ENZYMATIC AND ANTI MICROBIAL ACTIVITY OF PAPAYA (*Carica papaya* L.) LEAF, SEED AND FRUIT PULP EXTRACTS

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As thesis Research advisors, we hereby certify that we have read and evaluated this Thesis, prepared, under our guidance by Tsegaye Mekonnen entitled **Enzymatic and Antimicrobial Activities of Papaya (*Carica papaya* L.) Leaf Seed and Fruit Pulp Extracts**. We recommend that it be submitted as fulfilling the thesis requirement.

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DEDICATION

This thesis work is dedicated to my mother Selam Yosef and my father Mekonnen Libmogn to encourage and support me in different aspects.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this MSc thesis is my own work. I have followed all ethical and technical principles of scholarship in conducting studies, data collection, data analysis, and compilation of this thesis. Any scholar matter that is included in the Thesis has been given recognition through citation.

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ACRONYMS/ABBREVIATIONS

BITC	Benzyl Isothiocyanate
CPL	Carica Papaya Lipase
CRD	Completely Randomized Design
FAO	Food and Agricultural Organization
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum inhibitory concentration
NSAID	Non- Steroidal Anti inflammatory Drugs
NTAA	Nitrotri Acetic Acid
TPP	Three Phase Partitioning

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF TABLES IN THE APPENDIX

LIST OF FIGURE IN THE APPENDIX

Enzymatic and Antimicrobial Activities of Papaya (*Carica papaya* L.) Leaf, Seed and Fruit Pulp Extracts

ABSTRACT

In search of green technology, enzyme production has received much attention in industrial biotechnology processes with the objective of the substitution of chemical processes, with potentially adverse effects on humans and environment. Protease enzyme has been utilized widely in industries to engender a wide range of products such as, detergent, leather, waste management, brewing, meat softening, milk-clotting, food, pharmaceutical, cancer treatment, diagnostics, digestion, viral disorders and silver recovery. Therefore the present study was aimed to examine enzymatic and antimicrobial properties of protease from papaya (Carica papaya L.) Leaf, seed and fruit pulp. Enzyme extraction was conducted 10 mM Tris-Hydrochloride (Tris-HCl) buffer at pH 8.0 and 2MNaCl for three hours on an orbital shaker. The protein content was determined using Lowry assay using bovine serum albumin (BSA) as a standard protein. The optimum activity of the protease enzyme was done using PH and temperature. The antimicrobial activities of the enzyme extract were arranged as 3x4 completely randomized designed (CRD) factorial design in three replications. The result indicated that enzymatic extracts of papaya (Carica papaya L.) leaf, seed and fruit pulp presented significantly the highest protein content for leaf sample (11.67mg/ml). Significantly the highest protease activity was recorded for fruit pulp extract (42.28 U/ml). The optimum pH for protease activity was 7.5. The effect of incubation temperature on protease activity demonstrated that the maximum protease activity was observed at 35^0C. The strongest antibacterial activity with maximum zone of inhibition (17.50mm) at highest concentration (200mg/ml) of the enzyme extract was recorded for leaf enzyme extract against S. aureus. On

the other hand, the strongest antifungal activity with maximum zone of inhibition (18.50mm) was recorded for leaf extract against A. niger. The C. papaya leaf enzyme extract has exhibited strongest bactericidal activity with minimum inhibitory concentration (MIC 2.34mg/ml) and the corresponding minimum bactericidal concentration (MBC, 4.69 mg/ml) for leaf enzyme extract against S. aureus indicating that S.aureus was more susceptible than E. coli. Antifungal activity, the papaya leaf extract has presented strongest antifungal activity with MIC (4.69 mg/ml, the least value) and minimum fungicidal concentration (MFC, 7.81 mg/ml) against A. niger showing that A. niger was more susceptible to the enzyme extract than C. albicans. . It can be concluded that papaya enzyme extract has wide range of antimicrobial activities with the leaf extract with greatest antimicrobial potential while seed enzyme extract presented the least antimicrobial potential.

Keywords/Phrases: Bovine serum albumin, Diameter of zone of inhibition, Enzyme extract, **MIC**, *MBC, MFC, Protease activity, CRD, Antimicrobial*

1. INTRODUCTION

Papaya (*Carica papaya* L.) belongs to a small family caricaceae having four genera of which *Carica papaya* L. is the most widely cultivated and the best-known species (Jean *et al*., 2011).. The taxonomical classification includes Kingdom (Planate), Order (Brassicales), Family (Caricaceae), Genus (*Carica*) and Species (*papaya*). Papaya is probably originated in southern Mexico and Costa Rica, subsequently got introduced in Australia, Hawaii, Philippines, Sri Lanka, South Africa, India and all tropical and subtropical regions. It is growing both commercially and in home garden (Marotta *et al.,* 2006).

The papaya seed contain fatty acids, crude protein, crude fiber, papaya oil, carpaine, caricin, , benzyl Isothiocyanate, benzyl thiourea, hentriacontane, ß-sitostrol, caressing. The seeds and the pulp of *Carica papaya* contain benzyl glucosinolate which can be hydrolyzed by myrosinase to produce benzyl isothiocyanate. Seed extracts have profound bactericidal activity. The seeds of unripe fruits are rich in benzyl isothiocyanate, a sulphur containing chemical that has been reported to be an effective germicide and insecticide. These substances are important for plant natural defense mechanisms (El Moussaoui *et al.,* 2001). Medicinal uses of papaya seed are carminative, anti-fertility agent in males, counter irritant, as a paste in the treatment of ringworm, vermifuge, liver cirrhosis and abortifacient. Seed juice is used for bleeding piles, enlarged liver and pectoral properties. Seed paste is used as anthelmintic, stimulation of menstruation or abortion.

Chinoy *et al.*, (2006) proved the anti-fertility, anti-implantation and abortifacient properties of extracts from papaya seeds. It has been established in males that the seeds of *C. papaya* are potential anti-fertility drugs (Lohiya *et al*., 2005). Papaya seeds are used to produce an indigenous Nigerian food condiment called ‘daddawa’, the Hausa word for a fermented food condiment (Dakare, 2004). Fermented seeds have no effects on litters of rats (Abdul-Aziz *et al.,* 2009), whereas, those effects were apparent when the unfermented extract was administered (Abdulazeez, 2008). Anthelmintic activity of papaya seed has been predominantly attributed to carpaine (an alkaloid) and carpasemine (later identified as benzyl thiourea). Carpaine has an intensively bitter taste and a strong depressant action on health. Benzyl isothiocyanate (BITC), the main bioactive compound in *C. papaya* seeds

(Kermanshah *et al.*, 2001) has been shown to be responsible for the anti-fertility effect (Adebiyi *et al.*, 2003). BITC is capable of damaging the endometrial, making the uterus non-receptive and, thus, affecting adversely the implantation (Adebiyi *et al*., 2003).

Seeds are also a rich source of amino acids; scented oil was extracted, used in treatment of sickle cell disease and poisoning related disorders (Saran and Choudhary, 2013). Papa in is used in food processing to tenderize meat, clarify beer and juice, produce chewing gum, coagulate milk, prepare cereals, and produce pet food, also to treat wool and silk before dying, de-hair hides before tanning, adjunct in rubber manufacturing and photolytic enzymes (papa in and chymopapain). Papaya seeds are rich source of amino acids especially in the sarcotesta. A yellow to brown, faintly scented oil was extracted from the sundried, powdered seeds of unripe papayas at the Central Food Technological Research Institute, Mysore, India. White seeds yielded 16.1% and black seeds 26.8% and it were suggested that the oil might have edible and industrial uses. Air dried papaya seeds with honey showed significant effect on human intestinal parasites without significant side effect. Consumption of papaya seed is cheap, natural, harmless, readily available, and mono-therapeutic and prevent against intestinal parasitoids' especially in tropical communities (Saran and Choudhary, 2013).

Organic waste can be practically defined as any material or unused by-product from a process that is biodegradable and comes from either plant or animal. The main forms of organic waste are household food waste, agricultural waste, industrial waste and human and animal waste. Organic waste tends to be degraded over time by other organisms depending on its composition and moisture content. Agricultural and agro-industrial activities generate a lot of lignocelluloses' by-products such as abases, straw, stem, stalk, cobs, fruits peel and husk, among others. These wastes are mainly composed of cellulose (35-50%), hemicelluloses (25-30%), and lignin (25-30%) (Behera and Ray, 2016). Typically, in lignocelluloses' materials, the main cellulose constituent is glucose; hemicelluloses is a heterogeneous polymer that is mainly comprised of five different sugars (L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose) and some organic acids; whereas lignin is formed by a complex three-dimensional structure of phenyl propane units (Mussatto et al., 2012).

