Vol 1, No 1 (2015)

Antiproliferative activity of *Origanum compactum* extract on lung cancer and hepatoma cells

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Received: July 11th, 2015; Accepted: August 10th, 2015

Abstract: Natural products have been shown to present interesting biological and pharmacological activities and are used as cancer preventive and therapeutic agents. Plants have historically been used in treating cancer and are recognized for their ability to produce secondary metabolites.

Origanum compactum Benth. (Lamiaceae) is a well-known Moroccan plant with cancer-related ethnobotanical use. Previously, we demonstrated that ethyl acetate extract of *O. compactum* had antiproliferative 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 diverse human cancer cell lines such as A549 lung cancer and SMMC-7721 hepatoma cells. Furthermore, this study 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 protein expression analyses.

Our present work has shown that this extract remarkably inhibited proliferation of A549 (IC_{50}: 198 ± 12 µg/ml) and SMMC-7721 (IC_{50}: 266 ± 14 µg/ml) cells. The characterization of antiproliferative activity demonstrated that this extract was an apoptosis inducer in both cell lines tested. The results of protein expression analyses have shown in A549 cells that this extract activated caspase signaling triggered by the modulation of Bcl-2 family proteins. These results suggest that these natural extract-induced effects may have novel therapeutic applications for the treatment of various cancer types.

Keywords: Antiproliferative activity, apoptosis, cancer, caspase, *Origanum compactum*.

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1. Introduction

Over the past few years, cancer has remained a major cause of death. The number of persons affected by cancer is continuing to increase (Yildiz et al. 2013). Hence, a major part of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets (Xia et al. 2004). Due to enormous tendency of plants, which produce a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antiproliferative and anti-cancer activities (Kim et al. 2005; Chin et al. 2006).

The anti-cancer properties of a multitude of medicinal herbs mediated through different mechanisms include altered carcinogen metabolism, induction of DNA repair systems, induction of apoptosis, immune activation and suppression of cell cycle progression. While launching the mechanism of apoptosis could be considered a convergence point of all anti-neoplastic therapies, direct pro-apoptotic effects have been reported for bioactive phytochemicals (Taraphdar et al. 2001; Lee et al. 2003). Programmed cell death or 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 numerous pro-apoptotic molecules (Orrenius 2004). Most types of apoptosis induced by cellular stress (notably anticancer drugs) 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).

O. compactum belongs to the botanical family of Lamiaceae. It is very frequent around the Mediterranean basin. Throughout Morocco, it is known locally as "zaâtar" and is traditionally considered as a panacea for health. The aerial parts of this plant have been used as a vermifuge, aphrodisiac, condiment, antispasmodic, antidiarrheal, antiacid, sedative and to treat several solid tumors (Hmamouchi 1999; Merzouki et al. 2000; Chaouki et al. 2010).

Recently, we have showed that ethyl acetate extract of *O. compactum* has antiproliferative potential on human breast tumor MCF-7 cells (Chaouki et al. 2010). In order to know if the results obtained with extract of *O. compactum* on MCF-7 cells could be extended to other types of cancer cells, we have tested this plant extract on human A549 lung cancer and SMMC-7721 hepatoma cell lines. Moreover, to gain further insight into the mechanisms

involved in the effects of this natural extract on 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. Materials and Methods

2.1. Plant material

O. compactum was collected during March 2008 from its natural ground in Morocco and taxonomically identified at the National Institute of Medicinal and Aromatic Plants, Taounate. A voucher specimen (FMP-79) is deposited at the herbarium of the Faculty of Medicine and Pharmacy, Rabat.

2.2. Extraction of plant material

The dried aerial parts were crushed, and 500 g of powder were extracted as we described previously (Chaouki et al. 2010) on soxhlet over at least 48 h by four organic solvents with increasing polarity: hexane, chloroform, 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 3.33%.

2.3. 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 subconfluence. 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 *O. compactum* 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.

2.4. 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 cell 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 *O. compactum* 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 (5mg/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 experiment 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.

2.5. 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 generate a 50% reduction in cell proliferation (IC_{50}). The values of the IC_{50} obtained were confirmed by trypan blue exclusion assay data.

