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Research Article

# Anti-Inflammatory and Antioxidant Activity of an Extract of Luzula sylvatica in a Co-Culture Model of Fibroblasts and Macrophages

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## Abstract

**Background:** Plants are a promising source of innovative bioactive compounds for skin treatment and some species of the Juncaceae family have been used in folk medicine for the treatment of erysipelas or bleeding. We focused on *Luzula sylvatica*, a Juncaceae member that has sparked growing interest for its anti-inflammatory potential **Methods:** The antioxidant properties of an ethanolic extract of *Luzula sylvatica* (LS-E) were tested with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and Oxygen Radical Absorbance Capacity (ORAC) assay. The LS-E was then tested on human dermal fibroblast (HDFa) migration, THP-1 macrophage polarization, and a co-culture model of macrophages and fibroblasts **Results:** The LS-E showed strong DDPH and ORAC values (1,057 µM and 4,954 µM Trolox equivalent, respectively). The LS-E (50 µg/mL) significantly inhibited IL1β expression in pro-inflammatory polarized macrophages and decreased their secretion of PGE2. The co-culture of fibroblasts with inflammatory M1 macrophages increased proteases gene expression of the fibroblasts. The addition of LS-E dampened the expression of the MMP9 significantly whereas it increased COL1A2 by 2.3 **Conclusions:** *Luzula sylvatica* exhibited good antioxidant properties and altered the pro-inflammatory polarization of macrophages. By adding the inhibition of expression of proteases in fibroblasts, these results suggest that *Luzula sylvatica* shows interesting skin protective activity.

**Keywords:** Fibroblasts; Macrophages; *Luzula sylvatica*; Inflammation; Fibrosis; Skin

## Introduction

The healing of injured skin is a complex phenomenon. It involves numerous cell types such as keratinocytes, fibroblasts and immune cells. During the pro-inflammatory phase and the granulation phase, the macrophages and their interactions with other cells such as fibroblasts are central. Their phenotype changes over the course of wound healing, from pro-inflammatory M1 profile, characterized by the secretion of pro-inflammatory cytokines such as IL1β, IL6 or TNFα, to a M2 profile, which favor wound healing and angiogenesis. Macrophages are also involved in the capacity of fibroblasts and keratinocytes to produce matrix proteinases and collagens [1-3].

However, although inflammation is part of the normal response of an organism in the course of wound healing, a deregulation might impair the closure of the wound. An impairment in the polarization transition of macrophages, abnormal levels of proteases and inflammatory cytokines or the prolonged presence of neutrophils and an overproduction of Reactive Oxygen Species (ROS) may lead either to a chronic injury or to abnormal closure, with hypertrophic scar development [3,4].

Outside the context of an injury, the skin is also exposed to numerous external (ultraviolet radiation, cigarette smoke, pollution…) and internal (aging) stimuli leading to increased free radical production and inflammation, which cause skin damages and alter its aspect [5,6].

In this regard, plants are a promising source of innovative bioactive compounds for skin treatment and protection with some species or metabolites showing interesting antioxidant, anti-inflammatory and sometimes wound healing or anti-fibrotic activities [7-9]. Among them, the Juncaceae have shown to be an interesting family with anti-inflammatory potential, and some of them have been reported in folk medicine as treatment for skin diseases, such as erysipelas [10,11]. We chose to focus on *Luzula sylvatica.* This species, commonly named Great Wood-Rush, is widely spread in the Auvergne region of France and is found in spring and summer in cool humus-rich woods up to the base of the subalpine zone [12]. It has been little studied to date but showed antioxidant and anti-inflammatory effects on different cell lines, with notably an inhibition of superoxide anion generation [13,14]. In addition, Gainche et al. previously demonstrated that it contained interesting anti-inflammatory and antioxidant compounds, such as luteolin [14].

The aim of the present study was to assess the antioxidant and anti-inflammatory potential of an extract of *Luzula sylvatica* in skin inflammation *in vitro*. This was done with a model a macrophage polarization and a model of co-culture of macrophages and dermal fibroblasts.

## Materials and Methods

### Plant Material, Preparation of the Extract and Antioxidant Capacity

The specimens of *Luzula sylvatica* were collected at Marcenat, France, in August 2017. A voucher specimen (CLF 110940) was deposited after identification at the University of Clermont Auvergne herbarium. Dried aerial parts were powered and then extracted three times with an aqueous solution containing 80% of ethanol (LS-E) for 24 h with a plant/solvent ratio of 1:10 (mass:volume). After filtration, the extract was dried *in vacuo* to obtain a dry extract. For experiments with cells, the powder (LS-E dry extract) was dissolved extemporaneously in DMSO and then further diluted with appropriate culture medium. For control conditions, an equivalent amount of solvent was added in the medium.

