Acta Poloniae Pharmaceutica – Drug Research, Vol. 72 No. 4 pp. 719–725, 2015 ISSN 0001-6837
Polish Pharmaceutical Society

IMPACT OF GENISTEIN AND PHYTIC ACID ON THE VIABILITY AND PROLIFERATION ACTIVITY OF NASAL POLYPS' CELLS IN AN *IN VITRO* MODEL*

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Abstract: In developed countries, chronic rhinosinusitis with nasal polyps is one of the diseases that diminish patients' quality of life most significantly. Treatment of that often incurable disease is based on the steroids and surgery in patients who had failed thorough conservative management. It appears that the introduction of new treatment agents suppressing inflammation process and inhibiting cells' proliferation would be a valuable therapeutic option. The aim of the present study was to evaluate the *in vitro* effect of genistein and phytic acid on the viability and growth rate of fibroblasts derived from nasal polyps. Cells were incubated with various concentrations of genistein (5-500 μM) and phytic acid (100-20 000 μM). After 72 h incubation, cells survivability and cells' growth rate were estimated by combination of WST-1 and LDH methods. QRT-PCR technique was used to determine the expression of histone *H3, BCL -2, BAX* and *P53* genes. Caspase-8 and -9 expressions were evaluated by ELISA assay. Genistein and phytic acid significantly and in dose-specific manner decreased nasal polyps fibroblasts survivability and growth rate. Both agents in similar way decreased cell proliferation as measured by the expression of histone *H3*. They induce apoptotic machinery by modulating the expression of *BCL-2*, *BAX* and caspase-8 activity. Genistein and phytic acid have significant potential for a therapeutic role in the treatment of chronic rhinosinusitis.

Keywords: genistein, phytic acid, chronic rhinosinusitis, BAX, BCL-2, TP53, caspase, cytotoxicity, LDH, WST-1, QRT-PCR

Chronic rhinosinusitis (CRS) is considered to be one of the most common chronic diseases in developed countries. Besides significant reduction in patient's quality of life, negative effect on lower airway, cardiovascular system and general health CRS causes severe medical costs of treatment (1). The pathophysiology of CRS which is largely unknown involves eosinophils infiltration, with dysfunction of mucociliary apparatus, thinning of the epithelium, accumulation of fluid, pseudocyst and nasal polyps' (NP) formation. Eosinophils are the driving force of inflammatory process affecting the function of structural cells through the spectrum of mediators and cytotoxic granule proteins. Fibroblasts due to their secretory activity are also considered to play an important role in airways remodelling.

At present, topical or systemic glucocorticoids are the first line therapy for CRS that provide transient relief and require periodical reapplication. Therefore, medicines derived from plants are attracting increasing interest. Due to the often lower cytotoxicities they are often more suitable for long-term therapy. Proposed idea is to administrate into CRS management natural agents that inhibit or reduce cells' proliferation. The isoflavonoid genistein (4',5,7-trihydroxyisoflavone; GST), the aglycone of the heteroside genistin represents a major biologically active compound from soybean, the vegetal product from *Glycine max* (Fabaceae). This phytoestrogen being a non-specific inhibitor of tyrosine kinases is responsible for the health promoting effects of soy. The *in vitro* studies have shown that GST is a highly pleiotropic molecule capable of interacting with various molecular targets involved in inflammation, including transcription factors, growth factors and their receptors, cytokines, enzyme and genes regulating cell proliferation (2).

*Paper presented at IX MKNOL Conference, May, 2014

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Mechanisms underlying the anti-inflammatory action of GST were best identified in cancer where it has been reported to significantly inhibit cell growth, induce cell cycle arrest and apoptosis (3). Anti-inflammatory properties of GST were shown in asthma where it attenuated OVA-induced airway inflammation, decreased Th2-type cytokines and increased Th1-type cytokines (4).

Phytic acid, a hexaphosphorylated inositol (IP6) is a naturally occurring polyphosphorylated carbohydrate which has been found in whole grains, cereals, nuts leguments and oil seeds. IP6 has several roles in mammalian cells. It has been implicated in regulating growth, inflammation and neurotransmission, and is even thought to be involved in export of mRNAs from the nucleus (4). It might specifically bind to, and thus induce, conformational changes in a series of particular proteins, thereby altering their activity and substrate binding. Anti-proliferative and anticancer activity of IP6 has been demonstrated both *in vivo* and *in vitro* models (5).

