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Lectins from *Arbutus unedo*: Antitumor activity and a Possible Therapeutic Strategy in Pancreatic Cancer

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Ao meu avô Tó

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ABSTRACT

Pancreatic ductal adenocarcinoma is beyond any doubt the most common type of pancreatic cancer (covers about 90% of all pancreas neoplasms). It is still one of the deadliest cancers, with a 5-year survival rate below 10%, because it is likely to rapidly disseminate to the lymphatic system and distant organs. Furthermore, it is detected at such an advanced stage – between stages III and IV – that surgical and other treatments turn out not to be effective anymore. It is, hence, imperative to investigate further alternative strategies, in order to implement early detection and different therapies. Like most cancers, not only does pancreatic cancer have an accumulation of genetic mutations, but also there is a glycome aberration associated with it. The glycome of cancer cell surfaces include sialylation, fucosylation, *O*-glycan truncation, and *N*- and *O*-linked glycan branching.

Up until now, the most common biomarker is CA 19-9. Unfortunately, this tumor marker has poor predictive value of 0.5 – 0.9% in asymptomatic patients. Additionally, a percentage (10 – 20%) of the Caucasian population does not express this antigen, which may lead to false negative results.

Lectins are non-immune origin proteins, universally distributed in plants, animals, bacteria and fungi, that contain at least one non-catalytic domain which allows them to recognize and reversibly bind to specific carbohydrates present on glycoproteins and glycolipids, without changing the structure of the sugar. Plant extracts have the capacity to bind to the carbohydrate moieties on the surface of erythrocytes and agglutinate them, without altering their properties. This was first acknowledged in 1888 by Stillmark, by searching for toxicity factors in *Ricinus communis*. Only in 1954 Boyd and Shapleigh named these novel proteins lectins, considering their ability to selecting cellular types.

Plant lectins have innumerous biological functions, mainly they are present in the exposed organs of the plant as a way of protection. Due to their carbohydrate recognition abilities, they may have utility in pharmaceutical (based on its anti-inflammatory properties) and phytopharmaceutical industries, and as an alternative methods of plant health control. Furthermore, it is known that plant lectins have cytotoxicity properties that induce necrosis by increasing cytosolic calcium and reactive oxygen species, programed cell death – apoptosis, and autophagic cell death, by three kinds of mechanism, inactivating the ribosomes, binding to specific receptors on the cell surface or binding to mitochondria after endocytosis. Examples of such lectins are mistletoe lectins and concanavalin A, from jack beans.

Therefore, because these proteins gather such promising features, they may be a significant asset in the field of medical and pharmaceutical sciences, particularly as diagnosis and therapy methods for pancreatic cancer.

Arbutus unedo represents a great asset to human health. The leaves, rich in phenolic compounds and antioxidants, are also source of lectins with high anti-inflammatory and antitumor value.

This research analyzes the proteomic and glycoproteomic profiles of a total protein extract of *A. unedo* leaves and a semi-purified fraction unadsorbed by Q-Sepharose column of the protein extract, followed by the search for lectin activity. In addition, it investigates the affinity to the glycosylated receptors at MIA PaCa-2 (a pancreatic ductal adenocarcinoma cell line) cell surface, revealing possible lectin type proteins present in both protein fraction, with around 12 kDa, 15 kDa, 23 kDa, 44 kDa and 50 kDa, that bind to the isolated membranes under study. These membranes are also to be characterized in order to find new insight on their proteome and glycome. Furthermore, the work aims to assess, *in vitro*, the antitumor activity of both protein fractions from the strawberry tree leaves on the mentioned cell line.

The samples inhibited cell migration, though not through MMP inhibition. Results also indicate a considerable decrease in cell viability and proliferation, however, the semi-purified fraction presented a higher effect on the cells with a IC_{50} of 8.1 µg/mL compared to 47.6 µg/mL of the total extract.

All in all, the obtained results are good indicators of the presence of lectins in the *Arbutus unedo* leaves that exhibit binding abilities and antitumor activity toward MIA PaCa-2 cells. It should be considered that the fraction in which the lectin is more purified presented better results, making it interesting to further investigate the mechanism behind the antitumoral activity of the *A. unedo* lectin.

Key words: Lectins; pancreatic ductal adenocarcinoma; *Arbutus unedo*; aberrant glycosylation; antitumor activity.

RESUMO

O adenocarcinoma ductal pancreático é o tipo de cancro pancreático mais comum, representando cerca de 90% dos casos. É ainda um dos cancros mais letais com uma taxa de sobrevivência a 5 anos abaixo dos 10%, devido à sua tendência de disseminar para o sistema linfático e para órgãos distantes. Além disso, o diagnóstico é feito tardiamente, entre os estágios III e IV, o que torna a recessão e outros tratamentos ineficientes. É, então, imperativo investigar estratégias alternativas, procurando implementar métodos de deteção precoce e terapias eficazes. Como na maioria dos cancros, o pancreático não resulta apenas de processos mutagénicos como está associado a uma aberração glicómica.

O glicocálice das células tumorais inclui sialilação, fucosilação, O-glicanos truncados e N- e O-glicanos ramificados.

Até ao momento, o biomarcador mais comum é o CA19-9. Infelizmente este marcador tem uma previsão de 0,5 – 0,9% em pacientes assintomáticos. Adicionalmente, uma percentagem de cerca 10 – 20% da população caucasiana não expressa este antigénio, o que pode levar a resultados negativos falsos.

As lectinas são proteínas de origem não imune, ubiquamente distribuídas pelos seres vivos, que contêm pelo menos um domínio não catalítico que permite o reconhecimento e a ligação reversível a hidratos de carbono específicos presentes em glicolípidos e glicoproteínas, sem alterar a estrutura do açúcar. As lectinas vegetais têm a capacidade de se ligar a hidratos de carbono nas superfícies de eritrócitos e aglutiná-los sem alterar as suas propriedades. Este facto foi observado pela primeira vez em 1888 por Stillmark enquanto investigava fatores de toxicidade de *Ricinus communis*. Apenas em 1954 Boyd e Shapleigh atribuíram o nome de lectina a estas proteínas que demonstravam a capacidade de selecionar tipos celulares.

As lectinas de plantas possuem inúmeras funções biológicas; estão maioritariamente presentes em órgãos expostos como forma de proteção da planta. Devido ao seu reconhecimento de hidratos de carbono podem ser vantajosas na indústria farmacêutica e fitofarmacêutica. Além disso, as lectinas vegetais têm propriedades citotóxicas que induzem necrose aumentado o cálcio citosólico e espécies reativas de oxigénio, e induzem morte celular programada – apoptose e autofagia – por três diferentes tipos de mecanismo: Inativação ribossomal, ligação a recetores específicos na superfície celular, e endocitose e subsequente ligação à mitocôndria. A lectina do azevinho e a concanavalina A da *Canavalia ensiformis* são exemplos deste tipo de lectina. Então, devido às promissoras características destas proteínas, as lectinas poderão ser um grande benefício nas áreas das ciências médicas e farmacêuticas, idealmente como métodos de diagnóstico e terapêutica para o grande problema que é o cancro pancreático.

O medronheiro, *Arbutus unedo*, torna-se então uma vantagem na saúde humana, uma vez que as folhas, ricas em compostos fenólicos e antioxidantes, são também fontes de lectinas que exibem atividade anti-inflamatória e antitumoral.

Este trabalho tem por objetivo analisar os perfis polipeptídico e glicoproteico do extrato proteico total e de uma fração proteica semi-purificada não adsorvida à coluna de Q-Sepharose de folhas de *A. unedo*, evidenciando o perfil e o carácter de lectina dos mesmos. Pretende-se, também, investigar a ligação por afinidade das lectinas presentes, aos recetores glicosilados membranares das células MIA PaCa-2 (linha celular tumoral ductal pancreática), revelando possíveis lectinas presentes em ambas as frações proteicas em estudo, com cerca de 12 kDa, 15 kDa, 23 kDa, 44 kDa e 50 kDa. Realizou-se ainda a caracterização proteómica e glicómica das membranas das células MIA PaCa-2, demonstrando a localização de recetores glicosilados, possíveis biomarcadores capazes de serem reconhecidos e descodificados por lectinas.

Mais, esta investigação visou determinar in vitro a atividade antitumoral de ambas as amostras proteicas para as células MIA PaCa-2 em cultura. As frações proteicas inibiram a migração celular, não inibindo, porém, a atividade para as MMP. Os resultados mostram um decréscimo considerável na viabilidade e proliferação celular, sendo a fração semi-purificada mais eficiente com um IC_{50} de 8,1 µg/mL face ao IC_{50} de 47,6 µg/mL associado ao extrato total.

Em suma, os resultados obtidos indicam a presença de lectinas em folhas de *Arbutus unedo* que exibem capacidade de ligação aos recetores membranares das células MIA PaCa-2 do cancro pancreático exibindo atividade antitumoral. É de considerar o facto de a fração de lectina mais purificada apresentar melhores resultados, sendo interessante estudar melhor o mecanismo pelo qual esta(s) lectina(s) do *A. unedo* exerce(m) ação anti-tumoral.

Palavras-chave: Lectinas; adenocarcinoma ductal pancreático; *Arbutus unedo*; glicosilação aberrante; atividade antitumoral.

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LIST OF ABBREVIATIONS

5-FU 5-Fluorouracil
ACD Accidental cell death
AFL Atypical flat lesions
Apaf-1 Apoptotic protease activating factor 1
APS Ammonium persulfate
Asn Asparagine
ATP Adenosine 5'-triphosphate
ATV Trypsin-versene
β-ME β-Mercaptoethanol
BSA Bovine serum albumin
CA Carbohydrate antigen
CBB G-250 Coomassie Brilliant Blue G-250
CEA Carcinoembryonic antigen
CM Cell membrane
Con A Concanavalin A
CRD Carbohydrate recognition domain
CT Computed tomography
Cys Cysteine
DMEM Dulbecco's Modified Eagle Medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic Acid
DQ Dye quenching
DTT Dithiothreitol
ECM Extracellular membrane
EDTA Ethylenediaminetetraacetic acid
EEO Electroendosmosis
EGF(R) Epidermal growth factor (receptor)
EMT Epithelial-mesenchymal transition
ER Endoplasmic reticulum
ERCP Endoscopic retrograde cholangiopancreatography
FBS Fetal bovine serum
FDA Food and drug administration
Fuc L-Fucose
FUT Fucosyltransferase
GAG Glycosaminoglycan
Gal D-galactose
GalNac *N*-Acetyl-d-galactosamine
Glc D-Glucose
GlcA D-Glucuronic acid
GlcNAc *N*-Acetyl-d-glucosamine
GnT-V β1,6-Acetylglucosaminyltransferase V
GPC Glypican
H.U. Hemaggutination Unit
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER Human epidermal growth factor receptor
HSPG Heparan sulfate proteoglycans
IEF Isoelectric focusing
IL Interleukin
IPG Immobilized pH gradients
IPMN Intraductal papillary mucinous neoplasms
ITPN Intraductal tubulopapillary neoplasms
JNK Jun N-terminal kinase
Man D-mannose
MAPK Mitogen-activated protein kinase
MCN Mucinous cystic neoplasms
MIC-1 Macrophage inhibitory cytokine 1
ML Mistletoe lectin
MMP Matrix metalloproteinase
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
MUC Mucin
MW Molecular weight
Neu5Ac *N*-Acetylneuraminic acid
NR Non-reducing
OPN Osteopontin
PanIN Pancreatic intraepithelial neoplasia
PARP Poly(ADP-ribose) polymerase
PBS Phosphate-buffered saline
PCD Programed cell death
PDAC Pancreatic ductal adenocarcinoma
pH Potential of hydrogen
pI Isoelectric point
PMSF Phenylmethylsulfonyl fluoride
PTM Post translational modifications
PVPP Polyvinylpolypyrrolidone
R Reducing
Rib D-Ribose
RIP Ribosome inactivating protein
RNA Ribonucleic acid
ROS Reactive Oxygen Species
RTK Receptor tyrosine kinase
SDS-PAGE Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser L-Serine
SLe Sialyl Lewis antigen
SP Semi-purified through Q-Sepharose fraction of *Arbutus unedo*
TBS Tris-buffered saline
TE Total extract of *Arbutus unedo*
TEMED tetramethylethylenediamine
TGF Transforming growth factor
Thr Threonine
TNF Tumor necrosis factor
US Ultrasonography
WGA Wheat germ agglutinin
Xyl D-Xylose

I. INTRODUCTION

1 PANCREATIC CANCER

Cancer is the main cause of morbidity and mortality, representing one of the major public health problems worldwide (Mordente *et al.*, 2015). The term 'cancer' defines over one hundred different diseases that may appear from nearly any organ or tissue (Thangue and Kerr, 2011).

Pancreatic adenocarcinoma is the 4th most common cause of cancer related death (Talar-Wojnarowska and Malecka-Panas, 2006; Becker *et al.*, 2014) and is estimated to become the second leading cause of cancer-related deaths by 2030 (Haeberle and Esposito, 2019). Pancreatic cancer is composed of several kinds of cancers, being pancreatic ductal adenocarcinoma (PDAC) the most frequent, covering about 90% of all pancreas neoplasms (Sarantis *et al.*, 2020). For this reason, this exocrine tumor is commonly known simply as pancreatic cancer. It is one of the most lethal human cancers and continues to be a major unsolved health problem, with a 5-year survival rate below 10% (Munkley, 2019; Grossberg *et al.*, 2020; Sarantis *et al.*, 2020) and a disease-free survival of only 3%.

In spite of endeavors over the past 50 years, therapies like radiation, chemotherapy, surgery, or even a combination of these, have had little effect on the course of this powerful neoplasm due to, among other causes, the tremendous heterogeneity of genetic mutations and the dense stromal environment (Sarantis *et al.*, 2020). Almost every patient with pancreatic cancer develops metastases and die because of the debilitating metabolic effects of the unrestrained growth (Li *et al.*, 2004). In fact, there are four major challenges underlying the high mortality of PDAC (Grossberg *et al.*, 2020). The first one being the deep location of the pancreas, behind the stomach and between the aorta and its branches. The site of this organ, besides complicating the detection, hinders tumor resection due to the proximity to such major blood vessels (Li *et al.*, 2004). Secondly, this cancer is characterized by early metastasis. More than 50% of patients present metastatic disease and most of those who undergo resection surgery develop metastasis within 2 years, which suggests the presence of micro-metastasis in patients diagnosed with localized tumors (Conroy *et al.*, 2018; Grossberg *et al.*, 2020). Third, PDAC exhibits chemoresistance and low response to radiotherapy (Grossberg *et al.*, 2020). Lastly, the physiologic effects of pancreatic cancer take a toll on the patient's capability to endure the aggressive treatment. In fact, cachectic patients (80% of PDAC patients) present low treatment tolerance, increasing mortality after pancreatectomy or chemotherapy (Bachmann *et al.*, 2008; Kays *et al.*, 2018; Grossberg *et al.*, 2020).

Thus, one of the most promising ways to improve the prognosis of pancreatic cancer is to develop effective early detection strategies, namely plasmatic biomarkers. It is also undeniably vital to develop effective treatments capable of reversing the aggressive biology of this disease.

1.1 EPIDEMIOLOGY

Pancreatic cancer is a major cause of cancer mortality around the globe. There are two main tumor types of pancreatic cancer: the adenocarcinoma, which accounts for about 90% of pancreatic cancer cases, and the pancreatic endocrine tumors, that are make up for less than 5% of all cases) (Ilic and Ilic, 2016). Based on GLOBOCAN 2020 estimates, pancreatic cancer has ranked the 15th most incident cancer in the world counting 495 773 new cases and causing 466 003 deaths (4.7% of all deaths caused by cancer, ranking 9th among the deadliest cancers) (**Figure 1**). Worldwide incidence and mortality of pancreatic cancer, besides being on equal footing, they are correlated with increasing age, in fact almost 90% of all cases are diagnosed after the age of 55 (Ilic and Ilic, 2016). The lifetime risk of developing PDAC is 1.65% (Howlade *et al.*, 2020), meaning 1 in 61 people will probably be diagnosed with pancreatic

cancer. Additionally, this type of malignancy is also slightly more common in men than in women (Rawla, 2019). However, according to the World Health Organization (2020), the mortality rate of pancreatic cancer on women is 4.9% whilst in men is 4.5%.

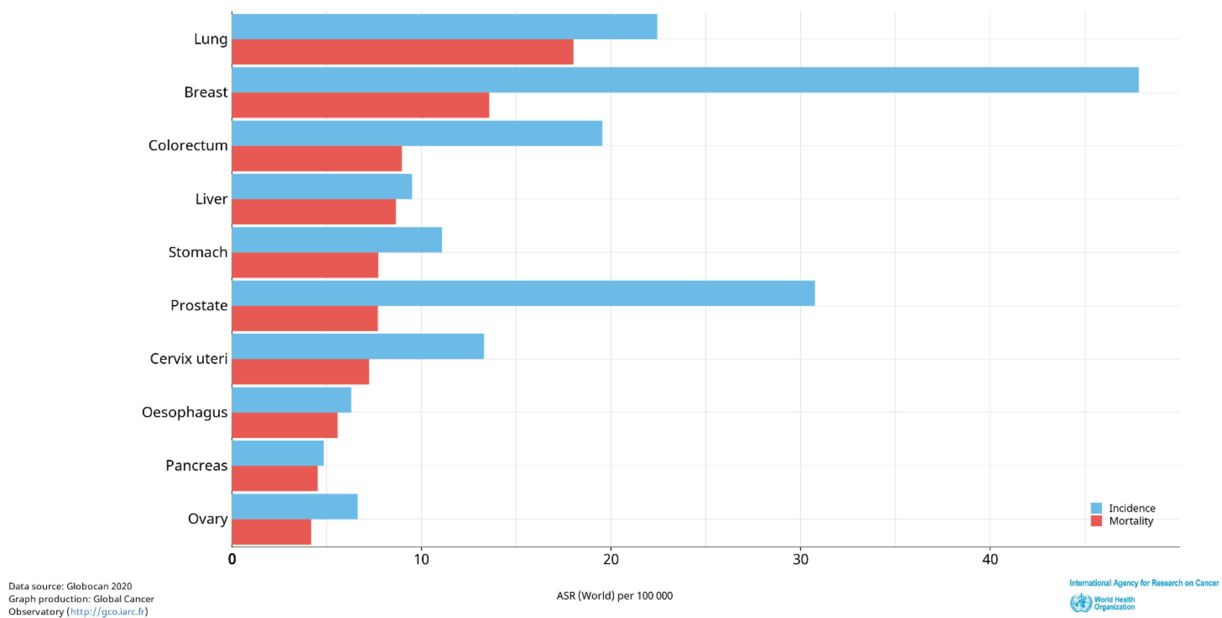

Figure 1: Estimated age-standardized incidence and mortality rates of pancreatic cancer in 2020, worldwide, both sexes, all ages. Adapted from https://www.iarc.fr/ (Ferlay, 2020)

The incidence of pancreatic cancer varies across regions and populations. There is a higher rate of incidence in developed countries. It should be noted that some differences in incidence of pancreatic cancer around the world may be attributed to the quality of registries, which coverage, completeness and accuracy varies by country. Having this into consideration, we can corroborate, in **Figure 2**, that incidence was highest in Europe and North America, followed by Asia and Oceania. The lowest rate was observed in Africa.

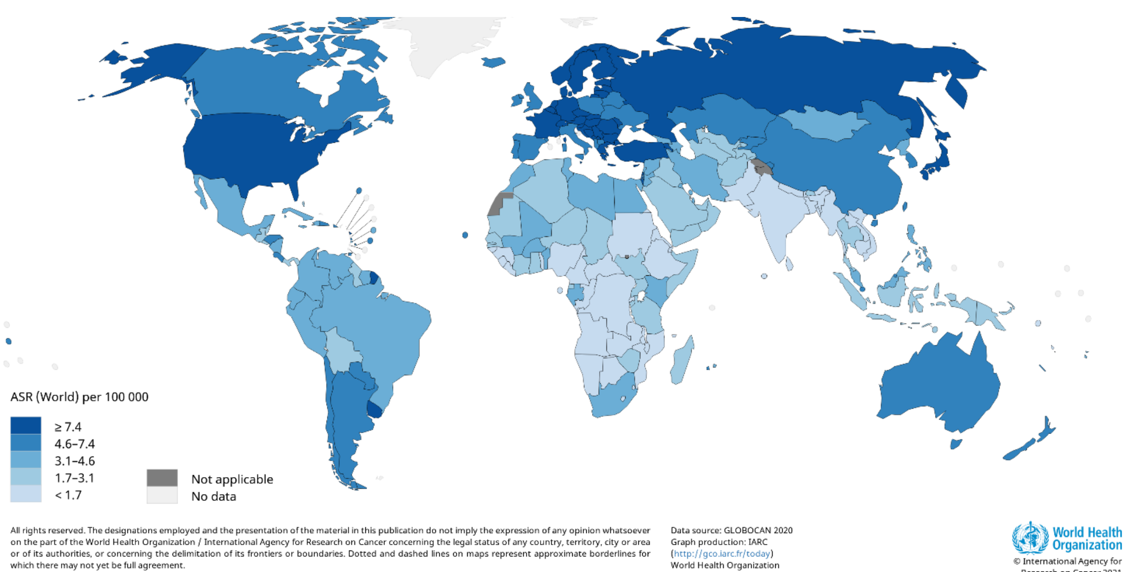

Figure 2: Geographical incidence rate of pancreatic cancer in 2020, worldwide, both sexes, all ages. Adapted from https://www.iarc.fr/ (Ferlay, 2020)

1.2 ETIOLOGY

Oncogenesis is a complex and dynamic process by which normal cells turn into cancerous cells, leading to cancer growth in the body. It can have innumerous causes and may involve lifestyle, namely diet and physical activity, environmental factors, and host factors, like inheritance or epigenetic changes.

As for pancreatic cancer, up until the current day, the causes are still unsatisfactorily known even though they have been extensively studied. However, certain risk factors have been identified and can be divided into two categories: **non-modifiable and modifiable risk factors**. The first ones being **age**, as mentioned before pancreatic cancer is predominately a disease of older individuals, in fact, the majority of the patients are diagnosed between the ages of 60 and 80 (Ferlay *et al.*, 2012); **gender**, since it is more common in men than women, with rates varying between 5 and 6.5 per 100 000, while in men 7 and 9 per 100 000 (Ferlay *et al.*, 2012, World cancer research fund international, 2018); **ethnicity**, for this cancer affects predominantly Caucasians and Asian patients were found to have less aggressive tumors than either white or black patients; **blood group**, because people with blood groups A, AB, or B have a higher risk of developing PDAC than people with blood group 0; **genetics** like in all other cancers, is fundamental for pancreatic adenocarcinoma development. Still, more than 90 per cent of pancreatic cancer cases are sporadic, due to spontaneous genetic mutations. Although a family history increases risk, particularly where more than one family member is involved (inherited mutations); chronic pancreatitis (Lowenfels *et al.*, 1993) or other previous **pancreatic diseases**; **diabetes mellitus**; and greater adult attained **height** may be related to increased exposure to endocrine and metabolic patterns, such as IGFs, in childhood and early adulthood (Gunnell *et al.*, 2001; Bray *et al.*, 2006), which have been associated with organ growth, greater cell division, and thus risk of cancer-initiating mutations. Moreover, the number of cells in taller people is higher, so there is a bigger probability for mutations that may lead to oncogenesis (World cancer research fund international, 2018).

The modifiable risk factors include **obesity**, due to activation of nuclear factor (NF)-κB signaling, increased production of proinflammatory cytokines and pancreatic infiltration of immunosuppressive cells. Furthermore, higher body fatness has been associated with increased levels of hormones such as insulin, which can promote cell growth and inhibit apoptosis (World cancer research fund international, 2018); **dietary factors** such as high consumption of red meat, processed meat, grilled food, foods containing saturated fatty acids, foods or beverage high in fructose, fried foods and sugar, and low intake of fruit, non-starchy vegetables, wholegrains, dietary fiber; **alcohol** (Secretan *et al.*, 2009) generating toxic metabolites such as acetaldehyde and reactive oxygen species (ROS); **infection**, i.e., the presence of bacterial DNA, namely *Enterococcus faecalis*, may be involved in the progression from pancreatitis to cancer; and **smoking** being the most important environmental risk factor for pancreatic cancer, for the pancreas is exposed to tobacco products indirectly – via blood stream or perhaps through exposure of the pancreas to either duodenal contents or bile (Teitelbaum *et al.*, 1993; Secretan *et al.*, 2009; Hidalgo, 2011). Other modifiable risks include physical inactivity, pharmacological drugs, including the ones used as antineoplastic agents in cancer therapy due to its unspecific genotoxicity, exposure to air pollution, all kinds of radiation, cyanide through drinking water, bisphenol A.

1.3 PATHOPHYSIOLOGY AND MOLECULAR BIOLOGY

The pancreas is a gland located behind the stomach. It is comprised of two types of tissue: exocrine and endocrine. While the exocrine pancreas is responsible for the production of digestive enzymes, the endocrine pancreas produces hormones, like insulin and glucagon, being therefore a part of glucose metabolism (World Cancer Research Fund, 2018).

As afore referred pancreatic cancer outcomes from hereditary germline or somatic acquired mutations in cancer-related genes (oncogenes, tumor suppressor genes, cell cycle genes, apoptosis and genome maintenance genes). Mutations also origin cancer progression and metastasis (Hidalgo, 2011; Goral, 2015). The tumor initiates in the ductal epithelium and evolves from premalignant lesions to fully invasive cancer. Such lesions are histologic precursor of pancreatic cancer and the pancreatic intraepithelial neoplasia, or PanIN, is the best characterized (Hosoda *et al.*, 2017) and they are usually located at the small pancreatic duct, increasing their prevalence with age (Hidalgo, 2011; Goral, 2015).

