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Chemopreventive Activity of Sesquiterpene Lactones (SLs) from Yacon against TPA-induced Raji Cells Deformation

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Abstract: Yacon is a medicinal plant used as a traditional medicine by the natives in South America. In Japan, it becomes popular as a health food. Sesquiterpene Lactones (SLs) from yacon leaves were investigated and the active SLs such as enhydrin, uvedalin and sonchifolin, bearing α -methylene- γ -lactone and epoxides as the active functional groups, were identified by ¹H-600MHz -NMR. Chemopreventive and cytotoxic activities were determined using different primary screening methods. In this study, all tested SLs strongly inhibited TPA-induced deformed of Raji cells. The IC₅₀ values of yacon SLs from anti-deforming assay were 0.04-0.4 μ M. Interestingly, yacon SLs showed more potential of chemo preventive activity than both curcumin and parthenolide. However, the cytotoxicity on Raji cells was observed at high concentration of yacon SLs. The degree of anti-deformation was ranked in order: enhydrin > uvedalin > sonchifolin > parthenolide > curcumin. As according to structure-activity relationship, the high activities of enhydrin, uvedalin and sonchifolin may be due to the 2-methyl-2-butenolate and its epoxide moiety.

Key words: Chemopreventive activity, epstein-barr virus, melampolides, sesquiterpene lactone, *Smallanthus sonchifolius*

INTRODUCTION

Cancer is a major health problem worldwide and its severity is at great concern (Haque *et al.*, 2010). During the past two decades, National Cancer Institute (NCI) has developed chemopreventive agents by collecting the basic experimental data, epidemiological data and finally, the clinical trials (Russo, 2007). The term chemoprevention refers to the application of natural products or pharmaceutical substances in order to suppress, arrest or reverse carcinogenesis process in its early stages (Sporn and Suh, 2002). Numerous phytochemicals found in many plants show chemopreventive properties or cancer prevention (Sale *et al.*, 2005; Manal *et al.*, 2007; Pugalendhi and Manoharan, 2010).

Yacon (*Smallanthus sonchifolius*) has been consumed for centuries among natives in Andes plateau as a staple food (Ojansivu *et al.*, 2011) and its tubers have become popular in Japan as a food supplement for diabetes mellitus patients (Valentova *et al.*, 2004). It was reported that yacon leaves possess the anti-diabetic property and it was consumed as tea from dried leaves. Research has shown that yacon leaves contain high amount of phenolic

compounds and Sesquiterpene Lactones (SLs) (Valentova and Ulrichova, 2003). SLs are known as major classes of natural products with a broad spectrum of biological activities including anti-inflammatory, neurocytotoxic and anticancer activities (Cho *et al.*, 2004). In previous study, we demonstrated that SLs extracted from yacon leaves are potent in induction of apoptosis-mediated proliferation inhibition via caspase 3/7 and deactivation of NF- κ B in cervical cancer cell line (Siriwan *et al.*, 2011). However, chemopreventive effects of yacon SLs in Raji cells have not been studied yet.

Previous studies has been proven that Epstein Barr Virus (EBV) genome carrying human lymphoblastoid cells (Raji), activated by 12-O-Tetradecanoyl-Phorbol-13-Acetate (TPA), has been used widely as a screening assay for the early detection of anti-tumor promoters (Kondo *et al.*, 1998). According to previous research, it was proven that adhesion and morphological changes of Raji cells induced by treatment of TPA can be used as a preliminary assay for detection of anti-tumor promoting activity or chemopreventive activity (Okazaki *et al.*, 2004).

This study aimed to determine the chemopreventive and cytotoxic activities of SLs from yacon leave extracts

using anti-deformation assay, which is based on morphological changes and CCK-8 assay (modified MTT assay) in virus-non producer Raji cells.

MATERIALS AND METHODS

This research was carried out from November 2008 to May 2009.

Plant material: were grown in Ato-cho, Yamaguchi Prefecture and provided by Nihonkaisui Co. Ltd. (Tokyo, Japan). The voucher specimens were deposited in the Department of Applied Biological Science, Kagawa University, Japan.

Extraction: The extraction of Sesquiterpene Lactones (SLs) from dried leaves of *Smilax sonchifolius* was described as our previous study (Siriwan *et al.*, 2011).

