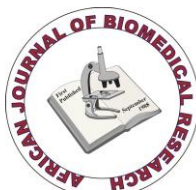

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Research Article

Phytochemical Investigation and Anti-Proliferative Effects of *Rauvolfia vomitoria, Calliandra portoriscensis* and *Anthocleista djalonensis* on Human Breast Cancer (MCF-7) Cell Line

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ABSTRACT

Breast cancer remains a global public health problem and natural products are increasingly being explored in search of new therapeutic agents with scientific evidence. This study investigated phytochemical composition, antioxidant and anti-proliferative activities of different solvent fractions of *Rauvolfia vomitoria*, *Calliandra portoriscensis*, and *Anthocleista djalonensis* in estrogen receptor positive MCF-7 breast cancer cell line ex vivo. Liguid-liquid partitioning separation technique was used to obtain the chloroform, ethyl acetate and aqueous fractions *Rauvolfia vomitoria*, *Calliandra portoriscensis*, and *Anthocleista djalonensis* from their crude methanolic extracts. Qualitative and quantitative phytochemical analysis were performed to determine the levels of total phenolics and flavonoids. Antioxidant activities of each extract fraction were determined using the DPPH and NO scavenging assays. The MCF-7 cell line was cultured in DMEM medium overnight, exposed to different concentrations (10-50 mg/mL) of each fraction for 48 h and growth inhibition was subsequently evaluated using the MTT assay. Phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins and phenols in the plants. The extract fractions of *C. portoriscensis*, *R. vomitoria* and *A. djalonensis* at 10, 20, 30, 40 and 50 ug/mL were found to scavenge DPPH free radical dose-dependently by 7.5 – 10.2%, 11.2 – 24.9%, 17.5 – 31.8 %, 27.8 – 47.1 % and 37.8 – 62.1%. The highest DPPH scavenging activity of 62.1%, 55.4% and 54.2% was elicited by the chloroform fractions of *A. djalonensis* and *C. portoriscensis* and ethylacetate fraction of *R. vomitoria* at 50 ug/mL Dose-dependent NO inhibition was also observed with the highest NO inhibition elicited by *R. vomitoria* chloroform fraction (61.7%), followed by *A. djalonensis* ethyl acetate fraction (55.9%) and *C. portoriscensis* chloroform fraction (49.6%) at 50 ug/mL the extract fractions also induced anti-proliferative effects on MCF-7 cell in a dose-dependent manner and significantly (P<0.05) with maximum elicitation observed at 96 h post treatment. On the whole, only the chloroform extract fraction of *A. djalonesis* elicited moderate anti-proliferative potency against MCF-7 cell line with an IC50 of 66.8 + 8.3 ug/mL, while other extract fractions have low to no potency with IC50 range of 237.4 – 605.7 ug/mL Unlike cisplatin (IC50 of78.4 – 108.3 ug/mL,), the proliferation of the normal Vero cell lines was less affected by the extract fractions (IC50 237.4 – 605.7 ug/mL). Findings in this study provide scientific evidence for chloroform fraction of *Anthocleista djalonensis* as a potential source of novel therapeutic agents for future breast cancer pharmacotherapeutic explorations.

Keywords: *breast cancer, cytotoxicity, anti-oxidants, medicinal plants, phytochemicals*

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INTRODUCTION

Breast cancer remains the most common type of cancer and cancer deaths worldwide with rising incidence and accounts for 30% of new cancers in women annually (Siegel, 2017). In Nigeria, Breast cancer presents as the most prevalent invasive cancer in women and the most frequent cancer in both sexes leading to a high disease burden to the society (Jedy-Agba *et al*., 2012). The three subtypes of breast cancer are hormone receptor positive (E.g. Estrogen receptor positive), human epidermal growth factor receptor positive (i.e. HER-2) and triple negative breast cancer (Yeo and Guani, 2017). Therefore, treatment modalities depend on the biology and stage of breast cancer (Yeo and Guani, 2017; Prat *et al*, 2015; Odle, 2017). Although various types of treatments such as surgery, radiation and systemic therapies (chemotherapy, hormonal therapy and targeted therapies) are available for breast cancer management, treatment outcomes still remain sub-optimal globally. The limited treatment success of orthodox cancer care has been attributed to drug toxicity and

resistance due to metastasis and inappropriate dosing (Ngamcherdtrakul & Yantasee, 2019; Soleja & Rimawi, 2016; Ballatori *et al*, 2017). Other factors include socioeconomic barrier to assess care due to high cost of anti-cancer drugs, which is not sustainable for low-income patients and those without health insurance or governmental support (Cabral *et al*, 2019; Forester *et al*, 2019). Multiples pathophysiological processes have been implicated in the pathogenesis of breast cancer. They include oxidative stress due to over production of free radicals such as nitric oxide and depletion of antioxidants (Hetcht *et al*, 2016; Basudhar *et al*, 2017). When overproduced, nitric oxide can interact with super oxide anion to form peroxynitrites and this can result in host cell DNA damage and impair DNA repair to set the stage for breast cancer development and drug resistance (Verenich and Gerk, 2019). Therefore, new therapeutic agents that are accessible, affordable and sustainable with ability to scavenge NO radicals and elicit anti-proliferation of breast tumors are urgently needed for breast cancer prevention and control in developing countries.

