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Original Study • DOI: 10.2478/ahem-2023-0008 • AHEM • Vol. 77 Issue 1/2023 • 49–58

AHEM

Advances in Hygiene and Experimental Medicine
Postępy Higieny i Medycyny Doświadczalnej

Anti-inflammatory activity of novel natural plant extracts composition—Levidor™

Original Study

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Received 14 October, 2022, Accepted 21 March, 2023

Abstract

Introduction. Due to the severe side effects of non-steroidal anti-inflammatory drugs, there is an urgent need to find new natural or synthetic chemicals that could act as selective inhibitors of cyclooxygenase-2 (COX-2). The most valuable source of new bioactive compounds and potential drugs are plants. The present study aimed at the investigation of Levidor™, a patented composition based on oil from the seeds of *Nigella sativa* and extract from the root of *Angelica archangelica,* regarding its potential anti-inflammatory effects on the molecular level.
Materials and Methods. Studies were carried out with the use of murine macrophages (RAW 264.7), human monocytes (U937), and rat pheochromocytoma (PC-12) cell lines. The cytotoxicity of the Levidor™ extract was evaluated by MTT assay. The concentration of pro-inflammatory cytokines (IL-6, TNF-*α*) was evaluated by ELISA. Moreover, the analysis of *COX-1* and *COX-2* mRNA expression levels by RT-PCR, as well as protein level by Western blot and immunofluorescent staining, were assessed.
Results. The Levidor™ extract did not impact cell growth and showed anti-inflammatory effect in the present study. It was able to decrease the level of IL-6, TNF-*α* and suppress transcription of COX-2, whereas it had no effect on COX-1.
Conclusions. Obtained results showed that Levidor™ exhibits strong anti-inflammatory properties and may provide a significant alternative to traditionally used medicines in acute pain and chronic inflammation.

Keywords

Nigella sativa • *Angelica archangelica* • phytochemicals • plant-based product • anti-inflammatory • cyclooxygenases

1. Introduction

Inflammation is a complex response of body tissues to harmful factors, such as pathogens, irritants, or damaged cells. It plays a protective role involving the local immune and vascular system as well as molecular mediators [1]. The primary aim of inflammatory response is fast elimination of the initial cause of injury and initiation of tissue repair. However, if the inflammation is prolonged, a state called chronic inflammation occurs. Over time, chronic inflammation can the trigger immune system to attack healthy tissue, leading to their damage. When left untreated, prolonged chronic inflammation can increase the risk of diseases such as diabetes, heart disease, or even cancer [1]. Among various inflammatory mediators implicated in pathological processes, cyclooxygenase (COX) and its downstream effector molecules are of greater significance. Cyclooxygenase 2 (COX-2) is a major enzyme involved in the modulation of inflammation and acts by catalyzing the rate-limiting step, leading to the production of prostaglandins (PGs) from arachidonic acid [2]. COX-2 is expressed at low levels under physiological conditions; however, it is highly and transiently expressed in response to the aforementioned harmful stimuli. This leads to a burst of PGs production. It has been proven that prolonged COX-2 expression may override immunological balance of the body, resulting in pathological states [3]. In different examples of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, or diabetes, the hyperactivation of such subpopulations of immune cells has been shown to be directly dependent on the over-expression of COX-2 [4]. The hyperexpression of COX-2 is the result of cross-action between broad range of inflammatory markers, namely interleukins and cytokines (i.e., IL-1, IL-6 or TNF-*α*), and it occurs via transcriptional activation [5].

The other key cyclooxygenase is COX-1, an enzyme that is constitutively and stably expressed at low levels in many tissues. Whereas COX-2 is predominantly implicated in inflammation, COX-1 is a critical regulator of homeostatic functions [4]. Protein COX-1 ensures the constant production of PGs, which contribute to the maintenance of important physiological functions, such as platelet aggregation, renal water balance, and most importantly, gastric mucosal protection [5]. Its expression is also found in fetal and amniotic cells, uterine epithelium in early pregnancy, and the central nervous system. It is believed to exert complex integrative functions [6].

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Consequently, strategies to decrease chronic inflammation in a broad range of disorders aimed at selective inhibition of COX-2 enzyme activity. Non-steroidal anti-inflammatory drugs (NSAIDs), which have their origin in the extracts of salicylate-containing plants, represent the first large class of inhibitors of COXs available on the market [7].

