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"*Identification and characterization of insect peptides with anticancer activity*"

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Cycle XXXVII

Table of contents

LIST OF ABBREVIATIONS

5- FU = 5- Fluorouracil

AAPs = Anionic Antimicrobial Peptides

ACPs = Anticancer Peptides

AIOM = Associazione italiana di oncologia medica

AIRTum = Associazione italiana registri tumori

AMPs = Antimicrobial Peptides

APL = Alkaline Phosphatase Level

APS = ammonium persulfate

ATCC = American Type Culture Collection

bFGF = basic Fibroblast Growth Factor

BFP = Blue Fluorescent Protein

BSA = Bovine Serum Albumin

BSF = Black Soldier Fly

CAPs = Cationic Antimicrobial Peptides

CBB = Coomassie® Brilliant Blue

COXIBs = Cyclooxygenase-2 Inhibitors

CRC = Colorectal Cancer Cells

CSC = Cancer Stem Cells

CST = Cell Signaling Technology

CTRL = control (untreated sample)

Cyt C = Cytochrome C

DAPI = 4′,6-diamidino-2-phenylindole

DDI = distilled, deionized

DMEM = Dulbecco’s Modified Eagle’s Medium

DMEs = Drug Metabolising Enzymes

DPBS = Dulbecco’s Phosphate Buffered Saline

E. coli = *Escherichia coli*

FBS = Fetal Bovine Serum

GC = Gastric Cancer

GCO = Global Cancer Observatory

GFP = Green Fluorescent Protein

H. illucens = *Hermetia illucens*

HBV = Hepatitis B Virus

hPBMC = Human Peripheral Blood Mononuclear Cells

HPV = Human Papillomavirus

IARC = International Agency for Research on Cancer

IDH2 = Isocitrate Dehydrogenase-2

IFN = Interferon

IMEM = Iscove's Modified Dulbecco's Medium

IRCCS CROB = Istituto di Ricovero e Cura a Carattere Scientifico

LPS = Lipopolysaccharide

M = metastasis

M. flavus = *Micrococcus flavus*

MAMT = Mosaic Analysis of Mutant Tumors

MDACC = MD Anderson Cancer Center

MDR = Multidrug Resistance

MSC = Mesenchymal Stroma/Stem Cells

MTT = Thiazolyl Blue Tetrazolium Bromide

N = nodes

NK = Natural Killer

NSAIDs = Non-Steroidal Anti-Inflammatory Drugs

OXA = Oxaliplatin

PA = Phosphatase Assay

PASSI = Progressi nelle aziende sanitarie per la salute in Italia

PBS = Phosphate Buffered Saline

PBS-T = Phosphate Buffered Saline-Tween

PFs = Peptide Fractions

PI = Propidium Iodide

PLGA = Polylactic-Co-Glycolic Acid

PMSF = Phenyl Methyl Sulfonyl Fluoride

*p*NPP = *para*-nitrophenyl phosphate

PRC2 = Polycomb Repressor Complex 2

PS = Phosphatidylserine

PVDF = polyvinylidene fluoride

QSAR = Quantitative Structure-Activity Relationship

SASP = Senescence-Associated Secretory Phenotype

SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SE = Standard Error

T = tumor

TAM = Tumor-Associated Macrophages

TBS = Tris Buffered Saline

TBS-T = Tris Buffered Saline-Tween

TEMED = N,N,N',N'-tetramethylethylenediamine

TME = Tumor Microenvironment

TNF = Tumor Necrosis Factor

VEGF = Vascular Endothelial Growth Factor

WHO = World Health Organization

ABSTRACT IN ENGLISH

Cancer is a leading cause of death worldwide, characterized by uncontrolled cell growth and multiple mutations. Chemotherapy is often associated with harmful side effects, and cancer cells may become resistant through various mechanisms. Solutions to this issue include enhancing drug accumulation, inhibiting DNA repair, and activating apoptotic pathways. Antimicrobial peptides (AMPs), naturally occurring peptides belonging to the innate immune system, have a wide spectrum of cytotoxic activity against cancer cells and are a promising solution. They are composed of short chains of amino acids, characterized by high hydrophobicity, and a net charge mainly positive, and interact electrostatically with cancer cell membranes to disrupt their integrity, leading to cell death. Indeed, Cationic Antimicrobial Peptides (CAPs), with positive net charge, selectively interact with the surface of cancer cells, sparing healthy cell with neutral charge, and disrupt their membranes through membranolytic actions (carpet model, barrel-stave model, aggregate channel model, and toroidal model). They also show non-membranolytic actions, including: inhibition of tumor angiogenesis, induction of tumor apoptosis, interference with functional proteins, induction of mitochondrial membrane disruption and subsequent release of cytochrome C, induction of cell cycle arrest, hemolytic activity. Insects, particularly the black soldier fly (BSF), *Hermetia illucens* L. (Diptera: Stratiomyidae), are among the richest and most innovative sources of AMPs, due to their ability to live in hostile environments. In this work the peptide fractions (PFs), rich in AMPs, extracted from the hemolymph of *H. illucens* larvae was tested on the HT29 and HCT116 human colorectal cancer cells, and on KATO III and AGS human gastric cancer cells. The PFs significantly inhibited tumor growth by inducing apoptosis, cell cycle arrest at the G2/M phase, and cytoskeleton reorganization leading to reduced motility. These effects, particularly evident in fractions obtained from *Escherichia coli*-infected larvae, also enhanced the efficacy of conventional chemotherapeutics, supporting the presence of biologically active molecules in the hemolymph of *H. illucens* and confirming insect-derived peptides as a promising research area in oncology.

ABSTRACT IN ITALIAN

Il cancro è una delle principali cause di morte in tutto il mondo, caratterizzata da una crescita incontrollata delle cellule e da molteplici mutazioni. La chemioterapia è spesso associata a effetti collaterali dannosi e le cellule tumorali possono diventare resistenti attraverso vari meccanismi. Le soluzioni a questa problematica comprendono l'aumento dell'accumulo di farmaci, l'inibizione della riparazione del DNA e l'attivazione di vie apoptotiche. I peptidi antimicrobici (Antimicrobial Peptides - AMPs), peptidi presenti in natura nel sistema immunitario innato, hanno un ampio spettro di attività citotossica contro le cellule tumorali e rappresentano una soluzione promettente. Si tratta di catene corte di aminoacidi, con un'elevata idrofobicità e una carica netta, per la maggior parte positiva, che interagiscono elettrostaticamente con le membrane delle cellule tumorali per interromperne l'integrità, portando alla morte cellulare. Infatti, i peptidi cationici antimicrobici (Cationic Antimicrobial Peptides - CAPs), a carica netta positiva, interagiscono con la superficie delle cellule tumorali, risparmiando le cellule normali a carica netta neutra, e di distruggere le loro membrane attraverso azioni membranolitiche (modello a tappeto, modello a botte, modello a canale aggregato e modello toroidale). Mostrano anche azioni non membranolitiche, tra cui: inibizione dell'angiogenesi tumorale, induzione dell'apoptosi tumorale, interferenza con proteine funzionali, induzione della rottura della membrana mitocondriale e conseguente rilascio di citocromo C, induzione dell'arresto del ciclo cellulare, attività emolitica. Gli insetti, in particolare la mosca soldato nera (BSF), *Hermetia illucens* L. (Diptera: Stratiomyidae), sono tra le fonti più ricche e innovative di AMP grazie alla loro capacità di vivere in ambienti ostili. In questo lavoro le frazioni peptidiche (PF), ricche di AMP, estratte dall'emolinfa delle larve dell'insetto *H. illucens* sono state testate su cellule umane di cancro del colon-retto HT29 e HCT116 e su cellule umane di cancro gastrico KATO III e AGS. Le PF hanno inibito significativamente la crescita tumorale inducendo l'apoptosi, arresto del ciclo cellulare nella fase G2/M e la riorganizzazione del citoscheletro con conseguente riduzione della motilità. Questi effetti, particolarmente evidenti nelle frazioni ottenute da larve infettate da *Escherichia coli*, hanno anche potenziato l'efficacia dei chemioterapici convenzionali, a sostegno della presenza di molecole

biologicamente attive nell'emolinfa di *H. illucens* e confermando i peptidi da insetto come una promettente area di ricerca in oncologia.

1. INTRODUCTION

1.1 Cancer

A neoplasia (from the Greek *nèos*, 'new', and *plásis*, 'formation') means a new growth, neoformation. A tumor mass consists of new, newly formed, non-pre-existing cells; a tumor (from the Latin word *tumor*, 'swelling'), refers to the macroscopic appearance of the tumor, which in most cases appears as a mass detected at the anatomical site of origin (benign, malignant) and indicates, in pathology, «*a mass of tissue that grows in excess and in an uncoordinated manner with respect to normal tissue, and persists in this state after the cessation of the stimuli that induced the process*», as clarified by the internationally accepted definition coined by the Australian pathologist Rupert Allan Willis (Kumar, Abbas, & Aster, 2007). Based on the studies of Hippocrates, the Roman doctor Galen (129-201 AD) attempts to reformulate the pathogenesis of tumours. In the second book of *De Naturalibus Facultatibus* he uses the term 'cancer' to refer to *«a disease characterised by an enlargement, a protuberance, and whose name derives from the resemblance that the veins swollen by the tumour have to the legs of the crab»*. It is, therefore, from the infiltrating appearance of the tumour, characterised by ramifications of the abnormal tissue starting from a central body and invading the surrounding tissue, that the similarity with the body and legs of the crustacean is derived, which is why the Galenic neologism descends from the Latin cancer (like the Greek *karkínos*, of which it is a calque) meaning «crab» (American Cancer Society, 2018).

The uncontrolled and uncoordinated growth of a group of cells, to the disadvantage of tissue homeostasis, is determined by alterations in their own genetic make-up and underlies a broad class of diseases, classified according to different characteristics, but mainly in three ways (Brábek *et al.*, 2010):

• according to the original histological type of the proliferating cells: mainly in epithelial, mesenchymal, blood cell or nerve tissue tumors (Liang & Kaufmann, 2023);

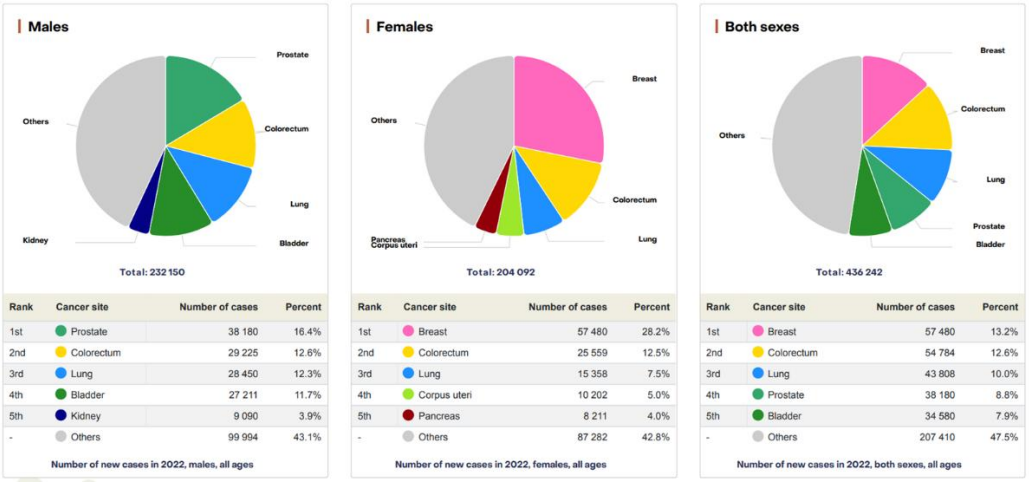
• according to aggressiveness and expected clinical course: into benign tumours (non-cancerous) and malignant tumours ('carcinogenic' or 'cancerous'); a degree of differentiation is

indicated on the basis of cytological atypia of the tumor cells by means of a grading (I-IV, according to severity) (National Cancer Institute, 2024b);

• according to tumor staging for malignant tumors, also called TNM classification, whereby the following are considered: primary tumour size in centimetres (T = tumor) T1-T4, regional lymph node status (N = nodes) N0-N3 and absence or presence of metastasis (M = metastasis) M0/M1 (National Cancer Institute, 2024a).

1.1.1 Incidence and risk factors of cancer

The report 'I Numeri del Cancro in Italia 2023', produced in collaboration with AIRTUM (Associazione italiana registri tumori), AIOM (Associazione italiana di oncologia medica), the AIOM Foundation and PASSI (Progressi nelle aziende sanitarie per la salute in Italia), shows that although cancer cases are on the rise, cancer is always easier to treat.
According to statistics, compared to 2020, the number of cancer cases in Italy in 2023 has increased by more than 18,000: from 376,600 new diagnoses (194,700 in men and 181,900 in women) to approximately 395,000 (208,000 in men and 187,000 in women) (Associazione italiana di oncologia medica (AIOM) and Associazione Italiana Registro Tumori (AIRTum), 2023) (Figure 1).



Rank	Cancer site	Number of cases	Percent
1st	Prostate	38 180	16.4%
2nd	Colorectum	29 225	12.6%
3rd	Lung	28 450	12.3%
4th	Bladder	27 211	11.7%
5th	Kidney	9 090	3.9%
-	Others	99 994	43.1%

Number of new cases in 2022, males, all ages

Rank	Cancer site	Number of cases	Percent
1st	Breast	57 480	28.2%
2nd	Colorectum	25 559	12.5%
3rd	Lung	15 358	7.5%
4th	Corpus uteri	10 202	5.0%
5th	Pancreas	8 211	4.0%
-	Others	87 282	42.8%

Number of new cases in 2022, females, all ages

Rank	Cancer site	Number of cases	Percent
1st	Breast	57 480	13.2%
2nd	Colorectum	54 784	12.6%
3rd	Lung	43 808	10.0%
4th	Prostate	38 180	8.8%
5th	Bladder	34 580	7.9%
-	Others	207 410	47.5%

Number of new cases in 2022, both sexes, all ages

Figure 1. Number of new cases, top 5 cancers in 2022 in Italy (Source: The Global Cancer Observatory).

Nonetheless, these somewhat discouraging numbers were expected since, during the Covid-19 pandemic, screening and data collection on cancer cases were suspended or slowed down.

These estimates indicate that in 2024 the most common cancers in Italy will be breast cancer (approximately 55,900 cases), colorectal cancer (approximately 50,000 cases), lung cancer (approximately 44,000 cases), prostate cancer (approximately 41,100 cases) and bladder cancer (approximately 29,700 cases).

The most frequent male tumour, accounting for almost 20% of all male cancers, appears to be prostate cancer, with about 41,000 new cases. It is followed by lung cancer, with about 29,800 new cases (about 14.3% of male cancers), colorectal cancer (with 26,800 cases, 12.9% of cancers in men) and bladder cancer (with about 23,700 new cases, about 11.4% of male cancers) (AIRC, 2024).

In women, breast cancer continues to predominate, with almost 56,000 new cases (about 30% of all cancers in women), followed by colorectal cancer (with about 23,700 new cases, 12.7% of cancers in women), lung cancer (about 14,000 new cases, 7.4% of cancers in women) and endometrial cancer (with 10,200 cases, about 5.5% of the total).

The absolute number of cancers is expected to increase by an average of about 1.3% for men and about 0.6% for women over the next 20 years (Associazione Italiana Registri Tumori (AIRTum), 2024) (Figure 2).

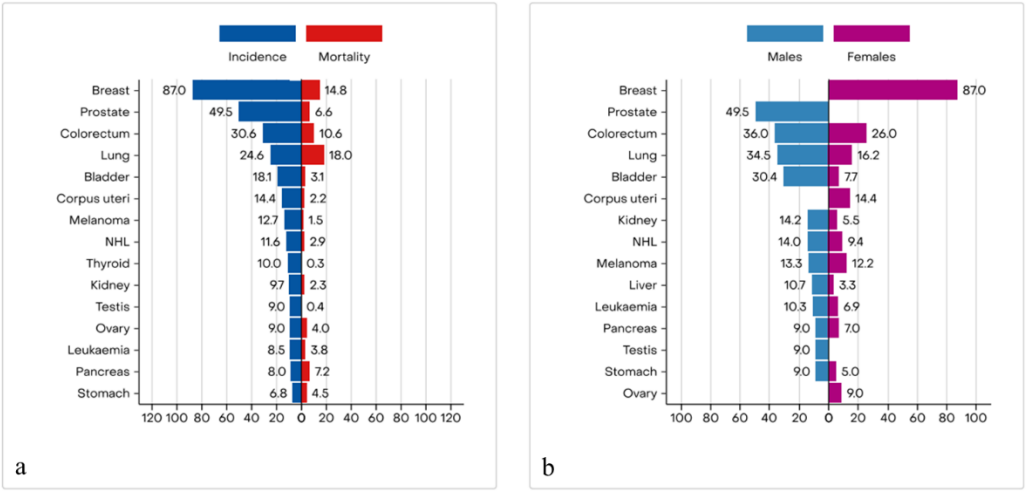


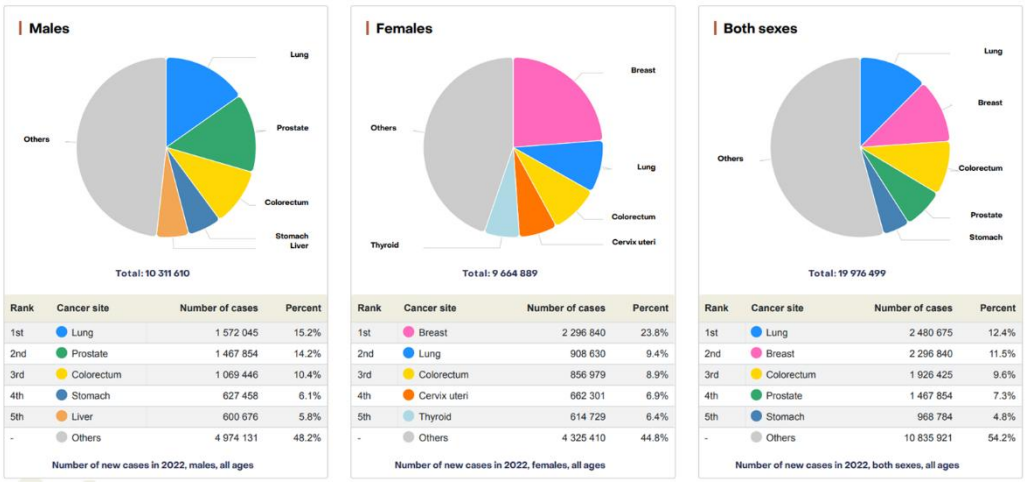
Figure 2. (**a**) Incidence and Mortality rates, top 5 cancers in both sexes in 2022 in Italy. (**b**) Incidence in Males and Females in 2022 in Italy (Source: The Global Cancer Observatory).

The World Health Organization (WHO)'s cancer agency, the International Agency for Research on Cancer (IARC), released the latest estimates of the global burden of cancer. WHO also published survey results from 115 countries, showing a majority of countries do not adequately

finance priority cancer and palliative care services, the growing burden of cancer, the disproportionate impact on disadvantaged populations and the urgent need to address cancer-related inequalities worldwide (Ferlay *et al.*, 2021).

Approximately 20 million new cancer cases and 9.7 million deaths occurred in 2022: 53.5 million people died within five years of a cancer diagnosis, about one fifth of people develop cancer during their lifetime, and about 1 in 9 men and 1 in 12 women die from the disease (Siegel *et al.*, 2023).

According to the latest estimates by the IARC Global Cancer Observatory (GCO), ten types of cancer will account for about two-thirds of new cases and deaths globally in 2022. Data covers 185 countries and 36 cancer typologies (Sung *et al.*, 2021) (Figure 3).



Rank	Cancer site	Number of cases	Percent
1st	Lung	1 572 045	15.2%
2nd	Prostate	1 467 854	14.2%
3rd	Colorectum	1 069 446	10.4%
4th	Stomach	627 458	6.1%
5th	Liver	600 676	5.8%
-	Others	4 974 131	48.2%

Number of new cases in 2022, males, all ages

Rank	Cancer site	Number of cases	Percent
1st	Breast	2 296 840	23.8%
2nd	Lung	908 630	9.4%
3rd	Colorectum	856 979	8.9%
4th	Cervix uteri	662 301	6.9%
5th	Thyroid	614 729	6.4%
-	Others	4 325 410	44.8%

Number of new cases in 2022, females, all ages

Rank	Cancer site	Number of cases	Percent
1st	Lung	2 480 675	12.4%
2nd	Breast	2 296 840	11.5%
3rd	Colorectum	1 926 425	9.6%
4th	Prostate	1 467 854	7.3%
5th	Stomach	968 784	4.8%
-	Others	10 835 921	54.2%

Number of new cases in 2022, both sexes, all ages

Figure 3. Number of new cases, top 5 cancers in 2022 in the World (Source: The Global Cancer Observatory).

With 2.5 million new cases, representing 12.4% of all new cases, lung cancer was the most common cancer globally. Second place is breast cancer in women with 2.3 million cases, 11.6%. It is followed by colorectal cancer (1.9 million cases, 9.6%), prostate cancer (1.5 million cases, 7.3%) and stomach cancer (1.9 million cases, 4.9%).

There were some differences by sex in incidence and mortality from the global total for both sexes. For women, the most commonly diagnosed cancer and leading cause of cancer death was breast cancer (in the vast majority of countries (157 of 185)), whereas it was lung cancer for men (Sung *et al.*, 2021) (Figure 4).

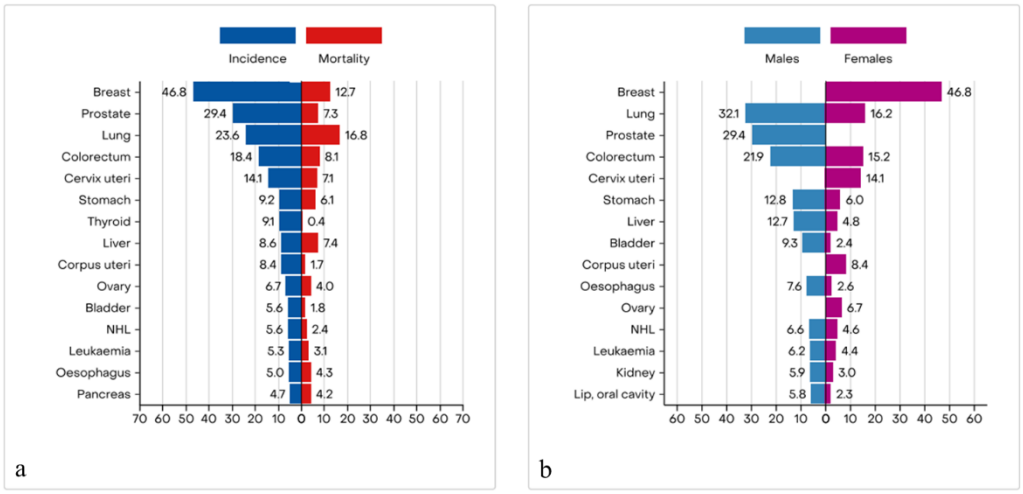


Figure 4. (**a**) Incidence and Mortality rates, top 15 cancers in both sexes in 2022 in the World. (**b**) Incidence in Males and Females in 2022 in the World (Source: The Global Cancer Observatory).

The incidence is directly related to the different risk factors leading to cancer (Arem & Loftfield, 2018). Usually, it is impossible to identify a specific reason why some people get cancer while others do not (Stein & Colditz, 2004). However, studies have indicated that a person's risk of acquiring cancer may be raised by certain risk factors (Khan, Afaq, & Mukhtar, 2010). There are also factors that are linked to a lower risk of cancer, these can also be referred to as protective factors (Wu *et al.*, 2016). Certain activities and exposure to chemicals or other substances are risk factors for cancer. They also include factors like age and family history that are beyond a person's control (Anand *et al.*, 2008). It is thought that abnormalities (mutations) affecting important genes have a role in the development of cancer. These genes generate proteins that affect cell division, growth regulation, and other fundamental cell characteristics (National Cancer Institute, 2024).

Cancer-causing gene alterations can be caused by exposure to harmful substances such as sunlight, chemicals, drugs, viruses, or other environmental factors (Lewandowska *et al.*, 2019). The two main gene categories linked to cancer are: oncogenes and tumor suppressor genes (Carbone, 2020). Oncogenes are mutations or amplifications of genes that normally control cell division. *HER2*, which causes breast cancer, and *EGFR*, which causes certain lung cancers, are examples of these oncogenes and some oncogenes cause cancer by misdirecting cell division into an unchecked growth (Haber & Settleman, 2007; Greenman *et al.*, 2007). Normally, tumor suppressor genes prevent the formation of malignant cells or repair damaged DNA by encoding proteins that inhibit the spread of cancerous cells. Damage to DNA alters the activity of tumor

suppressor genes, increasing the likelihood of cancer by enabling unchecked cell division. A specific number of occurrences, for example of breast cancer, which typically affect numerous family members and occur at a young age, may be caused by suppressor gene mutations inherited from a family member (Gale, 2023).
It could be possible to divide risk factors in two big groups:

1) factors that are known to increase the risk of cancer, like:

- cigarette smoking and tobacco use (Jacob *et al.*, 2018),

- infections like: Human Papillomavirus (HPV) increases the risk for cancers of the cervix, penis, vagina, anus, and oropharynx; Hepatitis B and hepatitis C viruses increase the risk for liver cancer; Epstein-Barr virus increases the risk for Burkitt lymphoma (Brady, MacArthur, & Farrell, 2008); *Helicobacter pylori* infection increases the risk for gastric cancer (Parsonnet *et al.*, 1991);

- radiations (Ultraviolet radiation from sunlight that is the main cause of nonmelanoma skin cancers; ionizing radiation including: medical radiation from tests to diagnose cancer such as x-rays, CT scans, fluoroscopy, and nuclear medicine scans and radon gas in our homes (Abalo *et al.*, 2021),

- immunosuppressive medicines after organ transplant (Mccullough, 2023);

2) factors that may affect the risk of cancer: diet, alcohol, physical activity, obesity, diabetes, environmental risk factors (being exposed to chemicals, pesticides or air pollution has been linked to lung cancers; drinking water that contains a large amount of arsenic has been linked to skin, bladder, and lung cancers (Osborne, Boyle & Lipkin, 1997).

1.1.2 Carcinogenesis and hallmarks of cancer

Tumor formation is a complex process that usually develops over decades. In a phenomenon known as tumor progression or carcinogenesis, normal cells transform into cells with an increasingly neoplastic phenotype (Rashid, 2017). This process can appear in many possible areas of the human body and develops progressively with age and occurs only on rare occasions until a visible tumor mass is produced, which can be detected (Vineis, 2003). A sequence of random mutations and epigenetic DNA alterations that affect genes regulating cell proliferation,

survival and other aspects related to the malignant cell phenotype determine tumor progression (Baylin & Jones, 2016).

The complexity of this process reflects the work of evolution, which has created many obstacles between normal cells and their neoplastic derivatives. Consequently, each stage of tumor progression can be seen as the overcoming of an additional barrier that has prevented a clone of pre-malignant cells from advancing (Nowell, 1976).

The accumulation of numerous genetic and epigenetic alterations during tumor formation could indicate that the cell of origin, the end product of tumorigenesis, is not very similar to its precursor (Sadikovic *et al.*, 2008).

However, it seems increasingly likely that much of the differentiation programme of a normal cell persists in its neoplastic progeny for years, if not decades. This leads to the conclusion that the complexity of this multi-step process reflects the existence of equally sophisticated defensive mechanisms that prevent tumors from appearing in healthy tissue (Jakóbisiak, Lasek, & Gołąb, 2003). Since an evolving population of cells will eventually acquire a completely malignant phenotype, all these defence mechanisms must be deactivated (Weinberg, 2021).

The process of carcinogenesis may be divided into at least three stages: initiation, promotion, and progression (Lawrence & Curtis, 2008). The first stage of carcinogenesis, tumor initiation, results from an irreversible genetic alteration, most likely one or more simple mutations, transversions, transitions, and/or small deletions in DNA, chromosomal rearrangements, gene amplification, insertional mutagenesis and damages to other cellular macromolecules that provide initiated cells with both an altered responsiveness to their microenvironment and a proliferative advantage relative to the surrounding normal cells (Haber & Settleman, 2007). The reversible stage of promotion does not involve changes in the structure of DNA but rather in the expression of the genome mediated through promoter-receptor interactions: this tumor promotion results in proliferation of the initiated cells to a greater extent than normal cells and enhances the probability of additional genetic damage, including endogenous mutations that accumulate in the expanding population (free-radical–induced DNA damage (Halliwell & Aruoma, 1991), DNA depurination (Lindahl & Nyberg, 1972), DNA polymerase infidelity (Preston, Albertson & Herr, 2010), and deamination of 5-methylycytosine (Lindahl & Nyberg, 1974) in addition to exposure to exogenous environmental carcinogens (Ames *et al.*, 1973).

The final irreversible stage of progression is characterized by karyotypic instability and malignant growth. Critical molecular targets during the stages of carcinogenesis include proto-

oncogenes, cellular oncogenes, and tumor suppressor genes, alterations in both alleles of the latter being found only in the stage of progression (Marusyk & Polyak, 2010).

According to the studies of Hanahan *et al.* (2000) the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: *self-sufficiency in growth signals* (Gschwind *et al.*, 2004; Slamon *et al.*, 1987), *insensitivity to growth-inhibitory (antigrowth) signals* (bypassing the activation of tumor suppressor genes) (Weinberg, 1995; Hannon and Beach, 1994), cell survival as a consequence of *evasion of programmed cell death (apoptosis)* (Wyllie, Kerr & Currie, 1980; Thornberry & Lazebnik, 1998) and of the development of a *limitless replicative potential* (Hayflick, 2000; Wright, Pereira-Smith & Shay, 1989), *sustained angiogenesis* (by induction of endothelial growth factors, in response to the necessity of tumors to find sustenance in the form of nutrients and oxygen, as well as the ability to evacuate metabolic waste and carbon dioxide) (Bouck, Stellmach, & Hsu, 1996; Kim *et al.*, 1993), and tissue remodelling through *invasion and metastasis* (Sporn, 1996; Johnson, 1991). Each of these physiologic changes represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues (Hanahan & Weinberg, 2000). The majority of human tumor types, if not all of them, share these six defensive mechanisms, which might account for why cancer is relatively rare over an average human lifetime (Figure 5).

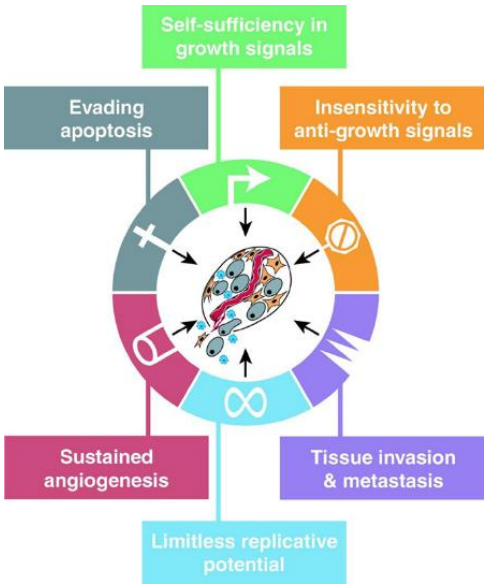


Figure 5. Acquired Capabilities of Cancer (Source: Hanahan *et al.,* 2000).

In the decade following the first information on the six essential alterations for tumor development, considerable progress has been made in understanding the mechanistic underpinnings of each hallmark (Hanahan & Weinberg, 2011). In Figure 6a are still shown these acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate and this acquisition is made possible by two additional hallmarks of cancer, labeled as emerging hallmarks, that are involved in the pathogenesis of some and perhaps all cancers: one involves the capability to modify, or reprogram, cellular metabolism (*deregulating cellular energetics*) (Reitman & Yan, 2010; DeBerardinis *et al.*, 2008) in order to most effectively support neoplastic proliferation; the second allows cancer cells to *evade immunological destruction*, in particular by T and B lymphocytes, macrophages, and natural killer cells (Luo, Solimini & Elledge, 2009; Biswas & Mantovani, 2010). Furthermore, two consequential enabling characteristics of neoplasia make it easier to acquire both core and emerging hallmarks: *genomic instability and* consequent *mutability* (Negrini, Gorgoulis & Halazonetis, 2010; Berdasco & Esteller, 2010), which give cancer cells genetic alterations that accelerate tumor progression, and *inflammation* (Colotta *et al.*, 2009; Mantovani, 2010) caused by innate immune cells, which are meant to fight infections and heal wounds, but may inadvertent support multiple hallmark capabilities, translating the effects of inflammation on tumor growth (Mantovani *et al.*, 2008; Yang, Pang & Moses, 2010) (Figure 6b).

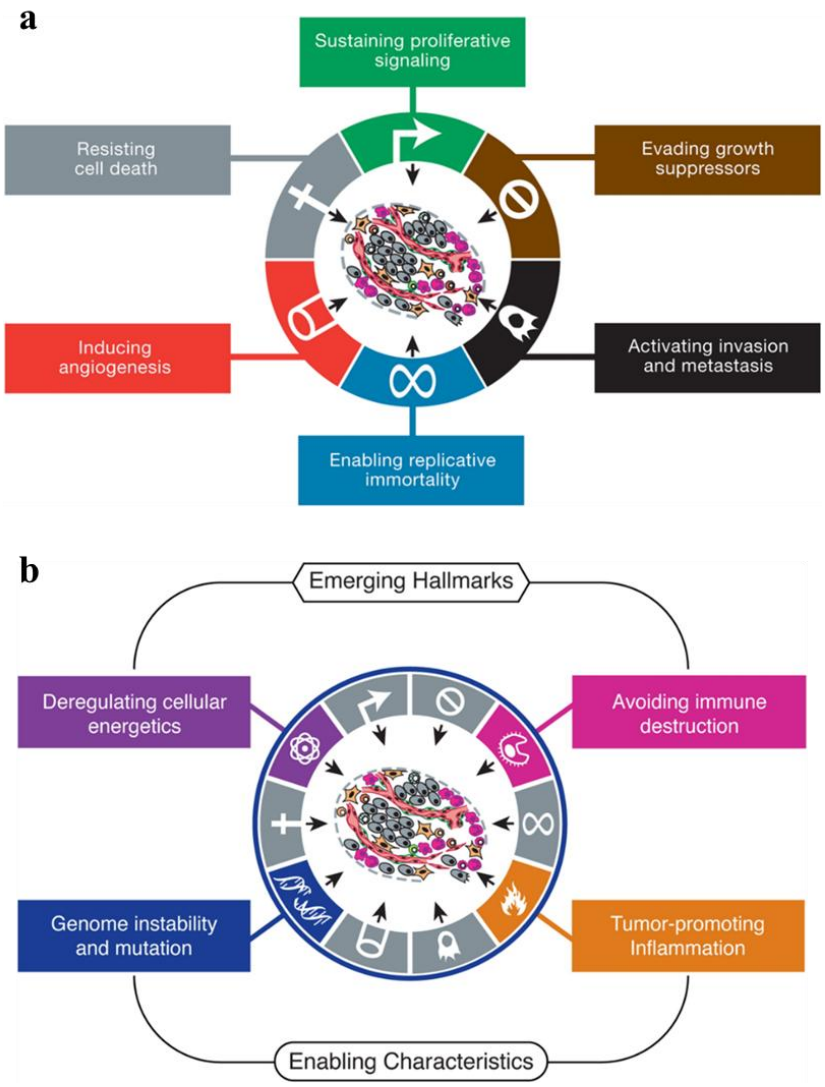


Figure 6. (**a**) The Hallmarks of Cancer. (**b**) Emerging Hallmarks and Enabling Characteristics (Source: Hanahan *et al.,* 2011).

