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# Research Article
# *Combretum farinosum* Extract Toxicity to Skin and Lung Cell Lines as Measured by the Methylthiazoletetrazolium Assay

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## Abstract

**Background:** The use of plants from the Combretaceae family in medicine has been of much interest to scientists. However, *Combretum farinosum* has little published research on its pharmacological properties, unlike many of its extensively studied congenerics. **Materials and Methods:** *Combretum farinosum* roots, fruits, leaves and stems were sequentially extracted via Soxhlet extraction using petroleum ether, acetone and ethanol solvents. A 3 day Wallert and Provost Lab™ 96-well plate MTT cell proliferation assay was performed on LL47 lung fibroblasts, H69 small lung cell carcinoma and BJ fetal foreskin cell lines. **Results:** *Combretum farinosum* roots petroleum ether extract showed the strongest anti-proliferation activity of all the extracts against the normal cell lines ($IC_{50}$ of 0.504 mg $mL^{-1}$ for the BJ cells and 0.608 mg $mL^{-1}$ for the LL47 cells). *Combretum farinosum* roots petroleum ether extract also showed the strongest anti-proliferation activity of all the extracts against the only cancer cell line tested ($IC_{50}$ = 0.642 mg $mL^{-1}$). **Conclusion:** Both the roots petroleum ether and roots acetone extracts may have potential use in targeting diseased non-cancerous tissue (e.g., benign tumors) due to their cytotoxicity to normal cell lines. Finally, the roots petroleum ether extract may be the most promising extract for potential use as an anticancer drug if active compounds can be more thoroughly isolated.

Key words: *Combretum farinosum*, cytotoxicity, MTT, methylthiazoletetrazolium, thiazolyl blue tetrazolium bromide, colorimetric assay, cells, cancer, cisplatin, doxorubicin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Combretum farinosum* is a member of the Combretaceae family. This family is pantropical and consists of 600 species placed in 18 genera[1]. Tropical Africa has plants from 11 of those genera[2]. The two largest genera are *Combretum*, with an estimated 370 species and *Terminalia*, which consists of approximately 200 species[1]. The use of plants from the Combretaceae family in medicine has been of much interest to scientists studying ethnopharmacology. Fossil evidence dates plant usage in healthcare as far back as 5,000 years[3]. Numerous studies have documented and examined the widespread use of plants in Africa's treatment for symptoms and diseases such as headaches, abdominal pains, diarrhea, skin infections and many other ailments[1]. In fact, Eloff *et al.*[4] estimated that plants in Combretaceae have been used for as many as 90 medical conditions. However, scientists have not been able to test all of Combretaceae for each of the ethnopharmacological uses and are still identifying the classes and chemical structures of compounds isolated.

Traditional healers tend to use plants grown in nature. Traditional healers perceive that any medicinal properties would be reduced or lost if the plant was grown in an isolated environment. Although the latter statement has not been empirically tested, some studies suggest that stress and competition can cause wild plants to form biologically active compounds[5]. Metabolites are chemicals that plants may use to prevent infections. If a metabolite is preformed in the plant it is called a prohibitin[6]. Phytoalexins are metabolites formed as a result of an infection. Additionally, phytoalexins can be triggered by herbivory, edaphic factors or environmental factors such as microclimate and UV irradiation[5,7]. Netshiluvhi and Eloff[5] examined annual rainfall to see if lack of rain could trigger an environmental stress that would result in the formation of active compounds in *Combretum* and *Terminalia*. However, it was determined that rainfall did not exclusively affect the antimicrobial properties of the plants tested[5].

The aim of this study was to investigate a novel species with little or no published scientific literature within the genus *Combretum* for its cytotoxicity to normal and cancerous cell lines with the intent of identifying potential novel anticancer drugs. Cytotoxicity of a compound against a cell line can be quantified by measuring the half maximal effective concentration to kill 50% of the cells ($EC_{50}$), the median effective dose to kill 50% of the cells ($ED_{50}$) or the concentration required to inhibit cell growth by 50% ($IC_{50}$). A drug may be cytotoxic to a cell line for various reasons, including its ability to trigger apoptosis by altering the regulation of apoptotic pathways[8]. By measuring the cytotoxicity of *Combretum* extracts their ability to kill cancer cells or tumors, or even damage healthy cells, can be quantified.

