



## Therapeutic role of protease inhibitors from plant source

### *Aegle marmelos in Breast Cancer treatment*

Shilpi Kumari<sup>1</sup> and Manjusa Dake<sup>2</sup>

Dr. D.Y. Patil Vidyapeeth, Pune, MH, India

---

#### Abstract

*The exact role of VE-cadherin in breast cancer is still unknown and the aims of this study is to prove whether reduced VE-cadherin expression can be a prognostic factor in patients with Breast cancer. Current research is aimed at enhancing the utility of medicinal therapeutic substances, which are widely available, safe, and cost-effective. Due to its medicinal properties such as antimicrobial, antiviral, radio protective, anticancer, chemo preventive, antipyretic, and anti-inflammatory properties etc. Aegle marmelos is an important traditional plant used to treat a variety of diseases, according to experimental and clinical evidence. The goal of this study was to develop novel lead compounds for the selective competitive inhibition of Ve-Cadherine protein which is key proteins in human cancer. The docking analysis was carried out at varied binding affinity levels to investigate the efficiency of indigenously Ve-Protein, to aegeline ligands. The maximum affinity for Ve-Cadherin protein (PDBID-5UJJ) was found in Threonine (7.7kcal/molwithRMSDvalueof2.38and 2.48). We believe that ligands, such as Aegeline, Imperatorin, and Palmitic acid are specific compounds that can be promising lead molecules to develop effective drug. Target Proteins Ve-Cadherin, will receive more attention in future research as a tumor suppressor in cancer cells.*

**Keywords:** Protease inhibitor, Adherensjunctions, Adhesion, Angiogenesis, Cadherine protein, Signaling, VE-cadherin

#### INTRODUCTION

Proteases are simply known as proteolytic enzymes essential for catabolism of protein and amino acid generation in primitive organisms. Proteases catalyse proteolytic cleavage of very

specific protein, to form smaller peptide and amino acids found in plants, animals as well as microbes.

Proteases monitor diverse cellular activities including protein modulation interactions, generation of molecular signal and new bioactive molecules, processing of cellular information, transduction and amplifications. Proteolytic enzymes play important role in cell growth and division, generation of blood vessels, production of nerve cells, reproduction process, wound healing, senescence, destruction of cellular components, blood clotting, and cell death due to diseased condition.

Protease inhibitors represent superior category of administrative molecules that, switches protein degradation reaction universally altogether in living entities (Kassel, 1970; Urnezawa, 1982). Most of the Protease inhibitors are valuable tools used for inactivation of target proteases directed to human related disease such as arthritis, thrombosis, pancreatitis, AIDS, muscular dystrophy, cancer (Johnson and Pellecchia, 2006). They have very high inhibitory activity against insect pest protease and thereby increase the crop production. Natural inhibitor increases storage span of various perishable seafood material. They inhibit protein hydrolysis by intracellular and extracellular proteolytic enzymes. So they are valuable in the food processing and preservation.

Plants are the major source of naturally occurring protease inhibitors that are characterized (Tamir *et al.*, 1996). They are serine protease inhibitors which inhibit the action of trypsin. Protease inhibitors are commonly found in the seed of plants, as well as in tubers and leaves. They are small molecular weight proteins with molecular weight ranging of 8000-10000. Plant's proteinase inhibitors (PIs) show defensive role against insect pests by anti-nutritional action, predators and pathogens and are induced in plant tissues by wounding and herbivory. They play an important role in plant nutrition and physiological and clinical processes. Protease inhibitors (PIs) have unique feature for the prime candidates to have large applications in life sciences. Apart from very common primary and secondary metabolites, protease inhibitors are most common groups of proteins. Most PIs form complex by binding with enzyme (Norton, 1991)

### **Classification of protease inhibitor:**

Protease inhibitors are also classified as:

1. Small molecule inhibitors
2. Proteinaceous inhibitors

#### **1. Small Molecule Inhibitors:**

Small molecule inhibitors derived from natural source includes pepstatin, bestatin, and amastatin, and synthetic inhibitors. SMIs are inhibitors including peptide and synthetic inhibitors are low molecular weight peptides. Naturally occurring SMIs produced by bacteria and fungi (Rawlings, 2010) and form transition state mimics as peptide aldehyde and boronates, peptidyl chromoethane and sulfonyl fluoride derivatives (Powers and Harper, 1986).

#### **2. Proteinaceous Inhibitors:**

Naturally occurring protease inhibitors originated from bacteria to animals and plants. They exhibit reversible or irreversible binding with proteases to restrict entry of substrate. e.g.

BPTI (Bovine Pancreatic Trypsin Inhibitor), alpha-1 PI (alpha-1 Proteinases Inhibitor),  $\alpha$ -2 macroglobulin.

**Classification of protease inhibitors based on the types; (Mechanism of inhibition) as-**

### **Serine Protease Inhibitor:**

Serine protease, a large family of naturally occurring inhibitors. Most serine protease inhibitors are low molecular mass molecules (3-25 kDa) inhibiting trypsin and chymotrypsin. Serine protease inhibitors are classified based on their substrate specificity and functional amino acid residues found in PI as either as positively charged or hydrophobic for trypsin, elastase, and chymotrypsin (Barrett *et al.*, 1998). Recently serine protease inhibitor LC-PI showed strong inhibition of the growth of *K.pneumonia* and *P. aeruginosa* (Syed Rakshanda *et al.*, 2012) which cause urinary tract infection, Pneumonia and septicemia in humans. Protease inhibitors are currently under the focus due to their potent anti- carcinogenic effect in various in vivo and vitro systems (Gillsefet *et al.*, 2007). Recent studies have reported that PI's are employed as new drugs in antiretroviral combination therapy which increase the expectancy in HIV patients (Bobbarala *et al.*, 2009). The PI's from the leaves of *Cocciniagrandis* found to exhibit to high degree of inhibition of growth in *K. pneumonia* (Satheesh Murugan 2001). Mainly the inhibitors are characterized from families like Leguminosae, Solanaceae and Gramineae (Gracias Olmedo *et al.*, 1987). The serine protease inhibitors can be classified into 13 structurally distinct families based on the source. Six families of SPIs are of mammalian or microbial origin as Hirudin, Bovine Pancreatic Trypsin Inhibitor (BPTI), Kazal, and the Chelonian in, the Streptomyces subtilisin inhibitor (SSI), Serpins. Seven families are of plant origin includes Cucurbit family, Bowman- birk family, the Cereal superfamily, the Potato serine protease inhibitors family, the Thaumatin family, the Kunit- type family.

