ISOLATION OF PURE CASSAVA LINAMARIN AS AN ANTI CANCER AGENT

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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ABSTRACT

Cassava is a known source of linamarin, but difficulties associated with its isolation have prevented it from being exploited as a source. A batch adsorption process using activated carbon at the appropriate contact time proved successful in its isolation with ultrafiltration playing a pivotal role in the purification process. Result revealed that optimum purification was obtained with increasing amount of crude cassava extract (CCE) purified. 60g of CCE took 32 mins, 80 g, 34 mins while 100 g took 36 mins of contact time, where 1.7 g, 2.0 g and 2.5 g of purified product were obtained, respectively. The purification process in batch mode was also carried out at different temperatures ranging from 25 to 65^{o}C. Results showed that purification increases with increase in temperature. In a bid to ascertain the moles of linamarin adsorbed per pore volume of activated carbon used, the composite isotherm was found to represent the measured adsorption data quite well. The adsorption of linamarin was used to study the goodness of fit criteria (R^2) for the entire process. Results showed that R^2 value was best with decreasing amount of CCE purified (R^2=1 for 60 g) at the temperature of 45^{o}C. Compound elucidation of purified product by Picrate paper test, IR and ^{1}HNMR confirmed the structure of linamarin. Cytotoxic effects of linamarin on MCF-7, HT-29, and HL-60 cells were determined using the 3 - (4, 5 – dimethylthiazol-2-yl) – 2, 5 – diphenyltetrazolium bromide (MTT) assay. Cytotoxic effects were significantly increased in the presence of linamarase, which catalysed the hydrolysis of linamarin to hydrogen cyanide. A 10–fold decrease in the IC_{50} values obtained for linamarin or crude extract in the presence of linamarase was determined for HL-60 cells. This study thus describes a method for the isolation and purification of linamarin from cassava, as well as the potential of this compound as an anticancer agent.

DEDICATION

This dissertation is exclusively dedicated to our Lord Jesus Christ who is, who was and who is to come in the glory of the Father Almighty God forever and ever. Amen.

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CONFERENCE ATTENDED

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LIST OF ABBREVIATIONS AND SYMBOLS

ATC	Activated carbon
BCT	Bulk cassava tissue
BCE	Bulk cassava extract
CCE	Crude cassava extract
Conc.	Concentration
Lnmr	Linamarin
$\frac{n_0 \Delta x}{m}$	Amount of linamarin adsorbed
1-x	Fraction of linamarin in solution
n_0^s	Pore volume efficiency of activated carbon
RPM	Revolution per minutes
HCN	Hydrogen cyanide
J	Coupling constant
S	Singlet peak
D	Doublet peak
T	Triplet peak
δ	Part per millions
D_2O	Deuterated water
IC_{50}	Inhibition concentration
n_0	Numbers of mole of linamarin
x	Mole fraction of linamarin
m	Mass of activated carbon used

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background and motivations

Cancer is known to be any disease of man or animals in which abnormal, unregulated proliferation of cells result in the formation of malignant tumor, which could either be carcinoma or sarcoma and benign or malignant as in the case of tumor (Singleton and Sainbury, 1997; Roger, 1996a). As the tumor grows, it tends to invade and destroy adjacent tissues and may shed cancer cells that metastasize to other areas of the body, resulting in the formation of secondary tumors (Holland and Frei, 1973; Singleton and Sainbury, 1997; Juan *et al*., 1977).

The causes of cancer are complex and appear to be multifactorial (Charles, 1995; Singleton and Sainbury, 1997) with varying degrees of morphologic disorientation, aggressive growth and invasion with ultimate destruction of the normal cell population. Extensive literature search has also revealed that cancer may be caused apparently by an almost limitless variety of physical, chemical and biological agents in which life style, nutrition, food additives, pesticides, ultraviolet radiation and variety of other environmental factors have been directly implicated (Roger, 1996a; Singleton and Sainbury, 1997; Holland and Frei, 1973; Shinikin and Triolo, 1969).

Cancer is known to rank second to cardiovascular disease as a cause of mortality with an over-all five year survival rate of less than 50% (Li *et al.,* 1999). Press release of 16 May, 2001 on improving performance of European cancer research, a report of cancer death annually in the EU was reported to be about 837,000 (Bill and Michel, accessed 28th June 2006). Report from the National Vital Statistics also showed that in the United State, the annual death rate for cancer is approximately 553,251 (NVSR, 2003) wherein, Robert Cathey Research source in the same vein revealed a figure of over 989,200 people to have died from cancer in the United State in 2005 in spite of the existing anti cancer drugs and treatment methods (Roger, 1996a). The true success of the “war against cancer” becomes a controversy over the high rate of incidence and mortality (Charles, 1995). This is quite alarming and provocative in getting this deadly hunt arrested.

Besides a much better knowledge of the natural history of many types of cancer and the establishment of different novel strategies based on it, yet the most efficient ways for treatment are those described as "classical" methods, such as surgery, radiotherapy, and chemotherapy (Hernandez-Alcoceba *et al.,* 1999). There is a general belief that development of new chemotherapeutic agents is probably the most reliable way to improve our success against cancer, and intelligent drug design is considered as a key factor for this end (Powis, 1994; Unger, 1996). However, no chronic or metabolic disease has ever found cure or prevention that is real except through factor essential to an adequate diet and/or normal to animal economy (Krebs Jr, 1970).

Cassava (*Manihot esculenta* Crantz) is a readily available source of food or diet across the globe most especially to the developing nations and contains cyanogenic compounds. In spite of the enormous biomedical potentials of this class of compound inherent in cassava, recent reviews have only dealt with the distribution, biosynthesis and toxicology of these compounds, rather than the practicality of isolation and identification thereof, due to the difficulties associated with these processes (Seigler, 1975). Crude cassava extract has been used to control one form of cancer and the other in time past (Iyuke *et al.*, 2004). Linamarin is the major cyanogenic component (93%) present in cassava relative to lotaustralin (7%) (Liangcheng *et al.,* 1995). The need to exploit and identify the colossal factor(s) inimical in its isolation and purification and to device a possible layout or process for this is thus very pertinent as well as to investigate its antitumoral candidature.

Separation of the components of a liquid solution (such as linamarin from cassava extract) has been one of the most frequently occurring problems in the field of chemical engineering, though, there are several general techniques available to the engineer to bring about this separation, some of which may be inapplicable as a result of certain physical properties that are unique to the system at hand (Treybal, 1951). Since it is required that components be recovered in nearly pure form, these techniques are usually physical rather than chemical operations. Many depend upon the tendency of a substance, when distributed between two insoluble phases, to come to different concentrations in each of the phases at equilibrium (Treybal, 1951). Impurities of organic origin are often more economically removed by the

use of a solid adsorbent in process supplementary to the economic utilization of the chemical methods (Mantell, 1951).

The use of ultrafiltration and adsorption process with activated carbon allow for the isolation and purification of this linamarin. Ultrafiltration functions well as a separation/purification technique, while the adsorption property of activated carbon thus mediates in the final purification of this compound. Since processing of liquids concern itself with maintaining composition within definite limits, then, raw material is subjected to stage treatment to obtain products of satisfactory purity. Each step effects increased concentration of material desired in the product and decreased concentration of undesired constituents (Mantell, 1951).

1.2 Research problem

In spite of the colossal bio-medical potentials of linamarin from cassava, it has up till date been known with an associated history of difficulty in isolation and perhaps purification. Therefore its direct application or usage has not been established.

Since it is strongly desirable that component be recovered in a pure state, conventional means (organics and hydrocarbon solvents) of isolating a desired compound in chemical process has more often than not compromised product's purity and integrity. Again, it suffices to express passionately that life is directly dependent on the environment and as such environmental impact of these organic waste disposal stands a major threat to the entire ecosystem. Therefore the need to strive for purity-sensitive and possibly environmental friendly processes is thus sternly important.

Cancer research has not found cure yet irrespective of the huge concern. The conventional therapies in use today are not only unable to cure cancer but also tend to induce more harm than cure (Roger, 1996a) and (Roger, 1996b).

Surgery and radiation therapies are described as forms of local control of diseases (Charles, 1995). Roger (1996a), reported that surgery does not cure the cancer; the excision of a lesion or affected organ does not cure the cancer since the causes of the initial development of the

cancer are still present in the body. Surgery also has the tendency of spreading cancer cells in the body resulting in secondary neoplasm. Radiotherapy on the other hand involves burning the cancer cells whereby, normal physiologic cells around and within its pathway will also be destroyed, and in strong consequence reducing the defense mechanism of the body, and subsequent exposure to opportunistic ailment(s) as well as further spread of the cancer (Roger, 1996a). Chemotherapy is known with indiscriminate killing of cells in the body once the poison is released with the implication of immunosuppression of the body system (Holland and Frei, 1973; Charles, 1995). Integrated therapy/immunotherapy also revolves in the same high degree of limitation. It is reported that hormonal therapy as a rule can not cure cancer, but long term effective control could be possible (Charles, 1995). Besides, these conventional therapies are believed to be associated with enormous side effects (Charles, 1995; Holland and Frei, 1973). Infact, studies have not only unveil their very expensive nature but has also shown that more than half the patients with cancer, the therapies available today are known to be inadequate, providing temporal control, but eventually failing. Such patients die of locally invasive tumor, or more often, of disseminated cancer (Holland and Frei, 1973). This has eventually left the true success of the 'war against cancer' a matter of controversy over the high rate of incidence and mortality (Charles, 1995).

In spite of all these odds, chemotherapeutic agents still have the general belief as the most probable way to improve our success against cancer (Powis, 1994; Unger, 1996), hence the need to strive and intensify research on getting an agent that is minimally immunosuppressive whenever possible and to design a possible process for it is thus very pertinent.

1.3 Hypothesis

A novel approach can be developed for the isolation and purification of linamarin from cassava, taking into consideration the physical and biochemical properties of this compound, and that of process potentials, which will not only provide the long expected remediation and cure for cancer and other ailments but can also be used as a basis for the isolation and purification of other cyanogenic compounds that are naturally occurring in hundreds and

thousands of plants that are available across the globe, since they have enormous potentials in bio-medical applications.

1.4 Justification of the study

Several research studies have been reported on the determination, and not on the isolation and purification of this group of compound known as beta-cyanogenic glucoside including linamarin in different naturally occurring plants using various quantitative and semi quantitative means because of the enormous difficulties associated with these processes (Seigler, 1975). Apart from using crude cassava extract for the control of one form of cancer or the other in time past (Iyuke et al., 2004), no work has been reported on the use of linamarin either from cassava or any other plant for the control or cure of one cancer or the other. This work is therefore expected to bring about significant technological, economical, medical and environmental benefits because of its environmental, medical and cost effectiveness. Furthermore, this research is of great value should it prove successful, in reducing the high rate of mortality posed by the ugly deadly hunt, CANCER that has long ridiculed and jettisoned the happiness of man's existence. It will also unlock the potentials of other medical, bio-chemical applications and research, wealth, social and job creation to nations because cassava is readily available across the globe and is a crop that can tolerate adverse environmental conditions.

1.5 Scope of the project

In order to establish a possible process for the isolation and purification of linamarin from cassava, the project is approached in four phases, namely, physicochemical and bio-chemical study of cassava linamarin, developed optimum process for its isolation and purification, characterisation by chemical elucidation and finally investigate its candidature on some cancer cell lines. These four stages involved sourcing for cassava stems and tubers, where cassava stems were planted in the university garden, the use of the tissues for extract, purification and characterisation. These were followed by sourcing and culture of cancer cell lines from Wits medical school which were screened with the analog (linamarin) purified from the cassava tissues.

1.6 Research questions

In an effort to devise and establish a possible process for the isolation and purification of cassava linamarin and to investigate its candidature on cancer cell lines, the following research questions have been considered.

- What is the chemical composition of linamarin
- How would its physicochemical properties influence its isolation and purification
- What could be responsible for its known difficulty in isolation and purification
- How possible and to what extent can ultrafiltration of membrane technology be useful in its isolation and perhaps purification
- Does the adsorption process using activated carbon have the potential for the purification of linamarin
- What is the candidature of linamarin on cancer cells

1.7 Purpose and aims

The main purpose of the research is to explore and establish a possible process for the isolation and purification of cassava linamarin and to tap its potential(s) directly by evaluating its candidature as an anti cancer agent. The research aimed to achieve the following objectives:

- To arrest the hydrolyzing enzyme (linamarase) responsible for the break down of linamarin
- The use of ultrafiltration in the isolation of linamarin component
- The use of activated carbon for the purification of impure linamarin
- Characterization of linamarin
- Screening of linamarin on different cancer cell lines

1.8 Expected contribution to knowledge

This work which is aimed at developing a process for the isolation and purification of linamarin from cassava and to ascertain its candidature on cancer cell is expected to provide:

- Information on the physicochemical characteristics of cassava linamarin
- Information on the bioactivities of cassava linamarin
- Information on the use of ultrafiltration for the isolation of linamarin component

- Information on the adsorption characteristics of activated carbon for the purification of linamarin
- Background and useful information for designing reactors and equipment for linamarin production from cassava
- Adequate information for the development and design of a new, better anticancer drug

1.9 Dissertation outline

Chapter 1

This chapter discuses the background and motivation of this study, research problem, hypothesis, justification of the study, scope of the project, research questions, purpose and aims and the expected contribution to knowledge.

Chapter 2

This chapter focuses on literature review and it will be discussed in four parts. The first part discusses linamarin as cyanogenic compound from cassava. The second part discusses the overview of ultrafiltration in membrane technology. The third part focuses on activated carbon, and finally, the MTT cancer assays as the fourth part of the chapter.

Chapter 3

This chapter explains the experimental procedure of isolation of cyanogenic component, purification by batch process with activated carbon adsorption, and procedure for cancer cell lines screening.

Chapter 4

This chapter discuses all experimental results.

Chapter 5

This chapter considers the conclusion and recommendation of the entire dissertation

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CASSAVA

Cassava (Manihot *esculenta* Crantz) is a storage root crop. It is widely cultivated in the tropics, primarily for the storage roots, although the young leaves are also eaten (Lancaster *et al.,* 1982). The strong ability of cassava to survive adverse environmental conditions makes it an important crop for food security in many of the developing countries. The carbohydrate-rich, low-in protein storage roots represent an important energy source and staple food stuff for more than 500 million people throughout tropical Africa, Latin America and parts of Asia (Yeoh *et al.,* 1998). Tetsu *et al.* (1996), reported it to be a staple food for about 800 million people in the world. Apart from being major source of carbohydrates, it is also a valuable source of calcium, iron, thiamine, riboflavin, niacin, dietary fibers, among others (Hansen, 1985; Oke, 1969). Figure 2.1 below shows cassava tubers (roots). Despite the fact that cassava leaves provide an inexpensive source of protein in the human diet, it is also an important source of animal feeds, industrial alcohols and other products (Poonam and Hahn, 1984).

There is a great interest among cassava growers and breeders to screen their germplasm collection for varieties with low linamarin (cyanide) content. Also, varieties with high linamarin content are also sought after as they tend to show greater resistance to pests and diseases, since there is strong evidence that cyanogenesis (the ability of some plants to synthesize cyanogenic glucosides, when enzymatically hydrolyzed, released cyanohydric acid, known as prussic acid) is one of the mechanisms that can serve to the plant as a protective device against predators such as herbivores (IIza and Mario, 2000).

Figure 2.1: Cassava tubers (roots)

2.1.1 Cassava as a cyanogenic plant

Cassava is a cyanogenic plant containing two cyanogenic glucosides, linamarin (2-β-D-glucopyranosyloxy-2-methylpropanenitrile) and lotaustralin [(2R)-2- β-D-glucopyranosyloxy-2-methylbutyronitrile] derived from valine and isoleucine, respectively (Peifan *et al.,* 2004; Koch *et al.,* 1992). The ratio of linamarin to lotuastralin in leaves and roots is reported to be about 93:7 (Liangcheng *et al.,* 1995). Although, these compounds have been reported to be present in all the tisues of the plant (Conn, 1980) with linamarin content varying widely in different tissues of the cassava plant: leaves (Figure 2.2), stem and where root peel contains higher level of the glucoside than the edible tuber (De Bruijn, 1973; Nambisan and Sandaresan, 1994). Different cassava varieties also show wide variation in tuber linamarin content (range, 25-450 µg cyanide equivalent/g), which could be due to different rate of biosynthesis, degradation or transport (Elias *et al.,* 1997). Enviromental factor, the cultivar and the growth condition have all been implicated as well (Cooke, 1978; Bradbury *et al.,* 1991).

Translocation of linamarin takes place from leaves to roots (Nambisan and Sandaresan, 1994). Santana *et al.* (2002) reported that cassava linamarin synthesis suggestes that the cyanoglucosides accumulate in roots and are synthesized in shoots and then transported to roots where they are stored. In contrast, Elias *et al.* (1997), reported that there is no progressive accumulation of linamarin in tuber which is an indication that linamarin is not stored passively in the tissue, but is mobilized and utilized.

2.1.2 Cyanogenocity of cassava (hydrolysis of linamarin)

Apart from the cyanogenic glucosides that cassava tissues are composed of, cassava tissues are also composed of an endogenous hydrolyzing enzyme known as linamarase (β-glucosidase). When cassava root tissue is damaged, mainly by mechanical action (e.g., during processing or preparation for consumption) or microbial action (e.g., during fermentation process or deterioration owing to poor-harvest storage), the enzyme (lianamarase) comes in contact with the linamarin, resulting in its hydrolysis and the subsequent release of hydrogen cyanide (cyanogenosity) (Yeoh *et al.,* 1998). The hydrolysis of linamarin is a two-step reaction involving the formation of an intermediate, acetonecyanohydrin, which breaks down spontaneously or by hydroxynitrilelyase action to form acetone and hydrogen cyanide (Yeoh *et al.,* 1998).

Figure 2.2a: Cassava planted at two months old (Wits garden)

Figure 2.2b: Cassava planted at eight months old (Wits garden)

Linamarin is not toxic in itself and is an unlikely source of cyanide exposure in humans (Mlingi *et al.,* 1992). It has been observed that part of ingested linamarin in cassava product has been found to pass through the human body unchanged within 24 hrs when excreted in human urine (Brimer and Roseling*.,* 1993; Carlson *et al.,* 1995). Since 1891 it has been known (Nestel*,* 1973), and it had been implicated as an aggravating factor in iodine deficiency disorder as a result of malnutrition. Its role in neurological disease and some tropical variants of diabetes mellitus has been reported (Grindley *et el.,* 2002). As cyanoglucoside, linamarin is chemically related to amygdalin (laetrile), although both are different in molecular weights. Linamarin has never been used in cancer metabolic therapy in the United State, but the use of amygdalin and linamarin derived from seed of almond, apricot and peach (Nahrstedt, 1987), and cassava extracts (Yeoh *et al.,* 1998), respectively for some forms of cancer control though not adequately proven scientificantly has been in existence in the Chinese herbal history (Iyuke *et al.,* 2004).

2.1.3 Linamarin as nitriloside

Linamarin is a member of a class known as beta-cyanogenetic glucosides, which also include others such as amygdalin, prunasin and dhurrin. Also called the'nitriloside' (Krebs Jr, 1970).

They have been defined as water soluble, essentially non toxic, sugary compounds found in plants, many of which are edible. They comprised molecules made of sugar, hydrogen cyanide, a benzene ring or an acetone (Krebs Jr, 1970), though linamarin does not have the benzene ring. This class of compound has been collectively considered to constitute vitamin B_{17} (Krebs Jr, 1970). Fourteen naturally occurring nitrilosides have been known to be approximately distributed in over 1,200 species of plants in which cassava has been highly implicated as a source of high nitriloside (linamarin) food amongst others such as millet, maize, and sorghum (Oke, 1969; Krebs Jr, 1974). Cassava has been reported on estimate to contain from 225 to 1830 mg/kg of the nitriloside linamarin (Culbert, 1983)

The metabolic breakdown of linamarin by beta-glucosidase results in the formation of sugar, a ketone and cyanide. Figure 2.3 shows the metabolic breakdown of linamarin. Cyanide is a potent cytotoxic agent that kills cells by inhibiting cytochrome oxidase of the mitochondrial electron transport chain (Saidu, 2004). But when ingested, cyanide activates the body own mechanisms of detoxification, resulting in the transformation of cyanide to thiocyanate, a compound that is less toxic (Tetsu *et al.,* 1996; Roger, 1996a; Roger 1996b; Saidu, 2004) and found in the serum, urine, sweat, saliva and tears of man and lower animals (Saidu, 2004). At the same time, it has been known as the precursor of HCN and supplier of the cyanide ion for the nitrilization of the precursor of vitamin B-12 (hydrocobalamin) to vitamin B -12 (cyanocobalamin) (Roger, 1996b). This gives a fact that HCN is a substance with fundamental physiological significance in man.

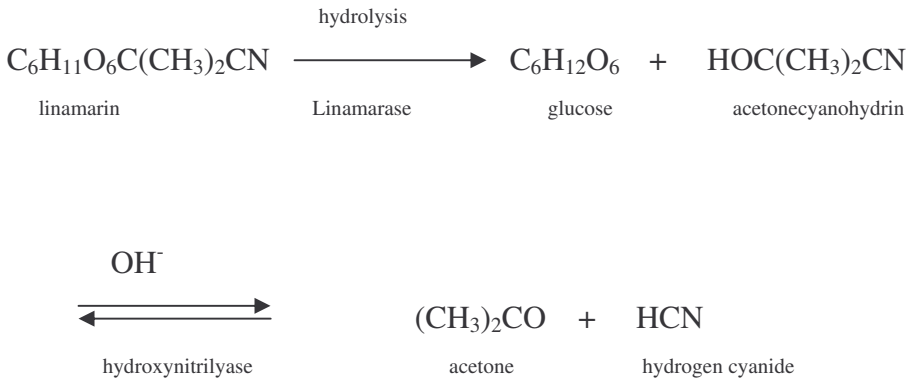


Figure 2.3: Linamarin hydrolysis

The principal detoxification pathway of cyanide is that catalysed by a liver mitochondrial enzyme, 'rhodanase' (sulphur transferase). It is widely distributed in both plants and animals species (Saidu, 2004). The major function of the rhodanase is cyanide detoxification which is highly specific in action. It is limited not merely to nitriles but only to those nitriloside which surrender free HCN ions upon hydrolysis such as linamarin (Roger, 1996b). However, development of thiocyanate in the presence of severe iodine deficiency has apparently been associated with goitrogenic effect in both human and animal populations. There has never been anything to suggest the possibility of any cumulative toxicity arising from the cyanide ion itself (Roger, 1996b). The nitriloside CN ion has been reported as a booster, raising both the red cell count and the total hemoglobin in animals and humans when small quantities of cyanides or various quantities of the nitriloside is given (Roger, 1996b).

2.1.4 Biosynthesis of linamarin

The biosynthesis pathway of linamarin has been proposed to involve two cytochrome P450s and a glucosyltransferase just like dhurrin (Peifen *et al.,* 2004; Anderson *et al.,* 2000). The first cytochrome P450 converts L-Valine to the corresponding oxime (2-methyl propanol oxime). The second cytochrome P450 converts the oxime to nitriles. The last step of adding a glucosyl group to 2-hydroxyisobutyronitril (acetone cyanohydrin) is catalysed by a UDP-glucose transferase. The conversion of Valine to 2-methylpropanal is the rate-limiting steps, and the substrate preference of the first committed cytochrome P450 is the major factor to determine the content of different cyanogenic glucosides accumulated in a given species (Peifen *et al.,* 2004). Anderson *et al.* (2004), has isolated two full length cDNA clones that encode cytochrome P450s that catalysed this reaction (Anderson *et al.,* 2004). Studies using seedlings indicated that linamarin is synthesized in the cotyledons and transported to other tissues of the cassava plant (Peifen *et al.,* 2004).

Koch *et al.* (1992), has demonstrated in vitro biosynthesis of cyanogenic glucosides in cassava using a microsomal system isolated from etiolated cassava seedlings (Koch *et al.,* 1992). It was found that the microsomal enzyme system catalyses the conversion of Valine and Isoleucine to the corresponding cyanohydrins which dissociated into the corresponding

aldehydes or ketones and hydrogen cyanide. *In invivo*, the cyanohydrins are glucosylated into linamarin and lotaustralin by a soluble UDP-glucosyltransferase (Nartey, 1978). Microsomal enzyme systems isolated from etiolated seedlings of sorghum and cassava has been used to demonstrate the involvement of cytochrome P450 in the biosynthesis of cyanogenic glucosides (Koch *et al.,* 1992). *De novo* synthesis of linamarin in cassava roots both *in vivo* and *in vitro* has shown also that at least part of the cyanogenic glucosides is synthesized in the roots (Liangcheng *et al.,* 1995).

