In vitro assessment of the cytotoxicity and anti-inflammatory properties of a novel dietary supplement

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Abstract. Studies on the immunopharmacological activities of various plant species have provided evidence for the high therapeutic potential of different extracts. These represent a promising alternative to reduce the inflammatory processes and, thus, diseases related to inflammation. Numerous scientific studies strongly suggest that diet plays an essential role in inflammation, and that certain dietary factors can act as preventive or treatment methods to lower inflammation. In the present study, a novel lingonberry-based dietary supplement was investigated for the ability to suppress the inflammatory response in activated monocytes/macrophages. Based on cell viability/proliferation and cytotoxicity tests, concentrations between 40 and 130 μg/ml of the extracts showed a high viability/proliferation effect and no cytotoxic activity in monocyte/macrophage cells. To further investigate the anti-inflammatory potential of our novel lingonberry-based dietary supplement, we studied the effect of the extract on the inflammatory response in lipopolysaccharide (LPS)-stimulated macrophages. We found that the extract exhibited a strong anti-inflammatory potential by inhibiting the expression of major inflammatory cytokines [interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)α] in activated monocyte/macrophage cells. The expression of IL-6 and IL-8 was subsequently validated by enzyme-linked immunosorbent assay (ELISA). In conclusion, we demonstrated that our product exhibits no cytotoxicity and suppresses inflammation,

and thus can be considered a natural important tool for inflammation control.

Introduction

Inflammation, a self-controlled immune process, occurs in response to different infections and other stimuli, or as a major component in wound and tissue repair. Orchestrated in a complex manner, inflammation can induce a dysregulated response, which underlies a disruption in the homeostasis of other physiological processes, that at first glance do not seem to be directly associated with classical inflammation triggers. However, chronic systemic inflammatory status could be settled, due to an elicited dysregulated inflammatory response (1,2). Inflammation is now considered a hallmark feature in various diseases, including diabetes, asthma, cardiovascular diseases and cancer (3,4).

Emerging evidence suggests that diet could be an important player in the onset and progression of inflammation, with a major impact on the inflammatory disease course.

Dietary components have gained key roles in promoting good health, with multiple mechanisms being proposed to reduce inflammation [e.g., concerning the nuclear factor (NF)-κB pathway] (5). Therefore, various plant-based diets have been suggested to be beneficial in alleviating the inflammatory response.

Studies have discovered that plants contain a wide range of biologically active substances, such as flavonoids, carotenoids, vitamins, minerals and fiber, which provide, individually or in a synergistic manner, important nutritional value and high protection against disease being used as a defense mechanism against environmental stress and pathogens. Among the great variety of biological functions, the anti-inflammatory properties are distinguished as being particularly important; inflammation standing out as a well-known protective and survival mechanism (6-10).

Taking into account the deleterious effects of chronic inflammation as a major component of almost all diseases, various fruit-derived products (extracts, nutraceutical supplements, juices) have been studied with an aim to analyze their anti-inflammatory effects (11) and to develop a new generation of therapeutic agents, especially designed for inflammation

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purposes (8). Based on their capability for producing secondary metabolites endowed with curative effects, medicinal plants have real potential in the development of new and potent drugs (12,13).

Widely studied for its anti-inflammatory and health-beneficial properties, lingonberry (*Vaccinium vitis-idaea*) has arisen as a promising dietary component (14-18). St. John's wort (*Hypericum perforatum*) is endowed with anti-inflammatory and antiviral properties, potentially used in inflammatory conditions, including inflammatory bowel disease, diarrhea, and respiratory infection (19-21). *Thymus vulgaris* is rich in phytocompounds with various pharmacological activities, including an anti-inflammatory effect (22,23).

Given the wide range of biological properties attributed to propolis and its complex composition rich in bioactive compounds, various studies have focused on investigating the benefits and pharmacological properties of propolis regarding its antioxidant, antimicrobial, anticancer and anti-inflammatory effects. The mechanisms proposed for propolis extract have highlighted pro-inflammatory cytokine suppression or metabolic reprogramming of lipopolysaccharide (LPS) activity in macrophage cells, underlying its potential immunomodulatory effect (24).

In view of all these aspects, a novel product has emerged, which is a dietary supplement containing powders from lingonberry fruit (*Vaccinium vitis-idaea*), thyme (*Thymus vulgaris*) and Saint John's wort (*Hypericum perforatum*), concentrated propolis tincture, ascorbic acid and volatile oils of thyme and rosemary (*Rosmarinus officinalis*), being rich in phytonutrients and bio-active compounds. Compared to marketed products, our dietary supplement has a complex formula, enriched in concentrated propolis tincture sprayed on the plant blend (known for its antibacterial, antiviral, antifungal, anti-inflammatory, anesthetic, analgesic, antibiotic and regenerator properties), and a combination of thyme and rosemary volatile oils (25).

Given this context, in the present study, we first aimed to establish whether the novel dietary supplement extract has cytotoxicity and, thus, we performed classical biocompatibility tests (LDH and MTS assays). Subsequently, our study focused on evaluating its anti-inflammatory properties, in order to extend the current understanding of the health benefits of the novel dietary supplemental extract.

Materials and methods

Plant collection and processing. Thyme (*Thymus vulgaris*) and Saint John's wort (*Hypericum perforatum*) were harvested from Hofigal's own culture (SC Hofigal Export Import SA, Bucharest). The aerial parts were dried under controlled conditions, at a maximum temperature of 40˚C, and then they were powdered in an industrial mill.

The lingonberry (*Vaccinium vitis-idaea*) fruits were purchased from a local supplier and picked from the spontaneous flora. They also were dried under controlled conditions, at a maximum temperature of 40˚C, and then were powdered in an industrial mill. The volatile oils of thyme and rosemary were obtained by steam distillation in Hofigal's own industrial plant from fresh plants, harvested from its own culture.

The concentrated propolis tincture was obtained by a 7-day simple maceration of a previously purified propolis, purchased from a local supplier, with 96% pharmaceutical ethylic alcohol, followed by a concentration to a dry mass of 27%.

Obtaining and characterizing the dietary supplement Sample preparation. For the determination of total polyphenol content (TPC), the total flavonoid content (TFC) and antioxidant activity, 1 g of the powdered samples was extracted with 50 ml of 50% (v/v) ethanol under a reflux condenser for 30 min.