Several observations, especially reported in asthma and allergic diseases, indicate that both agents through the modulation of immune responses and neutralization inflammatory processes may have positive therapeutic effects in CRS with nasal polyps (4, 6). The potential role of GST and IP6 in the treatment of CRS has not been examined systematically to date.

The aim of the present study was to analyze the effect of GST and IP6 on the viability and growth rate of cells derived from nasal polyps. The present study sought to elucidate the molecular mechanism of GST and IP6-induced apoptosis.

EXPERIMENTAL

Patients

The examinations were performed on the cells isolated from NP obtained from three patients with CRS during routine surgical procedure performed in the Deptartment of Otolaryngology, Wroclaw Medical University in Poland. All the subjects met diagnostic criteria for CRS as established by the European position paper on rhinosinusitis and nasal polyps (EPOS 2012). Patients had been free of any medication for at least 4 weeks before surgery and had bilateral polyps in the nasal cavities on endoscopic examination and computed tomography. The study was approved by Local Ethical Committee of Wroclaw Medical University.

Nasal polyps' specimens were immediately rinsed in phosphate buffered saline (PBS), cut into small fragments and placed into a sample tubes containing 1 mL of PBS. The tubes were directly transported on ice to the laboratory for further investigations. A part of each sample was fixed in 10% buffered neutral formalin (Chempur, Piekary Śl., Poland), processed routinely, and embedded in paraffin wax (Bio-Plast, Wrocław, Poland) for subsequent immunohistochemical examination to establish diagnosis and to exclude other pathologies.

Cell culture

Polyp tissues were milled mechanically and then separated in a solution containing 0.25% trypsin (Sigma-Aldrich, St. Louis, USA). The cells were placed in vessels with RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with

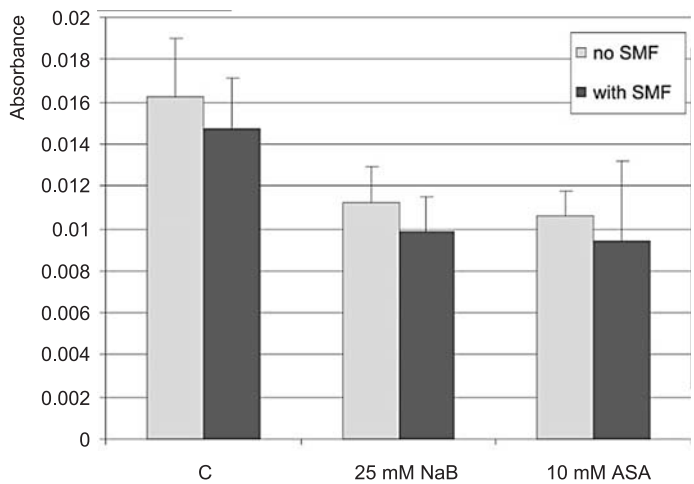

Figure 1. Influence of genistein (GST) on survivability and growth rate of NP fibroblasts determined by WST-1 assay. The cells were cultured for 72 h in the presence of GST at various concentrations. Data are shown as the mean percentages (± SD) of the control culture (C). *Significant difference from the respective untreated control culture ($p < 0.05$)

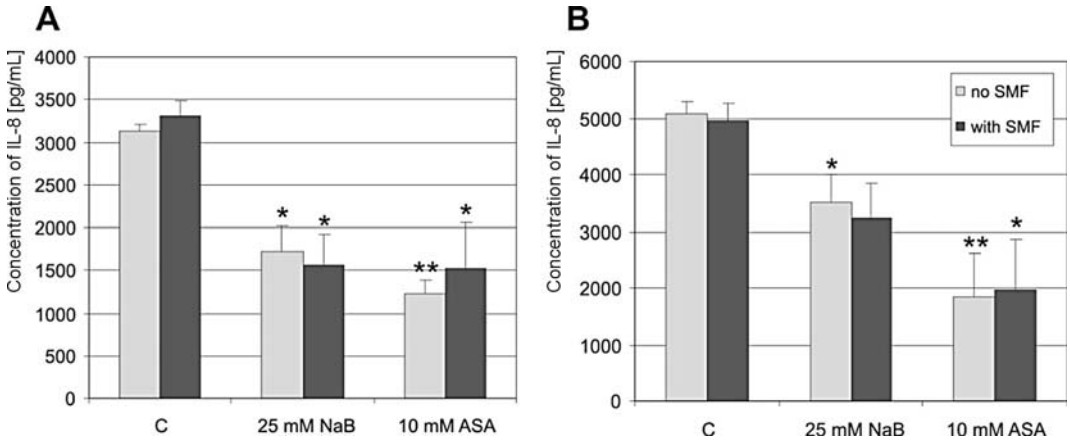

Figure 2. Influence of phytic acid (IP6) on survivability and growth rate of NP fibroblasts determined by WST-1 assay. The cells were cultured for 72 h in the presence of IP6 at various concentrations. Data are shown as the mean percentages (± SD) of the control culture (C). * Significant difference from the respective untreated control culture ($p < 0.05$)

