Research Article

Antioxidant and anti-inflammatory properties of active compounds from *Arnica montana* L.

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Abstract

Several plant products are used as antioxidant agents to treat inflammation-related disorders. In recent years, encapsulation of biologic active compounds in lipid vesicles was shown to improve their bioavailability and transdermal delivery. The aim of this study was to prepare two arnica extracts of polyphenols (Pf) and polysaccharides (Pz) and their liposomal formulations (L-Pf, L-Pz) and to characterize their antioxidant and anti-inflammatory effect. Cytocompatible concentrations of Pf (50 µg/ml), Pz (0.5 µg/ml), L-Pf and L-Pz were tested for cytoprotective and anti-inflammatory activity in a model of hydrogen peroxide-stressed fibroblast cell culture by assessment of lactate dehydrogenase activity, cell morphology and pro-inflammatory cytokines production. The results demonstrated that L-Pf and L-Pz protected better the cell membrane against oxidative damage and had significantly (p<0.05) higher anti-inflammatory activity compared to their non-encapsulated forms. All these findings showed that the liposomal formulations of arnica extracts could be useful in topical treatment of inflammatory skin disorders.

Keywords: arnica, liposomes, polyphenols, polysaccharides, cytotoxicity, free radicals, cytokines

Introduction

Inflammation plays an important role in the pathogenesis of severe diseases, such as rheumatoid arthritis, osteoarthritis, asthma, infections, skin disorders, cancer, diabetes, atherosclerosis or accelerated aging (LAVETI & al. [1]). The inflammation process occurs as a response to an infectious agent or tissue injury, recruiting immune cells (monocytes, mast cells, leucocytes) at the wounded site, which along with non-immune cells (fibroblasts, endothelial cells) regulate the production of various pro- and anti-inflammatory mediators, including cytokines (interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), etc), chemokines (CCL2, IL-8, etc), inducible enzymes (cyclooxygenase-2 and nitric oxide synthase) or receptor molecules for cytokines (EMING & al. [2]). In terms of redox disequilibrium, the reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical or singlet oxygen can accumulate at inflamed site and attack...
biological molecules (proteins, DNA) leading to cell and tissue injuries (ALCARAZ & al. [3]).

Several natural products and plant derived formulations are used as antioxidants and inhibitors of pro-inflammatory cytokines to treat inflammatory-related disorders. In recent years, many plant, herb and spice extracts have been screened in cell culture models for their anti-inflammatory activities (KIM & al. [4]). Curcumin, the natural yellow pigment from turmeric (Curcuma longa), capsaicin the active component of chili peppers (Capsicum spp.), or the polyphenolic constituents of green tea, named catechins, have been shown to modulate the NF-κB signaling pathway involved in the inflammation process (MENON & SUDHEER [5]; WANG & al. [6]).

Extracts of arnica (Arnica montana L.) flowers were used, as tincture or ointment, in traditional and homeopathic medicine for topical treatment of skin bruises, irritations, contusions and pain, presenting antiseptic, antiphlogistic, analgesic and anti-inflammatory properties (PUHLMANN & al. [7]). Among the biologic active constituents of arnica extracts, the phenolic acids (e.g., caffeic, chlorogenic) and flavonoids (e.g., quercetin, patuletin) were identified and quantified by micellar capillary chromatography (GANZERA & al. [8]), while a mixture of sesquiterpene lactones (e.g., helenalin, dihydrohelenalin and their esters) was shown to have important biological activity (JAGER & al. [9]). Arnica phenolic acids and flavonoids showed significant antioxidant and antibacterial activities (CRACIUNESCU & al. [10]; IAUK & al. [11]). Polysaccharides (Pz) from arnica had immunological properties that increased phagocytosis in cultured cells (PUHLMANN & al. [7]) and its sesquiterpene lactones exerted anti-inflammatory effects, mainly by preventing nuclear factor NF-κB activation, similarly to corticoid steroids (KLAAS & al. [12]). In its study, JAGER & al. (2009) [9] noticed a better activity of total plant extracts over pure compounds isolated from arnica flowers.

