Journal of Medicinal Plants Research Vol. 6(3), pp. 424-432, 23 January, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1125
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

In vitro antioxidant properties and induced G_2/M arrest in HT-29 cells of dichloromethane fraction from *Liriodendron tulipifera*

Lan Wang[1], Gui-Fang Xu[2], Xiao-Xia Liu[3], Ai-Xia Chang[4], Ming-Lu Xu[2], Amal Kumar Ghimeray[5], Jing-Pei Piao[5] and DongHa Cho[5]*

[1]College of Life Science, Henan Normal University, Xinxiang, Henan, 453-007, P.R. China.
[2]Henan Institute of Science and Technology, Xinxiang, Henan, 453-003, P.R. China
[3]School of Biology and Chemical Engineering, Jiaxing University, Jiaxing 314001, China.
[4]Institute of Tobacco, Chinese Academy of Agriculture Science, Qingdao 266000, China
[5]Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon, Gangwon-do, 200-701, Korea.

Accepted 9 December, 2011

The antioxidant and anti-proliferative effects of dichloromethane fraction from methanol extract of *Liriodendron tulipifera* were investigated in this study. Scavenging effects on the α,α-diphenyl-picrylhydrazyl (DPPH) and nitrite radicals, and protection against reactive oxygen species induced lipid, protein and deoxyribonucleic acid (DNA) damage were evaluated. The dichloromethane fraction of *L. tulipifera* exhibited high antioxidant activities to scavenge DPPH and nitrite radicals, ferric reducing power and protection against biological macromolecular oxidative damage. Furthermore, the dichloromethane fraction of *L. tulipifera* exhibits the anti-proliferative activity and induction of the G_2/M arrest in human colon tumor HT-29 cells. These results demonstrate potential antioxidant activities and anti-proliferative effect of *L. tulipifera*, as an antioxidant for diseases caused by reactive oxygen species.

Key words: Free radical, lipid peroxidation, protein oxidation, DNA damage, G_2/M arrest.

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in many physiological responses in the body. A wide variety of compounds with antioxidative properties have been used to attenuate ROS/RNS formation and protect DNA, lipid and protein damage against oxidative stress both *in vitro* and *in vivo*. However, some synthetic antioxidant compounds have found side effect. For instance, the oxidative characteristics and/or metabolites of butylated hydroxytoluene (BHT) may contribute to carcinogenicity or tumorigenecity (Umemura et al., 2002). Therefore, natural products from the plant source are growing interest of research. *Liriodendron tulipifera* L. belonging to family magnoliaceae is also known as Tulip or Yellow popler tree. This tree has an immense use in pharmaceutical industry due to the presence of phytochemical constituents including sesquiterpene and apophine alkaloids, etc (Muhammad and Hufford, 1989; Chen and Chang, 1978). The tree was used as a quinine replacement in the United States Civil War (Hasegawa, 2007). Specially, the bark of *L. tulipifera* was used by the Native Americans as a tonic, stimulant and febrifuge (Graziose et al., 2010), and was also used to treat the intermittent fevers associated with malaria (Rafinesque et al., 1828; Graziose et al., 2010). Recently, there are also reports of inhibitory effect of lipiferolide against farnesyl protein transferase (FPTase) (Moon et al., 2007). However, there is very limited research on anticancer and antioxidant activity by *L. tulifera*. Therefore, our aim of study was to examine the antioxidant and anti-proliferative activities of *L. tulipifera* extracts.

*Corresponding author. E-mail: chodh@kangwon.ac.kr. Tel: +82332506475. Fax: +82332506470.

MATERIALS AND METHODS

Preparation of sample extraction

The dried *L. tulipifera* stems were extracted three times with 70% MeOH, 85% MeOH, 95% MeOH sequencing at 45°C for 12 h, respectively, then filtered through filter paper (100 mm; Whatman, Maidstone, UK) and concentrated under reduced pressure by a rotary evaporator machine (CCA-1110; EYELA, Tokyo, Japan). The extracts were suspended in water and then partitioned and defatted three times with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. After removed the solvents, the dichloromethane fraction of *L. tulipifera* was used to study its bioactivities.

Total phenolic content (TPC)

Total phenolic content of the *L. tulipifera* was determined by the Folin-Ciocalteu method using gallic acid as a standard (Gutfinger, 1981). Briefly, 1 ml *L. tulipifera* was mixed with 1 ml of 50% Folin-Ciocalteu reagent and 1 ml of Na_2CO_3 (5%) and centrifuged at 5000 rpm for 5 min after 30 min of incubation at room temperature. The absorbance was measured with a spectrophotometer (Bio-Tek; Winooski, VT, USA) at 750 nm. TPC were expressed as gallic acid equivalents (GAE) in mg/g dw.

Total flavanoid content (TFC)

Total flavonoid content in the *L. tulipifera* was estimated by the aluminum colorimetric method using quercetin as a standard (Park et al., 1997). 0.5 ml LTD was mixed with 0.1 ml of aluminiumchloride hexahydrate (10%), 0.1 ml of potassium acetate (1 M), 2.8 ml of deionized water and 1.5 ml ethanol (95%). The absorbance was measured with a spectrophotometer (Bio-Tek; Winooski, VT, USA) at 490 nm after 40 min of incubation in the dark at room temperature. TFC were expressed as quercetin equivalents (QE) in mg/g dw.

