Analysis of Caffeic Acid Extraction From *Ocimum gratissimum* Linn. by High Performance Liquid Chromatography and its Effects on a Cervical Cancer Cell Line

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SUMMARY

Objective: *Ocimum gratissimum* is a herbal medicine and caffeic acid (3,4-dihydroxycinnamic acid) is one of its main components. Caffeic acid is known to control the levels of cholesterol and triglycerides, reduce the activity of cancer cells, and enhance immunity in the human body. The amounts of caffeic acid in herbal medicine and vegetable oils have not been reported in the literature since an analytical method has not yet been established. In this study, we explored the effects of caffeic acid treatment on anti-proliferation in HeLa cells.

Materials and Methods: This paper presents a method of extraction of caffeic acid from *O. gratissimum* and Ju ZenTa (*Ocimum basilicum* L.) using high performance liquid chromatography. Treatment of HeLa cells with the extracted caffeic acid (10 mM) was analyzed.

Results: We showed that caffeic acid isolated from several kinds of vegetables and from the herb of *O. gratissimum* had anti-proliferative effects on cervical cancer cell lines. Caffeic acid can significantly reduce the proliferation of HeLa cells in a time-dependent manner.

Conclusion: This paper shows that high performance liquid chromatography is a suitable analytical method for determining caffeic acid levels in *O. gratissimum*, Ju ZenTa, and several vegetable oils. Caffeic acid can suppress the proliferation of HeLa cells. [*Taiwan J Obstet Gynecol* 2010;49(3):266–271]

Key Words: anti-proliferation, caffeic acid, cervical cancer cell line, high performance liquid chromatography

Introduction

Natural products have anecdotally been considered effective for maintaining good health. For example, *Ocimum gratissimum* Linn grows in tropical areas [1] and is commonly used as a herbal medicine in Asia. Its major active ingredients are caffeic acid, ursolic acid, and ocimene [1]. Caffeic acid is a known plant phenolic acid, and the phenolic acids in plants are known as phytochemicals. Caffeic acid has also been reported to be abundant in curly kale [2].

The efficacy of caffeic acid has been previously reported in the literature. The structure of caffeic acid is shown in Figure 1. Caffeic acid can inhibit the absorption of cholesterol in the body and thus reduce the

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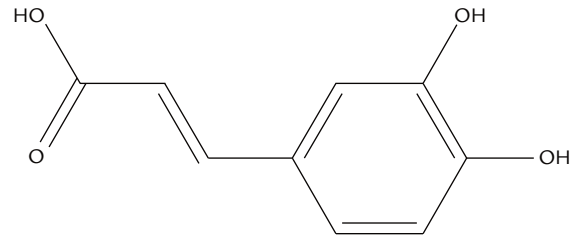

Figure 1. Chemical structure of caffeic acid.

levels of cholesterol, phospholipids, free fatty acids, and triglycerides in rats [3]. Anti-inflammatory function can be improved by caffeic acid [4], and it can reduce skin cancer [5] and oral cancer cell growth as well [6]. Caffeic acid can be found in vegetables and fruits [7]. The presence of caffeic acid has been reported to suppress colon carcinogenesis [8]. It can also be a factor in the formation of lymphocytes in the immunity process [9]. Caffeic acid can be found in vegetables such as *Origanum vulgare* L. It is used in experiments to inhibit soybean lipoxygenase [10].

O. gratissimum is called Chi ZenTa in herbal medicine, but Ju ZenTa, a similar herb, is ineffective in clinical studies. They are in commercial products available in the market. This paper reports an effective extraction process, high performance liquid chromatography (HPLC), and pertinent conditions to analyze caffeic acid in Chi ZenTa and vegetable oils. We report a method to determine the difference between the caffeic acid in Chi ZenTa and Ju ZenTa. We also demonstrated that caffeic acid from *O. gratissimum* can reduce the proliferation of HeLa cells significantly.

Materials and Methods

Apparatus and reagents

The chromatographic system included a gradient pump (Hitachi D-6500 model; Hitachi, Tokyo, Japan), a stainless steel injector (5 μL loop), and an ultraviolet-visible (UV-Vis) spectral detector (Jasco, Tokyo, Japan) operating at 280 nm to detect caffeic acid extracted from wheat germ oil, peanut oil, potato plants, curly kale, Ju ZenTa, and *O. gratissimum*. A Chromolith RP-18 column (Inertsil 7 ODS-3, 4.6 mm internal diameter, 250 mm; Merck) was used as the analytical column. The optimal composition of the mobile phase was 80% solution A (diluted water with 1% acetic acid) and 20% acetonitrile. The flow rate of the mobile phase was 1 mL/min and the column temperature was kept at 25°C. The sample solution and reagent solution were degassed before each run. A Soxhlet extractor apparatus was used to extract the caffeic acid from the desired samples. The UV-Vis spectra were generated using a DU-800 spectrometer (Beckman Coulter, USA). Unless otherwise specified, all reagents were HPLC grade (Merck) and included methanol, ethanol, acetonitrile, and potassium hydroxide (KOH). Petroleum ether was from BDH (Poole, UK). Reagents were degassed in an ultrasonic bath as required before injecting into the HPLC apparatus.