Clinically proven effectiveness of the aspirin led to the development of other classical NSAIDs. These pharmaceuticals belong to a group of carboxylic acids. Aside from aspirin, this group consists of naproxen, diclofenac, ketoprofen, flurbiprofen, ibuprofen, and indomethacin [8]. Eventually, these drugs hit the pharmaceutical markets. Unfortunately, the classical NSAIDs have varying selectivity towards COX-1. Thus, prolonged intake of NSAIDs was immediately recognized to cause serious side effects, such as damage to the gastric mucosa [9]. For this reason, patients taking NSAIDs for an extended period of time must use proton-pomp inhibitors to protect mucosa. The negative impact of NSAIDs on the gastrointestinal tract was related to co-inhibition of concomitant perturbation of COX-1 activity [10]. Also, preferential COX-2 inhibitors such as carboxamides (meloxicam), sulphonanilides (nimesulide) and naphthalenes (nabumetone), and selective COX-2 inhibitors called coxibs (celecoxib, etoricoxib, parecoxib, rofecoxib, valdecoxib, lumiracoxib) have been introduced into the market [8]. These drugs initially exhibited improved safety profiles regarding the health of the gastrointestinal tract as compared to the classical NSAIDs. Unfortunately, despite the early success of these drugs, many cardiovascular disorders have been reported when compared to classical NSAIDs. Rofecoxib and valdecoxib were withdrawn from the market because of severe side effects in the cardiovascular system. For the same reason, etoricoxib is not approved in several countries. Side effects affecting the liver caused the withdrawal from the market of drugs based on lumiracoxib. Currently, celecoxib is the safest NSAID and the only selective COX-2 inhibitor with market authorization both in the European Union (EU) and the United States. However, the labels of celecoxib-based drugs highlight the several potential adverse effects [11].

Due to the severe side effects of existing COX-inhibitors, there is an urgent need to find new natural or synthetic chemicals that could act as selective inhibitors of COX-2. The most valuable source of new bioactive compounds and potential drugs are plants. Historically, plant products with medicinal properties have been used to treat several inflammatory diseases [12]. These traditional anti-inflammatory remedies later become the basis for the production of aspirin, the first natural-product-derived synthetic anti-inflammatory drug [12]. The exploration of phytochemicals has been promising and resulted in the discovery of many plant secondary metabolites with therapeutic activities such as artemisinin, vinblastine, or pilocarpine [12]. Recently, attention has been paid to *Nigella sativa* and *Angelica archangelica*, which phytocomplexes are considered to exert potential anti-inflammatory activity.

Nigella sativa is a small flowering plant mainly grown in Central Asia, the Middle East, and North Africa [13–15]. It contains bioactive components such as fixed and essential (volatile) oil, proteins, amino acids, carbohydrates, alkaloids, organic acids, saponins, crude fibers, vitamins, and minerals [16,17]. Due to the presence of thymoquinone (TQ), thymol (THY), and *α*-hederin in the volatile and the fixed oil, the *N. sativa* exhibits several biological effects [13,18–20]. According to the literature, its seeds are used all over the world for the prevention and symptom reduction of numerous diseases including asthma, rheumatic illnesses, diarrhea, decreased blood pressure and dyslipidaemia, bronchitis, inflammation, eczema, fever, influenza, hypertension, cough, headache, dizziness, and diabetes. Additionally, it was determined that seeds show an analgesic, antipyretic, antimicrobial and antineoplastic effects [13,15].

The second plant exhibiting anti-inflammatory potential is *Angelica archangelica,* which is mainly grown in the north temperate and arctic regions and New Zealand [21]. According to the literature, the active components of *A. archangelica* exert many desirable biological effect related to chronic diseases, also those associated with inflammatory processes impairment such as rheumatic and gastrointestinal disorders, respiratory problems, migraine, bronchitis, chronic fatigue, and menstrual complaints [21–23]. Similarly to *N. sativa*, *A. archangelica* contains several bioactive phytochemicals such as imperatorin, xanthotoxin and xanthotoxol archangelenone (flavonoid), angelicin, osthol, bergapten, oreoselone, as well as oxypeucedanin; however, those of the most important biological importance are imperatorin and xanthotoxin [24–26]. Furthermore, all parts of this plants have been extensively used as food flavorings, spices, and condiments [21,25,26].

The studies showed that both plants have a strong analgesic and anti-inflammatory effect [13,16,17,18–26]. Recent investigations on bioactive phytochemicals showed that combinations of bioactive compounds such as different extracts may lead to the formation of a new preparation with modified biological properties due to interactions between phytochemicals [27]. Moreover, the extraction and fixation procedures have crucial impact on maintenance of bioactivity of isolated phytocomplex. The extraction method applied in the present study has been patented (P.412214, WO2016118027A1). For this reason, the present study aimed at the investigation of Levidor™, the composition based on oil from the seeds of *N. sativa* and extract from the root of *A. archangelica,* regarding its anti-inflammatory effects at molecular level.

2. Materials and Methods

2.1. Chemicals and reagents

The following chemicals were used for the study: lipopolysaccharide (LPS) from *Escherichia coli* (O111:B4), diclofenac (purity ≥ 98%, HPLC), sodium dodecyl sulfate (SDS; purity ≥ 98.5%, HPLC), and phorbol-12-myristate-13-acetate

