**Bryonia dioica** aqueous extract induces apoptosis through mitochondrial intrinsic pathway in BL41 Burkitt’s lymphoma cells

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**A R T I C L E   I N F O**

Article history:
- Received 20 August 2011
- Revised 16 January 2012
- Accepted 7 February 2012
- Available online 21 March 2012

**Keywords:**
- Bryonia dioica
- Apoptosis
- Burkitt’s lymphoma
- BL41 cell lines
- Mitochondrial pathway
- Caspase
- Phytochemicals

**A B S T R A C T**

**Ethnopharmacological relevance:** *Bryonia dioica* Jacq. is a climbing perennial herb with tuberous roots which is widely used in traditional medicine in Algeria for the treatment of cancers; it belongs to the genus *Bryonia* (Cucurbitaceae).

**Aim of the study:** To investigate the cytotoxic and apoptogenic activities, the phytochemical composition and acute toxicity of the aqueous extract of *Bryonia dioica* roots growing in Algeria.

**Materials and methods:** Dried roots of *Bryonia dioica* were extracted with water (decoction). The cytotoxic effects of the aqueous extract in the Burkitt’s lymphoma BL41 cell lines were evaluated by flow cytometry. Apoptosis induction was assessed by two corroborative assays: propidium iodide (PI) staining of cell DNA and flow cytometric light scatter analysis. The mitochondria membrane potential was investigated using a fluorescent dye DIOC6. The expression of caspases-3, -8, -9 and PARP was assessed by Western blot. The phytochemical screening of the roots of *Bryonia dioica* was performed using qualitative phytochemical standard procedures.

**Results:** The *Bryonia dioica* aqueous extract induced cell death in a dose-dependent manner. The IC50 of *Bryonia dioica* aqueous extract was estimated to be approximately 15, 63 μg/ml. This was accompanied by induction of apoptosis, activation of caspase-3 and -9, cleavage of PARP and loss of mitochondria membrane potential. Furthermore, the phytochemical screening of roots of *Bryonia dioica* showed the presence of various bioactive such as polyphenols, sterols and triterpenes, alkaloids, c-heterosides, carbohydrates and saponins.

**Conclusion:** The aqueous extract of *Bryonia dioica* induces apoptosis in the Burkitt’s lymphoma BL41 cell lines via the mitochondrial pathway. The flavonoids, sterols and triterpenes detected could be responsible for the cytotoxic and apoptogenic activities of the aqueous extract of *Bryonia dioica*. These findings suggest that *Bryonia dioica* could be considered as a promising source for developing novel therapeutics against Burkitt’s lymphoma.

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1. **Introduction**

Plants have played a vital role in the prevention and treatment of disease since prehistoric times (Assefa et al., 2010). According to the WHO, it is estimated that 60% of antitumoral medicines are derived, either directly or indirectly, from medicinal plants (Robinson and Zhang, 2011). A large majority of anticancer drugs used currently, or still undergoing clinical trials, are derived from natural sources (Effert et al., 2007; Laouié et al., 2008).

*Bryonia dioica* Jacq. a climbing perennial herb with tuberous roots which occurs in temperate Europe, North Africa, and western Asia (Sallam et al., 2010), belongs to the genus *Bryonia* in which some species may contain cytotoxic cucurbitacines (Frohne et al., 2009; Sallam et al., 2010). *Bryonia dioica* is used for both internal and external uses (Leporatti and Ghedira, 2009). It is taken orally in small quantities for the treatment of various inflammatory conditions, bronchial complaints, asthma, intestinal ulcers, hypertension and arthritis. Externally, it is applied as a rubefacient to muscular and joint pains and pleurisy. It has been reported that the plant is used in folk medicine as a drastic purgative, emetic, bitter tonic and anti-diabetic agent (Matsuda et al., 2010). In different European countries it is considered to be a cicatrising agent (Guarrera and Lucia, 2007). *Bryonia dioica*, popularly named in
Algeria “Fachira” and “queriou’āa” by the locals, is widely used in traditional medicine (Baba Aissa, 1999) to treat muscular–skeletal problems and cancer (González-Tejero et al., 2008).

There is an abundance of medicinal plants in Algeria but many of these plants have yet to be investigated for their phytochemical and biological properties (Rached et al., 2010). This study was undertaken to determine the scientific basis for the traditional uses of Bryonia dioica as an anticancer plant. The cytotoxic and apoptogenic activities of the aqueous extract of Bryonia dioica in Burkitt’s lymphoma BL41 cell lines were investigated. In addition, the phytochemical composition was studied.

