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In-vitro Antibacterial and Anticancer Evaluation of the Methanolic Root-Bark Extract of *Persea americana* Mill (Family Lauraceae)

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ABSTRACT

The medical plant, *Persea americana* is used in Nigerian traditional medicine for treatment of cancer, infections and other related diseases. We investigated the antibacterial activity, the cell proliferative and apoptotic effects of the crude extract of the plant using breast cancer cell line. Proximate analysis of the crude powdered drug was carried out using standard methods. The methanolic rootbark extract of *Persea americana* was subjected to an *in-vitro* antimicrobial susceptibility testagainst *Staphylococcus aureus Escherischia coli, Enterobacter aerogenes, Klebsiella pneumonia, Proteus vulgaris* and *Pseudomonas aeruginosa* using seeded agar diffusion technique. The crude extract was tested for antiproliferative and apoptotic activities against human breast adenocarcinoma (MCF-7) cell line. Proximate analysis revealed a moisture content of 7.50 ± 0.3412 %, total ash 4.7 ± 0.3801 %; acid and water insoluble 1.83 ± 0.8333 % and 2.5 ± 0.2887 % and Alcohol and water soluble extractive values of 6.13 ± 0.1250 % and 1 ± 0.2882 %. The extract demonstrated promising activity against the test organisms at different concentrations. *Escherischia Coli, Enterobacter aerogenes, Klebsiella Pneumonia* and *Proteus vulgaris* were resistant. There was a significant ($P < 0.01$) apoptotic effect of the crude extract compared to the DMSO control whereas the antiproliferative effect showed no difference in comparison with the control. The extract of *Persea americana* has antibacterial activity and demonstrated a significant apoptotic effect against human breast cancer cell line. This therefore supports the traditional medicinal use of the plant and suggests potential use of the plant extract or isolated compounds as an antibacterial and antitumor agent.

KEY WORDS: *Persea americana,* methanolicextract, antibacterial, anticancer.

INTRODUCTION

The need for continued research into herbal plants especially those used in developing countries of Africa, Asia, and South America, cannot be over-emphasized. For the past few decades, the traditional systems have gained importance in the field of medicine; World Health Organizationestimated that 85 % of traditional extract involves use of plant extracts. This means that 3.5 to 4 billion people (80 % of the world population) in the world rely on plants as source of drugs and primary health care [1]. Infact, it has been estimated that about 25 % of all prescribed medicines today are derived from plants [2]. Infectious diseases accounts for approximately one-half of all deaths in tropical countries, in industrialized countries, incidence of epidemics due to drug resistant micro-organisms also pose a public health concern [3]. Commercial antibiotics and synthetic antimicrobial agents have remained the main stay in combating infectious diseases. Attention has been drawn to the antimicrobial properties of plants and their metabolites due to the growing incidences of drug-resistant pathogens of both clinical and agricultural importance. Medicinal plants have their intrinsic ability to resist pathogenic microorganisms and this has led the researchers to investigate their mechanisms of action and isolation of active compounds. This has enabled exploitation of medicinal plants for the treatment of microbial infections of both plants and humans by developing new antimicrobial agents. Recently the acceptance of traditional medicine as an alternative form of

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health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants [4-7]. Medicinal plant- based antimicrobials represent a vast untapped source of pharmaceuticals and further exploration of plant antimicrobials need to occur for treatment of infectious diseases while simultaneously for mitigating many of the side effects that are often associated with synthetic antimicrobials [8].Cancer remains a leading cause of mortality in most, if not all, countries despite advances in diagnosis and therapy [9]. Natural products have played a significant role in the discovery and development of new anticancer agents, and they represent a rich source of biologically active compounds [10]. More than 79.8 % of anticancer drugs introduced from January 1981 to October 2008 were natural products, semisynthetic analogues, or synthetic compounds based on natural-product pharmacophores [11].

