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Chemical composition and cytoprotective and anti-inflammatory potential of *Sambucus ebulus* fruit ethyl acetate fraction

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Sambucus ebulus (SE) fruits are used in traditional medicine for immunostimulation and treatment of gastrointestinal disorders. We examined the anti-inflammatory potential of SE fruit ethyl acetate fraction (EAF) on a cell model of ethanol-induced cytotoxicity of 3T3-L1 preadipocytes. After fractionation the total polyphenol content (TPC) using Folin-Ciocalteu reagent and total antioxidant capacity (TAC) by $ABTS^+$ decolorization assay of SE fruit extract and fractions were measured. By thin-layer chromatography, the presence of selected polyphenols was analysed. Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide reduction assay. Cells were treated with SE EAF dissolved in ethanol or ethanol only. Transcription levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and glutamate-cysteine ligase catalytic subunit (GCLc) were measured using qPCR. Relative transcription levels were calculated using $2^{-\Delta\Delta}$ Ct method.

SE EAF exhibited the highest TPC and TAC among the analysed extracts. For the first time the presence of hyperoside, isoquercetin, isorhamnetin-3-O-β-glucopyranoside and traces of rutin and of 3,5-dicaffeoylqunic acid are reported for the SE EAF. SE EAF showed a cytoprotective effect, by increasing the ethanol-suppressed cell viability up to 2.4 times ($p<0.001$). Treatment with SE EAF (0.02% w/v) decreased ethanol-induced iNOS, COX-2, TNF-α and GCLc gene expression by 63% ($p<0.01$), 54% ($p<0.001$), 64% ($p<0.01$) and 65% ($p<0.05$), respectively, indicating anti-inflammatory potential of the fruit extract. Lower concentration (0.01% w/v) of SE EAF decreased IL-6 and GCLc gene expression by 71% ($p<0.01$) and 45% ($p<0.05$), respectively. This study provides first scientific evidences about the cytoprotective and anti-inflammatory potential of SE fruit EAF.

Keywords: *Sambucus ebulus*, 3T3-L1, ethanol, flavonoids, cytoprotective, anti-inflammatory

INTRODUCTION

S. ebulus L. (SE), widely used as a medicinal plant, is in fact a poorly studied species in regard to its biological effects. Fruits are rich in polyphenols, flavonoids and anthocyanins [1–3], sugars, valerianic acid, malic acid, tartaric acid, tannins, pectin, resins, vitamin C [4, 5]. In traditional medicine fruits are used to treat inflammation-related gastrointestinal disorders [6], tuberculosis [7] and rheumatoid arthritis [8].

Depending on the different phytochemical composition, different parts of the herb possess diverse biological activities. In support to folk medicine, scientific researches revealed high antioxidant, anti-inflammatory, antinociceptive, antiarthritic, and antimicrobial activities for SE flowers, fruits, leaves and roots [2, 3, 9–19] in a variety of models and scientific approaches. A recent study on the activities of SE fruit ethyl acetate fraction (EAF) suggested also possible anticancer activity [20].

Plant extracts with anti-inflammatory properties may provide a new useful therapeutic targeting inflammation for prevention or treatment of various diseases. The aim of the current study was to analyse SE fruit EAF cytoprotective and anti-inflammatory properties using a human physiology-relevant model of ethanol-induced inflammatory response in 3T3-L1 mouse preadypocytes. Chronic ethanol consumption is known to increase the IL-6 and TNFα production, and it is associated with insulin resistance in rats [21]. Ethanol treatment induces production of the inflammatory enzymes iNOS and COX-2 in animal models [22, 23], as well as in cell cultures [24], including 3T3-L1 preadipocytes [25]. There are scientific studies considering ethanol consumption as a reason for development of inflammation in adipose tissue [26]. Elevated adipose tissue IL-6 and TNF-α levels correlate with ethanol induced liver injury [26].

MATERIAL AND METHODS

Plant material

Sambucus ebulus fruits were collected in Northern Bulgaria in the period from late August to the end of September. Plant material was dried in shade at room temperature.