Since 2022 'cellular energetics' (now described more broadly as 'reprogramming cellular metabolism') and "avoiding immune destruction" have been sufficiently validated to be considered part of the core set, so the hallmarks of cancer currently embody eight hallmark capabilities and two enabling characteristics. To this revision other emerging hallmarks and enabling characteristics had been incorporated, involving (Hanahan, 2022) (Figure 7):

3) *unlocking phenotypic plasticity*. During cell development, cells differentiate to take over specific functions in tissues, often ceasing to proliferate and this antiproliferative process is a barrier to tumor formation; however, in cancer, cells can evade this terminal differentiation, reverting to progenitor-like states or maintaining a partially differentiated

state, thereby promoting tumor growth, so unlocking phenotypic plasticity is crucial in cancer pathogenesis (Yuan, Norgard & Stanger, 2019; Perekatt *et al.*, 2018).

4) *non-mutational epigenetic reprogramming*, or in other words, the completely epigenetic programming of distinct tumor phenotypes and the evolution of cancer that does not undergo mutations but rather is caused by environmental influences (Flavahan, Gaskell & Bernstein, 2017). For instance, hypoxia, caused by insufficient vascularisation, is a common feature of tumors and can influence the activity of TET demethylases, leading to significant changes in the methylome such as hypermethylation. Furthermore, this condition may reduce the availability of essential nutrients, impact translational control and increase the malignant phenotype of tumor cells (Thienpont, Van Dyck & Lambrechts, 2016; Gameiro & Struhl, 2018).
5) *polymorphic microbiomes*, i.e. evidence that the diversity of microbiomes between individuals can influence cancer phenotypes, and association studies on human and experimental manipulation in mouse models of cancer are identifying specific microorganisms, mainly bacteria, that may have positive or negative effects on cancer development, progression and response to therapy (Thomas *et al.*, 2017; Helmink *et al.*, 2019).
6) *senescent cells* (Gorgoulis *et al.*, 2019). The concept of cellular senescence refers to a typically irreversible proliferative arrest process that is believed to have evolved as a protective mechanism to maintain tissue homeostasis (Lee & Schmitt, 2019). Cellular senescence not only interrupts the cycle of cell division, but also causes changes in cell morphology and metabolism. Therefore, while cellular senescence has traditionally been considered a protective mechanism against cancer, as tumor cells induced to undergo senescence tend to limit malignant progression, nevertheless there is growing evidence showing that senescent cells may promote tumor growth and progression in some contexts, mainly through the activation of their senescence-associated secretory phenotype (SASP) (Birch & Gil, 2020; Faget, Ren & Stewart, 2019) which involves the release of many bioactive proteins such as chemokines, cytokines and proteases, the type of which depends on the type of senescent cell and tissue and which may transmit signals to neighbouring tumor cells and influence signalling networks and distinctive cell capacities (He & Sharpless, 2017; Kowald, Passos & Kirkwood, 2020; Wang, Kohli & Demaria, 2020) .

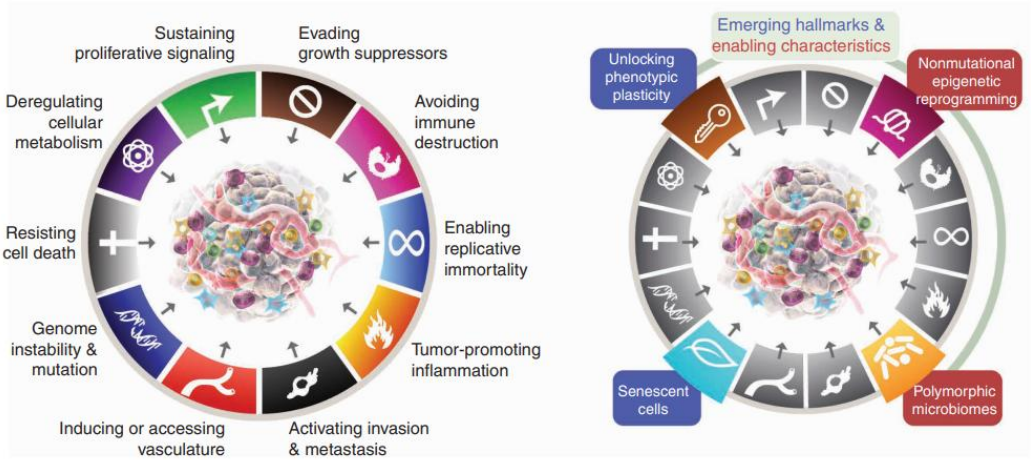


Figure 7. Left, the ten cancer hallmarks (eight distinctive abilities and two enabling characteristics). Right, the other two emerging hallmarks and two enabling characteristics (Source: Hanahan, 2022).

1.1.3 Cancer prevention and treatments

Cancer prevention comprises primary prevention (e.g. avoidance of cancer-causing substances in the environment or dietary elements associated with increased risk; dietary supplementation with putative protective agents) and secondary prevention (e.g. detection and removal of benign neoplasms, early detection of breast cancer by mammography) (Osborne, Boyle & Lipkin, 1997). The annual number of new cases is expected to rise from 14 million to 19 million in 2025, 21.7 million to 21.7 million in 2030, and up to 24 million in 2035 (Wiszniewska *et al.*, 2018; Fitzmaurice *et al.*, 2015) and due to this issue's worldwide significance, more and more focus is being placed on primary and secondary prevention through the pursuit of more potent preventive techniques (Lewandowska *et al.*, 2021).

The '1 + X' principle is used for the aetiology and prevention of cancer in which “1” indicates the primary risk factor for cancer (tumorigenesis) in a particular geographical area and “X” indicates the secondary risk factors for a particular type of cancer (progression) and it is possible that this principle could serve as a general guide for cancer prevention, which would probably reduce cancer incidence and mortality rates worldwide (Liu & Dong, 2021). In Figure 8 are reported some risk factors and prevention strategies for different cancers: lung cancer, gastric cancer, liver cancer, colorectal cancer, pancreatic cancer, prostate cancer, breast cancer, cervical cancer, skin cancer, nasopharyngeal cancer, oral cavity cancer and oesophageal cancer (Liu & Dong, 2021).

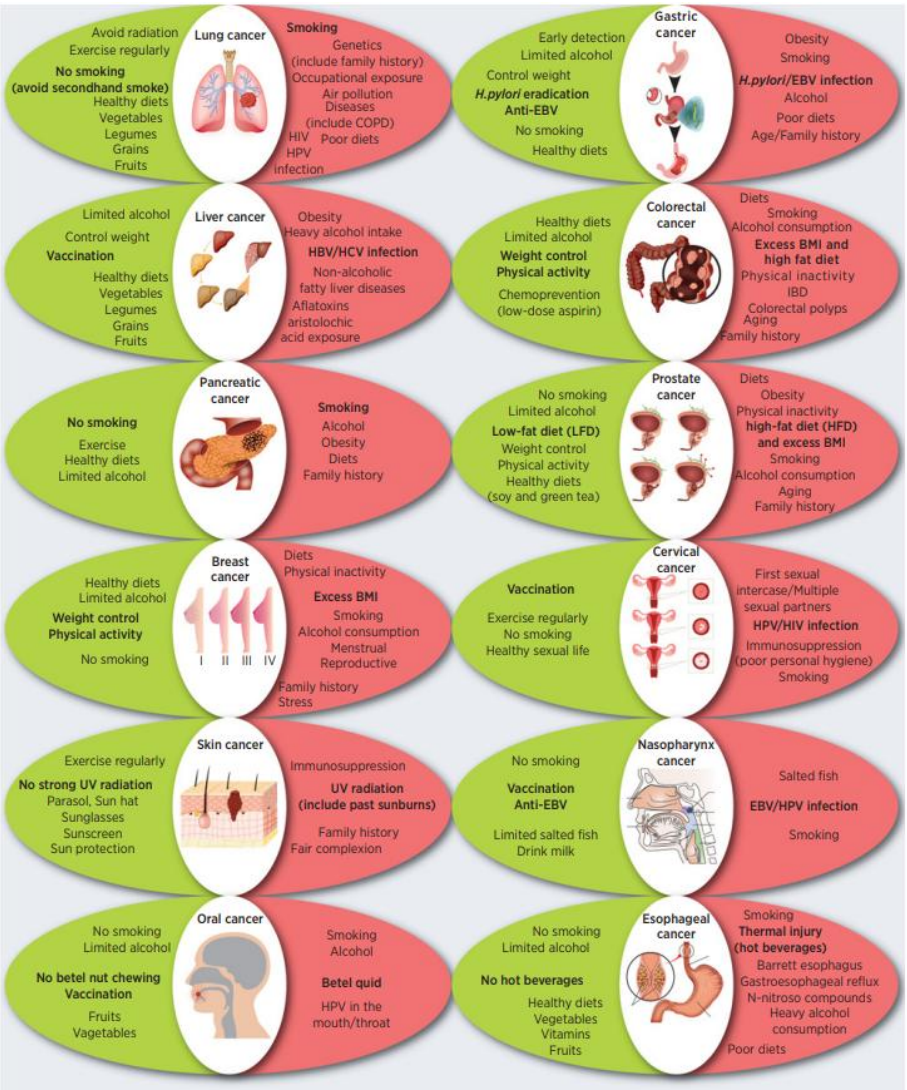


Figure 8. Etiology and prevention of different cancers. Risk factors in red and prevention strategies in green (Source: Liu *et al.*, 2021).

In most recent years, the cancer prevention had been divided not only in primary and secondary, but in particular (Loomans-Kropp & Umar, 2019) (Figure 9):

- primary, to prevent the onset of the disease as far as possible, adopting behaviours and lifestyles that can avoid or reduce the onset and development of a disease, thus keeping modifiable risk factors under control (Miller *et al.*, 2008; Forman *et al.*, 2004). Primary prevention also includes vaccines against certain viruses that increase the risk of cancer, such as the one for HPV, or Human Papilloma Virus, (cervical cancer) (Khan *et al.*, 2023; Kombe Kombe *et al.*, 2021) or for HBV, or Hepatitis B Virus (Chang, 2010; Rizzo, Cabibbo & Craxì, 2022) (stomach cancer). On the other hand, for people who, due to their family history, may be carriers of genes that predispose to certain types of cancer, it is advisable to assess with their

doctor the possibility of carrying out specific genetic tests and if the results are positive, the doctor may suggest preventive and surveillance examinations or surgery (Tamborero *et al.*, 2013; Nagpal *et al.*, 2016).

- secondary, for the early diagnosis of a disease already in progress; also known as *cancer screening*, refers to the early detection of a disease that allows rapid intervention, but does not prevent or reduce the likelihood of its occurrence; this improves treatment opportunities, progression and adverse effects by identifying precancerous lesions or tumors at a very early stage also with the use of specific drugs: e.g. long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 inhibitors (COXIBs) have been associated with a reduced risk of developing several gastrointestinal cancers, though an inhibitory effect has been observed consistently in colorectal cancer (Kuo *et al.*, 2018; Drew, Cao & Chan, 2016; Kraus, Sion & Arber, 2015; Nan *et al.*, 2013). In a few words, it is a matter of identifying the tumor between its biological appearance and the manifestation of the first symptoms, and today cancer screening is provided for cancers such as breast (Shieh *et al.*, 2017), cervix (Rayner *et al.*, 2023), prostate (Tidd-Johnson *et al.*, 2022) and colorectal (Avital *et al.*, 2013). Thus, early diagnosis is the tool of secondary prevention, which is not applicable to all cancers but is essential in treating the disease (Miller, 2012).

- tertiary, to reduce the probability of recurrence (Sagar & Lawenda, 2009). In this case, prevention concerns complications and the likelihood of recurrence of the previous disease. Therefore, it is related to the management of therapies and their correct intake, as well as the management of functional deficits and disabilities resulting from a pathological or dysfunctional state (Mahon, 2005). The aim of tertiary cancer prevention is also to reintegrate patients into their families and society in the post-tumor healing phase (Jacobsen & Andrykowski, 2015). The instruments of tertiary prevention are: adjuvant therapies (Dong & Gewirtz, 2022) (chemotherapy, radiotherapy and hormonal treatments) to prolong disease-free intervals, increasing survival (Anand *et al.*, 2023; Baskar *et al.*, 2012; Abraham & Staffurth, 2016); early diagnosis of recurrences through simple blood samples for the detection of tumor markers (e.g. CA-125 forovarian cancer and PSA for prostate cancer) (Sharma, 2009; Filella, Rodríguez-Garcia, & Fernández-Galán, 2023); other specific examinations depending on the type of tumor, such as computed tomography (CT), the radiography, colonoscopy or ultrasound (Tan, 2010; Loftus *et al.*, 1999; Sha *et al.*, 2020).

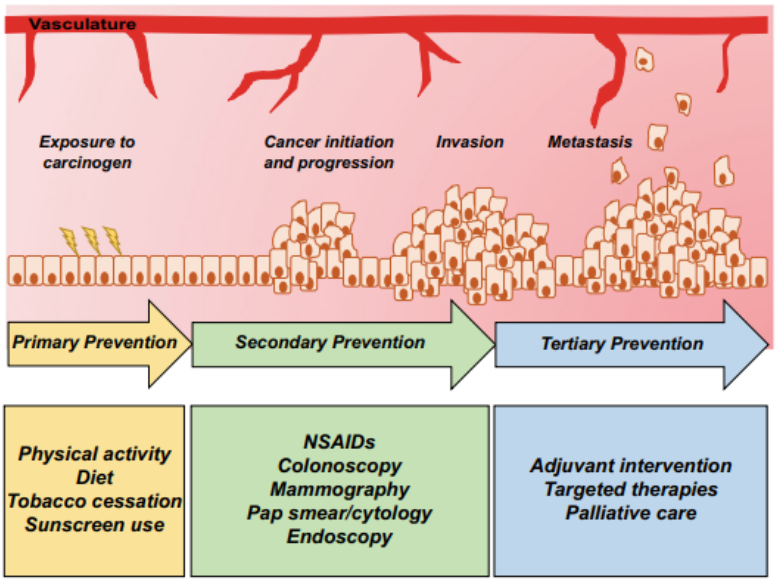


Figure 9. Cancer primary, secondary and tertiary prevention mechanisms (Source: Loomans-Kropp *et al*., 2013).

More specifically, systemic antineoplastic modalities include: hormone therapy (Abraham & Staffurth, 2016; Deli, Orosz & Jakab, 2020) (for certain tumors, such as prostate, breast or endometrium); immunotherapy (You *et al.*, 2024; Bergman, 2024), which may include interferons, monoclonal antibodies, biological response modifiers, and cell therapies; differentiating drugs, such as retinoids for acute promyelocytic leukaemia and isocitrate dehydrogenase-2 (IDH2) inhibitors for acute myeloid leukaemia; patient- and/or tumor-type-specific drug treatment (Zafar *et al.*, 2024; Adhikary *et al.*, 2024). Modalities are often combined: they may be used before or after primary treatment or concomitantly with it. Prevention and increased survival are the main goals of adjuvant therapy administered after and neo-adjuvant therapy administered before and many controlled research studies are ongoing and the choice of therapeutic approaches is constantly evolving (Zubair & Bandyopadhyay, 2023; Zhang *et al.*, 2021; Townsend *et al.*, 2021; Diepstraten *et al.*, 2022; Inai *et al.*, 2004). For three decades, the basis for considerable progress in research into the mechanisms of cancer pathogenesis has been the introduction of targeted therapies that target the mechanism of tumor proliferation. Many therapies have recently been introduced into clinical practice or are in development (Korde *et al.*, 2021; Untch *et al.*, 2014) (Figure 10).

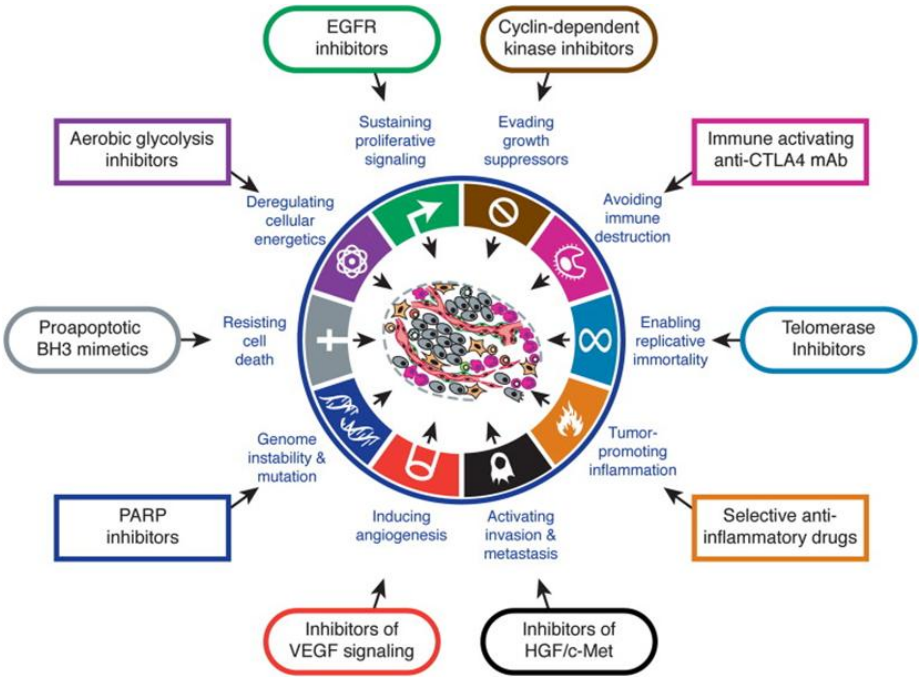


Figure 10. Therapeutic targeting of cancer hallmarks (Source: Hanahan *et al.*, 2011).

1.1.4 Resistance to cancer therapies

A variety of therapeutic approaches are available for the treatment of cancer, but many of these are ineffective, which is why it is referred to as multidrug resistance (MDR), which is one of the main obstacles to effective therapeutic interventions against cancer (Zahreddine & Borden, 2013). In oncology, MDR is defined as the ability of cancer cells to survive treatment with a variety of anti-cancer drugs, similar to the concept commonly applied to antibiotic treatment (Emran *et al.*, 2022). Drug resistance can result from the activation of acquired (caused by drugs) mechanisms referring to the decrease in the effectiveness of anticancer agents after repeated medication administrations or intrinsic (pre-existing) mechanisms prior to drug administration (Emran *et al.*, 2022), as shown in Figure 11.

An increasing amount of studies indicates that MDR is mediated by alterations in the tumor microenvironment (TME) that causes the enhanced efflux of chemotherapy drugs (which decreases the absorption of the drugs by cancer cells), changes in drug metabolism and drug targets, reduced drug efflux, apoptosis suppression, enhance of DNA repair mechanisms, oncogene mutations, tumor heterogeneity, target site mutations, or epigenetic modifications (Yeldag, Rice & Del Río Hernández, 2018; Mansoori *et al.*, 2017; Dagogo-Jack & Shaw, 2018).

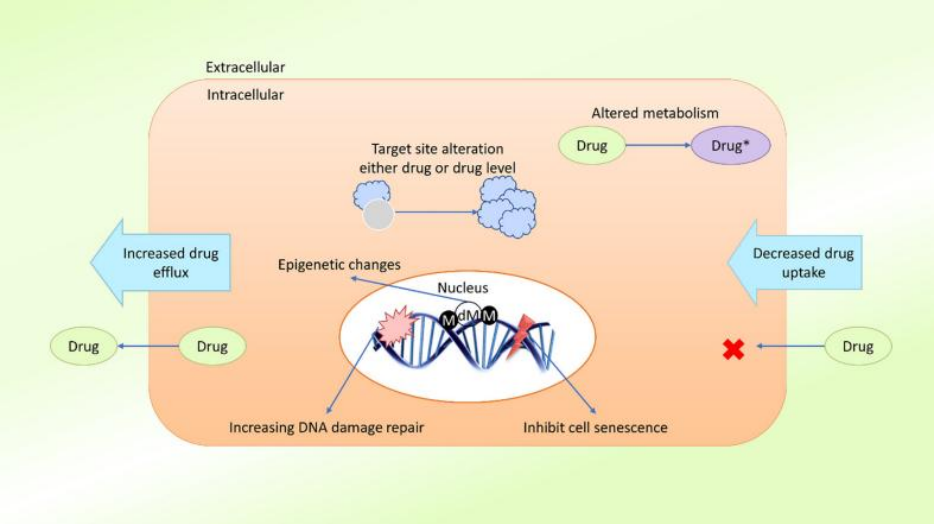


Figure 11. Mechanisms of drug resistance in cancer cells (Source: Emran *et al.*, 2022).

All of these molecular mechanisms of cancer drug resistance have different characteristics. In particular, drug metabolising enzymes (DMEs) control the activation and deactivation of several chemotherapeutic agents and one of the main mechanisms of chemoresistance in cancer is the dysregulation of metabolic signalling pathways and DMEs, which can cause drug detoxification or failure to convert drugs into active metabolites (Rahman & Hasan, 2015). In addition, drugs can pass through the plasma membrane via diffusion, endocytosis or transporters, and these processes can be influenced by the permeability, lipid composition and function or expression levels of the plasma membrane and the lipid composition of the plasma membranes of drug-resistant cancer cells differs from that of drug-sensitive parental cells: cholesterol and phospholipid levels are high, as is a 60% increase in the protein/lipid ratio (Zalba & ten Hagen, 2017). Therefore, the plasma membranes of MDR cells are more fluid and more permeable, which means they absorb less drugs, thus, changes in the structure of the plasma membrane may hinder the process of endocytosis (Peetla, Vijayaraghavalu & Labhasetwar, 2013). In addition to reduced drug absorption, drug efflux mediated by overexpression of ABC transporters is the main factor contributing to insufficient intracellular drug concentration: ABC transporters are mainly located on the plasma membrane and are activated upon drug binding by ATP hydrolysis; as a result, overexpression of these transporters has been associated with poor response to chemotherapy and poor prognosis for patients with various types of cancer (Wang *et al.*, 2021). It is well known that autophagy acts as a double-edged sword and plays multiple functions in both survival and cell death: autophagy has been

shown to contribute not only to the renovation of cells and tissues or the elimination of 'cellular waste', but also to the development of drug resistance in cancer cells. Consequently, autophagy plays a significant role in enhancing chemoresistance in cancer cells: it can improve survival under stressful conditions such as hypoxia, starvation and damage caused by therapeutic agents (White, 2012). In addition to autophagy, apoptosis processes also mediate cell death. Death occurs via internal and external pathways: ligands and receptors of cell death such as FAS, TNF-R, linker proteins and caspases-3, -6, -7 and -8 are involved in the external pathway; in the internal pathway, which occurs in the mitochondria, Bcl2 and AKT act as anti-apoptotic proteins, while for example Bax, Bak and caspase-9 act as pre-apoptotic proteins. Increased resistance to chemotherapy is related to up-regulation of anti-apoptotic genes and down-regulation of pre-apoptotic genes in tumor cells (Roos, Thomas & Kaina, 2016). Finally, the heterogeneity of the tumor is considered the main cause of the failure of ongoing anticancer therapies. This characteristic of tumors can be caused either by the clonal growth of cells with genetic mutations or by epigenetic alterations caused by physical and chemical signals found in the tumor microenvironment (TME). In addition to fibroblasts, endothelial and immune cells, mesenchymal stroma/stem cells (MSC) and tumor-associated macrophages (TAM) work closely with cancer cells and can have both anti- and pro-tumor effects: cancer stem cells (CSC) can be produced through the alteration of cellular phenotypes of cancer, increasing the plasticity of cancer cells (Hass, von der Ohe & Ungefroren, 2020). Epigenetic processes play a major role in controlling the development of CSCs and the changing of cancer cell phenotypes. These mechanisms involve making the chromatin either permissive or restrictive for certain transcriptional programs that are involved in cell differentiation or reprogramming (Easwaran, Tsai & Baylin, 2014). Active promoters, transcribed areas, and potential enhancers are identified by signature patterns of "active" chromatin, whereas other alterations show unique chromatin repression mechanisms, such as those mediated by the Polycomb repressor complex 2 (PRC2) (Varga & Greten, 2017). Even when there are no mutations in the tumor suppressor or traditional oncogene genes, epigenetic changes can nonetheless activate oncogenic pathways. Furthermore, certain chromatin configurations have the ability to limit tumor suppressor program activation and impede differentiation. On the other hand, permissive or "plastic" states, which arise from enhancer landscape reconfiguration during carcinogenesis, may enable treatment resistance in cancer cells as well as random oncogene activation or non-physiologic cell fate transitions (Fagnocchi, Poli & Zippo, 2018). Such dynamic pathways are

supported by TME-driven and epigenetic reprogramming, which favor tumor heterogeneity and cancer cell plasticity (Poli, Fagnocchi & Zippo, 2018).

1.2 Future perspectives in cancer treatments and possible solutions to cancer resistance

In recent decades, several strategies have been proposed to overcome resistance and improve the effectiveness of cancer treatments (Lei *et al.*, 2023).

Some general approaches against drug resistance are based on: early diagnosis of tumors allowing interception of tumors; adaptive monitoring during therapy; addition of new drugs and improved pharmacological principles resulting in more profound responses, as well as the possibility of combination therapy targeting multiple signalling pathways; integration of clinical-genomic data, identification of new targets and biomarkers that can predict drug response and resistance, as well as immunotherapy through the modulation of the TME (Lim & Ma, 2019; Ahronian & Corcoran, 2017; Bae & Park, 2020; Delou *et al.*, 2019; Gonzalez-Bosquet *et al.*, 2022; Sosinsky *et al.*, 2024).

But most importantly, and this is also what this work has focused on, is the interest in the development of new targeted drugs and molecules with targeted activity on cancer cells to reduce possible long-term and off-target toxic effects on healthy cells (Zhong *et al.*, 2021; Patwekar *et al.*, 2023) (Figure 12).

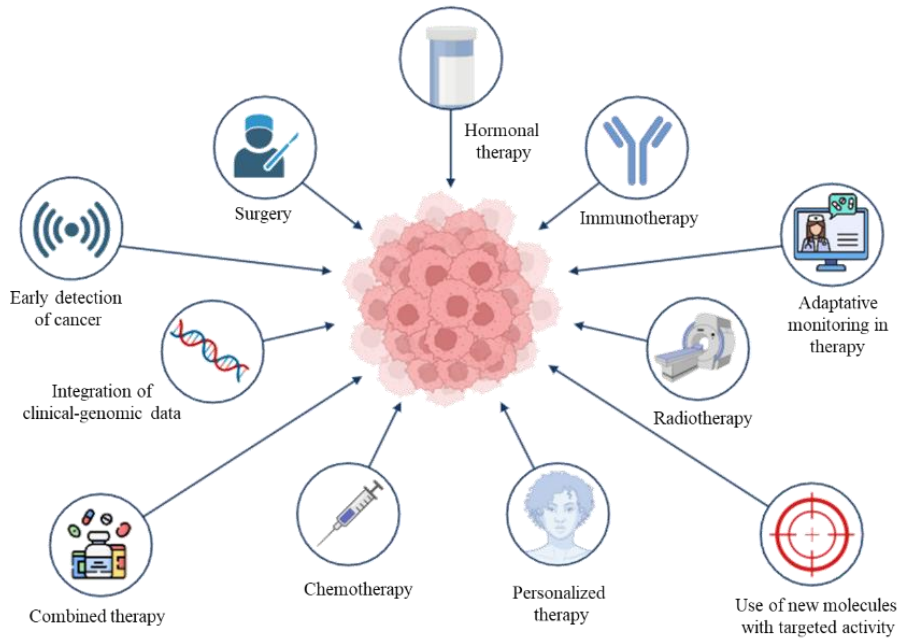


Figure 12. General solutions to drug resistance in cancer (Drawn by BioRender).

Also the development of drug delivery systems and new techniques are interesting in the treatment of cancer (Gao *et al.*, 2023; Elumalai, Srinivasan & Shanmugam, 2024; Sun *et al.*, 2023). For instance, nanoparticles have demonstrated promise in increasing the distribution of anticancer agents, decreasing systemic toxicity, improving therapeutic results, and delivering drugs to certain cells or tissues while minimizing damage to healthy tissues and they play a crucial role also in the effectiveness of immunotherapies, as their size, shape, and surface properties affect their interaction with immune cells (Trigueros, 2016). Stability and biocompatibility are crucial factors to minimise toxic effects and ensure long-term patient safety and biodegradable polymers like polylactic-co-glycolic acid (PLGA) and chitosan offer a safe and controlled release of therapeutic agents, allowing targeted delivery to specific immune cells or tumor sites (Jaiswal, Gidwani & Vyas, 2016).

Also the applications of CRISPR/Cas9 technology, the innovative genome editing, might completely change the field of cancer treatment and research, precisely targeting and edit specific genetic mutations that drive the growth and spread of tumors and in this way to have the possibility to develop more effective and personalized cancer treatments through the inactivation of oncogenes, the enhancement of immune response, the repair of genetic mutations or the delivery of cancer-killing molecules (Chehelgerdi *et al.*, 2024). Using a guide RNA, CRISPR-Cas9 targets a particular gene, and the Cas9 enzyme cuts DNA at that precise location like a molecular scissor: this break may then be repaired, allowing for precise gene editing or gene elimination. So CRISPR-Cas9 can be used in personalised cancer therapies to target and correct genetic mutations that cause cancer by identifying the specific genetic mutations that cause cancer and creating a personalized approach to disable or correct these mutations, effectively preventing the progression of cancer (Afolabi *et al.*, 2021; Zhi *et al.*, 2021).

1.3 Antimicrobial and Anticancer Peptides from insects

The ever-increasing incidence of antibiotic resistance and the continuing challenge in the effective treatment of cancer represent two of the major challenges in contemporary medicine and the growing problem of antibiotic resistance and the need for effective and less toxic treatments for cancer have prompted the scientific community to explore new sources of

therapeutic agents (Chinemerem Nwobodo *et al.*, 2022; Falzone, Salomone & Libra, 2018). In this context, antimicrobial peptides of natural origin emerge as a promising therapeutic resource and potential candidates to address these challenges and, among them, antimicrobial peptides (AMPs) stand out for their extraordinary efficacy in counteracting a wide range of pathogens, together with their ability to act as anti-cancer agents (ACPs) (Luong, Thanh & Tran, 2020; Mba & Nweze, 2022). AMPs are bioactive small proteins, naturally produced by all living organisms as important and indispensable components of their innate immune system, becoming the first-line defense against microbial attacks in Eukaryotes, or produced as a competition strategy in Prokaryotes, to limit the growth of other microorganisms (Lei *et al.*, 2019; Magana *et al.*, 2020).

These, which are also present in various insect species, represent a growing field of research, offering new perspectives for the development of highly innovative yet still less explored therapies (Moretta *et al.*, 2021). Despite the lack of an adaptive immune system, several species of insects can live in highly contaminated substrates and effectively face immune threats: this is possible thanks to the highly effective innate immune response, and AMPs are a crucial part of it and have considerable structural and functional diversity (Dho, Candian & Tedeschi, 2023).

The primary structure of insect AMPs is often characterised by a short amino acid sequence, usually between 10 and 50 residues, and a rich variety of secondary structures, such as α-helical AMPs, β-sheet cysteine-rich AMPs, proline-rich AMPs, and glycine-rich AMPs; they also have a net charge with values ranging from -5 (anionic antimicrobial peptides - AAPs) to + 10 (cationic antimicrobial peptides - CAPs, rich in residues of lysine and arginine) and a very strong heat stability (Wu, Patočka & Kuča, 2018; Deslouches & Di, 2017).

About the secondary structure, α-helical AMPs contain N-terminal amphiphilic α-helixes and C-terminal hydrophobic α-helixes and they belong to secreted proteins, and mature active AMPs are produced following the removal of signal peptides and cecropins and moricins are the most representative in this class (Yi *et al.*, 2014). Insect α-helical AMPs are produced (i) by the fat body (that serves as the functional analogue of the mammalian liver in metabolism and immunity) before being secreted into their body fluid (the hemolymph), (ii) by blood cells, or (iii) by certain epithelia and their activity is not restricted to pathogens, such as gram-negative (G–) and gram-positive (G+) bacteria, fungi, and protozoa, as some of the α-helical AMPs have

a tendency to be highly hemolytic, while others are good insecticides (Oren & Shai, 1998; Tossi, Sandri & Giangaspero, 2000) .