In search of green technology, enzyme production has received much attention in industrial biotechnology processes with the objective of the substitution of chemical processes, with potentially adverse effects on humans and environment. Various enzymes are emerging from biotechnology processes (Thomas et al., 2013). The extraction of protease enzyme from plants has increased significantly by considering its consequentiality (González-Rábade *et al*., 2011; Sun *et al*., 2016). Globally, the industrial production of proteases accounts for 60% of the enzyme sales economy (Kim *et al.*, 2016). Protease enzyme has been utilized widely in industries to engender a wide range of products such as, detergent, leather, waste management, brewing, meat softening, milk-clotting, food, pharmaceutical, cancer treatment, diagnostics, digestion, viral disorders and silver recovery (Kuddus, 2015). Proteases belong to a class of enzymes that can be classified based on their physical and biological property (Gupta *et al.*, 2012). Apart from this, the plant proteases have many biological roles, such as anti-cancer activity; avert edema, avails in the digestive process, procoagulant activity and many more. (Van deer Hoorn, 2008; González-Rábade *et al.*, 2011). However, the mechanism defining the biological activity of the enzyme remains obscure (Shivaprasad *et al.*, 2012). Therefore the present study was aimed to examine enzymatic and antimicrobial properties of crude enzyme from papaya (*Carica papaya* L.) Leaf seed and fruit pulp extracts.

General Objective

To investigate enzymatic and antimicrobial activities of papaya pulp, seed and leaf extracts

Specific Objectives:

- To extract and protease from pulp, seed and leaves of papaya;
- To investigate protease activity of pulp, seed and leaves of papaya;
- To optimize pH and temperature of crude enzyme extract ;
- To determine antibacterial and antifungal activities with respect to minimum inhibitory and bactericidal and fungicidal concentrations of the enzyme extract.

2. LITERATURE REVIEW

2.1. Botanical Description of Papaya (*Carica papaya L.*)

The papaya (*Carica papaya Linn.*) is an evergreen plant found in tropical regions (Jurandi *et al.*, 2011) originated from Central America and Mexico (Kaibing *et al.*, 2011). Few reports support that it is originated from the northwest of South America (Chan and Paull, 2008). Papaya is a herbaceous succulent plant that possesses a self-supporting stalk (Dick, 2003). It may be male, female, or hermaphrodite (Bruce and Peter, 2008) reproducing by self-pollination (Jeri, 2009) its fruit is a berry, produced from syncarpous superior ovary for export and household utilization. Papaya, an economically imperative crop of tropical and subtropical regions, is cultivated in over 50 different countries of the world. It is estimated that over 6.8 million tones of fruit were produced worldwide in 2004 on about 389,990 hectares of land. Globally, production of papaya has been raised to about 40% in a decade (1998–2008), with 9.1 million tones production during the year 2008. The leading producers of papaya are Brazil (world's biggest producer), Mexico, and Nigeria (FAO, 2007).

2.2. Nutritional and Photochemical Profile of papaya

The chemical evaluation of papaya revealed the presence of potassium (223 mg/100g of fresh fruit) along with sodium, calcium, phosphorus, zinc, iron, copper, manganese, and magnesium in appreciable amounts. Papaya occupies a key position among the fruits for vitamin A, C, B1, and B2, thiamine, foliate, riboflavin, niacin, calcium, potassium, iron, and fiber contents. It is low in calories, but rich in vitamins and minerals. About 60% of the ripe fruit is edible per 100 g of fruit. The energy value of papaya is 200 kJ/100 g.

The sensory traits, like taste and aroma, are due to volatile compounds, i.e., hydrocarbons, alcohols, terrenes, aldehydes, ketones, esters, benzyl isothiocyanate, and organic acids. (Almora *etal.* 2004). However, fruit aroma is related to ethyl hexaonate, ethyl 2-methylbutanoate and ethyl acetate (Balbontín *et al.*, 2007). Aliphatic and aromatic hydrocarbons are those groups that are found to be present at higher levels and are major contributors for aroma. Linalool is the papaya's highly abundant volatile and 94% in Solo papaya varieties. On the other hand, in Taiwan varieties the oxide cis-linalool is abundant;

linalool ranks second highest in concentration (Franco *et al.,* 1993) It has been investigated that aromatic compounds, such as 3-methylbutanol, butanol, terpineol, and benzyl alcohol, become abundant at the ripe stage (Almora *et al.*, 2004). It has been noted that among 103 esters, methyl butyrate is the peak in papaya. A variety of compounds other than linalool, are also present in fruit, i.e., benzyl isothiocyanate and terpene hydrocarbons. Butanol, 3-methylbutanol, benzyl alcohol, and α-terpineol show maximum concentration in the third maturation stage, in connection with fruit ripeness (Karina *et al.,* 2004).

The flesh color of papaya fruit is considered to be a significant nutritional quality attribute (Yan *et al.,* 2011). Softness and development of amber to orange color are characteristics of ripe fruit. Its flavor is alike to cantaloupe; it is sweet and juicy, includes some muskiness, (Morton, 1987) and its pulp can achieve 10–11.5% total soluble solids (TSS). Phenol compounds that are present in the fruit skin are likely to decrease as ripening progresses. The following compounds have been identified, i.e., ferulic acid (277.49 to 186.63 mg/100 g of fresh fruit), *p*-coumaric acid (229.59 to 135.64 mg/100 g fresh fruit), and coffin acid (175.51 to 112.89 mg/100 g). On the other hand, the following carotenoids in flesh along with vitamin C increases with ripening; lycopene (0.36 to 3.40 mg/100 g), β-criptoxanthin (0.28 to 1.06 mg/100 g), β-carotene (0.23 to 0.50 mg/100 g), and vitamin C (25.07 to 58.59 mg/100 g) are also present.

An array of dynamic compounds with versatile action are present in various tissues of the plant, i.e., linalool in fruit pulp (Winterhalter *et al.,* 1986), dehydrocarpaine I and II, alkaloids, carpaine, and pseudocarpaine in leaves (Khuzhaev and Aripova, 2000), glutamines cyclase in the latex; cysteine endopeptidases and class-II and III chitinase, (Azarkan, M.; Clantin, B.; Bompard, C.; Belrhali, H.; Baeyens-Volant, D. (Looze *et al.,* 2005) quercetin, and kaempferol in shoots (Miean and Mohamed, 2001); cyanogenic compounds in roots; and benzyl glucosinolate and its degradation product benzyl isothiocyanate in all tissues cumulatively. All of these compounds are identified by chemical characterization of extracted metabolite (Olafsdottire *et al.,* 2002).

2.3. Enzymes: Bioactive Moieties in Papaya

Numerous biologically active moieties are present in papaya. Papaya latex is a sap that is exuded from the point of plant damage caused either mechanically or by insect herbivore (Kotaro, 2011) and has been known to contain strong lipase activity. It is rich in cysteine endopeptidases having glycols endopeptidases, cysteine proteinases, serine proteinase inhibitor, glutamine cyclase characin, class II chitinase, papain, and chymopapain (Huet *et al.,* 2006). Studies report the identification of putative homologous lipase (a hydrolase and naturally immobilized biocatalyst) that is liable for the vital lipolytic activity of papaya latex (Dhouib *et al.,* 2011). *Carica papaya* lipase (CPL) has emerged as a protease having versatile biocatalytic properties, (de María et al., 2006), it finds abundant applications, such as fats and oils modification, facilitating a wide array of acids and alcohols as substrates for esterification and inter-esterification reactions and asymmetric resolution of different non-steroidal anti-inflammatory drugs (NSAIDs) and non-natural amino acids. Four types of cysteine proteases are present in papaya proteases, i.e., papa in (less than 10%), chymopapain A and B (26–30%), glycols endopeptidases III and IV (23–28%), and characin (14–26%). These form 69–89% of its total protein content. These proteases find wide application in medicine and the food industry. The method of three phases partitioning (TPP) can be effectively utilized for the extraction of proteases from papaya peels (Chaiwut *et al.,* 2007).