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Extraction and fractionation

Five grams of powdered dry fruits were extracted for 30 min with 100 mL of methanol at room temperature in a ultrasound chamber. The extract was filtered and the plant material was extracted another two times using the same procedure. All filtrates were combined and the crude methanol extract was evaporated to dryness under vacuum. The residue was dissolved in 50 mL of distilled water and the solution was further extracted consecutively with petroleum ether, chloroform, ethyl acetate and butanol (Fig. 1).

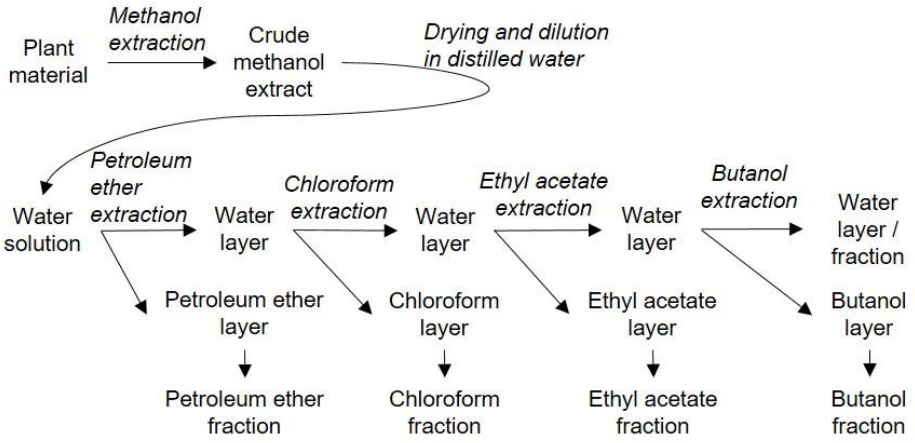

Figure 1. Extraction and fractionation procedure.

Measurement of total polyphenol content and total antioxidant capacity

Extracts and fractions were analysed immediately after their preparation. For the measurement of total polyphenol content (TPC) and total antioxidant capacity (TAC) 10 mg of fractionated material was dissolved in 1 mL of appropriate solvent: chloroform for petroleum ether and chloroform fraction; absolute ethanol for crude methanol extract, ethyl acetate and butanol fractions; distilled water for water fraction.

TPC was determined using Folin-Ciocalteu reagent [27]. Results were expressed as mmol/L quercetin equivalents (QE).

ABTS cation radical decolorization assay was performed to determine *in vitro* TAC of the extracts [28]. Results were expressed as mmol/L uric acid equivalents (UAE).

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed using silica gel plates (Merck, Germany), $CH_3COOCH_2CH_3$: HCOOH: CH_3COOH: H_2O (100:11:11:26) and TLC spots were visualized with NP/PEG reagent at 366 nm [29].

Cell culture

3T3-L1 preadipocytes from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle media (DMEM) (LONZA, Belgium) supplemented with 10% foetal bovine serum (SAFC Biosciences ™, USA), and 1% antibiotic (100 U/mL penicillin, 100 U/mL streptomycin sulphate) (LONZA, Belgium) at 37 ˚C in a humidified 5% CO_2 incubator.

Treatment scheme

Solutions for cell treatment were prepared as follows: 20 mg of SE EAF was dissolved in 0.5 ml of absolute ethanol (Scharlau Chemie S.A., Spain) and diluted with 0.9% NaCl up to 2 mL; 0.5 mL of absolute ethanol was diluted with 0.9% NaCl up to 2 mL; control cells were treated with culture medium only.

For cytotoxicity analysis cells were treated with ethanol (0-0.625% v/v) and SE EAF (0-0.025% w/v) dissolved in ethanol, in increasing concentrations for 24 hours.

To study SE EAF cell protective activity, the cells were co-treated with two different concentrations of SE EAF (0.01% and 0.02% w/v) and ethanol (0.25% and 0.5% respectively), or only with ethanol (0.25% and 0.5%) as control treatment. The volumetric ethanol concentrations of 0.25% and 0.5% correspond to 42.8 mM and 85.6 mM ethanol, respectively, and are physiologically relevant *in vivo* [30].

