

**Investigation of the abilities of cannabinoids to alter biomarker
production involved in inflammation and periodontal tissue
regeneration.**

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Dedication

To all patients, may the fruits of this research be of service to you.

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Equation 2: $Cv = \sigma / \mu$, (Cv= coefficient of variation, σ = standard deviation and μ =mean). (Canchola, 2017)..... 126

List of abbreviations:

AEA- anandamide

AM-251 – CB1 antagonist; GPR55 agonist

CB1- cannabinoid receptor 1

CB2- cannabinoid receptor 2

CBD- cannabidiol

CBG – canabigerol

c-DNA- complementary DNA

CIDs – Chronic immune diseases

Cox2 – cyclo-oxygenase 2

Ct- cycle threshold

ELISA- enzyme linked immunosorbent assay.

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

h-PDLc – human periodontal ligament cells

IL-8- interleukin 8

kDA- kilo Dalton

LPS- lipopolysaccharide

NSAIDS – non-steroidal anti-inflammatory drugs

OPG- Osteoprotegerin

RANK/RANKL- receptor activator of nuclear factor kappa beta (NFkB ligand)

RA- Rheumatoid arthritis

THC- Tetrahydrocannabinol

TIG-K- Telomerase inhibited gingival keratinocyte.

TLR4/5 – Toll like receptors

TNF-Alpha- Tumour necrosis factor alpha

Abstract

Periodontitis is a self-destructive, long-standing inflammation condition which has serious repercussions on general health and oral health, in particular. Periodontitis is caused by an immune response of periodontal cells with the noxious gram-negative bacteria. The chronic interaction between the immune cells, periodontal cells and bacterial virulence factors results in expression of cytokine cascade. It can result in destruction of periodontal apparatus which, includes the gingival recession and alveolar bone destruction: leading to periodontal pocket formation and could eventually lead to loss of tooth. Various treatment methods are available but still there is a need for robust treatment for periodontitis due to limitations and side effects of the available treatment methods. Cannabinoids have been proven to possess anti-inflammatory potential and antimicrobial capacity and hence could be found promising to address chronic diseases such as periodontitis. The aim of the present study is to evaluate the role of cannabinoids such as CBD and CBG to limit the over expression of self-destructive inflammatory cytokines in an in-vitro periodontitis model. This in-vitro periodontitis model comprises cells (Cell lines and primary cells) and inflammatory agents (LPS and Flagellin). In order to evaluate the efficacy of cannabinoids for their anti-inflammatory potential, Cell lines and primary cells were pre-treated with cannabinoids before presenting them with inflammatory stimulus such as LPS and Flagellin. The methods used include using different concentrations of CBD, CBG, A-251 and O-1602 therefore, the toxicity of these compounds was evaluated on the cells used in the study. The efficacy of the cannabinoids to modulate the release of cytokines such as IL-8, IL-6, IL-1 β and TNF- α was evaluated both at mRNA level and protein level. The results obtained showed that CBD was cytotoxic at concentration 100 μ M but not cytotoxic at

concentrations below 10 μ M on the cells used in this project. CBG, A-251 and O-1602 were not found to be cytotoxic for the concentration well above that used in the project. CBD and CBG were shown to modulate the expression and production of pro-inflammatory cytokines induced by LPS/flagellin; pre-exposure with CBD decreased the expression of IL-8 by 325 times at mRNA and by 60-70 % at protein level on TIGK cells. Whereas, pre-exposure with CBD decreased the expression of IL-8 approximately 60 % at m-RNA level and 25 % at protein level on primary periodontal cells. This study demonstrated the ability of cannabinoids to determine immunomodulatory effects suggesting possible therapeutic applications in the field of periodontal research.

Chapter 1 Introduction and literature review

General introduction

Available research literature informs us that periodontitis is a chronic condition which affects the health and wellbeing of an individual (Nazir., 2017). Poor periodontal health can cause problems with mastication (Palinkas et al., 2019), speech (Meusel et al., 2015), and aesthetics (Gkantidis et al., 2010). This causes undue burden not only to the individual but also to the dental clinics and the NHS (Zemedikun et al., 2021).

Periodontitis is a chronic inflammatory disease of the oral tissues surrounding and supporting the teeth in their alveolar sockets (Figure 1). The inflammation of the periodontium results from a chaotic interaction among inhabitant oral microorganisms (Hajishengallis, 2021), leading to an imbalance of the normal oral microbiome homeostasis (Mark Bartold & Van Dyke, 2013). The subsequent increase of specific microorganisms, especially Gram-negative pathogens result in chronic destructive inflammation of periodontal ligament and the supporting alveolar bone (Mark Bartold & Van Dyke, 2013). The result is gingival recession and the formation of periodontal pockets (Newman et al., 2018). The progression of periodontitis leads from mild to moderate and then to severe symptoms during progression of the disease. The initial periodontal destruction begins with gingival inflammation and loss of epithelial attachment from the tooth (Lang et al., 2009). This is because of the host inflammatory response against Gram-negative microorganisms and their toxins. During moderate progression of the disease, the destruction of periodontium progresses towards established chronic inflammation of the subgingival environment due to adaptive and innate immune responses (Fitzsimmons et al., 2018). Once established, the development and

progression of periodontitis towards its severe form is determined by the immune response and bacterial load on oral and general health (Cekici et al., 2014).

Experimental studies in animal models reported that activation of cytokine cascade is triggered when the animals were exposed to inflammatory agents. This results in bone loss during periodontitis (Alayan et al., 2007, Eskan et al., 2012). The pathological host immune response against local microbes or inflammatory agents includes expression of pro-inflammatory cytokines by activation of pattern recognition receptors on the periodontal cells (Pan et al., 2019). The expression of pro-inflammatory mediators, including Tumour Necrosis Factor - α (TNF- α), Interleukin-1b (IL-1b), Interleukin-6 (IL-6) and Interleukin-8 (IL-8), resulting from interaction of various cell types of periodontium and oral pathogens such as *Porphyromonas gingivalis* (*P. gingivalis*). The expressed cytokines are involved in recruiting and activating inflammatory cells to control the ensuing infection and restore the balance of tissue homeostasis. Hence, homeostasis facilitates tissue repair and regeneration. The host immune response can be either protective or destructive based on its stage of inflammation i.e., acute or chronic stage of inflammation (Marton, 2000).

The initial inflammatory response provides the protective self-defence mechanism. However, persistent local inflammation is a major factor in the destructive shift of the immune response. Persistent inflammatory cells contribute to systemic inflammation. Increased sub-gingival infection increases the risk of chronic diseases such as diabetes (Lalla et al., 2011), heart disease (Kebuschull et al., 2010), and rheumatoid arthritis (Lundberg et al., 2010). Periodontitis contributes to the additional systemic inflammatory burden (D'Aiuto et al., 2004) manifested in chronic diseases such as cardiovascular diseases (Genco et al., 2010) and physiological conditions such as pregnancy (Madianos et al., 2013).

Periodontitis is prevalent both in developed and developing countries and it affects approximately 11 % of the entire world population, making it the most prevalent chronic inflammatory disease (Eke et al., 2015).

Cumulative research reports that monocytes and macrophages are the major cell groups that elicit inflammatory responses in periodontal diseases. Furthermore, these cells are known to develop LPS tolerance and produce inflammatory cytokines albeit, transient. Nonetheless, gingival inflammation persists in the presence of bacterial virulence factors. Inflammatory responses persist in the presence of virulence factors which may be a result of gingival fibroblasts. It is now widely accepted that fibroblasts play an important role in chronic infections (Kang et al., 2016).

Although gingival fibroblasts are involved in the pathogenesis of chronic periodontal disease, such a role may not result from a direct stimulation by periodontopathic bacteria. This phenomenon is more likely to be mediated indirectly by immune system expressing IL-1 (Yamazaki' et al., 1992). If the immune response to periodontal pathogens is left unchecked, this leads to chronic unresolved inflammation, resulting in damage to the periodontal supporting tissues. Therefore, the host immune responses are critical in determining the overall periodontal tissue destruction.

The present study investigates the use of cannabinoids to reduce the expression of pro-inflammatory cytokines during periodontitis. Studies have confirmed that cannabinoids possess pro-inflammatory and anti-inflammatory properties which were shown to be effective in the treatment of chronic diseases (Klein, 2005). This is because cannabinoids have the capacity to bind G-binding protein

cannabinoid receptors (CB₂) via regulation of pro and/or anti-inflammatory markers (Scholten, 2006) (Ataei et al., 2022). Among various components of cannabis, psychoactive Tetrahydrocannabinol (THC) is identified as a psychoactive and an unstable component of hemp as it degrades at room temperature at the rate of 3 to 5 % per month and is less suitable for human consumption (Martínez et al., 2020), (Repka et al., 2006). Therefore, the current study aimed to identify the effect of non-psychoactive cannabinoids on inflammatory markers for the management of chronic periodontal diseases in humans.

Hypothesis and Aim(s)

This study hypothesises that the anti-inflammatory properties of cannabinoids have the potential to address self-destructive inflammation. The aim of the present study is to investigate the ability of cannabinoids to alter the production of the inflammatory biomarkers and their role during periodontal regeneration.

Thesis outline

Chapter 1 provides a literature review which supports the use of cannabinoids on periodontitis. Cannabinoids were found useful to address some chronic inflammatory diseases and the potential to arrest the destructive inflammation during periodontitis. Also included in this chapter is the aim and objectives of the study. Chapter 2 discusses the rationale for the use of the selected materials and methods. The choice of materials used in this project grounded in the literature. The principle of each and every method was outlined based on the selected methods after their standardisation. Chapter 3 details the experimental results of *in-vitro* laboratory work: A series of three experiments were conducted and the final results is a distillation of the three experiments. The results confirmed that non cytotoxic concentration of cannabinoids use altered the inflammatory biomarkers at both mRNA and protein level. In Chapter 4, the discussion focuses on rationale of selected methods and inference of the results obtained in this study. The results from this study correlated with existing work. Chapter 5 summarises the main themes and findings. In addition, it raises issues of future work.

1.1 Introduction

1.1.1 Periodontitis

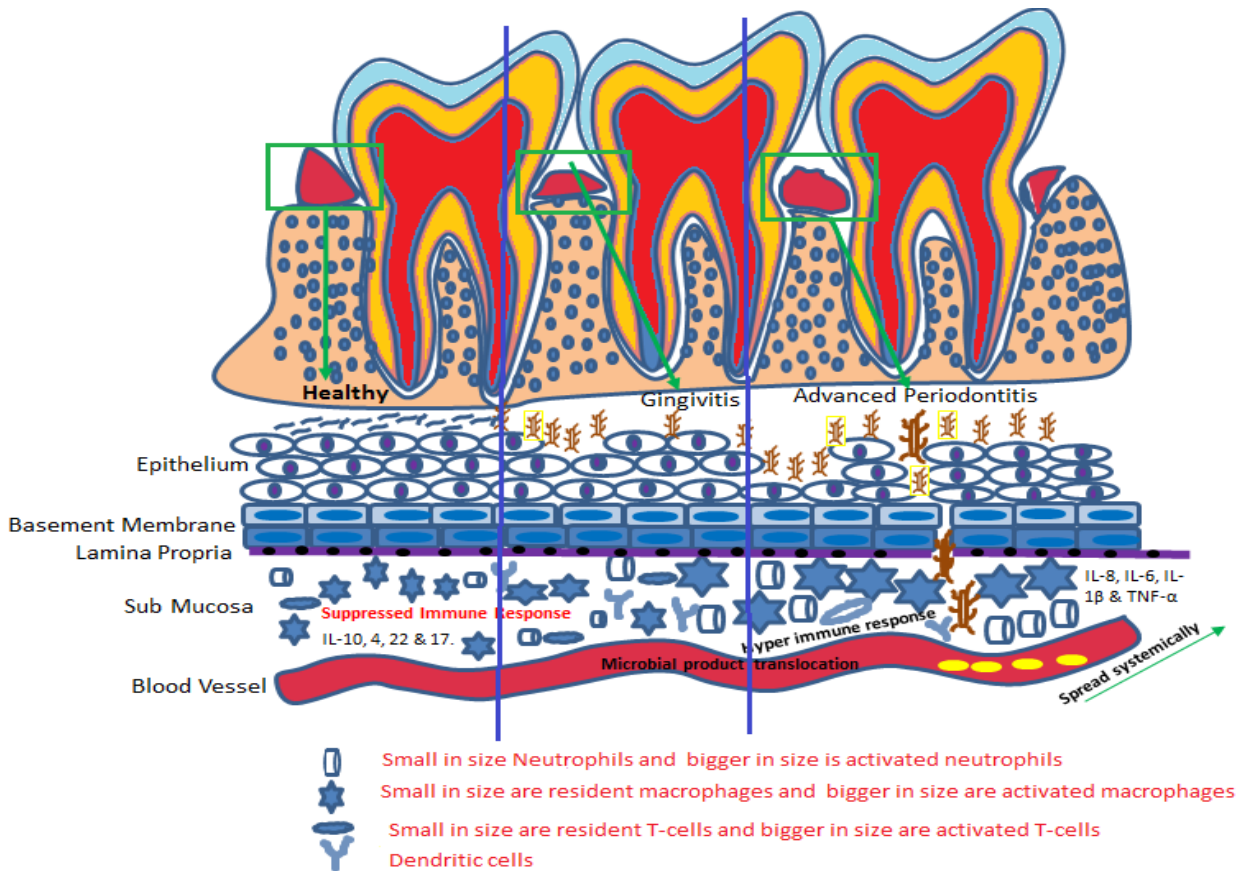
Periodontitis is an inflammatory gum disease caused by bacterial microorganisms (Siqueira Jr, 2007). It causes destruction of tooth-supporting oral tissues (Figure 1), which leads to loss of the tooth and a contributing factor in chronic conditions such as diabetes (Pejčić et al., 2006). It begins primarily with gingivitis, and if left unchecked affects adjacent tissues and could (Hajishengallis, 2021) cause loss of surrounding tissues. Studies inform us that poor oral hygiene leads to supragingival and subgingival plaque deposition, eventually resulting in mineralisation of plaque, and calculus formation (Lockhart et al., 2009) which then leads to periodontal diseases.

Periodontitis involves inflammation of gingiva and periodontal ligament. In the event of persistent inflammation, a catastrophic result is inevitable as shown in Figure 1. This diagram presents the cumulative physiological and pathological immune response during advancing periodontitis. It shows the gingival recession, destruction of alveolar bone and spread of infection systemically. The initial inflammation is a physiological response to bacterial invasion rather than pathological (bacterial and their toxins as microbial challenge), (Carranza and Camargo, 2006), the chronic inflammation caused by both physiological and pathological poses detrimental effects locally and systemically. In addition, local destruction of soft tissue (gingiva) and hard tissue (alveolar bone) has been linked to the overall chronic inflammation caused by release of bacterial toxins and the existing protective host immune mediated inflammation (Ramamurthy et al., 2014, Xu and Gunsolly et al., 2014). Eventually it leads to loss of the alveolar bone surrounding the tooth, thus results in increase mobility of the tooth which could lead to problems with mastication (De Freitas Borges et al., 2013), speech

(De Freitas Borges et al., 2013) and aesthetics(Gokturk et al., 2018). These detrimental effects could take a long time to develop, however, some adults can have the active form of periodontitis which affects the periodontium rapidly and acutely such as aggressive periodontitis (Teughels, 2014). Aggressive periodontitis has been known to be expressed differently to chronic periodontitis due to its hypothesised genetic aetiology, where the disease effects the dental tissue either locally or generally even in the absence of local factors (Könönen, 2014).



A



B

Figure 1: Mechanism of immune regulation in health versus immune pathological conditions. Figure A shows the alveolar bone loss in immune pathological condition as compared to healthy surrounding alveolar bone, Figure B represents cross sectional view of events or stages of periodontitis both clinically and molecular level differences occurring during the chronic periodontal diseases. (Adapted from Heron et al., 2017(B), created using Bio render.com(A)).

1.1.2 Classification of periodontal disease

Periodontitis can be classified based on several factors such as time (acute and chronic), extent (localised and generalised) and severity (mild, moderate and severe) of disease (Figure 2). Page & Schroeder in 1976 presented a classification of periodontal inflammation based on severity of inflammation (Page et al., 1976). They provide 4 stages of inflammation (Figure 2), the initial lesion, the early lesion, the established lesion, and the advanced lesion or the destructive phase. This last phase represents the transition from gingivitis to periodontitis (Page et al., 1976). In addition, the destructive phase can also be localised (<30%) or generalised (>30%), depending on the extent of the area affected in the oral cavity. This project focuses on the severity of the disease and investigating the ability of cannabinoids in limiting the damaging effects of periodontal inflammation at earlier stages of periodontal inflammation relating to mild periodontal stage.

FORMS OF PERIODONTITIS

1. Necrotising Periodontal Diseases

- a) Necrotising Gingivitis
- b) Necrotising Periodontitis
- c) Necrotising Stomatitis

2. Periodontitis as Manifestation of Systemic Diseases

Classification of these conditions should be based on the primary systemic disease according to the international statistical classification of Diseases and Related Health Problems (ICD) Codes.

3. Periodontitis

- a) **Stages:** Based on severity and complexity of management
 - Stage I:** Initial periodontitis
 - Stage II:** Moderate periodontitis
 - Stage III:** Severe periodontitis with potential for additional tooth loss
 - Stage IV:** Severe periodontitis with potential loss of dentition
- b) Extent and Distribution: Localised; Generalised; Molar-Incisor distribution
- c) Grades: Evidence or Risk of rapid progression, anticipated treatment response
 - i. **Grade A:** Slow rate of progression
 - ii. **Grade B:** Moderate rate of progression
 - iii. **Grade C:** Rapid rate of progression

Figure 2: Recent classification of periodontitis (Caton et al., 2018)

1.1.3 Predisposing factors

Common factors present in a person with increased risk of developing periodontal problems are poor oral hygiene leading to inflammation of gums (gingivitis) thereby increment of cytokines leading to destruction of surrounding tissue which leads eventually to advanced periodontitis (Figure 1). Predisposing factors include local and systemic factors or events, which trigger the onset or increase the susceptibility of an individual towards chronic periodontal diseases. These factors play a significant role in considering the management of more susceptible individuals, for instance patients with the specific risk factor IL-1 genotype were identified to need more regular oral care to address the identified genetic risk of moderate to severe periodontitis (Mcdevitt et al., 2000).

1.1.3.1 Local Factors

1.1.3.1.1 Acquired

Local acquired factors are the acquainted anatomical factors to restore the anatomy of tooth and habitual variations either due to inadvertent accidents or negligence of individuals such as dental restorations, trauma, caries, and poor oral hygiene and these were found responsible for accumulation of plaque inhabited by the bacterial species responsible for inflammatory periodontal disease (Matthews, 2004).

1.1.3.1.2 Anatomical

Local anatomical factors are developmental variation of dental tissue such as deviations from normal anatomical arrangement/structure of periodontal apparatus such as malposition of tooth, root concavities and furcation can cause difficulty in oral

hygiene maintenance and become one of the etiological factors for periodontitis (Ramfjord, 1952).

1.1.3.2 Systemic Factors

1.1.3.2.1 Nonmodifiable

These objective factors are inherent in people; these refers to genetics, age and hormonal variations. When treating chronic diseases such as periodontitis, health practitioners need to consider these factors (Michalowicz, 1994).

1.1.3.2.2 Modifiable

Systemic modifiable effects are either due to deleterious habits or associated with existing systemic diseases in an individual which have an impact on periodontal health. Certain modifiable factors incur extended risk of facing chronic periodontal diseases for example smoking, drugs (therapeutic and recreational), stress, obesity, diabetes, nutritional deficiency (e.g., vitamin C) and socio-economic status (Reynolds, 2014).

1.1.4 Aetiology

Periodontitis is a multifactorial chronic oral disease where bacterial plaque accumulation is the primary causative agent. Predisposing risk factors are extended aggravating factors, meaning they augment the disease progression, hence considered additional causative agents. Oral plaque serves as a breeding ground for a multitude of bacteria species. Two species of oral bacteria in particular *Porphyromonas gingivalis* (*P. gingivalis*) and *Treponema denticola* are associated with periodontal diseases (How et al., 2016). These two species belong to the Gram-negative bacterial species which express virulence factors responsible for destruction of periodontium either directly or indirectly by modulation of the host inflammatory response. This local tissue inflammatory response is thus responsible for the imbalance in bone remodelling and results in bone loss because of increased activity of osteoclasts (Y. Liu et al., 2010). Within periodontal lesions, the local immune response is amplified and dysregulated. In addition to the localised release of cytokines by local periodontal cells, there is a concurrent systemic release of cytokines. This synergistic release of cytokine is deleterious as it culminates in an overexpression of inflammatory cascade (Jewett, 1993) (Calder et al., 2001). LPS and Flagellin from *P. gingivalis* were used in this project to simulate the in-vitro periodontitis model in order to explore the release of cytokines by the periodontal cells.

Cytokines and chemokines are proteins which control and mediate the initiation and established stages of immune response. These proteins enable migration of immune cells to the site of infection and upregulate the immune response, manifested as an inflammatory cascade (Kouwenhoven, 2001). To date, more than 300 cytokines have been identified—including interleukins, chemokines, interferons and tumour necrosis

factors. The anti-inflammatory cytokines expressed during inflammatory response i.e., IL-1 β , IL-8, IL-6 and TNF- α were found to be associated with periodontitis and considered to play a key role in the destruction of periodontium during periodontitis ((Saito, 2001, Dinarello, 2000). These cytokines serve as biomarkers in disease activity, and estimation of expression of these biomarkers has therefore been suggested as a potential way to identify the severity of pathology to arrest chronic destructive inflammatory response (Deswal, 2001).

1.1.5 Prevalence

The World Health Organisation reported in 2022 that the approximately 3,5 billion people across the world suffer with oral diseases. Periodontal problems in particular constitute more than a billion cases (19% of the population worldwide) of periodontitis (Benzian et al., 2022). Severe periodontal disease i.e., periodontitis affects 10-15% of the population and moderate severity (i.e.) is comparatively widespread i.e., 40-60%, (Petersen et al., 2012, Preshaw et al., 2012, Papapanou et al., 2018). In United Kingdom almost 50 percent of the population suffers from some form of periodontal disease and its prevalence is linked to higher socio-economic status of the population (Gray-Burrows et al., 2017). Additionally, periodontitis has been reported as a manifestation of systemic diseases such as diabetes, haematological disorders e.g., neutropenia and genetic disorders e.g., Downs syndrome, papillon- Lefevre and chediak higashi syndrome (Tiedemann and Wetzel, 2001). Which is an additional factor to add up on the overall prevalence of the disease. Also, studies have reported that periodontal diseases increase the risk of systemic conditions such as pancreatic cancer (Michaud, 2007) increased risk of developing heart disease, diabetes, rheumatoid arthritis, dementia, problems in pregnancy, and oral and oesophageal cancer (Fitzpatrick et al., 2010) (NHS, Health risks of gum diseases. <https://www.nhs.uk/live-well/healthy-body/health-risks-of-gum-disease>, 2018).

1.1.6 Pathogenesis

During periodontitis, the chronic inflammation affects oral tissues and eventually acts as a risk factor for chronic systemic diseases, presenting initially as a mere mild erythema or inflammation of gingival tissue. Periodontium infection causes sequential changes such as gingival inflammation, destruction of connective tissue, periodontal

pocket formation and the loss of supporting alveolar bone around the teeth (Figure 2) (Kozono et al., 2010). Osteoblasts are cells that form bone tissue. Periodontal pockets result due to irreversible alveolar bone resorption. Osteoblasts express receptor activator of nuclear factor kappa-B ligand (RANKL) in response to bone-resorbing factors. Osteoblasts are also responsible for the production of osteoprotegerin (OPG). OPG is a decoy receptor for RANKL and prevents its binding to receptor activator of nuclear factor kappa-B (RANK). Such interaction suggests an intricate balance between bone formation and resorption (Kajiya, 2010). The increase in RANKL/OPG ratio serves as a biomarker that denotes the occurrence of periodontitis but may not necessarily predict ongoing disease activity (Belibasakis et al., 2012). The inflammatory cascade during periodontal disease involves the expression of proinflammatory cytokines such as TNF- α . Increased secretion of cytokines during chronic inflammatory bone diseases e.g., TNF- α was reported to induce bone resorption at the sites of chronic inflammation (Boyce et al., 2005).

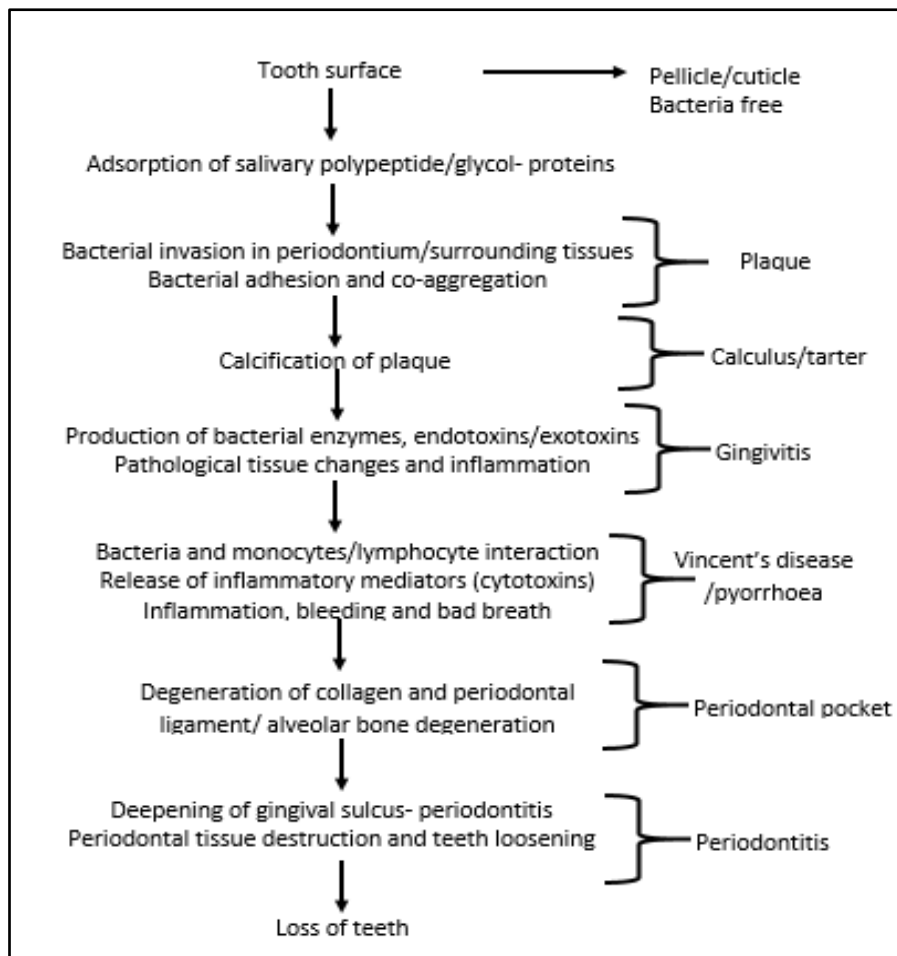


Figure 3: Flow chart representing pathogenesis of periodontal diseases. (Dusane et al., 2016).

Delay in receiving oral care such as regular maintenance of oral hygiene, or in identifying the underlying cause of oral pathology; or exposure of vulnerable immunocompromised individuals to pathogens may lead to a severe inflammatory condition i.e., chronic periodontitis or aggressive periodontitis. Periodontal disease or periodontitis is classified as a biologically complex chronic inflammation of the periodontium i.e., gum and its surrounding tissue. It can be compared with other chronic immune disorders (CIDs) such as diabetes mellitus where multiple factors (Figure 3) determine the resultant decline in immune fitness causing severe compromise in health and stability of the tooth (Munz et al., 2017).

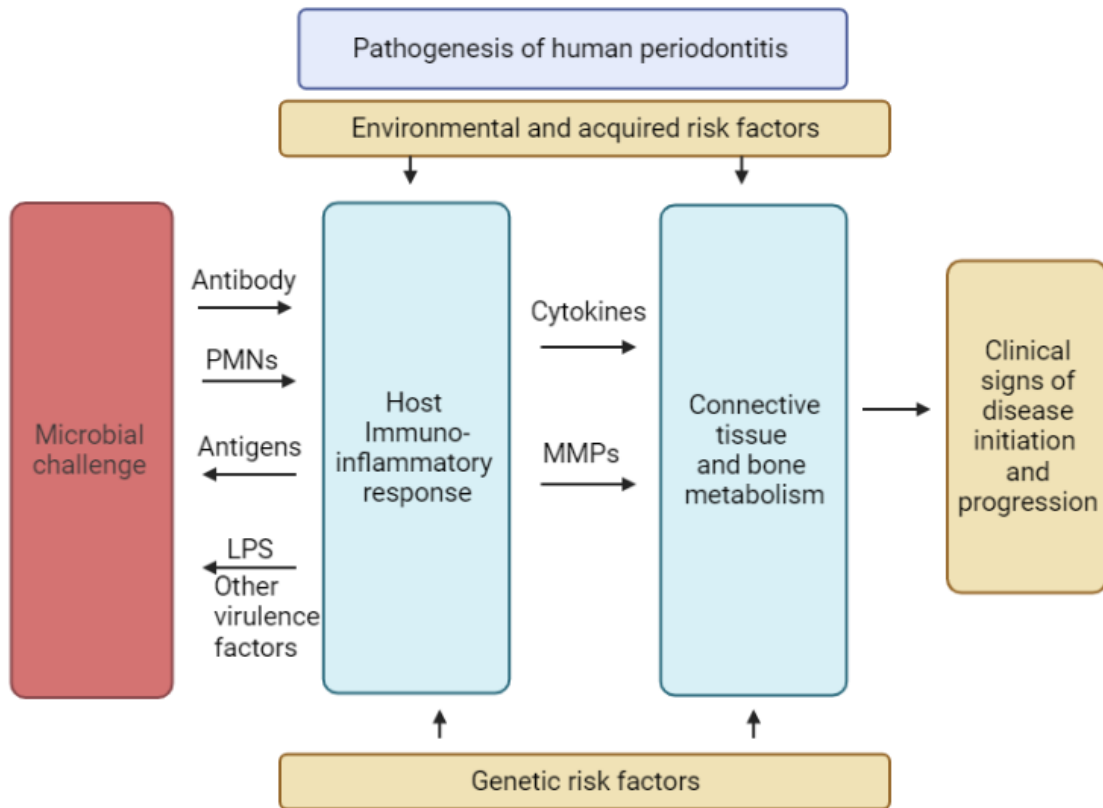


Figure 4: Page and Kornman model of pathogenesis of periodontitis illustrating the host microbe interaction promoting the induced expression of immunity molecules and their effect as leading to the initiation and progression of clinical signs of disease (designed by biorender.com)

The above flow diagram demonstrates the pathogenesis of periodontitis which involves the interaction of host immune system and oral microorganisms' interaction during their outcome as periodontal diseases. Collectively, the above-mentioned sequence of inflammation, gingival recession, and alveolar bone destruction (Figure 1) due to persistent poor oral hygiene disrupt the healthy and intricate arrangement of periodontium (Figure 3), leading to periodontitis.

1.1.7 Dental periodontium

Periodontium (peri = around, odontos = tooth) is a specialised structure that surrounds the tooth in its bony socket and assists dentition to perform essential functions including mastication, speech, and aesthetics. It includes various anatomical structures around the teeth such as gingiva, periodontal ligament, cementum, and alveolar bone (Figure 4).

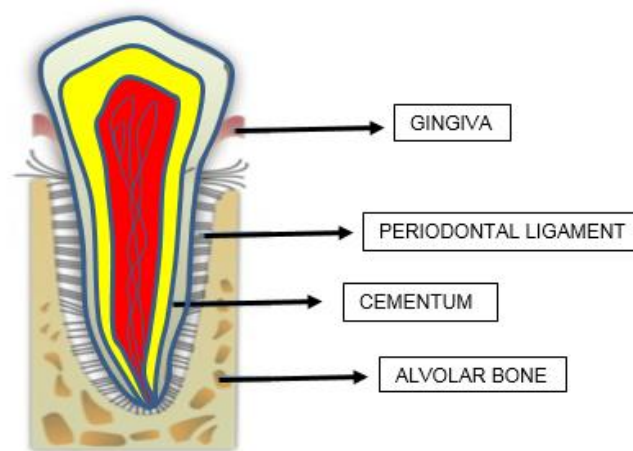


Figure 5: Anatomical description of periodontium: Representative cross-sectional image of a premolar showing the dental periodontium comprises gingiva, periodontal ligament, cementum and alveolar bone, Image adapted from (Edwards, 2010).

1.1.7.1 Gingiva

Gingiva is the anatomical term for gums surrounding the teeth. It consists of free marginal gingiva and attached gingiva separated by marginal groove (Figure 5). It lines the external surface of the periodontium and consists of stratified squamous epithelium supported by a thin layer of dense fibrous connective tissue. The gingival epithelium may be divided into keratinized and non-keratinized based on their presence. The unattached non-keratinized gingival epithelium lines the gingival sulcus (gingival crevicular surface) whereas the junctional epithelium which attaches the gingiva to the

tooth surface along with visible oral epithelium of gingiva is a keratinized structure and acts as impermeable barrier against harmful microbes (Figure 5). Bacteria and their toxins such as LPS were found responsible for transient bacteraemia of gingiva. Also, the inflammation caused due to bacterial, or their toxin insult causes the deterioration of tight junctions and make the gingival epithelium to become fragile (Vitkov et al., 2023). The inflamed gingiva becomes prone to rupture, such that an everyday activity like tooth brushing causes a break in the epithelial barrier.

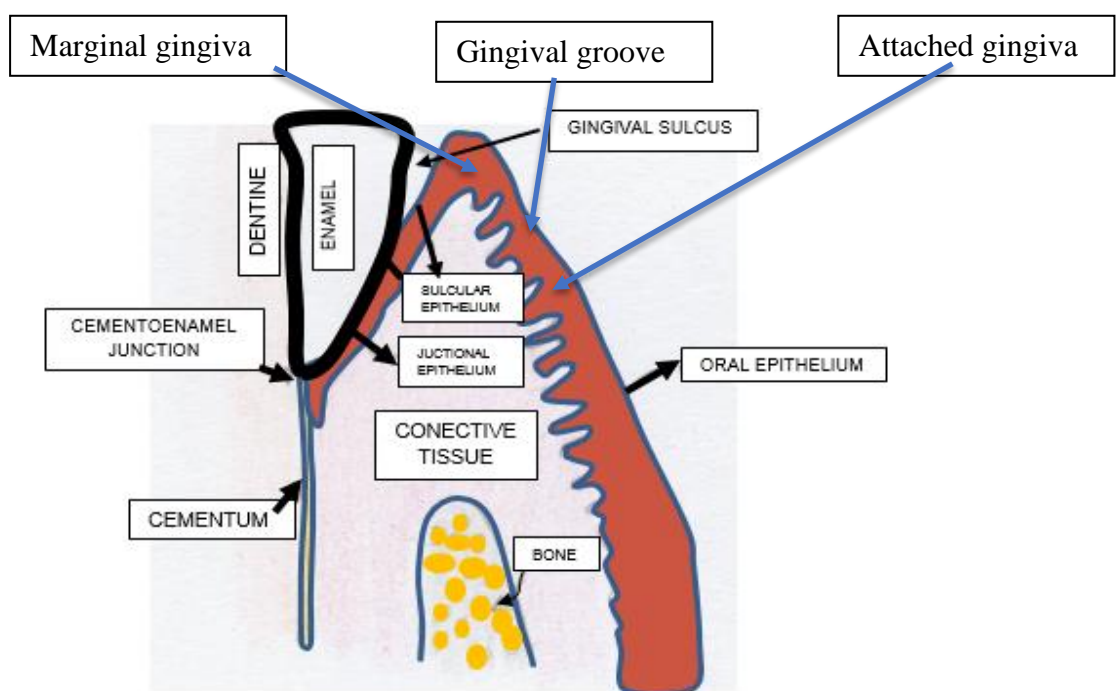


Figure 6: Periodontal apparatus attached to tooth, cross-sectional image showing attachment of junctional epithelium and surrounding hard and soft tissue descriptive image made using Biorender.com.

1.1.7.2 Periodontal Ligament

Periodontal ligament (PDL) (Figure 6) is a soft connective tissue that provides anchorage to cementum on one side and alveolar bone on the other side. It forms the gomphosis, which is a fibrous connective tissue joint and provides functional attachment of the tooth in its socket (Ho et al., 2007). PDL also provides a cushioning

effect which helps in dissipating the masticatory forces, alongside remodelling, and repair of adjacent tissues.

Periodontal fibres i.e., Sharpey's fibres bundles appear to arrange in different planes (Figure 6) and are classified based on orientation and location. There are five types of fibre attachment identified: trans-septal, oblique, horizontal, apical, and inter-radicular. They support the tooth to withstand masticatory forces, absorb and resolve tipping forces, rotatory forces, and the forces that could dislodge the tooth from its alveolus. These PDL Sharpey's fibres overlie the dentine of the root and are continuously deposited throughout life. PDL fibres have an enriched blood supply and reported previously that periodontal ligament fibre cells express cytokines at the site of inflammation. Hence, D. Jönsson et al (2011) hypothesised previously that future studies could investigate cytokines expression in addressing the periodontal diseases (D. Jönsson et al., 2011).

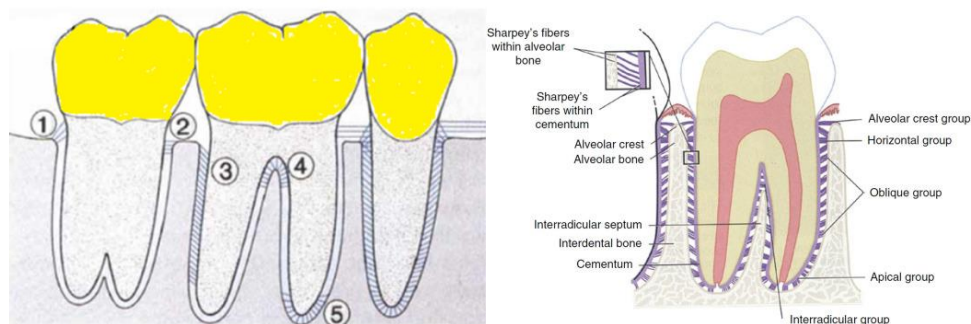


Figure 7: Diagrammatic representation of sagittal section (periodontal ligament space (0.2-0.4 mm)), Sharpey's fibers (periodontal fibers embedded in alveolar bone and cementum). 1. Alveolar crest fibers, 2. Horizontal fibers, 3. Oblique fibers, 4. Inter-radicular.

1.1.7.3 Cementum

Cementum is the mineralised tissue of periodontium covering the root dentine, providing anchorage, adaptation, repair, load absorption during mastication and anchorage to PDL. There are two types of cementum; (i) cellular or secondary cementum, which is relatively thick and covering apical root, and (ii) acellular or primary cementum, which is thin, covering cervical half of root and contains little to no cellular component. The primary role of cementum is to provide attachment to collagen fibres of PDL since these fibres do not innervate underlying root dentine. The cementum regeneration was considered an important factor for periodontal regeneration and hence a vital element to address while tackling periodontitis (Grzesik & Narayanan, 2002).

1.1.7.4 Alveolar bone

The second mineralised tissue of periodontium is alveolar bone. Alveolar bone houses the tooth by providing anchorage to PDL on its dense compact structure called lamina dura or bundle bone (Figure 8). It is a dynamic tissue and capable of rapid remodelling because of different cells which include osteoblasts, osteocytes, and osteoclasts. The remodelling capacity of alveolar bone is higher than that of any other skeletal bones (Huja et al., 2006). Occlusal stress stimulation and oral inflammatory process affect its remodelling (Gruber 2019; Lerner et al., 2019; Pandya, 2022) capacity. Previous studies stated that bacterial species in periodontal pocket were responsible for expression of cytokines such as IL-8, IL-6 and TNF- α . These cytokines were reported to have their role in activation of osteoclasts and inhibition of osteoblast hence resulting in alveolar bone resorption (Schwartz et al., 2000).

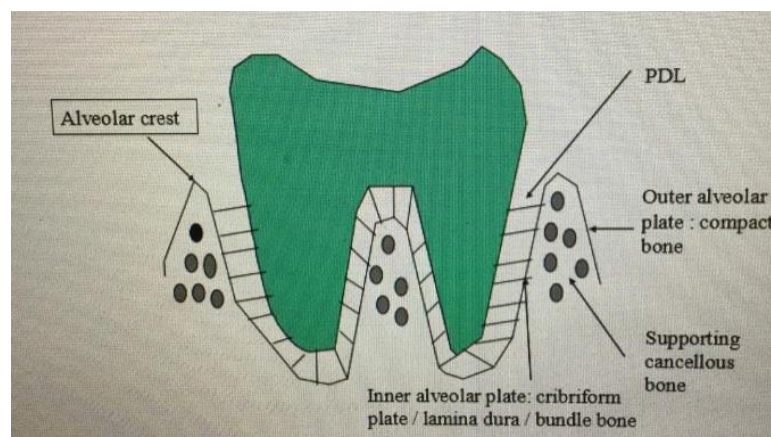


Figure 8: Alveolar bone structure formed by several different types of bone (compact bone, cancellous bone and bundle bone) and its relation to periodontal ligament (PDL), pictorial illustration of alveolar bone and its arrangement among periodontium (Drawn using Microsoft office paint).

1.1.7.5 Cells of periodontium

Periodontium is considered as a class of unique connective tissue as it performs vital functions such as proprioception and regeneration of cementum, PDL and bone. Bone remodelling cells such as osteoblast and osteoclasts reside in alveolar bone. Epithelial rests of Malassez and cementum formation cells (cementoblasts) are found at the root surface of the tooth. Endothelial cells present throughout the periodontal tissue are responsible for vascular supply to the various components of periodontium. Cells responsible for defence mechanism such as neutrophils, plasma cells and mast cells reside in epithelial cells (lamina propria) after migrating from the subepithelial vascular plexus and perform an active role during any insult to periodontium. Other important cells are fibroblasts, which are abundant in periodontal tissue (46 approx. 65 %) (Tzach-Nahman et al., 2017) plays a key role in the production of collagen and metalloproteinases, extracellular matrix and thus maintenance of homeostasis. Fibroblast cells of periodontal ligament are considered to have a fast turnover as compared to gingival fibroblasts (1/5th) and skin fibroblasts (1/15th) (Limeback, 1978). These cells are responsible for the continuous remodelling of periodontal tissue including remodelling of alveolar bone and cementum during repair or orthodontic treatment and passive eruption as a result of ageing (Vignery & Baron, 1980). Cells representing the osteoblasts such as G-292 cells, keratinised epithelial cells such as TIGK cells and non-keratinised epithelial cells such as A-2780 were used in this study along with the primary periodontal fibroblast cells harvested from extracted teeth.