Total polyphenol content (TPC). TPC was measured utilizing the Folin-Ciocalteu's method, as described in ISO 14502-1:2005(E) (26). Sample extract (1 ml) was mixed with 5 ml of 10% Folin-Ciocalteu's phenol reagent and allowed to stay for 3 to 5 min at room temperature. After incubation, 4 ml of 7.5% Na_2CO_3 was added, and the reaction mixture was mixed thoroughly and allowed to stay for 1 h at room temperature. The absorbance was measured at 765 nm against water, with a Jasco V-530UV-VIS spectrophotometer and total phenolic content was calculated using a gallic acid standard calibration curve with concentrations ranging from 1 to 5 μg/ml and expressed as gallic acid equivalent per gram.

Total flavonoid content (TFC). TFC was measured using the aluminium chloride colorimetric method as described in the Romanian Pharmacopoeia (27). Sample extract (10 ml) was diluted with methanol in a 25 ml volumetric flask; 5 ml of the diluted extract was mixed with 5 ml of 100 g/l sodium acetate, 3 ml of 25 g/l aluminium chloride hexahydrate and 12 ml methanol. The reaction mixture was mixed thoroughly and incubated for 30 min at room temperature. The absorbance was measured at 430 nm against a blank prepared with 5 ml diluted extract and 20 ml methanol, with a Jasco V-530 UV-VIS spectrophotometer, and TFC was calculated using a rutin standard calibration curve with concentrations ranging from 20 to 90 μg/ml and expressed as rutin equivalent per gram.

Antioxidant activity. The antioxidant activity was measured using the CUPRAC assay, as described by Apak *et al* (28). One milliliter of 10^{-2} M copper sulphate was mixed with 1 ml of 7.5×10^{-3} M neocuproine, 1 ml of 1 M ammonium acetate buffer with pH 7.0, 0.1 ml sample extract and 1 ml of water. The reaction mixture was mixed and incubated for 30 min at room temperature. The absorbance was measured at 450 nm against a blank prepared with water instead of sample extract, with a Jasco V-530 UV-VIS spectrophotometer, and the antioxidant activity was calculated using a trolox standard calibration curve with concentration ranging from 10 to 60 μg/ml and expressed as trolox equivalent per gram.

Procyanidin content. The procyanidin content was determined using the acid hydrolysis described in the European Pharmacopoeia (29). Powdered sample (1 g) was extracted with 30 ml of 70% (v/v) ethanol under a reflux condenser for 30 min and filtered. The residue was washed with 10.0 ml of 70% (v/v) ethanol; 15.0 ml of 250 g/l hydrochloric acid was added to the filtrate and 10.0 ml of water, then it was heated under a reflux condenser for 80 min. After filtration and

washing, the solution was diluted to 250 ml. Fifty milliliters were evaporated to approximately 3 ml and transferred to a separating funnel with 15 ml water. After extracting the water phase with butanol (three times with 15 ml), the combined organic phases were diluted to 100 ml. The absorbance of the solution was measured at 555 nm against butanol, with a Jasco V-530UV-VIS spectrophotometer and the procyanidin content was calculated as cyanidin chloride with the equation [A=absorbance at 555 nm; m=mass of sample (g)]: Content of procyanidins (mg/g)=(A x 500)/(1,200 x m).

Polysaccharides/mucilage determination. The mucilage content was measured using the method described by Deshmukh *et al* (30). Sample (1 g) was mixed with 50 ml of distilled water and boiled for 1 h at 100°C. The suspension was filtered. An equal volume of ethanol was added to the filtrate and the mixture was kept in a refrigerator for 24 h. The obtained precipitate was separated by filtration through a quantitative filter paper and dried in the oven at 50°C. The content of mucilage was calculated as the ratio between the dried precipitate and the mass of sample (30).

Cell line culture and treatment. The monocyte/macrophage cell line, originally derived from peripheral blood, was purchased from the American Type Culture Collection (ATCC 9855; ATCC/LGC Standards GmbH). As key regulators of the immune response, these type of cells are often used to predict *in vitro* cytotoxicity and, later on, the anti-inflammatory response.

The sample and control preparation was performed in accordance with the international quality standards ISO 10993-12: 2012 'Biological evaluation of medical devices-Part 12: Sample preparation and reference materials' and ISO 10993-5: 2009 'Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity' (31,32).

The cell cultures were grown in 25 cm^2 flasks in complete Iscove's modified Dulbecco's media (IMDM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% antibiotic-antimycotic mix solution (A5955; Sigma-Aldrich; Merck KGaA) and 10% fetal bovine serum (FBS) (F7524, non-USA origin, Sigma-Aldrich; Merck KGaA), 1% HT Supplement 0.1 mM hypoxanthine and 0.016 mM thymidine (Gibco; Thermo Fisher Scientific, Inc.), 0.1% β-mercaptoetanol (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere (95%) with 5% CO_2.

In order to assess the biocompatibility effect (cell viability/cytotoxicity) induced in monocyte/macrophage cells by the novel dietary supplement extract, the cells were seeded in 96-well plates at a density of 15x10^3 cells/well. Then, the novel dietary supplement extract was diluted in cell culture medium (4,000, 1,300, 400, 130 and 40 µg/ml from stock: 4 g sample/100 ml solvent ethanol for pharmaceutical use 50%), and incubated with the cells for 24, 48 and 72 h. The cell culture assays were performed in triplicates and subsequently, an additional test was performed with an extensive number of dilutions in order to confirm the obtained results.

To evaluate the anti-inflammatory effect induced in monocytes/macrophages by the novel dietary supplement extract, the cells were seeded in 24-well plates at a density of 1x10^5 cells/well, pretreated with LPS 50 ng/ml for 1 h, and then incubated with diluted extract in cell culture medium (40, 66 and 130 µg/ml; selected based on cytotoxicity tests) for 4 and 18 h. In order to evaluate the anti-inflammatory effects of the extract, we also used a positive control, 50 ng/ml LPS (L4391; Sigma-Aldrich; Merck KGaA), a negative control of inflammation (40 ng/ml dexamethasone sodium phosphate; E.I.P.I. Co.) and a mix of 50 ng/ml LPS and 40 ng/ml dexamethasone. Cell culture supernatants were collected and stored at -80°C until analysis.

