

Tropical Journal of Pharmaceutical Research May 2018; 17 (5): 905-912

ISSN: 1596-5996 (print); 1596-9827 (electronic)

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Available online at <http://www.tjpr.org><http://dx.doi.org/10.4314/tjpr.v17i5.22>

Original Research Article

Phytochemical profile, antioxidant, antimicrobial and antipancreatic lipase activities of fermented *Camellia japonica* L leaf extracts

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Sent for review: 9 January 2018

Revised accepted: 23 April 2018

Abstract

Purpose: To investigate the probable antioxidant, antimicrobial and antipancreatic lipase effects of fermented *Camellia japonica* leaf extracts.

Methods: *Camellia japonica* leaves fermented with Nuruk were extracted using methanol and ethanol. Total phenolic, flavonoid, carotenoid and L-ascorbic acid contents were determined by UV-visible spectrophotometry. The antioxidant activities of these extracts were determined by free radical scavenging, ferrous ion chelating and reducing power assays. Their antimicrobial properties against Gram-positive *Staphylococcus epidermidis* and *Bacillus subtilis*, and Gram-negative *Klebsiella pneumoniae* and *Escherichia coli* bacteria were evaluated by disc diffusion method. Inhibition of pancreatic lipase was measured based on the hydrolytic reaction of p-nitrophenyl butyrate with pancreatic lipase.

Results: The ethanol extracts of fermented *Camellia japonica* leaves exhibited higher phenolic (32274 mg GAE/100 g) and flavonoid (20519 mg RE/100 g) contents with higher superoxide ($IC_{50} = 0.23$ mg/mL), hydrogen peroxide ($IC_{50} = 0.28$ mg/mL) radical scavenging and ferrous ion chelating ($IC_{50} = 0.21$ mg/mL) activities than those of methanol. These ethanol extracts also showed higher antimicrobial activities against all bacterial strains tested with higher inhibitory effects on pancreatic lipase than methanol extracts.

Conclusion: The results highlight the possible use of fermented *Camellia japonica* leaf extracts as a source of antioxidant, antibacterial and antiobesity agents. Ethanol is recommended as solvent for extracting antioxidants, antibacterial and antiobesity agents from this plant.

Keywords: Antioxidant activity, Antimicrobial activity, Fermented *Camellia japonica* extracts, Pancreatic lipase inhibition

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INTRODUCTION

Tea is a commonly consumed beverage worldwide due to its health benefits for human

consumption. Most commercially prepared tea is obtained from differential preparation of *Camellia sinensis* (L.) Kuntze (apical buds and terminal leaves) [1]. Methods for obtaining *C. sinensis*

teas can be classified into non-fermented (green and white teas), semi-fermented (oolong and red teas) and fully fermented (black and pu-erh teas) [1]. Fully-fermented tea is fermented by microorganisms such as *Aspergillus*, *Penicillium*, *Rhizopus*, and yeast that determine its taste, color and fragrance as well as functional components [2-4]. In fermentation processes, monomeric catechins are oxidized by polyphenol oxidase leading to dimers and polymers such as theaflavin, thearubigin, and gallic acid [5]. These components are responsible for the dark coloration, lack of bitterness and health benefit of fermented teas.

Camellia japonica L. is a tree belonging to Theaceae family. It has been used in folk medicine in Korea [6]. Extensive studies have been made on constituents of *Camellia japonica*, including saponins in its fruits and seeds and triterpenes in its flowers and seed oil [6-8]. It has been reported that leaves of *Camellia japonica* possess antioxidant, antifungal, and cytotoxic properties [9-11].

Nuruk is a Korean traditional starter made from rice, corn or wheat fermented by various microorganisms such as fungi, yeast and lactic acid bacteria at low temperature with long periods. It has been used for Korean fermented food [12]. Many studies have been undertaken to investigate physiological activities of fully-fermented tea manufactured by using *Aspergillus* and *Rhizopus*. However, physiological and biochemical features of fully-fermented tea manufactured using *Nuruk* has not fully characterized yet. Therefore, the objective of this study was to evaluate antioxidant, antimicrobial and antipancreatic lipase properties of methanol and ethanol extracts of *Camellia japonica* leaves fermented with *Nuruk* as well as their bioactive contents including total flavonoids, total carotenoids, and L-ascorbic acid.