There are three grades of PanIN: PanIN-1, PanIN-2, and PanIN-3. PanIN-1 is divided into PanIN-1A that is a flat epithelial lesion that comprise tall columnar cells with regular nuclei in a basal location, and abundant supranuclear mucin, and PanIN-1B that has similar cytological characteristics, however, presents a papillary or micropapillary rather than flat architecture. PanIN-2 are papillary, micropapillary, or sporadically flat lesions, with mild to moderate nuclear atypia. They may present loss of polarity and pseudo-stratification with nuclear crowding, enlargement and hyperchromasia. PanIN-3 lesions are defined as in situ malignancy with an intact basement membrane and are papillary, micropapillary, or occasionally flat that display Cribriform growth, luminal necrosis, and detachment of small clusters of epithelial cells into the lumen of the gland. Cytologically, there is enlargement and hyperchromasia of nuclei with loss of polarity and increased nuclear/cytoplasmic ratios. Mitoses, including atypical forms, may be present and luminal (Scarlett *et al.*, 2011).

The evolution from a minimally dysplastic epithelium (PanIN-1) to a more severe dysplasia (PanIN-2 and PanIN-3) and then to an invasive carcinoma comes hand-in-hand with the successive accumulation of mutations. Sixteen mutated oncogenes have been identified in this disease (Goral, 2015), including the activation of the oncogene *KRAS* and inactivation of the tumor-suppressor genes *p53*, *BRCA2*, *p16 (CDKN2)* and *DPC4* (*SMAD4*) (Hidalgo, 2011; Goral, 2015). Biankin *et al.* (2012) also discovered novel mutated genes, such as genes involved in chromatin modification (*EPC1* and *ARID2*) and DNA damage repair, and other mechanisms (*ZIM2, MAP2K4, NALCN, SLC16A4*, and *MAGEA6*).

The formation of PanIN can be induced by the activation of the *KRAS* oncogene alone, as seen in **Figure 3**, and since this mutation is commonly restricted to the codon 12, it is looked upon as a "signature" of pancreatic cancer. While *KRAS* mutations are very frequently found in early and more advanced PanIN stages, mutations of *CKN2A*, *TP53* and *SMAD4* are usually only found in high-grade PanIN (Haeberle and Esposito, 2019).

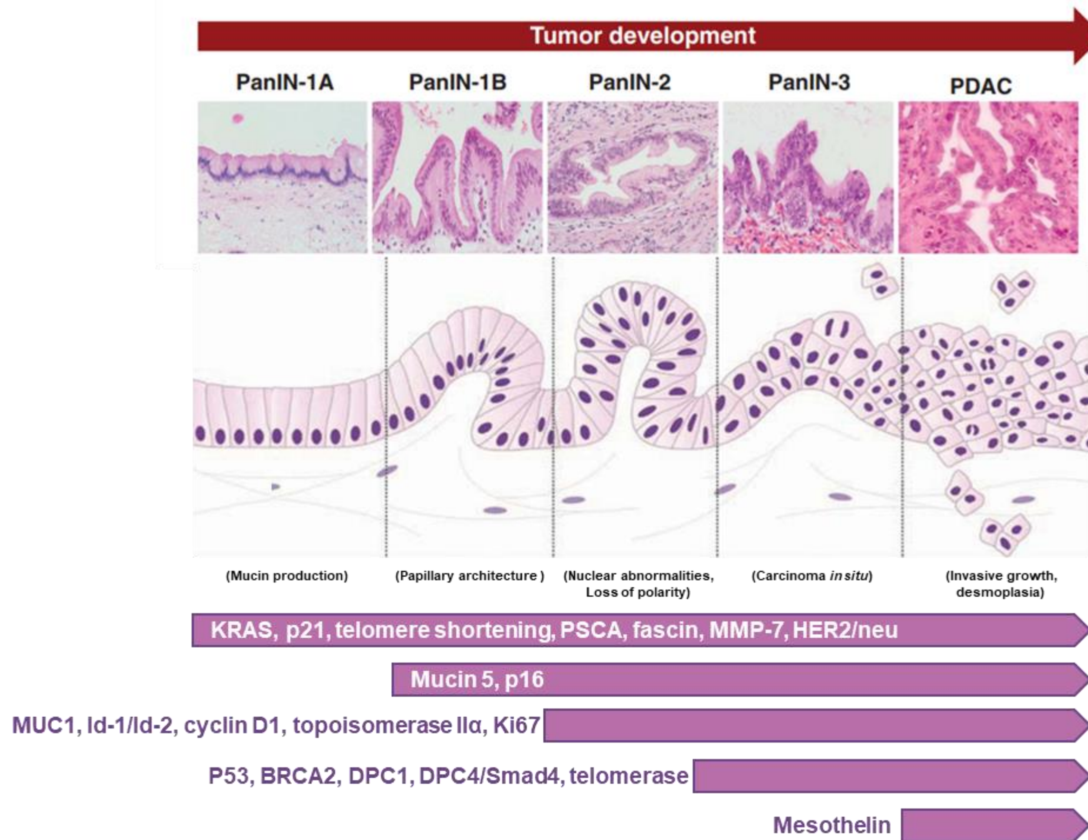

Figure 3: Genetic Mutations Detected in the progression from PanIN-1 to a fully developed PDAC. Adapted from Wörmann S. M. *et al.*, 2014; Goral V., 2015; Scarlett *et al.*, 2011.

Another microscopic precursor lesion of PDAC is atypical flat lesions (AFL), that consist in small tubular lesions. On the other hand, macroscopic precursor lesions of pancreatic cancer include intraductal papillary mucinous neoplasms (IPMN), mucinous cystic neoplasms (MCN) and intraductal tubulopapillary neoplasms (ITPN). Actually, 15% of pancreatic ductal adenocarcinomas are thought to result from mucinous pancreatic cysts. Very briefly, IPMN consists of mucinous papillary intraepithelial neoplasms with a diameter above 1 cm (Singhi *et al.*, 2019). Much like PanIN, IPMN is high-grade or low-grade giving the ranking of cytological atypia and besides *KRAS* mutations, *GNAS* mutations are representative for IPMN (Scarlett *et al.*, 2011; Basturk et al, 2016; Haeberle and Esposito, 2019). MCN are large cysts that mostly occur in the distal pancreas of middle-aged women. *KRAS* mutations are frequently found in early and advanced MCN. Just like IPMN, MCN may have mutations in *RNF43*, whereas mutated *CDKN2A, p53* and *DPC4* mutations are typically present in high-grade MCN. ITPN lesions are severe and unlike the other two mentioned macroscopic precursor lesions of pancreatic cancer, it doesn't harbor neither *KRAS* nor *GNAS* mutations yet *PIK3CA* mutations seem more frequent in ITPN (Scarlett *et al.*, 2011; Basturk et al, 2016; Haeberle and Esposito, 2019).

Oncogenes can be activated through different mechanisms including point mutation and amplification. *KRAS* is one of the most important oncogenes, for its mutation has been found in more than 92% of PDAC. This point mutation leads to an active form of the protein that binds to guanosine triphosphate (GTP) and leads to the arrest of GTPase activity of KRAS protein, resulting in the activation of MAPK signaling cascade promoting uncontrolled cell growth (Saiki and Horii, 2014; Grant *et al.*, 2016). The activation of the oncogenes *Notch* and *COX* also has a role in pancreatic tumorigenesis (Sarkar *et al.*, 2008). Notch protein, when activated, is cleaved releasing intracellular notch that translocates to the nucleus where it

associates with transcriptional factors, which regulate the expression of target genes, and thus plays an important role in both organ development and pancreatic carcinogenesis. High expression of Notch inhibits apoptosis, has been found in pancreatic cancers. The cyclooxygenase (COX) enzymes promote the formation of prostaglandins, leading to cell growth induction. It seems like there is a crosstalk between the isoform COX-2, RAS, NF-κB and Notch in cellular signaling driving the molecular pathogenesis of pancreatic cancer (Sarkar *et al.*, 2008). The amplification of the oncogenes, including *Akt-2, Myb, Src, Bcl-6, S100P, Cyclin D1,* results in its activation and contributes to the stimulation of cell growth and the progression of pancreatic cancers.

Inactivation of tumor suppressor genes is an important occurrence for pancreatic carcinogenesis. Tumor suppressor genes lead eventually to inhibition of cell growth, arrest of the cell cycle and induction of apoptotic cell death. These genes include *p16, p53, DPC4, PTEN*, which are inactivated, by mutation, deletion, or hypermethylation, in the majority of pancreatic cancers (Sarkar *et al.*, 2008).

Pancreatic cancer overexpresses many growth factors and their receptors, including the epidermal growth factor family (EGF), vascular endothelial growth factor, fibroblast growth factor, and many cytokines, such as transforming growth factor β, interleukin 1, interleukin 6, tumor necrosis factor (TNF), and interleukin 8 (Li *et al.*, 2004). The abundance of growth-promoting factors as well as the disruption of growth inhibitory factors lead to evasion of programmed cell death, self-sufficiency in growth signals, angiogenesis, and metastasis. Cytokines are key mediators of the innate and adaptive immune system and are involved in numerous pathologies including cancer. The epidermal growth factor receptor (EGFR) is a transmembrane protein, whose main ligands are EGF and TGF-α. Binding of a ligand to EGFR induces its dimerization and phosphorylation, ultimately activating molecules in different cell signaling pathways including PI3K, MAPK, STAT, giving rise to cell cycle progression, cell division, survival, motility, invasion, and metastasis (Li *et al.*, 2004; Sarkar *et al.*, 2008).

Overexpression of EGF and EGFR is a common feature of human pancreatic cancer. EGFR is also known as HER1 (human epidermal growth factor receptor 1) or erbB-1 (Talar-wojnarowska and Malecka-panas, 2006). In PDAC, overexpression of this receptor was found significantly more often in tumors of advances clinical stages and was linked to lower survival rate in patients carrying this pathology (Yamanaka *et al.*, 1993).

Deregulation of the Hedghog pathway is implicated in a variety of cancers including pancreatic cancer, for the overexpression of the Hedgehog ligand, sonic hedgehog (Shh), is suggested to be a mediator of pancreatic tumorigenesis, since Shh was aberrantly expressed in 70% of patients with pancreatic ductal adenocarcinoma, but none was found in normal pancreas, according to Thayer *et al.* (2013).

The NF-κB signaling pathway is utterly important in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling (Karin, 2006). **NF-κB is constantly activated in most human pancreatic cancer tissues and cell lines** but not in normal pancreatic tissues and cells, which suggests that the activation of this nuclear factor is involved in the formation of pancreatic cancer. Active NF-κB is translocated into the nucleus and binds to the promoters of target genes, controlling the expression of many genes that are involved in cell survival, apoptosis, invasion, metastasis, and angiogenesis.

1.4 DIAGNOSIS AND BIOMARKERS

PDAC poses a major challenge when it comes to diagnostic, for most of the patients present late, with either locally advanced or metastatic disease. In fact, they present non-specific symptoms or none at all, as well as close proximity of major blood vessels which can be invaded by the tumor (McGuigan *et al.*, 2018). Evaluation of a patient in whom pancreatic cancer is suspected should focus on diagnosis, staging of the disease, resectability and palliation of symptoms. Despite the advances in medical procedures and therapies for this pathology, PDAC is still a devastating malignancy. Many methods are used in the diagnosis of pancreatic cancer, like ultrasonography (US), endoscopic ultrasonography (EUS), helical computed tomography (CT), endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance (MR) and thin needle aspiration (Hidalgo, 2011), all of these with up to 98% of sensitivity and 80-90% of specificity (Chu *et al.*, 2017; Zhang *et al.*, 2018), but only EUS and CT have staging benefit. Most of these methods are invasive and have a high cost-to-benefit ratio, possibly causing some aversion on the patient (Goral, 2015) and not surprisingly, the sensitivity is higher for larger tumors compared to smaller tumors.

Although, tumor resection accompanied by chemotherapy can work, in a long term, for patients with early disease stages, only a small percentage of all PDAC patients are potential candidates for a tumor resection, because most patients are in an advanced, nonresectable tumor stage at the time of diagnosis (McGuigan *et al.*, 2018; Loosen *et al.*, 2019). Most patients with PDAC at an early stage are asymptomatic, so it is normally diagnosed at advanced stages, where it is associated with worse clinical prognosis. The terrible outcome is due, not only to late-stage diagnosis as mentioned, but also to limited and ineffective treatment options, early reappearance and absence of clinically useful biomarkers that can detect the cancer at the earliest stages or even in its precursor forms (Ballehaninna and Chamberlain, 2011; Loosen *et al.*, 2019). Thus, identifying robust biomarkers, i.e., cost-effective, with high specificity and sensitivity, and non-invasive, for earlier detection would allow for this cancer to be managed with curative intent, hence reducing PDAC associated mortality (**Figure 4**).

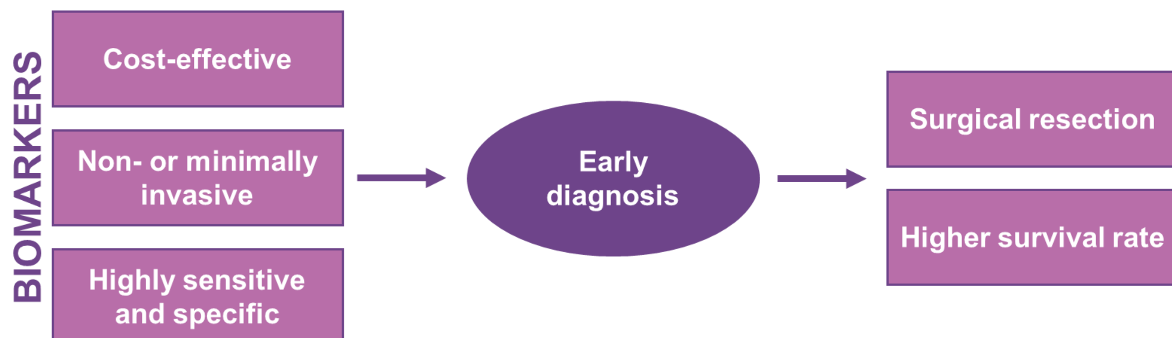

Figure 4: Pancreatic ductal adenocarcinoma biomarker characteristics.

MicroRNA (miRNAs) have been gaining attention as biomarkers for early detection of cancer. These non-coding RNA are involved in expression of post-transcriptional regulatory mechanisms and their dysregulation have been reported in pancreatic tissue, blood, stool, and saliva. Some are upregulated in pancreatic cancer and can differentiate carcinoma from precursor lesions, namely miR-21, miR-155 and miR-196 (Caponi *et al.*, 2013). Actually, miR-223 and miR-204 have been reported to distinguish early-stage cancer from chronic pancreatitis. Furthermore, miR-143, miR-223 and miR-30 are overexpressed in stage I PDAC (Debernardi *et al.*, 2015; Hasan *et al.*, 2019). Other **non- coding RNAs**, such as small ncRNAs (sncRNAs), long ncRNAs (lncRNAs), have been described to be good biomarkers (Kunovsky *et al.*, 2018).

The use of **cytokines** as tumor markers have also been studied. According to Yako *et al.* (2016), six cytokines, namely interleukin-1β, interleukin-6, interleukin-8, interleukin-10, vascular endothelial growth factor, and transforming growth factor, are increased in PDAC. The group concluded that the use of panel of cytokines may be a tool for distinguishing PDAC from other pancreatic benign diseases or healthy individuals. Macrophage inhibitory cytokine 1 (MIC-1) is an autocrine regulatory molecule, a distant member of transformer growth factor beta (TGF-β) superfamily. Serum levels of MIC-1 may be a good biomarker for PDAC since they are increased in the onset malignancy compared to healthy controls and benign pancreatic diseases, besides being related to tumor progression (Kunovsky *et al.*, 2018; Hasan *et al.*, 2019). In fact, MIC-1 can even outperform CA 19-9, although still lacking diagnostic specificity. Nevertheless, this may be improved combining the use of CA 19-9 with MIC-1 (Koopmann *et al.*, 2006).

PAM4 is a murine monoclonal antibody reactive to MUC5AC. This secretory highly glycosylated protein is over expressed in PDAC (Pan *et al.*, 2014). The expression of PAM4 is highly limited to early stages of neoplastic development in pancreas (e.g., IPMN, PanIN) and are not found in normal tissue. Moreover, it was reported a specificity of 85% for PAM4 compared with the 68% of CA 19-9 (Gold *et al.*, 2013).

Other potential biomarkers include **proteins** like C4b-binding protein α-chain (C4BPA), insulin-like growth factor-binding protein (IGFBP) 2 and 3 (Pan *et al.*, 2014), osteopontin (OPN). Some **metabolites** are also described as possible markers for PDAC, such as M2-pyruvate kinase (M2-PK), palmitic acid, glucitol, xylitol, inositol, histidine, proline, sphingomyelin, phosphatidylcholine, isocitrate, ceramide (Di Gangi *et al.*, 2016; Sakai *et al.*, 2016; Kobayashi *et al.*, 2013).

One of the characteristics of cancer is altered glycosylation, so it is undeniable that **glycoconjugates** can be useful in the detection of PDAC. Carbohydrate antigen (CA) 19-9 is an isolated form of Lewis antigen (SLe^a) and is the most common FDA-accepted diagnostic tumor marker and the only FDA approved marker for monitoring the disease, with a sensitivity of 68-81% and a specificity of 82-90% but, unfortunately, offers a poor predictive value in asymptomatic patients (0,5-0,9%) (Pan *et al.*, 2016). Additionally, this glycoprotein has some irregularities associated with the diagnosis: it can be elevated in other medical conditions, for example acute cholangitis, pancreatitis, and liver cirrhosis; about 10−20% of the pancreatic cancer patients who carry the Lewis-negative genotype, lacking fucosyltransferase activity, do not secrete the antigen. These inconsistencies contribute to false results (Nie *et al.*, 2014; Hasan *et al.*, 2019). Other carbohydrate antigens have also been studied extensively in order to be used in the diagnosis of pancreatic cancer including CA 242, CA 50, CA 195, CA 724, CEA and CA 125 (Goral, 2015; Hasan *et al.*, 2019). Carcinoembryonic antigen (CEA) is another extensively studied biomarker. It is a cell membrane-attached glycoprotein involved in cell adhesion and it is usually produced in intestinal tissue during fetal development, whose levels drop significantly before birth. In healthy adults, CEA serum levels are low, being overexpressed mainly in the presence of colorectal cancer, being used to monitor these patients, however it is also found in other carcinomas, including PDAC (Pan *et al.*, 2014). Serum levels of CA125, also known as MUC16, are particularly useful for evaluation of prognosis and for monitoring purposes (Rodrigues *et al.*, 2018). However, these molecules were found to be overall less sensitive than CA 19-9 (Goral, 2015; Hasan *et al.*, 2019). Thus, the need for a more reliable biomarker for early detection of PDAC is urgent.

Possible glycosylated biomarkers can be pointed out. PDAC cells present high levels of sialyl Lewis X (SLe^x) and lower levels of α2,6-sialic acid content on the α2β1 integrin-linked N-glycans leading to an invasive. SDC3 is also often expressed in this malignancy and drives to a poor prognosis (Rodrigues *et al.*, 2018). Glypican 1 (GPC1), a membrane anchoring protein, is overexpressed in pancreatic cancer (Hasan *et al.*, 2019). It has been detected in exosomes, which allows for differentiation between healthy individuals or with benign pancreatic disease from patients with early and late-stage PDAC (Rodrigues *et al.*, 2018; Hasan *et al.*, 2019). Additionally, MUC1 (Pan *et al.*, 2016), MUC3, MUC 4 (and its splice variants), MUC5AC, MUC5B, MUC6, MUC7, MUC13 (Singhi *et al.*, 2019), MUC15, MUC16, MUC17 (Kaur *et al.*, 2013), LGALS3BP (Pan *et al.*, 2014), CD73 (Haun *et al.*, 2015) are examples of potential glycosylated biomarkers. Moreover, MUC4 and MUC16 are reported to be 100% specific for PDAC and present 63 – 67% of sensitivity (Singhi *et al.*, 2019).

1.5 THERAPEUTICS

Pancreatic ductal adenocarcinoma, in spite of the remarkable progress in understanding tumorigenesis and identifying new therapies, is a lethal condition with a rising incidence. **Surgical resection** is still the only therapeutic approach that offers a possible cure of pancreatic cancer. It consists of the removal of the part of the pancreas where the tumor is located or the entire organ (McGuigan *et al.*, 2018). However, only about 10% of PDAC patients are suitable for this surgery (Neoptolemos *et al.*, 2004). **Chemotherapy** is defined as an anti-cancer drug administered intravenously or orally. It is frequently used as a treatment for any stage of pancreatic cancer. Chemo can be used before surgery as a neoadjuvant treatment (sometimes combined with radiation) in order to reduce tumors for removal or treat locally advanced cancer, it can be an adjuvant chemotherapy after resection, which has been shown to improve survival rates (McGuigan *et al.*, 2018), and when the tumor can't be completely removed with surgery, if surgery isn't an option, or if the cancer has metastasized. In neoadjuvant and adjuvant chemotherapy a combination of drugs is often used. Although, if the patient is not healthy enough to endure combined treatment a single drug, such as gemcitabine, 5-FU, or capecitabine, is administered. **Radiation therapy** uses high-energy x-rays (or particles) to treat pancreatic cancer. It can be used as neoadjuvant and adjuvant therapy, on locally advanced/unresectable cancer cases, and to relieve the symptoms in patients with advanced cancers or who aren't healthy enough for other treatments like surgery (American Cancer Society, 2020). The poor prognosis of PDAC is not only due to the lacking early detection methods, causing 80% of patients with pancreatic cancer to have metastatic disease when diagnosed, but also due to resistance to chemotherapy and radiation. Surgery, as afore mentioned, provides a better prognosis, yet the 5-year survival rate for patients who receive adjuvant therapy after resection is 20-25%, with a median survival of 12-22 months. **Immunotherapy** may also be used to treat pancreatic cancer, as it stimulates the patient's immune system to fight the carcinoma (American Cancer Society, 2020).

2 CELLULAR GLYCOSYLATION

2.1 CELL GLYCOME AND GLYCOSYLATION

Glycosylation plays important roles in the normal development and physiology of cells and is performed through several enzymes, including glycosyltransferases and glycosidases, producing glycosidic linkages of saccharides to specific sites in other saccharides, proteins, or lipids. There are several families of glycoconjugates, as illustrated in **Figure 5** (Reis, 2010; Pinho and Reis, 2015). The human genome contains around 700 genes (3-4% of the genome) that encode enzymes, transporters and chaperones required for cellular glycosylation, glycan modifications and degradation (Schjoldager *et al.*, 2020). More than 200 of these genes encode glycosyltransferases, and 173 establish the complex patterns of sugars found on glycoconjugates (Joshi *et al.*, 2018).

Proteins go through several post-translational modifications (PTM) such as phosphorylation, methylation, acetylation, and glycosylation, this last one being the most common alteration. Protein glycosylation takes place in the endoplasmic reticulum (ER) and Golgi, nucleus, cytoplasm, and mitochondria of all eukaryotic cells. The human glycome is built from ten monosaccharides D-glucose (Glc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), L-fucose (Fuc), D-glucuronic acid (GlcA), D-mannose (Man), *N*-acetylneuraminic acid (Neu5Ac), D-xylose (Xyl) and D-ribose (Rib) (Schjoldager *et al.*, 2020). This PTM is involved in processes such as cell-cell signaling, protein stability, interaction of the ligands with the receptors, activity, as well as properties like protein solubility and folding (Huang *et al.*, 2014; Clerc *et al.*, 2015). Proteins with the same amino acid sequence can hold diverse glycan structures, making different glycoforms of the same protein. These glycoforms have different properties resulting in implications for physiological processes, like protein folding and trafficking, cell–cell and cell–matrix interactions, cellular differentiation and immune response (Glavey *et al.*, 2015). Variation in oligosaccharide structures is related to many normal and pathological events: host-pathogen interactions, differentiation, migration, tumor invasion and metastasization, cell trafficking and signaling (Reis *et al.*, 2010). This glycan variation consists of differences in monosaccharide composition, linkage between monosaccharides, anomeric state, branching structures, in other substitutions and in linkage to their aglycone part (Pinho and Reis, 2015).

There are two main types of protein glycosylation: ***N*-glycosylation** and ***O*-glycosylation**. Because glycoconjugates are defined according to the nature of the linkage to their non-glycosyl moiety, glycoproteins are called *N*-glycans if the GlcNAc residue of a glycan links to the nitrogen of the amino acid asparagine in the sequence Asn-X-Ser/Thr, in which X represents any other amino acid except proline (seldom, the sequence Asn-X-Cys is also used), or *O*-glycans if a GalNAc residue binds to the hydroxyl group of a serine or threonine, creating the Tn antigen (Reis *et al.*, 2010; Lee *et al.*, 2010; Pinho and Reis, 2015; Rodrigues *et al.*, 2018).

