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RESEARCH ARTICLE

# Antioxidant and Antigenotoxic Activities of Ethanol Extracts from *Rhus chinensis* Mill Leaves

**Zhenyu Qiu, Mingli Tang, Guanjun Deng, Hao Yang, Xuan Zhang, Shengwei Huang, and Lifang Wu**

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**Abstract** Ethanol extracts were obtained from *Rhus chinensis* Mill (RCM) leaves and used for antioxidant and antigenotoxic activity assays. $IC_{50}$ values in DPPH assays were 15.96, 18.83, 20.43, 27.93, 37.43, 46.21, and 141.84 μg/mL for TPP, IPE, LLE, Vc, CE, BHT, and Trolox. Similar results were obtained using ABTS and FRAP assays. *In vivo* testing showed strong antioxidant activities that were positively correlated with polyphenol contents. Leaf tissue contained abundant polyphenols, and more than 10 phenolic compounds were detected in extracts. Quantitative results showed that quercetin-3-rhamnoside (26.4±0.76 mg/g of extract) was the most abundant ingredient, followed by hyperoside (15.2±0.42 mg/g of extract), quercetin (1.5±0.07 mg/g of extract), and kaempferol (0.48±0.05 mg/g of extract). This study increases the knowledge for possible uses of forest by-products as a substitute for gallnuts.

**Keywords:** *Rhus chinensis* Mill, polyphenols, antioxidant, antigenotoxicity

Zhenyu Qiu, Mingli Tang, Guanjun Deng, Xuan Zhang, Shengwei Huang, Lifang Wu (✉)
Bioenergy Forest Research Center of State Forestry Administration (Hefei) and Key Laboratory of Ion Beam Bioengineering, Chinese Academy of Sciences, Hefei 230031, Anhui, China
Tel, Fax: +86-551-65591413
E-mail: lfwu@ipp.ac.cn

Guanjun Deng, Xuan Zhang
School of Life Science, Anhui University, Hefei 230039, Anhui, China

Hao Yang
School of Life Science, South-central University for Nationalities, Wuhan 430074, Hubei, China

Lifang Wu
School of Life Science, University of Science and Technology of China, Hefei 230027, Anhui, China

## Introduction

*Rhus chinensis* Mill (RCM), a perennial deciduous shrub or small tree of Anacardiaceae known as Chinese Sumac, is widely distributed in China and East Asia. Gall-forming aphids injure leaves and petioles of *Rhus chinensis* and the plant responds by forming gallnuts, which are important crude materials in traditional Chinese medicine and are also the main materials used to produce gallic acid in China due to their high hydrolysable tannin content (50 to 70%) (1). The gallic acid extracted from gall nuts is broadly applied in the food and pharmaceutical industries in China (1,2). Historically, gallnuts and other plant tissues have been used for treating colds, fevers, cough, malaria, and other diseases (3). In recent years, related studies have made important progress in identification of the bioactive ingredients (polyphenols and flavonoids) from gall nuts and gallnut plants, and have studied related biomedical effects involving antitumor, antioxidant, antimutagenic, antiviral, and antibacterial activities (4-8). Previous research has shown that ethanol extracts from stems of *Rhus chinensis*, particularly the petroleum ether fraction, effectively suppressed HIV-1 activity *in vitro*, and mainly functioned during the late stages of the HIV-1 life cycle (8). Another study by Shim *et al*. revealed that aqueous extracts from gallnuts of *Rhus chinensis* had an activity similar to the activity of alpha-glucosidase which has anti-HIV, antiviral, and antibacterial activities (9). Production of gallnuts depends heavily on the climate and environmental conditions. The highest annual production is limited to 7,000 ton and the average annual output is about 5,000 ton in China at present (1). It is difficult to increase gallnut production for the market demands. The large amount of residue from annual plant tending and thinning processes, including leaves and twigs, offers abundant and cheap materials for target ingredient isolation and exploits a new method for

isolation of bioactive ingredients from these easily accessible resources as a substitute for gallnuts. Such a method is presented in this paper in which leaf tissues of *Rhus chinensis* (a gallnut host) were used in polyphenolic extraction. Antioxidant and antigenotoxic activities of extracted chemicals were detected *in vitro* and *in vivo* to evaluate the bioactivities of extracts based on the total phenolic content and component analysis. Results indicate a new method for comprehensive use of cheap and accessible forest residues as a substitute for gallnuts.

## Materials and Methods

**Chemicals and strains** Methanol and formic acid (chromatographic grade) used for preparing mobile phases were purchased from Merck (Darmstadt, Germany) and Tedia (Fairfield, OH, USA), respectively. The standards hyperoside, quercetin, kaempferol, quercetin-3-rhamnoside, and gallic acid used for calibration curves were obtained from Chengdu Purechem-Standard Co., Ltd. (Chengdu, China). Reagents used for measuring antioxidant activities, L-ascorbic acid, DPPH, Trolox, butylhydroxytoluene (BHT), and juglone were obtained from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and ABTS were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Folin-Ciocalteu's Reagent and tea polyphenols (TPP) were bought from Shanghai Labaide Biotechnology Co., Ltd. (Shanghai, China) and Ruibio (HeFei BoMei Biotechnology Co., Ltd., China), respectively. Mitomycin C (MMC) was obtained from Roche China (Shanghai, China). All other chemicals were of analytical reagent grade (AR).

