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Multivariate analysis on the relationship between radical scavenging activities and phenolic compounds of *baijiu* and its protective effect against LPS-induced inflammation in THP-1 cells

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Abstract

In previous studies, three phenolic compounds (vanillin, 4-methylguaiacol, and 4-ethylguaiacol) were identified in *baijiu*, and confirmed to possess antioxidant activity *in vitro*. However, the distribution of phenolic compounds in *baijiu*, and their associations with the functionality of this regimen have not been previously reported. In the present work, the antioxidant capacity and anti-inflammatory effect of baijiu were evaluated by DPPH$^{\bullet}$, ABTS$^{\bullet+}$, ELISA, and real-time PCR assays. The concentrations of vanillin, 4-methylguaiacol, and 4-ethylguaiacol (bioactive phenolic compounds) in 103 *baijiu* samples were confirmed by liquid-liquid extraction (LLE) combined with gas chromatography-mass spectrometry (GC-MS). *Baijiu* exhibited DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities, which positively correlated with the concentrations of vanillin, 4-methylguaiacol, and 4-ethylguaiacol. Moreover, ELISA and real-time PCR assays demonstrated that *baijiu* could relieve inflammation caused by LPS through the inhibition of NF-κB and AP-1 expressions, induction of Nrf2 expression, and repression of inflammatory cytokine secretion. These findings lay the foundation for further investigation on the health benefits of *baijiu* and its bioactive components by animal and human studies.

Introduction

Baijiu (literally means white wine) is a traditional indigenous distilled spirit prepared from grain fermentation, and has been used for almost 2,000 years, dating from the Western Han dynasty (Liu and Sun, 2018). As the national liquor of China, *baijiu* is popular among the Chinese. In 2018, the annual income and profit of *baijiu* were over USD 82.1 billion and USD 19.2 billion, respectively. Undoubtedly, the flavour of *baijiu* determines the consumers' acceptance, and majority studies have focused on the contribution of aroma components to *baijiu* flavour (Zheng *et al.,* 2016; Xiao *et al.,* 2016; Sha *et al.,* 2017; Sun *et al.,* 2018; Liu and Sun, 2018; Dong *et al.,* 2018; Li *et al.,* 2019). Currently, due to people's increasing health awareness, many studies have started to focus on the beneficial functions of *baijiu* on human health.

In China, *baijiu* is widely recognised as the essence of grain. The Chinese believe that moderate drinking may dulcify the mind and body. Bencao Gangmu, the Chinese *materia medica*, indicates that moderate consumption of *baijiu* may eliminate coldness, fatigue, and phlegm dampness (Zheng and Han, 2016). Recently, moderate alcoholic-beverage intake was reported to exert a protective effect on general health and cardiovascular-disease-specific mortality in the United States (Xi *et al.,* 2017). Additionally, it has been demonstrated that the *baijiu* extract could enhance inflammation resistance through the suppression of inflammatory cytokine production in mononuclear macrophages (RAW 264.7 cell line) (Liu *et al.,* 2018). The main components of *baijiu* are water and ethanol, while the remaining trace components are deemed as the key to the health functions and benefits of *baijiu*. This led to increased interest among researchers on the protective effects of the bioactive components of *baijiu* on human health.

A previous study identified a tetrapeptide (Ala-Lys-Arg-Ala) from the sesame aroma type of *baijiu*, and confirmed that this compound could exert preventive effects against 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH)-induced oxidative stress in HepG2 cells (Wu *et al.,* 2017). In

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another study, Sun *et al.* (2018) separated two tripeptides, namely Lys-Gly-Pro and Val-Pro-Asp from *jiupei* (the fermented raw material of *baijiu*) by LLE, ultrafiltration, macroporous resin chromatography, gel chromatography, and preparative liquid chromatography coupled with HPLC-Q-TOF-MS. These two tripeptides exerted antioxidant properties on the basis of *in vitro* chemical experiments and AAPH-induced HepG2 cells (Sun *et al.,* 2018). In consideration of the low volatility of peptides, the volatile compounds in *baijiu* are more likely to play beneficial functions. Therefore, volatile phenolic compounds in *baijiu* are receiving considerable attention in terms of their potential beneficial functions on human health (Jiang *et al.,* 2019). A recent study confirmed the potent cytoprotective effect of vanillin (VA), 4-methylguaiacol (4-MG), and 4-ethylguaiacol (4-EG) (Zhao *et al.,* 2018). Despite the beneficial components that have been investigated in *baijiu*, its function and the association between the concentration of the bioactive components and *baijiu* bioactivity are considered a puzzling question that requires further investigation.

Therefore, the present work aimed to explore the antioxidant properties and anti-inflammatory effects of *baijiu*, and to investigate the potential association between bioactive components (*i.e.,* VA, 4-MG, and 4-EG) concentration and *baijiu* bioactivity. The antioxidant activity of *baijiu* was evaluated by the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays. ELISA and real-time PCR assays highlighted the effects of *baijiu* on the protein expression of inflammatory cytokines and the gene expression levels of related regulatory proteins (NF-κB, AP-1, and Nrf2) in LPS-induced THP-1 human monocytic cells. The link between bioactive components (*i.e.,* VA, 4-MG, and 4-EG) and the antioxidant activity of *baijiu* was assessed with liquid-liquid extraction (LLE)-gas chromatography-mass spectrometry (GC-MS) and partial least squares regression (PLSR) analysis.

