JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Evaluation of Antibacterial and Anti-inflammatory Activities of Less Polar Ginsenosides Produced from Polar Ginsenosides by Heat-transformation

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Supporting Information

ABSTRACT: Ginsenosides are the major active constituents in both white and red American ginseng (AG), and their pharmacological effects on central nervous, cardiovascular, and endocrine systems have been well elucidated. However, the anti-*Propionibacterium acnes* (*P. acnes*) activity of them is still unknown. In this study, 5 ginsenosides enriched fractions were obtained from the total ginsenosides extract of AG roots (TAG) by resin adsorption and heat-transformation. Resin purification decreased the total polyphenol (TP) content in the fractions. However, heat treatment increased the TP content and induced extensive conversion of the polar ginsenosides (Rg_1, Re, Rb_1, Rc, Rb_2, Rd, and Gyp XVII) to less polar compounds (Rg_2, Rg_3, Rg_6/F_4, Rs_3, and Rg_5/Rk_1). Only the heat-treated fraction from 65% methanol elution of the HP-20 column (HPHF) showed antibacterial activity against *P. acnes* (ATCC11827, MIC, 128 μg/mL; ATCC6919 MIC, 64 μg/mL), *S. epidermidis* (MIC, 4100 μg/mL) and *S. aureus* (MIC, 15000 μg/mL). In the anti-inflammatory activity assay, 10 μg/mL of HPHF significantly ($P < 0.05$) inhibited NO production in LPS-stimulated RAW264.7 cells. These results suggest that less polar ginsenosides enriched fraction HPHF obtained from AG might be useful to develop new types of antibacterial substances and new skin care cosmetics for acne prevention and therapy.

KEYWORDS: *American ginseng, less polar ginsenosides, anti-P. acnes, anti-inflammation*

INTRODUCTION

Ginseng has been used for thousands of years as a traditional medicine in oriental countries. The traditional source of ginseng root has been Asian ginseng (*Panax ginseng* C.A. Meyer), however, American ginseng (AG) [*Panax quinquefolius* L. Araliaceae], a plant native to North America, is now also cultivated and becoming increasingly popular in the east.[1] Throughout the past few decades, the pharmacological activities of American ginseng have attracted a great deal of attention. Like Asian ginseng, American ginseng has been reported to have a wide range of pharmacological effects such as modulation activities on central nervous and immune system, protection activity on cardiovascular system, antidiabetes and antitumor activities. [2] It has been generally accepted that the triterpene saponins, also called ginsenosides, are the major active constituents in ginseng.

Recently, there have been some reports demonstrating that the biological activities of ginseng are potentiated by heat-treatment, and the resulting changes in biological activities are due to the formation of new type of ginsenosides or the increase in the content of particular kind of ginsenosides.[3] Until now, most researchers have been focused on the ginsenosides components and biological activities changes of the steamed or heat-processed ginseng roots. However, the steaming or heating treatment of ginseng roots is always accompanied with the Maillard reaction, which can form undesired color or toxicological compounds, or cause the loss of some biological components.[4] A possible way to resolve this problem is to extract and isolate ginsenosides from the roots and then perform the heat-transformation.

Acne is a follicular rash that can cause comedo, red papules, and pustule.[5] It affects 79% to 95% of adolescents.[6] *Propionibacteria* and *Staphylococci* species are the predominate bacterial group in pilosebaceous sites.[7] The excessive growth of the two major bacteria in the sites and the inflammation caused by reactive oxygen species (ROS) are two physiological factors in the pathogenesis of acne.[8] Azelaic acid, retinoids, and antibiotics are commonly used to treat acne.[9] However, there are some limitations for these chemical drugs, such as the widespread *P. acnes* resistance[10] and skin irritation.[11] Therefore, in recent years, there has been growing interest in alternative therapies and in the therapeutic use of natural products, especially phytochemicals with antibacterial and anti-inflammatory activities, for acne treatment.[12] The antibacterial activity of *Panax ginseng* against *P. acnes* and *Staphylococcus aureus* (*S.*

Received: July 23, 2013
Revised: November 30, 2013
Accepted: December 1, 2013
Published: December 2, 2013

dx.doi.org/10.1021/jf404461q | J. Agric. Food Chem. 2013, 61, 12274−12282

aureus) has been reported.[13] Norajit et al.[14] and Sung et al.[15] also found that red Korea ginseng possessed stronger antibacterial activity than the white one. However, to the best of our knowledge, the antibacterial activity of less polar ginsenosides, obtained by heat treatment from American ginseng, against *Propionibacteria* and *Staphylococci* species is not reported.

In this study, (1) three polar ginsenosides enriched fractions and two less polar ginsenosides enriched fractions were obtained from TAG with AB-8 or HP-20 chromatography and heat-transformation, respectively; (2) contents of the ginsenosides and total polyphenol, antibacterial activity of these fractions, and ginsenosides structures transformation during heat-treatment were investigated; (3) the anti-inflammatory activity of the fraction, which showed antibacterial activity, was examined by nitric oxide (NO) production assay using the murine macrophage RAW264.7 cell line. Our results will help to develop new natural cosmetic products for acne prevention and therapy.

MATERIAL AND METHODS

Chemicals and Reagents. Ginsenosides standards (Rg_1, Re, Rb_1, Rc, Rb_2, Rd, 20(*R*)-Rg_2, 20(*S*)-Rg_2, 20(*R*)-Rg_3, 20(*S*)-Rg_3) with >98% purity were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Erythromycin, retinoic acid, azelaic acid, Trolox, gallic acid, Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, liopopolysaccharides (LPS) from *Escherichia coli*, trypan blue solution, and Griess reagent were all purchased from Sigma-Aldrich (Shanghai, China). Agar, actinomycete broth medium (GAM broth), Muller−Hinton broth (MHB) medium, and Muller−Hinton agar (MHA) medium were purchased from Beijing Aoboxing Biotech Co., Ltd. (Beijing, China).

