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Antioxidant Activity of *Glossogyne tenuifolia*

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Glossogyne tenuifolia is a native traditional anti-inflammatory herb in Taiwan. It has previously been shown that the ethanol extract of *G. tenuifolia* (GT) inhibited the LPS-induced inflammatory mediator release from murine macrophage cell line and human whole blood. In the present work, the ethanol extracts of *G. tenuifolia* and its major constituent, luteolin-7-glucoside, were shown to be scavengers of 1,1-diphenyl-2-picrylhydrazyl, superoxide, and hydroxyl radicals. Moreover, copper-induced low-density lipoprotein oxidation was suppressed by GT and luteolin-7-glucoside as measured by decreased formation of malondialdehyde and conjugated diene as well as reduced electrophoretic mobility. GT and luteolin-7-glucoside were also against *N*-formyl-methionyl-leucyl-phenylalanine-induced reactive oxygen species (ROS) production in human polymorphonuclear neutrophils and peripheral blood mononuclear cells. In summary, these data indicated that GT is a potential ROS scavenger and may prevent atherosclerosis via inhibiting LDL oxidation or ROS production in human leukocytes. Moreover, luteolin-7-glucoside may serve as the active principal of GT.

KEYWORDS: ***Glossogyne tenuifolia*; reactive oxygen species; LDL; oxidation; luteolin; antioxidant**

INTRODUCTION

It has been well recognized that free radicals are formed in living organisms as a function of endogenous biochemical processes. Of particular relevance in aerobic systems are those reactive species derived from the metabolism of oxygen, which include hydrogen peroxide, singlet oxygen, superoxide radicals, and hydroxyl radicals, collectively known as the reactive oxygen species (ROS) (*1*). ROS are generated by several mechanisms, such as interaction of ionizing radiation with biological molecules, as well as produced by cellular respiration and phagocytic cells, namely, neutrophils and macrophages, which are crucial in the host innate immune system against invading microorganisms. Neutrophils and macrophages are readily mobilized to sites of infection and ingest microorganisms. Ingested bacteria are killed by ROS derived from superoxide produced by an activated, phagosome-bound NADPH-dependent oxidase (*2*, *3*). Superoxide anion is then quickly converted to hydrogen peroxide (H_2O_2) either spontaneously or via superoxide dismutase (SOD) (*4*). Although the production of ROS plays an important role in the killing of microorganism, excess production of these toxic metabolites damages the surrounding tissues during inflammation (*5*).

ROS have a huge potential for causing cellular components, such as DNA, protein, and lipid damages. It is widely acknowledged that the accumulation of oxidative damages in intracellular macromolecules is an essential element in the aging process and in certain degenerative diseases such as cancer, immune system decline, brain dysfunction, cataracts, and coronary heart disease (*6*). There is considerable evidence indicating that atherogenesis is initiated and promoted by lipid oxidation of low-density lipoprotein (LDL), ultimately leading to modification of apolipoprotein B such that the LDL particle becomes recognized by the macrophage scavenger receptor(s) and produces massive cholesterol loading and foam cell formation (*7*).

Lots of experimental and epidemiologic data have indicated that dietary antioxidants might reduce the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation. The antioxidants are not limited to well-known antioxidants such as ascorbic acid or α-tocopherol. Many natural components such as phenolic compounds are effective antioxidants (*8*).

Glossogyne tenuifolia Cassini (Hsiang Ju grass) is a plant native to Penghu, Taiwan. It is a popular herb tea for preventing sunstroke and has a long history of being used as an antipyretic, hepatoprotective, and anti-inflammatory remedy in folk medicine among local residents. Our laboratory has previously demonstrated that the ethanol extract of *G. tenuifolia* and its active components, oleanolic acid and luteolin-7-glucoside, inhibited the LPS-induced NO, PGE_2, IL-1β, IL-6, IL-12, and TNF-α synthesis in the murine macrophage cell line RAW 264.7 (*9*). The ethanol extract of *G. tenuifolia* also exhibited a strong inhibitory effect on the release of TNF-α, IL-6, and IFN-γ in activated human whole blood and on the secretion of hepatitis

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B virus surface antigen (HBsAg) in the hepatocellular carcinoma cell line PLC/PRF/5 (*10*).

It is well-known that a variety of anti-inflammatory natural components also possess antioxidant activity (*11*−*15*). In view of these, the possibility that *G. tenuifolia* has free radical scavenging activity arose. To better understand whether dietary supplement of *G. tenuifolia* could prevent oxidative stress, the biochemical properties of the ethanol extract of *G. tenuifolia* and its major components toward free radical production, LDL oxidation, and ROS release in activated polymorphonuclear neutrophils (PMN) and peripheral blood mononuclear cells (PBMC) were investigated. Results of the present study facilitate our understanding of the antioxidant effects of *G. tenuifolia* and its bioactive constituents.

MATERIALS AND METHODS

Materials. *G. tenuifolia* was obtained from the Kaohsiung District Agricultural Improvement Station in Penghu, Taiwan, and the voucher specimens were deposited in the herbarium of Chia-Nan University of Pharmacy and Science.

Ethanol Extraction and Fractionation of *G. tenuifolia*. The dry above-ground part (5.46 kg) was extracted with ethanol (10 L) at room temperature four times followed by filtration. The flow-through was concentrated in vacuo to yield a dark-brown syrup (623.4 g), which was diluted to lower concentrations with ethanol.

