Hepatoprotective Effect of Syringic Acid and Vanillic Acid on Concanavalin A-Induced Liver Injury

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The edible mushroom *Lentinula edodes* (shiitake) contains many bioactive compounds. In the present study, we cultivated *L. edodes* mycelia in solid medium and examined the hot-water extract (L.E.M.) for its suppressive effect on concanavalin A (ConA)-induced liver injury in mice. ConA injection into the tail vein caused a great increase in the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. The intraperitoneal administration of L.E.M. significantly decreased the levels of the transaminases. L.E.M. contains many bioactive substances, including polysaccharides and glucan, which could be immunomodulators. Since ConA-induced liver injury is caused by the activation of T cells, immunomodulating substances might be responsible for the suppressive effect of L.E.M. L.E.M. also contains phenolic compounds that are produced from lignocellulose by mycelia-derived enzymes. The major phenolics in L.E.M., syringic acid and vanillic acid, were intraperitoneally injected into mice shortly before the ConA treatment. Similar to L.E.M., the administration of syringic acid or vanillic acid significantly decreased the transaminase activity and suppressed the disorganization of the hepatic sinusoids. In addition, the inflammatory cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-6 in the serum increased rapidly, within 3 h of the ConA administration, but the administration of syringic acid or vanillic acid significantly suppressed the cytokine levels. Together, these findings indicate that the phenolic compounds in L.E.M. are hepatoprotective through their suppression of immune-mediated liver inflammation.

Key words hepatoprotection; *Lentinula edodes*; syringic acid; vanillic acid; concanavalin A

Many physiologically active hepatoprotective substances, such as those with antifibrotic activity, have been found in tea, fruits, and vegetables.[1,2] The edible mushroom *Lentinula edodes* (shiitake) contains several bioactive compounds, including compounds with immunoprotective and antiatherogenic activities and one compound with an anti-human immunodeficiency virus (HIV) effect.[3—5] The mycelia of *L. edodes* can be cultured in solid medium, and the extract obtained by hot-water treatment (L.E.M.) is commercially available as a nutritional supplement. In our previous study, we found that L.E.M. exerts a hepatoprotective effect on dimethylnitrosamine (DMN)-induced liver fibrosis and D-galactosamine-induced acute liver injury.[6,7] In the chronic liver injury model that uses DMN, the L.E.M. treatment suppressed the activation of hepatic stellate cells, which play a central role in liver fibrosis. The L.E.M. treatment also protected hepatocytes in the acute liver injury model that uses D-galactosamine. We also found that the oral or intraperitoneal administration of L.E.M. suppressed immune-mediated liver injury. Therefore, L.E.M. is a promising plant extract for the prevention of liver failure. With the aim of developing effective drugs for liver diseases, we examined the protective effect of a single L.E.M. component against liver injury.

The main components of L.E.M. are sugars, proteins, and polyphenolic compounds. The polyphenols act as antioxidants by scavenging reactive oxygen species (ROS), which produce oxidative stress and can adversely affect many cellular processes. Polyphenols have been proposed to protect against several diseases, including cancers, cardiovascular disease, and neurodegenerative disorders.[8—10] In our previous study, we found that the polyphenol-rich fraction of L.E.M. inhibits hepatic stellate cell activation, which is the main cause of liver fibrosis.[6] Among the polyphenols, syringic acid and vanillic acid are enriched in the solid medium of cultured *L. edodes* mycelia. *L. edodes* grown in lignocellulose secretes lignin-degrading peroxidase into the culture medium.[11] The mycelia-derived enzymes degrade the lignin to produce phenolic compounds, particularly syringic acid and vanillic acid. In the present study, we used a mouse model of liver injury to evaluate the hepatoprotective activity of these compounds.