2.6. LDH test

Cells were seeded in 96-well plates and treated without or with ethyl acetate extract of *O. compactum*. 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.

2.7. DNA fragmentation analysis

For detection of apoptotic DNA cleavage, the DNA degradation assay was performed using ladder DNA fragmentation assay. Briefly, A549 and SMMC-7721 cells were collected after treatment with IC_{50} values of ethyl acetate extract of *O. compactum* 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. Added 120 µl potassium acetate (pH

5.3) and stored on ice for 1 h after that centrifuged for 10 min at 4°C 12000 rpm. Added 2 µl (10 mg/ml) RNase to 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 dissoled pellet with distilled water. DNA fragmentation was visualized by electrophoresis in a 0.8% agarose gel containing ethidium bromide.

2.8. Apoptosis quantification by ELISA

Apoptotic cell death was quantitatively determined by "cell death" enzyme-linked immunosorbent assay (ELISA)(Cell Death Detection ELISAPlus, Roche Diagnostics). In brief, cells were cultured in 75 cm^2 tissue culture flasks. After ethyl acetate extract of *O. compactum* 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. 2009).

2.9. Protein expression analysis

Cells were cultured in 150 cm^2 tissue culture flasks. After treatment with IC_{50} values of ethyl acetate extract of *O. compactum*, 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.

2.10. Statistical analysis

The median and standard deviation (SD) were calculated using Excel (Microsoft Office, Version 2010). 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.

3. Results

3.1. O. compactum extract reduces proliferation of A549 and SMMC-7721 cancer 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) assay. In our experimental conditions, ethyl acetate extract of *O. compactum* could reduce proliferation for both tumour cell lines, especially at

times 48 and 72 h (Figure. 1A and B), and there were significant differences compared with the negative control ($P<0.001$). Ethyl acetate extract of *O. compactum* induced a remarkable decrease into cell proliferation in a dose and time-dependent manner.

The concentrations of plant extract required to generate a 50% reduction in cell proliferation (IC_{50}) were computed by a regression analysis using data from MTT assays (Table 1). For the the two cell lines, the IC_{50} values decrease according to time of treatment (data not shown). According to the comparison of IC_{50} values (Table 1), antiproliferative activity of ethyl acetate extract of *O. compactum* on A549 cell line (IC_{50}: 198 µg/ml at 48 h) was higher than SMMC-7721 cell line (IC_{50}: 266 µg/ml at 48 h).

Table 1: IC_{50} Values of ethyl acetate extract of *O. compactum* in tumor cell lines.
IC_{50} (inhibitory concentration 50%) and SD (standard deviation for 95% confidence): was determined from the linear regression curve of cell proliferation inhibition on A549 and SMMC-7721 cell lines versus plant extract. The range of concentrations assayed was 1 to 1000 µg/ml. Doxorubicin was used as a positive control and exhibited an IC_{50} value of less than 7 µg/ml for the three treatment times.

	IC_{50}-48 h (µg/ml)	
Tumor cell line	A549	SMMC-7721
O. compactum extract	198 ± 12	266 ± 14

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

3.2. O. compactum extract induced antiproliferative effect on A549 and SMMC-7721 cells via the process of apoptosis

In order to define the mechanism of the antiproliferative activity of *O. compactum* extract on proliferation of A549 and SMMC-7721 cells, plant extract-treated cells were subjected to 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 *O. compactum* respectively with concentrations of 198 and 266 µg/ml (IC_{50} values of each tumor cell lines), no cytotoxic effect was detected (data not shown). So, cell death induced by the *O. compactum* extract treatment for examined dose seemed to be not necrosis.

To know characteristic of antiproliferative activity of ethyl acetate extract of *O. compactum* on A549 and SMMC-7721 cells, plant extract-treated cells were subjected to apoptosis assays, including DNA fragmentation assay and ELISA assay, as described in Materials and Methods. At first, we have shown that no DNA fragment were found in untreated cells but DNA fragments were observed in A549 and SMMC-7721 cells (Figure. 2) treated with IC_{50} values of each cell lines, indicating that the both tumor cell lines underwent apoptosis.