On this basis, natural products have increasingly gained attention as veritable sources of therapeutic agents against breast cancer and other types of cancers globally. According to WHO, an estimated 80% of populations in developing countries use herbal medicines for their healthcare (WHO, 2002). It has also been reported that between 1981 and 2001, over 70% of anti-cancer drugs were derived from medicinal plants (Chloumessi *et al*, 2011). This has further made cancer research studies investigating anti-cancer properties of medicinal plants very important. Medicinal plants such as Vinca rosea, Viscum album, Allium sativum Curcuma longa, Xylopia aethiopica and Panax ginseng have been reported to elicit anticancer potency against cancer cell lines such as Hep2 cells (liver), A2780 (ovarian), H460 (lung), A431 (skin), MIA PaCa-2 (pancreas), HT29 (colon), BE2G (neuroblastoma), SMA (glioblastoma), MCF-7 (breast) and PC-3 (Prostate) (Pharm *et al*, 2019; Ferlenda *et al*, 2019; McGrowder *et al*, 2020; McCubrey *et al*, 2017; Adaramoye *et al*, 2017). The therapeutic usefulness of these plants has been linked to their unique phytoconstituent profiles, which enable their antioxidant, anti-proliferative, anti-angiogenesis potentials (Ferlenda *et al*, 2019; McGrowder *et al*, 2020; McCubrey *et al*, 2017; Adaramoye *et al*, 2017).

Rauvolfia vomitoria belonging to Apocynaceae family is used in traditional medicine in Nigeria and other African countries such as Democratic Republic of Congo to treat several types of ailments, including leprosy, psychotic disorder, jaundice, rheumatic pains and gastrointestinal disturbance (Bisong *et al*., 2011); Fetrov *et al*, 1999). *R. vomitoria* is well known for its alkaloid phytoconstituents such as reserpine and serpentine, which enables the plant to be useful for the treatment of hypertension and psychotic disorders (Amole and Onabanjo, 2002). A recent study by Oboh *et al* (2019) also showed that the phenolic fraction of the plant elicited phosphodiesterase inhibitory activity in vitro. *R. vomitoria* has also been found to elicit growth inhibition of pancreatic cancer, ovarian cancer and prostate cancer cell lines in vitro (Dong *et al*, 2018; Yu *et al*, 2014; Yu *et al*, 2013). Anti-prostate cancer effect of *R. vomitoria* has been linked to its beta carboline alkaloid, alstonine (Bemis *et al*, 2006). Calliandra portoricensis (Jacq.) Benth. belonging to the Mimosaceae family is used in traditional Nigerian medicine as a laxative/worm expeller and an abortifacient. The plant has also been reported to have anticonvulsant, antidiarrheal, antispasmodic, antipyretic, antirheumatic and analgesic activities in human beings (Orishadipe *et al*., 2010; Ogugua *et al*., 2012). The plant has also been shown to be active against prostate and rhabdosarcoma cancer cell lines as well promoting the opening of the mitochondria membrane permeability transition pore using the rat liver homogenate (Oyebode *et al*, 2017; Ogbole *et al*, 2017; Oyebode *et al*, 2012). The plant *Anthocleista djalonensis* belonging to Loganiaceae family is widely spread in tropical African countries such as Nigeria, Cameroon, Sudan, Sierra Leone and the Comoros island (Zirihi *et al*., 2005). This plant is one of the 14 species of the genus Anthocleista well known for its medicinal usefulness in Africa (Anyanwu *et al*, 2015). In Nigeria, the plant is used to treat wound, malaria, constipation, dysentery, diarrhoea, skin infection, hyperprolactinemia, chest pain, hemorrhoids, diabetes and hypertension (Edwin-Wosu *et al*., 2015); Anyanwu *et al*, 2015). The plant has also been reported to have pharmacological relevance in the traditional treatment of cancers. Different organic and inorganic solvents are used for preparation of medicinal plant extracts and this has been shown to have effects on their biological activities (Ho & Chun, 2019; Thouri *et al*, 2017).

Despite the therapeutic useful of *Rauvolfia vomitoria*, Calliandra portoricensis, and *Anthocleista djalonensis*, information on the anti-prolifefrative effects on breast cancer cells remain unclear. Therefore, this study was designed to determine the presence and abundance of phytoconstituents, antioxidant potency and anti-proliferative effects of different fractions of *Rauvolfia vomitoria*, Calliandra portoricensis, and *Anthocleista djalonensis* on estrogen positive breast cancer cell line, MCF-7.

MATERIALS AND METHODS

Chemicals: Ethanol, Ethyl acetate, and Chloroform of analytical grade were purchased from Carl-Roth, Germany. Dulbecco's Modified Eagle's Medium (DMEM), Foetal bovine serum (FBS), Phosphate buffer saline (PBS) without Ca2+ and Mg2+, Trypsin solution, Trypan blue, and Dimethylsulfoxide (DMSO) were purchased from ThermoFisher Scientific, USA. 2,2ediphenyl-1-picryhydrazyl (DPPH), ascorbic acid, quercetin and cathechin were purchased from British Drug House (BDH) chemical Limited (Poole, UK). Other reagents used in this study were of analytical grade and good quality. They were graciously provided by the Department of Biochemistry, College of Medicine-University of Lagos.

