Investigating herbs with anticancer properties in the Caribbean

Yuri Clement, PhD
Pharmacology Unit, Faculty of Medical Sciences, The University of the West Indies, Trinidad & Tobago
Introduction

• Over 80% of the developing world use herbal remedies as routine healthcare practice.

• Recently, there has been a resurgence in the use of herbal medicine in patient-directed complementary and alternative approaches.

• There is a heightened search for new drugs from natural sources, including plants.
Introduction

- Cancer is among the leading cause of death in the Caribbean and throughout the world.

- Chemotherapeutic approach based on the premise that drugs inhibit growth or induce death in neoplastic cells.

- The ideal anticancer drug would prevent cell growth or kill neoplastic cells with minimal effects on normal cells.

- The search for phytochemicals with anticancer properties has provided drugs which are widely used in clinical practice for various cancer types.
Podophyllum peltatum
(Mayapple plant, American mandrake)

Podophyllotoxins

Etoposide

Teniposide
Catharanthus roseus
(Periwinkle)

Alkaloids

Vincristine

Vinblastine
Taxus brevifolia
(Pacific Yew tree)
**Methods: Plant selection & extraction**

- Plant material authenticated at the National Herbarium
- Washed, oven dried <50°C and pulverized to 40µm
- Extracted in methanol over 3 days, filtered and rotavapored to dryness to produce the crude extract.

- *Solanum triste*
- *Pisonia cuspidata*
- *Swartzia pinnata*
- *Mollinedia laurina*
- *Andira inermis*
- *Guarea guidonia*
- *Psychotria marginata*
- *Spermacoce verticillata*
- *Flemingia strobilifera*
- *Ficus pumila*
- *Psychotria uliginosa*
- *Azadirachta indica*
- *Cecropia peltata*
- *Petiveria alliacea*
Methods: Preparation of crude extracts

- Fresh plant material
- Herbarium
  - Oven dried
  - Pulverized
- Extraction in MeOH
- Rotary Evaporation
- Extraction in H₂O
- Filtration
- Crude Extract
Methods: Fractionation

- Crude MeOH extract
  - Pet Ether Extraction
    - Pet ether
    - Chloroform
      - Chloroform Extraction
        - Ethyl Acetate Extraction
          - Ethyl acetate
          - Butanol
            - Butanol Extraction
            - Aqueous
            - Aqueous
            - Aqueous
Human T-cell Leukemia cell-line:

Determination of cell count & viability

Preparation of human leukemia cell line (MT-4)

The MT-4 human leukemia cell line - McKesson Bioservices, Rockville, MD, USA.

Growth medium: RPMI-1640, foetal bovine serum and penicillin/streptomycin.

Incubation in CO₂ at 37ºC; sub-cultured 2x weekly.

Cells harvested 24 hours after sub-culturing

Determination of cell count and viability

Cells prepared by aseptically transferring cryogenically-stored cells into a centrifuge tube and centrifuged for 5 minutes at 3000 rpm.

Supernatant discarded, pellet resuspended in 10 ml media.

For cell counting and viability test, 10 µl of cell suspension mixed with 90 µl of trypan blue, and then 10 µl of this mixture added to haemocytometer chamber.
Methods: *Cell culture*

- Crude MeOH extract/fraction dissolved in minimal DMSO and water.
- Serial dilutions of extract.
- 100µl extract (50 µg/ml to 500 µg/ml) added in triplicate to 96-well plate.
- For highly coloured extracts, sample blank prepared by adding 100µl extract to 100 µl growth media. Reading obtained from this coloured sample blank was subtracted from the corresponding sample value 4x10^5 cells/ml MT4 cells (in log phase).
- 100µl cell suspension added to wells containing extracts.
- Reagent blank, as well negative control (100µl cell suspension, 100µl media).
- Incubation over 3 days in CO₂ at 37°C.
Following incubation, 100 µl solution removed from each well and 10µl MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye added, plate incubated for further 4 hrs.

After, 150µl 0.04N HCL in isopropanol added to each well to dissolve formazan crystals to determine cell viability. Plate read on Multiskan® plate reader at 570nm.

Colour intensity directly correlated to the proportion of viable cells. Comparisons made with control to determine percent cell survival.

% Cell survival = \[\frac{\text{optical density of wells with treated cells}}{\text{optical density of control well}}\] x 100.
Microtitre plate after MTT Assay

Reagent
Blank

Sample 1
50ug/ml
100ug/ml
150ug/ml
200ug/ml

Sample 2
50ug/ml
100ug/ml
150ug/ml
200ug/ml

Sample 3
50ug/ml
100ug/ml
150ug/ml
200ug/ml
Preliminary screening –
Determination of 0% Survival Rates

Methanolic extracts of:
- Solanum triste
- Pisonia cuspidata
- Swartzia pinnata
- Mollinedia laurina
- Andira inermis
- Guarea guidonia
- Psychotria marginata
- Spermacoce verticillata
- Flemingia strobilifera
- Ficus pumila
- Psychotria uliginosa - Methanol extract partitioned into petroleum ether, chloroform and ethyl acetate fractions. These gave 0% survival rates at 1000 µg/ml, 250 µg/ml and 275 µg/ml respectively.

