Original article

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IN VITRO BIOLOGICAL ACTIVITIES OF *Lonicera japonica* FLOWERING BUD COMPARED TO CHLOROGENIC ACID, ROSMARINIC ACID AND CAFFEIC ACID

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ABSTRACT

Lonicera japonica Thub. or Japanese honeysuckle has been widely used in traditional medicine to treat many ailments. Chlorogenic acid, rosmaric acid and caffeic acid are hydroxycinnamic acid derivatives found in this plant part. This study investigated *in vitro* cytotoxic and antimicrobial of *L. japonica* flowering bud as well as chlorogenic acid, rosmarinic acid and caffeic acid. Dried *L. japonica* flowering bud was exhaustively extracted with 95% ethanol. Cytotoxic activities were performed by brine shrimp lethality assay, MTT cell viability assay (5 cancer and 1 normal cell lines) and comet assay. Antibacterial activities against 11 bacterial strains were determined. *L. japonica* flowering bud ethanolic extract showed non-toxicity on brine shrimp nauplii and 6 tested cell lines. Chlorogenic acid, rosmarinic acid and caffeic acid demonstrated toxicity against brine shrimp nauplii. They showed more cytotoxic potentials against tested cell lines than the extract but were still accepted as no cytotoxicity. The extract and 3 compounds showed human lymphocyte DNA damage by comet assay. They showed no inhibitory activities against tested microorganisms.

Keywords: *Lonicera japonica*, flowering bud, chlorogenic acid, rosmarinic acid, caffeic acid, cytotoxicity, antimicrobial

Introduction

Lonicera japonica Thunb. (Caprifoliaceae) is known as "Japanese Honeysuckle" and "Sai Nam Phueng" in Thai. The pharmacological studies of *L. japonica* flowering bud have demonstrated many biological activities, such as anti-inflammatory, antioxidation, hepatoprotective effects and so on. In Thailand, this plant is used for antipyretic, diuretic and antidiarrheal effects.[1] The indicator compound that is used as characteristics for the quality control of *L. japonica* is chlorogenic acid[2] (Figure 1A), which is hydroxycinnamic acids, one of two major groups of phenolic acid that demonstrates many biological activities such as anti-inflammatory, antioxidation, anti-melanogenic activities and so forth. Caffeic acid (Figure 1B) is one of the most common phenolic acid that is biosynthesized by hydroxylation of *p*-coumaric acid, more widely distributed in many plant species. Chlorogenic acid is an ester of caffeic acid and quinic acid. It is a kind of polyphenol derivative which is widely distributed in plants, especially in coffee, apples and pears. In addition, rosmarinic acid (Figure 1C), an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid, is commonly found in species of the Boraginaceae and Lamiaceae.[3] In our HPLC analytical study, chlorogenic acid, rosmarinic acid and caffeic acid contents in the ethanolic extract were found to be 0.302, 0.078 and 0.006 mg/mg, respectively and in *L. japonica* flowering bud were found to be 9.896, 2.543 and 0.195 g/100g of dried crude drug, respectively.[4]

The aims of the present study were to investigate *in vitro* biological activities of *L. japonica* flowering bud ethanolic extract compared to chlorogenic acid, rosmarinic acid and caffeic acid standard compounds.

