Original Article

Anti-nociceptive and Anti-inflammatory Effect of an Ethanol Extract Mixture of *Vitis amurensis*, *Aralia cordata*, and *Glycyrrhizae* radix

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***Vitis amurensis**, **Aralia cordata**, and **Glycyrrhizae** radix have been widely used in Korea, China, and Japan because of their anti-oxidative and anti-inflammatory activities. The present study investigated the anti-nociceptive and anti-inflammatory properties of an ethanol extract (SSB) of a mixture of three medicinal plants of **Vitis amurensis** (stem and leaf), **Aralia cordata** (stem and leaf), and **Glycyrrhizae** radix. Anti-nociceptive activity was determined using chemical (acetic acid and formalin) and thermal (hot plate) stimuli-induced algesia tests. Formalin-induced paw edema was evaluated for anti-inflammatory activity. SSB (25–100 mg/kg, p.o.) and ibuprofen (100 mg/kg, p.o.), a positive non-steroidal anti-inflammatory drug (NSAID), significantly inhibited the acetic acid-induced writhing response caused by peripherally mediated algesia, but failed to protect thermal nociception in the hot plate test that was employed for centrally mediated analgesic activity. However, morphine (5 mg/kg, s.c.) used as a positive opioid control alleviated the acetic acid-induced writhing response and thermal nociception in the hot plate test. In the formalin test, SSB (50 and 100 mg/kg, p.o.) inhibited the second phase response (peripheral inflammatory algesia), but not the first phase response (central algesia), whereas morphine inhibited both phases of the pain response. Both SSB (25-100 mg/kg, p.o.) and ibuprofen (200 mg/kg) caused significant reduction of the formalin-induced increase of paw thickness, which was the index of inflammation. These results suggest that SSB has a significant anti-nociceptive activity that seems to be peripheral, but not central. SSB also displays anti-inflammatory activity in an acute inflammatory model. The present study supports a possible use of SSB to treat pain and inflammation.*

Key words: *Vitis amurensis, Aralia cordata, Glycyrrhizae* radix, anti-nociception, anti-inflammation

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Introduction

Tissue injury results in inflammatory nociception, and various inflammatory mediators such as prostaglandins (PGs), leukotriens, histamine, bradykinin, and platelet-activating factor are involved in initiating and sustaining nociception and the inflammatory cascade [1, 2]. The classical symptoms of acute inflammation are fever, redness, swelling, and pain caused by increased blood flow. In inflammatory responses, arachidonic acid is transformed into PGs via the cyclooxygenase (COX) pathway [3]. Symptoms of the inflammation process can be alleviated by non-steroidal anti-inflammatory drugs (NSAIDs), which mainly inhibit COX and reduce synthesis of PGs, and by corticosteroids, which prevent the formation of PGs by inhibition of phospholipase A_2 (responsible for arachidonic acid release) [4]. However, both steroidal and NSAID medications can have undesirable side effects including gastric erosions [5]. Therefore, there is currently great interest in natural compounds that have been used for centuries to reduce pain and inflammation with few side effects.

Vitis species from the family Vitaceae have traditionally been used world-wide as medicinal herbs. Grape and its bioactive constituents have various pharmacological effects, including antioxidant, antibacterial, anticancer, anti-inflammatory and antidiabetic activities, as well as hepatoprotective, cardioprotective and neuroprotective effects [6]. *Vitis amurensis* is one of the most common wild grapes in Korea, Japan, and China. Bioactive compounds such as Vam3, resveratrol, and procyanidins from its root, stem, and seeds were found to have anti-inflammatory properties in in vitro experimental models [7-9].

Aralia species (Araliaceae) have long been recognized as therapeutic herbs with antinociceptive, antidiabetic, antioxidant, and anti-inflammatory activities. The roots of *Aralia cordata* have been shown to protect inflamma-

tory arthritis in rabbits [10]. In a previous study, we found anti-nociceptive and anti-inflammatory effects of the leaves and stems of *Aralia cordata* [11]. Potential anti-inflammatory candidates such as diterpenes, triterpenes, saponins, sterols, and polyacetylenes were isolated from the roots, leaves and stems of *Aralia cordata* [12, 13].