(PMA; purity ≥ 98%) from Sigma (USA). For cell culture, DMEM (HyClone, Cytivia, USA), RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA), penicillin and streptomycin (Sima-Aldrich, USA), fetal bovine serum (FBS; HyClone, Cytivia, USA), and donor heat-inactivated horse serum (Biowest, France) were utilized. Thiazolyl blue tetrazolium bromide (MTT) from Sigma-Aldrich (USA) was applied in the MTT test. The protein concentration was evaluated with the aid of BCA assay (Sigma-Aldrich, USA). Human ELISA sets (IL-6, TNF-*α*) were purchased from R&D Systems (USA). For gene expression analysis, Total RNA Mini Plus kit (A&A Biotechnology, Poland), Transcriba kit from A&A Biotechnology (Poland) and SYBR Green PCR reaction mix (SensiFAST SYBR No-ROX, Meridian Bioscience, USA) were purchased. For Western blot analysis, rabbit antibodies anti-COX-1 from Cell Signalling (USA), anti-COX-2 anti-*β*-actin, and vinculin from Sigma (USA) were obtained. Moreover, specific horseradish peroxidase-conjugated secondary antibodies from Jackson Immunoresearch (USA) were used. Fluorescent immunocytochemistry was performed with the aid of monoclonal antibodies against COX-1 and COX-2 from Thermo Fisher Scientific (USA). Phosphate buffered saline (PBS) and dimethyl sulfoxide used during experiments in cell culture were purchased from Sigma (USA). PBS solution was prepared by dissolving one tablet in 200 mL ultrapure water. Water was obtained using water purification system (HLP, Hydrolab, Poland).

2.2. Extract preparation

Plant preparations of Levidor™ were dissolved in DMSO at a concentration 20 mg/mL. The stock solution was stored in dark at 4 °C. For every experiment, extracts were taken from the stock vial and dissolved as needed. The extraction method has been patented (P.412214, WO2016118027A1). The extract has been phytochemically characterized previously [20].

2.3. Cell culture

The cell line of human monocytes U937 (ECACC 85011440) and murine macrophages RAW 264.7 (CLS 400319) cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The cell line derived from a rat pheochromocytoma PC-12 (CRL-1721) was cultured in RPMI-1640 medium. To make the complete growth medium, the following components to the base medium were added: heat-inactivated horse serum to a final concentration of 10% and FBS to a final concentration of 5%, 100 U/mL penicillin, and 100 µg/mL streptomycin**.** The cell lines were maintained at 37 °C under 5% CO_2 atmosphere in a cell incubator (Galaxy 170S, New Brunwick, Canada).

In the case of cytotoxicity assessment by MTT assay, the RAW264.37 and PC-12 cells were seeded at a density of 1 x 10^4 in 96-well plate. For determination of IL-6 and TNF-*α* protein levels by ELISA, the RAW 264.7 cells were seeded in 24-well tissue culture plates (5 x 10^5 cells per well). In turn, the evaluation of *COX-1* and *COX-2* gene expression and the concentration of proteins encoded by these genes were performed on U937 cells seeded at the density of 5 x 10^5 in 24-well plate. All cultures after seeding were left to settle for 24 h at 37 °C under 5% CO_2.

2.4. PMA stimulation

In the selected experiments, the human U937 cell line was applied. This cell line under appropriate stimulation is commonly used as a functional model of macrophages. For differentiation into macrophage-like cells, PMA at concentration 10 ng/mL in culture medium was added and cells were allowed to differentiate for 48 h at 37 °C under 5% CO_2 atmosphere in a cell incubator. Then the cells were incubated without treatment for 40 h. After that time, the cells were treated with extract and LPS stimulation was performed as described below.

2.5. LPS stimulation

In order to determine the influence of Levidor™ extract on the inflammatory response of RAW 264.7 or differentiated U937 cells, the extract at concentrations 1, 2.5, 5, 10, 25 and 50 µg/mL was added to cells for 4 h. Diclofenac was used as reference (50 or 100 µg/mL). After that time, the cells were stimulated with 1 µg/mL of LPS for 2, 4, 6, and 16 h when *COX-1* and *COX-2* gene expressions were investigated; for 24 h in the case of COX-1, COX-2, IL-6, and TNF-*α* protein levels by Western blot. The stimulation of LPS was performed to polarize macrophages towards the proinflammatory M1 subtype inducing the inflammatory response.

2.6. TNF-*α* stimulation

In the case of immunofluorescence staining performed to verify the level of COX-1 and COX-2 proteins under Levidor™ treatment, the U937 cells after 24 h pre-treatment with PMA (10 ng/mL), followed by 24 h incubation without treatment, were given 4 h treatment with 10, 25, and 50 µg/mL of Levidor™ extract and TNF-*α* addition (100 ng/mL) for 20 h to induce inflammatory response.

2.7. Cytotoxicity assessment

To determine the impact of Levidor™ extract on RAW264.37 and PC-12, MTT test was applied. The cells were treated for 72 h with concentrations ranging from 6.25 to 100 µg/mL of sample. After 72 h of incubation, 100 µL of MTT (1 mg/mL) were added to each well for 4 h. After that time, medium was aspirated from wells and formazan crystals were dissolved in 0.05 mL of DMSO. The absorption of the obtained solutions was measured at 540 nm with the aid of microplate reader Synergy™ HT, (Biotek Instruments, USA). The impact of investigated samples on cells growth was expressed as IC50.

