EXTRACT FROM NETTLE SEEDS (*URTICA DIOICA* L.) DECREASES THE SYNTHESIS OF LIPOXIN A_4 THROUGH INHIBITION OF THE DEVELOPMENT OF FLUORIDE-INDUCED INFLAMMATION IN THP1 MONOCYTES/MACROPHAGES

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ABSTRACT: This is the first study to investigate the effect of *Urtica dioica* L. (UD) seed extract on the synthesis of lipoxin A_4 (LXA_4) and its epimer (15-epi-LXA_4) in human monocytes/macrophages exposed to fluoride. Tests were performed on the THP1 monocytes which were incubated with fluoride solutions and/or the extract from nettle seeds. The prepared samples were concentrated by Solid Phase Extraction (SPE) and then analyzed by high performance liquid chromatography (HPLC). The mitochondrial generation of reactive oxygen species (ROS) was visualized by MitoSOX™ Red reagent and the cytoplasmic generation of ROS was visualized by fluorescent marker DCFH-DA. The levels of these receptor-active derivatives of arachidonic acid (AA), having immunomodulatory properties leading to the suppression of inflammatory responses, increased after the exposure to fluoride. Adding UD seed extract to the culture caused a reduction in LXA_4, increase in 15-epi-LXA_4, and a reduction in ROS, which indicates the anti-inflammatory properties of the studied extract. The use of an ethanol extract from nettle seeds reduced the activity of lipoxygenases involved in LXA_4 and 15-epi-LXA_4 synthesis pathways, which may indicate the protective properties of the compounds found in the nettle seeds against the development of inflammation induced by the toxic effects of fluoride.

Keywords: 15-epi-Lipoxin A_4; Fluoride-induced inflammation; Lipoxin A_4; Lipoxygenases; Macrophages; Monocytes; Nettle seeds; THP1 cells; *Urtica dioica* L.

INTRODUCTION

The common nettle (*Urtica dioica* L.) is one of 35 species of the genus *Urtica* (Urticaceae family). Many species of nettle are medicinal plants, yet only *Urtica dioica* is cultivated commercially for pharmaceutical purposes.[1] In folk medicine it has been used for over 3000 years.[2] Modern research has justified the use of nettle in traditional phytotherapy. The leaves of *Urtica dioica* have been confirmed to contain a number of biologically active compounds, including isorhamnetin, kaempferol, quercetin, vitamins B2, C, and K, as well as pantothenic acid, carotene, xanthophyll, and tannins. The common nettle also has a high content of essential elements such as magnesium, calcium, silicon, and iron.[3] Its roots are also valued in herbal medicine, containing many polysaccharides, including glucan, arabinogalactan, and glucogalakturonians, as well as sterols, fatty acids, tannins, ceramides, silica, monoterpenes, isolectins, and lignans.[3-4] Extracts of the common nettle have been applied, among others, as a diuretic in treating urinary, bladder, and kidney problems,

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and has exhibited antibacterial, antihyperglycemic, antioxidant, and anti-inflammatory properties.[5-7] The last two properties may be useful in offsetting the harmful effects of various environmental contaminants.[8]

Fluoride is a toxic element commonly present in the human environment. It readily penetrates biological membranes,[9] and by a cumulative effect causes toxic effects in the body even at relatively low doses with long term exposure.[10] By stimulating the synthesis of reactive oxygen species (ROS) it can activate the enzymes involved in the initiation and development of inflammation, including lipoxygenases (LOXs).[11] The activation of LOXs may also be responsible for inhibiting inflammation thanks to the synthesis of lipoxins,[12] with a major anti-inflammatory role played by lipoxin A_4 (LXA_4) and its epimer 15-epi-LXA_4.[13]

Herbal properties of the common nettle have been well researched, but available literature data offers little on the properties of its seeds. In some regions of Turkey nettle seed extract is used in folk medicine against advanced cancer.[14] Nettle seed oil may also be a potential agent in the treatment of colitis[15] and aflatoxicosis[16,17] with studies on cell cultures and rats also indicating hepatoprotective properties.[18] Nettle seed extract also have high-antibacterial activity.[19] Due to poor recognition of the therapeutic properties of nettle seeds, we aimed to investigate the effects of an extract (using *Urtica dioica*) on the synthesis of LXA_4 and 15-epi-LXA_4 in human monocytes/macrophages exposed to various concentrations of sodium fluoride (NaF).

MATERIAL AND METHODS

Research material: Tests were performed on the THP1 monocyte cell line (ATCC, Rockville, USA), a good model for researching immunomodulatory compounds in human monocytes and macrophages. As a culture medium we used RPMI medium (Sigma-Aldrich, Poznań, Poland) supplemented with 10% fetal bovine serum FBS (Gibco, Paisley, UK) with streptomycin and penicillin (Sigma-Aldrich, Poznañ, Poland). The cells were cultured under standard conditions (37°C/5% CO_2).