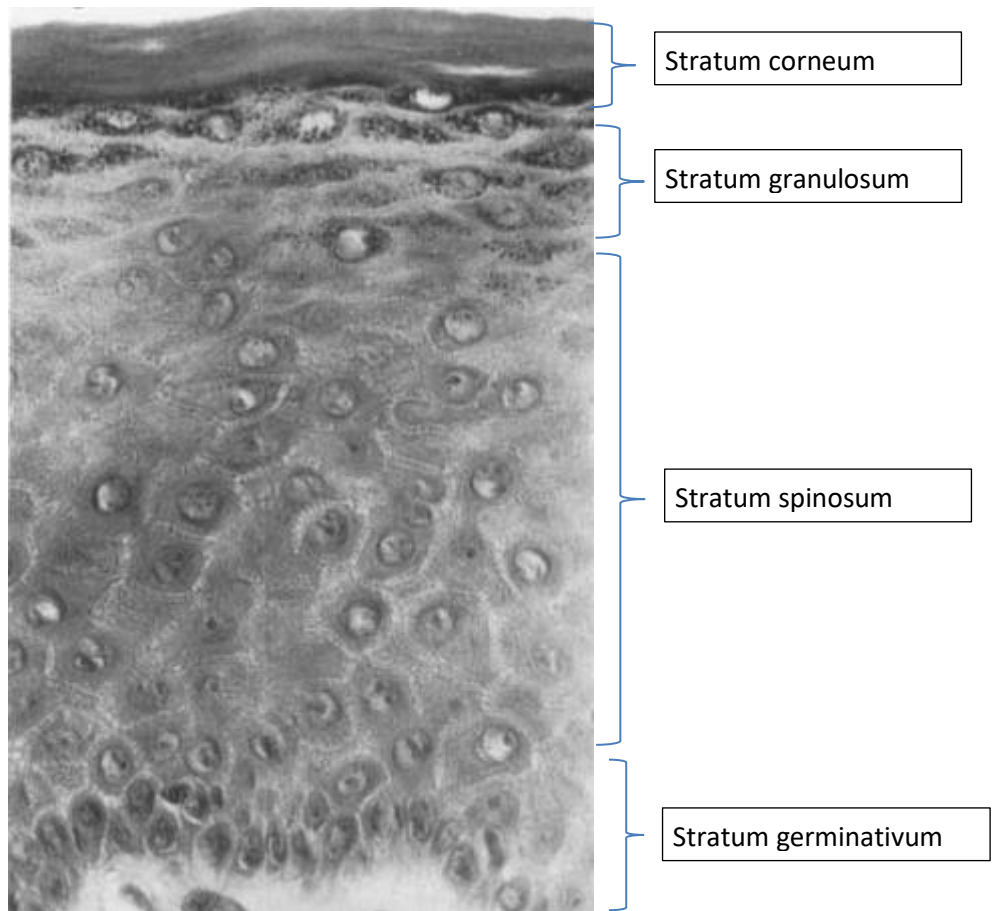


Figure 9: Light photomicrograph of the epithelium covering a normal, healthy human interdental papilla. The 4 classical epithelial strata are easily discernible. Intercellular bridges are clearly visible in the stratum spinosum (magnification ~43). (Menon, 2003)

1.1.8 Oral epithelium as barrier and role of dendritic cells

Periodontal apparatus interacts with oral environment by interaction of its outer layer epithelium (gingival, crevicular and junctional epithelium). Oral epithelium (Figure 9) is the first barrier to bacterial species; it provides primary protection against infection (bacterial & viral) and signals host response as expression of inflammatory cytokines (Dommisch et al., 2015). Bacterial adherence to epithelial cells is vital for bacterial colonization during periodontitis; oral bacteria penetrate the epithelial cells to evade the immune response by cells and spread into deeper oral tissue. Epithelia consist of four layers containing different cells (Figure 9), they are attached to each other by desmosomes and get flattened as we go towards surface stratum corneum. The tight junction of epithelial cells provides an impermeable barrier and prevents passing through of molecules (Pastar et al., 2014). Langerhans cells are dendritic cells residing in stratum spinosum and play an essential role as antigen-presenting cells for epithelial immune surveillance. They are considered as the connecting link between oral epithelium and immune system while demonstrating the inflammatory response to bacterial invasion (Wang et al., 2017). Other connective tissue cells which play a significant role during periodontitis are fibroblasts, fibrocytes, osteoblasts, osteocytes, cementoblasts, cementocytes; specialised cells such as epithelial cell rests of Malassez (ERM); immunological cells such as lymphocytes and plasma cells; defence cells such as neutrophils, natural killer (NK) cells, monocytes, macrophages, and mast cells.

During periodontal insult, the bacterial pathogens trigger the innate immune system and activate the protective white blood cells (pro-inflammatory cascade activation) to release the inflammatory mediators such cytokines and chemokines which play an

important role in the establishment and progression of chronic inflammation during periodontitis (Ramadan et al., 2020). Oral bacterial pathogens such as gram-negative porphyromonas gingivalis in the mouth make their way to infect the tissue surrounding the tooth, causing establishment of inflammation of periodontium and leading to chronic periodontal disease.

1.1.9 Oral microbiome

The oral cavity encompasses and is encircled by various hard and soft tissues, which play a vital role in the process of initial digestion. Along with hard and soft tissues, it harbours plenty of inhabitant symbiotic bacterial colonies that assist in preliminary digestion. In fact, in the human body, the oral cavity harbours the second largest microbiome compared to the largest gut microbiome. The National Human Genome Research Institute (NHGRI) in the US studied the human oral microbiome (Dewhirst et al., 2010), (Ley, 2006). It consists of microbial groups occurring at different habitats in the mouth such as teeth, tongue, cheeks, gingiva, palate, and tonsils (Moon & Lee, 2016). More than 1,000 bacterial species are colonising the mouth such as Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Spirochaetes. These microorganisms play a crucial role in maintaining a dynamic and delicate balance with host immune response and have a key role in maintaining oral homeostasis. Although the oral microbiome does play a vital role in maintaining health, certain ecological shifts in the microbiome can make way for some bacteria to develop different oral diseases such as periodontitis (Wade WG, 2013).

The precise amount and interactions between symbiotic and pathogenic bacteria and host immune system could be a potential factor in identifying and understanding the pathogenesis of chronic periodontal diseases. Dysregulation of the oral microbiome begins with the development, continued retention and maturation of biofilm.

The microorganism and microbial communities of the human body, cumulatively known as “Microbiota” (Lederberg and McCray, 2001), are commensal, symbiotic or pathogenic in nature. The oral microbiome constitutes a community of oral microorganisms, and it is different from gut and skin microbiomes. The symbiotic oral

microbiome has anti-inflammatory properties, suggesting a host defence system that confers resistance to colonisation of pathogens and possesses antioxidant activity (Kilian et al., 2016). Oral homeostasis is a controlled process: its harmonious function is maintained by numerous factors in the oral cavity such as the presence of saliva, which constitutes various protective elements, and the microbiota. When homeostasis is altered and the oral environment becomes dysbiotic, it leads to oral diseases like gingivitis and periodontitis.

1.1.9.1 Biofilm

A biofilm is a slimy layer that develops on non-shredding surface in non-sterile wet environment. Oral biofilm is a three-dimensional polysaccharide matrix harboring bacterial communities attached to hard surfaces in oral environment such as tooth enamel or implants (Zijnge, 2010). It contains 15-20% microorganisms enclosed in a 75-80% shaped matrix or glycocalyx (by volume) and is normally associated with a tooth surface (Socransky et al., 2000).

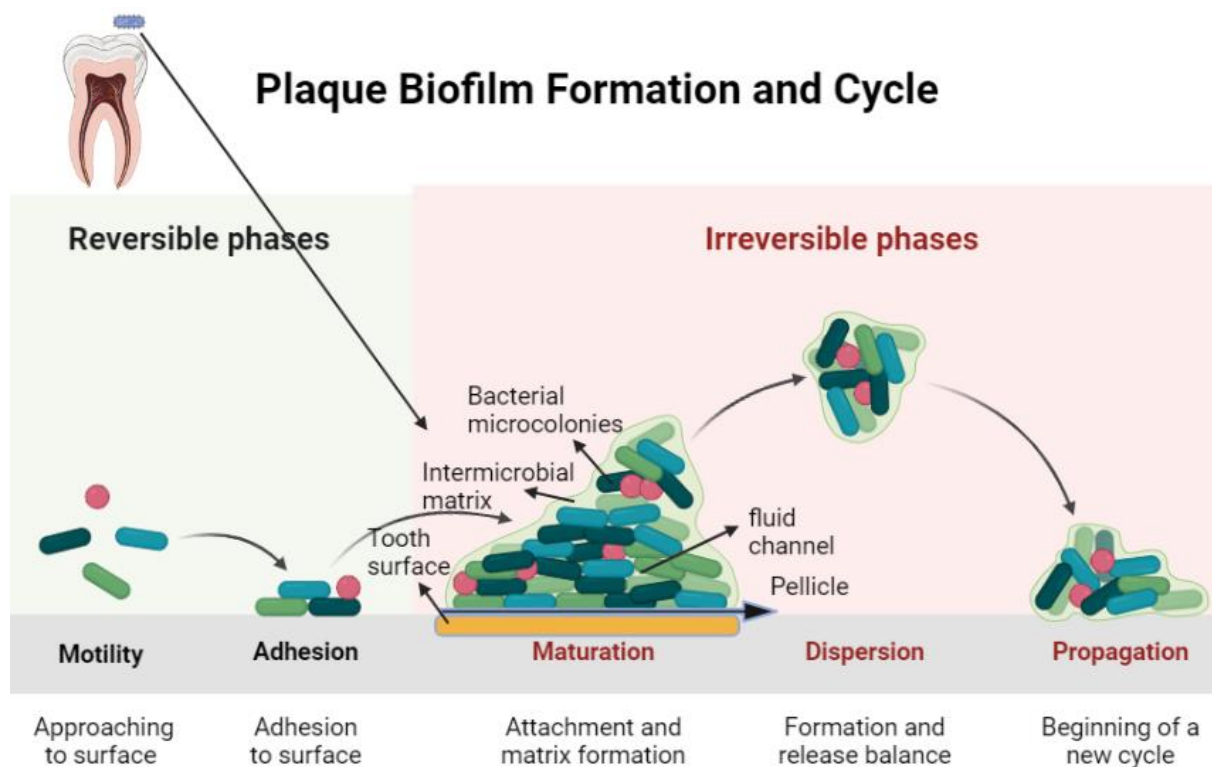


Figure 10: Artistic depiction of plaque biofilm formation and maturation on tooth biofilm and its mechanism (This diagram was created with BioRender.com)

1.1.9.1.1 Biofilm formation

Biofilm formation (Figure 10) begins with initial contact of moving planktonic bacteria and tooth surface; it is a reversible stage. Thereafter, the bacterial species interact with salivary proteins in order to establish their colonisation, evasion from host defence and nutrition. They then start forming a monolayer, producing an extracellular matrix for their protection from the oral environment. Eventually formation of bacterial colonies takes place, which exhibiting growth of biofilm and bacterial communication such as quorum sensing (Muras, 2020). The biofilm grows in a three-dimensional way and this attachment is now irreversible on its own. After maturation is completed, the biofilm starts to detach and disperse in the oral environment. Overall, it is a process where bacterial species attach reversibly in the beginning and grow on the tooth

surface in order to produce an extracellular matrix resulting in irreversible attachment along with the alteration of phenotypes of bacteria and gene transcription.

Newly formed biofilm contains various bacteria and fungi, and it forms a sticky odourless layer over teeth, restorations and dentures resulting in dental plaque. As a biofilm, dental plaque exhibits an open architecture (Figure 10) which means it consists of channels and voids, and the presence of these structures facilitates flow of nutrients, waste products, metabolites, enzymes, and oxygen supply for the inhabitants. This biofilm is a microbial homeostasis and provides a stable environment to over 700 species of aerobic and anaerobic bacteria (Marsh, 2006).

1.1.9.1.2 Quorum sensing (QS)

The term 'quorum sensing' describes intercellular bacterial communication, which regulates bacterial gene expression according to population cell density. Bacteria produce and secrete small molecules, named autoinducers (AIs), into the intercellular space. The concentration of these molecules increases as a function of population cell density. These autoinducers are signalling molecules, expressed in response to altered density of bacterial population. Once the concentration of AI threshold stimulation has been reached, alteration in gene expression occurs. Gram-positive and Gram-negative bacteria possess diverse types of quorum sensing systems. Periodontal pathogenic bacteria possess Autoinducer-2 (AI-2) quorum sensing systems. AI-2 QS is a process in which bacteria interact and communicate with each other (Figure 11). It is used for regulation of biofilm formation, iron uptake, stress response and virulence factor expression. A better understanding of bacterial communication mechanisms will allow the targeting of QS with QS inhibitors to prevent and control disease (Plančak et al., 2015).

In QS, bacteria secrete one or more AI agents depending on bacterial density in a biofilm/plaque, and thereby initiate gene expression to regulate cell behaviour. Gram-negative bacteria such as *P. gingivalis* use an acyl-homoserine lactone (AHL) as a signal molecule. Which was found to be responsible for bacterial plaque formation and potential causative of oral dysbiosis thus causing periodontal diseases (Muras et al., 2020).

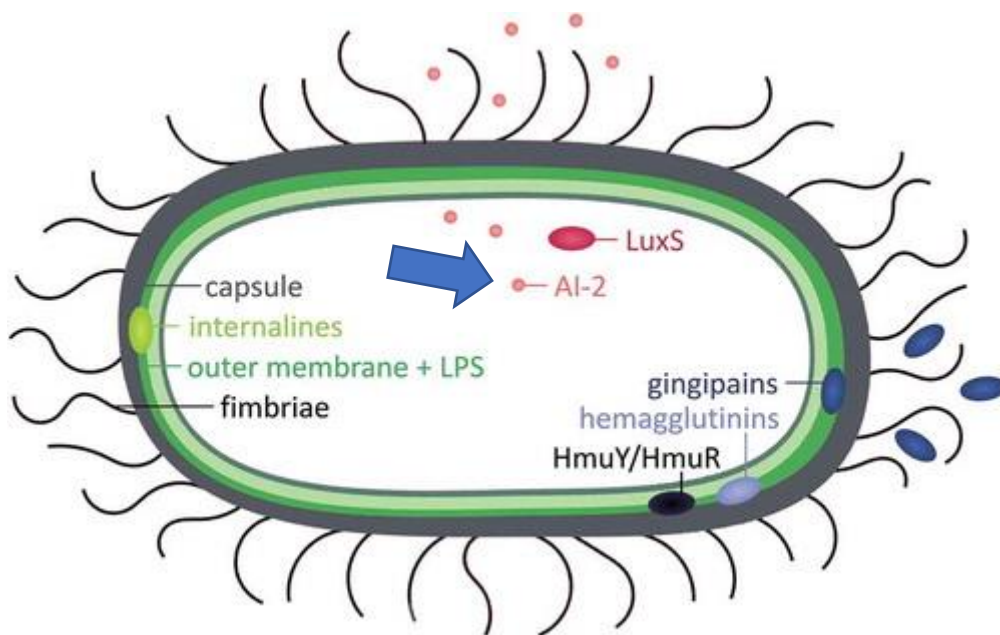


Figure 11: *P. gingivalis* autoinducer production (Autoinducer-2 (AI-2)) Image adapted from www.pinterest.co.uk.

1.1.9.1.3 Biofilm interactions

The mouth supports the growth of diverse communities of microorganisms – viruses, mycoplasmas, bacteria, fungi and protozoa (Wade, 2013). These communities persist on all surfaces as multispecies biofilms and form the resident oral microbiome, which exists in harmony with the host, and delivers important benefits that contribute to overall health and well-being.

The microorganisms found within these oral biofilms live in proximity with one another, resulting in a wide range of potential interactions, such as synergistic or antagonistic

(Figure 12). The inflammatory response can influence the subgingival microbiota in two ways: (i) via the impact of the host defences and (ii) by the resultant changes to the environment. The resultant change or dysbiosis or altered homeostasis in oral environment causes altered bacterial interactions and therefore causing oral imbalance thus to impact the oral health negatively.

Bacterial Interactions	
Synergistic	Antagonistic
Enzyme complementation/enzyme sharing	Bacteriocin production
Food chains (food webs)	Hydrogen peroxide production
Co-adhesion	Organic acid production/generation of inhibitory pH conditions
Cell–cell signalling	Bacteriophage release
Gene transfer	Competition for essential nutrients
Environmental modification	Predation

Figure 12:Types of synergistic and antagonistic microbial interactions that occur among oral microorganisms growing in dental plaque biofilms (Lamont, 2018).

More than 700 bacterial species or phylotypes are known to found in the oral cavity, of which over 50% have not been cultivated. The diversity of bacterial species is responsible for maintenance of oral-homeostasis, however the dysbiosis could result in altered ecological distribution thus altered oral-homeostasis leading to oral diseases.

Periodontitis is a type of bacterial disease resulting from a deranged interaction between the commensal microbiota, host susceptibility and environmental factors such as diet and smoking. Periodontitis results from a disproportionate inflammatory reaction to the normal microbiota, exacerbated by the presence of some disease-associated bacterial species (Wade, 2013). In addition, these pathogens can activate the acquired immune system that contributes to more and more progression of the inflammatory condition. As the immune response continues, released cytokines and chemokines can severely damage the gingiva, periodontal ligaments, and alveolar bone. This causes permanent bone damage and irreversible periodontal attachment loss, representing the characteristics periodontal disease symptoms.

1.1.10 Cytokines and chemokines in periodontitis

Cytokines and chemokines are secreted peptide mediators responsible for determination and regulation of immune response via cell signalling and their communications. Functions may vary from stimulation of cell migration, cell proliferation, control or expression of inflammatory and immune response. Cytokines are cell signalling small soluble protein molecules (5-20 kDa) produced by specific cells and mediate intercellular communications, orchestrate chemical regulations, and influence brain and behavioural characteristics in humans (D'Aiuto et al., 2004). Chemokines are relatively smaller cytokines (8-10 kDa) expressed by the nearby cells by chemotaxis.

Various cytokines are classified based on their activities and action performed by them such as effect on its own released cell (autocrine), effect on nearby cells (paracrine)

or acting on a distant cell via secreted molecules (endocrine). They perform certain vital actions such as chemokines performing cell migration (chemotaxis); immune response by interferons; and pro-inflammatory activity (lymphokines and TNF- α); and help to regulate cell to cell communications between white blood cells (Nyman, 2003). Chemokines perform the important function of coordination of leukotrienes recruitment and its activation which could result in immune system activation during inflammatory diseases.

Relatively smaller in size, chemokines are classified as inflammatory proteins which help to identify the antigens and facilitate the recruitment of protective response at the inflammatory site by signalling the molecules such as macrophages, lymphocytes and neutrophils. These chemokines are referred as chemotactic cytokines, produced by cells responding to traumatic or microbial challenge (Tonetti & Mombelli, 1999). During inflammatory cascade, in response to an injury or microbial confrontation, the cells produce a series of chemical signalling events in order to produce a protective inflammatory response. Periodontitis is classified as an example of recruitment of inflammatory response where periodontal cells are involved with expression of cytokines as inflammatory mediators. For instance, oral dendritic and Langerhans cells, which are found in the stratum spinosum of oral epithelium, (Figure 8) are considered as tolerogenic (Polak and Singh, 2021) and positively express the secretion of cytokines (IL-8, IL-6, IL-1 β and TNF- α) in response to bacterial interaction during periodontal insult.

1.1.10.1 Interleukin 8 (IL-8)

IL-8, identified in 1987 (Baggiolini et al., 1992), is a neutrophil chemotactic factor which induces chemotaxis, stimulates phagocytosis and promotes angiogenesis. It is 8-10 kDa proteins secreted by cells posing Toll-like receptors and responsible for eliciting innate inflammatory immune response. IL-8 and similar chemokines arise from chromosome 4, generated as a precursor of 99 amino acids and secreted after cleavage of single sequence 20 residues. The N-terminal extracellular processed biologically active isoform is with 72 and 77 peptides (Baggiolini & Clark-Lewis, 1992). This is expressed from different cells as inflammatory host response and secreted in the events of infection or disturbance of tissue homeostasis. Its expression is synergistically evident by the cells stimulated IL-1 or Tumour Necrosis factor. During periodontal inflammatory events IL-8 is responsible for accumulation of neutrophils.

1.1.10.2 Interleukin 6 (IL-6)

Interleukin 6 (IL-6) has been reported to act through cell surface receptors on various cells expressing β cell differentiation factor type 2 during inflammatory reactions (Schindler et al., 1990). It consists of 82 kilo Dalton (kDA) ligand binding glycoprotein chain (gp80) and signal transducing non-ligand binding (gp130) glycoprotein chain. It induces the maturation of B cells into antibody-producing cells (Erta et al., 2012). It is a pleiotropic cytokine which shows increased expression during inflammation. It consists of 184 amino acids and is expressed by either immune and/or non-immune cells. It induces different cellular responses when acting via classical or trans signalling (Figure 13). During classical signalling IL-6 stimulates the target cells by binding to membrane-bound isoform and forms a dimer complex to activate the signal transducing glycoprotein 130 (gp 130). In trans signalling it acts via soluble receptor

to form the dimer to activate the gp130. Proinflammatory cytokine expression is presented by trans signalling whereas classical signalling is responsible for regenerative activities of intestinal epithelial cells (Grivennikov et al., 2009). During periodontitis, the proinflammatory role of IL-6 was suggested at the tissue destruction site (Irwin, 2002).

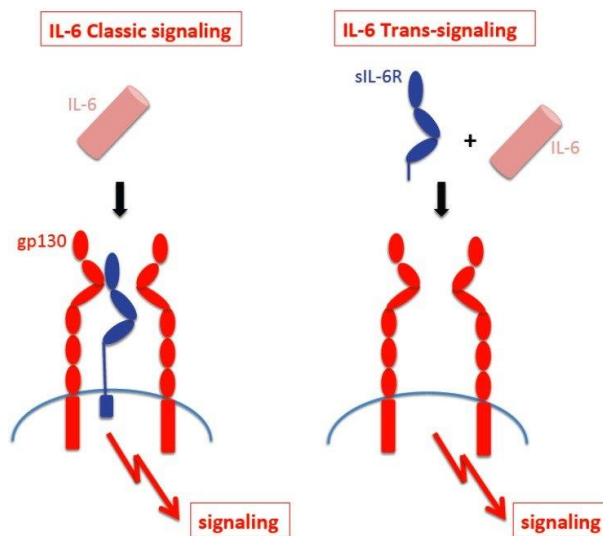


Figure 13: IL-6 classic signalling and IL-6 trans-signalling. IL-6 classic signalling requires membrane bound IL-6R and is restricted to hepatocytes, epithelial cells and leukocytes. (Rose-John, 2012)

1.1.10.3 Tumour Necrosis Factor Alpha (TNF- α)

Tumour Necrosis Factor α (TNF α) is a pro-inflammatory cytokine consisting of several transmembrane proteins which is used by the immune system for cell signalling and helps to alert cells to produce an inflammatory response. It is formed as a 27 kDa precursor, consists of 233 amino acids and exists in two forms i.e., transmembrane form and soluble form. Soluble TNF results from proteolysis of membrane TNF through substrate presentation (activation of TNF- α converting enzyme (TACE)). Released

157 amino acids TNF α weighs 17 kDa (Muhammad, 2019). TNF signals through two receptors, tumour necrosis factor receptor 1, (TNFR1) which is expressed by most cell types and tumour necrosis factor receptor 2 (TNFR2), expressed by endothelial, epithelial, and immune cells.

TNFR1 plays a role in pro-inflammatory response and apoptosis whereas TNFR2 is responsible for anti-inflammation and regeneration. Soluble forms of TNF α are a bioactive homotrimer which mediates its biological effects through tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2). Both receptors expressed in different cell types, and their activation induces various signalling pathways. In physiological conditions, TNF α is secreted by cells in low amounts to regulate physiological processes such as cell survival and homeostasis (Sedger et al., 2014). However, during pathological conditions such as periodontal inflammation, TNF α production is highly upregulated, which reinforces an inflammatory reaction resulting in increased production of pro-inflammatory factors such as cytokines and ROS/NOS.

1.1.10.4 Interleukin-1 β (IL-1 β)

Interleukin 1 is one of the pioneer cytokines to appear during periodontal inflammation and it induces secretion of pro-inflammatory mediators like tumour necrosis factor. IL-1 exists in two forms, IL-1 α and IL-1 β , among which IL-1 β found to be expressed ten-fold as compared to IL-1 α while expressing its catabolic effect in bone resorption. 1 β cytokine is produced as 31-kD pro-peptide termed pro-IL-1 β . Interleukin 1 β is expressed by genes on the long arm of chromosome 2 and consists of 269 amino acids. It is a pro-inflammatory cytokine which participates in immune regulation and bone resorption during periodontitis. Its biological effect is linked to higher concentration during periodontal diseases. It is secreted by endothelial cells as an inactive precursor and activates the inflammasome which converts pro-caspase to active caspase 1 (Frisch et al., 2019). To attain its potency, a precursor of IL-1 β is cleaved by an enzyme called cysteine protease caspase-1 (or interleukin-1 converting enzyme (ICE)) by formation of the inflammasome (a multiprotein complex). The inflammasome is responsible for initiating a pro-inflammatory response by recognising pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) molecules by activation of the ICE and IL-1 β (Lopez-Castejon & Brough, 2011). Elevated levels of IL-1 β are reported in patients with periodontitis.

Periodontitis spreads by aberrant immune response to bacterial biofilm. The exact mechanism has not yet been established, but several research studies state that bacteria and their products activate innate immunity which produces a series of inflammatory and immunological changes leading to destruction of connective tissue and bone (Kolenbrander, 2000) (Marsh, 2011).

1.1.11 Management of periodontal diseases

In addition to mechanical cleaning, various medicinal treatment methodologies have been evolved over time to address periodontitis. However, treatments available so far have had certain limitations and known side effects.

Considering microbial aetiology of periodontal diseases, systemic antibiotics have been administered (Walker et al., 1981). However, the development of antibiotic resistance, inadequate antibiotic concentration at the site of periodontal pockets and rapid decrease of sub-therapeutic antibiotic concentration has led to use of systemic antibiotics only for the treatment of rapidly progressing or refractory periodontitis. Several antibiotics and immunomodulatory drugs, including a tetracycline, doxycycline (antiproteinase), which can inhibit matrix metalloproteinases (MMPs); anti-inflammatory drugs (Non-Steroidal Anti-inflammatory Drugs (NSAIDs)) which block production of proinflammatory cytokines; and bone-sparing agents (bisphosphonates) which inhibit the activation of osteoclasts have been postulated for their therapeutic values as an adjunctive therapy in the management of chronic periodontitis (Badran, 2009). However, these treatments showed certain limitations such as antibiotic resistance, digestive problems like heart burn ulcers, and bone mineralisation issues. Therefore, the above-mentioned existing treatment methods are used as either for symptomatic relief or to cease the progress of periodontal diseases. Clinicians and researchers are working to find an alternative in order to reverse the damage to periodontal apparatus by periodontal diseases by harnessing the anti-inflammatory and regenerative properties of newer compounds. To date many studies have reported the association between periodontal diseases and the physiological endocannabinoid system. Anandamide, an endocannabinoid, was

found to be elevated in gingival crevicular fluid (GCF) during periodontitis. In contrast 2-arachidonoylglycerol (2AG), which is also an endocannabinoid, plays a role in antagonism to anandamide (Ozdemir et al., 2014).

This endogenous cannabinoid system (ECS) present in the human body is composed of endogenous lipid-based retrograde neurotransmitters, which bind to cannabinoid receptors in the central and peripheral nervous systems. ECS has been reported to possess anti-inflammatory effect owing to act directly on immune cells for inflammation reduction (Barrie and Manolios, 2017).

Reducing inflammation, achieved through improved oral care or by following special advice from clinicians such as proper brushing method, using dental floss and attend the advised scaling sessions when requested, have witnessed decrease in bone loss clinically. This reduction in bone loss could be an explanation of the regenerative potential of periodontium (Somerman et al., 1988, Karring et al., 1982, Isidor et al., 1986, Bowers et al., 1986). Various inflammatory cytokines such as IL-1, IL-6 and TNF- α have found to play significant role during bone remodelling whilst orthodontic treatment mostly in the direction of bone resorption.

Studies have shown that cannabinoids receptors such as CB₁ and CB₂ are seen to expressed during periodontal diseases. The above findings on an increased level of AEA (an endocannabinoid) following the expression of CB₁ and CB₂ receptors in periodontal tissue (Nakajima Y1 2006), the mechanism of action of cannabinoids through these receptors and their reducing inflammatory effect suggest a role for cannabinoids as a potential therapeutic agent in the treatment of periodontitis. Action of endocannabinoids on existing cannabinoids receptors and the potential of synthetic and Phyto cannabinoids to act through theses receptors was reported during in-vitro

anti-inflammatory studies (O'sullivan, 2007, Mechoulam and Parker, 2013, Mechoulam et al., 2014).

1.1.12 Limitations of the current treatments:

As described above, NSAIDs were used as anti-inflammatory agents in the treatment of periodontitis. These NSAIDs reduce inflammation through inhibiting cyclooxygenase 2 (COX2). However, this inhibition can cause potential damage, as COX2 participates in the maturation stage of the enamel organ. For instance, patients prescribed NSAIDs during pregnancy have shown to cause serious effects on organ formations including clefts (Østensen, 2004). Recent studies have shown that such inhibition of COX2 by NSAIDs can alter amelogenesis, producing hypo mineralisation (Serna Muñoz et al., 2018) and so leading to potential damage. Other potential side effects following exposure to NSAIDs, and antibiotics include-overdose, hepatic side effects and gastrointestinal intolerance (Bhatavadekar and Williams, 2009, Aimetti et al., 2012).

Recent literature has also shown an increase in the use of medicinal plants and other natural products for the treatment of chronic inflammatory diseases. Several studies with animal models have shown beneficial effects of various plants on periodontitis. All these plant products, such as terpenes, flavonoids, chlorophyll, and vitamins, have been shown to possess some sort of anti-inflammatory properties (de Figueiredo et al., 2016, Torres et al., 2016).

Although various treatments have been used, and are still in use, they exhibited limited outcomes regarding irreversible moderate to severe periodontal problems. Due to the

intricate multifactorial aetiology of periodontitis and the prominent role of immunogenic response, there remains a demand for a more effective clinical therapy for these types of periodontal diseases.

1.1.12.1 Bacterial and adjacent cells interactions

Various pathological bacterial species have been found to be associated with periodontal diseases and are claimed to be associated with destruction of host tissues. The interaction of host and bacteria is governed by host-related factors such as genetic susceptibility of the individual, environmental factors and the inflammatory immune response. Despite available periodontal therapeutic approaches, the complete eradication of bacteria is neither feasible nor desirable (Harper et al., 1987) (Magnusson et al., 1984). Induction of inflammatory cascade by the microorganisms lead to chronic periodontal pathologies. Therefore, an approach to eradicating such pathology in the treatment of periodontics is one of the expected outcomes to address periodontal diseases in this study (Hasturk et al., 2015). Another important factor considered while addressing chronic periodontitis is to tackle the inflammatory response by the cells affected by bacterial interaction. The inflammatory cascade initiated locally acts as focus of infection for its further transmission to adjacent tissue via cells and soluble inflammatory mediators (Hasturk et al., 2012).

1.1.12.2 Alternative approach

Chronic inflammatory diseases have a characteristic pattern whereby it manifests as a slow progression, and a long duration. This means it takes a long time for symptoms to be evident as in the case of periodontal diseases. Another characteristic feature is that it shares a similar outcome, meaning it affects multiple systems, and is typically non symptomatic, and at times associates with impairment of function. Treatment options which aim to resolve periodontal inflammation can be used to limit systemic inflammation and promote healing and regeneration of the periodontium (Hasturk et al., 2015). In the early 1990s (Mechoulam et al., 2014) the endocannabinoid system (ECS) was explored and found responsible for maintaining homeostasis, reducing pain and decreasing inflammation. Later in 1993 (Pertwee RG, 1997), the cannabinoid receptors 1 and 2 (CB₁ & CB₂) were identified as a possible site of action for endocannabinoids (Kogan et al., 2006).

Recent studies (Konermann et al., 2017) have reported changes in expression of both the receptors in mechanically loaded PDL cells and in periodontal tissues, affected by different entities of periodontal inflammation and mechanical stress. In addition to this, the phytocannabinoids were also found to act on these cannabinoid receptors, suggesting a potential function for the ECS in the regulation of periodontal pathophysiology. Therefore, the review and research presented in this thesis examine the effect of cannabinoids on periodontal cells to understand not only the self-damaging inflammation but also whether these cannabinoids play a role in periodontal regeneration and preventing bone demineralization (Lucaciu, 2019, Apostu et al., 2023).

1.1.13 Cannabinoids and periodontitis

Cannabinoids have recently been identified as a major target for cannabis-based medicines and therefore gained much attention as anti-inflammatory agents in the treatment of various diseases.

There are 3 types of cannabinoids, phytocannabinoids, synthetic cannabinoids and endocannabinoids. Phytocannabinoids are plant alkaloids, obtained from cannabis; they are produced in trichomes and protect the plant from ultraviolet light, predators, and dehydration (Gould, 2015, Turner et al., 2017). Alkaloids first mentioned in 1819 by W Meibner as the compounds appeared like alkali, plant alkaloids are biologically active compounds that contains nitrogen, and they may have some pharmacological activities and can be used for their medicinal and ecological use (Aniszewski, 2015). Cannabinoids such as cannabidiol (CBD), cannabigerol (CBG) and tetrahydrocannabinol (THC) (Figure 14), are reported to possess potent anti-inflammatory properties, and have been explored for their role in treatment of various chronic conditions such as cancer, diabetes and arthritis (McKallip et al., 2006, Weiss et al., 2008). In addition, these compounds have been successfully used in early healing of bone fracture (Singh, 2017). CBD has also been reported to exhibit protective effects in mouse models of A2A receptor mediated acute injury (Ribeiro et al., 2012) and chemotherapy induced diarrhoea via reducing inflammation (Fabisiak and Fichna, 2017).

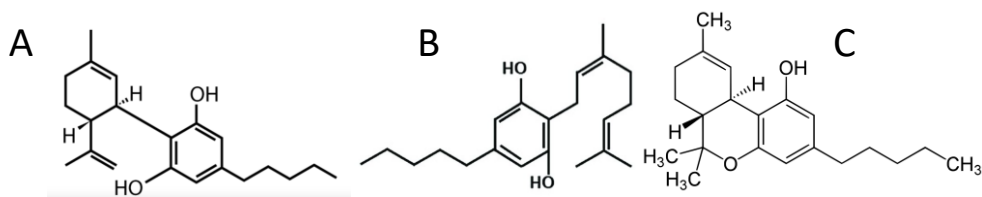


Figure 14: Chemical structures of A: Cannabidiol (CBD), B: Cannabigerol (CBG) and C: Tetrahydrocannabinol (THC) (Adams, 1940).

1.1.13.1 Physiology and pharmacology of endocannabinoids and their relation to Phyto cannabinoids.

The endocannabinoid system (ECS) is a biological system composed of chemical compounds naturally produced in the body. Endocannabinoids are neurotransmitter chemicals produced naturally within the body which bind to endocannabinoid receptors (ECRs), expressed throughout the central and peripheral nervous systems; whereas synthetic artificially made and Phyto cannabinoids are plant –based (cannabis) cannabinoids also found to activate the ECRs CR_1 and CR_2 in the body. ECs are mediators of various physiological processes and considered as local mediators like autacoids (prostaglandins) (Rodríguez Fonseca et al., 2005). The cellular effects of endogenous cannabinoids have a profound impact on the main physiological systems that control body functions (Table 1). Emerging evidence suggests that CB_1 and CB_2 receptor antagonists can exert analgesic and anti-inflammatory actions, possibly because endocannabinoids can behave as both pro- and anti-inflammatory mediators (Di Marzo, 2008). Alteration or addition of certain molecules to cannabinoids were advocated to possess different activities such as analgesics, anti-inflammation, anti-bacterial, anti-convulsion, neuroprotection, anti-

hyperglycaemic effects, anxiolytic and antidepressant actions (Wang, 2023). For instance, administration of a type of cannabinoid i.e., CB₁ receptor antagonist was reported to block cue-induced reinstatement to heroin and cocaine self-administration (De Vries et al., 2003). Also, a cannabinoid called cannabigerol (CBG) was shown to interfere with the transmission of the autoinducer signals and has been demonstrated as a potential anti-biofilm agent via inhibiting the QS cascade (Aqawi et al., 2020).

1.1.13.2 Cannabinoid receptors

As described earlier in this chapter, there are two types of cannabinoid receptors, termed CB₁ (Matsuda et al., 1990) and CB₂ (Munro et al., 1993). Different studies showed different expressions of these two receptors. It was reported that these receptors (CB₁ and CB₂) were upregulated during inflamed conditions within periodontal ligament and gingival fibroblasts (Kozono et al., 2010). However, in another study, only CB₂ expression was found to be upregulated in gingivitis and periodontitis (Nakajima et al., 2006). Furthermore, an increased expression of CB₂ mRNA was reported in periodontal cells treated with LPS (Kozono et al., 2010, Qian et al., 2010). Recently, an *in vivo* study has identified variations in expression of CB receptors; more expression of CB₁ than CB₂ in healthy periodontium, a decreased expression of CB₁ but an increased expression of CB₂ in bacteria-mediated inflammation. However, increased expression of both CB₁ & CB₂ were reported in sterile/aseptic inflammation (absence of bacterial involvement) caused by traumatic injuries (Konerman et al., 2017).

The human body is equipped with an endogenous cannabinoid system referred as the endocannabinoid system (ECS) ((Tam, 2006). The endocannabinoid system was explored in biological systems and lipid neurotransmitters were found to bind special

cannabinoid receptors including CB₁ (Matsudo et al., 1990) and CB₂ (Munro et al., 1993). In 2005, Nakajima et al illustrated the anti-inflammatory effect of endocannabinoid AEA (anandamide) in regulation of periodontal inflammation through NF-κB pathway inhibition and witnessed the expression of CB₁/CB₂ receptors in pathological conditions in fibroblasts, endothelial cells and macrophages. Recent studies have reported not only the presence of neurotransmitter receptors in periodontal ligament but also their differential expression in various inflammatory conditions (Nakajima et al., 2006, Konnermann et al., 2017).

1.1.13.3 Mechanism of action of cannabinoids

A major investigative effort on the mechanisms of action of cannabinoids was launched during the early nineties. Cannabinoids were found to act through the superfamily of G protein-coupled receptors (GPCRs) on cell membranes (Devane et al., 1988). However, the exact mechanism of action of cannabinoids is not fully understood. Cannabidiol (CBD) appears to act through inhibiting endocannabinoid AEA degradation, and subsequent enhancement of endogenous level of endocannabinoids (Kesner, 2020). It is documented that adenosine A_{2A} receptors participate in the anti-inflammatory effects of CBD, and A_{2A} antagonist (ZM241385) partially blocked the protective effects of CBD in the initial stages of inflammation.

It is also documented that CBD administration regulates bone homeostasis through inhibiting the expression of RANK/RANKL, which are markers of established bone resorption, suggesting its involvement in bone turnover during periodontitis (Napimoga

et al., 2009). In addition, the RANK/OPG ratio has been reported to increase with elevated levels of IL- β and TNF- α in arthritic bone resorption (Graves, 2011).

These findings suggest a role for cannabinoids as a potential therapeutic agent in the treatment of periodontitis through reducing inflammation and regulating bone homeostasis.

1.2 Aim

The aim of this *in vitro* study is to evaluate the ability of cannabinoids to alter biomarker production, involved in inflammation and periodontal tissue regeneration.

1.3 Objectives

This aim will be achieved through the following objectives:

- To Investigate the cytotoxicity of the cannabinoids on cell lines and primary periodontal cells procured from extracted teeth.
- To investigate the anti-inflammatory effect of cannabinoids.
- To assess the effect of cannabinoids on osteogenesis and chondrogenesis.
- To assess the regenerative potential of the primary periodontal cells

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Consumables

Consumable materials used during the study while performing the in-vitro study were procured from different suppliers as mentioned in the table below.

Table 1 : Consumables used during in in-vitro experiments during this project.

Items	Catalogue numbers	Suppliers
Adhesive PCR Plate Seals	AB0558	Thermo Fisher scientific
Cell culture flasks (T75, T175)	430641U, 431079	Corning incorporated
Clear flat bottom well plates (6 well, 24 well and 96 well)	3506, 3527, 3988	Corning incorporated
Disposable sterile syringes (10 mL)	S75101	Scientific laboratory supplies
DNase/RNase-free micro centrifuge tubes (1.5 mL)	AM12400	Thermo Fisher scientific
Eppendorf® micro centrifuge tubes (0.5 mL, 1.5 mL, 2 mL)	T8911, T9661, T2795	Sigma-Aldrich

Items	Catalogue numbers	Suppliers
FACS tubes	38007	STEMCELL™ Technologies
Falcon tubes (15 mL, 50 mL)	339650, 339652	Thermo Fisher Scientific
Glass coverslips	COV12440S	Solmedia
Microscopic glass slides	X-tra® Superfrost (3800050)	Leica Biosystems
PCR plates (96 well; Light cyclor type)	1402-9909	Star lab
PCR tubes (0.2 mL)	TF10201	Bio-Rad
Specimen pots	IR1381	Alpha Laboratories
SteriWare™ disposable tweezers	15849421	Thermo Fisher scientific
Syringe filters (0.2 µm)	725-2520	Thermo Fisher Scientific
White 96 well OptiPlate	6005290	PerkinElmer

2.1.2 General chemical reagents and kits

During this project the chemical reagents such as dyes, acids, ultra-pure water, culture media and kits were purchased from the suppliers mentioned below in Table 2.

Table 2: Chemical reagents and kits used throughout the research.

Chemical reagents/kits	Catalogue numbers	Suppliers
Alexa-Fluor® 488 Phalloidin	A12379	Thermo Fisher scientific
Alizarin red solution	TMS-008	Sigma-Aldrich
Alpha modified eagle's medium	BE12-169F	Lonza™
alamarBlue	DAL 1025	Invitrogen
Bovine serum albumin solution	A7979	Sigma-Aldrich
DAPI	D1306	Thermo Fisher scientific
Decal III	RRDC3-E	Atom Scientific
Deoxyribonuclease I	AMPD1	Sigma-Aldrich
Dimethyl sulphoxide	D2650	Sigma-Aldrich

Chemical reagents/kits	Catalogue numbers	Suppliers
DPX mounting medium	D/5319	Thermo Fisher scientific
Dulbecco's modified Eagle's media	D6546	Sigma-Aldrich
Ethanol	51976	Sigma-Aldrich
Ethylenediaminetetraacetic acid solution	03690	Sigma-Aldrich
FcR blocking reagent (Human)	130-059-901	Miltenyi biotec
bovine serum	F7524	Sigma-Aldrich
Giemsa solution	12-0440	Sigma-Aldrich
Harris's haematoxylin	HHS32	Sigma-Aldrich
High-Capacity RNA-to-cDNA™ Kit	4387406	Thermo Fisher scientific
Hydrochloric acid (6 M)	H/1200/PB17	Thermo Fisher Scientific
Haemocytometer	Z359629-1EA	Sigma-Aldrich
L-Glutamine solution	G7513	Sigma-Aldrich
Live/Dead Assay Kit	30002-T	Biotium
Magnesium chloride solution	M1028	Sigma-Aldrich

Chemical reagents/kits	Catalogue numbers	Suppliers
Mayer's hematoxylin	RRSP60-D	Atom Scientific
Neutral buffered formalin	BAF-6000-08A	Cell path
Nuclear Fast Red	N3020	Sigma-Aldrich
Nuclease free water	10336503	Thermo Fisher scientific
Oil red O solution	O1391	Sigma-Aldrich
Penicillin-Streptomycin solution	P4333	Sigma-Aldrich
Phosphate buffered saline	10010023	Thermo Fisher scientific
Phosphate buffered saline without calcium or magnesium	17-512F	Lonza
Picrosirius Red Stain Kit	24901-500	Polysciences, Inc.
QIAGEN DNeasy Blood and Tissue kit	69504	QIAGEN
Quant-iT PicoGreen dsDNA assay kit	P7589	Thermo Fisher scientific
Ribonuclease A	R4642	Sigma-Aldrich
Scott's tap water	EGW-0200-25A	CellPath

Chemical reagents/kits	Catalogue numbers	Suppliers
Sodium azide solution	S8032	Sigma-Aldrich
Sodium dodecyl sulphate	71736	Sigma-Aldrich
Sodium hydroxide (6 M)	S8045	Thermo Fisher Scientific
StemMACS AdipoDiff Media (Human)	130-091-677	Miltenyi biotec
StemMACS ChondroDiff Media (Human)	130-091-679	Miltenyi biotec
StemMACS OsteoDiff Media (Human)	130-091-678	Miltenyi biotec
TaqMan™ Fast Advanced Master Mix	4444557	Thermo Fisher scientific Ltd.
Tris-hydrochloride solution	T3038	Sigma-Aldrich
Tris-buffered saline	T5912	Sigma-Aldrich
Tris-buffered saline with 0.1% Tween® 20	T9039	Sigma-Aldrich
Tris-EDTA-based antigen unmasking solution	H-3301	Vector-Laboratories

Chemical reagents/kits	Catalogue numbers	Suppliers
Triton X-100	HFH10	Thermo Fisher scientific
Trypan Blue solution	T8154	Sigma-Aldrich
Trypsin-EDTA solution	T4049	Sigma-Aldrich
VECTASHIELD Antifade Mounting Medium with DAPI	H-1500	Vector-Laboratories

2.1.3 Materials used during specific techniques.

2.1.3.1 Cell culture

2.1.3.1.1 Inventory

During cell culture the disposable or reusable equipment and the media required for the culture and the required sterile conditions throughout the growth of the cells is mentioned in the table below.

Table 3: Disposable or reusable equipment and materials used for tissue culture.

