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The activation of the Nrf2/ARE pathway in HepG2 hepatoma cells by phytochemicals and subsequent modulation of phase II and antioxidant enzyme expression

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Abstract Previous studies have shown that naturally occurring phytochemicals, indole-3-carbinol, phenethyl isothiocyanate, protocatechuic acid, and tannic acid increased the activity and protein level of hepatic phase II enzymes in animal models. In order to further explore the mechanism of this activity, we investigated the effect of these compounds on the activation of nuclear factor erythroid-2-related factor 2 (Nrf2)-regulated transcription in human hepatocellular carcinoma HepG2 cells. Treatment with all the tested compounds resulted in the translocation from the cytosol and nuclear accumulation of active phosphorylated Nrf2. Furthermore, phenethyl isothiocyanate and indole-3-carbinol increased the transcript and protein levels of *GSTA*, *GSTP*, *GSTM*, *GSTT*, and *NQO1*. On the other hand, protocatechuic and tannic acids enhanced only the expression of *GSTA*, *GSTM*, and *GSTT*. The expression of genes encoding antioxidant enzymes *CAT*, *SOD*, *GR*, and *GPx* was increased after the treatment with all the tested phytochemicals. These results indicate that isothiocyanates/indoles and protocatechuic and tannic acids induce phase II and antioxidant gene expression in HepG2 cells through the Nrf2-Keap1-ARE signaling pathway. Moreover, the results of this study confirmed that the degradation products of glucosinolates are more effective inducers of phase II and antioxidant enzymes than protocatechuic and tannic acids.

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Abbreviations

ARE	Antioxidant response element
CAT	Catalase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione S-transferase
I3C	Indole-3-carbinol
Keap1	Kelch-like ECH-associated protein 1
NQO1	NADPH:quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid-2-related factor 2
PCA	Protocatechuic acid
PEITC	Phenethyl isothiocyanate
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TA	Tannic acid

Introduction

The induction of genes encoding phase II enzymes that protect against damage by electrophilic and reactive oxygen intermediates is considered as an important strategy in reducing the risk of cancer and other chronic degenerative diseases linked to oxidative stress [14, 32, 40]. The expression of phase II genes is controlled by

antioxidant response element (ARE) sequences which are regulated by nuclear factor erythroid-2-related factor 2 (Nrf2) signaling [26]. Inactive Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) molecules and is retained in the cytoplasm. Under oxidative stress or in response to other stimulating factors, Nrf2 dissociates from Keap1 and undergoes translocation to the nuclear compartment, where its accumulation correlates with binding to ARE regulatory DNA sequences and stimulation of target gene transcription [30].

Overexpression of Nrf2 has been reported to upregulate ARE-mediated gene expression that was inducible by various antioxidants and chemopreventive agents such as beta-naphtoflavone, tert-butyl hydroquinone, and phenethyl isothiocyanate [7]. The critical role of Nrf2 in the expression of antioxidant genes has been confirmed by using the *nrf2*-disrupted mice, which not only show reduced levels of antioxidant enzymes but are also more susceptible to carcinogens compared to wild-type mice [4]. Therefore, the regulation of Nrf2 is now of great interest. Treatment of cells with antioxidants and xenobiotics can lead to the release of Nrf2 from Keap1 [5].

Many naturally occurring compounds have been found to be potent cancer chemopreventive agents in cell lines and experimental animal models [36]. Of great interest are the isothiocyanates such as phenethyl isothiocyanate and indoles such as indole-3-carbinol which are the degradation products of glucosinolates found in *Brassica* vegetables. Despite the significant progress in the basic understanding of carcinogenesis, the molecular and cellular targets for the effective chemoprevention by these compounds are poorly characterized. Recent studies have reported the induction of Nrf2 by several chemopreventive compounds including sulforaphane and phenethyl isothiocyanate [10, 28]. Moreover, phenolic compounds, especially protocatechuic acid also increased phase II enzymes. Our earlier studies [8, 11–13] showed the increased activity of glutathione S-transferase (GST), which correlated with an enhanced level of GSTM isoform after treatment with this phenolic acid possessing a simple chemical structure. However, the questions concerning how different classes of natural chemopreventive compounds regulate the expression and stability of Nrf2 and how the regulation of Nrf2 by these compounds influences the expression of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and phase II enzymes—

GSTs or NAD(P)H:quinone oxidoreductase 1 (NQO1) remain largely unanswered. In addition, the relationship between Nrf2 stability and Keap1 in the presence of chemopreventive compounds remains unclear.

In the present study, we investigated the effect of different classes of naturally occurring chemopreventive agents, phenethyl isothiocyanate, indole-3-carbinol, and protocatechuic and tannic acids, on the expression of the enzymes controlled by the Nrf2/ARE pathway activation in human hepatocellular carcinoma HepG2 cells.

## Materials and methods

Chemicals and antibodies

Protocatechuic acid, tannic acid, indole-3-carbinol, and phenethyl isothiocyanate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical structures of the studied phytochemicals are presented in Fig. 1.

Dimethyl sulfoxide (DMSO), antibiotic solution (10^4 U penicillin, 10 mg streptomycin, 25 µg amphotericin B), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), radio-immunoprecipitation assay (RIPA) buffer, and Tris were obtained from Sigma-Aldrich (St. Louis, MO, USA). HepG2 (ATCC HB 8065) cells were provided by Prof. Zofia Mazerska from the Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, Poland. All the primers used in PCR reactions were obtained from oligo.pl (Warsaw, Poland). Protease inhibitor cocktail was obtained from Roche Diagnostics GmbH (Penzberg, Germany). Primary and secondary antibodies against CAT, SOD, GPx, GR, GSTA, GSTM, GSTP, GSTT, NQO1, Nrf2, Keap1, β-actin, and laminin were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Nrf2 (phospho Ser40) antibody was obtained from Abcam (Cambridge, UK). All the other chemicals were commercial products of the highest purity available.

