THE EFFECT OF CONCENTRATION ON THE CAPACITY OF FRESH *Azadirachta indica* (AI), *Persea americana* (PA) and *Mangifera indica* (MI) LEAVES EXTRACT TO INHIBIT PROTEINASE ACTIVITY

BY

ITAMAH, RUFINA ONOTIEMORIA

(FLS/BCH/19/51199)

A PROJECT SUBMITTED TO THE

DEPARTMENT OF BIOCHEMISTRY,

FACULTY OF LIFE SCIENCES,

AMBROSE ALLI UNIVERSITY,

EKPOMA, EDO STATE.

IN PARTIALFUFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE DEGREE IN BIOCHEMISTRY

MAY, 2024

Attestation

I hereby declare that this project was written by me and it is a correct record of my research work. All citations and sources of information are clearly acknowledged by means of references.

……..

Signature and Date

Itamah Rufina Onotiemoria

Certification

I declare that the work in this dissertation titled, '**The effect of concentration on the capacity of *Azadirachta indica* (ai), *Persea americana* (pa) and *Mangifera indica* (mi) leaves extract to inhibit proteinase activity**', was carried out by Itamah Rufina Onotiemoria with matriculation number, FLS/BCH/19/51199, Department of Biochemistry in the faculty of life sciences, Ambrose Alli University, Edo state. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other discussion.

_____ _____

DR. I. E. Ebhohimen Date

Supervisor

_____ _____

DR. I. E. Ebhohimen

Head of Department Date

_____ _____

External Invigilator Date

Dedication

This research work is dedicated to God, the one that has always being there for me. Also dedicating it to myself – Ono, for trying again and again, for not giving up, and for expending energy to see that this research work, comes alive.

Acknowledgement

This research work would not have been possible without God Almighty. I am grateful to him, for making it possible for me to carry out this research work.

I want to express my gratitude to my seminar supervisor – Dr I. E. Ebhohimen for his constructive criticism, intelligent corrections, support, care and encouragement towards the success of my seminar work.

I am especially indebted to my mum, the sweetest mum ever, Mrs Itamah, for her prayers, encouragement, care, love, and every other sweet thing one can think of. I love you so much, mummy. To my dad – Barr Itamah, thank you so much for all that you do. To my siblings – Ose, Enimien, and Omono, I love you all plenty. You all occupy all the spaces in my heart.

To my course-mates, friends, cousin, aunties, and to others who contributed immensely one way or the other, I am grateful. You all are the best!!

Special thanks and love goes to Ayokanmi Lamija, my friend, and senior colleague. Thank you for your selfless support, effort, and encouragement towards making this project work as a whole, worthwhile, and successful.

God bless you all.

Table of Contents

Attestation ii

Certification iii

Dedication iv

Acknowledgement v

Table of Contents vi

Abstract 1

Chapter One 2

1.1. Introduction 2

1.2. Aims and Objectives 5

1.3. Scope of Study 5

Chapter Two 6

2.1. Polyherbal Combinations 6

2.2. The Bioactive Compounds in Neem (*Azadirachta indica*) 7

2.3. Bioactive Compounds in *Mangifera Indica* 9

2.3. Mechanism of Proteinase Inhibition 11

2.4. pH Relevance of Plant Proteinases 18

2.5. Role of Ascorbic Acid in Studying the Inhibitory Effect of Polyherbal Extract on Proteinase Activity 19

Chapter Three 21

3.1. Apparatus 21

3.2. Chemicals 21

3.3. Collection of Plant Materials 21

3.4. Preparation of Plant Materials 21

3.5. Anti-proteinase Assay 22

3.6. Statistical Analysis of Data 22

Chapter Four 23

4.0. Results 23

Chapter Five 24

5.1. Discussion 24

5.2. Conclusion 25

References 27

Appendix 32

Abstract

Plants that inhibit proteinase activity have great potential for use in a variety of industries, including food preservation and medicine. In this study, the effect of concentration on the capacity of a polyherbal extract made from the leaves of *Mangifera indica* (Mango), *Pearsea americana (Avocado*), and *Azadirachta indica* (neem) to inhibit proteinase activity was studied. Standard procedures were followed to prepare the extract, and the anti-proteinase action was studied using spectrophotometric assay using ascorbic acid as standard control. The concentration range 100µg/ml - 1000µg/ml was studied both for the extract and control. The results obtained show that the polyherbal extract inhibited proteinase activity in a concentration-dependent manner. This result suggests that the polyherbal extract may contain useful anti-inflammatory compounds. Further research on the bioactive compounds and the elucidation of their mechanism of action is recommended.

Chapter One

1.1. Introduction

Proteinases also known as proteases likely arose at the earliest stages of protein evolution as simple destructive enzymes necessary for protein catabolism and the generation of amino acids in primitive organisms. For many years, studies on proteases focused on their original roles as blunt aggressors associated with protein demolition. However, the realization that, beyond these nonspecific degradative functions, proteases act as sharp scissors and catalyze highly specific reactions of proteolytic processing, producing new protein products, inaugurated a new era in protease research (Neurath and Walsh, 1976). The current success of research in this group of ancient enzymes derives mainly from the large collection of findings demonstrating their relevance in the control of multiple biological processes in all living organisms (López-Otín and Overall, 2002; Ciechanover, 2005). Thus, proteases regulate the fate, localization, and activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, transduce, and amplify molecular signals.

As a direct result of these multiple actions, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, hemostasis, blood coagulation, inflammation, immunity, autophagy, senescence, necrosis, and apoptosis. Consistent with these essential roles of proteases in cell behavior and survival and death of all organisms, alterations in proteolytic systems underlie multiple pathological conditions such as cancer, neurodegenerative disorders, and inflammatory and cardiovascular diseases. Accordingly, many proteases are a major focus of

attention for the pharmaceutical industry as potential drug targets or as diagnostic and prognostic biomarkers (Turk, 2006).

Proteases also play key roles in plants and contribute to the processing, maturation, or destruction of specific sets of proteins in response to developmental cues or to variations in environmental conditions (Garcia-Lorenzo *et al.,* 2006). Likewise, many infectious microorganisms require proteases for replication or use proteases as virulence factors, which has facilitated the development of protease-targeted therapies for diseases of great relevance to human life such as AIDS (Turk, 2006). Finally, proteases are also important tools of the biotechnological industry because of their usefulness as biochemical reagents or in the manufacture of numerous products (Saeki *et al.,* 2007).

Proteases were initially classified into endopeptidases, which target internal peptide bonds, and exopeptidases (aminopeptidases and carboxypeptidases), the action of which is directed by the NH_2 and COOH termini of their corresponding substrates. However, the availability of structural and mechanistic information on these enzymes facilitated new classification schemes. Based on the mechanism of catalysis, proteases are classified into six distinct classes, aspartic, glutamic, and metalloproteases, cysteine, serine, and threonine proteases, although glutamic proteases have not been found in mammals so far. The first three classes utilize an activated water molecule as a nucleophile to attack the peptide bond of the substrate, whereas in the remaining enzymes, the nucleophile is an amino acid residue (Cys, Ser, or Thr, respectively) located in the active site from which the class names are derived. Proteases of the different classes can be further grouped into families on the basis of amino acid sequence comparison, and families can be assembled into clans based on similarities in their three-dimensional structures.