Sample preparation

O. gratissimum was placed in an oven for 2–3 days. The dried plant was broken into pieces and powdered. A sample typically contained 100 g dried powder which was placed in the Soxhlet extraction setup for 48 hours to extract caffeic acid. A dark green oil was obtained after storing the extracted oil for 5 days. This oil was separated and 5 mL of aqueous KOH solution (10 M) was added. The upper layer of the liquid was obtained for the HPLC sample. Samples from potato plants, curly kale, and Ju ZenTa were obtained using the same process. Wheat germ oil and peanut oil were purchased from a supermarket in Taichung, Taiwan and the HPLC samples were obtained as above with extraction from solids after adding KOH. All samples were filtered as required before injecting into the HPLC apparatus. An authentic chemical sample of caffeic acid was purchased from Sigma-Aldrich Co. (NY, USA) and a concentration of 1 mg/mL was prepared by dissolving in methanol.

HeLa cells

The human cervical cancer cell line, HeLa, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 1% penicillin, and 100 μg/mL streptomycin (Life Technologies Inc.), and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. Caffeic acid was collected when *O. gratissimum* was analyzed and the concentration of the medium with caffeic acid was 10 mM.

MTT assay

Cells were seeded in a 96-well plate at 1,000 cells per well and cultured for 24 hours. Cells were then incubated with 10 mM caffeic acid for 48 hours. For the time course assay, the incubation times were 12, 24, and 48 hours. After incubation, MTT was dissolved in phosphate-buffered saline at 5 mg/mL and then added to the culture medium at a final concentration of 0.5%. After incubation at 37°C for 4 hours, the medium was gently aspirated and 150 μL DMSO was added to each well to dissolve any formazan crystals. The plate

was shaken for 10 minutes to allow complete solubilization and cell viability was determined spectrophotometrically by measuring the absorbance at 570 nm using a 96-well plate reader.

Results

Figure 2A shows the HPLC chromatogram for caffeic acid in the mobile phase with 100% solution A at pH 6.5 and the UV detector set at 280 nm. The retention time of caffeic acid in *O. gratissimum* was 38.83 minutes (Figure 2B) as confirmed by the standard solution in the chromatogram at the same conditions as in Figure 2A. The selectivity factor and the retention time could be adjusted by varying the composition of the mobile phase. Thus, 95% solution A and 5% acetonitrile as the mobile phase were adopted to run the standard solutions in Figure 3. A mixture of 80% solution A and 20% acetonitrile as the mobile phase were adopted to run the samples in Figure 4A. The retention times of caffeic acid were 29.75 minutes (Figure 3) and 7.75 minutes (Figure 4A), respectively. The retention time of caffeic acid was apparently reduced from 38.74 minutes (Figure 2A) to 7.75 minutes (Figure 4A). However, further adjustment of the volume ratio between solution A and acetonitrile could not be done because of solubility problems. Therefore, the optimal conditions for analyzing caffeic acid in *O. gratissimum* using HPLC was 80% solution A and 20% acetonitrile as the mobile phase with the other parameters described in the experimental section. The chromatogram of the sample solutions is shown in Figure 4B and the retention time was 7.81 minutes. This HPLC method was also applied to analyze samples obtained from Ju ZenTa but no peak was found for caffeic acid in its chromatogram. Thus, it can be said that this HPLC method is a good tool to identify the difference between Chi ZenTa and Ju ZenTa.

The Table shows the concentrations of caffeic acid in vegetable oils purchased in Taichung (Taiwan).

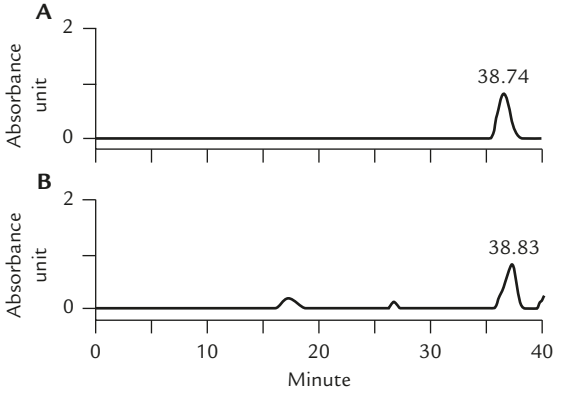

Figure 2. Chromatogram of (A) caffeic acid and (B) *Ocimum gratissimum*. The mobile phase was 99% diluted water with 1% acetic acid at pH 6.5. The analytical column was an Inertsil 7 ODS-3, 4.6 mm internal diameter × 250 mm; flow rate, 1 mL/min, 25°C, with an injection volume of 5 μL and detection at 280 nm.