Cell culture and treatment

HepG2 cells were maintained in DMEM containing 10 % fetal bovine serum and antibiotics. The cells were grown at 37 °C in a humidified atmosphere containing 5 % CO_2. To assess the effect of tannic acid, protocatechuic acid, indole-3-carbinol, and phenethyl isothiocyanate on the measured parameters, 5×10^5 cells

Fig. 1 The chemical structures of phenethyl isothiocyanate, indole-3-carbinol, protocatechuic acid, and tannic acid
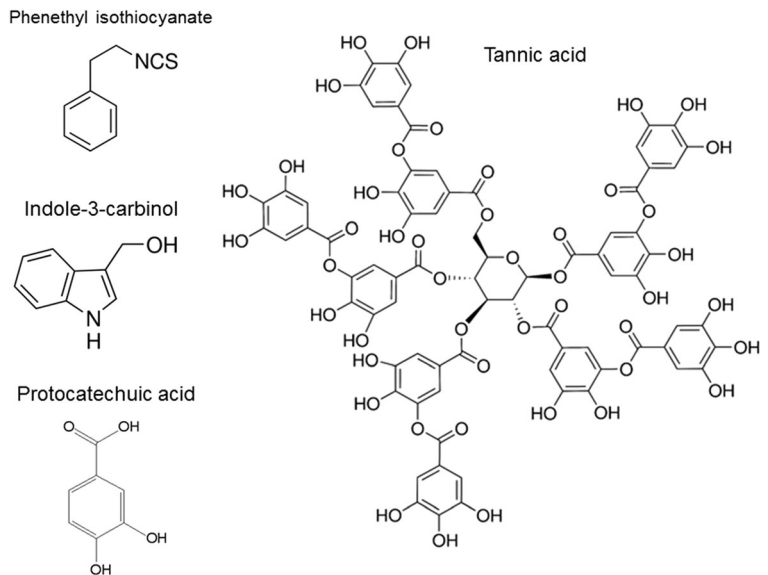
were seeded per 100 mm culture dish. After 24 h of initial incubation, the cells were treated with 2 and 10 µM tannic acid, protocatechuic acid, or indole-3-carbinol and 1 and 5 µM phenethyl isothiocyanate or 0.1 % vehicle (DMSO) control. The incubation was continued for subsequent 72 h, and cells were harvested.

Viability assay

The effect of phytochemicals on cell viability was assessed by the MTT assay, according to standard protocols. Briefly, 10^4 HepG2 cells were seeded per well in a 96-well plate. After 24 h of preincubation in complete medium, phytochemicals were added to culture medium in various concentrations and cells were incubated for subsequent 72 h. DMSO concentration did not exceed 0.1 %. After 72 h, cells were washed twice with warm PBS buffer and fresh medium containing MTT salt (0.5 mg/ml) was added. After 4 h of incubation, formazan crystals were dissolved in acidic isopropanol and absorbance was measured at 540 and 690 nm. All the experiments were repeated three times, with at least three measurements per experiment.

In all the subsequent experiments, non-toxic concentrations of phytochemicals (viability above 80 % based on the MTT assay) were used, ranging from 2 to 10 µM (tannic acid, protocatechuic acid, indole-3-carbinol) or 1 to 5 µM (phenethyl isothiocyanate).

Nuclear, cytosolic, and total protein lysate preparation

The cytosol and nuclear extracts from HepG2 were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Research, CA USA). Whole cell lysates were prepared from HepG2 using RIPA buffer.

RNA isolation

Extraction of total RNA from cells was performed using GeneMatrix Universal DNA/RNA/Protein Purification Kit (EurX, Gdańsk, Poland) according to the manufacturer's instructions.

Quantitative PCR

Total RNA was subjected to reverse transcription using RevertAid Kit (Fermentas, Burlington, Canada) followed by quantitative real-time PCR. For real-time analyses, the Maxima SYBR Green Kit (Fermentas) and a BioRad Chromo4 thermal cycler were used. The protocol started with 5 min enzyme activation at 95 °C, followed by 40 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 40 s and final elongation at 72 °C for 5 min. Melting curve analysis was used for product specificity verification. The estimation of the expression of TATA box binding protein (*TBP*) and porphobilinogen deaminase (*PBGD*) was used for data normalization. Primer sequences used for the analysis of *Nrf2*, *Keap1*, *GSTA*, *GSTM*, *GSTP*, *GSTT*, and *NQO1*

were described previously [9]. The sequences of primers used for the analysis of *SOD*, *CAT*, *GR*, *GPx* are listed in Table 1.

Western blot analysis

For the determination of the level of GSTA, GSTP, GSTM, GSTT, NQO1, Keap1, Nrf2, CAT, GR, SOD, and GPx proteins, the immunoblot assay was used. Protein content in the samples was determined by the Lowry method [20]. All the experiments were repeated three times. Whole cell lysates (GPx), nuclear extracts (Nrf2), or cytosolic proteins (Nrf2, Keap1, GSTA, GSTP, GSTM, GSTT, NQO1, SOD, CAT, GR) (100 μg) were separated on 12 or 10 % SDS-PAGE slab gels, and proteins were transferred to nitrocellulose membranes. After blocking with 10 % skimmed milk, proteins were probed with mouse monoclonal GSTA, rabbit polyclonal GSTM, goat polyclonal GSTP, mouse monoclonal GSTT, mouse monoclonal NQO1, goat polyclonal Keap1, rabbit polyclonal Nrf2, rabbit mono-clonal Nrf2 (phospho Ser40), goat polyclonal SOD, rabbit polyclonal GPx, rabbit polyclonal GR, goat poly-clonal CAT, rabbit polyclonal β-actin, and rabbit poly-clonal laminin antibodies. Either β-actin or laminin expression was used as an internal control. As the sec-ondary antibodies in the staining reaction, the alkaline phosphatase-labeled anti-goat IgG, anti-mouse IgG, or anti-rabbit IgG were used. Bands were visualized with BioRad AP Conjugate Substrate Kit NBT/BCIP. The amount of the immunoreactive product in each lane was determined using the Quantity One software (BioRad Laboratories, Hercules, CA, USA). Values were calcu-lated as relative absorbance units (RQ) per mg protein.

