SUPPLEMENTARY MATERIAL

Rhusflavanone and mesuaferrone B: tyrosinase and elastase inhibitory biflavonoids extracted from the stamens of *Mesua ferrea* L.

Khine Zar Wynn Myint[a,b], Takumi Kido[c], Ken Kusakari[a,*], Hari Prasad Devkota[a,b], Teppei Kawahara[a] and Takashi Watanabe[a]

[a]*Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan;* [b]*Program for Leading Graduate Schools, HIGO Program, Kumamoto University, Kumamoto, Japan;* [c]*Aging Research Center, Saishunkan Pharmaceutical Co. Ltd, Kumamoto, Japan*

*Corresponding author. E-mail: kusaken@kumamoto-u.ac.jp

Abstract

Chemical isolation and bioactivity studies were conducted on the stamens of *Mesua ferrea* L., which are being used in a traditional skincare formulation in Myanmar. Rhusflavanone and mesuaferrone B were obtained as the main biflavonoids together with lupeol, five common flavonoids, and five phenolic compounds. After being identified by NMR and other spectroscopic analyses, these compounds were evaluated for their 1,1-diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging, human leukocyte elastase inhibitory, and mushroom tyrosinase inhibitory activities. The two biflavonoids exhibited strong inhibitory activities against elastase and tyrosinase, but low DPPH-radical scavenging activities. The contents of rhusflavanone and mesuaferrone B in the stamens were 0.35 ± 0.04% and 0.55 ± 0.06%, respectively. Moreover, lupeol was considered to be a cosmetically important component of the stamens because of its high content and strong elastase inhibitory activity. Rhusflavanone was reported to be isolated from *M. ferrea* for the first time.

Keywords: *Mesua*; rhusflavanone; mesuaferrone; lupeol; elastase; tyrosinase; melanin

Experimental

Chemicals

MCI gel CHP20P (particle diameter: 75−150 μm) was purchased from Mitsubishi Chemical (Tokyo, Japan), Sephadex LH-20 from GE Healthcare (Chicago, IL, USA), Chromatorex ODS (30−50 μm) from Fuji Silysia Chemical (Kasugai, Japan), and silica gel 60 (0.040−0.063 mm) from Merck (Kenilworth, NJ, USA). Protein-R HPLC column, arbutin, and L-tyrosine were purchased from Nacalai Tesuque (Kyoto, Japan). Dacarpo DX-C18 HPLC column was purchased from Imtakt (Kyoto). Human leukocyte elastase and mushroom tyrosinase were from Sigma Aldrich (St. Louis, MO, USA), naringenin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Tokyo Chemical Industry (Tokyo), and Suc(OMe)-Ala-Ala-Pro-Val-MCA from PEPTIDE INSTITUTE (Osaka, Japan). 2-(*N*-morpholino)-ethanesulfonic acid (MES) and 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) were from Dojindo Chemical Research (Kumamoto, Japan). CD_3OD, pyridine-d5, and DMSO-d6 were from Cambridge Isotopic Laboratories (Andover, MA). Apigenin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and other chemicals were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka).

Instruments

Spectroscopic measurements were conducted using an AVANCE-I 600 NMR spectrometer (Bruker, Billerica, MA, USA), a Frontier FT-IR/NIR spectrometer (PerkinElmer, Waltham, MA), a V-550 UV/VIS Spectrometer (JASCO, Tokyo), a Xevo G2-XS QTof mass spectrometer (Waters, Milford, MA), an amaZon speed Ion-Trap mass spectrometer (Bruker), and a DIP-1000 polarimeter (JASCO). Chromatographic purifications were conducted using a Prominence HPLC system (Shimadzu, Kyoto).

Chromatographic analyses were performed with a Nexera X2 HPLC/UHPLC system (Shimadzu). An Infinite M-1000 and an F-200 PRO microplate reader (Tecan, Männedorf, Switzerland) were used in colorimetric and fluorometric assays.

Plant materials

M. ferrea L. stamens were purchased from Zay Cho market, Mandalay, Myanmar in May 2016. They were also collected from the cultivation site in Myayi Nandar Quarter, Chan Mya Tharzi Township, Mandalay in September 2018. The stamens were air-dried in dry atmosphere immediately after collection. A voucher specimen (MBK (MYN) 160530_1000) has been deposited in the Kochi Prefectural Makino Botanical Garden (Kochi, Japan), where the material was identified by Dr. N. Tanaka.

