THE EFFECT OF CANNABIS EXTRACTS ON THE EXPRESSION OF FIBROSIS-RELATED GENES

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DEDICATION

I dedicate this thesis to all people that supported me with love.

ABSTRACT

Fibrosis is a condition characterized by thickening and/or scarring of the respective tissue. Up till now, we can only try to prevent this condition, and unfortunately, it is almost impossible to reverse it. Therefore, new therapeutic interventions are urgently needed. It has been shown in the literature that cannabinoids, especially cannabidiol and Δ9-tetrahydrocannabinol and *Cannabis sativa* extracts have potent anti-inflammatory properties and thus may also exhibit anti-fibrotic effects. Our lab has developed several cannabis extracts that demonstrated anti-inflammatory properties. Here, we hypothesized that these high CBD extracts may also have anti-fibrotic action. In this research, we utilized commercially available 3D-tissues to induce fibrosis. Next, we studied the effects of cannabis extracts on the expression of genes involved in the major fibrosis-related pathways using RNA sequencing and qRT-PCR. We discovered that high CBD cannabis extracts downregulate the expression of several key fibrotic genes indicating their anti-fibrotic potential at the transcriptional level.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol
AEA: anandamide
ASK1: apoptosis signal-regulating kinase 1
CB1: cannabinoid receptors 1
CB2: cannabinoid
CBD: cannabidiol
DAMPs: damage-associated molecular patterns
ECM: extracellular matrix
ECS: endocannabinoid system
EMT: epithelial-to-mesenchymal transition
EndMT: endothelial-mesenchymal transdifferentiation
FSP1: fibroblast's specific protein
GPR18, GPR55, GPR119G: protein-coupled receptors 18, 55, 119
HPLC: high performance liquid chromatography
HSCs: hepatic stellate cells
IPF: idiopathic pulmonary fibrosis
IRE1: inositol-requiring enzyme 1
JNK: c-Jun N-terminal kinase
JNK: c-Jun N-terminal kinase
MMP: matrix metalloproteinase
MMT: mesothelial-to-mesenchymal transition
MSC: mesenchymal stem cells
NAFLD: non-alcoholic fatty liver disease
NG2: neural/glial antigen
OEA: oleoylethanolamide
PAMPs: pathogen-associated molecular patterns
PDGFR-β: platelet-derived growth factor receptor-β
PRRs pattern recognition receptors
TAFI: thrombin stimulates thrombin-activatable fibrinolysis inhibitor
TF: tissue factor
ββ: transforming growth factor-β
THC: tetrahydrocannabinol
TIMP: tissue inhibitor of metalloproteinase
TRPV1, TRPV2: transient receptor potential channels of the vanilloid subtype 1 and 2
VEGF: vascular endothelial growth factor
α-SMA: alpha-smooth muscle actin
PI3K: phosphatidylinositol 3-kinase
PRRs: pathogen recognition receptors
RLHs: RIG-like helicases
RPMI: Roswell Park Memorial Institute Medium
STAT: signal transducer and activator of transcription
Th1: T helper 1 cells
Th2: T helper 2 cells
THC: tetrahydrocannabinol

TLRs: Toll-like receptors
TNF-α: tumor necrosis factor alpha
TRPV1: transient receptor potential channels of the vanilloid subtype 1
TRPV2: transient receptor potential cation channel 2
TRPV2: transient receptor potential channels of the vanilloid subtype 2

1. Introduction

Fibrosis is a pathology associated with the replacement of parenchyma with connective tissue during the healing process. Fibrosis is defined as an excessive growth, stiffness, and sometimes scarring of different tissues or organs along with an over accumulation of extracellular matrix components and collagen [1]. Fibrotic illness is not well understood. It has a poor outcome and is mainly untreatable, all of which is compared to the terminal stage of cancer [2]. This condition is a lifelong pathological anomaly that may occur in various organs (Table 1), with a higher frequency in the skin, liver, heart, kidneys, and lungs.

Different types of fibrosis have been recognized based on anatomical location such as lungs (idiopathic pulmonary fibrosis (IPF), cystic fibrosis, emphysema), liver (cirrhosis, portal hypertension, hepatocellular carcinoma),skin (keloids, systemic sclerosis) and so on. The most studied example of fibrosis is IPF. This condition is lifelong and incurable that targets lungs. The disease usually affects middle-aged and older adults and is characterized by a long-lasting cough along with difficulties in breathing of an unknown origin, which in turn makes IPF diagnosis very difficult. Many IPF patients struggle with an acute worsening of breathing that is correlated with high mortality. The progression rate of this condition is highly unpredictable. Some patients can deteriorate very quickly, while others may remain asymptomatic for many years. The development of treatment is focused on fibroproliferation and fibrogenesis [1], [3], [4]. Because of insensitivity to pharmacological treatments, an average survival time is three years.

1.1 *Cannabis sativa* plant and cannabinoids

Various positive impacts of cannabis on humans were reported since the ancient period. Cannabis has been widely known as a plant with psychoactive properties. It includes over 500 compounds including cannabinoids, terpenes, terpenoids, fatty acids, and flavonoids. Cannabinoids (known as phytocannabinoids in contrast to endocannabinoids) act via modulating the endocannabinoid system. The most abundant and well-studied are cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) with numerous documented medicinal properties [5], [6]. The uniqueness of the effect of cannabis extracts is in its entourage effect, which means that the effectiveness of the whole extract is much more powerful, in comparison to its singular compounds, such as cannabinoids, terpenoids etc. Often, but not always, cannabis extracts have more profound effects on various diseases and conditions than isolated cannabinoids [7]. This is due to modifying effects of minor cannabinoids, terpenes, and other molecules that frequently act as amplifiers, acting on the same receptors. Looking at the enormous varieties of cannabis cultivars nowadays, it is clear that research in this field should continue to discover new possibilities of fibrosis treatment [8].

1.2 Endocannabinoid system

Recently, the endocannabinoid system (ECS) has received a significant attention from mainstream medical professionals, being viewed as an important therapeutic target for many pathological conditions. Human physiology significantly depends on a proper functioning of this system. The ECS has been established as an important homeostatic regulator of the human body. It regulates almost all functions of the body, for instance, reproduction, metabolic activities, etc. It consists of endocannabinoids, such as 2-arachidonoylglycerol and anandamide (2-AG, AEA), their metabolic enzymes and receptors, including cannabinoid receptors 1 (CB1), cannabinoid 2

(CB2), transient receptor potential channels of the vanilloid subtype 1 and 2 (TRPV1, TRPV2), G protein-coupled receptors 18, 55, 119 (GPR18, GPR55, GPR119) [9].

1.3 Fibrosis and Cannabis

1.3.1 Epidemiology, etiology, and pathogenesis of fibrosis

Epidemiological data on fibrosis in different organs is well documented in the literature. For example, an incidence of IPF varies between 0.6 and 17.4 per 100,000 population per year [10], two third of all patients were 60 years and above, and the highest prevalence was reported among patients of 80 years and above - 165.9 per 100,000 population [11]. In Caucasians, cystic fibrosis occurs roughly in 1 in 3,000-4,000 births; and among other races, cystic fibrosis is less frequent, 1 in 4,000-10,000 in Latin Americans and 1 in 15,000 – 20,000 in African Americans, and even less in Asian Americans [12]. As to liver cirrhosis, according to 2017 data, 112 million compensated cases were reported worldwide [13], and in patient who were more than 65 years old, a risk of severe liver fibrosis was 3.78 times higher [14]. Also, more than 100 million cases of keloid, a type of raised scar, are reported annually worldwide [15], [16].

Inflammation is body's defense response to external injury or pathogens. However, uncontrolled or chronic inflammation marks the first step in the etiology of fibrosis. There are two types of inflammation stimuli, infectious and non-infectious. Examples of the first group agents are viruses, bacteria, and/or other microorganisms. Non-infectious could be subdivided into biological, chemical, physical, and psychological. Injured cells are an example of biological subtype. Chemical examples are alcohol, chemical irritants, fatty acids, glucose, and so on.

Another category includes physical factors such as burns, frostbites, foreign bodies, lionizing radiation, physical trauma, and injury.

Table 1. Main types and causes of fibrosis

Skin	Hypertrophic scar Systemic sclerosis
Heart	Cardiac fibrosis Hypertrophic cardiomyopathy Cardiac dysfunction Valvular disease Arrhythmia
Bone marrow	Myelofibrosis Myelodysplastic syndrome Chronic myelogenous leukemia
Liver	Cirrhosis Portal hypertension Hepatocellular carcinoma
Retroperitoneum	Retroperitoneal fibrosis
Gut	Intestinal fibrosis Enteropathies Inflammatory bowel disease
Joint	Arthrofibrosis
Brain & Nervous system	Glial scar

	Alzheimer
Eye	Subretinal fibrosis Epiretinal fibrosis Vision loss
Lung	Idiopathic pulmonary fibrosis Cystic fibrosis Pulmonary hypertension Thromboembolic disease Emphysema
Mediastinum	Mediastinal fibrosis
Pancreas	Pancreabcfibrosis Cystic fibrosis Chronic pancreatitis Duct obstruction
Kidney	Renal fibrosis Cystic fibrosis Nephrogenic systemic fibrosis Chronic kidney disease Renal anemia

In most cases, fibrosis occurs after acute or more often chronic damage to tissues, followed by abnormal repair. There are two ways of repair of the injured tissues. The first one is the regeneration by the propagation of undamaged cells of parenchyma and the maturation of stem

cells – normal wound healing process. The second one is scar tissue formation through the accumulation of connective tissues – tissue fibrosis. The regeneration is a possibility of damaged tissues to be repaired and their defective elements to be restored. Cells that remain undamaged are able to proliferate and maintain the structure of the tissue. In some cases, fibrosis may occur due to a critical tissue injury or as a result of the inability of injured tissue to accomplish the repair. Fibrosis occurs due to either a large amount of collagen deposition associated with the long-lasting inflammation or ischemic necrosis. Cell proliferation is handled by growth factors, although the central role is played by extracellular matrix (ECM) and maturation of stem cells [17].

Different types of cells, such as fibroblasts, vascular endothelial cells, and some fragments of injured tissues proliferate along with the repair of damaged tissues. In fibrosis and scarring, tissue repair is characterized by the proliferation of connective tissues rather than parenchymal tissues that happens upon normal regeneration [18].

Classification of tissues by proliferative capacity

Different tissues have different proliferative capability [19]. The first group in the proliferative classification are labile cells (continuously dividing cells), which regularly die and can be restored with the help of tissue stem cells. They can regenerate fast after trauma, for example, regeneration of hematopoietic bone marrow cells, the transitional epithelium in the urinary tract, the columnar epithelium in the intestinal tract, the squamous epithelium of skin, mouth, vagina, and cervix.

The second group are stable cells that usually remain in the G0 stage (the resting phase) and have a low level of replication, but if the stimulation is present, they can return to the G1 phase and

proliferate. Examples of stable cells are the epithelium of kidney tubules, the alveolar cells of the lung, the parenchyma of the pancreas, liver fibroblasts, smooth muscles, and endothelial cells.

Finally, permanent cells are not able to proliferate; and after the damage, they repair by the connective tissue proliferation. Examples of these cells are cardiac and skeletal muscles, and neurons.

Phases of wound healing

Wound healing consists of four main phases, including hemostasis, inflammation, proliferation or granulation and remodeling or maturation, each phase lasting from days to months (Figure 1).

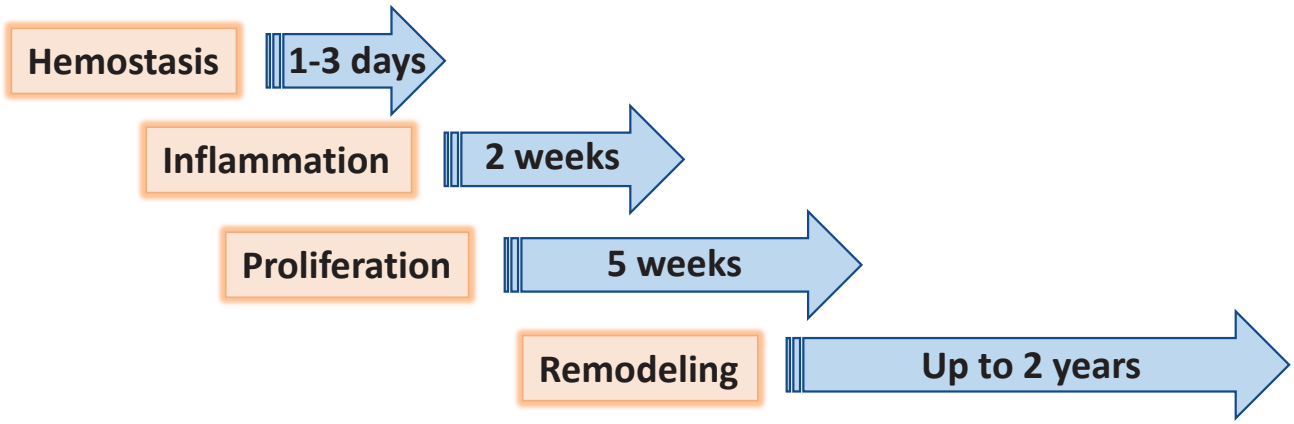


Figure 1. Phases of wound healing. Phase 1, hemostasis is the process of clot formation to stop bleeding, and includes steps such as vasoconstriction, aggregation of platelets and migration of leukocytes. Phase 2, inflammation is the process of cleaning the wound and preparing for the formation of new blood vessels. It includes processes such as release of antibacterial molecules by neutrophils, engulfing of pathogens and debris by macrophages, and release of angiogenic substances to stimulation angiogenesis and granulation. Phase 3, proliferation (or granulation) – the process allowing to bring the wound edges together and seal it. It includes proliferation of the wound by fibroblasts, with secretion of glycoproteins and collagen, followed by migration of epithelial cells from the wound edges and formulation of granulation tissues. Phase 4, remodeling (or maturation) phase is mostly a continuation of proliferation phase resulting in formation of proper tissue.

Abnormal wound healing resulting in scar formation also includes similar phases/steps, such as inflammation, cell proliferation, and remodeling, but is characterized by more extensive deposition of collagen, fibrin, fibronectin etc. [20].

The first phase – hemostasis

The most crucial step is not to restore a tissue but to stop bleeding from the injured place. Coagulation starts exactly after trauma and finishes within hours. Collagen assists this process in the damaged area. Hemostasis consists of two subphases, primary and secondary hemostasis. Primary hemostasis is the formation of a plug at the injured place where endothelial cells become exposed. In the secondary hemostasis, there are two main pathways of blood clotting: the extrinsic and the intrinsic pathways, and they come together in the common pathway. The extrinsic pathway is a primary stage in plasma mediated secondary hemostasis. Due to tissue damage, tissue factor (TF also known as platelet tissue factor or factor III) is released in the plasma, which results in binding of factor VIIa and calcium to boost the activation of factor X to Xa (Figure 2). The intrinsic pathway includes factors I (fibrinogen), II (prothrombin), IX (Christmas factor), X (Stuart-Prower factor), XI (Plasma thromboplastin), and XII (Hageman factor) [21]. The common pathway includes steps from the activation of factor X to the formation of active thrombin which brakes fibrin into a cross-linked complex.

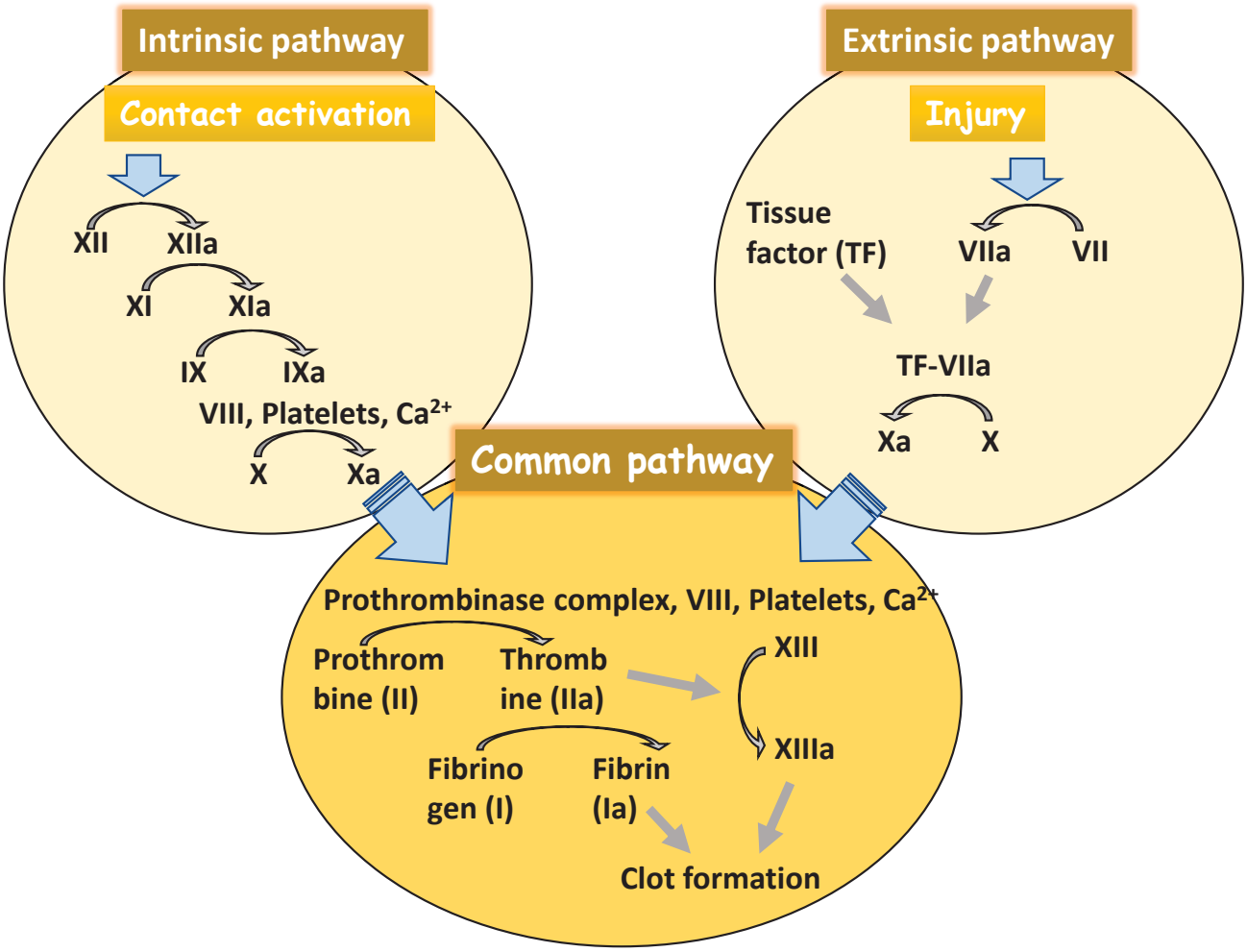


Figure 2. A clot formation cascade. There are three steps of the clotting (coagulation) cascade: the intrinsic pathway (factors XII, XI, IX, and VIII), the extrinsic pathway (factor VII), and the common pathway. During clotting, cascade factor X may be activated by the extrinsic and intrinsic pathways. The common pathway consists of steps from the activation of factor X to the clot formation. Factors that are activated are shown with a lowercase "a".

In current concepts of coagulation, there are several steps. The first step is an initiation which starts by the release of TF into the bloodstream and the formation of factor VIIa complex which leads to the activation of factor IX and X. Later on, factor Xa binds to factor II and forms thrombin (factor IIa). The next step is the amplification when thrombin activates factor V to

factor Va, factor VIII to factor VIIIa, then it activates factor XIa converting factor IX to factor IXa. Finally, platelets actively bind to factor Va, factor VIIIa and factor IXa. The propagation step is the activation of thrombin and platelets, thus leading to the activation of factor X which causes the formation of the prothrombin complex that converts prothrombin to thrombin. The stabilization step involves the formation of thrombin that activates factor XIII (the fibrin stabilizing factor) by attaching fibrin polymers and contributes to fibrin stability and strength of a platelet plug. Also, thrombin stimulates thrombin-activatable fibrinolysis inhibitor (TAFI), the primary function of which is to defend clot against fibrinolysis [22], [23], [24], [25].

The second phase - inflammation

Inflammation plays a central role in normal wound healing and fibrosis. There are two types of inflammation factors, infectious and non-infectious. Examples of the first group include viruses, bacteria, and/or other microorganisms. Non-infectious factors can be subdivided into biological, chemical, physical, and psychological ones. An example of a biological factor is damaged cells. Chemical factors are alcohol, chemical irritants, fatty acids, and glucose, while physical factors include burns, frostbites, foreign bodies, ionizing radiation, physical trauma, and injuries. The final agent is psychological excitement [26].

Tissue repair and regeneration also depend on the extent of injury and inflammation. When the injury is extensive in the presence of chronic inflammation, repair may predominate even when the damaged cells can regenerate.

