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ORIGINAL ARTICLE

Preparation of Diterpenes-Enriched Extract from *Croton stellatopilosus* Ohba Leaves using Enzyme- and Ultrasonic-Assisted Extraction

Wai Mi Aung, Supattra Limsuwanchote[1], Juraithip Wungsintaweekul

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, [1]Department of Pharmacology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand

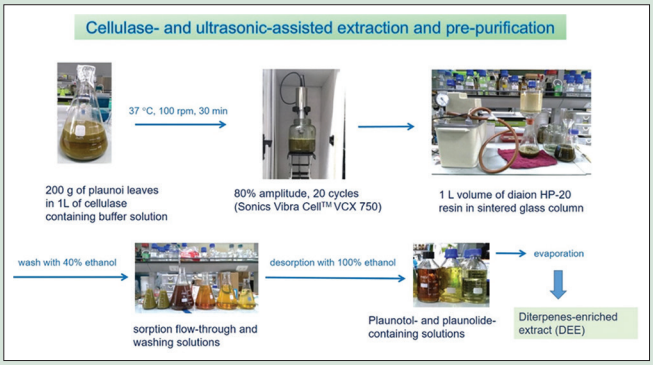
ABSTRACT

Background: *Croton stellatopilosus* Ohba accumulates several diterpenes. Among them, plaunotol, a cytoprotective agent, has been prescribed to treat peptic ulcers. **Objectives:** We aimed to prepare the diterpene-enriched extract (DEE) using enzyme- and ultrasonic-assisted extractions. **Materials and Methods:** The type and amount of pectinase and cellulase and the condition of ultrasonic treatment were optimized. A combination of enzyme and ultrasonic treatments was performed. The high-performance liquid chromatography (HPLC) method was validated and used for the determination of plaunotol and plaunolide contents in the DEE. **Results:** A combination of cellulase (10 units) and ultrasonic (at 80% amplitude) treatments enhanced the yield of plaunotol significantly. Application of the crude extract on diaion HP-20 resin afforded the DEE. With the validated HPLC method, the DEE contained 8.81% ± 0.02% w/w of plaunotol and 8.40% ± 0.92% w/w of plaunolide. **Conclusion:** We established an efficient method to prepare DEE based on green extraction. This method showed about eightfold enhancement purification factor compared to the original crude extract and about 90% yield recovery.

Key words: Cellulase-assisted, *Croton stellatopilosus,* diterpenes-enriched extract, plaunolide, plaunotol, ultrasonic-assisted

SUMMARY

- We applied a green extraction process to extract two active diterpenes. The risk of toxic solvent traces remaining in the extract had been mitigated and the environmental harassment was avoided. The present study suggested an alternative method of preparation of diterpene-enriched extract (DEE) from *Croton stellatopilosus* leaves. Possibly, all procedures can apply to the industrial scale in a cheaper and environmentally-friendly manner. Moreover, the DEE can safely be used further in pharmaceutical and cosmetic purposes.



Abbreviations Used: DEE: Diterpenes-enriched extract; HPLC: High-performance liquid chromatography; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; SEM: Scanning electron microscope.

Correspondence:
Dr. Juraithip Wungsintaweekul,
Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
E-mail: juraithip.w@psu.ac.th
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INTRODUCTION

Croton stellatopilosus Ohba (*Euphorbiaceae*) is a Thai indigenous plant that is locally known as plaunoi. This plant comprises several terpenoid compounds such as acyclic diterpene (plaunotol) and furanoditerpenes (plaunol A, B, C, D, and E and plaunolide).[1-4] Plaunotol and plaunolide [Figure 1] are the prominent diterpenes in the *C. stellatopilosus* leaves. The patented plaunotol has been launched in the pharmaceutical market as Kelnac®, indicated as an antipeptic ulcer agent.[5,6] Plaunotol has been approved for several pharmacological activities such as eradication against *Helicobacter pylori*,[7] antibacterial activity,[8-12] wound healing,[13] and anti-inflammatory activity.[14] Unlike plaunotol, plaunolide had only a report for anti-inflammatory activity.[14] Both diterpenes are promising to be functional ingredients for further pharmaceutical applications.

