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Effects of dwarf elder fruit infusion on nuclear factor kappa B and glutathione metabolism-related genes transcription in a model of lipopolysaccharides challenged macrophages

O. B. Tasinov*, Y. D. Kiselova-Kaneva, D. G. Ivanova

Department of Biochemistry, Molecular Medicine and Nutrigenomics, Faculty of Pharmacy
Medical University - Varna, 84B Tzar Osvoboditel Str., 9002 Varna, Bulgaria

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Sambucus ebulus L. (SE) is shown to be implicated in combating oxidative stress in inflammation and cell death. We aimed to analyse the effect of SE fruit aqueous infusion (FAI) in a model of lipopolysaccharides (LPS)-induced cytotoxicity in J774A.1 mouse macrophages. Transcription levels of NF-κB, antioxidant enzymes glutamate-cysteine ligase, catalytic subunit (GCLc) and glutathione peroxidase (GPx) were analysed. Cell viability tests showed that LPS (25-200 ng/mL) caused gradual cell death by up to 14.5%, whereas SE FAI (0.625%-12.5% in culture media) was non-toxic. Salicylic acid (SA) as a positive control (25-200 μM) gradually induced cell proliferation by up to 15%. Real-Time PCR analysis revealed that SE FAI alone significantly up-regulated NF-κB, GPx and GCLc mRNA levels (3.8, 3.04 and 9.57-fold, respectively). Pre-treatment with SE FAI significantly reduced LPS (200 ng/mL)-stimulated transcription of NF-κB and GPx by 70%, and GCLc by 37%. The effect of SE FAI/+LPS was similar to the effect of SA/+LPS. NF-κB mRNA levels significantly correlated with those of GCLc (r=0.66), and GPx (r=0.79). We conclude that SE FAI may exhibit its anti-inflammatory and antioxidant effects by altering transcription of LPS-stimulated oxidative stress and inflammation related genes.

Keywords: *Sambucus ebulus*; NFκB; glutathione; glutathione peroxidase; lipopolysaccharides.

INTRODUCTION

Although being traditionally used as a medicinal plant, *Sambucus ebulus* L. (SE, dwarf elder) is a poorly studied species. Dwarf elder fruits aqueous infusion (FAI) is rich in polyphenols, flavonoids and anthocyanins [1-4]. Fruits also contain sugars, valeric acid, methyl salicylate, citronellal, methyl palmitate, ursolic acid, malic acid, tartaric acid, tannins, pectin, resins, vitamin C [4, 5]. Folk medicine prescribes fresh berries or decoctions in cases of haemorrhoids, gastric pain [6], tuberculosis [7] and rheumatoid arthritis [8].

Dwarf elder is a plant which fruit and flower infusions and extracts exhibit high antioxidant activity *in vitro* [2, 9] due to high polyphenol content, and can modulate antioxidant enzymes expression in cell cultures [1, 10]. However, the mechanism of boosted antioxidant defense is not clear. Polyphenols are known to increase glutathione (GSH) levels by activating expression of glutamate cysteine ligase (GCL) [11, 12]. The cells respond to oxidative stress by increasing the expression of GCL [13]. Another enzyme, which activity is related to GSH levels, is glutathione peroxidase (GPx), and its expression is also modulated by polyphenols [14].

One of the main signalling cascades involved in initiation of inflammation process acts *via* activation of transcription factor NF-κB [15]. NF-κB controls gene expression of many inflammation and oxidative stress related proteins [16-18]. Lipopolysaccharides (LPS) activate gene expression of inflammation related cytokines, adhesion molecules and enzymes by activating NF-κB-dependent signal pathway [19-24]. Plant polyphenols may reduce LPS – stimulated NF-κB activity [25].

Inflammatory response involving oxidative stress in macrophages could be provoked by different stimuli such as high levels of free fatty acid (obesity), high glucose levels (diabetes) and bacterial endotoxins (infections) [26]. Earlier we reported the effects of SE FAI on GCL catalytic subunit (GCLc) and GPx genes expression in a cell culture model of *tert*-butyl hydroperoxide (*t*-BuOOH)-induced oxidative stress [27]. To reveal the potential mechanisms of anti-inflammatory and antioxidant activity of SE fruits now we used a cell-culture model of LPS-stimulated inflammatory response exploring the effect of SE FAI on the expression of GCLc, GPx and NF-κB in J774A.1 macrophages.