2.8. Determination of IL-6 and TNF-α protein levels by ELISA

After treatment of the U937 cells, cells were washed twice with PBS, collected and the protein concentration in the cell free supernatants was determined by BCA assay. The assay was performed according to the manufacture's instruction. Obtained material was stored in -80 °C. IL-6 and TNF-*α* protein levels were quantified by ELISA sets (R&D Systems) according to the manufacturer's recommendations. The intensity of the solutions color was measured with the aid of microplate reader Synergy™ HT (Biotek Instruments, USA).

2.9. Evaluation of COX-1 and COX-2 gene expression

After treatment, total RNA was extracted using Total RNA Mini Plus kit according to the supplier's protocol. The extracted RNA was quantified, and input amounts were optimized for each amplicon. Then, the RNA samples were reverse-transcribed with the aid of Transcriba kit, and the resulting cDNA samples were used as templates for real-time PCR analysis of selected gene expression using SYBR Green PCR reaction mix namely SensiFAST SYBR No-ROX and gene specific primers (Table 1).

Table 1. Forward and reverse sequences of applied primers

Gene	Forward	Reverse
GAPDH	5'-AACGACCCCTTCATT-GAC-3'	5'-TCCACGACATACTCAG-CA-3'
COX-1	5'-TCTCAGGCTA-CACCCTAGACCA-3'	5'-ATCGGGGTAGTCCGAG-TAACGT-3'
COX-2	5'-CAGCACTTCACGCAT-CAGTT-3'	5'-CGCAGTTTACGCTGTC-TAGC-3'

Reactions were conducted on an Miniopticon system (Biorad) with following stages: 95 °C for 3 min., 39 cycles of denaturation 95 °C for 10 sec. and a melting curve stage of 60 °C to 90 °C for 5 sec. Gene expression levels were determined by the $^{\Delta\Delta}C_t$ method. RT-PCR data was expressed as fold changes relative to control group. No-reverse transcribed mRNA served as a negative control. GAPDH was used as the housekeeping gene for normalization of the results.

2.10. Detection of COX-1 and COX-2 protein levels by Western blot

After treatment, the cells were lysed in RIPA buffer combined with homogenization. The protein concentration was evaluated by BCA assay. The lysates (30 µg of proteins) were fractionated by 7.5% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking with 5% fat-free milk, the membranes were incubated with primary antibodies: anti-COX-1 (1:1000), anti-COX-2 (1:1000), anti-*β*-actin (1:1000), and vinculin (1:5000) for 2 h at 20 °C followed by specific horseradish peroxidase-conjugated secondary antibodies for 1 h at 20 °C. Immunoreactive material was detected by chemiluminescence (Pierce Laboratories, Thermo Fisher Scientific) and imaged using Bio-Rad Imaging System (Bio-Rad).

2.11. Detection of COX-1 and COX-2 protein levels by immunofluorescence

Fluorescent immunocytochemistry was performed on U937 cells using monoclonal antibodies against COX-1 and COX-2. The cells were pre-treated with PMA (10 ng/mL). This step was followed by 24 h of incubation without treatment, 4 h treatment with Levidor™ at 10, 25, and 50 µg/mL as well as TNF-*α* addition (100 ng/mL) for 20 h. The immune staining was performed according to manufacturer's protocol.

2.12. Statistical analysis

All values are expressed as means ± SD of three independent experiments unless stated otherwise. The statistical significance of changes between samples and control was evaluated by nonparametric Mann-Whitney one-tailed statistical test. The level of statistical significance was set at $p \leq 0.05$. All statistical analyses were performed using Prism 4.0 software package (GraphPad Software, Inc., USA).

3. Results

3.1. Cytotoxicity assessment

The impact of studied extract on RAW264.37 and PC-12 was assessed by MTT test. The cells were treated with tested extract at concentrations ranging from 0.1 to 200 µg/mL. Table 2 presents IC50 determined after 72 h of exposure. The extract did not significantly influence the RAW264.37 cell growth at any of the investigated concentrations. In the case of PC-12 cell line, the IC50 at 193.1 µg/mL was observed. The obtained results indicate that Levidor™ extract does not exhibit cytotoxic activity.

Table 2. Values of IC50 obtained by MTT test

Cell line	IC50 [µg/mL]
RAW264.37	not cytotoxic at concentration range tested
PC-12	193.1

3.2. Evaluation of IL-6 and TNF-α protein levels

The RAW 264.7 cells were treated with tested extract at concentrations ranging from 1 to 50 µg/mL.

As shown in Fig. 1, control cells produced low levels of both tested proteins. The addition of LPS strongly induced production of both IL-6 and TNF-*α* as expected. Our results demonstrate that Levidor™ at the concentration of 25 and 50 µg/mL significantly decreased the production of both IL-6 and TNF-*α* ($p \leq 0.05$). In contrast, diclofenac at the same concentration did not impact cytokine level significantly.

