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Development of pressurized hot water extraction for five flavonoid glycosides from defatted *Camellia oleifera* seeds (byproducts)

Bing-Chung Liau[a,b], Vinoth Kumar Ponnusamy[c], Maw-Rong Lee[d], Ting-Ting Jong[d], Jung-Hui Chen[e,*]

[a] *Food Safety Analysis Laboratory, Chi Mei Inspection Tech Co. Ltd, No.5, 32nd Road, Taichung Industrial Park, Taichung City, 407, Taiwan, ROC*
[b] *Hung-Kuang University, College of General Education, No. 1018, Sec. 6, Taiwan Boulevard, Shalu District, Taichung City, 433, Taiwan, ROC*
[c] *Kaohsiung Medical University, Department of Medicinal and Applied Chemisry, No.100, Shih-Chuan 1st Road, Kaohsiung City, 807, Taiwan, ROC*
[d] *National Chung Hsing University, Department of Chemistry, 250 Kuo-Kuang Road, Taichung City, 402, Taiwan, ROC*
[e] *Nan Kai University of Technology, Department of Mechanical Engineering, Nantou County, Taiwan, ROC*

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ABSTRACT

In this study, five flavonoid glycosides were extracted from defatted *Camellia oleifera* seeds by using a pressurized hot water extraction (PHWE) method. The independent experimental factors (temperature, time and pressure) influencing the PHWE efficiency of the flavonoid glycosides were optimized and quantified using an orthogonal array design and high-performance liquid chromatography–negative mode electron spray ionization/mass spectrometry (HPLC/(−)ESI–MS-MS), respectively. Based on the result of experimental design, the maximum extraction efficiency of flavonoid glycosides were obtained by selecting temperature at 140 °C, pressure at 600 psi, and time for 10 min. The recovery and overall yield of the flavonoid glycosides were greater than those in ultrasonic extraction (UE) (78.5% vs 68.4% and 18.8 mg/g vs 15.91 mg/g). In particularly, compounds **1** and **2,** which are kaempferol derivatives, were higher 1.19–1.23 times than those of the compounds obtained through UE (6.63 mg/g vs 5.56 mg/g; 11.89 mg/g vs 9.66 mg/g, respectively). These two compounds have higher polarities and antioxidant capacities than the other three flavonoid glycosides. Thus, PHWE is an excellent alternative method for the extraction of flavonoid glycosides that are highly polar and potent antioxidants.

1. Introduction

The seeds of *Camellia oleifera* contain nutritious oils and numerous active compounds including oleic, linoleic and ketoprofen (Shyu et al., 1990; Cheng et al., 2004) that are beneficial to human health, and the seeds are commonly used in Asian countries, especially those in Southeast Asia. In particular, defatted seeds are the byproduct of the process of extracting and refining the oil from the seeds. This byproduct is typically used as a detergent or an organic fertilizer with low economic value, and if it were not completely recovered during the process, it could pollute rivers and soils. Therefore, the recovery, recycling, and upgrading application of defatted *C. oleifera* seeds are crucial in the oil refining process. Because the byproducts are an inexpensive residual resource that contain large amounts of active compounds, including flavonoids, saponins and polysaccharides (Wang and Wei 1990; Sugimoto et al., 2009; Chen et al., 2010) and the byproducts can be purified to obtain the biologically active compounds which are flavonoid glycosides and saponins, thereby increasing the economic benefits of the seeds of *C. oleifera*.

Flavonoids are a large group of natural products that contain a variety of polyphenolic compounds and exhibit a variety of pharmacological activities, including antioxidant, anticancer, and anti-inflammatory activities (Braca et al., 2002). Moreover, because of their ability to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidase activity (Montoro et al., 2005), flavonoids possess a protective effect in biological systems. Additionally, flavonoids have the ability to stimulate human protective enzyme systems. Numerous studies have demonstrated the protective effects of flavonoids against a variety of infectious (bacterial and viral) and degenerative diseases, including cardiovascular diseases, cancers, and other age-related diseases (Liu et al., 2014). The defatted seed cake of *C. oleifera* contains approximately 3% of flavonoids glycosides (Chen et al., 2009) which have potential for use in the formulation of nutraceuticals and preservation of food (Oboh and

* Corresponding author.
E-mail address: t115@nkut.edu.tw (J.-H. Chen).

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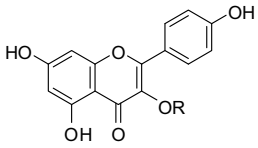
Ademosun, 2012). Therefore, the byproduct has commercial value in both agricultural and food industries. However, the defatted seed cake has a high oil content that could reduce the extraction efficiency of flavonoid glycosides in organic solvent extraction method.

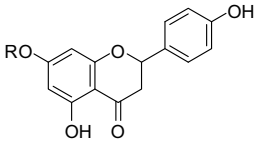
Extraction process is the key step in the recovery and purification of active substance from plant materials. Extraction techniques, including Soxhlet extraction, leaching, reflux extraction, and supercritical fluid extraction of anthocyanin and phenolic compounds (Prakash Maran et al., 2014), are laborious and require large amounts of solvents; moreover, they provide relatively low yields after long extraction times.