MATERIALS AND METHODS

Plant material

Sambucus ebulus L. fruits were collected from North-Eastern Bulgaria in the period August – September, 2014 and were dried at room

* To whom all correspondence should be sent:
E-mail oskan.tasinov@gmail.com

temperature. SE FAI was prepared from 150 mg finely ground dried fruits, vortex extracted three times with 3 mL of distilled H_2O for 3 min at room temperature. After centrifugation (5 min, 3500 rpm) the supernatants were collected and diluted to 15 mL with PBS buffer (pH=7.4).

Cell culture

J774A.1 mouse macrophage cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured according to the manufacturer's requirements. Cell counts were prepared in quadruplicate by 0.4% trypan blue exclusion dye (Chemapol, Czech Republic) using a counting Burker-chamber.

Cell viability test

Viability of treated cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [28]. Cells were seeded and after 24 h were treated with culture media containing SE FAI (0.625% – 12.5% v/v), or SA (25 μM – 400 μM), or LPS (25 ng/mL – 200 ng/mL). After 20 h 100 μL MTT solution (2 mg/mL) (AppliChem, Germany) was added and cells were incubated in dark for additional 4 h. Formazan crystals were solubilized with dimethyl sulfoxide (Scharlau Chemie S.A., Spain). Absorbance was measured using a multiwell scanning spectrophotometer (ELISA reader-Synergy 2, BioTek) at 550 nm. Untreated cells were used as control. Cell viability (%) was calculated as [(mean absorbance of the sample/mean absorbance of the control) × 100]. Treatments were performed in triplicate. Results are presented as mean ±SD.

Experimental design

Experimental model involved macrophage cells seeded in 6-well plates (2×10^5 cells/well), and allowed to adhere overnight. Cells were pre-treated with SE FAI dissolved in different concentrations (2.5%, 5% and 10% v/v) in DMEM (with 4.5 g/L glucose, w/o phenol red and L-glutamine) supplemented with 10% FBS, 100 U/mL penicillin/streptomycin mixture and 2 mM L-glutamine. After 24 h cells were treated with 200 ng/mL LPS (*Escherichia coli* 026:B6, Sigma-Aldrich, Germany) for additional 24 h. After LPS stimulation the cells were lysed and total RNA was extracted using TRI reagent (Ambion, USA) according to the manufacturers requirement. Non-treated cells were used as control. Salicylic acid (SA) (Merck, Germany), in concentration of 100 μM was used as a positive control. Treatments were performed in triplicate.

Gene expression analysis

GCLc, GPx, NF-κB and β-actin (internal control) genes expression was analysed using Real-Time PCR as previously described [29]. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [30]. The used primer sequences (Sigma-Aldrich, Germany) for each gene analysed are presented in Table 1. A denaturation step was added to the instrument's protocol to check for nonspecific amplification. Expression levels of mRNA are presented in relative units (RU) as compared to the control group, where the level of mRNA expression were considered to be equal to 1. Analyses were performed in triplicate.

Table 1. Primer sequences used in Real-Time PCR analysis.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-Actin	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAG
GCLc	AATGGAGGCGATGTTCTTGAG	CAGAGGGTCGGATGGTTGG
GPx	CCCCACTGCGCTCATGA	GGCACACCGGAGACCAAA
NF-κB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG

Statistical analysis

Graph Pad Prism 5.0 software was used to perform the statistical analyses (Student's *t*-tests). The values of $p<0.05$ were considered as significant.

RESULTS

Effects of SE FAI, LPS and SA on cell viability

SE infusion did not exert any cytotoxicity on macrophages cell culture when applied in increasing concentrations in the culture medium from 0.625% to 12.5% (Fig. 1A). What is more, a significant increase by 15% of cell viability was detected in the group treated with 1.25% SE FAI ($p<0.05$). The concentrations of 2.5%, 5% and 10% were selected for further testing of the SE FAI protective effect on J774A.1 macrophages in conditions of LPS-induced oxidative stress and inflammatory response.

The applied concentrations of SA from 25 μM to 400 μM showed no cytotoxic effect on treated macrophages (Fig. 1B). The presence of SA in the culture medium even induced cell proliferation. The

concentration of 200 μM induced cell proliferation in a statistically significant manner by almost 15% (p<0.001). SA in concentration of 100 μM in the culture medium was used in further cell culture treatments as a positive control.

