Original Paper

Chemical composition, anticancer, and antioxidant activities of essential oil obtained from lemon (*Citrus limon*) by-product

Lailatul Azkiyah[1,2], Yukihiro Yamamoto[1], Shinjiro Ogita[1] and Tomoyuki Yoshino[1,*]

[1] Program in Biological System Sciences, Graduate School of Comprehensive Scientific Research,
Prefectural University of Hiroshima
5562 Nanatsuka-cho, Shobara, Hiroshima, 727-0023, Japan
[2] Department of Agricultural Product Technology, The University of Jember
Jl. Kalimantan No. 37, Jember, Jawa Timur, 68121, Indonesia

(Received July 5, 2021; Accepted August 17, 2021)

ABSTRACT

The by-product of lemon (*Citrus limon)* is more than 0.8 million metric tons in 2020/2021 which consist mostly of the peel. This is a problem for management, pollution, and the environment due to microbial spoilage. Lemon peel is a good source of essential oil. Utilization of lemon essential oil (LEO) can provide an efficient and environmentally friendly material for many uses. LEO has GRAS (generally recognized as safe) status, which has generated considerable appeal from the food sector. However, in its implementation, it has to face many problems related to the volatility and variability of the composition.

This study was carried out to extract LEO from lemon peel by steam distillation, to determine its composition, to evaluate its antioxidant and anticancer activities, and to analyze the encapsulation effect of LEO in whey protein-pectin complex on these parameters. The LEO yield was 1.2 % (d.m). The main component in LEO was D-limonene (59.8 %) followed by ß-pinene (15.3 %), γ-terpinene (8.4 %), and α-pinene (3.0 %). Encapsulation of LEO in whey protein pectin-complex changed the composition of volatile compounds with an increase in D-limonene and γ-terpinene, and a decrease in ß-pinene and α-pinene. The LEO and LEO nanocapsules (LEONCs) showed a positive effect on the inhibition of colon-26 cells proliferation with IC_{50} of 105.9 µg/mL and 2.3 mg /mL, respectively.

Key words : Lemon essential oil, Encapsulation, Chemical composition, Anticancer, Antioxidant

Introduction

Citrus is one of the most popular fruit crops in the world due to their refreshing flavor and nutritional content. Lemon (*Citrus limon*) and limes are the third most-produced citrus species with over 8 million metric tons in 2020/2021, and of which 23.9 % are used for processing (USDA, 2021). Citrus fruit produces about 40−50 % by-products of the total bulk in the form of peels and membrane residues (Braddock, 1999). The major environmental problems associated with citrus peel is its highly fermentable carbohydrate content which accelerate its degradation when not carefully manage (Lin et al., 2013). The disposal issue has stimulated significant interest in developing more responsible ways of dealing with lemon waste products, and in the exploration of the bioactive compounds from the waste for the application in the production of high functional and economical food additives.

Lemon peel is a potential source of essential oil, which can be obtained by cold pressing or distillation technique. The total oil in whole lemon is 0.7 % and in lemon juice is 0.2 %, so in lemon peel is about 0.5 % (MEXT, 2021). Its mean there is a potential of 3400 metric tons of LEO from the processing activity in 2020/2021. Volatile and semi-volatile compounds represent 85−99 % of the citrus oil fraction (Dugo and Mondello, 2011). Previously study reported that LEO has many biological activities such as antioxidant and

***Corresponding author :** E-mail: yoshino@pu-hiroshima.ac.jp

antitumor activities (ChunYan et al., 2010). LEO has generated considerable appeal from the food sector because of its GRAS (generally recognized as safe) status (USFDA, 2020). However, the industrial uses of LEO had to face the problems of high volatility and composition variability. Encapsulation can make possible to immobilize the most volatile compounds of essential oil, to stabilize them and protect them as well as to modulate their release by prolonging the kinetics profile.