Items	Catalogue numbers	Suppliers
Specimen pots	IR1381	Alpha Laboratories
Class II safety cabinet	N4-437-400E	NUAIRE
Extraction forceps	DFO600	Dental directory
Tissue forceps	11512123	Thermo Fisher scientific
Scalpel blade handles	5334	Thermo Fisher scientific
Scalpel blades (Size 12,15)	53223	Thermo Fisher Scientific
Petri dish	430167	Corning
Cell culture flasks (T75, T175)	430641U, 431079	Corning incorporated
Inverted microscope	IX71	Olympus
Incubator	MCO-20AIC	Sanyo
Phosphate Buffer Saline	10010023	Thermo Fisher scientific
Cell culture media	D6546	Sigma-Aldrich
Foetal Bovine Serum	F7524	Sigma-Aldrich
Trypsin	T4049	Sigma-Aldrich
Penicillin/streptomycin	P4333	Sigma-Aldrich

Items	Catalogue numbers	Suppliers
Neubauer chamber	Z359629-1EA	Sigma-Aldrich
Stain	T8154	Sigma-Aldrich
Cryotubes (2 mL, 4 mL)	BR114832, BR114834	Sigma-Aldrich
Mr. Frosty™ freezing container	11315674	Thermo Fisher scientific Ltd.
Extraction forceps	FX73S	GDC
Fluorescence microscope	Axio imager Z1 with Epitome	Zeiss
Haemocytometer	Z359629	Sigma Aldrich
Confocal scanning laser microscope	Leica TCS SP8	Leica microsystems
Hot plate	E18.1 hotplate	Raymond A lamb
Inverted microscope	IX71	Olympus
Light cycler	Roche LC480	Roche LifeScience
NanoDrop™ 2000 spectrophotometer	Nc/2000	Thermo Fisher scientific Ltd.
Scanning electron microscope	S-3400N	Hitachi

2.1.3.1.2 Cell lines and Primary cells

The cells lines corresponding to different cells periodontium were used in the study along with the harvesting of primary periodontal cells from middle third of extracted teeth are the mentioned below in the table. The culture media required during their growth along with the required supplements was chosen based on the requirement of cell growth.

Table 4: Samples used during in-vitro experiments.

Cell types	Origin	Suppliers	Culture Media
G292	Human fibroblast like bone (osteosarcoma) cell line	Tissue bank oral biology	High glucose Dulbecco's modified eagle's medium (DMEM) supplemented with: - 10% (v/v) Foetal bovine serum (FBS) - 2 mM L-Glutamine - 100 U. mL-1 Penicillin and 100 µg. mL-1 Streptomycin

Cell types	Origin	Suppliers	Culture Media
A-2780	Adherent human ovarian epithelial cell line	University of Huddersfield Dr Fariedeh	<u>RPMI-1640</u> supplemented with: - 2 mM L- <u>Glutamine</u> -10% <u>Foetal Bovine Serum</u> (FBS)
TIGK	Gingival epithelial	Tissue bank oral biology	DermaLife K serum free culture medium (life cell technology).
PDLSCs	Human periodontal primary cells	Tissue bank oral biology	Low glucose Dulbecco's modified eagle's medium (DMEM) supplemented with: - 10% (v/v) Foetal bovine serum

2.1.3.1.3 Cannabinoids and Treatment reagents

Cannabinoids were procured in their purest form from the suppliers mentioned below. The dilution or reconstitution of the cannabinoids was performed as per the manufacturer's instructions.

Table 5: Cannabinoids and inflammatory agents used for in-vitro tissue culture.

Reagents	Supplier	Dilution/reconstitution sols
CBD (Cannabidiol)	TOCRIS Biotech Cat 1570	Ethanol
CBG (Canabigerol)	TOCRIS Biotech Cat 3021	Ethanol
AM-251	TOCRIS Biotech Cat 1117	Ethanol
o-1602	TOCRIS Biotech Cat 2797	Methyl acetate
Flagellin	Thermo Fisher	Endotoxin free water
LPS-P. gingivalis	InvivoGen	Endotoxin free water

2.1.3.2 ELISA

2.1.3.2.1 Antibodies

Sandwich ELISA technique was performed using the chemicals from oral biology laboratory, welcome trust, University of Leeds and the protocols were followed from the suppliers of the antibodies (Table3) used in this study.

Table 6: Antibodies used during sandwich ELISA for protein estimation.

ANTIBODY	CAPTURE	STANDARD	DETECTION
IL-8	Human CXCL8/IL-8 Antibody (Cat # MAB 208)	Recombinant human CXCL8/IL-8 (Cat # MAB 208-IL)	Human CXCL8/IL-8 Biotinylated Antibody (Cat # BAF 208)
IL-6	MQ2-13A5 antibody (Cat. No. 554543)	Recombinant human IL-6 (Cat. No. 550071)	Biotinylated MQ2 human IL-6 39C3 antibody (Cat. No. 554546)
IL-1b	H1b-27 antibody (Cat. No. 511602)	Recombinant human IL-1b (Cat. No. 579409/4pack)	Biotinylated Poly5174 (Cat. No. 517403)
TNF- α	Mab1 antibody (Cat. No. 551220)	Recombinant human TNF (Cat. No. 554618)	Biotinylated MAb11 (Cat. No. 554511)

2.1.3.2.2 General materials (ELISA)

2.1.3.2.2.1 Wash Buffer

For 1 litre of 10X wash buffer, the following components were added first to 800 ml

Millipore filtered water.

1. 80 g NaCl (1.37 M)
2. 2 g KCl (0.26 M)
3. 14.4 g Na₂HPO₄ (0.1 M)
4. 2.40 g KH₂PO₄ (0.176 M)
5. 5 ml of Tween-20 (0.5 (V/V))

After the solubilisation of the components, pH was adjusted to 7.4 and the volume was completed to make 1 litre.

2.1.3.2.2.2 Assay Buffer (10X)

1. 136.89 mM NaCl ($58.44 \times 0.1386 = 7.99$ grams)
2. 7.96 mM Na₂HPO₄ ($142 \times 0.00796 = 1.13$ grams)
3. 1.47mM KH₂PO₄ ($136.09 \times 0.00147 = 0.199$ grams)
4. 68 mM KCl ($74.55 \times 0.00268 = 0.199$ grams)
5. 0.5 (W/V) BSA 5 grams
6. (V/V) Tween 20 1ml
7. up to 10 ml water
8. PH 7.4

2.1.3.2.2.3 Coating Buffer (10X)

- 51.1mM NaHCO₃ (84.01×0.0511=4.2grams)
- 50mM Na₂CO₃ (105.99×0.5=5.29 grams)
- Up to 10 ml water
- PH 9.4

2.1.3.3 FLOW CYTOMETRY

2.1.3.3.1 Antibodies

Table 7: Flow cytometry antibodies

Antibodies-Conjugates	Catalogue numbers	Suppliers
Primary antibodies		
CD105 antibody-FITC	130-098-774	Miltenyi biotec
CD146 antibody-PE	130-092-853	Miltenyi biotec
CD90 antibody- PerCP-Vio700	130-114-864	Miltenyi biotec
CD45 antibody VioGreen	130-113-183	Miltenyi biotec
Control Isotypes		
Mouse IgG1-FITC	130-092-213	Miltenyi biotec
Mouse IgG1-PE	130-092-212	Miltenyi biotec
Mouse IgG1-PerCP-Vio700	130-097-561	Miltenyi biotec
Mouse IgG1-VioGreen	130-096-919	Miltenyi biotec

2.1.3.3.2 General materials

Lysis buffer

DI water

FACs tubes

Pastette

FACS buffer

2.1.4.3 WESTERN BLOT

2.1.4.3.1 General materials

Materials used during gel electrophoresis, protein transfer and labelling the antibodies are mentioned in the table below.

Table 8: Western blot experiment reagents and equipment

Cell extraction buffer	Sigma
Protease inhibitor	Santa Cruz
Phosphatase inhibitor	Santa Cruz
1.8cm blade cell scraper	Corning
-80°C freezer	Sanyo
6mm cone ball	Retsch
TissueLyser	QIAGEN
Bicinchoninic acid (BCA) assay kit	Thermo Fisher Scientific

DMX TC microplate reader	Dynex Technologies
Revelation software (v4.21)	Dynex Technologies
Sample buffer	Invitrogen
Reducing buffer	Invitrogen
Heating block	Fisher-Scientific
Bis-Tris polyacrylamide gel	Bio-Rad
Marker	Bio-Rad
Criterion cell tank Bio-Rad 2-(N-morpholino) ethanesulphonic acid/sodium	Invitrogen
dodecyl sulphate (MES SDS)	dodecyl Sulphate (MES SDS)
Polyvinylidene fluoride (PVDF) membrane	Merck Millipore
Metal stirrer	Star Lab
Methanol	Fisher Scientific
Magnetic rotary plate	Heidolph
Tris-buffered saline with Tween (TBST)	Sigma
KHK antibody	Sigma
Tubulin	Santa Cruz
Polyclonal rabbit anti-mouse	Dako

Polyclonal goat anti-rabbit	Dako
Cling film	Viking Direct
Strep-Tactin HRP	BIO-RAD
Immobilon western chemiluminescent	Merck Millipore
horseradish peroxidase (HRP) substrate	horseradish peroxidase (HRP) substrate
Camera scanner (Image Station 2000R)	Kodak
Restore PLUS stripping buffer	Thermo Scientific

2.1.4.3.2 Gel electrophoresis:

Gel electrophoresis is the standard technique to separate the proteins based on their size and thereafter the separated proteins can be transferred to a membrane before being identified by using the corresponding primary and secondary antibodies. Different chemical reagents and equipment are required during this process. The protocols and materials used during this process was kindly provided by Oral biology, University of Leeds.

Resolving (13%) gel

- Water (distilled) 650 ml.
- Glycerol 2.50 ml
- Gel buffer
- 1.5 M Tris pH 8.8 2.50 ml
- 30% Acrylamide 4.33 ml
- 10% APS 50 ml

- TEMED 5 ml

Stacking (4%) gel

- Water (distilled) 6.15 ml.
- Gel buffer
- 0.5 M Tris pH 6.8 2.50 ml
- 30% Acrylamide 1.33 ml
- 10% APS 50 ml
- TEMED 10 ml

Running buffer x10

- 30.3 g Tris FW 121.1
- 144.0 g Glycine
- 10.0 g SDS

into 1 litre of distilled water

2.1.4.4 Real Time Polymerase Chain Reaction

2.1.4.4.1 Taq Mans

Taqman gene expression assays used in this study were presented in the Table 3.

Table 9: Taq Man gene expression assays used in RT-qPCR.

Gene name/symbol	Description/base pairs	TaqMan® gene expression assay
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Housekeeping gene, 122 bp (Endogenous control)	Hs99999905_m1
IL-8	101 bp	Hs00174103_m1
IL-6	118 bp	Hs99999032_m1

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Introduction

Cell culture is a method by which living human cells are maintained and allowed to grow outside their natural environment. The controlled environment mimics the natural growth conditions where the essential nutrients, growth factors, hormones and required gaseous exchange are provided for their growth and multiplication. There are two types of cells: primary cells and cell lines. Primary cells are those which are extracted from the parent tissue. These cells grow efficiently without changing their original behaviour except for a few initial passages before entering senescence. Conversely, cell lines are genetically modified (cancerous cells) or transformed cells. These cells can multiply indefinitely if they are provided with the required growth conditions such as a high-glucose medium like DMEM.

Based on the medium on which they are cultured, it follows that there are two types of cells: those which need a surface upon which to thrive (monolayer cells), and those which require a medium to float in (suspension culture). Cells which require a surface to grow are called monolayer cells – these are the type, which is selected for this study, because periodontal cells are monolayer cells which need a surface to grow on.

2.2.1.2 Principle

The process of cell culture involves extraction of cells from tissues, maintaining them in an external aseptic environment, and growing them in vitro while providing almost similar growth conditions of their native environment. To culture any cells requires a specific culture condition such as low glucose medium for primary cells and high glucose medium for cell lines. High glucose medium is required for cell lines because they grow exponentially and therefore require nutrients at a higher rate. Nonetheless, despite providing all the required conditions cells cultured in vitro cannot be fully replicated compared with cells grown in vivo. In view of this of this challenge of in vitro culture of cells, a tightly regulated environment is required. This includes maintaining a high level of sterilisation, provision of optimal nutrients and most importantly, a high level of humidity, i.e., 95% and 5% of carbon dioxide for gaseous exchange.

2.2.1.3 Method

Cell lines and primary cells used in this research are listed below in table 2.3.2. Primary periodontal cells were isolated and harvested by using the explant method as mentioned below (2.1.2) from freshly extracted teeth. Ethical approval was obtained prior to their use for the experiments (**REF NO: 160418/SK/248**). The extracted teeth samples were procured from the tissue bank of oral biology, University of Leeds. Different types of culture media suitable for the cell type were used (Table 4). Foetal calf serum (FCS) and L-glutamine required for the cell type, were added to the cell culture media in a Class 2 laminar flow hood under aseptic conditions. It was stored in a fridge at a maximum of 4° Celsius for approximately up to two weeks. This ready to use culture media was equilibrated at 37° C. This was done by holding the stored media in a water bath at a temperature of 37°C before each use.

Table 10: Different culture media used during this study.

Cell type	Culture media
G-292	DMEM High glucose, Gibco
A-2780	DMEM High glucose, Gibco
TIGK	Complete medium, Lifeline
Periodontal cells	DMEM Low glucose, Gibco

2.2.1.3.1 Cell lines procurement and culture

Cell lines were provided by the Department of Oral Biology, School of Dentistry, University of Leeds (G292 and TIGK) and Department of pharmacy, School of Applied Sciences, University of Huddersfield (A2780). Those cell lines were stored and issued by the tissue bank, Oral Biology, University of Leeds.

2.2.1.3.1.1 G292

G292 is a cell line characterised as osteoblast-like human osteosarcoma adherent cells which originates from a 9-year-old Caucasian girl. This cell line was cultured with high glucose DMEM + 10% FBS + 1% L glutamine + 1% pen strep at 37 °C temperature, in a humid atmosphere containing 95% air + 5% CO₂. This was important to facilitate their attachment to the growing surface - either in a flask during passage or a well plate containing multiple wells according to experimental conditions. G292

cell line was chosen for this study to explore the effects of cannabinoids for in vitro experiments. This is because G292 was extensively used in several studies to explore the effects of various materials and chemicals on its osteoblastic phenotypes (Ren and Dziak, 1991, Stephan, 1998).

2.2.1.3.1.2 A2780 cell line

A-2780 is an ovarian cancer cell line which was used in this study. A-2780 possesses epithelial morphology which was initially established from an untreated adenocarcinoma case at Christie Hospital, Manchester. As this cell line was extracted from an untreated patient, this was found to be useful for comparative studies, especially during epithelial derangement cancerous growth (Han et al., 2013).

2.2.1.3.1.3 Human telomerase immortalised gingival keratinocytes (TIGK)

TIGK cells were gifted by Richer J. Lamont's Laboratory, School of Dentistry, University of Louisville, USA. TIGK cells are epithelial-like cells, derived from Homo sapiens gingival epithelium. Richer et al found that TIGK cells are resistant to senescence changes until 36 passages (Moffatt-Jauregui et al., 2013). TIGK cells are grown in DermaLife K complete medium with supplements (Lifeline® cell technology, USA) using standard aseptic conditions. No antibiotics were used, and the cells were incubated at 37°C, in 5% (V/V) CO₂ in air. The medium was changed every 2-3 days. Cells were passaged on observing 70-80% confluence. Cumulative studies have used gingival keratinocyte epithelial cells extensively to study the initial interaction of TIGK cells with bacterial encounter and their response during periodontitis (Moffatt-Jauregui et al., 2013). Therefore, this study follows the established method to investigate the effects of cannabinoids for in vitro periodontitis model.

2.2.1.3.1.4 Primary human periodontal cells (hPDLs) procurement

2.2.1.3.1.4.1 Introduction:

Various methods are available to procure primary periodontal cells from extracted human teeth (Frisbie and Smith, 2010, Koga et al., 2008, Lee et al., 2012). The explant method and enzymatic digestion are the two routinely used methods for periodontal cell harvesting from teeth. The explant method has been considered less invasive with less impact on cell viability. The explanted tissue contains native cells in their natural environment. Therefore, when we extract the periodontal cells by the explant method, it provides a growing environment like native periodontal tissue. In addition, the explant method is relatively convenient to harvest (Lee et al., 2011, Liu et al., 2009). The aforementioned reasons provide the rationale for selecting the explant method over the enzymatic method to harvest periodontal tissues from extracted teeth in this study.

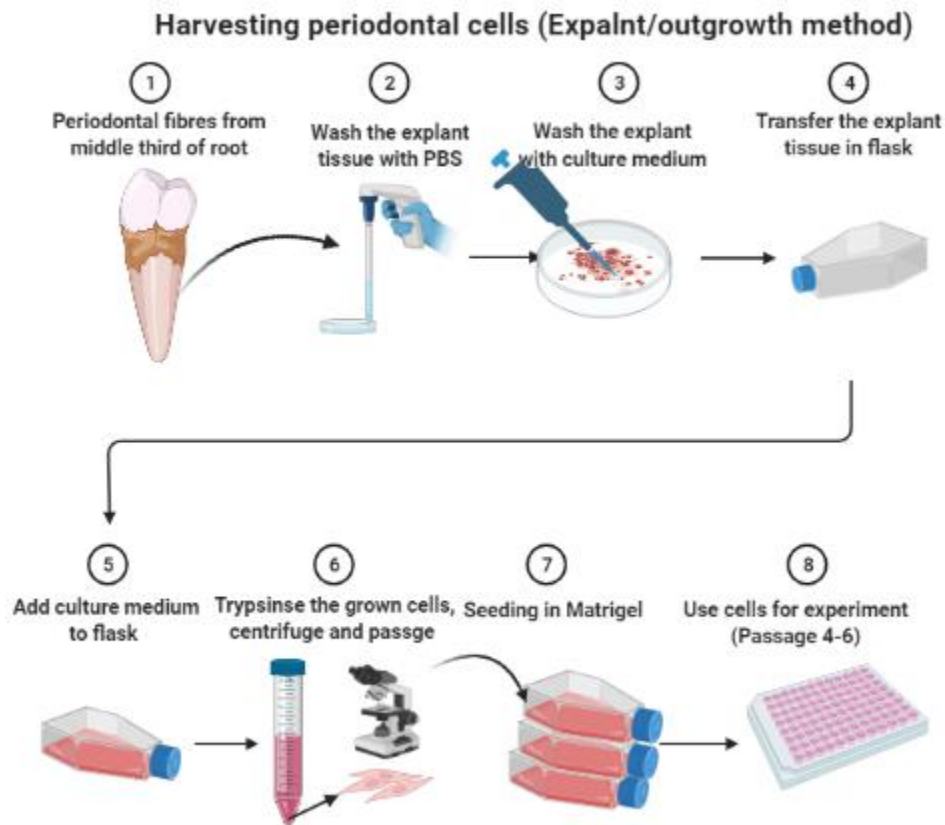


Figure 14: Harvesting and culture of periodontal ligament (PDL) cells. (Technique described by drawing using Biorender.com)

2.2.1.3.1.4.2 Isolation and culture of hPDLCs:

The isolation and culture of human periodontal ligament cells (hPDLCs) was carried out as described in Figure 14. The tooth extracted was obtained from human teeth extracted at Leeds Dental Institute. This sample was a by-product of a routine dental extraction procedure carried out on grounds of extensive dental caries which was beyond repair. As a result, these extracted teeth were given to the tissue bank for research purposes. Thereafter, it was issued to the researcher from the tissue bank as per tissue bank application **(REF NO: 160418/SK/248)**.

The teeth received from the tissue bank were immediately immersed in PBS (phosphate buffered saline D8662, Sigma UK). First, the tooth was washed and rinsed

thoroughly using PBS, then the periodontal ligament fibers were scraped from the middle third of the root using a surgical scalpel.

The tooth specimens were scraped from the middle third of the root. This was done in order to avoid any involvement of any gingival or periapical tissues. In the first instance, the harvested periodontal tissue was washed for 30 seconds with phosphate buffer saline (PBS, D8662, Sigma UK). This was followed by a further five washes, each wash lasting 30 seconds. Each wash involved the use of a cell culture medium (Dulbecco's Modified Eagles Medium (DMEM) (D6064, Sigma, Gillingham, UK) containing 10% foetal bovine serum (FBS DE14-801F, Lonza, Wokingham, UK) and 10% Penicillin/Streptomycin (15070-063 Gibco, Grand Island, UK).

Next, the explanted periodontal tissue was placed in a flask and left to dry until it stuck to the surface of the flask. The time taken for the tissue to stick to the surface of the flask was dependent on the thickness of the tissue. A thin piece of tissue specimen was noted to stick to the surface of the flask within 1-2 minutes whilst a relatively thick specimen took up to 5 minutes.

When the aforementioned periodontal tissue had attached to the surface of the flask, the culture medium (Dulbecco's Modified Eagles Medium (DMEM) (D6064, Sigma, Gillingham, UK) comprising 10% (v/v), Fetal Bovine Serum (FBS DE14-801F, Lonza, Wokingham, UK), 1% (v/v) L-Glutamine, and 1% Penicillin/Streptomycin (100 U. ml⁻¹/100µg.ml⁻¹) (15070-063 Gibco, Grand Island, UK) was poured onto the tissue. These flasks were then transferred to an incubator at this environment i.e., 37°C humidified atmosphere of 5% carbon dioxide and 95% air. Within 2-3 days, cells were seen to be

emerging from the specimens. This confirmed that migration of cells from tissue explants had occurred.

Tissue specimens growing in the flask were observed at 12-hourly intervals for cell outgrowth. Once cell growth occurred, the new cells were transferred to a different flask to prevent over-confluency of growing periodontal cells. Over-confluence retards cell growth and predisposes the cells to produce extracellular fluids. To mitigate this situation, the cells were passaged by trypsinising the sub confluent flask and the culture medium was changed every three days until the cell reached sub confluency. Thereafter, the cells were trypsinised either for experimental use or for storage for future use. PDL cells at passage number 4-6 were used for all the experiments. At passage 2, the cells were stored in liquid nitrogen for future experiments.

2.1.2 Cell culture passage and counting

Cell culture medium was aspirated carefully from the cell culture flasks (Corning®) and the monolayer cells were bathed twice with PBS (pH 7.4) without calcium and magnesium. Trypsin-EDTA solution (0.25%(v/v)) was then added to the cell culture flasks (5 ml for T75 and 10 ml for T175, size flasks) and incubated at 37°C for three to five minutes. Following incubation, due to the presence of trypsin in the flask, the monolayer cells became detached from the surface of the flask and were noted to float in the medium. This was seen using an inverted microscope.

On successful detachment of the cells from the flask surface, a proportional amount of complete cell specific medium, i.e., previously added trypsin was added to the flask in order to deactivate trypsin activity. Flask contents were then transferred into a universal tube (50 ml) and centrifuged at 150 g for five minutes. The process of centrifugation causes the cells to form into a pellet as they settle to the bottom of the

tube. The resultant supernatant mixture of culture media and trypsin was then discarded. After discarding the supernatant, the pellet was processed: 1) it was re-suspended in a 10 ml fresh media, 2) the cell number was counted using haemocytometer (see below) and 3) it was seeded in fresh new culture flasks (Corning® Costar®) or well plates (Corning® Costar®) at a required density.

The next paragraph details how the cells in the pellet were counted.

2.1.2.1 Cell counting using Neubauer chamber or hemocytometer:

The Neubauer chamber or haemocytometer is a device based on capillarity principle where a uniform cell suspension spreads across the counting area. This device contains 9 large squares with 1mm² area in each square. Cells were mixed with a dye called trypan blue and applied to the edge of the coverslip to be absorbed into the counting precision area under the coverslip by capillary action. The amount of cell suspension liquid is the same as the volume of counting place on haemocytometer, i.e., 1 mm width x1 mm height x0.1 mm depth = 0.1 mm³ = 1 x 10⁻⁴ ml (Strober). The number of cells counted in 4 each containing 16 small squares, the number of observed and counted cells must be averaged and then multiplied with a dilution factor to get the number of cells per ml.

Equation 1: Viable cell (%age) = $NV/NT \times 100$ (NV= number of viable cells, NT = total number of cells)

2.1.3 Cell resuscitation and maintenance

2.1.3.1 Principle:

Cells stored at -70 °C or in liquid nitrogen need defrosting before culture and further maintenance under controlled environment. Different cells require a specific culture nutrient, but the overall principle of in vitro cell culture growth is identical. Once cells are 'resuscitated', they need a sterile environment to grow in, and monolayer cells need to be attached to a substrate. In addition, a culture medium is required to 1) provide essential nutrients, 2) maintain the pH and 3) provide adhering proteins. Finally, a sterile incubator is vital for the maintenance of ambient temperature (37°C), humidity (95%) and optimal gaseous exchange (McAteer, 1994).

2.1.3.2 Method

A similar method was used to defrost cryopreserved primary cells and cell lines. The frozen cells were defrosted by adding the pre-warmed medium at 37°C to frozen vials and were then transferred into 10 ml of cell culture medium. Re-suspended cells in culture medium were then centrifuged at 1.5 g for 5 minutes at room temperature in order to create a cell pellet. The supernatant was then discarded, and the cell pellet again resuspended in 10 ml of fresh cell culture medium. Cells were then counted and transferred to required culture conditions. The cells were grown in incubator at 37°C with 95% relative humidity and 5% CO₂. The cell culture medium was changed every 3 days for primary cells. Conversely, for fast growing cells, the media was changed less than 3 days or earlier as required. Cells were monitored using an inverted microscope.

2.1.4 Cell harvesting using trypsin and cell passaging.

2.1.4.1 Principle

A proteolytic enzyme called trypsin along with a chelating agent, ethylene diamine tetra acetate (EDTA), was added to neutralise the calcium and magnesium ions and digest the adhesive proteins. This was done to help the growing monolayer cells detach from the surface of the flask. The process of cell detachment is vital to prevent density-dependent inhibition because it can result in diminished growth at later stages where they compete for space to grow (Pardee et al., 1978). Therefore, once approximately 80% coverage of cell growth in culture flask is reached, cells are trypsinised and passaged.

The periodontal primary cells are found to have a factor called P27 (an inhibitor of cyclin dependent kinase) (Seluanov et al., 2009). P27 is responsible for cessation of growth due to contact inhibition. When primary cells stop multiplying, it secretes extracellular matrix. This must be prevented in order to study the effect of cannabinoids on primary cells. Therefore, primary cells must be cultured to a maximum of 80% confluency and passaged thereafter. On the other hand, cell lines are resistant to contact inhibition due to hyaluronan (Itano et al., 2002). Hence, they show overlapping cell growth, leading to a formation of multilayer growth which is detrimental to experimental studies of cannabinoids on cell lines. In this study, this was averted by maintaining the maximum growth until 80% confluency.

2.1.4.2 Method

On observing approximate 80% confluent cells under microscope, the culture medium was discarded, and monolayer cells were washed twice with PBS. Subsequently the monolayer cells were incubated with trypsin at 37°C for approximately five minutes while observing their detachment from the culture surface flask which was tapped gently to prevent overexposure of cells with trypsin.

Trypsinised cells were observed under an inverted microscope for the confirmation of their dissociation from the flask. Thereafter, fresh culture medium including foetal bovine serum (FBS) was added to the trypsinised cells to deactivate the trypsin. The cell suspension containing trypsin and culture medium was then transferred to a corning falcon tube.

This falcon tube was then centrifuged for 5 minutes at 1.5 g at room temperature. Following centrifugation, the cell pellet was observed with naked eyes and the liquid supernatant was discarded carefully without disturbing the cell pellet. Cell pellet was then resuspended in 10 ml of culture medium and cell number was counted before seeding out for any experimental procedure. The remaining cell suspension was cultured again in a culture flask for further growth of the cells in an incubator at 37° C with relative humidity (95%) and CO₂ (5%).

2.1.5 Cell cryopreservation and storage

2.1.5.1 Principle

Cells can be preserved at a very low temperature, i.e. -80°C for about a week to maintain good viability. When the cells need to be preserved for an extended period, e.g., six months or longer, liquid nitrogen is the choice of preservation. This is because

liquid nitrogen has the capacity not only to preserve the viability of the cells, but also prevent any genetic change, and or contamination.

During this process, a cryoprotectant, DMSO, is used to prevent the formation of internal and external ice crystals which can damage cells. DMSO was found to be suitable for mammalian cell cryopreservation because it is less toxic to cells and also it is relatively inexpensive (Lovelock and Bishop, 1959). For this reason, DMSO was used in this study. The cells kept in the freezing medium which contains DMSO must be stored in the freezer immediately to prevent cell injury. Importantly, the freezing media contains a proportional mixture of nutrients (JH and US, 2017). Finally, the cells in the freezing vials were stored in a box called Mr Frosty, which contains isopropanol which facilitates temperature to drop at 1°C per minute.

2.1.5.2 Method

Primary cells and cell lines were both cryopreserved using similar techniques in this study. The freezing medium was prepared by adding 30% FBS and 10% sterile filtered DMSO to 60% cell culture medium. The cell pellet was suspended in 1 ml of freezing medium and transferred to a 1.5 ml sterile cryotube. Cryotubes were labelled with date, cell type, donor, passage number, name of the researcher and the source flask. Thereafter, the cryotube was transferred immediately into Mr Frosty containing isopropanol, and concurrently into a - 80°C freezer. Isopropanol is used to facilitate the gradual decrease in freezing temperature.

2.1.6 Characterisation of PDLCs by Flow cytometry

2.1.6.1 Principle

Flow cytometry is a technique using a laser beam to identify the physical and chemical properties of cells. The cells are to be labelled with fluorescent markers as they flow uniformly through the flow cytometer. Based on the fate of incident laser beam on cell sheath, the scattering of light is used to identify the cell characteristics. For example, the light scattered by the cells is directly proportional to the morphology and structural properties. When the scatter happens, it helps to identify the salient cell characteristics when the laser beam is scattered at a low angle, i.e., $0.5 - 10^0$, also known as forward scatter, it can help to reveal the size of the cells. When the laser beam is scattered at right angles (90^0), also known as side scatter, it reveals the granularity of the cells (Macey and Macey, 2007). Its application in cell biology is based on the fact that it is found suitable for the detection of cytoplasmic and nuclear antigens.

2.1.6.2 Method

In this study the surface characteristics of cells with and without treatment with cannabinoids were analysed using an acoustic focusing flow cytometer (Attune[®] NxT). Sample preparation included labelling of cells with fluorochrome conjugated antibodies. Cells with and without treatment were seeded based on the number of cells required for analysis. In a 5 ml FACS tube, about a million cells were suspended in 1 ml of FACS buffer (consisting of PBS supplemented with 0.5% (v/v) bovine serum albumin (BSA) and 0.1% (v/v) sodium azide (pH 7.2)). They were centrifuged at 350g for 5 minutes. The supernatant was discarded using pastette. The cell pellet was then added with approx. 2 ml FACS buffer without serum. The antibodies (Cell trace[™] Calcein violet stain (1 μ L stain / 1×10^4 cells) was added in cell suspension and left to

incubate for 45 minutes in the dark and on ice (4°C). After incubation the cells were centrifuged again at 350g for 5 minutes, and supernatant was discarded. This time the cell pellet was re-dissolved in staining buffer (100µL). Thereafter, it was blocked with fragment human crystallisable receptors (FcR) blocking reagent on ice (4°C) for 10 minutes at 10 µL reagent per 1X10⁴ cells. After incubation the cell suspension was added with two types of antibodies namely, control and primary antibodies as mentioned in table 2. The cell suspension with the antibodies were stored on ice in the dark for 45 minutes. The fluorochrome-conjugated primary and control isotype antibodies used in the flow cytometric analysis of cell surface markers are listed in Table 2. After incubation with antibodies, the cell suspension was washed twice and re-suspended with staining buffer (500µL). The cell suspension was then studied with flow cytometer.

The data obtained was analysed using cytoflex software *version 2.1*. Unstained or single colour-stained cells were used for compensation of fluorescence overlap. Non-stained and cells stained with isotype antibodies were used as negative controls.

2.1.7 Cell proliferation

Cell growth and differentiation are the processes during which the replication of its genetic material happens, and the cells divide into identical daughter cells. Cumulative studies on cell proliferation have provided vital information about cell behaviour during their growth (Holley, 1975, Schor, 1980). Cell growth can be studied by growing them either on their own or in the presence of chemicals such as cannabinoids (Adan et al., 2016). The behaviour of diseased cells differs from cells which undergo normal growth

and multiplication. Cell proliferation assays provide vital information regarding multiplication, rate of growth and proliferation.

2.1.7 Principle

To date, various cell proliferation assays are available and different assays use different ways of cell proliferation estimation. The proliferation is detected by measuring various assays such as 1) the DNA synthesis (³H thymine, bromodeoxyuridine), 2) the cell proliferation marker (phosphorylated histone H3), 3) ATP concentration (luciferase), 4) measuring the metabolic activity using bioluminescent dyes (AlamarBlue) and tetrazolium salts (LDH), and 5) with the help of spectrophotometers and enzyme-linked immunosorbent assays. In this study DNA estimation by using Picogreen Assay and the AlamarBlue assays were used.

2.2.8 Cytotoxicity assays

2.2.8.1 Viable cell counting using Trypan Blue dye.

2.2.8.1.1 Principle

Cell viability assays are an established method used to evaluate physical and physiological health of the cultured cells. The viability of cells is routinely checked during in vitro experiments investigating cell growth in different environmental such as chemical agents or therapeutic treatments. Trypan blue dye exclusion assay uses a dye to detect dead cells based on cell membrane integrity. If the cell membrane integrity is compromised by toxic materials such Triton X100, the cytoplasm of the cells is stained blue. Cells with intact membranes do not take up this impermeable dye and hence appear colourless. This assay is routinely used to determine cell viability

because it is inexpensive and not time consuming. The counting of live and dead cells needs to be carried out using a Neubauer chamber or haemocytometer, as mentioned above.

In this study, cell lines and primary cells were evaluated for their growth in the presence of cannabinoids.

2.2.8.1.2 Method

Cell viability was assessed using trypan blue dye and a haemocytometer (see section 2.1.2.). The cell suspension was mixed with the dye 1:1 (v/v) and mixed homogeneously before pipetting under a coverslip to ensure the even spread of cell suspension by capillary action. 20 μ L of the stained cell suspension liquid was infused under the coverslip.

The cell was then counted on 4 corner squares, each corner containing 16 small squares. The cells were observed under inverted microscope using 10 X objective lens. The number of cells counted in 4 corner squares were added, and then divided by the number of squares (4) and finally, multiplied by the dilution factor, i.e., 2. In order to get a total number of cells per millilitre of cell suspension, it was multiplied by 10,000 (Chamber). Viable cells appeared as bright white dots whereas the dead cells were dark blue dots.

2.2.8.2 Cell viability using Alamar Blue assay.

2.2.8.2.1 Principle

Alamar Blue is a blue water-soluble dye (resazurin) which is reportedly stable in culture medium, and also non-toxic and membrane permeable (Freddi, 2014). The dye helps to monitor the cellular cytoplasmic metabolic reducing environment. Once the dye is in contact with a reducing environment, the colour of the dye changes from blue to

pink. Colour change to pink represents cell activity, indicating the presence of increased numbers of viable cells. This is due to the development of a pink colour (resorufin) on accepting the electron from electron transport chain. The dye does not interfere with the normal function of cells, so it allows the monitoring of cell growth, cellular health and metabolic function over multiple cultures (Page et al., 1993). The absorption spectrum used to detect the change of colour in AlamarBlue was set at 570/600 nm or alternately 570/630 nm and 540/600 nm on the Spectro Photo meter. The fluorescence signals can be monitored at excitation (530/560 nm) and emission (590 nm). This assay of exploring cell viability is advocated largely for drug screening (Räz et al., 1997, Collins and Franzblau, 1997). This is due to ease of application, non-toxicity to cells and is largely preferred by laboratory technicians. Importantly, it is considered time efficient, easy at disposal and a possible long incubation with mammalian cells (Pettit et al., 2009).

2.2.8.2.2 Method

In this study Alamar Blue was used according to the manufacturer's instructions (Figure 15). The cell culture medium was aspirated with care, and the monolayer cells were washed twice with phosphate buffered saline without calcium and magnesium. Thereafter, the monolayer cell was introduced into the mixture of 10x AlamarBlue and culture medium. It contained 10 μ L AlamarBlue + 100 μ L of culture medium. Cells were incubated for 4 hours at 37°C in the presence of AlamarBlue and culture medium. The absorbance was measured using the plate reader at the given wavelengths mentioned above.

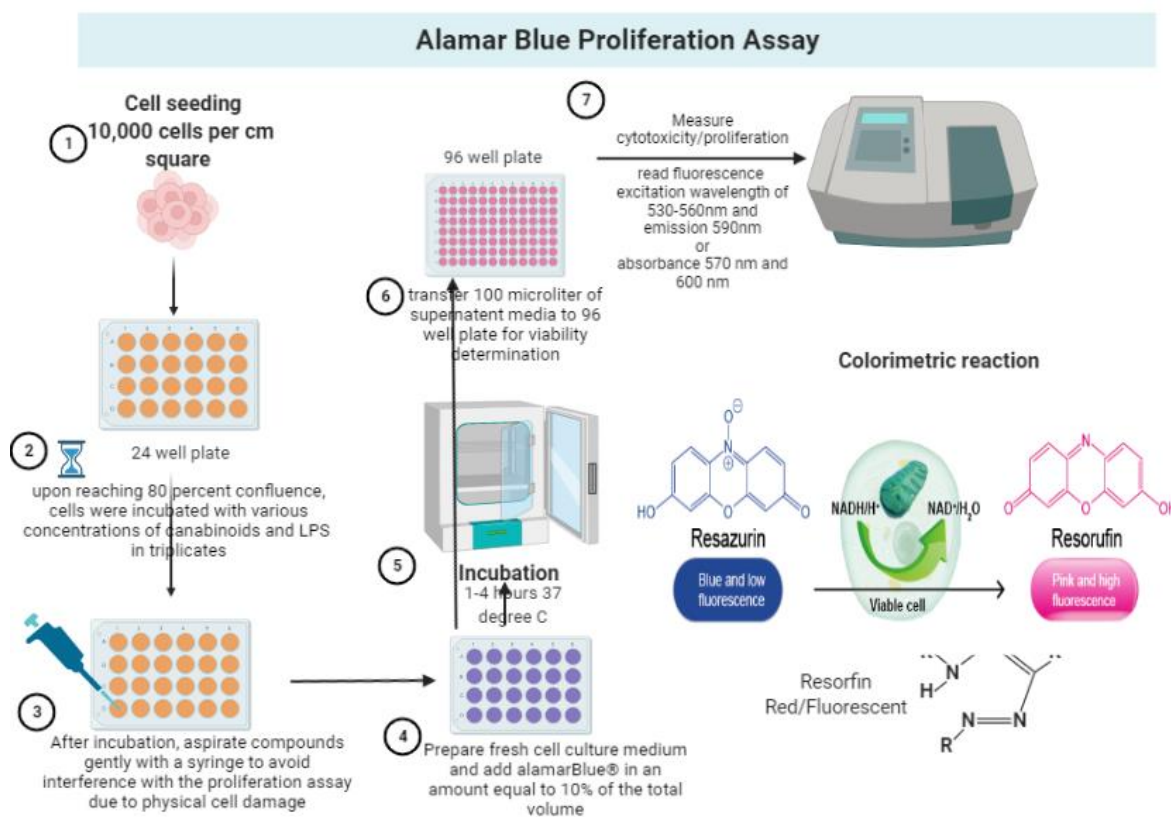


Figure 15: Schematic diagram showing stepwise Alamar Blue assay picture drawn using Biorender.com.

2.2.8.3 Cell viability/cell toxicity Assay (Live/Dead® assay)

2.2.8.3.1 Principle

Live/Dead is a fluorescence- assay based on plasma membrane integrity. In this assay an amine reactive dye (Ethidium homodimer III, Excitation/Emission 530/620) is used which is a cell membrane-impermeant nucleic acid which binds with cell DNA. It stains only damaged or dead cells and emits a bright red signal on excitation (Excitation/Emission wavelengths of 528/617 nm). On the other hand, cell membrane permeable dye (Calcein-AM) is a non-fluorescent chemical capable of passively crossing the cell membrane. On permeating the cell membrane, Calcein-AM gets hydrolysed by the cellular esterases. This hydrolysis cleaves the AM group and converts the non-fluorescent calcein-AM to a green, fluorescent calcein

(Excitation/Emission wavelengths of 494/515 nm). As the dead cell lacks active esterase, only live cells are labelled and produce a green colour when stained with calcein AM (Haugland et al., 1994).

2.2.8.3.2 Method

The Live Dead assay was carried out following the manufacturer's instruction. The staining solution was prepared by mixing 5 μ L of 4 mM Calcein and 20 μ L of 2 mM, Ethidium homodimer into 10 ml of serum free DMEM providing a final concentration of 2 μ M Calcein and 4 μ M Ethidium homodimer. The solution was then introduced onto the monolayer cells and incubated for 45 minutes in the dark at 37°C incubator which has 98 % relative humidity and 5% CO₂. Monolayer cells were then washed thrice with PBS for 10 minutes and viewed using confocal laser scanning microscope at wavelengths mentioned above. Images were taken using Leica Lax X software©.

2.2.9 Cell Morphology

The basic framework of the cells in relation to its appearance, form and phenotype characteristics is known as morphology, correlated with the function and cell health (Ambrosio, 2017). Every cell type possesses peculiar morphological features such as epithelial cells e.g., squamous, cuboidal or columnar, appears rectangular in shape, and fibroblasts appear flat, and spindle shaped: – this can be either bi or multi polar in shape. These epithelial and fibroblast cells need a surface to attach to, unlike lymphocytes which grow in suspension (Wu et al., 2020). Studies related to morphological characteristics like its shape, the size of the nucleus, its contents and membrane integrity can help identify the health of a cell growing on its own, and the effect of its surrounding environment. In this study the above-mentioned types of cells found in periodontium were chosen to investigate the interaction of cannabinoids with cells. Live cell morphology was determined by using inverted phase contrast

microscopy while growing in cell culture media with or without cannabinoids. The cell was stained with nuclear and cytoskeletal stains and were observed using a confocal microscope. Additionally, cells were fixed, sputter coated with gold and observed using a scanning electron microscope.

2.2.9.1 Inverted Microscope

2.2.9.1.1 Principle

The basic principle of any microscope is to enlarge a tiny object such as a cell many times in order to view its detailed structure. The working principle is similar to light microscope where light is focused on cells to form an image which is viewed by objective lens (Lund et al., 1958). Here, the light source and the condenser used to point the light on the specimen are found above the specimen stage. The objective lenses, which are located below the specimen, collect light from the condenser to provide the magnified view from its ocular lens corresponding to the objective lens used. The monolayer live cell growing on the surface of the culture flask can be viewed using an inverted phase contrast microscope at the desired magnification and recorded using the camera attached.

2.2.9.1.2 Method

Monolayer cells were cultured in the flask and well plates based on the experiments. Cells were observed under microscope for their morphologic appearance and compared with the cells growing under normal conditions of growth. The observed growth was then captured using the attached camera (Zeiss, AxioCam ICm1).

2.2.9.2 Confocal Microscope

2.2.9.2.1 Principle

This instrument is built around the basic principle of a light microscope where the light source is a laser, and it has sensitive photomultiplier tube detectors. A computer is used to capture and display images. A confocal microscope allows a chosen laser beam to pass through an objective lens to illuminate the sample. On hitting the specimen, it releases photons. They are directed to a pinhole before it is detected by the photomultiplier and sent to the attached computer for viewing and storing the images. It was measured in pixels. This technique allows filtering of focused light through a pinhole, hence providing a clear image of the sample (Gu, 1996).

2.2.9.2.2 Method

Cell morphology of monolayer cells was evaluated by staining the cells with cytoskeleton stain Alexa-Fluor 488 Phalloidin and the nuclear stain DAPI. Cells were cultured for 3 and 24 hours, with and without the cannabinoids, with different concentrations, i.e., 1 μ M and 10 μ M. After growing for the required time, i.e., 3 and 24 hours, the cells were washed with PBS twice and fixed with 10% NBF for 20 minutes at room temperature (25 °C).

Samples were then washed three times for approximately a minute each time and were presented with membrane permeabilisation agent 0.1% (v/v) Triton-X100 in PBS for 15 minutes at room temperature. The monolayer cells were washed again in PBS and incubated with Alexa fluor (1 :20 Alexa fluor: PBS) for 2 hours in the dark at room temperature. After incubation cells were washed again and incubated with DAPI in the dark at room temperature.