### **1.) Cysteine Protease Inhibitors (Cpi):**

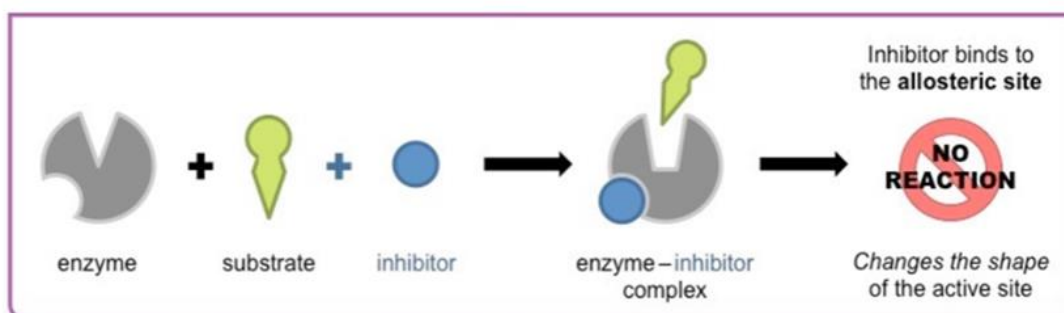
The class of cysteine PI is also called as cystatin super family, this super family excludes the members belonging to the Kunitz-type inhibitors and the clitocypin family. Cystatins and oryzacystatins inhibit the bacterial and viral replication (Tollinefet *et al.*, 2005). These inhibitors were reported for the first time by Khaner *et al.* (2013). In animals' cysteine protease inhibitors can be classified into the statin, cystatin and kininogen families according to their molecular mass and the presence or absence of cysteine residue.

### **2.) MetalloCarboxy Protease Inhibitors (Mcpis):**

MetalloCarboxy protease inhibitors from plants can be distinguished into two families. The MetalloCarboxy peptidase inhibitor isolated from potato and tomato plants (Rancor and Ryan, 1968) and cathepsin-D inhibitor isolated from potato. These inhibitors have 38-39 amino acid residues with three S-S bonds having the molecular weight of 42 kDa (Hass and Hermodson, 1981). MCPI's inhibit carboxy peptidase from both animals and microorganisms, (Havkioja andNeuvonen, 1985). The inhibitors that bind the metalloCarboxy peptidase have been identified in solanaceous plants and organisms like *Hirudo medicinalis* (Arolasefal., 2005). This inhibits the activity of thermolysin, matrilysin, collagenase, atrolysin C, stromelysin, carboxypeptidase A, and TNF- $\alpha$  convertase. The potato carboxy peptidase inhibitor (CPI) extracted from *Solanum tuberosum* inhibits thermolysin metalloCarboxy peptidase.

### 3.) Mechanism of Protease Inhibitors:

Protease inhibitors inhibiting the action of proteases depending on the mechanism inhibition and the type of protease. Understanding various mechanisms by which inhibitors function is valuable for therapeutic strategy. Reversible inhibitors bind by through noncovalent interactions.

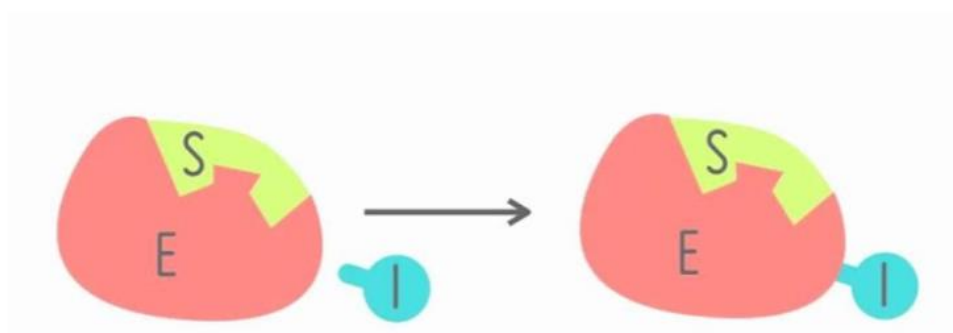


**Fig 1: Mechanism of Protease inhibitor interactions**

### 4) Competitive Inhibitors:

These resemble the substrate for binding with the enzyme active site. Competitive inhibitors resemble with the transition state of natural substrates. Therefore, compounds mimicking substrates bind with the enzyme, but could not undergo catalytic action. e.g.- Aprotinin which inhibits many serine proteases.

### 5) Uncompetitive Inhibitors:

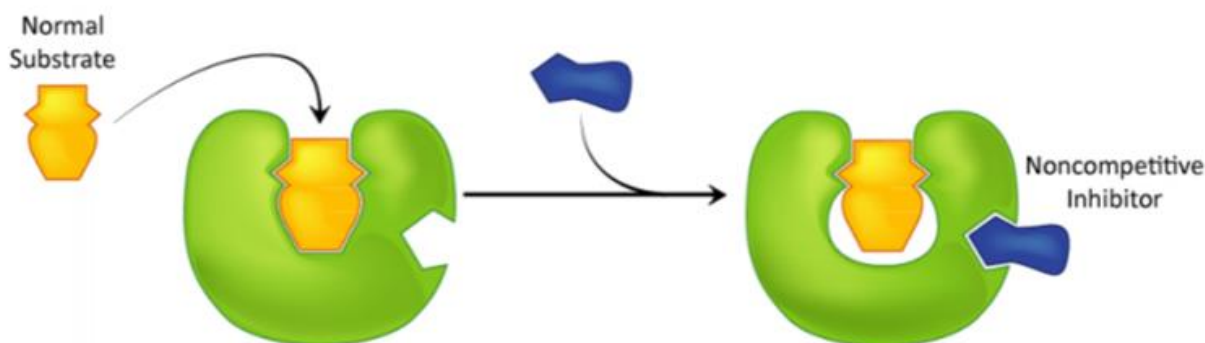


**Fig 2: Uncompetitive Inhibitors**

Bind to the enzyme at a site different from the enzyme active site. These inhibitors are effective against HIV-1 protease and the NS2B-NS3B proteinase of the west Nile virus.

## 6.) Non- Competitive Inhibitors:

Binds to the protease irrespective of substrate molecule with similar affinities using allosteric mechanism. e.g. BBI (Bovine-Birkprotease inhibitor), a trypsin inhibitor from the soybean and aminoglycoside inhibitor with non- competitive.



**Fig 3: Non-competitive Inhibitors**

## 7.) Irreversible Inhibitors

They function by destroying the active site functional group of enzymes through covalent binding. Some inhibitors showing higher affinity without covalent binding cannot be easily removed from the enzyme. Suicide inhibitors are examples of irreversible inhibitors. e.g. DIFP (Di-isopropylfluorophosphate) acts as a suicide inhibitor of chymotrypsin. Find out the role of matrix metalloproteases in cancer. Thereby screening of protease inhibitors against matrix metalloproteases will enable to formulate design novel drugs against the cancer disease.

## 8.) Sources of Protease Inhibitors

### 8.(A) Protease Inhibitors from Animals

Protease inhibitors in animals inhibit endogenous proteinases in tissues e.g. pancreatic trypsin inhibitors. Most animal produce a variety of protease inhibitors to prevent unwanted proteolysis through steric hindrance. Mammalian defense protease inhibitors with super families of serpins and cystatins have medicinal importance. Cystatin is a super family that regulates lysosomal cysteine protease from mammals and plants and known to act as a defensive agent against bacteria, viruses and plant eating insects.

