Research article

CANNABINOID-LIKE ANTI-INFLAMMATORY COMPOUNDS FROM FLAX FIBER

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Abstract: Flax is a valuable source of fibers, linseed and oil. The compounds of the latter two products have already been widely examined and have been proven to possess many health-beneficial properties. In the course of analysis of fibers extract from previously generated transgenic plants overproducing phenylpropanoids a new terpenoid compound was discovered.
The UV spectra and the retention time in UPLC analysis of this new compound reveal similarity to a cannabinoid-like compound, probably cannabidiol (CBD). This was confirmed by finding two ions at m/z 174.1 and 231.2 in mass spectra analysis.

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Abbreviations used: ALA – α-linoleic acid; CB1 – cannabinoid receptor 1; CB2 – cannabinoid receptor 2; CBC – cannabichromene; CBCA – cannabichromenic acid; CBD – cannabidiol; CBDA – cannabidiolic acid; CBGA – cannabigerolic acid; CBN – cannabinol; CHI – chalcone isomerise; CHS – chalcone synthase; CREB – cAMP response element-binding; DEG – differentially expressed genes; DFR – dihydroflavonol reductase; DMEM – Dulbecco's Modified Eagle Medium; EDTA – ethylenediaminetetraacetate; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; GC-MS – gas chromatography–mass spectrometry; GPP – geranyl pyrophosphate; HPLC – high-performance liquid chromatography; IL1β – interleukin 1β; IL6 – interleukin 6; IL8 – interleukin 8; IFN-γ – interferon γ; IPP – isopentenyl diphosphate; LPS – lipopolysacharide; MCP-1 – monocyte chemotactic protein 1; NFκB – nuclear factor κB; NHDF – normal human dermal fibroblasts; OLA – olivetolic acid; PBS – phosphate buffered saline; PKA – protein kinase A; PUFA – polyunsaturated fatty acids; RQ – relative quantification; RT PCR – real-time polymerase chain reaction; SD – standard deviation; SDG – secoisolariciresinol diglucoside; SE – standard error; SOCS-1 – suppressor of cytokine signaling 1; TFA – trifluoroacetic acid; THC – tetrahydrocannabinol; THCA – Δ9-tetrahydrocannabinolic acid; TLR4 – Toll-like receptor 4; TNF-α – tumor necrosis factor α; TNFR – TNF receptor; UPLC – ultra performance liquid chromatography

Further confirmation of the nature of the compound was based on a biological activity assay. It was found that the compound affects the expression of genes involved in inflammatory processes in mouse and human fibroblasts and likely the CBD from *Cannabis sativa* activates the specific peripheral cannabinoid receptor 2 (CB2) gene expression. Besides fibers, the compound was also found in all other flax tissues. It should be pointed out that the industrial process of fabric production does not affect CBD activity.

The presented data suggest for the first time that flax products can be a source of biologically active cannabinoid-like compounds that are able to influence the cell immunological response. These findings might open up many new applications for medical flax products, especially for the fabric as a material for wound dressing with anti-inflammatory properties.

Key words: Flax, *Linum usitatissimum*, Linen, Cannabinoid, Inflammation, Terpenoids, Flax fibers, Cannabinoid signaling

INTRODUCTION

Flax (*Linum usitatissimum*) is an annual plant widely distributed in the Mediterranean and temperate climate zone. It has a long history of cultivation, and great significance in medicine and industry. The main products obtained from flax are the fiber produced from the stem, the linseed and the oil.

Linseed products are highly recommended in our diet, as they are rich in secoisolariciresinol diglucoside (SDG), the main compound of flax lignans. Recent studies suggest that these are the most valuable flax compounds for human health [1]. SDG is an antioxidant [2] and has been shown to slow the progression of atherosclerosis, and to have anticancer properties, especially in breast and prostate cancer [3].

Flax oil is also a very valuable product as it is one of the richest sources of α-linoleic acid (ALA) and other polyunsaturated fatty acids (PUFA) among plant oils [4]. It has been widely proven that high levels of ALA can reduce the risks of cancer and cardiovascular diseases and decrease the production of arachidonic acids and other eicosanoids, which are known inflammation mediators [5, 6].

The results of our latest investigations suggest that not only can the flax seeds and the oil be used in medicine, but also the fibers appear to be a valuable material for wound dressing [7]. Analysis of previously generated transgenic plants, overexpressing genes from the flavonoid synthesis pathway, showed increased levels of many phenylpropanoids, resulting in higher antioxidant capacity, fungal resistance, oil stability and SDG content increase [8]. In the course of detailed analysis of transgenic flax, metabolites of the terpenoid pathway were also detected. Of this pathway attention has been caught by a terpenophenol, a cannabinoid-like compound, since we found a slight increase in the level of this compound in the transgenic seeds. Further analysis revealed that other parts of the transgenic plant, as well as the fabrics, also contain this compound but in an amount comparable to the control. The presence of

cannabinoid-like components in the flax fabric gives great opportunities for new medical applications of this flax product as an analgesic and anti-inflammatory agent. Cannabinoids are known to accumulate in the glandular trichomes of *Cannabis sativa* (*Cannabaceae*) and to date they have not been detected in other plant species. Almost 70 natural cannabinoids have been isolated, and the most common are Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabichromene (CBC). Chemically cannabinoids belong to a group of terpenoids, the terpenophenols. The biosynthesis of the terpenoid part of these molecules involves a common isoprenoid building block, isopentenyl diphosphate (IPP). The phenyl part of cannabinoids derives from olivetolic acid (OLA), whose biosynthesis is connected with the polyketide pathway and consists of the aldol condensation of hexanoyl-CoA and three molecules of malonyl-CoA [9]. It should be pointed out that this stage of cannabinoid synthesis is connected with the phenylpropanoid pathway. Malonyl-CoA is a key substrate for terpenophenol and phenylpropanoid biosynthesis pathways.

The enzyme catalyzing the alkylation of OLA with geranyl pyrophosphate (GPP) leading to cannabigerolic acid (CBGA) formation was purified and characterized recently. CBGA is further metabolized to Δ9-tetrahydrocannabinol acid (THCA), cannabidiolic acid (CBDA) or cannabichromenic acid (CBCA) by specific acid synthases. Those can be further modified to a broad variety of derivates [10, 11].

