General Introduction

Today, high-stressed lives paired with an increase in life expectancy lead to a natural increase in chronic diseases, which inexorably boosts medical and pharmaceutical costs worldwide. Despite the substantial advances in the treatment of complex, multigenic and chronic human diseases, their occurrence rate is still increasing significantly in the recent times. Also, many times traditional clinical treatments are not completely effective and have major disadvantages such as high cost and adverse side effects (Kunnumakkara *et al.*, 2016). Hence, the development of cost effective, readily available, non-toxic and highly effective agents for the management of different human diseases are a rapidly increasing and desired area of research.

1. Matrix metalloproteinases as therapeutic targets

Although complex diseases like cardiovascular diseases, metabolic diseases, cancer and neurological diseases occur due to perturbations of multiple signalling pathways (Bordoloi *et al.*, 2016), there are cases where targeting a single pathway among many may be effective in prevention or cure (Fingleton, 2008). Such is the case of matrix metalloproteinases (MMPs) pathways, which have been implicated in a number of different human diseases and are currently one of the actively pursued targets in drug discovery and development (Fingleton, 2008). MMPs are a family of zinc-dependent endopeptidases which possess the unique role of remodeling and degrading the molecules of the extracellular matrix (ECM) (Said, Raufman and Xie, 2014; Vandooren, Steen, Van den and Opdenakker, 2013; Zucker and Vacirca, 2004). MMPs cleave most components of the extracellular matrix including fibronectin, laminin, proteoglycans and type IV collagen (Heppner Goss, Brown and Matrisian, 1998). Because of this, they regulate a number of metabolic processes (Goldberg, 2015). In fact, proteolysis mediated by MMPs plays a key role in the modulation of cellular homeostasis as it can initiate, enhance or down-regulate signalling cascades involved in growth and inflammation, activate cytokines and release growth factors, by modifying the tissue architecture and degrading structural components of ECM (Kofla-Dłubacz *et al.*, 2014; Marshall *et al.*, 2015).

Although they are a major group within the proteome, being proteolytic enzymes (Puente *et al.*, 2003), the activity levels of proteases such as MMPs are tightly controlled (Folgueras *et al.*, 2004) in a way to maintain homeostasis. It is the deregulated MMP expression that is associated to multiple disease types. For example, an overexpression of MMPs followed by accelerated matrix degradation is related to several pathologies including cancer cell invasion and metastasis, the loss of cartilage in osteoarthritis, rheumatoid arthritis, cardiovascular diseases, acute lung injury, chronic obstructive pulmonary disease, eye and skin diseases and periodontitis (Fingleton, 2007). Due to this, in the last decades, MMPs inhibition quickly became an ideal drug target.

Being dependent on zinc for activity and up-regulated in diseases, MMPs seem a straightforward target. However, as it turned out, targeting MMPs is much more complex than initially realized (Fingleton, 2008). Although disease-associated, the ubiquitous roles of these enzymes make broad-spectrum inhibition unsuccessful; targeting the catalytic zinc with specificity is also difficult, since other related proteases as well as non-related proteins can be affected by some chelating groups (Fingleton, 2008). Hence, the early-generation MMP inhibitors were an overall disappointing failure, with dose-dependent toxicity and severe side-effects (Coussens, 2002; Ndinguri *et al.*, 2012). Nonetheless, there has recently been a wealth of studies examining the basic biology of MMPs which will greatly 'inform' new drug trials in this field (Fingleton, 2008). Because there is a vast number of MMPs within the family, with different sets of activities and specificities for substrates, it is apparent that the key for success may lie on specificity in targeting and the mode of action of the inhibitor itself.

2. Classification of MMPs

Usually, MMPs were classified into six main groups – collagenases, gelatinases, stromelysins, matrilysins, membrane type MMPs and others, unclassified to former groups (Grzela, Bikowska and Litwiniuk, 2011). However, increasing knowledge about their molecular structure, substrate specificity and mechanism of activation contributed to an arrangement of a new MMP classification. According to this classification, MMPs are divided into four groups: archetypal MMPs, matrilysins, gelatinases and furin-

activated MMPs (Fanjul-Fernandez *et al.*, 2010; Hadler-Olsen *et al.*, 2011), as described in Figure 1.

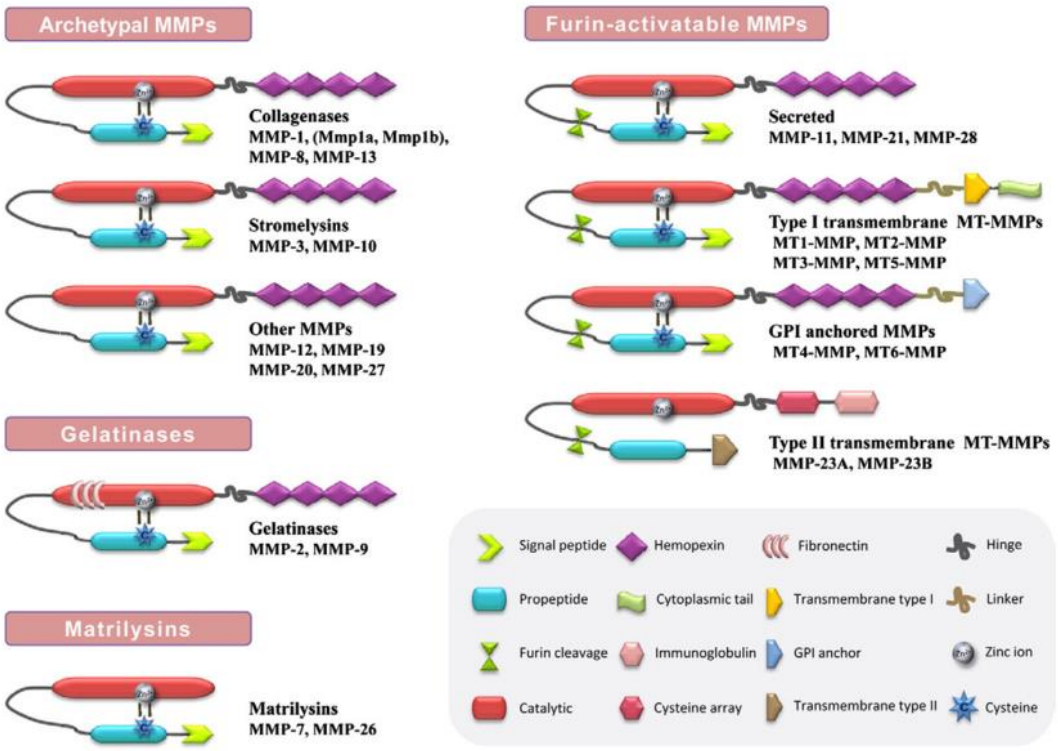

Figure 1. Structural classification of MMPs based on their domain arrangement. Almost all human MMPs contain the signal peptide, a propeptide domain, and a catalytic domain with a highly conserved zinc-binding site. A conserved cysteine in the propeptide domain coordinates with the zinc in the active site to maintain latency on the pro-MMPs. MMPs (except matrilysins, MMP-23A and -23B) contain in addition a hemopexin-like (PEX) domain connected to the catalytic domain *via* a hinge region. Adapted from Fanjul-Fernandez *et al.*, (2010).

MMPs (except MMP-23A and -23B) are characterized by an N-terminus that contains a signal sequence which directs the protein to the secretory pathway. This is followed by a pro-peptide which comprises 80 amino acid residues, containing the "cysteine switch" (Fanjul-Fernandez *et al.*, 2010; Grzela, Bikowska and Litwiniuk, 2011). MMPs have a catalytic site comprising a zinc metal binding domain and are initially synthesized as inactive zymogens (Farina and Mackay, 2014; Vandooren, Steen, Van den and Opdenakker, 2013). This pro-enzyme form is kept inactive by the cysteine- switch (Figure 2), a pro-peptide that binds to the nucleus of zinc via cysteines and must be later activated through proteolysis (Farina and Mackay, 2014).

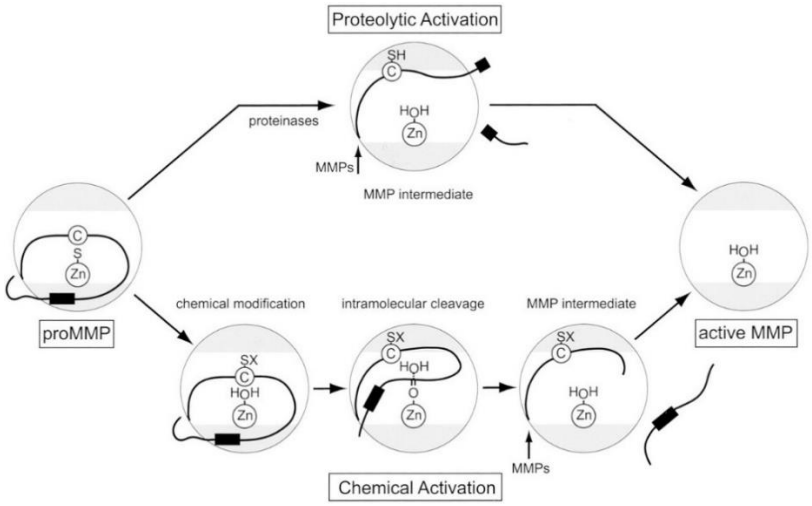

Figure 2. Stepwise activation of pro-MMPs. Pro-MMPs secreted as inactive zymogens can be activated by proteinases or by nonproteolytic agents. The catalytic domain is represented as a gray circle, with the active-site cleft shown in white (not to scale) containing the catalytic-site zinc (Zn). The propeptide is schematically shown as a black line containing the bait region (black rectangle) and the cysteine switch (C). SH indicates the sulfhydryl group of cysteine. Activation by the proteinases is mediated by cleavage of the bait region; this partly activates the MMP. Chemical activation relies on modification of the cysteine switch sulfhydryl (SX) resulting in partial activation of the MMP. Full activation of partially activated MMPs is achieved by completing the removal of the propeptide by intermolecular processing. (Visse and Nagase, 2003).

Most MMPs, excluding MMP-7, -23, -26, have one or more hemopexin domain. This domain is involved in substrate specificity and interacts with cell surface receptors (Fanjul-Fernandez *et al.*, 2010; Farina and Mackay, 2014; Vandooren, Steen, Van den and Opdenakker, 2013).

2.1. The gelatinases MMP-2 and MMP-9

Within the specific family of MMP enzymes there are two types of MMPs called gelatinases, MMP-2 and MMP-9 (gelatinases A and B, respectively) which contain a unique set of three repeats of fibronectin (Figure 3) that facilitate degradation of gelatinous substrates such as elastin, collagen type I and IV, gelatin and fibrinogen (Malla *et al.*, 2008; Toth and Fridman, 2012, Vandooren, Steen, Van den and Opdenakker, 2013).

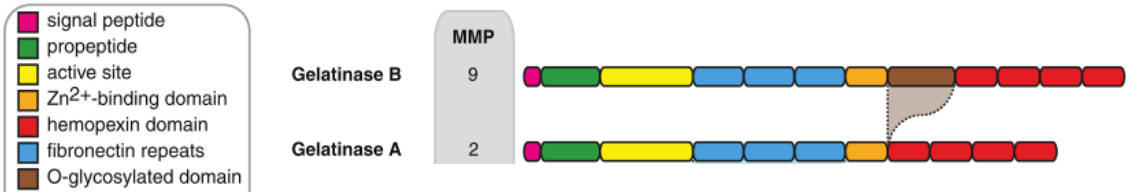

Figure 3. Domain structure of gelatinases group. The MMP-2 and MMP-9 uniquely contain three fibronectin repeats within their catalytic domain. (Vandooren, Steen, Van den and Opdenakker, 2013).

MMP-2 and MMP-9 can bind to inhibitors such as TIMP-1 and TIMP-3, to cell surface receptors and can even induce self-activation (Vandooren, Steen, Van den and Opdenakker, 2013). Exceptionally, MMP-9 contains an *O*-glycosylated (OG) domain before the hemopexin domain present at the carboxyl terminus, as shown in Figure 3 (Grzela, Bikowska and Litwiniuk, 2011; Vandooren, Steen, Van den and Opdenakker, 2013). This OG domain binds to the active site and to the hemopexin domain. Several studies indicate that this domain is essential for the correct functioning of the MMP-9, which might be important for specific inhibition of the enzyme (Vandooren, Steen, Van den and Opdenakker, 2013). Indeed, deletion of the OG domain dramatically reduces the efficiency of the degradation of large gelatinous substrates by MMP-9 (Vandooren, Steen, Van den and Opdenakker, 2013).

Despite the similarity in substrate selectivity for MMP-9 and MMP-2, they are differentiated by tissue specificity. In normal tissues, MMP-2 is constitutively expressed in fibroblasts, endothelial cells and epithelial cells, and may be up or down-regulated in response to inflammatory stimuli (Moore *et al.*, 2011). In contrast, MMP-9 expression is observed in a variety of immune cells, e.g., neutrophils, macrophages, lymphocytes and dendritic cells, as well as in other cells such as osteoblasts, fibroblasts and endothelial cells (Vandooren, Steen, Van den and Opdenakker, 2013).

3. *The role of MMP-9 and MMP-2 in cancer and inflammatory diseases*

3.1. Colorectal cancer and gelatinase activities

Colorectal cancer (CRC) is the second most common cancer diagnosed worldwide and after lung cancer is the most common cause of death in Europe (Ait Ouakrim *et al.*, 2015; Ferlay *et al.*, 2013). It is the second most common cancer in women, and the third in men (American Cancer Society, 2015). In 2012, 241,600 European men have been diagnosed with this type of tumour and 113,200 men died from the disease. In women, 205,200 cases of CRC and 101 500 deaths associated with the disease were reported in that same year (Ait Ouakrim *et al.*, 2015; Ferlay *et al.*, 2013). During the last decade intensive research has been made to develop novel anti-cancer drugs for CRC, both prophylactic and therapeutic. However, despite the significant advances in diagnosis, screening and treatment, the overall long-term outcome in patients has not significantly changed (Herszényi *et al.*, 2012). Under this context, there has been an intense search on various biological sources to discover novel anti-cancer drugs to combat this disease, which could be used in prevention, in aiding chemotherapy or even in preventing re-incidence (Champ, 2002; Clemente and Arques, 2014; Roy, Boye and Simpson, 2010).

It is currently known that it is not tumour itself that causes mortality and morbidity of patients suffering from CRC but its resulting metastases, which are responsible for 90% of CRC deaths, highlighting the importance of the process by which tumour cells spread from one tissue to another, through the bloodstream, thus colonizing other tissues in the body (Araújo Jr *et al.*, 2015; Guan, 2015). A myriad of studies have shown that MMP-2 and MMP-9 play an essential role in tumour invasion and metastasis (Figure 4) (Guan, 2015; Said, Raufman and Xie, 2014).

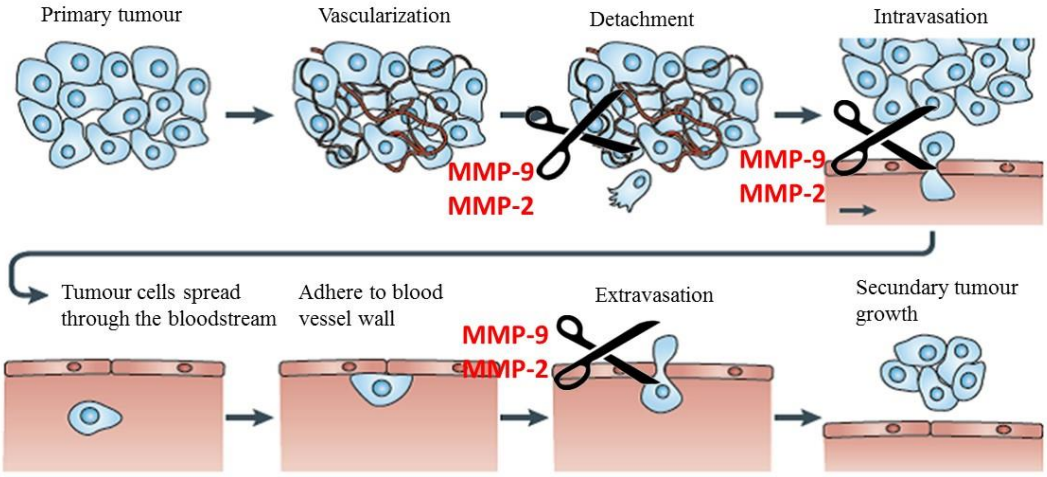

Figure 4. Schematic diagram of the role of MMPs in metastasis process. In this complex process, cells detach from a primary and vascularized tumour, penetrate the surrounding tissue, enter nearby in blood vessels (intravasation). Once at the bloodstream, cancer cell distribution is determined by blood flow and some of this cells adhere to the blood vessel walls and are able to extravasate and migrate into the local tissue, colonizing secondary organs. MMP-9 and MMP-2 have a crucial role in detachment, intravasation and extravasation. Adapted from Wirtz, Konstantopoulos and Searson, (2011).

The increase in expression levels of these two MMPs has been observed in several types of human cancer, as well as in tumour cell lines and animal models (Araújo Jr *et al.*, 2015). Consequently, their inhibitors (MMPIs) were demonstrated to be effective in reducing cancer progression/metastasis in *in vitro* assays and in animal models. In addition, they appear to be most effective at the early stages of cancer or in preventing development of undetected micrometastases after surgery (Coussens, 2002; Herszényi *et al.*, 2012; Mook, Frederiks and Noorden, Van, 2004; Yoon *et al.*, 2003; Zucker and Vacirca, 2004). Because of this, and in view of MMP-2 and MMP-9 involvement in various diseases, inhibition of specific MMPs up-regulation is believed to be able to improve clinical symptoms of patients. However, as with the rest of the MMP family, targeting gelatinases in disease treatment has proven itself difficult due to lack of specificity and previous efforts to inhibit their activity in the treatment of cancer patients yielded very unsatisfactory results with severe adverse side effects (Coussens, 2002; Ndinguri *et al.*, 2012).

3.2. Inflammatory bowel diseases (IBDs) and gelatinase activities

IBD are idiopathic and chronic inflammatory diseases of the gastrointestinal tract. These ailments encompass three types of diseases: Crohn's disease (CD), ulcerative colitis (UC) and indefinite inflammatory bowel disease (IBDU) (Matusiewicz *et al.*, 2014; Siloşi *et al.*, 2014), differing from each other by clinical symptoms, endoscopic findings, pathological features and immunopathophysiology (Siloşi *et al.*, 2014). In UC, the inflammatory process involves only the mucosa and extends continuously from the rectum (Owczarek *et al.*, 2016). In the CD case, the inflammatory process involves the entire wall of the gastrointestinal tract, from mouth to anus, occurring more frequently in the distal ileum and the colon, and being a transmural inflammation associated to other complications such as fistulas, abscesses, and stenosis (Cosnes *et al.*, 2011; Khor, Gardet and Xavier, 2011; Owczarek *et al.*, 2016). Being chronic, remitting and relapsing inflammatory disorders, IBDs have become almost a global disease, affecting millions of people of almost all ages (Cosnes *et al.*, 2011). Worldwide incidence and prevalence of these diseases have been dramatically increasing over recent times, evidencing their emergence as a global ailment (Cosnes *et al.*, 2011). Currently, the prevalence of IBD is more than 200 cases per 100,000 inhabitants in the West (Cosnes *et al.*, 2011), with a major impact on health care resources. Overall IBD clinical treatments are prone to induce side effects, affect unspecific targets, are extremely costly and their curative efficiencies are not acceptable (Cosnes *et al.*, 2011). Furthermore, with low mortality rates (Burisch *et al.*, 2013) and high incidence in the young (Lovasz *et al.*, 2013; Molodecky *et al.*, 2012), it is predicted that IBD globally will continue to increase substantially in the next decades (Cosnes *et al.*, 2011).

Finally, one of the most feared complications of IBD is carcinogenesis, because in the long-term, there is an increased risk of dying from UC-related causes such as, for example, lymphoma, skin cancer and especially colorectal cancer (Duricova *et al.*, 2010; Jess, Rungoe and Peyrin-Biroulet, 2012). Indeed, many of the molecular alterations responsible for sporadic CRC also play a role in colitis-associated colon carcinogenesis (Xie and Itzkowitz, 2008) and the risk of CRC increases with the duration and anatomic extent of colitis, whereas it decreases when patients are treated with anti-inflammatory agents (Wu *et al.*, 2016).

Numerous studies have documented the involvement of these MMPs in inflammatory processes in animal models, cell lines, altered tissue cultures and biopsies of patients (Baugh *et al.*, 1999; Lee *et al.*, 2013; Murphy and Nagase, 2009; Parks, Wilson and López-Boado, 2004). Although similar in their substrate selectivity, MMP-2 is constitutively expressed in fibroblasts, endothelial cells and epithelial cells and is only moderately involved in inflammatory diseases (Huhtala *et al.*, 1991), whereas MMP-9 expression is observed primarily in leukocytes (Steen, Van den *et al.*, 2002), being strongly induced in response to a variety of inflammatory pathologies (Steen, Van den *et al.*, 2002). MMP-9 has been pointed as the main metalloproteinase involved in IBD (Matusiewicz *et al.*, 2014; Moore *et al.*, 2011). As a downstream effector and an upstream mediator of pathways involved in growth and inflammation, MMP-9 expression was found to be elevated under a variety of inflammatory conditions, including ulcerative colitis (Baugh *et al.*, 1999; Liu *et al.*, 2013), showing a strong correlation to both the level of inflammation and to the early IBD development stages (Medina *et al.*, 2006). This makes the gelatinases a good therapeutic target to prevent CRC, as well as to reduce IBDs. However, studies relating MMP-9 inhibition to pre-clinical and clinical IBD reduction are very few.

4. MMP-9 as a therapeutical target

Overall, of all the MMPs, matrix metalloproteinase-9 (Figure 5) stands out as having an important role in wound healing, angiogenesis, inflammation, tumour invasion and metastasis (Ruhul Amin *et al.*, 2003). Considering the links between CRC and IBDs, MMP-9 has turned into a desirable therapeutic target in prevention and treatment. Hence in the last decade the development of synthetic MMP-9 inhibitors (MMPI) became an important branch of research in both academic and industrial settings. So far, numerous MMP inhibitors have been tested in different clinical trials (Hidalgo and Eckhardt, 2001).

