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Figure Legends

Fig. 1 Comparison of Urinary 8-OHdG Levels between the Normal and Depressive Symptoms by Menstrual Phases

The solid line indicates the results for normal individuals, the dotted line the results for individuals with depressive symptoms.

Two-way factorial analysis of variance with the presence/absence of depression and menstrual cycle as factors (adjusted for the age and BMI)

Depression: p=0.040; menstrual cycle: p=0.529; interaction: p=0.863

Adjusted mean: depressive: 7.01 ng/mL; normal: 3.98 ng/mL

Fig. 2 ROC Curve during Each Menstrual Phase

The solid line shows the results for the menstrual phase, the dashed line those for the proliferative phase, and the dotted line those for the secretory phase.

The AUCs (SDs) and p-values in each phase were 0.81(0.07), 0.005 (menstrual); 0.73 (0.90), 0.038 (proliferative); and 0.80 (0.72), 0.006 (secretory), respectively.

Table 1 Age, anthropometric variables, and SDS scores in the Normal and Depressive Groups

		Normal (n=47)			Depressive (n=10)			
		Mean	SD		Mean	SD		*p*
Age (years)		21.5	0.7		22.0	0.9		0.050
Height (cm)		158.6	4.6		160.1	5.1		0.361
Body weight (kg)		51.2	6.3		49.1	4.8		0.325
BMI (kg/cm^2)		20.3	2.3		19.1	1.3		0.118
SDS	Menstrual phase	42.2	6.2	†	55.1	4.2	‡	<0.001
	Proliferative phase	41.9	5.7		52.3	9.2		<0.001
	Secretory phase	41.4	5.7		52.9	7.8		<0.001

One-factor repeated measures analysis of variance: † p=0.865, ‡ p=0.361

SD: standard deviation

BMI: body mass index

SDS: self-rating depression scale

Table 2 Characteristics of the subjects of the Normal (SDS<53) and Depressive (53≦) groups with respect to the menstrual cycle

	SDS	
	<53	53≦
Menstrual phase	49	8
Proliferative phase	50	7
Secretory phase	48	9
Combination of Menstrual and Proliferative phases	55	2
Combination of Proliferative and Secretory phases	55	2
Combination of Menstrual and Secretory phases	54	3
Combination of all phases	54	3

SDS: self-rating depression scale

<53: SDS scale is less than 53 scores

53≦: SDS scale is 53 or more scores

Table 3 Relationship between the urinary 8-OHdG levels and SDS scores by menstrual phase (adjusted for age and BMI)

	partial correlation coefficient	*p* value
Menstrual phase	0.563	0.012
Proliferative phase	0.472	0.041
Secretory phase	0.56	0.013