The *Salmonella typhimurium* Sal94 (p*RecA*::*LuxCDABE tolC*$^c$ Cm$^R$Amp$^R$) bacterial strain was provided by Prof. Shimshon Belkin of the Hebrew University of Jerusalem, Israel. The N2 wild-type nematode and the *Escherichia coli* OP$_{50}$ bacterial strain were provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources of National Institutes of Health (NIH, Bethesda, MD, USA).

**Plant materials** RCM leaves were harvested from a field in a Hefei (China) suburb in July 2011, dried at 60°C and ground to a fine powder using an electric mill. The powder was stored in a freezer at −20°C until use.

**The extraction procedure for polyphenolic compounds** The optimized extraction process used 300 g of dried sample powder subjected to 60% ethanol for 2 h at 60°C at a solid-to-liquid ratio of 1:15. The mixture was then filtered through qualitative filter papers, and clarified by centrifugation at 5,000×*g* for 5 min. The supernatant was then divided into 3 parts. The first part was directly distillated in a reducing power and dried at low temperature (<50°C) to yield a crude extract (CE). The second portion of the supernatant was subjected to ethyl acetate extraction twice. The ethyl acetate phase was collected and distillated *in vacuo* to obtain purified extract (LLE). The third part was subjected to ion precipitation extraction (IPE).

**The total phenolic content quantification** The Folin-Ciocalteu method was used to measure the total phenolic content (10). Quantification was based on a standard gallic acid curve obtained from the same method and results were expressed in mg of gallic acid per g of dry extract powder.

**UHPLC and UHPLC-ESI-QTOF-MS analysis of phenolic compounds** Qualitative and quantitative analysis of polyphenolic compounds in CE was carried out using an Ultra-high performance liquid chromatography system (UHPLC; Waters, Milford, MA, USA) equipped with the UV-Vis photodiode-array detector (DAD). A sample of crude extract (0.1 g) was weighed and dissolved in 60% methanol (25 mL), followed by ultrasonic treatment (200 W, 40 kHz) for 30 min, after which the solution was cooled in room temperature and the loss volume was made up using 60% methanol, followed by purification using a C$_{18}$ Sep-Pack cartridge (Waters). Chromatographic separation was performed using an Acquity UHPLC BEH C$_{18}$ column (100×2.1 mm, 1.7 μm) with solvent A (methanol) and solvent B (0.2% formic acid) under gradient conditions of 15% A from 0-3 min; 25% A from 3-5 min; 35% A from 5-7 min; 40% A from 7-9 min; 50% A from 7-11 min; 100% A from 11-13 min; 15% A from 13-14 min; and 15% A from 14-16 min. The flow rate was 0.4 mL/min and the injection volume was 5 μL. The column was thermostatically controlled at 35°C. Further characterization of the phenolic composition was achieved using UHPLC coupled to a Xevo G2 QTOF mass spectrometer equipped with an orthogonal electrospray interface (ESI) and a Marker Lynx XS station (Waters). For polyphenol characterization, a capillary voltage of 2,500 V was used in the negative ion mode. Argon was used as a drying and nebulizing gas at a flow rate of 600 L/h. The desolvation temperature was set at 350°C and the nebulization pressure was 40-60 psi.

***In vitro antioxidant activity assay using DPPH***: The DPPH radical-scavenging activity was assessed according to the method of Katalinic *et al.* (11) and expressed as percent inhibition using the equation:

$$\%\text{Inhibition of DPPH radical} = (A_c - A_s)/A_c \times 100$$

where $A_c$ is the absorbance of a control and $A_s$ is the absorbance of the sample. An $IC_{50}$ (concentration of extract required for 50% reduction in absorbance) was calculated by interpolation from results of a linear regression analysis

and the obtained value was indicates with values for positive controls. A lower $IC_{50}$ value indicates a stronger antioxidant activity.

***ABTS assay***: The slightly modified ABTS method developed by Re *et al*. (12) was used. For plant extracts, the $ABTS^{+\bullet}$ solution was first diluted using ethanol to an absorbance of 0.70 (±0.02) at 734 nm using a spectrophotometer (UV-2550; Shimadzu, Tokyo, Japan). Then, 3 mL of prepared $ABTS^{+\bullet}$ solution and 200 μL of a sample solution (10-50 μg/mL) were mixed and incubated in a water bath at 37°C in the dark. The absorbance was measured exactly 5 min after the initial mixing. Appropriate controls were run in each assay. All measurements were carried out in triplicate at each concentration of the standards and samples. Percentage inhibition of the absorbance vs. the concentration was plotted and the $IC_{50}$ value was calculated.

