THE CYTOTOXIC EFFECT OF *Dioscorea hispida* ROOT EXTRACT ON CULTURED LEUKEMIC BLOOD CELLS

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ABSTRACT

An aqueous crude extract of *Dioscorea hispida* root was tested for its cytotoxic effect on cultured leukemic and normal lymphocytes. The cultured cells were exposed to tenfold serially diluted *D. hispida* extract prepared according to volume of the extract (µl) per volume of the solvent (ml) ratio. The set concentrations were: 111.11 µl/ml (10%), 250 µl/ml (20%), 428.57 µl/ml (30%), 666.67 µl/ml (40%), 1000 µl/ml (50%), 1500 µl/ml (60%), 2333.33 µl/ml (70%), 4000 µl/ml (80%), 9000 µl/ml (90%) and 500 µl pure extract (100%). Qualitative phytochemical analysis revealed the presence of alkaloid and saponin compounds. These substances may have caused the low percent survival of the cultured leukemic and normal blood cells *in vitro*.

INTRODUCTION

A. Background of the study

The plant *Dioscorea hispida* Dennst, locally known as *nami, bagai, gagos, kalut, karot, karoti, kayos, kalot, korot, kulot, mamo* or *orkot*, belongs to the largest genus *Dioscorea* of the yam family Dioscoreacea (Quisumbing, 1978). It is a spiny twiner with small flowers and hairy trifoliate leaves. It produces big, bunched, fleshy yellow roots covered with fine wiry smaller roots. The roots of *D. hispida* are commonly used as an anodyne and maturative for tumors and buboes and also against arthritic and rheumatic pains. They are also sources of edible food (Osagie, 1992). *D. hispida* contains the alkaloid dioscorine [1R-(1a,4a,5a)]-2,4'-dimethylspiro-[2-azabicyclo (2.2.2)octane-5,2'-(2H)-pyran]-6'-(3'H)-one (Nagata et al, 1999) and a steroid sapogenin (25R)-spirosten-3B,t4B-diol (Osagie, 1992). Sapogenin produces saponin in water (Ege, 1984). Studies regarding alkaloids and saponins have demonstrated that they are potent sources of anticarcinogenic agents. *D. hispida* root contains both alkaloids and saponin making it a possibly new source of anticarcinogenic agent. One way of assessing whether it is so is to test for its cytotoxic effects on cultured cancer cell lines.

B. Statement of the problem

Is *Dioscorea hispida* extract toxic to leukemic blood cells?

C. Hypotheses

Null: The crude extract of *Dioscorea hispida* root is not toxic to leukemic blood cells.

Alternative: The crude extract of *D. hispida* root is toxic to leukemic blood cells.

D. Objectives

The general objective of this study is to test for the cytotoxicity of aqueous crude extract of *Dioscorea hispida* root to the cultured leukemic blood cells *in vitro*.

Specific objectives:

1. To subject the crude extract from *Dioscorea hispida* root to qualitative phytochemical screening.
2. To test for the cytotoxicity of varying concentration of the *D. hispida* crude extract on leukemic and normal blood cells.
3. To estimate the 50% Lethal Concentration (LC_{50}) of *D. hispida* root extract on cultured leukemic and normal blood cells.

E. Significance of the study

New anticarcinogenic agents are needed due to the increasing resistance to currently used chemotherapeutic drugs. The study would determine a possible use of *Dioscorea hispida* as a new source of bioactive agents toxic to cancer cells. The cytotoxic assay would be performed on both leukemic and normal blood cells to test the toxicity and selectivity of the aqueous extract of *D. hispida* root.

F. Scope and Limitation

The study deals with the cytotoxicity of *Dioscorea hispida* root extract on cultured leukemic and normal blood cells. *D. hispida* root was mechanically extruded, resulting in an aqueous crude extract that can dissolve mostly polar compounds and some water-soluble organic, secondary metabolites. The extract was subjected to a qualitative phytochemical analysis limited only to a few compounds, with the revealed bioactive compounds not individually isolated. A cytotoxicity assay of the extract was performed specifically on cultured normal and leukemic lymphocytes with one set of replicates for each cell type.