Plant Materials and Sample Preparation. Four-year old cultivated American ginseng roots grown in Fusong (Jilin, China) were used in this experiment. The authenticity of those American ginseng roots were identified by Prof. Feng (The Ginseng and Antler Office of Jilin, Jilin, China). Dry roots were crushed into fine powder with a pulverizer and passed through a 60 mesh screen. The extraction and separation of ginsenosides protocol was slightly modified from that of Shehzad et al.[16] Briefly, 100 g power was immersed with 1500 mL 70% (v/v) ethanol−water solution in a flask. The extraction was performed on an ultrasonic cleaner (KQ-5000DE, 40 kHz, Beijing, China) under the conditions of ultrasonic power 500 W, extraction temperature 45 °C, and extraction time 1 h. Then, the flask was kept at 45 °C for 4 h in a water bath. The whole extraction was performed twice. After extraction, the mixture was filtered through Whatman (No. 2) filter paper under vacuum; the filtered solution was collected and then evaporated using a rotary evaporator (Buchi, Switzerland) at 45 °C to remove the ethanol, and lyophilized to obtain TAG. The dried TAG (85 g) was dissolved in distilled water and then loaded onto a Daion HP-20 open column (60 cm × 4.5 cm) (or a macroporous resin AB-8 open column (60 cm × 4.5 cm). The column was sequentially eluted with a methanol (or ethanol for AB-8 column) gradient from 0 to 15, 30, 65, and, finally, 80% (v/v). All the procedures were performed in triplicate. The results showed that, for the HP-20 column, the ginsenosides enriched fractions were eluted by 65% methanol (HPF1) and 80% methanol (HPF2); for the AB-8 column, the ginsenosides enriched fraction was only eluted by 65% ethanol (ABF). The fractions were evaporated, lyophilized, and stored at −20 °C until use.

Heat Transformation of Ginsenosides Enriched Fractions. ABF or HPF1 (14.0 g) was dissolved in distilled water (250 mL) and then subjected to an autoclave (MLS-3750; SANYO, Osaka, Japan) at 130 °C for 4 h. The heat-treated ABF or HPF1 fraction was loaded onto the AB-8 column and sequentially eluted with an ethanol gradient from 0 to 15, 30, 65, and finally 80%. All the procedures were performed in triplicate. The less polar ginsenosides enriched fraction was eluted by 65% ethanol. After evaporation and lyophilization, about 8.03 g of ABHF and 4.16 g of HPHF was obtained from ABF and HPF1, respectively. They were stored at −20 °C until use.

HPLC Analyses. Chromatographic analysis was performed using a SHIMADZU Prominence LC-20A HPLC instrument (Shimadzu Corporation, Kyoto, Japan) equipped with an YMC ODS-AM column (4.6 mm ×250 mm, YMC Co., Ltd., Kyoto, Japan). The detection wavelength was set at 202 nm. The mobile phase consisted of water (A) and acetonitrile (B). A gradient elution was used as follows: 20−26.5% B at 0−25 min, 26.5−38% B at 25−35 min, 38−80% B at 35−55 min, 80−100% B at 55−60 min. The flow rate was kept at 1 mL/min, and the injected sample volume was 20 μL.

HPLC-APCI-MS Conditions. The column effluent was introduced into an Esquire-LC-00054 (Bruker Daltonics, Massachusetts, USA) mass spectrometer equipped with an atmospheric pressure ionization source (APCI). Nitrogen was used as nebulizing gas and auxiliary gas. The parameters of APCI were slightly modified from the previous reported method.[17] The collision gas density was maintained at 70 molecule/cm^2; the coronal discharge was 2 μA; and the APCI source temperature was 450 °C. APCI-MS were acquired in negative mode to generate $[M\text{-}H]^-$ ginsenosides ions by scanning m/z over 50−2000 with unit mass resolution using a 1 ms dwell time.

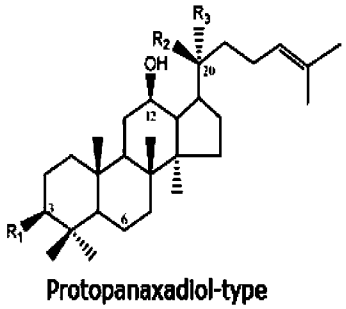
Preparation of Standard Curves. Stock solutions were prepared by dissolving Rg_1 (12.30 mg), Re (9.6 mg), Rb_1 (14.4 mg), 20(*S*)-Rg_2 (12.8 mg), 20(*R*)-Rg_2 (12.4 mg), Rb_2 (13.7 mg), Rd (10.6 mg), 20(*S*)-Rg_3 (10.3 mg), and 20(*R*)-Rg_3 (11.7 mg) in 70% (v/v) ethanol (10 mL), respectively. Then, the solutions were diluted with 70% (v/v) ethanol to give six different concentrations of working standard solutions. The working standard solutions were analyzed by the established method in triplicate. Calibration curves were constructed by plotting the peak area (y) versus the concentration of the nine ginsenosides standards (x), respectively. The content of ginsenosides in each sample was calculated using standard curves of each compound. Owing to the unavailability of reference compounds of Rg_6, F_4, Rs_3, Rg_5, and Rk_1; they were relatively qualified by 20(*S*)-Rg_3.[3]

Recovery Test. The recovery test was performed according to the method reported by In et al.[18] Three different concentrations of nine ginsenosides standards were added to known amounts of the preanalyzed ABHF sample solutions, respectively. The spiked samples were analyzed three times by the established HPLC method.

Determination of Total Polyphenol Content. Total polyphenol content (TPC) was determined according to the method of Singleton et al.[19] with some modifications. An appropriately diluted sample (1 mL) was mixed with 1 mL of Folin−Ciocalteau reagent. Then, 2 mL of a saturated sodium carbonate (Na_2CO_3) solution (10%) was added to the mixture. The mixture was subsequently brought to a final volume of 5 mL using distilled water. The absorbance was read at 765 nm (4802 UV/vis; UNICO) after 1 h of reaction. A standard calibration curve of gallic acid (0−100 μg/mL) was plotted. Results were expressed as μmol gallic acid equivalents (GAE)/g dried fraction. All determinations were performed in triplicate.