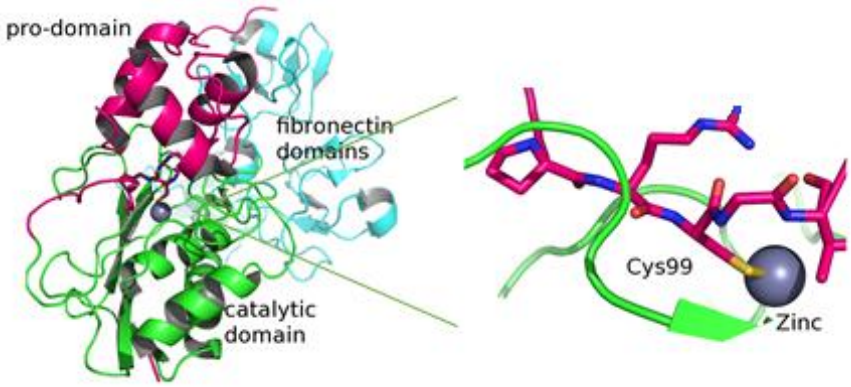

Figure 5. Tridimensional structure of MMP-9. Pro-MMP-9: pro-peptide (pink), catalytic domain (green) with fibronectin domains (cyan), with detail of the "cysteine switch" (from Protein Data Base entry 1L6J).

4.1. MMP-9 inhibition by TIMPs

The Tissue Inhibitors of MMPs (TIMPs) are naturally occurring proteins in the human body which inhibit MMPs. TIMP-1 is the most specific for MMP-9, by binding to the hemopexin domain. TIMPs are not only protein inhibitors but also play a role in cellular growth control, differentiation of oligodendrocytes and induction of erythropoiesis, for example (Vandooren, Steen, Van den and Opdenakker, 2013). Under normal conditions, there is a balance between MMPs and TIMPs. However, a disease condition is associated with imbalances between MMPs and TIMPs and can result in excessive proteolysis due to the presence of freely active MMPs or decreased proteolysis due to excessive inhibition of MMP activity (Vandooren, Steen, Van den and Opdenakker, 2013). Previous studies have attempted to manipulate TIMPs for a more specific and precise inhibition of MMPs but so far they were not successful (Brew and Nagase, 2010; Visse and Nagase, 2003).

4.2. Chemical inhibitors

Another type of inhibitors for MMPs are chemical inhibitors, such as tetracycline, doxycycline and minocycline. These inhibit MMP-9 by decreasing its secretion and consequently reducing the activity of gelatinase B (Vandooren, Steen, Van den and Opdenakker, 2013). Anti-inflammatory molecules such as Interleukin (IL)-4, IL-10, interferon-β (IFN-β) and retinoids (Kurzepa *et al.*, 2014) are also inhibitors of MMP-9

expression. Most of these inhibitors are toxic and non-specific in the human organism and display many adverse side effects.

4.3. Natural inhibitors

A major problem associated to unspecific inhibition of MMP-9 is that this enzyme is involved not only in various diseases but also in remodelling and scar tissue, so its complete inhibition causes several complications in the human body (AbdElazeem and El-Sayed, 2015; Marshall *et al.*, 2015). A distinct and more recent strategy in the search for novel MMP-9 inhibitors is to 'look' among the multitude of natural products that nature placed at our disposal, particularly if they are plant-foods and are already present in our regular diets. Plant organs and tissues, including seeds, have long been known to contain metabolites, peptides and proteins with an array of potentially useful bioactivities. One such bioactivity relates to their capacity to inhibit MMPs. For example, Cyr (2002) provides a huge list of plants (either stressed and non-stressed) whose aqueous, ethanolic and organic extracts exhibit inhibitory activity upon human MMP-2 and MMP-9 enzymes. These extracts encompass secondary metabolites and peptides such as withaferin A, a steroidal lactone derived from *Acnistus arborescens*, *Withania somnifera* and other members of *Solanaceae* family, as well as some of its stable derivatives (e.g. 3-azido withaferin A; Rah *et al.*, 2012), which abolish secretory MMP-2 expression and activity. The flavonoids chrysin, apigenin, genistein and their homoleptic copper(II) complexes have also been reported to attenuate the expression and secretion of the metastasis-relevant MMP-2 and MMP-9 (Spoerlein *et al.*, 2013). Many seeds have also been reported to contain MMPIs, such as those from grape (La *et al.*, 2009), soybean, and dried longan (*Euphoria longana* Lam.) (Panyathep *et al.*, 2013). In the case of grapevine, proanthocyanidins are the MMP inhibitors (Vayalil, Mittal and Katiyar, 2004), whereas in the case of soybean, the flavonoid genistein and the peptide lunasin seem to be the active ingredients (Kennedy, 1995; Seber *et al.*, 2012).

In summary, the presence of MMPIs of natural occurrence may be considered ubiquitous in plant tissues. However, virtually all these suffer from at least one of the following limitations: toxicity; chemical inactivation (e.g. denaturation) or destruction (e.g. proteolysis) during the digestive process; absorption into the blood stream, with or without triggering immunogenic (i.e. IgG) or allergenic (i.e. IgE) responses; destruction

during boiling (e.g. during cooking); no specificity towards the gelatinases and high dose requirements.

5. Legume seeds as a rich source of MMPIs

The Food and Agricultural Organization (FAO) has selected 2016 as the International Year of Pulses, which will highlight the health and environmental benefits they offer. There is now evidence that regular consumption of pulses has multiple health benefits including those on heart health, gastrointestinal health and cancer prevention with improved all-cause mortality (Kouris-Blazos and Belski, 2016; Lee *et al.*, 2016; Mudryj, Yu and Aukema, 2014).

For example, observational studies found that eating legumes is associated with a reduced risk of bowel cancer (Wang *et al.*, 2013). Two meta-analyses from 2009 (Yang *et al.*, 2009) and 2011 (Yang *et al.*, 2011) concluded that there is evidence that legumes provide protection against prostate cancer, lung cancer, breast and colorectal cancers, although much of the evidence is limited to the effect of soy intake. An analysis of the Nurses' Health Study found that those who consumed four or more servings of legumes a week had a lower incidence of colorectal adenomas than women who reported consuming one serving or less (Michels *et al.*, 2006). Researchers have attributed the anti-inflammatory and anticarcinogenic effects of pulses to the antioxidant activities of hydrophilic phenolic compounds, saponins and Bowman–Birk inhibitors (BBIs) (Clemente and Arques, 2014; Kennedy, 1995; Mudryj, Yu and Aukema, 2014; Scarafoni *et al.*, 2008).

5.1. A novel MMPI from **Lupinus albus**

Although there are many studies relating protease inhibitors such as BBIs to cancer inhibition (Roy, Boye and Simpson, 2010; Sang *et al.*, 2006; Seber *et al.*, 2012) few studies identified MMP inhibitors among legume seeds. However, recent work in our group (Lima *et al*., 2016) led to the discovery that the albumin and globulin fractions isolated from specific Mediterranean-type pulses presented high inhibitory activities towards MMP-9, particularly three species: *Cicer arietinum* L. (chickpea), *Glycine max* L, (soy bean), and *Lupinus albus* L. (lupin). However, lupin MMPI standed out for being

the best and its potential as a cancer suppressing agent was demonstrated in colon cancer cells (HT29 cells).

As one of the first protein MMPIs described in plants, we also found that the activity seemed to be stable at low pH values, which suggests it may pass unaltered through the human digestive tract. It also withstood boiling, implying a diet-associated usefulness of the edible seed it derives from. The protein or proteins responsible for this activity are therefore potential candidates for a novel and powerful anti-inflammatory nutraceutical. Nonetheless, much further work is required, such as its isolation and characterization, to ascertain its true efficacy *in vivo*, established required doses and MMP specificity.

6. Main Goals

Findings in our group led to the promising discovery of a mixture of water soluble polypeptides extracted from the edible seeds of *L. albus*, which exhibits a highly potent inhibitory activity towards MMP-9 and/or MMP-2 in colon cancer cells. This mixture of polypeptides is therefore an excellent candidate to become valuable anti-inflammatory nutraceutical agents. Hence, the goal of the present work was to ascertain if these polypeptides can be used as a nutraceutical and functional food in colon cancer and inflammation.

The overall work was aimed towards a more realistic diet approach, both curative as well as preventive, using cell and animal models and a multidisciplinary approach, through a network of collaborations. The overall main goals were:

- To develop a method of extraction for the MMPI protein(s) in *L. albus*, aiming towards feasibility to up-scale and to be used in dietary approaches;
- To identify and characterize the isolated MMPIs;
- Understand its potential as MMP-9 inhibitors, establishing dose-response effects;
- Evaluate its potential for cancer inhibition using cell models of cancer invasion;
- Assess its potential as anti-inflammatory agent, using animal models of colitis;
- To test different forms of administration;
- Finally, results obtained here will be used to submit a patent application.

These goals shall be addressed in three chapters, as follows:

Chapter 1: encompasses the method of extraction of the polypeptide fraction responsible for MMPI in *L. albus*, which was named deflamin;

Chapter 2: relates to the anticancer activities of deflamin, involving dose-response effects in colon cancer cells and the identification of the polypeptide mixture of deflamin;

Chapter 3: describes the anti-inflammatory activities of deflamin in animal models of colitis.

7. References

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Chapter 1

An up-scalable, cost effective methodology for isolating deflamin, an MMP-9 inhibitor polypeptide mixture from *Lupinus albus* seeds

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1. Introduction

Plants have long been utilized as an important natural source of compounds for medical therapy. It has been estimated that over the last 20 years, 25 to 30% of the new drugs entering the US market were discovered in plants (Bent, 2008; Ekor, 2014). Worldwide, the over-the-counter value of these drugs sustains multi-billion dollar incomes (Samy, Pushparaj and Gopalakrishnakone, 2008). During the last decades, intensive research has been made to develop novel anticancer and anti-inflammatory drugs, both prophylactic and therapeutic, from plant-food products or bioactive plant compounds (Su *et al.*, 2006). One attractive target for these bioactive compounds in cancer therapy is a group of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases (Herszényi *et al.*, 2012; Lee *et al.*, 2013), particularly two members of the MMP family, the gelatinases, MMP-9 and MMP-2, which have for long been recognized as important key-players in the degradation of extracellular matrix proteins during inflammation (Heimesaat *et al.*, 2011; Parks, Wilson and López-Boado, 2004) and in oncologic processes such as

tumourigenesis, cell adhesion and metastasis (Baugh *et al.*, 1999; Garg *et al.*, 2009). As a result, many synthetic MMP inhibitors (MMPIs) were developed as potential anticancer drugs. Well known examples are provided by tetracycline, zoledronate, ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, 2*S*,3*R*-3-amino-2-hydroxy-4-(4-nitrophenyl)butanoyl-L-leucine, and neovastat® (isolated from shark cartilage). Up to now, a myriad of MMPI has already been synthesized, some of which have been used as potential therapeutic agents to limit tumour progression (Bourguet *et al.*, 2012). However, only a few small MMPIs entered the clinical trial stage, most of which terminated prematurely either due to lack of benefits or to strong adverse side effects (Wang *et al.*, 2012). A distinct and more recent strategy is to search for MMPIs among the multitude of nontoxic natural products that nature placed at our disposal. There is abundant evidence in the published literature concerning the anticancer activities of many edible foodstuffs. Cyr (2002) provides a huge list of plants whose aqueous, ethanolic and organic extracts exhibit inhibitory activity upon human MMP-2 and MMP-9 enzymes. These extracts encompass secondary metabolites and peptides, such as withaferin A, a steroidal lactone derived from *Acnistus arborescens*, *Withania somnifera* and other members of *Solanaceae* family (Rah *et al.*, 2012), which abolishes MMP-2 expression and activity; the flavonoids chrysin, apigenin, genistein and their homoleptic copper(II) complexes, which have also been reported to attenuate the expression and secretion of MMP-2 and MMP-9 (Spoerlein *et al.*, 2013), as has curcumin from *Curcuma longa* (Jeong *et al.*, 2003). Many seeds have also been reported to contain MMPIs (La *et al.*, 2009; Panyathep *et al.*, 2013). Legume seeds are no exception and have been long recognized to contain a variety of proteinaceous enzyme inhibitors, such as the trypsin inhibitors and the Bowman-Birk inhibitors (BBIs; Clemente and Arques, 2014; Duranti *et al.*, 2008). However, although the presence of MMPIs of natural occurrence may be considered ubiquitous in plant tissues, virtually all these suffer from at least one of the following limitations, handicaps, disadvantages or weaknesses when we consider their possibility of clinical and/or nutraceutical application: toxicity; chemical inactivation (e.g. denaturation) or destruction (e.g. proteolysis) during the digestive process; absorption into the blood stream, with or without triggering immunogenic (i.e. IgG) or allergenic (i.e. IgE) responses; destruction during boiling (e.g. during cooking); low water solubility and the lack of a specific, low-cost and efficient method of isolation, that could

also be efficiently up-scaled to an industrial level (Jeong *et al.*, 2003; La *et al.*, 2009; Panyathep *et al.*, 2013; Rah *et al.*, 2012; Spoerlein *et al.*, 2013). This certainly explains, for the most part, why there is not yet a single, plant derived biological compound which found successful application in the realms of human health and nutrition at the level of MMP inhibition.

Lunasin from soybean is one of the first anticancer plant-based compounds (Jeong *et al.*, 2003). It is the smaller polypeptide chain of the larger cotyledon-specific 2S seed albumin (Gm2S-1) complex that has both anticancer and anti-inflammatory activities. Initially identified in soybean and then also in barley, wheat, rye, triticale, *Solanum nigrum* and *Amaranthus* seeds (Jeong *et al.*, 2007), lunasin´s large-scale animal studies and human clinical trials have been hampered by the cost of synthetic lunasin and the lack of a method for obtaining gram quantities of highly purified lunasin from plant sources (Seber *et al.*, 2012). A scalable method was developed that utilizes the sequential application of anion-exchange chromatography, ultrafiltration, and reversed-phase chromatography. This method generates lunasin preparations of 0.99% purity with a yield of 442 $mg.kg^{-1}$ defatted soy flour. Nonetheless, the proposed mode of lunasin action, as presented by Kyle, James and McPherson (2012), does not include a role in MMP inhibition. Rather, physical interactions seem to take place between this peptide with chromatin and histones (Jiang *et al.*, 2016). Hence, the isolation of an effective MMPI from plants remains to be achieved.

Recently, we compared the anti-proliferation and anti-invasion properties of protein extracts of the seeds from eight different legume species, which are usually consumed in Mediterranean diets, and demonstrated that their protein fractions can inhibit MMPs and cancer cell invasion (Lima *et al.*, 2016). Particularly, the water soluble albumin fraction of *Lupinus albus*, exhibited a strong inhibitory activity against MMP-9 and MMP-2 enzymes in HT29 cells, without exerting any apparent cytotoxicity (Lima *et al.*, 2016). Reverse zymography revealed this inhibitory activity could be related to small polypeptides/proteins present in the albumin fraction. Compared to other small MMPIs found in legume seeds, these polypeptides can offer various advantages, such as high specificity, low toxicity and easiness to isolate in larger amounts. Therefore, the isolation and characterization of the proteins/peptides responsible for such effect is of high importance and may open novel possibilities in the field of cancer treatment or

prevention. However, this remained to be attained (Lima *et al.*, 2016). Since preliminary results suggested that those *L. albus* polypeptides are resistant to heat and acid denaturation, an efficient method of isolation using heat, acid and sequential precipitations (appropriate for scaling-up to an industrial scale) was established. The activities of this isolated polypeptide mixture were compared to those already detected in the total extract.

2. Materials and Methods

2.1. Protein extraction isolation

Dry, mature seeds of *Lupinus albus* L. (lupin) were used in this work. The MMPI protein extract was isolated using its ability to resist boiling and acid denaturation, and by conjugation of several methods described and tested by Duranti *et al.* (2008) and Lima *et al.* (2016), with several modifications. Briefly, approximately 100 g ± 0.1 g of dry lupin seed (without embryo and tegument) was extracted using 50 mM of Tris-HCl buffer, pH 7.5 (1:10, w/v), The homogenate was centrifuged at 13,500 *g* for 30 min at 4 °C yielding the buffer extract (BE). The supernatant was collected, boiled for 10 min and centrifuged at 13,500 *g* for 20 min at 4 °C. The supernatant was collected and provided the heat treated extract (HT). Subsequently, the supernatant was made to pH 4.0 and centrifuged at 13,500 *g* for 20 min at 4 °C. The pellet was resuspended in 40% (v/v) ethanol containing 0.4 M NaCl, and centrifuged at 13,500 *g*, 30 min, 4 °C. The supernatant was made to 90% (v/v) ethanol and left overnight at -20 °C. The following day, the mixture was centrifuged at 13,500 *g* for 30 min at 4 °C. The pellet was suspended in 40% (v/v) ethanol containing 0.4 M NaCl, centrifuged at 13,500 *g*, 30 min, 4 °C and then made to 90% (v/v) ethanol and left once more overnight at -20 °C. Finally, after centrifuging the mixture at 13,500 *g*, 20 min, 4 °C, the pellet was resupended in the smallest possible volume of mili-Q water. The extract obtained, containing isolated deflamin, was stored frozen in falcon tubes at -20 °C.

2.2. In vitro *colon cancer cell assays*

2.2.1. HT29 cell cultures

The human colon adenocarcinoma cell line, HT29 (ECACC 85061109), established from a 44-year-old Caucasian female, and obtained from (ATCC; DSMZ….) was used throughout this work. HT29 cells were maintained in RPMI medium supplemented with 10% (w/v) of heat-inactivated fetal bovine serum (FBS), 200 mM glutamine, $2x10^{4}$ $UI.mL^{-1}$ penicillin and 20 $mg.mL^{-1}$ streptomycin at 37 °C, in a humidified atmosphere of 5% (v/v) CO_2.

2.2.2. Cell proliferation assay

HT29 cultured cells were seeded in 96-well plates ($2x10^{4}$ cells/well) and protein samples were added to the growth media at a 100 $\mu g.mL^{-1}$ concentration, and incubated for 24 h. After each treatment, the extracellular media was collected, and the wells were washed with phosphate buffer saline (PBS) in order to remove unattached cells. Cell proliferation and viability was determined using the standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay as described by Carmichael *et al.* (1987).

2.2.3. Cell migration assay

For cell migration analysis, the wound healing assay was performed. HT29 cells (5×10^{5} cells/well) were seeded in 6-well plates and allowed to reach 80% confluence. Wounds were performed by making a scratch with a pipette tip across the cell monolayer to create an open gap, mimicking a wound. Cells were then washed twice with PBS to remove floating debris. Each well was subsequently filled with fresh medium containing the protein fractions under study, in a concentration of 100 $\mu g.mL^{-1}$, and allowed to grow for 48 h. The invaded area after 48 h was calculated in each treatment and compared to the initial area at 0 h, to measure the area covered *de novo* by migrating cells into the denuded zone at the beginning of treatment. This comparison allowed us to assess the inhibitory effect (if any) exerted by each protein fraction on the HT29 cell migrating capacity.

2.3. MMP-9 and MMP-2 catalytic activities

2.3.1. Inhibition of gelatinolytic activity

The fluorogenic substrate dye-quenched (DQ)-gelatin was purchased from Invitrogen (Carlsbad, CA, USA) and dissolved in water at 1 $mg.mL^{-1}$. All solutions and dilutions were prepared in assay-buffer (50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% v/v Tween 20). A 96-well micro-assay plate (chimney, 96-well, black) was used. Each well was loaded with 0.1 mM MMP-9 (Sigma), to which 100 $\mu g.mL^{-1}$ of the protein fraction under study (for a final volume of 200 µL) was added, and the plate was incubated for 1 h at 37 °C. Subsequently, DQ-gelatin (at a final concentration of 2.5 $\mu g.mL^{-1}$) was added to each well and the plate was allowed to incubate again, for 1 h. Fluorescence levels were measured (ex. 485 nm/em. 530 nm). In each experiment, both positive (no protein fraction) and negative (no enzyme) controls were included for all samples, to correct for possible proteolytic activities present in the protein samples under analysis. All data were corrected by subtraction of their corresponding negative controls.

2.3.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples were treated with 100 mM Tris-HCl buffer, pH 6.8, containing 100 mM β-mercaptoethanol, 2% (w/v) SDS, 15% (v/v) glycerol and 0.006% (w/v) *m*-cresol purple, and heated at 100 °C for 5 min. One-dimensional electrophoresis was carried out, following the method described by Laemmli (1970) in a 12.5% (w/v) acrylamide resolving gel and a 5% (w/v) acrylamide stacking gel, and performed in a vertical slab electrophoresis unit at 100 V and 20 mA per gel. Gels were fixed for 20 min in 10% (w/v) Trichloroacetic acid (TCA), and stained in 0.25% (w/v) Comassie Brilliant Blue (CBB) R-250, 25% (v/v) 2-propanol and 10% (v/v) acetic acid. Destaining was carried in a solution of 25% (v/v) 2-propanol and 10% (v/v) acetic acid until polypeptide bands were clearly visible against a clear background.

2.3.3. Reverse gelatin zymography

Reverse zymography, used to detect and quantify MMPI proteins in different samples, was performed as described by Hawkes, Li and Taniguchi (2001), with some

modifications. Protein samples were treated with zymographic buffer (313 mM Tris-HCl buffer, pH 6.8, containing 10% w/v SDS, 50% v/v glycerol and 0.05% w/v bromofenol blue) and then loaded on SDS-polyacrylamide (12.5% w/v acrylamide) slab gels copolymerized with gelatin (1% w/v) and 1 $\mu mol.mL^{-1}$ MMP-9. Electrophoresis was performed as described in section 2.3.2 and the gels washed three times in 2.5% (v/v) Triton X-100, for 60 min each, to remove SDS. Gels were then incubated overnight at 37 °C, immersed in developing buffer (50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.01% w/v sodium azide), stained with CBB G-250 0.5% (w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, and destained with a solution of 50% (v/v) methanol and 10% (v/v) acetic acid. Dark bands visible against a white background marked the MMPI-mediated inhibition of gelatin degradation (Hawkes, Li and Taniguchi, 2001).

2.4. Statistical analysis

All experiments were performed in triplicate, in at least three independent times and the data are expressed as the mean ± standard deviation (SD). SigmaPlot software (version 12.5) was used for comparing different treatments, using one-way and two-way analysis of variance (ANOVA). Tukey's test was used to compare differences among groups and the statistical differences with *P* values lower than 0.05 where considered statistically significant.

3. Results and Discussion

There is much evidence suggesting that several compounds present in legume seeds, and especially in soybean can prevent cancer in many different organs (Kennedy, 1995), and that peptides such as BBIs have been reported to exhibit a benefic role at the levels of anticancer and antimetastasis in various animal models (Roy, Boye and Simpson, 2010). But none of these compounds has been effectively isolated for nutraceutical purposes and those which, such as lunasin, have no apparent effect on MMPs. Previous findings in our group (Lima *et al.*, 2016) led to the discovery of a promising water soluble polypeptide fraction present in the edible seeds of *Lupinus albus*, a legume species often neglected in this type of studies, which exhibits a highly potent inhibitory activity towards MMP-9

and MMP-2 in cultured colon cancer cells without exerting any significant cytotoxicity. Furthermore, this activity was seemingly higher in *L. albus* than in other legume seeds tested, namely soybean. This protein fraction presents high potential to be produced as an anticancer drug or nutraceutical. Hence, in this work, an efficient method to isolate it, in a matter which can be appropriate for scaling-up to an industrial level was attempted.

