THE EFFECT OF PHYTIC ACID ON THE EXPRESSION OF NF-κB, IL-6 AND IL-8 IN IL-1β-STIMULATED HUMAN COLONIC EPITHELIAL CELLS

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Abstract: Intestinal epithelial cells play an important role in the mucosal immune and inflammatory reactions *via* the expression and secretion of proinflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8). The expression of both interleukins is regulated by nuclear factor κB (NF-κB). Phytic acid (IP6) is an essential component of high fiber diet. It is physiologically present in the human large gut at concentrations reaching 4 mM. It exhibits pleiotropic health beneficial effects including anti-oxidant and anti-tumor activities. Recent studies showed that IP6 can modulate immune functions of intestinal epithelium through regulation of proinflammatory cytokines secretion. The aim of this study was to analyze the effect of IP6 on the expression of IL-6 and IL-8 as well as p50 and p65 subunits of NF-κB and its inhibitor IκBα in Caco-2 cells stimulated with IL-1β. A kinetic study of mRNAs expression in cells was performed after their treatment with 1 and 2.5 mM IP6 for 3, 6 and 12 h. Quantification of the genes expression was carried out using real time QRT-PCR technique. IP6 at all used concentrations had no influence on transcription of p65 gene and modulated expression of p50 and IκBα genes in Caco-2 cells. Treatment of cells with IP6 resulted in a marked decrease in both IL-6 (at 3 and 6 h) and IL-8 expression (3 h). The results of these studies suggest that IP6 may exert immunoregulatory effects on intestinal epithelium by influencing transcriptional activity of genes encoding p50 subunit of NF-κB, its inhibitor IkBα and proinflammatory cytokines IL-6 and IL-8.

Keywords: phytic acid, IL-8, IL-6, IL-1β, intestinal epithelial cells Caco-2, NF-κB, real-time QRT-PCR, mRNA quantification

Intestinal epithelial cells (IEC), in addition to their barrier role, appear to function as an integral component of the mucosal immune system (1). They may serve as targets for locally produced inflammatory mediators, such as interleukin-1β (IL-1β) (2). The levels of this cytokine are elevated in various intestinal pathologies including inflammatory bowel diseases (IBD) and intestinal neoplasia (3). IL-1β activates intracellular signaling cascades in IEC leading to the increase of expression and secretion of proinflammatory mediators (cytokines and chemokines), uncontrolled intestinal inflammation and disruption of epithelial function (2). A common effector of these pathways is nuclear factor κB (NF-κB), a pleiotropic transcription factor that regulates the expression of multiple genes involved in the inflammatory response (4, 5). The mammalian NF-κB family consists of five homologous subunits: RelA/p65, NF-κB1/p50, c-Rel, RelB, NF-κB2/p52. All members of this family form homo- or heterodimers that translocate to the nucleus and bind to DNA sequences on the regulatory regions of target genes and regulate their transcription. NF-κB signaling pathway is regulated by regulatory IκB proteins, which bind to NF-κB dimers and block their nuclear localization, thereby causing their cytoplasmic retention (6). Disturbances in NF-κB signaling pathway are known to contribute to pathogenesis of various diseases, also IBD (5, 6).

IEC also participate in mucosal immune and inflammatory response *via* the expression and secretion of cytokines and chemokines such as IL-8 and IL-6 (1). IL-8 is a potent chemotactic peptide for neutrophils and lymphocytes which infiltrate intestinal tissue in IBD (7). IL-6 is a multifunctional cytokine and one of the most important factors regulating immune response and inflammation. Increased expression and secretion of IL-8 and IL-6 is observed in acute and chronic inflammation and related cancers in the gastrointestinal tract (8, 9).

Recent evidence suggests that bioactive natural dietary products may play a role in the alleviation

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and control of inflammation-related diseases (10). Phytic acid (IP6) is an essential component of high fiber diet which is physiologically present in the human large gut at concentrations reaching 4 mM. It exhibits pleiotropic health beneficial effects including anti-oxidant and anti-tumor activities. IP6 has been shown to target cancer cells through multiple pathways, including modulation of cell signal transduction, inhibition of cell proliferation and cell cycle progression, activation of apoptosis and induction of cell differentiation (11, 12). Recent studies showed that IP6 may have an impact on the inflammatory response by modulating the expression and secretion of cytokines IL-8, IL-6, TGF-β (13, 14).