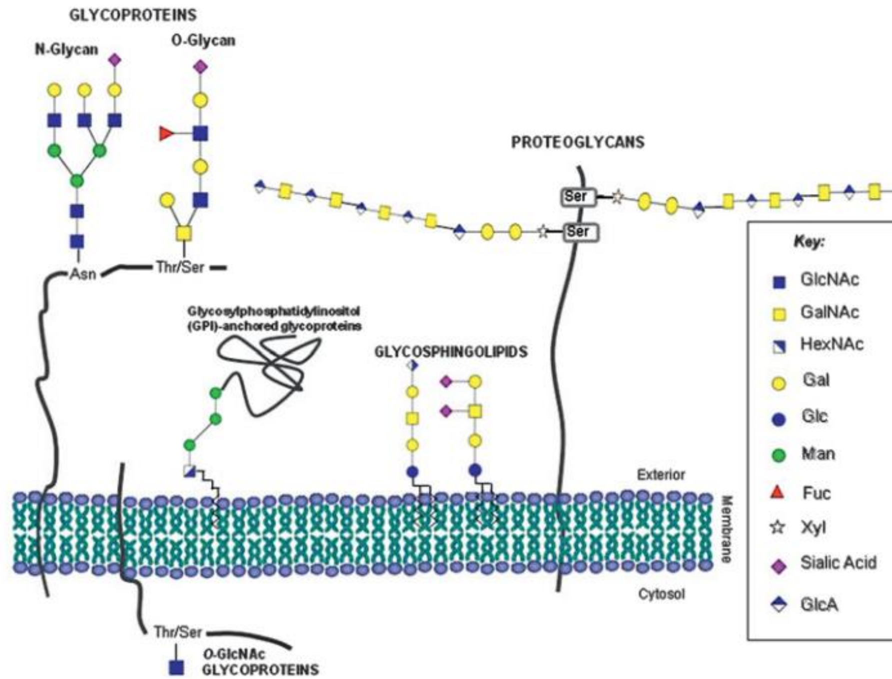

Figure 5: Common classes of glycoconjugates expressed in human cells. Adapted from Reis C, 2010

The formation of *N*-glycans requires the synthesis of an oligosaccharide precursor, containing two GlcNAc, nine Man, and three Glc, that is relocated to proteins with an available asparagine (Asn) in the ER. There, cycles of glucose removal and addition that contribute to protein folding stability, trafficking, localization, and oligomerization vital to cell–cell interactions and intracellular signaling occur (Reis *et al.*, 2010). *N*-glycans have GlcNAc core, a mannose residues core that can suffer elongation by GlcNAc, galactose, sialic acid (Sia) and fucose (Schachter, 1991). There are over two thousand human membrane-bound or secreted proteins harboring an amino acid motif suitable for *N*-glycosylation. Adhesion proteins including members of the immunoglobulin superfamily, CD44, integrins and cadherins, secreted proteinases (e.g., cathepsins, carboxypeptidase E, matrix metalloproteinases, PSA), receptors (e.g., EGFR, HER2/neu, TGF-β receptor, IGF2R), extracellular matrix (ECM) molecules (e.g., fibronectin, laminin), Wnt family are some examples of *N*-glycans (Oliveira-Ferrer *et al.*, 2017).

The biosynthesis of *O*-glycans takes place essentially within the Golgi apparatus and involves, as referred above, initial linkage steps between serine/threonine and Man, Xyl, GalNAc, Fuc, and Glc (Freeze and Ng, 2013). It counts on the activity of *N*-acetylgalactosaminiltranferases to form the Tn antigen and on the activity of Gal-transferase to add a galactose residue to GalNAc, assembling the T antigen (Reis *et al.*, 2010). This can be elongated with Sia, Gal, and later with GlcNAc (Orntoft and Vestergaard, 1999; Glavey *et al.*, 2015). The activity of several enzymes, receptors, transcription factors and structural proteins is affected by *O*-glycosylation. Many cell-surface or secreted proteins carry mucin-type *O*-glycans, which protects them against proteolytic degradation but also modulates recognition, adhesion, and cell-cell-communication functions. Some O-glycans include nuclear phosphoprotein c-myc which regulates cell proliferation, differentiation and apoptosis cell surface proteins like mucins, sialomucin, CD44, integrins and other cell adhesion proteins involved in cell-cell interaction, cell adhesion and migration, structural proteins (e.g., β-catenin), selectins (Oliveira-Ferrer *et al.*, 2017).

2.2 Glycosylation in Cancer

Glycans are involved in nearly every human disease, such as inflammation, infectious diseases, diabetes, neurodegeneration, and cancer (Llop *et al.*, 2018). Cancer is a complex heterogeneous illness and the leading cause of death worldwide. Disruption of the normal process of glycosylation of membrane associated glycoproteins, cell surface glycolipids and secreted glycoproteins is a characteristic of almost every cancer (Reis *et al.*, 2010). The two basic principles that phenotypically guide these changes are **incomplete synthesis**, at an early stage, and **neo-synthesis**, at an advanced stage, of cancer-associated cell surface glycans. Essentially, there is a shift from normal to aberrant glycosylation (Padler-Karavani, 2014). Aberrant glycosylation is the result of alterations in the cellular and molecular machinery, such as glyco-related gene expression, glyco-enzyme localization in the Golgi apparatus and availability of acceptor and donor substrates. Glycan alterations lead to impaired cell-cell adhesion, activation of oncogenic signaling pathways and induction of pro-metastatic phenotypes (Pinho and Reis, 2015; Carvalho *et al.*, 2016; Oliveira-Ferrer *et al.*, 2017). These alterations in malignant cells may include changes in the amount, linkage and acetylation of **sialic acids**, changes in the **branching of *N*-glycans** mediated by glycosyltransferases, under- or overexpression of glycosyltransferases, **altered glycosidase** activity, alterations in **expression of glycosaminoglycans (GAGs)** such as heparan sulfate, **improper function of the Golgi** structure, **truncated simple O-glycans** and **altered glycosylation of mucins**, which are highly glycosylated epithelial-derived proteins that are involved in some cancers (Padler-Karavani, 2014) (**Figure 6**). This was further corroborated since monoclonal antibody techniques have been used to analyze tumor-associated membrane antigens, therefore there is growing indications that aberrant glycosylation characterizes all human cancers (Hakomori, 1989), and contributes to many aspects of cancer development and progression, including proliferation, invasion, angiogenesis, metastasis, and immunity (Padler-Karavani, 2014).

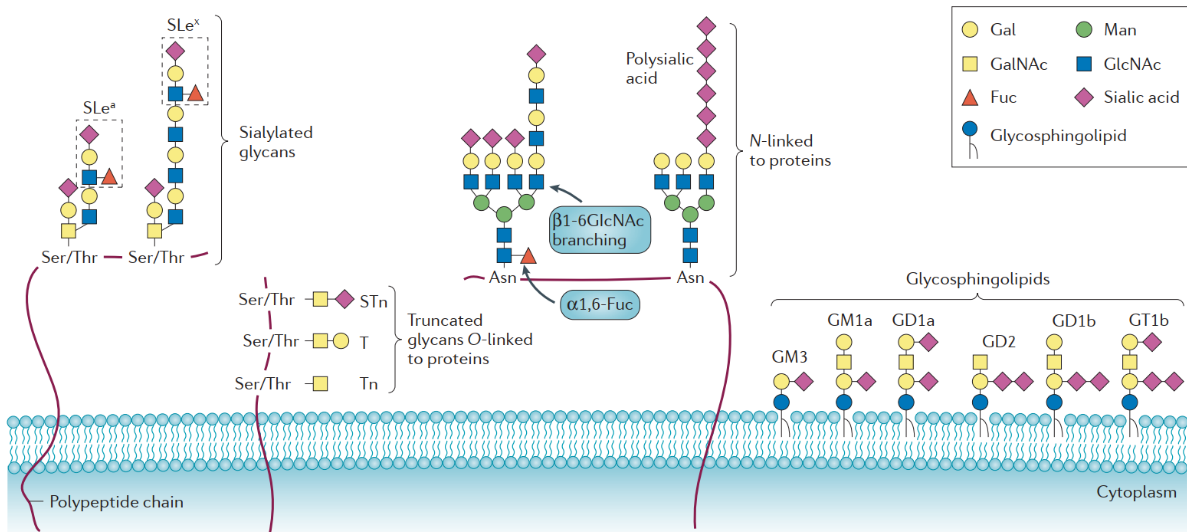

Figure 6: Glycans involved in tumor progression. Adapted from Pinho and Reis, 2015.

Aberrant glycosylation of receptor tyrosine kinases (RTKs), glycosylated transmembrane cell surface receptors that regulate cellular signaling (processes like cell division, differentiation, migration and angiogenesis), or transactivation of glycoconjugates lead to hyperactivation of RTK (Rodrigues *et al.*, 2018).

The ability of a tumor to overcome cell–cell adhesion and to attack the surrounding tissues is involved the development of malignant tumors. Glycans can affect tumor cell–cell adhesion by interfering with the transmembrane glycoprotein with key roles in epithelial to mesenchymal

transition (EMT), epithelial cadherin (E-cadherin). E-cadherin is a significant epithelial cell–cell adhesion molecule in cancer. The addition of *N*-glycans with β1,6-GlcNAc branching (mediated by β1,6-acetylglucosaminyltransferase V (GnT-V)) to E-cadherin leads to non-functional adherens junctions, compromising cell–cell adhesion and downstream signaling pathways (Pinho and Reis, 2015). These events contribute to an invading and metastatic tumor.

Sialylation, which is essentially the transfer of sialic acid – a nine-carbon acidic monosaccharide – to glycan chains, is a prominent alteration in most cancers (Coulibaly and Youan, 2017). Oncogenic cells produce augmented levels of sialylated glycans, which leads to the over-expression of tumor-associated antigens. This helps cell detachment from the tumor mass inhibiting and disrupting cell to cell adhesion. Moreover, these altered glycans, like sialyl-Lewis X (sLe^X) promote the adhesion of cancer cells to vascular endothelial cells by interacting with selectins, improving the chances of metastases (Hakomori, 2002). Another prominent glycosylation modification in cancer is the addition of a of fucose residues to the glycan moiety of glycoproteins or glycolipids – fucosylation – which increases dramatically during carcinogenesis (Coulibaly and Youan, 2017).

The ECM is a complex collection of glycoproteins, collagens, GAGs (ong unbranched polysaccharides of high molecular weight composed of either GlcNAc or GalNAc, and either uronic acid or galactose) and proteoglycans (a core protein onto which one or more GAGs are covalently attached), providing mechanical and structural support and room for signaling events to take place with consequences in tumor growth, maintenance of stem cell niches and cancer progression. Modifications in cell-ECM interactions are crucial for the acquisition of migratory and invasive behavior during carcinoma progression. Particularly, *N*-glycans modulate the function of integrins, which are surface receptors involved in the adhesion of cells to the ECM elements, regulating the migration capacity of tumor cells (Rodrigues *et al.*, 2018). Heparan sulfate proteoglycans (HSPGs), bound to the proteoglycans that are overexpressed in several cancers, are present on the cell surface, as well as in the ECM, and have the ability control cell growth and differentiation, also controlling embryogenesis, angiogenesis, and homeostasis (Pinho and Reis, 2015).

Tumor cell metastatic properties are also enhanced through the aberrant expression of selectin ligands at the cell surface, such as SLe^x, leading to a poor prognosis. Aberrantly overexpressed mucins and secreted N-glycosylated glycoproteins are high-affinity ligands for siglecs, sialic-acid-binding Ig-like lectins involved in immune evasion and tumor development. Due to the overexpression of ST6GAL1 during carcinogenesis, many galectins strongly bind to β1,6-branched glycans and reduced affinity to β-galactosides capped with α2,6-sialic acid. This may protect cancer cells from galectin-mediated apoptosis and promote survival of cancer cells (Rodrigues *et al.*, 2018). A key feature of cancer cell metabolism is a shift from oxidative phosphorylation to aerobic glycolysis, which is characterized by high rates of glucose uptake to cope with the increased energetic and biosynthetic needs to generate a tumor. To fulfil bigger biosynthetic demands, tumor cells also upregulate glutamine uptake (Warburg, 1956). The high levels of glucose in the cytoplasm increases glycolysis and the flux into the metabolic branch pathways, for instance the hexosamine biosynthetic pathway (HBP). The end-product of HBP is UDP-GlcNAc, which is then used for *O*-GlcNAcylation besides for *O*- and *N*-glycosylation (Wells *et al.*, 2001). Elevated *O*-GlcNAc contributes to tumor cell proliferation, survival, angiogenesis, invasion, and metastasis. Many oncogenes and tumor-suppressor genes products, like p53, were also shown to be modified by *O*-GlcNAc (Hart *et al.*, 2011).

Much like, to *O*-GlcNAcylation, *N*-glycan branching is nutrient sensitive. The nutrient flux that controls *N*-glycan biosynthesis manipulates the cellular response of malignant cells defining growth, invasion, and drug sensitivity (Pinho and Reis, 2015).

2.3 GLYCOME ABERRATION IN PANCREATIC DUCTAL ADENOCARCINOMA

In a healthy pancreas glycoproteins have crucial roles like including protection and lubrication of the pancreatic ducts. In PDAC the glycosylation of proteins becomes aberrant and may contribute to the progression and poor prognosis of this disease through changes in cellular adhesion, membrane trafficking, endogenous lectin binding, inflammation, immune function, and metastasis (Krishnan *et al.*, 2017; Munkley, 2019). Some alterations of the pancreatic cancer glycome include increases in the sialyl Lewis antigens (sLe^a and sLe^x), an increase in truncated *O*-glycans (Tn and sTn), increased branched and fucosylated *N*-glycans, upregulation of specific proteoglycans and galectins, and increased *O*-GlcNAcylation (Munkley, 2019).

Lewis-type blood group antigens, i.e., sLe^a and sLe^x, are expressed in cancer cells, mimicking their normal expression on blood cells and their potential for migration as they bind to endothelial cell selectins (Reis *et al.*, 2010). The overexpression of these antigens is associated with increased metastatic ability and poor survival of the patients. For instance, ST3 β-galactoside α-2,3-sialyltransferase III (ST3GAL3), a Golgi membrane enzyme that forms the sialyl Lewis epitope on glycoproteins (Hu *et al.*, 2011), is overexpressed in PDAC cells which promotes increased levels of SLe^x and lower levels of α2,6-sialic acid content on the α2β1 integrin-linked *N*-glycans, leading to FAK phosphorylation and augmented invasive phenotype (Rodrigues *et al.*, 2018). Mucins can be carriers of sialyl Lewis antigens (Reis *et al.*, 2010). In fact, the only FDA-approved and most relevant biomarker for pancreatic cancer, CA19-9, corresponds to a sLe^a antigen (Peixoto *et al.*, 2019). Increased expression sialylated antigens in pancreatic carcinomas is also associated with reduced levels of E-cadherin and altered pattern of E-cadherin localization in cancer cells (Carvalho *et al.*, 2016), and a tumor stroma containing several pro-inflammatory cytokines, such as IL-1β and IL-6 (Peixoto *et al.*, 2019). Another carbohydrate antigen expressed in glycoproteins such as mucins is sialyl-Tn (e.g., CA72-4) that is related with tumor recurrence and poor prognosis in pancreatic cancer. In this malignancy, the expression of the truncated *O*-glycans Tn and sialyl-Tn (sTn) are related to poor outcome, cancer progression and metastasis (Munkley, 2019). Additionally, knockdown of COSMC (a molecular chaperone) promotes aberrant O-glycosylation in pancreatic cancer, driving an anti-apoptotic and pro-metastatic cell behavior, reduced proliferation and increased migration. The enzyme *N*-acetylgalactosaminyltransferase III (GALNT3) is connected to the deviant production of tumor-associated *O*-glycans as well (Oliveira-Ferrer *et al.*, 2017; Munkley, 2019). The aberrant expression of mucins (*O*-glycosylated proteins) is also linked with this disease (Reis *et al.*, 2010). Furthermore, in PDAC integrins, ECM adhesion proteins and proteins involved in pathways like TGF-β, TNF, and NF-κB signaling present augmented levels of *N*-glycosylation (Munkley, 2019). Branched *N*-glycans are increased in tumor-associated carcinoembryonic antigen (CEA), a PDAC monitoring marker (Rodrigues *et al.*, 2018; Zhao *et al.*, 2018). N33, a suggested tumor suppressor, associated with oligosaccharyltransferase complex regulates *N*-glycosylation of specific substrates. The loss of N33 connects with, progression, lymph node involvement and poor survival (Oliveira-Ferrer *et al.*, 2017). Hyper-*O*-GlcNAcylation, *id est*, the increased addition of *O*-GlcNAc to proteins, can modify the hallmarks of pancreatic cancer by blocking apoptosis, leading to oncogenic activation of NF-κB signaling. Indeed, PDAC is associated with the alteration of several proteins by *O*-GlcNAc, such as the heat shock protein HSP70, the transcription factor Sp1, the Wnt signaling proteins β-catenin and LRP6, and the transcription factor Sox2 (Munkley, 2019). Also, high levels of *O*-GlcNAcylation lead to tumor cell survival by mitigating ER stress (Peixoto *et al.*, 2019).

3 LECTINS

Lectins were first described in 1888 by Stillmark who was working with an extract from beans of *Ricinus communis*. He reported the presence of a toxic proteinaceous factor in extracts from castor beans – a lectin – which he called hemagglutinin, because its ability of hemagglutination (Stillmark, 1888). In 1919, James B. Sumner purified a hemagglutinin from *Canavalia ensiformis* for the first time, concanavalin A (Sumner, 1919). Almost two decades later, Sumner and Howell reported that concanavalin A agglutinated cells, such as erythrocytes and yeasts, and precipitated glycogen in solution (Sumner and Howell, 1936). They also showed that hemagglutination by concanavalin A was inhibited by sucrose, demonstrating the sugar-binding specificity of lectins. The term lectin (from the Latin word 'legere' meaning 'to choose') was introduced by Boyd and Shapleigh in 1954 to describe blood group-specific proteins (agglutinins) from legumes that agglutinate erythrocytes through cross-linking reactions, that involve binding to exposed sugar residues (Boyd and Shapleigh, 1954). In 1980, Goldstein *et al.* updated the definition of lectin to "sugar binding proteins or glycoprotein of non-immune origin, without enzymatic activity, that agglutinate cells and/or precipitate glycoconjugates". For Peumans and Van Damme (2005) plant lectins can be defined as plant proteins that possess at least one noncatalytic domain that reversibly binds, through hydrogen bonds, Van der Waals, metal coordination and hydrophobic interactions, to a specific carbohydrate (Peumans and Van Damme, 1995; Elgavish and Shaanan, 1997).

They can bind to both free glycans and glycans attached to glycoconjugates, such as intact glycoproteins or even intact cells or viruses. Lectins bear at least one sugar-binding site and have complex specificities that can recognize not only different monosaccharides within the glycan chain, such as mannose, *N*-acetylglucosamine, sialic acid or galactose, but also different linkages between saccharide monomers or glycan branching (Goldstein *et al.*, 1980; Belický *et al.*, 2016). Therefore, lectins can distinguish types of cells, because they bind to these sugars and sugar chains on the surface of the cells that differ according to the cell type and species (Goldstein *et al.*, 1980; Ghannoum *et al.*, 2011). In addition, lectins should be soluble in biological fluids or membrane bound (Goldstein *et al.*, 1980). These proteins have been widely studied as tools in biochemistry, immunology, and cell biology along with cancer research (De Mejía and Prisecaru, 2005).

3.1 OCURRENCE

Lectins are ubiquitously distributed in living organisms, bacteria, fungi, plants, and animals (Mazalovska and Kouokam, 2020), and play innumerous roles in biological recognition phenomena involving cells, carbohydrates and proteins, in immune defense, in cell migration, in cell-cell interactions, in embryogenesis, in organ formation, and in inflammation (Mazalovska and Kouokam, 2020).

The focus, henceforth, will be on plant lectins. These lectins have been found in a wide variety of plant species and are present in every tissue, being generally most abundant in the seeds but they are also found in different vegetative tissues such as roots, leaves, barks, flowers, bulbs and rhizomes (Faheina-martins *et al.*, 2012). Lectins protect plants from insects and fungi and are also involved in sugar transport and storage. Moreover, some lectins are crucial for atmospheric nitrogen fixation (Mazalovska and Kouokam, 2020).

3.2 MOLECULAR STRUCTURE

Lectins are a structurally diverse group of proteins (Rini, 1995). The journey to study the structure of lectins started in the seventies with the crystal structure of concanavalin A, being the most studied lectin to date (Loris, 2002). Legume lectins, such as concanavalin A, from jack bean (*Canavalia ensiformis*), represent the largest and most meticulously studied family of lectins.

Typically, legume lectins comprise two or four very similar 25–30 kDa subunits, which are usually polypeptide chains of ≈250 amino acids. Each one of these subunits characteristically contains a carbohydrate-binding site, a strongly bound Ca^{2+} and a transition metal ion, usually Mn^{2+} or Mg^{2+}. Each subunit is arched and consist largely of antiparallel β-sheets. Virtually 60% of their secondary structure is comprised of β-sheets connected to each other by loops. The tertiary structure of all legume lectins known so far (**Figure 7**), is composed of two antiparallel β-sheets, a six-stranded flat "back" and a seven-stranded concave "front", associated by a five-stranded β-sheet, forming the “jellyroll” motif, also known as the “lectin fold”. The subunit structures of various legume lectins can overlap. Notwithstanding their similarities at the primary, secondary, and tertiary structural monomeric level, these lectins show major variations in their quaternary structure: little contrasts in the amino acid sequences at the monomer–monomer interfaces and the presence or absence of glycosylation influence the way the monomers interact. About 20% of amino acid residues in all legume lectins are conserved, especially those involved in the interaction with the saccharides – the carbohydrate recognition domain (CRD) – and the ones that coordinate metal ions, particularly those responsible for forming of the hydrophobic pocket (De Hoff *et al.*, 2009). Carbohydrate-binding sites are often shallow depressions on the surface of the protein composed invariantly of an aspartic acid, an asparagine, a glycine and an aromatic amino acid or leucine (Ambrosi *et al.*, 2005).

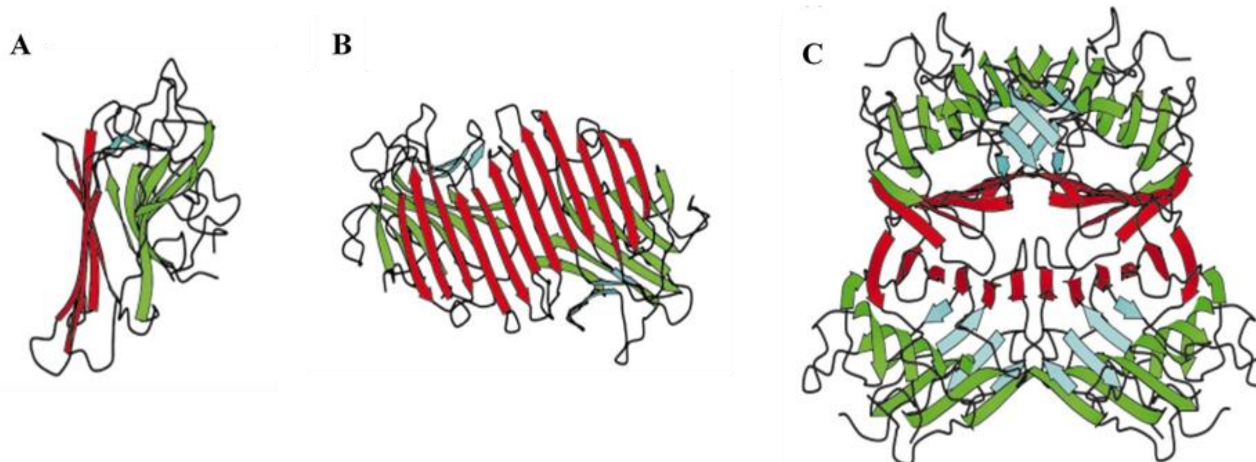

Figure 7: Representative structure of a legume lectin, Concanavalin A. A – Con A fold, tertiary structure of a monomer. B – Dimerization in Con A. C – Association of two dimers, tetramerization in Con A. Adapted from Srinivas *et al.*, 2001

Contrarily, the less widely portrayed family of proteins, cereal lectins, is considerably different from the leguminous plant lectins, for they are comprised of homodimers of two subunits of lower molecular weight (±18 kDa) (De Hoff *et al.*, 2009). This lectin family is highly homologous (Van Damme *et al.*, 2008).

3.3 CLASSIFICATION

Lectins are an immense group of 2-4 subunit proteins that generally differ in biochemical/physicochemical properties, molecular structure, the binding specificity to carbohydrates and biological activities (Sharon & Lis, 1990; Gallegos *et al.*, 2014). The classification of lectin families is still a challenge, nevertheless very important to provide a deeper knowledge and understanding of their functions. Hence, lectins can be classified based on their structure, evolutionary related families, and carbohydrate specificity.

3.3.1 Carbohydrate Specificity

Originally, lectins were classified based on their specificity for different blood groups (Bird, 1959), considering their hemagglutination capacity, and later the carbohydrate that binds to the lectin inhibiting its hemagglutination activity (Rego *et al.*, 2002).

As afore mentioned, carbohydrate specificity is associated to conserved domains within the lectin, distributed along the loops. The bivalent cations (Ca^{2+} and Mg^{2+}/Mn^{2+}) are extremely conserved in the loop, as well as the Asn residue. Water molecules are also crucial for the lectin-carbohydrate interaction.

The carbohydrate specificities of lectins are classified into five groups according to the monosaccharide for which they exhibit the highest affinity:

Group I – Mannose (Man)/Glucose (Glc) specific lectins. Interactions of monosaccharides with lectins of this group involve hydrogen bonds with conserved amino acid residues. The lectin Con A belongs to this group (Weatherman *et al.*, 1996).

Group II – *N*-Acetylgalactosamine (GalNAc)/Galactose (Gal) specific lectins

Most lectins belong to this group. These lectins have a lengthier D loop that those of group I or group IV. Examples are JCA (*Artocarpus integrifolia* lectin) and SNA-II (*Sambucus nigra* agglutinin) (Machuka *et al.*, 2003).

Group III – *N*-Acetylglucosamine (GlcNAc) specific lectins

There are only a few lectins that belong to group III, including BPA (*Bauhinia purpurea* agglutinin) (Wu *et al.*, 2004).

Group IV – Fucose (Fuc) specific lectins

The members of this group present differences in the sequence of D loop, but its dimension remains unaltered (Sharma and Surolia, 1997).

Group V – Sialic acid (Sia) or *N*-acetylneuramic acid (NeuNAc) specific lectins

An example of this type of lectin is MFA (*Maackia fauriei* agglutinin) (Kim *et al.*, 2004).

There is a sixth group (**Group VI**) that encompasses lectins specific for complex oligosaccharides and polysaccharides. There are very little lectins that react exclusively with oligosaccharides and polysaccharides. VL2, VL3 and VL4 are isolectins from *Acacia constricta* that do not react with AB0 system nor with simple sugars, however they are inhibited by complex carbohydrates from fetuin and thyroglobulin (Guzmán-Partida *et al.*, 2004).

