Food Sci. Biotechnol. Vol. 17, No. 4, pp. 713 ~ 717 (2008)

Food Science and Biotechnology

Aqueous Extracts of Walnut (*Juglans regia* L.) and *Nelumbo nucifera* Seeds Reduce Plasma Corticosterone Levels, Gastric Lesions, and c-fos Immunoreactivity in Chronic Restraint-stressed Mice

Dae Won Kim, In Koo Hwang[1], Ki-Yeon Yoo[2], Hua Li[2], Il-Jun Kang[3], Won-Kuk Moon, Moo-Ho Won[2], Seok Joong Kim[4], Daeseok Han[5], and Dong-Woo Kim*

Central Research Center, Natural F&P Co., Ltd., Chuncheon, Gangwon 200-163, Korea

[1]Department of Anatomy and Cell Biology, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul 151-742, Korea

[2]Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chuncheon, Gangwon 200-702, Korea

[3]Department of Food Science and Nutrition, Division of Life Science, Hallym University, Chuncheon, Gangwon 200-702, Korea

[4]Department of Food and Nutrition, Dongduk Women's University, Seoul 136-714, Korea

[5]Food Processing and Post-harvest Technology Division, Korea Food Research Institute, Seongnam, Gyeonggi 463-746, Korea

Abstract In the present study, chronic effects of the hot water extracts of walnut seed (*Juglans regia* L.) (WSE) and *Nelumbo nucifera* seed (NSE) were investigated in mice exposed to 2 hr of restraint stress each day for 4 weeks. Corticosterone levels in serum were significantly increased in the vehicle-treated stressed group (25 μg/dL) compared to that in the control group (13 μg/dL). This stress induced gastric redness and lesions. However, treatment with WSE and/or NSE significantly protected the stomach from this lesion by 50-60% compared to that in the vehicle-treated group. In the amygdala, the administration of WSE and/or NSE also reduced the immediate early gene (c-fos) expression by 70-90% vs. the vehicle-treated group. These suggest that WSE and/or NSE may reduce the appearance of symptoms induced by stress and these materials are useful as anti-stress foods, as natural products tend to be relatively safe compared to chemical products.

Keywords: restraint stress, corticosterone, gastric lesion, c-fos, medial amygdale

Introduction

Stress is a major problem in modern society. In the last one or two decades, the incidence of stress disorders has enormously increased in urban populations (1). Stress, depression, and associated mental problems have become important (2). In this regard, the market sizes of anti-anxiety drugs and foods have also significantly increased. It has been thought that antidepressant and antianxiety drugs are the best choice to reduce stress-induced mental changes. However, there are many problems with the abuse of psychoactive drugs. In recent years, many researchers have considered the use of plant-derived foods in the management of stress-linked mental health (3-6).

Ginseng extract, its constituents, which include ginsenosides, and some other plants are well-known to have anti-stress effects (3,5,6). Ginseng extract and some of its constituents have anti-stress activities in animals subjected to stressful stimuli such as foot-shock, cold, and heat (3,7-9). Walnuts are rich in polyunsaturated fatty acid and phytochemicals, and their consumption is linked to reduced coronary vascular disease (10,11). Fukuda *et al.* (12) recently isolated and characterized 16 polyphenols from walnut extracts, and found antioxidant effects of these polyphenols in *in vitro* experimental models of lipid peroxidation. On the other hand, it has been reported that extracts of *Nelumbo nucifera* seeds (NS) have free radical scavenging effects (13). In addition, *N. nucifera* extract (NSE) (rhizome and leaves part) shows antidiabetic effects (14,15). Although many studies have investigated the antioxidant effects of walnut (*Juglans regia* L.) seeds (WS) and NS, few studies have reported on the stress-lowering effects of these extracts. Therefore, in this study, the anti-stress effects of walnut seed extract (WSE) and/or NSE were investigated using a restraint stress model, which is a well-established method that does not utilize painful stimuli.

*Corresponding author: Tel: +82-33-258-6772; Fax: +82-33-258-6773
E-mail: drkimdw@hanmail.net
Received September 7, 2007; Revised January 15, 2008
Accepted January 15, 2008

Materials and Methods

Preparation of plants Walnut (*Juglans regia* L.) seeds (WS) and *Nelumbo nucifera* seeds (NS) were obtained from a local grocery store, Poongmul-sijang, Chuncheon, Korea. The WS (1 kg) and NS (1 kg) were washed with distilled water, defatted with *n*-hexane (5 L×3) and then extracted with 5 L of water at 100°C for 6 hr, respectively. The extraction procedure was repeated 3 times. The insoluble materials were removed through centrifugation at 10,000×g for 30 min at room temperature, and the resulting supernatants were freeze-dried. The extracted materials were evaporated, yielding 94 and 132 g, respectively. The WSE and NSE were dissolved in distilled water and then sterilized with a 0.45 μm syringe filter prior to use.