Cell viability assessment

Viability of treated cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [31]. 3T3-L1 cells were seeded in six well plates (2×10^5 cells/well) and allowed to adhere overnight. After 24 h the culture media containing SE fruit EAF (0.005–0.025% w/v), and ethanol (0.125-0.625%) were

replaced to each well and the cells were incubated for 20 h. To each well 100 μL of MTT (1.5 mg/mL) (AppliChem, Germany) was added and the plates were incubated in dark for another 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. Cell viability (%) was calculated as [(mean absorbance of the sample/mean absorbance of the control) × 100].

Gene expression analysis

Beta actin (β-actin), glutamate-cysteine ligase catalytic subunit (GCLc), inducible cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) genes expression were analysed using Real Time quantitative polymerase chain reaction (qPCR). Relative mRNA levels were calculated using $2^{-\Delta\Delta Ct}$ method [32].

Total RNA was extracted using TRI Reagent (Ambion, USA), and quantified by spectrophotometry at 260 nm (M501 Single Beam UV/Vis, Camspec, UK). cDNA was synthesized from 0.02 μg of total RNA by using First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's instructions. qPCR was performed using the PCR Master Mix with Eva Green dye (β-actin, GCLc, TNFα, COX-2, iNOS) (Genaxxon, Germany) and Probe/ROX qPCR Master Mix (Fermentas, Germany) (β-actin, IL-6) on an ABI Prism7500 Real-Time PCR System (Applied Biosystems, USA). As a reference β-actin gene was used in the comparative Ct method to determine the relative changes in the target samples. The primer sequences (Sigma-Aldrich, Germany) for each gene were: β-actin forward primer (FP) 5′ACGGCCAGGTCATCACTATTG3′, reverse primer (RP) 5′CAAGAAGGAAGGCTGGAAAAG3′, probe FAM5′ACGAGCGGTTCCGATGCCCTG3′TAMRA; IL-6 FP 5′CATCTGCTGGCCTTCTCCAA3′, RP 5′CAGGCTCTCTGGCTTCTG3′, probe FAM5′AGCTGCTCCCTGCCTCAGACCAGTG3′ TAMRA; GCLc FP 5′AATGGAGGCGATGTTCTTGAG3′, RP 5′CAGAGGGTCGGATGGTTGG3′; iNOS FP 5′GGCAGCCTGTGAGACCTTTG3′, RP 5′GCATTGGAAGTGAAGCGTTTC3′; COX-2 FP 5′TGAGCAACTATTCCAAACCAGC3′, RP 5′GCACGTAGTCTTCGATCACTATC3′; TNFα FP 5′CCCTCACACTCAGATCATCTTCT3′, RP 5′GCTACGACGTGGGCTACAG3′.

Statistical analysis

Results are presented as mean±SD for TAC, TPC and cell viability or ±SEM for relative units of mRNA. All measurements were performed in triplicate. GraphPad Prism 5.0 software was used to perform the statistical analyses. Differences between two groups were analysed applying two-tailed Student's *t*-tests. The values of $p<0.05$ were considered as significant.

RESULTS

TPC and TAC of SE fruit extract and fractions

TPC and TAC of different fractions isolated from SE fruit methanol extract are presented in Table 1. The correlation between TAC and TPC was significantly high (r=0.96) for all analysed extractions. Ethyl acetate fraction was with the highest TAC and TPC among the analysed extract and fractions.

Table 1. TPC and TAC of different fractions of *S. ebulus* fruit methanol extract. Data are presented as mean ±SD.