Thence, depending on the specificity toward a given saccharide, the lectin will selectively bind to one of these sugars which are typical constituents of eukaryotic cell surfaces (Lis and Sharon, 1998; Rego *et al.*, 2002).

3.3.2 Structure

In 1998, Els Van Damme and colleagues introduced a classification system built on the overall domain structure of mature plant lectins. This system divides lectins in four main classes: merolectins, hololectins, chimerolectins and superlectins (**Figure 8**).

Merolectins are proteins having a single carbohydrate-binding domain and are not able to precipitate glycoconjugates or agglutinate cells because they are monovalent by definition (Vasconcelos and Oliveira, 2004). An example is the chitin-binding protein from *Hevea brasiliensis* latex (Van Parijs *et al.*, 1991).

Hololectins comprise all lectins that have di- or multivalent carbohydrate-binding sites (Vasconcelos and Oliveira, 2004) and thus agglutinate cells and/or precipitate glycoconjugates. Most plant lectins are hololectins (Peumans and Van Damme, 1995).

Chimerolectins are proteins consisting of one or more carbohydrate-binding domain(s) plus an additional domain with catalytic (or another biological activity) working independently from the carbohydrate-binding site. They can behave like merolectins or hololectins hinging on the number of carbohydrate-binding sites. Type II ribosome inactivating proteins (RIP II) are examples of chimerolectins (Peumans and Van Damme, 1995; Vasconcelos and Oliveira, 2004).

Superlectins like hololectins possess at least two carbohydrate-binding domains but differ because their sites are able to recognize structurally unrelated sugars (Vasconcelos and Oliveira, 2004). An example of superlectin is the tulip bulb lectin that presents a mannose-specific and a *N*-GalNAc-specific carbohydrate-binding domain (Peumans and Van Damme, 1998). A novel superlectin, BC2L-C, from the opportunistic pathogen *Burkholderia cenocepacia* has been found to have dual carbohydrate specificity mediating the cross link between the host and the bacteria (Bermeo *et al.*, 2020).

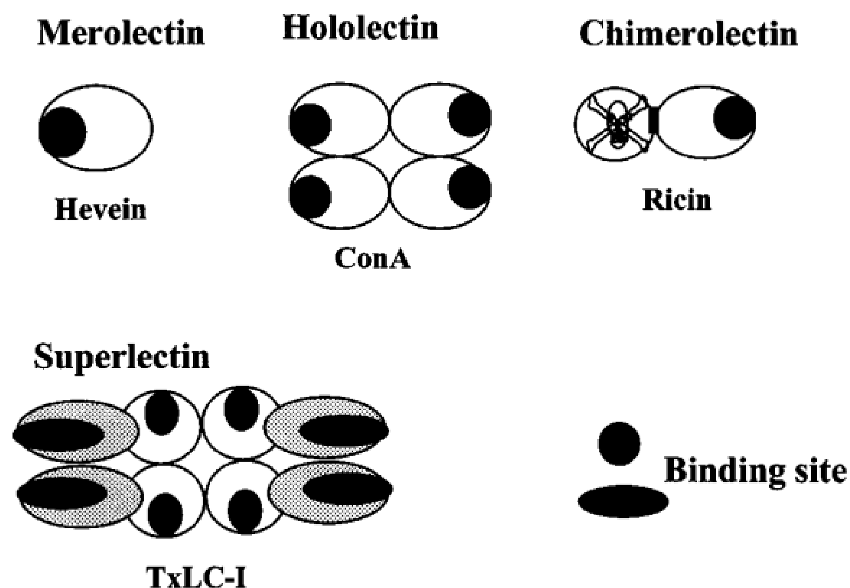

Figure 8: Lectin classification based on their structure: merolectin, hololectin, chimerolectin and superlectin. Adapted from Van Damme *et al.,* 1998.

3.3.3 Evolutionary Related Families

According to Van Damme *et al.*, 2008, the available structural information and molecular cloning of lectin genes the great majority of all known plant lectins can be subdivided into twelve families with evolutionarily and structurally related domains (Van Damme *et al.*, 1998), such as *Agaricus bisporus* agglutinin homologs, amaranthins, class V chitinase homologs with lectin activity, cyanovirin family, EEA family, GNA family, proteins with hevein domains, jacalins, proteins with legume lectin domains, LysM domain, nictaba family (formerly *Cucurbitaceae* phloem lectins), ricin-B family (Van Damme *et al.*, 1998; De Hoff *et al.*, 2009; Fu *et al.*, 2011), as shown in **Table 1**.

Table 1: Classification of plant lectins according to structural and evolutionary characteristics.

Family	Representative lectin	Abbreviation	Carbohydrate-binding specificity
***Agaricus bisporus* agglutinin homologs**	*Agaricus bisporus* agglutinin	ABA	Galactose
Amaranthins			
Class V chitinase homologs with lectin activity	Chitinase-related agglutinin	CRA	High-mannose *N*-glycans
Cyanovirin	Cyanovirin-N	CV-N	Mannose
EEA	*Euonymus europaeus* agglutinin	EEA	Mannose/galactose
GNA	*Polygonaturn cyrtonema* lectin	PLC	Mannose/sialic acid
Hevein domains	Wheat germ agglutinin	WGA	*N*-acetyl-D-glucosamine
Jacalins	Jacalin	JAC	Mannose
Legume domain	Concanavalin A	Con A	D-mannose
LysM domain			
Nictaba	*Cucurbitaceae* phloem lectin	CPL	
Ricin-B	European mistletoe lectin	ML-I	β-galactose

Adapted from Fu *et al.,* 2011.

3.4 ACTION MECHANISM

3.4.1 Carbohydrate Binding

Carbohydrates have a huge potential for encoding biological information in the position and anomeric configuration (α or β) of the glycosidic units. Besides the differences in the carbon backbone, length of the monomer, side group orientation and substitution, the carbohydrate often branches, further complicating structure of this diverse group of molecules. Therefore, just 4 different monosaccharides can create an astounding 35 560 tetrasaccharides. There is a great diversity of carbohydrate structures connected with soluble and surface-bound glycoconjugates, which turns out to be relevant, for carbohydrates are able to alter the activities of proteins to which they are linked and also serve as markers of cell differentiation, development, and pathological states as well as recognition signals (Mody *et al.*, 1995; Lis and Sharon, 1998; De Hoff *et al.*, 2009).

Proteins that interact with sugars noncovalently are common in nature, including carbohydrate antibodies and carbohydrate-specific enzymes (Lis and Sharon, 1998). In the last decades, research has turned its focus to lectins, non-immune origin proteins universally distributed in plants, animals, bacteria and fungi that contain at least one non-catalytic domain which allows them to recognize and reversibly bind to specific carbohydrates present on glycoproteins and glycolipids, without changing the structure of the sugar (Mody *et al.*, 1995). Hence, when discussing carbohydrates, lectins must be mentioned, since they bind to specific carbohydrate complexes present in glycoproteins and glycolipids, present in the cell membrane (**Figure 9**) or at the ECM. As mentioned before, carbohydrates have such complex structures that contain biologically significant information. Lectins are proteins that are able to discriminate the most elusive alteration of the oligosaccharide structure, as stated by Ambrosi *et al.* in 2005 "The ability of lectins to distinguish between subtle variations of oligosaccharide structure makes them perfectly suitable as decoders for such carbohydrate-encoded information. In other words, whilst sugars can carry the biological information, lectins are capable of deciphering this "glycocode"." (Ambrosi *et al.*, 2005).

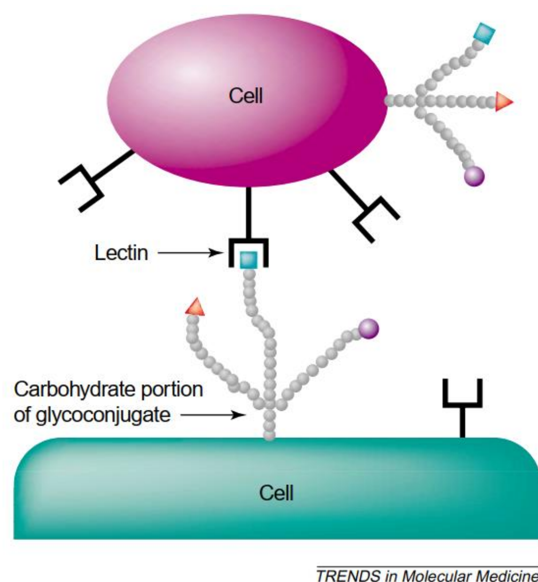

Figure 9: Lectin-mediated cell–cell interactions. Adapted from Nangia-Makker, P. *et al.,* 2002.

Lectins interact with carbohydrates at a binding site or carbohydrate binding domain. Each domain has its own characteristic overall fold with one or more sugar binding domains located at specific positions and consist of a shallow depression at the surface of the protein (Van Damme *et al.*, 1998; Ambrosi *et al.*, 2005). The recognition/binding of simple sugars primarily depends on the interaction of a few conserved amino acid residues located in the lectin carbohydrate binding domain with some hydroxyls of the glycans, the so-called "loop". The interaction includes the formation of hydrogen bonds, where water molecules also contribute, and is often reinforced by a hydrophobic stacking of the pyranose ring of the sugar to the aromatic ring of tyrosine or phenylalanine residues located in near the sugar binding site (Van Damme *et al.*, 1998). Even though Ca^{2+} and Mn^{2+}/Mg^{2+} (or other transition metal) are not always directly involved in sugar binding, the cations contribute to the amino acid residues that interact with the glycoside positioning (Weis & Drickamer, 1996; Lis and Sharon, 1998; Ambrosi *et al.*, 2005; Gabius, 2011). The presence of these bivalent ion triggers a lectin structure conformational alteration which will stabilize the lectin-carbohydrate binding (Sharon and Lis, 1990).

Although carbohydrates are polar molecules, the spatial arrangement of the hydroxyl groups promote hydrophobic zones, which contribute to sugar affinity and specificity through non-polar interactions, Van der Waals interactions (Toone, 1994). Lectins bind not only to oligosaccharides on cells but also to monosaccharides. This last interaction, nevertheless, is rather weak (Lis and Sharon, 1998). The reason for this difference in affinity is inherent in the atomic structure of the binding domains. The same carbohydrate specificity for different lectins doesn't necessarily imply the same level of affinity. In fact, affinity to the same sugar can be substantially different, suggesting structural differences between lectins that lead to diverse lectin-ligand complex affinity (Cavada *et al.*, 2001). Along these lines similar carbohydrate specificity lectins can hold distinct biological activities (Sanz-Aparicio *et al.*, 1997).

3.5 BIOLOGICAL FUNCTIONS, ROLE AND APPLICATION

It is no surprise that, in the past decade, lectins have been given the deserved attention and focus. In fact, these glycoproteins have countless applications depending on their assets, including insecticide, anti-fungal, anti-parasite, anti-microbial and antitumor, activities, immunomodulatory and HIV-1 reverse transcriptase inhibition (Ribeiro *et al.*, 2018). This has led to an emerging field of science known as "lectinology" or, in other words, the science of lectins (Coulibaly and Youan, 2017). These properties may be significantly useful in phytopharmaceutical and pharmaceutical industry (Hamid *et al.*, 2013).

Lectins are present in most organisms, ranging from viruses and bacteria to fungi, plants and animals (Ambrosi *et al.*, 2005). They are able to recognize specific oligosaccharides present at the cell membrane, that play important roles, like cell–cell and cell–molecule recognition and interaction, and cell adhesion, migration and invasion (Katink-Prastowska, 1999; Nangia-Makker *et al.*, 2002; Ribeiro *et al.*, 2012). Consequently, lectins have been explored in a way to benefit from them, for instance in the activation of specific cellular responses to host-pathogen interaction immunity (Eichinger, 2001), as cell agglutinants and mitosis inducers (Kabir, 1998), in blood group determinations (Cummings, 1996), as cancer therapy (Lord, 1987; Thrush *et al.*, 1996; Abdullaev and De Mejia, 1997; Wang *et al.*, 2000), in cell-cell recognition (Sharon and Lis, 1990; Sames *et al.*, 2001) and in cell death induction (Fu *et al.*, 2011; Oliveira *et al.*, 2019).

Plant lectins are defined as plant proteins that hold at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide by Peumans and Van Damme (1995). Most of these plant lectins not only play a role in the plant itself (e.g., to store nitrogen or as a specific recognition factor), but also interact with glycoconjugates of other organisms interfering with the normal functioning of that organism (Peumans and Van Damme, 1995). They are produced with a signal peptide and targeted via the secretory pathway into the vacuolar and extracellular compartments, being the first line of defense against plant eating organisms (Lannoo and Van Damme, 2010). Commonly, there's an abundance of lectins in the most exposed organs of the plant, such as seeds, particularly in the Leguminosae and Gramineae seeds (Rego *et al.*, 2002). Plants also express these proteins as a response to some stress factors and changing environmental conditions (Lannoo and Van Damme, 2010). Some plant lectins are also involved in symbiosis, like rhizobia binding to the lectin in plant roots to assist in the nitrogen fixation, by binding the lectin to carbohydrate moieties on the bacterial surface, or *ex planta* carbohydrate-based signal detection (De Hoff *et al.*, 2009).

Somewhat recent studies report that plant lectins can be used to recognize malignant tumors in diagnosis and prognosis of cancer. Conversely, these lectins also have antitumor activities, by aiming at programmed cell death (PCD), including apoptosis and autophagy (Oliveira *et al.*, 2019). Some examples are mistletoe lectin (ML), ricin (RCA) and *Arbutus unedo* lectin that induce cell death in cancer cells. As for Concanavalin A (Con A) and *Polygonatum cyrtonema* lectin (PCL) induce autophagy and subsequent cell death (Fu *et al.*, 2011).

3.6 LECTINS AS ANTITUMORAL AGENTS

One of the most important characteristics of cancer is the modification of cell glycosylation. This includes loss of expression or overexpression of certain glycan structures, incomplete, truncated, or novel structures and the accumulation of precursors (Reis *et al.*, 2010; Coulibaly and Youan, 2017; Rodrigues *et al.*, 2018). Aberrant glycosylation is, therefore, a major feature of cancer initiation, progression, invasion, and metastasis (Rodrigues *et al.*, 2018). Lectins have a tremendous potential in cancer diagnosis due to their high selectivity and specificity for certain carbohydrate structures, as referred to in I.3.4. Furthermore, most lectins are cytotoxic, therefore it's only logical to think of them as the perfect anti-cancer therapeutic candidate. The anti-cancer properties of lectins primarily consist of PCD (autophagic cell death and apoptosis) and inhibition of tumor growth and angiogenesis.

3.6.1 Cell Death Mechanisms

Tumorigenesis is driven by sequential genetic alterations that culminate in a malignant transformation of the cells, and by deregulation of specific biological processes that allow and maintain cancer progression (Labi and Erlacher, 2015). These regulatory processes include mitosis stimulation, whilst others aim to inhibit mitosis or induce apoptosis or other forms of programed cell death (PCD), such as vacuole-presenting cell death (autophagy, entosis, methuosis and paraptosis), mitochondrial-dependent cell death (mitoptosis and parthanatos), iron-dependent cell death (ferroptosis), immune-reactive cell death (pyroptosis and NETosis), lysosome-dependent cell death, or necroptosis (Kreuzaler and Watson, 2012; Galluzzi *et al.*, 2018; D'Arcy, 2019; Yan *et al.*, 2020).

Besides PCD, another type of cell death is accidental cell death (ACD) which, in contrast, is caused by severe insults, including physical, chemical, and mechanical stimuli. ACD includes cell death of the necrotic type (Galluzzi *et al.*, 2018; Yan *et al.*, 2020).

3.6.1.1 Accidental Cell Death

Necrosis is an energy independent accidental cell death stimulated by external factors like infection, toxins, and physical injury. Necrotic cells undergo morphological changes such as oncosis (a pre-lethal phase where the cytoplasm swells and it is caused by the disruption of ionic pumps such as Ca^{+} influx), plasma membrane rupture and the subsequent loss of intracellular organelles (Galluzzi *et al.*, 2019; Yan *et al.*, 2020). The release of cellular content causes damage to surrounding cells and an inflammatory reaction, leading to the upregulation of pro-inflammatory proteins and compounds (e.g., NF-κB) characteristic of this ACD (Ziegler and Groscurth, 2004; Galluzzi *et al.*, 2019).

3.6.1.2 Programed Cell Death

Cell death, as mentioned above, can be divided in accidental and programed cell death. Given the morphological characteristics and molecular mechanisms, PCD can be further categorized into apoptotic cell death and non-apoptotic cell death, like autophagic cell death.

Apoptosis involves a sequence of complex and tightly regulated events and is characterized by cell shrinkage, membrane blebbing, positional organelle loss, chromatin condensation and fragmentation. These events result in the formation of apoptotic bodies that are then engulfed by neighboring phagocytic cells (Cotter, 2009; Yan *et al.*, 2020).

The machinery of apoptosis requires the interplay of several of cysteine-aspartic proteases known as caspases – initiator and effector caspases. Once cell damage is detected, the initiator caspases (caspases-8 and -9) are activated and proceed to activate the effector caspases (caspases-3, -6 and -7) (D'Arcy, 2019). The effector caspases initiate a cascade of events that lead to nuclear condensation and fragmentation, externalization of membrane phospholipids, destruction of the nuclear proteins and cytoskeleton, crosslinking of proteins, the expression of ligands for phagocytic cells and the formation of apoptotic bodies that later are phagocytosed by macrophages (Boatright and Salvesen, 2003; D'Arcy, 2019).

Apoptosis can be initiated through three signaling pathways: the perforin/granzyme pathway, involving cytotoxic T-lymphocytes and perforin-granzyme-dependent killing of the cell (Elmore, 2007) (which will not be addressed in this work), the extrinsic pathway (cytoplasmic membrane receptors), or the intrinsic pathway (mitochondrial) (Yan *et al.*, 2020).

The **extrinsic pathway** of apoptosis is triggered by perturbations of the extracellular microenvironment. It is initiated by either death receptors that require ligand binding, or dependence receptors that are activated when the levels of their ligand drops below a certain threshold (Galluzzi, *et al.*, 2018). This allows the activation of the caspase cascade. The activation of effector caspases by initiator caspases lead to cleavage of specific substrates that belong to numerous functional classes, such as apoptosis signaling proteins (e.g., IAPs, Bcl-2), protein kinases (eg FAK, PKC), structural/cytoskeletal proteins (e.g., gelsolin, lamin), cell repair proteins (e.g., PARP, ATM), and cell cycle proteins (e.g., p21, Rb, p27) (Kreuzaler and Watson, 2012; Galluzzi, *et al.*, 2018; D'Arcy, 2019).

The **intrinsic pathway** – also known as mitochondrial – is initiated upon positive (e.g., hypoxia, toxins, radiation, reactive oxygen species, viruses, and toxic agents) or negative signals (e.g., absence of cytokines, hormones and growth factors that lead to the activation of pro-apoptotic molecules within the cell like Puma, Noxa and Bax). These factors lead to loss of cellular homeostasis, disruption of ATP synthesis and increased production of reactive oxygen species, and collapse of the mitochondrial inner membrane and subsequent rupture. This drives to loss of cytochrome c into the cytoplasm, which in the presence of ATP and apoptotic protease activating factor 1 (Apaf-1) activates the initiator caspase-9 and, consequently the effector caspase-3 (Labi and Erlacher, 2015; Galluzzi, *et al.*, 2018; D'Arcy, 2019).

Autophagy is a catabolic process where superfluous or damaged cellular components like macroproteins or organelles are seized into lysosomes for degradation. During autophagy, the components are encapsulated by cell membranes, forming autophagosomes. These will merge with lysosomes and the contents degraded by lysosomal hydrolases (Kelekar, 2005). This occurs in response cellular stress (e.g., caloric restriction, hypoxia, DNA damage or ER stress), to return cellular homeostasis (Kreuzaler and Watson, 2012). Autophagy not only recycles cellular components, but it is also associated with removal of senescent cells and destruction of neoplastic lesion. Thus, the lack of this event lead to some neurodegenerative conditions like Alzheimer’s disease and cancer (D'Arcy, 2019).

3.6.2 Antitumoral lectins

As mentioned earlier, some plant lectins have antitumor activities via targeting programmed cell death (PCD), such as apoptosis and autophagy, and inhibition of tumor growth. For instance, lectins from *Viscum album* (mistletoe) have been used for years as alternatives in cancer therapy, diminishing the hostile effects of the standard treatments (e.g., chemo and radiotherapy), improving patients' quality of life (Pervin *et al.*, 2015; Ribeiro *et al.*, 2018). Mistletoe lectins (ML), belong to the **ribosome inactivating proteins type II family** (RIP II) and are divided into three types: ML-I, ML-II and ML-III. ML-I binds to lactose, D-galactose and GalNAc, whilst MLII and III preferentially binds to GalNAc. MLs are comprised of 2 polypeptide chains, chain A and chain B. The first chain inhibits protein biosynthesis inside the cell by arresting elongation through the hydrolysis of the *N*-glycosidic bond at adenine-4324 in the 28S RNA of the 60S ribosomes. Chain B gives ML its immunomodulatory activity, by improving the secretion of cytokines and the activity of natural killer cells. MLs induce cancer cell death by an apoptotic pathway, activating several caspase-8, caspase-9, and caspase-3, that results in down-regulation of the pro-survival protein Bcl-2 and in inhibition of telomerase activity (Fu *et al.*, 2011; Coulibaly and Youan, 2017). ML-I activates the extrinsic apoptotic pathway through TNF-family death receptors. ML-I can induce apoptosis by impairing the mitochondrial membrane potential leading to cytochrome c release, increasing of the levels of ROS, and Apaf-1-dependent caspase activation (Hostanska *et al.*, 2003). ML-II activates MAPK signaling, leading to anti-proliferative and pro-apoptotic behaviors (Fu *et al.*, 2011; Coulibaly and Youan, 2017). This mistletoe lectin can also induce apoptosis through the activation of the SAPK/JNK and p38 pathways, as well as through inhibitions of the extracellular signal regulated kinase (ERK) 1/2 pathway (Pae *et al.*, 2001).

Ricinus communis agglutinin (RCA), also known as ricin, is a galactose-binding RIP-II extracted from castor beans. Ricin is a heterodimer that is also composed of two polypeptide chains. These chains are *N*-glycosylated and are linked by a disulfide bond. Chain A has an enzyme-like activity that inactivates 60S ribosomal subunits preventing them from binding to the GTP-binding translation elongation factor EF-2, which leads to averted protein synthesis. Chain B binds to β1-4-linked galactosyl containing glycoproteins causing the endocytosis of ricin (Coulibaly and Youan, 2017). RCA induces cell death also by apoptosis, through upregulating caspase-8 and its downstream caspase-3/7, and DNA fragmentation (Fu *et al.*, 2011).

Concanavalin A (*Canavalia ensiformis*) and *Sophora flavescens* lectin (SFL) both have **legume lectin domain**. Con A and SFL are able to activate the mitochondria mediated apoptotic pathway (Fu *et al.*, 2011; Yau *et al.*, 2015), mitochondrial membrane collapse, cytochrome c release and caspase-9/3 activation. Moreover, these lectins upregulate the pro-apoptotic protein Bax and downregulate the pro-survival proteins Bcl-2 and Bcl-xl. Besides, concanavalin A reduces the nuclear factor (NF)-κB, the ERK, and the c-Jun *N*-terminal protein kinase (JNK) levels, and contrarily increases tumor suppressor protein p53 and the cyclin-dependent kinase inhibitor p21 levels (Fu *et al.*, 2011; Coulibaly and Youan, 2017; Oliveira *et al.*, 2019). It is to note that *Sophora flavescens* lectin doesn't affect the levels of JNK. Furthermore, Con A can induce an autophagic cell death by binding to a mannose residue in cell membranes and it is then internalized to the mitochondria resulting in an impairment of mitochondrial membrane potential. In 2014, Roy *et al.* explored Con A capability to induce apoptosis regulated by autophagy downregulating the PI3K/Akt/mTOR pathway and upregulating the MEK/ERK pathway, thus leading to autophagy.

Polygonatum cyrtonema lectin (PCL), a mannose/sialic acid lectin, belongs to ***Galanthus nivalis* agglutinin (GNA) family** and induces tumor cell death by targeting caspase-dependent pathways, mitochondrial ROS-p38-p53 pathway, Ras-Raf and PI3K-Akt pathways. PCL activates caspase-9, caspase-8 and caspase-3. Inducing apoptosis (Fu *et al.*, 2011). *Polygonatum cyrtonema* lectin (PCL), a mannose/sialic acid lectin induces tumor cell death by targeting caspase-dependent pathways, mitochondrial ROS-p38-p53 pathway, Ras-Raf and PI3K-Akt pathways. PCL activates caspase-9, caspase-8 and caspase-3. Inducing apoptosis. This lectin is known to regulate Bax, Bcl-xl and Bcl-2 proteins, leading to the disruption mitochondrial membrane potential, which causes the activation of caspases and release of cytochrome c. Furthermore, PCL induces ROS accumulation in the mitochondria, which subsequently result in p38 and p53 activation (Fu *et al.*, 2011; Liu *et al.*, 2009).