The above information, provide the rationale behind the study that aims to extract LEO from lemon peel by steam distillation technique and to chracterize it by headspace gas chromatography-mass spectrometer (HS-GC-MS), while exploring its anticancer activity. In a second step, LEO was encapsulated in whey protein-pectin by complex coacervation. The chemical composition and anticancer activity of LEO and LEO nanocapsules (LEONCs) were determined.

Materials and Methods

Plant materials and LEO extraction

Lemons were collected in stage 1 (green color, immature) from farmers in Hiroshima prefecture, Japan. The lemon peel including flavedo (epicarp) and albedo (mesocarp) layers was carefully peeled and discarded. Extraction of LEO was carried out from fresh lemon peel without prior processing by steam distillation using special oil steam distiller (PureStiller type K-HJ200, Japan) for 2.5−3.0 h. The obtained LEO was dried over anhydrous sodium sulfates and stored at −20 ℃ in glass vials covered by aluminum foils until further analysis.

Encapsulation of LEO by complex coacervation

Encapsulated LEOs were prepared by the emulsion coacervation technique described previously by Ghasemi et al. (2018) with modifications. The biopolymer solutions of whey protein concentrate (4 % w/v), pectin (1 % w/v) and maltodextrin (50 % w/v) in a ratio of 1 : 1 : 1 (v/v/v) was mixed and stirred on magnetic stirrer at 1000 rpm, 37 ℃ for 30 min. Polysorbate 80 was added at a ratio of 5 % (w/w) of the total polymer. LEO as the core material was added to this solution gradually (10 % (v/w) of total polymers; density of LEO = 0.85 g/mL). Ultrasonic homogenizer was used to produce oil-in-water nanoemulsions. The pH level of the solution was adjusted at 3.0 to initiate the coacervation and then stirred at 1000 rpm, 37 ℃ for 30 min. The solution was centrifuged at 13000 rpm, 4 ℃ for 30 min. Pellet was collected as encapsulated LEO and then dried using a freeze dryer. The dried powder was collected and stored at −20 ℃ for further characterization. Encapsulation efficiency (%) was determined according to the method described by Bae and Lee (2008).

Gas chromatography/mass spectrometry (GC-MS) analysis

The volatile composition of the samples was analyzed using a headspace gas chromatography-mass spectrometer (HS-GC-MS, Shimadzu GCMS-QP-5050 series) auto-injector equipped with a DB-WAX column (60 m × 0.25 mm i.d. (inner diameter); film thickness = 0.25 mm). GC parameters were as follows: the carrier gas was helium and be set at a flow rate of 0.9 mL min^{-1}. The ion source temperature was 200 ℃, the interface temperature was 230 ℃, and the pressure was 100 kPa. The column oven temperature was initially set at 50 ℃ for 5 min, and then ramped to 160 ℃ at 3 ℃ min^{-1}, and after that, it was warmed up to 200 ℃ at 20 ℃ min^{-1}. MS parameters were as follows: the interface temperature was 230 ℃, elution time was 3 min, the full scan mode from *m/z* 50 to 250, the scan speed was 500, the threshold was 2,000, and the interval was 0.5 sec^{-1}. The identification of volatile compounds was based on a comparison of their GC retention time and mass spectra with the retention index of D-limonene (Wako, No. 124-03892), ß-pinene (Aldrich Chemistry, No. 402753), and α-pinene (Aldrich Chemistry, 147524) standard, and the reference spectra from the US National Institute of Standards and Technology (NIST) data base library.