The aim of this study was to examine the role of IP6 in the expression of genes encoding p65 and p50 subunits of NF-κB and its inhibitor IκBα as well as the expression of IL-6 and IL-8 genes in IL-1β-stimulated intestinal epithelial cells Caco-2.

EXPERIMENTAL

Cell culture

The Caco-2 human colon adenocarcinoma cells were purchased from the American Type Culture Collection (ATCC). The cells were routinely grown in RPMI 1640 medium (Sigma Aldrich) complemented with 10% fetal bovine serum (FBS; PAA), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma Aldrich) and 10 mM Hepes (Sigma Aldrich). The cell cultures were maintained at $37^\circ C$ in a humidified atmosphere containing 5% CO_2.

Cell culture for gene expression determination

To determine p50, p65, IκBa, IL-8 and IL-6 genes expression, Caco-2 cells were seeded into 6-well tissue culture plates (Nunc) at an initial density of 4.5×10^5 cells/well in 3 mL RPMI 1640 medium complemented with the components given above. After three days the culture media were changed to media with 2% FBS and cells were then cultured for 24 h. Afterwards, cells were stimulated with 1 ng/mL IL-1β (Sigma Aldrich) for 30 min. They were then treated with 1 and 2.5 mM IP6 as dipotassium salt (Sigma Aldrich) for 3, 6 and 12 h. As a control, Caco-2 cells were incubated under the same conditions without IP6. Control and the agent treatments were done in triplicate.

RNA extraction

Total cellular RNA was extracted from control and treated with IL-1β and IP6 cells with the use of TRIZOL® reagent (Invitrogen) according to the manufacturer's protocol. Integrity of isolated RNA was checked by electrophoresis in 1% agarose gel stained with subsequent visualization by ethidium bromide. The RNA concentration was determined spectrophotometrically on the basis of absorbance values at a wavelength of 260 nm using a GeneQuant pro RNA/DNA Calculator (Amersham Biosciences).

Real-time QRT-PCR assay

Transcriptional activity of examined genes was evaluated on the basis of copy number of mRNA related to 1 mg of total RNA by the use of real time QRT-PCR technique with Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research, Watertown, MA). The levels of the p50, p65, IκBa and IL-8 transcripts were evaluated with the use a SYBR Green (SYBR Green Quantitect RT-PCR Kit, QIAGEN, Valencia, CA, USA) as described previously (15). Oligonucleotide primers specific for p50, p65, IκBα and IL-8 mRNAs were presented elsewhere (16, 17). The expression of IL-6 mRNA was evaluated by the use of real time QRT-PCR TaqMan technique with commercially available kits of oligonucleotide primers and probe specific for IL-6 ((Hs 0017431_m1; Applied Biosystems) (18). QRT-PCR assay was performed in triplicate for each sample. The mRNA copy numbers of all examined genes were determined on the basis of the commercially available standards of β-actin cDNA (TaqMan DNA Template Reagent Kit, Applied Biosystems).

Statistical analysis

Statistical analysis was performed with the use of Statistica PL ver. 9.0 Software (StatSoft). All the results were expressed as the means ± SD. Student's *t*-test was used to assess statistical significances of difference between two groups. For multiple comparisons, one-way analysis of variance (ANOVA) was performed followed by *post-hoc* Tukey's test. Differences with a probability (p) value less than 0.05 were considered statistically significant.

RESULTS

Changes in the expression of p50, p65 and IκBα genes in IL-1β-stimulated Caco-2 cells treated with IP6

The expression of p50, p65 and IκBα mRNAs was detected in both control cells and cells treated with IP6 for 3, 6 and 12 h. No statistically significant changes in the p50 gene expression in cells treated with 1 mM IP6 for 3 h were found compared to control cells ($p > 0.05$; ANOVA) (Fig. 1A).

However, the level of p50 transcript in cells exposed to 2.5 mM IP6 was significantly lower than in the control (p = 0.013; ANOVA) (Fig. 1A). At 6 h in all cell cultures treated with IP6 the increase of p50 mRNA was observed, though with no statistically significant difference (p > 0.05; ANOVA) (Fig. 1A). The level of p50 expression in cells exposed to IP6 for 12 h was insignificantly lower than in untreated cells (1 mM IP6 vs. control p = 0.067; 2.5 mM IP6 vs. control p = 0.169; ANOVA) (Fig. 1A).