REVIEW OF RELATED LITERATURE

A. Leukemia

Leukemia is the sixth leading cause of cancer deaths among men and the seventh leading cause of cancer deaths among women. Each year, a high number of new cases of leukemia are diagnosed and the disease causes an estimated 22,000 deaths in United States alone. It accounts for about one-third of all cancers in children under age 15.

The bone marrow is an important tissue in the generation of immunocompetent and peripheral blood cells. The precursors of the hematopoietic cells in the bone marrow undergo continuous proliferation and differentiation (de Cavanagh et al., 2002). According to Encyclopædia Britannica, Leukemia begins when an immature blood cell in the marrow, the progenitor cell, divides uncontrollably. Over time, the marrow becomes crowded with cancerous cells, all of them descendants of the first abnormal cell. The malignant cells may also accumulate in a patient's lymph nodes, spleen, and elsewhere. At the time of diagnosis, up to a trillion leukemic cells may be present in the body. The mass of leukemic cells in the marrow suppresses the production of healthy blood cells, giving rise to the symptoms typical of leukemia. Pale skin, fatigue, and shortness of breath are signs of anemia, a decrease in the concentration of red cells in the blood. Nosebleeds, gum bleeding, a tendency to bruise easily, and pinhead-sized red spots on the skin reflect the decrease in the concentration of platelets in the blood. A lack of functional white cells makes patients with leukemia prone to infection.

The leukemias are classified based on the lineage of blood cell that becomes cancerous, and how rapidly the disease progresses. Leukemia is classified as myelocytic or myelogenous if the malignant cells have descended from the progenitors of red cells, granulocytes, macrophages, or platelets. If the leukemic cells have descended from a lymphocyte precursor cell, the leukemia is referred to as lymphocytic.

Leukemia may also be classified to as acute or chronic, depending on patient's life expectancy. If the disease remains untreated, acute leukemias result in death in a span of week due to severe suppression of normal blood cell production while in chronic leukemia, a patient may survive for several years since the leukemic cells develop more slowly and the effect is less severe. Leukemic cells in chronic myelocytic leukemia can often complete their development and become functional cells; in chronic lymphocytic leukemia, leukemic lymphocytes do not function normally but usually does not inhibit normal cell development severely. Further categorization of acute myelocytic, chronic myelocytic, acute lympocytic and chronic lympocytic leukemia may based on the appearance of malignant cell, presence of characteristic molecule on their surface, like that of hairy-cell leukemia in chronic lympocytic leukemia, or the stage of development.

There are several known causes of certain types of leukemia that were identified. Intensive radiation exposure or moderately intense exposure for long periods increases the risk of acute and chronic myelocytic leukemia and acute lymphocytic leukemia. These were demonstrated among survivors of the atomic bomb detonations at Japan. Exposure to certain chemicals like benzene and chemotherapy drugs used to treat breast and ovarian cancer, lymphomas, and certain other cancers also increase a patient's risk of later developing acute myelocytic leukemia. Human T-cell leukemia viruses (HTLV) I and II, are known to cause T-cell leukemia, a very rare form of lymphocytic leukemia, in humans. According to de Cavanagh (2002), genetic factors, such as alteration in DNA structure can cause mutations that may be carcinogenic.

There are several treatments employed in leukemia. The use of radiation wherein collection of leukemic cells accumulating in different parts of the body is made to shrink. Stem cell transplantation is also performed particularly in young patients, very intensive total body radiation or very high dose of chemotherapy are used. These are designed to destroy all leukemic cells but may also result in destruction of blood forming system in the patient's bone marrow so there is a need for the infusion of healthy stem cells. Immunotherapy that uses monoclonal antibodies that are highly specific, targets the molecule on the surface leukemic cells.

Cancer prevention by phytochemicals obtained from vegetables, fruits, spices, teas, herbs and medicinal plants is an effective approach. Phytochemicals such as carotenoids, phenolic compounds and terpenoids obtained from vegetables, fruits, spices, teas, herbs and medicinal plans have been proven to suppress experimental carcinogenesis in various organs such as skin and colon. Palm fruit carotenes were found to show anti-tumor promoting activity in a two-stage carcinogenesis experiment in skin. The tumor initiation was done by applying 390 nmol of dimethylbenz [a]- antracene (DMBA) on the shaved back of mice followed by the application of tumor promoter 12-)-tetradecanoylphorbol-13-acetate (TPA) at dose of 1.6 nmol, painted at the back twice a week after initiation for 20 weeks. The control group of mice developed 97% skin tumors after 20 weeks of study while the mice treated with palm fruit carotene suppressed tumor formation and no skin tumors developed during the whole period of the experiment (Nishino et al., 2002).

