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Subcritical water extraction of bioactive compounds from dry loquat (*Eriobotrya japonica*) leaves and characterization of triterpenes in the extracts

Erasto Mlyuka[1,2], Shuang Zhang[1], Zongping Zheng[1] and Jie Chen[1,3]*

[1]State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China.
[2]National Irrigation Commission, Ministry of Water and Irrigation, P. O. BOX 671, Mtwara, Tanzania.
[3]Synergetic Innovation Center for Food Safety and Nutrition, Jiangnan University, Wuxi, Jiangsu 214122, China.

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Medicinal properties of loquat leaf extracts (LLEs) are associated with their constituents of phenolic compounds and triterpenes. In this study, the efficacy of subcritical water extraction (SWE) technique was assessed by comparing with conventional solid-liquid extraction (CE) and Soxhlet extraction (SE). Results showed that the highest yields of total polyphenols were 82.7 ± 1.5 mgGAE/g leaf weight (LW), total flavonoids (54.1 ± 4.1 mgQE/g LW) and total triterpenoids (37.5 ± 3.2 mgUAE/g LW) were obtained by SWE compared to total polyphenols (61.8 ± 3.3 mgGAE/g LW), total flavonoids (43.2 ± 0.6 mgQE/g LW) and total triterpenoids (28.7 ± 2.3 mgUAE/g LW) extracted by SE and total polyphenols (50.3 ± 1.8 mgGAE/g LW), total flavonoids (40.4 ± 2.1 mgQE/g LW) and total triterpenoids (22.9 ± 3.2 mgUAE/g LW) obtained by CE. The extraction efficiency of triterpenes using SWE was about 1.7 times higher than those obtained using traditional extraction methods, and their main structural pattern of the cured extracts was comparable to the extracts obtained using traditional extraction methods.The infrared spectra obtained from the three extraction techniques appeared identical, but the variation in the intensity of the peak of absorption was visible among the three extraction techniques. The similarity of the infrared spectral pattern (peak coincided peak by peak) implies that the triterpenes in the extract obtained by the three techniques were identical by LC/MS. The findings of this study have demonstrated that SWE can be employed as an alternative green extraction technology to get important phytochemicals from plant sources.

Key words: Chinese loquat leaf, *Eriobotrya japonica*, subcritical water extraction, triterpene.

INTRODUCTION

Chinese loquat (*Eriobotrya japonica*) leaves have a high potential of bioactive compounds required in the growing pharmaceutical industries. They have been used as nutrition supplements for chronic bronchitis, coughs,

*Corresponding author. E-mail: chenjie@jiangnan.edu.cn. Tel: +86-510-85329032. Fax: +86-510-85919065.

asthma, phlegm, high fever and gastroenteric disorders (Hong et al., 2008a; Hong et al., 2008b; Chang et al., 2011). They also contain some phenolics and triterpenes which have promising potential for anticancer, anti-inflammation and hypoglycemia (Cha et al., 2011; Zong and Zhao, 2007).

Bioactive compounds from plant sources are extracted by various classical extraction techniques such as Soxhlet extraction, maceration and hydro distillation (Azmir et al., 2013). For the extraction of bioactive compounds from loquat leaves, conventional methods have been used for many years based on solid–liquid extraction using organic solvents (Thien et al., 2012). However, these techniques are time consuming, laborious, lack automation, and ultimately have low reproducibility. In addition, they are also less selective which leads to low extraction yields (Singh and Saldaña 2011). To address the shortcomings of classic extraction techniques, considerable research work by various researchers has been done on alternative extraction techniques, especially, non-conventional such ultrasonic-assisted extraction and microwave-assisted extraction (Vetal et al., 2012, Vetal et al., 2013, Bera et al., 2015), and combined ultrasonic/ microwave assisted extraction (Cheng et al., 2011). These techniques have been known to improve the extraction yields and extraction time. However, the use of conventional solvents like methanol poses environmental safety concern. In view of concerns and limitations associated with the use of solvents, the use of subcritical water extraction (SWE) is an attractive alternative to obtain bioactive compounds from loquat leaves.

To overcome the solvent limitations, the extraction of active ingredients from loquat leaves have necessitated the development of green and novel techniques which neither pollutes the environment nor damages the target bioactive compounds. In view of such development, SWE can be an ideal candidate. SWE is a technique which can extract both polar and non-polar compounds (Kwon and Chung, 2015; Luong et al., 2015), therefore it is an attractive alternative to obtaining bioactive compounds from loquat leaves. The SWE technique uses subcritical water (a green solvent) during the extraction process. The subcritical water refers to water at high temperature, mostly from its boiling point (100°C) to below the critical temperature (374°C), but with moderate pressure (below critical juncture at 22.4 MPa) that maintains it in the liquid phase.

