

Investigation of the cytotoxic effect of phytochemicals on commercially cultured *Hep G2* cells using MTT and neutral red assays

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Fokunang C, Estella TF, Bathelémy N, et al. Investigation of the cytotoxic effect of phytochemicals on commercially cultured *Hep G2* cells using MTT and neutral red assays. *J Anal Toxicol Appl.* 2017;1(1):2-6.

BACKGROUND: The use of plants in traditional medicine has been well documented, and hundreds of plant species and their extracts, are used in developing countries to treat numerous diseases despite the fact that only a small number are approved for therapeutic use by the FDA. However, the cytotoxic effects of these plants have not been studied in detail, nor have their molecular structures been identified.

AIM: This study was to investigate the cytotoxic effects of these medicinal plants using both MTT and Neutral red assays.

METHOD: The stem, leaf, bark and trunk extracts from each plant species were used to determine the cytotoxic effects of the plant species *Hep G2* cells were exposed to extracts taken from plant species at concentrations of 0.1,

1, 10 and 100 µg/ml and the cytotoxic effects determined using both MTT and Neutral Red assays.

RESULTS: Using both MTT and Neutral Red assays, the cytotoxicity of each plant species was determined. It was found that extracts of *F. ovata* incubated on cells resulted in no cytotoxic effect, but for cells treated with all other extracts of the plant species, cytotoxic effects were observed in at least one concentration tested.

CONCLUSION: The results obtained for cytotoxicity assays indicated that *F. ovata* may be suitable for use as medicinal agent as the extract tested did not infer cytotoxicity. However, the other plant species tested during this study may not be suitable for use in medicine because of their potential cytotoxic effects.

Key Words: Cytotoxic effect; CMV infection; Neutral red assays; *F. ovata*; T cells

Natural products have been used since millennia for the treatment of human diseases and as a result, a large proportion of current drugs in modern medicine have been developed from natural molecules. The search for new biologically active natural products continues to be an intense field of research (1,2). It has been reported that the high natural biodiversity represents a broad range of diverse chemical structures with potentially new molecules having promising biological activities for many therapeutic areas (3). Such new natural molecules can potentially serve as chemical entities for the design and the synthesis of novel drugs (4). Plants have historically proven their value as a rich source of molecules with therapeutic potential and many major current drugs are natural products-derived compounds (2,5). The natural products firstly commercialized for therapeutic use are morphine, isolated from *Papaver somniferum* (3,4) and aspirin (6), based on the natural product salicin from *Salix alba* (7).

For thousands of years, plants have been recognized to play a therapeutic role in curing diseases and infections, and currently, there is a wide range of herbal remedies available in health food shops and other outlets, which many people are using to treat numerous problems, despite the fact that most herbal and plant remedies have not been approved for use by the FDA (2,7). It is now realized that the therapeutic effects of plant products are mainly due to the phytochemicals found in these plants, as many are known to have a pharmacokinetic or pharmacodynamics interaction with certain drugs (8). Because of these potential benefits of using plants in medicine, many species are now being analyzed to identify the phytochemicals found in them.

Despite this, however, the safety of using plants in medicine is continuously being reviewed, and much work is being done to determine not only the efficacious effects of plants, but also their potential harmful effects (6,8). Although it has been purported by some herbal remedies that the use of plants as herbal medicine is safe due to the fact that it is a "natural" substance, there have been a number of publications that indicate otherwise (4-9). Some herbal medicines are known to have resulted in severe side-effects after ingestion, which may be due to the toxic properties of the herbs or plants used, while the interactions of the plants or herbal medicine with other drugs being used by the patient can also lead to adverse effects (6,10).

For example, a number of severe effects, including heart attack, stroke and even death have been reported following the use of products containing Ma huang (*ephedrine*) and kola nut due to the interaction of the caffeine in the kola nut and the ephedrine (11). Numerous members of the *Ficus* species have been documented to be used for both food and medicine and their use has been most widely documented in the Middle East, where many members of this species are known to grow.

