DETERMINATION OF LECTIN CONTENT IN MESOCARP OF RIPE AND UNRIPE PAWPAW (*Carica papaya* L.)

BY

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CERTIFICATION

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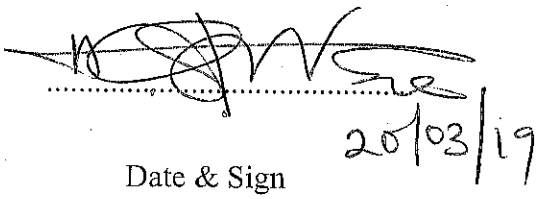

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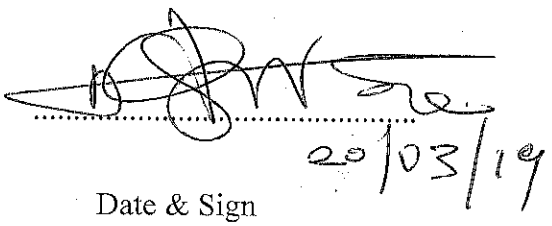

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DEDICATION

This report is dedicated to the Almighty God who made it possible for me to complete this research work.

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I appreciate the Almighty God who has preserved my life throughout this entire Bachelors' programme. To Him be glory, honour and adoration for seeing me through the completion of this project work. I am grateful to my supervisor and Head of the Department of Plant Science and Biotechnology, Dr. A.A Ajiboye for his encouragement, constant support and guidance which was of immense help during this project work.

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ABSTRACT

Lectins are glycoproteins which possess at least one non-catalyzing domain that specifically and reversibly binds to mono and oligosaccharides. Concentration of Lectin in the ripe and unripe *Carica papaya* were determined using the Glycoprotein Agglutinating property with carbohydrate coated surfaces (Erythrocytes) that can easily bind with lectin present in the ripe and unripe pawpaw. The research of the study was carried out to evaluate the quantities of lectin present in the mesocarp of ripe and unripe pawpaw fruit (*Carica papaya*).The fruits used in this study were gotten from the New market at Obafemi Awolowo University Ife, Osun State Nigeria. The fruits were identified at the Herbarium section of Botany, Obafemi Awolowo University Ife, Osun State. The study revealed that the unripe *Carica papaya* had a low Lectin content in the mesocarp. Comparatively, the ripe *Carica papaya* had high Lectin content in the mesocarp. Lowry Method of protein extraction and determination were used. The data assay showed that the unripe *Carica papaya* had high protein contents in the mesocarp (11.1 mg/ml). However, the ripe *Carica papaya* had low protein contents in the mesocarp (7.11 mg/ml). Furthermore, some tests were carried out on the Hemagglutinating Activity of Lectin and they are; Sugar Specificity Test which showed that glucose and sorbose sugar did not inhibit hemagglutination on the ripe and unripe mesocarp but Lactose, Maltose and Galactose had little or complete inhibition on ripe and unripe mesocarp. Effects of temperature, which showed that Lectin is heat stable from 30^0C to 50^0C in the ripe mesocarp but heat stable from 40^0C to 50^{0C} in the unripe. Effects of pH, which showed that Lectin was heat stable from pH 2-8 both in the ripe and unripe mesocarp and Denaturing agents such as Urea, Beta mercaptoethanol and guanidine-Hcl were used and the research showed that Urea and Guanidine-HCL denatures Lectin in the ripe mesocarp but Urea and Beta mercaptoethanol denatures Lectin in the unripe mesocarp. Further studies should be carried out on the genetic

compatibility of the ripe pawpaw in order to explore a viable product that would have optimum level of Lectin but high level of Protein contents.

CHAPTER ONE

1.1 INTRODUCTION

Pawpaw is a fruit with scientific name *Carica papaya* which belongs to the genus *Carica* and family Caricaceae. Papaya is one of native plants of Central America; however, it has been planted widely in most of tropical and subtropical countries.

Generally, the name pawpaw varies in different countries, for instance, papaya in Malaysia and Thailand, papaw / pawpaw in Australia; in Europe, papaya is also named "tree melon" etc. (Morton; 2006; Papaya, 2008).

In recent years, attention was given to papaya due to the nutritional and medical use, and most studies are concentrated on, roughly: papaya fruit and papaya latex. Papaya fruit is a good source of carbohydrate, as well as vitamins (C and A) and minerals (copper and magnesium) (Wall, 2006). Pawpaw, is a fruit that grows in tropical areas throughout the world. Not just the fruit itself, but other parts of the pawpaw are also beneficial. This fruit has a lot of hidden benefits for skin, health and hair. The pawpaw has the most health benefits of any fruit, from cardiovascular to colon health. Research has shown that the health benefits of the pawpaw may be even greater than its delicious taste. In fact, pawpaw and pawpaw-derived products are increasingly used for a variety of medicinal and therapeutic purposes and it is available all year round.

Lectin, a word of Latin origin, is a substance which is not triggered by antigenic stimulations in the immune system and which combines with an antigen in a way resembling that of an antibody. Lectins are multivalent proteins or glycoproteins of non-immune origin that reversibly and non-enzymatically bind carbohydrates with high specificity for the chemical structure of the glycan array without changing their structure (Sharon & Lis 2004). Peumans and

Van Damme (1995) extended the definition of lectins to include any protein that has non-catalytically carbohydrate binding domain (De Hoff *et al.*, 2009).

This study therefore aimed at making available information on the comparative assay of the presence of a protein precisely lectin in ripe and unripe pawpaw fruit and to ascribe possible roles for the proteins in the varieties they are present.

1.1 CHARACTERISTICS OF Pawpaw (*Carica papaya*).

STEM

Pawpaw is a fast-growing herbaceous like plant 7-20 meters in height. Papaya normally has a monaxial stem without branching but it has multi-stems when damaged. When the stem is wounded, white milky latex oozes from the wound. Although papaya can be up to 9 meters height, it is easily damaged and makes harvesting of fruit difficult (Nakasone & Paul, 1998).

LEAVES

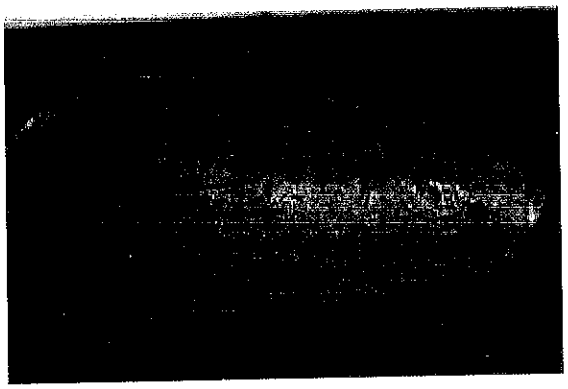
The cluster of leaves at the apex and along the upper of the stem makes up the foliage on tree. New leaves emerge from the apex and old leaves senescence and fall. Leaves are lobed with prominent venation; the blade is deeply divided into 7-11 segments and can measure 40-50 cm in diameter with 15 mature leaves per plant. The leaves contain white milk latex (Nakasone & Paul, 1998).

FLOWER

Papaya flowers are born in inflorescence which appear in the axils of leaves. It can be of female, male or hermaphrodite flowers. Female flowers are held close against the stem as single flowers or in cluster of 2-3 flowers. Male flowers are smaller and more numerous. Hermaphrodite (perfect) flowers are intermediate between the female and male (Nakasone & Paul, 1998).