Proteases extracted from papaya exhibit a broad specificity and thermo stability thus utilized in the meat industry for meat tenderization. Papaya proteases are of medicinal significance especially for gastroenterology, wound healing, anti-inflammatory, antitumor, anthelmintic, neurosurgery, ophthalmology and urology properties (Seki *et al.,* 2007). Anti-inflammatory properties of papaya proteases help to reduce pain and suffering from arthritis, edema, and osteoporosis. Papa in is a non-specific thiol protease with an action similar to that of pepsin in gastric juice, an excellent aid to digestion and pepsin dilapidation. Endopeptidases is a minor constituent (5–8%) (Pendzhiev, 2002)

2.4. Nutritional Status of papaya

Among fruits, papaya is greatly esteemed worldwide owing to its distinct pharmacological and functional attributes. It is an imperative fruit that reveals unique and exceptional nutritional worth. Hence, as a nutraceutical, its consumption may exert an anti-inflammatory response (Luximon-Ramma *et al.,* 2003). In contrast to various other fruits, papaya holds a higher quantity of carotene. The comparative low calories content (32 kcal/100 g of ripe fruit) and high in nutritive value makes papaya a preferred and excellent dietary article for obese and those who are on a weight reducing regime.

Papaya is placed in the top five fruits with guava, watermelon, grapefruit, and kiwifruit, based on nutritional scores. It is at second position owing to hydrogen peroxide and hydroxyl radical scavenging activity (Murcia *et al.,* 2001). Unfortunately, these nutritional aspects are exclusively ascribed to fresh fruit. Its seeds are edible with a sharp and spicy taste and are often found as a replacement for black pepper as an adulteration tactic. Papaya delivers a broad spectrum of phytochemicals together with polysaccharides, glycosides, enzymes, flavonoids, lectins, saponins, vitamins, steroids, etc. These fundamental nutritional facts reveal that papaya contains an assay of dietary articles that may be precious for fulfilling body nutritional needs and, hence, considered beneficial for overall health (Maragatham and Panneerselvam, 2011).

2.5. Ethanol Production from papaya

There exists a huge competition involving ethanol production by fermentation process and petroleum-based products although, on escalating the value of these petrochemicals production of ethanol by process of fermentation, it has attained high interest (Maragatham and Panneerselvam, 2011). Owing to the fact that usage of renewable materials is proven to be particularly cheaper as these are low in cost and easily accessible. Hence, its disposal might be problematic and challenging. However, this agro-waste can be converted into alcohol, which has vast industrial applications. It has been reported that the utilization of brewer’s yeast significantly increases ethanol yield as compared to baker’s yeast. The amount of yeast prejudices ethanol production (Jurandi and Angela, 2011). Additionally, sacchari8fication of waste significantly increases the reducing sugars from 7.6 to 13.6 g/100 g

after 48 h, while fresh waste has the lowest. The chemical analysis of papaya fruit extract reveals that it is a good source of energy and nutrients for the purpose of formation of cell mass with 9.8% saccharide content. Hence, by using it as a substrate the single cell protein can be produced by employing *Saccharomyces cerevisiae*, which is biochemically active to produce wine. The fermentation products of its seed may be beneficial for medicinal, industrial, and bio-fuel production (Aruoma *et al.* 2006).

2.6. Medicinal Properties of Papaya

Papaya has been known as a food or as a quasi drug. It has wide consumption owing to its pharmacological properties and can be used as a folk remedy for various disorders. It contains different kinds of immune-stimulating agents and antioxidants (Aruoma, *et al.*, 2006). Its pulp is utilized in hospitals of Africa for wounds healing as well as curing burns because management of chronic non-healing ulcers poses difficulty and many clinical problems. An amalgamation of papa in-urea has been proven effective in conducting enzymatic wound debridement (Hosamath *et al.*, 2011).

Papaya latex is very useful for curing dyspepsia and is externally applied to burns and scalds; it also cures diarrhea, bleeding hemorrhoids, and whooping cough. Papaya juice helps in alleviating infections of the colon by clearing away infection, pus, and mucus. Its ripe fruit is a carminative, diuretic, expectorant, sedative, and has preventive action against dysentery, skin diseases, psoriasis, and ringworm. Papaya also exhibits therapeutic assets against various pathological disorders. The unripe fruit is used as a remedy for ulcers and impotence (Elizabeth, 1994), it has the ability to exhibit bacteriostatic activity against human enteric pathogens; and it aids in reducing menstrual irregularities and promotes natural menstruation flow in women. It has been recommended for controlling the most ubiquitous problem of hair dandruff. The green leaf presents an imitable source of vital and essential nutrients while the yellow one provides iron (Ayoola and Adeyeye, 2010). It may have a synergistic action to reduce enlarged spleen and liver and it is used in snakebite to remove poison. Papaya fruit is thought to contain some immune stimulating and antioxidant agents; its juice is prescribed to cure gastrointestinal maladies.

2.6.1. Digestive Health and Celiac Disease Elimination

Celiac disease, a multisystem and immune-mediated enteropathy (Kimberly and Starch, 2011) of the small bowel, is caused by permanent sensitivity to dietary prolamins (protein and alcohol-soluble fraction of cereals) present in gluten (Janatuinen *et al.,* 2002), in genetically susceptible individuals. It is one of the most common food intolerances found in cereal-based communities globally. The gliding (a glycoprotein) fraction of gluten is accountable for the development of intestinal damage (Herbert and Peter, 2008). Typically, in celiac disease, an immune response against cereal-derived proteins affects the absorption of nutrients by the small intestine and leads towards loss of normal mucosal architecture, and consequently, subsequent clinical and metabolic complications occur. Its ingestion provokes a persistent inflammatory response that induces flattening of intestinal villi (Leon *et al.,* 2005).

Enzyme supplementation plays a vital role in the management of numerous digestive disorders, primarily with regard to protein intolerance. Recently, it is the fundamental hypothesis behind the alleviation of celiac disease symptoms by utilizing enzyme therapy. (Cornell and Stelmasiak, 2007) Plant-based enzymes, like papa in from papaya, serve as an effective digestive aid in the breakdown of proteins. It is a complex of various enzymes that have proteolytic, amylolytic, and weak lipolytic activity. From a therapeutic point of view, papa in is highly esteemed for its digestive properties, which help to digest the protein in food. Moreover, it has an action similar to that of pepsin in gastric juice. The observation that crude papa in obliterates the celiac activity of gluten (Cornell *et al.,* 2005).

The toxic exploitation of gluten is annihilated when it is digested with crude papa in. Thus, it can be prescribed for dyspeptic and celiac disease patients, who cannot digest wheat protein gliding but can tolerate it if it is treated with crude papa in. The pure papa in activity was rather low. The crude papa in is responsible for gilding detoxification. Additionally, utilization of crude papa in for hydrolyzing gluten is an appropriate and economic approach. It is observed that glutamine cyclotransferase is the factor in crude papain that abolishes celiac activity of gluten (Valko *et al.,* 2007).

2.6.2. Antioxidant Potential of papaya

Free radicals generate in the human body and possibly their production rate increases in a majority of diseases (Valko *et al.,* 2007). A synthetic amino-tricarboxilic acid, nitrilotriacetic acid (NTA) produces water-soluble composites in combination with iron. However, this complex is nephrotoxic and provokes renal proximal tubular damage linked with oxidative damage that ultimately leads to a high prevalence of renal cell carcinoma. Fe–NTA in company with H2O2 *in vitro* leads increased oxidative DNA damage and *in vivo*. Fermented papaya preparation (FPP) defends super-coiled plasmid DNA against Fe–NTA and H2O2 persuaded single and double strand breaks. Fe–NTA induces a dose dependent fragmentation of bovine serum albumin *in vitro* and diminishes cellular GSH levels in lymphocytes (Valko *et al.,* 2007). Rimbach *et al.* (2000) conducted electron paramagnetic resonance (EPR) spin trapping studies, which indicate that antioxidant assets of FPP are associated with both hydroxyl scavenging as well as iron chelating perspectives (Marchetti *et al.,* 2005). A high concentration of copper has been found in amyloidal deposits in Alzheimer's disease brains and in the postmortem brains of Parkinson disease patients. On the other hand, FPP has also been shown to calm the apoptosis stress (Wild *et al.,* 2004).

2.6.3. Papaya and Diabetes

According to Wild *et al.,* (2004) diabetes causes oxidative stress in patients encompassing a huge risk of developing lethal diseases like neuropathy disorders, retinopathy, cardiovascular risks, and inborn malformations. Glucose oxidation results in oxidative stress that is largely augmented in diabetics. Certain other issues involved are cellular redox imbalances and attenuation of antioxidant defense bearing a small level of antioxidants in cells along with a harnessed enzymatic activity, which takes part in scavenging free radicals. Diabetes can be remedied by adopting certain nutritional recommendations like increased intake of fiber, weight reducing strategies, and functional food incorporation in diet plans, e.g., pycnogenols, fruits, vegetables, and legumes that have greater aptitude towards forcing the action of insulin (Chan *et al.,* 2009).

