Bryonia dioica aqueous extract induces apoptosis and G2/M cell cycle arrest in MDA-MB 231 breast cancer cells

BACHIR BENARBA[1], ALMAHY ELMALLAH[2] and ATANASIO PANDIELLA[3]

[1]Laboratory Research on Biological Systems and Geomatics, Faculty of Nature and Life, University of Mascara, Mascara 29000, Algeria; [2]Zoology Department, Faculty of Science Beni-Suef University, Beni-Suef 62511, Egypt; [3]Instituto de Biología Molecular y Celular del Cáncer, CSIC-CIBERONC-Universidad de Salamanca, 37001 Salamanca, Spain

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Abstract. *Bryonia dioica* Jacq. is a climbing perennial herb with tuberous roots which is widely used in traditional medicine in Algeria for the treatment of cancer. The present study aimed to evaluate the apoptogenic activity and phytochemical composition of the aqueous extract of *B. dioica* roots growing in Algeria. The cytotoxic effect of *B. dioica* aqueous decoction against breast cancer MDA-MB-231 cells was evaluated by an MTT assay. Apoptosis induction was assessed by an Annexin V-fluorescien iosthiocyanate assay. Propidium iodide staining of cell DNA was used to assess the effects on the cell cycle. In addition to UV-Visible (UV-vis) analysis, the major compounds of the extracts were determined using liquid chromatography-mass spectrometric analyses. Our results showed that the *B. dioica* aqueous extract induced cell death in a time-dependent manner. The highest inhibitory effect was produced at concentrations of 50 μg/ml or higher after 72 h of treatment (91.15±0.71%). Furthermore, the extract induced apoptosis of MDA-MB-231 cells. At 250 μg/ml, 64.61% of the treated MDA-MB-231 cells were apoptotic. This was accompanied by cell cycle arrest at G2/M phase. The percentage of cells in G2/M increased from 15.7% (untreated cells) to 59.13% (50 μg/ml) and 58.51% (250 μg/ml). The UV-vis absorption spectra of *B. dioica* aqueous extract showed two absorption bands characteristic of the flavonol skeleton; 350-385 nm (Band I) and 250-280 nm (Band II). Myricetin (2,5,7,3,4,5-pentahydroxylflavonol) was found to be the major compound in the *B. dioica* aqueous extract. These findings suggest that *B. dioica* could be considered a promising source for developing novel therapeutics against breast cancer.

Correspondence to: Dr Bachir Benarba, Laboratory Research on Biological Systems and Geomatics, Faculty of Nature and Life, University of Mascara, B.P 305 Route de Mamounia, Mascara 29000, Algeria
E-mail: bachirsb@yahoo.fr

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Introduction

Breast cancer, considered heterogeneous cancer, both biologically and molecularly, is the most common malignancy in women worldwide (1). Breast cancer still remains the first cause of cancer-related deaths among women, especially in developing countries in which 60% of breast cancer-related deaths are reported (2). In spite of being treated by different therapeutic approaches including chemotherapy, radiotherapy, surgery, hormone therapy and other targeted therapies, breast cancer is facing important issues such as high long-term mortality, drug-resistance, and metastases (3). Thus, search for effective alternative therapies with fewer side effects has become necessary.

In Africa, up to 80% of the population uses traditional medicine for primary health care (4). Algeria, the biggest African country with a large variety of soils (littoral, steppe, mountains, and desert) and climates, possesses a rich flora (more than 3,000 species and 1,000 genders) (5). In Algeria, many patients use medicinal plants as a treatment for many ailments and serious diseases, such as cancer, diabetes and arterial hypertension, for several considerations: Historical, cultural and economic (6-8). Although few studies have been published on ethnobotanical and pharmacological properties of Algerian medicinal plants, several species have been found to be used by Algerian breast cancer patients such as *Aristolochia longa* L., *Berberis vulgaris* L., *Thymus vulgaris* L., *Prunus persica* (L.) Batsch, and *Artemisia herba-alba* L. (9). Previously, we have demonstrated that aqueous extracts of *Aristolochia longa* roots, an Algerian medicinal plant widely used in cancer therapy by local populations, induced apoptosis of Burkitt's lymphoma BL41 cell line by targeting the mitochondrial pathway (10). An aqueous extract of *Aristolochia longa* induced cell growth inhibition in HBL100 and MDA-MB-231 triple negative breast cancer cells in a dose-dependent manner (11). Other extracts of *Aristolochia longa* aerial parts exhibited promising antioxidant and anticancer activities in different cell lines (data not published).