High-quality natural products have witnessed a growing demand in the markets in recent years. Therefore, herbal drug production shifts the trend toward the preparation of active compounds based on safe and environmentally-friendly strategies. Meanwhile, the implementation of sustainable development or the concept of green chemistry becomes an urgent need for pharmaceutical products.[15,16] Natural materials such as leaves, barks, or roots should be kept in the proper system to preserve the active biomaterials. The particle size of the raw materials is essential to get enough contact surfaces with the solvents.[17] Energy consumption in the extraction process can be minimized by applying assisted extraction methods, including ultrasonic- and microwave-assisted extraction.[18] Many reports demonstrated that ultrasonic and microwave treatments could prepare the extract faster and easier.[19-22] Most researchers apply the mechanical process with enzymes to hydrolyze plant cells because of its ability to increase cell wall permeability.[23]

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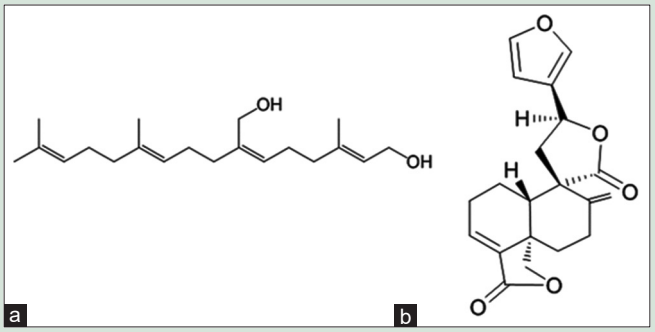

Figure 1: Chemical structures of (a) plaunotol and (b) plaunolide

Earlier, the isolation procedure of plaunotol from *C. stellatopilosus* leaves and stems was reported. Many purification steps have included hot extraction with methanol, removal of chlorophyll with activated charcoal, and partition with either chloroform, *n*-hexane, or diethyl ether to afford the pure plaunotol.[24-26] The mentioned solvents cannot be used in green extraction strategies.[27] Extraction with green solvents in the preparation of partially purified extract of herbal products is strongly needed, according to the requirement of the Food and Drug Administration.

To expand the utilization of *C. stellatopilosus* extract for pharmaceutical purposes, we aimed to prepare the diterpenes-enriched extract (DEE) of *C. stellatopilosus* leaves. Although most of the diterpenes are soluble in organic solvents such as acetone and hexane, we tried to treat plant leaves with enzymes and consequently extract with ethanol and ultrasonic treatment. Treatment of the extract with nonionic resin was performed to obtain the enriched extract. The established high-performance liquid chromatography (HPLC) was validated for simultaneous determinations of plaunotol and plaunolide contents.

MATERIALS AND METHODS

Materials

Tipco Biotech Company, Thailand, kindly provided dried *C. stellatopilosus* leaf powder and the hexane extract. Cellulase (E.C. 3.2.1.4; 1.14 unit/mg) from *Aspergillus niger* was purchased from Sigma Life Science (Hokkaido, Japan). Pectinase (E.C. 3.2.1.15; 1.10 units/mg) from *A. niger* was purchased from Sigma Life Science (Søborg, Denmark). Citric acid and sodium citrate (trisodium salt, dehydrate) were purchased from Bio Basic Canada Inc. (Ontario, Canada). The solvents applied in the isolation of plaunotol and plaunolide were analytical reagent grade and general purpose reagent grade, respectively. An HPLC grade acetonitrile was purchased from Lab-Scan Asia Co. Ltd (Bangkok, Thailand).

Isolation of plaunotol and plaunolide

Plaunotol was isolated from the hexane extract on silica gel column chromatography. The column (7 cm in diameter) was packed with 460 g of silica gel (SiliaFlash P60, Silicycle, Quebec, Canada). Twenty grams of the hexane extract was reconstituted with 10 mL of the mobile phase and loaded onto the packed column. The column was eluted with chloroform: *n*-propanol; 24:0.5. The fractions containing plaunotol were pooled and evaporated. The yield of plaunotol was about 30% w/w of the hexane extract. ^{1}H-NMR (Unity Inova, Varian, Germany) was performed and the purity of plaunotol was about 95%.

Plaunolide was isolated, as described previously, with modification.[14] Briefly, leaf powder (0.8 kg) was macerated with 3 L of dichloromethane three times. The filtrates were pooled and evaporated to dryness. The crude extract was loaded on top of the silica gel column (7 cm × 31 cm). The column was eluted with dichloromethane: ethyl acetate gradient from 9:1 to 2:8, followed with ethyl acetate: methanol gradient from 7:3 to 5:5. Fractions were collected and pooled as guided by thin-layer chromatography (TLC). Fractions containing plaunolide were pooled and re-crystallized with a mixture of dichloromethane and hexane, affording a white amorphous solid. The yield of plaunolide was about 7.5% w/w of the leaf powder's dry weight. ^{1}H-NMR (Unity Inova, Varian®, Germany) was performed and the purity of plaunolide was about 95%.

