Anti-inflammatory Principles from the Stem and Root Barks of *Citrus medica*

Yu-Yi CHAN,[a] Chan-Hao LI,[a] Yuh-Chiang SHEN,[b] and Tian-Shung WU*,[c,d]

[a] *Department of Biotechnology, Southern Taiwan University of Technology; Tainan, Taiwan 710, R.O.C.:* [b] *National Research Institute of Chinese Medicine; Taipei, Taiwan 112, R.O.C.:* [c] *Department of Chemistry, National Cheng Kung University; Tainan, Taiwan 701, R.O.C.: and* [d] *Department of Pharmacy, China Medical University; Taichung, Taiwan 401, R.O.C.* Received August 21, 2009; accepted September 28, 2009; published online October 29, 2009

Bioassay-guided investigation of the anti-inflammatory principles from the stem and root barks of *Citrus medica* L. var. *sarcodactylis* SWINGLE has led to the isolation of a new coumarin, namely citrumedin-B (1) and thirty known compounds. The anti-inflammatory components were xanthyletin (2), nordentatin (3), atalantoflavon (4) and lonchocarpol A (5) which displayed potent nitric oxide (NO)-reducing activity in microglial cells. The structure of this new compound was completely elucidated using a combination of 2D NMR techniques (correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC)) and HR-electrospray ionization (ESI)-MS analyses. The known compounds were identified by comparison of their spectroscopic and physical data with those reported in the literature. These results can be inferred from the treatment of allergic response and inflammatory properties of *Citrus medica* L. var. *sarcodactylis* SWINGLE in traditional Chinese medicine.

Key words *Citrus medica* L. var. *sarcodactylis*; Rutacae; coumarin; citrumedin-B; anti-inflammatory activity

Citrus medica L. var. *sarcodactylis* SWINGLE belongs to the genus *Citrus* (Rutacae) and is mainly distributed in Taiwan, the south of China, India, Vietnam and Malaysia. Various species of *Citrus* have been used as food, and their peel, leaves and root have been also used as folk medicine or spice in Taiwan.[1)] Species of *Citrus* have been reported to contain various bioactive coumarins,[2)] flavonoids,[3)] tetranortriterpenoids[4)] and monoterpenoids.[5)] Wu *et al.*[6—16)] and Furukawa and colleagues[17—21)] have reported the isolation of several acridone alkoids from the *Citrus* genus.

Flavonoids are one of the most important compounds present in the genus *Citrus*[22)]; more than 8000 compounds with a flavonoid structure have been identified. Four types of flavonoids (flavanones, flavones, flavonols, and anthocyanins) occur in *Citrus*. These compounds not only play an important physiological and ecological role but are also of commercial interest because of their multitude of applications in the food and pharmaceutical industries.[22—26)] Significantly, much of the activity of *Citrus* flavonoids appears to impact blood and micro vascular endothelial cells, and it is not surprising that the two main areas of research on the biological actions of *Citrus* flavonoids have been inflammation and cancer.[27,28)]

Inflammation is typically characterized by increased permeability of endothelial tissue and influxes of blood leukocytes into the interstitium, resulting in edema. Many different biological mediators influence each step of the inflammation cascade, and typically, anti-inflammatory agents exhibit therapeutic properties by blocking the actions or syntheses of these mediators. While inflammation is a normal response toward tissue injury, it is often uncontrolled in chronic autoimmune diseases, such as rheumatoid arthritis and Chrohn's disease, or when linked to allergic response like asthma and anaphylactic shock. In these cases, anti-inflammatory compounds are therapeutically administered to control the inflammation response. Plants rich in certain flavonoids have been traditionally used for their anti-inflammatory properties, and recently attention has been given to isolated flavonoids, including those in *Citrus*, as potential anti-inflammatory agents.[27,28)]

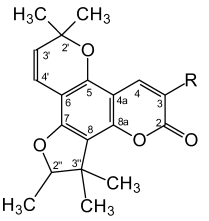
Citrus medica L. var. *sarcodactylis* SWINGLE is used as a folk medicine for the treatment of stomach ache, edema, headache, rheumatism, infectious hepatitis and arthritis in Taiwan.[29)] Continuation of our investigation on the bioactive compounds from natural resources, led us to study the constituents and anti-inflammatory components from the stem and root barks of *Citrus medica* L. var. *sarcodactylis* SWINGLE. In this paper, we report the isolation and structural determination of a new coumarin, citrumedin-B (**1**) together with seventeen known compounds from the root bark of this plant, and twenty three compounds from the stem bark.[30)] We also describe two coumarins, xanthyletin (**2**), nordentatin (**3**) and two flavonoids, atalantoflavon (**4**) and lonchocarpol A (**5**) as anti-inflammatory components from this plant.