2.1.5 Analysis of linamarin

Determination of cassava cyanogenic potential (linamarin) has been on the trend either with the use of an enzyme (linamarase) or sulfuric acid. Over the past 25years, many appropriate analytical methods of determining the cyanogenic potential of cassava have been well published (Yeoh *et al.,* 1998). Some of which are known to be quantitative methods (Bradbury *et al.,* 1991; Yeoh and Truong, 1993) while others are semi-quantitative (Indira *et al.,* 1969; Ikediobi *et al.,* 1980). Although the principle behind them has been reported to be the same (Yeoh *et al.,* 1998) in which linamarin is hydrolyzed by the enzyme (Linamarase). This is followed by detection of the hydrogen cyanide or glucose released. The semi-quantitative procedure involves direct damaging of the cassava root tissue by the addition of some organic solvents causing the endogenous enzyme to hydrolyze the linamarin. This is followed by alkaline picrate paper test and the detection of hydrogen cyanide liberated in the cause of the reaction (Egan *et al.,* 1998; Yeoh *et al.,* 1998). However, Yeoh *et al.,* (1998), had submitted that the reliability of such methods had often been questioned. The quantitative method of analysis involves extraction of linamarin from cassava roots and cassava processed products, and then hydrolysed by the addition of exogenous linamarase (Yeoh *et al.,* 1998). This is followed by the determination of hydrogen cyanide (linamarin equivalent) in several routs such as amperometry (Tetsu *et al.,* 1996), potentiometry (Yeoh and Truong, 1993), spectrophotometry (Cooke 1978; Bradbury *et al.,* 1991) and biosensory (Yeoh *et al.,* 1998). Although quantitative procedures can perform numerous analyses at one time, they are not designed to handle large numbers of samples. This is because the analysis

requires the use of cassava root extracts, and their preparation is a rate-limiting step (Yeoh *et al.,* 1998). Research findings and recommendation has been made on the newly modified picrate assay that, the newly modified picrate assay should be used for rapid screening of cassava cyanogens in all cases, except when significantly high proportion of low-cyanogen clones (0-50 $mgkg^{-1}$) are used, (there will be a high risk of the unintended rejection of low-cyanogen material) and where a very rapid result is required (Gerard *et al.,* 1994).

2.1.6 Anti cancer potential of nitriloside (linamarin)

An important exciting feature of the nitriloside has been unveiled exclusively with respect to malignant lesion that are known to be almost completely deficient of the detoxification enzyme, 'rhodenase' but rich in the hydrolyzing enzyme 'beta-glucosidase' or beta 'glucoronidase' (Roger, 1996a and Roger, 1996b). "Nitriloside is selectively hydrolysed at malignant lesion by beta-glucosidase in the rhodenase deficient lesion. By this way the CN ion is brought to the malignant cell in a highly concentrated and selective manner'' (Roger, 1996a). There are a number of normal tissues in the body that carry both beta-glucosidase and beta-glucoronidase but also carry equal concentration of rhodanase, which completely protects such normal somatic tissues from the action of any cyanide ion that the beta-glucosidase or beta-glucuronidase component of the tissue causes to be released from the hydrolysed nitriloside (Roger, 1996b). Figure 2.4 shows the detoxification reaction of cyanide by the rhodenase enzyme.

$$S_2O_3^{2-} + CN^- \xrightarrow{\text{rhodenase}} SCN^- + SO_3^{2-}$$

thiocvanate

Figure 2.4: Detoxification reaction of hydrogen cyanide by rhodenase

In a similar question asked by Dr. Krebs Jr; it is thus literally true to say that linamarin contains cyanide, a deadly poison. Yet it is also true to say that table salt, sodium chloride, contains the deadly poison, chlorine. Under normal conditions, the chlorine in salt and the cyanide in linamarin are tightly bound, in no danger of suddenly leaking out. In another sense

to the findings and report of Dr Krebs Jr, one may ask how can a compound that is totally non-toxic be relevant to a disease as serious as cancer, a disease perhaps as lethal as pernicious anemia once was? Would we not expect that very powerful cytotoxic compounds would be required to destroy cancer cells? Would these not be compounds like the nitrogen mustard, the antimetabolites, the cyclophosphoramides, methotrexate, 5-flourouracil, 6-chloropurine, 6-mercaptopurin, azaserine, triethylenphosphamide, the nitrosoguanidines, and countless other compounds so toxic that some kill almost 25 percent of the patients treated directly or indirectly through toxicity alone? Although for an agent to be effective it must be both non-toxic to normal somatic cells and yet present powerful cytotoxins to neoplastic cells (Krebs Jr, 1970).

Linamarin as a nitriloside containing vitamin B_{17} is expected on hydrolysis to release a specific and powerful cytotoxin, the HCN. Neoplastic (cancer) cell that is almost completely deficient of the detoxification enzyme (rhodenase) but highly rich in the hydrolyzing enzyme will be exposed to the lethal effect of the cyanide released thereby killing the cancer cell. If some cyanide “spill out” from the cancer cells, adjacent normal cells will then be able to detoxify it through their highly rich rhodenase enzymes (Figure 2.4) that are widely distributed in all the body tissues (Krebs Jr, 1975; Oke, 1969). If detoxification is equal to absorption, no death or injury occurs no matter the amount of cyanide absorbed (Oke, 1969).

The antineoplastic (cancer) potential of cassava linamarin can therefore never be undermined. In line with Krebs Jr., (1974) and according to fountain of life (http://www.thefountainnoflife.ws/cancer/nocancer.htm), “the Indians of North America are another people who are remarkably free from cancer. The Americal Association (AMA) went as far as conducting a special study in an effort to discover why there was little to no cancer amongst the Hopi and Navajo Indians. The February 5, 1949 issue of the journal of the America Association declared that they found 36 cases of malignant cancer from a population of 30,000. In the same population of white persons there would have been about 1800. Dr Krebs research later found that the typical diet for the Navajo and the Hopi Indians consisted of nitriloside-rich foods such as cassava. He calculated that some of the tribes

would ingest the equivalent of 8000 mg of vitamin B_{17} per day from their diet". Thus what can prevent has the potential to cure.

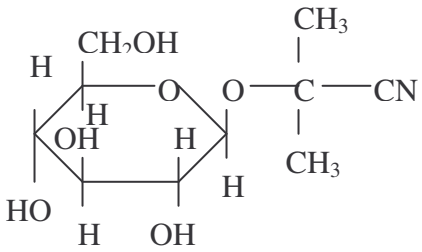
The chemical and structural nomenclature of linamarin 2-(-D-glucopyranosyloxy)-2-methylpropanitrile is relatively similar to those of amygdalin and methyl-α-glucoside due to the common location of glucose moiety as presented in Figure 2.5 (Iyuke *et al.,* 2004; Lei *et al.,* 1999; Zubay, 1993). One other main similarity between linamarin and amygdalin, both being cyanoglucosides, is that they do not contain free cyanide. Their cyanide ions (CN^-) only become freed when it undergoes hydrolysis. In the case of linamarin, the hydrolysis occurs when it comes in contact with linamarase to produce glucose, acetonecyanohydrin, which later decomposes to hydrogen cyanide and acetone as shown earlier in Figure 2.3. On the other hand, methyl-α-glucoside is being used to study the transport of glucose as a common carbon and energy source for many cells (Zubay, 1993). Taking the structural similarities of methyl-α-glucoside with linamarin, it is suggested that linamarin may transport glucose into cells as well (Iyuke *et al,* 2004). Methyl-α-glucoside is a non-mentabolisable analogue. But the study by Hagihira *et al.* (1963), involving glucose transport system of *Escherichia coli* by following intracellular accumulation of ^{14}C-labelled-α-methyl glucoside, it was observed that-α-methyl glucoside was partially phosphorylated. It was also highlighted that the glucoside was not incorporated into the cellular constituents or metabolized for energy. These observations could then be implied to have been resulted from the usual favoured bond split at α-positions leading into the glucose phosphorylation. Similarly therefore, linamarin molecules would be glucose transporters and the resultant bond cleavage is at the α-position, to produce glucose phosphorylation, and at the appropriate pH conditions, the nitrile component would dissociate to the usual hydrogen cyanide and ketone. The implication of this is that, when linamarin is transported into the cell, the cell will try to phosphorylate the glucose moiety by splitting the α–bond, and the cyanide ion will be deposited within the cytoplasm, in turn imposing a lethal effect on the cell, which will lead to cell apoptosis (Iyuke *et al.*, 2004). Therefore a neoplastic (cancer) cell which need a whole lot of glucose than normal cells to support it abnormal and rapid growth and proliferation is expected to be tremendously vulnerable to linamarin intracellular transport into the cancer

cells, simply described as a 'suicidal case'. In all, linamarin would be credited as a powerful arsenal with a strong weapon that arrests and destroys cancer.

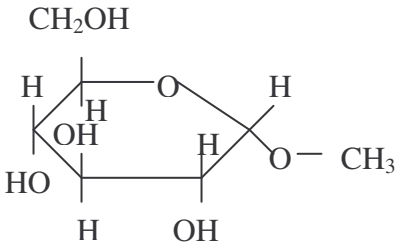
Recent reviews have dealt with the distribution, biosynthesis, toxicology and other aspects of this compound, rather than the practicality of isolation and identification thereof, due to the difficulties associated with these processes (Seigler, 1975), hence, no reference data to show up till date apart from the cyanogennocity test mentioned above. This study therefore is aimed to isolate pure cassava linamarin, screen it against cancer cell lines and investigate its candidature as anti cancer agent.

2.1.7 Physicochemical parameters of linamarin

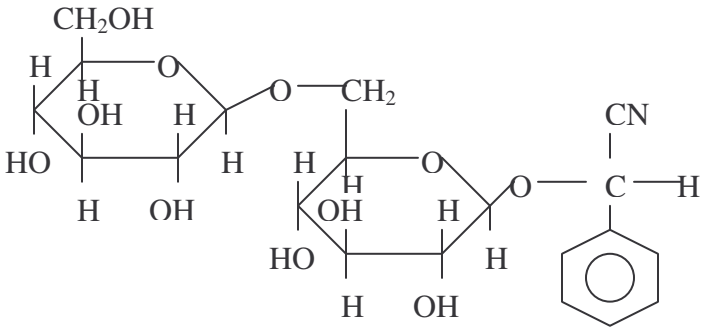
Linamarin [2-(-D-glucopyranosyloxy)-2-methylpropanenitrile] is also called phaseolunatin. It has an empirical formular of $C_{10}H_{17}NO_6$ with a molecular weight of 247.24 g/mol. It has an elemental composition of C 48.58%, H 6.93%, N 5.6% and O 38.83%, soluble in water, and appears as a white solid. Linamarin can be found in any of these families; Compositae, Leguminosae, Euphorbiaceae, Linaceae and Papaveraceae (Seigler, 1975) with the over all constitution of acetonecyanohydrin-glucoside.



Linamarin



Methyl-α-glucoside



Amygdalin

Figure 2.5: Structures of some related β-glucosidase compounds (Iyuke *et al.,* 2004)

2.2 OVERVIEW OF ULTRAFILTRATION IN MEMBRANE TECHNOLOGY

According to Mohr *et al.* (1989a), a membrane is defined as a thin barrier through which fluids and solutes are selectively transported when a driving force is applied across the barrier. Membrane technologies play an increasingly important role as unit operations for resource recovery, pollution prevention, and energy production, as well as environmental monitoring and quality control. They are also key component technologies of fuel cells and bioseparation applications (Wiesner and Shankararaman, 1999). The technology enables industrial users to simultaneously concentrate, fractionate and purify their products (Dziezak, 1990). The technology can be operated at ambient temperature and does not require phase change (Cheryan, 1986). Thus for heat sensitive products this becomes certainly very useful. Presently, the heart of membrane technology research is the formulation of the membrane themselves. The scope of applications is being enlarged, and costs are being reduced through the development of membranes that have reduced thickness, and greater compatibility with oxidants such as chlorine (Wiesner and Shankararaman, 1999).

Membrane separation technology involves the separation of components from a solution with mixed molecules sizes by flowing the solution under pressure over the surface of a membrane (Dziezak, 1990; Koseoglu *et al.,* 1991b). When feed stream enters the membrane system, a driving force is applied across the membrane such that solutes, whose sizes are greater than the pore size of the membrane, are retained and concentrated forming a liquid that can either be called the concentrate, retentate or residue. Water and solutes smaller than the pores pass through the membrane and are called the permeate (Srikanth, 1999; Woerner, 2004; Mohr *et al.,* 1989; Koseoglu *et al.,* 1991b). A simple membrane concept is shown in Figure2.6.

According to Woener, (2004) with respect to membrane basic principles, there are two keys to membrane filtration which differentiate membrane application from conventional filtration. First, the membranes are asymmetric with the small side of the pore facing the feed. This feature minimizes the pressure drop across the membrane, and eliminates any tendency to plug the membrane. Secondly, all membrane systems operate with a strong cross

flow over the surface of the membrane which limits the build up of a filter cake or concentration polarization layer in membrane to a few microns.

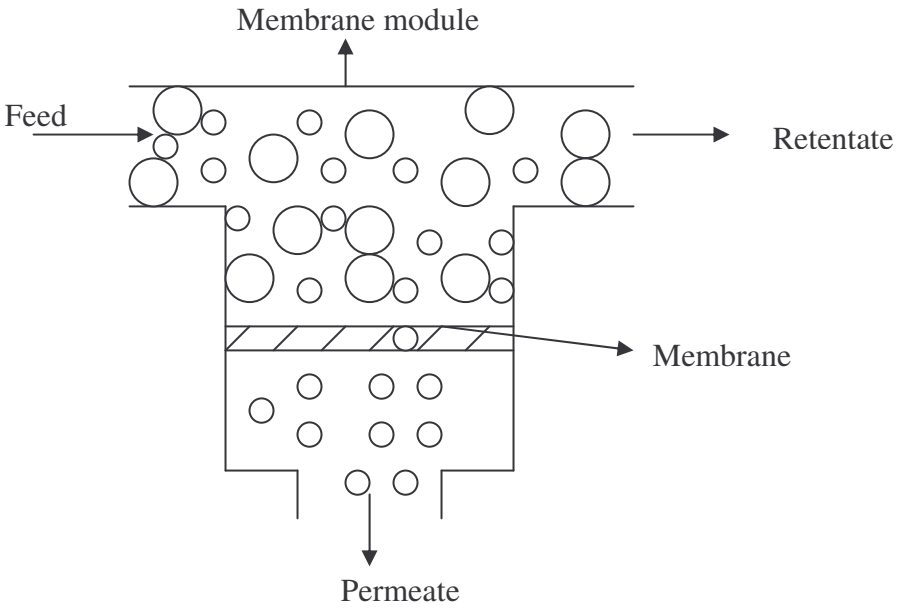
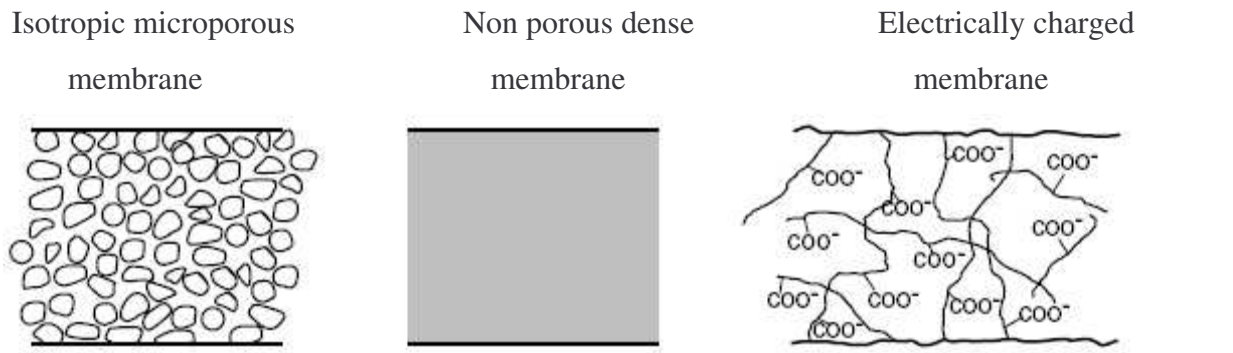


Figure 2.6: Simple membrane concept (Mohr *et al.,* 1989a)

The structure of membrane can be distinguished generally as either micro porous or asymmetric (Baker, 2004; Paulson *et al.,* 1984; Srikanth, 1999). A microporous membrane also called isotropic membrane behaves almost like a fibre filter and separates by sieving mechanism determined by the pore diameter and particle size distribution (Srikanth, 1999; Baker, 2004). Materials such as ceramics, graphite, metal oxides, and polymers e.t.c are used in making such membranes.

Asymmetric membrane also classified as the anitropic is the most important types comprising a thin (0.1-1.0 micron) skin layer on a highly porous (100-200 microns) thick substructure. These membranes combine the high selectivity of a dense membrane with the high permeation rate of a very thin membrane. The resistance to mass transfer is determined to a large extent by the thin top layer (Majid, 2001; Srikanth, 1999). Figure 2.7 below shows the schematic diagram of the principal membrane types.

SYMMETRIC MEMBRANES



ANITROPIC MEMBRANE

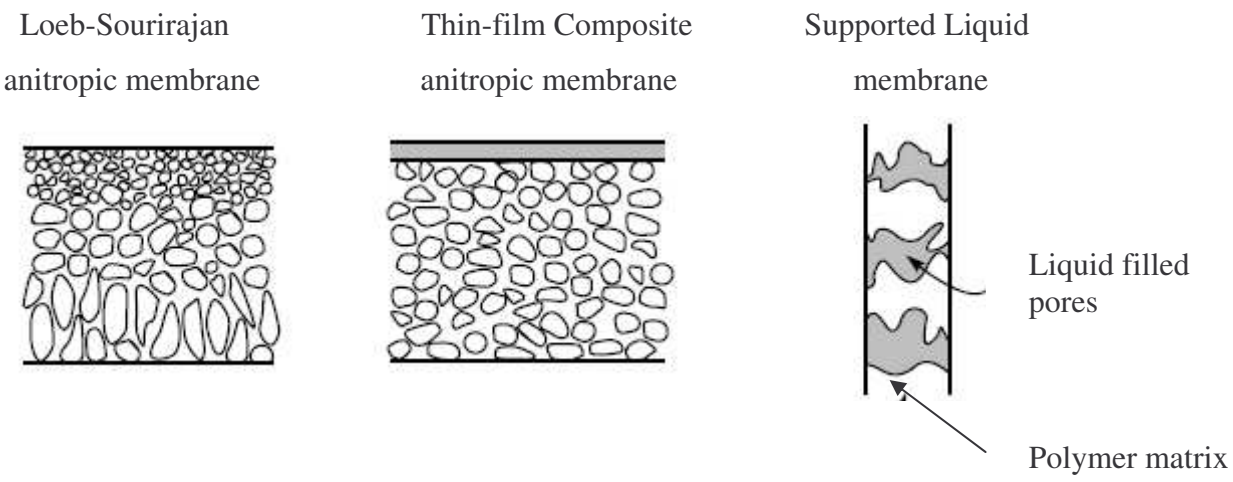


Figure 2.7: Schematic diagrams of the principal types of membrane (Baker, 2004).

The selection of the appropriate membrane requires consideration of operating conditions such as temperature, pressure, pH of the feed stream, and chemical compatibility of the membrane with the feed stream (Paulson *et al.,* 1984). Applegate (1984) also stated that performance of specific membrane system is affected by membrane composition, temperature, pressure, velocity of flow and interactions between components of the feedstock and with the membrane material. Thus, good membranes should have high permeability, proper size, and good mechanical stability.

The principle of membrane process involves separation of molecules on the basis of size and molecular weight (Dziezak, 1990). The four common pressure membrane processes include microfiltration, ultrafiltration, reverse osmosis and nanofiltration (Woerner, 2004; Srikanth, 1999; Koseoglu *et al.,* 1991b). These processes are all well established, and the market is served by a number of experienced companies (Baker, 2004). Although reverse osmosis, ultrafiltration and microfiltration constitute a conceptually similar process. The difference in pore diameter (or apparent pore diameter) produces dramatic differences in the way the membranes are used (Baker, 2004).

In context, ultrafiltration is most commonly used to separate a solution that has a mixture of some desirable components and some that are not desirable. Typical rejected species include sugars, biomolecules, polymers and colloidal particles (Srikanth, 1999). Ultrafiltration is somewhat dependent on charge of the particle, but is much more dependent on the size of the particles. Literature has revealed that ultrafiltration process operates at 1.03-13.8 bar, though in some cases up to 25-30 bar has been used, and the driving force for transport across the membrane is a pressure differential (Koseoglu *et al.,* 1991b; Srikanth, 1999).

Ultrafiltration separates dissolved solutes from 0.002 to 0.2 microns which corresponds to a molecular weight cut-off of approximately 500 to 300,000 MW (Dziezak, 1999; Paulson *et al.*, 1984). Cut-off is defined by the molecular size or weight of the components that are retained at 90 to 95% retention coefficient or rather more than 90% rejection i.e. if the components with a molecular weight of 50,000 daltons are retained at 90 to 95%, the cut-off

is 50,000 daltons (Srikanth, 1999; Koseoglu *et al.,* 1991b). Figure 2.8 shows a schematic diagram of ultrafiltration crossflow system.

It is found that, whenever the solvent of a mixture flows through the membrane, retained species are locally concentrated at the membrane surface, thereby resisting the flow. In the case of processing solution, this localized concentration of solute normally results in precipitation of a solute gel over the membrane. Then, when process is a suspension, the solids collect as a porous layer over the membrane surface. In view of this, it is clear that the permeate rate can be effectively controlled by the rate of transport through the polarization layer rather than by membrane properties. Hence, ultrafiltration throughput depends on physical properties of the membrane, such as permeability, thickness, process and system variable like feed consumption, feed concentration, system pressure, velocity and temperature (Srikanth, 1999). But it is important during ultrafiltration to balance speed with retention to obtain optimum performance. Operating parameters such as pressure, concentration, temperature, and pH and fouling affect the flux of a membrane, which is the flow rate divided by the membrane area. The flux is directly proportional to the pressure gradient across the membrane and inversely proportional to the viscosity and the resistance to flow of the membrane and the solid accumulation on the surface of the membrane.

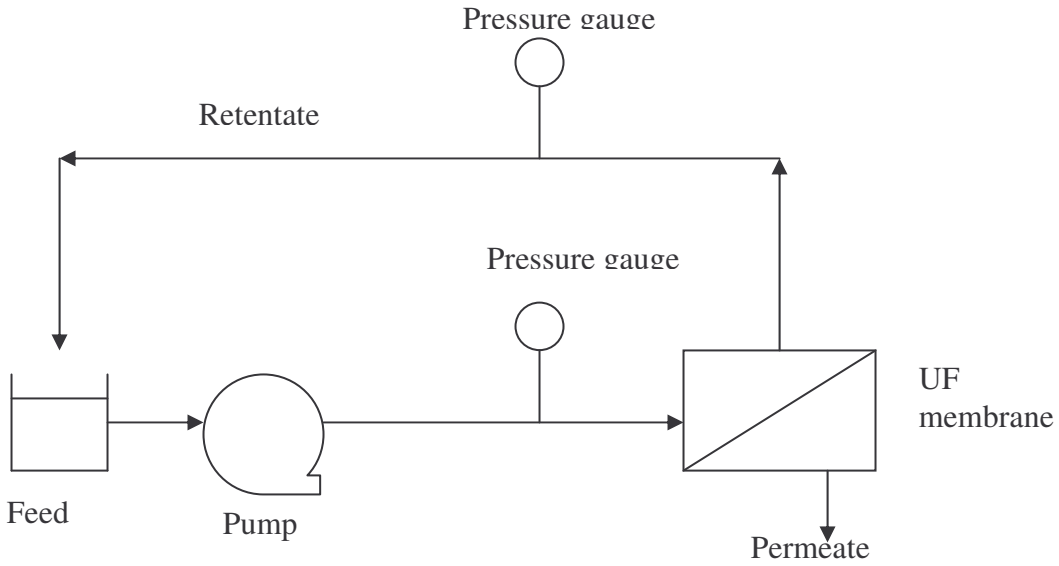


Figure 2.8: Schematic diagram of ultrafiltration crossflow system

The dual limiting factors of ultrafiltration can not be undermined. These are concentration polarization and membrane fouling, both of which have a detrimental influence on the permeation rate (Nakao and Kimuras, 1981; Haris *et al.,* 1986; Haris and Dobos, 1989). Concentration polarization is a boundary layer phenomenon (Majid, 2001). According to Field (1993), a boundary layer is that region within a fluid, adjacent to a surface, across which there is significant change in velocity, concentration or temperature. Koseoglu *et al.* (1991b) referred to concentration polarization as the collection of a layer of the solvent and its content on the surface of the membrane by hydrogen bonding or other attractive forces. Solving this problem, Smith and Gregorio (1970), suggested that polarization can be minimized by establishing turbulent mixing in the system to reduce the concentration profile along the flow channel. Although, attempt to control the polarization have included adoption of high tangential velocities and membrane configurations to increase surface turbulence and shear. This procedure includes the use of turbulence promoters, high flow rates, and operation at the maximum temperature permitted by the membrane materials and the membrane modules (Koseoglu *et al.,* 1991b; Mohr *et al.,* 1989a).