***FRAP assay***: The ferric reducing ability of the extracts was estimated following the procedure described by Benzie and Strain (13). The assay was preformed in triplicate and results were expressed as a FRAP value (mmol of Fe(II) per g of extract).

***In vivo ROS inhibition assay***: The wild type *Caenorhabditis elegans* strain ($N_2$) was synchronized to the L1 larvae stage and incubated in liquid S-basal medium containing the *E. coli* $OP_{50}$ strain at $10^9$ CFU/mL. L1 larvae were incubated with extracts at different concentrations of the solvent control for 72 h at 20°C. Adult worms were collected by centrifugation, and further incubated for 1 h in a liquid medium containing 150 μM juglone to generate oxidative stress (14). The worms were then washed twice with $M_9$ buffer (85 mM NaCl, 22 mM $Na_2HP_4$, 22 mM $KH_2PO_4$, 1 mM $MgSO_4$), and continuously incubated in $M_9$ buffer containing 10 μM 2,7-dichlorodihydrofluorescein-diacetate ($H_2$-DCF-DA) for 30 min at 20°C. Worms selected randomly from each group were mounted onto microscope slides coated with 3% agarose, anesthetized using 5 mM levamisole and the slides were capped with cover slips. Epifluorescence images were captured using a fluorescence microscope equipped with a CCD camera (DP72 system; Olympus, Tokyo, Japan) with excitation at 488 nm and emission at 510 nm. The relative fluorescence (IOD per worm) of the whole body was analyzed using Image-Pro Plus 6.0 software.

***Genotoxicity and antigenotoxicity assay***: SOS-Lux testing was used to determine the genotoxicity of extracts and antigenotoxicity effects induced using MMC based on the SOS response, according to the procedure described by Tang *et al*. with some adaptation (15). Briefly, an exponential phase culture of the Sal94 bacterial strain was centrifuged at 8,000 rpm/min for 5 min and the pellet was resuspended using 0.9% NaCl to the original volume. Then, 900 μL aliquots were distributed into glass test tubes containing different doses of purified extract and 50 μL of MMC (5 μg/assay) to a final volume of 1.2 mL. Positive and negative controls were prepared by exposure of bacteria to MMC or deionized water alone. The genotoxicity of extracts was also evaluated in the absence of MMC. After 1 h of induction at room temperature, the mixture was transferred to fresh LB medium and incubated for another 3 h with shaking at 26°C. An amount of 1 mL of the culture content was used for determination of luminescence (relative light units, RLUs) using a luminometer (GloMax 20/20; Promega, Fitchburg, WI). A spectrophotometer was used to measure cell growth at 600 nm. The SOS induction factor (IF) was calculated by dividing the mean luminescence ($RLUs/OD_{600}$) value of the induced sample by the value of the non-induced sample. Triplicate measurements were obtained for each sample and the result was considered positive when the IF value exceeded 2. Antigenotoxicity was expressed as percentage inhibition of the genotoxicity that was induced using MMC as:

$$\text{Inhibition (\%)}=[(100-(IF_1-IF_0)/(IF_2-IF_0)\times 100]$$

where $IF_1$ is the induction factor in the presence of both the phenolic extract and mutagen, $IF_2$ is the induction factor of the mutagen (positive control), and $IF_0$ is the induction factor of non-induced cells (negative control).

**Statistical analysis** All results are expressed as mean values±standard deviation (SD) ($n \geq 3$). The significance of differences was calculated using a one-way analysis of variance (ANOVA) and Duncan's multiple range test using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). Values of $p<0.05$ were considered to be significant.

## Results and Discussion

**The total phenolic content of the extracts** Polyphenols are important components in most plant extracts, and play a key role in antioxidant, antimicrobial, and antigenotoxic activities. The phenolic contents can be used as an important indicator of antioxidant activity, and are also used as a preliminary screening index for natural sources of antioxidants in functional foods. In this work, phenolic products were obtained using a direct ethanol extraction method and purified using procedures of ethyl acetate liquid-liquid extraction and ion precipitation extraction. The total polyphenol content of samples was detected using a Folin-Ciocalteu assay. IPE contained 727.45±6.17 GAE mg/g of extract, followed by LLE at 592.28±11.93 GAE mg/g, and CE at 383.02±3.60 GAE mg/g (Table 1). The total phenolic content increased 55 and 90%, respectively, using the 2 different purification treatments. Liquid-liquid extraction is mostly used for enriching polyphenols from

**Table 1. The total polyphenols, hyperoside, quercetin, and quercetin-3-rhamnoside contents of extracts from RCM leaves**

| Component | CE | LLE | IPE |
|---|---|---|---|
| Total phenolic (GAE mg/g)[1)] | 383.02±3.60 | 592.28±11.93* | 727.45±6.17* |
| Hyperoside (mg/g extracts)[2)] | 15.2±0.42 | - | - |
| Quercetin (mg/g extracts)[2)] | 1.5±0.07 | - | - |
| Quercetin-3-rhamnoside[2)] (mg/g extracts) | 26.4±0.76 | - | - |
| kaempferol (mg/g extracts)[2)] | 0.48±0.05 | - | - |