10% fetal bovine serum (FBS) (Biological Industries Ltd., Kibbutz Beit-Haemek, Israel), penicillin (10000 U/mL) and streptomycin (10 mg/mL) (Biological Industries Ltd., Kibbutz Beit-Haemek, Israel). Cell cultures were incubated in the laboratory incubator at 37°C in humidified atmosphere containing 5% CO_2. Cultures grew to confluence and then were passaged. Cells from IV-VI passage were used for further studies. Before the appropriate experiment, cells were stimulated in a medium with 10 ng/mL lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich, St. Louis, USA) for 24 h.

Survivability assay

The effect of increasing doses of GST (5-500 μM) and IP6 (100-20000 μM), both purchased from Sigma-Aldrich (St. Louis, USA) on NP cells survivability was evaluated by WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany) after 72 h treatment in medium without FBS. The intensity of the colorimetric reaction was measured by microplate reader UVM340 (Biogenet, Piaseczno, Poland) at 440 nm. The control group consisted of the cells incubated without GST and IP6 under the same conditions.

Growth rate assay

The effect of GST (5-500 μM) and IP6 (100-20000 μM) on the growth rate was assessed after 72 h of incubation with each agent in medium with FBS. The number of cells was examined by WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany) and the absorbance was measured using a microplate reader UVM340 (Biogenet, Piaseczno, Poland) at 440 nm. The control group consisted of the cells incubated without GST and IP6 under the same conditions.

Lactate dehydrogenase assay

Cytotoxic effect of GST (5-500 μM) and IP6 (100-20 000 μM) was determined by lactate dehydrogenase (LDH) release in culture medium with a diagnostic kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions after 72 h incubation. The activity of LDH was calculated after detection of absorption at 440 nm with an ELISA reader UVM340 (Biogenet, Piaseczno, Poland). All the assays were run in triplicate and averaged. The data were normalized to the control group and expressed as a percentage.

Transcriptional activity of histone *H3, BCL -2, BAX* and *TP53* gene

The evaluation of transcriptional activity of genes encoding histone H3, proteins involved in mitochondrial control of apoptosis signaling pathway (BCL-2 and BAX) and p53-cell cycle regulator was performed by QRT-PCR technique with SYBR Green I chemistry (QuantiTect® SYBR® Green RT-PCR Kit, Qiagen, Hilden, Germany). The analysis was carried out using Opticon™ DNA Engine (MJ Research, San Francisco, USA). The cells in the cultures were exposed to GST at 50 μM and IP6 at 5000 μM. RNA extraction was performed by column based method using Quick-RNA™ MiniPrep kit from Zymo Reseach (Irvine, CA, USA). RNA extracts were evaluated in terms of quality and quantity. The integrity of total RNA was checked by

electrophoresis on 1.2% agarose gel with ethidium bromide. The quantity and purity of total RNA in the extracts were determined by spectrophotometry with HP8452A spectrophotometer (Hewlett Packard).

Caspase-8 and -9 expression levels

The cells in the cultures were exposed to GST at 50 μM and IP6 at 5000 μM. Quantitative assessment of the presence of caspase-8 and -9 were performed using ELISA assays (BioVendor, Candler, NC, USA). The measurements were done with microplate reader UVM340 (Biogenet, Piaseczno, Poland).

Statistical analysis

Statistical analysis was performed with the use of Statistica PL 7.0 Software (StatSoft, Kraków, Poland). The data were presented as the mean ± SD. To demonstrate statistically significant differences in the numbers of cells, mRNA copy number of histone *H3, BCL-2, BAX*, *TP53*, caspase-8 and -9 levels between the control and the cells exposed to GST or IP6 one-way analysis of variance (ANOVA) and *post-hoc* Duncan test were applied. A level of $p < 0.05$ was considered to be significant.