Finally, the cells were given a wash with PBS again before viewing under the confocal laser scanning microscope (Leica® TCS SP8). The images were acquired using the

LasX software©. The wavelengths used were Alexa-Fluor 488 excitation /519 emission nm, DAPI 358 excitation / 461emission.

2.2.9.3 The scanning electron microscope (SEM)

2.2.9.3.1 Principle

The scanning electron microscope (SEM) produces the image by scanning the sample topography. It targets a focused beam of electrons and provides images based on signal emitted by the samples. These signals can be in the form of photons, light, secondary electrons (SE) and back scattered electrons (BSE). The signals produced by the SEM depend on the interaction of electrons and atoms at various depths of the sample. The signals produced by samples on interaction with focused electron beam are collected by detectors to produce the digital image (Svergun et al., 2013). The most used electrons to study the surface characteristics are the emitted secondary electrons. Back scattered electrons provide the description of different layers of the sample (Carrassi et al., 1987).

2.2.9.3.2 Method

The monolayer cells were grown on coverslips within the well plate. On observing approx. 80% confluency, the coverslips were taken out of the well plates with care and subjected to fixing using 4% formaldehyde at 4°C overnight. The cell surfaces were then washed with distilled water and subjected to dehydration with serial dilution of ethanol. The cells were then left to dry in order to get rid of any moisture before attaching the coverslip to a 1.2 mm aluminium stubs. Samples were then gold coated using an argon gas chamber before viewing under scanning electron microscope.

2.2.10 Gene Expression Analysis of Inflammatory Cytokines

following cannabinoids treatments:

2.2.10.1 Cells treatments with Cannabinoids:

The TIGK cells and PDLCS (1×10^4 cells/cm²) were exposed to inflammatory stimulants including LPS: (1µg/ml), flagellin (1µg/ml) at two time points i.e., 3 hours and 24 hours, with and without pre-exposure to cannabinoid (CBD, 1&10µM). Following this, a 3-hour and 24 hours cell free supernatants were collected using a micropipette, without disturbing the cell monolayer beneath, and stored at -80 °C for ELISA (section 2.2.11). The remaining corresponding cell monolayers were used to harvest the RNA for evaluation of gene expression.

2.2.10.1.1 Cytokines expression at mRNA level

2.2.10.1.1.1 Principle

Altered expression of such genes is studied using real time quantitative polymerase chain reaction (RT-qPCR).

Polymerase Chain Reaction (PCR): PCR is a sensitive technique (Figure 16) which relies on thermal cycling. It is used to detect, amplify and quantify a specific sequence of DNA or mRNA through repeated cycles of heating and cooling (RK, 1985, Cheng et al., 1994). The DNA amplification requires a mixture of reaction buffers which contain:

- 1) The sample DNA (DNA template that contains the DNA target region to amplify),
- 2) DNA building components (Deoxynucleotide triphosphates; Adenine, Thiamine, Cytosine and Guanine),
- 3) Buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase and reagents,

- 4) A heat-resistant DNA polymerase enzyme and
- 5) Forward & reverse primer sequences which are specific to targeted DNA fragments and complementary to 3' end.

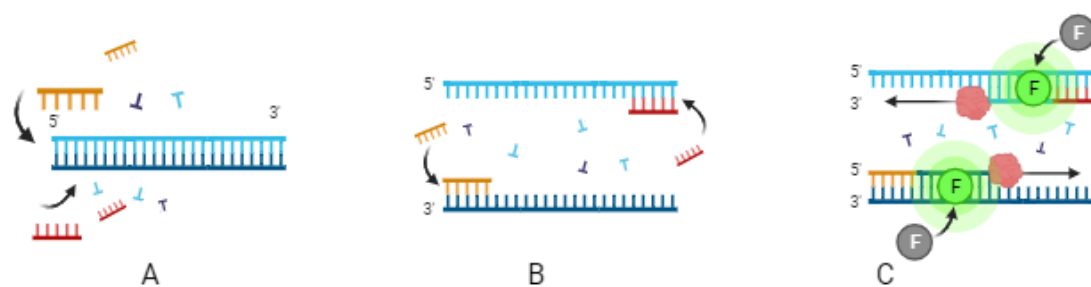
The PCR reaction consists of 20-40 thermal cycles: repeated cycles of heating and cooling, with each cycle consisting of three discrete temperature cycles. Each PCR cycle begins with initial increase of temperature of up to 95 °C for melting the double bonds by denaturation of hydrogen bonds of DNA template. This is followed by a decrease in temperature to 65 °C, called annealing temperature. The second cycle lasts 20-40 seconds which allows annealing of primers to single stranded DNA templates.

The annealing temperature was set at approximately 3–5 °C below the melting temperature (T_m) of the primers to achieve efficiency and specificity of the reaction. This process provides two separate copies of the original DNA sequence or gene, each of which is called an amplicon.

At the final stage, i.e., elongation or extension, the temperature is increased to 72 °C. Here, the DNA polymerase synthesizes a new DNA strand which is complementary to the DNA template strand by adding a free deoxynucleoside triphosphates (dNTP) from the reaction mixture. The newly formed DNA is complementary to the template in the 5' to 3' direction.

During real-time quantitative polymerase chain reaction, the (RT-qPCR) reaction product is quantified during PCR. This is achieved by using fluorescent dyes staining the nucleic acids like Cyber green or fluorescence based TaqMan probes (Ponchel et al., 2003).

Polymerase chain reaction



A Denaturation at 95-96⁰C

B Annealing at 68⁰C

C Elongation at 72⁰C.

**Figure 16: Pictorial diagram represents the sequential events during a polymerase chain reaction
Adapted from Glossary/Genomic science centre using Biorender.com.**

2.2.10.1.1.2 Method Real time PCR (RT-PCR)

Gene expression of inflammatory mediators was measured to determine whether the studied cannabinoids modulate their expression. The gene expression was analysed using total RNA prepared from cells (cannabinoids treated and untreated) and TaqMan gene expression assays (Applied bioscience UK) as per manufacturer's instructions.

Human TaqMan® Gene Expression Assays for the genes of interest (Table 3, section 2.1.4.4) were purchased from Applied Bio systems at ThermoFisher Scientific (General catalogue number :4331182). Each assay contains a specific forward and reverse primer. It contains a TaqMan® probe labelled with a fluorescent dye on the 5' end, and a minor groove binder and non-fluorescent quencher on the 3' end. Most of the assays were pre-designed and available from the Advanced Biosystems catalogue. The TaqMan gene expression assay reactions for qRT-PCR were prepared in a white 96-well PCR plate.

For each sample, TaqMan assay components were added to wells as described in Table 4 below. The plate was centrifuged for up to 5 seconds to spin down the contents before placing in a Light Cycler 480-II (Roche) which performed quantitative real-time PCR on the samples.

The cycle threshold (Ct) values were generated for gene of interest and for the housekeeping gene, otherwise known as control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), found consistently in all the samples (LightCycler® 480 software) (Table 5).

To perform RT-qPCR, firstly, the total RNA was extracted from cells. (Figure7) Secondly, the extracted RNA was subjected to reverse transcription to synthesise complementary DNA (cDNA) (Figure 8). Finally, the cDNA was used as a template for the PCR reaction.

2.2.10.1.1.2.1 Step 1: Extraction of RNA

This procedure is based on principle of RNA binding to the silica membrane of RNeasy column. The RLT lysis buffer provided in the RNA extraction kit was used for lysing the cells prior to RNA isolation (“Buffer RLT - Qiagen”). Buffer was used to inactivate the ribonucleases (RNases) and to preserve the integrity of extracted RNA. Furthermore, the addition of 70% ethanol helps the binding of RNA to silica membrane. During the process of washing, addition of deoxyribonuclease (DNase) is used to improve the yield of uncontaminated RNA with the genomic DNA.

RNA extraction (Figure 17) was conducted using RNeasy Mini Kit as per manufacturer’s instructions (RNeasy® Mini Kit Cat. No. 74104).

To outline the protocol briefly, monolayer cells were washed with PBS twice before adding RLT buffer solution (RLT buffer + β -mercaptoethanol). Addition of b-

mercaptoethanol aided the protection of RNA from being digested by inhibiting RNase enzyme. Following the addition of RLT buffer, 70% of RNase free ethanol (Sigma Aldrich Honeywell ethyl alcohol pure Cat. No 459844) was added, and the suspension was homogenised before transferring to spin column.

The spin column was centrifuged at 8000 x g for 15 seconds at room temperature. The flow-through was discarded. The step was repeated until all lysate for each sample was used. Thereafter, 350 μ L of buffer RW1 was added to each sample, it was centrifuged at 8000 x g for 15 seconds at room temperature and the flow-through was then discarded. After discarding the flow-through, DNA digestion was performed on silica membrane by incubating the samples with 80 μ L of DNase (10 μ L of DNase I + 70 μ L of buffer RDD) (RNase-Free DNase Set Cat. No. 79254) for 15 minutes at room temperature. Following DNA digestion, 350 μ L of buffer RW1 was added to each sample, centrifuged at 8000 x g for 15 seconds at room temperature and the flow-through was discarded. After discarding RW1, 500 μ L of buffer RPE was added and centrifuged at 8000 x g for 15 seconds at room temperature twice and flow-through was discarded. RNeasy columns were then transferred into new 1.5 ml RNase-free tubes. Then 30 μ L of RNase-free water was added over the transferred column and it was again centrifuged at 8000 x g for one minute to collect the eluted RNA. This time the column was discarded whilst the flow through was saved.

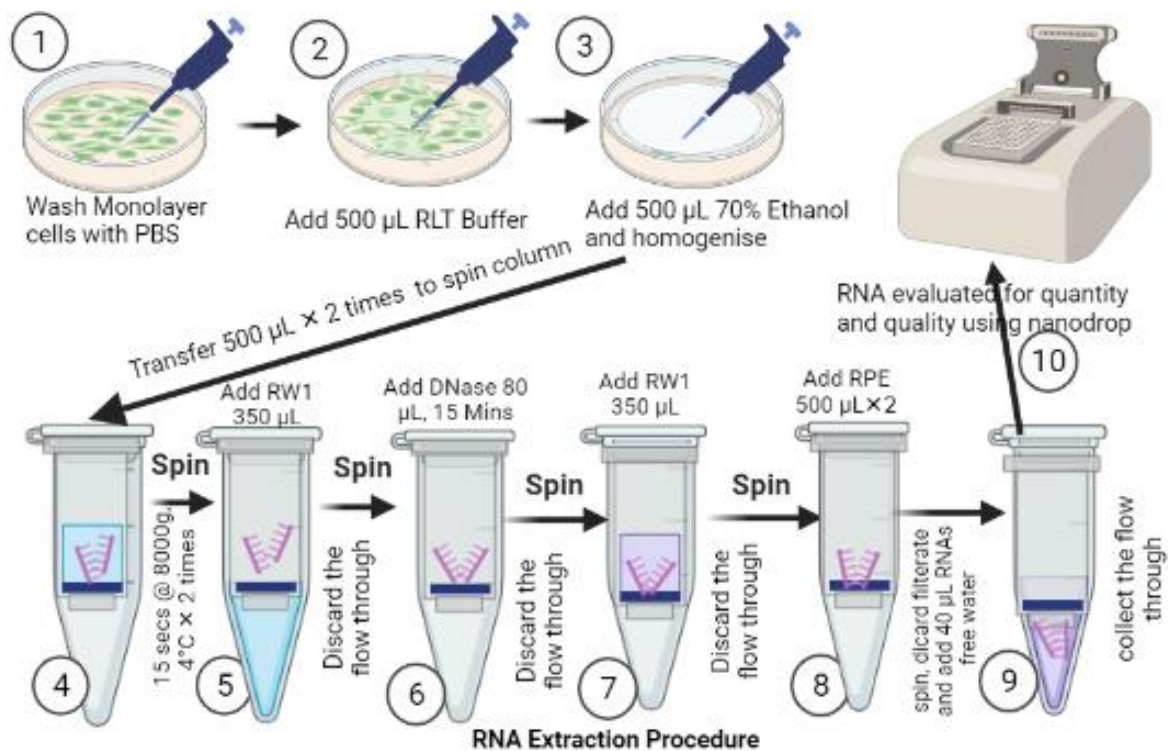


Figure 17: Schematic representation of RNA extraction using RNeasy Mini Kit (Figure adapted using Biorender.com)

The extracted RNA was then quantified using NanoDrop™-2000 Spectrophotometer (NanoDrop® Technologies Inc.) 2 μL volume from the extracted RNA was loaded on the sensor arm of the instrument. Absorbance was measured at 260 nm and the concentration of RNA was determined in $\text{ng} \cdot \mu\text{L}^{-1}$. The ratio obtained between 1.8 and 2.1 indicated good purity of RNA.

2.2.10.1.1.2.2 Step 2: cDNA synthesis

Copy DNA (cDNA) synthesis was carried out by RNA reverse transcription, using high-capacity RNA-to-c-DNA Kit (ABI Applied Biosystems) following manufacturer's instructions. Based on the yield of RNA detected by the NanoDrop spectrophotometer, 200 ng RNA was used for conversion to c-DNA. The reverse transcription protocol constituted an overall volume of 20 μL . This was prepared by mixing 10 μL of 2X RT buffer, 1 μL of 20X RT enzyme and 9 μL containing the above extracted RNA diluted in RNase-free water in a 0.2 mL RNase PCR tube (Table 5). The reaction mixture then

transferred to a thermal cycler (PTC-100) and incubated according to manufacturer instructions (Table 6). Negative controls were also included during every thermo cycler reaction. Prepared cDNA was then stored at -20°C/ -80°C until required for RT-qPCR reaction.

Table 11: Components of qRT-PCR reactions. NB: All steps were performed on ice, until ready to load the thermal cycler.

Component	Volume/reaction or well (µL)
2× RT Buffer	10.0
20×RT Enzyme Mix	1.0
Nuclease free water	Q.S* to 20µL (*Quantum satis)
Sample	Up to 9µL (max up to 2µg of RNA)
Total per Reaction	20.0

Table 12: Reverse transcription, different steps and conditions. Extracted RNA was subjected to reverse transcription using three different steps. The below temperatures and the durations used in these steps are mentioned in this table.

Condition	Step 1	Step 2	Step 3
Temperature (°C)	37	95	4

Condition	Step 1	Step 2	Step 3
Time	60 Mins	5 Mins	Final hold

2.2.10.1.1.2.3 Step 3: RT-qPCR reaction

The gene expression was analysed using TaqMan gene expression assays (Applied Bioscience UK). The polymerase chain reaction protocol was carried out using a light cycler (Roche LC480) following the manufacturer's instructions. To outline the protocol, required reagents for PCR reaction (Table 3) consisted of an overall volume of 20 μ L, which was prepared for each cDNA sample by making a homogeneous mixture of 10 μ L of TaqMan[®] fast advanced master mix, 1 μ L of TaqMan[®] gene expression assay and 9 μ L of diluted cDNA sample (concentration of cDNA 80-120 mg/mL).

Controls and samples were then added in a 96-well PCR reaction plate on ice in triplicates. The 96-well PCR plate was then sealed securely using an adhesive PCR plate sealer and centrifuged for 10 seconds to settle tube contents using PCR plate centrifuge (Labnet MPS 1000 compact). Thereafter, the PCR plate was transferred to a light cycler (Roche LC480). The amplification of sample DNA was carried out as per conditions mentioned in table below. Threshold cycle (Ct) values were automatically determined by the light cycler.

Human TaqMan[®] Gene Expression Assays for the genes of interest (Table 4) were purchased from Applied Biosystems. Each assay contains specific forward and reverse primers. It also contains a TaqMan[®] probe labelled with a fluorescent dye on the 5' end, and a minor groove binder and non-fluorescent quencher on the 3' end.

Most of the assays were pre-designed and available from the Advanced Biosystems catalogue.

The cycle threshold (Ct) values were generated for gene of interest and for the housekeeping gene otherwise known as the control gene (GAPDH) in all the samples (LightCycler® 480 software) based on following assay conditions (Table 9).

Table 13: Components of qRT-PCR reactions

Component	Volume/reaction or well(μ L)
TaqMan Gene Expression Master Mix(2X)	10
TaqMan Gene Expression Assay(20X)	1
c-DNA template +H ₂ O	9
Total per Reaction	20

Table 14: Details of TaqMan assays used in this study, including the assay ID and the product length.

Gene	Assay ID (Applied Biosystems)	Amplicon length
GAPDH	Hs99999905_ml	122 bp
IL-6	Hs99999032_m1	118 bp
IL-8	Hs00174103_m1	101 bp

Gene	Assay ID (Applied Biosystems)	Amplicon length
CNR2 (Cannabinoid Receptor 2)	HS00275636_m1	88 bp

Table 15: Real time PCR (TaqMan) assay conditions. *UDG (Uracil-DNA Glycosylase enzyme), **UP (ultra-pure polymerase suitable for Hot Start PCR)

Hold	*UDG incubation	50°C	2 minutes
Hold	AmpliTaq Gold, **UP enzyme activation	95°C	10 minutes
40 cycles	Denaturation	95°C	15 seconds
	Annealing/extension	60°C	1 minute

2.2.10.1.1.2.4 Step 4: Analysis of RT-qPCR data (Calculation of gene expression levels (delta-delta Ct))

RT-qPCR data was analysed using the comparative Ct method described by (Schmittgen and Livak, 2008). The ΔCt was calculated by normalising Ct value of each gene of interest to that of GAPDH determined for the same sample. Then, the relative change in gene expression was calculated using the $2^{-\Delta Ct}$. Finally, in order to determine the effect of inflammatory stimulant on gene expression compared to the control, the $2^{-\Delta Ct}$ value for each sample was normalised to the mean of the control sample (TIG-K cells cultured in medium). The $2^{-\Delta Ct}$ (fold change) \pm SD and were plotted for the sample

groups and analysed statistically using the ANOVA single factor test provided by Microsoft Excel 2013 software.

2.2.11 Cytokines expression at protein level

2.2.11.1 ELISA:

2.2.11.1.1 Principle:

Enzyme-linked Immunosorbent Assay (ELISA) is a plate-based immunological assay used to detect and quantify antibodies, antigens, and proteins in biological samples. This technique was developed in the 70s by two independent research groups: Peter Perlmann and Eva Engvall from Sweden; and Bauke van Weemen and Anton Schuurs from Holland (Engvall & Perlmann, 1971; Weemen & Schuurs, 1971). The basic principle states the use of ELISA is to utilise an enzyme in order to detect the antigen antibody binding (ag-ab binding).

This enzyme produces a colorimetric outcome as a product of ag-ab binding. Development of strong colour indicates more numbers of ag-ab binding. There are various types of ELISA reactions based on detection outcome, e.g., direct ELISA, indirect ELISA, competitive ELISA, and the most used, sandwich ELISA. It is basically a five-step procedure: first, antigen coating; second, blocking of unbound site; third, addition of primary antibody; fourth, conjugated secondary antibody addition; and fifth is substrate enzyme reaction to produce the coloured product for positive binding (Aydin, 2015).

2.2.11.1.2 Method:

Sandwich ELISA has been employed in this study as it quantifies antigen between capture and detection antibody. It allows amplification of a signal and leads to the highest sensitivity. Sandwich ELISA technique was used, utilising capture, detection antibodies and standards (Refer 2.1.4.1.1) antibodies for the targeted recombinant human cytokines such as IL-8, IL-6, IL-1 β and TNF- α . The antibodies were reconstituted as per the manufacturer's instructions. The expression of cytokines was compared after samples (cell lines and primary cells) were subjected to cannabinoids treatment. Controls were the samples not given any treatment and standards provided by the manufacturer. The absorbance reading was converted to concentrations (pg/ml) using the relation (Equation 2) obtained by standard curve.

Equation 2: $Cv = \sigma / \mu$, (Cv= coefficient of variation, σ = standard deviation and μ =mean). (Canchola, 2017)

In order to obtain accurate results, the samples were diluted with comparable standard proteins. The dilution factors were taken into consideration while analysing the results. A flat bottom clear Corning 96 well plate was coated with 1X coating buffer by incubating it overnight at room temperature (Figure 2). Then the plate coated with capture antibody was washed three times with washing buffer and the wells were blocked for non-specific binding antigens by adding the assay buffer for 2 hours at room temperature. Following this, the well plate was again washed three times. The prepared standard protein samples were then incubated for two hours at room temperature in order to let antigens and standards binds to capture antibody with high specificity. Thereafter, the unbound samples and standards were discarded. Washed

again 3 times as mentioned previously and well plated were added with detection antibody to allow avidin HRP binds to biotin for another two hours at room temperature. The 96-well plate was again washed three times and TMB developer was added. After incubating with TMB for 25 minutes the TMB was topped with 50 μ L of stop solution to inhibit the enzymatic reaction. The absorbance at 450nm was read using spectrophotometer Varioscan flash (Type 3001, REF 5250040) Thermo Fisher Scientific.

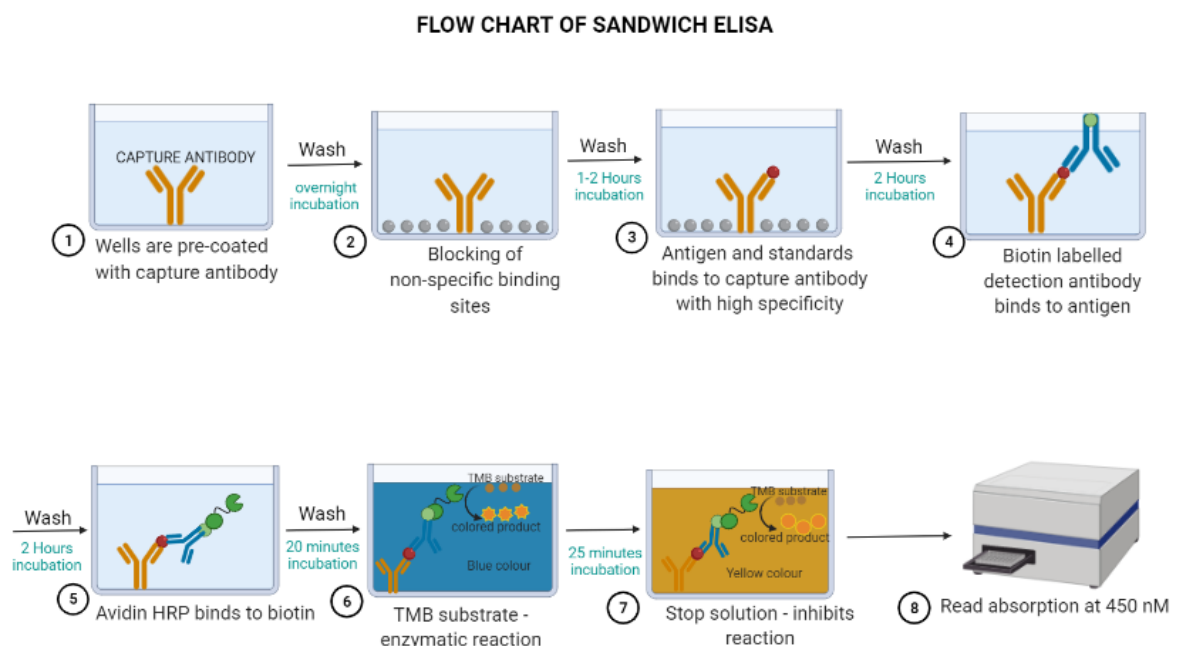


Figure 18: Figure shows the step-by-step illustration of process followed during enzyme linked immunosorbent assays, Drawings adapted using Biorender.com.

2.2.11.2 Western blot:

2.2.11.2.1 Principle:

Western blot is an analytical technique to detect and characterise protein molecules present in the samples tested. Principally this process is based on immunochromatography. It separates the proteins based on their size and molecular

mass and helps to transfer the separated proteins onto a hydrophobic membrane, e.g., nitrocellulose or PVDF, along with marking the proteins by utilising the corresponding specific primary and secondary antibodies labelled with an enzyme e.g., HRP (Kurien and Scofield, 2006). The proteins detected and bonded to the membrane are then visualised using chromogenic or chemiluminescent method. Overall, the procedure involves three steps: electrophoretic protein separation then transferring the isolated protein on to a membrane and finally labelling the transferred proteins (Ghosh et al., 2023).

2.2.11.2.2 Method:

In this research the method used followed the basic principles of the technique (Figure 20).

Preparation of cell Lysates:

For sample preparation, cells were seeded in a well plate (cell density 10,000 cells/cm²) and allowed to grow until required confluence level, washed thrice with neutral buffer saline and monolayer cells were then added with RIPA buffer for cell lysis. The cells suspension in RIPA buffer was then subjected to centrifugation for 10 seconds before putting the sample over a heating block for 5 minutes at 95^oC. Concentration of protein in samples was quantified using BCA assay.

2.2.11.2.2.1 Protein quantification by BCA Assay:

2.2.11.2.2.1.1 Principle:

It is colorimetric method of protein concentration estimation based on the fact that bicinchoninic acids sodium reacts with cuprous ions (Biuret reaction; formation of purple/blue coloured “Cu²⁺ protein complex” due to reaction of Cu(II) sulphate with peptides bonds, based on the protein concentration in the sample) to produce a deep

blue colour under alkaline conditions, detected using spectrophotometer at 562 nM wavelength with a detection range 0.2 -50 µg of proteins (Smith et al., 1985).

2.2.11.2.2.2 Method:

In this study the cultured untreated and treated cells were treated as stated above and samples (25µL) were added to 200 µL of working reagent (bicinchoninic acid) as per manufacturer's instructions in a well plate, allowing it to mix well by shaking the well plate over the plate shaker for about 30 seconds. The plate was then covered with cling film, incubated for 30 minutes at 37-degree temperature and left to cool at room temperature after the incubation. The absorbance was measured at 562 nM using a plate reader and the results were evaluated after blank subtraction. The standard curve (Figure 19) was determined using the absorbance of standards to evaluate the protein concentration of samples.

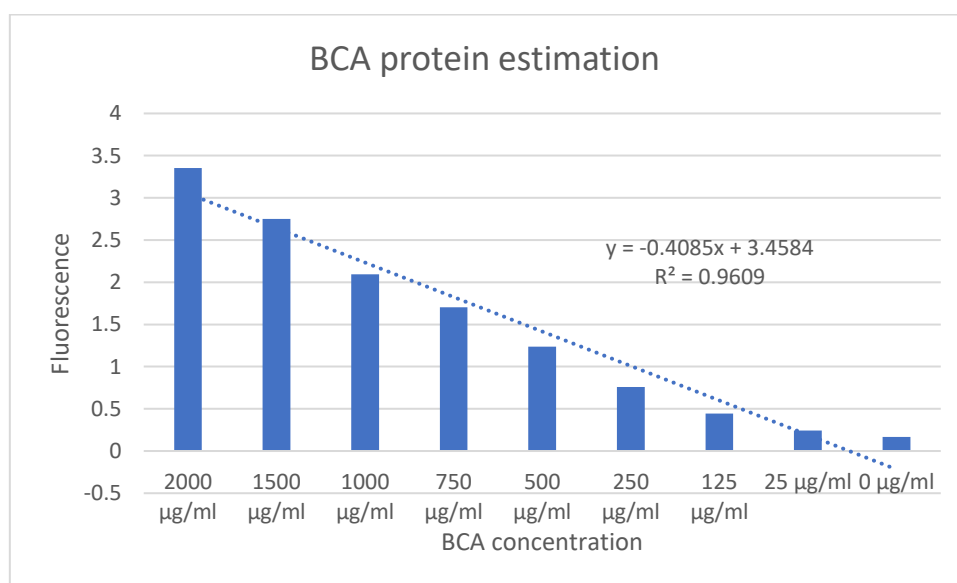


Figure 19: Standard curve to identify the amount of protein concentration in the samples.

Separation of proteins by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

After determination of protein concentration of the samples, the process of western blot was performed. Briefly, to begin with the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the gel prepared using 13% resolving and 6% stacking gel based on the molecular size of target protein molecules. Sample preparation involved the heating of sample and diluting the sample with sample buffer followed by loading the samples (15 μ L) and ladder into the wells which was dipped in electrophoretic tank containing running buffer. The voltage (100V) was then applied to help migration of proteins towards anode for about 20 minutes and observed that the proteins travelled up to the bottom end of the gel.

Western Blotting: Transferring departed proteins from gel to a membrane.

Subsequently the proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane. Step by step transfer protocol began with hydrating the membrane in milli Q water completely and left in transfer buffer. Similarly, we need a same size of filter paper hydrated before placing the hydrated membrane over it. Thereafter the gel with proteins was placed over the membrane followed by covering it with another hydrated filter paper. A wet sponge was then placed both sides of the above prepared paper membrane gel sandwich before holding the entire assemble into a transfer cassette. The transfer cassette was then gently transferred into a tank containing the transfer buffer. The transfer tank was then connected to electric current (90KV) for about one hour and 10 minutes.

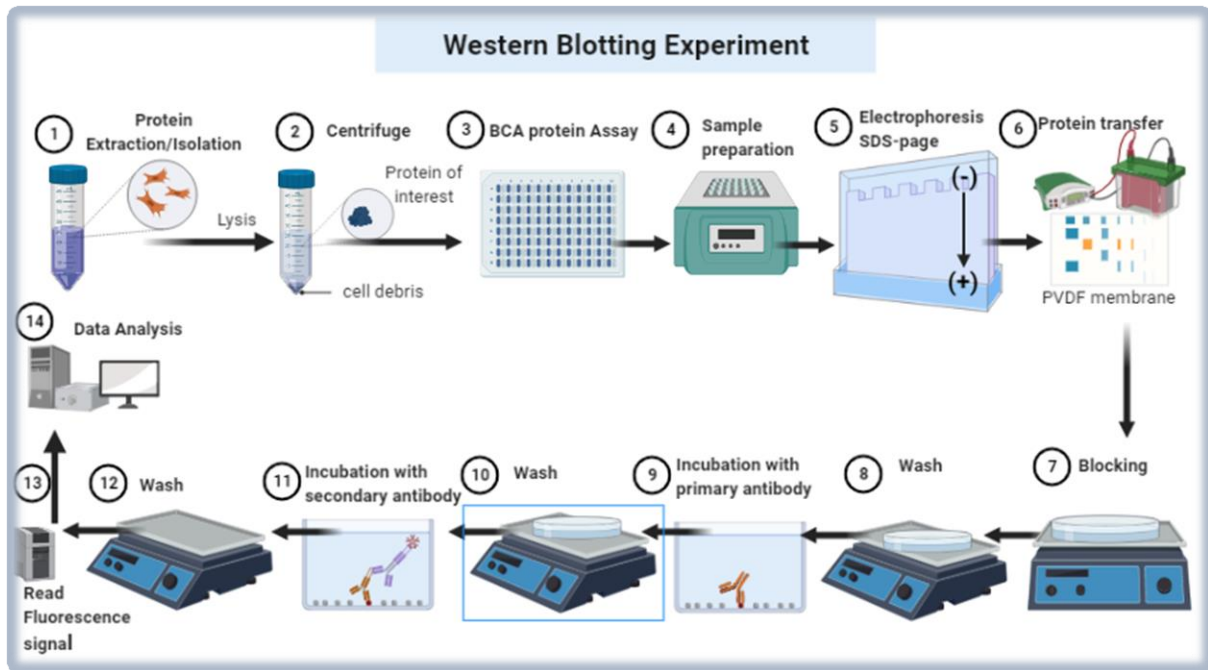


Figure 20: Diagrammatic illustration of step-by-step western blot procedure diagram made using Biorender.com.

Treating the membrane with Primary and secondary antibodies:

After the transfer the membrane was collected from the transfer apparatus and washed twice with Tris buffered saline containing Tween 20. Thereafter the membrane was blocked with blocking buffer diluted with Tween 20 (1:1) for an hour for blocking nonspecific binding proteins. The membrane was then washed three times as described previously (with Tris buffered saline containing Tween 20 (TBST)) and incubated with primary antibody (IL-8 IgG mouse) overnight at four-degree Celsius temperature over the shaker. After overnight incubation with primary antibody the membrane was washed again three times as above and incubated with secondary antibody (IRDye® 800 CW Goat anti-mouse IgG secondary antibody) for an hour at

room temperature. The membrane was then washed again twice and viewed using LICOR Odyssey. The molecular weight of the protein bands was analysed using Precision Plus Protein™ Dual Color Standards (Bio-Rad) and the density of proteins analysed by Image J (National Institutes of Health, USA) and normalised to β actin (Sigma-Aldrich).

2.2.12 Statistical analysis

Numerical data are presented as means, standard deviations and 95% confidence intervals. Statistical analyses were performed using Graph Pad Prism software (Version 9). Normality tests including D'Agostino & Pearson test and Shapiro-Wilk test were used to assess the distribution of the data. For normally distributed data, independent student's *t*-test and one-way or two-way analysis of variance (ANOVA) with Bonferroni correction were used to compare the means of two groups or more than two groups, respectively. The Kruskal-Wallis test with post hoc analysis was used to compare the mean rank of more than two groups of data not following normal distribution. The differences between the groups were considered significant when the *P*-value was less than 0.05.

The data were reported as mean \pm standard deviation and all the graphs were plotted using Microsoft Excel 2013. ANOVA single factor was applied for determining the statistical significance between the two groups at $p < 0.01$ and 0.001 ($n = 3$). All the experiments were performed at least in triplicate with three different samples.

Chapter 3. Results

This in-vitro project aimed to study the ability of cannabinoids to alter biomarker production, involved in inflammation and periodontal tissue regeneration.

This study evaluates the following:

- The cytotoxicity of inflammatory agents, flagellin and LPS, and cannabinoids.
- Expression of inflammatory biomarkers in the presence and absence of flagellin/LPS, these were evaluated for the effects of cannabinoids on the expression of inflammatory markers.

The protocols mentioned in Chapter 2 relate to the final distillation of various preliminary optimisation experiments. Cell lines and primary cells were both used to show the anti-inflammatory effects of cannabinoids on periodontal diseases. The preliminary experiments were performed using human cell lines including G292, gingival keratinocytes cell line (TIGK), and A-2780. The final experiments involve the use of primary periodontal cells.

3.1 Justification of cell used in the study.

The following paragraphs detail the reasons for the choice of these cell lines.

3.1.1 G292

Loss of bone mass is a distinctive feature of chronic periodontal diseases. Previous studies inform us that cannabinoid receptor agonist reduces the rate of bone degradation (Lozano-Ondoua et al., 2010). Therefore, G292, an osteosarcoma cell line, was found to be suitable and used for the preliminary cytotoxic experiments

during this study. The advantage on using G292, this is a cell line and there is no change in their behaviour on different passages.

3.1.2 TIGK-K

TIG-K cells were selected because they are gingival cell line. Also, the gingival cells are known to be involved in periodontal diseases. Importantly, these cells do not change due to ageing or undergo senescence at early passages (Lim et al., 2014). Hence, they were found suitable used during this study to identify the effect of cannabinoids on immortalized gingival keratinocytes during this study.

3.1.3 A-2780

A-2780 (ECACC catalogue no. 93112519) is an endometrium ovarian carcinoma cell line, subtype of epithelial cells tumour. It was selected in this study to identify the effects of expression of inflammation and the role of cannabinoid on the prevention of connective tissue destruction e.g., Junctional epithelium. During periodontitis, junctional epithelium recedes downwards to form periodontal pockets. This is because the protective signals are inhibited by the inflammatory cytokines during chronic periodontitis. As a result, the junctional epithelium migrates apically towards the root and the resultant pocket formation. Therefore, this extra oral (ovarian non-keratinised) epithelial cell line was suitably used to investigate the cytotoxic effect of the cannabinoids.

3.1.4 Primary periodontal cells

Primary periodontal cells were harvested from different donors to study the impact of inflammatory mediators (LPS P-gingivalis and flagellin) on expression of inflammatory

markers and the role of cannabinoids in alleviation of inflammation. Periodontal cells found to orchestrate the inflammatory events during the periodontal disease and could lead to loss of dental tissues including alveolar bone (Palioto, 2019). Also, previous research shows that the health of non-diseased periodontal cells has regeneration potential (Ripamonti, 1994). Therefore, the periodontal ligament cells from were chosen for this study to investigate the anti-inflammatory properties of cannabinoids.

Development of an in vitro mode to create inflammatory periodontal conditions:

Having made the decision on the choice of cells to study the effects of cannabinoids on inflammatory periodontal conditions, an in-vitro periodontitis model was selected. Here, cell lines and primary cells were presented with inflammatory agents such as LPS and Flagellin, to create a periodontitis model. Since we have recruited LPS and Flagellin to instigate the inflammation by cell to mimic the inflammatory condition, it is therefore vital to explore the toxicity of inflammatory agents. Cannabinoids were chosen to study their anti-inflammatory effects. Therefore, it is important to understand if the cannabinoids are toxic to these cells.

The following paragraphs discuss the extent of cannabinoids, LPS and Flagellin related to dosing and time.

3.2 Cytotoxicity effect of flagellin/LPS/cannabinoids

One of the aims of investigating cytotoxicity is to establish the optimal amount of concentration LPS, Flagellin which would cause inflammatory markers to induce periodontal-like conditions. For example, if an inadequate amount of LPS/ Flagellin is dosed on periodontal cells, it might not mimic the expression of actual periodontal

condition. Similarly, over-dosing the cells with LPS/Flagellin might result in a decrease in cellular efficacy to express the inflammatory biomarker. Likewise, we want to determine the optimal concentration of cannabinoids on periodontal cells to understand the anti-inflammatory properties to address destructive periodontal inflammation. Therefore, in-vitro periodontitis environment during cell culture helps to study normal cell growth, its replication and ideal morphological characteristics when exposed to pharmacological agents.

3.2.1 Measurements of cytotoxicity

Cytotoxicity can be measured using a number of different methods, including qualitative and quantitative methods. Identification of cell viability using vital dyes and measuring the ATP content are the commonly used methods. Alamar blue is a vital dye indicator of redox reaction where resazurin is reduced due to cell metabolism. On reduction of the amount of blue resazurin on the cells, the colour changes to pink. The pink gradually turns colourless, indicating depletion of oxygen in the growth medium (Twigg1945). Alamar blue is a quantitative way of measuring cell viability.

In this study cytotoxicity was tested on three cell lines including TIGK, A2780 and G292 and primary cells. These are different cell types i.e., 1) epithelial cell of oral origin, 2) epithelial cells of extra-oral origin 3) connective tissue cells and 4) primary cells.

Qualitative testing includes the identification of morphological characteristics in the absence or presence of pro-inflammatory and anti-inflammatory reagents such as flagellin and cannabinoids, respectively. Cell lines grew and maintained their morphology when cultured with the inflammatory agents and different cannabinoid concentrations used during the experiments. Quantitatively, Alamar blue method was

used to assess the cell viability and toxicity of compounds on the cells. This quantitative study was done by growing the cells with compounds and by assessing the post-exposure viability of the cultured cells.

3.2.1.1 Cytotoxicity assays in G-292:

3.2.1.1.1 Qualitative analysis (G-292)

The results of the cytotoxicity test on G- 292 are shown in figure 21 and figure 22. In the negative control group, under the normal culture condition, G-292 showed a confluent growth pattern and exhibited a fibroblastic spindle-shape morphology when cultured 4 Hours and 24 Hours (Fig 21A, 21B). On the contrary, the culture of G292 cell line in direct contact with the cytotoxic positive control (1%Triton x100) resulted in cell lysis and death as evidenced by the disappearance of almost all the cells from the well, with only a few alive exhibiting round morphology (Fig 21C). When the G-292 were cultured in the presence of cannabinoid, they maintained their morphology. Overall, the results of the cytotoxicity indicated no apparent cytotoxicity at selected concentration of cannabinoids at 1 μ M (Fig 21A) and 10 μ M (Fig 21B) at 24 Hours. Therefore, cannabinoids at lower concentrations (\leq 10 μ M) shown that they did not induce cytotoxicity of G-292 cells.

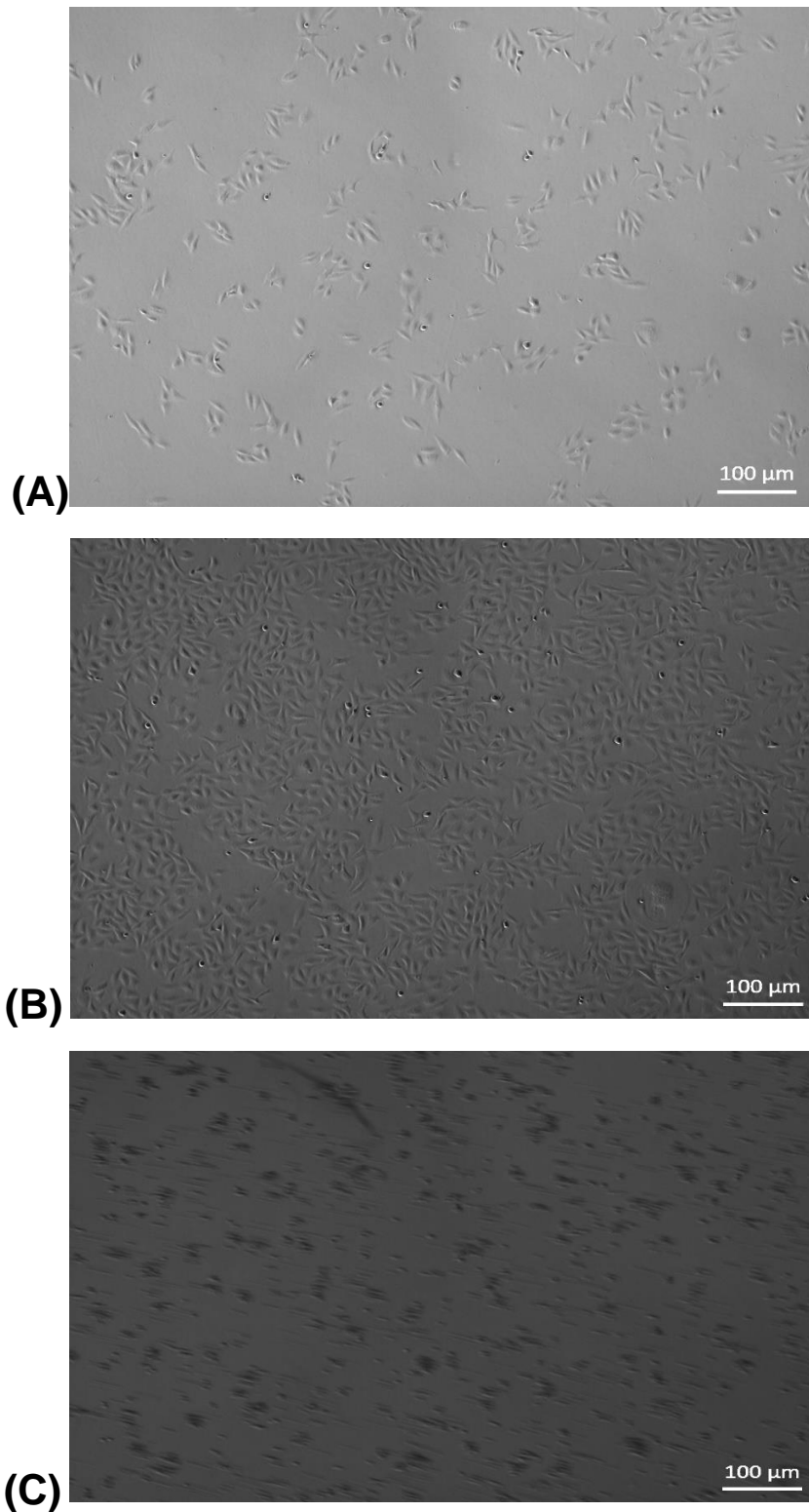


Figure 21: Appearance of monolayer cultured G292 cells (morphology). (A) 4 Hours (B) 24 Hours, cells only (25 X magnification), (C) cells with 1% triton x100 (25 X magnification).