3.3. Evaluation of COX-1 and COX-2 gene expression

The presented study aimed to elucidate the impact of Levidor™ on *COX-1* and *COX-2* gene expression in U937 cells differentiated with the aid of PMA. The. Fig. 2A presents modulation of *COX-*

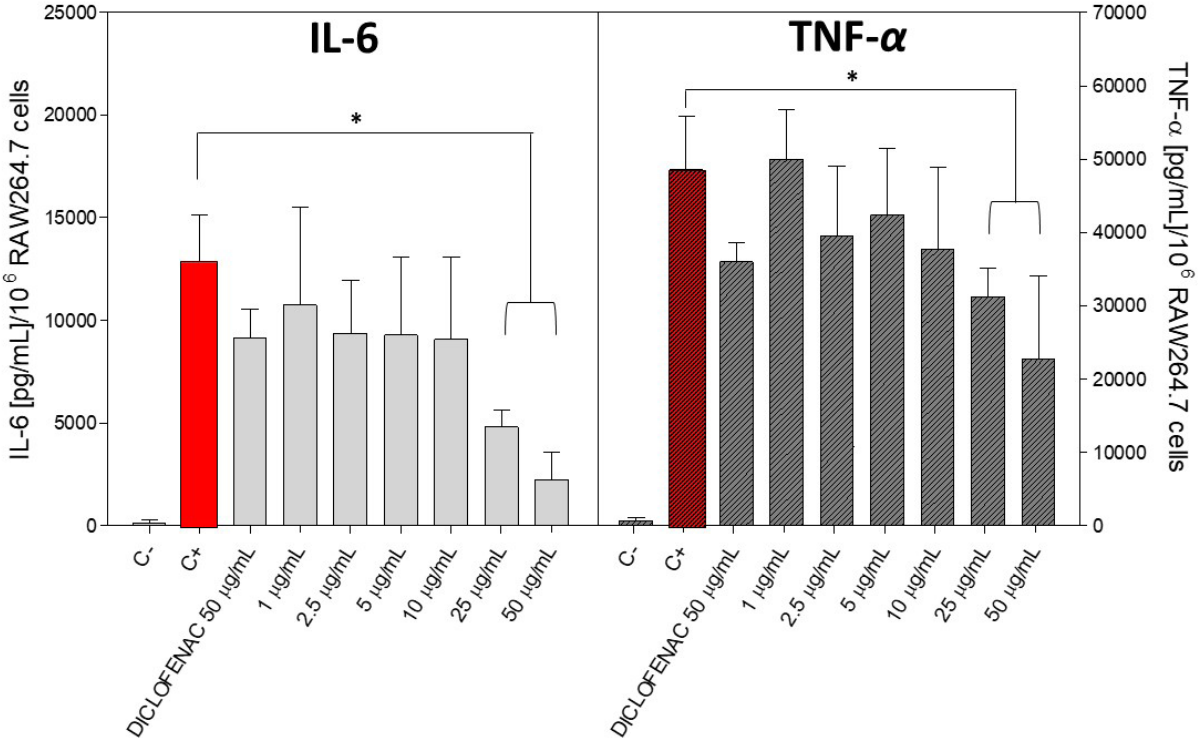


Fig. 1. The effect of Levidor™ extract on pro-inflammatory markers (IL-6, TNF-α) induced by LPS in the RAW267.7 cells. All results are given as means ± SD of three independent determinations. Negative control (C-) refers to cells treated with solvent, while positive control (C+) relates to cells treated with inflammatory inducer only. Significantly different values are marked as (*) $p \leq 0.05$

1 expression under different (ranged from 1 to 50 µg/mL) concentrations of Levidor™ extract. The study revealed slight impact of Levidor™ extract on *COX-1* expression when compared to *COX-2*. The down-regulation of *COX-1* was observed only at selected concentrations. Levidor™ extract tended to decrease *COX-2* expression (Fig. 2B) at all tested concentrations and time points, however the statistically significant drop in expression was observed for all tested concentrations after 4, 6, and 16 h following treatment with extract.

3.4. Detection of COX-1 and COX-2 protein levels

Subsequent to gene expression analysis, the determination of COX-1 and COX-2 protein level was performed (Fig. 3). The non- and differentiated U937 cells were treated with tested extract at concentrations ranging from 1 to 50 µg/mL. Western blot examination provided the confirmation of hypothesis that Levidor™ does not affect COX-1, while abundance of COX-2 protein decreases in a concentration dependent manner. Importantly, COX-1 level was not affected both in monocytes and macrophages. In turn, COX-2 level determined in macrophages treated with 50 µg/ml reduced protein level in comparison to negative control level. The concentrations 10 and 25 µg/ml of Levidor™ caused a similar effect on COX-2 as non-steroidal anti-inflammatory drug, namely diclofenac at 100 µg/mL. The lowest concentrations of the Levidor™ extract did not impact COX-2 protein level notably.

The two forms of COX-1 exist—glycosylated with a molecular weight of 70 kDa and deglycosylated with a molecular weight of 65 kDa. For this reason, two protein bands have been observed. The three protein bands for COX-2 derived from fully N-glycosylated COX-2 with a molecular weight of 72 and 74 kDa and the aglycosylated form (70 kDa) [29].

Immunofluorescence staining confirmed that Levidor™ extract did not cause drop in COX-1 protein level (Fig. 4). Conversely, it was observed that the extract potentiated fluorescence derived from COX-1. This effect was noted especially at 25 µg/mL. In the case of COX-2, the protein level was significantly decreased when compared to positive control. Such reduction exhibited dose dependent character similarly as it was observed in Western blot analysis.

