KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

DEPARTMENT OF HORTICULTURE

EFFICACY OF COCOA (*Theobroma cacao* L.) LEAF EXTRACT IN THE CONTROL OF STORAGE SPOILAGE MICROORGANISMS ASSOCIATED WITH EXPORT GRADE WHITE YAM (*Dioscorea rotundata* poir.)

BY

AMPOFO OWUSU SAMUEL

AUGUST, 2015

EFFICACY OF COCOA (*Theobroma cacao* L.) LEAF EXTRACT IN THE CONTROL OF STORAGE SPOILAGE MICROORGANISMS ASSOCIATED WITH EXPORT GRADE WHITE YAM (*Dioscorea rotundata* poir.)

A THESIS SUBMITTED TO THE SCHOOL OF RESEARCH AND GRADUATE STUDIES, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY (POST HARVEST PHYSIOLOGY) DEGREE

BY

AMPOFO OWUSU SAMUEL

AUGUST, 2015

DECLARATION

I hereby declare that this work herein submitted as a dissertation for a Master of Philosophy degree is the result of my own investigation. It contains no material previously published by another person for the award of any degree of the University. Work by others that served as useful source of information has been dully acknowledged by references to the authors.

……. ……

AMPOFO OWUSU SAMUEL Date
(STUDENT)

…… ……..

MR. PATRICK KUMAH Date
(SUPERVISOR)

…… ……..

DR. BEN BANFUL Date
(CO-SUPERVISOR)

…… ……..

DR. FRANCIS APPIAH Date
(HEAD OF DEPARTMENT)

DEDICATION

I dedicate this work to the almighty God, my caring parents and to my bosom family, especially Mr. and Mrs. S. K. Obeng.

ACKNOWLEDGEMENTS

Glory and praise be to the omniscient God, for granting me health, encouragement and the mental capacity to gather knowledge throughout my academic endeavour.

The bulk of my gratitude goes to West Africa Agricultural Productivity Progrmme (WAAPP) who worked tirelessly to see me through thick and thin as regards financial support for this programme. Special thanks also go to the Head and Staff Members of Department of Crop Services, Ministry of Food and Agriculture for their moral support to ensure my high placement in social and academic circles.

My deepest appreciation goes to my supervisors, Mr. Patrick Kumah and Dr. Ben Banful who assisted in the provision of support and direction. They also guided me through constructive criticisms and suggestion to complete this project.

Again, credit ought to be placed at the doorstep of my able lecturers who imparted knowledge, particularly my dutiful and conscientious Head of Department, Dr. Francis Appiah and Dr. Laura Atuah who instilled initiative and a sense of purpose in me throughout this study. I doff my hat for Prof. A. Y. Mensah and his students especially Jonathan Jato, Nana Ama Mireku-Gyimah and Kakraba Samuel from the Department of Pharmacognosy at KNUST, for their active support. In addition to that, my appreciation goes to Dr. C. Kwoseh, Mr. Faye Manneh and Mr. Malik Borigu from Department of Crop and Soil Sciences, for their sincere support. To Elizabeth Oppong-Kyekyeku, I say kudos for her invaluable assistance in data collection and data entry. Last but not least, I must say I cherish the tremendous support I enjoyed from friends who contributed their quota in diverse ways to lessen the dreaded stressful moments. Inasmuch as I wasn't found wanting but realized my dream, I thank you one and all for this success.

LIST OF ABBREVIATIONS

CL	Cocoa Leaf
DNA	Deoxiyribnuclei Acid
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FAO	Food and Agriculture Organization
FOB	Freight on Board
GDP	Gross Domestic Product
GSH	Glutathione
IEC	International Export Consultants
IITA	International Institute of Tropical Agriculture
KNUST	Kwame Nkrumah University of Science and Technology
MAMP	Microbe Associated Molecular Patterns
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAMP	Pathogen Associated Molecular Patterns
PCD	Programmed Cell Death
PDA	Potato Dextrose Agar
PDA	Potato Dextrose Agar
ROS	Reactive Oxygen Species
SAR	Systemic Acquired Resistance
USAID	United State Agency for International Development
UV	Ultraviolet
WAAPP	West Africa Agricultural Productivity Programme

ABSTRACT

Ghana is the largest exporter of yam in West Africa. There are constraints associated with yam storage as a result of yam rot during and after the pre-export handling period. The predominant method of control is the use of synthetic pesticides, which could pose health hazard to consumers. Therefore, this study aimed to test the efficacy of cocoa leaves (CL) extracts as an alternative to synthetic pesticides. A preliminary survey was conducted on pre-export handling operations. Fresh cocoa leaves were collected, steam-blanched, dried, powdered and qualitatively analyzed. Polyphenolic compounds were extracted using standard methods. Crude Aqueous CL Extract was also prepared by cold maceration for 7 days. Antioxidant assays conducted on the respective extracts such as total phenol content and total antioxidant capacity. Fungi were isolated and identified from yam collected from marketplaces and exporters' warehouses. Agar Well Diffusion and Broth Micro Dilution Assays were used to determine the antimicrobial activities and Minimum Inhibitory Concentrations (MICs) *in vitro*. *In vivo*, separately treated white yams with extracts were inoculated with test fungi. The experimental design was factorially arranged in CRD**.** Ethylacetate Purified CL Extract recorded the highest in both total phenol content and total antioxidant capacity whereas the Crude Aqueous CL Extract was the least active. The occurrences of *Apergillius flavus, Aspergillus niger, Rhizopus stolinifa and Penicillium spp*. isolated and identified from the samples collected, recorded 15.15%, 18.18%, 27.27% and 39.39% respectively. Among the extracts, Ethylacetate Purified CL Extract (5.4mm) was significantly ($p<$

0.5) effective followed by Acetone Purified CL Extract (3.13mm), while Crude Aqueous CL Extract (0.0mm) showed no inhibition against the four test fungi in the

Agar Well Diffusion Assay. Regarding the MICs, the most effective extract was obtained from Mancozeb (2mg/ml) followed Ethylacetate Purified CL Extract (2.5mg/ml) and the least was Crude Aqueous CL Extract (15mg/ml) *in vitro*. Crud Aqueous CL Extract and Distilled Water treated tubers significantly showed the highest and lowest weight losses respectively. Percentage incidence of rot was significantly higher on tubers treated with Distilled Water (6.94%), followed by Crude Aqueous CL Extract (4.1%), Ethylacetate Purified CL Extract (2.78%) and Mancozeb (2.78%). Hence cocoa leaf extracts especially Ethylacetate Purified CL Extract, which showed similar potential to that of Mancozeb, could be an ideal alternative to synthetic pesticide use in controlling rot-causing pathogens on export grade white yam.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICE

CHAPTER ONE

1.0 INTRODUCTION

Yam is classified as an important historical multipurpose staple food popularly grown in West Africa. Out of the six species commonly found in West Africa, *Dioscorea rotundata poir* is the most widely grown and generally considered to be the best in terms of food quality, thus commanding the highest market value (Markson *et al.*, 2010). As a matter of fact, the third largest producer of yam in the West Africa following Nigeria and Cote d'Ivoire is Ghana, however the largest exporter annually (FAO, 2005). Furthermore, yam ran second after pineapple in terms of volume and value of non-traditional export crops in Ghana (AsumingBrempong, 1991). In spite of it numerous importance, there are constraints associated with yam storage as a result of yam rot, caused by various pathogens that eventually depletes its quality during and after the pre-export (before export) period.

Pathogens, being one of the most significant causes of yam storage losses, are estimated to be as high as 40% - 50% (Olurinola *et al*., 1992; Osagie, 1992), although typical losses are assumed to be between 2% and 25% (Nwankiti and Arene, 1978). Many fungi attack yam tubers (Osagie, 1992) but the most common are *Fusarium spp, Botryodiplodia theobromae, Penicillium spp.,* and *Aspergillus spp* (Morse *et al.*, 1997). Pathogens cause rots through natural openings on white yam tubers and sometimes by the aid of pest or mechanical damage mostly at the time of harvest and transportation to the store (Osagie, 1992). Considering its easy susceptibility to infection, the need for proper postharvest handling and effective control of these pathogens from causing storage rot are important factors to be considered.

Meanwhile, prophylactic use of synthetic pesticides has been the readily available control measure against pathogen infestation during yam storage. Factually, the application of fungicides to the tubers at the onset of the storage period has also been practiced with varying degrees of success (Olurinola *et al.*, 1992). Nevertheless, synthetic pesticides are expensive, not environmentally friendly, induce pathogen resistance and cause health hazard to consumers. Gupta (2008) reported that complications or side effects on consumer's health caused by the use of synthetic pesticides may prevail irrespective of short, medium or long-term uses. The need to develop a substitute like cocoa leaves extract will be a breakthrough for mankind because it will be organic and cheap.

Moreover, cocoa is an important perennial tree crop widely produced in West African countries (like Ghana) and South America with so many industrial uses. Beside the uses of its beans for chocolate products, according to Osman *et al*. (2004), young cocoa leaves extracts contain polyphenols with high antioxidant and antimicrobial properties. Winkelhausen *et al*. (2005) also confirmed that extracted phenolic compounds have the potential properties of inhibiting the growth of pathogens like fungi. Polyphenols are large groups of natural compounds widely distributed in varieties of plants. They are among the phytochemicals produced or synthesized during secondary metabolism in plants. They are non-nutritive chemicals that have antimicrobial characteristics or disease preventive properties. The most important of these phytochemicals are alkaloids, flavonoids, tannins, and phenolic compounds (Izuka and Mbagwu, 2013). They seem to be connected in the protection of plants against infecting pathogens, such as bacteria, fungi, and viruses (Marsilio *et al.*, 2001). Currently, synthetic pesticides play a very important role in the defense of storage yam against pathogens (Copping

and Hewitt, 1998). In compliance with the growing public awareness of these hazards, there has been an increased in the search for novel naturally extracted products with antimicrobial properties that can compete with already existing pesticides, which are devastating to man eventually. Therefore, the main objective of this study was to investigate the effect of young cocoa leaves extract in the control of storage spoilage microorganisms on export grade white yam.

The specific objectives of this study, therefore, were to:

1. ascertain from yam retailer and exporters the handling practices and other causes of rot.
2. determine the phytochemical properties of cocoa leaves extracts and their efficacy in the control of identified spoilage microorganism *in vitro*; and
3. determine the most effective ways of applying the extracts to control the spoilage microorganism identified *in vivo*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ORIGIN AND DISTRIBUTION OF YAMS

Yams are important tuber crops widely cultivated throughout the world and they are of different species emanating from the genus *Dioscorea* of the family Dioscoreaceae (Adejumo, 2010). Several countries are available in effective yam tuber production, but Africa remains the largest continent producing yam from countries like Nigeria, Ghana, Ivory Coast, Cameroun, Togo, Benin Republic etc., which account for about 92% of the total world producing population and it also serves as a source of foreign exchange through export (Adejumo, 2010; Hahn *et al*., 1987). Amoo *et al*. (2014) reported that there are 600 species of yam, but only six are mostly grown as staple foods. These include *Dioscorea rotundata* (white yam), *D. alata* (water yam), and *D. cayenensis* (yellow yam), *D. esculenta* (Chinese yam), *D. bulbifera* (aerial yam) and *D. dumetorum* (trifoliate yam).

2.2 DESCRIPTION AND PRODUCTION OF WHITE YAM

Dioscorea rotundata, the white yam is mostly cultivated and mainly found in Africa. The white yam family comprises over 200 cultivated varieties. In Ghana, within the D. *rotundata* species, there is a class of cultivars commonly known as the "Pona" yam and it is rated superior to other white yam varieties such as "Dente", "Serwa", "Labreko", "Asobayere" and "Muchumudu", in terms of its cooking quality and thus commands a high price than other yams in the market. "Pona" and "Labreko" are sweet, floury and have fragrant tuber flesh and remain the most preferred cultivars by consumers in Ghana (Otoo *et al.*, 2009). Their tubers mostly often weigh about 2.5-5 kg (6-12 pounds). Yam tubers are mostly harvested between 7-12 months (Okigbo and Ikediugwu, 2000). White yam is a dry season tropical crop adapted to a high rainfall of about 1000-2200 mm and temperature of 20-32^{0}C. They thrive well in heavy and

loosely sandy soil that is not waterlogged. They possess tubers that are cylindrical with round-pointed ends and are smooth skinned with white flesh

(Adejumo, 2010).

2.3 VALUES AND USES OF YAM

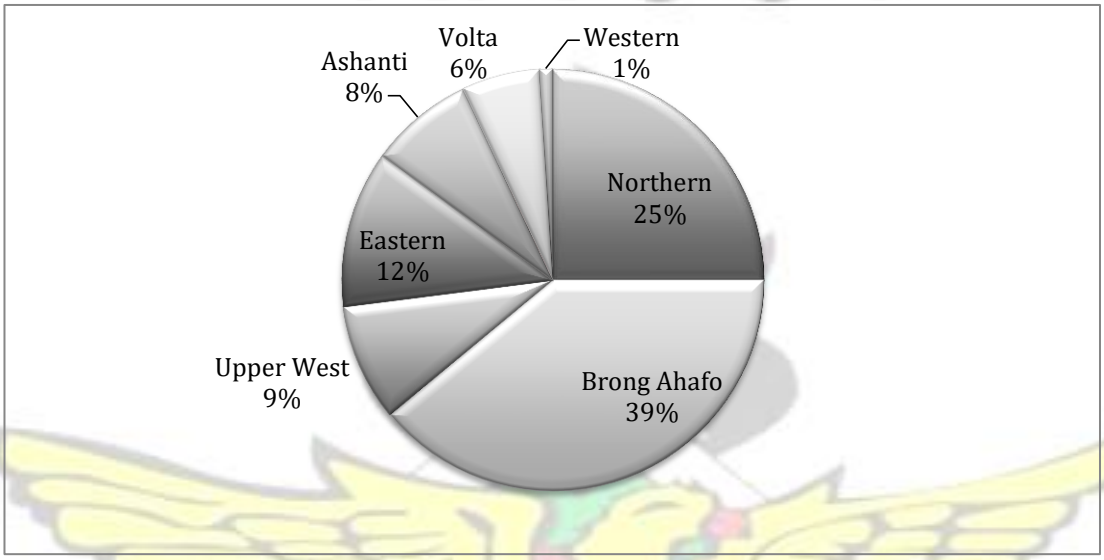
Yams are mainly used as a source of food, feed for animals, farmer's income generating crop and commodity for export to other countries for foreign exchange (Maalekuu *et al.*, 2014). In some parts of Southeastern Nigeria, the meals offered to gods and ancestors consist principally of mashed yam. Some traditional ceremonies are celebrated with yam as the major food item such as the New Yam Festival in parts of West Africa. In Ghana, traditional festivals such as Hogbetsotso, Apour, Homowo are new yam festivals accompanied with yam production and depict the high prestige given to the crop.

According to Baah (2009), a research conducted by International Export Consultants (IEC) exhibited that in early 1999, the international price of processed yam flour and yam chips/pellets ranged between $120,560 and $152,362 per tonne. Actually, these prices were even higher than those of cassava starch and cassava chips/pellets that sold between $90,000 and $105,000 for cassava chips/pellets and from $120,000 to $135,000 for cassava starch. Currently yam is now grown as a cash crop for both local consumption and export.

2.4 YAM EXPORT INDUSTRY IN GHANA

According to Anaadumba (2013), yam contributes 16 percent (16%) of Ghana's

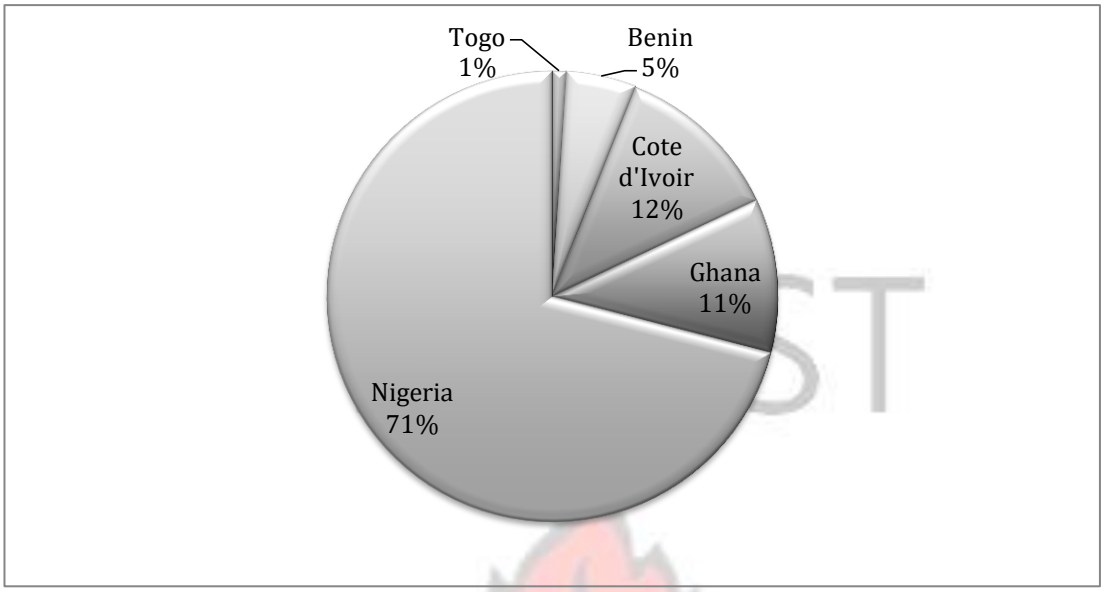
Agricultural Gross Domestic Product (GDP). The total production increased from 877,000 in 1990 to 5,960,490 tonnes in 2010 mainly by small-scale farmers. Several varieties of yam are produced throughout Ghana. These include “Pona”, “Labreko”, “Asobayere” and “Muchumudu”, “Dente”, “Asana” and “Serwa”. Figure 2.1 shows how yams are distributed among the various regions in Ghana.



Source: Anaadumba (2013)

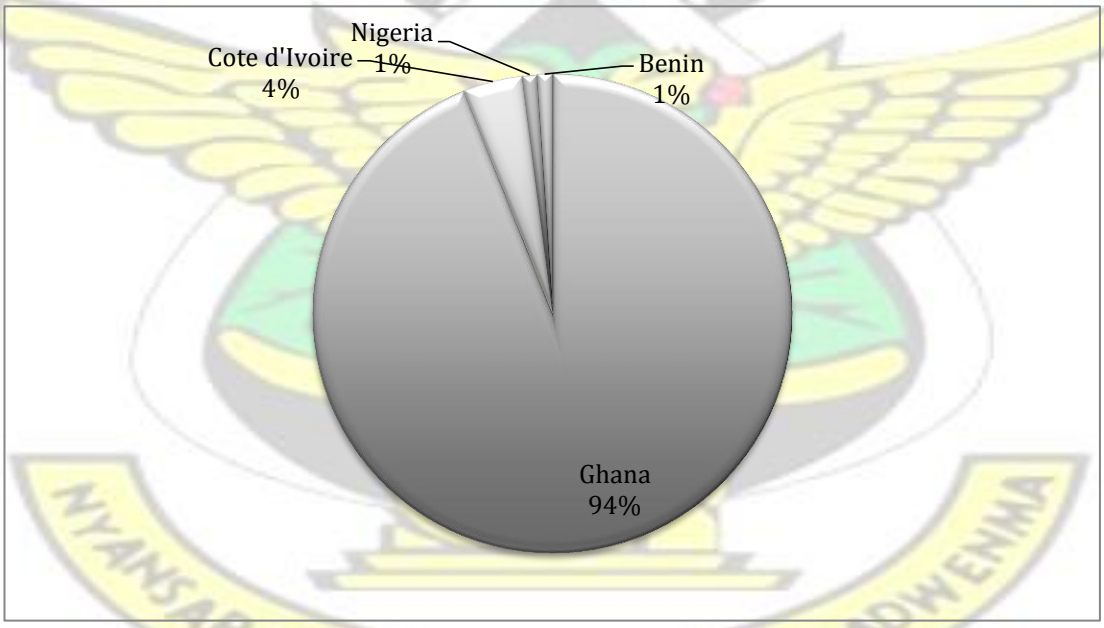
Figure 2.1: Distribution of Yam Production in Ghana, 2010

As shown in Figure 2.2 and Figure 2.3, Ghana is placed as the third largest yam producer in West Africa, following Nigeria and Cote d’Ivoire. On the contrary, Ghana is ranked as the leading exporter of yam, accounting for about 94% of total yam exports from the western part of Africa.



Source: Anaadumba (2013)

Figure 2.2: Top Yam Producers in West Africa (%) 2005-2010

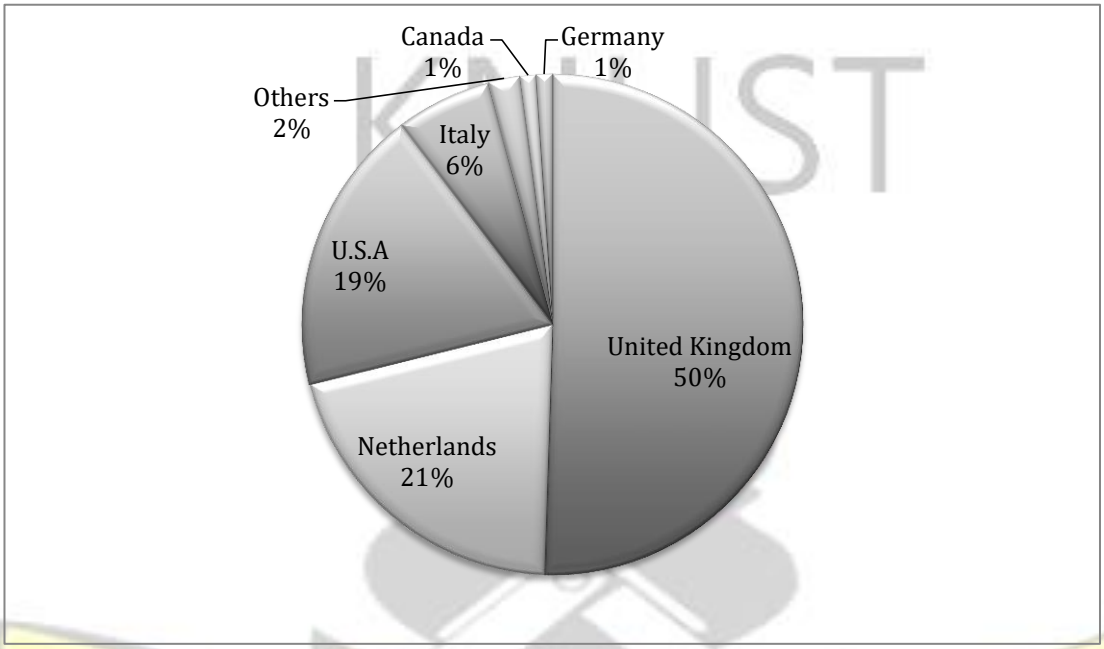


Source: Anaadumba (2013)

Figure 2.3: Top Yam Exporters in West Africa, 2005-2009

About 90 percent of Ghana's yams are exported into three (3) countries and they include United Kingdom, the United States and the Netherlands (Figure 2.4). The large

quantities of yam exported to these destinations are largely due to the high demand for yam by Ghanaians and other West Africans staying in these countries.



Source: Anaadumba (2013)

Figure 2.4: Ghana Yam Exports by Importing Country (%), 2008

2.4.1 Challenges Facing Yam Exporting Industry

Anaadumba (2013) further reported that white yam is claimed to be of high demand in Ghana, but its potential for income generation has not been fully realized due to problems and inefficiencies within the production, handling and trading systems in the country. For instance, in 2006, the yam export business suffered a huge set-back as a result of inadequate coordination among traders. This caused Ghana exporters to flood the international market with yam, leading to a major collapse in yam prices. However, things were rectified with the establishment of a pack-house, which is closer to the Tema port and coordinates all activities regarding exporting yam from Ghana. The

pack-house introduced an effective quota system that has assisted in rationing yam exports according to international demand.

Furthermore, the quality of yam produced in Ghana is sometimes compromised due to poor road transport network, harvesting practices, pathogen infestation and poor storage conditions. In most cases, these cause exported yam to rot before getting to EU and US markets. Consequently, exporters often do not get their payment for a shipment, which eventually results in their refusal to pay local producers and traders (Anaadumba, 2013).

Besides, quality of yam exports from Ghana is also compromised by fumigation practices and days spent at sea. Ghana yam is fumigated upon arrival at USA ports. This often decreases yam quality due to the high temperature at which fumigation takes place (USAID, 2005).

Moreover, despite the fact that yam is one of the most prominent export commodities for Ghana, information on the yam value chain is poor especially for aspects such as clear differences between yam varieties for the export market and those consumed locally. The difficulty to determine what specific types of varieties of yam are exported to the international market was a big challenge. Although white yam is mentioned as the preferred variety, it will be important to indicate if this is the main exported item as the FOB price as well as the domestic market prices may differ depending on the quality of yam (Anaadumba, 2013).

2.4.2 Marketing Channel of Yam in Ghana

Yam exporters normally operating in Accra, rather buy yam directly from small and large-scale farmers. They also sometimes buy yam from sedentary wholesalers at the main markets in Accra-Konkomba, Agbogbloshi and Baasare Markets. However, with systematic increase in competition in the yam export market, some exporters commission itinerant traders to purchase yam from major production areas (USAID, 2005)

2.5 STORAGE OF YAM

Baah (2009) reported that yam is a seasonal crop and mostly available during its harvesting period, but scarce and expensive during its planting and growing seasons. The tubers are stored under different conditions at the various growing areas. In principle, they must be stored in an accessible, adequately ventilated area protected against direct sunlight. The length of storage varies but yam can generally be stored from two (2) to four (4) months. Storage of yam for up to 9 months has been reported in Niger State (Alabadan, 2002). Stored yam tubers continue to respire at reduced levels after harvesting in the dormant state.

2.5.1 Storage Losses of White Yam

Yam tubers suffer considerable postharvest losses that can be as high as 60% (Asiedu, 1986; Alabadan, 2002). External agents such as insects, rodents, fungi, and bacteria, or physiological processes such as sprouting could cause these losses. Infection by microorganism that causes high storage rot is mostly bacterial, fungi as well as nematodes (Osagie, 1992; Amusa *et al.*, 2004).

2.5.2 Factors Contributing to Storage Losses of Yam Tubers

The principal causes of loss of yam are mechanical damage, respiration, transpiration, temperature, dormancy and rotting.

2.5.2.1 Mechanical damage

Yam tuber skin provides an effective barrier against most pathogen infestations that cause rotting of the tissues. Damage or injury caused to this barrier of yam skin will create access point of entry for infectious microorganisms and will eventually lead to physiological deterioration and dehydration. Damage that occurs on yam skin that is not visible could eventually lead to physiological deterioration and create an access point for microorganisms. (Coursey, 1967; Osagie, 1992).

2.5.2.2 Physiological factors

2.5.2.3 Respiration

According to Diop (1998), yams are living organisms and besides, they respire. The respiration process contributes in the oxidation of the starch (a polymer of glucose) contained in the cells of the tuber, which converts it into water, carbon dioxide and heat energy. During this conversion of the starch, the dry matter of the tuber is reduced. Respiration freely occurs when oxygen needed is supply and the resulting carbon dioxide and heat has to be removed from the products' surrounding. A limited amount of supply of oxygen and inadequate removal of carbon dioxide may lead to effective asphyxiation and the death of product tissue.

