In vitro anticancer potential of *Sacrocephalus latifolius* leaves extract and its fractions on human MCF-7 and NCI-H460 cancer cell lines.

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Abstract

Sacrocephalus latifolius is one of the recipes used in traditional medicine for the treatment of cancer ailment in South-western Nigeria. This work was aimed at evaluating the extract and fractions of this plant against human breast (MCF-7) and lung (NCI-H460) cancer cell lines as well as their antioxidant activity. While the ant-proliferative assay was done using the SRB-assay for the extract (1-250 µg/mL) and fractions (1-100 µg/mL), the DPPH antioxidant assay was recorded at concentrations between 25-400 µg/mL. Against the MCF-7 cell lines, the extract and ethyl acetate fractions recorded growth-inhibitory activities at all concentrations with GI_{50} of 73.02 and 63.38 µg/mL. While the chloroform fraction recorded cytotoxicities of -1.14 and -6.19 % at 50 and 100 µg/mL with GI_{50} and TGI of 43.18 and 89.70 µg/mL, the hexane and aqueous fractions were inactive. Similar pattern of activity was recorded against NCI-H460 cell lines with the chloroform fraction exhibiting a higher sensitivity with GI_{50} and TGI of 24.12 and 54.50 µg/mL respectively. The antiproliferative activity was further corroborated by the higher antioxidant activity (IC_{50} of 17.91 µg/mL), total phenolic (86.30 mg GAE/g) and flavonoid contents (72.74 mg QUE/g) of the chloroform fraction. The folkloric use of *S. latifolius* in the treatment of tumor ailments has been justified by this work.

Keywords: *Sacrocephalus latifolius*, anti-proliferative, cytotoxic, MCF-7, NCI-H460, DPPH, antioxidant, cancer, cell-lines, fractions, SRB-assay, DPPH

Introduction

Cancer, one of the leading cause of death, is a huge public concern worldwide with a rapid predominance in Africa, Asia and South America (Thun *et al.*, 2010). The treatment of this disease over the years has been the use of chemotherapy (Arruebo et al., 2011a). Unfortunately, this method of treatment has inflicted adverse side effects on the human tissues resulting in the inactivation of the bone marrow, vomiting, nausea as well as producing reactive oxygen species or free radicals in the body which causes oxidative stress which causes harm to many cellular components such as DNA, protein and lipid which are related to chronic debilitating diseases such as cancer, hypertention as well as diabetes (Nurgali *et al.*, 2018). The presence of antioxidant helps to mop up these ROS in the body.

The use of plants as an alternative form of treatment for this diseases has been recommended as a result of the less side effects coupled with the abundance of phytochemicals such as the phenolics which they contain, which are capable of inhibiting free radicals causing cancer and other diseases (Sofowora *et al.*, 2013). The search for alternative form of treatment of this disease has open a new horizon in to plant-based research for the discovery of anti-cancer and antioxidant agents (Choudhar *et al.*, 2020).

Sacrocephalus latifolius (Sm.) E. A. Bruce (Rubiaceae) is a plant with diverse medicinal values. It is locally known in some Nigerian speaking tribes as Egbesi , uburu Nbitinu inu and marga in Youruba, Igbo and Hausa tribes respectively. It is found in tropical areas of Africa. It has many irregular branches and thick foliage. The plant is widely distributed in Nigeria and mostly found in the south-east and south-south regions (Enemor and Okaka 2013).The antimalaria, antidiabetics (Gidado *et al*., 2005), and hepatoprotective, (Ngo Bum *et al.*, 2009) have been reported previously on the plant. The plant has been identified as one of the cancer treatment recipes in south-west Nigeria (Soladoye *et al.*, 2010).

While the plant is being recorded as an efficient medicinal plant, the *in vitro* anticancer studies against lung and breast cancers has never been reported. Our earlier work recorded higher cytotoxic and growth-inhibiting effects of the leaves over the root barks of the plant using simple bench-top assay involving the use of the radicles of *Sorghum bicolor* and tadpole of *Raniceps raninus* (Ikpefan *et al.*, 2014). The present studies take another look at the in vitro anticancer studies of the leaves extract and fractions against breast and lung cancer cell lines.

Methods

Collection, processing and extraction of plant sample

The leaves of *Sarcocephalus latifolius* were collected in Benin City and identified with the assistance of a Botanist/Pharmacognocist. Voucher specimen was sent to Forest Research Institute of Nigeria (F.R.I.N.) Ibadan, after authentication it was deposited under herbarium number (FHI 109707)..