Azadirachta indica, commonly known as neem, margosa, nimtree or Indian lilac is a tree in the mahogany family *Meliaceae*. It is one of two species in the genus *Azadirachta*. It is native to the Indian subcontinent and to parts of Southeast Asia, but is naturalized and grown around the world in tropical and subtropical areas. Its fruits and seeds are the source of neem oil. Products made from margosa trees have been used in the traditional medicine of India for centuries (Anna, 2006), for treating skin troubles and rheumatism (Tejesvi *et al.,* 2009), but there is insufficient clinical evidence to indicate any benefits of using margosa for medicinal purposes. In adults, no specific doses have been established, and short-term use of margosa appears to be safe, while long-term use may harm the kidneys or liver; in small children, margosa oil is toxic and can lead to death. Margosa may also cause miscarriages, infertility, and low blood sugar.

Mangifera indica, commonly known as mango, is a species of flowering plant in the family *Anacardiaceae* (Morton and Julia, 1987). It is a large fruit tree, capable of growing to a height of 30 metres (100 feet). There are two distinct genetic populations in modern mangoes – the "Indian type" and the "Southeast Asian type". The tree is more known for its fruit rather than for its timber. However, mango trees can be converted to lumber once their fruit-bearing lifespan has finished. The wood is susceptible to damage from fungi and insects. The wood is used for musical instruments such as ukuleles, plywood and low-cost furniture.

The avocado (*Persea americana*) is a medium-sized, evergreen tree in the laurel family (*Lauraceae*). It is native to the Americas and was first domesticated in Mesoamerica more than 5,000 years ago. Then as now it was prized for its large and unusually oily fruit. The tree likely originated in the highlands bridging south-central Mexico and Guatemala (Morton and Julia, 1987; Chen *et al.,* 2008). Its fruit, sometimes also referred to as an alligator pear or avocado pear, is botanically a large berry containing a single large seed (Storey, 1973). Sequencing of its

genome showed that the evolution of avocados was shaped by polyploidy events and that commercial varieties have a hybrid origin (Avocado trees are partly self-pollinating, and are often propagated through grafting to maintain consistent fruit output.

1.2. Aims and Objectives

- To investigate the inhibitory effect of polyherbal extract on proteinase activity.
- To determine whether the inhibitory effect of the polyherbal extract is concentration dependent.
- To compare the effectiveness of the polyherbal extract in inhibiting proteinase activity.

1.3. Scope of Study

This study is aimed at studying the effect of concentration on the capacity of AI:PA:MI leaves extract to inhibit proteinase activity.

Chapter Two

2.0 Literature Review

2.1. Polyherbal Combinations

Plants have a universal role in the treatment and prevention of diseases. In many instances phytomedicine relies on combinations rather than single entities. This concept of phytomedicine is often defined as "a polyherbal formulation". These combinations may be prepared from mixtures of many plant parts obtained from various plant species and families, and may contain multiple bio-active constituents (Van *et al.,* 2022).

The use of herbal combinations is part of traditional medicine in different cultures worldwide. The concept of combining two or more herbs in a formulation is based on a medicinal system that has existed since ancient times, often with the purpose of attaining better therapeutic efficacy and/or reduced profile of side effects, often favoured over single-herb treatment (Aslam *et al.,* 2106; Parasuraman *et al.,* 2014). The pharmacological activity of herbs and plant extracts is often explained by the phenomenon of synergy and polyvalence. Synergy is defined within medicine as an effect where the combination of substances exhibits a greater effect than the sum of the effects of each individual substance (Berenbaum, 1989; Wagner, 2009). While synergism only involves one specific type of interaction, polyvalence includes various forms of interactions also resulting in an overall enhancement of the pharmacological efficacy of the medicinal plants. On the other hand, the antagonistic effects of the herbs in a polyherbal drug formulation can also contribute to the favourable pharmacological effects. Therefore, inclusion of specific herbs, dosages, and the mixing ratio between the herbs cannot be arbitrarily made and are important for the pharmacological effects (Kiyohara *et al.,* 2004).

Almost 95% of the prescriptions in India are plant-based, and to date these systems focus on multicomponent mixtures (Van *et al.,* 2022). These polyherbal preparations combine anything from three to more than 20 plant species (Van *et al.*, 2022). The concept of polyherbal combinations is part of the Ayurvedic system where the belief to achieve greater therapeutic efficacy and reduce toxicity through synergistic effects is practiced. There is also evidence in Ayurvedic medicine on how sugar, honey, yogurt and milk have been used in plant preparations for various ailments (Akber *et al.,* 2011). In an African context, combining of plants in herbal mixtures is found to be a common practice for traditional healers with the purpose of obtaining an optimal outcome. Mixed plant remedies may be prepared as infusions (hot teas), decoctions (boiled teas), tinctures (alcohol and water extracts) and macerations (cold-soaking) (Van *et al.,* 2022). While combining plants is common practice, very little attention has been given to investigating this aspect in terms of establishing the different types of combinations used or validating the scientific role thereof.

2.2. The Bioactive Compounds in Neem (*Azadirachta indica*)

Multiple medically active compounds including terpenoids and steroids have been isolated from Neem. The most highly studied compounds are azadirachtin, nimbolinin, nimbin, nimbidin, and nimbidol, however, multiple other bioactive components have also been identified. The well documented flavonoid, quercetin, has also been identified in NLE (Schumacher *et al.,* 2011).

Azadiradione extracted from the seeds of Neem were found to be effective in ameliorating symptoms in mammalian and fly models of neurodegenerative diseases making it a potential candidate for consideration against neurodegenerative diseases (Nelson *et al.,* 2016). Additionally, Azadiradione and Gedunin were found to bind and inactivate human pancreatic α-amylase (a well-known anti-diabetic target) and thus are considered as lead drug candidates to

control post-prandial hyperglycemia (Ponnusamy *et al.,* 2015). Azadiramide B, isolated from the extracts of Bacillus subtilis-fermented Neem seeds was shown to selectively inhibit the growth of MDA-MB-231 a triple-negative breast cancer (TNBC) cell line with an IC_{50} value of 15.73±6.07 μM (Lin *et al.,* 2019). Azadirone was found to sensitize cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

Another Neem derivative, Catechin, was found to be one of the best phytocompounds against dental plaque forming bacteria and displayed promise as a drug for treating biofilm related acute infections (Lakshmi *et al.,* 2015; Lahiri *et al.,* 2021). Epicatechin was reported as a potent antibacterial agent in addition to its antioxidative activity (Lahiri *et al.,* 2021; Schumacher *et al.,* 2011). Epoxyazadiradione (EAD) extracted from the Neem seeds was found to induce mitochondrial apoptosis and inhibited NF-κB nuclear translocation in human cervical cancer cells (Shilpa *et al.,* 2017).