Bryonia dioica Jacq. (white bryony) a climbing perennial herb with tuberous roots is locally named in Algeria 'Fachira' and 'queriou'aa' by the locals. The species grows in North Africa, temperate Europe, and Western Asia (12).

The roots of *B. dioica* are characterized by the presence of cucurbitacins, oxygenated tetra-cyclic triterpenoids possessing anti-inflammatory, anti-infectious, cytotoxic and apoptogenic effects (9,13). The plant is used to treat several ailments such as asthma, bronchitis, hypertension, gastric ulcers, and diabetes (14). Important biological and therapeutic effects of *B. dioica* were demonstrated such as antidiabetic (15), antibacterial (16) and antioxidant activities (17). The plant possesses also important anticancer activities. Indeed, we have previously shown that *B. dioica* aqueous extract induced apoptosis in Burkitt's lymphoma BL41 cell line by triggering the intrinsic pathway (18). Besides, *B. dioica* aqueous extracts exhibited promising cytotoxic activities against different blood and breast cancer cell lines (data not published).

As part of our continuing work to evaluate the anticancer activity of Algerian medicinal plants used in cancer treatment, the present study aimed to evaluate apoptogenic activity and identify the major bioactive compound of *B. dioica*.

Materials and methods

Reagents. Cell culture media, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) and Propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC was from BD Biosciences (Franklin Lakes, NJ, USA). The other generic chemicals were from Sigma-Aldrich, Roche Applied Science (Mannheim, Germany) or Merck KGaA, Darmstadt, Germany.

Cells and culture conditions. The human triple-negative breast cancer MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in DMEM medium with Glutamax supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 in air at 37˚C. The experiments were performed three times using cells in the exponential growth phase.

Preparation of B. dioica aqueous extract. The roots of *B. dioica* were collected in March 2012 in Mascara, Algeria. Botanical identification and authentication were done by Dr Kada Righi (Department of Agriculture, Faculty of Nature and Life sciences, Mascara University, Mascara, Algeria). A voucher specimen of the plant (voucher no. LRSBG/B-2012/003) was deposited in the herbarium of the laboratory, Department of Biology, Faculty of Nature and Life sciences, Mascara University, Mascara, Algeria. The collected roots were dried at room temperature, pulverized and finely sieved. The *B. dioica* aqueous extract was prepared as follows: the dried roots were boiled for 20 min at 100˚C, cooled to room temperature, and then filtered. The filtered solution was collected, concentrated, lyophilized and stored in a desiccator at +4˚C until used.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The effects of the *B. dioica* aqueous extract on MDA-MB-231 cells viability were determined by the colorimetric MTT assay. Briefly, MDA-MB-231 cells were seeded at a density of $8x10^3$ cells/well in 96-well plates and incubated for 24 h at 37˚C. Thereafter, cells were treated with increasing concentrations (from 0 to 500 μg/ml) of *B. dioica* aqueous extract for 24, 48, and 72 h. At the end of the treatment, 50 μl of MTT (0.5 mg/ml) was added and the cells were incubated at 37˚C, 5% CO_2 for 1 h. After medium removal, 500 μl of DMSO was added to each well to dissolve the formazan formed during the reaction and the plate was then shaken for 10 min under obscurity. The absorbance was recorded at 570 nm using a 96-well plate reader (ASYS-UVM-340). All the experiments were performed in triplicate.

Detection of apoptosis-Annexin V-FITC/PI staining. Apoptosis induction was assessed using the Annexin V-FITC assay. Briefly, MDA-MB-231 were treated with 50 and 250 μg/ml of *B. dioica* aqueous extract for 48 h. Cells were harvested, re-suspended in the ice-cold 1x binding buffer, and then incubated with Annexin V-FITC and PI solutions for 15 min at room temperature in the dark. After incubation, the cells were analyzed using FACSCalibur, BD Biosciences (19).