Persea americana commonly called avocado tree has wide variety of uses in ethno-medicinal practices in Africa especially Nigeria. It is used locally among Edo peopleof Nigeria to prevent hypertension [12].The seed and the leaves are also used in folk medicine for the treatment of diarrhea and dysentery. The leaves and bark promote menstruation. The pulp is applied to shallow cuts and prevents infection. Previous scientific investigation has demonstrated that Persin, isolated from avocado leaves possess antifungal properties. Persenone A and B which can be extracted from avocado function as antioxidants, and may be effective chemo-preventive agents in inflammation-associated carcinogens [13]. The use of aqueous decoction from the seeds revealed that it has glyceamic properties [14]. The extract of the plant was found to have tumor-protective effect in chemically induced mammary cancer in rats due to high amounts of polyunsaturated acids (linolenic and alpha-linolenic acid) [15-17].

MATERIALS AND METHODS

Plant materials

The roots of *Persea americana* were collected from Nigeria Institute for Oil palm Research (NIFOR), Edo State in May, 2011. The roots were washed off of earthy materials and the bark carefully removed and air dried for ten days, after which it waspowdered with a milling machine. The powdered sample was kept in an air-tight container at room temperature.

Proximate analysis

The following quantitative parameters were carried out using standard methods [18].

Moisture content: The powdered drug (2 g) was weighed into a crucible of known weight and oven dried at 105^{o}C for 5 hours. The crucible was reweighed to determine weight loss in powder drug. The average percentage weight loss, with reference to the air dried powdered drug was determined in four replicates.

Total Ash Determination: The crucibles were washed thoroughly, dried in a hot oven at 100 ^{o}C, cooled in a desiccator and weighed. A 2 g portion of the sample was weighed into the crucible and put in the furnace. Heating was started gradually until a temperature of 600 ^{o}C was reached. This temperature was maintained for 6 hours. After washing the furnace switched off and the temperature allowed to drop before the crucible was removed and cooled in a dessicator. After cooling the sample was reweighed and the percentage ash calculated.

Acid Insoluble Ash value Determination: The crucible with ash of the powdered drug from the experiment above was transferred into a beaker containing 25 ml of dilute HCl. The beaker and its contents were boiled for 5 minutes and filtered through an ashless filter paper. The weight of a clean and heated crucible was accurately determined. The filter paper with the residue was gently heated until the filter paper was completely ashed, and then heated strongly for few minutes. The crucible and its content were cooled, weighed and the final weight was noted. The weight of the residue (ash) was then calculated. This was done by subtracting the constant weight of the crucible and ash. The weight of the ash was divided by the initial weight of the drug multiplied by a hundred was taken as the acid insoluble ash value.

Water Insoluble Ash value determination: The crucible with the total ash was transferred into a beaker containing 25 ml of distilled water. The beaker and its contents were boiled for 5 minutes and filtered through ashless filter paper. The weight of a clean and heated crucible was accurately determined. The filter paper with the residue was gently heated until the filter paper was completely ashed, and strongly heated for few minutes. The crucible and its contents were cooled, weighed and the final weight noted. The weight of the residue (ash) was then calculated. This was done by subtracting the constant weight of the crucible and the ash. The weight of the ash divided by the initial weight of the drug multiplied by a hundred was taken as the water insoluble ash value.

Alcohol Soluble Extractive value: 5 g of the powdered drug was weighed into a 250 ml stopper conical flask. 100 ml of 90 % ethanol was added into the conical flask and covered firmly. The flask was shaken in a mechanical shaker for 6 hours and then allowed to stand for 18 hours. The extract was filtered by suction filtration using a Buckner funnel. 20 ml of the filtrate was taken into a clean, dried and weighed crucible. The filtrate was evaporated to dryness at 100 ^{o}C. The residue was dried to constant weight and the final weight noted. The weight of the residue from the 20 ml extract was then determined by subtracting the constant weight of the crucible from the residue. The alcohol extractive was then calculated with reference to the initial weight of the powdered drug and expressed as percentage.

Water Soluble Extractive value: The above experiment was repeated using chloroform: water 400:1. The water extractive value was done for the powdered drug.