Glycyrrhizae radix, the root of *Glycyrrhiza uralensis*, has beneficial effects in brains such as memory enhancing effects [14, 15] and protection of ischemic brain damage in animal models [16]. These pharmacological activities are because of its anti-inflammatory ingredients, which include glycyrrhetinic acid, glycyrrhizic acid, and liquiritigenin [17-19].

In previous studies, the leaves and stems of *Vitis amurensis*, the aerial part of *Aralia cordata*, and *Glycyrrhizae* radix showed neuroprotection against amyloid β protein-induced and ischemic brain damage because of their anti-oxidant and anti-apoptotic effects [15, 20-23]. Furthermore, preparations that combined these three plants (SSB), have been demonstrated to have neuroprotective effects against amyloid β protein-induced neurotoxicity in mice [11] and ischemia-induced rat brain damage [24] via a synergistic effect. Since inflammation is a part of the complex series of pathophysiological events that contribute to neurodegenerative diseases such as Alzheimer's disease and stroke, it was hypothesized that neuroprotection of the SSB may be associated with its anti-inflammatory activity. The present study was conducted to investigate the properties of anti-nociception as well as anti-inflammation of SSB by employing chemical and thermal pain stimuli and formalin-induced paw inflammation in mice.

Materials and Methods

Plant materials, extraction and reagents

The leaves and stems of *Vitis amurensis* and the aerial parts of *Aralia cordata* were collected at Keryong Mountain in Daejeon, Korea, from May to July and *Glycyrrhizae* radix (Korean) was purchased from Daegu Oriental Pharm Co., Daegu, Korea. These plants were identified by Dr. Kyung-Sik Song, Kyungpook National University. Two-hundred grams of each of the three plants was mixed, extracted with ethanol (3 L × 24 h × 3) at room temperature, filtered, and concentrated under reduced pressure using a rotary evaporator to yield an ethanol extract (SSB, 64 g), which was stored at room temperature until required. Ibuprofen was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and morphine was acquired from Myungmoon Pharm. Co. (Gyunggi-Do, Korea).

Experimental animals

Male ICR mice (5 weeks old) were supplied by Samtako, Inc. (Gyunggi-Do, Korea) and housed in an environmentally controlled room at $22 \pm 2°C$ under a relative humidity of $55 \pm 5\%$ and a 12 h light/dark cycle, while provided with food and water *ad libitum*. The procedures involving experimental animals complied with the Animal Care Guidelines of the National Institutes of Health and the Animal Ethics Committee of Chungbuk National University.

Measurement of anti-nociceptive activity using acetic acid-induced writhing test

Acetic acid (0.8%) was intraperitoneally injected into mice 20 min after the administration of vehicle (normal saline, p.o.), SSB (25, 50, and 100 mg/kg, p.o.), ibuprofen (100 mg/kg, p.o.), or morphine (5 mg/kg, s.c.). Each mouse was then placed in an individual clear plastic observation chamber, and the total number of writhes made by each mouse was counted between 10 and 40 min after acetic acid administration. The counts of writhing response in the control group were regarded as 100%. For scoring purposes, a writhe was indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb [25].

Measurement of anti-nociceptive activity using the hot plate test

The test was conducted using a Hot Plate Analgesia Meter (IITC Life Science, Woodland Hills, CA, USA). The hot plate temperature was kept constant at $55 \pm 1°C$. The mice were gently placed on the hot plate and jumping with all four paws was considered as nociception. Only mice showing a pretreatment reaction time ≤ 5 s were selected. The mice were only kept on the hot surface for 20 s (cut-off time) to avoid any thermal injury [26]. The reaction time of jumping was measured before and repeatedly at different times (15, 30, 45, 60 and 90 min) after administration of vehicle (normal saline, p.o.), SSB (25, 50, and 100 mg/kg, p.o.), ibuprofen (100 mg/kg, p.o.), or morphine (5 mg/kg, s.c.).

Measurement of anti-nociceptive activity using formalin-induced algesia

Mice were placed in an open Plexiglas observation chamber for 30 min to acclimate to their surroundings, after which they were gently restrained while 20 μL of 5% formalin solution in saline was administered subcutaneously to the dorsal surface of the right hind paw using a 30 gauge needle [27]. Following formalin injection, mice were immediately returned to the observation chamber for a 30 min observation period. The time in seconds spent licking the injected paw as the nociceptive response for both the first neurogenic phase (0–5 min) and second inflammatory phase (20–30 min) was record-

ed with a chronometer [28]. Mice were treated with SSB (25, 50, and 100 mg/kg, p.o.), vehicle (normal saline, p.o.), ibuprofen (200 mg/kg, p.o.), or morphine (5 mg/kg, s.c.) 30 min before formalin.