Fouling on the other hand refers to the accumulation of impermeable particles onto the membrane surface which could be the possible crystallization and precipitation of smaller solutes that are normally permeable in the membrane pores (Moubois, 1980; Merin and Cheryan, 1980). The build-up of this deposit offers hydrodynamic on the stream and interfere with flux (Paulson *et al.,* 1984) and causes a continuous reduction in flux and separation. Foulants include inorganic salts, macro molecules, colloids and micro organisms (Majid, 2001). While fouling is an irreversible phenomenum, concentration polarization is a reversible phenomenum (Kun-pei and Munir, 1983).

Suggested methods in controlling and minimizing the extent of fouling include pretreatment, adjusting of membrane properties, membrane cleaning, modification of operating conditions and optimization of modules design (Mohr *et al.,* 1989a). But the main approach to the prevention/reduction of fouling has involved the hydrodynamic increase in shear at the membrane surface through an increase in cross-flow velocity or the use of turbulence promoters (Da Costa *et al.,* 1993; David *et al.,* 1971)

The important characteristics of membrane materials are porosity, morphology, surface properties, mechanical strength and chemical resistance. Polymeric materials such as polysulfone, polypropylene, nylon 6, polytetraflouroethylene (PTFE), PVC, acrylic copolymer etc., have been used successfully as ultrafiltration membranes. Inorganic materials such as ceramics, carbon based membranes, zirconia etc., have been commercialized by several vendors (Srikanth, 1999). Table 2.1 shows the characteristics of ultrafiltration membrane.

Table 2.1: Characteristics of ultrafiltration membrane

Process	**Membrane and pore radius**	**Membrane materials**	**Process driving force**	**Application**
Ultrafiltration	Asymmetric, micro-porous 1-10nm	Polysulfon, polypropylene, nylon, PTFE, PVC, Acrylic copolymer	Hydrostatic pressure, difference at approximately 0.1-1.0Mpa	Separation of macro molecular solutions

Membrane modules are available in four basic designs which include hollow fibre, spiral, plate and frame, and tubular (Srikanth, 1999; Mohr *et al.,* 1989a). The techno-economic factors for the selection, design and operation of membrane modules include cost of supporting materials and enclosure (pressure vessel), power consumption in pumping and ease of replaceability (Srikanth, 1999). Capillary membrane is also being largely used in industrial applications.

Ultrafiltration has a wide range of applications ranging from oil emulsion waste treatment, concentration of biological macromolecules, electro-coat paint recovery, concentration of textile sizing, concentration of heat sensitive proteins for food additives, concentration of gelatin, enzyme and pharmaceutical preparations, pulp mill waste treatment, production of ultra pure water for electronic industry, macro molecular separations replacing conventional change of phase method, refining of oil (Koseoglu, 1996b; Wiesner and Shankararaman,

1999) to bioprocess that involves the separation and concentration of biologically active components. (Koseoglu, 1991a). According to Haralson and Jondahl, (1983), large amount of spent ultrapure water from electronic plant can be purified for reuse by both reverse osmosis and ultrafiltration. The food industry has its precise application in the processing of meat, dairy, fruits/vegetable, sugar, grain mill, beverages and fats/oils in terms of protein concentration for gelatin manufacture, preconcentration of milk for cheese manufacture, fractionation and concentration of whey protein, clarification of fruit juices (apple, cranberry), concentration of tomatoes, pineapple and peach juice, natural colour recovery from cranberry wastewaster, recovery of sugar from confectionary equipement wastewater, preconcentration of dilute sugar juice prior to evaporation/crystallization, enzyme separations, removal of alcohol for low-or nonalcoholic beer and wine and vegetable protein preparation (Mohr *et al.,* 1989b).

Cleaning of the membrane involves four methods including hydraulic, mechanical, chemical and electrical cleaning. Back flushing is an example of hydraulic cleaning in which the process is carried out by reversing the direction of flow of the membrane. This dislodges the foulant from the membrane and enables the flux to be restored to a near initial value. Chemical cleaning involves exposing the module to the cleaning solution for a period of several hours (Majid, 2001). Cleaning the membrane with suitable acids, detergents or enzymes can loosen or dissolve fouling matter from the surface of the membrane or dislodge foulant from within the pores (Mohr *et al.,* 1989a; Porter, 1990). While mechanical cleaning involves the usage of oversized sponge balls which is only applicable in tubular systems. Electrical cleaning is the application of a pulsed electric field (Majid, 2001).

The enormous advantages of membrane technology can never be over emphasized. It is becoming increasingly attractive as a low-cost generic separation technique that enables processors to concentrate, fractionate, and purify their products (Dziezak, 1990; Koseoglu *et al.,* 1991b). This is purely an energy saving process technology that is void of phase change, heat source or any ancillary equipment, and it is a process that has a minimal effect on the micro environment of the product, such as its pH or ionic strength (Paulson *et al.,* 1984; Cheryan 1986).

2.3 ACTIVATED CARBON IN LIQUID-PHASE ADSORPTION

2.3.1 Theory of adsorption

The unit operation of adsorption is concerned with contacting a solid with a fluid mixture under such conditions that some of the fluid is adsorbed on the surface of the solid with a resulting change in composition of the unadsorbed fluid (George *et al.,* 1950). The adsorption process occurs at solid-fluid interfaces. There are two types of adsorption namely; Physisorption and chemisorption (Mantell, 1951; Ralph, 1987).

* Physisorption

Physisorption normally occurs as a result of energy difference and/or electrical attractive forces (weak van der Waals forces) such that the adsorbate molecules (liquid contaminant) become physically attached to the adsorbent molecules (solid surface). Adsorption of this type could be multilayered when each molecular layer forms on top of the previous layer with the number of layers proportional to contaminant's concentration.

Physisorption can be reversible (Mantell, 1951). But the reversibility of physisorption is dependent on the strength of attractive forces between adsorbent and adsorbate. Hence desorption is readily effected if these forces are weak (van der Waals force) (Hernning and Degel, 1990).

* Chemisorption

Chemisorption normally occurs when definite chemical bonds are produced between the atoms or molecules on the surface of the solid and the adsorbed atoms or molecules. Unlike physisorption, this process is one molecule thick and irreversible (Mantell, 1951) because energy is released to form the new chemical compound at the surface of the adsorbent.

Both methods take place when the molecules in the liquid phase become attached to the surface of the solid as a result of the attractive force at the adsorbent (Mantell, 1951), which thus overcome the kinetic energy of the adsorbate molecules.

This research focuses on physisorption of liquid purification by batch process where natural colours resulting from plant biological pigments (chlorophyll, xanthophylls or carotene) stand to be a predominant impurity.

The solid substrate on which adsorption occurs is called the adsorbent, or sorbent. The adsorbing species are the adsorptive, and the adsorbed materials are the adsorbate, or sorbate (Hernning and Degel, 1990; Mantell, 1951; Ralph, 1987). A schematic sketch of the mechanism of adsorption/desorption can be shown in Figure 2.9

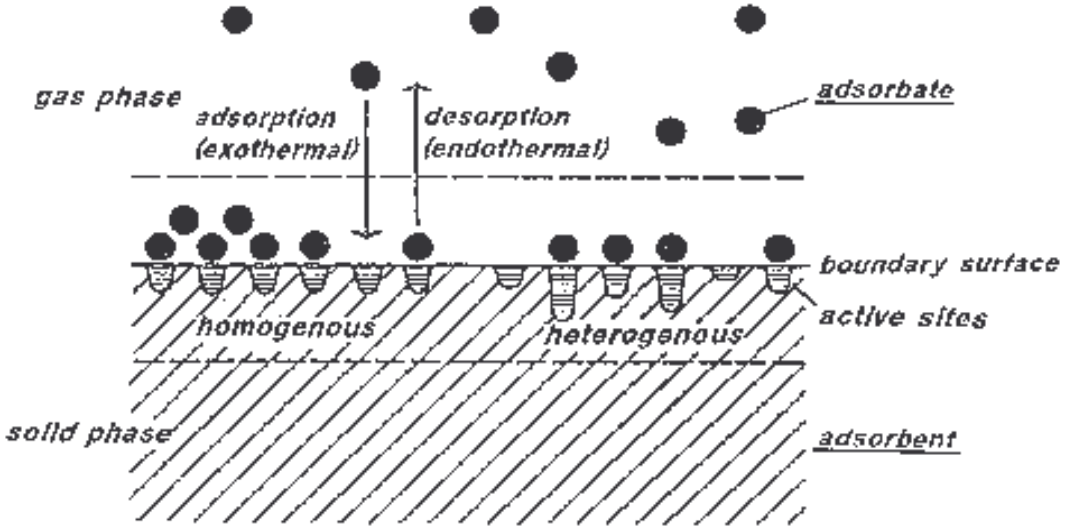


Figure 2.9: Mechanism of adsorption and desorption process (Hernning and Degel, 1990)

Generally, adsorption process involves the accumulation of molecules from a solvent onto the exterior and interior (i.e. pore) surfaces of the adsorbent. This surface phenomenon is a manifestation of complicated interactions among three components that involve the adsorbent, the adsorbate and the solvent (Ralph, 2003). Normally, the affinity between the adsorbent and the adsorbate is the main interaction force controlling adsorption. However,

the affinity between the adsorbate and the solvent (i.e. solubility) can also play a major role in adsorption (Norzilah, 2005; Mantell, 1951).

2.3.2 Activated carbon in adsorption

Odour, taste and colour are certain characteristics of organic compounds that present themselves as impurities to either a desired solution or product. Activated carbon has an affinity for organics and its use for organic contaminant removal from aqueous solutions, gaseous streams and wastewater is widespread.

The large surface area enhances the effectiveness of activated carbon for the removal of organic compounds from fluids by adsorption. The surface area of activated carbon typically ranges from 450-1,800m^2/g (Ralph, 2003; Norzilah, 2005). An example is shown in Table 2.2.

Apart from the surface area of activated carbon that enables its effectiveness, its chemical nature also plays a significant role. The chemical nature (polarity) varies with the carbon type and can influence attractive forces between molecules (Mantell, 1951). Alkaline surfaces are characteristic of vegetable carbon of origin whose surface polarity affects the adsorption of dyes, colours, and unsaturated organic compounds. Because of the nonpolar nature of activated carbon surface, it makes adsorption for organics easily affected but rather a difficult case for the adsorption of inorganic electrolytes (Mantell, 1951).

There are four factors that affect an adsorption and these are;

1. The physical and chemical characteristics of the adsorbent, i.e. surface area, pore size, particle size distribution and chemical composition.
2. The physical and chemical characteristics of the adsorbate, i.e. molecular polarity, concentration of the adsorbate in the liquid phase (solution) and chemical composition.
3. The characteristics of the liquid phase: pH and temperature and
4. The residence time of the system.

Table 2.2: A typically available surface area of activated carbon

Origin	Surface area (m^2/g)
Bituminous coal	800-1,400
Coconut shell	1,100-1,150
Pulp mill residue	550-1,100
Wood	700-1,400

Calgon Corporation 'Basic Concepts of Adsorption on Activated carbon', Pittsburg, Pennsylvania. In Norzilah (2005).

When a liquid containing impurities is brought into contact with carbon, the attraction of the carbon for impurity is greater than the attraction of the liquid for the impurities. The carbon therefore adsorbs the impurities, such as colouring matter, odour, and flavour, until equilibrium is reached, after which the carbon no longer removes these substances from that particular solution (Mantell, 1951).

The rate of removal of impurities from a solution by activated carbon is very rapid during the first interval of contact and gradually reaches a point where increased time of contact gives no further decolourization (equilibrium point). Colour removal is usually greater at higher temperatures. With time of contact and temperature both fixed, the percentage of total impurities removed varies logarithmically with percentage of carbon used (Mantell, 1951). However, adsorption is a complicated process depending on several interactions such as electrostatic and non-electrostatic (hydrophobic) interactions and much has been accomplished in terms of sorption properties and kinetics, hence much work is still necessary to identify the sorption mechanism clearly (Gregorio, 2006).

According to Mantell (1951), both the carbon and the impurity to be removed carry electrical charges such that in general the adsorption efficiency of carbons is dependent largely on the differences in electrical charges between the carbon and the particle, colloid, or colour, and ion to be adsorbed. In this case, the adsorption of nonelectrolytic materials, such as sugar, is a function of the activated carbon and is not affected by acidity or alkalinity. While electropositive materials (colours like ponceau red) are taking up more effectively in alkaline solution, electronegative materials (colours like methylene blue and most coloured

impurities) are removed by the carbon most effectively in acid solutions. And amphoteric substances such as colloids, proteins, and natural colours, which depend on the pH value of the solution, may act either as acids or as bases, adsorbed most effectively near isolectric point, where they show neither acidic nor basic properties (Mantell, 1951). With regard to natural products or solutions, one of the major contaminants results from biological colouring pigments present in plants such as chlorophyll, xanthophylls and carotene. Apart from materials or elements of trace impurities these make solution a 'crude' type and giving objectionable odour, taste and colour to meal and compounds of interest present in the solution. Activated carbon has been found very useful in the fractionation and removal of these biological pigments in the purification process and production of meal, beverages and alcoholic beverages such as wine (Mantell, 1951). Commercial activated carbon is known to be a preferred adsorbent for colour removal (Gregorio, 2006), an excellent adsorption capacity for organic compounds, e.g. phenolic compounds (Ru-Ling *et al.,* 2003). Report has shown the prolific use of activated carbon for waste water treatment and purification as well as solvent recovery and the purification of air (Dinesh *et al.,* 2002; Hernning and Dega 1990). Mahamed and Fahni (2002), has shown the favourable adsorption capacity of activated carbon of zinc from aqueous solution, though the primary use of activated carbon is the treatment of water, including potable water (24% of all use); wastewater (21%) and ground water remediation (4%) which accounts for approximately half of all the use in the US (Baker *et al.,* 1992). Non-agricultural ingredients such as enzymes are also often purified by the use of activated carbon (Aikat *et al.,* 2001). It suffices to know that activated carbon does not itself appear on any of the list of hazardous substances (US EPA, 1998). However, given that it is used to remove toxic substances from potable water, wastewater treatment, and hazardous waste effluent, spent activated carbon contaminated with toxic substances removed from production stream can be considered hazardous waste and fall under the EPA's authority under the Resources Conservation and Recovery Act's (40 CFR 264) (Shapiro, 1996). The application and use of activated carbon can improve, reduce or have no effect on the nutritional profile. This observation is further supported by Boulten *et al.,* (1968), who found that activated carbon does not adsorb sugar or amino acids which are highly water soluble in wine. However, activated carbon has been shown to remove some vitamins, which according to the Boulten reference may affect micro biological stability of

wine product. Wine fined with baker's yeast had comparable removal of phenols to activated carbon, with a taste panel detecting no significant difference in colour, flavour, and aroma (Bonilla *et al.,* 2001). Solid-phase extraction (SPE) technique with activated charcoal has been used to purify tomatoes and recovery of the pesticides; methanmidophos, acephate, malathion and methyl parathion (Beatriz *et al.,* 2001). Activated carbon is also shown to be a strong adsorber of phenols (Anderson, 1949) and should be efficient in removing tannins from grape juice. It is also reported to be used in the removal of ethylene from fruit storage facilities, particularly if brominated (Reid, 1985).

Activated carbon is generally considered non-toxic. Large doses are routinely given in human poisoning cases. The human dose for poisoning is 1 g activated charcoal per kg body weight (Scharman *et al.,* 2001, Minocha and Spyker, 1998).

The ability of carbon to purify some drugs such as Salicylic acid (aspirin), quinine and salts, caffeine, alkaloids and acetanilide has long been recognized (Mantell, 1951). Hence this study is to investigate the potential of activated carbon for the purification of linamarin, a cyanogenic compound from cassava, '*Manihot esculeta* Crantz'. Although many theories have been proposed to explain some of the adsorption phenomena, the simplest and best procedure to obtain information concerning the adsorption capacity of a particular solid is by direct experimental methods on the desired system. The experimental procedure need not always reproduce faithfully the proposed contacting method but it should make use of samples of the solid and fluid in question and should duplicate temperature and pressure (George *et al.,* 1950).

Finally, the environmental effect of using activated carbon is generally considered beneficial. The US EPA encourages the food industry to use activated carbon to treat wastewater effluent (US EPA, 2000) and volatile and green house gas air emissions (US EPA 1995).

2.3.3 Batch adsorption

Batch adsorption is often used to adsorbed solutes from liquid solutions when the quantity treated are present in small amount, such as in the pharmaceutical industry. Its purification process involves adsorption by solids from the liquid phase such that substances present in

low concentration are often preferentially adsorbed (Kipling, 1965). The efficiency of solute removal can be improved if the solution is treated using a number of batch stages rather than a single-stage batch process. Here the solution to be treated is mixed thoroughly and brought into intimate contact with a predetermined amount of activated carbon. The mass is brought up to the most suitable temperature and agitated with time. The carbon, now holding the impurities firmly adsorbed on its porous surface, is removed from the solution mixture by settling or simple filtration. For most efficient work, the manner and type of agitation to produce the proper contact between the activated carbon and the material treated are of great importance (Mantell, 1951).

Decolourizing, purifying, and deodourizing results are greatly affected by the degree of contact, which in turn is a function of the fineness of the carbon, its effective surface, and the manner and continuity of agitation (Mantell, 1951). Although equilibrium adsorption isotherm is fundamentally important in the design of adsorption systems, but adsorbents are characterized first by surface properties such as surface area and polarity (Motoyuki, 1990). A large specific surface area is preferable for providing large adsorption capacity, but the creation of a large internal surface area in a limited volume inevitably gives rise to a large numbers of small sized pores between adsorption surfaces. However the size of micropore determines the accessibility of adsorbates to the adsorption surface. Therefore, the pore size distribution of micropore is another important property for characterizing adsorptivity of adsorbents (Motoyuki, 1990).

2.3.4 Composite isotherm

Composite isotherm is a type of isotherm that experiences preferential or selective adsorption. This corresponds to the term ‘surface excess’ which is a measure of the extent to which the bulk liquid is impoverished with respect to one component, because the surface layer is correspondingly enriched, and thus ‘negative’ adsorption of component 1 means a preferential adsorption of component 2 (Kipling, 1965).The significance of the composite isotherm is known by deriving an equation though not based on any supposed mechanism of adsorption except to relate the preferential adsorption from a two-component mixture to the actual adsorption of each component (Kipling, 1965).

When a pore volume of a weight of solid m (cm^3/g) is brought into contact with n_o moles of liquid (initial concentration), the mole fraction of the liquid decreases by Δx with respect to component 1. This change in concentration is brought about by the transfer of n_1^s moles of component 1 and n_2^s mole of component 2 onto the surface of unit weight of the solid. At equilibrium, there remains in the solution phase n_1 and n_2 moles, respectively of the two components, giving a mole fraction, x with respect to component 1, the initial mole fraction having been x_o.

Then $$n_o = n_1 + n_2 + n^s{}_1 m + n^s{}_2 m \tag{2.1}$$

And $$x_0 = \frac{n_1 + n_1^s m}{n_0}, \qquad \text{x} = \frac{n_1}{n_1 + n_2} \quad \text{and 1-x} = \frac{n_2}{n_1 + n_2}$$

Therefore $$\Delta x = (x_0 - x) = \frac{n_1 + n_1^s m}{n_1 + n_2 + n_1^s m + n_2^s} - \frac{n_1}{n_1 + n_2} \tag{2.2}$$

Resolving equation (2.2)

$$\Delta x = \frac{n_1^2 + n_1 n_2 + n_1 n_1^s m + n_2 n_1^s m - n_1^2 - n_1 n_2 - n_1 n_1^s m - n_1 n_2^s m}{(n_1 + n_2)(n_1 + n_2 + n_1^s m + n_2^s m)} \tag{2.3}$$

But $n_o = n_1 + n_2 + n^s{}_1 m + n^s{}_2 m$

$$\Delta x = \frac{n_2 n_1^s m - n_1 n_2^s m}{(n_1 + n_2) n_0} \tag{2.4}$$

Therefore $$\frac{n_0 \Delta x}{m} = n_1^s (1 - x) - n_2^s x \tag{2.5}$$

Or $$\frac{n_0 \Delta x}{m} = n_1^s x_2 - n_2^s x_1 \qquad (2.6)$$

Where x_1 and x_2 refer to the mole fraction of component 1 and 2, respectively, in the liquid phase, and n^s is the total number of molecules in the adsorbed layer on unit weight of solid. The function $\frac{n_0 \Delta x}{m}$ (when moles and mole fractions are used) is plotted as linamarin 'adsorption' against 1-x 'linamarin remaining in solution' gives the composite isotherm. When a special case of pore filling occurs as in molecular sieve action or activated carbon (the sorption of one component to the complete exclusion of any others), then equation (2.5) becomes;

$$\frac{n_0 \Delta x}{m} = n_1^s (1 - x) \text{ (Adsorption isotherm of linamarin)} \qquad (2.7)$$

Because n_2^s (impurity) is now zero at a time t. Further, n_1^s becomes a constant for all values of x. Hence equation (2.7) describes a straight line which can be extrapolated to x = o, to give n_0^s, the mole of linamarin adsorbed per pore volume of the activated carbon (g mol/cm^3). This finally resolves equation (2.7) into equation (2.8).

$$\frac{n_0 \Delta x}{m} = n_0^s (1 - x) \qquad (2.8)$$

2.3.5 Types of composite isotherm

Basically there are three types of composite isotherms when classified according to shape is as shown in Figure 2.10

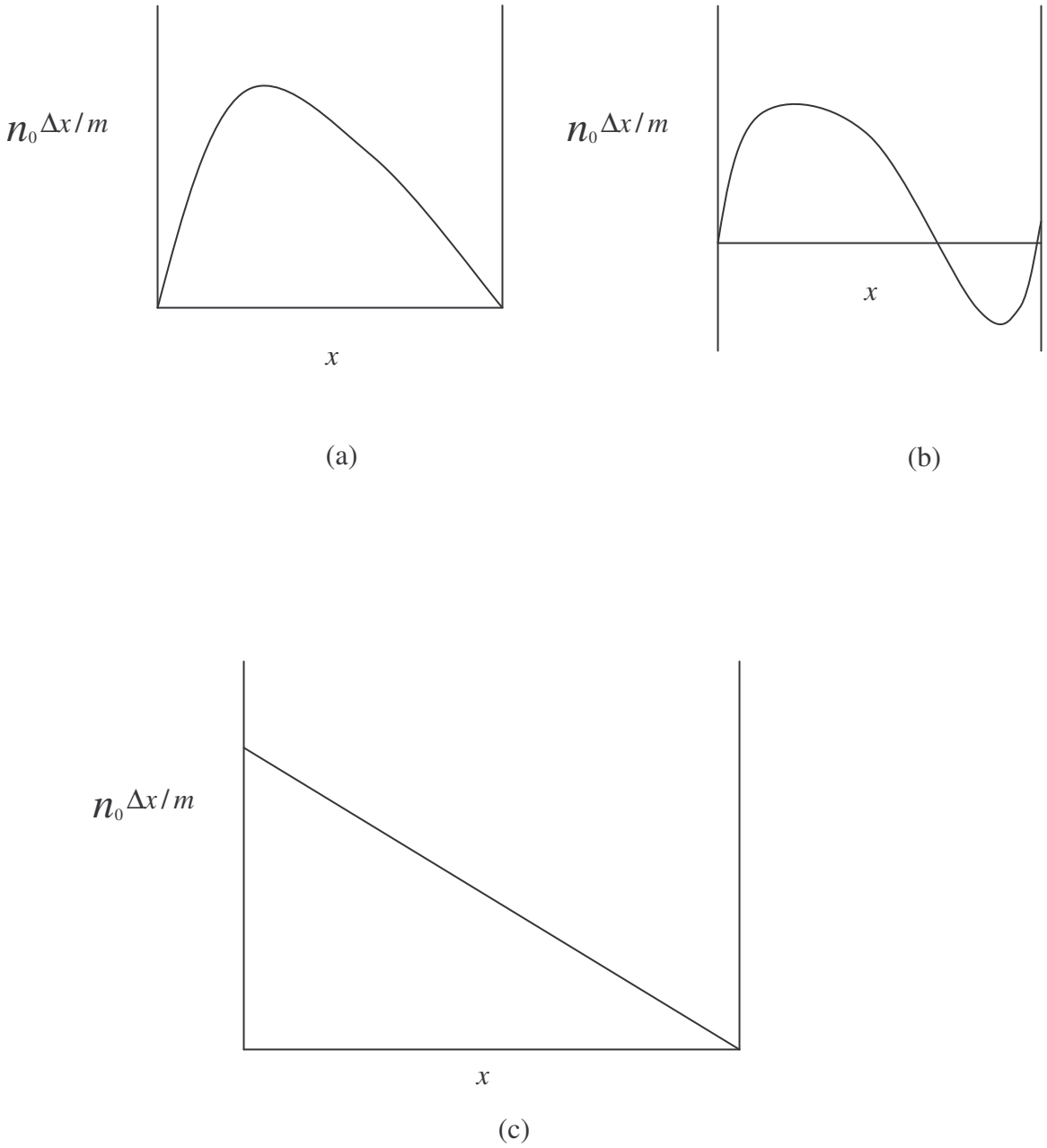


Figure 2.10: Mechanism of adsorption isotherms; (a) one branched or U-shaped (b) two branched or S-shaped (c) linear (Kipling, 1965)

2.4 *IN VITRO* EVALUATION OF ANTITUMORAL AGENT

The term *in vitro* evaluation means the test performed on samples taken from the body in order to ascertain parameters such as concentration of various chemical and biochemical compounds, microscopic examinations of cells, counting of cells, biological culturing and measuring of physical properties of a sample. Drug 'developability' assessment has become an increasingly important addition to traditional drug efficacy and toxicity evaluations, as pharmaceutical scientists strive to accelerate drug discovery and development process in a time-and –cost effectiveness manner (Sun *et al.,* 2004). Although scientific strategies for the *in vitro* evaluation of natural products with biological activity have changed in the past few years (Betancur-Galvis *et al.,* 1999). A recent development is that of automated bioassay screening based on colorimetric methods that quantify the proliferation of cell cultures (Mosman, 1983; Denizot and Lang, 1986). These techniques are considered to be quick and inexpensive for the evaluation of antitumor activity (Carmicheal *et al.,* 1987; Rubinstein *et al.,* 1990). A large number of natural product extracts, have also easily permitted to guide the isolation and purification of their biologically active principles (Cordell, 1995).

