Antidepressant-Like Effects of Sanggenon G, Isolated from the Root Bark of Morus alba, in Rats: Involvement of the Serotonergic System

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Depression is a chronic disease that has an enormous impact on society. According to the predictions of the World Health Organization, by the year 2020, depression will be the second leading global burden of illness.3,5 Despite recent progress in the development of clinically relevant antidepressant drugs, currently available antidepressant are not totally effective and are associated with many undesirable adverse effects.5 Because of the limitations of antidepressant drugs, alternative approaches, such as medicinal herbs, have been studied for their potential applications in the treatment of depression.3,5 Moreover, many herbal extracts have been shown to have antidepressant effects in a variety of animal models. For example, Hypericum perforatum, also known as St. John’s wort, is widely used for the treatment of mild to moderate depression.5 Panax ginseng, commonly known as Korea Ginseng, has been investigated experimentally and clinically for its stress-attenuating activity.6,7

The mulberry tree (Morus alba L.), one of the most well-known and widely distributed trees of the family Moraceae, is extensively cultivated in East Asia; the leaves of M. alba L. are an indispensable food source for silkworms, and the fruits are consumed in normal diets.8 The root bark of M. alba is commonly known by the Chinese name of “Sang-Bai-Pi” and is widely used for its anti-inflammatory, antihypertensive, diuretic, and antipyretic effects.9 M. alba extracts have also been reported to possess hypotensive,10 hypoglycemic,11 hepatoprotective,12 neuroprotective,13 and anti-inflammatory14 activities. However, while M. alba extracts have been reported to exert antidepressant or antistress activities in various animal models, little is known about the antidepressant-like effects of active compounds from the root bark of M. alba extracts.15–18

In the present study, the antidepressant-like effects of sanggenon G (Fig. 1), a major phenolic compound isolated from the ethyl acetate-soluble fraction of the root bark of M. alba, were investigated in response to the forced swim test (FST) in rats. Moreover, to determine the neurobiological effects underlying the antidepressant-like activity of the sanggenon G, corticosterone responses and c-Fos immunoreactivity were evaluated in rats exposed to FST. Finally, we also examined the receptors involved in the antidepressant-like effects of sanggenon G. Our study provides important

Fig. 1. Chemical Structure of Sanggenon G from the Root Bark of Morus alba

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insights into the mechanisms mediating the antidepressant-like effects of this important compound.

MATERIALS AND METHODS

Plant Materials and Solvent Extraction Dried root bark from M. alba L. was purchased from Kapdang Co. (Seoul, Korea). The sample was identified by Dr. Daeseok Han, and a voucher specimen (#NP-1207) was deposited at the facilities of the Research Group of Innovative Special Food, Korea Food Research Institute. Dried root bark of M. alba (10 kg) was prepared after immersion in 80% methanol (170 L) and shaking at room temperature for 24 h. The process was repeated, and the extracts were combined and filtered through a membrane filter (0.45 µm; Millipore, Billerica, MA, U.S.A.). After removing the solvent via rotary evaporation, the remaining extracts were vacuum dried to a yield of about 17% (w/w).

Isolation and Determination of Sanggenon G The EtOAc fraction (120 g) was subjected to SiO₂ column chromatography (CC; φ12.5×18 cm) and eluted with n-hexane–ethyl acetate (4:1–2:1–1:1, 27 L of each). The eluting solutions were monitored by thin layer chromatography to produce 41 fractions (MRE-1–MRE-41). Fraction MRE-37 (1.58 g; elution volume/total volume [Vᵣ/Vᵣ'] 0.80–0.86) was subjected to ODS CC (φ4×10 cm) and eluted with MeOH–H₂O (2:1, 4.2L), yielding 23 fractions (MRE-37-1–MRE-37-23), including the purified compound sanggenon G (MRE-37-17; Vᵣ'/Vᵣ, 0.28–0.44; 350 mg). The compound was identified as sanggenon G based on the results of several spectroscopic methods, including nuclear magnetic resonance (NMR), infrared spectroscopy (IR), and mass spectrometry (MS).