3.1. MMPI activity from L. albus *seeds is resistant to heat denaturation*

Preliminary findings showed that the MMPI polypeptide mixture may have a high stability to temperature (unpublished results). Considering the fact that lupin seeds are cooked before consumption, the maintenance of the MMPI activity after boiling is a preferable feature for its use in anticancer or cancer-preventive diets. To ascertain *Lupinus* seed MMPI activity after boiling, the polypeptide profile and the presence of MMPI bands by reverse zymography with MMP-9 included in the gel matrix were evaluated. Figure 1 shows a representative image of the polypeptide distribution between *L. albus* seeds simply extracted with buffer (buffer extraction; BE) or after heat treatment (HT), and visualized by SDS-PAGE (left) and by reverse gelatin zymography (right).

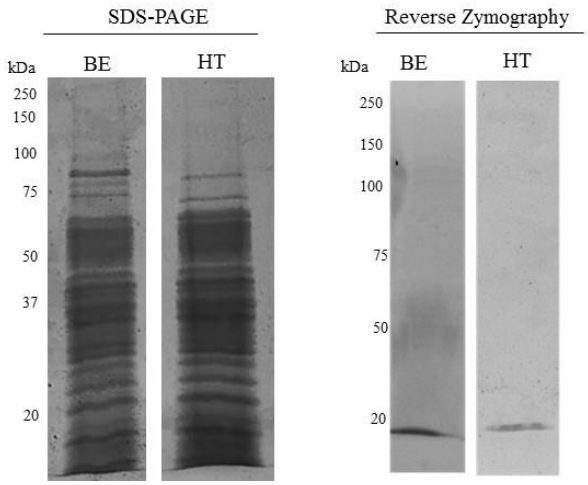

Figure 1. BE and HT extracts present MMPI activity. Representative images of the polypeptide distribution between *Lupinus albus* seeds simply extracted with buffer (buffer extraction; BE) or after heat treatment (HT), and visualized by SDS-PAGE (left) or by reverse gelatin zymography (right). Protein extracts (50 $\mu g.mL^{-1}$) were loaded onto 17.5% (w/v acrylamide) polyacrylamide slab gels, copolymerized with gelatin and MMP-9 in the case of reverse zymography.

SDS-PAGE profiles of the HT extracts show the presence of several polypeptide bands that survive heat denaturation at 100 ºC, whilst the reverse zymography reveals the presence of only one major band, which maintains its biological activity after the heat treatment. The polypeptide band visible in both lanes BE and HT presents a molecular mass lower than 20 kDa. In Lima *et al.* (2016), the presence of this band seemed to be related to the high MMPI activity present in *L. albus* seeds.

3.2. Sequential extractions allow the isolation of deflamin, which presents higher MMPI activity than the total extracts from which it derives

Taking advantage of the fact that the MMPI band is heat resistant and that this MMPI fraction is highly soluble in water, a method for its isolation with sequential precipitations (appropriate for scaling-up to an industrial scale) was attempted. This method was developed based on several protocols described before (Duranti *et al.*, 2008; Lima *et al.*, 2016), whilst introducing some modifications, mostly targeted at the lower molecular mass of the heat resistant soluble proteins from *L. albus* seeds. Hence, an initial total soluble protein extract was obtained and then boiled at 100 ºC to yield the heat resistant protein fractions. After acid precipitation, the more hydrophilic fractions were obtained by ethanol precipitation.

The representative images of the electrophoretic polypeptide profiles obtained after the several sequential extractions, including both the pellets and the soluble fractions throughout the established protocol are shown in Figure 2.

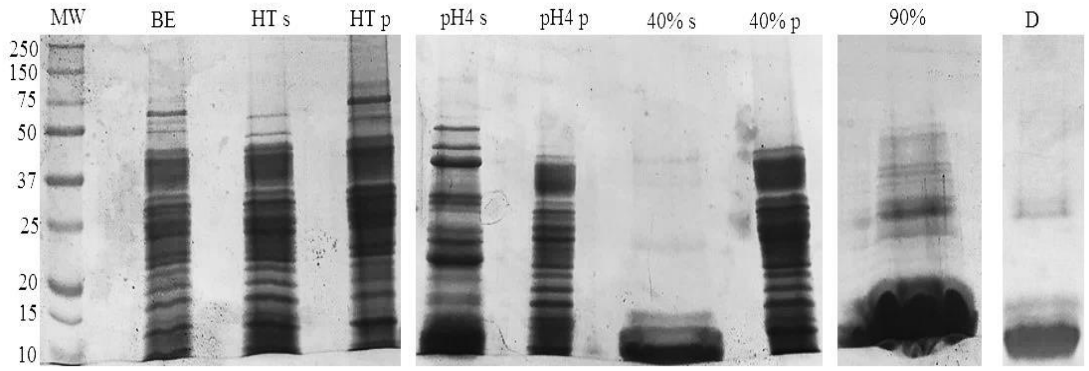

Figure 2. Representative images of the polypeptide profiles obtained after each step of the purification method as specified on the top of the gels. The protein samples (25 µg) were loaded onto 17.5% (w/v acrylamide)

polyacrylamide slab gels. MW- Molecular mass markers; BE – Buffer Extration; HT s – Heat Treatment, supernatant; HT p – Heat Treatment, pellet; pH4 s – Acid precipitation, supernatant; pH4 p – acid precipitation, pellet; 40% s – 40% v/v EtOH containing 0.4 M NaCl, supernatant; 40% p – 40% v/v EtOH containing 0.4 M NaCl, pellet; 90% - 90% v/v EtOH overnight at -20 ºC, pellet; and D - deflamin.

Analysis of the polypeptide profiles following each step of the isolation method depicted in Figure 2 revealed the gradual purification of a polypeptide fraction with a molecular mass below 20 kDa, which was termed deflamin and which matched the band obtained in Figure 1 right.

While the heat treatment allows the removal of a significant portion of soluble proteins, the use of acid precipitation followed by ethanol solubilization assures the removal of possible toxic contaminants such as saponins, phenolic compounds and other known secondary metabolites present in legume seeds (Xu and Chang, 2012). The final 90% (v/v) ethanol solubilization yields the lower molecular mass, more hydrophilic, polypeptide fraction. The presence of high molecular mass contaminants still present after 90% (v/v) ethanol precipitation was observed, which rendered the need to an additional precipitation with 40% (v/v) ethanol to obtain a purer fraction.

To determine if deflamin was indeed the responsible fraction for MMPI activities in *L. albus* seeds, we compared lupins previously detected activities (Lima *et al.*, 2016) in the different fractions. Results are shown in Figure 3. Buffer extraction (BE), heat treatment (HT) and deflamin (D) protein fractions were used to assess their inhibitory activity upon the proteolytic activity of MMP-9 on DQ-gelatin. The negative control (C) does not inhibit MMP-9, resulting in 100% proteolytic activity for this protease.

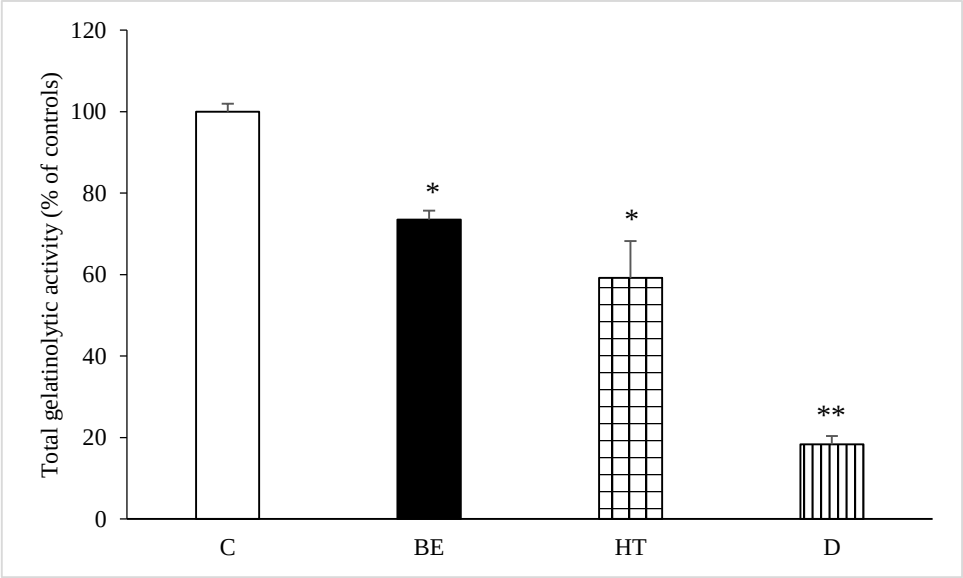

Figure 3. The effect of buffer extraction, heat treatment and deflamin on the proteolytic activity of MMP-9. Buffer extraction (BE), heat treatment (HT) and deflamin (D) protein fractions were obtained from *L. albus* seeds and used to assess their inhibitory activity upon the proteolytic activity of MMP-9. The negative control (C) does not inhibit MMP-9, resulting in 100% proteolytic activity for this protease. Protein samples were added at a concentration of 50 µg.mL^{-1} and gelatinolytic activity was measured by the DQ fluorogenic assay. MMP activities are expressed as relative fluorescence as a % of controls, and represent the averages of at least three replicate experiments (n = 3) ± SD. ∗ $P < 0.05$, ∗∗ $P < 0.001$.

At the protein concentration tested (50 µg.mL^{-1}), Figure 3 shows that all samples were able to significantly inhibit MMP-9 proteolytic activity. However, significant differences ($P<0.05$) were observed among the samples analysed, with the highest inhibition level ($P<0.05$) detected for deflamin, which induced a very significant ($P<0.001$) reduction of MMP-9 activity greater than 80%. In summary, as deflamin is gradually purified, its apparent inhibitory effect as an MMPI increases. Indeed, its effect is significantly higher than both BE and HT ($P<0.05$). This suggests that although *L. albus* total protein pool contains deflamin and can inhibit MMP-9 activities as described by Lima *et al.* (2016), isolated deflamin is most likely more effective that the consumption of lupin as a functional food. This effect is at least in part explained by the observation that deflamin specific activity increases from BE to HT to D. In other words, 50 µg of BE protein contains less deflamin than 50 µg of HT and so forth.

3.3. Deflamin is more effective in inhibiting colon cancer cell invasion

In Lima *et al.* (2016), 100 µg.mL^{-1} of total protein extract from lupin seed was able to reduce cancer cell invasion but was not cytotoxic. Hence, in this work, we further aimed to characterize deflamin´s ability to reduce cancer cell invasion in HT29 cells, while comparing it to the total extract and to the heat-treated extract of *L. albus*.

Figure 4 shows the effect of each protein fraction on HT29 cell migration after 48 h of exposure to the total extract, heat treatment and deflamin (50 µg protein.mL^{-1}).

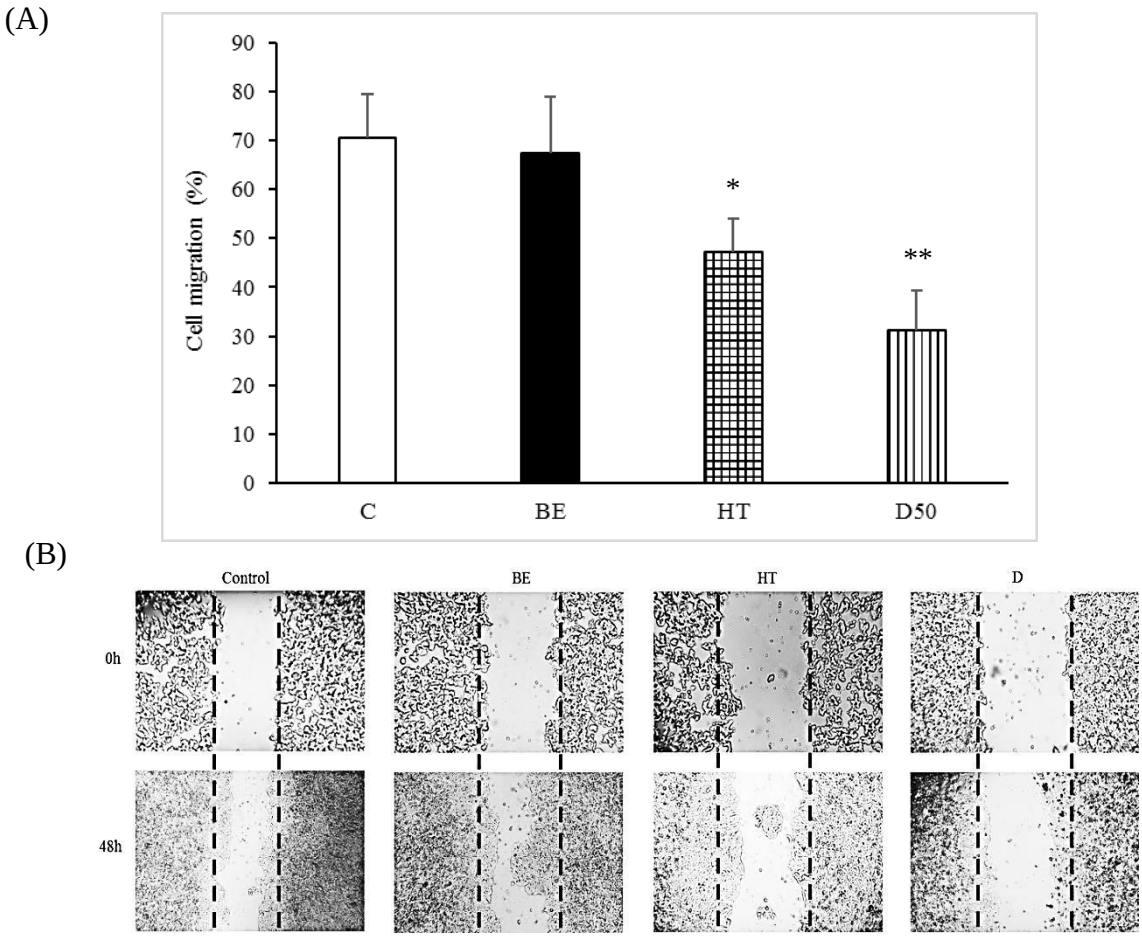

Figure 4. HT29 cell migration after exposure to Buffer Extraction (BE), Heat treatment (HT) and deflamin (D), as determined by the wound healing assays. (A) - Relative migration rates. Values are the means of at least three replicate experiments ± SD, and are expressed as a % the wound closure in relation to 0 h. (B) - Examples of cell migration showing the inhibitory effect of deflamin on HT29 cell migration. Cells were grown until reaching 80% confluence and the monolayer was scratched with a pipette tip (0 h). Cells were then exposed to 50 µg protein.mL^{-1} extract and cell migration was recorded after 48 h. ∗ $P < 0.05$, ∗∗ $P < 0.001$.

Results show that isolated deflamin presented the highest inhibition in migration rates when compared to the other deflamin-containing protein samples studied ($P<0.001$), inducing a 60% reduction in cell migration rates. Furthermore, at the concentration used, HT and deflamin were also statistically different from controls ($P<0.05$) whilst the BE sample remained statistically similar to controls ($P>0.05$), once again corroborating the higher efficiency of the isolated deflamin fraction, already noted in Figure 3. This means that, as the purification methodology proceeds from the initial total protein extract to isolated deflamin, its biological activity gradually increases, reaching a maximum with isolated deflamin. This result was expected given the concomitant increment in deflamin specific activity as the other proteins are gradually removed along the purification process. Indeed, as comparative tests performed after each purification step use identical amounts of proteins from each sample, as the degree of deflamin purification increases, the amount of deflamin relative to total protein in each fraction also increases, justifying the increment in deflamin bioactivity when one moves from less pure to purer deflamin fractions. This also justifies why the use of 50 µg protein.mL^{-1} reduced its ability to inhibit cell invasion, when compared to the 100 µg protein.mL^{-1} concentrations used by Lima *et al.* (2016). Being more diluted in the BE fraction, these results suggest a dose-dependent response action for deflamin.

3.4. Deflamin is the first proteinaceous MMPI which can be purified by a cost-effective and up-scalable procedure

Legume seeds have been long recognized by containing a variety of proteinaceous enzyme inhibitors, such as the trypsin inhibitors and the BBIs. However, although the presence of MMPIs of natural occurrence may be considered ubiquitous in plant tissues, all of them present several disadvantages when considering their production for clinical and/or nutraceutical purposes: toxicity; chemical inactivation (e.g. denaturation) or destruction (e.g. proteolysis) upon cooking and/or by the digestive process; the lack of a specific; and a high-cost and inefficient method of isolation, which prevent MMPIs in general to undergo efficient scaling-up to an industrial level . Isolated deflamin reported in this work surpasses all of these constraints, as it is resistant to boiling and is an enzyme inhibitor; on the other hand, the sequential precipitation method developed is simple, cost-effective and easily applied in an industrial context. As a mixture of edible polypeptides

which occur naturally in lupin seeds, it doesn't pose the problem of toxicity in higher doses, that most phenolic compounds and other bioactive secondary metabolites do, and the use of the acid and ethanol precipitations assures the removal of possible toxic contaminants as well as higher molecular mass proteins.

The yield of the extraction procedure is present in Table 1. Results show that per 100 g of dry seed yields 100 mg of deflamin, which correspond to around 0.5% of total protein content of the seed.

Table 1. Yield (expressed in %) of dry *L. albus* seeds in deflamin.

	Deflamin	% yield
Per 100 g of dry lupin seed	100 mg	0.1%
Per 100 mg of total protein	520 μg	0.5%

These results corroborate that deflamin is indeed present in very low concentrations in the seed, hence the lower activities observed in the BE fractions. It also suggests that the consumption of lupin alone may not provide enough deflamin to induce the same effects that its isolated form can provide.

It is important to notice that the low yields of the extraction procedure are not due to the method itself, but rather to the low amount of deflamin in the seed. Still, the relative ease of the procedure and the possibility to up-scale to larger amounts, in a cost-effective and simple manner, using filtrations and flow centrifugation as well as low cost reagents such as ethanol suggest a high potential for industrial production.

Furthermore, given the potential of deflamin, our developed procedure is also of particular importance to pursue a more thorough characterization of this proteinaceous fraction, such as its identity, dose-response effects and EC50, as well as clinical and pre-clinical studies. The fact that isolated deflamin is efficient in inhibiting MMP-9 and reducing cancer cell invasion suggests its high potential for a vast array of clinical uses. Since MMP-9 is closely involved in inflammation (Garg *et al.*, 2010; Heimesaat *et al.*, 2011) as well as in oncologic processes (Baugh *et al.*, 1999; Garg *et al.*, 2009), the MMPI

deflamin could possibly be used in both anticancer approaches as well as anti-inflammatory treatments, especially those related to the digestive tract, such as colorectal cancer (CRC) and inflammable bowel diseases (IBDs).

4. Conclusion

Here we report for the first time the isolation of deflamin, a potent MMPI proteinaceous fraction from *L. albus* seeds, which also inhibits cancer cell invasion, and assure its isolation by measuring the bioactivities of the various fractions along the purification process. This is, to the best of our knowledge, the first effective method for isolating a proteinaceous MMPI from food sources which is scalable to an industrial level, in a cost-effective manner. The facts that deflamin is an MMPI, is apparently resistant to digestion and derives from plant foods make it a perfect candidate to be used as a nutraceutical in cancer and inflammation preventing/curative diets, particularly in the case of colon diseases, where it can act *in situ*, without exerting side effects. Our developed method is not only useful for a future industrial production as a nutraceutical, but it also provides a way to isolate deflamin for further necessary studies.

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Chapter 2

Identifying deflamin, a specific polypeptide mixture from *Lupinus albus* seeds that reduces colon cancer cell migration through gelatinase inhibition

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Keywords: deflamin, isolation method, MMP-9 inhibition, colon cancer cells

This chapter will be submitted as a paper in Food Chemistry

1. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in the European Union, with an enormous health and economic burden (Ait Ouakrim *et al.*, 2015; Ferlay *et al.*, 2013). Despite the significant advances in diagnosis, screening and treatment, the overall long-term outcome in patients has not significantly changed in the last decades (Herszényi *et al.*, 2012). Intensive research has been made to develop novel anticancer drugs, both prophylactic and therapeutic (Su *et al.*, 2006).

A subgroup of matrix metalloproteinases (MMPs) called gelatinases (MMP-2 and MMP-9) was found to be largely implicated in CRC progression/metastasis in animal models and patients (Farina and Mackay, 2014; Lotfi *et al.*, 2015; Marshall *et al.*, 2015). Consequently, for over 30 years now, MMPs have been considered by researchers across the world as attractive cancer targets. Their inhibitors (MMPIs) were demonstrated to be effective in reducing cancer progression/metastasis in an array of *in vitro* assays, animal models and clinical trials and appear to be overall mostly effective at the early stages of

cancer or in preventing development of undetected micrometastases after surgery (Coussens, 2002; Herszényi *et al.*, 2012; Mook, Frederiks and Noorden, Van, 2004; Yoon *et al.*, 2003; Zucker and Vacirca, 2004). These observations could make them the perfect target for prevention or to limit tumour progression. Up to now, several MMPIs have already been synthesized, some of which have been used as potential therapeutic agents (Bourguet *et al.*, 2012). However, only a few small MMPIs entered the clinical trial stage, and most presented strong adverse side effects (Wang *et al.*, 2012). Thus, so far, most of their clinical trials in cancer have been rather disappointing.

Since tumour invasion from a primary site to progressively colonize distant organs is the major contributor to cancer mortality, one important therapeutic goal is the prevention of initial cancer cell invasion in high-risk patients as well as prevention of additional metastases in patients already with this disease (Steeg, 2016). One way to partly achieve this, particularly in CRC, might be by long-term ingestion of natural food-based MMPIs, which are colon-available, rather than serum-bioavailable, thus avoiding troublesome side-effects. There is indeed a substantial amount of research that has turned towards the discovery of novel plant-food derived MMPIs which are clinically active against various types of cancer cells and currently there is abundant evidence in the published literature concerning the anticancer activities of many edible foodstuffs. Legume seeds are a strong example, although most studies have focused primarily on soybean. There is much evidence suggesting that certain compounds present in soybean can prevent cancer in many different organ systems. Besides non-protein compounds such phytic acid, β-sitosterol, isoflavones (e.g. genistein and daidzein) and saponins (Kennedy, 1995), legume seed peptides and proteins (including Bowman-Birk inhibitors (BBIs) and other trypsin inhibitors) have been reported to exhibit anticancer and antimetastatic activities in various animal models (Roy, Boye and Simpson, 2010). For example, Champ (2002) reported that BBIs derived from soybean inhibited or prevented the development of chemically induced cancers of the liver, lung, colon, mouth and oesophagus in mice, rats and hamsters. However, very few studies demonstrate their effects on MMPs and animal studies and human clinical trials to determine their efficacy are usually hampered by the cost of their synthetic production and/or the lack of a method for obtaining gram quantities of highly purified forms of these compounds (Seber *et al.*, 2012). Also, most of them are destroyed during the digestive process and some are considered to be anti-

nutrients and can exert cytotoxicity. Hence, the identification of a non-toxic, digestion-resistant MMPI from a plant-food source can be of extreme potential for cancer prevention and cancer treatments. Its applications could range from developing novel anticancer drugs to combat metastasis formation, or to be used in diet prevention, or even aiding chemotherapy or to prevent re-incidence after surgery.