Antioxidant activity

The antioxidant activity is estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich). The DPPH assay was prepared according to the protocol described by Asikin et al. (2012) with modification. Briefly, 50 µL of samples at different concentrations was added with 150 µL of DPPH (0.1 mM). after incubation for 45 min in the dark at room temperature, the absorbance was measured at 517 nm using spectrophotometer. Ethanol served as negative control. Antioxidant activity is indicated by the percentage of inhibition of DPPH and calculated using following equation:

$$Inhibition\ (\%) = \frac{(Abs.\ control - Abs.sample)}{Abs.control} \times 100$$

Anticancer activity

Cell line: murine colon carcinoma (colon-26) cell line

was purchased from RIKEN BRC CELL BANK (Tsukuba, Japan). Colon-26 cells (RCB2657, RM092147) were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 % (v/v) fetal bovine serum (FBS). The cells were incubated at 37 ℃ in a 5 % CO_2 atmosphere. To investigate the cytotoxicity activity of samples, a CCK-8 (cell counting kit-8) assay (Dojindo, Japan) was performed. Colon-26 cells were seeded in a 96-well cell culture plate (Watson ® Bio Lab, Japan) at a density of 2×10^3 cells $well^{-1}$ 100 μL^{-1} and incubated at 37 ℃ in a 5 % CO_2 incubator for 24 hours. After incubation, each medium was replaced, and 100 µL of sample dissolved in the medium was added and then the cells were incubated for 24 h under the same condition as above. Finally, 10 µL of the CCK-8 reagent was added into each well and incubated at 37 ℃ in a 5 % CO_2 incubator for 6 h. The fluorescent intensity (FI) of the samples was recorded on a microplate reader (450 nm, Varioskan Flash from Thermo Scienctific, Waltham, MA, USA).

$$Cell\ viability\ (\%\ of\ control) = \frac{A\ sample - A\ color\ blank\ of\ sample}{A\ control - Acolor\ blank\ of\ control} \times 100$$

Antiproliferative activity (%) = 100 − cell viability. The IC_{50} value was calculated by plotting the sample concentration and antiproliferative activity.

Results

Extraction of LEO from fresh lemon peel by steam distillation produced a colorless and transparent LEO at a yield of 1.2 ± 0.1 % (d. m.). The encapsulation efficiency of LEO in whey protein-pectin complex was 77.4 ± 2.0 %. Chemical composition analysis showed that there was significant difference in the main volatile composition of LEO and LEONCs (Table 1).

Table 1. The predominant volatile compound of LEO and LEONCs.

Compound name	RT (min)	Area (%)	
		LEO	LEONCs
D-limonene	16.858	59.8 ± 1.4 [a]	74.0 ± 0.8 [b]
ß-pinene	12.717	15.3 ± 0.2 [a]	9.6 ± 0.1 [b]
γ-tepinene	18.975	8.4 ± 0.2 [a]	9.3 ± 0.1 [b]
α-pinene	9.542	3.0 ± 0.3 [a]	0.9 ± 0.0 [b]
		86.5 [a]	93.8 [b]

* The area (%) presented as Mean ± SD.
[a,b] Different words in each row represent of significant differences in one row ($p < 0.01$).

The antioxidant activity is shown in Fig. 1. LEO has an IC_{50} of inhibition of radical DPPH as 156.4 ± 2.3 µg/mL. LEONCs showed antioxidant activity higher than NCs at the same concentration 15 mg/mL).