Gene encoding p65 was found to be expressed at the similar level in both untreated and treated with IP6 cells at different concentrations. Transcriptional activity of this gene in cells treated with IP6 for 3, 6 and 12 h showed no statistically significant changes compared to control cells (p > 0.05; ANOVA) (Fig. 1B).

Short-term (3 h) treatment of cells with 1 mM and 2.5 mM IP6 decreased IκBα expression in comparison to untreated cells, but statistically significant reduction
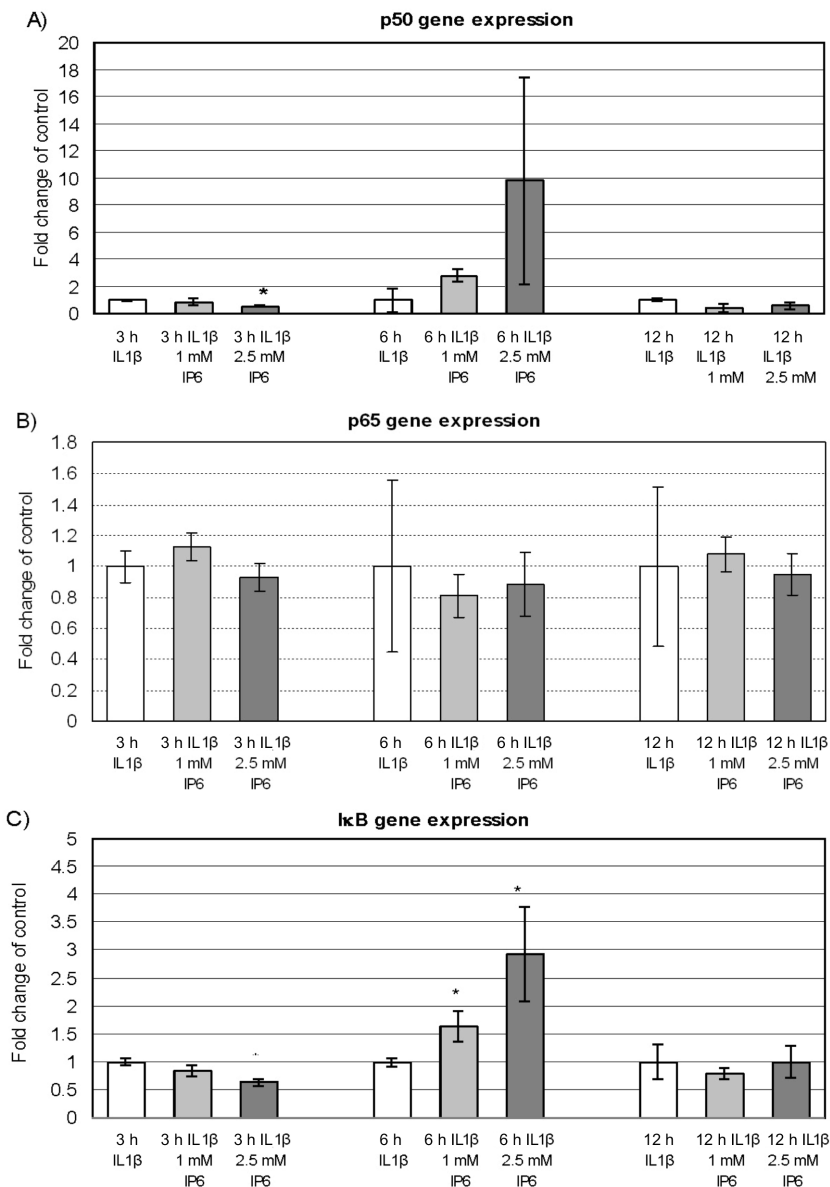

