Trop J Nat Prod Res, March 2021; 5(3):485-493

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Cytotoxic Activity of Combining Molecular Iodine and Dihydroartemisinin with Methanol Extracts of *Carica papaya* Linn and *Vernonia amygdalina* Delile Leaves against MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

Jadwiga Nowak[1]*, Charles Wambebe[1], Jackson Mukonzo[1], Esther Katuura[2]

[1]*Department of Pharmacology & Therapeutics, School of Biomedical Sciences, College of Health Sciences, Makerere University, Uganda*
[2]*Department of Plant Sciences, Microbiology and Biotechnology, College of Natural Sciences, Makerere University, Uganda*

ARTICLE INFO

Article history:
Received 01 December 2020
Revised 18 January 2021
Accepted 08 February 2021
Published online 01 April 2021

ABSTRACT

Carica papaya Linn (CP), *Vernonia amygdalina* Delile (VA), dihydroartemisinin (DHA), molecular iodine (I_2) were reported to have antiproliferative and cytotoxic activities on breast cancer, but their anticancer effect once combined had not been explored. This study assessed the cytotoxic effect of combining I_2, DHA, methanolic extracts of CP, VA leaves against MCF-7 and MDA-MB-231 breast cancer cell lines. Cytotoxic activity and cell viability of the plant extracts and combinations were determined using trypan blue assay with peripheral blood mononuclear cells(PBMCs) and doxorubicin (DOX) as negative and positive controls respectively. The study used SigmaPlot software to obtain IC_{50} values and drug interactions were determined using the isobologram and combination index (CI)(Chou-Talalay method).The combination of CP + VA revealed the strongest cytotoxic activity amongst tested combinations and individual extracts with CI < 1 and IC_{50} = 18.6 ± 2.5 on MCF-7. A combination of CP + VA + DHA with CI >1 was included in the study based on its possible multi-target mechanism of action. The cytotoxic effects of CP + VA, CP + VA + DHA were significantly lower when tested on PBMCs in comparison to activity against breast cancer cells. DOX showed higher cytotoxic activity on PBMCs when compared with the tested combinations. This study suggested CP + VA to be the most efficacious combination with strong antiproliferative effect on MCF-7 and lower toxicity to human PBMCs when compared to DOX. Each of the combination's components was found to be less cytotoxic against chosen cell lines.

Keywords: *Carica papaya* Linn, *Vernonia amygdalina* Delile, Dihydroartemisinin, Molecular Iodine, Breast Cancer Cells, Cytotoxic Activity.

Introduction

Breast cancer is a malignant tumour that originates in the cells of the breast and is one of the most widespread cancer among women worldwide.[1] It is the second most common cancer overall and a major cause of cancer-related deaths among women globally.[2]
Despite the progress in understanding the molecular basis, diagnosis, and treatment of breast cancer over the past several years, an outright cure remains elusive. Further, the management of breast cancer's malignancy continues to be very costly, particularly for African women[3] due to limited access to early diagnosis and effective treatment. Consequently, most breast cancer patients in Africa resort to the use of cheaper treatment alternatives including medicinal plants. Although plants have been used globally to treat many types of cancer, this practice has been abused due to inadequate scientific evidence on the safety and efficacy of such plant preparations. Many herbal preparations that are single botanical formulations have been studied but their synergistic effect has not been evaluated. In practice though, the synergistic use of herbs has been proven to be more effective.[4] This study explores the cytotoxic effects of combining extracts of CP and VA leaves with DHA and I_2 as possible agents to manage breast cancer. A plant extract is usually regarded as promising if its*in vitro* cytotoxic activity is IC_{50} < 30 µg/mL.[5] Similarly, for pure compounds, IC_{50} value of 4 µg/ml or less is acceptable for their further evaluation as chemotherapeutic agents.[6] Over the years, the screening of natural products by the National Cancer Institute (NCI) and other groups has resulted in the discovery of a few plant extracts with high anticancer activities. However, pure compounds from such extracts often exhibit severe side effects.[7] Therefore, it is highly reasonable to investigate natural products with IC_{50} values higher as stated above to ascertain if the extracts have the potential to kill selectively malignant cells with minimal harm, if at all, to the normal cells. Isobologram and CI analyses are the two most popular methods for evaluating interactions of combination drugs[8] and were adopted in this study to evaluate possible synergy between the chosen extracts and compounds.
CP is an evergreen tree-like herb that has been documented to have more than 5 000 compounds associated with anticancer properties. These compounds include phenolics, carotenoids, and glucosinolates.[9] The plant is rich in the enzyme papain that is effective against cancer cells.[10] Papain breaks down the fibrin of the cancer cell wall and protein into an amino acid. Further, CP contains lycopene that is highly effective against reactive oxygen and free radicals. CP also contains isothyocynate that is effective against breast, lungs, colon, pancreas, prostate cancer as well as leukaemia. These enzymes are capable of inhibiting both the formation and the development of cancer cells. The cytotoxicity was observed when a T47-D breast cancer cell line was treated with a protein fraction containing ribosome-inactivating proteins isolated from CP leaves with an IC_{50} value of 2.8 mg/ml.[10] The petroleum extract of CP's aerial parts was reported to have a significant anticancer effect on MCF-7 cancer cells.

*Corresponding author. E mail: Jagodanowak@hotmail.com
Tel: +256781703171

Citation: Al Nowak J, Wambebe C, Mukonzo J, Katuura E. Cytotoxic Activity of Combining Molecular Iodine and Dihydroartemisinin with Methanol Extracts of *Carica papaya* Linn and *Vernonia amygdalina* Delile Leaves against MCF-7 and MDA-MB-231 Breast Cancer Cell Lines. Trop J Nat Prod Res. 2021; 5(3):485-493. doi.org/10.26538/tjnpr/v5i3.12

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Therefore, CP's aerial parts can be helpful in cancer prevention and treatment.[11]

VA is a bushy shrub or small tree of 2–5 m with a petiolate leaf of about 6 mm diameter and an elliptic shape. It has been documented that this shrub has diverse therapeutic effects including anticancer properties. Coumarins, flavonoids, sesquiterpene lactones, and edotides may be the principal constituents in VAthat are responsible for its anticancer activity.[12] The previous findings from MTT assay have revealed that VA ethanol extract inhibits the proliferation of MCF-7 and MDA-MB-231 cell lines in both a time- and dose-dependent manner and induces apoptosis occurring through both extrinsic and intrinsic apoptotic pathways. Significantly, about 70% of diagnosed breast cancer express ER-α, and VA inhibits the expression of ER-α and its downstream player, Akt. Furthermore, when VA extract was combined with DOX, there was profound synergism suggesting that this combination could complement current chemotherapy.[13]

According to a study,[14] ancient Chinese medicine used a methyl ether derivative of artemisinin (ARTs), artemether injections to successfully inhibit proliferation in advanced breast cancer cases. A semisynthetic derivative of artemisinin, Dihydroartemisinin (DHA), has also been confirmed and documented to be an effective anti-cancer compound[15, 16]. Studies[17] have further established that DHA subdued breast tumor-induced osteolysis through inhibiting the propagation, migration and incursion of MDA-MB-231 cells. Studies have also yielded positive results on various combinations of DHA with other compounds.[15,18]

