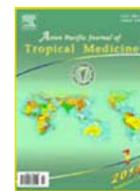


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Anticancer and antioxidant activities of methanol extracts and fractions of some Cameroonian medicinal plants

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ABSTRACT

Objective: To obtain a scientific basis of the use of plant-derived preparations by many rural people in Cameroon, for their primary health care needs in the treatment of diseases such as cancer.**Methods:** The antiproliferative effect of 11 plants methanol crude extracts on four cancer cells using sulforhodamine-B assay and their antioxidant activities using 1-diphenyl-2-picrylhydrazyl radical and nitric oxide radical scavenging ability were investigated. The *Ekebergia senegalensis* (*E. senegalensis*) and *Protea elliotii* (*P. elliotii*) extracts were selected based on their antioxidant and anticancer activities, and partition in hexane, dichloromethane, ethyl acetate, butanol and methanol was done. Each fraction was submitted to antioxidant and anticancer activities, and the effect of the dichloromethane fraction (the most antiproliferative fraction) on NCI-H460 cell cycle was determined by flow cytometry.**Results:** The most antiproliferative substances were found for the extracts from *E. senegalensis*, *P. elliotii*, *Terminalia macroptera* and *Vitellaria paradoxa*. Whereas the most antioxidant substances were found for the extracts from *Cissus populnea*, *E. senegalensis*, *P. elliotii*, *Terminalia macroptera*, *Vitellaria paradoxa*, and *Gardenia aqualla*. Dichloromethane fraction of *P. elliotii* was found to be highly antiproliferative to NCI-H460 cancer cells and showed S phase arrest cell cycle progression. Ethyl acetate *n*-butanol and methanol fractions showed quite strong antioxidant activity for both *E. senegalensis* and *P. elliotii*, as compared to that of gallic acid.**Conclusions:** Overall, the antiproliferative and antioxidant activities of some of the extracts lend some support to their use in the traditional medicine of Adamawa Region, Cameroon to treat cancer.

1. Introduction

Cancer is one of the most life-threatening diseases in which deregulating proliferation of abnormal cells invades and disrupts surrounding tissues^[1]. It constitutes serious

public health problems in both developed and developing countries. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treatment of cancer, indicates that there is an imperative need of alternative strategies in cancer management^[2].

Many natural products discovered from medicinal plants, or secondary metabolites such as terpenoids, phenolic acids, lignans, tannins, flavonoids, quinones, coumarins, alkaloids, which exhibit significant antioxidant and other

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activities, have played an important role in treatment of cancer[3]. Studies have shown that many of antioxidant compounds possess anti-inflammatory, antitumor, anti-mutagenic and anti-carcinogenic activities[4]. Medicinal plants have thus become a focal point to improve the present and future health needs against cancer. This is because medicinal plants secondary metabolites could maintain the health and cure various diseases including cancer with less harmful effects[5].

In Cameroon, leaves of *Senna siamea* (*S. siamea*) are used as antimalarial[6], while the fruits are used to charm away intestinal worms and to prevent convulsion in children[7]. The whole plant of *Centella asiatica* (*C. asiatica*) is used as a support for quick healing of small wounds and in the treatment of skin diseases such as eczema, leprosy, and psoriasis[8]. Roots of *Cissus populnea* (*C. populnea*) are useful in the treatment of male infertility as well as urinary tract infections[9]. The bark of *Ekebergia senegalensis* (*E. senegalensis*) is used in the treatment of ovarian cyst[10]. Leaves of *Eremomastax speciosa* (*E. speciosa*) are used to treat generalized pains, dermatitis, anemia, irregular menstruation, hemorrhoids and urinary tract infection[11]. Leaves of *Gardenia aqualla* (*G. aqualla*) are useful in the treatment of impotence[10]. *Lannea kerstingii* (*L. kerstingii*) bark is an antidiarrhea[12]. *Protea elliotii* (*P. elliotii*) barks are used in the treatment of carious teeth, hemorrhoids, sores, eyes worm diseases[10]. Roots of *Terminalia macroptera* (*T. macroptera*) are used by several traditional healers for the treatment of hepatitis[13]. Whereas *Vitellaria paradoxa* (*V. paradoxa*) barks are used for hypertension, incurable wounds, jaundice, hemorrhoids and fever[10]. However, although large sections of the rural population, through information provided by medicinal practitioners, use plant-derived preparations to cure some cases of diseases including lung, breast and prostate cancer, only a few studies have been reported for much variety of medicinal plants. It appears useful to obtain a scientific basis for the health properties of these plants of the northern part of Cameroon, in the treatment of cancer. Keeping this in view, the above plants were selected based on recorded ethno-botanical survey, and the antiproliferative effect of their crude methanol extracts on four cancer cells using sulforhodamine-B (SRB) assay as well as their antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical and nitric oxide radical scavenging ability were carried out.