The fraction of drug absorbed and the maximum absorbed dose (MAD) can be estimated from *in vivo* clinical pharmacokinetics, mass balance studies or in vivo drug permeability in humans by different calculation methods. Unfortunately, *in vivo* data are usually unavailable at the early stages of drug discovery and development, while in vitro screening for the permeability, solubility, activity and toxicity of a drug has become a routine measurement in drug discovery and development (Sun *et al.,* 2004). These *in vitro* data can be used to predict drug 'developability' with different calculation methods before selecting candidates for clinical evaluation (Sun *et al.,* 2004). Since the 1950s, a number of in vitro assays have been developed to predict the therapeutic outcome prior to the start of therapy. With the introduction of the human tumor stem cell assay in the 1970s, it was generally believed that oncology was on the threshold of entering an era of predictive *in vitro* chemosensitivity testing. Unfortunately, this assay was shown to have a number of technical drawbacks including the low plating efficiencies of many primary tumor samples which thus limits the percentage which can be evaluated, leaving us still at this threshold today (Bellamy, 1992). The ATP bioluminescence assays, and the fluorescent cytoprint assay that offer the promise

of rapid and sensitive result, the tetrazolium-based MTT and the sulphorhodamine blue assay appear to hold more promise in the screening and evaluation of potential new agents in established tumor cell lines than for evaluating chemosensitivity of clinical specimens (Bellamy, 1992).

A colorimentric assay using MTT was first introduced by Mosmann as a quantitative measure of mammalian cell survival and proliferation (Mosman, 1983). MTT is a yellow tetrazolium salt, which is converted into a blue formazan by dehydrogenase of a live cell (Dawit *et al.,* 2005). The assay is based on the principle that the amount of formazan product is directly proportional to the number of live cells (Mosman, 1983). The formazan can then be measured by a spectrophotometer after solubilisation (Denizot and Lang, 1986).

The tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is reduced by live but not dead cells, and this reduction is used as the end point in a rapid drug-screening assay. It can also be used for accurate determination of drug sensitivity but only if a quantitative relationship is established between cell number and MTT- formazan product (Plumb *et al.,* 1989). Plumb *et al.,* (1998), have shown that reduction of MTT to MTT- formazan product differs widely between cell lines. The absorption spectrum of MTT-formazan varies with cell number and pH such that at a low cell density or a high pH, the absorption maximum is at a wavelength of 560 to 570nm. However, at a high cell density or a low pH, there are two absorption maxima; one at 510nm and a second at about 570nm, and the addition of a buffer at pH 10.5 to the solubilised MTT- formazan product can overcome the effects of both cell density and culture medium on the absorption spectrum, provided that sufficient MTT is used and the pH of the MTT- formazan product is controlled (Plumb *et al.,* 1989). The main advantages of the MTT assay are its simplicity, rapidity, and the fact that the results are read automatically with a microplate spectrophotometer (Pieters *et al.,* 1988).

CHAPTER THREE

3.0 EXPERIMENTAL

3.1 Reagents and chemicals (All are of analytical grade of between 98-99.5% purity). The following chemicals were obtained from Sigma-Aldrich (USA): sodium carbonate, picric acid, 0.1 M phosphate buffered solution (PBS) (pH 6.0), 0.2 M PBS (pH 7.2) and 0.1 M acetate buffered solution (pH 5.5). Methanol and ammonium sulphate were obtained from Merck Chemicals (RSA). RPMI-1640 and foetal bovine serum (FBS) were obtained from Gibco (New Zealand), while Dulbeco Modified Eagle's Medium (DMEM), dimethylsulphoxide (DMSO), 100X non-essential amino acids, 10 mg/ml penicillin/10 mg/l streptomycin, trypsin/versene and 2 mM sodium pyruvate were purchased from Highveld Biological (RSA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from USB Corporation (USA). Commercial granular activated carbon (8-30 mesh), with an apparent density of 483 kg/m^3, moisture content of 2.4 wt% and iodine adsorption of 1257 mg/g was purchased from Associated Chemical Enterprises (RSA).

Ultrafiltration equipment

A MiniFlex UF system (Figure 3.1), built around a tubular module was obtained from Schleicher and Schuell (Germany), contained polyethersulfone membranes (0.2-0.45 μm) of polypropylene screens and silicone adhesives, with a nominal molecular weight cut-off of 0.2 μm, and membrane surface area of 2.4 mm^2. Other parts of the system included: 3R VL 100 constant pressure variable speed peristaltic pump, pressure gauge (bar) for indicating retention inlet and outlet pressure, connecting tubes for feed flow, 140 ml x 2 graduated container for feed and permeate, and a VOLTCRAFT switching power supply.

3.2 Preparation of alkaline picric paper

Alkaline sodium picrate solution was prepared by taking 25 g Na_2CO_3 and 5 g picric acid and dissolving it in 1litre of distilled water. Whatman no 1 filter paper were dipped into the solution, and immediately turned from white to yellow. After 30 mins the filter papers were allowed to air dry. Picrate papers were stored in a dark medium until use.

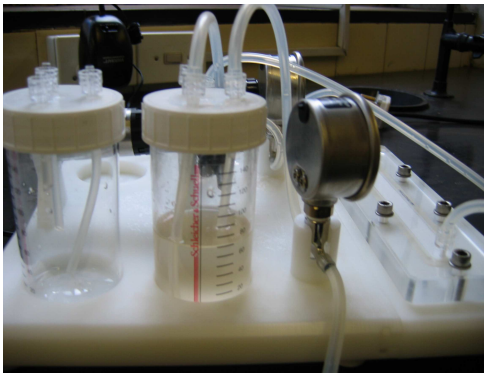


Figure 3.1: Laboratory set up of ultrafiltration

3.3 Preparation of hydrolyzing enzyme solution from cassava root peel

Linamarase (β-glucosidase) was prepared as described by Cooke (1978) with modification. Briefly, fresh cassava peel obtained from Wits University garden (250 g) was diced and homogenised in a Warring blender for 8 mins in 300 ml 0.1 M acetate buffer (pH 5.5). Preliminary separation of macro molecule was achieved by using an industrial mill bag that was cut to size. The solution was centrifuge at 10,000 rounds per minute (rpm) for 30 mins using the BECKMAN Model J2-2 and the supernatant was brought into saturated solution of ammonium sulphate (60% w/v) and held at 4^{o}C for 16 hrs. This solution was then centrifuged at 10,000 rpm for 1 hr. The precipitate thus obtained was dissolved in 50 ml 0.1 M PBS (pH 6.0), and was dialysed using 0.45 µm membrane pore size. Further purification was achieved using 0.2 µm membrane (ultrafiltration) that permeated cyanogenic component like linamarin, where the purified enzyme was collected as retentate. The enzyme solution was stored at 4°C.

3.4 Preparation of crude cassava extracts (CCE)

Cassava roots (1000 g) were diced and homogenised in boiling methanol (99.5%) using a Warring blender for about 40 mins. The bulk solution was squeezed out with the use of an industrial mill bag that was cut to size and allowed to settle for 60 mins in a 5 liters plastic bucket, after which relatively clear supernatant was decanted from the sediment. This was

followed by ultrafiltration (0.2 µm) set at 1.5 bars for the isolation of crude cassava extract (CCE). The isolated linamarin mixture was then evaporated using the BUCHI RE 120 model evaporator (Figure 3.2), set at 45^{o}C. A dark brown jellylike solid (60 g), was recovered. This preparation was repeated for 1500 g and 2500 g of cassava roots.

Figure 3.2: BUCHI rotary evaporator

3.5 Optimum amount of linamarase and linamarin determination

Varying volumes (2-13 ml) of linamarase were measured and placed in small flat bottom plastic vials (about 25 mm diam. 50 mm high) containing 1.5, 2.0 and 2.5 g of linamarin that was previously purified and characterised. According to the method of Egan *et al* (1998), this was followed by the addition of 10 ml of 0.2 M phosphate buffer (pH 7.2) and 11 ml hydrolyzing enzyme (linamarase) solution. A yellow picric paper cut to size was suspended above the samples and the vials were immediately stoppered. The vials were then incubated at 30^{o}C and left to stand overnight. The change in color from yellow to orange and to brown was observed as described by Egan *et al.* (1998). The papers were removed and immersed in 50 ml distilled water. After 30 mins the elutes were collected and the absorbance read at 510 nm against a picric paper blank using a UV Spectrophotometer (4802 UV/VIS). By means of a graph of absorbance vs. concentration (g/ml) of linamarin, the calibration curve of linamarin was determined in terms of HCN equivalent released.

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3.6 Determination of purification/adsorption point

A known amount (60, 80 and 100 g) of crude cassava extract (CCE) that was earlier isolated and evaporated was dissolved in a 250 ml distilled water, and was introduced into 80 g of activated carbon in a 800 ml Pyrex conical flask. The mixture was shaken (190 rpm) at room temperature (25^{o}C) using Labcon shaking incubator for varying contact times (mins.), until the solution turned colorless after filtering with Whatman 185 mm filter paper supported with vacuum pump. After each contact time and filtration, 4 ml filtrate was collected and added to 11 ml hydrolyzing enzyme solution and analyzed. This was repeated with longer contact times (62, 72, and 84 mins for 60, 80 and 100 g, respectively) until no more product was found after drying. With varying contact times, the highest concentration of linamarin obtained during purification before the concentration dropped to zero as a result of adsorption was taken as the purification/adsorption point with respect to the contact time of purification.

3.7 Purification of linamarin by batch adsorption with activated carbon

60 g dark brown crude cassava extract previously isolated and evaporated was dissolved in 250 ml distilled water, and was introduced into 80 g of activated carbon in an 800 ml Pyrex conical flask. The mixture was shaken (190 rpm) at room temperature (25^{o}C) using Labcon shaking incubator at varying contact time (mins.), until the solution turned colorless after filtering with Whatman 185 mm filter paper supported with vacuum pump. After each contact time and filtration, 4 ml filtrate was collected and added to 11 ml hydrolyzing enzyme solution and analyzed. At the end, colorless solution was solidified in a 250 ml round bottom flask using liquid nitrogen in an alpha 1.5 freeze drying machine and 1.7 g of white granules was recovered (Figure 4.8). The experiment was repeated for 80 and 100 g crude cassava extract and 2.0 g and 2.5 g of linamarin were also obtained, respectively.

3.8 Preparation of calibration curve

Different masses of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 of linamarin were weighed and dissolved in 1 ml distilled water in a clear plastic vial. This was followed by the addition of 11 ml hydrolyzing enzyme solution and 10 ml of 0.2 M PBS (pH 7.2) and analysed.

3.9 Determining the effect of temperature on the rate of purification/adsorption of linamarin

100 g of dark brown crude cassava extract that was earlier isolated and evaporated was dissolved in 250 ml distilled water, and was introduced into 80 g of activated carbon in an 800 ml Pyrex conical flask and corked. The mixture was shaken at different temperatures (25^{o}C, 35^{o}C, 45^{o}C, 55^{o}C and 65^{o}C) using Labcon shaking incubator at 190 rpm at varying contact time (mins.), until the solution turned colorless after filtering with Whatman 185 mm filter paper supported with vacuum pump. After each contact time and filtration, 4 ml filtrate were collected and analyzed.

3.10 Analysis of results

3.10.1 Infra-red spectra test

Infra-red spectra were recorded using a Bruker Vector 22 model. 2 mg of purified product was dissolved in 1 ml of deuterated water (D_2O) to form a film. The film was introduced onto sodium chloride plate, and the infrared spectra were recorded in the range of 1454-3419 cm-^{1}

3.10.2 Nuclear magnetic resonance test

Routine ^{1}HNMR spectra were recorded on Brucker 400 spectrometers. D_2O solvent was used to dissolve 2 mg of purified product and tetramethylsaline (TMS) was used as internal standard. ^{1}HNMR data were written in order: number of protons, multiplicity (b, broad; s, singlet; t, triplet; m, multiplet), coupling constant in hertz and assignment to respective protons

3.10.3 Melting point determination

A < 2 mg of purified product was introduced into a small capillary tube and inserted into BUCHI 510 melting point model. With increasing temperature, the melting (decomposition) temperature of the purified product was determined.

3.11 Cell culture

The human breast cancer (MCF-7) cell line was obtained from National Cancer Institute (NCI, USA), while the human colon adenocarcinoma (HT-29) and the acute myelogenous leukemic line (HL-60) were obtained from Highveld Biological, RSA.

MCF-7 cells were grown in RPMI-1640 supplemented with 5% FBS, 2% 100X non-essential amino acids and 2 mM sodium pyruvate, while HT-29 cells were grown in DMEM supplemented with 5% FBS. HL-60 cells were grown in RPMI-1640 medium supplemented with 10% FBS. 12 mg/ml stock solutions of crude cassava extract and purified linamarin contained not more than 1% DMSO were prepared. The final concentrations in the microtitre well ranged from 2.5 µg/ml to 300 µg/ml.

3.12 *In vitro* anticancer effect

MCF-7 and HT-29 cells were seeded at a density of 4000 cells/well, while HL-60 cells were seeded at 10 000 cells/well. The cells were exposed to crude cassava extract with and without enzyme and purified linamarin with and without enzyme at 37^{o}C for 24 hrs and 48 hrs. Cytotoxicity effects of the combinations were determined using the MTT assay as described by Mossman (1983). The plates were read at 540 nm using the Absorbance Labsystems Multiskan MS Version 2.4 against a DMSO blank.

3.13 Analysis of *in vitro* anticancer effect results

Results are expressed as mean ± standard deviation using ANOVA to determine significance of results (where $p < 0.05$ was considered significant). IC_{50} values were calculated using the Enzfitter (version 1.05).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Separation of linamarin from bulk cassava extract

For choice of membrane that suited this experiment, preliminary experiment with 0.45 µm, 0.2 µm and 50 kD membranes were carried out using 1000 g bulk cassava tissue (same stock). At 1.5 bar, the result gave different concentration of linamarin with different rate of isolation (0.04 g/ml and 1.52 ml/min, 0.07 g/ml and 1.44 ml/min and 0.08 g/ml and 0.62 ml/min for 0.45 µm, 0.2 µm and 50 kD, respectively). Since the concentration of linamarin for 0.2 µm is ± 0.01 of 0.08 (the highest concentration) but with higher rate of isolation, 0.2 µm membrane thus became the membrane of choice for this experiment. The bulk cassava extracts were earlier obtained from different weights (1000, 1500 and 2500 g) of bulk cassava tissue (BCT). Ultrafiltration using a 0.2 µm membrane was found very useful in the isolation of impure linamarin in the form of crude cassava extract (CCE). The presence of linamarin in the solution isolated was confirmed when portions of the samples isolated were treated with picrate paper test analysis as described in experimental, and the change in color from yellow to brown confirmed its presence. This is in agreement with the work and findings of Cooke (1978), Bradbury *et al.* (1991) and Egan *et al.* (1998). Figure 4.1 shows the kinetic of linamarin isolation using the 0.2 µm cross flow membrane (Figure 3.1), where it was high initially, but started to decrease after 250 mins, forming a plateau.

The rate of linamarin isolation from bulk of cassava extract was calculated from Figure 4.1 data (appendix 1) and was found to be higher (1.44 ± 0.01 ml/min) when 1000 g of bulk cassava tissue was used than when 1500 g (1.03 ± 0.01 ml/min) and 2500 g (0.8 ± 0.41 ml/min) were used. This could be as a result of high concentration of cassava extracts that reduced the membrane flux (Srikanth, 1999; Mohr *et al.,* 1989a). The gradual decrease of membrane performance as observed during the process can as well be attributed to membrane fouling and concentration polarization ((Nakao and Kimuras, 1981; Haris *et al.,* 1986; Haris and Dobos, 1989). These dual factors inevitably reduced linamarin isolation during the process with time.

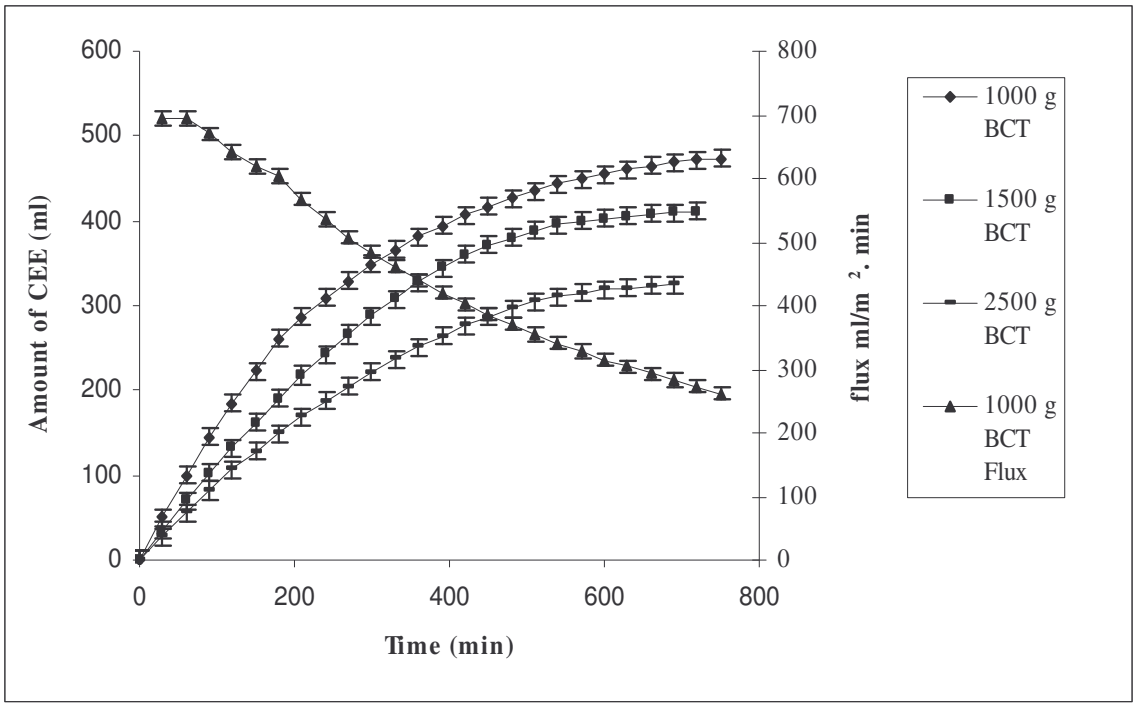

Figure 4.1: Kinetics of linamarin isolation (error bars represent standard deviation for 3 experiments)

4.2 Optimum amount of linamarase and linamarin determination

In order to study the effect of increasing the amount of enzyme upon the reaction rate, the substrate was made to be present in excess amount such that the reaction must be independent of the substrate concentration, linamarin. Therefore, the amount of linamarase present in the reaction was measured by the activity of linamarin it hydrolyses to release HCN, which was determined by the spectrophotometer method. Figure 4.2 shows the optimum activity of linamarase with respect to concentration used.

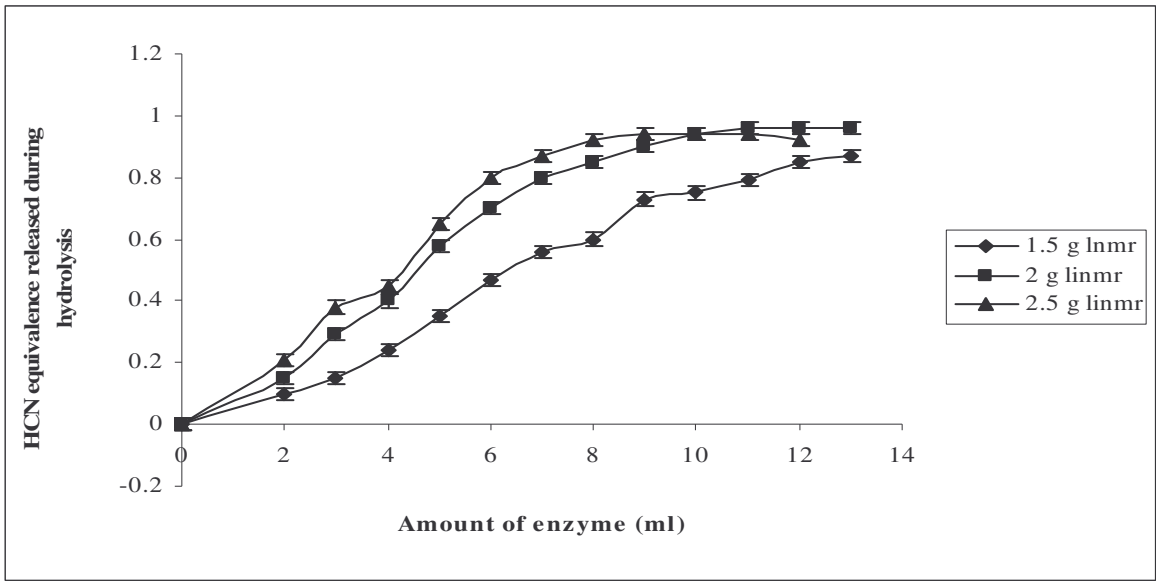

Figure 4.2: Optimum amount of enzyme determination (error bars represent standard deviation for 3 experiments)

The above figure confirms the activity of linamarase as an enzyme that breaks down linamarin by hydrolysis to release HCN (Yeoh *et al.,* 1998; Bradbury *et al.,* 1991) as shown in Figure 2.3 previously in literature review. The activity of linamarase on linamarin hydrolysis is proportional to the amount of the enzyme used. The highest activity was noted using 11 ml of enzyme solution with 2 g of linamarin, where it started leveling off at 0.96. This is regarded as the HCN equivalent concentration released (Egan *et al.,* 1998; Yeoh *et al.,* 1998; Bradbury *et al.,* 1991) from linamarin, even though the amount of enzyme was increased. Since linamarin was used in excess (2.5 g), 11 ml of the enzyme solution was determined to be the optimum hydrolyzing amount on linamarin in all processes run.

