



Pre-clinical *in vitro* investigation of the cytotoxic effect of *Ficus* species on hepatoma G2 cells using two standard toxicity assays

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ABSTRACT

Medicinal plants have had a long traditional use and gained integration into the primary health care through its recognition and standardization by WHO. Medicinal plant use 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. The objective of this study was to investigate the cytotoxic effects of *Ficus* species using two standard cytotoxic assays. 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. Using two standard assays; MTT and Neutral Red assays, the cytotoxicity of each plant species was determined. It was evident that extracts from *Ficus ovata* exposure to Hep G2 cell lines showed no level of cytotoxic effect, but for cells treated with all other extracts of the plant species, cytotoxic effects were observed in at least one concentration tested. This cytotoxicity assay study showed that *F. ovata* extracts at different concentrations exposed to Hep G2 cells indicated no observed cytotoxicity effect and by inference could be considered as a promising safe medicinal product. As safety was evaluated only at cell level there is need for more comprehensive *in vivo* toxicity testing to ascertain the safety level of the plant.

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INTRODUCTION

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 (Rout et al., 2009; Yapi et al., 2015). 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 (Sirisha et al., 2010; Awodiran et al., 2014; Nguele et al., 2016). Such new natural molecules can potentially serve as chemical entities for the design and the synthesis of novel drugs (Sivakuman et al., 2008; Zakari et al., 2016). 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 (Uttara, 2008; Adon et al., 2015; Ahouannon et al., 2017). The natural products firstly commercialized for therapeutic use are morphine, isolated from *Papaver somniferum* (Kantor, 2007; Goudjinou et al., 2017) and aspirin (Tembe et al., 2017), based on the natural product salicin from *Salix alba* (Devmurari et al., 2010; Vemo et al., 2017)..

For thousands of years, plants have been recognised 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 (Sirisha et al., 2010; Nikoloff et al., 2014; Adon et al., 2015). It is now realised 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

pharmacodynamic interaction with certain drugs (Kubmarawa et al., 2016). Because of these potential benefits of using plants in medicine, many species are now being analysed 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 (Kwete et al., 2014). Although it has been purported by some herbal remediests 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 (Lee, 2000; Dassa et al., 2017). 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 (Ao et al., 2008; Jiofack et al., 2010). 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 (Rao and Verma, 2008; Tsague et al., 2016). 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 objective of this study was therefore to investigate the cytotoxic effects of *Ficus* species using two standard cytotoxic assays

MATERIALS AND METHODS

Plant material

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 was incubated on HepG2 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/1mM EDTA was added. Cells were incubated at 37 °C and 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 minutes. 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 3 ml 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 minutes,

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, 6 ml 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 minutes. 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 minutes. 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 (Ngoutane et al., 2016).

$$\text{Cell Count} = \frac{104 \times \text{No. cells counted} \times \text{dilution factor}}{\text{No squares counted}}$$

Cytotoxicity testing

Methyl Tetrazolium (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 hrs (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 hrs (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 10 µl of MTT to each well and incubated for 3 hrs. After 3 hrs, 100 µl DMSO was added, and the plate was gently shaken to solubilise 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 (NR) Assay

Cells were seeded in a multi-well plate at the required density, and the plate was incubated for 24 hrs (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 hrs (37 °C, 5% CO₂). The medium and plant extract were then removed

from the cells, and replaced with 100 µl neutral red medium. The plate was incubated (37 °C, 5% CO₂) to allow uptake of dye into the cells. The neutral red medium was 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 minutes at room temperature. The absorbance was read at 540 nm using a Biotek Multiwell Plate Reader.

Statistical analysis

Results obtained for both MTT and Neutral Red assays were analysed statistically using student T-tests. Absorbance levels for control samples were compared with 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 with each other to determine if there were any significant differences between the different concentrations.

Table 1: Parts of the plant isolated from *F. ovata*.

Plant species	Part of plant isolated	Fractions isolated
<i>Ficus ovata</i>	Bark of stem	Hexane 50%, Methanol, CH₂Cl₂-MeOH 1:1
<i>Ficus lutea</i>	Bark of stem	CH₂Cl₂, Hexane fraction, Pyridine, hexane ethyl acetate fraction 50%
	trunk	Ethyl acetate methanol fraction 50%, methanol fraction 100%

Table 2: Plant species and plant part tested in cell cultures of cell line, HepG2 cells.