The critical point of water is at 22.4 MPa and 374°C and water below these critical points has demonstrated its ability to extract different classes of compounds depending on pressure and temperature programming (Duba et al., 2015; Liu et al., 2015). At subcritical condition, more polar compounds are extracted at low temperature while the less polar compounds are extracted at higher temperatures into subcritical water (Luong et al., 2015). The dielectric constants of liquid water change with temperature which in turn facilitates the release of different bioactive compounds in the plant material based on solubility. It is generally well recognized that loquat leaf extract consists of different kinds of bioactive constituents such as polyphenols, flavonoids, and triterpenes.

The aim of this current study was to compare the extraction yields of polyphenols, flavonoids, and triterpenes obtained by CE, SE and SWE, and the characterization of major triterpenes in the loquat leaf extracts obtained by SWE, which was then compared to other extraction techniques.

MATERIALS AND METHODS

Loquat leaves used in this study were bought from a local pharmacy store (Wuxi, Jiangsu, China).

Sample preparation

The dried loquat leaves were taken to the laboratory where they were ground using a grinder DYF-200 (Linda Machinery Company, Zhejiang, China). Grinding was done to attain appropriate particle size (80 mesh) for traditional extraction methods. The dried loquat leaves portioned for SWE, were crushed into the proper size of SWE filter (12 mesh). The samples were either used immediately or kept under low-temperature storage (-18°C) for further uses.

Standards and solvents

Standard CA, OA and UA with 99.9% purity and gallic acid (90%), quercetin (85%), and Folin-Ciocalteu reagent were purchased from Sigma (Shanghai, China). HPLC grade organic solvents such as methanol and ethanol were purchased from Amethyst Chemicals J & K Scientific Ltd (Beijing, China). Other chemicals used were of analytical grade and were purchase from a local chemical store (Wuxi, China). Double distilled water used in SWE technique was prepared within the laboratory.

Conventional solid-liquid extraction

Conventional solid-liquid extraction method reported by Singh and Saldaña (2011) was used with a slight modification as described in our previous work (Mlyuka et al., 2015). The dry loquat leaves powder was extracted twice with 90% ethanol (solid to solvent ratio of 1:20) at 80°C for 2 h. The supernatant from each extraction was mixed before centrifugations (6000 rpm for 15 min). After centrifugation, the supernatant was concentrated in a rotary evaporator at 60°C until dry and then the total extraction yield was obtained by the mean value of the total extracts divided by the mass of dry loquat leaves used.

Soxhlet extraction

Extraction was carried out using the method of Zhao and Zhang (2014) with a slight modification. 7.5 g of ground loquat leaves were placed in a cellulose thimble and transferred to a Soxhlet extractor. The extractor was filled with 150 mL of 90% ethanol heated for 24 h at 90°C. The extraction under the set conditions was performed in triplicate, and the combined extract was

concentrated in a rotary evaporator at 60°C until dry. The total extraction yield was obtained by the mean value of the total extracts divided by the mass of dry loquat leaves used.

Subcritical water extraction

Subcritical water extraction was carried using an extractor (Hangzhou Huali Co. Ltd, Hangzhou, China). The extractor sample cell had a capacity of 2000 mL. Samples were placed at the bottom of the extraction unit. The cell was then placed in the extractor followed by pressurizing the system at 10 MPa while heating, and the inlet valve remained opened until the temperature rose to the set temperature and for an additional of 5 min after the set temperature was attained (Luong et al., 2015; Mlyuka et al., 2016). The mode of extraction in this study was static, and water remained in the extraction unit for set durations. To investigate the effects of temperature, static extraction duration was fixed at 20 min for 100, 150, 180 and 200°C. To examine the effects of time, extraction at 200°C was also performed for 30, 45 and 60 min. At the end of the extraction time, both the outlet valve and point collection valve were opened simultaneously. The extract was collected in a one-liter glass bottle. Each subcritical water extract obtained were carried out in triplicate. The extract were then concentrated in a rotary evaporator at 60°C (at reduced pressure 72 mbar, with circulating cool water at 20°C) until dry and then the total extraction yield was obtained by the mean value of the total extracts divided by the mass of dry loquat leaves used.