Cell lines: The estrogen receptor positive MC7 and normal Vero cell lines were obtained from the department of Cell Biology and Genetics, University of Lagos and American Tissue Culture Collection (ATCC) (Manassas, USA). Each cell line was separately maintained in a 75 mL flask containing DMEM culture medium supplemented with 10% Fetal Bovine Serum (FBS), 100 unit/ml of penicillin and 100 ug/mL of

streptomycin incubated at 370C in a humidified 5% CO2 incubator

Collection and Identification of Plant Materials: Freshly collected leaves of Calliandra portoricensis, *Rauwolfia vomitoria*, and *Anthocleista djalonensis* were obtained from Mushin market, Lagos state, Nigeria. Samples of these plants were identified and authenticated at the Department of Botany, the University of Lagos and voucher specimen numbers LUH-6747, 6746, 6826 were deposited in the herbarium. The plants were air dried under shade for 3 weeks, powdered and weighed. The powdered leaves (500 g) were separately soaked in 2 L of 80% aqueous methanol at room temperature in air-tight glass jars for 7 days. The filtrates were concentrated in vacuo at 450C using a rotary evaporator (Bibi *et al*., 2012). This crude extraction process produced yields of 11.25% (56.25 g), 14.6% (73 g) and 10.8% (54 g) respectively for Calliandra portoricensis, *Rauvolfia vomitoria*, and *Anthocleista djalonensis*

Liquid-Liquid Partitioning of Crude methanol extracts Calliandra portoricensis, *Rauvolfia vomitoria*, and *Anthocleista djalonensis:* Partitioning of the crude extracts was carried out using organic solvents- chloroform and ethyl acetate. The extracts were transferred to a separatory funnel, 400 mL of chloroform added, and the resultant suspension was mixed vigorously and subsequently allowed to phase separate in order to separate the aqueous and organic fractions. After separation, ethyl acetate was added to the aqueous fraction for further partitioning. This process was repeated for all the crude extracts. All the fractions were oven dried at 50°C using Kottermann 2716 Oven (Bibi *et al*, 2012).

Preparation of Test crude extracts fractions: Prior to use 0.5 g of each fraction extract was dissolved in 1 ml of 5% DMSO overnight and subsequently diluted serially to 10 – 50 ug/ml with culture medium such that the concentration of DMSO in the culture medium was less than 0.02%. the prepared extraction solutions were then filter sterilized with 0.22 µm syringe filter and stored at -20oC until use. In this study the concentration range 10 -50 mg/mL was selected based on the previously reported anti-proliferative activity of *R. vomitoria* and C. portorisensis in prostate, pancreatic and cancer cell lines (cancer potency classification of medicinal plants as anti-cancer agents (Oyebode *et al*, 2017; Dong *et al*, 2018).

Phytochemical Analysis

Qualitative tests: Phytochemical screening for the presence of alkaloid, tannin, terpenoids, flavonoids, saponin Cardiac glycoside, reducing sugars and phenolic in the crude extract fractions was carried out using standard procedures (Trease and Evans, 1989; Sofowora, 1993)

Quantitative tests: Total Phenolic compounds: Total phenolic contents in the crude extract fractions were determined using Folin-Ciocalteau-sodium bicarbonate reagent (FCSB)with gallic acid as a standard (Chlopicka *et al*, 2011). The reaction mixture with gallic acid solution standard (control) or with extract fraction (1 ml of 50 mg/ml each with 5 ml of FCSB reagent) was incubated at room temperature for 1 h. Absorbance was then read at 765 nm with 1 ml of culture medium added to FCSB reagent used as blank. The total phenolic content was expressed as mg gallic equivalent per g extract. This procedure was carried out in triplicate per extract fraction.

Total flavonoid compounds: The total flavonoid content was determined using the aluminum chloride method according to Stankovic (2011) with quecertin as the standard in the range 20 – 80 ug/ml. the 4.5 mL composite aluminum chloride reagent comprised aluminum chloride (0.1 mL of 10%), potassium acetate (0.1 mL of 1M), 80% methanol (1.5 mL) and distilled water (2.8 mL). Each extract fraction (0.5 mL of 50 mg/mL) or quecertin standard (0.5 mL) was added to the composite aluminum chloride reagent to form the test reaction mixture while 0.5 mL of distilled water was used instand of the extract or standard to serve as the blank. After incubation at room temperature for 30 min, absorbance was read at 415 nm. The total flavonoid content was expressed as mg quercetin equivalent per g extract. This procedure was carried out in triplicate per extract fraction.

Antioxidant Assays

Determination of DPPH scavenging activity by the extract fractions of Calliandra portoricensis, *Rauvolfia vomitoria*, and *Anthocleista djalonensis* : The free radical scavenging activity of the extract fractions was caried out using the DPPH assay method (Koksal *et al*, 2011). The 3 mL reaction mixture consisted of 1 mL of 0.33M DPPH in methanol, 1 mL of methanol and 1 mL of extraction fraction (10 – 50 mg/mL). The reaction mixture was mixed and incubated in the dark for 10 min. ascorbic acid solution (10 – 50 mg/mL) was used in a parallel experiment as the positive control. The DPPH solution with the sample was as the negative control. Absorbance was read at 517 nm. The scavenging activity was determined according to the equation as follows:

Scavenging inhibition (%)

$$= \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ *= Absorbance of negative control;*
A_{sample} *= Absorbance of extract fraction or ascorbic acid*