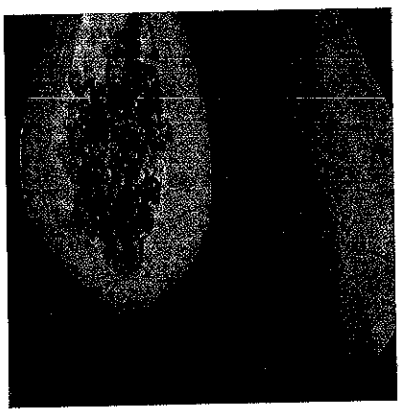
FRUITS

The fruit superficially resembles a melon pyriform, oval and elongated in shape. The fruits range in size from 7-30 cm. The ovary is normally composed of 5 carpels. Fruits from female trees are spherical whereas the shape of fruits from hermaphrodite trees is affected by environmental factors that modify floral morphology during early development of the inflorescence. Green fruits contain an abundance of milky latex. Ripe fruits have yellow-orange colored skin. Mature fruits contain numerous grey-black spherical seeds 5 mm in diameter (Nakasone & Paul, 1998).



A: Ripe Matured Pawpaw

B: Mesocarp and seed of ripe pawpaw



C: Mesocarp and seed of unripe Pawpaw

D: Unripe Pawpaw

Plate 1: Ripe and unripe fruits of *Carica papaya*

1.2 STATEMENT OF PROBLEM

Researchers have shown that Lectin has toxic effects on humans because of their ability to bind to sugar and other carbohydrate molecules, and because lectins are resistant to human digestion, it's believed that they interfere with the proper absorption of vitamins, minerals and some key proteins. The concern is that when we eat foods that are high in lectins, we are not getting the full benefit of the nutrients we eat and also cause inflammatory and digestive diseases in the body (Hamid *et al.*, 2013).

Research has shown that high amount of Lectin has a side effect on the human body, therefore it becomes important to know the one with high Lectin content between the ripe or unripe pawpaw.

1.4 OBJECTIVES OF THIS RESEARCH

This research was carried out to investigate the following:

Main Objective

- To determine the type of pawpaw with the highest Lectin level.

And Specific Objectives

- To assess and compare the protein concentration levels in the mesocarp of ripe and unripe pawpaw.
- To determine the level of Lectin content in the mesocarp of the ripe and unripe pawpaw.
- To compare the level of Lectin content in the mesocarp of the ripe and unripe pawpaw.

CHAPTER TWO

2.0 LITERATURE REVIEW

Carica papaya, Linn. "*Papaya*" is the most widely cultivated and best known species of *Carica*. It is cultivated nearly all over the tropics and subtropics for its luscious fruits and source of commercial papain. An alkaloid carpaine from *papaya* has been utilized as a diuretic and a heart stimulant (Singh *et al* 1983). *Papaya* is a fast growing, short-lived, single stemmed small tree, 7-10m in height with a straight, cylindrical, soft hollow grey trunk roughened by the presence of large leaf and inflorescence scars. Consumption of Papaya fruit is of high nutritive value to Human nutrition. The fruit crop has low calories of (32 kcal/100 g of ripe fruit) and rich in vitamins and minerals. Papaya is the leading fruit crops in Vitamin C, Vitamin A, Riboflavin, Folate, Calcium, Thiamine, Iron, Niacin, Potassium and Fiber. Papaya ability to supply the body with Vitamin A and C makes it a good fruit for improvement of eyesight.

2.1 NUTRIENT VALUE OF PAWPAW

TABLE 1: Nutrient composition of Pawpaw

NUTRIENT	COMPOSITION PER GRAMS
ENERGY	59.0 kcal
MOISTURE	84.4%
PROTEIN	1.0 g
FAT	0.1 g
CARBOHYDRATE	13.5 g
FIBRE	0.5 g
ASH	0.5 g
CALCIUM	31.0 mg
MAGNESIUM	0.8 mg
PHOSPHORUS	17.0 mg
IRON	1.0 mg
SODIUM	2.0 mg
POTASSIUM	337.0 mg
VITAMIN B1	0.08 mg
VITAMIN B2	0.15 mg
NIACIN	0.1 mg
ASCORBIC ACID(VITAMIN C)	69.3 mg
CAROTENE	2431.0 µg

(Source: Agric- Food Business Development Center)

TABLE 2: CHEMICAL COMPOSITION OF VARIOUS PARTS OF PAWPAW

PARTS	CONSTITUENT
FRUITS	protein, fat, fibre, carbohydrates, minerals, calcium, phosphorus, iron, vitamin C, thiamine, riboflavin, niacin, and caroxene, amino acid, citric acids and molic acid (green fruits), volatile compounds : linalol, benzylisothiocynate, cis and trans 2, 6-dimethyl-3,6 expoxy-7 octen-2-ol. Alkaloid, α; carpaine, benzyl-β-d glucoside, 2-phenylethl-β-D-glucoside, 4-hydroxyl -phenyl-2 ethyl-B-D glucoside and four isomeric malonated benzyl-β-D glucosides
JUICE	N-butyric, n-hexanoic and n-octanoic acids, lipids; myristic, palmitic, stearic, linoleic, linolenic acids-vaccenic acid and oleic acids
SEED	Fatty acids, crude proteins, crude fibre, papaya oil, carpaine, benzylisothiocynate, benzylglucosinolate, glucotropacolin, benzylthiourea, hentriacontane, β-sistosterol, caricin and an enzyme nyrosin
ROOTS	Arposide and an enzyme nyrosin
LEAVES	Alkaloids carpain, pseudocarpain and dehydrocarpaine I and II, choline, carposide, vitamin C and E
BARK	β-sitosterol, glucose, fructose, sucrose, galactose and xylitol
LATEX	proteolytic enzymes, papain and chemopapain, glutamine cyclotransferase, chymopapain A, B and C, peptidase A and B and lysozymes

(Source: Jean Bruneton. *Carica papaya*, In: Pharmacognosy, phytochemistry of medicinal plants, Tech Docu Fra 1999)

2.2 Importance of Pawpaw

Papaya is mainly cultivated for its edible fruits as a fresh fruit and for use in drinks, jams, candies and dried fruit. Ripe fruits are usually eaten fresh and green fruits are also used as a cooked vegetable. Papaya also has several industrial uses. Biochemically, its leaves and fruits produce several proteins and alkaloids with important medical and industrial application. The latex of green fruits contain a proteolytic enzyme, papain, used in the beverage, food and pharmaceutical industries for production of chewing gum, chill-proofing beer, tenderising meat, treat digestive disorders, degum natural silk, extracted fish oil. It is also used in the cosmetic industry, in soap, shampoo and face lifting preparations (Nuñez, 1982). Evolutionary, papain may be associated with protection from frugivorous predators and herbivores (Australian Government, Department of Health and Ageing, 2003).

2.2.1 Medicinal uses of Pawpaw

Over the years papaya fruits have always played a key role in biological systems, providing basic nutrients required for a healthy living. Many biologically active phytochemicals have been identified in Papaya. Seed of Papaya has been found to have the ability to act as an antimicrobial agent. A report by Calzada *et al.* (2007), has also shown to have bacteriostatic ability against enteropathogens such as *Bacillus subtilis, Escherichia coli* including *Salmonella typhi,* this view was confirmed by Osato *et al.* (1993)

Consumption of Papaya seeds has been reported by Okeniyi *et al.* (2007) to offer cheap, natural, harmless, readily available monotherapy and prevention strategy against intestinal parasitoids.