Determination of total polyphenol content

Total phenolic contents (TPC) in loquat extract were evaluated using the Folin-Ciocalteu assay, which was adopted from Khanizadeh et al. (2008) with some modifications as described by Khanam et al. (2012). The sample mixture was allowed to stand at room temperature for 30 min. Absorption was measured at 765 nm in an UV/Visible an Alpha-1102 spectrophotometer Laxco™ (Shanghai, China). Quantification was based on the standard curve generated with 15 to 200 µg/ml of gallic acid and the TPC was expressed as mg gallic acid equivalents (GAE) /g leaf weight (LW).

Determination of total flavonoid content

Total flavonoid contents (TFC) were determined using the aluminum ion colorimetric method (Kim et al., 2009a). 0.5 mL of leaf extract diluted with 90% ethanol was transferred to a test tube followed by 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After 30 min of incubation at room temperature (25°C), the absorbance of the reaction mixture was measured at 415 nm using a UV/Visible an Alpha-1102 spectrophotometer LaxcoTM (Shanghai, China). Quantification was based on the standard curve generated with 50 to 1000 µg/ml of quercetin and the TFC was expressed as mg quercetin equivalent (QE)/g LW.

Determination of the total triterpenes

The total triterpenes in crude loquat extract were determined by colorimetry (Fan and He, 2006; Grishkovets and Gorbacheva, 1997). The crude loquat extract was diluted to 5 mL with glacial acetic acid, and its absorbance was measured at 548 nm. Quantification was based on the standard curve generated with 100 to 1000 µg/ml of UA and the total triterpenes were expressed as mg ursolic acid equivalent (UAE)/g LW.

Characterization and separation of triterpene acids

Characterization and separation of triterpene acids were carried out by LC/MS analysis according to He et al. (2014) with slight modification. An individual triterpenoid was determined by an HPLC equipped with an MS/MS detector (Quattro micro API, United Kingdom) using Venusil XPC C18 column (100 mm × 2.1 mm) at 35°C. Chromatographic separation was carried out by using mass spectrometry (MS/MS) detection. It was obtained by electrospray ionization (ESI) source operated in negative ionization mode, and by using selected ion recording (SIR) function.Two channels were used: channel one and two were used to detect OA/UA, and CA, respectively. Data acquisition and analysis were performed using MassLynx 4.1 software with QuanLynx program.

Fourier transform infrared spectrometer analysis of loquat leaf extracts

Loquat leaf extracts were further examined by the Fourier transform infrared spectrometer (FTIR-650) technique to compare IR chromatograms of the extracts obtained by different methods (Soxhlet extraction, conventional solid-liquid extraction, and subcritical water extraction). Loquat leaf extracts from each of the methods were finely ground with powdered potassium bromide, followed by pressing the mixture under high pressure to produce KBr pellet which was inserted into a holder in the spectrometer. IR chromatograms were obtained using the FT-IR, the spectra corresponded to the sum of 32 scans at a 1.5 cm^{-1} spectral resolution.

Statistical analysis

Yields data were analyzed by one-way analysis of variance (ANOVA) with the statistical package for the social sciences (SPSS) version 19 (SPSS Inc., USA). Tukey method was used to determine significant differences of bioactive yields at 95% confidence interval. Sigma plot software version 10 was used in graphical data presentation.

RESULTS AND DISCUSSION

Total bioactive yields

Results for the extraction yields of different bioactive compounds obtained by various extraction techniques are presented in Figure 1. The total polyphenol yields in the extracts obtained by SWE at 200°C were comparatively higher than the yields achieved by the other techniques utilized in this study (CE & SE). The SWE increased the extraction rate of total polyphenol from loquat leaves when the extraction was performed at high pressure (10 MPa) and temperature. Additionally, a higher temperature at subcritical condition has been reported to affect the polarity of water (Carr et al., 2010). It also lowers the polarity of the solvent by weakening the hydrogen bonds. Furthermore, it has been reported to produce a series of effects including improved mass transfer and solubility (Carr et al., 2010) of the compounds in the extract. Moreover, it lowers the surface tension of the water leading to improved penetration into the sample matrix. Therefore, by careful and strategic temperature