Concanavalin A (ConA)-induced liver injury is a mouse model of immune-mediated liver injury that resembles viral and autoimmune hepatitis in humans.[12] The intravenous injection of ConA into mice increases the plasma alanine aminotransferase (ALT) level; simultaneously, activated T cells infiltrate the liver, and the apoptosis and necrosis of hepatocytes follows. The activation of T cells by ConA results in increased levels of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-6.[13] In the present study, we found that syringic acid and vanillic acid could suppress ConA-induced liver inflammation and damage in mice.

MATERIALS AND METHODS

Animals BALB/c mice were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 °C before the experiment. Hepatic injury was elicited in 6-week-old male mice by injecting ConA (20 mg/kg body weight) (Seikagaku Biobusiness, Tokyo, Japan) into the tail vein. L.E.M., syringic acid (WAKO, Osaka, Japan) or vanillic acid (WAKO, Osaka, Japan) was administered intraperitoneally just before the ConA adminis-

tration. L.E.M., syringic acid and vanillic acid were dissolved in sterilized phosphate buffered saline (PBS). Blood was collected from the orbital sinus 24 h after the ConA administration and analyzed for transaminases. The blood was sampled at 24 h because the transaminase levels peaked at 24 h after the ConA treatment. The animal experiments were conducted in accordance with the ethical guidelines of the Osaka University Graduate School of Pharmaceutical Sciences.

Analysis of Liver Enzymes　The serum aspartate aminotransferase (AST) and ALT levels were measured by using an assay kit (Transaminase C, WAKO, Osaka, Japan).

Cytokine Determination by ELISA　The IL-6, TNF-α, and IFN-γ levels in serum samples were determined by using a mouse enzyme-linked immunosorbent assay (ELISA) kit (Biosource, San Jose, CA, U.S.A.). Analyses were performed according to the manufacturer's instructions. The blood samples were collected at 3, 6, and 9 h because the cytokine levels increased more rapidly than the transaminases and returned to almost normal levels within 12 h.

Histological Analysis　Liver specimens were fixed in 4% paraformaldehyde and embedded in paraffin. The tissue blocks were cut into 3-μm sections that were mounted on slides and stained with hematoxylin–eosin.

DPPH Radical-Scavenging Activity　The free radical-scavenging activities of L.E.M., syringic acid, and vanillic acid were measured by using the 1,1-diphenyl-2-picrylhdrazyl (DPPH) method.[14] DPPH is a stable free radical that was used for evaluating the scavenging activity by end-point assay. Each compound at the concentration of 0.01 to 1.0 mg/ml was dissolved in ethanol and mixed with DPPH. The reaction was completed within a few minutes. After a 20-min incubation at room temperature in the dark, the absorbance of the sample was read at 517 nm by using a spectrophotometer. The scavenging activity was shown by the decrease in the absorbance at 517 nm.

Preparation of L.E.M.　L.E.M. was prepared as previously reported.[15] Briefly, L. edodes mycelia were cultivated in solid medium composed of sugarcane bagasse and defatted rice bran. To prepare the culture extract, hot water was added to the medium including the mycelia, and the extract was filtered and lyophilized before being used as the L.E.M. preparation.

Statistics　The data were analyzed for statistical significance by the non-parametric Steel–Dwass multiple comparison method. p values less than 0.05 were considered statistically significant.

RESULTS

Effect of L.E.M. on ConA-Induced Liver Injury　We examined the hepatoprotective effect of L.E.M. on ConA-induced liver injury in mice. Various amounts of L.E.M. were injected intraperitoneally just before the ConA injection. Twenty-four hours after ConA treatment, the activities of serum AST and ALT were greatly increased as compared to the untreated control (Fig. 1). The intraperitoneal administration of L.E.M. at 20 mg/kg body weight significantly decreased the AST and ALT levels. When administered orally 2 weeks before the ConA treatment, L.E.M. significantly suppressed the increase in transaminases (data not shown).
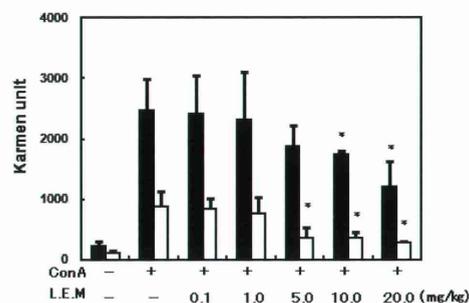