### 8.(B) Protease Inhibitors from Plants:

Plants are important sources protease and protease inhibitors with least 12 sub families with ten protease inhibitor families originating from plants seeds, leaves and the tubers including leguminous seeds (Olivia *et al.*, 2000) as Kunitz, Bowman-Brik, potato, squash, cereal super family, thaumatin-like and Ragi A1 inhibitors (Richardson, 1991). Plant protease inhibitors applied in agriculture insecticidal activity. Plants have protease inhibitors in excessive amounts to inhibit endogenous proteases. Protease inhibitors act against pest proteases (Lawrence and Koundal, 2002). Plant protease inhibitors have important role in their natural defense mechanism against herbivores or phytophagous insects by impairing protein

digestion (Broadway and Duffey, 1986; Ryan, 1990). Protease inhibitors from plants may serve as an important avenue of therapy because of their lesser side effects and lower cost as remedies.

### **8. (C) Protease Inhibitors from Microorganisms;**

The presence of diverse proteases in microbes and fungi also indicate them as a source of novel protease inhibitors. Marine microorganisms are potential source of enzymes inhibitors as low molecular weight compounds (Chiaki, 2004) having property like antibiotics inhibiting the activity of enzymes. The production of protease by microbes provides cell protection against its own proteolytic enzymes. The microbial and fungal protease inhibitors have unique inhibitory profiles that resist photolytic cleavages along with as well as high thermal tolerance along with broader pH range and stability, a useful property during immobilization under harsh conditions. Sea sponge is a source of novel metabolites including enzymes inhibitor (Lee *et al.*, 2001). e.g. *Aplysinaaerophobia*.

### **9.) Applications of Protease Inhibitors**

Protease inhibitors have applications in medical, agricultural and food sector. Proteolytic actions often cause food spoilage prevented by applying protease inhibitors. In surimi production, proteinases inhibitors minimize gel- forming ability caused due to the action of endogenous myosin degrading proteinases. Microbial food spoilage resulting the loss of 25% loss in food industry (Baird-Parker, 2003). Protease inhibitors are applied in preservation of seafood by preventing the proteolytic softening of fish, Mollusca and crustaceans by (Garcia-Carreio, 1996). This enhances the shelf life of seafood such as salted fish products.

### **10.) Protease Inhibitors as Defense Tools for Protection**

Prevention of crop pest involves use of chemical pesticides that leads to development of resistance by plant pathogens. In view of this a genetic approach is used to generate insect-resistant plants. The proteins originating from plants, exhibiting insecticidal effect includes protease inhibitors providing an effective strategy for insect pest control. Protease inhibitors enhance nutritional value of food (Lawrence and Koundal, 2002).

### **11.) Protease Inhibitor as Therapeutic Agents;**

Protease inhibitors have medicinal value with distinct role in all biological processes. Loss of control on proteolytic action impairs physiological processes like growth, cell division, DNA replication, blood clotting and immune response. Uncontrolled protease activity leads to diseases related to heart, brain, cancer etc. Thereby, proteases are considered to a prime target as therapeutics.

### **Serpin (Serine Pi) Family:**

A single gene in prokaryotes and multiple serpin coding genes are found (Irving *et al.*, 2002a), in eukaryotes.

### **Bowman Birk Inhibitors Family (Bbi) Family:**

BBIs are a first family of classic protease inhibitors recognized in soybean (*Glycine max*) (Birk *et al.*, 1963), legumes seeds and cereals (Laing and McManus 2002) and grass family poaceae (Odianiet *et al.*, 1986).

### **Kunitz Family:**

Protease inhibitors from kunitz inhibit serine class of proteases (Laing and McManus, 2002) and are common in plants including legumes, cereals and in solanaceous species (Ishikawa *et al.*, 1994).

### **Docking:**

In hit discovery and lead optimization, computational techniques that dock small molecules into the structures of macromolecular targets and their potential complementarity to binding sites are commonly utilised. Indeed, structure-based design and screening methodologies have had a significant impact on the creation of a number of medications, including HIV protease inhibitors. Molecular docking is a computational process that uses unbound structures, structures created from MD simulations, homology modelling, and other methodologies to predict non-covalent interactions between macromolecules or, receptor-ligand interactions, molecular conformations and binding affinity. Based upon this data, virtual screening of libraries of drug-like compounds as a lead compound for drug development is practised. The prediction of small molecule binding to proteins is of special practical importance. When experimental hollow structures are lacking, docking can be utilised to estimate the bound conformation of known binders. Because docking necessitates a substantial amount of computational power, it's preferable to increase the accuracy of these forecasts while reducing the amount of time they take up on the computer.

## **12. Auto DockVina**

Auto DockVina, a novel molecular docking and virtual screening software, is demonstrated. According to our experiments on the training set used in the development of Auto Dock 1.5.6, AutoDockVina delivers When compared to the molecular docking programme produced in our lab earlier, AutoDock 1.5.6 has a speedup of about two orders of magnitude (Auto Dock 1.5.6), while also considerably enhancing the accuracy of binding mode predictions. On multicore CPUs, parallelism and multithreading assist to speed up the process even further. Auto DockVina generates grid maps automatically and groups the results in a user-friendly manner.

## **REVIEW OF LITERATURE**

PIs (protease inhibitors) categorized based on the protease they inhibit, which consist of serine, cysteine, aspartic and metalloproteases. In which serine protease inhibitors form a major superfamily of PIs<sup>285</sup> and based on conserved functional motifs they can be classified into many classes, in which the Kunitz-type inhibitors, well characterized, due to their abundance. They have a vital role in a cell by constraining harmful proteolytic enzymes. In humans monitor molecular pathways associated with tissue homeostasis, and cellular defense. Few of them are involved as chaperons (HSP47), regulator of blood pressure (angiotensinogen SERPINA8) and hormone transporter proteins (thyroxine and cortisol-binding globulin). Therefore, *Capsicum frutescens* of interest to isolate and purify protease inhibitor. It comes under the Solanaceae

family and is a shrubby perennial plant. It's distributed throughout the warmer parts of India and cultivated. The fruits called capsaisin reduce pain sensations and effective indigestive disorders of stomach, intestine. It is also useful heart diseases and blood disorder. It can also be explored as a relief for toothache, seasickness, alcoholism, malaria and fever. A serine-protease inhibitor recognized from soybean (Bowman, 1940), purified by Birk in the 1960s termed Bowman-Birk inhibitor (BBI). According to studies it was observed that Japanese people suffering from breast, colon, and prostate cancer take soybean rich diet (61-63). BBI has anti carcinogenic properties at nanomolar concentrations(64).BBI are under clinical trial phase I for treating oral leukoplakia patients without toxic effects phase (65) Phase II clinical trial with 31 patients with oral leukoplakia by Armstrong and associates(66)reduced 24.2% in total lesion area. Currently, randomized, placebo-controlled trials are being performed. Plants have three types of multifunctional proteases target different enzymes and control more than one biological process.

