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Anti-inflammatory effects of hydrophobic constituents in the extract of the root cortex of *Paeonia suffruticosa*

Tatsuki Sato[1], Saki Shirako[1], Tetsuya Okuyama[2], Yukinobu Ikeya[3,4], and Mikio Nishizawa[1*]

[1]Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan; [2]Department of Surgery, Kansai Medical University, 2-5-1 Shinmachi, Hirakata, Osaka 573-1010, Japan; [3]Faculty of Pharmacy, Daiichi University of Pharmacy, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan; and [4]Ritsumeikan Asia-Japan Research Organization, 2-150 Iwakura-cho, Ibaraki, Osaka 567-8570, Japan

[*]**Corresponding author:** Professor Mikio Nishizawa, M.D., Ph.D., Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University; Kusatsu, Shiga 525-8577, Japan

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ABSTRACT

Background: The root cortex of *Paeonia suffruticosa* Andrews (Paeoniaceae), which is also called moutan bark, is known as *Botanpi* in Japan. This crude drug has been used in several Kampo formulas, such as *Daiobotanpito,* to treat menstrual disturbance and constipation by improving blood stasis and suppressing inflammation. However, the anti-inflammatory effect has not been well studied.

Objective: To clarify the anti-inflammatory effects of moutan bark, we isolated its constituents and investigated their activity.

Methods: Moutan bark was extracted and successively fractionated with ethyl acetate (EtOAc) and *n*-butanol. The constituents were analyzed using HPLC. The production of nitric oxide (NO), an inflammatory mediator, was measured in interleukin (IL)-1β-treated rat hepatocytes to identify active fractions or constituents.

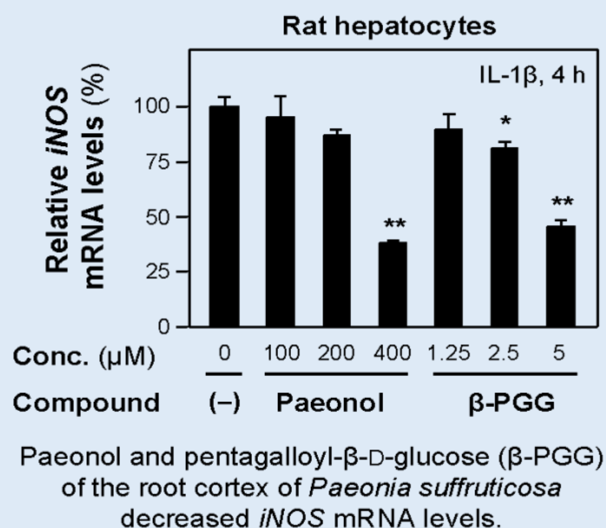
Results: The EtOAc-soluble fraction of moutan bark extract significantly inhibited NO production. Three hydrophobic constituents were isolated from this fraction and identified as paeonol (**1**), 1,2,3,4,6-pentagalloyl-β-D-glucose (β-PGG; **2**), and methyl gallate (**3**). Paeonol and paeoniflorin (**4**) were abundantly present in the EtOAc-soluble fraction and *n*-butanol-soluble fraction, respectively. The hydrophobic constituents suppressed NO production without exerting cytotoxicity and

reduced the protein and mRNA levels of inducible nitric oxide synthase in IL-1β-treated hepatocytes; β-PGG showed the highest potency. Furthermore, β-PGG, methyl gallate, and paeonol decreased the mRNA levels of tumor necrosis factor α and IL-1 receptor type 1, which are involved in inflammation.

Conclusion: These results suggest that hydrophobic constituents of moutan bark, such as β-PGG, methyl gallate, and paeonol, are involved in the anti-inflammatory effects of moutan bark.

Keywords: Galloylglucose, tannin, nitric oxide, Kampo medicine, cytokine.

Paeonia suffruticosa Andrews



Paeonol and pentagalloyl-β-D-glucose (β-PGG) of the root cortex of *Paeonia suffruticosa* decreased *iNOS* mRNA levels.

