

An *in vitro* inhibition of human malignant cell growth of crude water extract of cassava (*Manihot esculenta* Crantz) and commercial linamarin

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Abstract

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Epidemiological studies had shown a link between low incidences of cancer in communities where cassava intake is the main staple. The prophylactic action of cassava intake and its deleterious effect on humans are attributed mainly to the expressed toxicity of the aglycone moiety of the cyanogenic glucoside linamarin, when the latter breaks down. The effect of crude water extract of cassava leaf and that of commercial linamarin was investigated on two human tumor cell lines Caov-3 (ovarian), and HeLa (cervical adenocarcinoma) *in vitro*, using the MTT- assay for cell growth inhibition/cytotoxicity. The effect of the

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enzyme linamarase (EC 3.2.1.21), a β -glucosidase also tapped from the cassava plant and treated appropriately was tested on one of the cell lines. Crude water extract or commercial linamarin was added in a final concentration of 300 $\mu\text{g/ml}$ with or without the partially purified enzyme (10% added of total cell volume and culture medium) to the seeded cells. The water extract was more efficacious, eliciting an IC_{50} value of 38 $\mu\text{g/ml}$ and 57 $\mu\text{g/ml}$, respectively, for the ovarian and cervical cell lines, while the commercial linamarin shows a growth inhibition of 150 and 210 $\mu\text{g/ml}$, respectively, for the two cell lines. Inclusion of the enzyme on a linamarin treated cervical cell line improved the growth inhibition to 40 $\mu\text{g/ml}$. The antineoplastic effect exhibited by the two samples containing the cyanogenic glucoside is explained in terms of the activity of endogenous/exogenous linamarase, presence of dietary factors in the crude extract, and a possibly induced bystander effect. Tumors are unlikely to develop any resistance against hydrocyanic acid, due to its inhibitory role in oxidative phosphorylation. Additionally, an inbuilt mechanism *in vivo* ensures detoxification of excess cyanide that may seep to the surrounding normal tissues. Thus properly administered by increasing the prodrug concentration at the tumor site and decreasing its systemic level, treatment of cancer using the relatively non-toxic linamarin, with or without the enzyme, linamarase could be a favoured future treatment for a variety of cancers.

Key words : Epidemiological, cancer, linamarin, toxicity, antineoplastic, bystander effect

A study on the dietary habits of different population shows a strong connection between dietary factors (macro- and micronutrients) and carcinogenesis. This observation is reminiscent of those made in the etiology of various ailments linked to deficiency in certain dietary constituents. Thus, high intake of vegetables, fruits and whole grains are likely to stave off cancer; the inhibitory effect of these plants food is attributed to a variety of micronutrients and phytochemicals (Greenwald, 1999).

Cassava (*Manihot esculenta* Crantz) is a staple food in Africa, Asia and Latin America (Joseph *et al.*, 2001). The storage root crop is a good source of carbohydrate, and the leaves provide an inexpensive and rich source of protein (Rao and Hahn, 1984) albeit low in sulphur containing amino acids (Cliff *et al.*, 1985; Ngudi *et al.*, 2003). The edible root and leaves also contain vitamins, minerals, dietary fibres and cyanogenic glucoside, mainly linamarin (Bradbury *et al.*, 1988; Balagopalan *et al.*, 1988). The prophylactic action of cassava as it relates to cancer had been reported; the incidence of bowel cancer is said to be very low in communities where cassava meal contributes fairly appreciably to its nutritional need (Balagopalan *et al.*, 1988).

Cancer is a major public health problem in developed countries and is now emerging as a significant health concern in Asia (Smith, 2002).

Although there had been some success in the treatment of many haematological and childhood malignancies using chemotherapy (Zhitovovsky and Orrenius, 2003) most solid tumours continue to have a bad prognosis (Pietersz and McKenzie, 1992). The reason for this lack of efficacy is because effective concentration of these agents is usually not possible because of dose limiting systemic toxicity (Fainaro *et al.*, 2002) and the often resistance of tumours to this type of treatment (Zhitovovsky and Orrenius, 2003). The aberrance expressed by tumours, for example antigens on cell surface and neo-vascularization are now being exploited to improve specificity of delivery of anticancer agents.