The 1st is bilateral inhibitors which has two different target sites (Bowman- Birk inhibitors) and number of hydrolases and  $\alpha$ - amylase/ protease inhibitor and prevent seed predation as well as early germination. The 2nd type of multifunctional protease inhibitor has multi domain which develops any duplication of the region of the DNA and involve in tuber maturation and defense mechanism. The 3rdtype of multifunctional protease inhibitor consist of numerous inhibitory folds which looks like net traps e.g. Serpin regulating cell death, and  $\alpha$ - macro globulins. These have different loops which is present on the surface and are substrate specific (Hedstrom *et al.*,1992) and that's why it requires distinct inhibitory sites. Proteinases inhibitors found in plants and their belongings and a variety of systematic group, Solanaceae family has a high amount of proteinases inhibitor. Currently PIs are under focus due to their potent ability to inhibit carcinogenesis in vivo and in vitro systems. Since pathogenic microorganisms produces proteinases and its activity is suppressed by the inhibitory polypeptide which is synthesized by the plants e.g. tomato plant developed resistance against with *Phytophthora infestans*, elevation in trypsin and chymotrypsin inhibitors results increase in plant resistance towards pathogen. Potato tubers consists of 20-24 kDa serine protease inhibitor induced after mechanical injury and infection by P. infestans. The work of antimicrobial protein

A protease inhibitor acts as antimicrobial protein to overcome disease resistance derived from plants or natural products. M.M. Molin *et al.*, 2014, analyzed presence of protease inhibitor produced in plants during regular plant growth that prohibit enzymatic activity of phytopathogenic microorganisms. e.g. trypsin inhibitor, in the leaf of *Capsicumbaccatum*varpendulum- PepYMV.Leaf extracts isolated from plants accession number UNEF 1624 after 0, 24, 48, 72, 96,120- and 144-hourswere resistant to Pep YMV was collected. Protein extract from leaf sample has inhibitor activity against trypsin that was purified by reverse phase chromatography using C2/C18 column. Protease inhibitors play an important role in many biological processes such as formation and dissolution of blood clot and lysis of fibrin clots, transport and processing of secretary proteins and programmed cell death (Neurath, 1989). Most of the serine protease inhibitors apparently hinder proteases with alike standard competitive mechanism (Garcia-Oimededeo *et al.*, 1987).

For the screening, purification and classification of the protease inhibitor there are so many techniques and parameters are used. Crude samples extracted from the leaves of plant and the purification was done by ammonium sulphate precipitation for the further inhibitory activity. Partial purification with ammonium sulfate was done at different fraction to show the highest inhibition of protease inhibitor activity, and one of the fractions showing high estprotease inhibitor activity, was further purified. The crude extract was purified. Characterization for its



biophysical and physicochemical employing ammonium sulphate precipitation, dialysis and chemical properties like molecular weight, stability at different cellulose chromatography. SDS PAGE profile of the peaks is depicted protease inhibitor extracted from the leaves. Proteolysis is one of the most indispensable metabolic processes of protein processing and turnover. These enzymes are indispensable in developmental processes like programmed cell death. Proteases are highly substrate specific, with respect to structural and chemical properties at the active site. In the present study, the screening of plant was carried out based the inhibition assay using trypsin. Different plants were screened and subjected purified by salt precipitation method where highest activity has been observed. Herbal medicines have been vital treatments in India from ancient times and have made a significant contribution to traditional medicine for human health care. Future objectives for developing effective therapeutic molecules will be medicinal plants that are loaded with a significant number of secondary metabolites. In this light, current research is aimed at boosting the utility of medicinal therapeutic substances, which are widely available, safe, and cost-effective. Due to its medicinal properties such as antimicrobial, antiviral, radio protective, anticancer, chemo preventive, antipyretic, and anti-inflammatory properties etc. *Aegle marmelos* is an important traditional plant used to treat a variety of diseases, according to experimental and clinical evidence.

### **Cathepsins: role of cathepsin in cancer**

Cathepsins are lysosomal proteases that are categorised into cysteine, aspartate, and serine cathepsins based on their active sites (Brix K., 2005). Human cysteine cathepsins (Cts) have a cysteine as catalytic residue is highly conserved. Endocytosis, phagocytosis, and autophagocytosis are all required for the breakdown of proteins that are internalised in lysosomes (Pu J., 2016)

They also help in production of active proteases and extracellular matrix (ECM) remodelling (Fonović and Turk, 2014; Kukor Z. *et al.*, 2002). Cts are engaged in a variety of activities, including immune response, apoptosis, development, and differentiation, and are essential in maintaining tissue homeostasis under physiological settings (Reiser J *et al.*, 2010). Cts expression, localisation, and activity have all been linked to a variety of diseases, including cancer progression (Gocheva and Joyce, 2007; Sudhan and Siemann, 2013) and ectopic expression (Chen, 2017). Cts have been discovered in the cell cytoplasm, nucleus, mitochondria, and extracellular space in recent studies, showing that they have a broad biological action (Sever *et al.*, 2007; Goulet *et al.*, 2004; Cheng *et al.*, 2012). Secreted Cts contribute to tumour ECM degradation and remodelling during cancerogenesis, whereas intracellular cathepsins are key components of signalling networks that might promote cancer cell proliferation and inflammation (Koblinski *et al.*, 2000; Vidak *et al.*, 2019). Cts are also involved in the tumour microenvironments response to anticancer therapy, and they can play a key role in the establishment of therapeutic resistance (Olson and Joyce, 2015; Zheng *et al.*, 2004; Siu *et al.*, 2016). Human Cts have roles in tumour development, infiltration, mortality, and chemotherapeutic response regulation. The relationship between human Cts and cancer were demonstrated and also their involvement in tumor progression, infiltration, death, and their regulation in response to chemotherapeutics.

### **VE Cadherin: Role of cathepsin in cancer?**

VE-cadherin is endothelial specific adhesion molecule found at junctions between endothelial cells. Angiogenesis in response to tumor-derived signals is known as tumour-induced angiogenesis. Angiogenesis is essential for tumour progression, as tumours that lacking

angiogenesis are relatively harmless (Folkman, 2006). Tumors that engage in the angiogenic process, on the other hand, are significantly linked to advanced disease. Tumor-induced angiogenesis, like physiological angiogenesis, involves the encouragement of endothelial cells to break out of dormancy for proliferation and permeability (Carmeliet and Jain, 2011). Increased permeability initiates angiogenic process, necessary for outward movement of endothelial from the primary vessel for the attachment of breast cancer cells to endothelial cells Cai *et al.*, 1999).

The paracellular pathway controls the opening and closing of cell–cell junctions. To preserve vascular integrity, this function must be tightly regulated, and it necessitates the creation of sticky structures between adjacent cells, such as adherens junctions and tight junctions. The main constituent of adherens junctions, VE-cadherin (vascular endothelial cadherin), represent a family of calcium-dependent homophilic adhesion proteins to create a molecular link among extracellular space, plasma membrane, and cytoskeleton (Dejana, 2004).

Extracellular cadherin domains (EC-domain) characterise them, and they mediate adhesion through homophilic, Ca<sup>2+</sup>-dependent interactions. The majority of cadherins have five extracellular cadherin domains. Cadherins are divided into two groups: type I classical cadherins like E-cadherin, N-cadherin, and P-cadherin, and type II classical cadherins like VE-cadherin (CDH5) and OB-cadherin (CDH11) (Goodwin M and Yap S, 2004; Kemler R, 1992). The adhesion molecule vascular endothelial (VE)-cadherin is found at the junctions between endothelial cells and is totally endothelial specific. VE-cadherin regulates a variety of biological processes, including cell proliferation and death, as well as modulating the action of the vascular endothelial growth factor receptor. VE-cadherin may be linked to increased angiogenesis, which may have an impact on the patients' survival. Cadherin signalling disruption has a major impact on tumour genesis and progression. The cadherin switch has a significant impact on cancer invasion and spread. VE-cadherin aids tumour angiogenesis, which promotes tumour development.

