Srinakharinwirot Science Journal Vol. 37 No. 2 (2021) | 93-101

Research Article

Chemical Profiling and *in vitro* Testing for PCSK9 Inhibition of Coffee Cascara Extract

Kulvadee Dolsophon[1]*, Siritron Samosorn[1], Itthipol Sungwienwong[1], Nuttapon Apiratikul[1], Ramida Watanapokasin[2], Watcharaporn Poorahong[2] and Sukhumaporn Krajangsang[3]

Received: 17 November 2021
Revised: 4 December 2021
Accepted: 5 December 2021

ABSTRACT

Coffee cascara is a by-product generated from coffee processing. It has been discarded as an agricultural waste. In order to reduce the environmental problems caused by coffee processing, this study aimed to investigate the effect of fresh coffee cascara extract (CCE) on the inhibition of PCSK9 which is an enzyme that can increase low-density plasma lipoprotein (LDL) cholesterol by destructing LDL receptor. Moreover, the CCE chemical profile was identified by the thin-layer chromatography (TLC) technique together with diffusion-ordered NMR spectroscopy (DOSY). The chemical profile analysis results showed that trigonelline, caffeine, and chlorogenic acid were present in CCE, and its PCSK9 inhibitory activity screening showed that CCE at concentrations of 0.25 and 0.50 mg/mL reduced the amount of PCSK9 by 72 and 78%, respectively. These results revealed that coffee cascara provides novel applications in the nutraceutical industry.

Keywords: Cholesterol, Coffee cascara, LDLR, PCSK9, Chemical profiling

[1]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand

[2]Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand

[3]Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand

*Corresponding author, email: kulvadee@g.swu.ac.th

Introduction

One of the most popular beverages consumed by people across the world is coffee [1]. Coffee beverage is the product acquired from roasted and ground coffee plant seeds. Two important coffee species that have been widely used to make coffee beverage are *Coffea arabica* L. (Arabica coffee) and *Coffea canephora* (Robusta coffee). Arabica beans are more valuable than Robusta beans due to their better and more complicate flavor. There are three important processes including wet, dry, and semidry to produce coffee beans from coffee cherries. The wet process is commonly used in the production of Arabica coffee beans because it gave high quality of coffee beans. In wet process, after harvesting the coffee cherries, the skin and pulp covering the beans are removed. The waste or by-product generated during this process is coffee pulp (cascara). The mucilage is then eliminated by the action of microorganisms in fermentation process. Finally, the bean is washed, drained, and dried to obtain green coffee beans. The coffee process in both wet and dry processes produced massive amounts of cascara in 39-45 kg/100 kg of cherry [2].

One hundred and fifty three compounds have been so far isolated from *C. arabica* L., including alkaloids, flavonoids, terpenes, sterols, phenolic acids, and other compounds. *C. arabica* L. also exhibited the antioxidation, anti-inflammatory, antitumor, antidiabetic, liver protection, and neuroprotective activities [3]. However, after coffee process is complete, by-products including leaf, flower, cherry, husk, silver skin, and spent grounds are generated. There were many reports in the utilization of coffee by-products as novel foods. For example, leaf, flower and husk or cascara were used as beverages [4]. In 2016, Heeger discovered that cascara beverage from aqueous extraction of coffee cherry pulps, containing phenolic compounds and caffeine, showed antioxidant activity [5]. From the literature, the major constituents of cascara extract included chlorogenic acid, caffeine, and tannins [5-7]. Chlorogenic acids, the phenolic compounds in coffee, are formed by the esterification between quinic acid and cinnamic acid derivatives, exhibiting antioxidant, anti-inflammatory and antidiabetic activities, and lowering triglyceride and cholesterol concentrations [8-11]. In 2018, Ontawong *et. al*. discovered that *coffea arabica* pulp aqueous extract, consisting of chlorogenic acid as an active compound, showed cholesterol-lowering effect in *in vitro* and *in vivo* [12]. The most common form of chlorogenic acids found in the green coffee beans is caffeoylquinic acid, which is derived from quinic acid and caffeic acid.