Statistical analysis

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, with $p < 0.05$.

Results

The effect of phytochemicals on cell viability

The impact of tannic acid, protocatechuic acid, indole-3-carbinol, and phenethyl isothiocyanate on the viability of HepG2 cells was evaluated using the MTT assay. Within the concentration range 0.2–150 μM, tannic acid, protocatechuic acid, phenethyl isothiocyanate, and indole-3-carbinol reduced the viability of HepG2 cell in a dose-dependent manner (Fig. 2). The IC_{50} values obtained for phenethyl isothiocyanate, indole-3-carbinol, tannic acid, and protocatechuic acid were 15, 93, 42, and 150 μM, respectively. Based on these find-ings, all the subsequent studies were conducted at con-centrations of tannic acid, protocatechuic acid, and indole-3-carbinol of 2 and 10 μM while phenethyl iso-thiocyanate was applied at the concentrations of 1 and 5 μM.

The effect of phytochemicals on Nrf2 expression and phosphorylation

Quantitative analysis revealed a significant increase in the amount of *Nrf2* transcript in HepG2 cells as the result of phenethyl isothiocyanate treatment at the con-centrations of 1 and 5 μM and tannic acid, protocatechuic acid, and indole-3-carbinol at the con-centrations of 2 and 10 μM (Fig. 3a). Phenethyl isothio-cyanate was shown to be the most potent inducer of *Nrf2* transcription leading to an increase in its transcript level by 86–140 %. Nrf2 may induce gene expression only after its translocation into the nucleus. To investigate the translocation of Nrf2 upon phytochemical treatment, levels of Nrf2 in the cytosol and nuclear fractions of the cells were evaluated by Western blot analysis.

Table 1 The sequences of primers used in real-time PCR reactions

	Forward primer	Reverse primer	Product size
SOD	5′CGACAGAAGGAAAGTAATG	5′TGGATAGAGGATTAAAGTGAGG	146 bp
CAT	5′TGGACAAGTACAATGCTGAG	5′TTACACGGATGAACGCTAAG	144 bp
GPx	5′CAACCAGTTTGGGCATCAG	5′TTCACCTCGCACTTCTCG	120 bp
GR	5′ATCCCAAGCCCACAATAG	5′CTCCACAGCAATGTAACC	200 bp

Fig. 2 The effect of phytochemicals on the viability of HepG2 cells. Cell viability was expressed as the percentage of the optical density of phytochemical-treated cells in comparison with that of untreated control cells (100 % viability). Results were representative of three independent experiments (mean±SEM). *PEITC* phenethyl isothiocyanate, *I3C* indole-3-carbinol, *PCA* protocatechuic acid, *TA* tannic acid
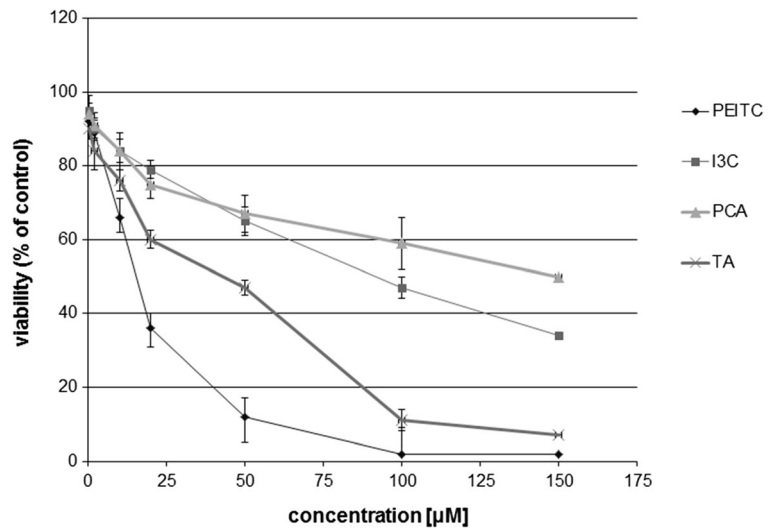
As shown in Fig. 3b, c, the Nrf2 protein was accumulated and phosphorylated at Ser 40 in the nucleus after exposure to all the studied phytochemicals; however, the highest level of Nrf2 phosphorylated at Ser 40 was induced by phenethyl isothiocyanate.

Since the activation of Nrf2 is generally accompanied by the degradation of Keap1, we also measured the amount of *Keap1* messenger RNA (mRNA) and cytosolic protein. However, the amount of neither *Keap1* mRNA nor the cytosolic protein was statistically significantly changed by phenolic acids, indoles, or isothiocyanates upon cell treatment (Fig. 3d, e).

The effect of phytochemicals on the expression and protein level of phase II enzymes

The expression of *GSTs* and *NQO1* was measured by quantitative PCR (Fig. 4). The level of *GSTA*, *GSTP*, *GSTM*, and *GSTT* mRNA transcripts was significantly increased after the treatment of HepG2 cells with indole-3-carbinol and phenethyl isothiocyanate. Protocatechuic acid in both tested doses enhanced only the expression of *GSTA*, *GSTM*, and *GSTT* while tannic acid increased the expression of *GSTT* in both tested doses and *GSTA* and *GSTM* only in a dose of 2 μM. On the other hand, the expression of *GSTP* was enhanced only by protocatechuic acid in both tested doses. Moreover, all the tested compounds at higher concentrations increased the expression of *NQO1* in HepG2 cells.