Water extracts of:
- Azadirachta indica
- Cecropia peltata
- Petiveria alliacea

0% survival rates >130,000µg/ml.
**Spermacoce verticillata**
*(Shrubby false buttonweed)*

**Medicinal Uses:**
- Vermifuge & Hemorrhoids (Brazil)
- Uterine fibroids (Dominican Republic)
Flemingia strobilifera
(wildhops, luck plant)

Medicinal Uses:
- Kidney stones (Trinidad)
- Epilepsy and hysteria (India)
**Ficus pumila**
(creeping fig or "climbing fig")

**Medicinal Uses:**
- Diabetes & hypertension  
  (Japan)
- Traditional Chinese Medicine for breast cancer
IC$_{50}$ for methanol crude extracts of *Spermacoce verticillata* (▲) 37 µg/ml, *Ficus pumila* (●) 23 µg/ml and *Flemingia strobilifera* (■) 97 µg/ml, respectively. *F. pumila* had highest level of cytotoxicity.
**Figure 2**

IC$_{50}$ for petroleum ether fractions of _Spermacoce verticillata_ (▲), _Ficus pumila_ (●) and _Flemingia strobilifera_ (■) to be 74 µg/ml, 304 µg/ml and 94 µg/ml, respectively. _S. verticillata_ and _F. strobilifera_ were more cytotoxic than _F. pumila_.

![Graph showing the concentration of compounds against percentage survival](image-url)
**Figure 3**

IC$_{50}$ for ethyl acetate fractions of *Spermacoce verticillata* (▲), >200 µg/ml, *Ficus pumila* (●) 255 µg/ml and *Flemingia strobilifera* (■) 203 µg/ml, respectively. All fractions had minimal cytotoxicity activity.
Figure 4

IC$_{50}$ for butanol fractions of *Spermacoce verticillata* (▲) 144 µg/ml, *Ficus pumila* (●) 26 µg/ml and *Flemingia strobilifera* (■) 87 µg/ml, respectively. *Ficus pumila* fraction had the most cytotoxic activity.
Figure 5

IC$_{50}$ for chloroform fractions of *Spermacoce verticillata* (▲) 37 µg/ml, *Ficus pumila* (●) 23 µg/ml and *Flemingia strobilifera* (■) 97 µg/ml. *Ficus pumila* chloroform fraction had greatest cytotoxic activity.
Table 1.
IC$_{50}$ values for the crude extracts and solvent fractions of *S. verticillata*, *F. pumila* and *F. strobilifera*

<table>
<thead>
<tr>
<th>Plant</th>
<th>Crude Extract</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Butanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. verticillata</em></td>
<td>89 µg/ml</td>
<td>74 µg/ml</td>
<td>37 µg/ml</td>
<td>&gt;200 µg/ml</td>
<td>144 µg/ml</td>
</tr>
<tr>
<td><em>F. pumila</em></td>
<td>131 µg/ml</td>
<td>304 µg/ml</td>
<td>23 µg/ml</td>
<td>255 µg/ml</td>
<td>26 µg/ml</td>
</tr>
<tr>
<td><em>F. strobilifera</em></td>
<td>81 µg/ml</td>
<td>94 µg/ml</td>
<td>97 µg/ml</td>
<td>203 µg/ml</td>
<td>87 µg/ml</td>
</tr>
</tbody>
</table>
Discussion

- Over 20 plant species native to Trinidad screened for cytotoxic activity against leukemia cell line.

- First study to show cytotoxic activity of *S. verticillata*, *F. pumila* and *F. strobilifera* against human leukemia cell line.

- Highest cytotoxic activity in chloroform and butanol fractions of *F. pumila*.

- Phytochemical analyses of *F. strobilifera* show presence of several compounds including chalkones, flavonoid glycosides, aurone glycosides and epoxy chromenes.

- Flemingiaflavanone and ß-sitosterol-D glucoside isolated in *F. strobilifera* roots. These compounds had significant antimicrobial activity against bacteria and fungi.
Discussion

- *F. pumila* leaves contain at least 5 flavonoid glycosides (including rutin). Rutin previously shown to prevent carcinogen-induced single-strand breakage in nuclear DNA in rats.

- Ethanolic extract of *Lactuca indica* (with polyphenolic compounds, including rutin) had cytotoxic effects against HL-60 human leukemia cell line by inducing apoptosis.

- We suggest that rutin, also found in *F. pumila*, is responsible for the observed cytotoxicity activity.

- 3 novel sesquiterpenoids glycosides isolated *F. pumila* fruit. In another study, sesquiterpenoids have been shown to possess cytotoxic activity against HL-60 human leukemia cell line, with one being three times more potent than etoposide.

- We suggest that *F. pumila* cytotoxicity may due to presence of sesquiterpenoids, probably concentrated in chloroform and butanol fractions.
Discussion

• Little published work on *S. verticillata*, but methanolic extracts of *S. exilis* and *S. articularis*, showed strong antioxidant and free radical scavenging properties.

• Polyphenolic compounds occurring in plants have received much attention as potential chemoprotective agents. We suggest that *S. verticillata* may possess similar properties, which may account for its cytotoxic activity.

• Bioactivity guided fractionation and isolation would identify which of these previously characterized compounds were responsible for the observed cytotoxic activity in *F. strobilifera* in our assay.

• For all 3 plants studied, phytochemical analyses indicate the presence of antioxidant compounds, including flavonoids and triterpenoids.

• Probable that antioxidant compounds trigger intracellular signaling pathways which induce apoptosis in the cancer cell line and may explain the observed cytotoxicity.
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