DPPH free radical scavenging assay

The DPPH radical scavenging activity of the *L. tulipifera* was evaluated by using the protocol of (Yoshie-Stark et al., 2004). In 0.2 ml *L. tulipifera* at different concentration (50, 100, 500 or 1000 µg/ml), 0.2 ml of 0.2 mM DPPH (methanolic solution) was added. Further, the mixture was incubated at room temperature for 30 min and the absorbance was measured with a spectrophotometer (Bio-Tek; Winooski, VT, USA) at 515 nm. The results were expressed as EC_{50}, that is, the concentration of antioxidant required to quench 50% of the initial DPPH radicals under the given experimental conditions. The results were compared with L-Ascorbic acid and BHA as standards.

Ferric-reducing activity

The reducing power of the *L. tulipifera* was determined according to the method of Yildirim et al. (2001). In brief, 1 ml of *L. tulipifera* in different concentration (10 to 100 µg/ml) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of (1% w/v) $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 30 min, followed by the addition of 2.5 ml trichloroacetic acid (10%, w/v). The resulting mixture was centrifuged at 3000 rpm for 10 min and 2.5 ml supernatant was mixed with equal volume of water. Finally, 0.5 ml of (0.1% w/v) $FeCl_3$ solution was added and the absorbance was measured at 700 nm. L-ascorbic acid was used as positive controls at the concentration of 50 µg/ml. Values presented are the means of triplicate analysis.

Ferrous ion chelating capacity assay

Ferrous ion chelating capacity was measured by the method of Dinis et al. (1994). Briefly, 0.1 ml *L. tulipifera* sample at different concentration (50 to 1000 µg/ml) was mixed with 0.1 ml (0.2 mM) $FeCl_2 \cdot 4H_2O$. The volume was raised by adding 3 ml deionized water and the mixture was stored at room temperature for 30 s. The reaction mixture was added with 0.1 ml 5 mM ferrozine and changes in the absorbance of the Fe^{2+}–ferrozine complex were monitored at 562 nm against a blank with a spectrophotometer after 10 min of incubation at room temperature. Ethylene diaminetetraacetic acid (EDTA) was used as a positive control.

Measurement of nitrite scavenging ability

The nitrite scavenging ability of the *L. tulipifera* was determined according to a method using Griess reagent (Kato et al., 1987). Briefly, 1 ml *L. tulipifera* was mixed with 1 ml of 1 mM nitrite sodium. Then the mixture was added to 8 ml of 0.2 M citrate buffer (pH 1.2), and incubated for 1 h at 37°C. After incubation, 1 ml solution/ supernatant was withdrawn and added to 2 ml of 2% acetic acid and 0.4 ml of Griess reagent (1% sulfanilic acid and 1% naphthylamine in a methanol solution containing 30% acetic acid). After vigorous mixing, the mixture was placed at room temperature for 15 min, and measured the absorbance at 520 nm. Quercetin was used as positive control.

Lipid peroxidation assay

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formation using egg yolk as lipid-rich media (Banerjee et al., 2005). 0.5 ml egg yolk homogenate (10% v/v) and *L. tulipifera* (50 to1000 µg/ml) were added to a test tube. To induce lipid peroxidation, $FeSO_4$ (0.07 M) were added and incubated at 37°C for 30 min. Then, 20% acetic acid (pH 3.5), 0.8% (w/v) thiobarbituric acid (TBA) (prepared in 1.1% sodium dodecyl sulfate), and 20% trichloroacetic acid (TCA) were added to the mixture and vortexed and heated in a boiling water bath for 60 min. After cooling, *n*-butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the upper layer was measured at 540 nm. BHT was used as a positive control to measure the lipid peroxidation inhibition.

Plasmid DNA damage

To determine the protect effect of *L. tulipifera* on the plasmid DNA, Oxidation of 2 µg of plasmid DNA (pET 28) by 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH, 10 mM) was incubated at 37°C for 30 min, with different concentrations of *L. tulipifera* in phosphate buffer saline (PBS) (10 mM, pH 7.4). Plasmid DNA was loaded onto a 1.2% agarose gel and visualized under ultraviolet (UV) light after staining with ethidium bromide. L-Ascorbic acid was used as a positive control.

Determination of protein oxidation

Protein oxidation was assayed as described by Kwon et al. (2000) with minor modifications. Oxidation of bovine serum albumin (BSA) in PBS was initiated by AAPH (20 mM) and incubated with various concentrations of the *L. tulipifera* or quercetin (standard). After incubation for 24 h at 37°C. 0.02% BHT was added to prevent the

Table 1. Total phenolics and flavonoid contents and DPPH radical scavenging ability (EC_{50}) of dichloromethane fraction of *L. tulipifera.*

Extract	Total phenolics (mg GAE/g extract)	Total flavonoids (mg QE/g extract)	DPPH EC_{50} (µg/ml)
L. tulipifera	103.56 ± 0.45	32.87 ± 0.71	119.15 ± 2.04
L-Ascorbic acid	–	–	12.81 ± 0.95
BHA	–	–	15.57 ± 0.63

Note: The results are presented as the mean ± SD. Different values indicate significance at $p<0.05$ value.

formation of further peroxyl radical. The proteins were then assayed with normal sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Biored, USA).

Cell lines and cell culture

The human colon tumor cell line HT-29 and HEK293 were obtained from the american type cell culture (Rockville, MD, USA). The HT-29 cells were cultured in RPMI-1640 medium (Gibco, MD, USA) and HEK293 cells were grown in dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 5% CO_2.

