Abstract. Background: The 5-year survival rate of the oral cancer patients has remained at approximately the 50% level during the past 30 years, possibly due to the poor tumor-selectivity of conventional anticancer drugs. This prompted us to search new plant extracts that have higher cytotoxicity against cancer cells than normal cells. Materials and Methods: Two human oral squamous cell carcinoma cell lines (HSC-2 and HSC-4) and two normal oral cells (gingival and periodontal ligament fibroblasts; HGF and HPLF) were incubated for 48 h with various concentrations of crude plant extract and the viable cell number was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The 50% cytotoxic concentration (CC50) was determined from the dose-response curve. Tumor-specificity (TS) was determined by the following equation: TS = mean CC50 (normal cells)/mean CC50 (cancer cell lines). Metabolic profiling techniques based on 1H nuclear magnetic resonance (NMR) were applied to gain the chemical structural insight for cytotoxicity induction. Results: Among 24 plant extracts, Camptotheca acuminate leaf, a well-known source for camptothecin, showed the highest TS value (88.3), followed by Vitis s.p.p. (>3.5), Sasa veitchii (>2.3) and Phellodendron amurense (>2.1), whereas other plant extracts showed much lower TS value (<2). These cytotoxic extracts made cluster on principal component analysis (PCA) score plot. Conclusion: The TS value determined by the present method seems to reflect the anti-tumor potential of each plant extract, while a part of the cytotoxic compounds present in these extracts may have common chemical structures.

Correspondence to: Professor Yoshiaki Shirataki, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai Sakado, Saitama 350-0295, Japan. Tel: +81 492717053, Fax: +81 492717984, e-mail: shiratak@josai.ac.jp

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Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 5-fluorouracil (5-FU) (Kyowa, Tokyo, Japan); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Inc., Osaka, Japan); DMSO-d$_6$ (Cambridge Isotope Laboratories, Inc., MA, USA).

Plant materials. Crude plant extracts used in this study are listed in Table I. These extracts have been already prepared and employed for various screenings in our laboratory. All extracts were stored at –30˚C before using. The original plants were mainly collected in various places in Japan. The specimens were proved and identified by Dr. Y. Shirataki and voucher specimens were also deposited in the Department of Pharmacognosy and Natural Medicines of Josai University.

NMR spectroscopy. $^1$H NMR spectra were recorded at room temperature on a 400 MHz Agilent-400MR-vnmrs 400 spectrometer (Agilent Technologies, CA, USA). Each spectrum consisted of 65,536 complex data points and a spectral width of 6,410.3Hz, obtained by 16 scans with a repetition time of 5.0 sec and a relaxation delay of 1.50 sec per scan. The detection pulse flip angle was set at 45˚.

NMR data reduction procedures and pattern recognition analysis. Each NMR spectrum was regimented into 375 regions, 0.04 part per million (ppm) wide, over the range –1.50 to 13.5 ppm. Each segment of the spectral regions (bucket) was integrated. Any integrated regions from 2.45 to 2.55 ppm that contained solvent signals were eliminated from the data table and then the total data were reduced to 372 regions. The remaining integral values of each spectrum were normalized over 100 total summed integrals to compensate for any differences in concentration between crude drug extracts. Spectral processing was performed using ALICE 2 for Metabolome version 5.0 software (JEOL Ltd., Akishima, Tokyo, Japan).

Preparation of crude drug extracts. Twenty-four kinds of crude plant extracts were prepared by extracting with MeOH under reflux.
for 3 h to give the methanolic extracts. These extracts were completely dried in vacuo and residues were dissolved in DMSO-$d_6$ at 10 mg/ml in concentration for $^1$H NMR measurement and dissolved in DMSO for cytotoxic evaluation.

**Cell culture.** Human oral squamous cell carcinoma cell lines (HSC-2, HSC-4) were purchased from Riken Cell Bank, Tsukuba, Japan. Normal human oral cells, gingival fibroblast (HGF) and periodontal ligament fibroblast (HPLF), were prepared from periodontal tissues, according to the guideline of the Intramural Ethic Committee (No. A0808), after obtaining the informed consent from a 12-year-old patient at the Meikai University Hospital (9). Since normal oral cells have a limited lifespan of 43-47 population doubling levels (PDL) (9), the cells at 8-15 PDL were used in this study. All the cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate. The normal cells were detached by 0.25% trypsin-0.025% EDTA-2Na in phosphate-buffered saline (PBS) without Mg$^{2+}$ and Ca$^{2+}$ (PBS(−)) and subcultured at a 1:4 split ratio once a week with a medium change in between the subcultures.