Recently, pressurized hot water extraction (PHWE), was developed and has become a popular green extraction method; it can be used to isolate different classes of compounds in numerous of types of matrices, such as environmental, food, and botanical samples. The main factors influencing the extraction efficiency of PHWE include temperature, extraction time, flow rate, and the addition of modifiers/additives, and among them, extraction temperature is the most critical (Plaza and Turner 2015; Teo et al., 2010). Phenolic compounds are polar antioxidant bioactive compounds that have been extracted from a wide variety of sources, such as plants and food-industry byproducts, through PHWE; generally, the ranges of extraction temperature and extraction time are 80–150 °C and 1–60 min, respectively (Plaza and Turner, 2015). To date, flavonoid glycosides (linking many sugars) extracted from plant or food-industry byproducts (and then identified) through PHWE have not been investigated in detail.

In this study, five flavonoid glycosides (linking many sugars) were extracted and identified from defatted *C. oleifera* seeds (byproduct) by using PHWE. Furthermore, the optimized experimental conditions for PHWE were obtained quickly by employing a multilevel orthogonal array design strategy. Additionally, the overall yields of the five flavonoid glycosides were quantitatively determined through high-performance liquid chromatography coupled with negative mode electrospray ionization and tandem mass spectrometry [HPLC/(−)ESI–MS-MS]. PHWE method were also compared with traditional ultrasonic extraction (UE) method in terms of the overall yield and recovery of the flavonoid glycosides from the defatted C. oleifera seeds (byproducts).



Compound	R
1	glc-[(1→2)-glc-(1→6)-rha]
2	glc-[(1→2)-xyl-(1→6)-rha]
6	rha



Compound	R
3	glc-(1→6)-xyl
4	glc-(1→2)-rha-(1→3)-glc
5	glc-[(1→2)-rha-(1→3)-glc][(1→6)-xyl]

Fig. 1. The chemical structures of **1-6.**
Compound **1**: kaempferol-3-*O*-[2-*O*-β-D-glucopyranosy-l-6-*O*-α-l-rhamnopyranosyl] −β-D-glucopyranoside
Compound **2**: kaempferol-3-*O*-[2-*O*-β-D-xylopyranosyl-6-*O*-α-L-rhamnopyranosyl] −β-D-glucopyranoside
Compound **3**: naringenin-7-*O*-β-D-xylopyranosyl(1 → 6)- β-D-glucopyranoside
Compound **4**: naringenin-7-*O*-[β-D-glucopyranosyl(1 → 3)-α-L-rhamnopyranosyl(1 → 2)]- β-D-glucopyranoside
Compound **5**: naringenin-7-*O*-[β-D-xylopy- ranosyl(1 → 6)][β-D-glucopyranosyl(1 → 3)-α-L-rhamnopyranosyl(1 → 2)]-β-D-glucopy-ranoside
Compound **6**: kaempferol-3-*O*-rhamnoside

2. Materials and method

2.1. Materials and reagents

A defatted seed cake of *C. oleifera* was purchased from a local natural and organic food store in Taichung city, Taiwan; it was dried overnight in a hot air-oven at 40 °C prior to extraction. The solvents used for the extraction and separation were methanol and ethanol (analytical reagent grade) and acetonitrile (high-performance liquid chromtography [HPLC] grade), all of which were purchased from Merck Chemicals (Germany). Ultrapure water (>18 MΩ) was obtained using an SG-Ultra water purification system (SG Water USA, USA), and was degassed under vacuum and filtered with a 0.22 μm membrane filter prior to use. The standard compounds were five flavonoid glycosides — kaempferol-3-*O*-[2-*O*-β-D-glucopyranosyl-6-*O*- α-L-rhamnopyranosyl]-β-d-glucopyranoside (**1**), kaempferol-3-*O*-[2-*O*-β-D-xylopyra- nosyl-6-*O*-α-L-rhamnopyranosyl]-β-d-glucopyranoside (**2**), naringenin-7-*O*-β-D-*xylo*- pyranosyl(1 → 6)-β-D-glucopyranoside (**3**), naringenin-7-*O*-[β-D-glucopyranosyl(1 → 3) −α-L-rhamnopyranosyl(1 → 2)]-β-D-glucopyranoside (**4**), naringenin-7-*O*-[β-D-xylopy- ranosyl(1 → 6)][β-D-glucopyranosyl(1 → 3)-α-L-rhamnopyranosyl(1 → 2)]-β-D-glucopy-ranoside (**5**); these flavonoid glycosides and the internal standard (IS) kaempferol-3-*O*-rhamnoside (**6**) were separated and purified to a purity exceeding 98%, and their chemical structures are shown in Fig. 1. These compounds (**1-5**) were isolated and indentified with existing literature (Chen et al., 2009, 2010). Compound **2** was chosen as a representative compound in the PHWE-optimization experiments because of two reasons: it was the most abundant among all the flavonoids in the defatted *C. oleifera* seeds and it exhibited high antioxidant capacity (Chen et al., 2009, 2010).

2.2. Ultrasonic extraction

Weighed samples (4.0 g) were taken in a 250 mL screw–cap glass bottle along with 200 mL of a solvent (60% ethanol solution). The glass bottles were then capped and placed in a water bath, and the mixture was subjected to ultrasonication extraction at 30 °C for 30 min by using an ultrasonicator (Bransonic ultrasonic cleaner, model 3510). After extraction, the extracts were filtered, and methanol was added to the filtrate to obtain a total volume of 200 mL. The extract solution was then stored at 4 °C until analysis. An appropriate volume (1 mL) of the extract solution was taken, diluted with methanol to an appropriate concentration, and filtered to obtain the stock solution. A total of 500 μL of the stock solution and 500 μL of the IS (50 ppb) were mixed for use as the work solution.

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