### **Aim and Objectives:**

The aim and objectives of this project are outlined below:

Aim: To study therapeutic role of protease inhibitors from plant source *Aegle marmelosin* for cancer treatment

### **Objectives**

- 1) Detection of antitumor metabolites in plant *Aegle marmelos*
- 2) To study molecular interactions of *Aegle marmelos* in tumor proteins by performing Docking studies.

### **Materials and Methods**

**Material Reagents:**

1 % Casein, Phosphate buffer: 0.1 M; pH 7.0, Standard Trypsin enzyme, 5 % Na<sub>2</sub>CO<sub>3</sub> Standard Tyrosine, TCA, 0.2 N HCl, Folin phenol reagent (make to be mentioned)

**Methods:****1. Determination of the activity Serratia peptidase**

**1A. Tyrosine value:** i. Standard solution- tyrosine solution 1 ml was taken + 2.5 ml of 5% sodium carbonate solution + 0.5 ml of dilute Folin's reagent. A suitable blank was prepared using 0.2 N HCl instead of Tyrosine. Incubate tubes at 37 °C for 30 mins. Take absorbance at 660 nm using water as blank.

**2. Enzyme Value:** i. Sample tube was prepared by addition of 1 ml of enzyme solution along with 3ml of casein solution. TCA was added after incubation at 37°C after 30 min. A suitable blank was prepared by following the same procedure. Reaction mixture from sample and blank was filtered to remove curdy white ppt. The resulting filtrate from sample & blank tube is used further to estimate tyrosine value. Filtrate was added with 2.5ml of Na<sub>2</sub>CO<sub>3</sub> solution + 0.5 ml of Folin's reagent and incubated further at 37°C for 30 mins. Absorbance was measured at 660 nm.

**Due to COVID 19 we had to stop our project and then our online project started**

- i. Search Target Protein of protease inhibitor:
- ii. A target protein was selected for the study was Cathepsin A
- iii. The structure of target protein was downloaded with the help of PubChem in PDB format
- iv. Then search for ligands of protease inhibitors was carried out and 3-D structures of ligands were downloaded from PubChem.

**Methodology**

1. Protein structure was downloaded from PDB
2. PubChem database was used to download the structure of ligand
3. Structure of protein was analysis from heteroatom
4. If found, heteroatoms, were removed
5. Protein and ligand files were prophase and then saved in PDBQT format
6. Active sites of protein were found earlier. From those active sites the dimension of grid box was obtained at X, Y and Z.
7. The configuration file was prepared by using grid box dimension.
8. Docking was done using command in PowerShell
9. The binding energies in kcal/mol, were recorded in log file along with RMSD
10. The specific location of ligand protein docking and interaction of residues was examined using PyMol.

11. For 2D/ 3D diagrams of interaction and publication quality photograph DSV was used.

Ligands: Structures of ligands were downloaded from PubChem

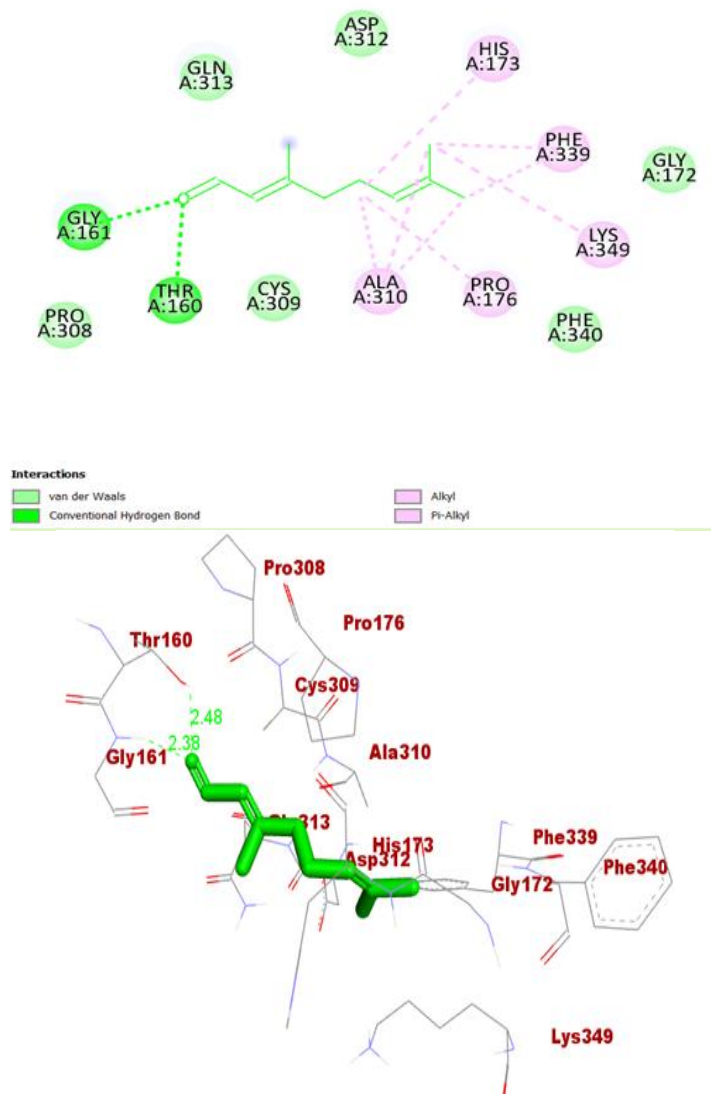
## **RESULT AND DISCUSSION**

The relationship between human Cts and cancer and their involvement in tumor progression, infiltration, death, and their regulation in response to chemotherapeutics were demonstrated. We have gone through the literature on the molecular effects of oncometabolites on cancer promoting proteins. According to profound cellular effects and nature of oncometabolites in cancer progression, we have proceeded to evaluate the relevance of intracellular oncometabolites with cancer promoting proteins using In Silico approach, with help of molecular docking and PyMol studies.