Determination of nitric oxide scavenging activity by the extract fractions of Calliandra portoricensis, *Rauvolfia vomitoria*, and *Anthocleista djalonensis* : The ability of the extract fractions to scavenge nitric oxide was determined using sodium nitroprusside-Griess reagent according to Jagetia and Baliga (2004). This method was based on the release of NO by sodium nitroprusside at physiological pH and subsequent reaction with oxygen to form nitrite ions, followed by quantitation based on diazotization with the Griess reagent. Briefly 2 mL of sodium nitroprusside (5 mM in PBS, pH 7.4) was mixed with 1 mL of each extract fraction (10 -50 ug/mL) and incubated at 250C for 150 min. This was followed by the addition of 2 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% naphthylethylenediamine Dihydrochloride). Absorbance was read at 546 nm after 30

min with the Griess reagent as blank, relative to the absorbance of standard solutions of potassium nitrite (10 – 50 ug/mL) treated with the Griess reagent as used as control. This procedure was done in triplicates. NO scavenging activity of extract fraction was measured according to the following equation

% NO Scavenging =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where Acontrol = Absorbance of potassium nitrite control; Asample = Absorbance of extract fraction

Effect of extract fractions on cell viability: Anti-proliferative effects of the extract fractions on MCF-7 cell line and the normal Vero cell line were determined by the MTT assay method (Srisawat *et al*, 2013). The MCF-7 cells or Vero cells were seeded into each well of the 96-well plate at a density of 104 cells per well per 100 uL culture preparation. After 24 hours of incubation, the cells were treated with different concentrations (10 – 50 ug/mL) of the extract fraction for 48 h. Thereafter, 10µL of MTT (5mg/ml) solution was added to each well containing no cells/treated/untreated cells and mixed by gently rocking the plate from side to side. The plate was incubated in a CO2 incubator set at 37oC for 3hours after which purple crystals were observed. A 100µl aliquot of MTT Solubilization buffer was added to each well and triturated to dissolve the crystals followed by gentle mixing on an orbital shaker. Cisplatin was used as a positive control. The absorbance was spectrophotometrically measured at a test wavelength of 570nm and 490nm with a reference wavelength of 630nm. The differences in the optical densities were derived; percentage cell viability was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\textit{optical density of treated cells}}{\textit{optical density of control cells}} * 100\%$$

IC_{50} was defined as the concentration at which 50% growth inhibition of the MCF-7 cells occurred in the culture medium. IC_{50} was determined based on the dose-response relationship of the extract fractions and cisplatin positive control.

Data Analysis: Data are summarized in Tables (phytoconstituent contents) and bar graphs (percent inhibition). They were expressed as mean + standard deviation of three determinations and disparity in values among the various fractions were analyzed by one-way analysis of variance (ANOVA) and Duncan post-hoc test. Statistical analyses were performed using SPSS statistical software version 22.0. Dose-response curve analysis was performed using the GraphPad Prism and IC_{50} values of the extract fractions and cisplatin against the MCF-7 and Vero cell lines were extrapolated. Extract fractions or control drug with IC50 values < 50 ug/mL, 50 – 200 ug/mL, 200 – 500 ug/mL and > 500 ug/mL were considered to elicit high, moderate, low and no anti-proliferative potency activity against the cell lines tested The Outcomes with P-value < 0.05 were considered to be significant.

RESULTS

Phytochemical Screening: In this study, the primary extraction with methanol produced 11.25%, 14.6% and 10.8% yields of *Calliandra portoriscensis*, *Rauwolfia vomitoria* and *Anthocleista djalonensis* leaf extracts that were subsequently used for solvent fractionation (Table 1). The phytochemicals present in the different solvent fractions of the leaf extracts of *C. portoriscensis*, *R. vomitoria* and *A. djalonensis* are also presented in Table 1. Apart from cardiac glycoside absence, other phytochemicals tested: alkaloid, tannin, terpenoids flavonoids, saponin, reducing sugar and phenolic were detected in the different solvent fractions of the leaf extracts studied. An exception was the absence of saponin in the chloroform fraction of *Calliandra portoriscensis* and *Anthocleista djalonensis* leaf extract (Table 1). Table 2 shows the quantitative Phytochemical analysis of extract fractions of *C. portoriscensis, R. vomitoria and A. djalonensis*

Table 1.
Qualitative Phytochemical analysis of extract fractions of *C. portoriscensis, R. vomitoria and A. djalonensis*

		General Phytoconstituents				
Plant	**Fraction**	**Alkaloid**	**Saponin**	**Phenol**	**Flavonoid**	**Tannin**
Calliandra portoriscensis	Ethylacetate	28.39 ±0.43	7.18 ±2.13	24.43 ±0.11	8.90 ± 0.51	1.59 ± 0.16
	Chloroform	23.93 ±0.32	-	13.11 ±0.11	11.72 ± 0.26	2.24 ± 0.20
	Aqueous	27.50 ±0.46	26.52 ±2.65	32.47 ±0.22	9.49 ± 0.32	3.38 ±0.20
Rauvolfia vomitoria	Ethylacetate	38.58 ±0.96	13.86 ±3.53	13.62 ±0.17	6.72 ± 0.13	1.16 ± 1.65
	Chloroform	6.20 ±0.22	13.37 ±2.85	19.83 ±2.10	14.58 ± 0.32	2.04 ± 0.16
	Aqueous	26.12 ±0.64	27.80 ±0.84	21.74 ±0.61	7.67 ± 0.45	2.47 ± 3.49
Anthocleista djalonesis	Ethylacetate	24.57 ±0.58	23.06 ±0.48	38.99 ±0.83	6.68 ±0.32	0.82 ±1.16
	Chloroform	30.02 ±0.26	-	9.13 ±0.22	9.04 ±0.32	1.42 ±2.01
	Aqueous	15.28 ±0.26	11.02 ±0.48	16.82 ±0.28	7.58 ±0.19	1.53 ±2.17

Values are Mean Percentage ±SD. No. of replicates, N=2.