Cell viability. Cell viability was measured after cell exposure to the novel dietary supplement extract by MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymet hoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] spectrophotometric test. Briefly, after the cells were seeded in 96-well plates at a density of 15x10^3 cells/well/200 µl, different dilutions of extract were added to the culture media and incubated for 24, 48 and 72 h. After each time interval, 20 µl of CellTiter 96® AQueous One Solution Reagent was added (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, G3580; Promega Corporation) into each well of the 96-well assay plate containing 100 µl of fresh culture medium. After that, the plates were incubated at 37°C for 3 h in a humidified 5% CO_2 atmosphere. Then, the absorbance of the formazan, produced by MTS reduction in metabolic active cells, was measured at 490 nm using a Microplate Multimode Detector Zenyth 3100 (Anthos Labtec Instruments GmbH). The results were calculated according to the formula: Viability (%)=100 x [Experimental value (OD490)-background average (OD490)]/Mean value of the untreated cells (OD490).

Cytotoxicity assay. The lactate dehydrogenase (LDH) amount released in the culture medium was assessed as a measure of the cell membrane integrity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1780, G1782; Promega Corporation). According to the manufacturer's instructions, lysis solution was used to generate a maximum LDH release control. After a 45-min incubation with lysis solution, the plates were centrifuged for 5 min at 600 rpm; 50 µl of culture supernatants from the same 96-well plates used for MTS test were transferred to a new 96-well flat clear bottom plate and 50 µl of the CytoTox 96® Reagent were added. The plates were incubated at room temperature in darkness, for 30 min. The reaction was stopped by adding 50 µl of Stop Solution, and the absorbance was read at 490 nm using Microplate Multimode Detector Zenyth 3100 (Anthos Labtec Instruments GmbH). The results were calculated according with the formula: Cytotoxicity (%)=100 x [Experimental LDH Release (OD490)-background average (OD490)/Mean value of Maximum LDH Release (OD490).

In vitro anti-inflammatory capacity assessment by xMAP array. Cell culture supernatants were obtained as mentioned above and assessed using HCYTOMAG Milliplex™ MAP 10-plex (Millipore) multiplex magnetic bead-based antibody detection kits according to the manufacturer's protocols. Briefly, the beads [interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, fibroblast growth factor (FGF)2, interferon (INF) γ, tumor necrosis factor (TNF)α, vascular endothelial growth factor (VEGF)a] provided within the kits were incubated with

buffer, cytokine standards (included in the kit), or samples in a 96-well plate at 4˚C overnight, with shaking at 800 rpm. All further incubations with detection antibodies and streptavidin phycoerythrin conjugate (SAPE) were performed at room temperature in the dark, with 800 rpm shaking. Multiplex data acquisition was achieved using the Luminex200 platform (Luminex Corp.) and the analysis was performed using the xPONENT 4.2 software (Luminex Corp.); the calibration curves were generated with a 5-parameter logistic fit. Duplicate samples were used for all specimens, and the average concentrations were used for statistical analysis.

Selection and validation of pro-inflammatory cytokines by ELISA. IL-8 and IL-6 were assessed using commercial Legend Max quantitative assays (BioLegend, Inc.). Standards and samples (cell culture supernatants) were added to the plates and incubated at room temperature (RT) for 2 h with 200 rpm shaking. All further incubations with detection antibody, avidin-HRP solution, substrate solution and stop solutions were performed according to the manufacturer's protocols. Samples were analyzed in duplicate, and the absorbance was read at 450 and 570 nm, using a Sunrise-Basic Tecan Microplate Reader (Tecan Group Ltd.). The absorbance at 570 nm was subtracted from the absorbance at 450 nm. The data were calculated with computer-based curve-fitting software (Magellan) using a 5-parameter logistics curve-fitting algorithm. IL-8 and IL-6 are expressed in picograms (pg) per ml of sample.

Statistical analysis. All tests were performed in triplicate, and the data are shown as mean ± standard deviation (SD). The statistical Student's t-test was performed for biological tests to analyze significant differences when comparing treated cells with the controls. The data were processed by one-way analysis of variance (ANOVA) test using Graph Pad Prism software (version 5; GraphPad Software, Inc.). The levels of statistical significance were considered at $P<0.05$.

Results and Discussion

Dietary supplement composition. The main bio-active compounds determined and quantified in the novel product are documented in Table I.

High cellular viability for 40-130 µg/ml concentrations of the dietary supplement. Our novel dietary supplement extract requires the simultaneous achievement of two conditions: Higher cell viability and low cytotoxicity.

In order to assess the cellular viability of the dietary supplement (DS) extract doses ranging from 40 to 1,300 µg/ml, we treated monocyte/macrophage cells for 24, 48 and 72 h. Untreated cells were used as maximum cell viability control, while the cells treated with lysis solution were used as minimum cell viability control; cells treated with different concentrations of ethanol were also used as extract vehicle controls.

At the highest dose of the novel dietary supplement extract (1,300 µg/ml), cell viability was decreased after 48 and 72 h exposure from 64 to 60%, the same trend being observed at the concentration of 400 µg/ml (Fig. 1). After the exposure

Table I. Bioactive compounds in the novel dietary supplement.

Bio-active compounds	Quantity
Polyphenols	146 mg/g gallic acid equivalents (GAE)
Flavonoids	9.5 mg/g rutin equivalents
Procyanidines	1.5 mg/g cyanidin chloride equivalents
Polysaccharides	105 mg/g expressed as mucilage
Vitamin C	90 mg/g
Trolox equivalent	326.5 mg/g

of monocytes to 40 and 130 µg/ml dietary supplement extract for 24, 48 and 72 h, it was observed that the cell viability was not significantly modified, with a mean of 87.3 and 94.6% respectively, compared to the control, suggesting high biocompatibility for these doses.

We also tested the extract vehicle [ethanol (EtOH)] and observed that the cell viability in this case was affected at the higher concentration 1,300 µg/ml (70%), but not at other concentrations, such as 400 (85%), 130 (95%) and 40 µg/ml (99%).

