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# Antiproliferative effect of extracts and fractions of the root of *Terminalia avicennioides* (Combretaceae) Guill and Perr. on HepG2 and Vero cell lines

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## Abstract

**Background:** *Terminalia avicennioides* Guill and Perr (Combretaceae) is an important West African medicinal plant. The plant is used locally against microbes and parasites in both humans and animals and studies have demonstrated its cytotoxicity potential. Thus, this study was carried out to test the cytotoxic effect of the extracts and fractions of the root of the medicinal plant *Terminalia avicennioides* Guill and Perr (Combretaceae) in two different cell lines.

**Methods:** Methanol, ethanol, 30 % ethanol, hot water and cold water extracts and ethylacetate, hexane, chloroform, butanol and residual water fractions, were evaluated at 1000, 750, 500, 250, 100 and 50 µg/mL concentrations, with doxorubicin as positive control. The cells were incubated with the extracts for 48 h at 37 °C in a 5 % CO<sub>2</sub> humidified incubator. The inhibition of cell viability, determined with the methyl blue thiazole tetrazolium bromide (MTT) assay, was used to assess the anti-proliferative effect of the extracts, in normal Vero Monkey kidney and human liver cancer (HepG2) cell lines.

**Results:** There was a concentration-dependent inhibition of cell viability in both the HepG2 and Vero cell lines. For HepG2 cells, antiproliferative effect was highest for the hexane fraction (viability ranged from 19.63 ± 1.10 % to 70.30 ± 1.78 % for 1000 and 50 µg/mL, respectively). For Vero cells, the highest antiproliferative effect, at 1000 µg/mL, was with hexane fraction (cell viability 21.37 ± 3.50 %), while at 50 µg/mL the chloroform fraction demonstrated the highest effect (viability of 86.10 ± 1.95 %).

**Conclusions:** The extracts and fractions from the root of *Terminalia avicennioides* have antiproliferative effect on the Vero and HepG2 cell lines tested. However, the extracts and fractions were not more toxic to the HepG2 than to the Vero cells. The cytotoxic effect of stem-bark and leaf extracts could be evaluated in the future to determine its anticancer potential.

**Keywords:** *Terminalia avicennioides*, MTT assay, HepG2 cell line, Vero cells, Cancer, Cytotoxicity

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## Introduction

Neoplastic disease (cancer) has persistently been a major health issue, being one of the most important causes of death globally, despite the different researches that have been conducted to reveal much about its pathology over the years [1]. The growth and progression of healthy cells depend on a proportionate regulation of growth stimulating and inhibiting pathways [2]. Alterations in the proto-oncogenes and tumour suppressor genes that encode for proteins, which regulate cell division, repair damaged DNA and cause apoptosis of cells, are understood to cause cancer [2]. These alterations may produce cells that do not need external signals for rapid increase, and will not perceive signals to curtail their division, causing uncontrolled cell growth [3]. Several genes may be altered and inherited by daughter cells that will not conform to normal growth restraints, thus resulting in benign or malignant tumour formation [4]. Increased oxidative stress may aid in causing genetic instability, producing new tumour phenotypes with a reduction in apoptosis and an increase in tumour progression [5]. Neoplastic cells have been able to invent the ability to elude apoptosis, through certain mechanisms, such as cellular transformation, apoptosis dysregulation, propagation, movement, angiogenesis as well as metastasis [6].

Patients with cancer are managed by orthodox surgical procedure, chemotherapy or radiotherapy [6]. Despite the enormous advancement made in anticancer therapy such as early diagnosis, improved treatment and prevention, thereby restoring health and prolonging survival rate, cancer remains an important cause of morbidity and mortality [7]. Rigorous and extensive scientific research has been conducted to produce newer anti-cancer agents [7].

Natural products that have been investigated for extended periods are ascertained to be active pharmacologically and harmless with prolonged use [7]. An enormous range of herbal phytochemicals and nutrients, consumed by humans, have been associated with good health and diminished hazard of certain types of cancer [8–12]. A lot of the agents used against cancer today are produced from plant materials. These include vincristine and vinblastine from *Catharanthus roseus*, taxol and docetaxel from *Taxus brevifolia*, as well as camptothecins from *Camptotheca acuminata* [1, 13].