Cell cytotoxicity assay

The cytotoxicity effect of *L. tulipifera* was measured using the standard 3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetra- zolium bromide (MTT) assay. Cells (2×10^5 cells/ml) were incubated in 96-well plates for 24 h and then exposed to 50, 100, 150, or 200 µg/ml of *L. tulipifera* for 48 h. Then, 50 µl MTT reagents (2 mg/ml in PBS) were added to each well. After 4 h incubation at 37°C the culture medium was removed and 180 µl dimethyl sulfoxide (DMSO) was added to each well. The metabolized MTT product was dissolved in DMSO and quantified by measuring the optical density at 550 nm on a microplate reader (Bio-Tek,Winooski, VT, USA). Data were calculated as the percentage of inhibition by the following formula:

$$\text{Inhibition (\%)} = (1- A_s/A_b)\times 100\%$$

A_s and A_b indicated the optical density of cell lines incubated with samples and blank control, respectively.

Cell cycle analysis

HT-29 cells (2×10^6 cells/ml) were seeded in 6-well plates for 24 h, then, were exposed to different concentration (50, 100, 150, or 200 µg/ml) of *L. tulipifera* for 48 h. Afterwards, cells were harvested, washed with cold PBS and fixed in 70% ethanol for 1 to 2 h at −20°C. The resulted sample were then stained with propidium iodide (PI) solution (20 µg/ml PI, 0.1 mM EDTA, 10 µg/ml RNase, and 1% Triton X-100 in PBS) for 30 min in the dark. DNA content was measured using a Facial Action Coding System (FACS) flow cytometer analysis system (Becton Dickinson Inc. USA), where at least 10,000 cells were analyzed for each experimental treatment. Data analysis was performed using WinMDI version 2.9 cell cycle analysis software (Scripps Research Institute).

Statistical analyses

All measurements were performed in triplicate and analyses of variance were conducted by the general linear model procedure using SPSS software. ANOVA one-way were used to test for significant differences between the mean values for the treatments ($P < 0.05$).

RESULTS AND DISCUSSION

Content of TPC, TFC and DPPH radical scavenging activity

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989). Recently, many reports have shown that the total phenol contents and the antioxidant have positive correlation (Xu et al., 2010; Gulcin et al., 2002).

Many antioxidants react with DPPH by hydrogen atom transfer or electron transfer (Niki, 2010). The inhibition or scavenging effect of antioxidant over DPPH free radicals can be monitored by observing the decrease absorbance in UV spectrometer at 517 nm. This is probably the simplest way to calculate the antioxidant potential of the sample. In our research, the total phenolics, total flavonoids content and free radical scavenging activity of *L. tulipifera* were given in Table 1. The amount of TPC and TFC was found to be 103.56 mg GAE/g extract and 32.87 mg QE/g extract, respectively. In this study, the EC_{50} of *L. tulipifera* was 119.15 µg/ml (Table 1). This result showed that the *L. tulipifera* has good potential to scavenge free radicals. The EC_{50} of L-ascorbic acid and BHA as positive standards were 12.81 and 15.57 µg/ml, respectively.

Ferric-ion reducing activity

The ferric reducing assay measures the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants. The reducers (that is, antioxidants) cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form.

In our research, the reducing ability of *L. tulipifera* was found to increase with the increasing concentration of *L. tulipifera*. The reducing powers of *L. tulipifera* from 10 to 100 µg/ml were 0.087 to 0.988, whereas, the reducing power of L-ascorbic acid (standard) was 1.127 at 50 µg/ml (Figure 1).

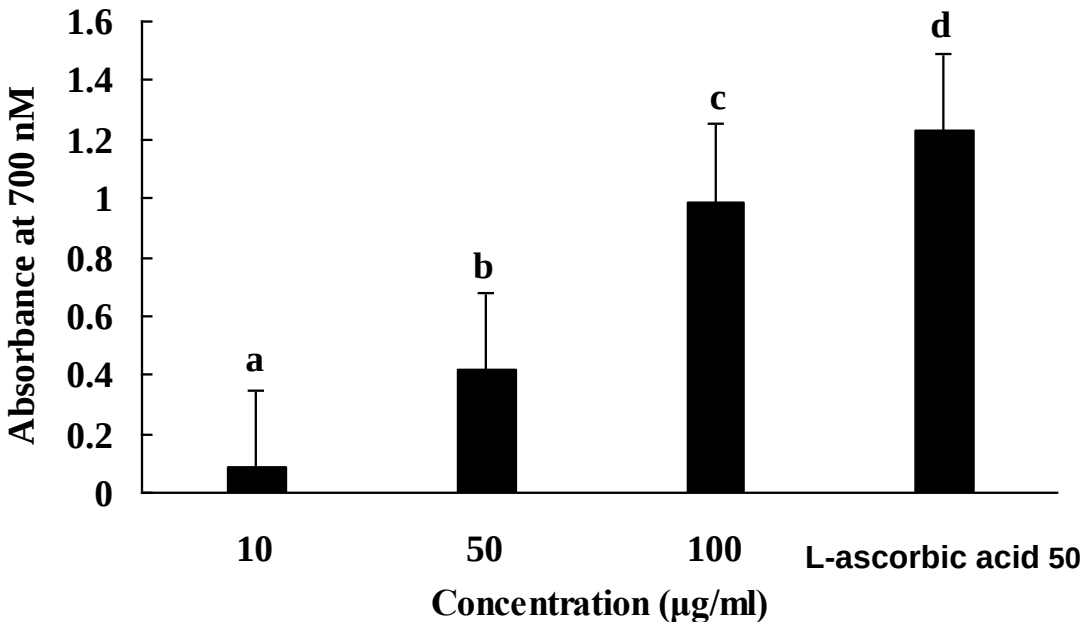

Figure 1. Reducing power of *L. tulipifera* (10 to 100 µg/ml) and L-ascorbic acid (50 µg/ml). Each value is expressed as mean ± standard deviation (n = 3).