### **Result of Site-Specific docking:**

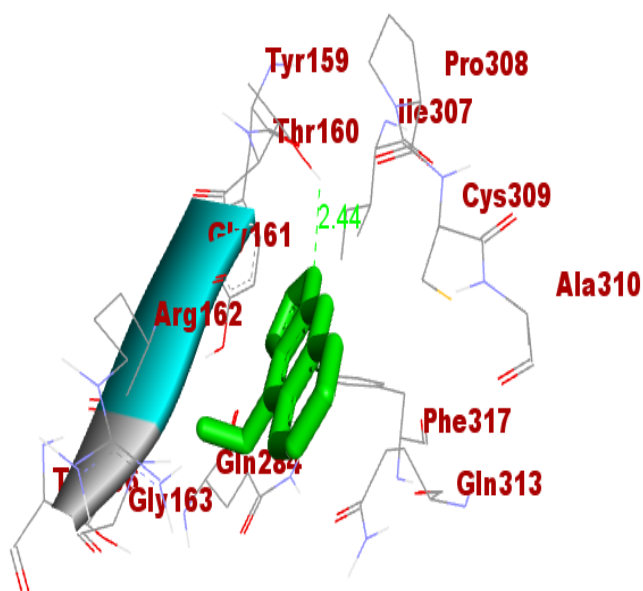
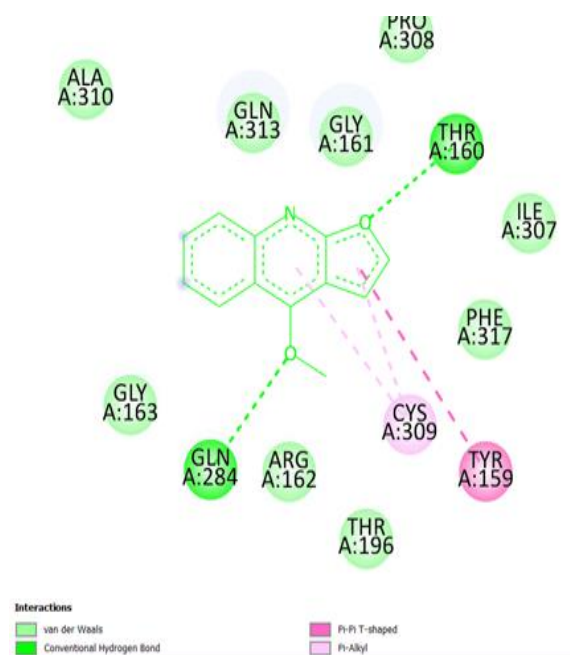
**Cathespine A:** First, we did the docking of cathepsin protein with twenty ligands of Aeglemarmelos but we did not get the desired result. So, we tried with another protein.

### **Ve-Cadherine Protein:**



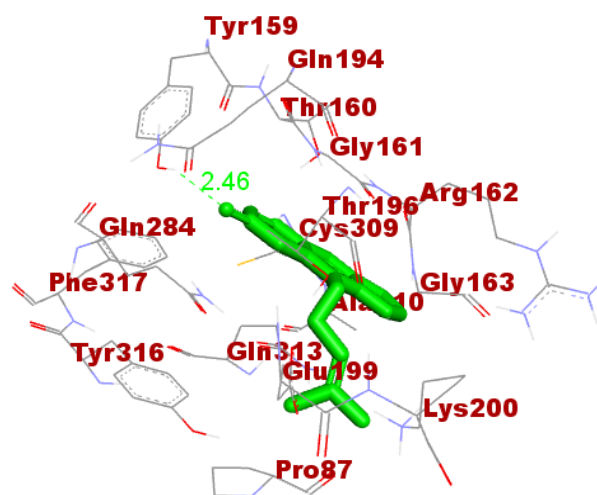
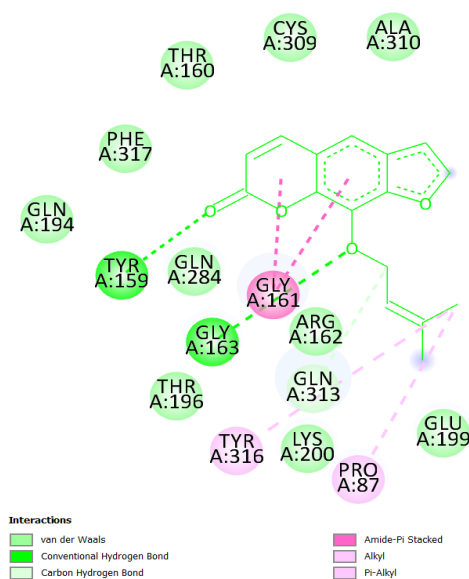
**Fig 4 (A): 2D Structure of Protein-ligand interaction. Fig No 4. (B)3D Structure of Protein-ligand interaction.**

The binding energy obtained for citral with ve-caderine Protein is -7.7 kcal/mol. Citral's intermolecular interactions with Ve-cadherin Protein are as follows: Conventional hydrogen bond formed by Thr 161 and Gly161 with a bond distance 2.38 Å and 2.48 Å. Phe340, Gln 313 engaged to form Van der waals bonding. Amide Pi-stacked formed by His 173, Phe339, Lys 349,Ala 310,Pro 176.



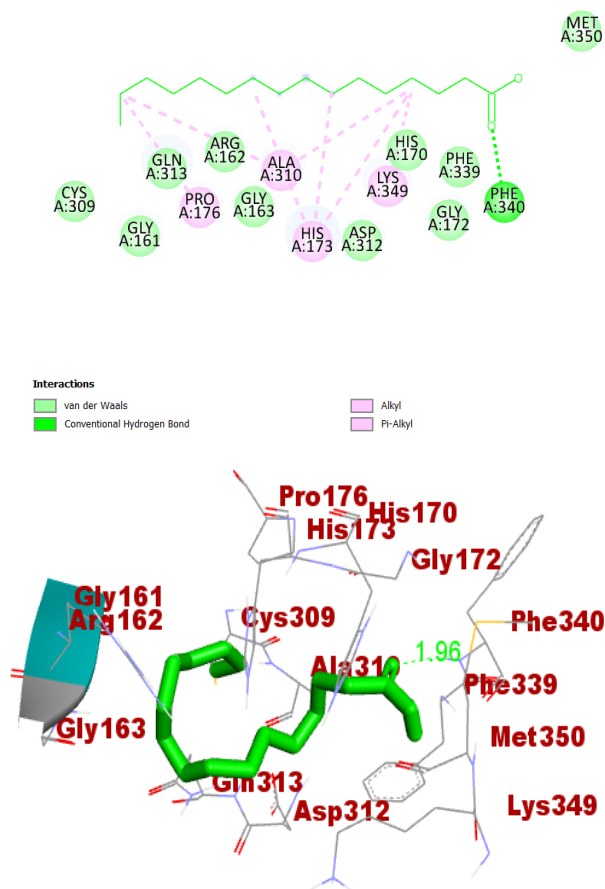
**Fig 5(A): 2D Structure of Protein-ligand interaction. Fig no 5.(B) 3D Structure of Protein-ligand interaction.**

The binding energy obtained for Dictamine with ve-caderine Protein is -7.7 kcal/mol. Dictamine's intermolecular interactions with ve-cadherin Protein are as follows: Conventional hydrogen bonds formed by Thr 160 with a bond distance 2.44Å. Gln 284 is also involved in hydrogen bonding with ligand. Gln 313, Gly 161, Pro 308 Gly 163, Arg 162, Phe 317, Ile 307, Thr 196, engaged to form Van der waals bonding. Amide Pi-stacked formed by Cys 309 and Tyr 159.