Extraction and isolation

Powdered *M. ferrea* stamens (2.4 kg) were extracted twice with 18 L of 70% methanol at room temperature by maceration for a week, with occasional stirring. The extracts were combined and concentrated *in vacuo* at 40 °C, resulting in 340 g of a dry extract. The extract was suspended in water, then applied to an MCI gel CHP20P column (8 × 35 cm) and fractionated by stepwise elusions with water, 40%, 60%, 80%, and 100% methanol/water and dichloromethane.

Compounds **1** and **2** were obtained from the 80% methanol fraction through further purification with Sephadex LH-20 column (eluent: 100% methanol), followed by preparative HPLC. Preparative HPLC was conducted with a Protein-R column (20 × 250 mm, 5 μm) under the following conditions: elution with methanol-water linear gradient (40:60 at 0 min to 100:0 at 30 min); 8 mL min^{-1} flow rate; detection by a PDA detector (200−360 nm).

Compound **3** was obtained from the 100% methanol fraction and the dichloromethane fraction through further purification using silica gel columns. Compounds **4**−**8** and **9**−**13** were obtained from the water fraction and the combined fraction of 40% and 60% methanol fractions, respectively, through further purification with Sephadex LH-20 (eluent: methanol-water), followed by purification using silica gel (eluent: dichloromethane-methanol-water) or ODS columns (eluent: methanol-water).

HPLC analyses

Powdered stamen samples (200 mg) were extracted with 10.0 mL of 70% methanol at room temperature for 2 days while shaking (100 rpm), after a 30-min sonication. After being centrifuged at 15000 rpm for 10 min, the supernatants were used for HPLC analyses, which were conducted with a Dacarpo DX-C18 column (2 × 100 mm, 2.5 μm) under the following conditions: elution with acetonitrile-H_2O linear gradient (10:90 at 0 min to 100:0 at 20 min) and 100% acetonitrile (from 20 to 30 min); 0.3 mL min^{-1} flow rate; detection with a PDA detector (200−360 nm); 2 μL injection. A sample HPLC chromatogram is shown in Figure S1.

Spectroscopic data

*Compound **1***

Light yellow powder, $[\alpha]_D^{23}$ −242° (c 0.5, MeOH); UV λ_{max} (MeOH) nm (ε): 216 (48671), 228 (46353), 278 *sh* (28726), 298 (34903), 332 *sh* (23654); Rν_{max}^{KBr} cm^{-1}: 3184 (br), 1594, 1151, 1089, 828; NMR δ_H and δ_C (CD_3OD): matched literature values (Lin and Chen 1973; Shrestha et al. 2012); ESI^+-HRMS *m/z* $[M + H]^+$ Calcd. for $C_{30}H_{23}O_{10}$: 543.1291. Found: 543.1298. ESI^--HRMS *m/z* $[M - H]^-$ Calcd. for $C_{30}H_{21}O_{10}$: 541.1135. Found: 541.1139.

*Compound **2***

Light yellow powder, $[\alpha]_D^{23}$ −198° (c 0.5, MeOH); UV λ_{max} (EtOH) nm (ε): 210 (62358), 228 (63914), 276 (42795), 298 (41705), 332 *sh* (31180); IRν_{max}^{KBr}cm^{-1}: 3094 (br), 1635, 1594, 1150, 829; NMR δ_H and δ_C (pyridine-d5): matched literature values (Raju et al. 1976; Shrestha et al. 2012); ESI$^+$-HRMS *m/z* $[M + H]^+$ Calcd. for $C_{30}H_{21}O_{10}$: 541.1135. Found: 541.1141. ESI$^-$-HRMS *m/z* $[M - H]^-$ Calcd. for $C_{30}H_{19}O_{10}$: 539.0978. Found: 539.0983.

Preparation of sample stock solutions

Dry extracts and the isolated compounds were dissolved in 100% DMSO at 10 g/L concentration, stored at −20 °C in the dark, and used as the sample stock solutions. They were dissolved well at room temperature by sonication prior to use.