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INTRODUCTION

Peony is a plant that is known for its beautiful flower and is part of the family Paeoniaceae, which includes *Paeonia suffruticosa* Andrews (formerly *Paeonia moutan* Sims). The root cortex of this species, also known as the moutan bark, is known as *Botanpi* in Japan [1]. This crude drug has been used in Japan and China [2] and included in several Japanese Kampo formulas. For example, *Daiobotanpito* improves blood stasis and suppresses inflammation in patients with menstrual disturbance and constipation. Moutan bark extract protects against oxidative injuries and inhibits the production of inflammatory mediators, such as nitric oxide (NO), interleukin (IL)-1β, and tumor necrosis factor α (TNF-α), in lipopolysaccharide (LPS)-induced RAW264.7 cells, which are a macrophage line [3]. NO plays a crucial role under physiological and pathophysiological conditions [4]. However, there have been few other reports on the anti-inflammatory effects of moutan bark.

The characteristic constituents of moutan bark are paeonol, paeoniflorin, and galloylglucose [2]. Paeonol, which is a major constituent, exerted anti-inflammatory effects and inhibited IL-1β-induced inflammation in human chondrocytes and osteoarthritis model mice [5]. Paeoniflorin, a monoterpene glucoside, exerted anti-inflammatory and immunoregulatory effects on animal models of autoimmune diseases [6]. Galloylglucose is a simple hydrolysable tannin, and the major galloylglucose

in moutan bark is 1,2,3,4,6-pentagalloyl-β-D-glucose (β-PGG) [7], which is the β-anomer of an ester of glucose with five gallic acid molecules. Moutan bark exerts a variety of pharmacological effects, such as antioxidative, anti-inflammatory, hepatoprotective, and antitumor effects [2]. It is unclear which constituent primarily contributes to these effects of moutan bark.

Foreign pathogenic bacteria, such as Gram-negative bacteria, induce inflammatory responses, including NO production, through the release of LPS from the outer membrane of dead bacteria. LPS activates resident liver macrophages, such as Kupffer cells, to induce the expression of inducible nitric oxide synthase (iNOS), which synthesizes NO. Kupffer cells secrete IL-1β, which binds to the IL-1 receptor type 1 (IL1R1) on hepatocytes to induce the expression of iNOS and proinflammatory cytokines through the IL-1 signaling pathway [8]. Primary cultured rat hepatocytes were used to assess anti-inflammatory activity because inhibiting IL-1β-induced NO production is thought to be an anti-inflammatory marker. To date, the anti-inflammatory activity of many constituents of crude drugs, such as the rhizome of *Cnidium officinale* [9] and the bark of *Prunus jamasakura* [10], has been evaluated and compared using this *ex vivo* system.

Here, we purified pharmacologically active constituents from moutan bark and measured NO production in IL-1β-stimulated hepatocytes. Then, the contents of the constituents were examined by HPLC. Finally, the anti-inflammatory effects of the constituents on the expression of *iNOS* and proinflammatory genes were examined.

MATERIALS AND METHODS

Plant materials: The root cortex of *Paeonia suffruticosa* Andrews was collected from Anhui Province, China, and purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co., Ltd.) authenticated it as *Botanpi*, according to the Japanese Pharmacopoeia [1]. The voucher specimen was deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University under RIN-PS-32.

Isolation of constituents from the bark of Paeonia suffruticosa: The dried root cortex of *Paeonia suffruticosa* (370.0 g) was pulverized and extracted with methanol (MeOH) under reflux [11]. As shown in Figure 1, the extract (95.91 g) was suspended in water and sequentially partitioned with ethyl acetate (EtOAc) and *n*-butanol. These layers were concentrated to prepare EtOAc-soluble, *n*-butanol-soluble, and water-soluble fractions. The EtOAc-soluble fraction (Fraction A; 19.98 g), which suppressed NO production, was further purified by silica gel column chromatography [6.5 cm internal diameter (i.d.) × 32 cm; Silica Gel 60, Nacalai Tesque, Inc., Kyoto, Japan] and was eluted with *n*-hexane:EtOAc (100:0 → 0:100), acetone, and MeOH to yield 12 subfractions (A1 to A12). Subfractions A2 and A10 were further purified. Subfraction A2 (300 mg) was further fractionated using silica gel column chromatography (1.2 cm i.d. × 9 cm; *n*-hexane:EtOAc = 80:20), resulting in one peak of Compound **1** (157 mg) as a white powder. Subfraction A10 was purified twice by ODS column chromatography (Cosmosil 75C18-OPN; 1.9 cm i.d. × 30 cm; Nacalai Tesque, Inc.) and eluted with water:MeOH (100:0 → 0:100) to yield Compound **2** (15.6 mg) as a brownish powder and Compound **3** (7.20 mg) as a brown powder.