4. Discussion

Over the last few decades, NSAIDs have been the drugs of choice for treating numerous inflammatory diseases. Unfortunately, the prolonged use of NSAIDs has been associated with severe and even life-threatening side effects. NSAIDs are inhibitors of cyclooxygenase enzymes (COX), which catalyze the conversion of arachidonic acid to inflammatory PGs. Unfortunately, their

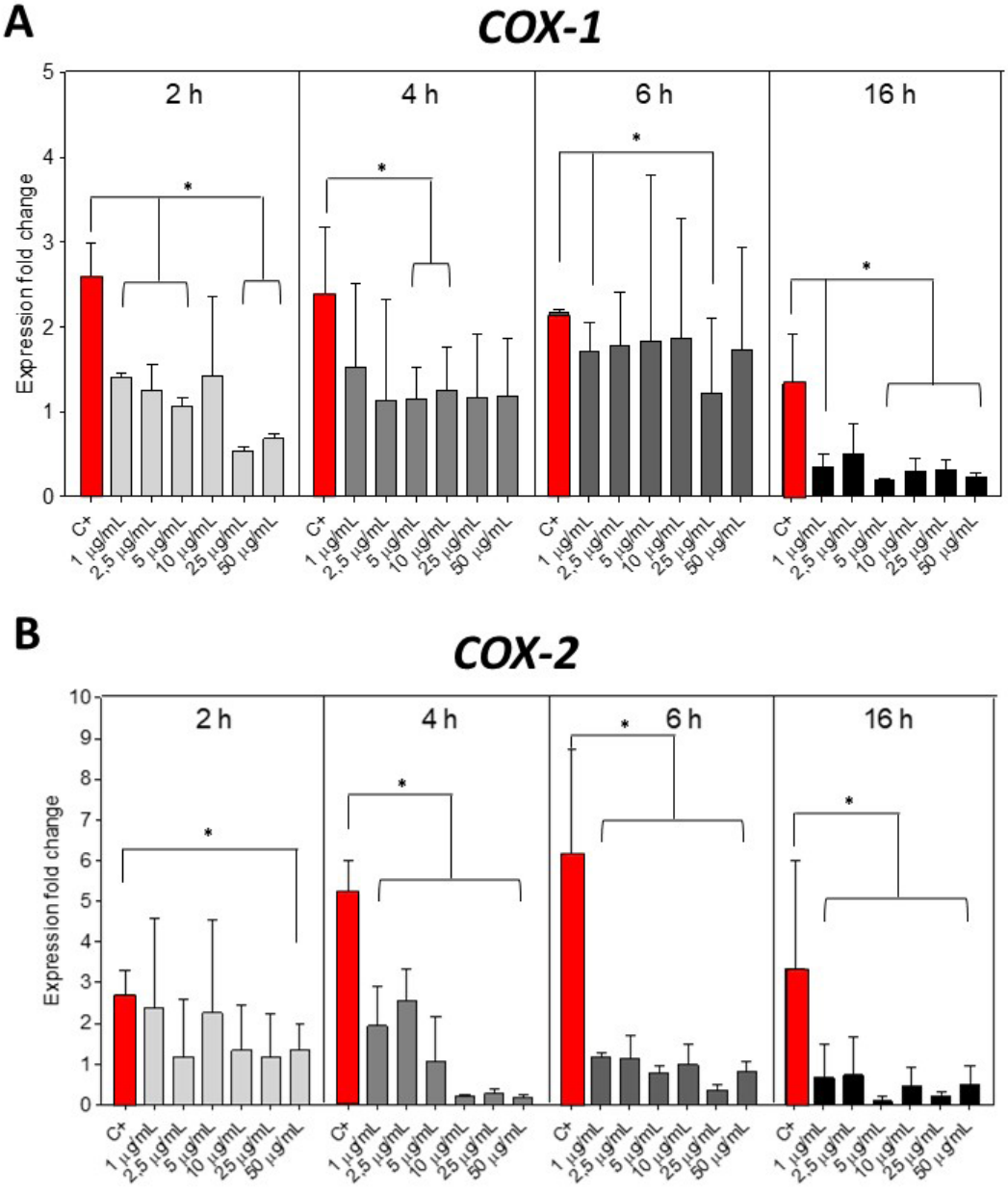


Fig. 2. Changes in COX-1 (A) and COX-2 (B) gene expression in the U937 cells after 48 h pre-treatment with PMA (10 ng/mL), followed by 40 h of incubation without treatment, 4 h treatment with 1, 2,5, 5, 10, 25, and 50 µg/mL of Levidor™ as well as LPS addition (1 µg/mL) for 2, 4, 6, and 16 h. Results are presented as fold changes in the expression of investigated genes. Negative control (C-) refers to cells treated with PMA, while positive control (C+) relates to cells treated also with inflammatory inducer. Significantly different values are marked as (*) $p \leq 0.05$. The results are calculated based on three independent experiments

use is associated with side effects such as gastrointestinal and renal toxicity due to the inhibition of COX-1. Thus, the need arises to find more specific substances, in which therapeutic anti-inflammatory action results from COX-2 inhibition efficiency with intact COX-1 pathway activity.