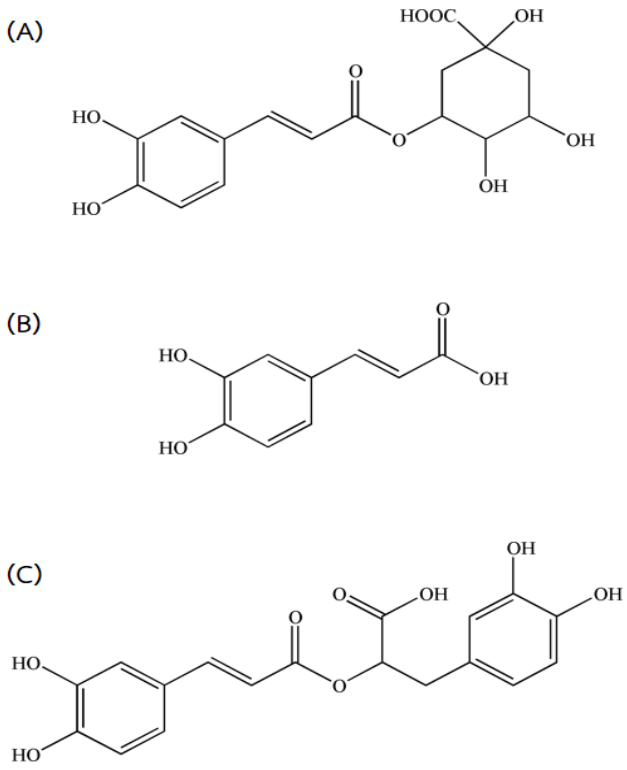

Figure 1 Structures of chlorogenic acid (A), caffeic acid (B) and rosmarinic acid (C)

Materials and method

Plant material and chemicals

Dried *L. japonica* flowering bud was purchased from a herbal drugstore and authenticated by one of the authors, Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. Standard chlorogenic acid (CAS no. 327-97-9), rosmarinic acid (CAS no. 20283-92-5) and caffeic acid (CAS no. 331-39-5) were purchased from Sigma-Aldrich, USA. The chemicals used were of analytical grade.

Plant extraction

The powders of the flowering bud were extracted with 95% ethanol by Soxhlet apparatus till exhaustion. The extract was filtered, evaporated to dryness and stored at -20 °C.

Brine shrimp lethality assay

According to Sowemimo *et al.* with minor modification,[5] ten nauplii were tested with flowering bud ethanolic extract (1,000 - 10,000 µg/ml) and 3 standard compounds (100 - 1,000 µg/ml) in 5 ml of artificial sea water. Each concentration was done in five replicates. The concentration that caused 50% of lethality (LC_{50}) at 24 h was determined.

MTT cell viability assay

Five human cancer cell lines including BT-474 (breast ductal carcinoma), ChaGo-K-1 (bronchogenic carcinoma), Hep G2 (hepatocellular carcinoma), KATO III (gastric carcinoma), SW620 (colorectal adenocarcinoma), and 1 human normal cell line as WI-38 (lung fibroblast) were purchased from American Type Culture Collection.

According to Sowemimo with minor modification,[5] each cell line was mixed with tested samples in DMSO and incubated at 37 °C with 5% (v/v) CO_2. After 72 h, 10 µl MTT solution (5 mg/ml) were added into each well and incubated for 4 h. The absorbance at 540 nm was measured. Doxorubicin and DMSO were used as positive control and negative control, respectively. Four replicates of each sample were performed. A percentage of cell survival was calculated from the ratio of sample and DMSO absorbance. The concentration that caused 50% of cell lethality was evaluated.

DNA damage by Comet assay

According to Cinkilic *et al.* with minor modification,[6] human lymphocytes were treated with tested samples in 2% DMSO at the concentration of 25, 50 and 100 µg/ml. DNA migration was performed in agarose slide by electrophoresis. The comet (migrated DNA) was stained with ethidium bromide and observed under a fluorescence microscope. The degrees of damage were categorized based on the size and intensity of the comet tail, into five classes of visual scoring. One hundred comets were scored from each slide to assign a value between 0-400 arbitrary units.

Antibacterial activity

The bacterial strains used were gram positive spore – and non spore – forming bacterial as well as gram negative bacterial as follows: *Bacillus cereus* ATCC 1177, *Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* (Isolates), *Salmonella typhimurium* ATCC 13311, *Shigella* spp. (Isolates), *Staphylococcus aureus* ATCC 6538P and *Staphylococcus epidermidis* (Isolates).