Figure 1. Comparison of (**A**) p50, (**B**) p65 and (**C**) IκBα gene expression in IL-1β-stimulated Caco-2 cells treated with 1 and 2.5 mM IP6 for 3, 6 and 12 h (the mean ± SD; * p < 0.05 vs. control)
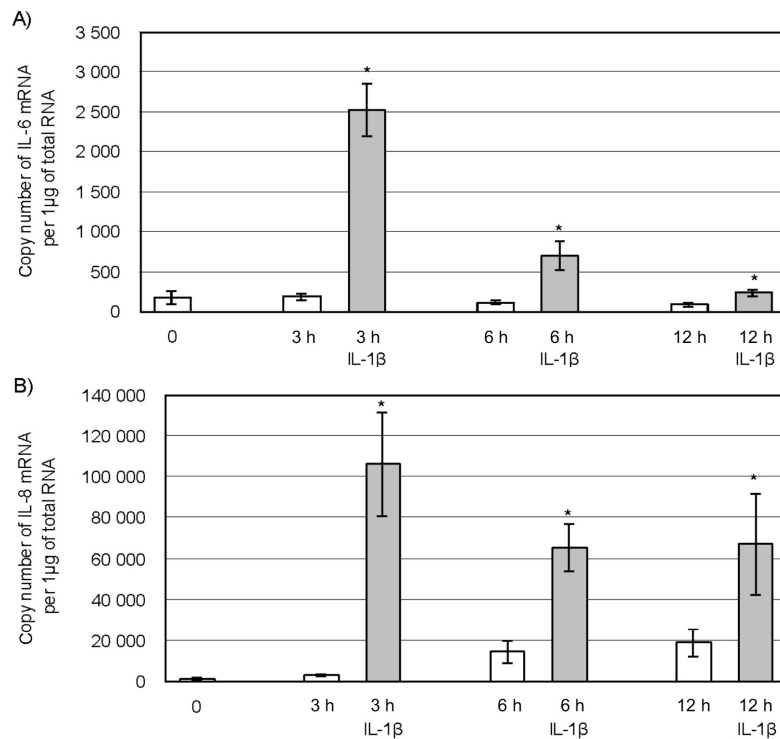
Figure 2. Comparison of (**A**) IL-6, (**B**) IL-8 gene expression in Caco-2 cells unstimulated and stimulated with IL-1β (the mean ± SD; * p < 0.05 *vs.* unstimulated cells)

of transcriptional activity of IκBα gene (p = 0.002) was achieved by the higher concentration of IP6 (Fig. 1C). As shown in Fig. 1C, after 6 h, the expression of IκBα gene tended to rise with increasing IP6 doses (p < 0.05; ANOVA). At 12 h incubation, the IκBα inhibitor did not show any significant changes in transcription in response to IP6 treatment in relation to the control cells (p > 0.05, ANOVA) (Fig 1C).

The influence of IL-1β on IL-6 and IL-8 genes expression

The IL-6 and IL-8 mRNAs were expressed in unstimulated Caco-2 cells. Their stimulation with 1 ng/mL of IL-1β for 3, 6 and 12 h resulted in up regulation of IL-6 gene as compared with unstimulated cells (Fig. 2A). The greatest, 13.3-fold increase in transcriptional activity of IL-6 gene following stimulation with IL-1β was observed after 3 h (p < 0.001). The prolonged incubation with IL-1β caused a 6-fold elevation of IL-6 expression after 6 h (p = 0,005) and its 2.5-fold increase after 12 h (p < 0.005). IL-1β also strongly enhanced IL-8 gene expression in cells exposed to it for 3–12 h (Fig. 2B). A 35-fold increase in IL-8 expression (p <

0.0001) was observed at 3 h. Weaker, but still statistically significant up-regulation of IL-8 gene was determined after 6 and 12 h stimulation with IL-1β, i.e, 4.5-fold (p < 0.005) and 3.5-fold (p < 0.05), respectively.

Changes in IL-1β stimulated expression of IL-6 and IL-8 in Caco-2 cells

A comparison of IL-6 transcript level in control cells and cultures treated with IP6 for 3 h showed statistically significant differences. The expression of IL-6 mRNA was down-regulated by 1 (p = 0.002; ANOVA) and 2.5 mM IP6 (p = 0.001; ANOVA) as compared to the control (Fig. 3A). The level of this gene transcript was insignificantly reduced by 1 mM IP6 (p > 0.05; ANOVA) and significantly decreased by 2.5 mM IP6 after 6 h treatment in comparison to control cultures (p = 0.046; ANOVA) (Fig. 3A). At 12 h IL-6 gene was found to be expressed at the similar level in both untreated cells and cells treated with IP6 at different concentrations (p > 0.05; ANOVA) (Fig. 3A).