EXPERIMENTAL

Fermentation of *Camellia japonica* leaves and sample preparation

Young leaves (third to fifth leaves from the top; light green) of *Camellia japonica* L. were harvested in May 2016 from Seogwipo-si, Jeju Island, South Korea and a voucher specimen (no. KCJYL-20160521) has been deposited in the herbarium of College of Applied Life Science, Jeju National University. Young leaves of *Camellia japonica* L. were identified by Professor Jung Hyun Kim from Cheju Tourism College. Young leaves of *Camellia japonica* L. were used in the fermentation process using a previously

described protocol [4] with some modification. Briefly, 1 kg of fresh picked young leaves of *Camellia japonica* were washed and soaked in tap water for 2 h. After draining the water, soaked leaves were steamed for 1 h. A traditional wheat-based nuruk was crushed and mixed with distilled water, followed by incubation at 60 - 62 °C for 5 h. After centrifugation, 100 mL of the resulting supernatant was added to steamed leaves, mixed well and transferred to a fermentation container. This mixture was then incubated at 50 °C for two weeks. Freeze-dried powder (200 g) of fermented *Camellia japonica* leaves was extracted with 1 L of 100 % methanol or 70 % ethanol at 25 °C for 72 h with constant shaking. The extract was then purified using a filter system (Corning, NY, USA), concentrated using a rotary evaporator (Buchi Rotavapor R-200, New Castle, DE, USA), freeze dried, and stored at -20 °C until further use. These dried extracts were reconstituted in dimethyl sulfoxide (DMSO, Amresco, Solon, Ohio, USA). DMSO acts as a solvent without changing any property of methanol or ethanol extracts.

Analysis of total phenolic and flavonoid contents

Contents of total phenolics and flavonoids were determined with published method [13]. For total phenol quantitation, 30 µL of methanol or ethanol extracts of fermented *Camellia japonica* leaves was thoroughly mixed with 30 µL of 95 % ethanol, 150 µL of distilled water, 15 µL of Folin-Ciocalteu reagent and 30 µL of saturated sodium carbonate solution (5 %). After 60 min of standing at room temperature, the absorbance was read at 725 nm against a blank in a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). Content of total phenolics was calculated based on gallic acid calibration curves. It was expressed as mg gallic acid per 100 g dry matter. To determine total flavonoids, each extract (15 µL) was mixed with 4.5 µL of 5 % NaNO₂, 60 µL of distilled water and 4.5 µL of 10 % AlCl₃. After incubation at room temperature for 6 min, 60 µL of NaOH solution (4 %) was added to the mixture to reach a final volume of 150 µL with distilled water. The absorbance was measured 15 min later at 510 nm. Quantitative determination of total flavonoids (mg rutin/100 g) was done based on a standard curve of rutin.

Evaluation of total carotenoid content

An aliquot of the extracts was used for quantification of total carotenoid content using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, USA). Total carotenoid content was calculated by measuring

the absorbance at 470, 653 and 666 nm. It was expressed as mg/100 g [14]. All operations were carried out on ice under dim light to prevent photodegradation, isomerizations, or structural changes of carotenoids.

Determination of ascorbic acid content

Ascorbic acid content of the extracts was determined following published procedures [15] with some modifications. Briefly, the extract (0.15 g) was treated with 10 mL of 1% metaphosphoric acid (pH 1.86) in a rotary mixer at 200 rpm for 45 min in the dark. After centrifuging at 1600 × g for 15 min at 4 °C, the supernatant was collected. A portion (25 µL) of the supernatant was mixed with 225 µL of 2, 6-dichloroindophenol (0.3 mg/mL), and the absorbance was measured at 515 nm within 15 s. The content of ascorbic acid (mg AA/100 g) was calculated based on the calibration curve of authentic L-ascorbic acid (0.0125-1 mM).

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging assay

DPPH radical scavenging assay was conducted with published method [13]. Briefly, 100 µL of fermented *Camellia japonica* leaf extracts at various concentrations was prepared in 96-well plates and equal volume of 0.4 mM DPPH was added to each well. The solution was kept in the dark for 10 min at room temperature and the absorbance of the solution was measured at 517 nm using a Spectra MR microplate reader (Dynex Technologies, Inc, Chantilly, VA, USA).

