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Research Article

Enhanced Antifungal Bioactivity of Coptis Rhizome Prepared by Ultrafining Technology

Ping-Chung Kuo,[1] Yao-Tung Lin,[2] Mei-Lin Yang,[3] Ming-Yuan Liao,[3] Guo-Feng Chen,[3] Po-Hsun Chen,[1] Lin-Fu Wong,[1] and Jenn-Wen Huang[4]

[1] *Department of Biotechnology, National Formosa University, Yunlin 632, Taiwan*
[2] *Department of Soil & Environmental Sciences and Nanoscience & Nanotechnology/Nano-Material Measurement Lab, National Chung Hsing University, Taichung 402, Taiwan*
[3] *Department of Chemistry, National Chung Hsing University, Taichung 402, Taiwan*
[4] *Department of Plant Pathology, National Chung Hsing University, Taichung 402, Taiwan*

Correspondence should be addressed to Ping-Chung Kuo; pcckuoo@sunws.nfu.edu.tw and Jenn-Wen Huang; jwhuang@dragon.nchu.edu.tw

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The aim of this study was to identify and quantify the bioactive constituents in the methanol extracts of Coptis Rhizome prepared by ultrafining technology. The indicator compound was identified by spectroscopic method and its purity was determined by HPLC. Moreover, the crude extracts and indicator compound were examined for their ability to inhibit the growth of *Rhizoctonia solani* Kühn AG-4 on potato dextrose agar plates. The indicator compound is a potential candidate as a new plant derived pesticide to control *Rhizoctonia* damping-off in vegetable seedlings. In addition, the extracts of Coptis Rhizome prepared by ultrafining technology displayed higher contents of indicator compound; they not only improve their bioactivity but also reduce the amount of the pharmaceuticals required and, thereby, decrease the environmental degradation associated with the harvesting of the raw products.

1. Introduction

Rhizoctonia solani Kühn causes serious damping-off disease in numerous crops in Taiwan; in particular, it is a major problem for the commercial production of vegetable seedlings grown in cell-plug systems [1, 2]. In order to control this pathogen effectively and to reduce the risk of development of resistance with chemical fungicides, more extensive studies related to biological agents were performed. It has been reported that the aqueous extracts of *Coptis chinensis* (goldthread), *Polygonum cuspidatum* (Japanese knotweed), *Cinnamomum cassia* (cinnamon), *Rheum officinale* (Chinese rhubarb), *Polygonum multiflorum*, and *Eugenia caryophyllata* (clove) showed inhibitory effects on conidial germination of *Oidium murrayae* [3]. For the purpose of discovering new plant derived fungicides for controlling *Rhizoctonia* damping-off in cabbage seedlings, the methanol extract of Coptis Rhizome was selected as the target. Herein we wish to explore the bioactive principles as well as determine the antifungal activities of the natural compound.

Recently, interests in the application of ultrafining technology to preparation of traditional Chinese medicine are increased [4–9]. Most of the important raw plant materials used are cultivated in the regions such as the Inner Mongolian grassland. The large-scale harvesting of the raw materials causes more and more serious environmental problems, such as dust storms. Therefore, the application of ultrafining technology to commonly used traditional Chinese medicine plant materials may improve their bioactivity and also reduce the amount of the plant materials required. It would decrease the environmental degradation associated with the harvesting of the raw products effectively. Thus, in the present study, the

powder of roots and stems of *C. chinensis* (Coptis Rhizome) prepared using ultrafining technology and traditional grinding methods were evaluated to compare the contents of the indicator compound.

2. Materials and Methods

2.1. General Procedure. All the solvents including the HPLC-grade methanol were purchased from Merck KGaA (Darmstadt, Germany). The chemical structure of the indicator compound was identified with the comparison of their spectroscopic and physical data reported in the literature. Plant materials were extracted using a Major Science LM-570R shaking incubator. High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-20AT series pumping system equipped with a Shimadzu SPD-20A UV-Vis detector, an Agilent HC-C8 column (4.6 mm × 250 mm, 5 μm), and a SIL-10AF autosampling system at ambient temperature. The particle size distributions were determined by dynamic light scattering (DLS, ZS-90, Malvern).

