ORIGINAL ARTICLE

Aristolochia longa aqueous extract triggers the mitochondrial pathway of apoptosis in BL41 Burkitt's lymphoma cells

Bachir Benarba[1], Gorbatchev Ambroise[2,3], Abdelkader Aoues[4], Boumedienne Meddah[1,5], Aimé Vazquez [2,3]

[1]Department of Biology, Laboratory for Research on Biological Systems and Geomatics, [5]Department of Biology, Laboratoire de Bioconversion, Génie Microbiologie et Sécurité Sanitaire, Faculty of Nature and Life Sciences, University of Mascara, Algeria, [2]Regulation of Cell Survival and Allograft, INSERM UMR-S 1014, Lavoisier Building, Paul Brousse Hospital, Villejuif, France, [3]Department of Medicine, Faculty of Medicine, Paris-Sud University, France, [4]Department of Biology, Laboratory of Experimental Biotoxicology, Biodepollution and Phytoremediation, Es-Senia University, Oran, Algeria

Aristolochia longa (from the family Aristolochiaceae) is widely used for cancer treatment in Algerian traditional medicine. The aim of the present study was to investigate the cytotoxic and apoptogenic activities. The phytochemical composition and acute toxicity of aqueous extracts of the roots of *A. longa* from Algeria. An aqueous extract (decoction) of dried *A. longa* roots was prepared. The cytotoxic effects of this aqueous extract were then evaluated in Burkitt's lymphoma BL41 cells, by flow cytometry. The induction of apoptosis was assessed in two corroborative assays: The propidium iodide staining of cell DNA and flow cytometry analyses of light scattering. Mitochondrial membrane potential was investigated with the fluorescent dye DIOC6. The expression and activation of capases-3, -8 and -9 were assessed by Western blotting. Acute oral toxicity was evaluated with a test dose limited to 5000 mg/kg. Phytochemical screening of *A. longa* roots was performed with standard qualitative procedures. The aqueous extract of *A. longa* induced cell death in a dose-dependent manner. The IC_{50} of *A. longa* aqueous extract was estimated at about 15,63 µg/ml. The extract-induced apoptosis, a loss of mitochondrial membrane potential and the activation of caspases-9 and -3 followed by PARP cleavage. *A. longa* could therefore be considered a promising source of novel treatments for Burkitt's and other lymphomas.

Key words: Apoptosis, *Aristolochia longa*, Burkitt's lymphoma BL41 cell lines, caspase, mitochondrial pathway

INTRODUCTION

Plants are the reservoirs of a large number of imperative organic compounds and they have long been used as the sources of medicines.[1] More than 3000 plants world wide have been reported to possess anticancer properties.[2] The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins.[3] Today, almost 60% of drugs approved for cancer treatment are of natural origin.[4]

A. longa belongs to the genus *Aristolochia* (Aristolochiaceae), which has up to 500 species, most of which are found in tropical, subtropical and Mediterranean regions of the world.[5,6] Anticancer activities have been reported for some species of *Aristolochia,* such as *A. mollissima*,[7] *A. acuminate*,[8] *A. constricta*,[9] *A. rotunda*[10] and *A. clematitis*.[11] *Aristolochia longa*, commonly known as 'Berrostom' to the local population in Algeria, is widely used in traditional medicine. It has been reported that the most widely uses of *A. longa* in Algeria are in cancer treatment.[12-14] The use of this plant as an anti-cancer has been also reported in Morocco.[15]

The aim of this study was to evaluate scientifically the traditional uses of *A. longa,* by studying its effects on the *in vitro* viability of tumour cells. We investigated the cytotoxic and apoptogenic activities of an aqueous extract of *A. longa* in the Burkitt's lymphoma BL41 cell line.

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MATERIALS AND METHODS

Chemicals

Anti-Puma (4976), anti-caspase-3 (9662) and anti-caspase-9 (9502) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), anti-caspase-8 (5F7) antibody from Upstate Biotechnology (Lake Placid, NY, USA), anti-PARP-1 antibody from BD Pharmingen (Franklin Lakes, NJ, USA), anti-tubulin (TU-02) antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Propidium iodide was obtained from

Address for correspondence: Dr. Bachir Benarba, Laboratory for Research on Biological Systems and Geomatics, Faculty of Nature and Life Sciences, University of Mascara, Algeria. E-mail: bachirsb@yahoo.fr
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Sigma Chemical Co (St Louis, MO, USA) and DIOC6 from Molecular Probes (Leiden, The Netherlands).