The major constituents of the LS-E were determined using LC-MS (UHPLC Ultimate 3000 RSLC chain) and an Orbitrap Q-Exactive (Thermo Scientific) with an Uptisphere C18-3 (250 x 4.6 mm, 5 µm) column from Interchim, by comparison with analytical standards (Extrasynthèse, France).

The measure of oxygen radical absorbance capacity (ORAC) was done in 96-well plates as described by Gillespie et al. (Gillespie et al., 2007). Fluorescein was used as the fluorescent probe and 2.2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) as the peroxyl radical generator. The decrease in fluorescence was measured (excitation/emission: 485/530 nm) using a microplate reader (TECAN infinite F200 PRO, Männedorf, Switzerland). The ORAC value of the extract was expressed as µmol of Trolox equivalent (µmol TE) per gram of dry extract.

The DPPH scavenging activity was evaluated according to the method of Meda et al. [15] with slight modifications. Briefly, 10 µL of a solution of the extract (1 mg/mL in methanol) was mixed with 2.5 mL of fresh DPPH solution (25 µg/mL in methanol). After 30 min of incubation, DPPH absorbance was recorded at 515 nm using a spectrophotometer (JASCO V-630). The results were expressed as µmol TE per gram of dry extract.

### *In vitro* Wound Healing (Scratch) Test

Human Dermal Fibroblast cells (HDFa, ATCC® PCS-201-012™) were grown in DMEM (GIBCO, ThermoFisher Scientific,

Waltham, MA) supplemented with 10% fetal bovine serum (FBS), 50 µg/mL gentamicin and 2 mM glutamine (Gln), at 37°C under 5% $CO_2$, and seeded in 24-wells plate (30 000 cells/well). A breach was made in each well by passing the tip of a sterile cone over the bottom of the well. The culture media was carefully removed, and wells were rinsed with Phosphate Buffer Saline (PBS). DMEM supplemented with 50 µg/mL gentamicin with or without the extract (50 µg/mL) was then added to the wells. Allantoin (50 µg/mL, Sigma-Aldrich, Saint Quentin Fallavier, France) was used as a positive control. The surface of the gap was immediately measured with a micrometric ocular and plates were put in an incubator (37°C, 5% $CO_2$) for 18 h. The surface was measured again, and the difference between the area at t = 0 h and t = 18 h was expressed as a percentage of recovered surface.

**THP-1 cell culture and M1 polarization**

THP-1 (ATCC® TIB-202™) monocytes were cultured at 37°C under 5% $CO_2$ in supplemented RPMI (GIBCO) (FBS 10%, gentamicin 50 µg/mL and Gln 2 mM) at 300 000 cells/mL. For differentiation, THP-1 cells were seeded at 300 000 cell/mL in 6-wells plates or in microporous inserts and incubated with PMA (10 ng/mL). After 3 days, the culture medium was renewed. Following differentiation, unstimulated macrophages (M0) were maintained without additional supplementation. Pro-inflammatory macrophages (M1) were obtained by incubation for 48 h in presence of LPS (1 µg/mL) and IFNγ (10 ng/mL). Thereafter, macrophages cultured in 24-wells plates (M0 or M1) were incubated with or without the extract (50 µg/mL). Supernatants and total RNA were collected for further analyses.

**HDFa cell culture and co-culture of macrophages with fibroblasts**

HDFa were grown in DMEM (GIBCO) supplemented with 10% FBS, 50 µg/mL gentamicin and 2 mM Gln, at 37°C under 5% $CO_2$, and coated in 12-wells plate (30, 000 cells/mL). Inserts containing M0 or M1 macrophages were then placed in wells with coated HDFa. Co-cultures were incubated at 37°C under 5% $CO_2$ for 24 h. Extract (50 µg/mL) was added or not and supernatants as well as the total mRNA of HDFa were harvested after 24 h.

**Analyses of supernatants**

The PGE2 secreted in the culture media of THP-1 macrophages co-cultured or not with HDFa was assessed with an ELISA kit, following the instructions of the manufacturer (R&D systems, Bio-Techne, Lille, France). Likewise, the collagen I (Pro-Collagen I alpha 1) and the TIMP-1 secreted in the culture media of co-cultures was assessed with ELISA kits (R&D systems).