Results and Discussion

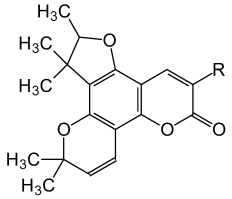
Citrumedin-B (**1**) was isolated as pale yellow syrup, $[\alpha]_D$ +14.4° ($CHCl_3$). The high resolution electrospray ionization-mass spectrum (HR-ESI-MS) of **1** showed a *pseudo* molecular ion peak ($[M+Na]^+$) at *m/z* 403.1887, which is in agreement with the molecular formula $C_{24}H_{28}O_4$. The IR spectrum revealed the presence of carbonyl group (1724 cm^{-1}). The UV spectrum appeared to show the maximum absorption at 225, 273(sh), 283, 302(sh) and 339 nm. In the ^{1}H-NMR spectrum, a singlet aromatic signal at δ_H 7.79 and a pair of AB type doublets at δ_H 6.45 and 5.53 (each 1H, d, J=9.8 Hz), accompanied by signals of two methyl groups attached to oxygenated carbon at δ_H 1.45 (6H, s), were assignable to β-proton on an α,β-unsaturated carbonyl system and two protons on the 2,2-dimethylbenzopyran ring system, respectively. The downfield shift of H-4 at δ_H 7.79 and the absence of other proton signals in the aromatic proton region in the ^{1}H-NMR spectrum, together with the results of the UV spectrum, suggested the presence of a 5,7-dioxygenated 3,6,8-trisubstituted coumarin skeleton[31)] having a dimethylpyran ring system in

* To whom correspondence should be addressed. e-mail: tswu@mail.ncku.edu.tw

the molecule. The remaining proton signals coupled with carbon signals in the ^{13}C-NMR spectrum were assigned to geminal methyls attached to a benzylic carbon [δ_H 1.47, 1.24 (each 3H, s) and δ_C 44.0] and a secondary methyl [δ_H 1.38 (3H, d, J=6.6 Hz)] attached to an oxygenated methine carbon [δ_H 4.45 (1H, q, J=6.6 Hz) and δ_C 90.9]. The appearance of H-C long-range correlations in the ^{1}H detected heteronuclear multiple bond connectivity (HMBC) spectrum between the oxygenated methine carbon at δ_C 90.9 and two methyl protons at δ_H 1.47 and 1.24, which further correlated to an aromatic carbon at δ_C 113.4 and a quaternary carbon at δ_C 44.0 indicated the presence of a 2,3,3-trimethyldihydrobenzofuran system in the molecule. Furthermore, the ^{1}H-NMR spectrum of **1** showed the signals at δ_H 6.19 (1H, dd, J=17.7, 11.4 Hz), 5.08 (1H, d, J=17.7 Hz), 5.06 (1H, d, J=11.4 Hz) and 1.45 (6H, s) due to a 1,1-dimethylallyl group. The cross peaks of δ_C 40.4 to δ_H 7.79, 5.08 and 5.06; δ_C 128.7 to δ_H 1.45 in the HMBC spectrum suggested the location of the 1,1-dimethylallyl group to be at C-3. Based on these spectral data together with the HMBC and nuclear Overhauser effect spectroscopy (NOESY) data shown by arrows in Figs. 1 and 2, the structure of citrumedin-B should be depicted by either formula **1** or **1a**. Comparison of the NMR data of citrumedin-B (**1**), citrusarin-A (**6**) and citrusarin-B (**6a**)[32] (Table 1), showed the similarity of the chemical shift of signal at δ_H 6.45 due to H-4′ on the pyran ring of citrumedin-B (**1**) to that of citrusarin-A (**6**). On the basis of the above results, the structure of citrumedin-B was assigned as formula **1**.