The main disadvantage of orally- and topically-delivered phytocconstituents is their low bioavailability that limits their clinical use. Encapsulation of biological active substances into lipid nanostructures with bilayered structure, such as liposomes, phytosomes, marinosomes, niosomes resulted in efficient dermal and transdermal drug delivery (HONEYWELL-NGUYEN & BOUWSTRA [13]; PIERRE & DOS SANTOS [14]). Liposomes (L) were shown to enhance drug permeation of skin by increasing the fluidity of lipids from stratum corneum, the main barrier to percutaneous absorption (MAESTRELLI & al. [15]).

The aim of this study was to prepare and characterize two arnica extracts of polyphenols (Pf) and Pz and their liposomal formulations useful in the treatment of skin inflammatory disorders. Comparative analysis of their in vitro antioxidant activity was performed both in a cell-free system against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and in a cell culture system by lactate dehydrogenase (LDH) activity and cell morphology assessment. Their cytotoxicity was evaluated in a mouse fibroblast L929 cell culture using Neutral Red (NR) assay and the anti-inflammatory activity was investigated by IL-6, IL-8 and TNF-α pro-inflammatory cytokines production in hydrogen peroxide-stressed cells.

Materials and Methods

Materials

Arnica montana L. was collected from Cluj county, Romania and authenticated by Dr. G. Coldea, Department of Taxonomy and Ecology, Institute of Biological Research Cluj-Napoca, Romania. Voucher specimen of A. montana L. (No. 647621) was deposited at Herb, Botanical Garden Cluj-Napoca, Romania. The arnica flowers were air-dried, at room temperature and then grounded.
Phosphatidylcholine (PC), dioleoyl-phosphatidyl-ethanolamine (DOPE), cholesterol (Chol), stearylamine (SA), hydrogen peroxide (H$_2$O$_2$), Neutral Red, Triton X-100, DPPH, D-glucuronic acid, Minimum Essential Medium (MEM), fetal bovine serum (FBS), L-glutamine and all other chemicals of analytical grade were purchased from Sigma-Aldrich Chemicals (Germany). Polycarbonate membrane filters with 0.2 µm pores were purchased from Avanti Polar Lipids Inc. (USA). Sterile membrane filters with 0.45 µm pores were purchased from Merck Millipore (Germany). A mouse fibroblasts cell line (NCTC clone L929) was purchased from ECACC (Sigma-Aldrich, Germany). LDH cytotoxicity assay kit was purchased from Cayman Chemical Co. (USA). Primary anti-IL-8 and anti-TNF-α antibodies, biotinylated secondary antibody and streptavidin coupled to alkaline phosphatase were purchased from R&D Systems Inc. (USA).

Preparation of phenolic and polysaccharidic extracts from A. montana

**Pf extraction.** Dried flowers of arnica (75 g) were extracted by maceration in ethanol/water (70/30, v/v), in a material/solvent ratio of 1:10 (w/v), on a shaker, at room temperature, for 8 h. The extract was separated from the material residue by filtration through Whatman No. 1 filter paper. The solvent was removed by evaporation *in vacuo*, at 40 °C, using a rotary evaporator (Heidolph VV Micro, Germany) and, finally, the extract was lyophilized in a Christ Gamma 1-16 LSC freeze dryer (Germany). The dry extract was weighed and stored in a sealed container until use.

The total phenolic content and total flavonoid content of Pf extract were determined using Folin-Ciocalteu assay (SINGLETON & al. [16]) and aluminium chloride colorimetric assay (EBRAHIMZADEH & al. [17]), respectively.

**Pz extraction.** Dried minced arnica flowers (10 g) were extracted three times successively, in 50 ml acetone to remove chlorophyll and then, in 50 ml methanol to remove polyphenols, at room temperature. After filtration, the vegetal residue was refluxed with water at 100 °C, for 1h in a Soxhlet apparatus. The procedure was repeated, in order to obtain a crude water-soluble Pz extract that was centrifuged and purified by supernatant precipitation with 75% (v/v) ethanol. The precipitate was dissolved in distilled water, lyophilized in a Christ Gamma 1-16 LSC freeze dryer (Germany), weighed and stored in a sealed container until use.

