Trop J Nat Prod Res, August 2021; 5(8):1445-1449

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Phytochemical Constituents and Antioxidative Activity of *Schleichera oleosa* Fruit

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ARTICLE INFO

Article history:
Received 07 July 2021
Revised 27 July 2021
Accepted 23 August 2021
Published online 02 September 2021

ABSTRACT

Schleichera oleosa (Lour) Oken is a plant in the family Sapindaceae found in Thailand. Although the fruit of this plant is popularly consumed, there is a lack of information about its biological activities. This study determined the antioxidant activity of this fruit and identified the active constituents. The peel, juice, and seed parts of the fruit were separated and extracted with ethanol. Total carotenoid, phenolic, flavonoid, and anthocyanin contents were determined by standard spectrophotometric methods. Ascorbic acid and beta-carotene contents were determined by HPLC. Antioxidative activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azobis (3-ethylbenzothialzoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), oxygen radical absorption capacity (ORAC), ferric thiocyanate, and metal chelating activity assays. The total carotenoid content was 11.90-343.57 mg/g extract as beta-carotene equivalents, total phenolic content was 4.06-9.91 mg/g extract as gallic acid equivalents, total flavonoid content was 2.19-9.18 mg/g extract as quercetin equivalents, and total anthocyanin content was 0.20-2.81 mg/g extract calculated as mg cyanindin-3-glucoside. Levels of ascorbic acid and beta-carotene were highest in the juice extract. Peel extract showed the highest antioxidative activity in the DPPH, ABTS, FRAP, and ORAC assays, while the juice extract showed the highest antioxidative activity in the ferric thiocyanate and metal chelating assays. Seed extract showed the least antioxidant activity. High levels of antioxidant compounds in extracts from the peel and juice parts of *S. oleosa* fruit corresponded with high antioxidant activities.

Keywords: *Schleichera oleosa*, Mark–Khro, Ta-Khro, Antioxidant.

Introduction

The use of natural products as health supplements has attracted interest due to the capability of various fruit and vegetable secondary metabolites to reduce oxidative stress. Several classes of secondary metabolites have been reported to have antioxidant properties such as phenolic compounds, flavonoids, carotenoids, and anthocyanins. Free radicals originating from oxidative stress play a critical role in aging and many human diseases, including cancer, cardiovascular disease, and Alzheimer's disease. Thus, the antioxidant components found in natural products may help to protect against the damage caused by oxidative stress.

Schleichera oleosa (Lour) (family Sapindaceae) is a tropical fruit commonly found in Southeast Asia. In Thailand, the fruit is called Ta-Khro or Mark–Khro and it can be harvested between March and July. *S. oleosa* is a medium to large sized tree up to 30 m in height. The leaflets are 20-40 cm long arranged in 2 to 4 pairs, elliptic-oblong shaped, spherical at the apex, and coriaceous. The flowers are tiny and yellowish-green.[1] The fruit is round with a thick skin and contains 1–2 brown, unevenly elliptic, oily, and slightly compressed seeds enclosed in a light-yellow aril (Figure 1). The flesh is juicy and sour. Many parts of this plant, such as the bark, leaves, fruit, and seed are used as feed for farm animals and the wood is used for charcoal. One study revealed anti-inflammatory and analgesic activities in bark extract from this plant.[2] The oil extracted from the seed, called kusum oil, is used for culinary and lighting purposes, and to treat itching, burns, acne, and for hair dressing. Moreover, it has been reported to possess anticancer, antioxidant, antimicrobial, antiulcer, and anti-inflammatory activities, and can be used in the production of biodiesel.[3,4] Phytochemical analysis of leaf extracts prepared in various solvents revealed the presence of saponins, tannins, steroids, terpenoids, carbohydrates, cardiac glycosides, and other bioactive compounds.[5,6] One study reported antimicrobial activity in the fresh juice extract of this fruit.[7] Although the fruit of this plant is commonly consumed, there is limited information about its biological activities compared to other commercially available fruits. Therefore, this study aimed to identify the bioactive constituents and determine the antioxidant activity of the fruit of this plant to provide opportunities for its development as a value-added health product. The total carotenoid, phenolic, flavonoid, and anthocyanin contents of the fruit as well as the levels of ascorbic acid and beta-carotene were determined. DPPH, ABTS, FRAP, ORAC, ferric thiocyanate, and metal chelating activity assays were used for the evaluation of the antioxidative activity of each part of *S. oleosa* fruit.