Reagents used: Dulbecco's modified Eagle's medium (D-MEM), Eagle's Minimum Essential Medium with Earle's salts (E-MEM) and DMSO were purchased from Wako chemical (Tokyo, Japan). Antibiotic-antimycotic was bought from Gibco-Invitrogen (CA., USA). Fetal bovine serum (FBS) was obtained from Biowest (Nuaille, France). 12-O-tetradecanoylphorbol 13-acetate (TPA), etoposide (VP-16), curcumin, mitomycin C (MMC) and parthenolide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit (CCK-8) and HEPES were purchased from Dojindo Molecular Technologies, Inc. (Tokyo, Japan).

Anti-deforming assay: This assay was modified from previous study (Kondo *et al.*, 1998). Raji cells in log phase at a density of 1×10^5 cells/mL were incubated in 1 mL of D-MEM supplemented with 10%FBS containing 10 mM of sodium butyrate, 40 ng mL⁻¹ of TPA with or without testing samples at 37°C for 48 h. Cells that showed detectable deformations were analyzed using a phase-contrast microscope (Nikon, Japan) at a magnification 200X. Deformed of Raji cells was determined by counting the number of deformed cells (dilation, flatness and a tree-branch like deformation) of the three areas of the counting plate in a random manner, which was conducted in triplicate. The inhibitory concentration 50% (IC₅₀) was calculated from the dose-response curve obtained by plotting the percentage of deformed Raji cells versus the concentrations using GraphPad Prism version 5 software.

Cell viability assay (CCK-8 assay): Raji cells (1×10^5 cells mL⁻¹) were exposed to testing samples with the different concentrations (0.1-2 µg mL⁻¹) for 48 h at 37°C under 5% CO₂ atmosphere. DMSO and butyrate were

served as the solvent control. After 48 h, 10 µL of CCK-8 (cell counting solution was added to each well and the 96-well plate was continuously incubated at 37°C for 4 h, then the OD values for each well were read at 450 nm wavelength using a microplate reader (MTP-450 Lab, Corona Electric, Japan). The cell viability of Raji cell line was expressed as the percent viability of treated cells compared with the untreated control.

Statistical analysis: Statistical analysis was performed using GraphPad 5.0 software (GraphPad Software Inc., La Jolla, CA). The results were derived from three independent experiments (each experiment was conducted in triplication). To determine the differences between control and treatments, one-way ANOVA followed by Dunnett's Multiple Comparison Test was applied. The p-values ≤ 0.05 from one-sided tests was considered as significantly different.

RESULTS AND DISCUSSION

In this study, we first demonstrated that three of SLs from yacon leaves extract showed chemopreventive activity against TPA-induced Raji cells deformation. Moreover, present results suggest that the efficiency of chemopreventive activity of yacon SLs was not only stronger than the well-known SLs parthenolide but also higher activity than curcumin.

The present results indicate that morphological changes could be induced in Raji cells by TPA and butyrate. The differences of morphology between untreated and TPA-treated Raji cells are shown in Fig. 1. In this study, untreated cells (only butyrate) are shown round shape as a typical morphology of lymphoblast similar phenomenon was observed in cells treated with TPA alone as well (data not shown). In contrast, TPA and butyrate-treated (positive control) cells were displayed many protrusions and adhere onto the surface of culture flask which mean synergism effects between TPA and butyrate (Fig. 1, positive control). The morphology of the cells in the positive control group was dilation, flatness and tree branch-like (Fig. 1). On the contrary, cells treated with yacon sesquiterpenes became more round shape and showed no dilation (Fig. 1a-c). It was noted that not only the number of deformed cells decreased but also the morphologic characteristics which were obviously similar, changed to negative control.

Previous studied showed that TPA and butyrate acted synergistically to cause the expression of the EBV early antigen (EA-D) (Jenson *et al.*, 1998). However, the relationship between morphological changes and the expression of EBV early antigen (EA-D) are remaining