An agar well diffusion method using a two-layer agar technique and a sterile 6 mm-diameter cork borer was performed for inhibition zone determination. Each well was filled with 20 µl of tested samples in DMSO (200 mg/ml). Ampicillin and amikacin (1 mg/ml) were used as a positive control and DMSO as a negative control. Each sample was tested in triplicate. Samples with zone inhibition were further investigated for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Broth microdilution method was performed in a sterile 96-well microplate. The samples were serially

diluted two-fold in DMSO. The microbial suspension (1×10^8 CFU/ml) in broth were added to each well containing tested samples and incubated at 37 °C for 18-24 h. The lowest concentration of samples which expresses growth inhibition detected by the lack of visual turbidity compared to the negative control was defined as the MIC. The broth from the wells without turbidity was streaked onto the agar plates and incubated at 37 °C for 18-24 h. The least concentration with no microbial growth on the plate was considered as MBC.[7]

Results

Brine shrimp lethality assay

The flowering bud ethanolic extract showed no toxicity to brine shrimp with the LC_{50} of 2,286.20 µg/ml. Chlorogenic, rosmarinic and caffeic acids showed toxic potential to brine shrimp with the LC_{50} of 266.05, 289.66 and 231.82 µg/ml, respectively.

MTT cell viability assay

The flowering bud ethanolic extract had no cytotoxic activity (IC_{50} > 100 µg/ml) against all tested cell lines. Chlorogenic acid showed no cytotoxic activity with IC_{50} of 78.72 and 69.59 µg/ml for ChaGo-K-1 and SW620 respectively, and another cell lines showed IC_{50} > 100 µg/ml. Rosmarinic acid showed no cytotoxic activity with IC_{50} of 49.71 to 88.54 µg/ml for 5 cancer cell lines, and IC_{50} > 100 µg/ml for a normal cell line. Caffeic acid showed no cytotoxic activity with IC_{50} of 7.02 to 84.46 µg/ml for 5 cancer cell lines, and 75.41 µg/ml for a normal cell line (Table 1).

Table 1 Cytotoxic activity (IC_{50}) against 5 cancer cell lines and 1 normal cell line

Tested samples	IC_{50} (µg/ml)					
	BT-474	ChaGo-K-1	Hep G2	KATO III	SW620	WI-38
L. japonica extract	>100	>100	>100	>100	>100	>100
Chlorogenic acid	>100	78.72	>100	>100	69.59	>100
Rosmarinic acid	88.54	82.98	53.33	62.43	49.71	>100
Caffeic acid	7.02	84.46	66.11	80.70	48.82	75.41
Doxorubicin	0.80	0.65	0.12	0.71	2.57	0.22

DNA damage (Comet assay)

The tested samples showed the dose-dependent relationship between the degree of DNA damage and concentration of the sample. Chlorogenic acid at 100 µg/ml showed the highest DNA damage. The ethanolic extract of flowering bud showed a potentiating effect on DNA damage more than rosmarinic acid and caffeic acid. The total scores of DNA damage showed in Figure 2.

Antibacterial activity

The ethanolic extract of flowering bud presented no inhibitory activity against all tested microorganisms. Chlorogenic acid, rosmarinic acid and caffeic acid showed an inhibition zone against most of the tested bacteria (Table 2). MIC and MBC of all compounds were found to be >4000 µg/ml (Table 3).