*Combretum farinosum* has little published research on its pharmacological properties, unlike many of its extensively studied congenerics in *Combretum*. Given bioactive compounds such as combretastatins and other stilbenoids, tannins, flavonoids and terpenoids have been isolated from *Combretum* spp., *C. farinosum* is expected to contain many bioactive compounds and should be studied as well[2,9,10]. The species chosen was predicted to show moderate to minimal cytotoxicity to the normal cell lines while showing strong cytotoxicity to cancer cell lines. *Combretum farinosum* was evaluated for cytotoxic effects against a small lung cell carcinoma line, a normal foreskin cell line and a normal lung cell line using the methylthiazoletetrazolium (MTT) cell proliferation assay, a colorimetric metabolism test which can quantify cell death.

## MATERIALS AND METHODS

**Sample collection and identification:** *Combretum farinosum* was collected and identified by Francisco Javier Rendón-Sandoval, a botanist affiliated with the University of Guadalajara. *Combretum farinosum* was harvested from its native habitat in the tropical dry forest on the coast of Jalisco, Mexico. The study was conducted from December, 2014-July, 2016 as part of a Master of Science thesis project conducted at Texas A and M International University, Laredo, Texas.

**Extraction process:** A separate sequential Soxhlet extraction was performed for each of the plant components[11]. Aluminum pans were used to separate the plant into roots, stems, fruits and leaves. Each plant component was then broken up by hand and ground to coarse powder with a mortar and pestle. Each plant component started with a petroleum ether extraction followed 24 h later by an acetone extraction and after more 24 h, a 90% ethanol/10% deionized water extraction was run. Each extract was then evaporated using a rotary evaporator (Heidolph Laborota™ 4000 series) to remove the solvent. The extracts were then redissolved in water, frozen and lyophilized using a FreeZone 2.5 L Benchtop System by Labconco™. Dimethyl sulfoxide (DMSO) was aseptically added to autoclaved vials under a Labconco™ class II biosafety cabinet to make stock concentrations with a volume of approximately 17 mL for the plant extracts listed

below. Due to low yields not all of the freeze-dried extract products were used. The stock concentrations for the ethanol: Deionized water (EtoH) extracts for *C. farinosum* are as follows: roots 70 mg $mL^{-1}$ (1.19 g of product), stems 125 mg $mL^{-1}$ (2.13 g of product), leaves 70 mg $mL^{-1}$ (1.19 g of product) and fruits 85 mg $mL^{-1}$ (1.45 g of product). The stock concentrations for the petroleum ether extracts for *C. farinosum* are as follows: roots 3 mg $mL^{-1}$ (0.052 g of product) and fruit 45 mg $mL^{-1}$ (0.77 g of product). The stock concentrations for the acetone extracts for *C. farinosum* are as follows: roots 5 mg $mL^{-1}$ (0.086 g of product), fruit 10 mg $mL^{-1}$ (0.17 g of product). The latter four extracts were sonicated in a Branson 5510 sonicator for approximately 1.25 h to breakup particulates of powdered extract. All extracts were aliquoted into 1 mL portions and stored in the dark at 10°C to prevent photo-degradation.

**Cell lines:** Three cell lines were used in this study: LL47, BJ and H69. The cell lines were purchased from the American Type Culture Collection (ATCC). Cell lines were cultured at 37°C and humidified with 5% $CO_2$. The LL47 and BJ cell lines were grown in a monolayer culture, while the H69 cell line was grown in suspension. The BJ cell line was used as a model for predicting cytotoxicity of extracts in topical applications. The LL47 and H69 cell lines were used as a model for predicting cytotoxicity to normal and cancerous organ tissues.

