Scientia Africana, Vol. 13 (No.1), June 2014. Pp 1- 8
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ANTIPROLIFERATIVE AND APOPTOTIC ACTIVITIES OF *Cola lepidota* AGAINST ESTROGEN RECEPTOR POSITIVE BREAST CANCER CELLS

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Received:31-01-14
Accepted:25-03-14

ABSTRACT

The stem bark, seed, roots and leaves of Cola lepidota have been used for various health conditions in Nigeria folk medicine, including treatment of cancer related ailments. The study evaluated the antiproliferative and apoptotic effects of extracts of the leaves and stem bark on breast cancer (MCF-7) cell line. Powdered stem bark and leaves were extracted with methanol and concentrated in vacuo. Extracts were partitioned with petroleum ether, chloroform and ethyl acetate successively. The extract and fractions were screened for antiproliferative and apoptotic activities using flow cytometry. The methanol leaf extract (CLL-M) demonstrated significant ($p < 0.01$) antiproliferative activity against estrogen receptor positive breast cancer cells (26.09 %), while the chloroform fraction of the stem extract (CLS-CH) showed significant ($p < 0.001$) apoptotic activity.
The study lends support to the ethnomedicinal use of Cola lepidota and indicates the potential of the plant as source of natural anticancer agent.

Key words: apoptosis, proliferative, anticancer, Cola lepiota, extract

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Jamine *et al*., 2007). About three quarter of the world's population relies on plants and plant extracts for their healthcare. The acceptance of traditional medicine as an alternative form of health care and the growing concern of cancer as a major public health problem worldwide with millions of new cancer patients diagnosed each year and many deaths resulting from this disease (Engel *et al*., 2011) has led to the screening of several medicinal plants for their potential antitumor activity. Natural products have played a significant role in drug discovery and development, especially agents active against cancer and infectious diseases (Butler, 2008). Deaths from cancer worldwide are projected to continue rising with an estimated 12 million deaths annually by 2030 (Wang *et al*., 2007).

Breast cancer is a cancer that starts in the tissue of the breast. It could be invasive or non invasive, estrogen receptor (ER) positive or ER negative. Chemotherapy remains the principal mode of treatment for various cancers. Fisher et al, (2005) reported the use of Tamoxifen a non-steroidal anti-estrogen agent in the treatment of estrogen receptor (ER) positive breast cancer patients The use of plants or plant products for cancer treatment could be due to several reasons such as availability of the materials, affordability, relatively cheap and little or no side effects (Engel *et al*., 2011).

World Health Organisation (WHO) supports the use of traditional medicines provided they are proven to be efficacious and nontoxic (WHO, 2003). It is well established that plants have been a useful source of clinically relevant antitumor compounds (Cragg, 1999). Herbs and food phytochemicals have been used in folk medicine for the treatment of cancer. The discovery of effective herbs and elucidation of their underlying mechanisms could lead to the development of alternative and complimentary method for cancer prevention and/or treatment, and provide bio-resource of drugs for traditional systems of medicine, modern medicines, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs.

*Cola lepidota*is a tree reaching 20 m tall and 20 cm diameter. Leaves are compound digitate, fruits unisexual, sub-cylindrical and about 20 cm long and 5 cm diameter, dark brownish-red, seeds are dark purple, flattened, and up to 5 cm diameter, with a thick, crisp white plant (Tripathi *et al*., 2005). The parts of *Cola lepidota* are commonly used in the treatment of various ailments such as pulmonary problems, as febrifuges and the seeds act as stimulants when chewed(Burkil, 1985). However it's claimed use for the treatment of cancer by traditional healers has not been reported in any scientific literature.

The present study was undertaken to evaluate the antitumour potential of the methanol extract and fractions of the stem bark and leaves of the plant by studying its inhibitory effects on cell proliferation and induction of apoptosis in human breast cancer (MCF-7) cell line measured by flow cytometry.

MATERIALS AND METHODS

Plant collection and identification

Fresh stem bark and leaves of *Cola lepidota* were collected in June 2011, at Umuosu Nsulu, Abia state, South Eastern Nigeria. The plant was identified and authenticated by Mr. Ugbogu O. A. and Shasanya O. S. of the Forest Research Institute of Nigeria (FRIN), Ibadan where specimen was deposited in the herbarium and voucher specimen number FHI 109560 was issued.

Processing of the plant material

Fresh sample of *Cola lepidota* leaves and stem were air dried and powdered using a mechanical blender. The powdered stem barks and leaves (1.2 kg each)was macerated with 9 litres of methanol for 72 hours. The filtered extracts were evaporated to dryness using a rota vapour at reduced pressure.