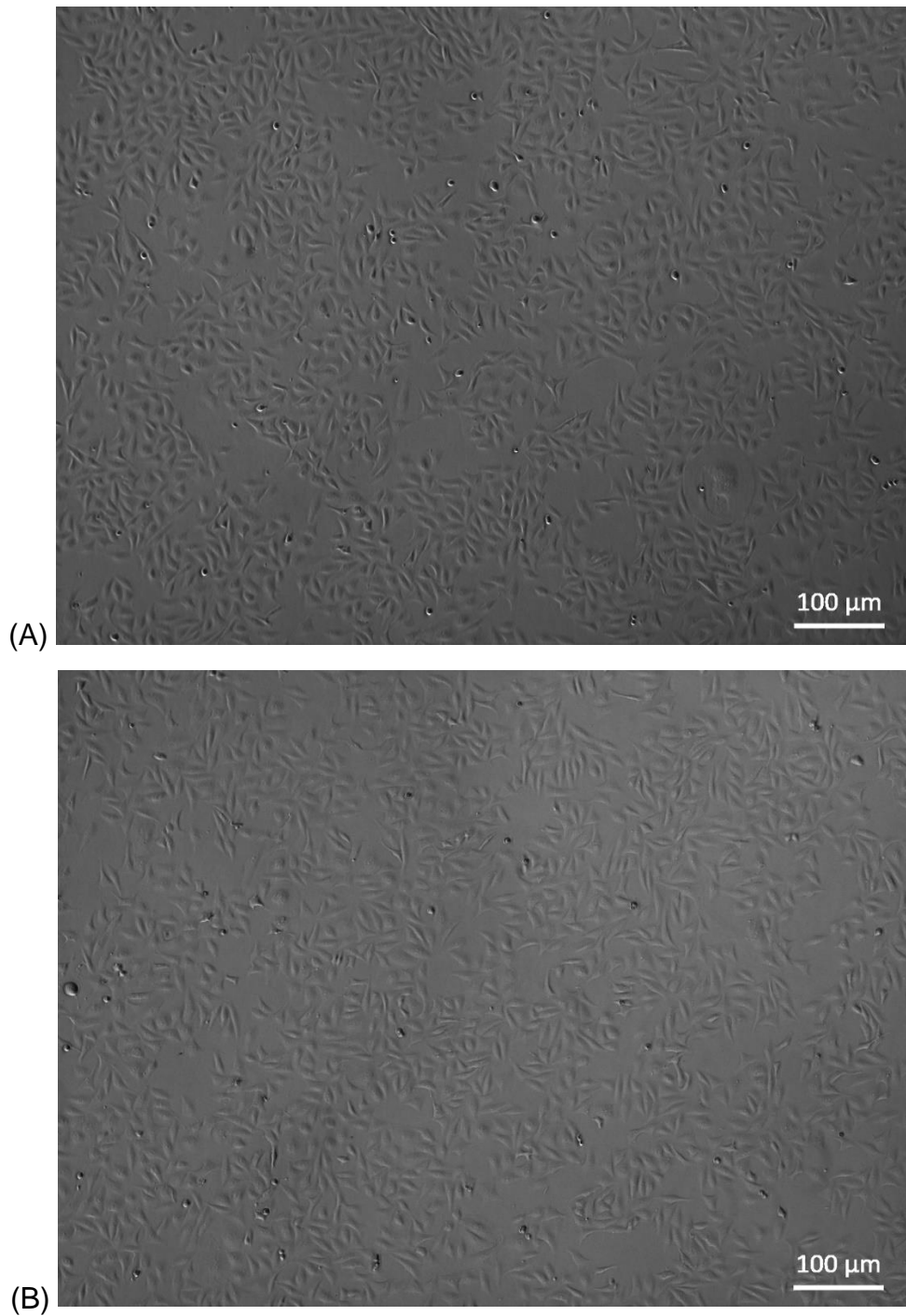


Figure 22: Images of G-292 cells following cultured with high glucose DMEM for 24 Hours viewed using a conventional light microscope CBD 1uM (A), CBD 10 μM (B). Representative images of the G-292 cells (A) CBD 1 μM and (B) after seeding with CBD 10uM. The images show morphology of the cells after presenting with CBD.

3.2.1.1.2 Quantitative analysis (G292)

The cytotoxicity of Cannabinoids (CBD and CBG), LPS and flagellin were quantitatively evaluated in in G292 cell line. The results are presented in the following paragraphs:

3.2.1.1.2.1 Cytotoxicity of LPS and flagellin

The cytotoxicity test in G292 cell line when cultured on its own with growth media i.e., negative control, and in the presence of Triton X100 i.e., positive control, was evaluated for the conversion of resazurin compound present in Alamar blue (cell viability indicator). The background fluorescence of the media itself was subtracted, and the fluorescence observed with the test conditions such as cells cultured in the presence of 1 µg/mL LPS or flagellin and two different concentrations of cannabinoids. Cells were exposed to LPS and Flagellin for 3 hours (Figure 10) and 24 Hours (Data not shown) as per the decided exposure time for the experiments. There was no cytotoxicity associated with the use of LPS and Flagellin when tested 1 µg/mL in the cells (Figure 23).

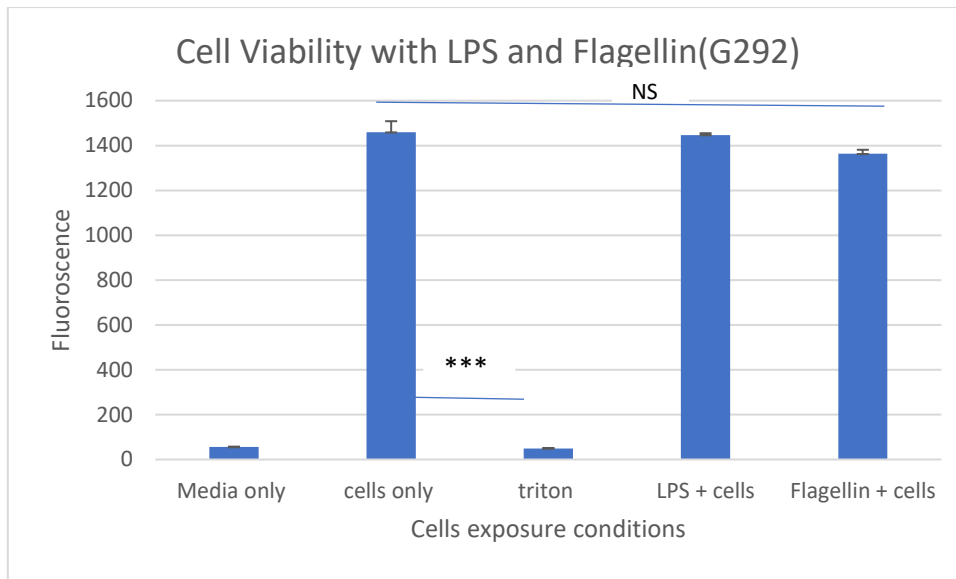


Figure 23:. The effect of 1 μg LPS and 1 μg flagellin on cell viability. Cells were exposed to LPS and Flagellin for 3 hours. Viability was assessed on G292 cells using Alamar-Blue assay. (***) significance scale used $p > 0.01$, $n=3$. Error bars: represent standard deviation ($n=3$). NS: no significance

3.2.1.1.2.2 Cytotoxicity of CBD

Treatment of the G292 cells with CBD (1 μM -100 μM) induced significant ($p < 0.001$) cytotoxicity only at concentration of 50 μM and higher, over 24 h exposure time (Figure 24).

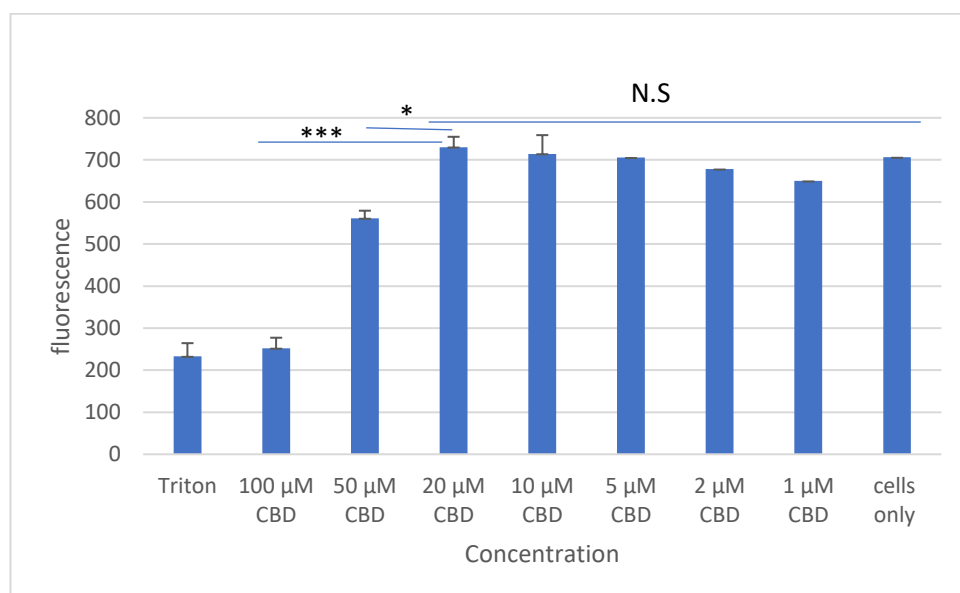


Figure 24: Evaluation of the cytotoxicity of CBD on G292 cells. G292 cells were incubated with different concentrations of cannabidiol (CBD) for 24 hours, then the cell viability was evaluated using Alamar-Blue. Negative control (culture medium only). Positive control (culture medium with triton 1%). Error bars = represent the standard deviation. (***) significance scale used $p < 0.001$, $n = 3$. NS: no significance

3.2.1.1.2.3 Cytotoxicity of CBG

Treatment with different concentrations of CBG did not induce a significant cytotoxicity in G292 cells at all concentrations tested (Figure 25) (1 μM to 100 μM).

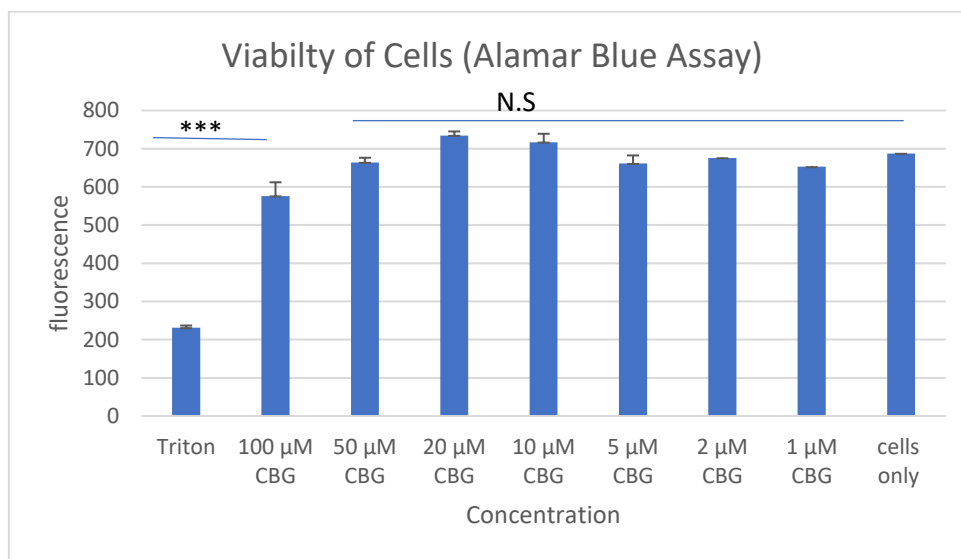


Figure 25: Evaluation of the cytotoxicity of CBG on G292 cells. G292 cells were incubated with different concentrations of cannabidiol (CBG) for 24 hours. The cell viability was evaluated using Alamar-Blue. Negative control (culture medium only). Positive control (culture medium with triton 1%). Error bars = represent the standard deviation. (***) significance scale used $p < 0.001$, $n = 3$. NS: no significance

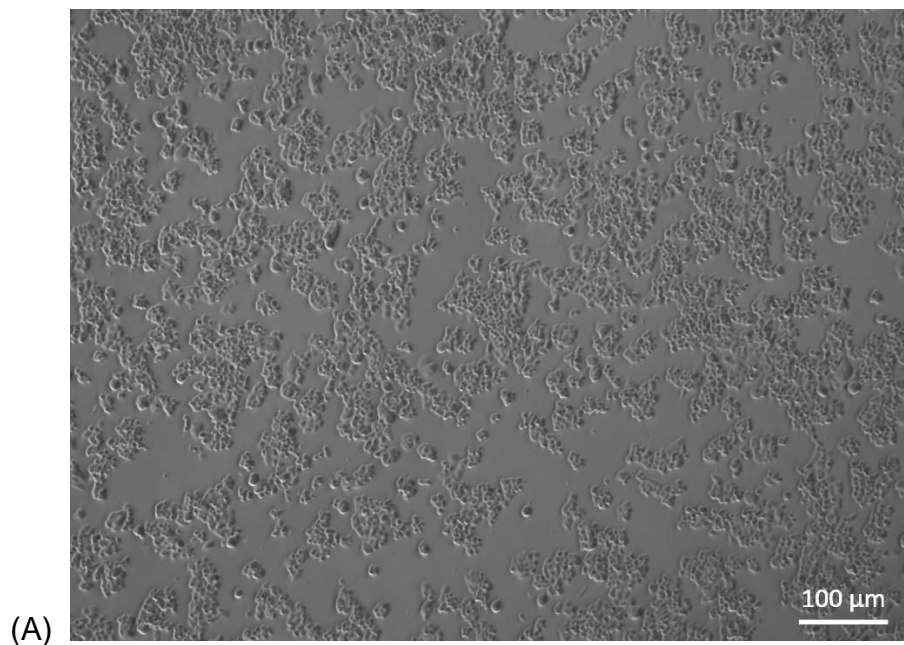
3.2.1.2 Cytotoxicity assays in A-2780 cells:

As stated earlier (section 3.3) this epithelial cell line originated from ovarian cancer and this was used to study the cytotoxic effect of cannabinoids on epithelial cells of non-oral region. The following paragraphs will illustrate the toxic effects of cannabinoids.

3.2.1.2.1 Qualitative analysis (A-2780)

A-2780 cells were cultured with and without cannabinoids, and morphology were assessed by comparing control with tests. This was verified by the existing literature on the appearance of cell morphology. A-2780 cells present round morphology and grow in clusters figure 28D and 28E (Haslehurst et al., 2012).

A-2780 cell line appeared to maintain their morphology, appeared to be round and clustered when they were grown in the presence of various concentrations of cannabinoids (only concentration 10 μm is shown, fig 28B).



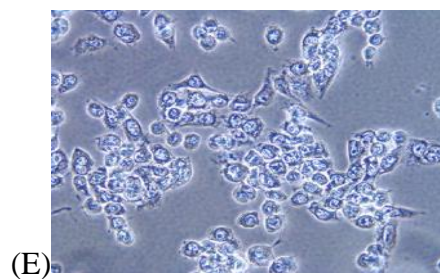
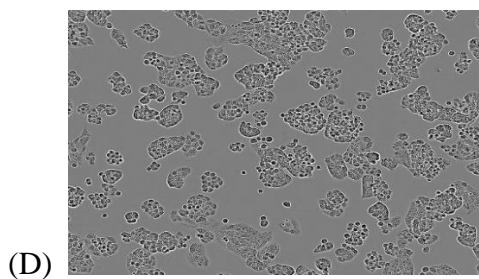
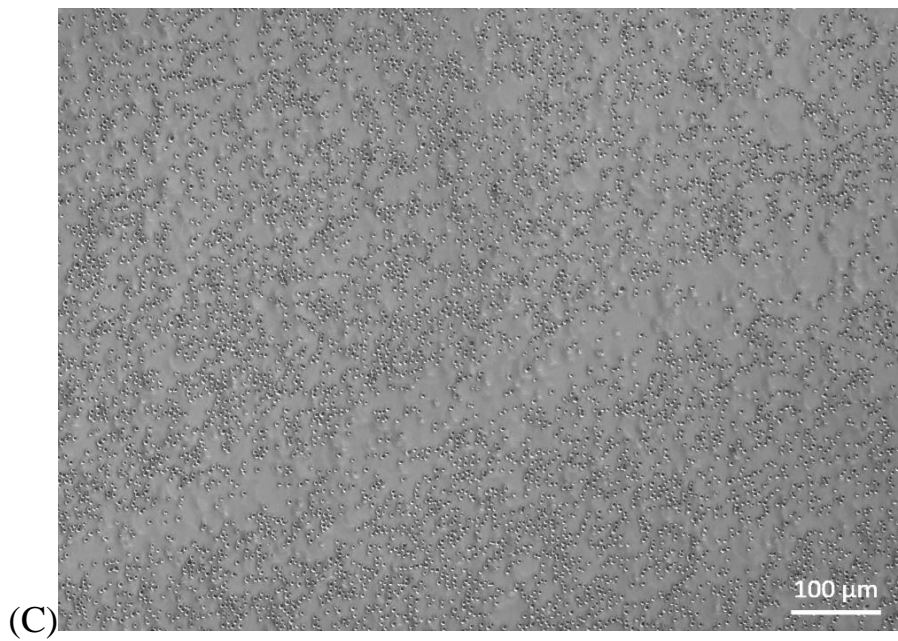
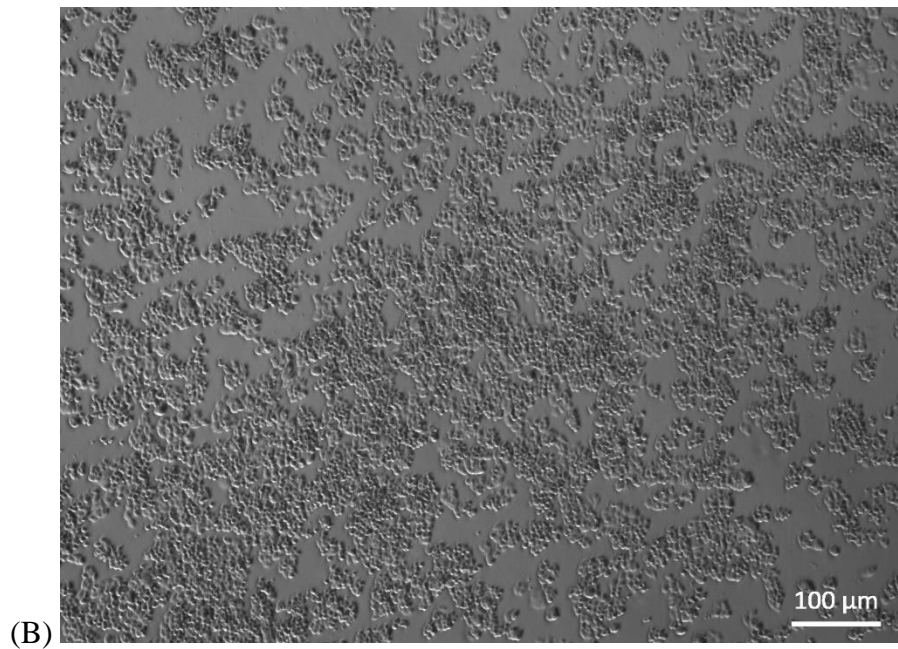


Figure 26: Images of A-2780 cells following cultured with high glucose DMEM for three days viewed using a phase contrast microscope (control (A), CBD 10 μ M (B), 1% Triton(C)) and compared with previous literature(D), (E)(Haslehurst et al., 2012)) (A-2780 PTX 64 resistance cell line, Ximbio).

Representative images of the A-2780 cells (A) before and (B) after seeding with CBD 10uM. (C) positive control (cells treated with Triton X-100).

3.2.1.2.2 Quantitative analysis (A-2780)

Evaluated cytotoxicity on the cells with inflammatory agents, Flagellin and LPS, and cannabinoids.

3.2.1.2.2.1 Cytotoxicity of LPS

Treatment of the cells with 1µg/mL LPS did not show toxicity as compared to the control cells (Figure 27).

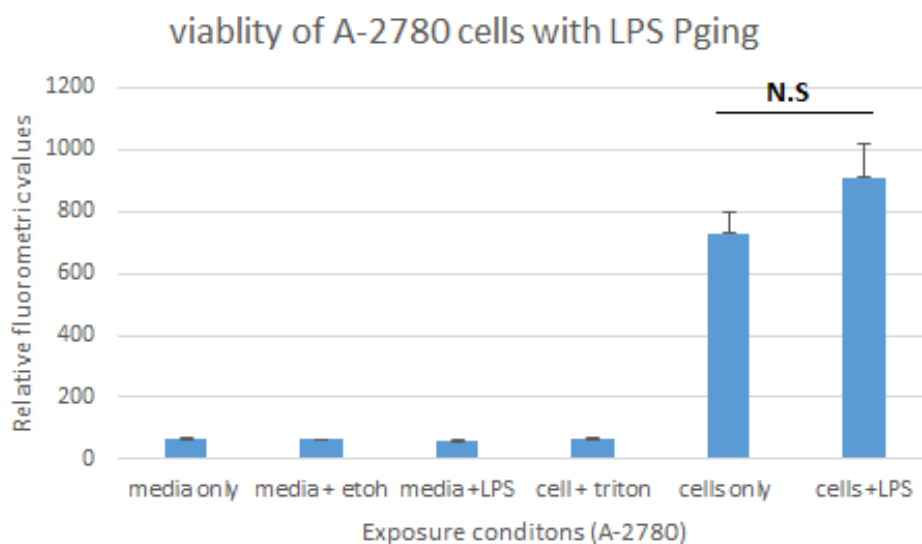


Figure 27: The effect of LPS on A-2780 cell viability at 24 hours. Viability was assessed on A-2780 cells using Alamar-Blue assay. ($p > 0.01$, $n = 3$), Error bars: represent standard deviation. NS: no significance.

3.2.1.2.2.2 Cytotoxicity of Flagellin

No cytotoxicity was observed in A-2780 cells when treated with 1µg/mL Flagellin, as compared to control cells (Figure 30).

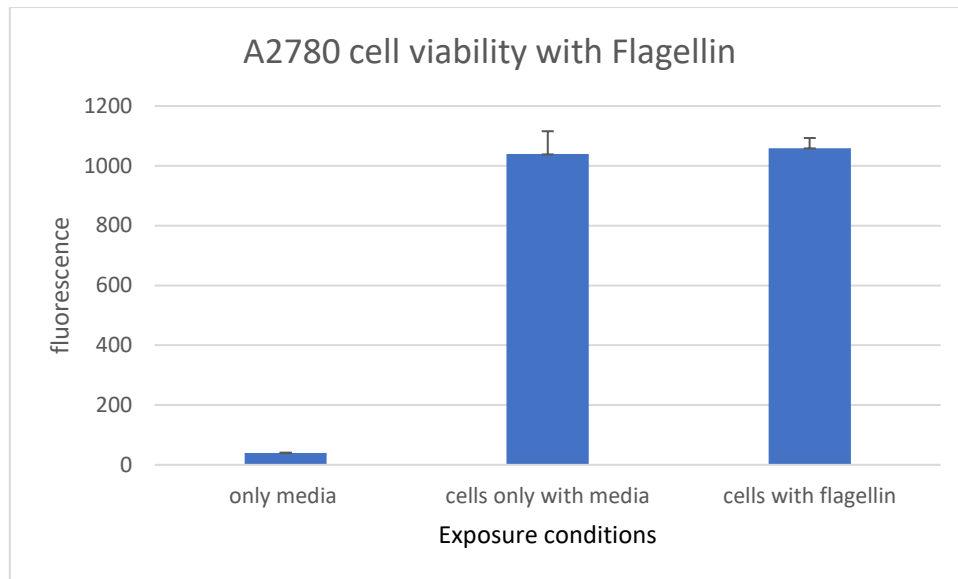


Figure 28: The effect of Flagellin on cell viability at 24 hours. Viability was assessed on A-2780 cells using Alamar-Blue assay. ($p > 0.01$, $n = 3$). Error bars: represent standard deviation ($n = 3$). NS: no significance.

Periodontal cells when treated with $1 \mu\text{g/ml}$ concentration of lipopolysaccharide did not show any cytotoxicity. Also, the cells were evaluated post exposure of LPS, no toxicity was observed (Results not shown).

3.2.1.2.2.3 Cytotoxicity of CBD

Treatment with CBD induced cytotoxicity in A-2780 cell line only at concentrations higher than $20 \mu\text{M}$ (Figure 29). CBD at concentration of $50 \mu\text{M}$ CBD induced 50% cytotoxicity as compared to control cells. However, when $100 \mu\text{M}$ of CBD was added to the A-2780 cell line, the cytotoxicity was equivalent to the positive control (almost 100% cytotoxicity).

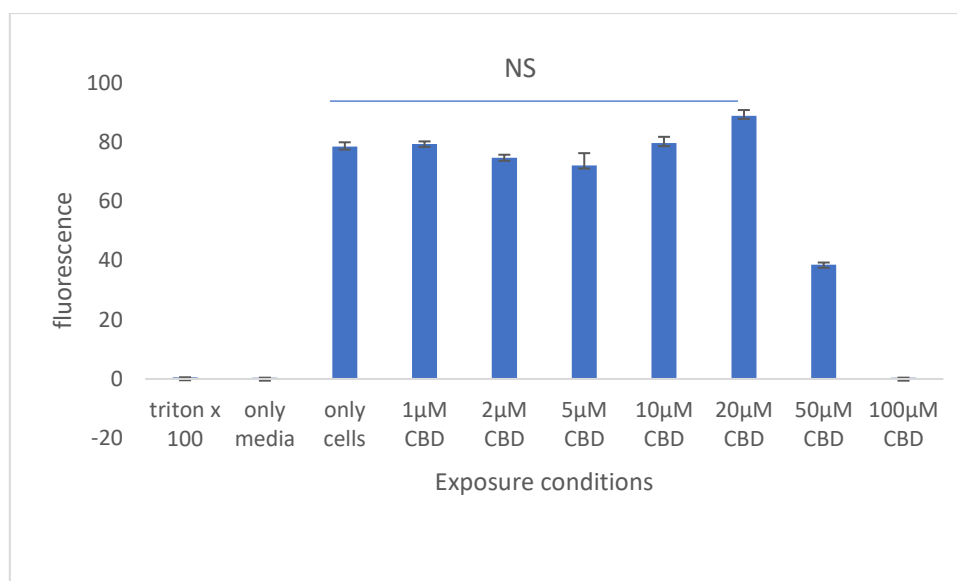


Figure 29: Alamar blue fluorescence ($p < 0.01$, $n = 3$). Error bars: represent standard deviation ($n = 3$). NS: no significance. The effect of different concentration of CBD on cell viability at 3 hours, no toxicity observed at 24 hours as well (Data not shown). Viability was assessed on A-2780 cells using Alamar-Blue assay.

3.2.1.3 Cytotoxicity assays in TIGK cells

3.2.1.3.1 Qualitative analysis

TIGK cells were evaluated for their changes in size, morphology and the growth inhibition, if any, when they were cultured in the presence of inflammatory agents and cannabinoids. The results presented in qualitative properties of TIGK are based on the size and shape of TIGK cells (Figure 30A, cells without CBD). The light microscopy analysis of TIGK cells cultured in the presence of CBD at concentrations 1 μM to 10 μM (only CBD at concentration 10 μM is presented (Figure 30B)) showed no changes on cell morphology compared to the negative control; however, the 100 μM concentration affected cell morphology, as cell were rounded shape instead of cobblestone appearance as in the negative control group Figure 30C (the image of CBD at concentration 100 μM is not shown).

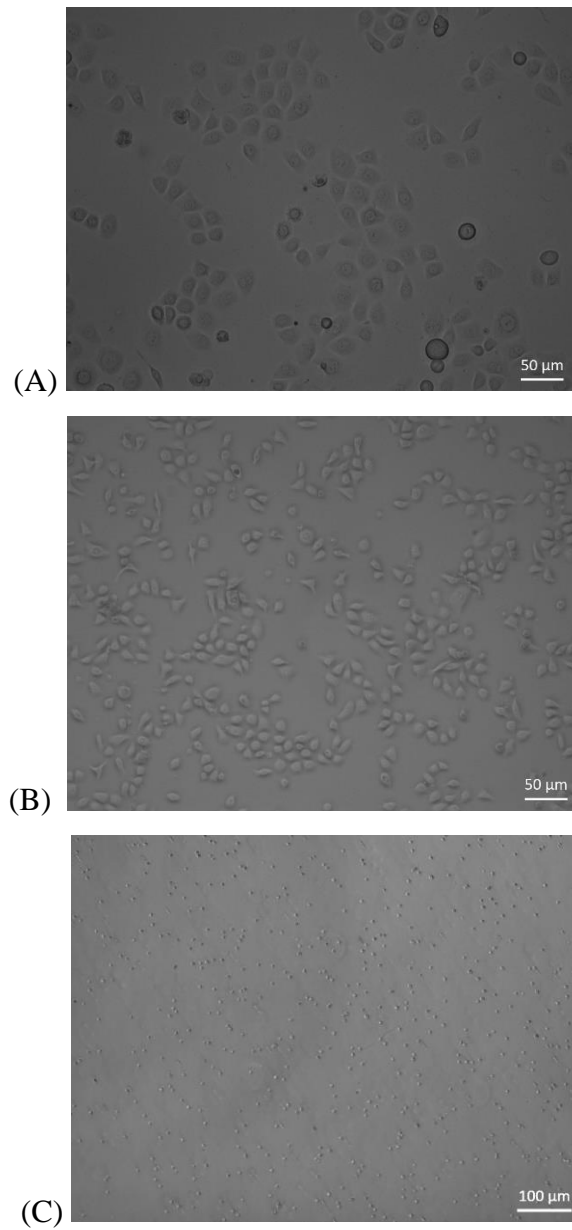


Figure 30: Images of TIG-K cells following cultured with high glucose DMEM for three days viewed using a contrast phase light microscope (negative control (A), CBD 10 μ M (B)) and positive control (C). Representative images of the TIG-K cells (A) before and (B) after seeding with CBD 10 μ M. The images show morphology of the cells before and after presenting with CBD. (C) Scale bars A , B 50 μ m and C 100 μ m.

To further monitor the effect of CBD on cells morphology, a higher resolution technique was included using confocal microscopy. The results presented in Figure 31A and Figure 31B showed that cannabidiol at 10 μ M seemed to alter the size of TIGK cells (Figure 31B). This is in line with previous studies where the CBD have shown to affect the size of glioma cell due to the formation of intracellular vesicles in the presence of 7.5 μ M CBD (Gross, 2021). Additionally, cannabidiol is reported to induce apoptotic changes and perturb the mitochondrial cells which might have an impact on actin arrangement within the cells. (Hohmann, 2019).

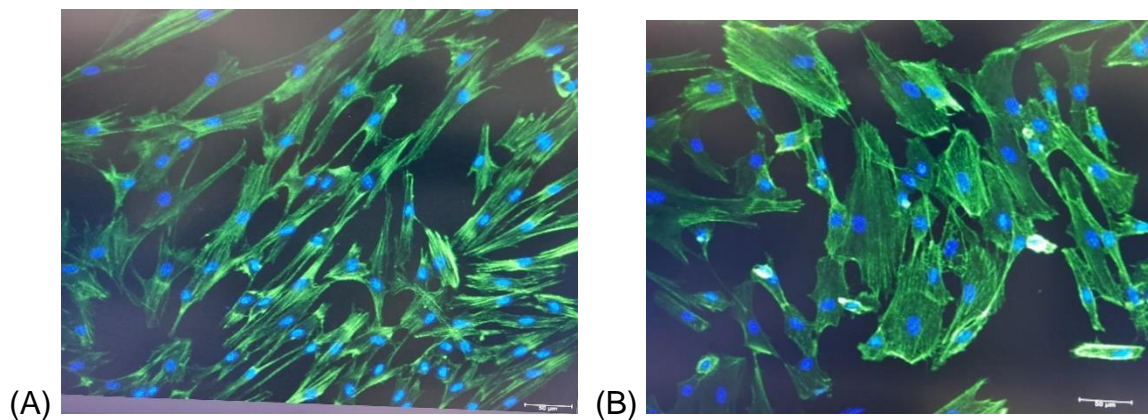


Figure 31: Morphology of TIG-K cells cultured without 10 μ M CBD (A) under confocal microscope after staining them with DAPI and Alexa-fluor dye showing surface attached spreading well over the surface grown, cone focal image showing oval shaped nucleus with cytoskeleton spreading overgrowth surface cells cultured with 10 μ M CBD (B). Magnification 20X (n=3).

3.2.1.3.2 Quantitative analysis

The cytotoxicity of LPS, Flagellin and different concentrations of cannabinoids CBD and CBG was further assessed on TIGK cells using Alamar-Blue assay. The results are presented in section 3.1.1 and section 3.1.2

3.2.1.3.2.1 Cytotoxicity of LPS and flagellin:

The cytotoxicity of 1µg/ml LPS (Figure 32) and 1µg/ml flagellin (results not shown) on TIG-K cells was evaluated after 24 hours of the treatment using Alamar-Blue assay. The result, presented in Figure 32, showed that LPS at 1µg/ml did not influence the viability of TIG-K cells. Flagellin at concentration of 1µg/ml also did not affect the TIGK cell viability.

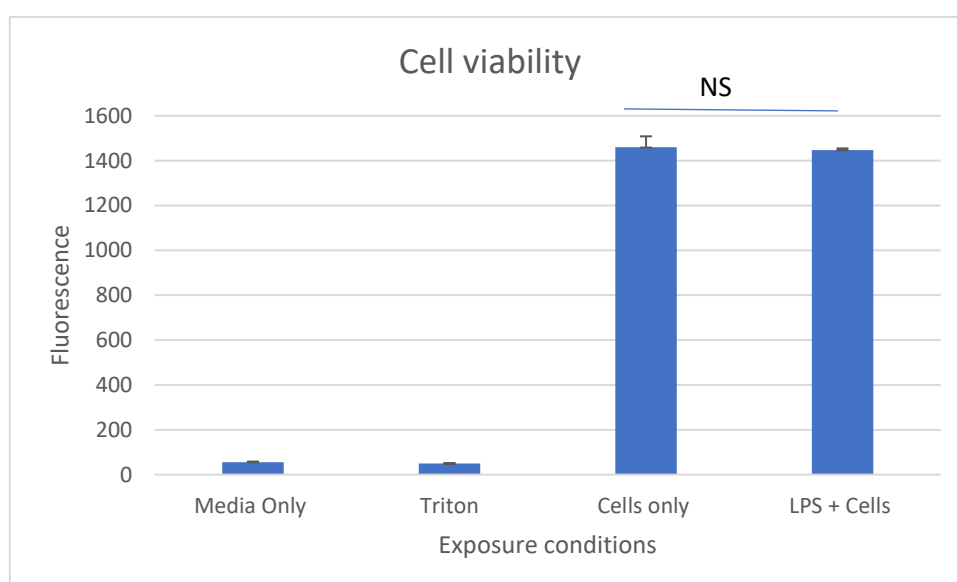


Figure 32: The effect of LPS on cell viability. Viability was assessed on TIGK cells (24 Hours) using Alamar-Blue assay. Significance scale used $p > 0.01$, $n = 3$. Error bars: represent standard deviation ($n = 3$). NS: no significance.

3.2.1.3.2.2 Cytotoxicity of CBD

The cytotoxicity of different concentrations of CBD was assessed on TIG-K cells after 24 hours of treatment using Alamar-Blue assay. Cell viability was investigated at the following concentrations of CBD: 100µM, 10 µM, 1 µM, 100nM, 10nM and 1nM. The results, presented in Figure 33, showed a significant decrease in cell viability at 100 µM concentration by around 95% compared to control. However, no difference was observed at concentrations tested including 10 µM, 1 µM, 100nM, 10nM and 1nM.

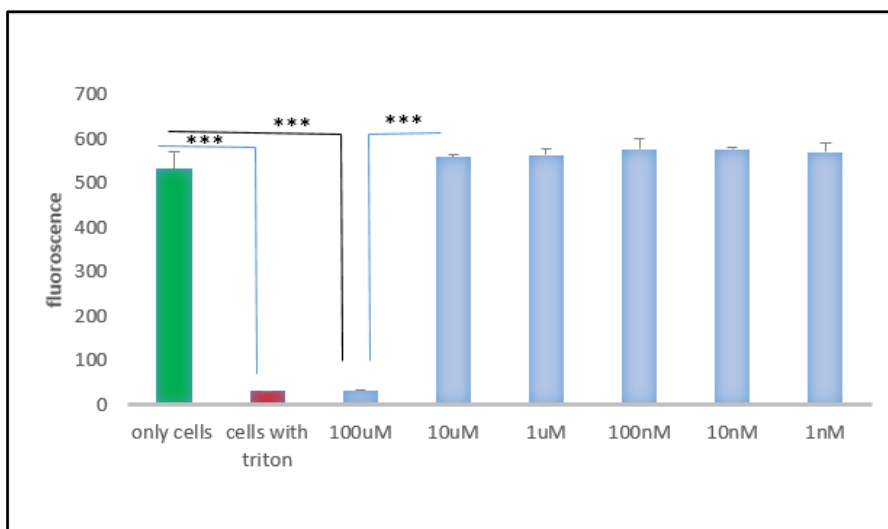


Figure 33: Evaluation of the cytotoxicity of CBD on TIG-K cells. TIG-K cells were incubated with different concentrations of cannabidiol (CBD) for 24 hours and the cell viability was evaluated using Alamar-Blue. Negative control (culture medium only). Positive control (culture medium with triton 1%). Error bars: represent the standard deviation. NS: no significance, (***) significance scale used $p < 0.001$, $n = 3$.

3.2.1.3.2.3 Cytotoxicity of CBG

The cytotoxicity of different concentrations of CBG was assessed on TIG-K cells after 24 hours of the treatment using Alamar-Blue assay.

The effect of CBG on cell viability was investigated at the following concentrations: 100 μ M, 10 μ M, 1 μ M, 100nM, 10nM and 1nM. The results, presented in Figure 34, showed the same pattern as the results obtained with CBD. A significant decrease in cell viability at 100 μ M concentration by around 95% was seen. Compared to control, no difference was observed at concentrations 10 μ M, 1 μ M, 100nM, 10nM and 1nM.

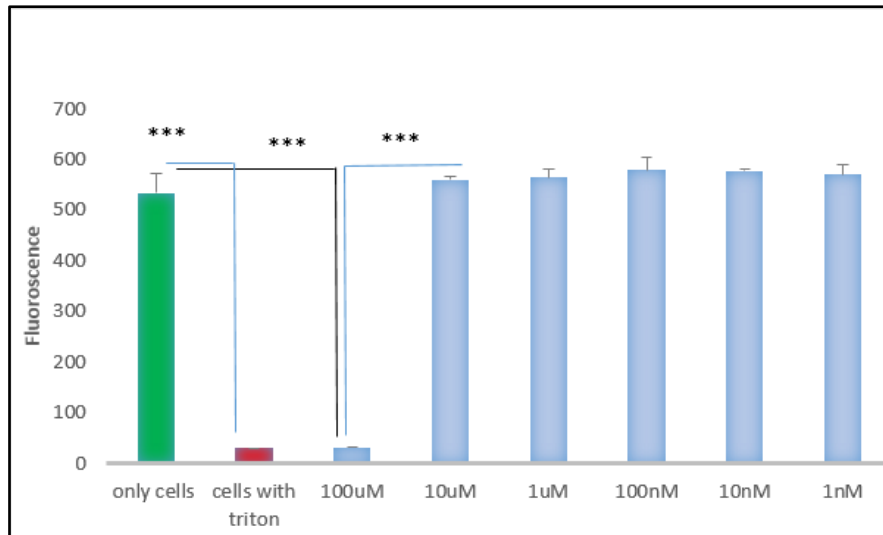


Figure 34: Evaluation of the cytotoxicity of CBG (cannabinoid receptor 1 antagonist) on TIG-K cells. TIG-K cells were incubated with different concentrations of cannabidiol (CBG) for 24 hours and the cell viability was evaluated using Alamar-Blue. Negative control (culture medium only). Positive control (culture medium with triton 1%). Error bars = represent the standard deviation. (*) significance scale used $p < 0.001$, $n = 3$.**

3.2.1.4 Primary periodontal cells

One of the cell types included in the periodontium are periodontal cells (PDLs). These were isolated from periodontal tissue harvested from freshly extracted teeth. The isolated PDLs were characterised before used in the study.

3.2.1.4.1 Periodontal cell harvesting

The periodontal tissue explants processed as mentioned previously in Chapter 2 section 2.2.1.3.1.4.2. The tissue explants provided an outgrowth of periodontal cells Figure 35 within 3-7 days from the periodontal tissue explant. When explant tissue was cultured, elongated fiber like cells were observed migrating from the explant tissue Figure 35. The growth was observed approximately from the third day of the culture from most of the donors and allowed to continue until cells were trypsinised to prevent overconfluent growth of the cells. These fibroblast cells varied in shape, ranging from spindle-shape to trapezoid-shape with a distinct nucleus (Figure 36B). The cells varied

in their behaviour as some cells remained attached to the explant whereas others migrated onto the surface of flask.

Eventually the cells formed colonies producing a storiform pattern. On observing the 80% confluency, the periodontal cells were harvested. It was either subcultured to further passages or stored for later use. Thereafter, the cells were seeded in a T-75cm² flask, and further passaged when seen at 80% confluence. Passage 4-6 were used during all the experiments.

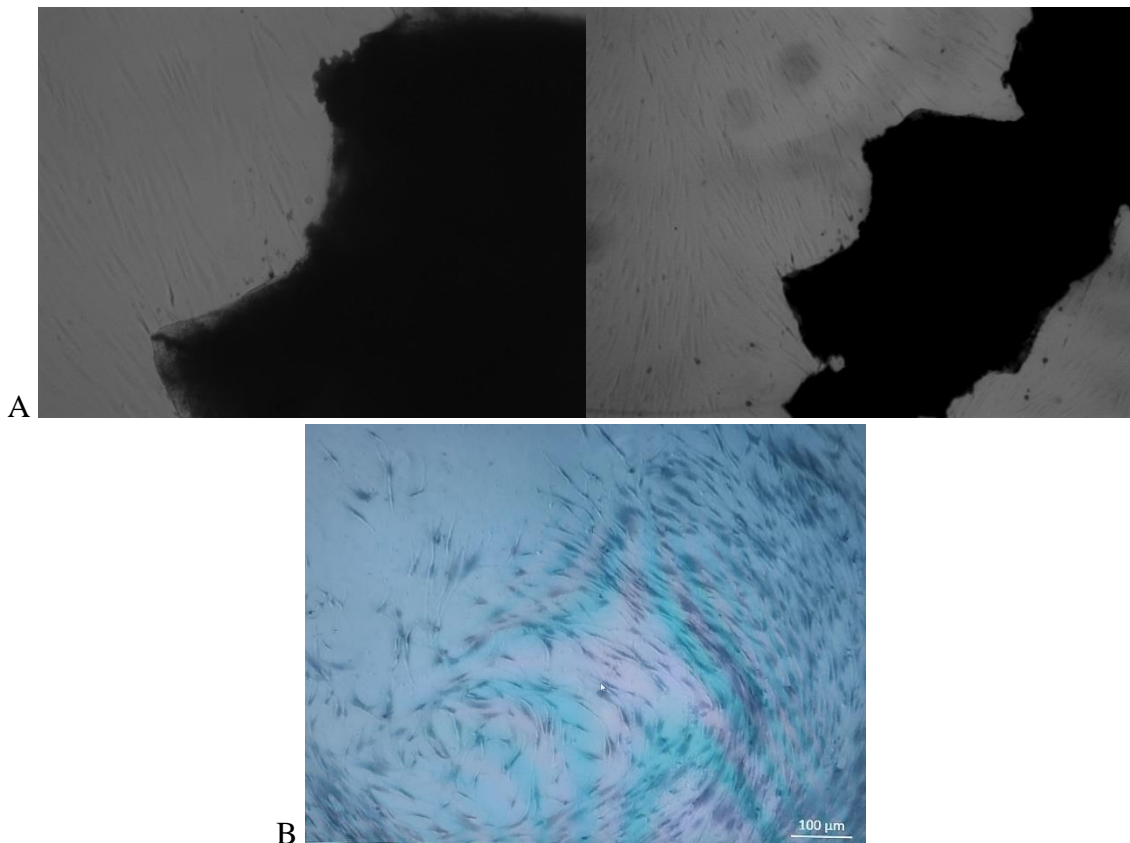


Figure 35:HPDL (Human Periodontal Ligament) cells outgrowth from tissue explant. Phase contrast micrograph showing spindle shape cells growing out of periodontal tissue implant. (Mag 30X) B Storiform pattern of the periodontal cell outgrowth.

