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Research article

Duguetia confinis Engl. & Diels (Annonaceae) inhibitory and cytotoxic effects on breast adenocarcinoma growth both *in vitro* and *in vivo*

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ABSTRACT

Background: Breast cancer is ranked as the most common malignant tumor in women globally with ~ 2.3 million new cases (11.7 %) diagnosed in 2020. The multiple drawbacks associated with treatments, prompt researchers and patients to search for alternative therapy. Plants continue to offer encouraging leads, in particular those of the Annonaceae family, to which belongs *Duguetia confinis,* used by Cameroonian traditional healers to fight cancers. This study was aimed at investigating the effect of *Duguetia confinis* against human breast cancer cells. This was carried out by investigating the cytotoxicity, underlying mechanism of action and chemopreventive potential of *D. confinis* on 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer.

Methods: To achieve this goal, the ethanolic extract of the bark of *D. confinis* was prepared and assayed for its ability to inhibit cell growth, cell proliferation and clone formation. Furthermore, cell death mechanisms, cell cycle progression and anti-metastatic potential were investigated. The *in vivo* study consisted in a once-off administration of 50 mg/kg BW DMBA (in olive oil, *s.c*) from the 10th day after pretreatment with *D. confinis* extract (50 and 100 mg/kg BW) or standards [tamoxifen (3.3 mg/kg) and letrozole (1 mg/kg)] or leaf extract of *Annona muricata* L. (200 mg/kg as pharmacological control). Normal and negative controls received vehicle (3 % ethanol). The treatment of animals was done for 20 weeks, followed by the assessment of the incidence, burden and volume of tumors, breast cancer biomarker (CA 15-3), antioxidant status, inflammatory status and histopathology profile. The LD_{50} of *D. confinis* extract was estimated according to OECD guideline 423.

Results: D. confinis displayed cytotoxicity at 80 μg/mL on all the tested breast cancer cell lines. It induced apoptosis and caused a blockade at G0/G1; S-phase of MDA-MB 231 cells, thus, suggesting anticancer potential. A significant concentration-dependent antimetastatic potential was

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observed with *D. confinis* extract at 50 ($p < 0.05$) and 100 ($p < 0.01$) μg/mL, evidenced by a reduction in cell migration, chemotaxis and increased adhesion to extracellular matrix. With respect to the chemopreventive study, *D. confinis* was able to prevent the onset of breast adenocarcinoma in Wistar rats by preventing the growth of tumor mass and volume, as well as the histopathological severity of the disease. This was achieved through the modulation of antioxidant parameters (SOD, CAT, MDA) and inflammatory parameters (IL-12, IL-6, INF- gamma, TNF). Also, the LD_{50} of *D. confinis* extract was greater than 2000 mg/kg, indicating low acute toxicity and thus, favorable for therapeutic use.

Conclusion: In summary, this study outlines for the first time the beneficial effect of *D. confinis* as a plant candidate in the fight against breast cancer just like other species of the Annonaceae family. However, further research studies are still warranted regarding its bioactive components, and in depth investigation of its anticancer mechanism of action are also needed.

1. Introduction

Cell cycle and programmed cell death (apoptosis) are vital aspects of cell biology, which in normal physiological conditions ensure growth and homeostasis of the organism. Alterations like reduced apoptosis or excessive proliferation may result in pathological conditions such as tumorigenesis [1]. Adding to this, unrepaired mutations resulting from the activation of proto-oncogenes into oncogenes, the inactivation of tumor suppressor genes and the inactivation of genes ensuring genome stability may give rise to clone cells capable of evolving at any time into the selection of a second population, more genetically redesigned than the first, thus leading to cancer [2]. Breast cancer, which is due to uncontrolled changes in the growth or function of breast tissue cells, is the most common malignant tumor in women worldwide and accounts for 36 % of oncological patients [3]. At present, it represents the most diagnosed cancer after lung cancer with an estimated burden of 2.3 million new cases (11.7 %) in 2020 [4]. It develops in three steps: initiation, promotion and progression; the later stage being characterized by the ability of tumors to leave the primary site and to expand to other parts of the body through its migratory and invasive abilities.

There are several factors that increase the risk of breast cancer among which are: age, hormonal factors (mainly related to the time of exposure to estrogens), procreative factors, (including the number of children born, the age of the first child birth, breastfeeding), the use of hormone replacement therapy, genetic factors, environmental factors, improper lifestyle and diet [3]. As far as the latter is concerned, the use of synthetic preservatives and chemical additives in foodstuffs has been linked to the ever-increasing incidence of new cases of cancer observed in recent years [5]. Although, about half of the cases worldwide arise in industrialized countries, as a result of the so-called Western lifestyle, the fate of this disease is more pronounced in developing countries due to late diagnosis, lack of resources to ensure adequate management of cancer patients and poverty [6]. In a developing country like Cameroon, most breast cancers are diagnosed at an advanced stage and as such, survival rate is low. In fact, according to studies by Ngowa et al. [7] and Zingue et al. [8], breast cancer screening in Cameroon mostly occurs at stages III and IV of the disease, and hence, treatment outcomes are poor. The therapeutic approaches to breast cancer includes: the use of selective estrogen receptor modulators such as tamoxifen, aromatase inhibitors such as letrozole, CAR-T "Chimeric Antigen Receptor T-cell" therapy, cytotoxic antibiotics, and surgery combined with radiation to minimize recurrence [9]. However, these therapies are inadequate due to the high treatment expenses, unpleasant side effects, recurrence, systemic toxicity and resistance of cancer cells to treatment [2]. Thus, the persistent search for effective and less harmful natural agents to improve breast cancer treatment is very essential [10].

Alternative and complementary medicine especially the use of herbal medicine for cancer patients is gaining grounds in recent years and 60 % of anti-cancer drugs used in the USA were isolated or derived from a natural source [11]. The anticancer effects of medicinal plants belonging to the Moraceae family have been demonstrated in some studies: *Ficus umbellata* [12], Mimosaceae family: *Acacia seyal* [13] and species of the Annonaceae family: *Annona muricata* which has distinguished itself in the fight against cancer [14,15]. Acetogenins and Alkaloids from *A. muricata* as well as the crude leave and fruit extracts have shown potent anticancer effects on numerous cell lines and in DMBA-induced breast cancer in female rats [15]. In this line, the interest of this study was on *Duguetia confinis*, a member of the Annonaceae family (subfamily of Annonoideae). The plant was evaluated for its *in vitro* and *in vivo* effects on breast cancer and its regulatory processes namely cell growth, proliferation, cell cycle progression, apoptosis, metastasis, inflammation, oxidative and histopathological grade together with its safety profile.

Duguetia confinis, also known as *Pachypodanthium confine* or "wild soursop", belongs to the genus Duguetia, one of the largest genera of Annonaceae, comprising around 100 species. Many of these species produce edible fruit and are widely used as anti-inflammatory, analgesic and anticancer agents [16]. In addition, Cameroonian healers from the rainforest of Cameroon use *Duguetia confinis* as well as *Annonidium mannii* and *Annona muricata* L to treat cancer. Although there is an abundance of literature on the ethnopharmacological uses of Duguetia species, only a handful of studies on their chemical and therapeutic properties have been published, making plants of this genus of interest to scientists. Like other Annonacece, its chemical composition shows that it has abundant alkaloids. In this respect, the alkaloids of *Duguetia pycnastera* Sandwith (Annonaceae) have shown cytotoxic activity against various cancerous and non-cancerous cell lines, with IC_{50} values around 25 μM [17]. Matos et al. [18] reported that (+)-allo-aromadendran-10,14β-diol, an aromadendrene-type sesquiterpene derived from the essential oil of *Duguetia glabriuscula* leaves was cytotoxic on Hep2 cells, with an IC_{50} value of 11.6 ± 2.3 μg/mL. Rodrigues et al. [19] demonstrated the antitumor properties of the essential oil of *Duguetia gardneriana* leaf extract *in vitro* on cancer cell lines B16-F10 (16.89 μg/mL), HepG2 (19.16 μg/mL), HL-60 (13.08 μg/mL) and K562 (19.33 μg/mL) and *in vivo* on B16-F10 carrier mice.

2. Methods

2.1. Chemicals and reagents

The above chemicals and reagents were purchased as follows.

Item	Use	Supplier (town, country)
Fetal bovine serum (FBS), streptomycin, 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethane sulfonic acid (HEPES), glutamax, and penicillin	Cell culture	Gibco/Invitrogen (Karlsruhe, Germany).
3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay kit	Cell growth/Viability	Roche Diagnostics (Penzberg, Germany)
BrdU cell proliferation enzyme-linked immunosorbent assay (ELISA) kit	Cell proliferation	Calbiochem/Merck Biosciences (Darmstadt, Germany)
Annexin V-FITC Apoptosis Detection	Cell death mechanism	BD Pharmingen (Heidelberg, Germany)
Cycle TEST PLUS DNA Reagent kits	Cell cycle	
Tamoxifen citrate (Mylan®) and Letrozole	Control positive in breast cancer rat model	Norvatis Access Laboratory (Barleben, Germany)
DMBA	Carcinogen in rat model	Sigma-Aldrich (Stanford, Germany)
Diazepam (Valium®)	Anaesthetic	Roche Pharma (Fontenay-sous-bois, France)
Ketamine	Anaesthetic	Rotex Medica (Tritau, Germany)
Amoxicillin 500 mg	Antiseptic	Sandoz Laboratories (Levallois-Perret, France)
Penicillin (xtapen®)	Antiseptic	CSPC Zhongnuopharmaceutical® (Shijiazhuang, China)
The MILIPLEX® cytokine assay kit for the luminex Xmap® technology	Determination of cytokine levels	Milipore (Minneapolis, USA)
The alanine transaminase (ALT) and creatinine reagent kits	Biomakers of liver and kidneys function	Fortress Diagnostics Limited® (Muckamore, UK)

2.2. Plant material

2.2.1. Collection and identification

Two different Annonaceaes: *Annona muricata* L. and *Duguetia confinis* were studied. The leaves of *A. muricata* L. were collected in August 2020 around 12 p.m. in Ngoa-Ekele (Center Region, Cameroon) and authenticated at the National Herbarium of Cameroon in Yaoundé in comparison with a specimen reference No. 32879/78HNC. On the other hand, the barks from the trunk of *D. confinis* were harvested in October 2020 at 1 p.m. in Akonolinga (Centre Region, Cameroon) and authenticated at the National Herbarium of Cameroon in Yaoundé in comparison with a reference specimen N° 29365/HNC.

2.2.2. Plant extraction

The botanical samples (*Annona muricata* leaves and *Duguetia confinis* barks) were cut and dried in a lighted shade for one week. After grinding 1.5 kg of each sample, the powder was soaked in 6 L each of 95 % ethanol for 72 h. Subsequently, the preparations were filtered through Whatman paper N°4. The filtrates were concentrated in a rotary evaporator under reduced pressure of 175 mbar at 40 °C. Quantities of 31.05 g (2.07 %) and 31.95 g (2.13 %) of ethanolic crude extracts were obtained from *D. confinis* and *A. muricata* L, respectively.

2.3. Animals

The animals consisted of 64 healthy female Wistar rats (*Rattus norvegicus*), of 5 weeks old, weighing between 55 and 65 g. These rats were obtained from the Animal Physiology Laboratory of the University of Yaoundé I, raised at room temperature, under natural light cycle, in plastic cages with sufficient ventilation. Access to tap water and a standard soy-free rat chow was *ad libitum* and the chow was made of: corn (42 %), bone meal (3 %), wheat flour (22 %), fish meal (19 %), crushed palm kernel meal (4 %), sodium chloride (0.75 %), peanuts (9 %) and multivitamin complex (Olivitazol® 0.5 %).

Housing and animal treatments was in accordance with the Joint Institutional Review Board Animal and Human Bioethics of the Faculty of Science (University of Yaoundé 1) reference # BTC-JIRB2021-010, which is in line with the regulations of the European Union on the care of animals (EEC Council 86/609).