Materials and methods

Sampling

A total of 103 *baijiu* samples were commercially obtained, and analysed in the present work. All samples were stored at 4°C until further analysis. In order to avoid commercial interests, the brand names of the *baijiu* samples were not revealed.

Materials and reagents

Absolute ethanol, dichloromethane, and sodium chloride (NaCl) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Vanillin, 4-methylguaiacol, and 4-ethylguaiacol were purchased from Ruiyuan Spice Co., Ltd. (Zaozhuang, China). 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), lipopolysaccharides (LPS), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, USA). 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\bullet+}$) was purchased from Biotop Life Sciences Co. Ltd. (Beijing, China). Potassium persulfate was purchased from Xilong Scientific Co. Ltd. (Beijing, China).

DPPH$^{\bullet}$ assay

The DPPH$^{\bullet}$ radical scavenging activity was determined following a previous method with slight modifications (Zhao *et al.,* 2017). Generally, 100 μL of *baijiu* sample was mixed with 100 μL of DPPH$^{\bullet}$ ethanolic solution (0.15 mM). A total of 100 μL of DPPH$^{\bullet}$ ethanolic solution (0.15 mM) was mixed with 100 μL of 50% ethanol solution, and used as the blank control. The mixture of 100 μL of ethanol and 100 μL of 50% ethanol solution was used to adjust the baseline. The reaction was conducted at room temperature for 40 min in a darkroom. A spectrophotometer (SpectraMax M2e) (Molecular Devices, USA) was used to monitor the decrease in the DPPH* radical concentration by measuring the absorbance at 517 nm.

ABTS$^{\bullet+}$ assay

The ABTS$^{\bullet+}$ radical scavenging activities were determined following a previous method (Zhao *et al.,* 2017). Briefly, 7 mM ABTS$^{\bullet+}$ ammonium and 2.45 mM potassium persulfate were mixed to prepare the ABTS$^{\bullet+}$ radical stock solution at room temperature for 16 h in a darkroom. Subsequently, the working solution of ABTS$^{\bullet+}$radical was obtained by diluting the ABTS$^{\bullet+}$ radical stock solution with 100 mM phosphate buffered saline (PBS) when its absorbance value was close to 0.70 (λ = 734 nm). Next, 50 μL of *baijiu* sample was mixed with 150 μL of ABTS$^{\bullet+}$ radical working solution. Then, 150 μL of ABTS$^{\bullet+}$ radical working solution was mixed with 50 μL of 50% ethanol solution, and used as the blank control. The mixture of 150 μL of 100 mM PBS and 50 μL of 50% ethanol solution was used to adjust the baseline. The reaction was conducted at room temperature for 30 min. A spectrophotometer (SpectraMax M2e) (Molecular Devices, USA) was used to monitor the decrease in the ABTS$^{\bullet+}$ radical concentration by measuring the absorbance at 734 nm.

Quantification of vanillin, 4-methylguaiacol, and 4-ethylguaiacol in baijiu samples

Vanillin, 4-methylguaiacol, and 4-ethylguaiacol in *baijiu* samples were extracted by LLE, and the concentrations were detected by an Agilent 7890B gas chromatograph, equipped with an Agilent 5977A mass-selective detector (Agilent Technologies, USA) as previously described (Zhao *et al.,* 2018). Generally, 2 mL of each *baijiu* sample was diluted to 15% ethanol with boiled Milli-Q water, saturated with NaCl, and extracted three times with freshly distilled dichloromethane (3 mL each time) by Vortex 2 (IKA, Germany). Subsequently, the extract was collected by centrifugation (6,000 rpm, 10 min), and concentrated to a final volume of 500 μL. Afterwards, the concentrated extract was analysed by GC-MS.

Stock solutions of vanillin, 4-methylguaiacol, and 4-ethylguaiacol were prepared in absolute ethanol. Afterwards, a series of working solutions (10,000, 5,000, 2,000, 1,000, 500, 200, 100, 50, 20, 10, and 1 μg/L) were prepared by diluting the stock solutions with 50% ethanol solution. Finally, 2 mL of each working solution was pre-treated and analysed using the same pre-treatment method as that for the *baijiu* samples.

Each *baijiu* sample extract (1 μL) or working solution extract (1 Ml) was injected in splitless mode, and analysed on a DB-FFAP column (60 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, USA). Helium was the carrier gas, and the flow rate was 1.0 mL/min. The injector temperature was 250°C. The temperature program of the oven was as follows: oven temperature was ramped from 40 to 50°C at a rate of 10°C/min, and held for 20 min, then raised at 1°C/min up to 70°C, and held for 10 min, finally raised at 3°C/min up to 250°C, and held for 15 min.

MS was conducted in electron ionisation (EI) mode at 70 eV. The temperatures of the interface and the ion source were 250 and 230°C, respectively. Vanillin, 4-methylguaiacol, and 4-ethylguaiacol were identified in a full scan mode with the mass range set from 45 to 350 amu, and quantified in selective ion monitoring (SIM). The selected ions (*m/z*) of vanillin, 4-methylguaiacol, and 4-ethylguaiacol were 151, 138, and 91, respectively.

The calibration curves were drawn by plotting the responses of vanillin, 4-methylguaiacol, and 4-ethylguaiacol against the corresponding concentrations. The concentrations of vanillin, 4-methylguaiacol, and 4-ethylguaiacol were calculated based on the calibration curves. The limit of detection (LOD) and limit of quantification (LOQ) were obtained from the lowest concentrations of the target compound working solutions based on a signal-to-noise ratio of 3 and 10, respectively.

