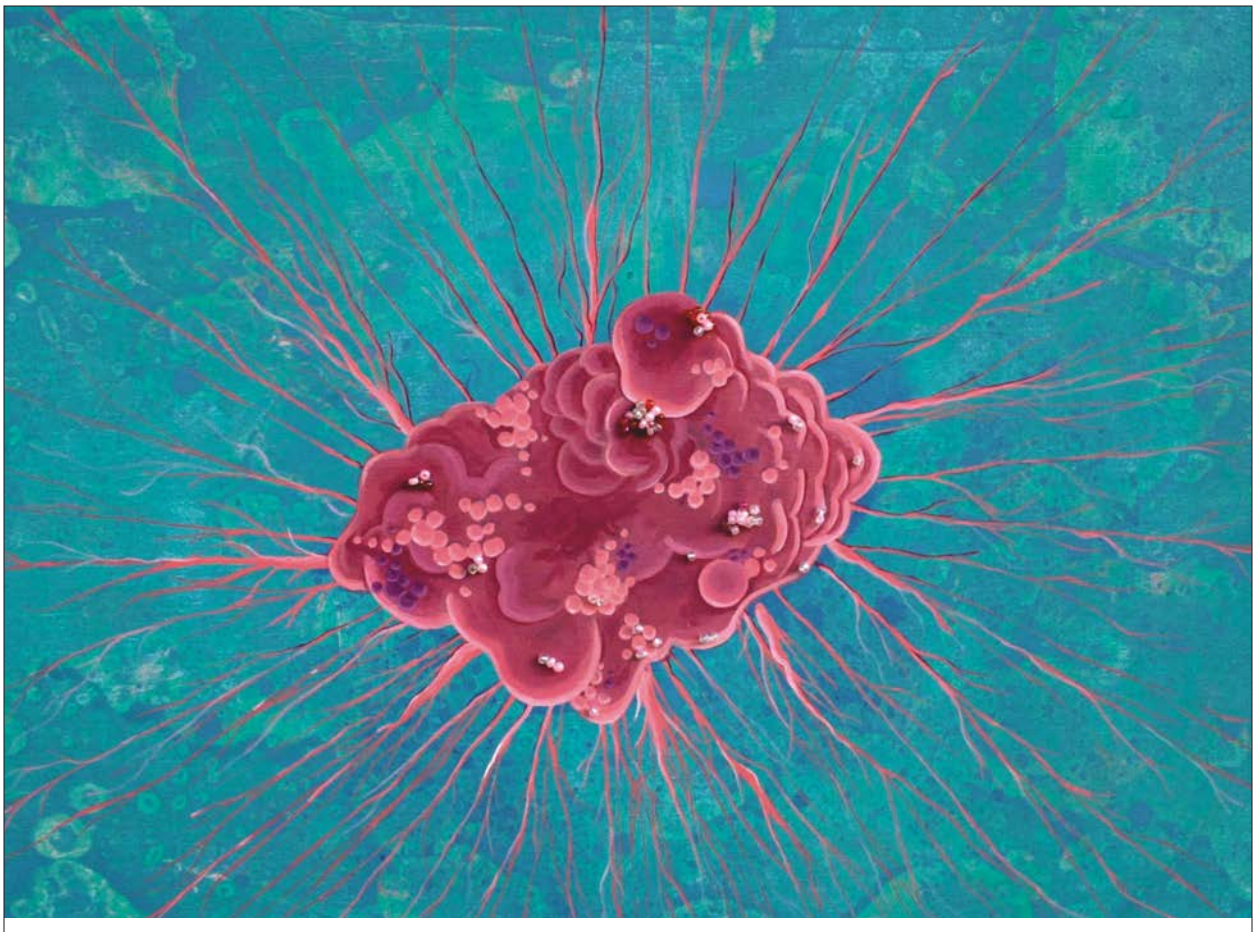

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Research Article
Evaluation of Cytotoxicity and Phytochemical Screening of *Pycnanthus angolensis* (Welw.) Warb Dichloromethane and Ethyl Acetate Stem Bark Extracts against HeLa Cells