2.4. Cell culture

MCF-7, MDA-MB-231, and 4T1 cells were grown and subcultured in RPMI-1640 medium supplemented with 10 % FBS. Penicillin 100 U/mL, streptomycin 100 g/mL, and HEPES 10 mM were also added to all cell cultures before incubating at 37 °C in a CO_2 5 % humidified atmosphere with a pH of 7.4. During each day's cell passage, 90 % of the supernatant was replaced with fresh medium. Prior to all experiments, the number of viable cells was determined using trypan blue method, and the cell count was determined using

automatic cell counter.

2.5. Cytotoxicity assay

The cytotoxic potential of *D. confinis* extract was assessed using the MTT dye reduction assay. Breast cancer cells (MCF-7, MDA-MB-231 and 4T1) were seeded (100 μL, 1×10^4 cells/mL) into 96-well tissue culture plates. The extract was prepared prior to the test by dissolving in DMSO (0.01 %) and the concentrations used were within the range 12.5–200 μg/mL, while controls were left untreated. MTT (0.5 mg/mL) was then added for 4 h after which cells were lysed in a 0.01 M HCl buffer containing 10 % SDS. The plates were incubated overnight at 37 °C, 5 % CO_2 after which each well's absorbance was measured at 570 nm using a microplate ELISA reader.

2.6. BrdU cell proliferation assay

Cell proliferation was assessed using a BrdU ELISA kit. MDA-MB 231 cells at a density of 1×10^4 cells/mL in 100 μL RPMI 1640 were seeded into 96-well culture plates and incubated with *D. confinis* extract (50 and 100 μg/mL) for 24 and 48 h. Then, at 0 h, 24 h, and 48 h, 20 μL of BrdU-labeling solution was administered per well, fixed and detected with anti-BrdU mAb at 450 nm using a microplate ELISA reader according to the manufacturer's instructions.

2.7. Clonogenic assay

Both treated and untreated MDA-MB-231 cells were transferred into 6-well plates at 500 cells per well. *D. confinis* at 50 and 100 μg/mL were then added. The obtained colonies were fixed and counted after 7 days of incubation. One colony was defined as clones with at least 50 cells. The number of clones on test group was compared to the number of clones of control cancer cells, which was set to 100 %.

2.8. Flow cytometry for detection of apoptotic cells

The type of cell death (necrosis or apoptosis) induced by *D. confinis* was assessed using the classical Annexin V-FITC assay. Breast cancer cells (MCF-7, MDA-MB-231 and 4T1) were treated for 24 h with *D. confinis* extract at 50 and 100 μg/mL. Next, cells were washed twice with PBS and incubated in the dark for 15 min with 5 μL Annexin V-FITC and 5 μL Propidium Iodide (PI). FACScalibur was used to analyze cells by flow cytometry (BD Biosciences, Heidelberg, Germany). The percentage of apoptotic (early and late), necrotic and viable cells was expressed as a percentage (%).

2.9. Flow cytometry for cell cycle analysis

Subconfluent cell cultures were used for cell cycle analysis. *D. confinis* (50 and 100 μg/mL) were introduced after replacing the medium and incubated for 24 h. MDA-MB-231 cells were stained with PI using a Cycle TEST PLUS DNA Reagent Kit, and then flow cytometry was performed using a FACScan (BD, Heidelberg, Germany) through which 10,000 events were collected for each sample. CellQuest software was used to collect data, and cell cycle distribution was computed using ModFit software. The percentage (%) of gated cells in G0/G1, G2/M, or S-phase was recorded.

2.10. Wound healing assay

Assay was done in 12-well plates, into which MDA-MB-231 cells at density of 200,000 cells/2 mL were seeded and treated with *D. confinis* (50 and 100 μg/mL) or control solvent (DMSO) for 6 h. The medium was decanted, a scratch wound was made with a 100 μL pipette tip and washed with PBS two times to remove detached cells. The cells were then cultured in RPMI-1640 medium free to FBS for 48 h. The variation in wound recovery by migrating cells was observed using a fluorescent microscope (40×) Zeiss Axio Observer.Z1 (Hallbermoos, Germany). Every 12 h, microphotographs were taken and the area of wound healing was assessed using ImageJ ® software.

2.11. Cell adhesion assay

Components of extracellular matrix, collagen or fibronectin were coated into 6 well plates overnight. The plates were then treated for 1 h with *D. confinis* (50 and 100 μg/mL) or control solvent (DMSO). The plates were washed with 1 % BSA in PBS to prevent non-specific cell adhesions. Then, for 60 min, triple negative MDA-MB 231 cells were added at 500,000 cells/2 mL to each well. The non-adherent tumor cells were then washed. To calculate the average cell adhesion rate, adherent cells were fixed with 2 % glutaraldehyde and counted in five different fields using a 20× objective microscope.

2.12. Cell invasion assay

D. confinis was tested for its ability to inhibit serum-induced cell migration in 6-well transwell chambers with 8 μM pores from Greiner (Frickenhausen, Germany). For 24 h, MDA-MB-231 cells in serum-free medium were seeded into the upper chamber, and RPMI-1640 serum plus 10 % FBS was poured in the lower chamber. Following incubation, the upper surface of the transwell membrane

was gently swabbed with to remove non-migrating cells and fixed with 2 % glutaraldehyde. Cells that migrated to the membrane's lower surface were stained with hematoxylin and counted separately under a microscope (20× objective).

2.13. Chemopreventive effect of D. confinis extract on a rat model of induced-breast cancer

2.13.1. Experimental study design

Sixty-four (64) Wistar rats of 45 days were acclimatized for 10 days before being randomly distributed into 8 groups of 8 rats each (n = 8). Group I served as the normal control (NOR), and Group II served as the negative control (DMBA), both groups received 3 % ethanol (vehicle). Positive controls were groups III and IV, which received tamoxifene at 3.3 mg/kg BW and letrozole at 1 mg/kg BW; while group V received *Annona muricata* L at 200 mg/kg BW as a pharmacological control. The ethanolic extract of *D. confinis* barks was administered to groups VI and VII at doses of 50 and 100 mg/kg BW, respectively. The group VIII consisted of a satellite group formed by normal rats receiving the ethanolic extract of *D. confinis*, only. Treatments began 10 days before cancer induction and continued for 20 weeks. Except for normal rats, all groups received a single doseonce-off treatment of the carcinogen DMBA (50 mg/kg BW) dissolved in 1 mL of olive oil *s.c.* The body weights of the rats were measured weekly, and their chests were palpated twice a week to detect any potential breast tumors. Animals that died during the experiment were subjected to autopsy.

2.13.2. Sacrifice and organ collection

After a 12-h fast, all survivors were sacrificed under anesthesia by decapitation at the end of the 20-week treatment. Following that, blood was drawn via intra-cardiac puncture and transferred into ethylene diamine tetraacetic acid (EDTA) tubes for hematological analyses and dry tubes (this blood was further centrifuged at 3000 rpm for 15 min and stored at −20 °C) for biochemical assays. Tumors, mammary glands, liver, spleen, lungs, and kidneys were harvested, weighed, and immediately fixed in 4 % formol containing 5 % NaCl for histological analysis. Breast tumor volume was calculated using Faustino Rocha et al. [20] formula (length × weight × height/π/6). The relative organ weights were computed using the formula:

Relative organ weight (mg/kg) = [organ weight (mg)/rat body weight (kg)] 10^6.

The tumor incidence was calculated as follows: Tumor incidence (%) = (rats with tumors/total number of rats) × 100.

The tumor burden was calculated as follows: Tumor burden (g) = total relative tumor mass in a group.

The reduction in tumor burden was calculated as follows: % Inhibition of tumor burden = (tumor burden in DMBA − tumor burden in test group)/tumor burden in DMBA) × 100.

2.13.3. Assessment of the breast cancer biomarker: CA 15-3

The Cancer Antigen 15-3 (CA 15-3) ELISA kit from Cell Biolabs was used to assess the level CA 15-3 in serum, which is one of the most widely used prognostic biomarkers in clinical practice. The CA 15-3 assay detects shed or soluble forms of the Mucin-1 (MUC-1) protein, a transmembrane protein composed of two subunits that form a stable dimer and is expressed at the apical plasma membrane of epithelial cells. The detection sensitivity limit of the kit is 4 U/mL CA 15-3. The assay was done following the manufacturer's instructions.

2.13.4. Histopathological analysis

Trimming, dehydration, and inclusion were the basic histological techniques used once the organs had been fixed as mentioned previously. The 5-μm sections of paraffin embedded tissues were stained with hematoxylin and eosin and mounted in resin. The changes in tumor and organ histoarchitecture were assessed using photomicrographs obtained with a light Axioskop 40× microscope equipped with a digital Celestro-44421 camera connected to a computer, where the images were transferred and analyzed with Image J software. The breast tumors were examined at the Department of Morphological Sciences and Pathological Anatomy, Faculty of Medicine and Biomedical Sciences (University of Garoua) using Russo and Russo's histopathologic criteria [21].

2.13.5. Determination of oxidative stress parameters levels

The main markers of oxidative stress and total protein levels in the mammary glands of normal (NOR) and treated groups, were measured. Reduced glutathione (GSH) level was determined using a modified Sehirli et al. [22] method, catalase by. Misra [23] method and superoxide dismutase (SOD) activities by Sinha [24] method. Malondialdehyde (MDA), a biomarker of lipid membrane peroxidation, was measured using Wilbur et al. [25] protocol. Total protein levels in samples were determined using the Gonal et al. [26] method.

2.13.6. Determination of inflammatory parameters levels

The magnetic luminex screening assay was used to measure serum levels of interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), epidermal growth factor (EGF), interleukin-6 (IL-6), interleukin-12 (IL-12), and fractalkine. This assay was performed on rat samples using a MILIPLEX® kit (Millipore, Minneapolis, USA) as directed by the manufacturer. In brief, 96-microplates were shaken horizontally with 50 μL/well of samples (previously diluted) and standard cytokines, as well as 50 μL of rat, magnetic, premixed, microparticle cocktail with antibodies specific for each cytokine. Following that, microplates were washed with a magnetic plate separator and incubated with a 50 μL/well cocktail of biotin-antibodies specific for each cytokine. The antibody-cytokine complexes were revealed using Streptavidin-PE and analyzed in a Luminex MAGPix Analyzer (XMAP Technology, SN, USA), and the results were expressed as median fluorescence intensity (MFI). The MFI was converted in cytokine relative concentration via a standard curve specific to each cytokine. The minimum concentration detectable of each cytokine was: 5.2 pg/ml (IFN- γ), 1.9 pg/ml (TNF-α), 0.3 pg/

ml (EGF), 0.7 pg/ml (fractakline), 0.2 pg/ml (IL-6), and 0.4 pg/ml (IL-12).

2.14. Statistical analysis

The results from *in vitro* assays were expressed as mean ± standard error of the mean (SEM) for triplicates from at least three independent experiments. Analysis was done using Sigma plot software version 11.00. One-way analysis of variance (ANOVA) was used for data involving 3 or more groups with one variable followed by Dunnett's post-hoc test for multiple comparisons. With the *in vivo* study, the ANOVA followed by the Dunnett post-hoc test was used to compare all groups against the negative control group (DMBA). The unpaired student's *t*-test was used to compare the differences between the negative control group (DMBA) and the normal control group (NOR). The significance was set at $p < 0.05$.

3. Results

3.1. D. confinis cytotoxic effect on breast cancer cell lines

Table 1 below depicts the CC_{50} values of *D. confinis* ethanolic extract and that of Doxorubucin; the positive control used in this study on breast cancer cell lines (MCF-7 and MDA-MB-231 and 4T1). *D. confinis* showed cytotoxic potential in a range of 85 μg/mL (MCF-7), 78 μg/mL (MDA-MB-231) and 79 μg/mL (4T1), respectively, while the CC_{50} of doxorubucin was ~ 2 μM.

3.2. Effect of D. confinis ethanolic extract on cell growth

D. confinis showed a significant inhibition of MDA-MB-231 ($p < 0.01$), MCF-7 cells ($p < 0.01$) and 4T1 ($p < 0.001$) cells at 50 and 100 μg/mL respectively. The magnitude of inhibition was time dependent (Fig. 1A–C).

3.3. Effect of D. confinis ethanolic extract on cell proliferation and clone formation

The effect of *D. confinis* on the proliferation of MDA-MB-231 cells after 24 and 48 h are shown on Fig. 2. We observed a significant ($p < 0.05$) inhibition of cancer proliferation at 100 μg/mL after treating for 24 and 48 h (Fig. 2A). A significant ($p < 0.001$) inhibition of MDA-MB-231 clone formation was observed after treatment with *D. confinis* extract at 50, 100 μg/mL and doxorubicin (Fig. 2B and C).

