Title page

Names of authors: Ken-ichi Kudo, Tadashi Hanafusa, Toshiro Ono*

Title: *In vitro* analysis of radioprotective effect of monoterpenes

Affiliation and address of the authors: Department of Radiation Research, Advanced Science Research Center, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan

*Corresponding author

E-mail address of the corresponding author: onot@md.okayama-u.ac.jp

In vitro analysis of radioprotective effect of monoterpenes

Ken-ichi Kudo, Tadashi Hanafusa, Toshiro Ono

Department of Radiation Research, Advanced Science Research Center,

Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan

Abstract

Monoterpenes are naturally occurring hydrocarbons composed of two units of isoprenes. They exhibit antioxidant activity to scavenge reactive oxygen species, such as hydroxyl radicals. We investigated the potential of monoterpenes such as thymol, linalool, and menthol to act as radioprotectants. The proliferation of EL4 cells, a mouse lymphoma cell line, treated with linalool at a concentration of 500 μM or more was not affected by X-ray irradiation. Plasmid-nicking assay performed using formamidopyrimidine-DNA glycosylase showed that linalool prevented single strand breaks and oxidized purines on pUC19 plasmid DNA. These findings indicate that linalool has the ability to scavenge reactive oxygen species and is a potential radioprotector.

Keywords

monoterpenes, linalool, X-ray irradiation, reactive oxygen species, SSB

Introduction

Ionizing radiation excites and decomposes water molecules to generate a variety of reactive oxygen species (ROS) in biological systems. ROS can react with cellular DNA molecules, resulting in a wide spectrum of cell death through single and double DNA

strand breaks [1]. An antioxidant molecule has the potential to quench ROS and act as a radioprotectant. A number of radioprotective molecules have been identified over more than 5 decades, such as vitamin E, melatonin, and thiol compounds [2-4]. However, few efficient radioprotectant molecules are available. Amifostine is the only drug approved by the U.S. Food and Drug Administration, in 1999, for use in radiation oncology clinics. However, its side effects, such as severe nausea, allergy, and acute hypertension, have prompted a continuing search for better radioprotective molecules [5].

A variety of plants have been used in folk medicine. Volatile plant extracts and essential oils are used in the food, cosmetic, and pharmaceutical industries on a large scale. They show low or negligible toxicity and have a range of biological activities, including anti-inflammatory and anti-bacterial activity [6,7]. Monoterpenes are the main components of essential oils and often show antioxidant activity [8-11].

In this study, we investigated the radioprotective activities of monoterpene molecules, thymol, linalool, and menthol, which are major components of monoterpenes. *In vitro* analysis was performed to assess cell viability and DNA strand break caused by X-ray radiation. The results showed that linalool acted as an effective radioprotectant molecule in the cells.

Experimental

Cell lines

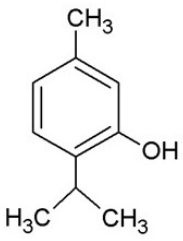
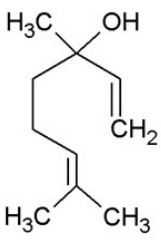
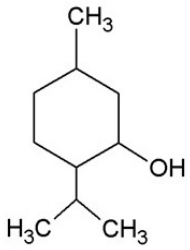
EL4 cells comprise lymphomas in the C57BL/6N mouse [12]. The cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10%

heat-inactivated fetal bovine serum (FBS) and antibiotics at 37°C under a humidified atmosphere of 5% CO_2.

Reagents

Three kinds of monoterpenes: thymol (CAS No. 89-83-8), linalool (CAS No. 78-70-6), and menthol (*l*-menthol, CAS No. 2216-51-5), were purchased from Wako Pure Chemical Industries (Osaka, Japan). Their physico-chemical characteristics are shown in Table 1. They were preserved at 4°C after preparing 1 M stock solution with acetonitrile. Formamidopyrimidine DNA glycosylase (Fpg) was purchased from New England Biolabs (Ipswich, MA, USA).

