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Antiproliferative and apoptotic potential of *Daphne gnidium* L. root extract on lung cancer and hepatoma cells

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Daphne gnidium L. (Thymeleacees) is a famous Moroccan plant with cancer-related ethnobotanical use. Previously, we demonstrated that ethyl acetate extract of *D. gnidium* had antiproliferative and pro-apoptotic potential on human breast tumor MCF-7 cells. The purpose of this study was to investigate if the antiproliferative effect of this extract was similar for different human cancer cell lines such as A549 lung cancer and SMMC-7721 hepatoma cells. Moreover, this work essentially focused on the intrinsic apoptotic signaling pathway. Antiproliferative activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide on A549 and SMMC-7721 cells. The characterization of the mechanisms involved in this effect was determined by lactate dehydrogenase test, apoptosis assays and western blot analyses. Our present study has shown that this extract strongly inhibited proliferation of A549 (IC_{50}: 213 ± 15 μg/ml) and SMMC-7721 (IC_{50}: 170 ± 13 μg/ml) cells. The characterization of antiproliferative effect demonstrated that this extract was an apoptosis inducer in both cell lines tested. The results of western blot analyses have shown in SMMC-7721 cells that this extract activated caspase signaling triggered by the modulation of Bcl-2 family proteins. These findings suggest that this natural extract-induced effects may have novel therapeutic applications for the treatment of different cancer types.

1. Introduction

The number of individuals affected by cancer is continuing to expand (Yildiz et al. 2013). Hence, a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets (Xia et al. 2004). Due to enormous propensity of plants, which synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumor and antiproliferative activities (Kim et al. 2005; Chin et al. 2006).
In cancer therapy, the focus is on strategies that suppress tumor growth by activating the apoptotic program in the cell (Fan et al. 1998). Many chemotherapeutic agents of kill tumour cells through launching the mechanism of apoptosis (Lee et al. 2003; Chaouki et al. 2009a). Apoptosis can be triggered by a death receptor-dependent extrinsic pathway and mitochondria-mediated intrinsic pathway (Kim 2005). Therefore, changes in expression of the Bcl-2 family members resulting in decreased anti-apoptotic (Bcl-2, Bcl-xL,...) and increased pro-apoptotic (Bax, Bak,...) proteins may cause mitochondrial release of several pro-apoptotic molecules (Orrenius 2004). Most types of apoptosis induced by cellular stress (notably anticancer treatment) involve caspase-3 as a major executioner, which upon activation cleaves cytosolic inhibitor of caspase-activated DNase (ICAD) to release and translocate caspase-activated DNase (CAD) to the nucleus for nuclear DNA fragmentation, the final event of apoptosis (Sakahira et al. 1998).
D. gnidium belongs to the botanical family of Thymeleacees. It is very frequent around the Mediterranean basin. In Morocco, the roots and leaves of this plant have been traditionally used to avoid hair loss, as a purgative, odontalgic, as a hair tonic and to treat jaundice and several solid tumours (Hmamouchi 1999; Merzouki et al. 2000).
Recently, we showed that an ethyl acetate extract of *D. gnidium* has antiproliferative and pro-apoptotic potential on human breast tumor MCF-7 cells (Chaouki et al. 2009b). In order to explore if the results obtained with extract of *D. gnidium* on MCF-7 cells could be extended to other types of cancer cells, we tested this plant extract on human A549 lung cancer and SMMC-7721 hepatoma cell lines. Moreover, to gain further insight into the mechanisms by which this natural extract induces apoptosis in human cancer cells, we have examined in this study the expressions of the apoptotic relative proteins like Bcl-2 family and caspases in the two human cancer cell lines (A549 and SMMC-7721 cells).

