Asian Journal of Chemistry Vol. 21, No. 1 (2009), 745-754

Cyclooxygenase Inhibitory Compounds with Antioxidant Activities from *Sophora subprostrata*

CHANGWEI AO, NOBUHARU ARAKI† and SHINKICHI TAWATA*
Department of Bioscience and Biotechnology, Faculty of Agriculture
University of the Ryukyus, 1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan
Fax: (81)(98)8958734; Tel: (81)(98)8958803; E-mail: aoweikun@yahoo.com

As a traditional Chinese medicine, *Sophora subprostrata* has been used as an antipyretic, antidote and analgesic. For the purpose of searching for bioactive compounds exerting antiinflammatory activity, two-quinolizidine alkaloids were isolated by cyclooxygenase (COX) inhibitory activity-directed chromatographic fractionation from the aerial portion of *S. subprostrata*. These alkaloids were identified as matrine and oxymatrine, respectively. Matrine showed significant COX-1 inhibitory activity (IC_{50}: 31.3 μM) and moderate COX-2 inhibitory activity (IC_{50}: 188.5 μM), whereas oxymatrine showed less COX-1 (IC_{50}: 197.8 μM) and COX-2 (IC_{50}: 385.1 μM) inhibitory activity. Both matrine and oxymatrine exhibited antioxidant activity in a dose-dependent manner by PMS-NADH system test (EC_{50} 192.5 and 275.8 μM, respectively). This is the first report on cyclooxygenase inhibitory and antioxidant activities from matrine and oxymatrine *in vitro* and provides certain scientific supports for the medicinal use of these two compounds from *S. subprostrata* for inflammatory disorders.

Key Words: *Sophora subprostrata*, Cyclooxygenase inhibition, Antioxidant, Matrine, Oxymatrine.

INTRODUCTION

Sophora subprostrata CHUN et T. CHEN (Chinese drug: Shan-Dou-Gen, Leguminosae) has traditionally been used as Chinese medicinal plant in southern China. It belongs to the category of heat eliminating and toxin removing herbs, whose roots have been used to treat pain, fever, peptic ulcers, inflammation and coughing[1]. Moreover certain alkaloids isolated from *S. subprostrata* have been confirmed to have some pharmacological effects, such as anti-pyretic[2], anti-tumoral[3] and anti-nociceptive effects[4]. As an important Chinese drug, extensive research about this herb has been performed in the past 50 years and a number of constituents have already been isolated from this plant, including alkaloids[5], flavonoids[6], saponins[7] and polysaccharides[8].

†J-DOLPH Pharmaceutical Co., Ltd., Osaka, Japan.

COXs play an important role in the synthesis of prostaglandin from arachidonic acid and have been widely used to evaluate the antiinflammatory activities of natural products[9,10]. There are two different forms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in various tissues, while COX-2 is induced in response to cytokines, growth factors and tumor promoters in inflammation. Many plant tissues contain a wide variety of compounds with antioxidant activity, such as flavonoids, lignans, alkaloids, terpenes and phenolics[11]. In addition to the protective effects of the endogenous antioxidant defense system, natural products with antioxidant activity could retard the oxidative damage of a tissue by increasing those defenses[12]. Some studies about the relationship of antioxidant and antiinflammatory have shown that oxygen free radicals produced in inflammation and antioxidant compound may block arachidonic acid metabolism by inhibiting lipoxygenase activity or may serve as a scavenger of reactive free radicals which are produced in arachidonic acid metabolism[13].

So far, little information on the *in vitro* COX inhibitory and antioxidant activities of *S. subprostrata* extracts has been reported. The purpose of present study was to isolate and identify major components from this herb by COX inhibitory activity-guided chromatographic fractionation methods. Two kinds of alkaloids, matrine and oxymatrine were purified from the active chloroform fraction of methanolic extract of *S. subprostrata*.

EXPERIMENTAL

Melting points were determined on a BUCHI 535 (BUCHI Co.). ^{1}H NMR and ^{13}C NMR spectra (ppm, J in Hz) were obtained using a JNM-A500 NMR spectrometer (Jeol) at 500 MHz and 125 MHz, respectively, with TMS as an internal standard.