Epidemiological studies indicate that supplementation of FPP being a novel, may cater to this problem. It has been envisaged that the oral administration of FPP has the ability to produce a

considerable decline in the level of plasma sugar in healthy persons as well as in patients with type-II diabetes (Danese *et al.,* 2006). Along with improvement of lipid profile, many efficacy studies demonstrated that administration of diabetic mice with FPP can exhibit an elevated profusion of not only CD68 but also CD31 on the site of wounds, which may propose efficient monocytes recruitment as well as the superior pro-antigenic response. It may also be visualized that FPP can alter genes controlling action and role for these proteins (Khanna *et al.,* 2010)

2.6.4. Inflammation and Immune Importance of Papaya

Nitric oxide synthase, that is macrophage inducible, has the ability of creating nitric oxide (NO), which can take part in the immune defense system of a host in opposition to bacteria and viruses. Monocytes macrophages can be triggered by the help of a bacterial wall constituent called lip polysaccharide (LPS) and cytokines like interferon (IFN)-inducible type of nitric oxide synthase enzyme; tumor necrosis factor (TNF) is a different central regulatory cytokines in macrophage antimicrobial action and synergy with IFN in nitric oxide (NO) amalgamation induction. FPP exerts both immune modulator and antioxidant action. (Rimbach *etal.* 2000). Supplementation on FPP up-regulates the IFN-induced NO production in a precise conduct, which is dose-dependent. Ethanol-induced gastric mucosal troubles can be effectively defended by supplementation of food with FPP. It has been observed that FPP affects redox status as well as on DNA smash-up in healthy persons (Herrera *et al.,* 2009).

2.6.5. Anticancer Perspectives of Papaya

Apart from its apparent nutritional portrayal, papaya may also have divergent therapeutic and chemo preventive properties owing to antioxidants or cyto toxic phytochemicals that may prove effective against some forms of cancer. Otsuki *et al.* (2010) demonstrated that papaya has been utilized for its indigenous activity and purported for the ostensible anticancer properties. Some constituents of its leaf include the fermenting agent myrosin, alkaloids, rutin, resin, tannins, carpaine, dehydrocarpaine and pseudocarpaine enzymes, ascorbic acid, and saponins that can be potentially exploited as having immune modulator and dramatic cancer-fighting properties against a broad spectrum of tumors; it acquires a status as a tumor-destroying agent (Okwu and Ekeke, 2003).

The presence of saponins supports the fact that papaya leaf has cytotoxic effects, while ascorbic acid leads the plant to be used in herbal medicine for treatment of prostate cancer. Moreover, its juice has been shown to have an ant proliferative effect on liver cancer cells. According to an anticipated biosynthetic trail, lycopene is the central and key compound (the most abundant carotenoids), which indicates high stimulation of its upstream steps during the stage of ripening (Okwu and Ekeke, 2003). It exhibits a potent antioxidant action that has the ability to neutralize free radicals, thereby conferring defense against different kinds of cancer like breast cancer, prostate cancer, atherosclerosis, and associated coronary artery disease as well (Namita and Umesh, 2011).

2.6.6. Anti-helmintics Activity of Papaya

Okeniyi *et al.* (2007) reported that papaya seed extract has anti-amoebic and anthelmintic activity. Anthelmintics often affect neurotransmitter receptors that are different in the parasite and host. It has been verified through laboratory investigations that papaya seeds are effective against helminthes efficiently *in vitro*. The anthelmintic activity is attributed to the presence of carpaine (an alkaloid) and carpasemine (later identified as benzyl thiourea), and benzyl isothiocyanate (BITC) and cysteine proteinase. BITC is derived from the action of enzyme myrosinase on benzyl glucosinolate that are found in separate compartments in the seed; enzyme is brought into contact with benzyl glusosinolate. It is considered to be a principal volatile compound and is found in papaya seeds, which shows profound activity against *C. elegans in vitro*, and is accountable for cytotoxic effect on vascular contraction (Kermanshah et al., 2001). It may inhibit movement and produce relaxation of strips of earthworm and at high doses may cause complete paralysis (Wilson *et al.*, 2002). Moreover, cysteine proteinase is found in papaya latex that has a potential anthelmintic property in mono gastric hosts (Wilson *et al.*, 2002).

2.7. Malaria and Dengue Fever Treatment using Papaya

Malaria, being one of the most prevalent disorders throughout the world, is caused by the parasites of genus plasmodium and can present itself with a myriad of distracted symptom (Laura et al., 2011). Saotoing et al. (2011) has reported that malaria can be effectively treated by using papaya leaves. This beneficial action is due to the presence of alkaloids in the leaves

since quinine is present in alkaloids, which proves to be an anti-malarial agent. Dengue fever, on the other hand, is the most up-and-coming viral disease of human beings, which has recently become an alarming global public health concern. It is anticipated that about 50 to 100 million cases of dengue fever occur every year that direly needs hospitalization. Dengue fever, caused by the dengue virus (belonging to the *Flaviviridae* family), is a mosquito-borne (*Aedes aegypti*) malady; it gets spread when a mosquito bites a tainted individual (Moreno-Sanchez *et al.,* 2006).

However, Nisar *et al.* (2011) conducted a scientific study whose results reveal that dengue fever can be ameliorated by using papaya leaf extract. Another infective agent, chikungunya vector, is also a potential risk to health so the use of papaya leaf extract in combination with spinosad (a bacterial insecticide) may have been proven to be a beneficial and eco-friendly approach for its remedy. It shows larvicidal and pupicidal action against this vector. Kalimuthu et al. (2011) explicated that leaf extract of methanol papaya has the highest larval and pupal mortality.

2.8. Papaya Leaf Extract against Various Maladies

Carica papaya (L.) leaves are popularly used as food and have many traditional claims for herbal medicine. Phenol compounds are important components in plant-derived foods for their beneficial effects on human health. A multitude of reports related to glycosides, phenolics, and composition of fruit and leaves of the papaya (Simirgiotis et al., 2009). In addition to these, it may contain various dynamic constituents, such as rutin, methyl salicylate, pseudocarpaine, benzylglucosinolate, tocopherol, linalool, resins, dehydrocarpaine, tannins, chymopapain, papa in, cyanogenic glycosides, carposide, glucosinolate, beta-carotene, cystatin, malic acid, myrosin, alkaloids, rutins, flavonoids, carpaine, and some fermenting agents. In addition, carapine is an alkaloid that has the capability to act as a heart depressant, amoebicide, and diuretic. It proves handy in the coagulation of blood, the apt functioning of heart and nervous system, and normal movement of muscles. Flavonoids are a group of widespread plant constituents. There is evidence those flavonoids rich products contribute to the protection of skin against UV-induced damage at the molecular and cellular

level. These attributes are comparable to those described for other dietary constituents, such as carotenoids and antioxidants (Wilhelm, 2011).

The regular use makes the skin supple. The role of antioxidants and phenol compounds has been supported by epidemiological facts that these compounds show greater activity to relieve the symptoms of asthma, nervous pains, gastric troubles, amoebic dysentery and cure sores, elephantoid growth, act as vermifuge, heal wounds, and are a remedy for various disorders and infectious diseases. It also plays a vital role in prevention of various chronic diseases, including cardiovascular problems, parasitic and bacterial disorders, diabetes, and cancer (Murakami *et al.*, 1994). The composition of leaves consists of the following: calories (74 g), water (77.5 g), protein (7 g), fat (2 g), total carbohydrates (11.3), fiber (1.8 g), ash (2.2 g), calcium (344 mg), phosphorous (142 mg), iron (0.8 mg), sodium (16 mg), potassium (652 mg), β-carotene equivalent (11,565 ug), thiamine (0.09 mg), riboflavin (0.48 mg), niacin (2.1 mg),ascorbic acid (140 mg) and vitamin E (136 mg) per 100 g. The presence of iron signifies that the leaves can be used against anemia, tuberculosis, and growth disorders. As a rich source of phytochemicals, they can be used as a cleanser in herbal remedies, coupled with the presence of essential vitamins and minerals, such as Ca, Mg, Mn, Fe, Zn, K, P, and vitamins like A, C, E, and B (Runnie *et al*., 2004).