Neem is known for its antibacterial and antifungal activity that is mainly attributed to its bioactive compounds such as nimbidin, nimbolide, mahmoodin, margolone, margolonone, and isomargolonone (Bansal *et al.,* 2019). Nimbidin is a mixture of tetranor-triterpenes extracted from Neem oil. It has been reported as a potent anti-inflammatory and anti-arthritic agent. Generally, Neem is accepted in the ayurvedic medical tradition as a therapy for various diseases as there are still many hurdles that limits large-scale use of Neem.

Neem has various components which contribute to the many dosage forms of *Azadirachta indica*. The most common used parts of Neem are the leaves and seeds. Components of Neem are often extracted with water or ethanol (Kumar *et al.,* 2006; Maurya, 2017). The extract can stay in an aqueous fluid or be further dried to make a powder (Kumar *et al.,* 2006; Kudom *et al.,*

2011). Seed oil can be extracted with ethanol and supercritical carbon dioxide (CO_2), among other solutions. Neem plant extracts (NPEs) have many indications. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles composed from Neem leaf-extracted nimbolide have anticancer effects. The PLGA polymer surface can be modified by conjugating polyethylene glycol with receptor binding ligands for targeted delivery of nimbolide in cancer treatments (Patra *et al.,* 2019).

2.3. Bioactive Compounds in *Mangifera Indica*

Apart from being important as a food, mango fruits as well as other parts of the plant are a source of bioactive compounds with potential health-promoting activity (Table 2.3.1). All parts of mango trees have been used in traditional South Asian medicine: kernels, flowers, leaves, gum, bark, and peel. Diseases commonly treated with herbal remedies obtained from parts of the mango tree include dysentery, diarrhea, urinary tract inflammation, rheumatism, and diphtheria. A number of these uses are supported by scientific evidence (Ross, 2003. Vimangs, an extract obtained from the stem bark of mango trees, shows in vitro and in vivo anti-inflammatory and antioxidant activities and is currently produced on an industrial scale in Cuba (Pardo-Andreu *et al.,* 2006). Aqueous decoctions of mango flowers showed potential gastroprotective and ulcer healing properties in the acute and subacute models of induced ulcer in mice and rats (Lima *et al.,* 2006). Extracts of mango leaves showed moderate larvicidal activity in experiments with Culex quinquefasciatus Say, the main mosquito vector of lymphatic filariasis, which is widely distributed in tropical regions (Rahuman *et al.,* 2008). The natural product used in Cuban traditional medicine, obtained from mango bark (MSBE), modulated the P450 enzymes in cultured cells, demonstrating inhibition of CYP1A2 and 2E1. The authors postulate that, by this mechanism, chemo-preventive properties could be attributed to the natural product, since both

P450 enzymes are involved in the bioactivation of mutagens and carcinogens (Rodeiro *et al.,* 2008). One study has indicated that peel extracts from mango had thyroid stimulatory effects on animals with induced hypothyroidism, and reduced lipid peroxidation in heart, liver, and kidney tissues (Parmar and Kar, 2008). Extracts from mango kernel showed superoxide anion scavenging activity in a cell-free system (Saito *et al.,* 2008), suggesting one possible bioactivity by antioxidant mechanism. Mangiferin, a xanthone present in mango, when administered at a dose of 100 mg/kg/d to rats subjected to experimental periodontitis, demonstrated an anti-inflammatory property, accelerating the processes of repairing and healing injured tissues (Carvalho *et al.,* 2009).

Table 2.3.1 Bioactive Compounds in Mango

BIOACTIVE COMPOUND	**PART**
Ascorbic and dehydroascorbic acids	Flesh
□-carotene	Flesh
Other carotenoids: mutatochrome, α-cryptoxanthin, violaxanthin, leutoxanthin, mutatoxanthin, autoxanthin	Flesh
Polyphenols: mangiferin, isomangiferin, homomangiferin, quercetin, kaempferol, anthocyanins	Flesh, bark, seed, peel, leaves, twigs

Phenolic acids: gallic, protocathechuic, ferulic, caffeic, coumaric, ellagic, 4-caffeoylquinic acids	Flesh, peel, seeds, kernel
Other Phenols: Alk(en)ylresorcinols	Peel, sap
Terpenoids: α-pinene, β-pinene, β-myrcene, limonene, *cis*-ocimene, *trans*-ocimene, terpinene, α-guainene, camphene, fenchene, α-humulene and others (lactones, aldehydes, acids, sesquiterpenes, esters and aliphatic alcohols)	Flesh, peel, sap



Figure 1: Neem, Avocado and Mango leaves. (Anna, 2006; Morton and Julia, 1987)

2.3. Mechanism of Proteinase Inhibition

Proteinase inhibitors, or antiproteases, are molecules that inhibit the function of proteases (enzymes that aid the breakdown of proteins). Many naturally occurring protease inhibitors are proteins (Roy *et al.,* 2022). Protease inhibitors may be classified either by the type of protease they inhibit, or by their mechanism of action. In 2004 Rawlings and colleagues introduced a classification of protease inhibitors based on similarities detectable at the level of amino acid sequence (Rawlings et al., 2004). This classification initially identified 48 families of inhibitors that could be grouped into 26 related superfamily (or clans) by their structure. According to the MEROPS database there are now 81 families of inhibitors. These families are named with an I followed by a number, for example, I14 contains hirudin-like inhibitors.

Classes of proteases are:

- Aspartic protease inhibitors
- Cysteine protease inhibitors
- Metalloprotease inhibitors
- Serine protease inhibitors
- Threonine protease inhibitors
- Trypsin inhibitors
- Kunitz STI protease inhibitor

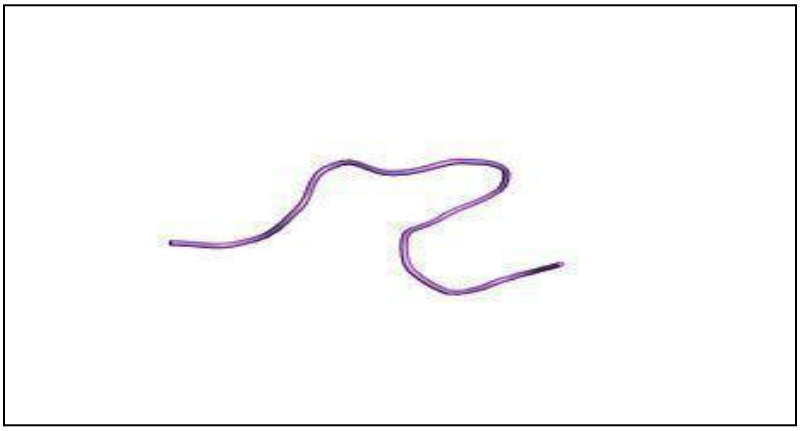
Classes of inhibitor mechanisms of action are:

- Suicide inhibitor
- Transition state inhibitor
- Protein protease inhibitor (see serpins)

- Chelating agents

Inhibitor I4: This is a family of protease suicide inhibitors called the serpins. It contains inhibitors of multiple cysteine and serine protease families. Their mechanism of action relies on undergoing a large conformational change which inactivates their target's catalytic triad.