Table 1 Physico-chemical characteristics of monoterpenes

Chemical name	Thymol	Linalool	Menthol
CAS number	89-83-8	78-70-6	2216-51-5
Chemical structure			
Molecular formula	$C_{10}H_{14}O$	$C_{10}H_{18}O$	$C_{10}H_{20}O$
Molecular weight (g mol^{-1})	150.2	154.3	156.3
Density (g cm^{-3})	0.97	0.87	0.89
Log K_{OW}	3.3	3.0	3.4
LD_{50} (mg kg^{-1} bw)	980 [a]	2800 [b]	2500 [c]

[a] Jenner et al. [13]

[b, c] Organisation for Economic Co-operation and Development (OECD) [14, 15]

Preparation of plasmid DNA

pUC19 plasmid DNA (2,686 base pairs) was isolated and purified from the *E. coli* JM109 strain (Takara Bio, Kusatsu, Japan) using a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany).

X-ray irradiation

EL4 cell suspension (500 μL) and pUC19 plasmid DNA solution (50 μL) in 1.5-mL microtubes were irradiated with 5 Gy and 10 Gy of X-rays, respectively. The X-ray irradiation was carried out using the X-ray generator MBR-1520R (HITACHI, Hitachi, Japan) at 125 kVp and 15 mA with a 0.5-mm Al + 0.2-mm Cu filter, and the dose rate was 0.85 Gy min^{-1}.

Measurement of cell viability

EL4 cells (1×10^6 cells mL^{-1}) were pretreated with 100-1,000 μM monoterpene solution (final acetonitrile concentration: 1%) in serum-free D-MEM medium in a 24-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 37°C under 5% CO_2 for 1 hr. Control cells were incubated in serum-free D-MEM containing 1% acetonitrile. The cells irradiated by 5 Gy of X-rays were immediately washed twice with D-MEM (10% FBS), and then plated in a 24-well plate. After 24-hr incubation at 37°C under 5% CO_2, cell viability was determined by the trypan blue exclusion assay.

Agarose gel electrophoresis for X-ray-irradiated plasmid DNA

pUC19 plasmid DNA (10 ng μL^{-1}) was pretreated with thymol, linalool, and menthol, respectively, at varying concentrations. Ten Gy of X-ray irradiation after, a total of 12 μL (100 ng) of plasmid DNA solution was separated by electrophoresis on a 1.2% (w/v) agarose gel containing ethidium bromide in TAE buffer at 50 V for 100 min. The intensity of the target bands was analyzed by Image-J software (National Institute of Health, Bethesda, MD). The intensity ratio of the closed circular form (CC) to the opened circular form (OC) was corrected to 1.4 [16].

Detection of oxidized purines formed on irradiated plasmid DNA

Plasmid DNA (10 ng μL^{-1}) treated with 100 μM thymol, linalool, and menthol for 1 hr was X-ray-irradiated at 1 to 10 Gy. After the X-ray irradiation, 1 unit of Fpg (1.95 ng μL^{-1}) was added to each plasmid DNA sample (200 ng). The mixture was incubated at 37°C for 1 hr. A total of 12 μL of samples was then separated by agarose gel electrophoresis. The band intensity of CC, OC, and linear form (L) was analyzed by Image-J software. A dose-response was determined from the logarithmic loss of CC plasmid DNA for the radiation dose. From the slope of this response, a D_{37} value was obtained which, assuming Poisson statistics for single strand break (SSB) induction, represents the radiation dose required to give on average one SSB per plasmid molecule. Using the D_{37} value, an average number of SSB (Gy^{-1} Da^{-1}) was calculated as:

$$\text{SSB number } \left(\text{Gy}^{-1}\ \text{Da}^{-1}\right) = \frac{1}{D_{37} \times 2{,}686 \times 660} \qquad (1)\ [16]$$

where 2686 is the pUC19 plasmid base pair number and 660 is the molecular mass of 1 base pair. The number of oxidized purines was calculated as:

$$\text{Oxidized purines } \left(\text{Gy}^{-1}\ \text{Da}^{-1}\right) = SSB_{Fpg} - SSB_0 \qquad (2)$$

where SSB_{Fpg} is the SSB number after Fpg treatment, and SSB_0 is Fpg non-treatment.