Extraction of Plant Material

700 g of the dried powdered root bark of *Persea americana* was weighed into a glass jar and macerated using 3.3 L of methanol at room temperature over a period of 72 hours. The extract was filtered and the filtrate was evaporated to dryness using a rotary evaporator at reduced pressure and the percentage yield calculated. The concentrated extract was kept in a refrigerator at 4 ^{o}C until ready for use.

Media and Chemicals

Nutrient broth (Micro master Maharashtra, India), Nutrient agar (Antec Diagnostic Products, United Kingdom). Methanol, absolute ethanol, chloroform, hydrochloric acid. All analytical grade solvents were obtained from Sigma-Aldrich, Germany.

Organisms

Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Enterobacter aerogenes and Klebsiella pneumoniae. All clinical isolates were obtained from Medical Microbiology laboratory, University of Benin Teaching Hospital, Benin City, Nigeria.

Media preparation

Both the Nutrient broth and the Nutrient agar were prepared aseptically according to the manufacturer's instruction. The medium was sterilized after preparation in the autoclave at 121 ^{o}C for 15 minutes. before use. The required quantity of agar was withdrawn using a sterile pipette into the Petri dishes and allowed to set.

Preparation of organisms

Each organism was inoculated in a slant medium and preserved by storing them in the refrigerator at 4 to 5 ^{o}C. Before every test, the organisms were aseptically sub-cultured into a sterile nutrient broth and incubated over-night at 37 ^{o}C.

Treatment with plant extract

Four Petri dishes were properly labeled with the different concentrations of the plant extract (25, 50, 75 and 100 mg/mL). The equivalent volumes of a stock of 500 mg/ml extract were pipetted into the Petri dishes and the needed volume of sterile nutrient agar was pipetted with a sterile pipette into the petri dishes to a final volume of 20 ml and rocked gently to ensure proper dispersion of the extract in the plates. Then the plates were allowed to set with the lid partially open within the sterile zone. Thereafter, the plates were dried in the oven at 50 ^{o}C for few minutes. Each of the plates base was divided into six portions using grease pencil with each portion having the name of an organism (*Escherichia coli, Staphylococcus aureus, Enterobacter aerogenes, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*). Finally each portion of the plate was inoculated with a drop of the corresponding over-night culture of the organisms. The plates were allowed to stand on the bench for thirty minutes to ensure absorption of the water in the bacteria culture. The plates were incubated right side up at 37 ^{o}C for 24 hours, and then examined for growth and the result recorded.

Cell culture

Human breast adenocarcinoma cell line (MCF-7) was obtained from the America Type Culture Collection, Manassas VA, USA. Cells were maintained at 37 ^{o}C and in a 5 % CO_2 atmosphere in a monolayer. Confluent cells were passaged by treating them with 0.05 % trypsin-0.02 % EDTA. The medium was changed every 48 hours.

Human mammary epithelial cell line

The oestrogen-sensitive human breast adenocarcinoma cell line MCF-7 (ATCC no. HTB-22) were cultured in Dulbecco's modified Eagle's medium with 10 % fetal bovine serum and 1 % gentamycin. As kind of control functions the non-tumorigenic epithelial breast cell line MCF-12A (ATCC no. CRL-10782) which was grown in Dulbecco's modified Eagle's medium Ham's F12 without phenol red (Invitrogen, Germany) containing 10% horse serum (PAA Laboratories GmbH, Germany), the Mammary Epithelial Cell Growth SupplementPack (Promo Cell, Germany) including Bovine Pituitary Extract 0.004 ml/ml, Epidermal Growth Factor (recombinant human) 10ng/ml, Insulin (recombinant human) 5 µg/ml,

Hydrocortisone 0.5 µg/ml and 1 % gentamycin (Ratiopharm, Germany).