The nettle seeds (*Urtica dioica* L.) used for preparation of the plant extract were taken from shoots in uncontaminated suburban green areas of the city of Szczecin (Zachodniopomorskie province, Poland) in late October/early November, when they contained fully mature seeds. Extraction used only panicles containing seed heads.

Preparation of the ethanol extract from the nettle seeds: Nettle seeds were freeze-dried and then pulverized by grinding in an agate mortar. 300 mL of 70% ethanol was added to 5 g of the resulting powder and the mixture was heated for 3 hr in a water bath (80°C). After cooling and filtration, the resultant layers were extracted with ether. The ether extract was discarded and the octanol layer distilled off under reduced pressure to remove the ethanol. The obtained distillation residue was dissolved in DMSO and stored in a plastic tube at –20°C until the experiments.

Cell culture in vitro: THP1 cells were cultured for 48 hr (37°C/5% CO_2) in RPMI medium (Sigma Aldrich, Poland) with 10% fetal bovine serum (Gibco, Poland), fluoride solution and/or ethanol nettle seed's extracts. Nettle extract was in final concentrations 10 μg/mL in accordance with Mohammadi et al.[20] These researchers

showed that this dose (10 μg/mL) causes the lowest cytotoxic effect, measured by the MTT test.[20] NaF was used in final concentrations of 1, 3, and 10 μM on the basis of results of fluoride concentration in human serum.[21] After incubation the cells were harvested by scraping and used for future analysis (n=7). The percent of the living cells was determined by trypan blue. Cell cultures with viability more than 97% were used for experiments. Protein concentration was measured by using the Micro BCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, USA) and Biochrom Asys UVM340 Microplate Reader.

Sample isolation and determination: The cell pellet was eluted with acetonitrile (100%) (Sigma-Aldrich, Poznań, Poland), internal standard prostaglandin B_2 was added (PGB_2; Sigma-Aldrich, Poznań, Poland), then vortexed and frozen at –20°C (10 min.). The samples were then centrifuged (3000 g for 20 min. at 4°C) and the supernatant adjusted to pH=3 with HCl (Sigma-Aldrich, Poznań, Poland).

The prepared samples were concentrated by SPE (J.T. Baker, Mallinckrodt Baker B.V., Deventer, Holland), and dissolved in a mixture of methanol, water and acetic acid (65/35/0.1, v/v/v; Sigma-Aldrich, Poland), and then analyzed by high performance liquid chromatography (HPLC) using an Agilent Technologies 1260 Infinity chromatograph. Separation of the desired compounds was achieved by the concentration gradient of the buffer under a constant temperature. The column used was C18 BDS HYPESIL manufactured by Fisher Scientific; the chromatography and data were processed in Agilent ChemStation software. In the identification of peaks and quantitative analysis we used a mixture of reference standards – external LXA_4 and 15-epi-LXA_4 (Sigma-Aldrich, Poznań, Poland), which allowed determination of a calibration curve. Absorption spectra of the peaks were analyzed to confirm identification of the analytes and to determine their quantities in samples.

Imaging of mitochondrial ROS generation: The mitochondrial generation of ROS was visualized by MitoSOX™ Red reagent (ThermoFischer Scientific, USA), a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells. MitoSOX™ Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria.[22] Cells were loaded with 5 μM MitoSOX™ reagent working solution and incubated for 10 min at 37°C, protected from light. The concentration of the MitoSOX™ reagent working solution should not exceed 5 μM. Concentrations exceeding 5 μM can produce cytotoxic effects, including altered mitochondrial morphology and redistribution of fluorescence to nuclei and the cytosol.[22] After incubation, the cells were washed gently three times with warm buffer at room temperature and the preparations were examined under a fluorescent microscope.

Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide (but not by other ROS- or reactive nitrogen species (RNS)-generating systems) and exhibits red fluorescence. MitoSOX™ Red reagent is readily oxidized by superoxide, and oxidation of the probe is prevented by superoxide dismutase. The oxidation product becomes highly fluorescent upon binding to nucleic acids (excitation at 510 nm, emission at 580 nm).

Imaging of cytoplasmic ROS generation: The cytoplasmic generation of ROS was visualized by fluorescent marker 2',7'-dichlorofluorescein diacetate DCFH-DA (Sigma-Aldrich, Poland). Fluorescein derivatives are the most common fluorescent reagents for biological research because of their high absorptivity, excellent fluorescence quantum yield, and good water solubility. Cells were loaded with 5 μM DCFH-DA. After incubation, the cells were washed with culture medium at room temperature and the preparations were examined under a confocal microscope. When DCFHDA is oxidized to DCF by hydrogen peroxide within the cell, it becomes fluorescent (excitation at 495 nm, emission at 525 nm).

Statistical analysis: Statistical analysis was performed using Statistica StatSoft 10.0 software. The results of a Kolmogorov-Smirnoff test demonstrated that the distribution of data differed from normal, so the parametric tests were rejected. To demonstrate the difference between the groups, non-parametric Mann-Whitney U tests were used. To examine the differences within specific groups, non-parametric Wilcoxon test sequence pairs were used. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

All samples showed the presence of LXA_4 and its epimer 15-epi-LXA_4.