Fig. 1.　Effect of L.E.M. on ConA-Induced Liver Injury

Mice received an intravenous injection of ConA (20 mg/kg) and an intraperitoneal injection of L.E.M. (0.1—20 mg/kg). Blood was collected to determine the serum levels of AST (solid columns) and ALT (open columns). The values are the means±S.D. (n=4). * p<0.05 as compared to ConA treatment alone.
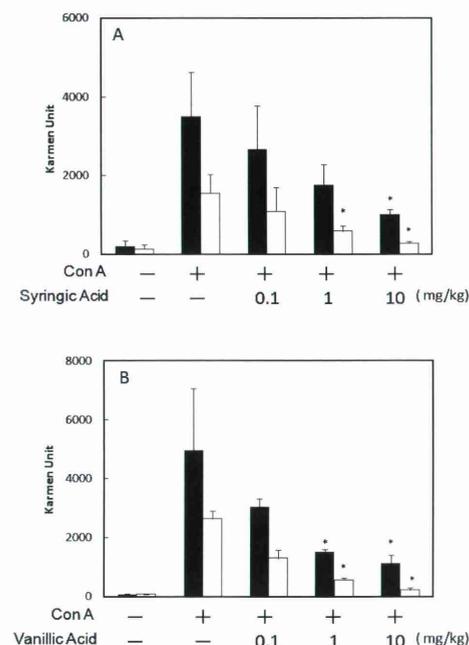

Fig. 2.　Effect of Syringic Acid or Vanillic Acid on Transaminase Leakage in ConA-Induced Liver Injury in Mice

Syringic acid (A) or vanillic acid (B) was injected intraperitoneally shortly before the intravenous injection of ConA (20 mg/kg). Blood was collected to measure the serum levels of AST (solid columns) and ALT (open columns). Values are the means±S.D. (n=4). * p<0.05 as compared to the ConA treatment alone.

These results indicate that L.E.M. has a protective effect against ConA-induced liver injury.

Effect of Syringic and Vanillic Acid on ConA-Induced Liver Injury　We next examined the hepatoprotective effect of syringic and vanillic acid on the ConA-induced liver injury in mice. Syringic or vanillic acid (0.1, 1.0, or 10.0 mg/kg body weight) was injected intraperitoneally just before the ConA injection. The intraperitoneal administration of syringic or vanillic acid dose-dependently decreased the activities of AST and ALT (Fig. 2). To obtain histological evidence for the protection from liver injury, liver sections were prepared and stained with hematoxylin and eosin; representative images are shown in Fig. 3. The structure of the hepatic sinusoids was normal in the sections from untreated mice. In contrast, the hepatic sinusoids were disorganized and inflammatory infiltration was present in the liver sections from the ConA-treated mice, showing that the liver was injured by the tail-vein injection of ConA. Although some hepatocytes
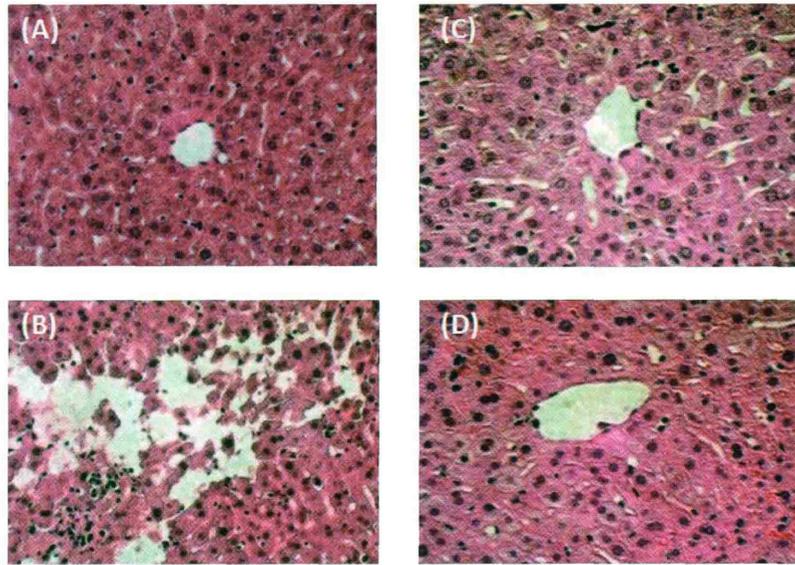
Fig. 3. Suppression of ConA-Induced Liver Injury in Mice That Received Syringic Acid or Vanillic Acid