Extraction procedures

Reflux method

Two grams of leaf powder was refluxed in 10 mL of ethanol at 80°C for 1 h and filtered. The marc was rinsed with 10 mL of ethanol three times, and the filtrates were pooled and evaporated to dryness.

Enzyme-assisted extraction

Stock solutions of cellulase and pectinase at 25 units/mL were prepared in 50 mM citrate buffer, pH 4.8 and the unit of the enzyme was adjusted by dilution with citrate buffer. There were six groups of treatments, comprising various unit ratios of cellulase and pectinase. The enzyme solution (20 mL) was added into two grams of leaf powder and incubated on incubator shaking at 100 rpm at 37°C for 30 min. Then, the mixture was centrifuged at 3000 rpm for 5 min and the supernatant was discarded. The marc was rinsed with 10 mL of ethanol three times, and the filtrates were pooled and evaporated to dryness.

Ultrasonic-assisted extraction

Two grams of leaf powder was suspended in 10 mL of ethanol. The ultrasonic processor (Sonics Vibra Cell™ VCX750, Sydney, Australia) was set at an amplitude of 80%, and the ultrasonic treatment was performed for 20 cycles during each period was composed of a run for 1 min and a pause for 1 min. The extract was filtered, the marc was rinsed with 10 mL of ethanol three times, and the filtrates were pooled and evaporated to dryness.

Enzyme- and ultrasonic-assisted extraction

There were six groups of combinations of enzyme and ultrasonic treatments. Two grams of leaf powder was treated with an enzyme solution at the unit in the total volume of 20 mL. After incubation at 37°C for 30 min, the mixture was centrifuged and the supernatant was discarded. 10 mL of ethanol was added to the marc and treated with ultrasonication for 20 cycles of 80% amplitude. Ethanol fraction was filtered out and rinsed the marc with 10 mL of ethanol three times, and the filtrates were pooled and evaporated to dryness.

Preparation of diterpenes-enriched extract

The DEE was prepared using enzyme- and ultrasonic-assisted extraction at the optimized condition. Cellulase solution (100 units of cellulase in 1 L of 50 mM citrate buffer, pH 4.8) was added to the leave powder (200 g) and mixed. The mixture was placed on the shaking incubator (100 rpm) and incubated at 37°C for 30 min. The enzyme solution was removed through the filter paper. Then, 1 L of ethanol was added to the marc, mixed, and treated with ultrasonication for 20 cycles of 80% amplitude. The mixture was filtered and the filtrate was collected. The marc was rinsed with 1 L of ethanol two times, and the filtrates were pooled. All eluates were combined and evaporated to dryness, affording the ethanolic crude extract.

Diaion HP-20 resin (Mitsubishi, Japan) was cleaned with ethanol and regenerated by soaking in 40% v/v ethanol in water overnight. The 1 L of regenerated resin was packed into 1 L capacity sintered glass funnel, connected with a vacuum pump. The crude extract was reconstituted with 1 L of 40% v/v ethanol and loaded onto the resin. The column was

pumped and the flow-through was discarded. The column was washed with 1 L of 40% v/v ethanol for 8 times to remove the impurity and the vacuum pump dried the resin. The column was eluted with 1 L of ethanol for four times for the desorption process, and the ethanol fractions were pooled and evaporated to dryness.

Scanning electron microscope

The leaf powder's surface morphology was examined by scanning electron microscope (Quanta 400® FEI [Czech Republic]) using the Everhart-Thornley detector. The sample was coated with gold particles, and the magnification powers were observed and captured.

Validation of the high-performance liquid chromatography method

The HPLC method was validated according to the ICH guideline Q2 (R1).[28] The chromatographic condition A was established for the quantification of plaunotol, while chromatographic condition B was established for plaunotol and plaunolide simultaneously. In this study, the HPLC systems were Agilent 1100 series and Shimadzu Prominence *i* HPLC (2030C 3D) attached with a photodiode array detector. The column was VertiSep™ UPS C_{18} HPLC column (4.6 mm × 250 mm, 5 µm particle size), the injection volume was 20 µL, the flow rate was 1 mL/min, and the wavelength was set at 220 nm.