1 R= $C(CH_3)_2CH=CH_2$
6 R=H



1a R= $C(CH_3)_2CH=CH_2$
6a R=H

The known compounds, xanthyletin (**2**),[13] nordentatin (**3**),[13] clausarin (**7**),[33] osthenol (**8**),[34] 6,7-dimethoxycoumarin (**9**),[35] erythrisenegalone (**10**),[36] lupinifolin (**11**),[36] β-sitosterol (**12**),[30] stigmasterol (**13**),[30] citrusarin-A (**6**),[32] oxanordentatin (**14**),[37] 7-demethylsuberosin (**15**),[38] 1-

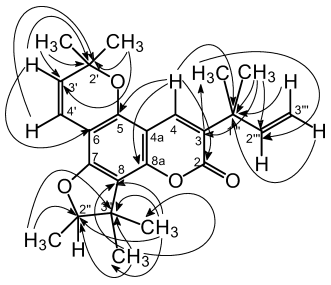


Fig. 1. HMBC Correlations of **1**

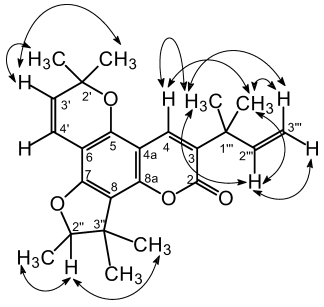


Fig. 2. NOE Correlations of **1**

Table 1. ^{1}H- and ^{13}C-NMR Spectra Data of **1**, **6** and **6a**

Carbon No.	**1**		**6**		**6a**	
	δ_H (ppm)	δ_C	δ_H (ppm)	δ_C	δ_H (ppm)	δ_C
2		159.8		161.3		161.4
3		128.7	6.07 (1H, d, 9.6)	109.8	6.11 (1H, d, 9.5)	110.5
4	7.79 (1H, s)	133.1	7.93 (1H, d, 9.6)	139.3	7.75 (1H, d, 9.5)	138.6
4a		103.7		103.6		99.1
5		149.9		150.2		156.2
6		101.7		101.8		117.9
7		157.2		158.1		153.2
8		113.4		114.2		102.9
8a		150.7		151.1		149.9
2′		77.7		77.9		77.4
3′	5.53 (1H, d, 9.8)	127.5	5.54 (1H, d, 9.9)	127.8	5.56 (1H, d, 9.9)	127.0
4′	6.45 (1H, d, 9.8)	116.1	6.45 (1H, d, 9.9)	115.9	6.80 (1H, d, 9.9)	115.6
2″	4.45 (1H, q, 6.6)	90.9	4.48 (1H, q, 6.6)	91.0	4.49 (1H, q, 6.6)	91.1
3″		44.0		44.0		44.1
1‴		40.4				
2‴	6.19 (1H, dd, 17.7, 11.4)	145.9				
3‴	5.08 (1H, d, 17.7)	111.7				
	5.06 (1H, d, 11.4)					
2′-CH_3	1.45 (6H, s)	28.1	1.46 (6H, s)	28.0	1.46 (3H, s)	28.1
		27.9			1.47 (3H, s)	28.1
2″-CH_3	1.38 (3H, d, 6.6)	14.2	1.39 (3H, d, 6.6)	14.2	1.40 (3H, d, 6.6)	14.3
3″-CH_3	1.47 (3H, s)	25.6	1.52 (3H, s)	25.5	1.42 (3H, s)	25.6
	1.24 (3H, s)	21.2	1.25 (3H, s)	21.2	1.18 (3H, s)	21.1
1″-CH_3	1.45 (6H, s)	26.3				