Sections of paraffin-embedded liver tissue were stained with hematoxylin–eosin. The liver was excised from normal (A), ConA-injured control (B), syringic acid-treated (C), and vanillic acid-treated (D) mice 24 h after ConA injection. Magnification for all photographs, ×400.
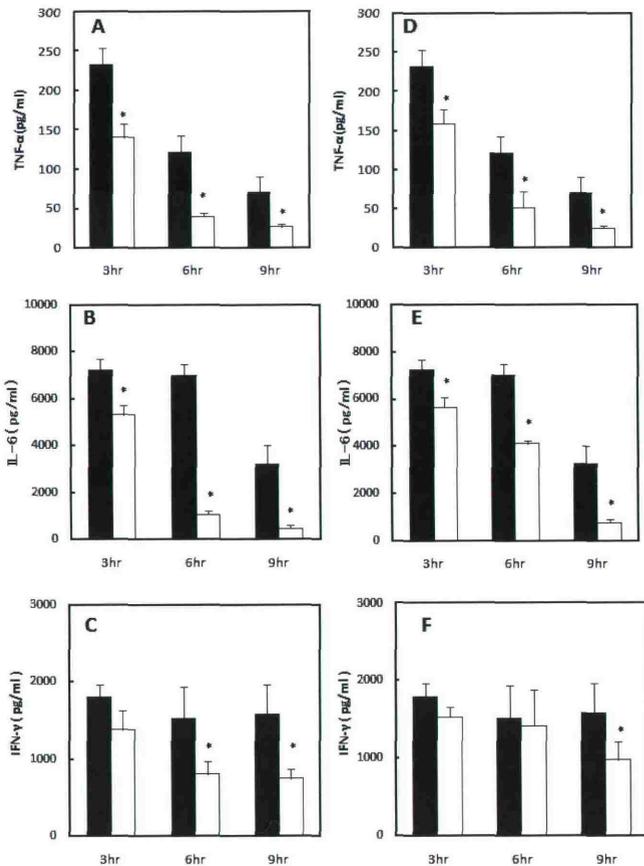

Fig. 4. Changes in Serum TNF-α (A, D), IL-6 (B, E), and IFN-γ (C, F) Levels Measured by ELISA

Mice received an intravenous injection of ConA and intraperitoneal injection of syringic acid (A, B, C) or vanillic acid (D, E, F). Cytokine levels were measured 3, 6, and 9 h after ConA treatment. Solid and open columns indicate the ConA treatment alone and the phenolics treatment, respectively. Values are the means±S.D. ($n=4$). *$p<0.05$ as compared to the ConA treatment alone.
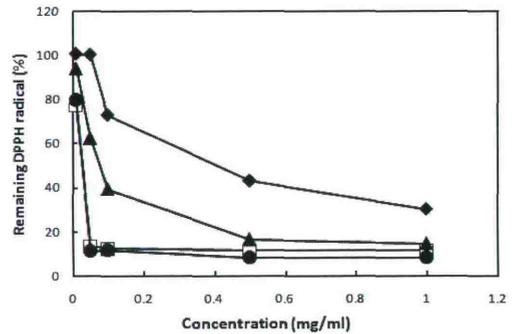