Table 2.
Quatitative Phytochemical analysis of extract fractions of *C. portoriscensis, R. vomitoria and A. djalonensis*

	Extract Fraction (50µg/ml)	DPPH (%)	Nitric oxide (%)	Reducing power (%)
Calliandra portoriscensis	Ethylacetate	43.28	25.16	0.241
	Chloroform	54.55	49.39	0.272
	Aqueous	37.15	36.46	0.308
Rauvolfia vomitoria	Ethylacetate	51.19	57.70	0.264
	Chloroform	43.08	61.48	0.287
	Aqueous	49.60	30.95	0.311
Anthocleista djalonesis	Ethylacetate	45.26	47.48	0.164
	Chloroform	56.13	38.84	0.183
	Aqueous	39.92	21.85	0.143

The levels of total phenolic and flavonoids in the various fractions of the crude methanolic extracts of the three plants are summarized in Table 3. The level of total phenolic was between 19.8 – 62.5 mg gallic acid equivalent per g extract with *Calliandra portoriscensis* ethyl acetate fraction and *Rauwolfia vomitoria* chloroform fraction (19.8 + 0.92-1.26 mg GAE per g extract), *Anthocleista djalonensis* ethyl acetate fraction (20.6 + 2.85 mg GAE per g extract) and *Calliandra portoriscensis* aqueous fraction (20.9 + 1.74 mg GAE per g extract) eliciting lower total phenolic level. In increasing order, *Anthocleista djalonensis* aqueous fraction (47.8 + 1.72 mg GAE per g extract), *Rauwolfia vomitoria* ethyl acetate fraction (50.3 + 1.51 mg GAE per g extract) and *Anthocleista djalonensis* chloroform fraction were found to elicit higher levels of total phenolics. For flavonoids, lower levels were found in *Anthocleista djalonensis* aqueous fraction (19.2 + 2.45 mg quercetin equivalent per g extract), *Rauwolfia vomitoria* chloroform fraction (19.6 + 0.52 mg quercetin per g extract) while the highest flavonoid level was found in chloroform fraction of *Anthocleista djalonensis* (38.3 + 0.73 mg quercetin per g extract), followed by the aqueous fraction of *Calliandra portoriscensis* (30.5 + 1.77 mg quercetin per g extract). On the whole, the disparity in the levels of these phytochemicals by the different fractions of the ethanolic leaf extracts of *C. portoriscensis*, *R. vomitoria* and *A. djalonensis* studied was found to be statistically significant (P<0,05) (Table 3)

Table 3
Analysis of Total Phenol, Flavonoid, and Antioxidant Capacity.

	Extract Fraction (50µg/ml)	Total Phenol	Total Flavonoid	TAC
Calliandra portoriscensis	Ethylacetate	41.90 ±0.72	9.56 ±0.39	17.75 ±0.64
	Chloroform	32.12 ±0.28	9.85 ±0.32	25.12 ±0.17
	Aqueous	55.82 ±0.44	12.72 ±0.39	19.69 ±0.23
Rauvolfia vomitoria	Ethylacetate	23.03 ±0.22	10.85 ±0.19	17.77 ±0.40
	Chloroform	32.63 ±0.33	8.95 ±0.71	30.64 ±2.08
	Aqueous	38.84 ±0.39	11.81 ±0.26	10.74 ±0.40
Anthocleista djalonesis	Ethylacetate	18.35 ±0.22	9.63 ±0.39	11.15 ±0.29
	Chloroform	22.83 ±0.39	14.35 ±0.51	14.50 ±0.64
	Aqueous	54.45 ±0.72	13.94 ±0.32	17.97 ±0.35

Values are Mean Percentage ±SD. No. of replicates, N=2.
TAC = Total Antioxidant Capacity

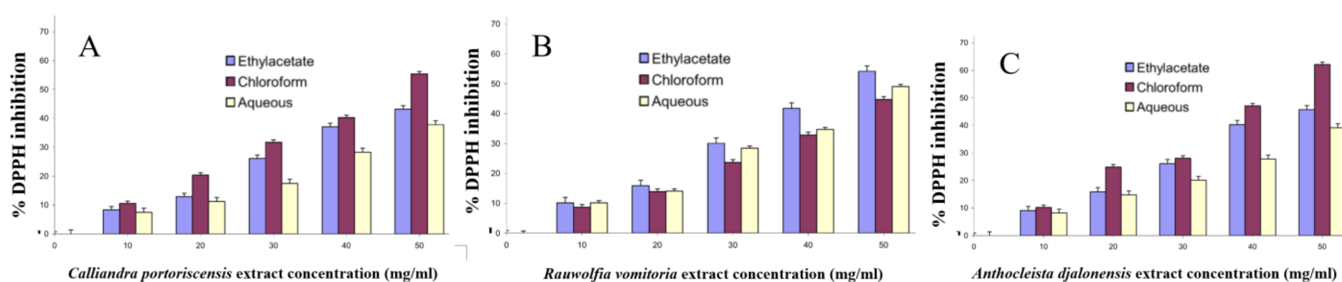

Figure 1
DPPH scavenging activities of *Calliandra portoriscensis, Rauwolfia vomitoria and Anthocleista djalonensis*