THP-1 cell culture

The human monocytic cell line (THP-1) was obtained from the National Institute for Communicable Disease Control and Prevention, Chinese Centre for Disease Control and Prevention. Briefly, THP-1 cells were cultured in a humidified incubator at 37°C in the presence of 5% CO_2 using Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen Life Technologies Inc.) supplemented with 10% (v/v) foetal-bovine serum (FBS, Gibco, USA). Prior to inflammatory stimulation, 80% confluent THP-1 cells were differentiated into a mature macrophage-like state by stimulation with 50 ng/mL PMA for 48 h.

Cell viability assay

The influence of *baijiu* on THP-1 cell viability was determined by the CCK-8 assay (Dojindo, Kumamoto, Japan). *Baijiu* sample (No. SA-3) was selected for the following experiments. Briefly, THP-1 cells (1×10^5 cells/mL) were seeded in a 96-well microplate, and treated with different *baijiu* dilutions in medium (37°C, 5% CO_2). Following treatment of the cells of 24 h, the CCK-8 reagent was added to each well, and the absorbance was measured at 450 nm with a spectrophotometer (SpectraMax M2e) (Molecular Devices, USA). Cell viability was expressed as the percentage of the absorbance estimated compared with that of the untreated control cells.

Inflammatory stimulation

THP-1 cells (5×10^5 cells/mL) were seeded into a 12-well microplate (for ELISA) or a 6-well microplate (for real-time-PCR). After the growth medium was removed, THP-1 cells were washed with PBS (Gibco) and treated with *baijiu* dilution (2%) for 1 h. Subsequently, THP-1 cells were incubated with LPS (1 μg/mL) for an additional 23 h (37°C, 5% CO_2). Cells treated with *baijiu* dilution alone were considered as the sample control group, whereas cells treated with LPS alone were considered as the LPS group. Cells treated with neither *baijiu* dilution nor LPS were considered as the control group.

Measurement of released inflammatory cytokines in cell supernatants by ELISA

Following inflammatory stimulation, the

supernatant of each well was collected for ELISA analysis. The concentrations of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), prostaglandin E_2 (PGE_2), and nitric oxide (NO) in the supernatants were measured using ELISA kits purchased from Abcam (Cambridge, UK) following the manufacturer's instructions. The results were shown as fold-changes relative to the control group.

Real-time PCR

Following inflammatory stimulation, the medium was removed, and the cells were washed with PBS. Afterwards, the total RNA was extracted by the Trizol reagent (Life Technologies, Shanghai, China) following the manufacturer's instructions. Subsequently, the M-MLV Reverse Transcriptase Kit (Promega, Madison, MI, USA) was applied to reverse-transcribe RNA samples into cDNA following the manufacturer's instructions.

The gene expression levels of p65 NF-κB, AP-1, and Nrf2 in THP-1 cells were monitored by the CFX96 Real-Time PCR detection system (Bio-Rad, CA, USA) with SYBR Green I gene expression assays. The sequences of the primers (forward and reverse) used for real-time PCR were as follows: p65 NF-κB: 5′-ATGTGGAGATCATT-GAGCAGC-3′ and 5′CCTGGTCCTGTGTAGC-CATT-3′; AP-1: 5′-TCCAAGTGCCGAAAAAG-GAAG-3′ and 5′-CGAGTTCTGAGCTTTCAAG-GT-3′; Nrf2: 5′-TCAGCGACGGAAAGAGTAT-GA-3′ and 5′-CCACTGGTTTCTGA CTGGAT-GT-3′. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method, and GAPDH was selected as the reference gene. The results were presented as fold-expression changes relative to the control group.

Statistical analysis

Two independent experiments were performed at least in triplicate. The quantitative data were expressed as mean ± standard deviation (SD). One-way ANOVA test was conducted to determine the significant differences between different groups by using the SPSS statistical program software version 22.0. $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered to indicate significant differences. PLSR was carried out to explore the potential relation between bioactive components (*i.e.*, VA, 4-MG, and 4-EG) and the antioxidant activity of *baijiu* using the Unscrambler software version 9.7.

Results and discussion

Antioxidant capacity of Baijiu

The antioxidant activities of the *baijiu* samples were determined by two different assays, namely $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Table 1). Generally, the radical scavenging activities of the *baijiu* samples ranged between 0.15 and 26.12% for DPPH*, and between 0.08 and 37.99% for $ABTS^{\bullet+}$. A previous study reported that *baijiu* exhibited an antioxidant effect by DPPH• assay ranging from 4.10 to 9.70% (Shi *et al.,* 2015).