2.5.2.3.1 Factor affecting the respiration rate

2.5.2.3.2 Skin permeability

Permeability of the white yam skin is a feature of its maturity and is a really crucial factor in the rate of respiration. The periderm of freshly harvested immature tubers is most permeable and thus permits greater levels of respiration than similarly harvested mature tubers (Burton, 1966).

2.5.2.4 Transpiration of water molecules from Yam

Transpiration is the loss of water molecules through the skin pores of the yam and it is attributed to evaporation. Weight loss of yam tuber could leads to economic loss to exporters when the market price of the exported yam is determine base on weight and attractiveness of yam tuber to potential consumers (Coursey, 1968).

2.5.2.5 Dormancy of white yam during storage

Dormancy is affected by many factors and they include temperature, moisture, oxygen and CO_2 content of the storage atmosphere, the extent of wounding and any disease of the tuber (Passam, 1982).

2.6 PATHOLOGICAL FACTORS

Diop (1998) further reported that because yams are living organisms, they are subjected to attacks by microorganisms like fungi, bacteria etc. When the protective barrier covering the yam is damaged, the yam tuber is eventually predisposed to pathogenic attacks.

2.6.1 Pathogenic Causes of Losses of White Yam Tubers

Pathogens are one of the common causes of loss during yam storage. This contributes to its easy susceptibility to infection by these microbial pathogens, especially fungi.

Many fungal pathogens such as *Fusarium spp, Botryodiplodia theobromae, Rhizopus stolonifer, Penicillium spp and Aspergillus spp* (Morse *et al.*, 1997) are found to cause rot to yam. Yam rot occurring at storage probably begins in the soil and progress to storage. This could occur when the infected yam tubers do not reveal any perceptible external symptoms (Okigbo and Ogbonnaya, 2006).

According to Aboagye-Nuamah *et al*. (2005), the differences in the occurrence of the organisms could be as a result of the localities where the tubers were cultivated.

2.6.2 Causal Agents of Yam Rot:

Bacteria, nematode, viruses, and fungi predominantly cause yams rots. *R. stolonifer* has been reported as a saprophyte that could initiate severe rot and decay in yam storage and has a wide host range (Aboagye-Nuamah *et al*., 2005). The presence of *Aspergillus flavus* can be hazardous to human and animal health. This is because *Aspergillus spp* such as *Aspergillus flavus* produce aflatoxins (G_1,G_2, B_1,B_2) of which aflatoxin B_1 is highly carcinogenic and causes hepatoma (Ogaraku, and Usman, 2008).

2.6.3 Types of Yam Rot

The storage rots of yam can be classified into three according the rot causing organisms and their respective symptoms.

2.6.3.1 Dry rot of yam

Dry rot is caused by *Penicillium oxalicum* and *P. cyclopium*. The infected yam part turns brown and becomes dry and hard but maintains their integrity except when the

tissues were infected by *Sphaerostilbe marcescens* (IITA, 1993). Tubers attacked by *A. niger* and *A. tamari* change the infected tissue into brown with yellowish margin. *Rosellina bunodes* and *B. theobromae* also creates dry black rots (IITA, 1993).

2.6.3.2 Soft rot of yam

Yams attacked by soft rot pathogens often turn brown, soft and turn wet due to rapid collapse of cell walls. Pathogens causing this form of rot are *Rhizopus sp.*, *Armillariella mellea, Mucor circinelloides and Sclerotium rolfsii,* (Aidoo, 2011). Soft-rot pathogens usually target pectins for digestion using specialized enzymes that attack cells to break them apart. Meanwhile pectins also form hydrated gels that help "cement" neighbouring cells together and regulate the water content of the wall (Freeman and Beattie, 2008).

2.6.3.2 Wet rot of yam

Wet rot of yam tuber is described and identified by the oozing of whitish fluid out of infected tissues when pressed. This symptom is normally caused by the bacterium, *Erwinia carotovora* (IITA, 1993).

2.7 CONTROL OF STORAGE YAM PATHOGENS

Reducing mechanical damage of tubers during and after harvesting basically controls rot and other losses of yam through storage at favorable environment and protecting tubers with fungicides such as benlate, sodium orthiophenylphenate, captan, borax, and macozeb. (Aidoo, 2011; Okigbo and Nmeka, 20051).

Okigbo and Nneka (2005) showed that extracts of *Zingiber officinale* and *Xylopia aethiopica* controlled post-harvest yam rot. Other treatments such as wood ashes, lime, are simple, and useful methods on the farm. It has also been reported that plant materials with fungicidal properties are very potent in inhibiting fungal growth *invivo* and *in-vitro* (Aidoo, 2011). *Crecentia alata* and *Dennettia tripetala* are included in the plants with such potentials (Khan *et al*., 2001).

2.7.1 Control of Pathogen by Synthetic Origin

Although synthetic fungicides are used to protect yams from deterioration, they are a health risk for humans and should be restricted to treatment of seed tubers. Farmers in developing economies such as Nigeria and Ghana have however hardly adopted these findings, because majority of them cannot afford the financial cost. Kuhn and Hargreaves (1987) observed that fungicidal substances works *in vitro* in most cases killed these fungi *in vivo*.

2.7.2 Control of Pathogen by Natural or Plant Origin

Plant extracts have been used to control many crop related diseases. Pesticides of plant origin are specifically more biodegradable, readily accessible, environmentally friendlier and cheaper than synthetic pesticides (Aidoo, 2011).

2.7.2.1 *Cassia alata*:

Cassia alata (family: Caesalpinaceae) is common at the tropics and an annual herb. It has been reported that their leaves, flowers and seeds contain phytochemical compounds that have been substantiated traditionally to treat certain skin diseases in humans and very effective control against mite infestations (Khan *et al*., 2001). So *C.*

alata extract possesses antimicrobial and insecticidal properties against wide ranges of pathogens.

2.7.2.2 *Zingiber officinale*

Zingiber officinale (common name; ginger) is a perennial plant, which had been reported by Aidoo (2011) to have various herbal properties of ginger. The crop contains volatile oil, phenols, alkaloid and mucilage. The herbal therapeutic importance of ginger is mainly due to the presence of volatile oils and the high oleoresin content.

A survey was done in Jaffna, Sri Lanka, to identify local medicinal uses of ginger. The fungicidal potential of ginger extract was also evaluated. The growth inhibition on *Fusarium* spp., *Colletotrichum* spp. and *Curvularia* spp. by ginger extract were indicated as 70.0%, 71.0% and 64.2%, respectively (Krishnapillai, 2003). Other researchers reported that Uda (*Xylopia aethiopica*) and ginger (*Zingiber officinale*) extracts inhibit the growth of fungi in culture and reduced rot initiation in yam tubers (Okigbo and Nmeka, 2005).

2.7.3 Side Effects of Pesticide of Synthetic Origin

Although, synthetic pesticides are essential for controlling storage pests, its effect on the health of consumers as a whole is a big challenge. Effects of these synthetic pesticides may be acute or chronic. Immediate effects may include headaches, eye irritation, skin rash, loss of consciousness, throat pain, coughing, rhinorrhea, vomiting, diarrhea and the like (Gupta, 2008). Long-term effects may increase risk of cancer, neurological impairment and damage certain internal organs (Gupta, 2008).

2.8 PHENOLIC COMPOUNDS AND POLYPHENOL OXIDASE IN YAM

Anthocyanins and carotenoids are pigments that do occur in yam to give characteristic colours to the flesh of the tuber. Xantophyll esters and and β- carotene in *D. cayenensis* is reported to be attributed to the yellow flesh of the species. The yam varieties with higher anthocyanin content are often prone to polyphenolic oxidation. The anthocyanin pigment in *D. alata* and *D. trifida* cultivars may lead to the appearance of the pink or purplish-red colour to the yam tissue, either the entire yam tissue or just under the skin of the yam can be affected. The anthocyanins consist of a mixture of cyanidin glucosides and can also occur in many intermediate forms (Baah, 2009). It is noted that polyphenolic compounds in yams undergo polyphenolic oxidase-catalyzed reactions to form o-quinones, their primary oxidation products. These react with other components to form brown polymeric compounds (Ozo, 1985).

The internal brown colour of some yams when cut indicates the presence of polyphenols and their oxidation reactions that occur in them (Farombi *et al*., 2000). The rate of browning in yam directly corresponds with the quantity of phenolic compounds and polyphenol oxidases in yam (Asemota *et al*., 1992; Muzac-Tucker *et al*., 1993). Muzac-Tucker *et al*. (1993) reported of a range of 0.061-10.50 g/100g dry weights phenolics in *D. alata* varieties while 0.023-0.034 g/100g dry weights were obtained for *D. rotundata* varieties. The vitamin C content of *D. rotundata* and *D. cayenensis* is enough to make these species fair sources, but is slightly higher in *D, rotundata* (6.5-11.6 mg/100 g) than in *D. cayenensis* (4.5-8.2 mg/100 g), according to Coursey (1967)

2.9 POLYPHENOLS

2.9.1 Chemical properties and benefits of Polyphenol

2.9.1.1 Chemical properties

Plant phenolics are usually soluble in organic polar solvents for instance glycosides. Water solubility increases with the number of hydroxyl groups present (Lattanzio *et al*., 2006) except in some few cases. As reported by Lattanzio *et al*. in 2001, phenolic compounds form one of the main classes of secondary metabolites. According to Lii (2012), polyphenols are catechins including （-）-EGCG,（-）-ECG ,（-）-EGC, （-）-EC; with basic structures as below:

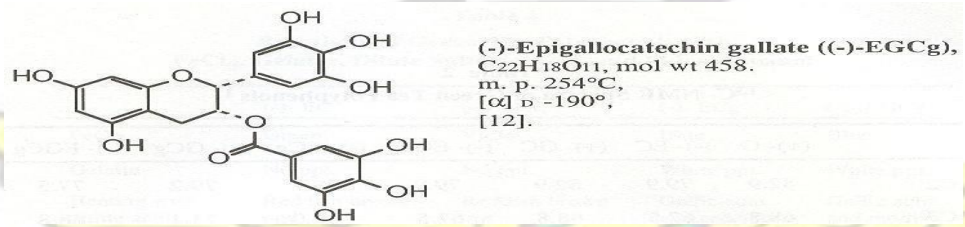


Figure 2.5: (-) - Epigallocatechin gallate ((-) – EGCg)

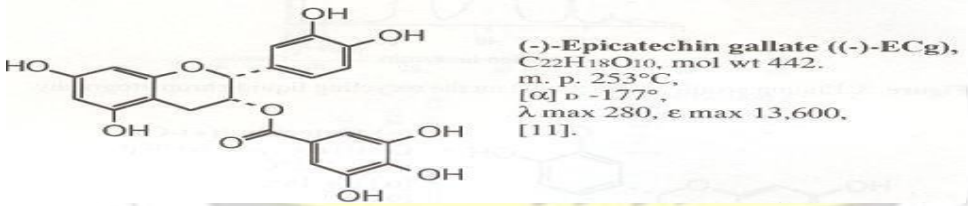


Figure 2.6: (-) – Epicatechin gallate ((-) – ECg)

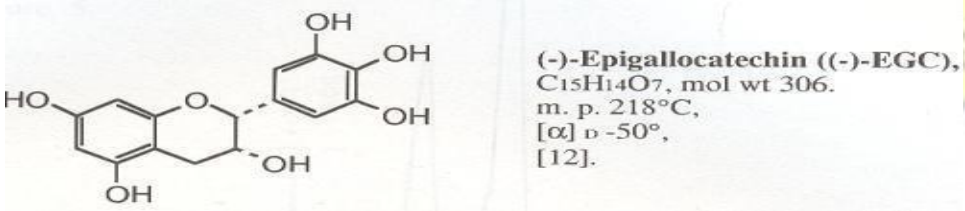


Figure 2.7: (-) – Epigallocatechin ((-) – EGC)

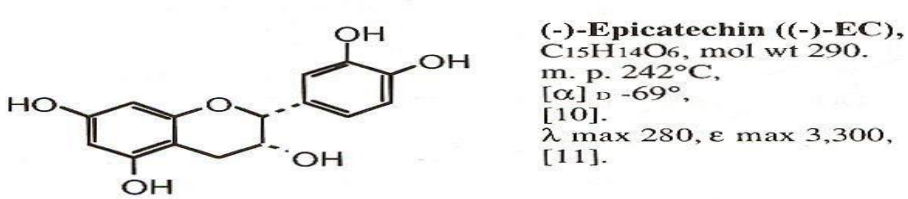


Figure 2.8: (-) – Epicatechin ((-) – EC)

2.9.1.2 Benefits and role of polyphenol in human

According to Lii (2012), polyphenol is a strong anti-oxidant, which has anti-bacterial and anti-viral functions. It helps to clear up free radicals and maintain the biological activities of DNA, preventing cardiovascular and cerebrovascular diseases, antitumor, anti-lipid per oxidation, as well as immunity-enhancing, anti-bacterial, antiviral, detoxifying, anti-aging and anti-radiation damage.

2.9.2 Effects of Polyphenol Antimicrobial Properties on Microorganisms

2.9.2.1 Secondary metabolites in plant defense mechanisms

Plants synthesize a greater amount of secondary compounds than animals because they cannot rely on physical mobility to escape their predators and have to therefore use them as chemical defense against such predator with polyphenol through secondary metabolism (Lattanzio *et al*., 2006; Freeman and Beattie, 2008).

2.9.2.2 Mechanism of defensive system of plant cell against pathogen (fungal)

Fungi depend on plants for their carbon and energy source, like most other organisms that are not able to photosynthesize. Mostly, relationships between them are mutual benefit. Furthermore, according to Freeman and Beattie (2008), basal resistance, which is also called innate immunity, is the beginning of pre-formed and inducible defenses that prevent invasion of certain pathogen in to the cells. Basal resistance could be prompted when cells detect microbe-associated molecular patterns (MAMPs). This procedure renders the plant cells strengthened defensively against external threats. It must however be indicated that, in certain plant species, pathogens have developed a rather strong aversion to effects of the basal resistance. When the basal defense is overcome by such pathogens, the plants also are able to come up with a different line

of defense: the hypersensitive response (HR). HR could be drastic when juxtaposed to the work of the basal resistance as it could save the other parts of the plant by immolating some cells in order to prevent pathogens' access to water and nutrients. Perhaps, the advantages of the HR over basal resistance include its ability to target specific pathogens and how it is quickly called into action when the presence of disease-causing effector molecules brought in by the pathogen in the affected plant are identified by gene products in the plant cell. Bacteria, viruses, fungi, and microscopic worms called nematodes are able to induce the HR in plants.

In the midst of the reactions indicated above in the work of the HR, a plant alert mode known as Systemic Acquired Resistance (SAR) is often activated. In this state, a hypersensitive response is activated and plant tissues could be highly resistant to a wide group of disease causing microorganisms for a considerable time. In the SAR mode, a heightened level of preparedness is activated in which plant resources are mobilized in case of further invasion. Through advancements in research, simulated approaches have been adopted through spraying the plants with chemicals called plant activators to spark off the work of the SAR. This procedure has gained much acceptance in the agricultural community because it possesses no deleterious consequences to the environment and humans as a whole as compared to antibiotics or fungicides and their protective effects can last much longer (Freeman and Beattie, 2008: Lattanzio *et al*., 2006).

2.9.2.3 Function of certain phytochemicals

2.9.2.3.1 Alkaloids

According to findings reported by Achakzai *et al*. (2009), stem and leaves of almost all plant species contain alkaloids. They also reported that alkaloids in stem and leaves

of plants significantly decreased as the plant continue to grow. This means alkaloids are present in all leaves but the appropriate quantities may differ in terms of age and type of plant. Plant species utilize alkaloids to protect themselves against invasion by herbivores and pathogen. Plants are easy preys to predators due their rooted to the spot characteristic. Therefore, plants utilize alkaloid as a chemical defense against all their enemies. This then becomes a natural source of insecticides and fungicides. Also, alkaloids assist in the storage of waste nitrogen, cationic balancing and protection against parasites biologically. Another fundamental action of alkaloids is on the nervous system that's why its high concentrations in plants secure them from grazing animals (Achakzai *et al*., 2009).

2.9.2.3.2 Saponins

The fundamental roles of saponins in plants appear to provide defense against invasion by pathogen. According to Izuka and Mbagwu (2013), saponins are suspected to show a broad range of biological properties as antibacterial and antifungal agents. This means plant parts containing saponins could also be exploited as natural fungicides.

2.9.2.3.3 Tannins

Basically, according to Izuka and Mbagwu (2013), the high levels of tannins present in *T. aphylla* could be one of the main reasons why such plant parts are not preferred by ruminants especially sheep and goats. Okwu (2004) stated that tannin aids in wound healing. Tannin also exhibits antimicrobial potential by the deposition of iron, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells (Schroeter *et al.*, 2006). Other findings reported by Tatiya *et al*. (2011), stated that tannins react in a complex manner with the cell wall of bacteria and the

extracellular enzymes secreted. Tannins act as growth inhibitors towards many microorganisms including bacteria, yeasts and fungi by inhibiting the transport of nutrients into the cell and retarding growth of the organism. When tannins are complexed with microbial proteins or polysaccharides, the interactions formed are often irreversible, and this characteristic confers fungicidal and fungistatic properties.

2.9.2.3.4 Flavonoids

According to Izuka and Mbagwu (2013), flavonoids that are part of the phytochemical constituents of cocoa revealed a wide range of biological activities and one of which is their ability to scavenge for hydroxyl radicals. Flavonoid available in *Theobroma cacao* seeds as used in the components of cocoa and chocolate preparations has been found as the key antioxidant components.

2.9.3 Types of solvent and methods for extraction

Types of solvent and method of extraction of plant material should be carefully and systematically selected in order to increase the concentration of secondary metabolites in the extract. Tatiya *et al*. (2011) reported that phenolic compounds in plant extracts are more often associated with other molecules like proteins, polysaccharides, terpenes, chlorophyll and inorganic compounds. Hence, it requires suitable solvent for extraction of tannins and other phytochemicals. They further reported that acetone extract of *B. retusa* contains higher amount of phenolics as compared to other solvents used in their work. They also stated that among all the solvents used in their experiment; acetone-water and ethanol-water were better solvents for effective extraction of tannins and other phytochemicals as compared to solvents like water, methanol, and ethanol. This may be because phenolics are often extracted in higher

amounts in polar solvents. Nsor-Atindana *et al*. (2012) also reported that the differences observed in yields of extracts could be related to the polarity of solvent used in the extraction. Since the least polar solvent such as acetone yields the higher contents of phenolics than that of the most polar solvent such as water, higher yields of phenolics are obtained with a decrease in polarity of the solvent in the extraction of phenolic compounds. Thus, polarities of solvents really need to be considered during selection of solvent and method of extraction. Igbinosa *et al*. (2009) revealed in their studies that aqueous extract was not active against any of the organism tested at 10 mg/ml which was the highest concentration tested. They conclusively reported that aqueous extracts of plants generally showed little or no antimicrobial activities. Goyal *et al*. (2008) also explained the use of organic solvents in the preparation of plant extracts as compared to aqueous extracts. The polarities of antibacterial compounds make them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial species. This suggests that organic solvents are clearly better solvents for antimicrobial agents extraction. The active phenolic compounds or other phytochemicals present in respective plant extracts are influenced by many factors which include the age of plant, extracting solvent, method of extraction and time of harvesting plant materials (Okigbo and Ajalie, 2005; Okigbo *et al*., 2005,

Koolen *et al.*, 2013).

2.9.4 Structural defenses of plant cell

According to Freeman and Beattie (2008), in order to help inhibit pathogen attachment, invasion and infection, all plant tissues contain pre-formed structural barriers. Protection against fungal and bacterial pathogens in plants has been a function of the cell wall, which works as a major line of defense. Its functions are of both physical and chemical essence as it offers a perfect structural barrier that also integrates a vast

variety of chemical defenses that can be quickly triggered at the detection of the presence of potential pathogens by cells. After the cell stops growing, primary cell walls also form a secondary cell wall that develops inside of the primary cell wall. The primary cell wall contains cellulose, a complex polysaccharide comprising thousands of glucose monomers combine to produce long polymer chains. Two groups of branched polysaccharides such as cross-linking glycans and pectins may be incorporated in the cell wall. Cross-linking glycans include hemicellulose fibers that provide strength to the wall via cross-linkages with cellulose. Pectins form hydrated gels that help "cement" neighboring cells together and regulate the water content of the wall. Soft-rot pathogens usually target pectins for digestion using specialized enzymes that cause cells to break apart.

Highly reactive oxygen molecules efficient at annihilating the cells of attacking organisms are secreted when enzymes catalyze an oxidative burst after a plant cell identifies the invasion of external threats in the form of potential pathogen. Strengthening of the cell wall is facilitated when the reactive oxygen molecules catalyze cross-linkages between cell wall polymers and also signal cells that are nearby of an impending attack. The epidermis, which comprises the outermost protective tissue system of leaves, floral parts, fruits, seeds, stems, and roots is the elemental line of protection against attacking pathogens and consists of both specialized and unspecialized cells. Further threat is however presented by certain fungal pathogens such as *Fusarium solani*, which make cutinases that exhaust the cuticle for eventual penetration of the epidermis by fungi (Freeman and Beattie,

2008).

2.10 APPLICATION OF ANTIMICROBIAL (ANTIFUNGAL) AGENTS

2.10.1 Conventional Anti-Infective

Antifungal agents are used to control and prevent fungi related diseases and to promote growth in plant. The mechanism of action of antifungal agents is to either kill (fungicidal) or inhibit the growth of pathogen (fungistatic). However, these actions are also based on the concentration, the phase of growth of the pathogen and the fungi species (Otoo, 2015).

2.10.2 Overview of *In Vitro*

2.10.2.1 Antimicrobial assays

Antimicrobial susceptibility tests have over some years been used to test and decide the potency of antimicrobials from plant extracts against most microorganisms. These tests have been very helpful in screening plant extracts for antimicrobial potency and also useful in determining the minimum inhibitory concentration (the barest concentration at which organisms or pathogens are inhibited) of the compounds present in the extract under study (Das *et al.*, 2010).

According to Tenover *et al.* (1995), diffusion and dilution methods are the main classifications for antimicrobial susceptibility tests. The diffusion tests include agar disc diffusion and agar well diffusion. However, dilution tests include agar dilution, broth micro dilution and broth macro dilution methods.

2.10.2.2 Diffusion methods

2.10.2.2.1 Agar disc diffusion assay

The technique of this assay is an antifungal impregnated disc (6mm sterilize filter paper discs – Whatmann No. 1), which is used to test the susceptibility of a particular microorganism. Disc are saturated in the antimicrobial agent at appropriate concentration and kept on seeded agar and incubated at 37^0C for 24 hours. The disc is soaked with the antimicrobial agent (antifungal) before or after being placed on the inoculated plate (Lourens *et al.*, 2004). As reported by Schmourlo *et al.* (2005) refrigeration of the plates containing the seeded agar and disc is mostly done for about a period of an hour in order to promote the pre-diffusion of the antimicrobial chemical from the disc into the agar before incubation. The antifungal agent diffuses radially outwards by becoming less concentrated as it does so. An obvious zone of inhibition appears where growth has been inhibited. The test microorganism is regarded as more susceptible to the disc with the larger zone of inhibition. The areas of inhibition are then recorded from the circumference of the disc to the
circumference of the inhibition area (Salie *et al.*, 1996).

The agar disc diffusion assay is normally qualitative as the amount of extracts that adhere to the disc is not quantitatively determined (Leite *et al*., 2006). This method is not appropriate for quantification of the bioactivity of the extract. Advantage is that smaller amount of extract is needed for the test as compared to the agar well method

2.10.2.2.2 Agar Well diffusion assay

In this assay, a methodized concentration of inoculums with stable volume is spread evenly on the surface of gelled agar plate. Cork borer (number 6 or 6mm) is used to make four-cup wells separate and equidistant from each other in the agar. Label the

cups with the intended concentrations of the antimicrobial agent. Fill each cup with its corresponding concentration to about three quarters full. Keep them on the bench at room temperature for 30 to 60 minutes for the drug to diffuse into the agar. Incubate at optimum temperature and duration depending on the test organism but usually 24 hours (Mbata *et al.*, 2008).

In testing for extracts that do not easily diffuse through the agar or non-polar extracts, the diffusion technique is not appropriate (Cos *et al*., 2006). Hence, before incubation, the plate has to be stored at low temperature for a number of hours in order to achieve the required level of diffusion.

2.10.2.3 Dilution methods

In order to know the Minimal Inhibitory Concentration (MIC), the dilution method is the principal alternative. The dilution techniques can be done with liquid or solid media and maturation of the pathogens could be known in various means. In the agar dilution method, the MIC refers to the lowest concentration able to inhibit any visible microbial growth (Cos *et al*., 2006).

Furthermore, as reported by Cos *et al*. (2006), in the liquid or broth micro dilution method, the growth of pathogens can be determined by turbidity and redox-indicators. After incubation, plates are scrutinized for possible alterations in turbidity as an index of growth. The first set of wells that appears clear is taken as the MIC of the test extracts. By measuring optical density at 405 nm, turbidity may also be established more perfectly. Redox indicator 3-(4,5-dimethylthiazol-2-yl)-2,5dipheny ltetrazolium bromide (MTT) is usually used to identify microbial growth. Broth dilution method

also helps to determine whether an extract has cidal (no growth) or static (inhibiting growth) actions at a particular concentration after incubation. Generally, dilution methods are ideal for testing polar and, non-polar extracts for the determination of MIC (Cos *et al.*, 2006).

2.10.2.3.1 Agar dilution method

Agar dilution susceptibility testing is seen as the gold standard for all other susceptibility-testing methods (Hendrisksen, 2003). This assay includes the integration of various concentrations of the test sample into a nutrient agar medium and then the application of a standardized number of cells to the surface of the agar plate (Wiegand *et al.*, 2008). This method uses a stock solution of the extract prepared in its extracting solvent, filter-sterilized (0.22um) and then integrated in molten agar, cooled to 50^0C in a water bath to attain different concentrations of the extract in the agar (Silva *et al.*, 2005). The microbial inoculum is standardized and inoculated on the surface of agar and incubated at 37^0C for 24 hours. The lowest concentration of antimicrobial test substance that inhibits seeable maturation on the surface of agar is taken as the MIC (Otoo, 2015).

The agar dilution method has also been proven and accepted to be an equally good and optimal technique as compared to broth micro dilution. However, the agar dilution method allows the determination of susceptibility and minimum inhibitory concentrations (MIC) whereas the broth micro dilution method can only be used for the determination of MIC. The agar dilution method has been recommended as an alternative to broth micro dilution (Barry *et al.*, 2001)

2.10.2.3.2 Broth micro dilution method

The micro-titre plate or broth micro dilution method has provided potentially applicable techniques to determine MIC value of large numbers of test samples. Its advantages over diffusion techniques include increased sensitivity for small quantities of extract; ability to differentiate fungicidal effects from fungistatic effects; and quantitative determination of the MIC (Langfield *et al.*, 2004). Several microorganisms can also be assessed with this method. Its other advantages include the fact that it is not costly and its results can be reproduced. Often, in the micro-titre plate method, a stock solution of the extract is first obtained in a solvent which is usually the solvent used for extraction (Grierson and Afolayan, 1999) or in DMSO (Sokmen and Ozbek, 2006).