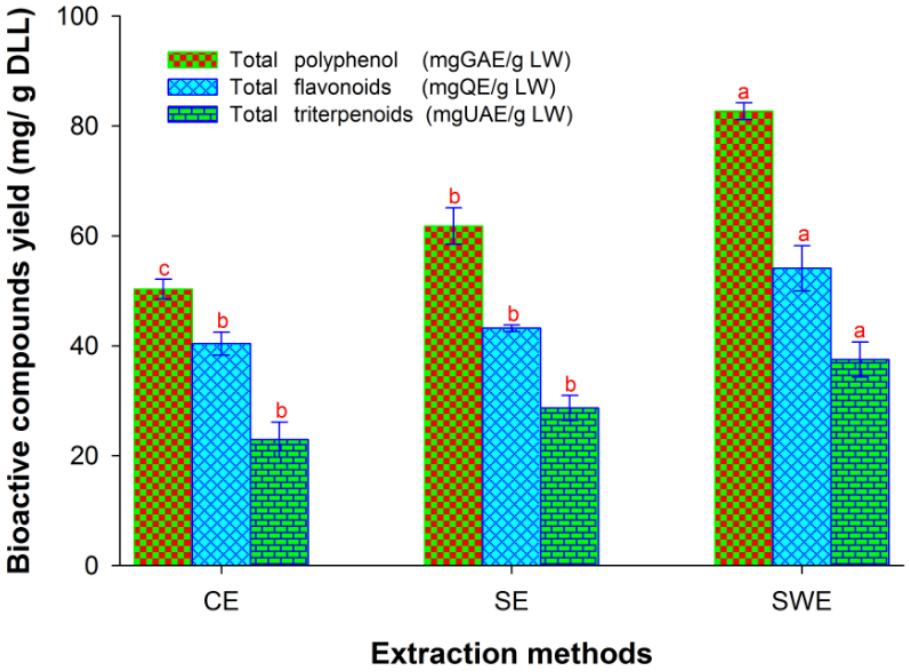

Figure 1. Extraction yield of bioactive compounds obtained from dry loquat leaves by different extraction methods. CE, Conventional extraction technique at 4 h; SE, Soxhlet extraction at 24 h; SWE, Subcritical extraction at 200°C for 20 min. GAE, Gallic acid equivalent; QE, Quercetin equivalent; UAE, Ursolic acid equivalent; LW, leaf weight. Extraction methods for similar phytochemical with the same letters are not significantly different according to the Tukey test ($p<0.05$).

programming, SWE can offer advantages when compared to the other extraction techniques tested in this study (Figure 1).

The time required to obtain total polyphenol yield from loquat leaves varied depending on the extraction technique used. The longest extraction time was spent when using SE (24 h) followed by CE (4 h) and SWE registered the shortest time (60 min), but within SWE duration variation did not result in a significant ($p>0.05$) yield of total polyphenol from loquat leaves (Figure 2). It was further found out that extending the extraction to 60 min resulted in a slight decline of total polyphenol yield which could be due to degradation of bioactive compounds at subcritical condition (Yang et al., 2007).

Results for the total flavonoids contents in the extracts obtained by CE, SE, and SWE at 200°C are presented in Figure 1. Total flavonoids obtained by CE and CE were not significantly different ($p>0.05$). In addition, these conventional methods used the same extraction solvent (90% ethanol) which might have influenced the performance of both CE and SE. In SWE, water was used as an extraction solvent. Water is considered safe for human use and has been employed previously for extraction of bioactive compounds from plant samples (Kim et al., 2009a; Mlyuka et al., 2016; Singh and Saldaña, 2011). The total flavonoids obtained by SWE were significantly ($p<0.05$) higher than the yields obtained by conventional extraction methods (Figure 1). This is because SWE is more selective and efficient technique. Tunable properties of subcritical water account for the selectivity of SWE. Furthermore, properties of water such as dielectric constant, surface tension, viscosity, and dissociation constant can be varied by adjusting extraction temperature at moderate pressure to keep water in the liquid state. Consequently, the maximum yield of total flavonoids was achieved when the SWE was performed at 200°C and 10 MPa for 45 min, but statistically ($p>0.05$) was not different from other tested times (Figure 2). This observation is consistent with what was reported by Kim and others on the extraction nutraceutical compounds from citrus pomaces (Kim et al., 2009a). It has been shown that bioactive compounds in plants could be extracted by subcritical water better than when using organic extraction techniques as demonstrated by the results of this study (Figures 1 and 2).