Genus	Species	Part of Plant Tested	Fraction
<i>Ficus</i>	<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%

RESULTS

Cytotoxicity

F. lutea, HepG2 cells treated with 0.1, 1, 10 and 100 µg/ml of the 100% methanol fraction of the stem bark exposed to the MTT assay, showed no significant differences compared with cells treated with culture media only (Figure 1). However, results of the MTT assay for *F. lutea* showed 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 and 100 µg/ml of extract, as well as with 1 µg/ml and 10 µg/ml of extract (Figure 2).

The Hep G2 cells exposed to 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml of the 100% methanol fraction exposed to Neutral Red assay, showed no significant differences between control samples and those treated with the different concentrations of plant extract (Figure 3). MTT assay results showed 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 3-4).

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 and 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).

The cytotoxic effects of the methanol fraction of the bark of the stem of *F. ovata* was also examined using HepG2 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 7-8). In addition, no significant differences were observed in either assay used between any of the different concentrations of extract tested (Figures 7-8).

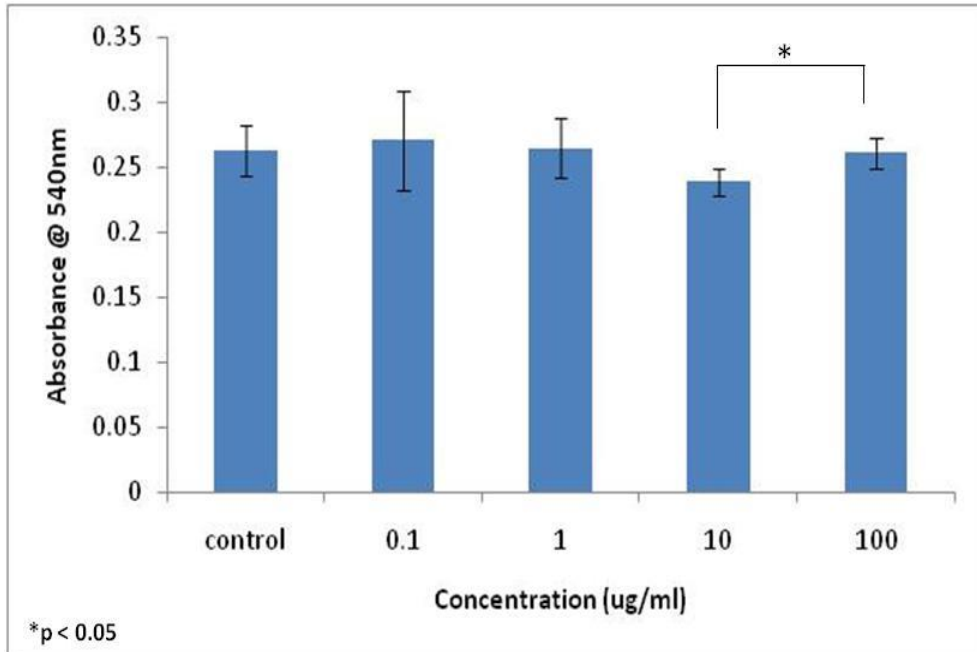


Figure 1: MTT assay results for Hep G2 cells exposed to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of the 100% methanol fraction of *Ficus. lutea* isolated from the stem bark.