Animals Forty male ICR mice were purchased from

Jackson Laboratory Co., Ltd. (Bar Harbor, ME, USA). They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and had free access to food and water. The animals were adapted to a chow diet for 1 week, and were then divided into 4 groups of 10 each using a randomized block design; (1) vehicle (water)-treated group, (2) 100 mg/kg WSE-treated group, (3) 100 mg/kg NSE-treated group, and (4) 100 mg/kg WSE/NSE-treated group. Extracts were orally injected using a feeding needle every day for 4 weeks. The procedures for the handling and care of the animals adhered to guidelines that are in compliance with the current international laws and policies (16). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Repeated restraint stress The restraint stress cages consisted of adjustable length (3 cm in diameter and 7 cm in length) Plexi-glass tubes with air holes in the front, top, and back. Repeated restraint-stressed animals were placed into restrainers for 2 hr a day (between 09:30 hr and 11:30 hr) for 4 weeks prior to sacrifice.

Blood, tissue sampling, and determination of gastric lesions Five animals in each group were anesthetized with sodium pentobarbital for blood and tissue sampling, and blood samples were collected from each mouse by cardiac puncture. Serum was separated from the blood by centrifugation at 1,100×g for 15 min at 4°C, and was kept at 80°C until analysis. The stomach was removed, and was subsequently opened along the greater curvature and gently placed on saline-moistened filter paper. The lesions were counted and measured with a stereomicroscope (Zeiss Stemi SV 11; magnification 6×, Carl Zeiss, Göttingen, Germany) by an observer who was blinded to the treatment. The sum of the lesion areas in each animal (in mm^2) was expressed as the mean gastric mucosal injury.

Analysis of serum corticosteroid levels Serum (50 μL) was added to 5 mL of methylene chloride and incubated at room temperature for 10 min. After filtration with cheesecloth, the mixture was combined with 2.5 mL of fluorescence reagent (7:3, sulfuric acid:absolute ethanol), vortexed vigorously, and incubated for 30 min at room temperature. After centrifugation, the lower layer was measured using a spectrophotometer (excited wavelength, 475 nm; emission wavelength, 530 nm).

Immunohistochemistry for c-fos To confirm the effects of WSE and/or NSE against stress-induced neuronal activation in the medial amygdala of the brain, vehicle-, WSE-, and/or NSE-treated animals (n=5 in each group) were anesthetized with sodium pentobarbital and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and cryoprotected by infiltration with 30% sucrose. Thereafter, frozen tissues were serially sectioned into 30-μm coronal sections on a cryostat (Leica, Wetzlar, Germany), and they were then collected in 6-well plates containing PBS. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) and 10% normal goat serum for 30 min, respectively. The sections were then incubated with diluted rabbit anti-c-fos (diluted 1:50; Upstate, NY, USA) at 4°C. Thereafter, the tissues were exposed to biotinylated goat anti-rabbit immunoglobulin (IgG) and streptavidin peroxidase complex (Vector, Burlingame, CA, USA), and were then visualized with 3,3'-diaminobenzidine in 0.1 M Tris-HCl (pH 7.2) buffer. After dehydration, the sections were mounted in Canada balsam (Kanto, Tokyo, Japan).

Quantification of data and statistical analysis All measurements were performed in order to ensure objectivity in blind conditions, by 2 observers for each experiment carrying out the measurement of experimental samples under the same conditions.

Measurement of neuronal number was performed using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA). The number of c-fos positive cells was counted in 25 sections/animal and they were compared to those of the vehicle-treated group.

Data are expressed as the mean±SEM. Differences among the means were statistically analyzed by 2-tailed Student t-test analysis of variance to elucidate differences between vehicle-treated and extract-treated groups.