Human periodontal cells were harvested from extracted healthy human teeth (n = 3), each one from a different donor, using an explant method as described in Chapter 2. Details of the teeth donors are described in Table 6.1. The received dental samples were all permanent healthy premolars removed for orthodontic treatment therefore the periodontal tissue scrapped from the middle third of root and made sure no involvement of either apical third or coronal third of periodontal tissue. All cell culture procedures were performed as described in chapter 2 section 2.1.2. The harvested cells were expanded for experimentation at passage 4 and characterised, as described below, by evaluating the cell attachment, proliferation, identification of cell surface markers and their potential to differentiate towards multilineage.

Table 6.1: Donors of human dental periodontal cells.

Donor	Age	Gender	Tooth condition and type
1	14	Female	Healthy lower second premolar
2	19	Female	Healthy upper first premolar
3	15	Female	Healthy upper first premolar

3.2.1.4.2 Basic characterisation of periodontal cells

The primary periodontal cells were isolated from the periodontal dental explant and these cells were characterised in order to confirm the types of cells outgrown out of the explant tissue. The periodontal fibroblasts of periodontium were found to be involved in periodontal disease and also possess regeneration potential. Therefore, the extracted cells were studied for their periodontal cell like characteristic behaviour.

3.2.1.4.2.1 Evaluation of cell adherence to the surface of the culture flasks

Cell adherence and growth were monitored using an inverted phase-contrast microscope, confocal microscope and electron microscope. The shape of images was captured using inverted phase-contrast and confocal microscope (Figure 36 A and B). Cell population growth and their division is an important factor to be witnessed during cell culture. The periodontal cells growth was observed during their passages and cells maintained their original fibrous shape during the growth, correlating the morphology of the cells originated from primary culture from the periodontal tissue explant Figure 35. The primary periodontal monolayer adherent cells attach, spread and grow during cell culture Figure 40 and were seen for their growth in cell culture flask, multiwell (12 wells) plates and on a coverslip seen using scanning electron microscope Figure 36C.

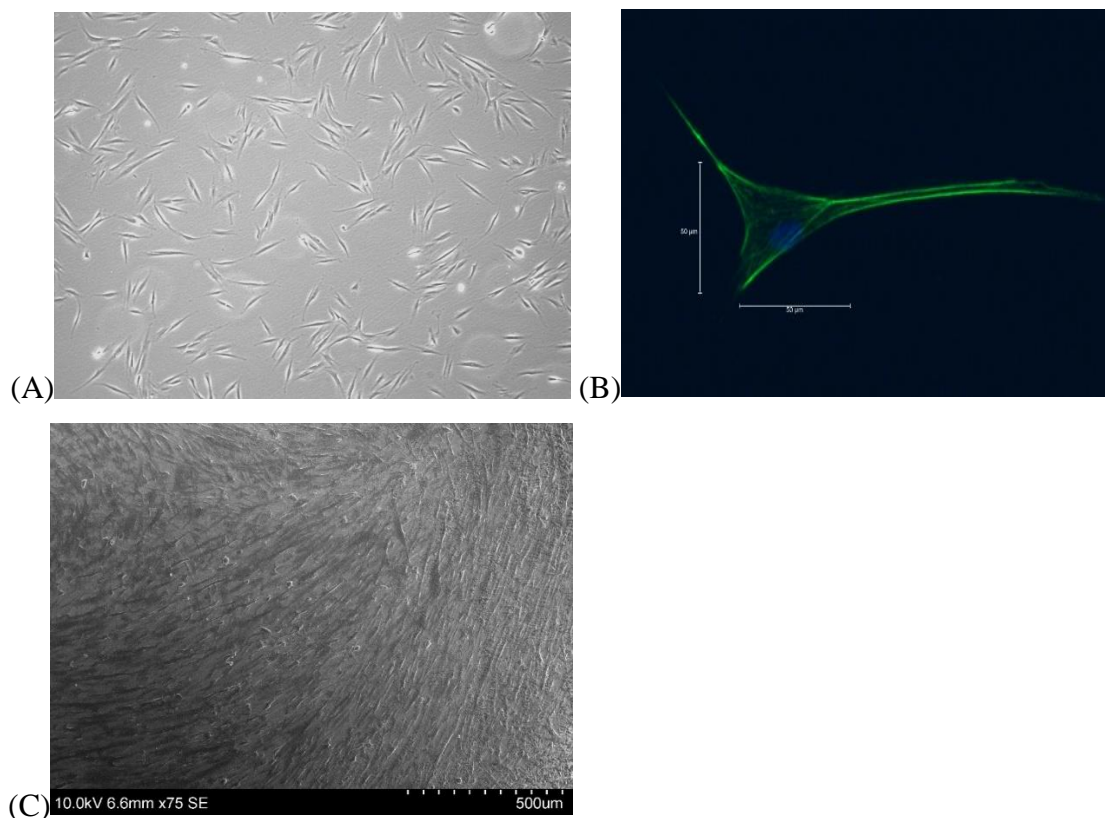


Figure 36: Periodontal cells growing on surface 25 X magnification (A), single cell appearance on cone focal scanning microscope with stained nucleus (DAPI) and cytoskeleton (Alexa fluor) (B). Scanning electron microscope analysis showed the periodontal adhesion capacity grown over coverslip surface (C).

3.2.1.4.2.2 Cell proliferation analysis

Cell proliferation of human periodontal cells with initial density of 20,000 cells/cm² were evaluated using Quant-iT™ PicoGreen® dsDNA assay. Briefly, periodontal cells were subjected to DNA extraction following 24, 48 and 72 hours of *in vitro* culture (n = 3 samples per time point) under basal conditions.

Calculation of the dsDNA content per sample dry weight

The fluorescence values of the test samples were used to determine the dsDNA concentrations in the samples using the standard curve as following:

$$dsDNA\ concentration\ (ng/mL) = \frac{Fluorescence\ value - 0.0334}{0.2157}$$

Periodontal cell proliferation was observed after seeding with initial density of 10,000 cells per cm square, the cell growth was observed until they reached the sub confluent level. The growth was observed using quantification of dsDNA using Pico green method (results not shown) of double stranded DNA estimation. The periodontal cells when cultured from frozen vials demonstrated a relatively slower but progressive increase in the double stranded DNA and hence the estimated growth was used in the experimental time points i.e., 3 and 24 hours.

3.2.1.4.2.3 Morphology (Fluorescent staining and confocal scanning laser microscopy).

Cell morphology and three-dimensional organisation of periodontal cells following seeding in different wells of 24-well plate was evaluated using Alexa-Fluor[®] 488 Phalloidin and DAPI using a confocal scanning laser microscope (Figure 42).

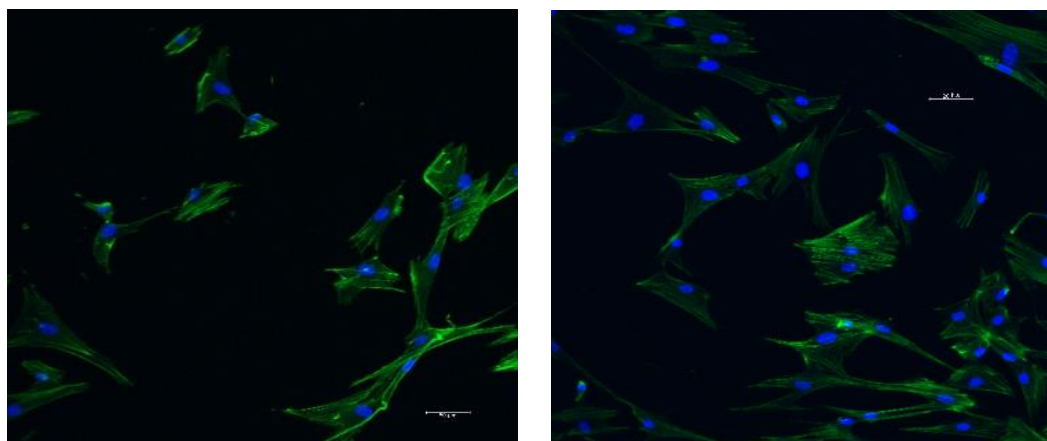


Figure 37: Periodontal cells images, stained with Alexa fluor 488 (cytoskeleton stained in green) and DAPI (Nucleus stained in blue) cultured with and without 10 μ M CBD and visualized using confocal laser scanning microscope.

Periodontal cell was treated with and without 10 μ M cannabidiol in order to observe the morphological changes due to the presence of cannabinoids, stained nucleus (DAPI) and cytoskeleton (Alexa Fluor 488) and imaged using confocal laser scanning microscope. The images show the cell morphological feature them growing as elongated fibroblastic shape cultured with and without 10 μ M CBD for 24 hours.

3.2.1.4.2.4 Evaluation of mesenchymal stem cell surface markers expression by the harvested cell populations

Human PDLCs from different donors were tested for positive expression of mesenchymal stem cell surface markers including CD105- endoglin; cell adhesion molecule, CD90 - Thy-1; cell adhesion molecule, and CD146- melanoma cell adhesion molecule. They were also tested for their negative expression of leukocyte marker, CD45.

The cells were subjected to cannabinoids as well to investigate their effect on the expression of the above markers. An example of a gating strategy is illustrated in Figure 38. A negative control gate was set where 98 percent of cell population was included for statistical relevant event used in the study. Periodontal cells were labelled with fluorochrome conjugate antibodies as negative control for the stains used.

The flowcytometry analysis of the PDLCs originated from three donors showed the positive stem cell markers for CD 105 and CD 90 and shown a negative expression for leukocyte marker CD 45. The expression for positive markers were also seen present on the cells subjected to cannabinoids treatment. The flow cytometry study was done for CBD and CBG treatments, however, only result of CBD is shown in figure 38.

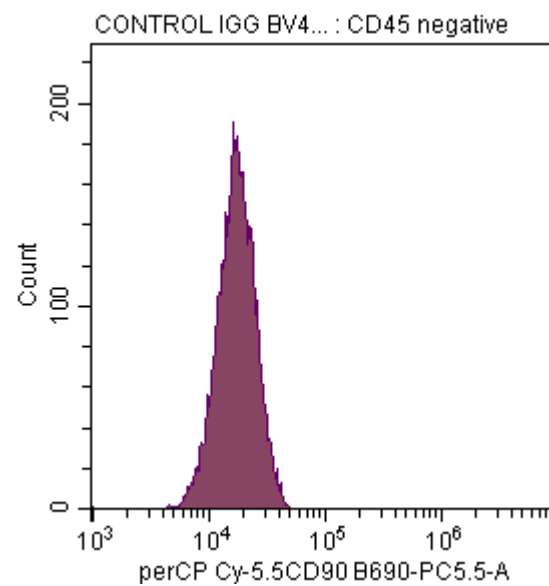
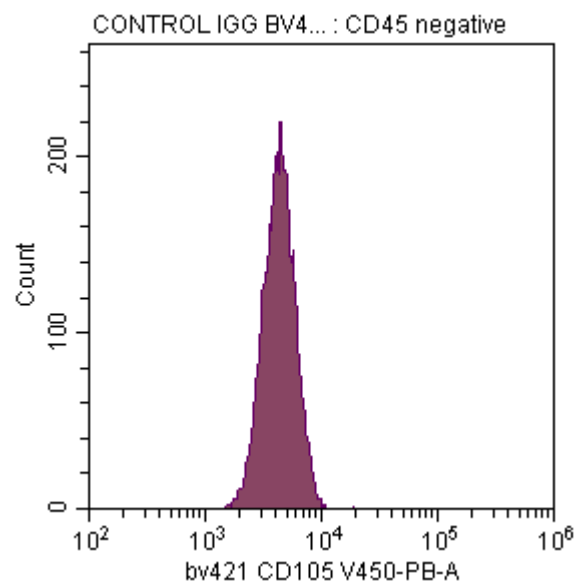
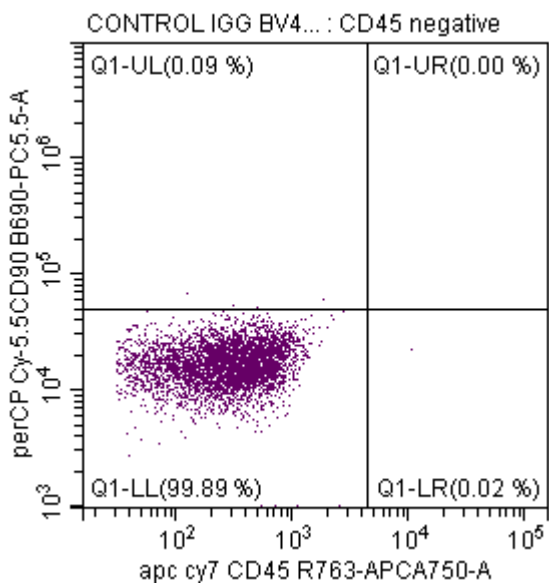
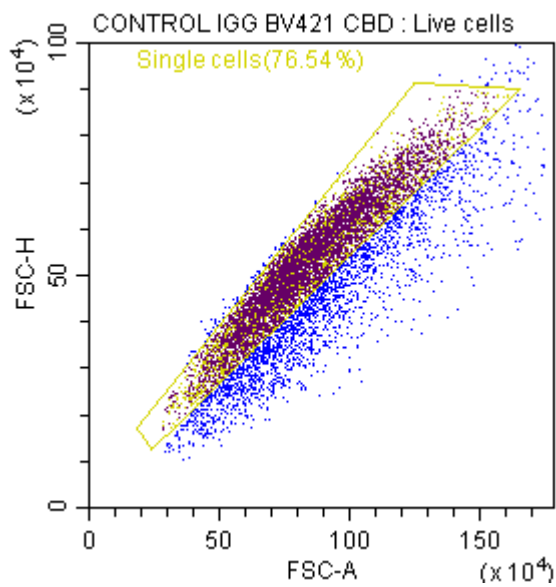
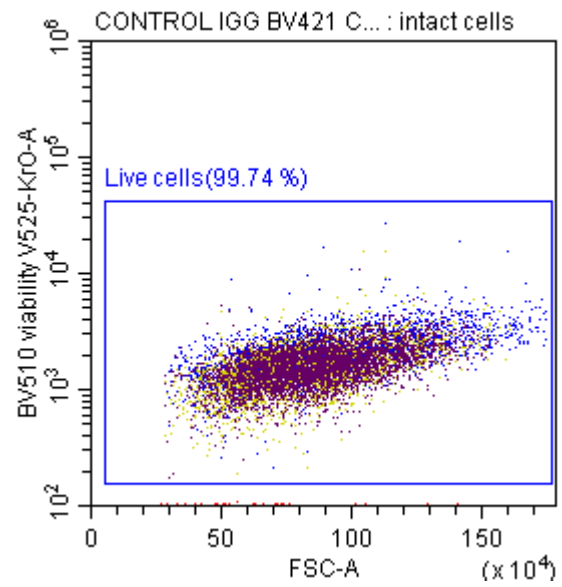
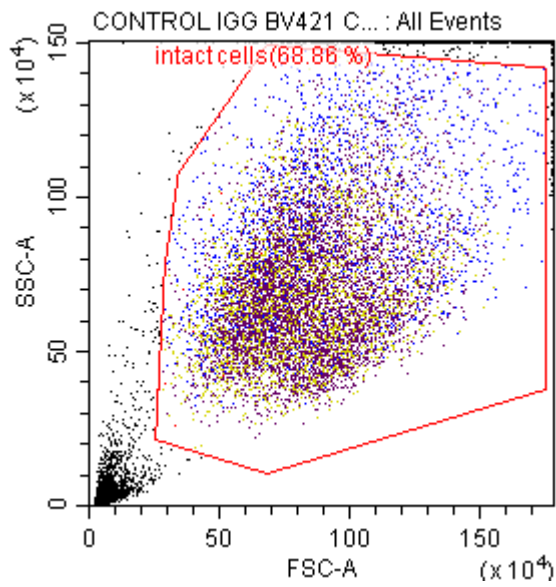


Figure 38: Cell surface marker expression on harvested primary periodontal cells. Gated population and marker histogram from 3 different representative donors. Control sample for setting gating strategy using IgG control antibodies for CD 45, CD105 and CD 90.

3.2.1.4.2.5 Evaluation of multilineage differentiation potential of the harvested cell populations

The harvested periodontal cells showed mesenchymal stem cell characteristics.

Human PDLCs from different donors were assessed for the multilineage differentiation potential. The osteogenic and chondrogenic cell differentiation potential were evaluated using Alizarin Red staining and Alcian Blue staining, respectively. The multilineage differentiation potential was observed after growing the cells for 21 days *in vitro* culture under basal osteogenic and chondrogenic conditions. Stained cultures were visualised using an inverted bright-field microscope and the images were captured digitally.

The osteogenic cell differentiation potential

The results have shown the deposition of calcium rich mineralised nodules in the cells cultured in the presence of osteogenic growth medium in all the tested culture conditions (Figure 39). Interestingly, the periodontal cell treated with both cannabinoids CBD and CBG (Figure 39 C, D, E, and F) have shown to deposit additional mineralised granules as compared to the cells not exposed to cannabinoids (Figure 39 A) and the cells which were only exposed to the inflammatory agent LPS (Figure 39 B).

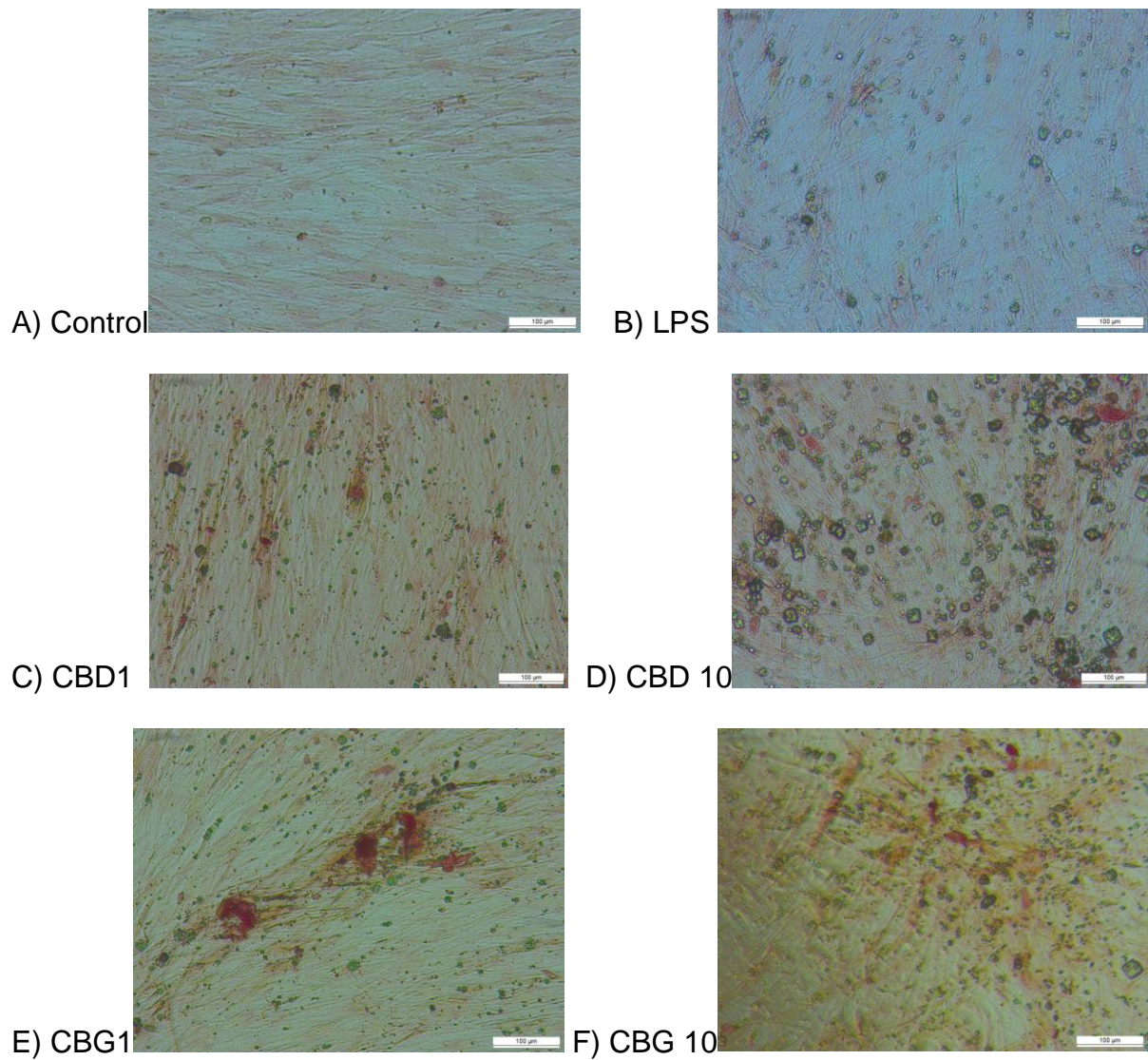
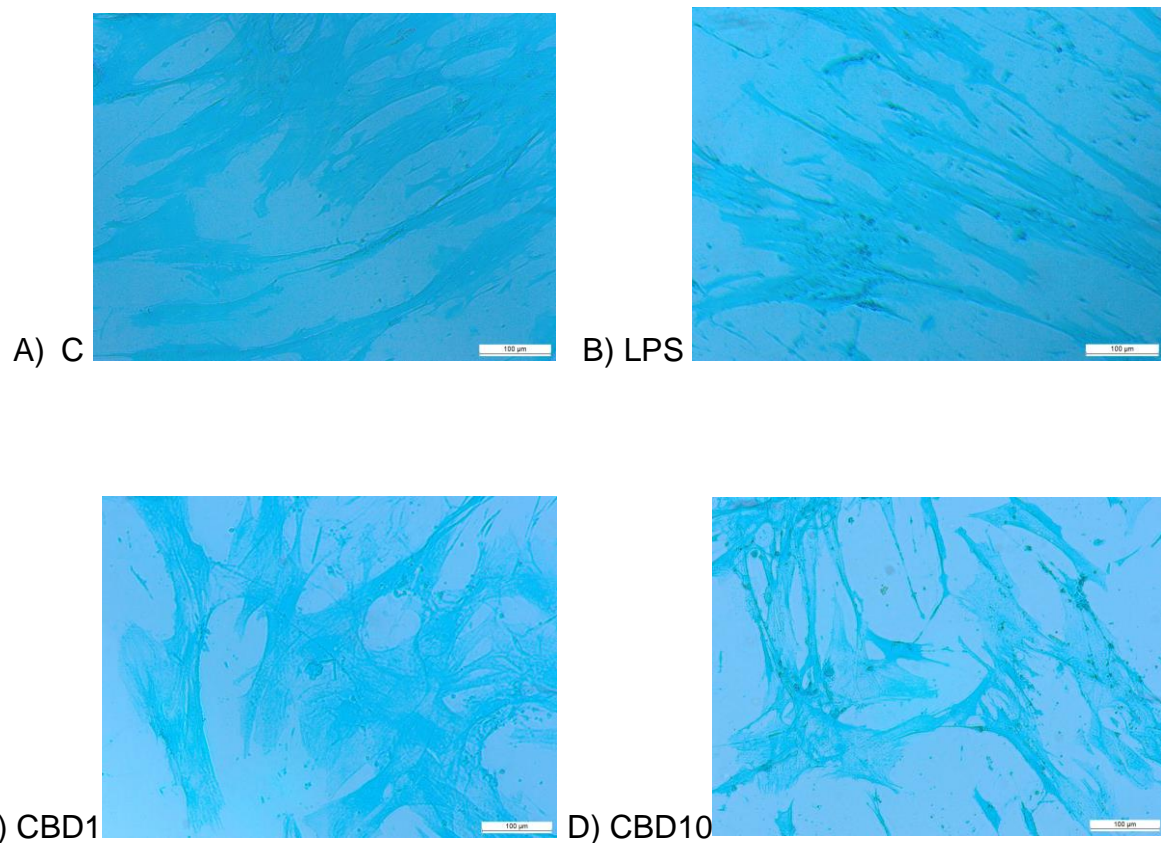


Figure 39: Bright Field microscopic image of Alizarin red staining of the periodontal cells either not or exposed to cannabinoids and flagellin following the three weeks of culture with osteogenic medium. Figure A represents the control cells only exposed to osteogenic medium, Figure B represents the cell culture with LPS for 3 hours before presenting them with osteogenic medium and Figure C, D, E, and F represents the cell pre-exposed to cannabinoids for an hour before presenting them with osteogenic medium.

The chondrogenic cell differentiation potential

PDLCs treated with chondrogenic media for 3 weeks have shown increased deposition of glycosaminoglycans and likewise the preexposure of cells with cannabinoids have shown to uptake the glycosaminoglycan stain more (Figure 40 C, D, E, and F).

The findings confirm the multilineage differentiation of the periodontal cells subjected to respective inductive environment and also it can be witnessed that the differentiation potential was enhanced due to the presence of lower concentration of cannabinoids.



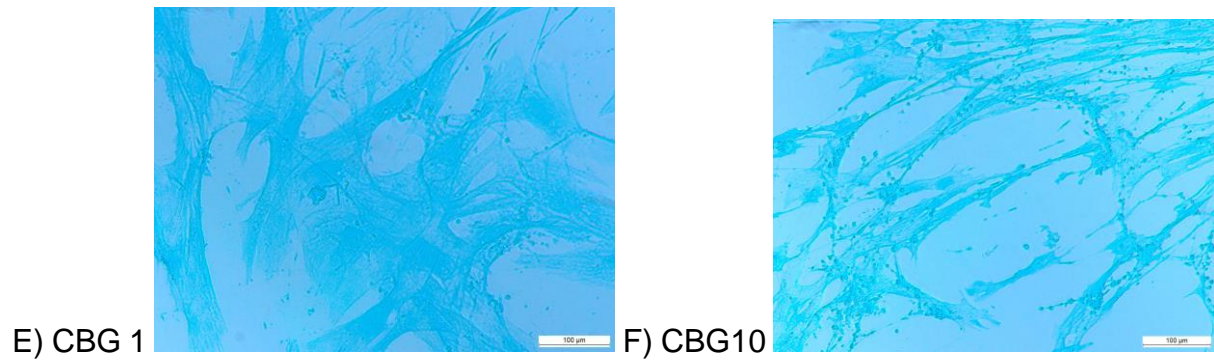


Figure 40: Bright Field microscopic image of Cells treated with or without cannabinoids were subjected to chondrogenic media. Figure A represents the control) cells only exposed to chondrogenic medium, Figure B represents the cell culture with flagellin for 3 hours before presenting them with osteogenic medium and Figure C,D,E,and F represents the cell pre-exposed to cannabinoids for an hours before presenting them with chondrogenic medium.

3.2.1.4.3 Cytotoxicity and Cell viability in PDLCS.

Cells maintained their viability when cultured with the cannabinoid concentrations used during the experiments.

3.2.1.4.3.1 Qualitative analysis:

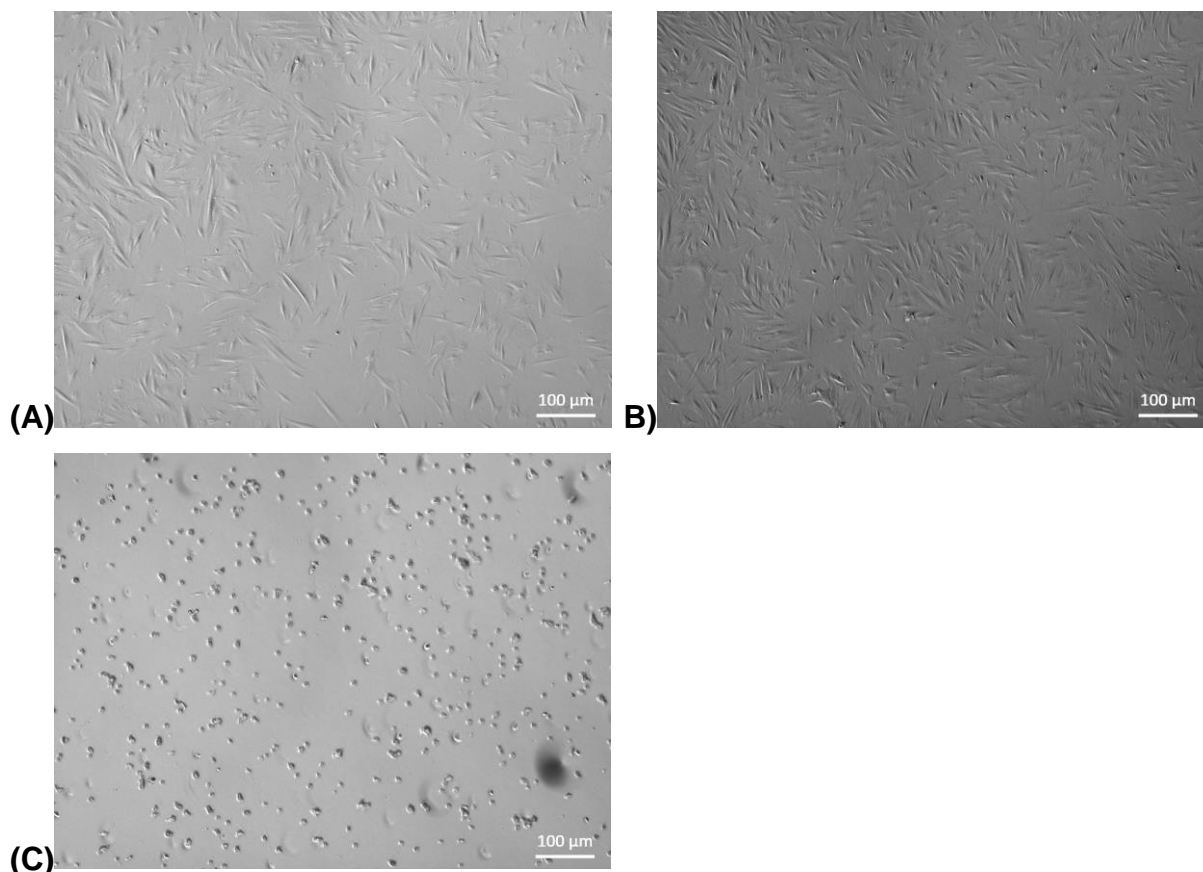


Figure 41: Images of periodontal cells following cultured with low glucose DMEM viewed using a contrast phase light microscope (negative control (A), CBD 10uM (B)) and positive control (C). Representative images of the periodontal cells with and without CBD 10µM. Scale bar 100µm.

3.2.1.4.3.2 Quantitative analysis: cytotoxicity of LPS

The cytotoxicity effects of LPS and cannabinoids on cell viability using Alamar blue assay was performed to examine if compounds show cytotoxic effects. Cells were incubated with above compounds, in the first instance hourly, followed by 3 and 24-hour stimulation with 1 µg/ml of LPS. Alamar blue assay revealed that LPS did not

affect the viability of cells at any of the tested concentrations. There was no statistical significance ($p=0.5$) in the viability of cells between negative control and LPS treated cells.

3.2.1.4.3.2.1 Cytotoxicity of LPS

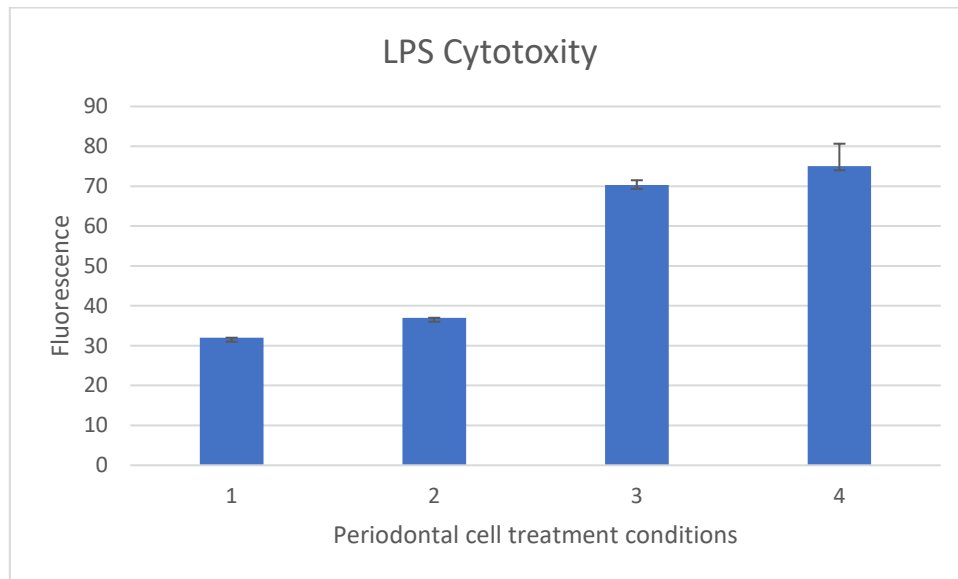


Figure 42: Cytotoxicity of the periodontal cells, cell treated with or without LPS 1 μ g/ml 1. 1. only media, 2. Cells treated with Triton-x 100, 3. cells cultured only in media, 4. cell cultured in media containing LPS (No significance observed when cells +/- LPS).

3.2.1.4.3.2.2 Cytotoxicity of cannabinoids CBD and CBG

The figure describes that the overall cell growth has shown no toxicity at 24 hours, except CBD 50 μ M and CBD 100 μ M. However, the cytotoxicity due to cannabinoids used in this study (1 and 10 μ M CBD) remained the same and did not show any cytotoxicity.

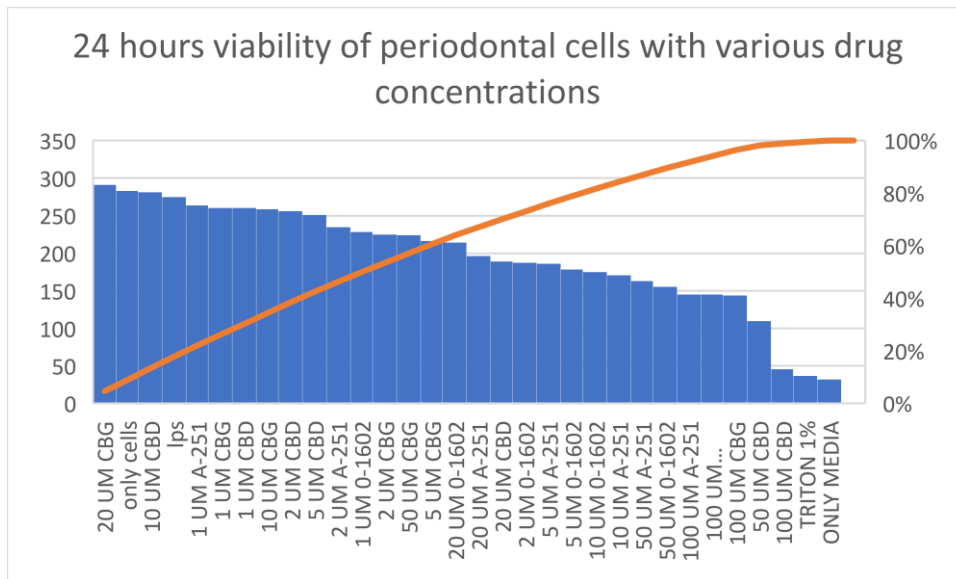


Figure 43: Periodontal cell viability using Alamar blue assay after 24 hours of exposure with various concentration of cannabinoids.

3.2.1.4.3.2.3 Cell viability analysis of PDLCs using live/dead® method.

Cell viability of cultured monolayer periodontal cell was evaluated using Live/Dead® cells assay, as described in (Stoddart, 2011), after 3 and 24 hours (n = 3 samples per time point) of *in-vitro* cell culture. The fixed and stained samples were visualised using a confocal scanning laser microscope (Leica TCS SP8) and the following dyes with specific wavelengths: Calcein AM (Excitation/Emission = 494/517 nm) and Ethidium homodimer III (Excitation /Emission = 530/620 nm).

Periodontal cells were evaluated for their viability by presenting them with chemicals used during experimental conditions and the time points. Cell was pre-exposed with cannabinoids for an hour before presenting with LPS for maximum of 24 hours in order to evaluate their viability cultured in media containing the inflammatory mediator and cannabinoids using Live/Dead® cell viability assay as shown in Figure 44. The results of experiment revealed the excellent viability in all the conditions following 24 hours of cell culture except the situation where cells were exposed to 100 µM CBD where few

dead cells were visible. Viable cells were stained green (Calcein-AM) in the 2D cell culture whereas the dead cells appeared stained red (Ethidium Homodimer). Periodontal cells appeared growing as spindle shaped cells spreading over two-dimensional surface. Cell treated with cannabinoids and LPS were seen growing without having any detrimental effect due to their presence for 24 hours, can be seen compared to control (Figure 44).

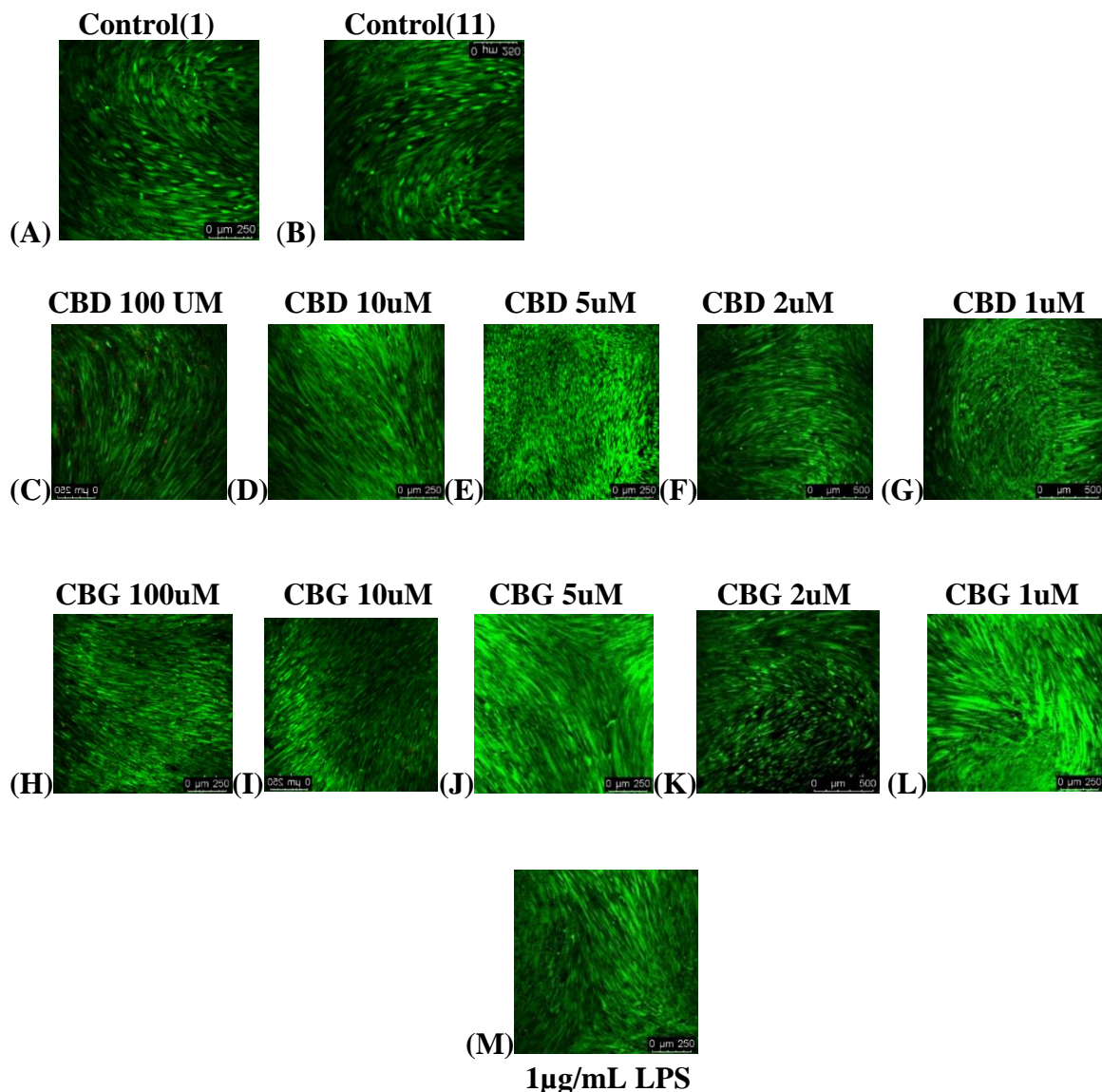


Figure 44: Images of periodontal cells seeded 24 well plate without “Control (A cells only with media, B cells with media + vehicle), (LPS 1 μ g/mL, M)” and with different concentrations of cannabinoids “CBD 100 μ M-1 μ M (C-G), CBG 100 μ M-1 μ M (H-L)” analysed using Live/Dead[®] staining and a confocal scanning laser microscope. Viable cells stained green and dead cells-stained red.

3.3 Study of the anti-inflammatory effect of CBD and CBG:

3.3.1 Effect of cannabinoid CBD and CBG on the expression of pro-inflammatory mediator

The effect of CBD and CBG on gene expression of pro-inflammatory mediators IL-6, IL-8 and TNF- α , induced by flagellin in TIG-K cells and LPS in PDLCs were examined at mRNA level and protein level.

For the gene expression studies at mRNA level, GAPDH was chosen as housekeeping as its expressions in both PDLCs and TIG-K showed good stability (no changes on its expression) under the experimental conditions with or without CBD or CBG.

The study of the anti-inflammatory effect of CBD and CBG was monitored by the evaluation of the expression of inflammatory biomarkers induced by LPS in PDLCs and in flagellin in TIGK.

The main biomarkers, expressed by cells and studied in the study are IL-8, IL-6, TNF- α and IL-1 β .

3.3.1 Study of the anti-inflammatory effect of CBD and CBG on TIGK cells and PDLCs:

3.3.1.1 Anti-inflammatory effect at m-RNA level

Validation of GAPDH for use as a house keeping gene

The expression of GAPDH was assessed in periodontal monolayers cells cultured *in vitro* under basal conditions. Threshold cycle (Ct) values of GAPDH expression were determined in all samples at each time point by the light cycler (Roche LC480). Data were then analysed using two-way ANOVA with Bonferroni correction ($P < 0.05$) and presented as the mean Ct value \pm standard deviation. Statistical analysis revealed no

significant differences ($P > 0.05$) between the mean Ct values of GAPDH expression in all samples across the examined time points. This indicates that GAPDH was consistently expressed in all experimental samples regardless of the changes in the culture conditions and duration. Based on these results, GAPDH was deemed suitable for use as a house-keeping gene RT-qPCR experiment.

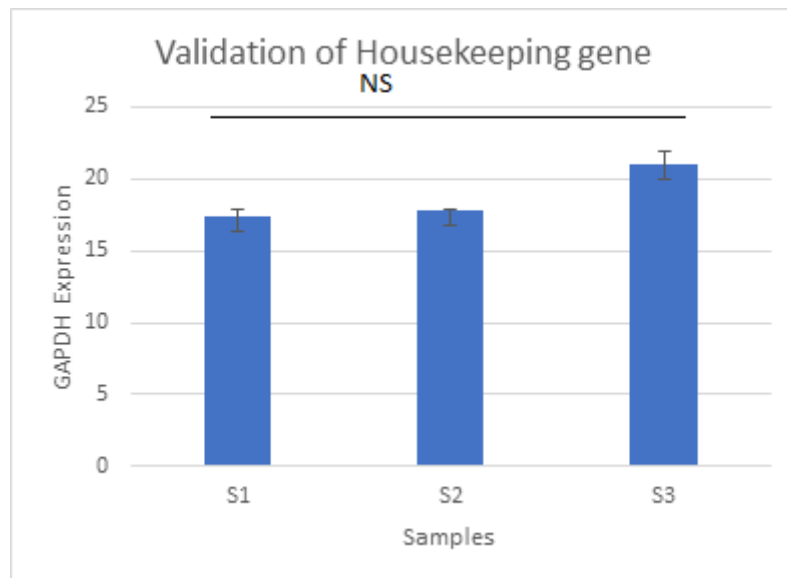


Figure 45: Validation of housekeeping gene (GAPDH), bar graph shows the Ct values of GAPDH expression on different periodontal samples. There was no significant difference between the GAPDH Ct values between different samples.

Effect cannabinoid (CBD) on gene expression of pro-inflammatory mediator IL-8 (TIGK cells)

TIG-K cells were pre-treated with 10 μ M, 1 μ M and 0 μ M CBD for 1 hour followed by incubation with flagellin (1 μ g/ml) for 3 hours. The control group was not treated with CBD nor with flagellin.