The crude ethanol extract of *G. tenuifolia* (GT) *(*623.4 g) was partitioned with hexane and 95% methanol. The 95% methanol layer was concentrated and then partitioned with ethyl acetate and water. The resulting aqueous solution was again partitioned with *n*-butanol and water. Oleanolic acid (17.1 g) was obtained by recrystallization of the insoluble particles existing in the hexane layer with acetone and methanol. Luteolin-7-glucoside (total, 4.92 g) existed widely in several partitions. It could be isolated from the ethyl acetate layer by passing through silica gel chromatography (70−230 mesh, Merck) followed by eluting with hexane/CH_2Cl_2 (1:1). It could also be obtained by recrystallization of the insoluble particles existing in ethyl acetate and water layers with acetone and methanol. These isolated compounds were identified by direct comparison (^{1}H, ^{13}C NMR[14]) with authentic samples (*9*). **Figure 1** shows the structures of oleanolic acid, luteolin-7-glucoside, and its aglycon, luteolin.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Capacities of GT and Its Major Constituents. The ethanol extract of *G. tenuifolia* and its major constituents, namely, oleanolic acid and luteolin-7-glucoside, as well as its aglycon, luteolin, were evaluated for activities to scavenge the stable DPPH radical (0.1 mM) according to the method of Dinis et al. (*16*). The affinity of the test material to quench the DPPH free radical was evaluated according to the equation

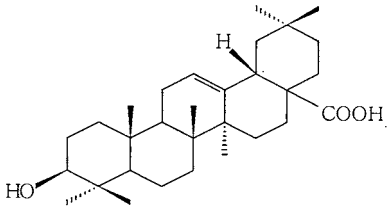
$$\text{scavenging \%} = \frac{A_c - A_s}{A_c} \times 100$$

where A_s and A_c are absorbance at 517 nm of the reaction mixture with samples and with vehicle control, respectively.

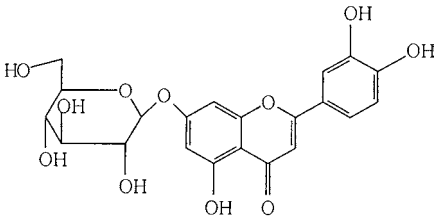
Superoxide Radical Scavenging Capacities of GT and Its Major Constituents. Superoxide scavenging effects of the ethanol extract of *G. tenuifolia* and its major constituents were measured using the modified lucigenin-enhanced chemiluminescence (CL) method (*17*). Briefly, the reaction was carried out in a mixture containing 80 μL of 1 mM lucigenin (in PBS) and 10 μL of vehicle, GT or its major constituents. Subsequently, 5 μL of xanthine oxidase (0.02 unit/mL) was added. The reaction was started by the addition of 5 μL of xanthine (0.03 M). The superoxide-induced lucigenin CL during the first 15 s was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated.

Hydroxyl Radical Scavenging Capacities of GT and Its Major Constituents. The hydroxyl radical scavenging effects of the ethanol extract of *G. tenuifolia* and its major constituents were measured using a modified luminol-enhanced CL method (*17*). Briefly, the reaction was carried out in a 100 μL mixture containing 5 mM luminol (in PBS), ferrous chloride (40 μM), 1% H_2O_2, and vehicle, various concentrations of GT or its major constituents. The hydroxyl-induced luminol CL during the first minute was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated.

Oleanolic acid



luteolin-7-glucoside



luteolin

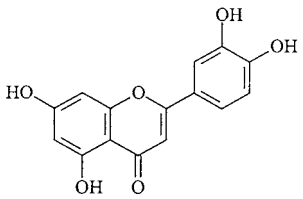


Figure 1. Structures of oleanolic acid, luteolin-7-glucoside, and luteolin.

Preparation and Oxidation of LDL. LDL ($d = 1.019-1.063$) was prepared from the plasma of healthy donors by sequential ultracentrifugation (*18*). Lipoprotein was desalted and concentrated by filtration (Centricon 4, Amicon, Beverly, MA) against PBS at 450g and 4 °C for 120 min. The protein concentration was measured according to the method of Bradford (*19*), using bovine serum albumin as a standard.

Oxidation of LDL was carried out by incubating EDTA-free LDL (0.1 mg/mL) with 10 μM Cu^{2+} in PBS in the presence of vehicle, different concentrations of the ethanol extract of *G. tenuifolia* or its major constituents, at 37 °C.

Analysis of LDL Oxidation. Peroxidation of LDL was measured by the determination of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents (*20*). The quantity of conjugated diene in LDL was assessed by monitoring the change at A_{234} (*21*). To measure the electrophoretic mobility, the ox-LDL was concentrated by filtration (Centricon 4) at 450g and 4 °C for 120 min. About 1∼2 μL of each concentrated sample was loaded onto Titan lipoprotein gel (Helena Laboratories, Beaumont, TX) and run at 80 V for 45 min. The electrophoretic mobility of LDL was determined by Fat Red 7B staining according to the manufacturer's instructions.

Preparation of Human PMN and PBMC. PMN and PBMC were isolated from heparinized blood donated by healthy volunteers using Ficoll−Paque (Amersham Pharmacia, Uppsala, Sweden) density gradient centrifugation according to manufacturer's instructions. The isolated PMN and PBMC were resuspended in RPMI-1640 medium containing 2 mM glutamine and 2.5% autologous plasma.