Cell cycle analysis. Following exposure to *B. dioica* aqueous extract (50 and 250 μg/ml) for 48 h, MDA-MB-231 cells were collected and fixed with cold 70% ethanol and stored overnight at -20˚C. Cells were washed, re-suspended in PBS and incubated at 37˚C for 30 min with 10 mg/ml RNase and 1 mg/ml propidium iodide (PI). DNA content was then determined using a FACSCalibur flow cytometer (BD Biosciences) (20).

UV-vis analysis. UV-vis analysis of *B. dioica* aqueous extract was performed on a Shimadzu spectrophotometer (λ=200-800 nm) as described by (21). The absorption peak values were recorded.

Chromatographic analyses. To determine the major compounds in the *B. dioica* aqueous extract, we performed liquid chromatography-mass spectrometric analyses using HPLC Agilent 1100 (Agilent Technologies GmbH, Waldbronn, Germany) coupled to an ultraviolet detector and to an Agilent Trap XCT mass spectrometer equipped with an electrospray (ESI) source with a nebulizer spacer as previously described (22).

Statistical analysis. Mean data values are presented, with their standard deviations (mean ± SD). The statistical comparisons were made by one-way analysis of variance followed by Bonferroni's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

B. dioica aqueous extract induced cell growth inhibition of MDA-MB-231. In the present study, we investigated the effects of an aqueous extract of *B. dioica* roots on cell viability *in vitro*, by incubating MDA-MB-231 cells with increasing concentrations (from 0 to 500 μg/ml) of the extract. After 24, 48 and 72 h, cell viability was determined by the colorimetric MTT assay. We determined survival as a percentage of that for untreated cells. As shown in Fig. 1, *B. dioica* aqueous extract induced cell growth inhibition in a time-dependent manner. At 50 μg/ml, *B. dioica* aqueous extract resulted in 50.36, 72.39 and 91.15% inhibition of cell viability of MDA-MB-231 cells after 24, 48 and 72 h, respectively.

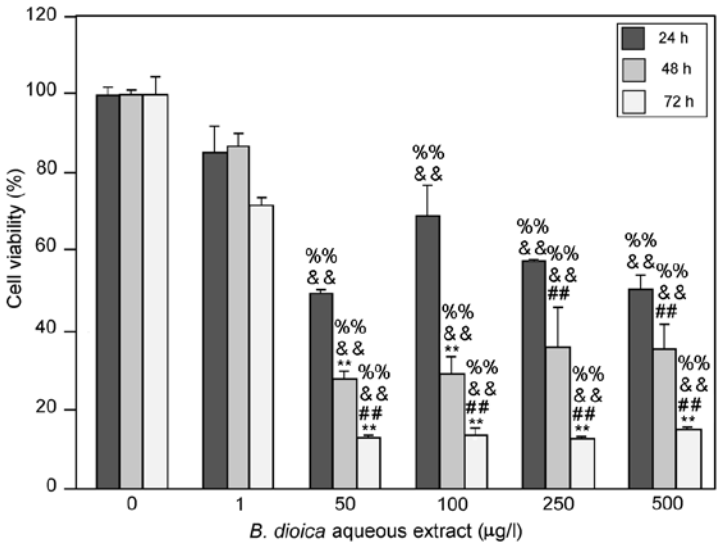

Figure 1. Effect of *B. dioica* aqueous extract on viability of MDA-MB231 cells. Cells were treated with increasing concentrations (between 0.00 and 500 μg/ml) of *B. dioica* aqueous extract for 24, 48 and 72 h; cell viability was measured by an MTT assay. Percentage of survival was determined as compared with the untreated cells. The experiments were performed in quadruplicate. Statistical comparisons were using the one-way analysis of variance followed by Bonferroni's post hoc test. **P<0.01 vs. respective 24 h; $^{\#\#}$P<0.01 vs. respective 48 h; $^{\&\&}$P<0.01 vs. respective group at 0 μg/ml *B. dioica*; $^{\%\%}$P<0.01 vs. 1 μg/ml *B. dioica*.

The highest inhibitory effect was produced at concentrations of 50 μg/ml or higher after 72 h of treatment (91.15±0.71%). Interestingly, this effect remains unchanged at higher concentrations (100, 250 and 500 μg/ml).