2. Materials and methods

2.1. Extract preparation and fractionations

The fresh sample of *E. senegalensis* (5092/SRFC), *P. elliotii*

(22522/HNC), *T. macroptera* (43688/HNC), *V. paradoxa* (50216/HNC), *E. speciosa* (23604/HNC), *C. asiatica* (20429/HNC), *Costus spectabilis* (32745/HNC) (*C. spectabilis*), *Cissus* (36962/HNC), *S. siamea* (25661/HNC), *G. aqualla* (36894/HNC) and *L. kerstingii* (52641/HNC) identified by the National Herbarium were collected during May 2013 in Adamawa Region of Cameroon. Ground and powdered dried part of each plant (500 g) were macerated daily with methanol (3 L) at room temperature for 3 d. The resulting methanol residue was evaporated at 40 °C using rotary evaporator and stored at –20 °C until further use.

Based on the anticancer and antioxidant activity, *P. elliotii* and *E. senegalensis* were selected and partitioned into fractions using separator funnel and solvents in increasing order of polarity. Thus methanol extracts of *P. elliotii* dried barks (150 g) were suspended in distilled water (500 mL) and separated into hexane (9 L), dichloromethane (6 L), ethyl acetate (6 L), *n*-butanol (4.5 L) and methanol (2 L). While methanol extracts of *E. senegalensis* dried barks (200 g) were suspended in 500 mL of distilled water and separated into hexane (9 L), dichloromethane (9 L), ethyl acetate (9 L), *n*-butanol (4.5 L) and methanol (3 L).

The resulting fractions were evaporated at 40 °C using rotary evaporator and stored at –20 °C until further use.

2.2. Antiproliferative effect of methanol extract of plants studied

The growth inhibitory activity of the methanol crude extracts and fractions were evaluated against four cell line panels consisting of NCI-H460 (lung cancer), MCF7 (breast cancer), PC3 (prostate cancer), HeLa (cervix cancer cell) and normal cell 3T3 (mouse cervical cells) using the SRB assay[14]. The cell lines were routinely maintained as monolayer cell cultures in Roswell Park Memorial Institute medium containing fetal bovine serum (10%), and glutamine, penicillin and streptomycin solution (1%, L-glutamine, penicillin and streptomycin).

The viability of the cells was determined by the trypan blue exclusion method with haemocytometer. Briefly, 100 µL of cell suspension (1 000 cells/well for MCF-7 and HeLa, 7 500 cells/well for NCI-H460 and PC3, and 5 000 cells/well for 3T3) were plated in each well of 96-well plates, and incubated for 24 h at 37 °C in a humidified CO₂ (5%) incubator. The stock solutions of methanol extract and fractions were prepared in dimethyl sulfoxide (DMSO) as a vehicle and various dilutions of the ethanol crude extract and fractions (250, 100, 50, 20, 10 and 2 µg/mL) were added (100 µL) in each well. After 48 h of incubation, cold (4 °C) trichloroacetic acid (50%, 50 µL) was added gently and left for 30 min at room temperature, followed by washing with distilled water and drying overnight at room temperature. To each well, SRB solution (0.4% w/v in 1% acetic acid, 100 µL)

was added and after 10 min, the unbound stain was washed off with acetic acid (1%) and air-dried at room temperature. The protein bound stain was solubilized with tris-base (pH 10.2) with shaking for 5 min followed by the measures of the absorbance at 515 nm using a microplate reader. The absorbance for the blanks including blank test substance and control (without drug) were used to calculate the growth inhibitory effect of the test compounds. GI_{50} which was the concentration of the extract or doxorubicin causing 50% growth inhibition of cells was determined.