Low cytotoxicity registered at 40-130 µg/ml concentrations of dietary supplement. In order to evaluate the possible cytotoxic effects induced by the novel dietary supplement extract on monocytes, the cell membrane integrity was evaluated at 24, 48 and 72 h, by measuring the level of LDH released in the cell culture medium. Under normal conditions, the LDH enzyme is located in the cytosol, but when the cell membrane integrity is affected, LDH released into the cell culture medium is assessed as a marker for membrane integrity (33).

The incubation of monocyte/macrophage cells with our novel dietary supplement extract induced the release of LDH in the cell culture medium (Fig. 2). Lysis solution was added as positive control wells, generating a maximum LDH release. A higher released LDH level was found in the supernatants of the monocyte/macrophage cells exposed to the high concentrations of our extract (400 and 1,300 µg/ml; LDH mean of 19 and 20%, respectively), than from cells treated with 40-130 µg/ml. A lower LDH level was released (11 and 10%), after incubation of cells with low concentrations of the dietary supplement: 130 and 40 µg/ml, respectively, compared to the positive control. In conclusion, the cell membrane integrity was unaffected at concentrations of 40-130 µg/ml, this result confirming the high biocompatibility of the novel dietary supplement extract.

In addition, when we tested the extract vehicle (EtOH), we noted that the cytotoxicity was higher at 400-1,300 µg/ml (19%). However, at 40 µg/ml (12%) and 130 µg/ml (11%) concentrations, no cytotoxicity was recorded.

For the first 24 h of treatment, a correlation between the concentrations of the bioproduct and the LDH release was noted. The lowest two concentrations (40-130 µg/ml) had similar LDH release, and similar to their corresponding vehicle-treated controls, suggesting a non-toxic effect on cell membrane permeability.
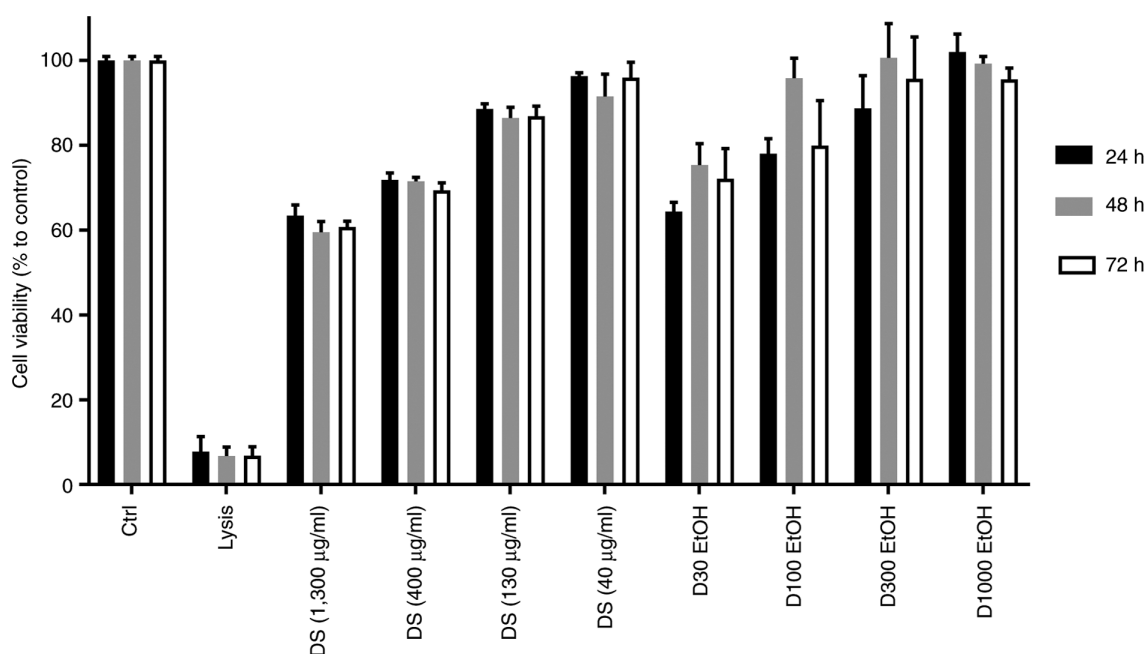
Figure 1. Cell viability of monocyte/macrophage cells after 24, 48 and 72 h of exposure to different doses (40-1,300 μg/ml) of novel dietary supplement extract (DS) and corresponding dilution of ethanol (EtOH)/vehicle. Data are expressed as the mean ± SD (n=3) and are represented as percentages of the untreated control (100% viability). For the same experimental situation, each bar represents a different time point (24/48/72 h).
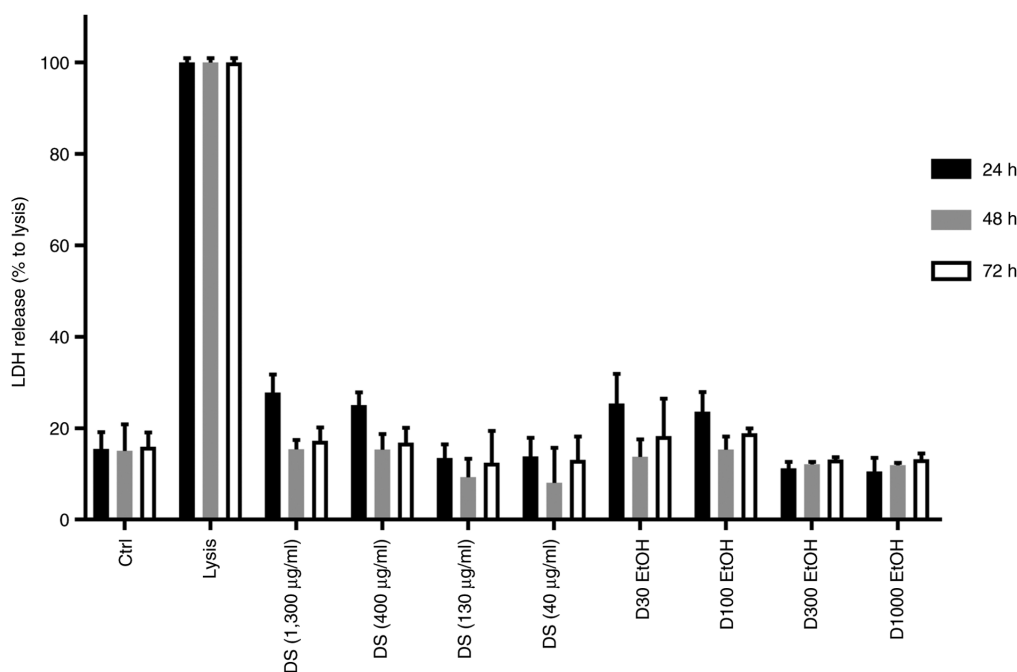