*Terminalia avicennioides* Guill and Perr (Combretaceae) is a medicinal plant of importance, in the West African sub-region, where it is found in abundance. The plant has been described to be active against microbes and parasites in both humans and animals. Similarly, studies have indicated that it exhibits cytotoxicity amongst other properties [14, 15]. This investigation was directed to appraise the cytotoxic effect of the extracts and fractions of the root of *Terminalia avicennioides* on

Vero monkey kidney and HepG2 human liver cancer cell lines, utilizing the methyl blue thiazole tetrazolium bromide (MTT) assay to assess cell viability.

## Methodology

### Chemicals and reagents

Gentamicin was from Virbac, Republic of South Africa. Trypsin-EDTA (cat no: BE17-16IF) and L-glutamine (cat no: BE-17-605E) were from Lonza (Verviers, Belgium). Dulbecco's Modified Eagle's Medium (DMEM, cat. No: D6546), MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide) reagent (cat. no: M5655), phosphate buffered saline (PBS, P4417) and Trypan blue (cat. no: T6146) were from Sigma-Aldrich (Darmstadt, Germany). Dimethyl sulphoxide (DMSO) (cat. no: SAAR1865000LP) was procured from Merck (Darmstadt, Germany). Analytical grade solvents were from Sigma-Aldrich/Merck (Darmstadt, Germany).

### Plant extract preparation

The roots of *Terminalia avicennioides* were collected in the wild, around Zaria (11° 04' N 7° 42' E) between the months of October and December (beginning of dry season in northern Nigeria). The plant material comprising the leaves, fruits and seeds were authenticated at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria. A verification number of VIN 900,239 was given. Sample of *T. avicennioides* roots collected was washed, allowed to dry in the shade and ground into powder using a laboratory mill. About 4 kg of the powdered material was put in a polythene bag and stored in a cabinet at room temperature. Two hundred and forty (240 g) gram of the powdered root of *T. avicennioides* divided into 6 parts, 30 g each, was extracted using acetone, absolute ethanol, 30 % ethanol and methanol, hot and cold water by maceration for 24 h. The different solutions were sieved through Whatman No. 1 filter paper. The filtrates were evaporated to obtain solid residues of the different extracts, respectively. Similarly, about 2 kg of the powder was extracted exhaustively using methanol by maceration for 24 h and filtered to obtain the methanol extract (ME). The ME was then concentrated *in vacuo* using a Rotary evaporator (Büchi® Rotavapor® R II, Vacutec, Switzerland) under lowered pressure to give the solid extract, which was kept for the fractionation process.

### Fractionation of extract

The crude ME was serially partitioned using solvents of decreasing polarity starting with butanol, chloroform and hexane. The procedure for each solvent was exhaustively carried out.

### Phytochemical analysis

The phytochemical composition of extracts was appraised by the methods of Trease and Evans [16].

#### Test for alkaloids

Two hundred milligram (0.2 g) of extract was placed in 10 mL acid alcohol, boiled and filtered. To 5 mL of the solution, 2 mL of dilute ammonia was added. Chloroform (5 mL) was added and the mixture agitated lightly. To this mixture, 10 mL of acetic acid was added. This was separated into two parts. Dragendorff's reagent was added to one part. The development of reddish-brown precipitate is positive for alkaloids.

#### Test for flavonoids

In 0.5 mL filtrate of extract, 5 mL dilute ammonia was added, followed by the addition of 1 mL concentrated sulphuric acid. The existence of flavonoids is identified via yellow colouration of the solution which vanishes on standing.

#### Test for phenolics

A tiny amount of extract was liquefied in 2 mL distilled water. Into it was added few drops of 10 % aqueous ferric chloride solution. Production of a blue or green colouration points to the existence of phenols.

#### Test for saponins

The extract was mixed with 5 mL distilled water in a test tube and agitated robustly for about 30 s. The development of a honeycomb-like froth that persists for 10–15 indicates the existence of saponins.