Papaya fruit exhibit antimalarial activity. This can be observed in petroleum ether extract of the rind of Papaya fruit. Several contribution of Papaya to human development has been recognized.

Papaya has been known to have antifungal properties, as observable in the latex of Papaya and Fluconazole, having synergistic action on the inhibition of *Candida albicans* growth (Giordani *et al.* 1997).

2.2.2 BOTANICAL CLASSIFICATION OF PAWPAW

Kingdom.....Plantae

Subkingdom.....Tracheobionta

Superdivision.....Spermatophyta

Division..... Magnoliophyta

Class.....Magnoliopsida

Subclass.....Dilleniidae

Order.....Violales

Family.....Caricaceae

Genus.....*Carica* Linn.

Specie.....*Carica papaya* Linn.

2.2.3 CLIMATIC AND SOIL REQUIREMENTS OF PAWPAW

Carica Papaya is a tropical crop, but it does well in a mild tropical climate. Papaya should not be grown in areas with strong, hot or dry winds. Dry climate at the time of ripening adds to the sweetness of fruits. Ideal temperature for growing of papaya is between 25-30^{0}C while temperatures below 10^{0}C delays ripening. High rainfall areas are not suitable for growing papaya due to the death of the plants by collar- rot disease.

Papaya grows well in well-drained rich sandy loam soils with a depth of 45cm or medium black soils free from water logging. Light soils with pH between 6.5 -7 are very good for papaya, if they are adequately manured.

2.2.4 METHODS OF PLANTING AND CARE OF PAWPAW

Growing papaya trees is generally done from seed that is extracted from ripe fruit. You should plant several seeds per pot to ensure germination. Under full sunlight, seedlings may emerge in about two weeks. Plants can be set out after they are a cm tall and spaced 8 to 10 cm apart. The seedlings will flower after five or six months. When considering the best papaya growing conditions in the home landscape, don't forget about planting location. The best place to plant a papaya is on the south or southeast side of a house with some protection from wind and cold weather. Papayas also grow best in full sun. Papayas like well-drained soil, and because of shallow roots, growing papaya trees will not tolerate wet conditions. In addition to proper papaya growing conditions, suitable care of papaya fruit trees is also important. In order for papaya trees to thrive, they require some fertilizer. Provide young plants fertilizer every 14 days using ¼ bag of complete fertilizer. Fertilize older trees with 1 to 2 pounds of fertilizer once a month. Also, be sure to take a soil sample and amend as necessary. Water trees frequently for best fruit production. Mulch trees with 4 inches of wood chips to help retain moisture, taking care to keep the mulch 8 to 12 inches from the trunk. Protect developing fruit from pests by placing a paper bag over them until they are ripe.

2.2.5 PEST AND DISEASES OF PAWPAW

TABLE 3: Pests affecting pawpaw and their control measures.

S/N	PEST	CONTROL MEASURES
1	Papaya mealybug	Mealybugs can potentially be controlled by natural enemies such as lady beetles but are commonly controlled using chemicals; chemical pesticides may also decrease populations of natural enemies leading to mealybug outbreaks.
2	Scale insects	Populations are often kept in check by natural enemies, including predacious beetles and some wasps - although broad-spectrum insecticides may result in outbreaks of scale by killing off populations of beneficial insects; trees can be sprayed with horticultural oils when dormant which effectively kill scales without damaging natural enemies.
3	Papaya fruit fly	Harvesting done before fruit turn yellow spray with dimethoate 0.1% or acephate

(Source: www.plantvillage.edu.com)

TABLE 4: Some diseases affecting pawpaw symptoms, control and method of application.

Diseases	Symptoms	Control
Black rot	Black sunken rot on young fruits originating from stem end or contact with a leaf; young fruit withering and dropping from plant; small, brown sunken lesions with light brown margins on ripening fruit.	Appropriate protective fungicides should be applied; dipping fruits in hot water at 48°C for 20 minutes reduces the incidence of the disease.
Black spot	Circular water-soaked or brown lesions on older leaves; centers of lesions become bleached as they mature; leaves curling and turning brown; raised lesions on trunks; sunken circular lesions on fruit.	Disease may require applications of appropriate fungicides for adequate control.
Cercospora black spot	Tiny black dots on fruit which enlarge to 3 mm across; spots are slightly raised and although indistinct on unripe green fruit, become visible on ripening to yellow; lesions on leaves are irregular in shape and gray-white in color; if infestation is severe, leaves	Applications of appropriate protective fungicides at intervals of 14 to 28 days provide satisfactory control of the disease.

	may turn yellow and necrotic and drop from plant.	
Powdery mildew	Infect all parts of tree. The infected leaves show white mycelial growth commonly on under surface, particularly near leaf veins. Sometime white mycelial growth can also seen on upper leaf surface. The infected area becomes light green and chlorotic (lesions) with dark green margin.	Remove the infected parts and dispose them properly. Avoid irrigating the trees by sprinkler. Provide proper nutrition to trees to withstand powdery mildew infection. If the disease is severe, apply suitable fungicides.

(Source: www.plantvillage.edu.com)

2.2.6 HARVESTING AND STORAGE OF PAWPAW

Maturity

Papayas are harvested when the first hint of yellow coloration appears.

Harvest Method

Fruits are hand harvested carefully to avoid scratching the skin, which would release latex and stain the skin.

Postharvest Handling

To reduce post-harvest fruit rot, papayas are commonly heat treated postharvest (110-120°F), then rinsed in cool water. Fungicides also may be used, generally in the wax applied during

packing. Radiation treatments such as "Sure Beam" are used to sterilize fruit fly eggs and larva in fruit intended for export. Fruit are packed into single-layer boxes (10-15 lbs.), often with tissue or foam padding to avoid bruising. Fruits can be cured at 85^0F and 100% humidity for better color expression prior to shipping.

Storage

Below 50°F, pawpaw experience chilling injury. C. *Papaya* are extremely perishable; shelf life at room temperature ranges from 3 to 8 days, depending on storage conditions.

2.3 LECTINS

Lectins are carbohydrate-binding proteins, macromolecules that are highly specific for sugar moieties. Lectins perform recognition on the cellular, molecular level and play numerous roles in biological recognition involving cells, carbohydrates, and proteins. Lectins also mediate attachment and binding of bacteria and viruses to their intended targets (Rutishauser and Leo Sachs, 1975). Lectins are ubiquitous in nature and are found in many foods. Some foods such as beans and grains need to be cooked or fermented to reduce lectin content, but the lectins consumed in a typical balanced diet are not harmful. Some lectins are beneficial, such as CLEC11A which promotes bone growth, while others may be powerful toxins such as ricin (Chan, Charles *et al.*, 2016). Lectins may be disabled by specific mono- and oligosaccharides, which bind to ingested lectins from grains, legume, nightshade plants and dairy; binding can prevent their attachment to the carbohydrates within the cell membrane.