The level of GSTA, GSTP, GSTM, GSTT, and NQO1 proteins in HepG2 cells was evaluated using

Western blot assay (Fig. 5). The cytosolic content of GSTA, GSTP, GSTM, and GSTT (Fig. 5) was significantly increased after treatment with indole-3-carbinol and phenethyl isothiocyanate. The similar effect was observed for protocatechuic acid with the exception of GSTP protein. The level of GSTA, GSTM, and GSTP was not statistically changed after treatment with tannic acid whereas the protein level of GSTT was enhanced after incubation with 2 and 10 μM of tannic acid. Indole-3-carbinol and phenethyl isothiocyanate only at their highest doses enhanced the level of NQO1. The similar effect was observed for phenolic acids.

The effect of phytochemicals on the expression and protein level of antioxidant enzymes

Figure 6 shows data on the effect of phytochemicals on antioxidant gene expression in HepG2 cells. The expression of *CAT*, *SOD*, *GR*, and *GPx* increased after treatment with indole-3-carbinol and phenethyl isothiocyanate. As shown in Fig. 6, a similar effect was observed for phenolic acids except for the expression of *SOD*.

The results of Western blot analysis detecting the level of CAT, SOD, GR, and GPx proteins are shown in Fig. 7. The CAT, GPx, and GR protein level increased after the treatment of HepG2 cells with all the tested compounds. The level of SOD was significantly enhanced by indole-3-carbinol and phenethyl isothiocyanate. By contrast, no significant increase in the SOD protein level was found in HepG2 as a result of the exposure to phenolic acids.
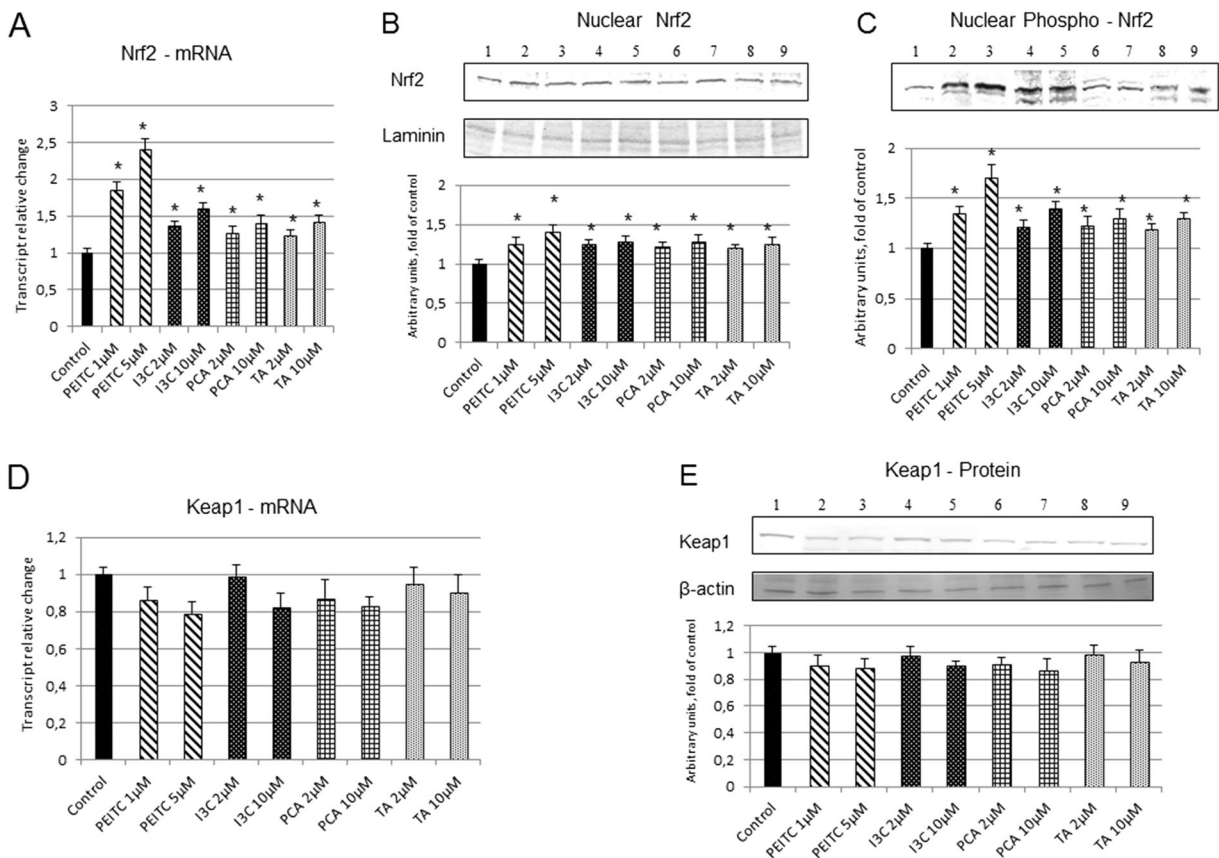
Fig. 3 The effect of phytochemicals on Nrf2 activation in HepG2 cells. **a** Nrf2 transcript levels. The values were calculated as mRNA level in comparison with control cells (expression equals 1). **b** Representative immunoblots for the analysis of the nuclear level of Nrf2 protein. *Lane 1* control, *lane 2* PEITC 1 μM, *lane 3* PEITC 5 μM, *lane 4* I3C 2 μM, *lane 5* I3C 10 μM, *lane 6* PCA 2 μM, *lane 7* PCA 10 μM, *lane 8* TA 2 μM, *lane 9* TA 10 μM. The level of laminin was used as a normalization control. Results of Western blot analysis of the nuclear content of the Nrf2 protein. **c** Representative immunoblots for the analysis of the nuclear level of the p-Nrf2 protein. Results of Western blot analysis of the nuclear content of the p-Nrf2 protein. The values were calculated as protein level in comparison with control cells (expression equals 1). **d** Keap1 transcript levels. **e** Representative immunoblots for the analysis of the cytosolic level of Keap1 protein. The level of β-actin was used as a normalization control. Results of Western blot analysis of the cytosolic content of Keap1 protein. The values were calculated as protein level in comparison with control cells (expression equals 1). The data are the means±SEM from three separate experiments. The *asterisk (*) above the bar* denotes statistically significant differences from the control group, $p < 0.05$

