JOURNAL OF FUNCTIONAL FOODS 8C (2014) 62–67

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Differentiation-inducing effect of piperitenone oxide, a fragrant ingredient of spearmint (*Mentha spicata*), but not carvone and menthol, against human colon cancer cells

Yasushi Nakamura [a,b,*], *Yuki Hasegawa* [a], *Koji Shirota* [b], *Noboru Suetome* [b], *Takako Nakamura* [a], *Mullika Traidej Chomnawang* [c], *Krit Thirapanmethee* [c], *Piyatip Khuntayaporn* [c], *Panida Boonyaritthongchai* [d], *Chalermchai Wongs-Aree* [d], *Shigehisa Okamoto* [e], *Tomoaki Shigeta* [f], *Tomoaki Matsuo* [f], *Eun Young Park* [a], *Kenji Sato* [a]

[a] *Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo-Hangi, Sakyo, Kyoto 606-8522, Japan*

[b] *Horticultural Division, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center, Amarube, Kameoka, Kyoto 621-0806, Japan*

[c] *Department of Microbiology, Faculty of Pharmacy, Mahidol University, 447 Sri Ayuthaya Rd., Bangkok 10400, Thailand*

[d] *Division of Postharvest Technology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, 49 Soi Thian Thale 25, Bang Khun Thain-Chai Thale Rd., Tha Kham, Bang Khun Thain, Bangkok 10150, Thailand*

[e] *Department of Agricultural Science, Kagoshima University, Korimoto 1-21, Kagoshima 890-0065, Japan*

[f] *Department of Biochemical Science and Technology, Kagoshima University, Korimoto 1-21, Kagoshima 890-0065, Japan*

ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 3 March 2014
Accepted 5 March 2014
Available online 28 March 2014

Keywords:
Spearmint
Terpenoid
Cancer
Colon
Differentiation
Mentha spicata

ABSTRACT

The *n*-hexane extract from spearmint (*Mentha spicata*) leaves was purified *via* a bioassay-guided fractionation scheme, which was based on the induction of differentiation in a RCM-1 human colon cancer cell line. One of the criteria of a differentiation marker is the formation of ducts determined visually using phase contrast microscopy, the most potent fraction contained piperitenone oxide, based on EI-MS, ^{1}H-NMR and ^{13}C-NMR spectra. Previously, the role of piperitenone oxide was considered as an odor producing compound in spearmint cultivars, but this study indicates the potential medical benefits of this compound. The effective dose of piperitenone oxide was lower than that of any differentiation-inducers found in the bioassay. Piperitenone oxide was categorized into monoterpene such as carvone and menthol, which are also familiar ingredients in *Mentha* genus plants. However, carvone and menthol did not possess the differentiation-inducing effect.

* *Corresponding author*. Tel.: +81 75 703 5406; fax: +81 75 703 5406.
E-mail address: yas@kpu.ac.jp (Y. Nakamura).

http://dx.doi.org/10.1016/j.jff.2014.03.005
1756-4646/

1. Introduction

One strategy for chemoprevention and chemotherapy would be to find chemicals that reduce the risk of cancer by prevention of mutational events in normal cells and induction of differentiation in cancer cells. In our previous study, a fragrant fraction of fully-ripened Katsura-uri, an heirloom vegetable in Kyoto (Japanese pickling melon; *Cucumis melo* var. *conomon*), exhibited both antimutagenic activity and differentiation-inducing effect (Nakamura, Suganuma, Kuyama, Sato, & Ohtsuki, 1998). One active compound, acetic acid 3-methylthio propyl ester (AMTP), exhibited antimutagenic activity, which was assessed by UV-induced mutation assay using *E. coli* B/r WP2 (Nakamura et al., 2010). Another active compound, 3-methylthiopropionic acid ethyl ester (MTPE), was identified as an anticarcinogenic compound as determined by its ability to induce differentiation in human colon cancer cells (RCM-1 cells) (Nakamura et al., 2008). AMTP and MTPE were isolated from the fragrant fraction of Katsura-uri by *n*-hexane extraction and silica-gel chromatography, and both of which have intense fruity and melon-like aromas (Nakamura et al., 2010). We therefore suppose that some fragrant ingredients in plants could potentially contribute to the chemoprevention and chemotherapy of cancer.