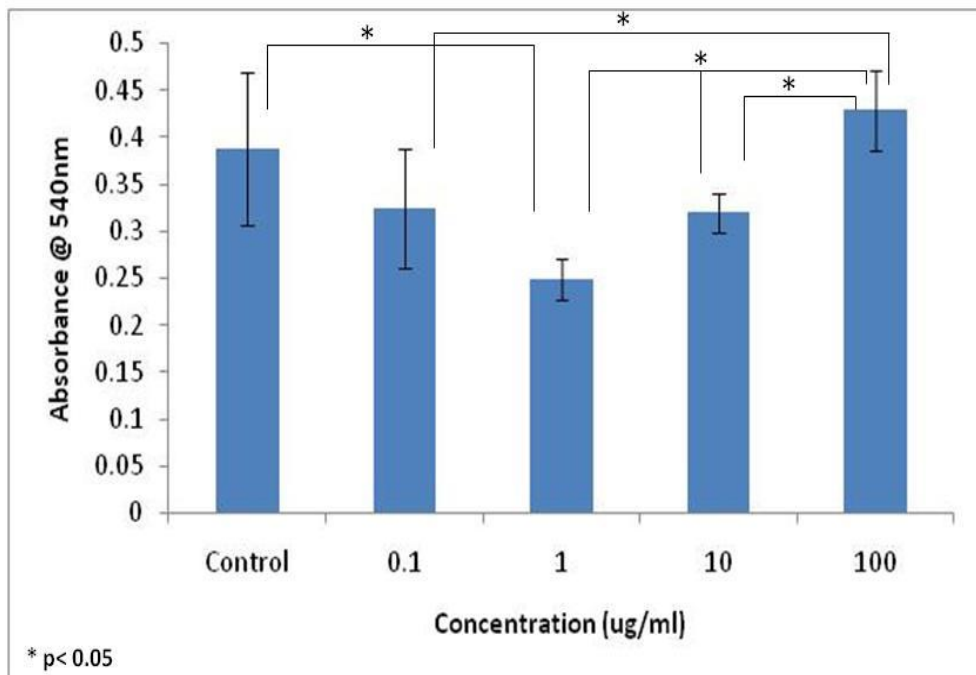


Figure 2: Neutral red results for Hep G2 cells exposed to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of the 100 % methanol fraction of *F. lutea* isolated from the stem bark.

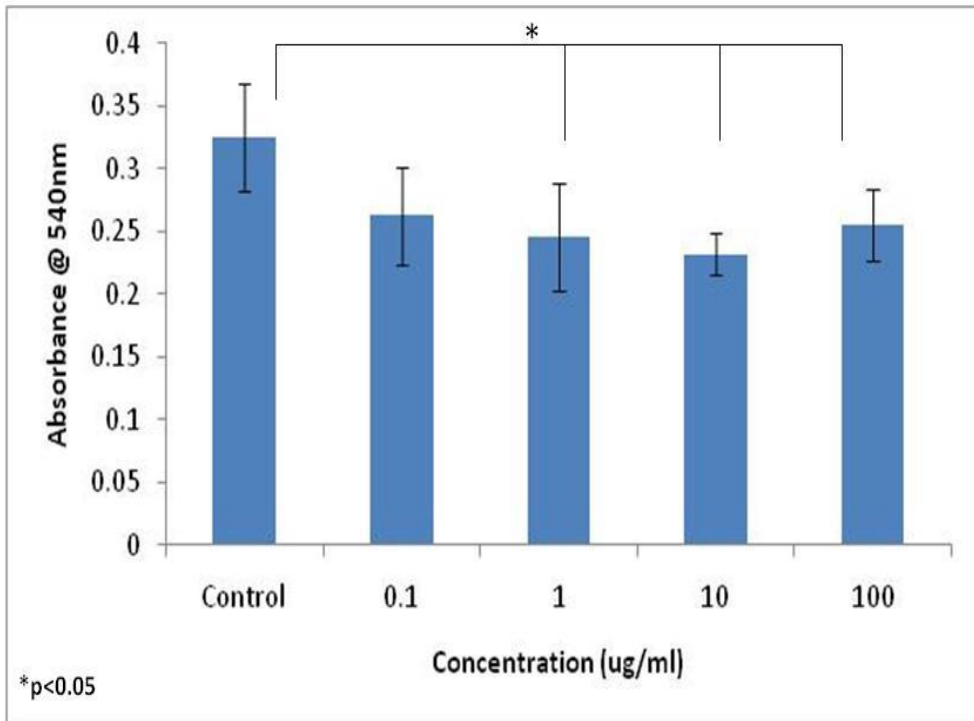


Figure 3: MTT assay results for Hep G2 cells exposed to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of the methanol crude extract of *F. polita* isolated from the leaf.