2. Materials and methods

2.1. Chemicals

Anti-caspase-3 (9662) and anti-caspase-9 (9502) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-caspase-8 (5F7) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-PARP-1 was from BD Pharmingen (Franklin Lakes, NJ, USA). (TU-02) antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Propidium iodide was from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cells and culture conditions

The Burkitt’s lymphoma BL41 cell line was provided by Dr. Aimé Vazquez (U1014, INSERM, Hopital Paul Brousse, Villejuif, France). The cells were cultured in RPMI 1640 medium with Glutamax supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, sodium pyruvate, and 1% nonessential amino acids (Life Technologies, Strasbourg, France) in a humidified atmosphere with 5% CO2 in air at 37°C.

2.3. Preparation of Bryonia dioica aqueous extract

The roots of Bryonia dioica were collected in March, 2010 in Mascara, Algeria. Botanical identification and authentication was done by Dr. Kada Righi (Department of Agriculture, Faculty of Nature and Life sciences, Mascara University, Algeria). A voucher specimen of the plant (voucher number: SNV/B-2010/019) was deposited in the herbarium of the Department of Biology, Faculty of Nature and Life Sciences, Mascara University, Algeria. The collected roots were dried at room temperature, pulverized and finely sieved. The Bryonia 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.

2.4. Detection of apoptotic cells

2.4.1. Analysis of dot-blot light scatter profiles

Cells were seeded in 96-well plates at a density of 1 × 10⁵ cells/ml and were incubated with Bryonia dioica aqueous extract (0–500 μg/ml) for 24 h. Apoptotic cells were detected by flow cytometry as described in Schrantz et al. (2001).

Cells were washed in phosphate-buffered saline (PBS), pelleted and resuspended in PBS. Their dot blot light scatter profiles were analyzed by flow cytometry using a FACSScan flow cytometer (Accuri C6, Accuri Cytometers Inc., Ann Arbor, MI, USA). Apoptotic cells cause lower forward light scatter (caused by cell shrinkage) and higher side scatter (caused by increased granularity of the cell, presumably as a result of chromatin condensation and fragmentation) than their viable counterparts (Shan et al., 1998). Shrunken cells with relatively high side-scatter and low forward-scatter properties were considered to be apoptotic and enumerated as a percentage of the total population.

2.4.2. Hypodiploid DNA

Following exposure to Bryonia dioica aqueous extract for 24 h, BL41 cells (10⁶) were washed in PBS and resuspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) as described by Clybouw et al. (2005). Samples were placed at room temperature for 1 h before flow cytometric analysis of the propidium iodide fluorescence of individual nuclei using a FACSscan flow cytometer (Accuri C6, Accuri Cytometers Inc., Ann Arbor, MI, USA). Debris was excluded from analysis by raising the forward scatter threshold. The DNA content of the intact nuclei was registered on a logarithmetic scale. Apoptotic cells were identified on the basis of their nuclei having hypodiploid DNA, emitting fluorescence in channels 10–200. These cells were counted, and their number was expressed as a percentage of the total population (Clybouw et al., 2005).

2.5. Analysis of mitochondrial membrane potential

The loss of mitochondrial membrane potential was assessed by flow cytometry according to the method of El Mchichi et al. (2007). Briefly, ΔΨm was evaluated by staining cells (10⁶) with DIOC6 at a final concentration of 40 nM (stock solution 40 mM in ethanol) for 15 min at 37°C in the dark. The fluorescence emitted by cells was analyzed with a FACSScan flow cytometer (Accuri C6, Accuri Cytometers Inc., Ann Arbor, MI, USA) using the fluorescence signal 1 channel. Cells with low ΔΨm were counted and their number expressed as a percentage of the total population.

2.6. Western blot analysis

Cytosolic protein extracts were prepared as described by Qi et al. (2010). Briefly, treated and untreated cells were collected by centrifugation at 300 × g for 5 min at 4°C, washed with icecold PBS twice and lysed with RIPALysis buffer (20 nM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 mM Protease Inhibitor Cocktail) for 30 min on ice, and debris was removed by centrifugation at 10,000 × g for 10 min. Aliquots of the supernatants were used for protein determination by Bradford method. Equal amounts of protein (30 μg) were subjected to SDS–PAGE, and the proteins were then electrophoretically transferred onto nitrocellulose membranes. The blotted membranes were blocked for 1 h with 5% on fat milk in Tris–buffered saline, 0.1% Tween 20. The membranes were then incubated overnight at 4°C with specific antibodies. Blots were washed three times for 10 min, in Tris-buffered saline, 0.1% Tween 20 and incubated for 1 h with peroxidase-labeled anti-mouse or anti-rabbit immunoglobulins. After washing three times in Tris-buffered saline, 0.1% Tween 20, images were captured using a DDC camera (LAS-1000; Fuji).