2. Investigations and results

2.1. Inhibitory effects of ethyl acetate extract of D. gnidium on proliferation of A549 and SMMC-7721 tumor cell lines

Cells were cultured in 10% CS-medium with or without 1-1000 μg/ml plant extract for 24-96 h and cell proliferation was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Under our experimental conditions, ethyl acetate extract of *D. gnidium*

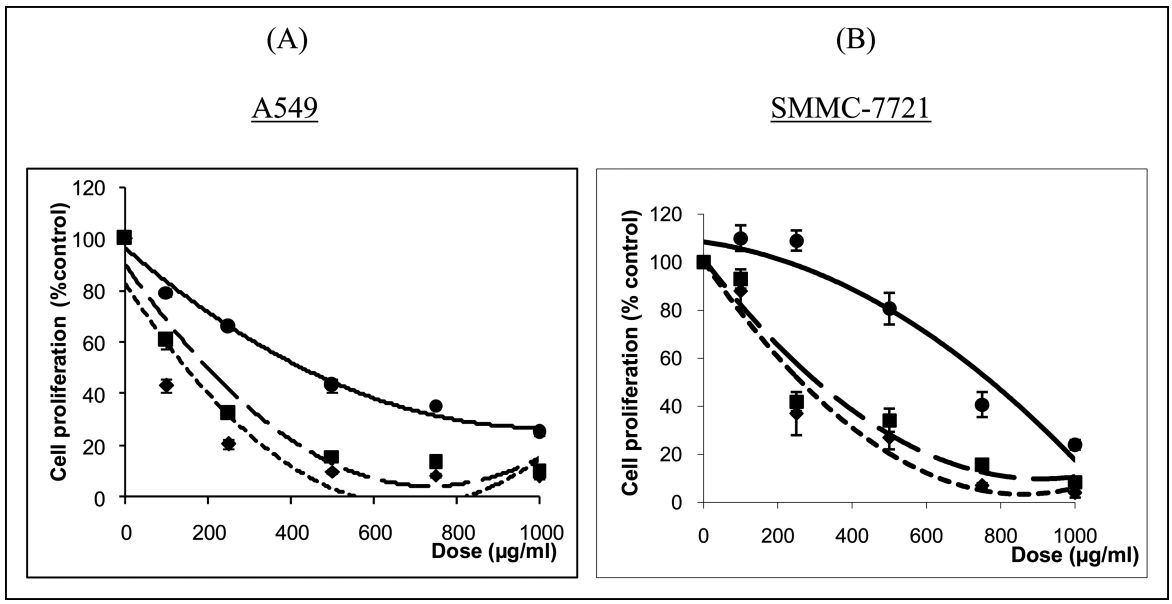

Fig. 1: Antiproliferative effect of ethyl acetate extract of *D. gnidium* on A549 (A) and SMMC-7721 (B) cancer cell lines. Cells were treated with various concentrations for 24 (⁻), 48 (- -), and 72 h (. . .). Results are presented as percentage of negative control (untreated cells) proliferation. Values were expressed as mean ± SD of six experiments (p-value relative to control group: $p < 0.05$).

could inhibit proliferation for both cancer cell lines, especially after 48 and 72 h (Fig. 1A and B), and there were significant differences compared with the negative control ($P < 0.001$). Ethyl acetate extract of *D. gnidium* induced a dramatic decrease into cell proliferation in a dose and time-dependent manner.

The concentrations of plant extract required to produce a 50% reduction in cell proliferation (IC_{50}) were calculated by a regression analysis using data from MTT assays. For the the two cell lines, the IC_{50} values decrease according to time of treatment (data not shown). The antiproliferative effect of ethyl acetate extract of *D. gnidium* on SMMC-7721 cell line (IC_{50}: 170 µg/ml at 48 h) was stronger than A549 cell line (IC_{50}: 213 µg/ml at 48 h).

The trypan blue exclusion study confirmed the IC_{50} values obtained for the two studied cell lines. We choose IC_{50}-48 h values of each cancer cell lines for following experiments.