The dried extracts were partitioned with different solvents; petroleum ether (40-60°C), chloroform and ethyl acetate in order of increasing polarity. The various fractions of both plant parts were concentrated to dryness *in vacuo* and kept at 4°C until use.

Scientia Africana, Vol. 13 (No.1), June 2014. Pp 1- 8
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Anticancer screening

Cell culture:The estrogen-sensitive human breast adenocarcinoma cell line MCF-7 (ATCC no. HTB-22) was obtained from the American Type Culture Collection, Manassas VA, USA. Cells were maintained in Dulbecco's modified Eagle's medium with 10 % fetal bovine serum (FBS) and 1% gentamycin at 37°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 2-3 days.

Treatment with plant extracts: Treatment with plant extracts was carried out as described previously (Nebe *et al*., 2011, Engel *et al*., 2011). The vehicle dimethylsulfoxide (DMSO) in final concentration of 0.1% was used. As negative control substance

Flow cytometric measurement of cell proliferation: Cell cycle analysis and apoptotic rates were estimated by flow cytometry as described previously(Engel *et al*., 2011). For statistical evaluation, the S-phase and G2/M-phase cells were defined as proliferative cells and cells with degraded DNA strands as apoptotic cells.

Statistical analysis: Every experiment was replicated 3 times 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

Figure 1 shows the percentage of proliferative (1A) and apoptotic (1B) MCF-7 cells after treatment with crude methanol extract of *C. lepidota* stem bark. There was a significant ($p < 0.01$) decrease in percentage proliferation when cells were treated with 10 µg/mL of extract compared to the DMSO control. The extract (CLS) showed significant alterations in the proliferative (G2+S) phases of MCF-7 cells.

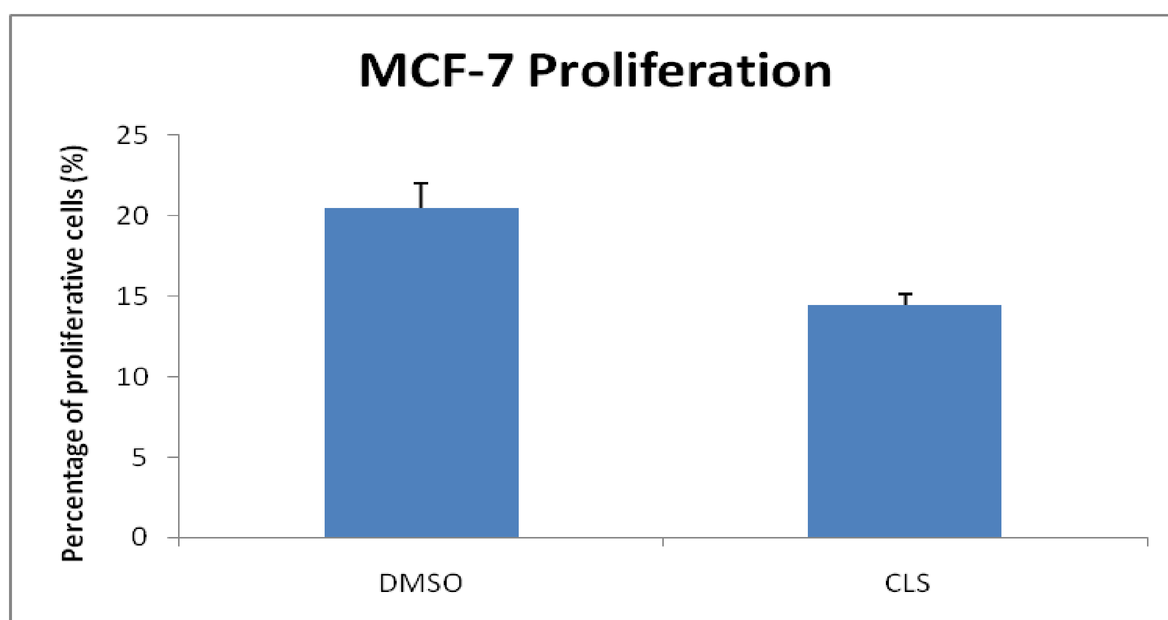

Figure 1 A: Proliferation rates (G2 + S phases) of MCF-7 cells after treatment with 10 µg/ml crude stem bark extract of *C. lepidota* (CLS) for 48 h. SE (n = 3). **$P < 0.01$.

