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Antinociceptive Effects of an Ethyl Acetate Soluble Fraction of *Spirodela polyrrhiza*

Sung Hoon Ko, Jin Kyu Kim, Jong Soo Kim, Jae Hyuk Lee[1], Sang-Youel Park[2] and Hoon Jeon*

College of Pharmacy, Woosuk University, Chonbuk 565-701, Korea
[1]Department of Oriental Pharmaceutical Development, Nambu University, Gwangju 506-706, Republic of Korea
[2]Korea Zoonoses Research Institute, College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Korea

Abstract – *Spirodela polyrrhiza* L. Schleid. (Lemnaceae), also known as 'duckweed', is a traditional medicine in Korea. The whole plant is used to treat many diseases, including the common cold, edema, acute nephritis, and urticaria. The present study investigated antinociceptive properties of the EtOAc soluble fraction of *S. polyrrhiza* (ESP). The antinociceptive activities of ESP were studied using experimental models of pain, including thermal nociception methods, such as the tail immersion test and the hotplate test. Moreover, we studied chemical nociception induced by intraperitoneal acetic acid and subplantar formalin in mice. ESP exhibited dose-dependent antinociceptive activity in both thermal and chemical pain models. In a drug combination test using the opioid receptor antagonist naloxone, diminished analgesic activities of ESP were observed, indicating that the antinociceptive activity of ESP is mediated by opioid receptors.
Keywords – *Spirodela polyrrhiza*, analgesic, antinociceptive, opioid receptor

Introduction

Despite recent advances in the development of antinociceptive therapies, there is still a need for effective painkillers. In this regard, new drugs originating from natural products have received much attention due to their high efficiency and low toxicity. In addition, many plant-derived compounds exhibit antinociceptive activities (Calixto *et al.*, 2000).

Spirodela polyrrhiza L. Schleid. (Lemnaceae), also called duckweed, is an aquatic plant that is distributed throughout Korea and China. The entire plant of *S. polyrrhiza* is an oriental drug used therapeutically to treat many diseases, including the common cold, edema, acute nephritis and urticaria. Previous phytochemical studies of this plant have shown the presence of sterol, anthocyanin and flavonoids such as vitexin, orientin, and cynaroside (Wallace 1975; Suh and Shin, 1969; Harborne, 1986). Pharmacological studies have indicated that *S. polyrrhiza* has anticoagulant (Choi and Sa, 2001), gastroprotective (Khasina *et al.*, 2003), immunomodulatory (Ovodova *et al.*, 2000), anti-inflammatory (Jeon, 2010) effects and inhibits immediate hypersensitivity (Kim and Ko, 2004).

However, there are no scientific reports on the antinociceptive properties of *S. polyrrhiza*. Thus, in this study, we evaluated the antinociceptive properties of ESP using various experimental pain models including thermal nociception and chemical nociception. We also did combination tests using naloxone to investigate possible involvement of opioid receptors.

Experimental

Plant materials – Plant materials were purchased from Wansanyakupsa (Jeonju, South Korea) in April 2007. A voucher specimen (WME048) was deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University.

Extraction and solvent fractionation – An extract was obtained twice from a dried sample of *S. polyrrhiza* (600 g) using 1.2 L of MeOH under ultrasonication for 2 h. The solvent was evaporated and the residue was then subjected to successive solvent partitioning to give n-hexane, CH_2Cl_2, EtOAc and *n*-BuOH soluble fractions. Each fraction was lyophilized and then stored at –20 °C until use. Preliminary experiments showed that among the above four fractions of *S. polyrrhiza*, the ethyl acetate fraction (ESP) had the most potent pharmacological potential, and therefore, further studies were conducted using ESP.