[1)]GAE, gallic acid equivalents; CE, crude extract; LLE, liquid-liquid extraction; IPE, ion precipitation extraction; the values are means of triplicate measurements±standard deviation (SD).
[2)]The values are means of triplicate measurements±SD; * $p<0.05$ in comparison with the CE group

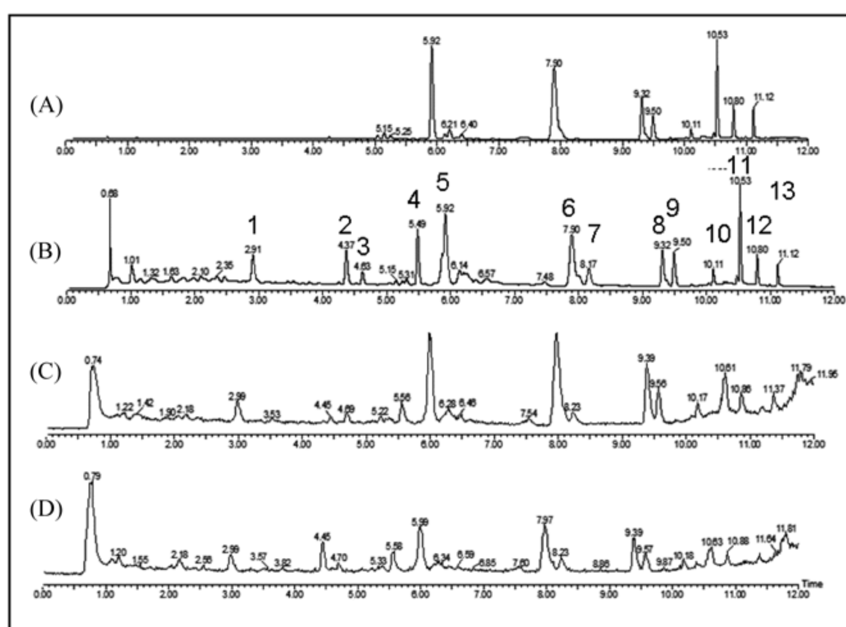


**Fig. 1. UHPLC chromatograms of a crude extract (CE) from RCM leaves at wavelengths of 370 nm (A) and 272 nm (B), and at collision energies of 40-60 V (C) and 0 V (D).**

plants. Ion precipitation is also an important method used to purify phenolic products, as phenols can easily react with the metal ions $Al^{3+}$, $Cu^{2+}$, and $Zn^{2+}$ to produce sediment that can be re-dissolved under certain concentrations of acid to release the phenolic compounds (16). Both the ethyl acetate liquid-liquid extraction and ion precipitation were efficacious phenolic enrichment methods. RCM leaf tissue is rich in phenolic contents at even higher concentrations than the well known plant species *Helichrysum methanolic* (66.74 to 160.63 GAE mg/g extract) and *Asteraceae* plants (2.9±0.1 to 404.3±27.3 GAE mg/g extract) (16,17). RCM residues should be considered as a potential substitute for gallnuts due to their phenolic contents, low cost, and easily accessible contents.

**Phenolic compound analysis of the crude extract** The composition of the ethanol extract was complex. In order to gain an insight into the phenolic compositions, the crude extract was subjected to UHPLC-MS analysis. Chromatographic profiles at 2 different detection wavelengths (370 and 272 nm) and QTOF mass spectrometry results for the total ion current using different energy collisions are shown in Fig. 1. The major phenolic compounds, corresponding to peaks 1 to 13 at 272 nm (Fig. 1B), were identified based on molecular ions, fragmentation patterns, and UV-Vis spectra. The major compounds in the extracts were polyphenols (compounds 1-4), flavonoid derivatives (compounds 5-6 and 8), flavonoids (compounds 9 and 10) and biflavonoids (compounds 11-13) (Table 2). Identities of hyperoside (peak 5), quercetin-3-rhamnoside (peak 6), quercetin (peak 9), and kaempferol (peak 10) were confirmed using parallel chromatographic analysis of molecular standards. Although the identity of peak 7 could not be confirmed using QTOF mass spectrometry and UV-Vis spectra, the compound was classified as a syringate derivative as the syringate chemical group was detected using mass spectrometry. Peaks 11, 12, and 13 were identified as biflavonoids with the same Mw. However, the fragment patterns of compounds 11 and 12 were similar, implying that they were isomers.

