RESEARCH ARTICLE

ISSN: 0331 - 670X
https://doi.org/10.51412/psnnjp.2021.5

The Nigerian Journal of PHARMACY
AS SEN OF HONOUR WE JOIN HANDS
THE OFFICIAL ORGAN OF THE PHARMACEUTICAL SOCIETY OF NIGERIA FOUNDED 1927

IN VITRO ANTIPROLIFERATIVE AND ANTIOXIDANT ASSESSMENT OF THE EXTRACT AND PARTITIONED FRACTIONS OF LEAVES OF EUPHORBIA GRAMINEA JACQ. (EUPHORBIACEAE)

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ARTICLE INFO

Article history:

Received 25 Aug 2020
Revised 11 Oct 2020
Accepted 8 Nov 2020
Online 31 Mar 2021
Published -

Keywords:

Cytotoxicity,
Growth Inhibitory,
Euphorbia graminea,
Antiproliferation,
Antioxidant,
Breast Cancer Lines (MCF-7),
Lung Cancer (NCI-H460)
Cancer Cells.

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ABSTRACT

Background: *Euphorbia graminea* Jacq. (Euphorbiaceae) is a medicinal plant with limited information on its biological applications and reported scientific studies.

Method: This work was aimed at investigating the *in vitro* antiproliferative and antioxidant effects of leaf the extract and fractions of *E.graminea* by employing the Sulforhodamine-B as well as the DPPH-RSA (2, 2-diphenyl-1-picrylhydrazyl) assays respectively. The extract and fractions of *E. graminea* displayed growth inhibitory and cytotoxic effects in a concentration dependent manner on the cell lines.

Results: At 250 µg/ mL, the extract recorded -3.1 and +75 % cytotoxic and growth inhibitory effects on MCF-7 and NCI-H460 cell lines. While the cytotoxic effect became more pronounced on MCF-7 cell lines as -2.19 and -9.6 % cytotoxicities were recorded by the chloroform fraction at 75 and 100 µg/ mL the ethyl acetate fraction recorded +58.72 and +89.33 % inhibitory effects at similar concentrations. The hexane and ethyl acetate fractions were inactive against NCI-H460 cell lines at all concentrations. The antioxidant activities further corroborated the antiproliferative activities as IC_{50} of 58.88 µg/mL was recorded by the chloroform fraction against 46.77 µg/mL by Quercetin.

Conclusion: This work has shown that the antiproliferative and antioxidant activities of *E.graminea* resides more in the chloroform phase and could be due to the active metabolites it contained. In continuation of this work, the Isolation and characterization of some of these metabolites will thus be carried out.

1. Introduction

The plant Euphorbia graminea Jacq. (Euphorbiaceae) is a new species which according to[1] was recently discovered in Africa and further explained that the plant probably came into existence in Nigeria in the early 1993 but became visible in the mid-2000s which is an indication that the plant might have been introduced into the country in the early 1990s. This claim was further corroborated by [2] in her account of newly introduced plant in Nigeria as she excluded *E. graminea* from her collections in 1993 due to unknown taxonomic identity. The plant is an annual plant which grows 15-30 cm tall but sometimes taller and often branching from the base and dichotomously branched distally. The leaves appear alternate and could be ovate or oblong, elliptical to linear with entire margin[3].

The dearth of information about *E. graminea* in literature is indicative of its current introduction into the Nigerian vegetation. However, species of the Euphorbia are known to have biological and ethnomedicaluses. The plant *E.graminea*, having fall under the genus Euphorbia, helps to determine the possible relationship between it and the phytochemical components and metabolites expected as most species of Euphorbia do . Among the possible ethnomedicinal uses of *E. graminea* include treatment of skin infections such as ulcers, cancers, tumours, warts, and other diseases as practiced by

Colombian traditional healers [4]. It has also been reported to be poisonous to ruminants [5,6]. In Nigeria, the plant has not been specifically indicated for the treatment of many disease condition. However, its uses may not be so much different from other Euphorbia species[7].