Animals – ICR mice (six-week-old males and females) weighing 20 - 22 g were supplied by Damul Science

*Author for correspondence
Tel: +82-63-290-1577; E-mail: hoonj6343@hanmail.net

(Dajeon, Korea). All animals were housed at 22 ± 1 ºC with a 12 h light/dark cycle and fed a standard pellet diet with tap water *ad libitum*. The experimental protocols complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

Grouping and drug administration – Animals were randomly assigned into groups, each consisting of eight or ten mice for analgesic testing. Negative controls were treated with the same volume of distilled water that was used for reconstituting the drug. Positive controls were treated with standard drugs: tramadol (i.p.) or indomethacin (p.o.). Treatment groups in each test were treated orally with different doses of ESP.

Acute toxicity test – To evaluate the possible toxicity of ESP, an acute toxicity test was carried out. Mice (n = 6) were tested by administering different doses of ESP and then increasing or decreasing the doses according to their responses (Bruce, 1985). The control group received equal volumes of distilled water. All groups were observed for a 24 h period for gross effects and mortality.

Tail immersion test – In the present study, the tail immersion test was performed according to the procedures used by Wang *et al*. (2000), with only minor modifications. Briefly, the lower two-thirds of a mouse's tail was immersed in a water bath set at 50 ± 0.2 ºC. The reaction time, i.e. the amount of time it takes the animal to withdraw its tail, was measured at 0, 30, 60, 90 and 120 min after the administration of ESP (250, 500 mg/kg; p.o.), tramadol (10 mg/kg; i.p.) and vehicle (distilled water). To avoid tissue injury, the cut-off time for the immersion was 20 s.

Hot plate test – Hot plate tests (Franzotti *et al*., 2000) were carried out on groups of male and female mice using a hot plate apparatus (JD-A-10A, Jungdo BNP, Korea), maintained at 55 ± 1 ºC. Only mice that showed initial nociceptive responses (licking of the forepaws or jumping) between 7 - 15 s were used for additional experiments. The chosen mice were pre-treated with ESP (250, 500 mg/kg; p.o.) or vehicle (distilled water), and measurements were taken at 0, 30, 60, 90 and 120 min after treatment. A tramadol (10 mg/kg; i.p.) treated animal group was included as a positive control. The cut-off time was set at 30 s to minimize tissue damage. The reaction time was calculated as described for the tail immersion test.

Acetic acid-induced writhing test – Antinociceptive activity of ESP was detected as previously described (Olajide *et al.*, 2000). The response to an intraperitoneal injection of an acetic acid solution (1% in 0.9% saline), which consisted of abdominal constrictions and hind limb stretching, was measured for each mouse starting 5 min after the acetic acid injection and continuing for 15 min. Each experimental group was treated orally with vehicle (distilled water), ESP (250, 500 mg/kg) or indomethacin (10 mg/kg) 1 h prior to the acetic acid injection.

Formalin test – In the formalin test (Santos and Calixto, 1997), groups of mice were treated orally with vehicle (distilled water) or ESP (250, 500 mg/kg). After 60 min, each mouse was given 20 μL of 5% formalin (in 0.9% saline, subplantar injection) into the right hind-paw. The duration of paw licking (measured in seconds) was used as an index of painful responses during the 0 - 5 min period (first phase, neurogenic) and the 20 - 35 min period (second phase, inflammatory) after formalin injection. Tramadol and indomethacin were used as positive controls and were administered 30 min before the test at a dose of 10 mg/kg, i.p. and p.o., respectively. To investigate the possible involvement of endogenous opioids in the anti-nociceptive activity of ESP, animals from each group were pretreated with naloxone (5 mg/kg; i.p.) 15 min prior to drug administration.

Statistical analysis –Results are expressed as the mean ± S.D. or mean ± S.E.M. depending on the experiment. Differences between groups were analyzed by Student's unpaired, two-tailed *t*-test, and p-values less than 0.01 were considered significant.