**Methylthiazoletetrazolium (MTT) assay:** The MTT assay is a 3 day procedure based upon a Wallert and Provost Lab™ protocol. The assay measures the absorbance of cell cultures treated with the yellow MTT salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The MTT salt was transported into all metabolically active cells and the tetrazolium ring was cleaved by mitochondrial dehydrogenase:

$$\text{MTT salt (yellow)} \xrightarrow{\text{Mitochondrial reductase}} \text{Formazan (purple)} \quad (1)$$

The salt, when cleaved was purple and by measuring the absorbance of the formazan in cell cultures treated with the salt, cell growth or death can be quantified by correlating absorbance with cell metabolism. Aseptic techniques were followed and cells were only treated while in a Labconco™ purifier class II biosafety cabinet. Each MTT test was performed in triplicate. The assay was performed in a 96-well plate and requires a blank, positive and negative control, untreated control cells and cells treated with plant extract.

Cell growth media was used as a blank and consisted of F-12K medium (Kaighn's modification of Ham's F-12 medium) supplemented with 15% Fetal Bovine Serum (FBS) for the normal lung cell line (LL47 (MaDo) ATCC CCL-135), RPMI supplemented with 10% FBS for the cancerous lung cells (H69 ATCC HTB-119) and EMEM supplemented with 10% FBS for the fetal foreskin cell line (BJ ATCC CRL-2522).

Either Cisplatin™ or Doxorubicin™ was used as a positive control. Both Cisplatin™ and Doxorubicin™ were purchased in a powdered form and were diluted in deionized $H_2O$ with 0.9% NaCl for a concentration of 1 mg Cisplatin™/1 mL deionized $H_2O$ or 2 mg Doxorubicin™/1 mL deionized $H_2O$ and then vacuum filter sterilized (0.2 µL filter). Cisplatin™ was stored in the dark at room temperature when not being used, while Doxorubicin™ was stored frozen in the dark (per manufacturer's guidelines).

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was used as a negative control for the assay. It was purchased in a powdered form and was diluted in deionized $H_2O$ for a concentration of 5 mg $mL^{-1}$ and then vacuum filter sterilized (0.2 µL filter). The MTT solution was stored frozen in the dark (per the manufacturer's guidelines).

The cell lines used in the assay were taken from a cell line during sub-culturing while they were in the logarithmic growth phase. During day one of the assay, cell lines were seeded onto the 96-well plate. To prepare the cells for seeding, cell culture was added to a 15 mL conical tube and spun down in a Beckmann CS15R™ centrifuge at 400 rpm for 10-15 min. The media was removed and the cells were resuspended by vortexing in 1 mL of complete media (either supplemented F12K, supplemented EMEM, or supplemented RPMI). The 96-well plate was then seeded with 100 µL cell culture per well (7,500 cells added per well). The small lung cell carcinoma line (H69) grows as a suspension with floating aggregates instead of a cell monolayer like the BJ and LL47 cell lines. Due to these unique cell growth properties, the cancerous lung cell line cannot be accurately counted (as the manufacturer's guidelines indicate). However, cell counting was used to verify the viability of the small lung cell line and to approximate the volume of cell culture (to dilute in fresh media) to use in the assay.

During day two of the MTT cell proliferation assay a dedicated set of cells were treated with an extract, a positive control, or were designated as control untreated cells. Both the positive control and the plant extracts were tested over three different concentrations (e.g., 3, 30, 60 µL/ 100 µL cells) to allow the calculation of $IC_{50}$.

During day three of the cell proliferation assay, each of the wells with cells were treated with 20 µL of MTT solution and then the 96-well plates were incubated at 37°C for 3.5 h. Afterwards, 150 µL of MTT solvent (DMSO) was added to each of the wells treated with MTT and then the 96-well plates were

gently agitated on an orbital shaker for 15 min at 130 rpm to dissolve the MTT crystals. The absorbance in each well was then read on a microplate reader (Bio-Rad™ model 680) at 570 nm with a reference filter at 655 nm.