4.3 Calibration curve

The working curve (Figure 4.3) is a plot of the analytical signal (absorbance) as a function of linamarin concentration. The working curve was able to measure the activity of linamarase from series of standards of known linamarin concentration. A plot of HCN released as absorbance (activity) versus concentration thus created a linear relationship which enabled interpolation of finding the concentrations of analytes.

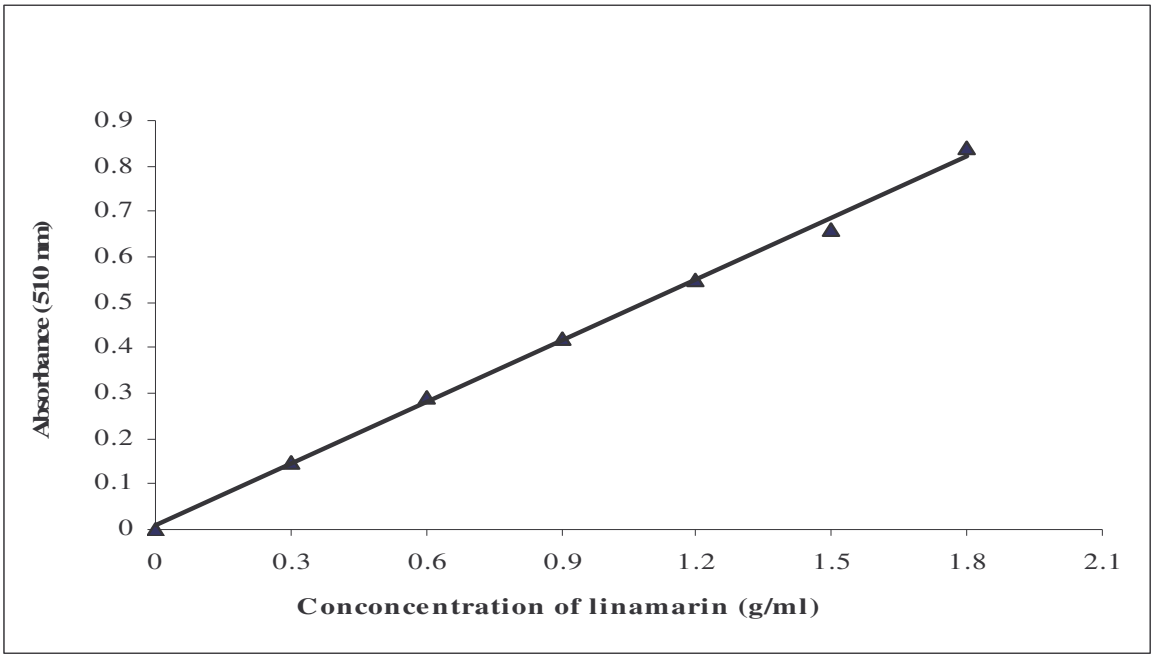

Figure 4.3: Linamarin calibration curve with standard deviation = 0.2

4.4 Determination of linamarin purification/adsorption point

In order to obtain purified product with a known weight of activated carbon, determination of the optimum purifying weight (60, 80 and 100 g) of activated carbon was carried out (Figure 4.4) with 100 g of crude cassava extract (CCE). It was observed from the figure that 60 g of activated carbon (ATC) got saturated before time of optimum purification by leveling off. On the other hand, 80 and 100 g ATC allowed for the optimum purification of linamarin with concentration difference of 0.02 g/ml. Since both showed similar result and for economic reason, 80 g ATC was chosen as the working purifying weight of activated carbon throughout the experiment. During the purification process of linamarin from the dark brown, CCE (Figure 4.5), a purification/adsorption point was obtained. This point characterises the adsorption of linamarin with preceding purification, signifying impurities removal and continued into linamarin consumption. The concentration of linamarin started depleting and at the end of the process, no product was recovered after drying. This created the awareness of preferential adsorption during the purification process. The determination of purification/adsorption points of linamarin purification is depicted in Figure 4.6, which presents curves of similar pattern for all concentrations of CCE used.

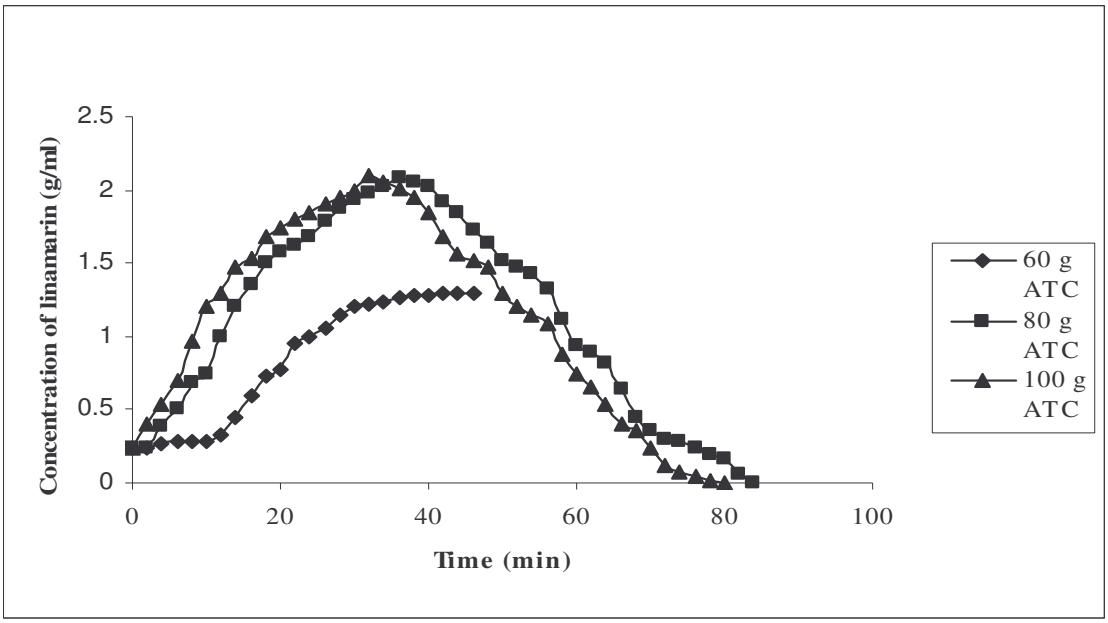

Figure 4.4: Preliminary determination of purifying weight of activated carbon

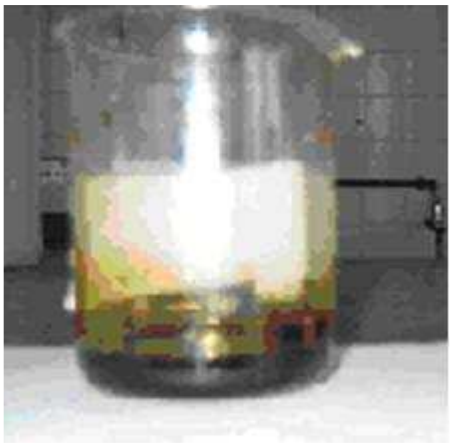

Figure 4.5: Evaporated sample (CCE)

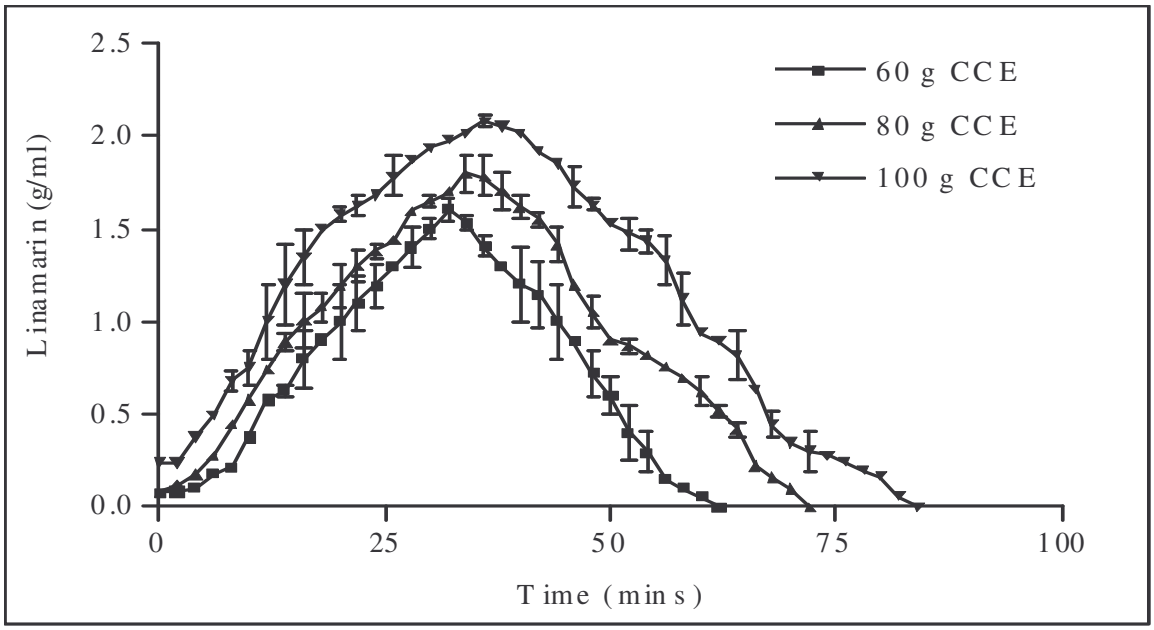

Figure 4.6: Determination of linamarin purification/adsorption point (error bars represent standard deviation for 3 experiments)

The above figure reveals the U-shaped isotherm as shown in literature review (Figure 2.10), which is classified as one of the types of composite isotherm according to Kipling (1965). The preferential adsorption encountered during the purification of linamarin corresponds to the term 'surface excess' which is a measure of the extent to which the bulk liquid is impoverished with respect to one component (linamarin), because the surface layer of the activated carbon used is correspondingly enriched with the sorbate (Kipling, 1965). In each of the different masses of crude cassava extract purified, it was observed that during the first 30 mins, impurities of organic and natural origin that constituted the cassava extract had preference for adsorption from the solution than for linamarin. The impurities may have had a stronger affinity for the activated carbon than the linamarin, since, the adsorption efficacy of carbons is largely dependent on the difference in electrical charges between the carbon and the particle, colloid, or colour to be adsorbed (Mantell, 1951). This also indicates that the end-product of linamarin hydrolysis is acidic, thereby confirming the release of hydrogen cyanide into the solution. Once solutions were purified, adsorption of linamarin occurred

with increasing contact times. For 60, 80 and 100 g CCE used, the removal of linamarin started from 34, 36 and 38 minutes, respectively.

These different purification/adsorption points of linamarin from solution that were determined were used as the optimum purification contact times with respect to the masses of crude cassava extract used. This relationship is shown in Figure 4.7 below, where linamarin concentration increases with time while adsorption of linamarin decreases with time. These findings are in agreement with the preferential adsorption of components from solution during purification process (Kipling, 1965). Normally, when a liquid (solution) containing impurities is brought into contact with a carbon, the attraction of the carbon for the impurity is greater than the attraction of the liquid for the impurities. The carbon under adsorption equilibrium therefore adsorbs the impurities such as colouring matter, odour, and flavour, etc., until all the carbon pores are filled, after which the carbon no longer removes these substances from that particular solution (Mantell, 1951). But in this study, activated carbon was employed in excess with respect to the amount of linamarin purified in order to tactically avoid saturation of the activated carbon before optimum purification was achieved.

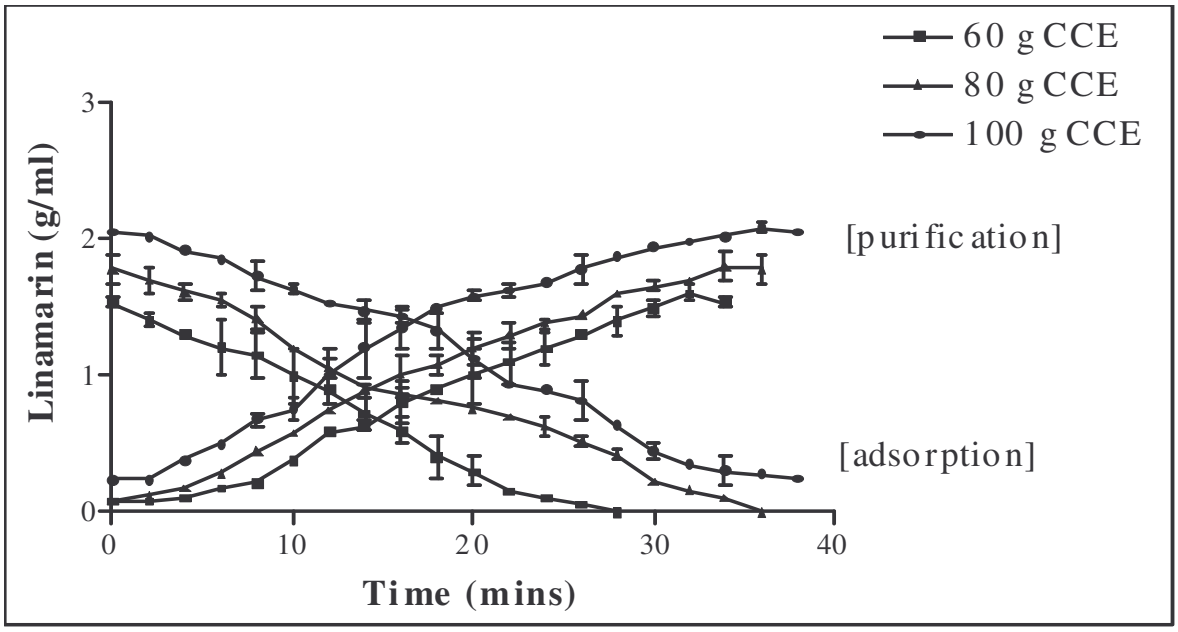

Figure 4.7: Purification and adsorption of linamarin (error bars represent standard deviation for 3 experiments)

From the above figure it was observed that concentration of linamarin increased with increase in contact time during the purification process. This shows that degree of contact time is a vital factor of linamarin purification during adsorption in batch process. According to Mantell (1951), since processing of liquids concerns itself with maintaining composition within definite limits, then, raw material is subjected to stage treatment to obtain products of satisfactory purity, such that each step effects increased concentration of material desired in the product and decreased concentration of undesired constituents. The increase in concentration from < 0. 25 g/ml to 1.6 g/ml, 1.8 g/ml and 2.08 g/ml for 60 g, 80 g and 100 g of CCE, respectively with increase in contact time, is an indication of a 'positive' purifying potential of activated carbon for linamarin. It can be seen that the amount of linamarin recovered at the end of the process is dependent on the amount of CCE purified since concentration increases with increase in the amount of CCE used. Figure 4.8 shows the purified product.

Figure 4.8: Purified product

4.5 Effect of temperature on purification/adsorption of linamarin

Temperature is known to be a crucial parameter in adsorption process. Determination of optimum conditions that will give optimum yield of product is thus very pertinent to the engineer in the design of any chemical process. Hence the effect of temperature on the

purification process of linamarin was determined. Although temperature has not been studied as relevant variable in biosorption experiments where the tests are usually performed at approximately 25-35^{o}C (Micheal *et al.,* 2005). Figure 4.9 shows the effect of temperature during linamarin purification where a composite (preferential) adsorption phenomenon as described in Figure 4.6 above was also obtained and helped to ascertain the optimum contact time of purification (where linamarin concentration is maximum) before linamarin depletion from solution. From Figure 4.9 it was observed that linamarin removal from solution with respect to contact time increases with increase in temperature. At 25, 35, 45, 55, and 65^{o}C, linamarin removal occurred at 36, 30, 26, 22 and 22 minutes, respectively at the rate of 0.045 g/min, 0.046 g/min, 0.046 g/min, 0.06 g/min and 0.08 g/min. Thus the effect of temperature on the purification/adsorption of linamarin in the aqueous solution is clearly display in Figure 4.9.

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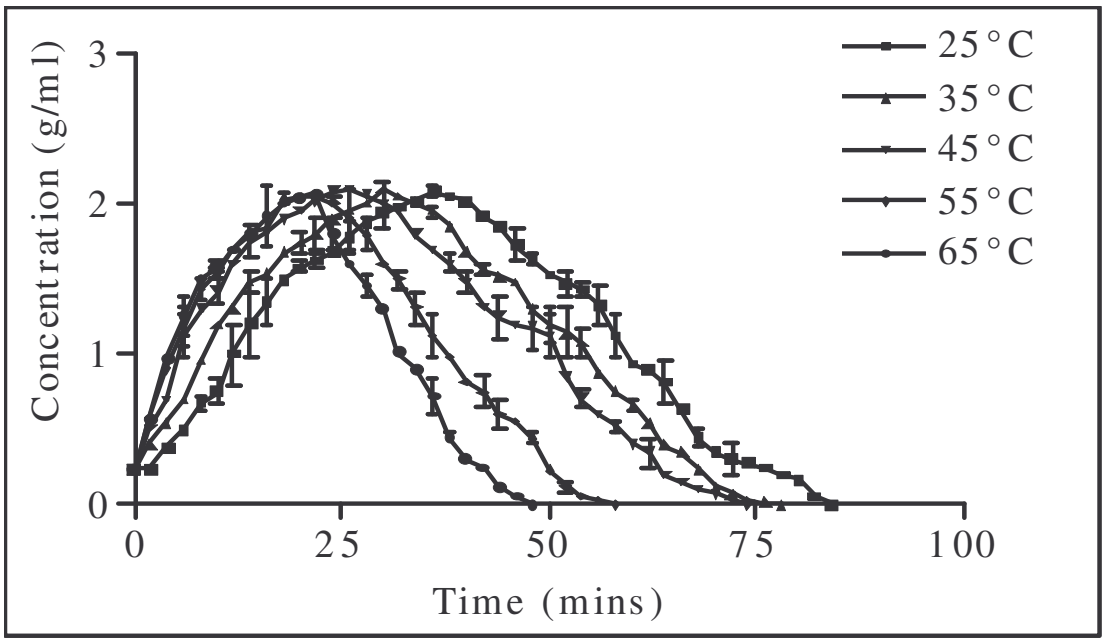

Figure 4.9: Effect of temperature on linamarin purification/adsorption kinetics (error bars represent standard deviation for 3 experiments)

When the purification phase was considered separatedly, data presented in Figure 4.10 showed that purification of linamarin with activated carbon increases with increase in

temperature, which is typical for the biosorption of natural colour impurities from their solution (Mantell, 1951). The rates of purification were found to be 0.06 g/min, 0.07 g/min, 0.08 g/min, 0.09 g/min and 0.09 g/min for 25, 35, 45, 55 and 65^{o}C, respectively. This increase in temperature with increase in purification rate shows the endothermic nature of the process. This observation agrees with the variant trend exhibited by activated carbon in adsorption theory that higher temperature allows the uptake of molecules into the pores more easily, causing adsorption to increase as temperature increases (Micheal and Ayebami, 2005). The maximum yields at temperatures of 25, 35, 45, 55 and 65^{o}C were 2.08, 2.1, 2.1, 2.06 and 2.06 g/ml, respectively. Thus the optimum yield of 2.1 g/ml at temperature of 45^{o}C and short time of 26 minutes. This could be that linamarin is sensitive at slightly high temperature even when literature reported melting points of between 139 to 148^{o}C (Hunsa *et al.,* 1995). Hence, its process determination and purification at low and at intermediate temperature remains a matter of controversy. Karin *et al.* (2004), carried its process determination by lyophilisation (freeze drying method below water freezing point) while Hunsa *et al.* (1995) attempted its process purification by evaporation at high temperature. But from this study such controversy can be put to rest since temperature ranges between 25 to 45^{o}c were seen as the favorable processing temperatures. This assertion should be true for impure linamarin in solution because observation also showed that pure linamarin, when stored above 10^{o}C, exhibited deliquescent characteristics and there was change in state. All these are recognized factors that must have contributed to its controversial nature in terms of instability with temperature.

4.6 Adsorption isotherm

Although equilibrium adsorption isotherm is fundamentally important in the design of adsorption systems, but adsorbents are characterized first by surface properties such as surface area and polarity (Motoyuki, 1990). A large specific surface area is preferable for providing large adsorption capacity, but the creation of a large internal surface area in a limited volume inevitably gives rise to a large number of small sized pores between adsorption surfaces. But, the size of micropore determines the accessibility of adsorbates to the adsorption surface. Therefore, the pore size distribution of micropore is another important property for characterizing adsorptivity of adsorbents (Motoyuki, 1990).

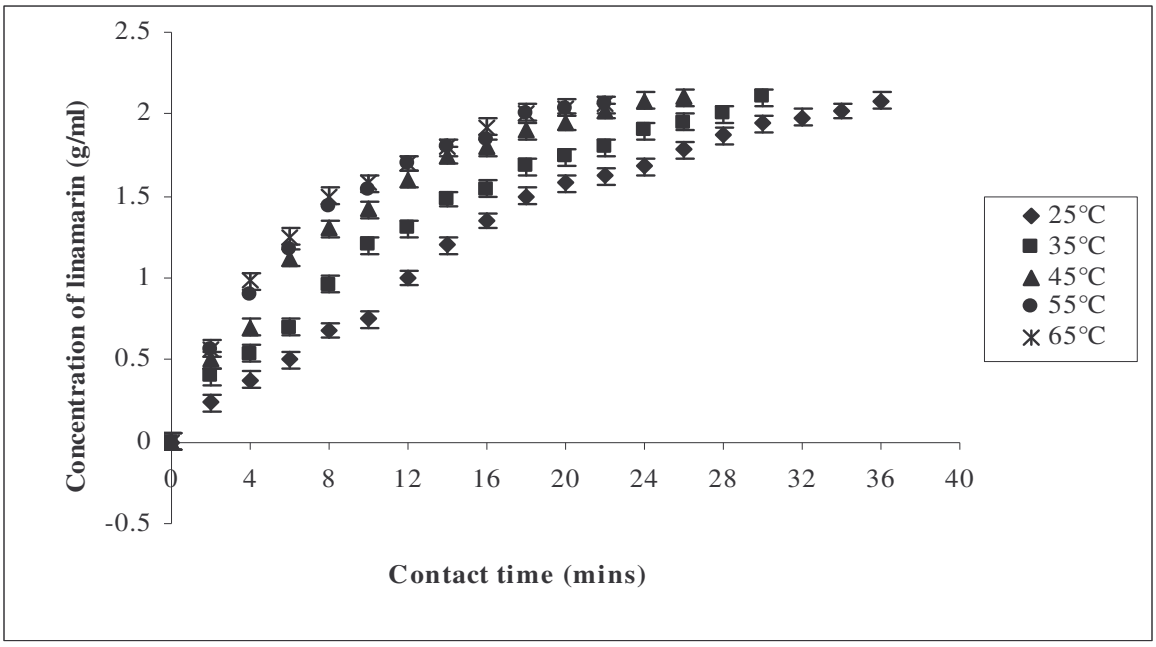

Figure 4.10: Effect of temperature on linamarin purification

Composite isotherm was used to characterise the pore volume of the activated carbon used. The fact that purification process proved to have exhibited composite adsorption relationship, the need to correlate experimental data with composite isotherm equation is thus very important for confirmation and to determine the pore volume efficiency of the activated carbon used. This involved the preliminary investigation of the rate of linamarin removal (adsorption) from solution and the effect of temperature on such in order to successfully correlate experimental data with composite isotherm. Figure 4.11 shows the kinetic of linamarin removal from solution with different amount of CCE used.

Figure 4.11 data (appendix 4, Table IV 4) was obtained from linamarin adsorption isotherm in Figure 4.6 data (appendix 4, Table IV 2). The rate of linamarin removal from solution was calculated and found to increase with increase in contact time which is true for typical adsorption process (George *et al.,* 1950; Mantell, 1951). The rate of linamarin removal was shown from the result to be dependent on the amount of CCE used (linamarin concentration). With 60 g of CCE the rate of linamarin removal was faster (0.05 g/min), followed by 80 g (0.045 g/min) and finally 100 g (0.04 g/min).