Figure 1: *Schleichera oleosa* (Lour) Oken fruits.

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Citation: Khamphukdee C, Chulikhit Y, Monthakantirat O, Maneenet J, Boonyarat C, Daodee S. Phytochemical Constituents and Antioxidative Activity of *Schleichera oleosa* Fruit. Trop J Nat Prod Res. 2021; 5(8):1445-1449. doi.org/10.26538/tjnpr/v5i8.20

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Chemicals and equipment

The M965+ microplate reader was from Metertech, Taiwan. The high performance liquid chromatograph and octadecylsilane column were products of Agilent Technologies, Netherlands. Folin-Ciocalteu's phenol reagent, beta-carotene, quercetin, gallic acid, ascorbic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (3-ethylbenzthiazoline-6-sulphonicacid), sodium acetate and TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) were purchased from Sigma-Aldrich (Steinheim, Germany). Aluminium chloride, sodium carbonate, potassium chloride, ferrous sulfate, potassium persulfate, and glucose were obtained from Univar (United States of America). Hydrochloric acid, sulfuric acid, ethanol, and methanol were purchased from Merck (Darmstadt, Germany).

Collection and identification of fruit material

S. oleosa fruits were collected from Ban Fang District, Khon Kaen, Thailand (March - July 2018. A specimen of the plant material (Voucher number: SD05) was identified and authenticated by Assistant Professor Dr. Prathan Luecha, Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand.

Preparation of crude extract

The fruits of *S. oleosa* were washed under running water until they were clean. The fruit was divided into peel and seeds that were dried at 50^{o}C, and juice that was freeze-dried. Dried materials (200 g) were macerated with 95% ethanol (1000 mL) twice within 7 days. The extracts were filtered and evaporated by a vacuum rotary evaporator. The crude extracts were stored at -20^{o}C until needed for analysis.

Determination of total carotenoid content

Total carotenoid content was evaluated by the spectrophotometric method adapted from Ashraf and co-worker's procedure.[8] The fruit extract (1 mg/mL) was dissolved in ethanol as a stock solution and diluted to 0.1 mg/mL. The absorbance was measured at 450 nm using a spectrophotometer. The total carotenoid content is expressed as beta-carotene equivalents. Samples were further diluted in cases where the absorbance value was above the linear regression values.

Determination of total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu phenol method modified from Singleton and Rossi using gallic acid as a standard.[9] Briefly, the fruit extract (1 mg/mL, 500 µL) was mixed with Folin-Ciocalteu's reagent (2.5 mL). After 5 min, sodium carbonate solution (2 mL) and deionized distilled water (5 mL) were added into the mixture then mixed carefully after being kept in the dark for 2 h. Absorbance was measured using a spectrophotometer at 760 nm. The results are reported in gallic acid equivalents.

Determination of total flavonoid content

An aluminium chloride colorimetric assay was performed to determined total flavonoid content.[10] The fruit extract (1 mg/mL, 500 µL) was mixed with aluminium chloride (0.4 mL) and sodium acetate solution (0.5 mL) followed by the addition of deionized distilled water (4 mL). The reaction was mixed thoroughly and kept at room temperature for 15 min. The absorbance was measured at 430 nm using a spectrophotometer. The data are expressed in quercetin equivalents.