Then, the apoptotic cell death induced by extract of *O. compactum* treatment was furthermore confirmed through ELISA assay. The treatment of A549 and SMMC-7721 cells at 48 h by the ethyl acetate extract with IC_{50} values respectively has revealed an increase in the apoptotic ratio of 2,99 and 1,96 times respectively compared to control (Figure. 3A and B). At this step of our research, results established that ethyl acetate extract of *O. compactum* was an apoptosis inducer in A549 and SMMC-7721 cells.

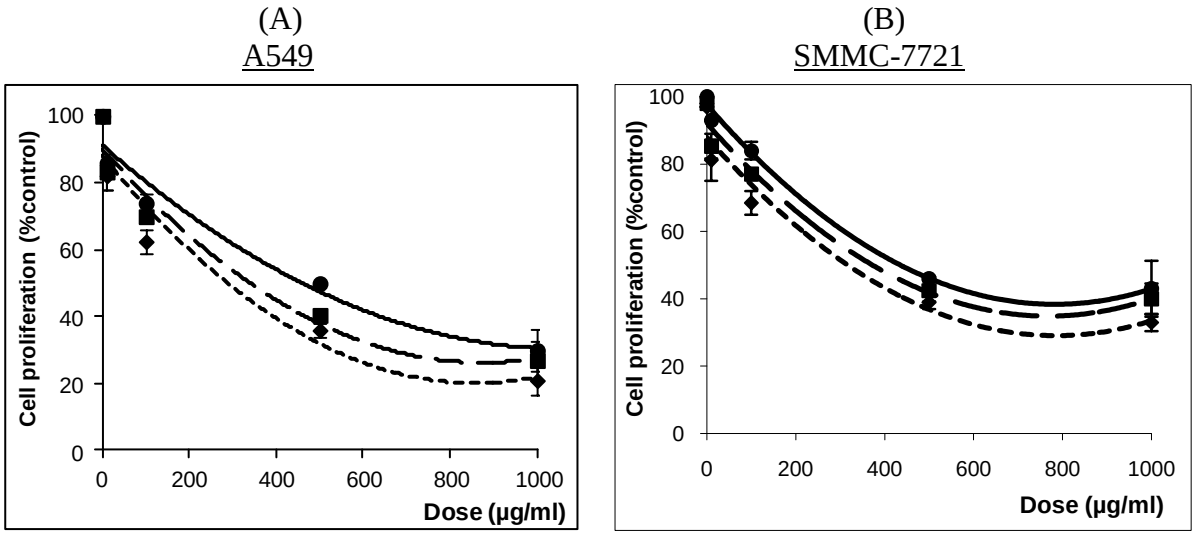

Figure. 1: Antiproliferative activity of ethyl acetate extract of *O. compactum* on A549 (A) and SMMC-7721 (B) cancer cell lines.
Cells were treated with various concentrations for 24 (—), 48 (- -), and 72 h (…). Results are presented as percentage of negative control (untreated cells) proliferation.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