2.2. Extract of D. gnidium induced antiproliferative effect on A549 and SMMC-7721 cells via the process of apoptosis

In order to better characterize the effect of *D. gnidium* extract on proliferation of A549 and SMMC-7721 cells, plant extract-treated cells were subjected to the lactate dehydrogenase (LDH) test. This test quantifies the LDH activity released from the cytosol of damaged cells into culture supernatant which evaluate the percentage of lytic cell death compared to maximum cell lysis obtained by Triton X-100 treatment. When A549 and SMMC-7721 cells were treated by ethyl acetate extract of *D. gnidium*, with concentrations of 213 and 170 µg/ml (IC_{50} values of each cancer cell lines), respectively no cytotoxic effect was detected (data not shown). Consequently, cell death induced by the *D. gnidium* extract treatment for the examined dose seemed to be not necrosis.

To understand the characteristics of the antiproliferative effect of the ethyl acetate extract of *D. gnidium* on A549 and SMMC-7721 cells, plant extract-treated cells were subjected to an apoptosis assays, including DNA fragmentation assay and ELISA assay, as described in the Experimental section. Initially, no DNA fragment was found in untreated cells (Fig. 2) but DNA fragments were observed in A549 (Fig. 2A) and SMMC-7721 cells (Fig. 2B) treated with IC_{50} values of each cell line, indicating that the both tumor cell lines underwent apoptosis.

The apoptotic cell death induced by *D. gnidium* extract was additionally confirmed through ELISA assay. The treatment of A549 and SMMC-7721 cells at 48 h by the ethyl acetate extract with IC_{50} values has shown an increase in the apoptotic ratio of 2.10 and 3.41 times, respectively, compared to control (Fig. 3A and B). At this step of our study, results demonstrated that ethyl acetate extract of *D. gnidium* was an apoptosis inducer in A549 and SMMC-7721 cells.

2.3. Extract of D. gnidium induced apoptosis is mediated by intrinsic mitochrondia-mediated pathway

To examine whether plant extract-induced apoptosis activates the caspase pathway or not, we incubated A549 and SMMC-7721 cells in the absence or presence of *D. gnidium* extract and then harvested the cells for western blot analysis. Because the mitochrondian pathway appears to be involved in the induction of intrinsic apoptosis, we measured the levels of anti- and pro-apoptotic protein levels which dysregulates mitochrondian balance. Incubation of SMMC-7721 cells with plant extract up-regulated the levels of pro-apoptotic protein Bax and down-regulates anti-apoptotic protein Bcl-2 in contrast to A549 cells (Fig. 4A and B), which indicates mitochrondia-mediated apoptosis in SMMC-7721 cells.

To further determine whether the extract of *D. gnidium* activates the caspase pathway or not, we incubated A549 and SMMC-7721 cells in the absence or presence of plant extract and then we measured the levels of cleaved caspase-3. Incubation of SMMC-7721 cells with extract of *D. gnidium* up-regulated the levels of the biologically active cleaved caspase-3 thereby activating the apoptotic cascade pathway (Fig. 4B), whereas the results for caspase-3 expression in A549 cells were inconclusive (Fig. 4A).

3. Discussion

Natural-derived products, regardless of crude extracts or isolated active compounds, have drawn growing attention as agents in cancer therapy, due to their ability to modulate apoptosis (Fulda 2010; Taraphdar et al. 2001).

