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ORIGINAL ARTICLE

Cytotoxicity Studies of the Extracts, Fractions, and Isolated Compound of *Pseudocedrela kotschyi* on Cervical Cancer (HeLa), Breast Cancer (MCF-7) and Skeletal Muscle Cancer (RD) Cells

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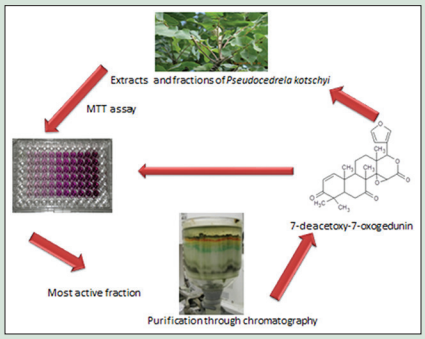
ABSTRACT

Background: This study determined the cytotoxic effects of root and stem bark extracts, fractions, and isolated compounds derived from *Pseudocedrela kotschyi* on HeLa, MCF-7, and RD cells. **Materials and Methods:** The cytotoxic activity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay against three cell lines (RD, HeLa, and MCF 7) at concentrations ranging from 0.01 to 1000 μg/mL. Isolation of crude saponin was done from the most active ethyl acetate fraction and further purified using vacuum liquid chromatography and preparative thin layer chromatographic techniques. **Results:** The cytotoxicity assay revealed that the methanol extract from the root bark and the ethyl acetate fraction from the stem bark exhibited marked anticancer activity with IC_{50} of 87.36 μg/ml and 21.53 μg/ml, respectively, on HeLa cancer cell line and 101.51 μg/mL and 38.46 μg/mL, respectively, on RD cell line. These values are comparable with that obtained from vinblastine and methotrexate used as standard drugs (IC_{50} values of 0.01 μg/mL and 0.05 μg/mL, respectively). The isolated crude saponins also gave IC_{50} values of 5.28 μg/mL and 81.52 μg/mL against the RD cell lines and IC_{50} values of 1.05 μg/mL and 86.8 μg/mL for the MCF 7 cancer cell lines. PTLC led to the isolation of a compound from the crude saponin which was identified as 7-deacetoxy-7-oxogedunin through spectroscopic analysis and comparison with literature data. **Conclusions:** *P. kotschyi* could be considered as a potential source of chemotherapeutic agent. However, further research to determine the exact mechanism of action needs to be carried out.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, 7-deacetoxy-7-oxogedunin, Cytotoxicity, HeLa, MCF-7, *Pseudocedrela kotschyi,* RD

SUMMARY

- *Pseudocedrela kotschyi* methanol extract from the root bark and the ethyl acetate fraction from the stem bark exhibited marked anticancer activity on HeLa, MCF-7, and RD cell lines
- 7-deacetoxy-7-oxogedunin isolated as a white crystalline substance from the most active ethyl acetate fraction contributed to the observed activity.



Abbreviations Used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TLC: Thin layer chromatography; VLC: Vacuum liquid chromatography; PTLC: Preparative thin layer chromatographic; NMR: Nuclear magnetic resonance; FBS: Fetal bovine serum; DMEM: Dulbecco's modified Eagle's medium; PBS: Phosphate buffer saline; FHI: Forest Herbarium Ibadan; DMSO: Dimethylsuphoxide; SEM: Standard error of mean

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INTRODUCTION

Cancer is one of the most prominent diseases in humans and is still not curable in most cases.[1] In the United States, about 1,665,540 new cancer cases and 585,720 cancer deaths are predicted in 2014.[2] A normal cell, in which the DNA or other components are externally damaged by various causes undergo apoptotic cell death which is a self-destructive metabolism according to the genetically encoded cell death signal.[3] Anticancer agents, however, are mainly related to their therapeutic role in a damaged system. The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells while disturbing their proliferation.[4] However, available drugs for the management of cancers are often limited by their nonspecific actions and their toxicity to normal cells.[5]

Currently, there is a considerable scientific and commercial interest in continuing the discovery of new anticancer agents from natural products[6] with the strategy of chemotherapy protocols being killing the cancer cells with no toxic effect on the host.[1] Many natural products have found usefulness as direct chemotherapeutic agents[7,8] while several others can decrease the resistance of cancer cells to chemotherapeutic drugs, improve the efficiency of chemotherapeutic agents, and reduce the adverse side effects experienced due to chemotherapy.[9] Although many anticancer drugs are in various stages of development with over 500 under clinical trials,[10] more effective and less toxic drugs are still needed.