3.4. Effect of D. confinis ethanolic extract on cell death and cell cycle

After treatment of the different breast cancer cell lines (MDA-MB-231, MCF-7 and 4T1) with doxorubicin, an increase in apoptotic cells (~30 %) was observed in comparison with the control. With *D. confinis* at 100 μg/mL, we equally observed a slight increase in apoptotic cell on the different breast cancer cell lines, a better increase being observed with 4T1 cells after treatment at concentrations of 50 and 100 μg/mL (Fig. 3A, B and C).

Regarding cell cycle progression, a decrease in G0/G1 phase, an increase in G2/M and a decrease of cell in the S-phase was seen after treatment of MDA-MB-231 cells with *D. confinis* at concentration 100 mg/kg (Fig. 3).

3.5. Effect of D. confinis ethanolic extract on cell migration

From the microphotographs depicted on Fig. 4(A-B), *D. confinis* extract significantly and concentration-dependently inhibited the migration of MDA-MB-231 cells. It significantly ($p < 0.05$ at 50 μg/mL and $p < 0.01$ at 100 μg/mL) inhibited MDA-MB-231 cell migration at 12 h and 24 h.

3.6. Chemotaxis assay

After treatment of MDA-MB-231 cells with *D. confinis* at 50 and 100 μg/mL, a significant concentration dependent inhibition of invading cells was observed ($p < 0.05$ at 50 μg/ml) and ($p < 0.01$ at 100 μg/ml) (Fig. 5A and B). However, this reduced invasion was less pronounced to that of doxorubicin in which there was significant ($p < 0.001$) inhibition of MDA-MB-231 invaded cells (Fig. 5C).

Table 1
CC_{50} values of *D. confinis* ethanolic extract on breast cancer cell lines.

	CC_{50} (μg/mL)		
D. confinis	MCF 7	MDA-MB-231	4T1
	85	78	79
Doxorubicin	3	2	2

D. confinis = ethanolic extract of *D. confinis*; **CC_{50}** = Concentration of *D. confinis* extract which results in 50 % of cell viability.

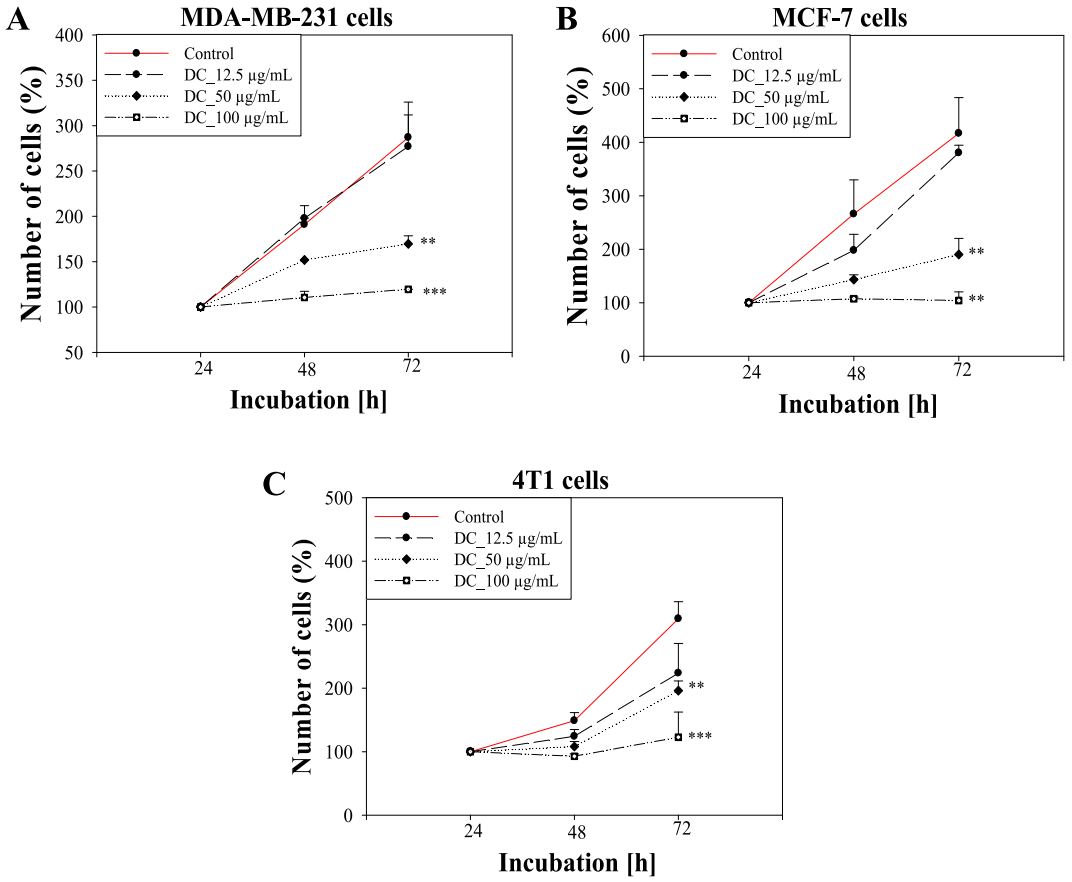

Fig. 1. Growth of MDA-MB-231 (**A**), MCF-7 (**B**) and 4T1 (**C**) cells treated with different concentrations of *D. confinis* after 24, 48 and 72 h. Controls remained untreated, (n = 3). Treated breast cancer cell cultures were compared to non-treated control cultures of the same passage and cell numbers per well. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control.

3.7. Effect of D. confinis ethanolic extract on cell adhesion

Fig. 5C depicts the effect of *D. confinis* on MDA-MB-231 cell adhesion. *D. confinis* at 100 µg/mL significantly ($p < 0.001$) inhibited cell adhesion both to collagen and fibronectin when compared with the control.

3.8. Chemopreventive effect of D. confinis ethanolic extract on a rat model of induced-breast

3.8.1. Effects of D. confinis on survival rate and body weight

As far as the survival rate (%) of animals during the 20 weeks of experimentation is concerned, the first deaths were observed in the DMBA group from week 1–5, tamoxifene (TAMOX), and letrozole (LTZ) groups, respectively. A There was a significant decrease ($p < 0.05$) in survival rate in the DMBA group compared to the normal group (NOR). The test groups treated with *D. confinis* (50 and 100 mg/kg) had the same survival rate as the DMBA group (62.5 %) at the end of the experiment, but later recorded more deaths than the test groups.

Fig. 6 shows the body weight curve of the animals throughout the 20 weeks of experimentation. It shows a normal and continuous growth of animals in all groups; except for the letrozole (LTZ) group, which shows a significant increase ($p < 0.01$) in the body mass of the animals compared to the DMBA group.

3.8.2. Effects of D. confinis on tumoral parameters

The effects of *D. confinis* extract on some tumor parameters are presented in Table 2. None of the animals in the NOR group developed tumors, while 100 % of animals (8/8) in the DMBA group showed at least one mammary tumor. The highest tumor burden was recorded observed in the DMBA group (97.28 g). The animals treated with the different substances showed a decrease in tumor burden compared to the DMBA group with the greatest percentage of tumor burden inhibition observed in the LTZ group (96.25 %) followed by DC 50 (94.95 %). The mean tumor mass was significantly ($p < 0.01$; $p < 0.001$) decreased in all treated groups compared to the DMBA group.

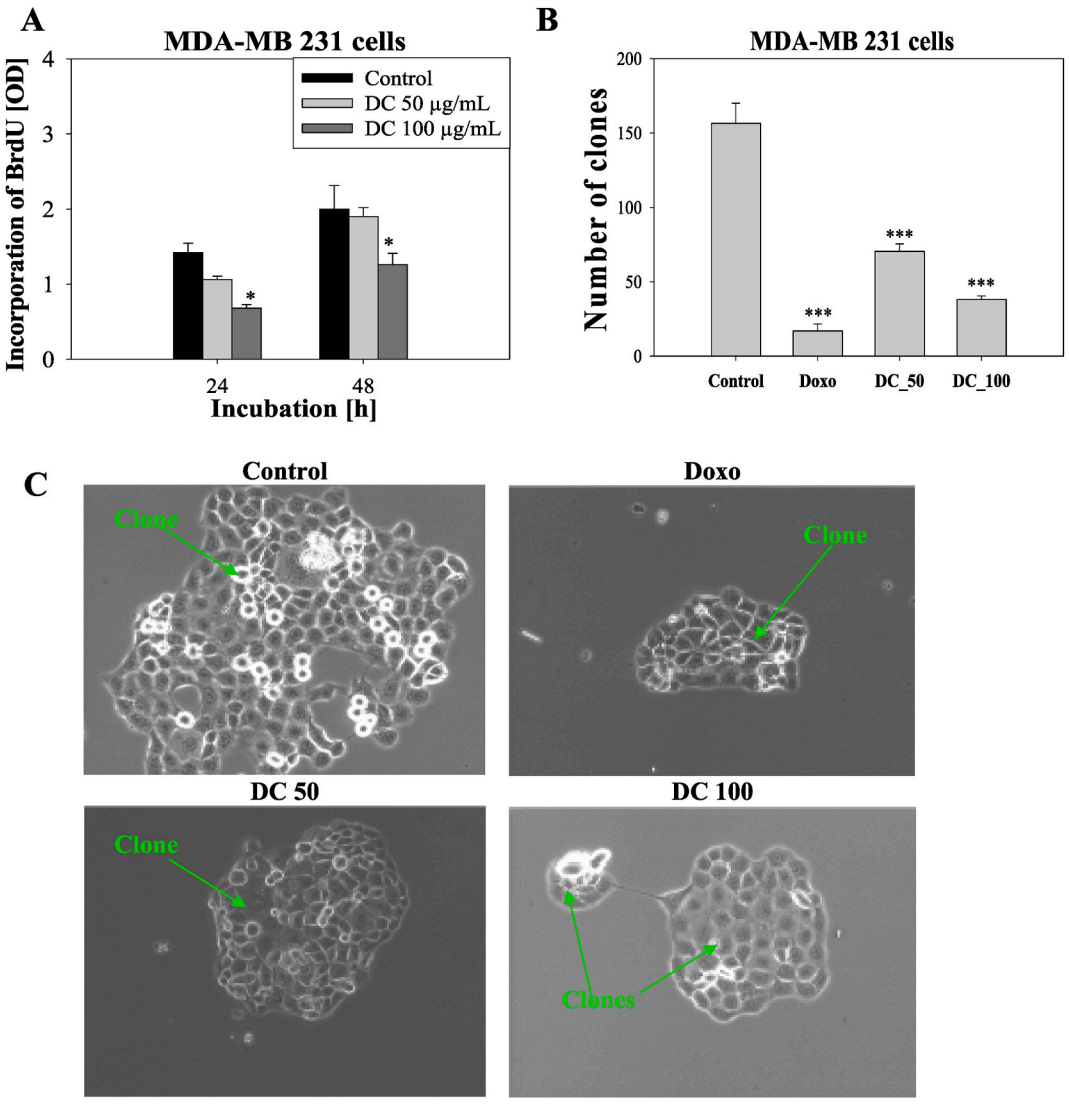

Fig. 2. Cell proliferation of MDA-MB-231 (**A**), after treatment with *D. confinis* at 50 and 100 µg/mL after 24 and 48 h. Clonogenic growth of MDA-MB-231 (**B**) cells with *D. confinis* at 50 and 100 µg/mL for 7 days. Photomicrographs (400×) of the clones obtained after 7 days of incubation (**C**). Control remained untreated. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control.

3.8.3. *Effects of D. confinis on tumor morphology and breast cancer marker*

A representation of tumor morphology image in each group are shown in (Fig. 7A). This is correlated with tumor volume (Fig. 7B). There was a significant ($p < 0.001$) decrease in tumor volume of animals in groups treated with Tamox, LTZ as well as with *D. confinis* extract at all doses compared to the DMBA group.

The serum concentration of CA15-3 (Fig. 7C) correlated with the level of tumor development, we observed a significant increase of this level in the DMBA group compared to the NOR group and a significant decrease of this marker in all treated groups compared to the DMBA group.