2.7. Phytochemical screening

The roots of Bryonia dioica were screened for the presence of phytochemical constituents such as alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins, steroids and glycosides using qualitative phytochemical standard procedures described by Tress and Evans (1983) and Harborne (1998).

2.8. Statistics

All statistical comparisons were made by Student’s t-test and statistical significance was defined as p < 0.05.
Aqueous extract induces apoptosis in BL41 cells. To investigate the effect of cytotoxic activity of aqueous extract of the *Bryonia dioica* on cell viability, BL41 cells were incubated with increasing concentrations. After 24 h, cell viability was determined by flow cytometry. Percent survival was determined as compared to untreated cells. Our results (Fig. 1) show that *Bryonia dioica* aqueous extract induced cell death in a dose-dependent manner.

Apoptotic cells were selected as shrunken cells with high side-scatter (SSC) and low forward-scatter (FSC) properties as assessed by flow cytometry. As shown in Fig. 2 (A), the proportion of apoptotic cells as assessed by cell dot-blot light scatter profiles and flow cytometry increased in a dose-dependent manner.

Propidium iodide (PI) staining of the BL41 cell lines exposed to 125 µg/ml of *Bryonia dioica* extract for 24 h revealed apoptotic induction. Fig. 2(B) showed that the proportion of apoptotic cells significantly elevated in BL41 (125 µg/ml)-treated cells from 2.7% in untreated cells to 15.7%.

Aqueous extract of *Bryonia dioica* induces mitochondrial transmembrane potential ΔΨm loss. BL41 cells with aqueous extract of *Bryonia dioica* at a dose of 125 µg/ml for 24 h. After staining with DiOC6(3), ΔΨm was assessed by flow cytometry, and cells with low ΔΨm were counted and their number expressed as a percentage of the total population.

Disruption of ΔΨm is one of the earliest intracellular events that occur following the induction of apoptosis (Qi et al., 2010). To confirm the involvement of mitochondria during *Bryonia dioica* aqueous extract induced apoptosis, we investigated the changes in mitochondria membrane potential using a fluorescent dye DiOC6 (3). Lower levels of DiOC6 fluorescence intensity suggested impaired mitochondrial membrane potential (Li et al., 2007).

*Bryonia dioica* aqueous extract induced-apoptosis as assessed by cell dot-blot light scatter profiles and flow cytometry (PI staining), was associated with a loss of ΔΨm, as quantified by staining with DiOC6(3) (32.9% vs. 11.6% in control cells) (Fig. 3).

Aqueous extract of *Bryonia dioica* induces cell death via mitochondrial pathway dependent on caspase cascade. To further investigate the molecular mechanism responsible for the *Bryonia dioica* aqueous extract induced apoptosis in BL41 cells, the activation of intrinsic and extrinsic caspase cascades, and other related proteins were investigated by Western blot. As shown in Fig. 4, the treatment of BL41 cells with aqueous extract of *Bryonia dioica* at a dose of 125 µg/ml for 24 h caused activation of caspases 3 and 9 (cleaved forms expressed) but not caspase 8. Activation of caspase-3 was detected as a double band representing the p19 proteolytic fragment, and the active subunit p17, respectively.

The activation of caspase-3 cleaves a variety of cellular proteins, among them PARP, one of the distinctive features of apoptotic cell death (Walczak and Haas, 1998; Karlsson et al., 2004). We investigated the cleavage of poly(ADP-ribose)polymerase (PARP) in untreated and treated cells by detecting the 116 kDa-intact and 89 kDa cleaved PARP identified by Western blotting.

3.2. Phytochemical screening of *Bryonia dioica*

The phytochemical screening of *Bryonia dioica* revealed the presence of polyphenols, sterols and triterpenes, alkaloids, c-heterosides, carbohydrates and saponins (Table 1).