(10→19)*abeo*-7α-acetoxy-10β-hydroxyisoobacunoic acid-3,10-lactone (**16**),[39] syringaresinol (**17**),[40] *cis*-*p*-coumaric acid (**18**),[41] nomilin (**19**)[30] and limonin (**20**),[35] were also isolated from the root bark of *Citrus medica* L. var. *sarcodactylis* SWINGLE. Furthermore, 23 compounds: xanthyletin (**2**),[12] nordentatin (**3**),[12] atalantoflavon (**4**),[30] lonchocarpol A (**5**),[42] 6,7-dimethoxycoumarin (**9**),[35] erythrisenegalone (**10**),[36] lupinifolin (**11**),[36] β-sitosterol (**12**),[30] stigmasterol (**13**),[30] *cis*-*p*-coumaric acid (**18**),[41] nomilin (**19**),[30] limonin (**20**),[35] lupeol (**21**),[30] 5,7-dimethoxycoumarin (**22**),[30] hiravanone (**23**),[30] citrumedin-A (**24**),[30] 7-methoxycoumarin (**25**),[30] leptodactylone (**26**),[30] citflavanone (**27**),[30] 7,8-dimethoxycoumarin (**28**),[30] citrusin (**29**),[30] limonexic acid (**30**)[30] and C-glycosylflavone-vitexin (**31**)[30] were also isolated from the stem bark of this plant. Their structures were identified by comparison of their spectroscopic data (UV, IR, NMR and mass spectrometry) with values in the literature.

Activated microglial cells play deleterious roles in mediating central nerve system (CNS) inflammatory responses by producing enormous amounts of nitric oxide (NO) and reactive oxygen species (ROS) through induction of inducible nitric oxide synthase (iNOS) and activation of nicotinamide adenine dinucleotide phosphate-oxidase (NOX), which results in neuronal damage by NO, ROS, and the more toxic metabolite peroxynitrite ($ONOO^-$).[43—45] We also reported that drugs with antioxidative and NO-reducing activity can prevent stroke-induced brain damage.[46] Therefore, inhibiting NO or ROS production is a useful strategy for treating inflammatory disorders such as cardiovascular diseases and neurodegenerative disorders.[44,47] The anti-inflammatory potentials of **2**—**5**, **16**, **19**—**22** and **29**—**31** (Fig. 3) were evaluated by examining their effects on LPS-induced iNOS-dependent NO production and NOX-dependent ROS production in microglial cells (Table 2). Among the compounds, xanthyletin (**2**), nordentatin (**3**), atalantoflavon (**4**) and lonchocarpol A (**5**) showed significant inhibited NOS activity with an IC_{50} value of 5.4, 10.2, 1.4 and 2.5 μM. Its potency was comparable to that of NG-nitro-L-arginine methyl ester (L-NAME) (IC_{50} 16.8 μM), a specific NOS inhibitor. NOX is the major ROS-producing enzyme in activated inflammatory cells.[48] Therefore, we also evaluated the above compounds for NOX activity in lysates of microglial cells.[49] Our data suggest that lonchocarpol A (**5**) and C-glycosylflavone-vitexin (**31**) are ordinary inhibitors of NOX, as compared to the specific NOX inhibitor di-phenyleneiodonium (DPI). In addition, the free radical-scavenging capacities of these compounds were examined in a cell-free 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution; none, however showed much free radical-scavenging activity.

Bioassay-guided fractionation of the extract of *Citrus medica* L. var. *sarcodactylis* SWINGLE finally was isolated and a

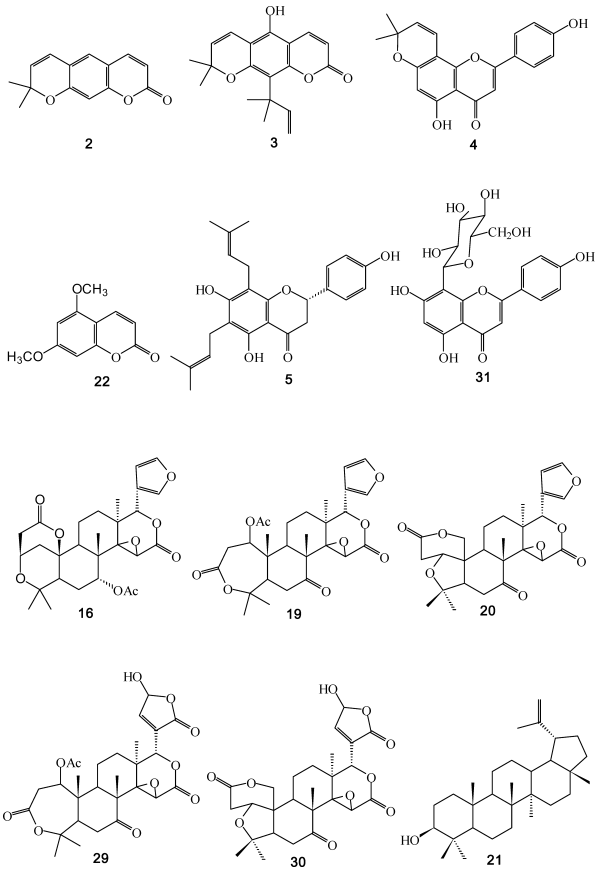


Fig. 3. Structures of Compounds **2**—**5**, **16**, **19**—**22** and **29**—**31**