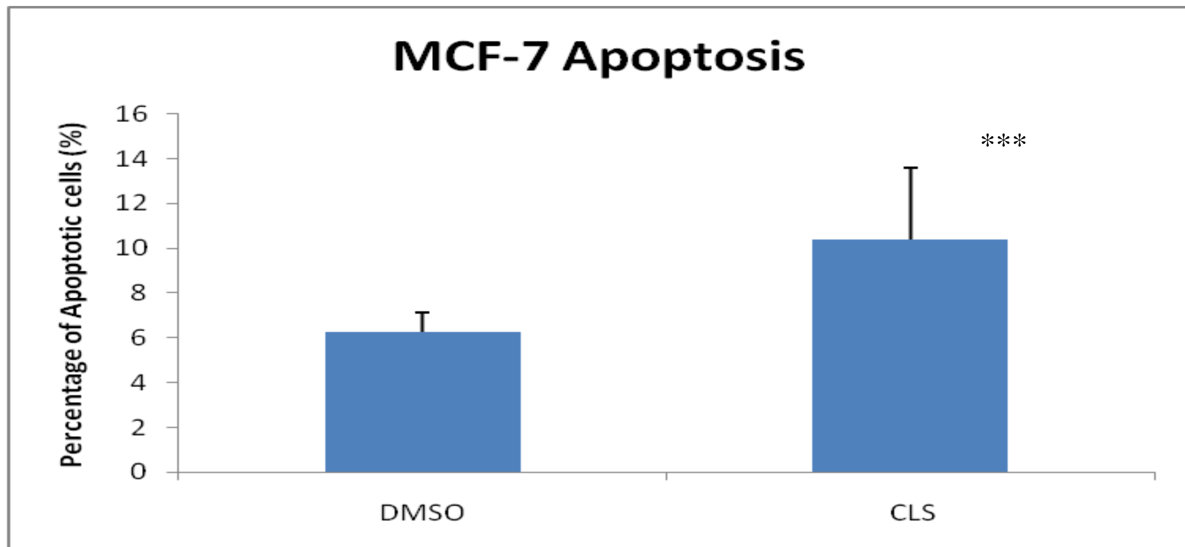

Figure 1 B: Apoptotic rates (degraded DNA) of MCF-7 cells after treatment with 10 µg/mL crude stem extract of *C. lepidota*(CLS) for 48 h. SE (n = 3). ***P < 0.001

As can be seen in figure 2A only fractions CLL-M, CLS-CH and CLL-EA caused significant ($p < 0.05$) alterations in the cell proliferative status of the breast cancer cell line when compared with the control. The proliferative (G2+S) phase was decreased about 26.09 %, 26.40 % and 26.92 % for CLL-M, CLS-CH and CLL-EA treated cells respectively. CLL-P, CLS-P and CLS-EA did not show marked inhibition of proliferation of the MCF-7 cell line. Figure 2B shows percentage of apoptotic cells of human breast cancer cell (MCF-7) after treatment with different fractions of the extract. Fraction CLS-CH exhibits significant induction of apoptosis ($p < 0.001$) about 10.52 % compared to DMSO (control) with percentage apoptosis of 6.26 %.

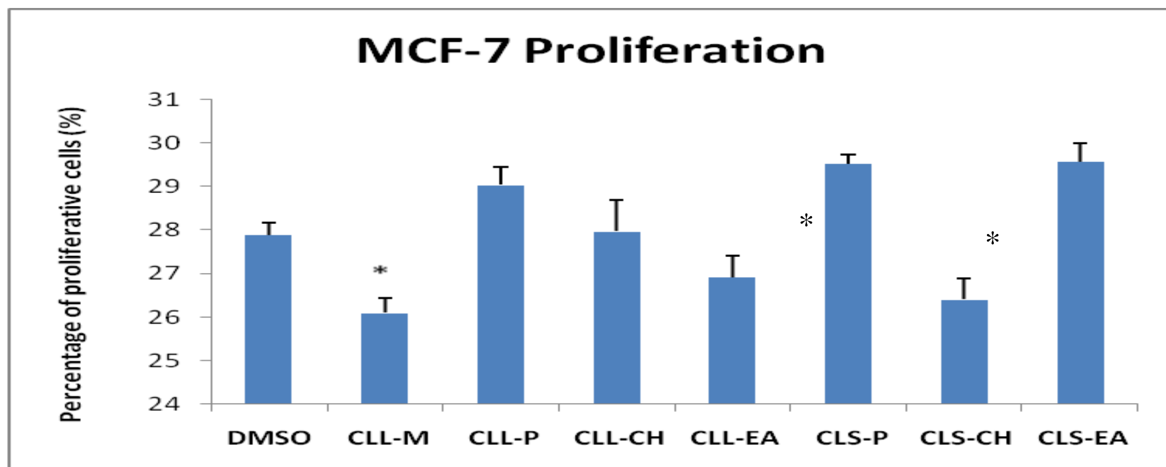

Figure 2 A: Proliferation rates (G2 + S phases) of MCF-7 cells estimated by flow cytometry. Cells were treated with 10 µg/ml of each fraction of the methanol extract of *C. lepidota* (CLL-leaves; CLS-stem bark) for 48 h. SE (n = 3). *p < 0.05, compared with DMSO-treated