Findings in our group led to the discovery of a group of water soluble polypeptides isolated from the edible seeds of *Lupinus albus*, which exhibit a highly potent inhibitory activity towards MMP-9 and MMP-2. This extract presented a high stability to pH and temperature, making it an excellent candidate to become a valuable anticancer nutraceutical agent. An efficient method to isolate this fraction was established and led to the isolation of deflamin, a specific polypeptide mixture from *L. albus* which presents high MMP-9 activity and was recently the subject of a patent application. Being proteinaceous in nature, cooking resistant and present in edible seeds, deflamin is likely to present no toxic effects and can be used safely in preventive diets. However, its effects on colon cancer cells, particularly its cytotoxic levels have yet to be proven. Also, the polypeptides that constitute deflamin remain to be identified.

The goal of the present work was to identify deflamin and to characterize its MMPI activities in colon cancer cells. Dose-response effects, cancer cell invasion and MMP minimal inhibitory concentrations were determined in colon cancer cells and the specific isolated polypeptide mixture were tentatively identified through mass spectrometry. Results show that deflamin is an excellent candidate to become a valuable anti-inflammatory and anticancer nutraceutical agents for colon diseases and possibly more.

2. Materials and Methods

2.1. Protein extraction isolation

Dry, mature seeds of *Lupinus albus* L. (lupin) were used in this work. The MMPI protein extract was isolated using its ability to resist boiling and acid denaturation, and by conjugation of several methods described and tested by Duranti *et al.* (2008) and Lima *et al.* (2016), with several modifications. Briefly, approximately 100 g ± 0.1 g of dry lupin

seed (without embryo and tegument) was extracted using 50 mM of Tris-HCl buffer, pH 7.5 (1:10, w/v), The homogenate was centrifuged at 13,500 *g* for 30 min at 4 °C yielding the buffer extract (BE). The supernatant was collected, boiled for 10 min and centrifuged at 13,500 *g* for 20 min at 4 °C. The supernatant was collected and provided the heat treated extract (HT). Subsequently, the supernatant was made to pH 4.0 and centrifuged at 13,500 *g* for 20 min at 4 °C. The pellet was resuspended in 40% (v/v) ethanol containing 0.4 M NaCl, and centrifuged at 13,500 *g*, 30 min, 4 °C. The supernatant was made to 90% (v/v) ethanol and left overnight at -20 °C. The following day, the mixture was centrifuged at 13,500 *g* for 30 min at 4 °C. The pellet was suspended in 40% (v/v) ethanol containing 0.4 M NaCl, centrifuged at 13,500 *g*, 30 min, 4 °C and then made to 90% (v/v) ethanol and left once more overnight at -20 °C. Finally, after centrifuging the mixture at 13,500 *g*, 20 min, 4 °C, the pellet was resupended in the smallest possible volume of mili-Q water. The extract obtained, containing isolated deflamin, was stored frozen in falcon tubes at -20 °C.

2.2. In vitro *colon cancer cell assays*

2.2.1. HT29 cell cultures

The human colon adenocarcinoma cell line, HT29 (ECACC 85061109), obtained from a 44-year-old Caucasian female, was used throughout this work. HT29 cells were maintained in RPMI medium supplemented with 10% (w/v) of heat-inactivated fetal bovine serum (FBS), 200 mM glutamine, $2x10^{4}$ $UI.mL^{-1}$ penicillin and 20 $mg.mL^{-1}$ streptomycin at 37 °C, in a humidified atmosphere of 5% (v/v) CO_2.

2.2.2. Cell proliferation assay

HT29 cultured cells were seeded in 96-well plates ($2x10^{4}$ cells/well) and protein samples were added to the growth media at a 100 $\mu g.mL^{-1}$ concentration, and incubated for 24 h. After each treatment, the extracellular media was collected, and the wells were washed with phosphate buffer saline (PBS) in order to remove unattached cells. Cell proliferation and viability was determined using the standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay as described by Carmichael *et al.* (1987).

2.2.3. Cell migration assay

For cell migration analysis, the wound healing assay was performed. HT29 cells (5×10^5 cells/well) were seeded in 6-well plates and allowed to reach 80% confluence. Wounds were performed by making a scratch with a pipette tip across the cell monolayer to create an open gap, mimicking a wound. Cells were then washed twice with PBS to remove floating debris. Each well was subsequently filled with fresh medium containing the protein fractions under study, in a concentration of 100 $\mu g.mL^{-1}$, and allowed to grow for 48 h. The invaded area after 48 h was calculated in each treatment and compared to the initial area at 0 h, to measure the area covered *de novo* by migrating cells into the denuded zone at the beginning of treatment. This comparison allowed us to assess the inhibitory effect (if any) exerted by each protein fraction on the HT29 cell migrating capacity.

2.3. MMP-9 and MMP-2 catalytic activities

2.3.1. Inhibition of gelatinolytic activity

The fluorogenic substrate dye-quenched (DQ)-gelatin was purchased from Invitrogen (Carlsbad, CA, USA) and dissolved in water at 1 $mg.mL^{-1}$. All solutions and dilutions were prepared in assay-buffer (50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% v/v Tween 20). A 96-well micro-assay plate (chimney, 96-well, black) was used. Each well was loaded with 0.1 mM MMP-9 (Sigma), to which 100 $\mu g.mL^{-1}$ of the protein fraction under study (for a final volume of 200 µL) was added, and the plate was incubated for 1 h at 37 °C. Subsequently, DQ-gelatin (at a final concentration of 2.5 $\mu g.mL^{-1}$) was added to each well and the plate was allowed to incubate again, for 1 h. Fluorescence levels were measured (ex. 485 nm/em. 530 nm). In each experiment, both positive (no protein fraction) and negative (no enzyme) controls were included for all samples, to correct for possible proteolytic activities present in the protein samples under analysis. All data were corrected by subtraction of their corresponding negative controls.

2.3.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples were treated with 100 mM Tris-HCl buffer, pH 6.8, containing 100 mM β-mercaptoethanol, 2% (w/v) SDS, 15% (v/v) glycerol and 0.006% (w/v) *m*-cresol purple,

and heated at 100 °C for 5 min. One-dimensional electrophoresis was carried out, following the method described by Laemmli (1970) in a 12.5% (w/v) acrylamide resolving gel and a 5% (w/v) acrylamide stacking gel, and performed in a vertical slab electrophoresis unit at 100 V and 20 mA per gel. Gels were fixed for 20 min in 10% (w/v) Trichloroacetic acid (TCA), and stained in 0.25% (w/v) Comassie Brilliant Blue (CBB) R-250, 25% (v/v) 2-propanol and 10% (v/v) acetic acid. Destaining was carried in a solution of 25% (v/v) 2-propanol and 10% (v/v) acetic acid until polypeptide bands were clearly visible against a clear background.

2.3.3. Reverse gelatin zymography

Reverse zymography, used to detect and quantify MMPI proteins in different samples, was performed as described by Hawkes, Li and Taniguchi (2001), with some modifications. Protein samples were treated with zymographic buffer (313 mM Tris-HCl buffer, pH 6.8, containing 10% w/v SDS, 50% v/v glycerol and 0.05% w/v bromofenol blue) and then loaded on SDS-polyacrylamide (12.5% w/v acrylamide) slab gels copolymerized with gelatin (1% w/v) and 1 $\mu mol.mL^{-1}$ MMP-9. Electrophoresis was performed as described in section 2.3.2 and the gels washed three times in 2.5% (v/v) Triton X-100, for 60 min each, to remove SDS. Gels were then incubated overnight at 37 °C, immersed in developing buffer (50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.01% w/v sodium azide), stained with CBB G-250 0.5% (w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, and destained with a solution of 50% (v/v) methanol and 10% (v/v) acetic acid. Dark bands visible against a white background marked the MMPI-mediated inhibition of gelatin degradation (Hawkes, Li and Taniguchi, 2001).

2.4. Minimal inhibitory concentrations (MICs)

Minimal inhibitory concentrations (MICs) were assessed in sterile 96-well plates (Greiner Bio-one, Germany), using the micro dilution method as described before (Bouhdid *et al.*, 2010). Briefly, 50 µL of RPMI medium was added to each well. Then, 50 µL of each sample was added to the first well and serially diluted 1:2 to each adjacent well, up to 10 dilutions. Subsequently, 50 µL of the HT-29 cell suspension with a concentration of 2 x 10^5 $cells.mL^{-1}$, was added to the wells. A positive control (50 µL RPMI medium + 50 µL

cell suspension) and a negative control (100 µL RPMI medium) were performed. Plates were incubated for 24 h, at 37 ºC, and cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Carmichael *et al.*, 1987). For MMP-9 MIC determination, the media from each well was collected and gelatinolytic activities were determined with DQ-gelatin, as described above.

2.5. Deflamin fractionation through High-Performance Liquid Chromatography

Deflamin samples were fractionated in a High-Performance Liquid Chromatography (HPLC) device (Waters 2695 Separations Module) equipped with a Waters 2998 Photodiode Array Detector. Protein samples were separated in a C18 reverse phase collumn, Zorbax 300SB 5 µm, 250 mm x 4.6 mm. The elution was made with eluent A (0.1 % (v/v) Trifluoroacetic acid (TFA)) and solvent B (acetonitrile in 0.1 % (v/v) TFA). Peak detection was made at 214 nm and 280 nm.

2.6. Mass spectrometry analysis

Selected isolated peaks were analyzed on a 5600 TripleTOF mass spectrometer (ABSciex®) in information-dependent acquisition (IDA) mode. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXPTM C18CL reverse phase column (300 µm ID × 15 cm length, 3 µm particles, 120 Å pore size, Eksigent®) at 5µL.min^{-1}. Peptides were eluted into the mass spectrometer with a multistep gradient: 0-2 min linear gradient from 5 to 10%, 2-45 min linear gradient from 10% to 30% and, 45-46 min to 35% of acetonitrile in 0.1% TFA. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, AB Sciex) with a 50 µm internal diameter (ID) stainless steel emitter (New Objective).

For information dependent acquisition (IDA) experiments the mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 100 MS/MS scans (100–1500 m/z from a dynamic accumulation time – minimum 30 ms for precursor above the intensity threshold of 1000 – in order to maintain a cycle time of 3.3 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected

before adding those ions to the exclusion list for 25 s (mass spectrometer operated by Analyst® TF 1.6, ABSciex®). Rolling collision was used with a collision energy spread of 5. Two IDA experiments were performed for each sample with the second analysis performed with an exclusion list of the peptides previously identified.

Protein identification was obtained using Protein Pilot™ software (v 5.0, ABSciex®) with the following search parameters: identification from uniprot database from March 2016, with no alkylation or digestion for the peptide samples. As a criteria for protein filtering we used 1.3 unused score value and a 95 % peptide confidence filtering and >0 contribution.

2.7. Statistical analysis

All experiments were performed in triplicate, in at least three independent times and the data were expressed as the mean ± standard deviation (SD). SigmaPlot software (version 12.5) was used for comparing different treatments, using one-way and two-way analysis of variance (ANOVA). Tukey's test was used to compare differences between groups and the statistical differences with *P* value less than 0.05 where considered statistically significant.

3. Results and Discussion

It has been predicted that the continued ingestion of functional foods and/or nutraceuticals with bioactivities will constitute the most effective human tool against the majority of the ailments that inflict today's modern societies (Das *et al.*, 2012). MMP-9 inhibitors (MMPIs) are considered anti-angiogenic agents for primary tumours and metastasis deterrents, and have also been demonstrated to effectively inhibit pre-cancer states such as colitis and other inflammatory bowel diseases (Bourguet *et al.*, 2012). In the last decade a substantial amount of research has turned towards novel plant foods presenting MMPIs, but targeting these gelatinases has proven itself difficult. In a previous work, we described the isolation of deflamin, a novel type of MMPIs that are proteinaceous in nature and which are potent inhibitors of the matrix metalloproteinases MMP-9 and MMP-2, exhibiting powerful anti-invasion activities. Hence deflamin has the potential to prove

useful as a nutraceutical or in functional foods in the prevention and/or treatment of a very wide array of MMP-9-related diseases. In the present work we ascertained deflamin effects on colon cancer HT29 cells and further characterized the polypeptide nature of deflamin.

3.1. L. albus *deflamin activities are dose dependent*

Previous works (chapter 1) showed deflamin's isolation and demonstrated it to be the MMPI fraction responsible for *L. albus* MMPI activities previously detected by Lima *et al.* (2016). A methodology was developed (see Chapter 1) to extract and isolate deflamin from seeds that is suitable to undergo up-scaling, allowing its mass production in industrial facilities. This methodology demonstrated to be highly efficient in isolating the MMPI fraction responsible for *L. albus* MMPI activities. In this work, we have therefore further tested if the effect of this fraction (i.e. deflamin) was dose-dependent. A set of four different deflamin concentrations (100, 50, 10 and 5 μg.mL^{-1}) were tested using the DQ gelatin method and the wound invasion assay in HT29 colon cancer cells and the results are expressed in Figure 1 and 2, respectively.

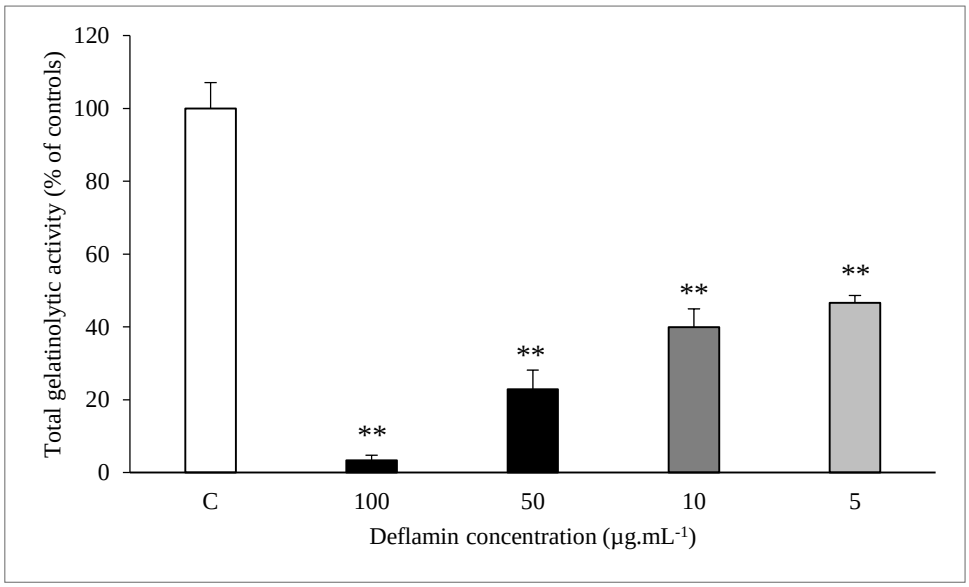

Figure 1. The effect of different concentrations of deflamin on gelatinase activities. Four different concentrations of deflamin were obtained from *L. albus* seeds and used to assess their inhibitory activity upon the proteolytic activity of MMP-9 on DQ-gelatin. The negative control (C) does not inhibit MMP-9, resulting in 100% proteolytic activity for this protease. Deflamin was added at concentrations of 100, 50, 10 and 5 μg.mL^{-1} and gelatinolytic activity was measured by the DQ fluorogenic assay. Gelatinase activities are expressed as relative fluorescence as a % of controls, and represent the averages of at least three replicate experiments (n = 3) ± SD. ∗∗ $P < 0.001$ when compared to controls.

Figure 1 shows that all concentrations tested (100, 50, 10 and 5 µg.mL^{-1}) were able to significantly inhibit gelatinase proteolytic activity (P<00.1), when compared to controls. However, the inhibition level in each concentration differed, in a dose-dependent manner, with the highest inhibition detected for 100 µg.mL^{-1} of deflamin, which induced a reduction greater than 90% of gelatinolytic activity.

Figure 2 shows that the capacity of deflamin to inhibit colon cancer cell invasion.

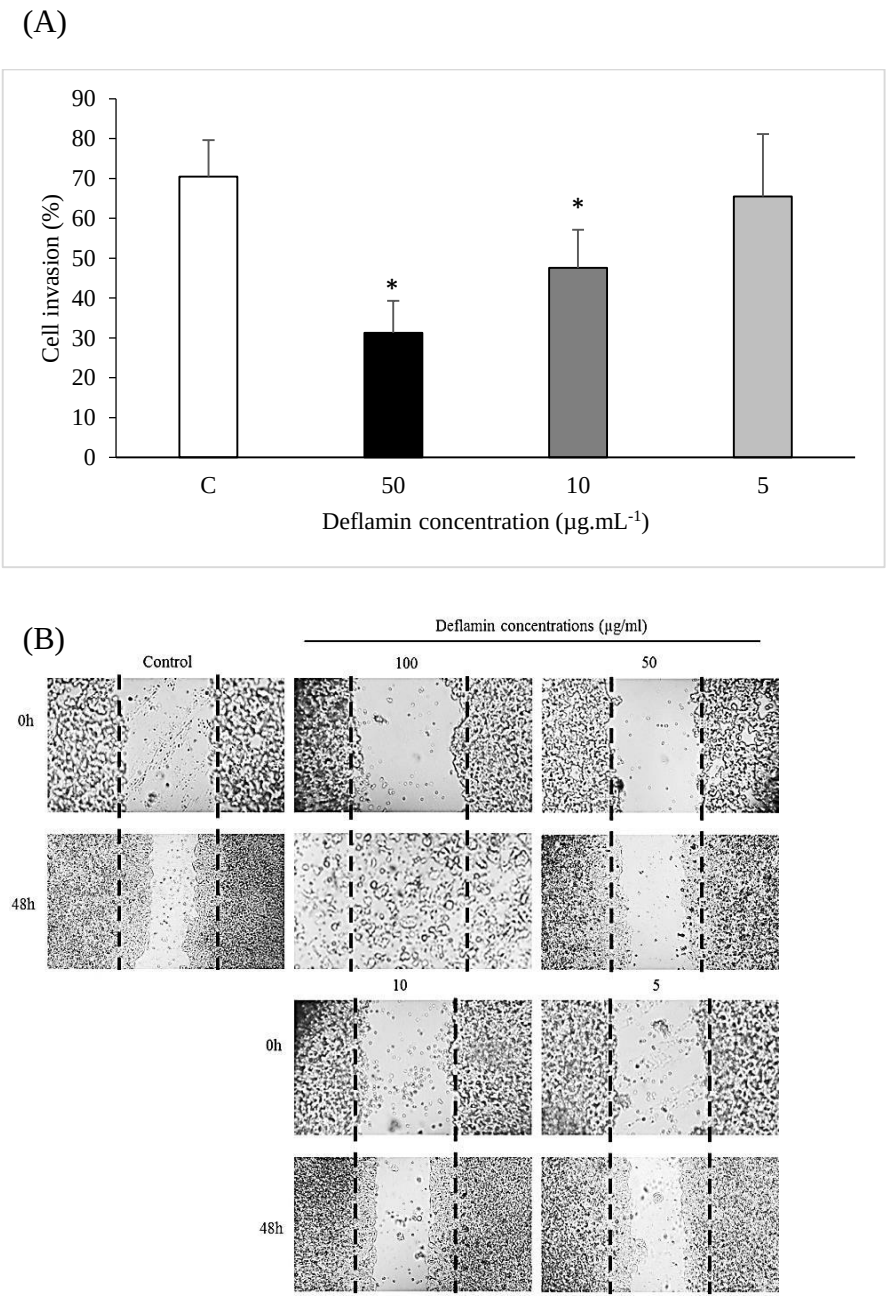

Figure 2. HT29 cell invasion after exposure to different concentrations of deflamin, as determined by the wound healing assay. (A) - Relative migration rates. Values are the means of at least three replicate experiments ± SD, and are expressed as %

wound closure in relation to time 0. (B) - Examples of cell migration obtained for the four deflamin concentrations tested plus the control. Cells were grown until reaching 80% confluence and the monolayer was scratched with a pipette tip (0 h). Cells were then exposed to 100, 50, 10 and 5 µg.mL^{-1} deflamin and cell migration was recorded after 48 h. ∗∗ represents $P<0.001$ and ∗ represents $P<0.05$ when compared to controls.

Although all concentrations inhibited cell invasion, unlike in the gelatinolytic activities in Figure 1, where all inhibitions were significant, in the invasion assay the lowest concentration (5 µg deflamin.mL^{-1}) was not significantly different from controls, whilst 10 and 50 µg deflamin.mL^{-1} were statistically and very significantly different from controls, respectively ($P<0.05$ and $P<0.001$). For the highest deflamin concentration studied (100 µg.mL^{-1}), a different and interesting result was obtained: HT29 cells were completely detached (see figure 1B), justifying the absence of this concentration in the graph from Figure 1A. The fact that higher doses of deflamin inhibits gelatinolytic activities and reduce the ability of HT29 cells to invade the scratch corroborates our previous results (chapter 1) and reveals a dose-response effect which can be useful for future studies; however, the detachment observed in the highest dose might suggest a cytotoxic effect, hence we proceeded for the evaluation of deflamin's effect on cell growth and metabolism.

3.2. Deflamin does not reduce cell growth and metabolism in colon cancer cells

To test whether deflamin was cytotoxic to HT29 cells, and if it influenced cell growth, we studied the same concentrations using a standard cell proliferation assay. Figure 3 illustrates the number of HT29 living cells after growth in the presence of different deflamin concentrations (100, 50, 10 and 5 µg.mL^{-1}), determined after staining with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (which can only be metabolized by living cells). The results show that a 2-day exposure to deflamin did not induce a significant reduction ($P>0.001$) in cell growth or in the number of living cells, when compared to controls. Furthermore, there were no visible cytotoxic effects (data not shown). This result indicates that deflamin is relatively non-cytotoxic to HT29 cells even at 100 µg deflamin.mL^{-1} and that it does not interfere with the normal cellular metabolism.