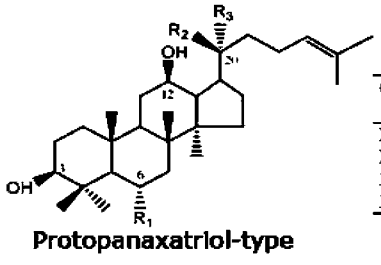
Antibacterial Activity Testing. Four bacterial species were used for the experiment: *S.aureus* (ATCC25923), *S. epidermidis* (ATCC12228), *P. acnes* (ATCC11827), and *P. acnes* (ATCC6919). *S. aureus* and *S. epidermidis* were cultured in MHB for 24 h, at 37 °C, and *P. acnes* was cultured in GAM broth at 37 °C for 24 h in YQX-II Anaerobic incubator (Shanghai, China) for further use. The anaerobic incubation atmosphere contained 5% (v/v) CO_2, 10% (v/v) H_2, and 85% (v/v) N_2. Cell suspensions were diluted in sterile MHB or GAM to provide initial cell counts of about 10^8 colony-forming unit per mL (CFU/mL). All antibacterial agents were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 200 mg/mL for the subsequent antibacterial experiments.

Determination of Growth Inhibition. The inhibition effect on the growth of *P. acnes* was determined by disk diffusion method. [20] Briefly, the bacteria were adjusted to the Mcfarland 0.5 standard and used to inoculate to GAM agar or MHA plates. The disk (6 mm in diameter) was impregnated with 5 μL of 200 mg/mL (1 mg/disc) samples, and placed on seeded agar. Erythromycin (6.4 μg/disc) was used as positive control. Discs contained DMSO only was used as negative



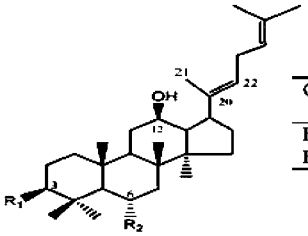
Protopanaxadiol-type

Ginsenoside	R_1	R_2	R_3	Formula	Molecular weight
Rb_1	-Oglc(2-1)glc	-Oglc(6-1)glc	$-CH_3$	$C_{54}H_{92}O_{23}$	1108
Rc	-Oglc(2-1)glc	-Oglc(6-1)araf	$-CH_3$	$C_{53}H_{90}O_{22}$	1078
Rb_2	-Oglc(2-1)glc	-Oglc(6-1)arap	$-CH_3$	$C_{53}H_{90}O_{22}$	1078
Rd	-Oglc(2-1)glc	-Oglc	$-CH_3$	$C_{48}H_{82}O_{18}$	946
Gyp XVII	-Oglc	-Oglc(6-1)glc	$-CH_3$	$C_{48}H_{82}O_{18}$	946
20(*S*)-Rg_3	-Oglc(2-1)glc	-OH	$-CH_3$	$C_{42}H_{72}O_{13}$	784
20(*R*)-Rg_3	-Oglc(2-1)glc	$-CH_3$	-OH	$C_{42}H_{72}O_{13}$	784
20(*S*)-Rs_3	-Oglc(2-1)glc-Ac	-OH	$-CH_3$	$C_{44}H_{74}O_{14}$	826
20(*R*)-Rs_3	-Oglc(2-1)glc-Ac	$-CH_3$	-OH	$C_{44}H_{74}O_{14}$	826

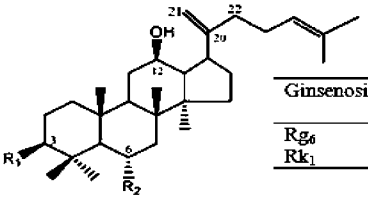


Protopanaxatriol-type

Ginsenosides	R_1	R_2	R_3	Formula	Molecular weight
Rg_1	-Oglc	-Oglc	$-CH_3$	$C_{42}H_{72}O_{14}$	800
Re	-Oglc(2-1)rha	-Oglc	$-CH_3$	$C_{48}H_{82}O_{18}$	946
20(*S*)-Rg_2	-Oglc(2-1)rha	-OH	$-CH_3$	$C_{42}H_{72}O_{13}$	784
20(*R*)-Rg_2	-Oglc(2-1)rha	$-CH_3$	-OH	$C_{42}H_{72}O_{13}$	784



Ginsenoside	R_1	R_2	Formula	Molecular weight
F_4	-OH	-Oglc(2-1)rha	$C_{42}H_{70}O_{12}$	766
Rg_5	-Oglc(2-1)glc	-H	$C_{42}H_{70}O_{12}$	766



Ginsenoside	R_1	R_2	Formula	Molecular weight
Rg_6	-OH	-Oglc(2-1)rha	$C_{42}H_{70}O_{12}$	766
Rk_1	-Oglc(2-1)glc	-H	$C_{42}H_{70}O_{12}$	766

C-20 dehydration group

Figure 1. Chemical structures of 17 ginsenosides in the fractions. glc, β-D-glucose; rha, α-L-rhamnose; arap, α-L-arabinose (pyranose); araf, α-L-arabinose (furanose); xyl, β-D-xylose. Ac, 6′-O-acetyl.

control. The MHA plates of *S. aureus* and *S. epidermidis* were incubated in an artificial climate chambers for 24 h and the GAM agar plates of *P. acnes* were incubated in the anaerobic incubator at 37 °C for 24 h (or 48 h). All determinations were performed in triplicate. The diameter of inhibition zone was determined.