2.3. Antioxidant activities of methanol extract of plants studied

2.3.1. DPPH free radical scavenging assay

The antiradical activities of the plant extracts and gallic acid (as a reference) were determined using DPPH, which is a stable free radical [15]. The solubilized test substances in DMSO were miscible in all proportions with water, while the DPPH was prepared in ethanol. Five microliters of each test substance was allowed to react with 95 μ L of DPPH (300 μ mol/L) at 37 °C for 30 min in a 96-well plate and absorbance was measured at 515 nm using an ELISA microplate reader (SpectraMAX-340 Molecular Devices, USA). The radical scavenging activity of the samples was determined in comparison with the vehicle control group (DMSO) and the inhibition (I%) of the free radical DPPH was calculated as follows:

$$I\% = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference.

2.3.2. Nitric oxide scavenging ability

The nitric oxide scavenging ability was determined according to the modified method described by Hemayet et al [16]. Briefly, the reaction mixture (150 μ L) contained 12 μ L of test sample (1.0 mmol/L in DMSO), 38 μ L of potassium phosphate buffer (10 mmol/L, pH 7.4) and 100 μ L of sodium nitropruside (10 mmol/L). Following incubation at 25 °C for 150 min, 50 μ L of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was added and allowed to stand for 5 min. To all the tubes, 50 μ L of N-(1-naphthyl) ethylenediaminedihydrochloride (0.1% w/v) was added, stirred, and allowed to stand for 1–2 min. The absorbance of pink-coloured chromophore solution was measured at 546 nm against the corresponding blank solution (DMSO) using ascorbic acid as the positive control.

The radical scavenging activity (RSA) was calculated according to the following equation:

$$\text{RSA} (\%) = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is

the absorbance of the extracts/reference.

2.4. Evaluation of the effect of *P. elliotii* on cell cycle

About 10^6 cells/well were plated into six-well plates for 24 h to allow monolayer formation, followed by treatment with the plant sample (1 μ g/mL, 3.5 μ g/mL and 25 μ g/mL) or doxorubicin (3.5 μ g/mL). After 48 h, cells were harvested using trypsin and re-suspended in phosphate buffer solution. After centrifugation (800 r/min) for 5 min, cold 70% ethanol was carefully added drop-wise carefully, while vortexing to avoid aggregation. The mixture obtained was kept at 4 °C for at least 12 h. After washing the cells twice with phosphate buffer solution, 10 μ L of RNase A (100 units/mL) was introduced and incubated for 30 min at 37 °C in water bath. Propidium iodide (150 μ L, 20 μ g/mL) was added and the mixture was incubated for 30 min at room temperature. The stained cells were analysed by flow cytometry (FACScalibur, Becton Dickinson, USA) and DNA content was quantified by using Flow Jo software.

2.5. Statistical analysis

The data from biological assays were subjected to the One-way analysis of variance (ANOVA) procedures which were presented as mean \pm SEM. The Dunnett's test was used to compare means. $P < 0.01$ was considered significant.

3. Results

3.1. Antiproliferative activity of methanol extract of plants studied

The screening of methanol crude extracts of 11 plants was done for their antiproliferative activity against four malignant cell lines: NCI-H460, MCF7, PC3, HeLa and one normal cell 3T3 using the SRB assay. This study was conducted with appropriate positive control (doxorubicin). After 48 h of treatment, some plant extracts exhibited an higher inhibitory effect against tumor cell growth, with varying efficiencies and selectivities while others caused marginal growth inhibition cell or not at all effective. The lowest GI_{50} (concentration of the extract causing 50% growth inhibition of the cells) values, corresponding to the most cytotoxic substances, were found for the extracts from *E. senegalensis*, *P. elliotii*, *T. macroptera* and *V. paradoxa* (Table 1). Very weak antiproliferative activity was observed in *P. elliotii* and *S. siamea*. The *G. aqualla* and *L. kerstingii* extracts showed a much reduced activity, reflected by large GI_{50} values, and we can consider that these extracts have no antiproliferative effect against this cancer cells (Table 1).

Table 1

Antiproliferative activity of methanol extract of plants studied.