The majority of previous studies focused on the antioxidant activities of fruit wine, beer, and *huangjiu* (literally means yellow wine) indicating potent antioxidant activities owing to their higher contents of phenolic compounds, whereas positive correlations were noted between antioxidant capacity and total phenolic contents (Ljevar *et al.,* 2016). Specifically, the $DPPH^{\bullet}$ radical scavenging activity of beer was estimated at 90.00%, followed by 73.80% (red wine) and 45.20% (*huangjiu*) (Fernández-Pachón *et al.,* 2004; Que *et al.,* 2006). Conversely, the radical scavenging activity of *baijiu* was lower than that of fruit wine, beer, and *huangjiu*. These findings may be attributed to the discrepancy in the contents of phenolic compounds caused by the different brewing methods. Fruit wine, beer, and *huangjiu* all belong to fermented alcoholic beverages without distillation, while *baijiu* is a type of distilled spirit, which renders the extraction of polyphenols particularly difficult. Hence, the contents of polyphenols with high boiling point in *baijiu* are far below those of fruit wine, beer, and *huangjiu*, which may result in lower radical scavenging activity of *baijiu*. Despite this, phenolic compounds in *baijiu* have been confirmed as potent antioxidants in a recent study. Notable compounds such as vanillin (VA), 4-methylguaiacol (4-MG), and 4-ethylguaiacol (4-EG) (Zhao *et al.,* 2017; 2018) were amongst the most common antioxidants identified. In addition, it is also worth noting that although polyphenols exhibit a potent antioxidant effect *in vitro*, their bioactivities are generally low. The molecular weights of phenols in *baijiu* are less than those of polyphenols. Therefore, phenolic compounds in *baijiu* may play a more direct role in biological activity than polyphenols; nevertheless, this requires further investigation. However, whether VA, 4-MG, and 4-EG contribute to the antioxidant properties of *baijiu* remains unknown.

Determination of vanillin, 4-methylguaiacol, and 4-ethylguaiacol in baijiu samples

Table 1. Radical scavenging activity of *baijiu* determined by DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays.

No.	Sample code	Aroma type	Radical scavenging activity (%)	
			DPPH$^{\bullet}$	ABTS$^{\bullet+}$
1	SA-1		5.03 ± 0.24	7.20 ± 0.35
2	SA-2		4.78 ± 0.23	12.52 ± 0.62
3	SA-3		7.49 ± 0.36	12.18 ± 0.60
4	SA-4		6.21 ± 0.30	12.94 ± 0.64
5	SA-5		4.64 ± 0.22	6.99 ± 0.34
6	SA-6		4.46 ± 0.21	5.24 ± 0.25
7	SA-7		7.67 ± 0.37	7.76 ± 0.38
8	SA-8		10.73 ± 0.53	30.92 ± 1.54
9	SA-9		1.19 ± 0.05	2.15 ± 0.10
10	SA-10		5.64 ± 0.27	10.44 ± 0.51
11	SA-11		3.61 ± 0.17	3.78 ± 0.18
12	SA-12		4.32 ± 0.21	3.64 ± 0.17
13	SA-13		5.67 ± 0.27	8.84 ± 0.43
14	SA-14		5.00 ± 0.24	9.74 ± 0.48
15	SA-15		1.79 ± 0.08	6.45 ± 0.31
16	SA-16		2.86 ± 0.13	9.04 ± 0.44
17	SA-17	Strong	4.50 ± 0.21	2.79 ± 0.13
18	SA-18		5.64 ± 0.27	0.59 ± 0.02
19	SA-19		2.97 ± 0.14	0.29 ± 0.01
20	SA-20		5.03 ± 0.24	5.05 ± 0.24
21	SA-21		3.86 ± 0.18	3.76 ± 0.18
22	SA-22		3.79 ± 0.16	2.54 ± 0.10
23	SA-23		4.93 ± 0.22	6.61 ± 0.30
24	SA-24		2.79 ± 0.11	2.52 ± 0.10
25	SA-25		3.61 ± 0.15	4.16 ± 0.18
26	SA-26		1.97 ± 0.07	2.29 ± 0.08
27	SA-27		4.07 ± 0.17	0.12 ± 0.01
28	SA-28		10.77 ± 0.51	3.38 ± 0.14
29	SA-29		2.40 ± 0.09	3.45 ± 0.14
30	SA-30		3.54 ± 0.15	2.41 ± 0.09
31	SA-31		9.66 ± 0.45	14.25 ± 0.68
32	SA-32		5.99 ± 0.27	6.02 ± 0.27
33	SA-33		4.57 ± 0.20	1.74 ± 0.06

34	SA-34		2.90 ± 0.11	4.02 ± 0.17
35	SA-35		3.93 ± 0.17	2.51 ± 0.10
36	SA-36		2.43 ± 0.09	4.33 ± 0.19
37	SA-37		3.68 ± 0.15	11.11 ± 0.53
38	SA-38		6.92 ± 0.32	6.88 ± 0.31
39	SA-39		8.02 ± 0.37	13.93 ± 0.67
40	SA-40		2.97 ± 0.12	0.59 ± 0.03
41	SA-41		5.85 ± 0.26	2.63 ± 0.10
42	SA-42		3.86 ± 0.16	0.94 ± 0.02
43	SA-43		4.25 ± 0.18	0.90 ± 0.01
44	LA-1		6.10 ± 0.28	4.65 ± 0.20
45	LA-2		6.07 ± 0.27	8.03 ± 0.37
46	LA-3		2.72 ± 0.11	0.08 ± 0.01
47	LA-4		6.24 ± 0.28	0.65 ± 0.03
48	LA-5		3.29 ± 0.13	0.40 ± 0.02
49	LA-6		0.83 ± 0.01	0.52 ± 0.03
50	LA-7		3.15 ± 0.13	4.35 ± 0.19
51	LA-8		2.43 ± 0.09	1.70 ± 0.05
52	LA-9		4.14 ± 0.18	3.06 ± 0.12
53	LA-10		2.43 ± 0.09	1.46 ± 0.04
54	LA-11		1.90 ± 0.06	8.71 ± 0.41
55	LA-12		4.57 ± 0.20	2.32 ± 0.09
56	LA-13	Light	4.61 ± 0.20	12.66 ± 0.60
57	LA-14		2.75 ± 0.11	6.18 ± 0.28
58	LA-15		0.37 ± 0.01	1.16 ± 0.03
59	LA-16		4.11 ± 0.18	2.54 ± 0.10
60	LA-17		10.34 ± 0.49	3.55 ± 0.15
61	LA-18		4.25 ± 0.18	4.77 ± 0.21
62	LA-19		5.57 ± 0.25	0.04 ± 0.01
63	LA-20		1.22 ± 0.03	1.58 ± 0.05
64	LA-21		4.57 ± 0.20	1.72 ± 0.06
65	LA-22		3.32 ± 0.14	1.45 ± 0.04
66	LA-23		11.30 ± 0.54	37.99 ± 1.87
67	LA-24		12.83 ± 0.61	8.75 ± 0.41
68	LA-25		7.95 ± 0.37	2.19 ± 0.08