Determination of MIC and MBC. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by microbroth dilution methods.[20,21] The diluted suspension of *S. aureus*, *S. epidermidis*, and *P. acnes* were inoculated into each well of a 96 well microplate, each containing a different concentration of the test samples. All the test broths and antibacterial agents-free broths contained 5% (v/v) DMSO. Agent-free broths were incubated as growth controls. The final range of test samples dilutions were 20 mg/mL to 0.0625 μg/mL in the MHB and GAM broth. The final bacteria concentration in each dilution was 1×10^5 CFU/mL. The plates of *S. aureus,* and *S. epidermidis* were incubated in an artificial climate chamber well, while the *P. acnes* were in the anaerobic incubator. They were all incubated at 37 °C for 24 h, but *P. acnes* ATCC6919 was incubated for 48 h. MIC was defined as the lowest concentration of antibacterial agents that inhibited bacterial growth, as indicated by the absence of turbidity. MBC was determined by inoculating a 10 μL of medium from each of the wells from the MIC test that showed no turbidity onto MHA or GAM agar plates and incubating for 24 h (or 48 h for *P. acnes* ATCC6919). MBC values were defined as the lowest concentration of antibacterial agents for which there was no bacteria growth on the plates. All determinations were performed in triplicate.

Anti-inflammatory Activity. *Cell Culture.* The murine macrophage cell line, RAW 264.7 (ATCC TIB 67), was obtained from Cell Resource Center (IBMS, CAMS/PUMC, Beijing, China). The cells were cultured in DMEM, which was supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin (10 000 U/mL)−streptomycin (10 000 U/mL)−amphotericin B (25 μg/mL). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell number and viability were assessed by the trypan blue dye exclusion method with a Neubauer hemacytometer.

NO Assay. One hundred microliters of RAW 264.7 cells (2.5×10^6 cells/mL) in RPMI-1640, which also supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin (10 000 U/mL)−streptomycin (10 000 U/mL)−amphotericin B (25 μg/mL), were seeded in a 96 well-plate. The macrophages were incubated in medium alone (control group) or pretreated with 0, 10, 50, and 200 μg/mL of HPHF for 2 h prior to addition of 1 μg/mL of LPS and then continued to incubate for 24 h. The NO production was measured by the method described by Azike et al.[22] Briefly, 50 μL of culture supernatant from each sample were transferred to wells of a 96-well U-bottom plate and equivalent amount of Griess reagent (containing 0.5% sulfanilic acid, 0.002% N-1-naphtyl-ethylenediamine dihydrochloride, and 14% glacial acetic acid) was added. The absorbance at 540 nm wavelength was measured using RT-6000 microplate reader (Rayto, Shenzhen, China). Nitrite concentrations were estimated from