COX (ovine) inhibitor screening assay kits and aspirin were purchased from Funakoshi Co., Japan, Cayman Chemical Company. Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Nacalai tesque, Japan, Sigma, Germany. Trolox, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3(2)-*t*-butyl-4-hydroxyanisole (BHA) and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

The dried aerial parts of *S. subprostrata* used in this study were purchased from the Herb Store in Jiang Kou, Guizhou province, China. The plant identity was confirmed by Dr. Xu Shengguo (Central South Forestry University, China).

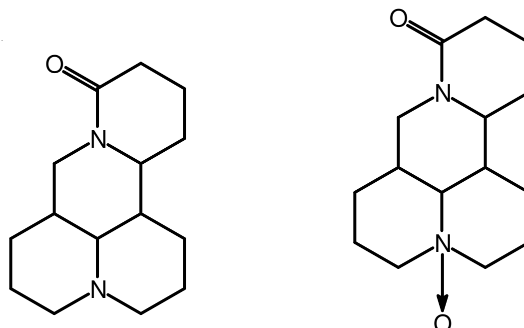
Extraction, isolation and identification: The dried aerial parts of *S. subprostrata* (235 g) were pulverized and extracted with methanol (3 × 900 mL, 72 h) at room temperature. The isolated crude extract was filtrated and then the solvent was removed at 40 °C under a vacuum and freeze-

dried to get 38.4 g crude extract. Dried crude extract was suspended in water (400 mL) and successively partitioned with hexane, chloroform and ethyl acetate. The obtained extract, in addition to the water solution remaining after extraction, was filtered and concentrated under reduced pressure to get 1.1, 5.1, 0.26 and 31.3 g of hexane, chloroform, ethyl acetate and water extracts, respectively.

The chloroform extract (5.1 g) was added with benzene (100 mL × 3 times) to obtain the benzene-soluble fraction (1.9 g) and benzene-insoluble fraction (3.1 g). Because benzene-soluble fraction showed much higher COX inhibitory activity than benzene-insoluble fraction, so an aliquot of the benzene-soluble fraction (105 mg, dissolved in 10 mL 50 % methanol) was applied to a C18 Sep-Pak column (Vac 35 mL, 10 g; waters) previously wet with 50 mL methanol and equilibrated with 50 mL of 50 % methanol. Then 50, 75, 90 % and absolute methanol (120 mL of each) were used in succession to elute the column. Four fractions were collected and named as fractions A1 (40 mg), A2 (16 mg), A3 (34 mg) and A4 (8 mg), respectively. Then, COX inhibitory and antioxidant activities were evaluated for these fractions. A1 and A2 fractions showed a high activity and were subjected to preparative HPLC purification.

Fractions A1 and A2, were further purified by preparative HPLC using a Shimadzu preparative HPLC equipped with SCL-10Avp system controller, LC-20AT pump, SPD-20A UV-Vis detector, FRC-10A fraction collector and Rheodyne Injector Model 7725i with 100 µL sample loop. Separation was achieved with a cosmosil preparative C-18 AR column (Nacalai Tesque company) (20 × 250 mm). The mobile phase consisted of acetonitrile : 0.01 M KH_2PO_4:triethylamine in a ratio of 20:8:0.01 (v/v/v). Two main peaks with retention times 21.6 (compound **1**) and 25.6 min (compound **2**) were collected with a 3.0 mL/min of flow rate and 208 nm detection. The pooled fractions were concentrated and then chloroform was added into these factions to extract the aim compounds. The two compounds have been identified as matrine (compound **1**) and oxymatrine (compound **2**) by comparison with previously published data. Matrine is a colourless glassy solid; m.p. 76 ºC; UV: (MeOH) λ_{max} 204 nm; MS (positive ion): m/z 249.4 [M+H]. Oxymatrine is a colourless glassy solid; m.p. 162-163 ºC; UV: (MeOH) λ_{max} 194 nm; MS (positive ion): m/z 265.4 [M+H]. ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) of matrine and oxymatrine also matched with published data[14]. Structures of the two compounds showed in Fig. 1.