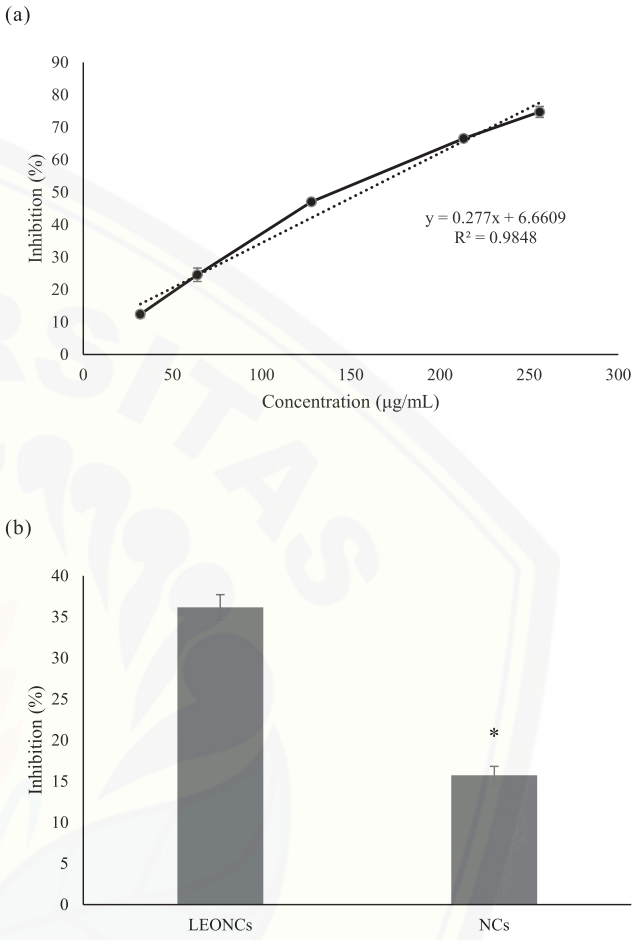

Fig. 1. Antioxidant activity of compounds by DPPH assay; (a) LEO and (b) LEONCs and NCs. Mean±SD, *significant difference ($p < 0.01$).

Antiproliferative activity against colon-26 cells is shown in Fig. 2. The IC_{50} of LEO and LEONCs was 105.9 ± 15.8 µg/mL and 2.3 ± 0.5 mg/mL, respectively. NCs at the same concentration as LEONCs did not have significant antiproliferative activity against colon-26 cells.

Discussion

In the present study, the yield of the LEO obtained from fresh lemon peel by steam distillation was 1.2 %. Previously study conducted by Bourgou et al. (2012) reported that the yield was 1.3 %, whereas Moosavy et al. (2017) reported a yield of 1.3 % with dried the peel before extraction. Encapsulation of LEO in whey protein-pectin complex had

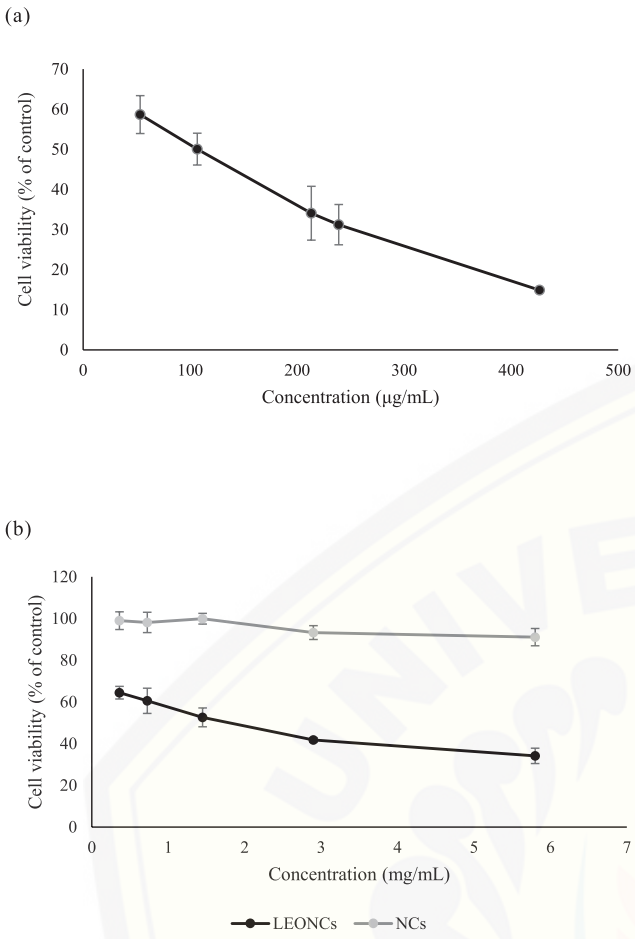

Fig. 2. Antiproliferative activity of compounds on colon-26 cell line by CCK-8 assay in vitro; (a) LEO and (b) LEONCs and NCs. Mean±SD.

an efficiency of 77.4 %. Ghasemi et al. (2018) reported that the encapsulation efficiency of orange peel oil in the whey protein-pectin complex was about 88 %.