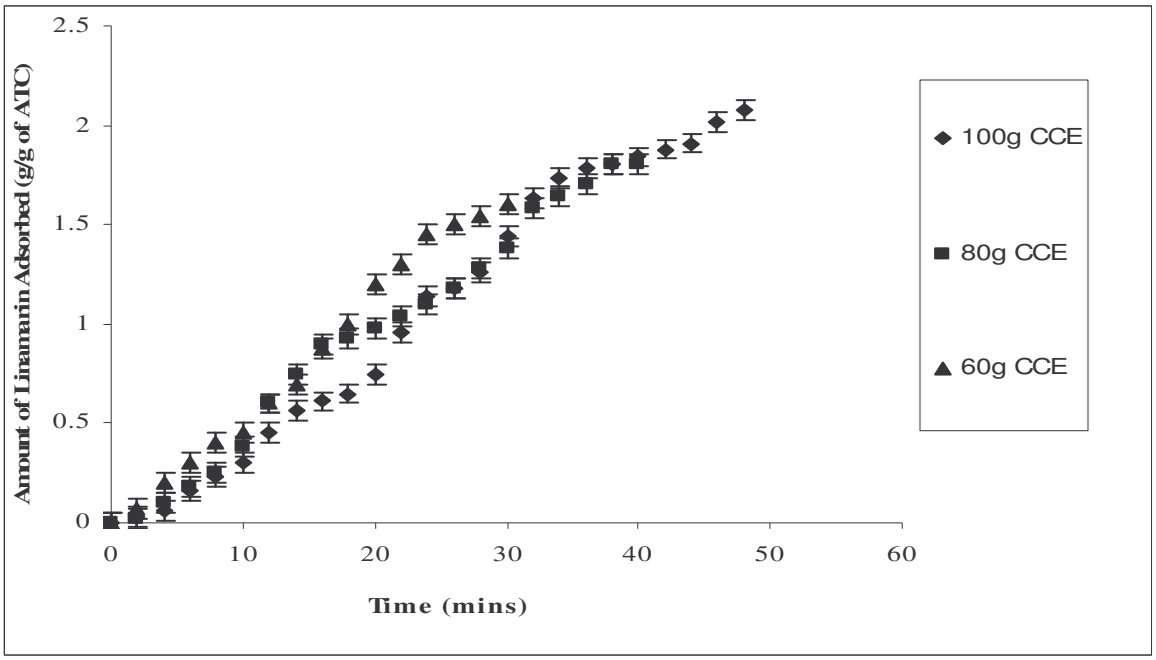

Figure 4.11: Amount of linamarin adsorbed from solution

4.7 Effect of temperature on linamarin removal from solution

The temperature range of 25– 65°C was used to study the effect of temperature on linamarin removal from solution when 100 g of CCE was considered as presented in Figure 4.12. Figure 4.12 data (appendix 4, Table IV 5) was obtained from Figure 4.9 data (appendix 4, Table IV 2) on the effect of temperature on linamarin purification.

From the figure it was observed that the kinetic of linamarin removal from solution was faster with temperature increase as from 45°C to 60°C. This is because, increasing temperature will increase molecular motion of linamarin thereby allowing the uptake of molecules into the pores of activated carbon more easily than at lower temperature (Micheal *et al.,* 2005). At 25°C to 45°C the rate of linamarin removal as obtained from appendix 4, Table IV V, was the same (0.048 g/min). A significantly higher rate of removal occurred at 55°C (0.063 g/min) and at 65°C (0.082 g/min.).

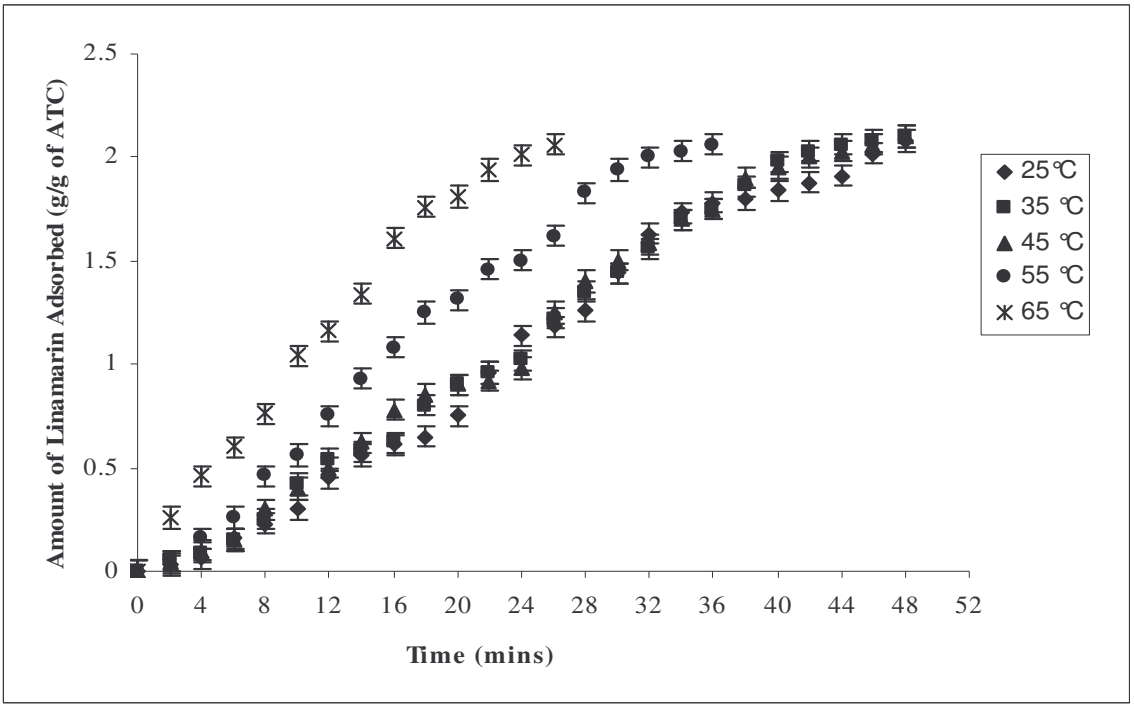

Figure 4.12: Temperature effect on linamarin adsorption.

4.8 Composite isotherm correlation

In order to correlate the conformity of experimental data with composite isotherm, a plot of amount of linamarin adsorbed $(\frac{n_0 \Delta x}{m})$ versus the amount of linamarin remaining in solution (1-*x*), was plotted as shown in Figure 4.13 which gave very reasonable straight lines, and their slopes gave the corresponding moles of linamarin adsorbed per pore volume of activated carbon. The R^2 values (goodness of fit criteria) computed by linear regression for the three different amounts of CCE purified and their corresponding moles of linamarin adsorbed per pore volume of activated carbon (n_0^s), are presented in Table 4.1.

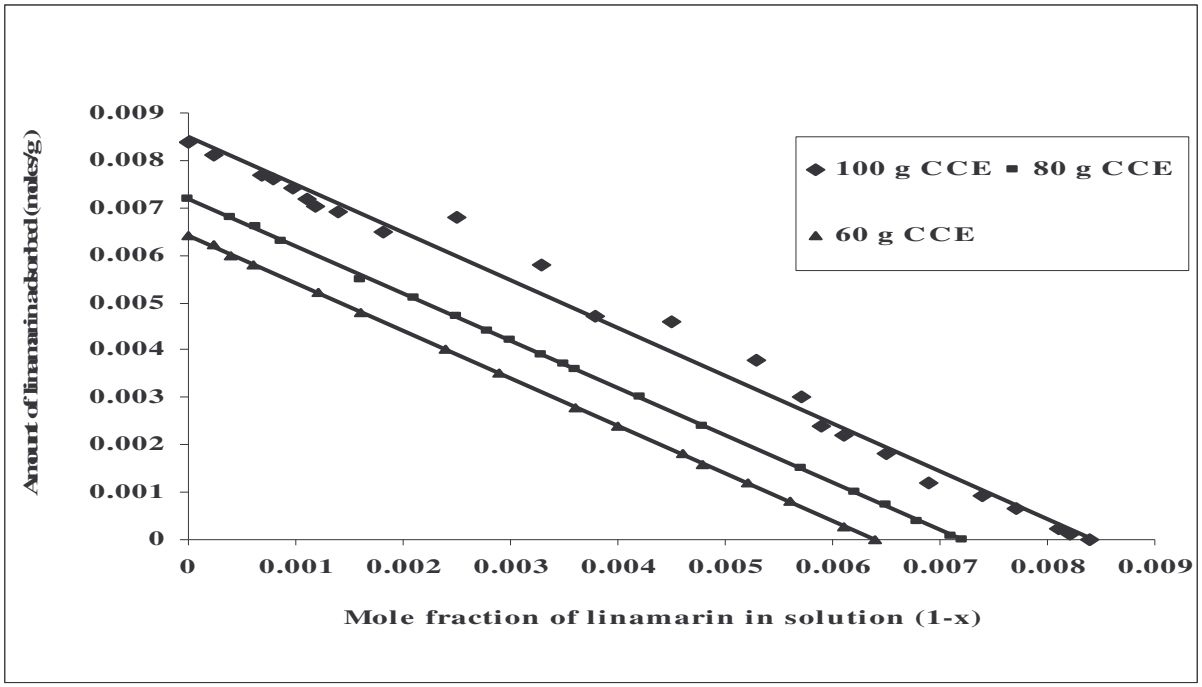

Figure 4.13: Adsorption isotherm of linamarin from solution [smooth lines, model; marks, experiments

Table 4.1: Adsorption data in composite isotherm

Amount of CCE purified (g)	**R^2 values**	**(n_0^s) (g mol/cm^3)**
60	1	1.00
80	0.999	1.00
100	0.988	1.01

These results show linear regression values ($R^2 > 0.98$) for the different amounts of CCE purified, as great conformity with the composite isotherm. This also indicates that the best fit of the isotherm is a function of the sorbent (activated carbon) and the amount of CCE used. Although no significant increase ($p > 0.05$) in the moles of linamarin adsorbed per pore volume of activated carbon (1.00 g mol/cm^3, 1.00 g mol/cm^3 and 1.01 g mol/cm^3 for 60, 80 and 100 g, respectively) for each CCE considered.

4.9 Effect of temperature on pore volume of activated carbon

In order to determine the moles of linamarin adsorbed per pore volume of activated carbon (n_0^s) with respect to temperature, the adsorption of linamarin from solution at 25°C, 35°C, 45°C 55°C and at 65°C were studied. Figure 4.14 shows the result of linamarin removal from solution with temperature at a fixed amount of CCE (100 g). The results showed very close similarities, and the goodness of fit (R^2) and their n_0^s are presented in Table 4.2. The composite isotherm for the five temperatures exhibited high R^2 values. Increase in temperature resulted into decreasing viscosity and increasing molecular motion at higher temperatures thereby allowing the uptake of molecules into the pore more easily, causing adsorption to increase as temperature increases (Micheal *et al.,* 2005). Hence Table 4.2, it was observed that the n_0^s of activated carbon increased from the temperature of 25°C to 45°C (0.99 g mol/cm^3 to 1.01 g mol/cm^3) and then decreased steadily from 1.01 to 1.00 g mol/cm^3 at 55°C and 65°C. The highest n_0^s of activated carbon for linamarin purification achieved at all temperatures studied is unity.

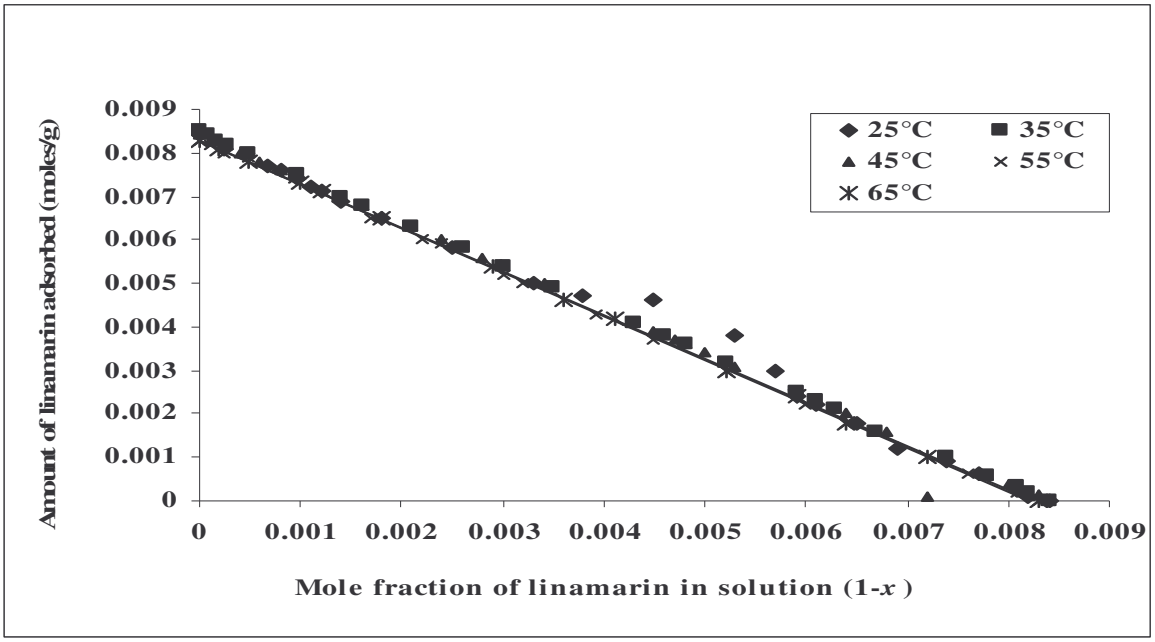

Figure 4.14: Effect of temperature on linamarin removal, using 100 g CEE [smooth lines: model; marks: experiments

Table 4.2: Composite isotherm result on effect of temperature using 100 g of CCE

Temperature (^oC)	R^2 values	(n_0^s) (g mol/cm^3)
25^{o}C	0.993	0.99
35^{o}C	0.999	1.00
45^o C	0.994	1.01
55^{o}C	0.999	1.00
65^{o}C	0.999	1.00

4.10 Compound characterisation

Characterisation of purified compound as linamarin was carried out by both chemical (picric paper test analysis) and physical (IR and ^{1}HNMR) method of structure elucidation. As seen earlier in Figure 4.2, it was observed that the activity of the enzyme on linamarin released the equivalent HCN from the solution. This therefore confirms the purified product to be linamarin.

4.10.1 Nuclear magnetic resonance test

Nuclear Magnetic Resonance (NMR) is another form of absorption spectrometry. This technique provides information on any nucleus possessing of a magnetic moment. But the application of NMR in natural product chemistry, however, has been limited almost exclusively to proton (^{1}H) spectra, since ^{12}C and ^{16}O have no magnetic moment, although ^{13}C and ^{17}O have magnetic moment. Figure 4.15 shows the ^{1}HNMR spectrum of purified product.

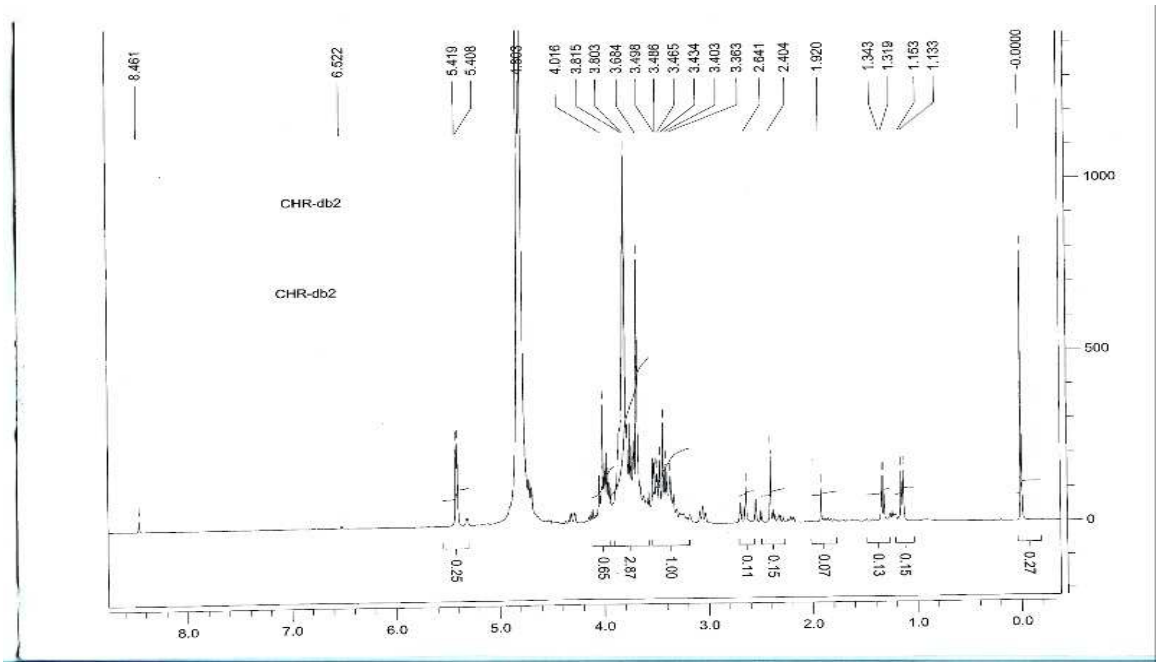

Figure 4.15: NMR spectrum of purified product

The ^{1}HNMR of purified product as shown in Figure 4.15 is comparable to that reported in Seigler, 1975, for cyanogenic glucosides (acecipetalin as a case study) in a non-polar solvent, tetrachloromethane (CCL_4). The characteristics difference are on the biggest absorption band (between 4.8-5.0 ppm) resulting from the effect of the different solvent (D_2O) used, and the effect of the presence of the two methyl (CH_3) present in linamarin at different site of the penultimate carbon atom, exhibiting doublet absorption band between 1.33-1.44 ppm. From the ^{1}HNMR (300 MHz, D_20): δ (ppm) = 1.44 (d, 3H, J = 5.9 Hz, CH_3), 1.33 (d, 3H, J = 6.9Hz, CH_3), 2.40 (s, 2H, CH_2OH), 3.06 (t, 1H, J = 8.12 Hz, 8.12, HCH_2OH), 5.41 (d, 4H, J= 3.58Hz, 4 * CHOH). This is the doublet corresponding to the anomeric proton which indicates linamarin as a β-glucoside (Clapp *et al.,* 1966). The remaining 4H band of the HOCH are inseparable from that of the OH of the D_2O (the solvent used) which appears as the biggest absorption band on the spectrum, indicating a total of 17 H atoms (H17). This is always the case for protons of NH and OH whenever D_2O is used as a solvent (Eberhard, 1993 Seiglar, 1975). This Figure thus satisfied all the protons (17 H) that constitute linamarin.

4.10.2 Infra-red spectrometer (IR) test.

Infra-red radiation in the range of about 10,000-100cm^{-1} (1-100μm) is absorbed and converted by an organic molecule into energy of molecular vibration. This absorption is also quantized, but vibrational spectra appear as band rather than as lines because a single vibrational energy change is accompanied by a number of rotational energy changes (Robert *et al.,* 1963). Band positions are represented as either wavenumbers or wavelength. It is more of functional group identification. Figure 4.16 is the IR spectrum of the purified product

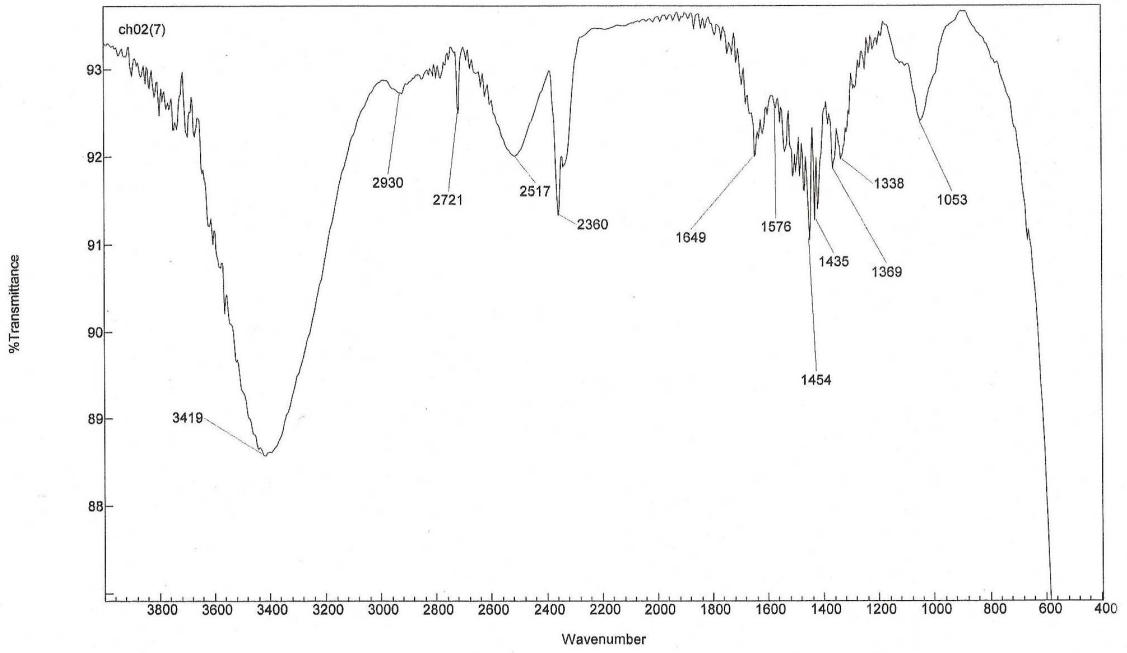

Figure 4.16: IR spectrum of purified product

From figure 4.16, the IR: Vmax wavelength (cm-^{1}) 3419 (O-H), 2930 (C-CH_3), 2721 (C-H), 1649 (-CN), 1576 (-CHO), 1454 (-CH_2). All the functional groups that constitute the linamarin as a compound were identified from the IR spectrum at their different wavelengths.

Integrating both ^{1}HNMR and IR results, $C_6H_{11}O_6C(CH_3)_2CN$ or $C_{10}H_{17}NO_6$, was obtained as a condensed formula, giving a molecular weight of 247.24 g/mol. The structure of linamarin thus determined is shown in Figure 4.17.

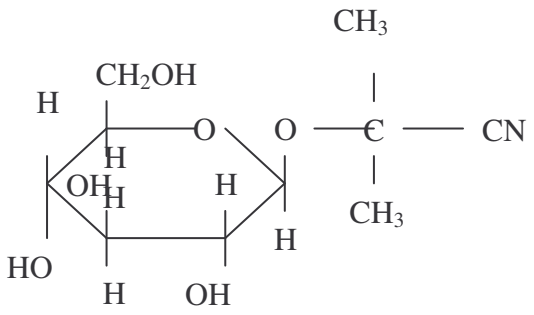

Figure 4.17: Linamarin structure

4.10.3 Melting point determination

BUCHI 510 melting point model was used to determine the decomposition temperature of purified product to be 138°C-140°C, which corresponds to values reported in the literature (Hunsa *et al.,* 1995).

Despite few unaccounted bands that were found in both spectra which is not something strange in compound analysis using these procedures, the physicochemical parameters of purified product obtained were consistent to a higher degree with that reported in literature. Therefore it becomes obvious to characterise purified product as linamarin since purity of a compound is relative (Robert *et al.,* 1963).

4.11 Cytotoxic effects of purified cassava linamarin and crude cassava extract

Figure 4.18a and 4.18b present the IC_{50} MTT cell assay for the purified linamarin and crude cassava extract at both 24 and 48 hrs exposures, respectively.