69	SEA-1	Sesame	5.50 ± 0.24	3.74 ± 0.16
70	SEA-2		5.18 ± 0.23	2.54 ± 0.10
71	SEA-3		4.39 ± 0.19	11.76 ± 0.56
72	SEA-4		0.15 ± 0.01	3.54 ± 0.15
73	SEA-5		4.11 ± 0.18	0.91 ± 0.02
74	SEA-6		5.57 ± 0.25	6.12 ± 0.28
75	SEA-7		3.32 ± 0.14	3.01 ± 0.12
76	SEA-8		1.04 ± 0.02	3.60 ± 0.15
77	SEA-9		2.11 ± 0.08	7.88 ± 0.36
78	SEA-10		3.50 ± 0.15	3.69 ± 0.15
79	SEA-11		10.16 ± 0.48	11.40 ± 0.54
80	SEA-12		2.79 ± 0.11	0.99 ± 0.02
81	SEA-13		6.21 ± 0.28	8.78 ± 0.41
82	SEA-14		8.27 ± 0.38	7.33 ± 0.34
83	SEA-15		6.71 ± 0.32	2.15 ± 0.09
84	SEA-16		4.32 ± 0.20	8.36 ± 0.40
85	SEA-17		10.41 ± 0.50	21.54 ± 1.06
86	SEA-18		12.26 ± 0.59	20.94 ± 1.03
87	SEA-19		20.63 ± 1.01	20.44 ± 1.00
88	SEA-20		13.83 ± 0.67	6.56 ± 0.31
89	SEA-21		26.12 ± 1.29	9.77 ± 0.47
90	SEA-22		14.33 ± 0.70	20.87 ± 1.02
91	SEA-23		6.78 ± 0.32	0.23 ± 0.01
92	SEA-24		4.75 ± 0.22	7.62 ± 0.36
93	SAA-1	Sauce	1.08 ± 0.03	7.14 ± 0.34
94	SAA-2		3.79 ± 0.17	8.49 ± 0.40
95	SAA-3		2.22 ± 0.09	3.58 ± 0.16
96	SAA-4		8.24 ± 0.39	7.77 ± 0.37
97	MA-1	Mixed	7.63 ± 0.36	15.74 ± 0.77
98	MA-2		13.08 ± 0.63	8.89 ± 0.42
99	CA-1	Chi	6.89 ± 0.32	3.12 ± 0.14
100	CA-2		11.84 ± 0.57	7.49 ± 0.35
101	RA-1	Rice	7.70 ± 0.37	2.66 ± 0.11
102	TA-1	Te	3.64 ± 0.16	0.73 ± 0.02
103	FA-1	Fuyu	9.73 ± 0.47	14.27 ± 0.69

The concentrations of VA, 4-MG, and 4-EG in 103 *baijiu* samples were quantified by LLE combined with GC-MS. 4-MG and 4-EG were detected in all 103 *baijiu* samples, and their concentrations ranged from 4.45 ± 0.02 to 1,575.41 ± 9.70 µg/L, and from 3.76 ± 0.13 to 2180.2 ± 20.42 µg/L, respectively. However, VA was only detected in 94 *baijiu* samples, and its concentration ranged from 0.54 ± 0.02 to 279.18 ± 5.19 µg/L.

On this basis, PLSR was conducted to investigate the association between phenolic compound concentration (*i.e.,* VA, 4-MG, and 4-EG) and the antioxidant activity of *baijiu*. The concentrations of phenolic compounds (*i.e.,* VA, 4-MG, and 4-EG) and the radical scavenging activities ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) of the corresponding *baijiu* samples were submitted to PLSR analysis.

VA, 4-MG, and 4-EG were marked with blue dots, while $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were marked with red dots (Figure 1). $DPPH^{\bullet}$, $ABTS^{\bullet+}$, 4-MG, and 4-EG appeared in the first quadrant, except for VA. It was suggested that the concentrations of 4-MG and 4-EG in *baijiu* positively correlated with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity, and notably with $ABTS^{\bullet+}$ scavenging activity. In addition, the concentration of VA also positively correlated with the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities of *baijiu*, but the correlation coefficient was low. It is interesting to note that the concentration of 4-MG highly correlated with that of 4-EG. Generally, the antioxidant activity of *baijiu* positively correlated with the concentrations of VA, 4-MG, and 4-EG in *baijiu*.