The results of the expression of IL-8 at mRNA level using real-time PCR are presented in Figure 46. TIG-K cells pre-treated with 0 μ M CBD was then incubated with only Flagellin for 3 hours. It showed a marked upregulation expression of IL-8 by around 350 times compared to control group. However, when TIG-K cells were treated with 10 μ M and 1 μ M CBD, the upregulation of IL-8 only increased by approximately 25 times compared to control group. Therefore, CBD attenuated the expression of IL-8 induced by flagellin whereas the expression of IL-8 was reduced by around 325 times.

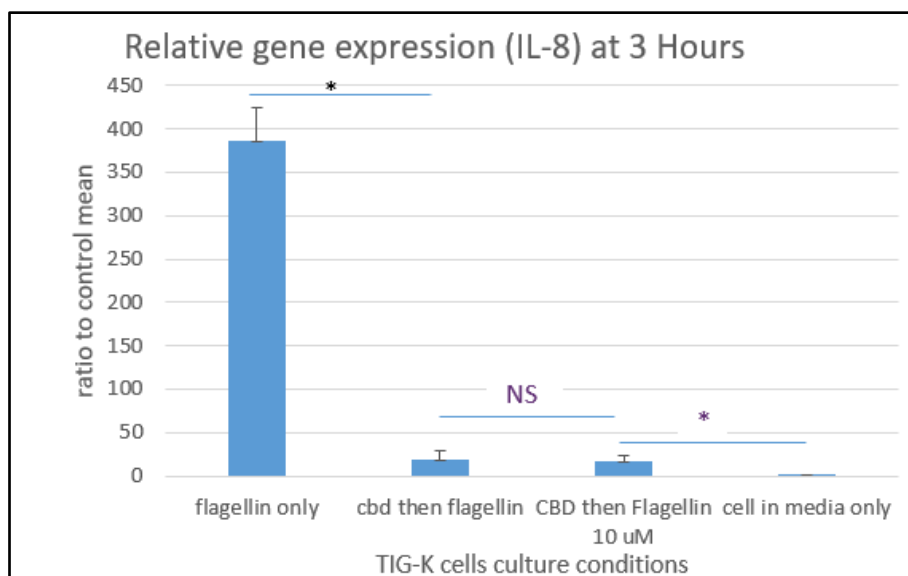


Figure 46: Relative expression of IL-8 as compared to GAPDH at 3 Hours. Effect of Flagellin and cannabinoids CBD on gene expression of pro-inflammatory mediator IL-8. Error bars: represent the standard deviation. NS: no significance, (***) significance used $p < 0.001$, $n = 3$.

3.3.1.2 Anti-inflammatory effect at Protein level (ELISA)

Effect of CBD on pro-inflammatory mediator IL-8 at protein levels in TIG-K

Cells stimulated by flagellin for 3 hours and 24 hours with or without preexposure to cannabinoids. TIG-K cells were pre-treated with 10 μ M, 1 μ M and 0 μ M CBD for 1 hour followed by incubation with flagellin (1 μ g/ml) for 3 hours or 24 hours. The control group was not treated with CBD nor with flagellin. The supernatants were analysed for the expression of cytokine IL-8 release using enzyme linked immunosorbent assay (ELISA). The results of the expression of IL-8 at protein level using ELISA are presented in Figure 47 and Figure 48.

Cells incubated with Flagellin for 3 hours showed a marked upregulation expression of IL-8 by around 500 times compared to control group. However, IL-8 expression only increased by 210 times and 90 times compared to the control group when treated with 1 μ M and 10 μ M CBD respectively. Therefore, the CBD attenuated the expression of IL-8 induced by flagellin; the expression of IL-8 was reduced by around 60% when TIG-K cells were pre-treated with 1 μ M CBD and by the 70% when pre-treated with 10 μ M.

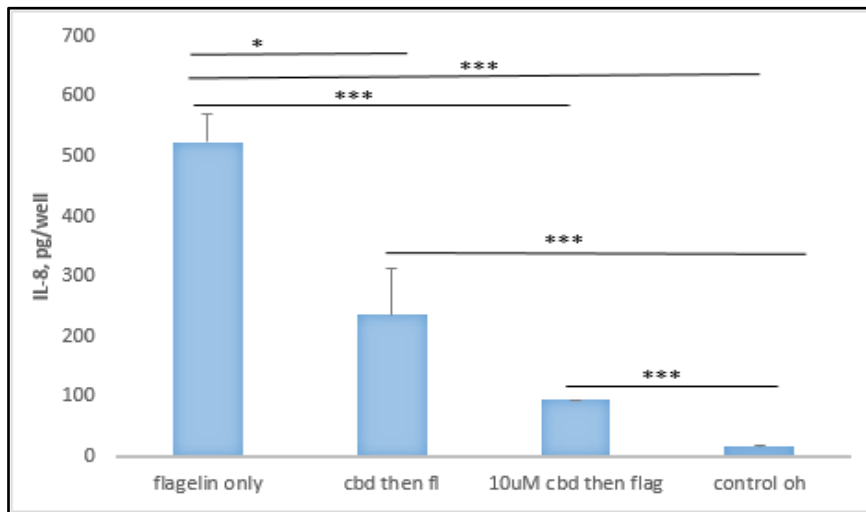


Figure 47: The effect of CBD at 1 μ M and 10 μ M on the TIG-K cell culture for three hours. Effect of Flagellin and cannabinoid CBD on gene expression of pro-inflammatory mediator IL-8. Error bars represent the standard deviation. NS: no significance, (***) significance used $p < 0.001$, $n = 3$.

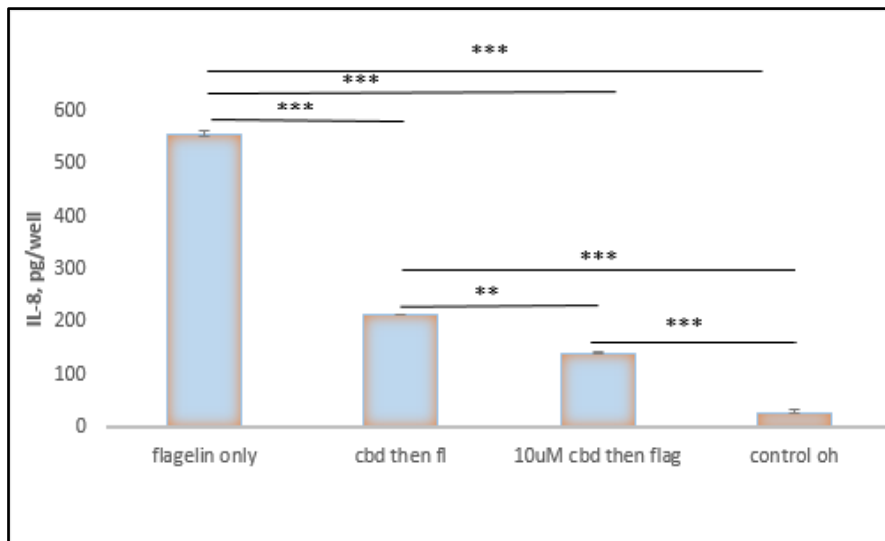


Figure 48: The effect of CBD at 1 μ M and 10 μ M on the TIG-K cell culture for 24 hours. TIG-K were stimulated with flagellin in the absence or presence of 10 μ M and 1 μ M CBD for 24 hours and the production of inflammatory mediators were evaluated using ELISA. (*= $p < 0.01$, **= $p < 0.001$ and ***= $p < 0.0001$, $n = 3$).

The supernatant harvested at 24 hours is also assessed for the pattern of cytokines on TIG-K cells. By stimulating the cells with flagellin, cytokine release was observed to be marginally higher (Figure 48) compared to a 3-hour result (Figure 47). The decrease in secretion of inflammatory cytokine following pre-treatment with 1 μ M and 10 μ M is marginally similar to the previous 3-hour result (Figure 47). However, at 24 hours, the attenuation of inflammatory reaction by 10 μ M CBD i.e., 80% decrease as compared to flagellin only, was reduced significantly i.e., 60% compared with cells treated with 1 μ M CBD.

Effect of CBD on pro-inflammatory mediator IL-8 at m-RNA levels in periodontal cells stimulated by LPS for 3 hours with or without preexposure to cannabinoids.

PDLCs were subjected to LPS. The results showed that LPS induced an increase of the expression of IL-8 by 33% compared to the control (LPS untreated PDLCs) Figure 49. When LPS treated PDLCs were pre-exposed to 1 μ M CBD the expression of IL-8 decreased by 61% approx. to approx. 1/3rd level of LPS stimulated IL-8. However, when LPS treated PDLCs were pre-exposed to 10 μ M CBD the expression of IL-8 decreased by around 56% to a similar level to 1 μ M CBD treatment.

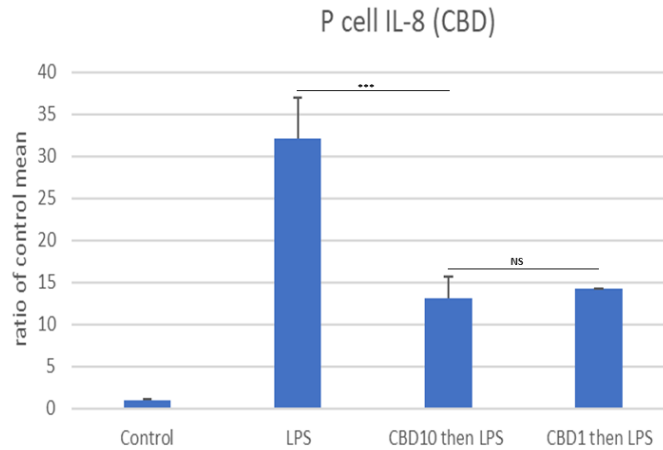


Figure 49: The effect of CBD at 1 μ M and 10 μ M on the periodontal cell culture for 3 hours. Effect of LPS and CBD on gene expression of pro-inflammatory mediator IL-8. Error bars: represent the standard deviation. NS: no significance, (*) significance used $p < 0.001$, $n = 3$.**

Effect of CBD on pro-inflammatory mediator IL-8 at protein levels in periodontal cells stimulated by LPS for 3 hours and 24 hours with or without preexposure to cannabinoids.

PDLCs were subjected to LPS. The results showed that LPS induced an increase of the expression of IL-8 by 30% compared to the control (LPS untreated PDLCs) Figure 50. When LPS treated PDLCs were pre-exposed to 1 mM CBG the expression of IL-8 decreased by 30% and brought back to the same level as the LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 mM CBG the expression of IL-8 decreased by around 70% bringing it at a level lower than the LPS untreated control.

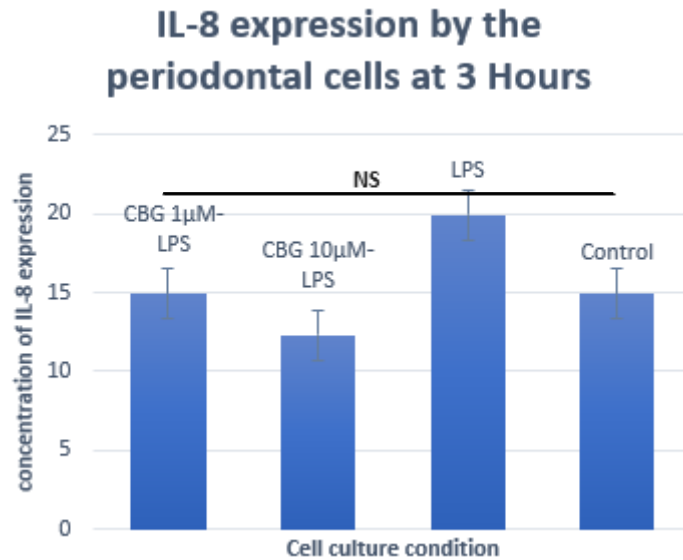


Figure 50: The effect of CBG at 1µM and 10µM on the periodontal cell culture for 3 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10µM and 1 µM CBG for 3 hours and the production of IL-8 were evaluated using ELISA (y axis, conc (µg/ml)).

PDLCs were subjected to three hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10µM CBG. The results showed that LPS induced an increase of the expression of TNF-α by approx. 33% compared to the control (LPS untreated PDLCs) Figure 51. When LPS treated PDLCs were pre-exposed to 1 µM CBG the expression of IL-8 decreased by 20% and brought back to the level just above the LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 the expression of IL-8 decreased by around 25% bringing it at a level lower than the cell when pre-exposed to 1 µM CBG (Not significant).

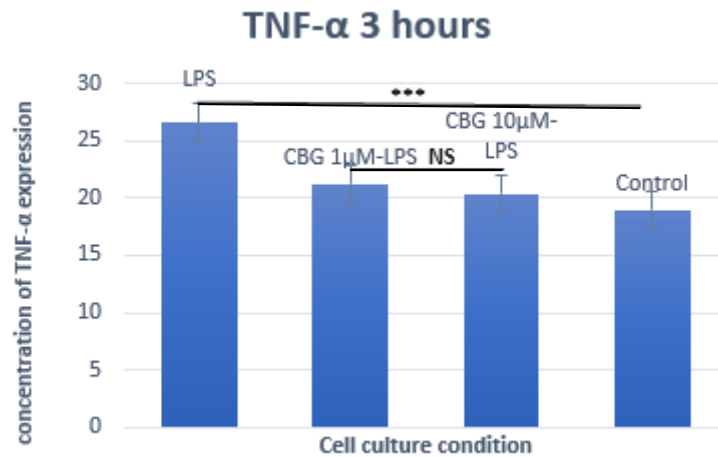


Figure 51: The effect of CBG at 1 μ M and 10 μ M on the periodontal cell culture for 3 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10 μ M and 1 μ M CBG for 3 hours and the production of TNF- α were evaluated using ELISA.

PDLCs were subjected to three hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10 μ M CBG. The results showed that LPS induced an increase of the expression of IL-6 by approx. 25% compared to the control (LPS untreated PDLCs) Figure 52. When LPS treated PDLCs were pre-exposed to 1 μ M CBG the expression of IL-6 decreased and was seen at the same level as the LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 the expression of IL-8 decreased by around 40% bringing it at a level lower than the cell when pre-exposed to 1 μ M CBG.

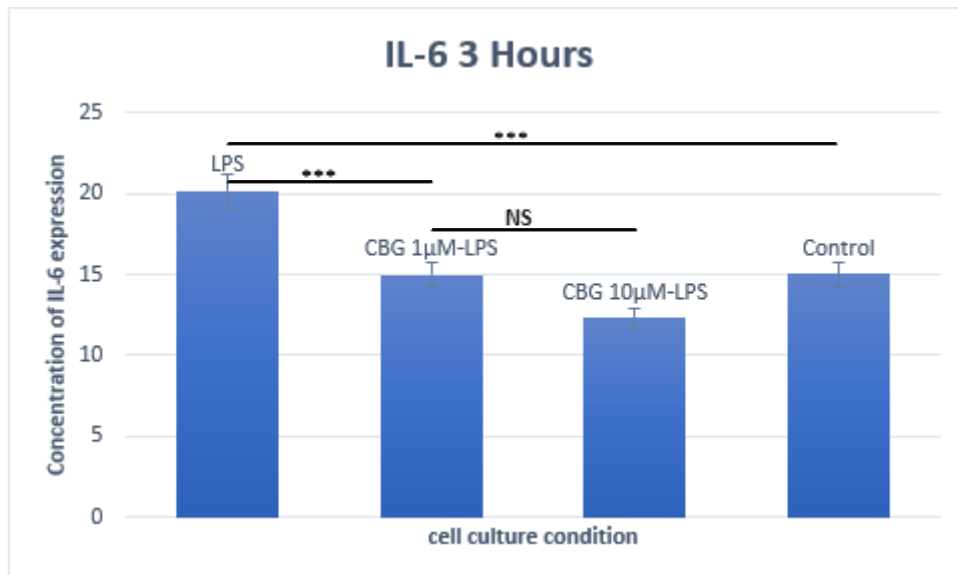


Figure 52: The effect of CBG at 1µM and 10µM on the periodontal cell culture for 3 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10µM and 1 µM CBG for 3 hours and the production of IL-6 were evaluated using ELISA.

PDLCs were subjected to 24 hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10µM CBG. The results showed that LPS induced an increase of the expression of TNF-α by approx. 36% compared to the control (LPS untreated PDLCs) Figure 53. When LPS treated PDLCs were pre-exposed to 1 the expression of TNF-α decreased by 22% and brought back to the level just above the LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 µM CBG the expression of TNF-α decreased by around 29% bringing it at a level lower than the cell when pre-exposed to 1 µM CBG (Not significant).

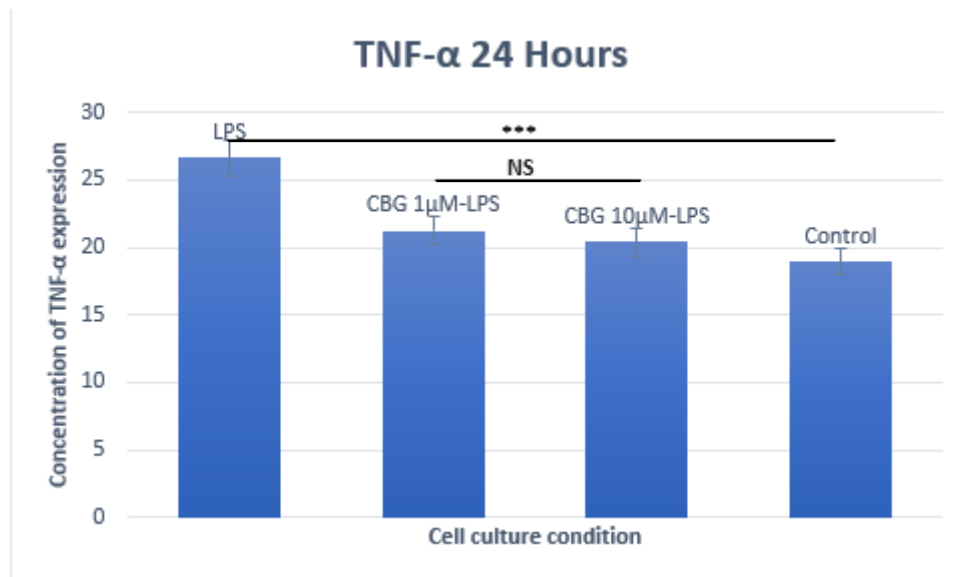


Figure 53: The effect of CBG at 1μM and 10μM on the periodontal cell culture for 24 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10μM and 1 μM CBG for 3 hours and the production of TNF-α were evaluated using ELISA.

PDLCs were subjected to 24 hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10 μM CBG. The results showed that LPS induced an increase of the expression of IL-1β by approx. 32% compared to the control (LPS untreated PDLCs) Figure 54. When LPS treated PDLCs were pre-exposed to 1 μM CBG the expression of IL-1β decreased merely 5% and was seen at the same level as the LPS treated cells. However, when LPS treated PDLCs were pre-exposed to 10μM CBG the expression of IL-1β decreased by around 37% bringing it at a level lower than the cell untreated with LPS (Control).

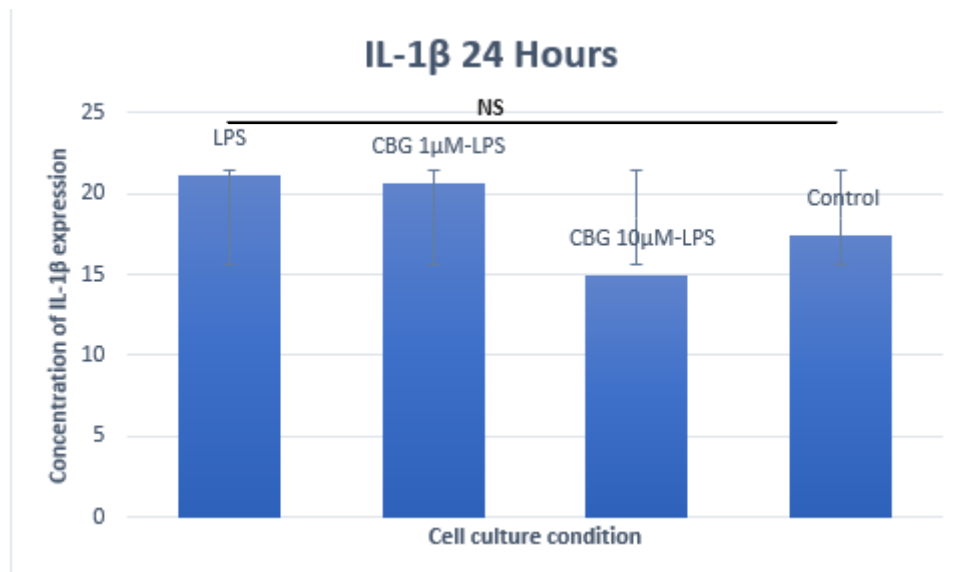


Figure 54: The effect of CBG at 1μM and 10μM on the periodontal cell culture for 24 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10μM and 1 μM CBG for 3 hours and the production of IL-1β were evaluated using ELISA.

PDLCs were subjected to 24 hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10μM CBG. The results showed that LPS induced an increase of the expression of IL-8 by approx. 25% compared to the control (LPS untreated PDLCs) Figure 55. When LPS treated PDLCs were pre-exposed to 1 μM CBG the expression of IL-8 decreased and was seen at the same level as the LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 the expression of IL-8 decreased by around Approx 38% bringing it at a level lower than the cell when pre-exposed to 1 μM CBG.

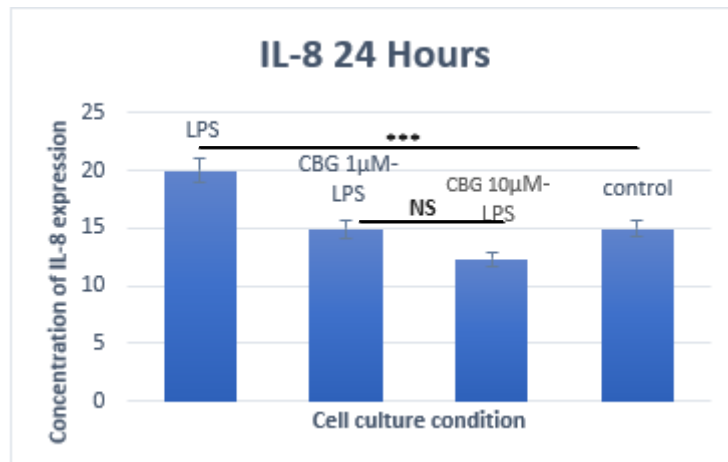


Figure 55: The effect of CBG at 1µM and 10µM on the periodontal cell culture for 24 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10µM and 1 µM CBG for 3 hours and the production of IL-8 were evaluated using ELISA.

PDLCs were subjected to 24 hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10µM CBG. The results showed that LPS induced no increase of the expression of IL-6 compared to the control (LPS untreated PDLCs) Figure 56. When LPS treated PDLCs were pre-exposed to 1 µM CBG the expression of IL-6 decreased by 12% and was seen at a level lower than the LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 the expression of IL-8 decreased by around Approx 6% bringing it at a level lower than the cell when pre-exposed to 1 µM CBG and the CBG untreated control cells.

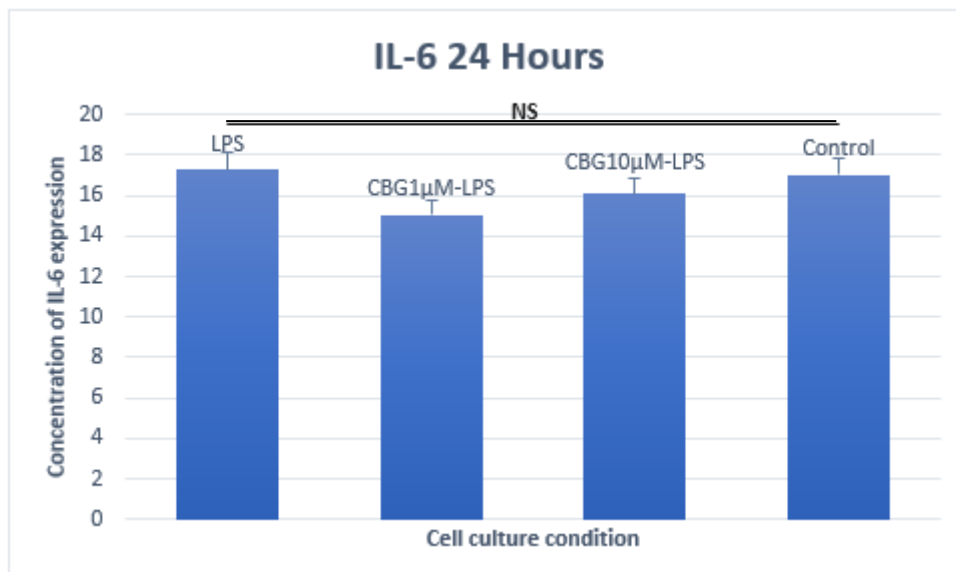


Figure 56: The effect of CBG at 1µM and 10µM on the periodontal cell culture for 24 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10µM and 1 µM CBG for 3 hours and the production of IL-6 were evaluated using ELISA.

PDLCs were subjected to three hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10µM CBD. The results showed that LPS induced an increase of the expression of IL-8 by approx. 52% compared to the control (LPS untreated PDLCs) Figure 57. When PDLCs were pre-exposed to 1 µM CBG the expression of IL-8 decreased by 33% and was seen at above LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 the expression of IL-8 decreased by around Approx 25% bringing it at a level lower than the cell when pre-exposed to 1 µM CBD.

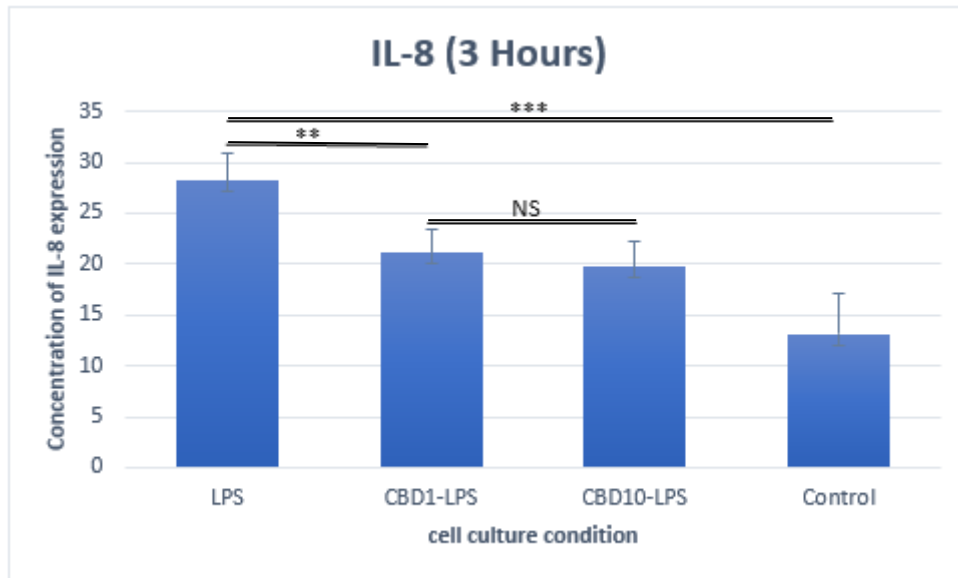


Figure 57: The effect of CBD at 1 μ M and 10 μ M on the periodontal cell culture for 3 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10 μ M and 1 μ M CBG for 3 hours and the production of IL-8 were evaluated using ELISA.

PDLCs were subjected to 24 hours of treatment with LPS with or without pre-exposing the periodontal cells with 1 and 10 μ M CBD. The results showed that LPS induced an increase of the expression of IL-8 by approx. 38% compared to the control (LPS untreated PDLCs) Figure 58. When LPS treated PDLCs were pre-exposed to 1 μ M CBG the expression of IL-8 decreased by only 8% and was seen at above LPS untreated control. However, when LPS treated PDLCs were pre-exposed to 10 the expression of IL-8 decreased by around Approx 24% bringing it at a level equivalent to the cells untreated with CBD.

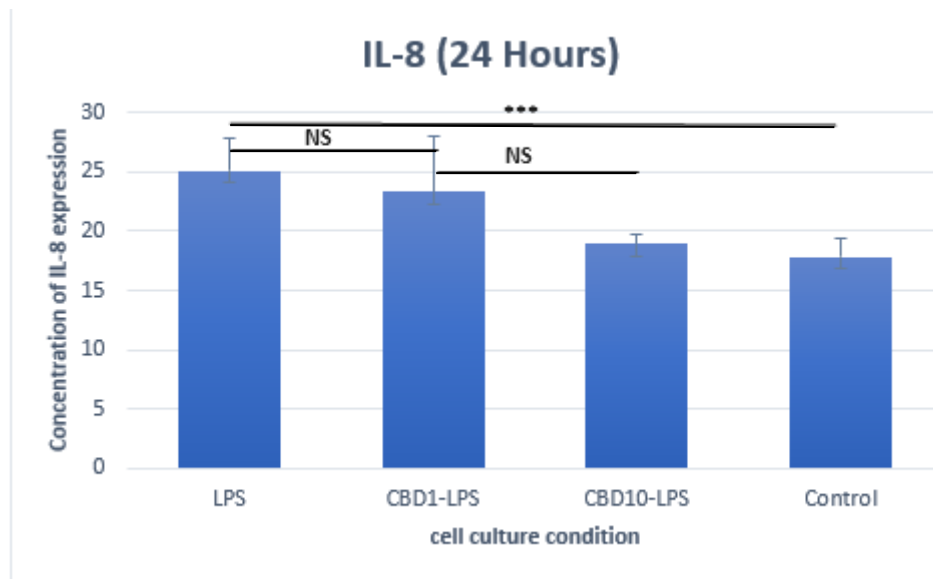


Figure 58: The effect of CBD at 1 μ M and 10 μ M on the periodontal cell culture for 24 hours. Periodontal cells were stimulated with LPS in the absence or presence of 10 μ M and 1 μ M CBG for 24 hours and the production of IL-8 were evaluated using ELISA.

3.3.1.3 Anti-inflammatory effect at Protein level (Western blot)

LPS induced expression of IL-8 cytoplasmic proteins and pre-treatment of periodontal cells showed reduction of expression of cytoplasmic IL-8 inflammatory proteins. Also, cell line G292 showed the expression of cytoplasmic protein IL-8 due to LPS and rescued by pre-exposure with cannabinoids for one hour (Figure 59).

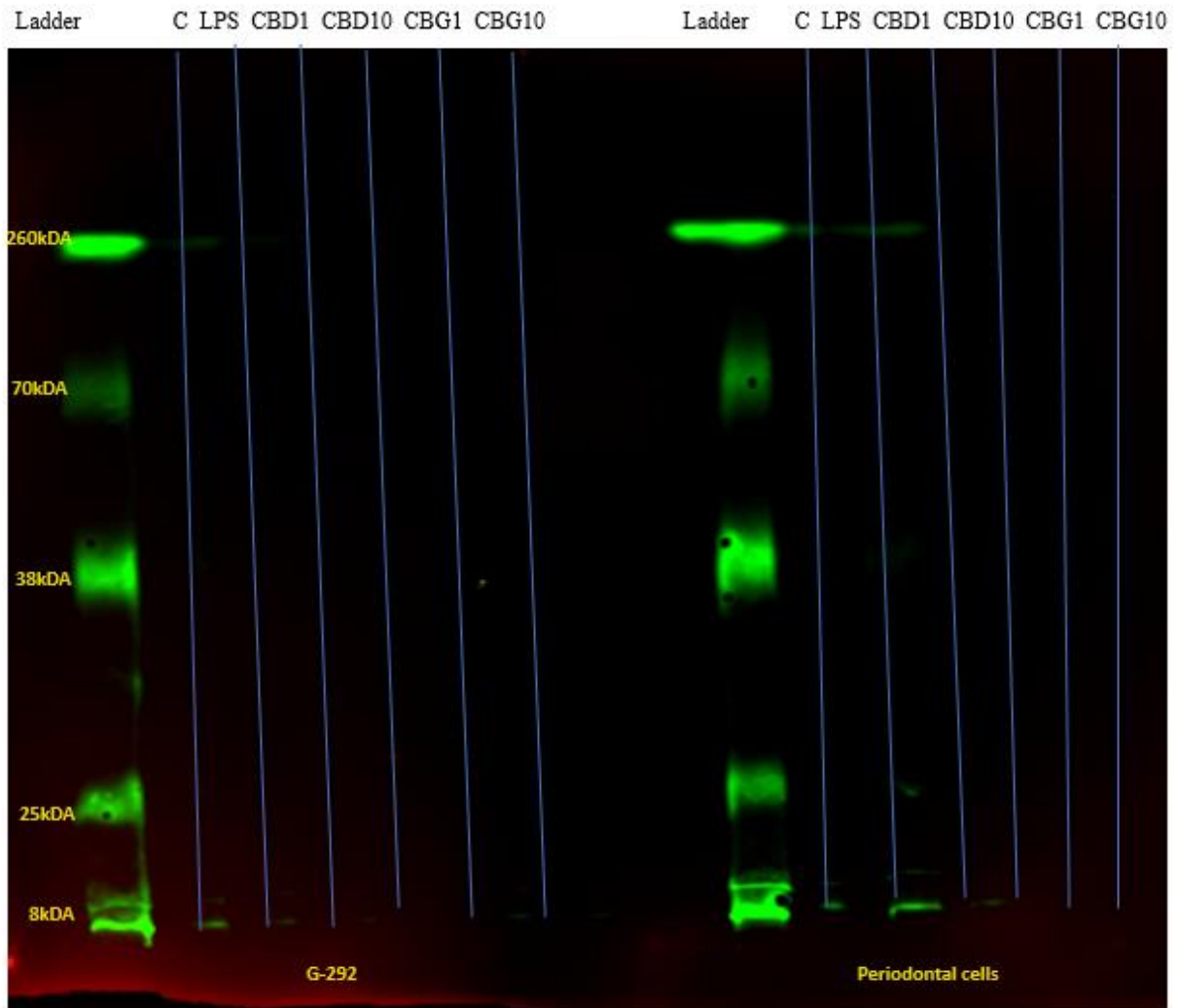


Figure 59: Intracellular localization of IL-8 (8kDA) in LPS stimulated G292 and periodontal cells. Cells were exposed to LPS with and without cannabinoids. Western blot analysis of the expression of IL-8 under the effect of CBD and CBG on periodontal cells and G-292. L= ladder (chameleon 800), C= Control LPS= Lipopolysaccharide, CBD1= Cannabidiol 1 μ M, CBD10= Cannabidiol 10 μ M, CBG1= Cannabegerol 1 μ M, CBG1= Cannabegerol 1 μ M, Image stained with revert stain LICOR.

Chapter 4 Discussion

4.1 Introduction

The aim of this project was to explore the role of the anti-inflammatory properties of cannabinoids with a view to attaining preliminary data to address chronic periodontal diseases. In addition, this project also aimed to investigate the role of cannabinoids in the treatment of periodontal diseases. Before looking at the anti-inflammatory effect of the cannabinoids, the cytotoxicity of the compounds used was tested on a variety of cells mimicking the cells of periodontal apparatus. For example, epithelial cells of oral and extra-oral origin (TIG-K, keratinised epithelium and A-2780, non-keratinised epithelium), connective tissue cells (G292) and primary periodontal cells from different donors. Chronic inflammatory disease such as periodontal disease is responsible for overreaction within the immune system (Nędzi-Góra et al., 2017), In some chronic diseases such as RA (Rheumatoid Arthritis) the immune system becomes hypersensitive resulting in auto destruction, meaning it attacks itself (Potempa et al., 2017). Periodontitis is considered a form of a multifactorial disease (Taba Jr, 2012), initiated by dysbiotic bacterial colonies in the plaque biofilm on the tooth (Wu et al., 2016), and the host's inflammatory response to changes in microbial colonies. This results in the destruction of tooth-supporting apparatus (Pihlstrom et al., 2005).

The cumulative inflammatory response results in cellular activation (Shi et al., 2020), expression of inflammatory mediators including cytokines (Yucel-Lindberg et al., 2013a), chemokines (Ramadan et al., 2020) and proteolytic enzymes (Yucel-Lindberg et al., 2013b) resulting in soft tissue destruction and resorption of surrounding alveolar bone. The pro-inflammatory cytokines expressed by the immune response which plays a key role are interleukin-1 (IL-1 β), IL-6, IL-8 and tumor necrosis factor alpha (TNF- α).

These interleukins are secreted by a variety of cell types comprising monocytes, macrophages, dendritic cells, epithelial cells, keratinocytes, and fibroblasts. This study provides in-vitro evidence that pre-exposure of periodontal cells with cannabinoids can help to significantly attenuate the expression of inflammatory cytokines induced by LPS/Flagellin. A previous study identified the beneficial effect of anti-inflammatory agents on the antihypertensive and immunosuppressive drugs induced periodontal diseases (Heasman, 2014).

Idiopathic or pathological trauma to periodontium induces gingivitis (Orban 1942). Significantly, prolonged and persistent gingival inflammation leads to periodontitis (Schätzle et al., 2003). In periodontitis, dysregulation of homeostasis leads to an increase in the numbers of non-symbiotic microbial flora, resulting in disturbance of the oral microbiome. One of the earliest signs of periodontitis is the acute inflammation of periodontal tissue. This is caused by the protective physiological response which is initiated by interaction with the microbial challenge. The bacterial toxins result in damage to the connective tissue, and it also triggers the protective junctional epithelium cells to produce cytokines. Destruction of the periodontium is caused by cumulative damage due to secreted bacterial toxins, and aberrant protective immune response.

The damage caused by periodontitis is not only confined to the loss of a tooth to its maximum effect, but it has also been linked to other chronic immune disorders (CIDs) (Velde AA et al., 2016). Studies inform us that periodontitis has been suggested as a potential source of infection and a risk factor in diabetes (Arigbede et al., 2012), atherosclerotic cardiovascular disease, respiratory diseases, musculoskeletal and disorders of the reproductive system.

To date, there are two main approaches to the management of periodontitis, including 1) Treatments aimed at the elimination of oral bacteria including professional scaling (Slots J, 2012), use of mouthwashes (Costa et al., 2017) and oral hygiene education (B. Jönsson et al., 2009), and 2) symptomatic treatments provided by a dental professional (Brunsvold et al., 1999). Cumulative studies demonstrate a paucity of treatment approaches to periodontitis, particularly in dealing with the damage caused by a dysregulated immune response in the oral microbiome (Gholizadeh et al., 2016).

The treatment options provided by dental professionals include:

- Scaling (Deas et al., 2016),
- Surgical methods such as flap surgery of infected periodontal tissue (Cortellini, 2017),
- Delivery of anti-microbials including local and systemic antibiotics (Xajigeorgiou et al., 2006),
- Use of drugs to inhibit the inflammatory pathway involved in tissue destruction during periodontitis such as NSAIDS (Howell & Williams, 1993),
- Antibiotic-free approach such as antimicrobial enzyme linked to nanoparticles (Sharmin et al., 2021, Mercado et al., 2019).
- Intra periodontal pocket drug delivery systems including fibers, strips, gels, films, microparticles, nanoparticles, and vesicular system.

However, the above dental treatment options are not risk-free. For example, scaling has been shown to result in gingival recession, sensitivity, and discoloration. These drawbacks are also seen in the use of surgical approaches including cost, the need for frequent follow-ups, patient compliance, and gingival recession.

With regards to the use of antibiotics, systemic anti-microbials were found to be more predictable than topical counterparts (Slots et al., 1990). However, its usage remains

challenging as patients can develop adverse reactions such as anaphylaxis, and more worryingly, the development of antibiotic resistance. Therefore, mechanical debridement by scaling and root planning is arguably the choice of treatment for mild to moderate periodontitis to avoid the over the prescription of antibiotics leading to the development of resistant subgingival microflora (Myszka et al., 2012).

As discussed earlier, the progression of periodontal diseases was accounted for due to the host's immune response to bacterial pathogens. The anti-inflammatory agents modulating the host response were considered to contain the damage of periodontal tissue due to inflammatory reaction. NSAIDs such as ibuprofen, naproxen, and ketorolac were used to inhibit prostanoid (inflammatory mediators) formation to prevent inflammation of periodontal tissue. However, the efficacy of NSAIDs to address periodontitis was not approved as the choice of treatment. Hence, we are still in need of a substantial solution to this chronic disease (Kraye et al., 2010).

To mitigate these side effects associated with the use of pharmacological agents or treatment approaches for periodontitis, medicinal properties of plant products may be used as an adjunct to address chronic periodontal diseases (Gościński et al., 2021). These plant-based therapies were reported to have anti-inflammatory and prostaglandin synthetase activity due to their phenolic compounds. Research informs us that plant products can prevent early inflammatory response in periodontal diseases and or other chronic inflammatory diseases such as RA (Ramesh et al., 2016, Kaur et al., 2012).

One of the plants identified i.e., *cannabis sativa* was reported to possess anti-inflammatory properties. This was found to be a potential alternative as it was shown to block the progression of inflammatory rheumatoid arthritis in a murine model (Malfait et al., 2000a). Cannabinoids obtained from plants called phytocannabinoids, of which

there are two types. These are either psychoactive (THC) or non-psychoactive (CBD, CBG)- differentiated by their effects on psychological dependence. Cannabidiol (CBD), which was first isolated in 1940 by Adams and co-workers. Since then, its therapeutic properties have been shown to be effective in the treatment of chronic conditions such as endocrine disorders e.g., diabetes, neurodegenerative conditions such as multiple sclerosis and certain end stage cancers.

On the other hand, CBG, obtained from hashish, a plant found in temperate countries by Gaoni and Mechoulam in 1964 has been identified as having anti-proliferative, and antibacterial activity. Alongside its anti-inflammatory properties, both CBD and CBG possess antibacterial activity against methicillin resistant *staphylococcus aureus* strains. It has been suggested that when these anti-inflammatory and antibacterial properties when harnessed in conjunction, it would be clinically relevant to address Periodontitis (Pan et al., 2019, Appendino et al., 2008).

These cannabinoids showed promising results in decreasing migration of neutrophils in gingival tissue and preventing progression of bone resorption in experimental periodontitis in rats (Napimoga et al., 2009). Therefore, the aim of this project was to investigate the recently explored anti-inflammatory properties and effects on bone mineralisation physiology of cannabinoids during periodontitis.

The presence of endocannabinoids and cannabinoid receptors (CB1 and CB2) in the human body including cells in the periodontium, acts as receptors for cannabinoids. Cannabinoids such as cannabidiol and cannabigerol are known to interact with endocannabinoids - a modulator of neuronal response involved in expressing the immune response. Cannabidiol was studied to act on endogenous endocannabinoid receptor or CB1 and CB2 receptor, however the mechanism of action is not fully

understood (Boehnke et al., 2022). It can be suggested that this is due to periodontal diseases being a multi-factorial condition.

These cannabinoids can be either synthetic, plant-based or produced in the human body. Significantly, it has been demonstrated that it possesses anti-inflammatory properties, and its efficacy has been proven in reducing inflammatory pain in RA (C Recio, Andujar et al. 2012). Moreover, cannabinoids have been proven to have an affinity to phytocannabinoid – a natural herb.

This discussion is focused on three sections using cell lines and primary periodontal cells:

- a) Evaluation of the cytotoxicity of various concentrations of cannabinoids, flagellin, and LPS.
- b) Expression of proinflammatory mediators at mRNA levels.
- c) Expression of proinflammatory mediators at protein levels.

In order to study the effect of cannabinoids on periodontal cells, an in-vitro periodontitis model was planned to include the culture of cell line or periodontal cells. Thereafter, the cells were exposed to inflammatory agents such as LPS and flagellin, known to cause damage to periodontium during periodontal diseases. Before exposure to inflammatory agents, cells were presented with cannabinoids. The aim was to investigate if they can rescue the expression of inflammatory biomarker which was expressed by the cells when they were presented with such inflammation inducing agents.

It is crucial to identify the effects of the compounds on the cells they were exposed to, in order to know whether the cells are having any toxic effect due to the presence of such compounds in the in-vitro culture medium. Basic science research has explored different in-vitro periodontitis models in order to address this multifactorial chronic

periodontitis (Aveic, 2021). Since periodontal regeneration and wound healing are a complex process, many in vitro models were used by previous researchers. For the purpose of this study, 2 dimensional in-vitro periodontitis model was used to evaluate the expression of inflammatory biomarkers. A simple model was used to address one aspect of periodontitis which is predominantly the inflammatory cascade. Hence, exploring the role of cannabinoids in addressing the damaging inflammatory process, (Weinreb, 2015).