Determination of total anthocyanin content

Total anthocyanin content was evaluated by the pH-differential method adapted from Lee and coworkers.[11] The absorbance of the extract (1 mg/mL) was measured at 700 nm and 535 nm at pH 1 and pH 4.5 to determine the anthocyanin content against a blank of the solvent used for extraction using a spectrophotometer. The total anthocyanin content was calculated and is expressed as cyanindin-3-glucoside equivalents. The absorbance (A) is related to the total anthocyanin content according to the following equation:

$$\text{Monomeric anthocyanin} = (\text{A} \times \text{MW} \times \text{DF} \times 1000) \div (\text{Molar absorptivity} \times 1)$$

Where,
A is the absorbance
MW is the molecular weight of a reference pigment (cyanindin-3-glucoside: 449.2 g/mol)
DF is dilution factor = 10
The molar absorptivity is the reference anthocyanin (extinction factor 26,900 L cm^{-1} mol^{-1})

HPLC analysis of ascorbic acid

Reverse-phase high performance liquid chromatography was applied to determine the amount of ascorbic acid in each extract. The analytical method for ascorbic acid was modified from the Asean Manual of Food Analysis, Institute of Nutrition, Mahidol University[12] and was validated for linearity, repeatability, intermediate precision, accuracy, limit of detection, and limit of quantitation. The analysis was carried out using HPLC with a diode array detector. The extract solution (10 mg in 3% phosphoric acid solution) was injected into a Hypersil ODS column (250 x 4.0 mm i.d.; 5 µm particle size). The mobile phase consisted of potassium dihydrogen phosphate (0.408 g) in 0.35% (v/v) phosphoric acid solution. Flow rate for all analyses was 1.0 mL/min. The absorbance was measured at a wavelength of 248 nm. The amount of ascorbic acid was determined by comparing the peak area of the extract solution with the peak area from the standard curve which ranged from 0.25-40 µg/mL.

HPLC analysis of beta-carotene

The analytical method for beta-carotene was modified from the study of Ahamad and coworkers[13] and was validated for linearity, repeatability, intermediate precision, accuracy, limit of detection, and limit of quantitation. The analysis was carried out using HPLC with a diode array detector and the extract solution (10 mg in 1 mL of 80% acetone) was injected into a Hypersil ODS column (250 x 4.0 mm i.d.; 5 µm particle size). The mobile phase consisted of acetonitrile, dichloromethane, and methanol in the ratio of 70:20:10. Flow rate for all analyses was 1.0 mL/min. The absorbance was measured at a wavelength of 450 nm. The amount of beta-carotene was determined by comparing the peak area of the extract solution with the peak area from the standard curve (0.25-30 µg/mL).

Determination of antioxidant activity using the DPPH free radical scavenging assay

The free radical scavenging (DPPH) method was applied according to the method of Brand.[14] In brief, the DPPH reagent was prepared by mixing DPPH with 100 mL ethanol, which was then stored at -20^{o}C prior to use. Fruit extract (10 mg/mL, 100 µL) and DPPH reagent (100 µL) were transferred into microplate wells and kept for 30 min at room temperature. Then, the absorbance was measured at 517 nm by UV/visible spectrophotometer. A calibration curve was plotted for 10-50 µM concentrations of trolox, the reference standard. The inhibitory percentage of DPPH was calculated by the following formula:

$$\%\text{inhibition} = [(\text{A}_{\text{DPPH}} - \text{A}_{\text{sample}}) \div (\text{A}_{\text{DPPH}} - \text{A}_{\text{blank}})] \times 100$$

Where A_{DPPH} is absorbance of DPPH radical solution (without sample or standard), and A_{sample} is absorbance of a DPPH solution (with sample or control).

