The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Retinoic acid dramatically enhances cytotoxicity of equol against human monoblastic U937 cells, but not against human peripheral neutrophils

Hidehiko Kikuchi[1], Kaori Harata[1], Chikage Kawai[2], Harishkumar Madhyastha[3], Akira Yamauchi[2] and Futoshi Kuribayashi[2]

[1]Department of Food and Nutrition, Shokei University Junior College, 2-6-78 Kuhonji, Chuo-ku, Kumamoto 862-8678, Japan
[2]Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama, 701-0192, Japan
[3]Department of Applied Physiology, Faculty of Medicine, University of Miyazaki, 5200, Kihara, Kiyotake, Miyazaki 889-1692, Japan

(Received May 11, 2020; Accepted May 16, 2020)

ABSTRACT — Since phytoestrogens (e.g. biochanin A, coumestrol, daidzein, genistein and glycitein) are structurally similar to estrogen, they mimic estrogen function. Phytoestrogens consist in certain edible plants, especially in soybeans. Among them, interestingly, equol that has the greatest estrogenic activity is generated from daidzein by gut microbes. Therefore, the relationship between phytoestrogens and gut (including intestinal cells and bacteria) is being watched with strong interest. In this paper, we revealed that all-*trans* retinoic acid (RA) dramatically enhances cytotoxicity of several phytoestrogens against U937 cells. While β-estradiol and phytoestrogens tested showed no effect on the viability of U937 cells in the absence of RA, 10 μM coumestrol, (±)-equol and genistein brought about remarkably reduced viability of U937 cells at 24 h (to ~15%, ~7% and ~35%, respectively) in the presence of 1 μM RA. In particular, the cytotoxicity of (±)-equol was drastically enhanced in the presence of RA. Moreover, very interestingly, (±)-equol showed no effect on the viability of human peripheral neutrophils even in the presence of RA. As is well known, human monoblastic leukemia U937 cells have been used as an *in vitro* model for macrophage that exists in intestine and plays significant roles to maintain intestinal homeostasis. These data suggest that equol not only can serve as an effective modifier in therapy for leukemia in combination with RA but also may affect the maintenance of intestinal homeostasis.

Key words: Phytoestrogen, Equol, Cytotoxicity, Macrophage, Neutrophil, U937

INTRODUCTION

Polyphenol compounds are secondary metabolites in plants, and protect them from various harms such as ultraviolet irradiation and numerous infections. They are contained in usual human diet and show various biological activities including nutritional effects. Therefore, polyphenols are increasingly gaining considerable attention for maintaining human health. Phytoestrogens belonging to plants-derived polyphenol compounds not only are structurally similar to β-estradiol (a typical estrogen) but also mimic its biological function (Schmitt *et al*., 2003; Desmawati and Sulastri, 2019; Kiyama, 2019; Hasan *et al*., 2020). As is well known, phytoestrogens (e.g. biochanin A, coumestrol, daidzein, genistein and glycitein) consist in certain edible plants, above all abundantly in

Correspondence: Hidehiko Kikuchi (E-mail: masakari@shokei-gakuen.ac.jp)

soy (Jargin, 2014). In addition, equol is generated from daidzein by gut microbes (Rafii, 2015; Mayo *et al.*, 2019). They show agonist or antagonist actions on estrogen receptor-mediated processes dependent on the cells or tissues. For example, phytoestrogens are known to modulate multiple target molecules in breast cancer cells (Basu and Maier, 2018; Molina *et al.*, 2018). In addition, phytoestrogens have also effects on anti-aging (Liu *et al*., 2020), immunomodulation (Soria-Jasso *et al.*, 2019), developing brain (Rosenfeld, 2019), colorectal neoproliferative lesions (Viggiani *et al.*, 2019), vaginal health and dyspareunia (Dizavandi *et al*., 2019), intestinal microbiome (Kolatorova *et al*., 2018; Rosenfeld, 2019), urinary incontinence, pelvic organ prolapse and fecal incontinence (Cardenas-Trowers *et al.*, 2018). While genistein, one of phytoestrogens, induces apoptotic cell death in various types of leukemia (Yamasaki *et al*., 2010, 2013; Li *et al.*, 2011; Hsiao *et al*., 2019), it also can induce infant leukemia cell death (Azarova *et al.*, 2010).