Fig. 5. DPPH Radical-Scavenging Activity of L.E.M., Syringic Acid, and Vanillic Acid

The amount of DPPH radicals was spectrophotometrically determined at 517 nm. Various concentrations of L.E.M. (diamond), syringic acid (open square), and vanillic acid (triangle) were tested. Ascorbic acid (circle) was used as a positive control. Values are the means±S.D. ($n=3$).

lacking nuclei were seen around the vessel, the disorganization caused by the ConA treatment was decreased in the sections from mice treated with 10 mg/kg of syringic acid or vanillic acid (Figs. 3C, D). Next, we measured the TNF-α, IFN-γ, and IL-6 levels in serum 3, 6, and 9 h after ConA treatment (Fig. 4). The intraperitoneal injection of 10 mg/kg of syringic or vanillic acid significantly decreased the cytokine levels in the serum. These results clearly indicate that syringic acid and vanillic acid have a protective effect against ConA-induced liver injury.

DPPH Radical-Scavenging Activity of L.E.M., Syringic Acid, and Vanillic Acid Figure 5 shows the radical scavenging activities of the samples with DPPH as the substrate. L.E.M. had a dose-dependent scavenging activity that was probably derived from the phenolic compounds including syringic acid and vanillic acid. Both syringic acid and vanillic acid had DPPH radical-scavenging activity; syringic acid had a much higher activity than vanillic acid. This anti-oxidation activity could potentially be effective for suppressing oxidative stress-derived liver injury.

DISCUSSION

This study showed that L.E.M., the hot water extract of cultured mycelia, had a hepatoprotective effect against ConA-induced liver injury in mice, a widely used model of viral hepatitis. Since ConA-induced liver injury is caused by the activation of T cells, the potential immunomodulators contained in the L.E.M., including polysaccharides, glucans, and eritadenine[4,16] could be responsible for the suppressive effect of L.E.M. L.E.M. also contains phenolic compounds that have antioxidation activity, and we previously reported that the administration of L.E.M. suppresses oxidative stress-induced liver injury.[6,7] In the present study, we found that the anti-oxidative phenolic compounds syringic acid and vanillic acid strongly suppressed ConA-induced liver injury in mice.

The physiological functions of plant-derived phenolic compounds have been previously described. Syringic and vanillic acid are reported to possess antimicrobial, anti-cancer, and anti-DNA oxidation activities.[17—19] The present study provides the first evidence that both of these compounds suppress transaminase leakage and inflammatory cytokine production in mice that have ConA-induced liver injury. When these phenolics are orally administered to hamsters, they are absorbed and appear in the plasma within 40 min.[20] Thus, although the phenolics were injected intraperitoneally in the present study, oral administration might also elicit a positive effect on liver injury. Furthermore, these compounds can be obtained in large amounts from inexpensive sources, such as sugarcane molasses. Therefore, syringic and vanillic acid might be promising internal medicines or supplements for suppressing the effects of immune-mediated liver injury, such as the persistent inflammation caused by hepatitis virus infection.