As yet cannabinoid biosynthesis in flax plants has not been reported. There is also no report on the presence of active genes in the flax genome critical for the compounds' biosynthesis. However, the pathways that provide the substrates for terpenophenol biosynthesis are fully active in those plants.

The biological activity of cannabinoids has been extensively investigated, and they have been shown to exhibit a wide variety of beneficial properties, inhibiting cancer, neuropathic pain, multiple sclerosis, Alzheimer's disease, atherosclerosis, rheumatoid arthritis, asthma and many inflammatory diseases [12-14]. Inflammation plays a crucial role in most of the mentioned health issues, and cannabinoids have been proven to influence these processes. Their biological activity is connected to the activation of specific receptors: CB1, expressed mostly in the central nervous system; and CB2, found mainly in peripheral tissues. It is thought that CBD, a non-psychoactive cannabinoid, is responsible for the anti-inflammatory activity of marihuana, acting mostly on the CB2 receptor in many peripheral tissues [15, 16], and immune cells [12, 16, 17]. Cannabinoid receptor activation suppresses inflammation through the cell-signaling pathway that inhibits pro-inflammatory and increases anti-inflammatory gene expression [18-20].

Since there is potential application of flax fabric as an anti-inflammatory wound dressing, this work focuses on the detection and biochemical and biological activity of the CBD-like compound extracted from this material.

MATERIALS AND METHODS

Plant material

Flax (*Linum usitatissimum cv. Linola*, Variety Registration Office, Plant Health and Production Division, Canadian Food Inspection Agency, registration number 5426) plants of the previously described transgenic line W92, expressing three cDNAs from *Petunia hybrida* – CHS (chalcone synthase), CHI (chalcone isomerase) and DFR (dihydroflavonol reductase) [8] – were grown either in a field or in tissue culture (grown under standard conditions on Murashige and Skoog medium). These and the non-transgenic control plants were the seeds and fabric source used for the extractions of terpenoid compounds. The field-grown plants were harvested 60 days after transfer to the soil and analyzed. Flax fiber was prepared via the standard dew retting process described previously [21]. The fabric was prepared from raw yarn using a standard weaving method. The linear mass of the warp and weft was 140 tex. The warp density was $65/dm^2$ and the linear density of the weft was 85/dm. The density of the final flax fabric was 220 g/m^2.

Extraction of terpenoids

For the UPLC analysis, 100 mg of ground dry plant parts, seeds or fabrics were extracted three times with chloroform, dried, and re-dissolved in methanol. The extracts were filtered through 0.25 μm Acrodisc and 10 μl was injected onto an Acquity UPLC BEH C18 column (2.1 mm x150 mm, 1.7 μm particles).

UPLC analysis

The analysis was performed using a Waters Acquity ultra performance liquid chromatograph with a PDA detector. The mobile phase was an acetonitrile-water gradient of 70:30 for 1 min followed by a gradient from 70% to 100% acetonitrile for 5 min, 100:0 till 10 min, then a 1-min return to 30:70 for a further 1 min (flow 0.4 ml min^{-1}). The addition of 0.05% trifluoroacetic acid (TFA) to both solvents eliminated the tailing of phenolic compounds. The detection and integration of the cannabidiol peak was performed at 230 nm in comparison to the compound standard (Sigma-Aldrich, 98.5% purity).

GC-MS analysis

The terpenoid extract was separated into fractions by a semi-preparative high-performance liquid chromatography (HPLC) method using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of two LC-10AD pumps, an SCTL 10A system controller and an SPD-M 10A photodiode array detector. The chromatography was carried out using a pre-packed semi-preparative LUNA column (10 × 250 mm, 5 μm; Phenomenx). Elution for 50 min in a gradient system of 60-100% acetonitrile in water adjusted to pH 2.5 with TFA was employed. The injection volume was 500 μl and the flow rate was 4 ml min^{-1}. Manually collected fractions were evaporated using a rotary evaporator.

Material obtained by semi-preparative HPLC dissolved in methanol was subjected to gas chromatography–mass spectrometry (GC-MS) analysis.

Chromatograms were generated with an Agilent Technologies 7890A gas chromatograph fitted with a 30 m x 0.25 mm HP-5ms column (Agilent Technologies), with a film thickness of 0.25 μm. Injector and detector port temperature were 250 and 300°C, respectively. Carrier gas (He) flow rate was 4 ml min^{-1}. The signal was detected with a 5975 C VL MSD (Agilent Technologies). The oven temperature was 90°C for 8 min, increased at 4°C min^{-1} to 300°C, and held for 35 min. A 0.5 μl volume of extract was injected into the column using splitless injection. The spectra library was used for compound identification. Mass spectra were recorded from *m/z* 50 to *m/z* 500.

Preparation of extracts for mouse fibroblast treatment
Unbleached linen fabric (4.5 g) was extracted three times with 15 ml of chloroform, the solutions were pooled, and after drying, the matter was extracted with 150 μl of ethanol; this preparation is called CBD^{I}. The solid remains were re-extracted with another 150 μl of ethanol and this preparation is called CBD^{II}. Both preparations were filtered through 0.25 μm Acrodisc and the concentration of cannabinoid-like compounds was measured by UPLC analysis. Calculations were based on a standard curve for CBD standard (Sigma-Aldrich, 98.5% purity). The two obtained preparations differ in CBD-like compound purity and were used for further analysis.

Mouse fibroblast culturing and treatment
Mouse fibroblast 3T3 Balb cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin until 90% confluence and then trypsinized (0.05% trypsin/1 mM EDTA in calcium- and magnesium-free PBS). For the treatment, the cells were grown in six-well plates (1.7 x 10^{5} cells/ml) for 24 hours. After fresh medium had been added, the cells were induced either with LPS (200 ng/ml final concentration in the culture) or TNF (50 ng/ml final concentration in the culture) in PBS, and incubated for 24 hours, prior to the CBD preparations (the final concentration of CBD-like compound was 130 nM or 31.4 nM) was added. For analysis of CB2 gene expression a positive control was included (pure CBD in a final concentration of 130 nM). The ethanol concentration was 0.1%. The cells were harvested after 6 hours of treatment and stored at -20°C in the presence of RNAprotect Cell Reagent (Quiagen).

Preparation of extracts for normal human dermal fibroblast treatments
Unbleached linen fabric (15 g) was extracted three times with 50 ml of chloroform, the solutions were pooled, and after drying in a nitrogen atmosphere, the matter was re-dissolved in 500 μl of ethanol. The extract was filtered through 0.25 μm Acrodisc and the concentration of cannabinoid-like compounds in the extracts was measured by UPLC analysis.