Due to the fact that plants are rich source of diverse bioactive compounds, it makes them a potential source of novel compounds with anti-inflammatory activity. Such examples may be *Nigella sativa* and *Angelica archangelica*, which phytocomplexes are considered to exert anti-inflammatory activity. For this reason, the patented composition based on oil extracted from the seeds of *N. sativa* and extract from the root of *A. archangelica* in the form of Levidor™ was the subject of presented research. It was hypothesized that the combination of these two natural

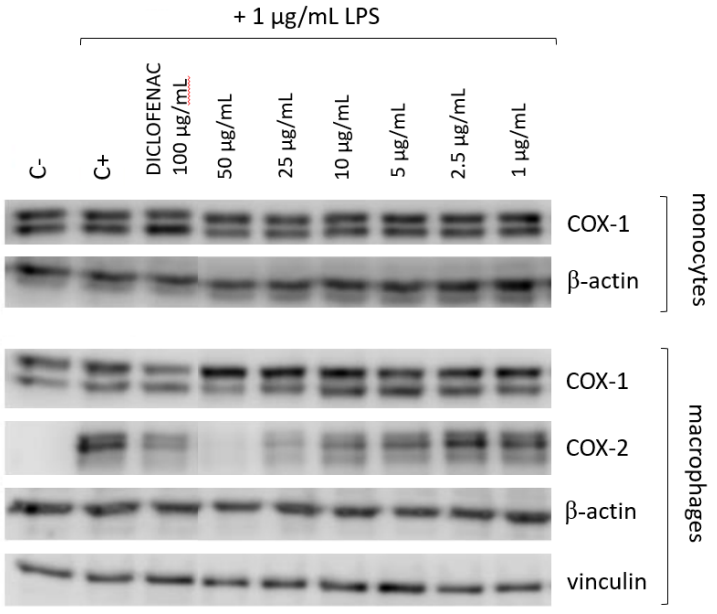


Fig. 3. Western blot analysis of the modulation of COX-1 and COX-2 protein abundance in the U937 after 48 h pre-treatment with PMA (10 ng/mL), followed by 40 h of incubation without treatment, 4 h treatment with 1, 2.5, 5, 10, 25, and 50 µg/mL of Levidor™ as well as LPS addition (1 µg/mL) for 24 h. Negative control (C-) refers to cells treated with PMA, while positive control (C+) relates to cells treated also with inflammatory inducer (LPS)

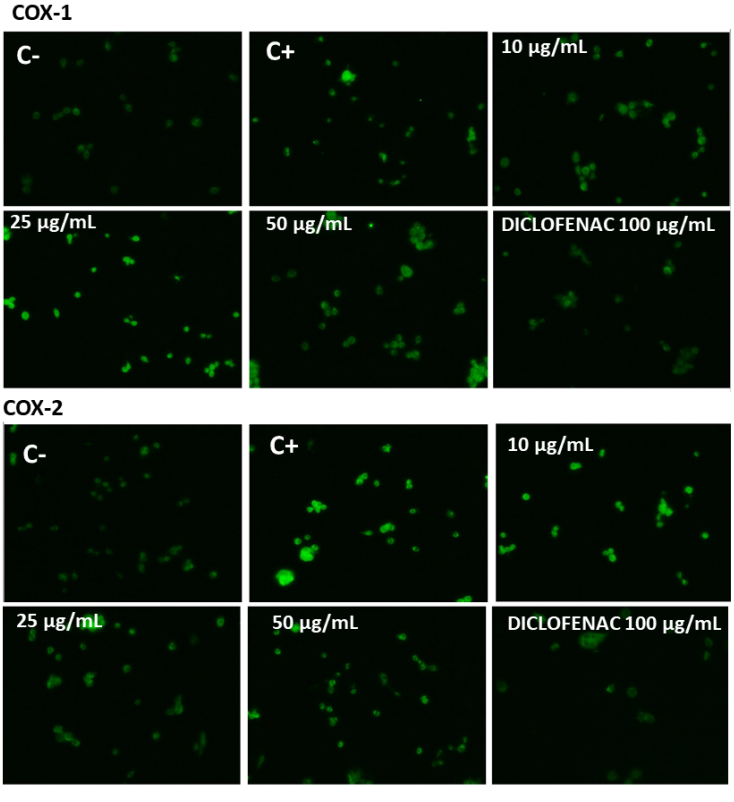


Fig. 4. Visualisation of COX-1 and COX-2 protein in the U937 cells after 24 h pre-treatment with PMA (10 ng/mL), followed by 24 h of incubation without treatment, 4 h treatment with 10, 25, and 50 µg/mL of Levidor™ as well as TNF-α addition (100 ng/mL) for 20 h. Negative control (C-) refers to cells treated with PMA, while positive control (C+) relates to cells treated also with inflammatory inducer (LPS)

phytocomplexes, extracted with optimized technology, will exert potentiated anti-inflammatory effect in relation to the individual phytocomplexes as well as commonly used anti-inflammatory drugs.