Syringic acid and vanillic acid significantly suppressed the increase in the inflammatory cytokines TNF-α, IFN-γ, and IL-6 elicited *in vivo* by the T-cell mitogen, ConA. Therefore, phenolics might alleviate the uncontrolled immune response through immunomodulation. Sharma *et al.* reported that the plant-derived antioxidant, chlorophyllin, inhibits ConA-induced lymphocyte proliferation *in vitro*.[21] Another antioxidant, resveratrol, is reported to inhibit the production of cytokines, such as IFN-γ and TNF-α, in ConA-treated spleen cells and macrophages.[22] Although chlorophyllin and resveratrol possess various activities that could be responsible for these results, their antioxidation activity could be a major contributor to the suppression of lymphocyte activation. Pani reported that the proliferation of mouse thymocytes in response to ConA treatment is strongly inhibited by the ROS scavenger, N-acetylcysteine, and by the inhibitor of NADPH oxidase, diphenyleneiodonium.[23] NADPH oxidase generates ROS after its activation in cells by various types of stimulation.[24] Therefore, the suppressive effect of syringic and vanillic acid on the ConA-induced liver injury might be due to their scavenging of ROS generated by activated NADPH oxidase in the lymphocytes. ConA induces a massive recruitment of activated T cells to the liver. Schwabe reported that ConA-induced liver injury is largely dependent on membrane-bound TNF-α on the infiltrating T cells. The TNF binds to its receptor on hepatocytes to induce ROS production.[24] Syringic acid and vanillic acid could scavenge the ROS to suppress hepatocyte death.

Although syringic and vanillic acids had almost the same effect on liver protection, syringic acid had stronger DPPH activity than vanillic acid. There might be alternative characteristic of these phenolic compounds that are responsible for their liver-protecting effect. Caffeic acid phenethyl ester (CAPE), which is an active phenolic compound contained in propolis, has immunomodulatory and anti-inflammatory properties.[25] Since DNA-binding and transcriptional activities of NF-κB are inhibited in CAPE-treated Jurkat cells, CAPE appears to suppress the proliferation of T cells. Curcumin, a phenolic compound with various biological activities including immunomodulation, suppressed TNF-induced NF-κB-dependent gene transcription.[26] Curcumin and CAPE covalently modify sulfhydryl groups by oxidation and alkylation, and the modification might be responsible for the inhibition of the NF-κB-dependent process. Syringic acid, vanillic acid, and curcumin are phenolic compounds that possess O-methoxy groups. Therefore, it is possible that the immunomodulatory effect of syringic and vanillic acids is mediated by inhibiting the NF-κB-dependent process. In the present study, we showed that the cytokine levels were lowered after the administration of syringic acid or vanillic acid in ConA-treated mice; however, the level of suppression was smaller than the anti-inflammatory effect. The inhibition of TNF-induced NF-κB-dependent processes might play an important role in protection against ConA-induced liver injury.

In addition to the ConA-induced acute liver injury, we found that syringic and vanillic acid extensively suppressed the liver fibrosis elicited by chronic treatment with carbon tetrachloride (to be published elsewhere). Thus, these phenolics appear to have physiologically versatile functions. Further studies on the bioavailability, toxicity, and stability of these compounds are underway. The contents of syringic acid and vanillic acid in L.E.M. are 450 and 378 μg/g, respectively. Thus, the contents are relatively small, and the phenolics might not play a major role in immunomodulation effect of L.E.M. However, these phenolics are small molecules that can be easily synthesized in large amounts by organic reactions. These characteristics have clear advantages over immunomodulating glucan or polysaccharide, which seem to be the major components in L.E.M. for drug development.

REFERENCES

1) Gebhardt R., *Planta Med.*, **68**, 289—296 (2002).
2) McKillop I. H., Schrum L. W., *Alcohol*, **35**, 195—203 (2005).
3) Suzuki H., Okubo A., Yamazaki S., Suzuki K., Mitsuya H., Toda S., *Biochem. Biophys. Res. Commun.*, **160**, 367—373 (1989).
4) Yamada T., Oinuma T., Niihashi M., Mitsumata M., Fujioka T., Hasegawa K., Nagaoka H., Itakura H., *J. Atheroscler. Thromb.*, **9**, 149—156 (2002).
5) Yamamoto Y., Shirono H., Kono K., Ohashi Y., *Biosci. Biotechnol. Biochem.*, **61**, 1909—1912 (1997).
6) Akamatsu S., Watanabe A., Tamesada M., Nakamura R., Hayashi S., Kodama D., Kawase M., Yagi K., *Biol. Pharm. Bull.*, **27**, 1957—1960 (2004).
7) Watanabe A., Kobayashi M., Hayashi S., Kodama D., Isoda K., Kondoh M., Kawase M., Tamesada M., Yagi K., *Biol. Pharm. Bull.*, **29**, 1651—1654 (2006).
8) Aggarwal B. B., Kumar A., Bharti A. C., *Anticancer Res.*, **23**, 363—398 (2003).

