

In Vitro Anticancer Assessment of *Annickia chlorantha* (Oliv.) Setten & Maas Stem (Annonaceae) Bark from Democratic Republic of Congo

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Abstract

In this study, we evaluated the anti-cancer property of five bark extracts and the isolates from chloroform and ethyl acetate of *Annickia chlorantha* by the tetrazolium salt method (MTT method). The anti-cancer activity was performed on human prostate cancer cell lines PC-3 and hormone-dependent breast cancer cell lines MCF-7. Results indicated that the two isolates displayed interesting cytotoxicity towards MCF-7 cell lines with CC_{50} of 3.84 CC_{50}/mL and 4.87 CC_{50}/mL for chloroform and ethyl acetate respectively; while the total bark extracts showed CC_{50} of 24.33 CC_{50}/mL , 36.49 CC_{50}/mL and 73.52 CC_{50}/mL for chloroform, ethyl acetate and methanol extracts respectively. By the other hand on PC-3, the CC_{50} of the isolates were higher than the one on MCF-7, more than 10 CC_{50}/mL for both chloroform and ethyl acetate isolates and 49.14 CC_{50}/mL , 77.33 CC_{50}/mL , 89.38 CC_{50}/mL and 92.37 CC_{50}/mL , respectively for chloroform, ethyl acetate and methanol soluble extracts. From this study, we identified that the two isolates had anti-cancer properties against MCF-7 cell lines.

Keywords

Annickia chlorantha, Cancer, MCF-7 Cell Lines, PC-3 Cell Lines, Anti-Proliferative Compounds

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1. Introduction

Cancer is considered as an important and large family of diseases, in which abnormal cells grow and form a subset of neoplasm, with the potential to spread to other parts of the body. This abnormal growth of cells can many times lead to death. This disease remains a major public health burden in both developing and developed countries and constitutes the second leading cause of death worldwide [1]. Due to the high death rate associated with cancer and also because of serious side effects of chemotherapy and radiation therapy, many of cancer patients seek for alternative treatment complementary methods. As a real dreadful one, combating this disease is of great importance to public health. The search for new compounds with effective bioactivity is necessary as the treatment of cancer with the available anticancer drugs is often unsatisfactory due to the cytotoxicity problem to the normal cells. Plants can then be considered as solution for anti-cancer drugs and have been used for treating diseases since time immemorial [2]-[9]. A high percentage (>50%) of modern drugs in clinical use are natural products. Plants are considered and known to possess anticancer activities against different cancer cell lines. Some phytochemicals agents from plants are used for cancer treatment, including: vincristine, taxol, vinblastine, camptothecin derivatives etc., are all over the world in clinical use. Others promising agents such as betulinic acid, flavopiridol, combretastatin, A-4-roscovitine and silvestrol are in clinical or preclinical development [10] [11].

In this paper, we are reporting for the first time the study based on anticancer properties of five crude extracts of *Annickia chlorantha*, from Democratic Republic of the Congo, which has been used in African traditional medicine as anti-malarial, anti-fever and for dyeing and back ache properties [12]-[14] and for two isolates obtained from the chloroform and ethyl acetate extracts of this plant.

Even this plant has shown an interesting *in vitro* activity on plasmodium strains, it is however cytotoxic and can be oriented to the treatment of cancer [15].

2. Material and Methods

2.1. Plant Material Collection and Identification

The tested plant material (stem barks) used in the present study was collected in Abumombazi (Nord Ubangi Province, Democratic Republic of the Congo) during a field work in March-April 2014 by Professor Kotote-Nyiwa Ngbolua and was authenticated by Mr B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques). Voucher specimen N0 Ngb020EQ is on deposit at the Bioprospection Laboratory of the Department of Biology (Faculty of Sciences, University of Kinshasa).

