Research Paper

Limoniastrum guyonianum extracts induce apoptosis via DNA damage, PARP cleavage and UHRF1 down-regulation in human glioma U373 cells

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ABSTRACT

Several studies have reported that plant-derived natural products have cancer chemopreventive and chemotherapeutic properties. The aim of the present study was to determine the anti-proliferative and pro-apoptotic potential of *Limoniastrum guyonianum* aqueous gall (G extract) and methanol stems (M extract) extracts on human glioma cancer U373 cell line and if so, to characterize the mechanism involved. The G and M extracts-induced proliferation inhibitory effect was associated with DNA damage as shown by the comet test. In addition, both extracts promoted in a concentration-dependent manner these cells towards apoptosis as indicated by the presence of cleaved PARP. In order to characterize the mechanism involved in the anti-proliferative and pro-apoptotic signaling pathway activated by G and M extracts, the expression of the anti-apoptotic protein UHRF1 was determined. The present findings indicated that G and M extracts caused a down-regulation of UHRF1. These effects could be ascribed to the presence of condensed tannins and flavonoids such as epicatechin and epigallocatechin gallate.

Keywords: M and G extracts; UHRF1; PARP cleavage; Comet assay.

INTRODUCTION

A large number of plants used in traditional medicines have been shown to possess anti-cancer activity. Many of these are being extensively explored for their potential use in the prevention\treatment of chronic diseases. While several types of anticancer agents are available, undesirable side effects often limit their use. Recently, complementary or alternative medicines have become popular for treating different immune disorders. Increasingly among these are extracts from medicinal plants.

Glioblastoma represent the most frequent primary tumors of the central nervous system and remain among the most aggressive human cancers as available

therapeutic approaches still fail to contain their invasiveness. Despite many efforts made in surgical management, radiotherapy and new combined chemotherapy protocols during the past three decades, the mean survival time still remains around 14 months (Mangiola, et al., 2010). Multiple reasons are behind this clinical failure including tumors heterogeneity, susceptibility to mutations, and a high tumor infiltration degree. Indeed, invasive brain tumor cells can escape surgical resection and be responsible for tumor recurrence (Van Meir, et al., 2010). Therefore, identifying new agents that target signaling pathways involved in proliferation and invasion processes can be of a great importance to reduce tumorogenicity and improve prognosis of patients with glioblastoma.

Limoniastrum guyonianum has been used in traditional medicines to treat gastric infections and as an anti-bacterial in the treatment of bronchitis (Le Floch, 1983). Recently, we have demonstrated that an aqueous gall extract (G extract) exhibited some powerful biological effects, including anti-oxidant, anti-mutagenic, and immunomodulatory activities (Krifa, et al., 2013b; c). Previous investigations revealed that G extract also induced apoptosis in human cervical cancer cells via a UHRF1/DNMT1 down-regulation that was related to $p16^{INK4A}$ re-activation (Krifa, et al., 2013a).

Many natural anti-oxidant substances have anti-cancer or anti-carcinogenic properties. However, little information is available on the apoptotic effect of *L. guyonianum* extracts, particularly against glioma. Owing to its frequent use in traditional medicine and in accordance with our previous study that demonstrated how the aqueous extract of *L. guyonianum* gall had potent biological activities and induced apoptosis in human cervical cancer cells (Krifa, et al., 2013a), we investigated the anti-glioma effects and mechanism of action of M and G extracts against U373 human cancer cells. To our knowledge, this is the first report of apoptotic activity via PARP cleavage and DNA damage of this plant collected from the Southern region of Tunisia.

MATERIALS AND METHODS

Materials: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was from Euromedex (Mundolsheim, France) and dimethylsulfoxide (DMSO) was from Sigma-Aldrich (St. Suentin Fallavier, France). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, and L-glutamine were purchased from Invitrogen Life Technologies (Cergy Pontoise, France).