A. Inhibitor I10: This family includes both microviridins and marinostatins. It seems likely that in both cases it is the C-terminus which becomes the active inhibitor after post-translational modifications of the full length, pre-peptide. It is the ester linkages within the key, 12-residue region that circularize the molecule giving it its inhibitory conformation.



Serine endopeptidase inhibitors

Figure 2: Solution structure of marinostatin, a protease inhibitor containing two ester linkages (Kanori *et al*., 2005).

B. Inhibitor I29: The inhibitor I29 domain, which belongs to MEROPS peptidase inhibitor family I29, is found at the N-terminus of a variety of peptidase precursors that belong to MEROPS peptidase subfamily C1A; these include cathepsin L, papain, and procaricain (Groves *et al.,* 1996). It forms an alpha-helical domain that runs through the substrate-binding site, preventing access. Removal of this region by proteolytic cleavage results in activation of the enzyme. This domain is also found, in one or more copies, in a variety of cysteine peptidase inhibitors such as salarin (Olonen *et al.,* 2003).

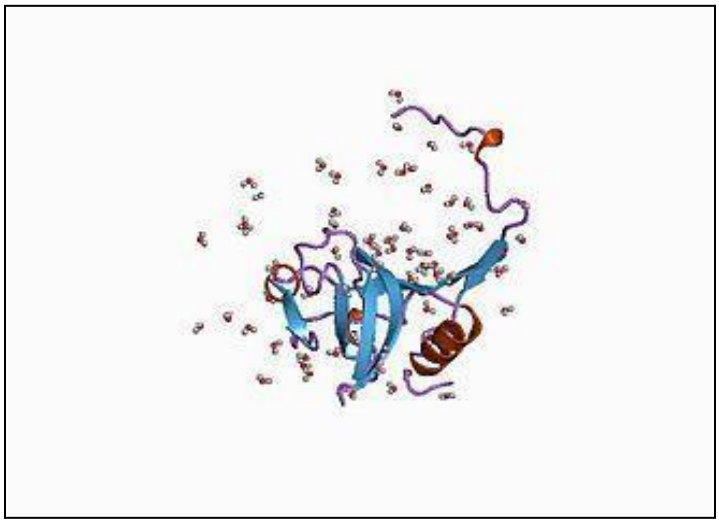


Figure 3: Crystal structure of a cysteine protease proform (Vicik *et al*., 2006).

C. Inhibitor I34: The saccharopepsin inhibitor I34 is highly specific for the aspartic peptidase saccharopepsin. In the absence of saccharopepsin it is largely unstructured, but in its presence, the inhibitor undergoes a conformational change forming an almost perfect alpha-helix from Asn2 to Met32 in the active site cleft of the peptidase.

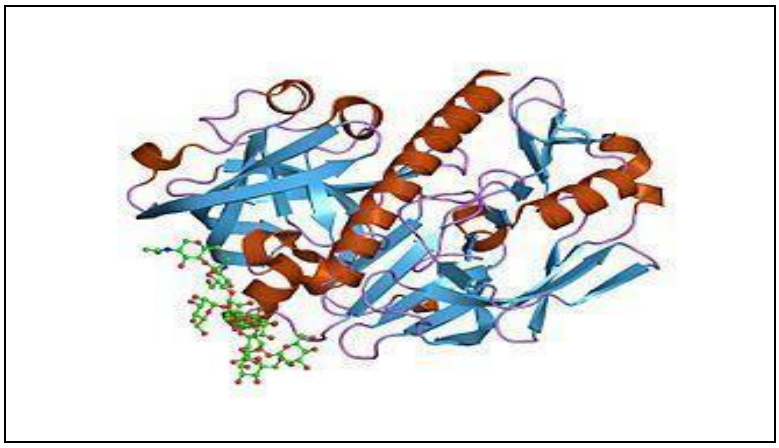


Figure 4: The structure of proteinase a complexed with an ia3 mutant inhibitor (Li *et al*., 2000).

D. Inhibitor I36: The peptidase inhibitor family I36 domain is only found in a small number of proteins restricted to Streptomyces species. All have four conserved cysteines that probably form two disulphide bonds. One of these proteins from Streptomyces nigrescens, is the well characterized metalloproteinase inhibitor SMPI (Tanaka *et al.,* 1990; Murai *et al.,* 1985).

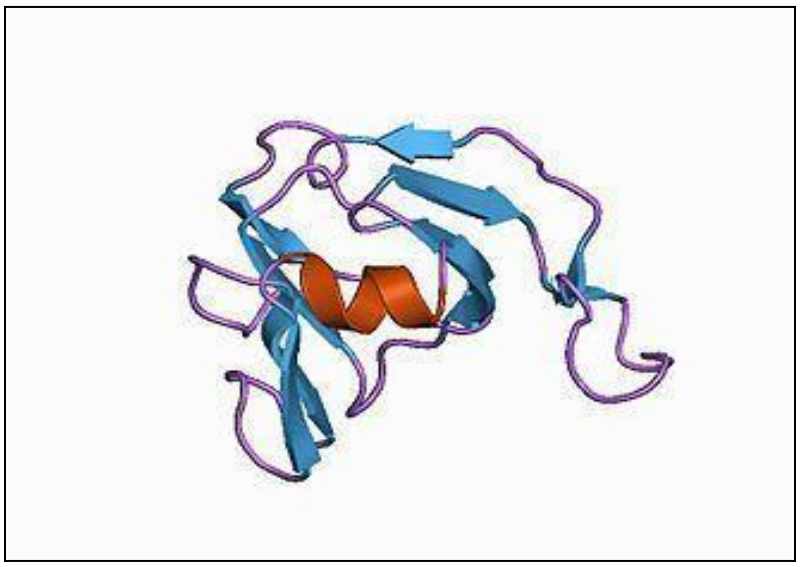


Figure 5: The 3d structure of the streptomyces metalloproteinase inhibitor, smpi, isolated from streptomyces nigrescens tk-23, nmr, minimized average structure (Murai *et al.,* 1985).

E. Inhibitor I42: Inhibitor family I42 includes chagasin, a reversible inhibitor of papain-like cysteine proteases (Monteiro *et al.,* 2001). Chagasin has a beta-barrel structure, which is a unique variant of the immunoglobulin fold with homology to human CD8alpha (Figueiredo *et al.,* 2007; Wang *et al.,* 2007).