Superoxide radical scavenging assay

Superoxide anion scavenging capacity of fermented *Camellia japonica* leaf extracts was analyzed by estimating the reduction product of nitroblue tetrazolium (NBT), as described previously [14]. The reaction mixture contained 50 mM Na₂CO buffer, 3 mM xanthine, 3 mM ethylenediamine tetraacetic acid, 0.5 mM NBT and bovine serum albumin solution. Test extracts were added to the reaction mixture and incubated at 25 °C for 10 min. The reaction was started by adding xanthine oxidase (XO) (0.25 units/mL). After incubation at 25 °C for another 25 min, absorbance was recorded at 560 nm using an ELISA reader, against blank samples, which did not contain XO enzyme.

Hydrogen peroxide radical scavenging assay

The hydrogen peroxide scavenging activity of methanol or ethanol extract of the fermented

Camellia japonica leaves was measured using a previous method [14].

Nitric oxide radical scavenging assay

Nitric oxide scavenging assay was performed by published method described by Im *et al* [14]. Briefly, the reaction mixture (100 µL) containing 10 mM sodium nitroprusside in phosphate-buffered saline (pH 7.0), with or without of fermented *Camellia japonica* leaf extracts at concentrations of 0.125, 0.25, 0.5, 1 and 2 mg/mL, was incubated at 25 °C for 3 h. After the incubation, reaction mixture was mixed with an equal amount of Greiss reagent (1 % sulfanilamide and 0.1 % N-1-naphthylethylene diamine dihydrochloride in 2.5% polyphosphoric acid) and, incubated at 25 °C for another 5 min and the absorbance at 540 nm was then measured using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, USA).

Ferrous ion chelating assay

The chelating ability was determined according to the method of Im *et al* [14]. An aliquot of 250 µL of each methanol and ethanol extract of fermented *Camellia japonica* leaves was mixed with 5 µL of 2 mM ferrous chloride (FeCl₂). The reaction was initiated by adding 10 µL of 5 mM ferrozine. After 10 min of incubation at room temperature, the absorbance was determined at 562 nm using a microplate reader.

Reducing power assay

Reducing power assay was carried out as described previously by Huang *et al* [16]. The methanol and ethanol extract solutions (concentration range 12.5 to 200 µg/mL) were mixed with 200 mM sodium phosphate buffer (pH 6.6) containing 1 % (w/v) potassium ferricyanide. After the mixture was incubated at 50 °C for 20 min, 10 % (w/v) trichloroacetic acid was added. The mixture was then added into each well of a 96-well plate, and the absorbance was measured at 700 nm. IC₅₀ value (mg/mL), the effective concentration at which the absorbance was 0.5 for reducing power, was obtained by interpolation from linear regression analysis.

Evaluation of antimicrobial activity

Bacterial strains used in this study included *Staphylococcus epidermidis*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Escherichia coli*. They were kindly provided by Prof. Ju Sung Kim (Jeju National University). Sterilized nutrient broth was inoculated with the test bacteria and incubated at 37 °C overnight. Disc diffusion assay was carried

out to detect the antimicrobial activity of fermented *Camellia japonica* leaf extracts using published method [17,18] with slight modification. Briefly, discs (8.0 mm) were overlaid with fermented *Camellia japonica* leaf extracts overnight and then dried overnight in a drying oven at 40 °C. Discs were placed on agar medium and incubated at 37 °C for 12 - 18 h. Antimicrobial activity was determined by calculating the diameter of the growth inhibition zone (mm) around the disc. Effects were compared with that of standard antibiotic ampicillin (10 µg/disc). Vehicle (DMSO) alone served as negative control.

Pancreatic lipase inhibitory assay

Lipase inhibitory activity of fermented *Camellia japonica* leaf extract was estimated based on ability to inhibit *in vitro* porcine pancreatic lipase activity as described previously [19] with slight modifications. Briefly, the reaction mixture consisted of 6 µL of 10 mg/mL of porcine pancreatic lipase and 170 µL of Tris buffer. Then 20 µL of fermented *Camellia japonica* leaf extract was added and incubated at 37 °C for 15 min. Four microliters of 10 mM p-NPB was then added and incubated at 37 °C for 60 min.

Lipase activity was determined by measuring the hydrolysis of p-NPB to p-nitrophenol at 400 nm using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, USA). IC₅₀, the concentration of a tested compound showing 50 % inhibition of the enzyme activity, was evaluated from the least square regression line of logarithmic concentration plotted against inhibitory activity. Hesperidin was used as a positive control [19].

