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Bertin Kipré Guédé
1: Département de Bactériologie/Virologie, Institut Pasteur de Côte d'Ivoire, Abidjan, Côte d'Ivoire
2: Unité de Formation et de Recherche des Sciences de la Nature, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire

Aya Nathalie Guessennd-Kouadio
1: Département de Bactériologie/Virologie, Institut Pasteur de Côte d'Ivoire, Abidjan, Côte d'Ivoire
2: Département de Bactériologie-Virologie, UFR-SMA, Université Félix Houphouët-Boigny, Abidjan, Côte d'Ivoire

Jules N'guessan. Kouadio
1: Département de Bactériologie/Virologie, Institut Pasteur de Côte d'Ivoire, Abidjan, Côte d'Ivoire
2: Unité de Formation et de Recherche des Sciences de la Nature, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire

Mamidou Witabouna Koné
1: Département de Bactériologie/Virologie, Institut Pasteur de Côte d'Ivoire, Abidjan, Côte d'Ivoire
2: Unité de Formation et de Recherche des Sciences de la Nature, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire
3: Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Abidjan, Côte d'Ivoire

Correspondence:
Bertin Kipré Guédé
Unité de Formation et de Recherche des Sciences de la Nature, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire
Côte d'Ivoire
BP 801 Abidjan 02, Côte d'Ivoire
Tel: +225 89264112
Email: bgkipre070[at]gmail.com

Cytotoxicity assessment of the stem bark of *Tieghemella heckelii* Pierre ex. A Chev. (Sapotaceae) towards Vero and RD human cancer Cell Lines

Bertin Kipré Guédé*, Aya Nathalie Guessennd-Kouadio, Jules N'guessan. Kouadio, Mamidou Witabouna Koné

ABSTRACT

The present study aimed at investigating the cytotoxicity of the stem bark of *Tieghemella heckelii* Pierre ex. A Chev (Sapotaceae). For this purpose, plant extracts were put into contact separately with the different Vero and human RD cancer cell lines diluted in a 96 wells microplate after 24-hour incubation at 37°C. Thereafter, the absorbance was measured every 24 hour for two days, using Elisa reader spectrophotometer. The IC_{50} values obtained from multi-dose testing of ethanol extract against Vero cell lines ranged from 0.051 to 0.192 mg/mL. The results also showed Vero cell viability of 80.2%, and a mortality rate of 94.9% against RD cell lines whereas methanol extract displayed for the same experiment an IC_{50} ranging from 0.018 to 2.98 mg/mL with a cell viability of 67%, and a mortality rate of 95.6%. From these results, it could be concluded that the methanol extract of the stem bark showed higher cytotoxic activity towards RD cell lines. As for the ethanol extract, it showed significant non-cytotoxicity towards the Vero cell lines. In the light of this evidence, it can be claimed that the plant exhibited non-cytotoxic patterns against Vero cells and has anticancer potential.

Keywords: *Tieghemella heckelii*, Sapotaceae, Cytotoxicity, Anticancer, Côte d'Ivoire.

INTRODUCTION

Since decades, plants have been used throughout the world for medicinal purposes [1], and 25% of the prescriptions against infectious diseases are plant-based [2]. Nevertheless, most of them carry along characteristics that could be either beneficial or bring about side-effects to human being or livestock. On the contrary, some groups of compounds which are derived from plants with known therapeutic virtues appeared to damage cell tissues of vital organs. For example, flavonoids which constitute a variety of active principles are made up of two groups of compounds. One is anti-viral, antimicrobial, anti-inflammatory [3], and known anti-cancer properties bearing compounds [4, 5], whereas the other one is a set of compounds, poisonous to cell mitochondria [6]. Saponins are another group of compounds, present in foodstuff, and could trigger hemolysis depending on the structural skeleton of the aglycone [7]. Coumarins also are composed of some groups of molecules which showed anti-inflammatory and antimicrobial activities [8] and anti-HIV replication [9-11], while other compounds displayed a cytotoxic activity due to phenyl group and ortho-dihydro substituents [9]. From this perspective, *Tieghemella heckelii*, identified as a medicinal plant was investigated to assess its toxicity towards normal Vero and human cancer RD cell lines. For this purpose, ethanol and methanol extracts were tested on the cells aforementioned in order to seek for apoptosis or change in cell morphology. Thus, the round shape and green color observation was an evidence of cell mortality. Also, the evaluation of the cytotoxicity was determined by the concentration of plant extract giving 50% survival (IC_{50}). This parameter was the key factor that could justify whether the plant was damageable to normal cell tissues or potentially anticarcinogenic. From the above arguments, the plant could be selected as a prospective candidate to improved traditional medicine. To begin with, plant samples were prepared, followed by the color development of cell lines, and cytotoxicity assays were carried out in the sterile 96 wells microplate. Additionally, phytochemical screening using thin layer chromatography, allowed searching for the secondary metabolites that might have been responsible for the properties displayed by the plant species.