4.2 Evaluation of cytotoxicity

“Primum non nocere” first do no harm is the basic principle to any research project (Gillon, 1985). The use of cannabinoids and inflammatory agents such as LPS and Flagellin on human tissues are known to be toxic (Guzmán, 2002). Nonetheless, cannabinoids and inflammatory agents were used in this study on different cells. The rationale underpinning its usage is to establish a dosage that would prove to be not toxic to healthy cells. In other words, this study will demonstrate that the compounds used are non-toxic to cell lines or primary cells.

4.2.1. LPS

Lipopolysaccharide (LPS) from *P.gingivalis* is responsible for expression of pro-inflammatory mediators by acting through a TLR-4 receptor (Nativel et al., 2017). When LPS from gram-negative bacteria acts on a cell, it is recognised by TLR4 receptor on the periodontal cells. Subsequently, cells activate the pro-inflammatory reactions by way of transient receptor potential cation channel subfamily V member 1 TRPV1 (López-Requena et al., 2017). G292 cell was confirmed as sensitive to TLR receptor and found to be highly responsive to IL-1 stimulation resulting in the

production of pro-inflammatory cytokines such as IL-6 (Rao et al., 2005). Therefore, G292 cells were evaluated in this study for any toxicity with LPS.

Previous studies have used various concentrations of LPS to illicit the inflammatory response by the G292 cells (Granger DN, 1998). This project advances the study conducted by Jones et al, 2010., using 1 µg/ml LPS P-gingivalis to induce inflammatory cytokine cascade; and as per Jones work, cytotoxicity due to 1 µg/ml LPS was absent. Previously LPS P-gingivalis was used at 10 µg/ml to study periodontitis in TLR-4 expressing cell line and they did not observe any toxicity (Yoshida et al., 2019). Thus, regardless of whether one is using 1 or 10 µg/ml of LPS P-gingivalis to establish cytotoxicity, it appears the different concentrations of LPS used by different researchers made no difference to toxicity. However, to date, no study has been conducted to justify the use of higher concentration of LPS. Conversely, it can be suggested that some primary cells or cell lines might need higher concentration to express the cytokine cascade. As per previous studies, for the purpose of this project, 1 µg/ml LPS P-gingivalis was used.

4.2.2 Flagellin

While LPS was recognised as TLR 4 receptors on the cells, another inflammation-causing agent which targets the TLR5 i.e., flagellin was used in this project. This is because different cells i.e., G292, A2780, and TIGK have different potential toll like receptor target hence flagellin was also used in this study. Flagellin, a gram-negative bacterial protein is recognised by the TLR-5 receptor on the gingival or periodontal cell surface during the bacterial invasion (I. A. Hajam et al., 2017). Flagellin was found to trigger an inflammatory response, either by activation of non-immune or immune cells by activation of TLR5 (Murthy et al., 2004, Gewirtz et al., 2001). Nakamura et al, 2012 proposed the activation of osteoblasts TLR5 receptors results in the expression of monocyte attractant chemoprotein 1 (MCP1), causing a chemokine/cytokine expression during periodontitis (Nakamura et al., 2012).

Expression of cytokines due to inflammatory response such as IL-6 was shown to be elevated in the presence of a higher concentration of Flagellin from 0.001 to 1µg/ml. In addition, the elevation of cytokine levels was also seen to be correlated to time-lapse i.e., from 3 hours until 48 Hours (Jeon et al., 2015). Flagellin orchestrates the activation of innate immunity and acquired immunity. It has been observed that antigen-antibody response is not dose-related in relation to its requirement for flagellin. This suggests that the induction of antibody response is not directly proportional to the strength of presenting the epithelial cells immune response. The immune expression in terms of cytokines expression by periodontal cells is caused by the effect of flagellin on a number of cells eg epithelial cells, lymphocytes, and lymph node stromal cell. The overall expression of cytokines in the presence of bacterial pathogens is crucial for presenting antigen-specific adaptive immune response (I. Hajam et al., 2017).

Cells used in this study.

Cell lines are considered the gold standard for studying the in-vitro effects of compounds or environments on the cells to evaluate the toxicity based on the amount of time exposed and dose-related toxicity levels.

Three types of cell lines were selected for this thesis, namely, i) the TIG-K cells, ii) G292, and iii) A2780. These were selected because the epithelial origin was in concordance with their role as the first line of protection against the establishment of an initial inflammatory response to pathogens. In addition, the cell line possesses the potential to multiply without changes in behaviour. This aspect is crucial to in-vitro study because uniformity must be maintained across the experiments. In this way, this study can demonstrate the reproducibility of research. Previous studies have used cell lines and primary periodontal cells to create in-vitro periodontitis models to investigate periodontitis, alongside varying concentrations of CBD and inflammatory agents. The aim was to evaluate cytotoxicity of various compounds using different methods. In this project, a periodontitis model was used to study the effects of cannabinoids and evaluated its cytotoxicity on the cells used to mimic periodontitis.

4.2.3 G292

G292 cell was selected as an in-vitro periodontal model to test any toxic effect in presence of G292 growth with LPS from *P. Gingivalis*. G292 was also used to explore the effects of prolonged culture of G292 with cannabinoids. This was done to investigate the effects of cannabinoids on the expression of cytotoxic effects due to the presence of cannabinoids during cell culture. Finally, the onus of selection of these cells in this study is 1) readily available from the tissue bank, 2) its homogeneity, and 3) its osteoblast-like characteristics (Peebles et al., 1978).

The sequential change in the morphology of G292 was observed from seeding until it reached 80 % confluence. Changes were not correlated to the concentration of CBD and CBG. In other words, regardless of whether 1 μM or 10 μM of CBD and CBG was used, it made no visible difference to the changes in the morphology. Similar to the present study other studies reported that a lower concentration 1-5 μM of CBD results in an increase of cell proliferation - when cultured for 72 hours, there was no cytotoxic effects on pulp cells (Qi, Liu et al. 2021). However, the present study aimed to culture the cells for 24 hours in order to witness the expression of cytokines when they are observing the peak expression of cytokines (Lambertsen, 2012). Rawal et al., 2012 determined the cytotoxicity of CBD (0.01-30 μM) and found no toxicity on gingival fibroblasts at any used concentration which included the observation of no cytotoxicity with 1% MeOH as dissolving agent (Rawal et al., 2012). In contrast, a study found a lower concentration i.e., 7 μM CBD was found cytotoxic to Sertoli cells after 48 hours of culture (Li et al., 2022). This could be due to the use of different origin cells i.e., germ cells.

4.2.4 A2780

A2780 cells were tested for their cytotoxicity in the presence of a different concentration of cannabinoids and the solvent/vehicle of cannabinoids i.e., ethanol. The concentration used for the experiments i.e., 1 and 10 μM for 3 and 24 hours, did not show any cytotoxicity. However, the cannabinoids were found toxic above those concentrations, IC₅₀ values of CBD and CBG on A-2780 – findings consistent with previous studies (Izzo et al., Ligresti et al., 2006). CBD and CBG when exposed for a longer duration 48 hours (about 2 days), showed increased toxicity on A-2780. Other

studies reported the role of cannabidiol 20 μ M by itself or in combination with other cannabinoids in reducing cell viability (Morelli et al., 2014). The study was conducted to rule out any cytotoxicity on A2780.

4.2.5 TIG-K

TIGK cells were tested for cytotoxicity of the compounds with which they were treated in this study. TIGK cells were treated with flagellin 1 μ g/ml for 3 and 24 hours and no cytotoxicity were found at this concentration and duration. TIG-K cells were previously used at a similar concentration. It was not found to be toxic to TIGK cells at the above concentration (Nadat, 2018). Therefore, in this study TIG-K cells were treated at the above concentration and for similar time periods. The concentration used for CBD and CBG were evaluated for any morphological changes in the cells over a period of time i.e., up to 24 Hours.

The changes in TIGK cell morphology were observed from their seeding point until they reached 80% confluent stage and during their exposure to the above-mentioned drugs.

4.2.6 PDLCs

Primary periodontal cells were also tested for any toxicity due to the compounds used in this study. PDLCs were treated with LPS 1 μ g/ml for 3 and 24 hours and no cytotoxicity were found at this concentration and duration. The toxicity of the used cannabinoids was assessed qualitatively and quantitatively during the cell culture.

Cell growth observation during cell culture:

Contact inhibition is the known regulatory mechanism to prevent the overgrowth of monolayer cells (Abercrombie M., 1970). Similar to TIGK cell lines such as G-292 are

devoid of such inhibitory mechanism and continue to grow layer by layer. Sudo et al reported a mosaic-like appearance on an ovarian cell line – the cells grow on top of the other due to over confluence (Sudo et al., 1983). The findings did not report mosaic appearance during growth of cell line (G292) reaching confluency. The cells were observed during growth and utilised for experiments before reaching 100 percent confluency. In contrast, primary periodontal cells were shown a reduced metabolism and proliferation due to contact inhibition, whereas the cell lines continue to grow in layers (Ruijtenberg and van den Heuvel, 2016).

The observation of cell behaviour during growth begins with observing the viability of the cells under culture system. The toxicity of the compounds in culture medium can alter the viability of cells. Therefore, observation of cell viability refers to the identification of ratio of live/Dead cell under a certain environmental condition.

The most common method to identify cell growth is to identify the cell population doubling time, whereas doubling time was not monitored during this study. The population size, that is the number of cells growing at a particular time, is counted manually (Neu bars chamber). In this project the initial seeding number was 10000 cells per cm^2 , and secondly, the confluency of initially seeded cells was observed in G292 was around 2 days, around 3 days for the A2780 and more than 3 days in case of immortalised TIGK cells based on the observation from initial seeding of 10,000 cells per centimeter square. Sudo et al., 1983 initial seeding density was 50-60K cells per cm^2 and the doubling times was 4 days. Variation in doubling time reflects that cell division cycle depends on extra-cellular signals, and intrinsic information in the cells. In contrast to TIGK cells, periodontal cell growth varied and depended on the culture conditions and the storage of the primary cells. Periodontal cells were

evaluated for their growth and doubling time, and it varied between approximately 5 to 10 hours and took more than cell lines for periodontal cells to reach up to the required confluency level from the initial seeding density of 10,000 cells per cm square. Developmental variations in cell cycle were reported to influence cell proliferation and differentiation (Ruijtenberg and van den Heuvel 2016).

4.2.7 Summary

To summarise, the discussion thus far on the evaluation of the cytotoxicity of various concentrations of cannabinoids, flagellin, and LPS has shown that the cells used in this study i.e., G292, TIGK and primary periodontal cells remained free from toxicity, except for CBD and CBG at higher concentration i.e., 100 μ M. G292 are osteosarcoma cells widely used for identification of expression of cytokines during inflammation. They are cell lines and homogeneous, hence results were reproducible while looking at the toxicity of compounds and their inflammatory potential used in this study. Hence before attempting the experiments, Cell viability was assessed with the decided concentration of compounds and their duration of exposure. Two different concentrations of cannabinoids were then tested for their anti-inflammatory effect at two different time points. At this time these cells remain free from toxicity. TIGK cells are telomerase inhibited gingival keratinocytes and they were used by various authors to mimic the in-vitro periodontitis model. For the above used concentration of cannabinoids and both the time periods, they were not at risk of viability issues.

LPS acts on TLR4 receptors whereas flagellin is recognised by TLR5 receptors on the cells. Their cytotoxicity effect of their presence during cell culture was evaluated at 1 μ g/ml concentration and showed no effect on the cell viability. The question now, Do

LPS and flagellin at 1µg/ml triggered an inflammatory response? The response is yes, and this will be discussed later.

4.3 Characterisation of primary cells

Primary periodontal cells go through replicative changes as they multiply i.e., senescence. This makes their isolation challenging and limits their indefinite growth. Therefore, the primary periodontal cell was used during their 4th -6th passages in this project. The primary cell culture requires regular monitoring and maintenance. Carrel and Burrows (1910) explained the method of isolating primary epithelial cells from their parent tissues by using the explant method. The current project followed the principle of periodontal cell harvesting using the explant method. In this study, periodontal cell growth was first seen from the explant periodontal tissue on the 3rd day. Previously, Orazizadeh et al., 2015 isolated the primary fibroblasts and observed the cell growth after 4 days of culture (Keira et al., 2004, Sriram et al., 2015). (Siengdee et al., 2018, Punita et al, 2018) witnessed the successful outgrowth from skin fibroblast during skin biopsy between 4 to 12 days. The fibroblast outgrown from the periodontal ligament tissue was found to be spindle shaped while observing the morphology under the light microscope.

Periodontal cells were characterised at single cell level, evaluated for their surface characteristics among the population of periodontal cells using multi parametric flow cytometry. The isolated cells showed mesenchymal cell characteristics including self-renewing capacity. Tri-lineage differentiation characteristics were evaluated during this study. Periodontal cells were subjected to growth under different conditioned culture media such as osteogenic media, chondrogenic media, and adipogenic media. This was done to assess their potential lineage to convert into the cells as required during regeneration.

Periodontal cells were cultured with different media as mentioned above and the cell was left to grow for 21 days (about 3 weeks). However, the media was changed as and when required or every third day, whichever arrives first. Periodontal cells from 3 donors used in this study showed the trilineage capacity in a study conducted by Szepesi et al, 2016 where the author characterized the cells from the periodontal ligament, adipose tissue, and Wharton jelly (Szepesi et al., 2016). They found a correlation between calcium formation and CD90 expression, and also confirmed that periodontal regeneration ought to be more osteogenic than adipogenic process. Similarly, Ma et al 2019 showed the multilineage properties of the cells grown from an amniotic membrane, umbilical cord and chorionic plate, and found similarly the cells showed a higher osteogenic potential by the cells (Hendrijantini and Hartono, 2019). Another study Heidari et al, 2013 observed the cells proliferation and multilineage potential by the liver, bone marrow, and spleen cells.

While the above studies advocated the use progenitor cells to promote the growth of stem cells towards a trilineage growth pattern aimed at the production of either osteogenic cells, or chondrogenic and adipogenic cells. They found that progenitor cells have more potential towards adipogenic growth. If this can be mimicked to produce periodontal cells, this can help to regenerate the loss of bone during periodontal diseases.

4.4 Expression of proinflammatory mediators.

This section compares and contrasts the expression of inflammatory biomarkers at mRNA level in the in-vitro periodontal model in this project. The cell lines and primary cells were stimulated with either TLR4/TLR5 agonist (LPS/Flagellin respectively) with

the aim to induce the expression of cytokines i.e., IL-8, IL-6, IL-1 β , and TNF- α . The different inflammatory markers studied include interleukin-8 (IL-8), interleukin 6 (IL-6), interleukin 1 beta (IL-1 β), and tumor necrosis factor-alpha (TNF- α).

The neutrophil function in its response to IL-8, IL6, IL-1 β and TNF- α is characterised by the destruction of connective tissue by cell migration and release of granular enzymes is recognised in periodontal diseases (Bikel M et al., 1993). Therefore, the role of cytokines in periodontitis is studied widely and presents its expression as an important etiological marker during chronic periodontitis.

IL-8 is a chemotactic cytokine that activates the neutrophils in the periodontal tissue to induce inflammation (Correa, 2010, Bastos et al., 2009). This is the highest expressed cytokine in response to inflammatory stimulation. In a study of 19 patients with periodontitis, the researchers reported similar but not identical overall excessive production (Noh, Jung et al. 2013). Another study informs us about the role of IL-8 in acute inflammation whereby anti-IL-8 treatment prevented neutrophil-dependent damage in conditions like arthritis and lung perfusion injury (Harada, Sekido et al. 1994). IL-8 is the cytokine produced by epithelial cells and is considered the first line of defence against pathological microorganisms (Baggiolini et al., 1989). Human gingival epithelial cells when exposed to 1- 10 μ g/ml LPS *P.gingivalis* for 24 hours, the expression of IL-8 was upregulated (dose and time dependent). A meta-analysis evaluating 31 studies till February 2016 showed ample literature exploring IL-8 expression in chronic periodontitis (Finoti et al., 2017). However, there is still a paucity in studies at gene level.

IL-6 is produced by fibroblasts, keratinocytes, epidermal cells, and mononuclear cells. It was reported that IL-6 is produced by both healthy cells and the cells undergoing periodontal diseases. Although it is produced by many cells, it needs a stimulus to get

expressed such as LPS or flagellin (Yamazaki et al., 1994). IL-6 is considered a bone-destructive cytokine and a dose-dependent bone inhibitor, secreted by osteoblasts on having their encounter with IL-1 β , TNF- α , Flagellin, or LPS (Irwin et al., 1998c). An in-vitro study looking at the effects of IL-6 during orthodontic treatment found that patients undergoing orthodontic treatment have elevated levels of IL-6 during periodontitis (Rath-Deschner et al., 2022). Zhang et al., 2008 preincubated THP-1 cells for 0.5 hours with 1 μ g/ml with LPS *P.gingivalis* and reported an increase in the expression of IL-6 (Zhang et al., 2008) without expressing any cytotoxicity. Similarly, Kent et al., 1999 explored the expression of IL-6 from diseased and healthy periodontal cells. They found that pre-treatment of diseased periodontal cells with IL-1 β followed by incubation with IL-1 β produced even higher IL-6 (Kent et al., 1999).

IL-1 β is a protective pro-inflammatory cytokine produced by an innate or non-specific immune system and is considered to have a role in the pathogenesis of periodontitis (Hönig, Rordorf-Adam et al. 1989). On stimulation of human periodontal ligament fibroblasts with LPS, it upregulates the OPG and RANKL expression by increasing the expression of IL-1 β (Wada, Maeda et al. 2004). IL-1 β is produced as a pro-peptide on the interaction of bacteria with TLR on cells while producing inflammatory signals. Once released by the cell, IL-1 β signals activation of pro-inflammatory cytokines release and initiates the inflammation (Tulotta and Ottewell 2018).

In this study, the identification of IL-1 β expression was evaluated based on stimulating the cell lines and primary cells with LPS. In this study the increased expression of IL-1 β was observed due to the presence of inflammatory mediators in the culture media. The pre-exposure of CBG showed the reduced expression of inflammation section 3.3. Similarly, Suzaki et al., 1999 studied the effects of the pre-treatment of monocytes with roxithromycin on their suppressive effect of IL-1 β expression on stimulating

monocytes with LPS (Suzaki et al., 1999). Therefore, the anti-inflammatory effect could be helpful in addressing chronic inflammation during periodontitis. During chronic lung disease, the continuous expression of cytokines plays an important role in pathogenesis, Literat et al., 2001 reported that pre-treated lung inflammatory cells with 0.5 and 20 μ M curcumin shows a decrease in the expression of IL-1 β (Literat, Su et al. 2001).

TNF- α is known as an adipokine or chemokine, produced by macrophages/monocytes involved in immune-mediated disorders such as rheumatoid arthritis. It is responsible for cellular events such as necrosis and apoptosis. It exerts its function by binding to a membrane receptor called TNFR-2 by polarising the macrophages towards pro-inflammatory actions to provide autoimmunity (Clarke 2021). TNFR-2 receptor was reported to be expressed by fibroblasts and neutrophils. It found modulating TNF- α mediated inflammatory response in severe periodontitis in a study on a Japanese population (Shimada et al., 2004). Yongchaitracul et al., 2006 pre-treated periodontal cells with 6 μ g/mL anti-TNFR-1 and 2 μ g/mL anti-TNFR-2 before exposing the cells to 1ng/mL TNF- α for 24 hours. They found that the induction of M-CSF and recruitment RANK through TNFR-1 facilitates osteoclast recruitment during periodontitis (Yongchaitrakul, Lertsirirangson et al. 2006).

It was suggested that secretion of the above immunomodulators (IL-8, IL-6, IL-1b, and TNF- α) could be determining factors to define the severity of the inflammation process by any tissue (Zaga-Clavellina et al., 2007).

Expression of these inflammatory markers (IL-8, IL-6, IL-1b, and TNF-a) can be monitored at both gene and protein level. Studies inform us of the infiltration of neutrophils to periodontal cells during periodontitis and when this happens, IL-1b & TNF- α are expressed in response to inflammation. Expression of inflammatory

markers such as IL-8 was found to be reduced when periodontal cells were exposed to CBD (Napimoga, Benatti et al. 2009). This finding is in accordance with the current study. Other studies have also shown that cannabidiol has the ability to arrest neutrophil migration to the periodontal cells. In other words, cannabidiol suppresses the expression of inflammatory cytokines in plasma (Weiss, Zeira et al. 2006). CBD was found effective either used orally or intraperitoneal in a mice model, and the lymph node of the mice model is found to drain less IFN- γ . As a result, there is a decrease in the expression of TNF α (Malfait, Gallily et al. 2000). This suggests the anti-inflammatory properties of CBD. Thus, cannabidiol treatment with up until 10 μ M was shown to suppress the TLR induced suppression of up to 25 % of IL-1 β production by monocytes (Sermet, Li et al. 2021). This project identified a decrease in IL-1 β production in the presence of 10 μ M CBG at protein level (ELISA).

A Randomized Controlled Trial (RCT) conducted in Belgium reported that mouthwash containing cannabidiol and cannabigerol was marginally more bactericidal compared with chlorhexidine mouthwash. A following study confirmed a previous finding where the author stated that CBD enhances the bactericidal efficacy of antibiotics by inhibiting the bacterial membrane vesicle formation in gram negative bacteria (Kosgodage et al., 2019, Vasudevan and Stahl, 2020).

With regards to cell lines TIG-K and periodontal primary cells were evaluated for their potential to be used as a cell model of inflammation replicating in-vitro periodontitis. The findings herein demonstrate that Flagellin can induce an inflammatory response in TIG-K cells alongside LPS found capable of induction of inflammatory response in the primary periodontal cells. It has been established that Flagellin is a virulent factor found in Gram-negative bacteria (Hayashi, Smith et al. 2001) reported to act as

obnoxious stimuli to provoke the innate immune system via toll-like receptors (Hajam et al., 2017).

The oral microbiome consists of approximately 6 billion bacteria, amongst which >25% are gram-negative bacteria. These Gram-Negative bacteria exist in harmony with other bacteria within the oral microbiome, if this homeostasis is breached and the gram-negative bacteria is outnumbered, it results in periodontitis. Out of these bacteria, *P-gingivalis* is considered one of the periodontal pathogens. The LPS – the outer membrane component of gram-negative bacteria, including *P gingivalis*, had been identified as a virulent, etiological factor in periodontal disease (Singhrao et al., 2015).

LPS acts through stimulation of inflammatory reaction by interacting with the Toll-like receptor (TLR 4), whereas Flagellin acts through receptor TLR5 (Hayashi et al., 2003) which are found on the cell membrane of periodontal cells. Various in-vitro model studies used different concentrations of flagellin and LPS to induce inflammation, such as in TIG-K cells (Guha Niyogi, 2016) and HPDLCs cells (Guo and Li, 2022). The concentration used to induce inflammation varied from high 5 µg/ml (Diomedea et al., 2017), 1 µg/ml to lower concentrations of 100 ng/ml (Quin et al., 2013).

It is well known that the inflammatory stimulation with LPS and Flagellin activates the adaptive immune system through the process of antigen presentation. Antigen presentation is a vital immune process essential for T cell immune response triggering. The release of bacterial LPS and Flagellin instigates expression of e-selectin, intercellular adhesion molecules (ICAMs), and interleukin-8 which facilitates migration of neutrophils from the vascularised gingival tissue to gingival crevice crossing the protective junctional epithelium (Darveau et al., 2010). IL-8 can be secreted by gingival cells which are a part of the innate immune system possessing TLRs (Roebuck 1999).

It increases intracellular calcium and induces chemotaxis to granulocytes and causes them to migrate toward the site of bacterial infection during periodontitis. By presenting the periodontal fibroblasts with LPS *P.gingivalis* or flagellin, the expression of IL-8 was increased thereby confirming the immune capability of the periodontal cells.

Based on a previous study (Shi H et al., 2016), the safest LPS dosage advised for induction of inflammation was 1 µg/ml. Therefore 1 µg/ml was selected in the present study for LPS and Flagellin as well. At this concentration, both LPS and Flagellin showed no cytotoxicity on TIG-K cells.

Flagellin was used in an in-vitro study to induce the inflammatory response and was evaluated for expression of IL-8, IL-6, and TNF-α. The amount of flagellin used was from 1 to 10µg. These doses initiate the expression of IL-6 and TNF-α from hour 1 and reach their peak of expression at 4 hours. It followed a progressive decrease in expression for about the next 20 hours. LPD was not effective in the expression of IL-8 whereas flagellin was found effective to express the inflammatory mediators in TIGK cells. This result was expected as LPS acts through the interaction with the Toll-like receptors (TLR-4), whereas Flagellin acts through TLR5 receptor (Hayashi, Means et al. 2003). Similarly, flagellin was used to elicit the inflammatory response due to its TLR5 response in a mice study to study acute lung inflammatory response (Liaudet et al., 2003).

In this study, the chosen time periods were at 3 hours and 24 hours post-stimulation either with LPS or Flagellin due to the fact the expression of cytokines were found to expressing at their higher concentration at these times (DeForge, 1991).

4.4.1 Influence of cannabinoids on the expression of inflammatory biomarkers

Cannabinoids are currently believed to be cytotoxic and even proven to be toxic over a certain amount or concentration (Nahler, 2022). In actuality, cannabinoids are

effective in downregulating the destructive effects of the cytokine cascade. Cannabinoids are otherwise found to show their regulation of immune response in protecting the tissue damage due to the autoimmune deleterious effect of inflammation. This can be witnessed while reviewing its reported benefits to address chronic inflammatory diseases such as multiple sclerosis, and rheumatoid arthritis.

The aforementioned paragraphs have provided compelling evidence of the usefulness and therapeutic effects of cannabinoids. Based on this premise, it is therefore important to identify the maximum effective concentration and at the same time keep it as low to have any toxic effects on cell growth. To this end, the project aimed to investigate the functional non-cytotoxic concentration of the cannabinoids used. As such, these compounds were tested for their cytotoxicity. Importantly, cumulative evidence shows that pre-treatment of 1 and 10 μM CBD or CBG on the three above-mentioned cell lines and primary periodontal cells rescued the apoptosis of the cells. In addition, non-psychoactive cannabinoids (CBD) were explored to identify their potential role in the regulation of inflammation in the prevention and treatment of periodontitis (Napimoga et al., 2009). An emergent question is whether CBD can reverse the induced inflammation in TIG-K cells by flagellin. Cannabinoids were found to have anti-inflammatory properties. Along with their anti-inflammatory properties, they were also found to possess antimicrobial, antioxidant, and immunomodulatory properties (Larsen and Shahinas 2020), (“Cannabidiol, Neuroprotection and Neuropsychiatric Disorders”)(Campos et al., 2016). However, others report that cannabis is the causative agent in periodontal diseases (Chisini, Cademartori et al. 2019). The conclusion was based on the findings that cannabis smoking involves the inhalation of non-purified smoke which contains ingredients other than CBD and CBG. It can be suggested that the inhalation of smoke is directly related to triggering

periodontitis. However, this report was challenged because the researchers inform us that smoking cannabis was not related to periodontitis in adolescents (López and Baelum 2009).

The aforementioned section discussed the effects of cannabinoids on different cells. When cells are cultured either with inflammatory agents such as LPS and flagellin or when it is pre-exposed to cannabinoids, the cells were evaluated for their efficacy in the production of biomarkers. If the efficacy is reduced, the resultant inflammatory biomarkers would be expressed less, this will be beneficial to prevent the cells from destructive autoimmunity. This means the detrimental effects of periodontitis can be prevented.

It has been established that when cells are cultured with inflammatory agents, biomarkers are expressed. The following section deals with firstly, estimating the expression of inflammatory biomarkers such as IL-8, IL-6, IL-1b, and TNF-alpha. Secondly, an exposition of the effect of pre-exposure to cannabinoids on the expression of inflammatory biomarkers by the cells. Since cells express inflammatory biomarkers at both the protein and mRNA levels, the last section discusses the expression of released biomarkers at the protein level and mRNA levels. This discussion is important as it reveals the effects of cannabinoids on the expression of protective inflammation by the cells.

Exposure of cannabinoids to the cells

As discussed previously in the introduction, the cannabinoids act on the cells via cannabinoid receptors, or by having their impact on the inflammatory pathways. The outcome of cannabinoid exposure depends on the length of time the number of cannabinoids presented to the cells. For example, in this study, CBD and CBG were

presented to the cells for 1 hour before presenting the inflammatory agent in order to mimic the in-vitro periodontitis model. This was done to evaluate the pro-inflammatory properties of the above-mentioned two cannabinoids. The cannabinoids, however, were presented to the different cells used in the study, for a longer duration too. This was done in order to evaluate their toxic effects due to a longer culture time, if any. Additionally, the cells cultured in the media containing cannabinoids were removed and cultured in the media without any cannabinoids. This was done to compare the cell growth of cell post exposure to cannabinoids as compared to the control cells cultured for a similar duration but had never been exposed to the cannabinoids. The reason underpinning this additional process was to evaluate the effect of post-exposure to cannabinoids on the cells. This is an important part of the experiment as it can confirm if a longer-duration culture shows any residual effects of cannabinoids. If the residual effect of CBD remains, then we might need not expose the cells to the same amount of the cannabinoid or for the same duration. Therefore, looking after the residual effect could be helpful in two ways; firstly, we can rule out the residual toxicity of the compound, and secondly in deciding the dosage of the compound for further treatment. This was not studied during this project as this is beyond the scope of the present study.

Previous literature shows the anti-inflammatory properties of cannabinoid receptor 2 agonist HU-308 where periodontitis was induced on a rat model by injecting the LPS in maxillary and mandibular gingiva. They treated the oral tissues in a rat model with LPS-induced periodontitis (Ossola, Surkin et al. 2016). The concentration of LPS used in the above study i.e., 1µg/ml, correlated with the concentration used in the present project.

The different cannabinoids used in this project are as follows:

1. Cannabidiol (CBD) - a non-psychoactive component of cannabis was procured from Tocris, USA and the vehicle was ethanol. One reason for its usage in this study is an experimental rat model of periodontitis reported that CBD can reduce bone resorption due to the fact that CBD possess anti-inflammatory properties (Napimoga, Benatti et al. 2009).
2. Cannabigerol (CBG) - a non-psychoactive component of cannabis was procured from Tocris, USA and the vehicle was ethanol. CBG when used as a concentration of 7.5 μ M possesses an antioxidant property and increases cell viability. Furthermore, when the NS3 motor neurons are pre-exposed with CBG it reduces the expression of inflammatory mediators i.e., IL-1b and TNF-a (Gugliandolo, Pollastro et al. 2018).

4.4.2 Expression of biomarkers at mRNA level

In this section, the gene expressions are summarised in relation to the biomarkers such as IL-8, IL-6, IL-1b, and TNF-a and their differential expression identified by various researchers. In this study, the expression of IL-8 was found to be consistently higher by the cell line TIGK (section 3.3) and primary cells (results not shown). A previous study showed that levels of IL-8 were higher in the saliva of patients with periodontitis as compared to their healthy counterparts (Huang, Tseng et al. 2021). While few studies found the opposite results and observed a lesser amount of IL-8 in the saliva of patients with periodontal disease (Mathur, Michalowicz et al. 1996, Chung, Grbic et al. 1997). At the mRNA level, our findings showed that the inflammatory flagellin response was attenuated when TIG-K cells were pre-treated with CBD; it reduced the expression of IL8 by 325 times when TIG-K cells were pre-treated for 1 hour with 1 μ M prior the treatment with flagellin for 3 hours. The increase

of CBD concentration to 10 μM , did not reduce further the effect of flagellin, as the reduction remained at the same level to that observed with 1 μM .

4.4.3 Expression of biomarkers at the protein level

When cells are exposed to any environment, they respond either by expressing the biomarkers or exhibiting a change in their behavior or health. In this project, different cells were treated with inflammatory agents such as LPS and Flagellin, and the level of expression of proteins was evaluated by estimating the markers expressed in the culture media and the expression of cytoplasmic proteins. The cytokines expressed and secreted in the supernatant culture media of the cells, when they were pre-treated with cannabinoids, and then exposed to inflammatory mediators, were evaluated by a technique such as enzyme-linked immunosorbent assay (ELISA). The cytokines expressed at cytoplasmic levels were also evaluated using western blot.

The expression of IL-8 at the protein level was observed at 3- and 24 hours post-exposure to flagellin. The choice of both post-exposure to flagellin times (3 and 24 hours) was dictated by the finding of a previous study which showed that IL-8 expression at an early stage of inflammation is biphasic; it increases in the first six hours and further increases from 12 to 24 hours (DeForge et al., 1992).

At the protein level, the finding in the present study showed a marked upregulation of IL-8 secretion by around 500 times by TIG-K cell exposed to flagellin for 3 hours. At 24-hour exposure to flagellin, the level of IL-8 secretion remained almost at the same level as that shown at 3 hours. In regard to cytoplasmic proteins the expression of IL-8 was observed in the presence of LPS, cannabinoids reduced the LPS induced IL-8 in cell line and primary periodontal cells. The importance of suppression of IL-8 has been studied widely, as it has been identified as an important factor during

periodontitis (Shindo, 2019). Therefore, this study aimed to witness and confirm the reduction of expression of cytoplasmic IL-8 due to pre-exposure of periodontal cells with cannabinoids.

Also, when TIG-K cells were pre-treated with CBD followed by incubation with flagellin; it reduced IL-8 secretion by 60 % when TIG-K cells were pre-treated for 1 hour with 1 μ M and 70 % when TIG-K cells were pre-treated for 1 hour with 10 μ M.

Periodontal cells when pre-exposed with cannabinoids the expression of secretory protein (IL-8) (ELISA) and cytoplasmic protein (western-blot) was found to be reduced. At the protein level, the finding in the present study showed a marked upregulation of IL-8 secretion by TIG-K cell when they were exposed to flagellin for 3 hours. An increase of IL-8 expression was found to be responsible for periodontal disease induction and progression (Dongari-Bagtzoglou, 1998).

4.5 Summary

In summary, this project investigated the role of cannabinoids to modulate the expression of pro-inflammatory biomarkers, namely IL-8 and IL-6, as a potential therapeutic target in preventing the potential damage of periodontal apparatus by the initial inflammation during periodontal disease. Bacterial toxins such as LPS and Flagellin are known to be associated with periodontal inflammation. To limit effects of bacterial toxins, inflammation within the periodontal cells is a manifestation of self-protection – this is orchestrated by periodontal cells. In order to mimic the periodontitis-like condition, cells were presented with bacterial toxins in a 2-dimensional cell culture. Therefore, cells of different origin and make were assigned having known the complexity and uniqueness of periodontal apparatus. This consists of cells of different origin, for example oral epithelial cells (TIGK), extra-oral epithelial cells (A-2780), non-

epithelial cells (G-292) and primary cells (Periodontal cells). Given that the oral environment is dynamic and various bacteria cause periodontal disease, two different inflammatory agents were used to trigger the inflammation. Cannabis alkaloids were hypothesized to prevent periodontitis (multifactorial chronic inflammatory disease), as previously cannabinoids were found useful during chronic inflammatory diseases such as multiple sclerosis and rheumatoid arthritis. CBD and CDB were used to assess its efficacy at reducing the expression of inflammatory biomarkers.

This thesis has demonstrated the absence of cytotoxicity due to the presence of experimental concentration of cannabinoids and inflammatory agents to the cells. To test the robustness of the results, various concentrations of CBD and CBG were applied to the test cells, in line with existing literature on cannabinoid toxicity. The decision to use varying levels of concentration of inflammatory mediators was maintained at 1µg/ml throughout the study. Throughout this thesis, the rationale for using this concentration has been discussed. With regards to the choice of cells used i.e., primary cells and cell lines, this was because these cells are consistent with the heterogeneity of periodontal cells, an important criterion for the study of periodontitis in general, and bone loss associated with periodontitis, in particular. Primary cells procured from extracted human teeth were then characterised to confirm the usage of periodontal fibroblasts. Different concentrations of cannabinoids was selected for pre-exposing the cells, showing its role in decreasing the expression of inflammatory biomarkers. The discussion showed the decrease of expression of biomarkers at mRNA level and protein level. The pre-exposure of cells to CBD and CBG was found to be effective in reducing periodontal inflammation. However, levels of reduction varied according to cell types.

4.6 Future directions

This study revealed that cannabinoids is effective in reducing the effects of the inflammatory expression during periodontal disease like conditions. Two-dimensional monolayer cells culture was used in this study. Arguably, future studies could advance this study by using 3d organoid culture areas to be a step closer to in vivo situation. Also, it can be recommended that in-vivo culture and studies performing the experiments may yield better results for wider application. One limitation of this study is that only four interleukins were used. It can be suggested that usage of a higher number of inflammatory interleukins, such as IL-17 would provide deeper insight to the disease and subsequently provide a more effective management option. A combination of cannabinoids such as CBD & CBG may have better anti-inflammatory effects therefore different combinations of cannabinoids are recommended in future studies. Finally, exploration of the mechanism of action of these cannabinoids in relation to reduction of inflammatory mediators' secretion would be possible if the knockdown studies are planned in future. The role of these cannabinoids holds promising future on clinical trials if the research is translated on animal models for their toxicity and minimal effective concentration are formulated. As discussed previously about these compounds having antimicrobial property, effect of these cannabinoids on over biofilm is highly recommended.

Chapter 5 Conclusion

To conclude, periodontitis is a chronic disease where chronic inflammation causes irreversible damage to the periodontium. The destruction of the periodontal apparatus is caused by the cumulative effect of periodontal pathogens such as *P gingivalis*, and the aberrant immune cascade of the host immune response. Cannabinoids were assessed and tested for their cytotoxicity on different cells and their anti-inflammatory potential.

This study investigated the anti-inflammatory potential of cannabinoids by pre-exposing the cells to different cannabinoids. The findings showed that these cannabinoids were effective in reducing the expression of pro-inflammatory biomarkers before the cells were exposed to periodontal disease-like situations.

This in-vitro study has investigated the effect of cannabinoids (CBD and CBG) on the expression of inflammatory biomarkers on different cell lines such as G292, A-2780, and TIG-K by observing the expression of inflammatory biomarkers. Prior to observing the expression of inflammatory biomarkers and the effect of cannabinoids on their expression, the cells were assessed for any toxicity due to the agents the cell were treated with such as LPS, flagellin, and Cannabinoids. Thereafter the primary periodontal cells were assessed for their expression potential of the biomarkers in the presence of bacterial toxins e.g., LPS and flagellin. This study draws the conclusion that the cells express manifold release of inflammatory biomarkers such as IL-8, IL-6, IL-1b, and TNF-a in interacting with the inflammatory mediators. The expression was further assessed by pre-exposing the periodontal cells with 1 and 10 μM concentrations of cannabidiol (CBD) and cannabigerol (CBG) for 1 hour followed by presenting the cells with LPS for 3 hours, and 24 hours. The reduction in expression

of inflammatory biomarkers was observed when the cells were pre-exposed to the above-mentioned cannabinoids.

The research outputs from this project were presented and published at scientific conferences and drafted to submit for publication in peer-reviewed journals.

The detrimental inflammation causing the destruction of periodontal apparatus during chronic periodontitis is worrying because it leads to the destruction of alveolar bone locally and has a negative impact on general health. This study can confirm the reduction of expression of the pro-inflammatory biomarker under the CBD and CBG pre-treatment. Cumulative research informs us that NSAIDs are effective in reducing the inflammatory cascade during periodontitis. However, the use of NSAIDs is limited due to their side effects and therefore, is not the recommended treatment for pathological chronic inflammation during periodontitis. Conversely, cannabinoids can be a potential anti-inflammatory agent to address periodontal problems. Additionally, cannabinoids were claimed to possess anti-bacterial properties as well, therefore further studies investigating the anti-bacterial effect alongside the anti-inflammatory potential can be recommended.

5.1 Strengths and limitations:

One of the strengths of this study is that it has assessed thoroughly the cytotoxicity of the compounds used on various cells. It has evaluated the effects of two inflammatory agents responsible for the induction of periodontal diseases. Furthermore, this project has included the evaluation of four different biomarkers expressed during periodontitis, whereby the cells were pre-exposed with two different cannabinoids to evaluate the potential anti-inflammatory action of these cannabinoids.

COVID-19 constituted a force majeure event impacting various aspects of this study. The outbreak of COVID-19 occurred during research affecting the timeline of the study, and the storage of reagents and cells. In addition, this research was self-funded, and many planned experiments were not possible due to the cost of experiments incurred. However, the research was carried out on time even with the required multiple repeats of many experiments.

In terms of limitations of this study, this is in-vitro, as such the scientist has not been able to observe the dynamics of the living environment i.e., periodontal cells multiplying in the human oral cavity. To this end, it is not possible to assess the effects of environmental factors such as interaction of saliva with cannabinoids, which contains antibacterial properties, and bacterial species which are responsible for the maintenance of homeostasis within the oral cavity. Saliva provides the medium for the growth of both synergistic protective and pathological bacteria which is the body's natural protective mechanism alongside the digestive ability of saliva. Therefore, the effects of cannabinoids observed in an in-vitro environment are not identical to the living environment. Nonetheless, future studies could employ the use of human saliva as a comparative study.

Another limitation of this project relates to its usage of two-dimensional monolayer cells. This is because it is not representative of the actual environment in which periodontitis unfolds. Whilst the three-dimensional cell was not planned as part of the study, it does not negate the use of two-dimensional cells culture. The results obtained with the use of two-dimensional cell culture have yielded novel results and also that which is consistent with the existing body of knowledge on the anti-inflammatory effects of cannabinoids.

Furthermore, the use of two-dimensional monolayer cell culture in contrast to three-dimensional cell growth arrangement in a living environment. The expression of cytokines from the 2-dimensional cell culture was represented by all the cells exposed to inflammatory mediators. However, in actuality, the cell grows in 3 dimensions and the outmost cells interact with the oral bacteria, and the adjacent cell impact the immune system only by receiving information from the front-line cells. This means the three-dimensional cell culture represents cells growing out of contact with inflammatory mediators that will play a passive role in the expression of the immune system. Therefore, future studies recommended to use of 3-dimensional cell culture to confirm the findings of the present study along with animal model studies and thereafter clinical trials based on confirmation of findings.

In terms of future perspectives, cannabinoids can be recommended for the use of periodontal diseases therapy in future studies to assess the effects of the anti-inflammatory properties of cannabinoids on the cells. Also, the investigation of additional inflammatory biomarkers could be helpful to confirm the inflammatory pathways during periodontitis. Additionally, confirmation of the present study using in-vivo models is recommended. This can confirm the expression of an inflammatory process in the presence of a living dynamic environment of the animals and the effect of cannabinoids on the overall dampening effect of the destructive inflammation. Finally, the use of antagonists of cannabinoids is recommended to confirm the effect of cannabinoids.

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Appendix: I

Below are the publications and academic outputs produced during my PhD. The paper published in materials represents a workflow that was not part of my core studies, as such this data is not found in this thesis.

Publications:

Iqbal, N., Braxton, T.M., Anastasiou, A., Raif, E.M., Chung, C.K.Y., Kumar, S., Giannoudis, P.V. and Jha, A., 2022. Dicalcium Phosphate Dihydrate Mineral Loaded Freeze-Dried Scaffolds for Potential Synthetic Bone Applications. *Materials*, 15(18), p.6245.

Attachment and morphological cytotoxicity characteristics of periodontal ligament cells towards CBD and CBG: An in-vitro periodontitis model study. (ongoing)

Presentations:

Poster Presentation: Effects of cannabinoids on chronic periodontal diseases. LIMM (Leeds institute of molecular medicine) symposium, St. James University Hospitals, Leeds 17 April 2018.

Poster: Anti-inflammatory effects of cannabidiol to modulate induced inflammation on gingival keratinocytes. Oral Medicine and Pathology prize BSODR 3-5 sept 2019.

Appendix II

Ethical approval as stated in section **2.2.1.3** was required for my project to receive and work with periodontal ligament for extracted teeth. Below is the e mail received prior to the commencement of my experimental confirming that all ethical considerations and requirements were met. I can confirm that the experimental conformed to the study design submitted.

DREC ref: **160418/SK/248**

Title: Identification of the abilities of cannabinoids to alter biomarker production involved in inflammation and periodontal tissue regeneration

Thank you for submitting the above Tissue Bank to the Dental Research Ethics Committee (DREC). Your application has been reviewed and I am pleased to inform you that it has been accepted for the collection of 20 fresh, sound teeth.

Should you require any further teeth after your allocated 20 have been collected, you will need to re-apply to DREC quoting the above reference number.

Documents reviewed:

Document name	Version number / date
Tissue Bank application form	Dated 11/04/2018
Protocol	Version 1 16/04/2018

With best wishes for the success of your project.

For and on behalf of
Dr Julia Csikar
DREC Chair