Results

Effect of monoterpenes on EL4 cells

To analyze the effect of monoterpenes on EL4 cells, the cells were incubated with thymol, linalool, and menthol at a concentration of up to 1,000 μM, respectively. At least 500 μM of menthol showed an effect to enhance proliferation. Linalool also enhanced proliferation at 1,000 μM. On the other hand, thymol markedly reduced proliferation at 500 μM (Fig. 1).

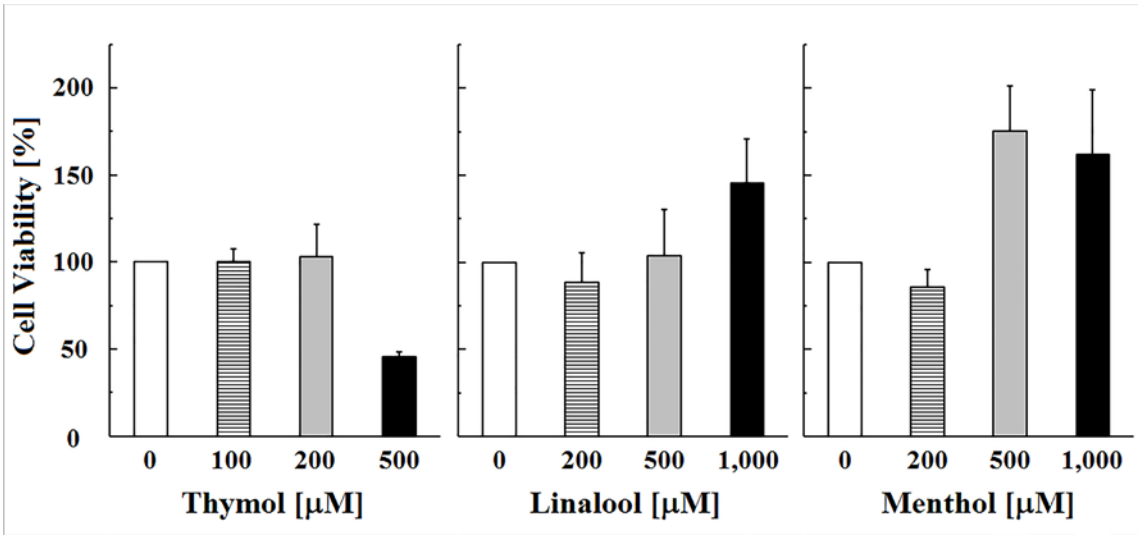


Fig. 1 Effect of monoterpenes on cell growth. Mouse lymphoma EL4 cells were treated by monoterpenes for 1 hr, and further incubated with the monoterpene-free medium for 24 hr. Cell viability was measured by the trypan blue exclusion test. Results represent the percentage relative to control cells without terpenoids, and show the means of three independent assays ± SD

Effect of monoterpenes on cell survival

Effects to protect against radiation were examined by analyzing the cell death rate after 5 Gy of radiation. Among the three kinds of monoterpene, linalool significantly reduced cell death in a dose-dependent manner. Nearly 80% of cell viability was observed at a concentration of 1,000 μM (Fig. 2). No effects to protect against radiation were observed for thymol or menthol.