The critical part of tissue regeneration and repair is the inflammatory response. Some of the cells are located in solid tissues, for instance, fibroblasts, macrophages, dendritic, and mast cells; others flow in the blood, for example, leukocytes, including monocytes, and neutrophils that can

detect cell injury or pathogen invasion. Primary initiating factor of inflammation is intracellular or surface-expressed pattern recognition receptors (PRRs). Cells that are injured can release damage-associated molecular patterns (DAMPs), while pathogens present in the wound can release pathogen-associated molecular patterns (PAMPs), and PRRs are capable of detecting them directly or indirectly [27]. When chronic inflammation is present, the cells responsible for the limitation of repair and tissue injury are enrolling to the place of infection or inflammation. Continuous DAMP release causes cellular stress [28]. Firstly, the DAMP release leads to vasodilatation and tissue edema stimulated by mast cells. Secondly, DAMPS suppress the T and natural killer effector cells and support the Th2 response. Besides PAMPs and DAMPs other elements such as gaseous mediators (NO and CO), reduction-oxidation reaction (redox), hypoxia, low or high pH and the degraded matrix components are involved in wound healing process. In normal circumstances, the inflammatory microenvironment quickly handles the damaged particles or pathogens. The essential factors of inflammation and fibrogenesis are summarized below (Table 2) [29] [30].

Table 2. Key mediators of inflammation and fibrogenesis

Profibrotic factors acting on fibroblasts	Substance	Production site	Effects
	TGFβ	White blood cells	Transformation of resident (subcutaneous, pulmonary etc.) fibroblasts to myofibroblasts. Stimulation of collagen and fibronectin transcription. Stimulation of resting monocytes and inhibition of activated macrophages.
	IL-1β	Fibroblasts, macrophages	Inflammation promotion and fibrotic responses (in part, through activation of TNFα).

	IL-6	T cells, skeletal muscle cells, macrophages	Regulation of inflammation (pro- and anti-inflammatory). Stimulation of cellular differentiation and fibrosis.
	IL-13	Mast cells, T lymphocytes, eosinophils and basophils	Stimulation of TGFβ production, proliferation of fibroblasts, collagen and MMP production.
	IL-33	Smooth muscle cells, epithelial and endothelial cells	Signals through ST2 to initiate and enhances profibrogenic cytokine production in a macrophage-dependent manner.
	TNFα	Macrophages, T lymphocytes, NK cells, mast cells, eosinophils	Stimulation of inflammation and fibrosis, in part through TGF-β signaling pathway, activation of myofibroblasts and increased secretion of MMPs.
	FGFs	Various parenchymal cells	Fibrosis enhancement through binding and activation of fibroblast growth factor receptor (FGFR).
	PDGF	Platelets, smooth muscle cells, endothelial cells and macrophages	Stimulation differentiation, proliferation, and ECM production via interaction with PDGFα and PDGFβ receptors on myofibroblasts.
	Leukotrienes (LTB4, LTC4, LTD4, LTE4)	White blood cells	Stimulation of fibroblasts proliferation and production of the matrix via modulation of the production of cyclic AMP by interaction with G-protein adenylate cyclase.
Profibrotic factors	VEGF	Macrophages, fibroblasts,	Angiogenesis promotion. Facilitates monocyte recruitment and infiltration

released from fibroblasts		platelets	of fibrotic tissues mediated through a VEGF-dependent sinusoidal permeability, leading either to resolution or promotion of fibrosis.
	IL-1	Fibroblasts	Facilitates inflammation and fibrosis through autocrine stimulation of IL-1 receptor.
	IL-6	Fibroblasts	Facilitation of inflammation and fibrosis through binding of IL-6 to IL-6Rα receptor, which then associates with the signal-transducing gp130 protein to facilitate phosphorylation of the transcription factor STAT-3. Phosphorylated STAT-3 regulates expression of pro-fibrotic genes.
	IL-33	Dermal and cardiac fibroblasts	Promotion of inflammation and fibrosis by signaling through ST2 and activating TGFβ production.
	Angiotensin II	Macrophages and myofibroblasts	Promotion of TGFβ mediated heart remodeling. Fibrosis enhancement via the angiotensin type 1 receptor (AT1).
	IGFII	Fibroblasts	Stimulation of fibrosis through mannose-6-phosphate/insulin-like growth factor receptor (M6P/IGFII receptor) in turn activating latent transforming growth factor β (L-TGF-β).
	IGFBP-3		Fibrosis initiation and enhancement by binding IGF-I and ECM components, inducing the production of extracellular matrix components such as collagen type I and fibronectin. Inhibit IGF mediated proliferation (via MEK/ERK and PI3K/AKT).
	IGFBP-5		
Antifibrotic factors acting on	PGE2	Almost all nucleated cells	Inhibition of fibroblast proliferation and suppression of collagen production. Promotion of normal fibroblast apoptosis through EP2/EP4

fibroblasts			signaling and a reduction in the Akt activity.
	HGF	Fibroblast	Prevents fibrosis and induces tissue repair acting through Met receptor and supporting the growth in epithelial and endothelial cells, but not in myofibroblasts.
	PPAR ligands	Expressed in almost all tissues	Potent antifibrotic effects, reduction of β-catenin levels. Regulate the fate determination of mesenchymal cell lineage.

The third phase - proliferation and granulation

Cell proliferation is an essential component of tissue repair, wound healing and fibrogenesis. There are several types of cells, such as epithelial cells, endothelial cells, and fibroblasts that participate in fibrogenesis and normal process of healing of the wound. Mesothelial cells originate from the embryonic mesoderm and play an essential role during trauma or infection. For instance, in pleural injuries, they assist in transporting white cells. Also, as a result of mesothelial-to-mesenchymal transition (MMT), these cells, might be genetically reprogrammed after the influence of specific stimuli. In a recent mouse model, the lineage analysis of stem cells demonstrated that MMT increased the proliferation of myofibroblasts and hepatic satellite cells during liver fibrogenesis [31].

Fibrocytes are of a mesenchymal origin and are phenotypically inactive due to a low amount of rough endoplasmic reticulum. These cells produce fibroblastic components such as collagen, fibronectin, and vimentin. When influenced by TGF-β, they can produce alpha-smooth muscle actin (α-SMA) which plays a role in angiogenesis and immunity. Fibrocytes can also migrate to the damaged area with blood flow [32].

Fibroblasts originate from the embryonic mesoderm tissues. Due to the chemotaxis feature, fibroblasts are able to migrate within tissue in response to chemical stimuli. In case of injury, they can cause contraction of the matrix that leads to the sealing of the open wound. Fibroblasts play an important role in fibrogenesis, for example, TGF-β1 dependent differentiation into myofibroblasts [33].

Epithelial cells are located in different areas of the body, such as skin, urinary tract, blood vessels, and internal organs. One of the critical features is their ability to differentiate into different types of cells. During epithelial-to-mesenchymal transition (EMT), epithelial cells become transited cells that become sensitive to the fibroblast's specific protein (FSP1). The plasticity of epithelial cells allows them to become a source of myofibroblasts in the damaged cells [34].

Endothelial cells are mainly responsible for the formation of a barrier in the endothelium of capillaries, venules, vein, arterioles, and arteries. Being stimulated by TGF-β, endothelial cells can release α-SMA and become able to convert into mesenchymal cells (endothelial-to-mesenchymal-transition, EndMT). It was demonstrated that EndMT could lead to fibrosis in the organs such as heart, kidney, and lungs [35].

Pericytes are fibroblast-like cells that surround endothelial cells in blood vessels. Pericytes are able to contract and consequently control blood flow. In the case study, it was suggested that this type of cells produce α–SMA, neural/glial antigen (NG2) and platelet-derived growth factor receptor-β (PDGFR-β). Moreover, they are a source of myofibroblasts in pulmonary tissues. Another study reported that Foxd1 progenitor-derived pericytes prominently lead to the lung fibrosis [35].

Vascular smooth muscle cells are responsible for the relaxation and contraction of blood vessels. As a result of the injury, they produce α–SMA, vimentin, desmin, and other compounds. It has also been shown that collagen type I is induced by bradykinin secretion in vascular smooth muscle cells through the TGF-β1 activation [37].

There are two main processes involved in proliferation phase of repair: formation of granulation tissues and wound contraction. Wound contraction usually starts on day 2-3 and is finished within two weeks. The primary cells that are responsible for this process are myofibroblasts, the unique cells that have features of fibroblasts and smooth muscle. The main role of these cells is the contraction of the wound by up to 80%. Granulation tissue is soft in touch and has a pink color. Granulation is a sign of tissue repair; it is formed by three steps: the inflammatory phase, the clearance phase, and the ingrowth of granulation tissue (Figure 3). During the inflammation phase, cells that are predominantly involved in the process are monocytes and neutrophils. The clearance phase is characterized by the release of autolytic enzymes from dying cells as well as enzymes from neutrophils, macrophages also clear necrotic debris. The final phase is the ingrowth of granulation tissue during which granulation tissue is formed. This phase can be divided into two processes: angiogenesis and fibrogenesis [38], [39], [40].

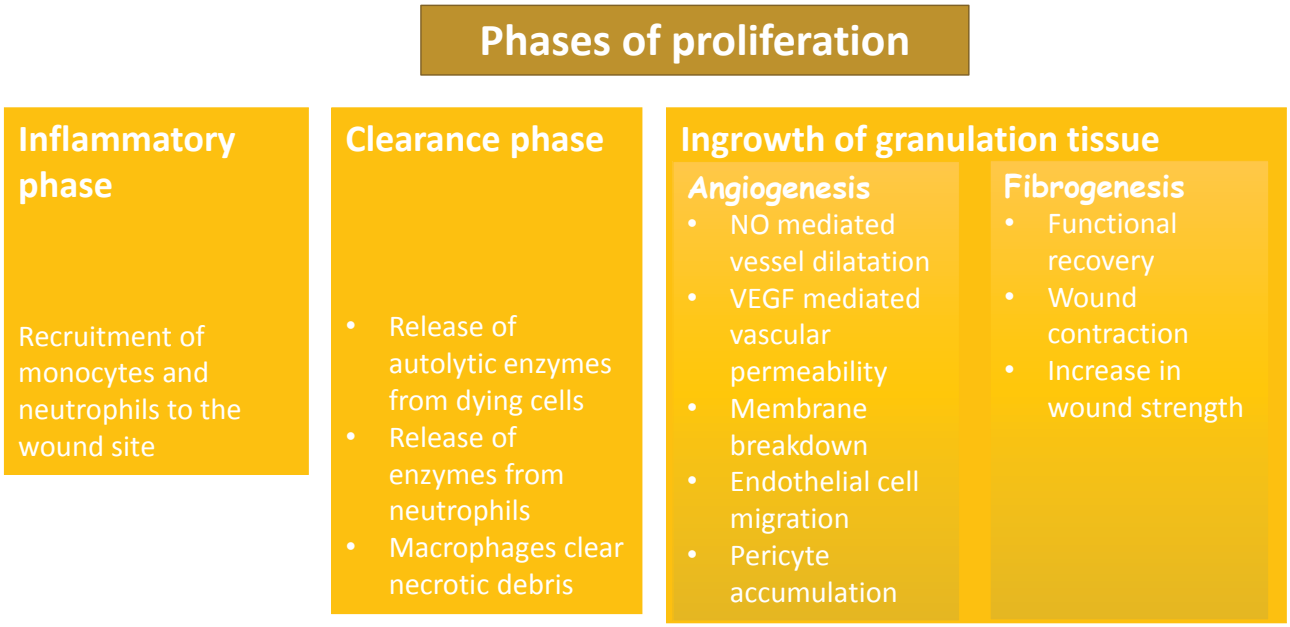


Figure 3. Phases of proliferation and granulation

Angiogenesis (neovascularization) is the development of blood vessels. Angiogenesis could be the result of sprouting either from pre-existing blood vessels or from stem cells. There are a few steps in angiogenesis from pre-existing blood vessels. The first one involves vessel dilation that is mediated by NO, and the second step includes an increased vascular permeability that is mediated by the vascular endothelial growth factor (VEGF). The next step is a breakdown of the basement membrane and the formation of a vessel sprout. The other step is the migration of endothelial cells toward chemotactic and angiogenic stimuli that cause a proliferation of endothelial cells and their maturation leading to capillary tube remodeling. The final phase of angiogenesis is the accumulation of periendothelial cells (pericyte) [41].

Angiogenesis from stem cells develops from endothelial precursor cells (EPC) stored in the bone marrow, and if needed, they migrate to the place of injury [42].

The fourth phase – healthy remodeling or remodeling with fibrogenesis

Remodeling (maturation phase) after injury usually takes place from several weeks to months or years and depends on what type of tissue is damaged, injury location, and the associated comorbidities (infections, arteriosclerosis, vein thrombosis, nutritional status, diabetes, and some drugs). During remodeling phase, rate of synthesis of collagen by fibroblasts exceeds the rate at which it is degraded, resulting in continuous increase in the amount of collagen. Remodeling includes three steps: functional recovery, wound contraction and an increased tensile strength of the wound [43]. The maturation phase is characterized by the formation of scar tissue as well as by the absence of inflammatory cells (neutrophils, macrophages) and the termination of blood vessel proliferation. Granulation tissue in the scar is replaced by dense collagen. The scar initially consists of a provisional matrix that contains fibrin, fibronectin, and collagen type III, but later on, collagen type III is replaced by collagen type I [44]. The next step is wound contraction, with the main goal being a reduction of a gap between two cut margins. Myofibroblasts play a key role during this phase. Figure 4 shows all major processes of differentiation, activation or transition of various cells into myofibroblasts. Collagen type I is responsible for the last step – an increase in the strength of the wound. The recovery of ~80% of the original tissue strength will usually take up to three months.

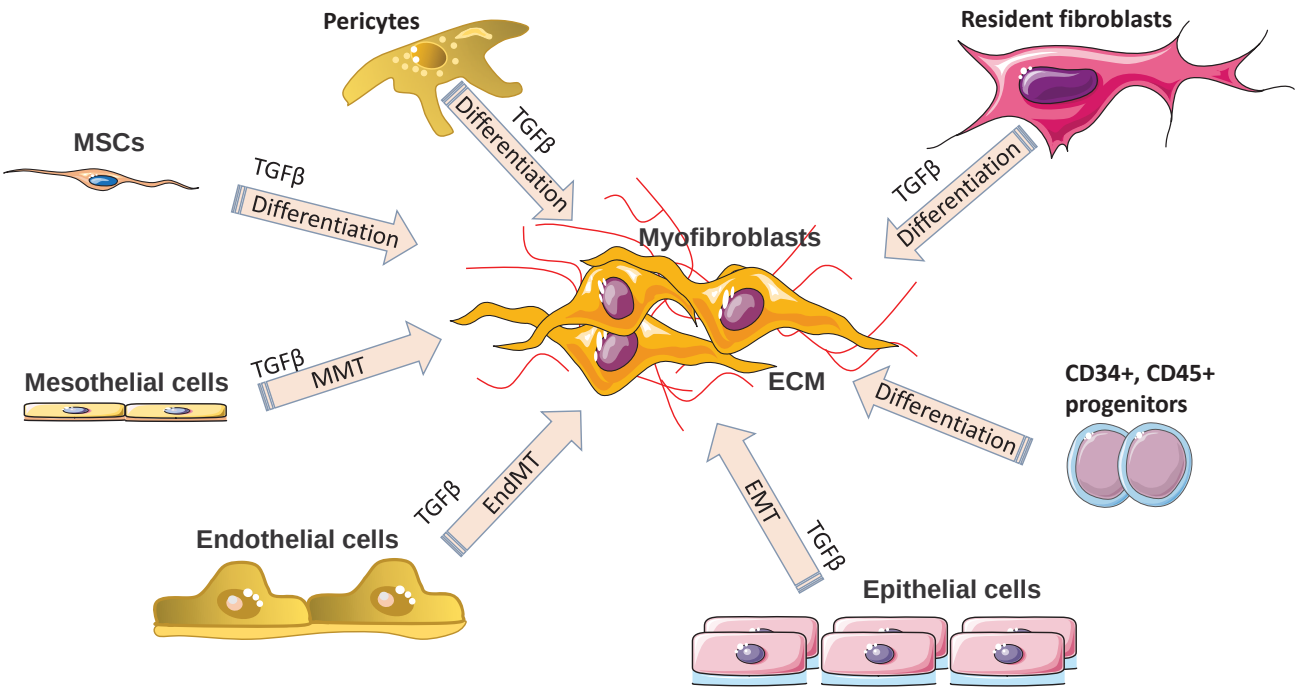


Figure 4. Myofibroblast origin in fibrosis. Resident fibroblasts, pericytes, circulating progenitor cells ($CD34^+$, $CD45^+$, bone marrow–derived mesenchymal stem cells (MSC) transition, mesothelial cells undergoing mesothelial-to-mesenchymal transition (MMT), epithelial cells undergoing epithelial to mesenchymal transition (EMT) and endothelial cells undergoing endothelial-mesenchymal transdifferentiation (EndMT) are all known sources of myofibroblasts in various fibrotic diseases. ECM, extracellular matrix; TGF-β, transforming growth factor-β.

Skin wound healing can be subdivided into primary and secondary unions [40]. By primary union (first intention), regeneration occurs with a minimum scaring tissue, for example, a clean surgical wound. By secondary union (secondary intention), the wound has the larger tissue defects with a wide distance between edges; wound healing by secondary intention occurs by regeneration and scarring. In some cases, due to abnormal wound healing, keloids or hypertrophic scars might occur. In a hypertrophic scar, there is a build-up of extra collagen fibers, which results in the elevation of the scar. Fibrillar collagen fibers are located parallel to the epidermis with a lumpy red scar, and they do not extend beyond the original scarring area. Usually, hypertrophic scars affect younger individuals with the delayed healing of wounds caused by underlying conditions such as infections, and usually, there is an improvement with treatment. Morphologically keloids

are characterized as eosinophilic, focally fragmented complexes of haphazardly arranged collagen. Also, in comparison with hypertrophic scars, one-third of keloids have α–SMA-expressing myofibroblasts. The scar tissue in keloids grows beyond the inflammation area, and it is difficult to treat [45].

Physiological injury healing vs. pathological fibrosis

Fibrosis of the organ tissues is caused by parenchymal cell destruction (alteration or injury phase); as a result of tissue trauma, macrophages become active and enter the damaged area. Also, local immune cells create chemokines and cytokines which activate mesenchymal cells located close to the injury area. The next step is the initiation of the production of extracellular matrix (ECM) and the elevated manufacturing of pro-inflammatory cytokines and angiogenic factors [33]. After trauma, cells produce inflammatory mediators that provoke the anti-fibrinolytic coagulation cascade, the first step of which is the coagulation. During this stage, known as inflammation stage, platelets are activated and form fibrin clots. Next, platelets liberate inflammatory chemokines. Then the infiltration of leukocytes happens into the injured site, and they excrete profibrotic cytokines (TGF-β and IL-13). Neutrophils are typically engaged in the infiltration process earlier than lymphocytes and macrophages [46].

The proliferation stage follows the inflammation stage; during this stage, fibroblasts become active, and myofibroblasts induce and deposit ECM that will be a framework through the tissue regeneration action. The last step is remodeling [47]. In physiological recovery, the extra volume of ECM is degraded, myofibroblasts and fibroblasts go through apoptosis, and inflammatory cells leave the recovered tissues. On the other hand, the fibrosis process extends inflammation, and

myofibroblasts stimulate the elevated accumulation of ECM which leads to the creation of a perpetual fibrotic scar. The contrasting features that distinguish fibrosis from normal wound healing are chronic inflammation, the persistence of myofibroblast activity, MMP-TIMP imbalance, and the excessive ECM deposition. These differences are very important to be understood from the therapeutic point of view because drugs can be prescribed to target these particular molecular disturbances.

Fibroblasts control synthesis and catabolism of collagen as well as an increase in collagen amount by MMPs and their inhibitors. Changing the balance between these mechanisms will cause the elevation or dropping of collagen amount inside the injured area. In addition, an increasing number of mesenchymal cells will aggravate response. During the remodeling phase, fibroblasts synthesize collagen at a higher rate than they degrade it, leading to the continuous accumulation of collagen. Generally, inflammation stimulates fibrosis. According to some reports, however, fibrosis is not always driven by inflammation. This fact clarifies the shortage of efficacy of anti-inflammatory mediators in the management of the fibrotic disease [48], [49].

1.3.2 Cannabinoids as anti-fibrotic agents

The imbalance in the ECS can significantly impact the proper functioning of the organism, including fibrosis and inflammation processes. For example, the activation of the CB1 receptor leads to fibrogenesis, while the enhancement of the CB2 receptor inhibits fibrosis progression [50]. In animal models, it was demonstrated that the deletion of CB1 caused an improvement of liver fibrosis, whereas CB2 deletion resulted in an elevated amount of collagen accumulation and an increased inflammation [51]. Concerning inflammation, the use of CB2 receptor agonists was

documented to inhibit the infiltration of inflammatory cells into liver tissue. In addition, CB2 receptor knockout mice had the more profound inflammation and damage to the liver than wild-type mice [52].