Treatment with plant extract

0.5 x 10^6 cells were seeded in a 6-well plate in regular culture medium for one day. Subsequently, cells were washed with phosphate buffered saline (PBS) and adapted to phenol-red-free Dulbecco's modified Eagle's medium (PAA Laboratories GmbH, Germany) with 10 % charcoal stripped fetal bovine serum (PAN Biotech GmbH, Germany) for 48 hours to avoid unspecific stimulation of endogenous hormones in the assay medium. Treatment with plant extracts (final concentration 10µg/ml) was carried out for 48 hours in assay medium. As negative control substancethe vehicle dimethylsulfoxide (0.1 %) was used in the same manner.

Flow Cytometric Measurement of Cell Proliferation

The extent of cell cycle progression and apoptosis in the cells was estimated by flow cytometric analysis after propidium iodide staining of cells. After plant extracts treatment, cells were trypsinized with 0.05 % trypsin-0.02 % EDTA for 5-10 min. The reaction was stopped with assay medium. Cells suspension was transferred to FACS tubes (BD Biosciences, USA) and fixed in 70 % ethanol for 12 hours at -20 °C. After washing with PBS, cells were incubated with RNase (1 mg/ml) at 37 °C for 30 minutes. Finally, cells were re-suspended in propidium iodide (50 mg/ml) for at least 3 hours at +2 to +8°C protected from light until flow-cytometric analysis [19].

Flow cytometric measurement was performed on flow cytometer BD FACS Calibur, equipped with an argon-ion laser of the wavelength 488 nm (BD Biosciences, USA). For data acquisition, the software CellQuest Pro 4.0.1 (BD Biosciences, USA) was used. A minimum of 15,000 ungated events was recorded. Double and clumps were excluded by gating on the DNA pulse width versus pulse area displays. For the analysis of cell proliferation, the cell cycle phases G0/G1, S and G2/M and calculated in percentages using ModFIT LT 3.0 for Power Mac G4 (BD Biosciences, USA). For statistical analysis, the S-phase and G2/M-phase cells were defined as proliferative cells.

Statistical analysis

Every experiment was done in duplicate with individual passaged cells and data sets were expressed as means ± standard deviations (SD). Statistical significance was determined by unpaired t-test (***P < 0.001, **P < 0.01, *P < 0.1).

RESULTS AND DISCUSSION

The percentage values of the parameters determined in the proximate analysis are shown in Table 1. The determination of the quantitative parameters of crude drugs is relevant in establishing the identity of plant especially as part of requirement for pharmacopoeia standards. The proximate analysis carried out in this study is used to establish the identity of the crude drug sample.

Table 1: Percentage (%) values of proximate analysis of the root barkpowder of *Persea americana*

Concentrations Parameter	Value ± SEM (%)
Moisture content	7.50 ± 0.3412
Total ash	4.70 ± 0.3801
Acid insoluble ash	1.83 ± 0.8333
Water insoluble ash	2.50 ± 0.2887
Alcohol extractive	6.13 ± 0.1250
Water extractive	1.00 ± 0.2887

A moisture content of 7.50 ± 0.3 412 % obtained from this study indicates that the crude drug has a good storage quality. The maximum range is between 6 – 8 % [18].

In the present study, we have evaluated the effect of the methanolic rootbark extract of *Persea americana* against *Staphylococcus aureus, Proteus vulgaris Pseudomonas aeruginosa, Escherichia coli, Enterobacta aerogene* and *Klebsiella pneumonia* at concentrations of 25 mg/ml, 50 mg/ml, 75 mg/ml and 100 mg/ml using the seeded agar diffusion assay.

Organisms	25 mg/Ml	50 mg/mL	75 mg/mL	100 mg/mL
Staphylococcus Aureus	-	-	-	-
Escherichia Coli	+	+	+	+
Enterobacter Aerogenes	+	+	+	+
Klebsiella Pneumoniae	+	+	+	+
Proteus vulgari	-	-	-	-
Pseudomonas aeruginosa	+	+	+	-

\+ -- Growth,
\- -- No growth

The antibacterial susceptibility test result obtained showed that the crude extract of *Persea america* has high activity against *Staphylococcus aureus* and *Proteus vulgaris* moderate activity against *Pseudomonas aeruginosa*. The other organisms (*Escherichia coli*, *Enterobacta aerogenes*

and*Klebsiella pneumonia*) were resistant to the extract even at the highest concentration of 100 mg/mL (table 2). It has been established in this present study that the rootbark extract of *Persea americana* possess antibacterial activity against three of the test organisms.