The amount of total hexoses was determined using the anthrone method (SCOTT & MELVIN [18]) and glucose as standard, while the uronic acid content was analyzed with orcinol reagent using D-glucuronic acid as standard (MOLDOVAN & al. [19]).

The extraction yield for each extract was expressed as percentage from the plant material dry weight. For cell culture experiments, the dry extracts were dissolved in phosphate buffered saline (PBS), pH 7.4.

**DPPH free radical scavenging activity**

The free radical scavenging activity of arnica extracts was determined against the stable radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) using a colorimetric assay, as previously described (HUANG & al. [20]). Briefly, increasing concentrations (0.01-1 mg/mL) of each plant extract were mixed with 0.1 M Tris-HCl buffer, pH 7.4 and then, with 0.25mM DPPH methanolic solution, in a ratio of 1:6:10 (v/v/v). The sample mixture was shaken vigorously and incubated at room temperature, for 20 min, in the dark. The optical density (OD) of the solution was read at an UV-VIS Jasco spectrophotometer (Japan), at 517 nm, against the DPPH methanolic solution (blank). The scavenging percentage was calculated using the following formula:

\[
\% \text{ scavenging} = \frac{\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{blank}}} \times 100
\]

The experiment was conducted in triplicate. The sample concentration that inhibited 50% of DPPH free radicals (IC$_{50}$, mg/mL) was calculated from the graph plotting inhibition
percentage against extract concentration by linear regression analysis. Butylated hydroxytoluene (BHT), an organic compound with potent antioxidant properties, was used as control.

Encapsulation of arnica extracts in liposomes
The liposomal formulations of arnica Pf and Pz extracts were prepared using a modified hydration method (TRIF & al. [21]), followed by sonication and extrusion of lipid vesicles, as previously described (CRACIUNESCU & al. [22]). Briefly, a mixture of phosphatidylcholine, dioleoyl-phosphatidyl-ethanolamine, cholesterol and stearylamine, in 4:2:3:1 molar ratio, was dissolved in a solution of chloroform:methanol (95:5, v/v). The solvent was removed in a Laborota-4000 rotary evaporator (Heidolph, Germany), at 40 °C, for 30 min, to obtain a thin, dry film. Residual organic solvents were removed by nitrogen exposure, to prevent lipid peroxidation and then, the film was hydrated with PBS, pH 7.4, containing arnica extracts, by vigorous shaking, to obtain liposomes entrapping Pf (L-Pf) and Pz (L-Pz), respectively, in the form of multilamellar large vesicles. In order to obtain small unilamellar vesicles, the resulting emulsion was sonicated and then extruded 10 times through 0.2 mm pore size polycarbonate membrane (Avanti Polar Lipids, USA). Before use in cell culture experiments, L-Pf and L-Pz were sterile filtered through 0.45 mm filters. The encapsulation efficiency was determined after liposomes solubilization in Triton X-100, as previously described (FRENCH & al. [23]).

Cell line and culture conditions
Mouse fibroblast cells (NCTC clone L929) were cultivated in Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 500 µg/mL neomycin. For experiments, cells were seeded in 24-well culture plates, at a density of 5x10^4 cells/well and maintained at 37 °C, in a humidified incubator with an atmosphere containing 5% CO2 – 95% air, for 24h, to allow cell adhesion.

Cytotoxicity of arnica extracts
In order to assess the cytotoxicity of arnica extracts, the culture medium in each well was replaced with MEM containing various concentrations of Pf and Pz extracts, in the range of 0.05-500 µg/mL. Also, L-Pf and L-Pz suspensions were added in other wells to give a final concentration of 50 µg/ml Pf and 0.5 µg/ml Pz, respectively, taking into account that the encapsulation efficiency was 68.5% Pf and 62.7% Pz. The culture plates were incubated in standard conditions, for 24 h and the cell viability was assessed by Neutral Red (NR) assay, as previously described with some modifications (FOTAKIS & TIMBRELL [24]). Briefly, the culture medium in each well was replaced with 50 µg/mL NR solution in MEM and the cells were incubated at 37°C, for 3 h. After incubation, the cells were rinsed and then a solution of 1% (v/v) acetic acid in 50% (v/v) ethanol was added. The plates were incubated on a shaker, for 15 min and the OD recorded at 540 nm in a microplate reader (Sunrise Tecan, Austria). Results were expressed as mean of three determinations ± standard deviation (SD). Untreated cells served as control considered as 100% viable cells.