Pseudocedrela kotschyi (*Meliaceae*) is a medicinal plant whose therapeutic value no doubt has a folkloric background as it has been used in the

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treatment of various diseases by traditional healers. In Togo, the root bark of *P. kotschyi* is used in treating gastrointestinal diseases, rheumatism, and as a febrifuge,[11] whereas the roots and leaves are used to treat rheumatism and dysentery in Nigeria, where it also serves as an ingredient for arrow poison in the Northern part.[12] In Ghana, the twigs and leaves are used for the treatment of malaria and stomach aches,[13] and for toothache and internal wound in North Côte d'Ivoire.[14] The decoction is also used for washing ulcers[15] as well as for the management of cancer.[16]

Various extracts of the plant have been reported to possess antidiabetic, antiepileptic, analgesic, antipyretic,[17,18] and antimicrobial properties.[14] Other biological activities reported for the plant include antinociceptive and anti-inflammatory[19] and *in vitro* growth inhibition of the schizont stage of *Plasmodium falciparum*.[20] The aqueous extract of the leaf reduced the onset and the duration of the sleeping time in rats.[21]

Several bioactive compounds have been isolated from the plant. These include pseudocedrelin from the bark with piscidal activity.[12] The leaves contain 3-*O*-rhamnosides of myricetin and quercetin, as well as their 3-*O*-glucosides (or galactosides).[22] Some limonoids, pseudrelones A, B, and C, and 7-desacetoxy-7-oxogedunin were isolated from the wood oil.[23,24]

In this study, we investigated the cytotoxic effect of extract, fractions, and isolated compound of *P. kotschyi* on cervical cancer (HeLa), breast cancer (MCF-7), and skeletal muscle cancer (RD) cells.

MATERIALS AND METHODS

Reagents

Fetal bovine serum, Dulbecco's modified Eagle's medium, and phosphate buffer saline were purchased from Gibco. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma. Vinblastine and Methotrexate were sourced locally. Human cancer cell lines (RD - skeletal muscle cancer cell lines, HeLa - cervical cancer cell line, and MCF-7 - breast cancer cell lines) were obtained from Tissue Culture Laboratory, Virology Department of College of Medicine, University College Hospital, Ibadan, Nigeria. All other solvents and reagents were of analytical grade.

Collection of plant material

The plants materials were collected in October, 2013 from Saki in Oyo State, Nigeria. The plant was identified and authenticated by Mr. Afilaka at Forest Herbarium Ibadan (FHI) where herbarium specimen with FHI Number 110,100 was deposited. Plant parts (Root and stem bark) collected were chopped and pulverized then stored in an appropriate container until required for use.

Preparation of extracts and fractions

Dried powdered stem bark (1.3 kg) and 1.6 kg of the powdered root bark of *P. kotschyi* were macerated separately with distilled methanol at a room temperature for 72 h. Each was filtered and the filtrates evaporated to dryness *in vacuo*. The resulting dried extracts were stored appropriately till further use.

Phytochemical screening

Preliminary phytochemical screening of the methanol extracts was done using standard procedures.[25] The extracts were tested for alkaloids, anthraquinones, tannins, saponins, cardiac glycosides, and flavonoids.