partial correlation coefficient: adjusted for the age and BMI

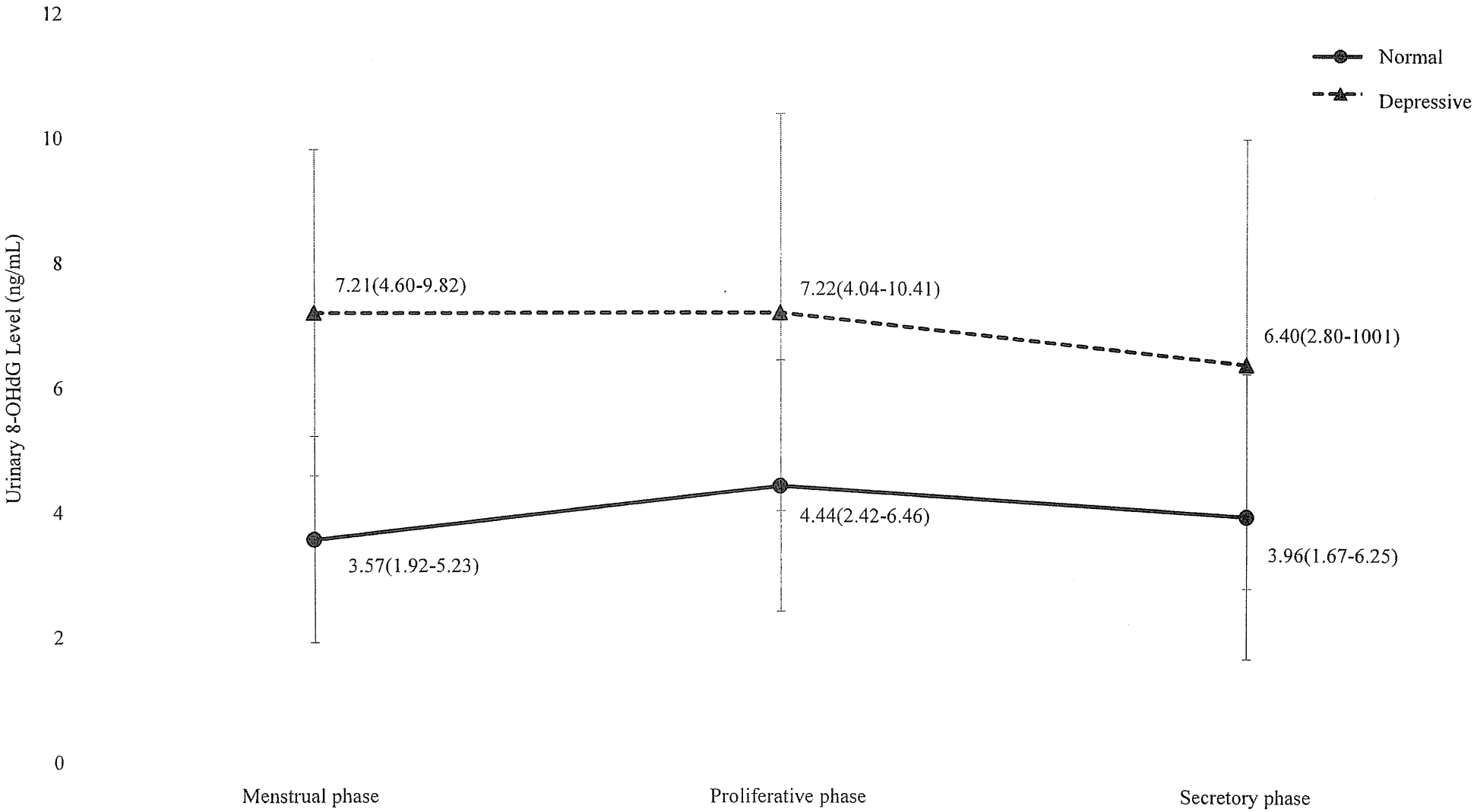
12
10
8
6
4
2
0
Urinary 8-OHdG Level (ng/mL)
Normal
Depressive
7.21(4.60-9.82)
7.22(4.04-10.41)
6.40(2.80-1001)
4.44(2.42-6.46)
3.57(1.92-5.23)
3.96(1.67-6.25)
Menstrual phase
Proliferative phase
Secretory phase

Fig.1

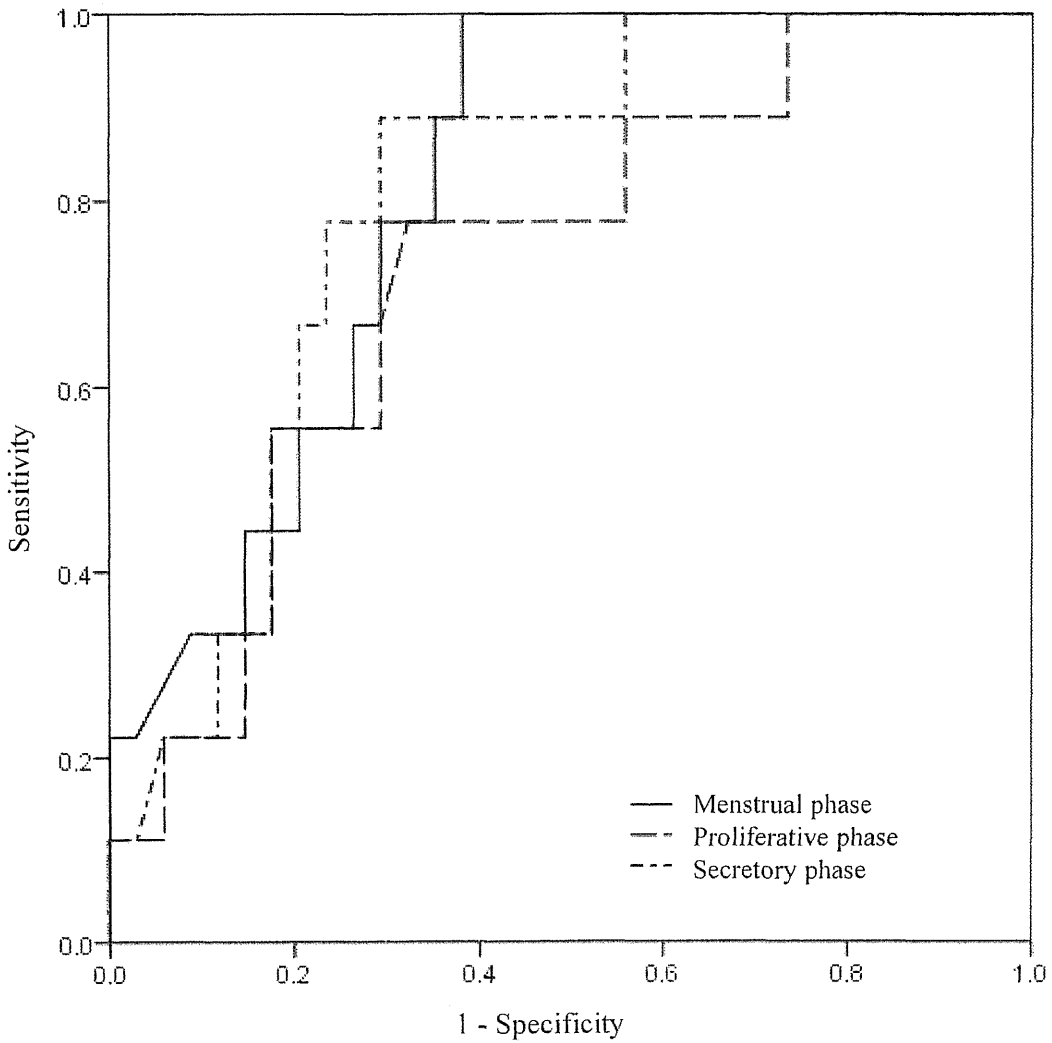
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
Menstrual phase
Proliferative phase
Secretory phase