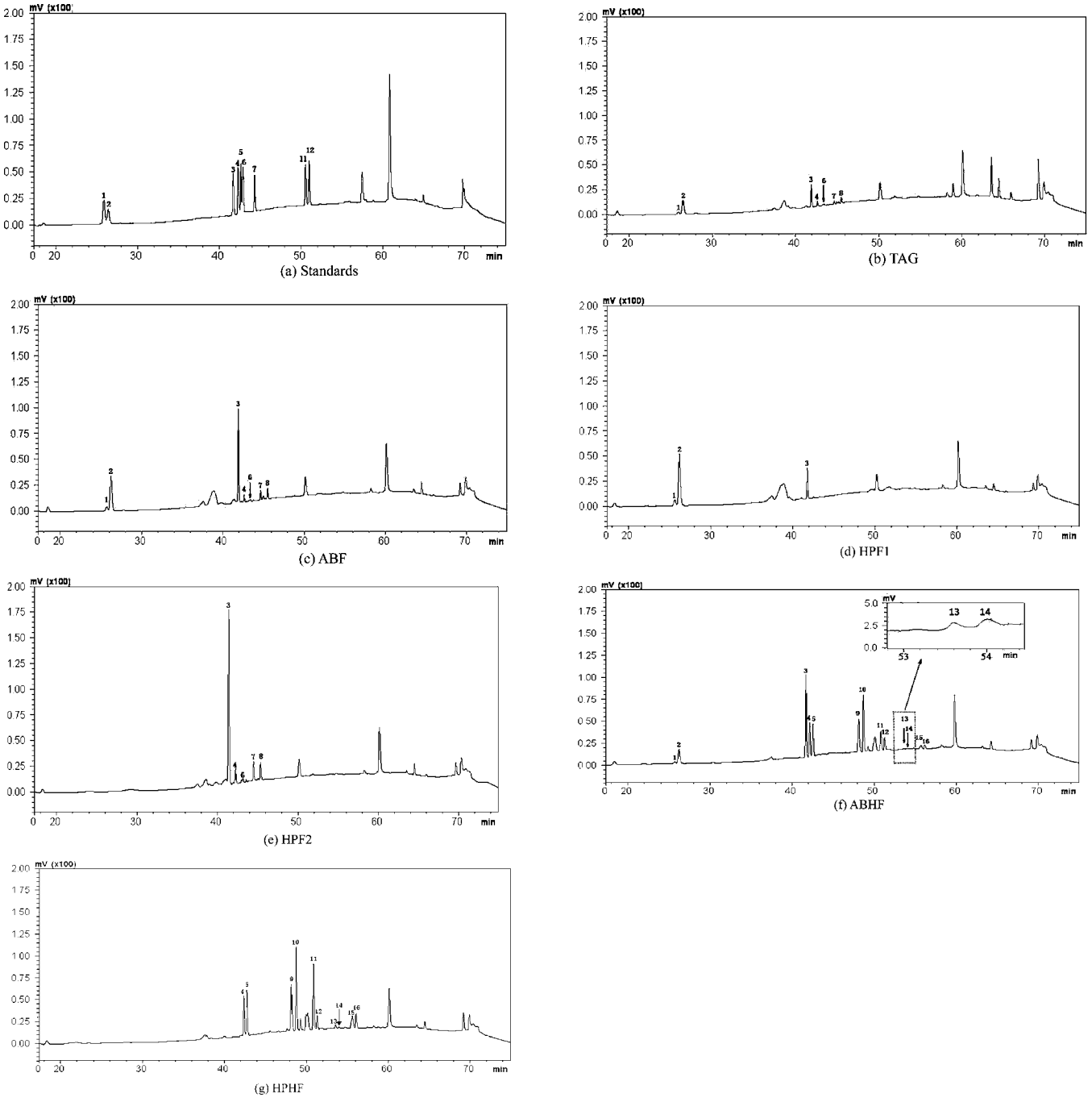


Figure 2. Typical HPLC-UV chromatograms at 202 nm of standards (a), TAG (b), ABF (c), HPF1 (d), HPF2 (e), ABHF (f), and HPHF (g). Ginsenoside peaks: (1) Rg_1, (2) Re, (3) Rb_1, (4)Rc/20(*S*)-Rg_2, (5) 20(*R*)-Rg_2, (6) Rb_2, (7) Rd, (8) Gyp XVII, (9)Rg_6/F_4, (10)F_4/Rg_6, (11)20(*S*)-Rg_3, (12) 20(*R*)-Rg_3, (13)20(*S*)-Rs_3, (14) 20(*R*)-Rs_3, (15) Rg_5/Rk_1, (16) Rk_1/Rg_5. TAG is the total ginsenosides extraction from American ginseng roots. ABF is the 65% ethanol eluted fraction from AB-8 column. HPF1 and HPF2 are 65% and 80% methanol eluted fractions from HP-20 column, respectively. ABHF and HPHF are heat-transformation fractions from ABF and HPF1, respectively.

a sodium nitrite standard calibration curve. All the experiments were performed in triplicate.

Statistical Analysis. Data were presented as mean ± standard deviation (SD). Statistical analyses were done using the statistical SAS 9.1 program, and the significance of each group was evaluated by one-way analysis of variance (ANOVA) followed by Duncan's test of $P < 0.05$.

RESULTS AND DISCUSSION

Identification of Ginsenosides. Sixteen ginsenosides were simultaneously identified according to the standards and HPLC-APCI-MS ions fragments. The results were consistent with previous studies.[23,24] Their chemical structures are shown in Figure 1. The typical HPLC-UV chromatograms of the standards and the ginsenosides enriched fractions are shown in Figure 2. The identification of ginsenosides Rg_1, Re, Rb_1, 20(*R*)-Rg_2, Rb_2, Rd, 20(*S*)-Rg_3, and 20(*R*)-Rg_3 was performed by comparing the retention times and $[M-H]^-$ with those of known standards (Table 1). Although ginsenosides Rc and 20(*S*)-Rg_2 had similar retention times, simultaneous determination could be accomplished by reference to the different MS

dx.doi.org/10.1021/jf404461q | J. Agric. Food Chem. 2013, 61, 12274−12282

Table 1. Ginsenosides and Total Polyphenol Contents in Different Fractions[a]

ginsenosides	time	$[M-H]^-$	TAG	ABF	HPF1	HPF2	ABHF	HPHF
Rg_1	24.656	799.5	0.38 ± 0.07 d	1.10 ± 0.04 b	2.38 ± 0.02 a	ND	0.66 ± 0.1 c	ND
Re	25.267	945.9	3.72 ± 0.11 d	17.11 ± 0.43 b	28.36 ± 0.51 a	ND	8.36 ± 0.08 c	ND
Rb_1	41.450	1107.6	3.47 ± 0.05 e	26.65 ± 0.36 c	8.55 ± 0.67 d	55.60 ± 0.6 a	29.33 ± 0.17 b	ND
Rc	41.913	1078.3	0.64 ± 0.01 c	1.98 ± 0.06 b	ND	4.44 ± 0.16 a	ND	ND
20(*S*)-Rg_2	41.913	783.7	ND	ND	ND	ND	9.3 ± 0.28 b	10.64 ± 0.37 a
20(*R*)-Rg_2	42.265	783.7	ND	ND	ND	ND	9.11 ± 0.16 b	12.13 ± 0.34 a
Rb_2	42.864	1077.5	0.16 ± 0.1 c	0.66 ± 0.09 b	ND	1.37 ± 0.08 a	ND	ND
Rd	44.205	945.9	0.35 ± 0.02 c	2.16 ± 0.25 b	ND	4.34 ± 0.03 a	ND	ND
Gyp XVII	45.225	945.9	0.70 ± 0.02 c	2.49 ± 0.17 b	ND	3.95 ± 0.04 a	ND	ND
Rg_6/F_4	47.683	765.7	ND	ND	ND	ND	10.04 ± 0.07 b	15.29 ± 0.25 a
F_4 /Rg_6	48.237	765.7	ND	ND	ND	ND	14.18 ± 0.17 b	22.4 ± 0.13 a
20(*S*)-Rg_3	50.380	783.8	ND	ND	ND	ND	3.66 ± 1.56 b	16.72 ± 1.14 a
20(*R*)-Rg_3	50.797	783.8	ND	ND	ND	ND	3.11 ± 0.36 a	3.15 ± 0.5 a
20(*S*)-Rs_3	53.541	825.8	ND	ND	ND	ND	0.24 ± 0.1 b	1.11 ± 0.03 a
20(*R*)Rs_3	53.979	825.9	ND	ND	ND	ND	0.24 ± 0.05 a	0.33 ± 0.09 a
Rg_5/Rk_1	55.124	765.8	ND	ND	ND	ND	1.58 ± 0.19 b	5.14 ± 0.30 a
Rk_1/Rg_5	55.597	765.8	ND	ND	ND	ND	1.1 ± 0.01 b	4.42 ± 0.01 a
total content			9.43 ± 0.17 e	52.15 ± 0.61 d	39.29 ± 0.73 c	69.71 ± 0.74 b	90.89 ± 1.14 a	91.35 ± 1.23 a
TP (μmol, GAE/g)			27.04 ± 1.55 b	15.51 ± 0.97 e	21.46 ± 0.75 c	5.61 ± 0.76 f	18.07 ± 1.25 d	30.80 ± 0.92 a