Plant names	Antiproliferative activity GI ₅₀ (µg/mL)				
	NCI H460	MCF-7	PC3	Hela	3T3
<i>L. kerstingii</i>	>250	>250	>100	>100	>250
<i>C. populnea</i>	>100	>100	>100	>100	>100
<i>C. asiatica</i>	>250	47.00±1.60	45.00±0.81	>100	>250
<i>G. aqualla</i>	>100	87.00±0.73	97.00±0.88	>100	>100
<i>E. speciosa</i>	98.00±0.77	44.00±0.77	>100	>100	>100
<i>S. siamea</i>	87.00±1.60	97.00±1.30	>100	>100	>100
<i>C. spectabilis</i>	13.00±0.80	20.00±0.80	22.50±0.81	10.30±0.26	6.72±0.05
<i>E. senegalensis</i>	20.00±0.04	13.50±1.04	16.18±1.34	17.86±0.74	28.84±0.10
<i>P. elliotii</i>	24.44±0.90	87.00±1.22	47.00±1.33	66.46±0.37	81.60±0.70
<i>T. macroptera</i>	31.00±1.50	34.00±1.05	42.37±0.31	68.46±0.18	64.42±1.30
<i>V. paradoxa</i>	27.00±0.90	24.00±0.19	67.00±0.09	66.46±0.37	30.67±0.50
Doxorubicin	0.020±0.001	0.220±0.053	0.29±0.12	0.62±0.15	0.70±0.19

Data are presented as mean±SEM. GI₅₀: Concentration of the extract or doxorubicin causing 50% growth inhibition of the cells.

3.2. Antioxidant activity of methanol extract of plants studied

In this study, the antioxidant activities of the plant extracts were evaluated using two different tests (DPPH and nitric oxide radical scavenging ability). The obtained results summarized in Table 2 revealed that for the DPPH method, the lowest area values corresponding to the most antioxidant substances were found for the extracts from *C. populnea*, *E. senegalensis*, *P. elliotii*, *T. macroptera*, *L. kerstingii* and *V. paradoxa*, while the highest area values (least activity) were those of *S. siamea*, *E. speciosa* and *G. aqualla*. No significant differences were observed between activity of gallic acid (reference drug) and those of the extracts. Except *C. asiatica*, *C. spectabilis*, *E. speciosa* and *S. siamea* with no nitric oxide radical scavenging ability, all other plants extracts (*C. populnea*, *E. senegalensis*, *G. aqualla*, *L. kerstingii*, *P. elliotii*, *T. macroptera* and *V. paradoxa*) showed lower activity compared to ascorbic acid which was used as positive control (Table 2).

Table 2

Antioxidant activity of methanol extract of plants studied.

Sample	Antioxidant activity	
	DPPH radical scavenging ability (IC ₅₀ µg/mL)	Nitric oxide radical scavenging ability (IC ₅₀ µg/mL)
<i>S. siamea</i>	236.70±3.80	NA
<i>C. asiatica</i>	NA	NA
<i>C. populnea</i>	15.72±1.20**	409.00±2.00
<i>C. spectabilis</i>	NA	NA
<i>E. senegalensis</i>	15.83±0.40	299.00±3.00
<i>E. speciosa</i>	454.00±2.54	NA
<i>G. aqualla</i>	105.90±2.10	278.00±1.56
<i>L. kerstingii</i>	34.40±2.20	253.00±2.00
<i>P. elliotii</i>	14.20±0.27**	306.00±2.50
<i>T. macroptera</i>	19.90±0.70**	205.00±0.90
<i>V. paradoxa</i>	22.14±0.39	290.00±2.04
Standard drug	22.67±0.40	108.00±2.00

Data are presented as mean±SEM. ***P*<0.01. IC₅₀: Concentration of the extract causing 50% of DPPH or nitric oxide radical scavenging ability. NA: Not active. Standard is gallic acid for DPPH radical scavenging ability and ascorbic acid for nitric oxide radical scavenging ability.

3.3. Antiproliferative and antioxidant activities of fractions of *E. senegalensis* and *P. elliotii*

Based on antioxidant and anticancer activities of the above extracts, *E. senegalensis* and *P. elliotii* were selected and partitioned in five different solvents with increasing polarity, and each fraction was submitted to the test. Dichloromethane fractions of these two plants were found to be highly cytotoxic to the cancer cells while other fractions caused marginal growth inhibition cell or not at all effective on NCI-H460 and MCF-7 (Table 3). Hexane fraction of *E. senegalensis* and dichloromethane fraction of *P. elliotii* were the most potent on MCF-7 and NCI-H460 cell lines respectively.