Iodine is a non-metallic element of the halogen group represented by the atomic symbol I. Its atomic number 53, and atomic weight is 126.90. The molecular effects of iodine and the growing evidence regarding these effects point to its possible role in the prevention of cancer, especially stomach and breast cancer. This is because of its antioxidant, anti-inflammatory, pro differentiating, and pro-apoptotic effects.[19] A study[19] suggested that Iodine's role in maintaining the health of breast tissue is because of its therapeutic effects on benign breast conditions. *In vitro* and *in vivo* studies suggest that the therapeutic form of iodine in breast cancer treatment is I_2.[19] Several studies indicate that I_2 can protect laboratory animals against mammary tumours.[20-22] In a chemical carcinogenesis model of mammary tumours using rats that were given methyl-nitrosurea, I_2 was given as a 0.05% of water source, and the rats were allowed unrestricted accessto the drug. The incidence of mammary tumours was reduced by 37.5% in the treated rats when compared to the rats in the control group.[20] A study [21] also explored the cytotoxicity of iodine on cultured human breast cancer cell lines *viz*, MCF-7, MDA-MB-231, MDA-MB-453, ZR-75-1, and T-47D. The findings revealed iodine-induced apoptosis in all of the tested cell lines except MDA-MB-231. Further, a study [22] suggested that iodine could affect the binding of oestrogen receptors to the steroid-binding element. Using MCF-7 breast cancer cells, this study demonstrated that Lugol's solution (5% iodine/10% iodide) affected 43 genes involved in cell cycle growth, proliferation, and differentiation.

In view of the above, this study tested alternative combinations amongst I_2, DHA, methanolic extracts of CP and VA leaves against MCF-7 and MDA-MB-231 breast cancer cell lines to assess their cytotoxic effects. Additionally, to confirm the IC_{50} values of Ugandan botanicals used in this study, single extracts of CP and VA were tested for their cytotoxic activity against the chosen cancer cell lines.

The two breast cancer cell lines, MCF-7 (hormone-dependant breast carcinoma) and MDA-MB-231 (hormone-independent breast carcinoma) used in this study were selected based on their prevalence, aggressiveness, and resistance to treatment.

Materials and Methods

Plant Material

The plant materials were collected from certified organic farms in Luwero, Uganda and taken to the Makerere University Herbarium (MHU) for authentication. The voucher specimens were allocated numbers JN/001 and JN/002 for VA and CP respectively. The materials for laboratory analysis were collected in the September 2017, and washed and air-dried in the shade at an ambient temperature of 25°C to 30°C for 6 (CP) and 10 (VA) days to constant weight. The dried material was pulverized (using mortar and pestle) and kept in an airtight container at room temperature until its extraction using methanol.

Preparation of methanol extracts

One kg of plant powder was soaked in 5 l of methanol for 3 days with occasional shaking every 8 hours. The acquired extract was filtered using the Whatman filter paper (No.1) and concentrated in a vacuum below 45^0C using a rotary evaporator. The extract was left standing in a sterile hood until all methanol evaporated to obtain dry crude methanol extract that was subsequently stored at -4°C until its use.

Cell lines and culture conditions

Two human breast cancer cell lines, MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC) (Virginia, USA). These cell lines were recently authenticated and cultured according to ATCC guidelines and were mycoplasma free. The MCF-7 cell line was grown in a complete growth medium, i.e., the Eagle's Minimum Essential Medium (EMEM) and supplemented with 10% FBS, 1% HEPES buffer, 1% penicillin/streptomycin, and insulin (10 µg/mL). The MDA-MB-231 cell line was grown in the Leibovitz L-15 Medium and supplemented with 10% FBS, 1% HEPES buffer, and 1% Penstrep. The cells were incubated at 37^oC in a 5% CO_2 incubator for MCF-7 and 37^oC in a non-CO_2 incubator for MDA-MB-231 as per the ATCC's cell culturing guidelines. The cells were sub-cultured at a confluence of 85% and a part of the cells was stored in liquid nitrogen until further use. The MDA-MB-231 cells were sub-cultured for 4 days, while the MCF-7 cells were sub-cultured for 10 days to attain enough cells for the cell viability assays. Due to experiments being performed in sterile conditions, all of the assays were free of mycoplasma contamination.

Reagents and chemicals

FBS, EMEM, L-15, human insulin, PBS, DMSO, trypan blue, penicillin/streptomycin, Hepes buffer, absolute ethanol, and CFSE were obtained from Sigma-Aldrich. EMEM, L-15, trypsin-EDTA (0.25% trypsin/0.53 mM EDTA) were obtained from the ATCC, DHA from the Cayman Chemical Company, USA, and Annexin V, PI, and I_2 from Biokom, Poland

Preparation of drugs

The plant extracts, DHA, and iodine were separately dissolved to obtain stock solution 1. This solution was prepared in a labelled centrifuge tube by weighing and separately dissolving 1 000mg of each herbal extract and 100 mg of DHA in 10 mL of DMSO and 10mg of I_2 in 10 mL of ethanol stock solution 2 was prepared from stock solution 1 by dissolving the latter in PBS, as per proportional sets formula: $C_1V_1 = C_2V_2$

where C1 is the concentration of the starting solution, V1 is the volume of the starting solution, C2 is the concentration of the final solution and V2 is the volume of the final solution. Obtained stock concentrations of VA = 1056 µg/mL, CP = 1600 µg/mL, DHA = 118.289 µg/mL, and I_2 = 16.24 µg /mL were based on 16x extrapolated IC_{50} value of each component taken from previous studies on cancer cell lines[5,10-12,14,18,20,22] to ensure adequate drugs concentration ranges in experimental assays. To prepare drug solution 1, stock solutions 2 were combined and mixed in a ratio of 1:1 in labelled centrifugal tubes to obtain the following six combinations: VA + CP, VA + DHA, VA + I_2, CP + DHA, CP + I_2, and DHA + I_2. Each of two extracts and six combinations were serially diluted to obtain 5 concentrations (Table 1). DOX stock solution was prepared at a concentration of 96 µg/mL to be used on cells in 5 concentrations as positive controls (Table 1). Based on the cytotoxicity of the most effective combinations and the possible wider mechanism of anticancer activity, a three-component combination of CP + VA + DHA was included for the cytotoxic assay. Drug solution 2 (CP + VA + DHA) was obtained by mixing three of the stock solutions 2 in a ratio of 1:1:1. The drug was serially diluted to obtain 5 concentrations (Tables 1, 2 and 3).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Concentrations of serially diluted extracts, combinations and DOX used in experiments on both cell lines

	Concentration µg/mL				
Drug	**1**	**2**	**3**	**4**	**5**
CP	800	400	200	100	50
VA	528	269	132	71	35.5
CP + VA	664	332	166	83	41.5
CP + DHA	428.572	214.786	107.393	53.696	26.848
CP + I	404.04	202.02	101.01	50.505	25.25
VA + DHA	293.572	146.786	73.393	36.695	18.348
VA + I	268.04	134.02	67.01	33.505	16.75
DHA + I	33.612	16.806	8.403	4.201	2.1
CP + VA + DHA	462.381	231.19	115.595	57.797	28.898
DOX	48	24	12	6	3

