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Optimizing CO_2-expanded Hexane for Enhanced Yield and Antioxidant Activity of Essential Oils from *Citrus reticulata*

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Abstract

Wide range applications of citrus essential oil (EO) and environmental issues resulting from citrus processing industries render in the evolution of extraction techniques. The efficacy of the CO_2-expanded hexane (CXH) extraction technique on mandarin orange EO, D-limonene, and total phenolic compounds (TPC) was evaluated and compared with conventional methods; hexane extraction and hydro-distillation. Moreover, the correlation of EO components with antioxidant activity was studied. It was revealed that CXH had better EO and D-limonene yields than conventional methods. The CXH optimal extraction conditions of 25 °C, 6.5 MPa, and 0.70 CO_2 mole fraction offered 0.155 g-EO/g-dry peel and 51% D-limonene in EO. Less total phenolic compounds and phospholipids were extracted using CXH compared to hexane extraction. However, a similar inhibition capability (12–54%) of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical was obtained regardless of the extraction technique. In addition, DPPH scavenging activity depended on the TPC composition. The results suggest the potential application of CXH for citrus waste valorization.

Keywords: *Citrus reticulata*; Essential oil; CO_2-expanded hexane; D-limonene; Antioxidant activity

Introduction

Global citrus fruit production in 2019 was 143.7 million metric tons, with orange and mandarin contributing about 76.3 and 37.4 million metric tons, respectively (FAO 2021). Other common citrus fruits include lemon, limes, and grapefruit. Mandarin has been projected to rise to 38 million metric tons in 2024 (USDA 2023). Citrus processing results in approximately 40–55% waste including peels, pulp membranes, and seeds (Zema et al. 2018). Peel wastes contribute about 65.5% of mandarin waste (Zema et al. 2018) resulting in more than 10 million metric tons of mandarin waste. However, citrus wastes contain beneficial compounds such as limonene, phenolic compounds, flavonoids, vitamins, and minerals (Zema et al. 2018, Siddigui et al. 2022, Singh et al. 2020, Czech et al 2020).

The presence of beneficial bioactive compounds in citrus fruit wastes fostered its valorization. The citrus EO has a wide range of applications including as a preservative in the food industry due to its antimicrobial properties (Settanni et al. 2012, Song et al. 2021, Zia-ur-Rehman 2006). In addition to medical applications (Czech et al. 2020, Adeniyi et al. 2017, Ke et al. 2020), citrus EO has been applied in cosmetics and perfumes,

aromatherapy, and environmental management through sanitizer and insecticide production (Palazzolo et al. 2013). Nevertheless, the conversion of citrus wastes to EO advocates for the environment and waste management.

Different methods have been deployed to address EO extraction from citrus fruit wastes, especially peels. Cold pressing was applied in lemon peels oil extraction (Ciriminna et al. 2014), and orange peels oil extraction resulted in a low yield of 0.3–0.6% dry peels (Mitiku et al. 2000). Conventional hexane extraction offers considerable yield but its toxicity downplays its application (Lopresto et al. 2014). Besides bio-based solvent (2-methyl-tetrahydrofuran) was found to outperform traditional hexane extraction (Ozturk et al. 2019). Despite natural deep eutectic solvent being safe, its performance was observed <70% of conventional hexane extraction (Panic et al. 2021). High-temperature extraction such as hydro and steam distillation are widely applied due to enhanced diffusion despite being energy intensive (Zema et al. 2018). To enhance solubility, high pressure and temperature extraction of limonene has yielded 3.56% of limonene of the dry lemon peels while saving power and time (Lopresto et al. 2014). The use of supercritical CO_2 afforded 3.3% limonene extraction from lemon peels (Lopresto et al. 2019).

The CO_2-expanded liquid is a solvent that offers high mass transfer, is less toxic and inflammable, has low volatility and is facile separated from extracts. Besides, it is tunable with the change in pressure and temperature. In addition, it operates in mild conditions. It has been reported to perform well in rice bran oil extraction (Mathias et al. 2024a, Okajima et al. 2021, Mathias et al. 2023). Moreover, CO_2-expanded ethanol was used to extract α-pinene and cis-verbenol from *Boswellia sacra* resin, yielding 2.2% EO (Al-Hamimi et al. 2016). However, there is no information about the potential use of CO_2-expanded liquids in citrus EO extraction, and its effect on TPC and antioxidant activity. Therefore, the current study aims to enhance EO yields using CXH. In addition, to analyze antioxidants and antioxidant activity as well as comparing the CXH method with hexane and hydro-distillation methods. The current study unravels the superiority of CXH over conventional extraction methods.