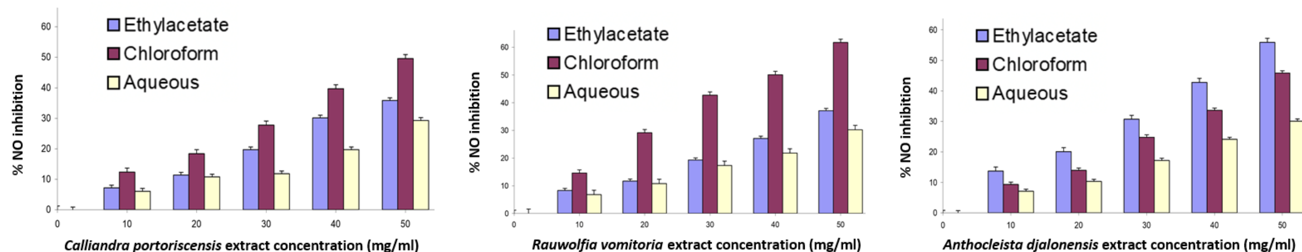

Figure 2
Nitric oxide scavenging activities of *Calliandra portoriscensis, Rauwolfia vomitoria and Anthocleista djalonensis*

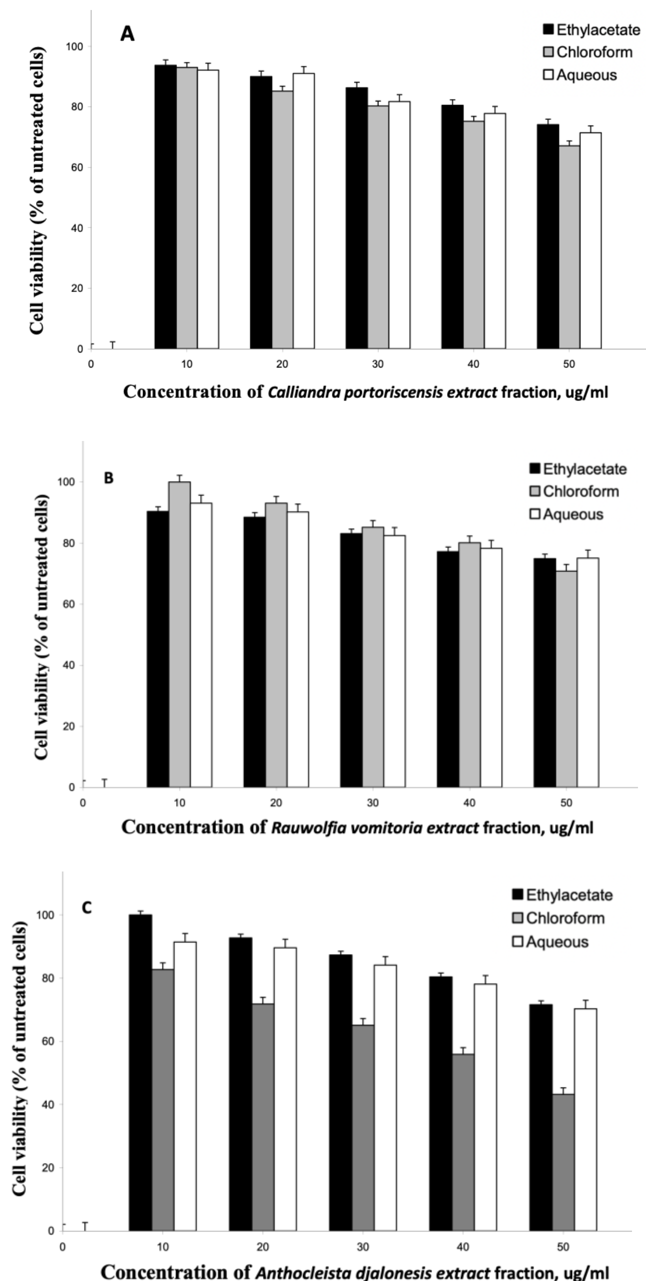

Figure 3
Effects of different concentrations of C. portoriscensis (A), R. vomitoria (B) and A. djalonensis (C) on MCF-7 proliferation. Data are mean ± SD of viability.

Antioxidant activities of the plant extract fractions: The extract fractions of *C. portoriscensis*, *R. vomitoria* and *A. djalonensis* at 10, 20, 30, 40 and 50 ug/mL were found to scavenge DPPH free radical dose-dependently by 7.5 – 10.2%, 11.2 – 24.9%, 17.5 – 31.8 %, 27.8 – 47.1 % and 37.8 – 62.1%. The highest DPPH scavenging activity of 62.1%, 55.4% and 54.2% was elicited by the chloroform fractions of *A. djalonensis* and *C. portoriscensis* and ethyl acetate fraction of *R. vomitoria* (Figures 1 a -c) at 50 ug/mL concentration. However, the DPPH radical scavenging activity elicited by each of the tested extract fractions tested was lower than 100% scavenging effect of ascorbic acid from 20 ug/mL concentration upwards (Figure 1d).