Table 2: Concentrations of stock solutions 2 of compounds, extracts, combinations and DOX used in experiments on both cell lines

Drug	**Concentration µg/ml**
DHA	118.289
I2	16.24
CP	1600
VA	1056
CP + VA	1328
CP + DHA	859.144
CP + I	808.08
VA + DHA	587.144
VA + I	536.08
DHA + I	67.224
CP + VA + DHA	924.762
DOX	96

Table 3: Concentrations (µg/mL) of individual components in combinations in 5 concentrations

CP + VA		**CP + DHA**		**CP + I**		**VA + DHA**	
CP	**VA**	**CP**	**DHA**	**CP**	**I**	**VA**	**DHA**
400	264	400	29.572	400	4.04	264	29.572
200	132	200	14.786	200	2.02	132	14.786
100	66	100	7.393	100	1.01	66	7.393
50	33	50	3.696	50	0.505	33	3.696
25	16.5	25	1.848	25	0.252	16.5	1.848

VA + I		**DHA + I**		**VA + CP + DHA**		
VA	**I**	**DHA**	**I**	**VA**	**CP**	**DHA**
264	4.04	29.572	4.04	176.59	266.8	19.74
132	2.02	14.786	2.02	88.29	133.4	9.87
66	1.01	7.393	1.01	44.14	66.7	4.93
33	0.505	3.696	0.505	22.07	33.35	2.46
16.5	0.252	1.848	0.252	11.03	16.67	1.23

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Cytotoxic activity of single extracts and combinations on cancer cells
The cells were removed from their culture flask using the trypsin EDTA treatment until they were dislodged with gentle aspiration into a single-cell suspension. The cell viability assay was performed in triplicate. The cell solution (2 ml) was seeded in a pre-labelled M6 tissue culture plate at a density of 200,000 cells/mL per well and incubated for 1 hour before exposure to the serial solutions of 2 mL drugs in each well. The non-stimulated cells and DOX were used as negative and positive controls respectively.The cytotoxicity was assessed at time intervals of 24 h, 48 h, and 72 h using trypan blue exclusion assay.

Isobologram and calculation of the CI
The drug interactions were determined using the isobologram and CI method derived from the median-effect principle of Chou-Talalay Method for drug combination[8]. The mathematical model that was established to assess drug-drug interactions, employed the median effect principle formula $f_a/f_u = [D/D_m]^m$ in which fa is the fraction of cells affected, $f_u = 1-f_a$ is the fraction unaffected, D equals the concentration of the drug, D_m is the drug dose required for 50% inhibition, and m is the slope of the median effect curve. The isobologram that provides a graphical representation of the pharmacological interaction is generated by a straight line connecting the Fa points against the fixed-ratio combinations of Drug 1 and Drug 2 on the x and y axes. The data points (Fa) on the lower-left, upper-right, or hypotenuse indicate synergism, antagonism, or additive effect respectively. As proposed by the Chou-Talalay method, the CI for the Loewe additivity dose-effect model[8]was adopted to determine the most synergistic formulation and was manually calculated following the equation stated below:

$$CI = \frac{CA,X}{ICX,A} + \frac{CB,X}{ICX,B}$$

where CA,X and CB,X are the concentration of the drugs A and B used in combination to achieve x % drug effect. ICX, A and ICX, B are the concentrations for single agents to achieve the same effect. A CI of less than equal to or more than 1 indicates synergistic, additive, or antagonistic effect respectively.

Assessment of viability of normal cells when treated with combinations
The isolated human PBMCs were used as a control to study the toxicity of combinations on human cells. The blood samples were collected with consent from a healthy volunteer in 8 ml ACD tubes. The cells were extracted from the whole blood by using the Ficoll layering technique. These cells were seeded in duplicate on M6 tissue culture plates and five concentrations of the serial dilutions of the drugs that worked best on the cell lines (CP + VA and CP + VA + DHA) were added. The non-stimulated cells and DOX were used as negative and positive controls respectively. The trypan blue assay was used to test the cell viability at time intervals of 24 h, 48 h, and 72 h.

Statistical analysis
The IC_{50} values were obtained using the SigmaPlot software analysis from the viability test data by plotting standard dose-response curves. To obtain the mean ± standard deviation (SD), the data from the different experiments were coded and entered using the IBM SPSS Statistics for Windows, Version 22 statistical software (Amonk,NY:IBM Corp). The main statistical procedure employed for the analysis was one-sample t-test to determine whether significant mean differences exist in the means. The mean differences were considered significant at $p \leq 0.05$.

Results and Discussion

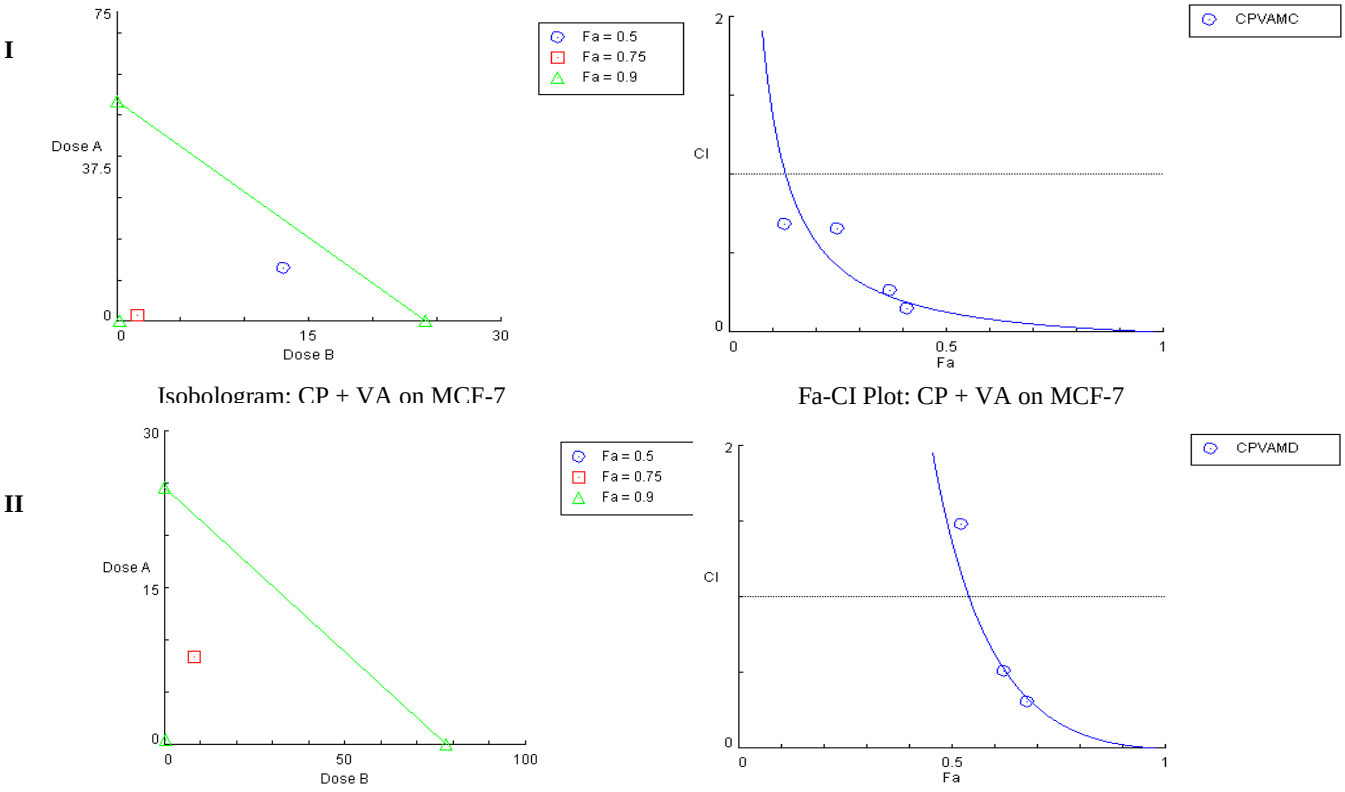
Cytotoxic activity of single extracts and combinations on cancer cell lines
Two breast cancer cell lines, i.e., MCF-7 and MDA-MB-231 were used to screen the *in vitro* cytotoxic activity of the two extracts and six combinations. The highest cytotoxic activity was observed at 72 h and expressed as IC_{50} values following the trypan blue viability test. Regarding the IC_{50} concentration of the individual drug components in their respective formulations and CI calculations, the combination of CP + VA with IC_{50} values of 18.6 ± 2.5 µg/mL revealed the strongest synergistic cytotoxic activities on MCF-7 (CI < l) and weaker additive cytotoxic activity with IC_{50}valueof 336.9 ± 13.6 on MDA-MB-231 (CI = 1) after a 72 h drug exposure in a concentration-dependent manner (Figure 1, 2).
In comparison, DOX showed cytotoxic activity with the values of IC_{50} = 6.6 µg/ml on MCF-7 and IC_{50} = 14.8 µg/ml on MDA-MB-231. The cytotoxicity of extracts from indigenous Ugandan *Carica papaya* Linn and *Vernonia amygdalina* Delile was less effective against the MCF-7 cell line compared to their combined effect (Table 4 and Figure 3,4).