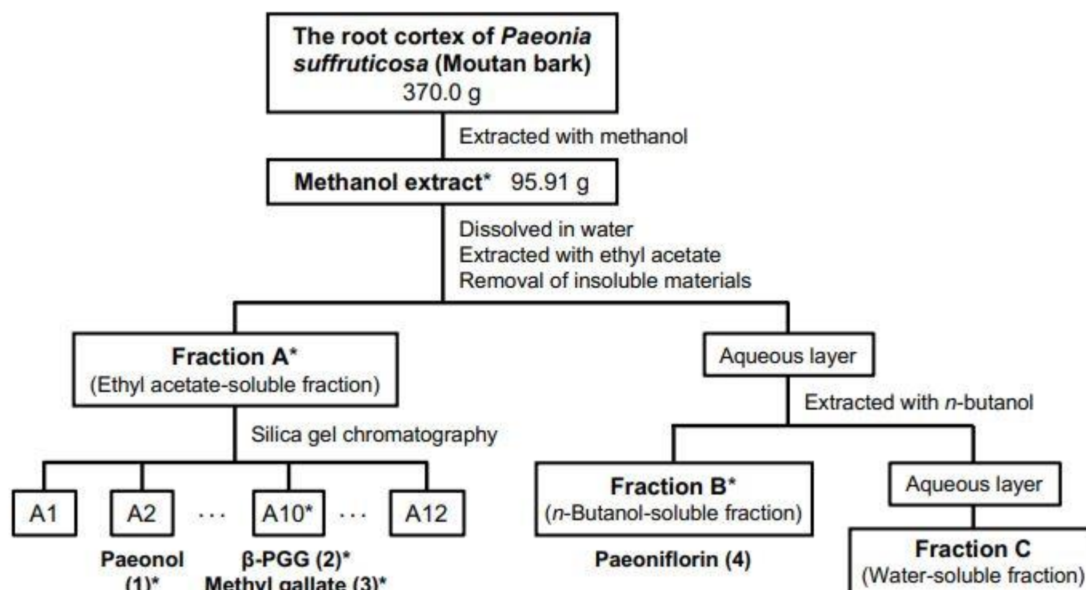

Figure 1. Purification of the constituents of the root cortex of *Paeonia suffruticosa*. A flowchart of the procedures used to fractionate the constituents of the root cortex of *Paeonia suffruticosa*. The yield of the extract (dried) was 25.9% of the plant material. The extract was separated into fractions A (hydrophobic fraction), B (amphipathic fraction), and C (hydrophilic fraction). Constituents included in the relevant subfraction are shown. * NO production was suppressed in hepatocytes in this study.

HPLC analysis of constituents: To measure the content of each constituent, an HPLC system equipped with an LC-20AD pump and an SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan) was used with a Cosmosil 5C18 AR-II column (4.6 mm i.d.×150 mm; Nacalai Tesque, Inc.). Paeonol, β-PGG, and methyl gallate were eluted at a flow rate of 1.0 mL/min by 0.1% (v/v) formic acid–MeOH (0 → 25 min; 80:20 → 0:100) and detected at a wavelength of 254 nm. Paeonol, β-PGG, and methyl gallate were accurately weighed and dissolved in MeOH to make stock solutions of 30 mM, 3 mM and 6 mM, respectively. Then, a series of paeonol (1.0, 2.0, 4.0 mM), β-PGG (0.0625, 0.125, 0.25 mM), and methyl gallate (0.05, 0.1, 0.2 mM) standard solutions were prepared by diluting the stock solutions to make calibration curves. A calibration curve for each standard compound was prepared by plotting peak areas (y) against a series of injection amounts (x µg). The calibration equation and correlation coefficient of the three standard compounds were as follows: paeonol, $y = 3878582x - 254175$ ($R^2 = 0.9994$); β-PGG, $y = 16418638x - 40279$ ($R^2 = 0.9999$); and methyl gallate, $y = 6733859x - 19490$ ($R^2 = 0.9999$). Fraction A was accurately weighed and dissolved in MeOH to make a 1.0 mg/mL sample solution. The peak areas of paeonol, β-PGG, and methyl gallate in the sample solution were fit to the calibration curves, and the amounts of each compound in 20 µL of the sample solution were calculated. The amounts of paeonol, β-PGG, and methyl gallate in 20 µL of the sample solution (20 µg of Fraction A) were calculated to be 6.5 µg, 2.3 µg, and 0.29 µg, respectively; therefore, the contents of these compounds in fraction A were 32.5%, 11.5%, and 1.47%, respectively.