**Data calculations:** The absorbance in each type of well, e.g., the treated, positive control and negative control was averaged across three trials for each test and those test averages were used for the calculations discussed below. Each extract was tested against each of the three cell lines at least 3 times. Percent inhibition, when calculable was measured using the following formula:

$$\text{Percent inhibition} = 100\times[(\text{XTC-UTC})/(\text{UTC})] \quad (2)$$

where XTC is the average absorbance in the extract treated cells and UTC is the average absorbance for the untreated control cells.

Standard deviation was calculated for the percent inhibition across each test's three trials. Inhibitory concentration 50 ($IC_{50}$) was calculated by graphing the average percent inhibition of the extracts (for the three trials) against the concentrations of the extracts in the cell culture (3, 30 and 60 µL extract/100 µL cells) and using linear regression to determine a line of best fit.

## RESULTS

The average $IC_{50}$ for the ethanol, acetone and petroleum ether extracts of the roots, fruits, leaves and stems were calculated for the BJ, LL47 and H69 cell lines (Table 1). Blank spots in the chart represent no measurable inhibition. Entries with no standard deviation showed measurable inhibition in only one test and no measurable inhibition in the other tests. The exception is Doxorubicin™, which was used as a positive control only once per cell line. The $IC_{50}$ values for the eight extracts, when used to treat the BJ cell line, ranged from a high of 222.70 mg $mL^{-1}$ to a low of 0.423 mg $mL^{-1}$, from 274.98 to 0.496 mg $mL^{-1}$ for the LL47 cell line and from 58.37 to 0.288 mg $mL^{-1}$ for the H69 cell line. The petroleum ether and acetone solvents yielded extracts with the smallest $IC_{50}$ values. The roots petroleum ether extract demonstrated $IC_{50}$ values smaller than the Doxorubicin™ control when applied to the BJ and LL47 cell lines. However, no extract had an $IC_{50}$ value smaller than the Cisplatin™ control.

## DISCUSSION

One of the many trends observed was that the extracts were more cytotoxic to BJ cells than they were to the LL47 cells. This is interesting because both of those cell lines were fibroblast cell types. However, the level of cytotoxicity most likely differs because fetal cells can be more sensitive to chemicals than the same type of cell in an adult[12]. Popovic *et al.*[13] noted that normal skin fibroblast cells were more susceptible to damage during cancer treatment than both normal keratinocytes and normal melanocytes. For instance, in support of the trend mentioned above, the $IC_{50}$ of the roots acetone extract for the BJ cells is about a tenth that of the LL47 cells. The fruit petroleum ether extract had an $IC_{50}$ approximately 33% smaller on average for the BJ cells than the LL47 cells. The positive control, Doxorubicin™ was also more cytotoxic to BJ cells than LL47 cells.

An additional trend was observed regarding polarity of the solvent used. The more polar the solvent used in the extraction, the less cytotoxic the extract. A similar trend in bioactivity and polarity was observed by Costa *et al.*[14] where

Table 1: Average $IC_{50}$ values in mg $mL^{-1}$

| Parameters | Skin | Lungs (normal) | Lungs (cancerous) |
|---|---|---|---|
| **Ethanol extract** | | | |
| Fruit | 46.270±16.79 | | 39.440±5.52 |
| Stem | 222.700±124.92 | 274.98 | 48.280±4.21 |
| Leaves | 74.370±28.63 | | 58.370±16.96 |
| Roots | | | 60.00 |
| **Acetone extract** | | | |
| Fruit | 16.150±19.11 | | 10.170±4.50 |
| Roots | 1.270±0.433 | 12.670±18.54 | 1.330±0.184 |
| **Petroleum ether extract** | | | |
| Roots | 0.504±0.141 | 0.608±0.440 | 0.642±0.154 |
| Fruit P. ether | 6.040±1.09 | 8.990±1.91 | 7.270±0.742 |
| Doxorubicin™ | 0.759 | 1.320 | 0.433 |
| Cisplatin™ | 0.423±0.123 | 0.496±0.0573 | 0.288±0.0368 |