LC/MS spectrometer: LC/MS spectra were obtained using a Sciex API 2000 LC-MS/MS System (Model Sciex API 2000, Applied Biosystems, Langen, Germany) coupled to a Agilent 1100 LC Binary pump equipped with a Agilent 1100 Thermo Auto-sampler, Agilent 1100 Column Oven



(**1**) Matrine: $C_{15}H_{24}N_2O$ (**2**) Oxymatrine: $C_{15}H_{24}N_2O_2$

Fig. 1. Structure of matrine (**1**) and oxymatrine (**2**) isolated from *S. subprostrata*

and Agilent 1100 Diode Array Detector in combination with a SYNERGI 4 u MAX-RP 80 A C18 reverse phase column (150 mm × 4.6 mm, Phenomenex Company, USA). 5 μL samples were injected for analysis. Draw speed and eject speed were 200 and 200 μL/min, respectively. UV detector spectral was recorded between 190-400 nm with a 2 nm step width. Flow rate was 200 μL/min, solvent A = ultra-pure water and solvent B = HPLC grade MeOH. Gradient program: 0 min (95 % A, 5 % B), 2 min (85 % A, 15 % B), 10 min (50 % A, 50 % B), 20 min (30 % A, 70 % B), 25 min (20 % A, 80 % B), 30 min (10 % A, 90 % B), 35 min (5 % A, 95 % B), 40 min (0 % A, 100 % B), 45 min (0 % A, 100 % B), 50 min (95 % A, 5 % B).

Mass spectra were obtained in ion spray voltage of 5000 V (positive mode) and a temperature of 450 °C using a Turbolon spray ion source. Spectra were recorded between m/z 100-900 with scan duration of 2 s/scan and an interscan time of 0.1 s. Spectra were processed using Biosystems/ MDS SCIEX instruments Analyst Software (version: Analyst 1.4).

Determination of COX inhibitory activity: The ability of the *S. subprostrata* extracts, matrine, oxymatrine and positive controls to inhibit ovine COX-1 and COX-2 was determined using a COX (ovine) inhibitor screening assay kit (Cayman Chemical Company, USA) according to the manufacturer's instructions. Briefly, 10 μL of different concentrations of test samples were added to series reaction buffer solutions (960 μL, 0.1 M *Tris*-HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 μL) enzyme in the presence of heme (10 μL). After solutions were incubated 5 min at 37 ºC, the reaction was initiated by adding 10 μL substrate solution (arachidonic acid, 100 μM). The reaction was stopped by the addition of 50 μL of 1 M HCl after 2 min and then saturated stannous chloride was added to reduce PGH_2 to PGF_{2a}, which was measured by enzyme immunoassay. All treatments were carried out in 3 replications. The inhibitory activity was calculated by comparing with a plotted standard

response curve. The concentration of the test compound causing 50 % inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve.

DPPH radical scavenging activity: Free radical scavenging activity of extracts was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method[15]. Different concentrations of extracts and positive control BHA in methanol solution (100 μL) were added to 4 mL of DPPH methanol solution (0.1 mM). An equal amount of methanol was used as a blank. After incubation for 0.5 h at room temperature in the dark, the absorbance was measured at 517 nm using a UV spectrophotometer (Jasco Ubest-50). Activity of scavenging (%) was calculated using the following formula:

$$\text{DPPH radical scavenging (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100\%$$

PMS-NADH system for scavenging superoxide radicals: The superoxide scavenging ability of extracts was assayed by the method of Lau *et al.*[16] with minor modifications. In the reaction mixture, 1.6 mL of phosphate buffer (0.1 M, pH 7.4) contained 105.6 μM β-nicotinamide adenine dinucleotide (NADH), 50 μM nitro blue tetrazolium (NBT) and samples in different concentrations dissolved in methanol. The reaction was initiated by adding 30 μM phenazin methosulfat (PMS) into the reaction mixture. Methanol was used as a control. After 10 min, the reaction mixture reached a stable colour; the absorbance was measured at 560 nm against a blank. The capability of scavenging superoxide radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100\ \%$$

Statistical analysis: Data were analyzed by SAS version 6.12 using ANOVA with the least significant difference (LSD) at the 0.05 probability level.