Specificity

The peak purity was checked using the diode array detector. Peak purity higher than 0.99 showed that the analytical procedure could discriminate between the target diterpenes and the impurities.

Linearity and range

The concentrations for plaunotol and plaunolide were prepared in the range of 7.8–250 µg/mL. Six injections from each level were analyzed. The linearity of the calibration curve was calculated by using the least square linear regression method. The correlation coefficient (R^2) should have been higher than 0.999.

Precision

The intraday precision was determined by injecting the standard concentration of 125 µg/mL (n = 6) on the same day. For interday precisions, three different standard concentrations (31.25, 62.5, and 125 µg/mL, n = 3) were injected into the HPLC system at 3-day interval. The intraday and interday precision results were expressed as a percentage of the relative standard deviation.

Accuracy

The percentage of the analyte recovered by the analysis was reported as accuracy. The known three different concentrations of the standard solutions (15.6, 31.25, and 62.5 µg/mL) were spiked into the samples. The area under the curve was recorded and used to calculate percentage recovery (n = 3).

Limit of detection and limit of quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) of plaunotol and plaunolide were determined. The LOD was performed to show that the detected lowest level of analytes was reliable. The LOQ was determined to confirm that the quantitated concentrations of analytes were acceptable for accuracy and precision. This study used a signal-to-noise (S/N) approach, and the calculation of the LOD was 3:1 of S/N ratio of analytes and that of the LOQ was 10:1.

Statistical analysis

Data were expressed as mean ± standard deviation. Software SPSS Ver 17.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The significant differences among mean values were analyzed with Duncan's multiple range test (DMRT) by setting the P value at 0.05.

RESULTS

This study aimed to explore the green process for the preparation of DEE. Enzyme- and ultrasonic-assisted extraction, together with a refinery on the hydrophobic resin, was investigated as the target approach. The herein validated method was used to determine the plaunotol and plaunolide contents.

Optimization of enzyme- and ultrasonic-assisted extraction

The enzyme- and ultrasonic-assisted extraction method was used to extract the ingredients from plant cells. The digestive enzymes were utilized to remove the plant cell walls and release the active components after the ultrasonic-assisted extraction. Table 1 summarizes the effect of the enzyme and ultrasonic treatments on plaunotol extraction compared with the maceration and reflux methods. The data show that cellulase exhibited similar potency to pectinase and that the plaunotol content did not differ from the combined cellulase and pectinase. In contrast, the ultrasonic-assisted group, set at 80% amplitude for 20 cycles, could extract a significantly higher yield of plaunotol than the enzyme-assisted group. Besides, the ultrasonic treatment has a more substantial effect on the plaunotol extraction compared to the reflux at 80°C for 1 h ($P < 0.05$). For the groups that employed the combination of cellulase- and ultrasonic-assisted extraction, the plaunotol content was in the same subset group according to the DMRT analysis by a P value at 0.05. Compared to the combination group, pectinase- and ultrasonic-assisted extraction released plaunotol with a lower yield compared to cellulase- and ultrasonic-treatment groups. Hence, the cellulase and ultrasonic treatment were applied for the extraction of diterpenes from *C. stellatopilosus* leaves.

Table 1: Plaunotol content in the extracts obtained from different extraction methods

Methods	Percentage w/w of plaunotol (mean±SD, n=3)
Enzyme-assisted extraction	
Cellulase (10 units)	0.147±0.006[a]
Pectinase (10 units)	0.141±0.003[a]
Cellulase: pectinase (10:10 units)	0.150±0.003[a]
Cellulase: pectinase (10:20 units)	0.159±0.003[a]
Cellulase: pectinase (20:10 units)	0.156±0.003[a]
Cellulase: pectinase (20:20 units)	0.163±0.003[a]
Ultrasonic-assisted extraction	0.386±0.011[c]
Enzyme-and ultrasonic-assisted extraction	
Cellulase (10 units)	0.512±0.029[d]
Pectinase (10 units)	0.415±0.010[e]
Cellulase: pectinase (10:10 units)	0.502±0.022[d]
Cellulase: pectinase (10:20 units)	0.508±0.013[d]
Cellulase: pectinase (20:10 units)	0.502±0.018[d]
Cellulase: pectinase (20:20 units)	0.510±0.018[d]
Reflux extraction: 80°C for 1 (h)	0.341±0.005[b]

n=Number of samples; Plaunotol content was determined by HPLC condition A; percentage w/w was calculated based on the dry weight of leaf powder. Plaunotol contents (percentage w/w, mean±SD) followed by the same superscript letters are not significantly different according to DMRT analysis by the setting of the P at 0.05. HPLC: High-performance liquid chromatography; DMRT: Duncan's multiple range test; SD: Standard devition