3.8.4. *Histopathological analysis of tumors and mammary glands*

The microarchitecture of the mammary glands of animals in the NOR group showed, a normal mammary parenchyma with mammary lobules containing mononuclear acinar cells surrounded by abundant adipocytes (Fig. 8). Animals in the DMBA group all had predominant cribriform or high-grade ductal carcinoma (SBR II or SBR III) with low lymphocytic infiltration (<10 %) and approximately 30 % comedonecrosis. Animals in the TAMOX and LTZ groups had low-grade fibrosarcoma (SBR I) without lymphocytic infiltration. However, only animals in the LTZ group had approximately 20 % comedonecrosis. The tumors found in the AM + DMBA group were of the cribriform ductal carcinoma type, grade SBR II, with 30 % lymphocytic infiltrate and about 30 % comedonecrosis. As for animals in the test groups, those in the DC 50 group had mostly SBR II fibrosarcoma, no lymphocytic infiltration and 30 %

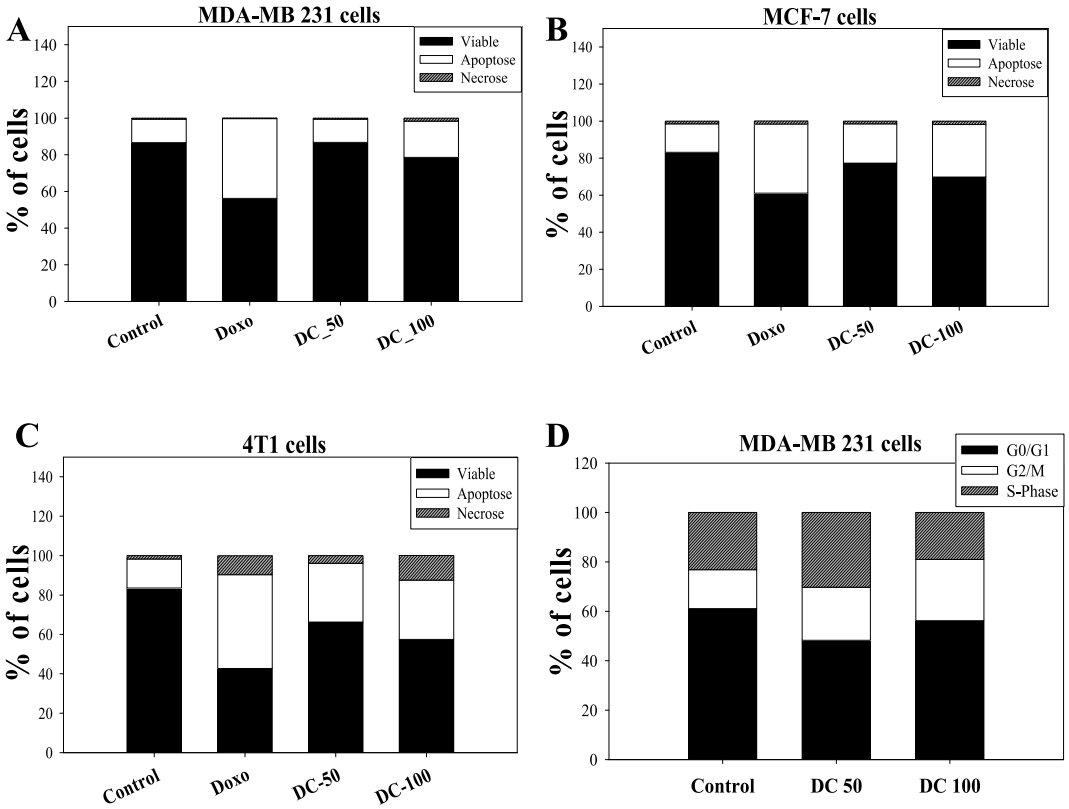

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Fig. 3. Apoptosis measurement by Annexin V-FITC/PI staining in MDA-MB-231 (**A**), MCF-7 (**B**) and 4T1 (**C**) cells after 24 h of treatment with *D. confinis* extract at 50, 100 μg/mL and Doxo (1 μM). Cell cycle analysis of MDA-MB-231 cells (**D**) following 24 h treatment of sub-confluent cultures with *D. confinis* extract 50 and 100 μg/mL. The different graphs are representation of the percentage of cells in each phase of three independent experiments.

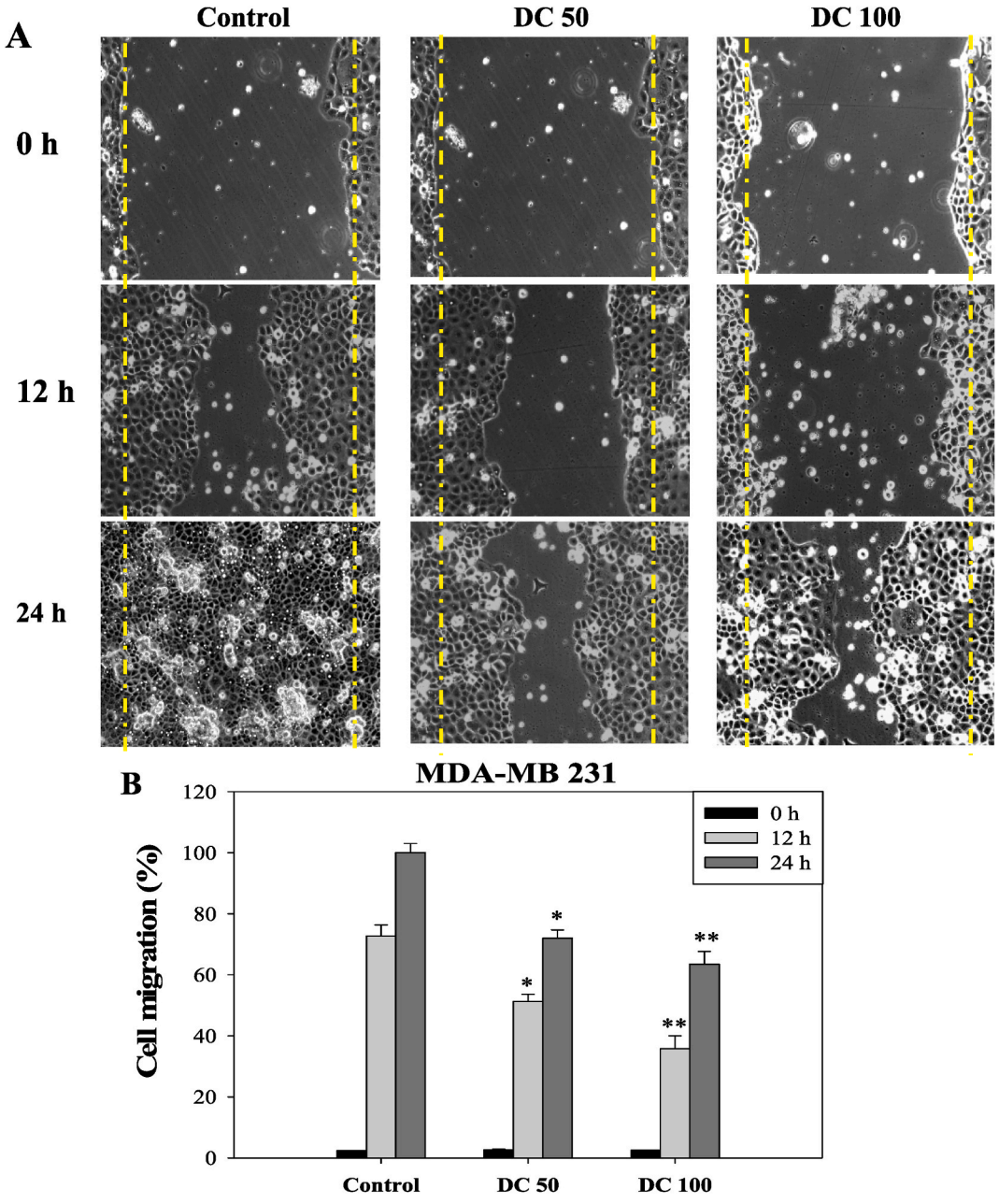

Fig. 4. Effects of *D. confinis* extract on MDA-MB-231 cells migration. Microphotographs of one assay (**A**) and graphical representation of three independent wound-healing assays (**B**) in MDA-MB-231 cells migration after 24 h of treatment. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with control.

comedonecrosis, whereas animals in the DC 100 group had mostly SBR I cribriform ductal carcinoma with 20 % lymphocytic infiltration and 20 % comedonecrosis.

3.9. Effect of D. confinis on inflammatory and oxidative stress parameters

3.9.1. Effect of D. confinis on oxidative parameters

Oxidative stress parameters and total protein in mammary gland and tumor homogenates are represented in Table 3. There was a significant decrease in GSH level ($p < 0.01$) and SOD ($p < 0.05$) activity, respectively in the DMBA group. SOD activity was significantly ($p < 0.05$) increased in all treated groups, while GSH level was significantly ($p < 0.01$) increased in groups treated with tamoxifen and *D. confinis* extract at 50 mg/kg.

With regards to MDA level, the DMBA group showed a significant increase ($p < 0.01$) in compared to the normal control (NOR)

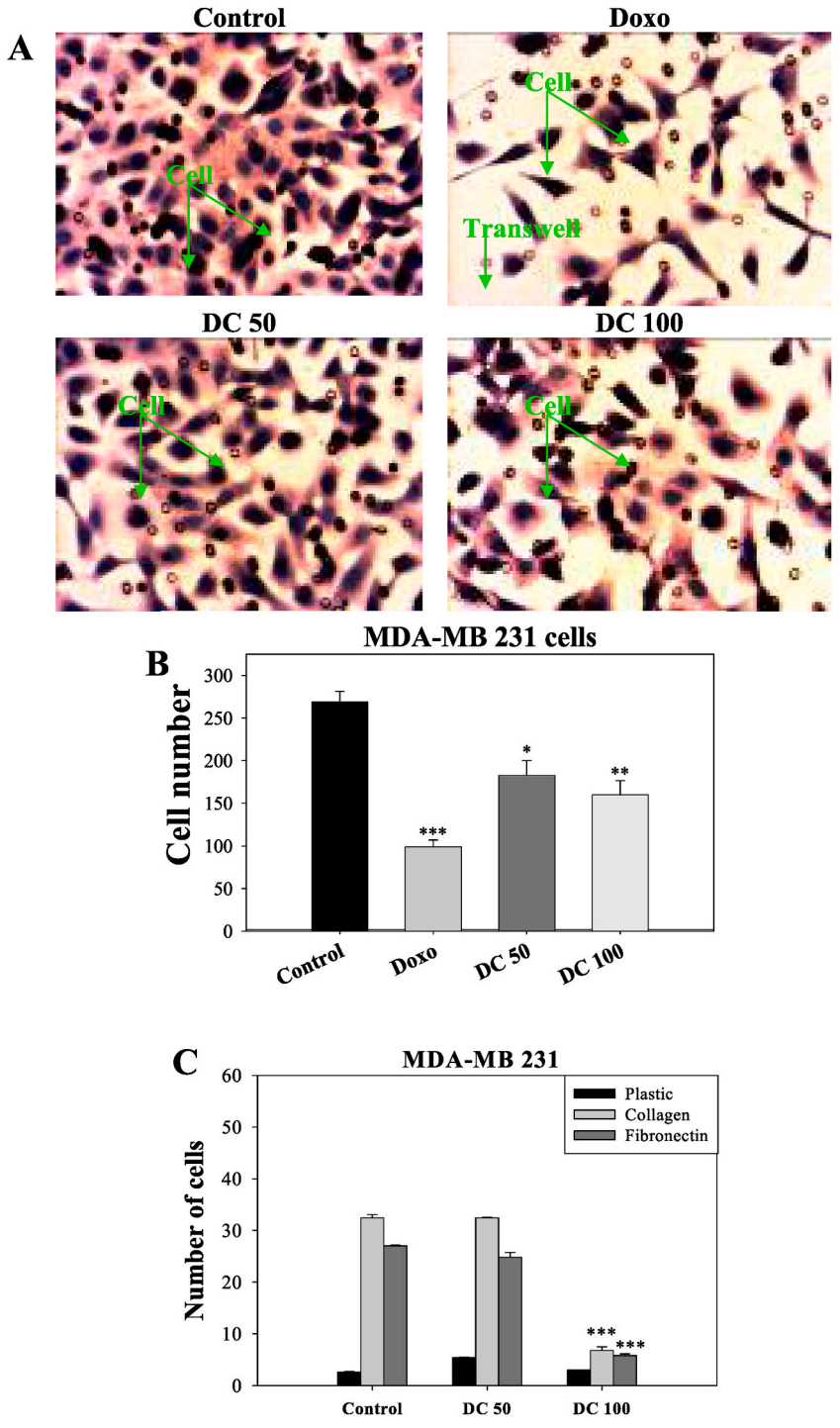

Fig. 5. Evaluation of MDA-MB-231 cell lines chemotaxis in the presence or absence (control) of *D. confinis* extract at 50 and 100 μg/mL: micro-photographs of one assay (**A**) and graphical representations of three independent assay (**B**). Serum-induced chemotactic movement was examined using six well transwell chambers with 8-μm pores. Adhesive behavior of MDA-MB-231 cell lines in presence of *D. confinis* (**C**). The plates were coated with extracellular matrix components (collagen and fibronectin) overnight $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison *to* control.

group. In the TAMOX, LTZ and DC50 groups there was at least, a significant inhibition ($p < 0.05$) of MDA level when compared to the DMBA group.