Extracts from various kinds of tea like the Japanese green teas and Chinese green teas or their major polyphenols inhibited the development of lung neoplasms chemically induced in mice. Male mice were initiated with 4-nitroquinone 1-oxide (4NQO) and promoted with glycerol (100%) for 25 weeks. Green tea polyphenols (GTPs), 0.05% in drinking water, were applied during tumor promotion period, without GTPs, 92% of the mice developed tumors while 71% of the mice developed tumors when GTPs were applied (Nishino et al., 2002).

B. *Dioscorea hispida* as a possible source of anti carcinogenic agent

Dioscorea hispida contains similar chemical compounds like ß-carotene that may act as an anticarcinogenic agent (Osagie, 1992; Nakamura et al., (2000).

Dioscorea hispida Dennst belongs to the largest genus *Dioscorea* of the family Dioscoreacea. It is a spiny twiner with small white, green, or red flowers arranged in clusters or spikes and hairy trifoliate leaves. It produces big, bunched, fleshy yellow roots covered with fine wiry small roots (Quisumbing, 1978).

The roots of *D. hispida* are commonly used as an anodyne and maturative for tumors and buboes and also against arthritic and rheumatic pains (Quisumbing, 1978). *D. hispida* root is also a rich source of carbohydrates and is cooked like potatoes (Osagie, 1992). It is sliced, blanched, and exposed for several days in gently running water before cooking to remove the bitter taste and toxins (Science Educ. Center, 1971). The nutritional value of *D. hispdia* includes ascorbic acid, ß-carotene, alkaloids, steroids, glycosides and saponins (Osagie, 1992).

The natural combinations of the above-mentioned phytochemicals exert cancer-protective effects via a decrease in lipid peroxidation (De et al, 2000). Ascorbic acid, ß-carotene, and saponin were recently evaluated as potential chemopreventors on the basis of involvement of leukocyte-derived reactive oxygen in carcinogenesis due to their strong inhibition of oxidative reactions both *in vitro* and *in vivo* (Nakamura et al, 2000).

De and collaborators (2000) studied the protective role of bitter gourd (*Momordica charantia*) and tomato (*Lycopersicon esculentus*). Bitter gourd contains glycosides, saponins, alkaloids, reducing sugars, resins, phenolic constituents, fixed oil and free acids whereas tomato has the phytochemicals alpha and beta-carotenes and lycopene (De et al, 2000). Oral administration of bitter gourd and tomato juice to both normal and carcinogen-treated groups of mice reduced their lipid peroxidation. Reduction in lipid peroxidation resulted in decreased cellular macromolecule damages that enhance carcinogenesis. Hence, target of lipid peroxidation maybe a way of exhibiting anticarcinogenic properties.

A toxic alkaloid known as dioscorine was extracted from *D. hispida* root and administered to three types of animals (Leyva and Gutierrez, 1937). Dioscorine was administered using different methods: adding to water ingested by mosquito fish; oral administration and injection to two white mice; and injection to monkey. This resulted in the death of the laboratory animals. The mechanism of action on the nervous system at the ion-channel level of dioscorine was studied based on its modulation of the nicotinic acetylcholine receptor, AChR (Nagata et al, 1999). The study was based on the effects of dioscorine on ACh-induced whole cell currents and on single channel currents. The whole cell current made used of whole-cell patch clamp technique while the single channel used the cell attached variation of the patch clamp technique. Dioscorine suppressed the ACh-induced whole-cell currents and shortened the open time of single channels, suggesting interference to the action of nicotinic (AChR). Other alkaloids derived from plants exhibited anticarcinogenic effects. Examples of these are the vincristine alkaloid of *Catharanthus roseus*, used as anti-leukemic drugs; and beta solamarine—the steroidal alkaloid glycoside of *Solanum dulcamara* recognized as having anti-tumor action (Hoffmann, n.y.; Sneden, 2003).