AMPs, such as drosomycins and defensins, contain conserved cysteine residues to create disulfide bonds and represent the β-sheet cysteine-rich subclass. Disulfide bridges stabilize the αhelixes and β-sheets which constitute these AMPs (Imler & Bulet, 2005). Numerous studies on them may be found in insect orders, including Lepidoptera, Hymenoptera, Diptera, and Coleoptera and they have activity against both Gram-positive and Gram-negative bacteria are effectively killed by them, and some of them, such as *Bombyx mori* (Insecta: Lepidoptera) defensin, *Galleria mellonella* (Insecta: Lepidoptera) gallerimycin, against fungi (Cociancich *et al.*, 1993; Vogel *et al.*, 2011; Kaneko *et al.*, 2008). Multiple proline residues, including lebocin, drosokine, and apidaecin, are present in the subclass of proline-rich AMPs, which is primarily found in lepidopteran insects. These proline residues undergo post-translational O-glycosylation, which is necessary to maximize their antibacterial and antifungal activity (Lele *et al.*, 2015). Glycine-rich AMPs are found in various insect orders, including Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera. The most common AMPs are attacin, coleopterin, tenecin, diptericin, hemiptericin, hymenoptaecin and gloverin: attacins are large peptides with six isoforms, divided into basic and acid forms and kill mainly Gram-negative bacteria in *Drosophila melanogaster*, but also fight Gram-positive bacteria and fungi in Lepidoptera insects; gloverins, found exclusively in Lepidoptera, show different activities against microbes; diptericins, found mainly in dipteran species such as *D. melanogaster*, *Sarcophaga peregrina*, *Mayetiola destructor* and *Phormia terranovae*, have activity against Gram-negative (Reichhart *et al.*, 1992a; Dimarq *et al.*, 1988; Hedengren, Borge & Hultmark, 2000; Ishikawa, Kubo & Natori, 1992).

AMPs also display an amphipathic structure, as they contain both hydrophobic and hydrophilic regions, that enable them to be soluble in aqueous environments (Boparai & Sharma, 2019) and, in terms of their net charge, less common class of AMPs is represented by the anionic AMPs (AAPs), which have a negative net charge ranging from -1 to -7 and have been identified in vertebrates, invertebrates and plants (Prabhu *et al.*, 2013). They include many negatively charged aspartic and glutamic acid residues, and in animals are found in various vital organs, including the brain, the epidermis, the respiratory and gastrointestinal tracts (Lakshmaiah Narayana & Chen, 2015). They show a different mechanism of action than the cationic ones. In order to facilitate their interaction with the target organism, some anionic AMPs use metal

ions to form cationic salt bridges with negatively charged constituents of microbial membranes, allowing their penetration into the cell. When they reach the cytoplasm, they may attach to ribosomes or inhibit ribonuclease activity (Jeżowska-Bojczuk & Stokowa-Sołtys, 2018).

Cationic AMPs (CAPs) are the most abundant AMPs and some of them have shown to act as anticancer agents while sparing normal cells by disrupting cancer cells membranes (membranolytic actions) and/or by entering the cells and interacting with intracellular components (non-membranolytic actions) (Schweizer, 2009). Increasing the number of positively charged amino acids or changing their position in the peptide chain can affect the secondary structure of the AMPs, thereby further affecting their antibacterial and anticancer activity (Wu, Patočka & Kuča, 2018).

1.3.1 Classification of Antimicrobial and Anticancer Peptides from insects

Insect AMPs are divided into three groups based on their amino acid sequence and structures: (a) Cecropins, the linear peptides with α-helix but lack cysteine residues; (b) Defensins with 6–8 conserved cysteine residues, have a stabilizing array of 3 or 4 disulfide bridges and 3 domains consisting in a flexible amino-terminal loop; and (c) peptides with an overrepresentation of Proline and/or Glycine residues (Makarova *et al.*, 2018; Zhang & Gallo, 2016).

In the research of Zhou *et al.* (2024), to explore the evolutionary history of AMPs across model insects, as well as their diversity and function, phylogenetic relationships analysis of 20 kinds of AMPs and lysozymes across model insects had been performed; they had been broadly clustered in four groups according to their structure and activity: group I contains eight kinds of AMPs such as moricin, cecropin, gambicin, diapausin, drosomycin, metchnikowin, cobatoxin, and bomanin; group II is clustered by lysozymes and four kinds of AMPs like defensin, gallerimycin, gloverin, and apismin; group III is composed of two kinds of AMPs that are attacin and diptericin and group IV includes six kinds of AMPs such as lebocin, drosocin, coleoptericin, hymenoptaecin, apidaecin, and abaecin (Zhou *et al.*, 2024).

The most explored insect AMPs are defensins, cecropins, attacins, lebocins, diptericins, ponericins and jelleines (Wu, Patočka & Kuča, 2018). In particular, defensins are a small family of arginine-rich, cationic peptides, consisting of 29–34 amino acids and are mostly stabilized by three disulfide bonds, with a prominent structural distinctive in a β-hairpin (Zhu & Gao, 2013). Defensins bind directly to the cell membrane and create pore-like membrane flaws that allow nutrients and vital ions to escape and are isolated from insect orders such as Diptera,

Hymenoptera, Coleoptera, Trichoptera, Hemiptera, and Odonata (Tay *et al.*, 2011). Cecropins were first isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* (cecropia moth), whence the term cecropin was derived and they are mostly isolated from various lepidopteran and dipteran species (Hultmark *et al.*, 1982). The main insect cecropins (A, B and D) consist of 35–37 residues without cysteine and they can lyse bacterial cellular membranes and can also inhibit proline uptake as well as cause leaky membranes (Bechinger & Lohner, 2006). Attacins are glycine-rich proteins that belong to the AMP group and they were initially discovered in the hemolymph of immunized pupae of *H. cecropia* (Carlsson *et al.*, 1991). Attacins are categorized into two groups based on their amino acid composition: attacins with a basic group, while attacins that contain acidic residues and the function of these proteins is to inhibit the synthesis of major outer membrane proteins, thereby compromising the integrity of the cell wall (Geng *et al.*, 2004). Lebocins are antibacterial peptides composed of 32 amino acids, identified in the hemolymph of the silkworm *Bombyx mori* after immunization with *Escherichia coli* and these peptides are proline-rich and O-glycosylated (Hara & Yamakawa, 1995). In contrast, diptericins are a family of glycine-rich antibacterial peptides, approximately 8 kDa in size, derived from Dipteran hemolymph proteins consisting of about 82 amino acids and they primarily target the cytoplasmic membrane (Reichhart *et al.*, 1992). Ponericins adopt α-helical structures within cell membranes, they are peptides extracted from the venom of the predatory ant *Pachycondyla goeldii* and exhibit hemolytic activity (Orivel *et al.*, 2001). Then jelleines are a family of peptides isolated from *Apis mellifera* royal jelly and, comprising 8–9 amino acids and bearing a +2 charge at the C-terminus, they exhibit antimicrobial activities against yeast, fungi, Gram-positive bacteria, and Gram-negative bacteria (Romanelli *et al.*, 2011).

1.3.2 Mechanisms of action of Anticancer Peptides from insects

Although anionic peptides with anticancer activity are found, CAPs are the most abundant AMPs and, some of them have shown to act as anticancer agents while sparing normal cells by disrupting cancer cells membranes (membranolytic actions) and/or by entering the cells and interacting with intracellular components (non-membranolytic actions) (Stowell, Ju & Cummings, 2015; Leuschner & Hansel, 2004).

Membrane interactions are mediated by electrostatic forces between positively charged AMPs and negatively charged microbial surfaces. The teichoic acids in the cell wall of Gram positive

bacteria and the lipopolysaccharide (LPS) in the outer membrane of Gram negative bacteria supply electronegative charge to the microbial surfaces, strengthening the interaction with AMPs (Boparai & Sharma, 2019). On the contrary, the outer layer of eukaryotic membranes is composed by zwitterionic phosphatidylcholine and sphingomyelin, which do not favor AMP interaction because of their neutral charge at physiological pH. Based on their mode of action, AMPs are divided into "membrane acting peptides", which destabilize bacterial membranes causing their disruption, and "non-membrane acting peptides", which are able to translocate across the membranes without damaging them but destabilizing normal cell functions (Boparai & Sharma, 2019).

Four models have been proposed to explain the permeabilization of membranes by AMPs, as shown in Figures 13 and 14: barrel stave model, carpet model, toroidal-pore model and disordered toroidal pore model (Raheem & Straus, 2019) (Figure 13). The barrel stave model describes the lateral insertion and diffusion of peptides through the lipid bilayer, where they arrange into helices and create barrel/stave-like channels that span the membrane (Sorochkina *et al.*, 2013); in the carpet model, peptides do not form pores but bind parallel to the membrane surface, forming a 'carpet' in association with other peptide monomers and the bilayers are disrupted and form micelles, destroying the membrane structure in a detergent-like manner at a certain peptide concentration (Gaspar, Veiga & Castanho, 2013); in the toroidal model peptide molecules maintain a predominantly parallel orientation to the membrane, and a water core forms the center of the pore, with the bioactive peptides and lipid head groups forming the wall of the pore (Ros & García-Sáez, 2015); at last, in the disordered toroidal pore model, after reaching a threshold concentration, a peptide rotates and insert into the membrane bilayer, inducing a fast variation in membrane conformation, forming a toroidal shaped pore (Dho, Candian & Tedeschi, 2023).

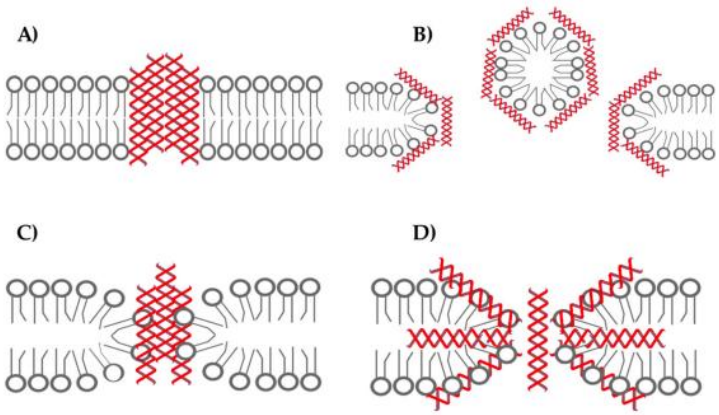


Figure 13. Different models to describe AMP pore-forming activity: (**A**) barrel-stave model; (**B**) carpet model; (**C**) toroidal pore model; (**D**) disordered toroidal pore model (Source: Dho *et al.*, 2023).

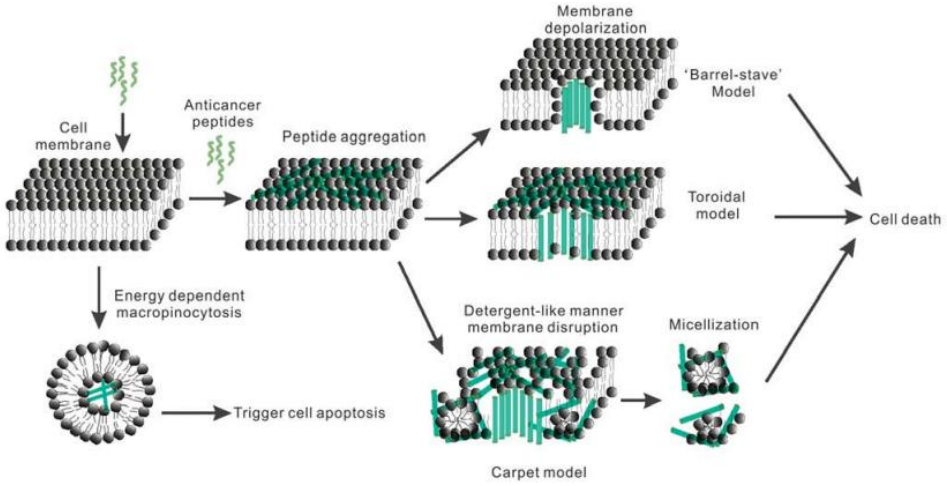


Figure 14. Schematic illustration of cell entry mechanisms of anticancer peptides (Source: Wang *et al.*, 2017).

Similarly to bacteria, cancer cells have a net negative charge due to the high expression of anionic molecules such as phosphatidylserine and O-glycosylated mucins on the outer sheet of the cytoplasmic membrane (Papo & Shai, 2005). These negatively charged molecules are not or less expressed on the outer surface of the cell membrane of healthy mammalian cells (Deslouches & Di, 2017; Leuschner & Hansel, 2004; Szlasa *et al.*, 2020). The net negative charge of cancer cells allows electrostatic interactions between CAPs and the surface of their membranes (Alves *et al.*, 2016). In addition, membrane fluidity is typically increased in cancer cells compared to their healthy counterparts (Manrique-Moreno, Santa-González & Gallego, 2021; Khan *et al.*, 2022), which can facilitate membrane destabilization by membrane-bound CAPs. Moreover, cancer cells tend to have more abundant microvilli on the cell surface than healthy cells, and this increase in the overall surface area may facilitate the interaction of CAPs with the surface of the cancer cell (Papo & Shai, 2005). Furthermore, the mitochondria of eukaryotic cells are negatively charged and have a highly negative transmembrane potential: once inside the cell, non-membranolytic CAPs can disrupt the integrity of negatively charged mitochondrial membranes (Chernysh *et al.*, 2002), resulting in the release of several mitochondrial proteins in the cytosolic compartment, which ultimately lead to apoptotic cell death (Manniello *et al.*, 2021). Therefore, it can be understood how, when referring to ACPs, it is possible to describe not only their extracellular activities, but also intracellular activities. Among these mechanisms there are: the regulation of apoptosis targeting vital molecules and

pathways involved in carcinogenesis (Paredes-Gamero *et al.*, 2012), such as mitochondria function and DNA replication; the activation of the mitochondria-dependent pathway generated by signals such as DNA damage, oxidative stress, and lack of growth factor, which simultaneously release pro-apoptotic proteins such as cytochrome C (Cyt C) from mitochondria to cytosol (Elena-Real *et al.*, 2018); the proteasome-modulation like inhibition of proteasome which cause blocking of cell cycle progression in multiple cancer cell lines (Su & Chen, 2017); the activation of the immune pathways such as Natural killer (NK) cells and Interferon (IFN) signaling pathway (interferons have antitumor activities by directly affecting tumor cell proliferation and indirectly affecting immunomodulatory and anti-angiogenic responses, while natural killer cells play important role in eliminating malignant cells by decreasing the expression of MHC class I molecules, presented on a variety of cancer cells) (Xie, Liu & Yang, 2020). Another important anticancer activity of ACPs could be the arrest of cell cycle after the formation of a complex made by the ACP with p53 (the transcription factor that maintains genome integrity and that mutated could result in uncontrolled cell division and proliferation) that prevents the inhibitory effect on the p53 tumor suppressor and the subsequent intracellular elevation of p53 arrests cell division cycle and brings tumor cells to the irreversible mitochondrial caspase-dependent and -independent apoptosis (Donehower *et al.*, 2019; Yamada *et al.*, 2009). Certain cationic peptides have been shown *in vitro* to be able to suppress both basic fibroblast growth factor (bFGF) and vascular endothelial growth factor VEGF, thus interfering with angiogenesis and this is possible due to the primary structure of certain peptides composed of the same amino acid residues as bFGF and VEGF, effectively competing with binding sites on endothelial cells or by the capacity of interrupting the interactions between growth factors and their receptors (Binetruy-Tournaire, 2000; Starzec *et al.*, 2006).

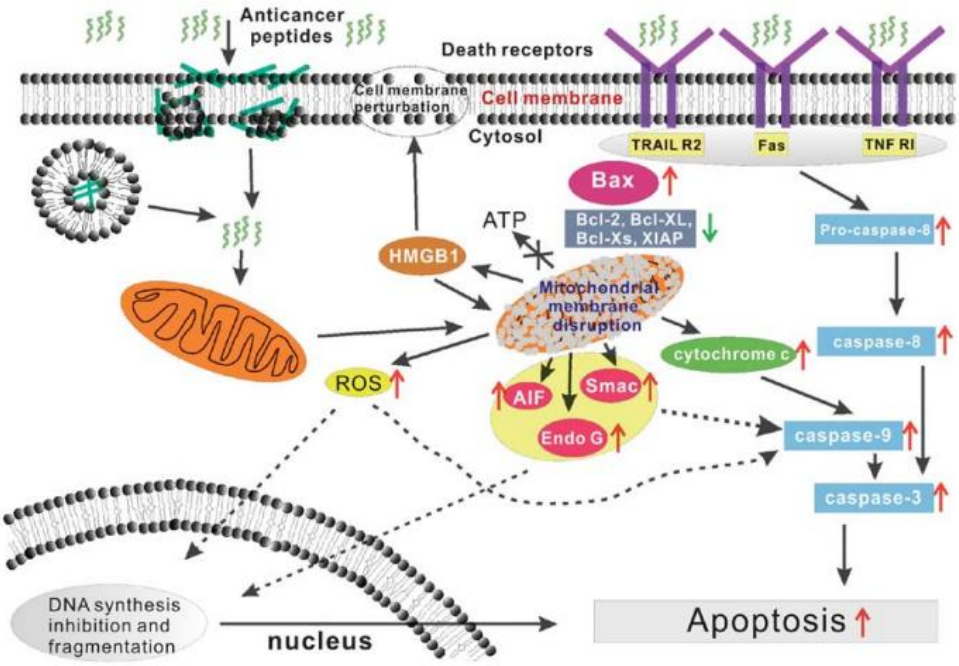


Figure 15. Mechanisms of action of anticancer peptides via mitochondrial-dependent pathways and death receptor-induced pathways (Source: Wang *et al.*, 2017).

1.3.3 *In vitro* and *in vivo* studies and current experimental contributions

Insect-derived AMPs have demonstrated a wide range of antimicrobial activity against pathogenic bacteria, fungi and viruses, making them promising as alternative antimicrobial agents to traditional antibiotics and their ability to act rapidly and with a broad spectrum of action makes them particularly suitable for combating the emergence of antibiotic resistance (Manniello *et al.*, 2021; Xia, Ge & Yao, 2021; Dho, Candian & Tedeschi, 2023; Oñate-Garzón *et al.*, 2017; Cederlund, Gudmundsson & Agerberth, 2011; Yi *et al.*, 2014; Lakshmaiah Narayana & Chen, 2015). Recent studies have also highlighted the potential of insect-derived AMPs as anti-cancer agents and these peptides have been found to induce apoptosis in tumor cells, inhibit their growth and proliferation, and in some cases even limit metastatic spread (Gaspar, Veiga & Castanho, 2013; Felício *et al.*, 2017; Mader & Hoskin, 2006). Their selectivity towards cancer cells compared to healthy cells, together with their relative lack of toxicity towards normal tissues, makes them particularly promising for the development of effective and safe anti-cancer therapies, and this has been demonstrated in numerous *in vitro* and *in vivo* (mice models) studies.

The review of Staczek al. (2023) underlines that some insect AMPs exhibit selective *in vitro* cytotoxicity against different cancer cell lines, e.g., melanoma, lymphoma, leukemia, breast

cancer, lung cancer, and bladder cancer (Tonk, Vilcinskas & Rahnamaeian, 2016; Suttmann *et al.*, 2008; Ramos-Martín, Herrera-León & D'Amelio, 2022) and, in addition to research conducted *in vitro* on the anticancer effect of insect AMPs, publications have shown the potential *in vivo* involvement of AMPs in antitumor action mostly based on research conducted using a *D. melanogaster* model. In particular, the study of Parvy et al. (2019) showed the anticancer effect of defensin: tumor cells in *D. melanogaster* body activate a cellular response, releasing Tumor Necrosis Factor (TNF) from hemocytes, so TNF exposes phosphatidylserine on tumor cells' surfaces, causing them to be targeted by cationic defensin and this interaction leads to cancer cell death and tumor regression (Parvy *et al.*, 2019).

Literature also indicates a link between anti-infection and anti-cancer mechanisms: Jacqueline et al. (2020) reported that oral infection with the Gram-negative bacterium *Pectobacterium carotovorum* accompanying tumor in *Drosophila* species decreased the growth of the tumor in comparison to insects that were not infected and this resulted in more tumor cells dying, which was linked to an increase in the expression of the genes producing drosomycin and diptericin AMPs (Jacqueline *et al.*, 2020). In the work of Jin et al. (2010) the antitumor activity of *Musca domestica* cecropin was examined and it had been found that it inhibited the proliferation of the human hepatoma BEL-7402 cells in dose- and time-dependent manners and did not affect the proliferation of normal liver cells, inducing also apoptosis of the human hepatoma cells probably by triggering extrinsic apoptotic pathway (Jin *et al.*, 2010).

According to Wang et al. (2008 and 2011) Polybia-MPI, an AMP purified from the venom of the brasilian wasp *Polybia paulista*, specifically destroys the membranes of prostate and bladder cancer cells, leading to the necrosis of different leukemia cells by creating transmembrane holes or rupturing membranes, while having no effect on normal murine fibroblasts (Wang *et al.*, 2008; Wang *et al.*, 2011).

In the studies conducted by Saini et al. (1999) and Killion et al. (1986) a synthetic melittin from *Apis mellifera* induced membrane permeabilization and phospholipase A2 and phospholipase D activation on U937 human monocytic leukaemia cells during cell lysis, causing pore formation and releasing lipids and enzymes (Saini, Chopra & Peterson, 1999; Killion & Dunn, 1986). An additional example concerns some studies in which cecropins A and B from *Hyalophora cecropia*, *M. domestica* derivatives Mdc, *B. mori* Cec XI and some chimeric derivatives were found to be active against a variety of human and rodent tumor cell lines *in vitro* (human gastric cancer BGC823 cells, MDA-MB-231 and M14K tumor cell lines, human

hepatocellular carcinoma cell line BEL-7402) and in addition, Cec XJ and Mdc were shown to hinder proliferation and support cellular apoptosis and none of the tested AMPs had a cytotoxic impact on normal cell lines when tested at the same concentrations (Brady *et al.*, 2019; Ghaly *et al.*, 2023; Hoskin & Ramamoorthy, 2008; Kordi *et al.*, 2023). Three novel structurally related pentadecapeptides, named lasioglossins, were isolated from the venom of the eusocial bee *Lasioglossum laticeps* and showed a high potency against leukaemia cells, against cells derived from solid tumors (PC12, HeLa S3 and SW480) and a lower toxicity was observed against the rat normal epithelial cells IEC-6 (Li *et al.*, 2012; Čeřovský *et al.*, 2009). Tonk et al. (2016) (Tonk, Vilcinskas & Rahnamaeian, 2016) in their mini-review listed, among all, some interesting studies in which some insect anticancer peptides had been tested on different cancer cell lines each of them demonstrating a very specific anticancer mechanism: in Kim et al. (2013) HaA4 ACP from *Harmonia axyridis* was cytotoxic on U937 and Jurkat cancer cells through the activation of necrosis and caspase dependent apoptosis pathways (Kim *et al.*, 2013); in Lee et al. (2015) CopA3 from *Copris tripartitus* induced apoptosis and necrosis on human gastric cancer cells (Lee *et al.*, 2015) and caspase-independent AIF-mediated apoptosis on human leukemia cells in the study of Kang et al. (2012) (Kang *et al.*, 2012); then Mastoparan from *Vespula lewisii* induced the activation of mitochondrial apoptosis pathway on B16F10-Nex2 melanoma cells in de Azevedo et al. (de Azevedo *et al.*, 2015).

There are many studies in the literature examining the anti-cancer properties of peptide fractions extracted from the haemolymph of various insect species, evaluating their efficacy and mechanism of action: haemolymph, the equivalent of blood in insects, contains a wide range of peptides that play crucial roles in their immune system and recent studies have suggested that some of these peptides possess anti-cancer properties. In particular, in the study of Chernysh et al. (2002) two peptides (variants of alloferons) were isolated from the hemolymph of the blow fly *Calliphora vicina* (Diptera), experimentally infected with heat-killed bacteria, with the following amino acid sequences: HGVSGHGQHGVHG (alloferon 1) and GVSGHGQHGVHG (alloferon 2) and *in vitro* and *in vivo* experiments demonstrated their antitumoral capabilities with their stimulation of natural cytotoxicity of human peripheral blood lymphocytes and induction of IFN synthesis in mouse and human models (Chernysh *et al.*, 2002). This study was confirmed by experiments of Chowański et al. (2017) in which they demonstrated that alloferon, isolated from the haemolymph of blow fly *C. vicina* larvae experimentally infected, with its structural analogue allostatine (HGVSGWGQHGTHG),

appears to be very interesting as potential anticancer drug decreasing the level of TNF-α (Tumor necrosis factor) and VEGF (vascular endothelial growth factor), simultaneously increasing the production of interleukin-2 (IL-2), which leads to cell apoptosis (Chowanski *et al.*, 2017; Kuczer *et al.*, 2016). Another interesting preliminary study is that from Januszanis et al. (2012) based on the effect of *Galleria mellonella* hemolymph polypeptides on a cancer cell line: in particular, methanolic extracts of hemolymph containing proteins of molecular mass below 30 kDa induced cell death, as some cells exhibited symptoms of apoptosis or necrosis, on human brain glioblastoma multiforme cells (T98G cell line) and it was detected that treatment of the cells with hemolymph of *E. coli* immunized larvae, enriched with inducible antimicrobial peptides, increased three times the number of the dead cells in comparison with the control culture and once in comparison with the hemolymph from non-immunized larvae (Januszanis *et al.*, 2012).

According to the study of Mahmoud et al. (2020), it was demonstrated the anticancer activity of the hemolymph of *Sarcophaga argyrostoma* (Diptera: Sarcophagidae) third larval instars against human breast cancer cell line (MDA-MB-231 cells) upon 24 hours of exposure (Mahmoud *et al.*, 2020) and also form the study of Mohamed et al. (2020), the crude hemolymph from the adult of *Trachyderma Philistina* (Coleoptera: Tenebrionidae) possessed antitumor activities were seen against different cancer cell lines like breast carcinoma cells (MCF-7), hepatocellular carcinoma (HEPG2) and *Mouse Myelogenous* Leukemia carcinoma cells (M-NFS-60 cell line) (Mohamed, El-Ebiarie & Essam, 2020).

The results of Orozco-Flores et al. (2020) it was shown that *Megalopyge opercularis* hemolymph extracts had antitumor activity against L5178Y-R murine lymphoma cells, involving apoptosis as the cell death mechanism, and also a potential activity in stimulating IL-1β, IL-6, IL-8, and TNF-α in Human peripheral blood mononuclear cells (hPBMC) and it is well known that cytokines are important to regulate host immune responses to infection, cancer, and tissue damage (Orozco-Flores *et al.*, 2020). Finally, according to the research of Zhao et al. (2021), the hemolymph of *Aspongopus chinensis* significantly inhibited *in vitro* tumor cell migration and viability while inducing apoptosis on murine 4T1 (TCM32) and human HCC1937 (TCHu148) breast cancer cell lines; furthermore, it inhibited *in vivo* tumor growth and metastasis with a minimal effect on mouse body weight, and did not induce liver or kidney damage (Zhao *et al.*, 2021).

1.3.4 Limitations of therapeutic applicability of ACPs and future perspectives

The capacity of antimicrobial insect peptides to both eradicate microbial infections and prevent the growth of tumor cells has generated interest in these molecules. This distinct characteristic offers a novel and complimentary viewpoint to established treatment approaches, opening up new avenues in the field of cancer therapy. Despite the increasing attention and optimism around these substances, their medicinal possibilities remain mostly unexplored and need an in-depth understanding of their mechanisms of action, structural characteristics, and prospective clinical uses (Ratcliffe, Azambuja & Mello, 2014). The delayed availability of AMPs for therapeutic application has occurred for a variety of reasons. One of these is the high production costs for the development of the AMPs as the cost of solid phase production is quite costly and they are limited naturally at low amounts (Ratcliffe *et al.*, 2011). Progress, however, is proceeding rapidly to reduce production costs, and numerous researches describe the synthesis of truncated synthetic analogues with higher killing activity and potential of lower production costs mainly through the use of modified, cost-effective recombinant technologies using *E. coli* or the methylotrophic yeast *Pichia pastoris* as vectors, to produce fully functional insect cecropins (Hansen *et al.*, 2012; Iwasaki *et al.*, 2009b; Bommarius *et al.*, 2010; Wang *et al.*, 2011). Furthermore, concerns relate to the stability and toxicity of ACPs for mammalian cells (Kang *et al.*, 2012; Ntwasa, Goto & Kurata, 2012). However, thanks to a better understanding of the structure-function relationships of AMPs and the introduction of computer modelling, it is now possible to design and synthesise AMPs with increased stability in serum and saline, without toxicity and with increased killing activity, by amino acid substitutions, sequence splicing, the *in silico* use of libraries of oligonucleotides coding for AMPs and advanced quantitative structure-activity relationship (QSAR) models and genetic engineering methods (Guralp *et al.*, 2013; Maccari *et al.*, 2013).

Research on ACPs is limited due to their complexity and lack of *in vivo* and clinical experiments, and the most significant obstacles to clinical use are: enzymatic degradation, inactivation of peptides through interactions with negatively charged serum proteins, haemolytic toxicity and ease of hydrolysis (Deslouches & Di, 2017). To address these issues, one can optimise the structure of ACPs, which is crucial for enhancing their anti-cancer effects, and design a new delivery and delivery system for ACP-based drugs using modern nanotechnology that protects them from enzymatic degradation and reduces their haemolytic

toxicity (Hu, Chen & Zhao, 2016; Dathe *et al.*, 2001). In addition, a specific ligand can be used to achieve direct tumor targeting of the antimicrobial peptide nanoparticles, reducing the amount of peptide required for therapeutic effect, avoiding serious side effects and releasing the drugs at the necessary time and site: this increases the concentration of the peptide at the tumor site, improves the anti-tumor efficacy of ACPs and reduces toxic side effects on normal tissues (Dong *et al.*, 2024; Diao *et al.*, 2012). Peptide chirality can be also altered to decrease the sensitivity to proteolytic degradation by substituting native L-aminoacids with their D-enantiomers, which are indigestible by serum proteases and because they have less conformational flexibility, cyclic peptides also lessen the possibility of degradation (Tornesello *et al.*, 2020; Uggerhøj *et al.*, 2015).

1.4 *Hermetia illucens* as an important source of Antimicrobial and Anticancer Peptides

AMPs are produced by all living organisms, but insects, that constitute about 55% of the total biodiversity on Earth, are among the richest and most innovative sources of AMPs (Chernysh *et al.*, 2002). In particular, as reported in many other papers, the insect *Hermetia illucens* L. (Diptera: Stratiomyidae), also known as Black Soldier Fly (BSF), native to the Americas, represents an extraordinarily rich source of AMPs probably due to its ability to live in hostile environments, feeding on decaying substrates, rich in microbial colonies, so is also an insect of significant ecological and economic importance (Manniello *et al.*, 2021; Moretta *et al.*, 2021; Moretta *et al.*, 2020; Scieuzo *et al.*, 2023b; Scala *et al.*, 2020; Di Somma *et al.*, 2022). In general, the larvae of *H. illucens* are notable for their ability to efficiently convert organic waste into high-quality protein and fat, making them invaluable in waste management and sustainable agriculture, but also in biotechnology and medicine, highlighting the multifaceted benefits of this remarkable insect, also as a crucial contributor to the development of innovative anticancer treatments (Scieuzo *et al.*, 2023a; Franco *et al.*, 2024; Scala *et al.*, 2020; Franco *et al.*, 2021).
It has reported an overview of *H. illucens* AMPs identified in different transcriptomes, that were *in silico* analyzed in order to characterize them for the chemo-physical properties and the putative biological activities and some of them were predicted as endowed with an anticancer activity (ACPs) (Moretta *et al.*, 2020).
AMPs are synthesized in different tissues of insects and are present in various anatomical structures and mainly in the hemolymph, the insect circulatory fluid where AMPs are released

and through which can reach all the organism to combat invading microbes (Stączek, Cytryńska & Zdybicka-Barabas, 2023; Zhou *et al.*, 2024).

Several research papers investigates various facets of antimicrobial peptides (AMPs), with a particular focus on those derived from the black soldier fly and underscores the potential of these AMPs as alternative therapeutic agents also against antibiotic-resistant bacterial strains. Choi et al. (2012) explores the antibacterial effects of extracts from *H. illucens* larvae against Gram-negative bacteria. Their study reveals that methanol extracts exhibit significant antibacterial activity, particularly against *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, and *Shigella sonnei*, indicating the potential of these extracts as alternative antimicrobial agents (Choi *et al.*, 2012). De Smet *et al.* (2018) delves into the genetic and evolutionary mechanisms of bacterial resistance to AMPs, stressing the urgent need for innovative treatments amidst rising antibiotic resistance (De Smet *et al.*, 2018). Di Somma *et al.* (2022) extends this by characterizing a new recombinant AMP from the BSF, demonstrating its structural properties and its efficacy in inhibiting *E. coli* growth, thereby highlighting the promise of insect-derived AMPs in medical applications (Di Somma *et al.*, 2022). Manniello *et al.* (2021) further examines the clinical potential of AMPs, particularly those effective against antibiotic-resistant pathogens, and discusses strategies for integrating these peptides into modern medical practice (Manniello *et al.*, 2021). The research from Elhag *et al.* (2017) focuses on identifying and characterizing AMPs from the BSF and, in particular, seven novel AMP genes were identified, named cecropinZ1, sarcotoxin1, sarcotoxin2a, sarcotoxin2b, sarcotoxin3, stomoxynZH1, and stomoxynZH1(a) and were cloned and expressed in *E. coli* to produce recombinant peptides, which were then purified and tested for antimicrobial activity: the study found that these AMPs exhibited significant bactericidal activity, particularly against Gram-positive bacteria such as *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*, as well as Gram-negative bacteria like *Pseudomonas aeruginosa* (Elhag *et al.*, 2017). According to Lee *et al.* (2020), whereas it is well known that the expressions of AMPs in the larvae of the black soldier fly is significantly increased by pathogen or stimulant induced innate immunity activation, it had been demonstrated that immunized *H. illucens* fifth instar larvae with five different *Lactobacillus* species (*Lactobacillus acidophilus*, *L. brevis*, *L. casei*, *L. fermentum*, or *L. delbrueckii*), induced the mass production of AMPs in the hemolymph and the extract had been evaluated against three salmonella species (*Salmonella pullorum*, *Salmonella typhimurium*, and *Salmonella enteritidis*) inducing cytotoxicity (Lee, Yun & Goo, 2020). These research works

underline the importance and interest of the immunisation process of *H. illucens* larvae in inducing immune responses and producing peptides capable of significant and various activities, highlighting their potential in the development of new therapies. Also in works of Park *et al.* (2014 and 2015), the process of immunization of the larvae is essential to first demonstrate that the larval extract possessed a broad spectrum of antibacterial activity, and then the possibility of induce and purify novel antimicrobial peptides using, for example, solid-phase extraction and reverse-phase chromatography (Park, Chang & Yoe, 2014; Park, Kim & Yoe, 2015).