To measure the paeoniflorin content, HPLC was performed. Paeoniflorin was eluted at a flow rate of 1.0 mL/min by 0.1% (v/v) formic acid–MeOH (72:28) and detected at a wavelength of 232 nm. Paeoniflorin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved

in MeOH, and a series of paeoniflorin standard solutions were prepared by diluting the stock solution to make calibration curves. The calibration equation and correlation coefficient of the paeoniflorin standard were $y = 360047900\,x - 1752.59$ ($R^2 = 0.9998$). Fraction B was dissolved in MeOH to prepare a 1.0 mg/mL sample solution and diluted to 0.5 mg/mL with 10% (v/v) acetonitrile containing 0.1% (v/v) formic acid. The peak areas of paeoniflorin in the sample solution were fit to the calibration curves. Because the amount of paeoniflorin in 20 µL of the sample solution (10 µg of Fraction B) was calculated to be 1.49 µg, the paeoniflorin content in fraction B was 14.9%.

Nuclear magnetic resonance (NMR), polarimetry, and mass spectrometry: A JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan) operated at 400 MHz (^{1}H) and 100 MHz (^{13}C) was used to record NMR spectra. Deuterated chloroform ($CDCl_3$), methanol (CD_3OD) and tetramethylsilane (an internal standard) were purchased from Eurisotop (Saint-Aubin, France). A DIP-1000 polarimeter (JASCO Corporation, Hachioji, Tokyo, Japan) was used to measure the optical rotations. Spectra using fast atom bombardment mass spectrometry (FAB-MS) were obtained with a JMS-700 MStation mass spectrometer (JEOL Ltd.).

Animal experiments: Specific pathogen-free male Wistar rats (5–6 weeks old; Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed at 21–23 °C with a 12 h light-dark cycle. The rats were fed a γ-ray-irradiated CRF-1 diet (Charles River Laboratories Japan, Inc.) and water *ad libitum*. All animal care and experimental procedures were performed in accordance with the laws and guidelines of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus (No. BKC2020-045).

Primary cultured rat hepatocytes: After at least one week of acclimatization, hepatocytes were isolated from the livers of Wistar rats [12]. Briefly, the liver was perfused with collagenase (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and the dispersed cells were centrifuged, resuspended, and seeded at 1.2×10^6 cells per 35 mm diameter dish. The cells were incubated at 37 °C for 2 h, and then the medium was replaced (Day 0). The hepatocytes were further incubated at 37 °C overnight for subsequent assays.

NO production and lactate dehydrogenase (LDH) activity: On Day 1, the hepatocytes were incubated with each fraction or compound with/without 1 nM IL-1β for 8 h. The Griess method [13–14] was used to measure nitrite, which is a stable metabolite of NO, in the medium in triplicate. The NO concentration in the medium alone was set as 0%, and the NO concentration in medium containing IL-1β was set as 100%. The IC_{50} values of nitrite were calculated for at least three different concentrations of a fraction or a compound, unless it showed cytotoxicity [14]. Loxoprofen sodium (Kolon Life Science, Inchon, South Korea) was used as a positive control [14]. Hepatocyte cytotoxicity was assessed by measuring LDH activity in the culture medium using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Otsu, Japan). To evaluate direct NO-quenching activity, each compound was added to medium containing 25 µM $NaNO_2$ and incubated at 37 °C for 1.5 h [15]. The medium was mixed with Griess reagent [13–14] and incubated at 20–23 °C for 5 min, and the absorbance at 540 nm was measured to determine the decrease in nitrite induced by the compound.

Western blot analysis: Hepatocytes were treated with 1 nM IL-1β and a compound for 8 h. Cell lysates (20 µg protein/lane) were resolved by a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred onto a membrane [16]. Blocking with 5% Difco skim milk (BD

Biosciences, San Jose, CA, USA) and immunostaining were carried out using antibodies against iNOS (BD Biosciences) and β-tubulin (Cell Signaling Technology Inc., Danvers, MA, USA; internal control) and horseradish peroxidase-conjugated anti-immunoglobulin Fc antibodies (Cell Signaling Technology Inc.). ECL Western Blotting Detection Reagents (Cytiva, Tokyo, Japan) were used to visualize the proteins, and chemiluminescence was then detected using an Amersham Imager 600 (Cytiva).