Colon cancer is one of the most widely distributed cancers in the world. Since most cases of colon cancer have few to no symptoms, the tumor is often diagnosed in the later stages of the cancer (Ferrández & DiSario, 2003; Telang, Li, & Katdare, 2006). Under the circumstances in colon cancer, an appropriate therapeutic strategy has been required. The conventional cancer therapy is surgery followed by the administration of anticancer drugs to kill any remaining tumorigenic cells (Jacob & Salky, 2005; Kahnamoui, Cadeddu, Farrokhyar, & Anvari, 2007; Umar, Viner, & Hawk, 2001). Since most patients unfortunately suffer from the side effects of these anticancer drugs, alternative chemotherapies, such as the induction of differentiation to cancer cells, are believed to be potentially better approaches. Therefore, we have focused on an identification of a differentiation inducer in RCM-1 human colon cancer cell line derived from a colon cancer tissue diagnosed as a rectum adenocarcinoma.

We have assessed the differentiation-inducing effect in human colon cancer cells (RCM-1 cells) focusing on the fragrant fraction of 44 kinds of vegetables, fruits, and herbal plants that emit strong aromas and flavors in Thai and Vietnamese cuisines. Some candidates can be listed to promote a chemical structure identification analysis, and it was used to identify potential new anticarcinogenic compounds from the fragrant fraction in spearmint (*Mentha spicata*) leaves.

In this paper, we used magnesium-silicate column and silica-gel thin layer chromatography procedures to purify the differentiation-inducers from an *n*-hexane extract of spearmint leaves *via* a bioassay-guided fractionation scheme. The bioassay system used a marker of differentiation (duct formation) in RCM-1 cells. This bioassay-guided fraction scheme allowed us to know the active ingredient, which was piperitenone oxide. Then, this compound was assessed for anticarcinogenic properties as compared to MTPE derivative, which is a potent differentiation-inducer in RCM-1 cells.

2. Materials and methods

2.1. Materials and reagents

Spearmint (*M. spicata*) leaves were harvested in August 2013 in an open field culture system at the Kyoto Prefectural Agricultural Research Institute (Kameoka City: *long.* 135°34′E, *lat.* 35°01′N, altitude of 110 m, annual mean air temperature of 14.6 °C, annual precipitation of 1590 mm), Japan. Spearmint leaves were cooled on harvest date in a refrigerator (4 °C), and overnight freighted to Kyoto Prefectural University for immediate extraction within 1 day after being held at 4 °C. *L*(-)-Carvone, and *L*-menthol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Assay of differentiation-inducing effect in human colon cancer cells

The RCM-1 colon cancer cell line was diagnosed as a well-differentiated rectum adenocarcinoma derived from a 73-year-old female human (Kataoka, Nabeshima, Komada, & Koono, 1989), and was obtained from Dr. H. Kataoka of the University of Miyazaki (Miyazaki, Japan). The RCM-1 cell line is characterized as a partially-differentiated type that is suitable to purify differentiation inducers, because the RCM-1 cells spontaneously differentiate as determined by the formation of ducts resembling villiform structures. Chemically induced differentiation further enhances the number and size of ducts above the background levels of the spontaneously differentiated RCM-1 cells (Nakamura et al., 2008). Thus, the real-time morphological observation of duct formation enables a simple assay for a rapid assessment of cell differentiation properties of potential chemopreventive compounds in plant extracts. RCM-1 cells (1×10^5) were each plated into 96-multi-well plastic culture plate with 0.2 ml of 45% RPMI1640 medium with 45% Ham's F12 medium (Gibco-Invitrogen Corporation, Grand Island, NY) and 10% fetal bovine serum (10% FBS-RPMI+F12). When RCM-1 cells were cultivated with samples for 2 days, the duct number was measured from digital photographs at 40× using a Zeiss Axiovert 25 microscope equipped with a CCD camera. Duct formation activity was calculated as the fraction of the solvent control. To identify an active fraction, we used a criterion that determined the lowest dose needed to induce a twofold increase of the number of ducts over the levels found in the control. A fraction was determined active if it met this criterion.