Regarding catalase activity, a significant increase ($p < 0.05$) was observed in groups treated with letrozole, *Annona muricata* (AM) and *D. confinis* 50 mg/kg compared to the DMBA group.

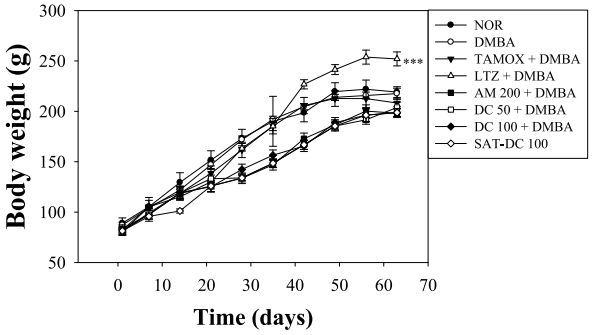

Fig. 6. Effects of *D. confinis* ethanolic extract on rats' body weight. NOR = Normal control, receiving 3 % ethanol in distilled water (vehicle); DMBA = Negative control, receiving vehicle; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg; LTZ = Positive control 2, receiving letrozole at 1 mg/kg; AM = Pharmacological control, receiving *Annona muricata* leaves ethanolic extract at the dose of 200 mg/kg; DC = Test groups, receiving *Duguetia confinis* ethanolic extract at the respective doses of 50 and 100 mg/kg. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. ##$p < 0.01$ is compared with the NOR group and **$p < 0.01$ is compared with the DMBA group.

Table 2
Protective effects of *D. confinis* ethanolic extract on some tumor parameters.

	Rats with tumors	Tumors incidence (%)	Tumor burden (g)	% Inhibition of tumor burden	Tumor mass (mg/kg)
NOR	0/8	0		/	/
DMBA	8/8	100	97.28 ± 31.32	/	23 ± 9.8
TAMOX	1/8	12.5	14.76 ± 1.1***	84.82	3,1 ± 0.1**
LTZ	3/8	37.5	3.65 ± 1.31***	96.25	0,9 ± 0.3***
AM	2/8	25	6.92 ± 1.16*	93.87	8,2 ± 2.2
DC 50	2/8	25	4.91 ± 1.81***	94.95	1,1 ± 0.17**
DC 100	3/8	37.5	6.23 ± 0.94***	93.59	1,2 ± 0.1**

NOR = Normal control, receiving distilled water; DMBA = Negative control, receiving distilled water; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg BW; LTZ = Positive control 2, receiving letrozole at 1 mg/kg BW; AM = Pharmacological control, receiving ethanolic extract of *Annona muricata* leaves at a dose of 200 mg/kg BW; DC 50 and DC 100 = Test groups, receiving ethanolic extract of *Duguetia confinis* barks at 50 and 100 mg/kg BW, respectively. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ are compared with the DMBA group.

Nitrite and total protein levels in tumor homogenates were significantly increased in the DMBA group ($p < 0.001$ and $p < 0.05$) respectively compared to the normal control. Treatments following LTZ, AM, DC 50 and DC 100, significantly ($p < 0.001$) prevented this increase compared to the DMBA group.

3.9.2. Effect of D. confinis on inflammatory parameters

Some inflammatory cytokines were measured and the results are presented in Table 4. This shows a significant ($p < 0.01$; $p < 0.001$) increase in serum levels of EGF, INF-γ, TNF-α, IL-6, and IL-12 in the DMBA group compared with the normal control. In all treated groups, there was a significant decrease in EGF, TNF-α, IL-6, and IL-12 levels and a significant increase ($p < 0.001$) in INF-γ levels compared with the DMBA group. No significant difference was observed between the different groups with regard to fractalkine level.

3.10. Toxicological assessment/safety profile of D. confinis

3.10.1. Effects of D. confinis on toxicological parameters and organs' relative weight

The relative weight of fresh organs is presented in Table 5. There was a significant increase in spleen ($p < 0.05$), liver ($p < 0.01$), lung and uterus ($p < 0.001$) relative weights in the DMBA group compared to the NOR group. In addition, compared with the NOR group, there was a significant increase ($p < 0.01$ and $p < 0.001$) in uterus and fresh liver relative weights respectively and a significant decrease ($p < 0.001$) of the lung's weight in the DC 100 mg/kg group. Compared to the DMBA group, relative liver weights were significantly decreased in the positive control (TAMOX and LTZ) and pharmacological standard (AM) groups, while lung and uterus weights were significantly decreased ($p < 0.01$) in all treatment groups. There was a significant decrease in kidney relative weights of animals treated with *D. confinis* extract at 50 ($p < 0.05$) and 100 ($p < 0.01$) mg/kg dose respectively. All treated groups prevented the DMBA-induced increase in spleen weight. A significant decrease ($p < 0.05$) of ovarian weight was observed in the DMBA group compared to the normal group (NOR). Conversely, animals treated with letrozole and all doses of *D. confinis* extract showed a significant increase ($p < 0.001$) in this parameter.

With the hepatic and renal biomarkers, we observed a significant ($p < 0.01$) increase in both ALT and creatinine levels in the DMBA group compared to the NOR group. In animals treated with *D. confinis* extract, we observed a significant decrease ($p < 0.05$) of both ALT and creatinine activities in DC 50 and 100 mg/kg.

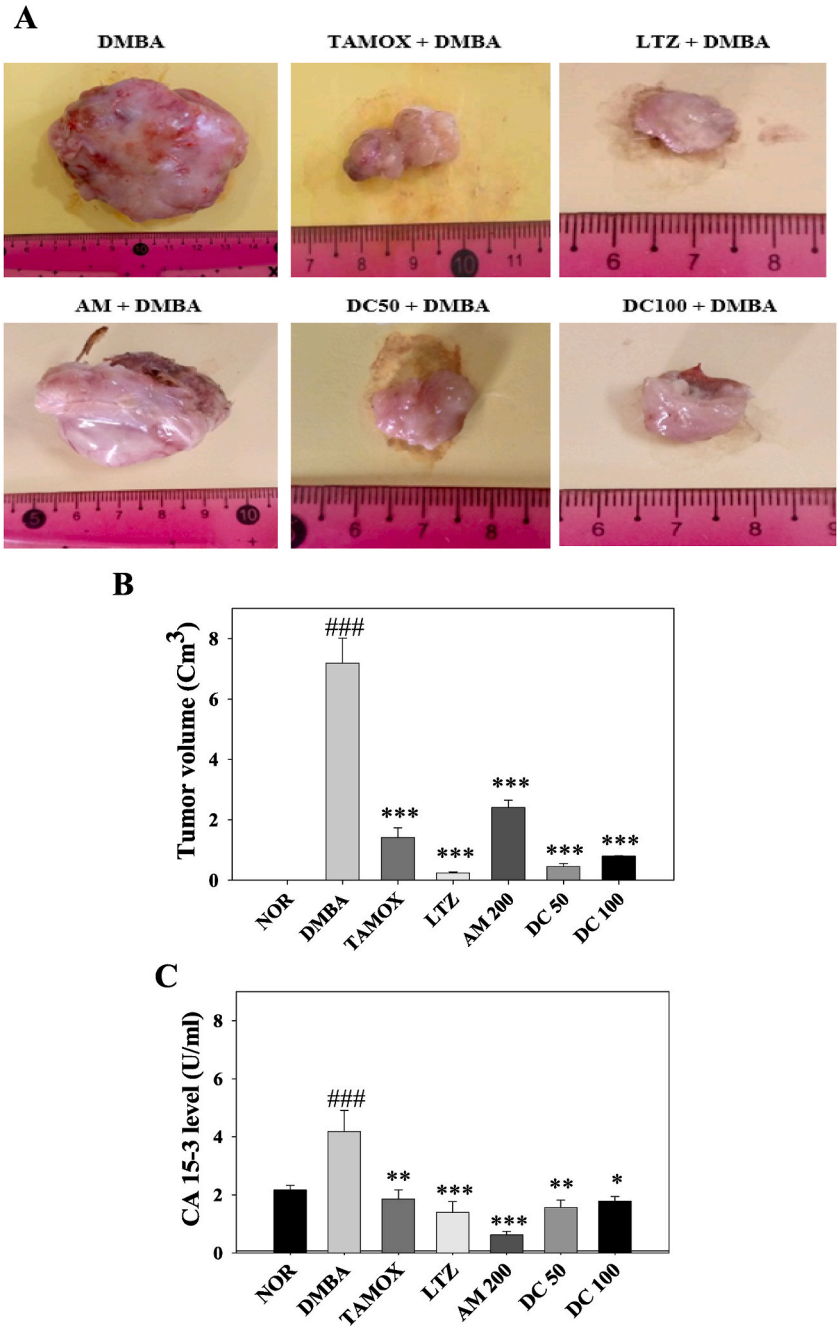

Fig. 7. Effects of *Duguetia confinis* ethanolic extract on tumor morphology (**A**), tumor volume (**B**), and levels of CA 15-3 (**C**). NOR = Normal control, receiving 3 % ethanol in distilled water (vehicle); DMBA = Negative control, receiving vehicle; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg; LTZ = Positive control 2, receiving letrozole at 1 mg/kg; AM = Pharmacological control, receiving *Annona muricata* leaves ethanolic extract at the dose of 200 mg/kg; DC = Test groups, receiving *Duguetia confinis* ethanolic extract at the respective doses of 50 and 100 mg/kg. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. ##$p < 0.01$ and ###$p < 0.001$ are compared with the NOR group and *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ are compared with the DMBA group.

3.10.2. Effects of D. confinis extract on hematological parameters

Table 6 shows the effects of the different treatments on some hematological parameters. There was a significant increase ($p < 0.01$) in WBC count in the DMBA group when compared to NOR group and a significant ($p < 0.05$) reduction of this parameter was observed in DC 50 mg/kg group. With red blood cells (RBC), we observed a significant ($p < 0.05$) decrease in RBC in the DMBA group and a significant ($p < 0.05$) increase of RBC in DC 100 when compared to NOR and DMBA, respectively.