#### Test for tannins

To about 2 mL of the extract, 3–5 drops of ferric chloride ( $\text{FeCl}_3$ ) solution was added. A blue or brownish-blue precipitate indicates the presence of hydrolysable tannins.

#### Test for terpenoids

One milliliter acetic anhydride was added to about 2 mL of extract, followed by the addition of concentrated sulphuric acid ( $\text{H}_2\text{SO}_4$ ). A pink to violet colour indicates the presence of terpenoids.

### Cell culture

HepG2 human liver cancer (ATCC<sup>®</sup> HB8065<sup>™</sup>) and Vero monkey kidney (ATCC<sup>®</sup> CCL-81<sup>™</sup>) cells were from Cellonex, South Africa. HepG2 cells were retained in DMEM high glucose (4.5 g/L) encompassing L-glutamine (4 mM) and sodium pyruvate (Hyclone<sup>™</sup>) supplemented with 10 % foetal calf serum (FCS). Vero cells were sustained in DMEM high

glucose (4.5 g/L) having L-glutamine in which 1 % gentamicin and 5 % FCS (Highveld Biological, South Africa) were added. Both cells were kept at 37° C in 175 cm<sup>2</sup> culture flasks in a 5 % CO<sub>2</sub> incubator (HERACELL 150<sup>R</sup>, Thermo-Electron Corporation). Cells were passaged three times a week and trypsin-EDTA was used to detach the cells. Counting of cells was with a haemocytometer and using trypan blue exclusion to define viable cells. Nunc 96 well microtiter plates were used for seeding cells.

### Evaluation of antiproliferative effect

Inhibition of viable cell growth was appraised utilizing the MTT assay [17]. Cells from a sub-confluent flask were collected and centrifuged at 200 x g for 5 min, then suspended in DMEM to  $1 \times 10^5$  cells/mL. One hundred microlitres (100  $\mu\text{L}$ ) of the cell preparation was seeded into wells of columns 2 to 11 of a sterile 96-well microtitre plate. DMEM (200  $\mu\text{L}$ ) was placed in wells of columns 1 and 12 to lessen the "edge effect" and sustain moisture. The plates were placed in the incubator overnight to allow for cell attachment and recovery. Test samples (methanol, hot water, cold water, ethylacetate, hexane, chloroform, butanol and residual water extracts) were liquefied in DMSO (making 100 mg/mL). Varying dilutions were made in DMEM starting with 1000  $\mu\text{g/mL}$ . Decreasing concentrations (100  $\mu\text{L}$  each) of the extracts (1000, 750, 500, 250, 100, 50  $\mu\text{g/mL}$ ) were pipetted into the matching wells and the plates incubated for 48 h. Cells maintained with growth medium only, representing 100 % viability, functioned as negative control whereas doxorubicin hydrochloride (Pfizer, South Africa) was used as positive control. After 48 h, the cells were washed with 200  $\mu\text{L}$  PBS, before 200  $\mu\text{L}$  fresh medium incorporating 30  $\mu\text{L}$  MTT (5 mg/mL in PBS), was added. Following 4 h incubation, the growth medium was cautiously aspirated, with a suction pump (Integra, USA) and the formed formazan crystals solubilized with 50  $\mu\text{L}$  DMSO. The plate was agitated lightly for 2 min. The MTT reduction was quantified instantly by reading the absorbance using a spectrophotometer (Synergy HT, BioTek<sup>®</sup> EL808, Winooski, Vermont, USA) at a wavelength of 570 nm and a reference wavelength of 630 nm. Each extract concentration was tested in quadruplicate and the assays repeated twice at weekly intervals.

Percentage cell viability was computed for each extract concentration and the inhibitory concentration 50 (IC<sub>50</sub>) values were determined with the straight line equation of the plots of viability (%) against log concentration.

### Data analysis

Data were presented as mean  $\pm$  SD (standard deviation). Cell viability as percentage of untreated negative control cells was computed with the equation:

$$\text{Cell viability (\%)} = (\text{Absorbance of extract treated cells} / \text{Absorbance of cells only}) * 100$$

Students' *t*-test was used to test differences in viability between Vero and HepG2 cell lines for each extract and concentration.