From the total triterpenoids yield comparison between extraction techniques (CE, SE & SWE), SWE obtained the highest yield (37.5 ± 3.2 mgUAE/g LW), which was significantly different ($p< 0.05$) from the other two techniques (Figure 1). These results indicated that SWE yields were strongly influenced by the temperature which is known to exert an effect on the dielectric constant of water (Kim et al., 2009a; Singh and Saldaña, 2011). The highest yields of total triterpenoids (41 ± 1 mgUAE/g LW)

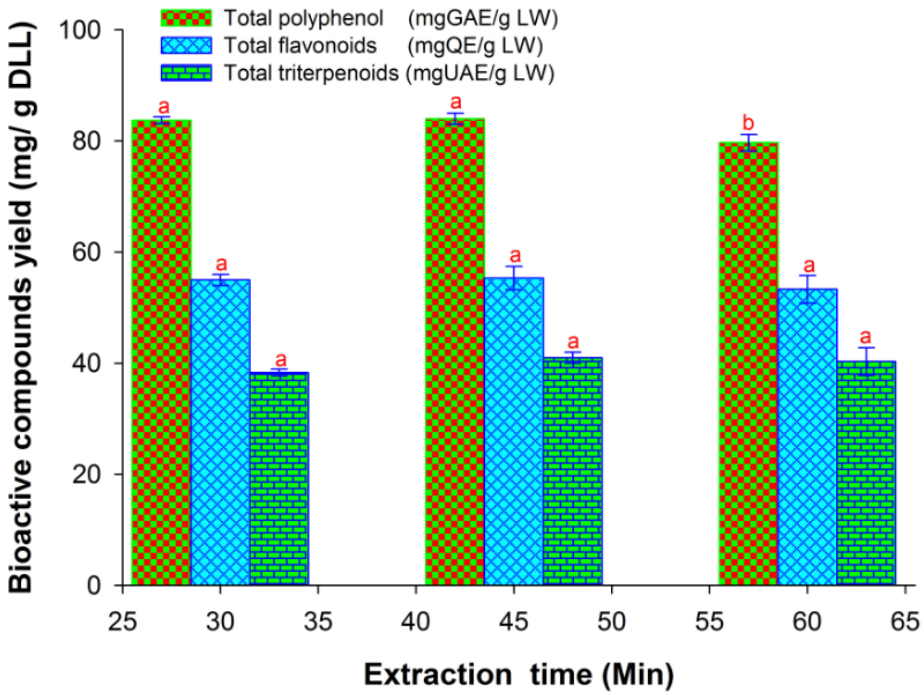

Figure 2. Extraction yield of bioactive compounds obtained from dry loquat leaves with subcritical extraction technique as a function of time. Extraction temperature and pressure were kept constant at 200°C and 10 MPa respectively. GAE, Gallic acid equivalent; QE, quercetin equivalent; UAE, ursolic acid equivalent; LW, leaf weight. Extraction times for similar phytochemical with the same letters are not significantly different according to the Tukey test ($p<0.05$).

were obtained at 200°C and 10 MPa for 45 min, but were not significantly different ($p>0.05$) from the other yields (40.3 ± 2.5 mgUAE/g LW) determined at the same temperature and pressure, but at different extraction time (60 min) as shown in Figure 2. This result is in agreement with the findings reported by other authors on the efficiency of SWE which was demonstrated in the process to obtain bioactive components yield from *Centellaasiatica* (Kim et al., 2009b). In our study, total triterpenoids increased with an increase in temperature (Figure 3) and time but slightly decreased at 60 min likely due to degradation of triterpenoids at high temperature and extended extraction time (Yang et al., 2007).

Characterization and separation of triterpene acids

Results on identification and quantification of triterpene acids are shown in Figure 4. HPLC conditions were optimized to provide reproducible separation triterpenes particularly CA, OA, and UA within a reasonable separation time. The separation of triterpenes was achieved after 16 min under optimal chromatographic conditions, compared to 22.5 and 25.03 min retention time previously reported by other authors (Olszewska, 2008; Xu et al., 2012). This separation time is consistent with the suitability property of fast separation of triterpene acids, which has recently been reported by Lesellier et al. (2012). Under current chromatographic conditions, OA and UA were not completely separated with CE and SE, but were separated at base level with SWE (Figure 4). It was not possible to completely separate OA from UA in loquat leaf extracts obtained by CE and SE. Therefore, only CA was successfully separated from OA/UA for the extract obtained by conventional extraction methods (Figure 4). The separation of OA from UA was not achieved for the extract obtained by CE and SE because these techniques likely were not able to initiate separation of isomeric triterpenes (UA and OA).