Four compounds (D-Limonene, ß-pinene, γ-terpinene, and α-pinene) were identified as the major compounds of LEO and LEONCs, represented 86.5 % and 93.8 % of all fractions, respectively. D-limonene was the most abundant volatile compounds in LEO which increase significant in LEONCs. Similar with our report, in many studies of lemon peel essential oil, D-limonene was found to be the predominant compound (Tisserand and Young, 2014). γ-terpinene ranks third also showed a percentage increase after encapsulation, which is 8.4 % and 9.3 % in LEO and LEONCs, respectively. Different results were shown on ß-pinene and α-pinene which decreased in percentage after encapsulation. Thus, encapsulation in whey protein-complex was better for D-limonene and γ-terpiene than for α-pinene and ß-pinene. The yield and composition of LEO depend on many factors such as such as plant variety, geographical region, age of the plant, stage of ripening, the drying and extraction methods, etc. (Bagamboula et al., 2004).

LEO was able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H with IC_{50} of 156.4 µg/mL. Frassinetti et al. (2011), demonstrated the scavenging abilities of Citrus spp. essential oil was ranging from 20 to 70 %. Another research by Moosavy et al. (2017) reported that antioxidant activity of essential oil from lemon was 55.1 %. The antioxidant activity is related to the chemical composition of LEO that rich in monoterpenes (D-limonene and α-pinene) (Wei and Shibamoto, 2007). D-Limonene, α-pinene, and ß-pinene individually tested do not have significant antioxidant activity compared to the same constituents when tested together (Ruberto and Baratta, 2000). LEONCs showed higher antioxidant activity than NCs at the same concentration (15 mg/mL). This indicated the LEO loaded in LEONCs. In addition, the capsule-forming polymer also contributes to the antioxidant activity of LEONCs as shown in the result of NCs. Whey protein, a compound of NCs, is well known to exhibit antioxidant activity (Corrochano et al., 2018).

The in vitro assessment of anticancer activity of LEO and LEONCs showed have a significant cytotoxic effect against colon-26 cell line, which IC_{50} value was 105.9 µg/mL and 2.3 mg/mL, respectively. The antiproliferation effect might be related to D-limonene in LEO, which reported can induce apoptosis by up-regulating of pro-apoptotic factors and down-regulating anti-apoptotic factors (Mukhtar et al., 2018). Other components of LEO, α-pinene, has been also reported involved in antiproliferative activity by stimulating apoptosis, proved by initial disruption of mitochondrial function, reactive oxygen species formation, improved caspase-3 properties, heterochromatin aggregation, DNA disintegration, and exposure of phosphatidylserine on the cell surface (Matsuo et al., 2011). This study found the potential antioxidant and anticancer activity of LEO and LEONCs which can be applied in food industry as functional food.

Acknowledgements

The Lailatul Azkiyah (L.A) gratefully acknowledge to the Directorate General and Higher Education, The Ministry of Education and Culture, The Republic Indonesia for the support through the IsDB Scholarship.

References

Asikin, Y., Taira, I., Inafuku, S., Sumi, H., Sawamura, M., Takara, K. and 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)**, C649–C675.

Bae, E. K. and Lee, S. J., 2008: Microencapsulation of avocado oil by spray drying using whey protein and maltodextrin. *Journal of Microencapsulation*, **25(8)**, 549–560.

Bagamboula, C. F., Uyttendaele, M. and Debevere, J., 2004: Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool, and p-cymene toeards Shigella sonmei and S. Flexneri. *Food Microbiology*, **21**, 33–42.

