Anti-Proliferative Effects of Plant Extracts from Zimbabwean Medicinal Plants against Human Leukaemia Cell Lines

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ABSTRACT

A selected group of 14 medicinal plants was screened for antiproliferative activity against two human leukaemia cell lines Jurkat T and Wil 2 in vitro. The Trypan Blue assay was used to assess antiproliferative and cytotoxic activity. The five most potent medicinal plants showed the following order of potency against Wil 2 cell line: Parinari curatellifolia > Aloe barbadensis > Croton gratissimus > Syzygium guineense > Vernonia adoensis with IG50s of 93, 115, 149.8 and 130 μg/ml, respectively. The plants had comparable proliferation inhibition to the cancer drug doxorubicin. From these species, high levels of cytotoxicity were detected in extracts from Parinari curatellifolia and Aloe barbadensis to Wil 2 cell line at concentrations of 500 and 1 000 μg/ml. Croton gratissimus, Syzygium guineense and Vernonia adoensis extracts were found to be antiproliferative and not cytotoxic at the same concentrations. P. curatellifolia extract at a concentration of 10 μg/ml reduced cell proliferation of Jurkat T cells by 70% after 48 h of incubation. Studies were also carried out where the extract from P. curatellifolia combined with doxorubicin at concentrations 10 to 0.50 μg/ml were tested for antiproliferative activity against a Jurkat T cell line. The top five plant extracts had IG50s of less than 150 μg/ml. The results show that plants used traditionally for treatment of diseases such as tuberculosis, mycosis and diarrhea can be used to inhibit cell proliferation in leukaemia cell lines. The extract from P. curatellifolia could be a potential source of lead compounds that may be used as anticancer drugs. The plant extracts that showed cytotoxicity and inhibition of cell growth will be further characterized to identify the active fractions and compounds.

Keywords: anticancer, Jurkat T cells, Parinari curatellifolia, proliferation, Wil 2 cells, Trypan blue
Abbreviations: ATCC, American type culture collection; DMSO, dimethyl sulphoxide FBS, foetal bovine serum; IG50, growth inhibition that bring about 50% reduction in the number of cells RPMI, Roswell Park Memorial Institute

INTRODUCTION

Leukaemia is a cancer of the blood-forming organs where white blood cells are created in very high numbers at the expense of other blood cells (Ozawa et al. 1984; Yan et al. 2011). The blood eventually becomes constituted of inadequate levels of cells such as red blood cells and platelets (Szczylwik et al. 1991). The body will have no ability to transport oxygen and nutrients around the body. Acute leukaemia is when the cancer causes the blood forming organs to produce white blood cells that are not fully differentiated and is fatal unless treatment is commenced upon diagnosis because the patient lacks functional white blood cells (Ross et al. 1994). This type of leukaemia occurs in children, especially infants. Chronic leukaemia mostly occurs in adults and in this case the blood forming organs retain their ability to over-produce fully differentiated cells despite the cancer (Ross et al. 1994). Leukaemia risk is reported to be increased for subjects with greater than 16 parts per million (ppm) cumulative exposure a year or with greater than 8 ppm intensity of benzene as shown by (Infante et al. 2006). There have been 415 reported cases of leukaemia recorded in Zimbabwe from a population of 12 million people. Zimbabwe has the 4th highest number of leukaemia cases in the southern Africa region (Chokunonga et al. 2005).