The present study also has evaluated the antiproliferative and apoptotic effects of the crude extract of *Persea americana* on human breast adenocarcinoma (MCF-7) cell line. The MCF-7 cells are an excellent *in vitro* model for studying the mechanisms of chemo-resistance because of its susceptibility to apoptosis [20]. The non-tumorigenic mammary epithelial cell line, MCF-12A was used as a control to exclude a negative influence on normal tissue. Cell cycle analysis via flow cytometry distinguishes between different cell cycle phases and detects apoptotic DNA fragmentation simultaneously so that the proliferative (S + G2/M) as well as the apoptotic (degraded DNA) effects of the extract can be measured [19].

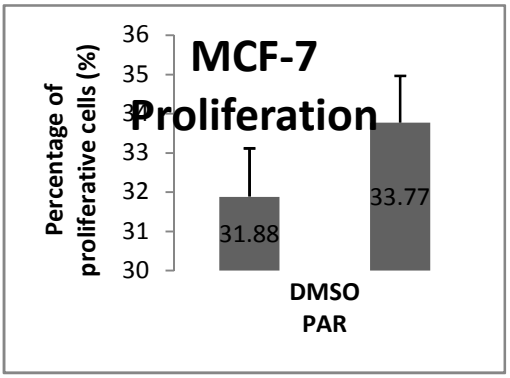

Figure 1: Proliferation (Growth after 48hrs stimulation with the extracts; 10 mg/ml finalconcentration)

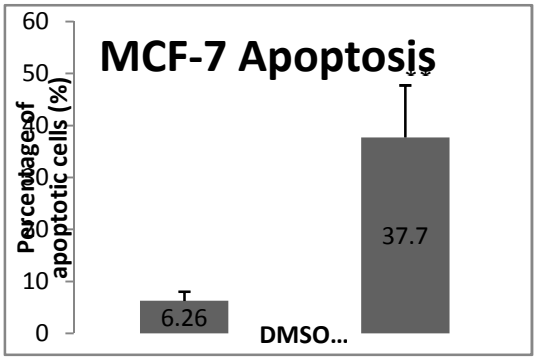

Figure 2: Apoptosis (dead cells after 48hrs stimulation with the extracts; 10 mg/ml final Concentration)
n = 2
* P < 0.1
**P < 0.01
***P < 0.001 compared to DMSO control
Key
DMSO: Dimethylsulphoxide
PAR: P*esea americana* root bark extract

As shown in figure 1, the percentage of proliferative cells of the extract treated cells is about 33.77 % and that of the DMSO control group is about 31.88 %. Statistical analysis shows that there was no significant (P < 0.15) difference between the two groups. However the extent of apoptosis in the extract treated cells (37.7 %) was significantly (P < 0.01) higher than that of the DMSO control group (6.26 %) (Figure 2).

The result therefore suggests that the extract could exact its antitumour effect via DNA fragmentation.

From the result obtained, it was established that *P. americana* extract in it crude or a more purified form could serve as a useful therapeutic agent in the treatment of infectious diseases caused by *Staphylococcus aureus, Proteus vulgaris* and *Pseudomonas aeruginosa*. It also has the potential for use as antitumour agent particularly against breast cancer. Future studies will focus on the isolation, characterisation and elucidation of the bioactive constituents of the extract.

CONCLUSION

Plants contain thousands of constituents and are valuable sources of new and biologically active molecules possessing antimicrobial and anticancer properties. The results obtained during this study indicated that the rootbark extract of *Persea americana* has antibacterial activity as a result of its ability to inhibit the growth of *Staphylococcus aureus, Proteus vulgaris and Pseudomonas aeruginosa*, it also possess good anticancer activity due to its apoptotic effect on MCF-7 cell line. The extract is a potential source of useful antiinfective and anticancer agent.

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