The level of IL-8 transcript after 3 h was significantly lower in cells exposed to 1 mM (p < 0.01;

ANOVA) and 2.5 mM IP6 (p < 0.05; ANOVA) than in control cells. No statistically significant changes in the IL-8 gene expression were observed between cultures treated with different doses of IP6 for both 6 and 12 h and the control cells (p > 0.05; ANOVA) (Fig. 3B).

DISCUSSION AND CONCLUSION

Extensive *in vitro* and *in vivo* research over the past half century and especially in recent years has revealed important antioxidant, anticancerogenic and immunomodulatory properties of dietary compounds. There is an increasing interest in using natural products to modulate immune responses and neutralize inflammatory processes in the intestine because of their fewer side effects and lower cytotoxicities (19).

Phytic acid, a common constituent of plant-derived foods like cereals or legumes, which are the main staple food of people in developing countries (20), has been the focus of interest in the latest years due to its anticancer properties and other beneficial for human health effects (20, 21). Recently, IP6 has been shown to possess inhibitory effect on IL-8 and IL-6 secretion by intestinal cells Caco-2 in response to IL-1β (14). The secretion of these cytokines is subject to complex intracellular events, which involve signal transduction pathways, activity of transcription factors, gene expression and posttranscriptional modifications. Caco-2 cell line is frequently used to study inflammatory response of human colonocytes. The concentration of IP6 (1 and 2.5 mM) used in the research corresponded to its concentrations in the human large gut.

The findings of the current study revealed that IP6 did not cause any alterations in p65 gene expression in IL-1β-stimulated Caco-2 cells, however, it changed transcriptional activity of p50 and IκBα genes in these cells. Incubation with 2.5 mM IP6 for 3 h resulted in a decrease in p50 and IκBα mRNA levels. An increase in transcriptional activity of IκBα was observed after 6 h incubation with IP6. These results can suggest that biological activity of IP6 in stimulated Caco-2 cells might by mediated through the modulation of IκBα at the mRNA level.
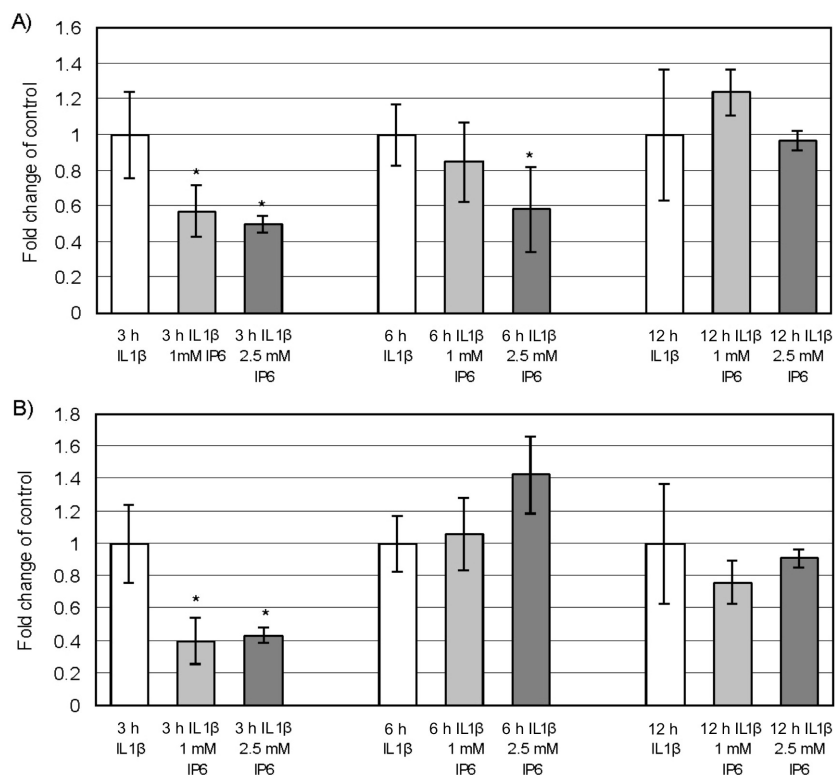