Extract/fraction	TPC [mmol/L QE]	TAC [mmol/L UAE]
Total methanol extract	1.24 ±0.05	6.58 ±0.64
Petroleum ether fraction	0.19 ±0.01	3.48 ±0.08
Chloroform fractions	0.36 ±0.01	0.04 ±0.001
Ethyl acetate fraction	6.04 ±0.22	29.31 ±0.69
Butanol fraction	4.12 ±0.20	17.41±0.50
Water fraction	3.14 ±0.10	8.91 ±0.09

TLC of SE fruit EAF

TLC comparison of SE fruit EAF with authentic standards confirmed the presence of hyperoside (quercetin-3-O-β-galactopyranoside), isoquercetin (quercetin-3-O-β-glucopyranoside), isorhamnetin-3-O-β-glucopyranoside and traces of rutin as well as 3,5-dicaffeoylqunic acid (Fig. 2).

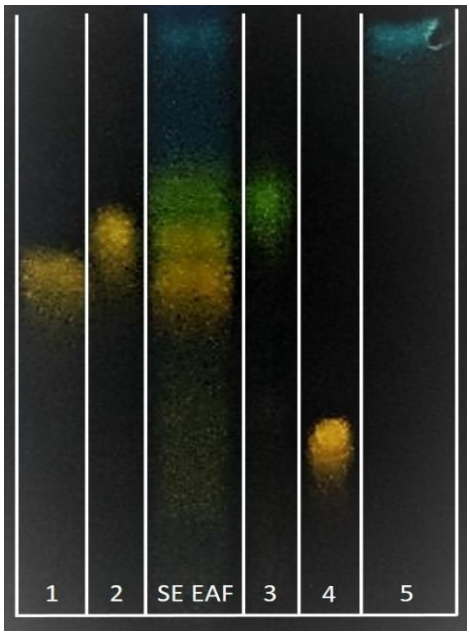

Figure 2. TLC of SE fruit EAF. Legend: standards 1 – hyperoside, 2 – isoquercetin, 3 – isorhamnetin-3-O-β-glucopyranoside, 4 – rutin, 5 – 3,5-dicaffeoylqunic acid.

Effect of SE fruit EAF on ethanol-induced cell death in 3T3-L1 preadipocytes

Using MTT assay we evaluated the cytoprotective effect of SE fruit EAF on ethanol-induced cytotoxicity in 3T3-L1 preadipocytes. Ethanol applied in final concentrations from 0.125 to 0.625% caused gradual decrease in cell viability, the latest causing 46% cell death (Fig. 3A). Co-treatment with 0.5% ethanol and 0.02% w/v SE fruit EAF significantly increased cell viability by 37% ($p<0.001$) *vs*. untreated cells and by 110% ($p<0.001$) *vs*. cells treated with 0.5% ethanol only (Fig. 3A). This effect was observed for all applied concentrations of SE fruit EAF when compared to respective ethanol control treatments, indicating its proliferative and cytoprotective effect on 3T3-L1 cells.

Effect of SE fruit EAF on ethanol-induced TNF-α, IL-6, iNOS, COX-2 and GCLc gene expression in 3T3-L1 preadipocytes

Ethanol applied in a concentration of 0.25% stimulated TNF-α (Fig. 3B) and IL-6 (Fig. 3C) transcription levels, vs. untreated cells by 1.2 ($p<0.05$) and 32.5 ($p<0.01$), respectively. Higher 0.5% ethanol concentration enhanced gene expression of TNF-α (Fig. 3B) and IL-6 (Fig. 3C), by 4.3 ($p<0.001$) and 38.3 ($p<0.001$) fold *vs.* untreated cells, respectively. The higher ethanol concentration induced also mRNA of COX-2 (Fig. 3D) and iNOS (Fig. 3E), by 6.8 ($p<0.05$) and 6.2 ($p<0.001$) fold *vs.* untreated cells, respectively. Treatment with 0.25% and 0.5% ethanol resulted in increased mRNA of GCLc, by 2.5 fold ($p<0.01$) and by 3.3 fold ($p<0.01$) *vs.* untreated cells, respectively, (Fig. 3F).