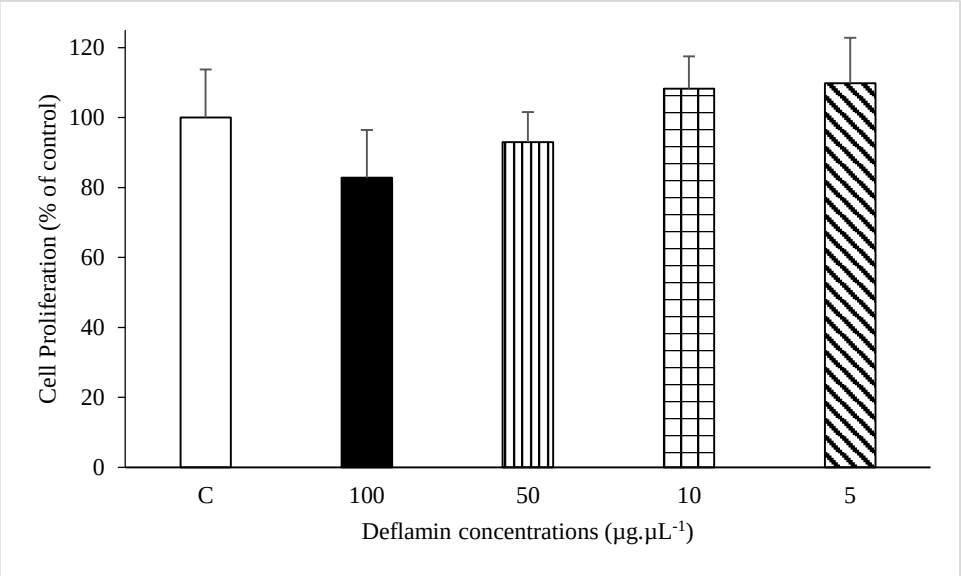

Figure 3. HT29 cell proliferation after a 24 h exposure to different concentrations of deflamin. Cells were grown for 24 h in the presence of 100, 50, 10 and 5 µg deflamin.mL^{-1} and stained with MTT. Values represented are the means of at least three replicate experiments (n = 3) ± SD and are expressed as a percentage of the control.

However, for the highest deflamin dose, HT29 cells were detached (Figure 1) which if not related to any degree of cytotoxicity, it might possibly be related to cell adhesion. It is known that cells adhere to a substrate via their integrins, i.e. transmembrane receptors that are the bridges for cell-cell and cell-extracellular matrix (ECM) interactions. One important function of integrins on cells in tissue culture is their role in cell migration. Recent studies demonstrated that integrins are modulated by tumour progression and metastasis and are tightly connected to both MMP-9 and MMP-2 activities (Hood and Cheresh, 2002) Nevertheless, few studies have shown a cell detachment effect in the presence of MMPIs. These results suggest that deflamin's mode of action might involve a broader mechanism than induces more than just gelatinase inhibition. The observation that the highest deflamin dose tested (*i.e.* 100 µg.mL^{-1}) causes no apparent cytotoxic effect suggests it is not harmful to the digestive system and may therefore be used in preventive diets, without any secondary effects.

Since cell growth was not impaired with deflamin, we set out to determine the minimal inhibitory concentrations (MICs) and the concentration necessary to induce 50% effect

(EC50) of deflamin, in the different tests: cell growth, cell invasion, cell detachment and MMP inhibition. Results are present in Table 1.

Table1. Determination of Minimal Inhibitory Concentrations (MICs) and the concentrations which induce a 50% effect (EC50) for deflamin bioactivities on cell growth, cell invasion, cell detachment and MMP inhibition. Results are expressed in $\mu g.mL^{-1}$. ND: not determined.

	MIC ($\mu g.mL^{-1}$)	EC50 ($\mu g.mL^{-1}$)
Cell Growth	>100	>100
Cell Invasion	<10	10
Cell Detachment	100	ND
MMP inhibition	<5	10

Under the conditions tested, MIC values for cell invasion and MMP inhibition were lower than the MICs found for the other parameters studied. A 10 $\mu g.mL^{-1}$ deflamin concentration was found enough to significantly inhibit 50% of cell invasion ($P<0.05$) making it the EC50 value for cell invasion. For MMP inhibition the EC50 is of 10 µg deflamin.mL^{-1} as well. This is in accordance to the high relation between MMP-9 activities and cell invasion, and corroborates that MMP inhibition is at least one of the major modes of action of deflamin. Nonetheless, the MIC levels determined for cell invasion were lower than 10 $\mu g.mL^{-1}$ but were not statistically significant ($P<0.05$) at 5 $\mu g.mL^{-1}$, whilst MMPs were already very significantly inhibited in the presence 5 $\mu g.mL^{-1}$, which is why the MIC values are lower than this concentration. With MIC values lower for MMP inhibition than for cell invasion, it is expected that MMP inhibition only induces a noticeable cell invasion reduction after a certain degree of inhibition. On the other hand, the MIC for cell detachment was only achieved for >100 $\mu g.mL^{-1}$, at the highest deflamin concentrations tested, at which no significant cell toxicity was detected.

Clearly, MMP inhibition and the reduction in cell invasion are the strongest activities of deflamin, when compared to cell growth impairment or cytotoxicity which were only affected in a very low degree. This could be of significant importance. MMPIs with high

specificity and low side effects have been very hard to find, and most clinical trials yielded unsatisfactory results. On the other hand, in cancer preventing diets, reducing MMP-9 and -2 activities in low amounts is desired but low toxicity levels against colon cells even in higher doses are a very important requirement. Compared to low molecular mass compounds such as polyphenols, polypeptide MMPIs may offer various advantages, such as high specificity and low toxicity. According to Park, Jeong and Lumen (2007), compared to the traditional cancer treatments such as chemotherapy or radioactive treatment, peptides and small proteins with high specificity against tumour promoters such as MMPs that simultaneously present low toxicity may represent the future in cancer treatment/prevention.

3.3. L. albus *deflamin is a mixture of low molecular mass polypeptides*

Isolated deflamin was analysed by denaturing electrophoresis, under reducing and non-reducing conditions to detect its polypeptide composition as well as to determine the potential presence of disulphide bonds. The results obtained are presented in Figure 4.

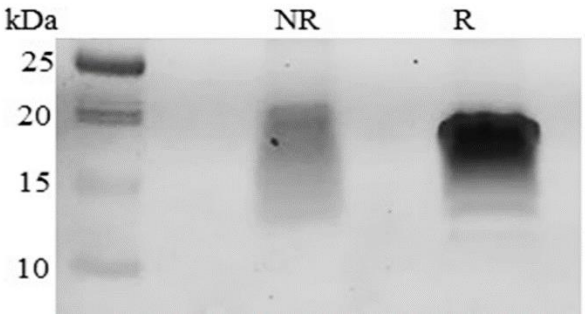

Figure 4. Deflamin polypeptide profile analysed by denaturing electrophoresis under reducing and non-reducing conditions. Representative image of the polypeptide distribution of isolated deflamin from *Lupinus albus* seeds separated by SDS-PAGE under reducing (R) and non-reducing (NR) conditions. Deflamin (50 µg.mL^{-1}) was loaded onto a 17.5% (w/v acrylamide) polyacrylamide gel with reducing buffer (100 mM Tris-HCl buffer, pH 6.8, containing 100 mM β-mercaptoethanol, 2% (w/v) SDS, 15% (v/v) glycerol and 0.006% (w/v) *m*-cresol purple) and non-reducing buffer (100 mM Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 15% (v/v) glycerol and 0.006% (w/v) *m*-cresol purple).

Results indicate the presence of different polypeptide bands in both conditions, suggesting the presence of disulphide bonds – possibly of an interchain disulphide bond,

as judged by the difference in polypeptide patterns between lanes R and NR in Figure 4., since the standard deflamin band (below 20 kDa) apparently does not present marked differences in mass when exposed to reducing or non-reducing conditions.

Deflamin was further analysed by reverse-phase HPLC, in order to separate its different polypeptide constituents. Figure 5 shows the chromatographic profiles obtained at 280 and 214 nm, and the respective electrophoretic patterns. Results show the presence of the deflamin standard bands (Figure 4), scattered throughout peaks 2 to 4.

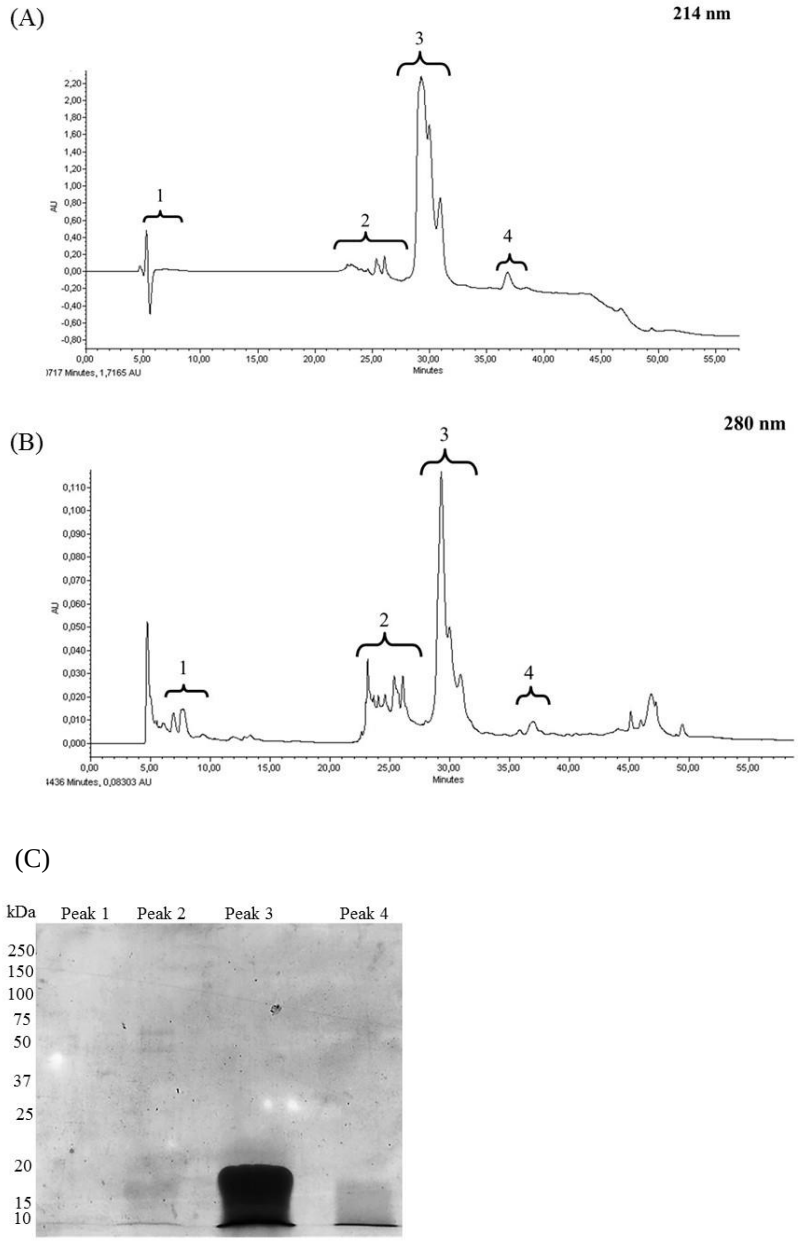

Figure 5. Deflamin analysis by RP-HPLC. Representative images of deflamin fractionation by RP-HPLC and SDS-PAGE into its constituent polypeptides. Deflamin was extracted and purified from *Lupinus albus* seeds by the

methodology developed and illustrated in Figure 1. (A) and (B) - Reverse Phase (RP)-HPLC chromatography of deflamin monitored at 214 nm (A) and at 280 nm (B). (C) Polypeptide profile of each peak collected from the HPLC run as visualized by SDS-PAGE (17.5% w/v acrylamide) performed under reducing conditions (R-SDS-PAGE). Protein peaks (50 µg) were loaded onto 17.5% (w/v acrylamide) polyacrylamide gels. Total polypeptides were stained with Coomassie Brilliant Blue.

In order to determine the peak fraction with the highest activity, we further determined the MMPI activities of the four HPLC peaks (Figure 5 A and B), using the DQ gelatin and the cell invasion assays. Results are shown in Figure 6 and 7, respectively. The 280 nm peak eluting from the HPLC reverse phase column at 45 to 50 min does not contain neither protein nor bioactivity. For this reason, its study it was not continued.

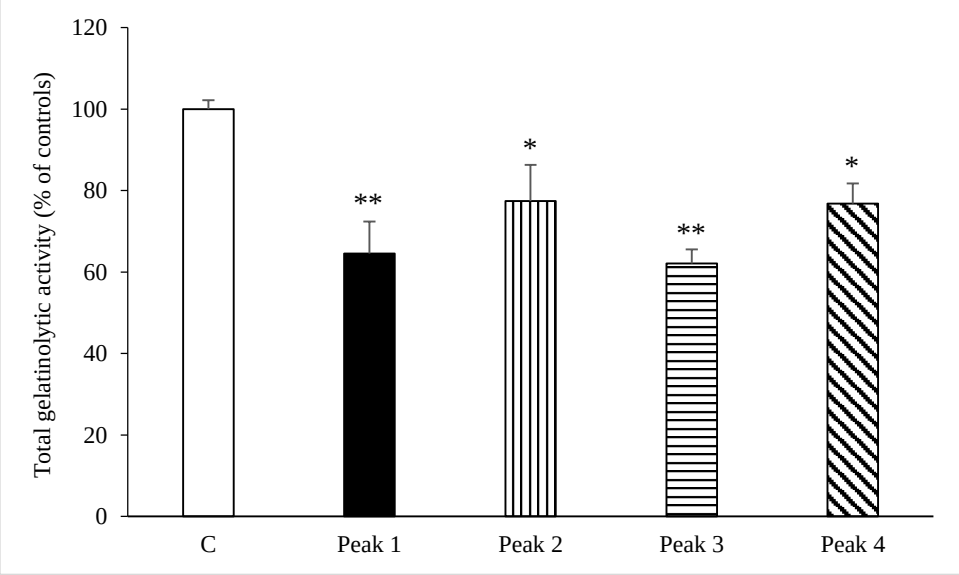

Figure 6. MMP-9 proteolytic activity of peaks 1 to 4 obtained by HPLC fractionation of deflamin. MMP-9 proteolytic activity of peaks 1 to 4 (Figure 5 A and B) obtained by HPLC fractionation of deflamin. Protein samples were added at a concentration of 25 µg.mL^{-1} and gelatinolytic activity was measured by the DQ fluorogenic assay. Results are expressed in arbitrary units of fluorescence and represent the average of at least three replicate experiments (n = 3) ± SD. ∗∗ represents P<0.001 and ∗ represents P<0.05 when compared to controls.

Results demonstrate that all the selected peaks presented some degree of gelatinolytic inhibition when compared to controls (P<0.05), but peak 1 and peak 3 stand out as presenting the highest MMPI activities, which were very significantly different from controls (P<0.001). Figure 7 presents HT29 cell migration after exposure to the selected deflamin peaks collected through RP-HPLC separation. With the cell invasion assay,

results differed significantly between peaks ($P<0.05$), as only peak 1 and peak 3 were able to significantly reduce cell invasion ($P<0.001$), whereas peaks 2 and 4 were not significantly different from controls ($P>0.05$).

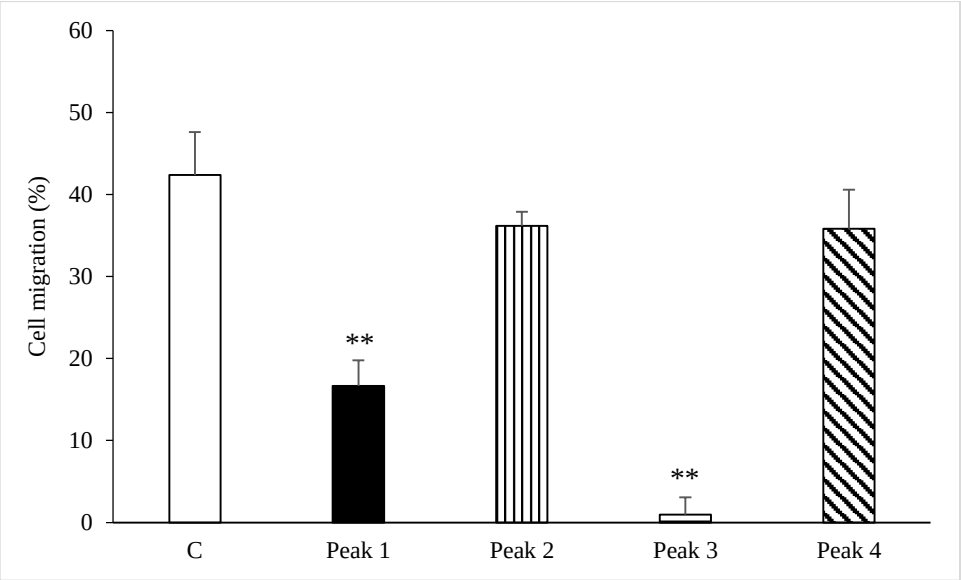

Figure 7. HT29 cell migration after exposure to the selected deflamin peaks collected after RP-HPLC separation - HT29 cell migration after exposure to each of the selected deflamin peaks collected after RP-HPLC fractionation (Figure 5 A and B) - Relative migration rates. Values are the means of at least three replicate experiments ± SD, and are expressed as % wound closure in relation to 0 h. Cells were grown until reaching 80% confluence and the monolayer was scratched with a pipette tip (0 h). Cells were then exposed to 25 µg protein.mL^{-1} extract and cell migration was recorded after 48 h. ∗∗ represents $P < 0.001$ when compared to the controls.

Therefore, the highest degree of activity seems to be related to the 15 kDa large band present in peak 3 and also to peak 1, the latter of which did not show any visible polypeptide bands in the electrophoretic pattern.

3.4. L. albus *deflamin mass is a mixture of β-conglutin and δ-conglutin large chain fragments*

The four selected peaks obtained from the HPLC separation illustrated in Figure 5 were further analysed by mass spectrometry, for identification. The results obtained are presented in Figures 8 A (peak 1), B (peak 2), C (peak 3) and D (peak 4).

The main seed storage proteins in lupins, referred to as conglutins, have been classified into four families: α-, β-, γ- and δ-conglutins. β-conglutin, a globulin and the main seed storage protein in lupins, is the vicilin or 7S member of the seed storage proteins, whereas α-conglutin, another globulin, is the legumin or 11S member of the seed storage proteins. In narrow-leafed lupin (*Lupinus angustifolius*), a total of three α-conglutin, seven β-conglutin, two γ-conglutin and four δ-conglutin encoding genes were previously identified (Foley *et al.*, 2011, 2015). These genes have been referred to as conglutin alpha 1, 2 and 3, conglutin beta 1, 2, 3, 4, 5, 6 and 7, conglutin gamma 1 and 2, and conglutin delta 1, 2, 3 and 4, respectively. In addition, the resulting polypeptides undergo extensive and complex processing and assembly processes, resulting in the high degree of microheterogeneity which characterizes these proteins.

In this work, mass sequencing of the four peaks revealed the presence of fragments of two of these major proteins, β- and δ-conglutin, but with a specific distribution:

Peak 1: fragments of conglutin beta 1, 2, 3, 4, 5 and 7 (Figure 8A). Note that β-conglutin fragments were detected which span the entire molecule. No δ-conglutin fragments were detected in peak 1.

Peak 2: fragments of conglutin beta 1, 2 and 6, and conglutin delta-2 large chain (Figure 8B). Note that β-conglutin and δ-conglutin fragments were detected which span the entire molecules of their precursors.

Peak 3: fragments of conglutin beta 1 and conglutin delta-2 large chain (Figure 8C). Also in here, note that β-conglutin and δ-conglutin fragments were detected which span the entire molecules of their precursors.

Peak 4: fragments of conglutin beta 1, 2, 3, 6 and 7 (Figure 5). Once more, note that β-conglutin fragments were detected which span the entire molecule and, as in peak 1, no δ-conglutin fragments were detected in peak 4. These results allow us to conclude that *L. albus* deflamin is composed of a complex mixture of β-conglutin and δ-conglutin fragments and that β-conglutin and δ-conglutin are both precursors of deflamin.