Results and Discussion

In this study we evaluated antinociceptive activity of the ethylacetate fraction of *S. polyrrhiza* (ESP). ESP showed strong analgesic activity against both central and peripheral nociception in thermal and chemical nociception tests in mice. To test for possible toxicity of ESP to animals, 2000 mg/kg of ESP was administered to mice. The treated mice did not exhibit any behavioral alteration, convulsion or death during the period of assessment (24 h). This result suggests that ESP is safe up to an oral dose of 2000 mg/kg of body weight.

Tail immersion and hotplate tests were used as thermal nociception models for the evaluation of central anti-nociceptive activity. In the tail immersion test, ESP caused a dose-dependent increase in the tail flick anti-nociceptive index (Fig. 1). At the 250 mg/kg dose of ESP, treated animals reached maximum anti-nociceptive activity 30 min after oral administration (23.48% increase in latency time). Pre-treatment with high concentrations of ESP (500 mg/kg) significantly delayed the reaction (increased the reaction time) to nociceptive stimuli 60 min after oral administration (48.31%, $p < 0.01$). Tramadol

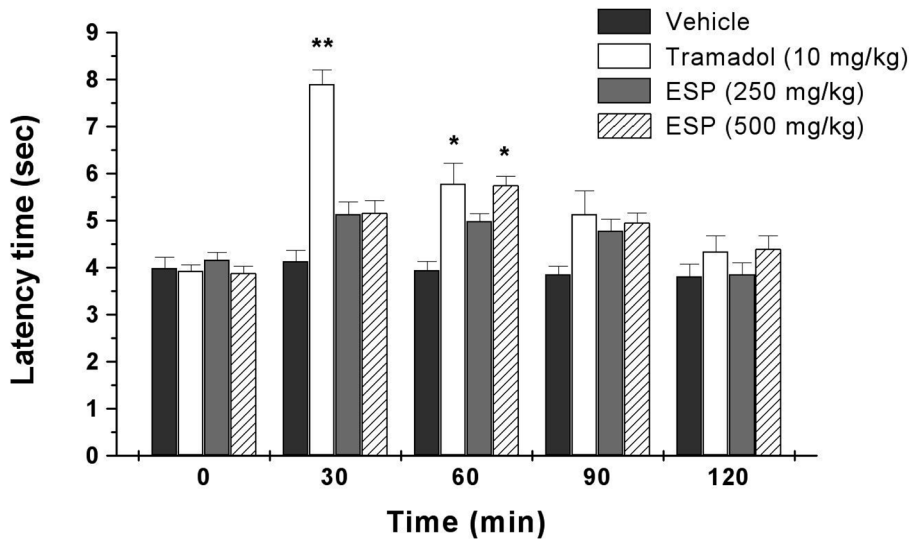

Fig. 1. The effect of ESP on nociceptive responses during the tail immersion test. Values expressed as mean ± S.E.M. and units are in seconds (n = 8 - 10). Differences between groups were statistically analyzed by the Student's *t*-test. *$p < 0.01$ and **$p < 0.001$ compared to the vehicle-treated group.