SE EAF treatment suppressed ethanol-induced expression of all analysed genes in 3T3-L1 mouse preadipocytes. Treatment with 0.02% w/v SE EAF decreased ethanol (0.5%) induced iNOS (Fig. 3E), COX-2 (Fig. 3D) and TNF-α (Fig. 3B) gene expression by 63% ($p<0.01$), 54% ($p<0.01$) and by 64% ($p<0.001$), respectively, indicating anti-inflammatory potential of the preparation. The lower concentration of 0.01% w/v SE EAF significantly decreased ethanol (0.25%)-induced IL-6 gene expression by 71% ($p<0.01$) (Fig. 3C). Both concentrations of the preparation 0.01% w/v and 0.02% w/v decreased ethanol-induced GCLc mRNA levels, by 44% ($p<0.05$) and 45% ($p<0.05$), respectively (Fig. 3F).

In all co-treated groups, we observed significant reduction in the transcription levels of analysed genes compared to ethanol controls, but still significantly higher compared to untreated cells (Figs. 3B, C, E and F), except those of COX-2 (Fig. 3D). This observation additionally confirms the strong inhibitory effect of SE fruit EAF on ethanol-induced COX-2 transcription.

DISCUSSION

Comparison of TPC and TAC of SE fruit extract and fractions

Fractionation procedure favours the comparison between different groups of plant-derived phytochemicals according to their biological activities. Measurement of TAC and TPC of the six different fractions indicated that the content of antioxidant substances was highest in the ethyl acetate fraction followed by butanol and water fractions (Table 1). Very high correlation ($r=0.96$) between TAC and TPC was established for all fractions. Results showed that polyphenols are mainly present in these three fractions. Considering these results we selected for further analysis the SE EAF as the one with the highest TAC and TPC.

Flavonoid glycosides in SE fruit EAF

We report for first time the presence of hyperoside, isoquercetin, isorhamnetin-3-O-β-glucopyranoside and traces of rutin and of 3,5-dicaffeoylqunic acid in the SE fruit EAF (Fig. 2). There are previous reports about the presence of hyperoside and rutin in ethanolic extracts of SE fruits [33], and about the presence of isorhamnetin-3-O-b-glucopyranoside and isoquercetin in SE leave methanol extract [34].

Cytoprotective effect of SE fruit EAF

We found that in 3T3-L1 cells SE EAF exerted cytoprotective effect in a cell model of ethanol-induced cytotoxicity (Fig. 2). Ethanol treatment in final concentrations of 0.125% up to 0.625% in the culture medium significantly decreased cell viability in a dose-dependent manner compared to untreated cells (Fig. 2). The highest ethanol concentration (0.625%) led to 46% cell death. In all co-treated groups SE EAF reversed ethanol-induced cell death, increasing proliferation up to 137%, as compared to untreated cells. In support to the cytoprotective effect of the extract, we detected a 2.4-fold increase in cell viability of SE EAF (0.025% w/v)- and ethanol (0.625%)- co-treated cells, compared to the control ethanol (0.625%) treatment. Cytotoxic or proliferative effects of the extracts are often concentration-related. Low concentrations often stimulate expression of genes to induce cell proliferation, while higher ones activate the caspase pathways initiating apoptosis [35]. This effect may also be dependent on the type of the extractor and on the type of compounds in the extracts, as well as on the type of the cell line.