### Results

Assay for phytochemical constituents revealed the existence of flavonoids, phenols, saponins and tannins in all the extracts and fractions tested.

In the human liver cancer cells treated with the different extracts of *T. avicennioides*, cell viability (percentage) inhibition was variable, manifesting a concentration dependent effect. At the highest concentration of 1000  $\mu\text{g}/\text{mL}$  tested, it ranged from  $19.63 \pm 1.10\%$  for the hexane fraction to  $47.54 \pm 1.22\%$  for the hot water extract (Fig. 1). At 50  $\mu\text{g}/\text{mL}$ , cell viability ranged from  $70.30 \pm 1.78\%$  to  $93.78 \pm 2.27\%$  for the hexane and chloroform extract, respectively.

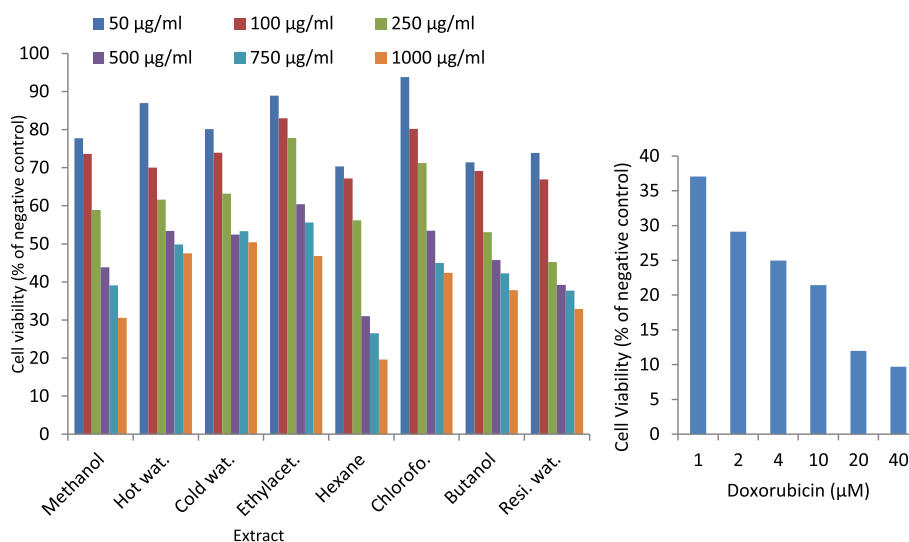
Inhibition of cell viability (percentage) by the different extracts of *T. avicennioides* against Vero monkey kidney cells varied. At 1000  $\mu\text{g}/\text{mL}$ , the hexane fraction showed highest inhibition of cell viability at  $21.37 \pm 3.50\%$  (Fig. 2). At the lowest concentration of 50  $\mu\text{g}/\text{mL}$  tested, the highest inhibition of cell viability was  $86.10 \pm 1.95\%$ , observed with the chloroform fraction (Fig. 2).

Furthermore, the effect of the different extracts on cell viability was concentration dependent as depicted in Fig. 2.

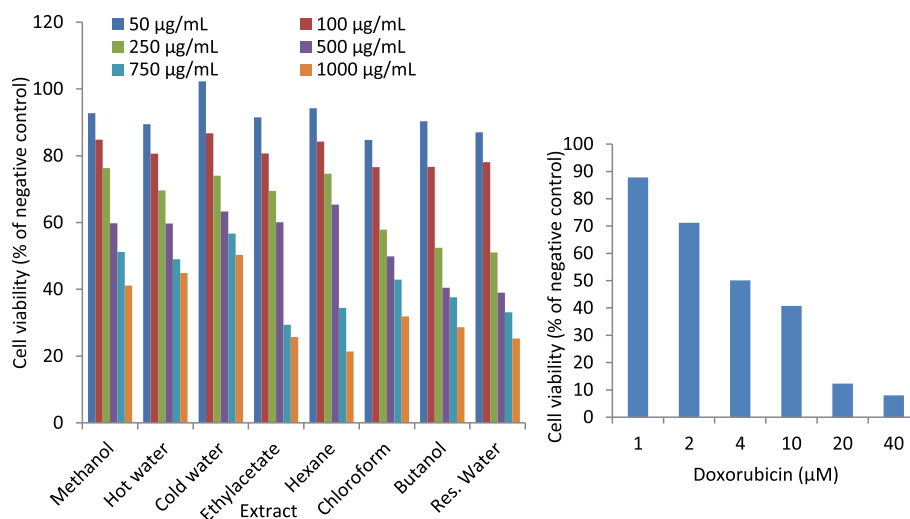
From the data summarized in Figs. 1 and 2, inhibitory or lethal concentrations ( $\text{IC}/\text{LC}_{50}$ ) were computed and are presented in Table 1.