2.2. Fungal Pathogen and Plant Materials. Rhizoctonia solani AG-4 isolate RST-04 was isolated from a seedling of Chinese kale (*Brassica alboglabra* Bailey) showing damping-off symptoms. The culture of *R. solani* AG-4 RST-04 was grown on agar (20 g of agar in 1 L of distilled water) slant. Dry powders of roots and stems of *C. chinensis* (Coptis Rhizome) prepared by either traditional grinding or ultrafining technology were generously provided by Diamond Nano-Biochem Company and the voucher specimens were deposited in the herbarium of the Department of Biotechnology, National Formosa University, Yunlin, Taiwan.

2.3. Characterization of Samples. The Brunauer-Emmett-Teller (BET) specific surface area (S_{BET}) of the samples was evaluated on the basis of nitrogen adsorption isotherms measured on a Micromeritics ASAP 2020 adsorption apparatus. All samples were degassed at 350 K for 6 h prior to nitrogen adsorption measurements at 77 K. The BET specific surface area was determined by a multipoint BET method using adsorption data in the relative pressure (P/P_0) range of 0.05–0.3. The surface morphologies and microstructures of the samples were analyzed by scanning electron microscope (SEM, JEOL JSM-6700F, Japan).

2.4. Extraction and Purification. The stems and roots of *C. chinensis* (1.8 Kg) were extracted with methanol under reflux (8 L × 8 × 8 h), and the crude extracts were concentrated *in vacuo* to give a brown syrup (570 g). The crude extract was acidified to afford the precipitates. The precipitates were dissolved in methanol and applied to a silica gel column and then eluted with chloroform and step gradient of methanol (10 : 1 to 1 : 1, v/v) to yield 9 fractions. Fraction 4 was further subjected to silica gel column chromatography eluted with chloroform and acetone (4 : 1, v/v) and recrystallized with chloroform-methanol to yield berberine chloride (5.0 g).

2.5. Sample Preparation for HPLC Analysis. Coptis Rhizome powder (1.0 g) prepared by traditional grinding or ultrafining technology was extracted with 10 mL methanol in a 100 mL flask, respectively. The samples were taken in a shaking incubator at temperature of 25°C and 150 rpm for 10, 30, 50, 70, or 90 min contact time period. The final volume was diluted to 50 mL after the solution was filtered and kept at 4°C temperature for the further analysis. The sample solutions were filtered through a membrane (0.45 μm) before their injection into a HPLC to quantify the bioactive constituent according to the following procedures.

2.6. Chromatography. HPLC analysis for berberine chloride in the methanol extracts of Coptis Rhizome was described according to the reported method [10]. The column was eluted with 0.5 mL/min of two solvent compositions, acetonitrile (solvent A) and potassium dihydrogen phosphate buffer solution (solvent B), and was detected by ultraviolet-visible spectroscopy (UV, Shimadzu, Japan) at 254 nm for berberine chloride (R_t, 26.40 min).

2.7. Method Validation. The reproducibility and precision were measured by repeatedly injecting a ready-made sample pool and expressed as the relative standard deviation of the results. Analyses with three different concentrations of berberine chloride were performed. To determine the intraday variance, the assays were carried out on the same samples at different times during 1 day. Interday variance was determined by assaying the spiked samples over three consecutive days at the same time each day. The limit of detection (LOD) was determined as the lowest concentration that could be detected with acceptable accuracy and precision, which was achieved from the plot three times above the noise level. The recovery from the samples was evaluated using three different concentrations of berberine chloride covering the linear range of the standard curve. After the samples were processed according to the methods mentioned earlier, the resulting peak heights were compared to the indicator compounds carried in mobile phase to provide the recovery values.

