Cytotoxic Activity of Selected West Indian Medicinal Plants against a Human Leukaemia Cell Line

G Ramcharan[1], YN Clement[1], AR Maxwell[2]

ABSTRACT

Objective: *To assess the cytotoxic activities of crude extracts and solvent fractions of* Spermacoce verticillata, Ficus pumila *and* Flemingia strobilifera *against a MT-4 human leukaemia cancer cell line.*
Methods: *Crude extracts of dried leaves of* S verticillata, F pumila *and* F strobilifera *were made by exhaustive methanol extraction, fractions were obtained from sequential extraction of the crude extract using solvents of increasing polarity. Dose responses corresponding to cell survival following 72-hour exposure to the extracts were determined using a leukaemia cancer cell line (MT-4). Cell viability was assessed using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay reading absorbances at 570 nm. Comparisons were made with controls and cell survival, in each sample well, was determined based on the ratio of the absorbance of the sample to the control.*
Results: *Crude extracts of* S verticillata, F pumila *and* F strobilifera *displayed cytotoxicity and the* IC_{50} *values were 89 µg/ml, 131 µg/ml and 81 µg/ml, respectively. The petroleum ether and chloroform fractions of the crude extracts of* S verticillata *and* F strobilifera *showed potent cytotoxic activity but the highest cytotoxic activity was found in the chloroform and butanol fractions of* F pumila *with* IC_{50} *values of 23 µg/ml and 26 µg/ml, respectively.*
Conclusion: *The crude extracts of* S verticillata, F pumila *and* F strobilifera *were shown to be cytotoxic to the leukaemia cell line, MT-4 and* IC_{50} *values were determined. Fractionation of the crude extracts by solvent-solvent extraction enabled determination of the active fractions and their* IC_{50} *values. We propose that cytotoxic activity may be due to antioxidant compounds previously isolated from these plants.*

Keywords: Cytotoxicity, leukaemia cell line, medicinal plants

Actividad Citotóxica de Plantas Medicinales de West Indies Contra la Línea Celular de la Leucemia Humana

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RESUMEN

Objetivo: *Evaluar las actividades citotóxica de extractos crudos y las fracciones solventes de* Spermacoce verticillata, Ficus pumila *y* Flemingia strobilifera *contra una línea celular de la leucemia humana MT4.*
Métodos: *Se obtuvieron extractos crudos de hojas secas de* S verticillata, F pumila *y* F strobilifera *mediante extracción exhaustiva con etanol, y se obtuvieron fracciones a partir de la extracción secuencial del extracto crudo mediante solventes de polaridad creciente. Se determinaron las respuestas a las dosis correspondientes a la sobrevivencia de las células luego de 72 horas de exposición a los extractos, usando una línea celular de leucemia (MT-4). La viabilidad celular fue evaluada usando lecturas de absorbancia a partir del ensayo MTT [3-(4, 5-dimetiltiazol-2-il)-2, 5-difenil tetrazolio bromuro] a 570 nm. Se hicieron comparaciones con los controles. La sobrevivencia celular en cada pozo de muestreo, fue determinada a partir de la tasa de absorbancia de la muestra con respecto al control..*

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Resultados: *Los extractos crudos de* S verticillata, F pumila *y* F strobilifera *mostraron citotoxicidad y los valores* IC_{50} *fueron 89 µg/ml, 131 µg/ml y 81 µg/ml, respectivamente. El éter de petróleo y las fracciones de cloroformo de los extractos crudos de* S verticillata *y* F strobilifera *mostraron una potente actividad citotóxica, pero la actividad citotóxica más alta fue hallada en las fracciones de cloroformo y butanol de F pumila con valores* IC_{50} *de 23 µg/ml y 26 µg/ml, respectivamente.*

Conclusión: *Los extractos de S verticillata,* F pumila *y* F strobilifera d*emostraron ser citotóxicos a la línea celular MT4 y* IC_{50} *se determinaron los valores. El fraccionamiento de los extractos crudos extractos mediante extracción solventes-solventes hizo posible la determinación de las fracciones activas y sus valores* IC_{50}*. Sugerimos que la actividad citotóxica puede deberse a compuestos antioxidantes previamente aislados a partir de estas plantas.*

Palabras claves: Citotoxicidad, línea celular de leucemia, plantas medicinales

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INTRODUCTION

The practice of herbal medicine continues to play a pivotal role in healthcare management throughout the developing world (1). Over the last few decades, there has been an increasing use of herbal preparations as patients become more involved in directing their health outcomes. There is also a heightened search for new drugs from natural sources including plants.