The quest for the discovery of new chemical entities, high throughput screening for biological activities of medicinal plants of pharmaceutical importance has increased in the last two decades in low income economies. This interest in finding phytochemical lead compounds have led to many plants identified with known pharmacological activities. However, with all the known despite the potential activities of these plants there is a gap of information on the safety of most of the potentially active plants. Within the motivation of establishing proof of efficacy (POE) and proof of safety (POS) of the medicinal plants in Cameroon the toxicology research group undertake toxicity studies to develop a safety platform for the potentially bioactive phytochemicals. The aim and objective of this study was therefore to investigate the cytotoxic effect of phytochemicals on commercially cultured *Hep G2* cells using MTT and neutral red assays. This study is the first study to investigate preclinical safety issues by *in vitro* oocyte toxicity cell culture standard regulatory assays on *Ficus ovata*.

MATERIALS AND METHODS

Plant materials

A number of species from the *Ficus* genus, along with species from the *Dorstenia* and *Triumfetta* genus were tested during this study. All plant species were collected from the jungle in Cameroon. The species studied are outlined in the Tables 1 and 2, in addition to the parts of the plant that were isolated and the fractions tested.

Cell culture

In this study, a human hepatocellular carcinoma cell line, *Hep G2*, was used to test the toxicity of each plant species. One sample from each plant species

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Received: September 08, 2017, Accepted: September 24, 2017, Published: November 06, 2017



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TABLE 1

Parts of the plant isolated from *F. ovata*

Plant species	Part of plant isolated	Fractions isolated
Ficus ovata	Bark of stem	Hexane 50%, Methanol, CH ₂ Cl ₂ -MeOH 1:1
	Bark of stem	CH ₂ Cl ₂ , Hexane fraction, Pyridine, hexane ethyl acetate fraction 50%
Ficus lutea	Trunk	Ethyl acetate methanol fraction 50%, methanol fraction 100%

TABLE 2

Plant species and plant part tested in cell cultures of cell line Hep G2 cells

Genus	Species	Part of Plant Tested	Fraction
Ficus	<i>polita</i>	Leaf	Crude extract in methanol
	<i>trichopoda</i>	Stem Bark	Ethyl acetate 100%
	<i>ovata</i>	Bark of Stem	Methanol
	<i>lutea</i>	Stem Bark	Methanol 100%

was incubated on Hep G2 cells at concentrations of 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml to determine if this fraction inferred any cytotoxic effects to the cells. Results were compared to control samples. The plant species tested, parts of the plant tested and extract solvent are outlined in Table 2.

Subculture of cells

Medium was removed from the dish and a solution of 0.25% trypsin/1 mM EDTA was added. Cells were incubated at 37°C/5% CO₂ for 10 minutes, and an equal volume of medium was added to deactivate the trypsin. The culture was transferred to a 50 ml conical tube and centrifuged at 2000 rpm for 5 min. Cells were re-suspended in medium and seeded into T 175 flasks. Cultures were replenished with medium every 3-4 days and passaged again when 90-95% confluent.

Preparation of cells from a monolayer culture for counting

Cells were first viewed to assess their morphology and their degree of confluence. Following this, medium was removed from the flask, and 3mls phosphate buffered saline (PBS) added to the flask, which was gently shook to wash the cells. The PBS was removed, and 6mls trypsin-EDTA added to the flask, which was again gently shook to soak the cells. The flask was then placed in an incubator (37°C, 5% CO₂) for 3-4 min and upon removing the flask the cells were observed under the microscope to assess their degree of detachment.

When cells were fully detached from the flask, 6mls fresh medium was added to the cells, mixed well and the cell suspension was then removed and placed in a universal. The cells were pelleted by centrifugation at 2000 rpm for 5 min. The supernatant was then removed, 2 ml fresh medium was added to the universal and the pellet was re-suspended in this fresh medium.

To count the cells, the haemocytometer and cover-slip were washed in 70% ethanol and wiped dry. The slide and cover-slip were moistened and placed in contact so that both grids on the slide were covered. 0.2 ml was taken from the cell suspension prepared in the universal, and mixed with 0.3 ml PBS and 0.5 ml 0.4% trypan blue (viability dye). This mixture was allowed to stand for 5 min. Following this, a small volume of the dyed cells was taken and added to both channels of the haemocytometer slide using a micropipette. All colorless cells were counted using the 5 large squares of the haemocytometer and this number was then placed into the following formula to determine cell density.