The collected leaves samples were air-dried in the laboratory for 3 days, and subsequently in an oven held for 24 hours at 45°C, during which they were ground to form powder using an electric milling machine in the laboratory (Chris Norris, England). A portion of the powdered sample (1.5 kg) was extracted with 90 % methanol using the cold maceration method previously described by Osama and Awdelkarim, (2015) and the extract obtained was weighed and recorded.

Fractionation of extract

A portion of the resulting leaves extract (55.0 g) was immersed in equal volumes of water and methanol (1:1, 400 mL) and subjected to hexane, chloroform and ethyl acetate-solvent partitioning in a separating funnel. The fractions were collected, concentrated, and weighed.

Qualitative determination of phytochemicals in the extract and fractions of *S. latifolius*

The screening of the extract and fractions of *S. latifolius* for the presence of phytochemicals was performed with standard methods (Khandewal, 2010)

Cell Lines and Cell Cultures

The PCMD Molecular Bank of the University of Karachi in Pakistan was the source of the human cancer cell lines MCF-7 (Breast) and NCI-H460 (lung). The cells were cultured and maintained following the guidelines of Geraghty *et al* (2014).

Anti-cancer studies of leaves and root barks extract of *Securinega virosa*

The antiproliferative activities of the extract and fractions of *S. latifolius* against the cell lines was carried using the SRB-assay method previously described by Shekan and Storeng (1990). Extract and fractional stock solutions of *S. latifolius* (40 mg / mL in DMSO) were prepared and the corresponding dilutions in RPMI-1640 were prepared, consisting of gentamicin (50 g / mL) and monolayer trypsinization, determination of cell viability as well as cell counting from the confluent flask (75 cm^2) were carried out. Monolayer formation of MCF-7 and NCI-H460 cells (10,000 cells / well/100 µL) was seeded in a CO_2 incubator at 37 ° C and incubated for 24 h. The various concentrations of methanol extracts of *S. latifolius* (1, 10, 50, 100, 200, and 250 µg/mL) and fractions (1, 25, 50, 75 and 100) were added (100 µL/well) in appropriate wells, followed by incubation for 48 h. Appropriate controls and blanks (drug and extract) were also prepared. At the end of 48 h, time zero-1 (Tz 1 plate) and time zero-2 (Tz 2 plate) cells (10,000 cells/well/100 µL for MCF-7 and NCI-H460) were seeded for monolayer formation and incubated in a CO_2 incubator at 37°C for 24 h.

Determination of total phenolic content

The Folin Ciocalteu method was used to assess total phenolic content (Chang *et al.*, 2002). Calibration curve was built using standard gallic acid and the total phenolic content is represented as mg equivalents of gallic acid (GAE)/g dry weight (DW).

Determination of flavonoid content

The Aluminum chloride colorimetric assay previously described by Chang *et al* (2002), was used to test the flavonoid content. The calibration curve was plotted using quercetin standards and the quality of flavonoids was expressed as equivalents of mg quercetin (QE)/g DW.

Determination of Antioxidant activity of extract and fractions of *S. latifolius*

The DPPH antioxidant-RSA for the extract and fractions of *S. latifolius* was performed using Osama *et al* (2017). Following the procedure, 1 mL (0,1 mM) of DPPH solution in methanol was mixed in water at various concentrations with 3 mL of sample solution. At last the absorbance was measured at 517 nm, after 30 min. Diminishing DPPH solution absorbance indicates an increase in DPPH's radical-scavenging activity. The IC_{50} scavenging assay values (concentration which can achieve 50 percent scavenging) are calculated by plotting the inhibition percentage against the concentration to quantify the activity. The experiment was done in replicate of three.
The radical-scavenging DPPH action was calculated on the basis of the following equation:

% Inhibition = $(A_0-A_1) / A_0 \times 100$.
Where, A_0 - the absorbance of the control;
A_1 - the absorbance of the test samples (extract or fractions).

Statistical Analysis
This was done using GraphPad Prism 7.0 and One-way analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparisons test were for the data analysis. The values were finally represented as Mean ± (SEM).

Results

Yield of the extract and fractions of *S. latifolius*
The 1.5 kg of the extracted powdered leaves of *S. latifolius* yielded 75.15 g of the extract which is equivalent to 5.00 % yield. Upon partitioning, a total of 2.85, 7.87, 9.08 and 16.60 g of hexane, chloroform, ethyl acetate and aqueous fractions were recorded which implies 0.19, 0.52,0.61 and 1.24 % yield.