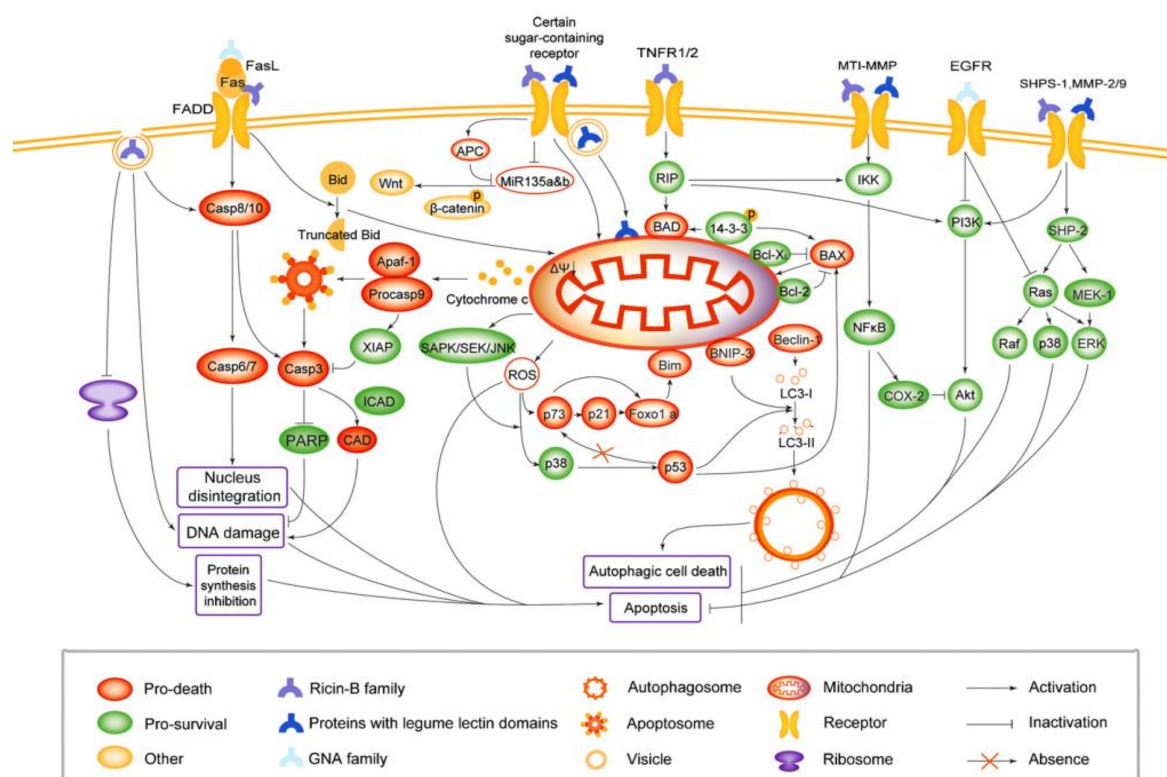

Figure 10: Effect of plant lectins on programmed cell death signaling pathways. Adapted from Fu *et al.* 2011

Considering the general overview of the cell death mechanisms inherent to plant lectins, as illustrated in **Figure 10**, it can be concluded that these proteins induce PCD by inactivating the ribosomes, by binding to specific receptors on the cells surface and by binding to mitochondria. These mechanisms are of the utmost importance given the great potential of lectins in the research for novel cancer therapeutic approaches.

3.6.3 *Arbutus unedo* L. lectins

Ancient civilizations soon realized plants therapeutic potential. Plant extracts and their bioactive compounds in health have been used since long ago as treatment to several conditions, being considered a source of new drugs. In fact, according to WHO, around 80% of health care of the world population is a consequence of plant-derived drugs. So, understandably, the study of potential medicinal plants and their bioactive content is of great importance nowadays.

Arbutus unedo L. (strawberry tree) (**Figure 11**), of the Ericaceae family, is a small evergreen bushy shrub that thrives in the Iberian Peninsula and in the Mediterranean basin, as well as in regions with hot summers and mild rainy winters (Pallauf *et al.*, 2008).

Figure 11: *Arbutus unedo*. A – Leaves. B – Fruits. C – Inflorescence.
Image by the author, captured on October 30th, 2021.

The orange-red spherical fruits are usually consumed as alcoholic beverages, as jams and, less frequently, as fresh fruit when ripe (Miguel *et al.*, 2014). Strawberry tree fruits contain several antioxidant molecules, including phenolic compounds (e.g., anthocyanins, gallic acid derivatives, tannins, and flavonoids), vitamin C, vitamin E and carotenoids (Oliveira *et al.*, 2009; Barros *et al.*, 2010), and have been used in folk medicine for their antiseptic, diuretic, and laxative properties. *A. unedo* leaves contain several phenolic compounds (e.g., tannins, flavonoids, phenolic glycosides) (Oliveira *et al.*, 2009; Erkekoglou *et al.*, 2017), as well as proteins, of which lectins (Ribeiro *et al.*, 2012). They are used as astringent, diuretic, urinary antiseptic, antidiarrheal, depurative and as a therapy for hypertension, diabetes, and some inflammatory diseases. Leaf extracts also act as good free radical scavengers (Oliveira *et al.*, 2009). This 3-6 m tree is associated with important qualities, such as its ornamental, economic and ecological value, and also the medicinal properties inherent to its organs (**Table 2**).

Table 2: Medicinal uses of different parts of *A. unedo* plant.

Plant organ	Medicinal uses
Roots	Gastrointestinal disorders, urological problems, dermatologic problems, cardio-vascular application, hypertension, cardiac diseases, diabetes, diuretic, anti-inflammatory, anti-diarrheal
Bark	Gastrointestinal disorders, urological problems, dermatologic problems, cardiovascular application
Fruits	Gastrointestinal disorders, urological problems, dermatologic problems, kidney diseases, cardio-vascular application
Leaves	Gastrointestinal disorders, urological problems, dermatologic problems, cardiovascular application, kidney diseases, hypertension, cardiac diseases, diabetes, antihaemorrhoidal, diuretic, anti-inflammatory, anti-diarrheal

Adapted from Oliveira *et al.*, 2011.

Indeed, *Arbutus unedo* represents a great asset to human health. The leaves, although rich in phenolic compounds and antioxidants, are yet to be exploited (Erkekoglou *et al.*, 2017). In 2012, Ribeiro *et al.* demonstrated that *A. unedo* leaves contain several lectins with high affinity to melezitose, methyl-α-D-mannopyranoside, D-glucosamine, D-galactosamine, GlcNAc, methyl-α-D-glucopyranoside, D-mannose, D-maltose, L-fucose and sucrose (Ribeiro *et al.*, 2012; Oliveira *et al.*, 2019). These lectins were found to bind to membrane receptors of HT29 colorectal cancer cells, inducing cell death and inhibiting cell migration and MMP-2 and MMP-9 activity (Oliveira *et al.*, 2019).

A lectin from the strawberry tree was purified by Oliveira (2017). This lectin was identified to be resistant to low pH of approximately 2.0, which spikes interest in pharmacological research since human stomach pH ranges from 1.5 to 3.5.

In vivo research by Monteiro (2017) found that the purified lectin of *A. unedo* has an anti-inflammatory effect on colitis in mice. The lectin presented a specificity for the glycosylation pattern at the surface of the diseased colon cells, for it did not affect normal cells.

Arbutus unedo lectins as anticancer and anti-inflammatory agents in both prevention as well as targeted therapy is then a strong possibility.

4 AIMS

Pancreatic cancer is still one of the deadliest malignancies with a 5-year survival rate below 10%, for it is prone metastasize. Furthermore, it is detected at a non-resectable stage and the therapeutic strategies turn out not to be effective. It is, henceforth, imperative to investigate further alternative strategies, in order to implement early detection and different therapies. Lectins are a considerable asset in those fields due to their carbohydrate binding characteristics, by which they can recognize different cells by decodifying membrane surface sugars.

Alterations in glycosylation are inherent to carcinogenesis, proliferation, invasion, and metastasis. Lectins, namely plant lectins, not only are able to specifically bind to these aberrant glycans and identify them as biomarkers, but also, they can revert the tumoral process by inducing cell death.

Accordingly, species from Mediterranean endemic flora have been studied as a source of antitumor lectins, particularly *Arbutus unedo* leaves. Extracts of these leaves have been found to possess lectins with anti-inflammatory and antitumor properties against HT29 colorectal cancer cells.

With this in consideration, the aim of this research, in a first approach, is to analyze the proteomic and glycoproteomic profiles of a total protein extract of *A. unedo* leaves and a semi-purified fraction unadsorbed by Q-Sepharose column of the protein extract, followed by the search for lectin activity. Membranes isolated from a pancreatic ductal adenocarcinoma cell line, MIA PaCa-2 (derived from a primary tumor at the body/tail of the pancreas), were also characterized in order to find new insight on their proteome and glycome. The protein fractions were then tested against the MIA PaCa-2 cell membranes to investigate the affinity to the glycosylated receptors at cell surface. Furthermore, it is intended to assess, in vitro, the antitumor activity of both protein fractions from the strawberry tree leaves on the mentioned cell line.

II. MATERIALS AND METHODS

1 BIOLOGICAL MATERIAL

In order to carry out this project, leaves from an endemic species, *Arbutus unedo* (strawberry tree), were used. Leaf samples were stored at –80 ºC until utilization.

Cells from a human ductal epithelial pancreatic cell line, MIA PaCa-2 (ATCC), were also used in this work.

Rabbit blood, in sodium citrate, for hemagglutination studies was purchased from Probiologica.

2 METHODOLOGIES

2.1 CHARACTERIZATION OF *ARBUTUS UNEDO* LEAF EXTRACTS

2.1.1 *Arbutus unedo* total protein leaves extraction

The total protein extract of leaves from *Arbutus unedo* was obtained through an optimized method. This methodology includes an extraction buffer containing 5% (v/v) glycerol and 1% (w/v) Triton X-100 (Silva and Souza, 2009). Because this method comprises Triton X-100, it has a much higher extraction ability than other methods, since Triton X-100 is a non-ionic detergent that dissolves cell membranes and solubilizes proteins, while preserving their native conformation. In the original protocol uses 4% (v/v) of this detergent however, this concentration was adjusted to 1% (v/v) to minimize cell lysis in assays involving erythrocytes and MIA PaCa-2 cells.

This process began by pulverizing 10 g of *A. unedo* leaves with liquid nitrogen, after discarding the stems and senescence zones. To the obtained powder it was added 250 mg polyvinylpolypyrrolidone (PVPP) per 500 mg of fresh leaves, in order to retain phenolic compounds. It was also added a protease inhibitor cocktail tablet (Roche), EDTA free to prevent the chelation of Ca^{2+} and Mg^{2+} ions necessary for the lectin activity, per 10 mL of extraction buffer (Tris-HCl 50 mM, pH 8.0, containing 5% (v/v) glycerol, 100 mM KCl and 1% (w/v) Triton X-100. The extraction per se was accomplished by adding 1 – 5 mL of extraction buffer per 0.5 g of leaves. Afterward, the resulting slurry was centrifuged at 14 000 *g* for 30 min, at 4ºC. The supernatant was then filtered through a Miracloth screen (Calbiochem) and desalted with PD-10 columns (GE Healthcare) equilibrated beforehand in Tris–HCl 50 mM, pH 7.5 buffer. The eluates were combined, homogenized, the protein concentration (as explained in II.2.1.3) was determined, and the total extract stored at -80ºC until further use. The total extract will be denoted TE.

Lastly, the PD-10 columns were regenerated through H_2O washes interspersed by a solution containing 1% (w/v) Triton X-100 and another one containing 2 M NaCl. At the end the columns are washed with Milli-Q water so that the matrix is equilibrated with the latter.

2.1.2 Purification of leaf extract in a Q-Sepharose column

The total protein extract from *A. unedo* leaves was subjected to a semi-purification through an anion exchange column of Q-Sepharose gel (quaternary ammonium salt from GE Healthcare). The resulting protein fraction will be referred to as SP.

A 20 mL of TE (obtained as described in II.2.1.1) in Tris-HCl 50 mM, pH 7.5 was injected in a Q-Sepharose column previously equilibrated with the same buffer. Pump A was also equilibrated with the same buffer, whereas pump B was equilibrated with Tris-HCl 50 mM, pH 7.5, 1 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$. The protein separation was performed in ÄKTA™

FPLC (Fast Protein Liquid Chromatography). The unadsorbed fraction was pooled and stored at -80 ºC to be used in the following assays.

Elution of the adsorbed proteins from the column was achieved by a step-by-step gradient and were also gathered and stored at -80 ºC. The chromatographic fractionation was done at a flow rate of 1 mL/min of buffer solution, with continuous reading of absorbance at 280 nm. The eluates were collected by monitoring of the chromatogram. All eluates were assayed for hemagglutination activity.

2.1.3 Determination protein concentration

The protein concentration was determined using the Bradford Method (1976). This technique comprises of a colorimetric assay that consists of the interaction of the dye Coomassie Brilliant Blue G250, in a solution of methanol and phosphoric acid (Bradford Reagent), with the basic or aromatic amino acid side chains. The red color of the reagent is converted into blue as the dye anionic form stabilizes (Ninfa *et al.*, 2010). The color is more intense the greater the concentration of protein present in the sample. This change in color is quantified spectrophotometrically by measuring the absorbance at 595 nm. This process is fast, sensitive, and highly reproducible.

The assay is performed considering a calibration curve. The curve is established in a 96-well plate, with a U-shaped bottom, using Bovine Serum Albumin (BSA) as the standard, in concentrations between 0 and 15 µg. The points of the curve were assayed in triplicate as well as the total extract sample. It was also added on the plate a sample of Q-Sepharose unadsorbed fraction of *A. unedo* leaves (Oliveira *et al.,* 2019). Lastly, Bradford reagent (BioRad) was added in all wells and incubated for 15 min, protected from light, at room temperature. Absorbance readings were measured at a wavelength of 595 nm (Software KinetiCalc – KC 4) and the protein concentration in the samples interpolated with the calibration curve.

2.1.4 Polypeptide profile evaluation

To analyze a protein sample, it is crucial to determine its electrophoretic profile. In order to do so, the proteins were separated on a polyacrylamide gel electrophoresis under reducing and denaturing conditions (SDS-PAGE-R) (Oliveira *et al.*, 2019).

The biological molecules, namely proteins and nucleic acids own electrical charge, which is conditioned by the surrounding environment, in particular by H^+ ion concentration (Plummer, 1987). Electrophoresis exploits this characteristic promoting separation of the molecules throughout a gel matrix, on the principle that an electrically loaded molecule, upon potential difference, moves toward the pole with a charge opposite to its own (from the cathode to the anode). The molecules, in this case the proteins, are subjected to an electric field promoting their mobility based primarily on their charge and size. Hence, a migratory activity according to the molecular weight of the proteins implies that all proteins have a constant charge density/mass quotient. This is attained by adding denaturing agents to the sample, e.g., SDS (sodium dodecyl sulphate), an anionic detergent that grants polypeptides a negative charge. This detergent can strongly bind to the proteins due to its apolar behavior (Robyt and White, 1990). This binding between SDS and proteins promotes the dissociation of protein oligomers and the disruption of the protein secondary structure. Furthermore, the saturation of the protein with SDS also leads to the camouflage of each protein charge which makes the migration through the gel to be entirely dependent on their molecular weight (Plummer, 1987). β-Mercaptoethanol (β-ME) is also used to reduce possible disulfide bonds (Robyt and White, 1990).

To improve the resolution, the Laemmli SDS-PAGE system (1970) was used where a discontinuous gel (16 cm × 18 cm × 0.75 mm) with an upper stacking gel, that concentrates the proteins, and lower resolving gel, which separates them (Gallagher, 2006). These gels have different pH values and polyacrylamide concentrations: the upper gel has a lower percentage of acrylamide and a more acidic pH to make the migration of the proteins quick and concentrate them into a tight band before entering the separation gel (Gallagher, 2006; Brunelle & Green, 2014). The separation gel consists of a 17.5% (w/v) acrylamide matrix, 0.1% (w/v) bisacrylamide, 375 mM Tris-HCl buffer, pH 8.8, 0.03% (w/v) ammonium persulfate (APS), and 0.05% (v/v) tetramethylethylenediamine (TEMED). The concentration gel entails 5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 125 mM Tris-HCl buffer, pH 6.8, 0.1% (w/v) APS, and 0.05% (v/v) TEMED.

Upon the vertical system assembly, the separation gel solution was carefully added, avoiding the formation of air bubbles, reaching approximately 2 cm bellow the top of the glass. Butanol (1 mL) was then added to the top of the gel to aid even polymerization, being discarded after this. The stacking upper gel was then added to the vertical system, where the comb was previously placed in order to form the wells of the gel. Once the polymerization is finished the gel apparatus was placed inside the electrophoresis chamber (HOEFER SE 600 Ruby, Amersham Biosciences) and added the electrophoresis buffer (200 mM glycine, 25 mM Tris-Base, 0.2% (w/v) SDS) immersing the electrodes. The SDS-PAGE was executed under reducing conditions, therefore the sample buffer contained 100 mM β-ME. In cases where there was a large volume of samples, these were precipitated with acetone (1:5) for 30 min at -20 ºC, followed by a centrifugation (Microcentrifuge VWR CT15RE), and the resulting precipitates were solubilized in sample buffer. All samples were denatured at 100 ºC for 4 min and applied to the gel. It was applied 40 µg of total extract (TE) and 30 µg of semi-purified fraction (SP). In the gel were also loaded 3 µL of molecular weight (MW) marker (BioRad), with a range between 10 and 250 kDa. The gel was then subjected to an electric current of 70 mA and a potential difference of 220 V (Pharmacia Gene Power Supply EPS 600) per gel unit. The run is over when the tracking marker (bromophenol blue) reaches the end of the gel.

2.1.5 Polyacrylamide gel staining by silver nitrate

The polyacrylamide gel was stained by silver nitrate ($AgNO_3$), according to Blum *et al.* (1987). This method is highly sensitive, about 100 times more sensitive than Coomassie Brilliant Blue R-250 (Creste *et al.,* 2001) and relatively low-priced (Chevallet *et al.,* 2006). It is based on the principle that proteins have the ability to bind to silver ions (Ag^+) that under the right conditions – in an alkaline environment, in the presence of sulfur from methionine and cysteine residues – can be reduced forming a stained complex. So, after the electrophoretic run, the gel matrix is saturated with Ag^+ ions, and since these binds to amino groups, any ion that does not bind to any amino acid is to be removed during the washing steps of the staining process.

The method started by immersing the gel in the fixing solution (50% (w/v) methanol, 12% acetic acid and 0.02% formaldehyde (H_2CO)) for at least 20 min. This step is crucial to prevent the diffusion of the already separated polypeptides, and to remove interfering substances. Three consecutive 10 minutes washes were performed with a 50% (v/v) ethanol solution and the gel was then incubated for 1 min with the pre-treatment solution (0.02% (w/v) sodium hyposulfite ($Na_2S_2O_3{\cdot}5H_2O$)). Afterwar, the gel was washed with Milli-Q water thrice, 20 s each, discarding the water after each wash. The staining solution (11 mM $AgNO_3$, 0.03% (v/v) formaldehyde) was then added for 10 min, followed by another 2 washes of 20 s with water. The gel was immersed in the developing solution (566 mM $NaCO_3$, 0.02% (v/v) formaldehyde, 16 µM $Na_2S_2O_3{\cdot}5H_2O$) for as long as necessary for the revealing of the bands/spots. Lastly, the latter solution was discarded and added a 50% (v/v) methanol and 12% (v/v) acetic acid solution to

arrest the staining reaction. Every step was performed in an orbital shaker, under slow agitation. From this point, after photographic capture, the gel may either be rejected or maintained by drying between two cellophane sheets upon dehydration, for around 20 min, in a 50% (v/v) methanol solution.

2.1.6 Glycoproteomic evaluation

The glycan moiety of a protein extract is key to its characterization since it allows for glycoprotein identification. A high-resolution gel (SDS-PAGE-R) is needed beforehand so that the polypeptides present in the sample are transferred through semi-dry electroblotting onto a nitrocellulose membrane that allows the detection of immobilized proteins (Kurien & Scofield, 2006).

2.1.6.1 Protein immobilization on nitrocellulose membrane

In this procedure a nitrocellulose membrane with 0.2 µm pores (Amersham Protran 0.2 NC 200mm×4m) was used. This membrane is composed of nitro groups bound to cellulose providing a matrix with high affinity to proteins. The electrotransferring itself is based on the same principle of electrophoresis, an electric field that varies according to the thickness of the gel.

In this particular case, a 0.75 mm gel was loaded with 12 µL of MW, 200 µg of TE and 100 µg of SP. Immediately after the electrophoresis, the gel, the membrane and 8 sheets of 3MM paper were incubated in transferring buffer (48 mM Tris-Base, pH 8.3, 39 mM glycine, 20% (v/v) methanol, 0.04% (w/v) SDS). Subsequently, the 'sandwich' was assembled by placing four 3MM sheets over the positive electrode of the apparatus (Trans-Blot Semi-Dry Transfer Cell, BioRad), ridding the paper of air bubbles not to compromise the results, the membrane was placed over the paper, followed by the gel, and topped by the remaining 4 3MM sheets, removing the air bubbles in each layer. The negative electrode was placed over the sandwich and the electrotransferring was executed for 50 min, at 15 V (no electric current limit).

Once the transferring is finished, the membrane is incubated for 5 min, under slow agitation, in a fixing solution (10% (v/v) acetic acid, 25% (v/v) 2-propanol) so that the proteins are immobilized on the surface and accessible to further methods. The membrane was then washed with Milli-Q water and stained with a solution containing Ponceau S (0.1% (w/v) Ponceau S, 25% (v/v) acetic acid) in order to assess the efficiency of the transferring. The membrane can be stored sheathed in 3MM paper or subjected to a glycan detection.

2.1.6.2 Chemiluminescent glycan detection

The differentiation of the glycoproteins immobilized on the nitrocellulose membrane was performed according to what was proposed in 1985 by Faye & Chrispeels.

After fixing the proteins onto the membrane, the latter was washed for 1 min in TBS (20 mM Tris, pH 7.4, 0.5 mM NaCl) and then blocked in TTBS (TBS, 0.1% (w/v) Tween-20), for 1 h.

The membrane was incubated with concanavalin A (Con A), 25 µg/mL, in TTBSS (TTBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$). The membrane was washed 4 times, 10 min each, with TTBSS followed by incubation with the conjugate avidin-peroxidase (Sigma), 50 µg/mL, in TTBS, for 1 h. Next, the membrane was subjected to another 4 washes (10 min) with TTBSS and a 5-minute wash with TBSS (TBS, 5 µL $CaCl_2$ 1 M, 5 µL $MgCl_2$ 1 M). Finally, the membrane was incubated with an enhanced chemiluminescent substrate SuperSignal West Femto (Thermo Fisher) and the results were observed through ChemiDoc XRS+ (BioRad) imaging system.

2.1.7 Lectin detection

To confirm the presence of lectins in the total extract and in the semi-purified fraction of *A. unedo* leaves (protein fraction not adsorbed by Q-Sepharose column), a hemagglutination assay was performed.

The erythrocyte is the primary model to study the animal cell membrane, for it is devoid of nucleus and organelles. Among the different constituents of the membrane, glycosylated receptors are involved in cell-cell communication, immunological recognition, and cell adhesion (Salmon *et al.,*1984). Blood group is species specific and is defined through the antigens present at the surface of erythrocyte membrane. These can be proteins, glycoproteins, or glycolipids. Blood group polymorphisms can be represented by the absence/presence of a macromolecule (e.g., Rh factor), of an amino acid (e.g., Fy^a/Fy^b), or by disparities between monosaccharides (e.g., AB0) (Daniels & Bromilow, 2007).

Hemagglutination is defined as the agglutination of erythrocytes visible to the naked eye. Lectins have this ability, so it is possible to detect the presence of these proteins through hemagglutination assays (Ribeiro *et al.,* 2012).

The protein samples being studied were subjected to a hemagglutination assay, by means of a 4% (v/v) rabbit erythrocyte solution in saline.

2.1.7.1 Erythrocyte solution

The rabbit blood was collected by Probiológica through cardiac puncture in the presence of anticoagulant sodium citrate. The technique began with a centrifugation of 5 mL of blood (3000 rpm, 6 min, 13 ºC) in order to separate the plasma from the sediment. The latter was washed 3 times with saline solution (0.9% (w/v) NaCl) previously autoclaved (40 mL per 5 mL of whole blood), through 3000 rpm centrifugation, for 6 min, at 13 ºC, discarding the supernatant. Afterward the pellet, which occupied a volume of 4 mL, was resuspended in 96 mL of saline solution. The resulting suspension was added 1% (w/v) trypsin (Sigma) and incubated at 37 ºC, for 1 h, under 120 rpm agitation. Subsequently, a new centrifugation was carried out as well as 3 washes similar to the ones performed earlier, to devoid the erythrocytes of trypsin, avoiding hemolysis. Finally, the pellet was resuspended in 96 mL of saline, thus obtaining the 4% (v/v) erythrocyte solution. This solution can used immediately or stored at 4 ºC (considered valid provided no signs of erythrocyte hemolysis are visible).

2.1.7.2 Hemagglutination assay

Hemagglutination activity was determined through microdilutions (serial dilutions in microplate) of the protein fractions (TE and SP) in saline, by means of a geometric progression of ratio 3, incubated with the 4% (v/v) rabbit erythrocyte solution obtained in II.2.1.7.1.

The hemagglutination activity is expressed in hemagglutination units (H.U.), which represents the minimal concentration of protein that exhibits hemagglutination activity and it is given by:

$$H.U. = \frac{[Total\ Protein]}{3^{n-1}} \qquad (1)$$

n = last dilution that still exhibits hemagglutination activity (Ribeiro *et al.,* 2012)

The assay was performed in a round bottom 96-well plate. To every well, 70 µL of saline solution containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was deposited. 70 µL of each protein sample containing 100 µg and 200 µg was added to the first well. From the first well 70 µL was removed and added to the saline on the second well, and so on until the end of the row.

To every well, 70 µL of the erythrocyte solution was added. In parallel, a negative control (C-) row of saline and a positive control (C+) row of 1 mg/mL concanavalin A was also assayed. The plate was incubated at 37 ºC, for at least 30 min.

The results were then analyzed and compared to C- and C+. A positive result corresponds to the adherence of the erythrocytes to the bottom of the well (**Figure 12.A**) being visible the formation of a coating that may even retract. Contrarily, a negative result is characterized by a red blood cell precipitate in a colorless supernatant (**Figure 12.B**).

Figure 12: Hemagglutination patterns. **A** – Presence of hemagglutination activity (positive). **B** – Absence of hemagglutination activity (negative).