Cell Count = $10^4 \times \text{No. cells counted} \times \text{dilution factor} / \text{No. of squares counted}$

TOXICITY ASSAYS

Methyl tetrazolium assay (MTT) assay

The MTT assay was used to determine the proliferation rate of cells treated with plant extracts at different concentrations taken from a number of different species. In this assay, metabolically active cells convert yellow tetrazolium salt MTT to purple formazan crystals, and the number of viable cells in the population is proportional to the amount of formazan produced. Cells were seeded in a 96-well plate at the required density, and incubated for 24 h (37°C, 5% CO₂) to allow the cells to adhere. 10 µl plant extract chemical was added to the cells at concentrations of 0.1, 1, 10 and 100 µg/ml. Untreated cells (control) received medium only. The plate was then

incubated for 24 h (37°C, 5% CO₂). The chemical and medium were both removed from the cells, which were then washed with 100 µl PBS. This was removed from the cells, and 100 µl fresh medium and 10µl of MTT to each well and incubated for 3 h. After 3 h, 100 µl DMSO was added and the plate was gently shaken to solubilize the dye. The absorbance was then read at 540 nm using a Biotek Multiwell Plate Reader. A set of negative controls (untreated cultures) was used in each experiment and all experiments were run in triplicate.

Neutral red assay

Cells were seeded in a multi-well plate at the required density, and the plate was incubated for 24 h (37°C, 5% CO₂) to allow the cells to adhere. 10 µl plant extract chemical was added to the cells at concentrations of 0.1, 1, 10 and 100 µg/ml. As a control, medium was added to the cells (with no plant extract). The plate was then incubated for 24 h (37°C, 5% CO₂). The medium and plant extract was then removed from the cells, and replaced with 100 µl neutral red medium. The plate was then incubated (37°C, 5% CO₂) to allow uptake of dye into the cells. The neutral red medium was then removed from all wells, which were washed with PBS that was then removed. 100 µl neutral red extract was added to the cells and incubated for 15 min at room temperature. The absorbance was then read at 540 nm using a Biotek Multiwell Plate Reader.

Statistical analysis

Results obtained for both MTT and neutral red assays were analyzed statistically using student T-tests. Absorbance levels for control samples were compared to the absorbance levels obtained for each concentration of extract used to determine if there were any significant differences between absorbance levels. In addition, results obtained for the concentrations of the extracts used were compared to each other to determine if there were any significant differences between the different concentrations.

RESULTS

Cytotoxicity assay results

Hep G2 cells were treated with extracts taken from each of the species tested to determine if they resulted in any cytotoxic effects to this cell line. Concentrations of 0.1, 1, 10 and 100 µg/ml of each plant species were prepared and dissolved in methanol, and incubated on the cells for 24 h. The toxicity of each species was determined carrying out both MTT and Neutral Red assays. For the control, cells were incubated with medium only for 24 h. Student T-tests were performed on the results obtained to determine if any significant differences could be detected between control samples, and those treated with plant extract and to determine if there were any significant differences between the various concentrations of plant extract used in the study.

For *F. lutea*, Hep G2 cells were treated with 0.1, 1, 10 and 100 µg/ml of the 100% methanol fraction of the stem bark. Significance tests showed for the MTT assay, no significant differences were observed between control samples treated with medium only and samples treated with varying concentrations of the plant extract (Figure 1). However, results of the MTT assay for *F. lutea* did show a significant difference in absorbance levels between cells treated with 10 µg/ml of extract and those treated with 100 µg/ml of extract (Figure

1). The neutral red assay results for *F. lutea* showed a significant difference in absorbance levels between control samples, and those treated with 1 µg/ml of sample (Figure 2). In addition, significant differences were observed between samples treated with 0.1 µg/ml of extract 100 µg/ml of extract, while significant differences were also detected between samples treated with 1 µg/ml of extract and 10 µg/ml of extract, and those treated with 1 µg/ml of extract and 100 µg/ml of extract (Figure 2).

Neutral red assay results for *Hep G2* cells exposed to 0.1, 1, 10 and 100 µg/ml of the 100% methanol fraction of *F. lutea* isolated from the stem bark.

The methanol crude extract fraction of the leaf of *F. polita* was also tested using *Hep G2* cells.