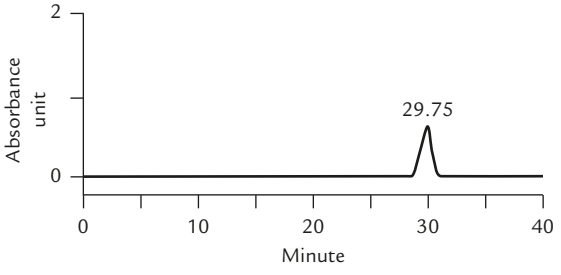

Figure 3. Chromatogram of caffeic acid. The mobile phase was 94% distilled water, 1% acetic acid, and 5% acetonitrile at pH 6.5. The analytical column and conditions used were the same as described above.

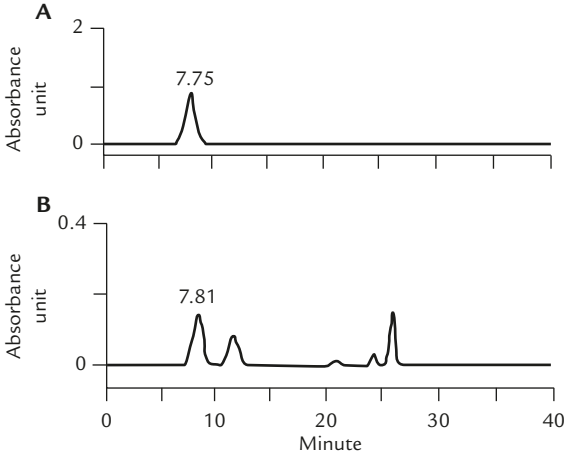

Figure 4. Chromatogram of (A) caffeic acid and (B) *Ocimum gratissimum*. The mobile phase was 79% distilled water with 1% acetic acid and 20% acetonitrile at pH 6.5. The analytical column and conditions used were the same as described in Figure 1.

Table. Amounts of caffeic acid in vegetable oils detected by high performance liquid chromatography

	Retention time (min)	Concentration (mg/mL)
Wheat germ oil	7.83	1.76
Peanut oil	7.54	1.27
Curly kale	7.78	0.71
Potato plants	7.69	0.81
Ocimum gratissimum	7.81	0.34