Statistical analysis

Results were expressed as mean ± standard deviation (SD) of triplicate values. They were analyzed with SPSS version 12.0 using a non-parametric test (Mann-Whitney U test). A dose-response curve was plotted to determine IC₅₀ values, defined as the concentration sufficient to obtain 50 % of the maximum scavenging

capacity. Correlations among data obtained were analyzed using Pearson's correlation coefficient. Significant difference was considered at $p < 0.05$.

RESULTS

Contents of total phenol, total flavonoid, total carotenoid, and L-ascorbic acid

Table 1 shows total phenolic, flavonoid, carotenoid and L-ascorbic acid contents in methanol and ethanol extracts of fermented *Camellia japonica* leaves. Contents of total phenols and flavonoids were significantly higher in ethanol extract (32274 and 20519 mg GAE/100 g, respectively) than those in methanol extract (27791 and 19273 mg GAE/100 g, respectively) ($p < 0.05$). However, methanol extract of fermented *Camellia japonica* leaves contained higher total carotenoids and L-ascorbic acid contents than ethanol extract ($p < 0.05$) (Table 1).

Antioxidant activities

Ethanol extracts of fermented *Camellia japonica* leaves exhibited significantly higher superoxide and hydrogen peroxide scavenging activities than methanol extracts ($p < 0.05$) (Table 2). However, methanol and ethanol extracts showed similar scavenging activities for DPPH and nitric oxide radicals (Table 2). Results of metal chelating ability of methanol and ethanol extracts of fermented *Camellia japonica* leaves are also shown in Table 1.

Chelating abilities of both extracts were increased with increasing in concentration. Metal chelating abilities of methanol and ethanol extracts at concentration of 0.5 mg/mL of ferrous ions were 85 % and 97 %, respectively. The IC₅₀ value of the chelating effect of methanol extract was 0.28 mg/mL, which was higher than that of ethanol extract (0.21 mg/mL) ($p < 0.05$) (Table 2). Ethylenediaminetetraacetic acid (EDTA), at 0.5 mg/mL used as positive control showed 99 % chelating ability on ferrous ion.

Table 1: Total polyphenol, flavonoid, carotenoid and L-ascorbic acid contents in methanol and ethanol extracts of *Camellia japonica* fermented leaves

Solvent	Total polyphenol (mg GAE/100 g)	Total flavonoid (mg RE/100 g)	Total carotenoid (mg/100 g)	L-Ascorbic acid (mg AA/100 g)
100% MeOH	27791±336	19273±416	1711±24	491±31
70% EtOH	32274±240 [†]	20519±291	1586±15 [†]	258±25 [†]

Abbreviations: GAE = gallic acid equivalent, RE = rutin equivalent, AA = L-ascorbic acid. Data were statistically different from the value of methanol extract ($p < 0.05$)

These results suggest that fermented *Camellia japonica* leaf extracts are capable of scavenging free radicals and preventing harmful effects of free radicals.

Reducing power of fermented *Camellia japonica* leaf extract was determined using ascorbic acid as a positive control. The reducing ability of extract was increased with increasing concentration. IC₅₀ values of the fermented *Camellia japonica* leaf extracts were 0.46 - 0.47 mg/mL (Table 2). The maximum absorbance for crude extract was 0.51 - 0.52 at 0.5 mg/mL compared to 0.64 for ascorbic acid at 0.25 mg/mL the positive control.

Correlation between antioxidant components and antioxidant activity

Knowledge about antioxidant properties and antioxidant components of fermented *Camellia japonica* leaf extracts is limited. Thus, linear correlation studies were carried out with results obtained from this study. As shown in Table 3, strong correlations between three antioxidant components (total polyphenols, total flavonoids and L-ascorbic acid), and free radicals (DPPH, hydrogen peroxide and nitric oxide) scavenging and chelating activities were found ($r^2 = 0.814$ - 0.999 , $p < 0.05$). In addition, total carotenoid was

weakly correlated with DPPH and nitric oxide scavenging activity ($r^2 = 0.761$ and 0.796 , respectively). On the other hand, all antioxidant components (total polyphenols, total flavonoids, total carotenoids and L-ascorbic acid) did not show correlation with reducing power activity with $r^2 < 0.3$. Furthermore, total polyphenol or total flavonoid content was not highly correlated with superoxide radical scavenging activity ($r^2 = 0.520$ and 0.667 , respectively).

Antimicrobial activity

Antibacterial activities of fermented *Camellia japonica* leaf extracts were assayed *in vitro* by agar disc diffusion against four bacterial species. Table 4 summarizes results of microbial growth inhibition by methanol or ethanol extract of fermented *Camellia japonica* leaves. Antimicrobial activities of both extracts were concentration-dependent (Table 4).