With regards to nitric oxide (NO) inhibition, dose-dependent inhibitory activity of the extract fractions was also observed. However, at 50 ug/mL concentration, highest NO inhibition was elicited by *R. vomitoria* chloroform fraction (61.7%), followed by *A. djalonensis* ethylacetate fraction (55.9%) and *C. portoriscensis* chloroform fraction (49.6%) (Figure 2 a - c).

Antiproliferative effects of the plant extract fractions on MCF-7 and Vero Cell lines

The proliferation of the MCF-7 cells was found to be dose-dependently inhibited by each of the tested extract fractions with maximum effect elicited after 96 h of treatment (Figure 3 a -c). Furthermore, at the maximum 50 ug/mL concentration tested, variations by extract and fraction types were also observed with *A. djalonensis* chloroform extract fraction eliciting the highest 56.8% growth inhibition, followed by *C. portoriscensis* chloroform extract fraction (32.9%) and *R. vomitoria* chloroform extract fraction (29.2%) (Figures 3 a – c). Further dose-response curve analysis revealed only the chloroform extract fraction of A. djalonesis to have moderate anti-proliferative potency against MCF-7 cell line with an IC50 of 66.8 + 8.3 ug/mL, while other extract fractions have low to no potency with IC50 range of 237.4 – 605.7 ug/mL (Table 4). On the contrary, the proliferation of the normal Vero cells was less affected by the extraction fractions with IC50 values observed at a range of 631.5 – 740.1ug/mL. However, the control drug Cisplatin elicited moderate anti-proliferative potency against both MCF-7 (IC50 of 78.4 + 6.1 ug/mL) and Vero cell lines (IC50 of 108.3 + 8.5 ug/mL) (Table 4).

Table 4
Cytotoxic effect of the different leaf extracts of on *C. portoriscensis, R. vomitoria and A. djalonensis* on MCF-7 breast cancer and normal Vero cell lines. At 96 h post-treatment

		Anti-proliferative activity (MCF-7)	***Anti-proliferative activity (Vero)***
Crude Plant extract (Yield, %)@ Fraction		IC_{50} value, μg/ml)	IC_{50} value, μg/ml)
Calliandra portoriscensis (11.25%)	*Ethylacetate*	*582.5* ± 11.6	728.5 ± 11.8
	Chloroform	*237.4* ± 6.3	731.7 ± 10.3
	Aqueous	*352.8* ± 7.6	737.2 ± 9.4
	P-value	*<0.05*	*> 0.05*
Rauvolfia vomitoria (14.6%)	*Ethylacetate*	*605.7* ± 9.3	740.1 ± 12.6
	Chloroform	*291.7* ± 6.2	732.5 ± 8.5
	Aqueous	*395.8* ± 9.1	737.8 ± 10.3
	P-value	*< 0.05*	*> 0.05*
Anthocleista djalonesis (10.8%)	*Ethylacetate*	*461* ± 10.3	640.2 ± 7.3
	Chloroform	*66.8* ± *8.3*	631.7 ± 10.2
	Aqueous	*305 + 6.3*	634.6 ± 9.2
	P-value	<0.05	> 0.05
Cisplatin		78.4 ± 6.1	108.3 ± 8.5

DISCUSSION

The burden of cancer is on the rise globally and is expected to reach 25 million cases by 2032 if urgent actions were not taken (WHO, 2016). In the past 2 decades, there has been a growing interest in the exploration of polyphenols as secondary metabolites from medicinal plants against all types of cancer, including estrogen positive breast cancer (Zhou *et al*, 2016; Braakhuis *et al*, 2016). Flavonoids and phenolic compounds represent the two major and widely distributed polyphenols found in medicinal plants (Neveu *et al*, 2010). This has been attributed to barriers of chemotherapy such as drug resistance, high treatment cost and severe adverse reactions (Singh *et al*, 2016) coupled with outcomes of several epidemiological studies supporting positive association between dietary polyphenol consumption and reduced cancer risk (Vanzour *et al*, 2010; Hui *et al*, 2013; Patrick *et al*, 2015).

In this study, variations in the total phenolics and flavonoids levels according to the solvent extraction process were observed. For *Calliandria portoriscensis*, higher total phenolics and flavonoids levels of 34.7 mg gallic acid equivalent per g of extract and 30.5 mg quercetin equivalent per g of extracts were respectively elicited in the chloroform and the aqueous fractions. This is different for *R. vomitoria* in which the observed high total phenolics of 50.3 mg gallic acid equivalent per g of extract was elicited in the ethylacetate fraction though its aqueous fraction retained the highest total flavonoid yield of 27.2 mg quercetin equivalent per g extract. A different scenario was also observed for *A. djalonensis* in which the highest yields of total phenolics and flavonoids of 62.5 mg gallic acid equivalent per g of extract and 38.3 mg quercetin equivalent per g of extract were both elicited using chloroform as the organic solvent. Our findings have further lent support to previous studies in which the importance of optimization of flavonoids and total phenolics based on different solvent extraction systems was demonstrated. agents (Iloki-Assanga *et al*, 2015; Igbai *et al*, 2012). Therefore, our findings suggest that for future exploration of *Calliandra portoriscensis*, *R. vomitoria* and *A. djalonensis* as anti-cancer agents, differential use of organic solvents such as chloroform (best for *A. djalonensis*), water and ethyl acetate (best for *R. vomitoria*) and chloroform and water (best for Calliandria portoriscensis) should be considered in the elicitation of total phenolics and flavonoids from these medicinal plants as this factor may influence their downstream biological activities.