This work was therefore aimed at determining antiproliferative and antioxidant potentials of the extract and fractions of the leaves of *E. graminea* for the first time which will provide information that will enhance its exploration in the field of cancer research.

Figure 1: *Photograph showing leaves of cultivated Euphorbia graminea in its natural habitat.*

2. Materials and Methods

2.1 Collection and processing of the plant.

The plants were collected at the University of Benin City (Capitol), Nigeria. The plant was identified by Dr Henry Akinnibosun and was authenticated at Forest Research Institute of Nigeria (F.R.I.N.) Ibadan where herbarium specimen number FHI 109024 was obtained. The leaves of the plant were air dried for 3 days and were further dried in an oven maintained at 40°C for 24 h. After this treatment, the plant material was powdered using a laboratory electric milling machine (Chris Norris, England) and kept in an airtight container until ready for extraction.

2.2 Extraction and fractionation of plant material

About 2.5kg of the powdered leaf sample was exhaustively extracted with a Soxhlet apparatus using aqueous Methanol (95%) as the extracting solvent. The extract was reduced to dryness in vacuo in a rotary evaporator (Buchi, R-300, Germany) sustained at 40°C. The extract was dissolved in equal amount of water -methanol (1:1, 300 mL) and was exhaustively partitioned using hexane, ethyl acetate and chloroform (600 mL X 5) in a 2 L separating funnel .

2.3 Phytochemical screening

The phytochemical screening of the samples (extract and fractions of *E.graminea*) was done in accordance with standard protocols [8].

2.4 Evaluation of Extracts for antiproliferative activity. Preparation of stock solutions

The stock solutions of plant extracts and fractions (40 mg/mL) were prepared in sterile DMSO (100 %). The stock solutions for doxorubicin (1 mM) were prepared in sterile distilled water and kept at -80 ºC until required. The dilutions were prepared in RPMI-1640 containing gentamicin (50 µg/mL) on the day of experiment.

2.5 Sulforhodamine-B assay

This was carried out using the method previously described by [9] with slight modifications. The cells lines MCF-7 (breast) and NCI-H460 (lung) lines contained in cryovials were maintained in water bath at 37°C and were transferred into a washing medium comprising of RPMI-1640: 98 %, fetal bovine serum (FBS): 1% and L-glutamine (2 mm), streptomycin (100 µg/mL), penicillin (100 U/mL) solution (L-GPSS): 1%] and the content slowly stirred and centrifuged for 10 min at 1000 rpm in centrifuge tubes (15 mL). The cells in the form of pellets were removed and resuspended in washing medium and the viability of the cells was determined using the trypan (10 µL, 0.4 % in 0.9 % saline) blue dye exclusion method with haemocytometer. The viable cells were cultured in 96 well plates and the various concentrations of the extract (1-250 mg/mL) and fractions (1-100 mg/mL) were added and the plates were incubated at 370C in CO_2 incubator. At the end of 48 hr, the plates were fixed with 50% trichloroacetic acid for 30 min after which the plates were turned upside down to remove fluids in the wells and were air died overnight at room temperature to remove moisture. This was followed by the addition of 100 µL of 0.4 % Sulforhodamine-B stain and 30 min incubation at room temperature. The stains were washed off the plates with 1% acetic acid, and the plates were dried overnight followed by addition of Tris base solution (100 µL of 10 mM) which solubilizes the stain.

The plates were shaken for 10-15 min in a plate shaker and their optical density measured at 515nm using spectrophotometer. The percentage, TGI (concentration of drug causing 100 % growth inhibition of cells) , GI_{50}(cell growth inhibition at 50%) and LC_{50} (concentration of drug that killed 50% cells) were calculated. The experiment was done in triplicate and was

repeated for the control.

2.6 Determination of total phenolics and flavonoid content.

(i) Total phenolic content (TPC)

This was determined for the extract and fractions of *E.graminea* using the Folin–Ciocalteu method using garlic acid as standard[10]. The test samples (1mL) was mixed with Folin–Ciocalteu reagent (5 mL) and $NaCO_3$ (4 mL of 0.7M) and the absorbance recorded using UV spectrophotometer (Beckman DU650 UV-Vis-NIR) after 2 h of incubation at 765 nm. The experiments were done in triplicates and the TPC in the test samples were measured as gallic acid equivalent (GAE)mg/mL of dry extract using the gallic acid calibration curve.