As mentioned above, intestinal bacteria converts daidzein to equol (Rafii, 2015; Mayo *et al*., 2019). Although equol is the phytoestrogen with the greatest estrogenic activity (Mayo *et al*., 2019), only about 30 ~ 50% of human can produce equol in the intestine (Rafii, 2015). Equol has great influences on atherosclerosis, arterial stiffness, decline/dementia (Sekikawa *et al*., 2019), developing brain, gut microbiota, neurobehavioral disorders (Rosenfeld, 2019). However, the understanding of the effects of equol against leukemia cells is still poor.

In this paper, we investigated the effects of β-estradiol and various phytoestrogens on viability of human monoblastic U937 cells, and revealed that all-*trans* retinoic acid (RA) dramatically enhances cytotoxicity of several phytoestrogens against the cells.

MATERIALS AND METHODS

Materials

Biochanin A, (±)-equol and genistein (Tokyo Chemical Industry, Tokyo, Japan), daidzein, β-estradiol and glycitein (Fujifilm Wako, Osaka, Japan), coumestrol (Cayman Chemical, Ann Arbor, MI, USA), RPMI-1640 culture medium and trypan blue solution (Gibco Laboratories, Gaithersburg, MD, USA), RA (Sigma, St Louis, MO, USA), fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) and plasmocin (InvivoGene, San Diego, CA, USA) were obtained from companies indicated respectively.

Cell culture and treatment with various reagents

Human monoblastic leukemia U937 cells (RCB0435) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin as described (Kikuchi and Imajoh-Ohmi, 1995; Kikuchi *et al*., 1996, 2011, 2018, 2019; Akiyoshi *et al*., 2019). Cells (1.0×10^6) in 5 mL of culture medium were incubated in the presence of 10 μM various reagents (biochanin A, coumestrol, daidzein, (±)-equol, β-estradiol, genistein or glycitein) and 1 μM RA for 24 hr. Concerning (±)-equol, cells (1.0×10^6) in 5 mL of culture medium were also incubated with 1 ~ 5 μM (±)-equol in the presence of 1 μM RA at 37°C for up to 48 hr. A hemocytometer was used to count total cells under the microscope. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi *et al*., 2016).

Peripheral neutrophils

Human neutrophils were prepared from peripheral blood of a healthy volunteer using Lymphoprep (Stemcell Technologies, Vancouver, Canada) followed by hypotonic treatment. Human neutrophils (1.0×10^6 cells/mL) were suspended in PBS containing 10% fetal calf serum (FCS) and 1 mM EDTA. In the absence or presence of 1 μM RA, human neutrophils (1.0×10^6 cells/mL) were cultured with 5 μM daidzein or 5 μM (±)-equol in the same buffer (PBS/10% FCS/1 mM EDTA) at 37°C for 24 hr. A hemocytometer was used to count total cells under the microscope. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi *et al*., 2016).

Statistical analysis

Data obtained with cell viability assays are presented as averages of four (U937 cells) or eight (neutrophils) separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

Ethical considerations

This experiment complies with the Declaration of Helsinki (adopted in 1964 and amended in 2013) and ethical approval for this study was obtained from the Research Ethics Committee of Kawasaki Medical School and Hospital (Approval Number: 3615).

RESULTS AND DISCUSSION

First, in order to study the effects of β-estradiol and various phytoestrogens (biochanin A, coumestrol, daidzein, (±)-equol, genistein and glycitein Fig. 1) on viability of U937 cells, the cells were treated with 10 μM