Results and Discussion

Stress can cause physical damage to the gastrointestinal tract, endocrine system, skin, and cardiovascular system (2,17-21). First, we assessed the serum glucocorticoid levels in the vehicle-, WSE-, NSE-, and WSE/NSE-treated stressed groups as well as the control group. In all the stress groups, serum glucocorticoid levels were significantly increased compared to that in the control group. The serum glucocorticoid level in the vehicle-treated group was approximately 25 μg/dL (Fig. 1). This result is supported by previous studies that the glucocorticoid secretion was significantly increased during and/or after stress (22,23) and that this secretion displayed a close correlation with the

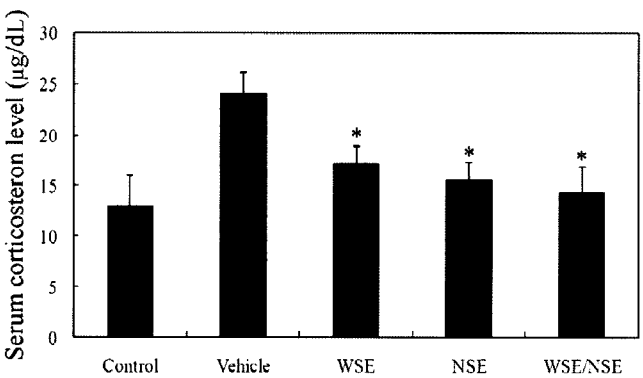

Fig. 1. Effects of walnut seed extract (WSE), *Nelumbo nucifera* seed extract (NSE), and WSE/NSE treatment on the serum corticosterone levels in mice exposed to 2 hr of restraint stress each day for 4 weeks. Differences among the means were statistically analyzed by Student's t-test (n=5 per group; *p<0.05, significantly different from vehicle-treated group). Bars indicate means±SEM.

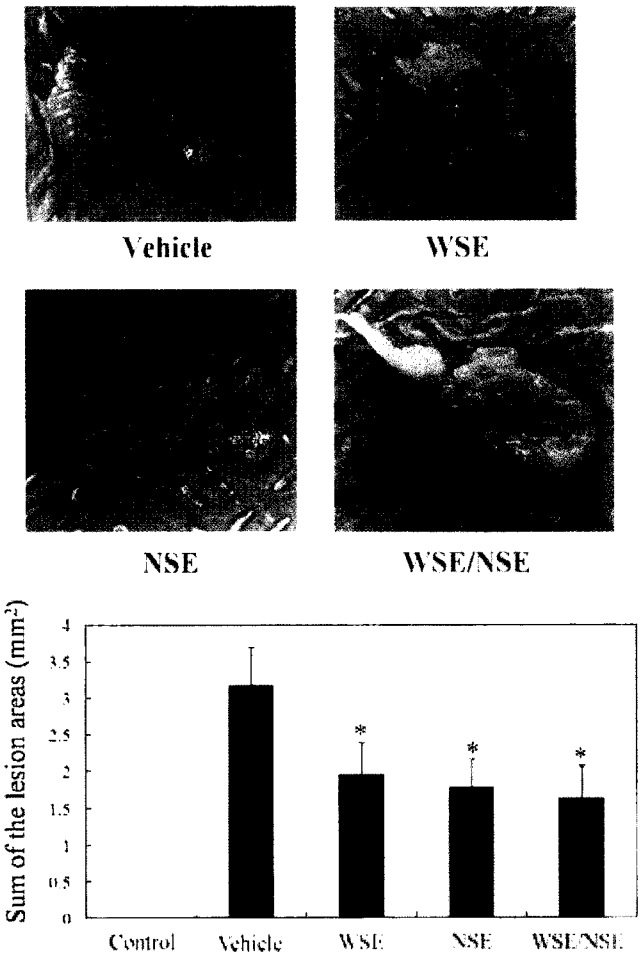

Fig. 2. Effects of walnut seed extract (WSE), *Nelumbo nucifera* seed extract (NSE), and WSE/NSE treatment on the gastric mucosal lesions in mice exposed to 2 hr of restraint stress each day for 4 weeks. In the vehicle-treated stressed group, the gastric mucosa shows lesions as well as redness. Note that WSE, NSE, and WSE/NSE treatment significantly reduces the number of gastric mucosal lesions as well as redness. Differences among the means were statistically analyzed by Student's *t*-test (n=5 per group; $*p<0.05$, significantly different from vehicle-treated group). Bars indicate means±SEM.