Additionally, OA and UA have been previously reported to always exist in the same plant (Lesellier et al., 2012; Olszewska, 2008), and are difficult to separate them completely by LC/MS (Lesellier et al., 2012), but in this study separation of the three triterpene acids was achieved by LC/MS only for the extract obtained by SWE (Figure 4). This separation was possible for the extract obtained by SWE which can be attributed to the fact that this technique probably initiated separation of the isomers at extraction stage as influenced by unique properties of water at higher temperatures. The selective extraction of triterpenes from dry loquat leaves was significantly influenced by SWE extraction mode and parameters (Liu

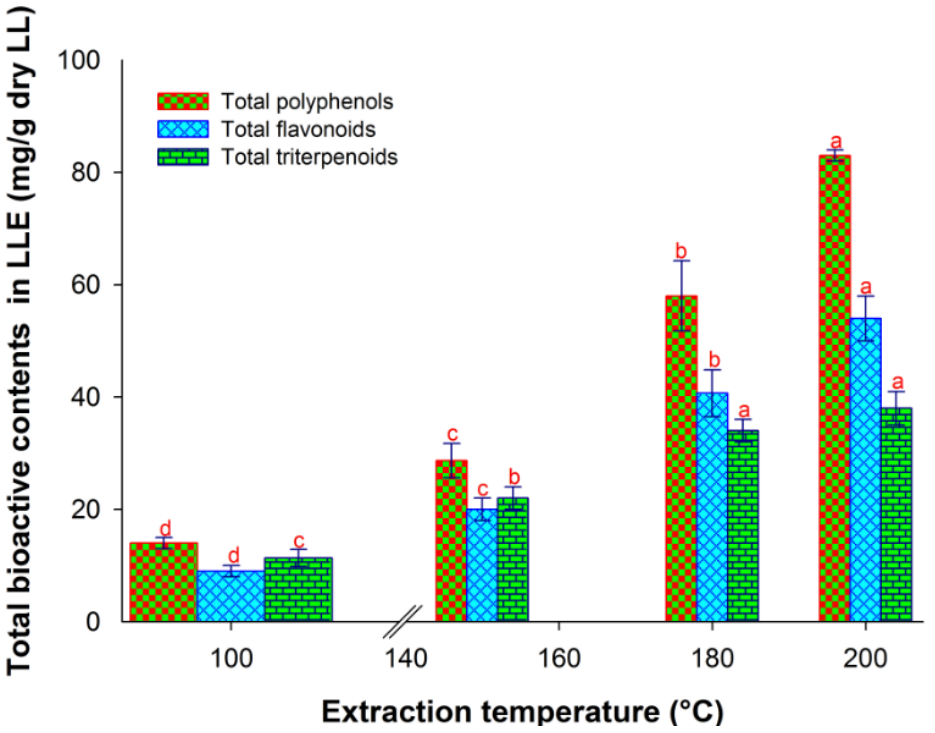

Figure 3. Total bioactive compounds of dry loquat extract by the subcritical water extraction as a function of temperature at 10 MPa and 20 min. LLE, Loquat leaf extract; LL, Loquat leaves. Extraction temperatures for similar phytochemical with the same letters are not significantly different according to the Tukey test ($p<0.05$).

et al., 2015).

The comparison of triterpenes contents of loquat leave extract with other plant materials

There has been a growing interest in triterpenes due to their beneficial health effects, such as anti-inflammatory, anti-diabetic and anti-tumoral (Lesellier et al., 2012, Giménez et al., 2015). They are widely found in more than 1620 plant species and resinous natural materials (Fujiwara et al., 2011; Pollier and Goossens, 2012, Rhourri-Frihet al., 2012). They have also been reported to be found mainly in the bark of trees such as plane, cork, and birch, liquorice roots, but also in the leaves (Rhourri-Frih et al., 2012). Triterpenes contents in extracts can vary due to plant species, geographical origin and extraction techniques used (Hong et al., 2008a; Huang et al., 2013). In our previous study, CA yield was found to be 9.63 ± 0.33 mg/g while UA was found to be 15.04 ± 0.81 mg/g as main PTTs extracted from dry loquat leaves at 180 and 200°C, respectively using static-dynamic mode (Mlyuka et al., 2016). The quantity of UA was higher than the quantity of CA at the same experimental condition and this might be attributed to the fact that dry loquat leaves have higher quantity of UA than CA. Similar results were reported by Olszewska (2008) on corosolic, oleanolic, and ursolicacids in *Prunus serotina* Ehrh. Other authors working on the process for the preparation of high purity corosolic acid and ursolic acid using traditional extraction method also reported higher UA proportional than CA in loquat leaves (Yoshida et al., 2012). In addition, Wei and Yang (2014) reported a 12% higher yield of OA and UA from *Hedyotisdiffusa* obtained by using the hyphenated ultrasound-assisted extraction. Vetalet al. (2012) reported the maximum yield of ursolic acid from *Ocimum sanctum* leaves (16.47 mg UA/g) produced at optimum extraction conditions (extraction time 12 min, solid to solvent ratio 1:30, temperature 45°C and frequency of 25 kHz). The observed differences of triterpenes from different plants source could be attributed to differences in plant species as well as the extraction technique employed. Therefore, extraction techniques have shown to have a significant impact on the yields of triterpenes from different plant sources.