Viability of PBMCs Treated with CP + VA and CP + VA + DHA
The combinations of CP + VA and CP + VA + DHA showed a significantly higher cytotoxic effect on breast cancer cell lines compared to their cytotoxic activities on human PBMCs (Table 5, Figure 5). At 72 h, the PBMC showed 99.4 ± 0.4 and 90.3 ± 2.6 viability when treated with combinations of CP + VA and CP + VA + DHA at their concentrations equal to IC_{50} = 18.6 ± 2.5 µg/mL against MCF-7 and IC_{50} = 149.3 ± 6.4 µg/mL against MD-MB-231 breast cancer cell lines respectively (Table 5). In contrast, DOX showed significantly higher cytotoxicity on human PBMCs than the most efficacious combinations. The PBMCs showed 73.89 ± 1.30 viability when treated with DOX at a concentration equal to its IC_{50} = 6.6 µg/ml value on MCF-7 and 48.82 ± 0.53 viability when treated with a concentration of IC_{50} = 14.8 µg/mL value obtained from the experiment on MD-MB-231 at 72 h (Table 5, Figure 5).
The adverse side effects of chemotherapy and radiotherapy in cancer treatment have been evident and documented in several studies[4,23]. These include hair loss, a sick feeling, radiation poisoning, or even death. The vast potential of plant-based medications in the fight against breast cancer remains a valid and viable option that is not only affordable and accessible but also has limited side effects[24,25]. In fact, a study[26] stated that in Uganda, in addition to the local herbalists, there are complementary practitioners of Chinese and Ayurveda medicines whose numbers have recently increased.[27] All these practitioners are adopting a combination of both allopathic and complementary forms of treatment. Several studies[24,27] have observed that most patients on allopathic cancer therapy are concurrently self-medicating with one or more complementary and alternative medicines (CAM), most common among them being herbal medicines[24]. The MCF-7 cell line has been documented to be the most common form of breast cancer that is responsible for about 83% of the cases,[29] while the MDA-MB-231 cell line is not as common but is much harder to treat[29]. Unlike other studies on the use of CP[9, 30], artemisinin[14,16], VA, [12,13] and iodine[21,22] to treat cancer, this study sheds light on the cytotoxic effect of combining the methanolic extracts of CP and VA with DHA and iodine against breast cancer cells. Some of the previously reported IC_{50} values of single-plant extracts and compounds include CP petroleum ether and aqueous methanolic extracts on MCF-7 breast cancer cell lines that were 100 µg/mL[31] and 130 µg/mL,[32] respectively. In case of VA it was IC_{50} = 56µg/ml on MCF-7 and 46 µg/mL on MDA-MB-231 cell lines respectively[33]; in case of DHA, it was IC_{50} = 111 nM on MCF-7 and 11.8 nM on MDA-MB-231 respectively;[34] and in case of I_2, it was IC_{50} = 2.7 and 3.1 µM on MCF-7 and MDA-MB-231 respectively.[35] It should be noted that in contrast to pure compounds, plant constituents vary both qualitatively and quantitatively according to the ecology, weather, age, and time of collection.[36] Extracts from Ugandan origin VA and CP were tested for their IC_{50} values in this study and hence, the results presented different cytotoxic profiles when compared to other studies. Although, several reviews have been written on the combination effects of complex mixtures,[30,37] it is still difficult to define the term synergy in this context, especially with regard to combining herbal extracts and pure compounds.[30,38,39] The additive and non-interactive combinations indicate that the combined effect of two substances is a pure summation effect, while an antagonistic interaction results in a less than additive effect. Positive interactions, known as potentiation or synergy occur when the combined effect of the constituents is greater than the expected additive effect.[30,37,38, 40-43]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The most popular model for studying combination effects is based on the Loewe additivity principle[30,40-44] and was used in this study. However, it is challenging to draw definite conclusions from the resulting data as, in some instances, the combination effects have been identified as synergistic by one model but antagonistic by another.[45] In view of the above, considering the IC_{50} values along with CI, CP + VA is potentially the most efficacious combination for both cell lines with strong synergistic activities (CI < 1) on MCF-7 (IC_{50} = 18.6 µg/mL) and additive activity (CI = 1) on MDA-MB-231 (IC_{50} = 336.9 ± 13.6 µg/ml). On the other hand, in this study, single extracts of CP and VA showed 623.9 ± 80.8, 144.8 ± 37.4 µg/ml IC_{50} values when used alone on MCF-7 and 321.5 ± 223.7, 354.6 ± 24.2 µg/ml IC_{50} values on MDA-MB-231 cell lines respectively. Hence, a combined application of two synergistically working extracts with a compound may lead to enhanced anticancer activity when compared to the use of a single plant extract or compound due to the multiple mechanisms by which cancer escapes regulated cell growth and avoids apoptosis. Thus, the combination CP + VA + DHA, despite its CI being more then 1 was chosen for cytotoxic assay against normal cells because of its encouraging IC_{50} values of 78.6 ± 5.2 and 149.3 ± 6.4 on MCF-7 and MDA-MB-231 respectively. The use of whole plant extracts and their combinations with or without pure compounds may provide an advantage over using one isolated plant or synthetic compound as the formulation contains several active components with different possible intracellular targets. According to a study[46], the mechanisms underlying synergistic therapeutic actions of herbal medicines are the following:

1. Different agents may regulate either the same or different targets in various pathways, and therefore cooperate in an agonistic, synergistic way;
2. Regulate the enzymes and transporters that are involved in hepatic and intestinal metabolism to improve oral drug bioavailability;
3. Overcome the drug resistance mechanisms of microbial and cancer cells; and
4. Eliminate the adverse effects and enhance pharmacological potency of agents by "processing" or by drug-drug interaction.