Table 2.1: Minimum inhibitory concentrations (MIC) of different plant extracts prepared against different pathogens

Plant Material	Part	Type of org.	Microorganism	Methanol	Ethanol	Acetone	Water	Reference
Adenophyllum aurantium	L	Fungi	*Fusarium. solani*	27.73 (mg/ml)				(Karla *et al*., 2014)
			Alternaria. alternata	27.73(mg/ml)				
Acalypha cuspidata	L		*Fusarium. solani*	12.50(mg/ml)				(Karla *et al*., 2014)
			Alternaria. alternata	12.50(mg/ml)				
Alloispermum integrifolium	L		*Fusarium. solani*	33.76(mg/ml)				(Karla *et al*., 2014)
			Alternaria. alternata	16.88(mg/ml)				
Echeveria acutifolia	L		*Fusarium. solani*	17.75(mg/ml)				(Karla *et al*., 2014)
			Alternaria. alternata	17.75(mg/ml)				
Galium mexicanum	L		*Fusarium. solani*	15.00(mg/ml)				(Karla *et al*., 2014)
			Alternaria. alternata	15.00(mg/ml)				
Lantana achiranthifolia	L		*Fusarium. solani*	12.50(mg/ml)				(Karla *et al*., 2014)
			Alternaria. alternata	6.25(mg/ml)				
Trema guineensis	L	Bacterial	S. enteritidis		31.5mcg/ml		32.5 mcg/ml	(Akinyemi *et al*., 2006)
			E. coli		31.5mcg/ml		31.5 mcg/ml	
			S. aureus		31.5 mcg/ml		33.5 mcg/ml	
Acalypha wilkesiana	L	Bacterial	S. enteritidis		45 mcg/ml		47.5 mcg/ml	(Akinyemi *et al*., 2006)
			E. coli		42 mcg/ml		40.4 mcg/ml	
			S. aureus		28. 5mcg/ml		28.5 mcg/ml	
Phyllathus discoideus	B	Bacterial	S. enteritidis		47.5 mcg/ml		49 mcg/ml	(Akinyemi *et al*., 2006)
			E. coli		37.8 mcg/ml		39.3 mcg/ml	
			S. aureus		27.0 mcg/ml		26.4 mcg/ml	
Callistemon viminalis			Bacillus cereus	0.8(mg/ml)			1.6(mg/ml)	(Delahaye *et al*., 2009)
			Escherichia coli	12.5(mg/ml)			12.5(mg/ml)	
			Pseudomonas aeruginosa	12.5(mg/ml)			12.5(mg/ml)	
			Shigella sonnei	12.5(mg/ml)			6.3(mg/ml)	
			Stephylococcus aureus	0.8(mg/ml)			1.6(mg/ml)	
			Candida albicans	1.6(mg/ml)			3.2(mg/ml)	
Clove			*L. monocytogenes*.			0.25(mg/ml)		Kaoutar *et al*., 2010)
Mint timija						0.315(mg/ml)		
Cinnamon						0.4(mg/ml)		
Cistus						0.52(mg/ml)		
Rose						0.9(mg/ml)		
Thyme						1.56(mg/ml)		
Wild thyme						2.15(mg/ml)		
Artemisia						3.75(mg/ml)		
Rosemary						5.25(mg/ml)		
Geranium						6.15(mg/ml)		
Camomile						6.75(mg/ml)		
Lavender						11.5 (mg/ml)		

Verbena	11.75 (mg/ml)

L – Leave, B – Back

2.11 ANTIOXIDANT

2.11.1 Oxidants and Antioxidants in Plant

2.11.2 The Role of Oxidant Stress in Plant Infection

Biotic and abiotic stresses such as UV stress, salinity, herbicide action, pathogen invasion (hypersensitive reaction), pH, and oxygen shortage, result in the overproduction of reactive oxygen species (ROS) in plants that are highly reactive and noxious. It also causes destruction to carbohydrates, DNA, lipids, and proteins that ultimately results in oxidative stress. ROS is made up of both free radical and non-radical (molecular) forms. The antioxidant defense machinery provides protection against oxidative stress damages. Plants possess very efficient enzymatic and non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds, alkaloids, non-protein amino acids and a-tocopherols) antioxidants that defend plant systems which work together to control the torrent of uninhibited oxidation and protect plant cells from oxidative damage by scavenging of ROS. ROS also affect the expression of a number of genes and therefore control the many processes like growth, cell cycle, programmed cell death (PCD), abiotic stress responses, pathogen defense, systemic signaling and development (Gill and Tuteja, 2010).

Several biotic and abiotic stress factors may alter the balance between the production and the scavenging of ROS. Such disturbances in equilibrium can lead to immediate increase in intracellular levels of ROS which then can cause serious damage to cell structures and it has been estimated that 1-2% of O2 consumption leads to the formation of ROS in plant tissues (Bhattacharjee, 2005).

2.11.2.1 ROS production in different organelles

The mitochondria seem to be the main ROS producers in the darkness. ROS production found to be as a result of an estimated 1-5% of the O2 consumption of isolated mitochondria (Moller, 2001).

2.11.3 Antioxidant defense mechanism

Effect of biotic and abiotic stresses on plant may elevate the production of ROS. However oxidative stress is a condition whereby ROS or free radicals are created extra- or intra-cellular that imposes their harmful effects to the cells. Cell membrane characteristics may be affected by these species and cause oxidative damage to nucleic acids, lipids, and proteins that may make them useless. Plant cells and its organelles like chloroplast, mitochondria and peroxisomes use antioxidant defense mechanisms to detoxify the harmful effects of ROS. They do this to shield themselves against these toxic oxygen intermediates. For protection against various stresses, the induction of the cellular antioxidant machinery is important. A great deal of research has corroborated that. (Gill *et al.*, 2011; Singh *et al.*, 2008).

Strengthened *in vivo* levels of antioxidant enzymes therefore can be a safe way to enhance plant stress tolerance. The significance of ROS detoxification for cellular survival is emphasized in the antioxidants found in almost all cellular compartments (Gill *et al.*, 2011).

2.11.4 Antioxidant Assay

2.11.4.1 Total antioxidant capacity

This assay is a spectrophotometric method outlined for the quantitative realization of antioxidant capacity (Prieto *et al.*, 1999). The test, in effect, depends on the reduction

of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate Mo (V) complex at acidic ph. The amount of phosphate Mo (V) complex formation is recorded by measuring the absorbance at 695 nm. A standard curve is prepared using solution of well-known antioxidant compounds (e.g. ascorbic acid, gallic acid, etc.). Total antioxidant values are expressed in terms of ascorbic acid or gallic acid equivalents (mg/g of dry mass).

2.11.4.2 Total phenol content

The estimation of the total phenol contents may be an indication of the antioxidant capacity of an extract. The Folin-Ciocalteu's reagent shows the total phenolic compound contents by measuring absorbance at 750 nm. Phenolic compounds go through complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reagent. This reaction is not peculiar to only polyphenols but to other compounds that could be oxidized by the reagent. Different scientists have reported poor specificity of the test (Escarpa and Gonzalez, 2001). Total phenol content is calculated as gallic acid/quercetin equivalent from the calibration curves.

2.11.5 Correlation Between Antioxidant and Phenolic Content

Phenolic compounds possess a wide spectrum of biocidal effects including antioxidant and free radical scavenging (Khanavi *et al*., 2009). Tatiya *et al*. (2011) confirmed that there might be a link between total phenolic and antioxidant capacity of different extracts. Moreover, antioxidant is not entirely dependent on phenolic content but it may be due to other phytoconstituents as tannins, triterpenoid or combined effects of them. Different types of phenolic compounds possess different antioxidant potentials. This is based mainly on their structures in the extract that exhibit various antioxidant capacities.

2.12 OSMOSIS

2.12 .1 Water Potential of Plant Tissues during Osmosis

The transfer of water molecules from a region of higher concentration to a rather lower concentration region through a semi-permeable membrane is what is termed osmosis. Thus, it requires a membrane that only permits water molecules but not solute molecules to penetrate it. Water molecules in distilled water are more concentrated than water in solutions because the solute has to occupy some room in the solution. When a cell is surrounded by distilled water or a solution with a lower concentration, osmosis will cause the cell to inflate through the flow of water from the surrounding solution into the cell. In such a situation, the external solution is said to be hypotonic to the solution in the cell (hypo meaning lower than and applies to the solute concentration). On the contrary, hypertonic solutions that surround cells possess concentrations that are higher and whose water potential is lower than that of the cell. This will cause water to flow out of the cell into the external solution. This implies that any hydrophilic solute (like sucrose or NaCl) will bind up hydrating water and restrict its movement. By building up turgor pressure, plant cells, with their thick cell walls, can resist the influx of water. Equilibrium is eventually achieved in a hypotonic solution through osmosis because as much water exits the cell due to turgor pressure, much enters (Kosinski, and Morlok, 2009).

2.13 COCOA (Theobroma *cacao L.*)

2.13.1 Botanical Description

Morgenstern, (2007) reported that out of about the 20 species of the genus Theobroma, *Theobroma cacao* is the most widely cultivated. To facilitate easy picking of the fruits,

cultivated trees are often kept low. They can enjoy a lifespan of over 200 years. Though commercially cultivated, they are known to be productive for about twenty-five years. Cocoa trees have a very distinctive appearance (average height of 10m to 20m). Cocoa has been found to never shed all its leaves. For this reason, young and mature leaves will be found growing together on the same tree. It is assumed that as a means of passive defense mechanism, the limp appearance of immature leaves discourage predators from attacking the plant as this could be an indicator that they are not worth consuming (Morgenstern, 2007).

2.13.2 Geographical Distribution

Brazil and Mexico have been identified as the origins of the tree. In its initial spread, the tree was sent into countries very close to the origins in the 15th century and this includes the regions across Central America and the Caribbean islands in the 16th century. The Indonesian islands received it from the Spaniards by 1560. The bean reached the West African Island called Fernando Po, from where it was later sent into the mainland (Meursing, 2008).

2.13.3 Ethnomedicinal Uses

The efficacy of cocoa in combatting enteric infections, diarrhea and to reduce secretions can be corroborated in folk medicine. It is also a good regulator of the thyroid gland and can also be used as a mild stimulant. Asthma, loss of appetite, poisoning, diarrhea, fractures, malaria parasites, pneumonia, cough, colic, general body weakness are some of the ailments that can be healed with a beverage made of beans and cocoa leaves. Skin conditions such as wounds, burns, split lip, fatigue etc. can also be treated with cocoa butter. Wounds can be disinfected with the young leaves (Kimball, 2012).

The alkaloids contained in the cocoa beans include caffeine, theobromine and theophylline. The caffeine it possesses does not however have a strong effect on the central nervous system like others. Its effect on the heart, kidney and muscles is however marked. Their ability to dilate the blood vessels makes them a good panacea for treating high blood pressure (Izuka, and Mbagwu, 2013).

2.13.4 Phytochemicals Activities in Cocoa Tree

2.13.4.1 Presence of phenols in cocoa

According to Hii *et al.* (2009), cocoa (*Theobroma cacao L.*) is rich in polyphenols and has been reported to possess higher amount of antioxidant potential than red wines and teas and the concentration of (-)-epicatechin varies from 2.66 mg/g to 16.52 mg/g for cocoa beans derived from different countries. However, Hii *et al.* (2009) further explained that processing of the beans affects the quantity of polyphenols constituted in the cocoa powder. Alkalized cocoa powder cannot compare with natural cocoa powder in polyphenol content. Othman *et al.* (2007) reported that geographical origin indicate diverse levels of antioxidant capacity but also depends on the type of solvent used in extraction. Ghanaian cocoa beans have been found to contain the highest levels of antioxidants. The next country whose beans possess high levels of antioxidants is Ivory Coast, followed by Malaysia and Sulawesi.

2.13.4.2 Cocoa leaves and their potential properties

According to Osman *et al.* (2004), cocoa leaves extracts contain phenolic compounds with high antioxidant and antimicrobial properties. Winkelhausen *et al*. (2005) confirmed that extracted phenolic compounds have the potential properties of

inhibiting the growth of fungi. Besides the antioxidant properties of phenol compound, these compounds possess antimutagenic, anticarcinogenic, antiglycemic (Visioli and Galli 1998) and antimicrobial characteristics. Defense in plants against pathogens including fungi, viruses and bacteria seems to be facilitated by these compounds (Marsilio *et al.*, 2001). Moreover, Osman *et al*. (2004) stated that blanching parameters were enough to deactivate peroxidase in the leaves. The deactivation of peroxidases is an important preliminary step in the process to change the leaves into a green tea-like product, unlike black tea, which allows extensive oxidation to occur.

Furthermore, Osman *et al*. (2004) have indicated that cocoa leaves extracts, particularly the cocoa shoot; possess high content of phenolic antioxidant compound than green tea. They therefore recommended that, through the use of proper pruning and management, ‘‘waste’’ leaves that are found in cocoa plantations could be judiciously utilized as a novel source of natural bioactive extract. Hence, the emergence of natural extracts that contain phenolic antioxidant and antimicrobial properties could help to reduce the modern dependency on synthetic chemical as a means of controlling white yam storage spoilage microorganisms.

2.13.5 Importance of Pruning

According to Flood (2003), Pruning is an essential element of cocoa management and it involves thinning of branches and removal of old, unproductive chupons or dead stems. Pruning serves many purposes including the determination of the shape of the tree; maximizing the nutrient distribution towards pods such as cutting away chupons on mature trees; and finally help to prevent certain pest problems such as pruning mistletoe infected branches.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SURVEYS ON PRE-EXPORT HANDLING PERIOD AND EXPORTERS KNOWLEDGE ON YAM ROT.

A field survey was conducted on ten exporters at the exporters' warehouses located at Agbogbloshi and Tema in the Greater Accra Region. This information was used to outline the various activities conducted during the pre-export period and the knowledge of exporters on yam rot. The survey was conducted between June and July 2014.

3.1.1 Questionnaire Construction and Administration

Relevant data was obtained through semi-structured questionnaire and observation. The exporters interviewed were randomly selected at their warehouses. The design of the questionnaire was based on the activities involved in the pre-export handling period and major challenges confronting exporters. Challenges linked to yam rot diseases as well as other relevant information pertaining to storage at pre-export handling period were collected. Semi-structured questionnaires were administered to exporters

engaged in yam export in the selected warehouse randomly. The criterion for selection of exporters was based on exporters who frequently export yam to UK, USA, Netherlands, Italy, Canada and Germany. Ten exporters were randomly selected based on this criterion.

3.2 LABORATORY EXPERIMENTS *IN VITRO*

The experiments were conducted in three (3) laboratories located at Department of Horticulture, Department of Pharmacognosy, and Department of Crop and Soil Sciences on Kwame Nkrumah University of Science and Technology (KNUST) campus, Kumasi - Ashanti region. The research was conducted between the September 2014 and June 2015.

3.2.1 Collection and Preparation of the Cocoa Leaves

Cocoa leaves were plucked (5^{th} leaf to the 8^{th} leaf) from cocoa plants at Juaso Cocoa Research Station. This was because Osman *et al.* (2004) reported that the young cocoa leaves (5^{th} leaf to the 8^{th} leaf) contained significantly higher phenolic compounds than cocoa shoot (the apex to 4^{th} leaf) and this may be due to accumulation of phenolic compounds in the "older". The leaves were generally processed in accordance with the principles of green tea processing. The fresh young cocoa leaves were steam-blanched (fixation stage) for four and half minutes, to inactivate peroxidases. The leaves were then dried in a convection oven (45 °C, air velocity 6.6-16.5m^3 min^{-1}, 18 h) until the moisture content reached 8% (w/w) (Osman *et al*., 2004). Dried cocoa leaves were then ground into powder using a grinding machine. The powdered cocoa leaves were sealed in plastic bag and stored in ambient temperature and humidity until use.

3.2.2 Phytochemical Screening (Qualitative Analysis)

Phytochemical tests were carried out, first, to establish the presence of some specific phytochemicals by protocols as outlined by Trease and Evans (1989).

3.2.2.1 Qualitative Test for flavonoids

Aqueous solution of the cocoa leaves extract was prepared by maceration; filtered and then a strip of filter paper was dipped in the aqueous cocoa leaves extracts. The filter paper was then dried, exposed to ammonia solution and an intense yellow colour was observed. The yellow colour disappeared when the same strip of filter paper was exposed to fumes of HCL. This indicates the presence of flavonoids (Trease and Evans, 1989).

3.2.2.2 Qualitative test for glycosides

200 mg of cocoa leaves powdered extract was warmed with 5 ml of dil. HCL in a water bath for 2 minutes and filtered. The filtrate was made distinctly alkaline by adding 5 drops of 20% NaOH. It was tested with a pH paper. 1ml each of Fehling's solution A and B were added to the filtrate and heated on the water bath for 2 minutes. A brick red precipitate was observed and this indicates the presence of reducing sugars (glycosides) (Trease and Evans, 1989).

3.2.2.3 Qualitative test for saponins

Powdered cocoa leaves were mixed with 5 ml of distilled water in a test tube and was shaken, filtered and the filtrate was shaken again. Froth persisted for 5 minutes after shaking. This indicates the presence of saponins (Trease and Evans, 1989).

3.2.2.4 Qualitative test for alkaloid

Powdered cocoa leaves were extracted with ammoniacal alcohol and filtered. The filtrate was evaporated to dryness. 1% H_2SO_4 was added to the cocoa leaves extract residue and filtered. The filtrate was rendered distinctly alkaline by adding dilute ammonia solution. It was then shaken with chloroform. The chloroformic extract was separated and evaporated off. The residue was dissolved in 1% H_2SO_4 and one drop of Mayer's reagent was added to the 1% H_2SO_4 extract and a buff precipitate was observed. This indicates the presences of alkaloids (Trease and Evans, 1989).

3.2.2.5 Qualitative test for triterpenoids

Chloroformic extracts were prepared from the powdered cocoa leaves. 5ml of the extract was added to concentrated sulphuric acid carefully down the side of the tube to form a lower layer. A cherry red colour was observed and this indicates the presences of triterpenoids (Trease and Evans, 1989).

3.2.2.6 Qualitative test for coumarins

Chloroformic extracts were prepared from the powdered cocoa leaves. 5ml of the cocoa leaves extract was evaporated to dryness and the residue was dissolved in 5 ml hot distilled water and cooled. 0.5ml of 10% ammonia solution was added to the cocoa leaves extract in a test tube. Bluish green fluorescence was observed under UV light and this indicates the presences of coumarins (Trease and Evans, 1989).

3.2.3 Preparation of Crude Cocoa Leaves Extract

3.2.3.1 Crude Aqueous CL Extract preparation

500g of the powdered cocoa leaves were subsequently extracted at room temperature with 4500ml of sterile distilled water for seven (7) days to obtain filtrate of cocoa leaves aqueous solution. Within the seven (7) days, it was stored in a refrigerator for five (5) nights. The filtrate obtained was concentrated in a water bath at 60°C to a reddish-brown crude extract.

3.2.4 Preparation of Refined Cocoa Leave Extracts

3.2.4.1 Ethylacetate Purified CL Extract preparation

The polyphenols were extracted in accordance to Todd and Paul (1996) method as described by Osman *et al.* (2004). The dried powdered cocoa leaves (100 g) were extracted with anhydrous methanol; enough water was added to keep the mass liquid.

After standing for 90 min, the methanol was evaporated using a rotary evaporator. Hexane (90 ml) was added to the mixture and agitated. The water-insoluble hexane phase was separated from the water phase and the water phase was again extracted with 30 ml of hexane. The hexane phase was separated when 10 g of sodium chloride was added to the water layer and the pH was adjusted to 3.5 with phosphoric acid. The aqueous phase was extracted twice with 150 ml of ethyl acetate. The ethyl acetate was evaporated until a dry solid catechin-rich fraction was obtained and kept in a capped bottle.

3.2.4.2 Acetone Purified CL Extract preparation

This was carried out by the method outlined by McMurrough (1996). 50g of finely powdered cocoa leaves were extracted with 200ml acetone-water (3:1) mixture for 48

hours at room temperature. The filtrate was saturated with 10g of sodium chloride to promote phase separation. The upper phase which contained the phenolics was retrieved and concentrated on a water bath at 70 °C.

3.2.5 pH Test of Extracts Prepared

Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract were respectively mixed with distilled water at a ratio of 2:5. The three (3) extracts turned blue litmus paper into red.

3.2.6 Antioxidant Assay of Extracts

3.2.6.1 Determination of total phenolic content of extracts

Total phenolic content determination of the plant material (Cocoa Leaves) was done according to the method outlined by McDonald *et al.* (2001) with slight modification. 0.5ml of each plant extract (0.03125-1mg/ml in methanol) was separately mixed with 0.1ml of Folin Ciocalteu's reagent and incubated for 15 minutes. Then 2.5ml of aqueous Na_2CO_3 (2 %w/v) was added to the mixtures and incubated at room temperature for 30 minutes. 200µl of the mixtures was picked into micro-titer plate and took absorbance at 760nm. 1 ml tannic acid was processed in the same way as the tested extracts and used as blanks. The standard curve was prepared by preparing tannic acid solutions (0.03-0.1 mg/ml in methanol). Total phenol content was calculated using calibration curve of tannic acid and total phenol values were expressed in terms of tannic acid equivalents (mg of tannic/g of dry material).

3.2.6.2 Determination of total antioxidant capacity of extracts

Total antioxidant capacities of the extracts were determined using the phosphomolybdate method by Prieto *et al.* (1999) with slight modification. 1ml of each extract (0.03125-1.0g/ml) was separately mixed with 3ml of reagent solution (0.6M sulphuric acid, 28nM Na_2HPO_4 and 4mM ammonium molybdate) which served as a positive control. The mixtures were incubated at 95°C for 90 minutes and then allowed to cool to room temperature. 200µl of mixtures were picked into microtitre plates and the absorbance determined at 695nm. Vitamin E was processed the same way as tested extracts and used as blanks. The standard curve was prepared using solutions of Vitamin E *(in methanol*). Total antioxidant capacity was calculated using calibration curve of Vitamin E and total antioxidant value are expressed in terms of Vitamin E equivalent (mg/g of dry extract).

3.2.7 Collection and Preparation of Spoilage Microbial Organism

Eighteen (18) white yam tubers (*Dioscorea rotundata)* with rots arising from natural infections were used for the study. Random sampling of infected white yam tubers was collected from marketplaces and exporters' warehouse. Ten (10) samples from marketplaces including Ejisu, Konongo and Tafo-Kumasi whereas the other eight (8) samples of spoilage white yam were from three (3) exporters located at Tema and Agbogbloshi. Samples were taken from the advancing regions of the rotten tissues of the tuber and each sample was individually wrapped in a plain polythene bag and sent to the laboratory. Samples were then preserved at 5°C –10°C until isolation and identification of the spoilage microorganisms (Aboagye-Nuamah *et al*., 2005).

3.2.7.1 Isolation and identification of spoilage microorganism

The microorganisms associated with the postharvest rots of white yams were isolated from the rotten white yam tubers using the methods of Aboagye-Nuamah *et al*. (2005). The rot causing pathogens were isolated from a sample of yam tissue segment measuring approximately 5 mm^3 taken from the advancing lesion margins of each rotting yam tubers with a sterilized scalpel. The surfaces of the tissue samples were cleaned in distilled water and then the surface was sterilized with 1% (w/v) sodium hypochlorite solution (Power zone bleach) for 2 min. The sterilized white yam tissues were kept on sterilized tissue paper in a petri dish to dry for 15–30 min under room temperature (28°C). Initial isolation of disease pathogen was done by inoculating a tissue sample from each tuber onto a plate of water agar (PDA). Three (3) replicate plates were used for each sample taken. All plates were kept under room temperature and the cultures were checked every 24h. Pathogens were sub cultured on potato dextrose agar (PDA). A series of sub-cultures were done until individual pure cultures were obtained (Aboagye-Nuamah *et al*., 2005). The identification of respective fungi was conducted based on their morphological features using light microscope (Model – Labomed, Magnification = x400, Eye piece = x10 and Objective lens = x40) to observe the characteristics of the colony on PDA and conidial structures. The characteristics of the cultures included growth rate, colour and gross colony morphology as described by Mathur and Kongsdal (2003) and Barnett and Hunter (1972). Data was taken on the source of tuber and the characteristics of each pathogen. Frequencies of occurrence of each spoilage microorganism were estimated as a percentage of the total isolates.

3.2.7.2 Preparation of fungi for antimicrobial assays

Preparation of solution of identified fungi at known concentration was done by method described by Kerry (1999) but with slight modification. 20 ml of the molten PDA each was poured in four (4) petri dishes. These were gently rotated to ensure even dispersion of the molten PDA and then allowed to solidify. A 6mm sterilized cork borer was used to pick each fungus from their respective cultured plates and placed each at the center of the four (4) petri dishes containing the PDA. They were left for seven (7) days to obtain pure cultures of each of the four test fungi. After seven (7) days, each plate was first examined for possible growth of contaminants and the absence of any growth confirmed that they could be used to prepare the various concentrations. 10ml of sterilized distilled water were poured on each of the cultured plates, mixed with mycelia on the PDA and poured in four (4) different beakers. Syringe was used to suction small amount of the concentrated solution of the fungi mycelia. This was poured on the hemacytometer and placed under the microscope (Model – Labomed, Magnification = x400, Eye piece = x10 and Objective lens = x40) to count their respective spores. Then 20ml of each fungus was prepared at a concentration of $1x10^5$ by using the spores counted, expected concentration, and final volume to calculate volume of stock of fungi. The volume of the stock determines the volume of distilled water to top up to the final volume to the expected concentration ($1x10^5$).

Below is the formula used;

$$Vs = \frac{Ce \times Vf}{Cc}$$

Top up volume of distilled water = Vf – Vs

Where by: Cc = spores counted, Ce = expected concentration, Vf = final volume fungi solution and Vs = volume of stock of fungi

3.2.8 Antimicrobial Assays of Extracts

In determining the antimicrobial susceptibility and minimum inhibitory concentration (MIC) of Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract, the agar well diffusion and broth micro dilution method were used according to Vanden-Berghe and Vlientinck (1991) and

Cos *et al.* 2006 protocols with slight modification.

3.2.8.1 Agar well diffusion method

The antimicrobial activities of Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract against *Aspergillus niger, Aspergillus flavus, Rhizopus stolonifer* and *Penicillium spp.* were determined using method outline by Vanden-Berghe and Vlientinck (1991) but with slight modification. 20 ml of the prepared potato dextrose agar (PDA) was measured into sterilized test tubes and mixed with 0.5ml each of the diluted microorganisms (each at a concentration of $1x10^5$) and then each poured into different sterilized petri dishes, which were labeled appropriately. The molten PDA mixed with the microorganisms were allowed to settle. A 6mm sterilized cork borer was used to create holes in which the concentrations of 40mg/ml-5mg/ml of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, Crude Aqueous CL Extract and Mancozeb (40mg/ml0.625mg/ml) were poured into respective holes in the PDA and labeled. The plant extracts were allowed to settle to enable them to diffuse before the plates were incubated for 24 hours at temperatures of 37°C according to antifungal assay.

Experiments were carried out in duplicates for the plant extracts.