Ethanol extract showed higher antimicrobial activities against both Gram-positive and Gram-negative bacteria than methanol extract. Ten micrograms of ampicillin (positive control) showed wide zones of inhibition against all test organisms (7.0-8.7 mm). DMSO negative control showed no zone of inhibition (Table 4).

Table 2: IC₅₀ values of antioxidant activity of *Camellia japonica* fermented leaf extracts

Solvent	IC ₅₀ (mg/mL)					Chelating activity on ferrous ions	Reducing power activity
	Radical scavenging activity						
	DPPH	Superoxide	Hydrogen peroxide	Nitric oxide			
100% MeOH	0.23±0.004	0.33±0.03	0.36±0.01	0.35±0.01	0.28±0.04	0.46±0.05	
70% EtOH	0.22±0.003	0.23±0.02	0.28±0.01	0.35±0.01	0.21±0.03	0.47±0.06	

IC₅₀ was obtained by interpolation from linear regression analysis. Each value is expressed as mean ± standard deviation ($n = 3$). Data are statistically different from the value of methanol extract ($p < 0.05$)

Table 3: Coefficients of correlation between antioxidant compounds and antioxidant activities of methanol and ethanol extracts of the fermented *Camellia japonica* leaves

Antioxidant	DPPH	Superoxide	Hydrogen peroxide	Nitric oxide	Chelating effect on ferrous ions	Reducing power
Total polyphenol	0.943*	0.520	0.994*	0.959*	0.999*	0.002
Total flavonoid	0.996*	0.667	0.969*	0.997*	0.933*	0.271
Total carotenoid	0.761	0.254	0.891*	0.796	0.941*	0.299
L-Ascorbic acid	0.814*	0.279	0.922*	0.855*	0.964*	0.196

All values are absolute value of correlation coefficients; $p < 0.05$ is considered statistically significant

Table 4: Antimicrobial activities of extracts of fermented *Camellia japonica* leaves against *S. epidermidis*, *B. subtilis* subsp., *K. pneumoniae* and *E. coli*

Antimicrobial	Inhibitory zone (diameter, mm)			
	<i>S. epidermidis</i>	<i>B. subtilis</i> subsp.	<i>K. pneumoniae</i>	<i>E. coli</i>
Ampicillin (10 µg) (positive control)	8.7±0.29 [*]	8.7±0.29 [*]	7.0±0.01 [*]	8.2±1.04 [*]
DMSO (negative control)	-	-	-	-
100% MeOH	4 mg	0.3±0.29	-	0.3±0.58
	8 mg	1.0±0.50	0.8±0.29	1.5±0.50 [†]
	16 mg	3.3±0.58	3.5±0.50	3.5±0.50
70% EtOH	4 mg	0.8±0.29	-	0.5±0.50
	8 mg	1.7±0.58	1.7±0.58	1.8±0.58 [†]
	16 mg	3.7±0.58	3.7±0.58	4.0±0.87 [†]

Each value is expressed as mean ± standard deviation ($n = 3$). Data are statistically different from the value of DMSO (negative control) ($p < 0.05$)

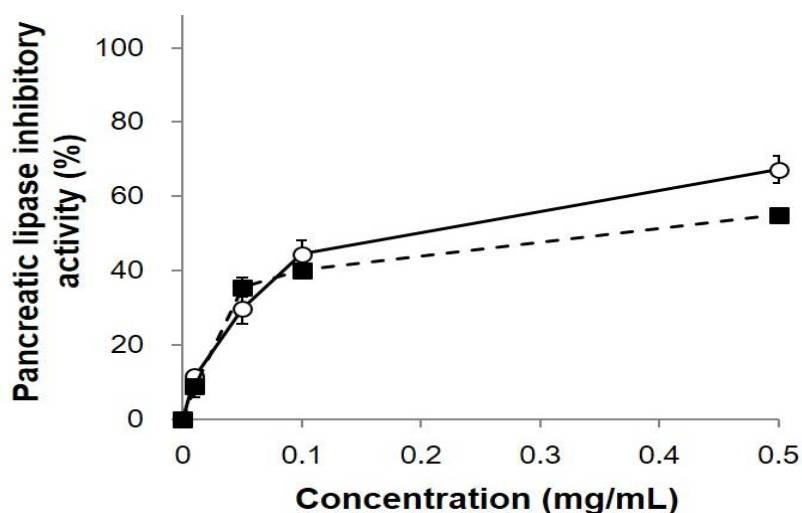


Figure 1: Pancreatic lipase inhibitory activity of methanol (○) and ethanol (■) extracts of fermented *Camellia japonica* leaves. Each value is expressed as mean ± standard deviation ($n = 3$). Data are statistically different from the value for ethanol extract ($p < 0.05$).