The current treatment of leukaemia is anticancer drug-based chemotherapy, biological therapy and bone marrow stem cell transplants (Liu et al. 2004). Chemotherapy uses one or more drugs to destroy cancer cells, and is often accompanied by the development of drug resistance and severe side effects (Yan et al. 2011). Therefore, it becomes imperative to develop other potential therapeutic agents for the treatment of the disease. One approach to discover novel lead compounds against cancer is the consideration of ancient ethno-medicinal knowledge and the investigation and screening of locally available natural resources (Özmen et al. 2010). Doxorubicin, vinblastine, vincristine and cis-diamminedichloroplatinum(II) (CDDP) represent some of the current standard chemotherapeutic drugs used in the treatment of solid and blood cancers (Glass et al. 2003). The drugs vinblastine and vincristine have been isolated from Catharanthus roseus (rose periwinkle), a wild plant native to Madagascar (Gaines 2004). The identification of plant compounds with anticancer properties is vital for cancer research. Although there are many therapeutic strategies to treat leukaemia, high systemic toxicity and drug resistance limit successful outcomes in most cases (Liu et al. 2004). Accordingly, searches have intensified to find novel compounds for leukaemia drug development. In drug discovery or drug assessment using cell lines, researchers aim to find compounds that lead to the triggering of apoptosis in diseased cells such as cancer or HIV infected cells (Klou et al. 2009). A candidate drug is, therefore, introduced to the cells and its effects ascertained. The most favourable is a compound that is potent at low concentrations and discriminates between diseased and normal cells (Cochrane et al. 2008).

The combination of phytochemicals with chemotherapeutic drugs which enhance drug efficacy while reducing toxicity to normal tissues could be one such approach to cancer treatment (Li et al. 2009). In addition, it has also been suggested that the combined effect of natural products may improve the treatment of proliferating cancer cells (Chen et al. 2009). Several herbs and plants with diver-
**Table 1 Plants that were used in this study, their ethnobotanical uses in Zimbabwe and other countries.**

<table>
<thead>
<tr>
<th>Family</th>
<th>Plant name and Authority</th>
<th>Vernacular name</th>
<th>Voucher number</th>
<th>Traditional medicinal uses of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabaceae</td>
<td>Cajanus cajan (Druce)</td>
<td></td>
<td>N8E7</td>
<td>Stomach ailments (Iwawela et al. 2007).</td>
</tr>
<tr>
<td>Myrtaceae</td>
<td>Callicton citrinus (Curtis Skeels)</td>
<td></td>
<td>U2Z9E7</td>
<td>Hemorrhoid treatment (Oyedeji et al. 2009).</td>
</tr>
<tr>
<td>Combretaceae</td>
<td>Terminalia prunoides (Lawson)</td>
<td>Mediziyave</td>
<td>N6E7</td>
<td>Diarrhea (Ruffo et al. 1991).</td>
</tr>
<tr>
<td>Asphodelaceae</td>
<td>Aloe barbadensis (Mill.)</td>
<td>Gavakava</td>
<td>N11E7</td>
<td>Sap is used to treat skin rashes and the leaves are prepared and used to treat tuberculosis (Chokunonga et al. 2004).</td>
</tr>
<tr>
<td>Combretaceae</td>
<td>Combretum apiculatum (L.)</td>
<td>Muruka</td>
<td>C9E7</td>
<td>Coughs, diarrhoea, snake bites stomach ache (Ruffo et al. 1991).</td>
</tr>
<tr>
<td>Araliaceae</td>
<td>Cassonia natalensis (Sond )</td>
<td>Mutolwi</td>
<td>U29Z9E7</td>
<td>Diarrhea (Ruffo et al. 1991).</td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td>Croton gratissimus (Burch)</td>
<td>Gunakira</td>
<td>U21Z3E7</td>
<td>Malaria, rabies, gonorrhea, wounds, Ascariasis, internal worms (Iwawela et al. 2007).</td>
</tr>
<tr>
<td>Chrysobalanaceae</td>
<td>Parinari curatellifolia (Planch ex Benth)</td>
<td>Muhachha</td>
<td>C6E7</td>
<td>Facilitates conception in women (Chigora et al. 2007).</td>
</tr>
<tr>
<td>Myrtaceae</td>
<td>Syzygium guineense (Will D.C)</td>
<td>Mokate</td>
<td>C12E7</td>
<td>Tuberculosis, fevers (Chigora et al. 2007).</td>
</tr>
<tr>
<td>Anacardiaceae</td>
<td>Rhus lancea (Barköy)</td>
<td>Muchokochiana</td>
<td>C11E7</td>
<td>Stomach ailments, fevers (Chokunonga et al. 2004).</td>
</tr>
<tr>
<td>Fabaceae</td>
<td>Xeroderris stuhlmannii (Mend)</td>
<td>Morumanya</td>
<td>C4E7</td>
<td>Stomach ailments (Iwawela et al. 2007).</td>
</tr>
<tr>
<td>Asteraceae</td>
<td>Vernonia adenos (Bip ex Walp)</td>
<td>Musikavakadzi</td>
<td>C1E7</td>
<td>Induce birth or carry out abortions (Iwawela et al. 2007).</td>
</tr>
</tbody>
</table>