Cancer is among the leading causes of death worldwide (2). The chemotherapeutic approach is based on the premise that anticancer drugs inhibit cell growth or induce cell death in neoplastic cells. The ideal anticancer drug would prevent cell proliferation or destroy neoplastic cells with minimal effects on normal cells.

The search for natural products with anticancer properties from plants has provided drugs which are widely used in clinical practice for the management of various cancer types. These include etoposide and teniposide which are semi-synthetic derivatives of naturally occurring podophyllotoxin found in the Mayapple plant (*Podophyllum peltatum*), vincristine and vinblastine are naturally occurring alkaloids in periwinkle (*Catharanthus roseus*) and paclitaxel was isolated from the bark of the Pacific Yew tree [*Taxus brevifolia*] (3).

In the present study, we assessed the cytotoxic properties of *Spermacoce verticillata, Ficus pumila* and *Flemingia strobilifera* against an MT-4 human leukaemia cell line. Traditionally, *S verticillata* has been used as a vermifuge and for treatment of haemorrhoids in Brazil (4) and also to treat uterine fibroids in the Dominican Republic (5). In Trinidad and Tobago, *F strobilifera* is used to eliminate kidney stones (6) and to treat epilepsy and hysteria in India (7). *Ficus pumila* is used for the control of diabetes and hypertension in Japan (8) and in a polyherbal formula known as Tian-Lou-Tang in traditional Chinese medicine for the treatment of breast cancer (9).

MATERIALS AND METHODS

Preparation of crude extracts

Fresh plant material was obtained from the Northern Range in Trinidad and the leaves and stems were used for all three plants. Samples were supplied to the National Herbarium at the University of the West Indies, St Augustine, Trinidad and Tobago, for identification and vouchering. The voucher numbers were assigned accordingly by the curator Mrs Yasmin Baksh-Comeau: *Flemingia strobilifera* (L.) Ait.f. (TRIN36513), *Spermacoce verticillata* L. (TRIN36514) and *Ficus pumila* L. (TRIN36515). The plant material was thoroughly washed under running tap water, oven-dried at 45ºC until constant weight, mill-ground (mesh size 40 µm) and exhaustively extracted with methanol. The mixture was then filtered by vacuum and the filtrate rotary evaporated to dryness at 50ºC to yield the methanol extract (10, 11).

The crude extract was initially dissolved in a methanol: water (80:20) v/v mixture and sequentially extracted with solvents of increasing polarity starting with petroleum ether, followed by chloroform, ethyl acetate and butanol. For each fractionation step, extraction was performed twice with 50 ml of solvent. The separated organic layers were dried with anhydrous sodium sulphate to remove traces of water and then rotary evaporated to dryness. This was done for subsequent fractions. The dried fractions were then assessed for cytotoxic activity against the cancer cell line (12).

The MT-4 human leukaemia cell line was obtained from McKesson Bioservices, Rockville, MD, United States of America (USA). Growth medium was RPMI-1640 with fetal bovine serum and penicillin/streptomycin was obtained from Invitrogen, New York, USA. Cells were incubated in a carbon dioxide incubator at 37ºC and sub-cultured twice weekly. Cells for experiments were harvested 24 hours following sub-culturing which corresponds to the log phase of growth. The cell line grows well in suspension and was easily cultured with a rapid doubling time. For long-term storage, cells were prepared in growth medium containing 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen (13).

Cells were counted and viability assessed using a Neubauer-type Haemocytometer and Nikon® inverted microscope. The cells were prepared for experimental work by aseptically transferring cryogenically-stored cells into a centrifuge tube and centrifuged for five minutes at 3000 rpm. The supernatant was discarded and the cell pellet resus-

pended in 10 ml of growth medium. For cell counting and viability assessment, 10 µl of cell suspension was mixed with 90 µl of trypan blue solution and then 10 µl of this mixture was added to the haemocytometer chamber.

Stock solutions of crude methanol extracts were made by initially dissolving known quantities in DMSO, the volume was then made up to a fixed volume with water and filter sterilized using a disc filter (0.2 µm). Dimethylsulfoxide concentrations in working plant extract solutions were maintained below 2% v/v to ensure minimal effects on cell viability. 100 µl aliquots of the working plant extract solutions were added in triplicate to a range of concentrations starting from 10 µg/ml to 500 µg/ml to 96-well plates followed by 100 µl of MT-4 cell suspension.

