Thai Journal of Pharmaceutical Sciences (TJPS)

5th International Conference on Pharmaceuticals,
Nutraceuticals and Cosmetic Science (IPNaCS 2017)

Determination of Caffeic Acid in Aqueous Extract of *Thunbergia laurifolia* Leaf Obtained by Using Different Extraction Methods and its Cytotoxic Effect in Hepatocytes

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Keywords: *Thunbergia laurifolia*, caffeic acid, HPLC, cytotoxic effect, hepatocytes

Introduction

Thunbergia laurifolia L., commonly called blue trumpet vine or laurel clock vine (Family Thunbergiaceae), is native to India.[1] In Thailand, it is known as Rang Chuet which has been used as a natural remedy for centuries and normally consumed as herbal tea. It is widely used in Thai traditional medicine for the protection against environmental toxins and thus well known for the detoxifying effects.[2] Rang Chuet are used as an antidote for poisons and drugs including the treatment of drug addiction.[1] The antidotal activity was used against pesticides, arsenic and strychnine poisonings.[3] Besides the detoxifying activity, Rang Chuet also possesses other biological activities such as antioxidant[4], antimicrobial[5], anti-proliferative[6], hepatoprotective[7], anti-inflammatory[8], anti-diabetic[9], anti-drug addiction[10], and wound healing activities.[11] The major phytochemical constituents found in Rang Chuet extract are phenolic compounds and chlorophylls.[2] Several phenolic compounds in the aqueous leaf extract, are identified namely, apigenin, apigenin glucosides, caffeic, gallic, and protocatechuic acid.[1] Phenolic compounds are secondary metabolites found in herbs, fruits, vegetables, cereals and beverages such as wine, coffee, cocoa and tea.[12,13] They are natural antioxidant capable of direct scavenging oxidants and free radicals, thereby contributing to the cytoprotective effects.

Recently, there has been increasing interest in functional foods and nutraceuticals industry in the search for detoxifying and cytoprotective agents from plant. Aqueous extract of *T. laurifolia* leaf have been studied with various techniques, such as HPLC by using flavonoids and phenolic acids as markers.[14] The HPLC methods described in the literature for the quantification of bioactive compounds in *T. laurifolia* leaf were inconvenient and involved sample pretreatment such as acid hydrolysis which may lead to the decomposition of bioactive compounds. Thus, the goal of this study was to determine the content of caffeic acid in aqueous extract of *T. laurifolia* leaf obtained by using different extraction methods. An RP-HPLC method with photodiode array detection for the determination of caffeic acid was developed and validated. The cytotoxic effect of aqueous extract of *T. laurifolia* leaf was also investigated in hepatocytes including HLC and HepaRG cell lines.

Methods

Chemicals

AR grade methanol, acetonitrile (Burdick & Jackson), glacial acetic acid (Merck) and sterile water (A.N.B) were filtered through 47 mm membrane filters nylon, 0.20µm (National Scientific) prior to use for HPLC analysis. The reference standard caffeic acid (purity ≥98%) was purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare Life Science), fetal bovine serum (FBS) (ThermoFischer Scientific), penicillin-streptomycin (ThermoFischer Scientific) and phosphate-buffered saline (PBS) (ThermoFischer Scientific) were used in the cell culture experiment.

Plant extraction

The fresh plants of *T. laurifolia* was collected in Bangkok, Thailand and authenticated by Prof. Dr. Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. Leaves were dried in a hot air oven at 60°C for 6 hours and were grounded in a blender to coarse powder, after which the powder was stored in vacuum packages at -20°C until further use. The dried powder was extracted by three different extraction methods. For maceration, the dried powder was macerated in hot water at 80-90°C

for 15 minutes in the shaking water bath at 25°C.[15] The marc was re-extracted twice. The water extract was filtered through Whatman filter paper No. 4 using aspirator. The water extract was further dried by freeze dryer (Labconco FreeZone 2.5 Liter Benchtop Freeze Dry System, USA). For decoction, the dried powder was decocted for 2 hours in hot water at 80-90°C using the slow cooker. The water extract was then filtered through Whatman filter paper No. 4 using aspirator and further dried by spray dryer (Buchi Mini Spray Dryer B-290, Switzerland). For infusion, this method was developed to simulate tea brewing. The dried powder was infused for two different timing (3 and 5 minutes) and each timing undergoes two different extractions (one-time extraction and three-time extraction). The water extract was then filtered through Whatman filter paper No. 4 using aspirator and further dried by spray dryer (Buchi Mini Spray Dryer B-290, Switzerland).