MATERIALS AND METHODS

Plant collection

The stem bark of *Tieghemella heckelii* (Sapotaceae) was collected within the period of September to October 2014 in the rainforest of Daloa (mid-western Côte d'Ivoire). The plant species was authenticated at the herbarium of *Centre Suisse de Recherches Scientifiques en Côte d'Ivoire, Adiopodoumé* and registered under Voucher number 3021. The barks were washed, air-dried and kept in paper bags in a storage chamber at a controlled temperature (10°C) and humidity (< 40 %). Shade dried plant material was ground in a traditional mortar, and a powder was obtained using a 2-millimeter sieve.

Plant extraction

All solvents were purchased from BDH Inc. (Toronto, Ont.). Approximately 200 mg of the ground plant material was extracted in 1L of hexane with 3 washes of 1L over 48 hours. The hexane extract was filtered using a 45 micrometer MILLEX GV®, and hexane was evaporated in an incubator at 40°C. The remaining marc residue was extracted 3 times with 1L of chloroform, and after filtration and evaporation of the solvent, chloroform extract was yielded. The fresh marc residue obtained was in turn submitted to the same procedure to prepare the ethyl acetate extract. Then, the ethyl acetate residue also went through the similar process using methanol as solvent. The final step was carried out by extraction of the methanol marc residue in the same manner using distilled water, except that the solvent was evaporated at 50°C to get the aqueous extract (Figure 1).