B. dioica aqueous extract induced apoptosis of MDA-MB-231. We then investigated whether this decrease in cell viability was associated with the induction of apoptosis by incubating MDA-MB-231 cells with 50 and 250 μg/ml of *B. dioica* aqueous extract for 48 h. The apoptotic cell death rate was estimated with Annexin V-FITC and PI double staining by flow cytometry. The results showed that *B. dioica* aqueous extract induced MDA-MB-231 apoptosis in a dose-dependent manner (Fig. 2). Indeed, apoptotic cells elevated significantly from 38.95 to 64.61% of the MDA-MB-231 cells exposed to 50 and 250 μg/ml, respectively. However, the apoptosis rate in untreated cells was only 0.68%.

B. dioica aqueous extract induced cell cycle arrest of MDA-MB-231. To examine the effect of *B. dioica* aqueous extract on the cell cycle progression, MDA-MB-231 cells were incubated for 48 h in the absence or presence of 50 and 250 μg/ml of the extract. Cell nuclei were stained with PI and the percentage of cells in each phase of the cell cycle were determined by flow cytometry. As shown in Fig. 3, our data revealed that treatment with *B. dioica* aqueous extract caused cell cycle arrest of MDA-MB-231 cells at G2/M phase. In fact, the percentage of cells in G2/M increased from 15.7% (untreated cells) to 59.13% (50 μg/ml) and 58.51% (250 μg/ml). Moreover, *B. dioica* aqueous extract resulted in a dose-dependent decrease in the population of cells in the G0/G1 phase from 65.82% (untreated cells) to 23.00, and 11.80% after treatment with 50 and 250 μg/ml, respectively.

Major compounds of B. dioica aqueous extract. The UV-vis spectrum of *B. dioica* aqueous extract was characterized by two major absorption bands: 350-385 nm (Band I, cinnamoyl system) and 250-280 nm (Band II, benzoyl system), corresponding to flavonol structure.

As shown in Fig. 4, peak 2 corresponded to the major compound found in the *B. dioica* extract. This compound was detected at 2.3 min of retention time and represented 75.3% of the extract.

The MS spectra (Fig. 5) of the major compound (peak 2) showed a molecular ion of m/z=318.5 and two main fragments (m/z=230.4 and 274.5). According to the obtained data (Table I) and in comparison with literature, we suggest that the major compound in *B. dioica* aqueous extract could be myricetin (Fig. 6).

Discussion

Natural products provide an appreciable percentage of new active lead molecules, and drugs despite competition from different methods of drug discovery (23). Medicinal plants constitute a common alternative for cancer treatment by providing cytotoxic and apoptogenic agents (24-27). We have previously found that the aqueous extract of *B. dioica* roots exerted a promising anticancer activity against Burkitt's lymphoma BL41 cells. This cytotoxic effect was accompanied by the induction of apoptosis (18). Likewise, we have demonstrated that the extract was able to inhibit different cancer cell lines growth including those of multiple myeloma, lymphoma and triple negative breast cancer (data not published). In the current study, we showed that *B. dioica* aqueous extract induced cell growth inhibition of breast cancer MDA-MB-231 cells in a time-dependent manner. At 50 μg/ml, the extract suppressed effectively the proliferation of MDA-MB-231 cells (91.15% inhibition of proliferation) after 72 h. Recently, Sahpazidou *et al* (28) evaluated the cytotoxic effects of polyphenolic extracts from grape stems against various cancer cell lines (breast, kidney, colon, and thyroid). After 72 h of treatment, MDA-MB-231 cells were the most sensitive to all