The nettle seed extract (Urtica dioica L.) can decrease LXA_4 synthesis in NaF-treated cells: In the group treated with NaF at three incremental concentrations of 1 μM, 3 μM, and 10 μM, there was a statistically significant increase in the concentration of LXA_4 compared to the controls ($p=0.037$ for 1 μM NaF; $p=0.005$ for 3 μM NaF; and $p=0.012$ for 10 μM NaF) (Figure 1).

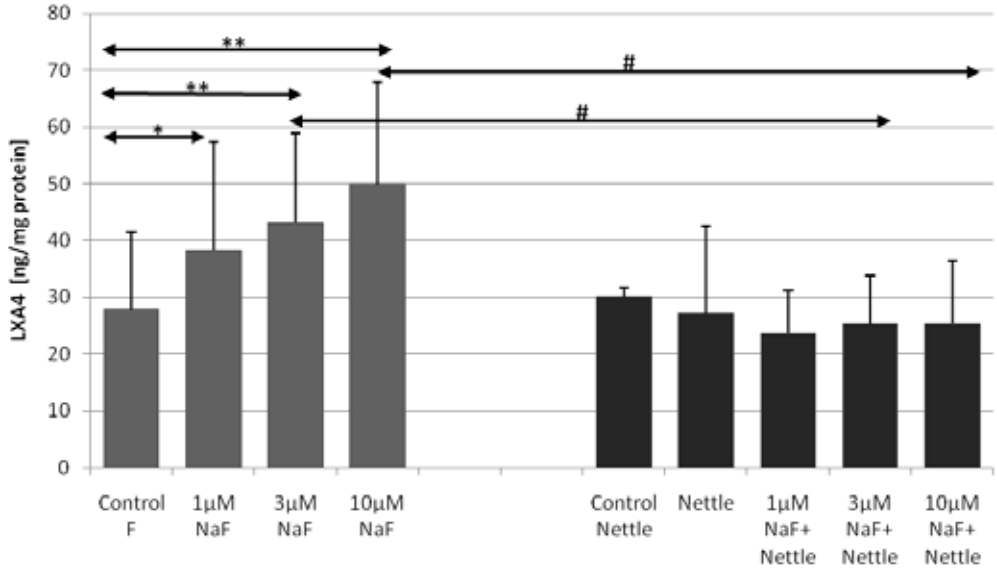

Figure 1. Influence of fluoride and/or nettle seeds extract on LXA_4 concentration in macrophages THP1. "Control F" defines cells cultured with distilled water, "1-, 3- and 10 μM NaF" define different final water solutions of NaF, "Control Nettle" defines cells cultured with DMSO and "Nettle" defines cells cultured with nettle seed extract solution in DMSO (10 μg/ml final solution); Mann-Whitney test, # = 0,05; Wilcoxon test, * p = 0,05, ** p = 0,01; n=7.

Comparing the samples exposed to NaF and those incubated in NaF and alcoholic extract from the seeds of the common nettle, we found statistically significant differences in LXA_4.

In the group exposed to 3 μM NaF and alcoholic extract of nettle seeds, LXA_4 was significantly lower (p=0.039) than in the samples without the addition of the seed extract. A decrease in LXA_4 was also observed for exposure to 10 μM NaF (p=0.041). There were no statistically significant differences in LXA_4 between the two groups at the lowest concentration of 1 ìM NaF.

Extract from nettle (Urtica dioica L.) seeds can increase synthesis of 15-epi-LXA4 in NaF treated cells: Analyzing the differences in the synthesis of 15-epi-LXA_4 in the fluoride-exposed group, we found a directly proportional increase in the concentration of the epimer relative to the concentration of fluoride added to the culture.

The levels of 15-epi-LXA_4 were significantly higher in both the samples exposed to 3 μM NaF and 10 μM NaF versus the controls (p=0.005 for 3 μM NaF, and p=0.002 for 10 μM NaF). For 1 μM NaF, there were no statistically significant differences in the concentration of 15-epi-LXA_4, either with respect to the controls or the samples incubated only with alcoholic extract of nettle seeds (Figure 2).

In the group treated with NaF and alcohol extract from the seeds of the common nettle there were significantly higher levels of 15-epi-LXA_4 in the case of 3 mM NaF and 10 mM NaF with respect to the controls (p=0.001 for 3 mM NaF, p=0.001 for 10 ìM NaF) (Figure 2).