The presence of putative ACPs in *H. illucens* was previously predicted in the study of Rajasekhar *et al.* (2020): in particular, crude protein extraction from *H. illucens* larvae showed inhibitory activity against MCF7 breast cancer cell line and HeLa cervical carcinoma cell line through cytotoxicity and cell cycle inhibition of G1 and S phase (Rajasekhar, Ramesh & Prashantha, 2020).

Being the AMPs preferentially released in the hemolymph in response to microbial infections, in this research study, the potential anticancer activity of peptide fraction (PF) extracted from hemolymph collected from *H. illucens* larvae uninfected or infected with two different bacteria was investigated. The results obtained using *in vitro* models of colorectal and gastric cancer cells suggest the presence of biologically active peptides in the hemolymph, thus confirming that the study of insect-derived peptides with anticancer activity is a promising area of research also in oncology with the potentiality to allow the identification of new effective anticancer agents to be used alone or synergistically with commercial drugs.

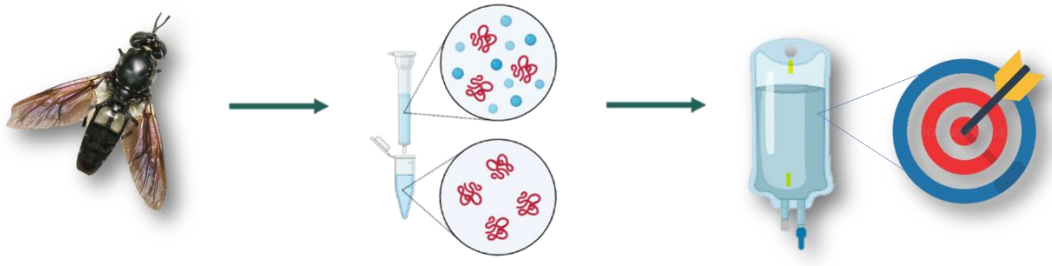


Figure 16. Application process of antimicrobial and anticancer peptides from *Hermetia illucens*. The insect represents a promising source of AMPs, which can be isolated from the hemolymph, purified, and subsequently used to develop novel targeted therapeutic strategies (Drawn by BioRender).

2. AIM OF THE STUDY

Cancer remains one of the leading causes of death worldwide, and conventional therapies are still limited by severe side effects and the development of multidrug resistance in tumor cells. Strategies to overcome these challenges include early diagnosis, adaptive monitoring, development of new targeted drugs, and combination therapies. In this context, antimicrobial peptides (AMPs), studied since 1957, have gained great relevance: some display cytotoxic activity against tumor cells and are termed anticancer peptides (ACPs). Insects, in particular, represent a rich yet underexplored source of AMPs.The principal aim of this experimental research project had been to identify, from the insect *H. illucens*, pharmacologically active principles of natural origin, deepening the understanding of their mechanisms of action, for use in the future development of alternative anticancer drugs or in support of conventional therapies already in use.

It is on these themes that the research group of the Laboratory of Physiology and Molecular Biology of Insects (Department of Science) at the Università degli Studi della Basilicata under the supervision of Prof. Patrizia Falabella has focused its attention to evaluate the activity of insect ACPs as potential anticancer compounds of natural origin, in collaboration and with the support of the research laboratory of Institute of General Pathology of Biological Institutes at Università Cattolica del Sacro Cuore in Rome under the supervision of Prof. Alessandro Sgambato, the Laboratory of Pre-clinical/Translational Oncology Research of the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS CROB) in Rionero in Vulture under the supervision of Prof. Geppino Falco and Dr. Simona Laurino, and the Department of Genitourinary Medical Oncology, Division of Cancer Medicine at the University of Texas - MD Anderson Cancer Center (MDACC) under the supervision of Prof. Giannicola Genovese and Dr. Luigi Perelli for the period abroad.

Specifically, through a combination of approaches, several experimental evaluations were performed on different cell lines with peptide fractions (PFs) derived from the hemolymph extracted from larvae of the insect *H. illucens*, phenotypically analysing the cellular response in order to set the ideal conditions for developing effective and safe therapies, thus satisfying the growing need for even less invasive solutions with fewer deleterious side effects.

This research work offers a general overview of the research context and objectives providing a detailed background of the topics covered, identifying the possible molecular functions of

ACPs from insects within tumor cells and evaluating their therapeutic potential in preclinical models, as well as suggesting their potential implications and future applications.

3. MATERIALS AND METHODS

3.1 Chemicals and Antibodies

The chemicals and antibodies used in for the present work are listed below :

Methanol, acetic acid and hexane were purchased from Merck Millipore, Burlington, MA, USA.

Dulbecco's Modified Eagle's Medium (DMEM), Penicillin-Streptomycin solution, and L-glutamine solution were purchased from EuroClone (Pero, MI, Italy).

Fetal Bovine Serum (FBS) was purchased from Gibco® by Life Technologies.

MTT [3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Merk Life Science (Darmstadt, Germany).

WesternBright TM ECL Kit for Western blotting detection was purchased from Advansta (Bering Drive San Jose, CA, USA).

Primary monoclonal antibodies purchased from Santa Cruz Biotechnology, Inc. included: mouse anti-human monoclonal GAPDH (H-284; sc-30220), mouse monoclonal anti-human β-Actin (C-4; sc-47778), mouse monoclonal anti-human PARP-1 (N-20; sc-1561), monoclonal anti-human p21 (M-19; sc-471), mouse monoclonal anti-human cyclin D1 (72-13G; sc-450). Primary monoclonal antibody Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (#9664) was purchased from Cell Signaling Technology (CST, Danvers, MA, USA).

Phosphatase assay kit was purchased from G-Biosciences (St. Louis, MO, USA).

TWEEN® 20 was purchased from PanReac Applichem (Darmstadt, Germany).

Dead Cell Apoptosis Kit with Annexin V FITC & Propidium Iodide for Flow Cytometry was purchased from ThermoFisher Scientific (Waltham, MA, USA).

Cell Cycle Kit was purchased from Beckman Coulter (Brea, CA, USA).

4X Laemmli Sample Buffer, Protein Assay Dye Reagent Concentrate (Bradford Reagent), 10X Tris/Glycine Buffer, and 10X Tris/Glycine/SDS Buffer were purchased from Bio-Rad Laboratories (Hercules, CA, USA).Thiazolyl Blue Tetrazolium Bromide (MTT), Skim Milk Powder, TWEEN® 20, and Phosphate buffered saline (PBS) washing buffer for peroxidase conjugates in Western Blotting 10x concentrate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4X Laemmli Sample Buffer, Protein Assay Dye Reagent Concentrate (Bradford Reagent), 10X Tris/Glycine/SDS Buffer, and Clarity™ Western ECL Substrate were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

0.05% Trypsin-EDTA 1X, Dulbecco's Modified Eagle Medium (DMEM) 1X, Dulbecco's Phosphate Buffered Saline (DPBS) 1X, Iscove's Modified Dulbecco's Medium (IMEM) 1X, Fetal Bovine Serum (FBS) Qualified, L–Glutamine 200mM (100X), and Penicillin-Streptomycin Solution were purchased from Gibco® by Life Technologies (Grand Island, NY, USA).

PageRuler™ Prestained Protein Ladder, Halt™ Protease Inhibitor Cocktail (100X), and Pierce™ RIPA Lysis and Extraction Buffer were purchased from Thermo Scientific™ (Waltham, MA, USA).

The FITC Annexin V Apoptosis Detection Kit I was purchased from BD Pharmigen™ (San Diego, CA, USA).

Primary antibodies: PARP (46D11) Rabbit mAb (#9532); Caspase-3 Antibody (#9662); Bcl-2 (50E3) Rabbit mAb (#2870); Cyclin E2 Antibody (#4132); p21 Waf1/Cip1 (12D1) Rabbit mAb (#2947); p27 Kip1 (D69C12) Rabbit mAb (#3686); β-Actin (8H10D10) Mouse mAb (#3700; dilution 1:1000; Cell Signaling Technology®, CST, Danvers, MA, USA) were purchased from Cell Signaling Technology® (CST, Danvers, MA, USA). Cyclin B1 (G-11) (sc-166757) and Vinculin (7F9) (sc-73614) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Secondary antibodies: Anti mouse IgG-HRP-linked (#7076), and Anti-rabbit IgG-HRP-linked (#7074) were purchased from Cell Signaling Technology® (CST, Danvers, MA, USA).

3.2 Cell lines and culture conditions

In this experimental work, two cell lines of colorectal carcinoma and adenocarcinoma and two cell lines of gastric carcinoma and adenocarcinoma were used for *in vitro* studies to test peptide fractions (PFs).

In particular HT29 (RRID:CVCL_0320) is a cell line with epithelial morphology that was isolated in 1964 from a primary tumor obtained from a 44-year-old, White, female patient with colorectal adenocarcinoma and HCT116 (RRID:CVCL_0291) cell line was isolated from the colon of an adult Caucasian male with colorectal carcinoma; both cell lines were obtained by American Type Culture Collection (ATCC, Manassas, VA, USA) and were used for *in vitro* studies with PFs extracted from the hemolymph of *Hermetia illucens* and propagated in

Dulbecco's Modified Eagle's Medium (DMEM, EuroClone, Pero, MI, Italy) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (Corning®), and 2 mmol/L *L*-glutamine (EuroClone, Pero, MI, Italy) at 37°C in a humid 5% CO_2 atmosphere.

The other two cell lines were KATO III (RRID:CVCL_0371) cells, that have spherical morphology and were established *in vitro* from a pleural effusion of a 55-year-old, Asian male patient with gastric carcinoma and AGS (RRID:CVCL_0139) cells that is a cell line exhibiting epithelial morphology that was isolated in 1979 from the stomach tissue of a 54-year-old, white, female patient with gastric adenocarcinoma. Both cell lines were purchased from ATCC (Manassas, VA, United States).

KATO III cells were propagated in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco™, Waltham, MA, USA) with *L*-glutamine and supplemented with 20% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin at 37°C in a humid 5% CO_2 atmosphere, and AGS cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) - low glucose (1 g/l) (Gibco™, Waltham, MA, USA) with *L*-glutamine and 110 mg/l Sodium Pyruvate and supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin at 37°C in a humid 5% CO_2 atmosphere.

Figure 16. Thermo Scientific™ Series 8000 Direct-Heat CO_2 Incubator.

The hemolymphatic extracts used in biological assays for the treatment of cells had an initial concentration of the stock solution of 2 µg/µl diluted in DMEM to obtain the desired final concentrations to be used. Prior to treatment on cells, the medium with the hemolymphatic

extracts was filtered using sterile 0.22 µm Millex-GS filters (Millipore®, Burlington, MA, USA).

3.3 Cell counting

The hemocytometer, also known as the Bürker cell counting chamber, was used for the seeding cells on plates for each type of experiment conducted in this research work.

Figure 17. Bürker cell counter (LO - Laboroptik Ltd, Lancing, UK).

It consists of a rectangular slide made of glass on which there are two incised areas, each divided into 9 squares of 1 mm, each with 16 squares of 0.2 x 0.2 mm and an area of 1/25 mm^2. A coverslip is placed above the chamber and a few microliters of cell suspension are transferred to both areas by placing the pipette tip on the edge, so that the liquid flows out through capillarity and without forming air bubbles. Since each square of the Burker chamber has a volume of 0,1 mm^3, equivalent to 1 ml, the number of cells per millilitre is obtained by dividing the number of cells counted by a manual cell counter by the number of squares where the count is made and multiplying this value by the cell suspension dilution factor and by 10^4, which is the conversion factor related to the chamber volume:

$$\text{cells/ml} = \text{Average number of cells} \cdot \text{dilution factor} \cdot 10^4$$

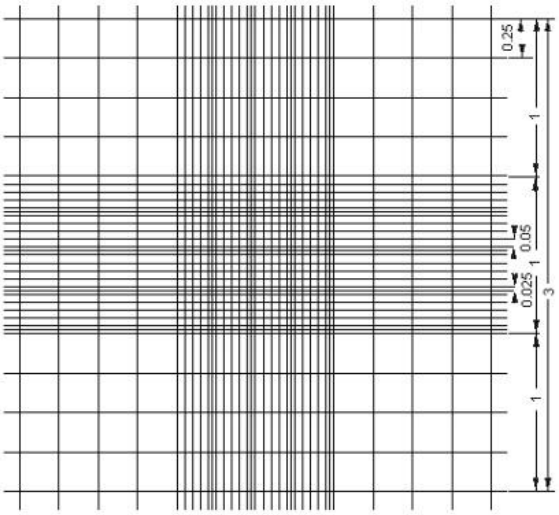


Figure 18. Cell counting chamber scheme.

The camera was placed on the stage of the inverted microscope and the 10X objective was inserted, focusing with the macrometric and micrometric screw.

Figure 19. Inverted Routine Microscope ECLIPSE Ts2 (Nikon®, Tokyo, Japan).

Figure 20. Manual cell counters.

Trypan blue was used to count KATO III cells, because they grow partly in suspension and partly in adhesion. It is a blue cell viability dye with formula: $C_{34}H_{24}N_6Na_4O_{14}S_4$, 0.4%, liquid and sterile filtered. Viable cells exclude dye, while nonviable cells absorb the dye and appear blue, and cells should be in suspension as single cells before counting.

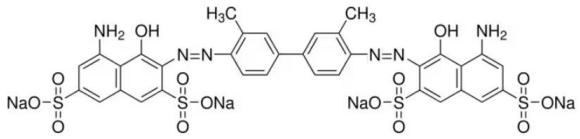


Figure 21. Trypan blue chemical structure.

3.4 Preparation of samples

3.4.1 Rearing of *Hermetia illucens*

The black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae) can be considered one of the most significant insect in the world of bioconversion (Makkar *et al.*, 2014), indeed this insect's larvae can digest a variety of organic substrates to produce body mass mostly made up of chitin, lipids, and proteins (Čičková *et al.*, 2015). The life cycle of *H. illucens* is brief, lasting between 40 and 45 days in optimal substrate and environmental conditions (Tomberlin, Sheppard & Joyce, 2002): a larva hatches from an egg after about four days, undergoes through five instars from thirteen to eighteen days, and then emerges as an adult fly after the prepupal and pupal phases. The adults survive for around five to eight days, during which time they reproduce. After that, the female deposits 500–900 eggs (De Smet *et al.*, 2018).

The capacity of *H. illucens* larvae to develop on a variety of organic substrates at varying stages of decomposition prompts interesting inquiries regarding the function of the microbiome in these processes and the defense systems in place in the insect gut that keep noncommensal bacteria from taking over the microbial population. BSF has immunological systems that allow it to survive and grow even on substrates heavily polluted with microbes. Because of this, the state of the art at the moment provides insight into the possibilities for using and modifying the *H. illucens* microbiome in support of improved zootechnical performance and insect-derived antimicrobial approaches (Nguyen, Tomberlin & Vanlaerhoven, 2015).

Sperimentally, *H. illucens* adults and larvae were raised in a colony kept at the Laboratory of Insect Physiology and Molecular Biology of University of Basilicata (Potenza, Italy). Larvae were reared under monitored conditions, with temperature 27 ± 1.0 ◦C, relative humidity of 70% ± 5%, and photoperiod of 12 L:12 D (light: dark, hours). The larvae were fed a conventional Gainesville diet (30% alfalfa, 50% wheat bran, 20% corn meal) 6 at 70% moisture (Hogsette 1992). The resulting pupae were raised in the same environment but were moved to a different room for their transformation into adults (Scieuzo *et al.*, 2021).

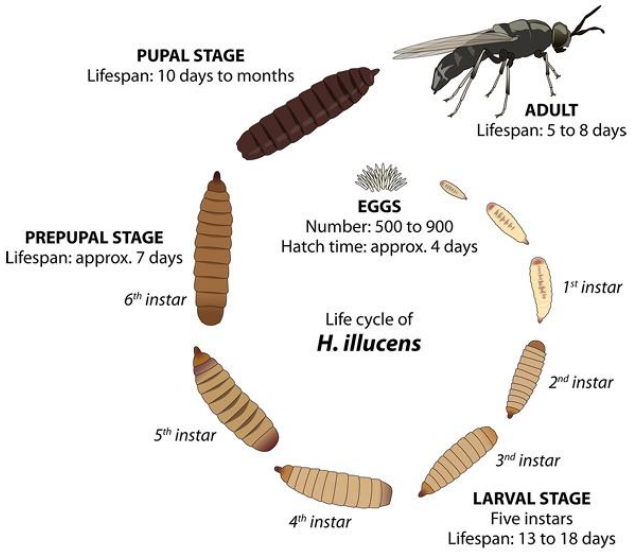


Figure 22. Life cycle of *H. illucens* (source: De Smet J. *et al*., 2018).

3.4.2 Infection of *Hermetia illucens* larvae and hemolymph extraction

The larvae of *H. illucens* (Diptera: Stratiomyidae) were washed with distilled water and 70% ethanol, and subsequently, to stimulate AMPs production, were infected with injection into the hemocelic cord with fine glass capillaries immersed in the suspension of the Gram positive bacteria *Micrococcus flavus* (DSM 1790; cultured to exponential phase OD600 = 1) or the Gram negative bacteria *Escherichia coli* (LGM 2092; cultured to exponential phase OD600 = 1) and incubated at 25 ºC and 70% of humidity for 24h. Uninfected larvae were considered as controls. After the incubation period, the hemolymph of infected and uninfected larvae was extracted in tubes containing 0,015 g L-ascorbic acid and kept on ice until the end of the procedure; L-ascorbic acid has been used to prevent the melanization process of hemolymph following contact with air. The tubes were then centrifuged at 10,000 rpm for 5 minutes at 4 ºC

to recover the plasma and remove the corpuscular part and any debris and kept at -80 ºC until their subsequent use (Scieuzo *et al.*, 2023b).

3.4.3 Isolation of Peptide Fraction via Organic Solvents Precipitation

The plasma was precipitated by organic solvents including methanol, acetic acid and distilled water, as reported in Scieuzo *et al*. (2023), obtaining a supernatant containing peptides with a molecular weight lower than 30 kDa and a pellet with proteins at higher molecular weight (Scieuzo *et al.*, 2023b). The organic solvents used for precipitation included methanol, acetic acid and distilled water in a 90:1:9 v/v ratio 298 and samples and solvent were mixed in a 1:9 v/v ratio. The sample was centrifuged for 45 min at 16,000 rcf at 4 ◦C. The obtained supernatant, containing compounds with a molecular weight lower than 30 kDa, was then vacuum dried to remove the organic solvents and resuspended in a volume of sterile Milli-Q® (Burlington, MA, USA) ultrapure water equal to the original plasma volume. The samples thus prepared were stored at -20 ºC until their subsequent use.

For convenience, samples of PFs from hemolymph extracts have been renamed with the following abbreviations: PF CTR, PF *E. coli* and PF *M. flavus*, which stand for peptide fraction from hemolymph extracted from uninfected larvae, from larvae infected with *E. coli* and from *M. flavus*, respectively.

3.4.4 Bradford Assay for the determination of protein concentration of peptide fractions

The protein content of PFs derived from hemolymph was measured according to the Bradford method (Kielkopf, Bauer & Urbatsch, 2020), a colorimetric assay based on the spectral properties of the Coomassie® Brilliant Blue G – 250 dye (Bio-Rad Protein Assay®, Hercules, CA, USA), which in free form absorbs at a wavelength of 465 nm, while binding to basic and aromatic residues of proteins forms a blue-colored complex whose optical absorption is measured at a wavelength of 595 nm.

The development of this colorimetric reaction enables the quantification of the protein content of an unknown sample, since the amount of dye binding and, therefore, blue color intensity, will be directly proportional to the amount of protein in the sample. Thus, Beer's law may be

applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

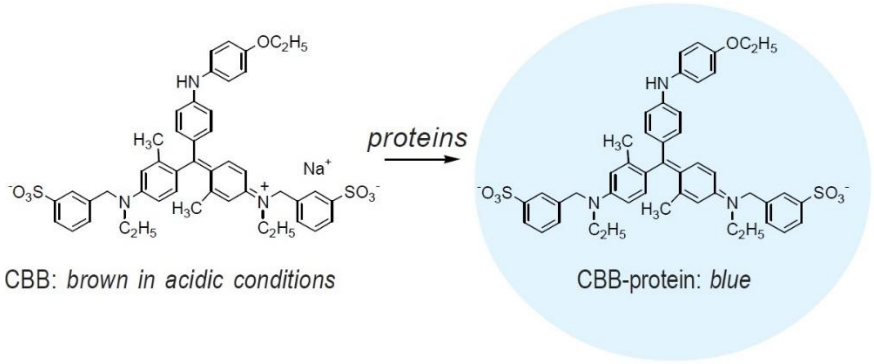


Figure 23. Chemical reaction of Coomassie® Brilliant Blue G – 250 dye (Bio-Rad Protein Assay®, Hercules, CA, USA).

Eight dilutions (0 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml) of Bovine Serum Albumin (BSA) protein standard in distilled water, representative of the protein solution to be tested, have been prepared in a total final volume of 500 µl.

BSA	***Abs***
0,25	0,05
0,5	0,08
1	0,12
2	0,175
4	0,258
8	0,518
16	0,9

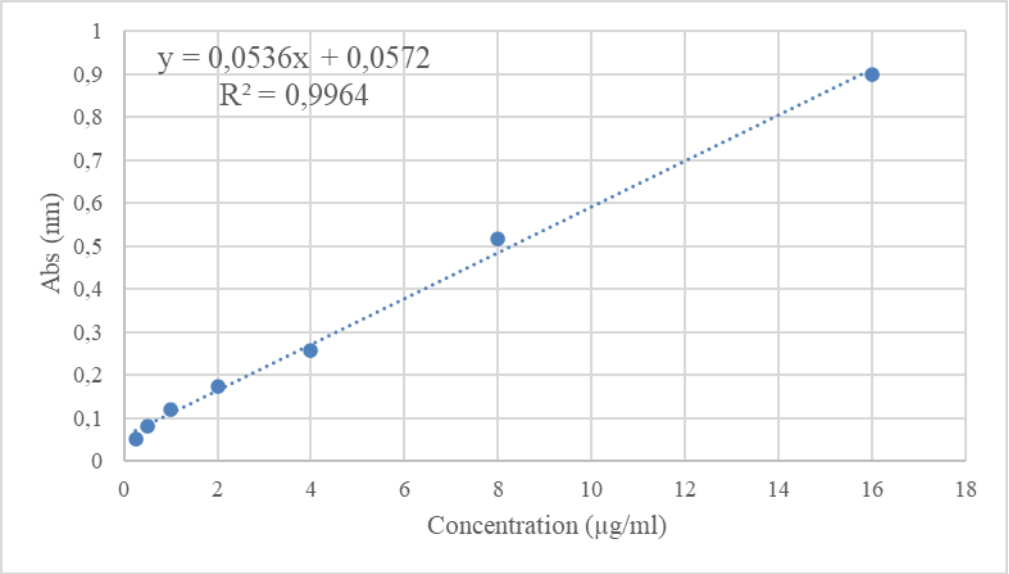


Figure 24. Standard calibration line for BSA in Bradford assay; (Abs)595nm.

Of these standard samples, absorbance values were measured to construct a calibration line from which protein concentrations of the unknown samples of peptide fractions prepared with 1 µl of sample and distilled water up to 500 µl were derived by extrapolation. This was made possible by adding, prior to spectrophotometer reading, to each known sample and each unknown sample of hemolymphatic extract, 500 µl of Bradford's reagent in water (by diluting 1 part Dye Reagent with 4 parts distilled, deionized (DDI) water), incubating for 5 min in the dark at room temperature and then proceeding to measure the absorbance in disposable plastic cuvettes at the wavelength of 595 nm with a NanoDrop™ One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Scientific™, Waltham, MA, USA). Once the values were obtained, they were interpolated in the calibration curve that made it possible to determine the protein concentration of the samples expressed in µg/µl and the concentration was standardized at 2 µg/µl for all samples used on colorectal cancer cell lines and at 1 µg/µl for all samples used on gastric cancer cell lines.

Figure 25. NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific™).

3.5 MTT Assay for measurement of Cell viability after treatment with peptide fractions

The MTT [3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to assess cytotoxicity levels following cell treatment with hemolymphatic extracts. MTT is a yellow compound that undergoes a reduction by mitochondrial dehydrogenases (to generate reducing equivalents of NADH or NADPH) present in live cells and therefore active at the metabolic level, which cut the tetrazole ring of tetrazolium salt, determining the opening and formation of the formazan salt, a blue/purple nitrogen chromogen compound.

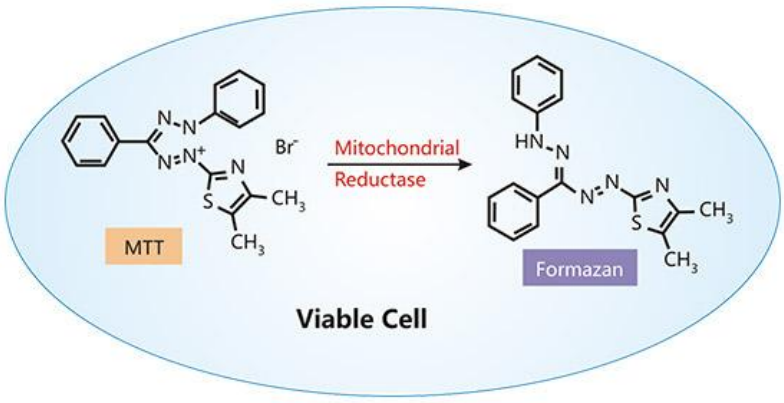


Figure 26. Chemical reaction of the MTT test (source: BroadPharm).

Formazan, present in the form of crystals of violet color in the intracellular environment and insoluble in an aqueous environment, has been solubilized following the addition of an organic solution (called stop solution) consisting of isopropanol with 0.04% of HCl. The intensity of staining, to be evaluated spectrophotometrically, is related to the amount of formazan that is produced in live cells and is, therefore, an index of the population of cells that remained viable following treatment at different concentrations of the substances tested, in comparison also with the viability index in controls of untreated cells.

Experimentally HT29 and HCT116 cells were seeded in 96-well microtiter plates (8×10^3 cells per well), KATO III cells and AGS cells were seeded in 96-well microtiter plates (5×10^3 cells per well) and incubated for 24 hours. Medium was then replaced to treat HT29 cells, HCT116 cells and AGS cells and added to the existing one to KATO III cells (cells in suspension) for 48 and 72 hours with different dilutions (1:6400, 1:3200, 1:1600, 1:800, 1:400, 1:200, 1:100, 1:50), corresponding to 40 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.62 µg/ml, 0.31 µg/ml, of PFs (2 µg/µl of stock solution) obtained from larvae infected with *E. coli* (PF *E. coli*), with (PF *M. flavus*) *M. flavus* and uninfected larvae (PF CTR).

The same method, at the same dilutions, HT29 cells and HCT116 cells were treated, as well as with PFs, also with Oxaliplatin (Selleckchem, Houston, TX, USA) and Fluorouracil (5-Fluoracil, 5-FU, Selleckchem, Houston, TX, USA) 300,301 at their IC_{30} concentrations (respectively 8 µM for Oxaliplatin and 48 µM for 5-Fluorouracil) (Longley, Harkin & Johnston, 2003; Shi *et al.*, 2002) to evaluate the possible synergistic effect of natural substances with chemotherapy drugs used in current therapies for the treatment of colorectal cancer. As negative control, fresh medium was added in control wells. At the end of the treatment, the medium was aspirated and the plate incubated for 4 hours in the dark at 37 °C and 5% CO_2 after adding to each well 100 µl of 0.75 mg/ml MTT (Sigma Aldrich-Merck®) solution starting from a stock solution of 5 mg/ml. After 4 hours, this solution was aspirated, noting the formation of formazan crystals remaining on the bottom, immediately solubilized in 100 µl of the stop solution (composed by isopropanol and DMSO in a 1:1 ratio + 1% Triton or by acidified isopropanol) for each well; the plate was left in the dark for an additional hour stirring at room temperature. The amount of MTT formazan crystals was evaluated by measuring absorbance with Tecan Spark® multimode microplate reader at a wavelength of 570 nm and a reference wavelength (background subtraction) of 630 nm. The 50% inhibitory concentration (IC_{50}) was calculated using GraphPad Prism software (San Diego, CA, USA).

From this point onwards, cells were treated for 48 h with concentrations of 40 µg/ml, 20 µg/ml and 5 µg/ml (for HT29 and HCT116 cells) and 20 µg/ml, 10 µg/ml and 2.5 µg/ml (for AGS and KATO III cells) of each sample in all subsequent experiments. The 48-hour treatment time was selected because it would allow significant effects of PFs on the cells to be observed, while maintaining a time window in which the cells themselves respond to the treatment without the effects becoming too extreme as in prolonged treatments. The concentrations selected were chosen to be close to the IC_{50}, with the aim of including a concentration capable of producing a significant effect, which would allow a clear response to be observed in the cells, a concentration in which the cells would give a response as close as possible to the untreated control and a concentration intermediate between the two previous mentioned.

Figure 27. Tecan Spark® (Männedorf, Switzerland) multimode microplate reader.

3.6 Observation of Morphological Changes

HCT116 and HT29 cells (from $3{,}5 \times 10^5$ to $4{,}5 \times 10^5$ cells), AGS ($2{,}5 \times 10^5$ cells per well) and KATO III (4×10^5 cells per well) cells were seeded into 6-well plates and cultured overnight. Then they were treated for 48 h with different concentrations of PFs (40 µg/ml, 20 µg/ml, 5 µg/ml for HT29 and HCT116 cells and 20 µg/ml, 10 µg/ml, 2.5 µg/ml for AGS and KATO III cells), using untreated cells as negative controls. The cellular morphology was observed using the inverted microscope ECLIPSE Ts2 (Nikon®, Tokyo, Japan) for HCT116 and HT29 cells and the inverted microscope Axio Vert.A1 (Zeiss™, Oberkochen, Germany) for AGS and KATO III cells.

Figure 28. Inverted research microscope Axio Vert.A1 (Zeiss™, Oberkochen, Germany).

3.7 Alkaline phosphatase (ALP) Assay

Alkaline phosphatase level (APL) activity was evaluated in HT29 and HCT116 cells using a Phosphatase assay kit (G-Biosciences, St. Louis, MO, USA), designed to measure the activity of phosphatases in biological samples and to screen for agonists and inhibitors of phosphatases, according to the manufacturer's instructions. This kit uses *para*-nitrophenyl phosphate (*p*NPP), a chromogenic substrate for most phosphatases, including alkaline phosphatases, acid phosphatases, protein tyrosine phosphatases and serine/threonine phosphatases; all these components remove the phosphate group to generate p-nitrophenol, which is deprotonated under alkaline conditions to produce p-nitrophenolate that has strong absorption at 405 nm (Lucchetti *et al.*, 2020).

Experimentally, cells were seeded in 12-well plates at $1{,}2 \times 10^5$ cells per well and treated for 48 hours with different dilutions of the PFs obtained from larvae infected with *E. coli*, with *M. flavus* and uninfected larvae (PF CTR) and then were lysed in ice with 1% Triton X-100 for 20 min. Then 50 µL of total cellular lysates were incubated for 30 minutes at room temperature with 50 µL of PA Substrate (Phosphatase Assay Substrate) and 50 µL PA Assay Buffer and, at the end of the incubation, the reaction was stopped with 50 µL of Stop Solution and the OD values were read at 405 nm for 1 h.

Spectrophotometric readings were taken on SpectraMax1 Plus 384 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) and were analyzed using the Softmax Pro

Software version 4.3 (Molecular Devices, San Jose, CA, USA) to obtain the Vmax values. Relative ALP activity was normalized by the total protein concentration.

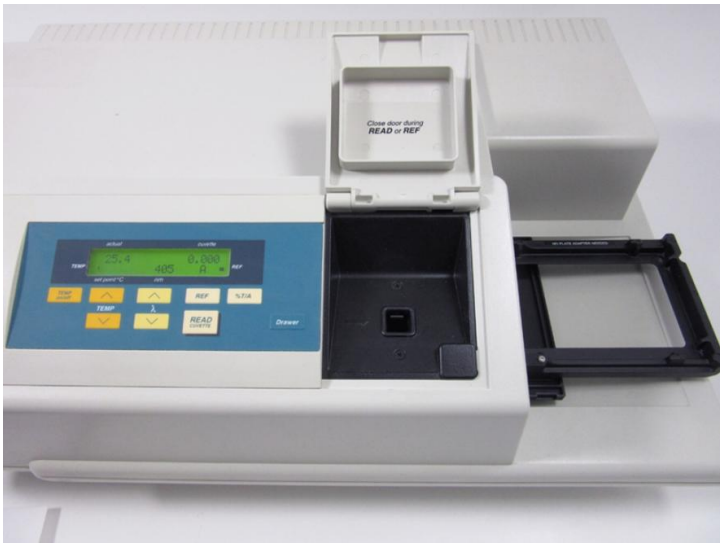


Figure 29. SpectraMax Plus 384 spectrophotometer (Molecular Devices, San Jose, CA, USA).

3.8 Cytofluorimetric Analysis for Apoptosis Assay

Apoptosis was evaluated on HT29 cells and HCT116 cells using a FITC Annexin V & Propidium Iodide/Dead Cell Apoptosis kit (ThermoFisher Scientific, Waltham, MA, USA), and AGS cells and on KATO III cells using a FITC Annexin V Apoptosis Detection kit I (BD Pharmigen™, San Diego, CA, USA), according to the manufacturer's instructions.

Phosphatidylserine (PS) is preferentially bound by a family of calcium-dependent phospholipid-binding proteins known as annexins (so called because their main property is to 'annex' to the cell membrane in a Ca^{2+}-dependent manner), having a conserved 'core' domain containing four to eight repeated units of 70 amino acids each. These repeated units are responsible for the annexins' ability to bind different phospholipids, while the binding specificity of each annexin is probably related to the N-terminal region that differs in the various proteins. In the plasma membrane's inner leaflet, PS is mostly found under normal physiological circumstances. The asymmetric distribution of PS throughout the phospholipid bilayer is lost during the initial stages of apoptosis, and it is translocated to the extracellular membrane leaflet, designating cells for phagocytosis. When PS reaches the membrane's outer surface,

fluorescently labeled Annexin V may detect it giving fluorescence to the apoptotic cell and maintaining its high affinity for PS (Rieger *et al.*, 2011).