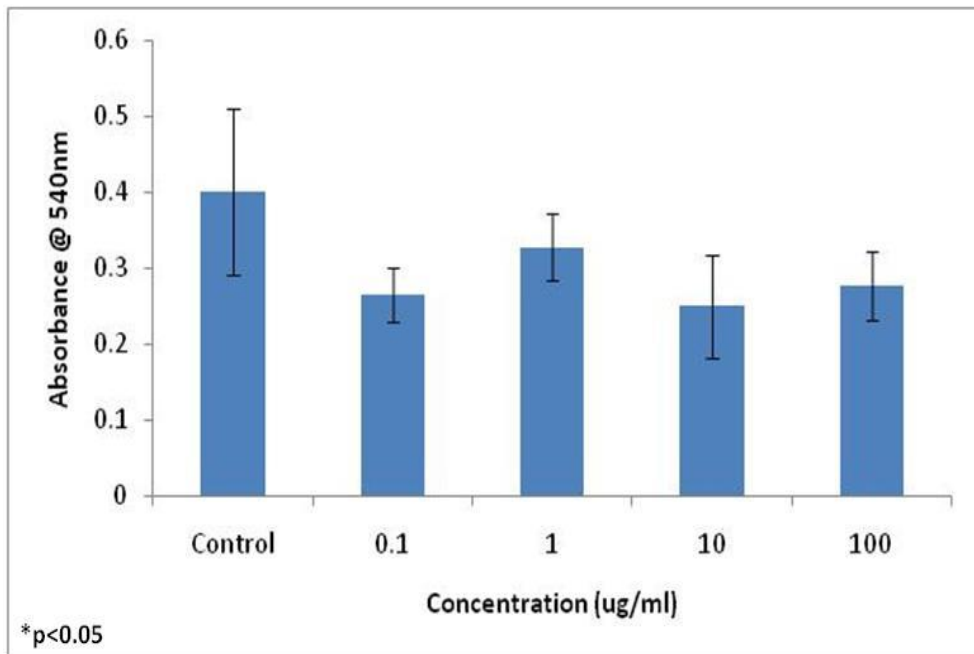


Figure 4: Neutral red assay results for Hep G2 cells exposed to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of the methanol crude extract of *F. polita* isolated from the leaf.

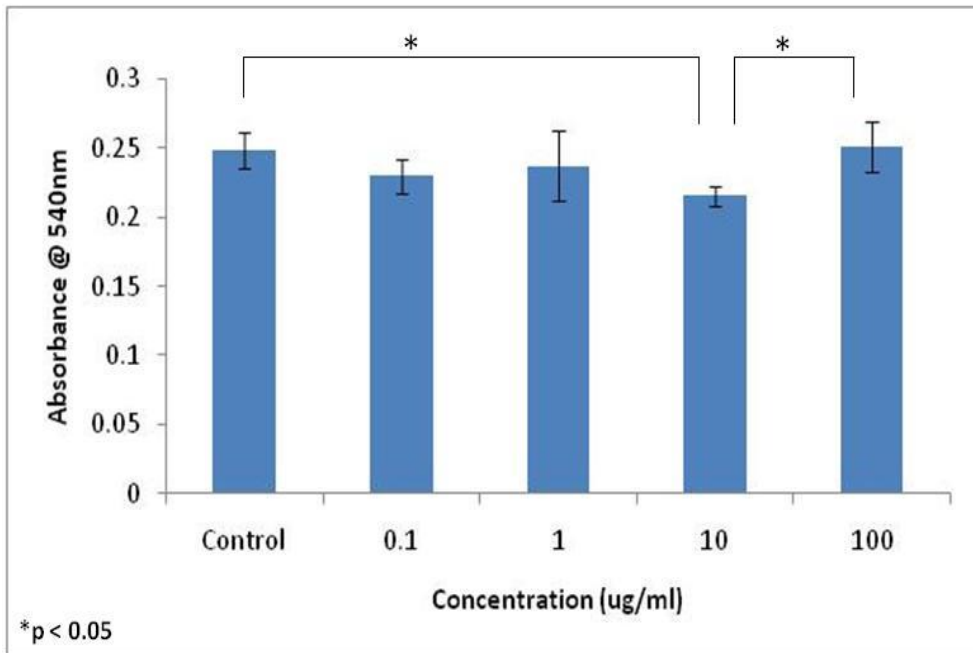


Figure 5: MTT assay results for Hep G2 cells exposed to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of the 100% ethyl acetate fraction of *F. trichopoda* isolated from the stem bark.