Results of phytochemical screening of extracts and fractions
The phytochemical screening of the extract and its respective fractions indicated the existence of some secondary metabolites, such as alkaloids, anthraquinones, saponins, terpenes, flavonoids, tannins and cardiac glycosides in different intensities (Table 1)

Table-1. Phytochemical screening results of extract and partitioned fractions of *S. latifolius*

Phytochemical groups	Extract	Solvent fractions			
		Hexane	Chloroform	Ethyl acetate	Aqueous
Alkaloids	++	+	+++	+	++
Anthraquinones	+	-	++	+	+
Tannins/Phenolic compounds	+	-	++	+	+
Flavonoids	+	-	++	+	-
Saponins	++	-	++	+++	+
Cardiac glycosides	+	-	+	++	++
Terpenes	+	+	++	++	+
Steroids	+	++	+	+	+

Key: +++: appreciable amount; ++: moderate amount; + : minute amounts; - : not detected

Results of the effects of the extract and fractions on MCF-7 and NCI-H460 cell lines.
Comparing the anti-proliferative activities of the extract and fractions of *S. latifolius* against the cell lines, showed a concentration based activities. The extract reported growth-inhibitory activities against MCF-7 cell lines at concentrations between 10-250 µg / mL with GI_{50} of 73.02 µg / mL. The activity was observed to increase after partitioning as the chloroform fraction became more cytotoxic as -1.14 and -6.12 % cytotoxicities were recorded at 50 and 100 µg / mL with GI_{50} and TGI of 43.18 and 89.70 µg / mL. The hexane and aqueous

fractions were inactive because they reported cell death of less than 50 percent, even at maximum concentrations (Table 2).

The experiment was observed against NCI-H460 cell lines, to follow the same trend as MCF-7. However, the chloroform and ethyl acetate fractions recorded higher sensitivity than that observed with MCF-7 cells. While the ethyl acetate fraction at 100 µg/mL gave cytotoxicity of -1.72 % as well as GI_{50} and TGI of 32.05 and 84.50 µg/mL, the chloroform fraction at similar concentrations gave cytototoxicity of -16.26 percent and GI_{50} and TGI of 24.12 and 54.50 µg/mL respectively (Table 3).

Table-2. Sensitivities of the extract and fractions of *S. latifolius* on breast (MCF-7) cancer cell lines

Conc. (µg/mL)	Extract	**Fractions % Growth inhibition/cytotoxicity**			
		Hexane	Ethyl acetate	Chloroform	Aqueous
1	-		+12.60±1.02	+53.18±0.14	
10	+21.26 ±0.13		+28.26±1.22	+64.10±1.90	
50	+53.11±1.49	< 50	+63.38±1.67	-1.14±0.02	
100	+62.97±3.28		+92.89±4.09	-6.19±2.10	< 50
200	+78.08±3.01				
250	+80.97±1.66				
GI_{50}	**73.02**	>100	63.38	43.18	>100
TGI	>250	>100	>100	89.70	>100
LC_{50}	>250	>100	>100	>100	>100

Control absorbance at 545 nm = 1.9 ± 0.1 n=3. Where "+" implies growth inhibition, "-" cytotoxicity, "GI_{50} "and "TGI" = concentration causing 50 % and 100 % growth inhibition of cells, while "LC_{50}"= concentration that killed 50% cells.

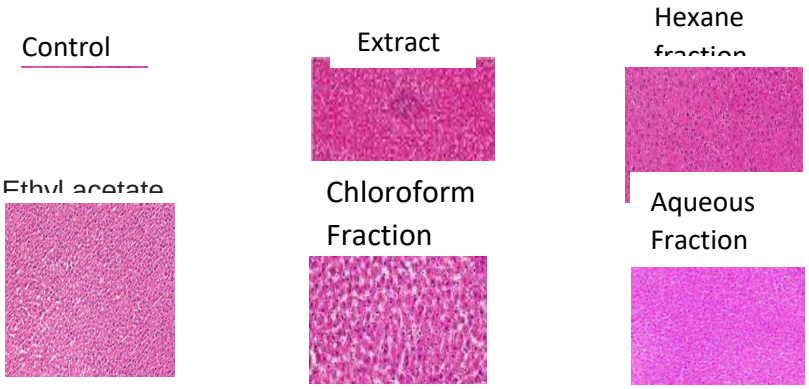

Figure-1. Some photograph showing the morphological distortions of extract and fractions of *S. latifolius* on MCF-7 cell lines under Inverted microscope (Nikon, objective 8×)

Table-3. Sensitivities of the extract and fractions of *S. latifolius* on lung (NCI-H460) cancer cell lines