2.3. Duct formation bioassay-guided fractionation of spearmint

Spearmint leaves (100 g) were extracted twice with *n*-hexane (500 ml) in a refrigerator (4 °C) for 12 h. The filtered solution was mixed and evaporated under 40 °C to dryness of *n*-hexane extract. The residual leaves were then extracted twice with chloroform (500 ml) in a refrigerator (4 °C) for 12 h. The filtered solution was mixed and evaporated under 40 °C to dryness of chloroform extract. The fractionation procedure for each fraction is summarized in Fig. 1. The fractions were weighed and examined for duct formation assay. Fraction A (*n*-hexane extract, 310 mg) showed an ability to enhance duct formation. It was

then divided into fractions C-G by 8 g of magnesium-silicate column chromatography, using 80 ml of each eluent: *n*-hexane; 4%, 8%, 16%, 32% acetone in *n*-hexane. Fraction C (112 mg), which showed an ability to enhance duct formation, was spotted on preparative-TLC plate (Merck; silica gel 60 F_{254}, 20 × 20 cm), developed with the solvent of 0.3% methanol in chloroform. The spot of the compound was detected visually by UVC-irradiation onto the TLC plate. The broad band of R_f value 1–0.52 in Fraction H; 0.52–0.42 in Fraction I; 0.42–0.39 in Fraction J; 0.39–0.29 in Fraction K; 0.29–0 in Fraction L were collected. The ability to enhance duct formation was only shown in Fraction K (15.7 mg).

2.4. *Gas chromatography-mass spectrometry (GC-MS), ^{1}H-NMR and ^{13}C-NMR spectrometry*

GC-MS analyses were performed on a model JEOL JMS-AMSUN200 mass spectrometer, coupled on a Hewlett-Packard 6890 gas chromatograph. The capillary column was a Rtx-5MS column (0.25 mm ID × 30 m, 0.25 μm film thickness; Restek, Pennsylvania, USA). The column oven temperature was held at 60 °C for 5 min and then was increased to 250 °C at 5 °C/min. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker NMR (AVANCE-400) using $CDCL_3$ as the solvent with TMS as internal standard.

3. Results

3.1. *Purification of differentiation-inducing compound from spearmint leaves*

An overall purification scheme for the compound from the leaves of spearmint is shown in Fig. 1. The activity of duct formation (as a differentiation-inducing marker) and the weight of the fractions are also shown in Fig. 1. The active fraction was defined as the fraction exhibiting a twofold increase in duct number. Based on this criterion, *n*-hexane extract of spearmint leaves (Fraction A) enhanced the number of ducts in RCM-1 cells at 20 μg/ml (Fig. 1). The chloroform fraction (Fraction B) also showed the activity in the induction of differentiation, but the concentration was needed 40 μg/ml (higher than 20 μg/ml of Fraction A), and the yield was 100 mg (lower than 310 mg of Fraction A) (Fig. 1). Thus further fractionation focused only on the *n*-hexane extract (Fraction A). The next fractionation step used magnesium silicate-gel column chromatography resulting in the highest activity in Fraction C (*n*-hexane elution) in which a dose of 12.5 μg/ml induced duct formation (Fig. 1). Fraction D also showed the activity in the induction of differentiation, but the concentration was needed 40 μg/ml (higher than 12.5 μg/ml of Fraction C), and the yield was 52.5 mg (lower than 130 mg of Fraction C) (Fig. 1). Thus, further fractionation focused