Cannabis extracts and single cannabinoids have been reported to be effective in reducing and preventing fibrosis in different organ types, such as liver, heart, lungs, skin [53]. They are able to inhibit the crucial proteins, such as *MMP-2, MMP-9, TGF-β, α-SMA, TNF-α, TIMP-1*, and in the main pro-fibrotic pathways, such as Notch, TGF-β/Smad, p38-MAPK, and in many *in vitro* and *in vivo* studies [54], [55], [56].

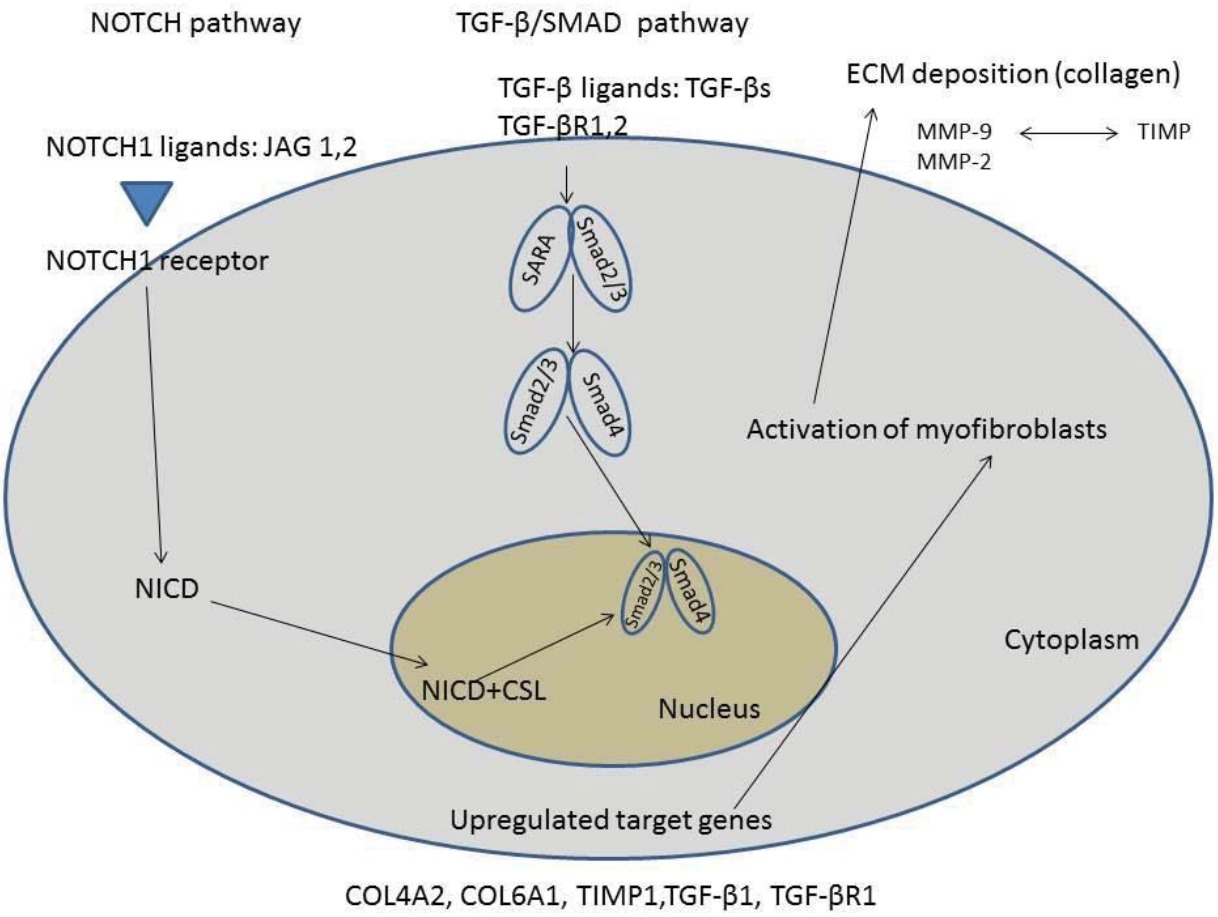


Figure 5. Main fibrotic pathways. The notch pathway consists of receptors such as Jagged 1 and 2. When ligands attach to the receptor, it causes disconnection of Notch intracellular domain

(NICD) and translocation to the cell nucleus where it binds to transcription factor-CSL (C-repeat/DRE binding factor 1 [CBF1]/suppressor of hairless/Lag1). Next, the expression of target genes is either stimulated or inhibited. TGF-β/Smad pathway requires phosphorylation and activation of Smad2 and 3 by the TGF-β receptor. Next, Smad4 attaches to Smad2/3, and this complex translocates to the nucleus for the transcription of specific genes. An excessive ECM production and its abnormal turnover cause fibrosis. Matrix metalloproteinases (MMPs) degrade collagen. Tissue inhibitors of metalloproteinases (TIMPs) are inhibitors of matrix metalloproteinases. An imbalance between MMPs and TIMPs is critical in fibrosis development. Myofibroblasts produce collagen, and their activation by upregulated expression of target genes from Notch and TGF-β/Smad pathways further enhances collagen production.

Table 3. Main function of genes involved in fibrosis.

Gene	Function
Collagen VI	This gene encodes the collagen type VI, which is the main structural component of extracellular matrix, particularly microfibrils.
Collagen IVa2	This gene encodes the collagen type IV, which is the main structural component of basement membrane.
JAG1	Ligand for the receptor NOTCH 1 in main pro-fibrotic pathway, Notch pathway. It encodes jagged 1 protein.
NOTCH 1	This gene encodes Notch 1 receptor proteins involved in Notch signaling pathway.
TGFβ	This gene encodes a ligand of the TGF-beta superfamily of proteins. Ligands of this family bind various TGF-beta receptors leading to activation of SMAD family transcription factors that regulate gene expression.
TIMP and MMP	The proteins encoded by the TIMP gene are natural inhibitors of MMPs, enzymes involved in degradation of the extracellular matrix.

A lot of research has already been done, and currently many studies are undergoing on the use of endo-, synthetic, and phytocannabinoids in the fibrosis field. In one of such *in vivo* studies where a mouse model of type I cardiomyopathy was used, it was demonstrated that CBD treatment diminished diabetes-associated cardiac fibrosis. A significant decrease of collagen deposition and the expression of profibrotic genes like *MMP-2, MMP-9, TGF-β,* connective tissue growth factor, fibronectin, and collagen-1 were noted [57].

Liver fibrosis is a usual complication of many long-lasting liver illnesses such as viral hepatitis B and C, non-alcoholic steatohepatitis, drug-induced liver injury, alcohol abuse, and autoimmune conditions. In long-lasting liver damage, the activated hepatic stellate cells (HSCs) and myofibroblasts are the main contributors to the development of liver cirrhosis and hepatocellular cancer [58]. An *in vitro* study performed on hepatic stellate cells (HSCs) documented that CBD induced the programmed cell death of these cells [59]. This effect was independent of cannabinoid receptors and was the result of endoplasmic reticulum stress induction. In addition, CBD enhanced the pro-apoptotic pathway IRE1 (inositol-requiring enzyme 1)/ASK1 (Apoptosis signal-regulating kinase 1)/c-Jun N-terminal kinase (JNK), which resulted in HSCs death. This CBD-induced programmed cell death of activated HSCs was confirmed *in vitro* in human, mouse and rat cell lines, but not in the quiescent cell lines. The well-known fact that the activated HSCs play a crucial role in the development and continuation of liver fibrosis supports the fact that cannabis extracts might be turned into promising antifibrotic drugs as they lead to the selective apoptosis of activated HSCs. The results of this study are very encouraging for further investigation of CBD *in vivo* [59]. In addition, a meta-analysis of nine studies performed on 5,976,026 patients concluded that marijuana did not elevate the prevalence or progression of liver

fibrosis in patients with hepatitis C or hepatitis C HIV co-infection. Also, it was noted that marijuana users had a reduced prevalence of non-alcoholic fatty liver disease (NAFLD). Furthermore, these patients consumed more carbonated drinks and alcohol, therefore healthy lifestyle was not a cause of the reduced prevalence of NAFLD. This effect might be induced by reducing fat depositions via increasing omega-3 fatty acids and the impact of CBD on insulin sensitivity [60].

Concerning the effect of THC, it has been shown that THC inhibits the proliferation of liver myofibroblasts and stellate cells via CB1 receptors and leads to their programmed cell death. Due to this, THC may also possess antifibrotic properties [61].

The endocannabinoid AEA also demonstrated anti-fibrogenic features by suppressing the proliferation of HSCs and induction of necrosis. The elevated AEA levels were documented in cirrhotic patients, which might be a response to fibrosis. This endogenous cannabinoid can trigger the topical inflammatory response and systemic dilatation of vessels, therefore the opportunity for fibrosis treatment was restricted [62]. Another endocannabinoid, 2-AG, was considered as a fibrogenic agent. When used in higher doses *in vitro* on HSC, it activated fibrosis via the membrane cholesterol-dependent mechanism [61]. Another endogenous cannabinoid, oleoylethanolamide (OEA), was used in a mouse model of hepatic fibrosis and showed the inhibition of collagen deposition and suppression of collagen type I and III gene expression, *α-SMA, MMP2, MMP9, and TIMP1*. These effects were mediated through the PPARα mechanisms [63].

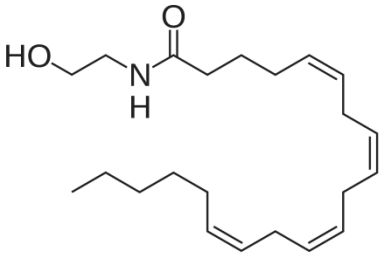
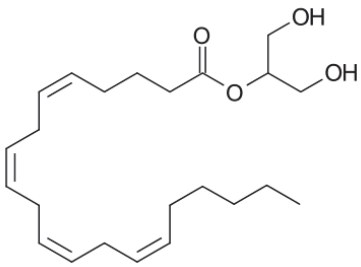
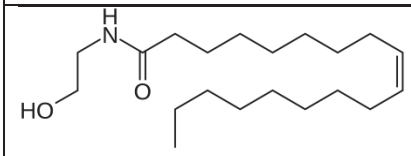
Synthetic cannabinoids were also shown to be beneficial for fibrosis treatment. An *in vitro* study performed on pulmonary fibroblasts demonstrated that JWH133, a CB2 receptor agonist,

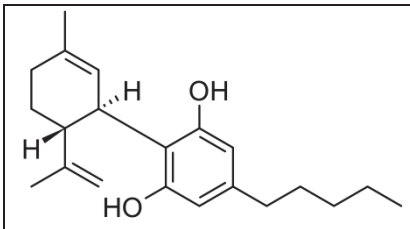
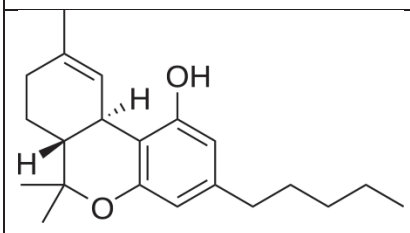
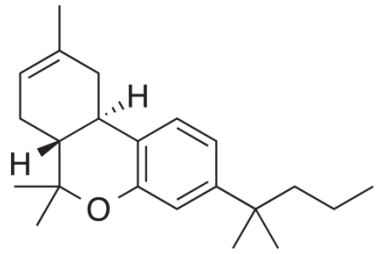
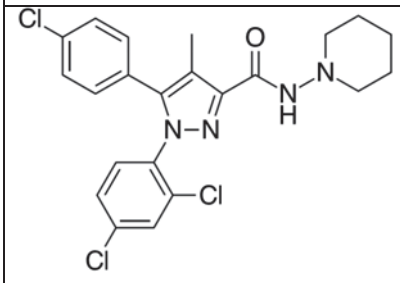
suppressed collagen type I and α-SMA and inhibited the proliferation and migration of fibroblasts. These effects were reversed by the use of a CB2 receptor antagonist, SR144528. *In vivo* studies on bleomycin-induced lung fibrosis in mice showed that JWH133 decreased the lung density, and the fibrotic score and histological results illustrated the suppression of collagen accumulation and inflammatory response. In both models, this particular synthetic cannabinoid inhibited the crucial pathway of fibrogenesis, TGF-β1/Smad2 [64]. WIN-55,212, a non-selective CB1 and CB2 receptor agonist as well as JWH133 were assessed on the mouse model of systemic sclerosis. They prevented the development of dermal and pulmonary fibrosis and inhibited the proliferation of fibroblasts. The $CB2^{-/-}$ mice developed a significantly enhanced skin and lung fibrosis compared with $CB2^{+/+}$ or wild-type mice, indicating significant influence of the CB2 receptor on fibrosis development [65]. Rimonabant, a CB1 receptor antagonist, was assessed on rat models of liver cirrhosis induced by carbon tetrachloride. Fibrosis was prominently suppressed by the use of this synthetic cannabinoid in rats compared with rats in the vehicle group. Rimonabant downregulated expression of the fibrogenic (*TIMP-1, TGF-β, MMP13, MMP2, MMP9, MMP1, MMP8*) and inflammatory mediator (*TNF-α, MCP-1*) genes. In addition, Rimonabant treatment induced a prominent increase in the expression of the CB2 receptor [66]. Another study demonstrated that chronic stimulation of CB2 receptor with selective CB2 receptor agonist, JWH-133, leads to regression of fibrosis in cirrhotic rats. This selective agonist suppressed the inflammatory infiltrate, decreased fibrosis, lowered the number of activated hepatic stellate cells, and improved arterial pressure in comparison to the vehicle group. In addition, JWH-133 reduced levels of α-SMA and collagen and elevated levels of MMP-2 in the liver tissue of rats with cirrhosis in comparison with untreated rats with cirrhosis. This data provided promising results for the possibility to use selective CB2 receptor agonists as a treatment modality of hepatic fibrosis in humans [67]. Another study tested the effect of a

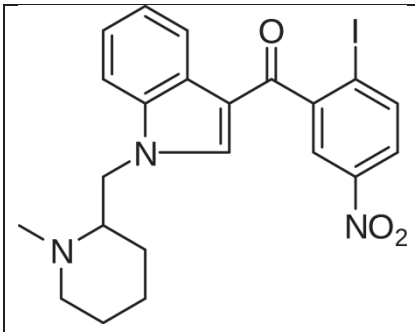
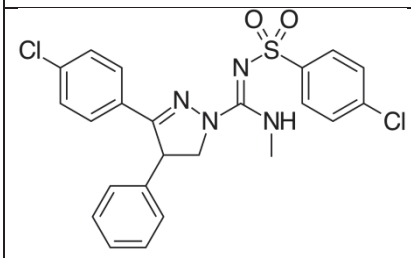
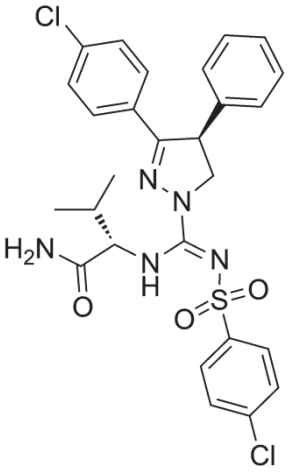
selective CB2 receptor agonist, AM1241, on myocardial fibrosis post-myocardial infarction in mice. The echocardiography results demonstrated that AM1241 significantly enhanced cardiac function; downregulated expression of collagen I, collagen III, TIMP-1, and plasminogen activator inhibitor (PAI)-1. When primary cardiac fibroblasts were exposed to hypoxia and serum deprivation to simulate ischemia, AM1241 was able to reduce α-SMA, collagen I and collagen III; this effect was partially abrogated by the Nrf2 siRNA transfection. Moreover, the CB2 receptor agonist, AM1241, activated and enhanced the translocation of Nrf2 to the nucleus and inhibited the TGF-β1/SMAd3 pathway. These data suggest that activation of the CB2 receptor might be one of the key targets to combat heart fibrosis after myocardial infarction [68]. The chronic peripheral pharmaceutical blockage of CB1 receptor (by SLV319 or JD5037 selective CB1 receptor antagonists) or genetic inactivation of CB1 receptors in the renal proximal tubule cells reduced kidney inflammation, suppressed tubulointerstitial fibrosis, and diminished diabetic-induced changes in the kidneys in mice. Also, the downregulation of the CB1 receptor suppressed glucose transporter 2, which resulted in reduced glucose reabsorption. These data supported the fact that peripheral CB1 receptor antagonists might be useful in treating patients with diabetic nephropathy [69]. Table 3 summarizes type of cannabinoids and their mechanism of action.

Table 4. Anti-fibrotic effect of cannabinoids

Compound	The mechanism of action
Endocannabinoids	

 AEA	Suppressing the proliferation of HSCs and induces their necrosis
 2-AG	Generally considered as a fibrogenic agent, however it is able to suppress fibrosis via the membrane cholesterol-dependent mechanism.
 OEA	Inhibition of collagen deposition and suppression of collagen type I and III gene expression, α-SMA, MMP2, MMP9, and TIMP1. These effects were mediated through the PPARα mechanisms.
Phytocannabinoids	

 CBD	Apoptosis induction of HSCs as result of the induction of endoplasmic reticulum stress and the enhancement of the pro-apoptotic pathway IRE1/ASK1/c-Jun N-terminal kinase.
 THC	Inhibition of myofibroblast proliferation and stellate cells, the induction of their apoptosis via CB2 receptors.
Synthetic cannabinoids	
 JWH-133	Suppression of collagen type I and α-SMA, inhibition of fibroblast proliferation and migration. The down-regulation of the TGF-β1/Smad2 pathway.
 Rimonabant	Suppression of expression of fibrogenic mediators (TIMP-1, TGF-β, MMP13, MMP2, MMP9, MMP1, MMP8, TNF-α, MCP-1)

 AM1241	Downregulation of expression of collagen I, collagen III, TIMP-1, and plasminogen activator inhibitor. The inhibition of the TGF-β1/SMAd3 pathway.
 SLV319	Suppression of glucose transporter 2; reduction in glucose reabsorption.
 JD5037	Suppression of glucose transporter 2; reduction in glucose reabsorption.

2. Hypothesis

We hypothesize that cannabis extracts have anti-fibrotic effects in tissue models of fibrosis via alteration of key fibrotic pathways.

The objective of my thesis is thus to test whether cannabis extracts can inhibit the expression of genes involved in pro-fibrotic pathways in 3D tissue models of fibrosis.

3. Materials and Methods

3.1 Plant growth, extract preparation

Cannabis cultivation and extract preparation was been previously described [70],[71]. *C. sativa* plants were grown in the licensed facility at the University of Lethbridge (license number LIC-62AHHG0R77-2019). *C. sativa* cultivars, 7 of them, were used for the experiments. Four plants per each cultivar were grown at 22°C 18 h light 6 h dark for 4 weeks and then transferred to the chambers with 12 h light/ 12 h dark regime to promote flowering, and flowers were harvested from mature plants and dried. Samples of flowers from four plants per each studied cultivar were pooled for extract preparation. Extract preparation was described in detail elsewhere [70],[71]. In brief, 3 grams of the powdered plant tissue per each line were used for Ethyl Acetate-based extraction. The extract stocks were made from the crude extracts by dissolving 3-6 mg of crude extract in DMSO (Dimethyl sulfoxide anhydrous, Life Technologies) to reach 60 mg/mL final concentration. Extracts were stored in -20°C. Appropriate cell culture media were used to dilute the 60 mg/mL stock to make working medium containing 0.01 mg/ml. Extracts were sterilized using 0.22 μm filter.

3.2 High performance liquid chromatography (HPLC)

Agilent Technologies 1200 Series HPLC system was used to analyze the levels of CBD and THC in the cannabis extracts. The acquisition of data, control of the instrument, and integration was done using ChemStation LC 3D Rev B.04.02 (Agilent Technologies). 230 nm and 280 nm were used for the detection of compound peaks. Two samples with two technical repeats for each sample was done per each cannabis cultivar.

3.3 Tissue models and treatments

Tissue models and treatments have been previously described by our laboratory [70]. In brief, human organotypic 3D tissue models - EpiOralTM, EpiGingivalTM, and EpiIntestinalTM were procured from Mattek Life Sciences (Ashland, MA), equilibrated and cultured according to manufacturer's instructions.

EpiOral Tissues (ORL-200) and EpiGingival (GIN-100) MatTek's EpiOral and EpiGIngival tissues are comprised of human-derived normal oral epithelial cells. The cells have been grown to form multilayered, highly differentiated models of the human buccal phenotypes (EpiOral) and gingival phenotypes (EpiGingival). The tissues are cultured on special cell culture inserts in a serum free medium and reach levels of differentiation on the cutting edge of *in vitro* cell culture technology. These well-established tissue models display *in vivo*-like morphological and growth characteristics which are uniform and highly reproducible (Mattek Life Sciences, MA). Two tissues (two independent biological replicates) were used per extract in experiments. Inflammation was induced with the help of LPS (10 ug/mL) for 20 min in EpiOral tissue, and TNF-α/IFN-γ (100 ng/mL) for 1 hour in EpiGingival tissue; control - no treatment at all [70]. The influence of different cannabis extracts was evaluated on this tissue [70]. For EpiOral 3D tissue treatment with extracts was given for 2 hrs; for EpiGingival 3D tissue extracts were given for 4 hrs. The extracts were given at a final concentration 0.015 ug/uL based on previous preliminary internal laboratory data. After treatment with extracts, RNA samples were collected. Two tissues were used per extract in experiments.