Figure 2. Level of lactate dehydrogenase (LDH) released from monocytes after 24, 48 and 72 h of exposure to different doses (40-1,300 μg/ml) of the novel dietary supplement extract (DS) and corresponding dilution of ethanol(EtOH)/vehicle. Data are expressed as the mean ± SD (n=3) and are represented as percentages of the positive control (100% cytotoxicity). For the same experimental situation, each bar represents a different time point (24/48/72 h).

These data were in accordance with the results of the MTS test and suggest that at 40 and 130 μg/ml concentrations, the novel dietary supplement extract is safe to use for further testing.

Inhibition of pro-inflammatory cytokine (IL-6, IL-8, TNFα) release by the novel dietary supplement extract. In response to various stimuli, such as LPS, cytokines or other chemical stimuli, macrophages exert an important role in the inflammatory response, mediating the secretion of a cascade of cytokines/chemokines, reactive oxygen/nitrogen species and growth factors (34,35).

Based on viability and cytotoxicity tests, our study continued with the inflammatory profile of the dietary supplement-treated cells, at concentrations ranging between 40 and 130 μg/ml, using xMAP array multiplexing technology.
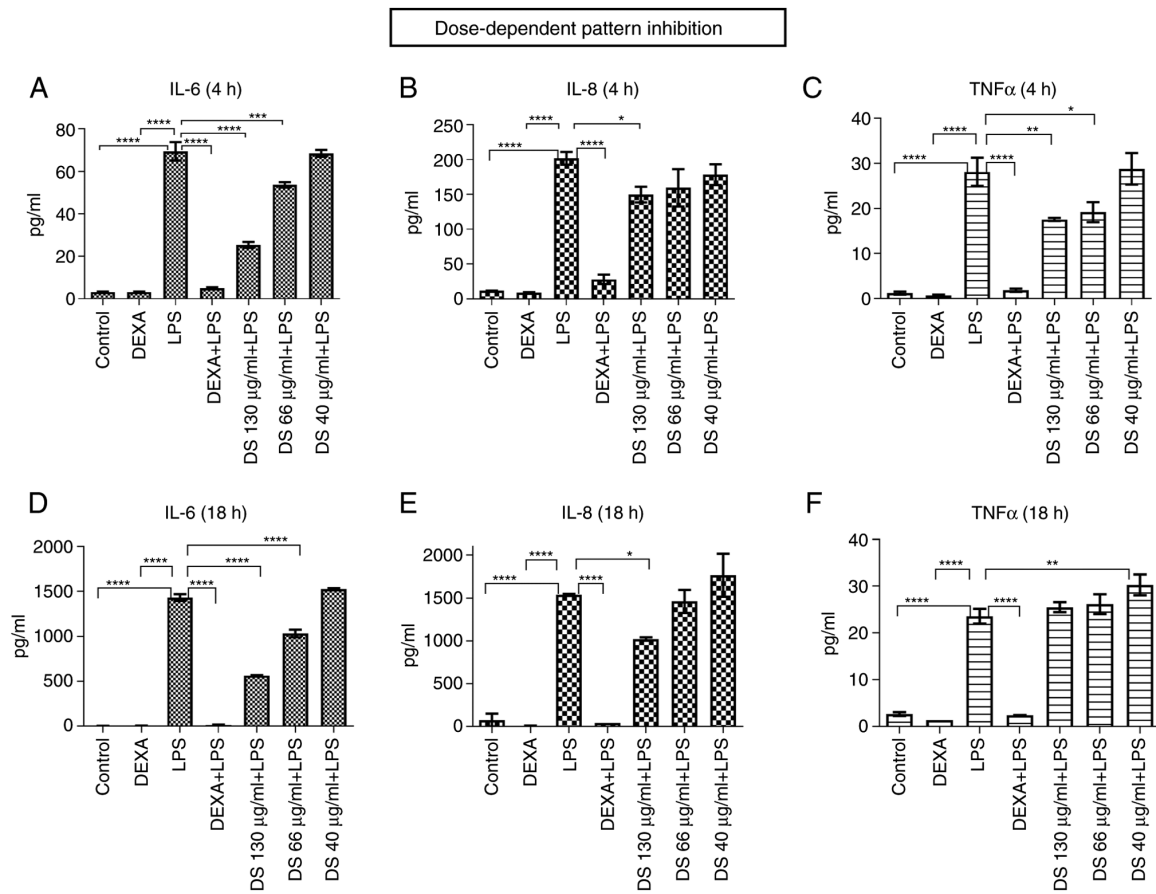
Figure 3. The dose-dependent inhibition pattern on pro-inflammatory cytokines (IL-6, IL-8, TNFα) released in the cell culture supernatant, following treatment with the dietary supplement (DS), at different concentrations (A-F). (A) IL-6, (B) IL-8 and (C) TNFα at 4 h of treatment; (D) IL-6, (E) IL-8 and (F) TNFα at 18 h of treatment. Bars represent average of duplicates ± SD. IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DEXA, dexamethasone. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Since the initial treatment of the monocytes using the dietary supplement did not generate an inflammatory response, we treated the cells with LPS, and afterwards applied the treatment with the dietary supplement at different concentrations. Following LPS stimulation, the anti-inflammatory response to our dietary supplement was also analyzed in comparison with dexamethasone and lingonberry extract (marketed products), widely used as anti-inflammatory treatments (in clinical practice). Due to the fact that our dietary supplement has a complex formula, consisting mainly of lingonberry (50% of all constituents), it was interesting to investigate the anti-inflammatory effect of lingonberry and the possible potentiation of this effect through its additional components.