Lectins have ubiquitous expressions in almost all living species involving plant kingdoms and animals. In plants their presence have been detected in bark, leaves, roots and seeds. Lectins have also been isolated from bacteria, fungi, snails and eels. Due to their ability to bind sugars, they can

precipitate various glycoconjugates, identify cell surface sugars and separate glycoproteins. (Awoyinka O.A *et al.*, 2013).

2.3.1 HISTORY OF LECTINS

In 1888 Peter Hermann Stillmark presented the earliest description of hemagglutinations. Stillmark isolated a protein from the seeds of castor tree (*Ricinus communis*) and he named it ricin which was highly toxic. Boyd and Reguera, (1949) and Renkonen, (1948) investigated saline extracts of hundreds of plants for hemagglutination activity, they demonstrated that some plant hemagglutinins are blood type specific. On this basis Boyd gave the term lectins to describe those blood type specific plant hemagglutinins (Sharon 2008).

2.3.2 ROLES OF LECTIN IN PLANTS

In spite of being the most thoroughly studied, the functions of the plant lectins remain enigmatic. Proposed functions for plant lectins include a storage or transport role for carbohydrates in seed, binding of nitrogen fixing bacteria to root hairs and inhibition of fungal growth or insect feeding. All cells are coated with sugars and many also express surface lectins (Sharon, 1988). Cell surface sugars and lectins on cell surfaces are believed to function as recognition determinants between cells, either homotypic or heterotypic. For instance, the interaction between lectins on bacterial surfaces and sugars on eukaryotic cells play a crucial role in the infection process. Lectins play a major role in nitrogen fixation in leguminous and non-leguminous plants. It has been suggested that root lectin recognized by bacterial receptor molecules is an important determinant of host plant specificity in Rhizobium legume symbiosis (Bohlool and Schmidt, 1978).

It can also be a tool in cancer research due to its ability to differentiate malignant (or transformed) cells from the normal ones (Smetana *et al.*, 2006). Lately, lectins have been used as Biotechnological tools in different studies (Kabir, 1998; Amadeo *et al.*, 2003; Texeira *et al.*, 2004; Ganiko *et al.*, 2005).

2.3.3 PLANT LECTINS AS PLANT DEFENSE PROTEIN

Plant lectins affect various biological parameters of insects including larval weight decrease, mortality, feeding inhibition, delay in total developmental duration, adult emergence and fecundity on the first and second generation (Powell *et al.*, 1993; *Habibi et al.*, 1993). Plant lectins can either directly or indirectly cause profound morphological and physiological modifications in the insect intestine. In insects, lectins bind to the midgut epithelium causing disruption of the epithelial cells, elongation of the striated border microvilli, swelling of the epithelial cells into the lumen of the gut lead to complete closure of the lumen and impaired nutrient assimilation by cells, allowing absorption of potentially harmful substances from intestine into circulatory system, fat bodies, ovarioles and throughout the haemolymph (Habibi *et al.*, 2000; Fitches *et al.*, 2001b). Beside their insecticidal properties, lectins have also been found to be effective against transmission of plant viruses. Certain plant viruses are transmitted by insects of the Hemiptera order (sap-sucking insects) including aphids, whiteflies, leafhoppers, planthoppers, and thrips. The mode of transmission can either by moving from the alimentary canal of the vector insect onto its hemocoel and through the salivary secretory system into the plant host during insect feeding or by associating with the cuticular lining of the insect mouthparts or foregut and directly releasing as digestive secretions onto the plant when insect begins to feed (Gray *et al.*, 2003; Hogenhout *et al.*, 2008). Lectins are able to recognize and bind to the viral glycoproteins thereby decreasing the binding of

virus with the receptor and subsequently avoid the transport of virus from gut to hemocoel of insect vector (Desoignies, 2008), finally suspending the virus transmission. The presence of lectins at relatively high concentrations in legume seeds has been associated with a possible role in plant defense. With the exception of some enzymes e.g. some type of chitinases, glucanases and glycosidases, lectins are the only plant proteins that are capable of recognizing and binding glycoconjugates present on the surface of microorganisms or exposed along the intestinal tract of insect or mammalian herbivores (Peumans and Van Damme, 1995). Molecular, biochemical, cellular, physiological and evolutionary arguments indicate that lectins have a role in plant defense. Most lectins are stable over a wide pH range, are able to withstand heat and are resistant to animal and plant proteases. Thus, they strongly resemble other defense related proteins such as some pathogenesis related proteins, protease inhibitors, chitinases, glucanases, RIPs, α-amylase inhibitors, antifungal proteins and thionins. Various plant lectins have shown insecticidal effects when fed to insects from coleoptera, homoptera, and lepidoptera (Balzanini *et al.,* 1992). Binding of plant lectins to bacterial cell wall peptidoglycans indicate that lectins strongly interact with muramic acid, N-acetyl muramic acid and muramyl dipeptide and play direct role in plants defense against bacteria. The definitive proof for antifungal activity of plant lectins was demonstrated by a purified lectin from stinging nettle (*Urtica dioica*) which inhibited the growth of *Botrytis cinerea, Trichoderma hamatum and Phycomyces blakeslecanus* (Broekaert *et al.,* 1992). Similarly, Hevein, a lectin from latex of rubber tree (Van Parijs *et al.,* 1991) and a lectin from seeds of *Amaranthus caudatus* (Broekaert *et al.*, 1992) have antifungal activities.

CHAPTER THREE

3.1 MATERIALS AND METHODS:

3.1.1 Collection of seed samples:

The samples used were obtained from New market at Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

3.1.2 Preparation of Crude Extract

The seeds and peeled mesocarp were homogenized in 500 ml of 0.15 M phosphate buffered saline (PBS), pH 7.2. The homogenate was kept overnight at 4°C and then centrifuged at 3500 rpm for 20 min. The crude extract was collected as clear supernatant.

3.2 Glutaraldehyde Fixation of Erythrocytes

The human red blood cells (A, B, O) were fixed with glutaraldehyde according to the method of Bing *et al.* (1967). Blood samples were collected into heparinized bottles and centrifuged at 3000 rpm for 15 minutes at room temperature using bench centrifuge. The erythrocytes were collected and washed five times with Phosphate buffered saline (PBS 7.2). Glutaraldehyde (25%) was diluted to 1% (v/v) with PBS and chilled at 4°C. The chilled glutaraldehyde-PBS solution was used to dilute the red blood cells to 2% (v/v). The suspension of cells and glutaraldehyde was incubated for 1 hour at 4°C with occasional mixing. The fixed cells were collected by centrifugation at 3000 rpm for 5 minutes and washed five times with PBS. The cells were suspended in PBS, containing 0.02% (v/v) and stored at 4°C until required.

3.3 Determination of Protein

3.3.1 Determination of protein by Lowery methods.

The protein concentrations were determined according to the method of Lowry *et al.* (1951) using Bovine serum albumin as standard.

REAGENTS

Reagent A: 2% Na2CO3 in 0.1 N NaOH

Reagent B: 1% NaK Tartrate in H2O

Reagent C: 0.5% CuSO4.5 H2O in H2O

Reagent D: 50 ml of A, 1 ml of B.