**Assay for cytotoxic activity.** The cells (3×10$^3$ cells/well, 0.1 ml/well) were seeded in 96-microwell plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and incubated for 48 h to allow cell attachment. Near-confluent cells were treated for 48 h with different concentrations of the test compounds in a fresh medium. The relative viable cell number of adherent cells was then determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (10). In brief, control and sample-treated cells were incubated for 4 h with 0.2 mg/ml of MTT in the culture medium. After removing the medium, the reaction product, formazan, was extracted with DMSO and the absorbance (the relative viable cell number) was measured at 540 nm by a microplate reader (Multiskan Bichromatic Labsystems, Helsinki, Finland). The 50% cytotoxic concentration (CC$_{50}$) was determined from the dose–response curve. The tumor specificity index (TS) was calculated by the following equation: TS = mean CC$_{50}$ (normal cells)/mean CC$_{50}$ (all tumor cell lines).

**Results**

**Cytotoxicity.** Several kinds of crude plant extracts showed higher cytotoxicity against human oral squamous cell carcinoma compared with human oral normal cells, yielding the tumor-specificity value of 1.0-88.3 (Table I). The extract of #10 (Camptotheca acuminata) showed the highest tumor-specificity, followed by #9 > #21 > #3 > #6 > #16.

**$^1$H NMR metabolomics.** To give the chemical structural feature of the active components, the crude plant extracts...
were subjected to NMR-based metabolomics. The $^1$H NMR spectra were subjected to principal component analysis (PCA) (Figure 1). All 372 variables in the bucketed regions (see Materials and Methods section) were equally accounted for in the data sets. The PCA score plot of principal component (PC) 1 (the greatest variance in the data) and PC2 (the second greatest variance in the data, orthogonal with PC1) showed that the crude plant extracts showing high TS value were gathered on PCA score plot (Figure 1, circled dots indicated the sample showing high TS value). The scores of PC1 and PC2 were 46.4, 31.8%, respectively.

Discussion

The present study demonstrated that some methanolic plant extracts showed tumor-specific cytotoxicity (TS=1.0-88.3) (Table I). Among these extracts, the extract of #10 (Camptotheca acuminata) showed the highest tumor specificity (TS=88.3). C. acuminata belongs to Nyssaceae and distributed in the center of South China. It is well-known that C. acuminata includes camptothecin, which has been the lead compound for irinotecan. Irinotecan shows tumor-specific cytotoxicity based on the inhibition of topoisomerase and is used for the clinical treatment of lung and cervical cancer. The present study demonstrated that the extract of C. acuminata indicated the highest TS value, in agreement with our recent report that camptothecin and SN-38, active metabolite of irinotecan, showed the TS value of 2561 and 808, respectively (11). This indicates that the TS value determined by the present method seems to reflect the anti-tumor potential of each plant extract, and may be a useful index for searching the new anti-tumor agents, regardless of the cell types [epithelia origin for oral squamous cell carcinoma (HSC-2, HSC-4) and mesenchymal origin for normal oral cells (HGF, HPLF)]. Although both cytotoxicity and TS values are inferior to those of #10, the extracts of #9 (Viis spp), # 21 (Sasa veitchii), #3 (Odontioda Marie Noel ‘Velano’), #6 (Odontogrossum Harvengtense ‘Tutu’) and #16 (Myrica rubra) indicated slightly higher cytotoxicity against human oral squamous cell carcinoma yielding modular TS values.

The values of PC1 and PC2 were used to give the information concerning chemical structure of active components in cytotoxic crude plant extracts. The score sum of PC1 and PC2 was 78.2%, which was sufficient to identify active components. Cytotoxic crude plant extracts indicating modulate TS value formed a cluster on PCA score plot. These results demonstrated that these extracts could contain common compounds with similar structure(s) for expressing cytotoxicity against tumor cells. Loading plot analysis and direct comparison of $^1$H NMR spectra indicated that aromatic signals on $^1$H NMR spectrum were detected commonly in cytotoxic extracts. Based on these results, we are planning to isolate and purify the active compounds and to elucidate those structures included in cytotoxic crude extracts.

References


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