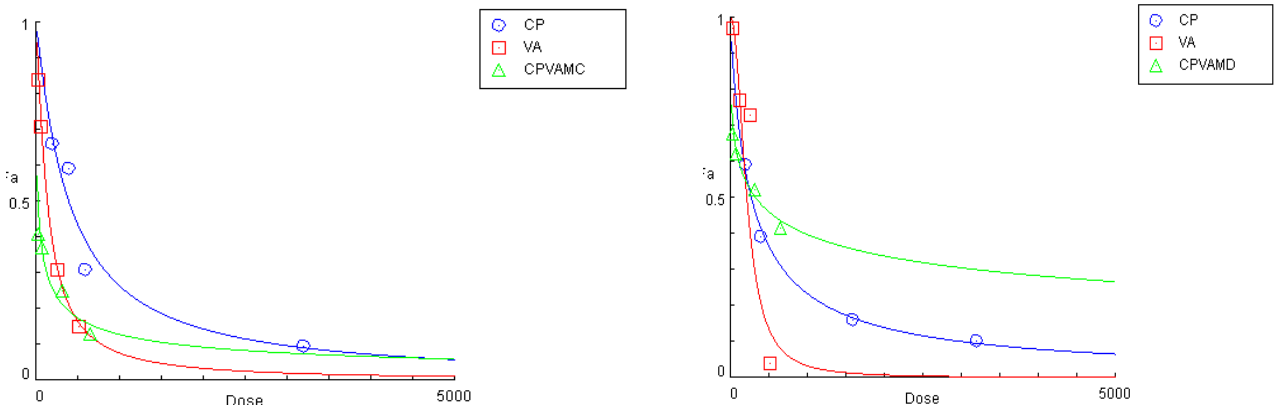


Isobologram: CP + VA on MCF-7 | Fa-CI Plot: CP + VA on MCF-7

Isobologram: CP + VA on MDA-MB-231 | Fa-CI Plot: CP + VA on MDA-MB-231

Figure 1: Isobolograms and Fa-CI Plots of CP + VA on MCF-7 (I) and MDA-MB-231 (II) Cell Lines.
Key: Fa = effect levels (fraction affected), Dose A = Concentrations (µg/ml) of VA, Dose B = Concentrations (µg/mL) of CP.
The MCF-7 and MDA-MB-231 cell lines were treated with a dose series of methanolic extracts of CP, VA, and their combinations. The dose ranges for individual and combined drugs were completed in triplicate. Isobologram and Fa-CI plot analysis were completed in accordance with the Chou-Talalay method using CalcuSyn. Isobologram: Fa on the lower-left, upper-right, or the hypotenuse indicate synergism, antagonism, or additive effect respectively. CP + VA proved to have a strong synergistic effect against MCF-7 showing three data points on the lower-left of the hypotenuse compared to the two data points against the MDA-MB-231 cell line. Fa-CI plot: The data points (simulated curves) below, above, or on the CI = 1 horizontal line indicate synergism, antagonism, or additive effect respectively. Among the four combination data points of CP + VA on MCF-7, all of them were on the synergy side (CI < 1). The same combination of MDA-MB-231 presents two data points on synergy and one on antagonism (CI > 1) sides.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)



I II

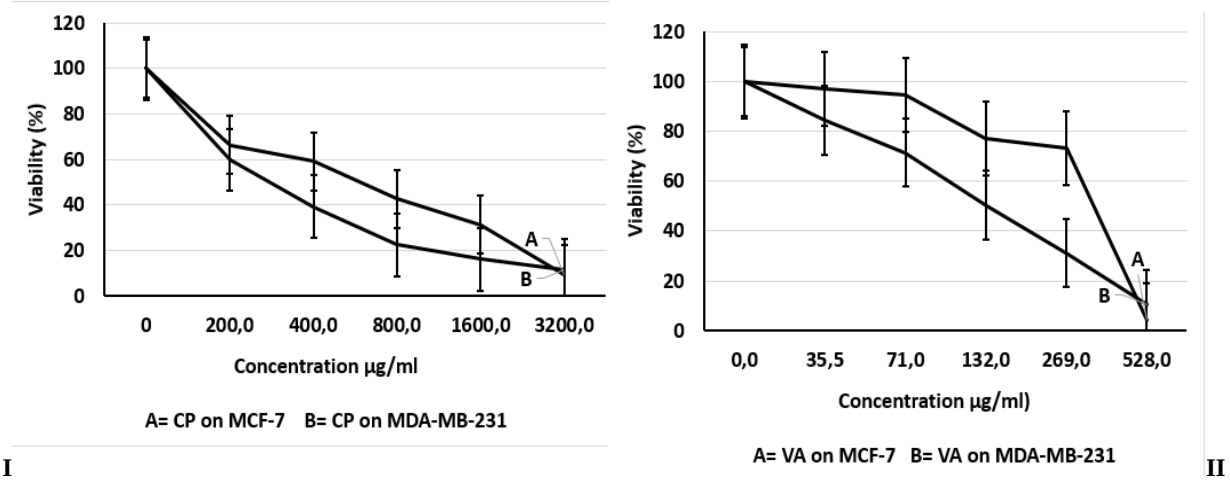
Figure 2: Dose-effect curve of single extracts (CP, VA) and its combination (CP+VA) against MCF-7 (I) and MDA-MB-231 (II) cell lines.

Key: Fa = fraction affected, Dose = concentrations of CP, VA, and CP + VA against cell lines.
The MCF-7 and MDA-MB-231 cell lines were treated with a dose series of methanolic extracts of CP, VA, and their combinations. The dose ranges for individual and combined drugs were completed in triplicate. The dose-effect analysis was completed in accordance with the Chou-Talalay method using CalcuSyn.

Table 4: *In vitro* cytotoxic activity of single extracts, combinations, and DOX against breast cancer cell lines at 72 h drug exposure

Drug	Cell line (IC_{50} µg/mL)		p-value
	MCF-7	MDA-MB-231	
CP	623.9±80.8	321.5±223.7	0.177
VA	144.8±37.4	354.6±24.2	0.001
CP + DHA	133.3 ± 8.3	138.7 ± 6.0	0.619
CP + VA	18.6 ± 2.5	336.9 ± 13.6	0.000
CP + I	388.2 ± 62.9	438.0 ± 3.9	0.584
VA + I	136.8 ± 22.9	348.9 ± 33.4	0.006
VA + DHA	51.4 ± 6.4	288.0 ± 7.9	0.000
DHA + I	53.4 ± 7.9	105.6 ± 28.6	0.258
CP + VA + DHA	78.6 ± 5.2	149.3 ± 6.4	0.001
DOX	6.6 ± 1.2	14.8 ± 1.0	0.003

Key: In vitro cytotoxic activity of the combinations and DOX against MCF-7 and MDA-MB-231 cell lines at 72 h drug exposure. The data in Table 4 are shown as means ± SD and are derived from three independent repeats after a 72 h exposure to the test extracts. The means are significantly different where $p \leq 0.05$ (independent samples t-test).