**Table 2. The main phenolic compounds in a crude extract (CE) from RCM leaves using UHPLC-MS analysis**

| Peaks | RT (min) | $[M-H]^-$ | $MS^2$ m/z | UV-Vis (nm) | Molecular formula | Compound name |
|---|---|---|---|---|---|---|
| 1 | 2.91 | 457.0779 | 125, 169 | 205, 273 | $C_{22}H_{17}O_{11}$ | Epigallocatechin gallate |
| 2 | 4.37 | 197.0442 | 124, 169 | 216, 271 | $C_9H_9O_5$ | Syringate |
| 3 | 4.63 | 441.0801 | 125, 169 | 276 | $C_{22}H_{18}O_{10}$ | Epicatechin gallate |
| 4 | 5.49 | 939.1135 | 125, 169, 469 | 217, 280 | $C_{41}H_{32}O_{26}$ | 1,2,3,4,6-O-Pentagalloylglucose |
| 5 | 5.92 | 463.0882 | 271, 316 | 261, 351 | $C_{21}H_{19}O_{12}$ | Hyperoside[2)] |
| 6 | 7.90 | 447.0921 | 271, 300 | 255, 349 | $C_{21}H_{19}O_{11}$ | Quercetin-3-rhamnoside[2)] |
| 7 | 8.17 | 349.0554 | 124, 197 | 215, 275 | $C_{16}H_{13}O_9$ | NA[1)] |
| 8 | 9.32 | 431.0973 | 255, 285 | 263, 345 | $C_{21}H_{19}O_{10}$ | Kaempferol 3-*O*-α-L-rhamnopyranoside |
| 9 | 9.50 | 301.0343 | 151, 271 | 205, 263, 351 | $C_{15}H_9O_7$ | Quercetin[2)] |
| 10 | 10.11 | 285.0369 | 169, 229 | 209, 265 | $C_{15}H_9O_6$ | Kaempferol[2)] |
| 11 | 10.53 | 537.0842 | 331, 375 | 205, 272, 335 | $C_{30}H_{17}O_{10}$ | Biflavanone (A) |
| 12 | 10.80 | 537.0823 | 331, 375 | 209, 269, 337 | $C_{30}H_{17}O_{10}$ | Biflavanone (B) |
| 13 | 11.12 | 537.0809 | 331 | 212, 269, 338 | $C_{30}H_{17}O_{10}$ | Biflavanone (C) |

[1)]The compound could not be completely identified by this method.
[2)]The compound identity was confirmed based on parallel chromatographic analysis of molecular standards.

Previous studies isolated more than 10 biflavonoids, including amentoflavone, robustaflavone, rhusflavanone, rhusflavone, and hinokiflavone from *Rhus* species (18-21). Among them, amentoflavone, hinokiflavone, rhusflavanone, and rhusflavone contain the most common components found in the different *Rhus* species (4). In this work, the 4 phenolic ingredients of hyperoside, quercetin-3-rhamnoside, quercetin, and kaempferol were identified based on comparisons with retention times ($t_R$) of authentic standards. Quantitative data were calculated from respective calibration curves using the UHPLC method (Table 1). Quercetin-3-rhamnoside (26.4±0.76 mg/g extract) was the most abundant ingredient, followed by hyperoside (15.2±0.42 mg/g extract), quercetin (1.5±0.07 mg/g extract), and kaempferol (0.48±0.05 mg/g extract). These results were used to verify that RCM leaf tissues are an ideal source of phenolic compounds. The qualitative results and the semi-quantitative results from the peak areas of the UHPLC (Fig. 1B) revealed that 1,2,3,4,6-O-Pentagalloylglucose, hyperoside, quercetin-3-rhamnoside, and biflavonoids were the predominant phenolic ingredients in CE. Gallic acid, which is the main hydrolyzate of gallnut, is a routine polyphenol in different Anacardiaceae species, including *Rhus chinensis* (19,20). However, gallic acid was not detected as a major ingredient of CE. In contrast, the compounds 1-4 and compound 7 derivatives of gallic acid were the main extract components.

***In vitro*** **antioxidant activities based on free radical scavenging assays** There are many methods to determine antioxidant activities that depend on different generators of free radicals through different mechanisms (22). To uncover the multiple aspects of antioxidant efficacy, 3 methods and 4 positive controls (TPP, Vc, Trolox, and BHT) were used *in vitro* to assess the antioxidant activity of extracts. The order of antioxidant activity from high to low based on $IC_{50}$ values was 15.96, 18.83, 20.43, 27.93, 37.43, 46.21, and 141.84 μg/mL for TPP, IPE, LLE, Vc, CE, BHT, and Trolox, respectively (Table 3). Similar results were obtained using the ABTS assay, for which the $IC_{50}$ values were 21.01, 27.31, 31.37, 32.86, 42.84, 50.85, and 52.95 μg/mL for TPP, IPE, Vc, LLE, CE, Trolox, and BHT, respectively (Table 3). This order was changed for FRAP detection results. The Vc control showed the highest antioxidant activity (2.37 mmol Fe(II)/g), followed by IPE (1.85 mmol Fe(II)/g), TPP (1.69 mmol Fe(II)/g), Trolox (1.31 mmol Fe(II)/g), LLE (0.79 mmol Fe(II)/g), CE (0.50 mmol Fe(II)/g), and BHT (0.28 mmol Fe(II)/g) (Table 3). This difference might be caused by an interaction between the Fe ions and polyphenols. Generally, based on $IC_{50}$ and FRAP value results, extracts showed higher antioxidant activities than the synthetic commercial antioxidants BHT and Trolox. The effects of both IPE and LLE were greater than or similar to the effect of Vc in both the DPPH and ABTS assays, and a little lower than the effect of Vc in the FRAP assay, indicating that the extracts possessed strong antioxidant activities.