3.2.8.2 Broth micro dilution method

Minimum inhibitory concentration (MIC) values of Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract were determined using broth micro dilution method outline by Cos *et al.* 2006 but with slight modification. Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract were used to prepare aqueous solutions through serial dilution at concentrations from 60 mg/ml to 0.625 mg/ml. Since respective concentrations of extracts were mixed with an equal volume of double strength nutrient broth, eventual concentrations of extracts in-well were between 30mg/ml to 0.3125 mg/ml. The sterile micro-titre plate containing 96 wells, and in each well was dispensed 100µL of double strength nutrient broth, 100µL each of the plant extract concentrations, and 20µL of the inoculums. The micro-titre plate was incubated at 37°C for 24 hours. Susceptibility or otherwise of the microorganisms were determined by 20µL of a 5% solution of MTT. Blue-black wells indicated the presence of growth of the microorganisms. The nutrient broth only and sterile distilled water served as negative controls and Mancozeb (40mg/ml -0.625mg/ml) served as positive controls. The experiment was carried out in triplicates for Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract fractions. The MIC values for Ethylacetate Purified CL Extract (as the best among the refined phenol extract) and Crude Aqueous CL Extract, which were lowest concentration of the extract that prevents fungal growth, were regarded as concentration applied for *in vivo* experiments.

3.2.9 Experimental Design

The experimental design was a 4 x 4 x 4 factorial arrangement in Completely Randomized Design (CRD) with three replications. The factors were Extracts at four levels; Ethylacetate Purified CL Extract, Acetone Purified CL Extract, Crude Aqueous

CL Extract and (Mancozeb (control); Inoculums at four levels; *Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer* and *Penicillium spp.* and Extract Concentration at four levels; 40mg/ml, 20mg/ml, 10mg/ml and 5mg/ml.

3.3 CONTROL OF ROT ON EXPORT GRADE WHITE YAM WITH COCOA LEAF EXTRACTS WITHIN PRE-EXPORT HANDLING PERIOD

Seventy-two export grade white yams were randomly collected from the exporters' warehouse. The tubers were taken through the same pre-export handling activities practiced by the yam exporters before shipment. They were stored for nineteen days, to depict the 2-3 weeks storage usually provided for yam during pre-export handling stage.

3.3.1 Collection and Preparations of White Yams at Pre-Export Handling Stage

Seventy-two tubers were randomly selected from the exporters' warehouse, packed in boxes and transported to the Department of Horticulture Laboratory, KNUST. During the selection, tubers with rotting spots or wrinkled skin (indicating nematode activity) were discarded. The tubers were brushed and thoroughly washed under running tap water. Tubers were air dried for a day on wooden pallets. The tubers were partitioned into three (3) replicates (24 tubers per replicate), while each tuber was placed in their respective cell created on the pallets to avoid contamination among tubers during treatment and inoculation (Plate 3.1). The tubers were labeled for ease of identification and weighed before the commencement of the experiment.

Empty Yam Cells on Pallet Filled Yam Cells on Pallet

Plate 3.1: Empty pallet used for storage and arrangement of treated yams on the pallet in their respective cell during pre-export storage period.

3.3.1.3 Isolation and identification of fungi pre-existed on the surfaces of the yam tubers

Twelve (12) yam tubers were randomly selected from the seventy-two (72) samples. They were sprayed with distilled water individually. The dripping water was collected separately from each of the twelve (12) sprayed tubers. 5ml of the drip from each tuber was poured on labeled plats containing solidified PDA and cultured for seven (7) days. Then identification of respective fungi was conducted based on their morphological features using light microscope (Model – Labomed, Magnification = x400, Eye piece = x10 and Objective lens = x40) to observe the characteristics of the colony on PDA and conidial structures. The characteristics of the cultures included growth rate, colour and gross colony morphology as described by Mathur and Kongsdal (2003) and Barnett and Hunter (1972). Frequencies of occurrence of each spoilage microorganism were estimated as a percentage of the

total isolates

3.3.2 Treatment of Yam Tubers with Crude Aqueous CL Extract and Ethylacetate Purified CL Extract under Different Method of Application. The seventy-two (72) tubers grouped into three (3) replicates (with 24 tubers per replicate) were divided into four (4) sets according to the number of treatments (Ethylacetate Purified CL Extract, Crude Aqueous CL Extract, Mancozeb, and Distilled Water), resulting into six (6) tubers per set. Each of these sets was further divided into two (2) subsets (three (3) tubers per set) based on the two (2) methods of application (i.e. dipping and spraying) of the extracts and controls.

At phase III, Ethylacetate Purified CL Extract and Crude Aqueous CL Extract were selected for the *in vivo* study. This was because among the refined extracts, Ethylacetate Purified CL Extract exhibited significant inhibition and better MIC value than Acetone Purified CL Extract at the phase II study. Also, Ethylacetate Purified CL Extract was the only extract in the study with the MIC value closest to that of Mancozeb. Based on this, the Ethylacetate Purified CL Extract was selected with Crude Aqueous CL Extract, which was economically cheapest and easiest to prepare. Mancozeb was used as positive control while Distilled Water was used as negative control. Concentrations of Ethylacetate Purified CL Extract, Crude Aqueous CL Extract and Mancozeb were deduced by doubling the concentrations obtained from the MIC values in the *in vitro* experiment at phase II (Table 3.1). After application of extracts to yam tubers, they were air dried for 24 hours.

Table 3.1: Concentration of extracts and volume of water used for each method of application *in vivo*

Quantity and Units	Ethylacetate Purified CL Extract	Crude Aqueous CL Extract	Mancozeb	Distilled Water
In vivo Concentration *(mg/ml)*	5	30	4	-

Volume Used in Spraying *(ml)*	300	300	300	300
Volume Used in Dipping *(ml)*	2700	2700	2700	2700

3.3.2.1 Preparing fungi to inoculate treated yam tubers

After tubers were thoroughly dried, inoculums of three (3) fungi (*Penicillium* spp., *Aspergillus flavus,* and *Rhizopus stolonifer*) out of the four (4) identified and used in the *in vitro* at phase II were selected for *in vivo* application. As indicated *in vitro*, *Penicillium* spp., *Aspergillus flavus, Aspergillus niger,* and *Rhizopus stolonifer* were isolated. Although, four (4) fungi were isolated, three (3) fungi including *Penicillium* spp., *Aspergillus flavus,* and *Rhizopus stolonifer* were selected for the phase III (*in vivo*) study. The three (3) were selected *in vivo* because *Penicillium* spp. was found to be associated with dry rot (IITA, 1993), and also recorded most virulent and prevalent among the fungi isolated in this study. *Rhizopus stolonifer* found to be associated with soft rot (Aidoo, 2011) was the second most virulent and prevalent among the isolated fungi. *Aspergillus flavus*, which least occurred among the fungi but considered as very hazardous to human and animal health because it produces aflatoxins (B1, B2, G1, G2). Aflatoxin B1 is known to be highly carcinogenic causing hepatoma (Ogaraku and Usman, 2008), so *Aspergillus niger* was excluded from the three (3) test fungi used as inoculums *in vivo*. 70ml of inoculums at a concentration of 1×10^5 of each fungus were prepared according to the same method outlined in phase II with microscope (Model – Labomed, Magnification = x400, Eye piece = x10 and Objective lens = x40) and Hemacytometer.

3.3.2.2 Inoculation of treated white yam tubers

Before applying the inoculum, the length of each tuber was measured. Each tuber was equally demarcated into three (3) sections namely anterior, middle, and posterior. Each

inoculum was applied separately on their respective group of tubers with syringe, brush (1/4 Inch) and PVC cover (0.15mm thick) bearing square hole (3cm x 3cm). On each tuber, the 1ml of the respective inoculum was applied to the anterior, middle, and posterior sections. Thus, each inoculum was applied to eight (8) tubers per replicates (i.e. 2 tubers per treatment per inoculum).

3.3.3 Parameters Monitored

The experiment consisted of monitoring the temperature and relative humidity of the pre-export environments and some quality parameters of pre-stored yam. The quality parameters determined were rot development (incidence and severity), diameter, length, weight loss, and rate of sprouting. The tubers were stored for two (2) weeks to meet all specification for export.

3.3.3.1 Environmental parameters

Temperature and Relative humidity were measured at every hour per day for even distribution of measurement within the storage period assumed to be the pre-export storage period (19^{th} day). Temperature and humidity readings were taken using Lascar Electronic EL-USB-2 Data Logger with EasyLog USB Version 5.40 software.

3.3.3.2 Physiological parameters:

Weight loss, rotting, diameter, length and sprouting were evaluated during the preexport period, which lasted for nineteen (19) days. To evaluate the weight loss the tubers were initially weighed before treatment. This was done every two (2) days starting from the first day of the storage period of the pre-export handling period. The percentage weight loss for each month was computed based on the initial tuber weight.

To assess sprouting rate, de–sprouting was carried out manually. This was done weekly. During weighing, the tubers were visually examined for rot development and the numbers of rot (incidence) and size of rot (severity) per section (anterior, middle, and posterior) were recorded. Differences in length and diameter of tubers within the period of the experiment were evaluated to determine size (circumference of rot infected area) of rot per tuber. The length and diameter were measured with measuring tape (cm) and digital caliper (mm) respectively at the commencement and ending of the experiment. The diameters of each tuber were measured three times (3x) at a specified point, marked respectively at the anterior, middle and posterior sections. At each point, the caliper was used to take three (3) readings around each tuber and the average reading of that point was taken as the diameter at that particular section of the tuber.

3.3.4 Isolation and Identification of Fungal Pathogen Associated with Initiation Rot on Infected Yam Tubers

Infected yam tubers were selected then they were cut opened with surface sterile knife to reveal the region of rot and with the aid of caliper, the size of rot was measured. A section of about 2×2mm was cut at the interphase between the healthy and infected portions of each tuber. The tuber pieces were surface sterilized by dipping completely in 1% (w/v) sodium hydrochlorite (NaOCl) solution for 2 minutes and rinsed once in sterilized distilled water. The sterilized white yam tissues were kept on sterilized tissue paper in petri dishes to dry for 15–30 minutes under room temperature. The Potato Dextrose Agar (PDA) was prepared according to the manufacturer's instruction and poured into sterile petri dishes for inoculation, after which each of the 2×2mm piece of tissues was inoculated onto PDA plate for 7 days. All plates were kept under room temperature and the respective cultures were checked in every 24 hours. Pathogens

were subculture on potato dextrose agar (PDA). A series of sub-cultures were done until individual pure cultures were obtained for identification (Aboagye-Nuamah *et al*., 2005).

The identification of respective fungi was conducted based on their morphological features using light microscope (Model – Labomed, Magnification = x400, Eye piece = x10 and Objective lens = x40) to observe the characteristics of the colony on PDA and conidial structures. The characteristics of the cultures included growth rate, colour and gross colony morphology as described by Mathur and Kongsdal (2003) and Barnett and Hunter (1972). Data was taken on the source of tuber and the characteristics of each pathogen. Frequencies of occurrence of each spoilage microorganism were estimated as a percentage of the total isolates.

3.3.5 Experimental Design

The experimental design was 4 x 3 x 2 factorial experiment arrangement in CRD. Four (4) treatments (two (2) Extracts (Ethylacetate Purified CL Extract and Crude Aqueous CL Extract), two (2) controls (Mancozeb (+), Distilled water (-), three (3) Inoculums (*Aspergillus flavus, Rhizopus stolonifer* and *Penicillium Sp*) and two (2) methods of application (spraying (S) and dipping (D)). All experiments were carried out in three replicates.

3.3.6 Data Analysis

3.3.6.1 Preliminary survey data analysis (Phase I)

Data collected from the warehouses were analyzed using Microsoft Excel version 14.5.3. The data output was presented in tables, flowchart and graphs.

3.3.6.2 *In vitro* data Analysis (Phase II)

Data collected was subjected to statistical analysis using Analysis of Variance (ANOVA). Statistical package used was Statistix version 9. Testing for differences between means was at 5% level ($P<0.05$). Data collected at the end of the antioxidants assays were analyzed using Graphpad. Version 5.01.

3.3.6.3 *In vivo* data Analysis (Phase III)

Data collected was subjected to statistical analysis using Analysis of Variance (ANOVA). Statistical package used was Statistix version 9. Testing for differences between means were at 5% level ($P<0.05$). Excel version 14.5 was used to develop graphs.

CHAPTER FOUR

4.0 RESULTS

4.1 PRELIMINARY STUDIES OF ACTIVITIES INVOLVED IN PREPARATION OF YAM FOR EXPORT

All persons interviewed under this survey were directly engaged in the exportation of yam. A total of ten (10) exporters were interviewed to ascertain their background information, activities involved in their pre-export handling period, varieties of yam ideal for export, sources of their yam supplies, and challenges. Figure 4.1 reveals that seventy percent (70%) of the respondents interviewed were male while thirty percent (30%) of them were female. The ages of exporters interviewed ranged between thirty-five (35) and fifty-four (54) years (Figure 4.2).

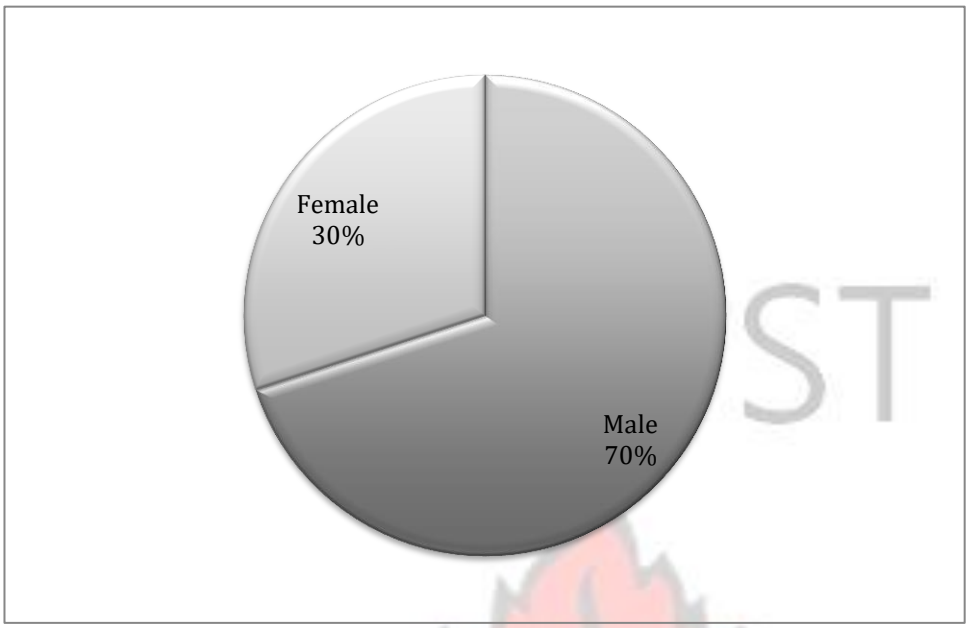


Figure 4.1: Gender of Exporters Interviewed During the Survey

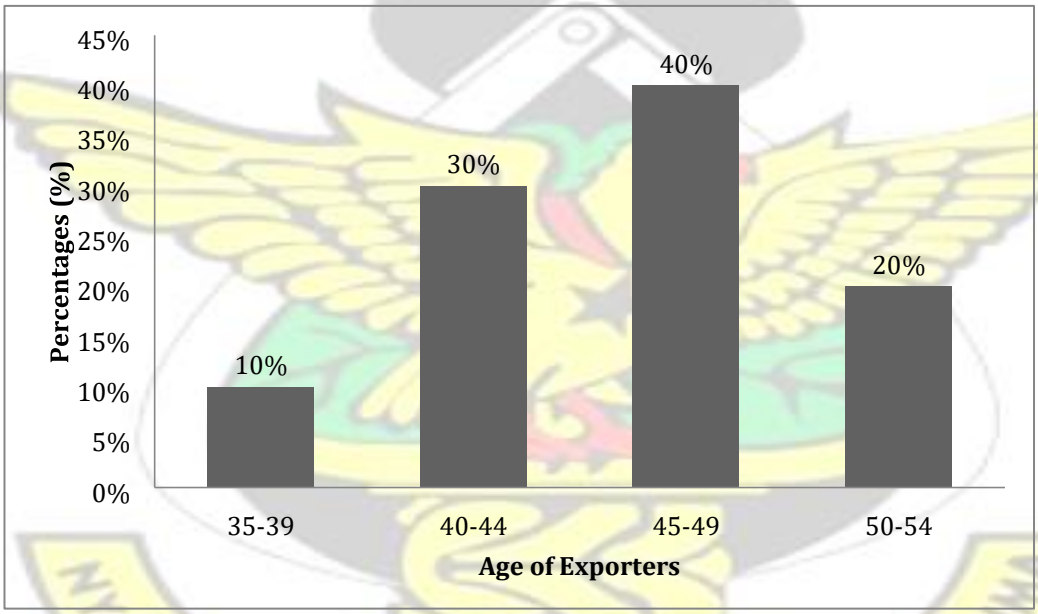


Figure 4.2: Age Range of Exporter Interviewed During the Survey

The survey revealed that white yam was mostly used for export but the varieties of white yam per export varied per season. The variation depended on the farming season and existing demand for yam at local and international market. It was realized that some of the white yam varieties mostly exported were ‘pona’, ‘dente’, ‘serwa’, ‘lily’,

'labreko', 'kwakaw', 'asobayere', and 'lawbayere'. Among these varieties, respondents ranked 'Pona' (40%) as the most preferred yam for export (Figure 4.3).

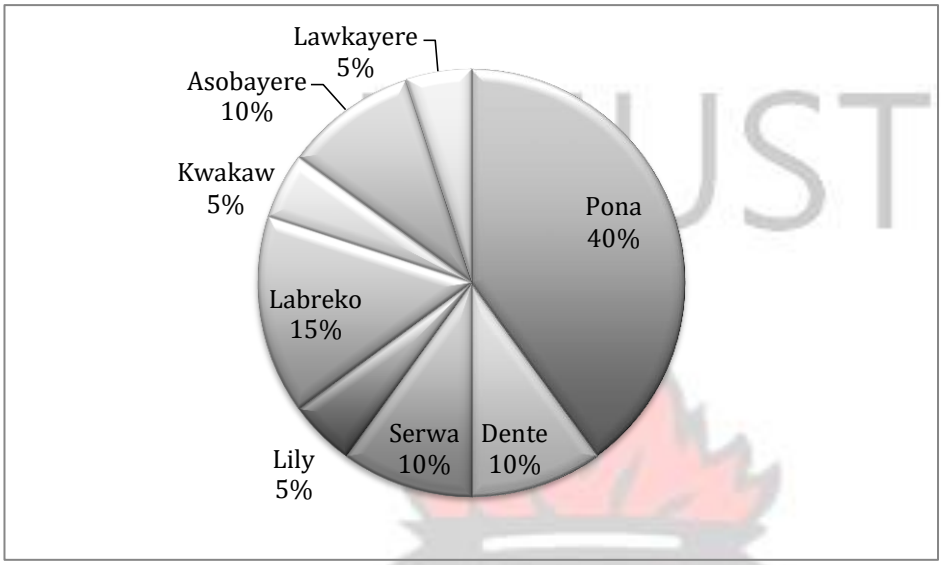


Figure 4.3: Most Preferred White Yam Varieties For Export in Ghana

4.1.0 Major Regions for Yam Production

The sources of yam for the export market are presented on figure 4.4. Most (40%) of the respondents indicated that they sourced their yams from Brong Ahafo Region followed by Northern Region (20%), Upper West (15%), Eastern (10%), Volta (10%) and Western (5%). None sources yam from Central, Greater Accra and Upper East Region of Ghana.

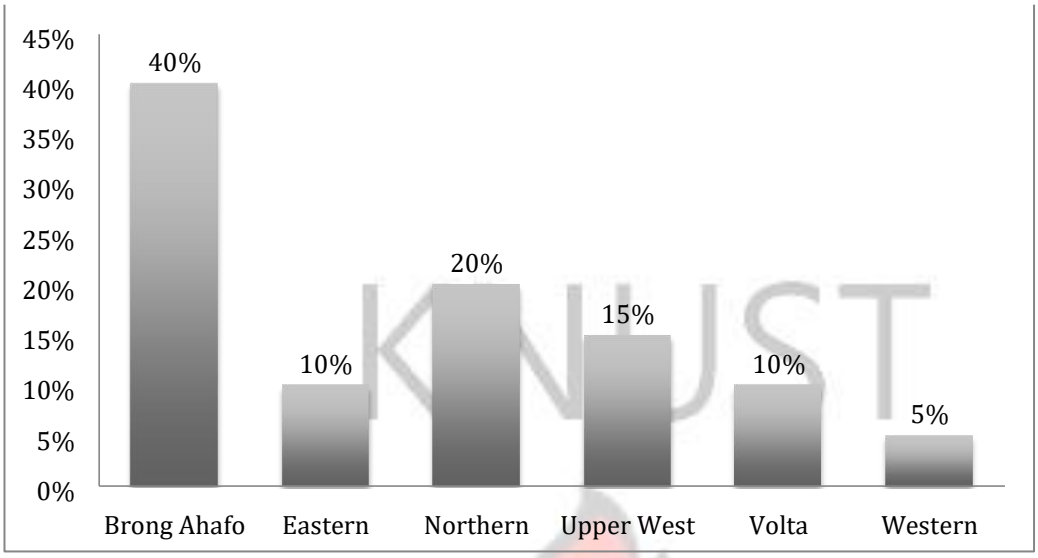


Figure 4.4: Sources of Exporters Yam Supply from Different Regions in Ghana

4.1.1 Pre-Export Handling Operations in Ghana

Figure 4.5 presents the linkages from farm gate to exporter's warehouses. Yams were purchased by both middlemen and exporter at the farm gate.

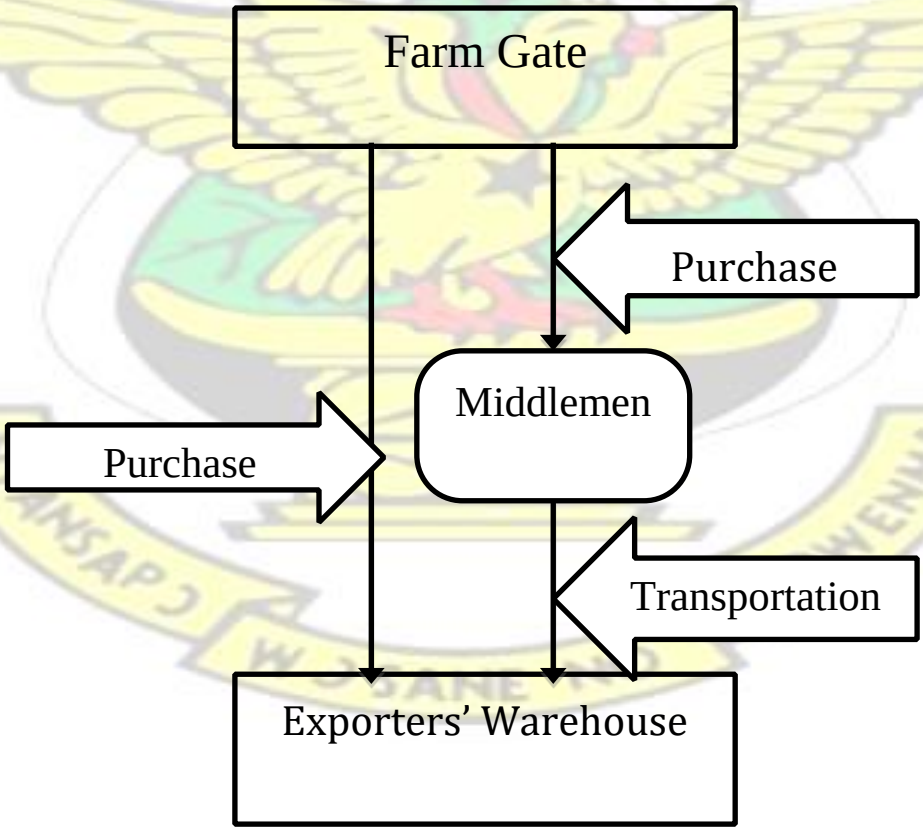


Figure 4.5: Distribution Value Chain for Export Grade Yam in Ghana

The descriptions of their activities involved during the pre-export handling period to shipment were orderly outlined and presented on Figure 4.6.

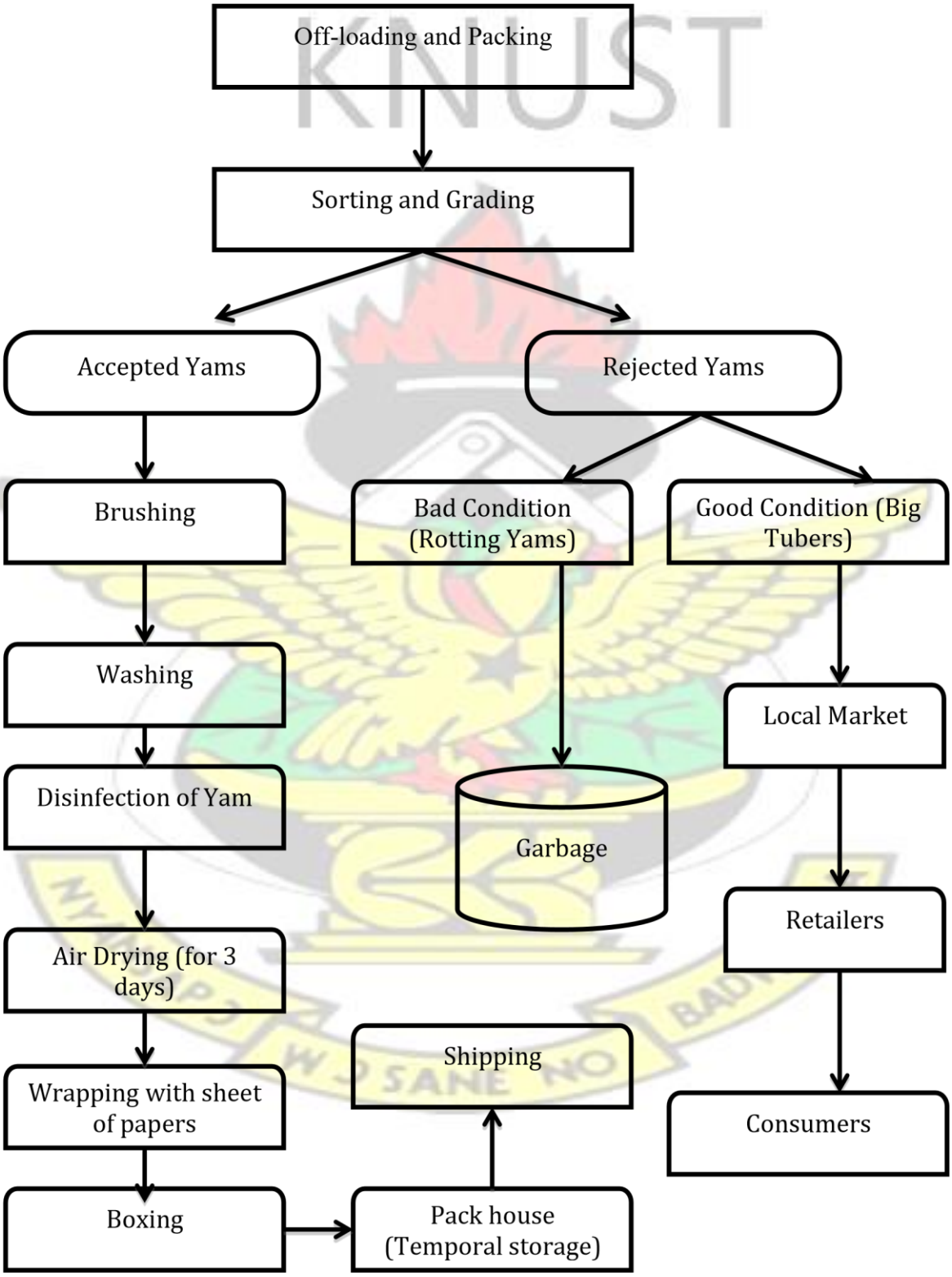


Figure 4.6: Pre-export handling operations of Export Grade yam in Ghana

4.1.2 Exporters' Understanding and Approaches on Yam Rot

All respondents had knowledge on yam rots; the impact of rot on yam export and the need to prevent them during the pre-export handling period. They said that yam rot occurred throughout the year but very prevalent at certain periods of the season. Most of the exporters (60%) agreed on the use of synthetic pesticides to prevent rot during the pre-export handling period since there were no better alternatives they knew (Figure 4.7).