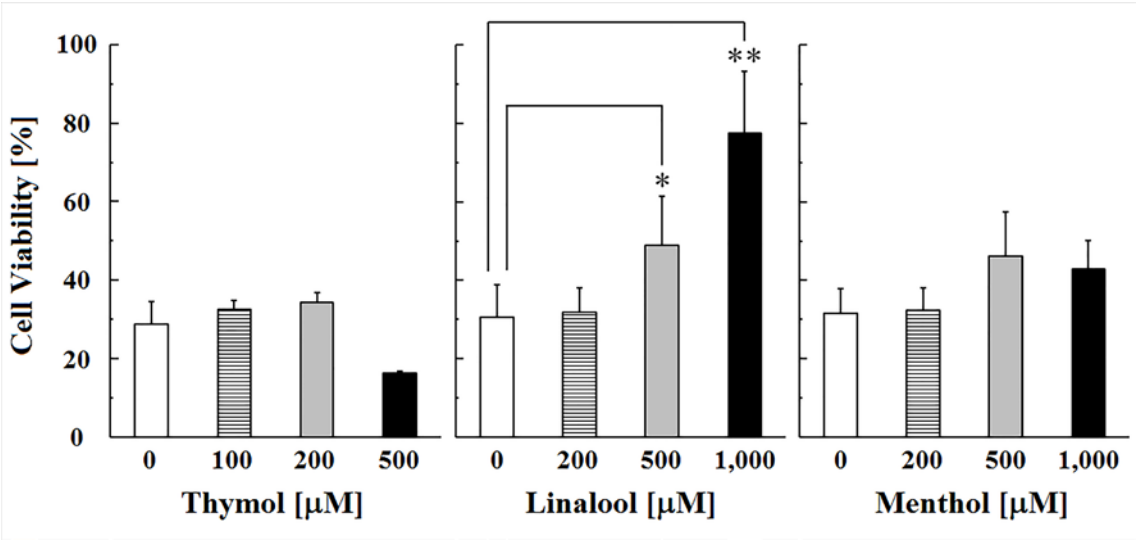


Fig. 2 Effect of X-ray irradiation on cell growth. EL4 cells were pretreated with monoterpenes for 1 hr prior to 5 Gy of X-ray irradiation. After the X-ray irradiation, the

cells were incubated with monoterpene-free medium for 24 hr, and cell viability was measured. Results represent the percentage relative to the monoterpene-free cells without irradiation, and show the means of three independent assays ± SD (* $p < 0.05$; ** $p < 0.01$, *t*-test)

Effect of monoterpenes on radiation-induced DNA damage

We then evaluated the effect of monoterpenes to protect against the radiation-induced DNA damage. pUC19 plasmid DNA in monoterpene solution was irradiated with 10 Gy. Ten Gy was the optimal dose for observing an efficient DNA break. The opened circular (OC) form, which resulted from DNA strand break, was analyzed by electrophoresis. As shown in Fig. 3, linalool significantly reduced the OC form in a dose-dependent manner, clearly indicating its effect to protect DNA from radiation. Thymol and menthol showed a limited protective effect even at 1,000 μM.