RESULTS

The effects of GST an IP6 on survivability of the NP cells

GST and IP6 decreased NP fibroblasts' survivability in dose-dependent manner in FSB-free medium cultures (Figs. 1, 2). Lack of growing stimuli (FBS) limited cells divisions. The results showed that NP cells survivability decrease significantly after exposure to GST at the concentration over 50 μM and IP6 over 5000 μM compared to the control ($p < 0.05$). The most pronounced decline in NP cells survivability was recorded to be 22% at 500 μM of GST and 20% at 20000 μM of IP6.

The influence of GST and IP6 on NP cells' growth rate

To evaluate not only cytotoxic properties of GST and IP6 but also the impact of both agents on cells' cycle progression and cells' divisions, WST-1 assay was performed after culturing the cells in medium with FBS (Figs. 1, 2). Under such conditions, the growth rate of NP cells was inhibited in a dose-dependent manner. Statistically significant decrease in cells number compared to the control was noted after exposing to GST at concentrations over 50 μM and IP6 over 100 μM ($p < 0.05$). The most pronounced decline in NP cells survivability was recorded to be 36% at 500 μM of GST and 28% at 10000 and 20000 μM of IP6. Both GST and IP6 at the lower concentrations (5, 10 μM and 100, 500, 1000 μM, respectively) suppressed cells number more efficiently in medium containing FSB than in FSB-free medium. At the higher concentrations (GST at 200 and 500 μM; IP6 at 10000 and 20000 μM) both agents revealed more pronounced cytotoxic effect.

Quantification of LDH release after treatment with GST and IP6

The lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme presents in all cells. LDH is rapidly released into the cell culture supernatant upon cell damage or lysis. Strong and concentration-

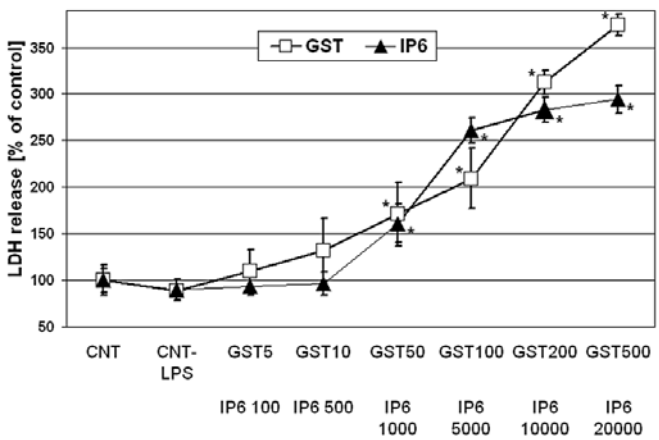

Figure 3. Quantification of lactate dehydrogenase (LDH) release in NP cell cultures after treatment with various concentrations of genistein (GST) and phytic acid (IP6). Data are shown as the mean percentages (± SD) of untreated control culture (CNT). CNT-LPS - CNT stimulated with LPS. * Significant difference from the respective control culture ($p < 0.05$)

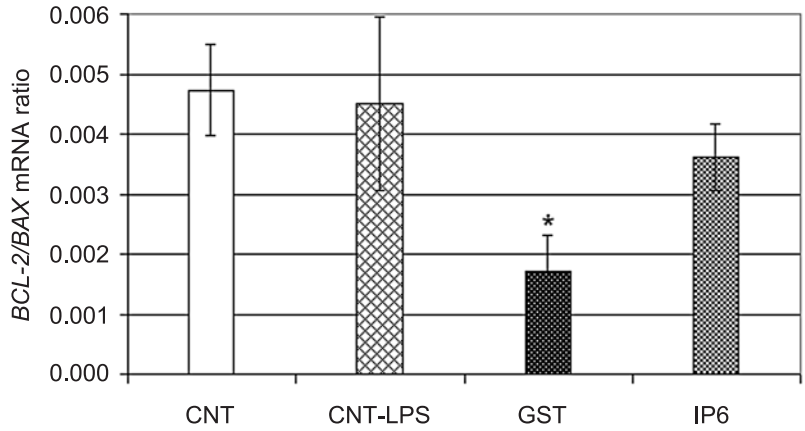

Figure 4. The effect of genistein (GST) at 50 μM and phytic acid (IP6) at 5000 μM on *BCL-2*/*BAX* mRNA ratio in NP fibroblasts. Data are shown as the mean (± SD). CNT-LPS - control stimulated with bacterial LPS. * Significant difference from the respective control culture (CNT) ($p < 0.05$)