sified pharmacological properties are known to be rich sources of chemical constituents that may have potential for the prevention and/or treatment of several human cancers (Mesquita et al. 2009). Humans have used plant products for centuries as spices and flavourings with no observed side effects (Iwawela et al. 2007). In recent years humans have incorporated the use of herbs and plant products in disease treatment (Iwawela et al. 2007). This mixture of some herbal medicines with conventional medicines tends to have negative results. Any potential drug must, therefore, be preclinically assessed for its interaction with the conventional medicine of that field (Szliska et al. 2008).

Natural plant product extracts have been identified as possible anticancer agents as they exhibit antimutagenic and antiproliferative characteristics (Pinmai et al. 2008). Most (70%) of all present antileukaemia drugs have been derived from plant compounds or their derivatives (Pujol et al. 2007). In order to find alternative ways to treat cancers, there is a need to evaluate plants with ethno-medicinal history and to examine the mechanisms responsible for the anticancer effects of plant-based drugs (Leong et al. 2011). The present study, therefore, was aimed at investigating the effect of 14 Zimbabwean medicinal plants on leukaemia cell lines Wil2 and Jurkat T-cells. This study was carried out in order to identify novel therapeutic compounds for possible leukaemia drug discovery and development.

**MATERIALS AND METHODS**

**Cells and reagents**

Two human cell lines were used for antiproliferative screening. ECACC strain Jurkat E6 (T-cell lymphocytic cell line) cells purchased from Sigma Aldrich, Germany and an ATCC strain Wil 2 (B cell lymphocytic cell line) was a kind gift from Professor Mamaru from the University of Limpopo, South Africa. The 14 plants listed in Table 1 were collected from three provinces in Zimbabwe namely, Mashonaland West, Mashonaland Central and Metropolitan and identified by a taxonomist, Mr Chris Chapano, of the National Botanical Garden of Zimbabwe. Voucher specimens were deposited at the Biochemistry Department at the University of Zimbabwe. Doxorubicin, RPMI 1640 Medium, penicillin streptomycin, dimethyl sulfoxide, methanol and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

**Cell culture**

Cell lines Wil 2 and Jurkat T were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin cocktail. Cells were inspected and counted using a haemocytometer daily for growth assessment and contamination checks. Cells were incubated in a 2400 Shel Lab carbon dioxide incubator ( Sheldon Mfg., Cornellius, USA) with 5% carbon dioxide at 37°C. The trypan blue dye exclusion assay was used to monitor cell viability. Cells that absorbed the dye and appeared blue under an inverted microscope were considered dead (Masters 2000).

**Preparation of methanolic extracts**

The samples were separated according to the part of the plant such as leaves, roots stem and bark. Samples were separately dried in an oven at 50°C and ground to a fine powder using a two-step electric blender (Cole Parmer Instrument Co., Vernon Hills, USA). The ground powder was placed in a 200-ml flask containing 100 ml of methanol. The flask was left shaking overnight in an incubator at 100 rpm for maximum extraction to occur (Cochrane et al. 2008). The sample was then filtered through Whatman 2® paper (Sigma Aldrich, Taufkirken, Germany). The filtrate was then filtered until only sludge remained. The sludge was weighed and then re-dissolved in 10 ml of DMSO and the concentration noted. The extract was then stored at -20°C until required.