Sanggenon G (Compound 5) Brown amorphous powder (MeOH); [α]₉⁰°= -277° (c=0.093, MeOH); electrospray ionization (ESI)/MS m/z 695.4 [M+H]+; ¹H-NMR (400 MHz, CD₃OD, δH) 7.60 (1H, d, J=8.0 Hz, H-30), 6.82 (1H, d, J=7.2 Hz, H-6′), 6.31 (1H, d, J=2.4 Hz, H-30), 6.30 (1H, dd, J=2.4, 8.0 Hz, H-32), 6.13 (1H, brs, H-24), 6.06 (1H, dd, J=2.4, 8.8 Hz, H-26), 6.41 (1H, dd, J=2.0, 7.2 Hz, H-5′), 5.96 (1H, d, J=2.0 Hz, H-3′), 5.87 (1H, s, H-8), 5.51 (1H, m, H-9), 5.38 (1H, m, H-2), 5.27 (1H, brs, H-10), 5.18 (1H, m, H-14), 4.27 (1H, m, H-20), 2.98 (1H, m, H-18a), 2.96 (1H, m, H-3a), 2.94 (1H, m, H-19), 2.60 (1H, m, H-18b), 2.57 (1H, m, H-3b), 2.14 (2H, m, H-13), 2.05 (2H, m, H-12), 1.68 (3H, s, H-16), 1.61 (3H, s, H-17). ¹³C-NMR (100 MHz, CD₃OD, δC) 198.59 (C-4), 198.25 (C-21), 165.80 (C-5), 165.61 (C-4′), 164.01 (C-8a), 163.28 (C-7), 159.63 (C-25), 159.57 (C-31), 157.12 (C-29), 157.11 (C-2), 156.59 (C-23), 136.84 (C-11), 134.14 (C-33), 131.89 (C-15), 128.80 (C-27), 128.86 (C-10), 125.54 (C-14), 125.12 (C-10), 122.90 (C-22), 117.91 (C-28), 116.22 (C-1), 110.38 (C-6), 108.15 (C-5), 96.12 (C-8a), 95.06 (C-9), 76.01 (C-2), 47.58 (C-19), 43.14 (C-18), 43.14 (C-13), 38.54 (C-12), 38.15 (C-20), 27.37 (C-13), 25.94 (C-16), 17.83 (C-17).

Animals Male Sprague Dawley (SD) rats (8 weeks old; Samtakio Bio Korea, Gyeonggi-do, Korea) weighing 180–210 g were housed at two rats per cage under a controlled temperature (23±1°C) and a 12-h light/dark cycle (lights on at 07:00 and lights off at 19:00). The rats were allowed at least 1 week for acclimatization before the experiments. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Food Research Institute.

Drugs and Treatment Imipramine hydrochloride, WAY100635, and SCH23390 (all from Sigma-Aldrich Co., St. Louis, MO, U.S.A.) were dissolved in 0.9% (w/v) saline solution. Sanggenon G was diluted in saline with 1% Tween 80. Sanggenon G (3, 10, or 30 mg/kg) and imipramine (30 mg/kg) were injected intraperitoneally (i.p., an injection volume of 0.1 mL/100 g body weight) 60 min before the FST. In order to investigate the possible involvement of 5-hydroxytryptamine (5-HT₁₆) and dopamine D₁ receptors in the antidepressant-like effects of sanggenon G, rats were pretreated with WAY100635 (1 mg/kg, i.p.) and SCH23390 (0.05 mg/kg, i.p.). Rats then received sanggenon G or vehicle injection 30 min later, and the FST was performed 60 min later.

FST The FST was carried out as previously described. Briefly, in the pretest session, rats were forced to swim for 15 min in a transparent Plexiglas cylinder (height 50 cm; diameter 20 cm) filled to a depth of 30 cm with water (temperature, 23–25°C). Twenty-four hours later, the procedure was repeated during a 6-min test session, and the immobility time during the last 4 min was measured by a SMART video tracking system (SMART v3.0, Panlab SL, Barcelona, Spain). Rats were considered immobile when they ceased struggling, remained floating motionless, and only made movements necessary to keep their heads above the water.

Serum Corticosterone Assay Blood samples were collected via the abdominal aorta after the FST. The serum samples were prepared by centrifugation of the collected blood samples for 15 min at 13000 g within 30 min and stored frozen (−80°C). The serum levels of corticosterone were measured using commercially available enzyme immunoassay kits (DetectX; Arbor Assays, Ann Arbor, MI, U.S.A.).

Immunohistochemistry Rats were sacrificed following the FST test, and their brains were fixed through the ascending aorta with 0.9% saline, followed by 500 mL of cold 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA). The fixed brains were cut into 30-µm sections on a cryostat (CM1850; Leica, Heidelberg, Germany). Immunohistochemistry staining was performed on 30-µm sections using polyclonal antibodies specific for c-Fos (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), followed by exposure to a biotinylated anti-rabbit antibody (1:500 dilution; cat. no. BA1000; Vector Labs, Burlington, ON, Canada). The sections were reacted with an avidin–bion–peroxidase complex (Elite ABC kit; 1:50 dilution; Vector Laboratories) at room temperature for 60 min, and the avidin–biotin complex was visualized with 0.05% 3,3-diaminobenzidine (DAB; Sigma) and 0.02% H₂O₂. Images of immunohistochemically stained sections were captured by a camera mounted on an Olympus BX-51 microscope (Olympus Optical, Tokyo, Japan).