The translocation of PS to the outer cell surface does not only occur in apoptosis, but also during necrosis. The difference is that in the early stages of apoptosis, the cell membrane remains intact, whereas during necrosis, the cell membrane loses its integrity. In order to distinguish living cells from apoptotic and necrotic ones, Annexin V is often used in double-labelling with propidium iodide (PI) (Vermes *et al.*, 1995). The latter is a synthetic dye characterised by low fluorescence (red-orange), capable of selectively binding to nucleic acids. It is useful for assessing the internucleosomal cleavage typical of apoptotic cells, as it binds stoichiometrically to the DNA double helix and provides information on the amount of DNA contained in the cells, in relation to the fluorescence intensity (the higher the DNA content, the higher the fluorescence). As apoptotic cells, once resuspended in an appropriate buffer, lose their small DNA fragments, a reduction in PI fluorescence can be observed in them that correlates with a decrease in DNA content. Indeed, viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI (Crowley *et al.*, 2016).

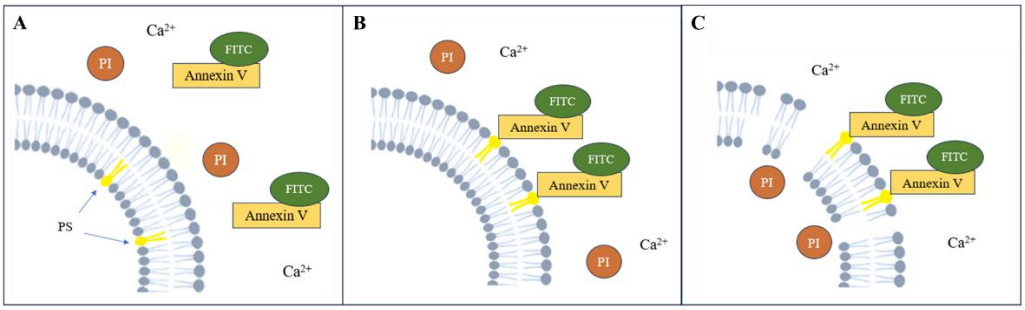


Figure 30. Schematic representation of FITC Annexin V/Propidium Iodide staining. Phosphatidylserines, typical constituents of the inner layer of the plasma membrane, are exposed on the outside of the membrane during the initial stages of apoptosis. In the presence of Ca2+ ions, Annessin V has a high affinity for phosphatidylserines and when conjugated with a fluorochrome marks apoptotic cells. Propidium iodide (PI) at low concentration enters and only marks necrotic cells. A. Normal cell, B. Early stages of apoptosis, C. Late stages of apoptosis (Drawn by BioRender).

The kits both contain recombinant annexin V conjugated to fluorescein (FITC annexin V), as well as a ready-to-use solution of the red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with FITC annexin V and PI in the provided binding buffer, apoptotic cells show green

fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.

Therefore, cells that are considered viable are negative for PI and FITC - Annexin V; cells that are in early apoptosis are positive for FITC - Annexin V and negative for PI, cells that are in late apoptosis are positive for both substances, and cells that are already in necrosis are only positive for PI.

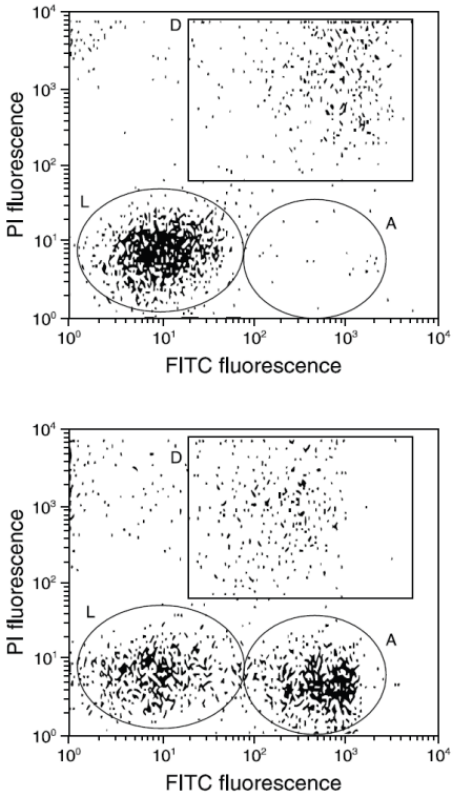


Figure 31. Example cytograms generated by the flow cytometric measurement of PI fluorescence versus FITC fluorescence of cells treated with the kit reagents. Cells treated with an apoptosis inducer (lower panel) have a higher percentage of apoptotic cells (indicated with an "A") than the basal level of apoptosis observed in control cells (upper panel). L = live cells, D = dead cells (source: Thermo Fisher Scientific).

Experimentally, HT29 and HCT116 cells were seeded in 60 mm plates (5×10^5 cells per plate) and incubated for 24 hours. After the period of incubation cells were treated with different dilutions (1:50, 1:100, 1:400), corresponding to 40 µg/ml, 20 µg/ml, 5 µg/ml, of PFs obtained from larvae infected with *E. coli*, with *M. flavus* and uninfected larvae. Untreated cells represented negative controls. After 48 hours of treatment, cells were washed once with cold

PBS, harvested, collected in new tubes in ice and were counted so that each sample reached the amount of one million cells. Each sample was resuspended in 100 µL of 1X Annexin Binding Buffer with 5 µL of FITC Annexin V and 1 µL of 100 µg/ml of PI Working Solution of the kit and incubated in the dark for 15 min at room temperature. Then, 400 µL of 1X Annexin Binding Buffer was added to each tube.

The analysis of samples was carried out by CytoFLEX flow cytometer (Beckman Coulter, Cassina de´Pecchi, Milano, Italy), measuring the fluorescence emission at 530 nm and > 575 nm. Data were analyzed with the Kaluza 2.1 analysis program.

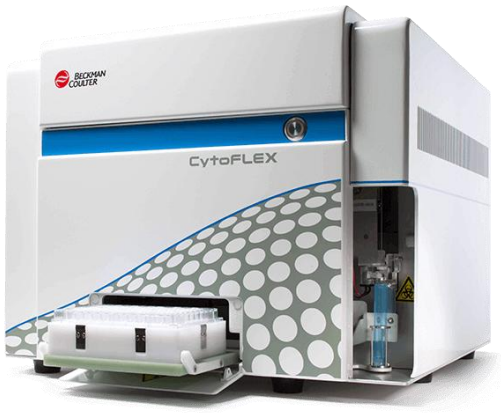


Figure 32. CytoFLEX flow cytometer (Beckman Coulter, Cassina de´Pecchi, Milano, Italy).

At the same way AGS cells (2,5 × 10^5 cells per well) and KATO III cells (4 × 10^5 cells per well) were seeded in 6 well plates and incubated for 24 hours. Cells were then treated with the same dilutions used for the other two cell lines (1:50, 1:100, 1:400), corresponding to 20 µg/ml, 10 µg/ml, 2.5 µg/ml, of PFs obtained from larvae infected with *E. coli*, with *M. flavus* and uninfected larvae. Untreated cells represented negative controls. After 48 hours of treatment, cells were washed with cold PBS, harvested, collected in new tubes and the pellet was resuspended in 100 µL of 1X Annexin Binding Buffer with 5 µL of FITC Annexin V and 5 µL of Propidium Iodide Staining Solution of the kit and incubated in the dark for 15 min at room temperature. Then, 150 µL of 1X Annexin Binding Buffer was added to each tube.

The analysis of samples was carried out by DxFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed with the Kaluza 2.1 software (Beckman Coulter Diagnostics, Brea, CA, USA).

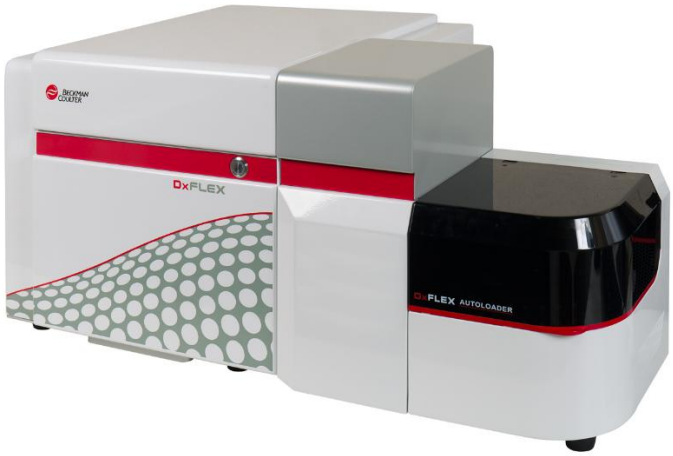


Figure 33. DxFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA).

3.9 Cytofluorimetric Analysis for Cell Cycle Assay

Using Flow cytometry to track the cell cycle is a quick method for studying the attributes of cell populations. The most commonly applied methods rely on the univariate analysis of cellular DNA content following staining with fluorescent DNA binding dyes, so, when stained with a cell cycle reagent, DNA in the cells bind the dye stoichiometrically (in proportion to the amount of DNA present in each cell) 308. Among those dyes, Propidium Iodide and DAPI (4′,6-diamidino-2-phenylindole) are commonly used for their ability to quantitatively bind to DNA; thus, the measurement of their fluorescence intensity allows the resolution of 3 major cell cycle phases, G0/G1, S and G2/M, which differ from each other by their DNA content. A histogram of the distribution of DNA content at each stage of the cell cycle is generated by the flow cytometric measurement of cell count versus linear fluorescence (Figure 34). The breakdown of cells in the G0/G1 phase vs S phase, G2, or polyploidy status of the cell population may then be established using standard modeling algorithms.

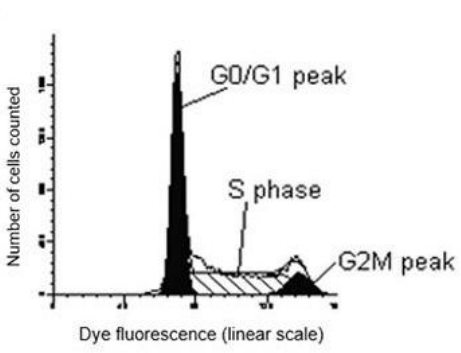


Figure 34. Histogram of the DNA content distribution across the steps of the cell cycle (source: Thermo Fisher Scientific).

Cell cycle assay was performed on HT29 and HCT116 cells using a Cell cycle kit (Beckman Coulter Life Sciences, Indianapolis, IN, USA), according to the manufacturer's instructions.
HT29 and HCT116 cells were seeded in 60 mm plates (5×10^5 cells per plate) and then incubated for 24 hours. The three different concentrations of PFs (40 µg/ml, 20 µg/ml, 5 µg/ml) obtained from *M. flavus*, *E. coli*, and from uninfected larvae (PF CTR), were used to treat cells for 48 hours after the incubation time. Negative controls were considered untreated cells. Then cells were harvested, added 1×10^6 cells per sample in new tubes in ice, filtered with 50 um filters and resuspended in ethanol 80% drop by drop. After the centrifuge at 1300 rpm for 5 minutes at 4°C, the pellets were washed with cold 1X PBS (Dulbecco's Phosphate Buffered Saline, EuroClone, Pero, MI, Italy) w/o calcium and w/o magnesium, resuspended in 0.5 mL of cell cycle kit (Cell Cycle Kit, Beckman Coulter, Brea, CA, USA) and incubated for 15 minutes at room temperature protected from direct light exposure.
The analysis of samples was carried out by CytoFLEX instrument (Beckman Coulter, Cassina de´Pecchi, Milano, Italy) set with a blue laser (488 nm) and detection filters 610/20 nm bandpass for PI. Data were analyzed with the Kaluza 2.1 analysis program.
AGS and KATO III cells were seeded in 6 well plates at a confluence of $2{,}5 \times 10^5$ and 4×10^5 cells per well, respectively, and incubated for 24 h at 37°C at 5% CO_2. After 48 h of treatment with PFs (20 µg/ml, 10 µg/ml, 2.5 µg/ml), cells were harvested, washed with PBS and fixed in ice-cold 70% ethanol for 1 h. Fixed cells were then incubated with PI/RNaseA staining solution for 30 min at room temperature and in the dark. The acquisition was performed by DxFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA) and the analysis was conducted using the Kaluza analysis software 2.1.

3.10 Western Blot Analysis

Using the immunochemical technique of Western blotting, a particular protein may be identified and separated from a complex mixture by means of its detection by certain monoclonal or polyclonal antibodies.

There are two primary procedures involved in this method:
(1) SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), is a qualitative analytical method in which proteins in a complex are separated based exclusively on their molecular weight;
(2) IMMUNOBLOTTING is the process of actually identifying the protein (antigen) using a highly specific antibody and chemiluminescence to detect its presence.

Prior to electrophoresis, the cells must be lysed in order to extract the protein content and quantify its amount.

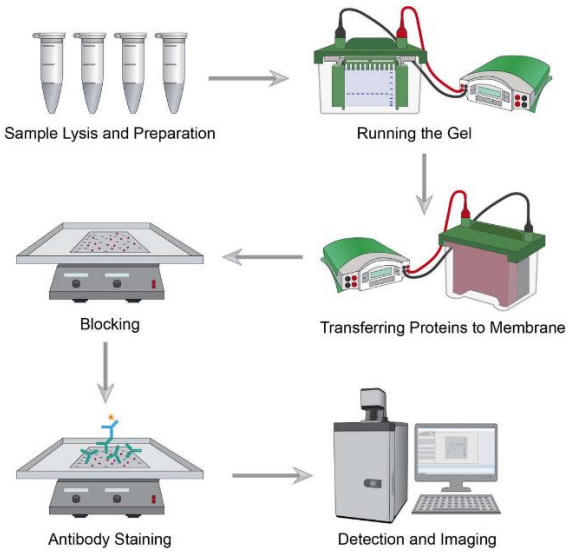


Figure 35. Workflow of Western Blot (source: Biolabs).

3.10.1 Cell lysis and protein dosage

HT29 and HCT116 cells were seeded in 60 mm Petri dishes for cell cultures (or six-well plates) at a density from $3{,}50 \times 10^5$ to $4{,}50 \times 10^5$ cells per dish or per well and treated for 48 h at the three principal concentrations for each sample of PFs. Treated cells were collected, pellets were washed in PBS and lysed for 30 min in ice-cold RIPA lysis buffer (Cell Signaling Technology,

Danvers, MA, USA) consisting of 50 mmol/L Tris-HCl pH 7.2, 5 mmol/L $MgCl_2$, 50 mmol/L NaCl, 0.25%, 0.1% SDS, and 1% Triton X-100 and supplemented with protease inhibitors (2 mmol/L phenyl methyl sulfonyl fluoride (PMSF), 10 mg/mL aprotinin, 2mg/mL leupeptin, 2 mmol/L Na_3VO_4, and 100 mmol/L NaF).

The cell pellets thus resuspended were vortexed and left on ice for 7 minutes, and these operations were repeated three times in sequence. If necessary, as an alternative to the previous cycles, the pellets could be sonicated for 30 seconds at 37% power. Subsequently, the resulting samples are centrifuged at 4°C at 13,000 rpm for 20 minutes to separate soluble proteins contained in the supernatant from insoluble ones and from cellular debris and un-lysed cells.

The protein concentrations of all samples were quantified with Bio-Rad Protein Assay, Dye Reagent Concentrate (Bradford protein assay kit II, Bio-Rad, Hercules, CA, USA), according to the Bradford method (Sissolak *et al.* 2019; Bradford, 1976) setting up a standard calibration using known concentrations of Bovine Serum Albumin (BSA) protein (Merck Millipore, Burlington, MA, USA), in particular: (0 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml) in a final volume of 800 µl with distilled water.

BSA	***Abs***
0,5	0,035
1	0,058
2	0,139
4	0,24
8	0,451
16	0,846

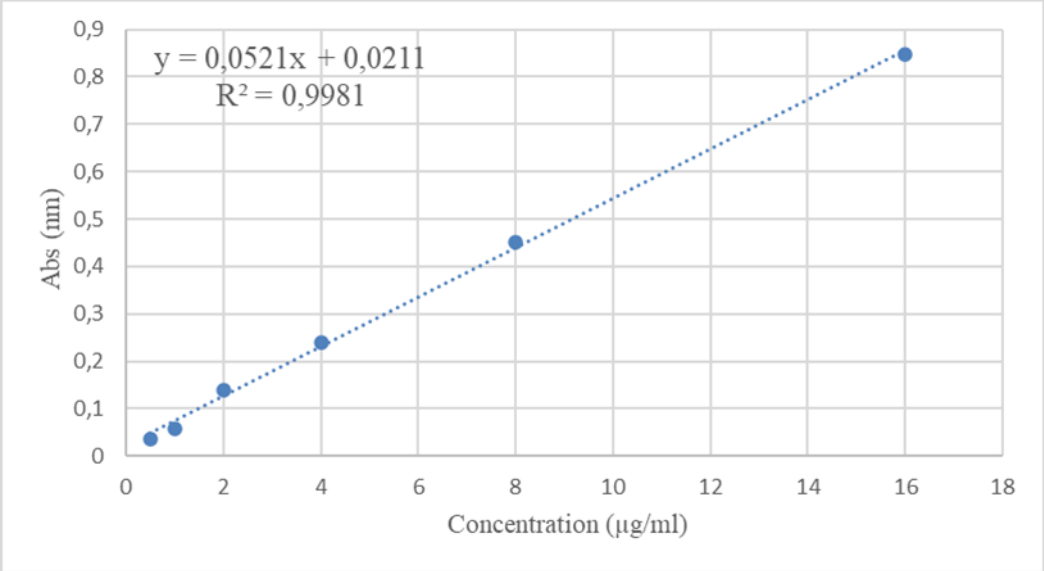


Figure 36. Standard calibration line for BSA in Bradford assay; (Abs) 595nm.

Each sample solution has been prepared with 2 µl of cellular lysates and distilled water up to 800 µl final volume into a clean, dry test tube (or alternatively 160 µl of each standard and sample solution into separate microtiter plate wells, adding 40 µl of dye reagent concentrate to each well and mixing the sample and reagent thoroughly using a microplate mixer). Then 200 µl of Bradford dye reagent concentrate (prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water) has been added to each tube, vortexed and incubated at room temperature for at least 5 minutes. Then 200 µl of the mix of each sample and reagent was added for each 96 plate well (triplicates or quadruplicates for each sample) and the absorbance was measured at a wavelength of 595 nm using Tecan Spark® (Männedorf, Switzerland) multimode microplate reader. Once the values were obtained, they were interpolated in the calibration curve that made it possible to determine the protein concentration of the samples expressed in µg/µl.

3.10.2 SDS-PAGE

SDS-PAGE, or Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, is a protein analysis technique conducted on a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). It's an analytical method that allows the separation of proteins based on their different molecular weights from a complex mixture. SDS, an anionic detergent, is crucial in this technique as it binds tightly to proteins, denaturing them by breaking non-covalent bonds. This results in all proteins acquiring the same negative charge density, causing them to move within the electric field at a speed dependent solely on their molecular weight (Roy & Kumar 2014).
The key components required for this technique include an inert support for protein migration (polyacrylamide gel), an electrophoretic cell, a power supply, a sample buffer for sample preparation, and a running buffer. The polyacrylamide gel used for electrophoretic migration is derived from the copolymerization of acrylamide monomers with N,N-methylenebisacrylamide. The acrylamide monomers polymerize head-to-tail and bind to bisacrylamide molecules, forming a well-defined gel matrix. The polymerization process is catalyzed by the radical initiator N,N,N',N'-tetramethylethylenediamine (TEMED) in the presence of ammonium persulfate (APS), generating sulfate radicals that initiate polymerization (Manns 2011).

The polyacrylamide gel consists of two parts:

- *Stacking gel* (*or concentration gel*): the upper part of the gel where sample wells are created. This gel has a low acrylamide concentration, resulting in large pores that facilitate protein stacking at the same migration front before entering the running gel.
- *Running gel* (*or separation gel*): the portion of the gel characterized by a higher acrylamide concentration, resulting in smaller pores that enable protein separation based on size and molecular weight.

The protein samples to be separated were treated with 1x sample buffer (4x Laemmli Sample Buffer, Bio-Rad Laboratories, Hercules, CA, USA) containing 5% SDS, 10% β-mercaptoethanol, 200 mM Tris-HCl pH 6.8, 0.25% Bromophenol Blue, and 20% glycerol. These components denature the proteins, disrupt disulfide bonds, provide tracking dye for electrophoresis, and aid in proper loading of samples into wells. Samples were denatured further at 95°C for 5 minutes, centrifuged, and loaded into the stacking gel wells. Prestained protein SHARPMASS™ VI Protein MW marker (EuroClone, Pero, MI, Italy) was loaded alongside the samples for reference.

Equal amounts of cell lysates (in µg) were resolved by SDS-PAGE and electrophoresis was conducted in running buffer consisting of 25 mM Tris, 200 mM glycine, and 0.1% SDS, applying a voltage of approximately 100 V for 90 minutes and a maximum of 300 mA.

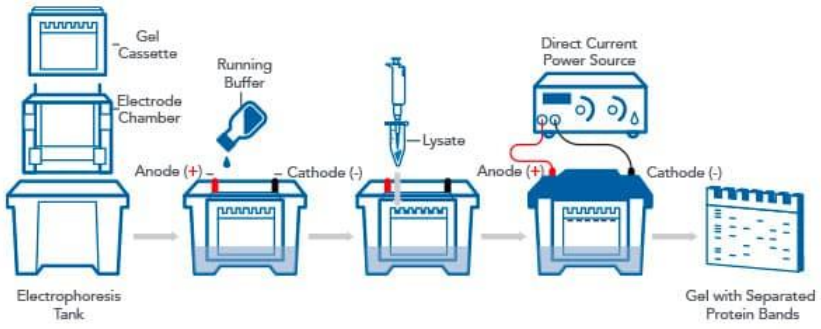


Figure 37. SDS-PAGE Gel Electrophoresis (source: Bio-Techne SRL).

3.10.3 Immunoblotting

After the separation by SDS-PAGE, protein transfer was carried out from the gel onto nitrocellulose blotting membranes (GE Healthcare, Solingen, Germany) in transfer buffer consisting of 25 mM Tris, 190 mM glycine pH 8.3 and 20% methanol.

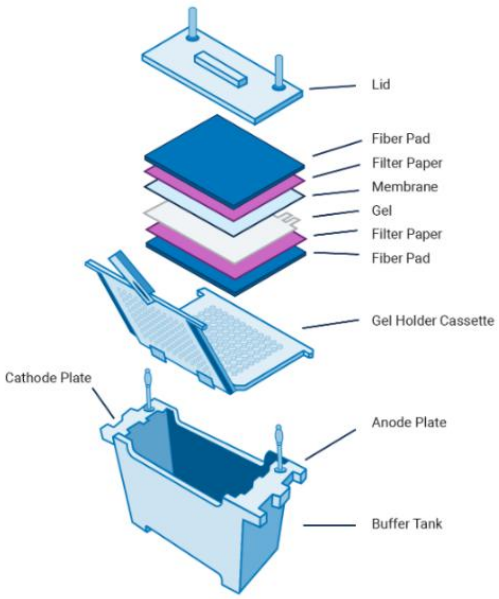


Figure 38. Western Blotting Transfer Technique (source: LI-COR Biosciences).

Once the transfer phase was completed, in order to verify that it had taken place correctly, the membrane was stained with a solution of Ponceau Red (Sigma-Aldrich, St. Louis, MO, USA, 0.1% Ponceau S [w/v] in 5% acetic acid [v/v]) and then decolored by performing washes of 10 min each in slow agitation with TBS-T (0.05% Tris Buffered Saline-Tween20).

Alternatively, for protein transfer from gel to membrane, the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA) was used, providing a rapid and efficient method for transferring proteins onto PVDF (polyvinylidene fluoride) membranes (Figure 39). After protein separation via gel electrophoresis, the PVDF blotting membranes were pre-equilibrated and placed in the transfer stack, ensuring optimal contact between the gel and membrane. The system was then run, according to the manufacturer's protocol, for approximately 7 minutes, significantly reducing the transfer time compared to conventional wet transfer methods enabling high-quality protein transfer for subsequent analysis.

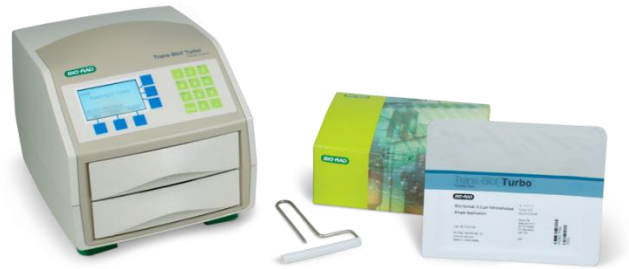


Figure 39. Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA).

After the decolorization of nitrocellulose membranes or the 7 minutes of Trans-Blot® Turbo™ Transfer System were completed, the membranes were blocked for 1h with 5% non-fat dry milk in TBS-T or PBS-T and then overnight probed with the primary antibodies of interest and analyzed using the enhanced chemiluminescence kit for Western blotting detection (Advansta, WesternBright TM ECL, Bering Drive San Jose, CA, USA) (Mahmood & Yang, 2012).

For HT29 and HCT116 cells, primary monoclonal antibodies were used following suppliers' instructions and included: mouse anti-human monoclonal GAPDH (H-284; sc-30220; dilution, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA), Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (9664; dilution 1:1000; Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-human PARP-1 (N-20; sc-1561; dilution 1:500; Santa Cruz Biotechnology, Dallas, TX, USA), monoclonal anti-human p21 (M-19; sc-471; dilution 1:500; Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal anti-human cyclin D1 (72-13G; sc-450; dilution 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), all diluted in BSA 5%/TBST + 0,05% NaN_3 or PBS-T (Table 1).

As secondary antibodies anti-mouse IgG-HRP-linked (7076; dilution 1:3000; Cell Signaling Technology, Danvers, MA, USA), and anti-rabbit IgG-HRP-linked (7074; dilution 1:3000; Cell Signaling Technology, Danvers, MA, USA) were used.

PROTEIN	PRIMARY ANTIBODY	DILUTION	SECONDARY ANTIBODY
GAPDH	α – GAPDH	1:500	α-Mouse 1:3000

Cleaved Caspase-3	α – cCaspase-3	1:1000	α-Rabbit 1:3000
PARP-1	α – PARP-1	1:500	α-Mouse 1:3000
p21	α – p21	1:500	α- Mouse 1:3000
Cyclin D1	α – Cyclin D1	1:200	α-Mouse 1:3000

Table 1. Primary antobodies with their corresponding dilutions used on membranes of HT29 and HCT116 cells.

For AGS and KATO III cells, primary monoclonal antibodies were used following suppliers' instructions and included: PARP (46D11) Rabbit mAb (#9532); Caspase-3 Antibody (#9662); Bcl-2 (50E3) Rabbit mAb (#2870); Cyclin E2 Antibody (#4132); p21 Waf1/Cip1 (12D1) Rabbit mAb (#2947); p27 Kip1 (D69C12) Rabbit mAb (#3686); cyclin B1 (G-11) (sc-166757); β-Actin (8H10D10) Mouse mAb (#3700), and Vinculin (7F9) (sc-73614), all diluted in PBST (Table 2).

As secondary antibodies anti-mouse IgG-HRP-linked (#7076), and Anti-rabbit IgG-HRP-linked (#7074) from Cell Signaling Technology® (CST, Danvers, MA, USA) were used.

PROTEIN	PRIMARY ANTIBODY	DILUTION	SECONDARY ANTIBODY
β-ACTIN	α – ACTIN	1:3000	α-Mouse 1:5000
Vinculin	α – Vinculin	1:3000	α-Mouse 1:5000
Full Caspase-3	α – Caspase-3	1:1000	α-Rabbit 1:3000
Full PARP-1	α – PARP-1	1:1000	α- Rabbit 1:3000
Bcl-2	α – Bcl-2	1:1000	α- Rabbit 1:3000
Cyclin E2	α – Cyclin E2	1:1000	α- Rabbit 1:3000
p21	α – p21	1:1000	α- Rabbit 1:3000
p27	α – p27	1:1000	α- Rabbit 1:3000
Cyclin B1	α – cyclin B1	1:1000	α-Mouse 1:3000

Table 2. Primary antobodies with their corresponding dilutions used on membranes of AGS and KATO III cells.

Visualization of the proteins of interest, incubated with the secondary antibody, was performed using a 1:1 mixture of two developing solutions from the ECL kit (WesternBright® ECL Chemiluminescent HRP Substrate, Advansta, San Jose, CA, USA) and a 1:1 mixture of two solutions with the TURBO EuroClone (Pero, MI, Italy) kit.

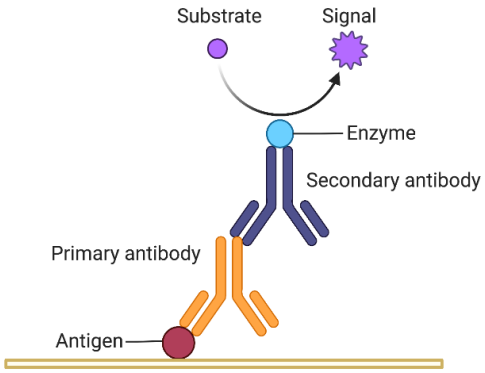


Figure 40. Illustration of detection in Western Blots (source: BioRender, Toronto, ON, Canada).

Detecting a signal that reflects antigen/antibody binding can provide semiquantitative or quantitative data about the target protein in simple or complex biological samples such as cell and tissue extracts (Taylor & Posch, 2014; Sule, Rivera & Gomes, 2023). The chemiluminescent signal was detected using the Alliance Cambridge Chemiluminescence Imager (UVITEC Cambridge) using UVITEC NineAlliance software and Chemidoc XRS detection system (BioRad, Hercules, California, USA) with the ImageLab software and quantified by densitometric band analysis using ImageJ software version 1.53t (NIH, Bethesda, Rockville, MD, USA) (Rinaldi *et al.*, 2021).

Figure 41. Alliance Cambridge Chemiluminescence Imager (UVITEC Cambridge) on the left and Chemidoc XRS detection system (BioRad, Hercules, California, USA) on the right.

3.11 Fluorescence Staining for Confocal Microscopy Coherency

HCT116 and HT29 cells were grown in 24 wells plates (5×10^4 cells/well) on glass coverslips previously washed with absolute ethanol for 24 hours. After the period of incubation cells were treated with PFs and untreated cells were used as negative control. After 48 hours of treatment cells were washed twice with cold PBS, fixed with 4% paraformaldehyde for 30 minutes and washed three times with cold PBS.

Coverslips, after permeabilization with triton X-100 0.1%/PBS for 5 min, were blocked with 1% BSA/PBS for 30 minutes at room temperature on shaking plate and washed twice with PBS. Subsequently, coverslips were incubated with fluorescein isothiocyanate–labeled phalloidin for 45 minutes at room temperature on shaking plate and in the dark. After a wash in PBS, the slides were assembled with DAPI (Fluoromount G with DAPI; Electron Microscopy Sciences, Hatfield, PA, USA) and left to dry in the dark.

In order to investigate if cytoskeleton organization was altered after treatment with hemolymph actin fiber coherency was evaluated. The coherence was calculated from the structure tensor of each pixel in the image and is bounded between 0 (isotropic areas) and 1 (highly oriented structures) (Lucchetti *et al.*, 2024; Jacquemet *et al.*, 2017).

Slides were examined using an inverted confocal microscope (Nikon Eclipse Ti2, Okolab, USA) showed in Figure 42. Images were taken using ImageJ software version 1.41 (NIH,

Bethesda, Rockville, MD, USA). Analyses of coherency were performed using the OrientationJ plugin for ImageJ/Fiji software.

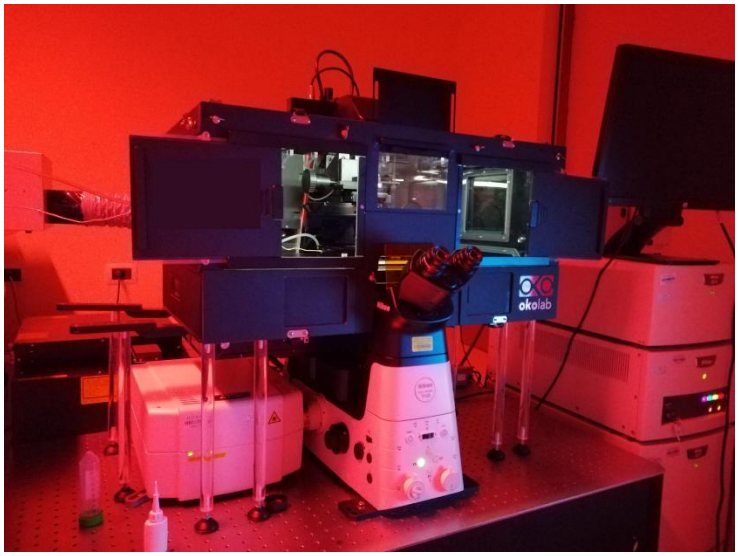


Figure 42. Inverted confocal microscope (Nikon Eclipse Ti2, Okolab, USA).

3.12 Scratch Test or Wound Healing Assay

According to the manual scratch test by Martinotti and Ranzato (Martinotti & Ranzato, 2020), HT29 and HCT116 cells were seeded (2×10^5 cells per well) in 24 well plates and allowed to reach 80% confluence (usually after 18-24 hours). A manual scratch wound was created in the center of each well scraping cell layer in a straight line using a 1 mm pipette tip and keeping it perpendicularly to the bottom of the well. After scratch, cell monolayer was gently washed with PBS Dulbecco's Phosphate Buffered Saline (EuroClone, Pero, MI, Italy) to remove detached cells, then the buffer was replenished with fresh medium for negative controls and with media with different dilutions of each PFs extracted from larvae infected with *E. coli*, *M. flavus*, and uninfected larvae (PF CTR).

Wounds were photographed with Eclipse TE2000-U inverted microscope (Nikon®, Tokyo, Japan) (Figure 43) on 10x magnification at five time points (0h, 6h, 12h, 24h, 48h) and analyses were performed using the Manual Wound_healing_size tool plugin from ImageJ/Fiji software version 1.41 (NIH, Bethesda, Rockville, MD, USA).

Figure 43. Eclipse TE2000 Inverted Microscope (Nikon®, Tokyo, Japan).

3.13 Statistical Analysis

Data are presented as means ± Standard Error (SE) of three technical replicates of three independent assays. Data were set to fit a normal distribution. Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test, performed using GraphPad Prism software (San Diego, CA, USA) version 5.00 for Windows (www.graphpad.com). The difference was considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$.