Proprotein convertase subtilsin-kexin type 9 (PCSK9), a hepatic protease, plays an important role in low-density lipoprotein cholesterol (LDL-C) metabolism by controlling the degradation of low-density lipoprotein receptors (LDLRs) in the hepatic cell. Typically, LDL-C is removed from the bloodstream by LDLR. When PCSK9 is activated, it binds to LDLR on the hepatic cell surface. It is then taken into the cells and released to the lysosome for degradation. Therefore, the inhibition of PCSK9 allows elevating the level of LDLR in the liver, resulting in more effective destruction of LDL-C from blood circulation for the treatment of hypercholesterolemia [13]. Over the past two decades, only two PCSK9 inhibitors (evolocumab and alirocumab) have been approved by Food and Drug Administration, and other PCSK9 inhibitors are still in early stage of development. Therefore, searching

for potential PCSK9 inhibitors has received much attention and natural compounds or extracts may be possible sources for PCSK9 inhibitors. In order to reduce coffee cascara waste obtained from coffee processing and provide new useful information of coffee cascara, the studies of their chemical profiles and biological activities would be required. It was previously demonstrated that coffee cascara aqueous extract has a cholesterol-lowering effect via inhibition of intestinal cholesterol absorption by down-regulating Niemann-Pick C1 like 1 protein (NPC1L1) [12]. However, none of the study has investigated the effect of fresh coffee cascara extract on lipid lowering via the inhibition of the attractive target PCSK9. This study thus aimed to explore chemical components in ethanolic extract of the fresh Arabica coffee cascara using thin-layer chromatography (TLC) technique, together with nuclear magnetic resonance spectroscopy (NMR) technique and to evaluate its PCSK9 inhibitory activity.

Materials and Methods

Sample preparation of CCE

Arabica coffee was collected from Chiang Mai Province, Thailand. Cascara was separated from coffee green bean and used for this study. Fresh cascara (1 kg) was extracted in 95% ethanol 3.5 L at room temperature for 2 days and kept in the dark. The extracted solvent was filtered through Whatman no. 1 filter paper, and then evaporated under reduced pressure to dryness. The crude extract, a brown oil, was weighed (100 g, 10%) and stored in a refrigerator.

The study of the chemical components of CCE

There are two methods to study the chemical components of the cascara extract. The first method was TLC technique using Merck silica gel 60 PF254. Spots or the dark areas were visualized under short wavelength ultraviolet lights (254 nm) and the plates were immediately sprayed with anisaldehyde spray reagents. Caffeine and chlorogenic acid (5-*O*-caffeoylquinic acid) were used as standards obtained from Sigma-Aldrich and TCI Chemicals, respectively. The mobile phase for TLC was toluene: ethyl acetate: water: formic acid (15:90:5:5) [14, 15] and R_f values, compared with the standards, were calculated from ratio between distance traveled by substances and distance traveled by solvent. The other method was NMR technique. NMR spectra were recorded on Bruker Avance NEO 500 MHz spectrometer in dimethyl sulfoxide-d_6 (DMSO-d_6). Chemical shifts were calibrated using the solvent signal. The cascara extract was weighed (38.8 mg) into glass vials. DMSO-d_6 was added to the extract. The sample was sonicated for 10 min at room temperature and transferred to NMR tube for NMR analysis. The identification and assignment of compounds were compared to reported assignments and were supported by 2D NMR experiment. The two NMR experiments used in this study are as follows: *Experiment 1*: ^{1}H NMR spectrum was obtained with cpmgpr1d pulse program in order to remove broadening of signals resulted from macromolecules. The parameters were set as follows: number of data points 64 k; number of scans 128 and 4 prior dummy scans. ^{1}H NMR spectrum was processed with 0.3 Hz line broadening. The phase and baseline were adjusted with the TOPSPIN 4.1.1 software (Bruker Biospin).