The antioxidant activities of extracts were positively correlated with the total polyphenol contents (Fig. 2). Coefficients of correlation between the total polyphenol content and the DPPH and ABTS radical scavenging activates were 0.91 and 0.98, respectively. Purified extracts had higher FRAP values and lower $IC_{50}$ values than CE (representing stronger antioxidant activities) (Table 1, 3), indicating that the polyphenols in RCM leaf extracts are responsible for the antioxidant activity. LLE and IPE, both with relatively lower phenolic contents than TPP, had antioxidant activities similar to TPP in DPPH and ABTS assays. The FRAP value of IPE (1.85 mmol/Fe(II) g) was

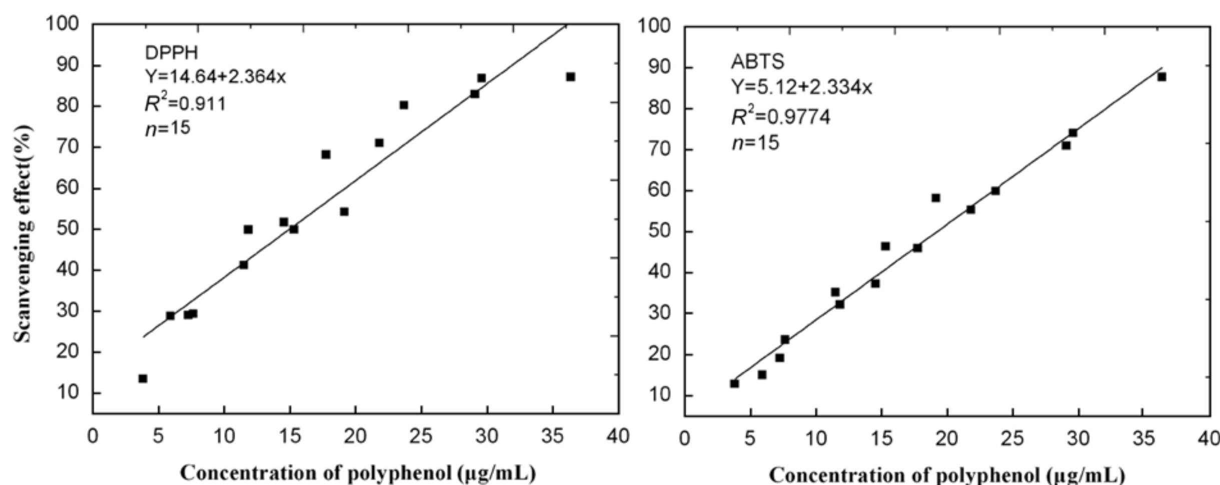


**Fig. 2. Correlations between the antioxidant activities and total polyphenol contents (GAE) of extracts from RCM leaves.**

**Table 3. Antioxidant activities of extracts from RCM leaves determined using DPPH, ABTS, and FRAP assays[1)]**

| Sample | $IC_{50}$ (μg/mL) | | |
|---|---|---|---|
| | DPPH | ABTS | FRAP (mmol/g)[2)] |
| CE | $37.43^C$ | $42.84^B$ | $0.50^F$ |
| LLE | $20.43^E$ | $32.86^C$ | $0.79^E$ |
| IPE | $18.83^F$ | $27.31^D$ | $1.85^B$ |
| TPP | $15.96^G$ | $21.01^E$ | $1.69^C$ |
| Vc | $27.93^D$ | $31.37^C$ | $2.37^A$ |
| Trolox | $141.84^A$ | $50.85^A$ | $1.31^D$ |
| BHT | $46.21^B$ | $52.95^A$ | $0.28^G$ |

[1)]The letters (A-G) indicate significant differences at a significance level of $p<0.05$.
[2)]FRAP values are expressed as mmol of Fe(II) per g of extract.

significantly higher than the value of TPP (1.69 mmol Fe(II) /g) ($p<0.05$). A comparison between the extracts and TPP suggested that the difference in antioxidant activities was probably due to the variety of phenolic compounds, as suggested by Rababah *et al*. (23). The predominent ingredients of CE were 1,2,3,4,6-O-Pentagalloylglucose, hyperoside, quercetin-3-rhamnoside, and biflavonoids, while TPP was mostly composed of catechins (60-80%), suggesting that both the amounts and varieties of polyphenols are related to the antioxidant effects of plant extracts. In a previous report, different polyphenol structures and flavonoid substances and derivatives resulted in variations in antioxidant activities in natural plant extracts (23).