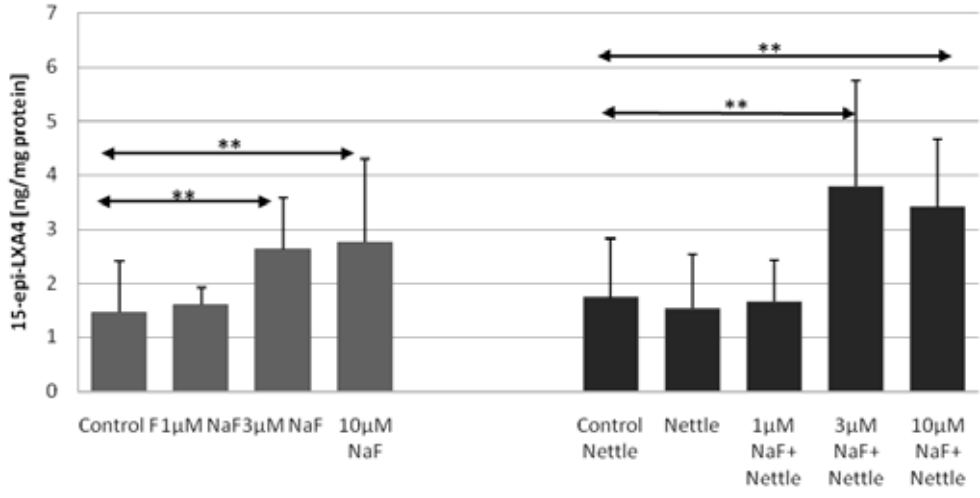

Figure 2. Influence of fluoride and/or nettle seeds extract on 15-epi-LXA4 concentration in macrophages THP1. “Control F” defines cells cultured with distilled water, “1-, 3- and 10 μM NaF” define different final water solutions of NaF, “Control Nettle” defines cells cultured with DMSO and “Nettle” defines cells cultured with nettle seed extract solution in DMSO (10 μg/ml final solution); Wilcoxon test, ** p = 0,01; n=7.

Extracts from nettle (Urtica dioica L.) fruit cluster can decrease cytoplasmic and mitochondrial superoxide amount in NaF-treated cells: Incubation of monocytes/ macrophages with increasing concentrations of NaF caused an increase in the cytoplasmic and mitochondrial superoxide synthesis in the dose dependent manner which is noticeable reduced after addition of ethanol extract from nettle to the culture.

DISCUSSION

To the best of our knowledge, this study was the first to investigate the influence of low levels of fluorides on the synthesis of lipoxin compounds with anti-inflammatory activity. It was also the first to test the effect of micromolar concentrations of NaF on LXA_4 levels in cells.

The incubation of monocyte macrophages with NaF caused a significant increase in LXA_4 concentration in the cells, which indicates that fluoride increased the activity of lipoxygenase pathways. Even at a low NaF concentration (1 μM blood concentration in people environmentally exposed to fluoride on the non-polluted areas), there was an elevated activity of the metabolic pathway of LXA_4 and 15-epi-LXA_4 production, involving lipoxygenases from the families 15-LOX, 5-LOX, and 12-LOX.[21] The correlation between the dose and effect was linear, i.e., the higher the fluoride concentration, the greater the production of LXA_4 and 15-epi-LXA_4 in the treated cells.

Lipoxygenases are responsible not only for the production of mediators with anti-inflammatory properties (such as lipoxins), but also for the production of inflammatory mediators (e.g., 15-HETE).[21] In addition, one of the LXA_4 pathways is based on the reaction of transcellular arachidonic acid oxidation, resulting in the formation of 15-HETE which is then converted into LXA_4.[23] Given the numerous scientific reports on the toxicity of fluoride,[18,21] it can be assumed that the appearance of lipoxins and its epimers indicates inflammation with its characteristic presence of both pro- and anti-inflammatory mediators. In this study, the cell culture incubated simultaneously with fluoride and nettle seed extract revealed a significantly lower level of LXA_4 compared to the culture incubated only with fluoride (there were no statistically significant differences in the levels of 15-epi-LXA_4), which can be attributed to the action of compounds contained in the extract that to some extent inhibited the development or initiation of inflammation.

It is likely that in fluoride-treated cells, LXA_4 initiates and enhances inflammation through an increase in the synthesis of ROS,[21] which was confirmed by tests under a fluorescence microscope (Figures 3–6). The mechanism of action is based on the action of ROS as intracellular modulators of signaling pathways that activate ROS-mediated NF-κB signaling pathways,[24] and thus involve the dissociation of NF-κB from IκBs – its inhibitor.[25] In most cells, NF-κB is sequestered in the cytoplasm due to interactions with the IκB family of inhibitory proteins. An increase in ROS activates IκB kinase (IKK)[25] which leads to a rapid phosphorylation, ubiquitination, and degradation of IκBs.[26] NF-κB is then released and translocated into the nucleus

where it activates the expression of genes of enzymes involved in inflammatory responses,[23] which include LOX that produces LXA_4.