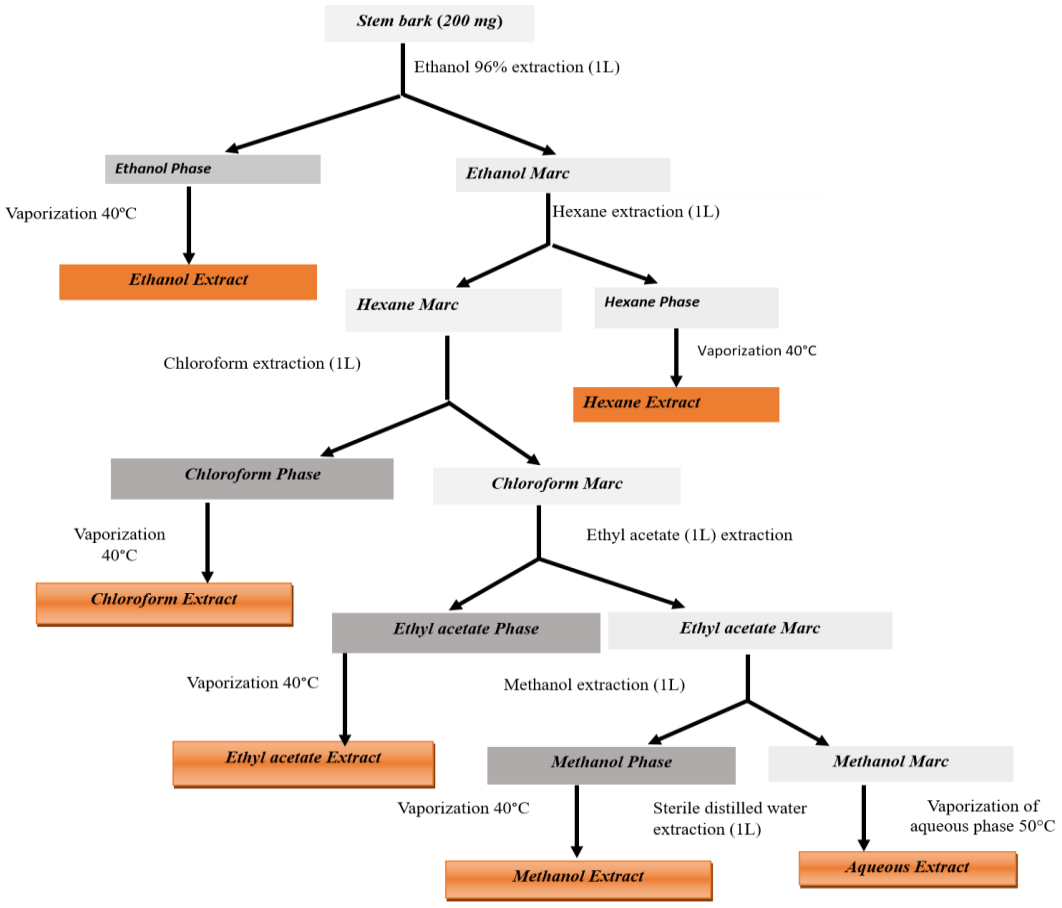

Figure 1: Synoptic scheme of stem bark extracts preparation

Assessment of plant extracts cytotoxicity

Two cell lines were used to screen plant extracts for toxicity towards cell tissues and anticancer effects: Vero E6 Dakar and Human Cancer RD CDC Atlanta. Cell lines were grown in minimum essential medium (MEM) with L-glutamine (Gibco BRL, Life Technologies, Burlington, Ont.), containing 10% fetal bovine serum (FBS) from Gibco BRL, in T-75 cm^2 tissue culture flasks and cultured at 37°C in humidified air and 5 % CO_2. The medium was changed twice a week and cells were stored in liquid nitrogen.

A stock solution of 5 mg/mL of each test extract was prepared in triplicate by completely dissolving 20 mg of extract in 0.1 mL DMSO, 20 µL Tween 80, and 3.9 mL MEM with L-glutamine (without FBS and antibiotics). Then, serial dilution of extract sample that ranged from 2.5 mg/mL to 0.0012 mg/mL was obtained from the stock solution across the 96-well microplate. Thus, the test took into account the concentration of plant extract fixed at 100 µg/mL [12], and showed how each extract reacted towards cell lines at minimum and maximum concentrations. The cytotoxicity of the plant extract was evaluated using the SRB assay [13]. The cells were fixed in 200 µL 10 % trichloroacetic acid for 30 min at 4°C, washed five times in distilled water and stained with 100 µL 0.1 % SRB in 1 % acetic acid for 15 min. The cells were washed four times in 1 % acetic acid and air-dried. At this colorimetric stable end-point, the microplates were stored at room temperature prior to absorbance measurement after 24 hour and 48 hour. The stain was solubilized in 200 µL 10 mM buffered Tris EDTA and absorbance was measured using a microplate reader at 450 nm, and 650 nm. The intensity of color is directly proportional to cell viability. The cell growth ratio in different

concentrations of each plant extract was calculated using the formula below:

$$B = \left(1 - \frac{A_b}{A_t}\right) \times 100$$

Where, B: Cell growth ratio of cell mortality (%).
At: Mean absorbance of treated cells
Ab: Mean absorbance of negative control.

The IC_{50} values of the plant extracts that produced the highest mortality rates were determined from a dose-response curve plotted in a concentration range of 2.5 to 0.0012 mg/mL. According to previous studies [14], the plant extract is cytotoxic when the IC_{50} value is lesser than 0.01 mg/mL, and non-cytotoxic in otherwise case.

Analysis of extracts of *Tieghemella heckelii* by thin layer chromatography

Analytical thin-layer chromatography (TLC) was carried out on aluminium-backed plates of silica gel 60 F_{254} (5x10 cm, 0.25 mm layer thickness; E. Merck, Darmstadt, Germany), and after elution with methanol/chloroform/water (65: 35: 5) solvent system, the plates were examined under UV light (254/366 nm). The plates were sprayed separately with Godin, Folin-Ciocalteu, lead acetate 5%, iron chloride 10%, aluminium chloride, potassium hydroxide, and Draggendorff staining reagent. Compounds were visualized after color development upon heating at 100°C for 10 min.

Statistical analysis

Results obtained from SRB assay were expressed as percentage of viable cells compared to untreated controls. Concentration response curves were generated by nonlinear curve fitting using the sigmoidal dose response, with variable slope (Graph Pad Prism 5.01 software, San Diego, CA). IC_{50} is the concentration of cytotoxic agent that led to a decrease of 50 % of signal. The logs IC_{50} of the data were compared using the Turkey test. The correlation between the cell concentration per well, and the optical density (absorbance and cell viability) was evaluated by a linear regression curve as a calibration plot in the SRB staining assay.

RESULTS

Extraction yield

The hydro-alcoholic extracts in the current study yielded a weight percentage of 7.8% for ethanol extract and 4.74% for methanol extract (Table 1).