A study by Ahn and collaborators (1997) on the inhibitory effect of saponins on adhesion of some solid tumor cells showed that extract from a *Dioscorea sp.* caused inhibition of cell line adhesion to plastic well surface by more than 50% as well as high cell death.

Though the insecticidal property of *D. hispida* is given more attention (Banaag et al, 1997; Banaag et al, 1998), an in-depth study of its nutritive properties might yield a more direct medicinal function. So far, much of its chemical make up points to its possibility as a chemopreventive /chemotherapeutic agent (Osagie, 1992; Nakamura et al, 2000; Nishino et al. 2000). Since cancer prevention may be effected by multiple mechanisms (De et al, 2000), *D. hispida* may contain a variety of potentially anticarcinogenic substances that may exert synergistic effects in preventing carcinogenesis.

MATERIALS AND METHODS

A. Preparation of *Dioscorea hispida* extract

A five-kilogram wild species of *Dioscorea hispida* root (Plate 1) was directly obtained from Mt. Palay-palay of Ternate, Cavite. A one-kilogram piece from the whole root was cut, peeled and washed. The peeled slices of the root were grated and squeezed through five layers of cheesecloth. The resulting 600 ml of aqueous extract was centrifuged for eight minutes at 1500 rpm at room temperature. The result was a 400 ml of extract designated as full (100%) concentration (Plate 2). Twenty milliliters (ml) of the supernatant was membrane-filtered through a pore size of 0.45 µm to minimize possible microbial growth. The membrane-filtered extract was diluted with distilled water according to the ratio: volume of the extract (µl)/volume of distilled water or solvent (ml), forming a 500 µl solution per concentration. The concentrations prepared were: 111.11 µl/ml, 250 µl/ml, 428.57 µl/ml, 666.67 µl/ml, 1000 µl/ml, 1500 µl/ml, 2333.33 µl/ml, 4000 µl/ml, 9000 µl/ml and 500 µl pure extract, representing 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%, respectively. These were used for cytotoxicity assay. About fifty milliliters of the supernatant was used for phytochemical screening. The remaining extract was refrigerated until needed.

B. Phytochemical screening (Ragragio and de los Reyes, 2001)

1.Test for glycosides

Two milliliters (ml) each of the *Dioscorea hispida* root crude extract was placed into two test tubes. One ml of 10% HCl was added to the first test tube and none for the second test tube. The test tubes were placed in boiling water bath for five minutes then cooled. The first solution was neutralized with anhydrous sodium carbonate until there was no effervescence, then one ml Fehling's solution was added. The two test tubes were re-heated in water bath for two minutes. Both solutions have brick red precipitate but no increase in the precipitate of the first solution indicated the absence of glycosides.

2.Test for alkaloids

One ml of *D. hispida* root crude extract was acidified with 1% HCl and added with a few drops of Wagner's reagent. The appearance of a white precipitate confirmed the presence of alkaloids.

3.Test for saponins

Two ml of *D. hispida* root extract was placed in a clean test tube and ten ml of distilled water was added. The solution was shook vigorously for 30 seconds, and then allowed to stand for a period of 30 minutes. The appearance and persistence of a three-centimeter or greater froth after 30 minutes above the surface of the liquid indicated the presence of saponin.

4. Test for tannins

A Few drops of ferric chloride and sodium carbonate were added to one ml of the *D. hispida* root crude extract. The lack of a blue-green to black precipitate indicated the absence of tannins.

5. Test for anthocyanins

One ml of 1% HCl was added to one ml of *D. hispida* root crude extract and boiled. Lack of an orange-red to blue-red coloration at boiling point indicated the absence of anthocyanins.

6. Test for flavonoids

One ml of 10% HCl and magnesium ribbon was added to one ml of *D. hispida* root crude extract. No change in coloration indicated the absence of flavonoids.

C. Cytotoxicity testing (Freshney, 1987; 2000)

The assay was performed at the Biological Research and Services Laboratory of Natural Science Research Institute (NSRI) at University of the Philippines, Diliman, Quezon City. The same institution provided the cultured blood cells (lymphocytes). Both cultured leukemic and normal blood cells were placed in a microtitration plate. Treatments for each blood cell type both consisted of one control well containing only the cultured blood cells and ten wells with the cultured cells added with *D. hispida* crude extracts serially diluted tenfold in distilled water ($V_{extract}$ in µl /$V_{distilled\ water\ or\ solvent}$ in ml). The extract concentrations were: 111.11 µl/ml (10%), 250 µl/ml (20%), 428.57 µl/ml

(30%), 666.67 μl/ml (40%), 1000 μl/ml (50%), 1500 μl/ml (60%), 2333.33 μl/ml (70%), 4000 μl/ml (80%), 9000 μl/ml (90%) and 500 μl pure extract (100%).