2.2. Preparation of Extracts and Isolation

The plant material of *Annickia chlorantha* (Oliv.) Setten & Maas stem (Annonaceae) was shade dried at $\pm 27^{\circ}\text{C}$ and 900 g of powder were extracted by maceration, sequentially with petroleum ether $60^{\circ}\text{C} - 80^{\circ}\text{C}$, chloroform, ethyl acetate, methanol and methanol 80%. The extraction was optimised by repeating the maceration twice and each solvent extract was concentrated under reduced pressure and allowed to dry at room temperature and weighed to give petroleum ether $60^{\circ}\text{C} - 80^{\circ}\text{C}$, dichloromethane, ethyl acetate, methanol and aqueous methanol-solubles, respectively.

Each soluble was weighed, coded and subjected to column chromatography over silica gel 60 (0.040 - 0.63 mm [230 - 400 mesh]). The column was eluted with the mixture of petroleum ether $60^{\circ}\text{C} - 80^{\circ}\text{C}$ -ethyl acetate and collected fractions were monitored on thin layer chromatography (TLC) (silica gel 60 F₂₅₄ aluminum barking from Merck, Germany).

Chromatographic techniques (thin layer and column chromatographies) were used to perform the separation.

2.3. Cytotoxicity Assay

Anticancer activities were assayed by the standard MTT colorimetric procedure against MCF-7 and PC-3 cell lines. MCF-7 and PC-3 cell lines were obtained from National Centre for Cell Science (NCCS), Pune. It was maintained in Roswell Park Memorial Institute (RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, amphotericin (3 $\mu\text{g}/\text{mL}$), gentamycin (400 $\mu\text{g}/\text{mL}$), streptomycin (250 $\mu\text{g}/\text{mL}$), penicillin (250 units/mL) and 1 mg/ml insulin in the incubator at 5% CO₂, 37°C. All extracts were dissolved in dimethylsulfoxide (DMSO) to give a stock concentration of 100 $\mu\text{g}/\mu\text{L}$. The stock solution was then serially diluted with culture medium. The test concentrations for the extracts were 0.5, 1, 5, 10, 50 and 100 $\mu\text{g}/\text{mL}$. The test concentrations for iso-

lates were 0.1, 1 and 10 µg/mL. The concentration of DMSO never exceeded 1% in any of the 96 wells.

The cells were trypsinized at sub-conflency and 2×10^4 cells/well were seeded in 96 well plates using culture medium, the viability was tested using Trypan blue dye with help of haemocytometer and 95% of viability was confirmed. After 24 hrs, the fresh medium with the extracts were added at respective wells and kept incubated for 72 hrs. After incubation the following assays were performed. The fresh medium was changed again for all groups and 10 µl of MTT (5 mg/mL stock solution) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100 µl of DMSO. The optical density was measured at 570 nm. The percentage toxicity was calculated as following.

$$\text{Percentage toxicity} = \left\{ 1 - \left[\frac{(At - Ab)}{(Ac - Ab)} \right] \right\} \times 100$$

where: At = Absorbance value of test compound,

Ab = Absorbance value of blank and Ac = Absorbance value of control.

The graph pad prism software was used to calculate CC_{50} of the extracts/isolates.

2.4. Chemical Screening

The phytochemical screening was performed on the isolates in order to identify their chemical groups.

3. Results and Discussion

The different extracts were concentrated under reduced pressure and yielded respectively (1.67 g, 0.19%), (2.17 g, 0.24%), (4.02 g, 0.45%), (7.55 g, 0.84%) and (1.81 g, 0.20%) of solvent-soluble, for petroleum-ether 60°C - 80°C, chloroform, ethyl acetate, methanol and methanol 80%.

For the separation of the chloroform extract, 2.10 g of the crude extract were eluted on the column and a total of 220 fractions of 10 mL volumes each were collected. Similar fractions according to the TLC analysis were pooled to give four (C_1 , C_2 , C_3 and C_4) combined fractions. The combined fraction C_1 eluted with petroleum ether 60°C-80°C-ethyl acetate (8:2) afforded one spot, white powder, of the major compound. Combined fractions C_2 , C_3 and C_4 eluted with petroleum ether 60°C-80°C-ethyl acetate (7:3), (6:4), (1:1) and (3:7), respectively afforded a mixture of spots each, in small quantities.