Cytotoxicity assay

The cytotoxicity assay of the samples was determined by MTT colorimetric assay method[26] with slight modifications. The test samples were diluted to obtain concentration range of 1000, 100, 10, 1, 0.1, and 0.01 µg/ml, respectively. To the confluent monolayer plate containing the cell lines that has been incubated for 24 h, 50 µl of 1 mg/ml of the stock test sample was added, 100 µl of growth medium was added, and 50 µl of the growth medium was used to make up the wells containing the test samples. After 72 h of incubation at 37°C in 5% CO_2 incubator, the supernatant was removed, and 25 µl of MTT reagent (2 mg/ml) was added into each well. After incubating at 37°C for 2 h, 125 µl of dimethylsuphoxide was added to each well to solubilize the formed formazan precipitate and then shaken for another 15 min. The absorbance was then determined by an ELISA reader at a wavelength of 490 nm. Control wells received only the media without the tested compound. The conventional anticancer drugs, vinblastine and methotrexate, were used as positive controls. The inhibition of cellular growth by the tested sample was calculated as the percent inhibitory activity and expressed as the IC_{50} value (concentration of the tested sample to inhibit 50% growth of the cells).

% inhibition = $(1 - [A_1/A_0] \times 100)$

where;

A_0 is the absorbance of the control and

A_1 is the absorbance of the extracts.

Statistical analysis

All assays were carried out in triplicates and data presented as mean ± standard error of mean (SEM). EXCEL package 2010 was used for the analyses of mean, SEM, and percentage inhibition while linear regression analysis was used to determine the IC_{50}.

Isolation of saponins

Saponin was isolated from the most active extract and fraction. This was done according to the standard method.[27] Twenty grams of the crude root extract was weighed and dissolved in a little amount of distilled methanol and120 ml of diethyl ether was added. The diethyl ether fraction was discarded. To the residue, 350 ml of N-butanol was added and the N-butanol fraction was collected. To this, 100 ml of 5% sodium chloride was added to wash, and the residue was collected and concentrated *in vacuo*. For the ethyl acetate fraction of the stem, 14 g of the fraction was weighed and subjected to the same procedure as described above.

Isolation of bioactive compound

Ten grams of the ethyl acetate fraction was subjected to vacuum liquid chromatography (VLC) on silica gel using a different ratio of N-hexane, ethyl acetate, and methanol successively in a gradient manner. Subfractions were collected and they were bulked into seven based on their thin layer chromatography (TLC) profile. The bulked fractions were further purified by preparative TLC (PTLC) leading to the isolation of compound 1.

Analysis of isolated compound

The isolated compound was subjected to spectroscopic analysis including 1H, 13C, to determine its structure.

RESULTS

Phytochemical screening

The results of the phytochemical screening which revealed the abundant presence of saponins and tannins are presented in Table 1.

Cytotoxicity results

Cytotoxicity results of methanol extracts and fractions of *P. kotschyi* on HeLa cell lines expressed as mean ± SEM.

Table 1: Phytochemical analysis of *Pseudocedrela kotschyi*

Test	Stem bark	Root bark
Alkaloids	–	–
Anthraquinones	–	–
Tannins	+++	+++
Saponins	+++	+++
Cardiac glycosides	–	–
Flavonoid	–	–
Phenol	+	+

The cytotoxicity results of methanol extracts and fractions of *P. kotschyi* on HeLa cell lines showed that the ethyl acetate fraction of the stem bark had the highest activity [Figure 1].

The cytotoxicity results of methanol extracts and fractions of *P. kotschyi* on RD cell lines expressed as mean ± SEM.

The cytotoxicity results of methanol extracts and fractions of *P. kotschyi* on RD cell lines revealed that the ethyl acetate fraction of the stem bark had the highest activity followed by the methanol extract of the root bark [Figure 2].

Cytotoxicity effect of the isolated crude saponins on MCF-7 cell lines: From the cytotoxicity activity of the isolated crude saponins on MCF-7 cell lines, it was observed that saponin isolated from the ethyl acetate fraction of the stem bark had better activity [Figure 3].

Cytotoxicity effect of the isolated crude saponins on RD cell lines: The cytotoxicity effect of the isolated crude saponins showed that saponin from the ethyl acetate fraction had a better activity on the RD cell lines [Figure 4].

