EFFECT OF THE EXTRACT FROM NETTLE (*URTICA DIOICA* L.) FRUIT CLUSTER ON THE SYNTHESIS OF PRO-INFLAMMATORY AGENTS IN HEPATOCYTES TREATED WITH FLUORIDE

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SUMMARY: Nettle, *Urtica dioica* L., is frequently used by humans for medicinal purposes and has antioxidant, anti-inflammatory, and hepatoprotective properties. As no data were available on the effect of nettle extract on the synthesis of pro-inflammatory agents in hepatocytes treated with fluoride, we decided to investigate this. Aqueous and ethanol extracts of nettle fruit clusters were prepared, after lyophilisation and homogenisation, and added to hepatocytes from HepG2 cell line, with and without NaF. After incubation, the concentrations of the pro-inflammatory end products of 15-LOX activity in hepatocytes (15-HETE, 12-HETE, 9-HODE, and 13-HODE) were determined by high performance liquid chromatography (HPLC), and apoptosis, necrosis, and mitochondrial superoxide generation were measured using immunohistochemical methods. Adding NaF increased the 15-LOX proinflammatory end products and these effects were decreased by both the ethanol and aqueous nettle extracts. Similarly, the ethanol extracts reduced the increased apoptosis, necrosis, and mitochondrial superoxide synthesis caused by NaF suggesting that nettle extracts may have hepatoprotective properties in fluoride intoxication.

Keywords: Anti-inflammatory properties; Environmental exposure; Fluoride; Hepatocytes; Hepatoprotective properties; Nettle; Pro-inflammatory agents; Stinging nettle; *Urtica dioica* L.

INTRODUCTION

Urtica dioica L. commonly known as nettle or stinging nettle is a plant frequently used by humans for medicinal purposes. This herb belongs to the family of *Urticaceae* and occurs as a perennial plant in temperate zones of Asia, America, and Europe. Raw material of the nettle herbal such as herbs (*Urticae herba*), leaves (*Urticae folium*), and roots (*Urticae radix*) are recommended as an adjunct supportive treatment for many illnesses and it is one of the most popular and valuable plants used in phytotherapy as both monotherapy and in combination therapy. It is recommended as an important medicinal herb in the human diet because it is a source of minerals (iron, manganese, potassium, calcium), chlorophyll, amino acids, lecithin, carotenoids, and vitamins as well as flavonoids, tanins, sterols, fatty acids, polysaccharides, and lectins.[1] The available literature shows that *Urtica dioica* L. has antioxidant,[2] anti-inflammatory, anti-ulcer,[3] anticancer, antimicrobial,[4] cardiovascular,[1] and hepatoprotective[2] properties.

Fluoride has not been demonstrated to be an essential trace element or to be required for the development of healthy teeth and bones and exposure to it in fluoride-contaminated areas, even at a low concentration, may lead to numerous metabolic disorders[5] and enhanced apoptosis[6,7] with increased oxidative stress[8,9] and changes in the activity of the enzymes involved in inflammatory processes.[5]

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Fluoride penetrates cell membranes relatively easily[10] and may affect the function of various organs, including the liver which has an important, detoxification function.

After finding no data available on the effect of the extract from nettle (*Urtica dioica* L.) fruit clusters on the synthesis of pro-inflammatory agents in hepatocytes treated with fluoride, we decided to investigate this.

MATERIALS AND METHODS

Plant material and preparation of extracts of nettle (Urtica dioica L.*) fruit clusters: Urtica dioica* L. material was collected in September and October from the province of West Pomerania in Poland. The collected nettle fruit clusters underwent lyophilisation in a lyophilisator (0.735 mm Hg, –20ºC; Alpha 1-2 LD plus) and then homogenisation by grinding in agate mortar to the form of powder.

Preparation of ethanol extract of nettle fruit clusters: About 5 g of powdered dried nettle material was transferred to 300 mL of 70% ethanol. This mixture was maintained for 3 hours at boiling point (80ºC) in a water bath under the condenser. In order to maintain a uniform process of boiling a few boiling stones were added to the flask. The mixture was then cooled down to room temperature and filtered. The transparent green alcohol filtrate obtained was extracted 4 times with petroleum ether. The collected alcohol layers were carefully extracted for a fifth time with petroleum ether. Then alcohol was evaporated under reduced pressure. The alcohol extract of nettle obtained was placed in a plastic vial and then stored at –20ºC until used.