Bourgou, A., Rahai, F., Ourghemmi, I. and Tounsi, M. S., 2012: Changes in peel essential oil composition of four Tunisian citrus during fruit maturation. *The Scientific World Journal*, **4**, 528–593.

Braddock, R. J., 1999: *Handbook of citrus Bi-Products and Processing Technology*. John Wiley & Sons, Inc. New York.

ChunYan, H., Hong, P., ZhenYu, Z. and Jing, S., 2010: Evaluation of antioxidant and antitumour activities of lemon essential oil. *Journal of Medicinal Plants Research*, **4(18)**, 1910–1915.

Corrochano, A. R., Buckin, V., Kelly, P. M. and Giblin, L., 2018: Invited review: whey proteins as antioxidants and promoters of cellular antioxidant pathways. Journal of Dairy Science, **101(6)**, 4747–4761.

Dugo, P. and Mondello, L., 2011: *Citrus oils: composition, advanced analytical techniques, contaminants, and biological activity*. CRC Press, Boca Raton, FL.

Frassinetti, S., Caltavuturo, L., Cini, M., Della Croce, C. M. and Maserti, B. E., 2011: Antibacterial and antioxidant activity of essential oils from Citrus spp. *Journal of Essential Oil Research*, **23**, 27–31.

Ghasemi, S., Jafari, S. M., Assadpour, E. and Khomeiri, 2018: Nanoencapsulation of D-limonene within nanocarriers produced by pectin-whey protein complexes. *Food Hydrocolloids*, **77**, 152–162.

Lin, C. S. K., Pfaltzgraff, L. A., Herrero-davila, L., Mubofu, E. B., Abderrahim, S., Clark, J. H., Koutinas, A. A., Kopsahelis, N., Stamatelatou, K., Dickson, F., Thankappan, S., Mohamed, Z., Brocklesbyc, R. and Luque, R., 2013; Food waste as a valuable resource for the production of chemicals, materials and fuels. *Energy Environmen. Sci*., **6**, 426–464.

Matsuo, A. L., Figueiredo, C. R., Arruda, D. C., Pereira, F. V., Scutti, J. A. B, Massaoka, M. H., Travassos, L. R., Sartorelli, P. and Lago, J. H. G., 2011: α-pinene isolated from Schinus terebinthifolius Raddi (Anacardiaceae) induces apoptosis and confers antimetastatic protection in a melanoma model. *Biochem Biophys Res Commun*, **411(2)**, 449–454.

MEXT, 2021: Food Composition Database - Lemon. https://fooddb.mext.go.jp/result/result_top.pl?USER_ID＝13133 (in Japanese), accessed on August 7, 2021.

Moosavy, M. H., Hassanzadeh, P., Mohammadzadeh, E., Mahmoudi, R., Khatibi, A. and Mardani, K., 2017: Antioxidant and antimicrobial activities of essential oil of lemon (*Citrus limon*) peel in vitro and in a food model. *Journal of Food Quality and Hazard control*, 42–48.

Mukhtar, Y. M., Adu-Frimpong, M., XU, X. and Yu, J., 2018: Biochemical significance of limonene and its metabolites: Future prospects for designing and developing highly potent anticancer drugs. Biosci Rep, **38(6)**, BSR20181253.

Ruberto, G. and Baratta, M. T., 2000: Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chem*., **69**, 167–174.

Tisserand, R. and Young, R., 2014: *Essential Oil Safety*, 2nd ed. Elsevier, New York, USA.

USDA (United States Department of Agriculture), Foreign Agricultural Services, 2021: Citrus: World Markets. *https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf.*, accessed on April 19, 2021.

USFDA (U.S. Food and Drug Administration), 2020: CFR-Code of Federal Regulation Title 21. *https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=182&showfr=1*, accessed on April 19, 2021.

Wei, A. and Shibamoto, T., 2007: Antioxidant activities and volatile constituents of essential oils. *J. Agric. Food Chem*., **54**, 1737–1742.