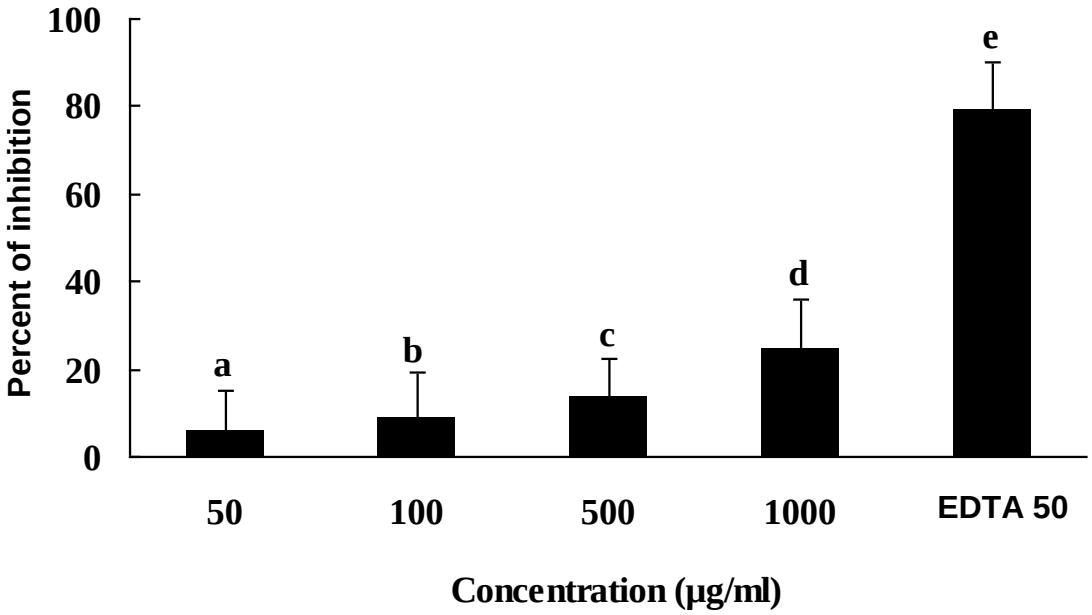

Figure 2. Ferrous ion chelating effect of *L. tulipifera* (50 to1000 µg/ml) in comparison with standard EDTA (50 µg/ml). Each value is expressed as mean ± standard deviation (n = 3).

Ferrous ion chelating activity

Ferrous ion chelating activity is also considered as one of the property of antioxidant. Iron is known as the most important transition metals in lipidoxidation prooxidant due to its high reactivity (Suganthy et al., 2010). In this study, ferrous ion chelating activities of *L. tulipifera* and standard (EDTA 50 µg/ml) were shown in Figure 2. The result showed that the ferrous ion chelating activity of *L. tulipifera* increased in a dose-depend manner. However, compared to the ferrous ion chelating of standard EDTA, the *L. tulipifera* could be considered as a moderate

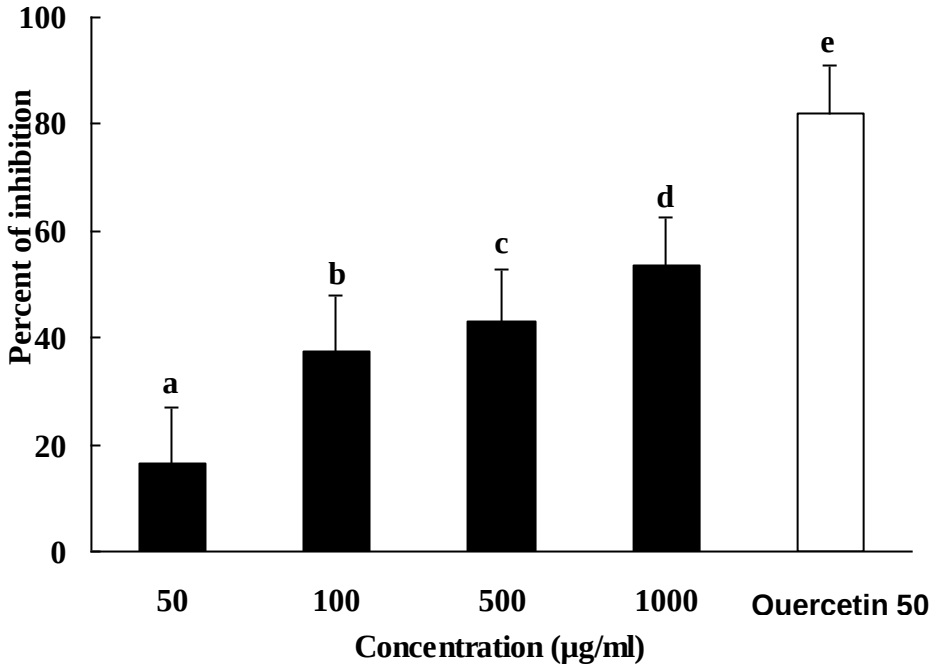

Figure 3. Nitric oxide scavenging activity of *L. tulipifera* (50 to 1000 µg/ml) and quercetin (50 µg/ml) as positive control. Each value is expressed as mean ± standard deviation (n = 3).

ferrous ion chelating agent.