Measurement of *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP)-Induced ROS Production in Human PMN and PBMC. ROS production was measured using a modified luminol-enhanced CL method (*22*). The CL response of PMN and PBMC was measured using a microtiter plate luminometer within 5 h after blood collection. Each well, which contained 7×10^5 PMN or PBMC, 1 mM luminol, and vehicle, various concentrations of the ethanol extract of *G. tenufolia* or its major constituents, was incubated at room temperature for 15

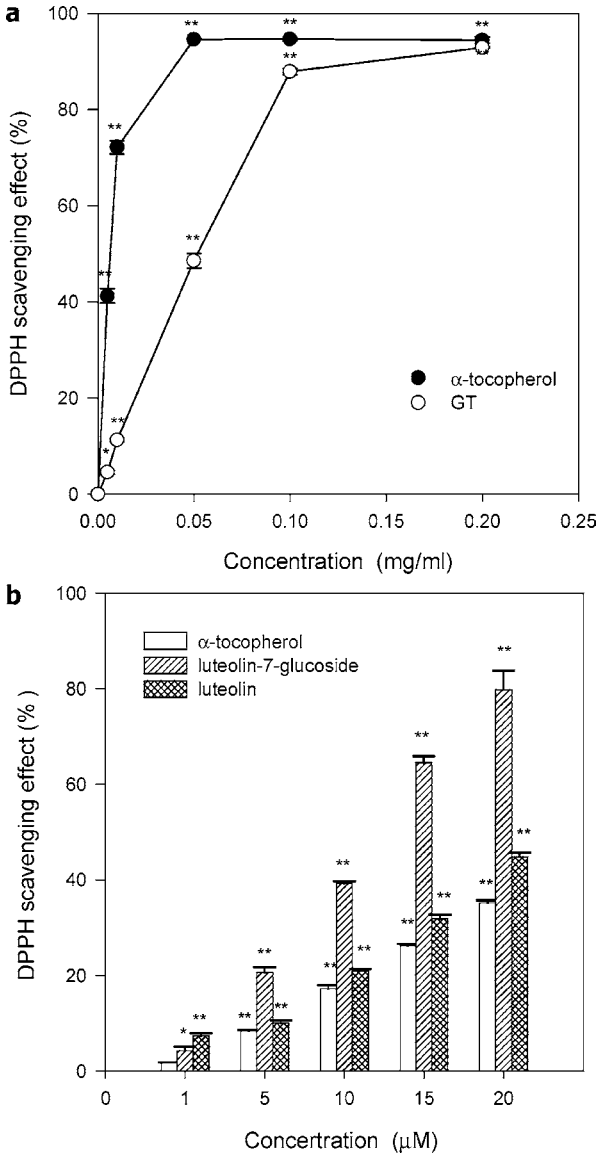


Figure 2. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as stable free radical scavengers: (**a**) DPPH scavenging effects of GT and α-tocopherol; (**b**) DPPH scavenging effects of α-tocopherol, luteolin-7-glucoside, and its aglycon, luteolin. The reaction was performed in 1 mL of solution containing 0.1 mM of freshly prepared DPPH in methanol and various concentrations of GT, luteolin, or luteolin-7-glucoside. After incubation at 37 °C for 30 min, the absorbance at 517 nm was measured in triplicate, and the scavenging effect was calculated against vehicle control. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

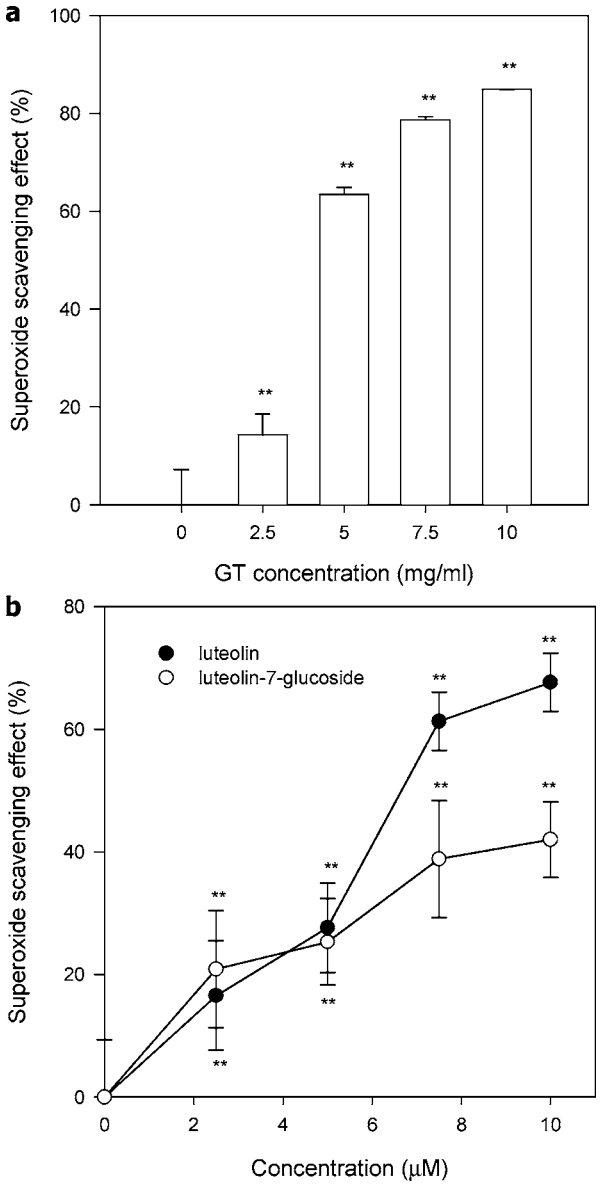


Figure 3. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as superoxide scavengers: (**a**) superoxide scavenging effect of GT; (**b**) superoxide scavenging effects of luteolin and luteolin-7-glucoside. Xanthine and xanthine oxidase were used to generate superoxide radicals, and their inhibition by GT was recorded by a luminometer. The lucigenin-enhanced superoxide chemiluminescence during the first 15 s was averaged. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period, and the kinetic curve was obtained.