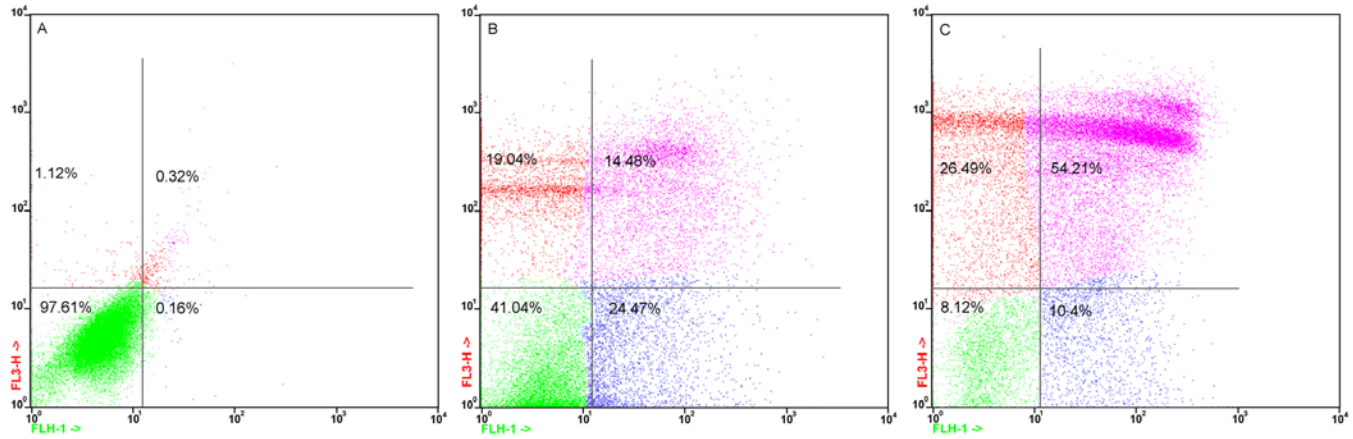

Figure 2. Apoptosis induction in MDA-MB-231 by *B dioica* aqueous extract as assessed by Annexin V/PI assay. Apoptosis in (A) control cells, and cells treated with (B) 50 and (C) 250 μg/ml *B. dioica*. Viable cells are AnnexinV-and PI-, early apoptotic cells are Annexin V+ and PI-, and late apoptotic cells are Annexin V+ and PI+. The experiments were performed in triplicate. PI, propidium iodide.

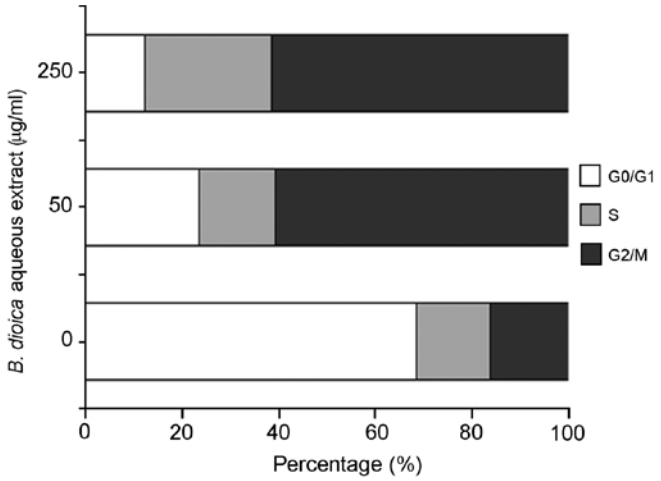

Figure 3. Effect of *B. dioica* aqueous extract on cell cycle progression. MDA-MB-231 cells were treated for 48 h with 50 and 250 μg/ml of *B. dioica aqueous* extract. Cell cycle analysis was conducted following propidium iodide staining. The experiments were performed in triplicate.

tested extracts, with IC_{50} values of 120 and 184 μg/ml. We then investigated the apoptotic effects of *B. dioica* aqueous extract in MDA-MB-231 cells.

Apoptosis is one of the main types of programmed cell death and can be triggered by a variety of stimuli received by the cells (29). Induction of apoptosis in the activated cancer cells may be an effective strategic approach for cancer therapy (30). We examined the induction of apoptosis with Annexin V-FITC and PI double staining by flow cytometry. Our results showed that the proportion of apoptotic cells significantly elevated in MDA-MB-231 (50 and 250 μg/ml)-treated cells from 0.68% in untreated cells to 35.95 and 64.61%, respectively. These results are in agreement with our previous study demonstrating that the *B. dioica* aqueous extract was able to induce apoptosis of Burkitt's lymphoma BL41 cells in a dose-dependent manner. Apoptosis induction was accompanied by triggering the intrinsic pathway (activation of caspase-3 and -9, cleavage of PARP and loss of mitochondria membrane potential) (18). Moreover, it has been demonstrated that several herbal extracts caused MDA-MB-231 cells growth inhibition through apoptosis induction at high concentrations (31,32). Our data showed that *B. dioica* aqueous extract was able to induce marked apoptosis at a lower concentration (50 μg/ml) in MDA-MB-231 cells, which are known to be resistant to apoptosis (33).