RESULTS AND DISCUSSION

COX inhibitory and antioxidant activities of methanol extract and sub-fractions from *S. subprostrata*: The methanolic extract of *S. subprostrata* was separated into hexane, chloroform, ethyl acetate and water fractions. Then, they were subjected to COX inhibitory, DPPH radical scavenging and PMS-NADH system antioxidant assays. The COX inhibitory and antioxidant effect of the methanolic extract and the fractionated extracts are shown in Figs. 2-4. These results indicate that the methanolic extract exhibited moderate COX-1 and COX-2 inhibitory activity and antioxidant effects. At the concentration of 45.5 μg/mL, the chloroform fraction showed the highest COX-1 and COX-2 inhibitory (41.1 and 30.8 %, respectively) in all fractions. Chloroform fraction also exhibited higher DPPH radical scavenging activity (EC_{50} 44.4 μg/mL) and superoxide radical scavenging activity (53.3 %)

compared with hexane, ethyl acetate and water fractions. The results suggested that COX inhibitory and antioxidant components most contented in chloroform fraction.

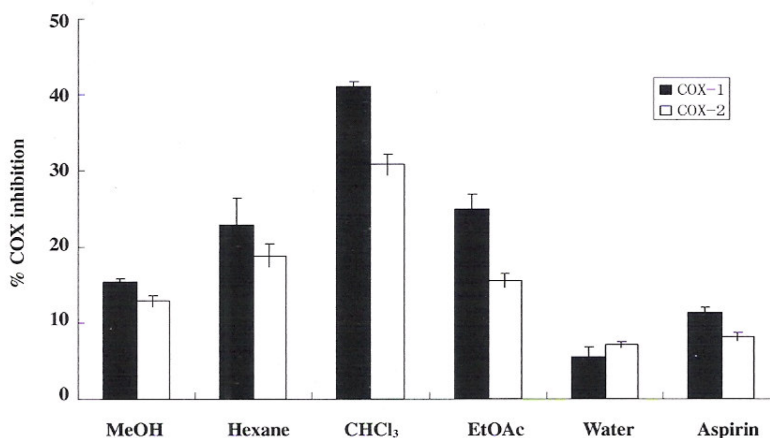


Fig. 2. Inhibitory activity of the extracts of *S. subprostrata* against COX. Effect of methanolic extract and sub-fractions of *S. subprostrata* for COX-1 and COX-2 inhibitory activities, samples concentration: 45.5 μg/mL. Aspirin was used as a positive control in the assay, the values represent means ± SD (n = 3), * $p < 0.05$ *vs.* methanolic extract

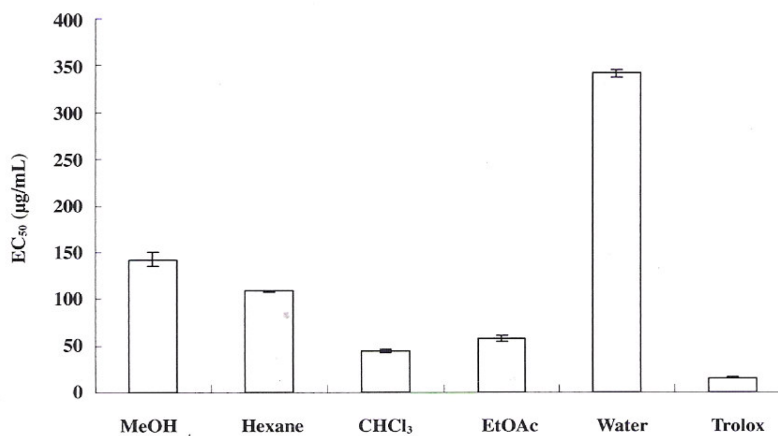


Fig. 3. DPPH radical scavenging activity (EC_{50}) of the extracts of *S. subprostrata*. Effect of the methanolic extract and sub-fractions of S. subprostrata on DPPH radical scavenging activities (EC_{50}); the values represent means ± SD (n = 3), *$p < 0.05$ *vs.* methanolic extract