A statistically significant decrease in macrophage cell viability by 14.5% (p<0.01) was observed upon treatment with 200 ng/mL LPS (Fig. 1C). In the range of 150-200 ng/mL LPS's effect on cell viability did not vary substantially, so the highest cytotoxic concentration applied, 200 ng/mL, was used for stimulation of macrophages in the next experiments.

The effect of SE FAI on gene expression of GCLc, GPx and NF-κB

GCLc mRNA levels were significantly induced by 2.5% and by 5% SE FAI up to 9.57 -fold(p<0.001) and 2 -fold(p<0.05), respectively, compared to untreated control cells (Fig. 2A). Pre-treatment with 2.5% and 5% SE FAI significantly reduced LPS-stimulated gene expression (3.68-fold, p<0.01 *vs*. control) of GCLc by 1.37 (p<0.05) and 0.68 (p<0.05)-fold, respectively. The effect of fruit infusion was similar to that of the positive control (100 μM SA), which reduced LPS-induced GCLc mRNA levels 2.12-fold (p<0.01)

Significant up-regulation of GPx mRNA levels was seen in groups treated with 2.5% and 5% SE FAI up to 3.04-fold (p<0.001) and 1.49-fold (p<0.05), respectively, as compared to untreated cells (Fig. 2B). LPS-induced enzyme gene expression (3.3-fold, p<0.01 *vs.* control) reduced by pre-treatment with 2.5% (1.4-fold, p<0.01), 5 % (2.33-fold, p<0.01) and 10% (1.75-fold, p<0.001) SE FAI in a culture medium (Fig. 2B). SA as a positive control significantly reduced (2.55-fold, p<0.001) GPx mRNA transcription, as compared to LPS group, similarly to the effect exerted by the fruit infusion.

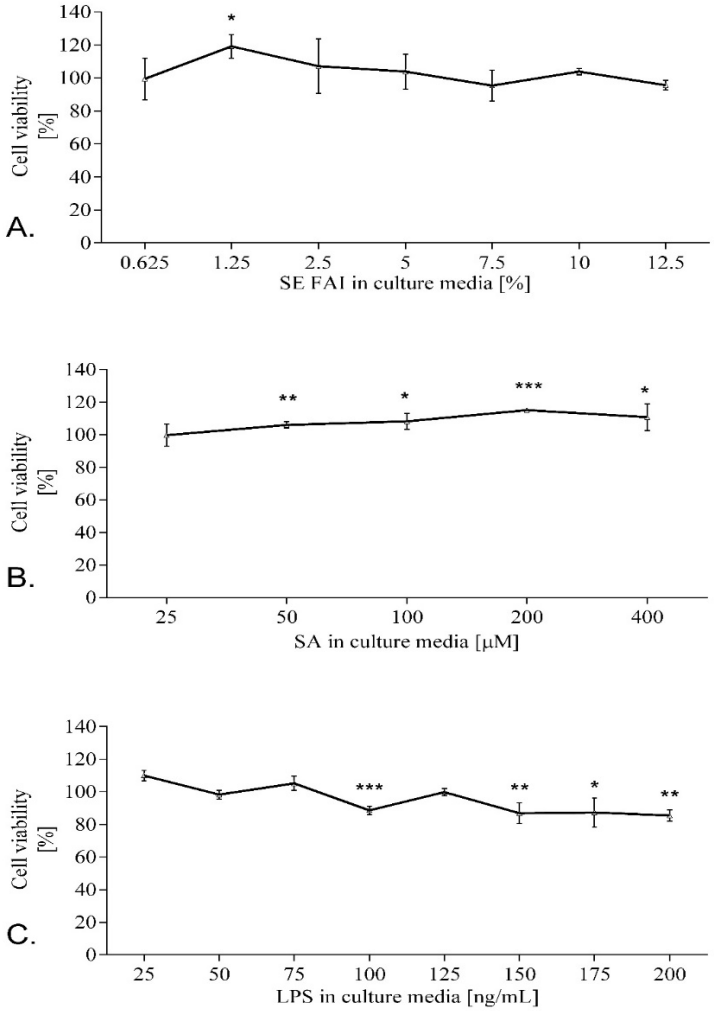

Figure 1. Effect of SE FAI (A), SA (B) and LPS (C) on J774A.1 cells viability. Data are presented as mean ±SD. *p<0.05, **p<0.01, ***p<0.001 *vs*. control (untreated cells).