Normal human dermal fibroblast cultivation and treatments
Normal human dermal fibroblasts were cultured in DMEM medium containing 1.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin until 90% confluence and then trypsinized (0.05% trypsin/1 mM EDTA in calcium- and magnesium-free PBS). For the treatment, the cells were grown in six-well plates (1.5 x 10^5 cells/ml) for 24 hours. After fresh medium had been added, the cells were induced with LPS (100 ng/ml final concentration in the culture) in PBS, and incubated for 6 hours, before the CBD preparations (the final concentration of CBD-like compound was 600 nM or 300 nM) were added. For analysis of the flax cannabinoid-like compound activity, a CBD standard positive control was included (pure CBD in a final concentration of 600 nM or 300 nM). The ethanol concentration was 0.1%. The cells were harvested after 24 hours of treatment and stored at -20ºC in the presence of RNAprotect Cell Reagent (Quiagen).

RNA isolation
Total RNA from the NHDF cells was isolated using an RNeasy Plus Mini Kit (Quiagen). The RNA quality and quantity were determined by absorbance measurements at 260 and 280 nm and by agarose gel electrophoresis.

Microarray experiments and data analysis
NHDF cell RNAs after the treatments with flax fiber extracts (600 and 300 nM) or CBD standard (600 nM) with respective controls (ethanol, LPS and negative) were prepared in three biological replicates. All samples were processed according to GeneChip 3' IVT Express Kit (Affymetrix) instructions, hybridized on the Human Genome U133A 2.0 Array (Affymetrix) and scanned. The quality of data obtained from the experiment was analyzed in Bioconductor. Annotation of the genes was based on the custom library using the Entrez Gene database. The data were normalized according to the GC-RMA method and differentially expressed genes (DEG) were identified using LIMMA software.

cDNA synthesis
To ensure no genomic DNA contamination, isolated RNA was DNase treated: 2 µg of RNA from each sample was treated with DNase I (Invitrogen), and directly used as a template for cDNA synthesis. cDNAs were synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Semi-quantitative PCR
Reactions were carried out using Phusion DNA polymerase (Finnzymes) on cDNA as a template, in a final volume of 25 µl, according to the manufacturer's instructions. Gene-specific oligonucleotides were obtained from Genomed (Warsaw, Poland). The sequence of the primers and reaction details are shown in Table 1. The reaction products were analyzed via agarose gel electrophoresis, and the products were visualized by ethidium bromide staining.

Real-time (RT) PCR
RT PCR reactions were carried out using a SYBR®Green Master Mix kit (Applied Biosciences) on an Applied Biosciences 7500 RT PCR cycler. The cDNA as template and priming oligonucleotides were the same as for the semi-quantitative PCR for mouse cell line treatments. Gene-specific primer sequences for human fibroblast treatments are shown in Table 2. The reaction conditions were designed according to the kit manufacturer's instructions, with an annealing temperature of 60ºC. The specificity of the primers at this temperature was confirmed by analyzing the products using the melting curve method. The reactions were carried out in five replicates. The ΔΔCt method was used to calculate the level of transcripts.

Table 1. Details of the semi-quantitative PCR reactions.

Gene	Primer sequence	Reaction details
GADPH	Fwd. GTGAAGGTCGGTGTGAACG Rev. AGATGCCTGCTTCCCATTC	55ºC annealing temp., 23 cycles
CB2	Fwd. TCTGTGTTACCCGCCTACCT Rev. GTGGGGAAAGCTCAGAGCAG	68ºC annealing temp., 35 cycles
SOCS-1	Fwd. GAGCTGCTGGAGCACTACG Rev. GAGGTCTCCAGCCAGAAGTG	68ºC annealing temp., 33 cycles
IL6	Fwd. GAGGATACCACTCCCAACAG Rev. AAGTGCATCATCGTTGTTCAT	62ºC annealing temp., 33 cycles
MCP-1	Fwd. CCTGCTGCTGGTGATCCTCTT Rev. CATGCTTCTGGGCCTGCTGTT	64ºC annealing temp., 33 cycles

Table 2. Human gene specific primer sequences for real-time PCR.

GAPDH	Fwd. AGGTCGGAGTCAACGGAT Rev. TCCGGAAGATGGTGATG
SOCS-1	Fwd. TTTTCGCCCTTAGCGTGAAG Rev. CATCCAGGTGAAAGCGGC
IL6	Fwd. CCAGGAGCCCAGCTATGAAC Rev. CCCAGGGAGAAGGCAACTG
MCP-1	Fwd. CCCCAGTCACCTGCTGTTAT Rev. AGATCTCCTTGGCCACAATG

RESULTS

Recently, flax plant transformed with three genes coding for key enzymes of the flavonoid biosynthesis pathway has been obtained (type W92). Selected transgenic flax showed increased content of antioxidative compounds and thus improved resistance against pathogen infection [22]. In this work transgenic and control plant parts and products were further analyzed for metabolite contents. The obtained data reveal the presence of a new terpenophenolic molecule in flax plants.

UPLC and MS analysis of flax extracts

The metabolomic analysis of generated transgenic plants, type W92, showed increased quantities of several flavonoid compounds, as reported recently [8, 22]. Also recently it has been demonstrated that manipulating expression (repression/overexpression) of a gene coding for an enzyme involved in a certain route of metabolite synthesis leads to redirection of the substrate to another route of the metabolic pathway [23]. This was a reason for quantity analysis of other compounds from neighbouring pathways such as the terpenophenol biosynthesis route [8]. Since the compounds are hydrophobic in nature the plant material or flax product was chloroform extracted. The UPLC chromatogram of hydrophobic compound analysis revealed peaks slightly differentiating transgenic plants from non-transformed ones (Fig. 1A). It was found that these peaks might reflect cannabinoid derivatives. Based on the retention time and UV spectra of standards, one of the peaks was tentatively identified as cannabidiol (Fig. 1B). This peak was accompanied by two additional peaks with almost the same UV spectra, suggesting the presence of CBD derivatives.