Preparation of an aqueous infusion of nettle fruit clusters: Distilled water at 90ºC was poured on about 1 g of powdered dried nettle material and the mixture, in a closed conical flask, was placed on a vortex mixer and gently shaken for 10 min. The mixture was cooled down to room temperature and filtered. The filtrate was evaporated under reduced pressure. The aqueous extract of nettle obtained was placed in a plastic vial and stored at –20ºC until used.

Cell culture and treatment: Hepatocytes from HepG2 cell line were cultured for 48 hr (37ºC, 5% CO_2) in EMEM medium (Sigma Oligarch, Poland) with 10% fetal bovine serum (Gibed, Poland), fluoride solution and aqueous or ethanol nettle seed extract. The nettle extracts were in final concentrations of 10 µg/mL for the ethanol extract and 15 µg/mL for the aqueous extract in accordance with Kadan et al.[11] NaF was used in final concentrations of 1, 3, and 10 µM on the basis of results of fluoride concentration in human serum.[12] After incubation, the cells were harvested by scraping and used for future analysis. The percent of the living cells was determined by trepan blue. Cell cultures with viability more than 97% were used for the experiments. The protein concentration was measured by using the Micro BCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, USA) and ELISA.

In vitro measurements of pro-inflammatory agents concentrations: 15-HETE, 12-HETE, 9-HODE, and 13-HODE: The activity of 15-LOX (15-lipoxygenase), the enzyme involved in the generation of pro-inflammatory agents, was measured

in vitro by measurement of the concentration of its products (15-HETE, 12-HETE, 9-HODE, 13-HODE). The cells were incubated for 48 hr with fluoride solutions and aqueous or ethanol nettle fruit cluster extracts, as described above. According to the manufacturer's instructions, SPE method and Baker bond columns were used for extracting 15-HETE, 12-HETE, 9-HODE, and 13-HODE from the cells. Residue after evaporation was reconstituted in 200 µL methanol/water/acetic acid (60/40/0.1, v/v/v; Sigma–Oligarch, Poland) and analyzed by high performance liquid chromatography (HPLC) method using the Gallant 1200 Gas Chromatograph (LiChrospher 100 RP-18 column: 250 mm length × 4 mm internal diameter, 5 µm particle size; column temperature: 25ºC; flow rate 1 mL/min; PGB_2 as internal standard). The chromatographic data were processed by Agilent Chemstation software.[12] For peak identification and quantitative analysis, a mixture of 15-HETE, 12-HETE, 9-HODE, and 13-HODE reference standards (Sigma–Aldrich, Poland) Absorbance spectra of peaks were analyzed to confirm the identification of analytes. The quantitation was based on peak areas with internal standard calibration.

Confocal microscopy: imaging in vitro of apoptosis process: Cells were incubated with fluoride solutions alone or with ethanol extract from nettle fruit clusters on microscope slides according to the aforementioned procedure. After 48 hr of cultivation, the cells were rinsed with PBS, suspended in a binding buffer and stained with 1 ng/mL Annexin V-FITC and 5 ng/mL propidium iodide for 30 min in the dark. Cells that are viable are Annexin V-FITC and PI negative; cells that are in early apoptosis are Annexin V-FITC positive and PI negative (green fluorescence); and cells that are in late apoptosis or already dead (necrosis) are both Annexin V-FITC and PI positive (red fluorescence). A dual-pass FITC/ rhodamine filter set was applied.

Imaging of mitochondrial superoxide generation: Cells were incubated with fluoride solutions alone or with ethanol extract from nettle fruit clusters on microscope slides according to the aforementioned procedure. After 48 hr of cultivation, visualization of mitochondrial superoxide generation in hepatocytes was performed using MitoSOX Red mitochondrial superoxide indicator (Invitrogen, UK) which is fluorogenic dye rapidly and selectively targeted to the mitochondria, for highly selective detection of superoxide produced in this organelle. The reagent is oxidized by superoxide but not by other reactive oxygen (ROS) or nitrogen (RNS) species. The oxidation product becomes highly fluorescent upon binding to nucleic acids. MitoSOX Red stock solution (50 mM in DMSO) was diluted in the incubation medium (BME) to a final concentration of 2.5 µg/mL. The cells were incubated in this solution for 10 min (humidified 95% air/CO_2 atmosphere at 37ºC). Next, cells were washed with BME at room temperature and were examined under the confocal microscope (Olympus, SV 1000).