**Analyses of gene expression**

Trizol (Invitrogen, Thermo Fisher Scientific) was used to collect total RNA and 1µg per sample was treated with DNAse I (Invitrogen, Thermo Fisher Scientific). The RT was undertaken with the MultiScribe reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Thermo Fisher Scientific) using the StepOne Real-Time PCR System (Applied Biosystems).

Gene expression analysis expression was done with the StepOne by qPCR, using SybRGreen reagent (Thermo Fisher Scientific). The analysis was conducted on 5 genes for the macrophages (IL1B, COX2, IL6, TNFA, IL10), 7 genes for the HDFa (MMP9, MMP2, MMP1, COL1A2, TNFA, CCL2, CXCL1) and 2 reference genes (18S rRNA; GAPDH) for both cell lines. The relative quantification method (RQ = 2-ΔΔCT) was used to compute the relative gene expression with ΔΔCT = [ΔCT (sample 1) - ΔCT (sample2)] and ΔCT = [CT(target gene) – geometric mean CT (reference genes)].

**Statistical analyses**

Results were expressed as mean +/- standard error. Statistical analyses were performed using R software (version 3.6.1). The normality of the variables was assessed by the Shapiro–Wilk test and their homoscedasticity by Bartlett's test. Comparisons between two groups were made by a t-test or the Wilcoxon-Mann-Whitney test when normality was rejected. Values with $p < 0.05$ were considered significant.

## Results and Discussion

**Composition and Antioxidant Properties of the LS-E**

During the inflammatory phase of wound healing, ROS are produced by leucocytes, especially neutrophils and macrophages, and by fibroblasts as well to ensure the clearance of pathogens. The production of ROS can also be increased with aging, as a chronic inflammation has been observed in the elderly population [5]. The detoxification of ROS in healing skin is of high importance, to avoid pathological scarring for example [16] and can also prevent several skin defects such as wrinkling, photoaging or drying [17]. We therefore measured the antioxidant capacity of the LS-E with an ORAC and a DPPH assay. The extract exhibited strong ORAC value (4954 ± 12.1 µM TE) and DPPH value (1057 ± 11.9 µM TE), hence showing a strong antioxidant capacity.

This is consistent with previous studies which have already highlighted the antioxidant properties of some Juncaceae species and their compounds, such as *Juncus acutus* and *Juncus maritimus* [18,19]. Moreover, the identification of major compounds of *Luzula sylvatica* shown in Table 1 highlighted the presence of

polyphenols, known for their antioxidant capacities [20]. Luteolin in particular was shown to be a flavone with good antioxidant and anti-inflammatory property as well [21]. Ananasate was also identified as an interesting antioxidant compound [14,22].

| N | Rt[a] (min) | M-$H_{exp}$ (m/z) | $MS^2$ fragment (m/z) | Compound |
|---|---|---|---|---|
| 1 | 3.85 | 191.0547 | 191[b] / 85 / 127 / 173 / 59 | Quinic acid |
| 2 | 3.93 | 341.1086 | 89[b] / 59 / 341 / 71 / 119 | Saccharose |
| 3 | 6.78 | 191.0189 | 111[b] / 87 / 85 / 191 / 129 | Citric acid |
| 4 | 12.97 | 353.0875 | 191[b] / 353 / 161 / 85 / 127 | Chlorogenic acid |
| 5 | 14.31 | 353.0876 | 191[b] / 353 / 161 / 85 / 127 | Cryptochlorogenic acid |
| 6 | 17.05 | 609.1459 | 300[b] / 609 / 271 / 255 / 179 | Quercetin-3-O-rutinoside |
| 7 | 18.45 | 463.0882 | 300[b] / 463 / 271 / 255 / 151 | Quercetin-3-O-glucoside |
| 8 | 19.00 | 447.0928 | 285[b] / 593 / 327 / 133 / 151 | Luteolin-7-O-glucoside |
| 9 | 35.99 | 415.1031 | 415[b] / 253 / 161 / 179 / 135 | Ananasate |
| 1 | 36.50 | 287.0563 | 151[b] /153 / 136 / 135/ 288 | 5,7,3',5'-tetrahydroxyflavanone |
| 11 | 38.35 | 285.0404 | 285[b] / 133 / 151 / 175 / 199 | Luteolin |

[a] Retention time; [b] Fragment with the highest relative intensity

**Table 1:** Major constituents of the extract of *Luzula sylvatica*. All compounds were identified according to standard data (reference).