**Fig 6(A): 2D Structure of Protein-ligand interaction. Fig no 6.(B) 3D Structure of Protein-ligand interaction.**

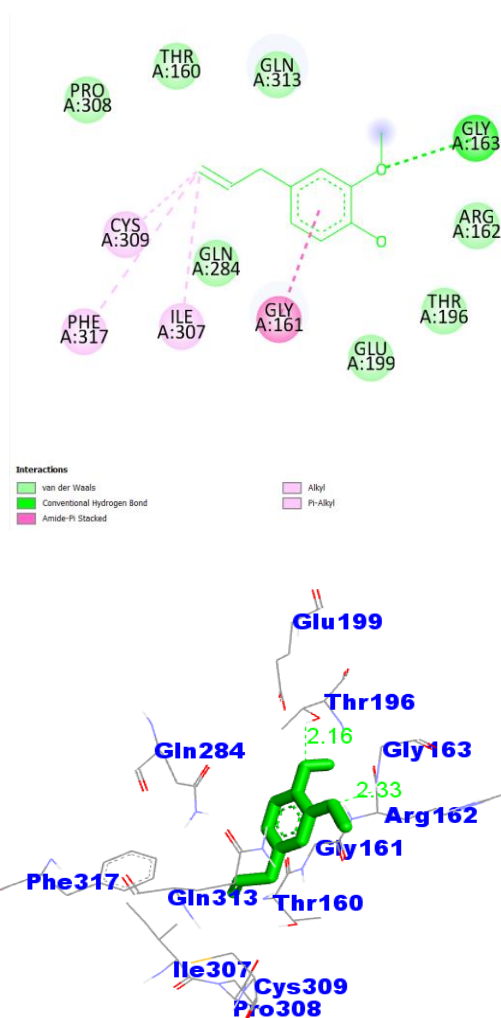
The binding energy obtained for Imperatorien with ve-caderine Protein is -6.9 kcal/mol. Imperatorien's intermolecular interactions with ve-cadherin Protein shows Conventional hydrogen bond formation with Tyr 146 with a bond distance of 2.46 Å and with Gly 163. Ala 310, Cys109, Thr 160, Phe 319, Gln 194, Gln 284, Arg 162, Gly 199, Thr 196 and Lys 200 are engaged to form Van der Waals bonding. Amide Pi-stacked formed by Gly 161, Tyr 316.



**Fig 7(A): 2D Structure of Protein-ligand interaction. Fig no 7.(B) 3D Structure of Protein-ligand interaction.**

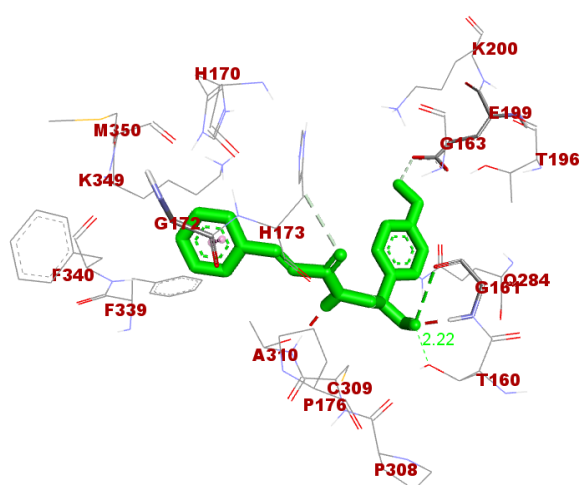
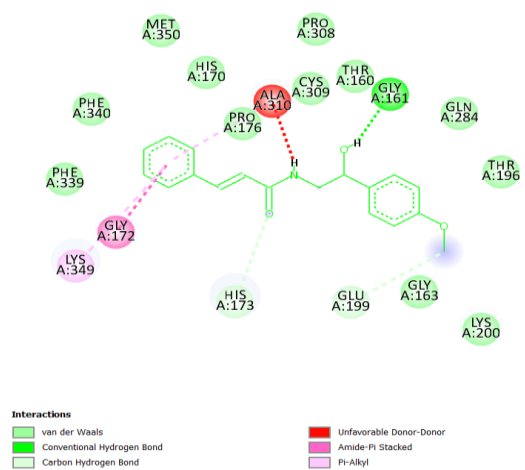
The binding energy obtained for Palmitic acid with ve-caderine Protein is -3.6 kcal/mol. Palmitic acid's intermolecular interactions with ve-cadherin Protein indicated formation of Conventional hydrogen bond with Phe 340 with a bond distance 1.96Å. Arg162, Gln313, Gly163, Gly161, Cys309, His170, Phe339, Gly 172 and Asp 312 are engaged to form Van der Waals bonding. Amide Pi-stacked formed by Ala 310, His 173, Lys 349 and Pro 176.





**Fig7(A): 2D Structure of Protein-ligand interaction. Fig no 7.(B) 3D Structure of Protein-ligand interaction.**

The binding energy obtained for Eugenol with ve-caderine Protein is -5.0 kcal/mol. Eugenol 's intermolecular interactions with ve-cadherin Protein displayed formation of conventional hydrogen bond with Gly 163 and Thr 196 with a bond distance 2.33 Å and 2.16 Å respectively. Gln 284, Gln313, Thr 160,Glu 199,Pro 308, Arg 162 and Thr 196 are engaged to form Van der waals bonding. Amide Pi-stacked formed byGLy 161,Cys 309,Phe 317and Ile 307.



**Fig 8(A): 2D Structure of Protein-ligand interaction. Fig no 8.(B) 3D Structure of Protein-ligand interaction.**

The binding energy obtained for Aegeline with ve-caderine Protein is -8.6 kcal/mol. Aegeline's intermolecular interactions with ve-cadherin Protein displayed formation of Conventional hydrogen bond with Gly 161 with a bond distance 2.22 Å His 173, Glu 199, Gly 163, Lys 200. Met 350, His 170, Pro 176, Pro 308, Cys 309, Thr 160, Gln 284, Thr 196, Phe 340, Ala 310, are engaged to form Van der Waals bonding. Amide Pi-stacked formed by, GLY 172 and Lys 349.

## Summary and Conclusion

Virtual Screening is the first and most crucial stage in the Molecular Docking process. We screened cathepsin A with 20 ligands of agelemarmelos. Docking process were done but no ligand bind with this protein. so we select another protein named Ve-Cadherine. Twenty ligands were selected for 32 docking studies. But only five ligands have a high affinity for the cancer target, and it is thought that further large-scale optimization of these likely leads will result in a powerful inhibitor for the target protein. Better scoring of the different conformations of the provided leads will be a part of the optimization process, which will take many days and a complex computational environment. For improved assessment of binding affinity and the search for broader conformational space, a combination of Docking programmes and scoring systems might be effective. The research on ligands and their physiochemical characteristics will be used further to optimize the process. It is certain that the suggested leads will produce a powerful inhibitor since their binding affinity is equivalent to that of an existing inhibitor of

the target protein. The goal of this study was to develop novel lead compounds for the selective competitive inhibition of Ve-Cadherine protein which is key proteins in human cancer. The docking analysis was carried out at varied binding affinity levels to investigate the efficiency of indigenously Ve-Protein, to aegeline ligands. The maximum affinity for Ve-Cadherin protein (PDBID-5UJJ) was found in Threonine (7.7kcal/mol with RMSD value of 2.38 and 2.48). We believe that ligands, such as Aegeline, Imperatorin, and Palmitic acid are specific compounds that can be promising lead molecules to develop effective drug. Target proteins VeCadherin, will receive more attention in future research as a tumor suppressor in cancer cells.