A reagent blank was prepared by adding 200 µl of growth medium per well. The control was prepared by adding 100 µl of cell suspension with the highest volume of solvent (DMSO) used in sample preparation and the total volume made up to 200 µl with growth medium. For coloured samples, a sample blank was prepared by adding 100 µl of sample extract to 100 µl growth media. The reading obtained from this coloured sample blank was subtracted from the corresponding sample value. The plate containing the reagent blank, sample blanks, control and plant extracts together with the cell suspension was incubated in a CO_2 incubator at 37ºC for three days.

Following incubation, 100 µl of solution was removed from each well and 10 µl MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] dye was added and the plate incubated for an additional four hours. After this period, 150 µl of 0.04N HCL in isopropanol was added to each well to dissolve formazan crystals which indicated cell viability. The plate was then read on a Multiskan® plate reader at 570 nm. The colour intensity correlated directly to the proportion of live cells in each well. Comparisons were made with the control to determine the per cent cell survival (14). The working cell concentration was $4x10^5$ cells/ml (15).

% Cell survival = [optical density of wells with treated cells/optical density of control well] x 100.

RESULTS

The cell survival rate (dose-response) curves for the crude extracts of the three plants are shown in Fig. 1. The IC_{50} values for the crude extracts of *S verticillata, F pumila* and *F strobilifera* were 89 µg/ml, 131 µg/ml and 81 µg/ml, respectively, as shown in Fig. 1. These results indicated that the crude methanolic extracts of *S verticillata* and *F pumila* possess similar cytotoxic potential, whereas that of *F strobilifera* had significantly greater cytotoxic activity against the MT-4 human leukaemia cell line.

The cell survival rate curves for the petroleum ether, ethyl acetate, butanol and chloroform fractions of the three plants are shown in Figs; 2, 3, 4 and 5, respectively. The IC_{50}

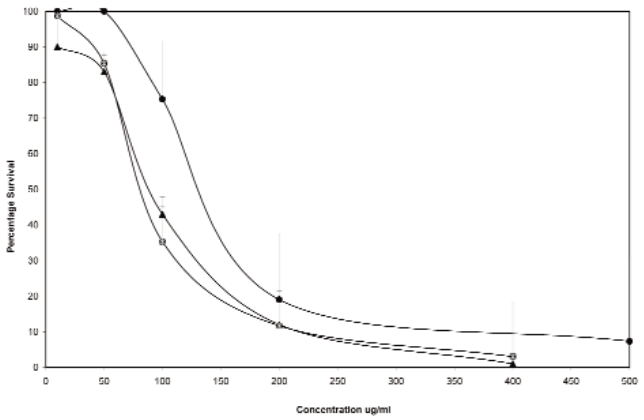

Fig. 1: Graph showing cell survival rates versus extract concentration (dose-response) for crude extracts of *Spermacoce verticillata* (▲), *Ficus pumila* (●) and *Flemingia strobilifera* (■). The IC_{50} values extracted from the graph for *S verticillata*, *F pumila* and *F strobilifera* were 89 µg/ml, 131 µg/ml and 81 µg/ml respectively. The crude extract of *F pumila* displayed the highest cytotoxicity.

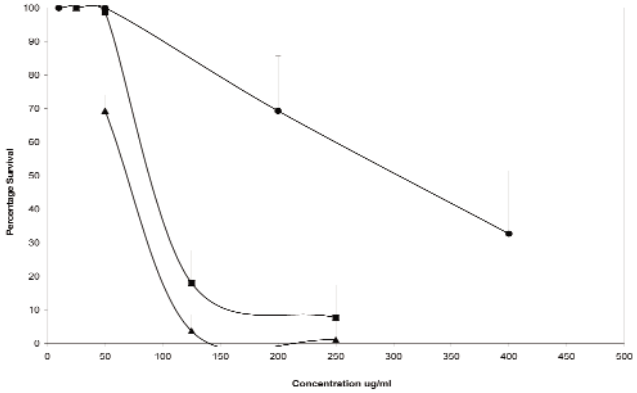

Fig. 2: Graph showing cell survival rates versus extract concentration (dose-response) for petroleum ether fractions of S*permacoce verticillata* (▲), *Ficus pumila* (●) and *Flemingia strobilifera* (■). The IC_{50} values extracted from the graph for *S verticillata, F pumila* and *F strobilifera* were 74 µg/ml, 304 µg/ml and 94 µg/ml respectively. The petroleum ether fractions of *S verticillata* and *F strobilifera* possessed greater cytotoxicity than *F pumila.*

