

INVITRO CYTOTOXIC ACTIVITY OF SOME MEDICINAL PLANTS USED IN TRADITIONAL MEDICINE FOR BREAST CANCER MANAGEMENT IN SOUTHWESTERN NIGERIA

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ABSTRACT: Joloo is a traditional herbal formulation used in the management of tumour of the breast in southwestern Nigeria. This study was conducted to assess the cytotoxic and antiproliferative effects of the seven individual plants used in the preparation of Joloo and five fractionated portions of Joloo (Chlorophorm, Hexane, Ethyl-acetate, Butanol and Water) for its safety and antitumour potentials on two cell lines. MTT assay was used to evaluate the cytotoxic and apoptotic activities of the seven individual plants used in the preparation of Joloo as well as five fractionated portions of Joloo formulation (Chlorophorm, Hexane, Ethyl-acetate, Butanol and Water) using three concentrations (1.0, 2.5, and 5.0 mg/ml) on VERO and CEF cells. In this study different solvent systems of Joloo caused significant apoptosis especially at 2.5 and 5.0mg/ml in VERO and CEF cell lines, with IC₅₀ ranges from 2.8-9.8 and 6.5-14.8 mg ml⁻¹ respectively, implying that VERO was more susceptible to cytotoxicity than CEF cell. *A.ascalonicum*, water and chloroform fractions exhibited the strongest cytotoxicity on the VERO cells than others, whereas water fractions and *S. longepedunculata* were the most potent in the CEF cells. However, *T. tetraptera* and chloroform fraction caused cell death at all the concentrations used in both cells. Based on the fact that Joloo exhibited mild cytotoxicity on the cells as observed in the high concentration required for IC₅₀, and was selective in its activities having high potency on VERO cells than CEF. This may be responsible for its selective effect on breast tumours, thereby justifying its traditional pharmacological claims.

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1.0 INTRODUCTION

Plants are invaluable in the quest for new drugs. There is a tremendous historical legacy in traditional uses of plants, parts of plants and isolated phytochemicals for the prevention, management and treatment of various health ailments. Scientific studies on plants used in ethnomedicine led to the discovery of many valuable drugs like taxol, vincristine and vinblastine (Sahoo *et al.*, 2010).

In our ongoing toxico-pharmacological investigation of Joloo (A traditional medicinal formulation used in the management of breast tumour in southwestern Nigeria), we now focused on the cytotoxic and antiproliferative activities of the formulation. Joloo was concocted from the following seven plants *Butyrospermum paradoxum* seed, *Securidaca longepunculata* bark, *Tetrapleura tetraptera* stem, *Hoslundia opposita* leaves, *Xylopi aethiopica* seed, *O lax subscorpioidea* stem and whole *Allium ascalonicum*. Some of the folkloric uses of the individual constituents of Joloo documented in literature include the following; the whole plant of *Allium ascalonicum* Linn. (*Liliaceae: Alliaceae*) has reported to have inhibitory effect on angiogenesis

(Seyfi *et al.*, 2010) and also possess some antitumor properties (Odugbemi, 2006). *Butyrospermum paradoxum* Gaertn (*Seapotaceae*) has been used as a stimulant, carminative, antihelminthic and antihypertensive in Nigeria (Burkill, 2000; Odugbemi, 2006). Burkill (1995) reported that *Hoslunda opposita* Vahl (*Labitae*) has antipyretic, diuretic, cholagogic, antimalarial and anticonvulsant properties while *O lax subscorpioidea* Olive (*Olacaceae*) is used traditionally as an anti-arthritic, antirheumatic (Burkill, 1997) and antimicrobial remedy (Ayandele and Adebisi, 2007). *Xylopi aethiopica* Dunal A. Richard (*Annonaceae*) has been associated with the treatment of cancer in Nigeria (Asekun and Adeniyi, 2004), while *Securidaca longepedunculata* Fresen (*Polygalaceae*) is reported to possess antirheumatic, antipyretic and anti-inflammatory properties (Asres *et al.*, 2001). The pod extract of *Tetrapleura tetraptera* Schum. and Thonn. (*Leguminosae: Mimosidae*) is used to treat chest pain, female sterility, ulcer, convulsion and arthritis (Burkill, 1995; Odugbemi, 2006).