Leukemic and normal lymphocyte cultures were grown in one liter standard medium containing RPMI 1640,10% fetal bovine serum, 2 g sodium bicarbonate, and 1 ml penicillin-streptomycin. Normal and leukemic lymphocytes had an initial count of $5x10^4$ cells/μl and $1.3x10^5$ cells/μl, respectively. Each culture was treated with the designated concentration of *D. hispida* root extract for four hours. Non-treated cultures served as controls. The lymphocytes were incubated at 37^0C.

Each test sample was added with twenty microliters of Trypan blue dye. Within an hour after the addition of the dye, an aliquot of twenty microliter from each culture were placed under the microscope to observe cell viability using hemocytometer. Non-dyed cells indicated an approximate density of viable cells.

The percentage of leukemic and normal blood cell mortality against the concentration of the extract was plotted. The 50% Lethal Concentration (LC_{50}) is the drug concentration required to reduce the viable cells to half that of the initial number of cells. This was determined by converting the data into percent mortality of the cells and using linear regression equation to obtain accurate values.

RESULTS

A. Phytochemical screening

Extraction of *Dioscorea hispida* root resulted in 400 ml of slightly turbid aqueous extract. When the extract was phytochemically analyzed for alkaloids, it produced white precipitate that indicated the presence of the said compounds. It also had a persistent froth when tested for saponins, indicating the presence of saponins. In contrast, the extract tested negative for glycosides, tannins, anthocyanin and flavonoids. The extract had no increase in brick red precipitate for glycosides test; it produced no precipitate when tested for tannins; it had no orange-red to blue-red coloration at boiling point for anthocyanin test; it had no color change for flavonoids test, indicating the absence of these compounds (Table 1, Plate 3).

B. Cytotoxicity assay

In the control group, no cell mortality in normal and leukemic blood cells was observed (Table 2). There was no *Dioscorea hispida* extract added to it. At the initial concentration of 111.11 μl/ml (10%) of the extract, there was 20% normal blood cell mortality while leukemic blood cells had 15.39% mortality. At 250 μl/ml (20%) concentration of the extract, normal and leukemic blood cells had 60% and 54% cell death, respectively. Mortality of normal blood cells at 428.57 μl/ml (30%) and 666.67 μl/ml (40%) concentration of the extract were both 80%, whereas leukemic blood cells at the same concentrations both had 92.31%. There was 100% cell mortality at both normal and leukemic blood cells at concentrations: 1000 μl/ml (50%), 1500 μl/ml (60%), 2333.33 μl/ml (70%), 4000 μl/ml (80%), 9000 μl/ml (90%) and 500 μl (100%) pure extract. Percent mortality of cells in both normal and leukemic blood cells increased in proportion to the increased concentration of the *D. hispida* extract (Figure 1). However, a 100% mortality of all the cells in both normal and leukemic blood cell groups was observed at 1000 μl/ml (50%) concentration onwards.

C. 50% Lethal Concentration (IC_{50})

The 50% Lethal Concentration (LC_{50}) of normal blood cells occurred at 2.17 (log of extract concentration) that is equivalent to 147.48 μl/ml concentration, while leukemic blood cells had LC_{50} at 2.14 equivalent to 137.39 μl/ml according to the plotted linear regression lines (Figure 2). Both LC_{50} occurred between 111.11 μl/ml and 250 μl/ml concentration.

DISCUSSION

In this study, the extract used was aqueous, where most of the compounds dissolved are polar and/or water-soluble organic, secondary metabolites. Also, the alkaloid and saponin contents of *D. hispida* root were not individually isolated in the extraction. The combination of these two toxic compounds and other unidentified chemical components may have been responsible for the cytotoxicity of the *D. hispida* root extract to both cultured leukemic and normal blood cells.