EpiIntestinal Tissues (SMI-100): EpiIntestinal tissues are 3D highly differentiated tissue models generated from normal, human cell-derived small intestine epithelial and endothelial cells and

fibroblasts. Grown at the air-liquid interface, EpiIntestinal tissue models are analogous to *in vivo* human epithelial tissues and present columnar shaped basal cells and Kerckring folds, as well as brush borders, functional tight junctions and mucous secreting granules (Mattek Life Sciences, MA). Fibrosis was induced with the help of TNF-α (40 ng/mL) and IFN-γ 5 ng/mL for 24 hrs. Control samples did not include any treatment at all. The influence of different cannabis extracts was evaluated on this tissue [70]. The extracts were given at a final concentration 0.015 ug/uL. Tissues were incubated with extracts for 24 hrs and flash frozen for RNA analysis. Two tissues were used per treatment.

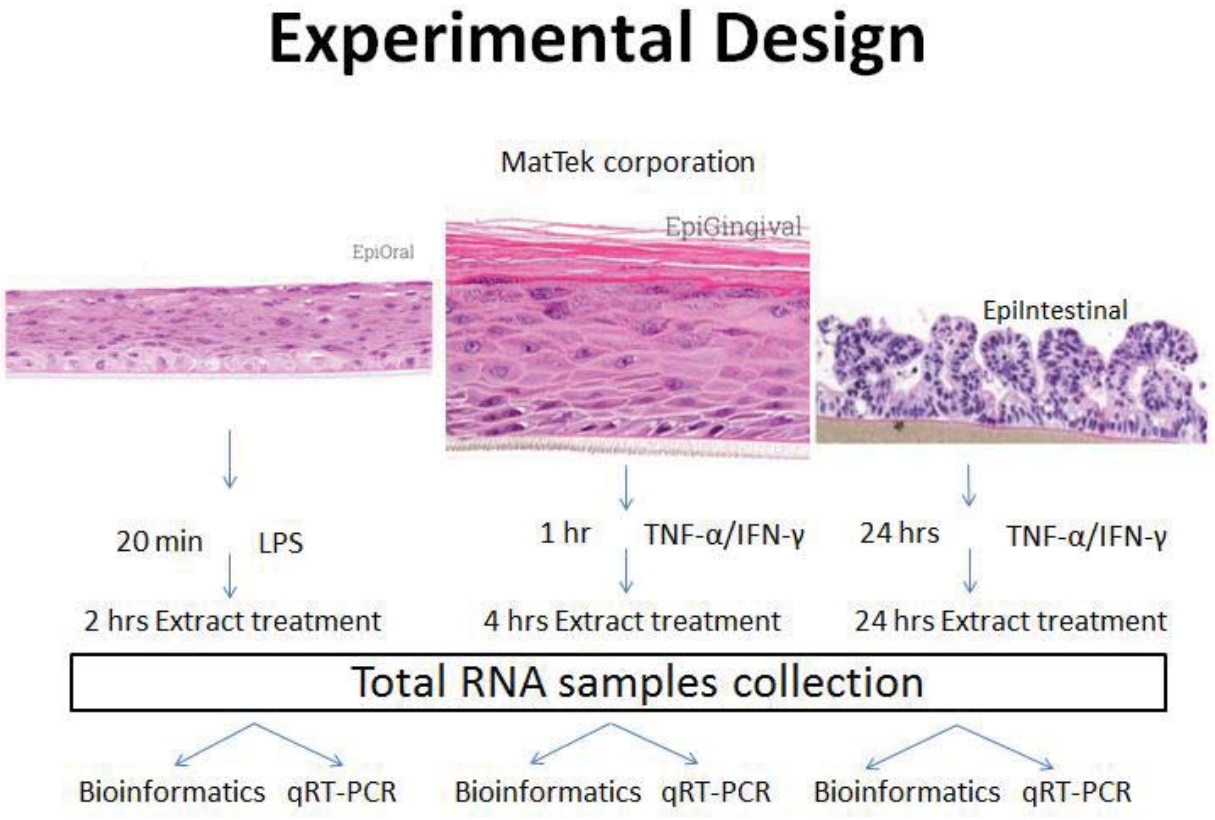


Figure 6. Experimental design. Images courtesy of the MatTek Life Sciences Inc. EpiOral, EpiGingival and EpiIntestinal 3D tissue were treated with LPS (10 ug/mL, 20 min), TNF-α/IFN-γ (100 ng/mL, 1hour), and TNF-α/IFN-γ (5 ng/mL/40 ng/mL, 24 hrs) respectively. Next, all 3D tissues were treated with selected cannabis extracts (0.015 ug/uL), followed by total RNA samples collection. Bioinformatics and qRT-PCR analysis were performed using total RNA samples.

3.4 Gene expression analysis

RNA extraction: Two tissues per group were utilised for the analysis of gene expression profiles1. RNA was prepared by TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

In brief, tissues (50 mg samples) were homogenized in 1 ml of TRIZOL reagent and incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. The next step was the separation phase, whereby 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added to each sample, samples were vortexed vigorously for 15 seconds and kept at room temperature for 5 minutes. Then, the samples were centrifuged for 15 min at 4°C at 12,000 x g. The aqueous phase was transferred to new vials, and RNA was precipitated using isopropyl alcohol and centrifuged. The supernatant was removed, and the RNA pellets were washed with 75% ethanol twice. Washed pellets were air-dried for 10 minutes. Next, RNA was further purified by an RNAesy kit (Qiagen), quantified on theNanodrop2000c (ThermoScientific), and quality-checked (RNA integrity and concentration) on the 2100 BioAnalyzer (Agilent) [70], [72].

Library construction and sequencing: The libraries for RNA sequencing were prepared with the NEBNext Ultra II mRNA library kit for Illumina (NEB). Importantly, the samples were processed by the same technician at the same time to avoid the introduction of technical batch effects, as previously described by Wag at el. [70]. The cDNA were sequenced using NextSeq500

sequencing analyzer (Illumina), and the samples were balanced evenly across the lanes of the sequencing flowcell.

Bioinformatics analysis: Base-calling and demultiplexing were performed using the Illumina CASAVA v.1.9 bioinformatics pipeline. The base qualities were evaluated using FastQC v.0.11.8. The adapters and low-quality bases were trimmed with the help of Trim Galore! v.0.6.4 https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/. Trimmed reads were mapped to the human genome version GRCh37 using HISAT2 version 2.0.5 [73]. Counts of reads that mapped to the gene as a meta-feature were obtained using featureCounts v.1.6.1 [74], considering the directionality of the sequencing libraries. Counts of reads mapping to features were loaded into R v.3.6.1 and normalized using DESeq2 v.1.24.0 Bioconductor package as described in the manual [70],[75].

Two samples were used per group. The differences between all experimental groups were analyzed using the likelihood ratio test (LRT) test applied in DESeq2. The reduced model included the intercept and the full model was the experimental group (Cannabis extracts and controls). Multiple comparisons adjustment of p-values was performed by Benjamini-Hochberg procedure [76]. Specific comparisons between groups were extracted using results() function with contrast argument specified. Genes with adjusted p-values below 0.05 were deemed significant [70].

3.5 cDNA synthesis

For the qRT-PC, cDNA was synthesized by reverse transcription using Zymo-Seq RiboFree Universal cDNA Kit following the manufacturer's instructions. The qRT-PCR was performed to

analyze the levels of expression of fibrosis-related using the CFX96 Touch™ Real-Time PCR Detection System. The sequences of gene-specific reverse and forward primers MMP-9, TGF-β1, COL4A2, COL6A1, TIMP-1, NOTCH1, JAG1 [77] are provided in the Table 5 below.

Table 5. List of primers

Gene	Sequence
COL4A2_1.for	**TATGCCAGCTCCATGTTCTC**
COL4A2_1.rev	**GACCTACCGCAGTGTGATTAT**
MMP9_1.for	**GGGCTTAGATCATTCCTCAGTG**
MMP9_1.rev	**GCCATTCACGTCGTCCTTAT**
Notch1_1.for	**ATGTGTTCTCGGAGTGTGTATG**
Notch1_1.rev	**AGGGACCAAGAACTTGTATAACC**
COL6A1_1.for	**CGCTGGTCAAGGAGAACTATG**
COL6A1_1.rev	**CAGGTGTAATCTGGACACTTCTT**
TGFB1_1.for	**CCTGCCTGTCTGCACTATTC**
TGFB1_1.rev	**TGCCCAAGGTGCTCAATAAA**
JAG1_1.for	**CTGCCGTTGCAGAAGTAAGA**
JAG1_1.rev	**CAGATCCAAGCCACAGTTAAGA**

TIMP1_1.for	**TCCCAGATAGCCTGAATCCT**
TIMP1_1.rev	**TGCTGGGTGGTAACTCTTTATT**
GAPDH_1.for	**CAGGAGGCATTGCTGATGAT**
GAPDH_1.rev	**GAAGGCTGGGGCTCATTT**

3.5 Statistical analysis

For qRT-PCR, the significance of differences was assessed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis t-test, performed with the Prism software package (GraphPad Software for Science, Inc., SanDiego, CA, USA).

For bioinformatics analysis, Benjamini-Hochberg procedure was done for multiple comparisons adjustment of p-values. The adjusted p-value <0.05were considered significant.

4. Results

Since the human organotypic artificial 3D tissue models of oral, gingival and intestinal tissues are well-established and accepted models for pathophysiology, inflammation, and fibrosis studies, we used these models to analyze the potential anti-fibrotic effects of novel *C. sativa* cultivars. We focused on the following fibrosis-related genes: *COL4A2,COL6A1, MMP9, TIMP1, JAG1, NOTCH1,* and *TGFB1*. *COL6A1* gene encodes the collagen type VI, which is the main structural component of extracellular matrix, particularly microfibrils. *COL4A2* gene encodes the collagen type IV, which is the main structural component of basement membrane. *JAG1* is a ligand for the NOTCH1 receptor in main pro-fibrotic pathway, Notch pathway; by binding to the receptor, it activates this pathway. *NOTCH1* gene encodes Notch 1 receptor proteins involved in Notch signaling pathway. *TGFB1* gene encodes a ligand of the TGF-β superfamily of proteins; ligands of this family bind various TGF-β receptors leading to activation of SMAD family transcription factors that regulate gene expression. *TIMP1* gene encodes TIMP1 protein that is natural inhibitor of MMPs. *MMP9* gene encodes MMP9 protein, which is an enzyme involved in degradation of the extracellular matrix.

4.1 Analysis of cannabinoids content using HPLC

Concentration of main cannabinoids (THC and CBD) in the flowers and the extracts (% from total dry weight) and molar concentrations in the studied extracts are reported in the Table 6.

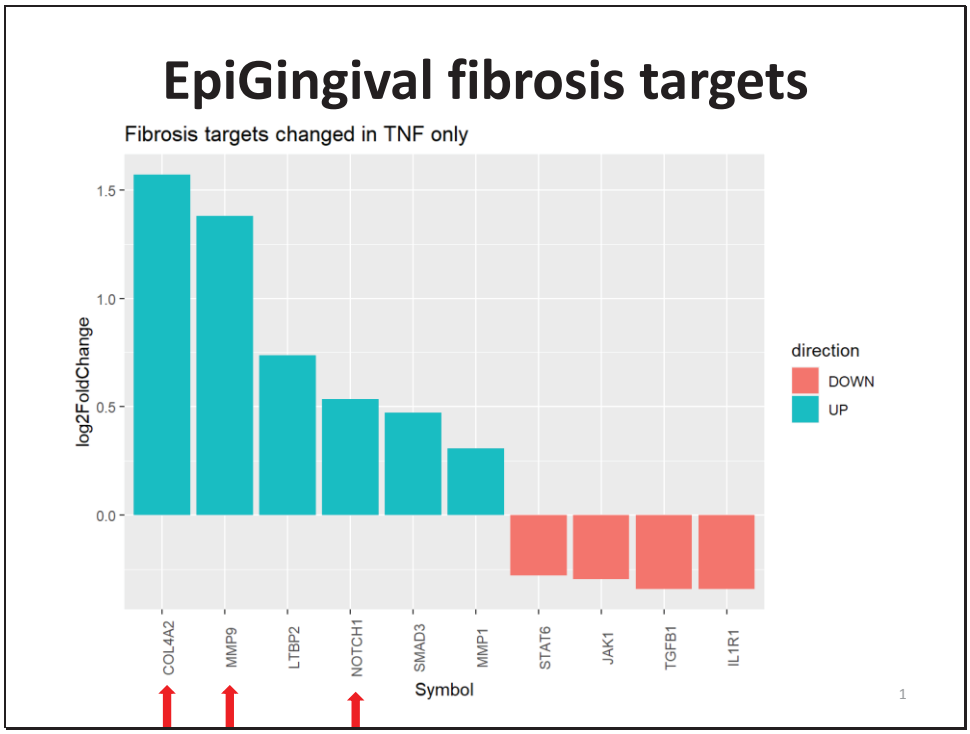
Table 6. Level of THC and CBD in flowers and extracts of selected *C. sativa* cultivars.

Extracts	Total THC, %	Total CBD, %	THC (µM)	CBD (µM)
1 (high CBD)	0.25	6.79	0.12	3.24
45 (high CBD)	0.29	9.54	0.14	4.55

115 (high CBD)	**0.2**	**1.88**	**0.09**	**0.896**
169 (high CBD)	**0.03**	**1.61**	**0.014**	**0.76**
IPB CBD4 (high CBD)	**0.86**	**2.63**	**0.4**	**1.25**
CBD Diamond (high CBD)	**0.46**	**11.81**	**0.2**	**5.63**
MSCnumber8 (high CBD)	**1.05**	**4.58**	**0.5**	**2.18**

4.2 Cannabis extracts modulate expression of fibrosis-related genes in inflammation-stimulated 3D tissues

We analysed the effect of cannabis extracts on the expression of fibrosis-related genes in inflammation-stimulated 3D tissues, since inflammation is a significant component of fibrosis.



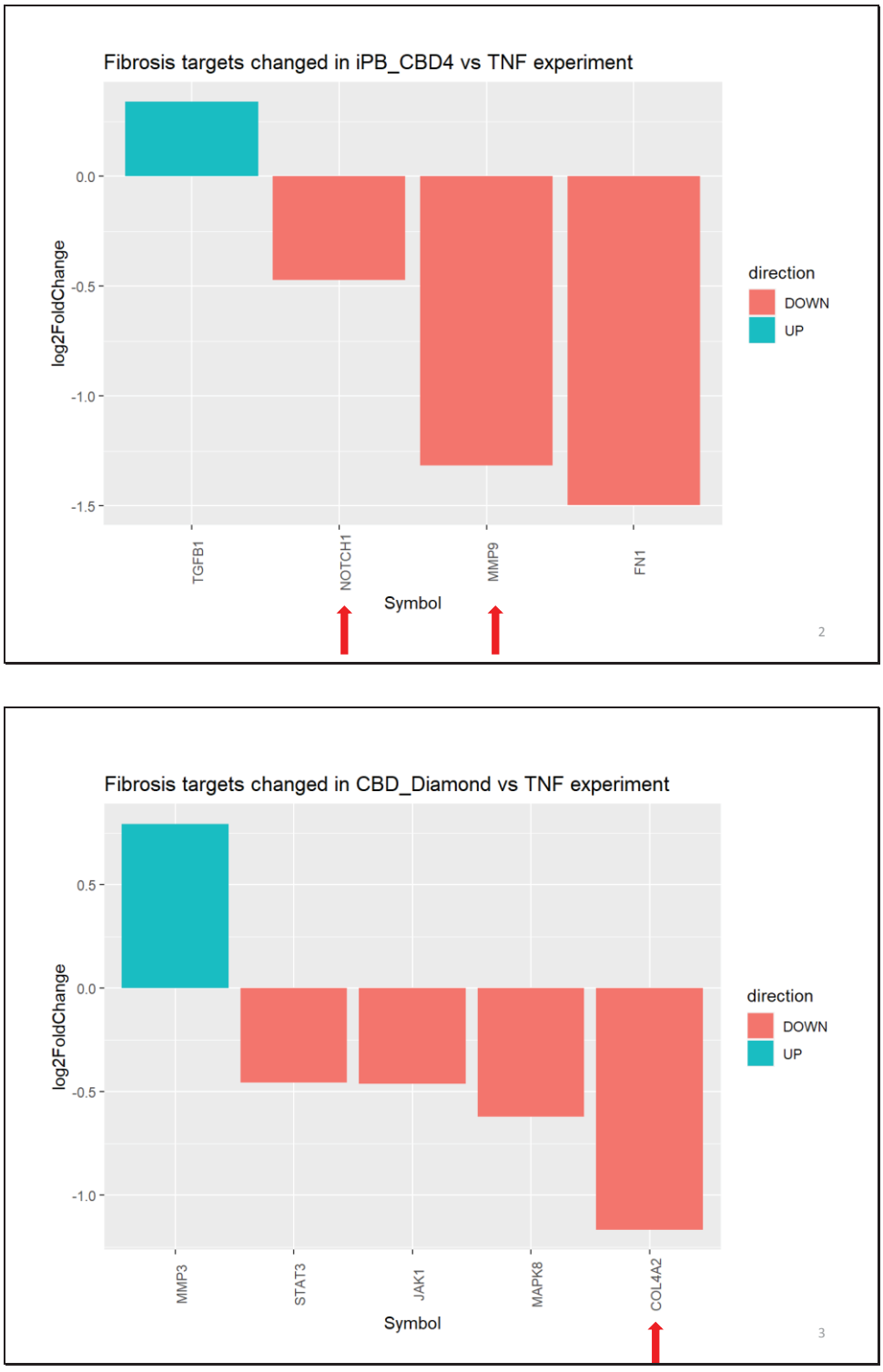


Figure 7. Expression of fibrosis-related genes in EpiGingival tissue. For inflammation induction, TNF-α/IFN-γ was used for 1 hour. Two extracts were used for treatment (4 hrs), IPB-CBD4, and CBD Diamond. Red arrows indicate fibrosis-related genes of interest. The likelihood

ratio test was used to examine the differences among all experimental groups. Benjamini-Hochberg procedure was done for multiple comparisons adjustment of p-values. P-values were considered significant when the value was below 0.05. The data is represented as log 2-fold changes. All the changes presented on the graphs are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

Epi-Gingival tissues: In EpiGingival tissues, inflammation was induced by exposure of tissues to TNF-α/IFN-γ (100 ng/mL), followed by application of extracts for 4 hrs. Bioinformatic analysis of EpiGingival tissue revealed, that TNFα/IFNγ application significantly upregulated the expression of pro-fibrotic genes, such as *COL4A2*, *MMP9*, and *NOTCH1*. The IPB CBD4 extract caused a significant downregulation of *NOTCH1* and *MMP9* genes expression, while CBD Diamond extract inhibited the expression of *COL4A2* gene.