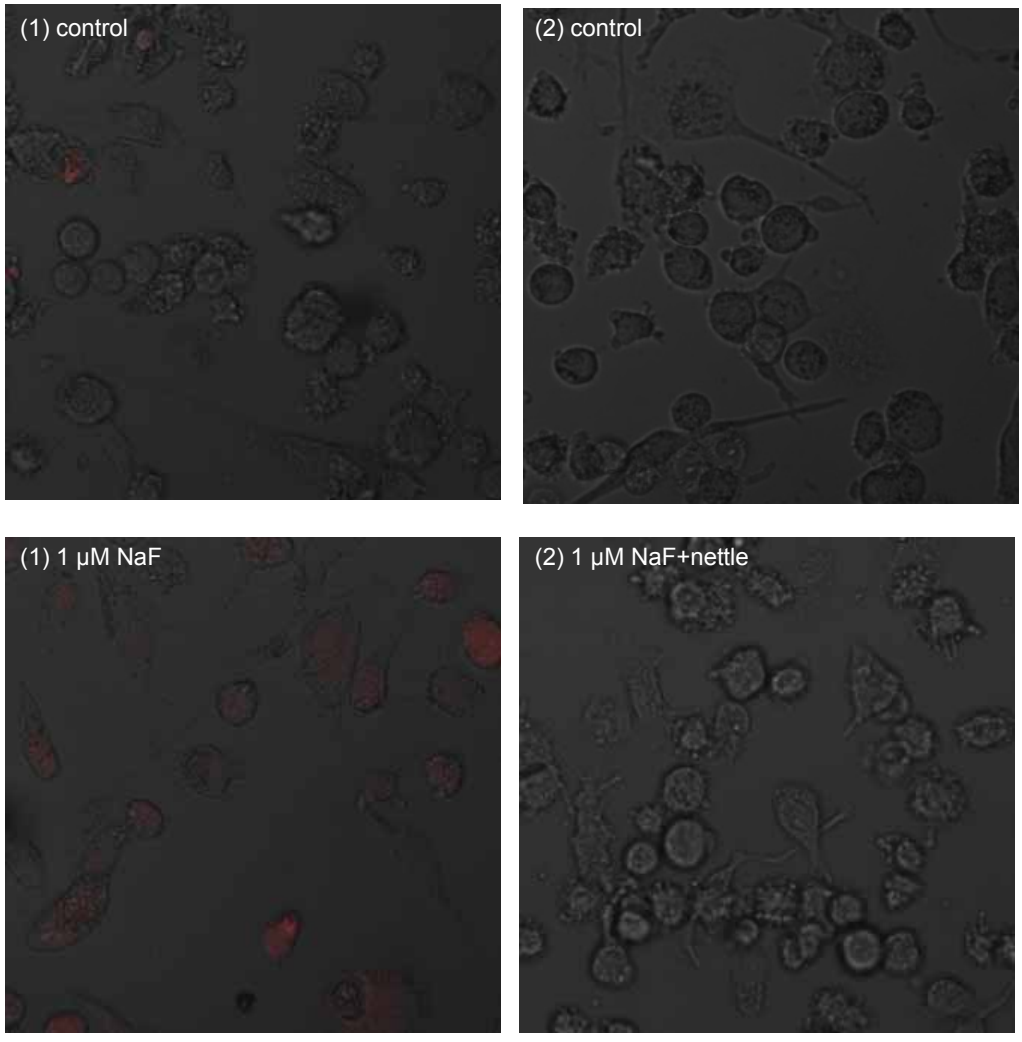

Figure 3. Imaging of mitochondrial ROS by confocal microscopy in macrophages cultured with NaF. Monocytes/macrophages were cultured with (1) NaF solutions (with control which contained distilled water as NaF solvent), (2) NaF solutions and the extract from nettle fruit clusters (with control which contained distilled water as NaF solvent and DMSO as extract solvent) for 48 hr. The histochemistry analysis was performed using 5 μM MitoSOX™ reagent solution (incubation for 10 min at 37°C, protected from light).