Discussion

Phase II along with antioxidant enzymes are responsible for the detoxification and elimination of xenobiotics and reactive metabolites including ROS that may cause damage to cells and tissues [21, 37]. The induction of these enzymes is now recognized as an efficient approach to prevent cancer. The key role in the induction of expression of phase II detoxifying enzymes as well as other cytoprotective proteins is played by the Nrf2–ARE pathway [3, 17].

The ability to activate Nrf2–ARE pathway and/or induce phase II enzymes by the breakdown products of glucosinolates such as isothiocyanates and indoles has widely been studied in different systems including HepG2 cells [6, 10, 14, 28]. In contrast, the effect of protocatechuic and tannic acids on phase II enzyme induction was evaluated only in animal models indicating that both compounds have the ability to enhance the activity and protein levels of phase II enzymes in mouse and rat liver [8, 10, 13].

The results of our current study indicate that protocatechuic and tannic acids induce phase II enzymes both on the transcriptional and protein level in human HepG2 cells in a manner similar to isothiocyanates/indoles, i.e., through the activation of Nrf2-ARE-
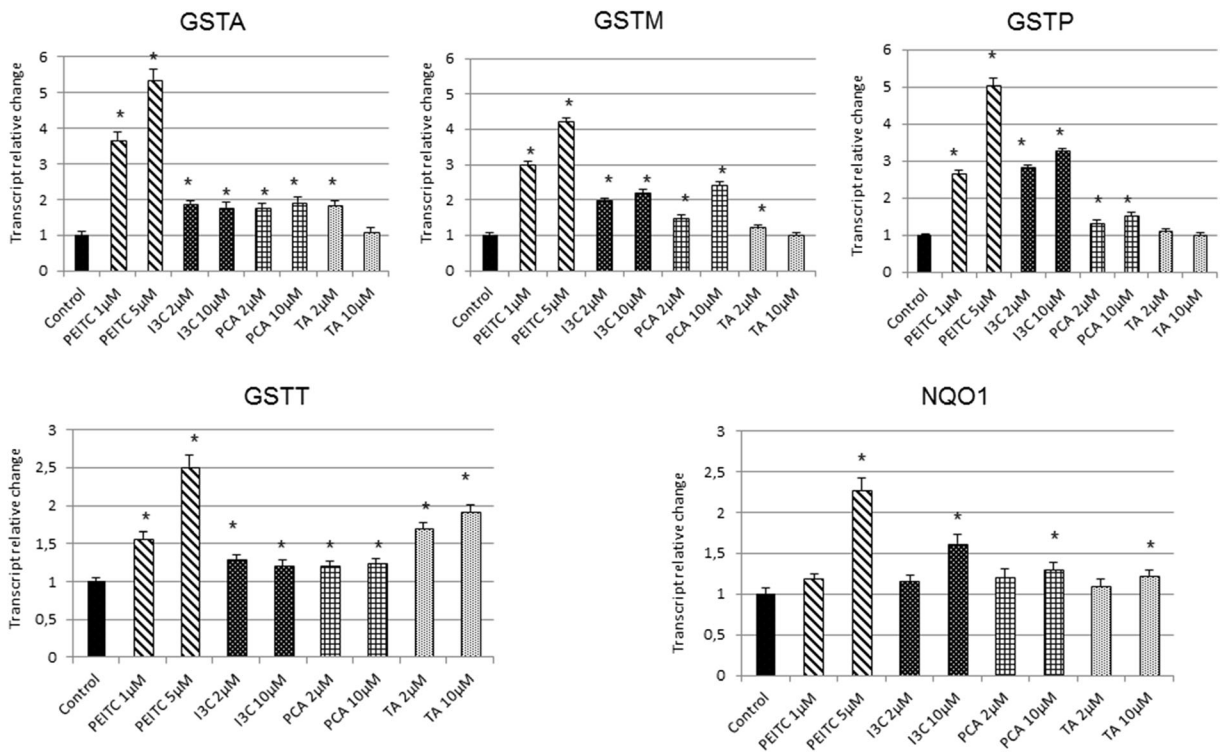
Fig. 4 The effect of phytochemicals on the expression of phase II enzymes in HepG2 cells. mRNA expression of *GSTA*, *GSTM*, *GSTP*, *GSTT*, and *NQO1*. The values were calculated as mRNA level in comparison with control cells (expression equals 1). Data (mean±SEM) from three separate experiments are presented; *asterisk above bar* denotes statistically significant differences from the control group, $p < 0.05$

pathway. Although characterized by relatively low levels of cytochrome P450 enzymes, HepG2 cells possess a complete set of phase II enzymes at the levels comparable to those found in primary human hepatocytes, with the exception of UDP-glucuronosyltransferases [33, 34]. Thus, the results obtained in this study might reflect the situation in normal human hepatocytes.