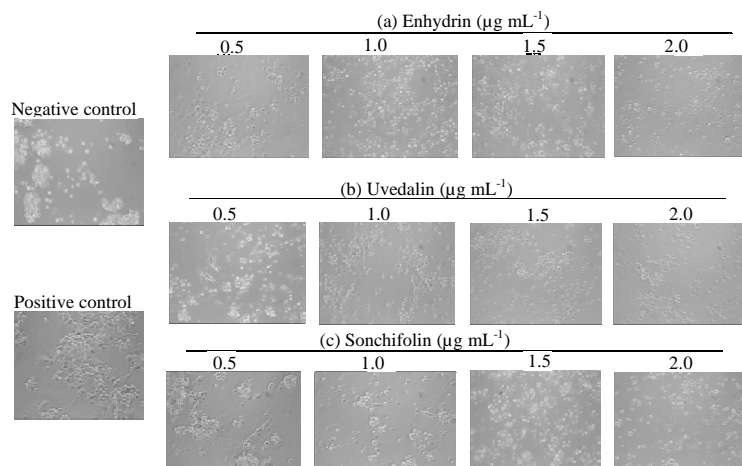


Fig. 1(a-c): Inhibitory effect of yacon SLs on the TPA-induced Raji cells deformation. Cells were treated with TPA ($0.04 \mu\text{g mL}^{-1}$) either alone or together with the $0.5\text{-}2.0 \mu\text{g mL}^{-1}$ of yacon chemicals. The negative control (0.1% DMSO and butyrate) and positive control (TPA only) were compared with treatment groups. (a) enhydrin, (b) uvedalin and (c) sonchifolin

unclear. In non-producer Raji cells, cellular morphology were obviously changed after cells were infected with EBV and the replication of EBV was occurred using electron microscope (Seigneurin *et al.*, 1977). The effect of TPA on cell membrane morphology was observed in another EBV-positive Burkitt lymphoma cell line (Patarroyo *et al.*, 1981). Recently, Raji cells, which treated with TPA and butyrate, are prone to adhere to the surface of the culture dish and this phenomenon was inhibited by addition of curcumin (Okazaki *et al.*, 2004). In addition, adherent Raji cells that treated with TPA also showed the expression of EBV early antigen. Base on previous findings, it may possible that TPA and butyrate induced activation of EBV virus and it specifically changes in cell membrane of Raji cells in our experiment.

The inhibitory effects of yacon SLs on TPA-induced the deformation of Raji cells and the 50% inhibitory concentration (IC_{50}) values are shown in Table 1. Curcumin has been proven as an anti-tumor activity *in vitro* and *in vivo* model (Shishodia *et al.*, 2007; Foda *et al.*, 2007). Parthenolide is a well-known SL which has been studied broadly in biological activities and now in cancer clinical trials (Ghantous *et al.*, 2010). Therefore, in anti-deformation assay, curcumin and parthenolide were used as a reference chemical. There were significant differences in the anti-deformation between the positive control and treatment group. As shown in Table 1 when the concentrations of enhydrin, uvedalin and sonchifolin were 0.1, 0.5, 1.0, 1.5 and $2.0 \mu\text{g mL}^{-1}$, significantly

($p \leq 0.05$) differences with positive control in the number of deformed Raji cells was observed (5-0 deformed cells for enhydrin, 7-0 deformed cells for uvedalin and 10-0 deformed cells for sonchifolin). The inhibitory effect against deformed of Raji cells was observed a dose-dependent fashion.

Interestingly, tested SLs exhibited higher inhibition with the lower of IC_{50} values ($0.04\text{-}0.40 \mu\text{M}$) than the reference chemicals, parthenolide (IC_{50} $2.20 \mu\text{M}$) and curcumin (IC_{50} $6.44 \mu\text{M}$). Among these SLs, enhydrin showed the highest activity with IC_{50} $0.04 \mu\text{M}$. The potency of the activity was ranked in an increasing order from enhydrin>uvedalin>sonchifolin>parthenolide>curcumin (Table 1). Furthermore, at the IC_{50} concentration of each yacon SLs, the viability of Raji cells was more than 60% (Table 1). Especially, sonchifolin is less toxic than enhydrin and uvedalin. However, toxicity on Raji cells was observed at high concentration ($1.0\text{-}2.0 \mu\text{g mL}^{-1}$) of yacon SLs (Table 1). A dose-dependent effect was also observed on cell viability, the inhibition of 50% cell viability of three SLs on Raji cells were in the range of $1.07\text{-}1.89 \mu\text{M}$ (Table 1). Among yacon SLs, enhydrin and uvedalin showed the highest toxicity with the IC_{50} value of 1.07 and $1.40 \mu\text{M}$. Whereas, sonchifolin gave the weakest toxicity with the IC_{50} value of $1.89 \mu\text{M}$. These results indicated that yacon SLs efficiently inhibited TPA-induced the deformation of Raji cells at low concentration but caused toxicity at high concentration.