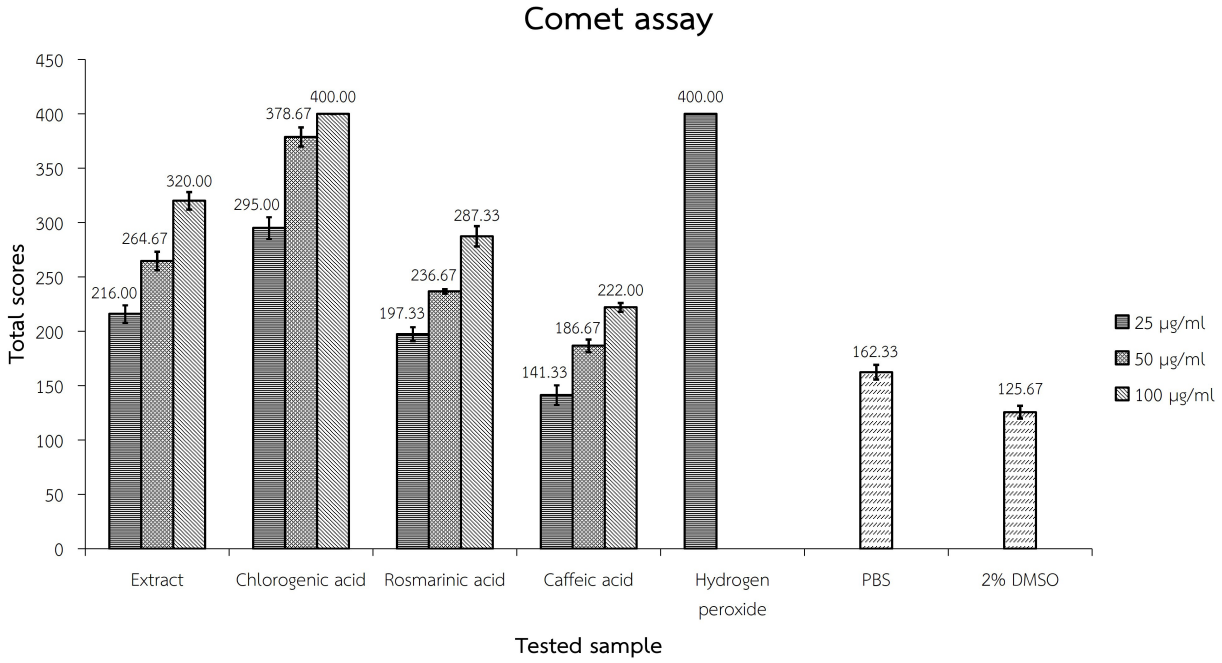

Figure 2 Human lymphocyte DNA damage score

Table 2 Antibacterial activity of *L. japonica* flowering bud ethanolic extract, chlorogenic acid, rosmarinic acid, caffeic acid, ampicillin and amikacin using agar well diffusion method

Microorganisms	Inhibition zone (mm)*					
	Extract	Chlorogenic acid	Rosmarinic acid	Caffeic acid	Ampicillin	Amikacin
Bacillus cereus	NA	NA	NA	NA	22.33 ± 0.58	22.00 ± 1.00
Bacillus subtilis	NA	10.00 ± 0.00	9.67 ± 0.58	10.33 ± 0.58	13.33 ± 0.58	21.00 ± 0.00
Enterobacter aerogenes	NA	7.33 ± 1.15	NA	9.67 ± 0.58	9.00 ± 0.00	16.33 ± 0.58
Escherichia coli	NA	6.67 ± 1.15	6.67 ± 1.15	9.33 ± 0.58	17.67 ± 0.58	18.33 ± 0.58
Kocuria rhizophila	NA	15.00 ± 0.00	16.33 ± 0.58	11.33 ± 0.58	40.67 ± 0.58	24.67 ± 0.58
Pseudomonas aeruginosa	NA	8.67 ± 0.58	8.67 ± 0.58	8.67 ± 0.58	NA	20.00 ± 0.00
Salmonella typhi	NA	8.00 ± 0.00	7.67 ± 1.53	9.67 ± 0.58	26.33 ± 0.58	18.33 ± 0.58
Salmonella typhimurium	NA	8.67 ± 0.58	7.67 ± 1.53	9.67 ± 0.58	30.33 ± 0.58	20.00 ± 0.00
Shigella spp.	NA	NA	9.33 ± 0.58	12.33 ± 0.58	23.33 ± 0.58	22.23 ± 0.58
Staphylococcus aureus	NA	9.67 ± 0.58	8.33 ± 0.58	9.33 ± 0.88	40.33 ± 0.58	12.33 ± 0.58
Staphylococcus epidermidis	NA	9.33 ± 0.58	8.33 ± 0.58	11.67 ± 0.58	25.67 ± 0.58	23.00 ± 0.00

*mean ± SD, NA = no activity, 6 millimeter of well. The tests were done in triplicate.

Table 3 Antibacterial activity of *L. japonica* flowering bud ethanolic extract, chlorogenic acid, rosmarinic acid, caffeic acid, ampicillin and amikacin using broth microdilution method.