Further investigations revealed the presence of CBD-like compounds in each analyzed flax plant's organs and products: seedcakes, leaves, stems, fibers and in flax fabric as well (Fig. 2). The quantity measurement was based on integration of the CBD peak from UPLC analysis. The highest quantity of CBD was detected in seeds and seedcakes. The transgenic plant with increased flavonoid content showed a slightly higher level of CBD-like compounds in some of the analyzed plant parts, but the differences were not statistically significant. The compounds were not detected in the flax oil. The level of CBD-like compound in the stem (the source of fiber) was comparable to that in flax fabric, suggesting that fibers processing only slightly affects the compound content.

Further identification of the CBD-like compound in flax was performed using GC-MS analysis. The HPLC purified CBD fraction that derives from the preparation CBD^{II} was analyzed by mass spectrometry. The analysis confirmed the presence of a compound with spectra corresponding to CBD. A compound with the retention time the same as for the CBD standard (32.5 minutes) was of particular interest. The MS spectra of CBD from flax and a commercially available standard (inset) are presented in Fig. 3; they are very similar and showed high similarity to those previously published by others [24].

The obtained spectra confirmed the data from UPLC analysis and strongly suggested that flax plant contains an active system for cannabinoid biosynthesis. However, it is as yet difficult to define the precise structure of the CBD-like compound from flax. The reason is that several cannabinoids have similar MS spectra in the measured range of m/z values and the ions 174, 193, 231 and 314 are present in the spectra of several of them [24]. However, the combination of ions (and their intensity) found in a spectrum from flax extract is characteristic of CBD. This taken together with retention time data from the UPLC analysis suggests that CBD is the major cannabinoid compound in flax extracts, but the presence of other derivates cannot be excluded. This finding is very interesting,
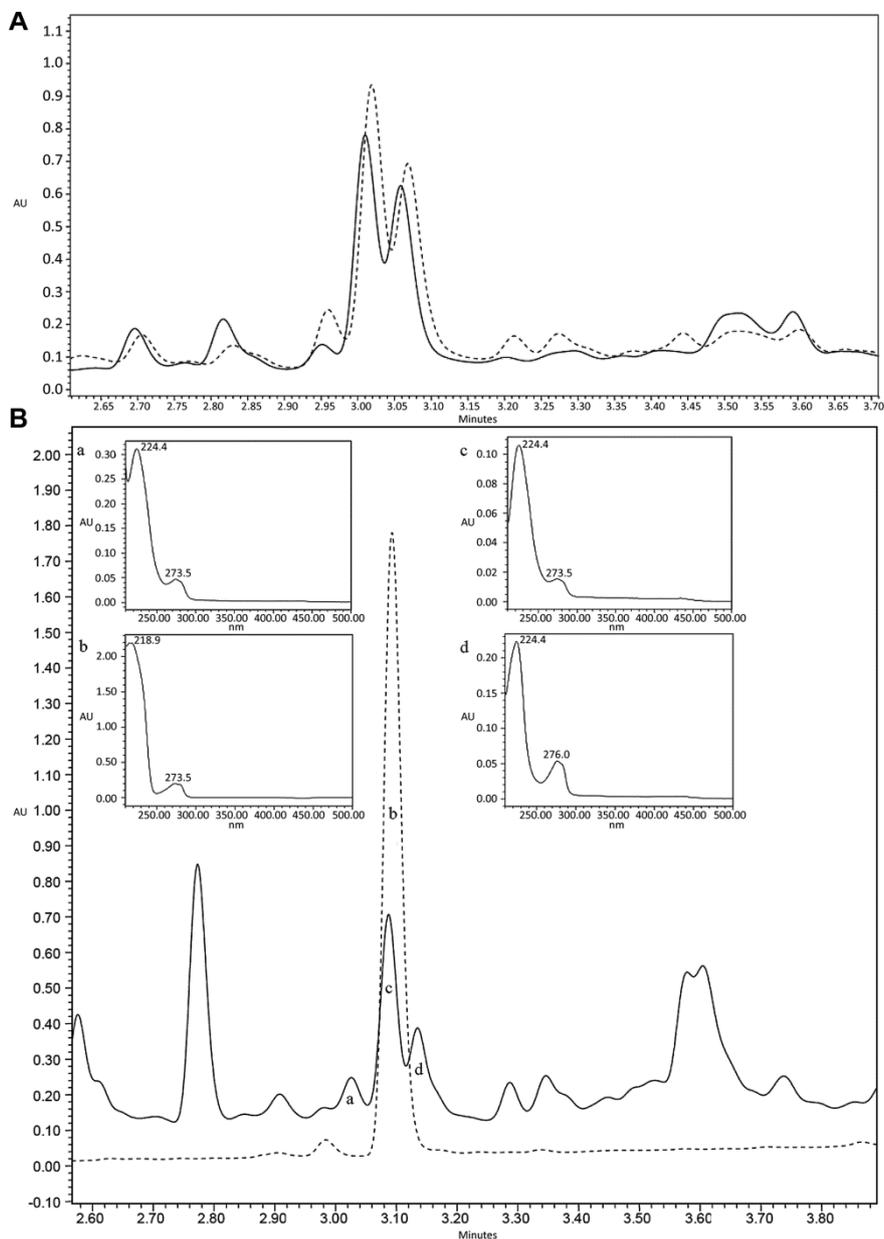
Fig. 1. The UPLC analysis of seed chloroform extract from control (solid) and W92-type transgenic plants (dashed) (panel A), and the UPLC profile of seed extract from W92 transgenic plants (solid) and the CBD standard (dashed) (panel B). The insets in B are the UV spectra of a group of three peaks (a, c and d), whose absorption spectra are similar to cannabidiol, and the retention time of the middle peak is exactly the same as for the CBD standard (peak b). The rest of the chromatogram showed the peaks of unknown compounds.