Statistical analysis: The obtained results were analyzed statistically using the software package Statistica 10 (StatSoft, Poland). The arithmetical mean and standard deviation (SD) were found for each of the studied parameters. As the

distribution of variables in most cases deviated from normal distribution (Shapiro–Wilk's test), non-parametric tests (Mann-Whitney U test) were used for further analyses. Tests were evaluated at the significance level p=0.05.

RESULTS

Extracts from nettle (Urtica dioica L.) fruit clusters can decrease synthesis of pro-inflammatory agents in NaF-treated cells: The increase in NaF concentration added to the culture was followed by an increase in 15LOX activity in hepatocytes and synthesis of its end products. The addition to the cells of both aqueous and ethanol extracts from nettle (*Urtica dioica* L.) seeds caused a significant decrease in 12-HETE, 15-HETE, 13-HODE, and 9-HODE concentrations (Figure 1). The significant differences noted were for:

- 12-HETE in cells incubated with 1 μM NaF (p=0.0007 for ethanol extract).
- 15-HETE in cells incubated with 1 μM NaF (p=0.009 for ethanol extract), 3 μM NaF (p=0.05 for ethanol extract), and 10 μM NaF (p=0.003 for ethanol extract and p=0.023 for aqueous extract).
- 13-HODE in cells incubated with 3 μM NaF (p=0.0007 for ethanol extract and p=0.023 for aqueous extract).
- 9-HODE in cells incubated with 1 μM NaF (p=0.0001 for ethanol extract and p=0.009 for aqueous extract), 3 μM NaF (p=0.023 for ethanol extract), and 10 μM NaF (p=0.05 for ethanol extract and p=0.023 for aqueous extract).

The analysis of the results indicated that the ethanol extract of nettle seeds had a stronger effect in lowering the concentration of proinflammatory compounds than the aqueous extract. The significant differences noted were for:

- 15-HETE in cells incubated with 3 μM NaF (p=0.05) and 10 μM NaF (p=0.023).
- 13-HODE in cells incubated with 3 μM NaF (p=0.009).
- 9-HODE in cells incubated with 1 μM NaF (p=0.0007) and 3 μM NaF (p=0.00001).

Extracts from nettle (Urtica dioica L.) fruit clusters can decrease apoptosis process in NaF-treated hepatocytes cell line HepG2: Increased NaF concentrations in the culture resulted in increased apoptosis and necrosis in the hepatocytes. Adding ethanol nettle seed extract to the culture decreased apoptosis and necrosis (Figure 2).

Extracts from nettle (Urtica dioica L.) fruit cluster can decrease mitochondrial superoxide amount in NaF-treated cells: Incubation of hepatocytes with increasing concentrations of fluorides increased mitochondrial superoxide synthesis in a dose dependent manner which was noticeably reduced after the addition of ethanol extract from nettle to the culture. (Figure 3).