As shown in Table 1, the cellulase- and ultrasonic-assisted extraction resulted in a higher yield of plaunotol than conventional reflux extraction. The morphological changes of the leaf powder from the various extraction procedures were assessed. The results indicated that the leaf powder's particle sizes in the ultrasonic extraction method were relatively smaller than those of the groups using the enzyme-assisted method alone [Figure 2]. It seems that sound waves reduced the powder's particle size, making it easier for the solvent to diffuse and penetrate the plant cells in this extraction method. The cellulase- and ultrasonic-assisted extraction showed the smallest particle size (<200 μm) among the various extraction methods.

Diterpenes-enriched extract of *Croton stellatopilosus*

The ethanolic crude extract was scaled up using the optimized conditions of the cellulase and ultrasonic treatment. Hydrophobic resins were chosen to enhance yield in the DEE, such as XAD-4 and diaion HP-20. After optimizing the type of resin and adsorption–desorption conditions, the diaion HP-20 was selected (data not shown). The diaion HP-20 was washed with ethanol before equilibration with soaking in 40% v/v ethanol in water and packed into the sintered glass funnel. The ethanol crude extract was then loaded on top, following the washing with 40% v/v ethanol, ultimately removing the impurities. After elution with ethanol, the filtrate was collected and evaporated to dryness.

Table 2 summarizes the amount of plaunotol and plaunolide contents in the crude extract and the DEE. The results showed that using diaion HP-20 for partial purification could increase diterpenes yield by about eightfold with a recovery of approximately 90%. The total percentages of plaunotol and plaunolide were enhanced from 2.32% w/w in the crude extracts to 17.21% w/w in the DEE by this procedure. The diaion HP-20 removed chlorophyll and improved the DEE's appearance, which is acceptable for the application.

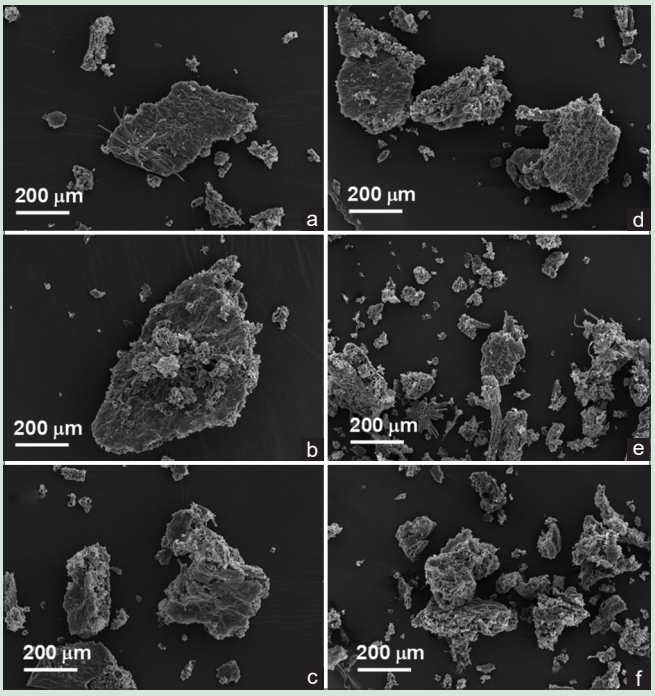

Figure 2: Scanning electron microscope images of plaunoi leaf powder after treatments. (a) with pectinase; (b) with cellulase; (c) with a combination of cellulase and pectinase; (d) with pectinase and ultrasonic treatments; (e) with cellulase and ultrasonic treatments; (f) with pectinase, cellulase, and ultrasonic treatments

Validation of high-performance liquid chromatography method for the quantifications of plaunotol and plaunolide

In the present study, we established the HPLC system connected with a UV detector and validated it to quantify plaunotol and plaunolide contents. The elution profiles of the mobile phase were isocratic for condition A (Agilent) and a linear gradient for condition B (Shimadzu) [Table 3].

Plaunotol was eluted by chromatographic condition A at 6.4 min.