Table 1: Extraction yield of the stem bark extracts of *Tieghemella heckelii*

Extracts	Weight (mg)	Yield (%)
Ethanol	15.60	7.8
Hexane	0.66	0.33
Chloroform	1.01	0.5
Ethyle acetate	0.97	0.48
Methanol	9.48	4.74
Aqueous	2.23	1.11

Cytotoxicity towards Vero Cell Lines

Calibration Curve

The initial calibration studies showed that at a concentration of 0.1% SRB, the assay was linear with respect to Cell number over a range of $0.5x10^4$ to $2.5x10^4$ Cells (Figure 2).

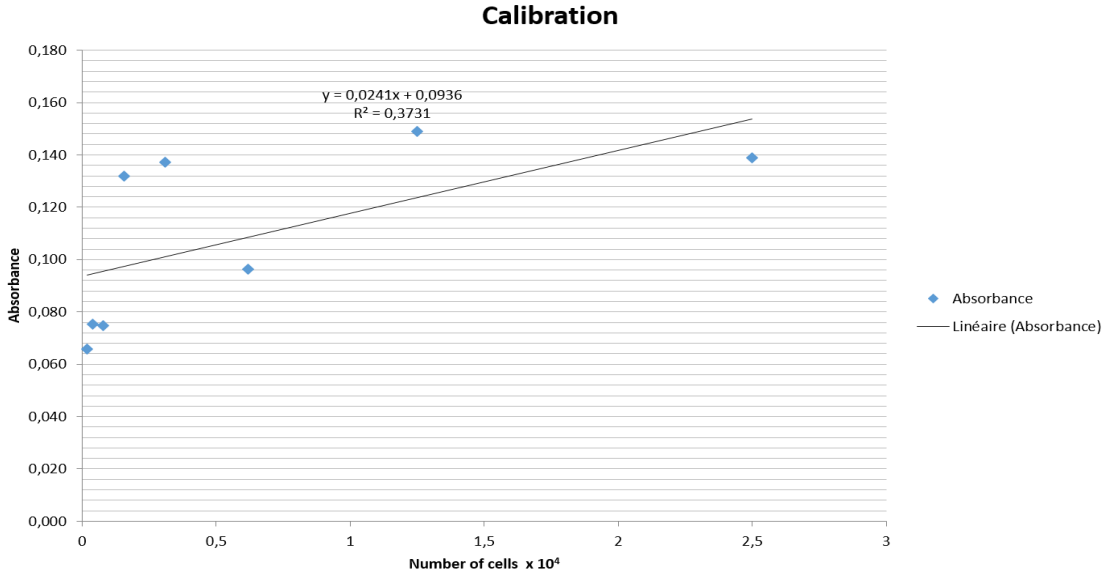

Figure 2: Vero Cells calibration curve

Cytotoxicity activity of ethanol extract towards Vero Cell Lines.

The Cytotoxicity was evaluated after a 24-hour incubation period at 37°C. The IC_{50} value obtained was 0.1922 mg/ml (Table 2) along with Cell viability of 80.2% at 0.100 mg/mL (Fig. 3). Additionally, the IC_{50} after 48-hour incubation was 0.1044 mg/mL (Table 3) with a Cell viability of 61.1% at a concentration of 0.100 mg/mL (Fig. 4)

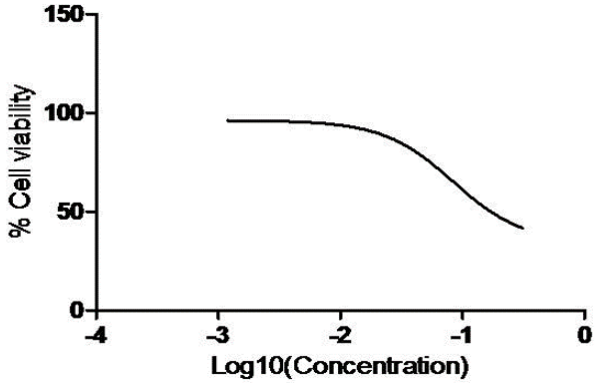

Figure 3: Vero Cells viability after 24-hour incubation at 37°C with ethanol extract

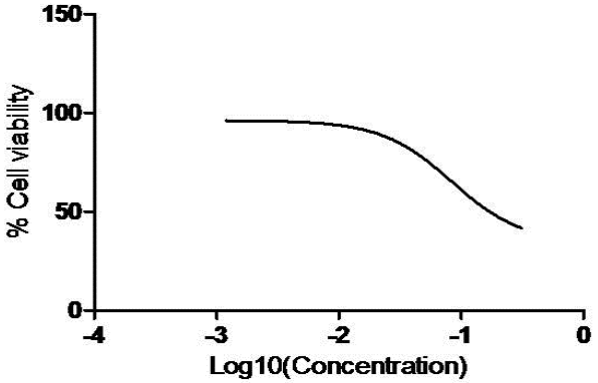

Figure 4: Vero Cells viability after 48-hour incubation at 37°C with ethanol extract