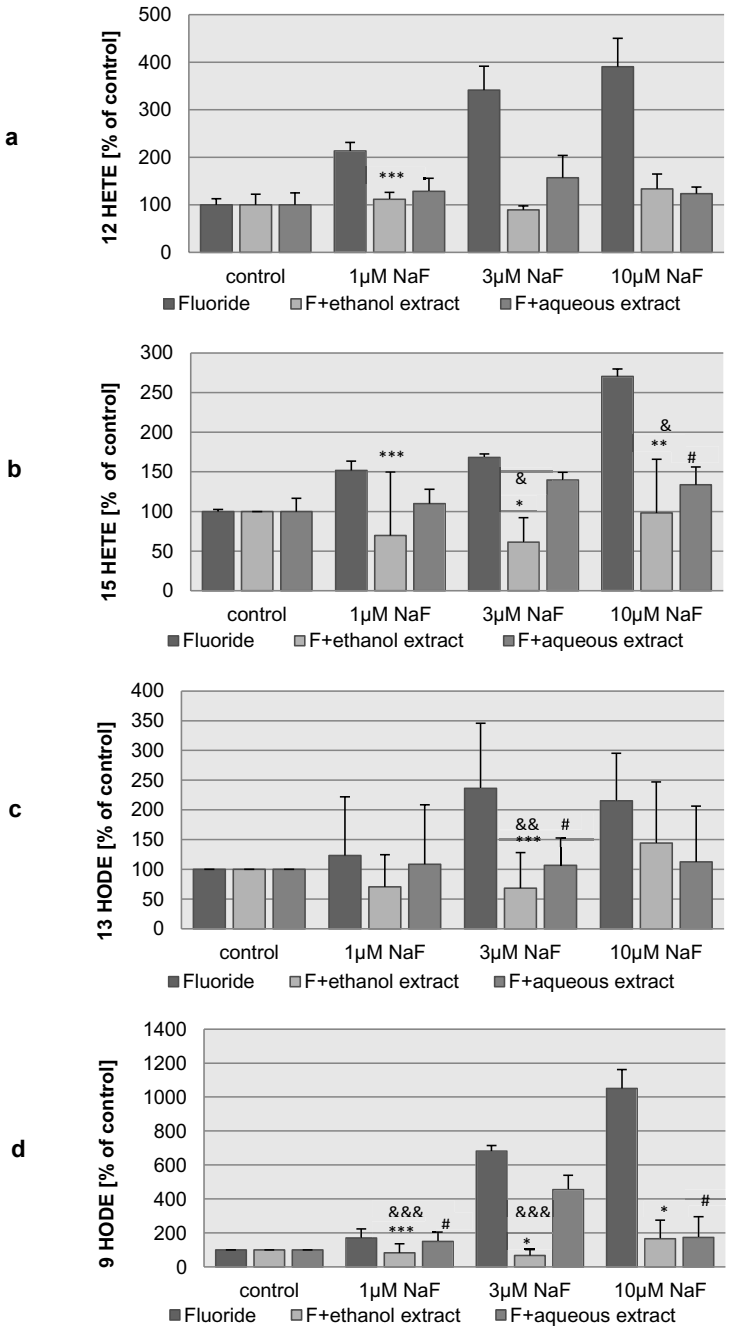

Figure 1. Effect of extracts from nettle (*Urtica dioica* L.) fruit cluster on (a) 12-HETE, (b) 15-HETE, (c) 13-HODE, and (d) 9-HODE concentrations in hepatocytes cultured with NaF. Hepatocytes were cultured with NaF solutions alone or with water / ethanol nettle fruit cluster's extract for 48 hr. After incubation, cells were harvested by scraping and concentration of 15 LOX enzyme's end products was measured by using HPLC method (n = 5).
*p<0.05; **p<0.01; ***p<0.001: statistically significant decrease vs results obtained for NaF (alcohol extract); #p<0.05; ##p<0.01; ###p<0.001: statistically significant decrease vs results obtained for NaF (water extract); &p<0.05; &&p<0.01; &&&p<0.001: statistically significant differences between results obtained for alcohol and water extracts