Nitric oxide scavenging activity

Sodium nitroprusside spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions in physiological pH that can be estimated using Griess reagent. NO react O_2^- to produce reactive peroxynitrite ($ONOO^-$), which causes serious damage to lipids, protein and nucleic acids (Moncada et al., 1991). Our data revealed that the *L. tulipifera* scavenged nitric oxide in concentration- dependent manner and inhibited almost 53% nitric oxide generation at 1000 µg/ml (Figure 3). The difference between *L. tulipifera* and quercetin was statistically significant ($P < 0.05$).

Inhibition of lipid peroxidation

Lipid peroxides are involved in numerous pathological events, like metabolic disorders, inflammation and aging. Lipid peroxidation leads to cell membrane disruption and cell damage (Upasani et al., 2001). Therefore, to prevent these, antioxidants have the capablity to scavenge peroxy radicals. It is known that, egg yolk lipids undergo rapid non-enzymatic peroxidation and the absorbance at 595 nm increased when incubated with ferrous sulphate. The effect of *L. tulipifera* on non-enzymatic peroxidation was shown in Figure 4. The results revealed that the *L. tulipifera* inhibited lipid peroxidation in a dose-dependently manner from 14.65 to 46.85% at 50 to 1000 µg/ml. BHT as the positive standard inhibited 53.87% lipid peroxidation at 50 µg/ml.

Protection of DNA oxidation

AAPH is a water–soluble initiator, which decomposes into alkyl radicals at physiological condition, then react to oxygen and produce alkyl peroxyl radicals to initiate DNA oxidative fragmentation (Cai et al., 2003). According to our result, as shown in Figure 5, when pET28 plasmid DNA were exposed to AAPH, some single-stranded nicked circular plasmid DNA were damaged, however, linear plasmid DNA was observed. Therefore, the results clearly showed the protective effect of *L. tulipifera* against oxidation of DNA at the concentration of above 100 µg/ml. This can be compared with L-ascorbic acid which exhibited good protection activity at 50 µg/ml.

Protection of protein oxidation

Under many pathological conditions cellular proteins get oxidized. The vulnerability of various amino acid residues of proteins to oxidation varies with reactive oxygen species (Ames et al., 1993). The protection against

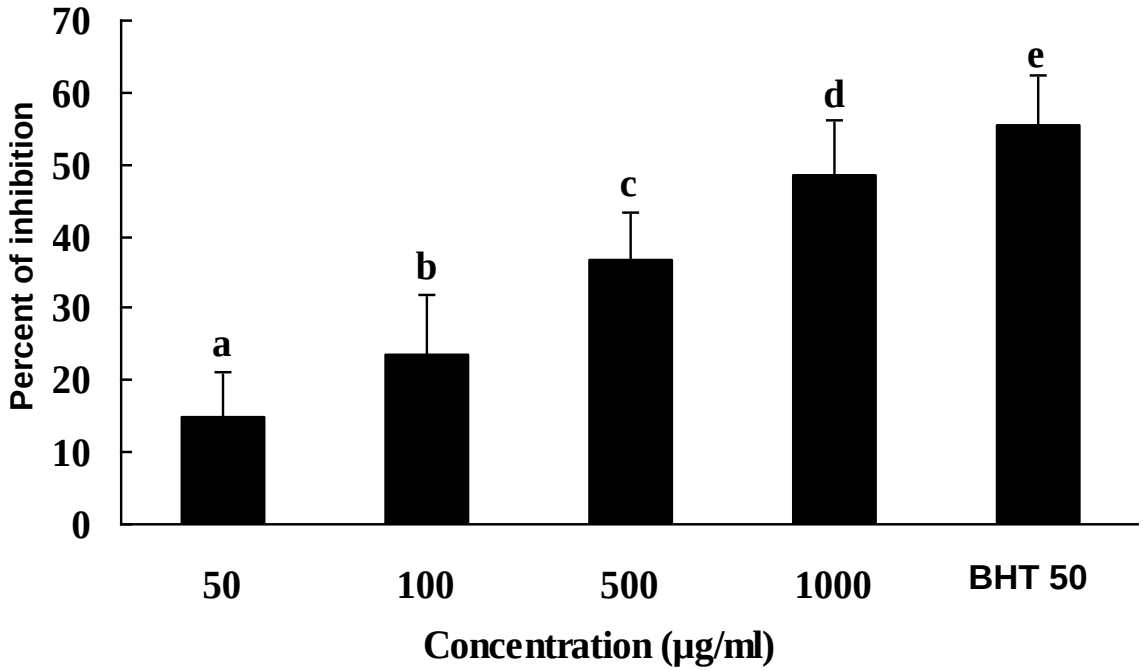

Figure 4. Inhibition of lipid peroxidation by *L. tulipifera* (50 to1000 µg/ml) and BHT (50 µg/ml). Each value is expressed as mean ± standard deviation (n = 3).