Preparation of plant extracts: *L. guyonianum* samples were collected from El Hamâ at Gabbes (a region situated in Southern Tunisia) in October 2009. Dr. Fethia Skhiri (Department of Botany, Higher Institute of Biotechnologie, University of Monastir) performed sample identification and verification according to the Tunisian Guide on Flora (Pottier-Alapetite., 1979). A voucher specimen (#L.g-10.09) was preserved for future reference. The aqueous gall extract (G extract) was prepared as previously described (Krifa, et al., 2013b). Furthermore, collected stems samples were shade-dried, powdered, and then stored in a tightly closed container. When needed, powdered stem was sequentially treated with solvents of increasing polarities, i.e., hexane, chloroform, ethyl acetate, and methanol. Stem powder was macerated at room temperature for 7 days with each solvent. After the final extraction (i.e., on Day 29), the extract was then passed through a filter paper. Collected filtrate was evaporated to dryness under vacuum at 40°C using a rotary evaporator and then placed at 4°C. The total methanol extract (M extract) concentrate yield (per gram dried plant material)

was determined using the formula [100 x weights (g) of dried extract]/dry stem weight (g); the actual percentage yield here was 12.4% (g/g).

Cell culture: The human glioma cancer cell line U373 was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and maintained in DMEM (Dulbecco's modified Eagle's medium) medium supplemented with 10% fetal bovine serum, in humidified incubator at 37°C and an atmosphere enriched with 5% CO_2. The culture medium was renewed every 5 days.

Cell viability assay: To control the impact of the tested concentrations of M and G extracts on cell viability, we estimated viability of U373 cells by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Cells were seeded in 96-well microtitration plates and twenty four hours after seeding, the test samples were added in serial dilutions before incubating the plates for an additional 24h and 48h. Cells were washed once before adding 10µl PBS containing 5mg/ml MTT in 100µl of medium. After one hour of incubation at 37°C, the medium was discarded, and the formazan blue formed in the cells was replaced by adding 100µl DMSO mixed with ethanol (1:1). Negative control without the tested extract was prepared in the same manner. Optical density (OD) was measured at 570nm. The cytotoxicity was expressed as IC_{50} which is the concentration which reduces the absorbance of treated cells by 50% with reference to the control (untreated cells). The IC_{50} values were graphically obtained from the dose-response curves. We determined IC_{50} values when activities resulted more than 50% at screening concentrations.

Comet assay: The Comet assay with U373 cells was used to detect DNA damage. Before each experiment, frosted microscope slides were precoated with 2 layers (100µl) of normal agarose (1% in milli-Q water) and left at room temperature to allow agarose to dry. The cells were treated during 24h and 48h by the different concentrations of extracts (100 and 200µg/ml. The cell dilution (500000 cells in 60µl) was mixed with an equal volume of low-melting-point agarose (1.2% in PBS). This agarose cell suspension (120µl) was spread onto each precoated slide, covered with a cover slip and for 10 minutes on ice. The cover slip was then gently removed, and the slides were placed in a tank filled with a lysate buffer (2.5mol/l NaCl, 100mmol/l EDTA, 10mmol/l acid Tris, 1% sodium sarcosinate pH 10, 1% of Triton X 100, and 10% DMSO). Slides were immerged for 1 h in this buffer (at 4°C, in the dark), then transferred into the electrophoresis buffer (NaOH 10N, EDTA 200mmol/l in deionized water) during 20min at room temperature in the dark. Electrophoresis was carried out for 15min at 25V, 300mA. Finally, the slides were gently rinsed with neutralization solution (0.4mol/l Tris MA Base, Sigma, pH 7.5) for 3 times, 5min each time. Staining of DNA was accomplished using 50µl of ethidium bromide solution at 20µg/ml in PBS per slide. The slides were examined using an epifluorescence microscope (Zeiss Axioskop 20; Carl Zeiss, Microscope Division, Oberkochen, Germany). A total of 100 comets on each scored slide for each concentration of extracts were visually scored according to the relative intensity of fluorescence in the tail and classified as belonging to one of five classes. We utilize three slides for each concentration, and the experiments were repeated three times. Each comet class was given a value of 0, 1, 2, 3, or 4 (from undamaged, 0 to maximally damaged, 4) as described by Collins, et al. (1996). The total score of DNA damage was calculated by the following equation:

Total DNA damage (TDD) = (percentage of cells in class 0×0) + (percentage of cells in class 1×1) + (percentage of cells in class 2×2) + (percentage of cells in class 3×3) + (percentage of cells in class 4×4).

- Consequently, the total score was ranging from 0 to 400.