Fourier transform infrared spectrometer analysis of loquat leaf extracts

Loquat leaf extracts obtained by different extraction techniques were further analyzed by FT-IR over a KBr window to compare the IR of the loquat extracted by various extraction techniques (SWE, SE and CE) as

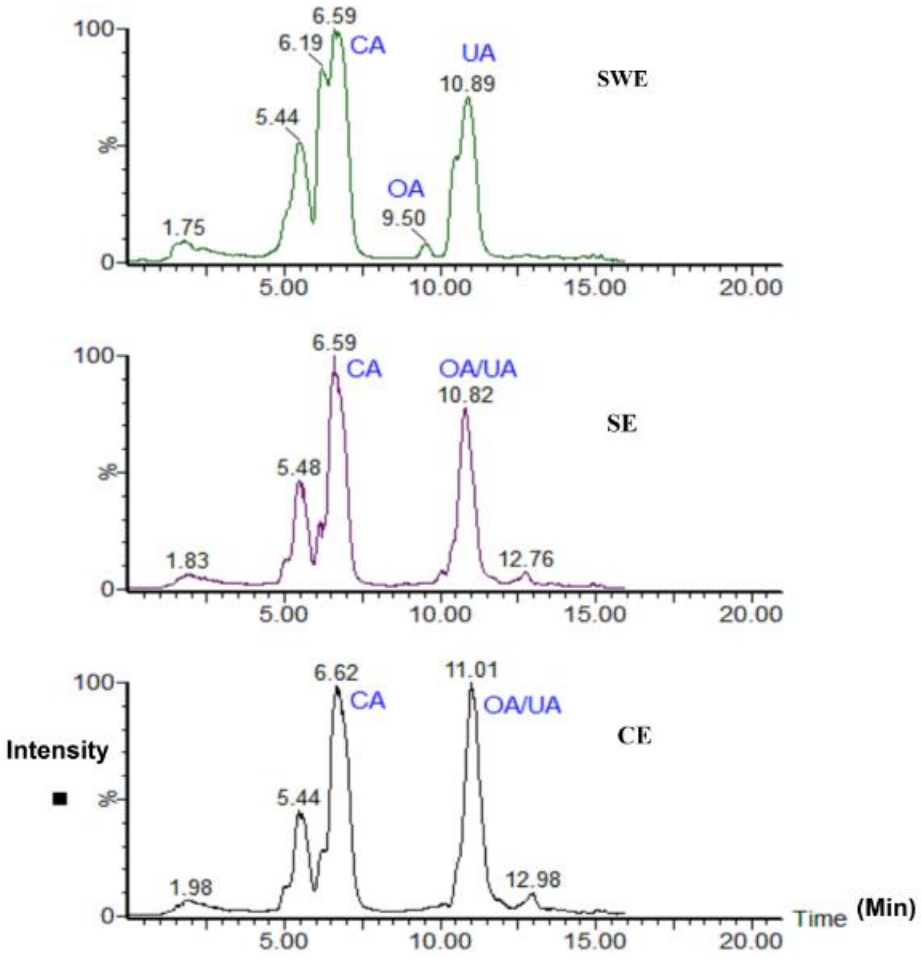

Figure 4. HPLC chromatograms of the triterpene acids from loquat leaf extract. SWE, subcritical water extraction: SE, Soxhlet extraction: CE, conventional extraction technique; CA, corosolic acid; OA, Oleanolic acid; UA, ursolic acid.