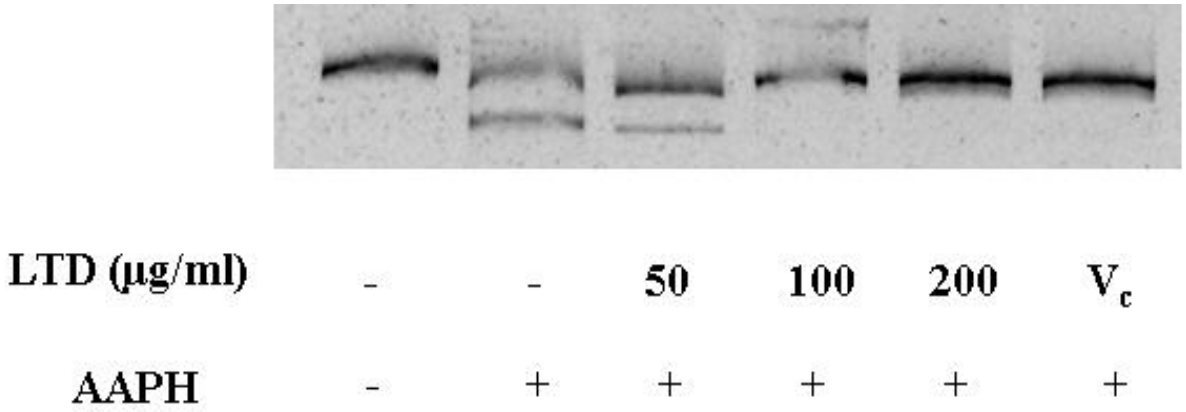

Figure 5. Protective activity of *L. tulipifera* against oxidation of DNA. Lane 1: non- treated with AAPH (control); Lane 2: without antioxidant (negative control); Lane 3 to 6: 50 to 200 µg/ml *L. tulipifera*; Lane 7: 50 µg/ml Vc (50) as positive control.

protein oxidative damage was determined by the oxidation of BSA initiated by AAPH. In this result, after the incubation of BSA (2 mg/ml) with different concentration of *L. tulipifera* (50, 100, 200 and 400 µg/ml) and L-ascorbic acid (standard) for 24 h at 37°C, the normal SDS–PAGE results demonstrated that, *L. tulipifera* exhibited significant protective effect against oxidation of BSA in a concentration depend manner (Figure 6A and B). The effect of *L. tulipifera* at the concentration of 50 µg/ml was similar with L-ascorbic acid at same concentration.

Cytotoxicity and anti-proliferation assay

Figure 7 showed the cytotoxicity and anti-proliferation activity of *L. tulipifera* in dose- dependent manner. The results showed that *L. tulipifera* has showed no cytotoxicity under 200 µg/ml in human cell lines (HEK293). However, the effect of *L. tulipifera* assessed

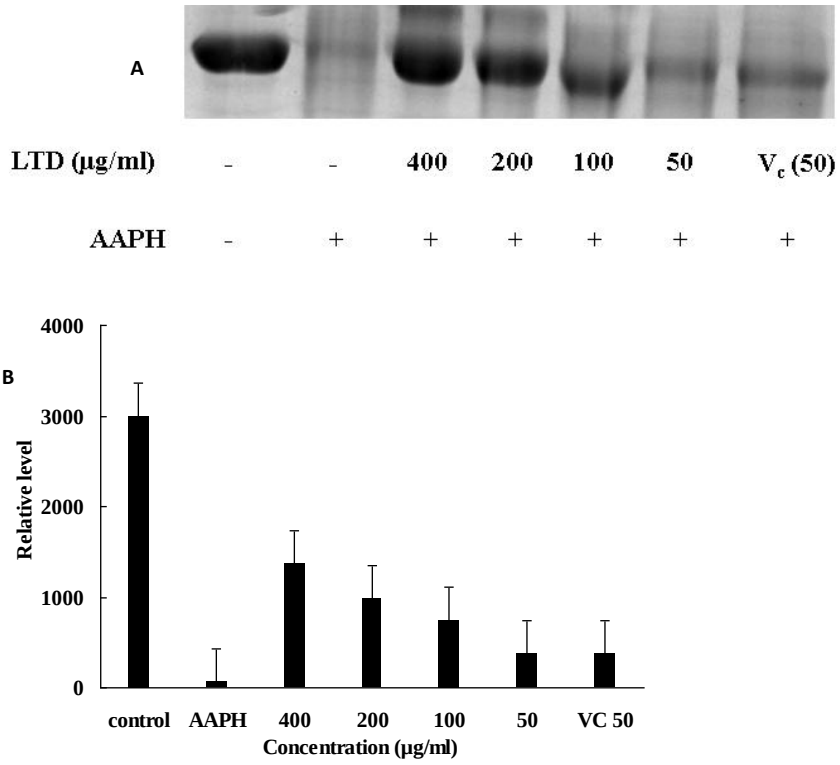

Figure 6. Protective activity of *L. tulipifera* against oxidation of BSA. Lane 1: non-treated with AAPH (control); Lane 2: without antioxidant (negative control); Lane 3 to 6: 400 to 50 µg/ml *L. tulipifera*; Lane 7: 50 µg/ml Vc as positive control.