4. RESULTS

4.1 Inhibitory effects of *H. illucens*-derived peptide fraction on CRC and GC cells viability

To evaluate the potential inhibitory effects of Peptide Fractions (PFs) on CRC cells, a dose-response experiment was performed by treating HT29 and HCT116 cells with concentrations of 40 µg/ml, 20 µg/ml, 10 µg/ml, 5 ng/ µl, 2,5 µg/ml 1,25 µg/ml, 0,62 µg/ml, 0,31 µg/ml of PFs obtained from larvae infected with *Escherichia coli*, larvae infected with *Micrococcus flavus* and uninfected larvae. Cell viability was assessed after 48h and 72h using the MTT test. A dose- and time-dependent inhibition of cell growth in both cell lines was observed, and this effect was stronger with the PFs from infected larvae compared to the PF from uninfected larvae, and to untreated control. Moreover, PF from larvae infected with *E. coli* displayed a higher ability to inhibit cancer cell growth compared to PF from larvae infected with *M. flavus* on both cell lines.

Results showed that PFs obtained from infected larvae significantly reduces cell viability in both CRC cell lines, and that the inhibitory effect of PF obtained from uninfected larvae, is less pronounced than the infected ones (Figure 44).

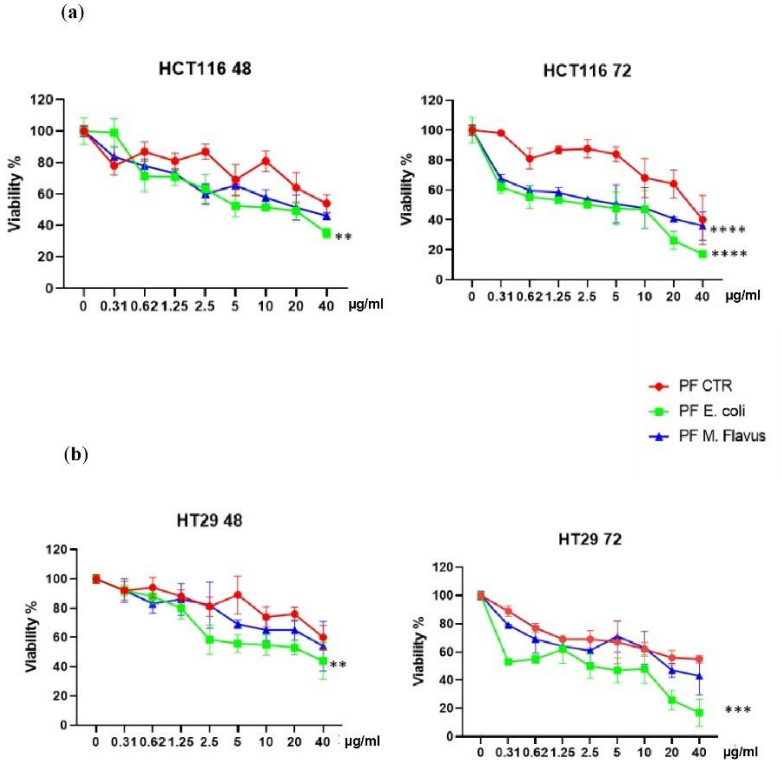


Figure 44. *H. illucens*-derived peptide fractions affect the viability and morphology of human CRC cells. Cell viability assay on (**a**) HCT116 and (**b**) HT29 cells. Cytotoxicity of the peptide fractions derived from uninfected and infected larvae with *M. flavus* and *E. coli* of *H. illucens* was assessed on HT29 and HCT116 cells treated with different concentrations of samples (from 0.31 to 40 µg/mL) for 48 h and 72 h. The percentage of viable cells was calculated as the ratio of treated cells to control cells. Data are presented as means ± Standard Error (SE) of three technical replicates of three independent assays. Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test (GraphPad Prism 5 software). The difference was considered statistically significant at ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

The activity of *H. illucens* PFs derived from larvae infected with *E. coli* (PF *E. coli*), *M. flavus* (PF *M. flavus*), or uninfected larvae (PF CTR), was evaluated through an MTT assay also on KATO III and AGS cells, treated for 24h, 48h and 72h with doubling concentrations of PFs (0.16 to 20 µg/ml). AGS cell viability was significantly reduced ($p < 0.01$) at 48h and 20 µg/ml (PF CTR 54.7%; PF *E. coli* 43.9% and PF *M. flavus* 50.9%). The same trend was observed at 72 hours ($p < 0.001$) with a viability of 42.3%, 35.6% and 39.8% for PF CTR, *E. coli* and *M. flavus* respectively (Figure 45a). On KATO III cells the activity was similar with a significant inhibition of proliferation ($p < 0.001$) at 10 µg/ml (PF CTR 56,8%; PF *E. coli* 63,9%; PF *M. flavus* 66,5%) and 20 µg/ml (PF CTR 56,3%; PF *E. coli* 55,5%; PF *M. flavus* 44,0%) after 48h

and at 10 µg/ml (PF CTR 64,2%; PF *E. coli* 69,9%; PF *M. flavus* 73,6%) and 20 µg/ml after 72h of treatment (PF CTR 45,5%; PF *E. coli* 48,6%; PF *M. flavus* 48,4%) (Figure 45b). Overall, PFs exhibited a dose- and time-dependent effect on cell viability in both cell lines.

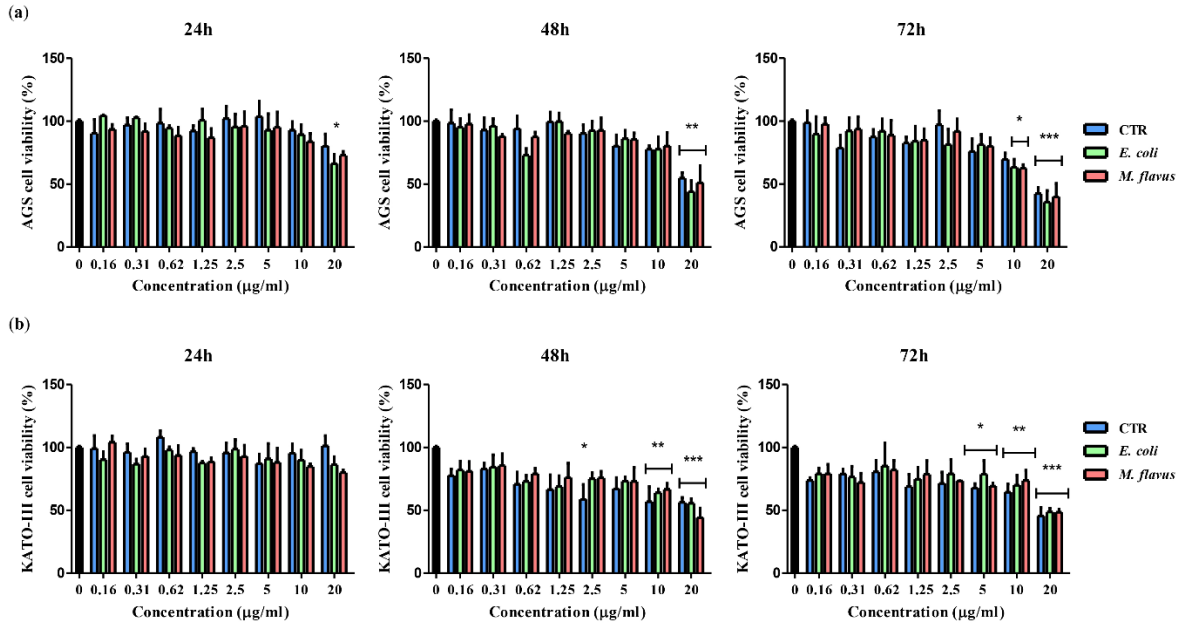


Figure 45. Evaluation of AGS and KATO III cell viability following exposure to PFs at varying concentrations over time. MTT assay was performed after incubation of (**a**) AGS and (**b**) KATO III cells with 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml 0.62 µg/ml, 0.31 µg/ml, 0.16 µg/ml PFs (PF CTR in blue, PF *E. coli* in green and PF *M. flavus* in red) for 24h, 48h and 72h. Cell viability was expressed as a percentage relative to control of untreated cells (black bar). The graphs represent means ± Standard Error (SE) of three independent experiments; differences between treatments groups were estimated by one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) using GraphPad Prism software 5.0. Significant differences were evaluated vs. untreated cells.

4.2 Changes in cellular morphology

Morphological changes of HT29 and HCT116 cells and AGS and KATO III cells were detected after treatment with PFs for 48 h. The microscopic analysis of HT29 and HCT116 cell cultures confirmed the inhibitory effect of the PFs and evidenced, as shown in Figure, that the treatment allowed to appreciate a dose-dependent change in cell morphology: already at the intermediate concentration used, the cells began to have different degrees of cellular deformation and distention, with cells losing their normal shape, appearing slimmer than control untreated cells

and jagged borders and numerous debris and cell fragments; while at the lower concentration used cells were similar to untreated ones (Figure 46).

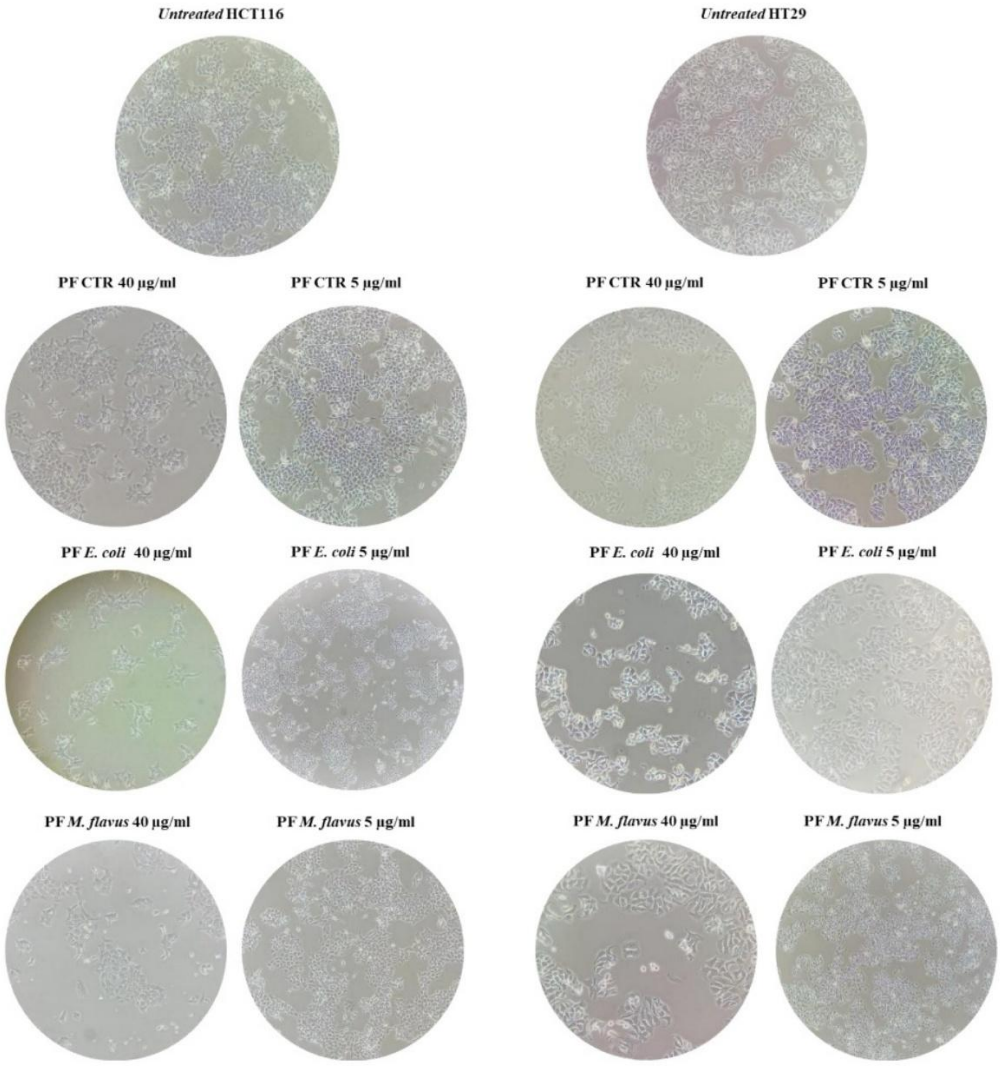


Figure 46. Morphological cell analysis. Microscopic analysis showing changes in the morphology of HT29 and HCT116 cells induced by the IC_{50} of peptide fractions after 48 h of treatment. Magnification: 10×; scale bars: 80 µm.

Morphological changes were observed when cells were exposed to 20 µg/ml of PF CTR, PF *E. coli* and PF *M. flavus*. These changes, compared to untreated cells, included reduced cell size, formation of vacuoles, increased cell detachment and rounded shape of AGS cells. At concentrations of 10 µg/ml and 2.5 µg/ml AGS cells were comparable to the untreated control

(Figure 47). No significant morphological alterations were observed in KATO III cells (Figure 48).

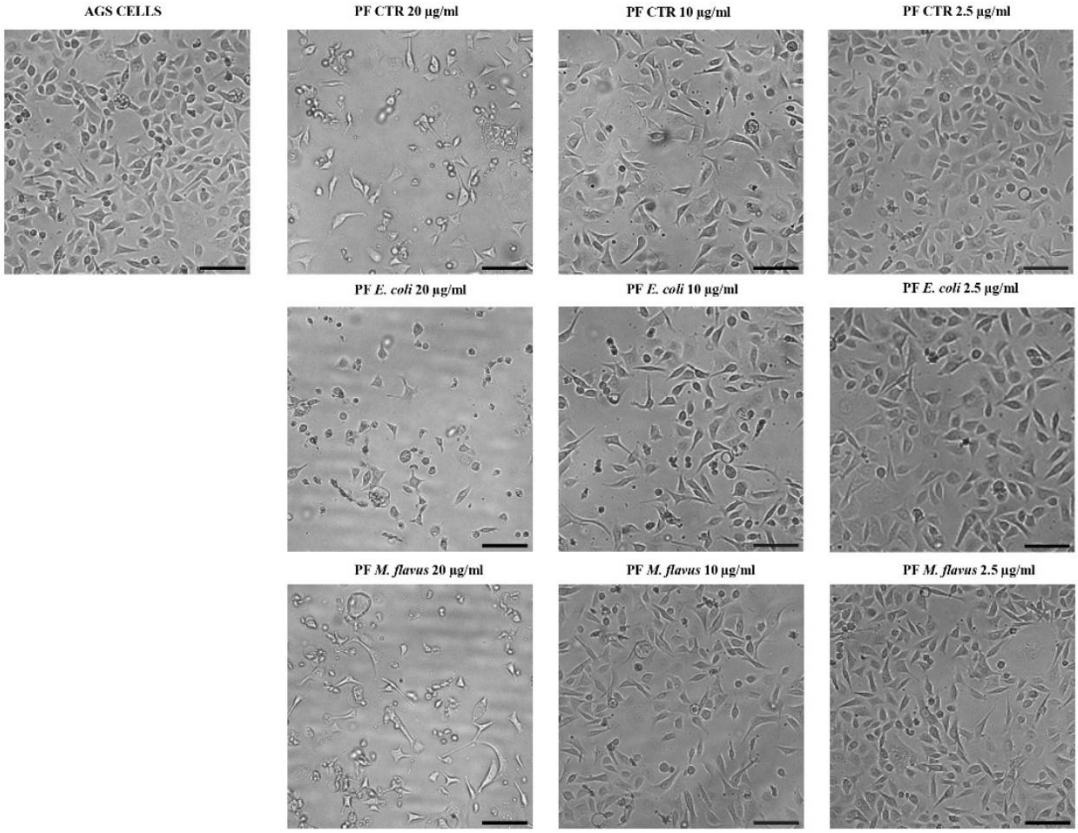


Figure 47. Microscopic analysis of AGS cells treated with PFs. Morphological cell analysis of AGS cell line treated for 48 h with 20 µg/mL, 10 µg/mL, 2.5 µg/mL PFs and untreated, observed through inverted phase contrast microscope. Images represent observations made on each sample in at least three separate fields of view. Magnification: 10×; scalebars:80µm.

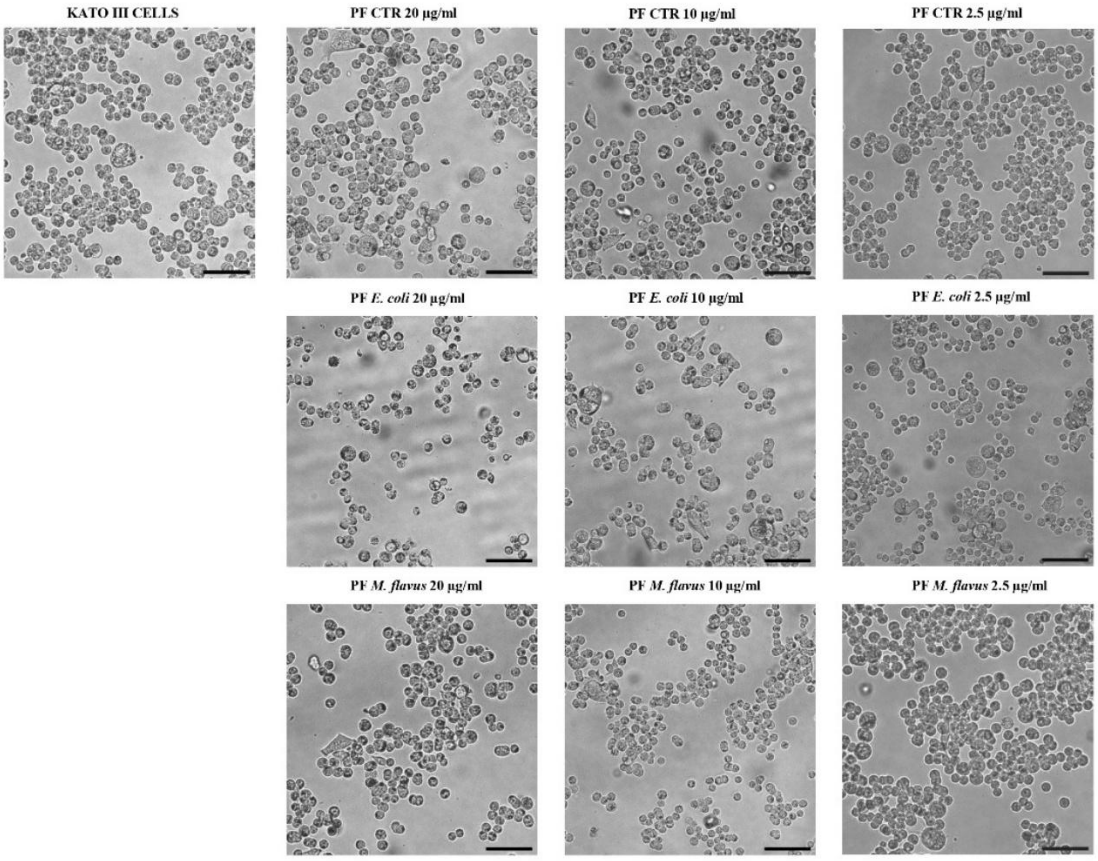


Figure 48. Microscopic analysis of KATO III cells treated with PFs. Morphological cell analysis of KATO III cell line treated for 48 h with 20 µg/mL, 10 µg/mL, 2.5 µg/mL PFs and untreated, observed through inverted phase contrast microscope. Images represent observations made on each sample in at least three separate fields of view. Magnification: 10×; scalebars:80µm.

4.3 Peptide fractions enhanced cytotoxicity of chemotherapeutics drugs in human CRC cells

The effects of chemotherapeutics drugs Oxaliplatin and 5-Fluorouracil, two well-known chemotherapeutics drugs widely used for treating CRC, alone or in combination with PFs, was evaluated by MTT assay on human colorectal cancer cell lines (HT29 and HCT116). The IC_{50} doses for Oxaliplatin and 5-Fluorouracil when used alone, and the IC_{30} when used in combination with 20 µg/ml of PFs obtained from infected and uninfected larvae have been chosen. As shown in Figure 49, combination with PFs significantly increased the growth-inhibitory effect of both drugs in a dose- and time-dependent manner.

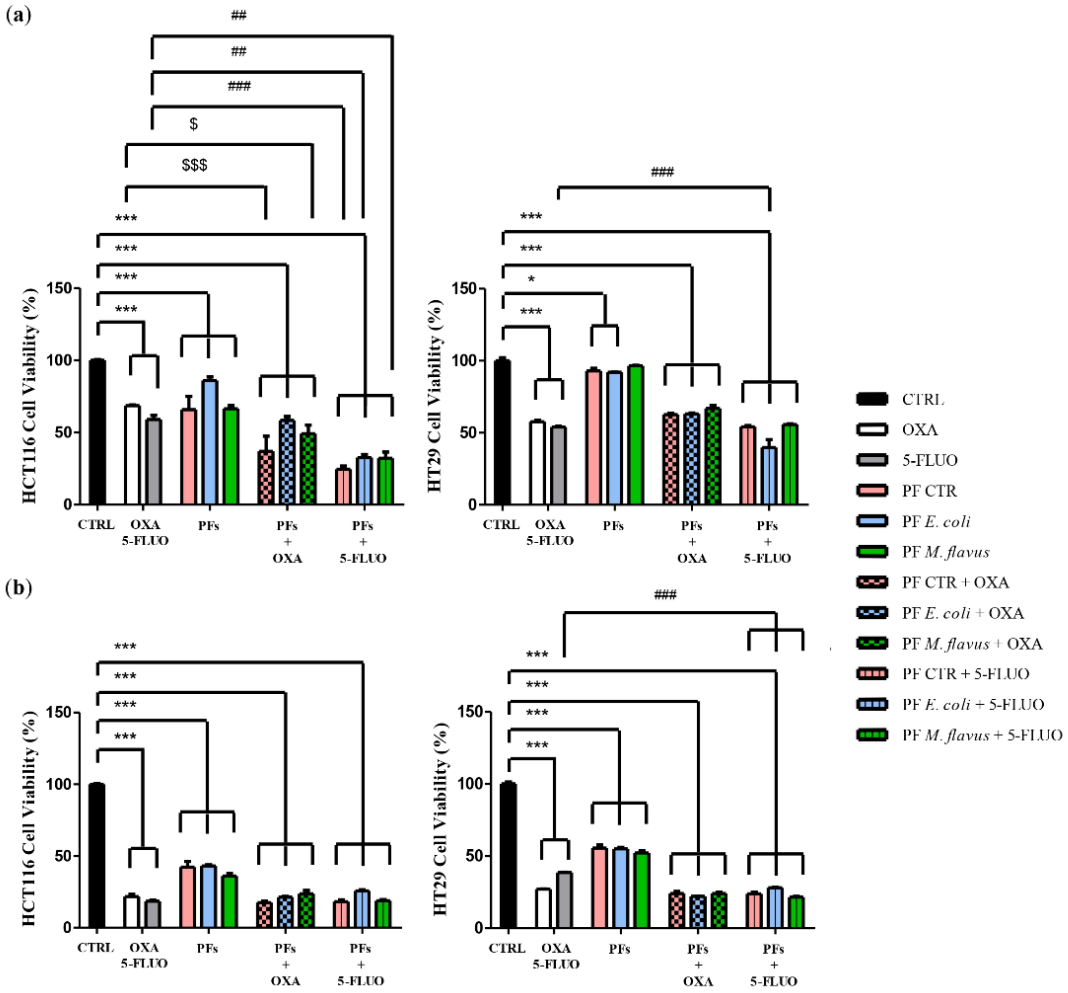


Figure 49. Effects of chemotherapeutic drugs and peptide fractions on HCT116 and HT29 cell viability. HCT116 (left) and HT29 (right) cells were exposed to Oxaliplatin (OXA) or 5-Fluorouracil (5-FU), alone or in combination with peptide fractions from larvae infected with *E. coli* or *M. flavus* and from uninfected larvae, and cell viability was assessed after (**a**) 48 h and (**b**) 72 h using the MTT assay. The percentage of viable cells was calculated as the ratio of treated cells to control cells. The data are expressed as means ± the Standard Error (SE) of three technical replicates of three independent assays, and statistical significance was evaluated (GraphPad Prism 5 software) with one-way ANOVA, followed by Dunnett's *post hoc* test. Significance: * $p < 0.05$, and *** $p < 0.001$ for all samples vs. CTRL; $ $p < 0.001$ for all PF samples in combination with OXA vs. OXA; and, ## $p < 0.01$, and ### $p < 0.001$ for all PF samples in combination with 5-FU vs. 5-FU.

4.4 Evaluation of Alkaline phosphatase activity in CRC cell lines

Alkaline phosphatase (ALP) activity, a marker of colon differentiation, was assessed by a kinetic assay measuring the conversion of the colorless *p*-nitrophenyl phosphate in *p*-nitrophenol, which is yellow in basic solutions. Briefly, total cellular lysates were prepared in phosphate-buffered saline with 10% Triton-X and aliquots of 100 mg of protein were assayed

for ALP activity by addition of p-nitrophenyl phosphate substrate. Spectrophotometric readings showed no differentiation in both cell lines (Figure 50).

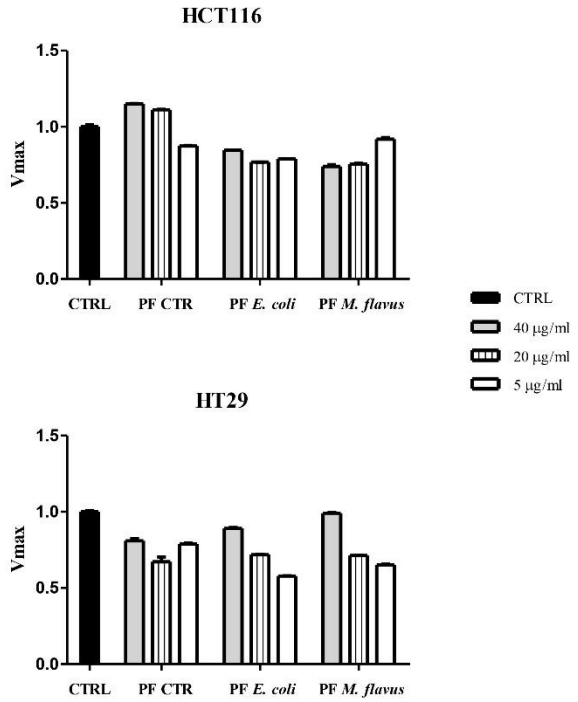


Figure 50. Alkaline phosphatase (ALP) activity showed an absence of cell differentiations in both HT29 and HCT116 cell lines. Spectrophotometric readings were taken on a SpectraMax1 Plus 384 spectrophotometer (Molecular Devices, San Jose, CA, USA) and were analyzed using the Softmax Pro Software version 4.3 (Molecular Devices, San Jose, CA, USA) to obtain the Vmax values. Data are expressed as means ± Standard Error (SE) of three replicates and statistical significance was evaluated (GraphPad Prism 5 software (San Diego, CA, USA)) with one-way ANOVA followed by Dunnett's *post hoc* test.

4.5 Apoptosis via Bcl-2/Caspase-3/PARP-1 pathway in HCT116 and AGS cell lines induced by Peptide fractions

To evaluate the potential effect of hemolymph-derived PFs, the occurrence of apoptosis in PF-treated cultures of both HT29 and HCT116 cell lines using the PI/AnnexinV double staining assay was analyzed. The results obtained confirmed the occurrence of apoptosis in treated HCT116, but not in HT29, cells. Indeed, the percentage of apoptotic cells after 48h treatment reached the early apoptosis values of 18%, 17%, 12% in the cultures treated with PFs obtained from larvae infected with *E. coli* and *M. flavus* and uninfected larvae, respectively (Figure 51a). Moreover, a percentage of late apoptosis values of 6%, 5% e 4%, respectively was detected. The apoptosis was confirmed by the dose-dependent increase of cleaved caspase-3 and cleaved

Parp1 in the HCT116 cells treated with an increasing amount of PFs compared to untreated cells (Figure 51b,c). No induction of apoptosis was observed in HT29 cells (Figure 52).

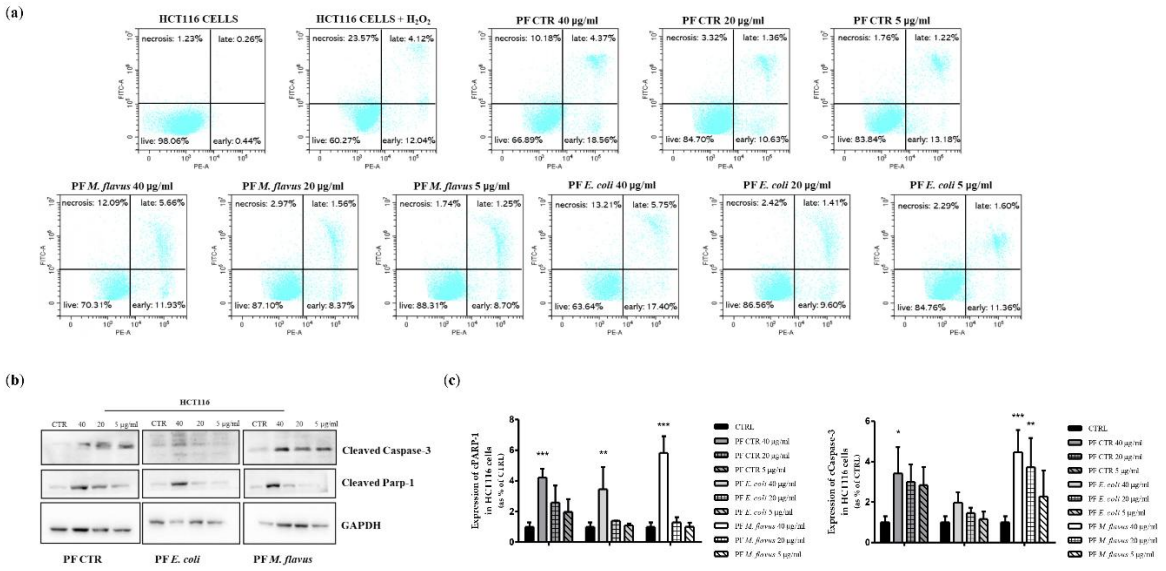


Figure 51. *H. illucens*-derived peptide fractions induced apoptosis in HCT116 human CRC cells. (**a**) Quantitative analysis of apoptosis. The quantitative evaluation of apoptosis after 48 hours of treatment of HCT-116 cells with the indicated increasing concentrations of peptide fractions showed a dose-dependent increase of the number of apoptotic cells. (**b**). Western blotting analysis of cell cy-cle-related proteins. Protein expression was evaluated by immunoblotting after 48 h treatment with the indicated peptide fractions at three different concentrations. The densitometric analysis (**c**) was performed by ImageJ software version 1.53t (NIH, Bethesda, Rockville, MD, USA) and reported by histograms Data are presented as means ± Standard Error (SE) of three replicates. Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test (GraphPad Prism 5 software). The difference was considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

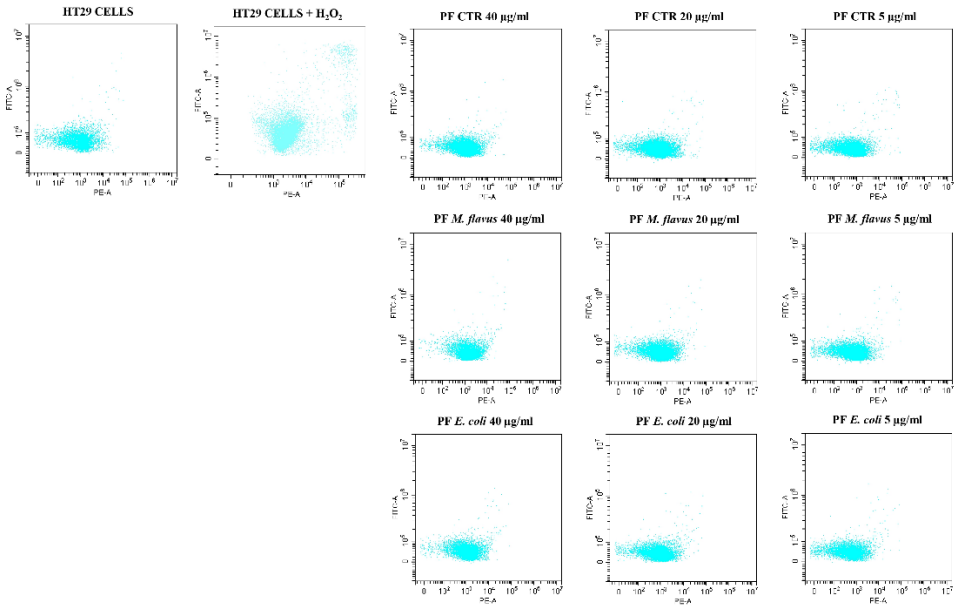


Figure 52. Flow cytometry analysis of apoptosis in HT29 cells treated with peptide fractions. HT29 cells were treated for 48 h with the indicated increasing concentrations of 40, 20, and 5 µg/ml of peptide fractions obtained from *Hermetia illucens* larvae infected with *Escherichia coli* (PF *E. coli*), *Micrococcus flavus* (PF *M. flavus*), or from uninfected larvae (PF CTR). Representative dot plots show no induction of apoptosis in HT29 cells upon treatment.

To evaluate the mechanisms of cell death a flow cytometry apoptosis assay was performed on AGS and KATO III cell lines treated with 20 µg/ml, 10 µg/ml and 2.5 µg/ml of PFs for 48h. Figure 53 shows an increasing number of apoptotic cells in a dose-dependent manner, in particular for cells treated with 20 µg/ml of PF *E. coli* (18,48%, $^{**}p < 0.01$) and of PF CTR (20,47%, $^{***}p < 0.001$), less for cells treated with 20 µg/ml of PF *M. flavus* (6,41%, $^{*}p < 0.05$), comparing with the untreated cells (5,93%). This effect was not observed in KATO III cells under the same conditions (Figure 54).