The first series of experiments concentrated on evaluation of cytotoxicity of the investigated preparation. The results of a MTT cell viability test performed in two employed cellular models revealed no substantial impact of Levidor™ on cell growth in a broad range of investigated concentrations. These determinations provided the first information about the safety of the new preparation. Another interesting finding was the desirable effect of treatment with Levidor™ on pro-inflammatory cytokine profiles. For instance, TNF-*α* is an important pro-inflammatory cytokine, inducing immune response by activating T cells and macrophages as well as stimulating secretion of other inflammatory cytokines, such as IL-6 and IL-1. In general, increased levels of IL-6 and TNF-*α* are associated with the amplified inflammatory response and also oxidative stress (expressed e.g., in the induction of acute phase proteins production CRP). The macrophages, which filtrate into an inflamed area, release a variety of cytokines including these two beforementioned proteins. Further, IL-6 and TNF-*α* initiate the pro-inflammatory and pro-apoptotic signalling cascades. Among these processes the induction of *COX-2* expression may appear. For this reason, both cytokines concentration (IL-6 and TNF-*α*) as well as gene expression and protein levels of cyclooxygenases (COX-1, COX-2) were investigated in the present study. The determinations carried out within the study scheme showed that Levidor™ exhibited impressive anti-inflammatory effects (Fig. 1). It was able to diminish the level of IL-6 and TNF-*α* at relatively low concentration, namely 25 µg/mL. The observed effect was even stronger than this observed for gold standard of NSAIDs such as diclofenac at 50 µg/mL. Moreover, Levidor™ extract strongly suppressed expression of *COX-2*, whereas it had only slight effect on *COX-1* (Fig. 2). Western blot and immunofluorescence analysis vividly supported the observation made for *COX-1* and *COX-2* gene expression pattern (Fig. 3–4). Here, we demonstrated the profound influence of Levidor™ extract on decreasing the level of COX-2 when compared to NSAIDs. Most importantly however, Levidor™ extract showed selective impact on COXs proteins. It did not significantly affect *COX-1* expression and protein level encoded by this gene. This observation is important as the majority of available anti-inflammatory drugs affect also COX-1 causing severe side effects.

The observed strong anti-inflammatory activity may be related to relatively high concentrations of bioactive compounds from both plants due to the applied extraction technology. Our study is in line with available scientific literature [30,31]. It has been shown that extract of *N. sativa* and *A. archangelica* individually as well as TQ administrated alone inhibited COX-2 expression in a mouse model of OVA-induced allergic airway inflammation [28]. Additionally, *N. sativa* bioactive components down-regulated the expression of pro-inflammatory mediators such as TNF-*α*, inducible nitric oxide synthase, 5-lipooxygenase, cyclin D1, and inhibited IL-6 signalling [30]. It has been even proved that TQ administered alone in diabetic rats at 3 mg/kg or added to cultured macrophages at 10 µM normalized the raised levels of the proinflammatory cytokines IL-1*β* and TNF-*α* [31].

In conclusion, Levidor™ exhibits strong anti-inflammatory properties in *in vitro* models and may provide a significant alternative to traditionally used substances in acute pain and chronic inflammation. Moreover, Levidor™ possesses the ability of selective influence of COX proteins. The next step of the Levidor™ investigation will embrace *in vivo* study and clinical trials in humans to support strong *in vitro* data for this combination of phytocomplexes.

Abbreviations

COX – cyclooxygenase
FBS – fetal bovine serum
IL-1 – interleukin 1
IL-1*β* – interleukin 1*β*
IL-6 – interleukin 6
LPS – lipopolisaccharide
MTT – thiazolyl blue tetrazolium bromide
NSAIDs – non-steroidal inflammatory drugs
PC-12 – cell line derived from a rat pheochromocytoma
PGs – prostaglandins
PMA – phorbol-12-myristate-13-acetate
RAW 264.7 – cell line of murine macrophages
SDS – sodium dodecyl sulfate
THY – thymol
TNF-*α* – tumour necrosis factor *α*
TQ – thymoquinone
U937 – cell line of human monocytes

Authors' contribution

Monika Kuczyńska: Writing - original draft; Writing - review & editing; Visualization. Paulina Kasprzyk: Writing - original draft. Magdalena Leszczyńska-Wiloch: Methodology; Investigation; Formal analysis. Joanna Bidzieńska: Methodology; Investigation; Formal analysis. Marcin Martyniak: Writing - review & editing. Alicja Wilandt: Conceptualization; Data curation; Funding acquisition; Project administration; Supervision. Krzysztof Lemke: Conceptualization; Data curation; Funding acquisition; Project administration; Supervision.

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Funding

This work was supported by EU and The National Centre for Research and Development grant: imNutra POIG.01.04.00-22-183/12 under Operational Programme Innovative Economy, 2007-2013, Priority 1.4.

Conflict of interest

The authors have no potential conflicts of interest to declare.

Ethics approval

Due to the nature of the research, the consent of the ethics committee was not required.

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