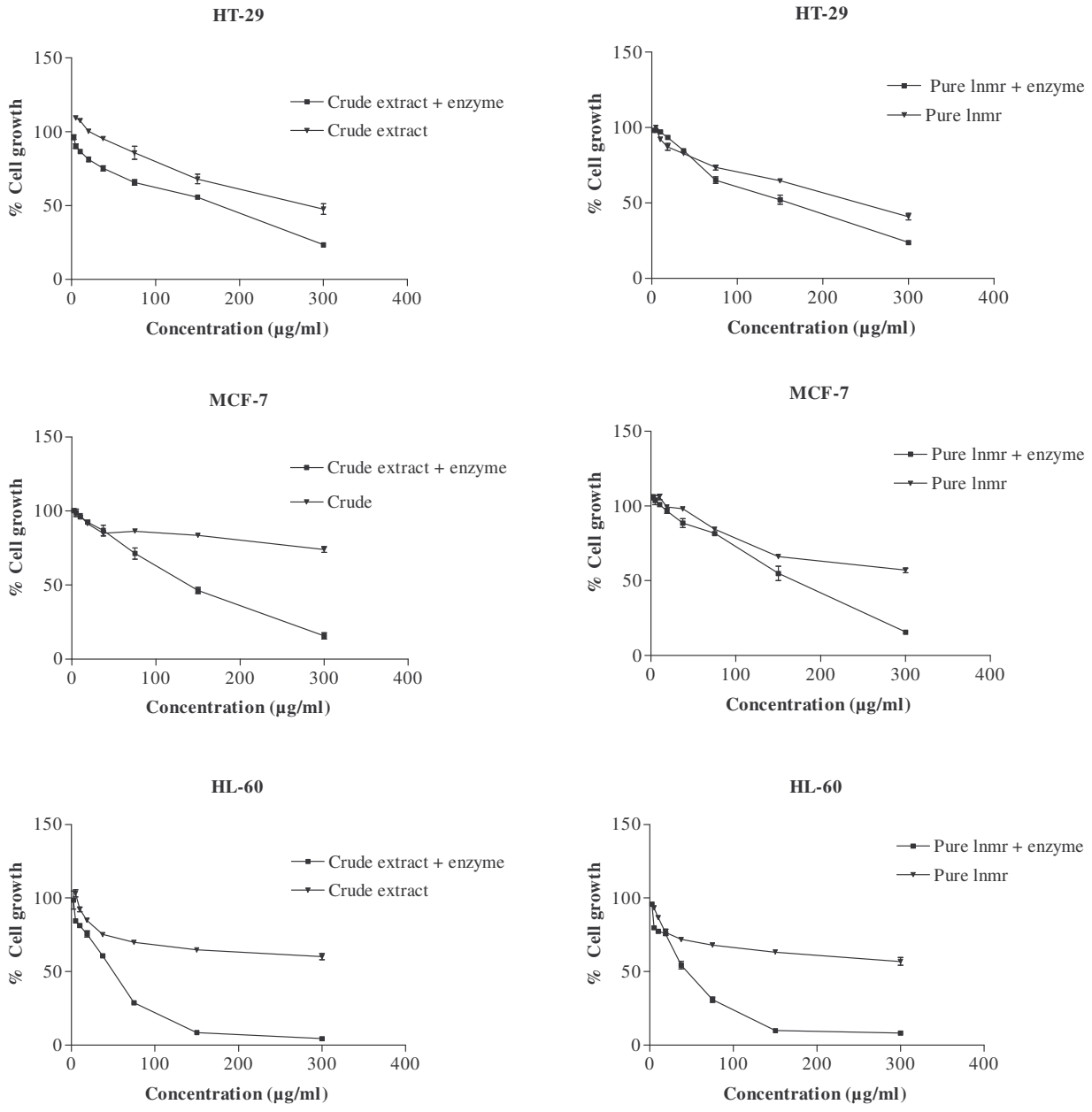

Figure 4.18a*:The effect of purified linamarin, purified linamarin + enzyme, crude extract, and crude extract + enzyme on cell viability in HT-29, MCF-7and HL-60 after 24 hr exposure period at 37°C.(error bars denote standard deviation for 3 experiments) Lnmr=linamarin,Enzyme=linamarase*

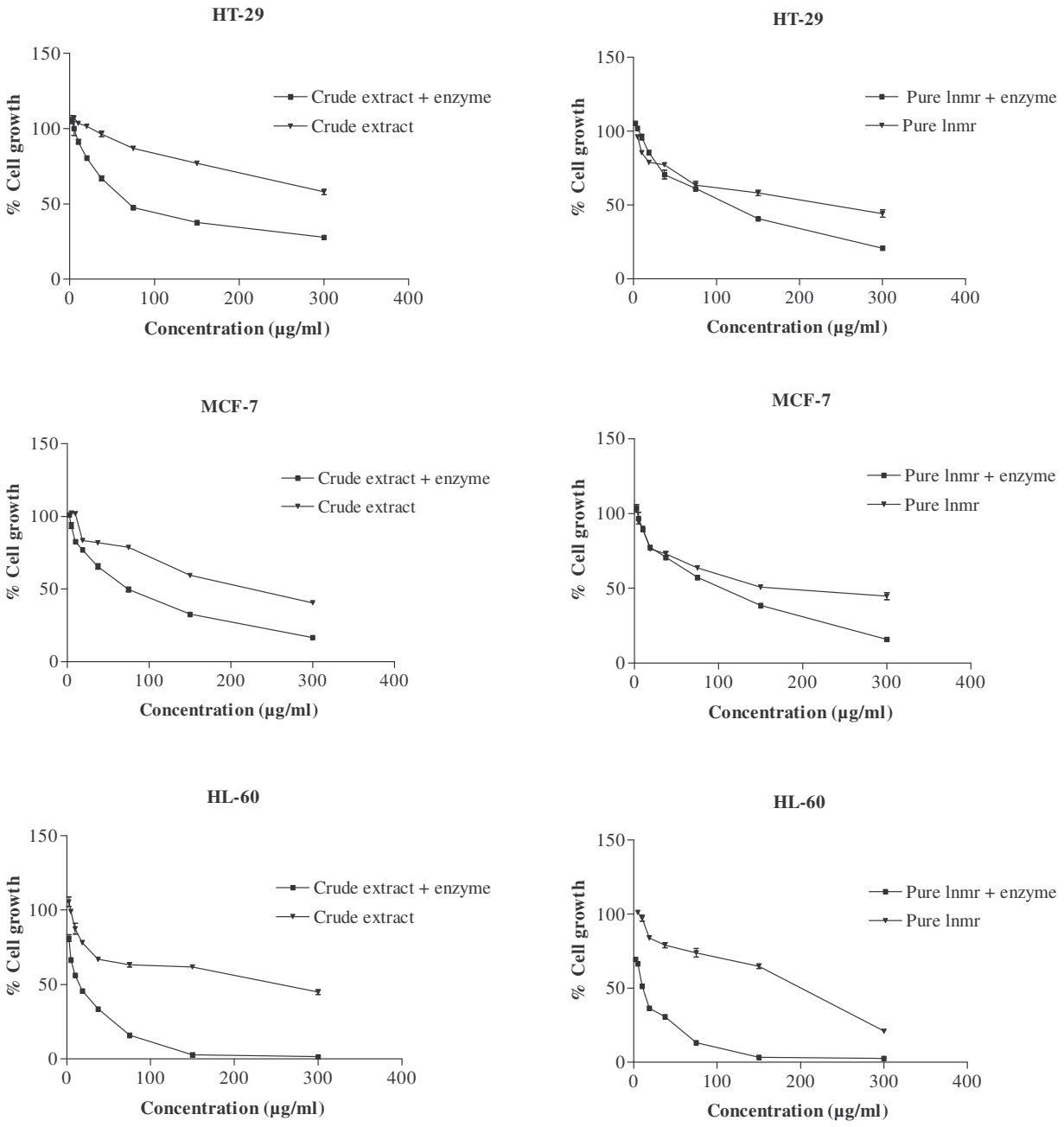

Figure 4.18b: *The effect of purified linamarin, purified linamarin + enzyme, crude extract, and crude extract + enzyme on cell viability in HT-29, MCF-7and HL-60 after 48 hr exposure period at 37^{o}C.(error bars denote standard deviation for 3 experiments) Lnmr=linamarin,Enzyme=lianamarase*

The potency of any drug depends on its efficacy on a defined disease or an ailment. The inhibitory concentration (IC_{50}) which is the concentration required for 50% inhibition was obtained for all cell lines used in order to quantify the efficacies of the purified linamarin and crude cassava extract with and without the enzyme, linamarase. Microdosing is a promising frontrunner in the search for alternatives to the use of animals in drug discovery and development. The European Medicine Agency (EMEA) maximum suggested dose of 100 micrograms has been reported likely too low to achieve the full potential of microdosing (NCB, 2005).

The anticancer potential of purified cassava linamarin and the crude cassava extract were determined on three cell lines representing some of the most common cancers affecting man. In previous studies conducted by Link *et al.* (2006), no significant cytotoxic effect of linamarase was found. They reported that response of non-cancerous cell of the Chinese hamster ovary cell (CHO-K1) to linamarin alone did not show any adverse impact on cell viability but there was effective killing when linamarin was administered at non-limiting and non-toxic concentration of 750 µg/ml in co-application with the enzyme linamarase (Link *et al.*, 2006). When HT-29 cells were exposed to 150 µg/ml crude cassava extract (in the absence or presence of the enzyme), a reduction in cell viability to 50% could not be attained after a 24 hr exposure period (Figure 4.18a). A reduction to 52.2 ± 5.2 % viability was noted for purified linamarin in the presence of linamarase. After 48 hrs, however, > 50% reduction in cell growth was found when the cells were exposed to either the purified linamarin or crude cassava extract in combination with the enzyme at 150 µg/ml (Figure 4.18b).

IC_{50} values obtained when MCF-7 were incubated with purified linamarin and the enzyme combination for 24 hrs was determined at 169.99 ± 4.99 µg/ml (Table 4.3), and for the crude extract and enzyme was 131.98 ± 8.61 µg/ml. This is greatly enhanced ($p < 0.05$) when compared to the pure linamarin and extract used in the absence of the enzyme (IC_{50} values > 300 µg/ml for both) (Figure 4.18a). At the higher concentrations (300 µg/ml), the effect is more pronounced, decreasing cell viability to ± 15% for the purified linamarin or crude extract in combination with the enzyme. Similarly, this is also seen after a 48 hr exposure

period (Figure 4.18b), with a decrease in the IC_{50} values to 78.10 ± 2.51 µg/ml and 63.28 ± 3.29 µg/ml, respectively (Table 4.3).

After 24 hr incubation of HL-60 cells and the purified linamarin or crude extract alone, a reduction in cell viability by 60% is noted at the highest concentration (300 µg/ml). The effect is greatly enhanced ($p > 0.05$) by the addition of the enzyme, with IC_{50} values in the low µg/ml range i.e. 49.14 ± 3.98 µg/ml and 49.82 ± 5.30 µg/ml (Table 4.3) for purified linamarin and crude extract, respectively (Figure 4.18a). At higher concentrations of crude extract in combination with the enzyme (150 µg/ml and 300 µg/ml), cell viability reduction in excess of 90% is seen (Figure 4.18a).

The efficacy of the 300 µg/ml extracts in combination with the linamarase is almost maximal after 48 hrs (Figure 4.18b). IC_{50} values for the purified linamarin or crude extract (202.11 ± 3.89 µg/ml and 246.51 ± 10.12 µg/ml, respectively) is significantly higher than when the enzyme was added (20.95 ± 4.51 µg/ml and 20.09 ± 4.04 µg/ml, respectively) ($p < 0.05$), whereby an approximately 10 times reduction in the IC_{50} values were seen. This shows that greater cytotoxic effects are noted when the extracts are broken down by linamarase to release HCN as the active component. HL-60 cells appear to be more vulnerable to both purified linamarin and the crude cassava extract in combination with the enzyme than the HT-29 and MCF-7 cells, with significantly lower IC_{50} values on both days ($p < 0.05$). Iyuke *et al*. (2004), determined that crude aqueous cassava extracts showed higher potency for all cell lines tested, (including a cervical cancer cell line) in comparison to commercial linamarin in aqueous solution. This could be as a result of some antioxidants present in the crude cassava extract that exhibit synergistic activity. It therefore implies that cassava linamarin, whether in crude extract or purified form, holds potential as a novel compound in the treatment and control of cancer.

Table 4.3: IC_{50} values obtained after exposure to linamarin and crude extract (in the presence and absence of linamarase) after a 24 hr and 48 hr exposure period at 37°C.

Treatment	**IC_{50} values (µg/ml)**					
	HT-29		MCF-7		HL-60	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
Pure linamarin	237.7 ± 7.92	243.05 ± 7.92	> 300	150.36 ± 0.37	> 300	202.11 ± 3.89
Pure linamarin and enzyme	139.16 ± 5.26	112.10 ± 5.33	169.99 ± 4.99	78.10 ± 2.51	49.14 ± 3.98	20.95 ± 4.51
Crude extract	> 300	> 300	> 300	235.96 ± 9.87	> 300	246.51 ± 10.12
Crude extract and enzyme	181.31 ± 9.31	82.35 ± 5.88	131. ± 8.61	63.28 ± 3.29	49.82 ± 5.30	20.09 ± 4.04

CHAPTER FIVE

5.0 CONCLUSION AND RECOMENDATION

5.1 Conclusion

The difficulties in isolating and purifying linamarin from cassava roots were overcome using membrane and adsorption with activated carbon. Degree of contact time with activated carbon during linamarin purification varies with the amount of crude cassava extract (CCE) used. 60 g of CCE took 32 mins, 80 g, 34 mins while 100 g took 36 mins of contact time of purification, respectively.

The MTT cell assay revealed that linamarin either in the form of extract or in a purified form is a good agent for the control and cure of human colon adenocarcinoma, human breast cancer and human leukemia cancer.

In the entire assay that was carried out, it was found that the crude cassava extract exhibited higher cytotoxic effect or rather higher anticancer efficacy than the purified form of linamarin. This could be as a result of the presence of some anti oxidants exhibiting synergistic characteristics.

The entire result also showed that linamarin either in the purified form or in extract is not toxic itself rather until it is broken down to release HCN which is the active component against cancer cells.

The strong anticancer candidature of linamarin when applied with linamarase as shown from the result, thus credited linamarin as a very strong ‘arsenal’ that releases a powerful weapon of cancer destruction.

5.2 Recommendations

1. From the enormous biomedical potential of linamarin as an anti cancer agent, and bearing in mind the very high threat that cancer poses to mankind over the centuries as an ugly 'hunt', further work on possible scaling up the process for economic production of cassava linamarin is thus very pertinent.

2. Because of the sensitive nature of Linamarin to the atmosphere (deliquescence) extreme handling care must be taken when processing it within the reported temperatures and storage must be below $6^{o}C$.

3. Future work on linamarin is aimed to encapsulate linamarin into nanocapsules for drug targeting and delivery, both *in vivo* and *in vitro.*

4. Stability testing on linamarin should be conducted.

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APPENDIX 1

KINETICS OF LINAMARIN ISOLATION BY ULTRAFILTRATON

Table I 1: Result of miniflex ultrafiltration in the isolation of linamarin in the form of crude cassava extract (CCE) at 1.5 bar with standard deviation for 3 experiments

BCE for bulk cassava extract; CCE for crude cassava extract.

Time (min)	**1000g BCE.**			**Ave.**	**1500g BCE**			**Ave.**	**2000g BCE**			**Ave.**
	CCE separated (ml)				**CCE separated (ml)**				**CCE separated (ml)**			
30	50	48	52	50	34	34	37	35	28	30	26	28
60	100	96	104	100	70	69	71	70	54	58	57	56
90	142	147	146	145	102	101	103	102	81	83	82	82
120	182	185	188	185	133	132	132	132	107	106	108	107
150	223	223	225	223	162	162	163	162	126	128	129	128
180	258	260	265	261	190	188	192	190	149	150	148	149
210	286	284	288	286	214	216	224	218	168	167	172	169
240	309	310	309	309	240	244	242	242	182	185	194	187
270	324	328	335	329	262	267	269	266	203	206	206	205
300	324	328	335	329	285	289	290	288	203	206	206	205
330	349	347	351	349	310	305	309	308	220	224	219	221
360	364	367	367	366	326	329	328	327	233	238	240	237
390	374	380	389	381	341	344	350	345	248	252	253	251
420	390	398	394	394	362	359	359	360	260	265	267	264
450	403	408	410	407	373	373	370	372	287	276	277	277
480	418	414	419	417	381	375	384	380	284	286	291	287
510	424	428	429	427	385	389	390	388	304	306	305	305
540	437	432	436	435	392	394	399	395	308	313	312	311
570	440	444	445	443	402	395	403	400	315	312	318	315
600	448	451	448	449	405	402	402	403	315	318	324	319
630	451	456	458	455	406	408	404	406	317	322	324	321

660	460	463	457	460	408	406	410	408	321	327	321	323
690	463	464	468	465	411	412	407	410	327	323	325	325
720	466	473	468	469	408	412	413	411				
750	470	472	474	472								
780	472	475	472	473								

APPENDIX 2

Table II 1: Optimum amount of enzyme determination with standard deviation for 3 experiments

Enzyme solution (ml)	**Concentration of HCN released**			**Average**
0	0	0	0	0
2	0.14	0.16	0.15	0.15
3	0.24	0.32	0.31	0.29
4	0.41	0.41	0.38	0.4
5	0.56	0.59	0.59	0.58
6	0.66	0.73	0.71	0.7
7	0.81	0.76	0.83	0.8
8	0.86	0.86	0.83	0.85
9	0.91	0.89	0.91	0.90
10	0.94	0.94	0.94	0.94
11	0.94	0.98	0.96	0.96
12	0.96	0.96	0.96	0.96
13	0.98	0.93	0.97	0.96

APPENDIX 3

CALIBRATION CURVE RESULT

UV Spectrometer model; 4802 UV/VIS Unico

Wavelength; 510nm

Table III 1: Calibration curve of linamarin, absorbance read to two places of decimal

Linamarin Conc. Used (g/ml)	0	0.3	0.6	0.9	1.2	1.5	1.8	2.1
Absorbance	0	0.15	0.29	0.42	0.55	0.66	0.84	0.97

APPENDIX 4

Table IV 1: Preliminary determination of purifying weight of activated carbon using 100 g crude cassava extract (CCE)

Contact Time (min)	60 g Activated Carbon	80 g Activated Carbon	100 g Activated Carbon
0	0.24	0.24	0.24
2	0.24	0.24	0.4
4	0.27	0.38	0.54
6	0.28	0.5	0.7
8	0.28	0.68	0.96
10	0.29	0.75	1.2
12	0.32	1	1.3
14	0.45	1.2	1.48
16	0.6	1.35	1.54
18	0.73	1.5	1.68
20	0.78	1.58	1.74
22	0.95	1.62	1.8
24	0.99	1.68	1.84
26	1.05	1.78	1.9
28	1.14	1.87	1.95
30	1.2	1.94	2
32	1.22	1.98	2.1
34	1.24	1.98	2.05
36	1.26	2.02	2.01
38	1.28	2.08	1.95
40	1.28	2.05	1.85
42	1.29	2.02	1.68
44	1.29	1.92	1.56
46	1.29	1.85	1.52
48		1.73	1.48
50		1.63	1.3
52		1.52	1.2
54		1.47	1.14
56		1.43	1.08
58		1.33	1.88
60		1.12	1.75
62		0.94	0.66
64		0.9	0.54
66		0.82	0.4
68		0.64	0.35
70		0.45	0.24

72		0.35	0.12
74		0.3	0.07
76		0.28	0.04
78		0.24	0.02
80		0.2	0
82		0.17	
84		0.06	
86		0	

Table IV 2: Determination of linamarin purification/adsorption point with standard deviation for 3 experiments

Contact Time (min)	60g CCE			Ave.	80g CCE			Ave.	100g CCE			Ave.
	Linamarin conc. (g/ml)				Linamarin conc. (g/ml)				Linamarin conc.(g/ml			
0	0.08	0.08	0.08	0.08	0.12	0.12	0.12	0.12	0.24	0.24	0.24	0.24
2	0.08	0.07	0.1	0.08	0.1	0.16	0.1	0.12	0.23	0.23	0.26	0.24
4	0.1	0.12	0.1	0.1	0.2	0.2	0.14	0.18	0.36	0.39	0.39	0.38
6	0.2	0.17	0.17	0.18	0.31	0.27	0.27	0.28	0.5	0.5	0.5	0.5
8	0.21	0.23	0.22	0.22	0.45	0.45	0.45	0.45	0.7	0.76	0.59	0.68
10	0.38	0.38	0.38	0.38	0.59	0.56	0.59	0.58	0.93	0.7	0.63	0.75
12	0.61	0.56	0.58	0.58	0.74	0.74	0.74	0.74	0.81	1.4	0.8	1
14	0.6	0.7	0.6	0.6	0.8	0.92	0.95	0.89	1.63	1	0.98	1.2
16	0.5	0.9	1	0.8	1.3	0.91	0.81	1	1.2	1.65	1.2	1.35
18	0.9	0.9	0.92	0.9	1	1.24	1	1.08	1.5	1.5	1.5	1.5
20	1.4	0.81	0.8	1	1.4	1.2	1	1.2	1.54	1.55	1.65	1.58
22	1	0.9	1.4	1.1	1.22	1.2	1.48	1.3	1.73	1.61	1.53	1.62
24	1.2	1.4	1	1.2	1.35	1.34	1.46	1.38	1.72	1.67	1.65	1.68
26	1.3	1.3	1.3	1.3	1.48	1.42	1.42	1.44	2	1.67	1.65	1.78
28	1.3	1.61	1.3	1.4	1.6	1.6	1.6	1.6	1.87	1.87	1.88	1.87
30	1.5	1.4	1.6	1.5	1.64	1.72	1.6	1.65	1.94	1.94	1.94	1.94
32	1.6	1.72	1.5	1.6	1.7	1.7	1.7	1.7	2.02	1.98	1.94	1.98
34	1.5	1.5	1.61	1.53	1.8	1.8	1.8	1.8	2.06	2.02	1.98	2.02
36	1.4	1.32	1.5	1.53	2	1.7	1.65	1.78	2.04	2.07	2.14	2.08
38	1.3	1.3	1.3	1.4	1.6	1.6	1.9	1.7	2.07	2.05	2.04	2.05
40	0.81	1.4	1.4	1.3	1.73	1.6	1.53	1.62	2.02	1.98	2.06	2.02
42	1.3	0.8	1.35	1.2	1.5	1.52	1.63	1.55	1.92	1.93	1.91	1.92
44	1.2	1.2	0.6	1.15	1.6	1.33	1.33	1.42	1.83	1.87	1.85	1.85
46	0.9	0.9	0.9	1	1.2	1.2	1.2	1.2	1.62	1.62	1.95	1.73
48	0.52	0.7	0.95	0.9	1	0.95	1.21	1.05	1.7	1.6	1.6	1.63
50	0.4	.07	0.7	0.72	0.92	0.89	0.9	0.9	1.5	1.54	1.56	1.53
52	0.22	0.3	0.7	0.6	0.8	0.9	0.92	0.87	1.6	1.32	1.5	1.47
54	0.18	0.52	0.2	0.4	0.84	0.82	0.8	0.82	1.48	1.5	1.32	1.43
56	0.15	0.15	0.15	0.3	0.74	0.8	0.74	0.76	1.59	1.2	1.2	1.33

58	0.08	0.15	0.08	0.15	0.7	0.7	0.7	0.7	0.99	0.98	1.4	1.12
60	0.07	0.08	0.03	0.1	0.7	0.47	0.7	0.62	0.92	0.93	0.97	0.94
62	0	0	0	0.06	0.48	0.5	0.58	0.52	0.9	0.9	0.9	0.9
64				0	0.37	0.5	0.4	0.42	0.81	0.83	0.82	0.82
66					0.2	0.27	0.2	0.22	0.68	0.62	0.62	0.64
68					0.15	0.17	0.17	0.16	0.35	0.42	0.58	0.45
70					0.08	0.08	0.15	0.1	0.34	0.37	0.34	0.35
72					0	0	0	0	0.52	0.21	0.18	0.3
74									0.28	0.28	0.28	0.28
76									0.25	0.24	0.24	0.24
78									0.2	0.2	0.2	0.2
80									0.16	0.18	0.17	0.17
82									0.02	0.08	0.06	0.06
84									0	0	0	0

Table IV 3: Effect of temperature on linamarin purification using 100 g crude cassava extract (CCE), with standard deviation for 3 experiments