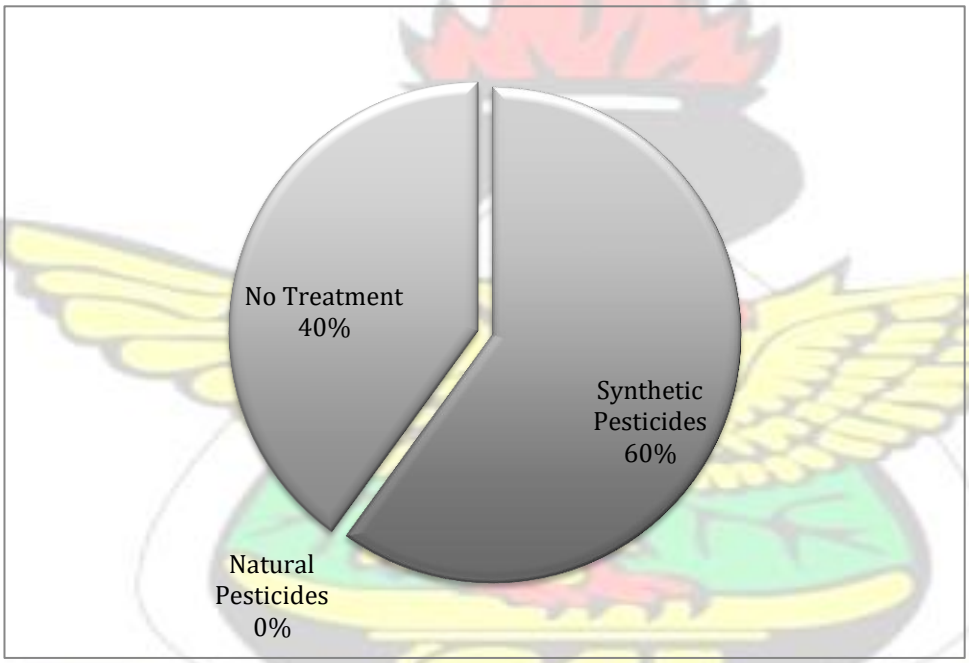


Figure 4.7: Treatment applied to yam tubers during pre-export period

4.2 PHYTOCHEMICAL SCREENING OF POWDERED COCOA LEAVES

Phytochemical screening of dry powdered cocoa (*Theobroma cacao)* leaves revealed the presence of various bioactive components (Table 4.1). It revealed the presence of phytochemical constituents such as Saponins, Tannins, Glycosides, Triterpenoids, Sterols, Coumarins, Flavonoids, and Alkaloids.

Table 4.1: Presence of phytochemicals in cocoa leaf powder

SAMPLE	PYTOCHEMICAL CONSTITUENT	OBSERVATION
Cocoa Leaves	Saponins	+
	Tannins	+
	Glycosides (Reducing sugar)	+
	Triterpenoids	+
	Sterols	+
	Alkaloids	+
	Coumarins	+
	Flavonoids	+

+ = Present

4.2.1 Effect of Solvent and Methods of Extractions on Yield of Cocoa leaf

Table 4.2 reveals resulting weights of yields (w/w) of extracts (Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract) obtained after extraction with different solvents and methods of extraction. The refined extracts (Ethylacetate Purified CL Extract and Acetone Purified CL Extract) resulted into higher percentage yields (w/w) than that of the crude extracts (Crude Aqueous CL Extract). The highest percentage yield (w/w) was recorded by Ethylacetate Purified CL Extract (57%), followed by Acetone Purified CL Extract (10%) and the lowest was recorded by Crude Aqueous CL Extract (6.7%).

Table 4.2: Percentage yield of extracts prepared from powdered cocoa leaves

EXTRACT	YIELD (W/W%)
Ethylacetate Purified CL Extract	57
Acetone Purified CL Extract	10
Crude Aqueous CL Extract	6.7

4.2.2 pH of cocoa leaf extracts pH of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude

Aqueous CL Extract acidic in nature (Table 4.3).

Table 4.3: pH test of extracts prepared

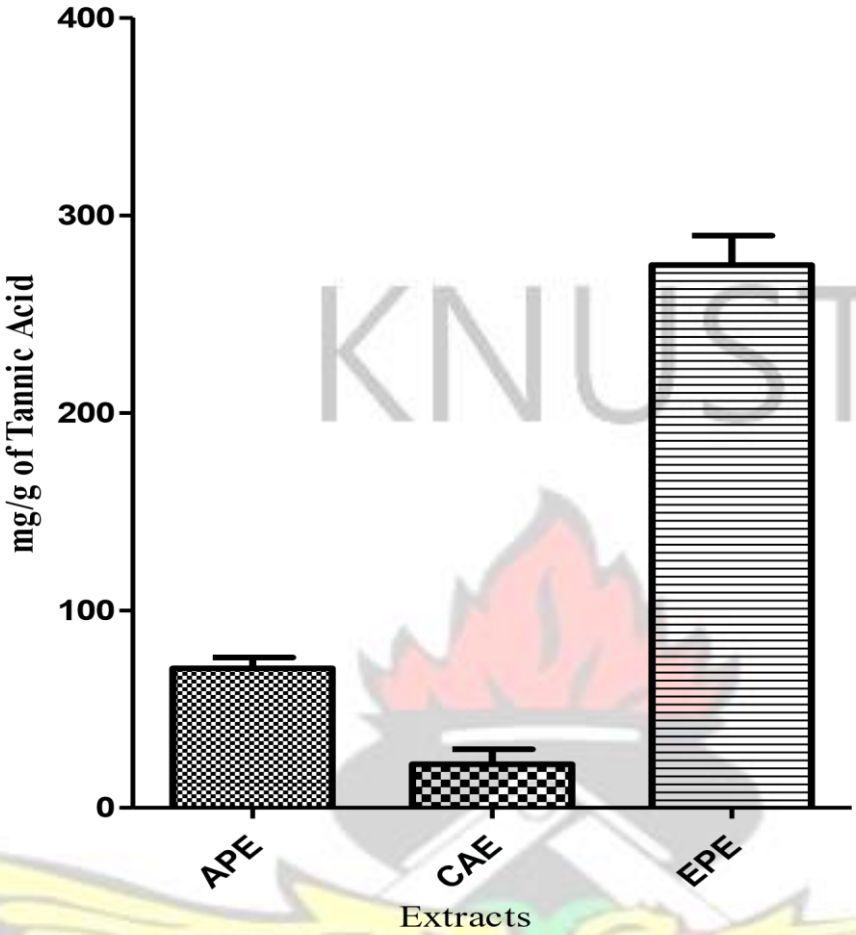
EXTRACT	Acidity	Basicity
Ethylacetate Purified CL Extract	+	-
Acetone Purified CL Extract	+	-
Crude Aqueous CL Extract	+	-

- = Absence, + =Presence

4.3 Antioxidant Activities

4.3.1 Total phenol content of extracts

Figure 4.8 shows the total phenol content of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract that were measured by Folin-Ciocalteau's reagent in terms of tannic acid equivalent (standard curve equation: $y = 0.9830x + 0.08750$, $r^2 = 0.9830$). The results obtained from the extracts showed that the total phenolics varied from 70.60mg/g to 274.90mg/g. Ethylacetate Purified CL Extract recorded the highest total phenolics (274.90mg/g), followed by Acetone Purified CL Extract (70.60mg/g). The lowest content of total phenolics was obtained with Crude Aqueous CL Extract (22.07mg/g). The calibration curve of Total Phenol Test of Extracts showed the line of best fit (Figure 4.9)



EPE - Ethylacetate Purified CL Extract (274.90mg/g), APE - Acetone Purified CL Extract (70.60mg/g), CAE - Crude Aqueous CL Extract (22.07mg/g)

Figure 4.8: Results of the total phenol test of extracts

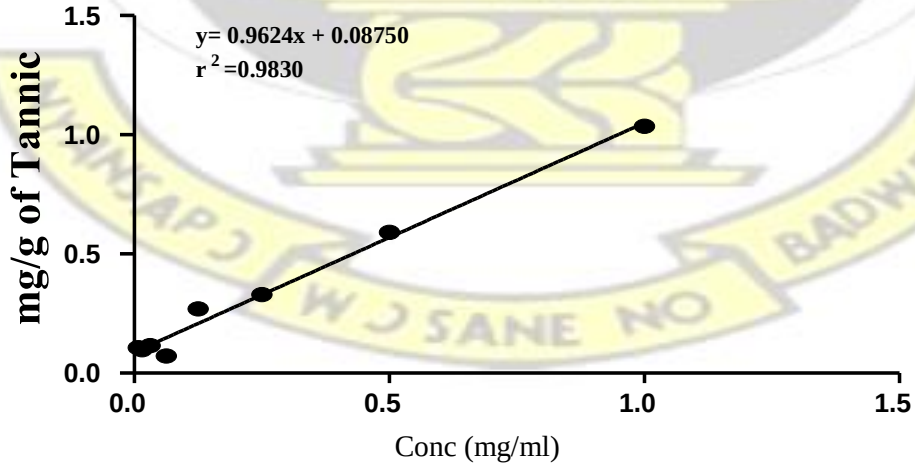
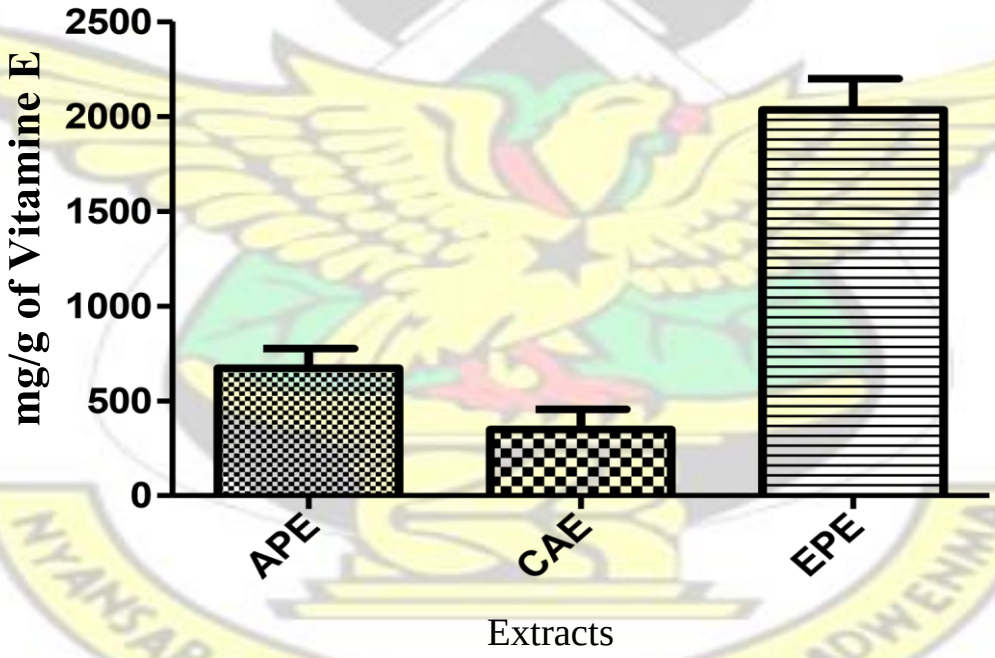


Figure 4.9: Calibration curve of total phenol test of extracts

4.3.2 Total Antioxidant Capacity of Extracts

Figure 4.10 shows the total antioxidant capacities of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract. They were determined in terms of vitamin E equivalent ($y = 0.02833x + 0.05843$, $r^2 = 0.9316$). The results showed that Ethylacetate Purified CL Extract (2034.72mg/g) was the most potent antioxidant of all studied extracts. Meanwhile, Crude Aqueous CL Extract (349.91mg/g) showed the least potency as compared with Acetone Purified CL Extract (671.77mg/g). Vitamin E served as a positive control. The calibration curve of the total antioxidant capacity of the extracts showed the line of best fit (Figure 4.11).



EPE - Ethylacetate Purified CL Extract (2034.72mg/g), APE - Acetone Purified CL Extract (671.77mg/g), CAE - Crude Aqueous CL Extract (349.91mg/g).

Figure 4.10: Result of total antioxidant capacities of Extracts

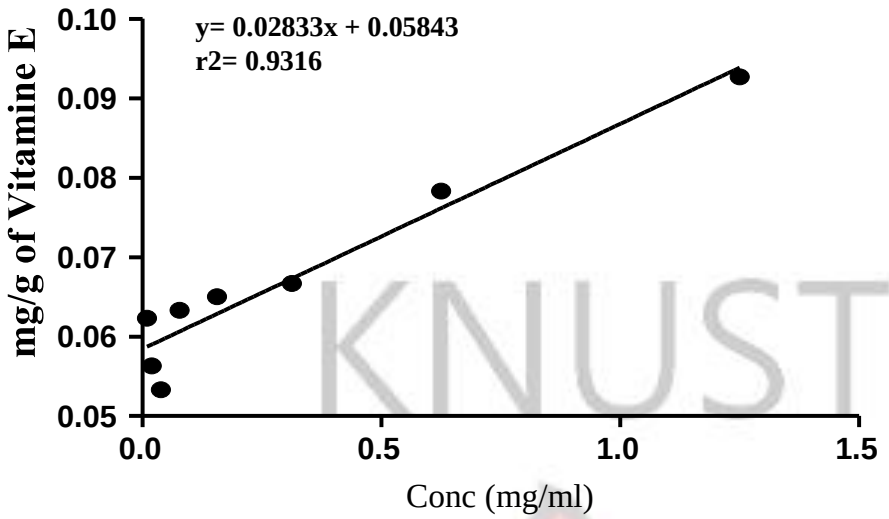
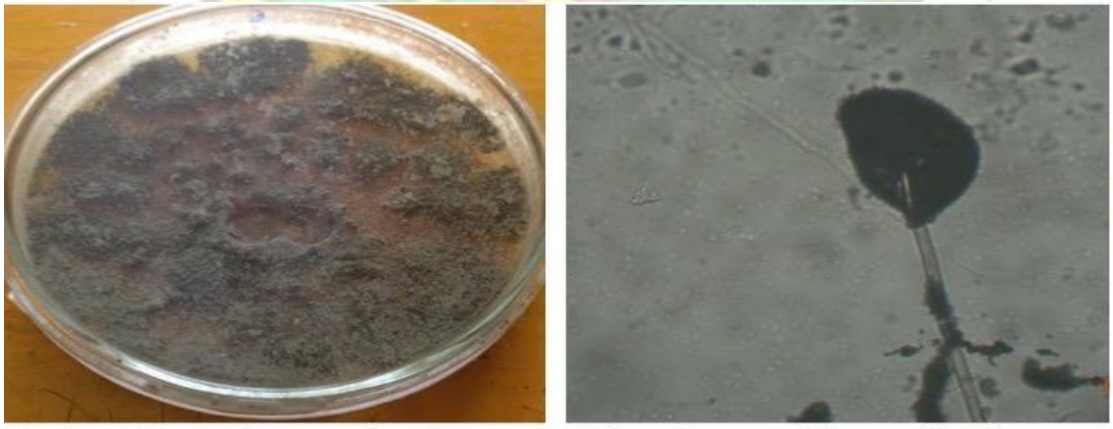


Figure 4.11: Calibration curve of total antioxidant capacity of extracts

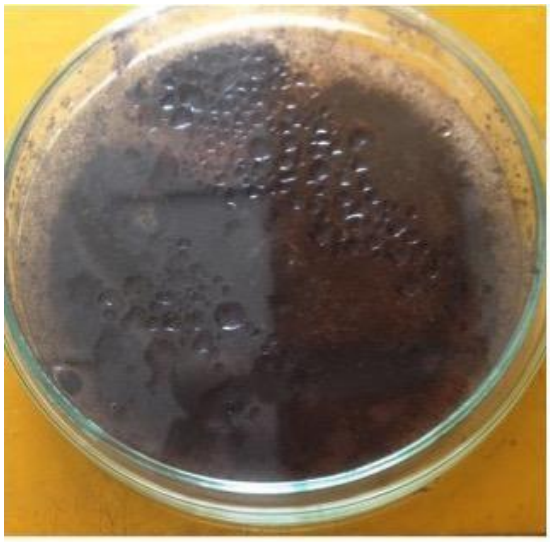
4.4 MORPHOLOGICAL STRUCTURE OF FUNGI ISOLATED

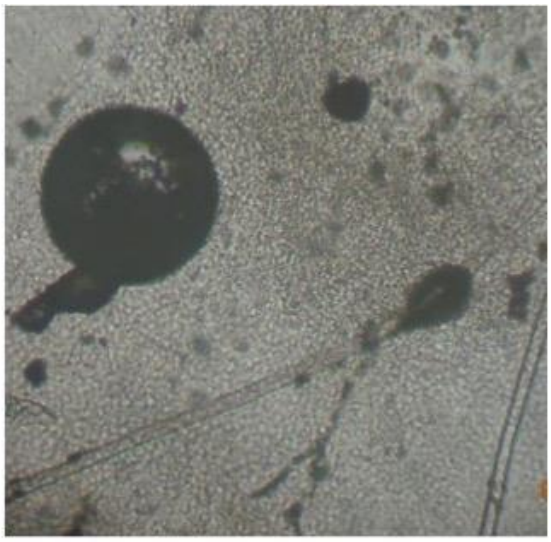
The isolation of four causative pathogens of the rotten yam samples were identified as *Aspergillus flavus* (Plate 4.1), *Aspergillus niger* (Plate 4.2), *Rhizopus stolonifer* (Plate 4.3) and *Penicillium spp.* (Plate 4.4) following descriptions of Mathur and Kongsdal (2003) and Barnett and Hunter (1972). These respective plates showed colour, conidia and morphology of test pathogens on PDA and under microscope.



Culture Plate of ***Aspergillus flavus*** **Micro Spores of** ***Aspergillus flavus***

Plate 4.1: Shows the Growth of *Aspergillus flavus* on PDA and microscopic structure of *Aspergillus flavus* under microscope.



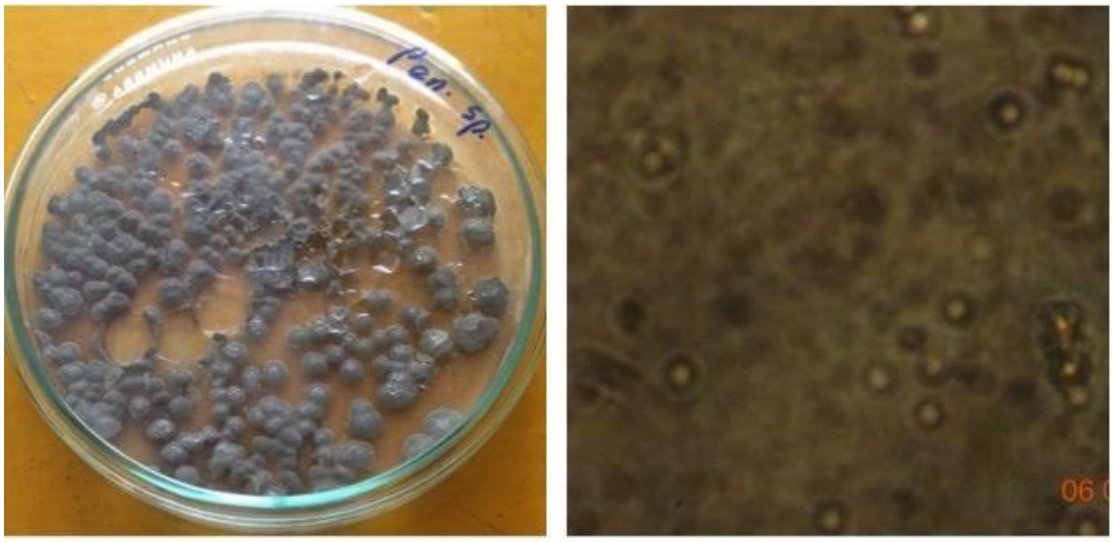


Culture Plate of *Aspergillus Niger* **Micro Spores of** *Aspergillus Niger*

Plate 4.2: Shows the Growth of *Aspergillus niger* on PDA and microscopic structure of *Aspergillus niger* under microscope.

Culture Plate of *Rhizopus stolonifa* **Micro Spores of** *Rhizopus stolonifa*

Plate 4.3: Shows the Growth of *Rhizopus stolonifer* on PDA and microscopic structure of *Rhizopus stolonifer* under microscope.



Culture Plate of *Penicillium Sp.* **Micro Spores of** *Penicillium Sp.*

Plate 4.4: Shows the Growth of *Penicillium spp.* on PDA and microscopic structure of *Penicillium spp.* under microscope.

Table 4.4 shows the percentage occurrence of fungi isolated and identified from plates obtained from white yam samples from the marketplaces and exporters' warehouses. The fungi isolated and identified were *Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer,* and *Penicillium spp.* Among the fungi isolated at the marketplace, *Penicillium spp*. (44.4%) recorded the highest percentage occurrence whereas *Aspergillus flavus* (16.7%) and *Aspergillus niger (*16.7%) had the lowest percentage occurrences. Meanwhile, samples obtained from the exporters' warehouse recorded the *Penicillium spp.* (33.3%) and *Rhizopus stolonifer* (33.3%) as the highest percentage occurrence whereas *Aspergillus flavus* (13.3%) recorded the lowest percentage occurrence.

Considering the total samples obtained from marketplaces and the exporters' warehouse, the percentage occurrences were 15.15%, 18.18%, 27.2% and 39.39% for *Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer* and *Penicillium spp.*

respectively. *Penicillium spp.* occurred most followed by *Rhizopus stolonifer* and the least occurred fungus was *Aspergillus flavus.*

Table 4.4: Percentage occurrences of fungi isolated at market place and exporter warehouse samples

		Fungi Isolated and Identified			
Plate	Number of Tubers	*Aspergillus flavus*	*Aspergillus niger*	*Rhizopus stolonifer.*	*Penicillium spp.*
MPS-1	2	+	+	-	+ +
MPS-2	2	+	-	+	+ +
MPS-3	2	+	-	-	+ +
MPS-4	2	-	+	+ + +	-
MPS-5	2	-	+	-	+ +
Total	10	3	3	4	8
% Occurrence		16.7%	16.7%	22.2%	44.4%
EWS-1	2	-	+	+ + +	+
EWS-2	2	-	+	-	+ +
EWS-3	2	+	-	+ +	+
EWS-4	2	+	+	-	+
Total	8	2	3	5	5
% Occurrence		13.3%	20%	33.3%	33.3%
Grand Total	**18**	**5**	**6**	**9**	**13**
% Occurrence		**15.15%**	**18.18%**	**27.27%**	**39.39%**

*MPS – Market Place Sample *EWS – Exporter Warehouse Sample, - = Absent, (+) = Present

4.5 ANTIMICROBIAL ACTIVITY

The *in vitro* antimicrobial activities of extracts (Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract) against the four (4) fungi isolated and identified were determined using the agar well diffusion and broth micro dilution method.

4.5.1 Agar Well Diffusion Assay

Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract were evaluated by the presence or absence of diameter of inhibition zone.

Table 4.5 depicts the antimicrobial activity of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract against the four test fungi expressed as inhibition diameter (mm). As the results indicate, all the extracts exhibited weaker antimicrobial activities against the test fungi compared to the positive control (Mancozeb). Table 4.5 indicated that there were significant differences ($p< 0.5$) among all the extracts means against test fungi. Mancozeb (11.46mm) recorded the highest mean, followed by Ethylacetate Purified CL Extract (5.41mm), while Crude Aqueous CL Extract (0.00mm) had the lowest mean that exhibited no inhibition.

The interactions between Ethylacetate Purified CL Extract, concentrations and microorganisms' showed significant differences ($p< 0.5$) in terms of diameter of zones of inhibition. At concentration 40mg/ml, the highest and lowest interactions on the diameter of zones of inhibition were 40mm and 20mm respectively. However, there were no interactive effects for the other concentrations (20mg/ml, 10mg/ml and 5mg/ml).

Statistically, the interactions between Acetone Purified CL Extract, concentrations and microorganisms' showed significant difference ($p< 0.5$) in terms of zone of inhibition. At concentration 40mg/ml and 20mg/ml, significant ($p< 0.5$) interactions existed on the diameter of the zones of inhibition with 30mm and 20mm respectively, while the others recorded the lowest. Crude Aqueous CL Extract did not show any significant ($p< 0.5$) interactive effect on zones of inhibition (Table 4.5).

The results of the interaction between mancozeb, concentration and microorganisms' showed significant differences ($p< 0.5$) in terms of zones of inhibition. At

concentration 40mg/ml the highest significant ($p< 0.5$) interaction was 30mm whereas the lowest was 24mm. The interaction between the concentration and microorganism revealed that the highest (20mm) zones of inhibition were recorded on *Aspergillus flavus* and *Penicillium spp.*, comparatively to the lowest zones of inhibition (15mm) on *Rhizopus stolonifer* at concentration 20mg/ml. At concentration 10mg/ml, there were significant differences ($p< 0.5$) in interactions between mancozeb, concentration, and microorganism on zones of inhibition. The highest significant ($p< 0.5$) zone of inhibition was 10mm whereas the lowest zone of inhibition was 7mm. At concentration 5mg/ml, there were no significant ($p< 0.5$) interactions between the mancozeb, concentration, and microorganism (Table 4.5).

Table 4.5: Result of Zones of Inhibition by Extracts

		Zones of Inhibition by Extracts (mm)															
		Ethylacetate Purified CL Extract				**Acetone Purified CL Extract**				**Crude Aqueous CL Extract**				**Mancozeb (*Positive control*)**			
Concentration (mg/ml)		40	20	10	5	40	20	10	5	40	20	10	5	40	20	10	5
Organism	*Aspergillus niger*	8gh	0i	0i	0i	0i	0i	0i	0i	0i	0i	0i	0i	24c	12f	8gh	0i
	Aspergillus flavus	20d	0i	0i	0i	0i	0i	0i	0i	0i	0i	0i	0i	20d	20d	10fg	0i
	Rhizopus stolonifer.	40a	0i	0i	0i	30b	20d	0i	0i	0i	0i	0i	0i	30b	15e	7h	0i
	Penicillium spp.	19d	0i	0i	0i	0i	0i	0i	0i	0i	0i	0i	0i	18d	20d	0i	0i
MEAN		5.41b				3.13c				0.00d				11.46a			

Lsd (concentration) = 0.36, Lsd (extracts) = 0.36, Lsd (microorganism) = 0.36, Lsd (interaction) = 2.06

4.5.2 Broth Micro Dilution Assay

Antimicrobial activities of Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract were reevaluated against the test fungi. Table 4.6 results reveal that all extracts had an inhibition against the four test fungi in attaining Minimum Inhibitory Concentrations (MICs). MICs were determined for Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract. The MIC values ranged from 15mg/ml to 2mg/ml (Table 4.6). All the extracts exhibited weak MICs against the test fungi as compared to the positive control (Mancozeb), which had the least MIC value of 2mg/ml. While the MICs of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract were observed to be 2.5mg/ml, 5mg/ml, and 15mg/ml respectively. Ethylacetate Purified CL Extract (2.5mg/ml) had the best MIC among the extracts and was close to that of the positive control (2mg/ml), followed by Acetone Purified CL Extract with MIC values of 5mg/ml against all the test fungi except *Rhizopus stolonifer* (7.5mg/ml). Crude Aqueous CL Extract (15mg/ml) showed an antimicrobial activity but with the lowest inhibition and weakest MIC value against the four test fungi among all the extracts.