**Effect of the LS-E on fibroblasts migration**

A scratch test was performed to assess the effect of the LS-E on fibroblast migration. The percentage of surface recovered was determined after 18 h of incubation (time needed for complete recovering in presence of allantoin 50 µg/mL). At that time, the breach in the cells incubated without the extract was reduced by around 25% (± 1%) (Figure 1). In presence of the LS-E (50 µg/mL) the area was reduced by 35.5% (± 5.9%). This is somehow comparable to what has been observed with other vegetal extracts. Schmidt et al. found that ethanolic extracts of Brazilian plants used in folk medicine for wound healing stimulated migration of mouse fibroblasts by 4.68 to 79.9% after 12 h of incubation, and some extracts even prevented fibroblasts migration [23]. Here the extract tended to improve the migration ($p = 0.1$), with a mean improvement of 10% compared to Control.

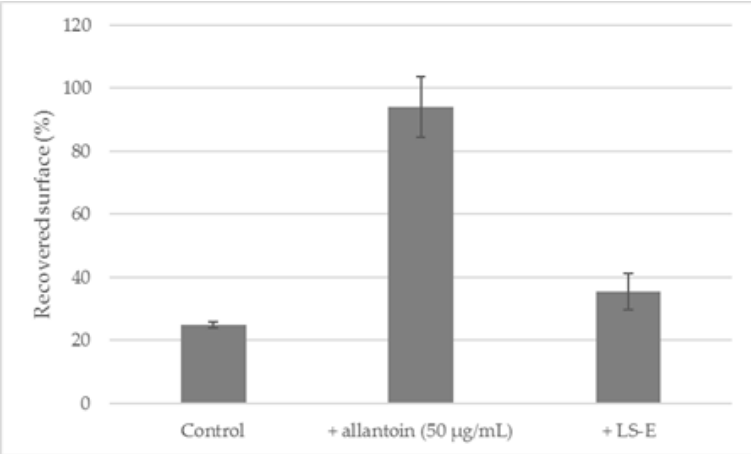



**Figure 1:** Migration of HDFa incubated with or without LS-E (50µg/mL) for 18 h. Allantoin (50 µg/mL) was used as a positive control. Results were expressed as mean % ± standard error of the initial breach area covered after 18 h of incubation, n = 3 independent experiments.

### The LS-E affected the macrophage polarization

We then decided to study the effect of the extract in a co-culture model of dermal fibroblasts and macrophages. The inflammatory phase of wound healing was characterized by a differentiation and polarization of newly recruited monocytes into inflammatory M1 macrophages. These M1 macrophages initiate matrix damage and inflammatory response. Without oversimplifying because polarization is more a spectrum than a binary M1 versus healing M2 status, the duration of M1 profile at the expense of M2 macrophages can impair the closure of the wound and delay termination of the inflammation [24,25].

The monocytic THP-1 cell line was used to obtain either activated neutral M0 macrophages or pro-inflammatory M1 macrophages. The expression of inflammatory markers was significantly upregulated in M1 macrophages compared to M0 (Figure 2A). IL1β had the strongest increase, its expression being multiplied by 121 in M1 compared to M0. The expression of COX-2 was increased by 3.6, and the expression of IL6 and TNFα by 45.5 and 47.3, respectively. The expression of IL10 was not affected by M1 polarization. Accordingly, the secretion of IL1β and PGE2 was increased in the culture media of M1 macrophages (Figure 3).

The LS-E was able to act on some of the main features of M1 induced THP-1 in our model. It strongly inhibited the expression of IL1β and tended to decrease TNFα expression as well (Figure 2B). Although no effect on COX-2 expression was detected, the LS-E decreased the secretion of PGE2 by M1 macrophages. The effect on the secretion of IL1β could not be determined, as its production quickly decreased at the end of polarization (Figure 3). The IL1β is a major pro-inflammatory cytokine and the PGE2 is an important mediator of inflammation [26,27]. Therefore, the LS-E showed a strong anti-inflammatory effect regarding macrophage polarization. Furthermore, we showed that the LS-E contained several compounds of interest, including luteolin. Luteolin has been found to inhibit IL1β expression in polarized RAW264.7 cells [28] and so the activity observed with LS-E might partly be allocated to the presence of luteolin.

Further analyses would be required to determine if it dampens the pro-inflammatory potential of M1 macrophages or is able to favor a M2-like phenotype, although we did not observe an effect on IL10 expression.