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Abstract

Background and Objective: Cancer is one of the major life threatening diseases in the world today. Medicinal plants are proven to be promising source of anticancer drugs. The objective of this study was to evaluate the phytochemicals and cytotoxic potential of dichloromethane and ethyl acetate stem bark extracts from *Pycnanthus angolensis* (Welw.) (*P. Angolensis*) Warb on cancer cell line-human cervix adenocarcinoma (HeLa) cells. **Methodology:** Based on resazurin assay, the cytotoxicity for dichloromethane and ethyl acetate stem bark extracts of *P. angolensis* was evaluated. The phytochemical screening of the extracts was analyzed using standard method. The *in vitro* cytotoxicity was assessed against human cervix adenocarcinoma (HeLa) cells using resazurin assay with the reference drug emetine. Statistical analysis of the data was carried out by one way ANOVA. Graphs were prepared by Prism software. **Results:** Phytochemical screening revealed the presence of glycosides, alkaloids, saponin, steroids, tannins, flavonoids and terpenoids. A value of $p<0.05$, <0.01 and <0.0001 were considered to be significant, very significant and highly significant. The results showed the significant decrease of the viability of the cells in a concentration-dependent manner. The ethyl acetate extract of *P. angolensis* showed a significant cytotoxicity with CC_{50} 90.27 ug mL^{-1}. The dichloromethane extract demonstrated a higher cytotoxic activity with CC_{50} 26.66 ug mL^{-1} <30 ug mL^{-1} a limit recommended for cytotoxicity for extract. **Conclusion:** The cytotoxicity study revealed *P. angolensis* extracts as having potential inhibitory effect on HeLa cells. Thus, extracts are promising cancer drug and their significance may increase in future in view of the lack of unwanted side effects characteristic for emetine compound currently in clinical use for treatment of cancer.

Key words: Cytotoxicity, HeLa cells, phytochemical screening, *Pycnanthus angolensis*, resazurin assay

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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

Since the dawn of human civilization, human beings have found remedies within their habitat and have adopted different therapeutic strategies depending upon climatic, phytogeographic, sociocultural, floral and faunal characteristics[1]. Traditional systems thus contain beliefs and practices in order to avoid, prevent, or avert ailments, which constitute traditional preventive medicine. The use of medicinal herbs and herbal medicine is an age-old tradition and the recent progress in modern therapeutics has stimulated the use of natural product worldwide for diverse ailments and diseases[1].

Cancer is one of the deadly diseases which severely effect the human population[2]. Statistical survey indicates that more than 11 million people are diagnosed with cancer each year and cancer accounts for about 7 million deaths/year (12.5% of deaths worldwide), making this disease a huge factor in worldwide mortality. The incidence of cancer is expected to increase continuously as the world population ages and it has been estimated that there will be 16 million new cancer cases every year by 2020[3].

Cervical-uterine cancer is the cause of many deaths worldwide. According to the world cancer statistics, cervical cancer is the fourth most common cancer affecting women globally and the second most common cancer in developing areas[4,5] with an estimated global incidence of 530,000 new cases and 270,000 deaths annually[6]. The preventive vaccination and organized screening programs are critical in identifying the cervical cancer before it enters advanced stages. Moreover, the treatments are often less effective in advanced stages compared with early interventions[7]. Thus, development of effective therapeutics with less side effect for early treatment of this life threatening disease is required.

Scientific and research interest is drawing its attention towards naturally-derived compounds as they are considered to have less toxic side effects compared to current treatments such as chemotherapy[2].

Natural products provide 70% anticancer compounds[8]. Medicinal plants have historically proven their value as a source of molecules with therapeutic potential and nowadays still represent an important pool for the identification of novel drug leads[9]. The Plant Kingdom are being investigated for their anticancer activities leading to the development of new clinical drugs[7].

The plant *Pycnanthus angolensis*, commonly known as African nutmeg, is a tropical plant belonging to the *Myristicaceae* family and having a geographical distribution stretching across Western Africa from Guinea to Cameroon, including the countries of Sierra Leone, Liberia, Cote D'Ivoire, Ghana, Togo, Benin, Nigeria, Equatorial Guinea, Angola and Uganda. The plant thrives well in secondary forests, growing up to 120 feet tall and produces fruit annually, typically between September and April. The oblong-shaped fruits, about 1.5 inches long, contain oil-rich seeds encased in a hard shell. The seeds are ready for harvest between December and April. The indigenous populations have devised a variety of uses for virtually all parts of the plant, ranging from incorporation of the plant in furniture, condiments, soaps and cattle feed to medicinal uses.

The ethnopharmacological survey of the plant, *Pycnanthus angolensis*, according to a study by Agyare *et al.*[10] confirms the potency of aqueous extracts of the plant for wound healing and it establishes antioxidant activities of the ethanolic extracts of the plant. The plant was reported to be good for stomach ulcer treatment due to its antiadhesive activity against *Helicobacter pylori* on human stomach cells[10]. Aside these reports and to the best of our knowledge, the pharmacological effect of the stem bark of the plant against HeLa cells has not been reported. The aim of this study was to evaluate for the first time, the effects of various plant extracts from *P. angolensis* on cancer cell line in order to further determine their usefulness.