Cytotoxicity activity of methanol extract towards Vero Cell Lines. The IC_{50} value obtained was 2.98 mg/mL (Table 4) with Cell viability of 67.4% at a concentration of 0.100 mg/mL (Fig. 5). In addition, the IC_{50} after 48-hour incubation was 0.5541 mg/ml (Table 5) with a Cell viability of 74.6% at 0.100 mg/mL (Fig. 6).

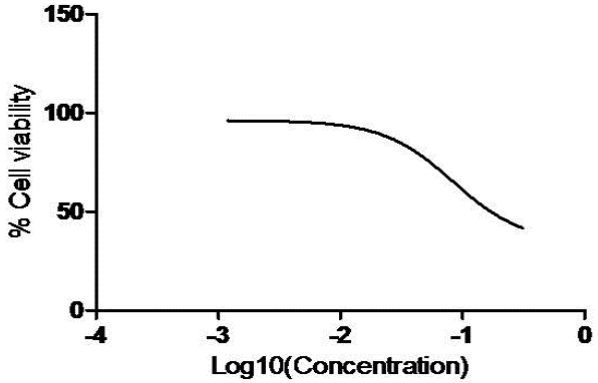

Figure 5: Vero Cells viability after 24-hour incubation at 37°C with methanol extract

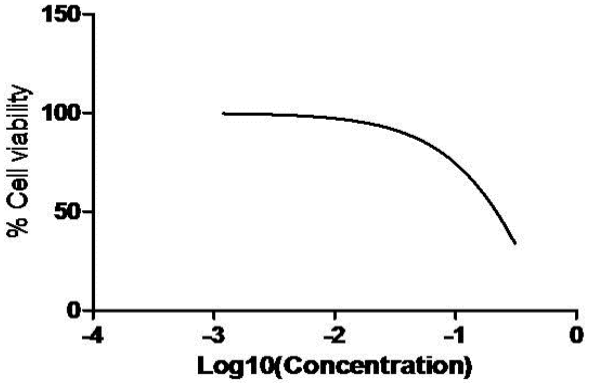

Figure 6: Vero Cells viability after 48-hour incubation at 37°C with ethanol extract

Cytotoxicity towards RD Cell Lines

Calibration Curve

Initial calibration studies, showed that at a concentration of 0.1% SRB, the assay was linear with respect to Cell number over a range of $0.5x10^4$ to $2.5x10^4$ Cells (Fig 7).