Experiment 2: 2D diffusion ordered spectroscopy (DOSY) was acquired with ledbpgp2s pulse program. This technique is based on their differing diffusion coefficients in different compounds in a mixture, depending on the size and shape of the molecules. Data was acquired using a 260 msec diffusion time (d20), diffusion gradient length 1 msec (P30), the gradient strength from 1 to 99%, number of scans 128 and 4 prior dummy scans.

Screening for PCSK9 inhibitory activity of coffee cascara extract

Cell culture and cytotoxicity assessment

Human Caucasian hepatocyte carcinoma (HepG2) cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI-1640 Medium (Roswell Park Memorial Institute 1640 Medium; HiMedia Laboratories, India) supplemented with 10% fetal bovine serum (FBS; HiMedia Laboratories, India) and 100 U/mL streptomycin-penicillin solutions. The cells were maintained in a humidified 5% CO_2 incubator at 37°C. Cells were seeded in culture plates and treated with medium only, 0.5% DMSO, and CCE at 0.25 and 0.50 mg/mL for 24 h. After that, the morphology of cells was observed under inverted microscope (X20) (Olympus, Tokyo, Japan). Then the non-toxic concentrations of CCE were chosen to carry out PCSK9 secretion assay.

PCSK9 ELISA assay

Coffee cascara extract (CCE) was screened for its PCSK9 inhibitory activity using a LEGEND MAX™ Human PCSK9 ELISA kit according to the manufacturer's instructions. Briefly, HepG2 cells were seeded for 10 x 10^4 cells/well in a 48-well plate, and then incubated at 37°C in an incubator with 5% CO_2 overnight. Subsequently, cells were treated with CCE at various concentrations for 24 h. The culture medium was centrifuged to remove cell debris at 10,000 rpm for 2 min. The human PCSK9 standard dilutions were prepared at 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47 ng/mL and the LEGEND MAX™ assay diluent D were used as the zero standard (0 ng/mL). The plate was washed four times, and then added 50 µL of the LEGEND MAX™ assay buffer A to each well. Afterwards, 50 µL of prepared standard dilutions or samples were added into the pre-coated well for 2 h incubation while shaking at speed higher than 200 rpm. Each well was washed four times, and then co-incubated for an hour with 100 µL of Human PCSK9 detection antibody solution at room temperature with shaking. For the PCSK9 quantification, 100 µL of Avidin-HRP solution was added to each well while shaking for 30 min. Each well was washed four times. Then wells containing human PCSK9 should turn to blue color with intensity proportional to concentration after adding 100 µL of substrate Solution F and incubated for 15 minutes in the dark. Finally, each well was added 100 µL of the stop solution, the color should change from blue to yellow. The optical density was measured at 450 nm. A 0.5% DMSO in culture media (v/v) was also used as a negative control.

Results and Discussion

The chemical components of coffee cascara ethanolic extract

Major components in ethanolic extract of coffee cascara were detected by TLC technique, using toluene: ethyl acetate: water: formic acid (15:90:5:5) as a solvent system. Standard caffeine (lane A) displayed the dark spot at the R_f = 0.36, whereas standard chlorogenic acid (lane B) showed a spot at the R_f = 0.11. By comparing with R_f values of standards, the TLC result under short wavelength of ultraviolet lights and after spraying with anisaldehyde reagents of ethanolic cascara extract showed that the two major spots at R_f values 0.36 and 0.11 (Lane C) were identified as caffeine and chlorogenic acid, respectively. The TLC result is depicted in Figure 1.