I II

Figure 3: *In vitro* cytotoxic effects of CP and VA extracts against MCF-7 and MDA-MB-231 cell lines; plates I–II

Key: Concentration-response curves of the effect of CP and VA on cell growth in MCF-7 and MDA-MB-231 cell lines: (I) CP on MCF-7 and MDA-MB-231, (II) VA on MCF-7 and MDA-MB-231. The cells were treated with various concentrations of drugs and the cytotoxicity was measured by trypan blue assay at 72 h. All the experiments were done in triplicates. The results were expressed as means ± SD (n = 3).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

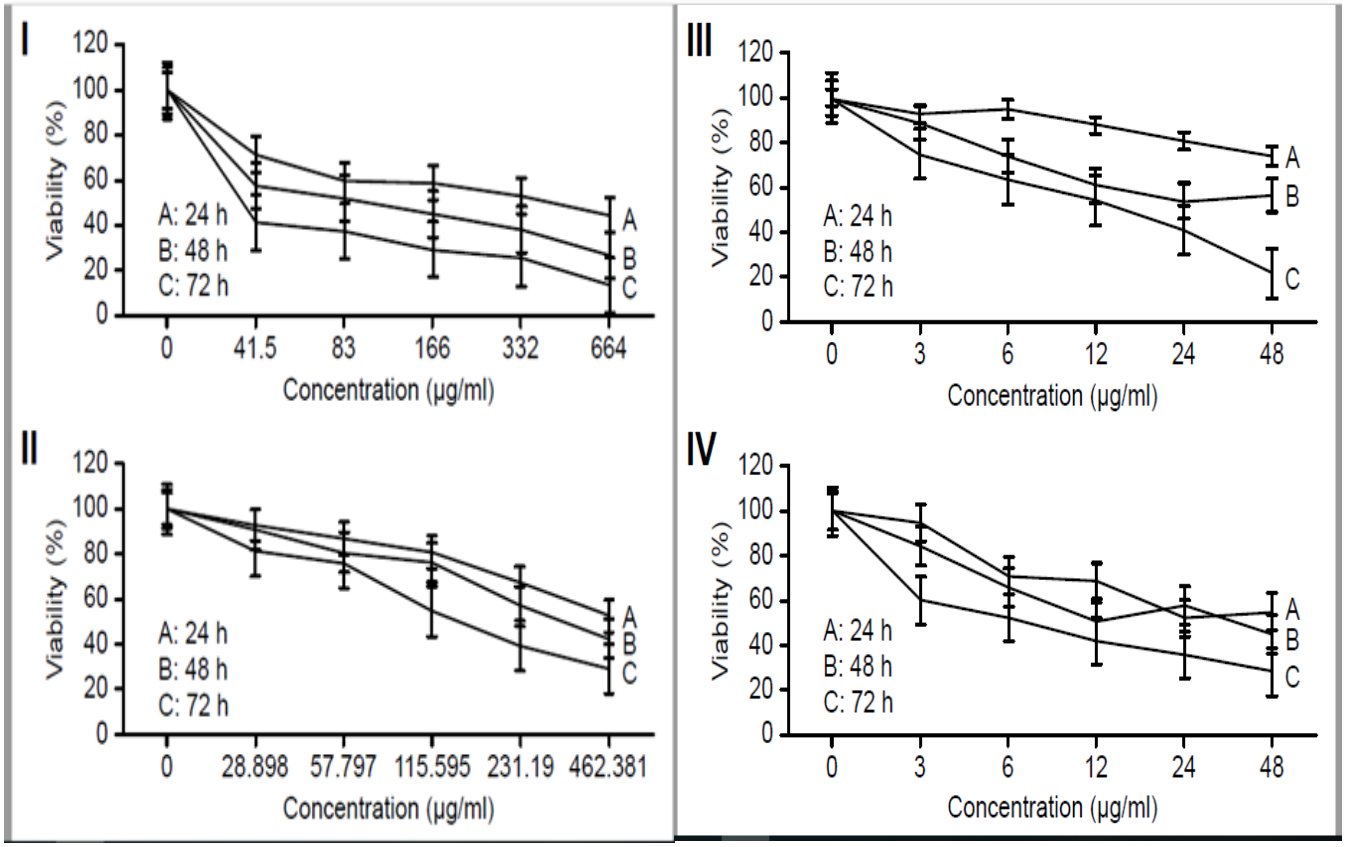


Figure 4: *In vitro* cytotoxic effects of combinations and DOX against MCF-7 and MDA-MB-231 cell lines; plates I–IV
Key: A = Concentration vs Average Viability at 24 h, B = Concentration vs Average Viability at 48 h, and C = Concentration vs Average Viability at 72 h. The concentration-response curves of the effect of CP + VA, CP + VA + DHA, and DOX on cell growth in MCF-7 and MDA-MB-231 cell lines: (I) CP + VA on MCF-7, (II) CP + VA + DHA on MDA-MB-231, (III) DOX on MCF-7, and (IV) DOX on MDA-MB-231. The cells were treated with various concentrations of drugs and the cytotoxicity was measured by trypan blue assay at time intervals of 24 h, 48 h, and 72 h. All the experiments were done in triplicates. The results were expressed as means ± SD (n = 3).

Table 5: *In vitro* viability of PBMCs treated with combinations and DOX

Drug	Reference	Viability	Mean Difference	p-value
CP + VA	Viability with reference to IC_{50} = 18.6 µg/ml (MCF-7)	99.4 ± 0.4	49.4	0.000
CP + VA + DHA	Viability with reference to IC_{50} = 149.3 µg/ml (MDA-MB-231)	90.3 ± 2.6	40.3	0.004
DOX	Viability with reference to IC_{50} = 6.6 µg/ml (MCF-7)	73.89 ± 1.30	23.9	0.003
	Viability with reference to IC_{50} = 14.8 µg/ml (MDA-MB-231)	48.82 ± 0.53	-1.2	0.154

Key: The PBMCs were treated with combinations and DOX at their respective IC_{50} concentration values on MCF-7 and MDA-MB-231 cell lines at 72h drug exposure. The data are shown as means ± SD and are derived from three independent repeats after 24h, 48h and 72h exposure to different concentrations of drugs on PBMCs. Apart from MDA-MB-231 when treated with DOX whose mean value of 48.8 ± 0.53 was insignificant (0.154 > 0.05), the statistical analysis for the rest of the means were significantly higher than the hypothesized value of 50% ($p < 0.05$; One sample t-test). That is, the predetermined value of 50 was significantly lower than the reported values.