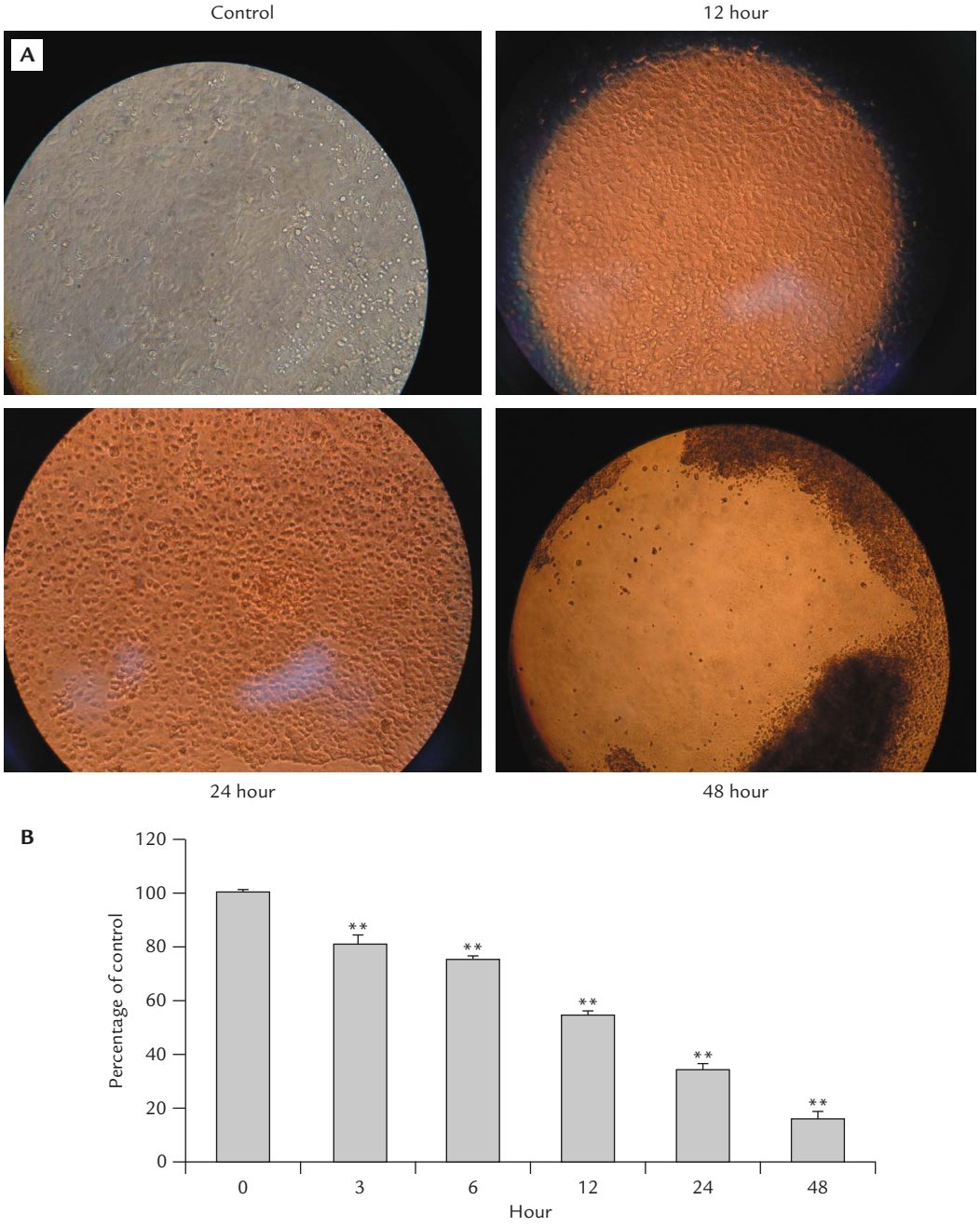

Figure 5. Effects of caffeic acid on cell morphology of HeLa cells. (A) 10 mM caffeic acid applied for 12, 24, and 48 hours directly observed under a microscope at 400× magnification. (B) MTT quantitative analysis showed that the effects of 10 mM caffeic acid are time-dependent. Data expressed as mean ± standard deviation, $*p < 0.01$.

The concentration of caffeic acid in *O. gratissimum* was 0.34 mg/mL. The concentrations of caffeic acid in wheat germ oil, peanut oil, curly kale oil, and potato plant oil were 1.76, 1.27, 0.71, and 0.81 mg/mL, respectively (Table). HeLa cancer cells were treated with caffeic acid from *O. gratissimum* and we found that the cervical cancer cells died (Figure 5A). MTT quantitative analysis showed that caffeic acid significantly inhibited the proliferation of HeLa cells in a time-dependent manner (Figure 5B).

Discussion

Caffeic acid is a phenol, and phenols in plants have been analyzed by HPLC-pulsed amperometric detector-atmospheric pressure chemical ionization-mass spectroscopy [11], and by liquid chromatography [12]. Fecka used thin layer chromatography to analyze polyphenols [13]. Wang et al used liquid chromatography in tandem with mass spectrometry to analyze the caffeic acid phenethyl ester in the plasma of rats [14]. Mader et al

analyzed phenolic compounds in the potato during commercial potato processing with an HPLC method [15]. DellaGreca et al used capillary gas chromatography-mass spectrometry (GC-MS) to analyze aromatic compounds in vegetable oils [16]. Skalicka-Woźniak et al used capillary gas chromatography and capillary gas chromatography-mass spectrometry methods to analyze caffeic acid from *Lavatera trimestris* L. and its antioxidative effects [17]. Turek and Cisowski used reversed-phase liquid chromatography to separate cinnamic acid derivatives [18]. Sha et al analyzed propolis by HPLC [19] and Zhong et al applied HPLC to analyze *Salvia miltiorrhiza* [20]. Hanganu et al applied HPLC to analyze *Melissa officinalis* L. [21]. HPLC methods have been widely used as they are non-destructive but have not been exploited in analyzing caffeic acid in herbal medicines and vegetable oils.

Wheat germ oil was found to be the richest (1.76 mg/mL) in caffeic acid content of all studied samples. *O. gratissimum* had the lowest content (0.34 mg/mL). The HPLC method presented here is rapid and can also be adopted to analyze caffeic acid in dairy products. We also found more caffeic acid in the other vegetable oils, such as peanut oil and curly kale, than in *O. gratissimum*. Therefore, this method can contribute to preventative and treatment effects.

The results were tested repeatedly, and the analysis parameter was 80% solution A and 20% acetonitrile as the mobile phase to achieve caffeic acid quickly. The caffeic acid content and the difference in content in *O. gratissimum* compared to Ju ZenTa can be detected. This analytical method can also be applied to determine the amounts of caffeic acid in vegetable oils. We found that caffeic acid can suppress the growth of HeLa cells, and it may act as an anti-cervical cancer reagent in the future. The molecular mechanism of the anti-proliferative effects of caffeic acid on HeLa is currently under investigation in our laboratory.

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