Quantitative reverse transcription-polymerase chain reaction (RT–PCR): Sepasol I Super G solution (Nacalai Tesque, Inc.) was used to extract total RNA from hepatocytes, which was then purified [17–18]. The resultant RNA was reverse-transcribed to cDNA, which was then amplified by PCR with primers [17–18]. Real-time PCR was performed with SYBR Green I and a Thermal Cycler Dice Real Time System (Takara Bio Inc.), and the mRNA levels were measured in triplicate. After calculation by the ΔΔCt method, Ct values were normalized to elongation factor 1α (EF) mRNA [9,17–18]. The mRNA levels that were normalized to the total RNA from hepatocytes in the presence of IL-1β alone were set as 100%.

Statistical analysis: The results are representative of at least three independent experiments that yielded similar findings. The values are presented as the mean ± standard deviation (SD). The differences were analyzed using Student's t test followed by the Bonferroni correction. Significance was set at $P < 0.05$ and $P < 0.01$.

RESULTS

Hydrophobic constituents in the EtOAc-soluble fraction of moutan bark extract: The root cortex of *Paeonia suffruticosa* (moutan bark) was extracted with MeOH and was successively fractionated with EtOAc and *n*-butanol based on hydrophobicity (Figure 1). NO production in hepatocytes was monitored, and the results demonstrated that the EtOAc-soluble fraction (Fraction A) and *n*-butanol-soluble fraction (Fraction B) of moutan bark extract decreased IL-1β-induced NO production, whereas the water-soluble fraction (Fraction C) did not affect NO production (data not shown). The LDH assay showed that cytotoxicity was not observed at the concentrations used (data not shown). As summarized in Table 1, Fraction A gave the highest IC_{50} value for NO production. Therefore, the hydrophobic constituents with NO-suppressing activity in this fraction were further purified.

Table 1. Fractionation of the extract from the root cortex of *Paeonia suffruticosa* (moutan bark) and its effects on NO production.

Fraction	Yield [%][a]	IC_{50} [µg/mL][b]
MeOH extract	100	36.2 ± 7.64
EtOAc-soluble fraction (Fraction A)	22.2	8.97 ± 0.06
***n*-Butanol-soluble fraction (Fraction B)**	9.93	141 ± 23.5
Water-soluble fraction (Fraction C)	67.9	NA

[a]The percentage was calculated by determining the weight of each fraction divided by the sum of three fractions. [b]The half-maximal inhibitory concentration of NO production in IL-1β-stimulated hepatocytes (mean ± SD). At least three experiments were performed to determine these values. NA, not applied due to low activity.

The constituents of Fraction A were purified by silica gel chromatography. Three hydrophobic constituents were purified from the subfractions that suppressed NO production, as described in the Materials and Methods. The constituents identified were paeonol (Compound **1**), 1,2,3,4,6-pentagalloyl-β-D-glucose (β-PGG; **2**), and methyl gallate (**3**). The values observed by the analyses are shown below. The chemical structures are shown in Figure 2.

Compound **1**: melting point (mp): 46–48 ℃; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 12.8 (1H, s, 2-OH), 7.63 (1H, d, *J* = 8.5 Hz, H-6), 6.46 (1H, d, *J* = 2.4 Hz, H-3), 6.43 (1H, dd, *J* = 7.3, 2.4 Hz, H-5), 3.84 (3H, s, 4-OMe), 2.56 (3H, s, H-8). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 202.6 (C-7), 166.1 (C-4), 165.3 (C-2), 132.3 (C-6), 113.9 (C-1), 107.7 (C-5), 100.8 (C-3), 55.6 (4-OMe), 26.3 (C-8). The ^{1}H NMR and ^{13}C NMR spectra of **1** were identical to the previously reported ^{1}H NMR and ^{13}C NMR spectra of paeonol [19]. The mp of **1** was similar to previously reported mp values (46–49 ℃) [20].