For the ethyl acetate extract, 3.71 g of the crude extract were eluted on the column and a total of 304 fractions of 10 mL volumes each were collected. Similar fractions according to the TLC analysis were pooled to give five (E_1 , E_2 , E_3 , E_4 and E_5) combined fractions. The combined fractions E_1 and E_2 eluted with petroleum ether 60°C - 80°C-ethyl acetate (9:1) and (7:3), respectively afforded one spot each of white powder. The fraction E_2 was in faint. Combined fractions E_3 , E_4 , E_5 eluted with petroleum ether 60°C - 80°C-ethyl acetate (6:4), (1:1), (4:6) and (2:8), respectively afforded a mixture of spots each, in small quantities.

The chemical screening performed on the isolates showed positive reaction only with Mayer and Dragendorff tests. This allowed concluding that the isolates could be alkaloids. This information is correlating with the literature review [16] [17].

Table 1 shows the CC_{50} in CC_{50}/mL obtained from the five bark extracts of *A. chlorantha* comparatively to the positive control-drug Paclitaxel.

By the one hand, from the analysis, as shown in **Table 2**, the chloroform and ethyl acetate isolates of *A. chlorantha* were found to be active against human breast cancer cells line (MCF-7), with interesting CC_{50} of 3.84 CC_{50}/mL and 4.87 CC_{50}/mL respectively. It can also be seen in the same table that these CC_{50} of the isolates and the one of the chloroform extract (24.33 CC_{50}/mL) were more less and then presenting a good cytotoxicity activity then the one of the positive control which was of 27.73 CC_{50}/mL .

However, the ethyl acetate and methanol 80% extracts showed middle IC_{50} of 36.49 CC_{50}/mL and 73.52 CC_{50}/mL , respectively and more than 100 CC_{50}/mL for methanol and petroleum ether 60°C - 80°C extracts. By the other hand on human prostate cancer cells (PC-3), the isolates' CC_{50} were higher than the one on MCF-7, more than 10 CC_{50}/mL for both chloroform and ethyl acetate isolates, and 49.14 CC_{50}/mL , 77.33 CC_{50}/mL , 89.38 CC_{50}/mL and 92.37 CC_{50}/mL respectively for chloroform ethyl acetate, methanol 80% and methanol and more than 100 CC_{50}/mL for petroleum ether 60°C - 80°C extract.

Further work is in progress to elucidate the chemical structures of constituents isolated in chloroform and ethyl acetate extracts. Indeed, Wall *et al.* [8] reported that any plant extracts with a CC_{50} value below CC_{50}/mL can be accepted as a potent cytotoxic extracts/compounds. At 10 CC_{50}/mL , the percentage cytotoxicity of chloroform and ethyl acetate isolates on MCF-7 cell lines were >90%.

Figure 1 shows the MCF-7 treated with DMSO (negative control), chloroform and ethyl acetate isolates and with positive control-drug Paclitaxel at 10 CC_{50} /mL for 48 hours respectively.

As shown in **Figure 1(a)**, which is the negative control, representing the treated cells with DMSO for 48 hours, most of the cells are damaged by the cancer: morphological deterioration; loss of the inhibition of the migration by contact; insensitivity of the proliferation to the cellular density (growth unlimited). It can then be seen

Table 1. Bioactivity (CC_{50} in CC_{50} /mL) of stem bark extracts and isolates from *Annickia chlorantha*.

Extract/Isolate	CC_{50} (CC_{50} /mL)	
	MCF-7	PC-3
Petroleum-ether 60°C - 80°C	>100	>100
Chloroform	24.33	49.14
Ethyl acetate	36.49	77.33
Methanol	>100	92.37
Methanol 80%	73.52	89.38
Chloroform isolate	3.84	>10.00
Ethyl acetate isolate	4.87	>10.00
Paclitaxel	27.73	22.34

Table 2. Percentage cytotoxicity of chloroform and ethyl acetate isolates on MCF-7 cell lines.