Preparation of *Aristolochia longa* Aqueous Extract

Roots of *A. longa* were collected in March 2009; in "Tissemssilet" (western Algeria). Dr. Kada Righi (Department of Agriculture, Mascara University, Algeria) was responsible for botanic identification and authentication. The collected roots were dried at room temperature, pulverized and finely sieved. The aqueous extract of *A. longa* was prepared as follows: The dried roots were boiled for 20 min at 100°C, cooled to room temperature and then filtered. The solution passing through the filter was collected, concentrated, lyophilized and stored in a desiccator at +4°C until use.

Cells and Culture Conditions

The Burkitt's lymphoma BL41 cells, provided by Dr. S. Sharma (Brown University, RI) 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, under a humidified atmosphere containing 5% CO_2 in air, at 37°C.

Detection of Apoptotic Cells

Analysis of dot-blot light scatter profiles

Cells were used to seed 96-well plates at a density of 1×10^5 cells/ml and were incubated with *Aristolochia longa* aqueous extract (0–500 µg/ml) for 24 h. Apoptotic cells were detected by flow cytometry, as previously described.[16] Briefly, cells were washed in phosphate-buffered saline (PBS), pelleted and resuspended in PBS. Their dotblot light scatter profiles were analyzed by flow cytometry, with a FACScan flow cytometer (Accuri C6, Accuri Cytometers Inc, Ann Arbor MI, USA). Shrunken cells with relatively high side-scatter and low forward-scatter properties were considered to be apoptotic and were counted. Apoptotic cell counts are expressed as a percentage of the total population.

Hypodiploid DNA

Following exposure to *A. longa* aqueous extract for 24 hours, BL 41 cells (10^6 cells) were washed in PBS and re-suspended in 1 ml of hypotonic fluorochrome solution (50 µg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100), as previously described.[17] Samples were placed at room temperature for 1 h before flow cytometry analysis of the propidium iodide fluorescence of individual nuclei in a FACScan flow cytometer (Accuri C6, Accuri Cytometers Inc, Ann Arbor MI, USA). The DNA content of the intact nuclei was recorded on a logarithmic scale. Apoptotic cells were identified on the basis of their nuclei having hypodiploid DNA.

Analysis of Mitochondrial Membrane Potential (ΔΨm)

The loss of mitochondrial membrane potential was assessed by flow cytometry, as described by.[18] ΔΨm was evaluated by staining cells (10^6 cells) 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 FACScan flow cytometer (Accuri C6), using the fluorescence signal 1 channel. Cells with low ΔΨm were counted and their number is expressed as a percentage of the total population.

Western Blot Analysis

Treated and untreated cells were collected by centrifugation at 300×*g* for 5 min at 4°C, washed twice with ice-cold PBS and lysed by incubation with RIPA lysis buffer (1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 mM Protease Inhibitor Cocktail in 20 mM Tris HCl buffer, p 7.5) for 30 min on ice. The debris was removed by centrifugation at 10,000×*g* for 10 min. Aliquots of the supernatants were used for protein determination by the Bradford method. Equal amounts of protein (30 µg) were subjected to SDS-PAGE, and the proteins were then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked for 1 hour by incubation with 5% nonfat milk powder in Tris-buffered saline supplemented with 0.1% Tween 20. The membranes were then incubated overnight at 4°C with specific antibodies. Blots were washed three times, for 10 min each, in Tris-buffered saline supplemented with 0.1% Tween 20, and were then incubated for 1 h with peroxidase-labeled anti-mouse or anti-rabbit immunoglobulins. They were washed a further three times in Tris-buffered saline supplemented with 0.1% Tween 20 and images were then acquired with a DDC camera (LAS-1000; Fuji).

RESULTS

We investigated the effects of an aqueous extract of *A. longa* roots on cell viability *in vitro*, by incubating BL 41 cells with various concentrations of *A. longa* aqueous extract or control extract. After 24 h, cell viability was determined by flow cytometry. We determined survival as a percentage of that