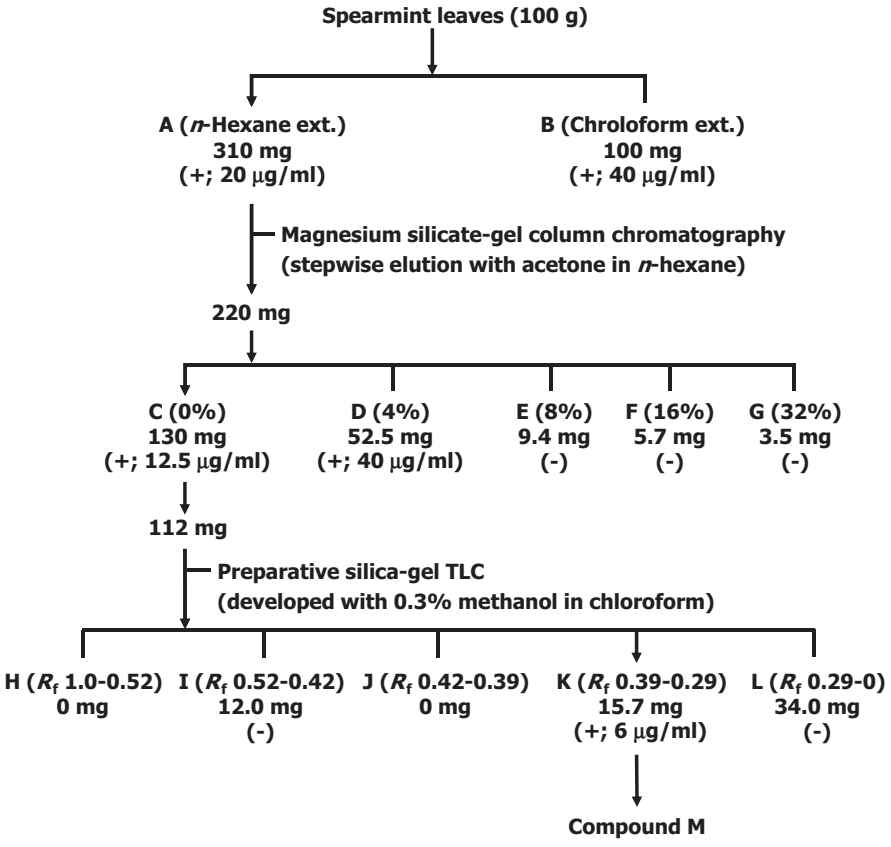

Fig. 1 – Duct formation bioassay-guided fractionation of spearmint leaves. The yield (weight) and activity (+; dose) or (–) was indicated directly under each fraction labeled A–L.

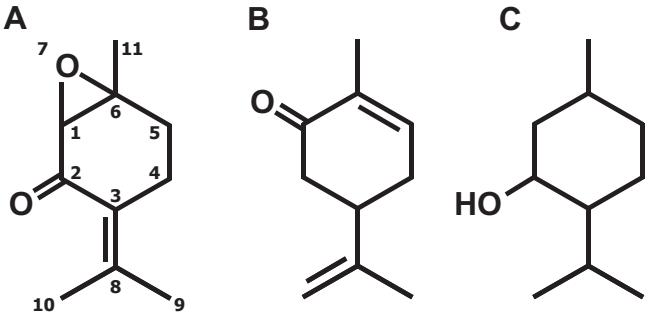

Fig. 2 – Chemical structure of 6-methyl-3-(1-methylethylidene)-7-oxobicyclo[4.1.0] heptone-2-one (piperitenone oxide), carvone, and menthol. (A) piperitenone oxide, (B) carvone, (C) menthol.

only on Fraction C. Fraction C (112 mg) was further purified using preparative silica-gel thin layer chromatography (TLC). The active fraction (6 μg/ml) that exhibited an increase in duct number, as compared to the untreated cells, was found in a band labeled Fraction K with an R_f value of 0.39–0.29 and a yield of 15.7 mg. In the purity analysis of Fraction K, both reverse phase HPLC and gas chromatography gave a single peak. In the series of purification steps, the active ingredient in spearmint leaves (Compound M) was successfully purified into Fraction K. Compound M was clear liquid at room temperature with a pale yellow color and had a slightly herbal aroma.

3.2. *Chemical component of the differentiation-inducing compound in Fraction K*

Fraction K gave one peak on the total ion chromatogram from the low-resolution-gas chromatography-electron ionization (LR-GC-EI) mass spectroscopy analysis. The compound (named Compound M), appearing at t_R 20.50 min, showed the ion peak at an *m*/z 166 $(M)^+$ and prominent fragment ions with masses of 151, 138, 123, 109, 95, 79, and 67. To validate our tentative identification, we analyzed Compound M with ^{1}H-NMR (400 MHz, $CDCL_3$), and the spectra were assigned: δ: 1.48 (3H), δ: 1.80 (3H), δ: 1.84–1.93 (2H), δ: 2.11 (3H), δ: 2.36–2.54 (2H), δ: 3.24 (1H). ^{13}C-NMR (100 MHz, $CDCl_3$) spectra were assigned: δ: 21.85 (C-11), δ: 23.15 (C-9, 10), δ: 23.17 (C-4), δ: 27.93 (C-5), δ: 63.43 (C-1), δ: 63.50 (C-6), δ: 127.69 (C-3), δ: 149.26 (C-8), δ: 198.47 (C-2). A literature retrieval was performed referring with the molecular weight, ^{1}H-NMR and ^{13}C-NMR spectra analyses, and the data of Compound M was corresponded with that of 6-methyl-3-(1-methylethylidene)-7-oxobicyclo[4.1.0]heptone-2-one (trivial name: piperitenone oxide) (Thach, Nhung, My, & Tran, 2013). The chemical structure of piperitenone oxide is shown in Fig. 2. These analytical data never show any negative proofs in the case, if Compound M were assigned as piperitenone oxide. We are now involving the detailed identification of the Compound M, including stereochemistry.