shown in Figure 5. The FT-IR employed in this study has emerged as an efficient tool for the characterization of extraction technique - matrix interaction (Lachos-Perez et al., 2015; Liu et al., 2014). The infrared spectra obtained from the three extraction techniques looks identical, but the variation in the intensity of the peak of absorption is visible among the three extraction techniques. The similarity of the peak pattern implies that the compounds in the extract obtained by the three techniques seem identical because their infrared spectra coincided peak for peak (absorption for absorption). Based on these observations, triterpenes could have contributed significantly to the signal recorded by FT-IR as supported by LC/MS results (Figure 4). These FT-IR results helped us to determine structural information about the molecules in the loquat leaf extracts. The absorptions of the double bond in triterpene acids have medium to weak absorption in the range of 1680 to 1600 cm^{-1}, as shown in Figure 5. All the three methods have shown characteristics absorption in this region, but SWE showed relatively stronger absorption when compared to the other extraction techniques employed.

Conclusions

In this study, the efficiency of SWE technique to obtain bioactive compounds yield from dry loquat (*Eriobotrya japonica*) leaves was compared with traditional extraction methods (CE and SE). The crude extracts obtained by both SWE technique and traditional extraction methods (CE and SE) mainly consisted of polyphenols, flavonoids as well as triterpenes. The extraction efficiency of triterpenes using SWE was found to be about 1.7 times higher than those obtained using the traditional extraction methods. In addition, the main structural patterns of the cured extracts obtained by SWE technique were comparable to extracts obtained using the conventional methods and the only extract obtained by SWE, UA and OA were able to separate completely using LC/MS.

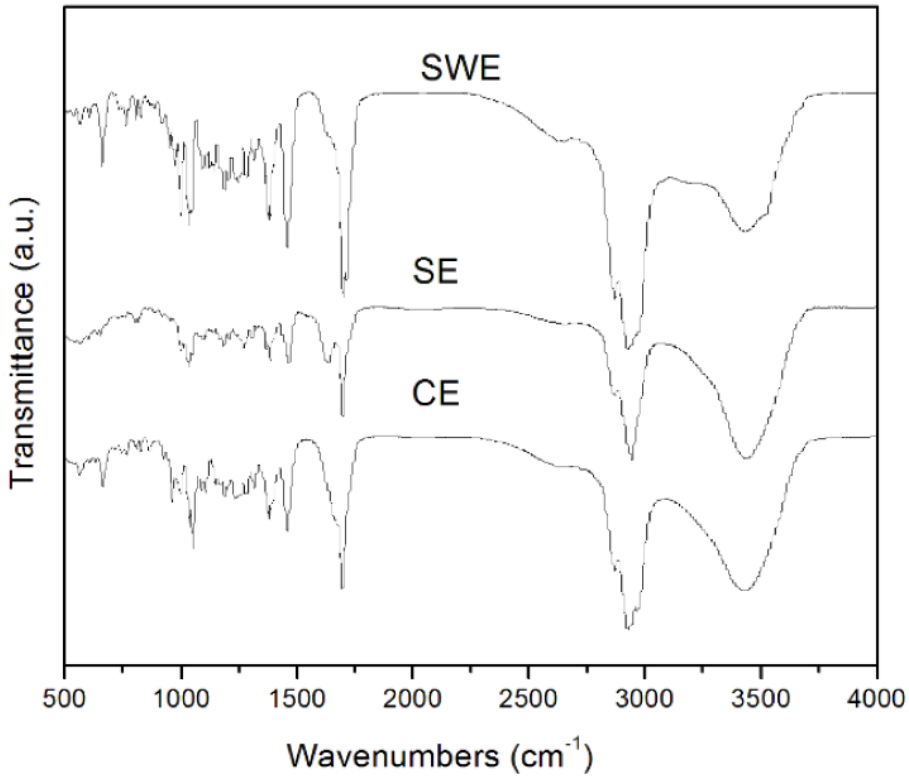

Figure 5. Fourier transform infrared spectra of dry loquat leaf extracts obtained by different extraction techniques. SWE, Subcritical water extraction; SE, soxhlet extraction; CE, conventional extraction.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

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Abbreviations

LLEs, Loquat leaf extracts; **CA,** corosolic acid; **DLL**, dry loquat leaves; **PTTs**, pentacyclic triterpenoids; **SWE**, subcritical water extraction; **CE**, conventional solid-liquid extraction; **UA**, ursolic acid; **LC/MS**, liquid chromatography-mass spectrometry; **SE**, soxhlet extraction; **TPC**, total phenolic contents; **OA**, oleanolic acid; **TFC**, total flavonoid contents; **FT-IR**, fourier transform infrared spectrometer.

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