Table 4.6: MIC Values of Plant Extracts against Test Fungal Organisms

Organism	**Plant Extracts** *(mg/ml)*			
	ETHYLACETATE PURIFIED CL EXTRACT	**ACETONE PURIFIED CL EXTRACT**	**CRUDE AQUEOUS CL EXTRACT**	**MANCOZEB (*Positive control)***
Aspergillus Niger	2.5	5	15	2
Aspergillus Flavus	2.5	5	15	2
Rhizopus stolonifer	2.5	7.5	15	2
Penicillium spp.	2.5	5	15	2

*The MIC values were in well concentration of antifungal extracts used

4.6 ISOLATION OF PATHOGENS FOUND ON THE SURFACE OF THE YAM BEFORE INOCULATION

Table 4.7 reveals microorganism pre-existed on the experimental export grade yam tubers, which were noted and isolated for identification. Based on cultural and microscopic characteristics of conidia, the five isolates of fungi obtained from surfaces of the 12 samples were identified as *Aspergillus flavus, Aspergillus niger, Penicillium spp., Rhizopus spp.,* and *Colletotrichum spp.* following descriptions of Mathur and Kongsdal (2003) and Barnett and Hunter (1972). Based on the results of the samples collected, *Aspergillus niger* had the highest percentage occurrence (52.17%) followed by *Rhizopus spp.* (26.10%). The *Colletotrichum spp*. and *Aspergillus flavus* had the same percentage occurrence as (8.7%). The lowest frequency of occurrence was observed on *Penicillium spp.* (4.35%).

Table 4.7: Results of Pathogens Found on the Surface of the Yam Samples

Sample	*Aspergillus niger*	*Aspergillus flavus*	*Rhizopus spp.*	*Penicillium spp.*	*Colletotrichum spp.*
1	+	-	+	-	-
2	+	-	+	-	-
3	+	-	+	-	-
4	+	-	-	-	-
5	+	+	+	+	-
6	+	-	-	-	-
7	+	+	-	-	+
8	+	-	-	-	-
9	+	-	-	-	-
10	+	-	-	-	-
11	+	-	+	-	-
12	+	-	+	-	+
Total	12	2	6	1	2
%	52.17%	8.70%	26.09%	4.35%	8.70%

- = Absent, (+) = Present

4.7 EFFECT OF ENVIRONMENTAL FACTORS WITHIN THE PRE-EXPORT HANDLING PERIOD

4.7.1 Trend of temperature and Relative Humidity fluctuations during the preexport period

Daily average temperature fluctuation within the pre-export handling period was recorded, including maximum and minimum temperatures. The temperature fluctuated between 26.5 ºC and 29 ºC with an average temperature value of 28.5 ºC. Meanwhile, relative humidity during the pre-export handling period varied from 75.5% to 82.4% with an average value of 78.5% (Figure 4.12).

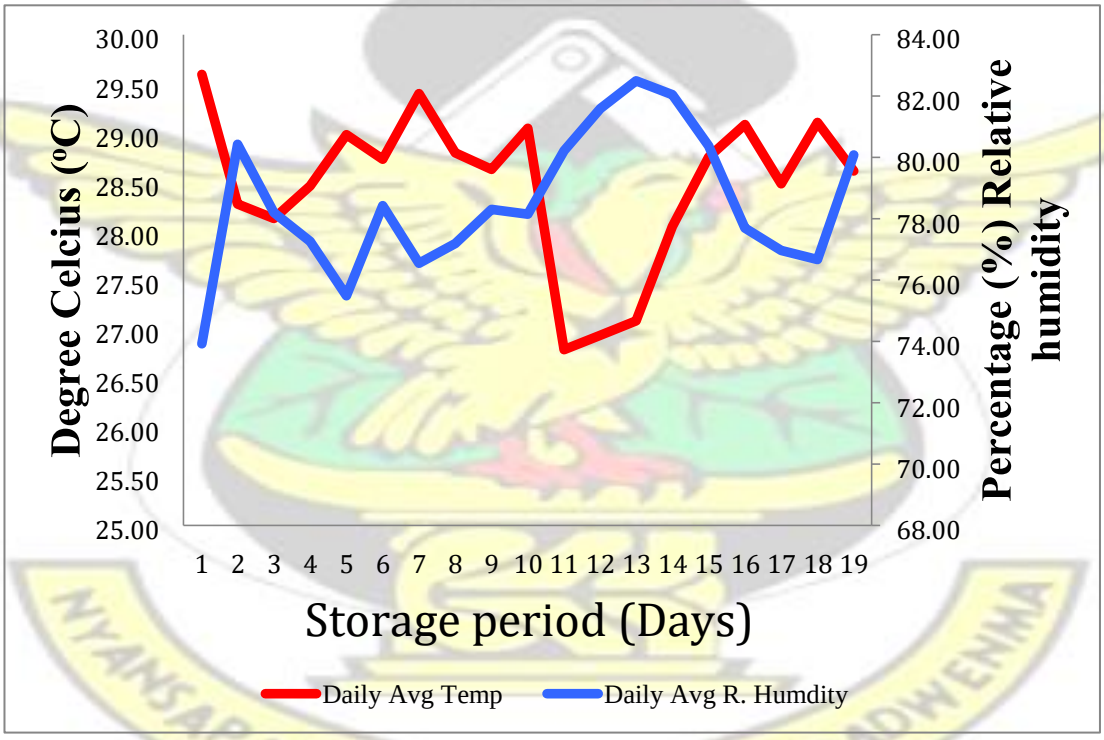


Figure 4.12: Temperature and relative humidity fluctuations within storage period.

4.8 EFFECT OF PHYSIOLOGICAL FACTORS WITHIN THE PRE-

EXPORT HANDLING PERIOD

4.8.1 Weight Loss

4.8.1.1 Trend of Effect of Extracts on Weight Loss of Yam Tuber over the Storage Period (Pre-Export Handling Period)

Trends of cumulative percentage weight loss observed during the storage period of the yam tubers are presented in Figure 4.13. The trend of value of cumulative percentage weight loss of Crude Aqueous CL Extract recorded the highest at 5.04%, followed by Ethylacetate Purified CL Extract, and Mancozeb at 4.46% whereas Distilled Water recorded the lowest at 3.89%.

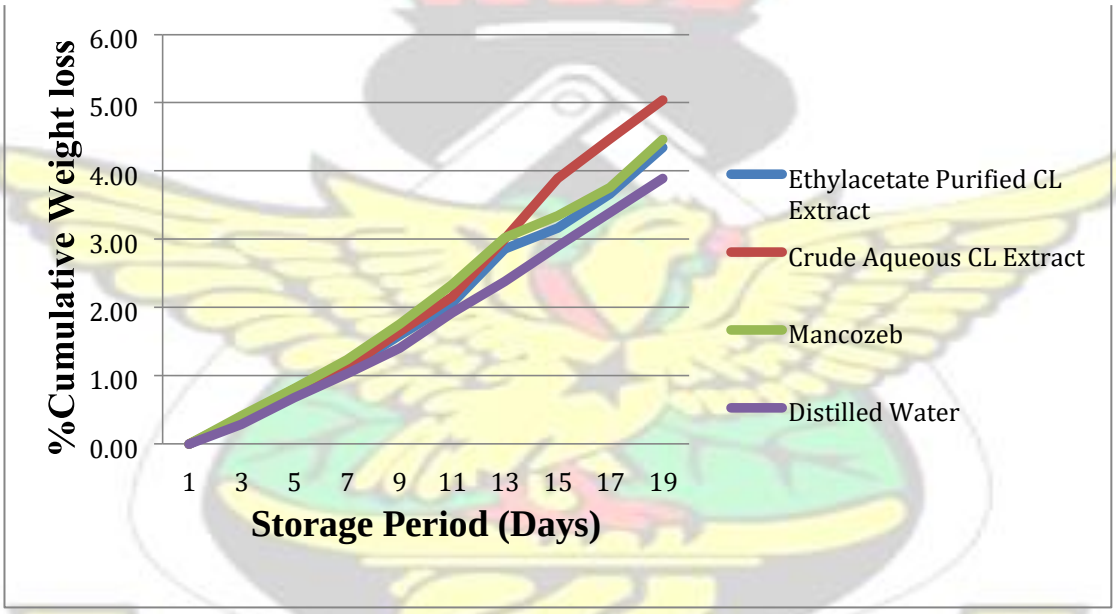


Figure 4.13: Cumulative (%) effect of extract on weight loss of yam during storage period.

4.8.1.2 Trend of impact of method of application of extracts on weight loss of yam during storage.

Trends of cumulative percentage weight loss observed during the storage period of the yam tubers are presented in Figure 4.14. The value of cumulative percentage weight

loss from spraying and dipping followed the same continuous increasing trend throughout the storage period.

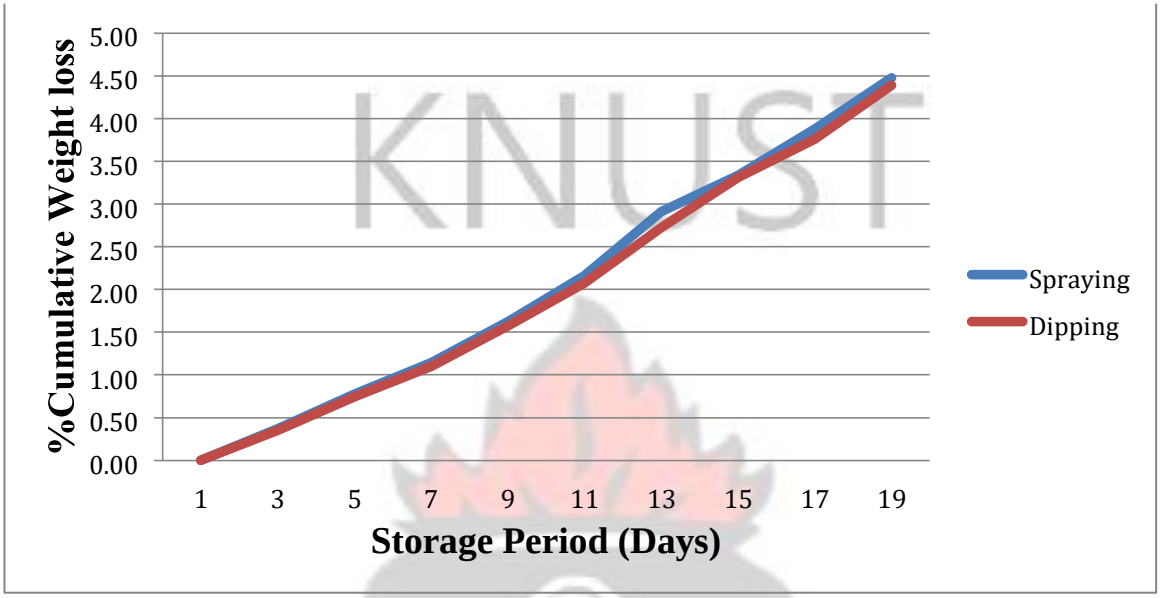


Figure 4.14: Cumulative (%) effect of method of application on weight loss of yam during Storage Period

4.8.1.3 Trend of effect of inoculums (test Fungi) on weight loss within the storage period

Trend of percentage cumulative weight loss observed during the storage period of the yam tubers is presented in Figure 4.15. The values of the cumulative percentage weight loss recorded 4.69% as the highest caused by *Penicillium spp.*, while both *Rhizopus stolonifer* and Aspergillus *flavus* recorded the lowest at 4.21%.

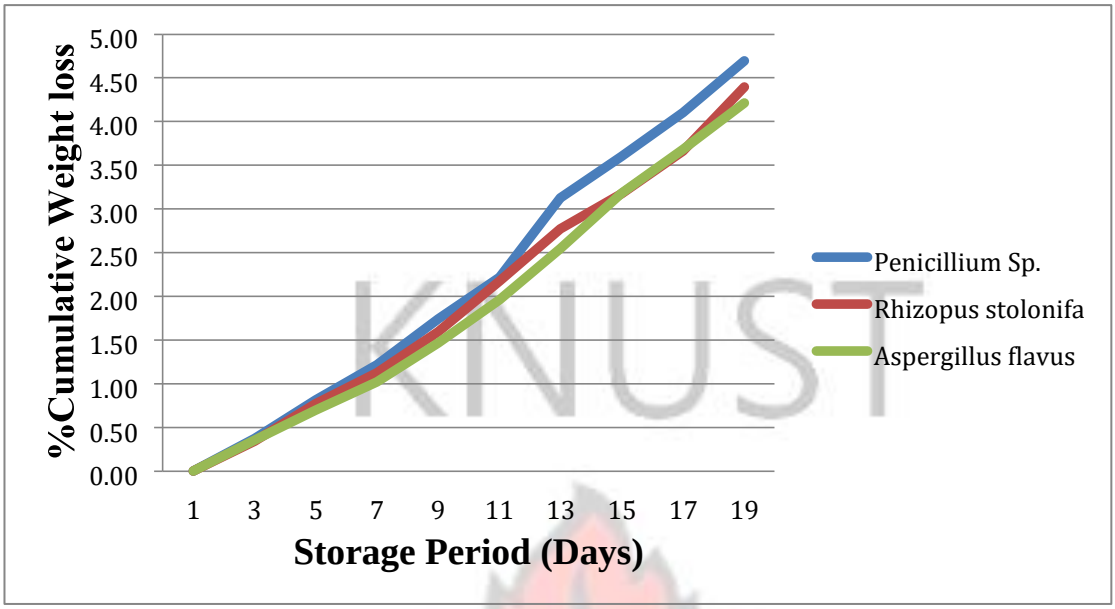


Figure 4.15 Cumulative (%) effect of test fungi on weight loss on yam during Storage Period

4.8.1.4 Effect of methods of application of extracts and microorganisms on weight loss (kg) of yam over the storage period

The effect of methods of application and microorganisms on the weight of yam was statistically evaluated (Table 4.8). Considering the interaction between methods of application and microorganisms on weight loss of yam during the storage period, there were no significant differences ($p < 0.5$) in the interaction of both spraying and dipping against all test microorganisms on weight loss. There were no significant differences ($p < 0.5$) between the means of both methods of application and microorganisms.

Table 4.8: Effect of Methods of Application of Extracts and Microorganisms on Weight loss (kg) of Yam during the storage period

METHOD OF APPLICATION			
MICROORGANISM	Spraying (Sp)	Dipping (Dp)	MEAN
Penicillium spp.	0.48a	0.45a	0.47a
Rhizopus stolonifa	0.46a	0.43a	0.45a
Aspergillus flavus	0.43a	0.41a	0.42a
MEAN	0.46a	0.43a	

Lsd (microorganism) = 0.09, Lsd (application) = 0.08, Lsd (interaction) = 0.13 Figures with same letters are not significantly different from each other whiles the figures with different letters are significantly different

4.8.1.5 Effect of extracts and microorganisms on weight loss (kg) of yam during the storage period

Table 4.9 exhibits the effect of extracts and microorganisms on weight loss of yam. Considering the interaction between extracts and microorganism on weight loss of yam, there was no significant difference ($p< 0.5$) in the interaction between the *Penicillium spp.* and *Aspergillus flavus* against four extracts on weight loss. Meanwhile, the interaction between *Rhizopus stolonifer* and the four extracts revealed significant differences ($p< 0.5$) on weight loss of yam tubers, where the Crude Aqueous CL Extract interactively recorded the highest weight loss of 0.56Kg whereas the interactions of other extracts (Ethylacetate Purified CL Extract, Mancozeb and Distilled Water) recorded the lowest weight loss under the *Rhizopus stolonifer*. Furthermore, there was no significant difference ($p< 0.5$) in the means of the microorganisms on weight loss. Besides, the means of the extracts showed significant difference ($p< 0.5$) on weight loss over the storage period. However, Crude Aqueous CL Extract and the Distilled Water recorded the highest (0.50kg) and lowest (0.39kg) mean values respectively.

Generally, in the interaction between extract and microorganism, Crude Aqueous CL Extract recorded the highest significant weight loss whereas Distilled Water recorded the lowest weight loss.

Table 4.9: Effect of extracts and microorganisms on weight loss (kg) of yam during the storage period

EXTRACT	MICROORGANISM			MEAN
	Penicillium spp	*Rhizopus stolonifer*	*Aspergillus flavus*	
Ethylacetate Purified CL Extract	0.51ab	0.40ab	0.40ab	0.43ab
Crude Aqueous CL Extract	0.5.ab	**0.56a**	0.45ab	**0.50a**
Mancozeb	0.44ab	0.42ab	0.49ab	0.45ab
Distilled Water	0.42ab	0.41ab	**0.34b**	**0.39b**
MEAN	0.47a	0.45a	0.42a	

Lsd (Extract) = 0.11, lsd (microorganism) = 0.15, lsd (interaction) =0.19 Figures with same letters are not significantly different from each other whiles the figures with different letters are significantly different.

4.8.1.6 Effect of extracts and method of applications of extracts on weight loss (kg) of yam during the storage period

Effect of extracts and methods of application of extracts on the weight loss of yam were statistically evaluated and are indicated on Table 4.10.

Interactively, there was no significance difference ($p<0.5$) between the spraying and the extracts on weight loss of the yam tubers but Mancozeb recorded the highest value among extracts. There was significant difference ($p<0.5$) in the interaction between the dipping method and the four extracts. However Crude Aqueous CL Extract was significantly different ($p<0.5$) among all the extracts interacted with dipping on weight loss. Crude Aqueous CL Extract (0.57kg) recorded the highest weight loss whereas Ethylacetate Purified CL Extract (0.42kg) and Mancozeb

(0.38kg) recorded the lowest weight loss interacting with the dipping method. Thus, Ethylacetate Purified CL Extract, Mancozeb, and Distilled Water were not significantly different ($p<0.5$) in the interaction with dipping but the Distilled water recorded the lowest weight loss value of 0.36kg. Furthermore, there were significant differences ($p<0.5$) among the means of the extracts; the Crude Aqueous CL Extract and Distilled Water recorded the highest (0.5kg) and the lowest (0.39kg) means respectively. Meanwhile, there were no significant differences ($p<0.5$) in the means of methods of application on weight loss. Generally, the Crude Aqueous CL Extract recorded the highest significant weight loss whereas Distilled Water recorded the lowest weight loss in the interaction between extracts and method of applications.

Table 4.10: Effect of method of applications of extracts and extracts on weight loss (kg) of yam over the storage period

EXTRACT	**METHOD OF APPLICATION**		**MEAN**
	Spraying (Sp)	Dipping (Dp)	
Ethylacetate Purified CL Extract	0.45abc	0.42bc	0.43ab
Crude Aqueous CL Extract	0.44abc	**0.57a**	**0.50a**
Mancozeb	0.51ab	0.38bc	0.45ab
Distilled Water	0.42abc	**0.36c**	**0.39b**
MEAN	0.46a	0.43a	

Lsd (Extract) = 0.11, lsd (Application) = 0.08, lsd (interaction) =0.15

Figures with same letters are not significantly different from each other whiles the figures with different letters are significantly different

4.8.2 Yam Rot

4.8.2.1 Percentage incidence of rot on yam against extracts

Table 4.11 shows percentage incidence of rots, which appeared at various respective sections (Anterior, Middle and Posterior) of infectious spots on yams that showed symptoms of rot. Twelve. (12) tubers out of the 72 tubers showed symptoms of rots,

which included rot that occurred on the positive (2 mancozeb treatment tubers) and negative (5 distilled water treatment tubers) controls. The highest percentage incidence of rots was observed at the posterior section (44.44%) whereas the lowest was observed at the middle section (16.67%).

Table 4.11: Incidence of rot at the various sections of the yam against extracts during the period of storage

		INCIDENCE OF ROT		
EXTRACTS	**Number of Rot Tubers**	**Anterior**	**Middle**	**Posterior**
Ethylacetate Purified CL Extract	2	1	0	1
Crude Aqueous CL Extract	3	3	1	1
Mancozeb	2	0	2	1
Distilled Water	5	3		8
Total	12	7	3	8
% Occurrences		38.89	16.67	44.44

Mancozeb – Positive control, Distilled water – Negative control

4.8.2.2 Effect of extracts against percentage rot of infectious yam tubers during the storage period

Table 4.12 depicts the statistical evaluation of efficacy of extracts on percentage rots of tubers in this experiment. A total of 16.67% (including positive and negatives) of yam tubers showed symptoms of rots. There were significant differences ($p<0.5$) between percentage rot of tubers and respective extracts used as treatment on them. Distilled water recorded the highest percentage rot of 6.94% significantly ($p<0.5$) greater than that of Crude Aqueous CL Extract, which recorded the lowest percentage rot of 4.17%.

Table 4.12: Efficacy of extract on percentage rot of yam tubers during storage

Extracts	**% Rot**
Ethylacetate Purified CL Extract	2.78c
Crude Aqueous CL Extract	4.17b
Mancozeb	2.78c
Distilled Water	6.94a
Lsd (0.01)	1.37

Mancozeb – Positive control, Distilled water – Negative control.
Figures with same letters are not significantly different from each other whiles the figures with different letters are significantly different.

4.8.2.3 Effect of extracts and methods of application on percentages severity of rots during storage period

The results of impact of extracts and methods of application on percentages of severity of rots are statistically represented on Table 4.13. There were no significant differences ($p<0.5$) with respect to the interactions between the extracts and methods of application, means of methods of application, and means of extracts on percentages of severity of rots. Relatively, among the mean values of the extracts, Distilled water (3.6%) recorded highest percentage of severity of rots whereas the lowest was recorded by Ethylacetate Purified CL Extract (0.27%).

Table 4.13: Effect of extract and method of application on percentages severity of rot during storage

EXTRACTS	**METHODS OF APPLICATION**		
	Spraying	**Dipping**	**MEAN**
Phenol-Methanol Extract	0.12a	0a	0.27a
Crude Aqueous CL Extract	0a	2.66a	1.33a
Mancozeb	3.1a	0a	1.81a

Distilled Water	4.22a	2.97a	3.60a
MEAN	1.99a	1.51a	

Lsd (application) = 3.7, lsd (extract)=5.23, lsd (application and extract) = 7.39
Figures with same letters are not significantly different from each other whiles the figures with different letters are significantly different.

4.8.2.4 Percentage occurrences of pathogen isolated from infected rot tubers after pre-export storage period

Table 4.14 reveals the percentages of occurrence of pathogens isolated from rot yam tubers. The percentage of occurrence was 8.33%, 41.67%, 16.67%, and 33.33% for *Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer* and *Penicillium spp.* respectively. *Aspergillus niger* occurred most followed by *Penicillium spp.* and the least occurred fungus was *Aspergillus flavus* among all the experimental yam tubers that showed rots.

Table 4.14: Percentage occurrence of pathogen isolated from infected rot tubers after pre-export storage period

Extracts	**Number of Rot**	***Penicillium spp.***	***Rhizopus stolonifa***	***Aspergillus flavus***	***Aspergillus niger***
Ethylacetate Purified CL Extract	2	+	-	-	+
Crude Aqueous CL Extract	3	+	-	-	+ +
Mancozeb	2	+	-	-	+
Distilled Water	5	+	+ +	+	+
Total	12	4	2	1	5
% Occurrence		33.33	16.67	8.33	41.67

- = Absent, (+) = Present

CHAPTER FIVE

5.0 DISCUSSION

5.1 PRELIMINARY STUDIES OF PRE-EXPORT HANDLING OPERATION

From the results, more males were engaged in the exporting of yam than females in the study area. The study also showed that people who usually engaged in yam exporting industry were within the economically active age group (35 – 54 years). This could be due to the capital-intensive nature of the yam export industry; the youth below 35 years could likely be beginners or work as middleman within the yam distributing chain. The survey also revealed that exporters could not clearly differentiate among the varieties of white yam (*Dioscorea rotundata*) they exported but they all attested that 'pona' was the most exported. Similar trend of assessment has been reported by Otoo *et al.* (2009) that 'Pona' yam is rated superior to the other white yam varieties in terms of its cooking quality and thus commands a high price than other yams on the market. Otoo *et al.* (2009) further explained that "Pona" and "Labreko" are sweet, floury and have fragrant tuber flesh and therefore remain the most preferred cultivars by consumers in Ghana. Moreover, in Ghana, the various farming seasons determine the variety of white yam available and this fact has been similarly reported by Baah (2009) who clearly stated that yam is a seasonal crop and most available during its harvesting period but scarce and expensive during its planting and growing seasons.

Exporters sometimes exported different varieties of white yam but varieties such as "Dente", "Serwa", "Lily", "Labreko", "Kwakwa", "Asobayere", and "Lawbayere" were not preferred. However, due to the nature of the farming season the exporters exported such varieties of white yam. This could also be due to the fact that the yam producers who are generally small-scale serve as the main sources of yam supply for

both the local and export (international) markets in Ghana. Exporters therefore depend on these small-scale farmers for their exports. Anaadumba (2013) stated that, despite the fact that yam is one of the most prominent export commodities for Ghana, information on the yam value chain is poor in aspects such as a clear distinction between yam varieties for the export market and those consumed locally. The difficulty in knowing what specific types of yam are being exported to the international market is a big problem.

Most of the exporters purchased their supplies from the middlemen or at the farm gate. USAID (2005) also reported that, yam exporters operating in Accra typically buy yam directly from small and sedentary wholesalers at the main markets in Accra. However, with increasing competition in the yam export market, some exporters commission itinerant traders to purchase yam from major production areas.

Before shipment, pre-export handling operations and activities were carried out starting with the offloading and packing of yams into their respective warehouses. The yam tubers were then sorted and graded into accepted and rejected tubers. The accepted tubers were brushed, washed, disinfected with a synthetic pesticide (fungicide) and allowed to be air dried for a day. The yam tubers were then wrapped and packed in boxes and temporarily stored in the pack house ready to be exported.

Yam rot was known to all the exporters even though they could not categorize the rots; they had their own traditional parameters used in identifying the type of rot. In the course of the pre-export operations, they rejected any yams that showed symptoms of rot. This is because they believed rot reduces the quality and contaminates the whole

batch of exported yam upon arrival at their respective destinations thus causing losses. To guard against rots, they treated the accepted yams with synthetic pesticide, a practice not preferred due to its numerous side effects such as polluting the environment and the propensity of causing diseases. Ghana yam is fumigated upon arrival at USA ports, but this often reduces yam quality due to the high temperature at which fumigation takes place (USAID, 2005).

Although the exporters recognised that the use of the synthetic pesticides were not the best due to their numerous side effects, they confessed that they usually used them depending on the seasons and distances of the countries the yam tubers were being exported to. The exporters could not also pin point any safer alternative like a natural pesticide that could replace the synthetic pesticides. Thus they insisted that the use of these harmful synthetic chemicals would continue until a safer replacement was found. Hence the need to substitute the synthetic pesticide with natural ones such as phenolic compound from cocoa (*Theobroma cacao)* leaves
which is the object of this study.