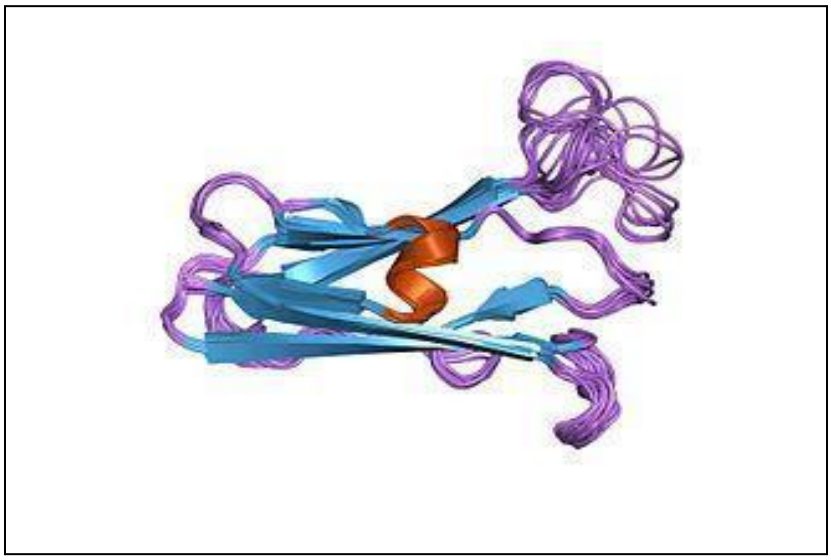


Figure 6: Solution structure of the trypanosoma cruzi cysteine protease inhibitor chagasin (Murai *et al.,* 1985).

F. Inhibitor I67: Bromelain inhibitor VI, in the Inhibitor I67 family, is a double-chain inhibitor consisting of an 11-residue and a 41-residue chain.

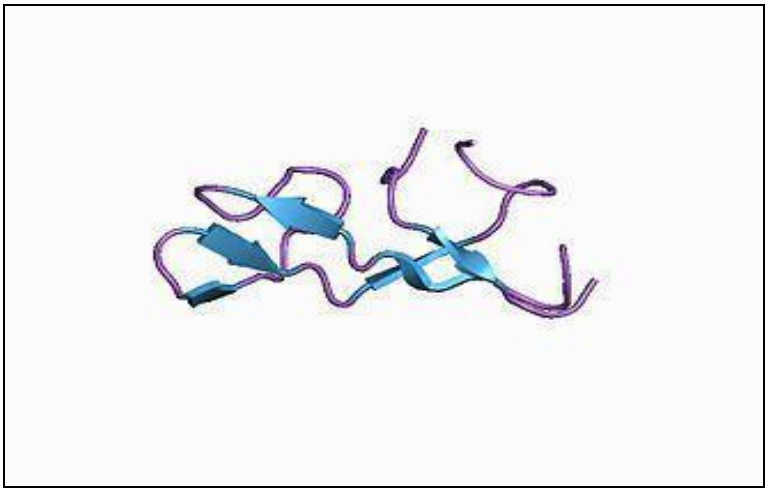


Figure 7: nmr structure of bromelain inhibitor vi from pineapple stem (Hatano *et al*., 1996)

Inhibitor I68: The Carboxypeptidase inhibitor I68 family represents a family of tick carboxypetidase inhibitors.

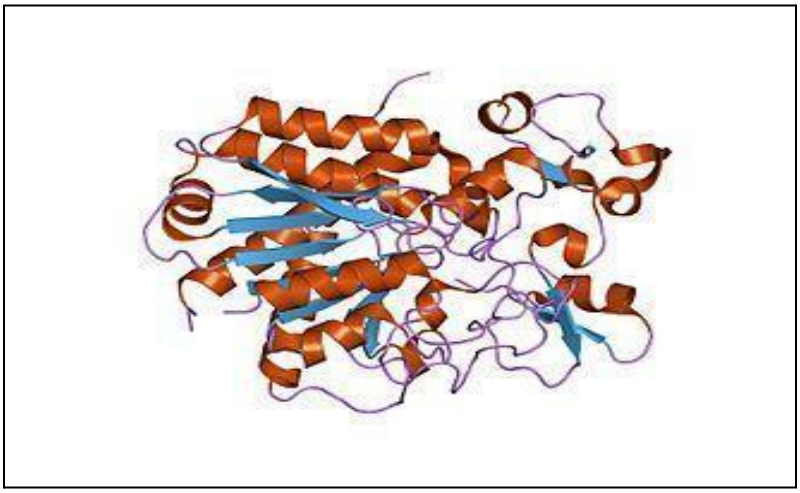


Figure 8: Crystal structure of the tick carboxypeptidase inhibitor in complex with human carboxypeptidase b (Richardson, 1977).

Plant proteinase inhibitors (PPIs) play an essential role by interacting with their proteinases counterparts forming protein complexes that modulate enzyme activity and consequently their

function (Richardson, 1977). The significance of PPIs as a relevant element controlling plant physiology is evident by their tremendous distribution among different species (Ryan, 1990). PPIs are represented by 19 families (Rawlings *et al.,* 2007).

A protease cleaves a peptide bond, called the scissile bond, between two amino acid residues named P1 and P1′ (Schechter and Berger, 1967). Residues on the N-terminal side of the scissile bond are numbered in the C to N direction, whereas residues on the C-terminal side of the scissile bond (the "prime" side) are numbered in the N to C direction (Figure 7). The substrate specificity of most endopeptidases is highly dependent on the nature of the side chain of the P1 residue, but the sequence of other residues near the scissile bond can also affect binding of the substrate to the active site and thus influence substrate specificity.

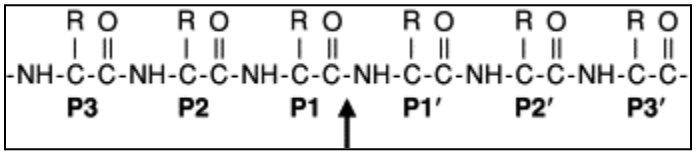


Figure 9: notation for protease cleavage sites. The arrow designates the scissile peptide bond between amino acid residues P1 and P1'(Schechter and Berger, 1967).

2.4. pH Relevance of Plant Proteinases

Antihelminthic and Digestive Aid Activity: The antihelmintic activity is a recognized feature attributed to vegetable cysteine proteinases. The antihelmin-tic efficacy of cysteine proteinases from various vegetable sources was established by using the rodent gastrointestinal nematode Heligmosomoides polygyrus in vitro assay. Each cysteine proteinase caused marked damage to H. polygyrus adult male and female worms, reflected by loss of the surface cuticle layer. The

effect was comparable in both sexes of worms and disappeared by enzyme treatment with E-64. Furthermore, plant cysteine proteinases attack structural proteins, decrease the worm burden and egg output of Trichuris muris. Hence, the efficacy of cysteine proteinases from plants against different gastrointestinal nematode species might represent a consistent alternative to currently available anthelmintic drugs. The usage of a glutamine-specific cysteine endoprotease (EP-B2) from barley seeds, in combination with a prolinespecific endopeptidase, was shown to rapidly accelerate gluten digestion in the mammalian gastrointestinal tract, alleviating the inflammatory immune response in intestinal lumen of celiac persons. The data suggest a promising potential of these proteinases for patients with celiac disease. In addition, plant proteinases are therapeutically relevant in the resolution of intestine obstruction. For instance, the meat tenderizer papain is effective relieving the symptoms of patients with phytobezoar without severe side effects.