Fig.2

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Letter

Estimation of endoplasmic reticulum stress-inducing ability of nobiletin, a citrus polymethoxyflavonoid, in SK-N-SH human neuroblastoma cells

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ABSTRACT — Nobiletin, a citrus polymethoxyflavonoid compound, has been considered useful in the development of drugs and functional foods for various diseases, including dementia and diabetes. It is therefore important to understand its toxic effects. We previously reported that nobiletin treatment at a dose of 100 μM induced the expression of DDIT3 and TRIB3 genes and proteins, which are well known to contribute to apoptosis caused by endoplasmic reticulum (ER) stress, commonly in three cell lines, such as SK-N-SH human neuroblastoma cells. Therefore, their increased expression raises concerns that nobiletin might exert a toxic effect by inducing ER stress. In the present study, SK-N-SH cells were treated with 100 μM nobiletin or 1 μg/mL tunicamycin, a potent inducer of ER stress, for 3, 6, 12, and 24 hr. The maximum expression of those proteins appeared later and was much weaker in the nobiletin-treated cells than in the tunicamycin-treated cells. The expression level of BiP protein, one of the chaperons, which increases in response to ER stress, was not changed in the nobiletin-treated cells, whereas it was strongly induced 12 and 24 hr after the onset of tunicamycin treatment. In addition, cleavages of caspase-3 and poly (ADP-ribose) polymerase occurred 24 hr after the onset of tunicamycin treatment, whereas cleavage did not occur at any point during nobiletin treatment. Therefore, although nobiletin has the ability to induce the expression of DDIT3 and TRIB3, those increased levels, at doses up to at least 100 μM, cannot be enough to lead to ER stress resulting in apoptosis.

Key words: Flavonoid, Nobiletin, Endoplasmic reticulum stress, DDIT3, TRIB3, Apoptosis

INTRODUCTION

Nobiletin (Fig. 1A), a polymethoxyflavonoid with six methoxy groups, is found at high concentrations in the peels of citrus fruits. Many studies using *in vivo* and *in vitro* systems suggested that nobiletin exerts a wide variety of beneficial activities, including those that prevent or alleviate dementia, carcinogenesis, obesity, hyperlipidemia, diabetes, and inflammation (Murakami *et al.*, 2000; Lee *et al.*, 2010; Yoshigai *et al.*, 2013; Ma *et al.*, 2014; Nakajima *et al.*, 2014). Nobiletin is thus a potentially useful compound in the development of drugs and functional foods for the treatment of these respective diseases. It has therefore become increasingly important to