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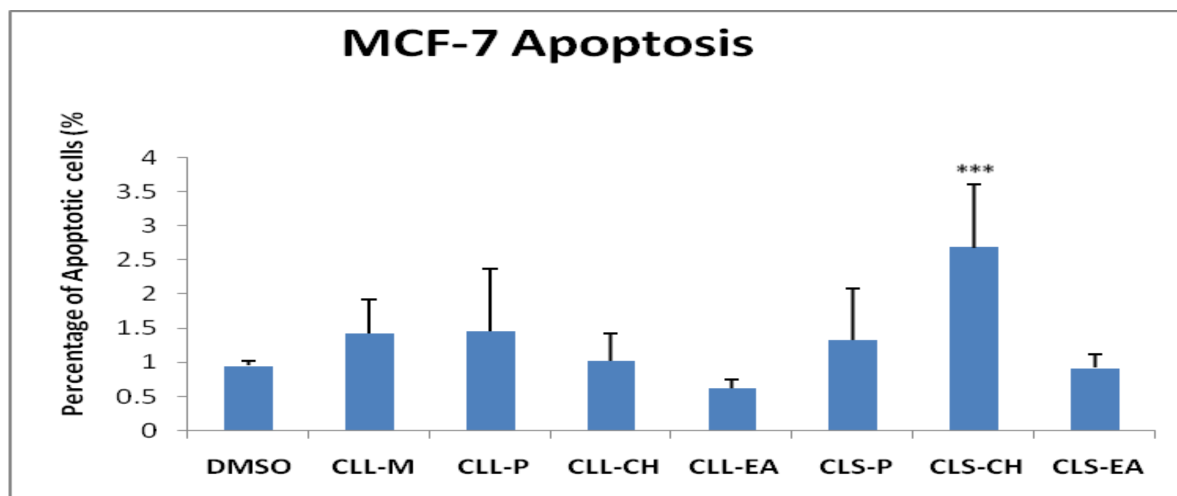

Figure 2 B: Apoptotic rates (degraded DNA) of MCF-7 cells. Cells were treated with a final concentration of 10 µg/ml of each fraction of the methanol extract of *C. lepidota* (CLL- leaves; CLS-stem bark) for 48 h. SE (n = 3). ***p < 0.001 compared with DMSO-treated control (unpaired t-test).

Explanatory notes to figures 2a and 2b:

CLL-M	Methanol extract of *C.lepidota* leaves
CLL-P	Pet.ether fraction of *C.lepidota* leaves
CLL-CH	Chloroform fraction of *C.lepidota* leaves
CCL-EA	Ethyl acetate fraction of *C.lepidota* leaves
CLS-P	Pet. ether fraction of *C.lepidota* stem bark
CLS-CH	Chloroform fraction of *C.lepidota* stem bark
CLS-EA	Ethyl acetate fraction of *C.lepidota* stem bark

Figure 3 shows the bright field images of MCF-7 cells treated with fractions CLL-M and CLS-CH and DMSO (0.1%). The bright field images of the cells obtained after 48 hours showed a pronounced clear field of the MCF-7 cells treated with CLL-M and CLS-CH compared to the control. These results indicate that the extracts of this plant includes components that affect the cell cycle phases of breast cancer cell lines to different degree.