Peak 1: Conglutin beta 1, 2, 3, 4, 5 and 7

N	Unused	Total	%Cov(95)	Accession	Name	Species	Peptides(95%)
1	70.99	70.99	55.41	P00761|TRYP_PIG	Trypsin	PIG	35
13	6.56	6.57	4.31	F5B8W1|CONB3_LUPAN	Conglutin beta 3	LUPAN	5
13	0	6.57	4.146	F5B8W0|CONB2_LUPAN	Conglutin beta 2	LUPAN	5
13	0	6.57	3.925	F5B8W3|CONB5_LUPAN	Conglutin beta 5	LUPAN	5
13	0	6.57	4.237	F5B8W2|CONB4_LUPAN	Conglutin beta 4	LUPAN	5
17	4	6.03	7.156	Q53HY0|CONB1_LUPAL	Conglutin beta 1	LUPAL	2
21	2	2.01	2.149	F5B8W5|CONB7_LUPAN	Conglutin beta 7	LUPAN	1

N13 - Conglutin beta 3

MAKMRVRFPTLVLLLGIVFLMAVSIGIAYGEKNVLKNHERPQEREQEERDPRQQPRPHHQEEQEREHRRESEESQ
EEEREQRREPRREREQEQQPQHGRREEEEEWQPRRQRPQSRREEREQEQGSSSSSRRQSGYERREQREEREQEQE
QGSRSDSRRQRNPYYFSSERFQTLYRNRNGQIRVLERFDQRTNRLENLQNYRIVEFQSKPNTLILPKHSDADYIL
VVLNGSATITIVNPDKRQSYNLENGDALRLPAGTTSYILNPDDNQNLRVVKLAIPINNPGNFYDFYPSSSKDQQS
YFSGFSKNTLEATFNTRYEEIQSILLGNEDEQEDDEQWHGQEQSHQDEGVIVRVSKEQVQELRKYAQSSSRKGKP
YESGPFNLRSNKPIYSNKFGNFYEITPDRNPQAQDLDISLTFIEINEGALLLPHYNSKAIFVVVVDEGEGNYELV
GIRDQQRQQDEQEVRRYSARLSEGDIFVIPAGHPISINASSNLRLLGFGINADENQRNFLAGSEDNVIRQLDREV
KGLIFPGSAEDVERLIKNQQQSYFANAQPQQQQQREREGRHGRRGHISSILSTLY

N17- Conglutin beta 1

MGKMRVRFPTLVLVLGIVFLMAVSIGIAYGEKDVLKSHERPEEREQEEWQPRRQRPQSRREEREQEQEQGSPSYP
RRQSGYERRQYHERSEQREEREQEQQQGSPSYSRRQRNPYHFNSQRFQTLYKNRNGKIRVLERFDQRTNRLENLQ
NYRIVEFQSKPNTLILPKHSDADYVLVVLNGRATITIVNPDRRQAYNLEYGDALRIPAGSTSYILNPDDNQKLRV
VKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRILLGNEDEQEYEEQRRGQEQSHQDEG
VIVRVSREQIQELTKYAQSSSGKDKPSQSGPFNLRSNEPIYSNKYGNFYEITPDRNPQVQDLDISLTFTEINEGA
LLLPHYNSKAIFIVVVGEGNGKYELVGIRDQQRQQDEQEEEPEEVRRYSARLSEGDIFVIPAGYPISVNASSNLR
LLGFGINAYENQRNFLAGSEDNVIRQLDREVKELTFPGSAEDIERLIKNQQQSYFANALPQQQQQSEKEGRRGRR
GPISSI

N21- Conglutin beta 7

MARMRVRFPTLVLLLGILFLMAVSIGIAYGEKDVIKNHERPGEREHEERDPRQQPRPRKQEEQEREHRREEEHDR
DPSRGRRESEERQEEERERRREPCREREQEQQPQHGRREEEEEEEEWQPRRLRPQSRKEEREQEQGSSSSSRKQS
GYERRQYHERREQRDEKEKEQDSRSDSRRQRNPYHFSSERFQTRYRNRNGQIRVLERFDQRTNRLENLQNYRIVE
FQSNPNTLILPKHSDADYILVVLNGRATITIVNPDKRQAYNLEYGDALRVPAGTTSYILNPDDNQNLRVVKLAIP
INNPSNFYDFYPSSTKDQQSYFSGFSKNTLEATFNTRYEEIQRILLGNEDEQEDEEQRRGQEQSYQDEGVIVRVS
KEQIQELRKHAQSSSRKGKPSESGPFNLRSNESIYSNKFGNFYEITPERNPQVQDLDISLTFTEINEGALLLPHY
NSKAIFIVVVDEGEGNYELVGIRDQQRQQDEQEEEEEEVRRYSARLSEGDIFVIPAGYPISVNASSNLRLLGFGI
NANENQRNFLAGSEDNVISQLDREVKELTFPGSAQDVERLIKNQQQSYFANAQPQQKQQREKEGRRGRRSLISSI
LSTLY

Figure 8A. Mass spectrometry analyses of the peak 1 component (see Figure 5) of *L. albus* deflamin. The fractionation of *L. albus* deflamin by RP-HPLC of deflamin resulted in four peaks, each of which contain polypeptides that were identified by mass spectrometry. Colour code indicates peptides confidence: **green** residues corresponds to peptides with **95% confidence**; yellow for peptides with confidence between 50 and 95%; red for peptides with confidence bellow 50%; and grey corresponds to unidentified residues.

Peak 2: Conglutin beta 1, 2 and 6 + conglutin delta 2 (large chain)

N	Unused	Total	%Cov(95)	Accession	Name	Species	Peptides(95%)
1	54.24	54.24	62.34	P00761|TRYP_PIG	Trypsin	PIG	27
3	14.97	14.97	11.11	Q53HY0|CONB1_LUPAL	Conglutin beta 1	LUPAL	11
10	3.14	3.14	42.5	P09931|CGD2L_LUPAN	Conglutin delta-2 large chain	LUPAN	3
17	2.43	9.79	2.53	F5B8W4|CONB6_LUPAN	Conglutin beta 6	LUPAN	1
82	1.73	7.4	8.63	Q6EBC1|CONB2_LUPAL	Conglutin beta 2	LUPAL	1

N3- Conglutin beta 1

MGKMRVRFPTLVLVLGIVFLMAVSIGIAYGEKDVLK**SHERPEEREQEEWQPR**RQRPQ**SRREEREQEQEQGSPSYP**
RRQSGYERRQYHERSEQREEREQEQQQGSPSYSRRQRNPYHFNSQRFQTLYKNRNGKIRVLERFDQRTNRLENLQ
NYRIVEFQSKPNTLILPKHSDADYVLVVLNGRATITIVNPDRRQAYNLEYGDALRIPAGSTSYILNPDDNQKLRV
VKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRILLGNEDEQEYEEQRRG**QEQSHQDEG**
VIVRVSREQIQELTKYAQSSSGKDKPSQSGPFNLRSNEPIYSNKYGNFYEITPDRNPQVQDLDISLTFTEINEGA
LLLPHYNSKAIFIVVVGEGNGKYELVGIRDQQRQQD**EQEEEPEEVRR**YSARLSEGDIFVIPAGYPISVNASSNLR
LLGFGINAYENQRNFLAGSEDNVIRQLDREVKELTFPGSAEDIERLIKNQQQSYFANALPQQQQQSEKEGRRGRR
GPISSI

N10- Conglutin delta-2 large chain

RHKSSQ**ESEE**SEELDQCCEQLNELNSQRCQCR**ALQQIYESQSEQCEGR**QQEQQLEGELEKLPR**ICGFGPLR**RCNI
NPDEE

N17- Conglutin beta 6

MIKMRVRFPTLVLLLGIVFLMAVSIGIAYGEKNVIKNHERPQEREQEERDPRQQPRPHHQEEQEREHRREEERDR
EPSRGRRESEESREEEREQRREPRREREQEQQPQHGR**REEEEEWQPR**RQRPQSRREEREQEQGSSSSSRRQSAYE
RR**EQREEREQEQEQGSRSDSR**RQRNPYYFSSERFQTLYRNRNGQIRVLERFDKRTDRLENLQNYRIVEFQSKPNT
LILPKHSDADYILVVLNGSATITIVNPDKRQSYNLENGDALRLPAGTTSYILNPDDNQNLRVVKLAIPINNPGNF
YDFYPSSSKDQQSYFSGFSRNTLEATFNTRYEEIQRILLGNEDEQEDDEQR**HGQEQSHQDEGVIVR**VSKEQVQEL
RKYAQSSSRKGKPSKSGPFNLRSNKPIYSNKFGNFYEITPNRNPQAQDLDISLTFIEINEGALLLPHYNSKAIFV
VLVDEGEGNYELVGIRDQQRQQDEQEVRRYSARLSEGDIFVIPAGHPISINASSNFRLLGFGINADENQRNFLAG
FEDNVIRQLDREVKGLTFPGFAEDVERLIKNQQQSYFANAQPQQQQQREREGRHGRRGHIFSILSTLY

Figure 8B. Mass spectrometry analyses of the peak 2 component (see Figure 5) of *L. albus* deflamin. The fractionation of *L. albus* deflamin by RP-HPLC of deflamin resulted in four peaks, each of which contain polypeptides that were identified by mass spectrometry. Colour code indicates peptides confidence: **green** residues corresponds to peptides with **95% confidence**; yellow for peptides with confidence between 50 and 95%; red for peptides with confidence bellow 50%; and grey corresponds to unidentified residues.

Peak 3: Conglutin beta 1 + conglutin delta 2 (large chain)

N	Unused	Total	%Cov(95)	Accession	Name	Species	Peptides(95%)
1	35.22	35.22	62.77	P00761|TRYP_PIG	Trypsin	PIG	17
3	12.48	12.48	36.25	P09931|CGD2L_LUPAN	Conglutin delta-2 large chain	LUPAN	8
98	2.13	2.22	8.286	Q53HY0|CONB1_LUPAL	Conglutin beta 1	LUPAL	3

N3- Conglutin delta-2 large chain

RHKSSQ**ESEESEELDQCCEQLNELNSQR**CQCRALQQIYES**QSEQCEGR**QQEQQLEGELE**KLPRI****CGFGPLR**RCNI
NPDEE

N2- Conglutin beta 1

MGKMRVRFPTLVL**VLGIVFLMAVSIGIAYGEKDVLKSHERPEEREQEEWQPRRQRPQ****SRREEREQEQEQGSPSYP**
RRQSGYERRQYHERSEQREEREQEQQQGSPSYSRRQRNPYHFNSQRFQTLYKNRNGKIRVLERFDQRTNRLENLQ
NYRIVEFQSKPNTLILPKHSDADYVLV**VLNGRATITIVNPDRR**QAYNLEYGDALRIPAGSTSYILNPDDNQKLRV
VKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNT**LEATFNTRYEEIQRILLGNEDEQEYEEQR****RGQEQSHQDEG**
VIVRVSREQIQELTKYAQSSSGKDKPSQSGPFNLRSNEPIYSNKYGNFYEITPDRNPQVQDLDISLTFTEINEGA
LLLPHYNSKAIFIV**VVGEGNGKYELVGIRDQQRQQD****EQEEEPEEVR**RYSARLSEGDIFVIPAGYPISVNASSNLR
LLGFGINAYENQRNFLAGSEDNVIRQLDREVKELTF**PGSAEDIERLIKNQQQSYFANALPQQQQQSEKEGR**RGRR
GPISSI

Figure 8C. Mass spectrometry analyses of the peak 3 component (see Figure 5) of *L. albus* deflamin. The fractionation of *L. albus* deflamin by RP-HPLC of deflamin resulted in four peaks, each of which contain polypeptides that were identified by mass spectrometry. Colour code indicates peptides confidence: **green** residues corresponds to peptides with **95% confidence**; yellow for peptides with confidence between 50 and 95%; red for peptides with confidence bellow 50%; and grey corresponds to unidentified residues.

Peak 4: Conglutin beta 1, 2 , 3, 6 and 7

N	Unused	Total	%Cov(95)	Accession	Name	Species	Peptides(95%)
1	177.74	177.74	21.9	F5B8W1|CONB3_LUPAN	Conglutin beta 3	LUPAN	103
2	91.12	173.11	40.3	Q53HY0|CONB1_LUPAL	Conglutin beta 1	LUPAL	50
3	22.91	22.91	51.08	P00761|TRYP_PIG	Trypsin	PIG	12
4	18.34	124.85	30.58	Q6EBC1|CONB2_LUPAL	Conglutin beta 2	LUPAL	13
5	15.16	74.85	20	F5B8W5|CONB7_LUPAN	Conglutin beta 7	LUPAN	12
8	4.68	78.45	17.88	F5B8W4|CONB6_LUPAN	Conglutin beta 6	LUPAN	4

N1- Conglutin_beta 3

MAKMRVRFPTLVLLLGIVFLMAVSIGIAYGEKNVLKNHERPQEREQEERDPRQQPRPHHQEEQEREHRRESEESQ
EEEREQRREPRREREQEQQPQHGRREEEEEWQPRRQRPQSRREERQEQEQGSSSSSRRQSGYERREQREEREQEQE
QGSRSDSRRQRNPYYFSSERFQTLYRNRNGQIRVLERFDQRTNRLENLQNYRIVEFQSKPNTLILPKHSDADYIL
VVLNGSATITIVNPDKRQSYNLENGDALRLPAGTTSYILNPDDNQNLRVVKLAIPINNPGNFYDFYPSSSKDQQS
YFSGFSKNTLEATFNTRYEEIQSILLGNEDEQEDDEQWHGQEQSHQDEGVIVRVSKEQVQELRKYAQSSSRKGKP
YESGPFNLRSNKPIYSNKFGNFYEITPDRNPQAQDLDISLTFIEINEGALLLPHYNSKAIFVVVVDEGEGNYELV
GIRDQQRQQDEQEVRRYSARLSEGDIFVIPAGHPISINASSNLRLLGFGINADENQRNFLAGSEDNVIRQLDREV
KGLIFPGSAEDVERLIKNQQQSYFANAQPQQQQQRERERGRHGRRGHISSILSTLY

N2- Conglutin_beta 1

MGKMRVRFPTLVLVLGIVFLMAVSIGIAYGEKDVLKSHERPEEREQEEWQPRRQRPQSRREEREQEQEQGSPSYP
RRQSGYERRQYHERSEQREEREQEQQQGSPSYSRRQRNPYHFNSQRFQTLYKNRNGKIRVLERFDQRTNRLENLQ
NYRIVEFQSKPNTLILPKHSDADYVLVVLNGRATITIVNPDRRQAYNLEYGDALRIPAGSTSYILNPDDNQKLRV
VKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRILLGNEDEQEYEEQRRGQEQSHQDEG
VIVRVSREQIQELTKYAQSSSGKDKPSQSGPFNLRSNEPIYSNKYGNFYEITPDRNPQVQDLDISLTFTEINEGA
LLLPHYNSKAIFIVVVGEGNGKYELVGIRDQQRQQDEQEEEPEEVRRYSARLSEGDIFVIPAGYPISVNASSNLR
LLGFGINAYENQRNFLAGSEDNVIRQLDREVKELTFPGSAEDIERLIKNQQQSYFANALPQQQQQSEKEGRRGRR
GPISSI

N4 - Conglutin_beta 2

MGKMRVRFPTLVLVLGIVFLMAVSIGIAYGEKDVLKSHERPEEREQEEWQPRRQRPQSRREEREQEQEQGSPSYP
RRQSGYERRQYHERSEQREEREQEQQQGSPSYSRRQRNPYHFSSQRFQTLYKNRNGKIRVLERFDQRTNRLENLQ
NYRIVEFQSKPNTLILPKHSDADYVLVVLNGRATITIVNPDRRQAYNLEYGDALRIPAGSTSYILNPDDNQKLRV
VKLAIPINNPGYFYDFYPSSTKDQQSYFSGFSRNTLEATFNTRYEEIQRIILGNEDEQEYEEQRRGQEQSDQDEG
VIVIVSKKQIQKLTKHAQSSSGKDKPSDSGPFNLRSNEPIYSNKYGNFYEITPDRNPQVQDLNISLTYIKINEGA
LLLPHYNSKAIYVVVVDEGEGNYELVGIRDQQRQQDEQEEKEEEVIRYSARLSEGDIFVIPAGYPISINASSNLR
LLGFGINADENQRNFLAGSKDNVIRQLDRAVNELTFPGSAEDIERLIKNQQQSYFANGQPQQQQQQQSEKEGRRG
RRGSSLPF

N5 - Conglutin_beta 7

MARMRVRFPTLVLLLGILFLMAVSIGIAYGEKDVIKNHERPGEREHEERDPRQQPRPRKQEEQEREHRREEEHDR
DPSRGRRESEERQEEERERRREPCREREQEQQPQHGRREEEEEEEEEWQPRRLRPQSRKEEREQEQGSSSSSRKQS
GYERRQYHERREQRDEKEKEQDSRSDSRRQRNPYHFSSERFQTRYRNRNGQIRVLERFDQRTNRLENLQNYRIVE
FQSNPNTLILPKHSDADYILVVLNGRATITIVNPDKRQAYNLEYGDALRVPAGTTSYILNPDDNQNLRVVKLAIP
INNPSNFYDFYPSSTKDQQSYFSGFSKNTLEATFNTRYEEIQRILLGNEDEQEDEEQRRGQEQSYQDEGVIVRVS
KEQIQELRKHAQSSSRKGKPSESGPFNLRSNESIYSNKFGNFYEITPERNPQVQDLDISLTFTEINEGALLLPHY
NSKAIFIVVVDEGEGNYELVGIRDQQRQQDEQEEEEEEVRRYSARLSEGDIFVIPAGYPISVNASSNLRLLGFGI
NANENQRNFLAGSEDNVISQLDREVKELTFPGSAQDVERLIKNQQQSYFANAQPQQKQQREKEGRRGRRSLISSI
LSSLY

Figure 8D. Mass spectrometry analyses of the peak 4 component (see Figure 5) of *L. albus* deflamin. The fractionation of *L. albus* deflamin by RP-HPLC of deflamin resulted in four peaks, each of which contain polypeptides that were identified by mass spectrometry. Colour code indicates peptides confidence: **green** residues corresponds to peptides with **95% confidence**; yellow for peptides with confidence between 50 and 95%; red for peptides with confidence bellow 50%; and grey corresponds to unidentified residues.

The presence of fragments of these two proteins is rather interesting. β-conglutin is a trimeric protein devoid of disulphide bridges in which the monomers consist of a very large number of polypeptides, glycosylated or not, ranging from 16 to over 70 kDa, but a large number of proteolytic processing sites give rise to the abundance of 7S mature

polypeptides observed (Duranti *et al.*, 1992). According to Duranti, Cucchetti and Cerletti (1984), the complete degradation post-germination strongly supports the storage function of β-conglutin. Interestingly, another fragment of this protein is known for its potent bioactivities against fungi: Blad, an abundant transient β-conglutin derived polypeptide chain of 20 kDa displaying lectin like activity (Ramos, *et al.*, 1997). Being highly reactive and with the presence of bioactive cupine domain, it is possible that there are many fragments of β-conglutin with specific uncharted activities yet to be discovered. Previous works revealed however that deflamin has no antifungal nor bactericide activity (results not shown), and the sequence of the β-conglutin fragments does not match that of BLAD.

δ-Conglutin belongs to the 2S sulphur-rich albumin family which might also have specific unknown bioactivities in lupine. *Lupinus* seeds 2S albumin, also termed δ-conglutin (Sironi *et al*., 2005), is a monomeric protein which comprises two small polypeptide chains linked by two interchain disulfide bonds: a smaller polypeptide chain, which consists of 37 amino acid residues resulting in a molecular mass of 4.4 kDa, and a larger polypeptide chain containing 75 amino acid residues with a molecular mass of 8.8 kDa (Salmanowicz and Weder, 1997). This later, larger polypeptide chain is somewhat similar to some of the polypeptide profiles obtained for deflamin, particularly in peak 3 (see Figure 5). The larger polypeptide chain contains two intrachain disulfide bridges and one free sulfhydryl group (Salmanowicz and Weder, 1997). This could tentatively explain the slight difference in apparent molecular mass detected between R- and NR-SDS-PAGE of deflamin (see Figure 4). This protein presents specific inherent unique features among the proteins from *L. albus*: besides its high cysteine content, it exhibits a low absorbance at 280 nm.

As far as the physiological role of δ-conglutin is concerned, a storage function has been proposed for this class of proteins. However, structural similarity with the plant cereal inhibitor family, which includes bi-functional trypsin/alpha-amylase inhibitors, may suggest a defense function for this protein in addition to its storage role and might corroborate its role as MMPI (Duranti *et al.*, 2008). The presence of free sulfhydryl groups in δ-conglutin could be related to a high degree of affinity towards the Zn^{2+} active site in MMPs, and could explain its mode of inhibition. Indeed, on way to isolate these conglutins is through Zn precipitation (Duranti *et al.*, 2008). Furthermore, its presence in

L. albus seeds was assessed to be around 3 to 4% of the seed weight (Sironi, Sessa and Duranti, 2005), which is consistent to the yields we obtained in chapter 1. Also, the *Lupinus* seed 2S albumin is typically present in both the albumin and the globulin fractions (Salmanowicz and Przybylska, 1994), thus explaining our results obtained previously for the MMP inhibitory activity in the two protein fractions (Lima *et al.*, 2016).

Nonetheless, we must not forget that the HPLC peaks 1 and 4 of deflamin (Figure 5) were only composed by β-conglutin fragments and still presented MMPI activities, albeit at lower levels. Therefore, the highest activity seems may be attributed to a specific mixture comprising fragments of both proteins, β- and δ-conglutins, and not to δ-conglutin exclusively. The fact that only the large polypeptide chain of δ-conglutin was found to be present in deflamin might suggest that its three sulfhydryl groups could be free to interact (this could explain the presence of a group of apparently three minor higher molecular mass bands which comprise deflamin) and that this complex holds the highest activity. Alternatively, δ-conglutin smaller polypeptide chain may be present in deflamin and the inability to detect its presence may be due to an artefact resulting from the techniques surrounding the mass spectrometry procedure. Future works would be necessary to fully characterize this polypeptide mixture.

4. Conclusion

In the last decade a substantial amount of research has turned towards the discovery of novel plant foods containing MMPIs, but few, if any present the potential of deflamin, as it is easy to isolate and displays high MMP-9 inhibitory activities. Here we have characterized deflamin as a complex mixture of soluble fragments from two specific protein precursors: δ- and β-conglutins. Overall, this polypeptide mixture was shown to be highly soluble in water; its bioactivities resist to boiling, to low pH values and possibly to digestive proteases; it strongly inhibits matrix metalloproteinase (MMP)-9 and/or MMP-2, *i.e.* it is an MMP inhibitor (MMPI) at low concentrations and in a dose-dependent manner, and it reduces the invasion capacity of the human colon adenocarcinoma cell line HT29 without exerting cytotoxicity. These features make deflamin a novel type of MMPI that can be used as a nutraceutical or as an ingredient of

functional foods in the prevention/treatment of tumourigenesis and cell invasion, as well as of any disease derived from them. As a potent inhibitor of the matrix metalloproteinases MMP-9 and MMP-2, deflamin may prove useful as a nutraceutical or in functional foods in the prevention and treatment of a very wide array of diseases related to MMP-9 activity. Its efficacy when administered orally and its capacity to survive the digestive process suggests that it may act efficiently in the colon, without exerting the deleterious side-effects which characterize the synthetic MMPIs, making deflamin an excellent candidate to be used in the prevention and treatment of colorectal cancer.

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Chapter 3

Anti-inflammatory effects of deflamin, a lupin seed-derived polypeptide mixture with a potent inhibitory effect on MMP-9 and MMP-2 activities

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1. Introduction

With the current general increase in the incidence of inflammatory diseases incurs an inevitable boost in medical and pharmaceutical costs. A well-known example is provided by the various inflammatory bowel diseases (IBDs) which have been dramatically increasing over time, considered as an emerging global disease (Burisch *et al.*, 2014; Molodecky *et al.*, 2012). Since mortality in IBDs is low (Burisch *et al.*, 2014; Duricova *et al.*, 2010) and the disease is most often diagnosed in the young (Burisch *et al.*, 2014; Loftus, Schoenfeld and Sandborn, 2002), it is predicted that the global prevalence of IBDs will continue to increase substantially in the next years (Abraham and Cho, 2009; Molodecky *et al.*, 2012). IBDs are mainly characterized by chronic mucosal inflammation

in pathologic histology of the gastrointestinal tract in susceptible individuals (Abraham and Cho, 2009; Danese, Sans and Fiocchi, 2004; Mikhailov and Furner, 2009; Podolsky, 2002). Overall, IBD clinical treatments are very prone to induce side effects, have very unspecific targets and are extremely costly, whilst presenting low success rates (Dyson and Rutter, 2012). Colitis-associated colorectal cancer (CAC) is a tumour that develops in the context of chronic inflammation, and is considered the most serious complication of IBD (Taleban *et al.*, 2016). Patients with ulcerative colitis (UC) are more likely (a 20 to 30-fold higher risk) to develop colorectal cancer (CRC) when compared to the general population (Romano *et al.*, 2016). In addition, the risk of developing CAC increases notably 8 to 10 years after diagnosis of IBD, being estimated that CRC accounts for 10 to 15% of IBD-related deaths (Taleban *et al.*, 2016).