Figure 3. Comparison of (**A**) IL-6 and (**B**) IL-8 gene expression in IL-1β-stimulated Caco-2 cells treated with 1 and 2.5 mM IP6 for 3, 6 and 12 h (the mean ± SD; * p < 0.05 *vs*. control)

The function of the IκB proteins in regulating NF-κB is well known, and the three most widely expressed members of this family (IκBα, IκBβ and IκBε) play very similar roles (22). Published reports have demonstrated decreased NF-κB activation in response to increased levels of IκBα (23, 24) and the role of newly synthesized IκBα in termination of transcription of inflammatory genes (25).

Previous study has shown that IP6 modulated transcriptional activity of p65 and IκBα genes in unstimulated Caco-2 cells and it did not affect the expression of p50 gene. IP6 significantly decreased expression of p65 gene at 1 h incubation and increased p65 mRNA level in 6 and 12 h lasting cultures. Dose dependent increase in IκBα expression in cells treated with IP6 was also observed (15).

Several studies confirmed the inhibitory effect of IP6 on NF-κB activation in human and mouse cells. Agrawal et al. (26) demonstrated a decrease in nuclear levels of p65 and p50 proteins in IP6-treated prostate cancer cells DU145. Inhibition of NF-κB activation was observed in cells treated with 1 mM IP6 for 12 h, and a decrease in its expression was more pronounced after 24 h incubation. The lowest activity of NF-κB was detected in cells exposed to 2 mM IP6 for 24 h. Furthermore, it has been shown that IP6 evoked a significant increase in total IκBα protein level (26). IP6 had the capacity of inhibiting insulin and TNF-α-induced NF-κB nuclear translocation and activity in HeLa cells (27). IP6 ability to decreasing constitutively high NF-κB activation was also confirmed in head and neck cancer cell lines (28). Another study on the SKH1 mouse model have also shown that IP6 strongly inhibited UVB-induced activation of NF-κB transcription and NF-κB DNA binding activities (29, 30).

In the present study, the transcriptional activity of IL-6 and IL-8 genes in unstimulated and stimulated with IL-1β Caco-2 cells under influence of IP6 was also evaluated. IL-6 and IL-8 mRNA was detected in both unstimulated and stimulated cells. IL-1β strongly elevated IL-6 and IL-8 transcript levels. The results remain in agreement with other findings which reported that IL-1β induced expression of IL-6, IL-8 and others inflammatory mediators in IEC (2, 14). The expression of IL-6 and IL-8 was markedly decreased by 1 and 2.5 mM IP6 after 3 h treatment and IP6 also reduced IL-6 transcript level after 6 h. These data indicate the capability of IP6 to inhibit IL-1β-induced IL-6 and IL-8 expression in Caco-2 cells at transcriptional level. Modulatory effect of IP6 on the expression and secretion of cytokines was reported by Cholewa et al. (31) who observed IP6-induced down-regulation of TNF-α

and TNFRII expression, which may reflect anti-inflammatory activity of this compound. Cherng et al. (32) demonstrated inhibition of IL-10 and activation of INF-γ secretion by IP6-treated human peripheral blood mononuclear cells (PBMC). The cited studies conclude that IP6 is most likely to be important in modulation of human immunity.

In conclusion, the results from this study suggest that IP6 at physiological concentration in the intestinal lumen, may have regulatory effects on inflammatory reactions in intestinal epithelium by influencing NF-κB pathway. IP6 could also exert anti-inflammatory effects by inhibiting IL-1β-stimulated expression of inflammatory mediators: IL-6 and IL-8 at the transcriptional level. Therefore, it is tempting to hypothesize that a diet rich in IP6 could be beneficial for preventing or reducing the inflammatory reactions in the intestine.