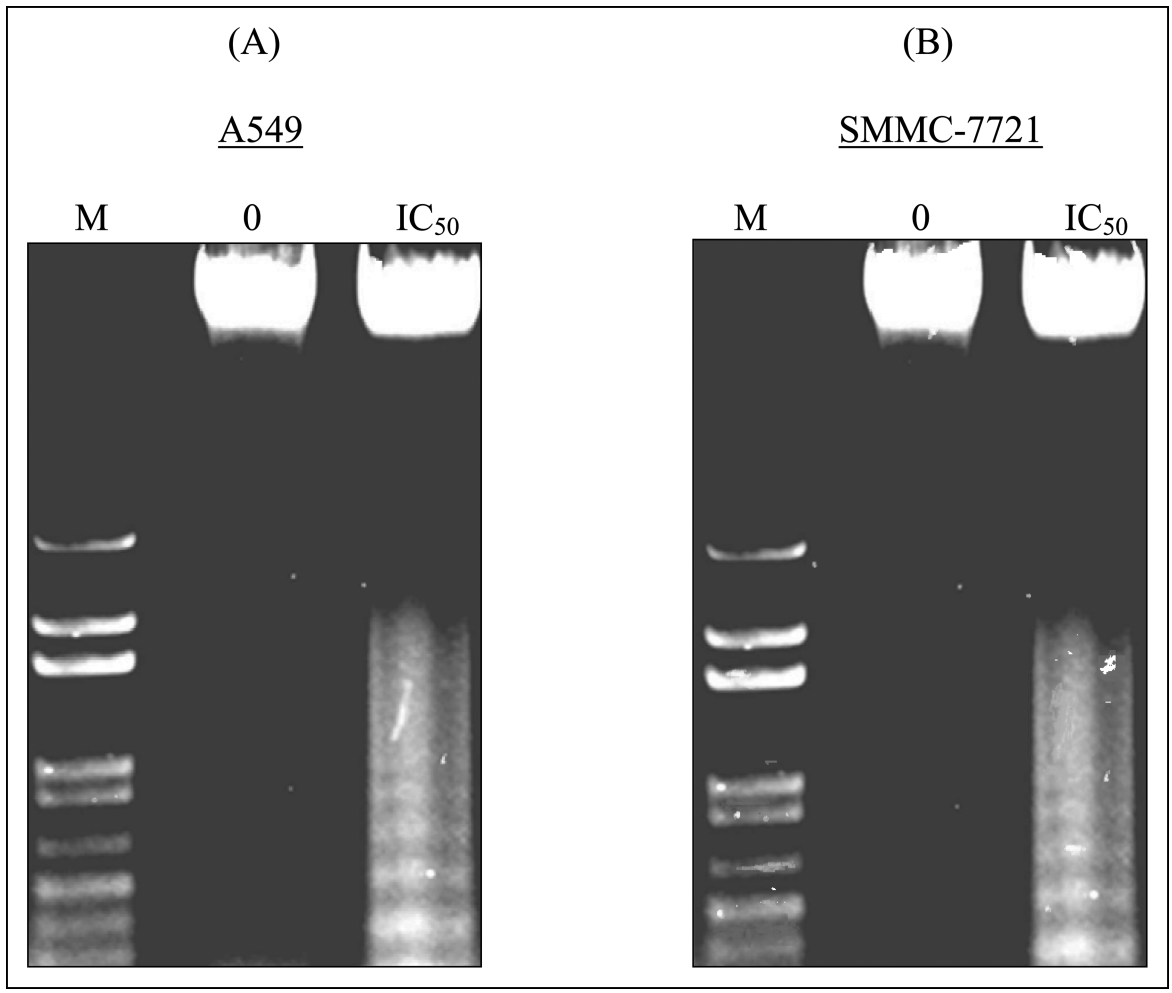

Fig. 2: Analysis of DNA fragmentation in A549 (A) and SMMC-7721 cells (B), using agarose gel electrophoresis. Cells were incubated without or with IC_{50} values of ethyl acetate extract of *D. gnidium* for 48 h. Genomic DNA was prepared as described in Experimental and analyzed by 0.8 % agarose gel electrophoresis containing ethidium bromide. DNA fragments were visualized under UV light. M indicates as a Marker. The figure is a representative of the results from three independent experiments.