Statistical Analysis Data analysis was performed using one-way ANOVA followed by Tukey’s post-hoc test using Prism 5 (GraphPad Software, Inc., San Diego, CA, U.S.A.) for multigroup comparisons. All data are presented as the mean±standard error of the mean (S.E.M.). Differences with p values of less than 0.05 were considered significant.
RESULTS

Effect of Sanggenon G on Depressant Behaviors in Response to the FST
First, we examined the antidepressant-like effects of sanggenon G in the FST. Sanggenon G treatment in rats reduced the duration of immobility, reducing immobility by a maximum of 25.8% when administered at a dose of 30 mg/kg. Sanggenon G also significantly increased the swimming time ($p<0.05$) without any significant change in climbing (Fig. 2). Imipramine (30 mg/kg), which was used as the positive control, markedly decreased immobility time and increased swimming time in the FST (both $p<0.05$ versus vehicle).

Effect of Sanggenon G on Serum Corticosterone Levels
Blood samples were collected via the abdominal aorta after the FST for corticosterone analysis. Columns show the mean±S.E.M. ($n=10$). *$p<0.05$ versus the control group.

Effect of 5-HT$_{1A}$ and Dopamine D1 Receptors in the Antidepressant-Like Activity of Sanggenon G
In order to examine the potential mechanisms through which sanggenon G mediated depressive symptoms in rats subjected to the FST, rats were pretreated with WAY 100635, a selective 5-HT$_{1A}$ receptor antagonist, or SCH23390, a selective dopamine D1 receptor antagonist, followed by sanggenon G treatment and the FST. As shown in Fig. 5, pretreatment with 1 mg/kg WAY100635 significantly inhibited the antidepressant-like effects of sanggenon G in the FST. However, SCH23390 did not alter the antidepressant-like effects of sanggenon G in the FST.

DISCUSSION

In the present study, we examined the antidepressant-like effects and mechanisms of sanggenon G in FST-induced depression in rats. Our results demonstrated that acute treatment with sanggenon G significantly decreased the immobility time in rats exposed to the FST. Moreover, sanggenon G decreased the hypothalamic–pituitary–adrenal (HPA) axis response to stress, as indicated by attenuation of the corticosterone response and decreased c-Fos immunoreactivity in the hypothalamic PVN. Preliminary analysis of the mechanism showed that pretreatment with a selective 5-HT$_{1A}$ receptor antagonist significantly inhibited the antidepressant-like effects of sanggenon G in the FST. Thus, our data provided important insights into the mechanisms through which sanggenon G exerts its antidepressant effects.

Continued and elevated glucocorticoid levels resulting from dysfunction of the HPA axis is one of the most prominent neurobiological findings in depression. Glucocorticoid receptors (GR) mediate the direct effects of the glucocorticoids that are released in response to stress and regulate the HPA axis via a negative feedback mechanism. Previous clinical studies have reported that depressed subjects exhibit down-regulation of GR expression, which subsequently leads to an increase in the activation of c-Fos was observed in the hypothalamic PVN following the FST in vehicle-treated rats. c-Fos expression in imipramine-treated rats was similar to that previously reported for stress-induced c-Fos expression in the hypothalamic PVN. Importantly, treatment with 30 mg/kg sanggenon G significantly inhibited c-Fos activity as compared with that in the vehicle-treated control group (Fig. 4).
endogenous levels of glucocorticoids.\textsuperscript{24)}

The HPA axis is responsible for initiation of glucocorticoid stress responses in all vertebrate animals. Activation of the HPA axis is regulated by diverse afferent input to the hypothalamic paraventricular nucleus PVN.\textsuperscript{25)} Animal models of depression play an important role in the screening and evaluation of antidepressants. The FST is an effective screening tool with good reliability and predictive validity\textsuperscript{26)}; the state of immobility in the FST is reported to mimic the symptoms of depression in humans and can be reversed by treatment with antidepressant drugs.\textsuperscript{27)} In our study, treatment with sanggenon G (30 mg/kg) decreased immobility and increased swimming time without any significant change in climbing. This pattern has also been observed by treatment with paroxetine and fluoxetine, selective serotonin reuptake inhibitors (SSRIs).\textsuperscript{28,29)} The antidepressant-like effects of sanggenon G were confirmed by quantitative analysis of c-Fos immunoreactivity and analysis of the activity of the HPA axis, which are both

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**Fig. 4. Effects of Sanggenon G on c-Fos Expression in the PVN**