Contact Time (min)	25°C			Ave.	35°C			Ave.	45°C			Ave.
	Linamarin conc. (g/ml)				Linamarin conc. (g/ml)				Linamarin conc.(g/ml			
0	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
2	0.23	0.23	0.26	0.24	0.41	0.39	0.42	0.4	0.48	0.51	0.53	0.5
4	0.36	0.39	0.39	0.38	0.53	0.53	0.59	0.54	0.72	0.68	0.71	0.7
6	0.5	0.5	0.5	0.5	0.7	0.7	0.7	0.7	0.96	0.99	1.42	1.12
8	0.7	0.76	0.59	0.68	0.98	0.95	0.95	0.96	1.3	1.31	1.3	1.3
10	0.93	0.7	0.63	0.75	1.2	1.2	1.2	1.2	1.33	1.33	1.6	1.42
12	0.81	1.4	0.8	1	1.3	1.3	1.32	1.3	1.6	1.6	1.6	1.6
14	1.63	1	0.98	1.2	1.6	1.35	1.5	1.48	1.95	1.7	1.6	1.75
16	1.2	1.65	1.2	1.35	1.55	1.5	1.57	1.54	1.8	1.78	1.83	1.8
18	1.5	1.5	1.5	1.5	1.69	1.65	1.7	1.68	1.9	1.9	1.9	1.9
20	1.54	1.55	1.65	1.58	1.9	1.63	1.7	1.74	1.98	1.94	1.94	1.95
22	1.73	1.61	1.53	1.62	2	1.7	1.7	1.8	2.01	2.05	2	2.02
24	1.72	1.67	1.65	1.68	1.9	1.9	1.9	1.9	2.14	2.05	2.06	2.08
26	2	1.67	1.65	1.78	1.97	1.94	1.94	1.95	2.1	2.1	2.1	2.1
28	1.87	1.87	1.88	1.87	2	2	2	2	2.08	2.05	2.05	2.06
30	1.94	1.94	1.94	1.94	2.15	2.08	2.08	2.1	2.3	1.8	1.9	2
32	2.02	1.98	1.94	1.98	2.08	2.04	2.04	2.05	1.94	1.97	1.97	1.95
34	2.06	2.02	1.98	2.02	2.01	2.03	2	2.01	1.8	1.8	1.8	1.8
36	2.04	2.07	2.14	2.08	1.9	1.94	2.02	1.95	1.69	1.72	1.69	1.7
38	2.07	2.05	2.04	2.05	1.83	1.88	1.84	1.85	1.71	1.51	1.6	1.6
40	2.02	1.98	2.06	2.02	1.67	1.67	1.71	1.68	1.63	1.45	1.36	1.48
42	1.92	1.93	1.91	1.92	1.52	1.54	1.63	1.56	1.34	1.31	1.31	1.32
44	1.83	1.87	1.85	1.85	1.5	1.54	1.53	1.52	1.4	1.4	0.96	1.25
46	1.62	1.62	1.95	1.73	1.48	1.48	1.48	1.48	1.2	1.2	1.2	1.2
48	1.7	1.6	1.6	1.63	1.3	1.3	1.3	1.3	1.35	0.9	1.3	1.18
50	1.5	1.54	1.56	1.53	1.2	1.4	1	1.2	0.97	0.99	1.4	1.12
52	1.6	1.32	1.5	1.47	1.3	0.81	1.31	1.14	0.8	0.88	0.87	0.85
54	1.48	1.5	1.32	1.43	0.99	1.25	1	1.08	0.7	0.62	0.8	0.7
56	1.59	1.2	1.2	1.33	0.84	0.9	0.9	0.88	0.6	0.6	0.6	0.6

58	0.99	0.98	1.4	1.12	0.73	0.79	0.74	0.75	0.58	0.51	0.48	0.52
60	0.92	0.93	0.97	0.94	0.7	0.7	0.59	0.66	0.4	0.4	0.4	0.4
62	0.9	0.9	0.9	0.9	0.58	0.52	0.52	0.54	0.18	0.52	0.35	0.35
64	0.81	0.83	0.82	0.82	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2
66	0.68	0.62	0.62	0.64	0.33	0.38	0.34	0.35	0.16	0.14	0.16	0.15
68	0.35	0.42	0.58	0.45	0.27	0.23	0.22	0.24	0.15	0.08	0.08	0.1
70	0.34	0.37	0.34	0.35	0.1	0.1	0.16	0.12	0.07	0.08	0.06	0.07
72	0.52	0.21	0.18	0.3	0.07	0.07	0.07	0.07	0.04	0.04	0.04	0.04
74	0.28	0.28	0.28	0.28	0.06	0.03	0.03	0.04	0	0	0	0
76	0.25	0.24	0.24	0.24	0.02	0.02	0.02	0.02				
78	0.2	0.2	0.2	0.2	0	0	0	0				
80	0.16	0.18	0.17	0.17								
82	0.02	0.08	0.06	0.06								
84	0	0	0	0								

Table IV 3 continued: Effect of temperature on linamarin purification using 100 g crude cassava extract (CCE), with standard deviation for 3 experiments

Contact Time (min)	**55°C**			**Ave.**	**65°C**			**Ave.**
	Linamarin conc. (g/ml)				**Linamarin conc. (g/ml)**			
0	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
2	0.61	0.56	0.54	0.57	0.53	0.62	0.57	0.57
4	0.92	0.9	0.9	0.9	0.97	0.97	1	0.98
6	1.32	0.92	1.31	1.18	0.98	1.39	1.39	1.25
8	1.47	1.54	1.31	1.44	1.5	1.5	1.5	1.5
10	1.5	1.53	1.6	1.54	1.65	1.55	1.55	1.58
12	1.82	1.6	1.7	1.7	1.72	1.71	1.67	1.7
14	1.79	1.8	1.82	1.8	1.8	1.8	1.8	1.8
16	1.86	1.84	1.82	1.84	1.65	2.3	1.81	1.92
18	2.1	2	2	2	2.04	2.01	1.99	2.01
20	2.06	2.05	2.02	2.04	2.02	2.06	2.05	2.04
22	2.08	2.04	2.06	2.06	2.05	2.06	2.08	2.06
24	2.01	1.99	2.04	2.01	1.66	1.74	2	1.8
26	1.6	2.3	1.8	1.9	1.6	1.61	1.6	1.6
28	1.75	2	1.65	1.8	1.35	1.6	1.44	1.46
30	1.6	1.6	1.6	1.6	1.3	1.3	1.3	1.3
32	1.4	1.6	1.5	1.5	1.02	1.02	1.02	1.02
34	1.53	1.2	1.21	1.31	0.89	0.92	0.9	0.9
36	0.99	1.42	0.98	1.13	0.95	0.52	0.7	0.72
38	0.98	0.98	0.98	0.98	0.4	0.5	0.45	0.45
40	0.82	0.82	0.8	0.81	0.3	0.3	0.3	0.3
42	0.96	0.59	0.7	0.75	0.24	0.25	0.26	0.25
44	0.7	0.7	0.4	0.6	0.16	0.1	0.1	0.12
46	0.55	0.55	0.58	0.56	0.05	0.07	0.04	0.05
48	0.44	0.39	0.5	0.44	0	0	0	0
50	0.27	0.23	0.2	0.23				
52	0.18	0.09	0.09	0.12				
54	0.07	0.08	0.03	0.06				
56	0.02	0.02	0.02	0.02				
58	0	0	0	0				

Table IV 4: Adsorption of linamarin from solution at room temperature (25^{o}C)

Contact Time (min)	**Amount of Linmr Adsorbed, Conc. (g/ml) 60g CCE (C_o-C_f)**	**Amount of Linmr Adsorbed, Conc. (g/ml) 80g CCE (C_o-C_f)**	**Amount of Linmr Adsorbed, Conc. (g/ml) 100g CCE (C_o-C_f)**
0	0	0	0
2	0.07	0.02	0.03
4	0.2	0.1	0.06
6	0.3	0.18	0.16
8	0.4	0.25	0.23
10	0.45	0.38	0.3
12	0.6	0.6	0.45
14	0.7	0.75	0.56
16	0.88	0.9	0.61
18	1	0.93	0.75
20	1.2	0.98	0.96
22	1.3	1.04	1.14
24	1.45	1.1	1.18
26	1.5	1.18	1.26
28	1.54	1.28	1.44
30	1.6	1.38	1.63
32		1.58	1.73
34		1.64	1.78
36		1.7	1.8
38		1.8	1.84
40			1.88
42			1.91
44			2.02
46			2.08

Table IV 5: Effect of temperature on linamarin adsorption from solution using 100 g crude cassava extract (CEE)

Contact Time (min)	**Amount of Linmr Adsorbed, Conc. (g/ml) 25^{o}C (C_o-C_f)**	**Amount of Linmr Adsorbed, Conc. (g/ml) 35^{o}C (C_o-C_f)**	**Amount of Linmr Adsorbed, Conc. (g/ml) 45^{o}C (C_o-C_f)**
0	0.03	0.05	0.04
2	0.06	0.09	0.1
4	0.16	0.15	0.15
6	0.23	0.25	0.3
8	0.35	0.42	0.4
10	0.45	0.54	0.5
12	0.55	0.58	0.62
14	0.61	0.62	0.78
16	0.65	0.8	0.85
18	0.75	0.9	0.9
20	0.96	0.96	0.92
22	1.14	1.02	0.98
24	1.18	1.22	1.25
26	1.26	1.35	1.4
28	1.44	1.44	1.5
30	1.63	1.56	1.58
32	1.73	1.7	1.7
34	1.78	1.75	1.75
36	1.8	1.86	1.9
38	1.84	1.98	1.95
40	1.88	2.03	2
42	1.91	2.06	2.03
44	2.02	2.08	2.06
46	2.08	2.1	2.1
48	0	0	0

Table IV 5 continued: Effect of temperature on linamarin adsorption from solution using 100 g crude cassava extract (CEE)

Contact Time (min)	**Amount of Linmr Adsorbed, Conc. (g/ml) 55oC (C_o-C_f)**	**Amount of Linmr Adsorbed, Conc. (g/ml) 65oC (C_o-C_f)**
0	0.05	0.26
2	0.16	0.46
4	0.26	0.6
6	0.46	0.76
8	0.56	1.04
10	0.75	1.16
12	0.93	1.34
14	1.08	1.61
16	1.25	1.76
18	1.31	1.81
20	1.46	1.94
22	1.5	2.01
24	1.62	2.06
26	1.83	0
28	1.94	
30	2	
32	2.03	
34	2.06	
36	0	

APPENDIX 5

COMPOSITE ISOTHERM DATA RESULT

Table V 1: Composite isotherm of linamarin adsorption

Contact Time (min)	**Amount adsorbed (moles),** $\frac{n_0 \Delta x}{m}$ **6 0g CCE**	**Amount remaining in solu.(moles) (1-x) 60g CCE**	**Amount adsorbed (moles),** $\frac{n_0 \Delta x}{m}$ **80g CCE**	**Amount remaining in solu.(moles)(1-x) 80g CCE**	**Amount adsorbed (moles),** $\frac{n_0 \Delta x}{m}$ **100g CCE**	**Amount remaining in solu.(moles) (1-x) 100g CCE**
0	0	0.0064	0	0.0072	0	0.0084
2	0.00028	0.0061	0.000080	0.0071	0.00012	0.0082
4	0.00080	0.0056	0.00040	0.0068	0.00024	0.0081
6	0.0012	0.0052	0.00072	0.0065	0.00064	0.0077
8	0.0016	0.0048	0.0010	0.0062	0.00093	0.0074
10	0.0018	0.0046	0.0015	0.0057	0.0012	0.0069
12	0.0024	0.0040	0.0024	0.0048	0.0018	0.0065
14	0.0028	0.0036	0.0030	0.0042	0.0022	0.0061
16	0.0035	0.0029	0.0036	0.0036	0.0024	0.0059
18	0.0040	0.0024	0.0037	0.0035	0.0030	0.0057
20	0.0048	0.0016	0.0039	0.0033	0.0038	0.0053
22	0.0052	0.0012	0.0042	0.0030	0.0046	0.0045
24	0.0058	0.00060	0.0044	0.0028	0.0047	0.0038
26	00050	0.00040	0.0047	0.0025	0.0058	0.0033
28	0.0062	0.00024	0.0051	0.0021	0.0068	0.0025
30	0.0064	0	0.0055	0.0016	0.0065	0.0018
32			0.0063	0.00088	0.0069	0.0014
34			0.0066	0.00064	0.0071	0.0012
36			0.0068	0.00040	0.0072	0.0011
38			0.0072	0	0.0074	0.0097
40					0.0076	0.00080
42					0.0077	0.00068
44					0.0081	0.00024
46					0.0084	0

Table V 2: Composite isotherm; effect of temperature on linamarin adsorption

Contact Time (min)	**Amount adsorbed (moles),** $\frac{n_0 \Delta x}{m}$, **25°C**	**Amount remaining in solu.(moles) (1-x) 100 g CCE**	**Amount adsorbed (moles),** $\frac{n_0 \Delta x}{m}$, **35°C**	**Amount remaining in solu.(moles) (1-x) 100g CCE**	**Amount adsorbed (moles),** $\frac{n_0 \Delta x}{m}$, **45°C**	**Amount remaining in solu. (moles)(1-x) 100g CCE**
0	0	0.0084	0	0.0084	0	0.0084
2	0.00012	0.0082	0.00020	0.0082	0.00016	0.0083
4	0.00024	0.0081	0.00036	0.0081	0.00040	0.0080
6	0.00064	0.0077	0.00060	0.0078	0.00060	0.0078
8	0.00093	0.0074	0.0010	0.0074	0.00012	0.0072
10	0.0012	0.0069	0.0016	0.0067	0.0016	0.0068
12	0.0018	0.0065	0.0021	0.0063	0.0020	0.0064
14	0.0022	0.0061	0.0023	0.0061	0.0025	0.0059
16	0.0024	0.0059	0.0025	0.0059	0.0031	0.0053
18	0.0030	0.0057	0.0032	0.0052	0.0034	0.0050
20	0.0038	0.0053	0.0036	0.0048	0.0036	0.0048
22	0.0046	0.0045	0.0038	0.0046	0.0037	0.0047
24	0.0047	0.0038	0.0041	0.0043	0.0039	0.0045
26	0.0058	0.0033	0.0049	0.0035	0.0050	0.0034
28	0.0068	0.0025	0.0054	0.0030	0.0056	0.0028
30	0.0065	0.0018	0.0058	0.0026	0.0060	0.0024
32	0.0069	0.0014	0.0063	0.0021	0.0063	0.0021
34	0.0071	0.0012	0.0068	0.0016	0.0068	0.0016
36	0.0072	0.0011	0.0070	0.0014	0.0070	0.0014
38	0.0074	0.0097	0.0075	0.00097	0.0076	0.00080
40	0.0076	0.00080	0.0080	0.00048	0.0078	0.00060
42	0.0077	0.00068	0.0082	0.00028	0.0080	0.00040
44	0.0081	0.00024	0.0083	0.00016	0.0082	0.00028
46	0.0084	0	0.0084	0.00080	0.0083	0.00016
			0.0085	0	0.0084	0

Table V 2 Continued: Composite isotherm; effect of temperature on linamarin adsorption

Contact Time (min)	**Amount adsorbed (moles), $\frac{n_0 \Delta x}{m}$, 55°C**	**Amount remaining in solu.(moles)(1-x) 100g CCE**	**Amount adsorbed (moles), $\frac{n_0 \Delta x}{m}$, 65°C**	**Amount remaining in solu.(moles)(1-x) 100g CCE**
0	0	0.0083	0	0.0083
2	0.00020	0.0081	0.0010	0.0072
4	0.00064	0.0076	0.0018	0.0064
6	0.0010	0.0072	0.0024	0.0059
8	0.0018	0.0064	0.0030	0.0052
10	0.0022	0.0060	0.0042	0.0041
12	0.0030	0.0052	0.0046	0.0036
14	0.0037	0.0045	0.0054	0.0029
16	0.0043	0.0039	0.0065	0.0018
18	0.0050	0.0032	0.0071	0.0012
20	0.0052	0.0030	0.0073	0.0010
22	0.0059	0.0024	0.0078	0.00048
24	0.0060	0.0022	0.0081	0.00020
26	0.0065	0.0017	0.0083	0
28	0.0074	0.0093		
30	0.0079	0.00048		
32	0.0080	0.00024		
34	0.0082	0.00011		
36	0.0083	0		

APPENDIX 6

MTT CELL ASSAY RESULT

Table VI 1: MCF-7 MTT assay for 24hr

	Purified linmr + Enzyme				purified linmr alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	16.75	16.4	14	15.71	57.61	59.44	54.01	57.02
150	61.48	45.48	58.01	54.99	67.21	64.63	66.7	66.18
75	83.22	80.82	81.65	81.65	83.15	84.2	85.82	84.39
37.5	91.22	82.68	91.75	88.55	99.46	96.52	98.12	98.03
18.75	94.15	99.62	96.29	96.68	97.83	99.5	100.5	99.27
10	101.98	102.02	99.16	101.05	105.68	103.2	109.54	106.14
5	104.2	102.69	103.79	103.79	98.27	108.2	105.09	106.82
2.5	102.96	106.13	108.65	105.91				

	Crude Cassava Extract + Enzyme				Crude Cassava Extract alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	13.07	19.86	14	15.64	74.11	70.55	77.5	74.05
150	50.14	45.88	42.94	46.32	82.12	83.4	85.3	83.60
75	66.01	69.48	78.71	71.4	86.27	87.75	85.3	86.44
37.5	92.64	80.55	87.45	86.88	82.68	83.75	88.49	84.97
18.75	93.35	91.49	92.69	92.51	90.69	92.02	92.05	91.58
10	96.34	97.09	95.22	96.21	96.02	97.09	97.38	96.83
5	103.61	95.35	97.38	98.78	98.02	97.89	100.79	98.9
2.5	100.2	102.57	98.15	100.30				

Table VI 2: MCF-7 MTT assay for 48hr

	Purified linmr + Enzyme				purified linmr alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	15.63	16.13	16.21	15.99	44.15	49.17	41	44.77
150	37.86	41.04	36.97	38.62	50.37	50.25	51.85	50.82
75	58.59	57.44	56.03	57.35	62.95	65.43	62.55	63.64
37.5	71.72	70.89	69.6	70.73	70.51	72.73	75.95	73.06
18.75	75.59	77.4	79.39	77.46	76.16	76.92	76.16	76.41
10	88.3	89.31	90.22	89.27	88.68	90.34	91.26	90.09
5	104.11	90.71	95.79	96.87	93.44	94.84	95.52	94.6
2.5	108.37	100.24	101.95	103.52				

	Crude Cassava Extract + Enzyme				Crude Cassava Extract alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	16.57	16.2	17.41	16.72	40.4	42.5	38.94	40.61
150	34.04	32.35	31.81	32.73	59.52	60.09	58.62	59.41
75	47.77	48.72	52.79	49.76	76.61	78.58	80.77	78.65
37.5	66.58	62.16	68.1	65.6	81.31	82.39	81.99	81.89
18.75	76.98	75.71	78.31	77	82.77	84.61	82.84	83.40
10	83.66	82.48	81.88	82.67	99.65	102.33	103.7	101.89
5	97.51	93.57	90.22	93.76	103.7	101	102.23	102.31
2.5	101.57	101.76	99.67	101				

Table VI 3: HT-29 MTT assay for 24hrs

	Purified lnmar + Enzyme				Purified linmar alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	23.81	23.08	24.46	23.78	36.59	42.8	43.49	40.96
150	52.13	57.46	47.01	52.2	65.98	63.55	64.63	64.72
75	69.23	64.39	61.96	65.19	73.09	70.93	76.59	73.53
37.5	85.32	86.63	82.34	84.76	81.37	84.95	82.29	82.87
18.75	91.76	95.23	93.60	93.60	82.66	89.91	88.91	87.16
10	97.5	97	97.2	97.23	92.22	93.36	91.3	104.17
5	99.44	97.83	97.48	98.25	100.03	100.56	100.12	100.23
2.5	100.68	96.26	97.94	98.29				

	Crude Cassava Extract + Enzyme				Crude Cassava Extract alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	23.81	24.39	22.53	23.57	40.65	51.76	50.75	47.72
150	53.33	56.45	57.83	55.87	64.63	65.14	74.58	68.11
75	66.75	62.24	68.22	65.73	84.86	78.78	93.92	85.85
37.5	77.69	71.71	76.4	75.26	97.73	93.55	95.05	95.44
18.75	81.59	84.02	78.61	81.40	101.5	100.19	99.58	100.42
10	88.32	85.76	90.38	86.75	107.2	107.57	107.76	107.51
5	92.9	87.76	90.38	90.34	107.2	109.06	111.87	109.37
2.5	94.3	99.53	95.25	96.36				

Table VI 4: HT-29 MTT assay for 48hrs

	Purified linmr + Enzyme				purified linmr alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	19.8	21.09	21.5	20.79	39.6	44.67	48.38	44.21
150	39.12	40.63	42.7	40.81	54.51	59.49	60.49	58.16
75	60.74	59.37	63.33	61.14	61.14	68.5	61.14	63.59
37.5	75.32	71.51	65.06	61.14	75.98	78.29	77.63	77.3
18.75	83	87.89	85.88	85.59	79.63	77.44	79.68	78.91
10	99.63	95.26	93.3	96.06	86.48	84.56	85.65	85.56
5	102.61	99.63	103.92	102.05	93.98	96.59	97.88	96.15
2.5	105.16	104.5	107.07	105.57				

	Crude Cassava Extract + Enzyme				Crude Cassava Extract alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	26.66	27.35	29.24	27.75	61.22	54.63	57.92	57.92
150	35.13	39	39.06	37.73	76.1	77.51	77.33	76.98
75	47.61	46.99	48.16	47.58	85.7	87.33	87.62	86.88
37.5	69.53	67.62	64.16	67.10	93.17	96.38	99.71	96.88
18.75	81.11	79.73	80.82	80.55	101.75	99.83	103.25	101.61
10	93.56	88.43	91.79	91.26	103.92	101.7	105.22	103.60
5	94.97	95.81	109.3	100.02	105.96	107.52	107.77	107.08
2.5	108.87	100.57	108.57	106.00				

Table VI 5: HL-60 MTT assay for 24hrs

	Purified linmr + Enzyme				Purified linmr alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	7.54	8.73	8.54	8.27	52.38	56.75	61.71	56.94
150	10.87	9.93	9.52	10.10	62.52	63.25	64.25	63.34
75	32.99	32.54	27.38	30.97	68.54	69.56	66.25	68.12
37.5	50	54.76	58.73	54.49	70.25	71.21	74.52	71.99
18.75	75.4	76.19	76.19	75.92	79.95	72.96	77.52	76.81
10	78.17	79.17	75.21	77.51	85.77	85.32	88.96	86.68
5	79.17	79.56	81.03	79.92	94.44	93.14	92.37	93.32
2.5	97.8	94.31	95.86	95.99				

	Crude Cassava Extract + Enzyme				Crude Cassava Extract alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave**
300	4.37	5.05	4.27	4.56	64.42	57.05	59.13	60.2
150	9.52	9.31	7.16	8.66	64.68	65.48	64.42	64.86
75	27.78	28.97	29.88	28.87	68.69	71.45	69.54	69.84
37.5	61.11	61.92	59.52	60.85	74.51	75.12	76.45	75.36
18.75	75.29	72.28	79.56	75.71	85.38	85.38	84.13	84.96
10	80.65	81.28	82.23	81.37	93.65	89.29	94.31	92.41
5	84.99	82.28	86.56	84.61	98.58	104.76	106.35	103.23
2.5	90.08	110.61	95.47	98.72				

Table VI 6: HL-60 MTT assay for 48hrs

	Purified linmr + Enzyme				Purified linmr alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	2.73	2.5	2.96	2.73	23.14	19.41	20.48	21.01
150	3.19	3.41	3.87	3.49	63.13	67.8	63.48	64.80
75	13.3	13.56	13.01	13.29	79.52	72.35	69.85	73.90
37.5	30.05	30.72	31.63	30.8	76.45	78.27	82.44	79.05
18.75	35.27	36.43	38.08	36.59	82.81	82.58	86.52	83.97
10	51.88	52.1	50.26	51.41	99.73	93.28	98.73	97.24
5	65.95	66.75	67.18	66.62	99.89	103.3	100.53	101.24
2.5	70.21	67.58	70.99	69.59				

	Crude Cassava Extract + Enzyme				Crude Cassava Extract alone			
Conc. (µg/ml)	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**	**Plate 1 % cell viability**	**Plate 2 % cell viability**	**Plate 3 % cell viability**	**Ave.**
300	1.59	1.82	1.14	1.51	41.75	45.96	47.1	44.93
150	2.93	2.66	2.93	2.84	63.83	61.25	60.25	61.77
75	15.42	16.15	16.38	15.98	60.52	63.83	65.69	63.34
37.5	35.1	32.31	33.67	33.69	67.35	67.8	65.68	63.34
18.75	46.54	46.64	44.41	45.86	76.22	78.73	79.52	78.15
10	58.51	56.2	54.23	56.31	84.3	83.24	94.88	87.47
5	67.82	64.09	67.92	66.61	98.66	99.2	99.25	99.03
2.5	77.39	80.58	85.63	81.2	110.1	99.46	107.17	105.5