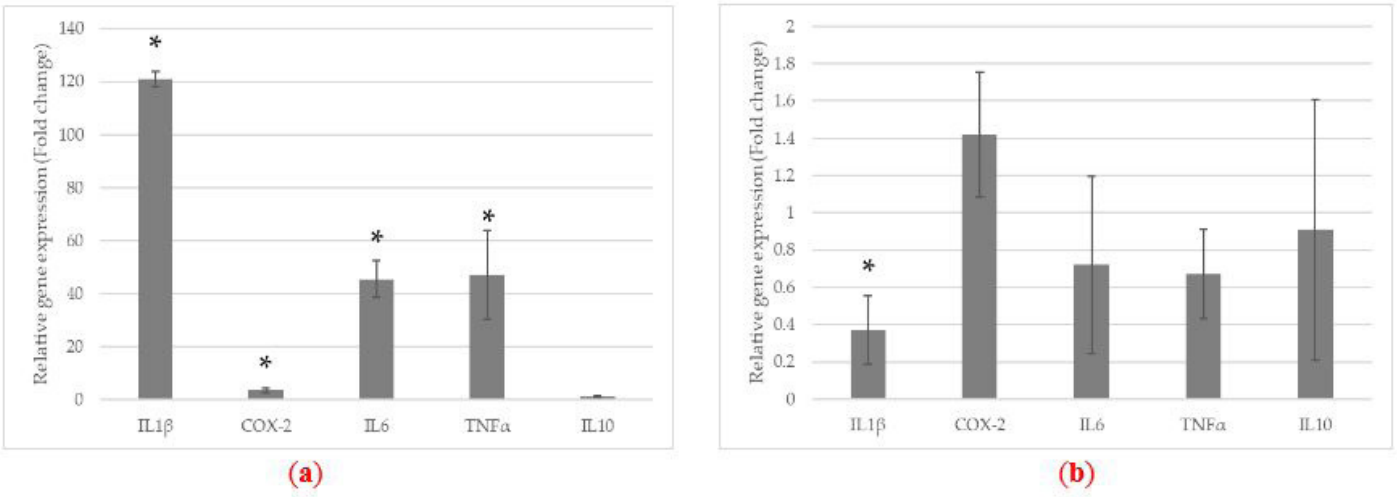



**Figure 2**: Relative gene expression of (a) M1 THP-1 macrophages vs M0 THP-1 macrophages after 72 h of polarization and (b) M1 THP-1 macrophages incubated with LS-E (50 µg/mL) for 24 h vs M1 THP-1 macrophages alone. Results were expressed as mean fold change ± standard error. *: $p < 0.05$, n = 4 independent experiments.