Extensive studies have been conducted on the acute, subchronic, geno-toxicity, Analgesic and anti-

inflammatory activities of Joloo (Oloyede *et al.*, 2008; 2009; 2011). The investigation by Oloyede *et al.* (2009) observing that Joloo possess some antimetabolic properties could also imply that it might be cytotoxic and antiproliferative as well. This study was, therefore conducted to assess the cytotoxic and antiproliferative effects of Joloo for its safety and antitumour potentials.

2.0 MATERIALS AND METHODS

The seven plant materials used for this study, *Butyrospermum paradoxum* seed (FHI 107924), *Securidata longepunculata* bark (FHI 103049), *Tetrapleura tetraptera* stem (FHI107984), *Hoslundia opposita* (PCGH 322;FHI 108121) leaves, *Xylopi aethiopia* seed (FHI107698), *Olax subscorpioidea* stem (FHI 107986) and *Allium ascalonicum* (FHI 107763) were collected, identified and authenticated by Pa Odewo and Pa Daramola in the Forestry Research Institute, Ibadan, Nigeria where herbarium specimens were deposited.

2.1 Extract preparation

The cocktail was prepared according to the ratio of 5:1: 3: 2: 4:1:3, respectively to produce the desired pharmacological action as reported by Oloyede *et al.* (2008). Samples were air dried, powdered and allowed to stand in 500 mL 95% cold ethanol for 72 h. They were thereafter decanted and filtered using a muslin cloth. The extract was further evaporated to dryness in an oven at 40°C. Finally, the dried extract weighed 19.2 g. The cocktail crude extract and the individual plants extracts (obtained by soxhlet extraction) used in this study were reconstituted to concentrations of 1.0, 2.5 and 5.0 mg mL⁻¹.

2.2 Fractionation of extracts

The combined ethanolic extract (44.8g) obtained above. The combined ethanol extract was then dissolved in 200ml of Ethanol: water (9:1), the mixture was then shaken with n-hexane and extract was evaporated under reduced pressure to yield Hexane fraction (2.6g). Ethanol was evaporated from the remaining extract and diluted with distilled H₂O to 200ml and further fractionated by successive solvent extraction with chloroform (4× 100ml), ethyl acetate (2×100ml) and n-butanol saturated with H₂O (3×100ml). Each extract was evaporated to dryness under reduced pressure to yield 'chloroform fraction (3.6g), ethylacetate fraction (1.45g), butanol fraction (12.6g) and the remaining H₂O fraction (23.5g).

2.3 Cell lines and cell cultures

African green monkey kidney cells – VERO, and chicken embryo fibroblast -CEF (provided by National Veterinary Research Institute, Vom Jos,

Plateau state, Nigeria) were grown in sterile DMEM (Dulbecco's modified Eagle's medium). The cells were maintained as monolayers in 25 cm² plastic tissue culture flasks at 37 °C in a humidified atmosphere containing 5% CO₂ in air. Exponentially growing cells were used in all the experiments. The fetal bovine serum in the medium represents a cocktail of most of the factors required for cell proliferation and maintenance. Penicillin and streptomycin were used to inhibit the growth of Gram-positive and Gram-negative bacteria respectively, which may contaminate the culture media.

2.4 Cytotoxicity assay

Cytotoxicity of the extracts was measured using the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as described by Kigundu *et al.* (2009), on African green monkey kidney cells (Vero) and chicken embryo fibroblast (CEF). The principle of cell viability determination is due to the cleaving of the MTT tetrazolium salt by mitochondrial Succinate-dehydrogenase enzyme of viable cells and converted to an insoluble purple formazan. 100 µL Containing 2 × 10⁴ of cell suspension was placed briefly in each of 96-well plates cells incubated at 37°C in 5% CO₂ for 24 h to attach. After attachment the medium was aspirated off carefully and cell cultures were treated with the extracts at concentrations of 1mg, 2.5mg and 5mg. Cyclophosphamide and cells without media were used as positive and negative controls respectively. Each sample was replicated 4 times and the cells incubated at 37°C in 5% CO₂ for 48 h. After 48 h incubation, 20 µL of the proliferation reagent MTT at concentration of 5 mg mL⁻² in phosphate-buffered saline (PBS, pH 7.4) was added to each well and the cells were incubated at 37 °C for 4 h in humidified atmosphere with 5% CO₂. At the end of the incubation period, the medium together with MTT was aspirated off from the wells; formazan precipitates were solubilized by addition of 100 µL of DMSO and the plate shaken gently for 5min. After 10 min at room temperature, the formazan salts were quantified by reading spectrophotometric absorbance at 492 nm of each well in a microculture plate reader {Lab systems (Multiskan EX) serial RS-232 C}.