While no significant differences were observed in the neutral red assay between control samples and those treated with the different concentrations of plant extract. MTT assay results did show a significant difference between control samples and those treated with 1, 10 and 100 µg/ml of plant extract (Figure 3). No significant differences were observed between the different concentrations of extract tested in either the MTT assay or neutral red assay (Figures 3 and 4). *Hep G2* cells were treated with various concentrations of the 100% ethyl acetate fraction of the stem bark of *F. trichopoda* and both neutral red and MTT assays used to determine any cytotoxic effects on the cells. Results obtained for the MTT assay indicated that a significant difference in absorbance levels was found between control samples, and those treated with 10 µg/ml of plant extract, but not samples treated with 100 µg/ml of plant extract (Figure 5). In addition, it was also determined that there was a significant difference in absorbance levels in cells treated with 10 µg/ml of the extract and those treated with 100 µg/ml of extract (Figure 5).

Results obtained for the neutral red assay showed a significant difference in absorbance levels between control samples and those treated with both 1 µg/ml of extract 100 µg/ml of extract (Figure 6). In addition, significant differences were observed in results obtained in the neutral red assay for cells treated with 0.1 µg/ml and 100 µg/ml of plant extract, and for cells treated with plant extract concentrations of 10 µg/ml and 100 µg/ml (Figure 6).

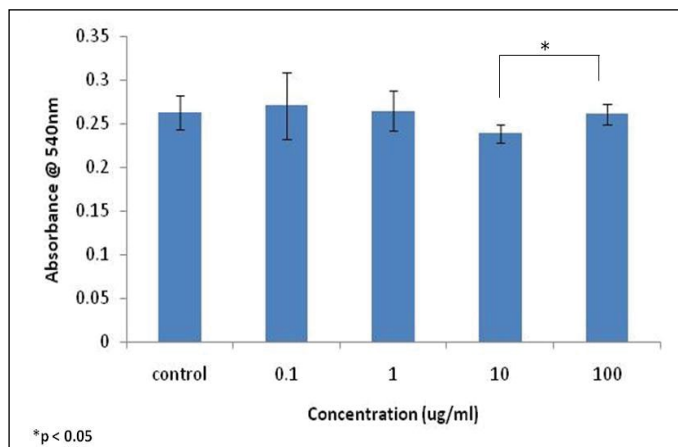


Figure 1) MTT assay results for *Hep G2* cells exposed to 0.1, 1, 10 and 100 µg/ml of the 100% methanol fraction of *Ficus lutea* isolated from the stem bark

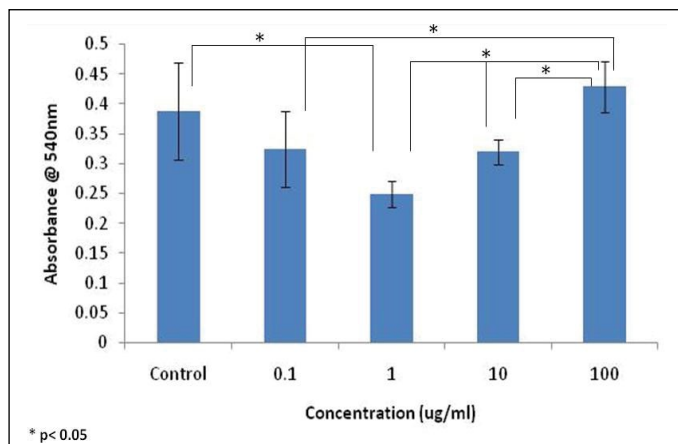


Figure 2) Neutral red results for *Hep G2* cells exposed to 0.1, 1, 10 and 100 µg/ml of the 100% methanol fraction of *F. lutea* isolated from the stem bark

The cytotoxic effects of the methanol fraction of the bark of the stem of *F. ovata* was also examined using *Hep G2* cells and the results obtained for both the MTT assay and neutral red assay showed that, for this plant species, no significant difference in absorbance levels between control samples and those treated with varying concentrations of plant extract was detected (Figures 7 and 8). In addition, no significant differences were observed in either assay used between any of the different concentrations of extract tested (Figures 7 and 8).