Materials and Methods

Materials

Mandarin orange fruits (*Citrus reticulata*) were sourced from the Entetsu store in Hamamatsu City, Japan to Okajima laboratory at Shizuoka University. The mandarin orange fruits were peeled and the peels were dried at 50 °C for 48 hours from 74.84% to approximately 2.80% moisture content using a hot air oven (VTN 113, Isuzu). Dried peels were crushed to 500 µM before storage at -18 °C. Fresh and dried mandarin orange peels were used during EO extraction.

Analytical grade reagents with purity >96% were used throughout this study including; n-hexane, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picryhydrazyl (DPPH), sodium sulfate, sodium carbonate, absolute ethanol, gallic acid, D-limonene standard, and hydrochloric acid, all from Fujifilm Wako Pure Chemicals (Osaka, Japan). The CO_2 gas was purchased from air Liquide (Tokyo, Japan), and the phosphorus standard was from AccuStandard (New Haven, CT, USA).

Methods

Extraction of EO with conventional hexane

Conventional hexane extraction of EO from mandarin orange peels was carried out according to Mathias et al. (2023). About 10 g of dried mandarin orange peels were weighed into a beaker. A ratio of 1:10 of solid to solvent was used and stirred for 24 hours. A watchman #1 filter paper was used to separate the mixture and the solids. Next was vacuum filtration to remove all particles in the filtrate into the pre-weighted flat-bottomed flask. The flat-bottomed flask containing oil was re-weighed and the oil yield was calculated as the amount of oil extracted divided by the amount of sample charged into a beaker.

Extraction of EO with Hydro distillation

Different mandarin orange peels samples (*Citrus reticulata*) were hydro-distilled following the method reported by Lopresto et al. (2019) with a slight modification. The modification involved the use of hexane to dissolve the organic layer to enhance immiscible layers separation. Briefly, approximately 50 g of either fresh or dried mandarin orange peels were charged into the distillation flask. Distilled water was used for distillation purposes. The distillation process was conducted at 100 °C for 3 hours. Hexane was added to the distillate to dissolve the EO, shaken vigorously, and left to settle. The organic layer was separated and anhydrous sodium sulfate was used to remove the remaining water from the organic portion. Vacuum separation was employed to remove hexane from the extract.

Extraction of EO with CO_2-expanded hexane (CXH)

Extraction procedures and details of extraction equipment were illustrated by Okajima et al. (2021). About 50 g of 500 µM pre-sieved sample of dried mandarin orange peels was extracted at 25–35 °C, 5.0–6.5 MPa, and 0.70–0.87 CO_2 mole fraction. During the extraction process, samples were collected at 3, 6, 10, 15, 30, 60, 90, 120, 150, and 180 minutes. The collected samples in pre-weighed flat-bottomed flasks were vacuum evaporated. The flat-bottomed flasks containing oils were re-weighed. Respective essential oils extracted were lump-summed to obtain the total amount of EO extracted and the yield.

D-Limonene quantification

D-limonene quantification in EO was conducted by using a Gas Chromatography-Flame Ionization Detector (GC-FID) per the Lopresto protocol (Lopresto et al. 2019) with a slight modification. In the current analysis, column and the initial column temperature were different due to availability and easy identification of volatile organic compounds, respectively. An EO sample of 200 µL weighing approximately 0.147 g was pipetted into a vial with 1.5 mL of hexane. The mixture was subjected to analysis in the Shimadzu GC-2010 system, equipped with an autosampler (AOC-20i auto-injector) together with Varian capillary column CP-Sim Dist (10 m×0.32 mm×0.1 µm #CP7521). The column temperature was kept constant at 35 °C for 3 min, heated from 35 °C to 140 °C at 5 °C/min, from 140 °C to 275 °C at 45 °C/min, and finally kept constant at 275 °C for 10 min. Injection of 5 µL of the sample was conducted at 280 °C in the 1:20 split mode. The carrier gas was helium (99.9995% purity) with a 1.5 mL/min flow rate. A calibration curve of D-limonene was established in the range of 100 to 4000 mg/L resulting in concentration in mg/L = 0.0002×Peak_area–8.5421 with R^2 = 0.9999.