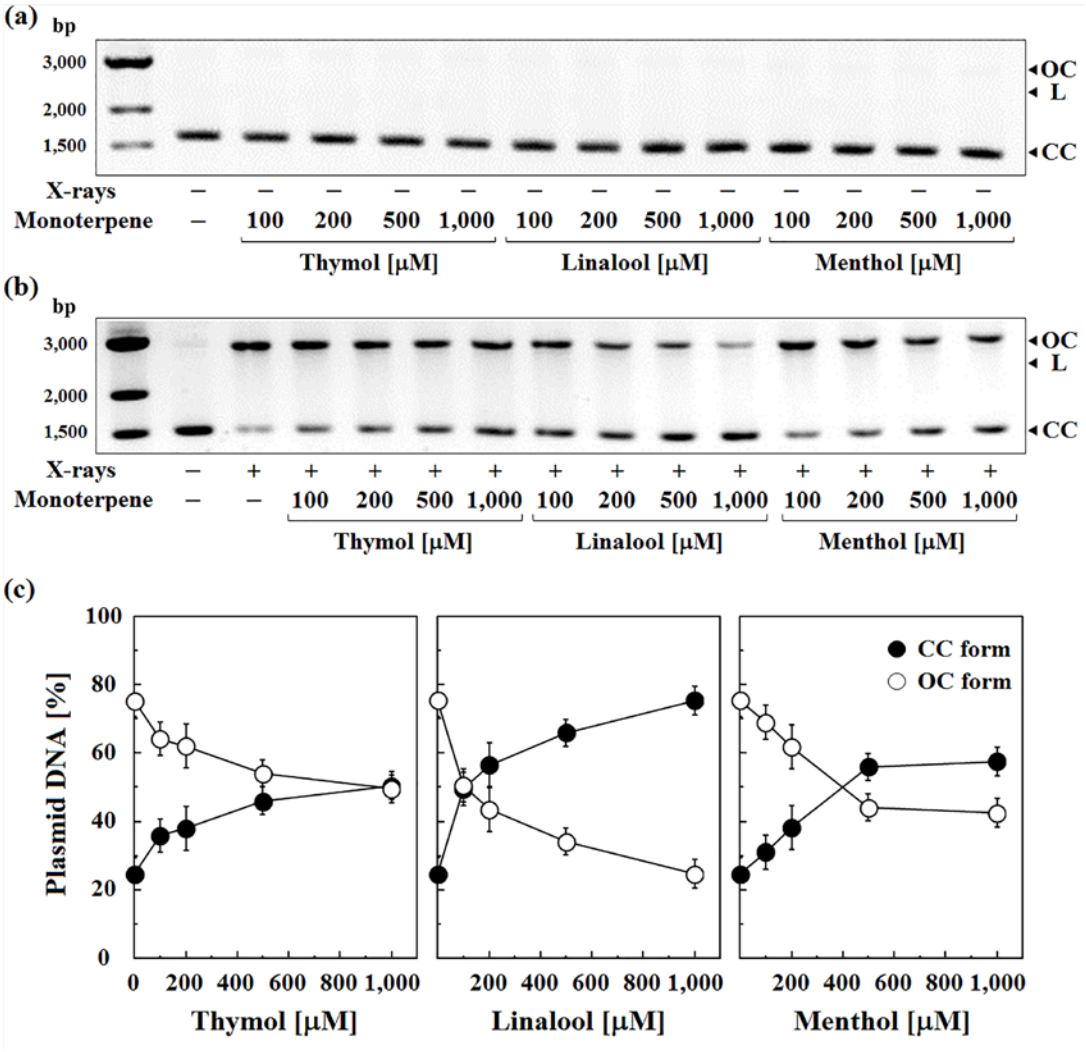


Fig. 3 a and **b** Analysis of radiation-induced DNA damage. Gel electrophoresis image of pUC19 plasmid DNA treated with monoterpenes. a. No X-ray irradiation; b. 10 Gy of X-ray irradiation; **c.** Percent relative quantities of CC and OC forms were calculated from **b**. Each result is the means of three independent assays ± SD

Quantitative analysis of effects to prevent SSB on radiation exposure

The effects of monoterpenes to protect against radiation were examined quantitatively by SSB production analysis. An oxidized damages of plasmid DNA is detected as SSB

using Fpg protein, that recognize and excise oxidized purine nucleotides [16-18]. The dose dependence of the CC DNA amount for X-ray irradiation of pUC19 plasmid DNA is shown in Fig. 4a and 4b. From the dose-dependent curves, the yields of SSB were calculated from the D_{37} values using equation 1. As shown in Fig. 4c, a significantly lower amount of the oxidized purines was found in the sample with linalool treatment, clearly showing the effectiveness of linalool to protect against radiation by an antioxidative mechanism. Thymol and menthol showed a limited effect on DNA cleavage.