3.3. *The ability of Compound M, carvone, and menthol to induce differentiation in human colon cancer cells*

Authentic piperitenone oxide (>98% purity) has not been commercial available, and we assessed the ability of piperitenone oxide to enhance the formation of ducts with Compound M being representative authentic piperitenone oxide. RCM-1 cells spontaneously formed a small number of duct-like structures (Fig. 3A). A derivative of MTPE was used as a differentiation-inducer for a positive control in this assay. The positive control is needed for the sufficient effect at 106 μg/ml to exhibit more than a twofold increase in duct number from 2.8 (vehicle control) to 12 on RCM-1 cells (Fig. 3B). Treatment of RCM-1 cells for 2 days with Compound M between the doses of 6 and 20 μg/ml progressively increased the number of the ducts (24.3) relative to the vehicle control (2.8), and also increased percent area occupied by duct structures in culture plate (Fig. 3C). Compound M did not show any increase in the duct number and the size at 3 μg/ml on RCM-1 cells, and showed cytotoxic effect at 40 μg/ml, as determined by the cell detachment from the culture plates (data not shown). In contrast to Compound M, carvone and menthol did not show any increase in the duct number and the size at 20 μg/ml (Fig. 3D and E), and showed cytotoxic effect at 40 μg/ml (data not shown). These results indicated that piperitenone oxide possessed potential anticarcinogenic properties by inducing differentiation in human colon cancer cells, but carvone and menthol did not.

4. Discussion

A *n*-hexane extract from spearmint leaves exhibited anticarcinogenic properties by inducing differentiation in a RCM-1 human colon cancer cell system. We used a bioassay-guided fractionation scheme that relied on the induction of differentiation of RCM-1 cells as determined by duct formation to identify the active ingredient in spearmint. Using this fractionation scheme (*n*-hexane extraction followed by magnesium-silicate-gel column chromatography and then silica-gel thin layer chromatography), we successfully purified the most potent fraction. Based on spectra (EI-MS, ^{1}H-NMR and ^{13}C-NMR) analyses with literature retrieval, the fraction contained 6-methyl-3-(1-methylethylidene)-7-oxobicyclo[4.1.0]heptone-2-one (piperitenone oxide). The IUPAC name of piperitenone oxide, 6-methyl-3-(1-methylethylidene)-7-oxobicyclo[4.1.0]heptone-2-one, is given to its planar structure. We are now involving the detailed identification of the compound, including stereochemistry.

On the bioassay-guided fractionation scheme, Fraction B and Fraction D also showed a differentiation-inducing effect (Fig. 1). To rank the differentiation-inducing potency, and determine which fraction should be advanced to the next purification step, we used the criterion that is 'yield/effective dose'. The criterion allowed us to compare the comprehensive activity on quantitative aspect of the fractions. On the basis of the criterion, great differences can be seen between Fraction A (15.5: 310/20) and Fraction B (2.5: 100/40); and between Fraction C (10.4: 130/12.5) and Fraction D (1.3: 52.5/40). Therefore, we promoted to purify the active compounds in Fraction A and C, and successfully reached piperitenone oxide that was considered to be a principal differentiation-inducing ingredient in spearmint. However, the possibility cannot be completely denied that Fraction B and Fraction D contain some active compounds