Microorganisms	Extract		Chlorogenic acid		Rosmarinic acid		Caffeic acid		Ampicillin		Amikacin	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Bacillus cereus	NA	NA	NA	NA	NA	NA	NA	NA	0.39	0.39	1.56	1.56
Bacillus subtilis	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	>200	>200	12.5	12.5
Enterobacter aerogenes	NA	NA	>4000	>4000	NA	NA	4000	>4000	>200	>200	25	25
Escherichia coli	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	6.25	25	25	200
Kocuria rhizophila	NA	NA	>4000	>4000	4000	>4000	4000	>4000	0.39	0.39	6.25	6.25
Pseudomonas aeruginosa	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	NA	NA	50	100
Salmonella typhi	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	1.562	50	200	>200
Salmonella typhimurium	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	0.781	6.25	50	100
Shigella spp.	NA	NA	NA	NA	>4000	>4000	4000	4000	6.25	12.5	50	50
Staphylococcus aureus	NA	NA	>4000	>4000	>4000	>4000	4000	>4000	0.39	25	6.25	100
Staphylococcus epidermidis	NA	NA	>4000	>4000	4000	4000	4000	>4000	12.5	12.5	3.125	12.5

*NA = no activity. The tests were done in triplicate.

Discussion

Brine shrimp lethality assay is simple, rapid and inexpensive assay which predicts the preliminary cytotoxicity. Toxic strength was classified as toxic if LC_{50} value <1000 µg/ml and non toxic if LC_{50} ≥1000 µg/ml.[8] Caffeic acid was demonstrated with highest toxicity against brine shrimp nauplii while the flowering bud ethanolic extract exhibited non toxicity. The previous study also reported LC_{50} of chlorogenic acid as 300 µg/ml that was related to this study.[9]

MTT cell viability assay is used to investigate the proliferation and cytotoxicity of medicinal agents based on the mitochondrial dehydrogenase enzyme activity in cells.[10] The U.S. National Cancer Institute (NCI) establishes the criteria of cytotoxicity that plant extract and pure compound, with IC_{50} value <20 µg/ml and <4 µg/ml respectively, are considered to have cytotoxic activity. All the tested samples demonstrated a cell viable inhibition potential. However, based on cytotoxic standard criteria, they were classified as no cytotoxicity. Park *et al*. reported that the extract of *L. japonica* did not significantly change WI-38 lung-derived cell line viability.[11] Moreover, caffeic acid also showed no cytotoxic activity to HepG2 cells (IC_{50} = 781.8 µg/ml).[12]

Comet assay is a rapid standard method to observe DNA damage in eukaryotic cells bases on the quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis. The flowering bud ethanolic extract and 3 standard compounds showed DNA damage potential. Estefanía *et al*. reported high level of DNA damage of K562 leukemia cells after exposure to chlorogenic acid (177 - 1,771 µg/ml) for 24 hours.[13] Devipriya *et al.* found that caffeic acid (10 µg/ml) demonstrated low DNA damage on human lymphocytes and no significant increase of comet formation compared to 0.2% DMSO.[14] Those results were related to the present study that chlorogenic acid showed high DNA damage potential and the lowest concentration of caffeic acid caused less DNA damage close to the negative controls.

The agar well diffusion assay showed antibacterial potential of chlorogenic acid, rosmarinic acid and caffeic acid. However, MIC was found to be >4,000 µg/ml representing low inhibitory potential against tested microorganisms. Zaixiang *et al*. reported MIC of chlorogenic acid by agar dilution method against *Bacillus subtilis* (ATCC 9372), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922) and *Salmonella Typhimurium* (ATCC 50013) as 40, 40, 80 and 40 µg/ml respectively, which seemed to be active potential to antimicrobial activity.[15] Fu *et al*. demonstrated MIC of *L. japonica* flowering bud aqueous extract against *E. coli* (ATCC 25922) as 500 mg/ml respectively.[16] The results from this study demonstrated less antibacterial potential than previous reports, probably attributed to different extraction methods and contents of chemical compounds in plant extract.

Conclusion

In vitro biological activities of *L. japonica* flowering bud and its active constituents revealed marginal potential on antibacterial activities. Cytotoxicity especially DNA damage by comet assay was demonstrated, therefore, this plant material should be concerned for a long-term treatment. Further DNA damage measurements are required, for example, DNA fragmentation test and mutagenicity by Ames test to establish scientific information that supported this study.