The average $IC_{50}$ values for each extract against the three different cell line, BJ (skin), LL47 (normal lung) and H69 (lung cancer)

the least polar solvent used yielded the highest number of total flavonoids, though the second most polar solvent yielded the highest number of total tannins and phenolics. In this study the ethanol/deionized $H_2O$ (most polar) solvent yielded extracts with the highest $IC_{50}$ values whereas the petroleum ether (least polar) solvent yielded extracts with some of the lowest $IC_{50}$ values. The ethanol/deionized $H_2O$ extracts consistently showed the highest $IC_{50}$ values within each cell line and in some instances showed no measurable inhibition (denoted by an asterisk in the absorbance column). For example, the roots EtoH extract showed measurable inhibition on one test with the H69 cells ($IC_{50}$ of 60 mg $mL^{-1}$) but showed no measurable inhibition in any of the repeated tests or in any of the tests with the LL47 or the BJ cell line.

The ethanol extracts were the least cytotoxic to the lung cell line. Though the fruit EtoH extract showed measurable inhibition with the BJ and H69 cell line, the extract showed no measurable inhibition with the LL47 cell line. Similarly, the leaves EtoH extract was more cytotoxic when applied to the BJ cell line and the H69 cell line than when applied to the LL47 cell line. When the extract was applied to the LL47 cell line there was no measurable inhibition. These results contrast with acetone and petroleum ether extracts, which have smaller and more consistent $IC_{50}$ values. For example, the roots petroleum ether extract, which had some of the smallest $IC_{50}$ values, had an average $IC_{50}$ of $1.27\pm0.433$ mg $mL^{-1}$ for the BJ cell line, $0.642\pm0.154$ mg $mL^{-1}$ for the H69 cell line and $0.608\pm0.440$ mg $mL^{-1}$ for the LL47 cell line.

Half of the extracts were more potent against H69 cells than the non-cancerous cells (LL47 and BJ). For instance, the fruit EtoH extract had an average $IC_{50}$ of 39.44 mg $mL^{-1}$ when applied to the H69 cells but an average $IC_{50}$ of 46.27 mg $mL^{-1}$ when applied to the BJ cells. Also, the stem EtoH extract had an average $IC_{50}$ of 222.70 mg $mL^{-1}$ for the H69 cells but an average $IC_{50}$ of 48.28 mg $mL^{-1}$ for the BJ cells. Yet, this trend did not hold for all of the extracts. For instance, the roots petroleum ether extract had a similar $IC_{50}$ value for all three of the cell lines though the $IC_{50}$ was the highest for the H69. For two of the extracts the average $IC_{50}$ value for the H69 cells fell between the $IC_{50}$ value of the BJ cells and the LL47 cells. Specifically, the fruit acetone extract had an average $IC_{50}$ of 10.17 mg $mL^{-1}$ for the H69 cells, no measurable inhibition for the LL47 cells and 16.15 mg $mL^{-1}$ for the BJ cells. The fruit petroleum ether extract had an average $IC_{50}$ of 7.27 mg $mL^{-1}$ for the H69 cells, 8.99 mg $mL^{-1}$ for the LL47 cells and 6.04 mg $mL^{-1}$ for the BJ cells.

Unlike in previous tests, i.e., when testing the BJ cell line and the LL47 cell line, the roots petroleum ether extract was found to be less cytotoxic than the positive control (Doxorubicin™) when applied to the H69 cells. On average, the roots petroleum ether extract was more cytotoxic than Doxorubicin™ for the BJ and LL47 cell lines. Interestingly, Doxorubicin™ was more potent against the H69 cell line ($IC_{50}$ 0.433 mg $mL^{-1}$) than it was against the normal cell lines (BJ $IC_{50}$ 0.759 mg $mL^{-1}$ and LL47 $IC_{50}$ 1.32 mg $mL^{-1}$). Yet, the roots petroleum ether extract was more potent against normal cell lines (BJ average $IC_{50}$ 0.504 mg $mL^{-1}$ and LL47 average $IC_{50}$ 0.608 mg $mL^{-1}$) than it was against the cancer cell line (H69 average $IC_{50}$ 0.642 mg $mL^{-1}$). Ideally, cancer drugs should be non-toxic to normal cell lines and highly toxic to cancer cell lines[15]. Doxorubicin™ more closely resembles this ideal pattern than the roots petroleum extract. Doxorubicin's™ cytotoxicity can be attributed to its ability to inhibit the enzyme topoisomerase II and intercalate DNA base pairs[16]. Cisplatin™ was also found to be more cytotoxic to the cancer cells (H69 $IC_{50}$ 0.288 mg $mL^{-1}$), than it was to the normal cell lines (BJ $IC_{50}$ 0.423 mg $mL^{-1}$ and LL47 $IC_{50}$ 0.496 mg $mL^{-1}$). Cisplatin works through its ability to damage DNA by forming intrastrand DNA adducts and is one of the first drugs of choice for treating non-small cell lung cancer so its level of cytotoxicity to the cell lines is not unexpected[17,18]. Unfortunately, the roots petroleum ether extract was not more cytotoxic to any of the three cell lines than Cisplatin™.