Generally, cell cycle arrest and induction of apoptosis are connected, an occurrence of cell cycle arrest leads to cell apoptosis (34). In the present study, the progression of cell cycle was assessed by propidium iodide (PI) staining of cell DNA after incubation of cells with *B. dioica* aqueous extract. Besides apoptosis induction, the cytotoxic effect caused by *B. dioica* aqueous extract was further due to a cell cycle arrest of MDA-MB-231 cells at G2/M phase. In fact, treatment with *B. dioica* aqueous extract resulted in an accumulation of MDA-MB-231 cells in the G2/M phase (15.7% of untreated cells vs. 59.1% of treated cells with 50 μg/ml). This effect may be attributed to the presence of flavonols (major compounds in the extract). It has been demonstrated that flavonols such as quercetin or kaempferol induce G2/M phase cell cycle arrest in different cancer cell lines (35). Zhang *et al* (36) demonstrated that three flavonols (Kaempferol, quercetin, and myricetin) exerted cytotoxic effects on a human oesophageal adenocarcinoma cell line (OE33) by inducing G2/M arrest. Similarly, quercetin, myricetin, laricitrin, and syringetin were capable of inhibiting the proliferation of colorectal epithelial adenocarcinoma cells via cell cycle arrest in the G2/M phase (37).

Taken together, our data demonstrate that *B. dioica* aqueous extract-mediated inhibition of MDA-MB-231 cell growth may be the result of apoptosis induction and cell-cycle arrest in the G2/M phase.

The UV-vis absorption spectra of *B. dioica* aqueous extract showed two absorption bands characteristic of flavonol skeleton, 350-385 nm (Band I) and 250-280 nm (Band II) indicating that the major compounds in the extract are flavonols (38). Flavonols are characterized by fully unsaturated C-rings that connect the A and B rings in a single conjugated system. All flavonoids have aromatic chromophores, as indicated by UV absorptions in the 250 nm region of their UV spectra (Band B) (39). Band A lies in the 350-385 nm range for flavonols (40). Flavonols, considered as the strongest antioxidant flavonoids, exhibit important antioxidant activity, mainly based on scavenging of oxygen radicals (41). Moreover,

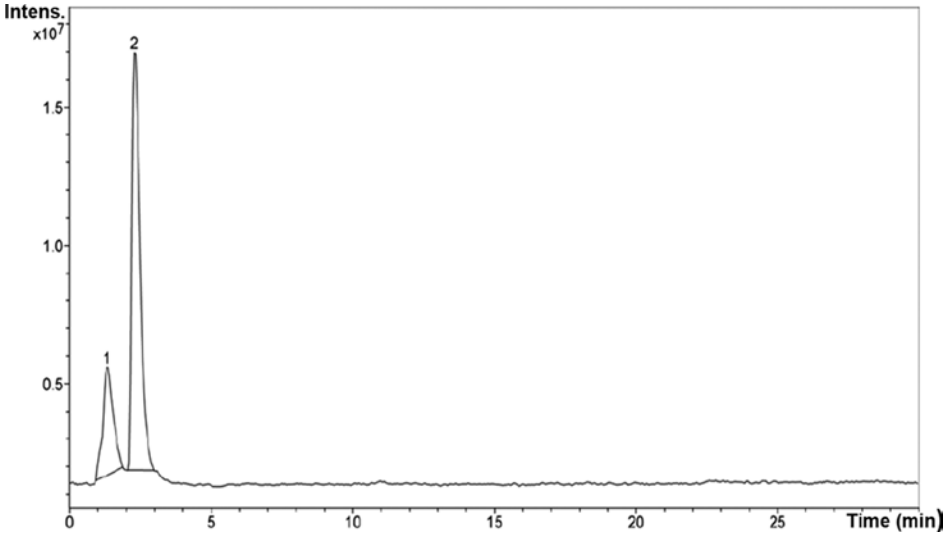

Figure 4. High-performance liquid chromatogram of *B. dioica* extract (254 nm).