2.5. Role of Ascorbic Acid in Studying the Inhibitory Effect of Polyherbal Extract on Proteinase Activity

Ascorbic acid, commonly known as vitamin C, plays a significant role in biological and biochemical studies due to its various properties and applications. In this context, studying the effect of ascorbic acid in controlling the capacity of plant extracts to inhibit proteinase activity is essential for understanding the mechanisms involved in enzyme inhibition and the potential applications in pharmacology and biochemistry.

- Antioxidant Properties: One of the many reasons for using ascorbic acid in this study is its potent antioxidant properties. Ascorbic acid acts as a scavenger of free radicals and reactive oxygen species (ROS) (Halliwell and Gutteridge, 1990). Proteinases, enzymes responsible for breaking down proteins, can be susceptible to oxidative damage, leading

to a loss of enzymatic activity. By including ascorbic acid in the experimental setup, researchers can mitigate the effects of oxidative stress on proteinases, ensuring accurate measurements of enzyme activity.

- Preservation of Extracts: Another crucial aspect is the role of ascorbic acid in preserving the activity of plant extracts. Plant extracts derived from *Azadirachta indica* (AI), *Persea americana* (PA), and *Mangifera indica* (MI) may contain bioactive compounds that could degrade over time due to enzymatic reactions or non-enzymatic processes. Ascorbic acid helps prevent enzymatic browning and degradation of these compounds, thereby maintaining the integrity and efficacy of the extracts during storage and experimentation (Schmedes and Hølmer, 1989).
- Stabilization of Enzymes: Ascorbic acid also plays a role in stabilizing enzymes, including proteinases. Enzyme stability is crucial for accurate measurements of enzyme activity and for studying enzyme kinetics. By preventing enzyme denaturation, ascorbic acid ensures that the proteinases under study retain their native structure and activity, providing reliable data for evaluating the inhibitory effects of plant extracts (Loewus, 1999).
- Experimental Control: Including ascorbic acid as a control in the experiments is essential for distinguishing between general effects on enzyme activity and specific inhibitory effects of the plant extracts. By comparing proteinase activity in the presence and absence of ascorbic acid, researchers can attribute changes in enzyme activity solely to the plant extracts' inhibitory properties. This control is fundamental in elucidating the precise mechanisms of enzyme inhibition by plant compounds.

Chapter Three

Materials and Methods

3.1. Apparatus

Sensitive weighing balance, mortar and pestle, conical flask, beaker, centrifuge, spectrophotometer, blender, test tubes, pipette, microplate reader, incubator, freezer, spatula, measuring cylinder, plastic containers, plastic buckets, face masks, gloves, reagent bottles.

3.2. Chemicals

Water, Tris-HCl, casein, trypsin, perchloric acid, DTT (dithiothreitol).

3.3. Collection of Plant Materials

The different plant materials, M. indica (MI), A. indica (AI) and P. americana (PA) leaves, were collected freshly from a garden in Ekpoma, Esan West Local Government Area of Edo State. The plant materials were identified and authenticated in the Department of Plant Science and Biotechnology, Ambrose Alli University Ekpoma, Edo State.

3.4. Preparation of Plant Materials

The leaves were air-dried at room temperature and homogenized using an electric blender. The polyherbal concoction was prepared by weighing and macerating 200g each of MI (*Mangifera indica*), AI (*Azadirachta indica*), and PA (*Pearsea americana*) leaves homogenate (ratio 1:1:1) in 4 L of distilled water (fresh sample). Another portion was prepared and cooked for 30 min with constant stirring of the boiling mixture (Ebhohimen *et al*., 2017). The mixtures were left to stand for 24 hours and then filtered with muslin cloth. The extracts were freeze-dried and stored in air-tight containers at -4°C until required for use.

3.5. Anti-proteinase Assay

The activity was performed by the protocol of Sakat *et al*. (2010) and Oyedepo *et al*. (1995) with some modifications. The reaction mixture (2 ml) containing 0.001% trypsin, 1 ml of 1mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100–500 µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.02% (w/v) casein was added. The mixture was incubated for an additional 20 min at 37°C. 2 ml of 2% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank.

The percentage inhibition of proteinase inhibitory activity was calculated. %inhibition = 100-

$$\frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

3.6. Statistical Analysis of Data

Data obtained was analyzed statistically using Microsoft excel 2019 and Graphpad prism 8 software. The results were presented as Mean ± standard deviation.

Chapter Four

4.0. Results

This research was conducted to ascertain the anti-proteinase activity of AI:PA:MI polyherbal extract. Different concentrations of the extracts were used for the study and results of the anti-proteinase assay conducted are presented in Table 4.1

Table 4.1: Effect of concentration on Anti-proteinase activity of AI:PA:MI polyherbal extracts

Conc (µg/ml)	Ascorbic acid	Experiment 1	Experiment 2	Experiment 3
100	[a]85.53 ± 2.08	26.58 ± 3.71	24.13 ± 2.77	19.22 ± 0.42
250	[b]87.04 ± 0.22	45.75 ± 1.67	32.52 ± 0.81	37.71 ± 3.51
500	[c]85.28 ± 1.64	59.45 ± 3.58	47.55 ± 0.31	55.60 ± 0.76
750	[d]84.78 ± 0.44	73.06 ± 0.69	58.38 ± 2.38	71.41 ± 0.56
1000	[e]82.14 ± 1.15	83.74 ± 2.93	75.87 ± 0.64	76.76 ± 2.59

Values are presented as mean ± SD of measurements made in triplicate (n=3). The % inhibition for control with a,b,c,d were significantly different from the test extract at $p<0.05$.

Chapter Five

Discussion and Conclusion

5.1. Discussion

The study evaluated the potential impact of polyherbal extract concentrations on proteinase enzyme inhibition. It was reported that the analysis process included several stages, such as obtaining samples and preparing the extracts. These were then examined using the anti-proteinase assay method described by Sakat *et al*, (2010) and Oyedepo *et al*, (1995). The practical was carried out in triplicates.

Results were gotten and taken in triplicate and the inhibition of the proteinase enzymes was calculated and presented in percentage inhibitory activity. This current study results depicts that, during the invitro study, the trypsin molecule is considered a mediator molecule for inflammatory reaction. The polyherbal extract used in the current study are anti-inflammatory agents providing a high level of protection acting as proteinase inhibitors. During the proteinase inhibitory assay, it is believed that the plants bind to the trypsin molecule, denoting that the inflammatory activity is to be subsided.

In this study, the potential anti-proteinase activity of the traditionally used plants *Azadirachta indica, Pearsean americana*, and *Mangifera indica.* It is observed that these plants have significant proteinase inhibitory activity. As shown in Table 4.1, samples with 100µg/ml showed percentage inhibitions within the ranges of 19 and 26%. However other samples with higher concentrations like that with 1000µg/ml showed percentage inhibition within the ranges of 76 and 84%. It showed that the higher the concentration, the more the percentage inhibition of proteinase activity measured.

Various recent studies have shown that the active compounds in the above mentioned polyherbal extract, contain anti-inflammatory potential by proteinase inhibitors. They've also shown that there's a binding mode and interaction of trypsin protein with the active ingredients in the polyherbal extracts, which is responsible for the anti-inflammatory activities.