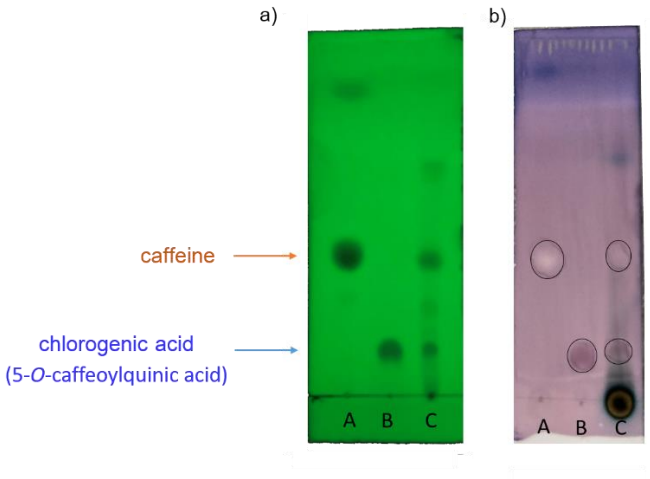

Figure 1 TLC chromatogram of standard caffeine (lane A), standard chlorogenic acid (5-*O*-caffeoylquinic acid) (lane B) and cascara extract (lane C) a) under short wavelength of ultraviolet lights and b) after spraying with anisaldehyde reagents.

The cascara extract was analyzed using the ^{1}H NMR technique in order to acquire more data on the chemical constituents of cascara extract. Structure identification of chemical composition in the extract was performed by comparing with previous ^{1}H NMR data reported [16] or pure standard. The ^{1}H NMR spectrum revealed that the chemical composition of cascara extract contained trigonelline, caffeine, and chlorogenic acid as shown in Figure 2. Trigonelline showed the ^{1}H NMR signal in the range of the chemical shift (δ_H) 8.0-9.2 ppm. Three *N*-methyl signals at δ_H 3.9, 3.4, and 3.2 ppm together with the signal at δ_H 8.0 ppm in the aromatic region were identified as caffeine. Moreover, the chemical shift range between 6.2 to 7.4 ppm was assigned as the proton signals of unsaturated carbons of chlorogenic acid.

The other 2D NMR technique to confirm chemical composition in the cascara extract was diffusion-ordered spectroscopy (DOSY). DOSY is the technique to separate the signals in mixture, based on their diffusion coefficients. 2D DOSY spectrum displayed chemical shifts along the F2 axis and diffusion coefficients along the F1 axis. Each compound had a different diffusion coefficient. In a mixture, the solid lines represented chlorogenic acid, trigonelline, and caffeine, respectively (Figure 3).