Determination of DPPH-radical scavenging activity

DPPH-radical scavenging activity was evaluated according to a previously reported method (Sharma and Bhat 2009), with minor modifications. Ethanol (50%) was used as the solvent for DPPH. Sample stock solutions were also diluted with 50% ethanol to obtain the final concentrations of 3, 10, 30, 100, and 300 mg/L in the assay mixtures. Each diluted sample (100 μL) was mixed with 50 μL of 0.2 mol/L MES buffer (pH 6.0) and 50 μL of 0.8 mmol/L DPPH solution in a well of a 96-well microplate. After incubation at 25 °C for 20 min in the dark, the absorbance at 520 nm (As) was measured with a TECAN infinite F-200 PRO plate reader. A blank solution (50% ethanol) was used to obtain the control absorbance (Ac). DPPH-radical scavenging activity was calculated using the following equation:

$$\text{DPPH-radical scavenging (\%)} = [(A_C - As) / A_C] \times 100$$

Determination of elastase inhibitory activity

Elastase inhibitory activity was evaluated according to a previously reported method (Castrillo et al. 1979), with minor modifications. A buffer containing 0.1 mol/L HEPES and 0.5 mol/L NaCl (pH 7.5) was used as the solvent for the enzyme and the substrate (Suc(OMe)-Ala-Ala-Pro-Val-MCA). Sample stock solutions were also diluted with the same buffer to obtain the final concentrations of 1, 3, 10, 30, and 100 mg/L in the assay mixtures. Each diluted sample (50 μL) was mixed with 25 μL of 0.1 mg/mL substrate and 25 μL of 0.01 units/mL human leukocyte elastase in a well of a 96-well microplate. After standing for 30 min at 37 °C, fluorescence intensity (Fs) was measured with a TECAN infinite M-1000 plate reader (excitation at 360 nm, emission at 460 nm). Fluorescence intensities in experiments without samples (Fc) and without the enzyme (F_B) were also measured. Elastase inhibitory activity was calculated using the following equation:

$$\text{Elastase inhibition (\%)} = [(F_C - Fs) / (F_C - F_B)] \times 100$$

To compensate the effect by DMSO carried over from the sample stock solutions, the concentration of DMSO was adjusted to 1% in all the reaction mixtures.

Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was evaluated according to a previously reported method (Jeong et al. 2009), with minor modifications. A phosphate buffer (50 mmol/L, pH 6.8) was used as the solvent for L-tyrosine and mushroom tyrosinase. Sample stock solutions were also diluted with the same buffer to obtain the final concentrations of 1, 3, 10, 30, and 100 mg/L in the assay mixtures. Each diluted sample (10 μL) was mixed with 120 μL of the phosphate buffer and 50 μL of 100 units/mL tyrosinase solution in a well of a

96-well microplate. After pre-incubation at 25 °C for 10 min, 2 mmol/L L-tyrosine (20 μL) was added to the mixture. The absorbance at 476 nm was recorded at 25 °C for 20 min at 2-min intervals. Tyrosinase inhibitory activity was calculated using the following equation:

$$\text{Tyrosinase inhibition (\%)} = [1 - \Delta S/\Delta C] \times 100$$

where ΔS and ΔC are the absorbance changes in the experiments with and without the test sample, respectively. The absorbance changes between 0 and 12 min were used for the calculations. To compensate the effect by DMSO carried over from the sample stock solutions, the concentration of DMSO was adjusted to 1% in all the reaction mixtures.

Calculation of EC_{50} and IC_{50}

EC_{50} for DPPH radical scavenging and IC_{50} for elastase and tyrosinase inhibitions were calculated with the following equation using two adjacent data sets across the 50% scavenging or inhibition point:

$$EC_{50}\ (mg/L) = 10\text{^} [\text{Log}\ (C_H/C_L) \times (50 - E_L) / (E_H - E_L) + \text{Log}\ (C_L)]$$

where E_H and C_H are the scavenging (%) and concentration (mg/L) data for the higher scavenging point, respectively, and E_L and C_L are those for the lower scavenging point. IC_{50} for elastase and tyrosinase inhibitions was also calculated using a similar equation:

$$IC_{50}\ (mg/L) = 10\text{^} [\text{Log}\ (C_H/C_L) \times (50 - I_L) / (I_H - I_L) + \text{Log}\ (C_L)]$$

where I_H and C_H are the inhibition (%) and concentration (mg/L) data for the higher scavenging point, respectively, and I_L and C_L are those for the lower scavenging point.

Table S1. NMR spectroscopic data for compounds **1** and **2**.