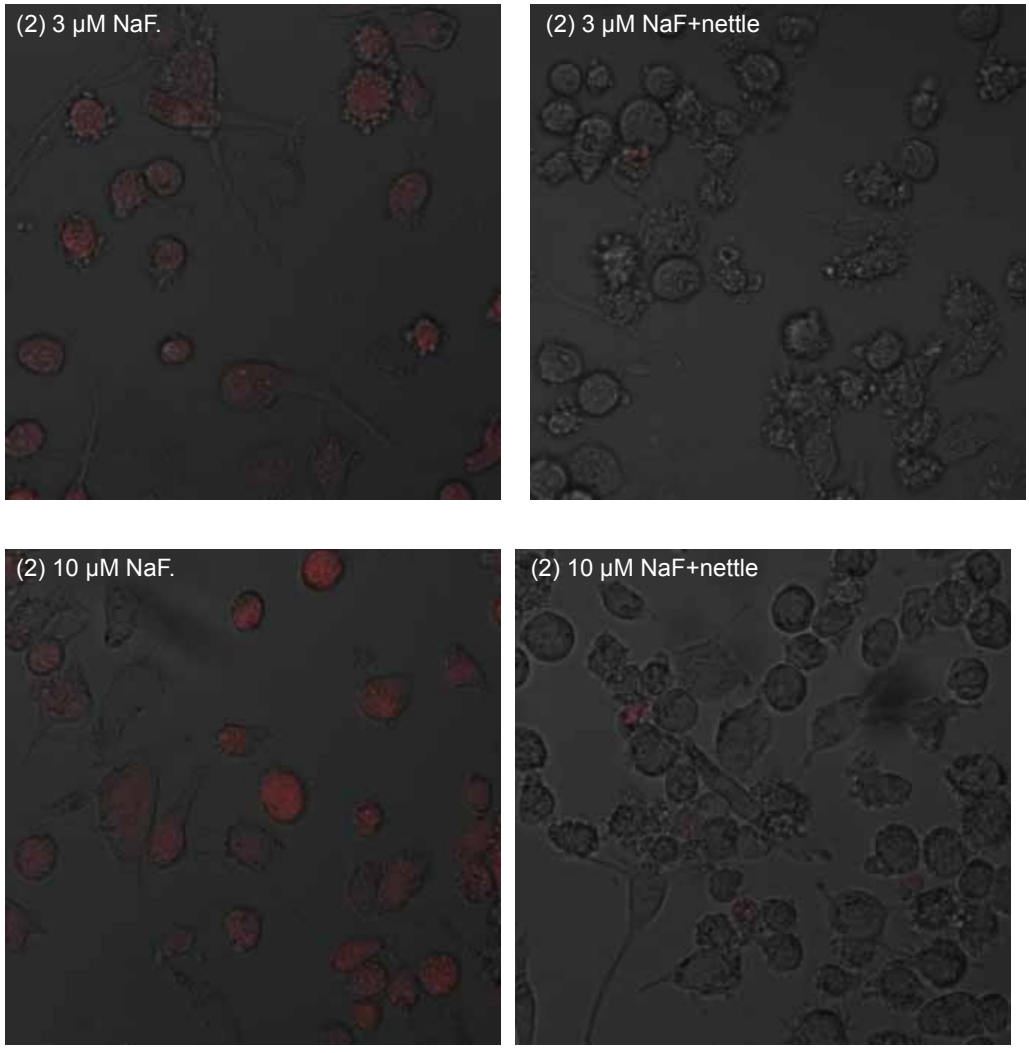

Figure 4. Imaging of mitochondrial ROS by confocal microscopy in macrophages cultured with NaF. Monocytes/macrophages were cultured with (1) NaF solutions (with control which contained distilled water as NaF solvent), (2) NaF solutions and the extract from nettle fruit clusters (with control which contained distilled water as NaF solvent and DMSO as extract solvent) for 48 hr. The histochemistry analysis was performed using 5 μM MitoSOX™ reagent solution (incubation for 10 min at 37°C, protected from light).