Statistical Analysis. All experiments were repeated at least three times. The results were analyzed by Student's unpaired *t* test, and a *p* value of <0.05 was taken to be significant.

RESULTS

Free Radical Scavenging Activities of GT. To evaluate the possible antioxidant activity of GT, we started by investigating stable free radical (DPPH), superoxide ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$) scavenging actions. **Figure 2a** demonstrates that the DPPH scavenging effect of GT was in a dose-dependent manner. The potency of GT was less than the positive control, α-tocopherol, at the same concentration. The estimated IC_{50} values for GT and α-tocopherol are approximately 55.2 and 6.7 μg/mL, respectively.

To further investigate the antioxidant activity of GT, lucigenin- and luminol-enhanced CL were employed to evaluate the superoxide and hydroxyl radical scavenging activities, respectively. Superoxide produced by xanthine/xanthine oxidase caused an increase in lucigenin-enhanced CL from a basal level of 100 to 3.61×10^5 RLU. The addition of GT (2.5−10 mg/mL) markedly inhibited superoxide radical induced lucigenin-enhanced CL in a dose-dependent manner with an IC_{50} of ∼5.18 mg/mL (**Figure 3a**).

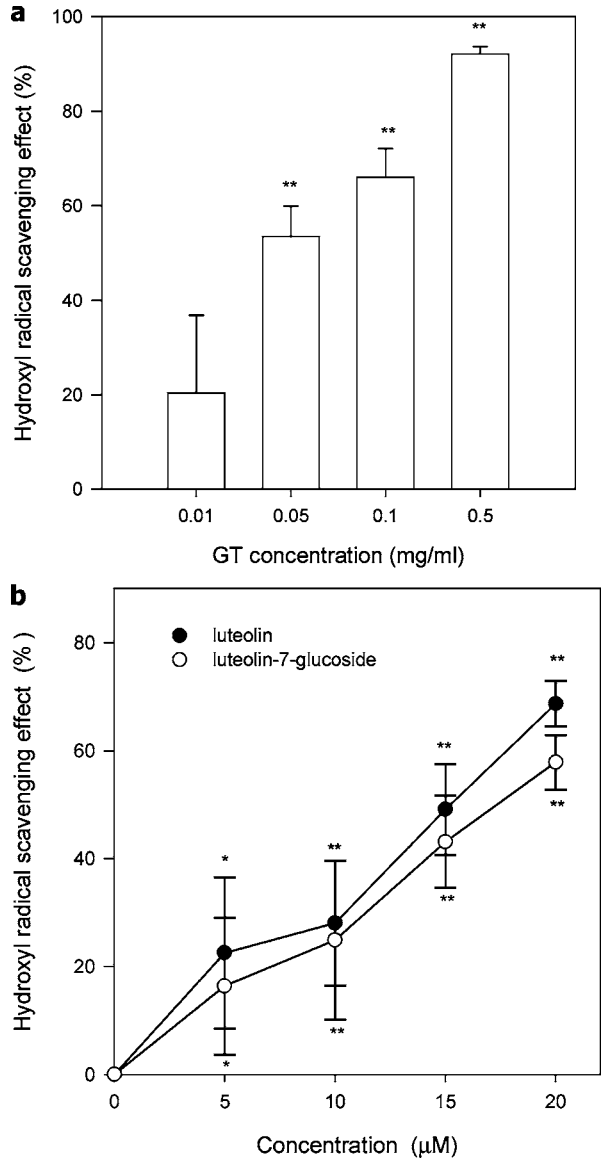


Figure 4. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside as hydroxyl radical scavengers: (**a**) hydroxyl radical scavenging effect of GT; (**b**) hydroxyl radical scavenging effects of luteolin and luteolin-7-glucoside. Reaction by H_2O_2 and $FeCl_2$ was used to generate hydroxyl radicals, and their inhibition by luteolin or luteolin-7-glucoside was recorded by a luminometer. The hydroxyl-induced luminol chemiluminescence (CL) was monitored every 5 s during the first 1-min observation period. The inhibitory efficiency in response to the CL of vehicle control was calculated. Data represent the mean ± SEM ($n = 12$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

Hydroxyl radical (•OH), which is formed nonenzymatically from hydrogen peroxide (H_2O_2) in a metal-dependent reaction, is the most reactive and toxic oxygen radical known to date (*23*). CL, a highly sensitive method, has been widely used for monitoring •OH production (*24*). In our experiment, hydroxyl radicals generated by H_2O_2- and $FeCl_2$-mediated Fenton reaction caused an increase of luminol CL from 87 to 6.1×10^5 RLU. **Figure 4a** shows that the addition of GT (0.01−0.5 mg/mL) exerted a dose-dependent scavenging activity on Fenton-mediated hydroxyl radicals with an IC_{50} of ∼60 μg/mL.

Free Radical Scavenging Activities of the Major Constituents of GT. We have previously identified oleanolic acid and luteolin-7-glucoside as the major constituents of GT (*9*). To test whether they are the active antioxidant principals, we performed the aforementioned tests. **Figure 2b** demonstrates that luteolin-7-glucoside possessed higher DPPH scavenging potency than its aglycon, luteolin, and a positive control, α-tocopherol. The estimated IC_{50} values for luteolin-7-glucoside and luteolin are 12.2 and 22.8 μM, respectively.