Both the DPPH and ABTS methods are recommended as easy and accurate for measuring the antioxidant activities of fruits and vegetables extracts. The FRAP assay is also a quick and simple method with high reproducibility that can be used for analysis of total antioxidant activities of plant extracts. The relative antioxidant activities varied between the detection methods in this study (Table 3), perhaps due to differences in mechanisms, such as different radicals and/or ions in the reaction systems. However, results of all 3 methods indicated that the extracts were capable of scavenging free radicals to prevent the initiation and propagation of free-radical-mediated chain reactions. Therefore, RCM ethanol extracts can be useful in preservation of foodstuffs, drug products, and cosmetics (24).

***In vivo* ROS inhibition effect** In order to evaluate antioxidant activities *in vivo*, the wild type *C. elegans* strain N2 was used to determine the reactive oxygen species (ROS) inhibition ability of ethanol extracts (LLE) from RCM leaves. An intensive whole worm green fluorescence was observed after treatment with juglone, used as a ROS generator, exhibiting a significant induction of the ROS level in *C. elegans* (Fig. 3A). In contrast, LLE inhibited ROS in a dose-dependent manner, resulting in a reduction of fluorescence (Fig. 3B, 3C). Quantitative analytical results of worm body fluorescence are shown in Fig. 3D. The mean fluorescence intensity of worms decreased from 0.099 IOD (positive control) to 0.081 and 0.071 IOD after treatment with LLE at concentrations of 50 and 100 μM. Both treatments showed significant ($p<0.01$) differences compared to the positive control.

In comparison with cell-free and cell-culture systems, use of *C. elegans* allows examination of a whole organism *in vivo* (25). These nematodes are easily handled and are sensitive to environmental stimuli and can be used for *in vivo* evaluation systems. Furthermore, *C. elegans* is similar to mammals with regard to pharmacological mechanisms due to strong conservation of biological principles between *C. elegans* and mammals as 60-80% human gene homologues have been identified in *C. elegans* (25).

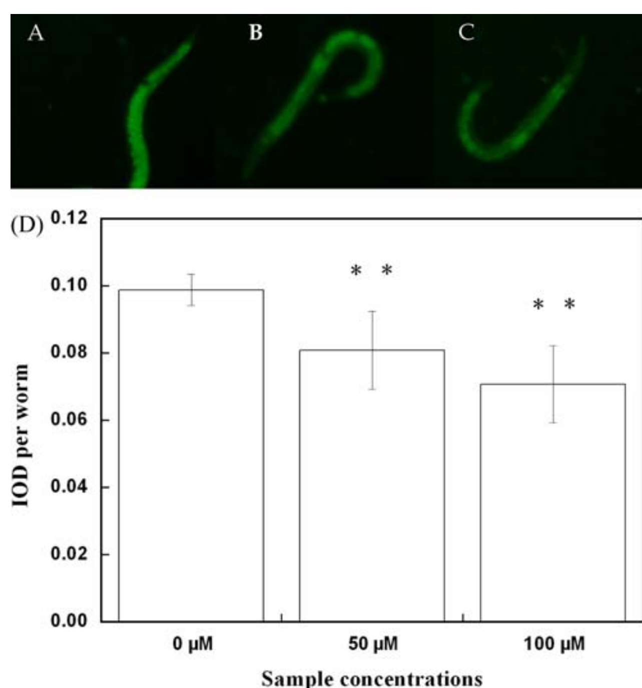


**Fig. 3. The ROS inhibition effects of a purified extract (LLE) from RCM leaves.** (A-C) Fluorescence images of ROS accumulation measured using a fluorescent probe (H2-DCF-DA) in *C. elegans* with and without LLE pretreatment. (D) Quantified fluorescence intensity in each group ($n$>20) determined using Image-Pro Plus 6.0 software. Results are expressed as mean±SD of relative fluorescence per worm (IOD per worm). **$p$<0.01 with control group

ROS are mainly generated in mitochondria during respiration due to incomplete reduction of oxygen. ROS are considered to be deleterious due to activities for reactions with adjacent biomolecules, such as lipids, carbohydrates, proteins, and DNA, which eventually lead to the loss of cellular integrity and functionality (26). Many environmental stresses, including drugs, metal ions, and radiation, can lead to induction of oxygen free radicals, thereby causing damage to cells and tissues. Recent studies of the lifespan of *C. elegans* showed that, in addition to SOD and catalyses, ROS are molecular targets for prevention of oxidative stress (27). Results from this study indicate that phenolic extracts from China Sumac have a protective effect against oxidative stress through *in vivo* ROS scavenging and, thus, might protect organs and tissues from damage. Ye *et al*. (28) reported that polyphenolic compounds can serve as an effective protection agent against the oxidative damage caused by ionizing radiation in *C. elegans*. Recent work has also indicated that phenolic compounds including hyperoside, quercetin, and quercetin derivatives, which were also identified in extracts in this study, have been demonstrated to have activities to reduce oxidative DNA damage and lipid peroxidation, and to quench free radicals (29).

