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Bio-guided fractionation of methanol extract of *Ziziphus mauritiana* Lam. (bark) and effect of the most active fraction on cancer cell lines

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PEER REVIEW

Peer reviewer

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Comments

This is an original work on the antioxidant and antiproliferative properties of extracts of *Z. mauritiana* barks evaluated in 4 different cancer cell lines. The experiments are well designed and the data are accurately described and discussed. The article is well-written in general.

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ABSTRACT

Objective: To investigate the anticancer and antioxidant potential of methanol bark extract of *Ziziphus mauritiana* (*Z. mauritiana*), which is used by traditional healers to cure some cases of cancer in Cameroon.

Methods: The methanol crude extract of *Z. mauritiana* has the antiproliferative activity on four cancer cell lines and its antioxidant activity. The extract was partitioned in five different solvents, and each fraction was tested. The effect of the most antiproliferative fraction on cell cycle was determined. Bio-guided fractionation was performed on the fraction with the highest antiproliferative and the highest antioxidant activities.

Results: *Z. mauritiana* methanol extract was active on all tested cells, and showed promising antioxidant activity. All fractions except hexane fraction were active with the dichloromethane fraction being the most active and showed S and G2-M phase arrest (P<0.01) on cell cycle progression of NCI-H460 and MCF-7, respectively. Bio-guided fractionation of the dichloromethane fraction led to lupeol and betulinic acid. The greatest antioxidant activity was recorded with ethyl acetate fraction and its fractionation led to catechin and epigallocatechin.

Conclusions: Overall, this study showed that *Z. mauritiana* barks has benefits as a chemoprevention agent cancer.

KEYWORDS

Ziziphus mauritiana, Anticancer, Antioxidant, Betulinic acid, Catechin.

1. Introduction

The incidence and mortality rates due to cancer are increasing compared to other disease even in developing countries where the rate of cancer is ranked as high. Although chemotherapy is one of the techniques used for cancer control and remains the treatment of choice, it offers limited results and often presents adverse effects. Thus, the development of new strategies in cancer control is vital. Plant extracts are showing interesting results such as vinblastine and vincristine isolated from *Catharanthus roseus* (a small plant)

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used in the treatment of various types of cancer[1].

Ziziphus mauritiana (*Z. mauritiana*) (Rhamnaceae) is a plant widely distributed in warm-temperate and sub-tropical regions throughout the world. In Nigeria, its fruits are traditionally applied on cuts and ulcers; used as anodyne, sedative, tonic anticancer, potent wound healer and against asthma. In India, the leaves are used in the treatment of asthma, fever[2], dysentery, liver ailments, pulmonary ailments and healing of fresh wounds[3]. The roots are used to prevent skin diseases, whereas its seeds are sedative and effective against nausea, vomiting and abdominal pains associated with pregnancy[4]. Its aqueous ethanol seed extract was found to markedly inhibit the proliferation of HL-60 cells, and induced apoptosis in a dose-dependent manner[5]. Extracts from the fruits and leaves exhibited antioxidant activity, while pulp was cytotoxic against different cancer cell lines[5]. In Northern Cameroon traditional healers use it for the treatment of incurable wounds, diabetes, lung, breast and prostate cancer (unpublished data). Although little information on its ethno-pharmacological is available in Cameroon, there is no scientific basis of its use in the treatment of cancer. However large sections of the population in northern Cameroon continue to rely on medicinal plants and herbal medicines as primary health care. It would be interesting to scientifically investigate the therapeutic properties of *Z. mauritiana*. This study was carried out to evaluate through bio-guided fractionation assay, the antiproliferative effect of methanol crude extracts of *Z. mauritiana* barks on cancer cell lines including MCF-7, PC3, HeLa, and NCI-H460 and its antioxidant properties.

2. Materials and methods

2.1. Extract preparation

The fresh barks of *Z. mauritiana* identified by the National Herbarium as 8868/SER/Cam were collected during May 2013 in the Adamawa region of Cameroon and dried at room temperature. Ground and powdered dried barks (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.

2.2. Antiproliferative effect of Z. mauritiana

The antiproliferative activity of the methanol crude extract and fractions of *Z. mauritiana* 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 sulforhodamine-B (SRB) assay[6]. The cell lines were routinely maintained as monolayer cell cultures in Roswell Park Memorial Institute medium containing fetal bovine serum (10%), and 1% of glutamine, penicillin and streptomycin solution.