Compound **2**: [*α*]20 D +31.0 (*c* 0.338, MeOH). FAB-MS *m/z* 963 [M+ Na]$^+$; HR-FAB-MS *m/z* 963.1076 [M+ Na]$^+$ (calculated for $C_{41}H_{32}O_{26}Na$; 963.1080); ^{1}H NMR (400 MHz, CD_3OD) *δ*: 7.11 (2H, s, H-2''''' and H-6'''''), 7.05 (2H, s, H-2'''' and H-6''''), 6.98 (2H, s, H-2' and H-6'), 6.95 (2H, s, H-2''' and H-6'''), 6.90 (2H, s, H-2'' and H-6''), 6.24 (1H, d, *J* = 8.6 Hz, H-1), 5.91 (1H, t, *J* = 9.6 Hz, H-3), 5.62 (1H, t, *J* = 9.6 Hz, H-4), 5.59 (1H, dd, *J* = 9.6, 8.6 Hz, H-2), 4.52 (1H, brd, *J* = 10.8 Hz, H_a-6), 4.42 (1H, m, H-5), 4.39 (1H, d, *J* = 10.8, 4.5 Hz, H_b-6). ^{13}C-NMR (100 MHz, CD_3OD) *δ*: 168.0, 167.3, 167.1, 167.0, 166.2 (C-7'/C-7"/C-7'''/C-7''''/C-7'''''), 146.6, 146.5, 146.5, 146.4, 146.3 (C-3' and C-5'/C-3" and C-5"/C-3''' and C-5'''/C-3'''' and C-5''''/C-3''''' and C-5'''''), 140.8, 140.4, 140.3, 140.2, 140.0 (C-4'/C-4"/C-4'''/C-4''''/C-4'''''), 121.1, 120.4, 120.3, 120.2, 119.8 (C-1'/C-1"/C-1'''/C-1''''/C-1'''''), 110.6, 110.5, 110.4, 110.4, 110.4, 110.4 (C-2' and C-6'/C-2" and C-6"/C-2''' and C-6'''/C-2'''' and C-6''''/C-2''''' and C-6'''''), 93.8 (C-1), 74.4 (C-5), 74.1 (C-3), 72.2 (C-2), 69.8 (C-4), 63.1 (C-6). The ^{1}H and ^{13}C NMR spectra of **2** were identical to the previously published ^{1}H and ^{13}C NMR spectra of 1,2,3,4,6-pentagalloyl-β-D-glucose (PGG) [21].

Compound **3**: ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.04 (2H, s, H-2/H-6), 3.81 (3H, s, H-8). ^{13}C NMR (100 MHz, CD_3OD) δ: 167.8 (C-7), 145.3 (C-3 and C-5), 138.6 (C-4), 120.2 (C-1), 108.8 (C-2 and C-6), 51.1 (C-8). The ^{1}H and ^{13}C NMR spectra of **3** were identical to the previously published ^{1}H and ^{13}C NMR spectra of methyl gallate [22].

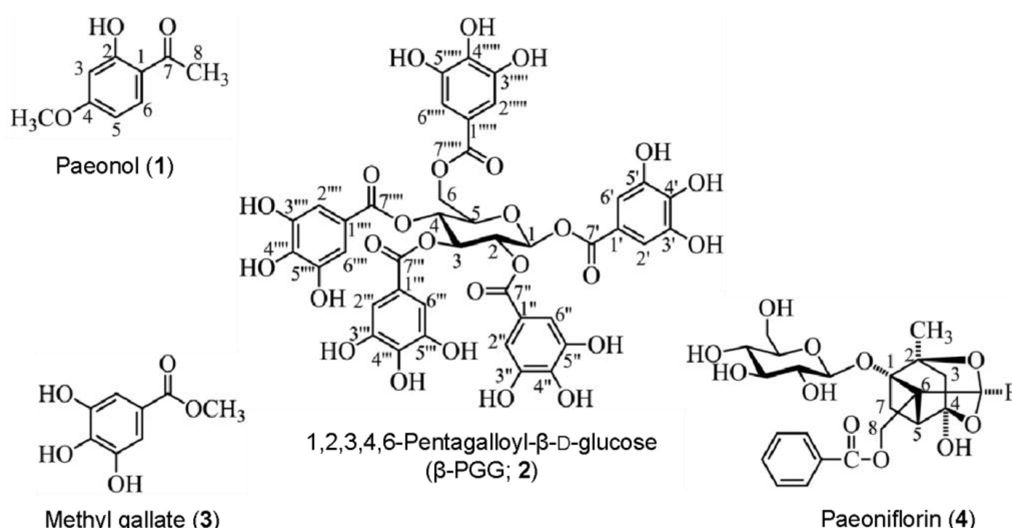

Figure 2. Constituents in moutan bark. The chemical structures of the constituents isolated from Fraction A of moutan bark extract (Compounds **1** to **3**) were determined by the spectral data. The chemical structure of paeoniflorin (Compound **4**) in fraction B is also shown.