5.2 PHYTOCHEMICAL SCREENING OF COCOA LEAVES

The phytochemical screening was useful for the evaluation of the qualitative assessment of the powdered cocoa leaves. Preliminary phytochemical analysis of the powdered cocoa leaves ascertained the presence of saponin, tannin, glycoside, triterpenoid, sterol, alkaloid, and coumarin. The presence of these phytochemicals in the cocoa leaves guaranteed that its extracts could contain polyphenols, which dominated in antimicrobial and antioxidant properties. Izuka and Mbagwu (2013) have also reported that phytochemicals such as flavonoid, tannin, saponin, alkaloid and

phenol have been found in *Theobroma cacao* seeds. The presence of these phytochemicals also indicated that *Theobroma cacao* leaves could be a major source of antioxidant and antimicrobial compounds. Extracts of the cocoa leaves exhibited a high antioxidant capacity and an ability to inhibit fungi growth. These facts have already been established by a lot of researchers; that phytochemical possesses various functions but with a common goal of defending or protecting the plants against invading pathogen (Lattanzio *et al*., 2006; Achakzai *et al*., 2009; Izuka and Mbagwu, 2013). Besides, Tatiya *et al*. (2011) also reported that tannins have an ability to act as growth inhibitors to many microorganisms, including fungi, by preventing the transport of nutrients into the cell therefore retarding growth of the organism. It can also undergo complex reactions with microbial proteins or polysaccharides where such interaction is often irreversible and this characteristic confers fungicide and fungistatic properties.

Moreover, the quantities of the above-mentioned phytochemicals in the cocoa leaves could depend on the quantity of preformed and induced compounds that existed before plucking the leaves. Naturally, depending on the type and intensity of invasion by pathogen on the cocoa plants, the cocoa plants provide resistance during the interaction by not supporting the lifestyle of invading pathogen or using the preformed chemical barriers to prevent invasion. When the preformed chemicals are not sufficient to stop the development of the infectious process, then the cocoa plant induces its endogenous multicomponent defense system (Lattanzio *et al*., 2006). This could mean that this potential phytochemical compounds are all over the cocoa plant or hidden in the cells of certain parts or organs of the cocoa plant like leaves, roots, skin of the stem or fruits. Therefore, these phytochemicals could be harvested when the right part of the plant

containing them is identified and collected. Then the appropriate methods of extraction and solvents could be used for extraction to obtain an optimum yield. The optimum yield obtained could serve as a source of antimicrobial agents like fungicides and antioxidants to reducing the effect of biotic and abiotic stress in postharvest storage.

5.3 EFFECT OF TYPES OF SOLVENTS AND METHOD OF EXTRACTIONS

The yields of extract and resulting biological activity of plant material are greatly influenced by the method of extraction and nature of the extracting solvent owing to the presence of different bioactive compounds of different chemical properties. Thus, differences in polarity of solvent determine the final yield and its constituents.

Oyedokun *et al*. (2011) similarly reported that plant material's biocidal components and the extracting solvents used contribute to the efficacy of the extracts. Though the leaves of *Theobroma cacao* were the only plant material used, due to the introduction of different types of solvents and methods of extractions for respective extracts, variations occurred to the yields and properties (potency) of the three (3) respective extracts obtained. Thus, Ethylacetate Purified CL Extract recorded the highest yield followed by Acetone Purified CL Extract and Crude Aqueous CL Extract recorded the lowest. Furthermore, solvents can be classified as polar and non-polar. Polyphenols are polar compounds that are soluble in polar solvent like water (Lattanzio *et al*., 2006). This implies that phenolic compounds could be best extracted with polar solvents.

Ethylacetate Purified CL Extract obtained the highest yield could be due to the series of solvents employed in this method of extraction. The second highest yield method of extraction used acetone (less polar) and water (more Polar) to obtain Acetone

Purified CL Extract while the Crude Aqueous CL Extract that recorded the lowest used only water (more polar). Hence, the differences in yield could have been attributed to the various type of solvents used with differences in their polarity. NsorAtindana *et al.* (2012) similarly reported that differences observed in yields of extracts could be related to the polarity of the solvent used in the extraction. From the study of Nsor-Atindana *et al.* (2012), the least polar solvent was acetone that yielded higher contents of phenolics than that of water (more polar). This means higher yields of phenolics are obtained with a decrease in polarity of the solvent in the extraction of phenolic compounds. This could be the reason why the ethyl acetate with less polarity than the acetone recorded the highest yield followed by the yield of the acetone and the least was recorded by water used in the Crude Aqueous CL

Extract.

5.4 ANTIOXIDANTS

The three (3) extracts exhibited antioxidant effects but there were variation in yields of total phenolic content and total antioxidant capacity. Thus, in both total phenol content assay and total antioxidant capacity assay, Ethylacetate Purified CL Extract recorded the highest total phenolic content and total antioxidant capacity, followed by Acetone Purified CL Extract while the least figures were recorded by Crude Aqueous CL Extracts. This variation in yields of phenolic or antioxidant potential could be based on different methods of extraction and extracting solvents, which could have influenced the differences in the proportion of phenolic compounds and other phytochemicals present in Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract. Nsor-Atindana *et al.* (2012) also reported that the resulting biological activity of plant material is greatly influenced by the nature of

the extracting solvent owing to the presence of different bioactive compounds of different chemical properties and polarities that may or may not be soluble in a particular solvent. Hence, considering the proportional mixtures of phenolic compound in Ethylacetate Purified CL Extract, Acetone Purified CL Extract and Crude Aqueous CL Extract, the Ethylacetate Purified CL Extract could have harvested the best proportional mixture of the active phenolic or other phytochemical compounds that could be utilized in several ways like storage of yam. Osman *et al*. (2004) revealed that antioxidant activities from cocoa leaf extracts could be attributed to the presence of epicatechin and other unidentified phenolics.

Epicatechin is known to possess strong antioxidant activity.

Yam respires through the skin thus making the permeability of the yam skin a really crucial factor in determining the rate of respiration (Diop, 1998; Burton, 1966).

During storage, the tubers' encountered biotic and abiotic stresses could possibly accelerate respiration. In the present study such biotic stress could have emanated from the activities of the invaded fungi (inoculum and saprophytes) causing rot. The abiotic stress could have resulted from the fluctuating temperatures and relative humilities within the storage period. These stresses could also lead to the overproduction of reactive oxygen species (ROS) that could be toxic to the yam cells. Gill *et al.* (2011) and Singh *et al.* (2008) reported that oxidative stress is a condition in which ROS are generated in extra or intra-cellular levels that exert their toxic effects on the cells.

The yam tubers on their own could also produce antioxidants as defense mechanism to reestablish the equilibrium (Freeman and Beattie, 2008; Gill *et al.*, 2011). During this period, polyphenolic compounds in yams undergo polyphenolic oxidase catalyzed reactions to form o-quinones, their primary oxidation products, which react with other

components to form brown polymeric compounds in yam (Ozo, 1985). The brown internal colour of certain yam when cut is as a result of the presence of polyphenol and the oxidation reaction that occur in them (Farombi *et al*. 2000). The rate of browning in yam has been positively correlated with the amount of phenolic compounds and polyphenol oxidases in the tuber (Asemota *et al*., 1992; MuzacTucker *et al*., 1993). The antioxidant potentials of the cocoa leaves extracts, especially Ethylacetate Purified CL Extract, could therefore be used to reduce ROS by applying them on the yam tuber as was done in the present study. The cocoa leaf extract could penetrate the microspore of permeable skin into the yam cells causing an increase in the scavenging of ROS, particularly where the yam cells are unable to balance the equilibrium or the level of production of ROS. Gill *et al.* (2011) reported that such plant stress tolerance might be improved by the enhancement of *in vivo* levels of antioxidant enzymes.

5.5 TYPES OF IDENTIFIED FUNGI AND THE ANTIMICROBIAL ACTIVITY OF EXTRACTS

Isolated and identified fungi found on rotten white yam samples collected for this study were *Aspergillus flavus, Aspergillus niger, Penicillium spp.* and *Rhizopus stolonifer. Penicillium spp.* was found to be very rampant from the samples randomly collected from the market places whiles at the exporters' warehouses *Penicillium spp* and *Rhizopus stolonifer* were the fungi found to be most rampant. From the above evaluations, *Penicillium spp.* followed by *Rhizopus stolonifer* fungi could be very prevalent and rampant in causing losses in white yam export. These fungal species isolated and identified in this study were similarly reported by Aboagye-Nuamah *et al*. (2005) and Aidoo (2011). The investigation of the rot yam samples collected for the study showed two (2) types (including dry rot and soft rot) out of the three (3) types

(dry rot, soft rot, and wet rot) of storage rot of yam reported by IITA (1993) and Aidoo (2011). Variations in the occurrence of the fungi in the market places and exporters' warehouses may be as a result of the localities where the tubers were cultivated (Aboagye-Nuamah *et al*., 2005).

5.5.1 Antimicrobial Activity of Extracts Based on Zone of Inhibition

The *in vitro* antimicrobial activity of extracts (Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and Crude Aqueous CL Extract) against *Aspergillus flavus*, *Aspergillus niger*, *Penicillium spp*., and *Rhizopus stolonifer* employed in the antimicrobial assays were evaluated by the presence or absence of inhibition zone. There were differences in the activities of the extracts against the test microorganism (fungi), so different means were observed among their respective zones of inhibition. Mancozeb, which was used as a positive control, expressed higher zones of inhibition against the four microorganisms (fungi) than all the other extracts. All the four selected concentrations of the Mancozeb used had antimicrobial activities against the four microorganisms (fungi). This implied that the active ingredient in the synthetic fungicide had antimicrobial effects against the four tested microorganisms (fungi). Also, the active ingredient could have had good diffusion rates in the agar.

Among the extracts, the Ethylacetate Purified CL Extract exhibited the highest antimicrobial inhibition in terms of the diameters of zones of inhibition against all the four tested microorganisms. Even though only the highest concentration among the four selected concentrations was able to inhibit all the four tested microorganisms. This could be due to the fact that the phenolic compounds and other phytochemicals were able to diffuse effectively throughout the agar to inhibit the microorganisms.

The method of extraction and the series of solvents used in the Ethylacetate Purified CL Extract could have also resulted in the higher potency and quantity of the phenolic compounds present. This suggested that some of the phytochemical compounds in the extract had antimicrobial effects against all four microorganisms.

The Acetone Purified CL Extract had significant zones of inhibitions under two concentrations on *Rhizopus stolonifer* only. This could be due to the fact that the series of solvents and the method of extraction employed could not have harvested enough phenolic compounds or other phytochemical compounds compared to the Ethylacetate Purified CL Extract. Moreover, it could not have had any antimicrobial activity against the other three tested microorganisms because the types of phenolics or other phytochemical compounds present in this extract, which diffused slowly in the agar.

The Crude Aqueous CL Extract exhibited no zones of inhibition against all the tested extracts. This might be due to the fact that the type and quantity of phenolic compounds present in this extract could have been very low to exhibit any antimicrobial activity against the four tested microorganisms. Also, the diffusion rates of the types of phenolic compounds or other phytochemical compounds in the agar medium could have been very low to defuse and inhibit the tested microorganisms. Igbinosa *et al*. (2009) reported that the highest concentration of aqueous extract was not active against any of the microorganism tested, so they concluded that aqueous plant extracts generally showed little or no antimicrobial activities. Furthermore, Goyal *et al*. (2008) also explained the use of organic solvents in the preparation of plant extracts as compared to aqueous extracts. The polarities of antimicrobial compounds make them

more readily extracted by organic solvents. Hence, organic solvents could clearly be better solvents for extracting antimicrobial agents.

Generally, Mancozeb significantly showed the highest antimicrobial potential than all the tested extracts at the four concentrations used. Comparatively, Ethylacetate Purified CL Extract showed similar antimicrobial potential to that of Mancozeb while the Acetone Purified CL Extract exhibited lower potential, and Crude Aqueous CL Extract showed no activity against all the tested fungi. Though the variations in the concentrations did not show any clear trend of antimicrobial potency, this could have been due to the types and polarities of active compounds and poor diffusion rate of the extracts for the Acetone Purified CL Extract and the Crude Aqueous CL Extract. However, information deduced earlier from the assays of the phytochemical screening, total phenol content, and the total antioxidant capacity proved that all the three extracts contained some types and quantities of phenolic and other phytochemical compounds.

The present study showed that agar well assay alone could not be used as the basis to conclude on the efficacy of antimicrobial potentials of the extracts, especially the Acetone Purified CL Extract and the Crude Aqueous CL Extract due to its limitations. Cos *et al*. (2006) reported that diffusion technique is not appropriate for testing non-polar extracts or extracts that do not easily diffuse through the agar. Hence, the broth micro dilution method was employed to help in justifying the efficacy of antimicrobial potency while determining the MIC of the three respective extracts. Cos *et al*. (2006) further suggested that dilution methods are appropriate for testing polar and non-polar, extracts for the determination of MIC.

5.5.2 Minimum Inhibition Concentration (MIC) of Cocoa Leaf Extracts

Broth micro dilution assay was the best approach used for the detection of inhibitory activity because it provided accurate results of inhibition at very low concentration (Nsor-Atindana, 2012). The re-evaluation of the antimicrobial potential of the extracts proved otherwise particularly, the Acetone Purified CL Extract and that of Crude Aqueous CL Extract. When determining the MICs of the respective extracts, Ethylacetate Purified CL Extract, Acetone Purified CL Extract and the Crude Aqueous CL Extract showed antimicrobial activity against all the tested microorganisms. This was contrary to the antimicrobial activities observed in the agar well diffusion method. This could be attributed to the fact that the extracts work better when in contact with the microorganisms than through diffusion in an agar medium.

In broth micro dilution assay, Ethylacetate Purified CL Extract showed the greatest antimicrobial activity with the least MIC value among the three extracts. Acetone Purified CL Extract followed in the levels of antimicrobial activity against all the tested microorganisms. The Crude Aqueous CL Extract also had antimicrobial activity against all four microorganisms but with the highest MIC value. All the three extracts showed higher MIC values as compared to the MIC value of Mancozeb, the positive control. This implied that Mancozeb was the most potent against the four microorganisms. The MIC of the Ethylacetate Purified CL Extract was closer to that of the positive control, Mancozeb. Therefore, the Ethylacetate Purified CL Extract could be a very potent natural antimicrobial agent.

The variations revealed in the antimicrobial activities and MIC of Ethylacetate Purified CL Extract, Acetone Purified CL Extract, and the Crude Aqueous CL Extract were

likely to be due to the type, proportion, and solubility of the active phenolic and phytochemical compounds present in the respective extracts. NsorAtindana (2012) reported that, variation in yield was based on different method of extractions, extracting solvents, and the concentration of the extracts that influenced the efficacy and/or biocidal activities of plant materials. Other researchers have similarly suggested that the presence of phenolic compounds or phytochemical compounds could be influenced by many factors, which include the age of plant, extracting solvent, method of extraction, and time of harvesting plant materials

(Okigbo and Ajalie, 2005; Okigbo *et al*., 2005, Koolen *et al.*, 2013).

5.6 EFFECT OF EXTRACTS IN CONTROLLING WHITE YAM WEIGHT LOSS AND ROT FUNGI *IN VIVO*

Ethylacetate Purified CL Extract and Crude Aqueous CL Extracts together with Mancozeb (positive control) and Distilled Water (negative control) showed significant differences on weight loss and rot of the white yam samples *in vivo*. Kuhn and Hargreaves (1987) reported that fungicidal substances found *in vitro* in most

cases killed the fungi *in vivo*. There were variations in potency among Ethylacetate Purified CL Extract, Crude Aqueous CL Extract, Mancozeb, and Distilled Water. Differences in compositions, polarities, and concentrations could have influenced the interactions of Ethylacetate Purified CL Extract, Crude Aqueous CL Extract, Mancozeb, and Distilled Water on weight losses and rots of the white yam samples.

Besides, before the application of extracts and inoculums, pathogens pre-existing at the surfaces of the white yam samples *in vivo* were isolated and identified. *Aspergillus flavus, Aspergillus niger, Penicillium spp., Rhizopus spp.* and *Colletotrichum spp.* were the pre-existed fungi isolated from white yam samples *in vivo*. *Aspergillus niger* recorded the most occurring fungus on the surfaces of the white yams *in vivo*. Identifying pre-existing pathogens on surfaces of white yam samples *in vivo* facilitated the documentation of all the yam pathogens that could have possibly caused rot in the present study.

5.6.1 Weight Loss

5.6.1.1 Effect of extracts on weight loss of treated yam tubers during storage The effects of Ethylacetate Purified CL Extract, Crude Aqueous CL Extract, Mancozeb and Distilled Water significantly showed different trends on weight loss. Tubers treated with Crude Aqueous CL Extract significantly exhibited the highest weight loss while yam tubers treated with Distilled water showed the lowest weight loss. This could have been the result of osmosis which facilitated the movement of water molecules from the distilled water into the yam tissues through a semi permeable yam peel (Kosinski and Morlok, 2009).

On the contrary, the respective solutions of Ethylacetate Purified CL Extract, Mancozeb and Crude Aqueous CL Extract could be referred to as hypertonic solutions (Kosinski and Morlok, 2009) but with different levels of concentrations and water potentials. The respective solutions surrounding the yam cell had higher concentrations and lower water potential as compared to that of Distilled Water.

Moreover, the Crude Aqueous CL Extract was highly concentrated than that of Ethylacetate Purified CL Extract and Mancozeb. Crude Aqueous CL Extract therefore possessed the lowest water potential as compared to Ethylacetate Purified CL Extract and Mancozeb. This could have rendered Crude Aqueous CL Extract treated yam tubers to encounter more water loss or weight loss than that of Ethylacetate Purified CL Extract and Mancozeb treated tubers. This could create a pathway for continuous flow out of water from the yam cells into the hypertonic solutions. Therefore, Crude Aqueous CL Extract was more hypertonic. Hence, Crude Aqueous treated tubers depicted highest weight loss as compared to weight loss with tubers treated with Ethylacetate Purified CL Extract and Mancozeb.

5.6.1.2 Effect of method of application on trend of weight loss of yam tubers during storage

Spraying and dipping, the two methods employed, showed similar trend in weight loss. This implied that both methods of applications could have similar efficacy on yam tubers. So exporters could use any of the two application methods.

5.6.1.3 Effect of microorganisms on trend of weight loss of yam tubers during storage

The microorganism showed similar trend on weight loss of yam tubers from the beginning of storage period until the thirteenth (13^{th}) day onwards where *Penicillium spp*. slightly departed from the trends of *Rhizopus stolonifer* and *Aspergillus flavus*. This could have been due to the rapid increase in relative humidity and decrease in temperature between the eleventh (11^{th}) and fifteenth (15^{th}) days, which enhanced the activities of *Penicillium spp.* on tubers, resulting in its slightly differential weight loss from the other fungi.

Considering the interaction between extracts and microorganism on weight loss of yam tubers, Rhizopus *stolonifer* showed significant weight loss against the tested extracts. Particularly, its interactions with Crude Aqueous CL Extracts attained the highest weight loss than the other extracts. This could be attributed to the increase in activities of *Rhizopus stolonifer* on the defensive system of the yam cells. *Rhizopus stolonifer* is known to cause soft rot to yam tubers (Aidoo, 2011). However, Freeman and Beattie (2008) reported that soft-rot pathogens often target pectins for digestion using specialized enzymes that cause cells to break apart. Meanwhile, pectins form hydrated gels that help "cement" neighboring cells together and regulate the water content of the wall.

5.6.2 Yam Rot

5.6.2.1 Effective control of yam rot during pre-export handling period To effectively control rot, all factors contributing to rot and the appropriate measures of controlling rot before export were critically evaluated in the present study. Okigbo

and Ogbonnaya (2006) reported that rot may probably start in the soil and progress during storage. Besides, wound cannot be entirely prevented especially during harvesting. The survey revealed that perceptible wounded yams were rejected during the pre-export handling operation. Unfortunately, the imperceptible wounds could still exist on the surfaces and lead to future infections. Hence, the need to protect the entire surface against rot pathogens with appropriate pesticide such as fungicide is very important. Aidoo (2011) also reported that rots are basically controlled by preventing wounds, storing at favorable environment, and treating yam tubers with fungicides such as Benlate, Captan, Shavit F71.5WP, and Mancozeb just after harvest.

As indicated in the survey, some exporters used synthetic pesticides as part of their pre-export handling operation. Though Mancozeb (positive control), used *in vitro* and *in vivo*, confirmed the effectiveness (potency) of synthetic pesticide (fungicide) in inhibition and protection against rot fungi. The effectiveness (potency) of synthetic pesticides cannot outweigh their numerous related side effects that could be exerted on consumers and the environment as a whole. They are also expensive to exporters. These side effects could result in acute or chronic diseases to consumers (Gupta, 2008). Therefore, substituting the synthetic pesticides with natural pesticides with stronger or similar potency as tested in this study could be a remedy for exporters.

Pesticides of plant origin are known to be specifically more biodegradable, readily available, cheaper and environmentally friendlier than synthetic chemicals (Aidoo, 2011). Extracts from cocoa leaves which could be composed of phytochemicals such as Saponin, Tannin, Glycoside, Triterpenoid, Sterol, Coumarin, Flavonoid, and Alkaloid were identified in the study. The cocoa leaves extracts therefore exhibited significant fungicidal properties against yam rot fungi both *in vitro* and *in vivo*. Other researchers have published the use of various plant extracts from different plant

material such as *Xylopia aethiopica, Zingiber officinale, Crecentia alata, Dennettia tripetala, Cassia alata Piper nigrum* etc. to control yam rot pathogens (Krishnapillai, 2003; Khan *et al*., 2001; Okigbo and Nmeka, 2005; Aidoo, 2011).

This study does not disregard the pragmatic processes the tubers used to defend themselves from pathogens like fungi. The cocoa leaves extracts applied on the surfaces (including the microspores of the yam skin) could rather boost the defensive system of the yam cells by increasing the levels of phenolic compounds and other phytochemical compounds. Reports by other authors confirmed the defensive mechanisms of phytochemicals such as polyphenol, anthocyanins, cyanidin glucoside, o-quinones, chlorogenic acid, leucoanthocyanidin etc. in yam cells (Ozo, 1985; Muzac-Tucker *et al*., 1993, Farombi *et al*., 2000; Baah, 2009). In circumstances where the growth rate of the microorganisms overcomes the defensive system of the yam cells, they could be overcome with the phytoconstituents in the extracts applied. This implied that the application of the cocoa leaves extracts could help the cells of the tubers to become fortified against fungi attack. Freeman and Beattie (2008) reported that the plant cells have a first line of preformed and inducible defenses that protect them against pathogens. This helps the plant cells to be highly resistant to a broad range of pathogens for an extended period of time. Hence, this induces the Systemic Acquired Resistance (SAR). This SAR represents a heightened state of readiness in the plant cells whereby resources (compounds) are mobilized in case of further attack. SAR can be artificially triggered by spraying plants with chemicals called plant activators. These substances are gaining favor because they are much less toxic to humans and wildlife than synthetic fungicides and their protective effects can last much longer (Lattanzio *et al*., 2006; Freeman and

Beattie, 2008).

The susceptibility of anterior, middle, and posterior sections of the yam tubers to rot differs. Therefore, variations in the incidence of rot at the various sections of the yam tuber were observed. This could be due to the differences in the morphology, moisture content, and chemical composition of the respective sections of the yam tubers. The posterior section exhibited the highest percentage incidence of rot. The softness of the posterior section could account for its high susceptibility to rot. A similar view reported by Aboagye-Nuamah *et al*. (2005) stated that the parenchyma, which constitutes the main part of the tuber, fades away progressively towards the distal extremity. This could be attributed to the sectional variation in the mineral composition of yam tubers and the peel and also, the differences in texture, density, moisture content, starch, glucose, a-amylase, protein, lysosome, cresolase and catecholase.

The efficacies of the Ethylacetate Purified CL Extract, Crude Aqueous CL Extract, Mancozeb, and Distilled Water in controlling white yam tubers rot fungi were significant. The Distilled Water recorded a higher significant rot than the Crude Aqueous CL Extract. This implied that the Crude Aqueous CL Extract could improve on the resistance of white yam tubers against rot fungi as compared to Distilled Water treated or untreated white yam tubers. Though the same amounts of Ethylacetate Purified CL Extract, Crude Aqueous CL Extract, Mancozeb and Distilled water were applied to boost the immunity of the yam cells, there were variations in their potencies. Variations in susceptibility of yam to rot could be due to the difference in potencies of the respective extracts applied. Ethylacetate Purified CL Extract and Mancozeb treated white yam tubers showed similar trend in potency against rot but higher potencies than the Crude Aqueous CL Extract. This could be due to the similar capabilities of Ethylacetate Purified CL Extracts and Mancozeb treated tubers in

controlling rot. This also means that Ethylacetate Purified CL Extract and Mancozeb could be used interchangeably. The causative fungi that caused rot on the white yam samples were evaluated at the end of the pre-storage handling period (on 19th day). In descending order of most occurring fungi, *Aspergillus niger*, *Penicillium spp.*, *Rhizopus stolonifer* and *Aspergillus flavus* respectively were identified as causative fungi that initiated rots. Among the causative fungi, *Aspergillus niger* was found to be most virulent in causing rot but was not part of inoculum applied. *Aspergillus niger* was confirmed most virulent because it was the most common fungi that pre-existed on the white yam samples before treatment. This could have resulted in its population being higher than the concentration of the inoculums applied. They could have already invaded the imperceptible wound and micro opens pre-existing or caused during postharvest handling. *Penicillium spp.* followed the *Aspergillus niger* in order of occurrence and was reported earlier in this study as most virulent to induce rot among the fungi initially isolated from samples collected from the market places and exporters' warehouses. This could imply that *Penicillium spp.* still stands at the position of being the most virulent to induce rot among the fungi inoculated (*Rhizopus stolonifer* and *Aspergillus flavus*) *in vivo*.

The variations in the respective test fungi to induce rots could be due to the specific abilities of the fungi to overcome the preformed and induced defenses of the yam cells and the extra defenses provided by the phytochemical compounds in the respective extracts applied. This had similarly been reported by Lattanzio *et al*. (2006) and Freeman and Beattie (2008). Similar trend of results has also been reported by Aboagye-Nuamah *et al*. (2005) who suggested that, once the spoilage organism penetrates the preformed and induced structural barriers of the tubers and establishes in the tissue, the tuber's biochemical resistance mechanism is unable to defend it

against the disease (rot). The variations in phytochemical compounds in the respective extracts can be related to the available concentrations of low polarity compounds due to the solvents and methods of extraction of the tested extracts (Goyal *et al*., 2008; Nsor-Atindana, 2012; Tatiya *et al*., 2011; Koolen *et al.*, 2013).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Ghana is the leading exporter of yam in West Africa. However, based on the survey, exporters are always unable to export the most preferred white yam varieties throughout the year because their supply depends on seasonal yam production and scattered small-scale farmers. The survey revealed that yam rot is one of main challenges facing the yam exporters and the most predominant method of control is the use of synthetic pesticides, which could pose health hazard to consumers.