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-7and 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 CO_2 (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 extract and fractions. GI_{50} which was the concentration of the extract or Doxorubicin causing 50% growth inhibition of cells was determined.

2.3. Antioxidant activities

2.3.1. DPPH free radical-scavenging assay

The antiradical activities of the plant extracts and gallic acid (as a reference) were determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), which is a stable free radical[7]. The solubilized test substances in dimethylsulfoxide (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 µL of DPPH (300 µmol/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:

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

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 Hossain *et al*[8]. Briefly, the reaction mixture (150 µL) contained 12 µL of test sample (1.0 mmol/L in DMSO), 38 µL of potassium phosphate buffer (10 mmol/L, pH 7.4) and 100 µL of sodium nitroprusside (10 mmol/L). Following incubation at 25 °C for 150 min, 50 µL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) was added and allowed to stand for 5 min. To all the tubes, 50 µL of N-(1-naphthyl) ethylenediamine dihydrochloride (0.1% w/v) was added, stirred, and allowed to stand for 1-2 min. The absorbance of pink-colored 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:

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

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 *Z. mauritiana* 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, 3.5 and 25 µg/mL) or doxorubicin (3.5 µg/mL). After 48 h, cells were harvested using trypsin and re-suspended in phosphate buffered saline. 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 buffered saline, 10 µL of ribonuclease-A (100 units/mL) was introduced and incubated for 30 min at 37 °C in water bath. Propidium iodide (150 µL, 20 µg/mL) was added and the mixture was incubated for 30 min at room temperature. The stained cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson, USA) and DNA content was quantified by using FlowJo software.

2.5. Fractionation and bio-guided isolation of methanol extract

Methanol extracts of *Z. mauritiana* dried barks (150 g) were suspended in distilled water (500 mL) and separated into fractions using separator funnel and solvents in increasing order of polarity *i.e.* hexane (9 L), dichloromethane (9 L), ethyl acetate (9 L), *n*-butanol (4.5 L) and methanol (2 L).

Dichloromethane and ethyl acetate fractions were subjected to further purification since they demonstrated highest antiproliferative and antioxidant activities. The thin layer chromatography (TLC) profile of these two fractions gave different profiles. The dichloromethane fraction (10.74 g) was subjected to bioassay-guided fractionation by column chromatography with hexane/acetone in different percentage. Aliquots of 50 mL were collected and monitored by thin layer chromatography. Aliquots showing the same components were combined and were subjected to another colum chromatography, using the same solvent system in different percentage. Two compounds were isolated into compound 1 from fractions 1–6 (30 mg) and compound 2 from fractions 7–40 (170 mg).

The ethyl acetate fraction (12.1 g) was subjected to bioassay-guided fractionation by column chromatography with dichloromethane/methanol system following the same procedure as described with the dichloromethane fraction, and two major sub-fractions containing slight impurity: fractions 7–16 (230 mg) and fractions 17–30 (385 mg) were obtained. HPLC technique was used to enhance the purification and obtain compound 3 and 4.

2.6. Statistical analysis

The data from biological assays were subjected to the One-way ANOVA procedures which were presented as mean±SEM (standard error of the mean). The Dunnett test was used to compare means. $P<0.01$ was considered significant.

3. Results

3.1. Antiproliferative activity of methanol extract of *Z. mauritiana*

Table 1 shows the effect of methanol crude extract and fractions of *Z. mauritiana* against four malignant cell lines: NCI-H460 (lung cancer), MCF7 (breast cancer), PC3 (prostate cancer), HeLa (uterus cancer) and normal cell 3T3 (mousse cervical cells) using a SRB assay. After 48 h of treatment, the plant extracts exhibited inhibitory effects against tumor cell growth at varying efficacies and selectivity. The lowest GI_{50} values (corresponding to the most antiproliferative substances) were obtained with NCI H460 and MCF-7 cell lines whereas PC3 and HeLa showed moderate activity (Table 1).

Table 1

Antiproliferative effects of crude methanol extract and fractions of *Z. mauritiana* and doxorubicin on NCI H460, MCF-7, PC3, Hela and 3T3 cells.