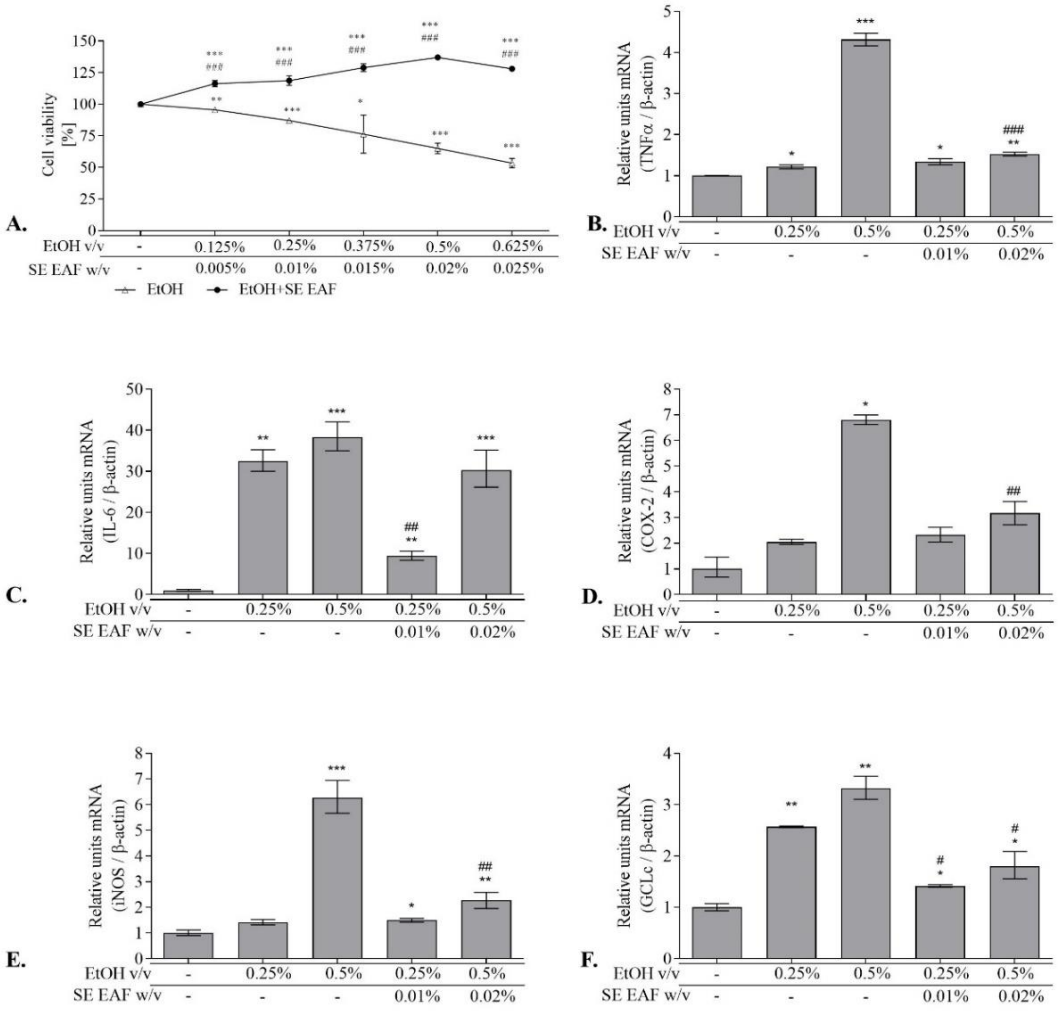

Figure 3. Viability of 3T3-L1 cells treated with different concentrations of ethanol and SE EAF dissolved in ethanol (data are presented as mean ±SD) (**A**) and changes in mRNA levels of TNFα (**B),** IL-6 **(C),** COX-2 **(D),** iNOS **(E)** and GCLc **(F**) in the cells upon treatment with increasing concentrations of ethanol and SE EAF+ethanol (data are presented as mean ±SEM; *p<0.05, **p<0.01, ***p<0.001 *vs.* untreated cells; #p<0.05, ##p<0.01, ###p<0.001 *vs*. respective treatment with ethanol). Legend: EtOH-ethanol.

Hyperoside and isoquercetin found in the SE fruit EAF by TLC are flavonoids known to suppress activation of caspase 3 cascade, thus increasing cell viability [36, 37] and their presence in the EAF could explain the established cytoprotective/proliferative effect of SE fruit extract, and specifically of its EAF.

SE fruit EAF alters ethanol-induced GCLc, TNF-α, IL-6, COX-2 and iNOS genes expression in 3T3-L1 preadipocytes

In individuals consuming alcohol in high doses [38] and even in these consuming lower amounts [39] the levels of cytochrome P450 2E1 are

increased. The high NADPH oxidase activity of P450 2E1 enzyme is associated with increased superoxide and H_2O_2 production. Since 1963 it is known that the oxidative stress is the main mechanism of ethanol toxicity [40] and thus we may assume that the cell model of ethanol induced cytotoxicity is also a model of ethanol-induced oxidative stress. We suggest that the availability of antioxidants of plant origin may reduce ethanol-induced oxidative stress and diminish the need for high supply with endogenous antioxidants, such as glutathione.