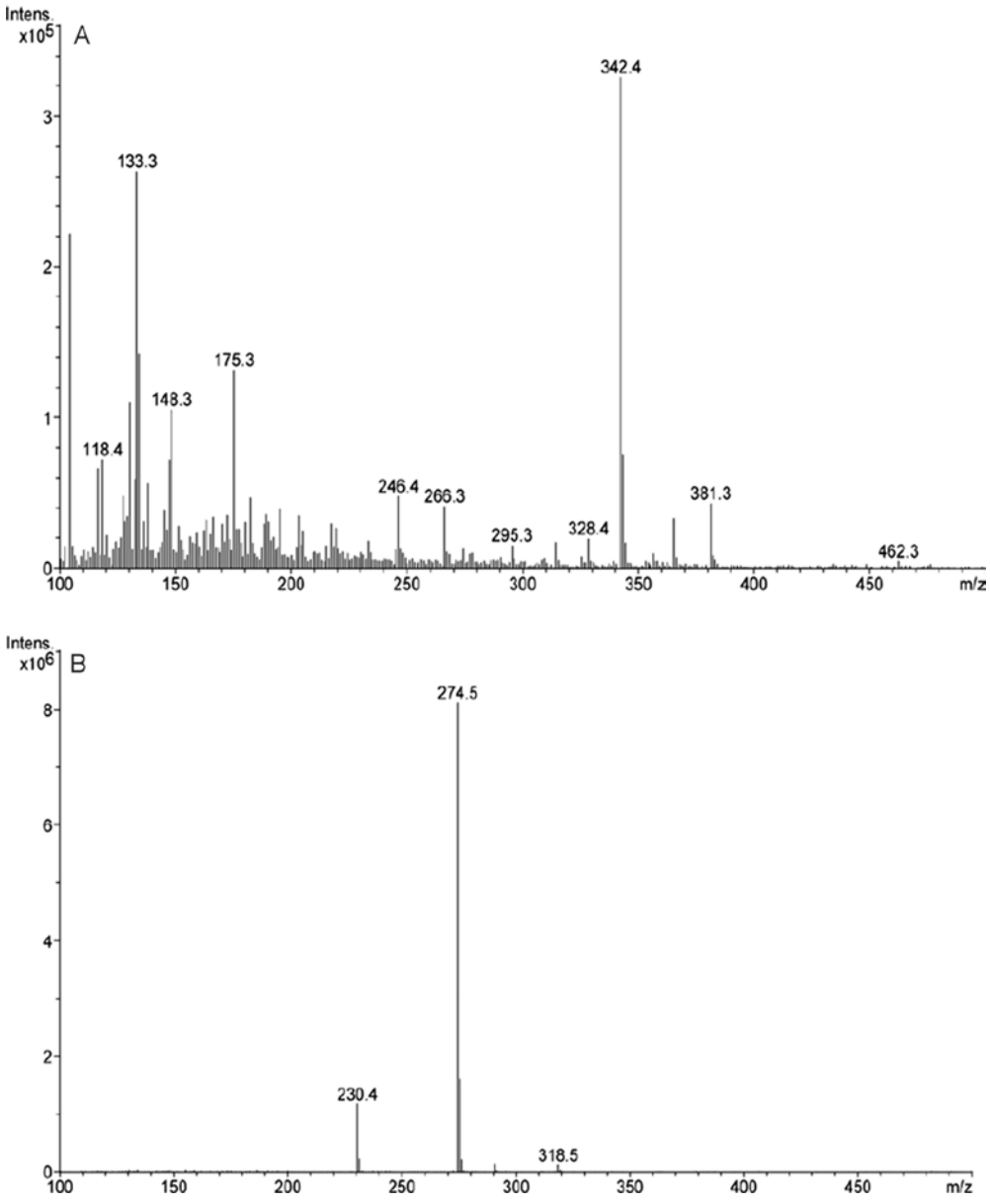

Figure 5. Mass spectroscopy of *B. dioica*. (A) MS spectra corresponding to the peak found at Rt=1.3 min. (B) MS spectra corresponding to the peak found at Rt=2.3 min. MS, mass spectrometry.