Sample	Antiproliferative activity (GI_{50}, µg/mL)				
	NCI H460	MCF-7	3T3	Hela	PC3
Doxorubicin	0.020±0.001	0.22±0.05	0.70±0.19	0.62±0.15	0.29±0.12
Methanol extract	16.000±0.590	29.00±0.90	42.53±0.48	37.10±0.47	39.87±0.19
Hexane fraction	18.000±0.940	96.00±0.01	66.00±0.93	NT	NT
Dichloromethane fraction	3.800±0.508	5.00±0.88	54.00±0.99	NT	NT
Ethyl acetate fraction	20.000±1.110	16.00±2.17	46.00±1.44	NT	NT
n-Butanol fraction	18.000±0.056	21.00±0.60	36.00±0.36	NT	NT
Methanol fraction	16.000±0.482	27.00±0.72	52.00±0.25	NT	NT

Data are presented as mean±SEM. NT: Not tested.

All fractions, except hexane, were active against MCF-7 and NCI-H460 cells. Furthermore, the growth of 3T3 was inhibited moderately compared to cancer cell lines. Of all these tested fractions, dichloromethane fraction showed the greatest antiproliferative effect on MCF-7 and NCI-H460 cells (Table 1). Although this activity was observed, the inhibitory effect of this methanol crude extract and fractions of *Z. mauritiana* was lower compared to that shown by doxorubicin (Table 1).

3.2. Antioxidant activity of crude methanol extract and fractions of *Z. mauritiana* methanol extract

Table 2 shows the antioxidant effect of methanol crude extract and fractions of *Z. mauritiana* evaluated using two different tests (DPPH and nitric oxide radical scavenging ability).

The obtained results revealed that, for the DPPH method, the lowest

area values corresponding to the most antioxidant substances were found for the methanol crude extract, ethyl acetate, *n*-butanol and methanol fractions. The hexane and dichloromethane fractions were found to be inactive. However, although the methanol extract and fractions showed more antioxidant activity than that of the positive control (gallic acid), the highest value was observed with ethyl acetate fraction (IC_{50}: 14.600±0.294 μg/mL).

Nitric oxide radical scavenging ability test of the crude methanol extract, dichloromethane, ethyl acetate, *n*-butanol and methanol fractions of *Z. mauritiana* showed weaker activities as compared to ascorbic acid (the positive control) while the hexane fraction was found to be inactive.

Table 2

Antioxidant effects of crude methanol extract and fractions of *Z. mauritiana*.

Sample	Antioxidant (IC_{50}, μg/mL)	
	DPPH radical scavenging ability	Nitric oxide radical scavenging ability
Methanol extract	19.05±0.50**	361.0±3.0
Hexane fraction	NA	NA
Dichlorométhane fraction	274.70±3.75	235.0±3.0
Ethyl acetate fraction	14.60±0.29**	264.0±0.3
n-Butanol fraction	16.70±0.40**	299.0±3.0
Methanol fraction	17.00±0.80**	306.0±2.5
Gallic acid	22.67±0.40	108.0±2.0

NA: Not active. The standards are gallic acid for DPPH radical scavenging ability and ascorbic acid for nitric oxide radical scavenging ability. Data are presented as mean±SEM. **: P<0.01.

3.3. Effect of dichloromethane fraction on cell cycle

The effect of dichloromethane fraction of *Z. mauritiana* on cell cycle progression was determined by flow cytometry method. NCI H460 (Figure 1) cells treated with various concentrations (Figure 1) of dichloromethane fraction of *Z. mauritiana*, showed S-phase arrest by increasing the population of S by 14% at GI_{50} value compared to that of the untreated control (Figure 1). The treatment of the same cell with the positive control compound (doxorubicin) showed arrested G2-M by increasing the population of G2-M by 23% compared to that of the untreated control.

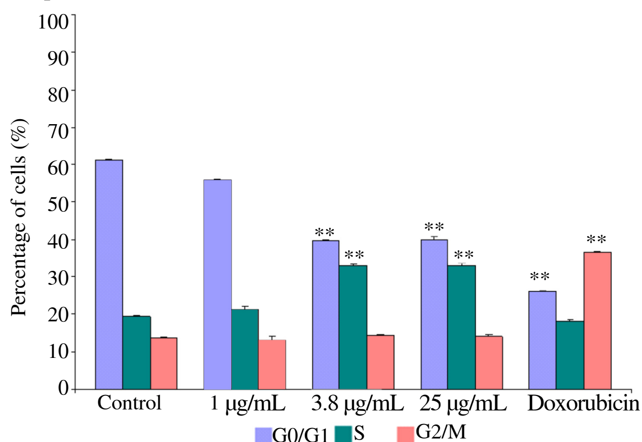

Figure 1. Effect of dichloromethane fraction of crude methanolic extract of *Z. mauritiana* on the cell cycle of NCI-H460 in various treatment doses.