2.2 Characterization of MIA PaCa-2 cell membranes

2.2.1 Cell culture

All cell manipulations concerning culture maintenance, trypsinization, plating and extract incubations were carried out under sterile conditions in a laminar flow chamber. Cell culture maintenance was performed in cell incubators at 37 °C under a humidified atmosphere of 5% (v/v) CO_2.

The MIA PaCa-2 cell line of pancreatic ductal adenocarcinoma was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Sigma), 2 mM Glutamine 0.5% (v/v), Penicillin (2×10^4 IU/mL) and 34 mM Streptomycin (Pen-Strep) and Amphotericin B 0,001% was filtered through a sterile microfilter (GVS Polyethersulfone Membrane 0.22 µm). The stock culture was maintained in T-flasks.

When cells reached the confluence of 80 – 100%, the medium was removed, and ATV (trypsin-EDTA) added to the flask for 10-15 min at 37 °C. The cellular medium was then removed and centrifuged at 500 *g*, for 5 min, and the supernatant discarded leaving only the cell corresponding volume (pellet), which was added approximately 2.7 mL of medium and homogenized. An aliquot of the cell homogenate was subjected to a colorimetric cell viability assay using trypan blue added to a Neubauer's chamber, in order to count the cells and calculated the necessary volume for subsequent assays. This was followed by dilution with DMEM and plating or added to 95% FBS and 5% DMSO to cryopreserve the cells for future use.

2.2.2 MIA PaCa-2 cell membrane isolation

The MIA PaCa-2 cell membranes were purified according to the protocol described in 2008 by Vercoutter-Edouart *et al.*.

The cells were unfrozen and added HES buffer (20 mM HEPES, pH 7.4, 250 mM Sucrose) and centrifuged for 10 min at 750 *g*, 21 ºC to remove the dimethyl sulfoxide medium. This process was repeated, and the pellet was resuspended in HES buffer with cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche). Cell lysis was achieved by cryolysis, comprising freeze-thawing in a total of four times for 30 min, at -20 ºC, and by undergoing 20 min of sonication with ultrasound, followed by a centrifugation at 960 *g* for 10 min, at 4°C, with the discard of the pellet, to remove non-lysed cells and nuclei. The final supernatant will be ultracentrifuged

(Beckman J2 -21M/E, Rotor JA 20 000) at 28 000 rpm for 45 min, 4°C (Vercoutter-Edouart *et al.,* 2008; Oliveira *et al.,* 2019). The pellet containing the cell membranes, will be solubilized in 2 mL of physiological saline (0.9% NaCl), containing 2 mM of $CaCl_2$ and 2 mM of $MgCl_2$ and will be divided into aliquots containing 1.3 mg protein determined by the Bradford method (1976), explained in II.2.1.3, and kept at -80°C, until further use.

2.2.3 Polypeptide profile evaluation

In order to characterize the proteomic profile of the MIA PaCa-2 cell membranes, a polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out in reducing (R), with 100 mM β-ME that promotes the reduction of possible disulfide bonds, and non-reducing (NR) conditions, as described by Oliveira *et al.* (2019). This method was executed similarly to II.2.1.4.

The membrane samples (13 µg) were incubated with sample buffer R and NR and denatured for 3 min at 100ºC. The treated samples were loaded into the gel and run at an electric current of 70 mA and a potential difference of 220 V.

The SDS-PAGE was stained through the silver nitrate method as described in II.2.1.5.

2.2.4 Glycoproteomic evaluation

A glycoprotein detection assay is crucial when characterizing cell membranes. Glycoprotein immobilization was done according to II.2.1.6.1.

Afterward, Faye and Chrispeels' glycoprotein detection methodology was applied. This method counts on concanavalin A (Con A), a lectin from *Canavalia ensiformis* (jack bean) to identify glycoproteins separated by electrophoresis, visualized due to a colored, insoluble reaction product of peroxidase (Faye & Chrispeels, 1985).

The membrane was washed in TBS (20 mM Tris, pH 7.4, 0.5 mM NaCl), for 1 min and then incubated in a blocking solution, TTBS (TBS, Tween-20 0.1% (w/v)), during 1 h. Afterward, the membrane was incubated, for 1 h, with TTBSS (TTBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$) containing 25 µg/mL Con A (Sigma), followed by 4 washes of 10 min each, with TTBSS. It was then incubated with 50 µg/mL horseradish peroxidase (Sigma) in TTBSS, for 1 h, washed another 4 times with TTBSS and once with TBSS (100 mL TBS, 100 µL $CaCl_2$ 1 M, 100 µL $MgCl_2$ 1 M). Finally, a dyeing solution (0.05% 4-Chloro-1-naphthol dissolved in 1.6% (v/v) methanol, 83.3% (v/v) TBSS e 0.1% (v/v) H_2O_2 30%) was added. The results appeared after a few minutes.

2.2.5 Two-dimensional proteomic analysis

A proteomic analysis of the MIA PaCa-2 cell membranes was performed, specifically a two-dimensional (2D) SDS-PAGE. It is a method with high resolution and sensitivity that separates proteins according to their isoelectric point (pI) by isoelectric focusing (IEF) in the first dimension, and to their molecular weight (MW), in the second dimension, by a polyacrylamide gel electrophoresis (SDS-PAGE-R) (O'Farrell, 1975; Celis and Gromov, 1999). The first dimension requires a polyacrylamide gel strip with a stable pH gradient, where the protein sample is placed and subjected to an electric field. The sample is freed of salts, detergents, phenols, nucleic acids and polysaccharides, compounds that compromise the migration of the polypeptides. Each protein migrates to and focuses on its corresponding pI (Vesterberg, 1972). The second dimension is an SDS-PAGE-R that does not require a concentration gel since the strip is placed directly over the separation gel.

The cell membrane samples (1400 µg for silver nitrate staining and 2000 µg for glycan detection) were treated by undergoing 3 to 4 washes, with Milli-Q water, by ultrafiltration in centricons with a 10 kDa cut-off (Microsep Advanced Centrifugal Devices, PALL). The samples were then precipitated with acetone and the pellets resuspended in solubilization buffer (7 M urea, 2 M thiourea, 2% (v/v) NP-40, 1% (w/v) DTT) and added IPG buffer (4 µL of IPG per 200 µL of solubilization buffer, for 11 cm IEF strips). The samples were then centrifuged at 21,500 *g*, for 10 min (VWR CT15RE). The protein content of the sample destined to the IEF strip has to be compatible with the size of the strip, type of sample, the staining method, and the goal of the study. Immobilin impregnated (IPG – Immobilized pH gel) 11 cm length 4-7 pH gradient IEF strips (BioRad) were used. The protein samples were loaded into the aisles of the IPGphor carrier and the IEF strips was placed on the samples guaranteeing that the gels and the samples were in contact. The strips were placed according to the respective poles of the focusing device holder (IPGphor). The carrier was placed in the focusing apparatus and the rehydration step was schedule overnight for 12 h at 30 V.h. Once the rehydration was finished, six steps followed: Step 1 - 250 V.h, 1 h; Step 2 - 500 V.h., 2 h; Step 3 - 1000 V.h, 2 h; Step 4 - 2500 V.h, 3.5h; Step 5 -8.000 V.h, 1h; Step 6 - 8,000 V.h, 25 min. Upon IEF completion the strips were incubated, for 15 min, at room temperature, under gentle agitation, in stabilization buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 305 (v/v) glycerol, 1% (w/v) dithiothreitol (DTT)). The latter solution was discarded, and the strips were incubated with a similar solution, but the DTT replaced by 2.5% (w/v) iodoacetamide. Thereafter, the strips were placed over a 17.5% (w/v) polyacrylamide gel, 1.5 mm thick, and sealed with a 0.5% (w/v) low electroendosmosis (EEO) agarose solution in Tris-HCl (50 mM), pH 8.8, containing 0.002% (w/v) bromophenol blue. 6 µL (silver staining) and 24 µL (glycodetection) of MW were loaded into a well molded with the agarose solution. The electrophoretic conditions were initially 15 mA and 220 V until the sample entered the gel (approximately 15 min) and switched to 30 mA and 220 V throughout the rest of the run. The gel with 1400 µg of cell membrane sample was subsequently stained by silver nitrate as described in II.2.1.5. and the gel destined to glycodetection was immediately electrotransferred onto a nitrocellulose membrane in order to profile the glycoproteins as depicted in II.342.1.6. In this case the transferring was performed during 65 min, 15 V.

2.3 *ARBUTUS UNEDO* LECTINS BINDING TO MIA PACA-2 CELL MEMBRANES

2.3.1 Incubation of MIA PaCa-2 cell membranes with the total extract and the unadsorbed Q-sepharose fraction of *A. unedo* leaves

The whole protein extract (TE) and the unadsorbed by Q-sepharose fraction (SP) from *A. unedo* leaves were treated in PD-10 columns with a saline solution containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$.

1200 µg of TE and 500 µg of SP were incubated with 1400 µg of MIA PaCa-2 cell membranes (CM) for 30 min, at 25 ºC, shaken gently. Thereafter, two centrifugations of 20 000 rpm, for 20 min, at 4 ºC, were accomplished (Beckman J2 -21M/E, Rotor JA 20.000). After each centrifugation the supernatant, containing the proteins that did not bind to the membranes, was discarded and 10 volumes of saline solution with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ were added for solubilization of the pellet. After the last centrifugation, the pellets were solubilized in 200 µL of the saline. In parallel, a blank assay was performed, replacing the samples by saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$.

The resulting protein content was quantified through Bradford Method as described in II.2.1.3.

2.3.2 SDS-PAGE analysis

The SDS-PAGE-R analysis was similar to II.2.1.4. The gel was loaded with 13 µg of MIA PaCa-2 cell membranes, 40 µg of TE and 30 µg of SP as comparison with the incubates. 13 µg of both CM+TE and CM+SP protein solutions resulting from the incubations were added, as well as 3 µL of protein ladder. After electrophoresis the gel was stained according to II.2.1.5.

2.4 ANTITUMOR ACTIVITY EVALUATION OF WHOLE EXTRACT AND SEMI-PURIFIED LECTINS OF *A. UNEDO* LEAVES IN MIA PACA-2 CELLS

2.4.1 Cell migration studies

2.4.1.1 Wound healing assay

In order to assess the effect of the samples on cell migration, a wound healing assay was performed. This *in vitro* assay relies on the ability cancer cells have to create cell-cell contacts in order to close the gap left by scratching the confluent cell monolayer. Monitoring of cell migration is accomplished by collecting photographs of different time points throughout the assay.

In a 24-well plate, 1 mL of a suspension of MIA PaCa-2 cells in complete medium was added to each well (2×10^5 cells/mL). The plate was incubated at 37 ºC, 5% CO_2, for 24 h or until the cells reach a confluence of 90% or higher. At this point, using a 200 µL pipette tip (P200), a scratch in the monolayer was created. After mild agitation, the medium was discarded, the wells were washed with PBS (0.8% (w/v) NaCl, 0.02% (w/v) KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) to remove non-adherent cells and added 1 mL of fresh medium. It was added 25, 35, 50 and 75 µg of whole extract to the plate, as well as 35 and 75 µg of semi-purified lectins of *A. unedo* leaves, removing the equivalent volume of medium from the respective well. A negative control was also used where it was added 100 µL of saline to a scratched well containing 900 µL of medium. The samples and controls were assayed in triplicates. The plate was placed in an incubator (Shel Lab CO2 Series, Shledon Mfg, Incorporated) at 37 ºC, with 5% CO_2 injection, for 48 h. The results were analyzed microscopically (Leica) and recorded photographically. The width of the scratch was measured (triplicate) at the beginning (0 h) and the end (48 h) of the experiment.

2.4.1.2 Determination of gelatinolytic activity

On account of cell migration inhibition after 48 h of incubation with TE and SP, the extracellular media was collected and subjected to gelatinolytic activity assessment assays.

2.4.1.2.1 Zymographic study

The zymograms were assembled using gelatin co-polymerized in 12.5% (w/v) acrylamide (0.1% (w/v) bisacrylamide, 370 mM Tris-HCl, pH 8.8, 1% (w/v) gelatin, 0.03% (w/v) APS, 0.03% (v/v) TEMED). The samples (cell secretome of wound-healing assay) were incubated in non-reducing buffer (63 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue) for 10 min, at room temperature, and applied to the gel. The gel was also loaded with 20 µL of MMP-9 and MMP-2 diluted 1:650 and 1:500, respectively. The concentration gel is comprised of 5% (w/v) acrylamide, 0.16% (w/v) bisacrylamide, Tris-HCl 700 mM, pH 6.8, 0.1% (w/v) APS and 0.5% (v/v) TEMED. The electrophoretic run was done at 20 mA, 200 V (Pharmacia/LKB, EPS 500/400), in a Mini Protean vertical system (BioRad) with electrophoresis buffer (25 mM Tris-Base, 200 mM Glycine, 0.02% (w/v) SDS).

In parallel, a 12.5% acrylamide control gel was made similarly, replacing the gelatin with Milli-Q H_2O. It was added 3 µL of molecular weight marker and 20 µL of MMP-2 diluted 500 times.

Once the run was concluded, the gels were rinsed with water and subjected to three 1 hour long washed with a 2.5% (v/v) Triton X-100 solution under gentle agitation, to remove the SDS activating the enzymes. Next, the gels were incubated in zymography buffer (50 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.01% (w/v) sodium azide), for 48h at 37 ºC, to develop proteolysis. Lastly, the gels were stained with Coomassie Brilliant Blue G-250 (CBB-250) (0.5% (w/v) Coomassie G-250, 30% (v/v) ethanol, 10% (v/v) acetic acid), during 24 h. Zymography resulted in transparent bands against a blue background marked the gelatinase activity of each proteinase (Toth *et al.*, 2012).

2.4.1.2.2 DQ-gelatin assay

The quantification of the gelatinolytic activity is possible thanks to a dye-quenching (DQ) assay consisting in adding to the sample a quenched fluorogenic gelatin, which is converted into fluorescent peptides once it is degraded. So, the inhibitory activity of the sample is known through the measurement of the fluorescence produced.

DQ-gelatin (Invitrogen, Carlsbad, CA, USA) was dissolved in water at 1 mg/mL. All solutions/dilutions were prepared in assay buffer containing 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 secretome was loaded (100 µL) into the plate and the DQ-gelatin, diluted 1:80 in assay buffer, was also added. The plate was incubated for 30 min at 37 ºC away from light. Fluorescence levels were measured (λ_{ex} 460 nm/ λ_{em} 528 nm). All data was corrected by the subtraction of their corresponding negative controls.

2.4.2 Cell viability

2.4.2.1 MTS metabolism assay

Cell viability was determined by measuring MTS metabolism in MIA PaCa-2 cells (5×10^3 cells per well), in a 96-well plate, exposed to 400 µg/mL, diluted serially (1:2) throughout 10 wells, of the total protein extract (TE) and the unadsorbed by Q-Sepharose fraction (SP) of *A. unedo* leaves and with saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ as a control. After 72h, the medium containing the samples was removed and added a solution of 100 µL of culture media, 19 µL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), and 1 µL of an electron coupling reagent, phenazine methosulfate (PMS) (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega). Metabolically viable cells reduce, via dehydrogenase enzymes, MTS into a formazan product that is soluble in culture medium turning it into a brown color directly proportional to the number of viable cells in culture. Then the cells were then incubated at 37 ºC for 20 min to 1 h. Changes in absorbance were measured at a wavelength of 490 nm, using a Model 96 microplate reader (GLOMAX, Promega).

2.4.2.2 Trypan Blue assay

The dye exclusion assays are used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, for example trypan blue.

Two 6-well plates were used, one for each tested sample (TE and SP). In each well, 1.5×10^5 MIA PaCa-2 cells were seeded and incubated at 37 ºC, in a humidified atmosphere with 5% CO_2, for 24 h. Three concentrations of each sample (½ IC_{50}, IC_{50}, and 2 IC_{50}) were tested against the cells. For each plate, a negative control was performed with 1 µL/mL saline solution containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$. After 72 h of incubation in the previously mentioned conditions, the extracellular medium was collected and the cells 'trypsinized' and saved. To 10 µL of each cell suspension 90 µL of trypan blue was added and an aliquot was loaded into a hemocytometer proceeding with the cell count. In summary, cells attached to the monolayer and cells in suspension in the surrounding medium were counted with the aid of the trypan blue dye after exposure to ½ IC_{50}, IC_{50}, and 2 IC_{50} of total extract (TE) and semi-purified fraction (SP) of *A. unedo* leaves.

2.5 STATISTICAL ANALYSIS

For gelatinolytic activity and wound healing assay, all experiments were performed in triplicate the data was 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.

For viability assays, best-fit IC_{50} values from at least three independent experiments were calculated using GraphPad Prism software (version 5.00; San Diego, CA, USA), using the log inhibitor) *vs* response (variable slope) function.

III. RESULTS AND DISCUSSION

1 CHARACTERIZATION OF THE PROTEIN EXTRACT AND THE SEMI-PURIFIED LECTIN FRACTION OF *ARBUTUS UNEDO* LEAVES

In order to obtain the best protein extraction conditions, a few important steps must be taken: cell walls must be fractured, which is done by immersing the plant tissue in liquid nitrogen and grinding with a mortar and pestle; the cell membranes are also destroyed to release the proteins; the extraction process has to be freed of protease activity that may degrade useful proteins. To guarantee so, the extraction buffer not only has an alkaline pH of 8, but it also contains protease inhibitor cocktail.

As mentioned in II.2.1.1 proteins from *Arbutus unedo* leaves were extracted with an extraction buffer containing 5% (v/v) glycerol, and 1% (w/v) triton X-100 in order to enhance the extraction of the proteins (Silva and Souza., 2009), the extraction yield was determined by the Bradford method (II.2.1.3). This method was also used to determine, in parallel, the protein content of the non-adsorbed to Q-Sepharose fraction stock.

1.1 POLYPEPTIDE PROFILE ASSESSMENT

The polypeptide profile of both samples was evaluated after electrophoretic separation (17.5% acrylamide) in the presence of an anionic detergent, in a reducing environment motivated by the reducing agent β-mercaptoethanol (SDS-PAGE-R), described in II.2.1.4. This along with the silver nitrate staining allows the proper assessment of the protein separation by their molecular weight (MW).

It is visible, in **Figure 13.A**, that the total protein extract from *A. unedo* leaves (TE) presents a simple profile with molecular weights under 75 kDa, although the majority of the bands are located below the 37 kDa mark. Regarding the non-adsorbed to Q-Sepharose fraction (SP) has a simpler profile with eight distinct polypeptides ranging between 10 kDa and 37 kDa, emphasizing band around 15, 16, 23 and 32 kDa. The mentioned protein fraction was obtained through a purification process comprising of an anionic exchange chromatography, shown by Oliveira (2017), where the proteins extracted from strawberry tree leaves were injected in a Q-Sepharose column and the unadsorbed proteins on the column were pooled and stored at -80 ºC. This procedure rids the extract of peptides and polypeptides that are negatively charged molecules that bind to the Q-Sepharose matrix, thus explaining how elementary this profile is when compared to the TE.

1.2 GLYCOPROTEOMIC PROFILE EVALUATION

The detection of the glycoproteins that compose the total extract and the semi purified fraction was performed after electrophoresis (SDS-PAGE-R) followed by electrotransferring and immobilization onto a nitrocellulose membrane, as depicted in II.2.1.6.1, and posterior glycoprotein detection by Concanavalin A – Peroxidase. In this case, the detection was achieved through chemiluminescence (II.2.1.6.2).

As proposed by Faye and Chrispeels in 1985, the Concanavalin A – Peroxidase method recognizes only mannose and glucose glycoproteins, since Concanavalin A (Con A) specifically binds to α-D-mannopyranosyl and α-D-glycopyranoside residues as well as to spatially related residues. Therefore, some glycans are not detected by this method, Con A still has a great detection potential of most glycoproteins, nevertheless (Ribeiro *et al.,* 2012).

According to the methodology in II.2.1.6 TE and SP revealed a glycoprotein band with 12 kDa and TE showed a 27 kDa glycoprotein (**Figure 13.B**).

In comparison to the SDS-PAGE-R stained by $AgNO_3$, the glycoproteome analysis presents a less complex profile. By transposing the information given by the Con A – Peroxidase method onto the SDS-PAGE, it can be extrapolated that the bands marked with an arrow in **Figure 13** correlate to glycoproteins.

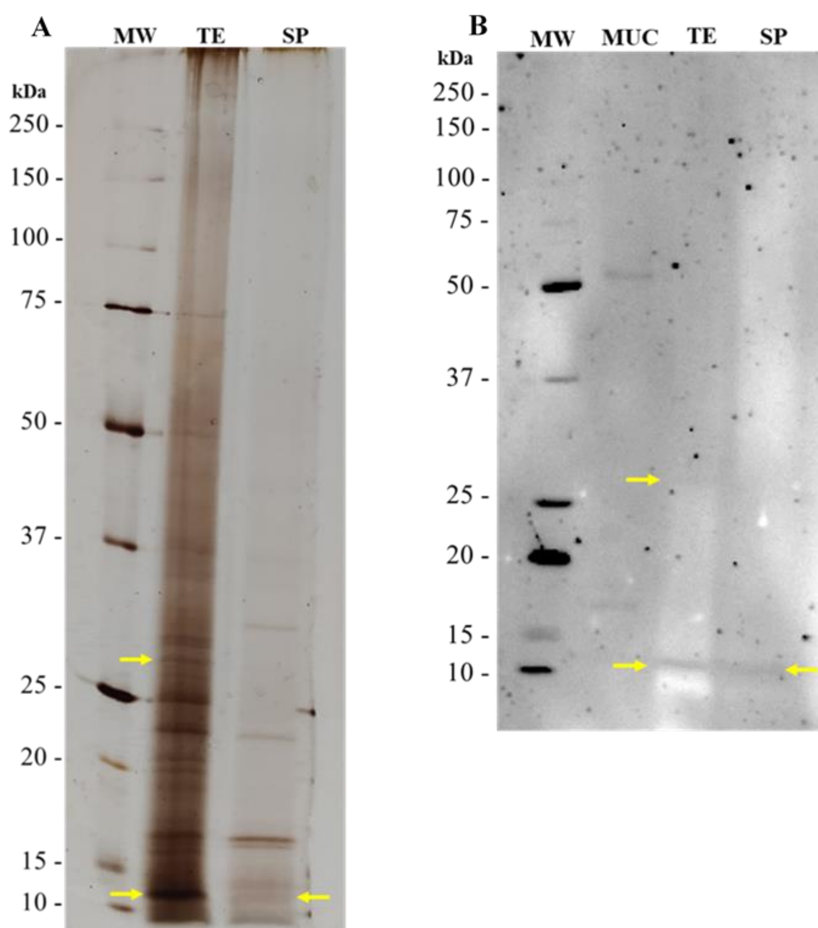

Figure 13: Characterization of protein extract (TE) and semi-purified protein fraction (SP) of *Arbutus unedo* leaves. **A** – SDS-PAGE R, 17.5% (w/v) acrylamide, stained with $AgNO_3$. It was applied 3 µL of protein ladder (MW), 40 µg of TE and 30 µg of SP. **B** – Glycoprotein detection by Con A-peroxidase method, in nitrocellulose membrane, revealed through chemiluminescence. It was applied 12 µL of the molecular weight marker (MW), 50 µg of the glycodetection control, mucin (MUC), 200 µg of TE and 100 µg of SP. The yellow arrows match the electrophoretic profile with the glycoprotein detection, that may be possible glycoproteins of interest.

1.3 LECTIN ACTIVITY

In order to determine the lectin behavior of the proteins present in the total extract and in the semi-purified fraction of *A. unedo* leaves, an assay with rabbit erythrocytes solution at 4% (v/v) was performed to assess the capacity of these samples to agglutinate the blood cells, revealing the lectin character of some of the polypeptides in the samples.

The hemagglutination activity was detected and the Hemagglutination Unit (H.U.) determined according to the equation $H.U. = \frac{[Total\ Protein]}{3^{n-1}}$ (1), of chapter II.2.1.7. This corresponds to the minimum concentration that is still associated with the hemagglutination of erythrocytes. There is an ideal stoichiometric relation between the erythrocyte membrane receptors and the lectin concentration present in the sample to exhibit this activity.

The assay was performed as described in II.2.1.7, for 100 µg and 200 µg of total extract (TE) and semi-purified extract (SP), serially diluted 1:3. A negative control (C-) with only saline solution (NaCl 0.9% (w/v); $MgCl_2$ 2 mM; $CaCl_2$ 2 mM) and a positive control (C+) of Con A (1 mg/mL).

The results indicate that the total protein extract from *A. unedo* leaves does not exhibit hemagglutination activity for either the tested concentrations (**Figure 14**). On the other hand, the semi-purified protein extract presents hemagglutination activity up to the dilution 1:3 when applied 100 µg of sample, revealing a H.U. of 33.3 µg and the dilution 1:9 when 200 µg, with a H.U. of 22.2 µg are applied. This means that the studied sample presents lectin activity as it binds to glycosylated receptors on red blood cells (**Table 3**).