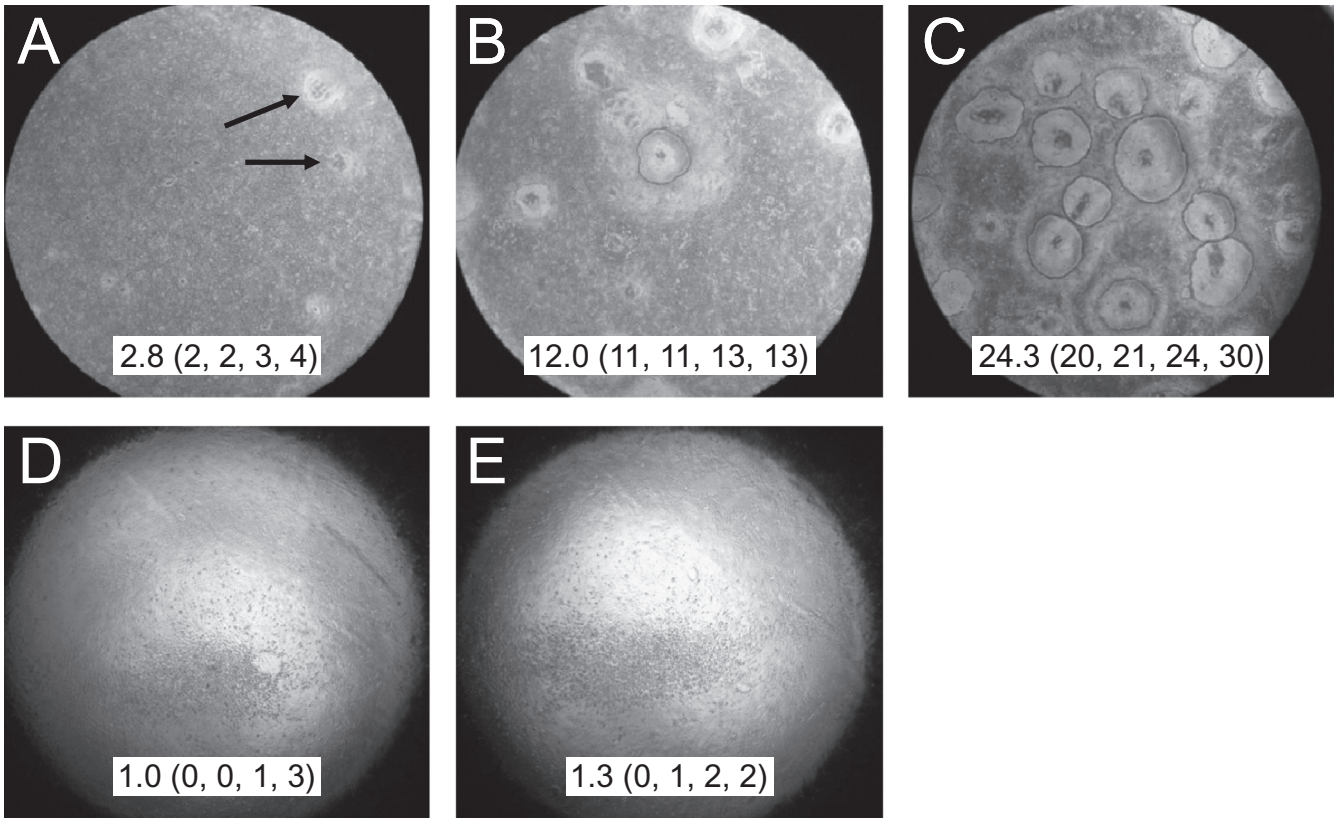

Fig. 3 – Enhancement of the duct formation of piperitenone oxide in RCM-1 cells. The differentiation potential was determined by seeding 1×10^5 RCM-1 cells into 96-multi-well plastic culture plate and then treated for 2 days with vehicle (A), positive control (106 μg/ml of MTPE derivative; B), piperitenone oxide (20 μg/ml; C), carvone (20 μg/ml; D), and menthol (20 μg/ml; E). The formation of ducts was visually determined using phase contrast microscopy. The values indicated under the photos represent averages of the number of ducts and each value of four replicates in parentheses. Arrows show an example of typical duct formation.

because the value of yield/effective dose is not zero in those fractions.

On a chemical aspect, piperitenone oxide was categorized into monoterpene such as carvone and menthol (Fig. 2B and C), which are familiar ingredients in *Mentha* genus plants. Carvone is principally found in some species of spearmint, and is widely used as an ingredient in toothpaste, spearmint chewing gum, and some foods. Menthol is mainly contained in peppermint (*Mentha* × *piperita* L.), American wild mint (*Mentha arvensis*) and Japanese mint (*Mentha canadensis*), and is included in many medical products such as balm, cough medicine, and poultice. However, both carvone and menthol do not possess a differentiation-inducing effect in human colon cancer cells on the duct formation assay. Therefore, not all monoterpens have the ability, and the active specificity of piperitenone oxide should be investigated. Future experiments determining the underlying mechanisms of how this compound induces differentiation will help us understand its anticarcinogenic properties. In addition, auraptene (7-geranyloxycoumarin) having a monoterpene moiety, and β-cryptoxanthin (one of tetraterpenes), increased antibody production by HB4C5 cells and mouse primary lymphocytes, and might promote immature B cells to differentiate plasma cells (Nishi, Muranaka, Nishimoto, Kadota, & Sugahara, 2012; Nishimoto, Muranaka, Nishi, Kadota, & Sugahara, 2012). Moreover, methanol extract of Canadian maple syrup induced cell cycle arrest with decreased levels of Cyclin A and Cyclin D1 in human colon cancer cells; some terpenoids in the extract might play a role in the differentiation-inducing effect (González-Sarrías, Li, & Seeram, 2012). Such food ingredients and extract may also serve as candidates for this RCM-1 bioassay in future.