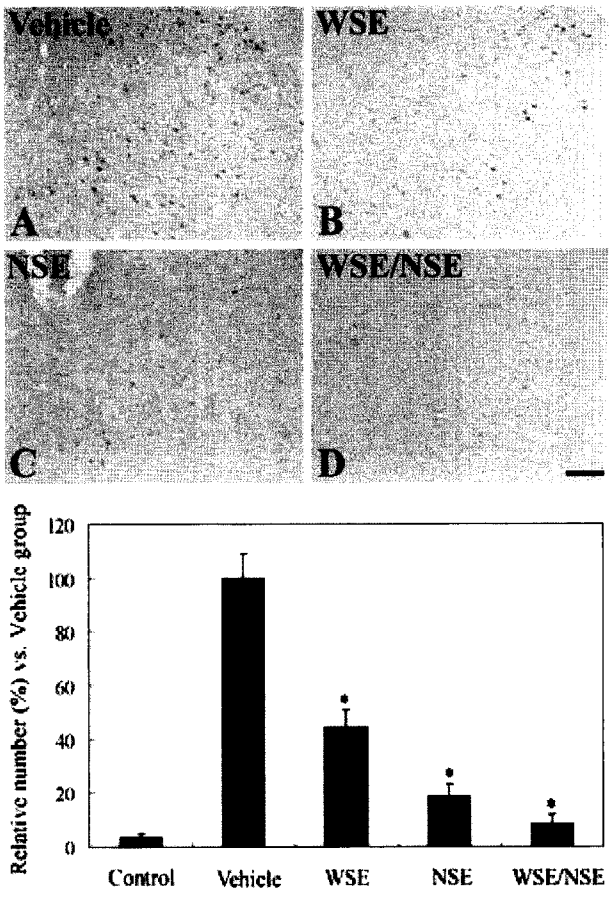

Fig. 3. Effects of walnut seed extract (WSE), *Nelumbo nucifera* seed extract (NSE), and WSE/NSE treatment on c-fos immunoreactivity in the medial amygdala of mice exposed to 2 hr of restraint stress each day for 4 weeks. In the vehicle-treated stressed group, c-fos immunoreactive neurons are abundant in the medial amygdala. Note that WSE, NSE, and WSE/NSE treatment significantly reduces the number of c-fos immunoreactive neurons in the medial amygdala. Differences among the means were statistically analyzed by Student's *t*-test (n=5 per group; $*p<0.05$, significantly different from vehicle-treated group). Bars indicate means±SEM.

intensity of the stressor (24,25). However, the serum glucocorticoid levels were significantly decreased in the WSE-, NSE-, and WSE/NSE-treated stressed groups. In the WSE-/NSE-treated stressed group, serum glucocorticoid level was reduced by 40% compared to the vehicle-treated stressed group.

In this study, we observed the gastric mucosal lesions in the vehicle-, WSE-, NSE-, and WSE/NSE-treated stressed groups (Fig. 2). Gastric lesions were not found in the control animals. However, gastric mucosal lesions were found in the vehicle-treated stressed group, and the size of gastric lesion was 3.17 mm^2 (Fig. 2). The administration of WSE-, NSE-, and WSE/NSE significantly reduced the size of gastric mucosal lesions by 50-60% vs. the vehicle-treated stressed group. There are some reports that extracts with antioxidant effects have protective effects against stress-induced gastric mucosal lesions in experimental animals (26-28).

Most immediate early genes, including c-fos, are transcription factors that regulate the expression of multiple genes assumed to be important for proper responses to stimuli. In addition, c-fos has been a major tool in the characterization of brain areas involved in the process of stressors (29-32). Amygdala is widely regarded as being involved in the emotion of fear and in both unconditioned and conditioned responses to fearful stimuli (29,31). In this study, we observed the changes of c-fos immunoreactivity in the medial amygdala. The c-fos immunoreactivity was significantly decreased in the amygdala of the WSE-, NSE-, and WSE/NSE-treated stressed groups compared to that in the vehicle-treated stressed group (Fig. 3). This result suggests that WSE, NSE, and/or WSE/NSE- treatment strongly reduce the stress-induced overactivation of neurons in the amygdala, as restraint stress induces c-fos immunoreactivity and protein levels in this region (33-35). In this study, we observed the anti-stress effects of WSE, NSE, and WSE/NSE in restraint-stressed mice. However, in this study, we did not find the functional components of WSE and/or NSE which have effects on stress-induced

damage. It has been reported that the main polyphenolic compound of walnut ethanol extract is pedunculagin (36). In the NSE, the main components are polyphenolic alkaloid such as liensinine, isoliensinine, and neferine (37).