The presence of alkaloid in the *D. hispida* root extract most likely contributed to the extract's toxicity to the cultured leukemic blood cells. Alkaloids are highly poisonous compounds that could cause physiological activities such as pupil dilation and vasoconstriction (Harrow and Apfelbaum, 1945; Richards, *et. al.*, 1967). Leyva and Gutierrez (1937) concluded in their study that the *D. hispida* root alkaloid, dioscorine, is highly toxic to mosquito fish, white mice and monkeys. Administration of 2-50 mg of extracted dioscorine from *D. hispida* root to these laboratory animals resulted in death after: 3 hours for the fish; 10-15 minutes for the white mice; and 30 minutes for the monkey. Based on their results, the computed lethal dose for a 50 kg man was 2.5-3 g of dioscorine with death after about four hours.

D. hispida root also contains sapogenin, which are toxic compounds that can produce saponin in water (Ege, 1984; Osagie, 1992). The saponin present in *D. hispida* root extract may be responsible for the cytotoxicity of the *D. hispida* root extract to the cultured blood cells. Ahn and his collaborators (1997) screened the saponin extract of 232 herbal plants for their capability to inhibit several cancer cell lines attached to the plastic well surface. Only six plants showed anti-cell adhesive activity by more than 50%, among these is a *Dioscorea* species, *D. tokoro*. This species is one of the three plants found to be cytotoxic to the cancer cell lines.

The assay confirmed the cytotoxicity of *Dioscorea hispida* root extract both on cultured normal and leukemic blood cells. The control/untreated leukemic and normal blood cells increased in number compared with their respective initial number of cells. But when added with the extract, both cell types had immediate cell death even at the lowest allotted concentration of 111.11 µl/ml (10%). 50% of cell mortality occurred in between concentrations 111.11 µl/ml and 250 µl/ml for both cell types, 147.48 µl/ml for normal blood cells and 137.39 µl/ml for leukemic blood cells. This implies that at 137.39 µl/ml concentration, half of the leukemic blood cells would be killed, whereas it would take an increase to 147.48 µl/ml concentration before 50% of the normal blood cells are killed. Thus, using 137.39 µl/ml concentration would harm more leukemic blood cells than normal blood cells, indicating selectivity of the extract at this concentration. Yet using concentrations higher than this would be detrimental to both leukemic and normal blood cells.

CONCLUSION

Cytotoxicity of *Dioscorea hispida* extract at the starting concentration of 111.11 μl/ml may be attributed to the presence of alkaloids and saponins. It could be inferred from the value of the 50% Lethal Concentration (LC_{50}), where leukemic blood cells had lower LC_{50} than normal blood cells, that the extract had a beneficial selectivity at 137.39 μl/ml concentration. Hence, using this concentration could cause higher leukemic blood cell mortality and less normal blood cell death *in vitro*. Concentration higher than the LC_{50} of leukemic blood cell would be detrimental to both leukemic and normal blood cells.

RECOMMENDATION

It is advisable for future researchers of the cytotoxicity of *Dioscorea hispida* to extract the organic compounds using other methods of extraction and to phytochemically analyze more compounds. When performing the cytotoxicity assay, it would be better to administer a set of concentration between 111.11 µl/ml (10%) and 250 µl/ml (20%) of *D. hispida* root extract in accordance to the 50% Lethal Concentration (LC_{50}). Adding more sets of replicates could strongly establish obtained data. Isolating the individual bioactive components of the *D. hispida* extract could help point the main cause of its toxicity to normal and leukemic blood cells. Applying the cytotoxicity test of the extract to other types of cancer cells is recommended.