Measurement of anti-inflammatory activity in formalin-induced paw edema

The same formalin-injected mice were utilized for measurement of paw edema volume. The vertical paw thickness of the injected paw and the contralateral non-injected paw was measured at the metatarsal level as the index of paw edema using a fine caliper (Vernier caliper 530 series, Mitutoyo, Japan) 18 h after formalin injection. Paw edema was calculated by subtracting the paw thickness of the contralateral non-injected side from that of the formalin-injected side and expressed as mm thickness [29].

Statistical analysis

Data were expressed as the means ± S.E.M. and statistical significance was assessed by the one-way analysis of variance (ANOVA) followed by Tukey's test. All statistical analyses were performed using Prism GraphPad v.5.0 (La Jolla, CA, USA). P values of <0.05 were considered to be significant.

Results

Inhibitory effect of SSB on acetic acid-induced writhing response

SSB (25, 50, and 100 mg/kg) significantly reduced acetic acid-induced writhing response to 53.6 ± 7.9, 45.2 ±

7.1 and 27.0 ± 5.3%, respectively. Ibuprofen (100 mg/kg) reduced the acetic acid-induced writhes to 40.4 ± 7.0%, and morphine (5 mg/kg) completely inhibited them (Fig. 1).

Inhibitory effect of SSB on thermal stimuli-induced nociception in the hot plate test

As shown in Fig. 2, SSB and ibuprofen did not increase the latency period of pain induced by nociceptive stimuli by the hot plate. These results indicate that SSB as well as ibuprofen did not exert anti-nociceptive effects on thermal stimuli-induced algesia. In contrast, the opioid drug morphine (5 mg/kg) significantly prolonged the latency of escape from the hot plate at 15, 30, 45, 60, and 90 min after its administration, indicating anti-nociceptive activity.

Inhibitory effects of SSB on formalin-induced inflammatory nociception

SSB (50 and 100 mg/kg) and ibuprofen (200 mg/kg) significantly decreased formalin-induced licking time relative to the vehicle-control (99.0 ± 8.9 s) in the second inflammatory phase (20–30 min), with values of 69.5 ± 4.6, 38.9 ± 7.6 and 52.3 ± 10.9 s, respectively, being observed. However, neither SSB nor ibuprofen inhibited the first phase of neurogenic nociception. Meanwhile, morphine (5 mg/kg) significantly inhibited both the first neurogenic and the second inflammatory nociception induced by formalin injection (Fig. 3).

Inhibitory effect of SSB on formalin-induced paw edema

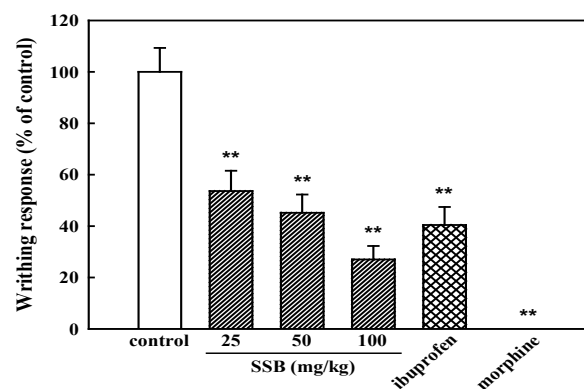Subcutaneous injection of formalin to the dorsal sur-

Fig. 1. Inhibitory effects of SSB on acetic acid-induced writhing response in mice. The number of writhes produced by 0.8% acetic acid was counted for 30 min. Stretching of the abdomen with simultaneous stretching of at least one hind limb was considered as a writhe. Results are expressed as means ± S.E.M (n= 13-19). *p<0.05, **p<0.01 vs. vehicle control.
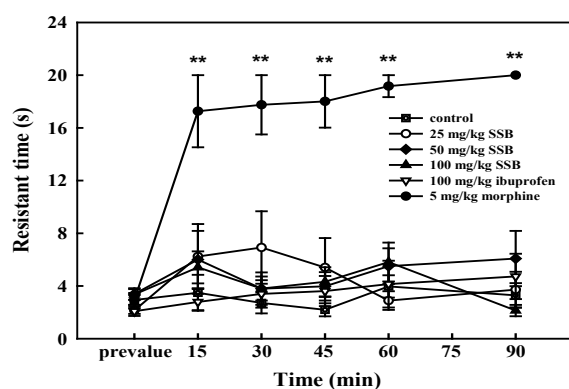