Phosphorus concentration analysis

The phosphorus concentration in EO was quantified according to Okajima et al. (2021). A 250 µL EO sample was weighted, heated in a furnace at 550 °C for 3 h, and digested in 20 mL of 1 M HCl for 16 hours. The contents were transferred to the 50 mL volumetric flask and deionized water was added up to the mark. After homogenization the solution was vacuum filtered and quantified using inductively coupled plasma (ICP) emission spectrometry (Optima 2100 DV). A calibration curve made of 0.1–5 ppm phosphorus solution in 1 wt% HCl was established. The concentration (mgL^{-1}) obtained in the ICP was recorded with an accuracy of 1.1%, and the phosphorus concentration in EO was estimated using the following formula.

$$\text{Phosphorus concentration (mg/Kg)} = \frac{\text{Concentration (mg/L)} \times \text{Dilution volume (mL)}}{\text{Weight of the sample (g)}}$$

Total Phenolic Compounds (TPC) analysis

The TPC quantification was obtained using the Folin-Ciocalteu colorimetric method (Mingyai et al. 2017). A sample of 200 µL reacted with 800 µL of water-diluted Folin-Ciocalteu reagent (1:10 v/v) in conjunction with 2 mL of 7.5% sodium carbonate. The mixture was diluted with distilled water to 7 mL. The reaction progressed for 2 hours in the dark at room temperature before absorbance was read at 765 nm using a UV-Vis spectrometer (Jasco V-550, Japan). The gallic acid calibration curve was prepared and

TPC was quantified as the gallic acid equivalent of essential oil (g-GAE/g-EO).

DPPH scavenging capacity analysis

The antioxidant activity of mandarin orange EO was estimated according to Mingyai et al. (2017), with slight changes. The reactants concentrations were changed to enhance absorbance readings. The 2, 2-diphenyl-1-picryhydrazyl (DPPH) was prepared by dissolving 0.20 g of DPPH into 1 L ethanol. The sample of 150 µL was pipetted to a test tube with 4 mL of DPPH solution. The mixture was incubated for 30 minutes in the dark at room temperature before absorbance was read at 517 nm using a UV-Vis spectrometer (Jasco V-550, Japan). Percentage inhibition of DPPH free radicals was estimated as described in Mathias et al. (2024b).

Statistical analysis

Half factorial of three factors (temperature, pressure, and CO_2 mole fraction) with two levels each was considered to study the effect of factors and their interactions. Analysis of variance and comparison between means using Tukey pairwise comparison in a Minitab 17.0 was conducted and the significance effect was evaluated at $p < 0.05$.

Results and Discussion

Mandarin orange fruits characterization

The mandarin orange fruits had an average weight of 108.54 g before juicing. The juice content was 46.07% and the peels composition was 18.04% whereas the membrane and pulp composed 35.89% of fresh mandarin orange fruit. Both fresh and dried mandarin orange peels were investigated.

Effect of extraction conditions on extraction kinetics

The CXH extraction process of mandarin oranges EO at different conditions is illustrated (Figure 1). The extraction curve indicates two phases, the washing stage at the early part of the extraction process governed by solubilization, and the depletion stage governed by diffusion at the later stage. Extraction process kinetics was enhanced significantly ($p < 0.05$) with either temperature (25–35 °C) or pressure rise. Enhanced mass transfer i.e. diffusion has promoted the extraction process by increasing the interaction of solvent molecules and EO (Lopresto et al. 2019). Also, high pressure (7–12 MPa) was associated with cell rupture of the solid matrices prompting high EO yield extraction (Raeissi and Peters 2005). The current finding suggests that 5.0–6.5 MPa is sufficient to enhance EO extraction.