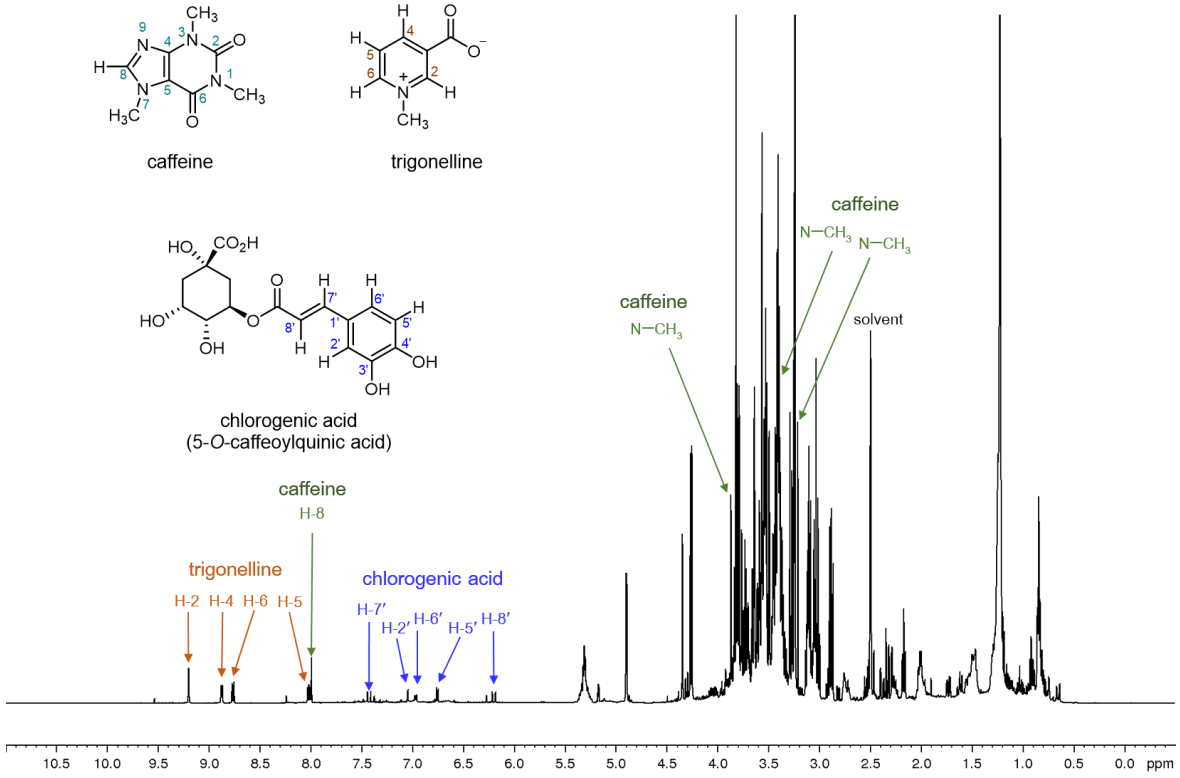

Figure 2 ^{1}H NMR spectrum and chemical identification of the cascara extract in DMSO-d_6.

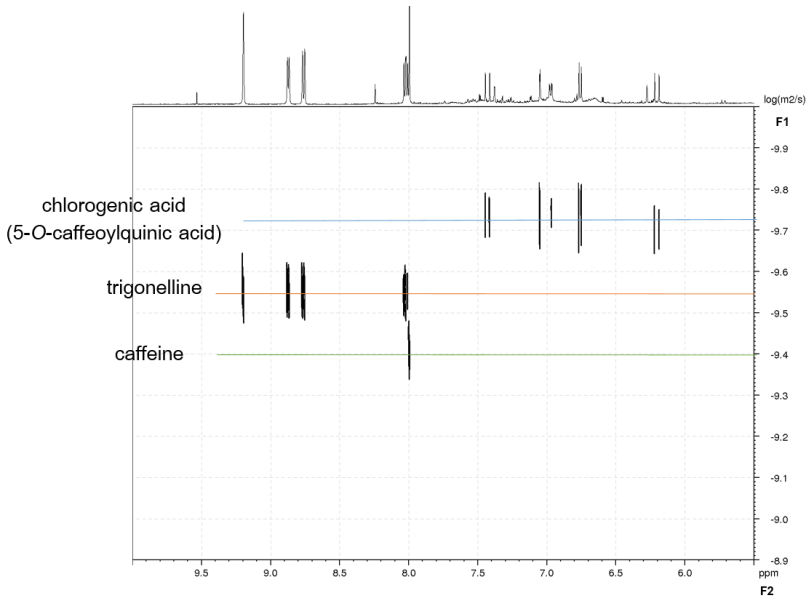

Figure 3 The diffusion ordered NMR (DOSY) spectra of cascara extract in the 5.0-10.0 ppm region.