Determination of antioxidant activity using ABTS radical cation decolorization assay

This method was adapted from the method of Ronald.[15] ABTS is oxidized to its radical cation $\text{ABTS}^{\cdot+}$, which is intensely coloured. The ability of test compounds to reduce the colour of $\text{ABTS}^{\cdot+}$ indicates a direct reaction with the $\text{ABTS}^{\cdot+}$ radical. The ABTS reagent was prepared by mixing the $\text{ABTS}^{\cdot+}$ and potassium persulfate then kept in the dark at room temperature for 12 hours. The $\text{ABTS}^{\cdot+}$ solution (1 mL) was diluted with ethanol (50 mL) to obtain an absorbance of 0.70 $\pm$ 0.02 at 734 nm. The fruit extract (10 mg/mL, 50 µL) and ABTS reagent (100 µL) were transferred into microplate wells and kept at room temperature for 2 hr. Then, the absorbance was measured at 734 nm by UV/visible spectrophotometer. The calibration curve was plotted for 10 - 50 µM concentrations of the trolox standard solution.

The inhibitory percentage of ABTS was calculated using the formula;

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

$$\% \text{ inhibition} = [(A_{ABTS} - A_{sample}) \div (A_{ABTS} - A_{blank})] \times 100$$

Where A is the absorbance

Determination of antioxidant activity using ferric reducing antioxidant power (FRAP)

This method was modified from previous methods.[16] Briefly, a working FRAP reagent solution composed of acetate buffer (100 mL), TPTZ solution (10 mL), and ferric chloride solution (10 mL) was kept at 37°C. Then fruit extract (10 mg/mL, 50 µL) and FRAP reagent (150 µL) were transferred into microplate wells and mixed well. The absorbance was read at 593 nm by UV/visible spectrophotometer. Results are expressed in µg Trolox equivalent/g extract.

Determination of antioxidant activity using ORAC assay

The ORAC assay was carried out according to the method of Dudonne.[17] The ORAC assay is based on the scavenging of peroxyl radicals generated by AAPH, which prevents the degradation of fluorescein, and accordingly inhibits the loss of fluorescence.

AAPH → ROO•

ROO• + fluorescein → ROO + oxidized fluorescein

ROO• + antioxidant (A) → ROOH + A•

The reaction was carried out in 75 mM phosphate buffer (pH 7.4). Fluorescein and AAPH solutions were also prepared in phosphate buffer. Twenty-five microliters of sample and 150 µL of fluorescein solution were mixed in a cuvette and preincubated for 5 min at 37°C. The APPH solution (25 µL) was then added. The fluorescence of the sample and a blank (25 µL of phosphate buffer in the reaction mix) was recorded for 60 min at excitation and emission wavelengths of 485 and 538 nm by spectrofluorometer microplate reader. All samples were analysed in triplicate. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The regression equation between net AUC and Trolox concentration was determined. The ORAC values were calculated as µg Trolox equivalents/g of plant extract.

Determination of antioxidant activity using ferric thiocyanate assay (lipid peroxidation assay)

This lipid peroxidation assay was modified from the method of Zhan, Dong and Yao.[18] The mechanism of action of the antioxidant compound is the inhibition of linoleic acid peroxidation, which can generate free radical (peroxide). Peroxide-free radical can oxidize ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) and a brownish-red complex (ferrothiocyanate compound) forms from the reaction between the ferric and thiocyanate ions. A reduction in the colour of the mixture is due to a reduction in the amount of ferrothiocyanate compound, which shows antioxidant capacity.

$ROO^{\cdot} + FeCl_2 \longrightarrow Fe^{3+}$

$Fe^{3+} + SCN^- \longrightarrow Fe(SCN)^{2+}$ (Ferrothiocyanate)

The thiocyanate method was applied to study the capacity of *S. oleosa* fruit extracts to inhibit linoleic acid peroxidation. The linoleic acid emulsion was prepared by mixing linoleic acid (0.280 4 g) and Tween 20 (0.280 4 g) in 50 mL of phosphate buffer (0.20 mol/L, pH 7.0). A reaction solution containing 0.5 mL of extract, 2.5 mL of linoleic acid emulsion, and 2.0 mL of phosphate buffer (0.20 mol/L, pH 7.0) was incubated at 60 °C for 8 h in the dark to generate the oxidation process. Sample solution (0.1 mL) was added into 4.5 mL of ethanol (75%) followed by 0.2 mL of ammonium thiocyanate solution (300 g/L) and 0.2 mL of the ferrous chloride solution (20.0 mmol/L in 3.5% hydrochloric acid solution) in sequence. The solution was stirred for 3 min. The absorbance was measured at 500 nm. All tests were performed in triplicate using Trolox as the positive control.