Spectroscopic data of compound 1

Nuclear magnetic resonance (NMR) data (500MHz, $CDCl_3$) are as follows: ^{1}H NMR; 7.10 (dd. H-1), 5.93 (d, H-2), 2.18 (dd, H-5), 2.94, 2.41 (dd, H-6), 2.21 (dd, H-9), 1.78, 1.98 (m, H-11), 1.48, 1.85 (m, H-12), 3.82 (s, H-15), 5.48 (s, H-17), 6.38 (dd, H-19), 7.41 (dd, H-20), 7.43 (dd, H-21), 1.15 (s, Me-22), 1.23 (s, Me-23), 1.37 (s, Me-24), 1.14 (s, Me-25), and 1.17 (s, Me-26). ^{13}C NMR; 155.91 (C-1),126.45 (C-2), 203.23 (C-3), 45.23 (C-4), 54.59 (C-5), 36.71 (C-6), 208.15 (C-7), 53.43 (C-8), 47.62 (C-9), 39.60 (C-10), 17.21 (C-11), 32.21 (C-12), 37.73 (C-13), 65.58 (C-14), 53.62 (C-15), 166.83 (C-16), 78.00 (C-17), 120.19 (C-18), 109.78 (C-19), 143.12 (C-20), 141.03 (C-21), 20.94 (C-22), 17.14 (C-23), 19.85 (C-24), 20.65 (C-25), 27.00 (C-26).

DISCUSSION AND CONCLUSIONS

Cancer is of great concern in both developing and developed countries of the world, and the current forms of management are chemotherapy, radiotherapy, and surgery. However, most of the chemotherapeutic agents in use today have several side effects such as hair loss, bone marrow suppression, gastrointestinal lesions, cardiac toxicity, and neurologic dysfunctions.[1,28,29]

Medicinal plants both from land and sea, as well as bioactive compounds isolated from them, have found usefulness in the management of several diseases including cancer.[30,31] Several active compounds such as flavonoids, diterpenoids, triterpenoids, and alkaloids have been shown to possess anticancer effects[32,33] Specifically, saponins, though extremely poisonous as they can cause hemolysis of blood cells and result in cattle poisoning,[34] are important therapeutic agents shown to have hypolipidemic and anticancer activities.[35] They are also necessary for activity of cardiac glycosides.

This study focused on the cytotoxicity assay of the extracts, fractions, and isolated compounds of *P. kotschyi*. The result obtained from the cytotoxicity activity carried out on the methanol extracts, and the fractions of the plant stem and root bark on HeLa cancer cell lines is expressed as IC_{50} values and presented in Figure 1. The lower the IC_{50} values the greater the activity, whereas the higher the IC_{50} values the

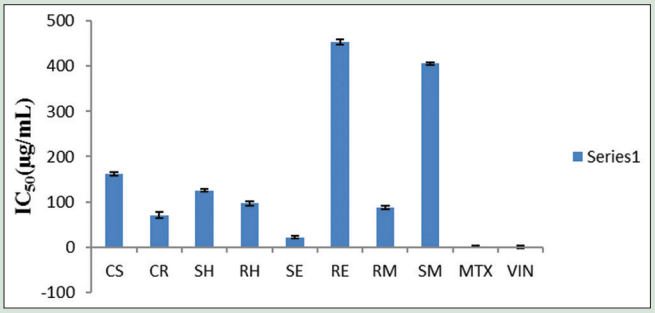


Figure 1: Cytotoxicity activity of methanol extract and fractions of the stem and root bark of *Pseudocedrela kotschyi* against HeLa cell lines. CS: Crude stem; CR: Crude root; SH: Hexane stem; RH: Root hexane; SE: Ethyl acetate stem; RE: Ethyl acetate root; RM: Methanol root; SM: Methanol stem; MTX: Methotrexate; VIN: Vinblastine