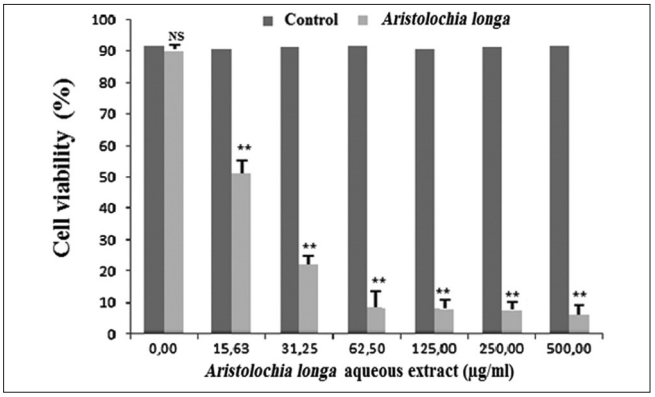

Figure 1: Effect of *Aristolochia longa* aqueous extract on the viability of BL 41 cells. **The difference in cell viability between untreated cells and cells treated with *Aristolochia longa* aqueous extract was highly significant (P<0.001)

for untreated cells. *A. longa* aqueous extract induced cell death in a dose-dependent manner [Figure 1]. By contrast, no decrease in cell viability was observed with the control extract. The IC_{50} of *A. longa* aqueous extract was estimated at 15.63 µg/ml. We then investigated whether this decrease in cell viability was associated with the induction of apoptosis. We used a flow cytometry-based approach to quantify apoptotic cells as shrunken cells with high side-scatter (SSC) and low forward-scatter (FSC) properties.[16] The proportion of apoptotic cells, as determined on the basis of cell dot-blot light scatter profiles, increased in a dose-dependent manner in the presence of *A. longa* aqueous extract. Stimulation with *A. longa* extract also promoted the appearance of nuclear features of apoptosis, such as hypodyploidy, as revealed by propidium iodide (PI) staining. Indeed, 20.7% of the BL41 cell exposed to 125 µg/ml *A. longa* extract for 24 hours had hypodiploid nuclei, versus only 2.7% of control cells [Figure 2]. We investigated the involvement of mitochondria in *A. longa*-induced apoptosis, by evaluating changes in mitochondrial membrane potential with the fluorescent dye DIOC6(3).[19] *A. longa* stimulation was associated with a loss of ΔΨm, (53 *vs*. 11.6% in control cells), indicating an ability of *A. longa* extract to induce mitochondrial dysfunctions in BL41 cells [Figure 3].

We further investigated the molecular mechanism responsible for the induction by *A. longa* aqueous extract of apoptosis in BL41 cells, by studying the activation of various caspases involved in the intrinsic (caspase-9) and extrinsic (caspase-8) pathways of apoptosis. The treatment of BL41 cells with *A. longa* aqueous extract at a concentration of 125 µg/ml for 24 hours led to the activation of caspases-9 and -3, as shown by the disappearance of the inactive proforms and the appearance of active cleaved forms [Figure 4]. By contrast, no activation of caspase-8 was observed in this context. Activated caspase-3 cleaves various cellular proteins, including PARP, this cleavage being one of the most distinctive features of apoptotic cell death.[20] For confirmation of the ability of *A. longa* extract to activate caspase-3, we investigated PARP cleavage in untreated and treated cells, by Western blot analysis of the 116 kDa-intact and 89 kDa cleaved PARP proteins. Activation by incubation with *A. longa* aqueous extract resulted in PARP cleavage [Figure 5], confirming the involvement of caspase-3 in this process.

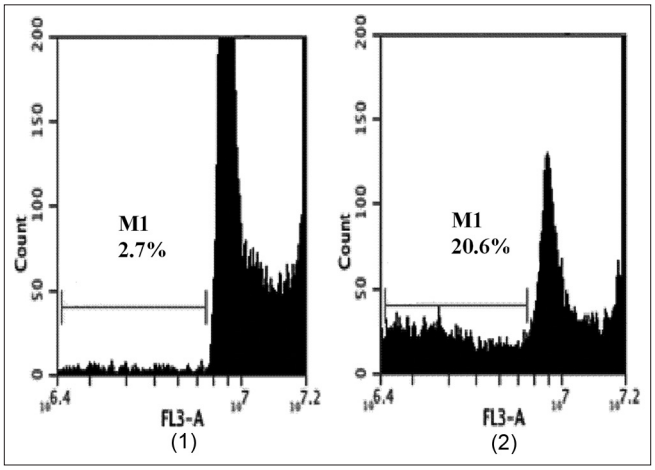

Figure 2: *Aristolochia longa* aqueous extract induces the apoptosis of BL 41 cells