In this study the chloroform fraction of *A. djalonensis* elicited the highest DPPH scavenging activity of 62% at 50 ug/mL concentration compared to *R. vomitoria* (54.2% for the ethylacetate fraction) and *C. portoriscensis* (55.4% for the chloroform) at the same concentration. Although this finding positioned *A. djalonensis* as the most potent reactive oxygen species (ROS) scavenger, followed by *C. portoriscensis* and *R. vomitoria*, organic solvent extraction process also seems to play an important role in the differential ROS scavenging property of the three plants. Moreso that these are the organic solvents that elicited highest yields of total phenolics (*A. djalonensis* and *C. portoriscensis*) and flavonoids (*R. vomitoria*) in the three studied plants. Both phenolics and flavonoids are well known for their free radicals scavenging activities due to preponderance of hydroxyl groups with inherent ability to donate hydrogen atoms to free radicals thereby terminating build up free radical in biological systems. In cancerous tissues, the proliferating tumor cells thrive under an oxidative stress environment characterized by high reactive oxygen species / antioxidant ratio. Therefore, the high antioxidant activity of *A. djalonensis* could play a role in the restoration of redox balance in cancerous tissues to inhibit the proliferation of tumor cells and induced anti-carcinogenic events such as inhibition of cell migration, tissue invasion, angiogenesis and induction of apoptosis as well as reduced expression of estrogen receptors in ER positive breast cancer cells (Ref). the relationship between antioxidant potency and anti-proliferative effects of medicinal plants has been previously reported by Adaramoye *et al* (2015 & 2017) for the methanolic extract of Xylopia aethiopica and *C. portoriscensis* against prostate cancer cell lines: PC-3 and LNCaP, by Georgive *et al* (2019) for aqueous, lectin free and polysaccharide fractions of Lycium barbarum against MCF-7 and by Correa *et al* (2020) for Psidium guajava fruit extract against MDA-MB-435 and MCF-7 breast cancer cell lines. In the work of Oyebode *et al* (2018), the presence of flavonoids and phenolics as polyphenol phytoconstituents in the methanolic extract of *Calliandra portoriscensis* was reported. But these phytoconstituents were not quantitated and free radical scavenging activity was not investigated. However, the investigators identified Afzelechin, a flavonoid and a combination of 2, 4, 5 trihydroxy cinnamic acid and gallic as phenolics present in the methanolic extract of the plant via liquid chromatography-mass spectrometry (LC-MS) fingerprinting. This was coupled with the observation of antiproliferative effect of the plant against LNCaP prostate cell line with an IC50 of 3.64 ug/mL and employing anti-carcinogenic mechanisms such as the opening of the mitochondrial permeability membrane pore, induction of cytokine release and Bax protein production and cell cycle arrest at the synthetic (S) phase. Although *C. portoriscensis* extract fractions were also less harmful to the normal Vero cell line (IC50 728.5 – 731.7 ug/mL) in this study as previously reported by Oyebode *et al* (2018), the plant seemed to elicit low to no potency against the MCF-7 cell line tested with an IC50 range of 237.4 – 582.5 ug/mL. This is so when compared with a much lower IC50 of 66.8 ug/mL elicited by the chloroform extract fraction of *A. djalonensis* and a similar higher IC50 range of 291.7 – 607.7 ug/mL elicited by the extract fractions of *R. vomitoria*. This finding further highlighted the need for consideration of solvent extraction system when exploring medicinal plants for their anti-cancer activity and provided a possible relationship between high phenolic and flavonoid content by solvent and anti-proliferative activity of medicinal plants.

In this study, the extract fractions were also found to elicit NO inhibition with the chloroform fraction of *R. vomitoria* eliciting maximum inhibition of 61.7%, followed by ethyl acetate extract *A. djalonensis* (55.9%) and chloroform extract fraction of 49.6% at 50 ug/mL concentration. This finding suggests a potential usefulness of these plants as a remedy against inflammatory diseases and vascular dysfunction mediated by NO dysregulation such as cancer. The relationship between NO inhibition and antiproliferative activity of medicinal plants has been reported by Carabajal *et al* (2020) for certain Argentinian plants such as Larrea

divaricata, Zuccagnia punctata and Larrea cuneifolia. This relationship has also been demonstrated for Sarcocephalus pobeguinii leave, root, bark and root extracts against MCF-7 and three other human non-breast cancer cell lines (Njoya *et al*, 2017).

However, this study has some limitations that need to be addressed prior to future exploration of *A. djalonensis* for breast cancer pharmacotherapy. First is the lack of understanding of the mechanisms of actions of *A. djalonensis* against MCF-7 and its anti-proliferative effects against other types of breast cancer cell lines, which were not investigated in the present study. The second limitation is with regards to the identity of the flavonoids and phenolic compounds responsible for anti-proliferative potency of *A. djalonensis*, which can be obtained vis LC-MS or HPLC fingerprinting in future studies.

Despite the above limitations of the present study, its findings have provided scientific evidence for chloroform fraction of *Anthocleista djalonensis* as a potential source of novel therapeutic agents for future breast cancer pharmacotherapeutic explorations with free radical scavenging and nitric oxide inhibition potentials.

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