The high concentration of Vitamins A and C is good for better eye-sight, prevention of early age blindness in children, and for those who frequently suffer from cold, cough, or flu, because intake of papaya boosts their immune system. Moreover, its regular consumption helps to relieve morning sickness, nausea, and overcomes constipation, while vitamin B1 fights against beriberi. It is eluded that its infusion may eradicate symptoms of gonorrhea. On the other hand, freshly obtained leaf latex contains 75% water, 4.5% caoutchouc-like substances, 7% pectinous matter, 0.44% salts, 5.3% papa in, 2.4% fat, 2.9% resins, and a significant amount of malic acid. The methanolic leaf extract reveals antioxidant and vasodilatatory characteristics; both of these properties are obliging in confiscating the cardiovascular risks (Runnie *et al.*, 2004).

Moreover, its antioxidants diminish the level of lipid per oxidation. Fresh, green papaya leaf is an antiseptic, while the brown dried leaf is the best tonic as a blood purifier. Papaya roots

demonstrate abortifacient action and act as a generative toxin to cure piles; they also show antibacterial and antifungal activity and purgative effect. Fresh roots are administered orally with sugarcane alcohol for alleviating rheumatism. Additionally, various parts of this precious species can be effectively utilized against viral contagion. The stem and bark can fight against jaundice and exhibit anti-hemolytic activity while its flowers are febrifuge and may show pectoral properties (Doughari *et al.*, 2007).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted at Biotechnology lab in School of Biological Sciences and Biotechnology, Haramaya University. The University is located at about 510 km East of Addis Ababa, between Dire Dawa and Harar cities and at an altitude of 2390 meters above sea level.

3.2. Research Design

The study was conducted to investigate enzymatic and antimicrobial activities of crude enzyme from papaya (*Carica papaya L.*) leaf, seed and fruit pulp. The fresh papaya samples of ripen fruits and leaves were collected from Home garden in Bihar Dar city. The protein content was determined using Lowry assay using bovine serum albumin (BSA) as a standard protein. The optimum activity of the protease enzyme was done using pH and temperature. The antimicrobial activities of the enzyme extract was arranged as 3*3*4*3 [3 source extracts of leaf seed and fruit pulp samples of papaya at three concentration levels, 4 test pathogens and 3 replications, a total of 72 experiments.) Completely randomized (CRD) factorial design. The antimicrobial activity was determined using disc diffusion and broth dilution methods.

3.3. Sample Preparation and Enzyme Extraction

The fresh papaya samples of ripen fruits and leaves were collected from Home garden in Bihar Dar city, Ethiopia. Fresh leaf, seed and pulp samples (60g) were pounded using 100ml acetone to remove fat content, using mortar and pestle. For enzyme extraction, samples were separately mixed with chilled 10 mM Tris-Hydrochloride (Tris-HCl) buffer at pH 8.0 and 2MNaCl for three hours on an orbital shaker. The samples were then centrifuged at 10,000 rpm for 10 minutes at 4°C and filtered through Whatmann filter paper; the filtrate was collected and used for enzyme assays.

3.4. Determination of Total Soluble Protein

Protein was determined by the modified method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as a standard protein. In this assay, three reactions solution were prepared as a Lowry solution (Soln A + Soln B + Soln C) with a ratio of 100:1:1 volume basis, respectively. For Sol A about 2.86 g of NaOH and 14.31 g of Na2CO3 were dissolved in 500 ml of de-ionized water. Soln B was prepared by dissolving 1.42 g of $CuSO4 \cdot 5(H_2O)$ in 100 ml of de-ionized water. Soln C was prepared by dissolving 2.86 g in 100mL of $Na_2Tartrate \cdot 2(H_2O)$ in 100 ml of de-ionized water. Folin Reagent was freshly prepared every assay by mixing 5 ml of 2N Folin and Ciocalteu' s Phenol Reagent with 6 ml of de-ionized water. The assay was initiated by mixing 0.5 ml sample with 0.7 ml of Lowry solution. Then the sample mixture was incubated for 20 min at room temperature in dark condition. After 20 min of incubation, the sample mixture was immediately added to 0.1 ml of Folin Reagent and mixed vigorously. Subsequently, the mixture was incubated for 30 min at room temperature in dark condition again. After the incubation, the mixture was vortexes briefly, and the absorbance was measured shortly. The protein content was estimated by measuring the absorbance at 750 nm using Varian Cary® 50 UV-visible spectrometers. The protein standard curve was established by serially diluted 2 mg ml-1 BSA protein standard.

$$\text{Conc. of protein in sample soln (mg/ml)} = \frac{\text{OD of test sample} - \text{OD of blank}}{\text{OD of std} - \text{OD of blank}} \text{ x conc of std BSA}$$

3.5. Crude Enzyme Assay

Protease activity was measured by caseinolytic assay using casein as a substrate. In this method, 1ml of enzyme solution was added to 2 ml of casein (1% w/v in 0.1N Glycine NaOH buffer pH 10) and the mixture was incubated for 15 min at 35^0C. The reaction was terminated by adding 3ml of 10% trichloroacetic acid and then centrifuged for 15 min at 10,000 rpm. Then, 2 ml of the supernatant (enzyme mixture) was mixed with 5ml of sodium carbonate and 0.5ml of Folin- reagent. Spectro photometric readings were determined at 660 nm. Enzyme activity was calculated for each saturation percentage, to measure the amount of active enzyme present. Similarly blank was carried out by replacing enzyme with distilled water. One unit enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine (as

a result of hydrolysis of casein) per ml per min under the assay conditions. The range of concentration 50 -250 µg of tyrosine was used as standard. All experiment was conducted in triplicate. After protease assay performed for samples, enzyme activity values for each sample were calculated using the standard formula (Kassell and Meitner, 1970).

Determination of Enzyme activity:

$$\text{PA (U/ml)} = \frac{(OD_{sample} - OD_{blank)(df)}}{(ODstd - ODblank)(t)(V_{enzyme})}$$

$$\text{df} = \frac{\text{final dilution volume}}{\text{volume of enzyme sample}}$$

$$\text{Protease specific activity (U/mg)} = \frac{\text{PA activity}}{\text{soluble protein concentration}}$$

Where, PA: protease activity; U: activity unit; t: time of incubation, (min); df: dilution factor

;

3.6. Optimization Crude Enzyme Activity

3.6.1. Effect of pH on Crude Enzyme Activity

Effect of pH on protease activity was measured using a Tris-HCl buffer. The pH was adjusted to 5, 6, 7, 8 and 9 respectively. Each of the six samples was mixed with Tris-HCl buffer in 1:1 ratio. After an incubation period of 24 hours at room temperature, protease assay was performed with each of the six samples at the pH mentioned above.

3.6.2. Effect of Incubation Temperature on Crude Enzyme Activity

The effect of temperature on protease activity was determined by testing enzyme activity at different temperatures (20-40^{0}C with an interval of 5^{0}C) with the pH and time of incubation remains constant at 35^{0}c and 24hr respectively. The protease assay was carried out to determine the concentration of the enzyme as per section 3.5 above.

3.7. Antimicrobial Activity of Crude Enzyme Extract

The experiment was arranged as 3*3 * 4*3 factorial designs (i.e. 3 enzyme extracts,3 different concentration, 4 test microbes (2 bacteria and 2 fungi) in three replications. A complete randomized design (CRD) was used to determine the antimicrobial activities using disc diffusion method. In addition, the least concentration of enzyme extract that show antimicrobial activity was selected for further determining the minimum inhibitory concentration (MIC) minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC).

3.7.1. Test Pathogens

Four test pathogens including two bacteria [*Escherichia coli* (gram negative) and Staphylococcus aureus (gram positive)], *two fungi (*Aspergullus niger and Candida albicans) were obtained from Ethiopian Public Health Institute (EPHI). The fungal and bacterial pathogens were sub cultured and maintained on nutrient agar and Potato Dextrose Agar

(PDA), respectively. In addition, the fungal and bacterial cultures were incubated for 70 hours at 27 ºC and for 24 h at 37 ºC, respectively.

3.7.2. Media Preparation and Standardization of Inoculums

Nutrient Agar (NA), Potato Dextrose Agar (PDA), and Muller Hinton agar (MHA) were used for sub-culturing of bacterial test organism, fungal test organism, and determination of antimicrobial activities, respectively. These media were prepared and sterilized using an autoclave according to the manufacturers' instructions. Two to three bacterial colonies on the plate were picked up with a sterile inoculating loop and transferred into a test tube containing sterile normal saline and vortexes thoroughly. The spores of the test fungi were harvested by washing the surface of the fungal colony using 5mL of sterile saline solution. This procedure repeated until the turbidity of each bacterial and fungal spore suspension matched the turbidity of 0.5 McFarland Standards as described by the Clinical Laboratory Standards Institute (CLSI, 2015). The resulting suspension will be used as inoculums for the test pathogen in the antimicrobial susceptibility test.