### Discussion

The study was directed to appraise the cytotoxic activity of the different extracts of *T. avicennioides* on normal Vero monkey kidney and human hepatocellular carcinoma HepG2 cells. Plants contain a variety of phytoconstituents that may not have direct effect on the plants' growth [18] but which are assumed to have biologic activities. The phytoconstituents in the extract and fractions of the root of *T. avicennioides* in this study are carbohydrates, saponins, alkaloids, tannins, flavonoids, anthracene, steroids, triterpenes, cardiac glycosides, which is in agreement with reports by Ukwade et al. [19] Barku et al. [20], Mann et al. [14] and Ahmadu et al. [21] where alkaloids, tannins, saponins, flavonoids, steroid and phenol were found in the extracts of the leaf, stem and root of *T. avicennioides*. These plant constituents are believed to be responsible for the cytotoxic effect observed. Tannins, isolated from different *Terminalia* species, including *Terminalia chebula*, were noted to exert selective cytotoxic effect, against human tumor cell lines [22, 23]. The aqueous extracts of *T. avicennioides* and *Anogeissus leiocarpus* root bark, contain alkaloids, which were described as having anaesthetic, stimulant, and anticancer activities [24]. Flavonoids also retain potent anticancer function while saponins are



**Fig. 1** Antiproliferative activity of *Terminalia avicennioides* extracts and fractions on HepG2 cell lines as inhibition of cell viability expressed as percentage (of negative control). Data represent the mean  $\pm$  SD (standard deviation) of two independent experiments. Data for doxorubicin as positive control are shown for comparison



**Fig. 2** Antiproliferative activity of *Terminalia avicennioides* extracts and fractions on Vero cell lines as inhibition of cell viability expressed as percentage (of negative control). Data represent the mean  $\pm$  SD (standard deviation) of two independent experiments. Values for doxorubicin as positive control are shown for comparison

cholesterol-lowering and cytotoxic [25]. Polyphenols could hinder tumor formation and disable carcinogenic and mutagenic agents [26, 27].

Medicinal herbs have been reported to cause lots of improvements in the management of cancer, which is attributable to the chemical constituents in them [28]. These constituents of higher plants exercise various actions on tumorigenesis, on cancer cells in vitro, on tumours in experimental animals in vivo and can act together with anti-cancer medications, thus influencing their efficacy, to safeguard non-diseased tissues from noxious effects of anti-cancer remedies [29].

The antiproliferative action of the extracts and fractions of the root of *T. avicennioides* observed in this study was concentration-dependent against both the

**Table 1** Inhibitory/Lethal concentration (IC/LC<sub>50</sub> in µg/mL) of extracts and fractions of the roots of *Terminalia avicennioides* and doxorubicin (positive control) in HepG2 human liver cancer and Vero monkey kidney cell lines

Cell line	HepG2 IC <sub>50</sub> (µg/mL)	Vero LC <sub>50</sub> (µg/mL)
Methanol	356.78	801.23
Hot water	706.17	796.60
Cold water	903.11	1068.37
Ethylacetate	1085.16	432.30
Hexane	213.16	441.21
Chloroform	636.34	429.11
Butanol	366.15	345.01
Residual water	265.58	310.04
Doxorubicin (µM)	0.14	5.05