Similar to the enzymes mentioned above, NF-κB transcription levels were up-regulated by 2.5%, 5% and 10% SE FAI treatments up to 3.8 (p<0.001), 2.3 (p<0.001) and 2.1-fold (p<0.01), respectively, as compared to untreated cells (Fig. 2C). Induced by LPS NF-κB transcription (3.7-fold, p<0.001 *vs.* control) was reduced by all applied SE FAI concentrations: 2.5% - 2.2-fold (p<0.001), 5 % - 2.6 –fold (p<0.01), and 10% - 2.2-fold (p<0.05). Expectedly, the effect of the fruits was comparable to that of the positive control, which significantly reduced (3.1-fold p<0.001) NF-κB mRNA levels as compared to LPS group.

Correlation analysis

Correlation analysis showed a highly significant linear dependence between mRNA levels of NF-κB and GCLc (r=0.66, p<0.05) and between NF-κB and GPx (r=0.79, p<0.01) in all treatment groups (Fig. 3).

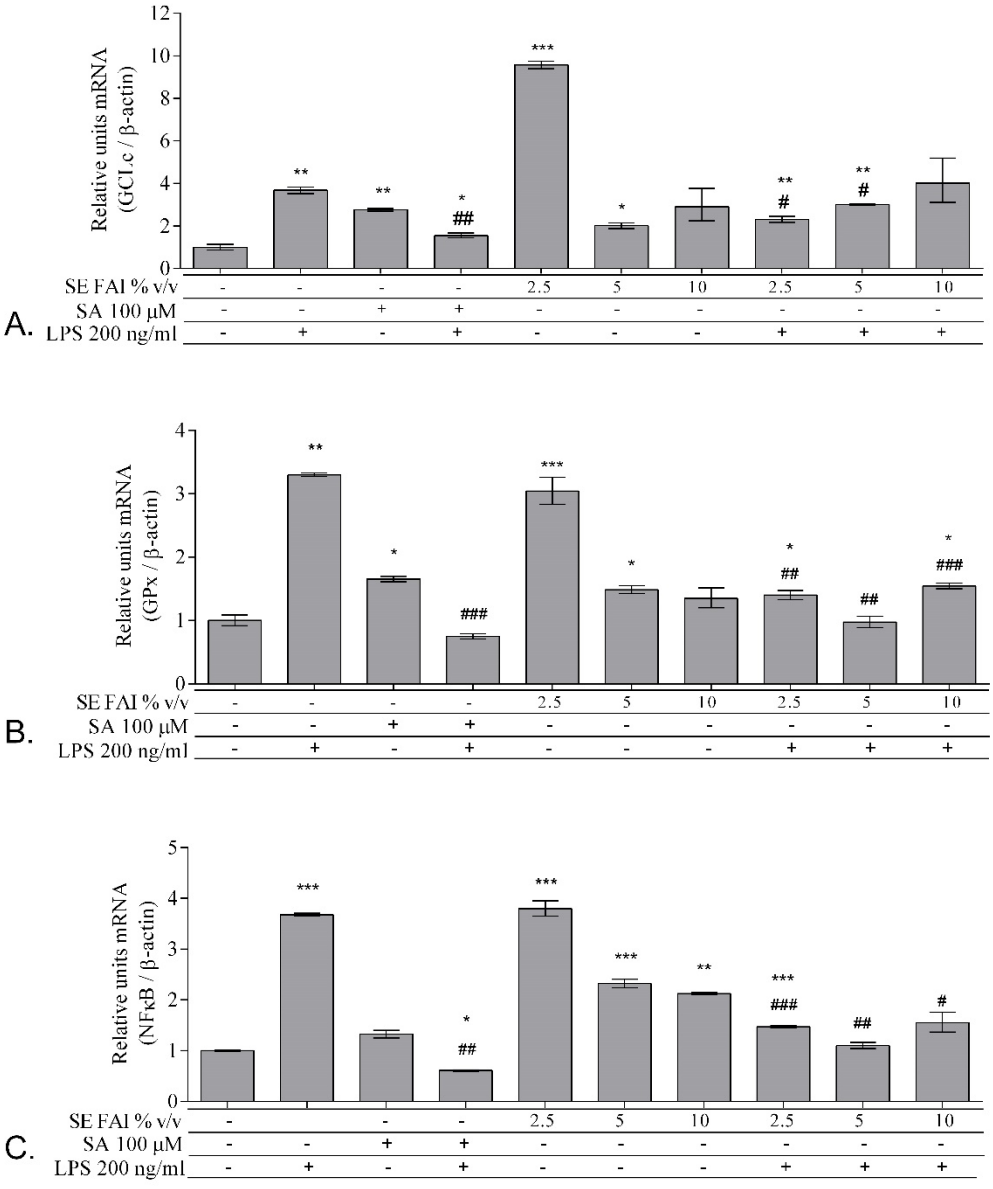

Figure 2. Changes of GCLc (A), GPx (B) and NF-κB (C) mRNA levels in J774A.1 cells pre-treated with increasing concentrations of SE FAI or SA, and stimulated with LPS. Data are presented as mean ±SEM. Legend: **C**-control (untreated cells); **SA –** 100 μM salicylic acid; **LPS –** 200 ng/mL lipopolysaccharides; **SE1 – 2.5**%, **SE2 – 5**%, **SE3 – 10**% SE FAI in culture medium. **p*<0.05, ***p*<0.01, ****p*<0.001 *vs.* **C**; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 *vs*. **LPS**.

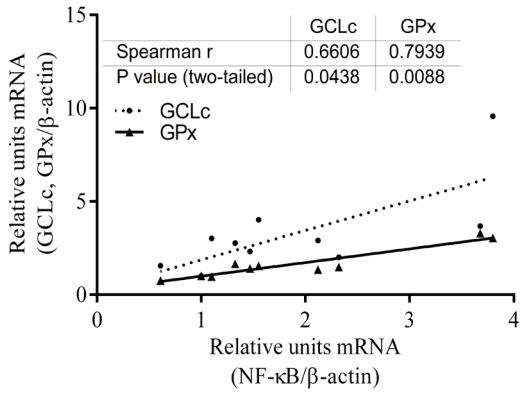

Figure 3. Correlation analysis between mRNA levels of NF-κB and GCLc, and of NF-κB and GPx in macrophages.

DISCUSSION