Determination of caffeic acid by High Performance Chromatography (HPLC)

HPLC analysis was performed on a Surveyor HPLC system (Thermo Finnigan, Germany). Reversed-phase column Merck LiChrospher C18 (4.6 × 250 mm, 5 µm) was used in the study with the mobile phase consisted of solvent A (0.5% v/v acetic acid in water) and solvent B (acetonitrile) in an isocratic condition (85:15).[14] The flow rate was 1.0 mL/min. Detection by UV was done by a Surveyor photodiode array detector (Thermo Finnigan) and the wavelength was set at 336 nm with the total run time of 30 minutes. The concentration of aqueous extract of *T. laurifolia* leaf was 20 mg/mL and the injection volume was 10 µL. All samples were filtered through 0.22 µm nylon membrane syringe filters and solvents were degassed in an ultrasonic bath for 30 minutes prior to use.

HPLC method validation

HPLC method was validated according to the ICH guideline.[16] The specificity was analyzed by injecting the aqueous extract of *T. laurifolia* leaf and standard solution of caffeic acid to compare the UV spectrum between the sample and the standard solution. The specificity was evaluated to ensure that there is no interference of impurities in the sample. Linearity was done in triplicate for 3 days in which the stock solution of caffeic acid was prepared by dissolving in 50% methanol in water (1 mg/mL). The solution was further diluted to obtain the concentration in the range of 10-100 µg/mL. The calibration curves were plotted with the peak areas versus the concentrations. Precision was determined by percent RSD (%RSD) which was calculated by three different concentrations with three replicate injections per each concentration. The standard solutions used for repeatability experiments were the same as used for the linearity experiment. For accuracy, the percent recovery was determined by standard addition method in which the aqueous extract of *T. laurifolia* leaf at the concentration of 20 mg/mL was spiked with three different concentrations of caffeic acid over the range of 50, 100 and 150%. The spiked samples were injected in triplicate for each concentration. Recovery was expressed as the percent mean ratio of the measured added concentration to the expected value.

Cytotoxicity test

In cytotoxicity test, hepatocytes were used to evaluate the toxic effect of aqueous extract of *T. laurifolia* leaf. Hepatocytes were kindly provided by Assist. Prof. Dr. Khanit Sa-ngiamsuntorn, Department of Biochemistry, Faculty of Pharmacy, Mahidol University. Hepatocytes were cultured in DMEM F-12 supplemented with 10% FBS and were maintained in 5% CO_2 atmosphere at 37°C. The hepatocytes used in the study were HLC (hepatocyte-like cells) and HepaRG (human hepatocellular carcinoma cell lines). HLC and HepaRG cells were treated with various concentrations of aqueous extract of *T. laurifolia* leaf in the range of 1.6-3.1 mg/mL. After treatment, the cells were incubated for two days in 5% CO_2 atmosphere at 37°C. The cytotoxic effect was examined under the inverted microscope to observe the change in cells morphology.

Results and Discussion

Plant extraction

The result showed that the most suitable extraction methods of *T. laurifolia* was infusion for 5 minutes (1-time extraction) which yielded the highest content of caffeic acid as shown in Table 1. Whereas, infusion for 3 minutes (1-time and 3-time extractions) revealed lower content of caffeic acid, which may be due to shorter duration used in the extraction methods, thus the content of caffeic acid were low when compared with infusion for 5 minutes. Unlike decoction which yielded the lowest content of caffeic acid, this might result from the longer duration of heat exposure to the bioactive compounds which lead to the degradation of caffeic acid in *T. laurifolia* leaf. From this result, heat and duration could have an effect on the content of the bioactive compounds during extraction methods.