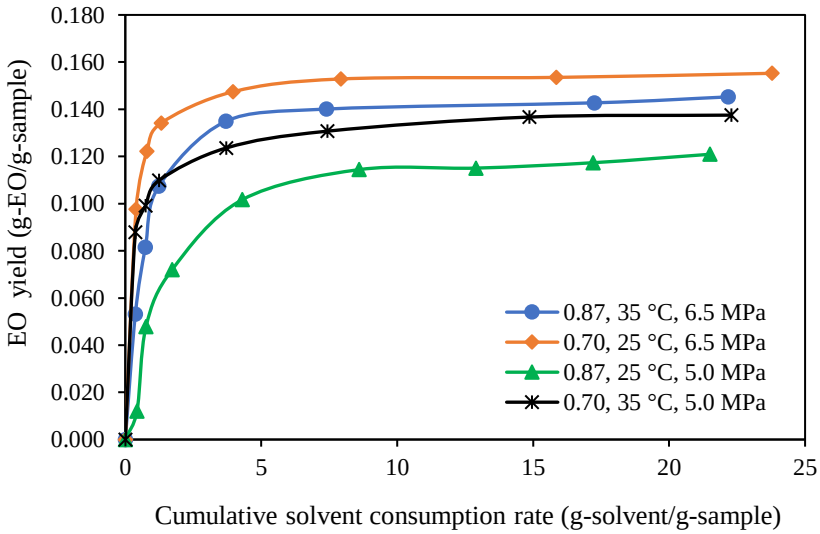

Figure 1: Extraction curve of essential oil at different extraction conditions. 0.70 and 0.87 are CO_2 mole fractions

Mutual interaction analysis demonstrated a synergistic effect between temperature and pressure, coincidentally with temperature and CO_2 mole fraction on EO yield (Figure 2a). However, there was no concerted effect between pressure and CO_2 mole fraction. Interestingly, all factor pairing synergized during D-limonene extraction (Figure 2b). This highlights the importance of regulating pressure, temperature, and CO_2 mole fraction to deploy the combined factors' effect on maximizing EO yield and D-limonene extraction.

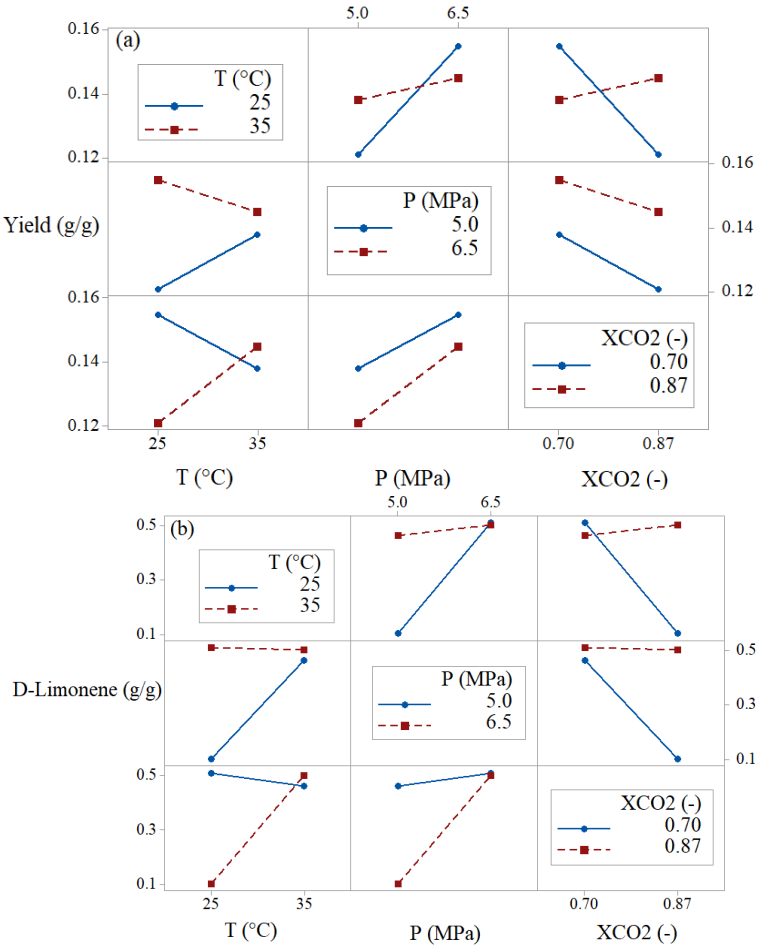

Figure 2: Interaction plots of extraction condition on (a) essential oil yield (g/g) where (g/g) is g-EO/g-dry peels (b) D-limonene yield (g/g) where (g/g) is g-D limonene/g-EO. T is temperature, P is pressure, and XCO_2 is CO_2 mole fraction.