The concentrations of 25, 3.8 and 1 μg/mL are GI_{75}, GI_{50} and GI_{25}, respectively. **: P<0.01.

MCF-7 cells treated with various concentrations of dichloromethane fraction of *Z. mauritiana* showed G2-M arrest by increasing the population of G2-M phase by 37% at GI_{50}, while doxorubicin also showed G2-M arrest by increasing the population of G2-M phase by 36% compared to that of the untreated control (Figure 2).

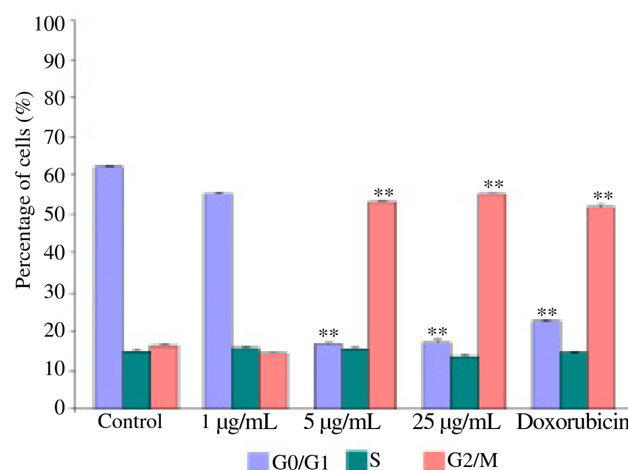

Figure 2. Effect of dichloromethane fraction of crude methanolic extract of *Z. mauritiana* on the cell cycle of MCF-7 according to various treatment doses.

The concentrations of 25, 5, and 1 μg/mL are GI_{75}, GI_{50} and GI_{25}, respectively. **: P<0.01.

3.4. Identification of the isolated compounds

The isolated compounds from *Z. mauritiana* were identified by the comparison of their spectral data with published values and were confirmed by comparing with authentic samples. Two compounds, isolated from dichloromethane fraction were identified as lupeol (compound 1), betulinic acid (compound 2), while two others, isolated from ethyl acetate fraction were identified as catechin (compound 3) and epigallocatechin (compound 4).

4. Discussion

The aim of this work was to investigate the anticancer potential of *Z. mauritiana* (bark), used by traditional healers in northern Cameroon for the treatment of some cancers. The screening of methanol crude extracts of *Z. mauritiana* bark was done for its antiproliferative activity against four malignant cell lines: NCI-H460, MCF7, PC3, HeLa and one normal cell 3T3 using an SRB assay. After 48 h of treatment with the methanol extract, it appeared that its antiproliferative activity was more effective against NCI H460 followed by MCF-7 breast cancer as oppose to PC3 and HeLa. The methanol crude extract was partitioned in hexane, dichloromethane, ethyl acetate, butanol and methanol and each fraction was submitted to the anticancer test. All the fractions (except hexane fraction which is generally rich in non-polar compounds) showed antiproliferative effects against NCI H460 and MCF-7, and greatest sensitivity was observed with dichloromethane fraction (Table 1).

Z. mauritiana has been shown to be rich in biologically active compounds such as triterpenes, cyclopeptide alkaloids and

flavonoids and exhibits inhibitory effects on histamine release, cyclooxigenase-1&2, activation of choline acetyl transferase activity[9], cytotoxic activity[5], and immunological adjuvant activity[9]. This immunological action of some constituent of *Z. mauritiana* can indirectly help in cancer cell proliferation through the activation of tumor associated macrophages and induction of apoptosis in tumor cells[10]. The antiproliferative effect of the crude methanol extract and fractions of *Z. mauritiana* on the above selected cancer cell lines, may be explained by a synergic effect of some of its constituents.

The observation of cancerous cell-death is a bioassay model that indicates the potential of a tested extract or compound 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 phases (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 either be apoptotic or necrotic[11]. Thus in our study, the S arrest of NCI H460 (Figure 1) and G2-M arrest of MCF-7 (Figure 2) could probably be one of the mechanisms used by the extract or fractions in inhibiting proliferation of cancer cells.