Table 1 Different extraction methods and caffeic acid content (ug/mg) in dry extract determined by HPLC analysis

Extraction methods	Drying methods	Caffeic acid (ug/mg dry extract)
Maceration-shaking, 15 min, 3-time extractions	Freeze dry	0.3509
Decoction, 2 hours	Spray dry	0.1292
Infusion, 3 min, 1-time extraction	Spray dry	0.3784
Infusion, 3 min, 3-time extractions	Spray dry	0.2493
Infusion, 5 min, 1-time extraction	Spray dry	1.1423
Infusion, 5 min, 3-time extractions	Spray dry	0.4621

HPLC method validation

The result showed that the calibration curve of caffeic acid exhibited good linear regression with R^2 0.9985 over a concentration range of 10-100 µg/mL. Moreover, the method used in this study was found to be precise as RSD values of repeatability and intermediate precision were less than 2 and 2.3%, respectively. In addition, the percent recovery of caffeic acid in the spiked samples revealed high recovery values (≥97%) which were in accordance with the recommendation of the Food and Drug Administration (FDA) guidelines.[17] This demonstrated that the method was successfully validated and could be further used in other applications for quantification of caffeic acid in functional foods and nutraceuticals developed from *T. laurifolia* leaf extract.

Cytotoxicity test

The results in Figure 1 and Figure 2 showed that there were no toxicity observed in the change of the cell morphology of the hepatocytes at the concentration range of 1.6-3.1 mg/mL of the aqueous extract of *T. laurifolia* leaf. This result indicated that the aqueous extract of *T. laurifolia* leaf were in a safe limit at this specific dose which could further be used for other experiments relating to the biological activities and gene expressions in hepatocytes. The possible reason that the extract possessed no toxicity was the extraction solvent used in this study which was water and the compounds that were released during extraction may be in suitable amount to cause any toxicity to the hepatocytes or the concentration of the extract used in this study were too low to cause a toxic effect to the hepatocytes.

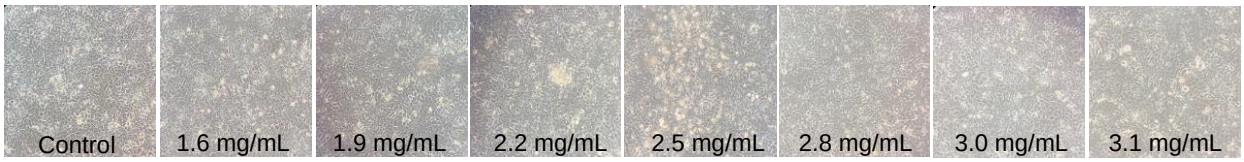

Figure 1 HLC treated with aqueous extract of *T. laurifolia* leaf (5-minute infusion, 1-time extraction)

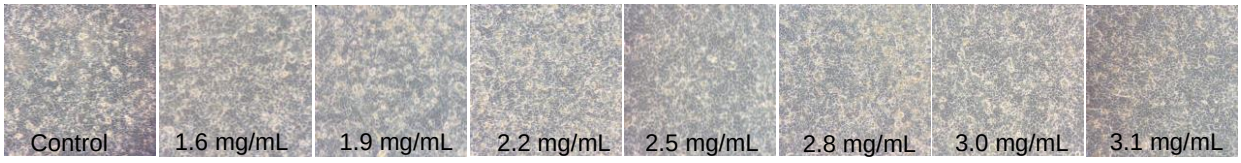

Figure 2 HepaRG treated with aqueous extract of *T. laurifolia* leaf (5-minute infusion, 1-time extraction)

Conclusion

In summary, infusion for 5 minutes with one time extraction was the most suitable extraction method to obtain the highest content of caffeic acid in *T. laurifolia* leaf. Since the developed extraction and HPLC methods is rapid and simple without using prolonged/high heat treatment and acid hydrolysis, the decomposition of bioactive compounds could be minimized. The HPLC method was successfully validated which could further be applied to quantify caffeic acid in functional foods and nutraceuticals developed from *T. laurifolia* leaf.

Acknowledgements

The authors would like to thank Assoc. Prof. Vimol Srisukh, Department of Food Chemistry, Faculty of Pharmacy, Mahidol University for the provision of the plant materials and Prof. Emeritus Dr. Nuntavan Bunyapraphatsara for the provision of laboratory equipments. This study was financially supported by Graduate Studies of Mahidol University Alumni Association.

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