All the phytochemicals tested in this study stimulated the translocation of the active form of Nrf2 into the cell nucleus in a concentration-dependent manner. However, Nrf2 activation by these compounds was not associated with any changes in the level of Keap1 expression. These observations suggests that the tested phytochemicals may react with redox reactive cysteines in Keap1 and disrupt the interaction between Nrf2 and Keap1, hence allowing its translocation [29]. This suggestion further supports the accumulation of Nrf2 phosphorylated at Ser 40 in the nuclear compartment correlating with its translocation. The latter is in agreement with the findings showing that Nrf2 phosphorylation is a critical event in the nuclear translocation of this transcription factor and is necessary for its transcriptional activity [27].

The majority of genes that encode phase II and antioxidant enzymes possess an ARE sequence in their promoter region [26]. Thus, the increased levels of the mRNA transcript and protein of phase II enzymes, GSTs, NQO1, and antioxidant enzymes, CAT, SOD, GPx, and GR as the result of treatment of HepG2 cells with the examined phytochemicals suggest that they may facilitate the binding of Nrf2 to this sequence.

Quantitative PCR analysis revealed the significant increase in the expression of all the tested isoforms of GST (GSTM, GSTT, GSTA, and GSTP) as the result of treatment of HepG2 cells with phenethyl isothiocyanate and indole-3-carbinol. Thus, the results of our present study at mRNA level confirm our earlier observations in in vivo model, showing increased protein levels of these isoforms in rat liver as the result of the administration of these *Brassicacae* components [10]. Moreover, similarly as in rat liver, phenethyl isothiocyanate was a stronger inducer of the expression of GST isoforms than indole-3-carbinol also in human cells. The earlier in vivo
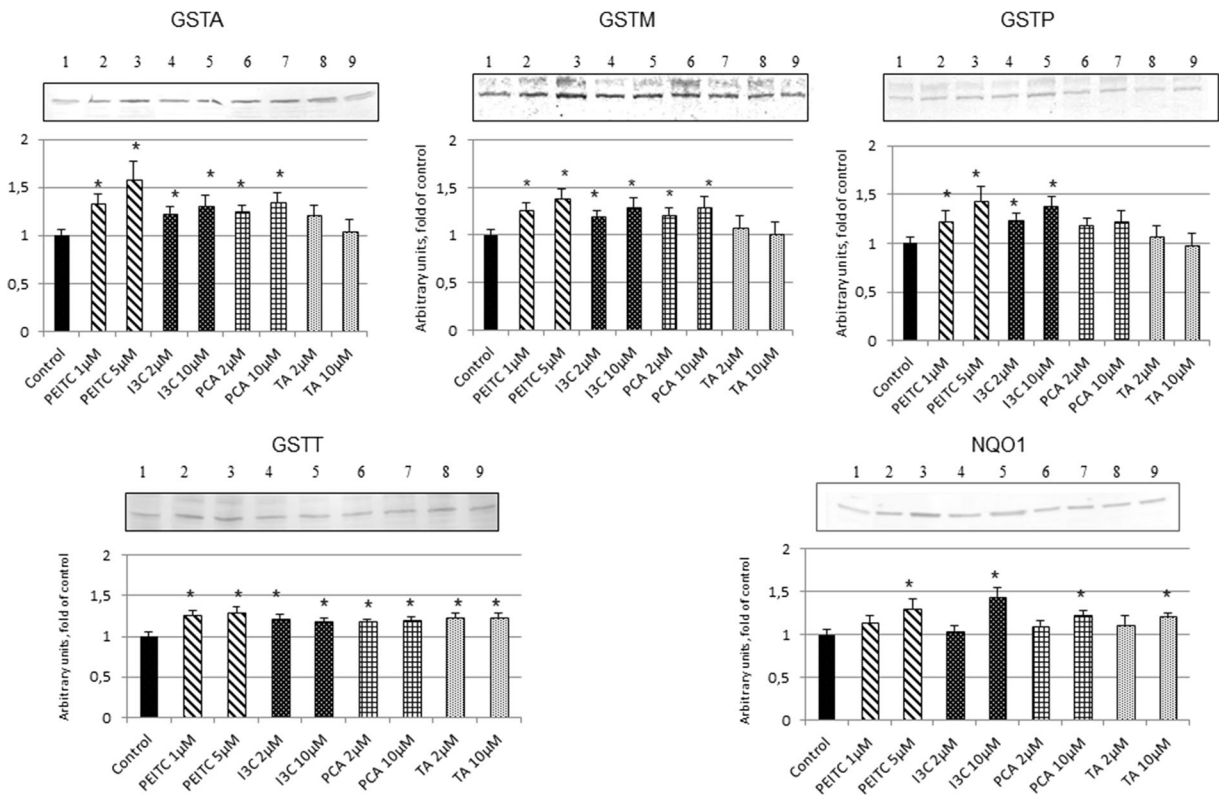
Fig. 5 The effect of phytochemicals on the protein level of phase II enzymes in HepG2 cells. Representative immunoblots for the analysis of GSTA, GSTM, GSTP, GSTT, and NQO1. *Lane 1* control, *lane 2* PEITC 1 µM, *lane 3* PEITC 5 µM, *lane 4* I3C 2 µM, *lane 5* I3C 10 µM, *lane 6* PCA 2 µM, *lane 7* PCA 10 µM, *lane 8* TA 2 µM, *lane 9* TA 10 µM. Results of Western blot analysis of the content of GSTA, GSTM, GSTP, GSTT, and NQO1. The values were calculated as protein level in comparison with control cells (expression equals 1). Data (mean±SEM) from three separate experiments are presented; *asterisk above bar* denotes statistically significant differences from the control group, $p<0.05$

observations in rat [8] and mouse liver [13] were confirmed by the results of the present study also in the case of protocatechuic acid. This compound similarly as in animal models significantly upregulated the expression of *GST* isozymes, particularly *GSTM*, in HepG2 cells.