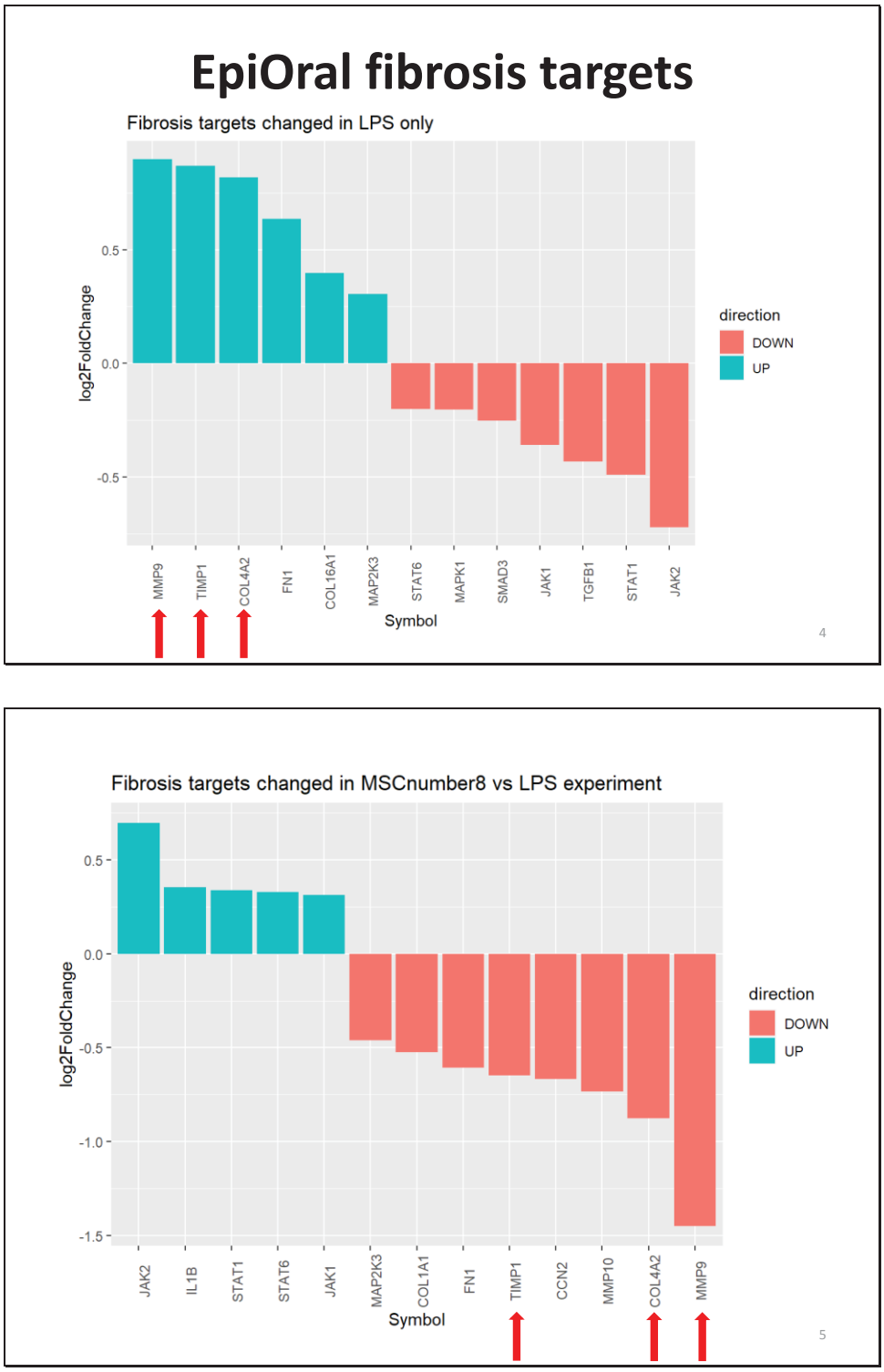
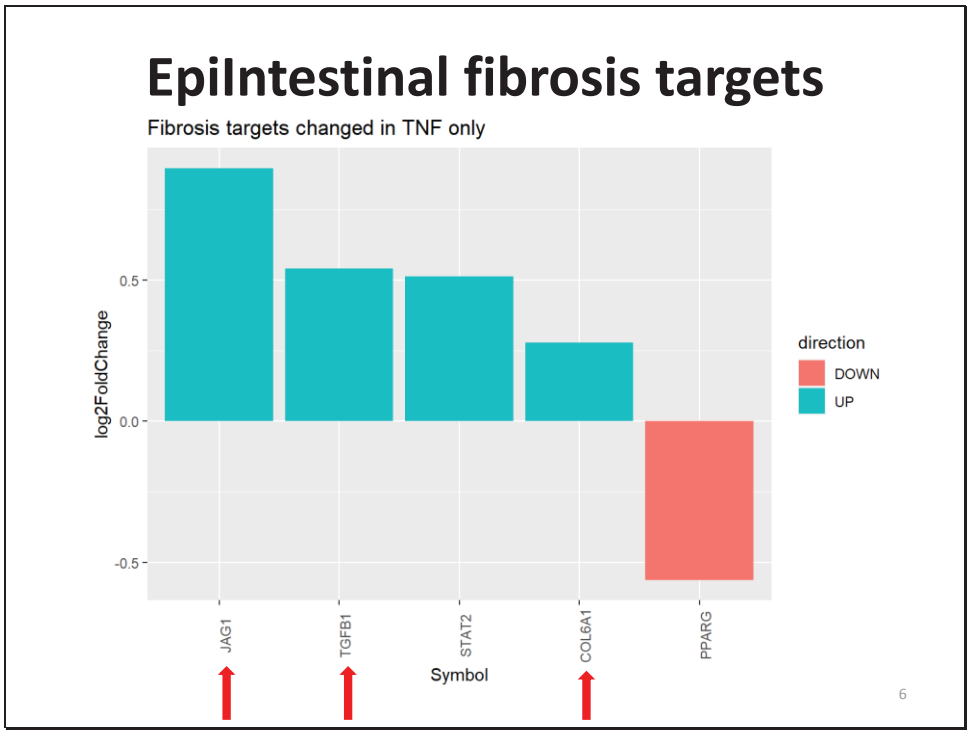
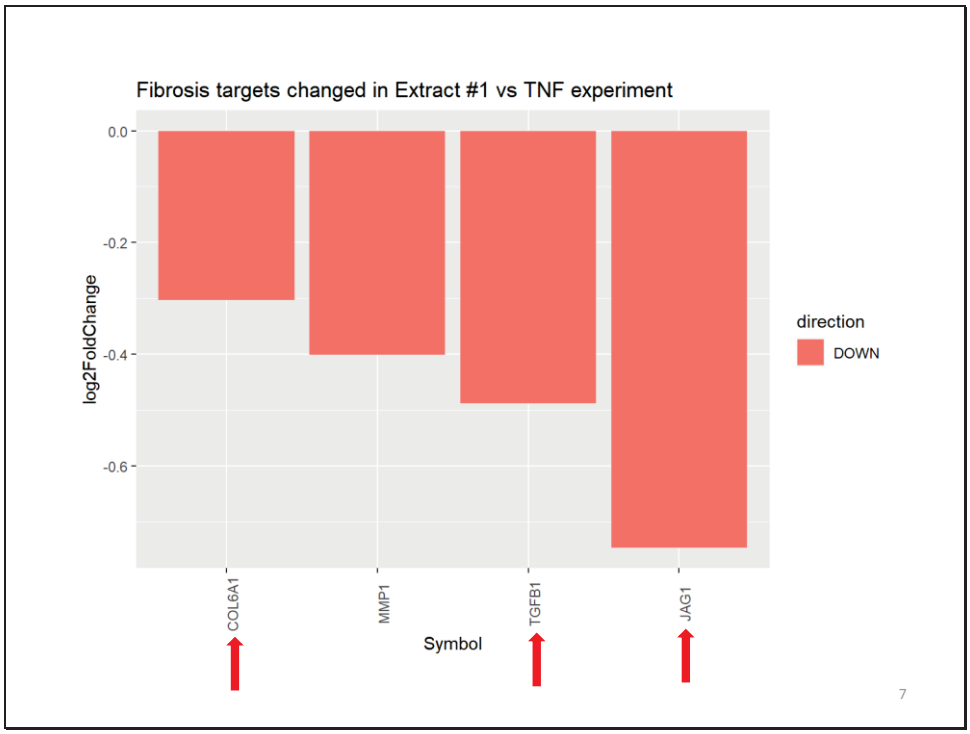


Figure 8. Expression of fibrosis-related genes in EpiOral tissue. For fibrosis induction, LPS was used for 20 min. The extract, MSC #8 was used for treatment (2 hrs). Red arrows indicate fibrosis-related genes of interest. The likelihood ratio test was used to examine the differences among all experimental groups. Benjamini-Hochberg procedure was done for multiple comparisons adjustment of p-values. P-values were considered significant when the value was

below 0.05. The data is represented as log 2-fold changes. All the changes presented on the graphs are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

Epi-Oral tissues: In EpiOral tissues, inflammation was induced by exposure of tissues to LPS (10 ug/mL) for 20 min, followed by application of extracts for 2 hrs. Bioinformatic analysis of EpiOral tissue data demonstrated that LPS treatment significantly elevated the expression of *COL4A2*, *TIMP1*, *MMP9* genes as compared to control. Application of the MSC #8 extract significantly inhibited the expression of these genes.




Fibrosis targets changed in Extract #1 vs TNF experiment
log2FoldChange
0.0
-0.2
-0.4
-0.6
COL6A1
MMP1
TGFB1
JAG1
Symbol
direction
DOWN
7

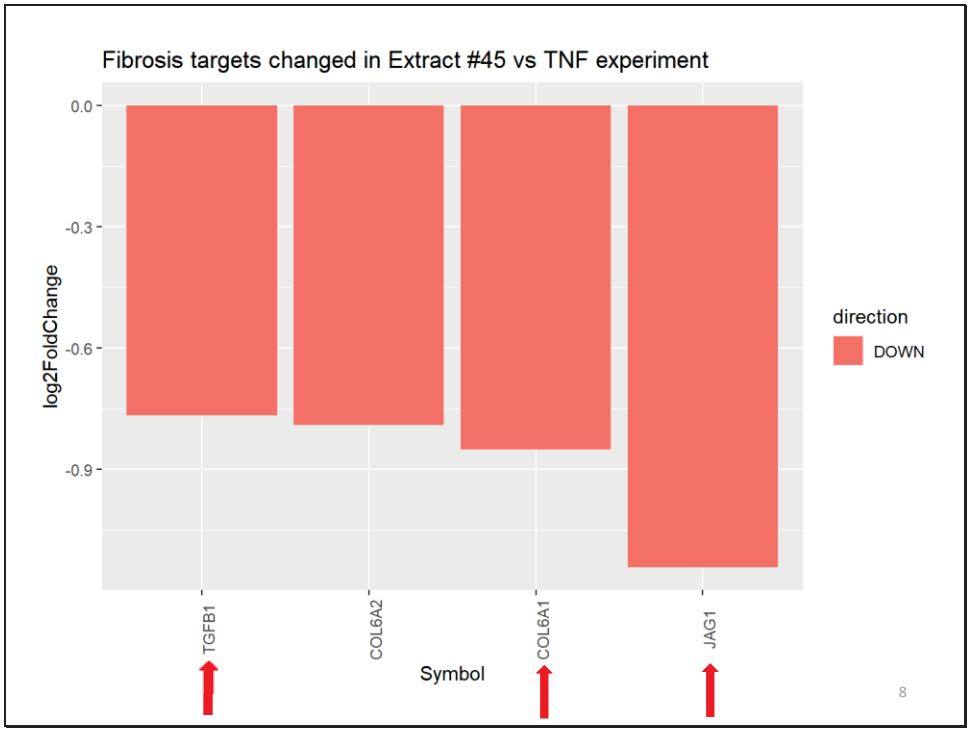

Fibrosis targets changed in Extract #45 vs TNF experiment
log2FoldChange
0.0
-0.3
-0.6
-0.9
TGFB1
COL6A2
COL6A1
JAG1
Symbol
direction
DOWN
8

Figure 9. Expression of fibrosis-related genes in in EpiIntestinal tissue. For fibrosis induction, TNF-α/IFN-γ was used for 24 hrs. Four extracts were used for treatment, #1, #45, #115, #169. Red arrows indicate fibrosis-related genes of interest. The likelihood ratio test was

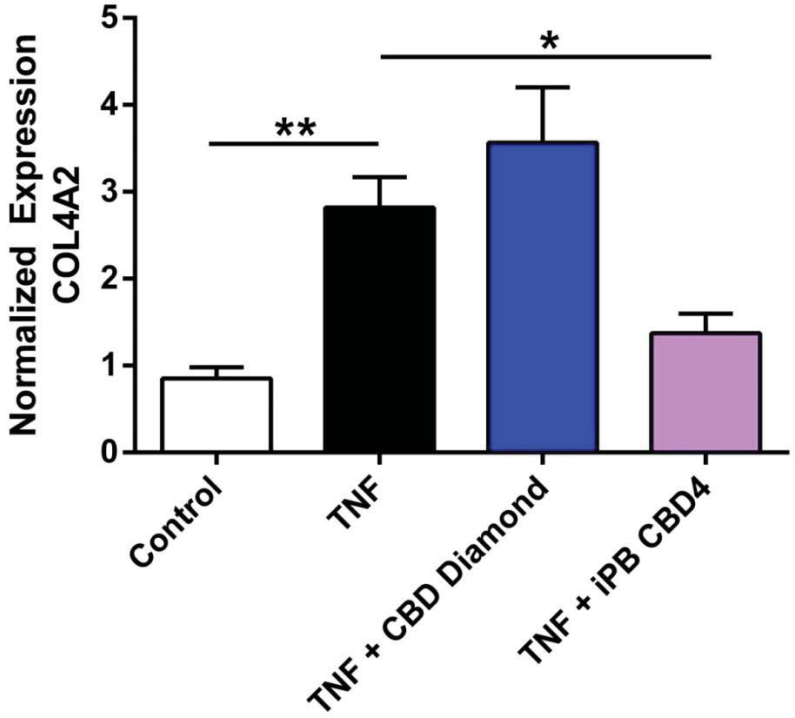
used to examine the differences among all experimental groups. Benjamini-Hochberg procedure was done for multiple comparisons adjustment of p-values. P-values were considered significant when the value was below 0.05. The data is represented as log 2-fold changes. All the changes presented on the graphs are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

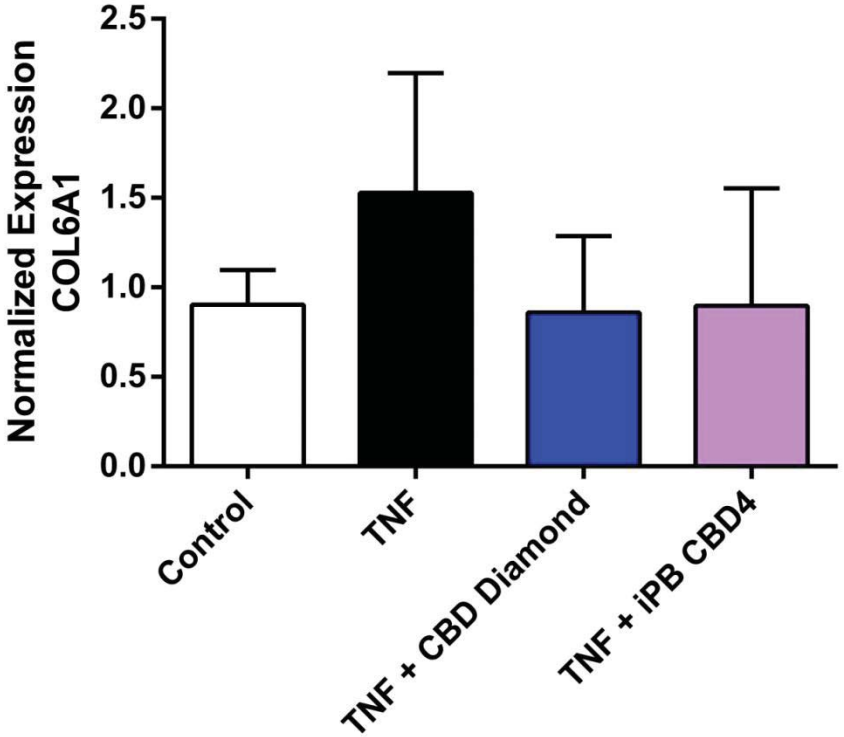
EpiIntestinal tissues: In EpiIntestinal tissues, inflammation was induced by exposure of tissues to TNF-α (40 ng/mL) and IFN-γ 5 ng/mL for 24 hrs, followed by application of extracts for 24 hrs. We noted that application of TNF-α/IFN-γ significantly upregulated the expression of *JAG1*, *TGFβ1*, and *COL6A1* in the Epi-Intestinal tissues. Application of all studied high-CBD extracts (#1, #45, #115, #169) significantly downregulated the expression of these genes.

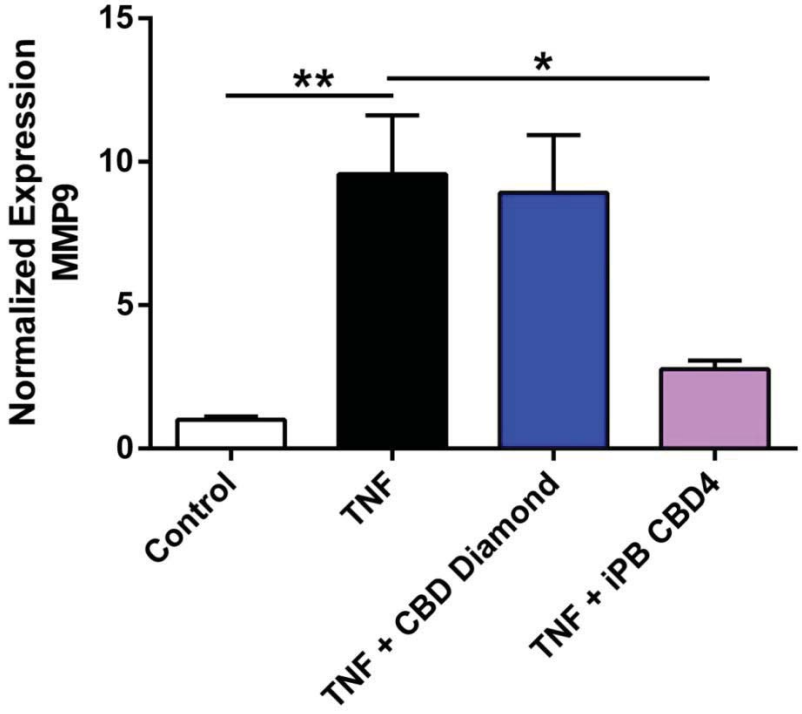
4.3 qRT-PCR-based data confirmation

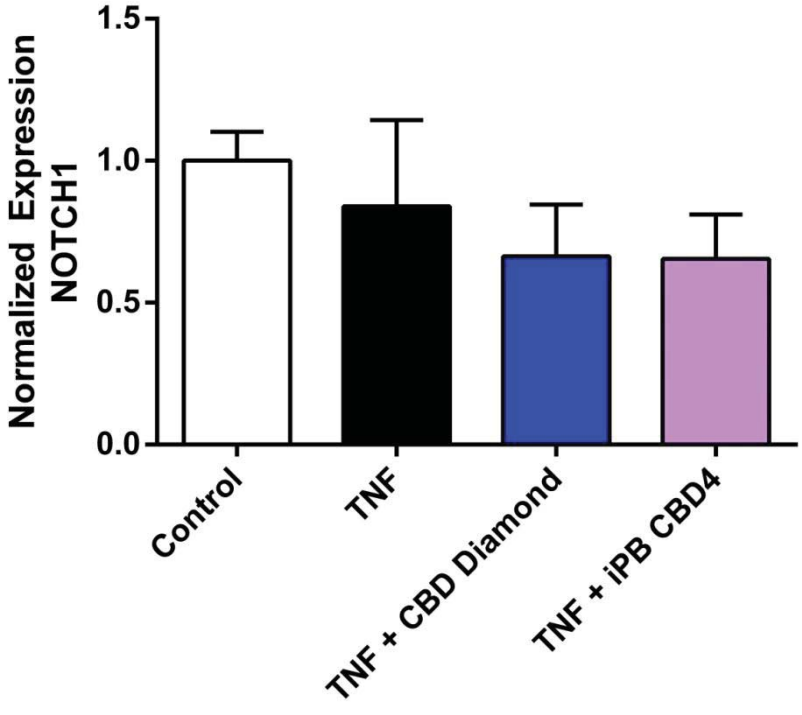
To further confirm the results of the global gene expression studies, we used qRT-PCR to analyze the expression of most interesting fibrosis-related genes as they are the most known to encode proteins that are critical regulators of fibrosis.