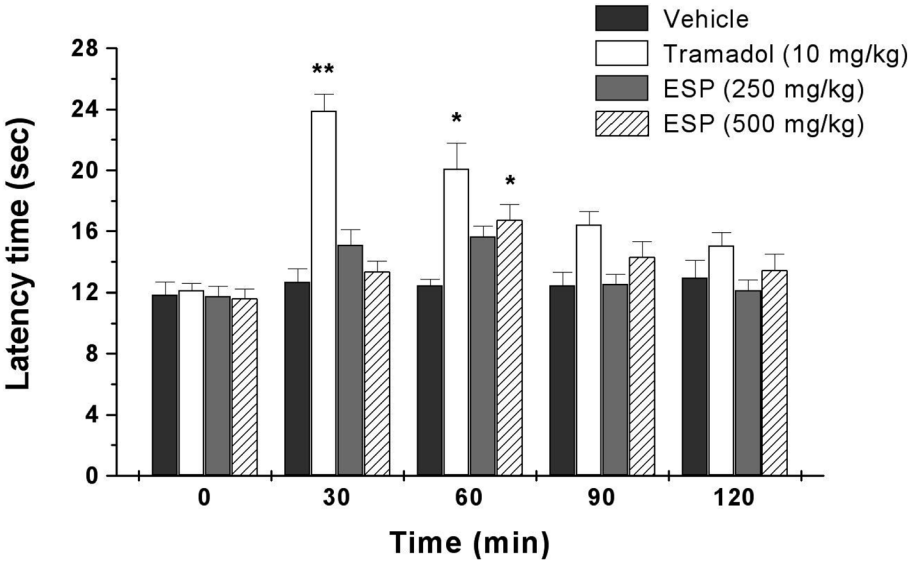

Fig. 2. The effect of ESP on nociceptive responses during the hotplate test. Values expressed as mean ± S.E.M. and units are in seconds (n = 11 - 13). Differences between groups were statistically analyzed by the Student's *t*-test. *$p < 0.01$ and **$p < 0.001$ compared to a vehicle-treated group.

(10 mg/kg), the reference drug, also exhibited powerful antinociceptive activity that was recorded 30 min after drug treatment (100.92%, $p < 0.001$). However, there was no significant difference in time latencies between tramadol and vehicle-treated groups. Similar results was also found for the hotplate test. As shown in Fig. 2, ESP (250 and 500 mg/kg) reached a maximum analgesia of, respectively, 33.02% and 43.78% at 60 min. Tramadol, the reference standard, also caused significant antinociception (96.84%, $p < 0.001$). ESP showed analgesic activities in both the tail immersion and the hotplate tests, implicating both spinal and supraspinal analgesic pathways. In both tests, tramadol exhibited rapid effects with a maximum peak similar to that of an opioid agonist (e.g., morphine). In contrast, ESP reached its highest analgesia level 60 min after drug administration. This difference in the maximum analgesic point could be explained by the methods of drug administration (i.p or

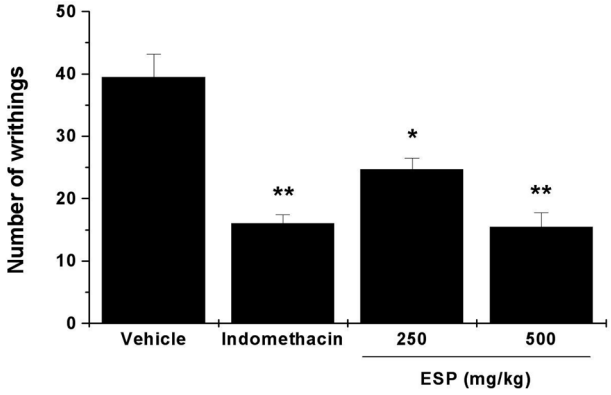

Fig. 3. The effect of ESP on nociceptive responses during the acetic acid-induced writhing test. Values are expressed as mean ± S.E.M. (n = 8). Differences between groups were statistically analyzed by the Student's *t*-test. *$p < 0.01$ and **$p < 0.001$ compared to the vehicle-treated group.

p.o.) or metabolic rate of each drug.