Lucigenin-enhanced CL showed that xanthine/xanthine oxidase-generated superoxide could be inhibited by luteolin and luteolin-7-glucoside (up to 10 μM) dose-dependently. Luteolin (>8 μM) showed a higher inhibitory potency than luteolin-7-glucoside did (**Figure 3b**). The estimated IC_{50} values are 11.0 and 7.1 μM for luteolin-7-glucoside and luteolin, respectively.

Figure 4b shows that luteolin inhibited Fenton-mediated hydroxyl radical production slightly but insignificantly stronger than luteolin-7-glucoside did. The estimated IC_{50} values are 17.6 and 15.0 μM for luteolin-7-glucoside and luteolin, respectively. On the other hand, oleanolic acid did not have any detectable DPPH, superoxide, or hydroxyl radical scavenging action (data not shown). This result indicated that the flavonoid instead of triterpenoid is the component responsible for the ROS scavenging activity of GT.

Inhibition of LDL Oxidation by GT. It is recognized that oxidatively modified low-density lipoproteins (ox-LDL) play an important role in the generation and progression of the atherosclerotic plaque (*25*). In vitro inhibition of the copper-catalyzed oxidation of LDL is a widely used strategy for the evaluation of antioxidant activity of natural components (*26*). To study the effect of GT as an antioxidant in preventing copper-induced oxidation of LDL, three different approaches were employed to measure changes in several parameters known to be associated with LDL oxidation: formation of TBARS and conjugated dienes during lipid peroxidation and increase in the electrophoretic mobility of LDL due to apolipoprotein B100 modification (*27*). As shown in **Figure 5a**, incubation of LDL (0.1 mg/mL) with Cu^{2+} (10 μM) at 37 °C for 3 h was responsible for the MDA formation increase from 0.35 ± 0.17 to 61.06 ± 1.28 nmol/mg of LDL. GT treatment produced dose-dependent reduction in MDA formation. GT (10 μg/mL) inhibited the MDA formation by ∼97.3% as compared with those of the vehicle group with an estimated IC_{50} of 7.5 μg/mL.

Oxidation of LDL is accompanied by an increase in absorbance at 234 nm, due to the formation of conjugated dienes in constituent polyenoic fatty acids (*28*). As shown in **Figure 6a**, LDL treated with GT, BHT, and α-tocopherol (20 μg/mL) showed a longer resistance to copper-initiated LDL oxidation, and this was indicated by a prolonged lag phase of lipid oxidation.

The ability of GT to inhibit the alteration in the surface charge of the apolipoprotein B100 when LDL was incubated with copper ions was monitored by observing the effects on electrophoretic mobility by agaorse gel electrophoresis. **Figure 7a** shows that treatment of native LDL (0.1 mg/mL) with copper (10 μM) at 37 °C overnight increased the electrophoretic mobility. LDL treated with GT (6.5−10 μg/mL) significantly reduced copper-induced LDL oxidation dose-dependently as indicated by decreased mobility shift. GT (10 μg/mL) reduced the electrophoretic mobility by ∼70%, as compared with the vehicle group.

Inhibition of LDL Oxidation by Major Constituents in GT. To study the possible phytochemicals present in GT responsible for inhibiting LDL oxidation, we analyzed copper-induced (10 μM) LDL oxidation in the presence of luteolin-7-

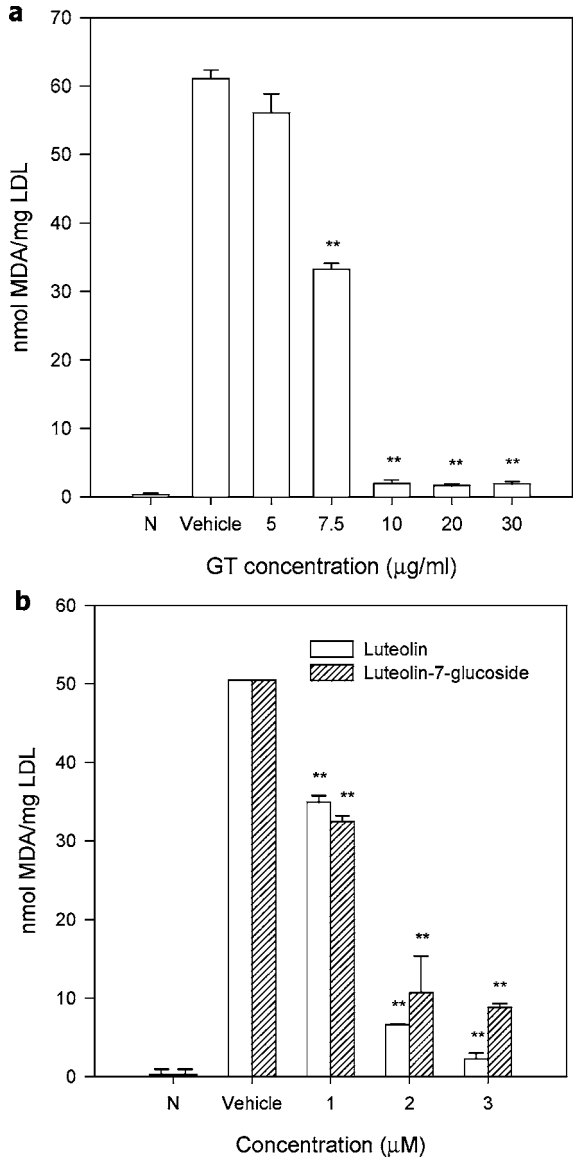


Figure 5. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit TBARS formation in copper-induced low-density lipoprotein (LDL): (**a**) GT inhibits TBARS formation in copper-induced LDL peroxidation; (**b**) luteolin and luteolin-7-glucoside inhibit TBARS formation in copper-induced LDL peroxidation. EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle and different concentrations of GT, luteolin, or luteolin-7-glucoside at 37 °C for 3 h. Peroxidation of the LDL was expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein. Data represent the mean ± SEM ($n = 3$) of vehicle control. *, $p < 0.05$, and **, $p < 0.01$, represent significant differences compared with vehicle control.