In conclusion, the administration of WSE, NSE, and WSE/NSE significantly reduced serum glucocorticoid levels, gastric mucosal lesions, and c-fos immunoreactivity in the medial amygdala. This result suggests that the WSE and NSE are natural substances that may be effective in lowering physical and psychological stress.

Acknowledgments

The authors would like to thank Mr. Suek Han, Mr. Seung Uk Lee, and Mrs. Hyun Sook Kim for their technical help in this study. This study was supported by the Technology Development Program for Agriculture and Forestry, the Ministry of Agriculture and Forestry (204010-03-3-HD120), Korea.

References

1. Andrews G, Sanderson K, Slade T, Isakidis C. Why does the burden of disease persist? Relating the burden of anxiety and depression to effectiveness of treatment. B. World Health Organ. 78: 446-454 (2000)
2. Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. J. Am. Med. Assoc. 267: 1244-1252 (1992)
3. Takahashi M, Tokuyama S, Kaneto H. Anti-stress effect of ginseng on the inhibition of the development of morphine tolerance in stressed mice. Jpn. J. Pharmacol. 59: 399-404 (1992)
4. Bhattacharya SK, Muruganandam AV. Adaptogenic activity of Withania somnifera: An experimental study using a rat model of chronic stress. Pharmacol. Biochem. Be. 75: 547-555 (2003)
5. Tan-Lee BS, Yu GY, Kim K, Han S, Han J, Lee GS, Kim ES, Lee CJ, Ryu JH, Cheong JH. Anti-stress effect of artichoke juice in SD rats and ICR mice. Food Sci. Biotechnol. 13: 302-308 (2004)
6. Lee GS, Choi JY, Ko HS, Tan-Lee BS, Yu GY, Jeong CW, Park HG, Kim MK, Ryu JH, Jung IK, Cheong JH. Stree-reducing effects of brown rice *koji*. Food Sci. Biotechnol. 15: 63-69 (2006)
7. Kaneko H, Nakanish K, Murakami A, Kaidoh H, Kuwashima K. The acute effects of massive dose of red ginseng on healthy adults under the condition of cold stress. Ginseng Rev. 22: 20-24 (1996)
8. Kim DH, Jung JS, Moon YS, Sung JH, Suh HW, Kim YH, Song DK. Inhibition of intracerebroventricular injection stress-induced plasma corticosterone levels by intracerebroventricularly administered compound K, a ginseng saponin metabolite, in mice. Biol. Pharm. Bull. 26: 1035-1038 (2003)
9. Choi SS, Lee JK, Suh HW. Effect of ginsenosides administered intrathecally on the antinociception induced by cold water swimming stress in the mouse. Biol. Pharm. Bull. 26: 858-861 (2003)
10. Xu R, Yokoyama WH, Irving D, Rein D, Walzem RL, German JB. Effect of dietary catechin and vitamin E on aortic fatty streak accumulation in hypercholesterolemic hamsters. Atherosclerosis 137: 29-36 (1998)
11. Davis P, Valacchi G, Pagnin E, Shao Q, Gross HB, Calo L, Yokoyama W. Walnuts reduce aortic ET-1 mRNA levels in hamsters fed a high-fat, atherogenic diet. J. Nutr. 136: 428-432 (2006)
12. Fukuda T, Ito H, Yoshida T. Antioxidative polyphenols from walnuts (*Juglans regia L.*). Phytochemistry 63: 795-801 (2003)
13. Sohn DH, Kim YC, Oh SH, Park EJ, Li X, Lee BH. Hepatoprotective and free radical scavenging effects of *Nelumbo nucifera*. Phytomedicine 10: 165-169 (2003)
14. Mukherjee PK, Saha K, Saha BP. Effect of *Nelumbo nucifera* rhizome extract on blood sugar level in rats. J. Ethnopharmacol. 58: 207-213 (1997)
15. Ono Y, Hattori E, Fukaya Y, Imai S, Ohizumi Y. Anti-obesity effect of *Nelumbo nucifera* leaves extract in mice and rats. J. Ethnopharmacol. 106: 238-244 (2006)
16. Bayne K. Revised guide for the care and use of laboratory animals available. The Physiologist 39: 208-211 (1996)
17. Selye H. History of the stress concept. pp. 7-36. In: Handbook of Stress. Goldberg L, Brenitz S (eds). The Free Press, New York, NY, USA (1993)
18. Hurst MW, Jenkins CD, Rose RM. The relatioin of psychological stress to onset of medical illness. Annu. Rev. Med. 27: 301-312 (1976)