values for the solvent fractions of the three plants are shown in the Table. The chloroform and butanol fractions of *F pumila* possessed significant cytotoxic activity with IC_{50} values of 23 µg/ml and 26 µg/ml, respectively. The chloroform fraction of *S verticillata* had significant cytotoxic effects with an IC_{50} value of 37 µg/ml. The cytotoxic constituents of *F strobilifera* appeared in the petroleum ether, chloroform and butanol fractions with IC_{50} values of 94 µg/ml, 97 µg/ml and 87 µg/ml, respectively. For *S verticillata,* the active constituents lie in the petroleum ether and chloroform fractions with IC_{50} values of 74 µg/ml and 37 µg/ml, respectively. The ethyl acetate fractions of *S verticillata, F pumila* and *F strobilifera* were effective only at high concentrations of > 200 µg/ml, 255 µg/ml and 203 µg/ml, respectively.

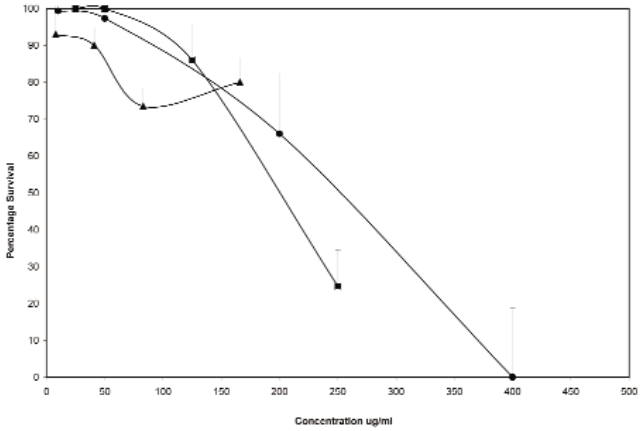

Fig. 3: Graph showing cell survival rates *versus* extract concentration (dose-response) for ethyl acetate fractions of *Spermacoce verticillata* (▲), *Ficus pumila* (●) and *Flemingia strobilifera.* The IC_{50} values extracted from the graph for *S verticillata, Ficus pumila* and *Flemingia strobilifera* were > 200 µg/ml, 255 µg/ml and 203 µg/ml respectively. The ethyl acetate fractions of all three plants demonstrated little cytotoxicity against the cell line.

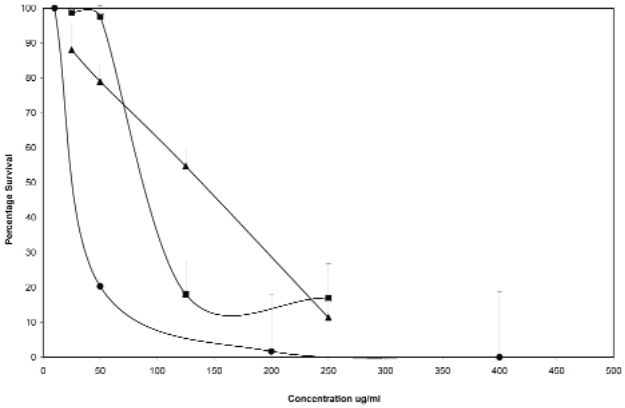

Fig. 4: Graph showing cell survival rates *versus* extract concentration (dose-response) for butanol fractions of *Spermacoce verticillata* (▲), *Ficus pumila* (●) and *Flemingia strobilifera* (■). The IC_{50} values were extracted from the graph for *S verticillata, F pumila* and *F strobilifera* were 144 µg/ml, 26 µg/ml and 87 µg/ml respectively. The butanol fraction of *Ficus pumila* demonstrated the greatest cytotoxicity.

These results suggest that *F pumila* possessed the greatest cytotoxic potential amongst the three plants based on the low IC_{50} values for its chloroform and butanol fractions.

DISCUSSION

To our knowledge, this is the first report demonstrating cytotoxic activity of crude extracts and solvent fractions of *S verticillata, F pumila* and *F strobilifera* against a human leukaemia cell line, with the highest anticancer activity found in the chloroform and butanol fractions of *F pumila.*

Phytochemical analyses of *F strobilifera* by other researchers have identified several compounds including chalkones (7), flavonoid glycosides (16), aurone glycosides (17) and epoxy chromenes (18). A recent investigation