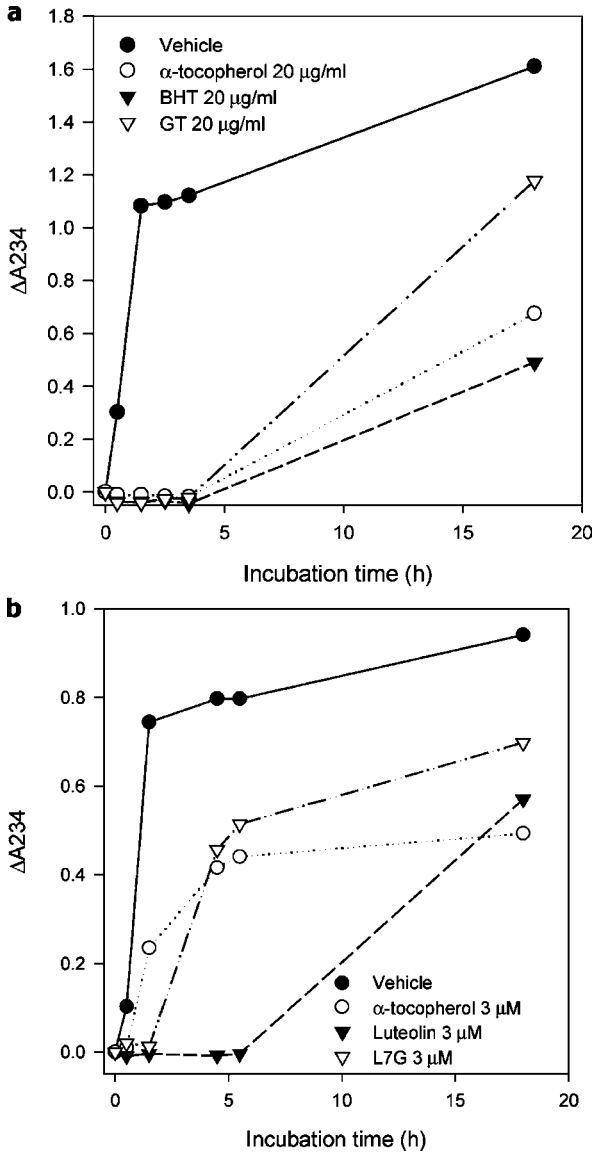


Figure 6. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit conjugated diene formation in copper-induced LDL: (**a**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle, α-tocopherol, BHT, or GT (20 μg/mL) at 37 °C for the indicated period; (**b**) EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle, α-tocopherol, luteolin, and luteolin-7-glucoside (L7G) (3 μM) at 37 °C for the indicated period. The formation of conjugated diene was measured by change in absorbance at 234 nm (ΔA_{234}). This experiment was repeated three times with similar results.

glucoside, luteolin, and oleanolic acid. As shown in **Figure 5b**, the MDA formation is inhibited by luteolin-7-glucoside and luteolin with estimated IC_{50} values of 1.52 and 1.34 μM, respectively. **Figure 6b** also demonstrates that the lag stage of diene formation were prolonged when luteolin-7-glucoside, luteolin, and α-tocopherol (3 μM) was added during LDL oxidation. The inhibitory effect was in the order of luteolin being greater than luteolin-7-glucoside followed by α-tocopherol. **Figure 7b** shows that luteolin-7-glucoside and luteolin reduced copper-induced LDL net charge change as compared with the vehicle group. Luteolin-7-glucoside and luteolin (3 μM) significantly reduced the electrophoretic mobility by about 41 and 63%, respectively, as compared with the vehicle group. The pattern of activities for luteolin and luteolin-7-glucoside was generally consistent with those seen for the lag-phase measurements. However, no detectable protection of LDL from copper-mediated oxidation could be found for the other major constituent, oleanolic acid (data not shown).

Inhibition of Oxidative Burst in PMN or PBMC by GT. Production of ROS by leukocytes is a potent microbicidal mechanism, but unrestrained production of these toxic metabolites has been indicated to mediate tissue damage. To examine whether GT could prevent ROS production by activated human leukocytes, we set up an in vitro method with luminol-enhanced CL to measure the ROS production induced by fMLP in PMN or PBMC. The chemoattractive peptide fMLP activates an oxidative burst by its binding to a membrane receptor and activating a signal transduction pathway that leads to an