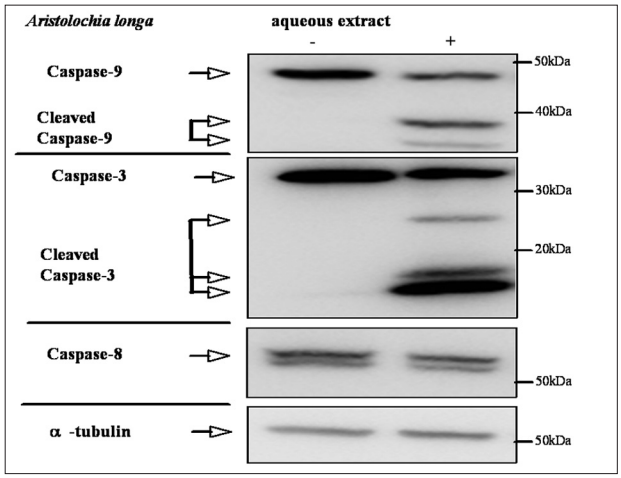

Figure 4: *Aristolochia longa* aqueous extract induces activation of the intrinsic/ mitochondrial apoptotic pathway

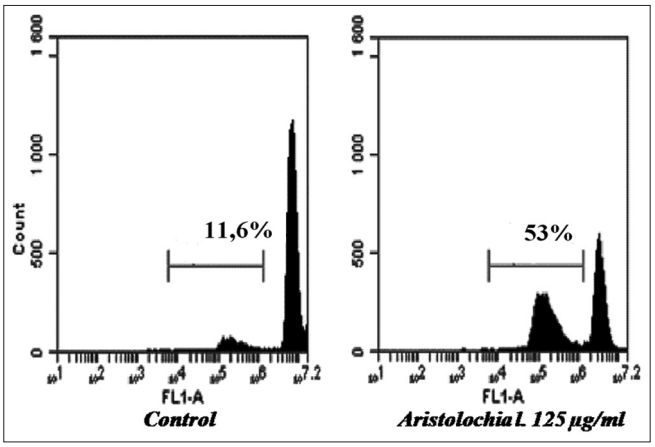

Figure 3: *Aristolochia longa* aqueous extract induces a loss of ΔΨm BL41 cells were incubated with *Aristolochia longa* aqueous extract

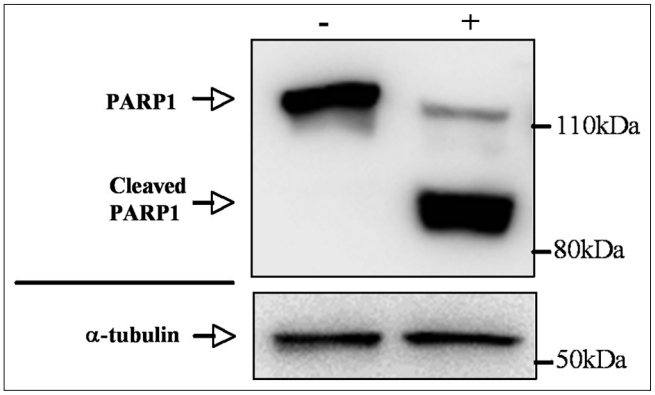

Figure 5: *Aristolochia longa* aqueous extract induced PARP cleavage

DISCUSSION

Searching for new biologically active compounds, novel chemotherapeutic agents derived from active phytochemicals, could be used to improve the anti-carcinogenicity of standard drug treatment.[21] According to the WHO, about 60% of anti-tumoral medicines are thought to have been derived, either directly or indirectly, from medicinal plants.[22] *A. longa* is widely used in traditional medicine in Algeria.[12-14] In this study, we investigated the cytotoxic and apoptogenic effects of *A. longa* aqueous extract in BL 41 cells. No previous study has investigated the cytotoxic and apoptogenic effects of this plant. We observed that *A. longa* aqueous extract caused marked cell growth inhibition of BL 41 cells in a dose-dependent manner. The administration of 62.50 µg/ml *A. longa* aqueous extract almost abolished the proliferation of BL 41 cells (91.2% inhibition of proliferation), and the use of this extract at a concentration of 500 µg/ml, the maximal dose used, resulted in 93.5% inhibition of the proliferation of BL 41 cells. We subsequently showed that the induction of apoptosis was responsible for the inhibition of tumor cell growth. The induction of apoptosis in tumor cells has been shown to be the most common anti-cancer mechanism conjoint by many cancer therapies.[23] We assessed apoptosis induction in two corroborative assays: The PI staining of cellular DNA after the incubation of cells with *A. longa* aqueous extract and flow cytometric light scatter analysis. In the PI staining assay, the percentage of apoptotic nuclei detected by PI staining and flow cytometry after 24 hours in culture was only 2.7% for control cells, but 20.7% for treated cells. Similar results were obtained in flow cytometric light scatter analyses.