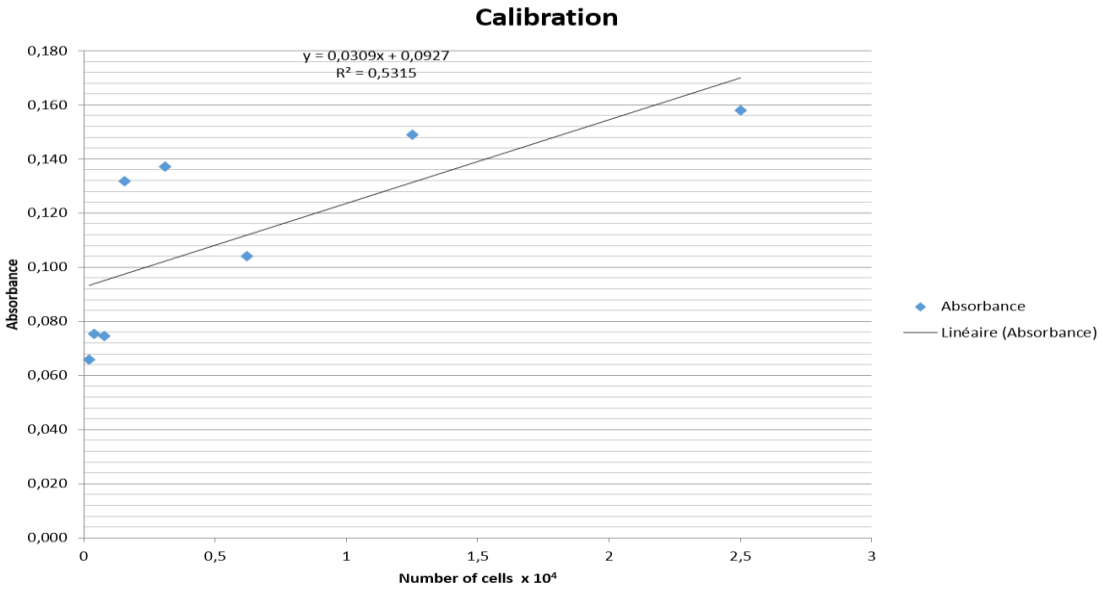

Figure 7: Human Cancer RD Cells calibration curve

Cytotoxicity activity of ethanol extract towards Human Cancer RD Cell Lines.

From the dose response curve, at a concentration of 0.100 mg/mL, the ethanol extract induced a Cell mortality of 94.9% (Fig. 8).

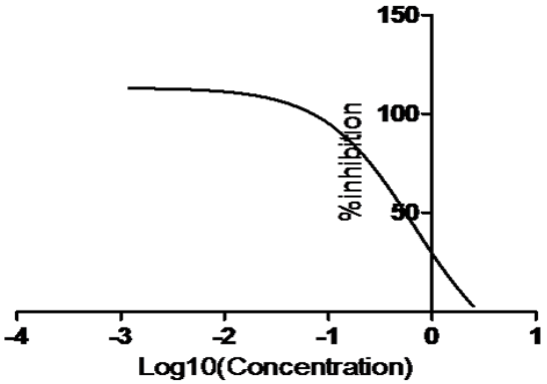

Figure 8: Human Cancer RD Cells inhibition at 37°C, 24 hour post-incubation with ethanol extract

Cytotoxicity activity of methanol extract towards Human Cancer RD Cell Lines.

From the dose response curve, at a concentration of 0.100 mg/ml, the ethanol extract induced a Cell mortality of 95.6% (Fig. 9)

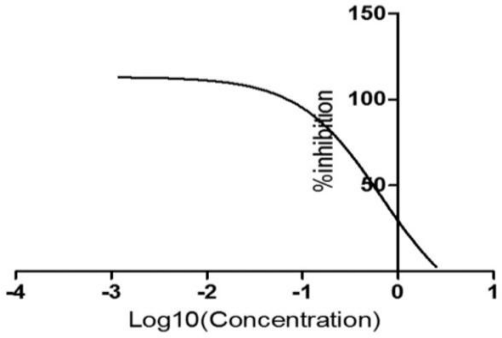

Figure 9: Human Cancer RD Cells inhibitionat 37°C, 24 hour post-incubation with methanol extract

Analysis of extracts of *Tieghemella heckelii* by thin layer chromatography

The phytochemical screening performed on methanol extract of the stem bark of *Tieghemella heckelii* revealed alkaloids, anthrones, coumarins, saponins, steroids and terpenes (Fig. 10 and 11). From chromatographic plates, the presence of anthrones was noticed with a yellow spot (Rf = 0.9 at 366 nm; Table 6); alkaloids with an orange spot (Rf = 0.8 in visible; Table 6); flavonoids with orange color (Rf = 0.37; 0.55; 0.63 at 366 nm; Table 6); saponins with blue spot (Rf = 0.3; 0.76 at 366 nm; Table 6); tannins with black spot (Rf = 0.81; 0.84 in visible; Table 6) and coumarins with green color (Rf = 0.41; 0.46 at 366 nm; Table 6).

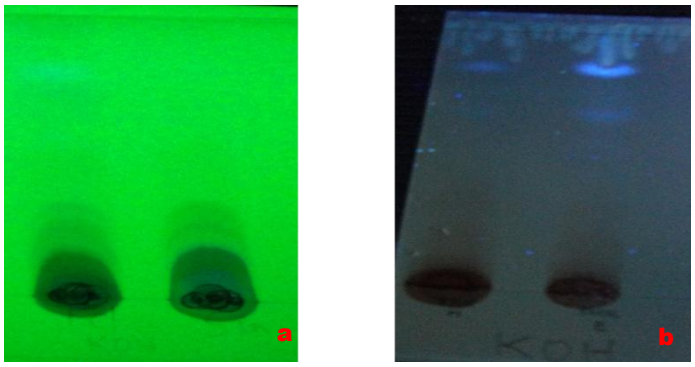

Figure 10: Chromatogram of 96% methanol extract of *Tieghemella heckelii* revealed with Potassium hydroxide reagent at 366 nm, (a) : before revelation, (b) : after revelation