2.7 Total flavonoid content (TFC)

This was done using the aluminium chloride calorimetric using Quercetin as standard[11]. By this method, 0.5 mL of the standard and test samples (extract and fractions) were mixed with 1.5 mL CH_3OH, 0.1mL 10 % $AlCl_3$, 0.1 mL 1M CH_3COOK (potassium acetate) and 2.8 mL distilled water and the reaction mixture was incubated for 30 min at room temperature and absorbance taken at 415 nm. The TFC of the extract and fractions of *E.graminea* were expressed as quercetin equivalent (QE) mg/mL dry extract.

2.8 DPPH radical scavenging activity

The method previously described by[12] was adopted with little modification in concentrations (31.25-250 µg/mL) with quercetin used as standard. By this method, 1mL 0.1 M DPPH ethanol solution mixed with 3mL of sample (extract/ fractions) solution in water at different concentrations of the samples and the absorbance of the mixtures were taken at 517 nm. The percentage ROS-activity was calculated in percentage using the formula below;

The IC_{50} values of the scavenging activity were calculated from a plot of % ROS-activity against the concentrations.

2.9 Statistical Analysis

The data were analysed using GraphPad Prism 7.0 and One-way analysis of variance (ANOVA) was used in data analysis. The data were represented as Mean±Standard Error of Mean (SEM) and were analysed using Tukey-Kramer Multiple Comparisons Test was used

3. Results

The 2.5kg of the powdered leaves yielded 216.11g which is equivalent to 8.64 % yield. The partitioned extract yielded 12.22g (0.06%), 21.04g (0.11%), 48.60g (0.24%) and 68.27g (0.34%) of hexane, ethyl acetate, chloroform and aqueous fractions respectively. The phytochemical screening of the extract and fractions revealed the presence of terpenes, tannins, saponins and cardiac glycosides (Table 1).

Table 1: *Phytochemical screening of extract and fractions of the leaves of E.graminea*

		Fractions			
Phytochemicals	Extract	n - Hexane	Ethyl acetate	Chloroform	Aqueous
Alkaloids	-	-	-	-	-
Anthraquinones	-	-	-	-	-
Tannins (Phenolics)	+	-	+	+++	+
Flavonoids	+	-	+	+++	+
Saponins	+	-	+	+	++
Cardiac glycosides	+	-	+	+	+++
Terpenes	+	++	+	++++	-

Key: - = absent or not detected; + =present in trace amount; ++ = present in moderate amount; +++ = present in appreciable amount

3.1 Results of the total phenolic (TPC) and flavonoid (TFC) contents of extract and fractions of *E.graminea*

The TPC and TFC of the extract and fractions were calculated from the regression equation of the calibration curves (y =0.043x + 3.588: R2 =0.997) and (y = 0.014x + 0.056; R2 = 0.999) with the chloroform fraction having the highest amount in both assays (Table 2)

Table 2: *Total phenolic and flavonoid contents of the extract and fractions of E.graminea*

Extract/fractions	**Total phenol (mg/GAE/g)**	**Total flavonoid (mg/QAE/g)**
Extract	92.22 ± 1.92[a]	56.20 ± 4.10[f]
Hexane	26.50 ± 0.36 [b]	11.18 ± 1.10[g]
Ethyl acetate	108.07 ± 5.84[c]	72.26 ± 1.90[h]
Chloroform	142.02 ± 2.56[d]	98.22 ± 0.68[i]
Aqueous	50.16 ± 1.70[e]	23.11 ±1.32[j]

These Values are expressed as Mean ± SEM. Statistically different values were evaluated using Tukey-Kramer Multiple Comparisons Test across column (ab,ad,ae,bc,bd,be,cd,ce,de p<0.001, acp<0.05); (fg, fh,fi,fj, gh,gi, hi,hj,ij p<0.001, gj, p<0.01)

The DPPH-RSA results of extract and fractions of E.graminea

The DPPH-RSA of the extract and fractions recorded a concentration dependent activity with the chloroform fraction recording the highest scavenging activities of 79.47 and 91.69 % at 250 and 500 mg/mL respectively. The hexane fraction was observed to be inactive at all concentrations (Table 3).