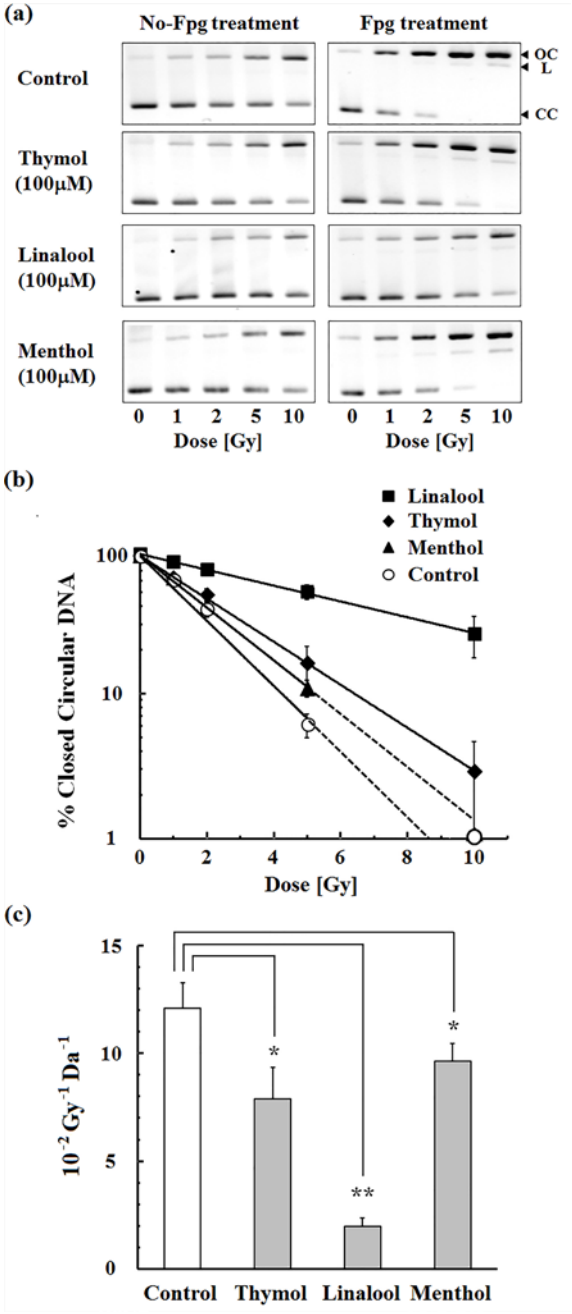


Fig. 4 Analysis of SSB of pUC19 plasmid DNA induced by X-ray irradiation. **a** Gel electrophoresis image showing the dependence of SSB induction in DNA on the X-ray dose following post-irradiation incubation in the presence or absence of Fpg. **b** X-ray dose-dependent curves of percent CC form of DNA calculated from the Fpg treatment group in Fig. 4a. **c** Yields of the oxidized purines produced in pUC19 plasmid DNA

after X-ray irradiation. Each result is the means of three independent assays ± SD (* $p < 0.05$; ** $p < 0.001$, *t*-test)

Discussion

Monoterpenes, which are the main components of aromatic essential oils, are abundant in flowers, leaves, fruits, and bark. They have a wide variety of bioactivities, such as antioxidant activity [8]. Antioxidant activities of essential oils extracted from coriander, eucalyptus, juniper, cumin, basil, and thyme have been reported [8-10].

In this study, three kinds of monoterpenes: thymol, linalool, and menthol, were analyzed to assess their radioprotective activities *in vitro*. Among them, linalool showed a potent radioprotective activity. It is abundant in lavender, coriander, cumin, cinnamon, and ginger, and has been commonly used in perfumed products. The proliferation of EL4 cells, a mouse lymphoma cell line, treated with linalool was not affected by X-ray irradiation. Linalool also markedly reduced SSB and oxidized purines induced on pUC19 plasmid DNA by X-ray irradiation. Radiation-induced radiolysis of water generates a variety of ROS which cause the direct damage of cellular DNA. Linalool may possess the ability to neutralize ROS, especially hydroxyl radicals, during irradiation and is considered to be an efficient radioprotector. On the other hand, thymol and menthol did not show any radioprotective activity. Thymol inhibited cell proliferation at 500 μM.

In addition to antioxidant activity, many volatile components in essential oils have been reported to possess anti-inflammatory and anti-cancer effects. Thymol, linalool,

and menthol exhibited anti-cancer effects against human tumor cell lines, such as prostate cancer [19,20], malignant melanoma [21], colon cancer [22], and gastric cancer [23]. They suppressed cell growth and induced apoptosis in tumor cells in a time- and dose-dependent manner. In our radioprotective study, EL4 cells were incubated with thymol, linalool, and menthol for 1 hr. Linalool and menthol enhanced the proliferation of EL4 cells in a dose-dependent manner. However, all 3 monoterpenes suppressed cell proliferation after 24 hr of incubation, being in agreement with the previous studies. Menthol showed marked inhibition at 1,000 μM (data not shown).