Except hexane fraction of *E. senegalensis* with no antioxidant activity, ethyl acetate *n*-butanol and methanol fractions obtained from crude methanol bark extract of *E. senegalensis* and *P. elliotii* showed significant antioxidant activity (Table 4), as compared to that of gallic acid. Dichloromethane fractions of *E. senegalensis* and *P. elliotii* showed weak antioxidant activity with IC₅₀ of (366.6±2.9) and (122.0±4.3) µg/mL respectively (Table 4).

Table 3Antiproliferative activity of fractions of *E. senegalensis* and *P. elliotii*.

Plant	Sample name	Antiproliferative activity GI ₅₀ (µg/mL)		
		NCI-H460	MCF-7	3T3
<i>E. senegalensis</i>	Hexane	30.000±0.100	10.000±0.800	45.000±0.220
	Dichloromethane	12.000±0.500	19.000±0.900	32.000±0.150
	Ethyl acetate	30.000±1.660	25.000±1.330	40.000±1.880
	<i>n</i> -Butanol	32.000±0.370	40.000±0.770	52.000±0.310
	Methanol	44.000±0.740	34.000±0.440	44.000±0.540
<i>P. elliotii</i>	Hexane	>100	>100	>100
	Dichloromethane	3.500±0.180	40.000±0.550	50.000±0.120
	Ethyl acetate	70.000±0.260	60.000±0.460	80.000±0.860
	<i>n</i> -Butanol	13.000±0.476	15.000±0.370	55.000±0.760
	Methanol	12.000±0.100	92.000±0.800	48.000±0.500
	Doxorubicin	0.020±0.001	0.220±0.053	0.700±0.190

Data are presented as mean±SEM. GI₅₀: Concentration of the extract or doxorubicin causing 50% growth inhibition of the cells.

Table 4Antioxidant activity of fractions of *Ekebergia senegalensis* and *Protea Elliotii*

Plant	Sample name	DPPH radical scavenging ability	
		Scavenging ability (%)	IC ₅₀ (µg/mL)
<i>E. senegalensis</i>	Hexane	NA	NA
	Dichloromethane	61.81±0.70	366.60±2.90
	Ethyl acetate	95.45±1.01	14.00±0.20**
	<i>n</i> -Butanol	94.63±1.58	19.12±0.50**
	Methanol	93.41±0.90	20.16±1.30*
<i>P. elliotii</i>	Hexane	91.92±0.80	122.00±4.30
	Dichloromethane	92.74±0.70	104.30±1.80
	Ethyl acetate	94.64±2.02	24.10±0.18
	<i>n</i> -Butanol	94.71±0.30	16.50±0.70**
	Methanol	96.05±0.50	15.30±0.20**
	Gallic acid	96.67±0.80	22.67±0.40

Data are presented as mean±SEM. ***P*<0.01. IC₅₀: Concentration of the extract causing 50% of DPPH radical scavenging ability; NA: Not active. Standard is gallic acid.

3.4. Effect of the most active fraction of *P. elliotii* on cell cycle

The effect of dichloromethane fraction of *P. elliotii* on cell cycle progression was determined by flow cytometry. NCI–H460 cells (Figure 1), treated with varying concentration (25 µg/mL, 3.5 µg/mL, and 1 µg/mL which were respectively GI_{75} , GI_{50} and GI_{25}) of dichloromethane fraction of *P. elliotii*, showed S arrest by increasing the population of S phase from 14.76% to 26% at GI_{50} value compared to that of the untreated control (Figure 1). The treatment of the same cell with doxorubicin (0.02 µg/mL) that was positive control, showed G2–M arrest by increasing the population of G2–M phase from 13.56% to 36.5% compared to that of the untreated control.

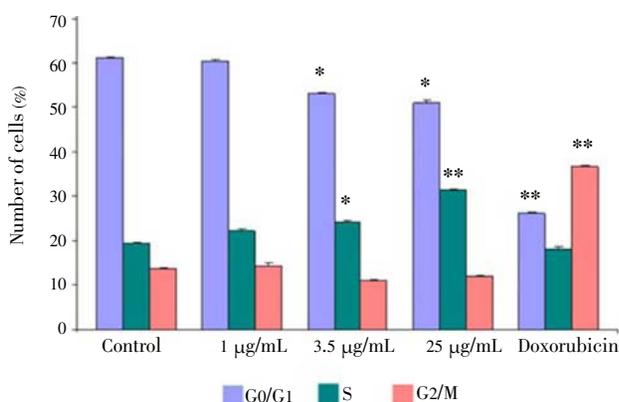


Figure 1. Effect of dichloromethane fraction of crude methanolic extract of *P. elliotii* on the cell cycle of NCI–H460 according to various treatment doses. 25 µg/mL, 3.5 µg/mL, and 1 µg/mL are GI_{75} , GI_{50} and GI_{25} respectively. * $P < 0.05$, ** $P < 0.01$.