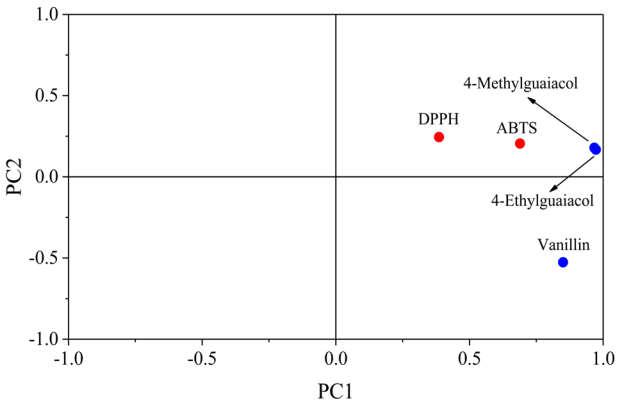

Figure 1. PLSR loading plot of the concentrations of phenolic compounds (X-variables) and the radical scavenging activities of corresponding *baijiu* samples (Y-variables) on the first and second PLS components (PC1 and PC2). PC1 explained 87% X-variables and 38% Y-variables, PC2 explained 11% X-variables and 5% Y-variables.

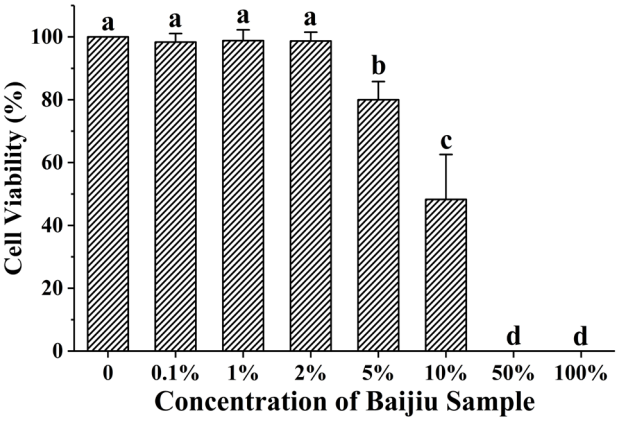

Figure 2. Cytotoxicity of *baijiu* sample (No. SA-3). Significant difference between different doses of *baijiu* sample (No. SA-3) is indicated by different letters ($p < 0.05$).

CCK-8 assay

In order to determine the suitable treatment dose for the following experiment, the effects of *baijiu* sample (No. SA-3) on THP-1 cell viability were examined (Figure 2). No cytotoxic effect was noted when *baijiu* dilutions were below 2% since no significant difference was found between the control and treatment groups ($p > 0.05$). Hence, 2% *baijiu* dilution (2%) was selected as the treatment dose to be used for subsequent studies.

Inhibition of the production of LPS-induced inflammatory cytokines in THP-1 cells by baijiu

LPS-induced THP-1 cells were selected as a model to characterise the anti-inflammatory profile of *baijiu*. In view of the key role of inflammatory cytokines in the inflammatory response, the effect of *baijiu* on LPS-induced THP-1 cells was studied. As shown in Figure 3, the expression levels of the inflammatory cytokines extremely increased following treatment of LPS in comparison to the control group ($p < 0.001$). The secretion levels of the inflammatory cytokines increased by 2.20-fold (TNF-α), 0.90-fold (IL-1β), 4.70-fold (IL-6), 10.04-fold (IL-8), 3.00-fold (IL-10), 1.94-fold (NO), and 1.55-fold (PGE_2). However, *baijiu* was able to ameliorate the LPS-induced inflammatory response. Specifically, the overproduction of inflammatory cytokine stimulation by LPS significantly decreased by 69.12% (TNF-α, $p < 0.001$), 59.88% (IL-1β, $p < 0.001$), 72.85% (IL-6, $p < 0.001$), 63.39% (IL-8, $p < 0.001$), 30.54% (IL-10, $p < 0.001$), 45.90% (NO, $p < 0.001$), and 7.36% (PGE_2). Recent studies have also confirmed that *baijiu* can alleviate inflammation by inhibiting the TNF-α and NO expression in LPS-induced RAW 264.7 macrophages (Liu *et al.,* 2018).