Table 2: Diterpenes in diterpene-enriched extract after diaion HP-20 treatment

Diterpenes	Amount (w/w)		Percentage relative recovery
	In crude extract	In DEE	
Plaunotol	1.11±0.18	8.81±0.02	93.31±3.32
Plaunolide	1.22±0.14	8.40±0.92	89.93±10.38
Total diterpenes	2.32	17.21	

Percentage (w/w) was defined based on the percentage of plaunotol or plaunolide in the crude extract and the diterpenes-enriched extract. Data were expressed in mean±SD. The samples were performed in triplicates. DEE: Diterpene-enriched extract; SD: Standard deviation

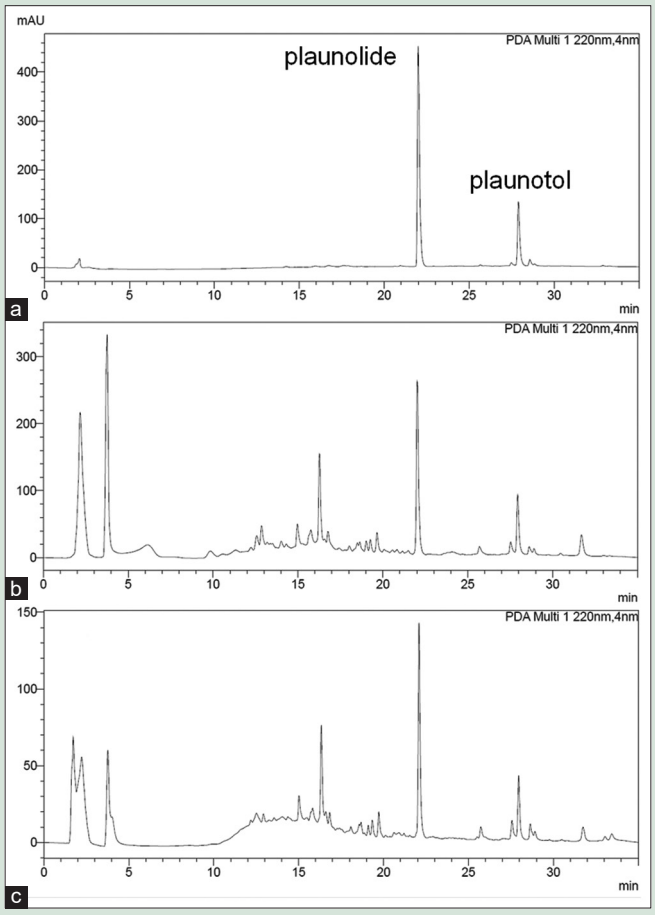

Figure 3: High-performance liquid chromatography-ultraviolet chromatograms using condition B. (a) authentic plaunotol and plaunolide; (b) ethanol extract obtained from cellulase- and ultrasonic-assisted extraction; (c) diterpenes-enriched extract after diaion HP-20 treatment

Table 3: High-performance liquid chromatography-ultraviolet method validations for quantification of plaunotol and plaunolide contents

Validation parameters	Results		
	Condition A (Agilent)[a] Plaunotol	Condition B (Shimadzu)[b]	
		Plaunotol	Plaunolide
Linearity			
y = ax + b linear model	y = 12.258x + 178.42	y = 12550x + 13478	y = 38775x + 55717
The correlation coefficient (R^2)	0.9991	0.9992	0.9994
Range (μg/mL)	7.8-125	7.8-125	7.8-125
Precision (% RSD)			
Intraday precision (125 μg/mL, *n*=6)	1.34	0.78	0.57
Interday precisions			
31.25 μg/mL (*n*=3)	0.90	0.99	0.09
62.5 μg/mL (*n*=3)	1.14	1.17	0.04
125 μg/mL (*n*=3)	1.24	1.78	0.59
Accuracy (percent recovery)			
31.25 μg/mL (*n*=3)	100.04%±0.98%	99.78%±0.43%	96.23%±1.02%
62.5 μg/mL (*n*=3)	99.99%±1.54%	96.32%±0.14%	98.56%±1.39%
125 μg/mL (*n*=3)	101.07%±2.06%	95.85%±0.79%	95.97%±0.93%
LOD (μg/mL)	0.90	1.95	0.48
LOQ (μg/mL)	1.90	3.9	1.95
Total run time	10 (min); isocratic elution	35 (min); linear gradient	