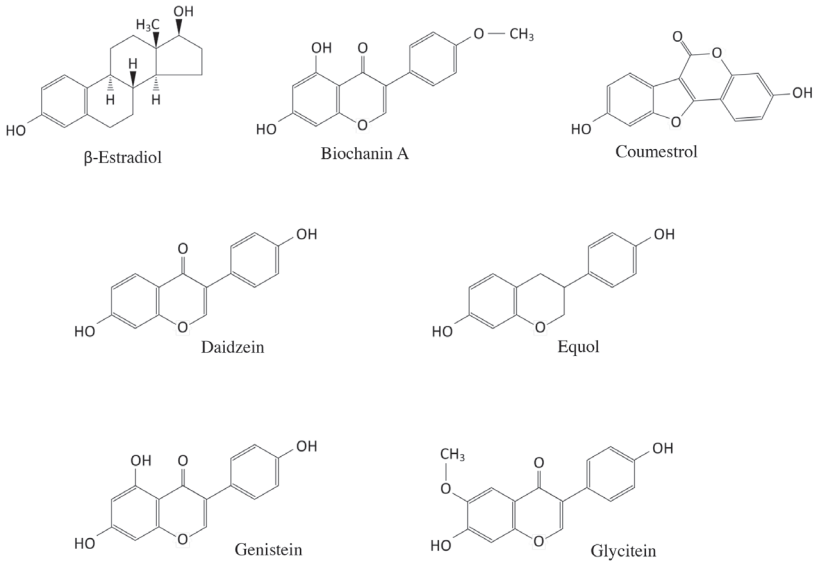

Fig. 1. Chemical structures of estrogen and phytoestrogens.

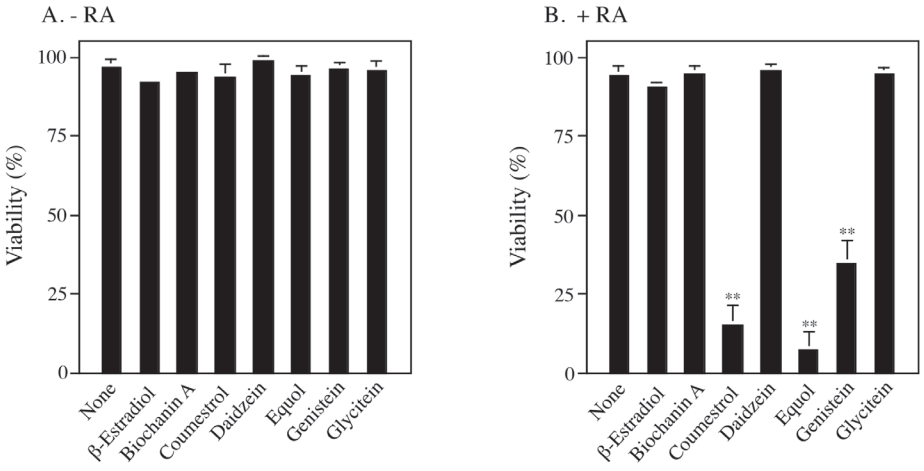

Fig. 2. Effects of RA on cytotoxicity of estrogen and phytoestrogens against U937 cells. (A) Cytotoxicity of β-estradiol and phytoestrogens in the absence of RA. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated with 10 μM of each reagent at 37°C for 24 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of four separate experiments. Statistical differences were calculated using Student's *t* test. Error bars indicate standard deviation. (B) Cytotoxicity of β-estradiol and phytoestrogens in the presence of RA. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated with 10 μM of each reagent in the presence of 1 μM RA at 37°C for 24 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of four separate experiments. Statistical differences were calculated using Student's *t* test. Error bars indicate standard deviation. **, $p < 0.01$ compared with the data without β-estradiol or phytoestrogens (reffered to as "None").

of each reagent. β-Estradiol and phytoestrogens tested showed no effect on the viability of U937 cells in the absence of RA at 24 hr (Fig. 2A). On the other hand, coumestrol, (±)-equol and genistein brought about remarkably reduced viability of U937 cells (to ~15%, ~7% and ~35%, respectively) in the presence of RA (Fig. 2B). In contrast, β-estradiol and other phytoestrogens used showed no effect on the viability of U937 cells even in the presence of RA (Fig. 2B).