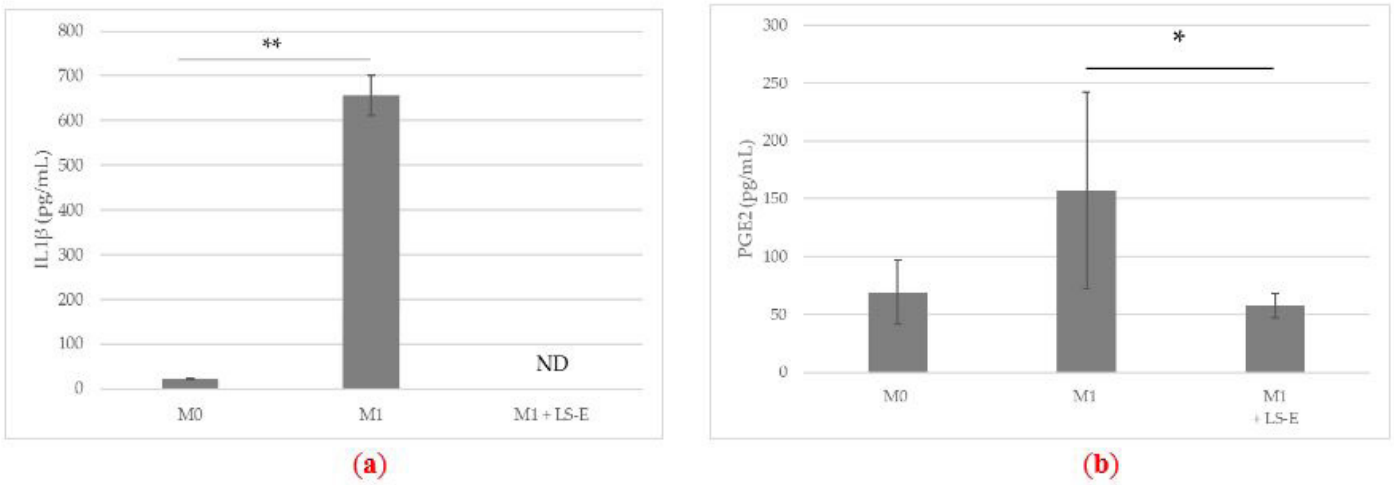
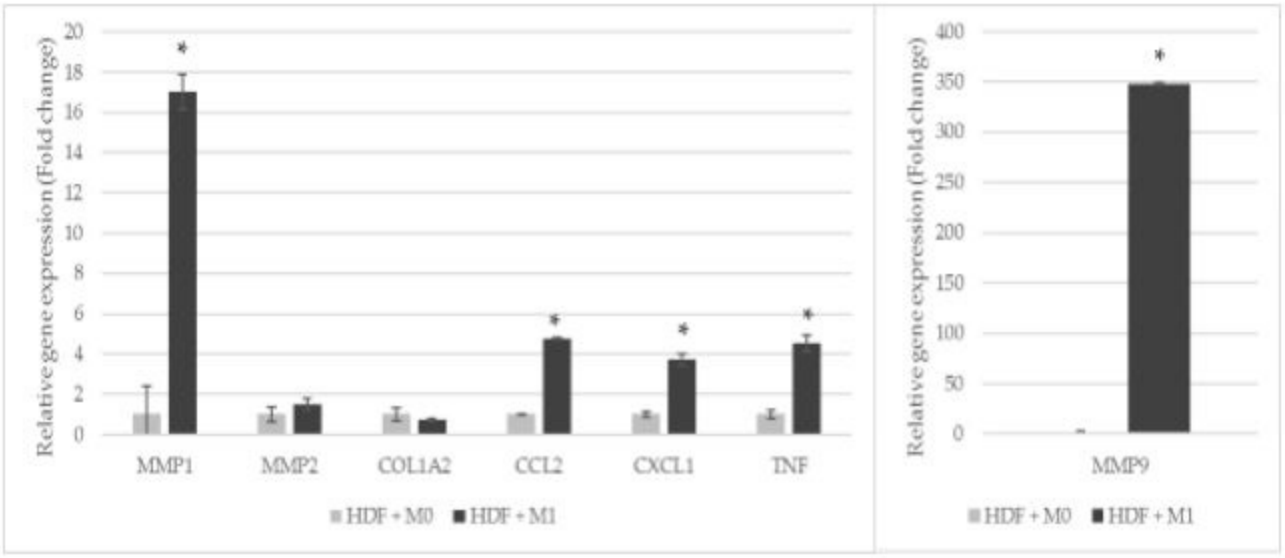



**Figure 3:** (**a**) IL1β concentration and; (**b**) PGE2 concentration in the culture media of M0 or M1 THP- 1 macrophages incubated with or without LS-E (50 µg/mL) for 24 h. Results were expressed as mean ± standard error .*: $p<0.05$, **: $p < 0.01$ n = 3-4 independent experiments. ND: Not Detected.

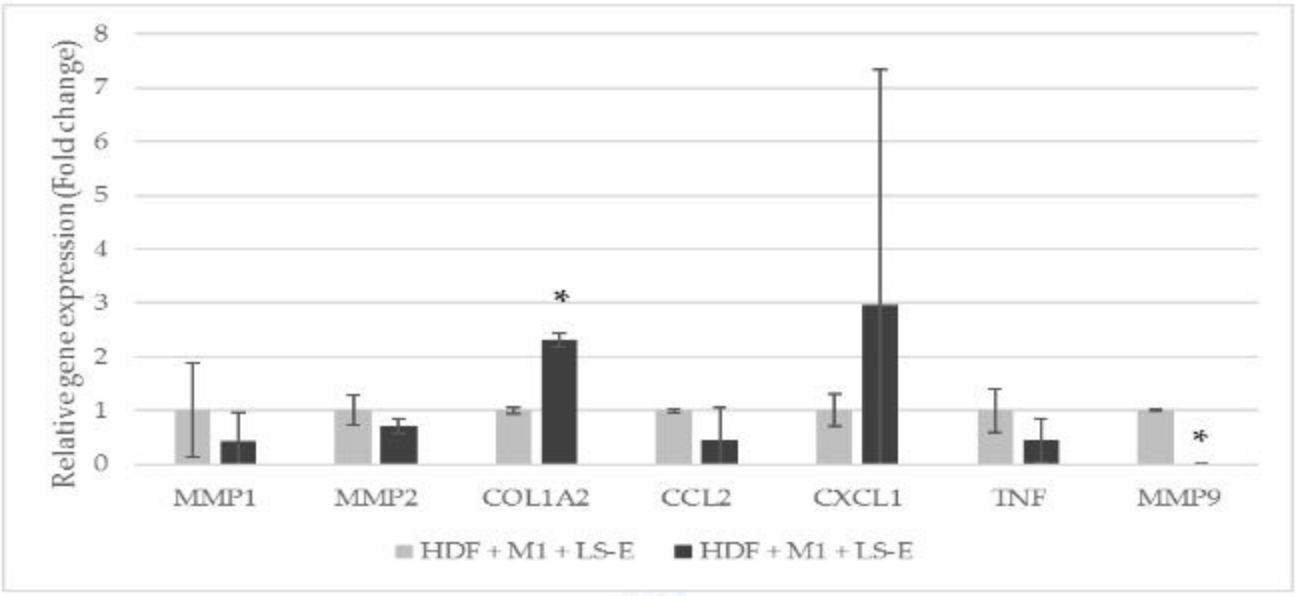
**Anti-inflammatory effect in a fibroblasts-macrophages co-culture model**