Hydrogen peroxide-stressed cells and treatment with arnica formulations
To examine the effect of arnica extracts rich in Pf and Pz, respectively, and their liposomal formulations on inflamed cells, we used an experimental model of L929 fibroblast cells stressed with H_2O_2, as previously described (CRACIUNESCU & al. [10]). Briefly, cells cultivated at a density of 5x10^4 cells/well were treated with 50 µg/mL Pf and 0.5 µg/mL Pz extracts and the same concentrations of their liposomal formulations, for 24 h. Then, the cells were exposed to 50 µM H_2O_2 and the culture plate was incubated in standard conditions, at
37°C, for 24 h. LDH activity, cell morphology and cytokines production were evaluated. Untreated cells and cells treated with 50 µM H₂O₂, cultivated in the same conditions, were used as controls.

**Determination of LDH activity**
LDH activity, as an indicator of cell membrane damage was measured using CytoTox 96 kit (Promega, UK), according to the manufacturer’s protocol. Briefly, an aliquot of 100 mL culture supernatant was incubated with 100 mL mixed reaction solutions, with gentle shaking, at room temperature, for 30 min. OD was measured at 490 nm using a 96-well plate reader (Sunrise Tecan, Austria). LDH activity (mU/ml) was calculated from the built standard curve and the results were reported as arbitrary units (a.u.) against the untreated control considered equal to 1. Data were expressed as mean of three determinations ± SD.

**Light microscopy**
Cells from the same experiment were examined for their morphology after fixation in methanol and Giemsa staining. Light micrographs of the treated cell culture were acquired at a Zeiss AxioStar Plus microscope equipped with a digital camera driven by AxioVision 4.6 software (Carl Zeiss, Germany).

**Cytokines determination by ELISA**
The culture media of cells pretreated with arnica formulations and stressed with 50 µM H₂O₂ were collected, centrifuged at 400g, for 10 min and the supernatants were stored at -20 °C. In order to assess the level of IL-6, IL-8 and TNF-α pro-inflammatory interleukins, specific and sensible ELISA sandwich assay (R&D Systems, USA) was performed in triplicate, as previously described (LEUNG & al. [25]). Results were expressed as mean ± SD.

**Statistical analysis**
Data were expressed as mean ± SD (n=3). Statistical analysis of the data was performed using the one-tailed paired Student’s test, on each pair of interest. Differences were considered statistically significant at p<0.05.

**Results and Discussions**

**Characterization of arnica extracts**
In our study, two arnica extracts of phenolic and polysaccharidic compounds were prepared using simple and efficient techniques resulting in extraction yields of 18.44% Pf (w/w) and 0.36% Pz (w/w) (Table 1).

| Table 1. Main constituents content, extraction yield and IC₅₀ value of Pf and Pz arnica extracts. Data are expressed as mean ± SD. |
|-------------------------------------------------|-------------------------------------------------|
| **Pf extract** | **Pz extract** |
| Total phenolic content (mg/g dry extract) | 97.16 ± 1.37 | Total hexose content (mg/g dry extract) | 466.64 ± 9.23 |
| Flavonoid content (mg/g dry extract) | 38.62 ± 1.50 | Uronic acid content (mg/g dry extract) | 147.55 ± 3.01 |
| Extraction yield (%) | 18.44 ± 0.83 | Extraction yield (%) | 0.36 ± 0.04 |
| IC₅₀ (mg/mL) | 0.66 ± 0.07 | IC₅₀ (mg/mL) | 1.71 ± 0.31* |

*p<0.05, compared to IC₅₀ of Pf extract
The analyzed Pf extract had a content of total phenolic compounds of 97.16 mg/g dry extract and a content of total flavonoid compounds of 38.62 mg/g dry extract (Table 1). In our previous study, HPLC analysis of arnica Pf extract identified 10 main phenolic and flavonoid compounds and quantified high levels of quercetin (1.881 mg/g dry extract), rutin (1.186 mg/g dry extract), apigenin (0.501 mg/g dry extract) and chlorogenic acid (0.329 mg/g dry extract) (CRACIUNESCU & al. [10]). The water-soluble Pz extract was characterized in terms of total sugar content (466.64 mg/g dry extract) and total uronic acids content (147.55 mg/g dry extract) (Table 1).