Representative photomicrographs show c-Fos-positive nuclei in the PVN of normal (non-exposed to FST) (A), control (B), imipramine (30 mg/kg)-treated (C), and sanggenon G (30 mg/kg)-treated (D) rats exposed to the FST. Columns show the mean±S.E.M. (n=5). *p<0.05 and **p<0.01 versus the control group. ##p<0.01 versus the normal group. PVN, hypothalamic paraventricular nucleus (−1.80 to −1.88 mm from the bregma), PV, periventricular parvocellular subdivision, DP, dorsal parvocellular subdivision, MN, magnocellular part of PVN, MP, medial parvocellular subdivision, VP, ventral parvocellular subdivision.
associated with high corticosterone production.\textsuperscript{30} c-Fos is an immediate-early gene that is rapidly expressed in response to neuronal activation\textsuperscript{31} and has been widely used as a marker for neuronal activation and to explore the effects of external stimuli on neuronal circuits.\textsuperscript{32} Previous studies involving acute or chronic stress states have demonstrated that profound alterations in the expression of GR mRNA are closely associated with elevated corticosterone production and c-Fos expression.\textsuperscript{33} Antidepressant drugs, including SSRIs, compensate for impaired feedback inhibition by regulating GR levels in the hippocampus.\textsuperscript{34} Therefore, it seems that decreasing neural activity in the hippocampal regions normalizes via altered HPA activity. Consistent with this idea, we found that acute treatment with sanggenon G at a dose of 30 mg/kg blocked the increase in c-Fos-positive cells in the hypothalamic PVN associated with stress-induced depression and reduced the HPA axis response to stress and corticosterone levels. Although we did not determine GR phosphorylation and GR-dependent transactivation in hypothalamic PVN, our findings suggest that sanggenon G reverse the downregulation of hippocampal GR expression in rats exposed to the FST.

Exposure to a stressor increases secretion of corticosterone through the action of the postsynaptic 5-HT\textsubscript{1A} receptors in the corticotrophin-releasing hormone (CRH) neurons of the hypothalamus.\textsuperscript{35} CRH neurons in PVN were also reported to be hyper-activated in major depression.\textsuperscript{36} Serotonin 5-HT\textsubscript{1A} receptors, which are abundantly expressed in the brain, are thought to be essential for antidepressant responses.\textsuperscript{37} 5-HT\textsubscript{1A} receptors are involved in the pathophysiology of depression and have been shown to act as targets for antidepressants in various \textit{in vivo} models of depression.\textsuperscript{39} For example, 5-HT\textsubscript{1A} receptor-knockout mice cannot be rescued by administration of antidepressants in the tail suspension test.\textsuperscript{40} 5-HT\textsubscript{1A} receptor agonists (buspirone, ipsapirone, and gepirone) significantly decrease immobility time in the FST.\textsuperscript{41,42} Moreover, treatment with 5-HT\textsubscript{1A} receptor antagonists, such as WAY100635, results in complete inhibition of the antidepressant activity of combined SSRIs and 5-HT\textsubscript{1A} receptor agonists.\textsuperscript{43} Deficiencies in the function and expression of 5-HT\textsubscript{1A} receptors are important factors in the development of depression.\textsuperscript{44} In our study, the antidepressant-like effects of sanggenon G were significantly blocked by WAY100635 treatment. These results strongly suggested that the antidepressant-like effects of sanggenon G are likely mediated via 5-HT\textsubscript{1A} receptors. However, a secondary mechanism is also possible because sanggenon G affects 5-HT\textsubscript{1A} receptors indirectly through another

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\textbf{Fig. 5.} Effects of WAY 100635 and SCH23390 on the Sanggenon G-Induced Reduction in Immobility Time (A) and Serum Corticosterone Levels (B) in Rats Subjected to the FST

WAY 100635 (1 mg/kg) and SCH23390 (0.05 mg/kg) were injected 30 min before treatment with sanggenon G or imipramine. The FST test was then performed 60 min after sanggenon G or imipramine treatment. Columns show the mean±S.E.M. (n=10). *p<0.05 and **p<0.01 versus the control group.
target. Therefore, further studies are needed to determine the specific mechanisms through which sanggenon G affects the 5-HT1A receptor.

In conclusion, our results demonstrated that treatment with sanggenon G significantly decreased immobility time and the HPA axis response in rats exposed to the FST, as indicated by attenuation of the corticosterone response and decreased c-Fos immunoreactivity in the hippocampal hypothalamic PVN region. In addition, this was the first study to show that sanggenon G causes antidepressant-like effects through interaction with the serotonergic system.

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Conflict of Interest  The authors declare no conflict of interest.

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