**Antiproliferative and cytotoxic assays in vitro**

Wil2 and Jurkat T cells were seeded in 6 well COSTAR® culture plates, Cornell, St Louis, USA) at a density of 500,000 cells/well. The methanolic extracts were then dissolved in DMSO to the concentrations of 0, 50, 100, 250, 500 and 1000 μg/ml. Doxorubicin as the positive control was used at 10 μg/ml. The various concentrations were added to the culture wells in triplicate. Samples were collected at 1, 3 and 6 h and viable cells counted using the trypan blue dye exclusion assay. Plant extract effectiveness was assessed by the IG₅₀ to determine the concentration for 50% growth inhibition. These are interpolated values from the dose-response graphs. Preliminary anticancer screening results showed that the extract from Parinari curatellifolia had the highest antiproliferative activity at a concentration of 50 μg/ml. Further work was then carried out to elucidate the lowest concentration of this extract that could be potent against the leukaemia cell lines. Methanolic extracts from P. curatellifolia were dissolved in DMSO into test concentrations ranging from 0.50 to 10 μg/ml. The different concentrations of extracts from P. curatellifolia were screened for their ability to inhibit Jurkat T and Wil2 cell growth as described above. The positive control was doxorubicin used at concentrations of 0, 0.5, 1.25 and 10 μg/ml. The Trypan blue dye exclusion assay was used for counting viable cells (Naranayan et al. 2005). The response parameter IG₅₀ was calculated to determine the concentration for 50% growth inhibition of tumor cells.

**Drug and plant extract combinations**

Once cell population reached 2 × 10⁶ cells/ml, cells were seeded in 6-well COSTAR culture plates (COSTAR, St Louis, USA). The
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Fig. 1 Wil 2 antiproliferation activity of plant extracts. Parinari curatellifolia (A), Aloe barbadensis (B), Syzygium guineense (C), Croton gratissimus (D) and Vernonia adoensis (E) on Wil2 cells over a period of 24 h (A, B, C, D and E respectively). Statistics: t-test, * P < 0.05, ** P < 0.01, *** P < 0.001. Readings were carried out in triplicate and each point represents the mean ± standard deviation.

Table 2 The effects of combining Parinari curatellifolia extract and doxorubicin on Jurkat T cells.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Index</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.00</td>
<td>Synergism</td>
</tr>
<tr>
<td>1.25</td>
<td>0.60</td>
<td>No interaction</td>
</tr>
<tr>
<td>1.00</td>
<td>0.54</td>
<td>No interaction</td>
</tr>
<tr>
<td>0.50</td>
<td>0.60</td>
<td>No interaction</td>
</tr>
</tbody>
</table>

Key: values < 0.5 synergism, > 0.5 to 1.0 no interaction and 1.0 to 4.0 antagonism (Zhao et al. 2004).

RESULTS

Antiproliferative activity in vitro

Of the 14 plant extracts screened, 5 were found to have the highest proliferation inhibition activity. The criterion for potency was considered as the lowest extract concentration that could inhibit proliferation by at least 50% in the shortest period of time. Upon observing that this criterion was met after 3 h of incubation as shown in Fig. 1, the cut-off period was then set at 3 h. The 5 extracts where found to be most cytotoxic to Wil 2 cells inhibiting proliferation by 100% as drug, doxorubicin and the extract from P. curatellifolia were added to each flask at 10, 1.25, 1, 0.5 and 0.5 μg/ml and placed in the incubator for 48 h. The cells were then counted using the Trypan blue dye exclusion assay. The combination index (CI) was then calculated using the formula:

CI = Inhibition of plant extract + doxorubicin x 100 %

Inhibition of doxorubicin

The CI values were then interpreted as follows: < 0.5: synergism; > 0.5 to 1.0: no interaction; and 1.0 to 4.0: antagonism (Zhao et al. 2004).

Statistical analysis

Linear regression analyses were used to determine the IG50 of the plant extracts, the concentration that inhibits cell proliferation by half. Numerical data for treatment of cells were analysed using the Student’s t-test using Graphpad™ version 4 for Windows (Graphpad™ Software Inc., San Diego, California, USA). P values of 0.05 or less were reported as significant.
shown in Fig. 3A and 3B. The extracts from C. gratissimus, S. guineense and V. adoens inhibited Wil 2 cell proliferation by 96, 59 and 51% at 1000, 200 and 50 μg/ml, respectively. Extracts from C. gratissimus, S. guineense and V. adoens exhibited a dose- and time-dependent effect on proliferation of the Wil 2 leukaemia cell lines with IG50s of 148, 149 and 130 μg/ml, respectively, as shown in Fig. 3C-E and Table 2.