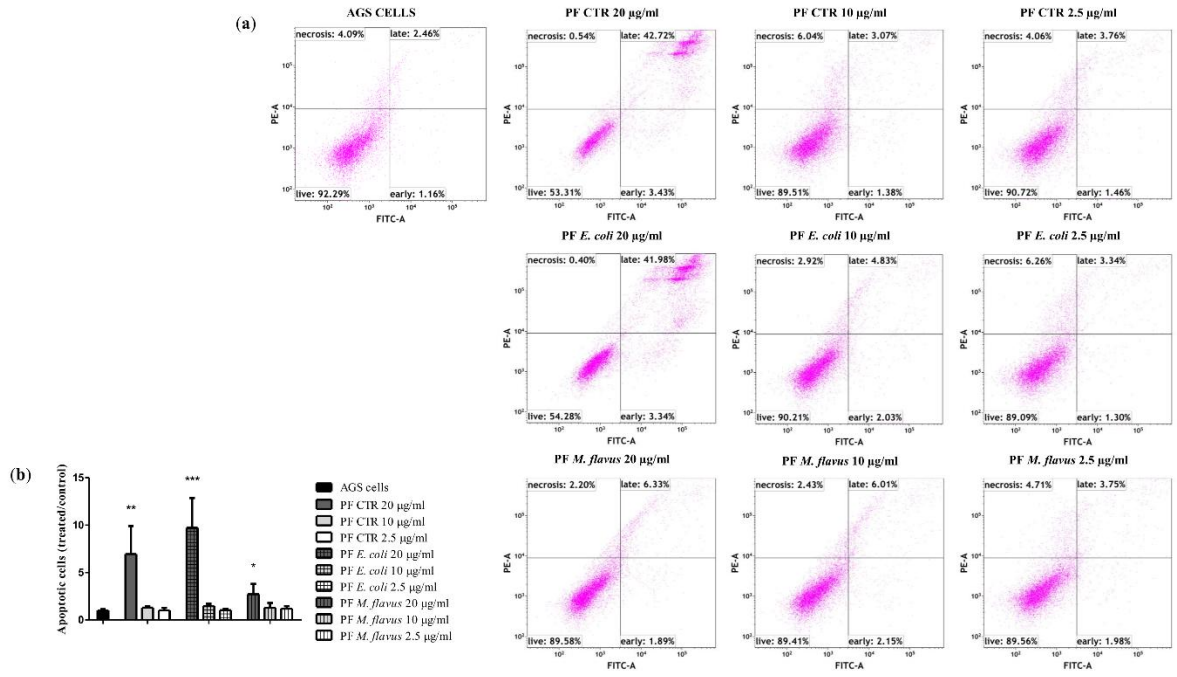


Figure 53. FACS analysis for apoptosis on AGS cell line treated for 48h with 20 µg/ml, 10 µg/ml, 2.5 µg/ml PFs and untreated. (**a**) The FACS profiles represent 10,000 events and is indicative of the patterns seen in three replications and (**b**) the resultant histogram analysis shows data as means ± Standard Error (SE) of three independent experiments and statistical significance was evaluated (GraphPad Prism software 5.0) with one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significant differences were evaluated vs. untreated cells (AGS cells bar).

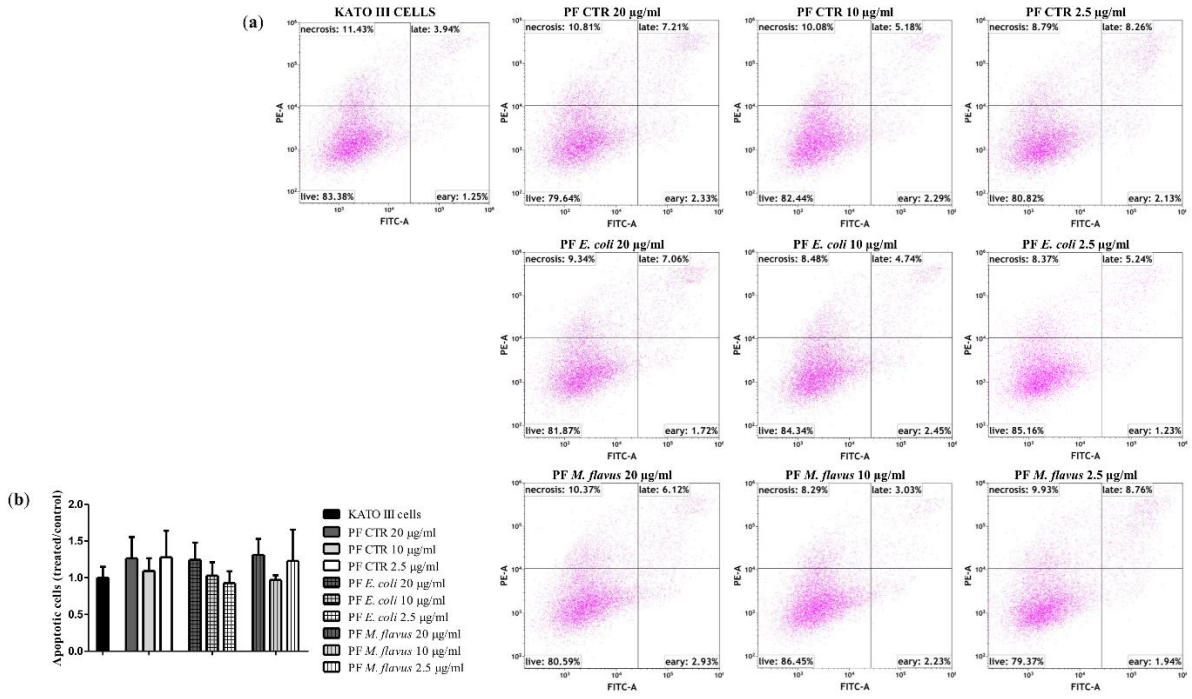


Figure 54. FACS analysis for apoptosis on KATO III cell line treated for 48h with 20 µg/ml, 10 µg/ml, 2.5 µg/ml PFs and untreated. (**a**) The FACS profiles represent 10,000 events and is indicative of the patterns seen in three replications and (**b**) the

resultant histogram analysis shows data as means ± Standard Error (SE) of three independent experiments and statistical significance was evaluated (GraphPad Prism software 5.0) with one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significant differences were evaluated vs. untreated cells (KATO III cells bar).

Apoptosis induction was further assessed via western blot by evaluating the expression levels of full-length (116 kDa) nuclear poly (ADP-ribose) polymerase (PARP-1), full length (35 kDa) cysteine-aspartic acid protease 3 (Caspase 3) and the anti-apoptotic protein Bcl-2. In AGS cells (Figure 55) both full length-PARP-1 and full length-Caspase 3 (35 kDa) decreased in a dose-dependent manner.
Additionally, Bcl-2, known as a cell survival protein that inhibits apoptosis, significantly decreased in a dose-dependent manner.

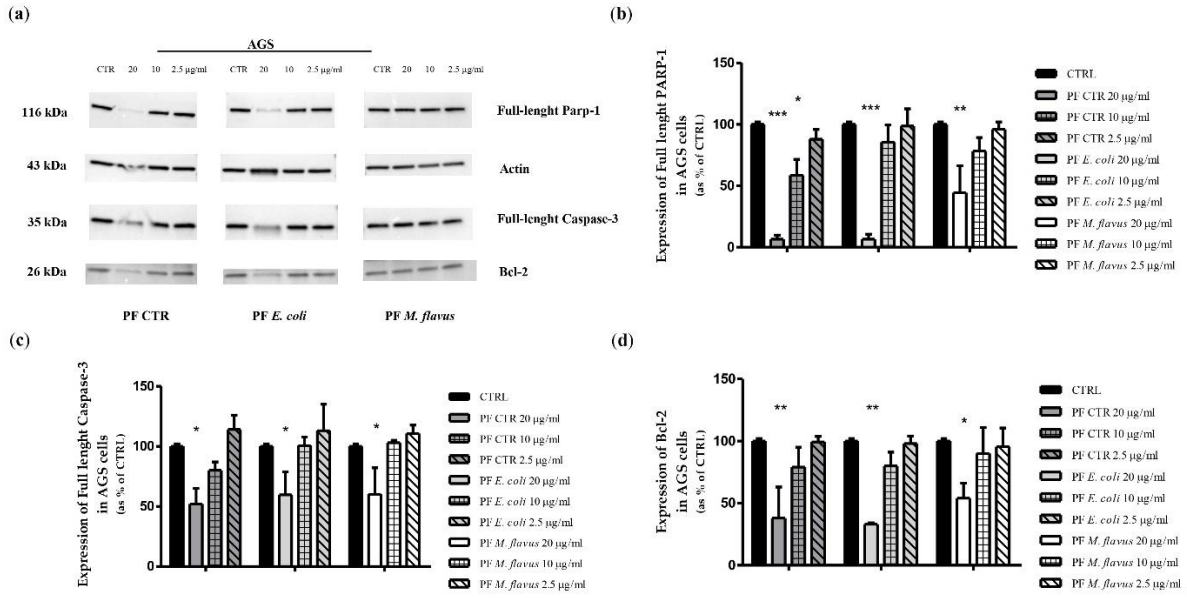


Figure 55. Western Blotting analysis of apoptosis-related proteins on AGS cells. (**a**) Western blot analysis showing the expression levels of protein related to apoptotic pathway (**b**) PARP1, (**c**) Caspase-3 and (**d**) Bcl-2) on AGS cell line after exposing cells for 48h with 20, 10 and 2.5 µg/ml of PFs. The relative expression levels were normalized to the control actin housekeeping. The data are expressed as means ± Standard Error (SE) of three independent experiments and statistical significance was evaluated (GraphPad Prism software 5.0) with one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significantly differences were evaluated vs. untreated cells. Significant differences were evaluated vs. untreated cells (CRTL).

On the contrary, in KATO III cells (Figure 56) there was no significant change in PARP-1, caspase 3 and Bcl-2 expressions, in line with results from the cytofluorimetric analysis.

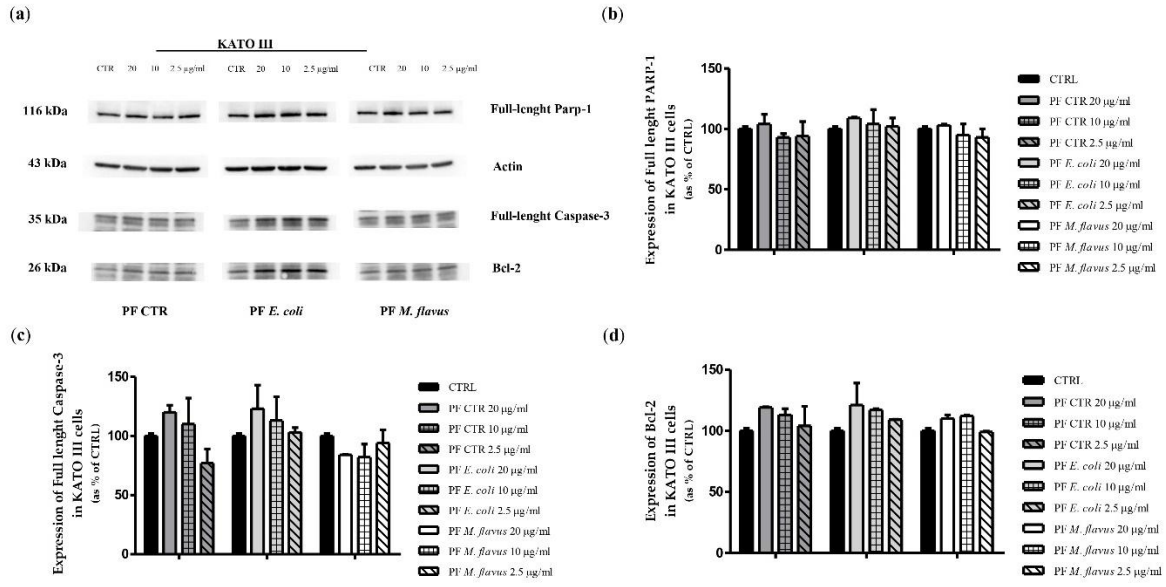


Figure 56. Western Blotting analysis of apoptosis-related proteins on KATO III cells. (**a**) Western blot analysis showing the expression levels of protein related to apoptotic pathway (**b**) PARP1, (**c**) Caspase-3 and (**d**) Bcl-2) on KATO III cell line after exposing cells for 48h with 20, 10 and 2.5 µg/ml of PFs. The relative expression levels were normalized to the control actin housekeeping. The data are expressed as means ± Standard Error (SE) of three independent experiments and statistical significance was evaluated (GraphPad Prism software 5.0) with one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significant differences were evaluated vs. untreated cells (CTRL).

4.6 Peptide fractions induced G2/M phase arrest in human CRC cells and S phase and G2/M phase arrest in GC cells

In order to further understand the mechanisms mediating the observed inhibition of cell growth, the distribution of cells within the phases of the cell cycle was analyzed using flow cytometry in both HCT116 (Figure 57a) and HT29 (Figure 58a) cell cultures treated with the PFs. Cell cycle analysis demonstrated that the PFs induced a cell cycle arrest. In fact, after 48h hours of treatment, a dose-dependent accumulation of cells in the G2/M phase of the cell cycle was observed in both cell lines and with all the treatments, compared to control untreated cells. Similarly to the growth inhibition, this effect was stronger in the cultures treated isolated from infected larvae than the uninfected counterpart and was more evident with the PF obtained from larvae infected with *E. coli* (Figures 57a and 58a).

In the cultures treated with the PF obtained from larvae infected with *E. coli*, the percentage of cells in the G2/M phase of the cell cycle reached the 74% and the 58% in the HT29 and the

HCT116 cells, respectively, compared to about 33% in the control untreated cells. A slighter effect (37% and 44%, respectively in HT29 and HCT116 cells) was observed with the PF obtained from larvae infected with *M. flavus* (Figures 57a and 58a).

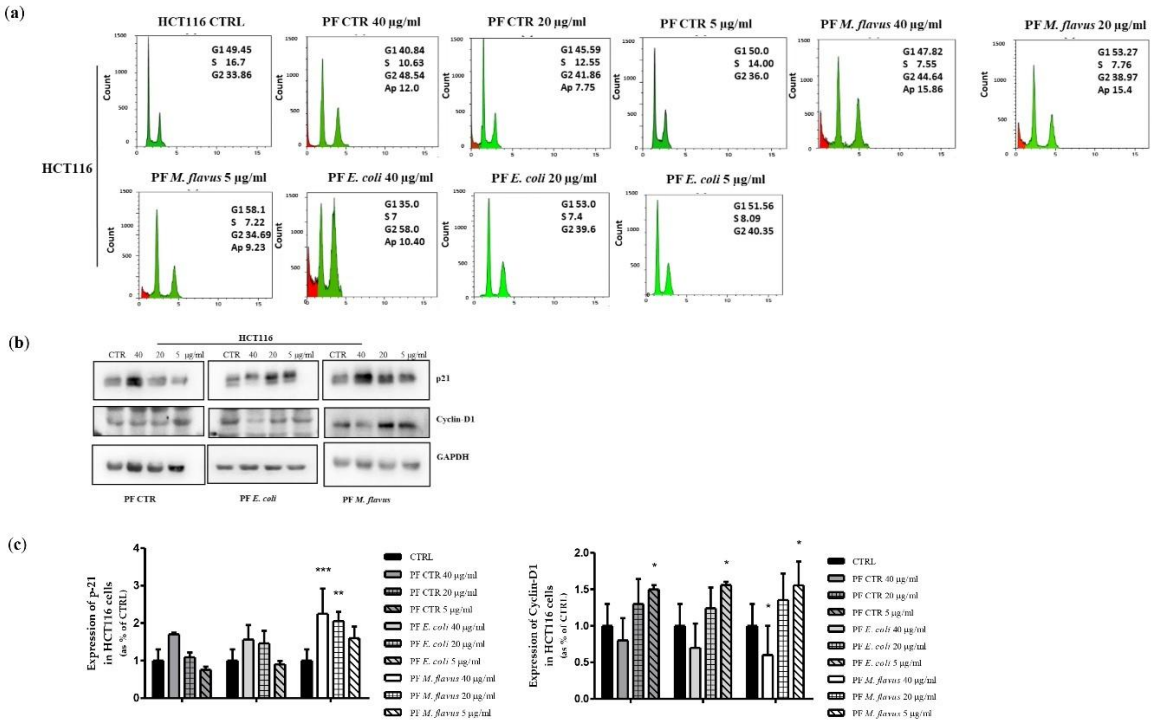


Figure 57. *H. illucens*-derived peptide fractions affect cell cycle distribution in HCT116 human CRC cells. (**a**) Flow cytometry analysis. Cell cycle analysis demonstrated a dose-dependent accumulation of cells in the G2/M phase of the cell cycle in all treatment groups after 48 hours of treatment compared to control cells. This effect was stronger in the cultures treated with peptide fractions from infected larvae than the uninfected counterpart and was more evident with the peptide fraction obtained from larvae infected with *E. coli*. (**b**) Western Blotting analysis of cell cycle-related proteins. Protein expression was evaluated by immunoblotting after 48 h treatment with the indicated peptide fractions at three different concentrations. The densitometric analysis (**c**) was performed by Im-ageJ-win64 software and reported by histograms. Data are presented as means ± Standard Error (SE) of three technical replicates of three independent assays. Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test (GraphPad Prism 5 software). The difference was considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To investigate the molecular mechanisms underlying the results obtained, the effects of PFs on the expression of proteins known to be involved in the regulation of the cell cycle were investigated. An increase of the expression levels of p21 protein (CDK inhibitor) and a decreased expression of cyclin-D1 was detected in both cell lines compared to untreated cells (Figures 57b,c and 58b,c). This effect was highest with the PF obtained from larvae infected with *E. coli*. Flow cytometry analysis also revealed the appearance of a sub-G1 peak, indicative

of apoptotic cells, in the HCT116 treated with the PFs obtained from larvae infected with *E. coli* or *M. flavus*, which reached the value of about 10% and 16% at 48 hours, respectively (Figure 57a). A sub-G1 peak was not evident in the cultures of treated HT29 cells (Figure 58a).

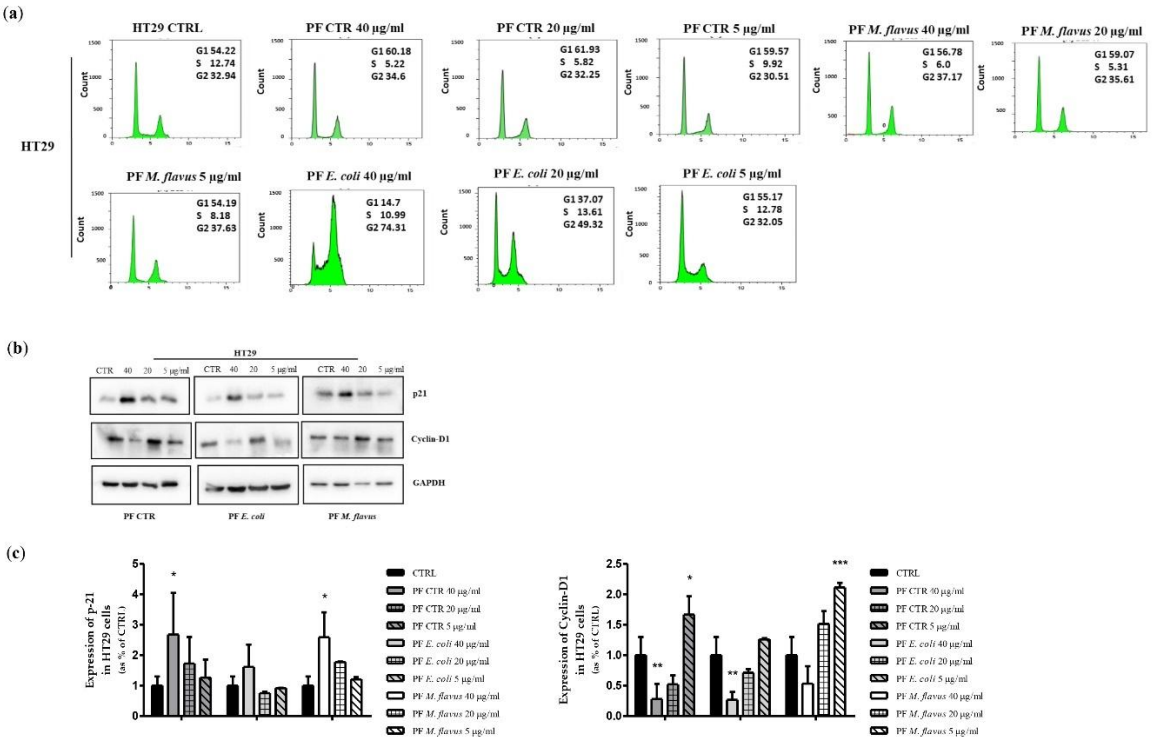


Figure 58. *H. illucens*-derived peptide fractions affect cell cycle distribution in HT29 human CRC cells. (**a**) Flow cytometry analysis. Cell cycle analysis demonstrated a dose-dependent accumulation of cells in the G2/M phase of the cell cycle in all treatment groups after 48 hours of treatment compared to control cells. This effect was stronger in the cultures treated with peptide fractions from infected larvae than the uninfected counterpart and was more evident with the peptide fraction obtained from larvae infected with *E. coli*. (**b**) Western Blotting analysis of cell cycle-related proteins. Protein expression was evaluated by immunoblotting after 48 h treatment with the indicated peptide fractions at three different concentrations. The densitometric analysis (**c**) was performed by ImageJ software version 1.53t (NIH, Bethesda, Rockville, MD, USA) and reported by histograms. Data are presented as means ± Standard Error (SE) of three technical replicates of three independent assays. Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test (GraphPad Prism 5 software). The difference was considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To verify whether the viability reduction after PFs treatment was due to cell cycle arrest, instead of apoptosis in KATO III or in addition to apoptosis in AGS, flow cytometry evaluations were performed on both cell lines (Figures 59 and 60).

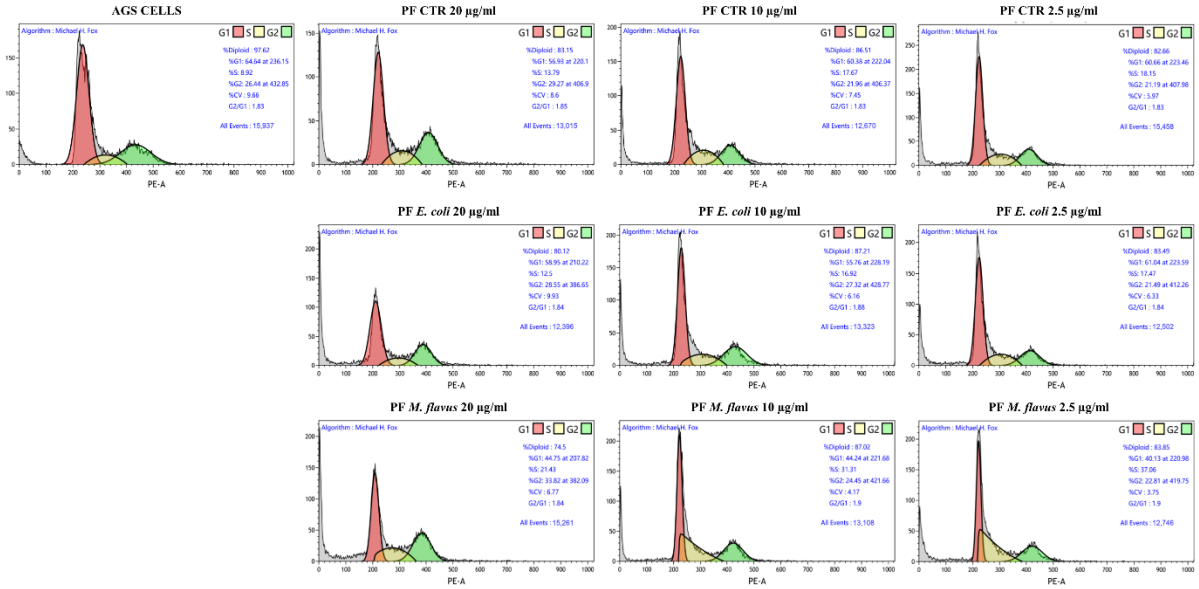


Figure 59. Representative plots (10,000 events) of cell cycle analysis by flow cytometry on AGS cell line. Dot plots report the distribution of cell cycle phases of AGS cells treated with PFs for 48h at concentrations of 20, 10 and 2.5 µg/ml and untreated.

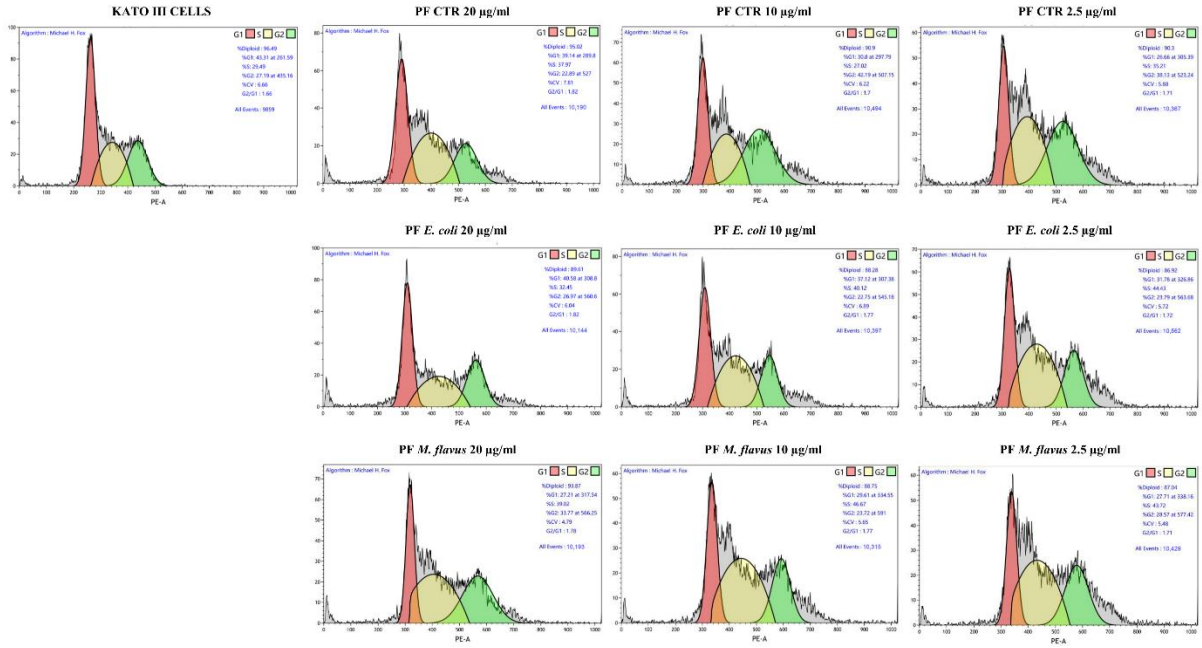


Figure 60. Representative plots (10,000 events) of cell cycle analysis by flow cytometry on KATO III cell line. Dot plots report the distribution of cell cycle phases of KATO III cells treated with PFs for 48h at concentrations of 20, 10 and 2.5 µg/ml and untreated.

As shown in Figure 61a, the cell cycle analysis highlighted an accumulation of AGS in G2/M phase (*p < 0.05 significance), with a consequent decrease of G0/G1 phase ($^{\$}$ p < 0.05, $^{\$\$}$ p < 0.01 significance), after 48h of treatment (20 and 10 ng/µl of PF CTR and PF *E. coli*), while PF *M. flavus* also determined a significant increase of cells in S phase ($^{\#}$ p < 0.05 significance). Western blot analysis on AGS cells lysate (Figure 61b) reported a dose-dependent decrease in cyclin B1, a regulatory protein involved in mitosis and mainly expressed during the G2/M phase of the cell cycle, with the highest concentration of both PF CTR and PF *E. coli*, confirming a G2/M arrest in AGS cell line. The same trend, under the same conditions, has been observed for the p21 protein. PF *M. flavus* treatment induced a minor reduction of cyclin B1 levels but higher levels of p21 than other PFs were observed.

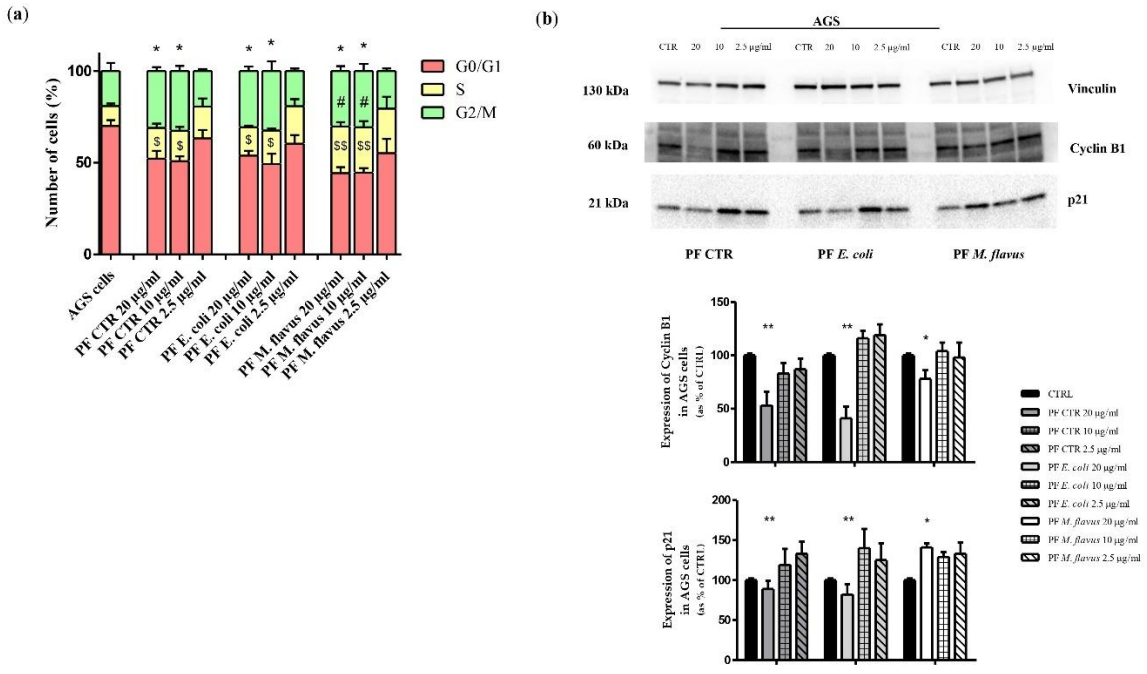


Figure 61. Cell Cycle Analysis in AGS cells. (**a**) Histogram reports the mean ± Standard Error (SE) of percentage of AGS cells treated with PFs for 48h at concentrations of 20, 10 and 2.5 µg/ml and untreated in each phase of the cell cycle (G0/G1, S, and G2/M) from three separate experiments. (**b**) Western blot analysis reporting the expression levels of protein related to cell cycle mechanisms (Cyclin B1 and p21) on AGS cells treated with PFs for 48h at concentrations of 20, 10 and 2.5 µg/ml and untreated. The relative expression levels are normalized to the control vinculin housekeeping. The data are expressed as means ± Standard Error (SE) of three independent experiments and statistical significance was evaluated (GraphPad Prism software 5.0) with one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ or \$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$). Significant differences were evaluated vs. untreated cells (CTRL).

As regards KATO III cells (Figure 62a), an accumulation of cells in the S phase after treatment for 48h with all three types of PFs occurred (*p < 0.05, **p < 0.01, ***p < 0.001 significance). The Figure 62b shows western blot on KATO III cells, in which densitometric analysis revealed that there was an increase in cyclin E in cells treated with all PFs. In particular, for cells treated with PF CTR and PF *E. coli* samples a dose-dependent increase was observed, while those treated with *M. flavus* did not. In addition, a similar trend was observed for the accumulation of p27 protein levels.

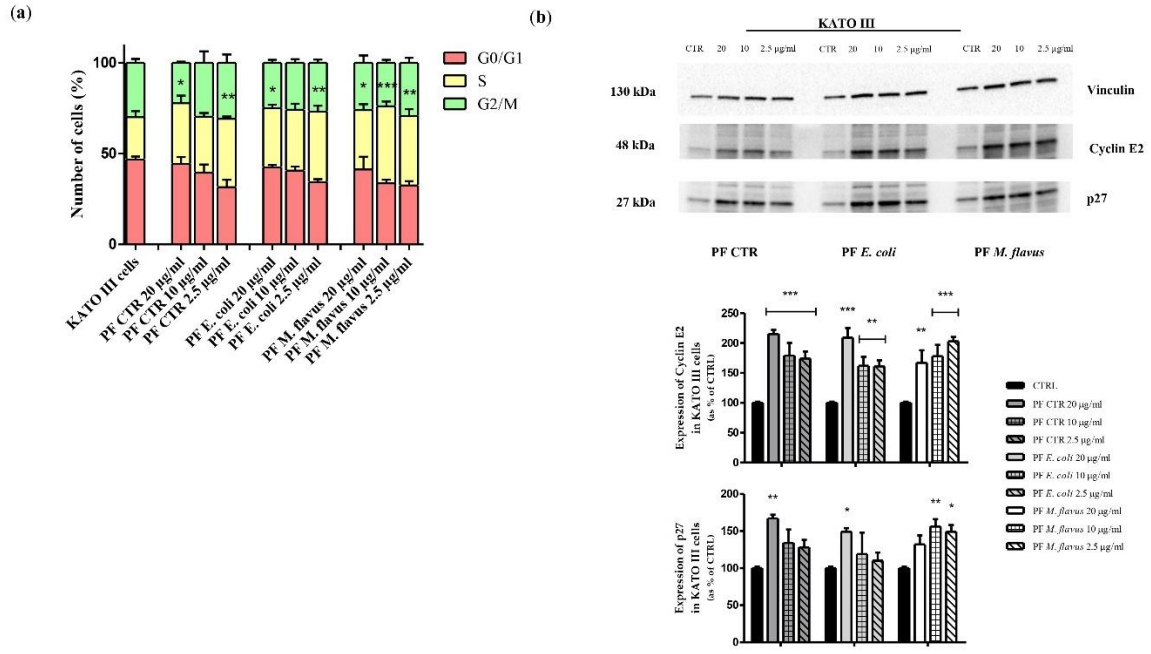


Figure 62. Cell Cycle Analysis in KATO III cells. (**a**) Histogram reports the mean ± Standard Error (SE) of percentage of treated KATO III cells treated with PFs for 48h at concentrations of 20, 10 and 2.5 µg/ml and untreated in each phase of the cell cycle (G0/G1, S, and G2/M) from three separate experiments. (**b**) Western blot analysis reporting the expression levels of protein related to cell cycle mechanisms (Cyclin E2 and p27) on KATO III cells treated with PFs for 48h at concentrations of 20, 10 and 2.5 µg/ml and untreated. The relative expression levels are normalized to the control vinculin housekeeping. The data are expressed as means ± Standard Error (SE) of three independent experiments and statistical significance was evaluated (GraphPad Prism software 5.0) with one-way ANOVA followed by Dunnett's *post hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significant differences were evaluated vs. untreated cells (CTRL).