Apoptosis pathways can generally be divided into signaling via the death receptors (extrinsic) or the mitochondria (intrinsic) pathways.[24] The extrinsic pathway is characterised by the activation of cell surface death receptors following binding of their specific ligands. It is mediated by death receptors, a subgroup of the tumour necrosis factor (TNF) receptor superfamily, including CD95 (also known as Fas), TNF-related apoptosis-inducing ligand (TRAIL), and TNF-R1.[25] The activation of procaspase-8 results in the formation of the death-inducing signaling complex (DISC), which attaches to procaspase-8, leading to autoproteolytic cleavage and the release of active caspase-8.[26] Active caspase-8 then activates effector caspase-3 and/or -7.[27] The intrinsic pathway centers on mitochondria as initiators of cell death. Multiple signals converge on mitochondria, including DNA damage, microtubule disruption, and growth-factor deprivation, causing these organelles to release cytochrome c (cyt c) and other apoptogenic proteins (Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G) into cytosol.[28,29] Released cytochrome c together with cytoplasmic protein Apaf-1 form a complex called APOPTOSOME, where procaspase 9 undergoes activation, which is the main initiator caspase of mitochondrial pathway.[30] Activated caspase-9 can then cleave and activate downstream executioner caspases such as caspase-3. At the onset of apoptosis, activated caspase-3 cleaves PARP, resulting in the formation of PARP cleavage products, a hallmark of apoptosis.[31]

We investigated the molecular mechanism involved in the apoptosis induced by *A. longa* aqueous extract, by assessing the levels of apoptosis-related proteins — caspase-3, caspase-8, caspase-9, and PARP— in BL 41 cells. We found that the apoptosis induced by *A. longa* aqueous extract was accompanied by the activation of caspase-3 and caspase-9 with the cleavage of PARP, suggesting that the aqueous extract-induced apoptosis involves a caspase-dependent mitochondria pathway. The activation of caspases-9 and -3 implies a role for the mitochondrial pathway.[25] *A. longa* aqueous extract also induced the loss of mitochondrial membrane potential ($\Delta\Psi m$), implicating the mitochondrial signaling pathway in the apoptosis induction. We show here that *A. longa* aqueous extract triggers the mitochondrial pathway of apoptosis.

However, caspase-8 was not activated. Caspase-8 is considered to be a key caspase involved in signaling in the extrinsic pathway;[32] we can thus rule out death receptor-induced apoptosis as a cause of the mitochondrial activation observed in this study. We propose below a mechanism by which *A. longa* aqueous extract induces apoptosis in BL 41 cells, based on the findings of this study [Figure 6]. In the acute toxicity study (data not shown), in which doses of up to 5000 mg/kg (body weight) were administered, the *A. longa* aqueous extract was found to be safe.

The present evidence suggests that *A. longa* aqueous extract can induce apoptosis in Burkitt's lymphoma BL41 cells, by

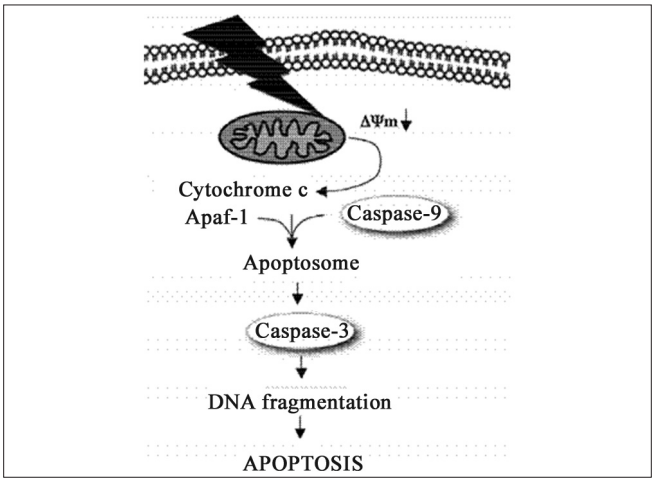

Figure 6: Proposed model for the apoptogenic activity of *Aristolochia longa* aqueous extract on the Burkitt's lymphoma cell lines BL41

triggering the mitochondrial pathway (disruption of ΔΨm, activation of caspases-9 and -3 and PARP cleavage). Thus, *A. longa* may be considered a promising source of new drugs for treating Burkitt's lymphoma and other lymphomas.

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