Whether a plant extract would have a cytotoxic or proliferative effect depends on the concentration administered. The type of extracting agent and substances contained in the extract, as well as the cell type are factors that may determine the effect on cell viability. In general, low concentrations stimulate the expression of genes associated with proliferation, and high concentrations activate caspase cascades that initiate apoptosis [31]. SE FAI did not exhibit any cytotoxicity in concentrations of 0.625% to 12.5% in the culture medium. Indeed, a significant increase in cell proliferation was established upon treatment of macrophages with 1.5% SE FAI. Similar results have also been reported for aqueous extracts of other medicinal plants [32]. Probably the biologically active substances contained in the Dwarf elder fruit aqueous extract affect the proliferation of the cells. Low concentrations of the aqueous extract had a cytoproliferative effect, whereas, with the increase in the concentration in the nutrient medium, cell vitality gradually declined.

Factors triggering cellular immune response, such as LPS, activate a respiratory burst that produces a large amount of reactive oxygen species (ROS) and develops oxidative stress [26]. Induced oxidative stress requires the activation of the endogenous antioxidant defence mechanisms, including the antioxidant enzymes. As a response to the increasing concentrations of free radicals in cells, in particular peroxides, in LPS-treated macrophages, the levels of the enzymes involved in the synthesis of the major cellular antioxidant glutathione (GCLc) and the enzyme reducing peroxides (GPx) increased significantly (p<0.01), (Fig. 2 A and B).

In RAW 264.7 macrophages, LPS treatment induced nitric oxide (NO) production [33], associated with production of peroxynitrites (ONOO.) [34, 35]. This enhances the oxidative stress in cells, thus explaining the activation of intracellular antioxidant defence, in particular, the enzymes GPx and GCLc.