The study of anti-proteinase activity is crucial in understanding and managing inflammatory responses, disease progression, and the development of targeted therapies. It also plays a significant role in investigating the anti-inflammatory properties of various substances (Pederzoli-Ribeil *et al.,* 2011).

5.2. Conclusion

In conclusion, this study; the effect of concentration of the polyherbal extract on the inhibitory activity of proteinase enzyme shows the dependence of both parameters on each other. Hence, the invitro study, shows that these active ingredients potentially inhibit the proteinase activity and could further be used as an anti-inflammatory drug and further used to design and develop anti-inflammatory compounds. While the need for further studies is important to get a more reliable result and better understanding of the process, the results from this study concludes that the higher the concentration of the polyherbal extract, the more their ability to inhibit proteinase enzymes.

References

Aslam, M.S., Ahmad, M.S., Mamat, A.S., Ahmad, M.Z., & Salam, F. (2016). An update review on polyherbal formulation: A global perspective. Syst. Rev. Pharm., 7, 35–41.

Bansal, V., Gupta, M., Bhaduri, T., et al. (2019). Assessment of Antimicrobial Effectiveness of Neem and Clove Extract Against Streptococcus mutans and Candida albicans: An In vitro Study. Niger Med J, 60, 285-289.

Berenbaum, M.C. (1989). What is synergy? Pharmacol. Rev., 41, 93–141.

Carvalho, R. R., Pellizon, C. H., Justulin, L., Jr., Felisbino, S. L., Vilegas, W., Bruni, F., Lopes-Ferreira, M., & Hiruma-Lima, C. A. (2009). Effect of mangiferin on the development of periodontal disease: Involvement of lipoxin A4, anti-chemotaxic action in leucocyte rolling. Chemico-Biological Interactions, 179, 344–350.

Chen, H., Morrell, P. L., Ashworth, V., de la Cruz, M., & Clegg, M. T. (2008). Tracing the Geographic Origins of Major Avocado Cultivars. Journal of Heredity, 100(1), 56–65. doi:10.1093/jhered/esn068. PMID 18779226.

Ciechanover, A. (2005). Nat. Rev. Mol. Cell Biol., 6, 79–87. [PubMed] [Google Scholar]

De Leo, F., Volpicella, M., Licciulli, F., Liuni, S., Gallerani, R., & Ceci, L.R. (2002). PLANT-PIs: a database for plant protease inhibitors and their genes. Nucleic Acids Res., 30, 347-348.

Figueiredo da Silva, A. A., de Carvalho Vieira, L., Krieger, M.A., Goldenberg, S., Zanchin, N.I., & Guimarães, B.G. (2007). Crystal structure of chagasin, the endogenous cysteine-protease inhibitor from Trypanosoma cruzi. J. Struct. Biol., 157(2), 416–423.

Garcia-Lorenzo, M., Sjodin, A., Jansson, S., & Funk, C. (2006). BMC Plant Biol., 6, 30.

Groves, M.R., Taylor, M.A., Scott, M., Cummings, N.J., Pickersgill, R.W., & Jenkins, J.A. (1996). The prosequence of procaricain forms an alpha-helical domain that prevents access to the substrate-binding cleft. Structure, 4(10), 1193–1203.

Halliwell, B., & Gutteridge, J.M.C. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. Methods in Enzymology, 186, 1–85.

Hatano, K. I., Kojima, M., Tanokura, M., & Takahashi, K. (1996). Solution Structure of Bromelain Inhibitor VI from Pineapple Stem: Structural Similarity with Bowman− Birk Trypsin/Chymotrypsin Inhibitor from Soybean. *Biochemistry*, *35*(17), 5379-5384.

Kanaori, K., Kamei, K., Taniguchi, M., Koyama, T., Yasui, T., Takano, R., ... & Hara, S. (2005). Solution structure of marinostatin, a natural ester-linked protein protease inhibitor. *Biochemistry*, *44*(7), 2462-2468.

Kiyohara, H., Matsumoto, T., & Yamada, H. (2004). Combination Effects of Herbs in a Multi-herbal Formula: Expression of Juzen-taiho-to's Immuno-modulatory Activity on the Intestinal Immune System. Evid.-Based Complement. Alternat. Med., 1, 83–91.

Kudom, A.A., Mensah, B.A., & Botchey, M.A. (2011). Aqueous neem extract versus neem powder on Culex quinquefasciatus: implications for control in anthropogenic habitats. J Insect Sci, 11, 142.

Lahiri, D., Nag, M., Dutta, B., et al. (2021). Catechin as the Most Efficient Bioactive Compound from Azadirachta indica with Antibiofilm and Anti-quorum Sensing Activities Against Dental Biofilm: an In Vitro and In Silico Study. Appl Biochem Biotechnol, 193, 1617–1630.

Lakshmi, T., Krishnan, V., Rajendran, R., et al. (2015). Azadirachta indica: A herbal panacea in dentistry - An update. Pharmacogn Rev, 9, 41–44.

Li, M., Phylip, L. H., Lees, W. E., Winther, J. R., Dunn, B. M., Wlodawer, A., ... & Gustchina, A. (2000). The aspartic proteinase from Saccharomyces cerevisiae folds its own inhibitor into a helix. *Nature structural biology*, *7*(2), 113-117.

Lin, P., Fan, X., Lu, X., et al. (2019). Four limonoids from Bacillus subtilis-fermented neem seeds and their cytotoxic activity. Fitoterapia, 135, 73–78.

López-Otín, C., & Overall, C. M. (2002). Nat. Rev. Mol. Cell Biol., 3, 509–519.

Maurya, M. (2017). Antinociceptive Activity of Ethanolic Neem (Azadirachta Indica) Leaf Extract (Enle) Through Opioid Pathway in Adult Rats. J of Pharmacol & Clin Res, 3, 555603.

Monteiro, A.C., Abrahamson, M., Lima, A.P., Vannier-Santos, M.A., & Scharfstein, J. (2001). Identification, characterization and localization of chagasin, a tight-binding cysteine protease inhibitor in Trypanosoma cruzi. J. Cell Sci., 114, 3933–3942.

Morton, J. F. (1987). Mango (Mangifera indica L.). In Fruits of Warm Climates; New Crop Resource Online Program, Center for New Crops and Plant Products, Purdue University (pp. 221–239). Retrieved 24 December 2021.

Murai, H., Hara, S., Ikenaka, T., Oda, K., & Murao, S. (1985). Amino acid sequence of Streptomyces metallo-proteinase inhibitor from Streptomyces nigrescens TK-23. J. Biochem., 97(1), 173–180.

Nelson, V.K., Ali, A., Dutta, N., et al. (2016). Azadiradione ameliorates polyglutamine expansion disease in Drosophila by potentiating DNA binding activity of heat shock factor 1. Oncotarget, 7, 78281–78296.

Neurath, H., & Walsh, K. A. (1976). Proc. Natl. Acad. Sci. U. S. A., 73, 3825–3832.