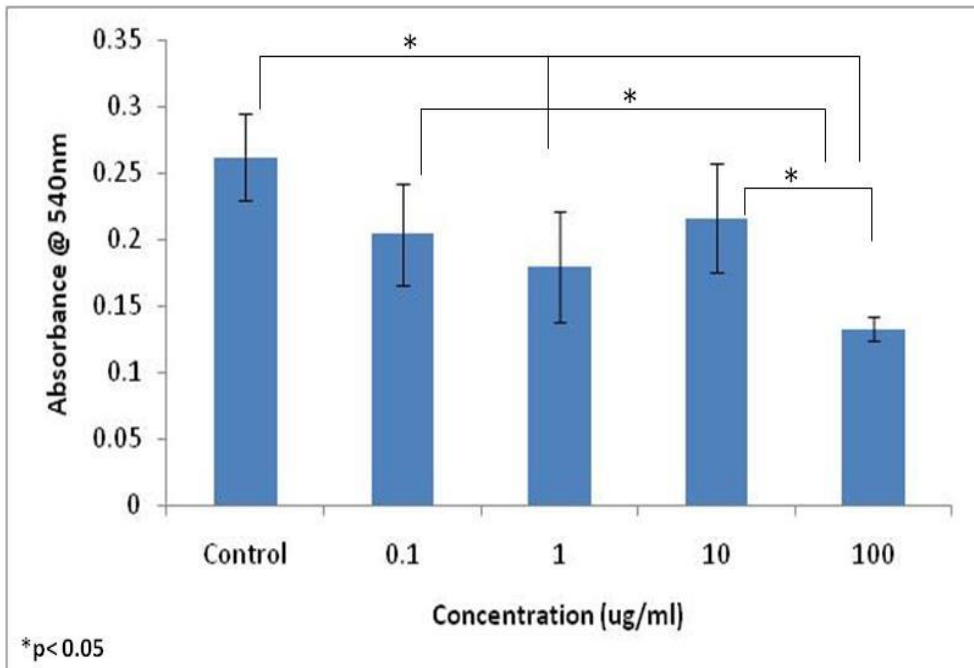


Figure 6: Neutral red assay results for Hep G2 cells exposed to 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of the 100% ethyl acetate fraction of *F. trichopoda* isolated from the stem bark.

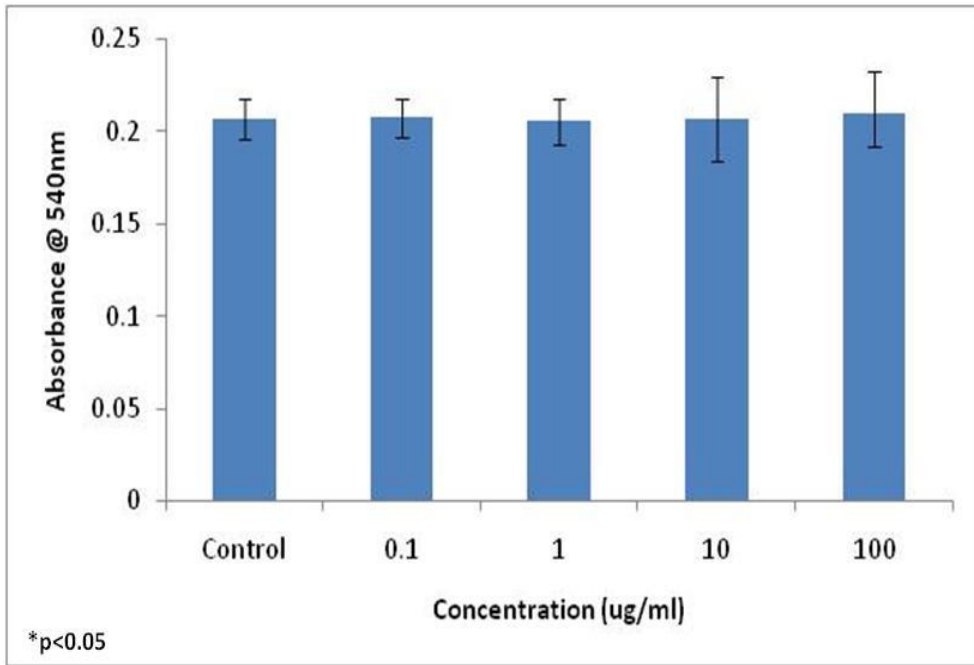


Figure 7: MTT assay results for Hep G2 results exposed to 0.1, 1, 10 and 100 µg/ml of the methanol fraction of *F. ovata* isolated from the bark of the stem.