The cyanogenic glucoside linamarin, and its hydrolytic enzyme linamarase, a beta-glucosidase [EC 3.2.1.21], are compartmentalized in the intact tissues of the cassava. Upon damage to the tissue, the two come together to give glucose and its aglycone moiety; the latter degenerates further under an alkaline/neutral environment to give hydrogen cyanide and ketone (Cooke, 1978). The reaction sequence of linamarin hydrolysis is illustrated in Figure 1.

While toxicity associated with linamarin had not been reported (Cooke, 1978; Cereda and Mattos, 1996), hydrogen cyanide is a powerful metabolic poison (Keresztessy *et al.*, 2001). Using gene and antibody guided therapy, the reaction in

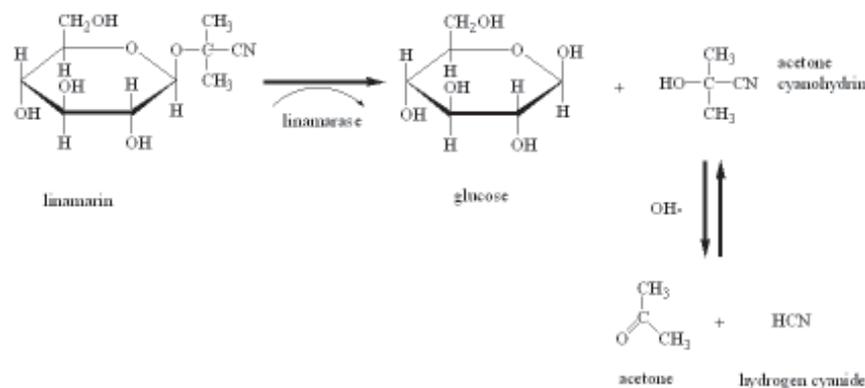


Figure 1. Hydrolysis of linamarin to give hydrogen cyanide.

Figure 1 was mimicked by two different research groups to kill tumour cells using the lethality of hydrogen cyanide. One group engineered an antibody that can carry the enzyme linamarase (Kousparou *et al.*, 2002) and another group constructed the enzyme in a retrovirus (Cortes *et al.*, 1998). Both techniques are based on enzyme-prodrug approach and *in vitro* and *in vivo* tests have been reported by both groups with varying success.

In our research, we seek to employ biodegradable polymers for selective delivery of linamarin, with or without the enzyme linamarase. It has been clearly demonstrated that polymers of nanoparticle size can accumulate in tumours by a phenomenon known as "the enhanced permeability and retention effect" (EPR), which is a consequence of the neo-vascularization arisen from tumor angiogenesis. This effect ensures a gradual increase in concentration of the polymers from leaky blood vessels through passive diffusion (Fainaro *et al.*, 2002; Connors *et al.*, 1995; Matsumura and Maeda, 1986). A variety of studies have shown the effectiveness of this approach (Brigger *et al.*, 2002; Soppimath *et al.*, 2001); also biocompatibility of the polymers, their non-immunogenicity and sustained release are some of the advantages offered by the technique.

In this paper however, we present report on a preliminary investigation of the cytotoxicity of a) a crude water extract of fresh cassava leaves, representing a dietary exposure to cyanogenic

glucoside, b) commercial linamarin and c) a combination of commercial linamarin and crude exogenous linamarase, against ovarian cancer (Caov-3) and cervical adenocarcinoma (Hela) cell lines. The ability of the tested compounds to inhibit the growth or kill human cancer cells in culture is taken as indication of potential value as an anti-cancer agent *in vivo* (Lieberman *et al.*, 2001).

Materials and Methods

Reagents and Chemicals

RPMI-1640 culture medium, L-glutamine, and Phosphate Buffered Saline (PBS), Etoposide and Tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Trypsin-EDTA solution (0.25% w/v) was obtained from PAA Laboratories (Austria). D-glucose and Sodium bicarbonate were from BDH Limited (Poole, England). Tetrazolium dye 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was bought from Amresco (Ohio, USA). Commercial linamarin was purchased from A.G. Scientific, Inc. (USA) and Australian National University supplied picrate paper kit. All other reagents used were analytical grade.