19. Barsky AJ, Wyshak G, Klerman GL. Medical and psychiatric determinants of outpatient medical utilization. Med. Care 24: 548-560 (1986)
20. Breier A, Albus M, Picker D, Zahn TP, Wolkowitz OM, Paul SM. Controllable and uncontrollable stress in humans: Alterations in mood and neuroendocrine and psychophysiological function. Am. J. Psychiat. 144: 11-19 (1987)
21. Balzer D, Hughes D, George LK. Stress life events and the onset of a generalized anxiety syndrome. Am. J. Psychiat. 144: 9-18 (1987)
22. López-Calderón A, Ariznavarreta C, González-Quijano MI, Tresguerres JA, Calderón MD. Stress induced changes in testis function. J. Steroid Biochem. 40: 473-479 (1991)
23. Millán S, González-Quijano MI, Giordano M, Soto L, Martin AI, López-Calderón A. Short and long restraint differentially affect humoral and cellular immune functions. Life Sci. 59: 1431-1442 (1996)
24. Armario A, Restrepo C, Castrellanos JM, Balasch J. Dissociation between adrenocorticotropin and corticosterone responses to restraint after previous chronic exposure to stress. Life Sci. 36: 2085-2092 (1985)
25. Djordjeviæ J, Cvijiæ G, Davidoviæ V. Different activation of ACTH and corticosterone release in response to various stressors in rats. Physiol. Res. 52: 67-72 (2003)
26. Akpinar D, Yargicoglu P, Derin N, Aslan M, Agar A. Effect of aminoguanidine on visual evoked potentials (VEPs), antioxidant status, and lipid peroxidation in rats exposed to chronic restraint stress. Brain Res. 1186: 87-94 (2007)
27. An SM, Park CH, Heo JC, Park JY, Woo SU, Seo JH, Lee MS, Cho KJ, Cho HS, Shin HM, Lee SH. *Gastrodia elata* Blume protects against stress-induced gastric mucosal lesions in mice. Int. J. Mol. Med. 20: 209-215 (2007)
28. Ohta Y, Chiba S, Tada M, Imai Y, Kitagawa A. Development of oxidative stress and cell damage in the liver of rats with water-immersion restraint stress. Redox Rep. 12: 139-147 (2007)
29. Liu S, Zhou W, Liu H, Yang G, Zhao W. Electroacupuncture attenuates morphine withdrawal signs and c-fos expression in the central nucleus of the amygdala in freely moving rats. Brain Res. 1044: 155-163 (2005)
30. Trnecková L, Armario A, Hynie S, Sída P, Klenerová V. Differences in the brain expression of c-fos mRNA after restraint stress in Lewis compared to Sprague-Dawley rats. Brain Res. 1077: 7-15 (2006)
31. Badowska-Szalewska E, Ludkiewicz B, Domaradzka-Pytel B, Dziewiatkowski J, Spodnik JH, Moryœ J. The immunoreactivity of c-Fos, NGF and its receptor TrkA after open-field exposure in the central and medial nuclei of the rat amygdala. Folia Morphol. (Warsz) 65: 145-151 (2006)
32. Rotllant D, Nadal R, Armario A. Differential effects of stress and amphetamine administration on fos-like protein expression in corticotropin releasing factor-neurons of the rat brain. Dev. Neurobiol. 67: 702-714 (2007)
33. Chen X, Herbert J. Regional changes in c-fos expression in the basal forebrain and brainstem during adaptation to repeated stress: Correlations with cardiovascular, hypothermic, and endocrine responses. Neuroscience 64: 675-685 (1995)
34. Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64: 477-505 (1995)
35. Kubo T, Okatani H, Nishigori Y, Hagiwara Y, Fukumori R, Goshima Y. Involvement of the medial amygdaloid nucleus in restraint stress-induced pressor responses in rats. Neurosci. Lett. 354: 84-86 (2004)
36. Fukuda T, Ito H, Yoshida T. Effect of the walut polyphenol fraction on oxidative stress in type 2 diabetes mice. BioFactors 21: 251-253

(2004)
37. Chen Y, Fan G, Wu H, Wu Y, Mitchell A. Separation, identification, and rapid determination of liensine, isoliensinine, and neferine from embryo of the seed of *Nelumbo nucifera* GAERTN. by liquid chromatography coupled to diode array detector and tandem mass spectrometry. J. Pharmaceut. Biomed. 43: 99-104 (2007)