Second, to investigate the influences of these three

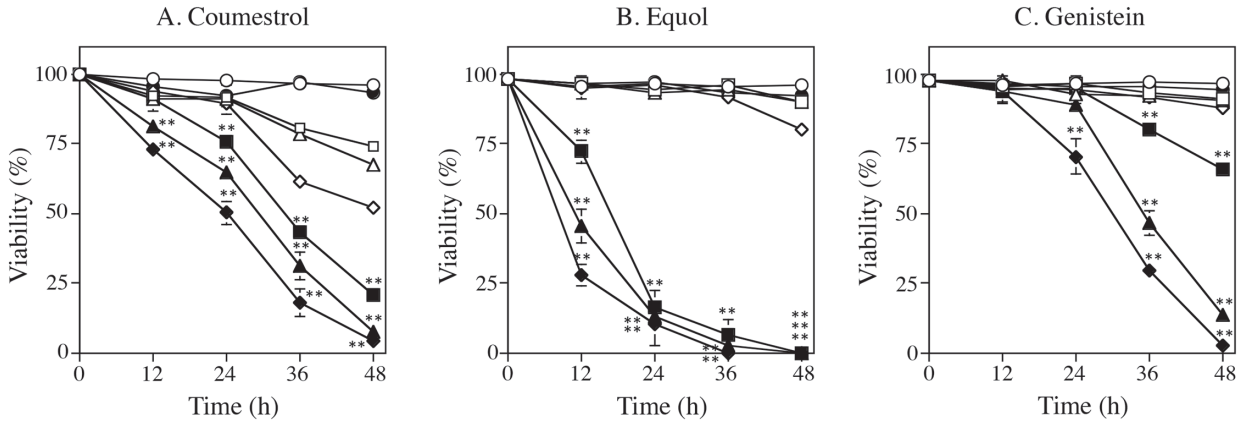

Fig. 3. Influences of RA on cytotoxicity of coumestrol, (±)-equol and genistein. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated with coumestrol (A), (±)-equol (B) and genistein (C) (0 μM: circles, 1 μM: squares, 2 μM: triangles, 5 μM: lozenges) in the absence (open symbols) or presence (closed symbols) of 1 μM RA at 37°C for up to 48 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of four separate experiments. Statistical differences were calculated using Student's *t* test. Error bars indicate standard deviation. **, $p < 0.01$ compared with the data of without RA at each time point.

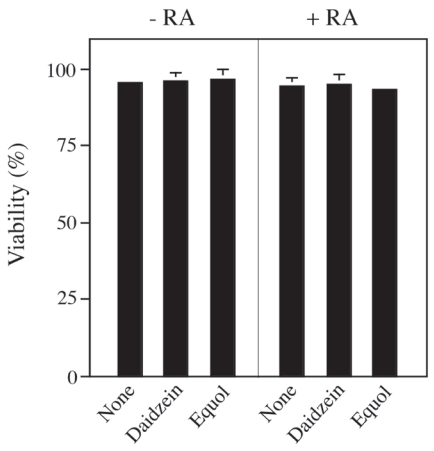

Fig. 4. Effects of combination of RA and daidzein or (±)-equol on cytotoxicity against human peripheral neutrophils. Human peripheral neutrophils (1.0 x 10^6 cells/mL) were cultured with 5 μM daidzein or 5 μM (±)-equol in the absence or presence of 1 μM RA at 37°C for 24 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the average of eight separate experiments. Statistical differences were calculated using Student's *t* test. Error bars indicate standard deviation. Either with or without RA, there were no significant differences between without daidzein or (±)-equol (reffered to as "None") and with daidzein or (±)-equol.

phytoestrogens on cytotoxicity in the presence of RA in more detail, we examined the dose-dependency of them on viability. As shown in Fig. 3, in the presence of RA, three phytoestrogen tested showed remarkable cytotoxicity in a dose-dependent manner. In particular, the cytotoxicity of (±)-equol was drastically enhanced in the presence of RA (Fig. 3B). While daidzein showed little cytotoxicity with or without RA, interestingly, (±)-equol generated from daidzein displayed very strong cytotoxicity in the presence of RA. As is well known, (±)-equol is generated from daidzein by intestinal bacteria. These findings suggested that conversion from daidzein to (±)-equol by intestinal bacteria brings about drastic strong cytotoxicity against monoblastic U937 cells that have been used as an *in vitro* model for macrophage in the presence of RA. In other words, intestinal bacteria can affect the intestinal cells including macrophages through conversion from daidzein to (±)-equol.

Finally, to examine the effects of equol on cytotoxicity against normal cells (not leukemia cells) in the presence of RA, human peripheral neutrophils were incubated with 5 μM daidzein or (±)-equol in the presence of 1 μM RA for 24 hr. Very interestingly, not only daidzein but also (±)-equol showed no effect on the viability of human peripheral neutrophils with or without RA (Fig. 4). These results suggested that the combination of RA and equol can selectively induce cell death of leukemia cells without killing normal leukocytes.