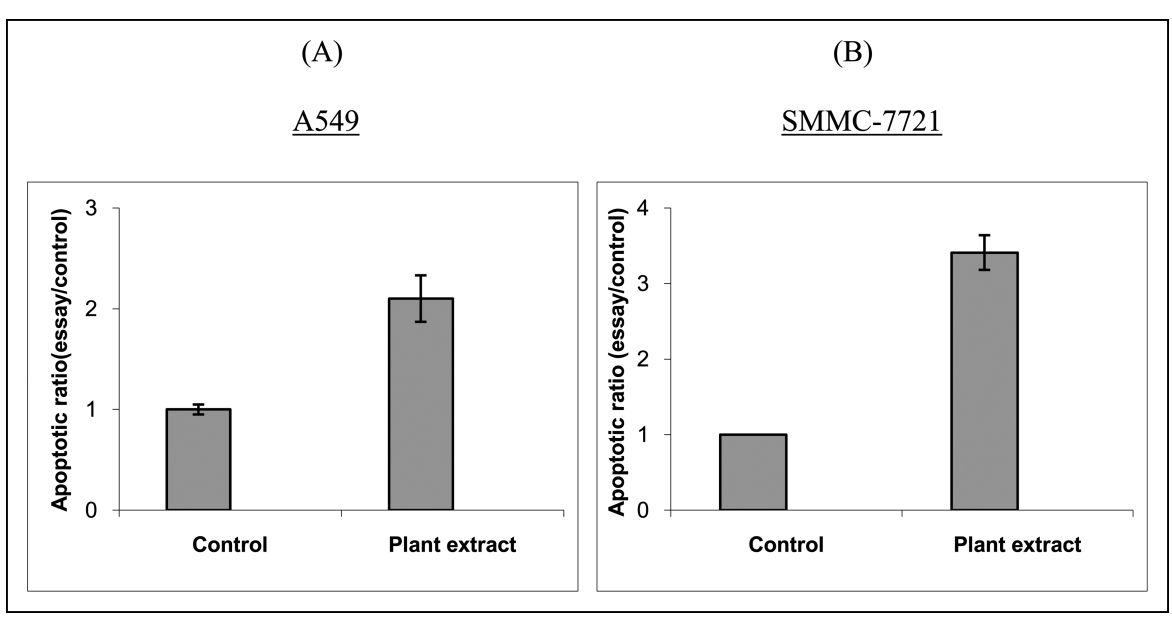

Fig. 3: Quantification of DNA fragmentation after *D. gnidium* extract treatment. A549 (A) and SMMC-7721 (B) cells were treated or not (control) for 48 h with IC_{50} values. Apoptosis was quantified on floating and adherent cells using "cell death" ELISA kit (Cell Death Detection ELISA^{Plus}, Roche Diagnostics). Apoptotic ratio was determined as sample absorbance/control absorbance. Values were expressed as mean ± SD of three experiments.