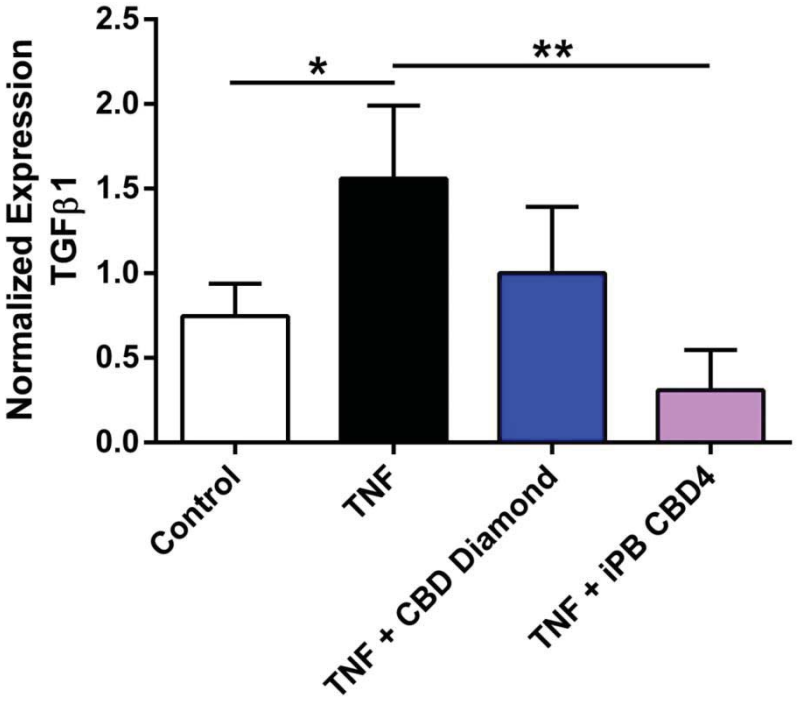
EpiGingival tissue

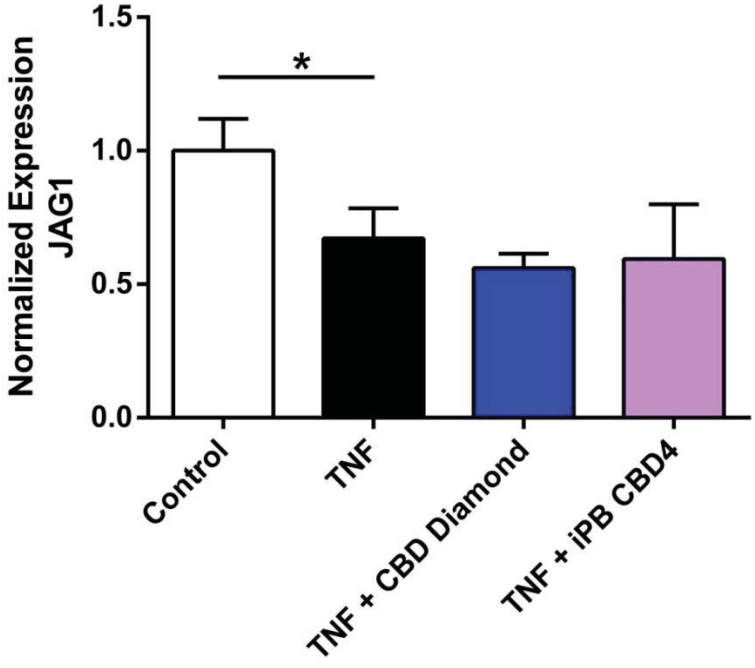

Normalized Expression
COL4A2
5
4
3
2
1
0
**
*
Control
TNF
TNF + CBD Diamond
TNF + iPB CBD4


Normalized Expression
COL6A1
2.5
2.0
1.5
1.0
0.5
0.0
Control
TNF
TNF + CBD Diamond
TNF + iPB CBD4


Normalized Expression
MMP9
15
10
5
0
**
*
Control
TNF
TNF + CBD Diamond
TNF + iPB CBD4


Normalized Expression
NOTCH1
1.5
1.0
0.5
0.0
Control
TNF
TNF + CBD Diamond
TNF + iPB CBD4


Normalized Expression
TGFβ1
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
TNF
TNF + CBD Diamond
TNF + iPB CBD4


Normalized Expression
JAG1
1.5
1.0
0.5
0.0
*
Control
TNF
TNF + CBD Diamond
TNF + iPB CBD4

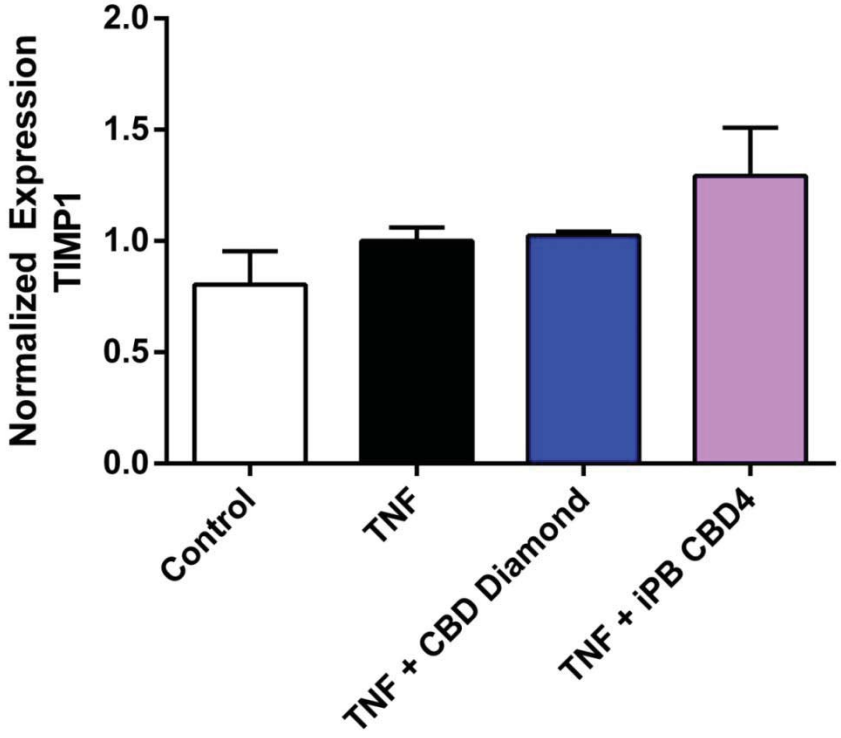


Figure 10. The expression of fibrosis-related genes as per qRT-PCR analysis in EpiGingival tissue. The following genes were analysed: *COL4A2* , *COL6A1* , *MMP-9* , *NOTCH1* , *TGFβ1* , *JAG1*, *TIMP1* . Statistical analysis was performed by ANOVA followed by Tukey post-hoc multiple comparison test (two biological replicates and four technical replicates were used). A value of $p < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. For simplicity, TNF-α is denoted as TNF here.

The expression of *COL4A2* gene was significantly elevated in the TNF-α/IFN-γ group in comparison to control. The application of IPB CBD4 extract significantly suppressed the expression of this gene upon TNF-α/IFN-γ stimulation. Application of the CBD Diamond extract

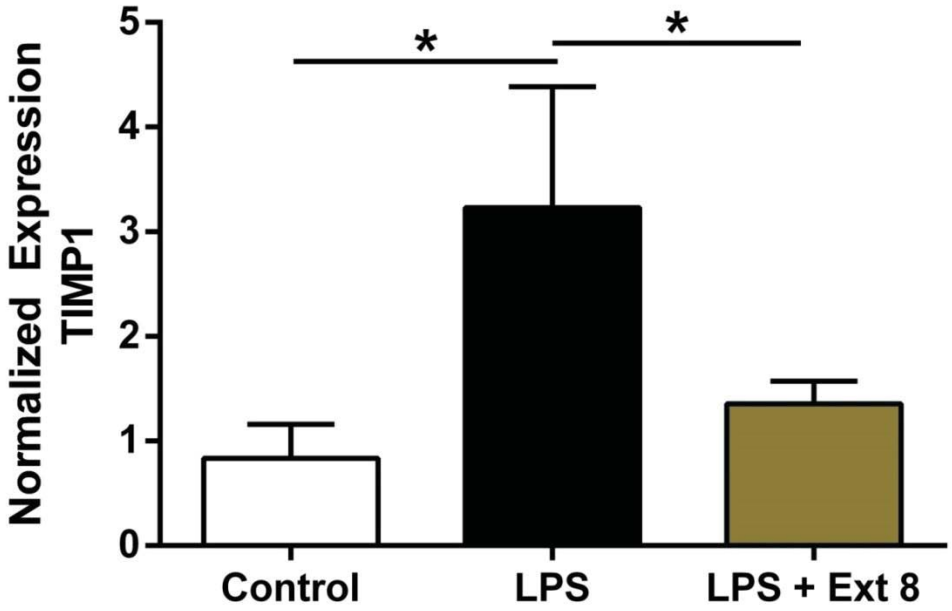
to the TNF-α/IFN-γ -stimulated tissues did not show any significant changes in the expression of *COL4A2* gene.

The expression of *COL6A1* gene showed a tendency to upregulation in the TNF-α/IFN-γ group, while both extracts, CBD Diamond and IPB CBD4, after TNF-α/IFN-γ stimulation demonstrated a tendency to downregulate the expression of this gene, however this effect did not reach the significance (p value between TNF vs TNF + CBD Diamond = 0.447, p value between TNF vs TNF + IPB CBD4 = 0.4913).

TNF-α/IFN-γ treatment significantly elevated the expression of *MMP9* gene in comparison to the control, while iPB CBD4 extract after TNF-α/IFN-γ significantly inhibited the expression of this gene significantly as compared to TNF group. The other studied extract, CBD Diamond did not have any effect on the expression of this gene.

Analysis of the expression of *NOTCH1* gene by qRT-PCR data did not reveal any statistically significant changes. The expression of *TGFβ1* gene was significantly upregulated in TNF-α/IFN-γ group compared to control group. The IPB CBD4 extract significantly downregulated the expression of this gene in comparison to the TNF-α/IFN-γ group. Also, quite unexpectedly, the expression of *JAG1* gene was significantly lower in the TNF-α/IFN-γ group in comparison to control. Furthermore, no significant changes were noted in the he expression of *TIMP* gene.

EpiOral tissue


Normalized Expression
TIMP1
5
4
3
2
1
0
*
*
Control
LPS
LPS + Ext 8

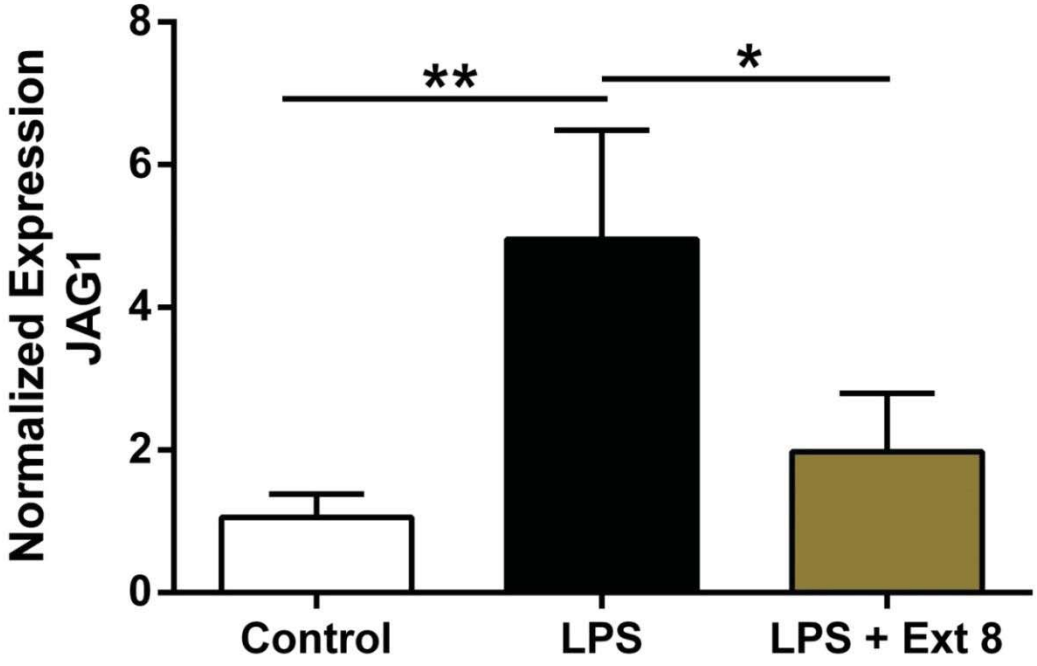
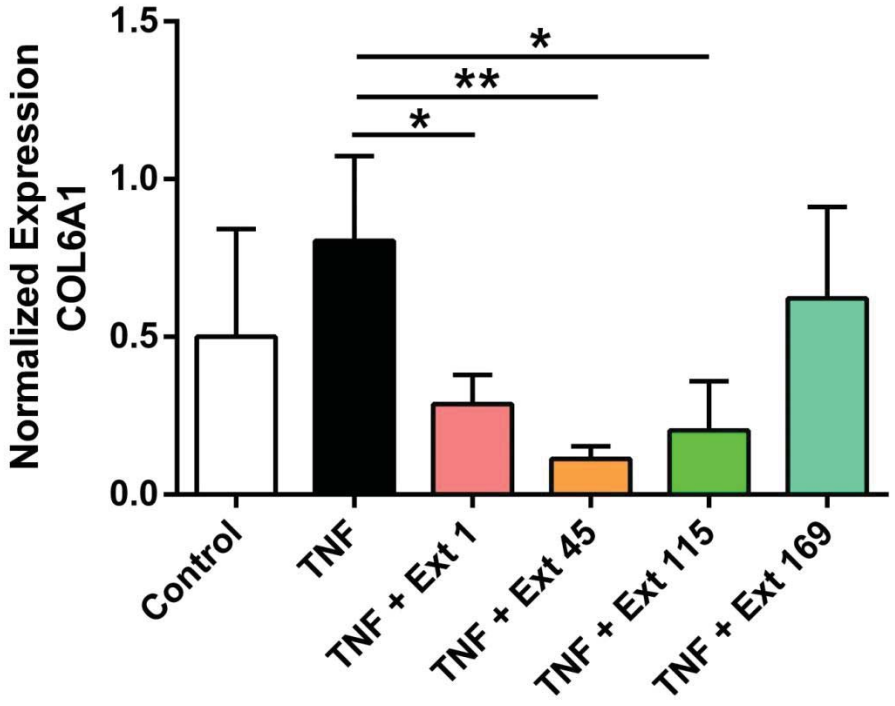

Normalized Expression
JAG1
8
6
4
2
0
**
*
Control
LPS
LPS + Ext 8

Figure 11. The expression of fibrosis-related genes as per qRT-PCR analysis in EpiOral tissue. The following genes were assessed: *TIMP1*, *JAG1*. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test (two biological replicates and four technical replicates were used). A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The expression of *TIMP1* and *JAG1* genes was significantly upregulated in the LPS group as compared to the control. Application of extract #8 after LPS stimulation significantly downregulated the expression of these genes indicating the anti-fibrotic potential.

EpiIntestinal tissue



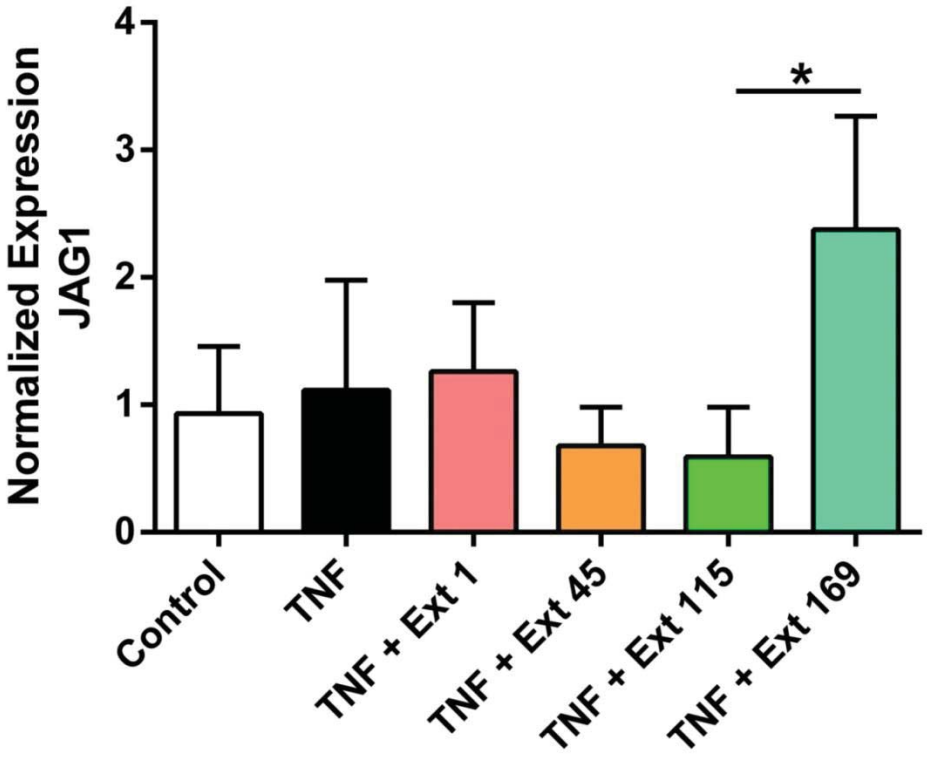
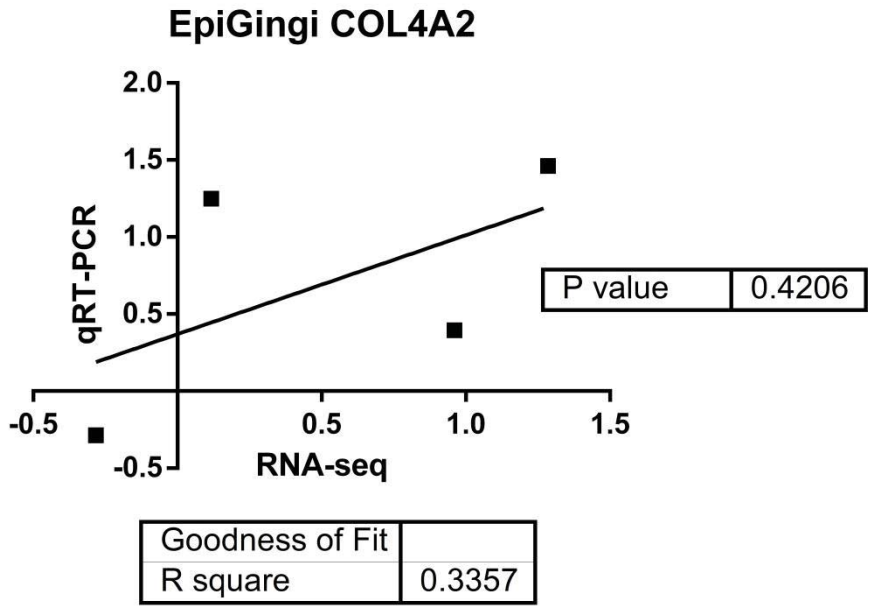


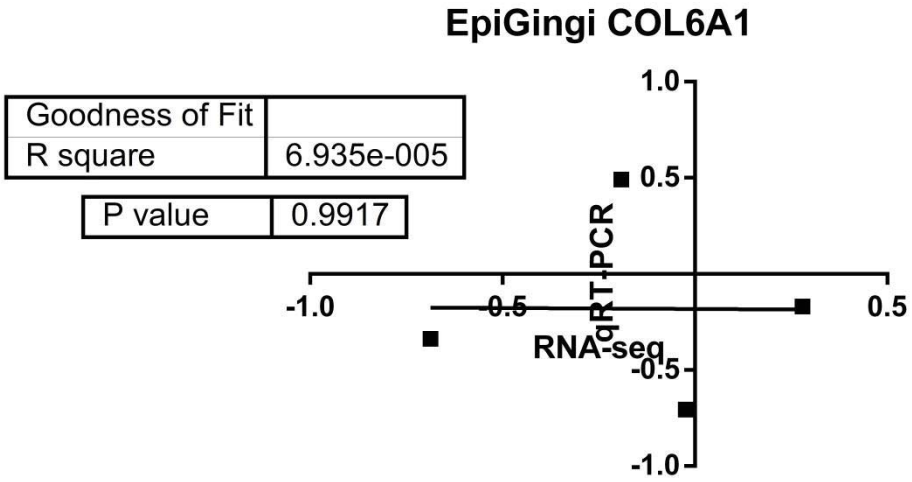
Figure 12. The expression of fibrosis-related genes as per qRT-PCR analysis in EpiIntestinal tissue. The following genes were assessed: *COL6A1*, *JAG1*. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test (two biological replicates and four technical replicates were used). A value of $P < 0.05$ was considered to be significant. Significant differences between groups are marked with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The expression of *COL6A1* gene showed a tendency to upregulation in TNF-α/IFN-γ group in comparison to control, while extracts #1, #45, and #115 after TNF-α/IFN-γ stimulation significantly downregulated the expression of this gene in comparison to TNF-α/IFN-γ only.

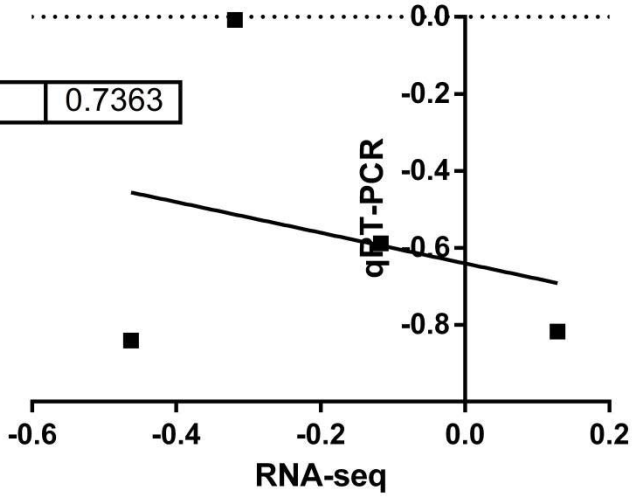
JAG1 expression showed a trend of higher expression after TNF-α/IFN-γ treatment, while extract #45 and #115 demonstrated a tendency to reduce its expression (p value between TNF vs extract #45 + TNF = 0.9512, TNF vs extract #115 + TNF = 0.9019).