Our results revealed that RA dramatically enhances

cytotoxicity of some phytoestrogens, especially (±)-equol, against human monoblastic leukemia U937 cells that have been used as an *in vitro* model for macrophage. As is well known, macrophages exist in intestine and play significant roles to maintain intestinal homeostasis (Bain and Schridde, 2018). These findings suggested that conversion from daidzein to equol may affect the intestinal homeostasis. Moreover, these results in this paper propose that combination of RA and (±)-equol has the potential to serve as an effective tool in therapy for leukemia. However, further studies are needed before practical application as therapy for leukemia. Studies on cytotoxicity of phytoestrogens may be more important for both maintenance of intestinal homeostasis and development of tumor treatment in the future.

ACKNOWLEDGMENTS

We thank R. Madhyastha for editorial reading of the manuscript. This work was supported in part by JSPS KAKENHI Grant Number 19K02329 (to H. K.) and 18K07804 (to F. K.).

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Akiyoshi, S., Kikuchi, H., Kuribayashi, F. and Madhyastha, H. (2019): Sulforaphane displays the growth inhibition, cytotoxicity and enhancement of retinoic acid-induced superoxide-generating activity in human monoblastic U937 cells. Fundam. Toxicol. Sci., **6**, 319-325.

Azarova, A.M., Lin, R.K., Tsai, Y.C., Liu, L.F., Lin, C.P. and Lyu, Y.L. (2010): Genistein induces topoisomerase IIbeta- and proteasome-mediated DNA sequence rearrangements: implications in infant leukemia. Biochem. Biophys. Res. Commun., **399**, 66-71.

Bain, C.C. and Schridde, A. (2018): Origin, differentiation, and function of instetinal macrophages. Front. Immunol., **9**, 2733.

Basu, P. and Maier, C. (2018): Phytoestrogens and breast cancer: *in vitro* anticancer activities of isoflavones, lignans, coumestans, stilbenes and their analogs and derivatives. Biomed. Pharmacother., **107**, 1648-1666.

Cardenas-Trowers, O., Meyer, I., Markland, A.D., Richter, H.E. and Addis, I. (2018): A review of phytoestrogens and their association with pelvic floor conditions. Female Pelvic Med. Reconstr. Surg., **24**, 193-202.

Desmawati, D. and Sulastri, D. (2019): Phytoestrogens and their health effect. Open Access Maced. J. Med. Sci., **14**, 495-499.

Dizavandi, F.R., Ghazanfarpour, M., Roozbeh, N., Kargarfard, L., Khadivzadeh, T. and Dashti, S. (2019): An overview of the phytoestrogen effect on vaginal health and dyspareunia in peri- and post-menopausal women. Post Reprod. Health, **25**, 11-20.

Hasan, M., Kumolosasi, E., Jasamai, M., Jamal, J.A., Azmi, N. and Rajab, N.F. (2020): Evaluation of phytoestrogens in inducing cell death mediated by decreasing annexin A1 in annexin A1-knockdown leukemia cells. DARU, **28**, 97-108.

Hsiao, Y.C., Peng, S.F., Lai, K.C., Liao, C.L., Huang, Y.P., Lin, C.C., Lin, M.L., Liu, K.C., Tsai, C.C., Ma, Y.S. and Chung, J.G. (2019): Genistein induces apoptosis *in vitro* and has antitumor activity against human leukemia HL-60 cancer cell xenograft growth *in vivo*. Environ. Toxicol., **34**, 443-456.

Jargin, S.V. (2014): Soy and phytoestrogens: possible side effects. Ger. Med. Sci., **12**, Doc18.

Kikuchi, H. and Imajoh-Ohmi, S. (1995): Activation and possible involvement of calpain, a calcium-activated systeine protease, in down-regulation of apoptosis of human monoblast U937 cells. Cell Death Differ., **2**, 195-199.

Kikuchi, H., Iizuka, R., Sugiyama, S., Gon, G., Mori, H., Arai, M., Mizumoto, K. and Imajoh-Ohmi, S. (1996): Monocytic differentiation modulates apoptotic response to cytotoxic anti-Fas antibody and tumor necrosis factor α in human monoblast U937 cells. J. Leukoc. Biol., **60**, 778-783.

Kikuchi, H., Kuribayashi, F., Kiwaki, N., Takami, Y. and Nakayama, T. (2011): GCN5 regulates the superoxide-generating system in leukocytes via controlling gp91-phox gene expression. J. Immunol., **186**, 3015-3022.

Kikuchi, H., Kuribayashi, F., Mimuro, H. and Imajoh-Ohmi, S. (2016): GCN5-deficiency remarkably enhances the sensitivity of B cells in response to 4-nitroquinoline 1-oxide. Fundam. Toxicol. Sci., **3**, 137-142.