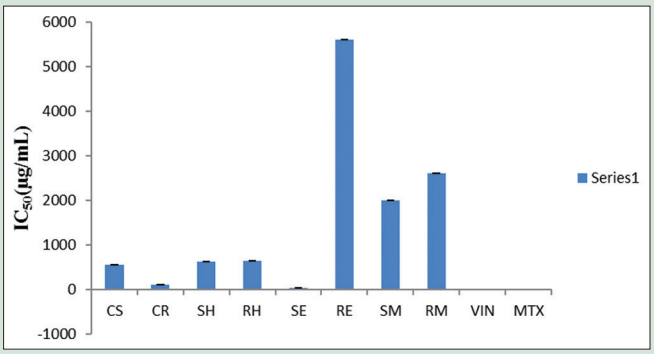


Figure 2: Cytotoxicity activity of methanol extract and fractions of the stem and root bark of *Pseudocedrela kotschyi* against RD cell line. CS: Crude stem; CR: Crude root; SH: Hexane stem; RH: Root hexane; SE: Ethyl acetate stem; RE: Ethyl acetate root; RM: Methanol root; SM: Methanol stem; MTX: Methotrexate; VIN: Vinblastine

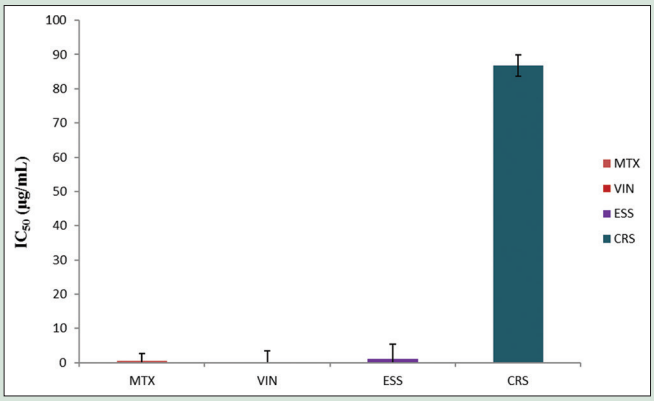


Figure 3: Cytotoxicity effect of the isolated crude saponins from the ethyl acetate fraction of the stem and methanol root bark of *Pseudocedrela kotschyi* on MCF - 7 cell lines. MTX: Methotrexate; VIN: Vinblastine; ESS: Ethyl acetate fraction saponin; CRS: Methanolic extract saponin (root)

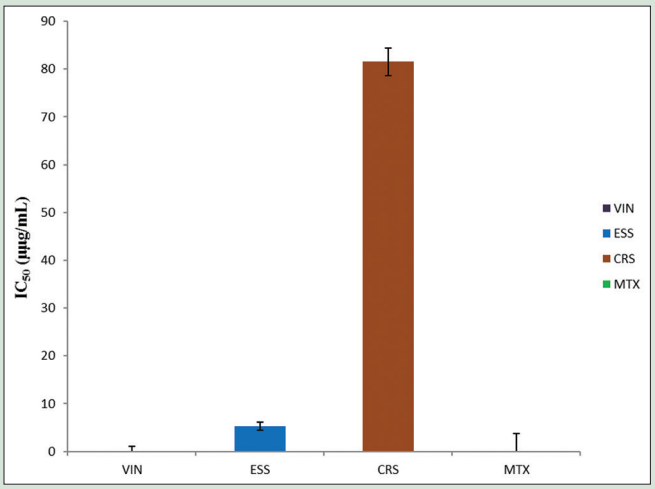


Figure 4: Cytotoxicity effect of the isolated crude saponins from the ethyl acetate fraction of the stem and methanol root bark of *Pseudocedrela kotschyi* on RD cell line. MTX: Methotrexate; VIN: Vinblastine; ESS: Ethyl acetate fraction; CRS: Methanolic extract saponin (root

lower the anticancer activity. The result revealed that the ethyl acetate fraction of the stem bark of the plant showed the highest anticancer activity with IC_{50} value of 21.53 µg/ml, whereas the ethyl acetate fraction of the root showed the lowest anticancer activity with IC_{50} value of 453.3 µg/ml though the methanol extract of the root bark had IC_{50} of 70.03 µg/ml. These results are comparable to the IC_{50} values obtained from the standard drugs such as methotrexate and vinblastine which were 0.53 µg/mL and 0.03 µg/mL, respectively. This implies that the methanol extract of the root bark had a greater anticancer activity on HeLa cell lines than the fractions, whereas the ethyl acetate fraction from the stem bark has a greater anticancer activity than the methanol extract.

