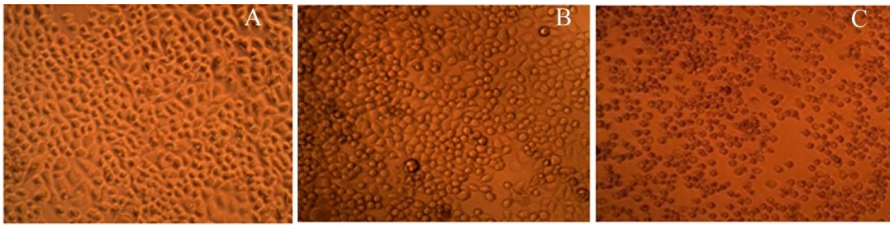

Figure 4. HeLa cells treated with fukugiside under phase contrast microscope: (A) HeLa cells treated with DMSO (0.01%); (B) DLA cells treated with fukugiside (50 µg/mL); (C) HeLa cells treated with fukugiside (150 µg/mL)

To corroborate that apoptosis has been induced by fukugiside, HeLa cells were analysed in the presence of acridine orange and ethidium bromide staining (AO/EB staining). Five concentrations of fukugiside used in MTT assay (1µg, 10 µg, 50 µg and 100 µg and 150 µg) were chosen for this experiment. HeLa cells cultured in complete media and stained with AO/EB (**Figure 5**) were used as control.

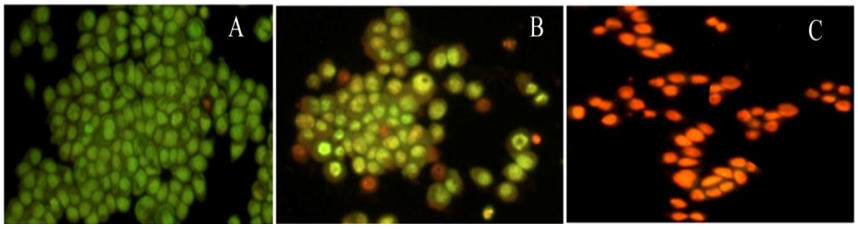

Figure 5. HeLa cells stained with acridine orange-ethidium bromide under fluorescent microscope: (A) HeLa cells treated with DMSO (0.01%) appeared in green color (live), (B) DLA cells treated with fukugiside (50 µg/mL) appeared in slight yellowish (early apoptotic cells), (C) HeLa cells treated with fukugiside (150 µg/mL) appeared in yellowish red (dead cells)

Figure 5 shows that the fukugiside at tested doses induced apoptosis after 48 hours incubation. Cells stained green represent viable cells (**Figure 5A**), whereas yellow staining represented early apoptotic cells (**Figure 5B**) and yellow to reddish orange staining represents late apoptotic cells (**Figure 5C**). As shown in **Figure 5**, HeLa cells treated with 150 µg/mL of fukugiside showed changes in cellular morphology, including chromatin

condensation and membrane blebbing. Stronger apoptosis signal was induced in HeLa cells with higher concentrations of fukugiside.

Effect of fukugiside on cell cycle distribution by flow cytometry

Considering that fukugiside decreased cell proliferation and induced cell death as evident from MTT assay and apoptotic induction by staining experiments, the effect of this molecule on cell cycle distribution was analysed by flow cytometry. Flow cytometric analysis was carried out on HeLa cells treated with 100 µg/mL of fukugiside for 48 hrs. In HeLa cells, 100 µg/mL of fukugiside induced accumulation of cells in S phase concurrently to a significant decrease in G0/G1 cells (**Figure 6**).

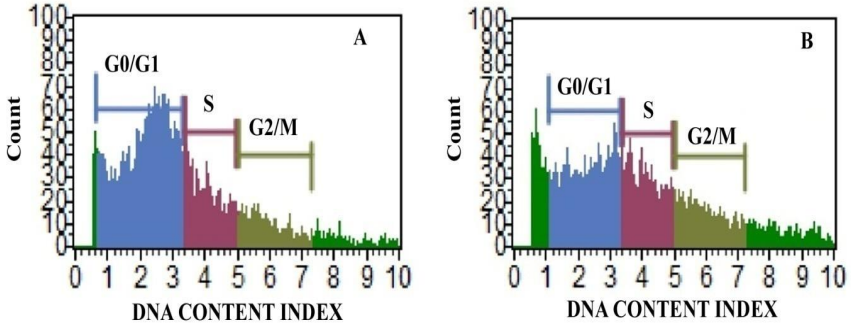

Figure 6. Comparison of DNA content in control (0.01% DMSO) and fukugiside (100 µg/mL) treated HeLa cells by flow cytometry

Deregulation of cell cycle is one of the critical events that drive cancer cells into uncontrolled proliferation (Evan and Vousden, 2001). Molecular changes, including the over expression of cyclins and CDKs and the loss of CDK inhibitors and tumor suppressor proteins resulting from gene mutations or epigenetic inactivation, are frequently detected in tumor cells (Sherr, 1996; Malumbres and Barbacid, 2001). Because of the important roles of cell cycle deregulation in tumorigenesis and tumor progression, molecules involved in cell cycle regulation also serve as potential targets for therapeutic intervention in cancers. Modulation of p21, and MAPK/ERK pathway can have a potent role in inhibiting cells at S phase. In the present study, addition of the compound fukugiside induced significant change in cell proliferation and the cells were found to be arrested in S phase compared to untreated control. The results were comparable with previous reports regarding inhibtion of MCF 7 cells by resveratrol and other flavonoid compounds in S phase (Joe *et al*., 2002).

Effect of fukugiside on the expression of MAPK p38 in HeLa cells

In continuation with the studies on cell cycle deregulation seen in S phase by fukugiside, the effects of fukugiside on the level of MAPK p38 in HeLa cells were examined. A series of time course experiments were conducted to analyse the expression of Erk in HeLa cells treated with fukugiside, where DMSO served as control. Reverse transcription polymerase chain reaction (RT-PCR) followed by agarose gel electrophoresis demonstrated that the expression levels of MAPK was decreased after 48 hrs of treatment with fukugiside. The intensity of the bands were analysed by ImageJ analyser and the results revealed that,

treatment with fukugiside lead to inhibition of MAPK expression by 26.15 % compared to untreated control (**Figure 6**).

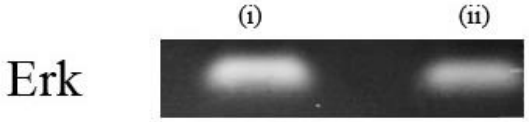

Figure 6. Intensity of MAPK p38 expression in agarose gel electrophoresis; **(i)** control, **(ii)** fukugiside

Conclusions

Garcinia species are well known for the diversity of secondary metabolites and potential bioactivities. The biflavonoid fukugiside has been identified as the major antioxidant component in *G. travancorica* through *in vitro* free radical scavenging assays and reducing power assay. Further, the antitumor properties of the molecule in different human cancer cell lines were also checked. Fukugiside caused a dose dependent cancer cell growth inhibition in A431 and HeLa cells, and the antiproliferative effect appears to be due to its ability to induce S-phase arrest and apoptotic cell death. In HeLa cells, fukugiside down regulated the MAPK p38 expression compared with untreated control. The study highlights fukugiside as a potential candidate for drug development.

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