The effects of ESP on peripheral nociception was determined using the acetic acid-induced writhing model, which is frequently used to estimate both central and peripheral analgesic effects of drugs (Fukawa *et al.*, 1980). Intraperitoneal injection of 1% acetic acid into mice caused an average of 39.42 ± 3.74 writhings, while mice treated with ESP showed a significant decrease in the mean number of writhings (Fig. 3). The data showed that the antinociceptive activity of ESP was dose-dependent with a 37.43% ($p < 0.01$) reduction of abdominal constriction observed for the 250 mg/kg dose and 60.82% ($p < 0.001$) seen for the 500 mg/kg dose. The reference drug, Indomethacin (10 mg/kg), caused 59.42% ($p < 0.001$) inhibition, which is slightly lower than that of the higher concentrations of ESP. The acetic acid-induced writhing test has been associated with increased levels of prostaglandins (PGs), especially PGE_2, in peritoneal fluids (Derardt *et al.*, 1980). PGs induce abdominal constrictions via the activation and sensitization of peripheral chemosensitive nociceptors (Dirig *et al.*, 1998), which are largely associated with the development of inflammatory pain (Bley *et al.*, 1998). Therefore, one of the possible analgesic mechanisms of action that could explain the results of this test may include the inhibition of the COX enzyme. Non-steroidal anti-inflammatory drugs exert their peripheral analgesic effects as a consequence of the inhibition of PG synthesis, and in the present study indomethacin produced a significant decrease in writhing responses. ESP also caused potent inhibition of acetic acid-induced abdominal constrictions and this antinociceptive action could be explained by its inhibition of COX-2 expression, as previously reported (Jeon, 2010).

Because the acetic acid-induced writhing test can not distinguish between central and peripheral antinociceptive activity, a formalin test was conducted. The subcutaneous injection of formalin, as a peripheral noxious stimulus, causes a biphasic nociceptive response involving two different mechanisms (Hunskaar *et al.*, 1985). The first phase (neurogenic pain) is caused by the direct chemical stimulation of nociceptive afferent fibers, predominantly C fibers, which can be suppressed by opiates such as morphine (Amaral *et al.*, 2007). The second phase (inflammatory pain) results from the action of inflammatory mediators such as prostaglandins, serotonin, histamine and bradykinin in peripheral tissues (Hunskaar and Hole, 1987), and of functional changes in the spinal dorsal horn (Dalal *et al.*, 1999). As shown in Fig. 4, the reference drug, Tramadol, significantly blocked the pain of the formalin-response during both phases (first-phase, 48.16%; second-phase, 77.47%). Indomethacin was significantly effective (50.71%) only during the second phase. These results agree with many reports that suggest that the drugs that primarily act on the central nervous system cause inhibition equally in both phases, while peripherally acting drugs, such as steroids and NSAIDs, mostly cause slight inhibition of pain during the early phase of the formalin test (Vontagu et al., 2004; Trongsakul *et al.*, 2003). In this test, ESP-treated mice demonstrated antinociceptive activities during both the early phase (29.00 and 35.88%) and the late phase (26.68% and 38.16%) of the pain response, at the tested doses (250 and 500 mg/kg, respectively) compared to the vehicle group. This suggests the inhibition of both neurogenic and inflammatory nociception. This test provides further confirmation of the central effect of ESP suggested by the tail-flick and hotplate tests. Furthermore, ESP also caused dose-dependent peripheral analgesia , similar to the results of the acetic acid test. Moreover, when the animals were pre-treated with naloxone, a non-selective opioid receptor antagonist, the antinociceptive action of tramadol was reduced substantially. Naloxone pretreatment did not change the licking time for the group treated with indomethacin. Interestingly, naloxone pretreatment antagonized ESP (500 mg/kg) induced antinociception activity during both first (–30.20%) and second phases (–12.17%). Therefore, it is clear that the central and peripheral antinociceptive action of ESP involves, at least in part, the opioid system.

In summary, the present results showed that ESP exhibited potent anti-nociceptive activities both centrally and peripherally by acting as a partial opioid receptor