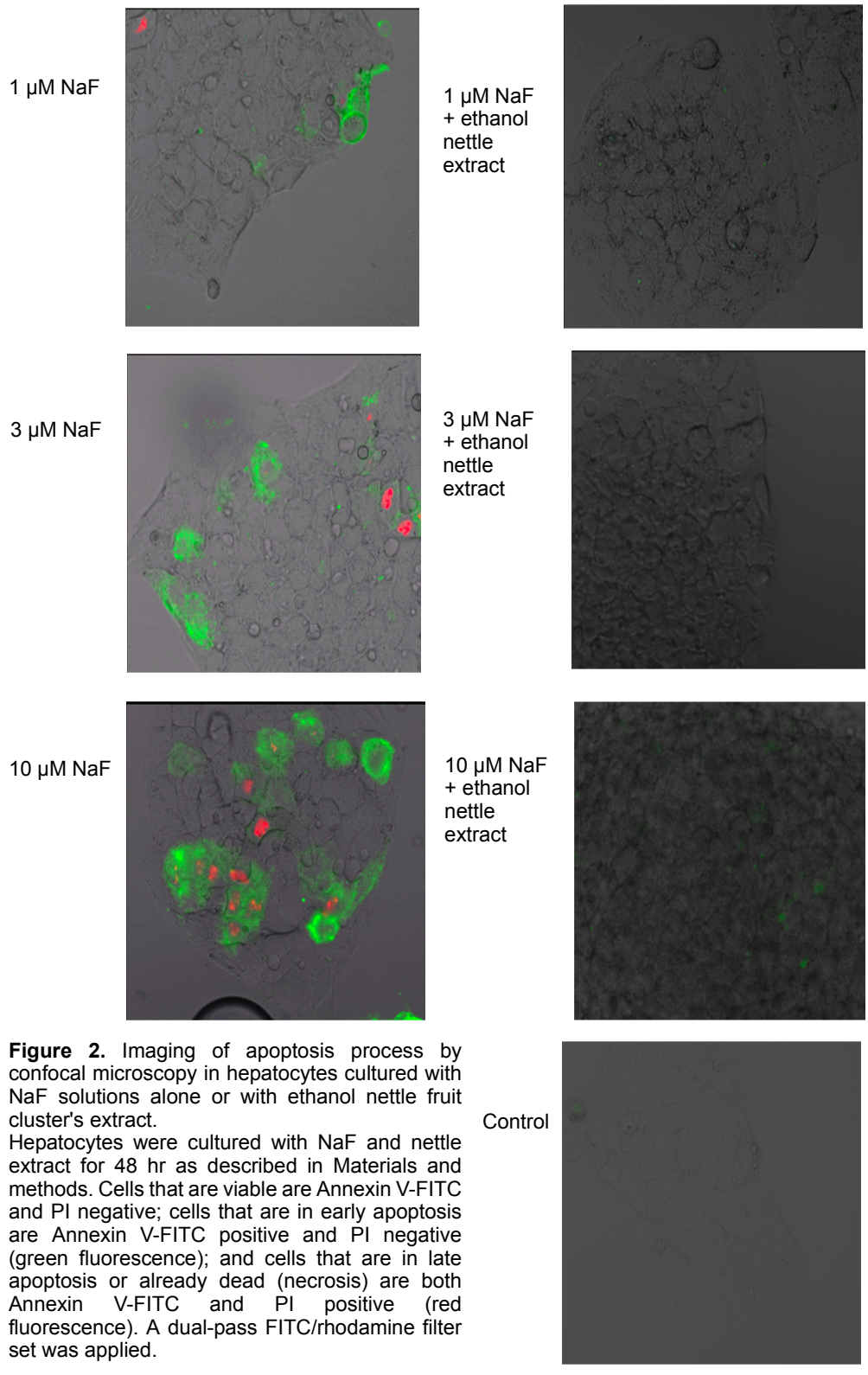

Figure 2. Imaging of apoptosis process by confocal microscopy in hepatocytes cultured with NaF solutions alone or with ethanol nettle fruit cluster's extract.
Hepatocytes were cultured with NaF and nettle extract for 48 hr as described in Materials and methods. Cells that are viable are Annexin V-FITC and PI negative; cells that are in early apoptosis are Annexin V-FITC positive and PI negative (green fluorescence); and cells that are in late apoptosis or already dead (necrosis) are both Annexin V-FITC and PI positive (red fluorescence). A dual-pass FITC/rhodamine filter set was applied.