[a]Agilent 1100 series was equipped with DAD G1315B detector and analyzed with Agilent Chemstation; [b]Shimadzu prominence *i* 2030C was equipped with built-in detector and analyzed with LabSolutions software. *n*=Number of samples; RSD: Relative standard deviation; LOD: Limit of detection; LOQ: Limit of quantitation; DAD: Diode array detector

Linearity (R^2 > 0.999) was in the range of 7.8–125 μg/mL of plaunotol. Table 3 summarizes the validation parameters of the chromatographic condition A. Besides, we tried to find the HPLC system for the simultaneous determination of plaunotol and plaunolide. By the chromatographic condition B, the retention times of plaunolide and plaunotol were 22 and 28 min, respectively [Figure 3]. The validated HPLC-UV methods of chromatographic conditions A and B are in agreement, according to the ICH guidelines 2005.[28] These HPLC conditions were used for monitoring plaunolide and plaunotol contents during extraction as appropriate.

DISCUSSION

In the present study, we focus on the green extraction and preparation of the enriched fraction of plaunotol and plaunolide in the DEE. The medicinal relevance of plaunotol and *C. stellatopilosus* extract and the broad spectrum of their biological activities make them widely applied in pharmaceutical products. The HPLC condition was established and validated for the first time in this study. Formerly, plaunotol was quantified based on gas chromatography-flame ionization detector (GC-FID) and thin layer chromatography (TLC)-densitometric method, which cannot apply for plaunolide determination.[29-31]

For the green extraction, ethanol, a bio-solvent, could extract plaunotol from the *C. stellatopilosus* leaves, which allows avoiding the toxic organic residues in the extraction step. Enzyme-assisted extraction is an eco-friendly extraction method. Cellulase can break down beta-1,4-linkages of cellulose in plant primary cell walls. Pectinase acts on pectic compounds in the middle lamella of the primary cell walls.[32] The ultrasonic-assisted extraction process has two enhanced physical effects. First, the solvent passes through the cell walls, and then, the cell contents are washed out.[32] Plaunotol is mainly located in the upper palisade cells of *C. stellatopilosus* leaf.[33] Therefore, the cellulase was suitable for pretreatment in plaunotol extraction for breaking down the cell walls of the *C. stellatopilosus* leaves. Plaunotol was readily extracted with ultrasonication by diffusing the solvent mechanically into the cells and by washing the components of the cell. The combined use of cellulase- and ultrasonic-assisted extraction showed a higher plaunotol yield percentage. Our findings are also in line with previous reports. In their researches, the utilization of carbohydrase enzymes improved the extraction of polyphenol contained in green yerba mate from 38.67% to 52.08%.[34] Pudziuvelyte *et al*. reported the ultrasonic-assisted power to extract 13 phenolic compounds and six volatile compounds from *Elsholtzia ciliata*.[35]

In this study, we successfully used a green extraction technology to prepare the DEE. The effectiveness of diaion HP-20 for the prepurification of diterpenes has been demonstrated. Methanolic extract of *Vitex rotundifolia* was loaded onto a diaion HP-20 resin and eluted with a step gradient of methanol and acetone. It afforded the eight labdane-type diterpenes and two abietane-type diterpenoids.[36] The diaion HP-20 resin was successfully used to remove chlorophylls and some pigments from the extract of *Taxus baccata* L. Then, this partially purified mixture was again refined using silica-based solid-phase extraction. After two purification steps had been performed, paclitaxel and 10-deacetylbaccatin III were achieved at 8 and 3 times more compared to their traditional extraction methods.[37] All the findings observed herein support that the combination method of cellulase- and ultrasonic-assisted extraction increased the yield of plaunotol and plaunolide and resulted in the enriched extract. Likewise, the utilization of diaion HP-20 resin enhanced the plaunotol and plaunolide contents in the DEE.

CONCLUSION

The present study chose the green extraction method to prepare the diterpene-enriched extract (DEE) as a safe extraction method for consumers and the environment. According to the plaunotol contents, the combination of cellulase and ultrasonic-assisted extraction methods was the most appropriate method for the extraction. The high-performance liquid chromatographic method was validated for the determination of plaunotol and plaunolide contents simultaneously. In summary, the green extraction method could prepare the crude extract and subsequently pre-purified by resin to obtain the DEE. The scaling-up may require further optimization steps for amounts of needed enzyme and resin selection.

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Conflicts of interest

There are no conflicts of interest.

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