$$\text{Cell viability \%} = \frac{\text{Absorbance of experimental wells}}{\text{X 100}}$$

$$\frac{\text{Absorbance of control wells}}{\text{(Sreeja and Sreeja, 2009)}}$$

2.5 Statistical analysis

Data are presented as mean±S.E.M. for at least four replicates. Statistical analysis was

performed using Student t-test with significant level set at $P < 0.05$. The IC_{50} values were obtained by linear regression analysis using the SPSS program.

3.0 RESULTS

3.1 Cytotoxicity

Table 11: Cytotoxic activity of Joloo and its constituents on VERO and CEF cell-lines

Plants	VERO 492nm				CEF 492nm			
	Treatment (mg)			IC ₅₀	Treatment (mg)			IC ₅₀
	1	2.5	5		1	2.5	5	
<i>Securidaca longepedunculata</i>	0.370±0.02 ^b	0.358±0.02 ^b	0.287±0.01 ^c	8.5	0.191±0.02	0.176±0.00	0.133±0.00 ^c	8.7
<i>Hoslunda opposita</i>	0.408±0.02 ^a	0.299±0.02 ^c	0.278±0.02 ^c	6.5	0.181±0.01	0.161±0.00	0.138±0.00 ^c	10.5
<i>Olox subscopiiidea</i>	0.326±0.03 ^b	0.294±0.03 ^c	0.231±0.01 ^c	5.6	0.197±0.00	0.177±0.00	0.164±0.00	15.3
<i>Xylopiya aethiopia</i>	0.380±0.01 ^b	0.264±0.01 ^c	0.257±0.00 ^c	5.9	0.164±0.00	0.149±0.01 ^b	0.129±0.00 ^c	10.6
<i>Butyrospermum paraxodum</i>	0.448±0.04	0.441±0.02	0.351±0.02 ^b	4.9	0.163±0.01	0.156±0.01	0.134±0.01 ^c	12.5
<i>Tetrapleura tetraptera</i>	0.321±0.01 ^b	0.293±0.03 ^c	0.282±0.01 ^c	11.7	0.147±0.01 ^b	0.143±0.01 ^b	0.130±0.01 ^c	16.8
<i>Allium ascalonicum</i>	0.460±0.03	0.258±0.04 ^c	0.219±0.02 ^c	4.5	0.252±0.016	0.210±0.01	0.182±0.01	10.8
Joloo Cl	0.388±0.01 ^b	0.300±0.05 ^c	0.197±0.01 ^c	4.5	0.141±0.01 ^b	0.127±0.01 ^c	0.124±0.01 ^c	15.7
Joloo Bu	0.613±0.03	0.407±0.04 ^a	0.403±0.04 ^a	8.3	0.149±0.01 ^b	0.131±0.00 ^c	0.125±0.01 ^c	12.7
Joloo E	0.493±0.02	0.348±0.04 ^b	0.225±0.02 ^c	4.9	0.202±0.00	0.197±0.01	0.181±0.01	19.3
Joloo He	0.692±0.01	0.562±0.04	0.410±0.03 ^a	7.7	0.156±0.01	0.137±0.01 ^c	0.135±0.01 ^c	16.1
Joloo W	0.447±0.01	0.296±0.06 ^c	0.201±0.02 ^c	4.5	0.152±0.00 ^a	0.129±0.01 ^c	0.106±0.00 ^c	7.1
Cyclophosphamide			0.289±0.06				0.129±0.00	-
Control			0.433±0.01				0.159±0.00	-

Average of three independent determinations; four replicates; values are mean ± SEM. ^{abc}significantly different from control $p < (0.05, 0.01, 0.001)$ respectively.