DISCUSSION

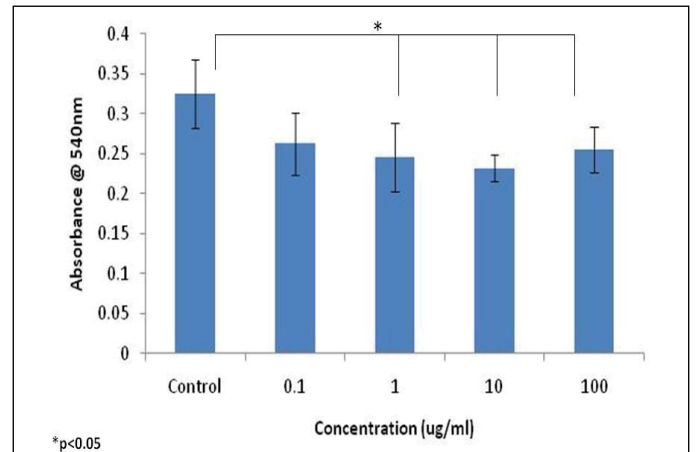


Figure 3) MTT assay results for *Hep G2* cells exposed to 0.1, 1, 10 and 100 µg/ml of the methanol crude extract of *F. polita* isolated from the leaf

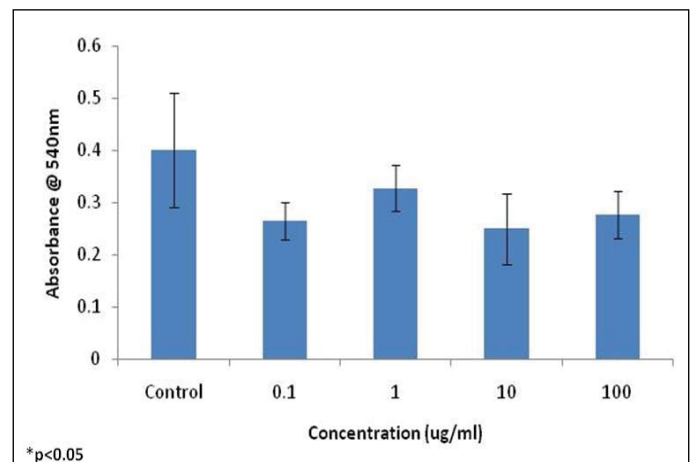


Figure 4) Neutral red assay results for *Hep G2* cells exposed to 0.1, 1, 10 and 100 µg/ml of the methanol crude extract of *F. polita* isolated from the leaf