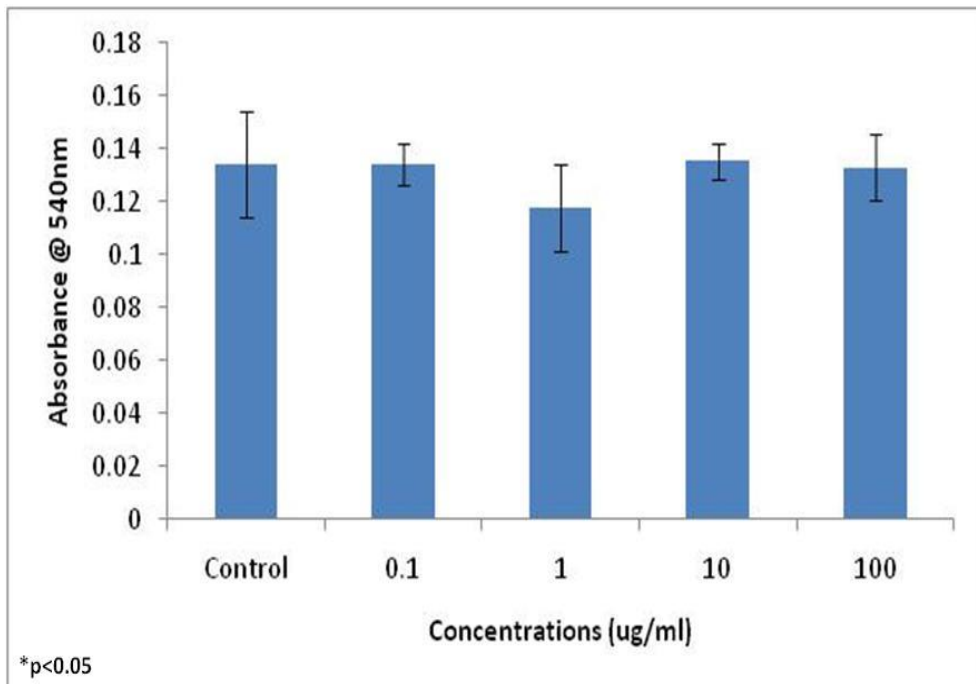


Figure 8: Neutral red assay results for Hep G2 cells exposed to 0.1, 1, 10 and 100 µg/ml of the methanol fraction of *F. ovata* isolated from the bark of the stem.

DISCUSSION

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 (Avisi and Nyadedzorl, 2003; Mevy et al., 2006; Bessa et al., 2017). It was found that significant differences in cytotoxicity were observed in cell samples treated with 100 µg/ml of plant extract when compared with 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 (Frogoso et al., 2008; Dias et al., 2012; Falade et al., 2014). Studies showed in some cases that *Ficus thonningii* and *F. platyhylla* showed very weak cytotoxicity with IC50 values 1500 g/ml on NBMH mammalian cell lines (Galati et al., 2001; Jiofack et al., 2010). 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 lg/mL, for extracting solvents, toluene, ethyl acetate, and butanol, respectively (Newman and Cragg, 2016; Mbosso et al., 2017).

Some cytotoxic effects were observed on HepG2 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 (Pistelli et al., 2000; Truong et al., 2012).

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 HepG2 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 antiplasmodial and very important antitrypanosomal activities associated with a low cytotoxicity (Sivakuman et al., 2008; Zakari et al., 2016). The comparison between the cytotoxicity effects suggests that the decreased viability of parasites may not be caused by a general cytotoxicity of the extracts (Sirisha et al., 2010; Adon et al., 2015). 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 (Pistalli et al., 2000; Uttara, 2008). 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

This cytotoxicity assay study showed that *F. ovata* extracts at different concentrations exposed to Hep G2 cells indicated no observed cytotoxicity effect and by inference could be considered as a promising safe medicinal product. As safety was evaluated only at cell level, there is need for more comprehensive *in vivo* toxicity testing to ascertain the safety level of the plant.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

EAT, CNF, SB and GH contributed in the conception of the protocol, laboratory analysis and statistics, BN, DG, BTN, participated in manuscript writing and data mining. PT the main investigator and project

sponsor. All the authors participated in the review of the manuscript.

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