Traditional medicinal plants have been used for pharmaceutical and dietary therapy for several millennia in East Asia. Several studies indicated the usefulness of some medicinal plants and their extracts as radioprotective reagents. Extract from plants, such as ginseng, Citrus, and Mentha, have been found to have radioprotective effects in mammals [24-26]. The treatment of Chinese hamster lung fibroblasts with thymol (25 μg/mL) prior to 10-Gy gamma radiation protected the cells against radiation-induced cytotoxity [27].

Taken together, our *in vitro* analysis of monoterpenes in cells as radioprotectors against X-ray radiation revealed that linalool has a potent radioprotective effect. Linalool may be used as a radioprotector or radiorecovery reagent during both planned and unplanned radiation exposure.

Conclusions

In this study, we showed notable radioprotective effects of monoterpenes. By the cell

proliferation assay and the plasmid-nicking assay, linalool revealed a strongest radioportective effect among three monoterpenes investigated. As we also confirmed low cytotoxicity of linalool, we concluded that linalool can be used as a potential radioprotector. Further studies will be needed for radioprotective effects of a variety of monoterpenes..

Acknowledgements

We thank the staff of Department of Animal Resources, Advanced Science Research Center, Okayama University for use of X-ray generator MBR-1520R. We also thank Ms. T. Terada for preparation of the manuscript. This work was supported in part by the Grant for Strategies for the Efficient Operation of Okayama University.

References

1. Roots R, Okada S (1975) Estimation of life times and diffusion distances of radicals involved in X-ray-induced DNA strand breaks or killing of mammalian cells. Radiat Res. 64: 306-320
2. Erol F S, Topsakal C, Ozveren M F, Kaplan M, Ilhan N, Ozercan I H, Yildiz O G (2004) Protective effects of melatonin and vitamin E in brain damage due to gamma radiation. Neurosurg Rev. 27: 65-69
3. Koc M, Taysi S, Buyukokuroglu M E, Bakan N (2003) Melatonin protects rat liver against irradiation-induced oxidative injury. J Radiat Res. 44: 211-215

4. Hofer M, Falk M, Komůrková D, Falková I, Bačíková A, Klejdus B, Pagáčová E, Štefančíková L, Weiterová L, Angelis K J, Kozubek S, Dušek L, Galbavý S (2016) Two new faces of amifostine: protector from DNA damage in normal cells and inhibitor of DNA repair in cancer cells. J Med Chem. 59: 3003-3017
5. Koukourakis M I, Kyrias G, Kakolyris S, Kouroussis C, Frangiadaki C, Giatromanolaki A, Retalis G, Georgoulias V. (2000) Subcutaneous administration of amifostine during fractionated radiotherapy: a randomized phase II study. J Clin Oncol. 18 (11): 2226-2233
6. De La Luz Cadiz-Gurea M, Borras-Linares I, Lozano-Sanchez J, Joven J, Fernandez-Arroyo S, Segura-Carretero A. (2017) Cocoa and grape seed byproducts as a source of antioxidant and anti-inflammatory proanthocyanidins. Int J Mol Sci. 18 (2): 376
7. Lang G, Buchbauer G (2012) A review on recent research results (2008-2010) on essential oils as antimicrobials and antifungals. A review. Flavour Frag J. 27: 13-39
8. Wei A, Shibamoto T (2010) Antioxidant/Lipoxygenase inhibitory activities and chemical compositions of selected essential oils. J Agric Food Chem. 58: 7218-7225
9. Tomaino A, Cimino F, Zimbalatti V, Venuti V, Sulfaro V, De Pasquale A, Saija A (2005) Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. Food Chem. 89: 549-554
10. El-Ghorab A, Shaaban H. A, El-Massry K F, Shibamoto T (2008) Chemical composition of volatile extract and biological activities of volatile and less-volatile

extracts of Juniper Berry (*Juniperus drupacea* L.) fruit. J Agric Food Chem. 56: 5021-5025