Table 2. Effects of Tested Compounds on Nitric Oxide (NO) Production and NADPH Oxidase (NOX) Activity in Murine Microglial Cells, and DPPH Free Radical Scavenging Activity[a]

Compound	IC_{50} (μM) in NO production	NOX	DPPH
Xanthyletin (**2**)	5.4±1.6*	NA	NA
Nordentatin (**3**)	10.2±0.3*	NA	53.6±0.2
Atalantoflavon (**4**)	1.4±0.2*	NA	NA
Lonchocarpol A (**5**)	2.5±0.4*	24.4±3.9*	NA
1-(10→19)*abeo*-7α-Acetoxy-10β-hydroxyisoobacunoic acid-3,10-lactone (**16**)	23.4±1.4	NA	NA
Nomilin (**19**)	32.8±6.5	NA	NA
Limonin (**20**)	NA	NA	NA
Lupeol (**21**)	24.0±2.4	NA	NA
5,7-Dimethoxycoumarin (**22**)	16.7±0.8	NA	NA
Citrusin (**29**)	44.0±5.7	NA	NA
Limonexic acid (**30**)	39.5±6.3	NA	NA
C-Glycosylflavone-vitexin (**31**)	20.2±3.9	35.5±1.6*	NA
Positive control	16.8±1.5 (L-NAME)	0.8±0.4 (DPI)	28.0±4.2 (Trolox)

a) NO production was measured in the presence of 1—50 μM of test compound. L-NAME (a non-selective NOS inhibitor), DPI (a NOX inhibitor) and trolox (a Vit. E analogue) were included as positive controls. Data were calculated as 50% inhibitory concentration (IC_{50}) and expressed as the mean±S.E.M. from 3—10 experiments performed on different days using microglial cells from different passages. $*p<0.05$ as compared with L-NAME. N.A., value not available (not effective).

new coumarin identified, citrumedin-B (**1**), together with 30 known compounds. In addition, biological screening of the pure compounds in Table 2 has revealed that xanthyletin (**2**), nordentatin (**3**), atalantoflavon (**4**) and lonchocarpol A (**5**) display more potent NO-reducing activity in microglial cells than that of positive control. Interestingly, these compounds have the dimethylpyran ring at the C-6 and C-7 positions in the coumarin skeletal structure and have the prenyl group at C-8 in the flavonoids. The major difference between **4** and **5** is that compound **4** has a dimethylpyran ring, which is formed by prenyl moiety cyclization with *ortho*-phenolic hydroxyl groups. Thus, we suggest that the dimethylpyran ring is the reason that compound **4** has better activity than compound **5**. These results can be inferred with the treatment of allergic response and inflammatory properties of *Citrus medica* L. var. *sarcodactylis* Swingle in tradition Chinese medicine.

Experimental

General Melting points were determined on a Yanagimoto MP-S3 apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR and UV spectra were recorded on Shimadzu FTIR-8501 and Hitachi UV-3210 spectrophotometers, respectively. ESI-MS was obtained on a Bruker APEX II mass spectrometer. The ^{1}H- and ^{13}C-NMR, DEPT, COSY, HMQC, NOESY, and HMBC experiments were recorded on a Bruker AMX-400 spectrometer. Standard pulse sequences and parameters were used for the NMR experiments and all chemical shifts were reported in parts per million (ppm, δ). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70—230 mesh and 230—400 mesh, E. Merck).