Reagent E: 1 part Folin-Phenol [2 N]: 1 part water

BSA Standard - 1 mg/ ml

Procedure:

- 0.2 ml of BSA working standard was put in 7 test tubes and made up to 1ml using distilled water.
- The test tube with 1 ml distilled water served as blank.
- 2.5 ml of Reagent A was added and incubated for 10 minutes.
- After incubation 0.25 ml of reagent B was added and incubated for 30 minutes
- The absorbance at 660 nm was measured and the standard graph was plotted.
- The amount of protein present in the given sample from the standard graph was estimated.

TABLE 5: Protocol for Protein Estimation Using Lowry's Method

Tube Number	Std. BSA (ml)	Distilled Water (ml)	Solution C (ml)	Solution D (ml)
1	0	1.0	2.5	0.25
2	0.2	0.8	2.5	0.25
3	0.4	0.6	2.5	0.25
4	0.6	0.4	2.5	0.25
5	0.8	0.2	2.5	0.25
6	1.0	0	2.5	0.25
7	0	0	2.5	0.25

All samples were incubated for 30 minutes. Absorbance reading was taken at 660 nm. The protein concentration in the test samples was calculated.

3.4 DETERMINATION OF LECTIN

3.4.1 Assay of Hemagglutinating Activity

Agglutination of the red blood cells by the crude extract was carried out as described by Wang *et al.* (2000). 100µl of PBS was delivered sequentially into wells arranged in rows (each row contained 12 wells) on a U-shaped microtiter plate. 100µl of the crude extract was added into first well to obtain a two-fold dilution. A serial dilution was made by transferring 100µl of the diluted sample in a particular well into the next well containing 100µl of PBS. Aliquots (50µl) of the 2 % erythrocyte suspension were added to each well and the microtiter plates were left undisturbed for 1 hour. The titer value was taken as the reciprocal of the highest dilution of the extract causing visible hemagglutination. Specific activity is the number of hemagglutination units per mg protein expressed as hemagglutinating units (HU)/mg.

3.4.2 Sugar Specificity Test

The sugar specificity of the lectin was investigated by the ability of simple sugars to inhibit the agglutination of erythrocytes (Goldstein and Hayes, 1978). In this study, the sugars tested were: glucose, sorbose, lactose, galactose, and maltose. 0.2 M of each sugar in PBS was prepared. A serial dilution of the sample was made until the end point causing hemagglutination was obtained. 50µl of the sugar solution was added to each well (each row was assigned to each sugar) and allowed to react for 30 min at room temperature. The control well had no sugar added to it. 50µl erythrocyte suspension was then added and the mixture left for 1 h. The hemagglutinating titer that was obtained was compared with the control.

3.4.3 Effect of Temperature on Hemagglutinating Activity

The effect of temperature on hemagglutinating activity was carried out as described by (Sampaio *et al.*, 1998). Aliquots of the purified lectin sample were incubated in a water bath for 30 min at different temperatures (30 – 90°C). After 30 min, the samples were removed from water bath and rapidly cooled on ice and assayed for hemagglutinating activity. Results were expressed as a percentage of the hemagglutinating shown by a control kept at 20°C for 30 min.

3.4.4 Effect of pH on Hemagglutinating Activity

The effect of pH on haemagglutinating activity of the purified lectin was determined by incubating the lectin sample in the following buffers at different pH: pH 2–6, 0.2 M citrate buffer; pH 7-8, 0.2 M Tris-HCl buffer; and pH 9–11, 0.2 M glycine-NaOH buffer. Equal volumes of protein sample and buffer solution were mixed and incubated at room temperature for 30 min. The solution was neutralized and the hemagglutination assay was performed. The hemagglutination titre of the protein sample incubated in PBS, pH 7.2, served as the control. Percentage of hemagglutinating activity was calculated (Nakagawa *et al.*, 1996).

3.4.5 Effect of Detergents on Lectin-induced Haemagglutination

The effect of denaturing agents: urea, beta-mecaptoethanol and guanidine-HCl at a specific concentration in PBS was carried out on lectin activity by incubating 1 ml of each denaturant solution with an equal volume of lectin sample at 37°C for 1h. Lectin sample in PBS served to estimate as control which was considered to be 100% activity.

3.4.6 Precautions taken during the Protein and Lectin Analysis of pawpaw.

Samples were stored in the freezer before the extraction process. Phosphate buffer saline was added to homogenized samples to resist changes in pH of protein solution, else, there might be denaturing or precipitation of protein. Apparatus like mortar and pestle were refrigerated to allow reduced denaturing of extracts. Crude extracts were kept under refrigeration to stop proteolytic activity of proteases. Centrifuged samples were kept in a cold box to prevent denaturing by heat. Stopwatch was used in ensuring accurate timing. The cuvette of the UV spectrophotometer was rinsed in distilled water and cleaned with tissue paper before each sample was poured and analyzed.

CHAPTER FOUR

4.1 RESULTS

3.1.1 Protein concentration determination

Protein determination was evaluated, the mesocarp of unripe *Carica papaya* had high protein concentration than that of the mesocarp of ripe *Carica papaya.*

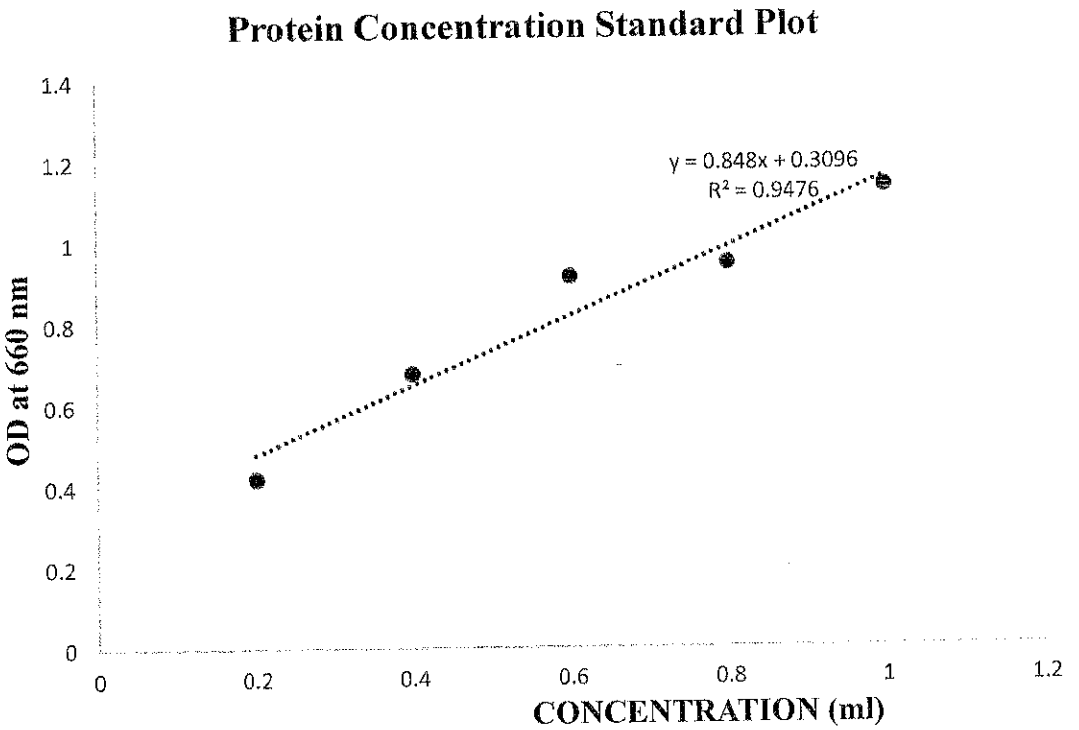


Fig 1: Protein concentration of the ripe and unripe mesocarp of Pawpaw

- O.D- Optical density

Note: Protein concentration standard plot is a plot where the protein content can be determined. The protein concentration was extrapolated from the plot using the equation from the plot.