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TABLES

TABLE 1. Qualitative test of *Dioscorea hispida* root crude extract

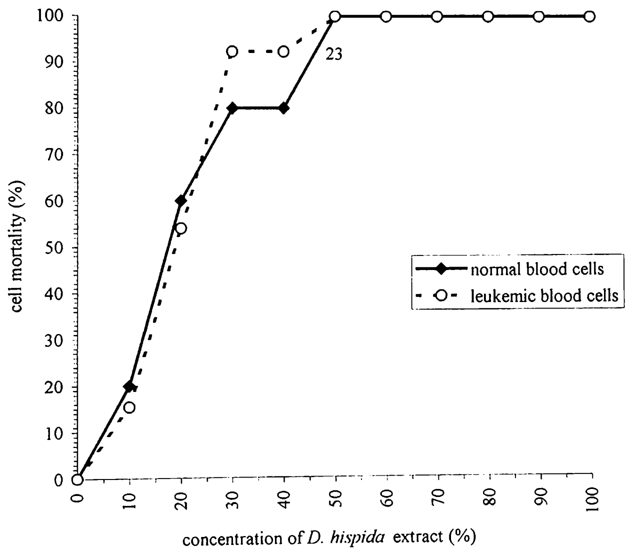
Chemical Compounds	**Reaction**
Glycosides	-*
Alkaloids	+**
Tannins	-
Saponins	+
Anthocyanins	-
Flavonoids	-

* (-) absent
** (+) present

TABLE 2. Cytotoxicity of *Dioscorea hispida* root crude extract on leukemia lymphocytes

Concentration of *Dioscorea hispida* root extract		**Cell Mortality (%)**	
%	µl /ml	**Normal Blood Cells**	**Leukemic Blood Cells**
0	Negative Control	0	0
10	111.11	20	15.39
20	250	60	54
30	428.57	80	92.31
40	666.67	80	92.31
50	1000	100	100
60	1500	100	100
70	2333.33	100	100
80	4000	100	100
90	9000	100	100
100	500 µl pure extract	100	100

FIGURES



FIGUIRE 1. Comparison of normal and leukemic blood cell mortality plotted against the concentration of *Dioscorea hispida* root extract

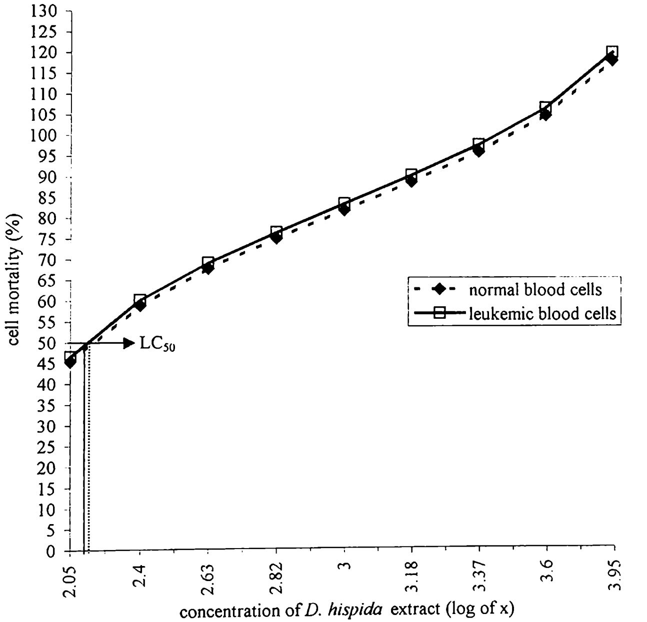


FIGURE 2. Estimation of 50% Lethal Concentration (LC_{50}) through linear regression

PLATES

PLATE 1. *Dioscorea hispida* root

PLATE 2. 100% concentration of *D. hispida* root extract

PLATE 3. Results of phytochemical analysis

APPENDICES

BIOLOGICAL RESEARCH AND SERVICES LABORATORY
Natural Sciences and Research Institute
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Date: Jan. 15, 2003

Cytotoxicity Analysis

Sample/s: Nami root extract

Changes in cell density denote changes in cell proliferation. A positive change corresponds to cell proliferation while a negative change denotes cell death. Trypan Blue exclusion test was used to determine cell viability. The Trypan Blue exclusion test relies on the ability of viable cells to prevent the permeation of the dye into their cytoplasm.

Methodology:

Lymphocytes were isolated using Histopaque technique. Leukemic and normal lymphocyte cultures were grown in standard media (RPMI 1640 + 10% fetal bovine serum + 2g sodium bicarbonate + 1ml penicillin-streptomycin = 1 L solution of media). Each type of culture was treated with varying concentrations of the above sample (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%). Non-treated cultures served as negative controls. The lymphocyte cultures were incubated at 37°C. Twenty (20) microliter aliquots from the maintained leukemic and normal lymphocyte cultures were acquired 4 hours from the time of initiation and observed under the microscope for changes in cell viability. Observations were made using a hemocytometer within an hour after the addition of twenty (20) microliters of Trypan blue for each test sample. The number of non-dyed cells observed in the hemocytometer is an approximation of the density of viable cells maintained in culture.