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Result</th>
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<tbody>
<tr>
<td>Phenolic compounds</td>
<td>+</td>
</tr>
<tr>
<td>Gallic tannins</td>
<td>−</td>
</tr>
<tr>
<td>Catechic tannins</td>
<td>−</td>
</tr>
<tr>
<td>Sterols and triterpenes</td>
<td>+</td>
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<tr>
<td>Alkaloids</td>
<td>+</td>
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<tr>
<td>Quinones</td>
<td>−</td>
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<tr>
<td>Carotenoids</td>
<td>−</td>
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<tr>
<td>Anthocyaines</td>
<td>−</td>
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<tr>
<td>Coumarins</td>
<td>−</td>
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<tr>
<td>Anthraquinones</td>
<td>−</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>C-heterosides</td>
<td>+</td>
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<td>O-heterosides</td>
<td>+</td>
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<td>Saponins</td>
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*: presence; −: absence.

4. Discussion

In much of the developing countries, 70–95% of the population rely on traditional medicines for primary care, and between 70% and 90% of populations in industrialized world use traditional medicines under the titles “complementary”, “alternative”, or “nonconventional” (Robinson and Zhang, 2011). Herbal plants and plant-derived medicines have been used as the source of potential anticancer agents in traditional cultures all over the world (Shoeb, 2006).

In the present study, we showed that *Bryonia dioica* aqueous extract treatment of human Burkitt’s lymphoma cells BL41 induced cytotoxic effects in a dose dependent fashion. *Bryonia dioica* aqueous extract at 250 µg/ml suppressed effectively the proliferation of BL41 cells (81.8% inhibition of proliferation), and at 500 µg/ml, the maximal inhibitory dose on the cell proliferation, resulted in 93.5% inhibition of proliferation of BL41 cells. We then investigated the apoptotic effects of *Bryonia dioica* aqueous extract in BL41 cells.

Apoptosis plays a central role in cancer, since its induction in cancer cells is critical to a successful therapy (Bunz, 2001). A wide variety of natural substances have been recognized to have the ability to induce apoptosis in various tumor cells of human origin (Taraphdar et al., 2001). In the current study, apoptosis induction was assessed by two corroborative assays; propidium iodide (PI) staining of cell DNA after incubation of cells with *Bryonia dioica* aqueous extract and flow cytometric light scatter analysis. Flow cytometry data of PI single staining showed that the percentage of apoptotic nuclei (detected by PI staining and flow cytometric analysis after 24 h in culture) increased from 2.7% in the control to 20.7% in treated cells. The flow cytometric light scatter analyses

confirmed these findings. These observations are consistent with cell death by apoptosis (Darzynkiewicz et al., 1994; Ferlini et al., 1996). Our findings suggest that *Bryonia dioica* aqueous extract causes cell death in BL41 cells by inducing apoptosis.

There are two main signaling pathways that are involved in apoptosis, termed intrinsic and extrinsic pathways, regulated via caspase-9 and -8, respectively (Hui et al., 2011). Apoptosis activation by the extrinsic pathway involves the binding of extracellular death ligands (such as TNF ligand/TRAIL) to death receptors, provoking the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD) and recruiting caspase-8 (Dai et al., 2009). Once activated, caspase-8 triggers a caspase cascade and subsequent cell death by activating downstream executioner caspases, such as caspase-3 (Jiang and Wang, 2004).

In the intrinsic pathway, DNA damage induces release of cytochrome c from the intermembrane space leading to failure in maintaining the mitochondrial membrane potential and ATP synthesis (Wang, 2003). The apoptotic protease activating factor 1 (Apaf-1) activates caspase-9 through cleavage, and caspase-9 generates a signaling cascade that results in direct DNA fragmentation (Speirs et al., 2011). Released cytochrome c interacts with Apaf-1, pro-caspase 9 and dATP to form the apoptosome (Thorburn, 2004), which activates downstream effector caspases such as caspase 3 (Schafer and Kornbluth, 2006; Wu et al., 2006). Caspase-3 has been
implicated in the execution phase of apoptosis cleaving over 100 substrates, including PARP, an abundant DNA-binding enzyme that detects and signals DNA strand breaks (Koh et al., 2005; Nawab et al., 2011). At the onset of apoptosis, activated caspase-3 cleaves PARP, which results in the formation of PARP cleavage products, a hallmark of apoptosis (Cohen, 1997; Yang et al., 2008).

To elucidate the molecular mechanism involved in apoptosis by *Bryonia dioica* aqueous extract, expression of apoptotic-related proteins, caspase-3, caspase-8, caspase-9 and PARP were assessed in BL41 cells. The present data showed that the *Bryonia dioica* aqueous extract induced-apoptosis was accompanied by the activation of caspase-3 and caspase-9. The aqueous extract of *Bryonia dioica* resulted in the cleavage of PARP as shown in Fig. 5 confirming the involvement of caspase 3 in the apoptotic events in treated cells.