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References

1. Chuakul W, Soonthornchareonnon N, Ruangsomboon O. *Lonicera japonica* Thunb. ex Murray Bogor, Indonesia: PROSEA (Plant Resources of South-East Asia) Foundation; 2001 [cited 2017 23 March]. Available from: http://proseanet.org/prosea/e-prosea_detail. php?frt=&id=1179.
2. Chaowuttikul C, Palanuvej C, Ruangrungsi N. Pharmacognostic specification, chlorogenic acid content, and In vitro antioxidant activities of *Lonicera japonica* flowering bud. Pharmacognosy Res. 2017;9(2):128-32.
3. Taofiq O, González-Paramás A, Barreiro M, Ferreira I. Hydroxycinnamic acids and their derivatives: cosmeceutical significance, challenges and future perspectives, a review. Molecules. 2017;22(2):281.
4. Chaowuttikul C, Palanuvej C, Ruangrungsi N. Chlorogenic, rosmarinic and caffeic acids contents in selected Thai medicinal plants by HPLC-DAD. Manuscript in preparation. 2017.
5. Sowemimo A, Venables L, Odedeji M, Koekemoer T, van de Venter M, Hongbing L. Antiproliferative mechanism of the methanolic extract of *Enterolobium cyclocarpum* (Jacq.) Griseb. (Fabaceae). J Ethnopharmacol. 2015;159:257-61.
6. Cinkilic N, Cetintas SK, Zorlu T, Vatan O, Yilmaz D, Cavas T, et al. Radioprotection by two phenolic compounds: Chlorogenic and quinic acid, on X-ray induced DNA damage in human blood lymphocytes in vitro. Food Chem Toxicol. 2013;53:359-63.
7. Chansukh K, Charoensup R, Palanuvej C, Ruangrungsi N. Antimicrobial activities of selected thai medicinal plants bearing quinonoids. Res J Pharm Biol Chem Sci. 2014;5(2):425-32.
8. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982;45(05):31-4.
9. Wang L-N, Wang W, Hattori M, Daneshtalab M, Ma C-M. Synthesis, Anti-HCV, antioxidant and reduction of intracellular reactive oxygen species generation of a chlorogenic acid analogue with an amide bond replacing the ester bond. Molecules. 2016;21(6):737.
10. Li Y, Huang W, Huang S, Du J, Huang C. Screening of anti-cancer agent using zebrafish: Comparison with the MTT assay. Biochem Biophys Res Commun. 2012;422(1):85-90.
11. Park KI, Park H, Nagappan A, Hong GE, Yumnam S, Lee HJ, et al. Polyphenolic compounds from Korean Lonicera japonica Thunb. induces apoptosis via AKT and caspase cascade activation in A549 cells. Oncol Lett. 2017;13(4):2521-30.
12. Magnani C, Chiari BG, Isaac VLB, Correa MA, Salgado HRN. In vitro safety evaluation of caffeic acid. Athens J Health. 2014;1(3):181-8.
13. Burgos-Morón E, Calderón-Montaño JM, Orta ML, Pastor N, Pérez-Guerrero C, Austin C, et al. The coffee constituent chlorogenic acid induces cellular DNA damage and formation of topoisomerase I– and II–DNA complexes in cells. J Agric Food Chem. 2012;60(30):7384-91.
14. Devipriya N, Sudheer AR, Menon VP. Caffeic acid protects human peripheral blood lymphocytes against gamma radiation-induced cellular damage. J Biochem Mol Toxicol. 2008;22(3):175-86.
15. Lou Z, Wang H, Zhu S, Ma C, Wang Z. Antibacterial activity and mechanism of action of chlorogenic acid. J Food Sci. 2011;76(6):M398-M403.
16. Fu L, Li Z, Xie Z, Tan Z, Zhang Q. The in vitro antibacterial activity of dried flowers used in Guangdong traditional soup. Int J Food Nutr Saf. 2015;6:106-16.