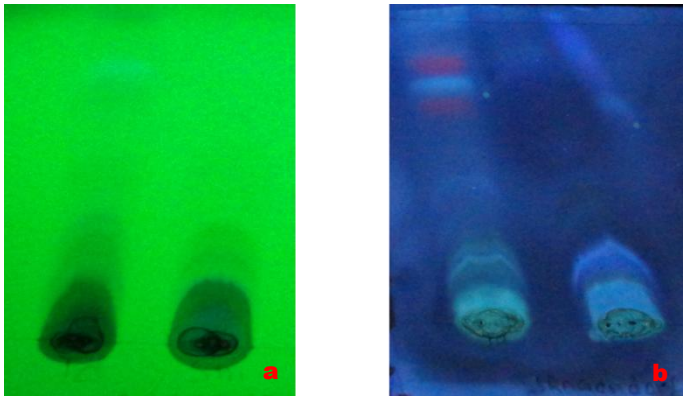

Figure 11: Chromatogram of 96% methanol extract of *Tieghemella heckelii* revealed with Draggendorff reagent at 366 nm, (a) : before revelation, (b) : after revelation

DISCUSSION

The present study was carried out based on beneficial aspects of plants as source of anticancer agents [15], and adverse effects of some natural compounds used in traditional medicine. Aside these, *in vitro* cytotoxicity data of *Tieghemella heckelii* were assumed to be currently non-existent. Therefore, the investigation performed was the first of its kind. From the results obtained, the stem bark of the plant did not show significant cytotoxicity towards the Vero cell line, by displaying an IC_{50} value higher than 0.01 mg/mL [14]. This negative result held only the first indication that the plant matrix was free of harmful substances or had an insufficient quantity of them to cause damage to Vero cells. On the other side, it showed a similarity with the stem bark methanol extract of *Manilkara discolor* (Sond) J.H.Hemsl (Sapotaceae). In fact, this medicinal plant used in Kenya showed higher value of IC_{50} greater than 0.100 mg/mL, meaning its non-cytotoxicity towards Vero cell lines [16]. Another characteristic showed by the plant in the current work was, a growth inhibition rate of 95 ± 0.07 % at 0.100 mg/mL, for both ethanol and methanol extracts towards human cancer cell line RD. This activity proved the plant to be a potential anticancer agent. The latter result confirmed the anticancer cytotoxicity of plants from Sapotaceae family, in early studies [17] on anticancer activity of ethanol extract of *Manilkara zapota* (Sapotaceae) towards breast cancer (MCF-7), and the outcome was an IC_{50} value of 0.012 mg/mL demonstrating a slight activity. Other researchers [18] studied the anticancer effect of Argania *spinosa* (L) Skeels (Sapotaceae), which inhibited leukemia cell lines at a dose of 0.100 mg/mL.

The additional experiment conducted to screen out the prospective compounds responsible for anticancer cytotoxicity revealed alkaloids, saponins, steroids, terpens, tannins, and polyphenols. This finding confirmed the existence in plant extracts of known active principles

like steroids [19], triterpenoid saponins [20, 21], flavonoids [4, 5], and tannins [22].

Table 2: Inhibition Concentration 50% of the ethanol extract towards Vero Cells, 24 hour post-incubation at 37°C

Suspension concentration (Cells/mL)	Ethanolic extract concentration (mg/mL)	95% confidence Interval	Optimum IC_{50} (mg/mL)	R^2
1.25×10^4	$2.5 \leq C \leq 0.0012$	$0.00024 < IC_{50} < 21.89$	0.0729	0.4175
0.62×10^4	$2.5 \leq C \leq 0.0012$	$0.02143 < IC_{50} < 1.724$	0.1922	0.9337
0.31×10^4	$2.5 \leq C \leq 0.0012$	$0.1832 < IC_{50} < 3.547$	0.8061	0.8945
0.155×10^4	$2.5 \leq C \leq 0.0012$	$0.00035 < IC_{50} < 7.685$	0.0519	0.4472

Data shown are mean ± SEM of three independent experiments

Table 3: Inhibition Concentration 50% of the ethanol extract towards Vero Cells, 48 hour post-incubation at 37°C