Compound **1**				Compound **2**			
Position	^{13}C	^{1}H	*J* (Hz)	Position	^{13}C	^{1}H	*J* (Hz)
2	79.92	5.30[a] (dd*)	12.7, 3.5	2	79.79	5.36 (dd)	13.1, 3.0
3	43.84	3.00 (dd) 2.73 (dd)	17.0, 12.8 17.0, 3.1	3	43.31	3.22 (dd) 2.84 (dd)	17.1, 13.1 17.1, 3.0
4	198.34	-	-	4	196.60	-	-
5	162.87[a]	-	-	5	163.57	13.24 (br.s, OH)	-
6	102.46	-	-	6	95.96	6.63 (s)	-
7	166.86[b]	-	-	7	167.45	-	-
8	95.89	6.01[b] (s)	-	8	102.21[a]	-	-
9	164.14[c]	-	-	9	163.79	-	-
10	103.74[d]	-	-	10	103.02[a]	-	-
1'	131.47[e]	-	-	1'	129.73	-	-
2'	128.64[f]	7.16 (d)	8.5	2'	128.83[b]	7.51 (d)	8.5
3'	116.24[g]	6.71 (d)	8.5	3'	116.45	7.25 (d)	8.5
4'	158.53[h]	-	-	4'	159.58	-	-
5'	116.24[g]	6.71 (d)	8.5	5'	116.45	7.25 (d)	8.5
6'	128.64[f]	7.16 (d)	8.5	6'	128.83[b]	7.51 (d)	8.5
2''	80.56	5.31[a] (dd*)	12.8, 3.2	2''	164.73[c]	-	-
3''	44.14	3.09 (dd) 2.63 (dd)	17.0, 13.2 17.1, 2.9	3''	103.75	6.90 (s)	-
4''	197.96	-	-	4''	183.20	-	-
5''	163.32[a]	-	-	5''	164.78[c]	13.85 (s, OH)	-
6''	96.85	6.03[b] (s)	-	6''	99.99	6.94 (s)	-
7''	166.83[b]	-	-	7''	164.78[c]	-	-
8''	101.28	-	-	8''	100.72	-	-
9''	164.89[c]	-	-	9''	156.69	-	-
10''	103.39[d]	-	-	10''	105.39	-	-
1'''	131.29[e]	-	-	1'''	122.88	-	-
2'''	129.14[f]	7.30 (d)	8.5	2'''	128.86[b]	7.98 (d)	8.8
3'''	116.39[g]	6.80 (d)	8.5	3'''	116.93	7.16 (d)	8.8
4'''	158.97[h]	-	-	4'''	162.56	-	-
5'''	116.39[g]	6.80 (d)	8.5	5'''	116.93	7.16 (d)	8.8
6'''	129.14[f]	7.30 (d)	8.5	6'''	128.86[b]	7.98 (d)	8.8

CD_3OD was used as the solvent for compound **1**, while pyridine-d5 was used for compound **2**.
Values with the same superscripts are interchangeble within the column.
*overlapping signals

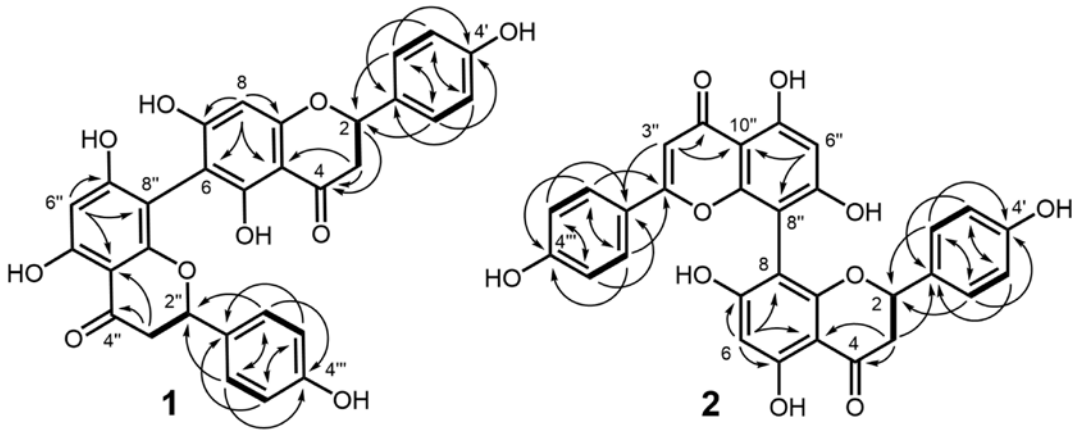

Figure S1. HMBC (arrows) and ^{1}H-^{1}H COSY (bold lines) correlations in compounds **1** and **2**.