3.7.3. Disc diffusion Method

Discs of 6 mm diameter was prepared from sterile filter paper cut into small, circular pieces of equal size by a perforator and then impregnated each of them were impregnated with 0.01 ml of the prepared test extract ethyl acetate solution. The extract impregnated discs were placed onto MHA plates evenly inoculated with test pathogens (Morshed et al., 2012).

3.7.4. Inoculation of Mueller Hinton Agar (MHA) Plates

After adjusting the turbidity of the suspension of inoculums within 15 minutes, a sterile cotton swab was dipped into adjusted suspension and rotated several times by pressing firmly on the inside wall of the tube above the fluid level. This removes excess fluid from the swab. Then, the dried surface of Mueller Hinton Agar plates were inoculated by streaking using the swab three times over the entire surface and rotating the MHA plates approximately 60° each time to ensure an even distribution of the inoculums. Then, the MHA plates were left open for three to five minutes to allow for any excess surface moisture to be absorbed (CLSI, 2015).

Following this step, the impregnated discs were dispensed onto the surface of the inoculated agar plates using sterile forceps. Each disc was pressed down to ensure complete contact with the agar surface. The discs were distributed evenly so they were not closer than 24 mm from center to center (CLSI, 2015). Discs of commercial gentamycin (1mg/disc) and fulconazole (1mg/disc) were used as positive controls for bacterial and fungal pathogens, respectively and distilled water impregnated discs were used as negative controls.

Then the MHA plates were sealed with par film and incubated at 37°C for 24 hrs and 27°C for 72 hrs for bacterial and fungal pathogens, respectively. After incubation, the diameters of the zone of inhibition around each disc were measured to the nearest millimeter along two axes (i.e. 90° to each other) using a transparent ruler and the means of the two readings were be recorded. For each selected pathogen the experiment was carried out with three replications.

3.7.5. Determination of Minimum Inhibitory Concentration (MIC)

The enzyme extracts that showed significant antimicrobial activity in the antimicrobial activity tests were selected for determination of MIC based on the method used by Morshed et al (2012) . The MICs of the oil extracts were determined by broth dilution method. In the broth dilution method, the extract solution for example at 100mg/ml (w/v) was serially diluted in a two-fold dilution as 1mg/ml, 0.50 mg/ml, and 0.25 mg/ml 0.125 mg/ml 0.0625 mg/ml concentrations. Two milliliter of nutrient broth and potato dextrose broth for bacteria and fungi respectively were added into all test tubes and 0. 1 ml of the prepared concentration of each enzyme extract was mixed with the nutrient broth and potato dextrose. Thereafter, standardized inoculums of 0.1 ml of the respective test pathogens were dispensed into the test tubes containing the suspensions of the broth and the enzyme extract. Then, all test tubes were properly corked and incubated at 37°C for 24 hrs for bacteria and 27°C for 72 hrs for fungi. After that, they were observed for absence or presence of visible growth. The experiment was carried out for each test organism in triplicates.

3.7.6. Determination of Minimum Bactericidal (MBC) and Fungicidal Concentrations (MFC)

For the determination of the MBC and MFC, fresh nutrient agar and potato dextrose agar plates were inoculated with one loop full of culture taken from each of the broth cultures that showed no growth in the MIC tubes. That is MBC/MFC values were determined by sub-culturing from respective MIC values. If for example MIC=1 mg/ml (w/v) sub-culturing was performed as 0.50 mg/ml, 1.00 mg/ml, 1.50 mg/ml, 2.00 mg/ml up to four acceptable concentration levels. Since antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC (CLSI, 2015). MBC/MFC is the amount of the extract that kills microbial growth. While MBC assay plates were incubated for 48 h, MFC assay plates were incubated for 3 days. After the incubation periods, the lowest concentration of the extract that did not allow any bacterial or fungal growth on solid medium was regarded as MBC and MFC for the extract (CLSI, 2015). This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation for bacteria or spore germination after 3 days of incubation for fungi. Fulconazole (1mg/disc) disc was applied as positive control and distilled water served as negative control for incubation of fungi while gentamycin (1mg/disc) was served as positive control and demonized water was served as negative control for bacterial pathogens.

3.8. Data Analysis

All data were entered into Microsoft excel. Mean comparison and Analysis of variance (ANOVA) was carried out using SAS version 20 software package. Statistically significant differences were indicated by $p<0.05$ and $p<0.01$.

4. RESULT AND DISCUSSION

4.1. Crude Enzyme Activity Assay of Papaya (*Carica papaya* L.) Leaf, Seed and Fruit Pulp Enzymatic Extracts

Enzymatic extracts of papaya (*Carica papaya* L.) leaf, seed and fruit pulp presented significantly the highest protein content for leaf sample (11.67mg/ml), followed by seed extract (10.69mg/ml) and the least protein content was recorded for fruit pulp (8.73mg/ml). Significantly the highest protease activity was recorded for fruit pulp extract (42.28 U/ml) followed by leaf sample (25.33 U/ml) and the least protease activity (18.05 U/ml) were observed for papaya seed extract. Likewise, significantly the highest protease specific activity (4.85U/mg) was recorded for fruit pulp followed by leaf extract (2.17 U/mg) and the least protease specific activity (1.69 U/mg) was recorded for seed extract.

Table 1. Protease activity of papaya leaf, seed and fruit pulp enzymatic extracts

Source of Enzyme	Protein content	PA	PAspa
Leaf	11.67±0.08a	25.33±0.57b	2.17±0.04b
Seed	10.69±0.21b	18.05±1.94c	1.69±0.34b
Pulp	8.73±0.21c	42.28±1.24a	4.85±0.14a

PA: Protease activity; PAspa: Protease specific activity.

4.2. Optimization of Crude Enzyme Activity

4.2.1. The effect of pH on crude enzyme activity

The optimum PH for crude enzyme activity was 6.5 to 8 with maximum activity at PH 7.5 (Fig. 1 and Appendix Table 1). It was also found that crude enzyme activity was the highest for papaya leaf extract, followed by fruit pulp and the least crude enzyme was observed for papaya seed extract.

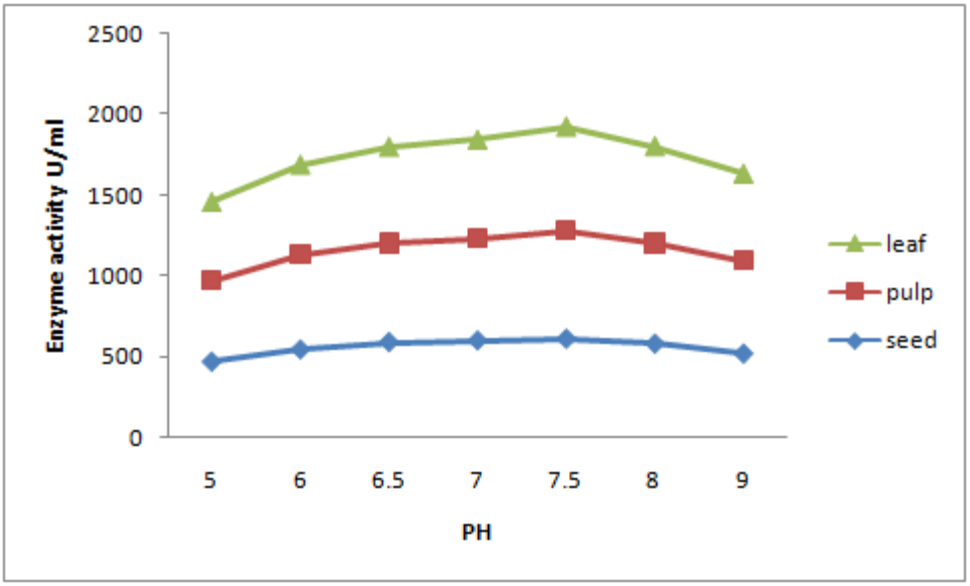

Figure 1. The effect of pH on crude enzyme activity

4.2.2. The effect of incubation Temperature

The effect of incubation temperature on crude enzyme activity demonstrated that the maximum protease activity was observed at 35^0C (Fig 2 and Appendix table 2). The working temperature for crude enzyme extract from papaya might be from 30 to 40^0C. The highest crude enzyme activity was observed for leaf extract while the lease crude enzyme activity was recorded for seed extract.