Acknowledgment

REFERENCES

1. Stadnyk A.W.: Can. J. Gastroenterol. 16, 241 (2002).
2. Bosani M., Ardizzone S., Porro G.B.: Biologics 3, 77 (2009).
3. Epstein J., Docena G., MacDonald T.T., Sanderson I.R.: Br. J. Nutr. 103, 824 (2010).
4. Jijon H.B., Panenka W.J., Madsen K.L., Parsons H.G.: Am. J. Physiol. Cell Physiol. 283, C31 (2002).
5. Tak P., Firestein G.: J. Clin. Invest. 107, 7 (2001).
6. Wang S., Liu Z., Wang L., Zhang X.: Cell. Mol. Immunol. 6, 327 (2009).
7. Son D.O., Satsu H., Kiso Y., Totsuka M., Shimizu M.: Cytokine 42, 265 (2008).
8. Rizzo A., Pallone F., Monteleone G., Fantini M.C.: World J. Gastroenterol. 17, 3092 (2011).
9. Mudter J., Neurath M.F.: Inflamm. Bowel Dis. 13, 1016 (2007).
10. Maldonado-Rojas W., Olivero-Verbel J.: J. Mol. Graph. Model. 30, 157 (2011).
11. Vucenik I., Shamsuddin A.M.: Nutr. Cancer 55, 109 (2006).
12. Fox C.H., Eberl M.: Complement. Ther. Med. 10, 229 (2002).
13. Węglarz L., Parfiniewicz B., Mertas A., Kondera-Anasz Z., Jaworska-Kik M.,

Dzierżewicz Z.: Pol. J. Environ. Stud. 17, 283 (2008).

14. Węglarz L., Wawszczyk J., Orchel A., Jaworska-Kik M., Dzierzewicz Z.: Dig. Dis. Sci. 52, 93 (2007).

15. Kapral M., Węglarz L., Parfiniewicz B., Lodowska J., Jaworska-Kik M.: Folia Microbiol. 55, 657 (2010).

16. Inoue M., Matsuoka M., Yamaguchi K., Takatsuki K., Yoshida M.: J. Cancer Res. 89, 53 (1998).

17. Rostkowska-Nadolska B., Kapral M., Frączek M., Kowalczyk M., Gawron W., Mazurek U.: Auris Nasus Larynx 38, 58 (2011).

18. Kapral M., Wawszczyk J., Smolik S., Weglarz L.: Acta Pol Pharm. Drug Res. 67, 701 (2010).

19. Tsai J.T., Liu H.C., Chen Y.H.: Mediators Inflamm. 293642 (2010).

20. Haefner S., Knietsch A., Scholten E., Braun J., Lohscheidt M., Zelder O.: Appl. Microbiol. Biotechnol. 68, 588 (2005).

21. Matejuk A., Shamsuddin A.: Curr. Cancer Ther. 6, 1 (2010).

22. Claud E.C., Lu L., Anton P.M., Savidge T., Walker W.A., Cherayil B.J.: Proc. Natl. Acad. Sci. USA 101, 7404 (2004)

23. Dobrovolskaia M.A., Medvedev A.E., Thomas K.E., Cuesta N., Toshchakov V., Ren T., Cody M.J. et al.: J. Immunol. 170, 508 (2003).

24. Riedemann N.C., Guo R.F., Bernacki K.D., Reuben J.S., Laudes I.J., Neff T.A., Gao H. et al.: Immunity 19, 193 (2003).

25. Ghafoori P., Yoshimura T., Turpie B., Masli S.: FASEB J. 23, 2226 (2009).

26. Agarwal C., Dhanalakshmi S., Singh R.P., Agarwal R.: Anticancer Res. 23, 3855 (2003).

27. Ferry S., Matsuda M., Yoshida H., Hirata M.: Carcinogenesis 23, 2031 (2002).

28. Yu Z.H., Huang F., Xu N., Zhao D.M., Hu F.A., Liu J., Liu H.F.: J. Immunoassay Immunochem. 32, 47 (2011).

29. Chen N., Ma W.Y., Dong Z.: Mol. Carcinog. 31, 139 (2001).

30. Kolappaswamy K., Williams K.A., Benazzi C., Sarli G., McLeod C.G. Jr, Vucenik I., DeTolla L.J.: Comp. Med. 59, 146 (2009).

31. Cholewa K., Parfiniewicz B., Bednarek I., Świątkowska L., Jezienicka E., Kierot J., Węglarz L.: Acta Pol. Pharm. Drug Res. 65, 75 (2008).

32. Cherng J.M., Chiang W., Chiang L.C.: Food Chemistry 104, 613 (2007).