Determination of antioxidant activity using metal chelating activity assay

The metal chelating activity assay was slightly modified from the method of Dinis, Madeira and Almeida.[19] Ferrozine-ferrous complexes (red colour) are the product from the reaction of ferrozine and ferrous ion (Fe^{2+}). Reduction in the amount of the red-coloured complex results from the capacity of antioxidant compounds to compete with the ferrous ion (Fe^{2+}).

Fe^{2+} + Ferrozine ⟶ Fe^{2+}-Ferrozine complex (red pink)

Fe^{3+}-Ferrozine complex + antioxidant ⟶ Fe^{3+}-Ferrozine complex (pale pink)

Sample solution (0.1 mL) and $FeCl_2$ (2 mM, 0. 5 mL) were mixed and 0.2 mL of ferrozine (5 mM) was added after 15 minutes. Then the mixture was left to stand for 10 minutes at room temperature in a dark room. The absorbance of the Fe^{2+}-ferrozine complex red-violet colour was measured at λmax = 562 nm. The percentage inhibition was calculated**.** Distilled water was used as a control**.**

Statistical analysis

All results are expressed as mean ± standard deviation (SD) for three replicates of the antioxidant activity assays and the determination of the active content of the samples. One-way analysis of variance at a significance level of 0.05 was used for comparison of active content.

Results and Discussion

The total active contents, including carotenoid, phenolic, flavonoid and anthocyanin contents and ascorbic acid and beta-carotene levels, of ethanolic extracts of the peel, juice, and seed parts of *S. oleosa* fruit are presented in Table 1 and the total antioxidant capacity of each part is reported in Table 2. The peel of *S. oleosa* contained the highest carotenoid, phenolic, flavonoid and anthocyanin contents, while the juice contained the highest ascorbic acid and beta-carotene contents, as shown in Table 1. The lowest active content level and lowest antioxidative capacity was found for the seed extracts. HPLC determination of ascorbic acid and beta-carotene were performed and chromatograms are shown in Figures 2 and 3. The validation results of the HPLC method for determination of ascorbic acid and beta-carotene were satisfactory for accuracy (percent recovery 93.37-109.27% for ascorbic acid, 86.09-110.14% for beta-carotene), precision (percent relative standard deviation < 2%), linearity (R^2 0.99), limit of detection (0.1 µg/mL), and limit of quantitation (0.25 µg/mL). *S. oleosa* peel extract showed the highest antioxidant activity in the DPPH, ABTS, ORAC, and FRAP assays, while the juice part showed the most activity in the ferric thiocyanate and metal chelating assays. This suggests that the active compounds in these two parts may have different antioxidative mechanisms, which could be applied to study the role of different oxidative mechanisms in diseases, such as oxidative stress in Alzheimer's disease.

Together, the results of this study reveal a relationship between the antioxidative capacity and the phytochemical content of this fruit, with the peel and juice parts exhibiting the highest potential activity. The carotenoid, phenolic and flavonoid contents of each *S. oleosa* fruit part were directly related to the antioxidant activities determined in the DPPH, ABTS, FRAP, and ORAC assays, whereas the anthocyanin content showed no correlation with antioxidant activity. However, for the ferric thiocyanate and metal chelating activity assays, it was the ascorbic acid and beta-carotene content that showed a direct relationship with antioxidant activity. These results support the observation that the active constituents in *S. oleosa* fruit perform antioxidant activities via different mechanisms including radical scavenging, chain breaking, and metal chelation. Although some active compounds have been found in kasum oil, which is prepared from the seed part of *S. oleosa* fruit, the use of ethanol extraction in the current study is likely to have resulted in the recovery of only low levels of active constituents from *S. oleosa* seeds. In this study, ethanol was selected as the extraction solvent because the extracts would be safe enough to prepare as food supplements or to include in herbal formulations.[20] Thus, the peel and juice parts of *S. oleosa* fruit should be further investigated as a source of natural antioxidants for the development of value-added nutraceutical products.