Researchers generally establish an arbitrary cut-off concentration for $IC_{50}$ of either 100 μM or 100 μg $mL^{-1}$ for extracts. Though, researchers typically work with either an isolated compound or perform an extraction with multiple solvents at a time rather than a sequential Soxhlet extraction. Extracts with $IC_{50}$ values larger than the cut-off are not considered very potent and the $IC_{50}$ is simply reported as >100 (μg $mL^{-1}$ or μM)[19-23]. However, the arbitrary cut-off of 100 μg $mL^{-1}$ may not be appropriate for this experiment given that the positive controls (known anti-cancer drugs such as Doxorubicin™) had $IC_{50}$ values above the cut-off. Thus, one can expect the unrefined extracts to similarly have $IC_{50}$ values above the arbitrary cut-off (100 μg $mL^{-1}$). Nevertheless, the level of cytotoxicity of the *C. farinosum* extracts was somewhat expected. Given *C. farinosum* is traditionally taken as a tea (hot water extraction) and ingested, it can reasonably be concluded that its level of toxicity is low when taken orally[24]. However, it is worth noting that *C. farinosum*'s low toxicity in teas may instead be due to the extraction technique used by those preparing the traditional medicine. Finally, its toxicity may vary if it is administered through a different route, e.g., topically or intravenously[25]. Given that neither of the extracts nor the positive controls tested had an $IC_{50}$ less than 100 μg $mL^{-1}$ and in two cases (LL47 and BJ cell lines) an extract was more cytotoxic than the positive control, the $IC_{50}$

values, though larger than seen in other studies, should not be arbitrarily dismissed.

Results from this study showed that extract prepared by extracting the roots of *C. farinosum* with petroleum ether particularly was more cytotoxic than Doxorubicin™ (a known anticancer agent) when tested against BJ and LL47 cell lines. This result will serve as basis for isolation, characterization and further cytotoxic assay of the pure isolates from the roots petroleum ether extract of *C. farinosum*. The potential synergistic or antagonistic effects of various isolates on each other will be studied in the quest to discover novel compound(s) in the treatment of various cancers.

## CONCLUSION

*Combretum farinosum* roots petroleum ether extract showed the strongest anti-proliferation activity of all the extracts tested. The roots acetone extract was the second most cytotoxic extract to the normal skin and cancerous lung cell line yet the fruit petroleum ether extract was the second most cytotoxic against the normal lung cell line. Some of the *C. farinosum* extracts (e.g., fruit EtoH, stem EtoH and fruit acetone) were more cytotoxic against the H69 cell line than they were against the normal cell lines, for other extracts the trend was reversed. The inhibition of cell growth after treatment with *C. farinosum* extracts was noteworthy as several of the extracts tested may have potential use as anti-cancer drugs if the active components can be isolated and purified.

## SIGNIFICANCE STATEMENT

This study provides information on the cytotoxicity of various extracts of *Combretum farinosum. Combretum farinosum*, whose cytotoxicity against various cell lines has not been studied, belongs to a plant family that has history of producing anti-cancer drugs. This study lays preliminary results for phytochemical studies on particular plant extracts with the potential of isolating anti-cancer drug candidates.

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