By using Luminex xMAP array multiplexing technology, the response to dietary supplement treatment was investigated by analyzing a 10-plex inflammatory cytokine panel. Dietary supplement treatment demonstrated a relevant dose-dependent inhibitory pattern for the most important pro-inflammatory cytokines: IL-6, IL-8, TNFα, at 4 and 18 h of treatment (Fig. 3).

The trend registered in cytokine level following treatment with the new dietary supplement could suggest its potential anti-inflammatory role. Given the significant amount of flavonoids contained in our dietary supplement (derived from lingonberry, thyme, St. John's wort and propolis tincture), its natural anti-inflammatory effect may be facilitated by the potential of flavonoids for inhibiting enzymes or transcription factors, important for mediating the inflammatory response. Increasing evidence has revealed that polyphenolic compounds, including flavonoids, originating from fruits and vegetables may have anti-inflammatory properties and the potential to hinder the onset and development of inflammatory diseases (36,37).

As shown in Fig. 4, the inhibition of pro-inflammatory cytokines released into the supernatants of cell samples treated with dietary supplement (DS)+LPS is depicted, compared to LPS only. After 4 h of treatment, the fold-change decrease was 0.36 for IL-6 (P=0.005), 0.74 for IL-8 (P=0.03), and 0.62 for TNFα (P=0.04). After 18 h of treatment, the fold-change decrease was 0.39 for IL-6 (P=0.0009) and 0.66 for IL-8 (P=0.0008), while for TNFα there was no significant decrease compared to the control.

In the case of the lingonberry extract, known for its anti-inflammatory properties in the different experimental models (11-15), we observed that a fold-change decrease of 0.925 for IL-6 (P=0.30), 0.825 for TNFα (P=0.10), and a fold-change increase of 1.325 for IL-8 (P=0.01) was registered at 4 h. The fold-change measurements at 18 h revealed that none of the released cytokine levels was below the control level.

Our study revealed that the pro-inflammatory cytokine release following lingonberry treatment was increased in the case of all analyzed concentrations as compared to our dietary
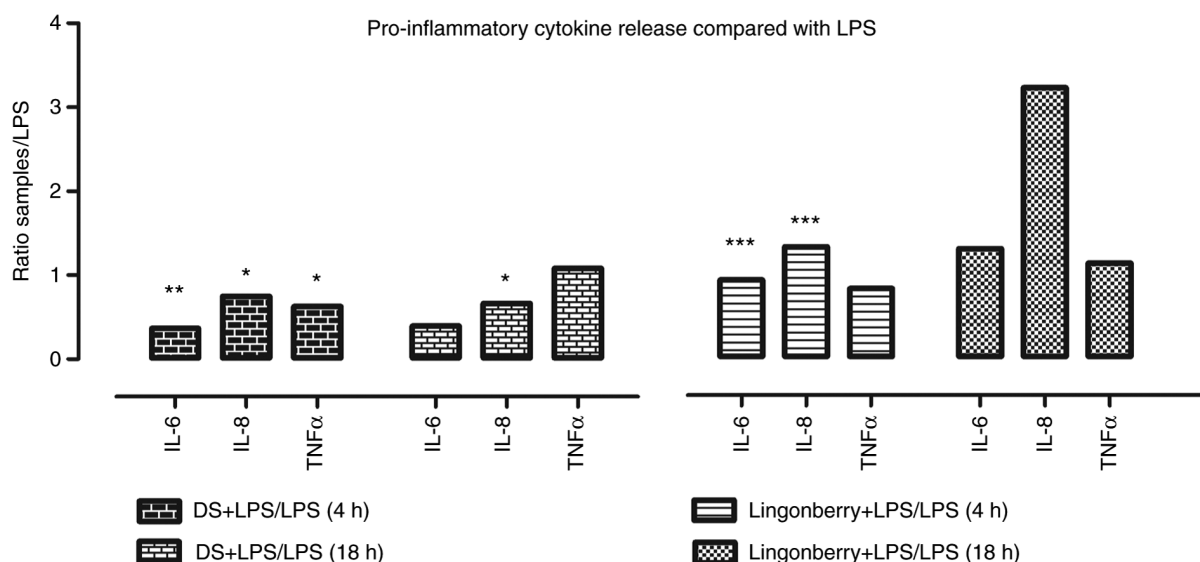
Figure 4. The inhibitory effect of dietary supplement (DS) and lingonberry extract (130 μg/ml concentration) on pro-inflammatory cytokine release, compared with the positive control (LPS). IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide. *P<0.05, **P<0.01, ***P<0.001.
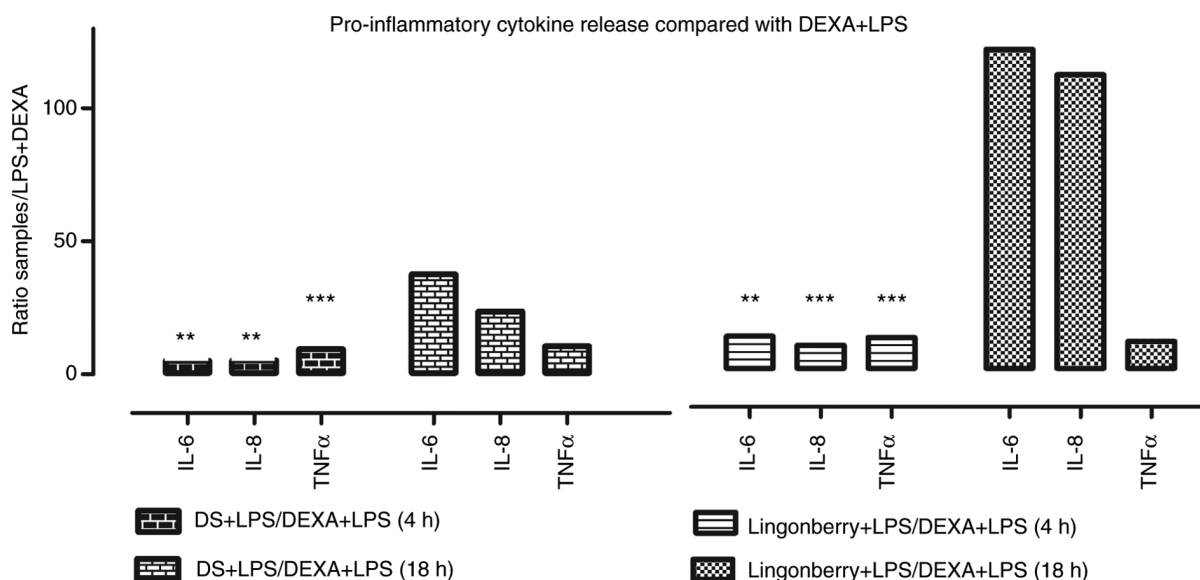

Figure 5. Dietary supplement (DS) and lingonberry inhibitory effect on pro-inflammatory cytokine release (IL-6, IL-8 and TNFα), compared with the effects of DEXA+LPS control. IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DEXA, dexamethasone. **P<0.01, ***P<0.001.

supplement, which in turn developed a more pronounced anti-inflammatory activity.