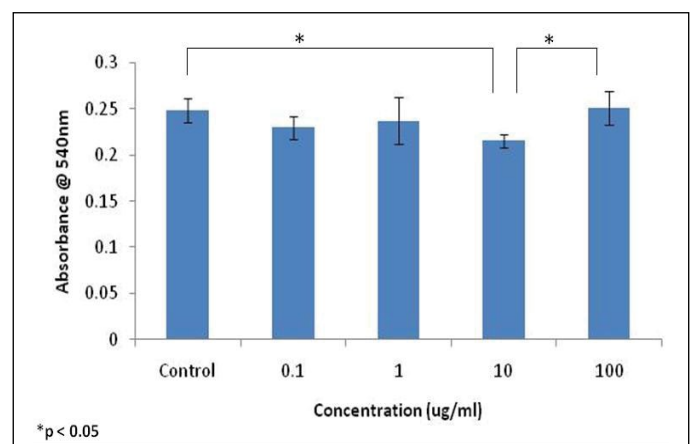
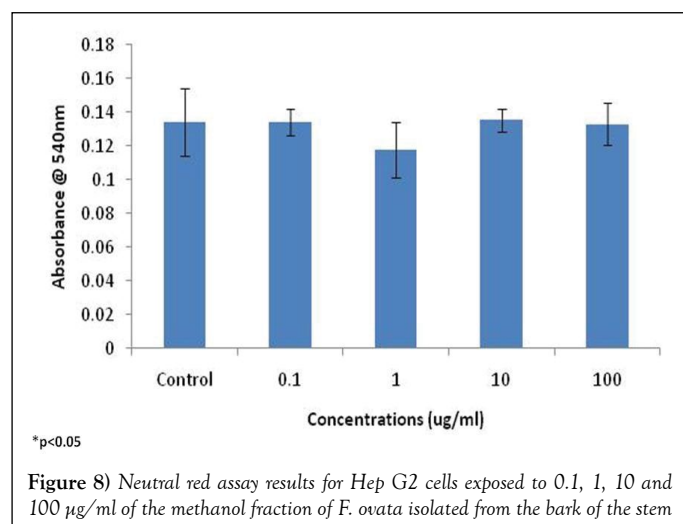
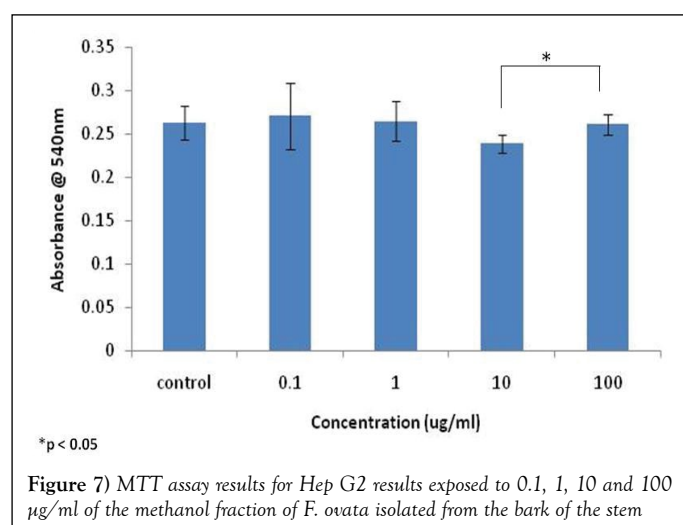
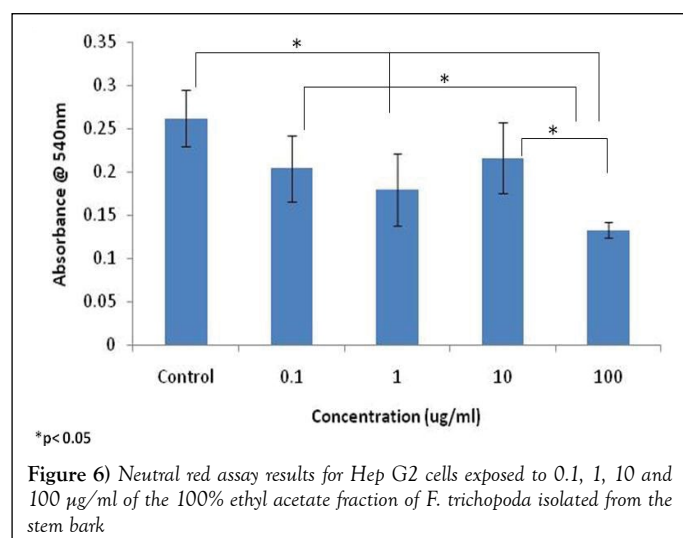


Figure 5) MTT assay results for *Hep G2* cells exposed to 0.1, 1, 10 and 100 µg/ml of the 100% ethyl acetate fraction of *F. trichopoda* isolated from the stem bark



In order to determine the cytotoxic effects of each of the species, a number of concentrations were tested on Hep G2 cells, and their cytotoxic effects determined using both MTT and neutral red assays. It was found that for *F. ovata*, no cytotoxic effects on Hep G2 cells were observed for any concentration of the species used. The highest concentration of the extract tested for each species was 100 µg/ml, and much higher concentrations may need to be tested on cells in order to observe any cytotoxic effects.

For cells treated with extracts of *F. lutea*, the only significant difference observed in cytotoxic effects in the MTT assay was between cells treated with 10 µg/ml and 100 µg/ml of extract. However, Neutral Red assay results for

this species in relation with other studies indicated that higher concentrations of this species may contribute to significant cytotoxicity (12,13). It was found that significant differences in cytotoxicity were observed in cell samples treated with 100 µg/ml of plant extract when compared to those treated with 0.1, 1 and 10 µg/ml, indicating that the bark of the stem of *F. lutea* may not be suitable for medicinal purposes at higher concentrations.

Leaf extracts of *F. polita* may also result in some cytotoxic effects. MTT assay results for this species indicated that significant differences in cytotoxic effects were observed between control samples, and those treated with 1, 10 and 100 µg/ml of plant extract. However, neutral red assay results did not show significant differences in cytotoxicity between control samples and the different concentrations of extract tested, which may indicate that the neutral red assay was not as sensitive for detecting cytotoxic effects as the MTT assay for this species.