Fig. 2. The UPLC-based measurement of CBD-like compound content in different flax organs and products. WT – wild-type plants, W92 – the transgenic line with overexpression of flavonoid synthesis genes. The values are mean ± SD of three replicates.
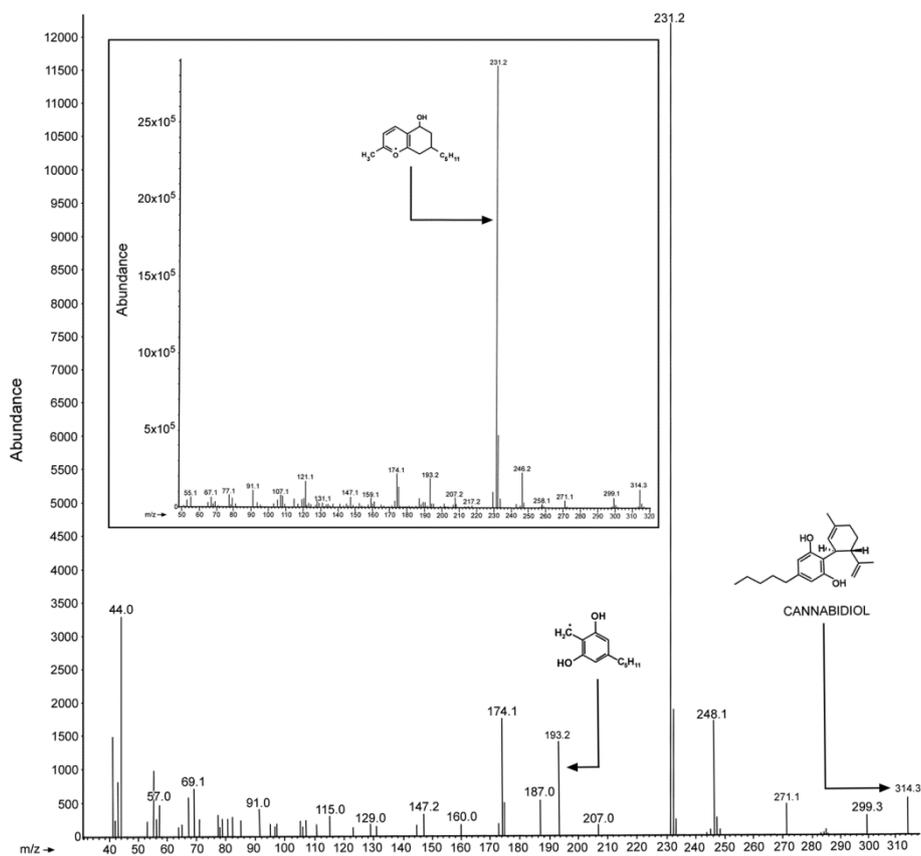

Fig. 3. The mass spectra of CBD from flax and respective standard (inset).

since the presence of cannabinoids has so far been reported only for *Cannabis* plants. Synthesized from isoprenoid and phenolic compounds, they can form many possible derivates, such as THC, CBD, cannabinol (CBN), CBC and their acidic forms. Of these, CBD is a non-psychoactive agent, with a variety of pharmacological activities [11].

Additional confirmation of the molecule's features was based on biological assay. The assay consisted of mouse and human fibroblast treatment and measurements of specific transcript level, proven to change after cannabinoid exposure.

For the mouse fibroblast treatment, two preparation of CBD from flax fabric (CBD^I, CBD^{II}) were used. In the CBD^I preparation the concentration of the compounds was much higher (130 μM) than in CBD^{II} (31.4 μM), but the second fraction showed higher CBD compound purity (about two-fold) in relation to other cannabinoid-like compounds (Fig. 4).
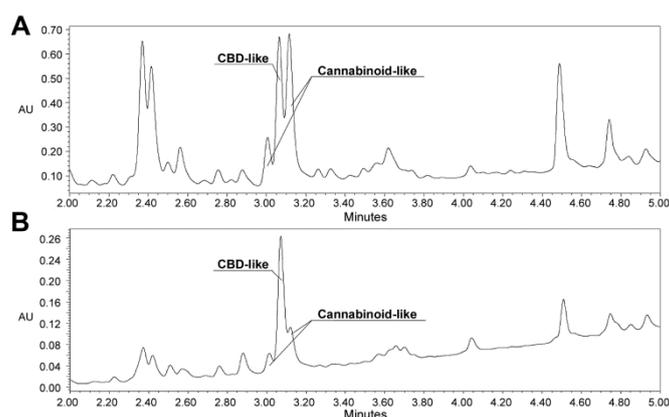

Fig. 4. The UPLC analysis of flax fabric extracts used for mouse fibroblast treatment. The preparation CBD^I with compound concentration 130 μM (panel A). The preparation CBD^{II} with compound concentration 31.4 μM (panel B).

The reason for selecting the fabric extract as a source of CBD was that it contains a relatively high concentration of CBD when compared to other compounds present in the extract and also that flax fabric can be directly used as a biomedical product, for example for chronic wound treatment.

Flax CBD biological activity assay

The cannabinoid biological activity assay was based on measurements of expression of selected genes, known to be key regulators of inflammation-related processes, these being MCP-1, IL6 and SOCS-1. Cannabinoid receptor 2 gene expression was monitored to ensure that the experimental conditions were optimal for the cells analyzed. In mouse cell treatments, inflammation was induced by LPS or TNF-α, and the gene transcript levels were measured in the presence of flax fabric extracts (Fig. 5). The expression of a housekeeping gene (GAPDH) was used as a control. The data from semi-quantitative PCR analysis

(Fig. 5A) suggest that all cells express the CB2 receptor gene in the experimental conditions. We have observed an increase in CB2 mRNA level in TNF-α-induced cells treated with flax extract.
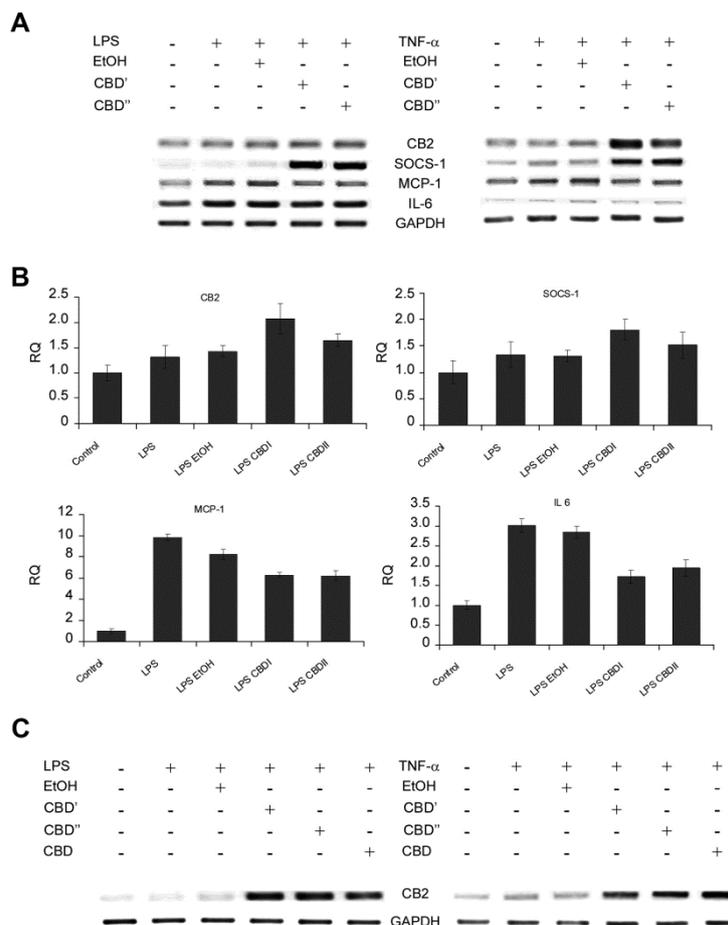