Plant material

Cassava sticks were sourced locally and planted on a farm acquired from the Faculty of Agriculture University Putra Malaysia. Five months after planting, young and matured leaves were

collected as samples for the preparation of the crude extract.

Extraction, and sample preparation for testing

About 350 g of the fresh cassava leaves were soaked in 2 L of hot water (about 85°C) to inactivate the enzyme linamarase. The mixture was allowed to cool at room temperature and left overnight for maximal extraction. The crude solution was filtered, concentrated and freeze-dried; the yield was 23 g of dried extract, which was stored at -20°C until needed.

The presence of endogenous cyanogenic glucoside from the water extract was expressed using the modified method of Bradbury *et al.* (1999). The cyanide liberated by enzyme hydrolysis of the glucoside was quantified by eluting the colour formed by the cyanide picrate complex on the alkaline picrate paper and measured spectrophotometrically at 510 nm.

Stock solutions of 30 mg/mL of the extract and commercial linamarin were prepared separately in distilled water. These stocks were stored at -20°C. Before preparation of the sample dilutions in cell culture medium, the frozen concentrates were thawed at room temperature and diluted appropriately with complete growth medium. The two samples were tested at five three-fold dilutions in triplicate with an upper limit of 300 µg/mL.

Extraction of the enzyme linamarase (E.C.3.2.1.21).

The enzyme was extracted from cassava latex as described by Nambisan, (1999) and modified by Haque and Bradbury (1999). Latex taken from petioles of cassava leaves that were broken off from the plant was dissolved in distilled water at 10 mg/mL. The crude aqueous mixture was filtered first using Whatman No.1 filter paper followed by a 0.22 µm filter; this preparation was stored at -20°C until required.

Positive control

Two positive controls, Tamoxifen and Etoposide were used in this study. They were also tested at five three-fold dilutions in triplicate with

an upper limit of 300 µg/mL.

Cell culture

The cell lines from human ovarian adenocarcinoma (Caov-3) and cervical adenocarcinoma (HeLa) were obtained from America Type Culture Collection (ATCC, USA). Cells were maintained as sub confluent monolayers in 75 cm² culture flasks (Costar-Corning) supplemented with 10% fetal bovine serum (FBS) and L-glutamine (2mM) at 37°C under humid atmosphere of 5% CO₂.

In vitro anti-tumoral assay

Both cell lines were trypsinized from culture plates with 0.25% trypsin EDTA; RPMI medium was added to inactivate the trypsin. Detached cells were freed into single cell suspension and counted on a haemocytometer using trypan blue exclusion. Cells were further diluted with medium to give a final cell concentration of 3.0 x 10⁴ cell/mL. 100 µL of these cells were inoculated into 96-well microtiter plates, a 24-h incubation period was allowed for the cells to stabilize; thereafter 100 µL of the experimental samples of final concentration ranging from 1.0 µg/mL to 300 µg/mL were added to the appropriate microtiter wells in triplicate with or without the enzyme linamarase. The positive control were also tested in a similar manner. After an additional 4 d of incubation at 37°C, 5% CO₂ and humid atmosphere, MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide) was added; this reagent would be cleaved by mitochondrial dehydrogenases produced by viable cells, to a blue formazan product. The absorbance was measured at a wavelength of 570 nm and cytotoxicity in treated cells was expressed as a percentage of control cell growth (Fonseca *et al.*, 2002). IC₅₀ (inhibition concentration that inhibits or kills 50% of the cells) was evaluated from the MTT dose response curve of cell viability against drug concentration.