MATERIALS AND METHODS

Raw materials and chemicals

Plants: The stem bark of *Pycnanthus angolensis* was obtained from Zakibiam in Benue State, Nigeria. The plant taxanomic identification was established by Mr Ibe Ndukwe of the Forestry department, Michael Okpara University of Agriculture Umudike, Abia state, Nigeria in Jan, 2016. Voucher samples of the plants were deposited in the Herbarium of Michael Okpara University of Agriculture Umudike, Abia state, Nigeria. The study was carried out in the Department of Chemistry, Rhodes University, Grahamstown. The duration of study was between March-July, 2016. The stem bark was dried under a shade for 3 weeks and were milled at the Chemistry Department, University of Agriculture Makurdi, Benue State, Nigeria, using Thomas model 4 Willey Mill.

Preparation of extracts: *Pycnanthus angolensis* (640 g) was macerated in methanol analytical grade (99.8%) for 5 days and concentrated on a rotary evaporator at 35°C separately. The TLC was done on the concentrates obtained to give 58.20 g for *P. angolensis* (black coloured). The crude extract was labeled PF00.

Maceration of crude extract: *Pycnanthus angolensis* crude extract (58 g) was extracted successively with hexane (4×100 mL), dichloromethane (4×100 mL), ethyl acetate (4×100 mL), acetone (4×100 mL) and ethanol (4×100 mL) by maceration to obtain PF01, PF02, PF03 and PF04, respectively.

Phytochemical screening: The crude stem bark extracts were later screened qualitatively for the phytochemical constituents utilizing standard methods of analysis[11-13].

Cell culture and treatment: Human cervix adenocarcinoma cell (HeLa) obtained (from ATCC CCL-2 LGC standard Wesel, Germany) were cultured in a 5% CO_2 incubator at 37°C in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin/fungizone). The cells were split every 3-5 days (when the cells have reached close to full confluency): The cells were detached from the culture flask surface using trypsin/EDTA and the majority aspirated off. Medium was added to the flask and the remainder of the cells and the flask returned to incubation. The confluency and state of the cells were regularly assessed using an inverted light microscope. Cells were cryopreserved by detaching the cells from the culture flask in trypsin/EDTA, pelleting the cells, transferring them to cryotubes in 10% DMSO in fetal bovine serum and placing the tubes in a -80 freezer. For the cytotoxicity assay a range of concentrations of extract (1-250 μg mL^{-1}) was used for 24 h treatment for the determination of CC_{50}.

Test for cytotoxicity: Cytotoxic activity was determined by resazurin reduction based assay[14].

Resazurin based assay: HeLa cells were used for the determination of the CC_{50} value of the cytotoxicity of the *Pycnanthus angolensis* stem bark extracts. To assess the overt cytotoxicity of the compounds, extracts were incubated at various concentrations in 96-well plate containing HeLa (human cervix adenocarcinoma) cells for 24 h. The numbers of cells surviving drug exposure were also determined by using the resazurin based reagent and reading resorufin fluorescence in a multiwell plate reader. Reagent was prepared by dissolving high purity resazurin in DPBS (pH 7.4) to 0.15 mg mL^{-1}. The resazurin solution was filtered and sterilized through a 0.2 μm filter into a sterile, light protected container. The resazurin solution was stored and protected from light at 4°C for frequent use or at -20°C for long term storage. Cells and test compounds were prepared in opaque-walled 96-well plates containing a final volume of 100 μL/well. An optional set of wells were prepared with medium only for background subtraction and instrument gain adjustment. This was incubated for desired period of exposure. Twenty microliter resazurin solution was added to each well. This was incubated for 1-4 h at 37°C. The fluorescence was recorded using a 560 nm excitation/590 nm emission filter set.

Statistical analysis: Data represent the mean±standard error (SEM) of the indicated number of experiments. Graphs were prepared by Prism software. Statistical analysis of the data was carried out by one way ANOVA (Graph Pad Prism 5.02 Software). A value of $p<0.05$, <0.01 and <0.0001 were considered to be significant, very significant and highly significant, respectively. Linear regression analysis was used to calculate CC_{50}.

RESULTS

Phytochemical screening: The phytochemical test of the dichloromethane and ethyl acetate stem bark extracts of *Pycnanthus angolensis* stem revealed the presence of glycosides, alkaloids, saponins, steroids, tannins, flavonoids and terpenoids (Table 1). However the test for steroids was negative for ethyl acetate extract.