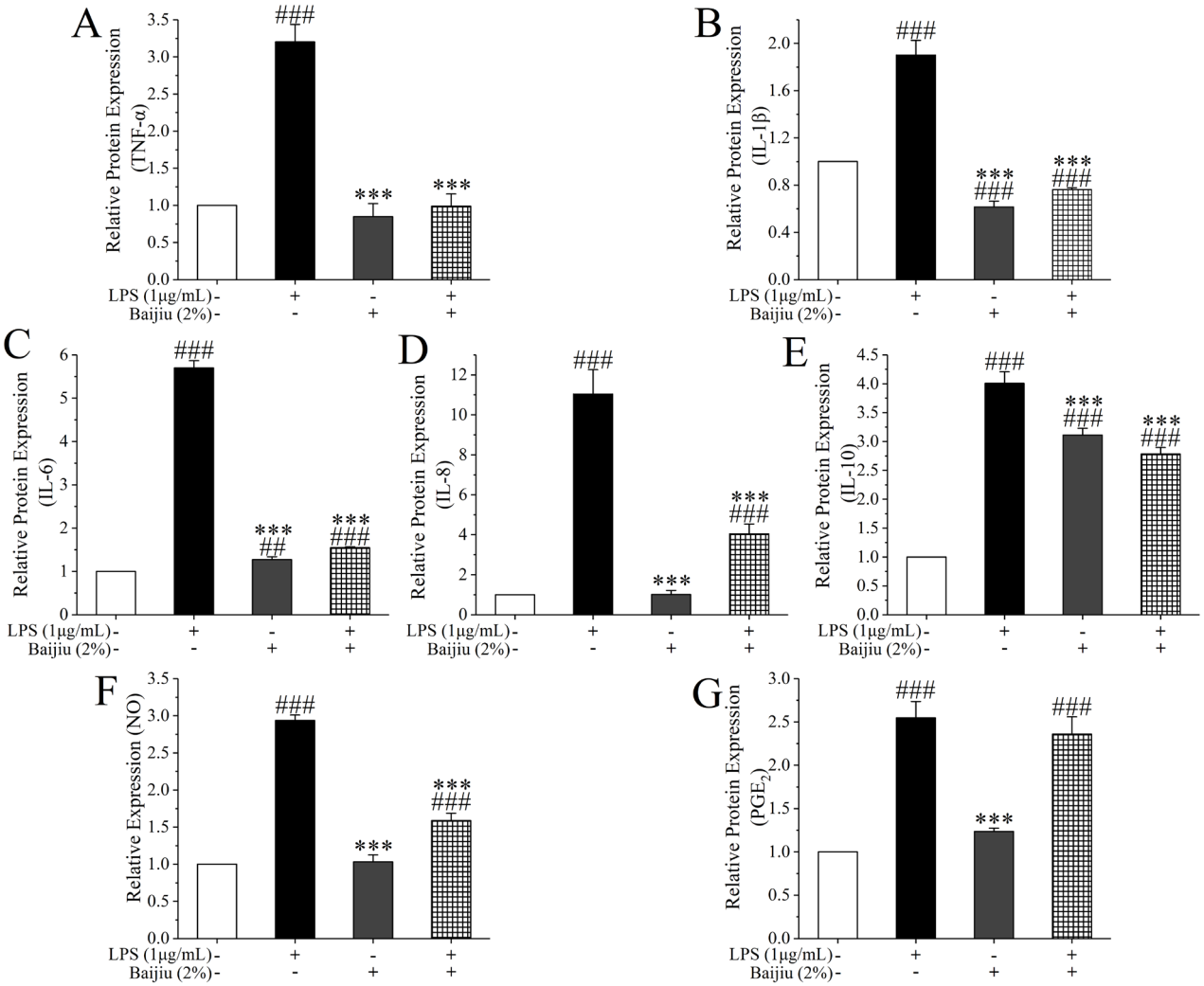

Figure 3. The effect of *baijiu* sample (No. SA-3) on the production of LPS-induced inflammatory cytokines in THP-1 cells. Significant differences among different groups are indicated by ### : $p < 0.001$, ## : $p < 0.01$, and # : $p < 0.05$ vs control group; *** : $p < 0.001$, ** : $p < 0.01$, and * : $p < 0.05$ vs LPS group.

Currently, compelling evidence has been reported that demonstrates the involvement of chronic inflammation with a variety of diseases such as rheumatoid arthritis, gastroenteritis, and Alzheimer's disease (Teng *et al.,* 2017; Liu *et al.,* 2017). Inflammatory cytokines play important roles in the inflammatory response (Walker *et al.,* 2017), and it has been shown that their overproduction promotes the development of inflammation (Walker *et al.,* 2014). Therefore, the inhibition of the secretion of inflammatory cytokines is helpful for inflammation anesis. In the present work, *baijiu* treatment decreased the production of pro-inflammatory cytokines (*i.e.,* TNF-*α*, IL-1*β*, and IL-6), which were able to lead to serious inflammatory response (Yang *et al.,* 2016; Tang *et al.,* 2017). Particularly, the expression levels of TNF-*α* returned to those similar of the control group, whereas the expression levels of IL-1*β* following the treatment with *baijiu* were significantly lower than those of the control group ($p < 0.001$). In addition, the expression levels of chemokines (*i.e.,* IL-8) were also downregulated following treatment of the cells with *baijiu*. IL-8 released by macrophages could induce chemotaxis in primary neutrophils, causing them to migrate toward the site of infection and eventually lead to the activation of the inflammatory response (Zhang *et al.,* 2017). Moreover, a previous study reported that NO and PGE_2 were critical inflammatory mediators that could aggravate the inflammatory response (Bi *et al.,* 2018). In the present work, LPS stimulation significantly upregulated the production of NO and PGE_2. However, *baijiu* treatment notably inhibited LPS-induced overexpression of NO and decreased the expression levels of PGE_2. Based on these findings, it could be assumed that *baijiu* exhibited an anti-inflammatory effect by inhibiting the production of inflammatory cytokines.