Fig. 5. Gene expression in 3T3 BALB mouse fibroblasts treated with CBD preparations from flax fibers. Semi-quantitative PCR showing expression of selected inflammation-related genes (panel A). Real-time PCR showing expression of selected inflammation-related genes in cells untreated (Control) and treated as indicated; data are the mean values ± SE of five replicates (panel B). Semi-quantitative PCR showing expression of CB2 receptor gene in cells treated with flax CBD preparations in comparison to the CBD standard (panel C). LPS – lipopolysaccharide; TNF-α – tumor necrosis factor α; CBD[I] with compound final concentration 130 nM, CBD[II] with compound concentration 31.4 nM, CBD – cannabidiol standard of 130 nM final concentration; CB2 – cannabinoid receptor 2; SOCS-1 – suppressor of cytokine signaling 1; MCP-1 – monocyte chemotactic protein 1; IL6 – interleukin 6; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; RQ – relative quantification.

Semi-quantitative and real-time PCR results shown in Fig. 6 suggest the anti-inflammatory activity of preparations from flax fabric. The mRNA level for SOCS-1 increases in LPS- and TNF-α-induced cells upon flax extract treatment. The expression of two pro-inflammatory genes, MCP-1 and interleukin 6, induced by LPS and TNF-α, was also affected by the CBD preparation treatment (Fig. 5). In the LPS treatment the changes were not so obvious (Fig. 6A), but a more detailed real-time PCR analysis showed the influence more clearly (Fig. 5B). The results also suggest that the changes in gene expression level upon flax cannabinoid-like compound treatment are concentration dependent, and that the 31.4 nM level of the compound does not cause an effect, while the 130 nM final concentration induces small but noticeable changes. The level of mRNA for the CB2 receptor in LPS-induced cells also increases after the treatment, when measured by real-time PCR.
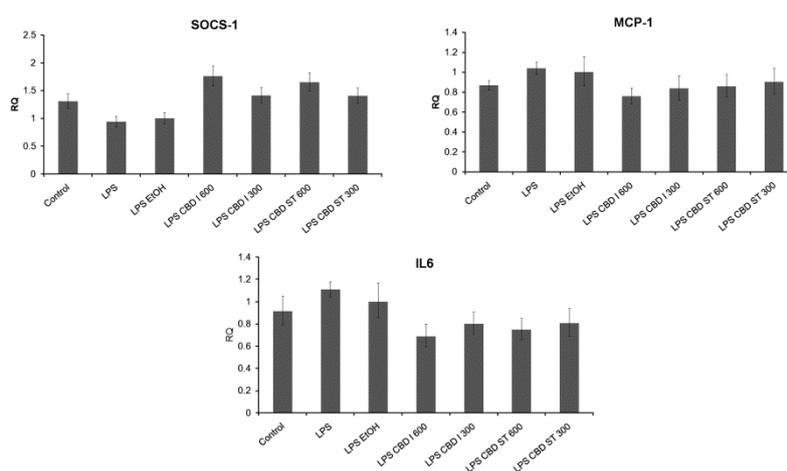

Fig. 6. The impact of flax fiber preparations on gene expression in normal human dermal fibroblast. Real-time PCR showing expression of selected inflammation-related genes in cells untreated (Control) and treated as indicated; data are the mean values ± SE of five replicates. LPS – lipopolysaccharide; $CBD^{I\ 300}$ and $CBD^{I\ 600}$ – flax fiber CBD preparations with compound final concentration 300 and 600 nM, respectively; $CBD^{ST\ 300}$ and $CBD^{ST\ 600}$, pure CBD standard with 300 and 600 nM concentration, respectively; SOCS-1 – suppressor of cytokine signaling 1; MCP-1 – monocyte chemotactic protein 1; IL6 – interleukin 6; RQ – relative quantification.

In both LPS and TNF-α induced cells we found for the first time the activation of CB2 receptor gene expression after treatment with flax preparations. In order to verify this new finding we examined the action of CBD standard on mouse fibroblasts. For that reason cells treated with 130 μM CBD concentration in ethanol were analyzed. The analysis of CB2 gene expression was based on semi-quantitative PCR and data presented in Fig. 5C. The results strongly suggest that the impact of the flax preparation on CB2 gene expression in the mouse fibroblastic cell line is due to the CBD compound. It should be pointed out that

this is the first report on CB2 gene expression activation upon CBD compound action. However, the mechanism of this induction remains as yet unknown.

Further investigation of the cannabinoid compound's biological activity was conducted on normal human dermal fibroblast (NHDF) cells. The cells were treated with flax fabric CBD[1] preparations and compared to CBD standard action. In this experiment a higher concentration of CBD was used since the concentrations that affected mouse fibroblast gene expression were not effective enough. Thus after immunological induction with LPS, NHDF cells were treated with concentrated fabric preparations (300 nM and 600 nM concentration of CBD-like compound in cell culture). The real-time PCR analysis of the genes of interest corresponding to those investigated in mouse fibroblasts are presented in Fig. 6. Significant induction of human SOCS-1 gene expression and decrease in Il6 and MCP-1 transcript levels were observed. The gene expression changes were CBD specific and compound concentration dependent.

In summary, the biological activity assay positively proved the identification of CBD-like compounds in flax fabric extract suggested by UPLC and GC-MS analysis. The mRNA level increase for the SOCS-1 gene, and decrease in MCP-1 and IL6 genes upon human and mouse fibroblast treatment with fabric preparations indicate the biological activity of the CBD-like molecules. CB2 receptor expression changes in the mouse cell line also suggest that the compound is indeed CBD, though the mechanism of this activation is as yet unknown. Comparison of the changes induced by flax preparation in human dermal fibroblast gene expression levels with those of the CBD standard confirm the presence of the cannabinoid-like anti-inflammatory activity of the newly identified compound.