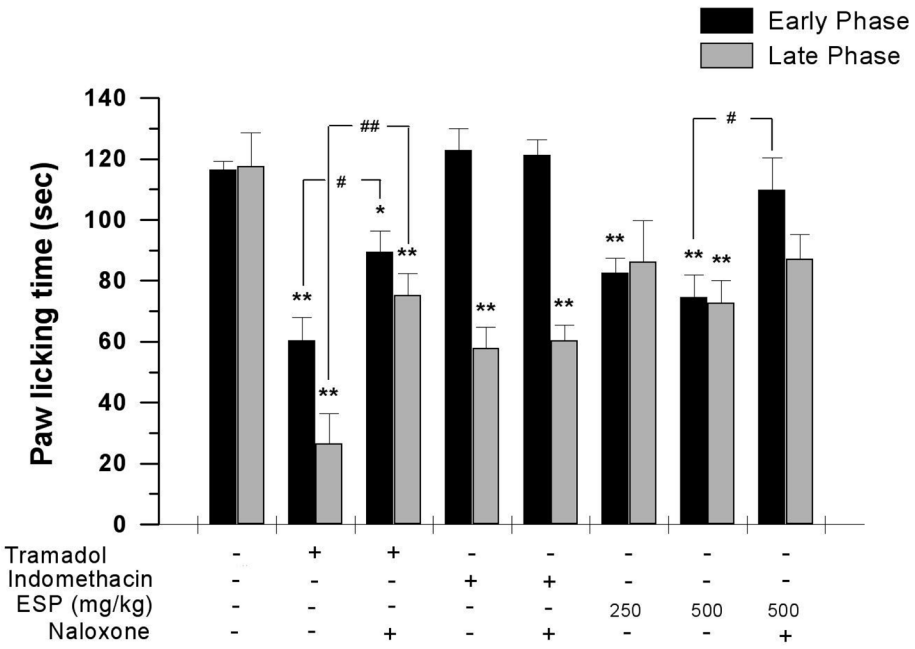

Fig. 4. The effect of ESP on nociceptive responses in the formalin test. Values are expressed as mean ± S.E.M. (n = 8 - 10). Naloxone (5 mg/kg) was given 15 min prior to drug administration. Differences between groups were statistically analyzed by Student's *t*-test. $^{*}p < 0.01$ and $^{**}p < 0.001$ are for comparisons to the vehicle-treated group. $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ are for comparisons to the naloxone-untreated ESP group.

agonist. Based on these findings, ESP holds great promise for use in many medical situations as an effective analgesic agent.

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References

Amaral, J.F., Silva, M.I.G., Neto, M.R.A., Neto, P.F.T., Moura, B.A., Melo, C.T.V., Araújo, F.L.O., DeSousa, D.P., Vasconcelos, P.F., Vasconcelos, S.M., and Sousa, F.C.F., Antinociceptive effect of the monoterpene R-(−)-limoneneinmice. *Biol. Pharm. Bull*. **30**, 1217-1220 (2007).

Bley, K.R., Hunter, J.C., Eglen, R.M., and Smith, J.A., The role of IP prostanoid receptors in inflammatory pain. *Trends Pharmacol. Sci*. **19**, 141-147 (1998).

Bruce, R.D., An up-and-down procedure for acute toxicity testing. *Fundam. Appl. Toxicol.* **5**, 151-157 (1985).

Calixto, J.B., Beirith, A., Ferreira, J., Santos, A.R., Filho, V.C., and Yunes, R.A., Naturally occurring antinociceptive substances from plants. *Phytother. Res*. **14**, 401-418 (2000).

Choi, H.S. and Sa, Y.S., Fibrinolytic and antithrombotic protease from Spirodela polyrrhiza. *Biosci. Biotechnol. Biochem*. **65**, 781-786 (2001).

Dalal, A., Tata, M., Allegre, G., Gekiere, F., Bons, N., and Albe-Fessard, D., Spontaneous activity of rat dorsal horn cells in spinal segments of sciatic projection following transections of sciatic nerve or of corresponding dorsal roots. *Neuroscience* **94**, 217-228 (1999).

Derardt, R., Jougney, S., Delevalcee, F., and Falhout, M., Release of prostaglandins E and F in an algogenic reaction and its inhibition. *Eur. J. Pharmacol*. **51**, 17-24 (1980).