The effect of plant extracts was also investigated on Jurkat T cells. Lower concentrations of P. curatellifolia extract inhibited proliferation of Jurkat T cells after 24 h of incubation as shown in Fig. 4. Concentrations of P. curatellifolia extract below 50 μg/ml exhibited potency after longer periods of incubation as compared to the effects seen with the high concentrations as shown in Fig. 4. The extract from C. cajan was not effective in terms of inhibiting Jurkat T cells. Lower concentrations of the extract below 50 μg/ml exhibited proliferation of the Jurkat T cells. Higher concentrations of 1000 μg/ml after 24 h of incubation.

Fig. 2 IG50s of extracts from Parinari curatellifolia, Aloe barbadensis, Syzigium guineense, Croton gratissimus and Vernonia adoens.

Fig. 3 The antiproliferative and cytotoxic effect of extracts from Parinari curatellifolia and Aloe barbadensis on Wil 2 cells in comparison to anticancer drug doxorubicin (A, B respectively). The antiproliferative effect of Croton gratissimus, Syzigium guineense and Vernonia adoens on Wil 2 cells (C, D and E, respectively). Statistics: t-test, * P < 0.05, ** P < 0.01, *** P < 0.001. Number of readings (n = 3) and each point represents the mean ± standard deviation.

Fig. 4 The effect of fixed concentrations of Parinari curatellifolia extract and Doxorubicin on Jurkat T cells after 24 h of incubation. Each reading was taken three times: (n = 3) and each point is the mean ± standard deviation.

Fig. 5 The effect of plant extract on Jurkat T cell viability and antiproliferative effect of extracts from Cajanus cajan and Euphorbia tiracalli on A, B and C respectively. Statistics: t-test, * P < 0.05, ** P < 0.01, *** P < 0.001. Number of readings (n = 3) and each point represents the mean ± standard deviation.
doxorubicin alone as shown in Fig. 4. The extract from *P. curatellifolia* was not as effective at inhibiting proliferation at low concentrations of 10, 1.25, 1.00 and 0.50 μg/ml as the effectiveness of doxorubicin at the same concentrations.

**In vitro combination assay**

Synergism was observed at concentrations of 10 μg/ml of *P. curatellifolia* and doxorubicin while the other concentrations showed no interaction in terms of proliferation inhibition.

**DISCUSSION**

Ethnobotanical knowledge is an important tool in drug discovery and development (Roberson 2008). For example, although the lower concentrations inhibited proliferation, the effects of these compounds may explain the lethality observed in this study. Diterpenoids have been shown to inhibit cell cycle progression from the stem bark have been shown to have moderate activity against ovarian cancer cell lines (Khalafalla et al. 2009). Diterpenes from the dichromethane extract of the plant, which have been isolated from this genus (Giddy et al. 2007). It is possible that the diterpenoid content could be responsible for the antiproliferative activity observed in this study. Diterpenoids have been shown to inhibit cell cycle progression of breast cancer cell lines in vitro (Block et al. 2002). Few studies are reported in the literature on the antiproliferative effects of *C. gratissimus*. Cembranolides from the dichromethane extract of the leaves were shown to have cytotoxic activity on HeLa cells (Okonk and Nwafor 2010).

The extract from *C. gratissimus*, also known as *C. zam-bescius*, at 1000 μg/ml had an antiproliferative activity 1.2-fold more than that of doxorubicin (Fig. 1C). *C. gratissimus* belongs to the family Euphorbiaceae which has been found to have anti-inflammatory and pain-relieving properties (Iwawela et al. 2007). The Stem of *A. barbadensis* is well known for its diterpenoid content and different types of diterpenes (phorbol esters, clerodane, labdane, kaurene, trachylobane, and lupeol) have been isolated from this genus (Iwawela et al. 2007). It has been reported that the diterpenoid content could be responsible for the antiproliferative activity observed in this study. Diterpenoids have been shown to inhibit cell cycle progression of breast cancer cell lines in vitro (Block et al. 2002). Few studies are reported in the literature on the antiproliferative effects of *C. gratissimus*. Cembranolides from the stem bark have been shown to have moderate activity against ovarian cancer cell lines (Khalafalla et al. 2009). Diterpenes from the dichromethane extract of the leaves were shown to have cytotoxic activity on HeLa cells (Okonk and Nwafor 2010).