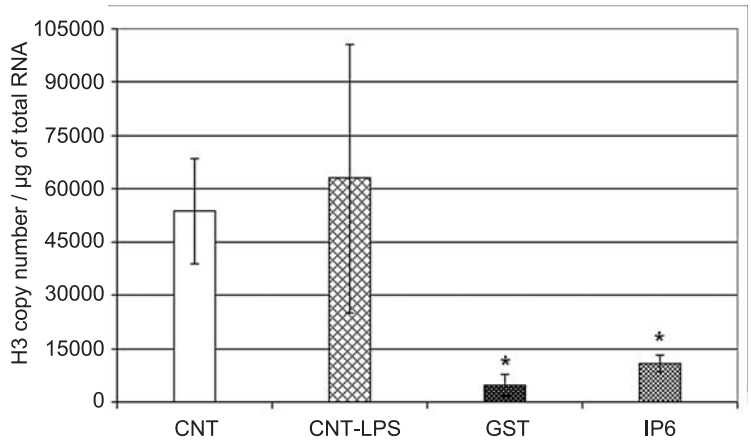

Figure 5. Histone *H3* mRNA copy number in NP fibroblasts after exposure to genistein (GST) at 50 μM and phytic acid (IP6) at 5000 μM. Data are shown as the mean (± SD). CNT-LPS - control stimulated with LPS. * Significant difference from the respective control culture (CNT) ($p < 0.05$)

dependent increase of LDH release from NP fibroblasts exposed to GST and IP6 was measured (Fig. 3). Number of death cells statistically grew after treatment with higher concentrations of GST and IP6 (GST at 50, 100, 200 and 500 μM, IP6 at 1000, 5000, 10000 and 20000 μM) ($p < 0.05$) as measured by LDH leakage. LDH content rose to 374% of the control at 500 μM of GST and to 294% of the control at 20000 μM of IP6.

Quantification of *BCL-2*, *BAX*, histone *H3* and *TP53* mRNA copy number after treatment with GST and IP6

The levels of pro-apoptotic *BAX* and anti-apoptotic *BCL-2* mRNA expression in NP cells following treatments with IP6 and GST were determined (Fig. 4). *BAX* was upregulated and *BCL-2* was downregulated at the mRNA level after incubation with GST at 50 μM in NP cells. Treatment with IP6 caused minor *BCL-2* and major *BAX* gene expression increase. The alterations in expression levels of two members of the Bcl-2 family may direct the cells to the path leading to apoptosis. Herein, both GST and IP6 treatment caused *BAX*/*BCL-2* mRNA ratio decrease, however only GST modulated the ratio significantly compared to the control ($p < 0.05$).

Analysis of histone *H3* mRNA level is considered to be a valuable marker of cells proliferation. Our results showed that both GST at 50 μM and IP6

at 5000 μM decreased significantly histone *H3* mRNA level ($p < 0.05$) (Fig. 5).

TP53 gene that codes for p53 protein through the regulation of crucial cell cycle events is involved in growth arrest and apoptosis. GST at 50 μM and IP6 at 5000 μM showed significant decrease of *TP53* transcripts compared to the control ($p < 0.05$). GST and IP6 revealed similar effect and reduced the number of *TP53* transcripts by half from 96635 to 50116 and 51641, respectively.

The effects of GST an IP6 on caspase-8 and -9 levels

To determine which apoptotic pathway is involved, the levels of caspase-8 and -9 mediated by GST and IP6 was examined. Both GST and IP6 treatment led to an increase of caspase-8 expression measured by ELISA assay but only IP6 at 5000 μM was sufficient to cause significant change (twofold) ($p < 0.05$). Neither GST at 50 μM nor IP6 at 5000 μM influenced the caspase-9 level significantly. GST upregulated the caspase-9 level from 11790 in the control to 12370 in NP. Contrary, IP6 slightly decreased the caspase-9 level to 10330.

DISCUSSION AND CONCLUSION

In the present paper, for the first time, we revealed significant antiproliferative, cytotoxic and pro-apoptotic effects of both GST and IP6 against nasal polyps' fibroblasts.