Linamarase Assay

To check the appropriate volume that will give optimal enzymatic activity. Cells of the density as described above were incubated to a final drug

concentration of 100 µg/mL except the control wells. Aliquots of 5, 10, 15, 20, 25 and 30 µL of the enzyme preparations (10 mg/mL) were added to different cell wells including one segment of the control. The other control segment contains only the cells and the dilution medium. After four days incubation, MTT was added to assess the growth inhibitory and or cytotoxic activity of linamarin-linamarase. 20 µL of the aliquot was found to have about the same enzymatic effect as 25 or 30 µL, hence 20 µL was chosen as the working volume.

Statistical analysis

The % control absorbance data was expressed as the means ± S.D. and the IC₅₀ values were obtained from the single replicate experiments. The student independent t-test using SPSS package was used to evaluate the significance of the differences between the control and treated cells in the cytotoxicity analysis, as well as to compare the significant differences of the tested samples between the two cell lines.

Results

Cell growth inhibition

The three experimental samples were tested at a concentration of 1.0 µg/mL to 300 µg/mL in triplicate. Cell growth inhibition/cytotoxicity were

measured using the MTT assay. The crude water extract and commercial linamarin showed cytotoxic activity against ovarian cancer (Caov-3) to be IC₅₀ of 38 and 150 µg/mL, respectively, and against cervical adenocarcinoma to be 57 and 210 µg/mL respectively (Table 1). Addition of exogenous linamarase augments the potency of the commercial linamarin on the cervical cell line with an IC₅₀ calculated at 40 µg/mL. These values are much higher than those obtained for the positive control. For tamoxifen the IC₅₀ are 11.82 and 10.85 µg/mL, respectively, while etoposide was 17.76 µg/mL for the ovarian cell line.

Exogenous linamarase

In vitro activation of linamarin having a β-glucoside moiety using extracellular β-glucosidase should provide information on the inhibitory role of the prodrug alone. 20 µL (10 mg/mL) of the cassava latex was shown to be optimal for the activation of linamarin (data not shown); this represents about 10% of total cell volume and drug dilution. The activity of latex enzyme solution is said to be about the same order of magnitude as those obtained from cassava leaves after partial purification using a Phenyl Sepharose short column (Haque and Bradbury, 1999). In this study the absorbance of control wells containing cells with linamarase or cells alone were not significant. Test

Table 1. IC₅₀ values of tested samples and positive control (single replicate)

Sample	IC ₅₀	
	Caov-3	HeLa
Crude WE Linamarin**	38	57
Etoposide	17.76	b
Linamarin*	150	210
Linamarin + Linamarase**	b	40
Tamoxifen	11.82	10.85

1. The significance of the tested samples i.e. the crude water extract (WE) of linamarin, commercial linamarin and linamarin + linamarase on the two cell lines were compared with the control cells using the independent student sample t-test, *P<0.05 when linamarin alone is used as the experimental drug compared with the control and **P<0.01 when either the crude water extract of linamarin or linamarin + linamarase are used as the experimental drug compared with the control.

2. The significance of the tested samples and the positive control were compared between the two cell lines, P>0.05 indicating that the effect of the tested samples including the positive controls on both cell lines are not significantly different.

b. Not tested

for the presence of linamarin in latex solution had also been reported to be negative (Haque and Bradbury, 1999). Any trace of linamarin present in the latex may have been sequestered during the preparation of the latex solution as both substrate and enzymes are very soluble in water.