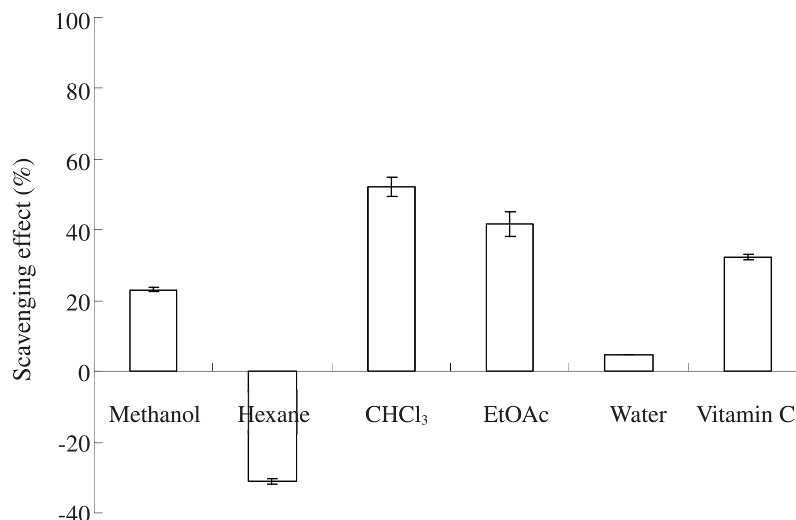


Fig. 4. PMS-NADH system antioxidant activity of the extracts of *S. subprostrata* Effect of the methanolic extract and sub-fractions of *S. subprostrata* for PMS-NADH system antioxidant activities; samples concentration: 30 µg/mL, the values represent means ± SD (n = 3), *p < 0.05 *vs.* methanolic extract

Chemical composition in the extracts of *S. subprostrata* by LC/MS: LC/MS analysis of the methanolic extract and sub-fractions of *S. subprostrata* resulted in the identification of two compounds, matrine and oxymatrine. LC/MS chromatograph of methanolic extract showed in Fig. 5. According to the analysis results of different fractions by LC/MS, the content of matrine and oxymatrine were gradually increased following COX inhibitory activity-directed chromatographic fractionation. As showed in Table-1, the matrine and oxymatrine present in methanolic extract were 2.7 and 11.6 %, respectively and in the chloroform fraction were 12.0 and 60.1 %, respectively. The contents of matrine and oxymatrine in the chloroform fraction were much higher than in the methanolic extract. The amount of matrine contented in benzene-soluble fraction was increased to 35.8 %, but the amount of oxymatrine was decreased to 38.4 %. The matrine composition in the A2 fraction was *ca.* 53.0 %. Based on the results of preliminary COX inhibitory and antioxidant activities testing, these activities might be due to the presence of matrine and oxymatrine and we also concluded that matrine exhibited higher COX inhibitory activity than oxymatrine. These two compounds were purified from the A1, A2 fractions by successive preparative HPLC.

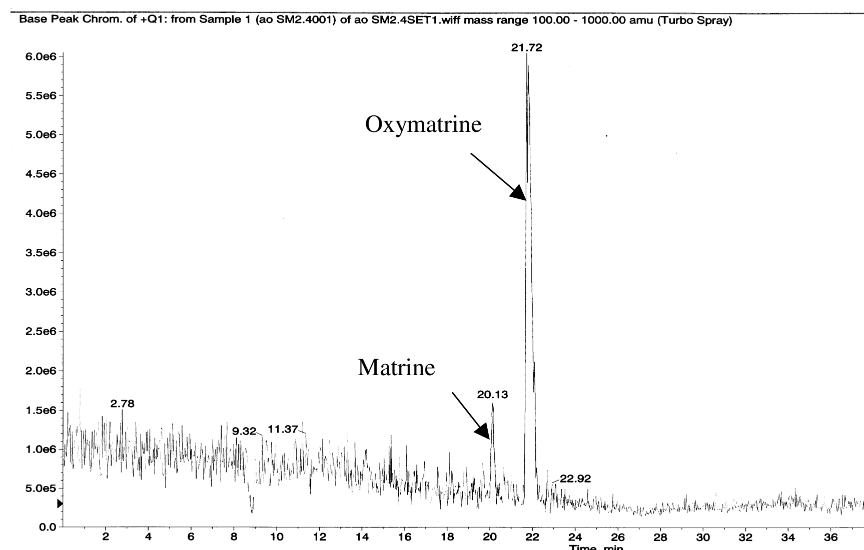


Fig. 5. LC/MS chromatograph of *S. subprostrata* methanol extract

TABLE-1
MATRINE AND OXYMATRINE COMPOSITION IN EXTRACTS OF *S. subprostrata* BY LC/MS

Extracts	Composition (%)	
	Matrine (20.5-21.0)[a]	Oxymatrine (21.5-22.7)
Methanol extract	2.7	11.6
$CHCl_3$ fraction	12.0	60.1
Benzene-soluble fraction	35.8	38.4
A1 fraction	38.7	18.2
A2 fraction	53.0	6.8

[a]Retention time (min).