HepG2 and Vero cells. Similar reports have been described elsewhere. Saluja et al. [23] reported a decline in cell viability, suppression of cell proliferation, and induction of cell demise in a dose-reliant fashion on numerous malignant cell lines by the fruit methanolic extract of *Terminalia chebula*. It was further observed to induce apoptosis at lesser doses, but necrosis at greater concentrations. Similarly, Chen et al. [30] described the water extract of the leaf of *Terminalia catappa*, including its constituent punicalagin, having effect on bleomycin prompted genotoxicity, in Chinese hamster ovary cells. In addition, the leaf extract of *T. catappa* was observed to exert a dose-reliant antiproliferative action, on the incursion and mobility of metastatic A549 and Lewis lung carcinoma cells [31]. As well, the ethanol extract of *T. catappa* leaves inhibited the relocation capability of oral squamous cell carcinoma cells [32]. The crude extract of the bark of *Terminalia bellerica* displayed a significant cytotoxic action towards brine shrimp nauplii [33]. Plants effects on cancer cells are mediated by suppressing the enzymes stimulating cancer, repairing damaged DNA, triggering the synthesis of anticancer enzymes in the cell, raising the body's immune response, and bringing about antioxidant actions [28].

The American National Cancer Institute proffered the IC<sub>50</sub> (cytotoxic activity) for crude extracts to be less than 30 µg/mL (i.e. IC<sub>50</sub> < 30 µg/mL), as the higher benchmark considered as possibly effective after treatment for 3 days [34]. The IC<sub>50</sub>s observed in this study were relatively high. The lowest value was 213.16 µg/mL observed with hexane fraction of the extract on HepG2 cells thus, having the greatest cytotoxic effect. Steenkamp and Gouws [35], reported a higher IC<sub>50</sub> value of greater than

50 µg/mL for the extracts of *Bidens pilosa*, *Centella asiatica*, *Cnicus benedictus*, *Hypoxis hemerocallidea* and *Sutherlandia frutescens* except for *Dicoma capensis* against DU-145 prostate cancer cells, MDA-MB-231 and MCF-7 breast cancer cells and a nonmalignant breast cell line, MCF-12 A. Although the extracts appeared to be less toxic to Vero cells compared to the cancer cells in this study, it is not statistically significant. The LC<sub>50</sub> values were generally higher for all the extracts on Vero cells, except with ethylacetate fraction, where the IC<sub>50</sub> value for HepG2 cells (1085.16 µg/mL) was higher than that for Vero cells (432.30 µg/mL). Njoya et al. [36] reported that the leaf, root and bark extracts of *Sarcocephalus pobeguini* demonstrated significant anti-cancer activity against four human cancer cells (MCF-7, HeLa, Caco-2 and A549 cells) while the fruit extract of the same plant showed higher toxicity against Vero cells than on the human epithelial colorectal cancer cells.

In conclusion, this study has established that the different extracts of *T. avecinnoides* evaluated have cytotoxic effect on the Vero monkey kidney and HepG2 human liver cancer cell lines. The extracts were not more toxic to the cancer cells than to the normal Vero cells. Since different parts of a plant elaborate varying types and amounts of phytoconstituents and the root extracts were used in this study, it is plausible that the leaf or stem-bark extracts might yield more selective toxicity against the cancer line. However, this is to be determined in future studies.

#### Abbreviations

DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethyl sulphoxide; DNA: Deoxyribonucleic acid; EDTA: Ethylene diamine tetraacetic acid; FCS: Foetal calf serum; IC50: Inhibitory concentration 50; LC50: Lethal concentration 50; ME: Methanol extract; MTT: Methyl blue thiazole tetrazolium bromide; PBS: Phosphate buffered saline; SD: Standard deviation

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#### Authors' contributions

This work was carried out in collaboration by all authors. Author HAA designed and coordinated the project. HII and EMN performed the in vitro experiments and analysed the data. Author HAA prepared the extracts, conducted the preliminary screening and wrote the original draft of the manuscript. HII and EMN revised the manuscript. LJM participated in interpretation of data, provided the bench space, supervised the work and revised the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

The datasets generated and/or analysed during this study are not publicly available but can be made available from the corresponding author upon reasonable request.

## Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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