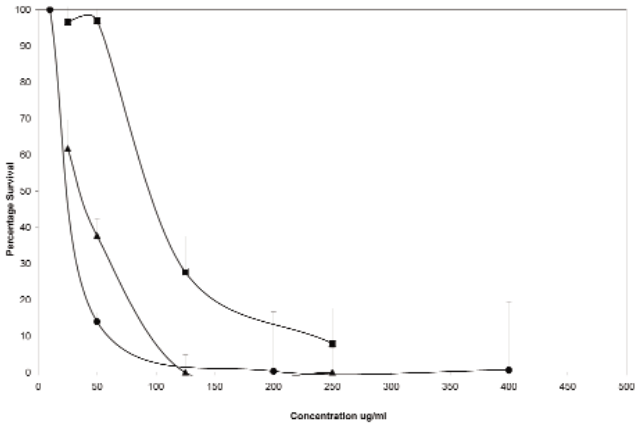

Fig. 5: Graph showing cell survival rates *versus* extract concentration and for chloroform fractions of *Spermacoce verticillata* (▲), *Ficus pumila* (●) and *Flemingia strobilifera* (■). The IC_{50} values were extracted from the graph for *S verticillata, F pumila* and *F strobilifera* were 37 µg/ml, 23 µg/ml and 97 µg/ml respectively. The chloroform fraction of *F pumila* demonstrated the greatest cytotoxic activity.

Table: IC_{50} values for crude extracts and solvent fractions of *S verticillata, F pumila and F strobilifera*

Plant	Crude Extract	Petroleum ether	Chloroform	Ethyl acetate	Butanol
S verticillata	89 µg/ml	74 µg/ml	37 µg/ml	>200 µg/ml	144 µg/ml
F pumila	131 µg/ml	304 µg/ml	23µg/ml	255 µg/ml	26 µg/ml
F strobilifera	81 µg/ml	94 µg/ml	97 µg/ml	203 µg/ml	87 µg/ml

showed that flemingiaflavanone and ß-sitosterol D-glucoside isolated from the roots of *F strobilifera* exhibited significant antimicrobial activity against Gram-positive (*S aureus, S epidermidis,* methicillin-resistant *S aureus*) and Gram-negative bacteria (*Ps aeruginosa, E coli*) and fungi [*C elegans*] (19). Bioactivity guided fractionation and isolation would identify which of these previously characterized compounds were responsible for the observed cytotoxic activity in *F strobilifera* in the assay.

The leaves of *F pumila* were shown to contain at least five flavonoid glycosides (including rutin – a polyphenolic compound) which exhibited strong antioxidant activity and radical scavenging activity (20). Rutin was also previously shown to prevent carcinogen-induced single-strand breakage in nuclear DNA in rats (21). An ethanolic extract of *Lactuca indica* (which contains polyphenolic compounds, including rutin) was shown to exhibit significant cytotoxic effects against the HL-60 human leukaemia cell line by causing induction of programmed cell death (22). We suggest that rutin, previously isolated in *F pumila*, may be partly responsible for the observed cytotoxicity activity against MT-4 cells in our assay and bioactivity guided fractionation and isolation would definitely determine its role.

Other phytochemical analyses of *F pumila* have also isolated α-tocopherol, a related compound, two known ster-

ols, fifteen known triterpenoids and five known flavonoid glycosides (23). These classes of compounds are known to possess potent antioxidant characteristics. Three novel sesquiterpenoids glycosides were also isolated from the fruit of *F pumila* (24). Other researchers have isolated sesquiterpenoids from *Tithonia diversifolia* and have demonstrated cytotoxic activity against the HL-60 human leukaemia cell line. One of the isolated sesquiterpenoids had three times more cytotoxic activity than etoposide (25). We suggest that the cytotoxic activity observed in *F pumila* against the MT-4 cell line may also be attributed to the presence of related sesquiterpenoids and postulate that these compounds may likely be found in the chloroform and butanol fractions of *F pumila.*

There is little published work on *S verticillata*, but methanolic extracts of *S exilis* and *S articularis*, which belong to the same genus as *S verticillata,* showed strong antioxidant and free radical scavenging properties (26). The accumulation of reactive oxygen species (ROS) is the hallmark of oxidative stress (27) and has long been associated with intracellular events leading to protein, DNA and lipid damage (28). The induction and propagation of carcinogenesis has long been strongly correlated with oxidative stress-related intracellular events (29). Polyphenolic compounds occurring in plants have received much attention as potential chemoprotective agents. We suggest that *S verticillata* possess similar properties to other members of the genus and this accounts for its cytotoxic activity.

In all three plants studied, previous phytochemical analyses have indicated the presence of compounds with antioxidant properties, including flavonoids and triterpenoids. It is probable that these antioxidant compounds trigger intracellular signaling pathways which induce programmed cell death in the cancer cell line and may explain the observed cytotoxicity.

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