In this bioassay, MTPE was first identified as a differentiation-inducing chemical of Japanese heirloom pickling melon (*Cucumis melo* var. *reticulatus*) fruit (Nakamura et al., 2008). Following the report, methylthioacetic acid ethyl ester (MTAE) was found in the same to be the second differentiation-inducing chemical (Nakamura et al., 2010). Thereby, derivatives of MTPE have been synthesized, and some sulfur-containing chemicals are known to possess the effect (our unpublished data). Thus, all chemicals presently known to induce differentiation in RCM-1 cells are sulfur-containing chemicals. Piperitenone oxide is therefore a novel differentiation-inducing compound that does not contain sulfur atom in its chemical structure. On a biological aspect in this bioassay, we have used MTPE derivative as a positive control, because the chemical induces differentiation at

the lowest dose range between 27 and 212 μg/ml among chemicals identified so far. In this study, the dose range of piperitenone oxide was between 6 and 20 μg/ml, which is lower than that of MTPE derivative.

The potential medical benefits of piperitenone oxide have not been realized. Few reports described relaxant and depressant activities in the intestinal smooth muscle of guinea pig, and a decrease in aortic pressure and heart rate by intravenous treatment in normotensive rat (Lahlou, Carneiro-Leão, Leal-Cardoso, & Toscano, 2001; Sousa, Magalhães, Lima, Oliveira, & Leal-Cardoso, 1997). The results of this study indicating that piperitenone oxide can induce differentiation of the RCM-1 human colon cancer cells is the first report of anticarcinogenic properties for this compound. In addition, oral administration of MTPE derivative delays the expression of papilloma and decreases the number in two-stage mouse skin carcinogenesis model in our promoting study. Piperitenone oxide would therefore be expected the effects in the same model with a lower dose of administration.

Piperitenone oxide is a common component in spearmint leaves, but is not considered to be distributed to only spearmint cultivars, because hybridization between some mint species easily occurs in open field. Thereby, piperitenone oxide has been also found in the leaves of other mint species, such as horse mint (*Mentha longifolia*), apple mint (*Mentha suaveolens*), pineapple mint (*Mentha suaveolens* cv. 'Variegata'), and Bowles' mint (*Mentha* × *villosa* var. *alopecuroides*) (Ghoulami, Idrissi, & Tetouani, 2001; Ohkubo, 2012; Venskutonis, 1996). Presently, piperitenone oxide is considered to be widely distributed in *Mentha* genus. However, regional difference in spearmint (supplied at local supermarket) was not observed on the ability of differentiation-inducing effect among three cities: Kyoto, Japan; Bangkok, Thailand; Ho Chi Minh City, Vietnam; and all of which contained piperitenone oxide (data not shown). Since these results gave a novel aspect of functional food for spearmint, public understanding of science and education of such results would be an important matter in future. For realizing the matters, one of the authors established a science soup cafe named 'Shizuku-ya' at Nishiki-ichiba market known as 'Kyoto's kitchen' in the center of Kyoto city in 2012. One of the roles that the science soup cafe can play would be to supply spearmint soup with relevant instruction about the result presented here about spearmint for promoting human health. Epidemiological studies and animal experiments on the anticarcinogenic effects of piperitenone oxide need to be performed; and to identify mint species with high piperitenone oxide content for being efficacious and for effective utilization of spearmint in the future.

REFERENCES

Ferrández, A., & DiSario, J. A. (2003). Colorectal cancer: screening and surveillance for high-risk individuals. *Expert Review of Anticancer Therapy*, *3*, 851–862.

Ghoulami, S., Idrissi, A., & Tetouani, S. (2001). Phytochemical study of *Mentha longifolia* of Morroco. *Fitoterapia*, *72*, 596–598.