Earlier studies with other *Ficus* species showed that these plants have rather a great significance for their traditional use in the treatment of other pathologies than cancer (14-16). Studies showed in some cases that *Ficus thonningii* and *F. platyhylla* showed very weak cytotoxicity with IC50 values 1500.0 µg/ml on NBMH mammalian cell lines (16,17). The ethanolic extracts from *F. bryopteris* did not display significant cytotoxic activity against the rat skeletal myoblast cell line (L-6 cells) with IC50 values >90, 32.6 and >90 µg/ml, for extracting solvents, toluene, ethyl acetate and butanol, respectively (5,18).

Some cytotoxic effects were observed on Hep G2 cells when treated with extracts of *F. trichopoda* at varying concentrations. Results of the Neutral Red assay indicated that at concentrations of both 1 µg/ml and 100 µg/ml of extract, cytotoxic effects were observed on the cells when compared to control samples. However, no cytotoxic effects were observed between control samples and those treated with 10 µg/ml of extract, indicating that this experiment may need to be repeated again to ensure accurate results. In addition, Neutral Red assay results also indicated that at a concentration of 100 µg/ml of extract, significant differences in cytotoxic effects were observed on the cells when compared to samples treated with both 0.1 µg/ml and 10 µg/ml of extract. These results indicate that high concentrations of the ethyl acetate fraction of the stem bark of *F. trichopoda* may cause cytotoxic effects and so its use as a medicinal plant should be limited this in contrast with other studies that showed that cytotoxicity of *Ficus* sp. to be dose dependent (7,19). MTT assay results also showed a similar trend in results for cells treated with extracts of *F. trichopoda*. These results indicated that cytotoxicity was observed when Hep G2 cells were treated with 10 µg/ml of plant extract when compared to control samples. However, since no significant difference was observed between control samples and those treated with 100 µg/ml of extract, this experiment should be repeated to ensure the accuracy of the results. MTT assay results also indicate that significant differences in cytotoxicity were observed in cells treated with 10 µg/ml of plant extract and those treated with 100 µg/ml of extract. Taken together, both the MTT and neutral red assay results indicate that some cytotoxic effects may be observed when higher concentrations of extracts of *F. trichopoda* are used, and so if used as a medicinal plant, low concentrations of extract should be used.

Other studies have demonstrated that the crude extracts of the wood of *F. elastica* aerial roots and *S. vogelii* leaves presented low anti-plasmodial and very important anti-trypanosomal activities associated with a low cytotoxicity (3,20). The comparison between the cytotoxicity effects suggests that the decreased viability of parasites may not be caused by a general cytotoxicity of the extracts (21,22). These results indicate that the selected medicinal plants should be explored more actively in order to isolate the main compounds responsible for the pharmacological action (22,23). It is important to mention that to the best of our knowledge, this study represents the first report on cytotoxic, evaluation for extract of *Ficus ovata*. The obtained results support to some extent the safe traditional uses of these plants for the treatment of some poverty related diseases in folk medicine. Isolation, purification, and structure elucidation of constituents from these plants are important to support discovery of new chemical entities for biological activities.

CONCLUSION

The results obtained for cytotoxicity assays indicated that *F. ovata* may be suitable for use as medicinal agent as the extract tested did not show any cytotoxicity potential. The two assays used were regulatory preclinical toxicity testing assays and the proof of non-cytotoxicity is an indication of proof of safety (POS) and indicator for a potential his selection for pharmacological activities and galenic formulation of *Ficus ovata* for category II improve traditional phytomedicine.

ACKNOWLEDGEMENT

The authors are thankful to the Centre for Biopolymer and Bio-molecular Research, Athlone Institute of Technology, County Westmeath, and Republic of Ireland for providing the reagents and laboratory facilities for the study. The natural product chemistry, Faculty of Science of the University of Yaoundé 1 for the extraction and phytochemical screening of the plant materials.

SOURCES OF FUNDING

This work was funded by the Centre for Biopolymer and Bio-molecular Research, Athlone Institute of Technology, County Westmeath, Republic of Ireland, and the research mobilization grant from the Ministry of Higher Education, Cameroon.

POLICY ISSUES

All statements and opinions are the responsibility of the authors. All authors have participated in the research, and have reviewed and agree with the content of the article.

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