The HDFa were then co-cultured with M0 or M1 THP-1 macrophages and the effect of the LS-E (50 µg/mL) was observed.

First, the impact of the co-culture and LS-E on fibroblasts gene expression was determined (Figure 4). As expected, the dialogue between M1 macrophages and HDFa increased the expressions of MMP and chemokines among the latter (Figure 4a). The expression of the proteases MMP1 and MMP9 was higher in HDFa incubated with M1 compared with HDFa incubated with M0 (x17 and x348), as well as the expression of CCL2, CXCL1 and of the cytokine TNFα (x4.8, x3.7, x4.6, respectively). This is consistent with what was observed by Ploeger et al.: when they stimulated HDF with paracrine factors from M1 macrophages, the expression of MMP1 and CCL2 was increased [29]. The addition of LS-E drastically decreased the fibroblasts expression of the MMP9 (x0.02), which was previously significantly increased by the presence of M1 macrophages (Figure 4b). The addition of LS-E also decreased the expression of other MMP, although not significantly and increased the expression of COL1A2. The effect of the extract on MMP9 could be attributed to the presence of luteolin and quercetin, as both have previously been shown to inhibit this protease expression on various cell lines [30,31]. The dampening of MMP expression could indicate a matrix protection role of the LS-E, as the expression of proteases is involved in the degradation of the skin matrix during inflammation and skin aging [32,33]. In addition, the expression of MMP9 is in particular associated with non-healing wounds [34]. Thus, the LS-E could be of interest for the prevention of skin matrix degradation by inhibiting proteases expression while increasing that of collagen (COL1A2, x2.1 compared to HDFa with M1 and without LS-E).




(a)




(b)

**Figure 4.** Relative gene expression in HDFa incubated with M0 or M1 THP-1 macrophages and with or without LS-E (50 µg/mL) for 24 h. (a) HDF + M1 vs HDF + M0 and (b) HDF + M1 +LS-E vs HDF +M1. Results were expressed as mean fold change ± standard error, *: $p < 0.05$, n = 4 independent experiments.

Finally, we analyzed the supernatants of the co-culture of fibroblasts with M0 macrophages *versus* with M1 macrophages. The co-culture with M1 macrophages and the addition of the LS-E did not seem to affect the production of TIMP-1, a major inhibitor of MMPs and of collagen 1 (Figure 5b and 5c). The lack of concordance between COL1A2 expression and collagen 1 secretion might be due to a delay between gene induction and protein secretion, as previous studies showed it may took up to 48h of delay before observing a change in the protein secretion [35], although it should be investigated further before concluding on this point.

The secretion of PGE2 was increased in the medium of HDFa incubated with M1 compared to incubation with M0 macrophages. It was also higher than in the culture media of M1 grown alone (1655 pg/mL vs 157 pg/mL) (Figures 5a and 5b). The PGE2 secretion was strongly dampened by the LS-E, although we could not determine if that was the result of its action on THP-1, as previously observed, or a direct effect on fibroblasts. However, this demonstrates a strong anti-inflammatory effect of the LS-E with regards to the secretion of lipid mediator, as the secretion of PGE2 is linked to the exacerbation of the inflammatory response [36].