COX inhibitory activities of matrine and oxymatrine: The isolated matrine and oxymatrine from *S. subprostrata* were tested for COX-1 and COX-2 inhibitory activities. The concentrations of each of the two compounds assayed were 365.1, 182.6 and 91.3 μM for the assays. These two compounds showed concentration-dependence in COX-1 and COX-2 inhibition assays. The inhibition of cyclooxygenase activities (IC_{50}) for the two compounds was shown in Table-2. Standard antiinflammatory compounds, aspirin and NS-398, were used as positive controls, when aspirin showed minor inhibitory activity against COX-1 and COX-2 (IC_{50} 1.04 and 2.23 mM, respectively) and NS-398 inhibited excellent COX-2 inhibitory activity (IC50 5.6 μM). Matrine showed high COX-1 inhibitory activity (IC_{50} 31.3 μM) and moderate COX-2 inhibitory activity (IC_{50} 188.5 μM). Oxymatrine had weaker inhibitory

activities against COX-1 (IC_{50} 197.8 μM) and COX-2 (IC_{50} 385.1 μM) compared with matrine. Both matrine and oxymatrine showed higher COX-1 inhibitory activity than COX-2 inhibitory activity.

TABLE-2
COX INHIBITORY AND ANTIOXIDANT ACTIVITY OF MATRINE AND OXYMATRINE

Samples	COX inhibitory activity IC_{50} (μM)		PMS-NADH assay EC_{50} (μM)
	COX-1	COX-2	
Oxymatrine	197.8 ± 2.4b	385.1 ± 2.7b	275.8 ± 3.5a
Matrine	31.3 ± 0.6c	188.5 ± 3.7c	192.5 ± 2.3b
Aspirin	1041.1 ± 8.5a	2228.1 ± 18.4a	NT
NS-398	NT	5.6 ± 0.1d	NT
Trolox	NT	NT	126.6 ± 1.1c

Values represent means ± SD (n = 3); Values sharing the same superscript letter in same column are not significantly different ($p < 0.05$); NT = not tested.

Antioxidant activity of matrine and oxymatrine: To examine the antioxidant activity effects of the isolated matrine and oxymatrine, the PMS-NADH system superoxide radicals scavenging methods were used. As indicated in Table-2, matrine and oxymatrine exhibited moderate antioxidant activities when assayed by radical scavenging (EC_{50} 192.5 and 275.8 μM, respectively), while EC_{50} of positive control Trolox was 126.6 μM.

Shibata and Nishikawa[5] reported that matrine and oxymatrine were first isolated from the roots of *S. subprostrata*. An intensive investigation into the pharmacology and clinical applications of these two alkaloids had been performed in the past and still remained one of the focal points of medical research. The main clinical applications of matrine and oxymatrine were treatment of tumour[3], viral hepatitis[17,18], ocular inflammation[19], ulcer[20], cardiovascular and nociceptive diseases[4,21]. Matrine was also reported to be used as a non-steroidal antiinflammatory drug (NSAIDs)[4]. Hong *et al.*[22] have reported that the methanolic extract of *S. subprostrata* showed COX-2 and iNOS inhibitory activities; however they did not isolate active compounds. One research showed that matrine exhibited selective inhibitory effect on functional activity of COX-2 in the HT-29 cell line[23]. These results are accordance with present study. The presence of matrine and oxymatrine were reported in many leguminous plants. Some researchers isolated these compounds from the root of *S. flavescens*, the aerial parts of *S. alopecuroides*[19,24]. However, there are few reports about these alkaloids isolated from the aerial parts of *S. subprostrata*. The mechanism of antiinflammatory activity of matrine and oxymatrine was not well understood. Therefore, it is necessary to clarify the COX inhibitory effect by these compounds. The present study reports for the first time the COX-1 and COX-2 inhibitory activities *in vitro*

of matrine and oxymatrine isolated from *S. subprostrata* and also give some biological effects of its superoxide radical scavenging activities. The findings provide some scientific supports for the traditional use of *S. subprostrata* for controlling inflammatory disease.