Pancreatic lipase inhibition activity

In the current study, methanol and ethanol extracts of the fermented *Camellia japonica* leaves inhibited the lipase activity in a dose-dependent manner in the assay system with p-NPB as a substrate (Figure 1). Methanol extract showed higher activity than ethanol extract ($p < 0.05$). Methanol extract showed significantly higher lipase inhibition effect ($IC_{50} = 0.308$ mg/mL) than ethanol extract ($IC_{50} = 0.397$ mg/mL) (Figure 1) ($p < 0.05$). Nevertheless, its activity was weak (IC_{50} value of 0.308 mg/mL) than that of hesperidin (IC_{50} value of 0.032 mg/mL), the positive control.

DISCUSSION

Nuruk is a dry cake of wheat, barley, and rice that hosts a variety of wild yeasts, bacteria, and

koji mold spores. It is useful for saccharification of starch during fermentation [20].

In the current study, amounts of total phenolics (32274 mg/100 g) and flavonoids (20519 mg/100 g) in ethanol extract of fermented *Camellia japonica* leaves were higher than those in methanol extract (Table 1). This indicates that ethanol is a relatively more efficient extraction solvent for polyphenolic compounds from fermented *Camellia japonica* leaves. In a previous study, ethanol extracts of pu-erh tea fermented by *M. pilosus* contained 12500 mg/100 g of total phenolics [21]. In the present study, 70 % ethanol extract contained phenolic compound of 32274 mg/100 g (Table 1). Phenolic compounds are aromatic secondary plant metabolites. They mainly include flavonoids, carotenoids, and L-ascorbic acid with high antioxidant activity. Their consumption has

been linked to decreased risk of chronic and degenerative diseases [22].

Results from superoxide- and hydrogen peroxide-scavenging activity and metal chelation assay revealed that fermented *Camellia japonica* leaf extracts could act as antioxidants (Table 2). Moreover, high correlations between antioxidant capacities and total phenolic and flavonoid contents of fermented *Camellia japonica* leaf extracts were observed (Table 3). The antioxidant activity of phenolics is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have metal chelating potential [23].

Some commercial antibiotics used today have unwanted side effects or antimycotic resistance [24]. Thus, there is a need to search for natural products with antimicrobial activity. Plants remain the most common source of antimicrobial agents [25]. Many aromatic plants have been used traditionally in folk medicine as well as in food preservatives to extend shelf life and improve the safety of food by inhibiting the growth of bacteria, fungi, and yeast [25]. In the present study, fermented *Camellia japonica* leaf extracts had antimicrobial activity all bacteria strains tested (Table 4). In a previous study, antimicrobial effects of pu-erh tea extracts on *S. aureus* were 64%, whereas those of *B. subtilis* were 50% [26]. These antimicrobial activities of tea might be affected by bacteria strains tested. Therefore, further studies are necessary to investigate antimicrobial activity of fermented *Camellia japonica* leaf extracts with more diverse strains.

Obesity is associated with increased risks of metabolic syndrome, diabetes mellitus, hypertension and some cancers [27]. Obesity is a common, serious, and costly health problem in developed nations, it accounts for 2 – 6 % of total health care costs [27]. *Orlistat*, a potent competitive inhibitor of gastric and pancreatic lipase, has been approved by FDA as an antiobesity drug [28]. However, this medication is associated with side-effects such as intestinal flatulence, borborygmic, and abdominal cramps [28]. Therefore, searching potent lipase inhibitors from natural plant extracts might be a useful approach to treat obesity.

CONCLUSION

Camellia japonica leaves fermented with *Nuruk* were shown to possess potential antioxidant, antimicrobial and lipase inhibitory effects. Thus, they might have, potential in pharmaceutical and food applications. Additional studies are needed

to elucidate the detailed mechanism underlying the effect of fermented *Camellia japonica* leaves.

DECLARATIONS

Acknowledgement

This research was supported by the 2018 scientific promotion program funded by Jeju National University.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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