***In vitro* cytotoxicity in HeLa cells by resazurin assay:** A significant decrease ($p<0.05$) of viability of the cells was observed for all extracts. Dichloromethane and ethyl acetate stem bark extracts of *P. angolensis* showed significant cytotoxicity against HeLa cells with CC_{50} 26.08 and 90.10 ug mL^{-1}, respectively (Table 2). A higher cytotoxicity was exhibited by the dichloromethane stem bark extract more

Table 1: Phytochemical screening of dichloromethane and ethyl acetate stem bark extracts

	Extracts	
Constituents	DCM	EA
Glycosides	+	+
Alkaloids	+	+
Saponins	+	+
Steroids	+	-
Tannins	+	+
Flavonoids	+	+
Terpenes	+	+

+: Presence, -: Absence , DCM: Dichloromethane, EA: Ethyl acetate

Table 2: The CC_{50} of dichloromethane, ethyl acetate extracts and emetine drug

Extracts	Cytotoxocity CC_{50} (ug mL^{-1})
Dichloromethane	26.10
Ethyl acetate	90.27
Emetine	0.014049

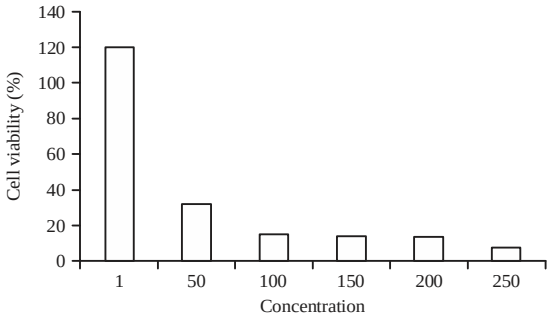

Fig 1: Activity of dichloromethane stem bark extract on HeLa cells at different concentration (µg mL^{-1})

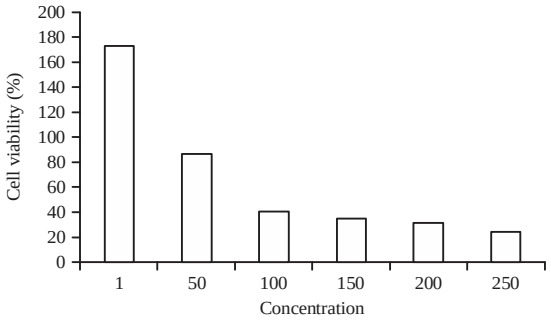

Fig 2: Activity of ethyl acetate stem bark extract on HeLa cells at different concentration (µg mL^{-1})

than the ethyl acetate stem bark extract (Fig. 1and 2). The CC_{50} of positive control (emetine) was 0.01049 ug mL^{-1} higher in potency than the extract (Table 2).

DISCUSSION

There are several medicines available in the market to treat the various types of cancer but no drug is found to be fully effective and safe. The major problem in the cancer chemotherapy is the toxicity of the established drugs. However plants and plant-derived products have proved effective and safe in the treatment and management of cancers. These days most of the research study on cancer drugs is targeted on plants and plant-derived natural products. Many natural products and their analogues have been identified as potent anti-cancer agents[15]. This report tried to evaluate the cytotoxicity of *Pycnanthus angolensis* against HeLa cells *in vitro*.

The results obtained in the present study indicate that *Pycnanthus angolensis* extracts exhibited potent cytotoxic activity against HeLa cells *in vitro*. The activities might be attributed to its polyphenolic content and other phytochemical constituents.

Several compounds belonging to various classes have been previously reported in *P. angolensis*. These include flavonoids[16], terpenoid[17] and steroids[18]. Thus, supporting the validity of the phytochemical screening. Masoor *et al.*[19] and Masoor *et al.*[16], reported the higher apoptosis activity demonstrated by the plant derived compounds (lignans and flavonoids) when compared with the control. Flavonoids and lignans are polyphenolic compounds which display a remarkable spectrum of biological activities, including those that might influence the processes that are dysregulated during cancer development. This includes antiallergic, anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic and modulation of enzymatic activities[20] and are therefore considered as chemotherapeutic agents with various mechanisms against cancer[21].

The criteria of cytotoxicity activity for the crude extracts, as established by the American National Cancer Institute NCI) is an IC_{50} <30 µg mL^{-1} in the preliminary assay[22]. The dichloromethane stem bark extract with CC_{50}<30 ug mL^{-1} suggests the extract as promising against cancer. Its significance may increase in future in view of the lack of unwanted side effects characteristic for emetine compound currently in clinical use for treatment of cancer.

CONCLUSION

Pycananthus angolensis bark extracts exhibited activity against HeLa cells with the dichloromethane extract showing the highest cytotoxicity. Further studies on the isolation of chemical constituents active against cancer should follow. This should provide lead compounds of high therapeutic efficacy for cancer ailment. Transformation of the lead through synthesis will generate many more active recipes for the disease. Cytotoxicity of the plant extracts on other cancer cell lines should also be studied.

SIGNIFICANCE STATEMENTS

This study discovers extracts from *Pycnanthus angolensis* to contain active constituents that can be beneficial for treatment of cancer diseases. This study will help the researcher to uncover the critical area of cervical cancer that many researchers were not able to explore. Thus a new theory on developing a lead compound from this plant with multidimensional functions of high therapeutic efficacy and hopefully little or no side effect may be arrived at.

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