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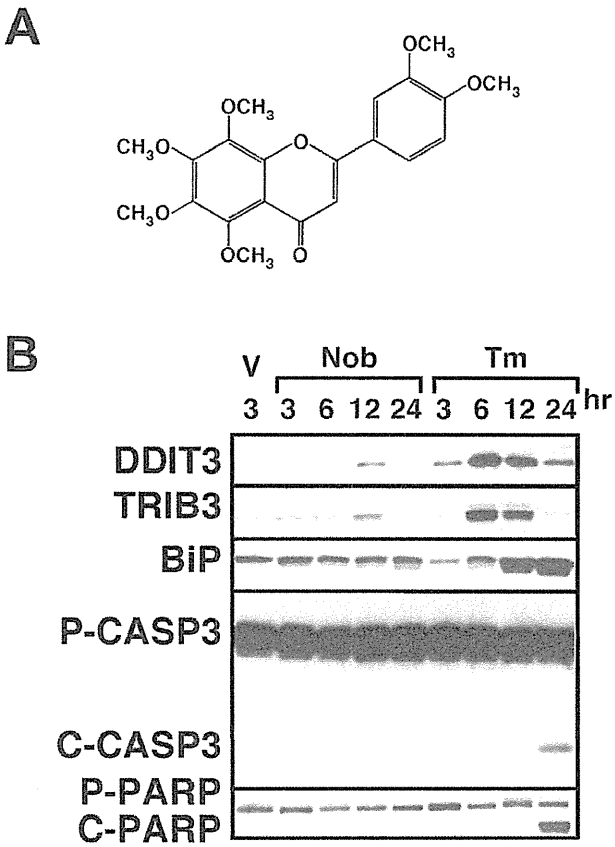

Fig. 1. Effects of nobiletin (A: chemical structure) or tunicamycin on the expression of DDIT3, TRIB3, and BiP proteins and the generation of cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) in SK-N-SH cells. The cells were treated with 0.1% DMSO (vehicle: V), 100 μM nobiletin (Nob), or 1 μg/mL tunicamycin (Tm) for the indicated periods. The expression levels were assessed by Western blotting (B). P-CASP3, C-CASP3, P-PARP, and C-PARP represent pro-caspase-3, cleaved caspase-3, full-length PARP, and cleaved PARP resulting from caspase cleavage, respectively.

clarify the mechanisms of nobiletin-mediated biological effects, including adverse (toxic) effects.

We previously found the upregulated expression of DDIT3 (DNA-damage-inducible transcript 3, also known as CHOP or GADD153) and TRIB3 (tribbles homolog 3 protein, also known as TRB3) genes and proteins, which are well known to contribute to apoptosis caused by endoplasmic reticulum (ER) stress (Tabas and Ron, 2011), commonly in 100 μM nobiletin-treated SK-N-SH human neuroblastoma, HuH-7 human hepatoma, and 3Y1 rat fibroblast cell lines (Nemoto *et al.*, 2013). Prolonged or excessive ER stress is thought to be a trigger for the cell loss and cell damage found in several common human diseases, including type 2 diabetes and neurodegeneration, as well as in chemical-induced toxicity (Sano and Reed, 2013). Therefore, there is concern that the increased expression of DDIT3 and TRIB3 genes and proteins induced by nobiletin treatment might be involved in the adverse (toxic) effect of nobiletin through the induction of ER stress. In conflict with that, we also found that 100 μM nobiletin treatment suppressed the induction of apoptosis by 1 μg/mL tunicamycin, a potent inducer of ER stress, in SK-N-SH cells, suggesting that this suppression of apoptosis could be a mechanism of nobiletin's beneficial effects (Ikeda *et al.*, 2013). Accordingly, it is of considerable importance to cautiously examine whether the nobilitin-induced expression of DDIT3 and TRIB3 genes and proteins possesses the ability to induce apoptosis caused by ER stress.

In the present study, we intended to consider whether nobiletin is a possible inducer of ER stress by comparing the expression levels of DDIT3 and TRIB3 proteins between nobiletin- and tunicamycin-treated SK-N-SH cells.

MATERIALS AND METHODS

Nobiletin

Nobiletin was extracted and isolated from *Citrus reticulata* peels as described previously (Nagase *et al.*, 2005).

Cell culture

Human SK-N-SH neuroblastoma cells were basically grown in α-Minimum Essential Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a saturated humidity atmosphere of 95% air and 5% CO_2. For chemical treatments, stock solution (in DMSO) of 100 mM nobiletin or 1 mg/mL tunicamycin (Sigma, St. Louis, MO, USA) was added to the culture medium by a 1,000-fold dilution.