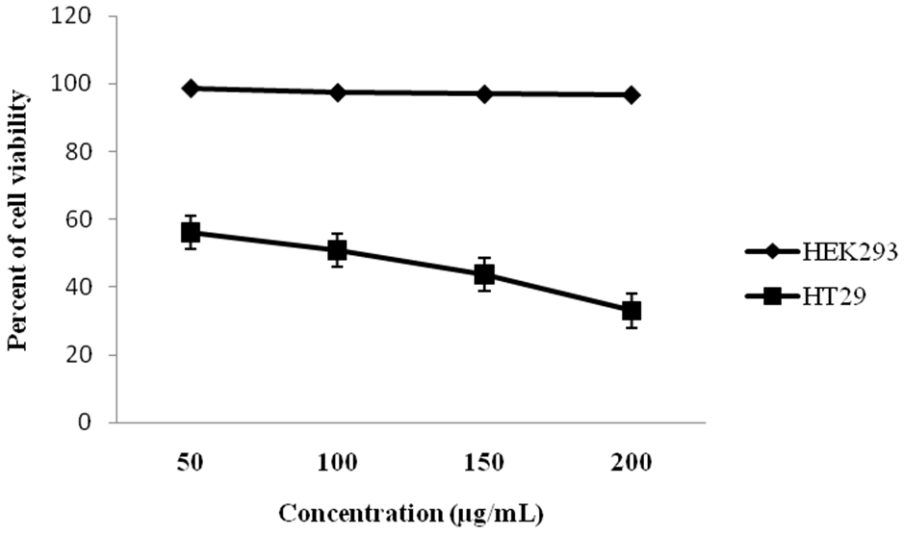

Figure 7. The cell viability of HEK293 and HT-29 cells by *L. tulipifera*. Data are the mean ± SD of three independent experiments.

on the viability of HT-29 cells was decreased in a dose-dependent manner (Figure 7). This showed a potent anti-proliferative effect on HT-29 cancer cells by *L. tulipifera*.

Cell cycle analysis

Further to prove the anti-proliferative effect of *L. tulipifera*

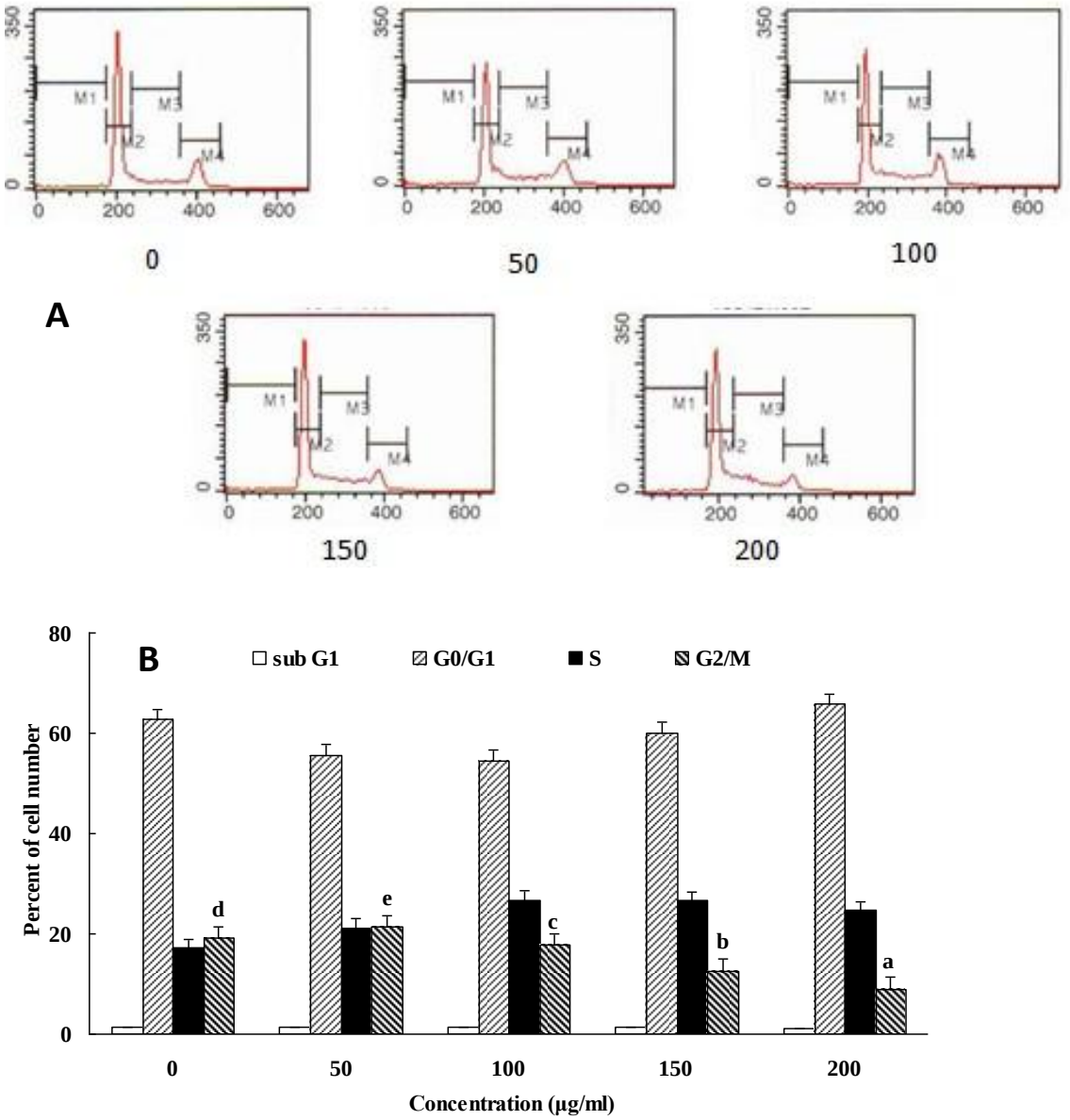

Figure 8. Flow cytometric analysis of cell cycle progression after *L. tulipifera* treatment for 48 h. Data are the mean ± SD of three independent experiments.

we performed cell cycle analysis. After treatment of HT-29 cells with *L. tulipifera* at different concentration, the percentage of the G_2/M phase of cells decreased in a concentration-dependent manner, indicating that it inhibited the cellular proliferation of HT-29 cells via G_2/M phase arrest of the cell cycle (Figure 8A and B). Overall, it can be concluded that *L. tulipifera* has a potent antioxidant capacity, and also has an anti-proliferative effect by inducing cell cycle arrest at G_2/M phase in HT-29 cells. So far, we know this is the first report on anti-proliferation effect of *L. tulipifera* extract, these findings are useful in the treatment of oxidative stress and ROS related diseases. However further studies are required to explore the mechanisms of its action.