**DPPH radical scavenging activity**

The stable DPPH free radical has been extensively used to evaluate reducing substances. In our study, the scavenging activity of DPPH radical was investigated for different concentrations of arnica Pf and Pz extracts in the range of 0.1-1 mg/ml. The inhibition versus concentration curves are shown in Fig. 1.

It was found that inhibition of DPPH radical increased in a dose-dependent manner (Fig. 1). The DPPH free radical scavenging capacity of Pf extract, calculated as IC$_{50}$, was 0.66 ± 0.07 mg/ml, while the IC$_{50}$ value of Pz extract was 1.71 ± 0.31 mg/ml (Table 1). The antioxidant capacity of arnica extracts was compared to that of BHT (0.28 ± 0.05 mg/ml), a strong synthetic antioxidant agent. A small value of IC$_{50}$ indicates a high antioxidant activity. Arnica Pf extract had an IC$_{50}$ value below 1 mg/ml, representing a significant activity towards free radicals scavenging, which correlated with its high content in polyphenols. The value calculated for Pz extract was significantly (p<0.05) higher compared to that of Pf extract and BHT, showing a lower antioxidant activity.

Previous studies reported that both Pf and Pz extracts from algae exhibited significant, dose-dependent antioxidant capacity towards DPPH radical (MAHENDRAN & SARAVANAN [26]). For several traditional medicinal plants it was shown a linear relationship between the antioxidant capacity value and their phenolic and flavonoid content (MOLDOVAN & al. [27]).
Evaluation of *in vitro* cytotoxicity of arnica extracts

The cells from mouse fibroblast L929 cell line were exposed to increasing concentrations of Pf and Pz extracts, L-Pf and L-Pz, for 24 h and their viability was assessed using the vital stain NR. The results are presented in Fig. 2.

**Fig. 2.** Cell viability of L929 fibroblast cells cultured with increasing concentrations of arnica Pf and Pz extracts, L-Pf and L-Pz, evaluated by NR assay. Data are presented as mean ± SD (n=3). *p<0.05 compared to control cells

Data showed that the cytotoxicity of Pf and Pz arnica extracts was dependent on their concentration. It was observed that the percentage of cell viability was above 80% (non-cytotoxic) within 0.05-50 μg/ml Pf and 0.05-0.5 μg/ml Pz ranges of concentrations. Higher concentrations were cytotoxic and caused a significant (p<0.05) decrease of the cell viability to 21.5%. From the non-cytotoxic concentrations, 50 μg/ml Pf extract and 0.5 μg/ml Pz extract determined the highest values of cell viability and were also tested as liposomal formulations. L-Pf and L-Pz were non-cytotoxic, presenting cell viability values close to that of the untreated control (100%).

The extensively used MTT colorimetric assay led to false results when tested in our experiments, due to tetrazolium dye interaction with phenolics from arnica extracts and reduction to formazan in the absence of cells, as previously reported for *H. perforatum* and *C. racemosa* extracts (BRUGGISER & al. [28]). For this reason, we used NR uptake assay to quantify the number of viable cells after cultivation with arnica extracts. Previous studies of WOERDENBAG & al. [29] showed moderate to low cytotoxicity of most flavonoids present in arnica species and a strong cytotoxicity of the sesquiterpene lactone helenalin in GLC4 cell lung carcinoma cell line. Due to this toxic compound, arnica preparations are usually restricted to topical applications for treatment of skin lesions, eczema, oedema, or tendon/joint inflammation; if taken internally, it could cause serious side effects (KLAAS & al. [12]; AULD & al. [30]). No reports on cytotoxicity of arnica Pz extracts were found.
Cell membrane protection against peroxide-induced oxidative stress

A cell culture model using H₂O₂ as damaging agent induced a variety of processes and biological effects, similar to an *in vivo* oxidative stress environment (GUTIERREZ-VENEGAS & al. [31]). In our study, Pf and Pz arnica extracts and their liposomal formulations were comparatively tested in a model developed in L929 fibroblast cell culture, in order to evaluate their cytoprotective effect. Cells pre-treated with arnica extract formulations were exposed to H₂O₂ treatment and investigated for cell membrane integrity by LDH activity measurement in the culture medium. The results showed that all arnica formulations were able to reduce the induced oxidative damage, resulting in significantly (p<0.05) decreased values of LDH in comparison with H₂O₂-treated cells (9.55 a.u.) (Fig. 3).