Fig. 2. Inhibitory effects of SSB on thermal pain in hot plate test in mice. Pre-values were determined before administration of vehicle, SSB, ibuprofen, and morphine, and post latencies were obtained at 15, 30, 45, 60, and 90 min after administration. Values are expressed as the means ± S.E.M. (n= 7–8). **p< 0.01 vs. vehicle control.

face of the paw elicits significant inflammation (e.g., paw edema) [29, 30]. To investigate whether the apparent attenuation of formalin-induced nociceptive behavior during the second phase reflected an inhibitory effect of SSB on the peripheral inflammation produced by formalin injection, the change in thickness of the hind paw edema was compared between vehicle-treated and SSB-administered mice. As shown in Fig. 4, SSB (25, 50, and 100 mg/kg) and ibuprofen (200 mg/kg) significantly inhibited formalin-induced paw edema, showing 0.48 ± 0.06, 0.43 ± 0.06, 0.37 ± 0.07, and 0.36 ± 0.06 mm paw thickness, respectively, when compared with the control group (0.72 ± 0.03 mm, thickness).

Discussion

The acetic acid-induced writhing test is a typical model for measurement of inflammatory pain that is commonly used to screen new agents with peripheral anti-nociceptive and anti-inflammatory properties [31]. Intraperitoneally injected acetic acid causes tissue damage and triggers the liberation of a variety of mediators such as bradykinin and PGs, as well as some cytokines such as TNF-α, IL-1β, and IL-8 [32, 33]. PGs are generated during metabolism of arachidonic acid by the COX pathway and activate chemosensitive nociceptors that contribute to the development of inflammatory pain [3]. Thus, NSAIDs such as ibuprofen or indomethacin, COX inhibitors, are commonly used as analgesics and anti-inflammatory agents [2]. SSB as well as ibuprofen used as a reference NSAID significantly inhibited the acetic acid-induced writhing response, suggesting a mechanism involved in

the anti-nociceptive effect by the inhibition of mediator release of the inflammatory process in mice.

The hot plate test using thermal stimuli is employed to investigate the anti-nociceptive activity mediated by central mechanisms observed in opioid-like analgesics, while peripheral compounds are inactive in this kind of painful stimulus [34]. The involvement of endogenous substances such as PGs may be minimized in this model [35]. The hot plate test was performed to determine whether the activity of the SSB on the acetic acid-induced writhing test was associated with central anti-nociceptive activity or not. While morphine increased the escape latency from the hot plate, both SSB and ibuprofen failed to increase the escape latency. This result supported that anti-nociceptive activity of SSB in the acetic acid-induced writhing test may be related to a peripheral mechanism.

Unlike traditional reflex tests of nociception such as tail-flick and hot-plate, pain produced by the hind paw injection of formalin results from persistent tissue damage and thus more closely resembles clinical pain conditions [29]. Subcutaneous injection of formalin into the back of the hind paw produces a distinct biphasic response of the first phase, which begins at the time of injection and lasts for about 10 min, and a second phase, which starts at 10 min postinjection and lasts for about 50 min. The first phase is considered to be a direct result of stimulation of nociceptors in the paw that reflects centrally mediated pain. The second phase is pain due to inflammation with a release of serotonin, histamine, bradykinin, and PGs. As a result, different analgesics may act differently in the first and second phases of this test; therefore, this test can be used to clarify the possible mechanisms of an anti-
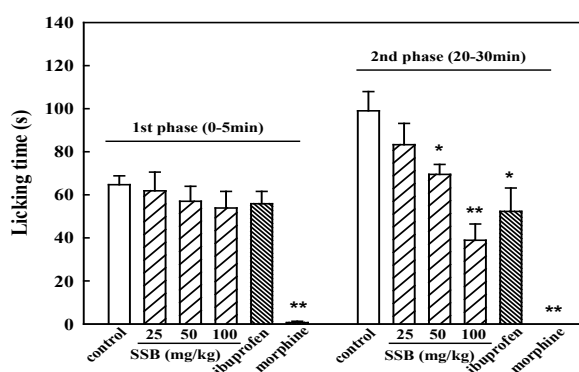