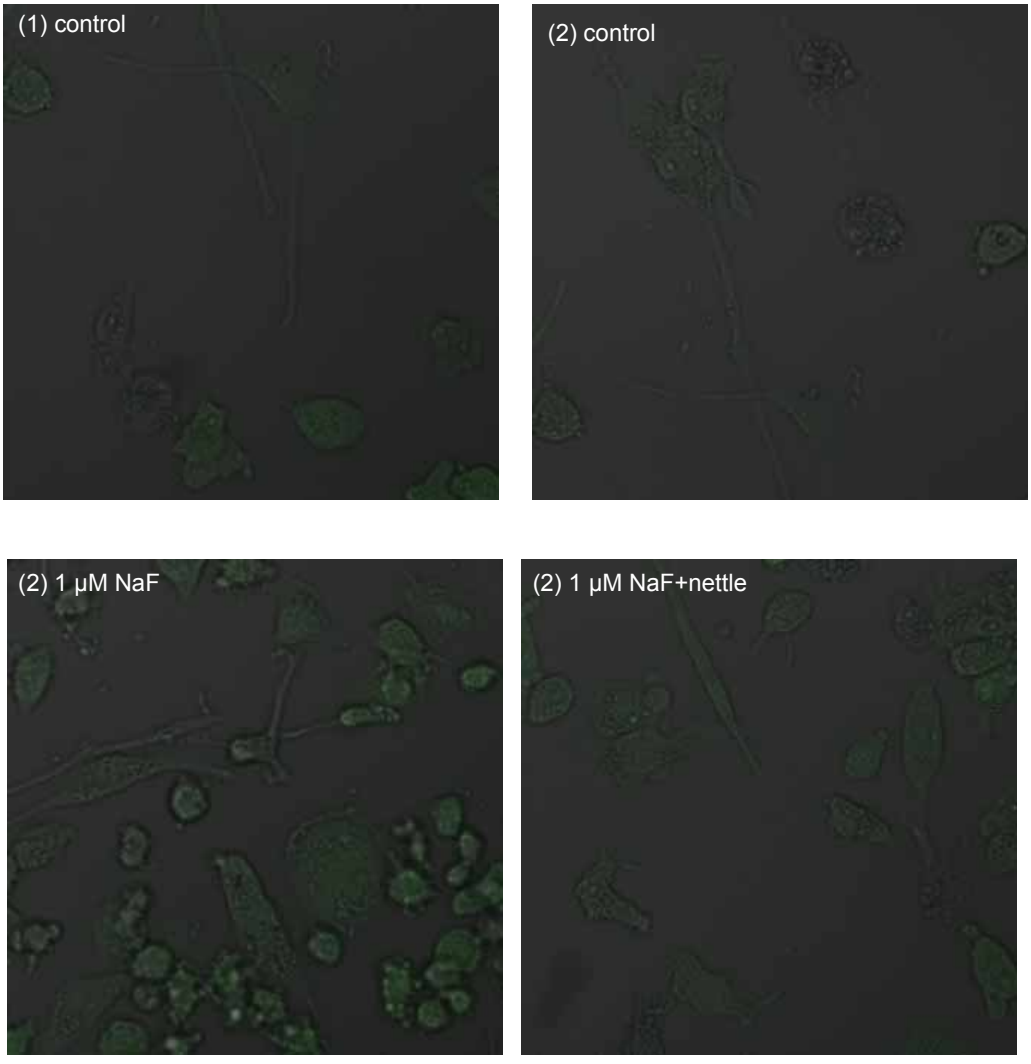

Figure 5. Imaging of cytoplasmatic ROS by confocal microscopy in macrophages cultured with NaF. Monocytes/macrophages were cultured with (1) NaF solutions (with control which contained distilled water as NaF solvent), (2) NaF solutions and extract from nettle fruit clusters (with control which contained distilled water as NaF solvent and DMSO as extract solvent) for 48 hr. The histochemistry analysis was performed using 5 μM DCFH-DA. After incubation, the cells were washed with culture medium at room temperature and the preparations were examined under a confocal microscope (excitation at 495 nm, emission at 525 nm).

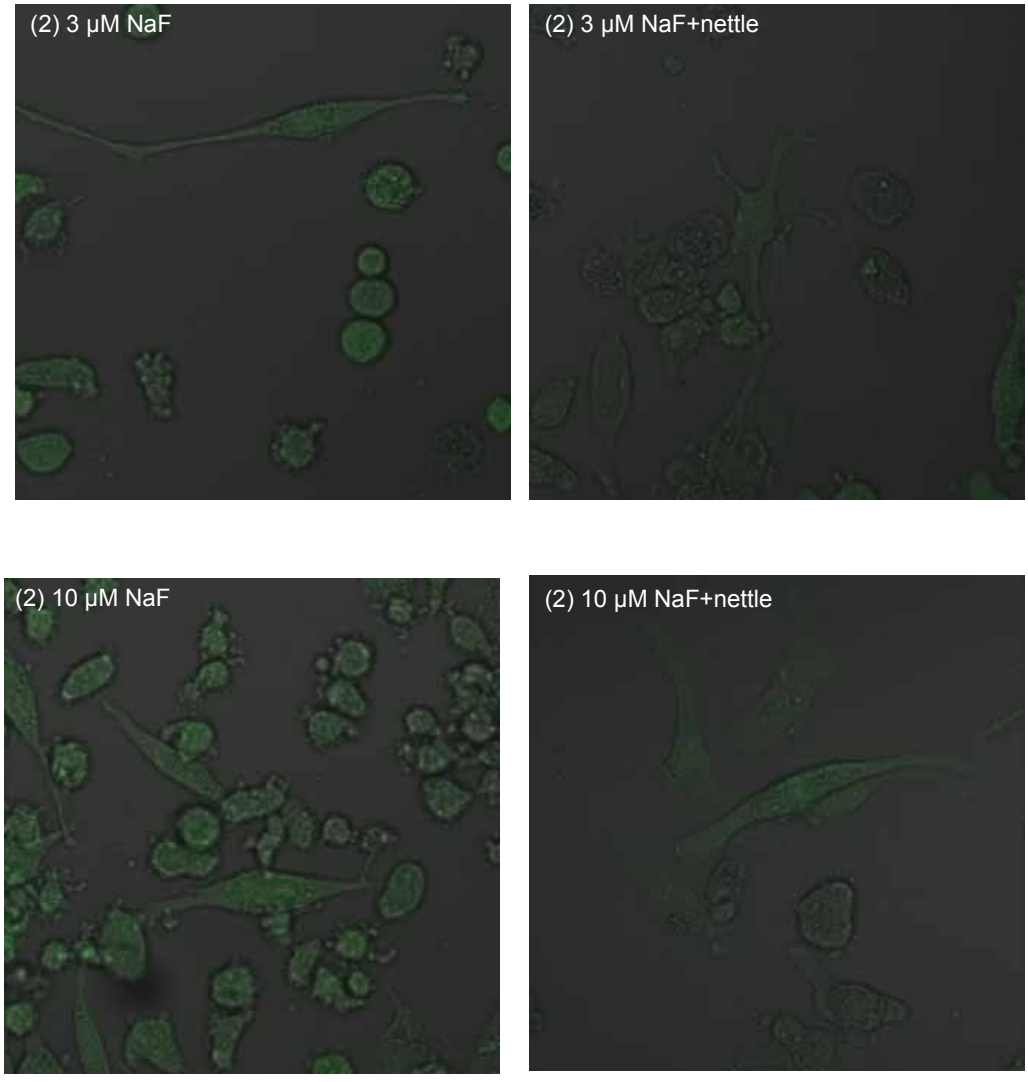

Figure 6. Imaging of cytoplasmatic ROS by confocal microscopy in macrophages cultured with NaF. Monocytes/macrophages were cultured with (1) NaF solutions (with control which contained distilled water as NaF solvent), (2) NaF solutions and extract from nettle fruit clusters (with control which contained distilled water as NaF solvent and DMSO as extract solvent) for 48 hr. The histochemistry analysis was performed using 5 μM DCFH-DA. After incubation, the cells were washed with culture medium at room temperature and the preparations were examined under a confocal microscope (excitation at 495 nm, emission at 525 nm).