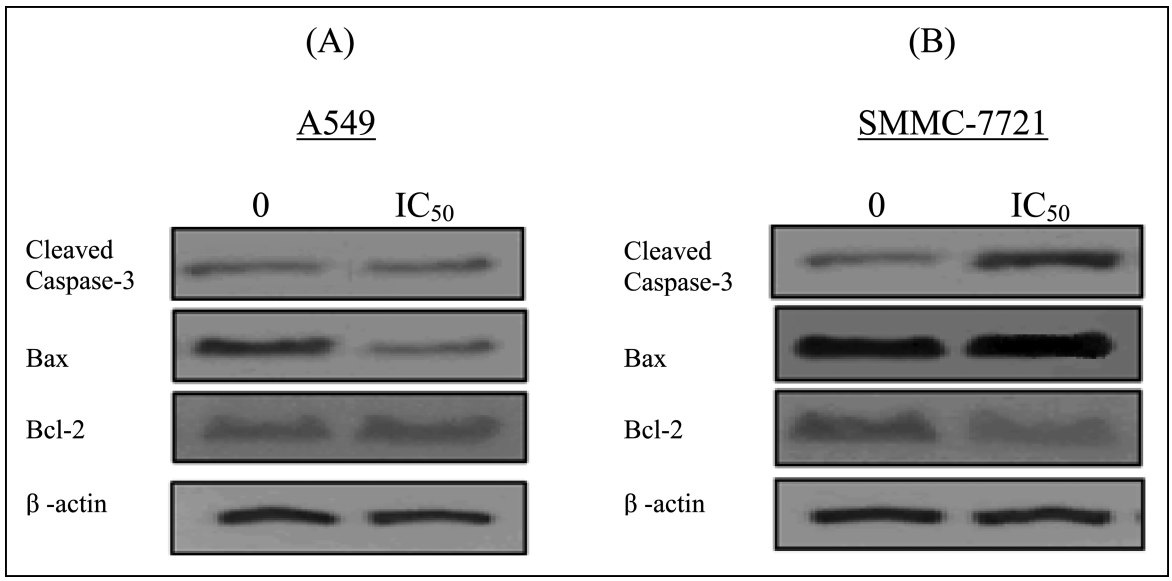

Fig. 4: Bax, Bcl-2 and caspase-3 analysis expression in A549 and SMMC-7721 cells. (A and B), Western blot analysis. A549 and SMMC-7721 cells were treated or not for 48 h with IC_{50} values of ethyl acetate extract of *D. gnidium.* Total protein was extracted from the cells and separated on 15% SDS-PAGE gel. Cellular expression of Bax, Bcl-2, β-actin and cleaved caspase-3 were estimated using mouse anti-human antibodies. Quantification of each band was performed by densitometry analysis software. One of three representative experiments is shown.

Recently, we have shown that an ethyl acetate extract of *D. gnidium* had antiproliferative and pro-apoptotic effect on human breast tumor MCF-7 cells (Chaouki et al. 2009b). We not tested this plant extract on human A549 lung cancer and SMMC-7721 hepatoma cell lines. Our results showed that this extract strongly inhibited the proliferation of A549 and SMMC-7721 cells and induced apoptosis as previously described in MCF-7 cells (Chaouki et al. 2009b). Our findings were in agreement with a previous study showing that another Chinese plant of the same botanical family, named *Daphne genkwa* exhibited anti-neoplastic effects on P-388 and A-549 cell lines (Liou et al. 1982; Zhan et al. 2005). According to the comparison of IC_{50} values, the degree of antiproliferative activity and apoptosis on SMMC-7721 cell line is stronger than on A549 cell line. This selectivity could be related to the sensitivity of the cell line to the active compounds in the extract.

The purpose of this study was also to investigate whether the ethyl acetate extract of *D. gnidium* affects the apoptosis of A549 and SMMC-7721 cells through the activation of caspases, which might explain mechanisms underlying the apoptosis and antiproliferative effect of cancer cells.

Apoptosis, as a regulable biological mode of cell death, is mediated by two major types of pathways, namely, the death-receptor-mediated extrinsic pathway and the mitochondria-dependent intrinsic pathway (Wyllie 1997; Ashkenazi and Dixit 1998). Bcl-2 family proteins, as critical checkpoints, play important roles in controlling the mitochondria-dependent intrinsic pathway (Kroemer 1997). So far, more than 20 members of Bcl-2 family have been identified in human including anti-apoptosis proteins (such as Bcl-2) and pro-apoptosis proteins (such as Bax) (Antonsson and Martinou 2000). Bcl-2 and Bax proteins, as two major members of the Bcl-2 family, may form a heterodimer complex to cause mutual neutralization of their functions which resulting in apoptosis triggering (Nascimento Pde et al. 2004). Therefore, the balance between the expression levels of Bcl-2 and Bax is critical in determining the fate of cells, survival or death. In this study, the protein level of Bcl-2 was down-regulated and that of Bax in SMMC-7721 cells was up-regulated. Hence, increasing Bcl-2 family protein expression (Bax and Bcl-2 ratio) might indicate that the ethyl acetate extract of *D. gnidium* induced apoptosis in SMMC-7721 cells, and strongly correlates with the intrinsic mitochondrial apoptotic signaling pathway in contrast to A549 cells. The detailed pathways and mechanisms of apoptosis induction *D. gnidium* extract in A549 and SMMC-7721 cells warrant further research.