4.4 Linear regression method-based correlation analysis of gene expression between RNA-seq data and qRT-PCR data





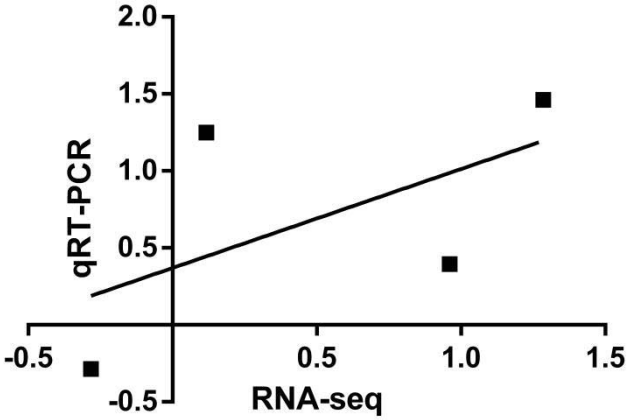
EpiGingi JAG1



P value	0.7363

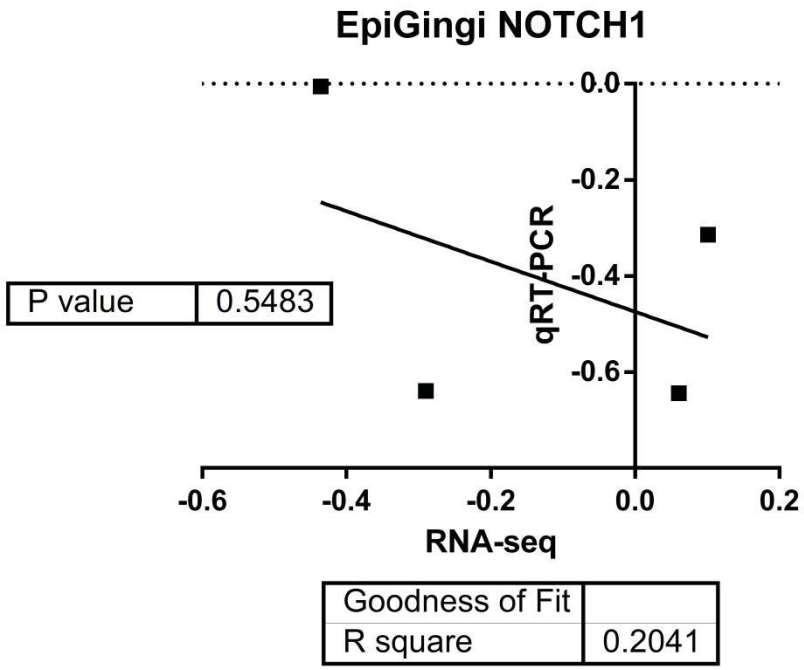
Goodness of Fit	
R square	0.06956

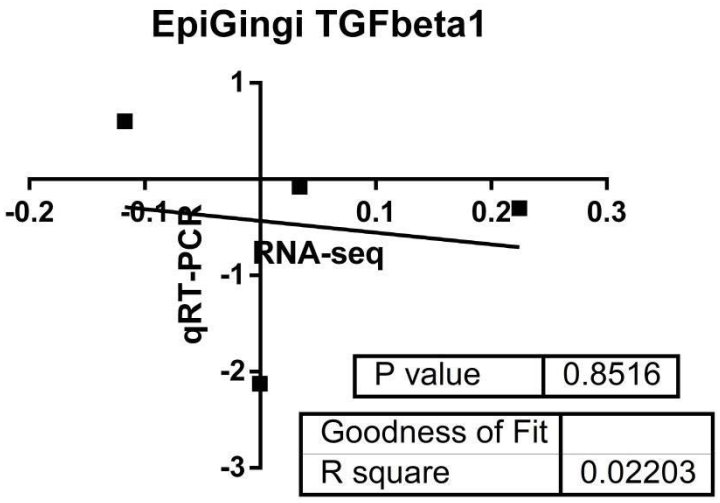
EpiGingi MMP9



Goodness of Fit	
R square	0.3357

P value	0.4206


EpiGingi NOTCH1
0.0
-0.2
-0.4
-0.6
qRT-PCR
-0.6
-0.4
-0.2
0.0
0.2
RNA-seq
P value
0.5483
Goodness of Fit
R square
0.2041


EpiGingi TGFbeta1
1
-1
-2
-3
qRT-PCR
-0.2
-0.1
0.1
0.2
0.3
RNA-seq
P value
0.8516
Goodness of Fit
R square
0.02203

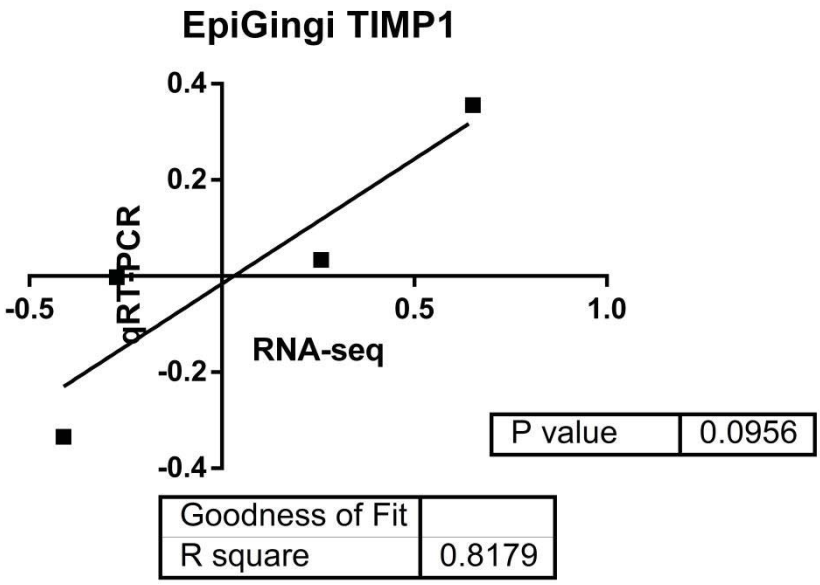
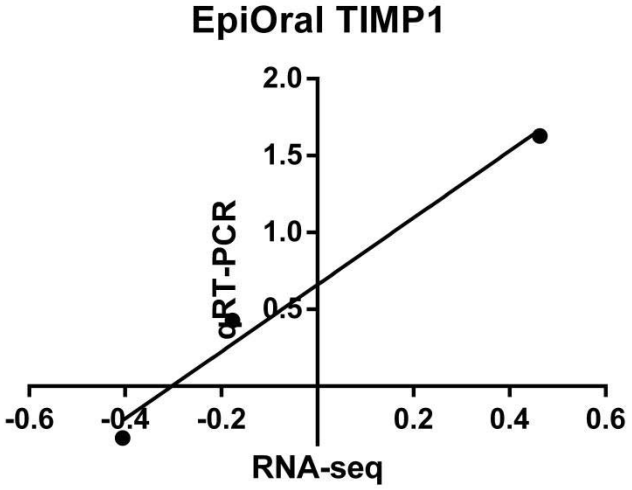


Figure 13. Correlation analysis of gene expression between RNA-seq data and qRT-PCR data for EpiGingival tissue. For performing correlation analysis, log 2-fold values were plotted to calculate the correlation between RNA-seq vs qRT-PCR. On the X-axis, there are log 2-fold values from RNA-seq data, and on the Y-axis, there are log 2-fold values from qRT-PCR data. Simple linear regression was performed using GraphPad Prism 6.0 software.

Epi-Gingival tissue: For *COL4A2*, the correlation analysis showed that R square value was 0.3357 which demonstrated a weak, positive linear association between analyzed data. For *COL6A1*, R square value was 6.935e-005 which showed complete lack of relationship between two groups. The R square value for *JAG1* gene correlation analysis was 0.069, which showed no association between analyzed groups.

Correlation analysis for *MMP9* showed a weak positive linear relationship between examined groups (R square value 0.3357); for *NOTCH1* – weak positive linear association (R square

value was 0.2041); for *TGFβ1* – no linear relationship was found (R square value – 0.02203); for *TIMP1* – strong positive linear regression (R square value – 0.8179).



Goodness of Fit	
R square	0.9805

P value	0.0893

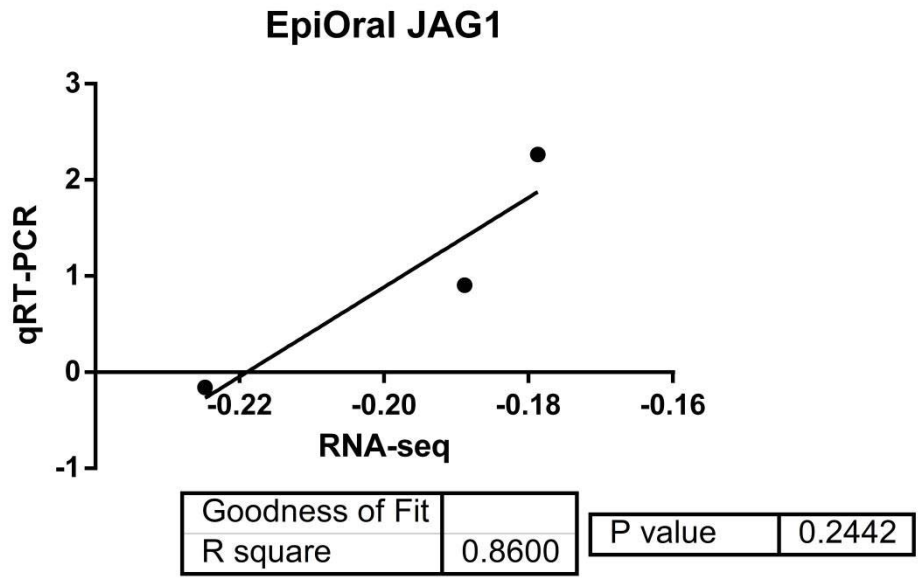


Figure 14. Correlation analysis between RNA-seq data and qRT-PCR data for EpiOral tissue. For performing correlation analysis, log 2-fold values were plotted to calculate the correlation between RNA-seq vs qRT-PCR. On the X-axis, there are log 2-fold values from RNA-seq data, and on the Y-axis, there are log 2-fold values from qRT-PCR data. Simple linear regression was performed using GraphPad Prism 6.0 software.

Epi-Oral tissue: Correlation analysis for *TIMP1* gene revealed a perfect uphill linear regression (R square value 0.9805); for *JAG1* – strong positive linear relationship (R square value 0.8600)

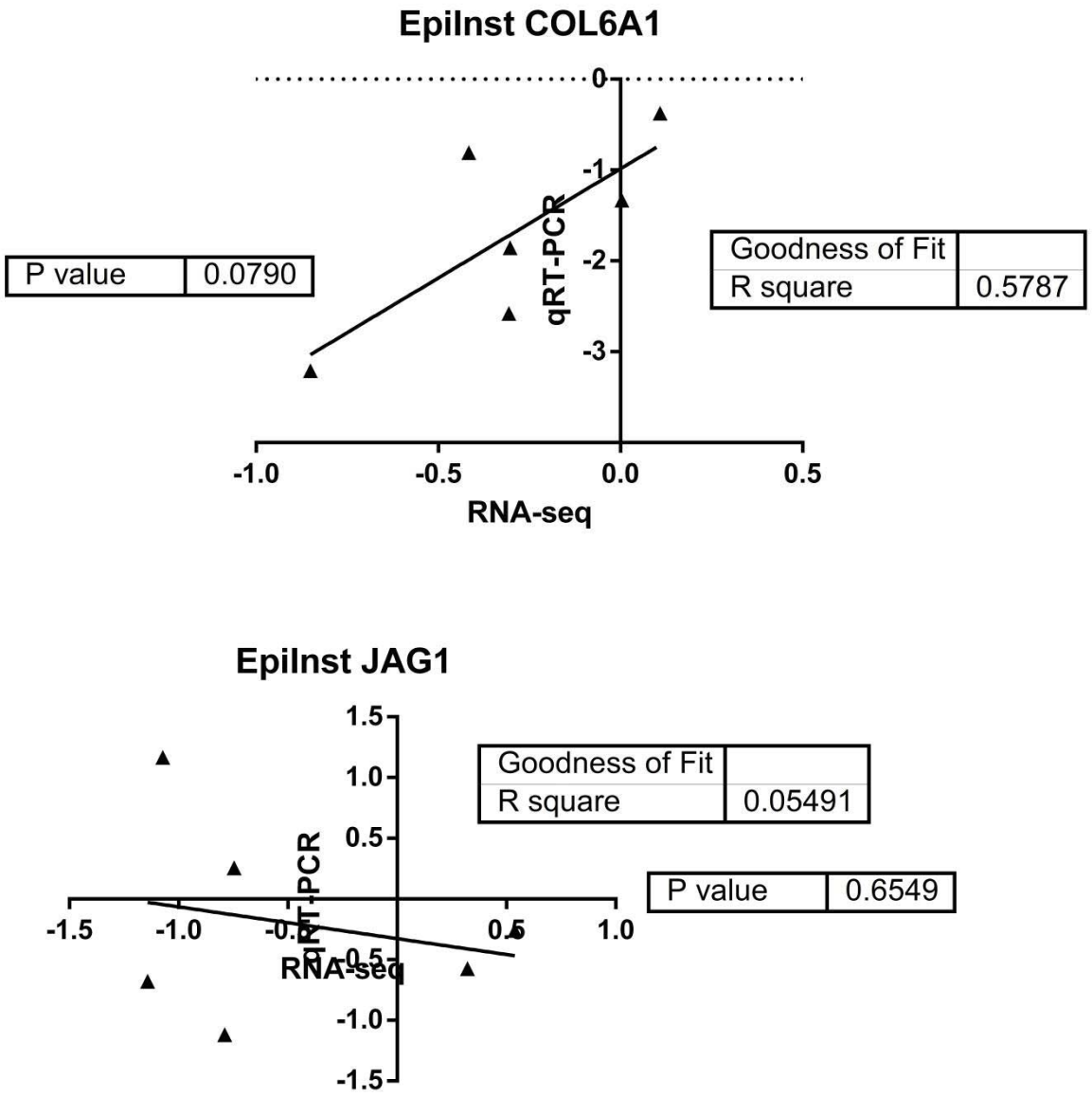


Figure 15. Correlation analysis between RNA-seq data and qRT-PCR data for EpiIntestinal tissue. For performing correlation analysis, log 2-fold values were plotted to calculate the correlation between RNA-seq vs qRT-PCR. On the X-axis, there are log 2-fold values from RNA-seq data, and on the Y-axis, there are log 2-fold values from qRT-PCR data. Simple linear regression was performed using GraphPad Prism 6.0 software.

Epi-Intestinal tissue: The correlation analysis for *COL6A1* demonstrated a moderate positive relationship (R square value 0.5787); for *JAG1*- strong uphill linear relationship (R square value 0.6549).

5. Discussion

The objective of this research was to analyse the anti-fibrotic potential of several selected novel high CBD *C. sativa* extracts using human organotypic 3D tissue models and inflammation inductions. Our previous studies showed that application of TNF-α/IFN-γ or LPS caused inflammatory response in the Epi-Oral, EpiGingival and EpiIntestinal tissues [70], [78]. Inflammation is closely related to fibrosis, and fibrosis is a detrimental outcome and complication in many inflammatory conditions and disorders. Anti-fibrotic therapies are scarce, hence there is an urgent need for the new anti-fibrotic agents.

To that effect, we evaluated the anti-fibrotic potential of several high cannabidiol cannabis extracts to model medical delivery, such as topical or oral applications. Our study showed that cannabis extracts high in CBD significantly downregulated the expression of certain pro-fibrotic genes, however not all extracts showed such effect. Out of six studied genes, in Epigingival *COL4A2, MMP9, TGFβ1*, in Epioral *TIMP1, JAG1* and in EpiIntestinal *COL6A1* genes were affected.

The results of our study are in agreement with previously published works in the same field. For instance, CBD inhibited the expression of pro-fibrotic genes like *MMP-9, TGF-β*, and *collagen-1* in a mouse model of type I cardiomyopathy [57]. Endogenous cannabinoid, OEA, suppressed the expression of *collagen-1*, *MMP9, and TIMP1* in a mouse model of hepatic fibrosis [63]. A selective CB1 receptor antagonist, rimonabant downregulated the expression of many pro-fibrotic genes, such as, *TIMP-1, TGF-β,* and *MMP9* on rat models of liver cirrhosis [66]. JWH-133, a selective CB2 receptor agonist, reduced collagen expression in rats with liver cirrhosis [67]. AM1241, selective CB2 receptor agonist, downregulated the expression of *collagen-1, collagen-*

3, and *TIMP1* on myocardial fibrosis mouse model [68]. Synthetic cannabinoid, WIN55,212-2, was able to prevent skin fibrosis development in mouse model of scleroderma and it efficiently suppressed the expression of *TGF-β,* and inhibited phosphorylation of SMAD2/3 [79]. In addition, synthetic CB1 agonists, WIN55,212-2 and Arvanil demonstrated to reduce the expression of pro-fibrotic genes, such as collagen IV and fibronectin significantly in 3D bioengineered human trabecular meshwork tissue construct [80].

The fibrotic triggers used in our study were similar to other studies. For example, LPS has been used to induce endothelial fibrosis [81], pulmonary fibrosis [82], liver fibrosis [83], and gingival fibrosis [84]. On the other hand, there is a limited data of the established models using of TNF-α/IFN-γ as a pro-fibrotic trigger. More studies are needed in the future to further validate and substantiate this model.

It is very prominent that all the high CBD extracts studied showed some effects on the expression of pro-fibrotic genes. These results must be further substantiated, and the changes in specific fibrotic pathways need to be analyzed in detail. Furthermore, the studies have to be further expanded to include *in vivo* analysis using animal models of fibrosis. Once further confirmed, high CBD cannabis extracts may be brought to clinical trials to investigate their anti-fibrotic potential. As CBD does not have any significant psychotropic effects [85], such cannabis extract can then further be validated in clinical trials in patients with fibrotic diseases [86].

Intestinal tissue in our study was less prone to the development of fibrosis-related changes as compared to Gingival and Oral tissues as per our qRT-PCR data. This can be explained from the point of view that intestine is the most highly regenerative part of human body and when the injury is initiated, the regeneration process happens much faster in comparison to oral mucosa

[87], [88]. Even though it is difficult to judge because the fibrotic trigger was given only for a short period of time, the explanation from pathophysiology side is most logical.

In the future, it would be important to increase the sample sizes of the studies and include the other tissue models, such as skin and lung tissues, as well as liver tissues. Human organotypic full thickness skin and lung epithelial models are well-established and available from MatTek [70],[78]. Liver fibrosis is a bit more difficult to study and requires an animal model.

Tissue fibrosis is one of the important processes associated with aging [89], [90]. It would be important to analyze the effects of CBD and high CBD extracts in context of aging-associated fibrosis.

6. Limitations and future studies

Fibrosis is a pathological process that affects many organs. Significant improvement in the understanding of tissue fibrosis pathways may give us an opportunity in the future to discover an effective antifibrotic treatment. Many studies have been performed to understand the molecular mechanisms behind the pathogenesis and development of fibrosis in human organs, however the data are contradicting. For instance, cytokines like TNF-α play a key role in the establishment of inflammation and pathogenesis of fibrosis. At the same time, TNF-α may be used a therapeutic agent that can resolve the established pulmonary fibrosis [91], further confirming that we do not have a clear picture on the pathophysiological mechanisms of fibrosis.

In most tissues and organs, the fibrosis mechanisms are similar, but the regeneration and regression processes are different across organs and tissues. Mainly, this diversity is due to the difference in the regenerative capacity of each tissue or organ [92].

The future studies might include more research of newly identified discoidin domain receptor 1 (DDR1), which is a kinase target implicated in fibrosis [93]. It would be interesting to check if cannabis extracts can modulate the expression of this fibrotic target.

Based on the results of our study, we conclude that cannabis extracts high in CBD can positively interact with the key profibrotic genes. In comparison with modern antifibrotic medications, they have fewer side effects on the patient's health when used under the medical supervision.

Our study had several crucial limitations. Firstly, the fibrotic agents used to induce fibrosis in 3D tissues were different and were used at different times. If the same fibrotic inducing agent was used for a specified time in all 3D tissues after optimization experiments, then it would have been easier to compare the results. Secondly, for all experiments, only two biological replicates were used while there is a clear consensus of using 3-4 biological replicates among the current

scientific community. Hence, we used four technical replicates of two biological replicates which limits the significance of this study. Thirdly, the tissue samples that we used were about three years old which may explain lack of correlation between RNA-seq and qRT-PCR data, albeit tissues were stored in -80°C, and RNA integrity was good. Lastly, due to ambiguity in the RNA-seq and qRT-PCR, it has become even more difficult so as to which fibrotic pathways should be pursued further. Lastly, no protein samples were available to study respective fibrotic pathways and cannabinoid receptor expression using ELISA, western blotting, and other experimental approach, hence it is impossible to predict whether the changes in the gene expression would translate into alterations in the respective pro-fibrotic pathways.

Nevertheless, we conclude that modulation of ECS should be used for the treatment of different fibrotic conditions. This aspect of treatment has not been sufficiently studied. More detailed research should be performed to find a patient-oriented treatment.

The future studies can include studying *in vivo* the models of oral submucous fibrosis and gingival fibromatosis in view of prevention oral squamous cell carcinoma [94], [95] as well as *in vivo* model of inflammatory bowel disease for preventing the colorectal carcinoma development [96]. When fibrosis is looked from a pre-malignant scope of view it will gain more attention from researchers worldwide and more studies will be performed.

References

[1] Y. Liu, "Cellular and molecular mechanisms of renal fibrosis," *Nat. Rev. Nephrol.*, vol. 7, no. 12, pp. 684–696, 2011.

[2] G. Wernig *et al.*, "Unifying mechanism for different fibrotic diseases," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 18, pp. 4757–4762, 2017.