The cells treated with L-Pf, L-Pz and Pf extract secreted small quantities of LDH into the culture medium (<4 a.u.), while cells treated with Pz extract presented a significantly increased (p<0.05) value of LDH (6.18 a.u.), compared to the control sample (1 a.u.) (Fig. 3). Comparing the liposome-entrapped extracts to non-encapsulated ones, it was noticed a significantly (p<0.05) better cell membrane protection against oxidative stress provided by L-Pf and L-Pz than by Pf and Pz, respectively. The improvement of this activity was more visible for Pz liposomal formulation.

![Fig. 3. Modulation of LDH activity secreted by L929 fibroblast cells cultivated in the presence of arnica extract formulations. Data are presented as mean ± SD (n=3). *p<0.05 compared to hydrogen peroxide-treated cells; #p<0.05 compared to L-Pf-treated cells; §p<0.05 compared to L-Pz-treated cells.](image)

In certain conditions, H₂O₂ is a powerful oxidant resulting in production of ROS and other cytotoxic agents that damage an inflamed site (BARAN & al. [32]). Previous studies showed that crude extracts of *A. montana* and *P. vulgaris* protected fibroblast cells against H₂O₂-induced oxidative stress (CRACIUNESCU & al. [10]; ZDARIOVA & al. [33]).

A recently growing opinion is that encapsulation of antioxidant compounds into liposomal systems allows a rapid intracellular delivery and direct interaction with ROS involved in several pathological conditions, improving their therapeutic potential (SUNTRES [34]). It was reported a better control of cell growth and metabolic activity of H₂O₂-stressed fibroblasts when cultivated with liposomes entrapping chondroitin sulfate than with free drug.
Antioxidant and anti-inflammatory properties of active compounds from *Arnica montana* L. (CRACIUNESCU & al. [22]). Encapsulation of N-acetylcysteine in liposomes modulated mitochondrial dehydrogenases activity in a culture of A549 epithelial cells, improved cytoprotection and ameliorated the cellular redox status of the stressed cells (MITSOPoulos & SUNTRES [35]). Also, plant extracts, like *K. galangel*, *T. cucumerina*, *A. precatorius* improved their bioavailability and biologic activity after complexing with phosphatidylcholine (KUSUMAWATI & YUSUF [36]; SANDHYA & al. [37]). In our study, liposomal formulations of arnica Pf and Pz extracts had a better cytoprotective effect than the non-encapsulated extracts. This could be due to positive effects of both liposomes involved in preservation of cell membrane composition and arnica extracts presenting free radical scavenging activity.

**Cell morphology**

Light microscopy images were taken for inflamed cells pretreated with arnica samples for 24h (Fig. 4). Giemsa staining allowed observation of cell morphology changes induced in the cell membrane, cytoplasm and nuclei by H$_2$O$_2$, in the presence (Fig. 4A, C-F) or the absence (Fig. 4B) of arnica samples, on one hand, and in the cell density after 24h of stress, on the other hand. The micrographs of the control culture plate showed that the cells had a normal fibroblastic phenotype, with euchromatic nuclei and 1-2 nucleoli, clear cytoplasm and cytoplasmic extensions (Fig. 4A). The morphology of L929 cells pre-treated with arnica L-Pf (Fig. 4C), L-Pz (Fig. 4D), Pf (Fig. 4E) and Pz (Fig. 4F) and stressed with H$_2$O$_2$ was similar to that of control after 24h of cultivation. Moreover, the density of treated cells was similar to that of the untreated control, reaching an almost complete monolayer (95% surface covered by cells) (Fig. 4A-E). Slightly lower values were observed for Pz-treated cells (~80% surface covered by cells) (Fig. 4F).