11. Asikin Y, Taira I, Inafuku S, Sumi H, Sawamura M, Takara K, Wada K (2012) Volatile aroma components and antioxidant activities of the flavedo peel extract of unripe Shiikuwasha (*Citrus depressa* Hayata). J Food Sci. 77 (4): 469-475
12. Goper P A (1950) Studies in antibody response of mice to tumour inoculation. Br J Cancer. 4:372-379
13. Jenner P M, Hagan E C, Taylor J M, Cook E L, Fitzhugh O G (1964) Research section Food flavourings and compounds of related structure I Acute oral toxicity. Food Cosmet Toxicol. 2: 327-343
14. OECD Screening Information DataSet (SIDS) (2002) High production volume chemicals (processed by UNEP chemicals), SIDS Initial Assessment Report For SIAM 14
15. OECD Screening Information DataSet (SIDS) (2003) High production volume chemicals (processed by UNEP chemicals), SIDS Initial Assessment Report For SIAM 16
16. Gulston M, Fulford J, Jenner T, De Lara C, O'Neill P (2002) Clustered DNA damage induced by gamma radiation in human fibroblasts (HF19), hamster (V79-4) cells and plasmid DNA is revealed as Fpg and Nth sensitive sites. Nucleic Acids Res. 30 (15): 3464-3472
17. Tchou J, Bodepudi V, Shibutani S, Antoshechkin I, Miller J, Grollman A P, Johnson F (1994) Substrate specificity of Fpg protein. J Biol Chem. 269: 15318-15324

18. Kudo K, Ito H, Ihara S, Terato H (2015) Quantitative analysis of oxidative DNA damage induced by high-voltage pulsed discharge with cavitation. J Electrostat. 73: 131-139

19. Kim S H, Nam J H, Park E J, Kim B J, Kim S J, So I, Jeon J H (2009) Menthol regulates TRPM8-independent processes in PC-3 prostate cancer cells. Biochim Biophys Acta. 1792: 33-38

20. Wang Y, Wang X, Yang Z, Zhu G, Chen D, Meng Z (2012) Menthol inhibits the proliferation and motility of prostate cancer DU145 cells. Pahol Oncol Res. 18: 903-910

21. Kijpornyongpan T, Sereemaspun A, Chanchao C (2014) Dose-dependent cytotoxic effects of menthol on human malignant melanoma A-375 cells: correlation with TRPM8 transcript expression. Asian Pac J Cancer Prev. 15 (4):1551-1556

22. Iwasaki K, Zheng Y W, Murata S, Ito H, Nakayama K, Kurokawa T, Sano N, Nowatari T, Villareal M O, Nagano Y N, Isoda H, Matsui H, Ohkohchi N (2016) Anticancer effect of linalool via cancer-specific hydroxyl radical generation in human colon cancer. World J Gastroent. 22 (44): 9765-9774

23. Kang S H, Kim Y S, Kim E K, Hwang J W, Jeong J H, Dong X, Lee J W, Moon S H, Jeon B T, Park P J (2016) Anticancer effect of thymol on AGS human gastric carcinoma cells. J Microbiol Biotechnol. 26 (1): 28-37

24. Ivanova T, Han Y, Son H J, Yun Y S, Song J Y (2006) Antimutagenic effect of polysaccharide ginsan extracted from Panax ginseng. Food Chem Toxicol. 44: 517-521

25. Hosseinimehr S J, Tavakoli H, Pourheidari G, Sobhani A, Shafiee A (2003) Radioprotective effects of citrus extract against g-irradiation in mouse bone marrow cells. J Radiat Res. 44: 237-241

26. Samarth R M, Goyal P K, Kumar A (2004) Protection of swiss albino mice against whole-body gamma irradiation by *Mentha piperita* (Linn.). Phytother Res. 18: 546-550

27. Archana P R, Nageshwar Rao B, Ballal M, Satish Rao B S (2009) Thymol, a naturally occurring monocyclic dietary phenolic compound protects Chinese hamster lung fibroblasts from radiation-induced cytotoxicity. Mutat Res. 680: 70-77