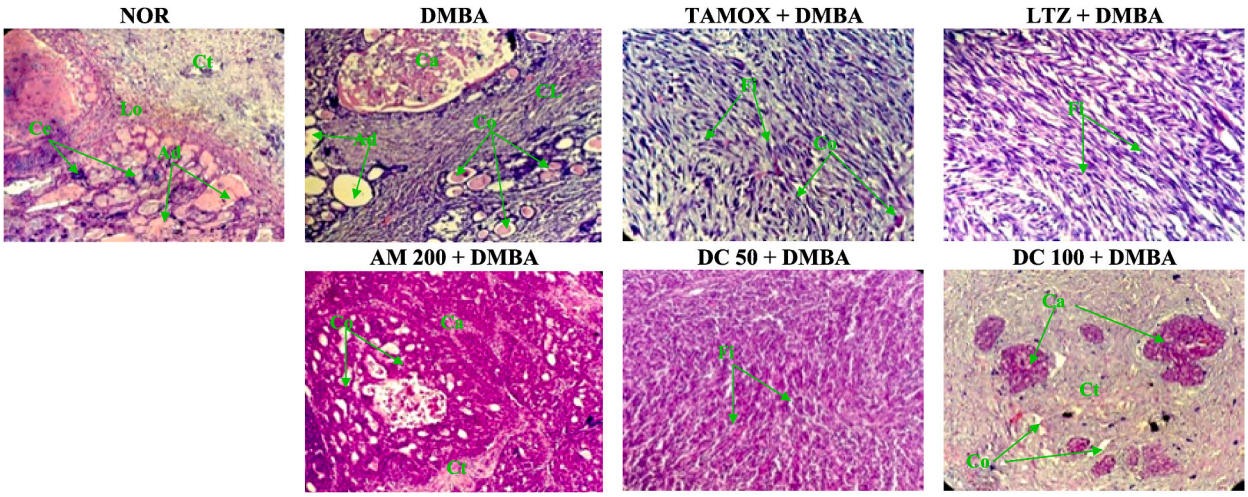

Fig. 8. Effects of *Duguetia confinis* ethanolic extract on tumors and mammary gland histoarchitecture. NOR = Normal control, receiving 3 % ethanol in distilled water (vehicle); DMBA = Negative control, receiving vehicle; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg; LTZ = Positive control 2, receiving letrozole at 1 mg/kg; AM = Pharmacological control, receiving *Annona muricata* leaves ethanolic extract at the dose of 200 mg/kg; DC = Test groups, receiving *Duguetia confinis* ethanolic extract at the respective doses of 50 and100 mg/kg. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. Ad = adipocytes; Lo = mammary lobule; Ct = connective tissue; Ce = normal mammary cell; Ca = carcinoma; CL = cribriform lobule; Fi = fibrous structures of the mammary gland; Co = comedonecrosis.

Table 3
Effects of *D. confinis* ethanolic extract on oxidative stress parameters and total proteins level.

Parameters	NOR	DMBA	TAMOX	LTZ	AM	DC 50	DC100
Oxidative stress							
GSH (mol/g of tissu)	25.3 ± 2.8	11.3 ± 1.6##	25.8 ± 2.4**	14.6 ± 0.1	12.1 ± 1.5	23.9 ± 2.4 **	19.1 ± 3.1*
MDA (µmol/g of proteins)	9.3 ± 0.9	16.2 ± 1.0##	13.1 ± 1.0*	11.3 ± 4.34**	14.3 ± 4.2	11.1 ± 2.6*	14.2 ± 2.4
SOD (U/mg of proteins)	1.3 ± 0.2	0.6 ± 0.3 #	1.71 ± 0.2*	1.3 ± 0.4*	1.3 ± 0.2*	1.3 ± 0.3*	1.3 ± 0.1*
Catalase (nM/min/mg)	2.4 ± 0.4	4.6 ± 0.3	3.7 ± 0.7	7.3 ± 0.9*	8.3 ± 1.5*	7.1 ± 1.7*	8.1 ± 2.7***
NO	47.3 ± 4.2	427.6 ± 36.8##	355.4 ± 15.8	212 ± 14.7***	154.8 ± 2.3***	318.8 ± 18.5**	155-3 ± 13.9***
Total proteins (g/l)	0.3 ± 0.1	0.9 ± 0.1#	0.7 ± 0.1	0.4 ± 0.1*	0.2 ± 0.1***	0.3 ± 0.1**	0.6 ± 0.1*

NOR = Normal control, receiving distilled water; DMBA = Negative control, receiving distilled water; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg BW; LTZ = Positive control 2, receiving letrozole at 1 mg/kg BW; AM = Pharmacological control, receiving ethanolic extract of *Annona muricata* leaves at a dose of 200 mg/kg BW; DC 50 and DC 100 = Test groups, receiving ethanolic extract of *Duguetia confinis* barks at 50 and 100 mg/kg BW, respectively. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. $\#p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ are compared with the NOR group and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ are compared with the DMBA group GSH: Reduced Glutathione, MDA: Malondialdehyde, NO: Nitric oxide, SOD: Superoxydedismultase.

Table 4
Effects of *D. confinis* ethanolic extract on some cytokines and growth factors involved in breast cancer.

	EGF (pg/ml)	INF-γ (pg/ml)	TNF-α (pg/ml)	FKN (pg/ml)	IL-6 (pg/ml)	IL-12 (pg/ml)
NOR	211.6 ± 10.8	304.3 ± 8.3	221.4 ± 9.8	10.5 ± 0.4	508.2 ± 6.8	403.7 ± 8.8
DMBA	262.3 ± 18.3#	315.2 ± 24.7###	525.1 ± 10.1##	9.6 ± 0.7	567.6 ± 5.4###	477.1 ± 6.8##
TAMOX	109.2 ± 10.9***	529.1 ± 31.4*	170.1 ± 11.4***	9.9 ± 0.7	512.1 ± 7.5***	411.0 ± 1.6
LTZ	40.5 ± 7.2***	498.6 ± 19.7	180.1 ± 12.4***	10.1 ± 0.3	501.4 ± 5.4	398.4 ± 4.3
AM	110.8 ± 14.8***	646 ± 16.7***	337.2 ± 18.9***	9.9 ± 0.8	521.4 ± 5.5***	446.8 ± 5.5**
DC 50	52.6 ± 13.0***	570 ± 49.3**	238 ± 11.8***	10.2 ± 0.4	521.9 ± 3.7***	437.3 ± 7.6***
DC100	180.2 ± 27.9#	550.9 ± 21.8	224 ± 15.2***	10.5 ± 0.3	–	–

NOR = Normal control, receiving distilled water; DMBA = Negative control, receiving distilled water; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg BW; LTZ = Positive control 2, receiving letrozole at 1 mg/kg BW; AM = Pharmacological control, receiving ethanolic extract of *Annona muricata* leaves at a dose of 200 mg/kg BW; DC 50 and DC 100 = Test groups, receiving ethanolic extract of *Duguetia confinis* barks at 50 and 100 mg/kg BW respectively. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. $\#p < 0.01$, $\#\#p < 0.01$ and $\#\#\#p < 0.001$ are compared to the NOR group and $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ are compared to the DMBA group. EGF: Epidermal growth Factor, FKN: Fractalkine, IL-6: Interleukin-6, IL-12: Interleukin-12, INF-γ: Interferon gamma, TNF-α: Tumor Necrosis Factor alpha.

Table 5
Effects of *D.confinis* ethanolic extract on relative weight of some toxicity, lymphoid and metastasis organs.

Item	NOR	DMBA	TAMOX	LTZ	AM	DC 50	DC 100	SAT-DC 100
Liver	30381 ± 624.7	35214.6 ± 1331.6y	30922.6 ± 235.4*	29049.9 ± 488.3**	30794.4 ± 908.1**	34582.0 ± 882.7	35944.4 ± 1875.0	35981.0 ± 732.7##
Lungs	6315.3 ± 115.6	8483.6 ± 303.7###	6372.5 ± 55.5***	5696.4 ± 74.8***	5882.7 ± 243.2***	5118.1 ± 86.7***	6075.0 ± 206.6***	5526.9 ± 101.0###
Kidney	5618 ± 113.6	5780.8 ± 322.3	5902.1 ± 83.6	4851.5 ± 103.1**	5767.0 ± 136.3	5471.3 ± 76.4*	5266.6 ± 279.7**	5810.0 ± 102.8
AG	321.2 ± 17.5	287.4 ± 14.8	423.0 ± 3.6***	198.7 ± 15.1***	360.7 ± 19.2**	301.5 ± 5.3	196.7 ± 12.9***	204.1 ± 4.1###
Spleen	6000 ± 85.5	7127.4 ± 710.6#	4335.3 ± 44.5***	3055.7 ± 19.9***	3413.2 ± 69.1***	4825.7 ± 293.3***	7058.6 ± 502.2	4255.7 ± 244.7##
Femur	2569.7 ± 84.7	2157 ± 106.0#	3044.2 ± 41.1***	2647.83 ± 91.1*	3217.7 ± 92.5***	2589.5 ± 56.7	2613.5 ± 54.2*	3278.7 ± 68.9##
Thymus	1396.7 ± 40.3	768 ± 40.3###	1423.7 ± 20.9***	976.4 ± 43.2**	439.3 ± 27.1***	1564.0 ± 78.8***	1401.0 ± 76.2***	1690.5 ± 93.5#
Ovaries	518.3 ± 15.1	471.2 ± 14.1#	462.1 ± 4.0	823.2 ± 13.0***	632.8 ± 27.1***	680.3 ± 31.9***	645.3 ± 32.5***	607.8 ± 35.7#
Uterus	1616.4 ± 76.2	2281.9 ± 84.0###	1206.8 ± 17.2***	640.1 ± 14.1***	1338.9 ± 36.3***	1132.7 ± 28.8***	1835.5 ± 105.4*	2069.5 ± 87.7##
ALAT	230.6 ± 35.6	362.3 ± 11.6#	259.0 ± 36.9*	282.3 ± 45.4*	259.0 ± 25.8*	260.3 ± 9.9*	226.9 ± 27.3*	230.0 ± 27.1
Creatinine	0.33 ± 0.1	0.75 ± 0.2##	0.6 ± 0.1	0.5 ± 0.1*	0.43 ± 0.1	0.5 ± 0.1*	0.6 ± 0.1*	0.7 ± 0.1#

NOR = Normal control, receiving distilled water; DMBA = Negative control, receiving distilled water; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg BW; LTZ = Positive control 2, receiving letrozole at 1 mg/kg BW; AM = Pharmacological control, receiving ethanolic extract of *Annona muricata* leaves at a dose of 200 mg/kg BW; DC 50 and DC 100 = Test groups, receiving ethanolic extract of *Duguetia confinis* bark at 50 and 100 mg/kg BW, respectively. SAT-DC 100 = Satellite control, normal rat treated with ethanolic extract of *Duguetia confinis* bark at 100 mg/kg BW. All animals except the NOR and SAT-DC 100 groups received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. #$p < 0.05$, ##$p < 0.01$, and ###$p <$ 0.001 are compared to the NOR group and *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ are compared to the DMBA group. ALT: Alanine aminotransferase; AG: Adrenal glands.

3.10.3. Acute toxicity in accordance to OECD guidelines

The single oral dose of *D. confinis* ethanolic extract at 2000 mg/kg BW, did not cause mortality, changes in general behavior or any clinical signs of toxicity in animals throughout the 14 days observatory period. In addition, no significant differences were noted between control and DC 2000 mg/kg groups with regards to body weight profile; relative weight of organs, hematological parameters, and histopathological examinations of the major toxicity organs (liver, lungs and kidneys) (Fig. 9). According to the OECD flow chart

Table 6
Effects of *D. confinis* ethanolic extract on some hematological parameters.