DISCUSSION

Traditionally flax fibers were only used for textile production. Recently however, by supplying new metabolites (polyhydroxybutyrate) or by increasing the content of endogenous compounds, a new area of fiber application has appeared. Those with polyhydroxybutyrate were proposed for use as a component of tissue engineering scaffold (implant) and data from a successfully used new implant for muscle tissue repair in rats were recently reported [25]. Also those enriched with phenylpropanoid compounds with high antioxidant potential were successfully used for wound dressing production and chronic wound healing [7]. In the course of detailed metabolic analysis of flax fiber new compounds were detected. Based on retention time, UV spectra and mass spectrometry analysis, these new compounds were identified as terpenophenols. This finding is interesting for two reasons. First, to this date this kind of compound was detected only in *C. sativa* and never found outside of this species. Second, the presence of cannabinoids in fiber strongly suggests the active pathway of their synthesis in flax and generates the possibility of its manipulation for increasing the content of these compounds in future. Increasing the compound content

might enhance the anti-inflammatory and anti-analgesic properties of wound dressing based on flax fibers. These two reasons were the direct stimuli for detailed analysis of cannabinoid-like compounds from flax with respect to their biological activity. It has been proven in both *in vitro* and *in vivo* studies that plant-derived cannabinoids regulate immunological responses at the molecular level [19, 20, 26, 27].

Two cannabinoid receptor types have been found so far: cannabinoid receptor 1 (CB1) [28] and 2 (CB2) [29]. The CB1 receptor was first found in the cortical rat brain, and soon afterward in the analogous human and mouse tissues, while the CB2 receptor is expressed mainly in peripheral tissues and also tumor cells [12]. Both molecules are $G_{i/0}$ protein-coupled receptors that inhibit adenylate cyclase, PKA and CREB activity. By this mechanism they can directly change the expression of particular proteins, thus explaining their influence on inflammatory agent production [30, 31].

Inflammatory processes play an important role in a broad spectrum of medical disorders, including neuropathic pain, multiple sclerosis, Alzheimer's disease, atherosclerosis, rheumatoid arthritis, asthma and allergies. The primary process activator is bacterial cell wall lipopolysaccharide (LPS), which activates the TLR4 receptor, leading to NFκB transcription factor family activation and the expression of pro-inflammatory intracellular signal transducers (cytokines and chemokines) such as interferon γ (IFN-γ), interleukin 1β (IL1β), interleukin 6 (IL6), interleukin 8 (IL8), monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor α (TNF-α) [32-34]. TNF-α, through activating the respective receptor (TNFR), partially involves the same signaling pathway, thus acting as an additional endogenous signal transducer with an effect similar to LPS [35]. The opposite effect is produced by suppressor of cytokine/chemokine signaling 1 (SOCS-1), which specifically suppresses LPS-induced cytokine and chemokine production. SOCS-1 can bind to the NFκB subunit, inducing its degradation [36].

In order to analyze further the CBD-like compounds from flax, two experiments were performed. In the first one the fibroblasts were treated with a CBD preparation and the gene expression profile was analyzed by microarray technique using Affymetrix Human Genome U133A 2.0 Array. It was found that 210 genes were expressed differentially after treatment with the flax CBD preparation (130 of them were identical to those that responded to the CBD standard). Of those, 106 genes were activated and the expression of 104 genes was repressed. The affected genes could be grouped into several types. The selected genes affected by the flax CBD treatment are depicted in Table 3.

It is interesting that among activated genes were those involved in anti-inflammatory signaling and those that were repressed belong to the group of genes that promote inflammation. The second experiment was based on the result from the first and concerned detailed analysis of expression of genes in fibroblasts upon treatment with LPS or TNF-α. In cells treated with LPS or TNF-α in the presence of flax CBD the expression of genes which are known to be involved in inflammation signaling or development (SOCS-1; MCP-1; IL6)

or respond directly to cannabidiol (CB2 receptor) was measured. The CBD activates cannabinoid receptor CB2 and strongly affects other signaling pathways such as those that lead to inflammation propagation.

Table 3. The genes affected by flax CBD[II] treatment as revealed by microarray analysis.

	Genes inhibited
Inflammation	TNFα-induced protein 3; CD83 molecule; TNF (ligand) sf, member 10; IFN-induced protein with tetratricopeptide repeats 1; chemokine (C-X-C motif) receptor 7; chemokine (C-X-C motif) receptor 4; IL 3 regulated NF; interleukin 15; prostaglandin G/H synthase and cyclooxygenase; prostaglandin E synthase; cytokine receptor-like factor 1; CD59 molecule; complement regulatory protein; CD97 molecule
G proteins	GPCR 37; GPCR 64; GPCR, fam C, gr 5, mbr A; regulator of G-protein signaling 2; A kinase (PRKA) anchor protein 12; CREB3-like 2; CREB reg transcription coactivator 3; Rho GTPase act protein 12; Rho/Rac guanine nt exchange factor (GEF) 18; GTP-binding protein 8; GTP cyclohydrolase 1; annexin A6; Ras homolog gene family, member B and C
Signaling pathways	phospholipase A2, gr IVA (cytosolic, Ca-dependent); PKC delta binding protein; ser/thr kinase 38; ser/thr kinase 19; ser/thr kinase 16; WNT1 inducible sign pathway protein 2; protein phosphatase 1 inhibitor subunit 3C; protein phosphatase 4 catalytic subunit and reg subunit 2; serum/glucocorticoid reg kinase 1; MAPK kinase 6; immediate early resp 2
Growth factors	connective tissue growth factor; early growth response 1 and 2; TGFβ receptor III; latent TGFβ binding protein 1
Extracellular matrix	collagen I, α1 and 2; collagen IV, α 2; collagen V, α 1; procollagen C-endopeptidase enhancer 1 and 2; mucin 16; cell surface associated melanoma CAM; chondroitin sulfate N-acetylgalactosaminyl transferase 1; ADAM metallopeptidase with thrombospondin type 1 motif 1
Cell cycle	CDK inhibitor 1C (p57, Kip2); CDK inhibitor 1A (p21, Cip1); CDK 9; cyclin M3; cyclin E2 S-phase response centromere protein F (mitosin); centromere protein M; CDC 45 homolog; cell cycle associated prot 1 growth arrest and DNA-damage-inducible, beta
Steroid metabolism	sterol-C4-methyl oxidase-like; 7-dehydrocholesterol reductase; 24-dehydrocholesterol reductase; squalene epoxidase; LDL receptor; sterol regulatory element binding transcription factor 1
	Genes activated
Inflammation	TNF receptor-associated factor 2; IFN-related development regulator 1; TNF receptor sf, member 21; IFNγ-inducible prot 30; Ig sf member 3; IFNα 7; interleukin 24; chemokine (C-X-C motif) ligand 3
G proteins	G protein α; inhibiting activity polypeptide 1; G protein γ 5; Ras-related GTP binding C; Ras interacting protein 1
Signaling pathways	MAPK 6; MAPKK kinase 14; NFKB-p105; protein kinase, X-linked (cAMP dependent); phosphodiesterase 2A; cGMP-stimulated C/EBP β and δ
Growth factors	growth differentiation factor 15; vascular endothelial growth factor C
Extracellular matrix	MMP 9 (gelatinase B, type IV collagenase); TIMP metallopeptidase inhibitor 4; adhesion molecule with Ig-like domain 2; dermatan sulfate epimerase; CD302 molecule
Cell cycle	spindle and kinetochore associated complex subunit 1; cyclin L1; G0/G1switch 2; CDKN1A interacting zinc finger protein 1 (DNA replication initiation); CDC 37 homolog like 1; CDK 7; cyclin D1; BCL2-associated X protein
ROX metabolism	SOD 1, soluble; NAD(P)H quinone dehydrogenase 1 and 2; NADH dehydrogenase (ubiquinone) 1 α and assembly factor 4; thioredoxin; glutathione S-transferase mu 3; cyt P450, fam 1, subfam B, polypeptide 1 thioredoxin reductase 1; epoxide hydrolase 1 microsomal (xenobiotic); metallothionein 1E; metallothionein 4; NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)