Table I. Compounds identified in *B. dioica* aqueous extract in positive-ion mode.

Peak number	RT, min	Area fraction percentage	(M-H)+(m/z)	MS^2 Characteristic ions (m/z)	Compound
1	1.3	24.7	-	-	-
2	2.3	75.3	318.5	318.5, 274.5, 230.4	Myrecitin

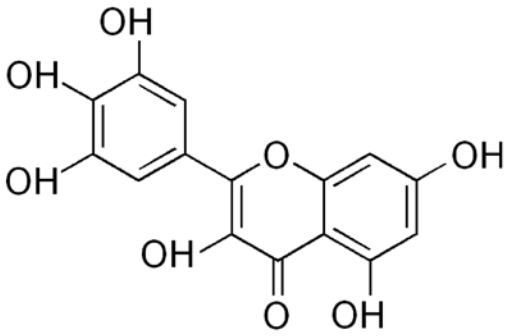

Figure 6. Chemical structure of myricetin.

their increased intake has been correlated with a reduced risk of various cancers such as ovarian, breast, prostate, lung and liver cancers (42). These cancers were found to be of the most frequent malignancies in Algeria. Previously, Barros *et al* (2011) (43) reported the presence of one flavonol in *B. dioica* roots: Kaempferol 3, 7-di-O-rhamnoside. Kaempferol has been demonstrated to possess anticancer and apoptogenic activity against a variety of cancer cell lines such as myelogenous leukemia cell line K562, promyelocytic human leukemia U937 (44), Human lung non-small carcinoma H460 cell line (45), breast cancer MDA-MB-231 cell line (46) and oral cancer cell lines (SCC-1483, SCC-25 and SCC-QLL1) (47).

In the present study, we identified Myricetin (2,5,7,3,4,5-pentahydroxylflavonol) as the major compound in *B. dioica* aqueous extract. Myricetin the most common flavonoid found in herbs, vegetables, and fruits, has been shown to possess important biological activities including antioxidant, antimicrobial, antidiabetic and anticarcinogenic effects (48). Anticancer and apoptogenic activities of myricetin have been demonstrated against several cancers such as colorectal cancer (49), ovarian cancer (50,51), leukemia (52) or lung cancer (53). Furthermore, it has been reported that myricetin was able to cause cell death in different cancer cell lines by arresting the cell cycle at different phases (54). In fact, Myricetin arrested the cell cycle of cancer cells by triggering CDKs and cyclins (55,56).

Myricetin was found to be the major component in different herbal extracts inducing apoptosis and/or cell cycle arrest in human breast cancer cells (57). *In vivo*, myricetin was found to be more effective than vincristine in chemoprevention of dimethyl benzanthracene-induced-breast cancer in female Wistar rats (58). Interestingly, myricetin was not cytotoxic towards normal breast cells, justifying its ability to be considered for *in vivo* studies (59).

Recently, a second-generation myricetin analog: Oncamex has been demonstrated to induce apoptosis and cell cycle arrest in MCF-7, MDA-MB-231, BT-549 and HBL-100 breast cancer cell lines. In addition, the myricetin analog exhibited important anti-breast cancer activity in mice implanted with MDA-MB-231 xenografts (60).

In conclusion, we demonstrate a strong cytotoxic effect of the *B. dioica* aqueous extract against breast cancer MDA-MB-231 cells line. *B. dioica* aqueous extract was able to induce marked apoptosis and cell cycle arrest at G2/M phase of MDA-MB-231 cells at a lower concentration (50 μg/ml). The phytochemical study (UV-vis and LC-MSD-Trap-XCT) revealed the presence of myricetin as the major compound that may contribute to the apoptogenic activity of the *B. dioica* aqueous extract. Thus, *B. dioica* could be considered as a promising source for developing novel therapeutics against breast cancer.

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Availability of data and materials

The datasets used, generated and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BB and AP designed the study. BB and AE performed the experiments. BB wrote the manuscript. BB and AP contributed to the manuscript revisions.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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