REFERENCES

1. Z.Y. Jiang, *Zhong Yao Tong Bao*, **7**, 29 (1982).
2. C.H. Cho, C.Y. Chuang and C.F. Chen, *Planta Med.*, **5**, 343 (1986).
3. R. Kojima, S. Fukushima, A. Ueno and Y. Saiki, *Chem. Pharm. Bull.*, **18**, 2555 (1970).
4. J. Kamei, P. Xiao, M. Ohsawa, H. Kubo, K. Higashiyama, H. Takahashi, L. Jiashi, H. Nagase and S. Ohmiya, *Eur. J. Pharmacol.*, **337**, 223 (1997).
5. S. Shibata and Y. Nishikawa, *Yakugaku Zasshi*, **81**, 167 (1962).
6. K. Kyogoku, K. Hatayama, K. Suzuki, S. Yokomori, K. Maejima and M. Komatsu, *Chem. Pharm. Bull.*, **21**, 1777 (1973).
7. S. Sakamoto, M. Kuroyanagi, A. Ueno and S. Sekita, *Phytochemistry*, **31**, 1339 (1992).
8. D. Qun, J. Yao and J.N. Fang, *Carbohydrate Polym.*, **54**, 13 (2003).
9. Y. Noreen, T. Ringbom, P. Perera, H. Danielson and L. Bohlin, *J. Nat. Prod.*, **61**, 2 (1998).
10. C.L. Kuo, C.W. Chi and T.Y. Liu, *Cancer Lett.*, **203**, 127 (2004).
11. Y. Cai, Q. Luo, M. Sun and H. Corke, *Life Sci.*, **74**, 2175 (2004).
12. T.B. Ng, F. Liu and Z.T. Wang, *Life Sci.*, **66**, 709 (2000).
13. P. Trouillas, C.A. Calliste, D.P. Allais, A. Simon, A. Marfak, C. Delage and J.L. Duroux, *Food Chem.*, **80**, 399 (2003).
14. D.C. Chen, Chinese Chemistry Standard (Manuals of Work), Medicinal Technology Press, Beijing: China 2000, pp. 112-142.
15. K. Saha, N.H. Lajis, D.A. Israf, A.S. Hamzah, S. Khozirah, S. Khamis and A. Syahida, *J. Ethnopharmacol.*, **92**, 263 (2004).
16. K.M. Lau, Z.D. He, H. Dong, K.P. Fung and P.P.H. But, *J. Ethnopharmacol.*, **83**, 63 (2002).
17. B.E. Wang, *J. Gastroenterol. Hepatol.*, **15**, 67 (2000).
18. P.F.L. Zheng, Niu, W.Z. Liu, S. Yao and L.G. Lu, *World J. Gastroenterol.*, **11**, 4912 (2005).
19. C.Y. Chuang, J.G. Xiao and G.C. Chiou, *J. Ocular Pharmacol.*, **3**, 129 (1987).
20. M. Yamazaki, *Yakugaku Zasshi*, **120**, 1025 (2000).
21. J.W. Wei, J.F. Liao, C.Y. Chuang, C.F. Chen and P.W. Han, *Life Sci.*, **9**, 215 (1985).
22. C.H. Hong, S.K. Hur, O.J. Oh, S.S. Kim, K.A. Nam and K.S. Lee, *J. Ethnopharmacol.*, **83**, 153 (2002).
23. J. Huang, M.J. Zhang and F.M. Qiu, *Zhongguo Zhong Xi Yi Jie He Za Zhi*, **25**, 240 (2005) (In Chinese).
24. S. Ohmiya, K. Saito and I. Muakoshi, *Yakugaku Zasshi*, **120**, 923 (2000).

(*Received*: 13 February 2008; *Accepted*: 5 September 2008) AJC-6825