Table 1: Anti-deforming activity of SLs from yacon leaves and reference chemicals

Chemical	Anti-deforming activity (The number of deformed cells) Concentration (times/40 ng TPA)								IC ₅₀ of deformed cells (μM)	IC ₅₀ cell viability (μM)
	2.5	12.5	37.5	50	100	150	200	250		
	0.1 μg mL ⁻¹	0.5 μg mL ⁻¹	1.0 μg mL ⁻¹	1.5 μg mL ⁻¹	2.0 μg mL ⁻¹	4.0 μg mL ⁻¹	6.0 μg mL ⁻¹	8.0 μg mL ⁻¹		
Enhydrin	5* (82)	3* (46)	0* (15)	0* (6)	0* (4)	nd ^a	nd ^a	nd ^a	0.04 (80)	1.07
Uvedalin	7* (92.5)	4* (73)	2* (18)	0* (10)	0* (5)	nd ^a	nd ^a	nd ^a	0.07 (90)	1.40
Sonchifolin	10* (98)	10* (86)	4* (20)	1* (14.5)	0* (8.2)	nd ^a	nd ^a	nd ^a	0.40 (96)	1.89
Parthenolide	22* (99)	15* (94)	4* (51.2)	1* (23.2)	0* (5.6)	nd ^a	nd ^a	nd ^a	2.20 (98)	4.15
Curcumin	28* (98.8)	24* (97.5)	23* (98.2)	20* (96.2)	17* (94.6)	3* (87.4)	1* (39)	0* (19.4)	6.44 (70)	16.00

^aNot determined. *Significant difference ($p \leq 0.05$) from number of deformed cells in positive control (28 deformed cells). Values in parentheses are % cell viability of Raji cells

According to cytotoxicity of SLs, many researchers have been focusing on anti-cancer or anticancer drugs development, approximately 1500 articles have reported in Medline and EMBASE databases (Ghantous *et al.*, 2010). However, there have been few studies of chemopreventive effects in plants rich in SLs. Previous study have documented that SLs from *Saussurea salicifolia* showed the cancer chemopreventive activity via quinone reductase assay in Hepalcl7 cells (Kang *et al.*, 2007). Iranshahi *et al.* (2010) found that four new SLs namely, diversolide A, D, F and G strongly inhibited the EBV-EA activation induced by TPA in Raji cells and two-stage mouse skin model (Iranshahi *et al.*, 2010). It was obviously shown that the antitumor promoter activity of diversolides A was stronger than curcumin (Iranshahi *et al.*, 2010). In this study, we also found that all tested SLs from yacon were inhibited adhesion and deformation of Raji cells stronger than curcumin (IC₅₀ = 6.44 μM).

The biological activities of SLs can be affected by many chemical properties such as alkylating center reactivity, side chain and lipophilicity or molecular geometry (Ghantous *et al.*, 2010). Our previous research has shown that epoxides and α -methylene- γ -lactone moiety are important for anti-cervical cancer activity (Siriwan *et al.*, 2011). Present study, enhydrin, uvedalin and sonchifolin have a stronger chemopreventive activity than parthenolide. It means that epoxide on the 10-membered ring has a positive effect but small effect on chemopreventive activity. However, 2-methyl-2-butenate moiety of uvedalin and its epoxide of enhydrin influenced a lot on anti-tumor promotion of Raji cells. From these results, 2-methyl-2-butenate may be converted to 2,3-epoxy-2-methylbutanoate like an aflatoxin which, is done to the epoxide and then the epoxide of 2,3-epoxy-2-methylbutanoate may be more reactive than the epoxide on the 10-membered ring in order to form a cross-linkage of proteins and DNA. This is the first report that shows evidence of the importance of 2-methyl-2-butenate and 2,3-epoxy-2-methylbutanoate moieties of SLs from yacon leaves.

CONCLUSION

Taken all the results together, the present research have shown that yacon SLs has chemopreventive effect on Raji cell line. In addition, anti-deformation assay can be used as a screening or preliminary assay to detect the potential of chemopreventive activity. However, further studies such as mouse model or different types of cancer are required to confirm their chemopreventive activity as well as the molecular target of these SLs.

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