4.7 Confocal Microscopy and evaluation of coherency in CRC cells

Cell morphology differences had been more visible in immunofluorescence images: it appeared that the cytoskeleton and membrane of the treated cells have been reorganized and there are

several structures present, including protrusions or elongations of the filopodial type and lamellipodial protrusions that the cell uses to control its ability to migrate and invade. In particular, to investigate whether cytoskeleton organization was affected by treatment with PFs, actin fiber coherency, in control and treated cultures, was analysed. Motile cells must assemble their cytoskeletal actin filaments in a spatially organized way, such that net filament growth and cell protrusion occur at the front of the cell. The coherence was calculated from the structure tensor of each pixel in the image and is bounded between 0 (isotropic areas) and 1 (highly oriented structures). Results showed that PFs induced an important reorganization of actin filaments of cells cytoskeleton with coherency increasing at increasing concentrations of samples and this effect was highest with the PFs obtained from infected larvae compared with lower concentrations of samples and control cells in both HCT116 and HT29 cells (Figure 63).

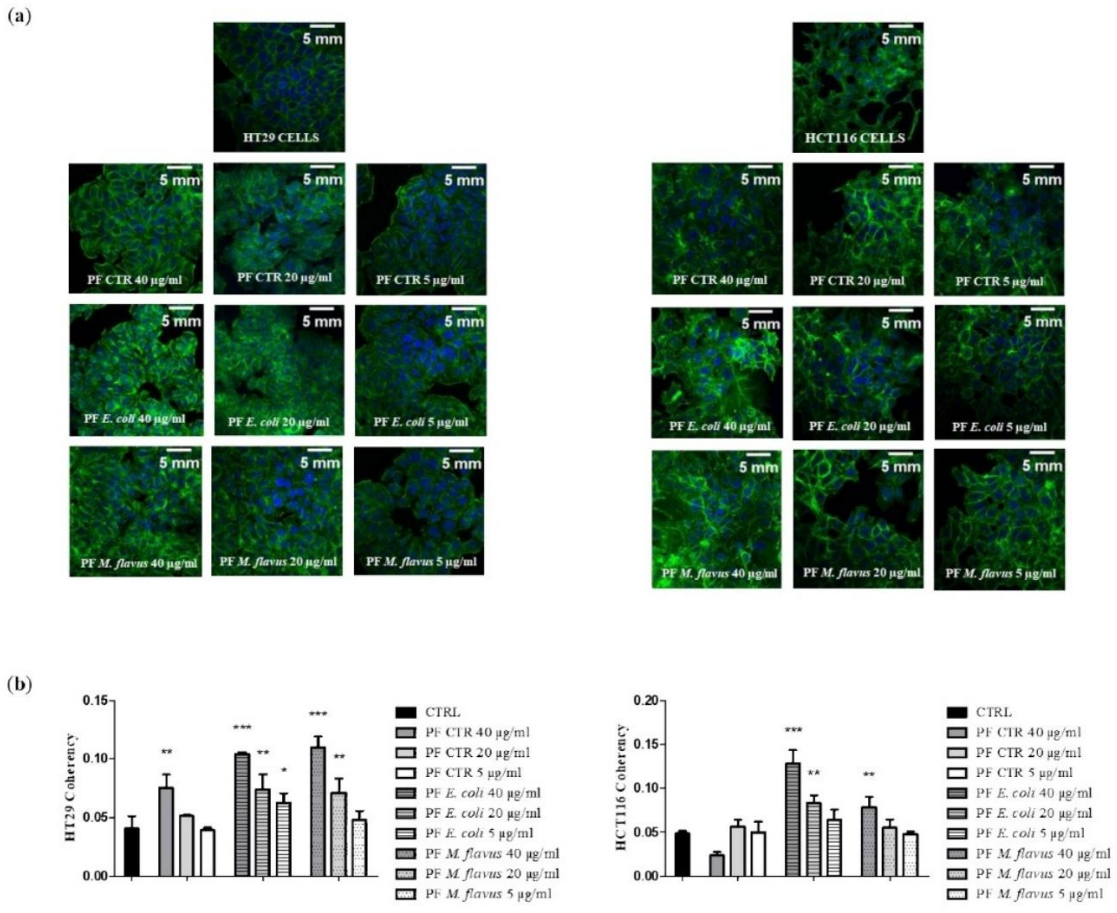


Figure 63. *H. illucens*-derived peptide fractions increased cytoskeleton coherency of human CRC cells. Cytoskeleton organization was affected by treatment with peptide fractions at different time points for both cell lines, as assessed by analyzing the actin fiber coherency in the control and treated cultures. Coherence was calculated from the tensor structure of each pixel in the image and is bounded by 0 (isotropic areas) and 1 (highly oriented structures), and the analysis was conducted on the

original confocal images, according to the procedure described in the Materials and Methods Section. (**a**) Representative images of a cytoskeleton after 48 h of treatment: actin filaments are shown in green, while cell nuclei are stained in blue. Scale bars: 5 mm. (**b**) Data are presented as means ±the Standard Error (SE) of three replicates of three independent assays. Statistical significance between groups was evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test (GraphPad Prism 5 software). The difference was considered statistically significant at * $p < 0.01$, and *** $p < 0.001$.

4.8 Wound Healing evaluation in CRC cells

To further investigate the effects of PFs on the phenotype of human CRC lines, the scratch-wound migration assay was used to analyze two-dimensional cell migration in control and treated HCT116 and HT29 cell cultures (Figure 64) at different time points. The results showed that PFs slowed down wound closure compared to untreated cells, in a dose-dependent fashion with the time needed for wound healing increasing at increasing concentrations of PFs and this effect was highest with the PF obtained from larvae infected with *E. coli* (Figure 65).

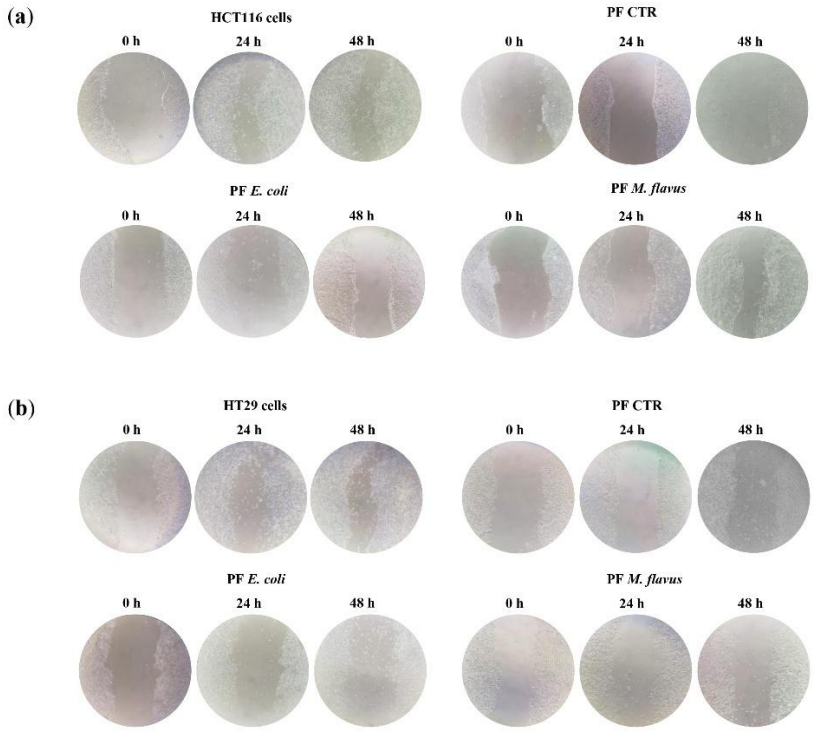


Figure 64. *H. illucens*-derived peptide reduced the motility of human CRC cells. Scratch test analysis. A total of 2×10^5 cells per well of (**a**) HCT116 and (**b**) HT29 cell lines were plated in 24-well plates and were allowed to reach confluence. A scratch wound was made using a pipette tip. Media were replaced, and cultures were exposed to peptide fractions and were

photographed at different time points. The samples delayed the closure of the wound in a dose-dependent manner. Magnification: 10×; scale bars: 80 µm.

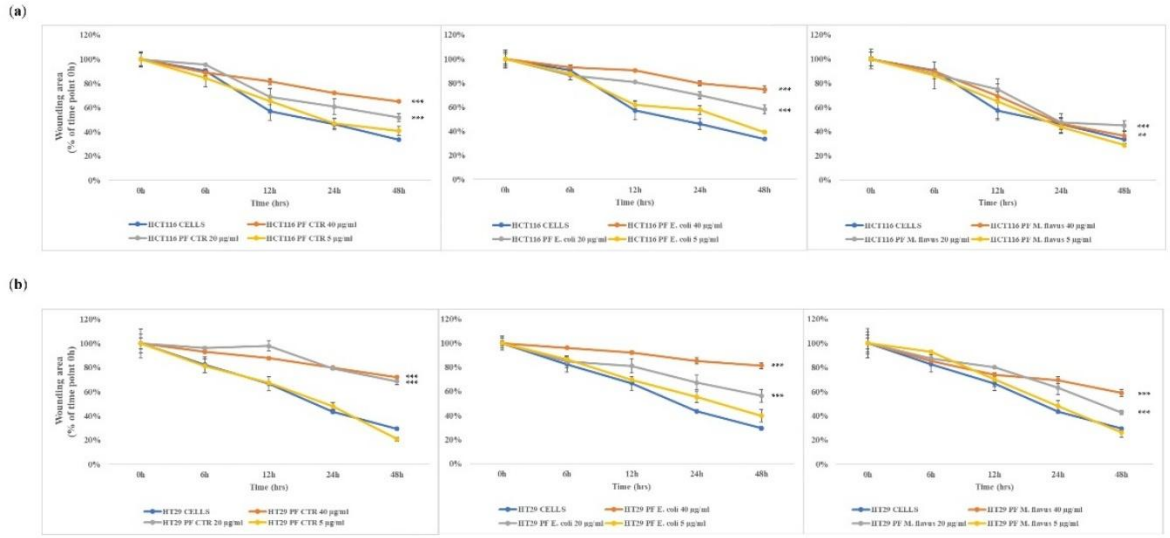


Figure 65. Wound area analysis of HCT116 and HT29 cells over time. The wound area for both (**a**) HCT116 and (**b**) HT29 was calculated by the ImageJ software version 1.53t (NIH, Bethesda, Rockville, MD, USA) at different time points (time: 0 h, 6 h, 12 h, 24 h, and 48 h). Statistical significance between groups was evaluated by two-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test (GraphPad Prism 5 software). The difference was considered statistically significant at ** $p < 0.01$, and *** $p < 0.001$.

5. DISCUSSION

Cancer is one of the leading causes of mortality worldwide and more and more cases of treatment resistance among patients are occurring. Antineoplastic resistances represent one of the most urgent therapeutic challenges worldwide and there is a great need for the development of novel alternative treatments (Vasan, Baselga & Hyman, 2019).

In particular, the approach to treat CRC (colorectal cancer) patients depends on tumor staging and Fluorouracil (5- FU)-based therapy is generally the first-line systemic treatment used, in combined regimens with oxaliplatin or irinotecan (Chau & Cunningham, 2009). Although these drugs have improved the CRC patient's overall survival, the therapeutic efficacy is hampered by CRC resistance and the undesired toxicity (Mármol *et al.*, 2017). New approaches able to tackle both the toxicity issues of chemotherapy and the resistance of CRC is of primary relevance (Punt, Koopman & Vermeulen, 2017).

Chemotherapy for GC (gastric cancer) patients instead of a single drug, it often consists of a combination of different drugs (e.g. epirubicin, cisplatin and fluorouracil are often used) (Puhr *et al.*, 2020), although surgical removal of the tumor mass is usually the first and most important intervention, depending on the severity of the disease and the degree of stomach involvement (Smyth *et al.*, 2020).

Unfortunately, as is the case with most cancers, the use of chemotherapeutic drugs for the treatment of stomach cancer has proved to be highly controversial: it seems, in fact, that the administration of some drugs is mainly useful for neoadjuvant purposes, i.e. prior to surgery, and that they may be useful in reducing the tumor extension, increasing the chances of successful treatment. Therefore, even in this case, targeted therapy with fewer side effects can be a hope for patients (Punt, Koopman & Vermeulen, 2017).

In the past screening of natural products has allowed the identification of some of the most active antitumor compounds (i.e. anthracyclines, Vinca alkaloids, epipodophyllotoxins, taxanes). Thus, although current research in this area is more focused on the development of drugs specifically targeting molecular mechanisms involved in tumorigenesis, the screening of natural products might still represent a valid alternative. Moreover, natural products could represent an inspiration source for the development of new antitumor drug candidates able to override resistance to currently available drugs and potentially characterized by an increased selectivity on tumor cells and reduced side effects on healthy cells (Wu *et al.*, 2023).

Active molecules derived from insects have been used in traditional medicine for centuries and still provide a valuable supply of healing substances in less-developed countries. The search for sustainable sources of biomolecules that find application in cancer fields is currently a goal of increasing importance. Insects constitute about 55% of the total biodiversity on Earth and are among the richest and most innovative sources of antimicrobial peptides (AMPs). Several insect-derived AMPs have been reported in the literature and also suggested as promising anti-cancer peptides (ACPs) potentially lacking toxicity to healthy cells and unaffected by common mechanisms of resistance (Slocinska, Marciniak & Rosinski, 2008; Chowanski *et al.*, 2017).

In this research work the hemolymph extracts were obtained from *Hermetia illucens* L. (Diptera: Stratiomyidae) larvae infected with strains of *Escherichia coli* or *Micrococcus flavus* and uninfected larvae and precipitated to obtain peptide fractions (PFs) containing AMPs with a molecular weight from 30 kDa and below, which, according to numerous published studies in literature, are the most active among all the pool of AMPs in terms of both their antimicrobial effect (Van Moll *et al.*, 2022; Erdem Büyükkiraz & Kesmen, 2022; Kleino & Silverman, 2014) and their toxicity towards tumor cells (Iwasaki *et al.*, 2009; Tian *et al.*, 2018; Leuschner & Hansel, 2004).

Insect PFs could be representing a large reservoir of unexplored compounds with possible anticancer activity (ACPs). While the anticancer activity of natural ACPs isolated from other sources has been previously explored, less extensive is the literature specifically regarding the therapeutic potential of ACPs isolated by insects (Felício *et al.*, 2017; Gaspar, Veiga & Castanho, 2013; Hoskin & Ramamoorthy, 2008; Mader & Hoskin, 2006; Koszałka *et al.*, 2011; Borrelli *et al.*, 2018).

The class of insects, which is distinguished by the abundance and variety of its molecules and activities, is one of the greatest sources of AMPs. Because they have an array of cellular and humoral responses from their innate immune system, insects are exceptionally well-adapted organisms to a wide variety of settingsFare clic o toccare qui per immettere il testo.. Insects can also consume materials that vary in their degree of contamination, therefore in order to combat these infections and endure hazardous environments, they produce AMPs (Kleino & Silverman, 2014).

In this study, the efficacy of PFs from infected and uninfected larvae of *H. illucens* as an anticancer drug on CRC and GC cells was evaluated and characterized, also considering for both type of cancer a carcinoma and an adenocarcinoma cell line.

Firstly, we observed that peptide extract from larvae of *H. illucens* strongly affects cell cycle progression due to a block in G2/M phase, showing a similar overall effect in all the cell lines tested. Furthermore, PFs induced an important reorganization of the cytoskeleton, leading to a reduced motility. These effects were more evident with the PF obtained from larvae infected with *E. coli.* Similarly, in gastric cancer (GC) cells, in particular AGS cells, a notable increase in G2/M phase accumulation was noted after treatment, highlighting a potential mechanism of action through the disruption of mitotic progression and inhibition of uncontrolled proliferation. Interestingly, in KATO III gastric cancer cell line, PFs did not induce significant apoptosis but led to cell cycle arrest in the S phase, suggesting differential cell line-specific responses to the treatment. Morphological alterations were observed in AGS cells, such as reduced cell size, vacuolization, and increased detachment, further indicating cytotoxic stress induced by the treatment. However, in KATO III cells, no significant morphological changes were detected, highlighting the importance of cellular context in determining peptide efficacy. Also the microscopic analysis of the CRC cell cultures confirmed the peptide fractions' inhibitory effect and evidenced different degrees of cellular deformation and distention

Apoptosis is induced only for HCT116 and not for HT29 cancer cell line by activation of Caspase-3 and cleaved Parp-1. HCT116 is known to be a highly aggressive cell line with little or no capacity to differentiate compared to HT29 and these different features, also in molecular point of view (HT29 is Kras wild type while HCT116 carrying Kras mutation), justify the different cellular behavior in response to PFs. Similarly, in GC cells, apoptosis was significantly induced, with the most pronounced effects observed in *E. coli*-derived PFs. Western blot analysis revealed a downregulation of the anti-apoptotic protein Bcl-2 and an upregulation of cleaved Caspase-3, supporting an apoptotic pathway activation. The differential apoptotic response observed between cell lines further underscores the complexity of mechanisms of action of ACPs, which may involve selective intracellular interactions and signaling modulations.

The anticancer activity of ACPs towards CRC cells is probably exerted by different cell biochemical pathways giving rise to multiple biological effects. Still little is known about the molecular effects of insect protein extracts on cancer cells. So it is possible that the identification and purification of specific ACPs from PFs could improve their anticancer effects. Our data lay the foundations for undertaking new studies to identify new natural substances deriving from an inexhaustible source such as that of the world of insects.

These findings collectively reinforce the therapeutic potential of insect-derived peptides in oncology. The selective cytotoxicity of these peptides toward cancer cells, combined with their ability to induce apoptosis, arrest the cell cycle, and impair migration, positions them as promising candidates for further preclinical development. Moreover, their efficacy in combination with traditional chemotherapeutics suggests potential synergy in overcoming drug resistance mechanisms. Future research should focus on elucidating the specific peptide compositions responsible for these effects, optimizing their stability and delivery, and exploring their application in combinatorial treatment strategies.

6. CONCLUSIONS

In conclusion, data from this thesis provide compelling evidence that *H. illucens*-derived peptide fractions possess anticancer activity through multiple mechanisms, including cell cycle arrest, apoptosis induction, and motility inhibition. While further investigations are necessary to fully characterize these peptides and their therapeutic potential, these findings highlight the untapped reservoir of bioactive molecules within the insect kingdom, offering novel avenues for the development of innovative anticancer treatments.

The immune stimulation of larvae using different microbial strains resulted in peptide fractions with distinct biological activities, suggesting that the nature of the immune challenge plays a critical role in shaping the peptide repertoire. This highlights the potential for modulating the insect immune response to enhance or diversify the therapeutic properties of hemolymph-derived extracts. In this context, comparative proteomic analyses and transcriptomic profiling could serve as valuable tools to elucidate the molecular composition of the most active fractions.

Importantly, the observed enhancement of chemotherapeutic efficacy in cancer cells also when combined with peptide fractions suggests a promising avenue for the development of adjuvant therapies. These findings support the integration of insect-derived peptides into combinatorial treatment strategies, potentially enabling dose reduction of conventional drugs and lowering the associated toxicity.

Beyond their anticancer properties, the peptides characterized in this study originate from a highly sustainable biological source. The use of *H. illucens*, a species already exploited in bioconversion and waste valorization systems, aligns with the principles of circular economy and green biotechnology. This represents a significant advantage in terms of production scalability and environmental impact, making insect-derived molecules particularly attractive for future pharmaceutical development.

Looking forward, future research should focus on the isolation and structural characterization of individual peptides responsible for the observed bioactivities. *In vivo* validation of efficacy and safety, evaluation of pharmacodynamics and pharmacokinetics, and formulation studies for drug delivery will be crucial steps toward clinical translation. Additionally, engineering

strategies such as peptide optimization or synthetic analog design may further enhance therapeutic performance and stability.

Overall, this thesis contributes to a growing body of literature demonstrating the vast, and still largely unexplored, potential of insects as sources of novel bioactive compounds. It paves the way for innovative, sustainable, and effective therapeutic strategies, particularly in the fight against cancer.

The reported data led to the publication of two scientific articles:

1. **Rinaldi R**, Laurino S, Salvia R, Russi S, De Stefano F, Galasso R, Sgambato A, Scieuzo C, Falco G, Falabella P. 2025. Biological Activity of Peptide Fraction Derived from *Hermetia illucens* L. (Diptera: Stratiomyidae) Larvae Haemolymph on Gastric Cancer Cells. *Int. J. Mol. Sci.* 26, 1885. https://doi.org/10.3390/ijms26051885
2. Lucchetti D, **Rinaldi R**, Artemi G, Salvia R, De Stefano F, Scieuzo C, Falabella P, Sgambato A. 2025. Peptide Fractions Extracted from the Hemolymph of *Hermetia illucens* Inhibit Growth and Motility and Enhance the Effects of Traditional Chemotherapeutics in Human Colorectal Cancer Cells. *Int. J. Mol. Sci.* 26, 1891. https://doi.org/10.3390/ijms26051891

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Appendix 1. – Publications

1) Scieuzo, C.; Giglio, F.; **Rinaldi, R.**; Lekka, M.E.; Cozzolino, F.; Monaco, V.; Monti, M.; Salvia, R.; Falabella, P. "*In Vitro* Evaluation of the Antibacterial Activity of the Peptide Fractions Extracted from the Hemolymph of *Hermetia illucens* (Diptera: Stratiomyidae)". *Insects*. **2023**; *14*(5):464. https://doi.org/10.3390/insects14050464.
2) Franco, A.; **Rinaldi, R.**; Giglio, F.; Ianniciello, D.; Boschi, A.; Scieuzo, C.; Salvia, R.; Falabella, P. "Edible Insects: An Overview on Farming, from Processing Procedures to Environmental Impact, with a Glimpse to Traditional Recipes and to Future Cultured Meat". *Entomologia Generalis* **2024**, *44*(4):813–831. https://doi.org/10.1127/entomologia/2024/2651.
3) Ianniciello, D.; Boschi, A.; **Rinaldi, R.**; Franco, A.; Giglio, F.; Scieuzo, C.; Salvia, R.; Letcher, S.; Kaplan, D.; Falabella, P. "A Comprehensive Review of Entomophagy under Legal, Historical, Safety, and Nutritional Profile". *Entomologia Generalis* **2024**, *44*(4):833–851. https://doi.org/10.1127/entomologia/2024/2524.
4) Scieuzo, C.; **Rinaldi, R.**; Giglio, F.; Salvia, R.; AlSaleh, M.A.; Jakše, J.; Pain, A.; Antony, B.; Falabella, P. "Identification of Multifunctional Putative Bioactive Peptides in the Insect Model Red Palm Weevil (*Rhynchophorus ferrugineus*)". *Biomolecules* **2024**, *14*(10):1332. https://doi.org/10.3390/biom14101332.
5) Lucchetti, D.; **Rinaldi, R.**; Artemi, G.; Salvia, R.; De Stefano, F.; Scieuzo, C.; Falabella, P.; Sgambato, A. "Peptide Fractions Extracted from the Hemolymph of *Hermetia illucens* Inhibit Growth and Motility and Enhance the Effects of Traditional Chemotherapeutics in Human Colorectal Cancer Cells". *Int. J. Mol. Sci.* **2025**, *26*, 1891. https://doi.org/10.3390/ijms26051891.
6) **Rinaldi, R.**; Laurino, S.; Salvia, R.; Russi, S.; De Stefano, F.; Galasso, R.; Sgambato, A.; Scieuzo, C.; Falco, G.; Falabella, P. "Biological Activity of Peptide Fraction Derived from *Hermetia illucens* L. (Diptera: Stratiomyidae) Larvae Haemolymph on Gastric Cancer Cells". *Int. J. Mol. Sci.* **2025**, *26*, 1885. https://doi.org/10.3390/ijms26051885.
7) Scieuzo, C.; **Rinaldi, R.**; De Stefano, F.; Di Fazio, A.; Falabella, P. "The Contribution of Molecular Biology to Forensic Entomology". *Insects* **2025**, *16*, 694. https://doi.org/10.3390/insects16070694.
8) **Rinaldi, R.**; Scieuzo, C.; De Stefano, F.; Masucci, L.; Falabella, P. "Vectors and Vector-Borne Diseases: Biology, Epidemiology, and Integrated Control Strategies". *BioFactors*

2025. UNDER SUBMISSION.

Appendix 2. – Abroad period

Introduction

The research period abroad was conducted at the Department of Genitourinary Medical Oncology – Research, Division of Cancer Medicine, University of Texas – MD Anderson Cancer Center (Houston, Texas, US), under the supervision of Prof. Giannicola Genovese. Immersed in this high-profile and internationally recognized research environment, the activities were aimed at consolidating advanced expertise in molecular cancer biology and functional genetic engineering. The main focus was placed on the role of epithelial-to-mesenchymal transition (EMT) in liver fibrosis and cancer progression, together with the study of clonal dynamics and the selective pressures that influence tumor evolution.

The host laboratory's program integrates molecular biology, functional genomics, and systems biology approaches with genetically engineered mouse models (GEMMs) to dissect the influence of specific genomic alterations on metastatic evolution and tumor biology. Key lines of investigation include:

- the characterization of tumor suppressor gene alterations, such as mutations or deletions;
- the analysis of oncogenic dependencies on activating mutations in KRAS, particularly KRAS^G12D or KRAS^G12V, which represent early and decisive events in tumorigenesis;
- the study of chromosomal imbalances, with particular attention to amplifications or deletions of the 9p21 locus, relevant in pancreatic, renal, and other solid malignancies.

A central aspect of the research involves the combined use of conventional GEMMs and somatic mosaic GEMMs (SM-GEMMs) with *in vivo* genome editing strategies. These are enabled by conditional Cas9 alleles (e.g., H11-LSL-Cas9) and the use of viral vectors such as adeno-associated viruses (AAVs) to deliver sgRNAs directly to target tissues. This technology allows for the induction of somatic mutations *in vivo*, thereby creating faithful models of tumor initiation and progression, closely mirroring patient-derived disease (Gurreri et al., 2023; Perelli et al., 2023; Perelli et al., 2025).

Materials and Methods

The methodological training was broad and multifaceted, covering both animal colony management and advanced molecular biology tools.

Mouse colony management

The training included the maintenance of large-scale mouse colonies, involving routine procedures such as ear or toe clipping for identification, weaning at ~21 days to separate sexes, genotyping via PCR-based assays, and strategic breeding to obtain specific allelic combinations. Detailed colony inventories were maintained to track genotypes, parental lineages, and experimental status, ensuring robust experimental reproducibility.

Genotyping strategies

PCR-based genotyping was routinely used to identify alleles essential for experimental models. These included recombinase drivers (Alb-Cre, Sox2-Cre, Pax8-Cre), oncogenic alleles (e.g., KRAS^LSL), conditional Cas9 alleles (H11-LSL-Cas9), fluorescent reporters (R26^LSL-FSF-tdTomato), and mesenchymal markers (Vim^FLEX-GFP/HSV-TK). PCR reactions were optimized per allele, with results analyzed by agarose gel electrophoresis.

Chemical induction of liver injury and tumorigenesis

To model chronic liver damage and multistep hepatocarcinogenesis, mice were subjected to combined treatment with diethylnitrosamine (DEN) as a mutagen and carbon tetrachloride (CCl_4) to establish persistent inflammation and fibrosis. These chemical insults provided a pathological background in which to interrogate EMT dynamics and genetic interactions.

Lineage tracing and selective ablation

Dual recombinase systems enabled high-resolution lineage tracing. The R26^LSL-FSF-tdTomato allele provided permanent hepatocyte labeling following Cre recombination, while the Vim^FLEX-SA-GFP-HSV-TK cassette specifically traced EMT-derived mesenchymal lineages. The inclusion of HSV-TK allowed selective ablation of these lineages upon ganciclovir administration, facilitating functional interrogation of their contribution to fibrosis and tumorigenesis.

sgRNA design and viral vector construction

A core methodological component involved the design of sgRNAs targeting tumor suppressors (Cdkn2a/b, Nf2, Setd2), oncogenic drivers (KRAS), or immune regulators. Guide RNAs were designed using bioinformatic pipelines to minimize off-target effects and cloned into viral backbones under U6 promoters. Both single and multiplex sgRNA constructs were generated to allow simultaneous perturbation of multiple loci.

Viral production and *in vitro* validation

The sgRNA-containing plasmids were packaged into adeno-associated viral (AAV) vectors or, for some applications, into lentiviral particles. Packaging was carried out by co-transfection of HEK293T cells with helper plasmids. Viral supernatants were collected, concentrated by ultracentrifugation, and titrated. *In vitro* validation included infection of target cell lines to confirm Cas9-mediated cleavage and loss of protein expression prior to *in vivo* application.

Somatic genome editing *in vivo*

For *in vivo* knockout studies, viral preparations carrying sgRNAs were delivered to mice harboring conditional Cas9 alleles (e.g., H11-LSL-Cas9). Tissue-specific editing was achieved through systemic injection or local delivery, depending on the target organ. This approach generated somatic mosaic GEMMs (SM-GEMMs), in which targeted mutations arose stochastically across hepatocytes, closely mimicking the heterogeneity observed in human tumors.

Clonal analysis (MAMT platform)

The Mosaic Analysis of Mutant Tumors (MAMT) platform would be used to monitor clonal competition and selection. By integrating Cre/Flp dual recombinase systems with fluorescent reporters (GFP, BFP, tdTomato), distinct mutant clones could be stochastically labeled and followed over time. When combined with CRISPR-mediated knockout of tumor suppressors within the 9p21 locus (Cdkn2b, Mtap, Ifnb1), this system would allow real-time visualization of clonal expansion, cooperation, and selective advantage *in vivo*.

Necropsy and histopathology

At experimental endpoints, necropsies were performed to recover tumors and organs. Macroscopic data included tumor size, weight, and metastatic burden. Tissues were processed for histology, including H&E staining, immunohistochemistry, and immunofluorescence. Frozen samples were collected for molecular analyses, and fresh tissues were used for organoid generation.

Immunofluorescence and molecular assays

Training also encompassed immunofluorescence for fluorescent reporters (tdTomato, GFP) and pathway markers (e.g., phospho-ERK), as well as molecular biology techniques including PCR, Western blotting, sgRNA design, and cloning. These assays enabled the integration of molecular readouts with lineage-tracing and clonal dynamics analyses.

Results (formative outcomes)

The main outcomes of this research period were educational and methodological rather than quantitative. Nevertheless, important conceptual insights were gained.

The models employed allowed the study of:

- EMT dynamics *in vivo*, including the contribution of EMT-derived hepatocytes to fibrotic remodeling and neoplastic transformation.
- Clonal competition and selective pressures, with particular attention to the impact of Cdkn2a/b, Nf2, and Setd2 loss, and the activation of KRAS^G12D.
- The power of dual recombinase systems and fluorescent reporters for stable and heritable lineage tracing of hepatocytes undergoing EMT.
- The potential of HSV-TK/ganciclovir-mediated ablation to directly assess the causal role of EMT-derived lineages in fibrosis and tumorigenesis.
- The stochastic labeling of genetically distinct clones through the MAMT system, which will enable real-time monitoring of clonal expansion, competition, and selection pressures during tumor evolution.

From a practical perspective, the training provided advanced expertise in mouse colony management, *in vivo* experimental techniques, molecular cloning, viral vector design, and the principles of CRISPR-based somatic mosaic modeling. These skills are essential for translational cancer biology and directly applicable to other biological contexts.

Discussion and Conclusions

This research period represented a pivotal step in scientific training, providing not only practical expertise but also a conceptual framework for addressing fundamental questions in oncology. The combination of GEMMs, SM-GEMMs, lineage tracing, and CRISPR/Cas9 genome editing offered a unique opportunity to dissect the molecular mechanisms of EMT, clonal dynamics, and tumor heterogeneity in physiologically relevant settings.

The methodological training acquired is of particular translational value:

- **Genome editing *in vivo***: strategies for somatic gene targeting and the generation of complex mutational landscapes.
- **Lineage tracing and functional interrogation**: tools for mapping cellular plasticity, assessing lineage fate, and selectively ablating populations to test functional contributions.
- **Clonal analysis and competition**: models to evaluate the selective pressures and evolutionary trajectories that shape tumor heterogeneity.

Beyond the oncological context, this experience highlighted the broader applicability of these approaches. CRISPR/Cas9 genome editing and lineage-tracing tools are increasingly being exploited in entomology to investigate the role of antimicrobial peptides (AMPs) in innate immunity and tumor suppression. Insects produce a wide range of AMPs, such as cecropins and defensins, which display selective cytotoxic activity against mammalian tumor cells. For example, cecropin B derivatives have been shown to exert antitumor effects on leukemia and bladder carcinoma cell lines, while *Drosophila* defensin promotes tumor regression *in vivo* (Parvy et al., 2019). In *Drosophila*, CRISPR/Cas9 knockout of immune-inducible AMP genes (attacins, diptericins, drosocin, defensin) has revealed their tumor-suppressive roles through modulation of apoptotic pathways and immune signaling (Hanson et al., 2019). Similarly,

CRISPR-based functional genomics in *Aedes aegypti* has been employed to manipulate immune regulators such as AaRel1 (Toll pathway), with applications ranging from antiviral resistance to immune effector modulation (Jaiswal et al., 2023). Novel delivery systems, such as peptide-mediated Cas9 ribonucleoprotein transduction (ReMOT Control), further expand the versatility of these tools by enabling heritable genome editing without embryonic microinjection (Chaverra-Rodriguez et al., 2018).

Final remarks

In conclusion, the research period abroad provided in-depth training in genetic engineering, *in vivo* tumor modeling, and advanced functional genomics. It also emphasized the transferability of cutting-edge tools, such as CRISPR/Cas9 editing and lineage tracing, from cancer biology to other disciplines, including entomology and antimicrobial peptide research. The formative value of this experience lies not only in the acquisition of technical and conceptual skills but also in the establishment of research avenues whose results, currently in progress, are projected to generate relevant publications contributing to both basic and translational oncology.