[3] N. Hoyer, T. S. Prior, E. Bendstrup, T. Wilcke, and S. B. Shaker, "Risk factors for diagnostic delay in idiopathic pulmonary fibrosis," *Respir. Res.*, vol. 20, no. 1, pp. 1–9, 2019.

[4] H. Fujimoto, T. Kobayashi, and A. Azuma, "Idiopathic Pulmonary Fibrosis Treatment – OFEV® (nintedanib) Capsules," vol. 9, pp. 179–185, 2015.

[5] R. B. Zurier and S. H. Burstein, "Cannabinoids, inflammation, and fibrosis," *FASEB J.*, vol. 30, no. 11, pp. 3682–3689, 2016.

[6] G. Lafaye, L. Karila, L. Blecha, and A. Benyamina, "DialoguesClinNeurosci-19-309," pp. 309–317, 2017.

[7] O. Kovalchuk and I. Kovalchuk, "Cell Cycle Cannabinoids as anticancer therapeutic agents Cannabinoids as anticancer therapeutic agents," 2020.

[8] E. B. Russo, "The case for the entourage effect and conventional breeding of clinical cannabis: No 'Strain,' no gain," *Front. Plant Sci.*, vol. 9, no. January, pp. 1–8, 2019.

[9] C. Laezza *et al.*, "The endocannabinoid system: A target for cancer treatment," *International Journal of Molecular Sciences*, vol. 21, no. 3. MDPI AG, 01-Feb-2020.

[10] B. Ley and H. R. Collard, "Epidemiology of idiopathic pulmonary fibrosis," *Clin. Epidemiol.*, vol. 5, no. 1, pp. 483–492, 2013.

[11] K. Raimundo, E. Chang, M. S. Broder, K. Alexander, J. Zazzali, and J. J. Swigris, "Clinical and economic burden of idiopathic pulmonary fibrosis: A retrospective cohort study," *BMC Pulm. Med.*, vol. 16, no. 1, 2016.

[12] D. B. Sanders and A. K. Fink, "Background and Epidemiology," *Pediatr. Clin. North Am.*, vol. 63, no. 4, pp. 567–584, 2016.

[13] S. G. Sepanlou *et al.*, "The global, regional, and national burden of cirrhosis by cause in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017," *Lancet Gastroenterol. Hepatol.*, vol. 5, no. 3, pp. 245–266, 2020.

[14] I. H. Kim, T. Kisseleva, and D. A. Brenner, "Aging and liver disease," *Curr. Opin. Gastroenterol.*, vol. 31, no. 3, pp. 184–191, 2015.

[15] G. G. Gauglitz, H. C. Korting, T. Pavicic, T. Ruzicka, and M. G. Jeschke, "Hypertrophic scarring and keloids: Pathomechanisms and current and emerging treatment strategies," *Mol. Med.*, vol. 17, no. 1–2, pp. 113–125, 2011.

[16] X. Li, L. Zhu, B. Wang, M. Yuan, and R. Zhu, "Drugs and targets in fibrosis," *Front. Pharmacol.*, vol. 8, no. NOV, 2017.

[17] N. L. Occleston *et al.*, "Therapeutic improvement of scarring: Mechanisms of scarless and scar-forming healing and approaches to the discovery of new treatments," *Dermatol. Res. Pract.*, vol. 2010, no. 1, 2010.

[18] B. Galliot, M. Crescenzi, A. Jacinto, and S. Tajbakhsh, "Trends in tissue repair and

regeneration," *Dev.*, vol. 144, no. 3, pp. 357–364, 2017.

[19] K. P. Krafts, "Org0604_0225," no. December, pp. 225–233, 2010.

[20] C. Profyris, C. Tziotzios, and I. Do Vale, "Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics: Part I. the molecular basis of scar formation," *J. Am. Acad. Dermatol.*, vol. 66, no. 1, pp. 1–10, 2012.

[21] S. Robertson and M. R. Miller, "Ambient air pollution and thrombosis," *Part. Fibre Toxicol.*, vol. 15, no. 1, pp. 1–16, 2018.

[22] Y. Feng *et al.*, "Direct and indirect roles of macrophages in hypertrophic scar formation," *Front. Physiol.*, vol. 10, no. AUG, pp. 1–7, 2019.

[23] S. P. Grover and N. Mackman, "Intrinsic pathway of coagulation and thrombosis: Insights from animal models," *Arterioscler. Thromb. Vasc. Biol.*, vol. 39, no. 3, pp. 331–338, 2019.

[24] R. Chaudhry and H. M. Babiker, "Physiology, Coagulation Pathways," *StatPearls*, pp. 3–5, 2018.

[25] S. Palta, R. Saroa, and A. Palta, "Overview of the coagulation system," *Indian J. Anaesth.*, vol. 58, no. 5, pp. 515–523, 2014.

[26] L. Chen *et al.*, "Inflammatory responses and inflammation-associated diseases in organs," *Oncotarget*, vol. 9, no. 6, pp. 7204–7218, 2018.

[27] D. Tang, R. Kang, C. B. Coyne, H. J. Zeh, and M. T. Lotze, "PAMPs and DAMPs: Signal 0s that spur autophagy and immunity," *Immunol. Rev.*, vol. 249, no. 1, pp. 158–175, Sep. 2012.

[28] D. L. Rosin and M. D. Okusa, “Dangers within: DAMP responses to damage and cell death in kidney disease,” *Journal of the American Society of Nephrology*, vol. 22, no. 3. American Society of Nephrology, pp. 416–425, Mar-2011.

[29] K. Newton and V. M. Dixit, “Signaling in innate immunity and inflammation,” *Cold Spring Harb. Perspect. Biol.*, vol. 4, no. 3, 2012.

[30] R. T. Kendall and C. A. Feghali-Bostwick, “Fibroblasts in fibrosis: Novel roles and mediators,” *Front. Pharmacol.*, vol. 5 MAY, no. May, pp. 1–13, 2014.

[31] M. Tirado and W. Koss, “Differentiation of mesothelial cells into macrophage phagocytic cells in a patient with clinical sepsis,” *Blood*, vol. 132, no. 13. American Society of Hematology, p. 1460, 27-Sep-2018.

[32] R. C. de Oliveira and S. E. Wilson, “Fibrocytes, wound healing, and corneal fibrosis,” *Investigative Ophthalmology and Visual Science*, vol. 61, no. 2. Association for Research in Vision and Ophthalmology Inc., 01-Feb-2020.

[33] R. Weiskirchen, S. Weiskirchen, and F. Tacke, “Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications,” *Mol. Aspects Med.*, vol. 65, no. March 2018, pp. 2–15, 2019.

[34] I. G. Macara, R. Guyer, G. Richardson, Y. Huo, and S. M. Ahmed, “Epithelial homeostasis,” *Curr. Biol.*, vol. 24, no. 17, pp. R815–R825, 2014.

[35] N. Sakai and A. M. Tager, “Fibrosis of two: Epithelial cell-fibroblast interactions in pulmonary fibrosis,” *Biochim. Biophys. Acta - Mol. Basis Dis.*, vol. 1832, no. 7, pp. 911–921, 2013.

[36] D. Öztekin Long, Nicole, M and Badre, "基因的改变NIH Public Access," *Bone*, vol. 23, no. 1, pp. 1–7, 2008.

[37] Z. Liu *et al.*, "Vascular disease-causing mutation, smooth muscle α-actin R258C, dominantly suppresses functions of α-actin in human patient fibroblasts," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 28, pp. E5569–E5578, 2017.

[38] J. Baum and H. S. Duffy, "CityPaws Animal Hospital 1823," *J. Cardiovasc. Pharmacol.*, vol. 57, no. 4, pp. 376–379, 2011.

[39] M. L. Bochaton-Piallat, G. Gabbiani, and B. Hinz, "The myofibroblast in wound healing and fibrosis: Answered and unanswered questions," *F1000Research*, vol. 5, no. 0, pp. 1–8, 2016.

[40] M. Alhajj, P. Bansal, and A. Goyal, "Physiology, Granulation Tissue," *StatPearls*, pp. 4–7, 2020.

[41] M. Papetti and I. M. Herman, "Mechanisms of normal and tumor-derived angiogenesis," *Am. J. Physiol. - Cell Physiol.*, vol. 282, no. 5 51-5, 2002.

[42] T. Aldair and J. Montani, "Chapter 1. Overview of Angiogenesis," *Angiogenesis*, pp. 1–10, 2010.

[43] L. Cañedo-Dorantes and M. Cañedo-Ayala, "Skin acute wound healing: A comprehensive review," *Int. J. Inflam.*, vol. 2019, 2019.

[44] J. M. Reinke and H. Sorg, "Wound repair and regeneration," *Eur. Surg. Res.*, vol. 49, no. 1, pp. 35–43, 2012.

[45] S. S. Moshref, S. T. Mufti, S. S. Moshref, and S. T. Mufti, “Keloid and Hypertrophic Scars: Comparative Histopathological and Immunohistochemical Study,” *JKAU Med. Sci*, vol. 17, no. 3, pp. 3–22, 2010.

[46] C. Rosales, “Neutrophil: A cell with many roles in inflammation or several cell types?,” *Front. Physiol.*, vol. 9, no. FEB, pp. 1–17, 2018.

[47] A. C. D. O. Gonzalez, Z. D. A. Andrade, T. F. Costa, and A. R. A. P. Medrado, “Wound healing - A literature review,” *An. Bras. Dermatol.*, vol. 91, no. 5, pp. 614–620, 2016.

[48] J. Kryczka and J. Boncela, “Leukocytes: The Double-Edged Sword in Fibrosis,” *Mediators Inflamm.*, vol. 2015, no. January 2016, 2015.

[49] T. A. Wynn, “Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases,” *J. Clin. Invest.*, vol. 117, no. 3, pp. 524–529, 2007.

[50] A. Mallat, F. Teixeira-Clerc, V. Deveaux, S. Manin, and S. Lotersztajn, “The endocannabinoid system as a key mediator during liver diseases: New insights and therapeutic openings,” *British Journal of Pharmacology*, vol. 163, no. 7. Wiley-Blackwell, pp. 1432–1440, Aug-2011.

[51] E. Patsenker and F. Stickel, “Cannabinoids in liver diseases,” *Clinical Liver Disease*, vol. 7, no. 2. John Wiley and Sons Inc., pp. 21–25, 01-Feb-2016.

[52] S. Batkai *et al.*, “Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury,” *FASEB J.*, vol. 21, no. 8, pp. 1788–1800, 2007.

[53] Vaccarino, “基因的改变NIH Public Access,” *Bone*, vol. 23, no. 1, pp. 1–7, 2008.

[54] B. Hu and S. H. Phan, “Notch in fibrosis and as a target of anti-fibrotic therapy,” *Pharmacol. Res.*, vol. 108, pp. 57–64, 2016.

[55] H. H. Hu *et al.*, “New insights into TGF-β/Smad signaling in tissue fibrosis,” *Chem. Biol. Interact.*, vol. 292, no. 229, pp. 76–83, 2018.

[56] J. Lee, J. N. An, J. H. Hwang, H. Lee, J. P. Lee, and S. G. Kim, “P38 MAPK activity is associated with the histological degree of interstitial fibrosis in IgA nephropathy patients,” *PLoS One*, vol. 14, no. 3, pp. 1–19, 2019.

[57] F. Montecucco and V. Di Marzo, “At the heart of the matter : the endocannabinoid system in cardiovascular function and dysfunction,” *Trends Pharmacol. Sci.*, vol. 33, no. 6, pp. 331–340, 2012.

[58] R. Fu *et al.*, “Targeting transforming growth factor βRII expression inhibits the activation of hepatic stellate cells and reduces collagen synthesis,” *Exp. Biol. Med.*, vol. 236, no. 3, pp. 291–297, Mar. 2011.

[59] M. P. Lim, L. A. Devi, and R. Rozenfeld, “Cannabidiol causes activated hepatic stellate cell death through a mechanism of endoplasmic reticulum stress-induced apoptosis,” *Cell Death Dis.*, vol. 2, no. 6, pp. e170-11, 2011.

[60] M. T. Farooqui *et al.*, “HHS Public Access,” vol. 31, no. 2, pp. 149–156, 2019.

[61] J. Tam, J. Liu, B. Mukhopadhyay, R. Cinar, G. Godlewski, and G. Kunos, “Endocannabinoids in liver disease,” *Hepatology*, vol. 53, no. 1, pp. 346–355, Jan. 2011.

[62] A. Parfieniuk and R. Flisiak, “Role of cannabinoids in chronic liver diseases,” *World Journal of Gastroenterology*, vol. 14, no. 40. pp. 6109–6114, 2008.

[63] B. L. McVicker and R. G. Bennett, “Novel anti-fibrotic therapies,” *Front. Pharmacol.*, vol. 8, no. MAY, pp. 1–21, 2017.

[64] Q. Fu, Y. Zheng, X. Dong, L. Wang, and C. G. Jiang, “Activation of cannabinoid receptor type 2 by JWH133 alleviates bleomycin-induced pulmonary fibrosis in mice,” *Oncotarget*, vol. 8, no. 61, pp. 103486–103498, 2017.

[65] A. Servettaz *et al.*, “Targeting the cannabinoid pathway limits the development of fibrosis and autoimmunity in a mouse model of systemic sclerosis,” *Am. J. Pathol.*, vol. 177, no. 1, pp. 187–196, 2010.

[66] F. A. Giannone *et al.*, “Reversal of liver fibrosis by the antagonism of endocannabinoid CB1 receptor in a rat model of CCl 4-induced advanced cirrhosis,” *Lab. Investig.*, vol. 92, no. 3, pp. 384–395, 2012.

[67] J. Muñoz-Luque *et al.*, “Regression of fibrosis after chronic stimulation of cannabinoid CB2 receptor in cirrhotic rats,” *J. Pharmacol. Exp. Ther.*, vol. 324, no. 2, pp. 475–483, Feb. 2008.

[68] X. Li *et al.*, “Activation of Cannabinoid Receptor Type II by AM1241 Ameliorates Myocardial Fibrosis via Nrf2-Mediated Inhibition of TGF-β1/Smad3 Pathway in Myocardial Infarction Mice,” *Cell. Physiol. Biochem.*, vol. 39, no. 4, pp. 1521–1536, Sep. 2016.

[69] L. Hinden *et al.*, “Modulation of Renal GLUT2 by the Cannabinoid-1 Receptor: Implications for the Treatment of Diabetic Nephropathy,” *J. Am. Soc. Nephrol.*, vol. 29, no. 2, pp. 434–448, Feb. 2018.

[70] A. Kovalchuk *et al.*, “Fighting the Storm: Could Novel Anti-Tnfá And Anti-Il-6 C. Sativa Cultivars Tame Cytokine Storm in COVID-19?,” *Aging (Albany. NY).*, vol. 13, no. 2, pp. 1571–1590, Jan. 2021.

[71] B. Wang *et al.*, “In search of preventive strategies: novel high-CBD Cannabis sativa extracts modulate ACE2 expression in COVID-19 gateway tissues,” *Aging (Albany. NY).*, vol. 12, no. 22, pp. 22425–22440, Jan. 2020.

[72] M. Grinstein, H. L. Dingwall, R. R. Shah, T. D. Capellini, and J. L. Galloway, “A robust method for RNA extraction and purification from a single adult mouse tendon,” *PeerJ*, vol. 2018, no. 4, pp. 1–17, 2018.

[73] D. Kim, B. Langmead, and S. L. Salzberg, “HISAT: A fast spliced aligner with low memory requirements,” *Nat. Methods*, vol. 12, no. 4, pp. 357–360, 2015.

[74] Y. Liao, G. K. Smyth, and W. Shi, “FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features,” *Bioinformatics*, vol. 30, no. 7, pp. 923–930, 2014.

[75] M. I. Love, W. Huber, and S. Anders, “Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2,” *Genome Biol.*, vol. 15, no. 12, pp. 1–21, 2014.

[76] Y. Benjamini, Y. Hochberg, and H. Y. Benjamini, Yoav, “Benjamini and Y FDR.pdf,” *Journal of the Royal Statistical Society. Series B (Methodological)*, vol. 57, no. 1. pp. 289–300, 1995.

[77] R. Illescas-Montes *et al.*, “Human Fibroblast Gene Expression Modulation Using 940 NM

Diode Laser," *Sci. Rep.*, vol. 9, no. 1, pp. 1–7, 2019.

[78] B. Wang *et al.*, "In search of preventive strategies: novel high-CBD Cannabis sativa extracts modulate ACE2 expression in COVID-19 gateway tissues," *Aging (Albany. NY).*, vol. 12, no. 22, pp. 22425–22440, 2020.

[79] E. Balistreri *et al.*, "The cannabinoid WIN55,212-2 abrogates dermal fibrosis in scleroderma bleomycin model," *Ann. Rheum. Dis.*, vol. 70, no. 4, pp. 695–699, Apr. 2011.

[80] "Distinct pharmacological activities between cannabidiol (CBD) and cannabidiol analog (CBDVHS) in a 3D human trabecular meshwork model | IOVS | ARVO Journals." [Online]. Available: https://iovs.arvojournals.org/article.aspx?articleid=2768071. [Accessed: 17-Jun-2021].

[81] C. Echeverría *et al.*, "Lipopolysaccharide induces a fibrotic-like phenotype in endothelial cells," *J. Cell. Mol. Med.*, vol. 17, no. 6, pp. 800–814, Jun. 2013.

[82] H. Li *et al.*, "Rapid pulmonary fibrosis induced by acute lung injury via a lipopolysaccharide three-hit regimen," vol. 15, no. 3, pp. 143–154, 2009.

[83] Y. Liu *et al.*, "A role for intestinal alkaline phosphatase in preventing liver fibrosis," vol. 11, no. 1, pp. 14–26, 2021.

[84] Y. Sakurai, M. Yamaguchi, and Y. Abiko, "Inhibitory effect of low-level laser irradiation on LPS-stimulated prostaglandin E2 production and cyclooxygenase-2 in human gingival fibroblasts," *Eur. J. Oral Sci.*, vol. 108, no. 1, pp. 29–34, Feb. 2000.

[85] D. Perry, J. Ton, and G. M. Allan, "Evidence for THC versus CBD in cannabinoids," *Canadian Family Physician*, vol. 64, no. 7. College of Family Physicians of Canada, p.

519, 01-Jul-2018.

[86] C. Larsen and J. Shahinas, “Dosage, Efficacy and Safety of Cannabidiol Administration in Adults: A Systematic Review of Human Trials,” *J. Clin. Med. Res.*, vol. 12, no. 3, pp. 129–141, 2020.

[87] W. D. Rees, R. Tandun, E. Yau, N. C. Zachos, and T. S. Steiner, “Regenerative Intestinal Stem Cells Induced by Acute and Chronic Injury: The Saving Grace of the Epithelium?,” *Frontiers in Cell and Developmental Biology*, vol. 8. Frontiers Media S.A., p. 1333, 12-Nov-2020.

[88] C. Politis, J. Schoenaers, R. Jacobs, and J. O. Agbaje, “Wound healing problems in the mouth,” *Frontiers in Physiology*, vol. 7, no. NOV. Frontiers Media S.A., p. 507, 02-Nov-2016.

[89] A. Zhavoronkov and P. Mamoshina, “Deep Aging Clocks: The Emergence of AI-Based Biomarkers of Aging and Longevity,” *Trends Pharmacol. Sci.*, vol. 40, no. 8, pp. 546–549, 2019.

[90] N. Bhatia-Dey, R. R. Kanherkar, S. E. Stair, E. O. Makarev, and A. B. Csoka, “Cellular senescence as the causal nexus of aging,” *Front. Genet.*, vol. 7, no. FEB, pp. 1–14, 2016.

[91] E. F. Redente *et al.*, “Tumor necrosis factor-α accelerates the resolution of established pulmonary fibrosis in mice by targeting profibrotic lung macrophages,” *Am. J. Respir. Cell Mol. Biol.*, vol. 50, no. 4, pp. 825–837, 2014.

[92] S. L. Friedman, “Clarity and Challenges in Tissue Fibrosis,” in *Innovative Medicine*, Springer Japan, 2015, pp. 187–194.

[93] A. Zhavoronkov *et al.*, "Deep learning enables rapid identification of potent DDR1 kinase inhibitors," *Nat. Biotechnol.*, vol. 37, no. 9, pp. 1038–1040, 2019.

[94] D. Ghartimagar *et al.*, "Squamous cell carcinoma arising in familial gingival fibromatosis—a rare case report," *J. Surg. Case Reports*, vol. 2017, no. 10, pp. 1–4, Oct. 2017.

[95] A. R. Gadbail *et al.*, "Oral squamous cell carcinoma in the background of oral submucous fibrosis is a distinct clinicopathological entity with better prognosis," *J. Oral Pathol. Med.*, vol. 46, no. 6, pp. 448–453, Jul. 2017.

[96] H. C. Chang *et al.*, "Irritable bowel syndrome and the incidence of colorectal neoplasia: A prospective cohort study with community-based screened population in Taiwan," *Br. J. Cancer*, vol. 112, no. 1, pp. 171–176, Jan. 2015.