2.8. Assay of Antifungal Activity. The methanol extract of Coptis Rhizome and purified berberine chloride was dissolved in distilled water. The assay of the antifungal activity against *R. solani* AG-4 RST-04 was carried out on 100 mm × 15 mm Petri dishes each containing 10 mL of potato dextrose agar (PDA). The Petri dishes were sealed with parafilm and incubated at 27°C temperature. In order to determine the antifungal activity, the tested samples were added separately to PDA at 45°C temperature, mixed immediately, and poured into small Petri dishes (90 mm × 15 mm). When the agar became solid, the mycelial disc (6.5 mm in diameter) of *R. solani* AG-4 RST-04 precultured on PDA plate was placed on the center of each plate for 3 days. The PDA plates added with distilled water were served as the control, and all the plates were incubated at 27°C. When the mycelial growth of control treatment had covered the entire surface of the plate, then the areas of the mycelial colonies were measured, and the

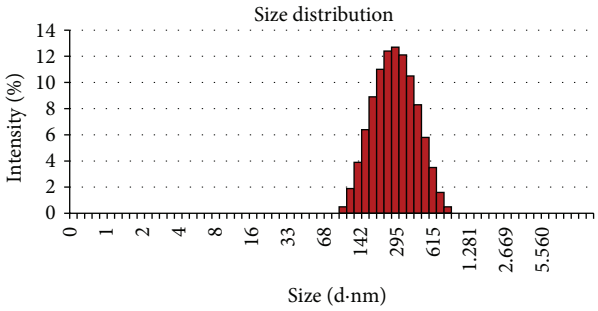


Figure 1: The particle size distribution of Coptis Rhizome powder prepared by ultrafining technology.

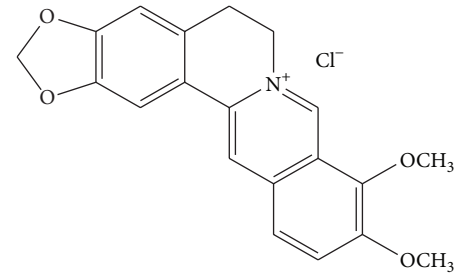


Figure 2: Chemical structure of berberine chloride.

Table 1: Calibration curve parameters and limits of detection (LOD) of the HPLC method developed for Coptis Rhizome.

Calibration curve	Correlation coefficients (r^2)	LOD (ng/mL) ($n = 3$)
$y = 97645x + 375695$	0.9999	16.95

Table 2: Precision and recovery of the HPLC method developed for Coptis Rhizome.

Concentration (μg/mL)	Intraday precision	Interday precision	Recovery (%)
	Mean ± SD (RSD %)		
24.19	25.30 ± 0.09 (0.34)	25.21 ± 0.45 (1.80)	98.92 ± 1.10 (1.11)
48.38	48.87 ± 0.44 (0.89)	47.94 ± 1.13 (2.35)	104.32 ± 0.59 (0.57)
96.75	97.64 ± 0.45 (0.46)	97.87 ± 0.64 (0.66)	103.92 ± 0.64 (0.62)

inhibition of fungal growth was determined by calculating the percentage of reduction in the area of the mycelial colony [11].

2.9. Comparison of Antifungal Activity of Coptis Rhizome Powder Prepared by Traditional Grinding or Ultrafining Technology. The Coptis Rhizome powder prepared by traditional grinding or ultrafining technology (1 g, 2.5 g, 3.5 g, and 5 g) was extracted with methanol (10 mL, 25 mL, 35 mL, and 50 mL), respectively. The solutions were filtered and concentrated to dryness, and the resulting powder was added separately to PDA at 45°C, mixed immediately, and poured into small Petri dishes (90 mm × 15 mm) to afford the examined solution (2%, 5%, 7%, and 10%, resp.). The assay of the antifungal activity against *R. solani* AG-4 RST-04 was carried out according to the procedures described above and the inhibition of fungal growth was also determined by calculating the percentage of reduction in the area of the mycelial colony.

2.10. Statistical Analysis. Data were analyzed with Duncan's multiple range test by a standard statistical package (SAS/STAT) (version 9.1, SAS institute Inc., NC, USA) to assess the statistical significance between each plot.