4.1.2 DETERMINATION OF LECTIN CONTENT

HEMAGGLUTINATING OF THE SAMPLES

Both the mesocarp of the ripe and unripe pawpaw samples showed the ability to hemagglutinate human red blood cells (Erythrocytes). The ability of these samples to hemagglutinate was determined by its ability to suspend red blood cells in a buffer, done through their ability to bind to two red blood cells and to form a lectin erythrocyte –lectin matrix, leading to their clumping. When agglutinating occurs, cross linked red blood cells form a network that prevents the red blood cells from sedimenting to the bottom of the well. They appeared like a carpet that covers the whole microtiter plate well. When there was no agglutinating the red blood cells sedimented and formed a button on the bottom of the well.

TABLE 6: Hemagglutination affinity extracted from mesocarp of ripe and unripe pawpaw.

BLOOD SAMPLES	RIPE MESOCARP HEMAGGLUTINATING TITRE	UNRIPE MESOCARP HEAMAGGLUTINATING TITRE
BLOOD GROUP A	2^5	2^4
BLOOD GROUP B	2^5	2^5
BLOOD GROUP O	2^6	2^5

NOTE: In the ripe mesocarp, the blood group A and B had only 5 wells with agglutination while the blood group O had 6 wells with agglutination. In the unripe mesocarp, the blood group A had only 4 wells with agglutination while the blood group B and O had only 5 wells with agglutination.

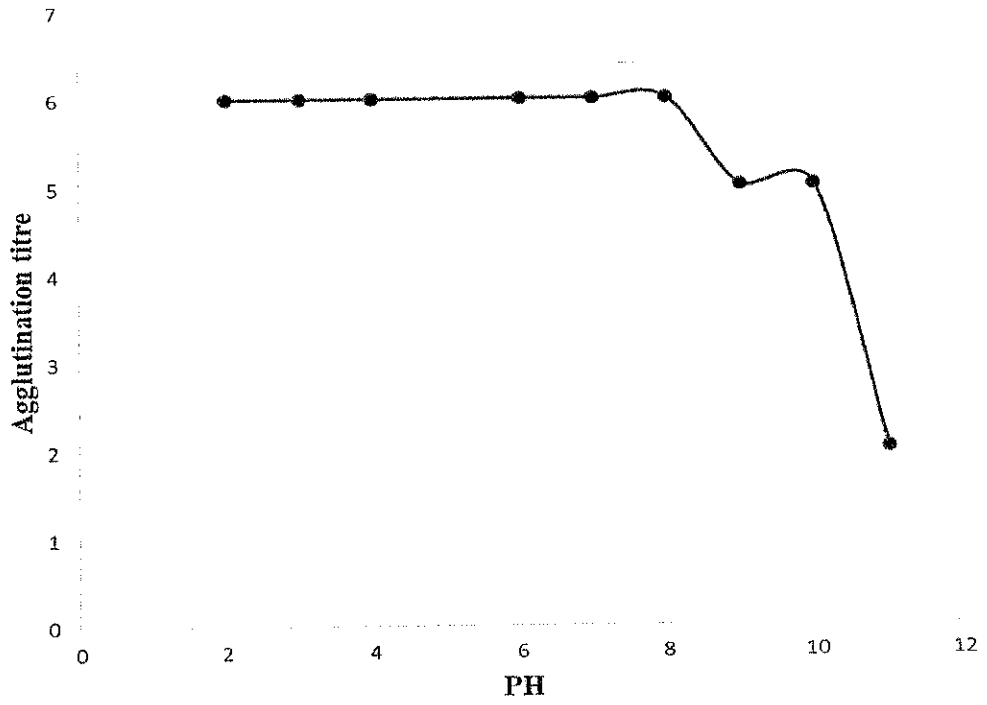


Fig 2: Effects of pH on the Lectin Activity on the ripe mesocarp of Pawpaw.

The optimum pH was determined using pH 2–6, 0.2 M citrate buffer; pH 7-8, 0.2 M Tris-HCl buffer; and pH 9–11, 0.2 M glycine-NaOH buffer. The optimum pH was 8 as shown in the pH activity curve.

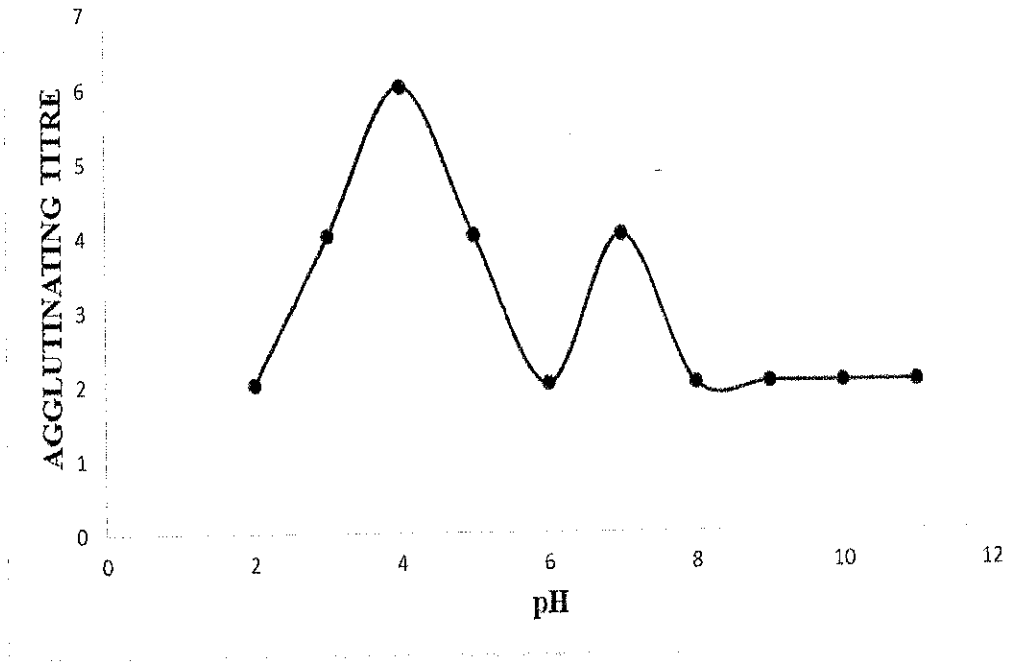


Fig 3: Effects of pH on the Lectin Activity on the unripe mesocarp of Pawpaw

The optimum pH was determined using pH 2–6, 0.2 M citrate buffer; pH 7-8, 0.2 M Tris-HCl buffer; and pH 9–11, 0.2 M glycine-NaOH buffer. The optimum pH was at 4 and 7 as shown in the pH activity curve.