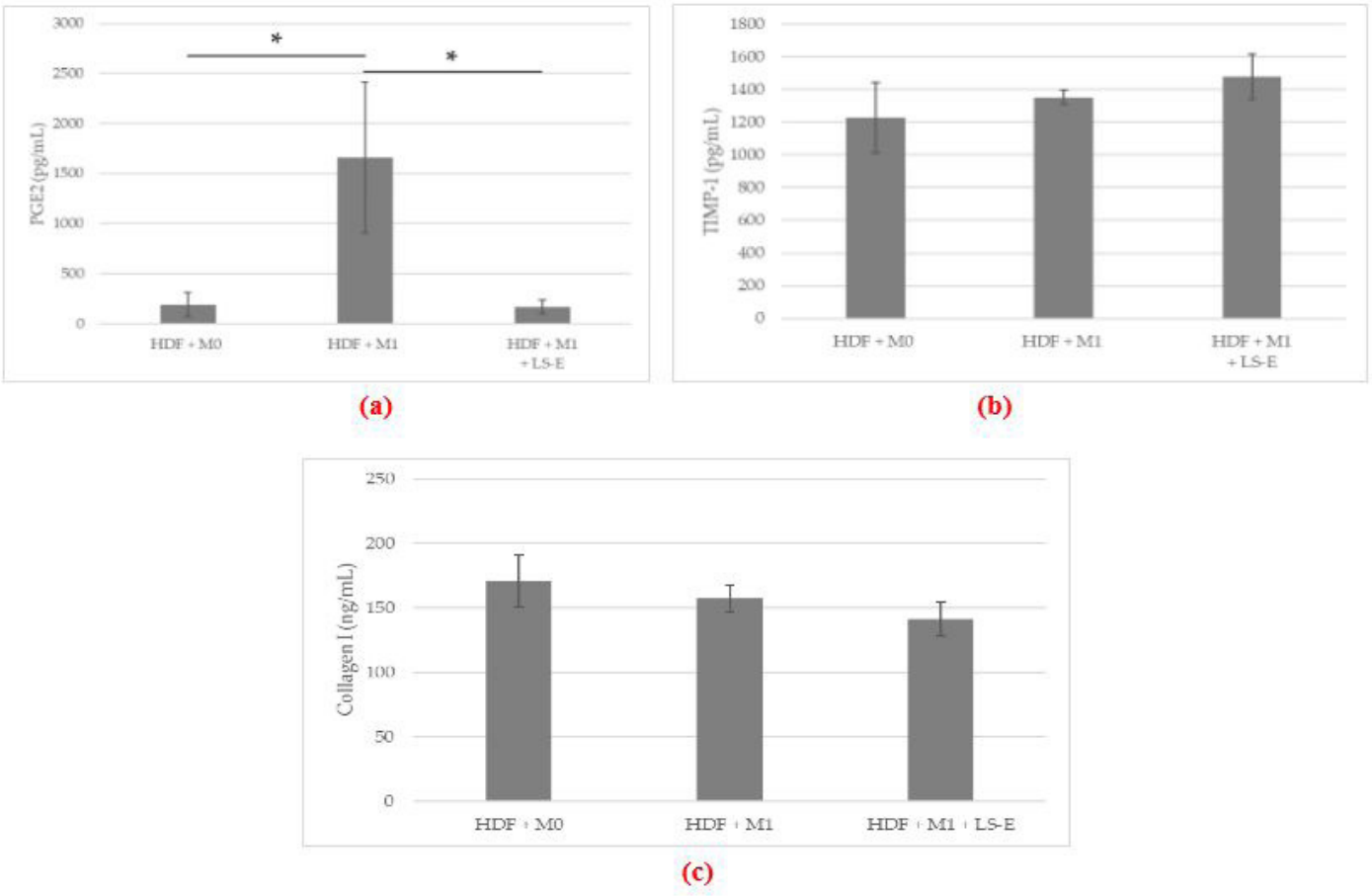



**Figure 5:** Concentration of: (**a**) PGE2; (**b**) TIMP-1 and (**c**) collagen I in the culture media of HDFa incubated with M0 or M1 THP-1 macrophages and with or without LS-E (50 µg/mL) for 24 h. Results were expressed as mean ± standard error, *: $p < 0.05$, $n = 3$ independent experiments.

## Conclusions

To conclude, the LS-E showed excellent antioxidant potential regarding the DPPH and ORAC values obtained as well as interesting anti-inflammatory properties, with an effect on macrophage polarization and on the co-culture of fibroblasts and macrophages. Thus, *Luzula sylvatica* might be of great interest in the inhibition of skin inflammation and the protection of skin matrix via notably the inhibition of the MMP9 expression and of PGE2 secretion. These properties might be attributable at least in part to the presence of luteolin and quercetin in our extract, as they are already known for various anti-inflammatory effects.

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