3. Results and Discussion

3.1. Characterization of Samples. The particle size distribution and polydispersity of Coptis Rhizome powder prepared by ultrafining technology were determined by dynamic light scattering. The particle size distribution was in the range between 80 and 600 nm and average size was 183.7 nm. The polydispersity of Coptis Rhizome powder prepared by ultrafining technology was 0.209 (Figure 1).

3.2. Identification of Indicator Compound. The indicator compound berberine chloride (Figure 2) was purified and characterized by comparison of their spectral and physical data with those reported in the literature [12, 13]. The purity of berberine chloride as determined by HPLC was better than 98.1%.

3.3. Optimization and Validation of the HPLC Method. The optimized HPLC analytical conditions for the medicinal plant extracts were designed as displayed in the experimental section. The calibration curve parameters and LOD for the indicator compound are displayed in Table 1. The precision of the HPLC method was evaluated through the intraday and interday experiments. Among the linear ranges, the RSDs for berberine chloride of the intraday and interday precisions were found to be less than 0.89 and 2.35%. The mean recovery rate was found to be in the range of 98.92 to 104.32% with satisfactory relative standard deviations (RSDs) in the range between 0.57 and 1.11% (Table 2).

3.4. Quantitative Determination of the Indicator Compound in the Methanol Extracts of Coptis Rhizome Powder Prepared by Traditional Grinding or Ultrafining Technology. The developed HPLC chromatographic analytical method was applied to assess the contents of indicator compound in the Coptis Rhizome extracts [10] to evaluate the differences between the traditional grinding method and ultrafining technology. Chromatograms of the two samples of Coptis Rhizome powder and the indicator compound are shown in Figure 3. The contents of berberine in the Coptis Rhizome samples prepared by ultrafining technology and traditionally grinding were quantified and compared in Figure 4. Although the concentration of berberine was found as high as 32.4 mg/g in the sample prepared by ultrafining technology after 10 min