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(a)

(b)

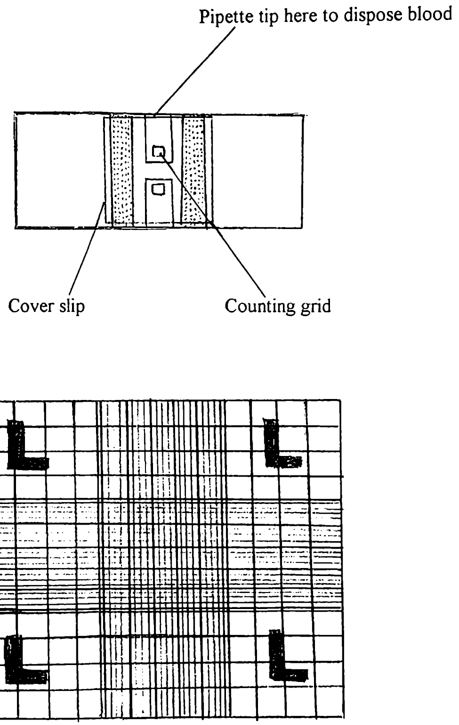

Illustration of: (a) the hemocytometer for blood cell counting. (b) Magnified view of the counting grid for lymphocytes (indicated by **L**). The number of cells is counted in each of the four large squares at the corners of the ruled area, averaged, and multiplied by 200.

BIOLOGICAL RESEARCH AND SERVICES LABORATORY
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Quezon City, Philippines 1101

Nov. 11, 2002

Here are the data on the cytotoxicity assays performed on leukemic and normal lymphocyte cultures dated October 25, 2002 using varying concentration of extracts given by Ms. King and Ms. Ureta.

Table 1. Cytotoxicity Test of Varying Concentration of Extracts on Leukemic and Normal Lymphocytes (Total # of viable cells = $n \times 5 \times 10^4 \times DF$; where DF = 4 for all)

Compound	n (CENTRAL SQUARE)	
	Normal (initial n = 5)	Leukemic (initial n= 13)
Negative control	9	27
10% extract	4	11
20% extract	2	6
30% extract	1	1
40% extract	1	1
50% extract	0 (all blue)	0 (all blue)
60% extract	0 (all blue)	0 (all blue)
70% extract	0 (all blue)	0 (all blue)
80% extract	0 (all blue)	0 (all blue)
90% extract	0 (all blue)	0 (all blue)
100% extract	0 (all blue)	0 (all blue)

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Linear Regression

A. Normal Blood Cell

log x	M (y)	xy	x2	y2			
2.05	20	41	4.2025	400		SP	111
2.4	60	144	5.76	3600		SSx	2.8636
2.63	80	210.4	6.9169	6400			
2.82	80	225.6	7.9524	6400		b	38.7624
3	100	300	9	10000		a	-34.065
3.18	100	318	10.1124	10000			
3.37	100	337	11.3569	10000		**LE**	**y= 38.7624x-34.065**
3.6	100	360	12.96	10000		**LC_{50}**	**50=38.7624x-34.065**
3.95	100	395	15.6025	10000			**=147.48**
27	740	2331	83.8636	66800			
3	82.22222						

x = extract concentration (µl/ml)
y = cell mortality (%)
LE = Linear Equation
LC_{50} = 50% Lethal Concentration

B. Leukemic Blood Cell

log x	M (y)	xy	x2	y2			
2.05	15.39	31.5495	4.2025	236.8521		SP	112.209
2.4	54	129.6	5.76	2916		SSx	2.8636
2.63	92.31	242.7753	6.9169	8521.136			
2.82	92.31	260.3142	7.9524	8521.136		b	39.18459
3	100	300	9	10000		a	-33.7749
3.18	100	318	10.1124	10000			
3.37	100	337	11.3569	10000		**LE**	**y=39.18459x-33.7749**
3.6	100	360	12.96	10000		**LC_{50}**	**50=39.18459x-33.7749**
3.95	100	395	15.6025	10000			**=137.39**
27	754.01	2374.239	83.8636	70195.12			
3	83.77889						