Dose (CC_{50} /mL)	Chloroform isolate	Ethyl acetate isolate
0.1	10.58	0.00
1.0	57.82	34.73
10.0	96.95	92.69

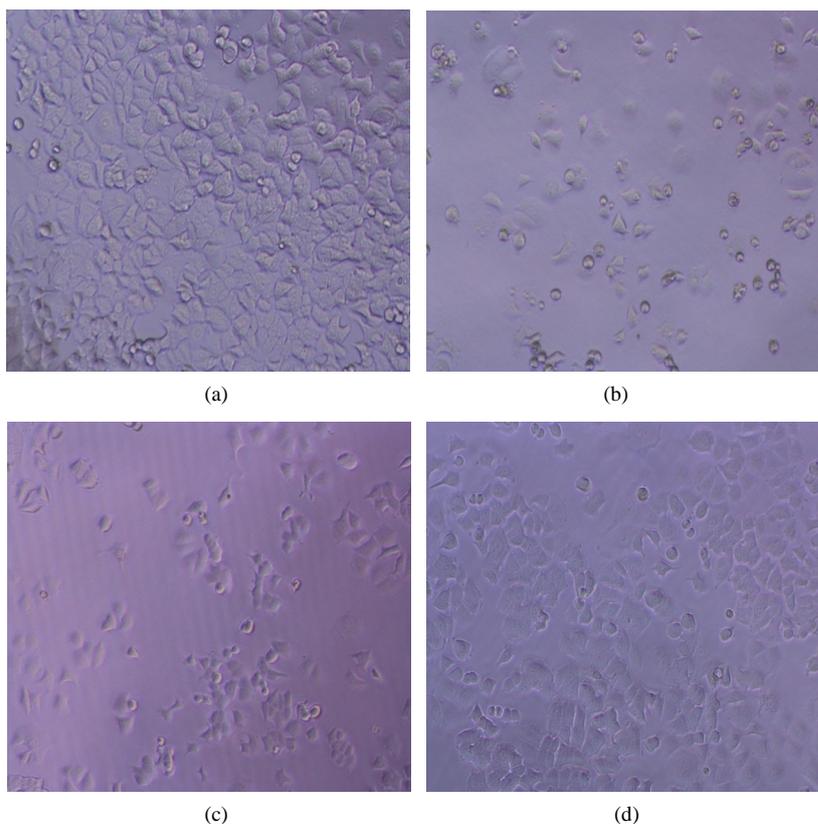


Figure 1. Morphology of untreated (a) and treated MCF-7 cells ((b)-(d)) ($\times 10$). (a) DMSO (negative control), (b) ethyl acetate isolate, (c) chloroform isolate, (d) paclitaxel (positive control).

in **Figure 1(b)** to **Figure 1(d)** that the density and the cells proliferation were decreased in comparison to the DMSO control. The morphology of the treated cells was changed and the dead cells were more in the treated group (anti-proliferative effects) compared to the negative control. We can then conclude that the isolates and the positive control have an inhibitory effect on the MCF-7 cell lines.

Figure 2 shows the morphology of PC-3 cell lines treated with DMSO (negative control), chloroform and ethyl acetate isolates and with the positive control-drug Paclitaxel at 10 CC₅₀/mL for 48 hours respectively

In **Figure 2(a)**, which represents negative control, it can be seen that most of the cells are damaged by cancer. In **Figure 2(b)** and **Figure 2(c)** which represent the treated cells at 10 CC₅₀/mL for 48 hours by the two isolates; most of the cells remain damaged and metabolically active indicating that these isolates are inactive towards PC-3 cell lines.

However in **Figure 2(d)**, representing the treated cells by the positive control-drug Paclitaxel at 10 CC₅₀/mL for 48 hours, it can be seen that the density of the damaged cells was decreased in comparison to the negative control cells. The morphology of the treated cells was changed and the dead cells were more in the treated group compared to the negative control.