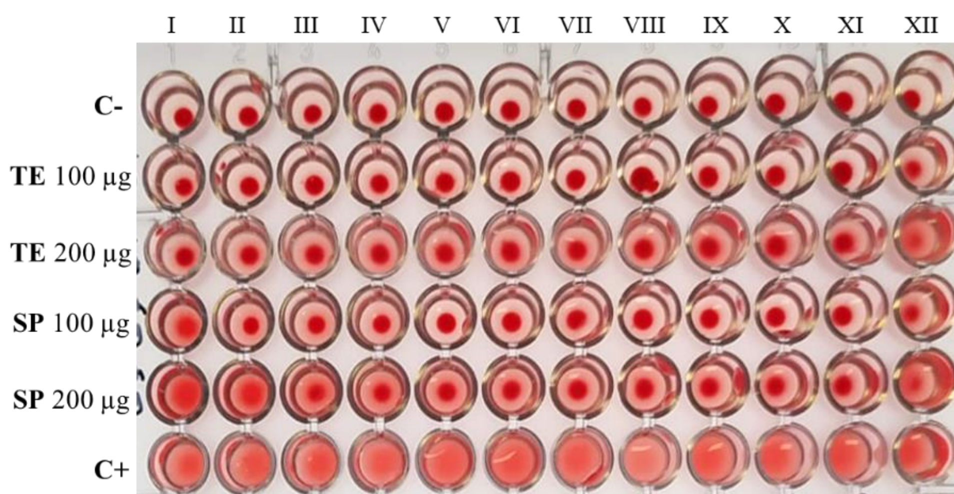

Figure 14: Hemagglutinating activity of total extract (TE) and unadsorbed by Q-Sepharose fraction (SP) of *Arbutus unedo* leaves. Two concentrations of each sample were assayed, 100 µg and 200 µg. Each sample was added to the first well and subjected to 11 serial dilutions of 1:3, followed by the addition of an erythrocyte solution 4% (v/v). The results were revealed after 30 min at 37 ºC. C- – negative control (saline); C+ – positive control (Con A).

The apparent incoherence between each sample, even though they are from the same species, suggests the possibility of having extracted from plant tissue, not only the desired lectins, but also natural inhibitors of these proteins. These inhibitors may be peptides or polypeptides that, when the extract was subjected to a purification process, were eliminated allowing the lectins to freely perform their activity. It is important to note that plant physiology and molecular biology changes in response to its environment fluctuations (e.g., daily and seasonal cycles, biotic and abiotic stress) (Gaspar *et al.,* 2002). Since lectins play a protective role in plants, it is only logical that the content of these proteins oscillates in response to external and internal conditions (Babosha, 2007).

Table 3: Assessment of lectin activity through determination of the hemagglutination unit (H.U.). H.U. was calculated through the quotient of protein concentration by 3^{n-1}, n being the last dilution which still exhibits hemagglutinating activity (Ribeiro *et al.,* 2012).

Protein Fraction	Concentration (µg)	Hemagglutination Unit (H.U.) (µg)
TE	100	-
	200	-
SP	100	33.3
	200	22.2

2 CHARACTERIZATION OF MIA PACA-2 CELL MEMBRANES

Disruption of the normal process of glycosylation of membrane associated glycoproteins, cell surface glycolipids and secreted glycoproteins is a characteristic of almost every cancer (Reis *et al.,* 2010). This event is called glycome aberration and is expressed in all tumor cell membranes.

MIA PaCa-2 cell line, from human pancreatic adenocarcinoma, was used in this study. This cell line is originated from a primary tumor (Ling *et al.,* 2013) located at the body and tail of the pancreas which makes it harder to diagnose leading to a poor survival rate.

Furthermore, body/tail pancreatic cancer has a high tumorigenicity, local infiltration, distant metastasis potential and chemoresistance (Ling *et al.,* 2013).

2.1 PROTEOMIC ANALYSIS

2.1.1 One-dimensional polypeptide profile

The polypeptide profile of the MIA PaCa-2 cell membranes was assessed through an SDS-PAGE, with and without the action of β-mercaptoethanol (R and NR, respectively), as can be observed in **Figure 15.A**. The analysis of the profiles stained with silver nitrate suggests the presence of disulfide bonds (NR) that were broken down into a more complex profile (R) with an increased number of polypeptide bands that are more prominent between 27 – 130 kDa. There are a few representative bands in the non-reducing (NR) profile that seem to be repeated in the reducing (R) one, around 15, 35, 50 and 58 kDa.

2.1.2 Two-dimensional polypeptide profile

To have a more in-depth characterization of MIA PaCa-2 membranes proteomics a two-dimensional electrophoresis (2D IEF/SDS-PAGE-R), followed by silver nitrate staining, was performed. This grants the possibility to separate polypeptides according to MW and isoelectric point (pI), revealing isoforms otherwise concealed.

It is visible, when analyzing **Figure 15.B**, that the crowded 2D SDS-PAGE is well distributed throughout the assayed pH range (4-7), however, it is more condensed in the acidic zone, having pIs of 4.5 – 6.

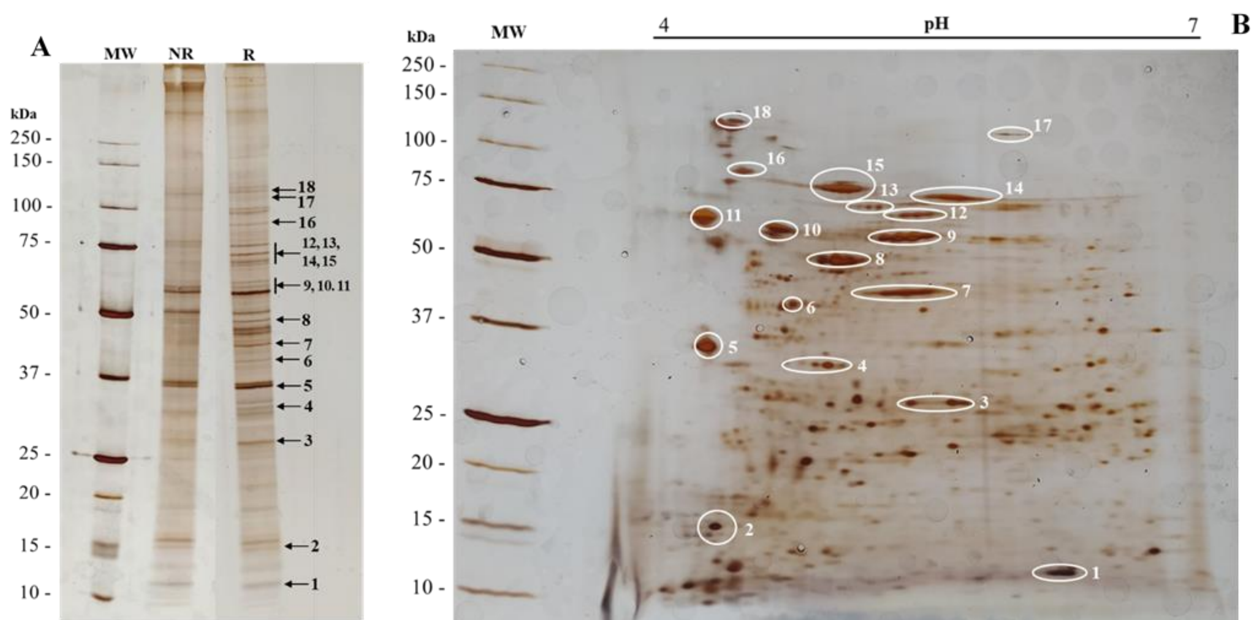

Figure 15: Proteomic profile of MIA PaCa-2 cell membranes. **A** – SDS-PAGE, 17.5% acrylamide, of 13 µg MIA PaCa-2 cell membranes in the presence (R) and absence (NR) of β-ME, stained with silver nitrate. It was applied 3 µL of molecular weight marker (MW) **B** – Two-dimensional electrophoresis (IEF/SDS-PAGE), 17.5% acrylamide and $AgNO_3$ staining. It was applied 6 µL of MW and 1400 µg of cell membranes.

On the comparative study between panel A and B, presented in **Table 4**, it is possible to conceive a parallel between representative bands and the respective peptide spots and isoforms.

Table 4: 1D/2D polypeptide matching analysis by molecular weight (MW) and Isoelectric point (pI).

Number code	MW (kDa)	pI	Number code	MW (kDa)	pI
1	11	6.1 – 6.3	**10**	59	4.8
2	15	4.4	**11**	60	4.3
3	27	5.4 – 5.8	**12**	65	5.4 – 5.6
4	32	4.8 – 5.1	**13**	66	5.2 – 5.4
5	35	4.4	**14**	70	5.5 – 5.9
6	40	4.8	**15**	73	4.9 – 5.3
7	43	5.2 – 5.7	**16**	82	4.6
8	48	4.9 – 5.3	**17**	110	5.9 – 6.1
9	58	5.3 – 5.6	**18**	120	4.5

2.2 GLYCOPROTEOMIC PROFILE EVALUATION: 1D AND 2D ANALYSIS

The method used to evaluate the glycoproteomic composition of the MIA PaCa-2 membranes was, once more, the Con A – Peroxidase method.

The cell membrane samples were subjected to a 1D-SDS-PAGE R and NR (**Figure 16.A**) and a 2D IEF/SDS-PAGE-R (**Figure 16.B**), the resulting gels were transferred onto nitrocellulose membranes and the proteins immobilized, as described in II.2.2.4. The results were observed thanks to a colorimetric method (Con A – Peroxidase).

Figure 16.A shows that from the complex polypeptide profile of MIA PaCa-2 membranes it is possible to discern glycoproteins with high molecular weights, in a simpler profile, meaning that glycosylated proteins represent a smaller portion of the polypeptide composition of MIA PaCa-2 cell membranes.

We can see, in **Figure 16.B**, that much like the 1D analysis, the glycoproteins at the surface of MIA PaCa-2 cells have molecular weights above 45 kDa. In this case, since the SDS-PAGE was preceded by an isoelectric focusing, it's clear that these glycoproteins have isoelectric points within pH 4.5 to 6.

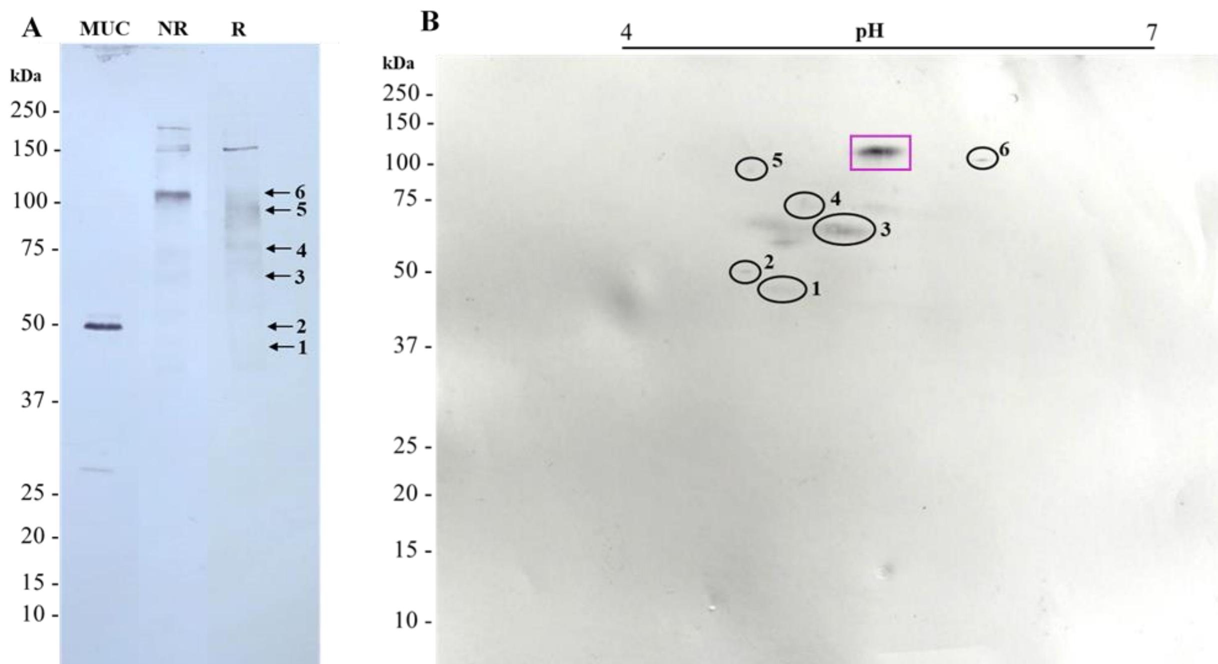

Figure 16: Glycoproteomic profile of MIA PaCa-2 cell membranes. A – One dimensional glycoprotein detection in nitrocellulose membrane revealed through the Con A-Peroxidase method. It was applied 150 µg of MIA PaCa-2 cell membranes. B – IEF/SDS-PAGE transferred onto a nitrocellulose membrane. Glycan detection through the Con A-Peroxidase method. 2000 µg of sample was applied.

The comparative analysis in **Table 5** shows the matching glycoproteins from panel A and B of **Figure 16**.

Table 5: 1D/2D glycoprotein matching analysis highlighting representative molecular weight (MW) and Isoelectric point (pI).

Number code	MW (kDa)	pI
1	48	4.8 – 5.0
2	50	4.6
3	66	5.1 – 5.4
4	73	5.0
5	95	4.7
6	110	6.0

When comparing with the one-dimensional silver-stained gel, as portrayed in **Figure 17**, it can be clearly identified matching glycoproteins that stand out around 210 (red), 175 (green), 150 (yellow), 120 (pink) and 100 (blue) kDa, under non-reducing conditions. Some of these glycosylated proteins are overexpressed in PDAC, with potential for being used as biomarkers. **Table 6** presents possible glycosylated biomarkers that match some of the molecular weights pointed out in **Figure 17**. Interestingly, it was identified a glycoprotein with 210 kDa (red arrow) that may correspond to the already FDA approved PDAC biomarker CA19-9.

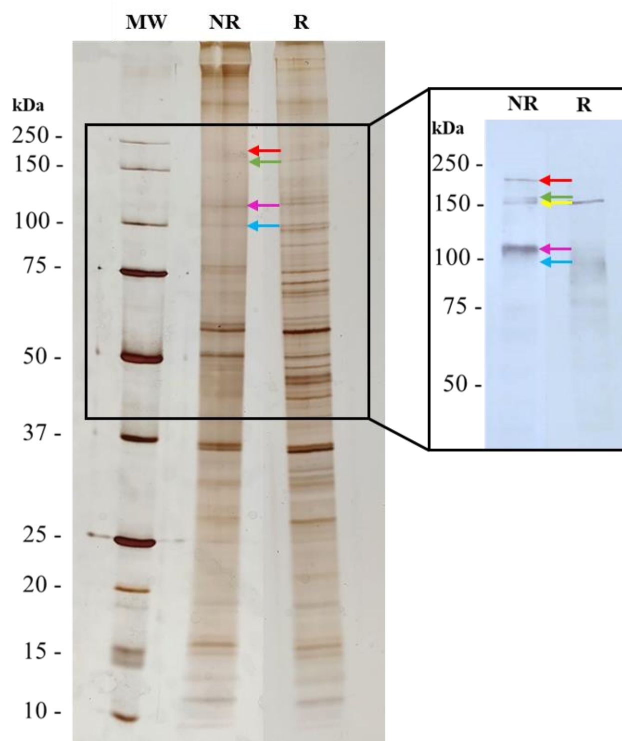

Figure 17: SDS-PAGE NR and R of MIA PaCa-2 cell membranes, 13 µg, stained by $AgNO_3$, matching glycoproteins, 150 µg, in nitrocellulose membrane stained by Con A-Peroxidase. Arrows indicate the match of the glycoproteins on the polypeptide profile.

Table 6: Possible corresponding glycosylated PDAC biomarkers with the colored arrows in Figure 17.

Color code	Glycoprotein	MW (kDa)	References
Red	CA19-9	210	Pan *et al.*, 2016
Green	MUC13	175	Maher *et al.*, 2011; Kaur *et al.*, 2013
Yellow	CEA	150 – 200	Pan *et al.*, 2014; Miyoshi and Nakano, 2008; Rückert *et al.*, 2010
Pink	MUC15	120	Huang *et al.*, 2009; Kaur *et al.*, 2013
Blue	CD73/NT5E	80 – 100	Haun *et al.*, 2015

The comparison between **Figure 15.B** and **Figure 16.B** culminated in four glycoproteins common to both the proteomic and the glycoproteomic profiles (**Table 7**). In panel B of **Figure 16**, a glycoprotein with 115 kDa and a pI of 5.3 – 5.6 stands out for its representativity, marked with a purple rectangle in **Figure 16.B**. Although, there is no match on the two-dimensional SDS-PAGE. It is, however, clear that the protein in question is highly glycosylated, which could be a good target for biomarker research.

Table 7: Match analysis of the 2D glycoproteomic profile of MIA PaCa-2 cell membranes with the 2D polypeptide profile.

2D proteomic analysis	2D glycoproteomic analysis	MW (kDa)	pI
8	1	48	4.9 – 5.0
13	3	66	5.2 – 5.4
15	4	73	5.0
17	6	110	6.0

3 *A. UNEDO* LECTINS BINDING TO MIA PACA-2 CELL MEMBRANES

The binding capacities of *Arbutus unedo* lectins with MIA PaCa-2 cell membranes glycosylated receptors were analyzed by 1D SDS-PAGE-R, stained by $AgNO_3$ (**Figure 18**). The analysis reveals at least 3 bands that may correspond to lectins of TE and SP bound to the glycosylated receptors of the cells, ≈15 kDa (blue), ≈44 kDa (yellow) and ≈50 kDa (red). Actually, *Arbutus unedo* leaf extract has been known to bind to HT29 cell membranes (a colorectal carcinoma cell line), with peptide spots around 44 kDa and 15 kDa, as reported by Oliveira *et al.* in 2019. A peptide of the semi-purified fraction, with ≈23 kDa, has been found to bind to the cell membranes. Lastly, a polypeptide with approximately 12 kDa present in TE and SP, when incubated with MIA PaCa-2 cell membranes, was only revealed in CM+SP, meaning that the binding was compromised when the membranes were incubated with TE. This result is in accordance with the hemagglutination assay where the whole leaf protein extract did not show lectin activity. Moreover, the glycoprotein detection depicted in III.1.2 indicates that this ≈12 kDa protein is glycosylated and confirms the fact that it is present in both samples. These results are presented in **Table 8**, providing a simpler analysis.

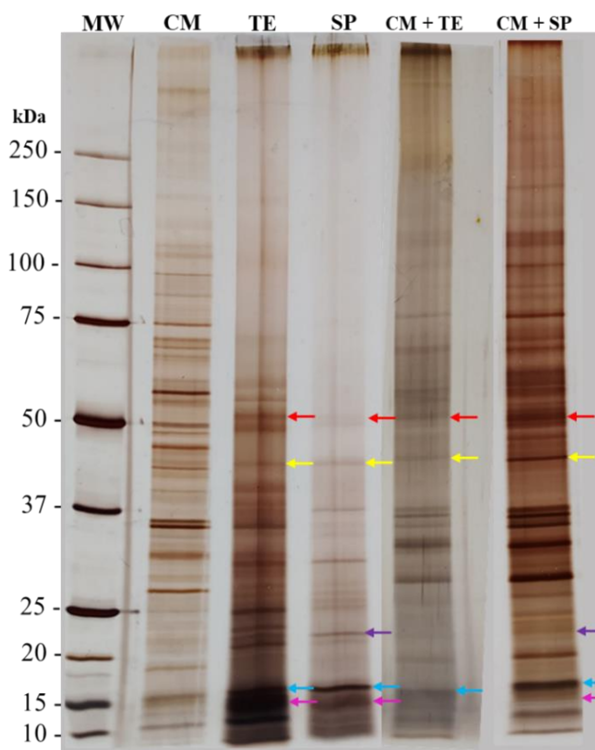

Figure 18: Binding of lectin type proteins of the total extract (TE) and the unadsorbed by Q-Sepharose fraction (SP) to MIA PaCa-2 cell membranes (CM). SDS-PAGE-R, 17.5% (w/v) acrylamide, stained with $AgNO_3$. It was applied 3 µL of MW, 13 µg of CM, 40 µg of TE and 30 µg of SP. Regarding the incubations, it was applied 13 µg of both CM+TE and CM+SP. The colored arrows mark the lectins present in the protein fractions bound to the glycosylated receptors present at the surface of MIA PaCa-2 cells.

This leads us to assume that there is in fact involvement of the lectins or lectin subunits present in *A. unedo* in binding to the membrane glycosylated receptors on this pancreatic cell line. As the glycome profile of MIA PaCa-2 cell membranes reveals some glycoproteins it is possible that this lectin binds to these epitopes. This ability to recognize cell epitopes is a characteristic of lectins that may allow the detection of pancreatic tumor cells in an early stage where these cells begin to express an aberrant glycocalyx.

It is important to note that, with this methodology only allows to study the lectins that bind to receptors on the membranes, yet lectins can be internalized by the cell, acting in a mitochondrial pathway, which is impossible to visualize in this polyacrylamide gel (Fu *et al.*, 2011).

Table 8: Schematic representation of lectin type proteins of the total extract (TE) and the unadsorbed by Q-Sepharose fraction (SP) binding to MIA PaCa-2 cell membranes (CM). + and – correspond to the presence and absence, respectively, of a polypeptide band.

Color code	MW (kDa)	TE	SP	CM + TE	CM + SP
Red	50	+	+	+	+
Yellow	44	+	+	+	+
Purple	23	-	+	-	+
Blue	15	+	+	+	+
Pink	12	+	+	-	+

4 ASSESSMENT OF ANTITUMOR ACTIVITY

4.1 CELL MIGRATION

Most patients with pancreatic cancer do not resist to metastatic disease. Yet there is little knowledge concerning the mechanisms that regulate PDAC cell motility and metastasis (Roy *et al.*, 2015).

4.1.1 Wound healing assay

In order to evaluate the effect of the total extract and the unadsorbed by Q-Sepharose fraction of strawberry tree leaves on pancreatic cancer cell migration the wound healing assay was performed as described in II.2.4.1.1. The inhibition of cell migration can be assessed in vitro after producing a wound on the MIA PaCa-2 cell monolayer, establishing a prognosis of the metastasis formation capacity in a living organism. When the monolayer is interrupted, the cells tend to “close in” the gap until new cell-cell contact is recovered.

Figure 19 shows the wound 48 hours of exposure to different concentrations of TE and SP. The migration inhibition capacity was determined by comparison with the 0 hours of exposure and the negative control. The values presented in **Figure 20** are the mean of the migration percentage after 48 hours of exposure to the different concentrations of both compounds in relation to the saline control, being demonstrative of a tumor invasion inhibition. The dimensions of each wound were measured when the fractions were added to the cells and again after 48 h, the average of the triplicates was calculated.

Considering the control with saline (C) had 0% of inhibition, TE was more effective at 35 µg/mL and 75 µg/mL, inhibiting approximately 54% and 45% of the cell migration, respectively. These results are consistent with previous studies on HT29 cells exposed to *A. unedo* total leaf protein extract (Oliveira *et al.,* 2019), which means that *Arbutus unedo* leaf samples demonstrate to have an effect on these two cells line. SP presents an inhibition of around 88% when administered 75 µg/mL (**Figure 20**). We can infer that both protein fractions have a suppressive effect on MIA PaCa-2 cell migration. Furthermore, the semi-purified fraction displays a higher inhibition of wound healing, when added to the culture medium in concentrations of 75 µg/mL, than the same concentration of the total extract.

The inhibition of cell invasion can occur through synthesis or inactivation of metalloproteinases MMP-9 and MMP-2, growth factor or other promigratory factors inhibition, presence of bioactive compounds (e.g., lectins) that lead to the release of mediators that impair adhesion and invasion of the cells.

Because they have such an important role in tumor progression and are upregulated in PDAC (Slapak *et al.,* 2020), we decided to study the behavior of the gelatinases MMP-9 and MMP-2. Hence, proteolytic activity inhibition assays were accomplished.

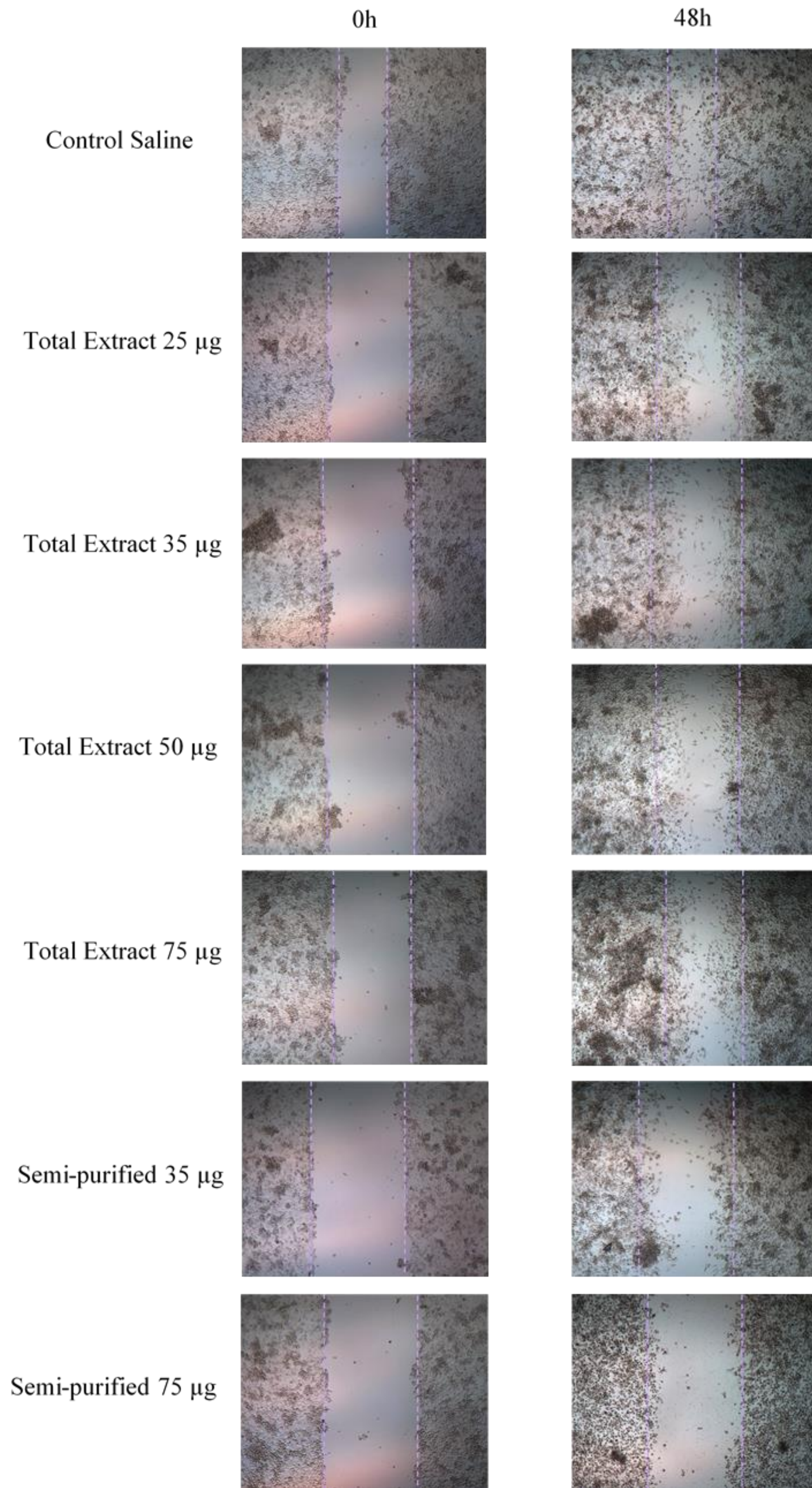

Figure 19: Photographic record of MIA PaCa-2 cells exposed to TE and SP for 48h. On the right it is portrayed the moment the samples were added to the cells after the scratch on the monolayer. It was assayed 25, 35, 50 and 75 µg/mL of TE, and 35 and 75 µg/mL of SP, besides a saline control. The left panel of photographs shows the same fields after 48h of exposure.