**Genotoxicity and antigenotoxicity assay** The toxicity and side effects of traditional Chinese medicines and extracts remain a controversial subject. Rapid, economic, sensitive, and intuitive methods are needed to determine these possible effects. In this work, the *S. typhimurium* Sal94 bacterial strain containing the recA'::lux-bearing plasmid was used to detect an SOS response that was directly induced using RCM extracts in order to evaluate the genotoxicity that is induced by MMC for evaluation of the antigenotoxicity of RCM extracts. The results of genotoxicity and antigenotoxicity analyses are shown in Table 4 and Fig. 4. Almost no fluorescence was induced by LLE at a dosage of 2,500 μg/assay (Fig. 4A) with an induction factor of 0.85 (Table 4). Thus, the extract exhibited no detectable genotoxicity at the tested concentration.

MMC is a potent antibiotic used in combined chemotherapy for prostate, breast, and lung cancer. Upon bioreductive activation, inter-strand crosslinks are formed, preferably at guanines of the CpG island. An intensive blue fluorescence was observed, indicating strong expression of recA'::lux in Sal94 bacteria due to MMC treatment (Fig. 4C). In contrast, LLE inhibited MMC-induced SOS gene

**Table 4. The genotoxic and the antigenotoxic effects of RCM leaf extracts on the mitomycin C (MMC) induced SOS response in the *S. typhimurium* Sal94 bacterial strain**

| Sample | Dose (μg/assay) | $RLUs/OD_{600}$ ($\times 10^6$) | IF | % of inhibition |
|---|---|---|---|---|
| LLE | 2,500 | 12.29±0.12 | 0.85 | - |
| NC[1)] | 0 | 14.54±0.16 | 1.00 | - |
| PC(MMC)[2)] | 5 | 163.74±1.82 | 11.26 | - |
| PC+LLE[3)] | 500 | 137.87±0.96* | 9.84 | 17.34 |
| PC+LLE | 1,000 | 78.59±1.14* | 5.40 | 57.07 |
| PC+LLE | 1,500 | 43.53±0.69* | 2.99 | 80.57 |
| PC+LLE | 2,000 | 30.78±1.13* | 2.12 | 89.11 |
| PC+LLE | 2,500 | 27.72±0.27* | 1.91 | 91.17 |

[1)]Negative control (without MMC and LLE)
[2)]Positive control (with 5 μg/assay of MMC alone)
[3)]Treatment groups (with 5 μg/assay of MMC and different concentrations of LLE). *$p$<0.05 with NC and PC group

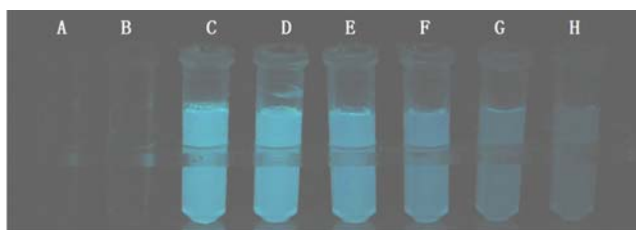


**Fig. 4. Genotoxicity and antigenotoxicity of LLE displayed in fluorescence images of SOS expression in the *S. typhimurium* Sal94 strain.** (A), genotoxicity of LLE (in the presence of LLE alone); (B), negative control (in the absence of both LLE and MMC); (C), positive control (in the presence of MMC alone); (D-H), antigenotoxic effects of an RCM extract (in the presence of MMC and different concentrations of LLE)

expression, resulting in a reduction in luminescence (Fig. 4D-4H). This is the first study to display the results of cytotoxic and genotoxic effects using imaging, although SOS-Chromotest and SOS-Lux testing are frequently used in investigations of environment stress with plant extracts (30). A dose-related inhibitory effect on the genotoxicity induced by MMC was demonstrated (Table 4). The inhibition ratio was increased from 17.34 to 91.17% at LLE dosages from 500 to 2,500 μg/assay. A positive correlation ($R^2$=0.96, data not shown) was identified between the antigenotoxic activities and the total phenolic contents, indicating that polyphenols are the major components responsible for the antigenotoxicity of the extracts, consistent with the results of Nuria *et al*. (31) using phenolic compounds from different common bean cultivars.

In conclusion, China Sumac extracts had strong *in vitro* and *in vivo* antioxidant activities, and showed strong antigenotoxicity, but had no genotoxicity based on SOS-Lux and Ames-Lux testing. Polyphenols were responsible for the multiple bioactivities of the extracts, suggesting that China Sumac leaves can be used as a cheap and accessible source material for production of bioactive compounds with antioxidant, antimicrobial, and antigenotoxic activities.

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