Western blotting

The chemically treated cells were lysed with lysis buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing phenylmethylsulfonyl fluoride (PMSF), aprotinin, sodium orthovanadate, and protease inhibitor cocktail. After 15 μg per lane of total protein was subjected to SDS-PAGE (12.5% e-PAGEL; ATTO, Tokyo, Japan), it was transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Waukesha, WI, USA). The primary antibodies used in Western blot

analysis were monoclonal anti-DDIT3 (Cell Signaling Technology, Beverly, MA, USA), monoclonal anti-TRIB3 (Epitomics, Burlingame, CA, USA), polyclonal anti-BiP (Cell Signaling Technology), polyclonal anti-caspase-3 (Cell Signaling Technology), polyclonal anti-PARP (Cell Signaling Technology), or polyclonal anti-β-ACTIN (Cell Signaling Technology) antibodies. The secondary antibodies were anti-mouse IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology) for DDIT3 and anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) for TRIB3, BiP, caspase-3, PARP, and β-ACTIN. Immunoreactive bands were visualized by the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

RESULTS AND DISCUSSION

In our previous study, the generation of cleaved caspase-3 as an indicator of apoptosis was assessed in SK-N-SH cells treated with tunicamycin (0.1, 1, 5, and 10 μg/mL) for 24 hr (Ikeda *et al.*, 2013). The results showed that 1-10 μg/mL tunicamycin treatment generated the cleaved caspase-3, suggesting that the apoptosis induced through ER stress occurred as a result of treatment at those concentrations. Therefore, we decided in the present study to use a 1 μg/mL concentration of tunicamycin to compare the expression levels of various proteins in 100 μM nobiletin treatment with those in tunicamycin treatment.

Expression of DDIT3 and TRIB3 proteins was assessed by Western blot analysis in SK-N-SH cells treated with nobiletin or tunicamycin for 3, 6, 12, and 24 hr (Fig. 1B). Nobiletin treatment only for 12 hr allowed us to clearly detect DDIT3 and TRIB3 protein expression, while tunicamycin treatment for 6 or 12 hr induced very strong expression levels of both proteins. Thus, nobiletin appears to be considerably weaker than tunicamycin at inducing the expression of these proteins. In addition, we evaluated the expression of BiP protein, one of the chaperons, whose expression increases in response to ER stress (Oslowski and Urano, 2011). Nobiletin treatment had little influence on its expression, whereas tunicamycin treatment highly induced its expression (Fig. 1B). This result strongly suggested that nobiletin treatment at a concentration of 100 μM is scarcely able to induce ER stress in SK-N-SH cells. It was confirmed that the cleavages of caspase-3 and poly (ADP-ribose) polymerase, as indicators of apoptosis, occurred 24 hr after the onset of tunicamycin treatment, while cleavage did not appear at any point during nobiletin treatment (Fig. 1B).

The present study showed that the levels of DDIT3 and TRIB3 protein expression induced by 100 μM nobiletin treatment were remarkably lower than those induced by 1 μg/mL tunicamycin treatment, and that 100 μM nobiletin treatment did not change the expression level of BiP protein as a marker of ER stress. Therefore, although nobiletin has the ability to induce the expression of the DDIT3 and TRIB3 genes and proteins, those increased levels, at doses up to at least 100 μM, cannot be enough to lead to potent ER stress resulting in apoptosis.

Suppression and inhibition of apoptosis can be regarded as beneficial actions from the perspective of the improvement and prevention of cellular damage, including neuronal degeneration and pancreatic β-cell death accompanied by hyperglycemia. Conversely, the exclusion of cancer cells by apoptosis is useful for thinking about cancer therapy and prevention. In fact, there is accumulating evidence that nobiletin treatment at doses around 100 μM can cause apoptosis in various cancer cell lines (Moon *et al.*, 2013; Chen *et al.*, 2014; Ma *et al.*, 2014), supporting the idea that nobiletin has chemopreventative potential in cancer treatment. Therefore, nobiletin might act on apoptosis as a double-edged sword. The binary choice between induction and inhibition of apoptosis might depend on cell type (e.g., cancer or noncancer types) and/or sensitivity to cellular stress, including ER stress. Our previous (Nemoto *et al.*, 2013) and present studies demonstrated that nobiletin has a potential to induce the expression of DDIT3 and TRIB3 genes and proteins. Therefore, it will be important to assess the intensity of nobiletin-induced expression of those genes and proteins in cancer cell lines in which nobiletin has been reported to induce apoptosis.

Conflict of interest---- The authors declare that there is no conflict of interest.

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