Table 3: *Results of the DPPH antioxidant assay of the extract and fractions of E.graminea*

Conc. (µg/mL)	Extract	Fractions				**Control** Quercetin
		Hexane	Ethyl acetate	Chloroform	Aqueous	
31.25	12.14 ± 2.06	-	18.67 ±2.98	29.16 ± 3.57	19.11 ± 4.08	41.11±5.84
62.5	26.07 ± 1.98	-	34.92 ± 1.75	46.93 ± 2.12	26.19 ± 3.26	56.15 ± 4.57
125	32.11 ± 1.11	-	51.50 ± 1.26	79.47 ±	32.66 ± 3.47	81.64 ± 4.22
250	61.03 ± 0.68	-	69.74 ± 1.01	91.69 ±	48.21 ± 5.20	98.06 ± 6.07
IC_{50} (µg/mL)	190.55	-	120.23	58.88	>250	46.77

3.2 Effects of the extract and fractions of *E. graminae* on MCF-7 and NCI-H460 Cell Lines

The extract and fractions of exhibited growth inhibitory and cytotoxicity on MCF-7 cell lines. While the growth inhibitory activity were recorded at concentrations between 1-250 µg/mL, the cytotoxicity of –3.15 % was recorded at 250 µg/mL with GI_{50}, LC_{50} and TGI of 78, 51.33 and 200 µg/mL respectively against MCF-7 cell lines. The chloroform fraction having produced growth inhibitory effects at 1-75 µg/mL gave cytotoxicity of -9.60 % at 100 µg/mL with GI_{50}, LC_{50} and TGI of 48, 16.98 and 97.32 µg/mL. However, the aqueous and hexane fractions were inactive as they recorded less than 50 % cell death (Table 4).

Unlike the results of MCF-7, the extract and chloroform fractions were observed to be less sensitive as they exhibited growth only inhibitory activities. The hexane, ethyl acetate and aqueous fractions were observed to be inactive (Table 5).

Table 4: *Sensitivities of the breast cancer cell-lines (MCF-7) to the methanol extract and fractions of E.graminea.*

Test samples	**(µg/mL)**	**% Growth inhibition/ cytotoxicity (MCF-7)**	**GI_{50}**	**LC_{50}**	**TGI**
			(µg/mL)		
Extract	1.0	+9.10± 0.02			
	10	+15.50± 0.26	78.00±3.54	51.33	200
	50	+35.11± 1.42			
	100	+87.60± 5.5			
	200	+98.30± 2.6			
	250	–3.15± 0.60			
Fractions		**% Growth inhibition/ Cytotoxicity (MCF-7)**			
n-Hexane		-	>100	>100	>100
Ethyl acetate	1.0	-			
	25	-			
	50	+15.07±1.62	94.67±6.72	>100	>100
	75	+58.72±2.22			
	100	+89.33±5.01			
Chloroform	1.0	+ 6.1± 0.04			
	25	+15.78 ± 0.7			
	50	+42.10± 2.01	48.00± 2.31	16.98	97.32±4.62
	75	+96.19± 3.0			
	100	–9.60 ± 5.0			
Aqueous		<50	>100	>100	>100

Control absorbance for MCF-7 at 545 nm = 1.9 ± 0.1 n=3. "+" = growth inhibition, "-" =cytotoxicity, "GI50 "and "TGI" = concentration of drug causing 50% and 100 % growth inhibition of cells, while "LC50"= concentration of drug that killed 50% cells.