Fig. 5 presents the pro-inflammatory cytokine profile (IL-6, IL-8 and TNFα) in the supernatants of cells treated with our dietary supplement, lingonberry extract and LPS, compared with those treated with LPS and dexamethasone (DEXA) (anti-inflammatory steroid).

In order to investigate the beneficial anti-inflammatory effect of our dietary supplement, we compared its inflammatory profile with that of a lingonberry-based marketed product, and to dexamethasone, a well-known anti-inflammatory treatment in clinical practice.

When compared to LPS and dexamethasone (negative control), at which the anti-inflammatory effect was maximum, that was registered at 4 h, in the case of the lingonberry

marketed product, a higher fold-change release in IL-6 (12.93) (P=0.003) was noted compared to only 5.09 (P=0.002) in the case of our product. As for the IL-8 fold-change release, we found an increase of 9.47 (P=0.0005) (lingonberry marketed product) vs. 5.34 (P=0.005) (our dietary supplement), and for TNFα the data showed 12.48 (P=0.0003) vs. 9.44 (P=0.0004) fold-change. At 18 h, the anti-inflammatory release for IL-6 was 3.3 times lower in the case of our product compared to the lingonberry marketed product, while for IL-8 it was 4.84 times lower, and 1.04 times lower in the case of TNFα (Fig. 5).

There are various studies in the literature which have focused on the anti-inflammatory effects of lingonberry under different medical conditions. Madduma Hewage et al investigated the role of lingonberry supplementation in the inhibition of inflammatory cytokine expression, which prevents high-fat
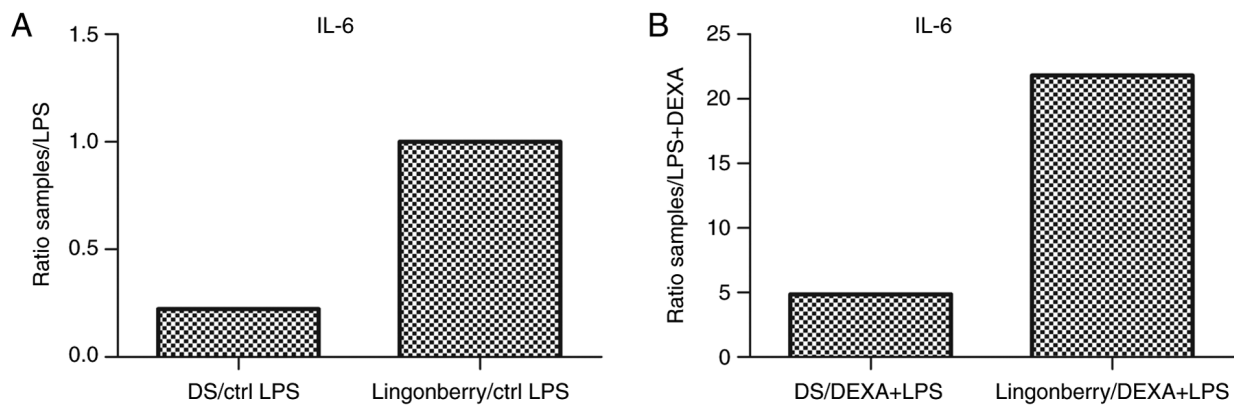
Figure 6. Release of the pro-inflammatory cytokine IL-6 into the cell culture supernatant at 18 h after treatment with dietary supplement and lingonberry, compared to the positive control: LPS (A) or to the negative control: Dexamethasone+LPS (B). IL, interleukin; LPS, lipopolysaccharide; DEXA, dexamethasone.

diet (HFD)-induced kidney injury. They found a significant elevation in renal TNFα, IL-6, and MCP-1 mRNA expression, and a significant elevation in inflammatory cytokines (TNFα and MCP-1) in the plasma of mice fed an HFD. Lingonberry supplementation reduced these inflammatory cytokine levels in the circulation. Several lines of evidence from their study suggest that NF-κB activation may play an important role in HFD-induced renal inflammatory response (38). Kowalska *et al* found that lingonberry fruit extract exhibited a high anti-inflammatory potential in a macrophage (RAW 264.7) cell culture by downregulating the expression of proinflammatory mediators (IL-6, TNFα, IL-1β, MCP-1, COX-2, iNOS) (39). It is worth mentioning that lingonberry (*Vaccinium vitis-idaea*) and cranberry (European *Vaccinium oxycoccos* or North American *Vaccinium macrocarpon*) are both members of the Vaccinium family, and the specific characteristic that contributes the most to their differentiation is their polyphenol and phospholipid contents (40). An interesting study conducted by Kylli *et al* highlighted the beneficial properties of lingonberry phenolics, which inhibited IL-6 and TNFα production at a concentration of 100 µg/ml in murine macrophages and human promonocytes stimulated by LPS (41). The potential health benefits of lingonberries are attributed to their diverse polyphenol composition, such as flavonoids, phenolic acids, anthocyanins and procyanidins, as well as organic acids and different vitamins (A, B1, B2, B3 and C) (42).

As revealed by our study, the treatment of cells with the new dietary supplement following LPS treatment led to a significant inflammatory suppression, which was enhanced as the concentration of the analyzed product increased, with the best-case scenario at 130 µg/ml concentration. Our study showed that the inflammatory suppression occurred at all analyzed concentrations for both lingonberry and our dietary supplement, compared to the control, but in case of the dietary supplement, the anti-inflammatory effect was more pronounced.