Fig. 3. Inhibitory effects of SSB on formalin-induced nociceptive response in mice. Licking time of the injected paw at 0–5 min (first phase) and 20–30 min (second phase) after formalin injection was measured in seconds. Results are expressed as the means ± S.E.M (n= 4-10). *p<0.05, **p<0.01 vs. vehicle control.
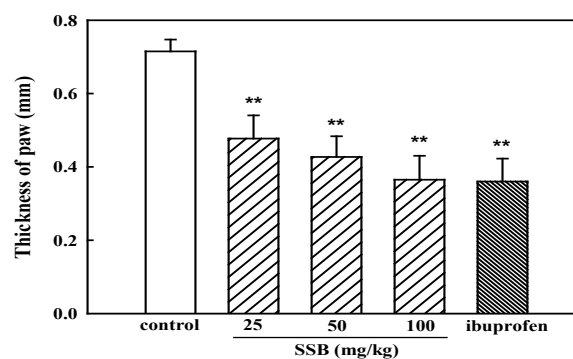

Fig. 4. Inhibitory effects of SSB on formalin-induced paw edema in mice. Vertical thickness of the injected paw at the metatarsal level was measured using a caliper. The basal value of vertical thickness of the paw was measured before injection, and then formalin-induced edema was measured 18 h after the injection. The change in paw thickness was calculated by subtracting the pre-thickness from the post-thickness of the injected paw and considered paw edema. Results are expressed as the mean ± S.E.M (n= 5-8). *p<0.05, **p<0.01 vs. vehicle control.

nociceptive effect of a test compound [36, 37]. Centrally acting drugs such as opioids inhibit both phases equally, but peripherally acting drugs such as NSAIDs and corticosteroids only inhibit the second phase [37, 38]. In the present study, the first and the second phase responses were observed 0–5 min and 20–30 min after formalin injection, respectively. Pretreatment orally with SSB (50 and 100 mg/kg) showed a significant inhibitory effect on the licking activity in the second phase, but not in the first phase. Similarly, ibuprofen produced a marked inhibition of the inflammatory nociception of the second phase only. However, morphine inhibited both phases of the formalin test. These results suggested that the anti-nociceptive effect of the SSB was specific to the inflammatory pain, possibly through inhibition of PGs synthesis, but not to the neurogenic pain.

Paw injection of formalin in rodents causes local tissue injury of the paw, which results in inflammatory pain and edema [37]. Its main characteristics are the exudation of fluid and plasma proteins and the emigration of leukocytes, predominantly neutrophils, caused by vasoactive biogenic amines such as bradykinin, histamine, serotonin and PGs [30]. SSB as well as ibuprofen showed significant inhibition of formalin-induced paw edema, further supporting that SSB could inhibit peripheral inflammatory pain in the present study.

In previous reports, we demonstrated an anti-nociceptive and anti-inflammatory effect and inhibitory effect on COX-1 and COX-2 of the leaves and stems of *Aralia cordata* and isolated diterpenes, triterpenes, and a saponin as active constituents of the pharmacological activity [11, 13]. Studies demonstrated a variety of anti-inflammatory components from *Vitis amurensis* and *Glycyrrhizae* radix, suggesting anti-inflammatory effect of these plants [8, 9, 17, 19]. However, no previous studies have investigated the anti-nociceptive and anti-inflammatory effects of the leaves and stems of *Vitis amurensis* and *Glycyrrhizae* radix. Mice treated with 50 mg/kg SSB showed 45.2 ± 7.1% of the writhes observed in control mice, whereas mice treated with 200 mg/kg *Vitis amurensis* and *Glycyrrhizae* radix showed 49.9 ± 19.4% and 59.1 ± 14.9%, respectively, (data not shown). In a previous study, the leaves and stems of *Aralia cordata* at 200 mg/kg resulted in 54.7 ± 5.0% of the writhes observed in a control [11]. Therefore, the results of the current study indicate that the three medicinal plants acted synergistically to produce anti-nociceptive and anti-inflammatory activity. In conclusion, these results suggest that SSB exerted anti-nociceptive and anti-inflammatory effects. The anti-nociceptive effects of SSB may be mediated through its anti-inflammatory action through inhibition of inflammatory mediators such as PGs. Overall, this study supports a potential use of SSB as a therapeutic agent for the alleviation of inflammation and inflammatory pain.