In our experiments SE FAI alone induced significant gene expression of both enzymes GCLc (p<0.001) and GPx (p<0.001), as well as that of transcription factor NF-κB (p<0.001) in J774A.1 macrophages (Fig. 2). This effect could be attributed to the substances contained in the extract (e.g. polyphenols) activating redox-sensing transcription factors such as Nrf2 and NF-κB, which, on their turn, induce expression of cellular antioxidant enzymes and thus exhibit protective antioxidant and immunomodulatory action. Earlier we observed induction of GCLc and GPx genes expression in SE FAI-treated mouse 3T3-L1 preadipocytes [27]. Fruit extract rich in polyphenols such as quercetin and ellagic acid, can induce GCL transcription *in vitro* and *in vivo*, thus increasing GSH levels [11, 37]. The transcription of GCLc gene is controlled by response element binding sites including NF-kB and antioxidant response elements/electrophile response elements (AREs/EpREs) [11, 38]. Flavonoids, such as quercetin, established in SE fruits [4, 39, 40], activate GSH synthesis by AREs/EpREs in the promoter of the GCLc gene [11].

An oxidative stress associated transcription factor Nrf2 [41] is able to transfer to the nucleus and bind to AREs/EpREs element. Scientists claim that some quinones react with thiols [42], therefore, it can be assumed that quercetin oxidized to quinone by ROS, oxidizes thiols in the Keap1 protein, leading to Nrf2 release and to the activation of GCLc gene expression [10]. GPx is a second phase enzyme of xenobiotic metabolism, which expression, similarly to GCLc, is positively influenced by activation of the Nrf2 transcription factor [43, 44].

Cell glutathione levels and redox potential also increase as a result of NF-κB activation. As mentioned above, GCLc promotor contains NF-κB binding site [38]. We suggest that relationship exists between the activation of the NF-κB signaling pathway [45] and increased transcription levels of GCLc. These studies are in support to the observed high positive correlation between the transcriptional levels of NF-κB and those of the two enzymes GCLc and GPx (Fig. 3), indicating one of the possible mechanisms of GCLc and GPx induction by SE FAI itself.

In macrophages pretreated with SE FAI, there was a reduction in mRNA levels of GCLc and GPx enzymes, as compared to cells exposed to LPS only (Fig. 2 A and B). Similar effect was observed in SE

FAI-pre-treated mouse preadipocytes, in conditions of *t*-BuOOH-induced oxidative stress [27]. This effect may be explained by the *in vitro* antioxidant properties of the aqueous extract [1] in conditions of LPS stimulated oxidative stress. Flavonoids and anthocyanins found in SE fruits are able to bind and neutralize free radicals. On the other hand, SE FAI itself induces the gene expression of both enzymes from GSH metabolism, as discussed above. Thus, to a certain extent, SE FAI bioactive compounds compensate for the need to induce antioxidant protection as seen in cells pre-treated with SE FAI and subsequently stimulated with LPS.

NF-κB transcription factor plays a key role in the induction of inflammation and oxidative stress, for example in obesity and development of insulin resistance [26, 46, 47]. In LPS stimulated macrophages, transcriptional levels of NF-κB increased more than 3-fold, in contrast to cells where pre-treatment with SE FAI significantly reduced LPS-stimulated expression of the transcription factor (Fig. 2C).

In a model of LPS-induced inflammation in human monocytes, plants rich in anthocyanins have been shown to inhibit the activity of NF-κB [25]. Ursolic acid found in SE fruits [4] is known to exert anti-inflammatory action in various cell types inhibiting NF-κB activation [48]. Suppression of transcriptional levels of NF-κB might be a probable mechanism, by which SE fruits exhibit anti-inflammatory activity suggesting their use in the prevention of acute and chronic inflammation states.

In this aspect, quite convincing is the fact that the effect of SE FAI on gene expression in all experiments followed the same direction as the effect of SA (Fig. 2). Salicylates are well known inhibitors of NF-κB activation, thus inhibiting LPS-stimulated inflammatory response [48-50]. Probably the unique combination of active compounds is in the basis of the biological effects of SE fruits. Further experiments and detailed analysis of SE FAI immunoregulatory potential involving transcriptomics and proteomics in animal models of inflammation or human intervention studies may help to elucidate the mechanism of action.

CONCLUSIONS

These findings are in support to folk medicine traditions where the SE fruits are used for immunostimulation. Our study provides first scientific data on the effects of SE FAI in a model of LPS-stimulated cells. SE fruits alter GCLc and GPx expression levels in a model of oxidative stress and inflammation, possibly by modulating NF-κB mRNA expression. Data reveal the potential of SE fruits as a natural source for the development of new products for prevention of inflammation and oxidative stress related disease states.

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Conflict of Interest: *The authors declare that there are no conflicts of interest.*

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