Paramaters	NOR	DMBA	TAMOX	LTZ	AM	DC 50	DC 100	SAT-DC 100
WBC ($\times 10^3 \mu L^{-1}$)	12.5 ± 0.54	15.4 ± 0.5##	12.8 ± 0.7	6.9 ± 0.6**	9.9 ± 1	8.5 ± 0.6*	13.4 ± 1.8	8.7 ± 0.7###
Lymphocytes (%)	69.8 ± 5	66.0 ± 0.4	79.7 ± 2.5*	83.6 ± 1.2**	73.7 ± 3.9	68.7 ± 3.4	62.6 ± 2.9	79.3 ± 1.5
Monocytes (%)	5.2 ± 0.5	6.3 ± 0.9	4.6 ± 0.9	3.8 ± 0.7	13.3 ± 4.2	11.1 ± 1.1	32.5 ± 2.4***	6.5 ± 1.2
Granulocytes (%)	18.4 ± 1.5	27.6 ± 1.3##	16.8 ± 1.4***	13.2 ± 0.7***	9.9 ± 1.3	12.8 ± 1.1***	23.1 ± 1.9	14.2 ± 0.5###
RBC ($\times 10^3 \mu L^{-1}$)	8.0 ± 0.5	5.6 ± 0.8#	7.8 ± 0.4*	7.5 ± 0.8*	6.6 ± 0.4	7.3 ± 0.1	7.6 ± 0.3*	7.5 ± 0.3
Hématocrite (%)	44.2 ± 0.9	30.0 ± 1.2###	52.3 ± 0.6***	46.5 ± 2.0***	37.5 ± 1.7*	43.3 ± 0.6***	44.7 ± 1.8***	45.1 ± 1.9
MCV (fL)	64.8 ± 0.3	64.7 ± 0.8	66.2 ± 0.5	56.9 ± 0.8***	60.6 ± 0.9*	59.2 ± 0.2***	61.8 ± 1.4	61.0 ± 0.6###
Platelets ($\times 10^3 \mu L^{-1}$)	379.0 ± 0.6	292.0 ± 10.7###	893.0 ± 45.9***	627.0 ± 32.6***	650.0 ± 28.4***	605.5 ± 12***	463.3 ± 39.0***	543.0 ± 27###
MCH (pg)	17.2 ± 0.4	16.6 ± 0.3*	16.8 ± 0.6	17.6 ± 0.2*	17.6 ± 0.3*	17.9 ± 0.2**	16.8 ± 0.3	17.5 ± 0.3
Hemoglobin (g/dL)	12.3 ± 0.2	8.0 ± 0.3***	12.4 ± 0.3***	14.2 ± 0.7***	12.3 ± 0.5***	13.0 ± 0.3***	13.5 ± 0.3***	12.7 ± 0.3
MCHC (g/dL)	27.9 ± 0.4	25.7 ± 0.2***	26.4 ± 0.2	31.6 ± 0.4***	29.8 ± 0.5***	30.2 ± 0.2***	28.4 ± 0.3***	28.4 ± 0.5

NOR = Normal control, receiving distilled water; DMBA = Negative control, receiving distilled water; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg BW; LTZ = Positive control 2, receiving letrozole at 1 mg/kg BW; AM = Pharmacological control, receiving ethanolic extract of *Annona muricata* leaves at a dose of 200 mg/kg BW; DC 50 and DC 100 = Test groups, receiving ethanolic extract of *Duguetia confinis* barks at 50 and 100 mg/kg BW, respectively. SAT-DC 100 = Satellite control, normal rat treated with ethanolic extract of *Duguetia confinis* barks at 100 mg/kg BW. All animals except the NOR and SAT-DC 100 group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. #$p < 0.05$, ##$p < 0.01$ and ##"$p <$ 0.001 are compared with the NOR group; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ are compared with the DMBA group. WBC: White blot cell, RBC: Red blot cell, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, MCV: Mean Corpuscular Volume.

recommendations, the LD_{50} was estimated greater than 2000 mg/kg.

4. Discussion

Breast cancer represents a serious health problem worldwide and actually is the most diagnosed cancer [4]. In Cameroon, its incidence in 2020 was estimated to be 34.1 % [27]. Just like in other developing countries, insufficient resources to ensure adequate

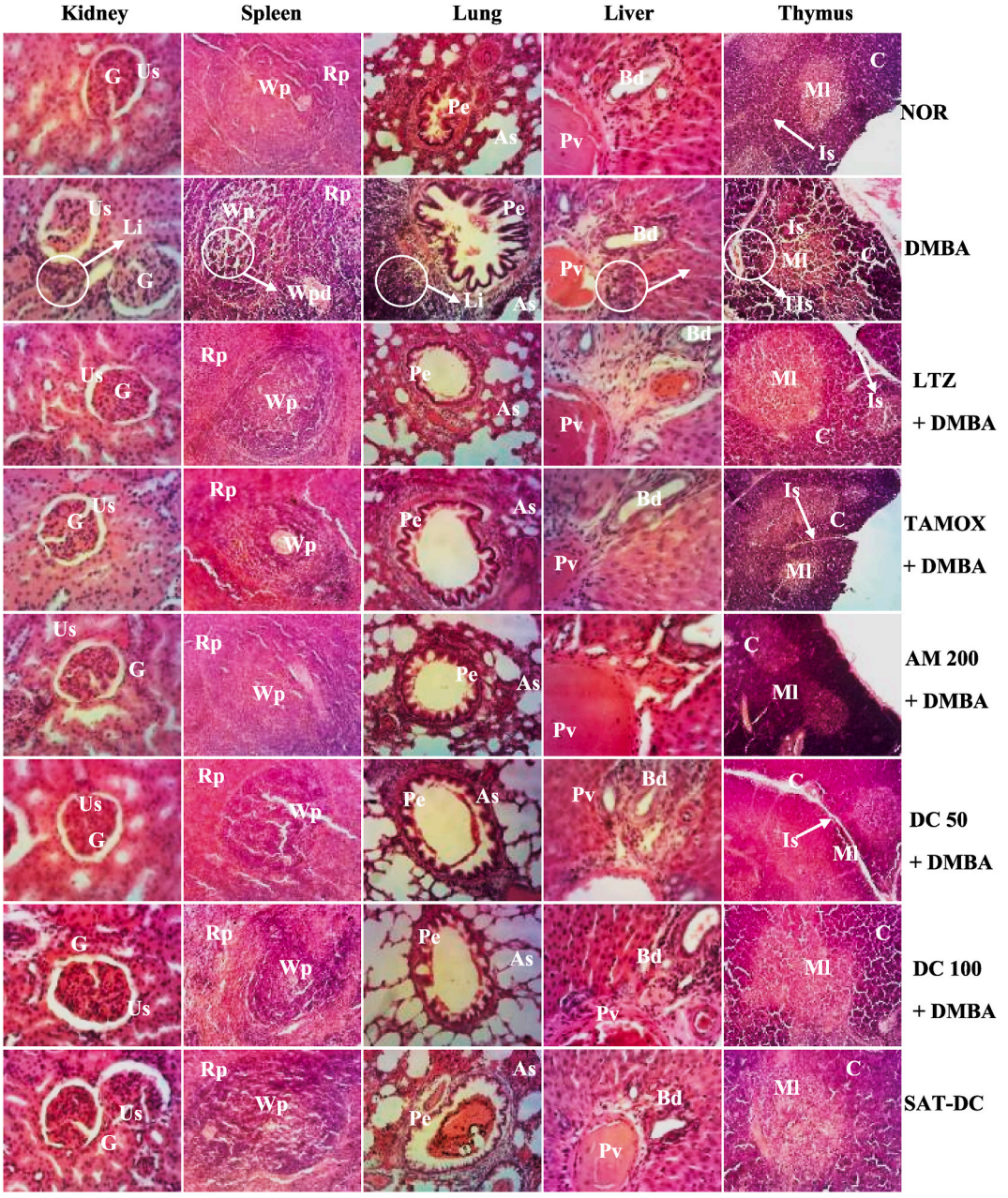

Fig. 9. Effects of *Duguetia confinis ethanolic extract* on the microarchitecture of kidney (×200), spleen (×100), lung (×100), liver (×200) and thymus (×400) following a H&E staining. NOR = Normal control, receiving 3 % ethanol in distilled water (vehicle); DMBA = Negative control, receiving vehicle; TAMOX = Positive control 1, receiving tamoxifen at 3.3 mg/kg; LTZ = Positive control 2, receiving letrozole at 1 mg/kg; AM = Pharmacological control, receiving *Annona muricata* leaves ethanolic extract at the dose of 200 mg/kg; DC = Test groups, receiving *Duguetia confinis* ethanolic extract at the respective doses of 50 and100 mg/kg. SAT-DC 100 group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. All animals except the NOR group received DMBA at a dose of 50 mg/kg *in situ* intraperitoneally. Us = urinary space, G = glomerulus, Li = leukocyte infiltration, Wp = white pulp, Rp = red pulp, DWp = Disorganization of white pulp, Pe = pulmonary epithelium, As = alveolar sac, Pv = portal vein, Bd = bile duct, Ml = medullary layer, Is = Interlobular septum, C = cortex, TIs = Thickening of the interlobular septum.

management of cancer patients, late diagnosis, fear of the unknown constitute factors which contribute to its rise [8]. To overcome this, patients mainly rely on traditional plants as preventive measures, for their primary care and even for treatment. Among these traditional plants, *Annona muricata* (Annonaceae) is well known and used as anticancer agent in Asia and Africa. However, there are studies which argue the safety profile of *Annona muricata* and the use of plants in drug development make research on anticancer substances a great necessity. To the best of our knowledge no anticancer effect of *Duguetia confinis* has been demonstrated yet. Moreover, being rich in bioactive compounds able to modulate various signaling pathways of cancer caught our attention. Thus, the present study was aimed at evaluating the effect of *Duguetia confinis* Engl. & Diels (Annonaceae) on breast cancer growth and prevention both *in vitro* and *in vivo*. To accomplish this, *D. confinis* cytotoxic potential was evaluated on 3 cancer cell lines using the well-known MTT reduction dye and CC_{50} values of 85 μg/ml (MCF-7), 78 μg/ml (MDA-MB-231) and 79 μg/ml (4T1), were obtained respectively. This result corroborates many studies that reported the CC_{50} of various Annonnaceae (*Annona muricata*, *Anonidium mannii*, *Xylopia eapithicum*) in the same range [14,28]. Although the phytochemical investigation was not performed in this study, the observed effect could be due to the presence of common bioactive compounds, mainly acetogenins and alkaloids, which could vary from one species to other, or in the same species depending on the area of cultivation [29].

Uncontrolled cell proliferation and deregulation of apoptotic mechanisms are characteristic features of cancer. That is, impairment of the cell cycle results in uncontrolled cell proliferation, growth, and tumor progression [15]. The cell cycle has two major phases called interphase and mitosis. Interphase is divided into G1, S, and G2 phases which are important to cell division and maintenance of genetic material. However, cells destined to die and no longer divide may enter into the G0 phase [30]. In the present study, *D. confinis* at concentration of 100 mg/kg was able to significantly ($p < 0.05$) inhibit cell proliferation, suggesting a, cytostatic or cytotoxic effect. This inhibition could pass through cell cycle blockade. As observed in this study, *D. confinis* at concentration of 100 mg/kg reduced the number of cells in the G0/G1 and S phases. This feature is a characteristic of blockage at G1 and S phases. Previous studies have shown that *A. muricata* extracts, and acetogenins are capable of regulating the cell cycle machinery, thus, causing cell cycle arrest and inhibition of cell proliferation through the downregulation of EGFR expression. Ethanol extracts of roots, fruits, or leaves of *A. muricata* inhibited proliferation via G0/G1 cell cycle arrest in leukemia HL-60 cancer cells [31]. This corroborates with another study, carried out by Zorofchian et al. [32] where *A. muricata* leaf extracts were reported to induce cell cycle arrest at the G0/G1 phase in MDA-MB-468 breast cancer cells. There has been an increase of apoptotic cells in all breast cancer cell lines after treatment with *D. confinis* at concentrations of 50 and 100 μg/mL, meaning that it triggers apoptosis. Apoptosis defined as regulated cell death is a process important to maintain the homeostasis of an organism and compounds able to be pro-apoptotic have been considered as anti-cancer drugs. *D confinis* extract could mediate its effect through the regulation of caspases or members of the Bcl-2 family (not investigated in this study). However, it could act like *A. muricata* Leaf extract, which induced apoptosis in breast cancer cells (MDA-MB- 468) via activation of caspase-3 [32]. This effect being associated with the suppression of PCNA and Bcl-2 proteins and an augmentation of Bax protein. The pro-apoptotic feature of *D. confinis* could also pass through the Endoplasmic reticulum (ER) stress pathway as shown by other Annonaceae [33].