ACKNOWLEDGMENTS

This work was partially supported by a grant from Institute of Bioscience and Biotechnology of Kangwon National University.

REFERENCES

Ames BN, Shigenaga MK, Hagen TM (1993). Oxidants, antioxidants and the degenerative diseases of aging. Proc. Natl. Acad. Sci. U.S.A., 90: 7915-7922.

Banerjee A, Dasgupta N, De B (2005). *In vitro* study of antioxidant activity of *Syzygium cumini* fruit. Food Chem., 90: 727-733.
Cai YJ, Fang JG, Ma LP, Li Y, Liu ZL (2003). Inhibition of free radical induced peroxidation of rat liver microsomes by resveratrol and its analogues. Biochim. Biophys. Acta, 1637: 31-38.
Chen CL, Chang HM (1978). Lignans and aporphine alkaloids in bark of *Liriodendron tulipifera*. Phytochemistry, 17: 779-782.
Dinis TCP, Madeira VMC, Almeida LM (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch. Biochem. Biophys., 315: 161-169.
Gulcin I, Oktay M, Kufrevioglu OI, Aslan A (2002). Determination of antioxidant activity of Lichen *Cetraria islandica* (L). J. Ethnopharmacol., 79: 325-329.
Gutfinger T (1981). Polyphenols in olive oils. J. Am. Oil Chem. Soc., 58: 966-968.
Hasegawa GR (2007). Quinine substitutes in the confederate army. Mil. Med., 172: 650-655.
Hatano T, Edamatsu R, Mori A, Fujita Y, Yasuhara E (1989). Effect of interaction of tannins with co-existing substances. VI. Effects of tannins and related polyphenols on superoxide anion radical and on DPPH radical. Chem. Pharm. Bull., 37: 2016-2021.
Kato H, Lee IE, Chuyen NV, Kim SB, Hayase F (1987). Inhibition of nitrosamine formation by nondialyzable melanoidins. Agric. Biol. Chem., 51: 1333-1338.
Kwon HY, Choi SY, Won MH, Kang TC, Kang JH (2000). Oxidative modification and inactivation of Cu, Zn-superoxide dismutase by 2,20-azobis (2- amidinopropane) dihydrochloride. Biochim. Biophys. Acta, 1543: 69-76.
Moncada S, Palmer RMJ, Higgs EA (1991). Nitric oxide: physiology, pathology and pharmacology. Pharmacol. Rev., 43: 109-142.
Moon MK, Oh HM, Kwon BM, Baek NI, Kim SH, Kim JS, Kim DK (2007). Farnesyl protein transferase and tumor cell growth inhibitory activites of Lipiferolide isolated from Liriodendron tulipifera. Arch. Pharm. Res., 30: 299-302.
Muhammad I, Hufford CD (1989). Phenylpropanoids, sesquiterpenes, and alkaloids from the seeds of *Liriodendron tulipifera*. J. Nat. Prod., 52: 1177-1179.
Niki E (2010). Assessment of antioxidant capacity *in vitro* and *in vivo*. Free Rad. Biol. Med., 49: 503-515.
Park YK, Koo MH, Ikegaki M, Contado JL (1997). Comparison of the flavonoid aglycone contents of *Apis mellifera* propolis from various regions of Brazil. Arquiv. Biol. Technol., 40: 97-106.
Rafinesque CS, Atkinson A (1828). Medical Flora, or, Manual of the Medical Botany of the United States of North America. In: Rafinesque, C.S., Atkinson, Alexander (Eds.), Alexander, Philadelphia.
Suganthy N, Arif NS, Karutha PS, Pandima DK (2010). Antioxidant and metal chelating potential of the solvent fractions of *Gelidiella acerosa*, the red algae inhabiting South Indian coastal area. Biomed. Pharmacother., pp. 1-6.
Umemura T, Kodama Y, Hioki K, Nomura T, Nishikawa A, Hirose M, Kurokawa Y (2002). The mause rasH2/BHT model as an *in vivo* rapid assay for lung carcinogens. Jpn. J. Cancer Res., 93: 861.
Upasani CD, Khera A, Balaraman R (2001). Effect of lead and vitamin E, C or spiruline on malondialdehyde, conjugated dienes and hydro peroxides in rat. Indian J. Exp. Biol., 39: 70-74.
Xu ML, Wang L, Hu JH, Lee SK, Wang MH (2010). Antioxidant activities and related polyphenolic constituents of the methanol extract fractions from *Broussonetia papyrifera* stem bark and wood. Food Sci. Biotechnol., 19: 677-682.
Yildirim A, Mavi A, Kara AA (2001). Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. J. Agric. Food Chem., 49: 4083-4089.
Yoshie-Stark Y, Wäsche A (2004). *In vitro* binding of bile acids by lupin protein isolates and their hydrolysates. Food Chem., 88: 179-184.