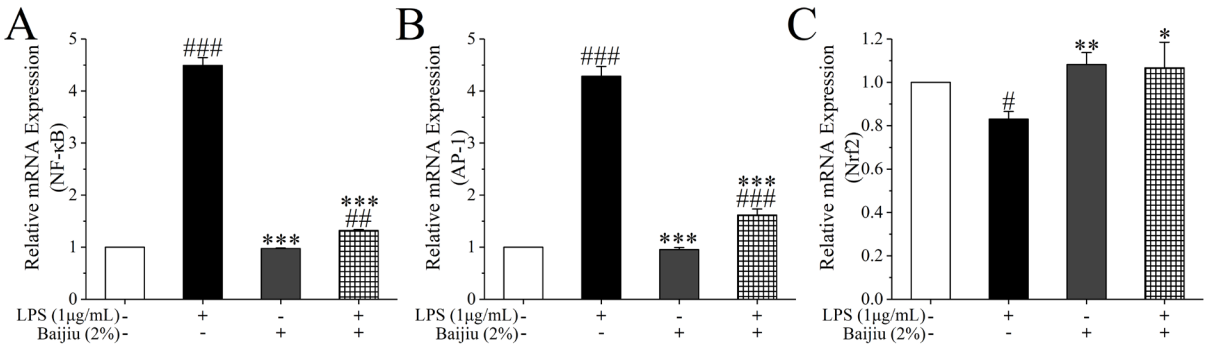

Figure 4. The effect of *baijiu* sample (No. SA-3) on the expression of NF-*κ*B, AP-1, and Nrf2 in LPS-induced THP-1 cells. Significant differences among different groups are indicated by ### : $p < 0.001$, ## : $p < 0.01$, and # : $p < 0.05$ vs control group; *** : $p < 0.001$, ** : $p < 0.01$, and * : $p < 0.05$ vs LPS group.

Effect of baijiu on the expression levels of NF-κB, AP-1, and Nrf2 in LPS-induced THP-1 cells

It has been shown that NF-*κ*B along with AP-1 play a fundamental role in the development of the inflammatory response and related pathologies (Walker *et al.,* 2014; 2016; 2017). By contrast, the over-secretion of the inflammatory cytokines can activate the NF-*κ*B and AP-1 signalling pathways, leading to the aggravation of inflammation. Therefore, suppression of NF-*κ*B and AP-1 expression is crucial for alleviating the inflammatory response.

As Figure 4 shows, LPS treatment alone stimulated a significant increase in the gene expression of NF-*κ*B and AP-1 when compared with the corresponding levels in the control group ($p < 0.001$). The expression levels of NF-*κ*B and AP-1 increased by 3.49- and 3.28-fold, respectively. Of note, *baijiu* treatment markedly restored LPS-induced changes in the gene expression of NF-*κ*B and AP-1 ($p < 0.001$). The expression levels of NF-*κ*B and AP-1 decreased by 70.60 and 62.18%, respectively. A previous study has also confirmed the inhibitory effect of *baijiu* on the expression of NF-*κ*B in LPS-induced RAW 264.7 macrophages (Liu *et al.,* 2018). Combined with these findings, it could be inferred that *baijiu* may contribute to ameliorating inflammation by inhibition of NF-*κ*B and AP-1 activation.

Recently, increasing studies have indicated that Nrf2 is not only responsible for relieving oxidative stress but is also involved in the inhibition of inflammatory response (Albishi *et al.,* 2013). Previous research has shown that Nrf2 exhibits an anti-inflammatory effect by relieving oxidative stress and interacting with the NF-*κ*B signalling pathway (Teng *et al.,* 2017). Therefore, increased expression levels of Nrf2 contribute to ameliorating inflammation. In the present work, LPS treatment alone significantly inhibited the expression of Nrf2 ($p < 0.05$). The expression levels of Nrf2 were 17.95% lower than those of the control group. This finding is in agreement with the results reported in previous studies (Zhao *et al.,* 2019a; 2019b; 2019c). However, *baijiu* treatment notably upregulated Nrf2 expression levels by 28.45% in comparison to the LPS group ($p < 0.05$).

Previous studies have indicated that the bioactive components (*i.e.,* VA, 4-MG, and 4-EG) in *baijiu* possess potent antioxidant and anti-inflammatory effects (Zhao *et al.,* 2017; 2018; 2019a; 2019b; 2019c). VA, (4 mg) and 4-EG can inhibit the inflammatory response by inducing the Nrf2 signalling pathway and its downstream antioxidant enzymes, thus inhibiting the activation of NF-*κ*B and AP-1, and suppressing the secretion of inflammatory cytokines. Therefore, the anti-inflammatory properties of *baijiu* may be attributed to VA, 4-MG, and 4-EG. Nevertheless, the anti-inflammatory effects of these compounds require further investigations in future studies.

Conclusion

In summary, the present work investigated the antioxidant and anti-inflammatory effects of *baijiu* and the potential association between the concentration of bioactive components (*i.e.,* vanillin, 4-methylguaiacol, and 4-ethylguaiacol) and *baijiu* bioactivity. Based on the results, the radical scavenging activity (*i.e.,* $DPPH^{\bullet}$ and $ABTS^{\bullet+}$) of *baijiu* positively correlated with the concentrations of vanillin, 4-methylguaiacol, and 4-ethylguaiacol in *baijiu*. Furthermore, LPS treatment could stimulate a significant increase in the gene expression of NF-κB and AP-1, while *baijiu* was confirmed to possess an anti-inflammatory effect in LPS-induced THP-1 cells by inhibition of gene expression of NF-*κ*B and

AP-1, inhibition of induction of Nrf2 expression, and repression of inflammatory cytokine secretion. These findings preliminary uncovered the beneficial effect of *baijiu*, and the association between bioactive components (*i.e.* vanillin, 4-methylguaiacol, and 4-ethylguaiacol) and *baijiu* bioactivity.

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