Comparison of CXH with conventional extraction methods

The EO yields from mandarin orange peels extracted with different methods are shown in Table 1. The CXH had the highest EO yield of >13% hexane extract and 300% hydro-distilled extract. High EO yield was associated with improved mass transfer through enhanced diffusion of CXH solvent and solubilization by the CO_2 gas addition. The extract of EO with the highest yield (5.9–9.7%) has been reported with the hydro-distillation method of *Citrus reticulate* dried peels (Shaw et al. 2023). Moreover, it has

been reported that depending on the solvent, the EO yield from *Citrus sinensis* dry peels varied from 0–54% (Liewi et al. 2018). The finding shows that the EO yield depends on the type of citrus peels and the extraction method.

Table 1. Yields of essential oil and its D-limonene composition

CODE	T(°C)	P (MPa)	XCO_2 (-)	Essential oil yield (g-EO/g-dry peels)	**D**-Limonene yield (g-**D**-limonene/g-EO)
CXH1	25	5.0	0.87	0.121	0.1018 ± 0.0035^{c}
CXH2	25	6.5	0.70	0.155	0.5113 ± 0.0212^{ab}
CXH3	35	6.5	0.87	0.145	0.5017 ± 0.0021^{ab}
CXH4	35	5.0	0.70	0.138	0.4639 ± 0.0095^{ab}
CXH5	25	5.0	0.82	0.124	0.5566 ± 0.0113^{a}
CXH6	25	5.0	0.70	0.132	0.1632 ± 0.0069^{c}
HDF	100	0.1	0.00	0.057	0.3136 ± 0.0130^{b}
HDD	100	0.1	0.00	0.040	0.6276 ± 0.0339^{a}
HE	25	0.1	0.00	0.107	0.5489 ± 0.0325^{a}

CXH is CO_2-expanded hexane, HDF is hydro-distillation of fresh peels, HDD is hydro-distillation of dried peels, EO is essential oil, and HE is hexane extraction. Means with the same letter in the column are not significant different ($p < 0.05$).

The major component of citrus fruit extract has been demonstrated to be D-limonene with over 50% composition (Liewi et al. 2018, Lota et al. 2000, Lota et al. 2001). The finding in this study indicates that D-limonene in the EO from mandarin orange peels was 10–63% (Table 1). During CXH extraction D-limonene extraction has been affected significantly by CO_2 mole fraction, pressure, and temperature. The change in extraction condition changed the density of the CXH resulting in altered mass transfer properties. All three extraction methods had a similar efficacy on D-limonene extraction. Regarding the high extract yield and D-limonene extraction, CXH is superior to hydro-distillation and hexane extraction methods. Besides, 25 °C, 6.5 MPa, and 0.70 CO_2 mole fraction offered the highest EO yield (15.5% dry peels) and D-limonene composition of 51% D-limonene in EO.

Composition of mandarin orange essential oil

The optimum condition for EO and D-limonene extraction was illustrated and its D-limonene composition to dry peels charged was compared with conventional extraction methods. The 100 g dry mandarin orange peels offered 7.9% D-limonene during CXH extraction (Figure 3a). Hexane extraction contained 5.9% while hydro-distillation offered <2.6%. The effect of pressure 5.0–6.5 MPa was associated with increased D-limonene yield. A similar elevated pressure and temperature effect was reported to enhance D-limonene yield (Lopresto et al. 2014). This result stresses further that at optimum CXH extraction conditions, CXH offers more D-limonene than conventional methods.