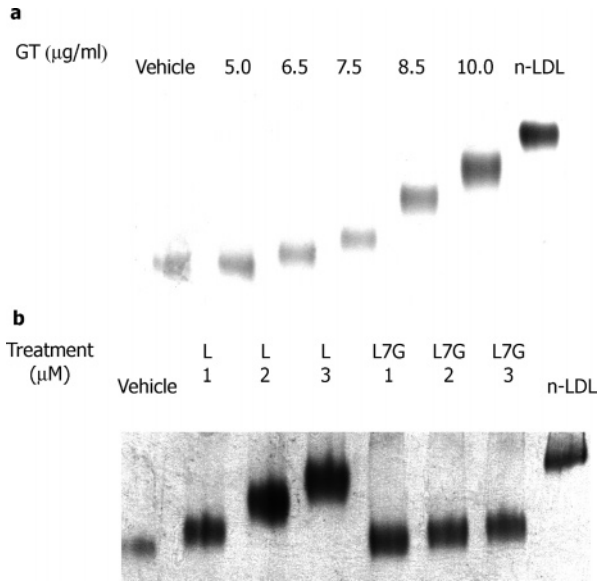


Figure 7. Ethanol extracts of *G. tenuifolia* (GT), luteolin, and luteolin-7-glucoside inhibit electrophoretic mobility in copper-induced LDL: (**a**) native LDL (n-LDL) (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentrations of GT for 24 h; (**b**) n-LDL (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentrations of luteolin (L) or luteolin-7-glucoside (L7G) for 24 h. Concentrated LDL (1–2 μL) was separated on Titan lipoprotein gel and stained with Fat Red 7B.

oxidative burst being induced (29). **Figure 8a** shows the kinetic profile of fMLP-stimulated ROS production in PMN. GT (0.25−2.5 mg/mL) significantly inhibited ROS generation in a dose-dependent manner. The reaction curve of fMLP-induced ROS production in PBMC (**Figure 8b**) showed a slower and longer response than fMLP-stimulated PMN. GT (0.25−3.75 mg/mL) treatment dose-dependently inhibited ROS production in PBMC.

Inhibition of Oxidative Burst in PMN or PBMC by Major Constituents in GT. To elucidate whether luteolin-7-glucoside can inhibit ROS production in phagocytes, the aforementioned procedure was performed, and the peak CL values were used for calculating IC_{50}. **Table 1** demonstrates that both luteolin-7-glucoside and luteolin exhibited slightly stronger inhibitory effects against fMLP-triggered ROS production in PBMC than in PMN.

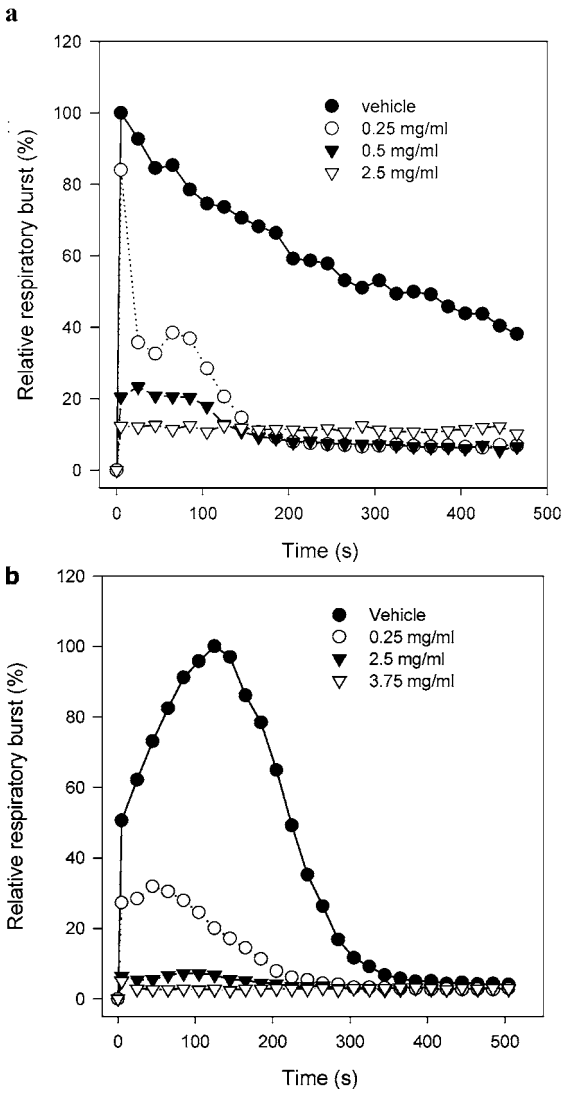


Figure 8. Ethanol extract of *G. tenuifolia* (GT) inhibits fMLP-induced ROS production in human leukocytes: (**a**) PMN; (**b**) PBMC. Each well containing 7×10^5 PMN or PBMC, 1 mM luminol, and vehicle or various concentrations of GT was incubated at room temperature for 15 min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period.

Table 1. Effects of Luteolin-7-glucoside and Luteolin on the Inhibition of FMLP-Induced ROS Production in Human Leukocytes

IC_{50} (μM) for fMLP-induced ROS[a]	PMN	PBMC
luteolin-7-glucoside	14.8 ± 0.4	6.4 ± 0.2
luteolin	11.7 ± 0.2	5.2 ± 0.2

[a] Each well containing 7×10^5 PMN or PBMC, 1 mM luminol, and vehicle or various concentrations of luteolin or luteolin-7-glucoside was incubated at room temperature for 15 min. Activator, 2 μM fMLP, was then added, and the light emission, expressed as relative light units (RLU), was monitored every 30 s for 5 s during a 10-min observation period and the peak CL values were used to calculate IC_{50}.

DISCUSSION

Herbs have played a significant role in maintaining human health and improving the quality of life for thousands of years. Many active phytochemicals, including flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides, have been identified (*30*). Numerous in vitro studies have shown that some of the phytochemicals are potent antioxidants, metal chelators, or free radical scavengers, which may account for their health-promoting properties (*31*). To evaluate the antioxidant potency of *G. tenuifolia*, scavenging effects against free radicals including DPPH, superoxide, and hydroxyl radical were analyzed in this study. Our current results demonstrate that the ethanol extracts of *G. tenuifolia* and its major flavonoid, luteolin-7-glucoside, as well as its aglycon, luteolin, are strong DPPH scavengers with IC_{50} values of 55.2 μg/mL,12.2 μM, and 22.8 μM, respectively.