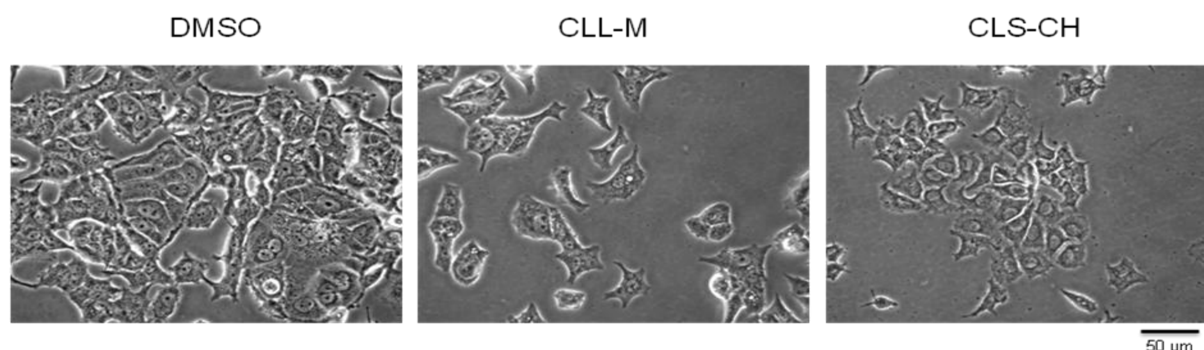

Fig. 3 Morphological changes of the MCF-7 cells after 48 h treatment with the solvent (0.1% v/v DMSO) only and with 10 µg/mL of the fractions CLL-M and CLS-CH viewed under bright field.

DISCUSSION

Cell proliferation is one of the fundamental processes required during tumour progression and metastasis (Edward and Alan, 2004). This study evaluated the anticancer properties of the crude extract of *C. lepidota* leaves (CLL) and stem bark (CLS) and different fractions (CLL-M, CLL-P, CLL-CH, CLL-EA, CLS-P, CLS-CH, and CLS-EA) on breast cancer cell line (MCF-7) by cell cycle analysis via flow cytometry. The MCF-7 cells are an excellent breast cancer cell model for *in-vitro* anticancer studies due to its susceptibility to endogenous human estrogen, plant-derived estrogens and ability to undergo DNA fragmentation (Simstein *et al.*, 2003). Cell cycle analysis via flow cytometry distinguishes between different cell cycle phases and detects apoptotic DNA fragmentation so that the proliferative (S + G2/M) and the apoptotic effects of compounds or natural extracts can be measured simultaneously (Nunez, 2001).

In validating the anticancer potential of the plant, MCF-7 cells were treated with the extract and fractions (diluted to a final concentration of 10 µg/mL). A reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death (apoptosis) or a combination of these two mechanisms. In contrast to the DMSO control we observed a decreased proliferation rate after exposure to 10 µg/mL of the CLL-M fraction (figure 2A) and an enhanced apoptosis after treatment with the CLS-CH fraction (figure 2B). These observations were not only seen in the distribution of the cell cycle phases but also visible under bright field microscopy (figure 3). Both treatments (CLL-M and CLS-CH) caused higher detachment of the MCF-7 cells so that the cells became rounded and lost their adhesion to the culture dishes. It is possible that this effect on cell adhesion is due to the inhibition of integrin expression which mediates cell-cell and cell-extra cellular matrix interactions and subsequently regulates cell growth in some tumours (Assoain and Klein, 2008; Vellon *et al.*, 2006; Gianotti and Ruooslanhti, 1999). Cell detachment from the extra cellular matrix or the loss of contact with the neighbouring cells was a clear indication of induction of an apoptotic process termed anoikis (Frisch and Francis, 1994).In contrast to the well spread cells under control conditions, cells treated with CLL-M and CLS-CH were smaller and showed some morphological changes. The fractions especially the CLS-CH caused apoptotic alterations of the MCF-7 cells presumably due to cell dehydration, an early event in apoptosis (Hu and Kong, 2004).

Phytochemicals have been shown to induce cell cycle arrest, cause apoptosis and affect the differentiation and proliferation of cells mediated by the effect of intracellular reactive oxygen species on the signal transduction pathway (Hu and Kong, 2004). Plant extracts with cytotoxic activities may be able to play a significant role in the treatment of breast cancer by being incorporated into conventional treatment regimen which can improve overall treatment efficacy and/or may reduce toxicity. Future research work is in progress to isolate, identify and characterize the compounds responsible for the anticancer activity.

The study revealed that the methanol and ethyl acetate fractions of the leaves and chloroform fraction of the stem bark of *Cola lepidota* were efficient in inhibiting breast cancer cell line proliferation.

Scientia Africana, Vol. 13 (No.1), June 2014. Pp 1- 8
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The chloroform fraction showed a demonstrated significant induction of apoptosis.

Special gratitude to the Department of cell Biology, Biomedical Research Center, University of Rostock, Rostock, Germany. Special thanks to Rev. Kelechi Orji and Rev. Joseph Agbai for collection of plant materials, and VC.23 URPC, TETFUND2013 Research grant, University of Benin, Benin City, Nigeria.

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