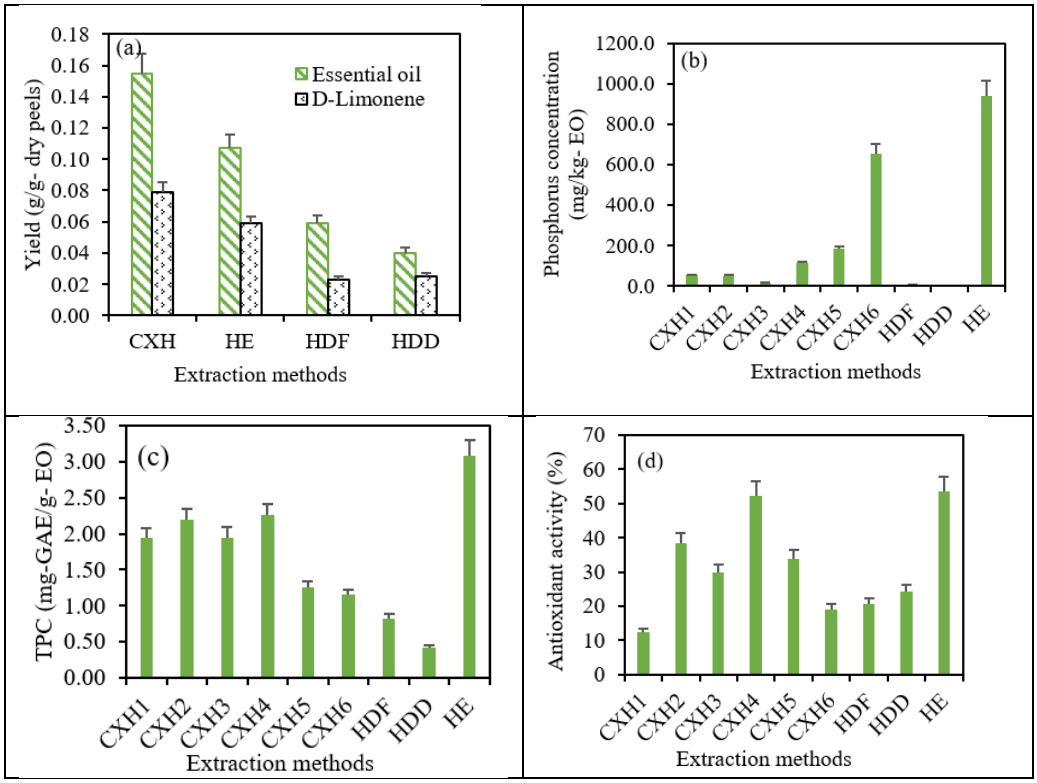

Figure 3: Effect of extraction methods on EO compositions and antioxidant activity. CXH is CO_2-expanded hexane, HDF is hydro-distillation of fresh peels, HDD is hydro-distillation of dried peels, HE is hexane extraction, and EO is essential oil.

Phospholipids are impurities in oils that cause changes in taste, odor, and color (Ambrosewicz-Walacik et al. 2015). Phospholipids were estimated in terms of phosphorus concentration. CXH extract had an intermediate phosphorus concentration of 14–651 mg/kg-EO compared to hydro-distillation (2.4–4.1 mg/kg-EO) and hexane extraction method of 939.5 mg/kg-EO (Figure 3b). Hydro-distilled extract had the lowest phosphorus concentration because it is difficult to diffuse phospholipids in steam than to dissolve it in the solvent owing to long molecular chain. CO_2 addition in hexane improved the selection of EO from solid matrices. Hence, CXH offers an intermediate selection of phospholipids in comparison to conventional hexane.

The EO was found to contain TPC in the range of 1.15–2.26 mg-GAE/g-EO during CXH extraction, reduced from 3.01 mg-GAE/g-EO of hexane extraction (Figure 3c). However, hydro-distillation method offered the lowest TPC, 0.42–0.83 mg-GAE/g-EO. The CO_2 addition in hexane reduced the CXH dissolution power of TPC resulting in low TPC extraction compared to hexane extraction. On the other hand, the presence of hexane in CO_2 improved the dissolution power of TPC in comparison to hydro-distillation extraction. Besides, TPC has been associated with antioxidation properties (Singh et al. 2020, Liew et al. 2018). Moreover, polar solvents such as methanol and ethanol have been reported to extract more TPC than less polar solvents (Singh et al. 2020, Zia-ur-Rehman 2006, Liew et al. 2018, Saleem et al. 2023).