9) Aviram M., Dornfeld L., Kaplan M., Coleman R., Gaitini D., Nitecki S., Hofman A., Rosenblat M., Volkova N., Presser D., Attias J., Hayek T., Fuhrman B., *Drugs Exp. Clin. Res.*, **28**, 49—62 (2002).

10) Levites Y., Weinreb O., Maor G., Youdim M. B., Mandel S., *J. Neurochem.*, **78**, 1073—1082 (2001).

11) Forrester I. T., Grabski A. C., Mishra C., Kelley B. D., Strickland W. N., Leatham G. F., Burgess R. R., *Appl. Microbiol. Biotechnol.*, **33**, 359—365 (1990).

12) Tiegs G., Hentschel J., Wendel A., *J. Clin. Invest.*, **90**, 196—203 (1992).

13) Cao Q., Batey R., Pang G., Russell A., Clancy R., *Immunol. Cell Biol.*, **76**, 542—549 (1998).

14) Velazquez C., Navarro M., Acosta A., Angulo A., Dominguez Z., Robles R., Robles Zepeda R., Lugo E., Goycoolea F. M., Velazquez E. F., Astiazaran H., Hernandez J., *J. Appl. Microbiol.*, **103**, 1747—1756 (2007).

15) Mizoguchi Y., Katoh H., Kobayashi K., Yamamoto S., Morisawa S., *Gastroenterol. Jpn.*, **22**, 459—464 (1987).

16) Wasser S. P., Weis A. L., *Crit. Rev. Immunol.*, **19**, 65—96 (1999).

17) Aziz N. H., Farag S. E., Mousa L. A., Abo Zaid M. A., *Microbios*, **93**, 43—54 (1998).

18) Guimaraes C. M., Giao M. S., Martinez S. S., Pintado A. I., Pintado M. E., Bento L. S., Malcata F. X., *J. Food Sci.*, **72**, C039—C043 (2007).

19) Kampa M., Alexaki V. I., Notas G., Nifli A. P., Nistikaki A., Hatzoglou A., Bakogeorgou E., Kouimtzoglou E., Blekas G., Boskou D., Gravanis A., Castanas E., *Breast Cancer Res.*, **6**, R63—R74 (2004).

20) Chen C. Y., Milbury P. E., Kwak H. K., Collins F. W., Samuel P., Blumberg J. B., *J. Nutr.*, **134**, 1459—1466 (2004).

21) Sharma D., Kumar S. S., Sainis K. B., *Mol. Immunol.*, **44**, 347—359 (2007).

22) Gao X., Xu Y. X., Janakiraman N., Chapman R. A., Gautam S. C., *Biochem. Pharmacol.*, **62**, 1299—1308 (2001).

23) Pani G., Colavitti R., Borrello S., Galeotti T., *Biochem. J.*, **347 Pt 1**, 173—181 (2000).

24) Bedard K., Krause K. H., *Physiol. Rev.*, **87**, 245—313 (2007).

25) Marquez N., Sancho R., Macho A., Calzado M. A., Fiebich B. L., Munoz E., *J. Pharmacol. Exp. Ther.*, **308**, 993—1001 (2004).

26) Aggarwal S., Ichikawa H., Takada Y., Sandur S. K., Shishodia S., Aggarwal B. B., *Mol. Pharmacol.*, **69**, 195—206 (2006).