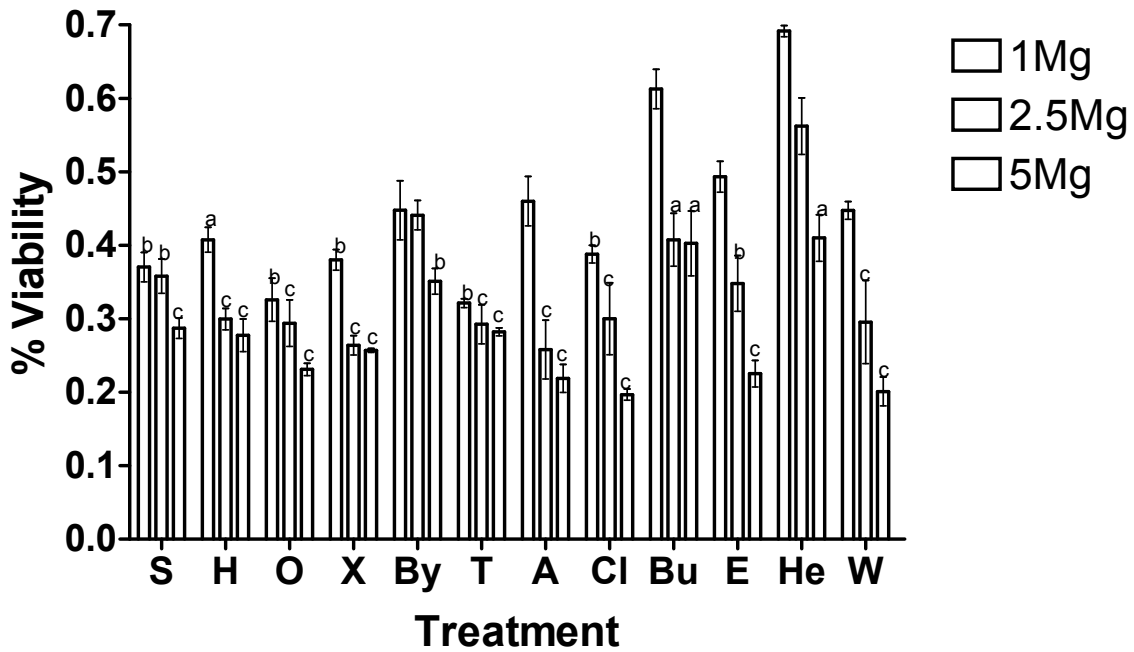


Fig. 2: Cell viability of VERO cells at different concentrations.

Data are presented as mean \pm SEM for quadruplicates. Value is statistically significant compared to control (student's t- test), ^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.001$

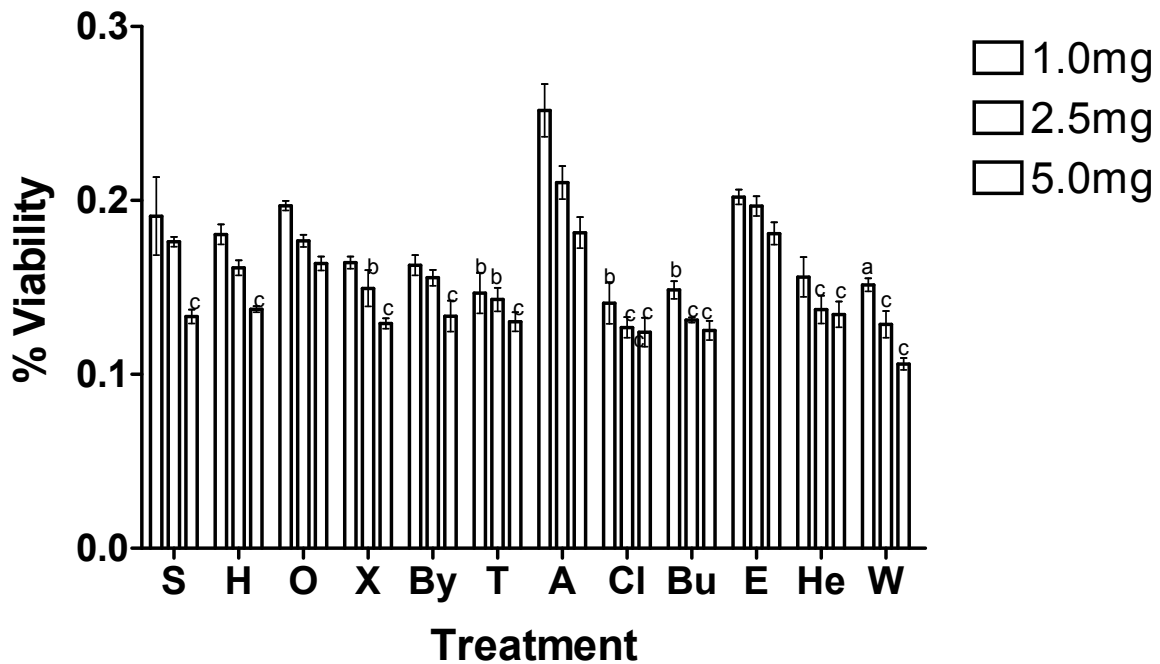


Fig. 3: Cell viability of CEF cells at different concentrations.