The antioxidation capacities of mandarin orange essential oils from different extraction methods are shown in Figure 3d. The DPPH scavenging capacity was 12–54% for CXH,

21–24% for hydro-distillation, and 54% for hexane. The current study with <54% inhibition of 0.5 mM DPPH is in agreement with mandarin orange EO having 65–74% inhibition of 0.25 mM DPPH (Casquete et al 2015) and orange peels EO with 68–98% inhibition of 0.1 Mm DPPH (Shaw et al. 2023). The findings support that mandarin orange EO has antioxidation properties.

Influence of essential oil composition on antioxidant activity

It has been hypothesized that the presence of the bioactive compounds in EO bolsters its antioxidant activity. The Pearson correlation analysis between D-limonene composition in EO and antioxidation activity revealed a moderate correlation, r = 0.37 (Figure 4a). Hence, D-limonene has little influence on the oxygen inhibition process. On the other hand, it was revealed that TPC influences the antioxidation activity strongly by having a Pearson correlation coefficient of r = 0.73 (Figure 4b). The current finding coincides with the study highlighted that the phenolic compounds influence the antioxidation process (Singh et al. 2020, Liew et al. 2018).

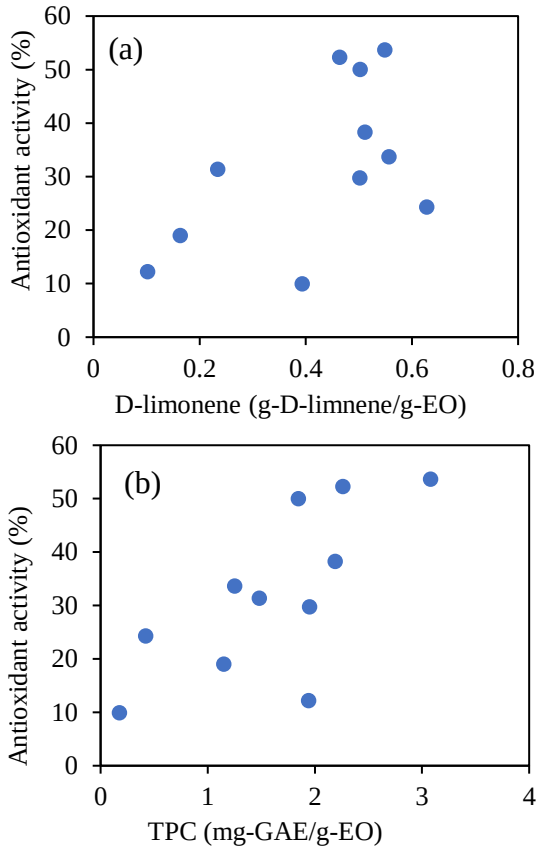

Figure 4: Correlation of antioxidant activity with (a) D-limonene (b) Total phenolic content. EO is an essential oil.

Conclusions

CO_2-expanded hexane had three times the EO extraction capability over hydro-distillation and >13% of hexane extraction owing to CO_2 addition which enhanced the mass transfer of hexane. The EO yield and D-limonene composition were affected by extraction conditions. The CXH optimum condition for mandarin orange EO extraction was 25 °C, 6.5 MPa, and 0.70 CO_2 mole fraction offering 0.155 g-EO/g-dry peels and 51% D-limonene in EO. Long molecules;

phospholipids and phenolic compounds were less extracted with CXH than with hexane extraction owing to low dissolution power. Moreover, the DPPH scavenging activity was unaltered and correlated with TPC content. Therefore, CXH is an alternative novel EO extraction technology.

Statements and declarations

Conflict of interest

The authors declare no conflict of interest

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Author contributions: Conceptualization, Data curation, investigation, Methodology, Visualization, Writing-original draft, and Writing-review6editig; Mathayo Gervas Mathias: Funding acquisition, Methodology, Supervision, and Writing-Review & editing by Idzumi Okajima. Both authors reviewed and edited previous manuscript versions and approved the final manuscript.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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