González-Sarrías, A., Li, L., & Seeram, N. P. (2012). Anticancer effects of maple syrup phenolics and extracts on proliferation, apoptosis, and cell cycle arrest of human colon cells. *Journal of Functional Foods*, *4*, 185–196.

Jacob, B. P., & Salky, B. (2005). Laparoscopic colectomy for colon adenocarcinoma: an 11-year retrospective review with 5-year survival rates. *Surgical Endoscopy*, *19*, 643–649.

Kahnamoui, K., Cadeddu, M., Farrokhyar, F., & Anvari, M. (2007). Laparoscopic surgery for colon cancer: a systematic review. *Canadian Journal of Surgery*, *50*, 48–57.

Kataoka, H., Nabeshima, K., Komada, N., & Koono, M. (1989). New human colorectal carcinoma cell lines that secrete proteinase inhibitors in vitro. *Virchows Archiv. B, Cell Pathology Including Molecular Pathology*, *57*, 157–165.

Lahlou, S., Carneiro-Leão, R. F., Leal-Cardoso, J. H., & Toscano, C. F. (2001). Cardiovascular effects of the essential oil of *Mentha* x *villosa* and its main constituent, piperitenone oxide, in normotensive anaesthetised rats: role of the autonomic nervous system. *Planta Medica*, *67*, 638–643.

Nakamura, Y., Nakayama, Y., Ando, H., Tanaka, A., Matsuo, T., Okamoto, S., Chang, C.-C., Upham, B. L., Trosko, J. E., Park, E. Y., & Sato, K. (2008). 3-Methylthiopropionic acid ethyl ester, from Katsura-uri (Japanese pickling melon, *Cucumis melo* var. *conomon*), enhanced differentiation in partially-differentiated human colon cancer cells. *Journal of Agricultural and Food Chemistry*, *56*, 2977–2984.

Nakamura, Y., Suganuma, E., Kuyama, N., Sato, K., & Ohtsuki, K. (1998). Comparative bio-antimutagenicity of common vegetables and traditional vegetables in Kyoto. *Bioscience, Biotechnology, and Biochemistry*, *62*, 1161–1165.

Nakamura, Y., Watanabe, S., Kageyama, M., Shirota, K., Shirota, K., Amano, H., Kashimoto, T., Matsuo, T., Okamoto, S., Park, E. Y., & Sato, K. (2010). Antimutagenic; differentiation-inducing; and antioxidative effects of fragrant ingredients in Katsura-uri (Japanese pickling melon, *Cucumis melo* var. *conomon*). *Mutation Research*, *703*, 163–168.

Nishi, K., Muranaka, A., Nishimoto, S., Kadota, A., & Sugahara, T. (2012). Immunostimulatory effect of β-cryptoxanthin *in vitro* and *in vivo*. *Journal of Functional Foods*, *4*, 618–625.

Nishimoto, S., Muranaka, A., Nishi, K., Kadota, A., & Sugahara, T. (2012). Immunomodulatory effects of citrus fruit auraptene *in vitro* and *in vivo*. *Journal of Functional Foods*, *4*, 883–890.

Ohkubo, N. (2012). Analysis and classification of emitted scent compounds in leaves of *Mentha* cultivars. *Bulletin of the National Institute of Floricultural Science* (in Japanese), *12*, 103–112.

Sousa, P. J. C., Magalhães, P. J. C., Lima, C. C., Oliveira, V. S., & Leal-Cardoso, J. H. (1997). Effects of piperitenone oxide on the intestinal smooth muscle of the guinea pig. *Brazilian Journal of Medical and Biological Research*, *30*, 787–791.

Telang, N. T., Li, G., & Katdare, M. (2006). Prevention of early-onset familial/hereditary colon cancer: new models and mechanistic biomarkers (review). *International Journal of Oncology*, *28*, 1523–1529.

Thach, L. N., Nhung, T. H., My, V. T. N., & Tran, H.-A. (2013). The new rich source of rotundifolone: *Mentha aquatica* Linn. var. *crispa* oil from microwave-assisted hydrodistillation. *The Journal of Essential Oil Research*, *25*, 39–43.

Umar, A., Viner, J. L., & Hawk, E. T. (2001). The future of colon cancer prevention. *Annals of the New York Academy of Sciences*, *952*, 88–108.

Venskutonis, P. R. (1996). A chemotype of *Mentha longifolia* L. from Lithuania rich in piperitenone oxide. *The Journal of Essential Oil Research*, *8*, 91–95.