Conc. (µg/mL)	Extract	**Fractions % Growth inhibition/cytotoxicity**			
		Hexane	Ethyl acetate	Chloroform	Aqueous

1	+18.13±2.30		+32.01±0.12	+78.67±2.98	
10	+56.10 ±1.08		+48.12±0.16	+94.26±2.63	
50	+78.11±0.92	< 50	+82.11±3.56	-11.28±0.71	< 50
100	+87.06±2.32		-1.72±2.38	-16.26±1.26	
200	+92.26±1.20				
250	+98.24±1.28				
GI_{50}	**46.10**	>100	32.05	24.12	>100
TGI	>250	>100	84.50	54.5	>100
LC_{50}	>250	>100	>100	>100	>100

Control absorbance for NCI-H460 in 545 nm = 2. 0± 0.1, n=3.

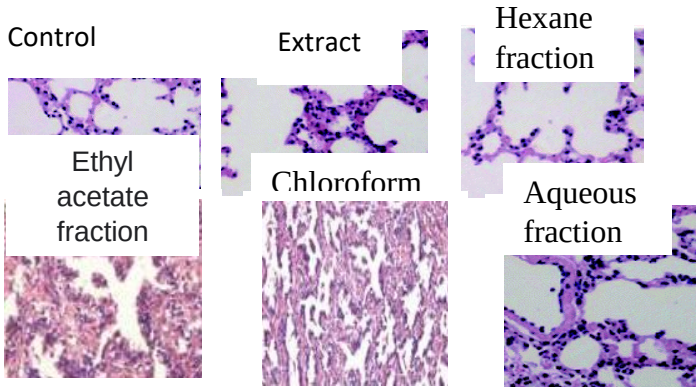

Figure-2: Photograph showing the morphological effects of extract and fractions of *S. latifolius* on NCI-H460 cell lines under Inverted microscope (Nikon, objective 8×)

Result of the evaluation of the total phenolic and flavonoid contents.

Using the equations (y = 0.0250x + 0.0009, R2 = 0.980; y= 0.022x +0.0005, R2=0.654) the total phenolic and flavonoid content of the *S. latifolius* extract and fractions was determined using the normal gallic acid and quercetin curve respectively. Among the test samples, the chloroform fraction recorded the highest (86.30 ±0.16 mg GAE/g and 72.74 ±0.17 mg QE/g) amount of total phenolic and flavonoid content followed by the ethyl acetate fraction (83.20 ±1.01 mg/GAE/g and 69.49 ±2.31 mg QE/g).

Table-4. Results of the total phenolic and flavonoid contents of the extract and fractions of *S. latifolius.*

Test samples	Total Phenol (mg/GAE/g)	Flavonoid content (mg QUE/g)
Extract	61.10 ±0.07^{a}	54.11 ±0.94^{f}
Hexane fraction	46.72 ±1.28^{b}	31.52 ±1.32^{g}
Chloroform fraction	86.30 ±0.16^{c}	72.74 ±0.17^{h}
Ethyl acetate fraction	83.20 ±1.01^{d}	69.49 ±2.31^{i}
Aqueous fraction	81.11 ±2.10^{e}	52.19 ±1.42^{j}

Values are mean ± SEM for triplicate measurements $^{ab\text{-}e}$ p<0.001, $^{bc\text{-}e}$ p<0.001, $^{cd\text{-}e}$ p>0.05, $^{d\text{-}e}$ p>0.05 ns, $^{fg\text{-}i}$ p<0.001, $^{i\text{-}e}$ p>0.05 ns (not significant)

Results of DPPH-Antioxidant studies of the extract and fractions of *S. latifolius*

The DPPH-RSA results for the extract and fractions of *S. latifolius* showed variations in activity among the test samples with the chloroform fraction recording the highest RSA-activity corresponding to IC_{50} of 17.91 µg/mL which was found lower when compared to that of quercetin (12.62 µg/mL).

Table-5: Results of DPPH-RSA activity of the extract and solvent fractions of *S. latifolius*

Conc. (µg/mL)	Extract	Solvents-solvent fractions				
		n-hexane	Ethyl acetate	Chloroform	Aqueous	Quercetin
25	26.10±1.10	18.20±2.64	31.01±1.14	42.50±2.30	22.11±3.94	56.25±4.41
50	39.00±1.06	31.92±1.97	42.62±2.92	56.31±3.78	34.72±1.64	78.62±2.36
100	51.03±0.12	45.60±3.21	66.73±2.92	73.81±2.56	50.24±1.65	92.10±1.62
200	69.32±4.19	52.10±1.92	72.50±2.33	86.76±3.92	52.60±2.05	96.42±3.76
400	76.11±2.50	58.05±4.01	83.24±3.11	91.30±1.94	64.12±4.00	99.08±2.53
IC_{50}	87.10□□	88.31□□	63.01□□	17.91□□	151.36□□	12.62

□□Significantly different from control (Quercetin) P<0.01

Discussion

The rising cases of cancer globally and the several side effects associated with the current forms of treatment (chemotherapy, radiation, surgery, etc.) of the disease is of great concern to the international community (Arruebo *et al*., 2011 b).