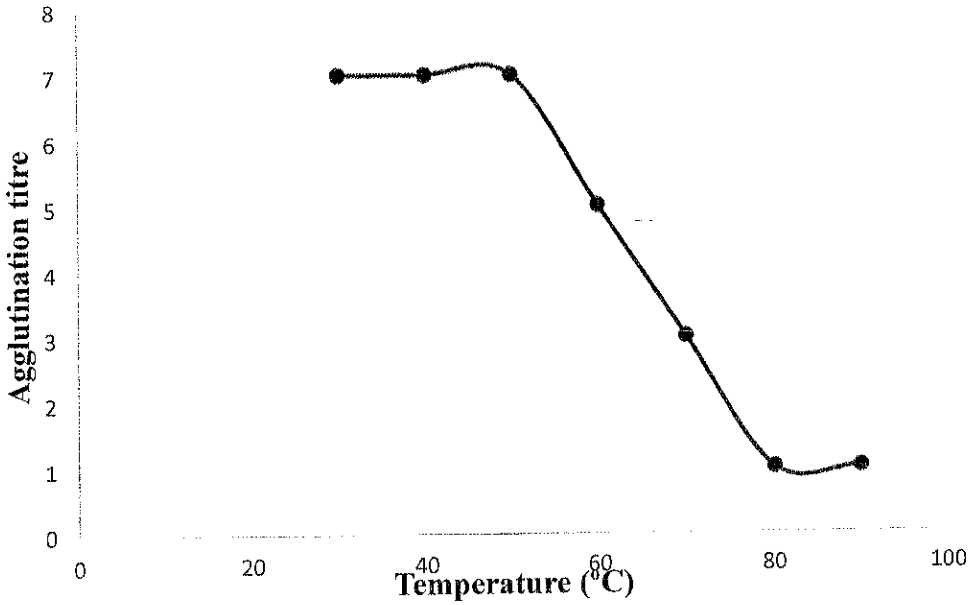


Fig 4: Effects of Temperature on the lectin Activity of Ripe mesocarp Pawpaw.

Lectin activity was at its optimum at 50°c and at its lowest at 90°c in the mesocarp of ripe pawpaw as shown from the curve.

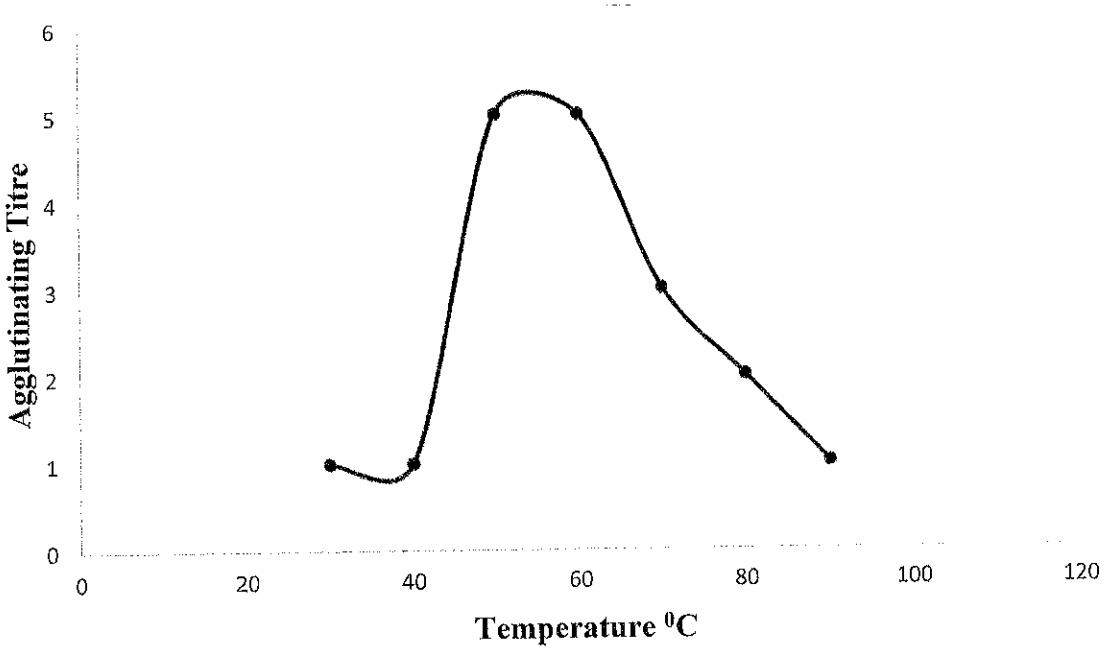


Fig 5: Effect of Temperature on the Lectin Activity of Unripe mesocarp Pawpaw

Lectin activity was at its optimum at 40°c and at its lowest at 90°c in the mesocarp of unripe pawpaw as shown from the curve.

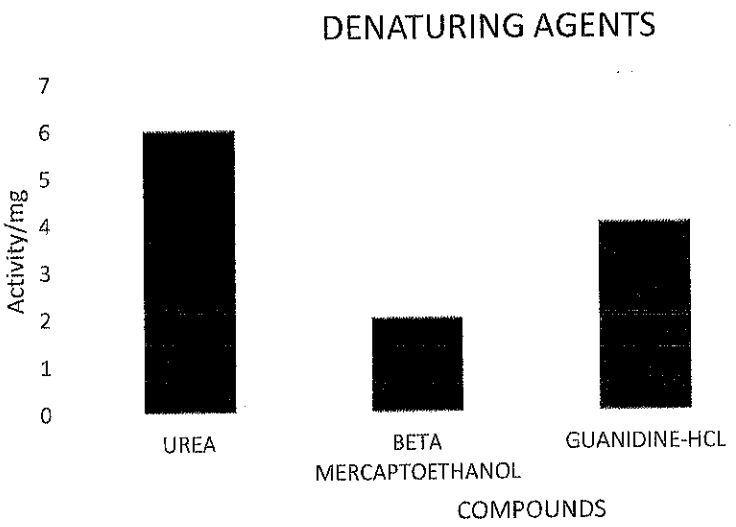


Fig 6: Effect of Denaturing Agents on the Lectin Activity of Ripe mesocarp of Pawpaw.

Lectin activity was denatured by beta mercaptoethanol in the mesocarp of ripe pawpaw. Urea on the other hand enhanced the activity of Lectin.

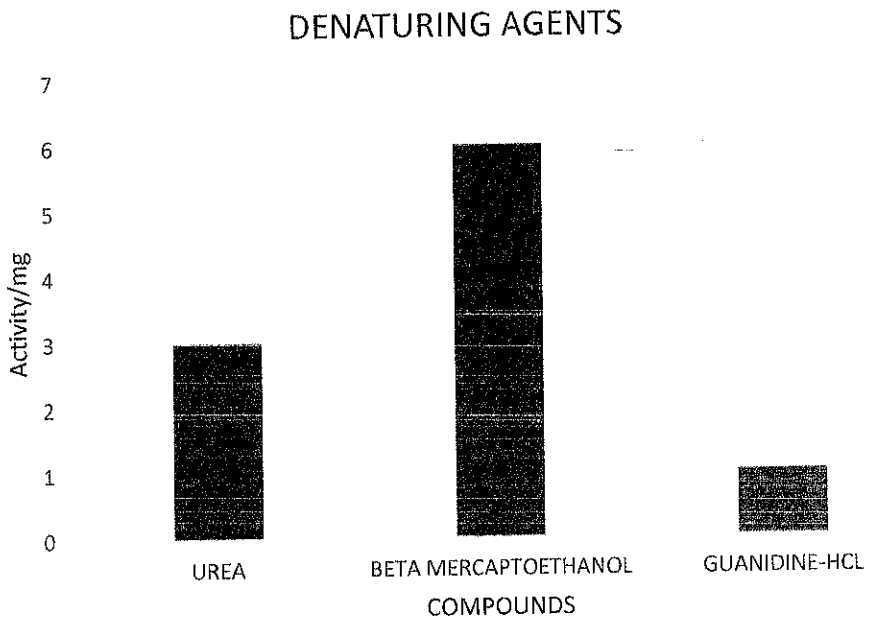


Fig 7: Effects of Denaturing Agents on Lectin Activity of Unripe mesocarp of Pawpaw.