The activation of caspases 9 and 3 suggests that the aqueous extract of *Bryonia dioica* induced apoptosis through the intrinsic pathway. Mitochondria play an essential role in the propagation of apoptosis, and the disruption of the mitochondrial membrane potential is a critical step occurring in cells undergoing apoptosis (Cui et al., 2007). In the intrinsic or mitochondrial pathway of apoptosis, caspase activation is closely connected to the permeabilization of the outer mitochondrial membrane (Samm et al., 2011). The changes in mitochondria membrane potential were investigated using a fluorescent dye DIOC6 (3). We found that the aqueous extract of *Bryonia dioica* induced loss of mitochondrial membrane potential (ΔΨm), which indicated that the mitochondrial apoptotic death-signal pathway plays a critical role in *Bryonia dioica* aqueous extract-induced apoptosis in BL41 cells.

However, caspase-8 was not activated. Caspase-8 is considered as a signaling and key caspase in extrinsic pathway (Tang et al., 2006); thus, death receptor-induced apoptosis could be ruled out as cause for the mitochondrial activation in the present study.

Taken together, our results demonstrate that *Bryonia dioica* aqueous extract induces apoptosis of BL41 cells through a mitochondria-dependent pathway.

Based on the results of the present study, the mechanism by which *Bryonia dioica* aqueous extract induces apoptosis in BL41 cells is summarized in Fig. 6.

The phytochemical screening of roots of *Bryonia dioica* showed the presence of polyphenols, sterols and triterpenes, alkaloids, c-heterosides, carbohydrates and saponins. However, catechic tannins, gallic tannins, anthocyanes, coumarins, anthraquinones and o-heterosides were not detected. The biological or therapeutic activities of medicinal plants are closely related to their chemical compounds (Hashemi et al., 2008), thus the cytotoxic activity of the *Bryonia dioica* aqueous extract shown in this study.
5. Conclusion

Our present findings provide evidence that the *Bryonia dioica* aqueous extract can induce apoptosis in Burkitt’s lymphoma cells line BL41 by triggering the mitochondria mediated pathway (the disruption of $\Delta \Psi _m$, the activation of caspase-3 and -9, the cleavage of PARP and degradation of PUMA) (Fig. 6). The phytochemical screening revealed the presence of bioactive compounds that may contribute to the apoptogenic activity of the *Bryonia dioica* aqueous extract such as flavonoids, triterpenoids and sterols. Thus, *Bryonia dioica* could be considered as a promising source for developing novel therapeutics against Burkitt’s lymphoma.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

The authors would like to thank Dr. Aimé Vazquez (U1014, INSERM, Hopital Paul Brousse, Villejuif, France) for expert technical assistance.

References


may be attributed to the detected compounds. Our data showed the presence of sterols and triterpenoids. Akhihsa et al. (1996a,b) have isolated and characterized from the roots of *Bryonia dioica* eight sterols, and four triterpenoid alcohols along with forty-one known sterols and triterpenoid alcohols. It was reported that triterpenoids possess strong anticancer activity (Raturi and Sharma, 2011). The flavonoids detected could be responsible for the cytotoxic and apoptogenic activities of the aqueous extract of *Bryonia dioica*. Recently, Barros et al. (2011) have studied the phenolic profile of *Bryonia dioica* and found that the main compounds identified were C-glycosylated flavonoids, five flavones and one flavonol: luteolin 6-C-glucoside-7-O-glucoside, apigenin 6-C-glucoside-7-O-glucoside, luteolin 6-C-glucoside, apigenin 6-C-glucoside, kaempferol 3,7-di-O-rhamnioside, apigenin C-hexoside-O-rhamnosyl-hexoside. The results of the quantification of the phenolic compounds showed a higher content of total flavonoids (2410 mg/kg) and that apigenin 6-C-glucoside-7-O-glucoside was the major compound (1550 mg/kg). Apigenin was found to induce apoptosis through the mitochondria mediated pathway in human lung cancer A549 cells (Lu et al., 2010), human lung cancer H460 cells (Lu et al., 2011), human breast cancer MDA-MB-453 cells (Choi and Kim, 2009), and human osteosarcoma U-2 OS cells (Huang et al., 2010). Marfe et al. (2009) reported that treatment of the chronic myelogenous leukemia cell line K562 and promyelocytic human leukemia U937 with 50 µM kaempferol resulted in induction of mitochondrial release of cytochrome c into cytosol and significant activation of caspase-3, and -9 with PARP cleavage.