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References

1. Maslinska D, Gajewski M. Some aspects of the inflammatory process. Folia Neuropathol 1998;36: 199-204.

2. Vane J, Botting R. Inflammation and the mechanism of action of anti-inflammatory drugs. FASEB J 1987; 1:89-96.

3. Scott KF, Bryant KJ, Bidgood MJ. Functional coupling and differential regulation of the phospholipase A2-cyclooxygenase pathways in inflammation. J Leukoc Biol 1999;66:535-541.

4. Higgs GA. Arachidonic acid metabolism, pain and hyperalgesia: the mode of action of non-steroid mild analgesics. Br J Clin Pharmacol 1980;10 Suppl 2: 233S-235S.

5. Takeuchi K. Pathogenesis of NSAID-induced gastric damage: importance of cyclooxygenase inhibition and gastric hypermotility. World J Gastroenterol 2012;18:2147-2160.

6. Nassiri-Asl M, Hosseinzadeh H. Review of the Pharmacological Effects of Vitis vinifera (Grape) and its Bioactive Constituents: An Update. Phytother Res 2016;30:1392-1403.

7. Bak MJ, Truong VL, Kang HS, Jun M, Jeong WS. Anti-inflammatory effect of procyanidins from wild grape (Vitis amurensis) seeds in LPS-induced RAW 264.7 cells. Oxid Med Cell Longev 2013;2013: 409321.

8. Huang KS, Lin M, Cheng GF. Anti-inflammatory tetramers of resveratrol from the roots of Vitis amurensis and the conformations of the seven-membered ring in some oligostilbenes. Phytochemistry 2001; 58:357-362.

9. Xuan L, Jiang R, Wu Z, Yi H, Yao C, Hou Q, Qu C. Vam3, a Compound Derived from Vitis amurensis Rupr., Attenuated Colitis-Related Tumorigenesis by Inhibiting NF-kappaB Signaling Pathway. Front Pharmacol 2016;7:311.

10. Park DS, Huh JE, Baek YH. Therapeutic effect of Aralia cordata extracts on cartilage protection in collagenase-induced inflammatory arthritis rabbit model. J Ethnopharmacol 2009;125:207-217.

11. Jang JY, Seong YH. Anti-nociceptive and anti-inflammatory effect of an ethanol extract of the leaf and stem of Aralia cordata. Natural Product Sciences 2014;20:

301-305.

12. Dang NH, Zhang X, Zheng M, Son KH, Chang HW, Kim HP, Bae K, Kang SS. Inhibitory constituents against cyclooxygenases from Aralia cordata Thunb. Arch Pharm Res 2005;28:28-33.

13. Lee IS, Jin W, Zhang X, Hung TM, Song KS, Seong YH, Bae K. Cytotoxic and COX-2 inhibitory constituents from the aerial parts of Aralia cordata. Arch Pharm Res 2006;29:548-555.

14. Dhingra D, Parle M, Kulkarni SK. Memory enhancing activity of Glycyrrhiza glabra in mice. J Ethnopharmacol 2004;91:361-365.

15. Lee HK, Yang EJ, Kim JY, Song KS, Seong YH. Inhibitory effects of Glycyrrhizae radix and its active component, isoliquiritigenin, on Abeta(25-35)-induced neurotoxicity in cultured rat cortical neurons. Arch Pharm Res 2012;35:897-904.

16. Hwang IK, Lim SS, Choi KH, Yoo KY, Shin HK, Kim EJ, Yoon-Park JH, Kang TC, Kim YS, Kwon DY, Kim DW, Moon WK, Won MH. Neuroprotective effects of roasted licorice, not raw form, on neuronal injury in gerbil hippocampus after transient forebrain ischemia. Acta Pharmacol Sin 2006;27:959-965.

17. Chang YL, Chen CL, Kuo CL, Chen BC, You JS. Glycyrrhetinic acid inhibits ICAM-1 expression via blocking JNK and NF-kappaB pathways in TNF-alpha-activated endothelial cells. Acta Pharmacol Sin 2010;31: 546-553.