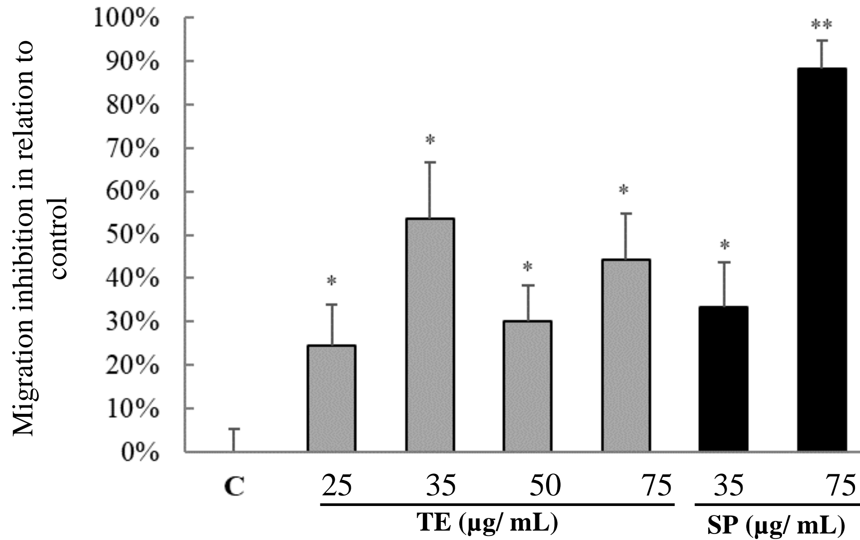

Figure 20: MIA PaCa-2 cell migration inhibition after 48 h of exposure to TE and SP, through the wound healing assay. The values are the mean of replicates ± SD and are expressed in % of wound closure inhibition in relation to 0 h (C). * $P<0.05$; ** $P<0.001$, when compared to the controls.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are involved in the remodeling of connective tissue in numerous natural physiological processes (Sternlicht and Werb, 2001; Lima *et al.,* 2017; Slapak *et al.,* 2020). The abnormal expression of these proteases can lead to several pathological processes characterized by the degradation of the extracellular matrix (ECM) (Wu *et al.,* 2002). MMPs invariably have a pro-domain maintaining the enzyme inactive. The MMP is activated when the pro-domain is cleaved, leading to a conformational change (McCawley and Matrisian, 2000). A subgroup of MMPs, the gelatinases, comprised of MMP-2 and MMP-9, are important mediators of inflammation and oncologic events such as tumorigenesis, cell adhesion and metastasis (Lima *et al.,* 2017). MMP-2 is ubiquitously expressed as a 72 kDa proenzyme (proMMP-2) that is cleaved into a 64 kDa active enzyme. Regarding MMP-9, in its inactive form it is a 92 kDa proenzyme (proMMP-9) whose active form is an 83 kDa mature enzyme (Kumar and Patnaik, 2018).

4.1.2 Gelatin Zymography

Metalloproteinase expression can be analyzed through a substrate zymographic assay which identifies MMPs by their molecular weight and the degradation of their preferential substrate (Snoek-Van Beurden and Von De Hoff, 2005).

In this study, we pretend to assess the gelatinolytic activity of MMP-2 and MMP-9 in the protein fractions. Firstly, a zymography was performed – consisting of an SDS polyacrylamide gel co-polymerized with gelatin, which is degraded by the enzymes present in the sample during the incubation time. The gel is afterward stained with Coomassie Brilliant Blue G-250, revealing the proteolysis sites as transparent bands over a blue background. The method was accomplished as described in II.2.4.1.2.1.

The control SDS-PAGE (**Figure 21.A**) of the secretome of all samples, shows the presence of peptide bands between 64 and 72 kDa that match to MMP-2 and its inactive form. Additionally, there is an 83 kDa band corresponding to MMP-9, which is noticeable mainly in the saline control (C) and 75 µg/mL SP, and a 92 kDa one belonging to proMMP-9, visible in every concentration of both samples but rather tenuous in the control.

The results of the substrate zymography in **Figure 21.B** do not reveal the presence of active MMP-2, yet proMMP-2 occurs equally in every sample including in C. It is evident the presence of proMMP-9 which appears as a light band in the control lane but becomes heavier the bigger the concentration of the fractions. This event is common to both SP and TE. The matrix metalloproteinase 9, though difficult to perceive, is more appreciable in the saline control compared to the extracellular medium after incubation with the protein fractions. There's a clear decrease in representativity of the protease MMP-9 when the cells were incubated with 25 µg/mL of TE and becomes progressively lower to the point where in the 50 and 75 µg/mL the band is nonexistent. The same happens when the cells were incubated with SP: at a concentration of 35 µg/mL MMP-9 gelatin degradation is barely noticeable, however at 75 µg/mL is absent.

Through this analysis it can be concluded that there is no considerable inhibition of the gelatinases by the total extract and semi-purified fraction of *A. unedo* leaves since no significant changes were observed regarding the saline control where no protein fraction was added.

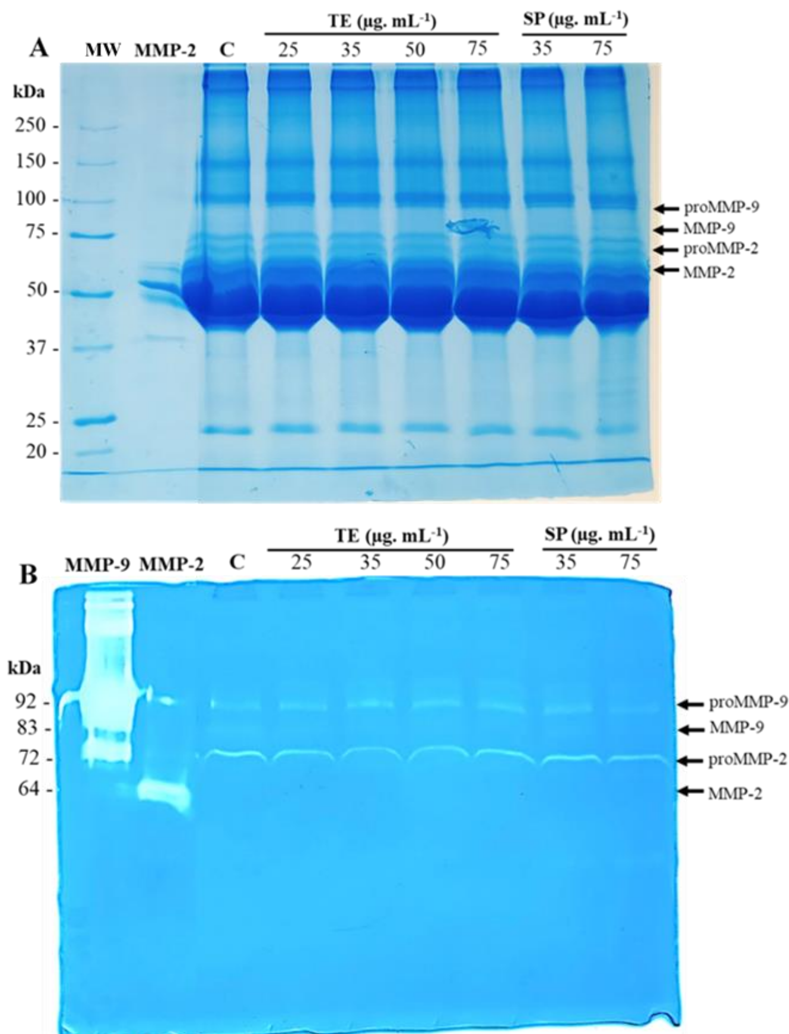

Figure 21: Gelatinolytic activity of MMP-2 and MMP-9 when MIA PaCa-2 cells were incubated for 48 h with total extract (TE) and semi-purified fraction (SP) of *A. unedo* leaves. **A** – SDS-PAGE-NR control, 0.75 mm, 12.5% (w/v) acrylamide, stained by Coomassie Brilliant Blue G-250 (CBB G-250) of the extracellular medium. It was loaded 3 µL of MW, 20 µL of MMP-2 diluted 1:500, and 20 µL of extracellular medium for each sample, including the saline control (C). B – Zymography, 0.75 mm, 12.5% (w/v) acrylamide with 1% (w/v) gelatin, stained by CBB G-250, of the extracellular medium samples collected at the end of the wound healing assay. It was added to the gel 20 µL of MMP-9 and MMP-2 diluted 1:650 and 1:500, respectively, and 20 µL of the extracellular medium. The black arrows indicate the molecular weights associated with MMP-2, proMMP-2, MMP-9 and proMMP-9.

4.1.3 Inhibition of gelatinolytic activity

To better understand how the protein fractions being studied influence gelatinolytic activity of MMP-2 and MMP-9, the compounds were tested using the DQ-gelatin method, that upon proteolytic activity the dye quenched gelatin is converted into bright fluorescent peptides, described in II.2.4.1.2.2.

The results shown in **Figure 22** reveal that 25 and 35 µg/mL of TE aren't statistically different form the control (C) while 50 µg/mL is statistically significant (p<0.05). The concentrations 50 and 75 µg/mL of TE, and 35 and 75 µg/mL of SP are highly significant (p<0.01). However, no sample in any concentration decreased the gelatinolytic activity any lower than 80% which leads to assume that the *Arbutus unedo* fractions under study do not have a considerable inhibitory effect on gelatinase activity of MIA PaCa-2 cells. Thus, it can be affirmed that the samples inhibit cell migration yet not via MMP, in contrast to what Oliveira *et al.* (2019) found to happen in HT29 colorectal cancer cells. The *A. unedo* extract inhibited migration of HT29 and gelatinolytic activity of MMP-2 and MMP-9 that are directly involved in the breakdown of the ECM.

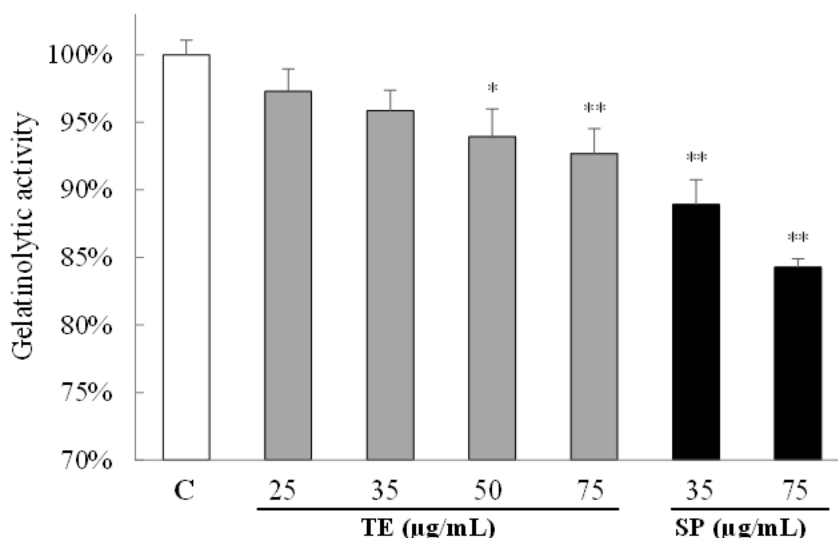

Figure 22: Effect of the total extract (TE) and the semi-purified fraction (SP) of *A. unedo* leaves on gelatinolytic activity of MMP-2 and MMP-9 measured by the DQ fluorogenic assay. TE and SP and well as the negative control (C) were collected at the end of the wound healing assay. C does not inhibit the gelatinases which results in 100% proteolytic activity. MMP activities are expressed as relative fluorescence as a % of controls, and represent the means of at least three replicates ± SD. * P <0.05, ** P < 0.001

4.2 CELL PROLIFERATION AND VIABILITY

4.2.1 MTS metabolism assay

To test if TE and SP were cytotoxic to MIA PaCa-2 cells an MTS metabolism assay was performed as depicted in II.2.4.2.1.

A series of concentrations of TE and SP were tested. TE, which is the total protein extract of the strawberry tree leaves, revealed an IC_{50} of 47.6 µg/mL (**Table 9**). The semi-purification through a Q-Sepharose column (SP) led to a 6-fold increase in cytotoxicity, revealing an IC_{50} of 8.1 µg/mL. It can be taken from these results that as we come closer to have the purified lectin of *A. unedo* the cytotoxic activity increases. This may happen for two reasons: in the total extract in question the lectin may be inactive due to inhibitors present on the sample; and/or since SP is freed of many of the compounds that occur in TE, leading to a higher concentration and availability of the *A. unedo* lectin.

Table 9: IC_{50} values of total extract (TE) and unadsorbed by Q-Sepharose fraction (SP) of strawberry tree leaves in MIA PaCa-2 PDAC cells. The necessary concentration of the protein samples to inhibit 50% of cell growth (IC_{50}) was determined by the MTS metabolism of cells exposed to TE and SP, for 72 h, with a confidence (CI) of 95%.

Protein Fraction	IC_{50} (µg/mL)	95% CI
TE	47.6	42.39 – 53.44
SP	8.1	6.76 – 9.71

4.2.2 Trypan Blue assay

To further investigate cell viability and cell proliferation, an assay using trypan blue staining was designed as described in II.2.4.2.2. Three concentrations of each sample were tested on MIA PaCa-2 cells for 72 h, ½-fold IC_{50}, IC_{50}, and 2-fold IC_{50}, alongside a negative control (saline solution).

This assay provided the number of total, alive and dead cells in the monolayer and in the extracellular medium, for each of the tested concentration for either TE and SP, and the respective control. The values of proliferation inhibition were obtained through the following equation:

$$Prolif.\ inhib. = \frac{TS_{sample} + TA_{sample}}{TS_{negative\ control} + TA_{negative\ control}} \quad (2)$$

Note: "A" – attached; "S" – in suspension.
TS – Total cells in suspension
TA – Total cells attached

Cell viability was determined through:

$$Cell\ viability = \frac{LA_{sample}}{LA_{negative\ control}} \quad (3)$$

LA – Living cells attached

As highlighted in **Figure 23.A** there was a decrease of cell proliferation as the concentrations of TE the cells were exposed to increased. Cell incubated with ½ IC_{50}, IC_{50}, and 2 IC_{50} proliferated 65%, 55% and 36%, respectively, in relation to the control, behaving in a rather streaked way. On the other hand, the values for cell viability were more erratic (**Figure 23.B**). Since the objective is to have antitumor activity, IC_{50} was the concentration that produced less cytotoxicity, however it can be assumed that both ½ IC_{50} and IC_{50} aren't significantly different for they show 27% and 31% of cell viability. In this case, 2 IC_{50} of TE presented the best results inhibiting cell proliferation and decreasing cell viability to only 5%.

Analyzing the same parameters when MIA PaCa-2 cells were incubated with ½ IC_{50}, IC_{50}, and 2 IC_{50} of SP (**Figure 23.A**) it's noticeable that in terms of proliferation the concentration IC_{50} had a higher inhibitory effect, with a proliferation rate of 43%, compared to 2 IC_{50} (whose proliferation was 53%) and especially compared to ½ IC_{50} which revealed a cell proliferation rate of 96% in relation to the control, inhibiting only 4%. This latter concentration presented almost 70% of cell viability decreasing abruptly to 12% when testing IC_{50} of SP. 2 IC_{50} also had good results with a cell viability of 6% compared to the control, as portrayed in **Figure 23.B**.

Comparing the two protein fractions it is safe to assume that, considering the aim of the experiment, TE seems to present better results in lower concentrations, yet it must be considered the disparity of IC_{50} values for each sample. Thus, the concentration IC_{50} of the unadsorbed by Q-Sepharose fraction, or rather 8.1 µg/mL, showed better antitumor activity.

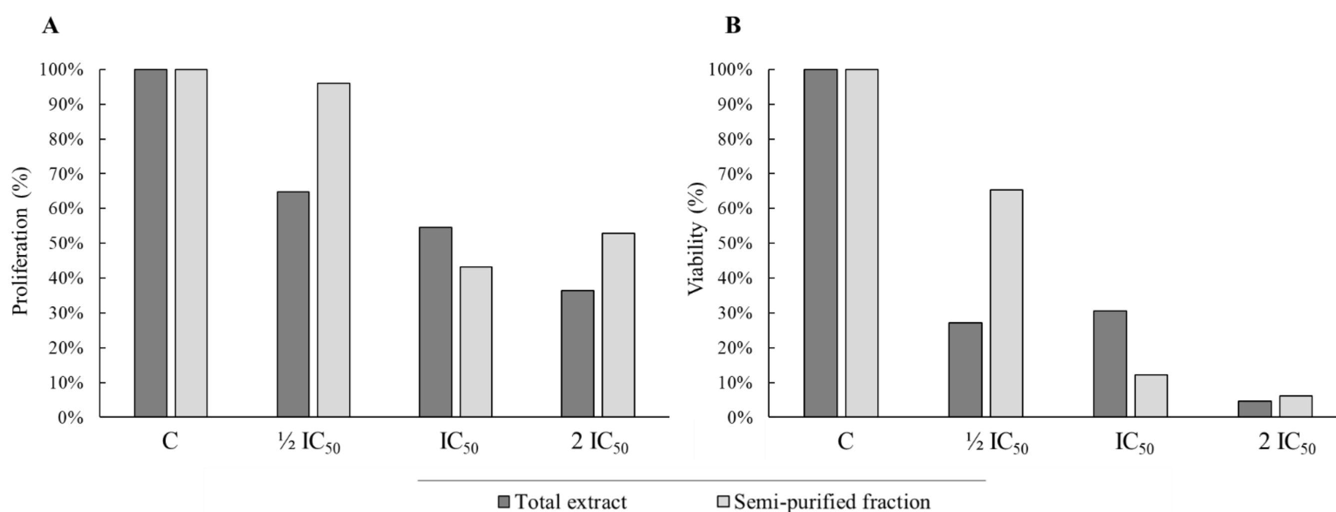

Figure 23: Percentage, in relation to control, of cell proliferation (**A**) and viability (**B**) after 72 h exposure of MIA PaCa-2 pancreatic cancer cells to a ½ IC_{50}, IC_{50} and 2 IC_{50} of TE (dark gray) and SP (light gray). Cell count in Neubauer chamber aided by trypan blue dye.

To scrutinize the cell viability situation a different data analysis approach was taken. The previous result presents the percentage of live cells after exposure to each concentration of each sample over the live cells in the control. **Figure 24** illustrates the percentage of non-viable cells after incubation with ½ IC_{50}, IC_{50}, and 2 IC_{50} of TE and SP, i.e., the percentage of dead cells attached to the monolayer, dead cells in suspension in the extracellular medium and living cells in suspension in the extracellular medium. The values were obtained through:

$$Non-viable\ cells = \frac{LS + DS + DA}{TS + TA} \qquad (4)$$

LS – Living cells in suspension
DS – Dead cells in suspension
DA – Dead cells attached

Incubation with ½ IC_{50}, IC_{50}, and 2 IC_{50} of TE resulted in 60%, 46% and 88% of non-viable cells, respectively. The concentration 2 IC_{50} produced better results than the other studied concentrations, for the percentage of non-viable cells exceeded the control by approximately 29 times. This last conclusion is also true regarding SP, as non-viable cells in the control were surpassed by around 22 times. However, a dose-dependency may be observed in this case.

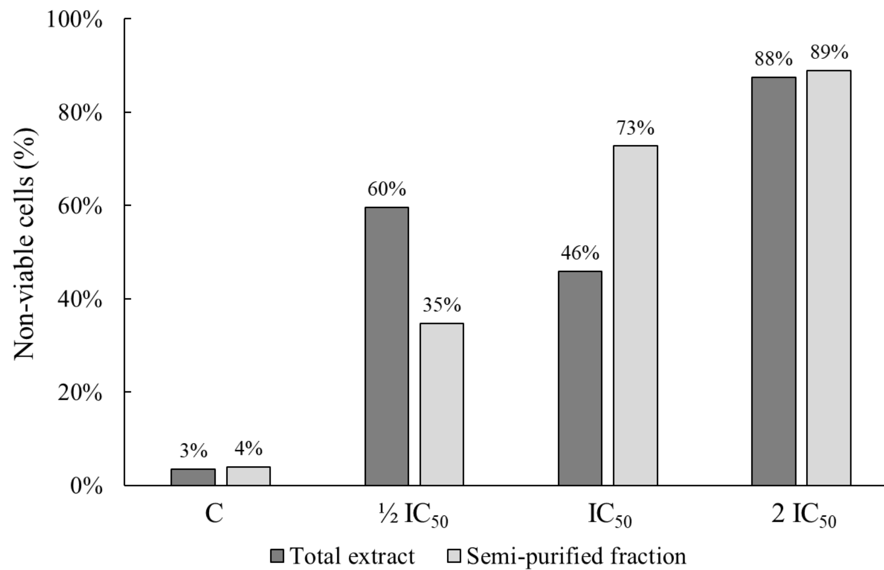

Figure 24: Percentage of non-viable MIA PaCa-2 cells after 72 h of incubation with ½ IC_{50}, IC_{50} and 2 IC_{50} of TE and SP. Cell count in Neubauer chamber aided by trypan blue dye. Dead cells in suspension and in the monolayer, as well as living cells in suspension are considered non-viable cells.

As expected, the semi-purified fraction proved to be more effective, since a lower concentration, in particular 8.1 µg/mL (IC_{50}) offers a higher inhibition of cell proliferation while reducing cell viability.

IV. FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

Pancreatic ductal adenocarcinoma is an aggressive malignancy with a high death rate. In fact, 80% of patients with pancreatic cancer have metastatic disease when diagnosed, besides the radio- and chemoresistance linked to the diagnosis of PDAC, due to the lack of early detection methods and effective therapeutic approaches.

With this in mind, this work is relevant insofar as it aims to identify a lectin type protein in a total protein extract (TE) and a semi-purified protein fraction (SP) of *Arbutus unedo* leaves that binds to the glycosylated receptors on the membranes of a pancreatic malignant cell line, MIA PaCa-2, inducing cell death.

Results show the presence of two glycoproteins in TE and one in SP worth studying, when comparing the proteomic and glycomic profile. In contrast to TE, SP presented hemagglutination activity. It must be considered that TE is a whole leaf extract and as such contains the protein content of strawberry tree leaves, including natural inhibitors of the lectin activity.

The MIA PaCa-2 cell membranes were isolated and analyzed according to their proteomic and glycoproteomic profiles. The results indicate a complex profile of polypeptides ranging more prominently from 27 kDa to 130 kDa, with isoelectric points between 4.5 – 6. From this it is possible to discern glycoproteins with high molecular weights, in a simpler profile, within the same pH range. A few glycoproteins could be discriminated, including CD73 (80 – 100 kDa), MUC15 (120 kDa), CEA (150 – 200 kDa), MUC13 (175 kDa) and the FDA-approved biomarker CA19-9 (210 kDa). Besides, a conspicuous glycoprotein spot was identified with 115 kDa and a pI of 5.3 – 5.6, making it possible to argue the potential as a glycosylated biomarker.

The presence of glycoproteins at the surface of the MIA PaCa-2 cell membranes imply the possibility of lectin recognition and binding. The results of the incubation assays highlight at least 3 bands that may correspond to lectins of TE and SP bound to the glycosylated receptors of the cells, around 15 kDa, 44 kDa and 50 kDa. Oliveira *et al.*, 2019 reported the binding of two polypeptides of 15 kDa and 44 kDa of *A. unedo* leaves to HT29 cell membranes corroborating these results. Additionally, another 23 kDa polypeptide of the SP bound to the MIA PaCa-2 cell membranes. Furthermore, a glycopolypeptide with approximately 12 kDa present in TE and SP revealed to bind to CM+SP which did not occur in CM+TE in accordance with the hemagglutination assay where TE did not show lectin activity. This leads us to assume that there is in fact involvement of the lectins or lectin subunits present in *A. unedo* in binding to the membrane glycosylated epitopes on this pancreatic cell line.

Lastly, cell migration is inhibited by TE although at a higher concentration than SP. By assessing the gelatinolytic activity in the secretome, there is visible evidence that the samples inhibit migration, though not via MMP. Results indicate a considerable decrease in cell viability and proliferation when cells were exposed to TE and SP, also revealing IC_{50} of 47.6 and 8.1, respectively. This evidence highlights the promising antitumor potential of lectins extracted from the leaves of *A. unedo*, yet signifying that a purified lectin, i.e., devoid of compounds that compromise its activity, should be more effective as a possible therapeutic strategy.

It would be interesting to further explore the lectins of this species present in the leaf not only as possible therapeutic approach for carcinomas as is or as a mediator for drugs, but also as detection methods (e.g., serum biomarker detection, probes). The great potential of these lectins in human health could motivate the research for stimuli that induce the production of lectins by the plant, in particular the leaf.

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