4. Discussion

Large sections of the rural population in Cameroon still rely on medicinal plants and herbal medicines as primary health care, however only a few studies have been reported for much variety of medicinal plants of the Northern Cameroon. In an attempt to contribute towards the latter, we examined some Northern Cameroonian medicinal plants for their antiproliferative activity against four malignant cell lines.

The highest inhibitory effect on cell growth was observed from *C. spectabilis* which was active against all cancer cell lines compared to all other extracts. Greatest sensitivity was observed for 3T3, Hela and NCI–H460 cell lines, but the fact that the noncancerous cell line 3T3 was susceptible to this extract showed that it has no selectivity between cancer and non–cancer cells, denoting an increased toxicity of the plant.

The crude methanol extracts from *E. senegalensis*, *P. elliotii*, *T. macroptera* and *V. paradoxa* exhibited a high or moderate inhibitory effect on cell growth, but was very effective against the NCI–H460 cell lines in which GI_{50} values was (20.00±0.04), (24.44±0.90), (31.0±1.5) and (27.0±0.9) µg/mL, respectively. The lowest area of antioxidant (DPPH test) values, corresponding to the most antioxidant substances were found for the extracts from *C. populnea*, *E. senegalensis*, *P. elliotii*, *T. macroptera*, *L. kerstingii* and *V. paradoxa*, while the highest area values (least activity) were those of *S. siamea*, *E. speciosa* and *G. aqualla*. The observed activity of the methanol extract may be due to the secondary metabolites components of the plants, since earlier studies describe the presence of saponins, steroids, alkaloids and flavonoids in *V. paradoxa*^[17], saponins, alkaloids and tannins in *C. populnea*^[18], saponins, alkaloids, tannins and flavonoids in *E. senegalensis*^[19]. Indeed, many of plant secondary metabolites such as phenolic components are potential antioxidants and free radical terminators^[20]. These compounds are the main agents that can donate hydrogen to free radicals and thus inhibit their harmful effect on living system. By their phenolic hydroxyl groups, flavonoids, which are the most important natural phenolic group, possess a broad spectrum of chemical and biological activities as well as radical scavenging properties^[21]. They can scavenge radicals such as singlet hydroxyl, superoxide and oxygen radicals.

Based on antioxidant and anticancer activities of these extract, *E. senegalensis* and *P. elliotii* were selected and partition in hexane, dichloromethane, ethyl acetate, butanol and methanol was done. Each fraction was submitted for antioxidant and anticancer activities using the model of scavenging of the stable DPPH radicals which is a widely method to evaluate the antioxidant activity of the investigated sample in relatively short time. Although hexane fraction of *E. senegalensis* and dichloromethane fraction of *P. elliotii* were the most potent on MCF–7 and NCI–H460 cell lines respectively, no significant difference was observed between crude extract and fraction for antioxidant activity.

The observation of cancerous cell–death is a bioassay model that indicates the potential of the fraction to inhibit the progression of cancer. The progression of cell proliferation is halted by the arrest of the cell division cycle at one of the checkpoints (G0/G1, S or G2/M phases) in the cell division cycle. The arrest is mainly triggered by the irreparable or repairable damage in the cell's DNA. In case of an irreparable DNA damage, the cell death pathways are triggered. The cell death could be either apoptotic or necrotic^[22]. Thus in this study, the S arrest ($P < 0.01$) shown by dichloromethane fraction of *P. elliotii* on NCI–H460

is probably one of the mechanisms used by extracts or fractions in the inhibition of cancer cells proliferation.

Overall, the results of the present study provided evidence for the antioxidant and anticancer activities of some plant extracts studied in the traditional medicine of Adamawa Region of Cameroon, and brought supportive data for future investigations that will lead to their use in cancer and oxidative stress therapy. A bio-guided fractionation will be performed on fraction with the highest antiproliferative activity, in order to elucidate the active principle.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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