Triple negative breast cancer is associated with severe adverse effects and poor prognosis compared to hormonal/ER responsive breast cancer cell lines. Thus, the MDA-MB-231 cell lines was further considered in underlying mechanism of action. The metastatic spread of breast cancer is responsible for 90 % of cancer-related deaths [34]. Thus, for a compound to be considered as an effective drug against breast cancer, it must possess the capacity to inhibit the metastases of breast cancer since the progression of tumor is also dependent metastases. Metastasis (migration and invasion of tumor cells) constitute a formidable barrier for successful treatment [14]. It is a multi-cascade of events which involve distinct stages: cell migration, invasion, attachment, and angiogenesis and is facilitated/enhanced by members of the MMPs (Matrix metallo proteinase) family [35,36]. Studies have shown that MMPs proteins are regulated by proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukins (IL)-1, and IL-6 [37]. Thus, in the present study, wound healing abilities and the ability of *D. confinis* to inhibit invasion were assessed. *D. confinis* at 50 and 100 μg/mL were able to significantly inhibit cell migration and invasion, respectively. On the other hand, cell adhesion to the extracellular matrix such as collagen or fibronection are important characteristic of cell migration. Thus, the ability of a substance to adhere is inversely proportional to its ability to migrate. In the present study, *D. confinis* significantly inhibited ($p < 0.001$) MDA-MB-231 cells ability to migrate. This anti-metastatic potential could be due to the ability of *D. confinis* to inhibit IL-6, IL-12 and TNF-alpha. As observed in this study, *D. confinis* at 50 and 100 mg/kg significantly ($p < 0.01$) inhibited the levels of these cytokines *in vivo*.

The *in vitro* studies showed beneficial effects on breast cancer, but considering the fact that drug metabolism can affect its efficacy, *in vivo* chemopreventive study was further assessed in DMBA-induced breast cancer in Wistar rats. 7,12-dimethylbenz(α)anthracene (DMBA), is a widespread genotoxic and tumorigenic environmental pollutant [38]. The animal model of tumor induced by it showed a typical pattern of anatomy, pathology, and growth of premalignant and malignant hyperplastic lesions that is similar to human breast cancer [39]. Once-off treatment of the rats with DMBA (50–80 mg/kg BW has been reported to induce the development of breast cancer [40–42]. Indeed, DMBA metabolizes to a mutagenic epoxide intermediate under the action of cytochrome P450 enzymes (CYP1A1 and CYP1B1), which induce breast cancer in preclinical models by activating the cellular cytosolic receptor, aryl hydrocarbon receptor (AhR). When AhR is activated, it is translocated into the nucleus and combines with the AhR nuclear translocation protein. The AhR/ARNT complex induces gene transcription by binding to specific DNA recognition sites upstream of AhR-responsive genes forming DNA adducts [43]. *D. confinis* and *A. muricata* extracts and the positive controls (tamoxifen and letrozole) significantly reduced the tumor incidence, tumor volume, relative tumor mass, total protein levels in tumors and CA 15-3 levels compared to the DMBA group. These results suggest a protective effect of the tested substances on breast carcinogenesis. In fact, Mucin-1 is a transmembrane protein found on plasma membranes of epithelial cells, it is overexpressed in breast cancer and thus, serve as a biomarker with a prognostic value [44]. The increase in Mucin-1 protein expression in this study is consistent with the significant increase of tumor growth observed in the DMBA group. Animals treated with *D. confinis* significantly inhibited this increase in CA 15-3 level. The

selective estrogen receptor modulator (tamoxifen) and aromatase inhibitor (letrozole), used as positive controls in this study significantly reversed carcinogenesis as they are known to prevent cancer cell multiplication by inhibiting estrogen synthesis or competing with endogenous estrogens for binding to their receptors in the mammary glands [45]. These effects are in agreement with numerous studies, which have shown the protective effect of tamoxifen and letrozole in DMBA-induced breast cancer in rats [28,46].

According to Ngowa et al. [7], breast cancer screening mostly occurs at stages III and IV of the disease in Cameroon. Both CA 15-3 and SBR grade are generally associated with high cell proliferation rates and hence, are important prognostic factors of breast cancer [47]. Numerous studies have shown the potential of *A. muricata* L. to inhibit induced breast cancer in rats. In one study, it was shown that *A. muricata* L. leaves are able to inhibit SBR III grade invasive ductal breast cancer developed in DMBA group, improve histological changes and reduce proliferation indices at the dose of 300 mg/kg [48]. Another example that was done on DNA smears obtained from agarose gel electrophoresis showed that, the extract of *A. muricata* L. leaves prevented DMBA-induced DNA damage [49]. These chemopreventive effects of *A. muricata* L. are due to its bioactive metabolites specially acetogenins, phenolic compounds and megastigmanes which either block the cell cycle of cancer cells, or act via the antioxidant pathway, or antagonize estrogens for receptor binding [50]. These different inhibitory pathways such as cell cycle blockade demonstrated in this study could be characteristic of *D. confinis*, which is also an annonaceae, rich in phenylpropanoid derivatives as well as several alkaloids [16,51].

To better understand the possible mechanism by which *D. confinis* inhibits DMBA-induced mammary tumorigenesis in rats, oxidative stress status was evaluated. It is well known that DMBA triggers tumorigenesis through the production of reactive oxygen species (ROS). In fact, high level of ROS is responsible for the damage of many biomolecules and the exertion of various cellular and molecular effects, including cytotoxicity, and mutagenicity that can lead to the initiation and promotion of carcinogenesis. Antioxidants interfere with the sequence of free radical chain by donating hydrogen atoms. The radicals formed are stabilized by the delocalization of resonance electrons within the aromatic ring [52]. *D. confinis* at both 50 and 100 mg/kg and *A. muricata* (200 mg/kg) extracts as well as letrozole induced significant ($p < 0.05$) increase in SOD and catalase activities and a decrease in MDA levels in contrast to observations made in the DMBA group. It should be noted that SOD and catalase belong to the first line of defense family of antioxidant enzymes, against ROS in the biological system [53]. SOD is a major antioxidant enzyme, which scavenges free radicals by decomposing them into hydrogen peroxide and oxygen, which are then removed by catalase and glutathione peroxidase. Catalase on the other hand is a hemoprotein known to protect tissues from highly reactive hydroxyl radicals by decomposing hydrogen peroxides [54]. We also observed in this study a significant ($p < 0.01$) decrease in GSH level of the DMBA group in comparison to the NOR group whereas, a significant increase of this parameter was observed in groups treated with *D. confinis* extract (50 and 100 mg/kg) and letrozole as shown by Faheem et al. [55]. Indeed, glutathione is an antioxidant that is characterized by its ability to prevent the harmful effects of reactive oxygen species such as free radicals, heavy metals, peroxides and lipid peroxides [56]. The increase in MDA levels observed in the DMBA group could be explained by the presence of oxidative DNA damage that stimulates breast cancer induction [56]. Both morphologically and biochemically, the results of this study show desirable anticancer effect of *D. confinis* extract in DMBA-induced breast cancer model.

The increased production of ROS triggers a cascade of reactions leading to the onset of an inflammatory response that can be chronic and lead to diseases such as cancer [57]. This inflammatory response is closely associated with all stages of malignant development and progression of most types of cancer, as well as with the efficacy of cancer therapies [58]. Some researchers believe that key molecules involved in the inflammatory response to cancer are pro-inflammatory cytokines such as IL-12, IL-6, TNF-α, Fractacline and IFN-γ [59]. Administration of DMBA to rats resulted in increased levels of IL-12, IL-6, TNF-α, EGF and IFN-γ. Indeed, IFN-γ is a group of cytokines synthesized by the cell in response to foreign intrusion with antiviral, immunomodulatory and anti-proliferative activities, used in anti-cancer therapy. Its elevated serum level is usually correlated with tumor growth and its decrease is a good prognosis in cancer management [60]. Pro-inflammatory mediators such as TNF-α and IL-6 are the main ones responsible for cancer-related inflammation and suppression of these cytokines could protect against chemically induced breast tumors [61]. *D. confinis* and *A. muricata* L. extracts as well as letrozole and tamoxifen significantly reduced the levels of pro-inflammatory cytokines. *A. muricata* L. is thought to induce its effects via annonacin, annomuricin E, muricoreacin, quecertin, and murihexocin C, its secondary metabolites are well known for their anti-inflammatory activity [62]. This reduction in the secretion of pro-inflammatory cytokine observed with *D. confinis* ethanolic extract maybe as a result of their ability to suppress lipopolysaccharide (LPS), LPS-induced NF-KB and MAPK pathways, and levels of p-JNK, p-ERK1/2, and p-p38 in the MAPK pathway [63]. Nevertheless, the assessment of tumor infiltrating lymphocytes (TILs) would have brought some insight into the understanding of the anticancer effect of *D. confinis* extract.

Relative organ weight is considered a key indicator of the toxic effects of xenobiotics. Significant weight reduction was observed in the heart, femur, ovary, and adrenal glands of rats in the DMBA group. These results are in line with those reported by Zingue et al. [13], and could be explained by the toxic effect of DMBA which causes apoplexy (hemorrhagic injury) in different organs of the rats [38]. The weights of liver, lung, and uterus were increased significantly in the DMBA group compared to the normal control. Liver and lung are target organs in toxicology [64]. Fresh spleen weight was increased significantly in DMBA-exposed rats in this study; as observed by Zingue et al. [8], this decrease in spleen weight is correlated with a significant ($p < 0.05$) decrease in red blood cells and all hemoglobin-related parameters in the DMBA group compared to normal animals. The significant decrease in red blood cell count and the significant increase in white blood cell count in the DMBA group compared to normal rats confirmed the hematotoxicity of this carcinogen. Indeed, the hematopoietic system is one of the most sensitive targets of xenobiotics and is therefore an important indicator of physiological and pathophysiological status [65]. For example, DMBA-induced lesions in rats are usually followed by anemia, increased platelet counts, white blood cell counts, and transaminase activity [66]. *D. confinis* just like the positive controls protected the rats from DMBA-induced decrease in red blood cells. Alanine aminotransaminase activity was increased in DMBA-treated animals, suggesting damage in the cells [8]. Despite an increase in creatinine level no difference was observed in kidney mass of animals in the DMBA group. However, the observed increase in creatinine level is consistent with the disorganization of the renal parenchyma and

leukocyte infiltration during histopathological analysis. The ethanolic extract of *D. confinis* bark prevented the increase in kidney mass and creatinine level suggesting a protective effect of this extract in the kidneys. Nevertheless, investigation into the longer-term safety, efficacy, and potential side effects in humans is required, before human clinical trials can be conducted to validate *D. confinis* as a preventative measure for breast cancer.

5. Conclusion

Research on anticancer drug is of important necessity due to the ongoing surge of this disease. This study reports for the first time the anticancer effect of *D. confinis* (Annonaceae) *in vitro* and *in vivo*. It can be seen that *D. confinus* possesses cytotoxic and preventive potential against breast cancer and is able to regulate many processes involve in carcinogenesis, therefore, justifying its use by traditional healers in Cameroon. However, further research studies are still warranted regarding its bioactive components and anticancer mechanism of action.

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Ethics approval and consent to participate

Animal handling and treatments were approved by the Joint Institutional Review Board on Animal & Human Bioethics of the Faculty of Science (University of Yaoundé 1), which is in line with the European Union on the care of animals (EEC Council 86/609).

Availability of data and materials

The data and materials used in this study are available upon request from the corresponding author (stephane.zingue@fmsb-uy1.cm).

CRediT authorship contribution statement

Ornella Bernie Kami Nkuimi: Investigation. **Kevine Kamga Silihe:** Writing – original draft, Investigation, Data curation, Conceptualization. **Yves Omgba Tabi:** Writing – original draft, Investigation. **Judith Christiane Ngo Pambe:** Methodology, Investigation, Data curation. **Dieudonné Njamen:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Stéphane Zingue:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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Abbreviations

ATCC	American Type Culture Collection
ALT	Alanine transaminase
AR	Androgen receptor
BD	Becton Dickinson
BSA	Bovine Serum Albumin
BW	body weight
CA 15-3	cancer antigen 15-3
CAR-T	Chimeric Antigen Receptor T-cell
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
DMBA	7,12-dimethylbenz(a)anthracene
EDTA	ethylenediaminetetraacetic
EGF	Epidermal Growth Factor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor

FBS	fetal bovine serum
Hb	hemoglobin
IL-6	Interleukin-6
IL-12	interleukin-12
IFN-γ	interferon gamma
LTZ	letrozole
MDA	malondialdehyde
MTT	3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide
MFI	median fluorescence intensity
NHC	National Herbarium of Cameroon
NOR	normal
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PI	propidium iodide
RBC	red blood cell
RPMI 1640	Roswell Park Memorial Institute 1640
ROS	reactive oxygen species
SBR	Scarff-Bloom -Richardson
SEM	standard error of mean
TAM	tamoxifen
TNF-α	tumor necrosis factor
WHO	World Health Organization

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