It is generally accepted that luminol-enhanced CL can be used to measure the ROS production in vivo (*32*) and in neutrophils (*29*) or of hydroxyl radicals in a cell-free system (*33*). On the other hand, lucigenin reacts only with superoxide, hydrogen peroxide, or singlet oxygen (*34*, *35*). Using lucigenin- and luminol-enhanced CL, we discovered herein that GT is a superoxide and hydroxyl radical scavenger in vitro with IC_{50} values of 5.18 and 0.06 mg/mL, respectively. There is consider-

able difference between superoxide and hydroxyl radical scavenging potencies of GT in vitro. On the other hand, luteolin-7-glucoside, one of the major constituents of GT, exhibited compatible superoxide and hydroxyl radical scavenging effects with IC_{50} values of 11.0 and 17.6 μM, respectively. It is possible that the free radical scavenging effects of GT are attributable to synergistic interactions among all natural phytochemicals. The discrepancy between superoxide and DPPH/hydroxyl radical scavenging potencies of GT may be caused by adverse effects of other unidentified phytochemicals, which could disrupt the superoxide scavenging effect of luteolin-7-glucoside.

We also found that luteolin-7-glucoside exerted a stronger DPPH quenching activity than its aglycon, luteolin. On the other hand, luteolin-7-glucoside had weaker or compatible superoxide and hydroxyl radical scavenging potencies than luteolin. The scavenging potencies of flavonoids against free radicals are thought to be closely linked to their structures and to depend on the nature of the radicals. The reaction mechanism between free radicals and flavonoids is influenced by the presence of glycosidic moieties, the position of glycosylation, and the number and positions of hydroxy groups in flavonoids. Thus, the antioxidant effectiveness will be determined by a combination of all structural elements.

A substantial body of evidence has indicated oxidation of LDL to be one of the major mechanisms for the pathogenesis of atherogenesis. Some flavonoids possess antioxidant properties and have been shown to be potent inhibitors of LDL oxidation in vitro (*26*). From our current data, GT demonstrated abilities to inhibit copper-induced peroxidation of LDL in a dose-dependent manner (7.5−30 μg/mL) during the propagation phase (**Figure 5a**) and to extend the lag phase to oxidation at 20 μg/mL (**Figure 6a**). Moreover, GT (6.5−10 μg/mL) inhibited an increase in the net negative surface charge of apolipoprotein B 100 as measured by decreased electrophoretic mobility (**Figure 7a**). The effective concentration of GT for inhibiting copper-induced LDL oxidation was slightly lower than that for scavenging hydroxyl radical (IC_{50} of 60 μg/mL) and differed by 2 orders of magnitude as compared with that for scavenging superoxide (IC_{50} of 5.18 mg/mL). It has been demonstrated that in vitro copper-mediated oxidation of LDL was not involved in the generation of detectable superoxide or H_2O_2 (*36*). These results suggest the contribution of metal-sequestering rather than superoxide scavenging functions to the antioxidant activity of GT against LDL oxidation. We also found that luteolin-7-glucoside demonstrated a dose-dependent reduction of LDL oxidation. It has been reported that luteolin-7-glucoside had copper-chelating properties, which suggest a potential role of metal chelating in the antioxidant effects of GT (*37*).

We also found that luteolin-7-glucoside was significantly less effective in inhibiting copper-mediated LDL oxidation than its aglycon, luteolin. It has been reported that the reactivities of flavonoids in protecting LDL against copper-induced oxidation are dependent on their structural properties in terms of the response to copper ions, whether chelation or oxidation, their partitioning abilities between the aqueous compartment, and the lipophilic environment within the LDL particle, as well as their hydrogen-donating antioxidant properties (*38*). It is possible that the higher effectiveness of luteolin in inhibiting LDL oxidation may be attributed to its higher hydrophobicity compared with luteolin-7-glucoside.

Due to its chemical structure, luminol can cross biological membranes and therefore allow for the detection of both extra- and intracellular production of ROS (*39*). In this paper, fMLP stimulated PMN to generate ROS, which exhibited biphasic shapes as was observed by other authors in experiments with human blood neutrophils. It has been speculated that the first and second peaks of the CL reaction would reflect extra- and intracellular ROS production, respectively (*40*). GT and luteolin-7-glucoside showed concentration-dependent inhibition of fMLP-induced ROS production in PMN and PBMC, indicating that they could serve as potent antioxidants against oxidative stress in human primary cells.

Previously, we have demonstrated that GT inhibited pro-inflammatory cytokine release from human whole blood and PBMC and HBsAg expression in PLC/PRF/5 cells (*10*), which suggests that the health promotion effect of GT may be the result of combinatory activities, including antioxidant, anti-inflammatory, and antiviral. It has been proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their antioxidant and anticancer activities (*41*). In conclusion, we demonstrated for the first time that the ethanol extract of *G. tenuifolia* exhibited strong ROS scavenging activity in both cell-free and cell-based systems. In addition, GT also exhibited antioxidant activity against copper-induced LDL oxidation. Overall, the results demonstrate that the antioxidant activity of GT relates in part to its constituent, luteolin-7-glucoside, which acts as free radical and copper scavenger.

ABBREVIATIONS USED

GT, ethanol extract of *Glossogyne tenuifolia*; CL, chemiluminescence; LDL, low-density lipoprotein; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

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