However, tannic acid with a more complex structure being a polymer of digallic acid and glucose increased only the transcript and protein level of GSTT. This observation is in contrast to the results obtained in animal models [8, 13], in which tannic acid decreased the protein level of GSTT and GSTA. This discrepancy might result from the differences in the bioavailability of this complex molecule in vivo and in vitro. The available data indicate that in rats following oral administration at a dose of 1.0 g/kg, ~60 % of tannic acid remained unchanged, but some was hydrolyzed to gallic acid by tannase in the intestine and further metabolized to 4-O-methylgallic acid, pyrogallol, and resorcinol [24].

In vitro bioavailability of tannic acid was evaluated in ligated rat small intestine segments showing 50 % uptake but not complete transfer through the gut wall [2]. Thus, further studies are required in order to explain the fate of tannic acid in HepG2 cells and the mechanism of GST isoform induction by this compound.

GSTT which is induced in HepG2 cells by tannic acid acts as a scavenger of electrophiles, such as epoxides; however, it may also metabolically activate halogenated compounds, thus producing a variety of intermediates that can potentially damage DNA and cells [16]. Thus, its induction in cancer cells might be considered as ambiguous.

Another important observation of this study was the increased expression of GSTP induced by phenethyl isothiocyanate and indole-3-carbinol. This GST isoform protects cells against the adverse effects of such compounds as toxins, carcinogens, and ROS [19]. An increase in GSTA and GSTM levels by the same
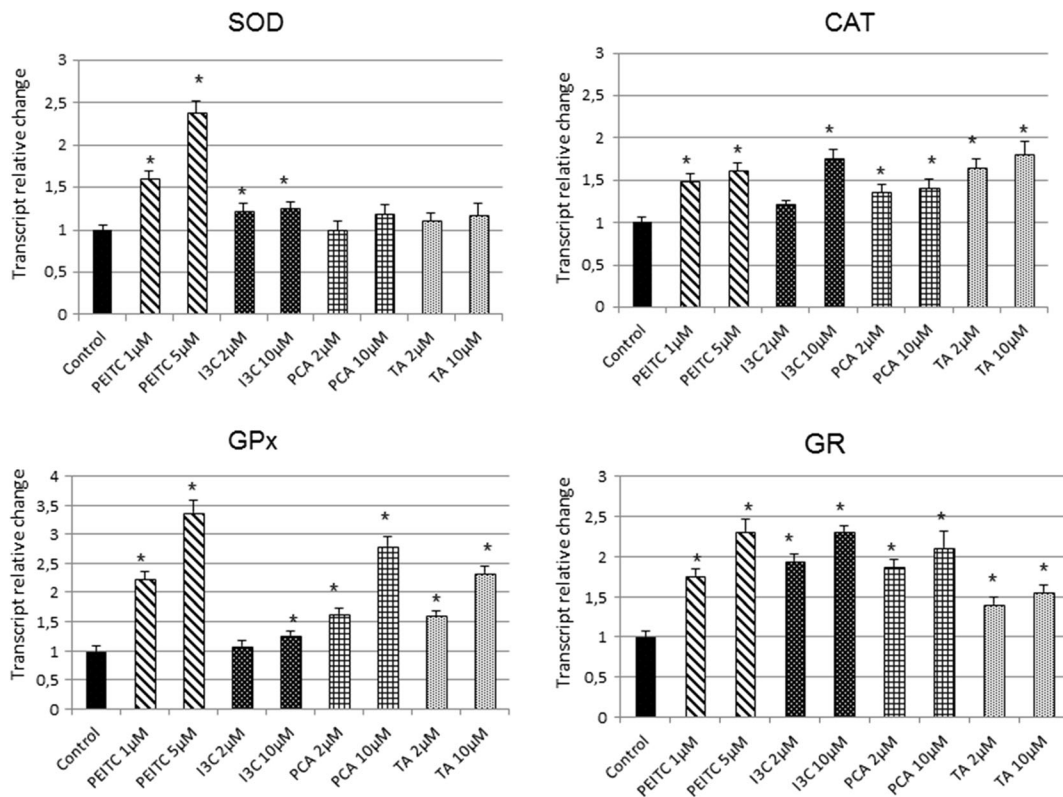
Fig. 6 The effect of phytochemicals on the expression of antioxidant enzymes in HepG2 cells. mRNA expression of SOD, CAT, GPx, and GR. The values were calculated as m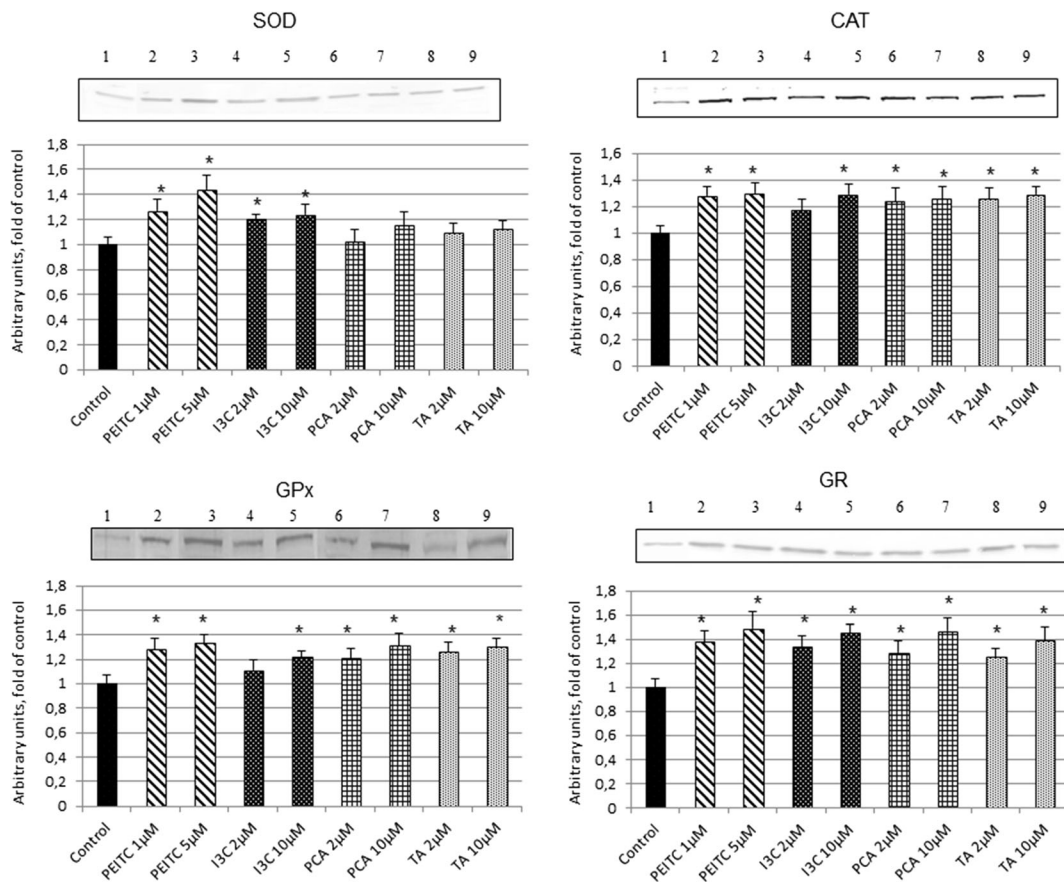RNA level in comparison with control cells (expression equals 1). Data (mean±SEM) from three separate experiments are presented, *asterisk above bar* denotes statistically significant differences from the control group, $p < 0.05$