18. Kim YW, Zhao RJ, Park SJ, Lee JR, Cho IJ, Yang CH, Kim SG, Kim SC. Anti-inflammatory effects of liquiritigenin as a consequence of the inhibition of NF-kappaB-dependent iNOS and proinflammatory cytokines production. Br J Pharmacol 2008;154:165-173.

19. Wang W, Luo M, Fu Y, Wang S, Efferth T, Zu Y. Glycyrrhizic acid nanoparticles inhibit LPS-induced inflammatory mediators in 264.7 mouse macrophages compared with unprocessed glycyrrhizic acid. Int J Nanomedicine 2013;8:1377-1383.

20. Cho SO, Ban JY, Kim JY, Ju HS, Lee IS, Song KS, Bae K, Seong YH. Anti-ischemic activities of aralia cordata and its active component, oleanolic acid. Arch Pharm Res 2009;32:923-932.

21. Jeong HY, Kim JY, Lee HK, Ha do T, Song KS, Bae K, Seong YH. Leaf and stem of Vitis amurensis and its active components protect against amyloid beta protein (25-35)-induced neurotoxicity. Arch Pharm Res 2010; 33:1655-1664.

22. Cho SO, Ban JY, Kim JY, Jeong HY, Lee IS, Song KS, Bae K, Seong YH. Aralia cordata protects against amyloid beta protein (25-35)-induced neurotoxicity in cultured neurons and has antidementia activities in mice. J Pharmacol Sci 2009;111:22-32.

23. Kim JY, Jeong HY, Lee HK, Kim S, Hwang BY, Bae K, Seong YH. Neuroprotection of the leaf and stem of Vitis amurensis and their active compounds against ischemic brain damage in rats and excitotoxicity in cultured neurons. Phytomedicine 2012;19:150-159.

24. Choo HR, Jang JY, Song KS, Seong YH. Protective effect of an ethanol extract mixture of Vitis amurensis, Aralia cordata, and Glycyrrhizae radix against ischemia-induced brain injury In rats and excitotoxicity in cultured neurons. Journal of Biomedical and Translational Research 2016;17:13-19.

25. Collier HO, Dinneen LC, Johnson CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. British Jorunal of Pharmacology and Chemotherapeutics 1968;32:295-310.

26. Eddy NB, Leimbach D. Synthetic analgesics. II. Dithienylbutenyl- and dithienylbutylamines. J Pharmacol Exp Ther 1953;107:385-393.

27. Capone F, Aloisi AM. Refinement of pain evaluation techniques. The formalin test. Ann Ist Super Sanita 2004;40:223-229.

28. Udobang JA, Nwafor PA, Okokon JE. Analgesic and antimalarial activities of crude leaf extract and fractions of Acalypha wilkensiana. J Ethnopharmacol 2010;127: 373-378.

29. Fu KY, Light AR, Maixner W. Long-lasting inflammation and long-term hyperalgesia after subcutaneous formalin injection into the rat hindpaw. J Pain 2001;2: 2-11.

30. Suleyman H, Buyukokuroglu ME. The effects of newly synthesized pyrazole derivatives on formaldehyde-, carrageenan-, and dextran-induced acute paw edema in rats. Biol Pharm Bull 2001;24:1133-1136.

31. Collier HO, Dinneen LC, Johnson CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br J Pharmacol Chemother 1968;32:295-310.

32. Ikeda Y, Ueno A, Naraba H, Oh-ishi S. Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice. Life Sci 2001;69: 2911-2919.

33. Ribeiro RA, Vale ML, Thomazzi SM, Paschoalato AB, Poole S, Ferreira SH, Cunha FQ. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. Eur J Pharmacol 2000;387:111-118.

34. Connor J, Makonnen E, Rostom A. Comparison of analgesic effects of khat (Catha edulis Forsk) extract, D-amphetamine and ibuprofen in mice. J Pharm Pharmacol 2000;52:107-110.

35. Bachhav RS, Gulecha VS, Upasani CD. Analgesic and anti-inflammatory activity of Argyreia speciosa root. Indian J Pharmacol 2009;41:158-161.

36. Hunskaar S, Fasmer OB, Hole K. Formalin test in mice,

a useful technique for evaluating mild analgesics. J Neurosci Methods 1985;14:69-76.

37. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987;30:103-114.

38. Sevostianova N, Zvartau E, Bespalov A, Danysz W. Effects of morphine on formalin-induced nociception in rats. Eur J Pharmacol 2003;462:109-113.