The combined applications of studied herbal extracts and compounds may address many causes of breast cancer simultaneously and improve the effectiveness of allopathic chemotherapeutic breast cancer treatments. In addition, this study has proven the most efficacious combinations to be less toxic to normal cells at their respective IC_{50} values when compared to DOX thereby also showing their activity to be more selective towards cancer cells. The strongest cytotoxic activity of the tested combinations was observed at 72 h suggesting that their cytotoxic effect increases in both a time- and concentration-dependent manner. The effect of the test combinations on the cell lines could be explored for a longer time in continuous cultures.

The exploration of the cytotoxic effects of combining studied herbal extracts and bioactive compounds against breast cancer cell lines may help researchers in discovering new phytomedicines or drug combinations. This study validates and recommends that further investigation is called for on cancer cell lines, using an *in vivo* animal model to establish mechanisms of action as well as cytotoxic effects of combinations on growth, migration, and invasion of breast cancer in a live host.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

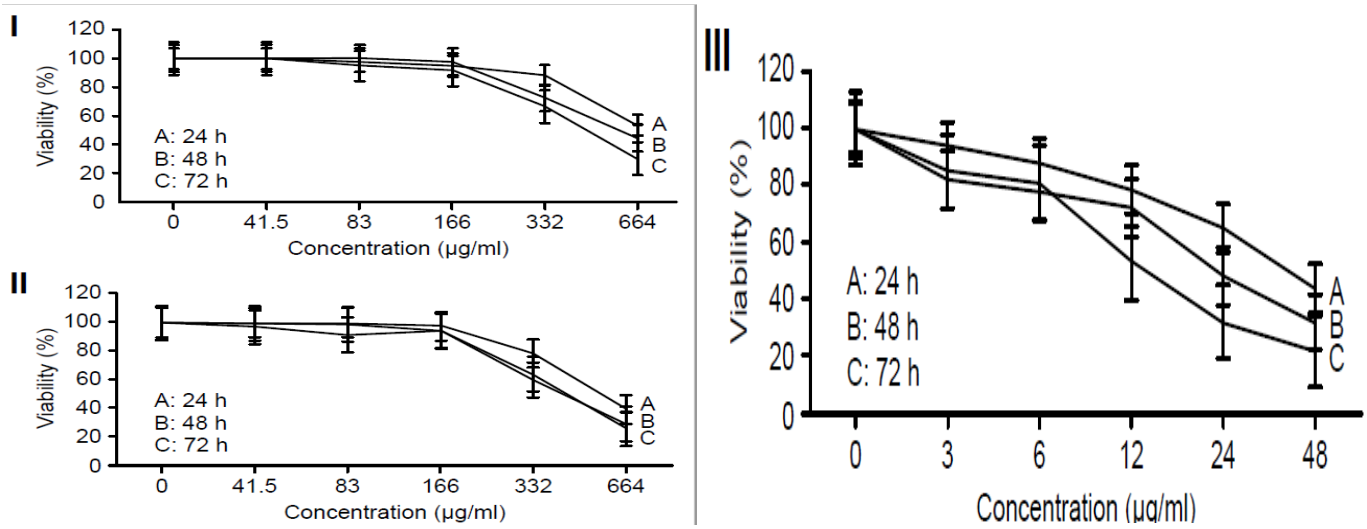


Figure 5: *In vitro* cytotoxic effects of CP + VA, CP + VA + DHA, and DOX on PBMCs; plates I–III
Key: A = Concentration vs Average Viability at 24 h, B = Concentration vs Average Viability at 48 h, and C = Concentration vs Average Viability at 72h.The concentration-response curves of cytotoxic activity of CP + VA (I), CP + VA + DHA (II), and DOX (III) on PMBCs were treated with various concentrations, and the cytotoxicity was measured by trypan blue assay at time intervals of 24 h, 48 h, and 72 h. All the experiments were done in triplicates. The results were expressed as means ± SD (n = 3).

Conclusion

This study shows the cytotoxic activity of combining CP, VA, DHA, and I_2 against breast cancer cell lines. Based on the CI calculations, the combination of CP + VA revealed the strongest cytotoxic and synergistic activity against MCF-7 and additive activity on MDA-MB-231 cell lines respectively. In comparison, single extracts have shown to be less and similarly cytotoxic when tested against MCF-7 and MDA-MB-231 cell lines respectively. Due to the potential wider mechanism of its cytotoxic activity, the combination of CP + VA + DHA was also considered for further testing. All the combinations had a minimal cytotoxic effect on PBMCs. In contrast, DOX showed higher toxicity against both tested cells and PMBCs, thus proving that the cytotoxicity of most efficacious formulations is more selective towards cancer cells.
It is suggested that CP + VA and CP + VA + DHA be further explored in relation to their drug resistance, mechanism of action, safety profile, immune system stimulatory effects, and *in vivo* tumour cytotoxicity using animal models.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors sincerely thank Francis Wasswa for helping with the laboratory experiments and Muhammad Ssekumba for providing the statistical software.

References

1. Akram M, Iqbal M, Daniyal M, Khan AU. Awareness and current knowledge of breast cancer. Bio res. 2017; 50(1):33.
2. Apuri S. Neoadjuvant and Adjuvant Therapies for Breast Cancer. Southern Med J. 2017; 110(10):638-642.
3. Lukong KE, Ogunbolude Y, Kamdem JP. Breast cancer in Africa: prevalence, treatment options, herbal medicines, and socioeconomic determinants. Breast Cancer Res and Treat. 2017; 166(2):351-365.
4. Pezzani R, Salehi B, Vitalini S, Iriti M, Zuñiga FA, Sharifi-Rad J, Martorell M, Martins N. Synergistic effects of plant derivatives and conventional chemotherapeutic agents: An update on the cancer perspective. Medicina. 2019; 55(4):110.
5. Boyd MR. The NCI *in vitro* anticancer drug discovery screen. In Anticancer drug development guide 1997. 23-42 p.
6. Likhitwitayawuid K, Angerhofer CK, Cordell GA, Pezzuto JM, Ruangrungsi N. Cytotoxic and antimalarial bisbenzylisoquinolme alkaloids from Stephania erecta. J Nat Prod. 1993; 56(1):30-38.
7. Mensah ML, Komlaga G, Forkuo AD, Firempong C, Anning AK, Dickson RA. Toxicity and safety implications of herbal medicines used in Africa. Herb Med. IntechOpen. 2019. 63-86 p.
8. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer res. 2010; 70(2):440-446.
9. Pinnamaneni R. Nutritional and medicinal value of papaya (Carica papaya Linn.). World J Pharm and Pharmaceut Sci. 2017; 6(8):2559-2578.
10. Saha S and Giri TK. Breaking the Barrier of Cancer through Papaya Extract and their Formulation. Anti-Cancer Agents in Med Chem (Formerly Current Med Chem-Anti-Cancer Agents). 2019; 19(13):1577-1587.
11. Rahmani AH and Aldebasi YH. Potential role of carica papaya and their active constituents in the prevention and treatment of diseases. Int J Pharm Pharm Sci. 2016; 8(1):11-15.
12. Kaur D, Kaur N, Chopra A. A comprehensive review on phytochemistry and pharmacological activities of Vernonia amygdalina. J Pharmacogn and Phytochem. 2019; 8(3):2629-2636.
13. Wong FC, Woo CC, Hsu A, Tan BK. The anti-cancer activities of *Vernonia amygdalina* extract in human breast cancer cell lines are mediated through caspase-dependent and p53-independent pathways. PLoS One. 2013; 8(10):e78021.