It was reported that plant-derived extracts showing cytotoxicity toward tumor cells could contain antioxidant activity[12]. In this study, DPPH radical scavenging activity of the tested extract and fractions increase compared to that of gallic acid (a phenolic compound which acts as an antioxidant and helps to protect our cells against oxidative damage). Compounds with antioxidants activity may function as free radical scavengers, form complexes with pro-oxidants metals, reducing agents, and quenchers of single-oxygen formation or reactive oxygen species, thereby protecting the body from degenerative diseases such as cancer. The reactive oxygen species are harmful by-products generated either during normal cellular metabolism or from toxic insult. They lead to a state of oxidative stress that contributes to the pathogenesis of some human diseases by damaging lipids, proteins and DNA[13]. The exhibition of high DPPH radical scavenging activity found in crude methanol extract and its fractions could be attributed to the phenolic compounds present in this plant.

In order to elucidate the active principle responsible for the antioxidant and antiproliferative activities of *Z. mauritiana* methanol extract, its dichloromethane and ethyl acetate fractions were subjected to an isolation procedure. One of the triterpenoid isolated from the dichloromethane fraction was found to be lupeol (compound 1) and the other was betulinic acid (compound 2). The ethyl acetate fractionation led to the isolation of catechin (compound 3) and epigallocatechin (compound 4). All of these isolated compounds have been found to have enormous biological activities.

The antitumor and cytotoxicity activities of betulinic acid have been extensively studied on many cancer cell lines, primary tumor samples and xenograft mouse models. While initial reports suggested that betulinic acid was selectively cytotoxic against melanoma cell lines[14], its anticancer activity recorded against other types of human cancers including neuroblastoma, glioblastoma, medulloblastoma, Ewing sarcoma, leukemia as well as several carcinoma, *i.e.* head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovary or cervix[15]. The molecular mechanism of betulinic acid-mediated antitumor activity is based on its ability to trigger the mitochondrial pathway of apoptosis in cancer cells. The apoptosis pathway is triggered by activation of the mitochondrial pathway, induction of mitochondrial outer membrane permeabilization or by modulation of NF-κB activity[16].

Many researchers have revealed various important pharmacological activities of lupeol *in vitro* and *in vivo*, including anti-inflammatory, anti-microbial, anti-protozoal, anti-tumor and anti-angiogenic[17]. Lupeol has been reported to inhibit growth and induce apoptosis in pancreatic cancers, while G2/M cell cycle arrested cancer cells by inhibiting the cyclin regulated signaling pathway[18].

Catechin and epigallocatechin are two natural antioxidant compounds belonging to the flavonoid group of plant polyphenols. Catechin can scavenge superoxide and hydroxyl radicals, as well as the DPPH radical[19], peroxyl radicals[20], nitric oxide and peroxynitrite by preventing the nitration of tyrosine[21]. Catechins chelate metal ions such as copper (II) and iron (III) to form inactive complexes and prevent the generation of potentially damaging free radicals[22]. Another mechanism by which the catechins exert their antioxidant effects is through the ultra-rapid electron transfer from catechins to reactive oxygen species-induced radical sites on DNA[23].

Overall, this study showed that extracts of *Z. mauritiana* may be used as a chemoprevention agent because of its antioxidant effect or in the treatment of cancer due to its antiproliferative effect on cancer cell lines (NCI-H460 and MCF-7). The use of animal models would be beneficial for improving the understanding of the pharmacological actions of this plant and its active compounds.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Comments

Background

This is an interesting investigation concerning the potential anticancer properties of methanol extracts of *Z. mauritiana* barks, used in traditional medicine in Cameroon. The development of new

strategies in cancer control is vital. Plant extracts, especially from African countries, display promising anticancer properties.

Research frontiers

Through a rational scientific paradigm the data of this work allows the proposal that extract of *Z. mauritiana* may be used as a chemoprevention agent because of its antioxidant effect or in the treatment of cancer due to its antiproliferative effect on cancer cell lines.

Related reports

The material and methods utilized in these studies are accurately chosen and the experiments are well-conducted and interpreted. They are in conformity with the experimental approaches found in the literature in comparable studies.

Innovations & breakthroughs

This study brings novel scientific basis on a plant used in traditional medicine in Cameroon. This study was carried out to evaluate the antiproliferative and antioxidant properties through bio-guided fractionation assay of methanol crude extracts of *Z. mauritiana* barks on 4 cancer cell lines including MCF-7, PC3, HeLa, and NCI-H460.

Applications

Novel potential anticancer strategies can apply to prevent or treat cancer.

Peer review

This is an original work on the antioxidant and antiproliferative properties of extracts of *Z. mauritiana* barks evaluated in 4 different cancer cell lines. The experiments are well designed and the data are accurately described and discussed. The article is well-written in general.

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