Medicinal plants have shown significant application in the management of numerous diseases including cancer (Desai *et al.,* 2008). This is as a result of the active principles such as alkaloids, flavonoids, saponins, terpenes which fights against these diseases including cancer. Our study showed the presence of some of these active principles in both the extract and fractions of *S. latifolius*.

The solvent-solvent partitioning of the extract was necessitated from the fact that the chemical constituents in plant exist in different polarities, hence the hexane, chloroform and ethyl acetate as well as the aqueous fractions are therefore separated into non-polar, semi-polar and polar phases of the extract respectively. This explains the variation in anti-proliferative activity against the cell lines as well as the antioxidant activity. For example, at 100 µg/mL, the extract +78.08 and +87.06 % growth-inhibiting effects on the cell lines (MCF-7 and NCI-H460). The activity later increased and became cytotoxic as the chloroform fraction recorded -6.19 and -16.26 % cytotoxicities at the same concentration. The chloroform fraction exhibited higher antiproliferative activity followed by the ethyl acetate fraction which is in line with the work of Memariani *et al* (2016). The higher sensitivity of NCI-H460 cell lines to the test materials agree with the findings of Al-Oqail *et al* (2015) where A-549 lung cancer cells were more sensitive to all the tested extract and fractions of *Nepeta deflersiana* compared to MCF-7 cells. However, this does not agree with our previous work (Ikpefan *et al.,* 2019).

By nature, plants are foreseeably the largest sources of antioxidants which are protective against several diseases e.g cancer and cardiovascular disease (Zhang *et al*., 2015). Our studies have shown the extract and fractions of *S. latifolius* to be a rich source of antioxidants. The variation in the total phenolic (TP) and flavonoid (TF) contents among the extract and fractions of *S. latifolius* could accounts for the variations in the antioxidant activities among the test samples. For example, the chloroform fraction which recorded the highest TP (86.30 ±0.16 mg/GAE/g) and TF (72.74 ±0.17 mg QE/g) also recorded the most effective antioxidant activity having recorded an IC_{50} of 17.91µg/mL against the 12.62 µg/mL recorded by quercetin. This pattern of results have previously been reported by Cho *et al* (2011) where the chloroform fraction recorded higher phenolic and flavonoid contents as well as antioxidant activity among the other fraction. However, Abdel-Gawad *et al* (2014) and Oresanya *et al* (2020) have reported higher phenolic, flavonoid content and antioxidant activity of ethyl acetate fraction of six Allium species and *Musa acuminate* fruits and leaves. The therapeutic activity of plants is mainly due to polyphenolic metabolites, especially the flavonoids and phenolic contents. Our results showed a higher reduction in cell growth in the MCF-7 and NCI-H460 cancer cell lines as well as the DPPH-antioxidant activity of the of the chloroform fraction of *S. latifolius*. The higher phenolic and flavonoid content in the chloroform fraction could be responsible for the antiproliferative activities recorded, hence could serve as a potential source of anticancer agents.

Conclusion

Our results have demonstrated a considerable antiproliferative and antioxidant potentials of the leaves of *S.latifolius* and the activities reside more in its chloroform fraction. This further justifies the folkloric use of this plant as one of the recipes used by some Nigerian herbalist in treating cancer patients. However, isolation of the active compounds and *in vivo* antiproliferative studies involving the use of other cell lines as well as their mode of action will form the basis of the next phase of the work.

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Contribution of Authors

Ikpefan EO: Conceived concept, research methodology developed, data collection, literature review, data analysis, final reading and acceptance of manuscripts.

Ayinde BA: Principal overseer. Supervised compilation of data, literature review, data processing, final reading and acceptance of manuscripts.

Ikpefan JO: Participated actively in the extraction and fractionation of the extract, proof reading, final reading and endorsement of manuscripts.

Mudassir A: Design the anticancer methodology and cell culture study and analyzing of data.

Farooq, AD: Co-supervised the anticancer study.