Suspension concentration (Cell/mL)	Ethanol extract concentration (mg/mL)	95% confidence Interval	Optimum IC_{50} (mg/mL)	R^2
1.25×10^4	$2.5 \leq C \leq 0.0012$	$0.02811 < IC_{50} < 0.2512$	0.08403	0.9589
0.62×10^4	$2.5 \leq C \leq 0.0012$	$0.08235 < IC_{50} < 0.1324$	0.1044	0.9911
0.31×10^4	$2.5 \leq C \leq 0.0012$	$0.07984 < IC_{50} < 0.1331$	0.1031	0.9914
0.155×10^4	$2.5 \leq C \leq 0.0012$	$0.07407 < IC_{50} < 0.1203$	0.09439	0.9915

Data shown are mean ± SEM of three independent experiments

Table 4: Inhibition Concentration 50% of the methanol extract towards Vero Cells, 24 hour-post-incubation at 37°C

Suspension concentration (Cell/mL)	Methanol extract concentration (mg/mL)	95% confidence Interval	Optimum IC_{50} (mg/mL)	R^2
1.25×10^4	$2.5 \leq C \leq 0.0012$	$0.1686 < IC_{50} < 11.73$	1.406	0.9990
0.62×10^4	$2.5 \leq C \leq 0.0012$	$2.2063 < IC_{50} < 4.5 \times 10^3$	2.98	0.2812
3.1×10^3	$2.5 \leq C \leq 0.0012$	$0.000146 < IC_{50} < 1.8 \times 10^4$	0.8415	0.9900
1.55×10^3	$2.5 \leq C \leq 0.0012$	$0.000126 < IC_{50} < 1.4 \times 10^5$	0.01849	0.9416

Data shown are mean ± SEM of three independent experiments

Table 5: Inhibition Concentration 50% of the methanol extract towards Vero Cells, 48 hour-post-incubation at 37°C

Suspension concentration (Cell/mL)	Methanol extract concentration (mg/mL)	95% confidence Interval	Optimum IC_{50} (mg/mL)	R^2
1.25×10^4	$2.5 \leq C \leq 0.0012$	$0.1241 < IC_{50} < 7.193$	0.9449	0.9819
0.62×10^4	$2.5 \leq C \leq 0.0012$	$0.00045 < IC_{50} < 6.708$	0.5541	0.9870
0.31×10^4	$2.5 \leq C \leq 0.0012$	$0.1162 < IC_{50} < 0.1495$	0.1318	0.9975
0.155×10^4	$2.5 \leq C \leq 0.0012$	Nd	Nd	Nd
0.0775×10^4	$2.5 \leq C \leq 0.0012$	Nd	Nd	Nd
0.03875×10^4	$2.5 \leq C \leq 0.0012$	Nd	Nd	Nd

Data shown are mean ± SEM of three independent experiments. Nd: Not detected

Table 6: Thin layer Chromatography of hydro-alcoholic extracts of *Tieghemella heckelii*

	Before revelation				Godin			Folin-Ciocalteu		Draggendorff		Compounds
Extracts	Visible	254nm	366nm	Rf	Visible	366 nm	Rf	Visible	Rf	Visible	Rf	
										orange	0.8	Alkaloids
MeOH 96%		blue		0.9						yellow	0.75	Nd
								blue	0.4			Polyphenols
						purple	0.37					Terpens
			blue	0.72	blue		0.3					Saponins
EtOH 80%				0.34	blue		0.76					Saponins
						blue	0.4	blue			0.37	Polyphenols

Table 6: Thin layer Chromatography of hydro-alcoholic extracts of *Tieghemella heckelii* (followed)

	Lead acetate 5%			Iron Chloride 10%			Aluminium Chloride			Potassium hydroxyde		Compounds
Extract	Visible	366nm	Rf	Visible	366 nm	Rf	366 nm	Rf	Visible	366 nm	Rf	
										yellow	0,9	Anthrones
				black		0,84						Tannins
MeOH 96%							yellow	0,63				Flavonoïds
		green	0,41									Coumarins
				black		0,81						Tannins
							yellow	0,55				Flavonoïds
EtOH 80%										blue	0,57	Anthrones
		blue	0,46									Coumarins
							yellow	0,37				Flavonoïds

CONCLUSION

Overall, the present study proved that *Tieghemella heckelii* was free of compounds that could damage Vero cell lines, and to some extent human normal cells. Additionally, the plant was revealed to be a potential anticancer agent by showing cytotoxicity towards human cancer cell lines RD. Nevertheless, final determination on its toxicity against healthy cells could only be made after examining tissues, organs, or the entire organism.

Acknowledgement

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Ethical approval

Experimental protocols were approved by the Ethical Committee of Health Sciences of Félix Houphouët-Boigny University.

Conflicts of interests

There are no conflicts of interest.

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