[a]Data were expressed as means ± SD (mg/100 mg of dry weight, n = 3). ND, Not detected. TP: Total polyphenol content, GAE: Gallic acid equivalents. TAG is total ginsenosides extraction from American ginseng roots. ABF is 65% ethanol eluted fraction from AB-8 column. HPF1 and HPF2 are 65% and 80% methanol eluted fractions from HP-20 column, respectively. ABHF and HPHF are heat-transformation fractions from ABF and HPF1, respectively. Rc was relatively qualified by 20(*S*)-Rg_2. Gyp XVII is relatively qualified by Rd. Rg_6, F_4, Rs_3, Rg_5 and Rk_1 are relatively qualified by 20(*S*)-Rg_3. Values in each row followed by different letters are significantly different ($P < 0.05$).

of their molecular ions. In the chromatogram of TAG, ABF (65% ethanol eluted fraction from AB-8 column), and HPF2 (80% methanol eluted fraction from HP-20 column) (Figure 2b, c and e), the fourth peak was identified as Rc on the basis of the presence of $[M-H]^-$ ion at m/z 1078.3. However, in the chromatogram of ABHF (heat-transformation fraction from ABF) and HPHF (heat-transformation fraction from HPF1) (Figure 2f and g), the fourth peak was identified as 20(*S*)-Rg_2 on the basis of the presence of $[M-H]^-$ ion at m/z 783.7. Moreover, ginsenosides with the same molecular weights such as Re/Rd/Gypenoside XVII (Gyp XVII), Rg_2/Rg_3, Rg_6/F_4/Rg_5/Rk_1, could be distinguished by their different chromatographic retention times.

Method Validation. In order to validate our analysis method, the recoveries for the nine ginsenosides were calculated from nine different analyses. The linearity and recovery results are listed in Supporting Information. The correlation coefficients were greater than or equal to 0.9911, which showed a good linearity. The average recovery of the ginsenosides ranged from 98.9 to 102.3% with RSD less than 3.0%. These results demonstrated the accuracy and repeat ability of the method.

Yield and Ginsenosides Content. Two ginsenosides enriched fractions, HPF1 (65% methanol eluted fraction from HP-20 column) and HPF2, were eluted from HP-20, while only one fraction (ABF) was eluted from AB-8. About 45.8 ± 2.36 g of TAG, 5.34 ± 1.12 g of ABF, 2.83 ± 0.83 g of HPF1, 2.03 ± 0.35g of HPF2, 2.13 ± 0.74 g of ABHF, and 0.84 ± 1.03 g of HPHF were obtained from each 100 g of ground American ginseng roots. The total ginsenosides content in TAG was only 9.43 ± 0.17 mg/100 mg. After purification by AB-8, or HP-20, the total ginsenosides content in ABF, HPF1, and HPF2 was increased to 52.15 ± 0.61, 39.29 ± 0.73, and 69.71 ± 0.74 mg/100 mg, respectively (Table 1). After heat-transformation, the content in ABHF and HPHF was further increased to 90.89 ± 1.14 and 91.35 ± 1.23 mg/100 mg (Table 1). It indicated that the purification steps used in this study significantly improved the purity of the ginsenosides in each fraction. Rg_1 and Re were only found in HPF1. Rc, Rb_2, Rd, and Gyp XVII were only found in HPF2. Rb_1 was found both in HPF1 and HPF2, but it was about 6.5 times higher in the latter than in the former. This result was inconsistent with the previous report by Shehzad et al.[16] (90 g dry extract was loaded onto a 100 cm × 10 cm HP-20 open column) that Rb_1 was only found in the HP-20 80% (v/v) methanol eluted fraction. One of the possible reasons for such a difference might be that more TAG was loaded on to the HP-20 column in our experiment.

Structural Changes in the Heat-transformation Process. To remove the pigments and monosaccharides produced during heat-transformation, ABF and HPF1 were purified by AB-8 to get ABHF and HPHF, respectively. After purification, Rg_1, Re, and Rb_1 were not detected in HPHF (Figure 2g), while there were still 0.66 ± 0.1 mg/100 mg of Rg_1, 8.36 ± 0.08 mg/100 mg of Re, and 29.33 ± 0.17 mg/100 mg of Rb_1 in ABHF (Figure 2f and Table 1). New types of ginsenosides 20(*S*)-Rg_2, 20(*R*)-Rg_2, 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rg_6/F_4, 20(*S*)-Rs_3, 20(*R*)-Rs_3, and Rk_1/Rg_5 were observed in ABHF and HPHF (Figure 2f and g). This was due to the conversion of polar ginsenosides to less polar ones by high temperature and pressure.[25,26] The 20(*S*) and 20(*R*)-ginsenoside compounds represented typical stereoisomers formed by the selective attack of hydroxyl group after elimination of glycosyl at C-20. The ginsenosides structural changes during the heat-transformation process were schemed in Figure 3. During the process, the protopanaxiadiol (PPD) group (e.g., ginsenosides Rb_1, Rc, Rb_2, and Rd) were easy to selectively eliminate the carbon-20 sugar chain to produce 20(*S*)/(*R*)-Rg_3 (Figure 3a). The generated content of 20(*S*)-Rg_3, however, was higher than that of 20(*R*)-Rg_3 (Table 1), which was consistent with the previous reports.[3,27] This was more obvious in HPHF than in