Discussion

Growth was inhibited in the ovarian and cervical cancer cell lines by exposure to the test samples in varying degrees, with the crude samples showing greater efficacy than the commercial linamarin. In the present study an IC_{50} of 150 (0.6mM) and 210 (0.85mM) $\mu\text{g}/\text{mL}$ were recorded as the expressed toxicity of linamarin on the ovarian and cervical cancer cell lines, respectively. When the cervical cancer was exposed to linamarin and the beta-glucosidase linamarase, the potency of the prodrug was enhanced five fold to give an IC_{50} of 40 $\mu\text{g}/\text{mL}$ (0.16mM). These results indicate a higher level of sensitivity of the cell lines to the toxic insult of linamarin compared to other cell lines previously reported. Kousparou *et al.* (2002) have used a colorectal cancer cell line (LS174T), in an approach based on antibody enzyme-prodrug therapy with linamarase as the enzyme and linamarin the prodrug. An IC_{50} of 6mM was reported after a two-day incubation period, and where the cells were incubated with linamarin alone, the value was above 100mM. In a similar approach, amygdalin, a naturally occurring cyanogenic glucoside, which also hydrolyses to hydrogen cyanide, was used as a prodrug on bladder cancer cells (HT1376) by Syrigos *et al.* (1998). An IC_{50} of 40.4mM was obtained when amygdalin was used alone after 24-h incubation and when activated by a β -glucosidase conjugated to a bladder associated monoclonal antibody, the IC_{50} was 1.1mM. In yet another approach, also based on enzyme - prodrug therapy, a human glioblastoma brain tumor cell line (U-373 MG) was transduced with retroviral vectors expressing the cassava linamarase gene (Cortes *et al.*, 1998). After incubation with linamarin for two days, the toxic index was 143 $\mu\text{g}/\text{mL}$ (0.58mM); the non-transduced cell line exhibited no apparent

toxicity.

Two suggestions are, therefore, put forth to explain the greater inhibitory effect of linamarin on the cell lines used in this study. The first, is the possible presence of trace amount of intracellular enzymes that is able to activate the prodrug, and the second, a prolong incubation period of four days at the physiologic temperature which could provoke greater degradation of the prodrug.

Human endogenous β -glucosidase that can cleave the β -glucoside moiety in cyanogenic glucoside has been reported to be present in the cytosol of the liver, spleen, and intestinal cells and lymphocytes (Graaf *et al.*, 2003). Cellular uptake of linamarin and other glucose analogues have already been reported via the glucose transporter (Sreeja *et al.*, 2003; Hagihira *et al.*, 1963; Smith and Titley, 1999). There are also reports of stock solution of linamarin stored at 4°C hydrolysing slowly (<10%) after 3 d of storage (Rao and Hahn, 1984). We, therefore, hypothesize that the greater sensitivity of the cell lines as witnessed in this work could be attributed to the presence of extracellular linamarin in the culture media which degrades or decomposes slowly during 4d of incubation at 37°C, and the likely presence of endogenous β -glucosidase that is able to activate intracellular linamarin taken up by some of these cells via the glucose transporter. These factors may have contributed to the release of both extracellular and intracellular HCN, lethal enough to induce toxicity on the cells. Furthermore, the diffusivity of the cyanide may have provoked a bystander effect on neighbouring cells, thus augmenting the overall effect of the test samples. It is also intriguing to suggest the path of glycolysis for the breakdown of linamarin to release HCN, although glucose analogues can be partially phosphorylated, they are however not substrates for hexokinase activity (Hagihira *et al.*, 1963; Smith and Titley, 1999).

The cells are refractory to lower doses of linamarin due to the protection provided by the mitochondrial rhodanese in its ability to detoxify the metabolic poison to a certain extent (Oke, 1979, 1980; Balagopalan *et al.*, 1988). Once the threshold

is exceeded, however, toxicity is expressed. The absence of this inbuilt intracellular mechanism for detoxification of the positive controls may, therefore, be the reason for their greater efficacy compared with the test samples.

The Caov-3 cell line tends to be more sensitive than the HeLa, possibly because of the higher turnover rate of the latter than the ovarian cell line. Besides, high glucose requirement of Caov-3 cell line implies faster cellular uptake of glucose and its analogues, more so as tumours exhibit greater rate of glycolysis than normal tissue (Smith and Titley, 1999).

Cassava consumption provides a classical example of a dietary exposure of humans to cyanogenic glucoside. The crude water extract was confirmed in this study to contain linamarin as expressed by the picrate paper test. There are reports in the literature of the presence of β -carotene, vitamin C and free Ca in both roots and leaves of the cassava plant in appreciable quantity (Balagopalan *et al.*, 1988; Bradbury and Holloway, 1988). The roles of these micronutrients in the etiology of various cancers are well documented (John *et al.*, 1996; Manju *et al.*, 2002; Kallay *et al.*, 1999; Nesaretnam *et al.*, 2000).