Testing for PCSK9 inhibitory activity of coffee cascara extract

As the expression of PCSK9 was investigated on HepG2 cells, cytotoxicity of CCE at 0.25 and 0.50 mg/mL was tested against HepG2 cells to ensure that the test compounds were not harmful to the HepG2 cells. The result demonstrated that cells in 0.5% DMSO, and both concentrations of CCE comparing morphological changes with the cells in culture medium presented no dead cells or unhealthy cells such as cells floating and the disturbing of cell membrane integrity [17]. It also indicated that both concentrations of CCE were non-toxic to the HepG2 cells (Figure 4). Screening for PCSK9 inhibitory activity was subsequently performed. Treatment of HepG2 cells with 0.25 and 0.50 mg/mL of CCE led to a concentration-dependent reduction of PCSK9 expression (Table 1). Chemical profiling of CCE confirmed the presence of trigonelline, caffeine, and chlorogenic acid in the cascara extract, suggesting these compounds as promising agents to reduce LDL-C levels. To the best of our knowledge, this is the first preliminary study to reveal that ethanolic extract of fresh Arabica coffee cascara had an effect on lowering LDL-cholesterol via the inhibition of PCSK9.

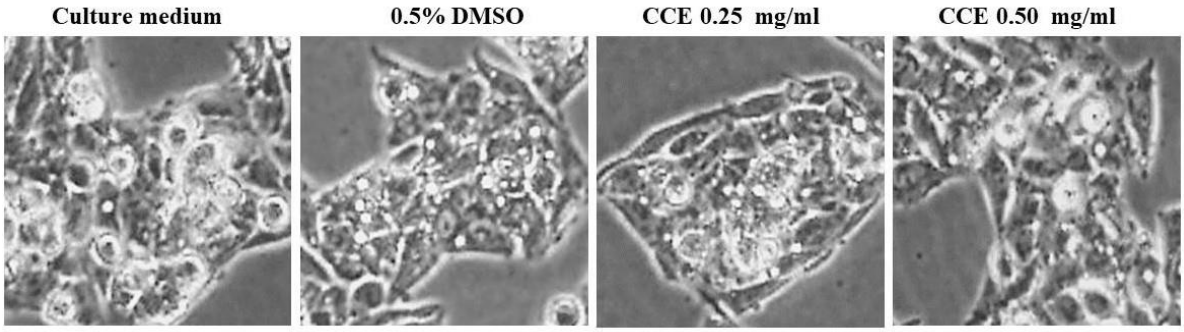

Figure 4 Cell morphology of HepG2 cells after treating with CCE for 24 h under inverted microscope (X20).

Table 1 Testing of CCE (0.25 mg/mL) on inhibition of PCSK9 secretion in HepG2 cells compared with control (0.5% DMSO in culture media (v/v))

Compound	Concentration (mg/mL)	Secreted Human PCSK9 Level (ng/mL)	Relative Percentage of Secreted Human PCSK9
CCE	0.25	1.59	28.5
	0.50	1.26	22.6
Control	0.5%DMSO	5.58	100.0

Conclusions

This study demonstrated that the chemical composition of ethanolic extract of fresh coffee cascara contained trigonelline, caffeine, and chlorogenic acid that was confirmed by both TLC and NMR techniques. This extract exhibited a dose-dependent on PCSK9 inhibition. However, there has been no data available so far on the effect of trigonelline, caffeine, and chlorogenic acid on PCSK9. To clarify the effect of these compounds on PCSK9 inhibition, we need to further investigate single compounds.

Acknowledgements

This research was supported by Kasetsart University Research and Development Institute (KURDI) grant no. FF(KU)18.64 under coffee innovation research unit at Srinakharinwirot University grant no. 608/2563. The authors would like to thank Department of Chemistry and Microbiology, Faculty of Science and Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University for the laboratory facilities. Moreover, we would like to thank Miss Shanya Taratikundech and Miss Thitirat Tirattankul, Q-arabica grader team, to support our knowledge on arabica coffee process.