14. Singh NP and Panwar VK. Case report of a pituitary macroadenoma treated with artemether. Integrative cancer therapies. 2006; 5(4):391-394.
15. Luo H, Vong CT, Chen H, Gao Y, Lyu P, Qiu L, Zhao M, Liu Q, Cheng Z, Zou J, Yao P. Naturally occurring anti-cancer compounds: shining from Chinese herbal medicine. Chinese med. 2019; 14(1):48.
16. Jiang C, Li S, Li Y, Bai Y. Anticancer effects of dihydroartemisinin on human esophageal cancer cells in vivo. *Analy cell pathology*. 2018; 2018:7.
17. Feng MX, Hong JX, Wang Q, Fan YY, Yuan CT, Lei XH, Zhu M, Qin A, Chen HX, Hong D. Dihydroartemisinin prevents breast cancer-induced osteolysis via inhibiting both breast cancer cells and osteoclasts. Sci reports. 2016; 6:19074.
18. Zhao J, Pan Y, Li X, Zhang X, Xue Y, Wang T, Zhao S, Hou Y. Dihydroartemisinin and curcumin synergistically induce apoptosis in SKOV3 cells via upregulation of MiR-124 targeting midkine. Cell Physiol and Biochem. 2017; 43(2):589-601.
19. Kaczor T. Iodine and cancer: a summary of the evidence to date. Nat Med J. 2014; 6(6).
20. Kato N. Suppressive effect of iodine preparations on proliferation of DMBA-induced breast cancer in rat. J Jpn Soc Cancer Ther. 1994; 29:582.
21. Shrivastava A, Tiwari M, Sinha RA, Kumar A, Balapure AK, Bajpai VK, Sharma R, Mitra K, Tandon A, Godbole MM. Molecular iodine induces caspase-independent apoptosis in human breast carcinoma cells involving the mitochondria-mediated pathway. J Bio Chem. 2006; 281(28):19762-19771.
22. Stoddard II FR, Brooks AD, Eskin BA, Johannes GJ. Iodine alters gene expression in the MCF7 breast cancer cell line: evidence for an anti-estrogen effect of iodine. Inter J med sci. 2008; 5(4):189.
23. Li S, So TH, Tang G, Tan HY, Wang N, Ng BF, Chan CK, Yu EC, Feng Y. Chinese Herbal Medicine for Reducing Chemotherapy-Associated Side-Effects in Breast Cancer Patients: A Systematic Review and Meta-Analysis. Frontiers in Oncology. 2020; 10: 599073.
24. Shahid U. Herbal treatment strategies for breast cancer. OMICS group of ebooks. 2013.
25. Grady D. Uganda fights stigma and poverty to take on breast cancer. The New York Times. 2013; 10:15.
26. Kiwanuka F. Complementary and alternative medicine use: influence of patients' satisfaction with medical treatment among breast cancer patients at Uganda Cancer Institute. Advances in Biosci and Clin Med. 2018; 6(1):24-29.
27. MoH. Annual health sector performance report. Kampala: Ministry of Health. 2011.
28. Rockwell S, Liu Y, Higgins SA. Alteration of the effects of cancer therapy agents on breast cancer cells by the herbal medicine black cohosh. Breast Cancer Res and Treat. 2005; 90(3):233-239.
29. Holliday DL and Speirs V. Choosing the right cell line for breast cancer research. Breast cancer research. 2011; 13(4):1-7.
30. Patil T, Patil S, Patil A, Patil S. Carica papaya leaf extracts–An Ethnomedicinal boon. Inter J Pharmacogn and Phytochem Res. 2014; 6(2):260-265.
31. Rockwell S, Liu Y, Higgins SA. Alteration of the effects of cancer therapy agents on breast cancer cells by the herbal medicine black cohosh. Breast cancer Res and treat. 2005; 90(3):233-239.
32. Noor Atiqah AA, Maisarah AM, Asmah R. Comparison of antioxidant properties of tamarillo (Cyphomandra betacea), cherry tomato (*Solanumly copersicum* var. cerasiform) and tomato (*Lyopersicon esulentum*). Inter Food Res J. 2014; 21(6):2355-2362.
33. Wong FC, Woo CC, Hsu A, Tan BK. The anti-cancer activities of *Vernonia amygdalina* extract in human breast cancer cell lines are mediated through caspase-dependent and p53-independent pathways. PLoS One. 2013; 8(10):e78021.
34. Zhao J, Pan Y, Li X, Zhang X, Xue Y, Wang T, Zhao S, Hou Y. Dihydroartemisinin and curcumin synergistically induce apoptosis in SKOV3 cells via upregulation of MiR-124 targeting midkine. Cellular Physiol and Biochem. 2017; 43(2):589-601.
35. Lai HC, Singh NP, Sasaki T. Development of artemisinin compounds for cancer treatment. Investigate new drugs. 2013; 31(1):230-246.
36. Borges CV, Minatel IO, Gomez-Gomez HA, Lima GP. Medicinal plants: Influence of environmental factors on the content of secondary metabolites. In Medicinal Plants and Environmental Challenges [Online]. 2017 [cited 13 Jan. 2021]. Available from: http://hdl.handle.net/11449/175954
37. American Cancer Society. *Breast cancer facts & figures 2017–2018*. American Cancer Society Inc.: Atlanta, 2017.
38. Yedjou CG and Tchounwou P. Mechanisms of Vernonia amygdalina-induced apoptosis of human breast adenocarcinoma (MCF-7) cells. Cancer Res. 2014; 74:127.
39. Efferth T and Koch E. Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Current drug targets. 2011; 12(1):122-132.
40. Lin NU, Vanderplas A, Hughes ME, Theriault RL, Edge SB, Wong Y, Blayney DW, Niland JC, Winer EP, Weeks JC. Clinicopathological features and sites of recurrence according to breast cancer subtype in the National Comprehensive Cancer Network (NCCN). J Clin Oncology. 2009; 27(15):543.
41. Van Vuuren S and Viljoen, A. Plant-based antimicrobial studies–methods and approaches to study the interaction between natural products. *Planta medica*. 2011; *77*(11):1168-1182.
42. Wagner H and Ulrich-Merzenich G. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine. 2009; 16(2-3):97-110.
43. Pemovska T, Bigenzahn JW, Superti-Furga G. Recent advances in combinatorial drug screening and synergy scoring. Current opinion in pharmacol. 2018; 42:102-110.
44. Tang J, Wennerberg K, Aittokallio T. What is synergy? The Saariselkä agreement revisited. Frontiers in pharmacology. 2015; 6:181.
45. Berenbaum MC. What is synergy?. Pharmacol reviews. 1989; 41(2):93-141.
46. Rather MA, Bhat BA, Qurishi MA. Multicomponent phytotherapeutic approach gaining momentum: Is the "one drug to fit all" model breaking down?. Phytomedicine. 2013; 21(1):1-4.