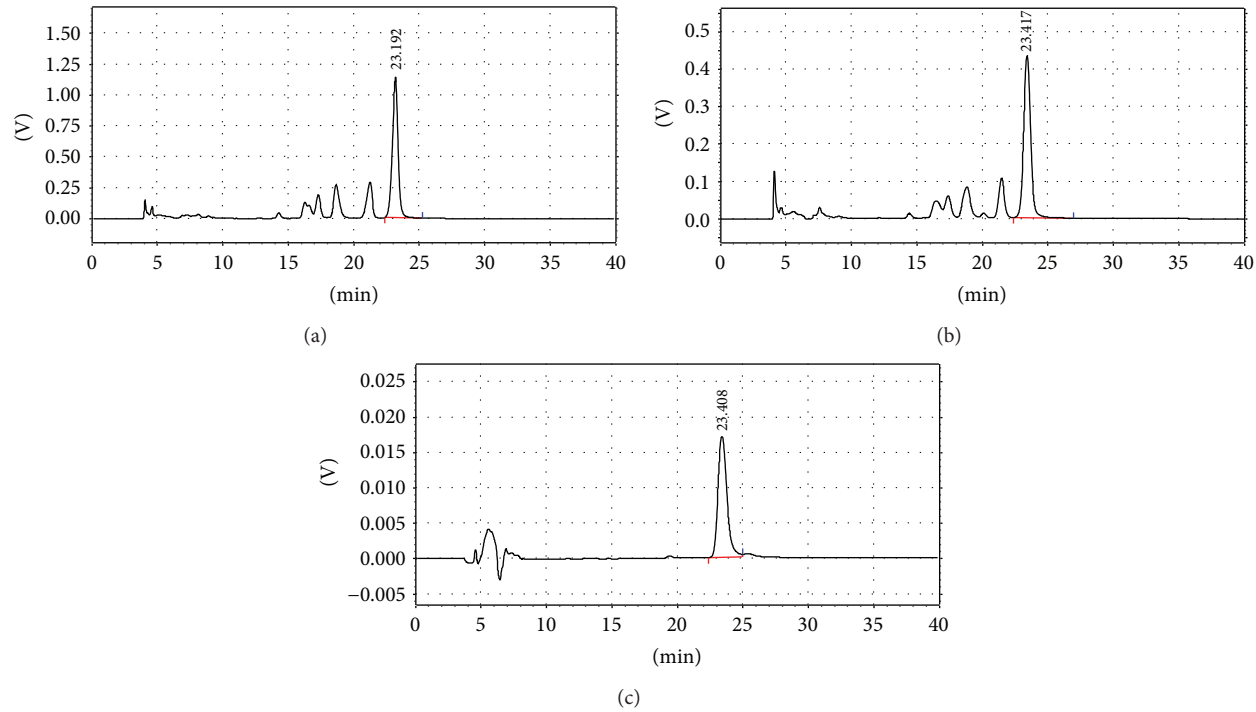


Figure 3: HPLC profiles of (a) extracts of Coptis Rhizome powder prepared by ultrafining technology; (b) extracts of Coptis Rhizome powder prepared by traditional grinding; and (c) reference compound berberine chloride.

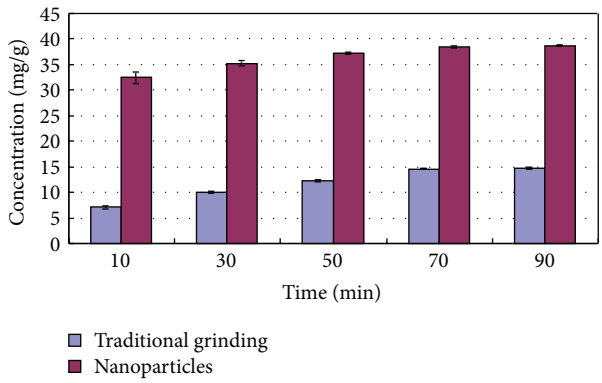
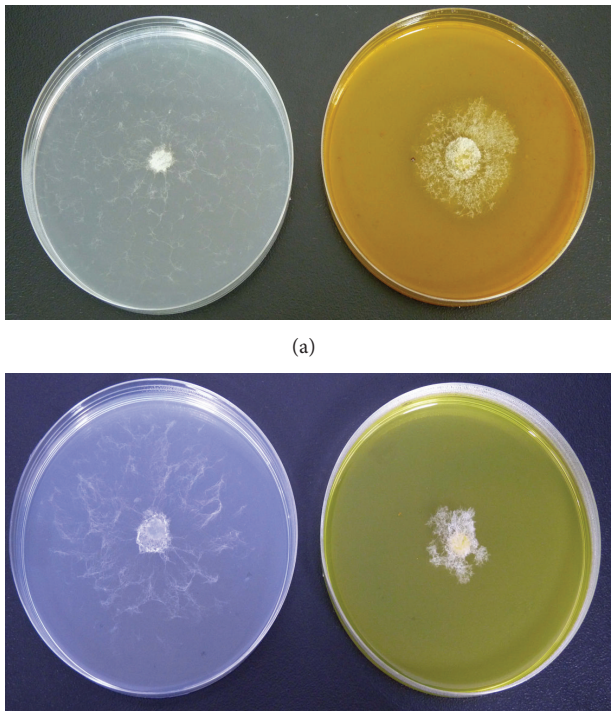


Figure 4: Comparison of the contents of berberine in the Coptis Rhizome samples prepared by ultrafining technology or traditional grinding.

of extraction, the concentration did not increase with further extraction. By contrast, the concentration of berberine increased with extraction time in samples powdered using the traditional grinding procedures. However, the concentration did not exceed that of the ultrafining samples. Only 14.8 mg/g berberine was found in the traditional grinding samples after 90 min of extraction. Based on the results described above, it appears reasonable to suggest that the release of the constituents will be more rapid and more complete in the sample of Chinese medicine plants prepared by ultrafining technology as compared to those prepared using the traditional methods.