![Fig. 4. Light micrographs of L929 fibroblast cells pre-treated with arnica extract formulations, L-Pf (C), L-Pz (D), Pf (E), Pz (F), for 24h and then treated with hydrogen peroxide, for 24h.](image-url)
Cells cultured on plastic were negative control (A) and cells cultured with 50μM H₂O₂ were positive control (B). (Giemsa staining) (Bar = 10 µm)

The quantitative results concerning the cell membrane integrity (LDH assay) correlated well with cell morphology observations (light microscopy) and all these findings showed the cytoprotective activity of arnica extract formulations.

**Anti-inflammatory activity**

In the same *in vitro* experimental model, we measured the production of IL-6, IL-8 and TNF-α pro-inflammatory cytokines secreted in the culture medium of H₂O₂-stressed cells pretreated with liposomal formulations or non-encapsulated Pf and Pz arnica extracts. The results of specific ELISA determination are presented in Fig. 5. TNF-α recorded the highest production increase by 14 times in H₂O₂-treated cells, while IL-6 and IL-8 secretion augmented by 1.2 and 1.4 times, respectively, compared to untreated cells (control). In the presence of liposomal formulations, the secretion of IL-6 and IL-8 pro-inflammatory cytokines decreased close to control level (1.1-1.2 times), while TNF-α was reversed by 2.5-3 times, compared to H₂O₂-inflamed cells. The non-encapsulated Pf and Pz arnica extracts reduced the secretion of IL-6 cytokine by 1.1 times, IL-8 production was lowered by 1.26 and 1.15 times, respectively, while the secretion of TNF-α decreased by 2 and 1.44 times, respectively. These results demonstrated that the inflammatory process could be limited through regulation of pro-inflammatory cytokines by Pf and Pz arnica extracts, encapsulated or not in liposomes. Moreover, the results showed that L-Pf and L-Pz had higher anti-inflammatory activity compared to Pf and Pz non-encapsulated extracts, respectively. Arnica extract liposomal formulations were most efficient in TNF-α cytokine inhibition in L929 inflamed cells. These effects could be due to liposomes ability to achieve intracellular delivery of their content after easily passing through the cell membrane.

![Graph showing cytokine production](image)

**Fig. 5.** Influence of arnica extract formulations on the secretion of pro-inflammatory cytokines in inflamed fibroblast cells, as determined by ELISA assay. Data are presented as mean ± SD (n=3).

*p<0.05 compared to hydrogen peroxide-treated cells; #p<0.05 compared to L-Pf-treated cells; §p<0.05 compared to L-Pz-treated cells.
Antioxidant and anti-inflammatory properties of active compounds from *Arnica montana* L.

Previous studies showed that arnica ethanolic extracts inhibited TNF-α and nitric oxide production and reduced the protein levels of inducible nitric oxide synthase and cyclooxygenase-2 in J774 murine macrophage cells treated with lipopolysaccharide (VERMA & al. [38]; CAPELARI-OLIVEIRA & al. [39]). This anti-inflammatory activity was mainly attributed to its sesquiterpene lactones of helenanolid type, which had the capacity to inhibit the transcription factor NF-κB (EKENAS & al. [40]). Studies on macromolecular polyphenolic-polysaccharidic conjugates isolated from *A. montana* showed that they inhibited oxidative stress in blood platelets and might have anti-inflammatory properties (SALUK-JUSZCZAK & al. [41]).

Conclusions

The Pf and Pz extracts obtained in this study from arnica flowers presented high content in phenolics and carbohydrates, respectively, had a good scavenging activity against DPPH free radicals and they were non-cytotoxic up to 50 µg/ml Pf and 0.5 µg/ml Pz. The extracts encapsulated in lipid vesicles had better cytoprotective effect against cell membrane oxidative damage and higher anti-inflammatory activity, compared to their free forms, in a H₂O₂-stressed fibroblast cell culture model. All these results suggested that arnica extract liposomal formulations could be further tested and included in ointments for topical treatment of inflammatory skin disorders.

Acknowledgments

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