Glutathione consumption by glutathione peroxidase, responsible for H_2O_2 neutralization, drives up mechanisms responsible for recovery of reduced/oxidised glutathione ratio and *de novo* biosynthesis of glutathione. Transcription levels of the regulatory enzyme GCL of glutathione biosynthesis are stimulated by many phenolic compound. Plant extracts rich in flavonoids, including quercetin, activate gene promoter leading to an increase in glutathione levels in COS-1 and HepG2 cells [41]. Fruits rich in polyphenols and ellagic acid may also induce transcription levels of GCL in mouse [42]. In a previous study we showed that SE fruit extract rich in anthocyanins induces the gene expression of GCL in 3T3-L1 preadipocites [43]. The quercetin glycosides found in SE fruit EAF (fig. 2) are direct antioxidants [36] and modulators of GCL gene expression [43], GPx and catalase activity [36], thus having the potential to reduce ethanol-induced oxidative stress. We may suggest that reduction of ethanol-induced oxidative stress by the presence of plant-derived antioxidants, reduces the needs of more glutathione production, respectively, the GCLc transcription, as observed in the current study.

In response to ethanol treatment the expression levels of proinflammatory cytokines IL-6 and TNF-α, and enzymes iNOS and COX-2 are increased in rat and mouse models [22–24, 44]. There are few analyses of ethanol effect on adipose tissue cells, such as preadipocytes. Chronic ethanol intake *in vivo* in rats induces macrophage infiltration in adipose tissue, thus revealing its link to the development of insulin resistance [21]. The mRNA expression of COX-2, iNOS, TNFα and IL-6 increased significantly, probably also because of ethanol-induced oxidative stress. At the same time transcription levels of all above-mentioned proteins was significantly decreased in cells co-treated with SE EAF and ethanol vs. control ethanol treatment. The strong inhibitory effect of SE fruit EAF on ethanol-induced inflammatory response was additionally supported by the reduction of COX-2 transcription levels close to those of untreated cells.

Generation of oxidative stress leads to induction of inflammation including induction of related inflammatory proteins [45]. Production of peroxynitrite ONOO− as a result of iNOS induction additionally stimulates gene expression of COX-2 and production of prostaglandin E2 [46]. We could suggest that the antioxidant potential of SE fruit polyphenols to scavenge NO [16] and to neutralise free radicals such as ONOO−, is one of the possible mechanisms by which the anti-inflammatory potential of SE fruit EAF is realised, altering the inflammation progression related to cytokines release.

Increased production of proinflammatory cytokines and chemokines such as, IL-6, TNFα, IL-1β and MCP-1 by the adipocytes is related to fat tissue low-grade inflammation and insulin resistance [47–49]. The suppression of TNFα activity improves insulin sensitivity in obese individuals and rat model of obesity [50, 51]. Since ethanol may additionally provoke inflammation and insulin resistance in adipose tissue [21-24, 44], an herb extract as SE fruit EAF able to suppress ethanol-induced transcription of proinflammatory proteins may have protective effects in such conditions.

It has been established that quercetin glycosides present in SE fruit EAF reduce the levels of proinflammatory cytokines by suppressing NF-κB activation [37]. Anthocyanin-rich plant consumption inhibits the activity of NF-κB transcription factor and reduces plasma concentrations of its target gene products such as IL-6 and CRP [52, 53].

CONCLUSIONS

A possible mechanism of the protective potential of SE fruits is suggested in support to its use by folk medicine as a therapeutic and preventive tool. SE fruit EAF may overcome ethanol toxicity by activating cell proliferation and reducing the mRNA expression of proinflammatory proteins. Additionally, it prevents ethanol-induced antioxidant GCLc gene expression, revealing a possible mechanism by improving cellular redox balance. This makes SE fruits a good potential source for the development of new therapeutic remedies for the prevention and control of oxidative stress and inflammation.

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