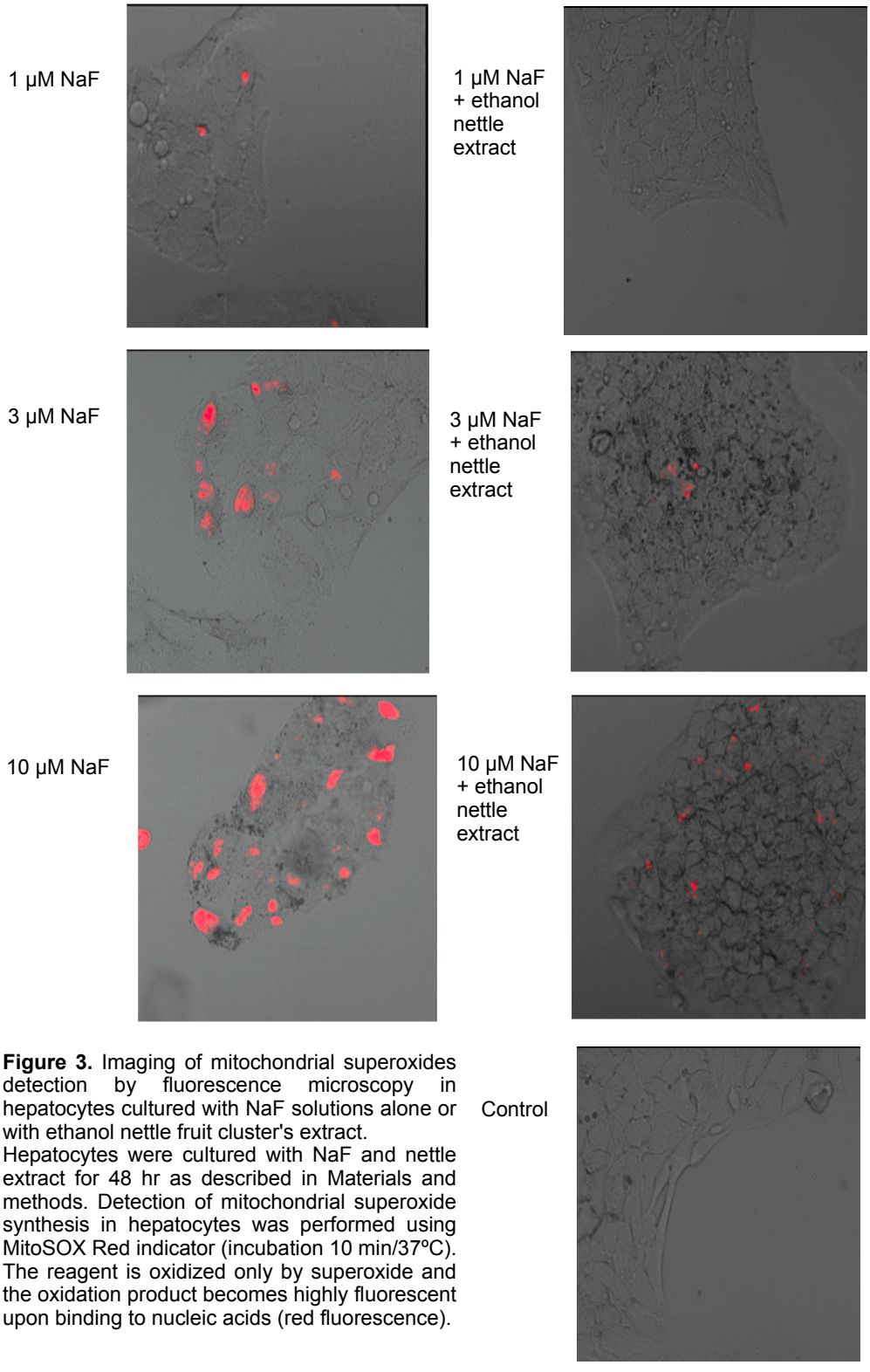

Figure 3. Imaging of mitochondrial superoxides detection by fluorescence microscopy in hepatocytes cultured with NaF solutions alone or with ethanol nettle fruit cluster's extract.
Hepatocytes were cultured with NaF and nettle extract for 48 hr as described in Materials and methods. Detection of mitochondrial superoxide synthesis in hepatocytes was performed using MitoSOX Red indicator (incubation 10 min/37ºC). The reagent is oxidized only by superoxide and the oxidation product becomes highly fluorescent upon binding to nucleic acids (red fluorescence).