Table 5: *Sensitivities of the lung cancer cell-lines (NCI-H460) to the methanol extract and fractions of E.graminea.*

Test samples	**(µg/mL)**	**% Growth inhibition/ cytotoxicity NCI-H460**	**GI_{50}**	**LC_{50}**	**TGI**
			(µg/mL)		
Extract	1.0	-			
	10	-			
	50	+15.93 ± 1.86	148.17 ±3.54	-	-
	100	+33.24 ± 1.77			
	200	+54.26 ± 3.0			
	250	+75.42 ± 0.6			
Fractions		**% Growth inhibition/ Cytotoxicity NCI-H460**			
n-Hexane		<50	>100	>100	>100
Ethyl acetate		<50	>100	>100	>100
Chloroform	1.0	+ 10.00± 1.68			
	25	+28.06 ± 2.18			
	50	+44.51± 2.34	91.91± 2.31	47.64	>100
	75	+64.59± 1.53			
	100	+93.45 ± 0.84			
Aqueous		<50	>100	>100	>100

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

4. Discussion

Among the leading causes of death, cancer accounts for about 7.6 million deaths globally per annum [13]. Chemotherapeutic agents used for the treatment of this disease over the years, have recorded undesirable side effects such as musculoskeletal pain in breast and lung cancer patients [14]. Such side effects are less often associated with plant-derived anticancer drugs such as vinblastine and vincristine from *Catharanthus roseus* and ectoposide and teniposide from Podophyllum Linn respectively.

The antiproliferative potential of medicinal plants stems from the presence of certain bioactive metabolites in them [15]. The phytochemical screening of the extract and fractions of *E.graminea* showed the presence of flavonoids, alkaloids, tannins, glycosides, terpenes, in varying intensities. The higher activities shown by the chloroform fraction could be as a result of the abundance of one or more of these phytochemical groups as some of them act as anti-radicals in the body, hence preventing carcinogenesis and other diseases [16]. Among the extract and fractions, the chloroform fraction which recorded an IC_{50} of 58.88 ug/mL comparable to 46.77 ug/mL of quercetin, had the highest total phenolic content (142.02 µg/mL) accompanied by ethyl acetate fraction (108.07 µg/mL). The total phenolic content was observed to correlate the antioxidant activity as the chloroform fraction recorded the highest activity. The higher TPC and DPPH-RSA of the chloroform fraction have previously been reported by [17] where the chloroform fraction recorded strong DPPH, phenolic and flavonoid contents which is in line with our work. The DPPH-RSA technique, unlike other antioxidant assays, allows the screening of lipophilic and hydrophilic samples *in vitro* at moderate concentrations [18], hence is the most used. The extract and fractions of *E.graminea* similarly gave a concentration-dependent growth-inhibitory and cytotoxic activity on the cell lines. The liquid-liquid partitioning further enhanced the action of the extract against the cell lines. For example, at 100 ug/mL, the extract gave growth-inhibitory event of +87.60 % against MCF-7 cell lines while the chloroform fraction at the same concentration gave cytotoxicity of -3.15 %. The chloroform fraction recorded the highest activity more than the extract and respective fractions. These variations in the activities could be due to the differences in polarities as well as the phytochemicals they contain. This further goes to show that the active constituents of this plant reside more in the chloroform phase.

The antiproliferative potential of chloroform fractions of medicinal plants have previously been reported. For example, [19] has reported the potent activity of the chloroform fraction of *Butea monosperma* leaf against A-549 (human lung cancer) cell lines. The significant effect of the chloroform fraction of *Allium bakhtiaricum* on MDA-MB-231 (human breast adenocarcinoma), MCF-7 (human breast adenocarcinoma), HT-29 (human colorectal adenocarcinoma), HepG2 (liver hepatocellular carcinoma), 4T1 (mouse mammary tumor,) and NIH3T3 (mouse embryonic fibroblasts) has also been reported [20]. These findings are in line with our present work, hence further corroborating the efficiency of the chloroform fraction in reducing tumour growth.

5. Conclusion.

The plant *E. graminea* has demonstrated a significant *in vitro* antiproliferative activity against breast and lung cancer lines as well as DPPH radical antioxidant potentials. The chloroform fraction among the other fractions of *E. graminea* will be a suitable target for further bioassay-purification process that could lead to the isolation of the active antiproliferative and antioxidant constituents which could play vital role in cancer therapy.

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