Dirig, D.M., Isakson, P.C., and Yaksh, T.L., Effect of COX-1 and COX-2 inhibition on induction and maintenance of carrageenan-evoked thermal hyperalgesia in rats. J *Pharmacol. Exp. Ther*. **285**, 1031-1038 (1998).

Franzotti, E.M., Santos, C.V., Rodrigues, H.M., Mourao, R.H., Andrade, M.R., and Antoniolli, A.R., Anti-inflammatory, analgesic activity and acute toxicity of Sida cordifolia L. (Malva-branca). *J. Ethnopharmacol*. **72**, 273-277 (2000).

Fukawa, K., Kawano, O., Hibi, M., Misaki, N., Ohba, S., and Hatanaka, Y., A method for evaluating analgesic agents in rats. *J. Pharmacol. Methods* **4**, 251-259 (1980).

Harborne, J.B., The natural distribution in angiosperms of anthocyanins acylated with aliphatic dicarboxylic acids. *Phytochemistry* **25**, 1887-1894 (1986).

Hunskaar, S., Fasmer, O.B., and Hole, K., Formalin test in mice, a useful technique for evaluating mild analgesics. *J. Neurosci. Methods* **14**, 69-76 (1985).

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

Jeon, H., Anti-inflammatory and Radical scavenging Effects of *Spirodela polyrrhiza*. *Natural Product Sciences* **16**(2), 111-115 (2010).

Khasina, E.I., Sgrebneva, M.N., Ovodova, R.G., Golovchenko, V.V., and Ovodov, Y.S., Gastroprotective effect of lemnan, a pectic polysaccharide from Lemna minor L. *Dokl. Biol. Sci.* **390**, 204-206 (2003).

Kim, Y.H. and Ko, W.S., Inhibitory effect of Spirodela polyrrhiza on the mast cell-mediated immediate hypersensitivity. *J. Oriental Physiology and Pathology* **18**, 919-923 (2004).

Olajide, O.A., Awe, S.O., Makinde, J.M., Ekhelar, A.I., Olusola, A., Morebise, O., and Okpako, D.T., Studies on the anti-inflammatory, antipyretic and analgesic properties of Alstonia boonei stem bark. *J. Ethnopharmacol*. **71**, 179-186 (2000).

Ovodova, R.G., Golovchenko, V.V., Shashkov, A.S., Popov, S.V., and Ovodov, Iu.S., Structure and physiological activity of lemnan, Lemna minor L. pectin. *Bioorg. Khim*. **26**, 743-751 (2000).

Santos, A.R.S. and Calixto, J.B., Further evidence for the involvement of tachykinin receptor subtypes in formalin and capsaicin models of pain in mice. *Neuropeptides* **31**, 381-389 (1997).

Suh, S.S. and Shin, J.S., Studies on phytosterols. *Yakhak Hoe Chi* **13**, 144-146 (1969).

Trongsakul. S., Panthong. A., Kanjanapothi. D., and Taesotikul. T., The analgesic, antipyretic and anti-inflammatory activity of Diospyros variegata Kruz. *J. Ethnopharmacol*. **85**, 221-225 (2003).

Vontagu, H., Abbah, J., Nagazal, I.E., Kunle, O.F., Chindo, B.A., Otsapa, P.B., and Gamaniel, K.S., Anti-nociceptive and anti-inflammatory activities of the methanolic extract of Parinari polyandra stem bark in rats and mice. *J. Ethnopharmacol*. **90**, 115-121 (2004).

Wallace, J.W., Biosynthetic studies on flavones and C-glycosylflavones: B-ring oxidation patterns. *Phytochemistry* **14**, 1765-1768 (1975).

Wang, Y.X., Gao, D., Pettus, M., Phillips, C., and Bowersox, S.S., Interactions of intrathecally administered ziconotide, a selective blocker of neuronal N-type voltage-sensitive calcium channels, with morphine on nociception in rats. *Pain* **84**, 271-281 (2000).

Zimmermann, M., Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**, 109-110 (1983).

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