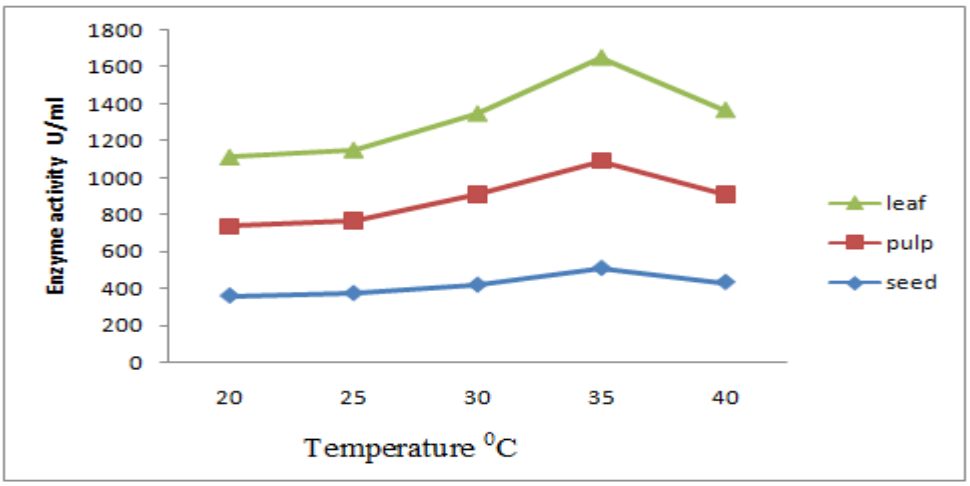

Figure 2. The effect of temperature on crude enzyme activity

4.3. Antimicrobial Activities of Papaya (*Carica papaya* L.) Leaf, Seed and Fruit Pulp Enzymatic Extracts

The diameter of zone of inhibition measured based on disc diffusion, for papaya (*Carica papaya* L.) leaf, seed and fruit pulp enzymatic extracts (Table 2) demonstrated significant antimicrobial activities of the enzyme extracts against tested bacteria and fungi. The mean zone of inhibition at highest concentration (200mg/ml) against bacterial test pathogens ranged from 14.00±0.50 to 17.50±0.50 mm, while 12.73±0.64 to 18.50±0.55mm against fungal test pathogens. The strongest antibacterial activity with maximum zone of inhibition (17.50mm) at highest concentration (200mg/ml) of the enzyme extract was recorded for leaf enzyme extract against *S. aureus* while the weakest antibacterial activity (14.00mm) was recorded for seed enzyme extract against *E. coli* indicating that *S. aureus* was more susceptible than *E. coli.* On the other hand, the strongest antifungal activity with maximum zone of inhibition (18.50mm) was recorded for leaf extract against *A. niger,* but the weakest antifungal activity with minimum zone of inhibition (12.73mm) was recorded for seed enzyme extract against *C. albicans* indicating that *A. niger* was more susceptible to the enzyme extract while *C. albicans* was more resistant to the enzyme extract. It can be concluded that papaya enzyme extract has wide range of antimicrobial activities with the leaf extract with greatest antimicrobial potential while seed enzyme extract presented the least antimicrobial potential.

Table 2. Antimicrobial activity of based on diameter of zone of inhibition of papaya (*Carica papaya* L.) leaf, seed and fruit pulp enzymatic extracts

Test path.	Enzyme extract	Concentrations of enzyme extract			Gentamycin (100mg/ml)
		100mg/ml	150mg/ml	200mg/ml	
E. coli	Seed	10.53±0.50dD	12.83±0.76dC	14.00±0.50dB	18.17±0.29aA
	Pulp	12.17±0.76bcC	15.17±0.28aB	15.58±0.80cB	18.83±0.35aA
	Leaf	11.83±0.78cD	13.83±0.71cC	16.23±0.25bcB	18.83±0.58aA
S. aureus	Seed	13.00±0.56abBC	14.00±0.50bcB	15.50±0.45cB	18.50±0.50aA
	Pulp	12.83±0.76abcD	14.50±0.50abcC	16.63±0.71abB	18.50±0.06aA
	Leaf	13.50±0.50aB	14.92±0.38abB	17.50±0.50aA	18.83±0.76aA
					Fulconazole (100mg/ml)
A. niger	Seed	9.40±0.53bcD	13.10±0.36cC	15.03±0.45dB	18.00±0.50aA
	Pulp	8.83±0.76cD	13.00±0.50cC	15.50±0.45dB	18.17±0.76aA
	Leaf	10.50±0.40aC	13.50±0.45cB	18.50±0.55aA	18.00±0.50aA
C. albicans	Seed	0.00±0.00dD	10.57±0.51dC	12.73±0.64eB	18.17±0.29aA
	Pulp	10.17±0.76abC	15.43±0.51aB	16.50±0.55cB	18.00±0.50aA
	Leaf	11.17±0.75aC	14.43±0.51bB	17.57±0.40bA	18.50±0.87aA

Means followed by same letter within a column were not significantly different at 0.05 probability level based on LSD (Least Significance difference) test. Small letters: significance within column; capital letters: significance across row. *E. coli: Escherichia coli, S. aureus: Staphylococcus aureus; A.niger: Aspergillus niger; C. albicans: Candida albicans.*

4.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), Minimum Fungicidal Concentration (MFC) of Papaya (*Carica papaya* L.) Leaf, Seed and Fruit Pulp Enzymatic Extracts

The effectiveness of the enzyme extract against pathogenic microbes was further assessed using MIC, MBC and MFC as in Table 3. The *C. papaya* leaf enzyme extract has exhibited strongest bactericidal activity with MIC (2.34mg/ml) and the corresponding MBC (4.69 mg/ml) for leaf enzyme extract against *S. aureus* while the weakest bactericidal activity with MIC (18.75mgl/ml, the largest value) and MBC (37.50mg/ml) was recorded for seed enzyme extract against *E. coli* indicating that *S.aureus* was more susceptible than *E. coli.*

Table 3. Minimum inhibitory concentration (MIC), Minimum Bactericidal Concentration (MBC) minimum fungicidal concentration (MFC) of the enzyme extract

Test pathogens	Source of Enzyme	MIC (mg/ml)	MBC/MFC (mg/ml)
E. coli	Seed	18.75	37.50
	Pulp	12.50	37.50
	Leaf	9.38	18.75
S. aureus	Seed	9.38	18.75
	Pulp	9.38	12.50
	Leaf	2.34	4.69
A. niger	Seed	37.50	62.50
	Pulp	12.50	25.00
	Leaf	4.69	7.81
C. albicans	Seed	37.50	75.00
	Pulp	18.75	37.50
	Leaf	9.375	18.75

For antifungal activity, the papaya leaf extract has presented strongest antifungal activity with MIC (4.69 mg/ml, the least value) and MFC (7.81 mg/ml) against *A. niger* while the weakest antifungal activity with MIC (37.50mg/ml) and MFC (75 mg/ml) was recorded for seed enzyme extract against *C.albicans* showing that *A. niger* was more susceptible to the enzyme extract than *C. albicans*.

5. SUMMARY, CONCLUSION AND RECOMMENDATION

5.1. Summary

In search of green technology, enzyme production has received much attention in industrial biotechnology processes with the objective of the substitution of chemical processes, with potentially adverse effects on humans and environment. Protease enzyme has been utilized widely in industries to engender a wide range of products such as, detergent, leather, waste management, brewing, meat softening, milk-clotting, food, pharmaceutical, cancer treatment, diagnostics, digestion, viral disorders and silver recovery. Therefore the present study was aimed to examine enzymatic and antimicrobial properties of crude enzyme from papaya (*Carica papaya* L.) Leaf, seed and fruit pulp extracts.

Crude enzyme has been utilized widely in industries to engender a wide range of products such as, detergent, leather, waste management, brewing, meat softening, milk-clotting, food, pharmaceutical, cancer treatment, diagnostics, digestion, viral disorders and silver recovery. Crude enzyme belongs to a class of enzymes that can be classified based on their physical and biological property. Apart from this, the plant crude enzyme has many biological roles, such as anti-cancer activity; avert edema, avails in the digestive process, procoagulant activity and many more.