Western blot analysis: U373 cells were seeded into culture dishes. After 24 hours, cells were treated with different concentrations of G or M extract for 48 hours. Cells and culture medium were then harvested, washed twice with PBS, and finally lysed using lysis buffer including protease inhibitors. After 15 minutes on ice, lysates were centrifuged at 20,000g for 10 minutes. Protein concentration was determined with the BCA kit (Sigma-Aldrich). The lysates (30µg of the total protein) were resolved on 10% SDS-PAGE (polyacrylamide gel electrophoresis) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for 1 hour with 5% nonfat dry milk, dissolved in TBS solution. Membranes were then successively incubated overnight at 4°C with the following primary antibodies: entire and cleaved poly(ADP-ribose) polymerase (PARP) (cell signaling, France), polyclonal β-actin antibody (Santa Cruz, France), monoclonal UHRF1 antibody (Proteogenix, Oberhausbergen, France), and a monoclonal β-tubulin antibody (Proteogenix, France) in TBST (TBS with 0.1% Tween 20) with 5% nonfat dry milk. After washing in TBST solution, the secondary antibody (anti-rabbit or -mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotechnology), diluted in TBST/5% nonfat dry milk, were added for 45 minutes at room temperature. Membranes were rinsed with TBST before being developed by an enhanced chemiluminescence detection system (ECL; GE Healthcare).

Statistical analysis: Data were analyzed with student's t-test and presented as mean value±SEM of three independent measurements in separate experiments.

RESULTS

Cell viability assay: The MTT assay is a commonly used method to study the action of natural products on cell proliferation, viability and cytotoxicity. This assay is based on the reduction of a tetrazolium salt to a purple insoluble formazan by metabolically active cells (Mosmann, 1983). The absorbance of the solubilized formazan is taken as a measure of the number of living cells. As illustrated in table 1, incubation of *L. guyonianum* extracts with U373 cell lines inhibited cell proliferation in a dose-dependent manner. The IC_{50} values were determined graphically and the inhibition percentages were calculated.

Results of this experiment demonstrated that M extract exhibited the highest anti-proliferative effect against U373 cells when compared to G extract with estimated IC_{50} values of 98.52µg/ml and 220µg/ml after 48 hours treatment of M and G extracts, respectively. Inhibition of proliferation of U373 cells reached a maximum of 94.15 % and 92.22 % at a concentration of 400µg/ml after 48 hours of incubation with M and G extracts, respectively (table 1).

Comet assay: The induction of DNA damage in human glioma U373 cells after exposition to different concentrations of both extracts (100 and 200µg/ml), for 24h and 48h was studied using the Comet assay. This method was reported to be sensitive for detecting DNA strand breaks in individual cells (Collins, et al., 1996). Quantification of the comet data is reported as Total DNA damage (TDD) in table 2.

A significant difference was detected between the TDD of the U373 treated with 200µg/ml of G extract for 48h (TDD = 273.35) on one hand, and the negative

control (non-treated cells; TDD= 136.18) on the other hand. Indeed, a significant increase of the Total DNA damage was observed in U373 cells exposed to 100 or 200µg/ml of methanol extract for 24h and 48h, compared to the untreated cells. The TDD reached a maximum (302) after treatment of 200µg/ml of methanol extract for 48h suggesting significant induction of DNA breakage in glioma human cells.

Methanol and aqueous gall extract induce PARP cleavage via UHRF1 down regulation: In order to evaluate whether G and M extracts decrease cell viability through apoptosis activation, the cleavage of the PARP was examined by Western blot. This enzyme is one of the first proteins identified as a substrate for caspases. PARP is involved in the repair of DNA damage and functions by catalyzing the synthesis of poly(ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented after cleavage of PARP by caspase-3. Existing as a 116-kDa nuclear protein, PARP is cleaved in two fragments of approximately 89 and 26 kDa. U373 cells were treated with different concentrations of G or M extracts for 48 hours, and the cleavage of PARP was monitored by Western blot analysis. Our findings revealed that G and M extracts treatment during 48h of cells induced PARP cleavage in a dose dependent manner. The band intensities corresponding to the characteristic proteolytic PARP fragments of 89 kDa and 26 kDa respectively, increased after a 48-hours treatment. These results confirmed that the cell death induced by G and M extracts on U373 cells is due to apoptosis (figure 1A).

In order to identify the mechanisms implicated in PARP cleavage, we tested the effects of G and M extracts on UHRF1 (ubiquitin-like containing PHD and RING finger domains, 1) expression. In fact, a recent study demonstrated that apoptosis via PARP cleavage and may be caused by UHRF1 depletion (Tien, et al., 2012). UHRF1 is a 90-kDa nuclear protein that plays an important role in cancer progression through the epigenetic regulation. High levels of UHRF1 are identified as an important factor that can keep the cancer cells in a proliferated state and prevent them from differentiation.