It is thus proposed (Fig. 7) that CBD acts in two ways: the first is direct activation of its receptor (CB2), which results in reducing pro-inflammatory gene expression; and the second is a direct impact on inflammatory regulator (SOCS-1) gene expression, leading to the same result.
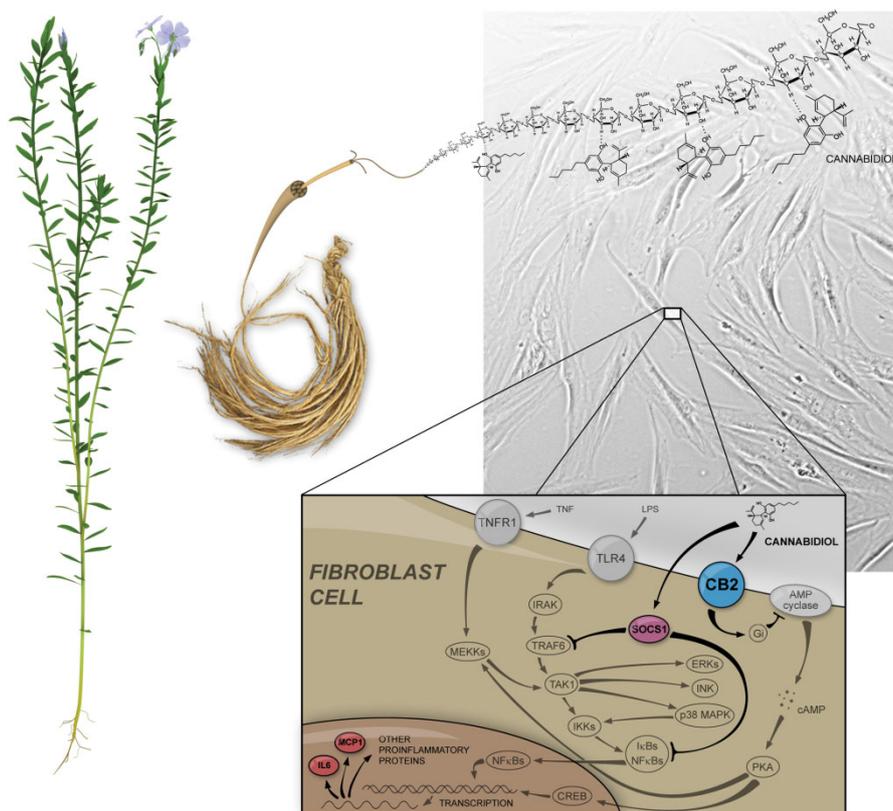

Fig. 7. The scheme of CBD binding to cellulose polymer and its impact on the genes involved in inflammatory signal transduction. The general pathways of inflammatory signaling were adopted from the literature and the genes whose expression was measured are marked in color.

From a biochemical point of view the important question concerns the nature of CBD binding to the fiber constituents. Although this is not yet known, we speculate that the compound binds directly to cellulose polymer by hydrogen bonds. It is established that the compound is released from fibers by treatment with methanol (data not shown), which is an agent known to break hydrogen bonds. This needs however to be investigated in more detail.

The results demonstrate the existence of active cannabinoid in flax plant products. To the best of our knowledge, this is the first report providing evidence for the presence of active cannabinoid in plants other than *C. sativa*.

The cannabinoid synthesis pathway enzymes have not yet been identified in flax plants, but the main and critical substrates for their synthesis have been identified in flax. The presence of fully active machinery of terpenoid and phenolic part of terpenophenol (CBD) synthesis in flax strongly supports this view.

The presence of cannabidiol in flax is interesting not only in the aspect of its so far unique natural source (*Cannabis* plants) but primarily for its natural synthesis in fibers which when used for wound dressing production might offer anti-inflammatory and anti-analgesic activity. Recently, the treatment of patients suffering from chronic wounds of different aetiology with flax wound dressing resulted in inflammation reduction [7]. The results of the clinical study are perhaps now clearer; the presence of CBD within fiber compounds might be at least partially responsible for this positive effect.

It should be pointed out that the biological activity of the CBD preparation from flax was the same as the pure compound isolated from *C. sativa* and commercially available. Of great importance is that this bioactive anti-inflammatory component is present in the final industrial flax product which is fabric based on fibers. Therefore the identification of cannabinoids in flax is important not only because they are present in plants other than *Cannabis*, but also as a ready to use, non-psychoactive, anti-inflammatory component of innovative flax fiber product for medical applications.

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