Supplementation of soy isoflavones and IP6 was shown to lower the risks of some cancers, inflammatory diseases, cardiovascular, allergic and metabolic disorders (3, 4, 6, 7, 8). IP6 has been demonstrated to prevent the mucosa from inflammatory state or may even contribute to the clearance of infection (9). Subcutaneous injection of GST exerted anti-inflammatory properties on collagen-induced arthritis (10). Although the influence of both substances has been tested on many models, new attempts are worth to undertake due to the fact that cells from different origins show different sensitivities to IP6 and GST. Both agents hypothesized to target inflammation tissue through multiple pathways, including inhibition of cell cycle progression and activation of apoptosis, what might be confirmed by the outcomes of our study. GST and IP6 significantly increased the number of nasal polyps' cells alive and impaired cells proliferation as was measured indirectly by histone *H3* expression. The precise molecular pathways mediating GST and IP6 action have remained obscure. Inhibition of the transcriptional activity of NF-κB, a key regulator of the inflammatory response, is thought to explain the effects of GST on cell proliferation and cell cycle progression (11). Activation of NF-κB is regarded as an important event in the airway inflammation, including regulation of multiple pro-inflammatory genes likely to participate in the etiology of CRS (12). Another transcription factor inhibition of which may lead to decreased proliferation of NP fibroblasts is an activator protein 1 which has been recognized to be a molecular target of both soya flavonoids and IP6 (13, 14). Recently, IP6 has been showed to reduce the cell number by modulating the wnt/β-catenin pathway (15).

Using WST-1 assay we showed in the present study that GST and IP6 at lower concentrations affect very efficiently dividing cells but at the higher concentrations both agents revealed more pronounced cytotoxic effect what fits also the results from the LDH assay.

GST-induced apoptosis in cancer cells has been shown to be due to the ability to control expression of apoptosis-related genes including *BCL-2* family members (16). The ratio between *BCL-2* and *BAX* is a reliable indicator of enhanced apoptotic processes and is more important than the expression levels. Herein, GST and IP6 modulated expression of pro-apoptotic *BAX* and anti-apoptotic *BCL-2* levels cause a decrease in *BCL-2*/*BAX* ratio what may initiate the intrinsic mitochondria-apoptosome pathway. In such a way both GST and IP6 cause the collapse of mitochondrial membrane potential ($\Delta\Psi m$), resulting in the release of cytochrome c in combination with caspase-9, -3 activation and induction of DNA fragmentation (17). In our study, GST treatment increased and IP6 decreased the caspase-9 level insignificantly compared with the control.

The second extrinsic pathway for apoptosis or cell death receptor pathway is mediated distinctively by active caspase-8 that is characterized by binding cell death ligand and cell death receptors followed by activation of caspase-8 and -3. Our data showed that caspase-8 is involved in both GST and IP6 induced apoptosis in NP fibroblasts. This is consistent with report by Shafie et al. (18), who described significant increase of caspase-8 and *BAX* after treatment with IP6 in HT-29 cells. GST has been also reported to promote apoptosis *via* activation of casapse-8 in ovarian cancer and neuroblastoma cells (19, 20). Although some reports indicated that GST and IP6 could induce apoptosis through both extrinsic and intrinsic pathways, the results of our study suggest that in NP fibroblasts tested agents act rather *via* death receptor dependent pathway.

We did not observe up-regulation of the pro-apoptotic *TP53* gene, which was described to be component of GST and IP6 mediated apoptosis (21). Activated p53 upregulates expression of downstream target genes, including *BAX*, *P21*/WAF1, proapoptotic proteins such as NOXA and PUMA (21, 22). It might be partially explained by the insufficient tested agents' concentration. Data would be consistent with observations where apoptosis was seen only at higher concentrations of GST or even no induction of apoptosis was observed like in melanoma and leukemia cells (23, 24).

An attempt to complement the treatment of chronic inflammatory diseases with biologically active plant chemicals is consistent with present trends in prevention and management of CRS (25). In some of the clinical entities, simple diet modification according to the concept of functional foods can improve not only patient overall health but also reduce symptoms. In patients with asthma, condition having a great similarity with CRS, consumption of moderate to high amounts of soy GST was associated with better lung function and better asthma control (26).

The anti-proliferative and pro-apoptotic effects of GST and IP6 towards NP fibroblast presented herein may attenuate the re-growth of polyps. Further studies are mandatory to assess the ability of those agents to modulate immune responses and neutralize inflammatory processes within the paranasal sinuses and the influence of GST and IP6 supplementation in the diet.

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Received: 19. 10. 2014