U373 cells were treated with different concentrations of G and M extracts for 48h and the expression of UHRF1 was monitored by Western blot analysis. As shown in figure 1B, when cells were treated with the different concentrations of G extract (100, 200 and 300µg/ml), the bands at 90 kDa corresponding to UHRF1 significantly decreased after 48h. Indeed, treating cells with M extract induced a dose down-regulation of UHRF1. UHRF1 expression was decreased after treatment with 50µg/ml of M extract and approximately disappeared at 200µg/ml after 48 hours (figure 1B).

DISCUSSION

Chemotherapy is considered the most effective method of cancer treatment, but most chemotherapeutic drugs severely affect normal cells. Therefore, developing safe and powerful anticancer agents from medicinal plants has become very attractive.
Many extracts obtained from some medicinal plants have been shown to possess cytotoxic effects on some cancer cells, such as colon, cervical, ovarian, esophageal cancer cells… but the studies about the cytotoxic effects of *L. guyonianum* G and M extracts on human glioma cancer (U373) cells were not reported. In this study, we examined the cytotoxic and genotoxic potencies of the aforementioned extracts on this cell line. Our results showed that M and G extracts were able to induce cytotoxicity and DNA damage in a concentration and time dependant manner.

To examine the mechanisms by which cytotoxicity is induced, the cleavage of poly (ADP-ribose) polymerase, key enzyme in the apoptosis process (Cain, et al., 2002; Virag and Szabo, 2002), was evaluated. PARP is a single-strand break-repair enzyme (116 kDa). It is known that upon activation of the apoptotic pathway, caspase cleaves PARP into 89 and 26-kDa polypeptides. By immunoblotting using PARP, we found that the addition of G or M extracts led to prominent PARP cleavage, resulting in accumulation of the 89 kDa and 26-kDa product. PARP cleavage in U373 cells was observed after treatment for 48 hours. Such results were in agreement with findings of other researchers who reported on the apoptotic activity of some plant extracts. For example, Etebari, et al. (2012) showed that hydro-alcoholic and aqueous extracts of *E. amoenum* and *N. Jatamansi* can cause cytotoxicity and DNA damage in HepG2 cells. This activity was attributed to the presence of flavonoids. In the same way, other studies demonstrated how some flavonoids (i.e luteolin and apigenin) could induce cytotoxicity, DNA damage and PARP cleavage in human cancer cells (Kilani-Jaziri, et al., 2011).

In order to identify the mechanisms implicated in PARP cleavage, we tested the effects of G and M extracts on an anti-apoptotic protein UHRF1. Thus, UHRF1 plays a fundamental role in the inheritance of the DNA epigenetic marks from the mother cell to the daughter cells. It also appears that preventing the transmission of these marks via knock-down of UHRF1, leads to an activation of pro-apoptotic pathways (Avvakumov, et al., 2008; Karambataki, et al., 2010). Inhibiting UHRF1 expression would be an efficient way to block the development of a tumor by blocking the transformation and the proliferation of the cells, as well as the vascularization of the tumor (Alhosin, et al., 2011).

Our findings demonstrate that the pro-apoptotic activity of G and M extracts in U373 cells is linked to a repression of UHRF1. These results were in agreement with our previous studies that demonstrated how the aqueous extract of *L. guyonianum* gall had potent biological activities and induced apoptosis in human cervical cancer cells via a UHRF1/DNMT1 down-regulation that was related to p16^{INK4A} re-activation (Krifa, et al., 2013a).

Regarding the chemical composition of M and G extracts, we could hypothesize that their apoptotic effects might be dependent on the presence of tannins and flavonoids (Krifa, et al., 2013a). Chemical analysis using MALDI-TOF demonstrated that G extract contains epicatechin and especially epigallocatechin gallate (data not shown). This natural product is known to exhibit cytotoxic activities against several cancer cells. It is able to eliminate cancer cells by inhibiting cell-cycle progression and/or causing apoptosis (Jin, et al., 2013).