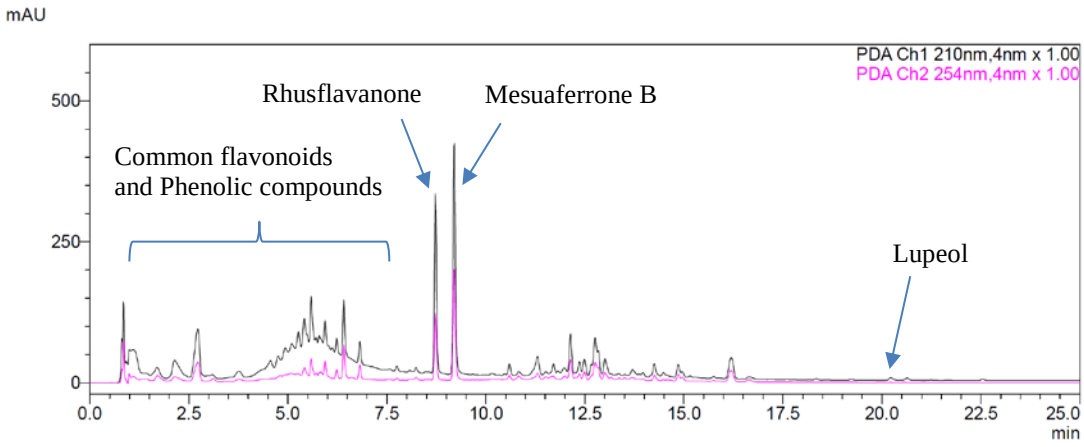

Figure S2. HPLC chromatogram for the 70% MeOH extract of *M. ferrea* stamens.

The peak of lupeol was very small due to its low UV absorption.

Table S2. Yield and MS data of isolated compounds

Compound	Compound name	Yield (mg)	Observed ions (ion-trap MS)[b]	References
1	Rhusflavanone	121[a]	*m/z* $[M + H]^+$: 543.06, *m/z* $[M - H]^-$: 541.18, *m/z* $[2M - H]^-$: 1083.35	Lin and Chen 1973; Shrestha et al. 2012
2	Mesuaferrone B	681[a]	*m/z* $[M + H]^+$: 541.06, *m/z* $[M - H]^-$: 539.18, *m/z* $[2M + H]^+$: 1080.48, *m/z* $[2M - H]^-$: 1079.44	Raju et al. 1976; Shrestha et al. 2012
3	Lupeol	4800	*m/z* $[M + H - 18]^+$: 409.33	Kang et al. 2013
4	Gallic acid	558	*m/z* $[M + H]^+$: 171.11, *m/z* $[M - H]^-$: 169.15, *m/z* $[2M + H]^+$: 341.02, *m/z* $[2M - H]^-$: 339.08	Sarria Villa et al. 2017
5	Protocatechuic acid	337	*m/z* $[M + H]^+$: 155.12, *m/z* $[M - H]^-$: 153.18, *m/z* $[2M + H]^+$: 309.09, *m/z* $[2M - H]^-$: 307.12	Nguyen et al. 2013
6	Benzyl-*β*-D-glucopyranoside	465	*m/z* $[M + H]^+$: 155.12, *m/z* $[M - H]^-$: 153.18, *m/z* $[2M + H]^+$: 309.09, *m/z* $[2M - H]^-$: 307.12	Ly et al. 2002
7	*p*-Hydroxybenzoic acid	155	*m/z* $[M + H]^+$: 139.15, *m/z* $[M - H]^-$: 137.20	Wang et al. 2000
8	Luteolin 8-*C*-glucoside	18	*m/z* $[M + H]^+$: 449.06, *m/z* $[M - H]^-$: 447.17, *m/z* $[2M + H]^+$: 896.71, *m/z* $[2M - H]^-$: 895.31	Joshi et al. 2015
9	Protocatechuic acid methyl ester	60	*m/z* $[M + H]^+$: 169.10, *m/z* $[M - H]^-$: 167.15	Lyu et al. 2009
10	Quercetin 3-*O*-rhamnoside	135	*m/z* $[M + H]^+$: 449.01, *m/z* $[M - H]^-$: 447.20, *m/z* $[2M - H]^-$: 895.35, *m/z* $[M + H - 146]^+$: 303.03	Lee et al. 2014
11	Kaempferol 3-*O*-glucoside	63	*m/z* $[M + H]^+$: 449.04, *m/z* $[M - H]^-$: 447.18, *m/z* $[2M - H]^-$: 895.32, *m/z* $[M + H - 162]^+$: 287.06	Seo et al. 2016
12	Apigenin 8-*C*-glucoside	445	*m/z* $[M + H]^+$: 433.08, *m/z* $[M - H]^-$: 431.20, *m/z* $[2M - H]^-$: 863.30	Joshi et al. 2015
13	Kaempferol 3-*O*-rhamnoside	244	*m/z* $[M + H]^+$: 433.02, *m/z* $[M - H]^-$: 431.16, *m/z* $[2M - H]^-$: 863.30, *m/z* $[M + H - 146]^+$: 287.05	Lee et al. 2014