DISCUSSION

An increasing number of reports have demonstrated harmful effects of fluorides on hepatocytes[13] and on liver function in animals[9,10] and humans.[14] Due to the ability of fluorides to accumulate in the soft tissues, the prolonged exposure to fluorides, even at low doses, can cause changes in the activity of enzymes essential for the proper functioning of the liver[9,14] and to an increased reactive oxygen species (ROS) production that can enhance inflammatory reactions and apoptosis.[10,13]

Eicosanoids synthesis in hepatocytes is controlled by the activity of phospholipase A2 (PLA_2), which affects the release and availability of free arachidonic (AA) and linoleic (LA) acids.[15] Our earlier study[15] and the study of other authors[16] confirmed that fluoride, even in small concentrations, contributes to increasing release AA and LA from cell membranes. This result suggests that fluoride may not only increase the amount of secreted phospholipase A2 ($sPLA_2$) in cells but also the activity of the enzyme, causing increased AA and LA release.[15] Probably fluoride acts by stimulation of ROS production[6], which are associated with protein tyrosine phosphorylation and phospholipase A_2 (PLA_2) activation.[17] Released fatty acids may be converted by 15LOX to lipid mediators[7] which take part in the development of inflammation and the disruption of liver function. Therefore, the inhibition of 15LOX is an important mechanism for decreasing hydroperoxide synthesis with resulting liver protection. It seems that extracts from *U. dioica* fruit clusters may be one of the agents able to influence this process. Our study demonstrated that the addition of nettle extract to the NaF-treated cells significantly decreased the synthesis of 15LOX end products and this process was independent of the concentration and type of added nettle seeds extract (aqueous or ethanol). It seems that ethanol extract (or rather compounds soluble in ethanol) caused a greater reduction in hydroperoxide synthesis than the aqueous extract. Probably the anti-inflammatory properties of nettle fruit clusters extracts result from decreasing ROS production in hepatocytes through the increase of activity of glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT).[18,19] In addition, lower activity of cytochrome P450 (cyt P450), lactate dehydrogenase (LDH), NADPH-cytochrome P450 reductase (cyt P450 R) may result in a further decrease of ROS in cells.[18]

The results of this study confirm that nettle fruit clusters extract reduced the amount of mitochondrial ROS generated in fluoride-treated hepatocytes. Similar results were obtained by Toldy et al.,[20] who noted that dried nettle (*Urtica dioica* L.) leaf supplementation decreases the level of reactive oxygen species in rat brain. The data reveal that nettle supplementation has an effective antioxidant role. Probably compounds contained in the extract of nettle seeds down-regulate the inflammatory transcription factors NFκB.[20]

Mitochondria are the main energy source in hepatocytes and play a major role in normal liver function including the extensive oxidative metabolism which occurs. Impaired mitochondrial functioning affects cell survival and mitochondria have a

gateway function at the center of the signaling pathways that mediate hepatocyte injury and also determine hepatocyte survival.[21] A key role in hepatocyte survival is played by ROS[22] generated from several intracellular sources, including cyclooxygenases, lipoxygenases, NADPH oxidase and mitochondrial respiration.[22] ROS in small concentrations may function as a second messenger and regulate multiple cell functions, e.g., cellular differentiation, intracellular signaling,[22] gene expression regulation and (through activation of caspases or Fas receptors) in apoptotic stimulation.[23] However, increased ROS production has been implicated in the development of inflammation and oxidative stress leading to impaired liver function[13] as noted in our research on hepatocytes treated with NaF. The results of the present study confirm that fluoride may cause apoptosis of hepatocytes, with the rate increasing with increasing concentrations of the added fluoride solution. The increase in the cells apoptotic process was probably due to increased ROS production. The addition to the medium of the nettle seeds extract decreased the apoptosis process, probably, at first, by causing by a decrease in the cell ROS concentration and then by decreasing the 15-lipoxygenase end products.

Enhanced fluoride-dependent ROS synthesis can induce lipid peroxidation which leads to DNA damage and cell death.[24] Lipid peroxidation and apoptosis may co-exist at the beginning when the cells generate a lot of free radicals that may be sufficient to cause apoptosis.[24] The pro-apoptotic properties of fluoride and the mechanism of F-induced apoptosis have been well described[22,24] but there are still only a few articles about anti-apoptotic properties of nettle[18,19] which indicate an ability of *Urtica dioica* L. to intensify the anti-oxidative systems in cells.[18] Futhermore, nettle extract significantly increases the mitotic and proliferation indexes, decreases apoptosis, and attenuates hepatic vacuolar degeneration and sinusoidal congestion in rat liver.[19]

CONCLUSION

The results obtained in this research suggest that *Urtica dioica* L. fruit clusters' extracts could protect liver cells from the negative effects of fluoride.

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