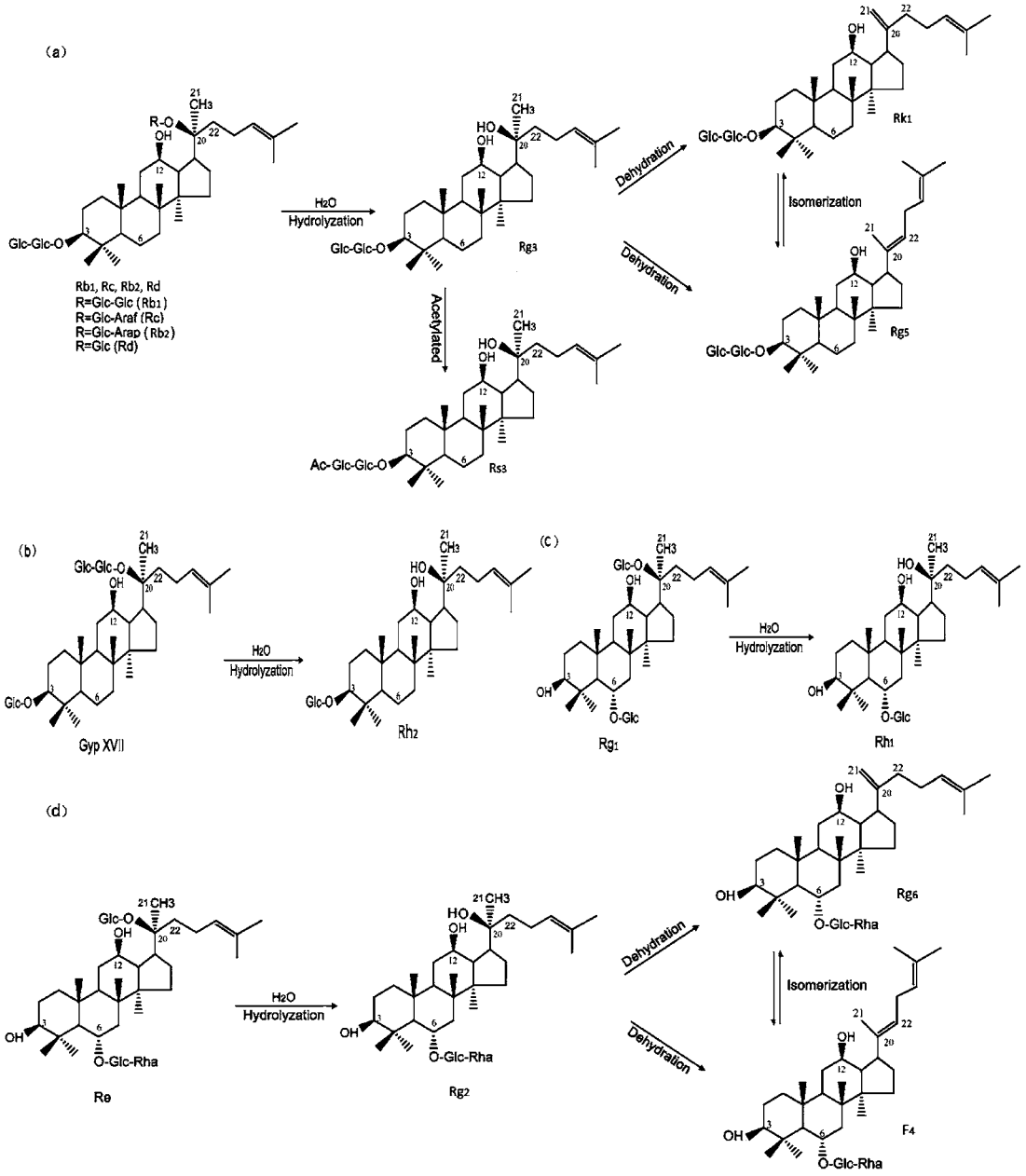


Figure 3. Proposed major structural changes of polar ginsenosides to less polar ones during heat-transformation. glc, β-D-glucose; rha, α-L-rhamnose; arap, α-L-arabinose (pyranose); araf, α-L-arabinose (furanose); xyl, β-D-xylose; Ac, 6′-O-acetyl.

Table 2. Growth Inhibition of the Ginsenosides Enriched Fractions and the Positive Control Erythromycin

	diam. of inhibition zone, $\sigma \pm$ SD (mm) (at 1 mg/disk)			
	P. acnes[a]	*P. acnes*[b]	*S. epidermidis*[c]	*S. aureus*[d]
HPHF[e]	13.0 ± 0.06 b	22.5 ± 0.5 a	8.5 ± 0.62 c	7.78 ± 0.51 c
erythromycin[f]	22.2 ± 0.29 c	25.1 ± 0.21 a	18.3 ± 0.25 d	23.3 ± 0.25 b

[a]*Propionibacterium acnes* (ATCC11827). [b]*Propionibacterium acnes* (ATCC6919). [c]*Staphylococcus epidermidis* (ATCC12228). [d]*Staphylococcus aureus* (ATCC25923). [e]HPHF is heat-transformation fractions from HPF1. Values in each row followed by different letters are significantly different ($P < 0.05$). [f]The content of erythromycin is 6.4 μg/disk.

ABHF. 20(S)/(R)-Rg_3 could be further transformed to two geometric isomers, which represent positional isomers of the double bond at carbon-20(21) or carbon-20(22), namely Rk_1 and Rg_5, by dehydration.[27] Furthermore, 20(S)/(R)-Rg_3 could also be converted to 20(S)/(R)-Rs_3 by acetylation at glycosides.[28,29] Previous report found that Gyp XVII could be transformed to Rh_2 (Figure 3b) by losing the carbon-20 sugar chain.[30] As shown in Figure 3c and d, the protopanaxatriol (PPT) group, Rg_1 and Re, tended to first lose (20) glc residue to form Rh_1 and 20(S)/(R)-Rg_2, respectively; 20(S)/(R)-Rg_2 could be further converted to F_4 and Rg_6 by dehydration.[31] However, in the HPLC analysis, Rh_2 and Rh_1 were not

Table 3. MIC and MBC of HPHF, Retinoic Acid, Azelaic Acid, and Erythromycin

	P. acnes[a]		*P. acnes*[b]		*S. epidermidis*[c]		*S. aureus*[d]	
antibacterial agent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
HPHF[e]	128	512	64	64	4100	15000	15000	20000
retinoic acid	4	16	8	8	8	16	16	16
azelaic acid	5000	5000	5000	5000	2048	2048	2500	2500
erythromycin	0.0625	0.125	0.25	0.25	0.25	0.25	8	8

[a]*Propionibacterium acnes* (ATCC11827). [b]*Propionibacterium acnes* (ATCC6919). [c]*Staphylococcus epidermidis* (ATCC12228). [d]*Staphylococcus aureus* (ATCC25923). [e]HPHF is the heat-transformation fraction from HPF1.

detected. One possible reason was that Rh_2 and Rh_1 could be easily decomposed by high temperature treatment (130 °C for 4 h).