Given these observations, we can conclude that the potentiated anti-inflammatory effect may be the result of the complex formula of our dietary supplement, as well as by the addition of propolis, whose effects in reducing the inflammatory response are well known. The main proposed mechanisms underlying the anti-inflammatory effects of

propolis are, among others, the decrease in inflammatory cytokine concentration and immunosuppressive activity (43).

Selection and validation of pro-inflammatory cytokines IL-6 and IL-8. Considering the xMAP analysis results, our study continued with the selection and validation of pro-inflammatory cytokines IL-6, IL-8 and TNFα, which exhibited the most prominent activity. Pro-inflammatory cytokine expression was assessed using cell culture supernatant samples harvested at 4 and 18 h, under the same experimental condition, as used in the xMAP analysis.

As for the TNFα level, its expression was insignificant, so that it should be further validated. TNFα was not detectable by the method chosen for any of the samples tested, including the LPS-treated cells.

Following 18 h of treatment with the dietary supplement (DS), a decrease in IL-6 release was observed [0.22 fold-change decrease compared to the positive control (LPS)], while in the case of lingonberry extract treatment, IL-6 release was 1 fold-change increased compared to the positive control (Fig. 6A). For IL-6, a ratio of 4.87 was observed in the case of the dietary supplement treatment compared to the negative control (DEXA+LPS), and 21.8 in case of lingonberry extract treatment (Fig. 6B). Therefore, the dietary supplement exerted a more distinct inhibitory effect on the release of the pro-inflammatory cytokine IL-6, compared to the lingonberry extract.

As for IL-8, a decreased level in the supernatant at 18 h of treatment with dietary supplement (DS) was observed, with a ratio of 0.96 compared to the positive control (LPS), while for the lingonberry extract the ratio was 1.6. (Fig. 7A). When comparing the amount of IL-8 released in the culture medium to the negative control (DEXA+LPS), a ratio of 16.11 was found in the case of the dietary supplement treatment, and 26.6 in the case of lingonberry extract treatment (Fig. 7B). Consequently, the novel dietary supplement extract recorded a notable inhibitory effect on the release of the pro-inflammatory cytokine IL-8, compared to lingonberry extract.

Regarding the expression levels of IL-6 and IL-8 released at 4 h of treatment, the results recorded were below the detection limit.

Not all studies in the literature have reported a decline in inflammatory marker expression. A study conducted by
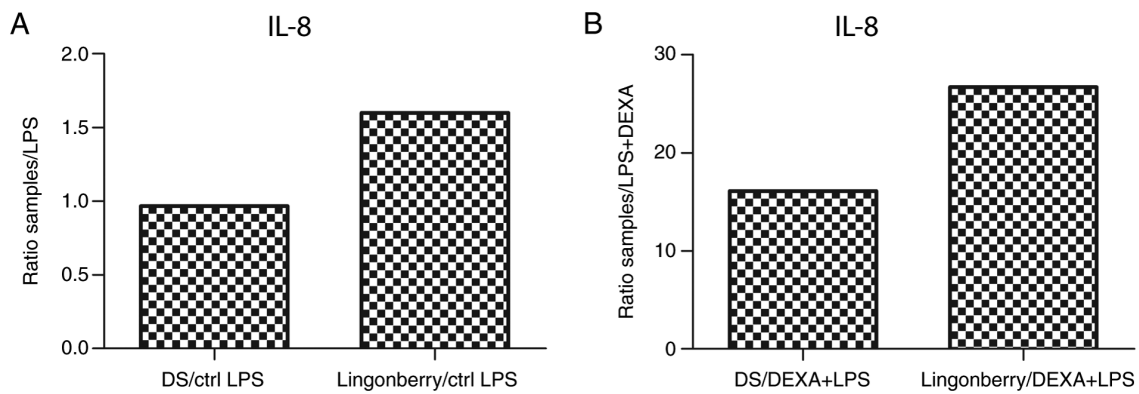
Figure 7. Release of the pro-inflammatory cytokine IL-8 into the cell culture supernatant at 18 h after treatment with dietary supplement and lingonberry, compared to the positive control: LPS (A) or to the negative control: Dexamethasone+LPS (B). IL, interleukin; LPS, lipopolysaccharide; DEXA, dexamethasone.

Zhao *et al* observed a significant decrease following treatment with Brazilian green propolis for TNFα, while for IL-1β and IL-6 a significant increase was recorded. It was hypothesized that propolis, through its components, actively stimulated the release of a cascade of cytokines, including IL-1β, and the subsequent pro-inflammatory action induced by IL-1β release was probably hampered by the anti-inflammatory effects of IL-6. As a result, the combined action of propolis components on the inflammatory process was favorable (44).

Based on the present data, the precise mechanisms underlying the novel lingonberry-based dietary supplement effects are still to be elucidated; therefore, further studies are needed in order to reveal the beneficial effects for each of the active compounds included in our dietary supplement.

To conclude, in the present study, we investigated the potential role as an inflammation suppressor for a novel dietary supplement containing powders from lingonberry fruits, thyme and Saint John's wort, concentrated propolis tincture, ascorbic acid and volatile oils of thyme and rosemary. The results of our biological studies revealed that the treatment with our dietary supplement at concentrations of 40-130 μg/ml recorded a cell viability of over 85% and did not exhibit cytotoxicity. Moreover, the treatment with the novel dietary supplement induced a significant inflammatory suppression in monocyte/macrophage cells, highlighting an inhibition of pro-inflammatory cytokine (IL-6, IL-8) release, at the same concentrations. In addition, the anti-inflammatory response was more prominent for our dietary supplement compared to lingonberry extract (from marketed products), being a potential successful candidate as an inflammation suppressant in various diseases in which the inflammatory component is important. Nevertheless, further studies are needed in order to elucidate the beneficial role of our novel dietary supplement, unravelling potential therapeutic agents of new generation, especially designed for inflammation purposes.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IDP, EC and SM equally contributed to the conception and design of the study, and they were involved in the interpretation of the results and in the writing of the manuscript. CL and MN were involved in the novel dietary supplement description and characterization. CT critically revised the manuscript. All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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