Furthermore, the result of the anticancer activity of the methanol extracts and fractions of the plant on RD cancer cell lines shows that the ethyl acetate fraction from the stem bark had the highest anticancer activity with IC_{50} value of 38.46 µg/ml followed by the methanol extract from the root bark with IC_{50} value of 101.5 µg/ml [Figure 2]. This result is also comparable with that of the standard drugs that were used in this assay methotrexate and vinblastine with IC_{50} values of 0.05 µg/ml and 0.07 µg/ml, respectively.

These results are consistent with the ethnomedicinal use of *P. kotschyi* in the treatment of cancer as earlier reported,[36] and also with the data collected from our ethnobotanical survey done in Oke-Ogun region of Oyo State reporting the use of the plant in the treatment of tumors. The result also suggests that the plant can serve as a lead in the development of new anticancer agent.

As a result of the observed high activity in the ethyl acetate fraction of the stem bark (IC_{50}-21.53 µg/ml and 38.46 µg/ml) and the methanol extract of the root bark (IC_{50}-70.03 µg/ml and 101.51 µg/ml) on HeLa and RD cell lines, respectively, and coupled with the fact that the phytochemical screening revealed high level of saponins in the plant, direct isolation of saponin using standard method was done. The crude saponins obtained were then subjected to anticancer assay against RD and MCF-7 cancer cell lines.

The anticancer assay of the isolated crude saponins on MCF-7 cancer cell lines shows that saponin from the ethyl acetate fraction demonstrated high anticancer activity comparable to that of the standard drugs [Figure 3]. Saponin from the ethyl acetate had IC_{50} value of 1.05 µg/ml, whereas saponin from the methanol extract of the root had IC_{50} value of 86.8 µg/ml. The two standard drugs such as methotrexate and vinblastine had 0.03 µg/ml and 0.53 µg/ml, respectively.

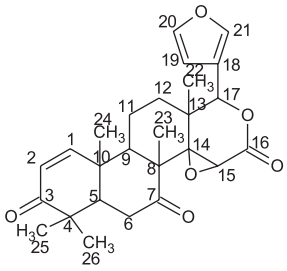
Saponins were also tested on RD cancer cell line and the result showed that saponin from the ethyl acetate fraction had IC_{50} value of 5.28 µg/ml while that from the methanol extract of the root had IC_{50} value of 81.52 µg/ml [Figure 4] with the standard drugs such as methotrexate and vinblastine having IC_{50} values of 0.05 µg/ml and 0.01 µg/ml, respectively.

In general, it was consistently observed that the stem bark had better anticancer activity than the root bark. This is also reflected in the ethyl acetate fractions as well as in saponins isolated from the two samples. As earlier mentioned, saponins have been well implicated in cancer chemotherapy.[35] Thus, the activity of this plant could be attributed to the abundant presence of saponins as revealed by the phytochemical screening [Table 1].

The most active ethyl acetate fraction was subjected to VLC using gradient solvent elution. Several fractions pooled into seven based on TLC pattern were obtained. From the pooled fraction, one major compound was isolated using PTLC.

Compound 1 was isolated as white crystals. The 13C NMR data showed that there were 5 CH3, 3 CH2, 9 CH, and 9 Cq. Thus, compound 1 is a C-26 carbon compound. Of diagnostic importance is the quaternary carbons C-3, C-7, C-16, and C-18 resonating at δc 203.23, δc 208.15, δc 166.83, and δc 120.19 as well as the methine carbons C-1, C-2, C-19, C-20, and C-21 resonating at δc 155.91, δc 126.45, δc 109.78, δc 143.12, and δc 141.03. On analysis of the full spectra of compound 1 and comparison with already published data,[37] it was identified as 7-deacetoxy-7-oxogedunin.

This compound, which is a limonoid, has been previously isolated from the plant[24] and has been reported for several activities including antiplasmodial,[38] insecticidal,[39] and anti-HIV.[40] However, this report represents the first association of the compound with cytotoxic activity.



7-deacetoxy-7-oxogedunin

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Conflicts of interest

There are no conflicts of interest.

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