CONCLUSION

This study showed that the G and M extracts of *L. guyonianum* imparted a significant anti-glioma effect *in vitro*. These extracts could dose-relatedly enhance apoptotic effects in U373 cells via DNA damage and PARP cleavage. This work also demonstrated that G and M extracts might be implicated in the epigenetic regulation by inhibiting UHRF1 expression and might someday be utilized as a good natural anticancer agent in the clinical setting.

Declaration of interest: The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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Table- 1: Aqueous gall (G) and methanol (M) extracts inhibit U373 cell proliferation.

Extracts	Concentration (µg/ml)	24h	48h
M	0	100.00	100.00
	50	96.35 ± 3.8	87.22 ± 2.1
	100	85.67 ± 1.5	52.14 ± 3.1*
	200	43.88 ± 1.9*	11.95 ± 2.7*
	300	14.08 ± 2.3*	5.93 ± 1.1*
	400	13.38 ± 3.6*	5.84 ± 1.5*
G	0	100.00	100.00
	100	99.28 ± 2.9	99.81 ± 3.7
	200	88.52 ± 2.1	63.86 ± 3.2*
	300	36.25 ± 2.2*	9.54 ± 1.5*
	400	15.03 ± 3.4*	7.78 ± 1.4*

- Values are means ± S.E.M of three independent experiments.
- Statistically significant, *$P < 0.05$ (versus the corresponding untreated group).

Table- 2: Genotoxic effect of M extract (M) and G extract (G) on U373 cells.

Extracts	Concentration (µg/ml)	24h	48h
M	0	139.51 ± 9	139.19 ± 7
	100	241.25 ± 7*	257.45 ± 9*
	200	283.61 ± 13*	302.92 ± 12**
G	0	136.18 ± 7	136.18 ± 10
	100	147.31 ± 11	182.14 ± 13
	200	184.28 ± 8	273.35 ± 7*

- Values are means ± S.D of three independent experiments.
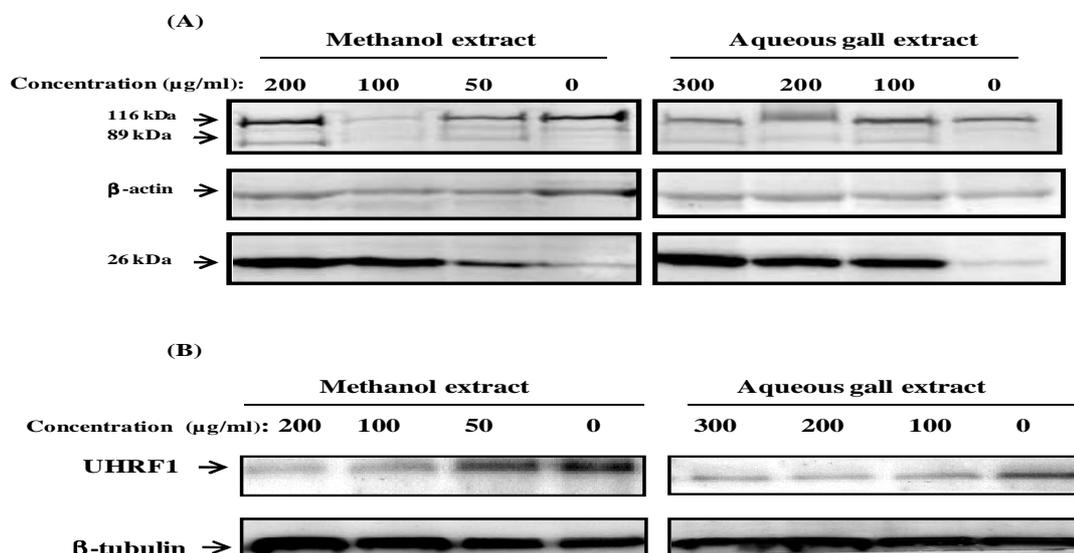- Statistically significant, *$P < 0.05$, **$P < 0.01$ (versus the corresponding untreated group).

Figure -1: Apoptotic activity of M and G extracts.
- **M and G extracts treatment-induced PARP cleavage.**
- **Effects of M and G extracts on UHRF1 expression.**
- U373 cells were incubated with different concentrations of these extracts for 48 hours. Then, cleaved PARP and UHRF1 expression levels were examined by Western blot. Each membrane was reported with anti–β-actin or anti–β-tubulin antibody to confirm equal protein loading. A representative blot is shown from three independent experiments with identical results.