## References:

1. Barrett, A. J., & McDonald, J. K. (1986). Nomenclature: Protease, proteinases, and peptidase. *Biochemical Journal*, 237(3), 935–937.
2. Hörger, A. C., & van der Hoorn, R. A. L. (2013). The structural basis of specific protease-inhibitor interactions at the plant-pathogen interface. *Current Opinion in Plant Biology*, 16(4), 451–459. <https://pubmed.ncbi.nlm.nih.gov/23937902/>
3. Angelova, L., Dalgarrondo, M., Minkov, I., Danova, S., Kirilov, N., Serkedjieva, J., Chobert, J.-M., Haertlé, T., & Ivanova, I. (2006). Purification and characterization of a protease inhibitor from *Streptomyces chromofuscus* 34-1 with antiviral activity. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1760(8), 1210–1216. <https://pubmed.ncbi.nlm.nih.gov/16730414/>
4. Ahn, Y.-B., Rhee, S.-K., Fennell, D. E., Kerkhof, L. J., Hentschel, U., & Häggblom, M. M. (2003). Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba*. *Applied and Environmental Microbiology*, 69(7), 4159–4166.
5. Baird-Parker, T. C. (2003). The production of microbiologically safe and stable foods. In B. M. Lund, T. C. Baird-Parker, & G. W. Gould (Eds.), *The microbiological safety and quality of food* (pp. 3–18). Aspen Publishers. <https://www.researchgate.net/publication/284469963>
6. Bijina, B., Chellappan, S., Basheer, S. M., Elyas, K. K., Bahkali, A. H., & Chandrasekaran, M. (2011). Protease inhibitor from *Moringa oleifera* leaves: Isolation, purification, and characterization. *Process Biochemistry*, 46(11), 2291–2300. <https://www.researchgate.net/publication/251668229>
7. Bhattacharyya, A., Mazumdar, S., Leighton, S. M., & Babu, C. R. (2006). A Kunitz proteinase inhibitor from *Archidendron ellipticum* seeds: Purification, characterization, and kinetic properties. *Phytochemistry*, 67(3), 232–241. <https://pubmed.ncbi.nlm.nih.gov/17097298/>
8. Bode, W., & Huber, R. (2000). Structural basis of the endoproteinase–protein inhibitor interaction. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1477(1–2), 241–252. <https://pubmed.ncbi.nlm.nih.gov/10708861/>
9. Brix, K., & Saftig, P. (2005). Lysosomal proteases: Revival of the Sleeping Beauty. In *Madame Curie Bioscience Database* [Internet]. Landes Bioscience. <https://www.ncbi.nlm.nih.gov/books/NBK6348/>
10. Chen, S., Dong, H., Yang, S., & Guo, H. (2017). Cathepsins in digestive cancers. *Oncotarget*, 8, 41690. <https://doi.org/10.18632/oncotarget.16677>
11. Cheng, X. W., Shi, G.-P., Kuzuya, M., Sasaki, T., Okumura, K., & Murohara, T. (2012). Role for cysteine protease cathepsins in heart disease: Focus on biology and

- mechanisms with clinical implication. *Circulation*, 125(12), 1551–1562.  
<https://doi.org/10.1161/CIRCULATIONAHA.111.066712>
12. Fonović, M., & Turk, B. (2014). Cysteine cathepsins and extracellular matrix degradation. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1840(8), 2560–2570. <https://doi.org/10.1016/j.bbagen.2014.03.017>
  13. Gocheva, V., & Joyce, J. A. (2007). Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle*, 6(1), 60–64. <https://doi.org/10.4161/cc.6.1.3669>
  14. Goulet, B., Baruch, A., Moon, N.-S., Poirier, M., Sansregret, L. L., Erickson, A., Bogyo, M., & Nepveu, A. (2004). A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. *Molecular Cell*, 14(2), 207–219. <https://pubmed.ncbi.nlm.nih.gov/15099520/>
  15. Goodwin, M., & Yap, A. S. (2004). Classical cadherin adhesion molecules: Coordinating cell adhesion, signaling and the cytoskeleton. *Journal of Molecular Histology*, 35(8–9), 839–844. <https://doi.org/10.1007/s10735-004-1833-2>
  16. Pynam, H., & Dharmesh, S. M. (2018). Antioxidant and anti-inflammatory properties of marmelosin from bael (*Aegle marmelos* L.): Inhibition of TNF- $\alpha$  mediated inflammatory/tumor markers. <https://pubmed.ncbi.nlm.nih.gov/29957472/>
  17. Kim, J.-Y., Park, S.-C., Hwang, I., Cheong, H., Nah, J.-W., Hahm, K.-S., & Park, Y. (2009). Protease inhibitors from plants with antimicrobial activity. *International Journal of Molecular Sciences*, 10(6), 2860–2872.  
<https://pmc.ncbi.nlm.nih.gov/articles/PMC2705521/>
  18. Sabočič, J., & Kos, J. (2012). Microbial and fungal protease inhibitors—Current and potential applications. *Applied Microbiology and Biotechnology*, 93(4), 1351–1375.  
<https://doi.org/10.1007/s00253-011-3834-x>
  19. Otlewski, J., Polanowski, A., & Leluk, J. (1984). Trypsin inhibitors in summer squash seeds: Isolation, purification and partial characterization of three inhibitors. *Acta Biochimica Polonica*, 31(3), 351–358. <https://pubmed.ncbi.nlm.nih.gov/6524213/>
  20. Kim, J.-Y., Park, S.-C., Hwang, I., Cheong, H., Nah, J.-W., Hahm, K.-S., & Park, Y. (2009). Protease inhibitors from plants with antimicrobial activity.  
<https://pmc.ncbi.nlm.nih.gov/articles/PMC2705521/>
  21. Shah, K. R., Patel, D. K., Pappachan, A., Prabha, C. R., & Singh, D. D. (2015). Characterization of a Kunitz-type serine protease inhibitor from *Solanum tuberosum* having lectin activity. <https://pubmed.ncbi.nlm.nih.gov/26645142/>
  22. Kukor, Z., Mayerle, J., Krüger, B., Tóth, M., Steed, P. M., Halangk, W., Lerch, M. M., & Sahin-Tóth, M. (2002). Presence of cathepsin B in the human pancreatic secretory pathway and its role in trypsinogen activation during hereditary pancreatitis. *Journal of Biological Chemistry*, 277(24), 21389–21396.  
<https://doi.org/10.1074/jbc.M200878200>
  23. Kemler, R. (1992). Classical cadherins. *Seminars in Cell Biology*, 3(3), 149–155.  
[https://doi.org/10.1016/S1043-4682\(10\)80011-X](https://doi.org/10.1016/S1043-4682(10)80011-X)
  24. Koblinski, J. E., Ahram, M., & Sloane, B. F. (2000). Unraveling the role of proteases in cancer. *Clinica Chimica Acta*, 291(2), 113–135. <https://>