The higher efficacy of the crude water extract compared to the commercial linamarin may be due to the combined effect of these micronutrients and the presence of linamarin. Linamarase was deactivated by heat during the preparation of the crude extract in order to get linamarin; however, enzymes denatured by heat can be reversed if the enzyme is returned to a moist environment having a pH and temperature within its stability range (DeSantis, 1983). Thus, a fraction of the deactivated linamarase could have been reactivated, under the in vitro condition employed in this work (pH=7.3, T=37°C), to enhance the hydrolysis of linamarin. The optimal pH for the activity of linamarase has severally been reported to range from 7-8 (Bradbury *et al.*, 1999; Nambisan, 1999; Haque and Bradbury, 1999).

There are reports suggesting that the cyanogenic glucoside in food broken down in the gut flora leads to the formation of free radicals that

initiate damage to cells through lipid peroxidation, an occurrence that could initiate carcinogenesis (Grindley *et al.*, 2002; Ragoobirsingh *et al.*, 1993). However, epidemiological studies have shown certain deleterious ailments like tropical ataxic neuropathy (TAN), konzo and iodine deficiency to be the most prevalent disorders in communities that subsist on a monotonous diet of cassava or cassava related food (Balagopalan *et al.*, 1988; Oke, 1979, 1980; Osuntokun, 1981). In certain areas, endemic in TAN, daily intake as high as 50 mg HCN equivalent from cassava derivatives (Osuntokun, 1981) had been reported. Cancer is rarely mentioned in the etiology of diseases linked to prolonged ingestion of cassava products.

The main biochemical process of cyanide metabolism leading to cellular injury is its participation in oxidative phosphorylation. The preponderance of free radicals in the catabolism of cyanogenic glucoside on the scale mentioned in these reports (Grindley *et al.*, 2002; Ragoobirsingh *et al.*, 1993) is, therefore, unlikely. The main limiting factor in diseases linked to high ingestion of cassava products are the deficiency in sulphur containing amino acid and, in some cases, certain vitamins like riboflavin (Oke, 1979, 1980; Osuntokun, 1981).

Resistance of tumor cells to a single anti-cancer agent and the subsequent resistance to other agents have been a bane in cancer chemotherapy (Burke *et al.*, 1996). Tumor resistance to the insult of the cyanide is unlikely because it acts by blocking uptake of oxygen to cells leading to hypoxia and eventual cell death (Balagopalan *et al.*, 1988).

The MTT dose response curves (Figures 2, 3) illustrate the cytotoxicity profiles of the experimental drugs on the two cell lines. These profiles exhibit a measure of sustained release over an appreciable period of drug exposure. Such features illustrate a good therapeutic index and a likelihood of minimal side effect. The mechanism of action of these experimental drugs may start with the initiation of a series of cellular processes, leading to inhibition of growth, followed by the induction of cell death (Saleem *et al.*, 2002). Depending on the intensity of the injury, a cell can

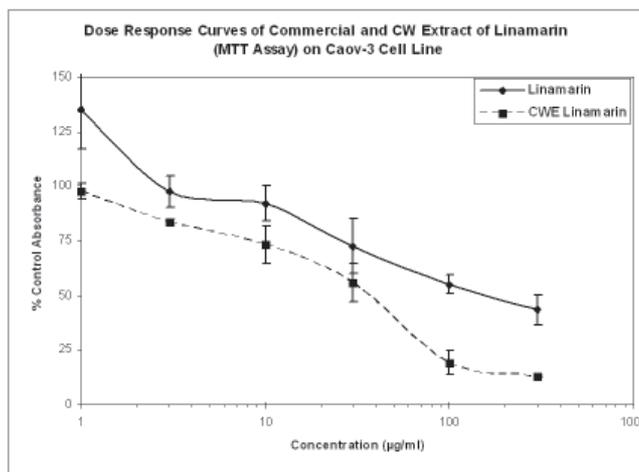


Figure 2. Dose response curves of the MTT assay in the ovarian cell line using crude water extract from cassava leaf and commercial linamarin as the experimental drugs. The y - axis depicts the optical density as a percentage control of the absorbance. The IC₅₀ values are given in Table 1.