Plant Material The stem and root bark of *Citrus medica* L. var. *sarcodactylis* Swingle was collected from Changhua Hsien, Taiwan, in September 2002 and verified by Prof. C.-S. Kuoh. A voucher specimen (TSWu 91023) has been deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Isolation The dried and powdered root bark of *Citrus medica* L. var. *sarcodactylis* Swingle (174 g) was extracted with acetone at room temperature and concentrated under reduced pressure to give a dark brown syrup (11 g). The syrup was partitioned successively between H_2O and $CHCl_3$. The $CHCl_3$ layer (2.5 g) was chromatographed directly on silica gel and eluted with a gradient of *n*-hexane and EtOAc to afford 4 fractions. Fraction 1 was rechromatographed on silica gel and eluted with $CHCl_3$–Me_2CO (30 : 1, v/v) to give **1** (1 mg), **2** (36 mg), **3** (25 mg), **7** (2 mg), **8** (1 mg), **9** (1 mg), **10** (2 mg), **11** (2 mg), **12** and **13** (3 mg), successively. Fraction 2 was rechromatographed on silica gel and eluted with *n*-hexane–Me_2CO (4 : 1, v/v) to give **6** (1 mg), **14** (1 mg), **15** (1 mg), **16** (8 mg), **17** (1 mg), **18** (1 mg). Fraction 3 underwent a series of chromatographic separations on silica gel using benzene–EtOAc (2 : 1, v/v) as eluent to afford **19** (7 mg). Fraction 4 underwent chromatography on silica gel using $CHCl_3$–EtOAc (3 : 1, v/v) as eluent to afford **20** (6 mg).

The dried and powdered stem bark of *Citrus medica* L. var. *sarcodactylis* Swingle (1.34 kg) was extracted with acetone at room temperature and concentrated under reduced pressure to give a dark brown syrup (87 g). The syrup was partitioned successively between H_2O and $CHCl_3$. The $CHCl_3$ layer (19.4 g) was chromatographed directly on silica gel and eluted with a gradient of *n*-hexane and EtOAc to afford 10 fractions. Fraction 4 was rechromatographed on silica gel and eluted with *n*-hexane–EtOAc (10 : 1, v/v) to give **21** (37 mg). Fraction 6 was rechromatographed on silica gel and eluted with *n*-hexane–Me_2CO (5 : 1, v/v) to give **2** (28 mg), **5** (22 mg), **10** (2 mg), **11** (3 mg), **12** and **13** (312 mg), **22** (43 mg) and **23** (1 mg). Fraction 7 underwent a series of chromatographic separations on silica gel using $CHCl_3$–EtOAc (49 : 1, v/v) as eluent to afford **3** (1 mg), **4** (82 mg), **9** (3.5 mg), **18** (1 mg), **24** (1 mg), **25** (1 mg), **26** (1.5 mg) and **27** (1 mg), successively. Fraction 8 underwent chromatography on silica gel using $CHCl_3$–Me_2CO (20 : 1, v/v) as eluent to afford **28** (2 mg). Fraction 9 was also separated by chromatography on silica gel using $CHCl_3$–Me_2CO (15 : 1, v/v) to give **19** (834 mg), **20** (726 mg), **29** (56 mg) and **30** (27 mg), respectively. The H_2O layer (67.6 g) was subjected to column chromatography on silica gel, eluted with a gradient of EtOAc–Me_2CO and gave 25 fractions. Fraction 2 was separated by column chromatography on silica gel using $CHCl_3$–MeOH (6 : 1, v/v) as eluent to give **31** (13 mg).

Citrumedin-B (**1**): Pale yellow syrup, $[\alpha]_D$ +14.4° (c=0.03, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 1724, 1604. UV λ_{max} (MeOH) nm (log ε): 225, 273(sh), 283, 302(sh), 339. ^{1}H- and ^{13}C-NMR data, see Table 1. ESI-MS *m*/*z*: 403 ([M+Na]$^+$), 381 ([M+H]$^+$), 353. HR-ESI-MS *m*/*z*: [M+Na]$^+$ (Calcd for $C_{24}H_{28}O_4Na$: 403.1885; Found: 403.1887).

Measurement of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical-Scavenging Capacity DPPH radical-scavenging capacity assay was performed as in our previous report.[49]

Microglial Cell Culture and Measurements of Nitric Oxide (NO) The murine microglial cell line (BV2) was cultured and production of NO was measured by the methods described in our prior report.[46]

Measurement of NADPH Oxidase (NOX) Activity NADPH oxidase activity was measured as described previously.[46]

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