Many cancer cells circumvent normal apoptotic mechanisms to prevent their self-destruction. However, caspase is one of the key executioners of apoptosis (Budihardjo et al. 1999). In particular, caspase-3 plays a pivotal role in the terminal and execution phases of apoptosis induced by diverse stimuli (Thornberry and Lazebnik 1998). Upon activation, initiator caspase-9 triggers the proteolytic activation of the executioner caspase-3/-7 and caspase-8 in a process that results in the cleavage of PARP and subsequent DNA fragmentation and apoptotic death (Cain et al. 2002). In our study, treatment of SMMC-7721 cells with *D. gnidium* extract resulted in a dramatic increase in the proteolytic activation of caspase-3 (cleaved caspase-3), which is the main executioner of apoptosis, in contrast to A549 cells. As expected in SMMC-7721 cells, caspase-3 degraded clearly which correlated to a caspase signaling pathway and apoptosis that finally fragment DNA.

In conclusion, we demonstrated that an ethyl acetate extract of *D. gnidium* caused an inhibition of cell growth by apoptosis induction in different human cancer cell lines (lung cancer and hepatoma cells). Moreover, a large part of our work essentially focused on the intrinsic apoptosis pathway. As far as we know, this is the first report to demonstrate that this plant extract activated caspase signaling triggered by the modulation of Bcl-2 family proteins which results in the accumulation of fragmented DNA in SMMC-7721 cells. These findings suggest that *D. gnidium* may serve as a potent chemosensitizer in the treatment of human cancers. Therefore, these results warrant further investigation of this plant as a source of pharmacologically active agents and the assessment of their anti-tumor therapeutic efficacy *in vivo*.

4. Experimental

4.1. Plant material

D. gnidium was collected during March 2004 from its natural location in Morocco and taxonomically identified at the National Institute of Medicinal and Aromatic Plants, Taounate. A voucher specimen (FMP-49) is deposited at the herbarium of the Faculty of Medicine and Pharmacy, Rabat.

4.2. Extraction procedure

The dried roots were crushed, and 100 g of powder were extracted as described previously (Chaouki et al. 2009b) in soxhlet apparatus for at least 48 h by four organic solvents with increasing polarity: hexane, dichloromethane, ethyl acetate and methanol. The recovered extracts were concentrated by the rota-vapor and were stored at -20 °C until analysis. The percentage yield of the ethyl acetate extract was 7.09%.

4.3. Cell lines, cell culture and treatment

A549 and SMMC-7721 cells were cultured in RPMI-1640 (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% calf serum (Gibco BRL), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL). Cultures were maintained in a humified atmosphere with 5% CO_2 at 37 °C to sub-confluence. Fresh medium was supplied every 72 h. The doubling time of A549 and SMMC-7721 cells were estimated respectively to be 22 and 24 h by growth curve studies.
Cells were allowed to grow for 24 h in culture medium prior to exposure to ethyl acetate extract of *D. gnidium* for 24, 48, and 72 h. A stock solution of 50 mg/ml of extract was prepared in DMSO (Sigma) and diluted in culture medium to give a final concentration 1 to 1000 µg/ml. The same amount of DMSO was added to control cells.

4.4. Cell proliferation assay

Medium was aspirated from cells grown to about 90% confluence. Cells were washed with PBS, trypsinized, counted with a hemocytometer and subcultured into 96-well plates with 10^4 cells per well in a 100 µl medium. After 24 h incubation at 37 °C in a 5% CO_2 incubator, the seeding medium was removed and replaced by ethyl acetate extract of *D. gnidium* diluted in medium to a final concentration ranging from 1 to 1000 µg/ml. Measurement of the cell proliferation was determined after 24, 48, and 72 h of the treatment using the MTT assay. MTT experiments were performed in six assays as previously described (Moalic et al. 2000). Briefly, 10 µl of the MTT solution (5 mg/ml in distilled water) were added on cultured cells. After 4 h of incubation at 37 °C in wet atmosphere, at the darkness, 100 µl of a lysis solution (SDS: 10%; HCl: 0.01N) were added into the wells, and the plate was then incubated at 37 °C overnight. The absorbance was read at wavelength of 550 nm using a microtiter plate reader (Multiskan EX, Labsystems).
Negative and positive controls of the test correspond respectively to untreated A549 and SMMC-7721 cells and cells treated by doxorubicin (Cooper Maroc Laboratoires, Morocco); only exposed to vehicle (DMSO). Results are expressed as a percentage of negative control proliferation:
[Mean DO essay/Mean DO negative control (= 100 % proliferation)] ± standard deviation.