This synthesis appears to be a defense of the cells against the increasing inflammation, as some studies have revealed that LXA_4 acts as an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and thus it can block the production of intracellular ROS. On the other hand, LXA_4 can also block neutrophil-platelet interactions, which reduces neutrophil-derived ROS, characteristic of inflammation.[27] As a result of ROS synthesis inhibition, lipoxins –

the products of enzymatic conversion of arachidonic acid by the lipoxygenase – act as "stop signals" in inflammation by inhibiting the NF-κB pathway.[28] On one hand, this inhibits the penetration of neutrophils and eosinophils into the epithelium (anti-inflammatory action); and on the other, it promotes the phagocytosis of neutrophils[29] and macrophages in which lipoxin inhibits the cell cycle and increases apoptosis.[30]

Studies in HepG2 hepatocytes incubated with NaF (1 μM, 3 μM, 10 μM), in which the proinflammatory activity of 15LOX was measured by measuring the concentrations of 15,12-HETE and 9,13-HODE by HPLC,[18] also showed a statistically significant increase in their concentrations. Moreover, already at 1 μM NaF there was an increased synthesis of mitochondrial superoxide anions compared to the controls, and severe hepatocyte apoptosis transforming into necrosis.[18] These studies confirm the pro-inflammatory action of fluoride via activation of the 15-LOX pathway.[21] Therefore, increased synthesis of LXA_4 and 15-epi-LXA_4 appears to be due more to a defensive reaction aiming at neutralizing immune reaction.

Numerous studies have shown that lipoxins (LXs) have potent anti-inflammatory properties in many acute inflammatory disorders, and probably insufficient LXs is an important mediator for these chronic diseases, such as asthma, rheumatoid arthritis, etc., in which a lot of chronic inflammatory disorders are caused by a decrease in the levels of LXs, as has been proven in many articles.[31] In contrast, LXs could be involved in the activation and regulation of the immune response and loss of control of the immune response, especially through the activation of infiltrative cells like macrophages, where the increased activation of specialized pro-resolving mediator biosynthesis, including LXs,[32] may be the primary cause of inflammatory protraction. Our research showed that the nettle seed extract (*Urtica dioica* L.) could influence the production of LXA_4 and its epimer and thereby regulate inflammation.

As mentioned earlier in this study, the addition of nettle seed extract to the cell culture statistically significantly decreased the concentration of LXA_4 compared to the samples treated only with fluoride. Similar results were obtained in studies performed in hepatocytes,[18] where the addition of an extract of nettle seeds to cell cultures incubated with fluoride significantly reduced the synthesis of the final products of lipoxygenase (LOX-15) activity: 12,15-HETE and 9.13-HODE.[18] It is likely that through the reduction of ROS levels in cells,[18] the alcoholic extract of nettle seeds inhibited signaling pathways (such as NF-κB) leading to the activation of lipoxygenases. As evidenced by the work of other researchers, it may be due to the extract-induced increase in activity of antioxidant enzymes: glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT), which reduce inflammation in the body through a substantial reduction in ROS.[33] Toldy et al.[34] found in a study on rats given dried leaves of nettle, that their brain ROS levels were significantly reduced. That data confirms that a nettle supplementation may reduce oxidative stress.[34]

In addition, our results showed an increase in the amount of synthesized LXA_4 epimers (15-epi-LXA_4), when cell cultures exposed to fluoride were treated with the the nettle seed extract, although that increase was not statistically significant. 15-epi-LXA_4 is synthesized from arachidonic acid by the enzyme system which includes

COX-2 and 5-LOX. However, acetylated COX-2 (e.g., under the action of acetylsalicylic acid (ASA)) can catalyze such reactions.[13] The increase in the concentration of this compound in the fluoride-exposed group may have been due to the stimulating effect of fluoride on the factors acetylating COX-2. This resulted in an increase in the synthesis of 15-epi-LXA_4 in response to the growing inflammation caused by the increasing concentration of fluoride added to the cell cultures. The addition of the nettle seed extract at higher fluoride concentrations increased epimer synthesis even further, which could have been caused by the acetylating activity of compounds found in nettle seeds, such as benzoic acid (with salicylic acid as its derivative).

CONCLUSION

In summary, the use of an ethanol extract from nettle seeds reduced the activity of lipoxygenases involved in LXA_4 and 15-epi-LXA_4 synthesis pathways, which may indicate the protective properties of the compounds found in the nettle seeds against the development of inflammation induced by the toxic effects of fluoride.

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