*S. guineense* is traditionally used to treat stomach aches and diarrhea (Tsakala et al. 1996; Hami et al. 2000). Ten novel triterpenoids such as betulinic acid, oleandrin, asiatic acid, arjunolic acid and hydroxyasiatic acid have been isolated from *S. guineense* (Djoukend et al. 2005). Betulinic acid is one of the triterpenes that have been isolated from *S. guineense* to date; it is a natural product with a range of biological effects and potent antitumor activity. Betulinic acid has been shown to induce apoptotic cell death by triggering the mitochondrial pathway of apoptosis (Fulda et al. 2008). The extract from *S. guineense* has the ability to reduce hyperglycaemia, a property that has also been found in the extract of *Catharanthus roseus*. *C. roseus* is the source of the highly effective antileukaemia drugs, the
vinca alkaloids vincristine and vinblastine (Roberson 2008). Therefore, S. guineense might possess similar compounds to C. roseus that are responsible for anticancer activity. The high levels of triterpenoids may play a role in the antiproiferative effect observed as triterpenoids have been found to have high anticancer against breast, lung and colon cancer cell lines (Fernandes et al. 2000).

Cajanus cajan at 1000 mg/mL was cytotoxic against Jurkat T cells. In this study, C. cajan, commonly known as pigeon pea, has edible pods that are eaten in tropical areas. An antifungal peptide has been isolated from C. cajan; this peptide has an antiproliferative effect toward leukemia cells (Shirataki et al. 2004). Two isoprenylated isoflavone phytoalexins have been isolated from C. cajan. Isoflavones have been shown to inhibit proliferation of cancer cells (Dahiya et al. 1984). Isoflavones from Saphora sp. have shown cytotoxicity and tumor specificity against squamous cell carcinoma and submandibular gland carcinoma (Shirataki et al. 2004). The isoflavone, genistein inhibits cell growth in breast and prostate cancer cells in vivo and in vitro (Sakar et al. 2006). Genistein has been found to regulate genes that are critical for the control of cell proliferation, cell cycle, apoptosis, transcription regulation and cell signal pathways (Shirataki et al. 2004).

E. tirucalli extract inhibited proliferation of Jurkat T cells in a dose-dependent manner as shown in Fig. 4. E. tirucalli, commonly known as pencil plant or milk bush, has been found to have ingonol esters which are polyfunctional triterpenoids. The extract has been used traditionally for treatment of warts and cancers in Brazil, Indonesia, Halabar and Madagascar (Cataluna et al. 2000). Major constituents are diterpenes from the tiglane (phorbol ester) and from the ingenane (ingenol ester). The plant contains terpenoids such as cycloartenol, euphorinol and euphorinogin (Khaleghian et al. 2010). These terpenoids are antileukemic agents (Valadares et al. 2006). E. tirucalli ethanol extract has been found to modulate myelopoiesis and reduce spleen colony formation of tumors (Valadares et al. 2006). E. tirucalli reduction of tumours may be related to its antitumor activity. Extract from Euphorbia peplus tested against cancer cells taken from 8 patients with acute myeloid leukemia killed 56% and 95% of cancer cells at top and end of the scale (Yadav et al. 2002). Euphorbia peplus methanolic extract, known as petty sugar, has been found to activate protein kinase C which triggers controlled cell death in acute mye- loid leukemia cells (Yadav et al. 2002).

The plants whose extracts exhibited the most antiproliferative activity were collected from the Mashonaland Province of Zimbabwe against leukaemia cell lines (Fernandes et al. 2000). Antiproliferogenic activity and brine shrimp lethality studies on methanol extract of Cajanus cajan (L.) Millsp. leaves and roots. Advances in Natural and Applied Sciences 4, 311-316