[a]Purified by HPLC.
[b]Detected with a Bruker amaZon speed mass spectrometer.

References

Castillo MJ, Nakajima K, Zimmerman M, Powers JC. 1979. Sensitive substrates for human leukocyte and porcine pancreatic elastase: A study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. Anal Biochem. 99(1):53–64.

Jeong SH, Ryu YB, Curtis-Long MJ, Ryu HW, Baek YS, Kang JE, Kang JE, Lee WS, Park KH. 2009. Tyrosinase inhibitory polyphenols from roots of *Morus ihou*. J Agric Food Chem. 57(4):1195–1203.

Joshi KR, Devkota HP, Nakamura T, Watanabe T, Yahara S. 2015. Chemical constituents and their DPPH-radical scavenging activity of Nepalese crude drug *Begonia picta*. Records of natural products. 9(3):446–450.

Kang SC, Lim SY, Song YJ. 2013. Lupeol is one of active components in the extract of *Chrysanthemum indicum* Linne that inhibits LMP1-induced NF-κB activation. PLoS ONE. 8(11): 1–11.

Lee SY, So YJ, Shin MS, Cho JY, Lee J. 2014. Antibacterial effects of afzelin isolated from *Cornus macrophylla* on *Pseudomonas aeruginosa*, a leading cause of illness in immunocompromised individuals. Molecules. 19(3):3173–3180.

Lin YM, Chen FC. 1973. Rhusflavanone - A new biflavanone from *Rhus succedanea*. Tetrahedron Letters. 14 (48):4747–4750.

Ly TN, Yamauchi R, Shimoyamada M, Kato K. 2002. Isolation and structural elucidation of some glycosides from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). J Agric Food Chem. 50(17):4919–4924.

Lyu HN, Lee DY, Cho MH, Jeong TS, Kim IH, Lee CH, Baek NI. 2009. Inhibition on LDL-oxidation by phenolic compounds from the fruit body of *Phellinus linteus*. J Appl Biol Chem. 52 (3), 147–150.

Nguyen DMC, Seo DJ, Kim KY, Park RD, Kim DH, Han YS, Kim TH, Jung WJ. 2013. Nematicidal activity of 3,4-dihydroxybenzoic acid purified from *Terminalia nigrovenulosa* bark against *Meloidogyne incognita*. Microb Pathog. 59–60:52–59.

Raju MS, Srimannarayana G, Rao NVS, Bala KR, Seshadri TR. 1976. Structure of mesuaferrone-b a new biflavanone from the stamens of *Mesua ferrea* linn. Tetrahedron Lett. 17(49):4509–4512.

Sarria-Villa RA, Gallo-Corredor JA, Páez MI. 2017. Isolation of catechin and gallic acid from Colombian bark of *Pinus patula*. Chem Sci J. 8:174.

Seo KH, Jung JW, Thi NN, Lee YH, Baek NI. 2016. Flavonoid glycosides from the flowers of *Pulsatilla koreana* nakai. Nat Prod Sci. 22(1):41–45.

Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chem. 113(4):1202–1205.

Shrestha S, Park JH, Lee DY, Cho JG, Cho S, Yang HJ, Yong HI, Yoon MS, Han DS, Baek NI. 2012. *Rhus parviflora* and its biflavonoid constituent, rhusflavone, induce sleep through the positive allosteric modulation of GABA A-benzodiazepine receptors. J Ethnopharmacol. 142(1):213–220.

Wang M, Kikuzaki H, Zhu N, Sang S, Nakatani N, Ho CT. 2000. Isolation and structural elucidation of two new glycosides from sage (*Salvia officinalis* L.). J Agric Food Chem. 48(2):235–238.