The study was conducted to investigate enzymatic and antimicrobial activities of crude enzyme from papaya (*Carica papaya L.*) leaf, seed and fruit pulp. The fresh papaya samples of ripen fruits and leaves were collected from Home garden in Bihar Dar city, Ethiopia. Fresh leaf, seed and pulp samples (60g) were grounded in 100ml acetone to remove fat content, using mortar and pestle. For enzyme extraction was conducted 10 mM Tris-Hydrochloride (Tris-HCl) buffer at pH 8.0 and 2MNaCl for three hours on an orbital shaker. The protein content was determined using Lowry assay using bovine serum albumin (BSA) as a standard protein. Then protease activity was determined. The optimum activity of the protease enzyme was done using PH and temperature. The antimicrobial activity of the enzyme extract was arranged as 3x4 completely randomized (CRD) factorial design in three replications. The antimicrobial activity was determined using disc diffusion and broth dilution methods.

The result indicated that enzymatic extracts of papaya (*Carica papaya* L.) leaf, seed and fruit pulp presented significantly the highest protein content for leaf sample (11.67mg/ml), followed by seed extract (10.69mg/ml) and the least protein content was recorded for fruit pulp (8.73mg/ml). Significantly the highest protease activity was recorded for fruit pulp extract (42.28 U/ml) followed by leaf sample (25.33 U/ml) and the least protease activity (18.05 U/ml) were observed for papaya seed extract. Likewise, significantly the highest protease specific activity (4.85U/mg) was recorded for fruit pulp followed by leaf extract (2.17 U/mg) and the least protease specific activity (1.69 U/mg) was recorded for seed extract.

The optimum PH for protease activity was 7.5. The effect of incubation temperature on protease activity demonstrated that the maximum protease activity was observed at 35^0C.

The strongest antibacterial activity with maximum zone of inhibition (17.50mm) at highest concentration (200mg/ml) of the enzyme extract was recorded for leaf enzyme extract against *S. aureus* while the weakest antibacterial activity (14.00mm) was recorded for seed enzyme extract against *E. coli* indicating that *S. aureus* was more susceptible than *E. coli.* On the other hand, the strongest antifungal activity with maximum zone of inhibition (18.50mm) was recorded for leaf extract against *A. niger,* but the weakest antifungal activity with minimum zone of inhibition (12.73mm) was recorded for seed enzyme extract against *C. albicans* indicating that *A. niger* was more susceptible to the enzyme extract while *C. albicans* was more resistant to the enzyme extract. It can be concluded that papaya enzyme extract has wide range of antimicrobial activities with the leaf extract with greatest antimicrobial potential while seed enzyme extract presented the least antimicrobial potential.

The *C. papaya* leaf enzyme extract has exhibited strongest bactericidal activity with MIC (2.34mg/ml) and the corresponding MBC (4.69 mg/ml) for leaf enzyme extract against *S. aureus* while the weakest bactericidal activity with MIC (18.75mgl/ml, the largest value) and MBC (37.50mg/ml) was recorded for seed enzyme extract against *E. coli* indicating that *S.aureus* was more susceptible than *E. coli.*

Antifungal activity, the papaya leaf extract has presented strongest antifungal activity with MIC (4.69 mg/ml, the least value) and MFC (7.81 mg/ml) against *A. niger* while the weakest antifungal activity with MIC (37.50mg/ml) and MFC (75 mg/ml) was recorded for seed enzyme extract against *C.albicans* showing that *A. niger* was more susceptible to the enzyme extract than *C. albicans.*

5.2. Conclusion

The diameter of zone of inhibition measured based on disc diffusion, for papaya (*Carica papaya* L.) leaf, seed and fruit pulp enzymatic extracts demonstrated significant antimicrobial activities of the enzyme extracts against tested bacteria and fungi. The mean zone of inhibition at highest concentration (200mg/ml) against bacterial test pathogens ranged from 14.00 to 17.50mm, while 12.73 to 18.50mm against fungal test pathogens. It is well-known that proteins are used for various purposes in industrial, biological and medical applications. Protease is a commercial enzyme with wide range of applications. The crude enzyme was successfully extracted leaves, seed and fruit pulp of papaya. The determination of pH plays a vital role in enzyme production for large scale of industrial interest. The successful identification of crude enzyme from papaya fruit and leaves will be beneficial to mankind. As humans consume papaya as food, the effect of protease in human metabolism may play a key role. Studying metabolic role of crude enzyme will help in better understanding of proteases in human health and diseases.

5.3. Recommendation

The present study had assessed crude enzyme activity from papaya leaf, seed and fruit pulp. Crude enzyme was characterized with respect to its activity, optimum pH, temperature, and antimicrobial activities. Further studies are required to

- Purify crude enzyme from papaya leaf, seed and fruit pulp extracts.
- Optimize crude enzyme activities with respect to various parameters;
- using different extraction methods so as to get the most efficient plant enzyme;
- qualitative and quantitative study of crude enzyme at different stages of fruit ripening, and plant growth stages;
- the antimicrobial experiment need to be conducted for various types of gram stain bacteria, and also common pathogenic fungi;

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7. APPENDICES

Table 1. Data for the effect of pH on crude enzyme extract activity

Enzyme extract	Rep	Protease activity (U/ml) at PH						
		5	6	6.5	7	7.5	8	9
Seed	1	470	545	590	600	610	580	520
Seed	2	465	540	600	595	620	590	530
Pulp	1	500	585	610	630	670	620	575
Pulp	2	495	580	620	620	680	630	570
Leaf	1	490	560	600	615	645	605	540
Leaf	2	480	570	610	610	650	610	550

Table 2. Data for the effect of temperature on crude enzyme extract activity

Enzyme extract	rep	Temperature ^{0}C				
		20	25	30	35	40
Seed	1	350	370	410	520	430
Seed	2	360	374	420	512	435
Pulp	1	385	390	500	576	470
Pulp	2	380	395	490	580	475
Leaf	1	375	385	440	560	460
Leaf	2	370	380	420	550	450

Table 3. Data for antibacterial activity of the enzyme extract

Test pathogens	Enzyme extract	Rep	Concentrations of enzyme extract			Gentamycin (100mg/ml)
			100mg/ml	150mg/ml	200mg/ml	
E. coli	Seed	1	10	13	14	18
E. coli	Seed	2	11	13.5	14.5	18.5
E. coli	Seed	3	10.6	12	13.5	18
E. coli	Pulp	1	13	15	15	19
E. coli	Pulp	2	12	15.5	16.5	18.4
E. coli	Pulp	3	11.5	15	15.25	19
E. coli	Leaf	1	12.1	14	16	18.5
E. coli	Leaf	2	12.5	14.5	16.5	18.5
E. coli	Leaf	3	11	13.1	16.2	19.5
S. aureus	Seed	1	13.1	13.5	16	18
S. aureus	Seed	2	12.4	14.5	15.5	18.5
S. aureus	Seed	3	13.5	14	15.1	19
S. aureus	Pulp	1	13.5	15	16	18.5
S. aureus	Pulp	2	12	14.5	16.5	18.4
S. aureus	Pulp	3	13	14	17.4	18.5
S. aureus	Leaf	1	13	15	17	18
S. aureus	Leaf	2	13.5	14.5	17.5	19
S. aureus	Leaf	3	14	15.25	18	19.5

Table 4. Data for antifungal activity of the enzyme extract

Test pathogens	Enzyme extract	Rep	Concentrations of enzyme extract			Fulconazole (100mg/ml)
			100mg/ml	150mg/ml	200mg/ml	
A. niger	seed	1	9	13	14.6	17.5
A. niger	seed	2	10	13.5	15.5	18.5
A. niger	seed	3	9.2	12.8	15	18
A. niger	pulp	1	9	13	16	18
A. niger	pulp	2	8	13.5	15.5	17.5
A. niger	pulp	3	9.5	12.5	15	19
A. niger	leaf	1	10	13	18	18
A. niger	leaf	2	10.5	14	19	18.5
A. niger	leaf	3	11	13.5	18.5	17.5
C. albicans	seed	1	0	11	12	18
C. albicans	seed	2	0	10	13	18.5
C. albicans	seed	3	0	10.7	13.2	18
C. albicans	pulp	1	10	15	16	18
C. albicans	pulp	2	9.5	16	16.5	17.5
C. albicans	pulp	3	11	15.3	17	18.5
C. albicans	leaf	1	0	14	18	19
C. albicans	leaf	2	0	14.3	17.5	17.5
C. albicans	leaf	3	0	15	17.2	19

Figure 1. Sample preparations

Figure 2. Separations of seed and pulp

Figure 3. Measuring samples

Figure 4. Antimicrobial test against test

Figure 5. Observations for growth of test pathogens