Olonen, A., Kalkkinen, N., & Paulin, L. (2003). A new type of cysteine proteinase inhibitor—the salarin gene from Atlantic salmon (Salmo salar L.) and Arctic charr (Salvelinus alpinus). Biochimie, 85(7), 677–681. https://doi.org/10.1016/S0300-9084(03)00128-7

Oyedepo O. O. and Femurewa A. J. (1995). Anti-protease and membrane stabilizing activities of extracts of Fagra zanthoxiloides, Olax subscorpioides and Tetrapleura tetraptera. Int J Pharmacong ;33:65-9

Parasuraman, S., Thing, G.S., & Dhanaraj, S.A. (2014). Polyherbal formulation: Concept of ayurveda. Pharmacogn. Rev., 8, 73–80.

Pardo-Andreu, G.L., Dorta, D.J., Delgado, R., Cavalheiro, R.A., Santos, A.S., Vercesi, A.E., & Curti, C. (2006). Vimang (Mangifera indica L. extract) induces permeability transition in isolated mitochondria, closely reproducing the effect of mangiferin, Vimang's main component. Chemico-Biological Interactions, 159, 141–148.

Pederzoli-Ribeli, M., Gabillet, J., & Witko-Sarsat, V. (2011). Proteases from inflammatory cells: regulation of inflammatory response. Proteases and their receptors in inflammation, 73-100.

Ponnusamy, S., Haldar, S., Mulani, F., et al. (2015). Gedunin and Azadiradione: Human Pancreatic Alpha-Amylase Inhibiting Limonoids from Neem (Azadirachta indica) as Anti-Diabetic Agents. PLoS One, 10, e0140113.

Porter, A. H. (17 April 2006). Neem: India's tree of life. BBC News.

Rawlings, N.D., Tolle, D.P., & Barrett, A.J. (2004). Evolutionary families of peptidase inhibitors. Biochem. J., 378(Pt 3), 705–716.

Richardson, M. (1977). The proteinase inhibitors of plants and microorganisms. Phytochemistry, 16, 159–169.

Rodeiro, I., Donato, M.T., Lahoz, A., Lavaut-Laguna, A., Castell, J.V., Delgado, R., & Gomez-Lechón, M.J. (2008). Modulation of P450 enzymes by Cuban natural products rich in polyphenolic compounds in rat hepatocytes. Chemico-Biological Interactions, 172, 1–10.

Ross, I.A. (2003). Medicinal plants of the world: Chemical constituents, traditional medicinal uses. Totowa, NJ: Humana Press Inc.

Roy, M., Rawat, A., Kaushik, S., Jyoti, A., & Srivastava, V.K. (2022). Endogenous cysteine protease inhibitors in upmost pathogenic parasitic protozoa. Microbiological Research, 261, 127061.

Ryan, C.A. (1990). Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol., 28, 425–449.

Saeki, K., Ozaki, K., Kobayashi, T., & Ito, S. (2007). J. Biosci. Bioeng., 103, 501–508.

Saito, N.K., Kohno, M., Yoshizazaki, F., & Niwano, K. (2008). Extensive screening for edible herbal extracts with potent scavenging activity against superoxide anions. Plant Foods for Human Nutrition, 63, 65–70.

Sakat S., Juvekar A. R., and Gambhire M. N. (2010). In vitro antioxidant and antiinflammatory activity of methanol extract of Oxalis corniculata Linn. Int J Pharm Pharm Sci; 2:146-55.

Schmedes, A., & Hølmer, G. (1989). A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. Journal of the American Oil Chemists' Society, 66(6), 813-817.

Schumacher, M., Cerella, C., Reuter, S., et al. (2011). Anti-inflammatory, pro-apoptotic, and anti-proliferative effects of a methanolic neem (Azadirachta indica) leaf extract are mediated via modulation of the nuclear factor-κB pathway. Genes Nutr, 6, 149–160.

Storey, W. B. (1973). What kind of fruit is the avocado? California Avocado Society 1973–74 Yearbook, 57, 70–71.

Tanaka, K., Aoki, H., Oda, K., Murao, S., Saito, H., & Takahashi, H. (1990). Nucleotide sequence of the gene for a metalloproteinase inhibitor of Streptomyces nigrescens (SMPI). Nucleic Acids Res., 18(21), 6433.

Tejesvi, M. V., Tamhankar, S. A., Kini, K. R., Rao, V. S., & Prakash, H. S. (2009). Phylogenetic analysis of endophytic Pestalotiopsis species from ethnopharmaceutically important medicinal trees. Fungal Diversity, 38, 167–183.

Turk, B. (2006). Nat. Rev. Drug Discov., 5, 785–799.

Van Vuuren, S.F., Motlhatlego, K.E., & Netshia, V. (2022). Traditionally used polyherbals in a southern African therapeutic context. Journal of Ethnopharmacology, 288, 114977.

Vicik, R., Busemann, M., Baumann, K., & Schirmeister, T. (2006). Inhibitors of cysteine proteases. *Current topics in medicinal chemistry*, *6*(4), 331-353.

Wagner, H. (2009). Synergy research: A new approach to evaluating the efficacy of herbal mono-drug extracts and their combinations. Nat. Prod. Commun., 4, 303–304.

Wang, S.X., Pandey, K.C., Scharfstein, J., Whisstock, J., Huang, R.K., Jacobelli, J., Fletterick, R.J., Rosenthal, P.J., Abrahamson, M., Brinen, L.S., Rossi, A., Sali, A., & McKerrow, J.H. (2007). The structure of chagasin in complex with a cysteine protease clarifies the binding mode and evolution of an inhibitor family. Structure, 15(5), 535–543.

Appendix

	First Technical Replicate		
Conc (µg/mL)	Absorbance		
100	0.11	0.098	0.083
250	0.16	0.17	0.171
500	0.223	0.226	0.202
750	0.264	0.267	0.269
1000	0.299	0.3	0.318
Control	0.365		
	First Biological Replicate		
Conc (µg/mL)	Absorbance		
100	0.088	0.078	0.07
250	0.107	0.108	0.103
500	0.155	0.156	0.154
750	0.188	0.184	0.199
1000	0.245	0.248	0.249
control 1	0.326		
control 2			

	Second Biological Replicate		
Conc (µg/mL)	Absorbance		
100	0.052	0.054	0.052
250	0.093	0.105	0.112
500	0.15	0.154	0.153
750	0.196	0.197	0.194
1000	0.209	0.204	0.218
control 2	0.274		
	Ascorbic control		
Conc (µg/mL)	Absorbance		
100	0.044	0.038	0.033
250	0.035	0.034	0.034
500	0.037	0.044	0.036

750	0.041	0.041	0.039
1000	0.044	0.05	0.048
		0.265	

Preparation of Reagents:

Using a weighing balance, 0.4g of casein was weighed into a beaker. 200ml of water was added to the beaker.

Perchloric acid (8mls) was measured using a measuring cylinder and was diluted using 400ml of water.

Trypsin (0.2 g) was measured into a beaker containing 200ml of Tris-HCl Buffer