It can then be confirmed that the positive control-drug Paclitaxel has anti-proliferative effect on PC-3 cell lines. However the two isolates and the negative control didn't show effective effect on the PC-3 cell lines.

The Control of cell proliferation is considered to be a potentially effective strategy for the control of tumor growth [18] [19]. The present study revealed that treatment of MCF-7 cell lines with isolates resulted in significant inhibition of the cell proliferation. The reduction of the metabolically active proliferating cells number (the loss of viability) is a good indicator of chemotherapeutic activity of *A. chlorantha* isolates and might be the result of induction of apoptosis and DNA fragmentation. At biochemical level, these compounds could inhibit proliferation of cancerous cells (MCF-7 cell lines) by down-regulation of estrogen receptor and NF- κ B signaling pathways [20]. As the conventional cancer therapies fail to completely fulfill the criteria for a successful cancer therapy due to certain limitations and side effects, the use of naturally occurring anticancer compounds could be an alternative that is safe, low-cost and convenient [21].

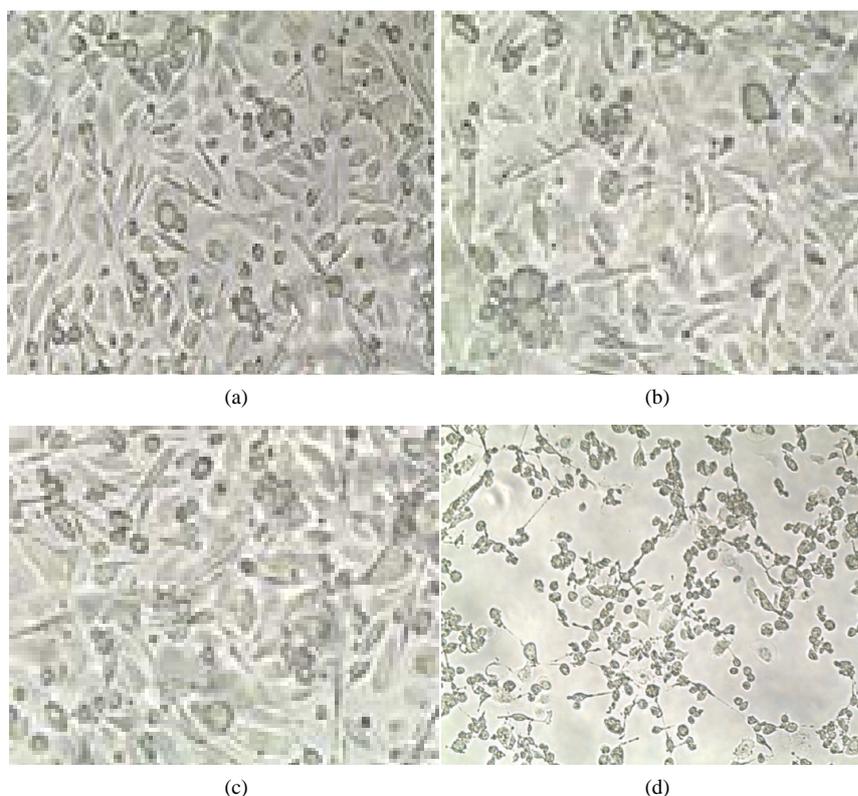


Figure 2. Morphology of untreated (a) and treated PC-3 cells ((b)-(d)) ($\times 10$). (a) DMSO (negative control), (b) ethyl acetate isolate, (c) chloroform isolate, (d) paclitaxel (positive control).

4. Conclusion

The aim of this work was to evaluate the anticancer properties of five crude extracts of *Annickia chlorantha*, and two isolates obtained from the chloroform and ethyl acetate extracts of this plant species. We have shown that the isolates have an inhibitory effect on the MCF-7 cell lines and only the positive control-drug Paclitaxel itself has anti-proliferative effect on the damaged cells PC-3; however the two isolates don't have any effect on the PC-3 cell lines. It can be suggested that *Annickia chlorantha* can be considered as a possible therapeutic agent against human breast cancer.

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