One important link between CRC and IBD is the involvement of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases which are engaged in the remodeling of connective tissue (Baugh *et al.*, 1999; Lee *et al.*, 2013; Markle, May and Majumdar, 2010; Murphy and Nagase, 2009; Parks, Wilson and López-Boado, 2004), mostly the gelatinases MMP-9 and MMP-2, which play important and well-recognized roles in inflammation (Malla *et al.*, 2008) and also in several oncologic processes, such as tumourigenesis, cell adhesion and metastasis (Herszényi *et al.*, 2012). Although similar in their substrate selectivity, the two gelatinases differ in their roles in IBDs. While MMP-2 is only moderately involved in inflammation (Huhtala *et al.*, 1991), MMP-9 expression is observed primarily in leukocytes (Vandooren, Steen, Van den and Opdenakker, 2013), being highly induced during ulcerative colitis and other IBDs (Garg *et al.*, 2009; Moore *et al.*, 2011). Recently, the involvement of MMPs in inflammatory processes has been observed both in animal models of induced IBD and in lines of intestinal cells and tissue cultures (AbdElazeem and El-Sayed, 2015; Kofla-Dłubacz *et al.*, 2014; Siloşi *et al.*, 2014). Although studies relating MMP-9 inhibition to pre-clinical and clinical IBD reduction are very few, they already suggest that applying MMP inhibitors (MMPIs) can decrease the incidence of IBDs (Matusiewicz *et al.*, 2014), similarly to what happens to the oncological disease (Spoerlein *et al.*, 2013).

Although pharmacological and clinical treatments of IBDs are known to be fairly unsuccessful so far, the ingestion of functional foods and/or nutraceuticals that reduce

inflammation has been suggested to be an effective human tool against these diseases. In the last decade a substantial amount of research has turned towards discovering novel plant foods containing MMPIs (Jeong *et al.*, 2007; Su *et al.*, 2006; Vayalil, Mittal and Katiyar, 2004). Targeting MMP-9 in IBDs could be achieved, at least partly, through the long-term ingestion of natural food-born specific MMP-9 inhibitors that are colon-available, rather than serum-bioavailable, resistant to digestion and non-toxic, which is not always the case for most of the natural MMPIs discovered in several, studies such as phenolic compounds and other secondary metabolites (Kennedy, 1995; Xu and Chang, 2012).

Previous work in our lab has led to the isolation of deflamin, a novel proteinaceous MMPI from *Lupinus albus*, which presents a high inhibitory action against MMP-9 and that has been the subject of a patent application. This proteinaceous mixture is also resistant to heat and acid denaturation, suggesting it may survive the digestive process without reducing its activity. Deflamin has been shown to decrease MMP-9 activity in colon cancer cells and in enzymatic studies, but its effects on IBDs and on *in vivo* animal models has never been tested.

In the present work, we aimed to test the anti-inflammatory effects of deflamin, using *in vivo* experimental animal models of acute inflammation, and with different administration procedures. With that in mind, we evaluated its effect on a model of 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice, a widely used animal model of experimental IBD. Gelatinolytic activity was also evaluated to ascertain if results observed were related to MMP inhibition. The expression of inflammatory biomarkers such as cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) were also evaluated.

2. Materials and Methods

2.1. Materials, solvents and reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS) 5% (w/v) aqueous solution was purchased from Sigma Chemical Co. Ketamine (Imalgene® 1000) and xilazine (Rompun® 2%) were purchased from Bio2 *Produtos Veterinários* (*Lisboa, Portugal*). All other reagents

were purchased from Sigma-Aldrich (St. Louis, USA). Dye-quenched (DQ)-gelatin was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Sampling / Preparation of the lupin extract

Seeds lupin (*Lupinus albus* L.) was used in this study. The lupin extract was prepared by conjugation of several methods (Duranti *et al.*, 2008; Lima *et al.*, 2016), with some modifications. Briefly, approximately 200 g ± 0.1 g of dry lupin seed (without embryo and tegument) was extracted using 50mM of Tris-HCl pH 7,5 (1:10, m/v). The extracted sample was centrifuged at 13500 *g* for 30 min at 4 °C. The supernatant was collected and boiled for 10 min and centrifuged at 13500 *g* for 20 min at 4 °C. After, the supernatant was reduced to pH4 centrifuging again at 13500 *g* 20 min 4 °C. The pellet was resuspended in 40% (v/v) ethanol and 0.4M NaCl, centrifuging it 13500 *g* 30 min 4 °C. Is added 90% (v/v) ethanol to the supernatant leaving overnight at -20 °C. The next day centrifuged at 13500 *g* for 30 min at 4 °C and was again added 40% (v/v) ethanol and 0.4M NaCl, centrifuging again at 13500 *g* 30 min 4 °C and added 90% (v/v) ethanol leaving again overnight at -20 °C. Finally, after centrifugation 13500 *g* 20 min 4 °C, the pellet was resuspended in the smallest possible volume of mili-Q water. The extract obtained was stored in falcon tubes at -20 °C.

2.3. Experimental colitis model

2.3.1. Animals

Male CD-1 mice, 25 to 30 g in weight and 5 to 6 weeks of age (Harlan Iberica, Barcelona, Spain), were housed in standard polypropylene cages with *ad libitum* access to food and water, inside a controlled environment room kept at 22 °C ± 1 °C with a 12 h light, 12 h dark cycle at the Faculty of Pharmacy Central Animal Facility, University of Lisbon.

2.3.2. Animal care and maintenance for the in vivo *experiments*

Experiments were conducted according to the Home Office Guidance in the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London, UK and the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996), as well as to the currently adopted EC regulations

(Directive 2010/63/EU). The studies were performed in compliance with the ARRIVE Guidelines for Reporting Animal Research summarized at http://www.nc3rs.org.uk. The experimental protocol was also endorsed by the Ethics Committee of the Faculty of Pharmacy, University of Lisbon.

2.3.3. Induction of colitis

TNBS was instilled as an intracolonic single dose as previously described (Impellizzeri *et al.*, 2014). Briefly, mice were left unfed during 24 h. On the induction day (day 0), mice were anesthetized with ketamine 100 $mg.kg^{-1}$ + xilazine 10 $mg.kg^{-1}$. Then, 100 µL of TNBS solution was administered through a catheter carefully inserted until 4.5 cm into the colon. Mice were kept for 1 min in a Tredelenburg position to avoid reflux. Four days after induction, mice were anesthetized and blood samples were collected by cardiac puncture. Mice were euthanized by cervical dislocation and necropsied. The abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues and opened longitudinally for observation and classification of diarrhea severity. Afterwards, the colon was washed with PBS for macroscopical observation of the tissue and subsequently fixed in paraformaldehyde for further processing.

2.3.4. Experimental groups

Animals were randomly allocated into four experimental groups as described:

1. Sham group (n = 6): animals were subjected to the procedures described above except the intracolonic administration was with 100 µL of saline solution. During the 4 days of the protocol the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
2. Ethanol (EtOH) group (n = 6): animals were subjected to the procedures described above except the intracolonic administration was with 100 µL of 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
3. TNBS group (n = 10): animals were administered with 100 µL of 2.5% (w/v) TNBS in 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.

4. TNBS + deflamin p.o. (n = 9): animals were administered with 100 µL of 2.5% (w/v) TNBS in 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with deflamin (15 $mg.kg^{-1}$).
5. TNBS + deflamin i.p. (n = 10): animals were administered with 100 µL of 2.5% (w/v) TNBS in 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered intraperitoneal injection with deflamin (10 $mg.kg^{-1}$).

Oral administrations were performed daily, starting from 3 h after the initial administration of TNBS, by gastric gavage.

2.3.5. Macroscopic evaluation of colitis severity

After colon removal, a longitudinal incision was performed for observation of content and classification of diarrhea severity by an observer blinded regarding the experimental groups. Afterwards, the colon was rinsed with saline and analyzed with a surgical microscope for closer observation of the tissue and capture of lesion pictures. The colon was then measured, as well as the extent of injury (if present).

2.3.6. Histology and immunohistochemistry procedures

Colons were removed, fixed in 4% (w/v) paraformaldehyde in PBS for 72 h at room temperature, dehydrated through a graded ethanol series and embedded in paraffin (n = 3 per group). Hematoxylin & Eosin (H&E) staining was performed as previously described (Rocha *et al.*, 2015) and images were acquired using a bright field Axioscop microscope (Zeiss, Göttingen, Germany).

The degree of inflammation and colon damage on microscopic cross-sections was graded semi-quantitatively from 0 to 3, normal colon with no lesions, mucosa of uniform thickness, crypts straight, normal crypt architecture, no cellular infiltration, edema, or exudate meaning no signs of inflammation; 1, colon with mild lesions, mucosal erosion and small superficial ulcers scattered along the length of the colon, with slight crypt loss and mononuclear cell infiltration; 2, colon with moderate lesions, intestines with extensive erosion and ulceration, with moderate crypt loss and neutrophil infiltration; 3, colon with very severe ulceration, thin mucosa with loss of crypts and markedly increased infiltration of neutrophils and acute inflammatory exudate.

For immunostaining, 6 µm thick colon sections were submitted to antigen retrieval in 20 mM citrate buffer containing 1.5% (v/v) H_2O_2 for 15 min at room temperature in the dark, incubated for 10 min in Tris/EDTA buffer at 84 °C and blocked for 1 h at room temperature with 1% (w/v) bovine serum albumin (BSA) in PBS. Primary antibodies [rabbit anti-COX2 (Cell Signaling #4842, 1:100) and mouse anti-iNOS (BD Transduction Laboratories #610328, 1:100)] were used in 0.5% (w/v) BSA in PBS overnight at 4 °C. After washing in PBS, sections were incubated for 1 h at room temperature with anti-rabbit antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, 1:5000) in 0.5% (w/v) BSA in PBS, incubated for 10 min in SIGMAFAST DAB with Metal Enhancer (Sigma, USA) and mounted with Entellan (Merck, Germany). Tissue sections were visualized with a AxioScope brightfield microscope (Zeiss, Göttingen, Germany).

2.3.7. Preventive effects vs curative effects of deflamin.

To compare the preventive effect of deflamin with its curative effect, mice were maintained and treated as described (2.3.2 and 2.3.3) above and randomly allocated into four experimental groups:

1. Sham group (n = 6): animals were subjected to the procedures described before (2.3.3) except the intracolonic administration was with 100 µL of saline solution. During the 4 days of the protocol the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
2. Ethanol (EtOH) group (n = 6): animals were subjected to the procedures described above except the intracolonic administration was with 100 µL of 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
3. TNBS group (n = 10): animals were administered with 100 µL of 2.5% (w/v) TNBS in 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with 10 $mL.kg^{-1}$ of distilled water.
4. TNBS + deflamin p.o. (n = 9): animals were administered with 100 µL of 2.5% (w/v) TNBS in 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with deflamin (15 $mg.kg^{-1}$)
5. TNBS + deflamin preventive treatments (n = 10): Three days before of TNBS induction, animals were administered orally with deflamin (15 $mg.kg^{-1}$).

Animals were administered with 100 µL of 2.5% (w/v) TNBS in 50% (v/v) ethanol. During the 4 days of the protocol the animals were administered orally with deflamin (15 $mg.kg^{-1}$).

After the 4 days experiment, macroscopic evaluation of colitis severity was performed as described above (2.3.5).

2.4. The effect of deflamin on MMP-9 and MMP-2 catalytic activities

2.4.1. Total gelatinolytic activity from colonic tissues

Combined MMP-9 and MMP-2 activities were determined in the colons of the mice subjected to deflamin extract treatments, as described in Garg *et al.*, (2009) and Medina *et al.*, (2006), with few alterations. Briefly, colonic tissue was homogenized in a 1/100 ratio (weight/vol) in 50 mM Tris HCl (pH 7.6), 150 mM NaCl (samples were sonicated three times for 10 s each, at 1 min intervals). After 10 min on ice, protein extracts were centrifuged for 10 min at 13,000 *g* at 4 °C, the supernatants were preserved, and protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951). Samples were stored at -80 °C until assayed. Protein extraction of each colon, as described above, was used to quantify the respective gelatinolytic activities. The fluorogenic substrate dye-quenched (DQ)-gelatin purchased from Invitrogen (Carlsbad, CA, USA) was used to quantify MMP-9 and MMP-2 activities. DQ gelatin was dissolved in water at 1 mg. mL^{-1} as per the manufacturer's instructions. All solutions and dilutions were prepared in assay-buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% v/v Tween 20). A 96-well micro-assay plate (Chimney, 96-well, black) was used. Each colonic tissue supernatant from each treatment was loaded (100 µL). Subsequently, DQ-gelatin (at a final concentration of 2.5 $\mu g.mL^{-1}$) was added to each well and the plate was allowed to incubate for 1 h. Fluorescence levels were measured (ex. 485 nm/em. 530 nm). All data were corrected by subtracting of their corresponding negative controls.

2.4.2. Gelatin zymography of colon extracts

To determine the specific metalloproteinase activities in colon extraction supernatants, a gelatin-zymography was performed according to standard methods (Toth, Sohail and Fridman, 2012), with the following modifications: SDS-polyacrylamide gels (12.5% w/v acrylamide) were copolymerized with 1% (w/v) gelatin. Colon extraction supernatants

was treated with a non-reducing buffer containing 62.6 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue were loaded into each well of the SDS-gel. Electrophoresis was carried out as described before (Laemmli, 1970) in a 12,5% (w/v) acrylamide resolving gel and a 5% (w/v) acrylamide stacking gel, performed in a vertical electrophoresis unit at 100 V and 20 mA per gel. After electrophoresis, gels were washed three times in 2.5% (v/v) Triton X-100 for 60 min each, to remove the SDS. Gels were then incubated two days with developing buffer (50 mM Tris-HCl pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.01% w/v sodium azide), stained with Coomassie Brilliant Blue G-250 0.5% (w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid, for 30 min, and destained with a solution of 50% (v/v) methanol, 10% (v/v) acetic acid. White bands visible against a blue background marked the gelatinase activity of each proteinase (Toth, Sohail and Fridman, 2012).

2.5. Statistical analysis

For the animal colitis model, all results were expressed as mean ± SEM of *n* observations, where *n* represents the number of animals studied. Results were compared using a one-factorial ANOVA test, followed by a Bonferroni's post hoc test using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). For gelatinolytic activities, all experiments were performed in triplicate, in at least three independent times and the data were expressed as the mean ± standard deviation (SD). SigmaPlot software (version 12.5) was used for comparing different treatments, using one-way and two-way analysis of variance (ANOVA). Tukey's test was used to compare differences between groups and a *P* value less than 0.05 was considered to be statistically significant.

3. Results and Discussion

MMP-9 inhibitors (MMPIs) are mostly regarded as anti-angiogenic agents for primary tumours and metastasis deterrents, but they have also been demonstrated to effectively inhibit pre-cancer states such as colitis and other inflammatory bowel diseases. For over 30 years now, MMPs have been considered by researchers across the world as attractive therapeutic targets, for cancer as well as inflammation. As a result, a myriad of MMPI has already been synthesized, some of which have been used as potential therapeutic

agents (Bourguet *et al.*, 2012), but only a few small MMPIs entered the clinical trial stage, most of which terminated prematurely either due to lack of benefits or to strong adverse side effects (Wang *et al.*, 2012). Ideally, for a specific MMP-9 inhibitor to be successfully used in IBD treatments as a dietary supplement, it should be colon-available, rather than serum-bioavailable, resistant to the digestion process and also non-toxic for colon cells. Previous works on deflamin, a novel protein MMP-9 and MMP-2 inhibitor isolated from *Lupinus albus* showed that small doses of this protein complex can significantly reduce cancer invasion and gelatinolytic activities in colon cells, and in a dose-dependent manner (chapters 1 and 2). Being an MMP-9 inhibitor, it is very plausible that it can be an anti-inflammatory agent. Furthermore, although no *in vivo* assays have been performed on deflamin yet, previous results also showed that deflamin is acid-resistant, suggesting it can possibly survive the digestive process. Hence, in this work we tested the anti-inflammatory effects of deflamin in an *in vivo* model of colitis.

3.1. Deflamin administration reduces the macroscopical and functional signs of colitis injury

In order to ascertain the anti-inflammatory effects of deflamin, we tested its effects on mice with TNBS-induced colitis, using two types of administrations, oral administration (p.o.) and intraperitoneal injection (i.p.), applied 3 hours after induction. Figure 1 and 2 show the effect of deflamin on the length of colons (cm) and on the extent of intestine injury (cm), respectively-.

The animals in the Sham and Ethanol groups exhibited no macroscopical signs of colon injury, and presented no mortality, whilst intracolonic injection of TNBS/EtOH led to a very significant ($P<0.05$) decrease in colon length and an increase in the extent of visible injury (ulcer formation).

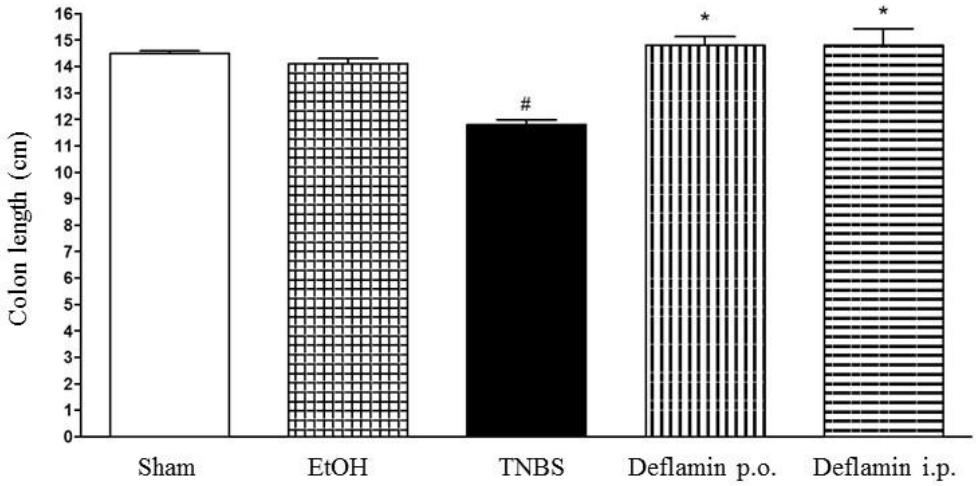

Figure 1. Effect of deflamin administration on the length of colon (cm). Sham group (n=6), EtOH group (n=6), TNBS group (n=10), TNBS+deflamin p.o. (15 mg.kg^{-1}; n=9), TNBS+deflamin i.p. (10 mg.kg^{-1}; n=10). $^{\#}$P<0.001 vs Sham group, *P<0.001 vs TNBS group.

In the deflamin-treatment group (both p.o. and i.p.) all of the macroscopical signs of colon injury were significantly reduced, comparing to the TNBS group (Figure 1 and Figure 2).

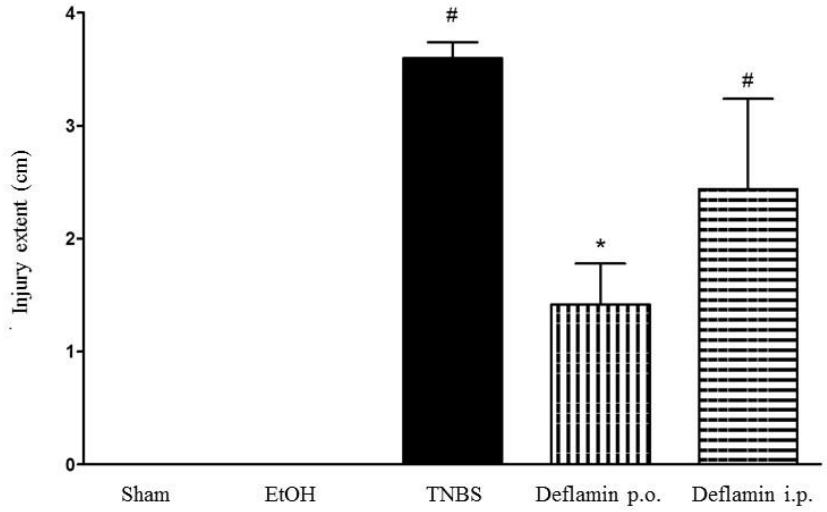

Figure 2. Effect of deflamin administration on the extent of intestine injury (cm). Sham group (n=6), EtOH group (n=6), TNBS group (n=10), TNBS+deflamin p.o. group (15 mg.kg^{-1}; n=9), TNBS+deflamin i.p. (10 mg.kg^{-1}; n=10). #P<0.001 vs Sham group, *P<0.01 vs TNBS group.

Results presented here corroborate the anti-inflammatory activities of deflamin *in vivo*, and also that it maintains its biological activity throughout the digestive process, showing

that the administration of deflamin (both i.p. as well as p.o.) led to an overall reduction in colon inflammation, and in the case of p.o., a significant ($P<0.05$) attenuation of colon length reduction, a significant reduction ($P<0.05$) in the extent of visible injury (ulcer formation). These differences can be easily perceived with a macroscopical observation of the fresh and rinsed colons immediately after colon collection at the end of the experiments. Figure 3 and 4 demonstrate representative pictures of these macroscopic observations obtained by a bench surgical macroscope of the colons isolated from the different treatments groups. Four days after intra-colonic administration of TNBS, the colons appeared flaccid and filled with liquid stool. Observations of images show a clear attenuation of colon injury in animal treated with deflamin when compared to the TNBS-induced colitis (Figure 3 and 4).

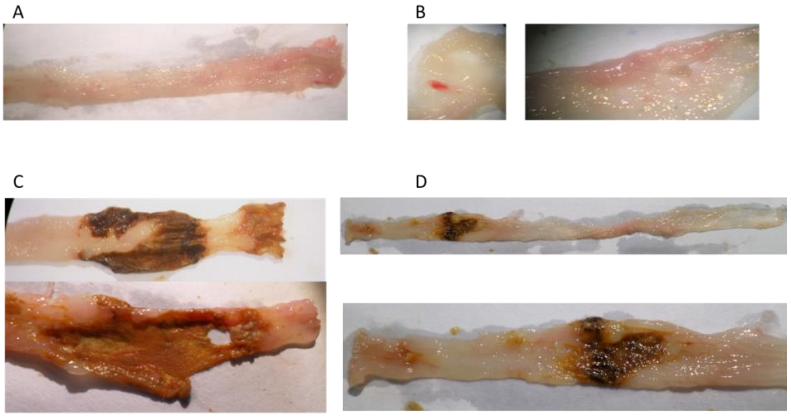

Figure 3. Effect of deflamin administration on the macroscopic observation of colon. (A) Sham group (n=6), (B) EtOH group (n=6), (C) TNBS group (n=8), (D) TNBS+deflamin p.o. group (15 $mg.kg^{-1}$; n=9).