Data are presented as mean \pm SEM for quadruplicates. Value is statistically significant compared to control (student's t- test), ^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.001$.

4.0 DISCUSSIONS

Joloo herbal formulation is primarily used against breast cancer. It is a promising antiproliferative preparation whose mechanism is largely unknown.

Apoptotic induction has been a continuous effort in cancer treatment. Huang *et al.*, (2003) reported that reduction in cell growth and induction in cell death are two major means to inhibit tumour growth. In this study different solvent systems of Joloo caused significant growth inhibition in VERO and CEF cell lines with IC₅₀ ranges from 2.8-9.8 and 6.5-14.8 mg ml⁻¹ respectively. From the IC₅₀ observed between treatments on the two cell lines, VERO was more susceptible to cytotoxicity than CEF cell lines. However the result obtained by the MTT test, which monitors a reduction of yellow tetrazolium salt by mitochondrial dehydrogenase enzyme of metabolically active (viable) cells to purple formazan crystals with different concentrations of different solvent systems of Joloo extract (Joloo combined, fractionated portions and constituents individual plant extracts) exhibited varying degree of dose-dependent cytotoxicity on both VERO and CEF cell lines. *A. ascalonicum*, water and chloroform fractions exhibited the strongest cytotoxicity on the VERO cells than others, whereas in the CEF cells water fractions and *S. longepedunculata* were the most potent. Generally *T. tetraptera* and chloroform fraction caused cell death at all the concentrations used on both cells. Apoptosis is one of the most potent defenses against cancer, since this process eliminates potentially deleterious, mutated cells (Martin, 2006). The mechanisms of apoptosis are mainly implicated in two signal pathways; the mitochondrial pathway and the cell death receptor pathway (Lui *et al.*, 2006). The main occurrence in the mitochondrial pathway is the migration of cytochrome c from the mitochondria to the cytosol. Immediately cytochrome c arrives the cytosol, together with Aparf-1 activates caspase-9, which then activates caspase-3 (Kidd, 1998). The cell death receptor pathway is characterized by binding of cell death ligands and cell death receptors and the subsequent activation of caspase-8 and 3 (Nijhawan *et al.*, 1997). All the treatments on VERO cells showed some degree of cytotoxicity especially at 2.5 and 5.0 mg ml⁻¹ except *B. paradoxum* and hexane fraction that showed a very weak activity inducing apoptosis only at 5.0mg ml⁻¹ concentration. This apoptotic effect of *A. ascalonicum* juxtapose with the anti-angiogenesis result of Seyfi *et al.*, (2010) as tumor growth and metastasis are angiogenesis-dependent (Hanahan, 1998). *Allium* plants have also been reported to possess anti-oxidative and anti-tumour properties (Ogra *et al.*, 2005). *S.*

longepedunculata, *O. subscopioidea*, *A. ascalonicum* and ethylacetate fraction did not produce any apoptotic effect on CEF implying that Joloo could be selective in affecting cells. This result is an indication that Joloo may possibly localize on tumour cells and induce the release of cytochrome c from mitochondria. It may also trigger caspase independent apoptosis if some of the cytochrome c released from mitochondria accumulates in the nucleus (Liu *et al.*, 2006). Cell death is also regulated by a series of genes (Nakagawa *et al.*, 2001). Overexpression of Bax has been linked to the induction of the release of cytochrome c from mitochondria (Kluck *et al.*, 1997). The cytotoxic extracts of Joloo may have serious effect on these proteins by inducing increased expression of Bax and suppress release of Bcl-2.

Conclusion

The study infers the presence of mild cytotoxicosis considering the large concentrations observed in their IC₅₀. Although the apoptotic effect of Joloo towards cancer cell lines invitro has not been investigated, but the differential effect of Joloo and its constituents on VERO and CEF showed that it can be pharmacologically useful in the management of tumour if the doses are properly enhanced and hence justifies the folkloric claims of its pharmacological uses.

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