Kikuchi, H., Mimuro, H. and Kuribayashi, F. (2018): Resveratrol strongly enhances the retinoic acid-induced superoxide generating activity via up-regulation of gp91-phox gene expression in U937 cells. Biochem. Biophys. Res. Commun., **495**, 1195-1200.

Kikuchi, H., Mimuro, H., Madhyastha, H. and Kuribayashi, F. (2019): Chalcone skeleton promotes transcription of gp91-phox gene but inhibits expression of gp91-phox protein, and hydroxyl groups in hydroxychalcones participate in the stable expression of gp91-phox protein. Microbiol. Immunol., **63**, 438-443.

Kiyama, R. (2019): Estrogenic activity of coffee constituents. Nutrients, **11**, E1401.

Kolatorova, L., Lapcik, O. and Starka, L. (2018): Phytoestrogens and the intestinal microbiome. Physiol. Res., **67**, S401-S408.

Li, W., Frame, L.T., Hoo, K.A., Li, Y., D'Cunha, N. and Cobos, E. (2011): Genistein inhibited proliferation and induced apoptosis in acute lymphoblastic leukemia, lymphoma and multiple myeloma cells *in vitro*. Leuk. Lymphoma, **52**, 2380-2390.

Liu, T., Li, N., Yan, Y.Q., Liu, Y., Xiong, K., Liu, Y., Xia, Q.M., Zhang, H. and Liu, Z.D. (2020): Recent advances in the anti-aging effects of phytoestrogens on collagen, water content, and oxidative stress. Phytother. Res., **34**, 435-447.

Mayo, B., Vazquez, L. and Florez, A.B. (2019): Equol: A bacterial metabolite from the daidzein isoflavone and its presumed beneficial health effects. Nutrients, **11**, E2231.

Molina, L., Bustamante, F.A., Bhoola, K.D., Figueroa, C.D. and Ehrenfeld, P. (2018): Possible role of phytoestrogens in breast cancer via GPER-1/GPR30 signaling. Clin. Sci. (Lond.), **132**, 2583-2598.

Rafii, F. (2015): The role of colonic bacteria in the metabolism of the natural isoflavone daidzein to equol. Metabolites, **5**, 56-73.

Rosenfeld, C.S. (2019): Effects of phytoestrogens on the developing brain, gut microbiota, and risk for neurobehavioral disorders. Front. Nutr., **6**, 142.

Schmitt, E., Metzler, M., Jonas, R., Dekant, W. and Stopper, H. (2003): Genotoxic activity of four metabolites of the soy isoflavone daidzein. Mutat. Res., **542**, 43-48.

Sekikawa, A., Ihara, M., Lopez, O., Kakuta, C., Lopresti, B.,

Higashiyama, A., Aizenstein, H., Chang, Y.F., Mathis, C., Miyamoto, Y., Kuller, L. and Cui, C. (2019): Effect of S-equol and soy isoflavones on heart and brain. Curr. Cardiol. Rev., **15**, 114-135.

Soria-Jasso, L.E., Carino-Cortes, R., Munoz-Perez, V.M., Perez-Hernandez, E., Perez-Hernandez, N. and Fernandez-Martinez, E. (2019): Beneficial and deleterious effects of female sex hormones, oral contraceptives, and phytoestrogens by immunomodulation on the liver. Int. J. Mol. Sci., **20**, E4694.

Viggiani, M.T., Polimero, L., Di Leo, A. and Barone, M. (2019): Phytoestrogens: dietary intake, bioavailability, and protective mechanisms against colorectal neoproliferative lesions. Nutrients, **11**, E1709.

Yamasaki, M., Mukai, A., Ohba, M., Mine, Y., Sakakibara, Y., Suiko, M., Morishita, K. and Nishiyama, K. (2010): Genistein induced apoptotic cell death in adult T-cell leukemia cells through estrogen receptors. Biosci. Biotechnol. Biochem., **74**, 2113-2115.

Yamasaki, M., Mine, Y., Nishimura, M., Fujita, S., Sakakibara, Y., Suiko, M., Morishita, K. and Nishiyama, K. (2013): Genistein induces apoptotic cell death associated with inhibition of the NF-κB pathway in adult T-cell leukemia cells. Cell Biol. Int., **37**, 742-747.