4.5. Determination of IC_{50} concentration of plant extract

The absorbance values obtained per treatment were converted to percentage proliferation. Regression analysis was performed on MTT assay proliferation data and the resulted equation was used to calculate the inhibition concentration required to produce a 50% reduction in cell proliferation (IC_{50}). The values of the IC_{50} obtained were confirmed by trypan blue exclusion assay data.

4.6. LDH test

Cells were seeded in 96-well plates and treated without or with ethyl acetate extract of *D. gnidium*. Cytotoxicity detection kit (Boehringer Mannheim) was used to measure the LDH activity released from the cytosol of damaged cells into the supernatant which evaluated the percentage of lytic cell death compared to maximum cell lysis obtained by Triton X-100 treatment according to manufacturer's protocol.

4.7. Detection of DNA fragmentation

For detection of apoptotic DNA cleavage, the DNA fragmentation assay was performed using ladder DNA fragmentation assay. In brief, A549 and SMMC-7721 cells were collected after treatment with IC_{50} values of ethyl acetate extract of *D. gnidium* for 48 h and washed in PBS. The cells were then lysed with 500 µl of genomic DNA extraction buffer (0.1 M Nacl, 10 mM EDTA, 0.3 M Tris- HCl, 0.2 M sucrose, pH 8.0). The lysate was incubated with 20 µl of 10% SDS solution and incubated at 65 °C for 30 min mixed with 120 µl potassium acetate (pH 5.3) and stored on ice for 1 h. After that the solution was centrifuged for 10 min at 4 °C 12000 rpm. Added 2 µl (10 mg/ml) RNase were added to the supernatant, and incubated for 30 min at room temperature. The DNA was extracted by washing the resultant pellet in phenol/chloroform extraction and precipitation by ethanol and then by dissolving the pellet in distilled water. DNA fragmentation was visualized by electrophoresis in a 0.8% agarose gel containing ethidium bromide.

4.8. Quantification of apoptosis

Apoptotic cell death was quantified by "cell death" enzyme-linked immunosorbent assay (ELISA)(Cell Death Detection ELISAPlus, Roche Diagnostics). Briefly, cells were cultured in 75 cm^2 tissue culture flasks. After *D. gnidium* extract treatment, cell lysates were obtained from pooled floating and adherent cells, according to the manufacturer's protocol. Apoptosis from control and treated cells was then measured as previously described (Chaouki et al. 2009a).

4.9. Western blot analysis

Cells were cultured in 150 cm^2 tissue culture flasks. After treatment with IC_{50} values of ethyl acetate extract of *D. gnidium*, adherent cells were trypsinized and pooled with the floating cell fraction. Western blot analysis was performed as previously described (Moalic et al. 2001) using the primary monoclonal antibodies β-actin (Sigma), Bcl-2 (Dako), Bax (Immunotech), Caspase-3 (Santa Cruz Biotechnology) and respective secondary polyclonal antibodies conjugated with peroxidase (Dako). Blots were visualized using enhanced chemiluminescence reagents (Amersham Biosciences) and immediately exposed to X-ray films.

4.10. Statistical analysis

Median and standard deviation (SD) were calculated using Excel (Microsoft Office, Version 2007). Statistical analysis of differences was carried out by analysis of variance (ANOVA). A P- value of less than 0.05 was considered to indicate significance.
Conflict of Interest: The authors have no known conflicts of interest associated with this study and there has been no significant financial support for this work that could have influenced its outcome.

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