Isolated and identified fungi occurred on infected white yam samples collected were *Aspergillus flavus, Aspergillus niger, Penicillium spp.* and *Rhizopus stolonifer*. *Penicillium spp.* was found to be very rampant from the samples randomly collected from the market places whiles at the exporters' warehouses *Penicillium spp* and *Rhizopus stolonifer* were the fungi found to be most rampant. So *Penicillium spp.* followed by *Rhizopus stolonifer* were found to be very prevalent and rampant fungi causing rot among the isolated fungi and variations in the occurrence of the fungi were due to localities where the tubers were cultivated.

Ethylacetate Purified CL Extract was significantly effective followed by Acetone Purified CL Extract, while Crude Aqueous CL Extract (0.0mm) showed no inhibition

against the four test fungi. Regarding the MICs, the most effective extract was obtained from Mancozeb followed Ethylacetate Purified CL Extract and the least was Crude Aqueous CL Extract *in vitro*. The types of solvent and methods of extraction employed in the study could have accounted for the significant unequal distribution of phenolics and other phytochemical compounds present in cocoa leaf extracts. This contributed to the variation in the efficacies of the respective extracts in terms of antioxidant and antimicrobial potentials in inhibition of fungi growth and determination of MICs *in vitro*.

Both Spraying and dipping methods of application showed no significant differences and therefore similar efficacies on application of extracts on yam tubers. So exporters can use the two methods of application interchangeably during pre-export handling period.

Ethylacetate Purified CL Extract and Mancozeb treated yam tubers showed lower weight loss as compared to Crude Aqueous CL Extract treated yam tubers. This could be due to the fact that solutions prepared from Ethylacetate Purified CL Extract and Mancozeb contain lesser quantities of extract (solute) and high water potential and that made their respective solutions less hypertonic as compared to Crude Aqueous CL Extract. However, Distilled Water treated yam tubers showed the lowest weight loss as compared with all the extracts because Distilled Water was hypotonic with the highest water potential.

Therefore, cocoa leaves extracts especially Ethylacetate Purified CL Extract which recorded the highest antioxidant and antimicrobial potential among the extracts, can be used as a natural pesticide as compared to Crude Aqueous CL Extract. This can be

an ideal alternative for synthetic pesticide used in controlling rot-causing pathogens on export grade white yam in Ghana.

6.2 RECOMMENDATIONS

- Further studies should be done on the exact varieties of yam exported from Ghana to the international market especially varieties with high international value.
- Further studies should be done on the potency of Cocoa leaves extracts especially duration of breaking down.
- Future studies should be done on the use of antioxidants from cocoa leaves extracts in reducing oxidative stress resulting from abiotic and abiotic stress stored yam.
- Further studies should be done on the isolation and identification of active compounds possessing antimicrobial properties in respective cocoa leaves extracts in order to maximize the potentials of pesticidal properties.
- Further investigation has to be carried out on the best extracting solvents for different herbal plant materials for effective control of rot causing pathogens.

REFERENCES

Aboagye-Nuamah, F., Offei, S. K., Cornelius, E. W., & Bancroft, R. D. (2005). Severity of spoilage storage rots of white yam (Dioscorea rotundata Poir.). *Annals of Applied Biology*, *147*(2), 183–190.

Achakzai, A. K. K., Achakzai, P., Masood, A., Kayani, S. A., and Tareen, R. B. (2009). Response of plant parts and age on the distribution of secondary metabolites on plants found in quetta. *Pakistan Journal of Botany*, *41*(5), 2129–2135.

Adejumo, A. S. (2010). *Growth And Yield Performance Of White Yam (Dioscorea Rotundata Poir) Cultivars In White Yam/Sweet Potato Intercrop*. University Of Agricuture, Abeokuta, Ogun State, pp. 49

Aidoo, A. K. (2011). *Yam Tuber rot: identification and Control of Pathogens in Storage* (Doctoral dissertation, KNUST), Kumasi, pp. 60

Alabadan, B. A. (2002). Assessment of storage losses in roots and tubers in Niger State, Nigeria. *African Journal of Root and Tuber Crops* 5(1): 49-52.

Amoo N. A. R., Wireko-Manu, F., Baah, O. I., (2014). Physicochemical and Pasting Properties of Starch Extracted from Four Yam Varieties. *Journal of Food and Nutrition Sciences*. Vol. 2, No. 6, pp. 262-269.

Amusa, N. A., Adigbite, A. A., Muhammed, S., and Baiyewu, R. A. (2004). Yam diseases and its management in Nigeria. *African Journal of Biotechnology*, *2*(12), 497-502.

Anaadumba, P. (2013). Analysis of incentives and disincentives for yam in Ghana. *Technical notes series, MAFAP, FAO, Rome*, (Draft Version), pp. 41

Asemota, H. N., Wellington, M. A., Odutuga, A. A., & Ahmad, M. H. (1992). Effect of short_term storage on phenolic content, o _diphenolase and peroxidase activities

of cut yam tubers (Dioscorea sp). *Journal of the Science of Food and Agriculture*, 60(3), 309-312.

Asiedu, J. J. (1986). Processing and Physical/chemical properties of Tropical products. Centaurus-Verlagsgesellschaft, Pfaffenweiler. pp. 377-398.

Asuming-Brempong, S. (1991). Yam for foreign exchange: potentials and prospects in Ghana. In *Symposium on Tropical Root Crops in a Developping Economy 380* (pp. 382-387).

Baah, F. D. (2009). *Characterization of water yam (Dioscorea alata) for existing and potential food products* (Doctoral dissertation, department of Food Science and Technology, Kwame Nkrumah University of Science and Technology). Kumasi. 134pp

Barnett, H. L., and Hunter, B. B. (1972). *Illustrated genera of imperfect fungi*. (3rd ed). Burgess Publishing Company. 241 pp.

Barry, A. L., Fuchs, P. C., and Brown, S. D. (2001). Identification of β-lactamasenegative, ampicillin-resistant strains of Haemophilus influenzae with four methods and eight media. *Antimicrobial agents and chemotherapy*, *45*(5), 1585-1588.

Bhattacharjee, S. (2005). Reactive oxygen species and oxidative burst: roles in stress, senescence and signal. *Curr. Sci*, *89*, 1113-1121.

Burton, W. G. (1966). The potato. A survey of its history and of factors influencing its yield, nutritive value, quality and storage. *The potato. A survey of its history and of factors influencing its yield, nutritive value, quality and storage.*, (Edn. 2 (revised)). Netherlands. 339pp

Copping L. G., and Hewitt, H. G. (1998). *Chemistry and Mode of Action of Crop Protection Agents,* The Royal Society of Chemistry, Cambridge, 1998, pp. 1–113.

Cos, P., Vlietinck., A. J., Berghe, D. V. and Maes, L. (2006). Anti-infective potential of natural products; how to develop a stronger in vitro 'proof-ofconcept'. *Journal of ethnopharmacology* 106(3): 290-302

Coursey, D. G. (1967). Yams. An account of the nature, origins, cultivation and utilisation of the useful members of the Dioscoreaceae. *Yams. An account of the nature, origins, cultivation and utilisation of the useful members of the Dioscoreaceae.* Longmans, London, 230 pp.

Coursey, D. G. (1968). Low temperature injury in yams. *International Journal of Food Science & Technology*, *3*(2), 143-150.

Das, K., Tiwari, R. K. S., and Shrivastava, D. K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: current methods and future trends. *Journal of Medicinal Plants Research*, Vol. 4(2); 104-111

Diop, A. (1998). Storage and processing of roots and tubers in the tropics. FAO Corporate Document Repository No. 45. Rome

Escarpa, A., and González, M. C. (2001). Total extractable phenolic chromatographic index: an overview of the phenolic class contents from different sources of foods. *European Food Research and Technology*, *212*(4), 439-444.

FAO, (2005). *FAO Annual Report*. Food and Agriculture Organization Production Year Book, FAO Rome Vol. 9, ISSUES 7-8, 692-692pp

Farombi, E. O., Britton, G., and Emerole, G. O. (2000). Evaluation of the antioxidant activity and partial characterization of extracts from browned yam flour diet. *Food Research International*, *33*(6), 493-499.

Flood, J. (2003). *Discovery Learning about Cocoa: An inspirational guide for training facilitators*. J. G. Vos, & B. J. Ritchie (Eds.). Surrey, United Kingdom: CABI Bioscience. 111 pp

Freeman, B. C., and Beattie, G. A. (2008). *An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor. doi: 10.1094*. PHI-I2008-0226-01.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, *48*(12), 909–930.

Gill, S. S., Khan, N. A., Anjum, N. A., and Tuteja, N. (2011). Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. *Plant Stress*, *5*(1), 1-23.

Goyal, P., Khanna, A., Chauhan, A., Chauhan, G., and Kaushik, P. (2008). In vitro evaluation of crude extracts of Catharanthus roseus for potential antibacterial activity. *International Journal of Green Pharmacy*, *2*(3), 176-181.

Grierson, D. S., and Afolayan, A. J. (1999). Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. *Journal of Ethnopharmacology*, 66(1): 103-106.

Gupta, A. (2008). Impacts of pesticides on human and ecosystem health, *72*, 61–72.

Hahn, S. K., Osiru, D. S. O., Akoroda, M. O., and Otoo, J. A. (1987). Yam production and its future prospects. *Outlook on Agriculture*, 16 (3), 105-110

Hendrisksen, R. S. (2003). MIC susceptibitlity testing of Salmonella and Campylobacter. *Laboratory protocols*, Level 2 Training Course, 4th Edition, 2-28pp

Hii, C. L., Law, C. L., Suzannah, S., and Cloke, M. (2009). Polyphenols in cocoa (Theobroma cacao L.). In *Asian Journal of Food and Agro-Industry.* Bangkok. Vol. 2, No. 4, pp. 702-722.

Igbinosa, O. O., Igbinosa, E. O., and Aiyegoro, O. A. (2009). Antimicrobial activity and phytochemical screening of stem bark extracts from Jatropha curcas (Linn). *African Journal of Pharmacy and Pharmacology*, *3*(2), 058-062.

IITA, (1993). *Crops Improvement Division/ Tuber Root Improvement Program* Archival Reports (1989-1993). Part III yam. Ibadan. Pp 20-85

Izuka, C. M. and Mbagwu, F. N. (2013). Phytochemical Screening on the Seed of Theobroma Cacao L. (Sterculiaceae). *Global Research Journal of Science* 2(2):127-135.

Kerry B. R. (1999) Progress towards biological control strategies for plant-parasitic nematodes. In: Proceeding of the 1998 Broughton Conference-Pest and Diseases. BCPC, Farnham, UK, pp. 751-752.

Khan, M. R., Kihara, M., and Omoloso, A. D. (2001). Antimicrobial activity of *Cassia alata*. *Fitoterapia*, *72*(5), 561-564.

Khanavi M, Hajimahmoodi M, Cheraghi-Niroomand M, Kargar Z and Ajani Y, (2009). Comparison of the antioxidant activity and total phenolic contents in some *Stachys* species, *Afr J Biotechnol*, 8(6), 1143-1147

Kimball, Ralph, (2012). Cocoa health benefits. Pharmacognosy - Medicinal Plants Kimball University. Available online at; http://www.medicinalplantspharmacognosy.com/herbs-medicinal-plants/cocoa-cacao/cocoa-healthbenefits/ (accessed 2014 December 10)

Koolen, H. H., da Silva, F. M., Gozzo, F. C., de Souza, A. Q., & de Souza, A. D. (2013). Antioxidant, antimicrobial activities and characterization of phenolic compounds from buriti (Mauritia flexuosa L. f.) by UPLC–ESI-MS/MS. *Food Research International*, *51*(2), 467-473.

Kosinski Robert J., and Morlok Kaighn C., (2009). Challenging Misconceptions about Osmosis. Association for Biology Laboratory Education (ABLE) 2008 Proceedings, Vol. 30:63-87.

Krishnapillai, N. (2003). Medicinal value of Ginger (Zingiber officinale) in Jaffna. In *III WOCMAP Congress on Medicinal and Aromatic Plants-Volume 6: Traditional Medicine and Nutraceuticals 680* (pp. 83-86).

Kuhn, P. J., and Hargreaves, J. A. (1987). Antifungal substances from herbaceous plants. In *Symposium series-British Mycological Society*. CambridgeUni. Press. 48-129 pp.

Langfield, R. D., Scarano, F. J., Heitzman, M. E., Kondo, M., Hammond, G. B., and Neto, C. C. (2004). Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant Peperomia galioides. *Journal of ethnopharmacology*, *94*(2), 279-281.

Lattanzio, V., Di Venere, D., Linsalata, V., Bertolini, P., Ippolito, A., and Salerno, M. (2001). Low temperature metabolism of apple phenolics and quiescence of Phlyctaena vagabunda. *Journal of agricultural and food chemistry*, *49*(12), 5817-5821.

Lattanzio, V., Lattanzio, V. M., and Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in research*, *661*, 23-67.

Leite, S. P., Vieira, J. R. C., de Medeiros, P. L., Leite, R. M. P., de Menezes Lima, V. L., Xavier, H. S., and de Oliveira Lima, E. (2006). Antimicrobial activity of Indigofera suffruticosa. *Evidence-Based Complementary and Alternative Medicine*, *3*(2), 261-265.

Lii, R., R., (2012). *Tea, Physical Health And Mental Health. Training Course on Pollution-Free Tea Production Technology for Developing Country*. Zhangzhou College of Science and Technology. Reference Material for ChinaAid Training Programs. Fujian province.

Lourens, A. C. U., Reddy, D., Başer, K. H. C., Viljoen, A. M., and Van Vuuren, S. F. (2004). In vitro biological activity and essential oil composition of four indigenous South African Helichrysum species. *Journal of Ethnopharmacology*, *95*(2), 253-258.

Maalekuu, B. K., Saajah, J. K., and Addae, A. K. (2014). Effect of three storage methods on the quality and shelf-life of white yam (*Dioscorea rotundata*) cultivars pona and tela. *Journal of Agricultural Science*, *6*(7), 221pp.

Markson, A. A., Omosun, G., Madunagu, B. E., Amadioha, A. C., and Wokocha, R. (2010). Physicochemical alteration of tissues of white yam (Dioscorea rotundata Poir) tubers incited by Botryoiplodia theobromae Pat. *International Journal of Current Research*, *4*, 055-061.

Marsilio, V., Campestre, C., and Lanza, B. (2001). Phenolic compounds change during California-style ripe olive processing. *Food Chemistry*, *74*(1), 55-60.

Mathur, S. B., & Kongsdal, O. (2003). Common laboratory seed health testing methods for detecting fungi published by the International Seed Testing Association (ISTA). *Bassersdorf, CH., Switzerland*, 89pp.

Mbata, T. I., Debiao, L. U., & Saikia, A. (2008). Antibacterial activity of the crude extract of Chinese green tea (Camellia sinensis) on Listeria monocytogenes.*African Journal of Biotechnology*, *7*(10).

McDonald, S., Prenzier, P. D., Autolovisch, M. and Robards, K. (2001). Phenolic content and antioxidant activity of olive extracts. *Food Chem 73:73-84.*

McMurrough, I., Madigan, D., & Smyth, M. R. (1996). Semipreparative chromatographic procedure for the isolation of dimeric and trimeric proanthocyanidins from barley. *Journal of Agricultural and Food Chemistry*, 44(7), 1731-1735.

Meursing, E. H. (2008). Dezaan CoCoa & ChoColate Manual. ADM Cocoa International, 40 th Anniversary Edition. Rolle, Switzerland, 169pp.

Moller, I. M. (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual review of plant biology*, *52*(1), 561-591.

Morgenstern, Kat, (2007). Plant Profile - Cacao. Sacred Earth. 326 Mitchell Street, Ithaca, NY 14850, USA. Available online at: http://www.sacredearth.com/ethnobotany/plantprofiles/cacao.php (accessed 2014 November 15)

Morse, M., Acholo, S., McNamara, N., Flegg, L., and Oliver, R. P. (1997). Aetiology of yam (*Dioscorea rotundata*) tuber rots held in traditional stores in Nigeria: Importance of *Fusarium spp*. and yam beetle. *Microbiological Research* 152, 1-6.

Muzac-Tucker, I., Asemota, H. N., and Ahmad, M. H. (1993). Biochemical Composition and Storage of Jamaican Yams (*Dioscorea* sp). *Journal of the Science of Food and Agriculture* 62:219-224

Nsor-Atindana, J., Zhong, F., Mothibe, K. J., Bangoura, M. L., and Lagnika, C. (2012). Quantification of total polyphenolic content and antimicrobial activity of cocoa (Theobroma cacao L.) Bean shells. *Pakistan Journal of Nutrition*, *11*(7), 574–579.

Nwankiti, A. O., and Arene, O. B. (1978). Diseases of yam in Nigeria. Pest Articles and News Summaries 24 (4), 486-494.

Ogaraku, A.O. and Usman H. O (2008). Storage Rot of Some Yams (*Dioscorea Spp*) In Keffi and Environs, Nasarawa State, Nigeria. PAT; 4 (2): 22-27 ISSN: 0794-5213 Available online: patnsukjournal.com/currentissue (accessed 2015 March 9)

Okigbo R. N., Mbajuka C., and Njoku C. O. (2005). Antimicrobial potential of (UDA) *Xylopia aethopica* and *Ocimum gratissimum L.* on some pathogens of man *Int. J. Mol. Med. Adv. Sci. (Pakistan)* 1(4): 392 – 394.

Okigbo, R. N., & Ogbonnaya, U. O. (2006). Antifungal effects of two tropical plant leaf extracts (Ocimum gratissimum and Aframomum melegueta) on postharvest yam (Dioscorea spp.) rot. *African Journal of Biotechnology*, *5*(9).

Okigbo, R. N., and Ajalie, A. N. (2005). Inhibition of some human pathogens with tropical plant extracts–Chromolaena odorata and Citrus aurantifolia, and some antibiotics. *International Journal of Molecular Medicine and Advance Sciences*, *1*(1), 34-40.

Okigbo, R., N., and Nmeka, I., A. (2005). Control of yam tuber rot with leaf extracts of Xylopia aethiopica and Zingiber officinale. *African Journal of Biotechnology*, *4*(August), 804–807

Okigbo, R.N. and F.E.O. Ikediugwu, (2000). Studies on biological control of post harvest rot of yam (*Dioscorea* sp.) with *Trichoderma viride*. *J. Phytopathology*, 148: 351-355.

Okwu, O. E. (2004). Phytochemical and Vitamin content of Indigenous Spices of South Easterm Nigeria. *Journal of Sustain Agricultural Environment. Vol. 6:* Pp. 30-34

Olurinola, P., Ehinmidu, J., and Bonire, J. (1992). Antifungal activity of normaltributyltin acetate against some common yam rot fungi. *Applied and Environmental Microbiology* 58, 758-760.

Osagie, A. U. (1992). *The Yam Tuber in Storage*. Postharvest Research Unit, University of Benin, Benin City, pp. 107–173.

Osman, H., Nasarudin, R., and Lee, S., L., (2004). Extracts of cocoa (*Theobroma cacao* L.) leaves and their antioxidation potential. *Food Chemistry*, *86*(1), 41–46.

Othman, A., Ismail, A., Abdul Ghani, N., Adenan, I., (2007). Antioxidant capacity and phenolic content of cocoa beans. *Food Chemistry*, 100(4), 1523-1530.

Otoo, A. (2015). *Pharmacognostic studies and anti-infective properties of hilleria latifolia (lam.) h. walt.(phytolaccaceae)*. KNUST, Kumasi. 70pp

Otoo, E., Akromah, R., Kolesnikova-Allen, M., Asiedu, R., (2009). Delineation of Pona complex of yam in Ghana using SSR markers. *International Journal of Genetics and Molecular Biology*, 1 (1): 6-16.

Oyedokun, A. V., Anikwe, J. C., Okelana, F. A., Mokwunye, I. U., & Azeez, O. M. (2011). Pesticidal efficacy of three tropical herbal plants' leaf extracts against Macrotermes bellicosus, an emerging pest of cocoa, Theobroma cacao L. *Journal of Biopesticides*, *4*(2), 131-7.

Ozo, O. N. (1985). Analytical procedure for the polyphenols and polyphenol oxidase of yam tubers. *Advances in yam Res. ed. Godson Osuji*, 165-212.

Passam, H. C., (1982). Dormancy of Yams in Relation to Storage. *In Yams. Ignames*. Oxford: Clarendon Press. 285-293pp.

Prieto, P., Pineda, M., Aguilar, M. (1999). Spectrophotometeric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical biochemistry* 269 (2): 337-341.

Salie, F., Eagles, P. F. K., Lens, H. M. J. (1996). Preliminary Antimicrobial Screening of four South African Asteraceae Species. *Journal of Ethnopharmacology*, 52(1) 27-33.

Schmourlo, G., Mendonça-Filho, R. R., Alviano, C. S., and Costa, S. S. (2005). Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. *Journal of Ethnopharmacology*, *96*(3), 563-568.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., and Kelm, M. (2006). (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(4), 10241029.

Silva, M. T., Simas, S. M., Batista, T. G., Cardarelli, P., and Tomassini, T. C. (2005). Studies on antimicrobial activity, in vitro, of Physalis angulata L.(Solanaceae) fraction and physalin B bringing out the importance of assay determination. *Memorias do Instituto Oswaldo Cruz*, *100*(7), 779-782.

Singh S., Anjum N., A., Khan N. A., Nazar R., (2008). Metal-binding peptides and anti- oxidant defense system in plants: significance in cadmium tolerance. In: N.A. Khan, S. Singh, (Eds.) Abiotic Stress and Plant Responses, IK International, New Delhi, pp. 159-189.

SOKMEN, M., and OZBEK, T. (2006). Biological activities of the essential oil and methanol extract of Achillea biebersteinii Afan.(Asteraceae). *Turk J Biol*, *30*, 65-73.

Tatiya, A. U., Tapadiya, G. G., Kotecha, S., and Surana, S. J. (2011). Effect of solvents on total phenolics, antioxidant and antimicrobial properties of Bridelia retusa Spreng. stem bark. *Indian Journal of Natural Products and Resources*, *2*(4), 442–447.

Tenover, F. C., Swenson, J. M., O'Hara, C. M., and Stocker, S. A. (1995). Ability of commercial and reference antimicrobial susceptibility testing methods to detect vancomycin resistance in enterococci. *Journal of clinical microbiology*, *33*(6), 1524-1527.

Todd, Jr., and Paul, H. (1996). Lipid-soluble green tea catechin antioxidant solutions. United States Patent No. 5, 527-552. Washington, D. C: U. S. Patent and Trademark Office.

Trease, G. E. and Evans, W. C. (1989). *Pharmacognosy*. 13th edition. ELBS/Bailliere Tindall Ltd, London, 345-6

USAID, (2005). *West Africa Trade Hub: Export Development Feasibility Study*.

Vanden-Berghe D. A., and Vlientinck A. J. (1991). Screening Methods for Antibacterial and Antiviral Agents from Higher Plants. In: Hostettmann, K. (Editor). Methods in Plant Biochemistry VI. Assays for Bioactivity, Academic Press: London, 47-69.

Visioli F., and Galli, C. (1998). Olive oil phenols and their effects on human health, *J. Agric. Food Chem.*, 46, 4292–4296.

Wiegand, I., Hilpert, K., Hancock, R. E. (2008). Agar and both dilution Methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols, 3(2): 163-75.

Winkelhausen, E., Pospiech, R., & Laufenberg, G. (2005). Antifungal activity of phenolic compounds extracted from dried olive pomace. *Bulletin of the Chemists and Technologists of Macedonia*, *24*(1), 41-46.

APPENDICES

Appendix 1: Sample Questionnaire Administered to Exporters

SURVEY ON PRE-EXPORT HANDLING OPERATION, THEIR CONSTRAINTS AND YAM ROT DISEASE AT MAJOR EXPORTERS' WAREHOUSES IN GHANA

Name.....
.
Location..... Sex.....
Age.....Date.....

1) How long have you been an exporter?.....
2) What varieties of yam do you export?.....
3) What is the most preferred variety for export?.....
4) Why is that variety the most preferred for export? .....

5) Do you always export the most preferred variety?.....
6) What is your source of supply for export?…….....
7) Who are your suppliers?.....
8) How do you get your supply of yam for export?.....
9) Can you describe the linkage between your source of supply and your suppliers?
…….
…….
……
10) What are the major constraints involved in getting your supply?
.....
.....
.....
..
11) Can you outline the activities involved in pre-export handling operation?.....
.....
.....
.....
12) Can you describe the importance of these activates involved in pre-export handling operation?
…….....
.....
.....
.....
.....
.....
13) What is yam rot?.....
14) Which yam rot do you know?.....
15) What causes the rot in export grade yam?.....
.....
.....
16) Do these rots have an effect on the export? Yes / No
17) If yes, what effect?.....
.....
.....
.....
18) Do you know any microorganism that causes yam rot? Yes / No 19) If yes, can you mention the name of any yam rot-causing microorganism?
……
……
……

……

20) Do you do anything to prevent these yam rots? Yes / No
21) If yes, what are some of the control measures you use to prevent yam rots?.....
22) Have you tried any synthetic chemical for treating yam tubers prior to export before?.....
23) If yes, what chemicals do you usually use? .....
24) How do you apply these chemicals on the yam tubers? ……..
25) Do you have any idea of the side effects of these chemicals? Yes / No 26) If yes, what are some of these side effects?
.....
.....
.....
.

27) Do you know of any substitute for chemicals you use to control rot-causing microorganism?.....
.....

Appendix 2: Analysis of Variance Table for Weight Loss

Source	DF	SS	MS	F	P
Rep	2	0.05773	0.02887		
Applicati	1	0.01201	0.01201	0.47	0.4952
Extract	3	0.11882	0.03961	1.56	0.2123
MicroB	2	0.02583	0.01291	0.51	0.6050
Applicati*Extract	3	0.17373	0.05791	2.28	0.0920
Applicati*MicroB	2	0.00081	0.00040	0.02	0.9842
Extract*MicroB	6	0.09163	0.01527	0.60	0.7281
Applicati*Extract*MicroB	6	0.09454	0.01576	0.62	0.7133
Error	46	1.16920	0.02542		
Total	71	1.74429			
Grand Mean 0.4438	CV 35.93				

Appendix 3: Completely Randomized AOV for Percentage Rot

Source		DF	SS	MS	F	P trt
3	34.6112	11.5371	46.13	0.0000		
Error		8	2.0006	0.2501		
Total		11	36.6118			
Grand Mean 4.1675		CV 12.00				

Appendix 4: Analysis of Variance Table for Severity Rot

Source		DF	SS	MS	F	P Rep
2	3.522	1.7611				
applic		1	1.368	1.3680	0.15	0.7063
extract		3	34.732	11.5773	1.25	0.3287
applic*extract		3	31.233	10.4109	1.13	0.3723
Error		14	129.494	9.2496		
Total		23	200.349			
Grand Mean 1.7487		CV 173.91				

Appendix 5: Analysis of Variance Table for Zone of Inhibition

Source	DF	SS	MS	F	P
Rep	2	1.8	0.89		
Conc	3	4878.8	1626.26	5094.87	0.0000
Extract	3	3379.2	1126.39	3528.83	0.0000
Micro	3	982.7	327.58	1026.28	0.0000
Conc*Extract	9	3667.2	407.47	1276.54	0.0000
Conc*Micro	9	1576.5	175.16	548.76	0.0000
Extract*Micro	9	912.1	101.34	317.49	0.0000
Conc*Extract*Micro	27	1631.5	60.43	189.31	0.0000
Error	126	40.2	0.32		
Total	191	17070.0			
Grand Mean 5.0000	CV 11.30				