3.5. Antifungal Activity of the Methanol Extracts and Purified Compounds. The methanol extracts of Coptis Rhizome were subjected into the antifungal examinations with the procedures described above. 1% methanol extracts of Coptis Rhizome at 84 h showed 56.35% of inhibition against the growth of *R. solani* AG-4 RST-04 (Figure 5). Furthermore, the purified indicator compound was examined for its antifungal activities. The 0.1% indicator compound berberine chloride at 84 h inhibited the growth of the mycelial colony of *R. solani* AG-4 RST-04 with 62.80% of inhibition (Figure 5). The results of present study suggested the berberine chloride was involved in the effect of the methanol extract of Coptis Rhizome against the mycelial growth of *Rhizoctonia solani* AG-4 RST-04.

3.6. Comparison of Antifungal Activity of Coptis Rhizome Powder Prepared by Traditional Grinding or Ultrafining Technology. The 2%, 5%, 7%, and 10% sample solutions of Coptis Rhizome powder prepared by traditional grinding or ultrafining technology were subjected to the antifungal examinations with the procedures described above. 2% sample solution of Coptis Rhizome powder prepared by traditional grinding at 60 h displayed almost no inhibition against the growth of *R. solani* AG-4 RST-04 (Figure 6). Comparatively, 2% sample solution of Coptis Rhizome powder prepared by ultrafining technology at 60 h exhibited 20.8% inhibition against the growth of examined fungus. 5%, 7%, and 10% sample solutions of Coptis Rhizome powder prepared by traditional grinding and ultrafining technology have also shown the similar tendency. All the solutions of Coptis Rhizome powder



(a)

(b)

Figure 5: The mycelial growth of *R. solani* AG-4 RST-04. (a) Left side: control; right side: methanol extract of Coptis Rhizome powder at 1%. (b) Left side: control; right side: berberine chloride at 0.1%.

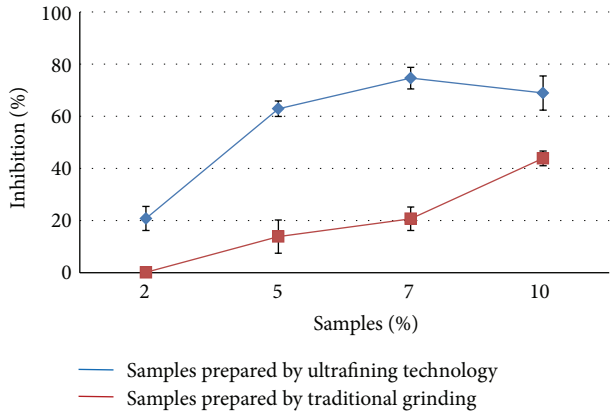


Figure 6: Comparison of antifungal activity of Coptis Rhizome powder prepared by traditional grinding or ultrafining technology.

prepared by ultrafining technology have more significant inhibitory activities against the fungus growth than the same concentration of solutions of powder prepared by traditional grinding. The above results indicated that the higher contents of berberine chloride released from the ultrafining Coptis Rhizome powder samples significantly enhanced the inhibitory effects against the growth of *Rhizoctonia solani* AG-4.

According to the experimental data in the present study, the methanol extracts of Coptis Rhizome powder and the indicator compound berberine chloride have the potential to be explored as new plant derived pesticides to control *Rhizoctonia* damping-off in vegetable seedlings. Although these diseases could be successfully controlled by the synthetic chemical fungicides, the utilization of synthetic fungicides led to the development of resistance and environment pollution. On the contrary, the herbal extracts would be safer and less dangerous to the ecosystem. These traditional Chinese medicines could be studied further for their cytotoxicity and synergistic effects of different combinations. It would be also potential to study the antifungal mechanism in the future.

4. Conclusion

It has been observed from the present data provided in our study that the alkaloid berberine chloride is a potential new plant derived pesticide to control *Rhizoctonia* damping-off in vegetable seedlings. In addition, application of ultrafining technology in Coptis Rhizome preparations may significantly increase the bioactive constituents and bioactivity. Hence it could reduce the amount of raw materials as well as contribute to decrease the environmental degradation. These results would encourage researchers to study the advances of bioactive constituents and related bioactivities as the ultrafining technology applied in the preparation of herbal medicines.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ping-Chung Kuo and Yao-Tung Lin contributed equally as the first authors of this work.

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