References

1. Samoggia, A., & Riedel, B. (2019). Consumers' perceptions of coffee health benefits and motives for coffee consumption and purchasing. *Nutrients, 11*, 653.
2. Iriondo-DeHond, A., Iriondo-DeHond, M., & del Castillo, M. D. (2020). Applications of compounds from coffee processing by-products. *Biomolecules, 10*, 1219.
3. Shen, X. J., Zhou, Z. B., Nie, F. Q., Zi, C. T., & Fan, J. P. (2020). Progress in phytochemical and bioactivities of *Coffea arabica* L. *Medicine Research, 4*, 200012.
4. Klingel, T., Kremer, J. I., Gottstein, V., Rezende, T., Schwarz, S., & Lachenmeier, D. W. (2020). A review of coffee by-products including leaf, flower, cherry, husk, silver skin, and spent grounds as novel foods within the European Union. *Foods 9*, 665.
5. Heeger, A., Cagnazzo, A. K., Cantergiani, E., & Andlauer, W. (2016). Bioactives of coffee cherry pulp and its utilisation for production of cascara beverage. *Food Chemistry*, 969–975.
6. Janissen, B., & Huynh, T. (2018). Chemical composition and value-adding applications of coffee industry by products: A review. *Resources, Conservation & Recycling*, *128*, 110–117.
7. Murthy, P. S., & Naidu, M. M. (2012). Recovery of phenolic antioxidants and functional compounds from coffee industry by-products. *Food and Bioprocess Technology*, *5*, 897–903.
8. Cho, A. S., Jeon, S. M., Kim, M. J., Yeo, J., Seo, K. I., Choi, M. S., & Lee, M. K. (2010). Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food and Chemical Toxicology 48*, 937–943.
9. Farah, A., Monteiro, M., Donangelo, C. M., & Lafay, S. (2008). Chlorogenic acids from green coffee extract are highly bioavailable in humans. *The Journal of Nutrition, 138*(12), 2309–2315.

10. Pimpley, V., Patil, S., Srinivasan, K., Desai, N., & Murthy, P. S. (2020). The chemistry of chlorogenic acid from green coffee and its role in attenuation of obesity and diabetes. *Preparative Biochemistry & Biotechnology*. doi: 10.1080/10826068.2020.1786699.
11. Vinson, J. A., Chen, X., & Garver, D. D. (2019). Determination of total chlorogenic acids in commercial green coffee extracts. *Journal of Medicinal Food, 22*(3), 314–320.
12. Ontawong, A., Duangjai, A., Muanprasat, C., Pasachan, T., Pongchaidecha, A., Amornlerdpison, D., & Srimaroeng, C. (2019). Lipid-lowering effects of *Coffea arabica* pulp aqueous extract in Caco-2 cells and hypercholesterolemic rats. *Phytomedicine, 52*, 187–197.
13. Melendez, Q. M., Krishnaji, S. T., Wooten, C. J., & Lopez, D. (2017). Hypercholesterolemia: The role of PCSK9. *Archives of Biochemistry and Biophysics, 625–626*, 39–53.
14. Adham, A. N. (2015). Simultaneous estimation of caffeic and chlorogenic acid content in *Ammi Majus* seed by TLC and HPLC. *International Journal of Pharmacy and Pharmaceutical Sciences,* 7(6), 263–267.
15. Kiattisin, K., Nantarat, T., & Leelapornpisid, P. (2016). Evaluation of antioxidant and anti-tyrosinase activities as well as stability of green and roasted coffee bean extracts from *Coffea arabica* and *Coffea canephora* grown in Thailand. *Journal of Pharmacognosy and Phytotherapy, 8*(10), 18–192.
16. Li, C. Y., Lin, C. H., & Wu, T. S. (2005). Quantitative analysis of camptothecin derivatives in *Nothapodytes foetida* using ^{1}H-NMR method. *Chemical and Pharmaceutical Bulletin, 53*(3), 347–349.
17. Tsuji, T., Morita, S. Y., Nakamura, Y., Ikeda, Y., Kambe, T., & Terada, T. (2021). Alterations in cellular and organellar phospholipid compositions of HepG2 cells during cell growth. *Scientifc Reports*, *11*, 2731.