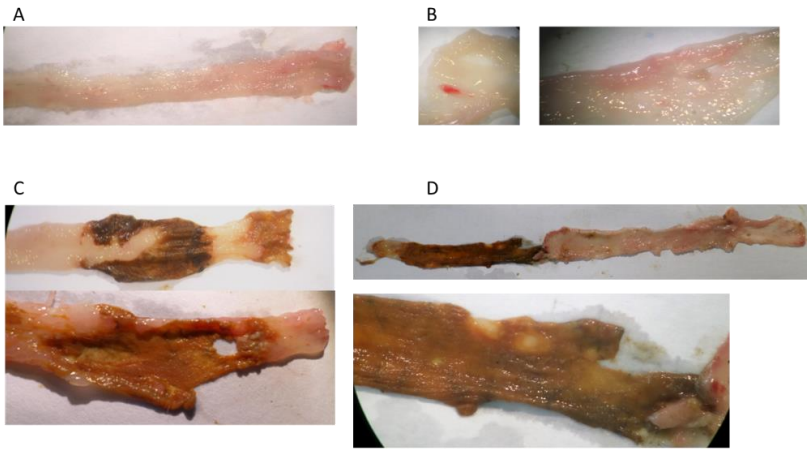

Figure 4. Effect of deflamin administration on the macroscopic observation of colon. (A) Sham group (n=6), (B) EtOH group (n=6), (C) TNBS group (n=8), (D) TNBS+deflamin i.p. group (10 $mg.kg^{-1}$; n=10).

Concerning the type of administration, some differences were observed between the two types of administration when comparing the morphological signs of colitis and the extent of the colonic injury which were higher in i.p. administrations (Figure 2) ($P<0.05$). The macroscopical observations also corroborate this trend between administrations (Figure 3 and 4).

3.2. Deflamin reduces the expression inflammatory markers involved in the inflammatory signaling cascade.

In order to enlighten the mechanisms responsible for the effect of deflamin, we analyzed the severity of histological injuries and also determined the presence of specific markers of inflammation and cancer progression, COX2 and iNOS.

Histological evaluations are present in Figure 5. As expected, while control samples exhibit a normal colon with no lesions, a mucosa with uniform thickness, normal crypt architecture and no signs of inflammation, in the TNBS treatments the colons exhibited a severe ulceration with crypt loss and a thinner mucosa with a marked neutrophil infiltration, equivalent to a score 3.

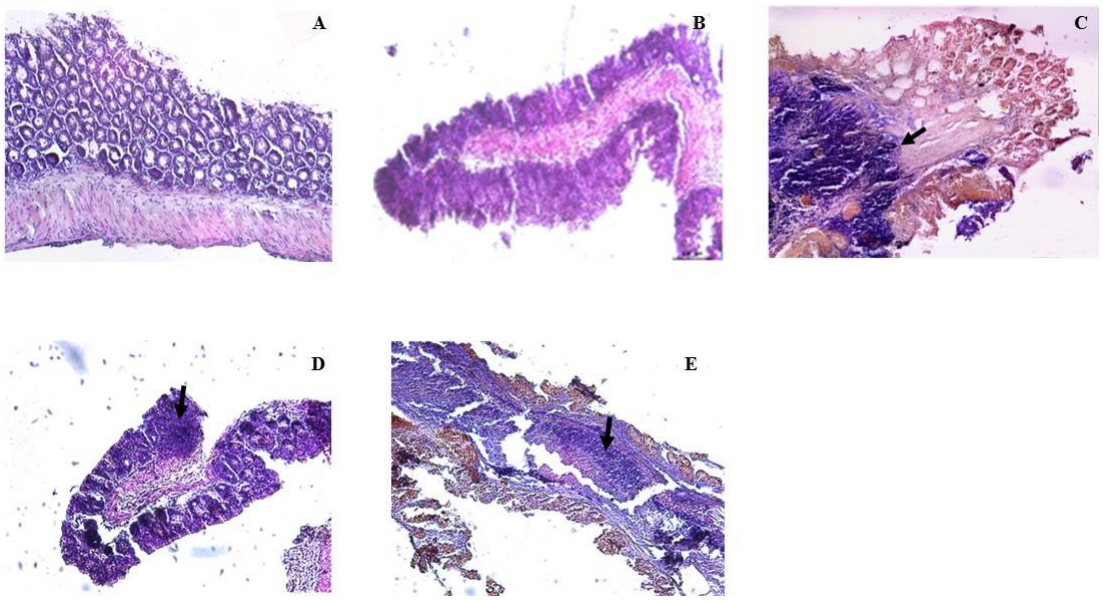

Figure 5. Effect of deflamin administration on the histological features of colon inflammation. (A) Sham group (n=6), (B) EtOH group (n=6), (C) TNBS group (n=8), (D) TNBS+deflamin p.o. (15 $mg.kg^{-1}$, n=9), (E) TNBS+deflamin i.p. (10 $mg.kg^{-1}$, n=10).

In comparison, the samples from animals administered with deflamin show more moderate lesions with partial intact crypts and some neutrophil infiltration indicating a lower damage score of 2, particularly in the p.o. administrations.

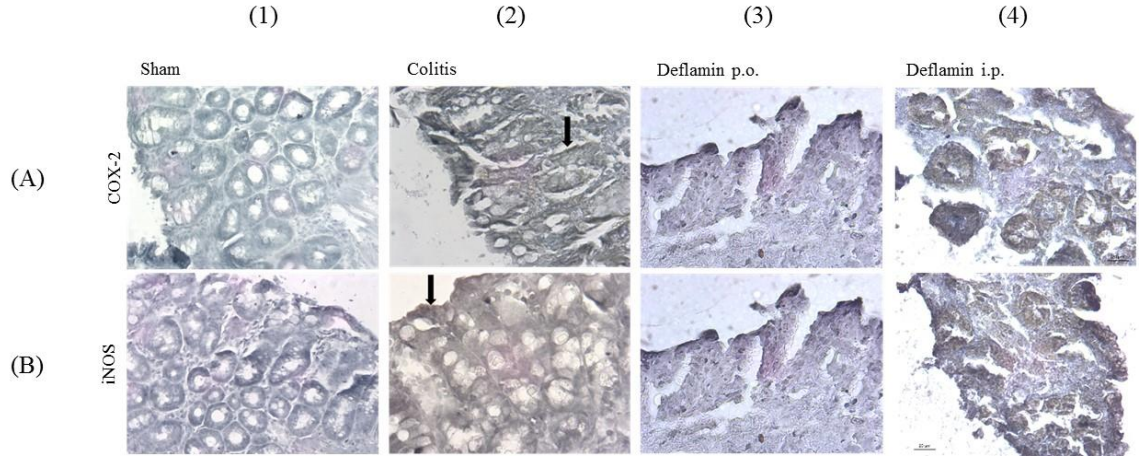

Figure 6. Effect of deflamin administration on the colon tissue expression of inflammation markers COX-2 and iNOS. (A) – COX-2 expression: **(**1) Sham group, (2) TNBS group, (3) TNBS+deflamin p.o. group, (4) TNBS+deflamin i.p. group; (B) – iNOS expression: **(**1) Sham group, (2) TNBS group, (3) TNBS+deflamin p.o. group (15 $mg.kg^{-1}$, n=9), (4) TNBS+deflamin i.p. group (10 $mg.kg^{-1}$, n=10).

Figure 6 shows COX2 and iNOS expression in colonic tissues. Overall, the histological analysis and the expression of important markers of inflammation also corroborated the anti-inflammatory effects of deflamin. Results show that TNBS treatment induced a marked increase in COX2 and iNOS expression along the remaining crypts, indicated by brown color when compared with control samples (Figure 6). This is in accordance to clinical and epidemiologic studies that prove the important role of COX-2 and prostaglandins in the progression of intestinal inflammation in patients with IBD (Ogasawara, Matsunaga and Suzuki, 2007) and (Chen *et al.*, 2014). Also, specifically in the intestine, the up-regulation of the production of nitric oxide is produced and released locally in much greater quantities in the inflamed gut than in the non-inflamed gut, being suggested as a novel clinical biomarker for diagnosis and monitoring of IBD patients (Lee *et al.*, 2013).

Administration of deflamin led to a reduced staining for COX-2, indicating that it impaired the expression of COX-2 in the injured intestinal tissue. Similarly, to COX-2, immunostaining assays in the experimental colitis study showed that there was in fact an

increased expression of iNOS in animals subjected to colitis induction (Figure 6). Again, deflamin administration, particularly via p.o., was able to reduce iNOS expression and therefore contribute to impairment of the inflammatory process in the colon.

When comparing both types of administrations, better results were again obtained for p.o. when compared to intraperitoneally. For obvious reasons, oral administrations are a far easier and practical method of administration than i.p. administrations. Many bioactive compounds have to be injected intraperitoneally or intravenously because their biological activities do not survive the digestive tract. Our results suggest that deflamin not only survives the digestion process, oral administration was found to be a superior method for reducing colon inflammation compared with intraperitoneal applications. This could be related to a higher concentration in the colons, acting *in situ* or because in i.p. administrations the protein complex in deflamin can trigger an immune response. In any case, in the follow-up work, we decided to use only oral administrations instead of i.p.

3.3. Deflamin's effects are evident in preventive as well as in curative treatments

Since p.o. administrations induced better results, we further tested if a preventive approach through an oral diet supplementation with deflamin, rather than a just a curative approach would present similar effects. Table 1 shows the morphological and functional signs of colitis in both treatments, curative (D – p.o. administration of deflamin 3 h after TNBS induction) and preventive (Dp – p.o. administration of deflamin 3 days prior to TNBS administration).

The animals in the Sham and Ethanol groups exhibited no macroscopical signs of colon injury, and presented no mortality, whilst intracolonic injection of TNBS/EtOH led to a very significant ($P<0.05$) decrease in colon length and an increase in the extent of visible injury (ulcer formation) and diarrhea severity, exhibiting a mortality rate of 50%.

Table 1. Morphologic and functional observations of the colon, immediately after collection in both treatments, curative (D – p.o. administration of deflamin 3 h after TNBS induction) and preventive (Dp – p.o. administration of deflamin 3 days prior to TNBS administration). *P < 0.001 versus Sham group; #P < 0.05 versus TNBS group and ϕP < 0.05 D versus Dp group.

Animal Group	Length of colon (cm)	Extent of injury (cm)	Presence/consistency of diarrhea	Mortality (%)
Sham	14.5 ± 0.08	0	0	0%
EtOH 50%	14.1 ± 0.20	0	0	0%
TNBS	11.8 ± 0.19*	3.6 ± 0.14*	3*	50%
NBS + D	14.8 ± 0.33#	2.44 ± 0.84#	1.13 ± 0.35#	11%
TNBS + Dp	13.1± 1.98#	2.48 ± 1.24#	1.63 ± 0.74#	12%

Results show that the administration of deflamin, both curative as well as preventive, led to an overall reduction in colon inflammation, with a significant ($P<0.05$) attenuation of colon length reduction, a significant reduction ($P<0.05$) in the extent of visible injury (ulcer formation). Also, a significant decrease ($P<0.05$) in diarrhea severity, mortality rates and a reduction of general histological features of colon inflammation were observed when compared to the TNBS group.

Interestingly, a preventive approach, with a more prolonged dietary administration of deflamin was not significantly different from the curative approach, were deflamin was administered 3 h after TNBS induction as there were no significant differences ($P>0.05$) observed between preventive and curative approaches. Also there were no differences between both deflamin treatments and the controls. These results suggest that deflamin can act quickly an inflammatory deterrent when in the presence of acute inflammation, but is also effective in preventing it from arising when administered continuously before the inflammatory episode, suggesting it can be used in both curative as well as preventive approaches.

3.4. Deflamin reduces MMP-9 activity in vivo

Previous results obtained in our groups had shown that deflamin inhibits MMP-9 and MMP-2 in colon cells (see chapter 2) *in vitro* assays, and that it was resistant to heat and acid denaturation, making it a good candidate to become a nutraceutical for IBDs and colon cancer. However, *in vivo* tests were required to further determine its effectiveness

after digestion and corroborate its potential as a nutraceutic. In order to corroborate if the anti-inflammatory effects observed in the deflamin treatments were due to MMP-9 and -2 inhibition *in situ*, the gelatinolytic activities of MMP-9 and MMP-2 in the fresh colon tissue of the different experimental groups (curative and preventive) were tested, using the DQ-gelatin kit and zymographic assays. Figure 7 shows the total gelatinolytic activity in the colon samples, quantified by the quenched-dye DQ-gelatin method.

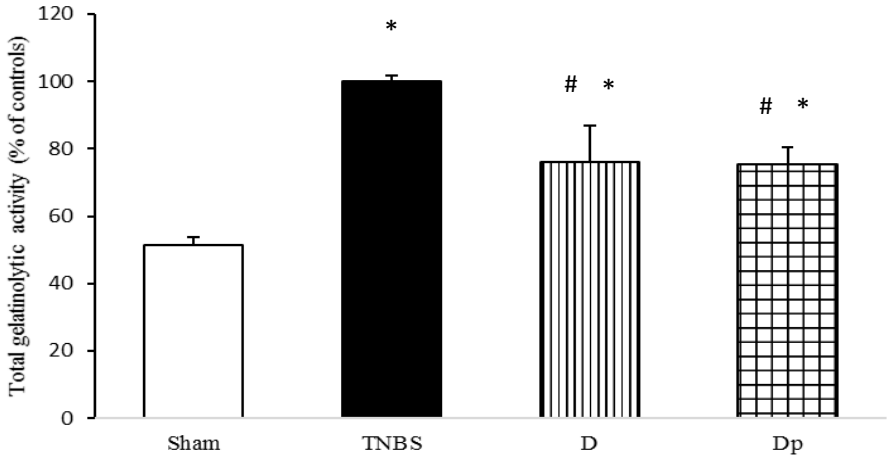

Figure 7. Effect of deflamin administration on the colon tissue gelatinase activities of MMP-2 and MMP-9 from colitis-induced mice. Proteolytic activity of the gelatinases presents in colons quantified by the DQ fluorogenic method. Results are expressed as relative fluorescence as a % of controls and represent the average of at least three replicate experiments (n=3) ± SD. Sham group (n=6); TNBS group (n=10); D=Curative treatments with deflamin (15 $mg.kg^{-1}$; n=9, p.o.) and Dp=Preventive treatments with deflamin (15 $mg.kg^{-1}$, n=9, p.o.). *P < 0.001 versus Sham group; #P < 0.05 versus TNBS group and ϕP < 0.05 D versus Dp group.

Results obtained in Figure 7 show that the TNBS induced a very significant increase in gelatinolytic activities, when compared to controls (P<0.001), whereas both curative and preventive deflamin administrations reduced significantly (P<0.05) the total MMP-9 and MMP-2 activity when compared to the TNBS treatment, but there were no significant differences (P>0,05) between the curative and preventive administrations.

Given that the DQ-gelatin assay provides evidence for total gelatinolytic activity in the colon tissue, we further tested its specificity through substrate zymography, where MMP-9 and MMP-2, in their active and non-active forms can be separated through electrophoresis. Figure 8 shows an example of a zymographic profile of the protein

extract from the colonic tissues in the different experimental groups. White bands show the gelatinolytic activity of the specific bands.

The zymographic profile show how TNBS increased MMP-9 and MMP-2 activities both in the active as well as in the inactive forms of the enzymes, when compared to controls where there is low activity of the active forms of MMP-2 and MMP-9. However, in deflamin treatments there was an evident reduction in MMP-9 and MMP-2 activities (active and inactive forms), when compared to the TNBS group.

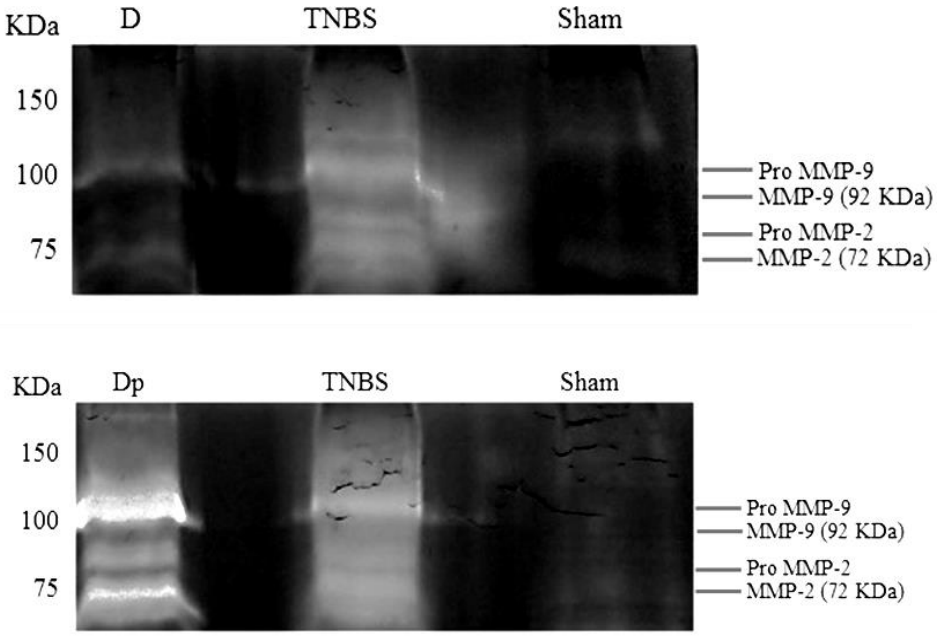

Figure 8. Effect of deflamin administration on the colon tissue gelatinase activities of MMP-2 and MMP-9 from colitis-induced mice. Representative image of the zymographic profiles of MMP-9 and MMP-2 activities of the colons. Protein extracts of the colon were loaded on 12.5% (w/v acrylamide) polyacrylamide gels co-polymerized with 1% (w/v) gelatin. Sham group (n=6); TNBS group (n=10); D=colon from animals treated with deflamin in curative treatments (15 $mg.kg^{-1}$, n=9, p.o.) and Dp=colon from animals treated with deflamin in preventive treatments (15 $mg.kg^{-1}$, n=9, p.o.)

Interestingly, although there were no morphological and functional differences observed between preventive and curative treatments, there were significant differences between the specific inhibitions in both enzymes in the zymographic assays. Whilst in the curative treatments both MMP-2 and MMP-9 were reduced in a similar fashion, (and in both forms), this trend was not the same in the preventive approach, where the active MMP-9

was more visibly inhibited than the pro-MMP-9, whereas the pro-MMP-2 was inhibited, but the active MMP-2 was not (Figure 8). MMPs are usually synthesized as zymogens (pro-MMPs), with their catalytic activity blocked by a cysteine switch and are only activated by its removal, through proteolysis. In the zymography, the pro-gelatinases also become active because they are denaturated by the SDS exposing the catalytic site (hence the slightly higher mass of the pro-enzymes in the zymography that still maintain the short amino acid sequence of the cysteine switch). The fact that only the active MMP-9 is inhibited by deflamin suggests that it has a certain degree of specificity towards this form, perhaps to the catalytic site, only exposed in the active form.

On the other hand, pro-MMP-2 seems to be inhibited and not MMP-2. Because MMP-2 is one of the proteases that activate MMP-9, it seems plausible that in a more prolonged exposure to deflamin, a high inhibition of MMP-9 would induce, through feedback, a higher activation of MMP-2 to activate MMP-9.

Although a more prolonged exposure to deflamin seems to suggest more profound effects in the synthesis and activation of the gelatinases, results suggest that its administration in a curative approach is just as effective in reducing colitis injuries as the preventive. The higher specificity towards MMP-9 is nonetheless important because it insures low side-effects, as opposed to the majority of broad-range MMPIs used in clinical trials.

4. Conclusion

Deflamin is a novel digestion-resistant gelatinase inhibitor that reduces colitis injury through oral supplementation

Results presented here clearly show that deflamin survives the digestion process and is able to attenuate the lesions provoked by TNBS-induced colitis, leading to a reduction in several functional and histological markers of colon inflammation, namely: attenuation of colon length decrease, reduction of the extent of visible injury (ulcer formation), decrease in diarrhea severity, reduced mortality rate, reduction of mucosal hemorrhage and reduction of general histological features of colon inflammation. Moreover, this effect was more evident in the oral treatments corroborating its potential use in a dietary approach. Furthermore, the overall physiological and morphological results presented

here corroborated that oral administration of deflamin can indeed inhibit the colitis-induced rise in MMP-9 and MMP-2 activities observed in animal models, leveling them to physiological and morphological levels closer to that observed in healthy controls. Besides being a potent inhibitor of the gelatinases and exhibiting powerful anti-inflammatory activities, deflamin was efficient both in the prevention and curative treatments of inflammation. Under the current context of the fast growing incidence of inflammatory diseaes worldwide, deflamin may be of enormous clinical potential as a nutraceutical or as an additive in functional foods, targeted at the prevention or treatment of IBDs and also other MMP-9-related diseases.

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General Conclusions

Although the presence of MMPIs of natural occurrence may be considered ubiquitous in plant tissues, virtually all of them suffer from limitations, handicaps, disadvantages or weaknesses when one considers the possibility of clinical and/or nutraceutical applications.

In this work we report the isolation of deflamin, a potent polypeptide mixture from *Lupinus albus* seeds, which inhibits cancer cell invasion and colitis, but that doesn´t present most of those handicaps: it is highly soluble in water; its bioactivities resist to boiling, to low pH values and to the digestive process; it strongly inhibits MMP-9 and/or MMP-2 at low concentrations and in a dose-dependent manner, without exerting any apparent cytotoxicity. Its bioactivities include a strong reduction of the invasion capacity of the human colon adenocarcinoma cell line HT29 and it inhibits the colitis-induced lesions observed in animal models, leveling colons to physiological and morphological levels similar to those of the controls. Additionally, it is more effective when administered orally and is equally actived in curative or preventive approaches. Nevertheless, deflamin may also be administered intraperitoneally and topically with good results. This work presents, as far as we are aware, the first effective method for isolating proteinaceous MMPI fraction from food sources, which is scalable to an industrial level, in a cost-effective manner. Although much work is still needed to properly assess deflamin full potential, the fact that it is an MMPI, derives from plant foods, and can be taken orally makes it the perfect candidate to be used as a nutraceutical or as food additive in preventing/curative diets, particularly in the case of colon diseases such as CRC and IBDs, where it can act *in situ*, without exerting side effects. Looking over the last decade, where a substantial amount of research has turned towards novel plant foods presenting MMPIs, few (if any!) compounds present the potential of deflamin.

Hence, under the current worldwide context of the increasingly higher incidence of cancer and inflammatory diseases worldwide, the overall activities of deflamin may be of enormous clinical potential, targeted at the prevention or treatment of MMP-9-related diseases