Several studies have revealed that the antioxidative activities of fruits are due to phytochemical constituents such as carotenoids, tocopherols, and ascorbic acid.[21] Ascorbic acid is present in many fruits and vegetables such as apples, oranges, carrots, beets, cherries, and tomatoes.[22] A previous phytochemical analysis of *S. oleosa* fruit by Toobpeng *et al*[7] showed high levels of phenolics and antioxidant activity against DPPH radicals. These results are confirmed and expanded by the present study to identify additional antioxidant compounds (flavonoids, carotenoids, anthocyanins, ascorbic acid, and

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

beta-carotene) that are found in *S. oleosa* fruit and may act synergistically with each other. The potential health benefits of *S. oleosa* fruit should be further investigated and the cultivation of this plant should be encouraged as a natural source of phytonutrients and for sustainable development in the region.

Table 1: Active constituents in each part of *S. oleosa* fruit

fruit part	Active constituent (mg/g extract, n=3)					
	total carotenoid content[a]	total phenolic content[b]	total flavonoid content[c]	total anthocyanin content[d]	ascorbic acid content	beta-carotene content
peel	343.57 ± 1.27	9.91 ± 0.22	9.18 ± 0.08	2.81 ± 0.01	0.07 ± 0.00	0.15 ± 0.00
juice	74.27 ± 1.01	6.01 ± 0.17	5.07 ± 0.12	0.23 ± 0.02	0.14 ± 0.01	0.44 ± 0.01
seed	11.90 ± 1.09	4.06 ± 0.31	2.19 ± 0.06	0.20 ± 0.01	*	*

*Could not detect
a- mg beta-carotene equivalent to g extract, b- mg gallic equivalent to g extract, c-unit mg quercetin equivalent to g extract, d- mg cyanindin-3-glucoside equivalent to g extract

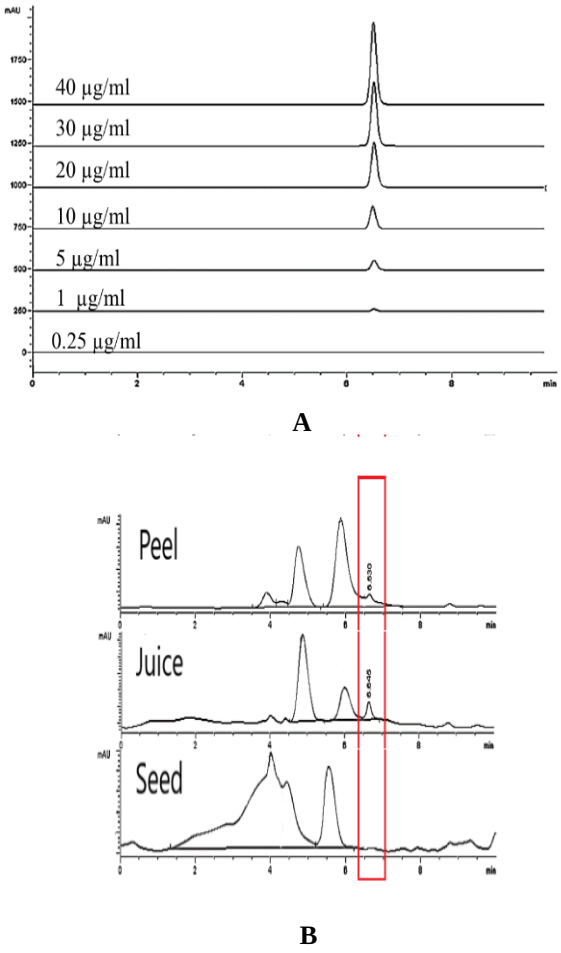

Figure 2: HPLC chromatograms of standard ascorbic acid solution (concentration 0.25-40 µg/mL, A) and the extract solutions of each part of *S. oleosa* fruit (B).