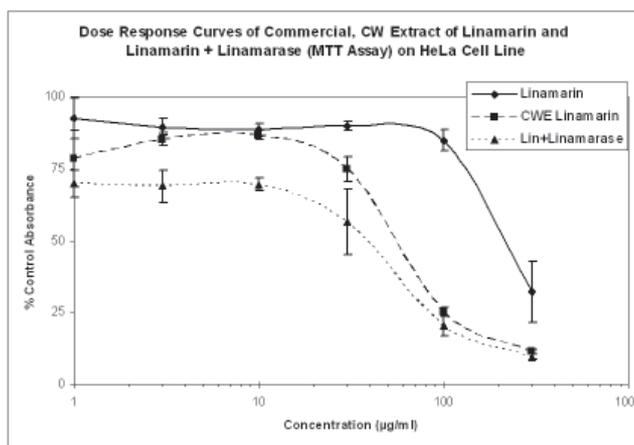


Figure 3. Dose response curves of the MTT assay in the cervical cell line using crude water extract from cassava leaf, commercial linamarin and linamarin + linamarase as the experimental drugs. The y - axis depicts the optical density as a percentage control of the absorbance. The IC₅₀ values are given in Table 1.

die by necrosis or apoptosis (Renvoize *et al.*, 1998).

As a follow up to this study, the experimental drugs will be delivered to tumors using nanoparticles of biodegradable polymers in a system called PDPT (polymer directed prodrug therapy) or if a combination therapy is desired, PDEPT (polymer directed enzyme prodrug therapy).

In the PDPT system, the polymer containing the prodrug will be administered, for example, intravenously. The polymer will circulate and will eventually be trapped in the tumor tissue through the EPR effect. Biodegradation, controlled release of the prodrug and eventual activation will lead to the environment of the tumor being toxic. On the other hand, PDEPT will be a two-step anti-tumor

approach in which the encapsulated plant derived linamarase enzyme is sent after the prodrug to mediate its activation (Fainaro *et al.*, 2002). The activation of the polymer-carrying drug at the tumor site, specifically, should result in the malignant cells being killed without the systemic toxicity often seen in chemotherapy. Additionally, since the EPR effect occurs only at the tumor site, minimal systemic toxicity is achieved even if a human homologue of the linamarase enzyme with similar activity is present in other tissues, hence, there is less chance of non-specific prodrug activation. The cyanide that diffuses to healthy neighbouring tissue is likely to cause less harm because their level should be below the threshold.

Genetic engineering could be quite cumbersome; inserting a linamarase gene into a retrovirus or fabricating a fusion protein incorporating an antibody that is specific for the enzyme linamarase and certain antigen portends that this therapy could be costly. Polymeric drug carrying nanoparticles on the other hand, are easier to prepare, have versatile route of administration, and would be convenient for the patient. It, therefore, represents a strategy for making cheaper, faster and more effective drug delivery.

Cyanide has a well-documented metabolic pathway and toxicokinetics in humans and animals (Oke, 1979, 1980). The lethal dose range for humans of HCN taken by mouth is given as 0.5-3.5-mg/kg-body weight (Bradbury *et al.*, 1988), which is between 500 to 1000mg linamarin (Cortes *et al.*, 1998). The IC_{50} values obtained in this work using pure linamarin alone or in combination with exogenous linamarase are far below the lethal dose.

The ovarian and cervical cancer cell lines are mere prototype in this study and more cell lines are being studied and their mode of cell death is also being investigated. The fact that the cause of cell death is at the level of the mitochondria poses an intrigue, because this organelle plays an important role in the cell death machinery.

Linamarin has the potential of a better drug compared to both tamoxifen and etoposide because of the diffusivity of its toxic metabolite and the ability of healthy surrounding cells to detoxify

small doses of it. Carefully designed, therefore, linamarin can be used to inflict its redox insult in such a manner that tumor cells are systematically eliminated by their own default process without disturbing the homeostatic balance of the healthy surrounding tissue.

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