Polyphenol Content. The highest polyphenol content was found in HPHF, followed by TAG > HPF1 > ABHF > ABF > HPF2 (Table 1). This indicated that the polyphenol content could be decreased by macroporous resins but increased by heat-transformation. The polyphenol content of 70% v/v ethanol extract (TAG) was comparable to the gastrointestinal mimic extraction of American ginseng roots.[32]

Anti-*P. acnes* Activity. Results of the disk diffusion method testing (Table 2) showed that HPHF had an inhibition zone at 1 mg per disk against the four test bacteria, but the inhibition zone of TAG, ABF, HPF1, HPF2, and ABHF were not detected (results were not shown).

The MIC and MBC of six ginsenosides fractions against the four bacterial strains, along with azelaic acid, retinoic acid, and erythromycin, are shown in Table 3. Similar to the inhibition zone results, the MIC and MBC of TAG, ABF, HPF1, HPF2, and ABHF (results not shown) were all not detected against the four test bacterial strains even when the concentrations were at 20 mg/mL. *P. acnes* ATCC6919 was mostly susceptible to HPHF, with the MIC and MBC both of 64 μg/mL. The MIC of HPHF (128 μg/mL) against *P. acnes* ATCC11827 was lower than that the clove essential oil (310 μg/mL), which is always acting as defensive substance against microorganisms.[33] HPHF, with MIC values of 4100 μg/mL and 15 000 μg/mL against *S. epidermidis* and *S. aureus,* exhibited less potent activity than the ginsenosides from Korean red ginseng, which showed the same MIC value of 100 μg/mL against the two bacterial strains.[15]

It seems that the less polar ginsenosides, such as Rg_2, Rg_3, Rg_6/F_4, Rs_3, and Rg_5/Rk_1, in HPHF might contribute to the antibacterial activity. One possible reason for this is that less polar ginsenosides are more lipophilic than polar ones and more easily interact with the bacterial cell membranes or pass through cell membranes to influence metabolism of the bacteria. However, future research should be conducted to prove their effectiveness by using pure less polar ginsenoside and to clarify the antibacterial mechanism. However, ABHF, which also contained less polar ginsenosides, showed no antibacterial activity. In ABHF, the polar ginsenosides (Re, Rg_1, and Rb_1) and the less polar ones (Rg_2, Rg_3, Rg_6/F_4, Rs_3, Rg_5/Rk_1) accounted for 38.35% and 52.56% (Table 1), respectively. It has been reported that some ginsenosides showed opposite activity on central nervous system, such as Rg_1 (enhance effect) and Rb_1 (inhibit effect).[34] It might be concluded that polar ginsenosides in some case had opposite effect on the antibacterial activity of less polar ginsenosides.

Effect of HPHF on NO Production. As shown in Figure 4, NO production of cells stimulated by LPS (19.1 ± 1.6 μM) was significantly ($P < 0.05$) higher than that of the control (10.4 ± 0.71 μM). However, NO production of cells pretreated with HPHF evidenced a concentration-dependent reduction in the after LPS stimulation. The NO production of HPHF at 200 μg/mL showed insignificant difference ($P > 0.05$) with that observed in control. For the dose 200 μg/mL is out of physiological range, HPHF might be only considered for topical therapeutic use.

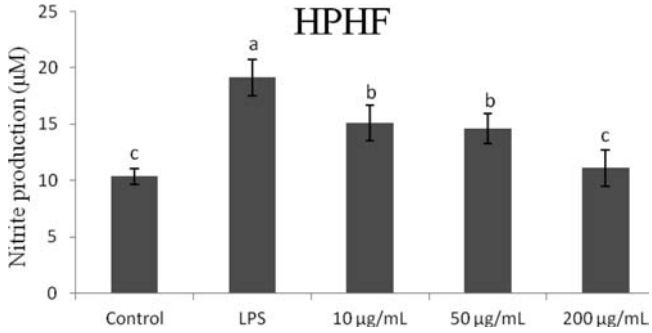


Figure 4. Suppressive effect of HPHF on the up-regulation of macrophage NO production by LPS. HPHF is the heat-transformation fraction from HPF1. Experiments were performed in triplicate and the data are expressed as mean ± SD. Values sharing different letters are expressed as significantly different ($P < 0.05$).

Inflammation component is a prominent clinical feature in many acne patients. It has been reported that *P. acnes* is an important factor in acne inflammation.[35] Tsai et al.[36] also reported that *P. acnes* could increase NO production by increasing ROS accumulation. A low level of ROS and NO is necessary in the inflammatory process. However, when large amounts of ROS and NO are produced, induced, or generated in the human body, it can lead to apoptotic or necrotic cell death, tissue destruction, and occasionally, shock and death.[37] After infection of *P. acnes*, our body always overproduces the ROS and NO, which outweighs the defense mechanism.[38] The antibacterial and anti-inflammatory activities of HPHF could guarantee its medicinal use in the treatment of acne. Ginsenosides can be absorbed in gastrointestinal tract and then deglycosylated by intestinal microflora after oral consumption of ginseng. However, the oral bioavailability of ginsenosides is generally very low (less than 18%).[39] As acne vulgaris is a skin disease, many antibiotics, such as erythromycin, clindamycin, doxycyline, are used in the topical treatment of acne.[40] Besides, some kinds of herbs that show antibacterial and anti-inflammatory activities have already been used in topical treatment of acne.[12] Our research suggests that American ginseng can also be used in the topical treatment of acne.

■ ASSOCIATED CONTENT

Supporting Information

Linear equations of nine ginsenosides standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study is supported by the International S&T Cooperation Project (No. 2011DFG32500) and the Agricultural Science and Technology Innovation Program of CAAS for Wang Tianyu (Crop Germplasm Resources Identification and Discovery).

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