compounds might be beneficial as protection against ROS and the products of lipid peroxidation, such as 4-hydroxynonenal [38].

Besides the influence on GSTs, the phytochemicals tested in our study enhanced both the mRNA and protein level of NQO1 in HepG2 cells. The induction of NQO1 which coincided with increased activity and protein level of NQO1 was observed in our earlier study [10] in rat liver as the result of oral administration of phenethyl isothiocyanate and indole-3-carbinol. In this regard, the results of the present in vitro study confirmed those obtained in the animal model. Again, the results of this study showed that phenethyl isothiocyanate and indole-3-carbinol were also more potent inducers of this enzyme than protocatechuic and tannic acids. These compounds possess totally different chemical structures. Isothiocyanates are characterized by the presence of sulfur containing N=C=S functional group, which is a Michael reaction acceptor [31]. This functional group plays an important role in the induction of phase II enzymes.

Nrf2 transcription factor controls also the expression of the antioxidant enzymes, GR, GPx, CAT, and SOD, playing a crucial role in the defense against oxidative stress [1]. Our study showed that isothiocyanates and indoles induced all these enzymes in HepG2 cells. A similar effect in the case of SOD expression was found in Hepa1c1c7 cells by Saw and Kong [29] for phenethyl isothiocyanate. This compound was also shown to increase the activity of the antioxidant enzymes GR and CAT in hepatocytes isolated from Wistar rats [15].

Interestingly, the results of the present study showed also that protocatechuic and tannic acids were equally potent inducers of the expression of antioxidant enzymes (except SOD) in HepG2 cells as isothiocyanates/indoles. These in vitro observations showing the ability of protocatechuic and tannic acids to induce antioxidant enzymes are also in agreement with recently published data on the effect of these compounds in

Fig. 7 The effect of phytochemicals on the protein level of antioxidant enzymes in HepG2 cells. Representative immunoblots for the analysis of SOD, CAT, GPx, and GR. *Lane 1* control, *lane 2* PEITC 1 μM, *lane 3* PEITC 5 μM, *lane 4* I3C 2 μM, *lane 5* I3C 10 μM, *lane 6* PCA 2 μM, *lane 7* PCA 10 μM, *lane 8* TA 2 μM, *lane 9* TA

10 μM. Results of Western blot analysis of SOD, CAT, GPx, and GR. The values were calculated as protein level in comparison with control cells (expression equals 1). Data (mean±SEM) from three separate experiments are presented; *asterisk above bar* denotes statistically significant differences from the control group, $p<0.05$

animal models [35, 39]. These studies showed that treatment of rats with both protocatechuic acid and tannic acid induced the activity of GPx and CAT.

It has to be stressed that the doses used in our in vitro study were comparable to those which might be obtained in human serum after oral administration of indole-3-carbinol [23], isothiocyanate phenethyl [18], protocatechuic acid [22], or tannic acid [25]. Thus, the results of our studies might partly reflect possible in vivo effects.

In summary, we demonstrated that isothiocyanates/indoles and protocatechuic and tannic acids induce phase II and antioxidant genes in vitro through the Nrf2-Keap1-ARE signaling pathway and that the mechanism of this induction might result from their interaction with the Keap1 protein.

Moreover, the results of this study confirmed that protocatechuic and tannic acids are less effective than the degradation products of glucosinolates—phenethyl isothiocyanate or indole-3-carbinol in the induction of phase II enzymes. However, protocatechuic and tannic acids induced antioxidant enzymes to the same extent as isothiocyanates/indoles in HepG2 cells.

This activity may be a part of their chemoprotective activity against injury by toxicants and/or oxidants. Thus, our results contribute to a better understanding of the effect of these chemically diverse phytochemicals, which are common ingredients of daily human diet.

Conflicts of interest All authors declare no conflicts of interest.

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