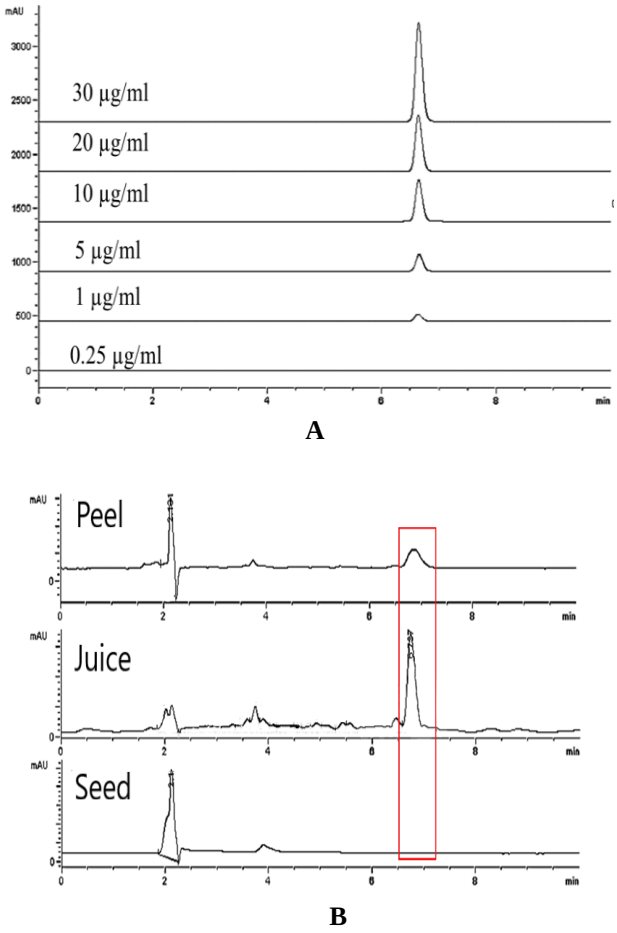

Figure 3: HPLC chromatograms of standard beta-carotene solution (concentration 0.25-30 µg/mL, A) and the extract solutions of each part of *S. oleosa* fruit (B

Table 2: Total antioxidative capacity in peel, juice, and seed extracts of *S. oleosa* fruit.

Extract	Antioxidative capacity					
	DPPH assay IC_{50}[a]	ABTS assay IC_{50}[a]	FRAP assay[b]	ORAC assay[b]	Ferric thiocyanate assay[c]	Metal chelating activity assay IC_{50}[a]
peel	510.70 ± 1.59	784.74 ± 3.11	173.10 ± 0.67	784.76 ± 3.05	19.71 ± 1.11	602.71 ± 2.78
juice	779.72 ± 2.80	825.79 ± 2.61	62.79 ± 0.73	518.65 ± 2.18	43.30 ± 1.16	506.39 ± 4.11
Seed	>1000	>1000	*	283.09 ± 5.38	8.05 ± 0.10	942.73 ± 3.26

*could not detect

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Conclusion

Extracts from the peel and juice parts of the fruit of *S. oleosa* showed high content of active compounds, which corresponded with antioxidative activity. The peel extract showed the highest antioxidant activity in the DPPH, ABTS, ORAC, and FRAP assays, while the juice extract showed the highest antioxidant activity in the ferric thiocyanate and metal chelating assays. Seed extracts showed the lowest levels of active compounds and lowest antioxidative activity. These results could be used as a basis for further research and development of value-added products for consumers in the future.

Conflict of Interest

The authors declare no conflicts of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

This study was supported by Khon Kaen University (KKU Research Program 2021: RP64-4/009). The authors thank Dr Glenn Borlace, Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, for English language assistance and also thank Assist. Prof. Prathan Luecha and Ms. Buntarawan Turaphra for their kindly help.

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