Lectin activity was denatured by Guanidine-HCL in the mesocarp of unripe pawpaw. Beta mercaptoethanol on the other hand enhanced the activity of lectin.

SUGAR SPECIFICITY

The sugar specificity of the lectin was investigated by the ability of simple sugars to inhibit the agglutination of erythrocytes (Goldstein and Hayes, 1978). In this study, the sugars tested were: glucose, sorbose, lactose, galactose, and maltose. 0.2 M of each sugar in PBS was prepared. A serial dilution of the sample was made until the end point causing hemagglutination was obtained. 50µl of the sugar solution was added to each well (each row was assigned to each sugar) and allowed to react for 30 min at room temperature. The control well had no sugar added to it. 50µl erythrocyte suspension was then added and the mixture left for 1 h. The hemagglutinating titer that was obtained was compared with the control.

Table 7: Effects of different simple sugars on the Lectin Activity of Pawpaw

RIPE MESOCARP

SUGAR	HEAMAGGLUTINATING TITRE
GLUCOSE	2^6
SORBOSE	2^6
LACTOSE	2^2
GALACTOSE	2^1
MALTOSE	2^3
CONTROL	2^7

UNRIPE MESOCARP

SUGAR	HEAMAGGLUTINATING TITRE
GLUCOSE	2^7
SORBOSE	2^7
LACTOSE	2^0
GALACTOSE	2^0
MALTOSE	2^0
CONTROL	2^7

DISCUSSION

Hemagglutination of lectin extracted from the mesocarp of ripe and unripe *Carica papaya* (Cp (mg/mL) was used as the positive control. Negative control (N) contained buffer only. Each well of the microtiter plate contains 100uL of sample, 50uL erythrocytes of 2% suspension of human erythrocytes. The agglutination of samples form a carpet that covers the whole well; where no agglutination occurs, red blood cells form a button at the bottom of the well. (John shi *et al.* (2007).

The agglutination was used quantitatively to provide evidence for the presence of lectin in isolated fractions in the mesocarp of ripe and unripe pawpaw.

John Shi *et al.*, (2007) stated that the amount of lectin present in a sample were used in differentiating two forms of beans where the Lectin content was much lower in the canned sample than in the dry raw bean sample. In the affinity extracted, the amount of Lectin present was much in the mesocarp of the ripe C. *papaya*, while unripe C. *papaya* had Low Lectin content.

More so, Lectin was found to be stable at two optimum ranges of pH 2-4 and pH 6-8 (Fig 2) in the ripe but in the unripe it was found to be stable at pH 3-5 and pH 6-8 (Fig 3). This results suggest that the protein has two binding sites, both sites which are active. This result is however, in contrast with that of Alexander *et al.* (2004) in which Manila clam lectin activity was stable between pH 6 and pH 9 and was temperature-dependent.

Lectin in this study was found to be heat stable from 30°C to 50°C (Fig.4) in the ripe but was found to be heat stable at 40^0C to 50^0C in the unripe (Fig.5) thus they are heat labile and not thermophilic, a further increase in the temperature from 60^0C-90^0C reduced the hemagglutinating activity of Lectin. In comparison Lectin extracted from C. *annuum* was found to be stable between 20°C to 30°C (Alexander *et al.*, 2004; Kuku *et al.*, 2009).However, the hemagglutinating activity from the *Pterocladiella capillacea* lectin was affected only by exposure to a temperature of 70°C. This

occurs in some plant lectin (Cavada *et al.*, 1996) and marine algae (Benevides *et al.*, 1998). Lectin from *Ganaderma caperise* is not affected after exposure to the temperature at and above 70^0C for 60 minutes (Ngai and Ngi, 2004).

Hemagglutinating activity of lectins from *Pterocladiella ostreatus* is reduced at or above 40°C (Wang *et al.*, 2000). The difference in all these values may be supported by the submissions of Okomato *et al.*, (1990) and Leiner (1994). The former reported that heat stability of this protein differs from Lectin to Lectin while the latter, reported that Lectins are known to be unstable and their activity can be decreased by heat treatment. Hence, a decrease in hemagglutination activity of Lectins as temperature increases showed that its activity depends on the native conformation of the protein.

Addition of Lowry reagent to the extract from both the ripe and unripe pawpaw indicated that the mesocarp of the unripe pawpaw contains high protein content than that of the ripe which contains low protein concentration.

Out of the five sugars tested, only glucose and sorbose did not inhibit Lectin-induced hemagglutination in the unripe mesocarp while maltose, lactose and galactose inhibited hemagglutination. In the ripe glucose and sorbose sugars did not inhibit lectin but maltose, lactose and galactose inhibited the lectin. These suggested that glucose and sorbose sugars did not inhibit the activity of the lectin found in the ripe and unripe pawpaw. Thus, they might be free sugar binding lectins as described by Okamoto *et al.*, (1990) in their report on numerous marine algal lectin such as *Gracilaria bursa-pastoris*. The presence of these sugars in the diet would still probably enable the lectin under study to prevent adhension of the pathogenic bacteria at the gastro intestinal tract.

All protein denaturing agents (Urea, Beta Mercaptoethanol and Guanidine-HCl) used were chaotropic agents and could prevent the hemagglutination activity of lectin. The high concentration of denaturing agents were supposed to allow water molecules to disrupt the hydrophobic interactions in the interior of lectin that supported its native conformations. Little loss of hemagglutination activity of lectin by guanidine-HCl than urea and beta mercaptoethanol indicates guanidine-HCl to be more potent denaturing agent for the unripe pawpaw lectin while guanidine-HCL and beta mercaptoethanol was found to be more potent in the ripe pawpaw Lectin.

CONCLUSION AND RECOMMENDATIONS

This study indicated that lectins extracted from ripe and unripe pawpaw have varying rate of hemagglutinin activity; although they agglutinate all blood groups.

Also, this study indicated that hemagglutinating activity of lectin from pawpaw towards human erythrocytes was found to be selective to types of blood groups. It can be deduced from the study that it may be beneficial health wisely to consume more of the unripe pawpaw because it contains high protein content than the ripe pawpaw.

Although, according to the findings the mesocarp of the ripe pawpaw has a high content of lectin it is advisable to reduce drastically the rate of consumption as a result of its health implications, which include inflammatory, intestinal disorder and other related diseases (Hamid *et al.*, 2013). Whereas the rate of consumption of the unripe pawpaw should be encouraged among people due to its high level of protein and low lectin content.

Subsequent research should be done using erythrocytes from other animals as well as evaluating the effects of heat treatments, storage conditions, level of maturity and other factors on hemagglutinin activity of pawpaw.

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