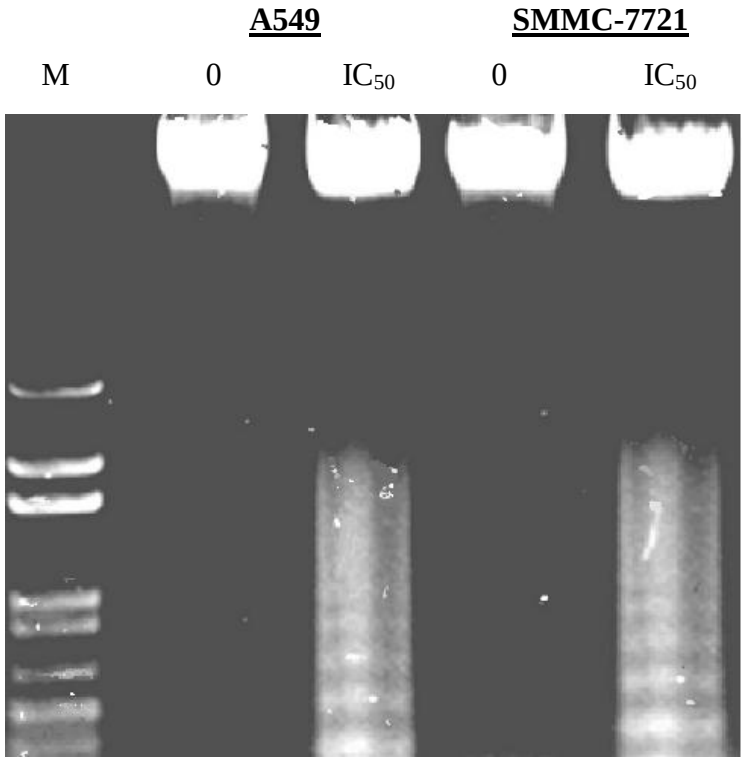

Figure. 2: Analysis of DNA fragmentation in A549 and SMMC-7721 cells, using agarose gel electrophoresis.
Cells were incubated without or with IC_{50} values of ethyl acetate extract of *O. compactum* for 48 h. Genomic DNA was prepared as described in Materials and Methods and analyzed by 0.8 % agarose 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.

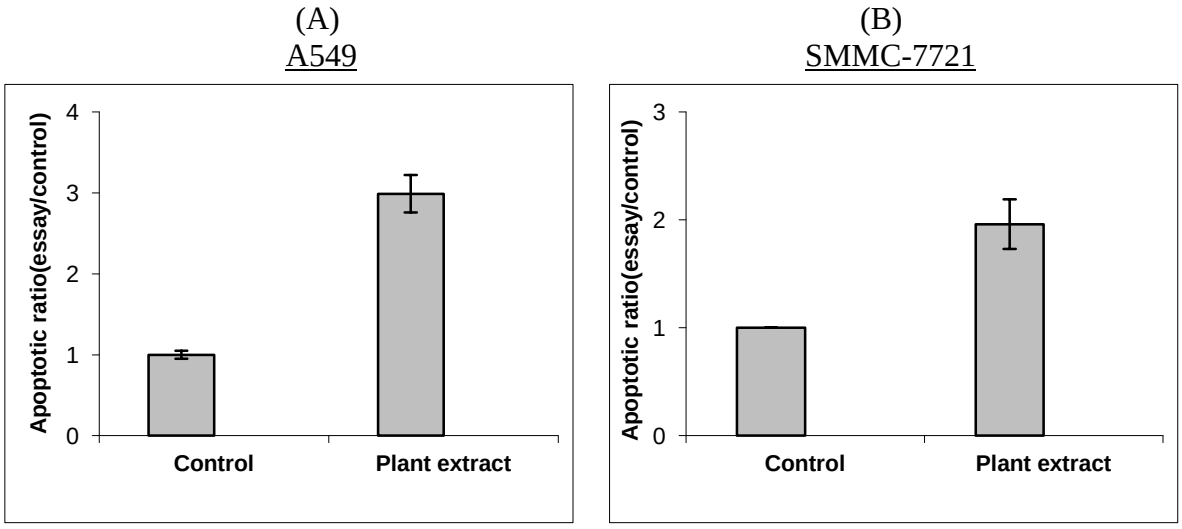

Figure. 3: Quantification of DNA fragmentation after *O. compactum* extract treatment.
A549 (A) and SMMC-7721 (B) cells were treated or not (control) for 48 h with IC_{50} values. Apoptosis was quantified on floating and adherent cells using "cell death" ELISA kit (Cell Death Detection ELISA^{Plus},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

3.3. O. compactum extract 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 extract of *O. compactum* and then harvested the cells for protein expression analysis. For the reason that mitochrondian pathway appears to be implicated in the induction of intrinsic apoptosis, we measured the levels of anti- and pro-apoptotic protein level which dysregulates mitochrondian balance. Incubation of A549 cells with plant extract up-regulated the levels of pro-apoptotic protein Bax and down-regulates anti-apoptotic protein Bcl-2 on the contrary to SMMC-7721 cells (Figure. 4A and B), which indicates mitochrondia-mediated apoptosis in A549 cells.

To further determine whether extract of *O. compactum* 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 A549 cells with extract of *O. compactum* up-regulated the levels of the biologically active cleaved caspase-3 thereby activating the apoptotic cascade pathway (Figure. 4A), whereas the results for caspase-3 expression in SMMC-7721 cells were inconclusive (Figure. 4B).

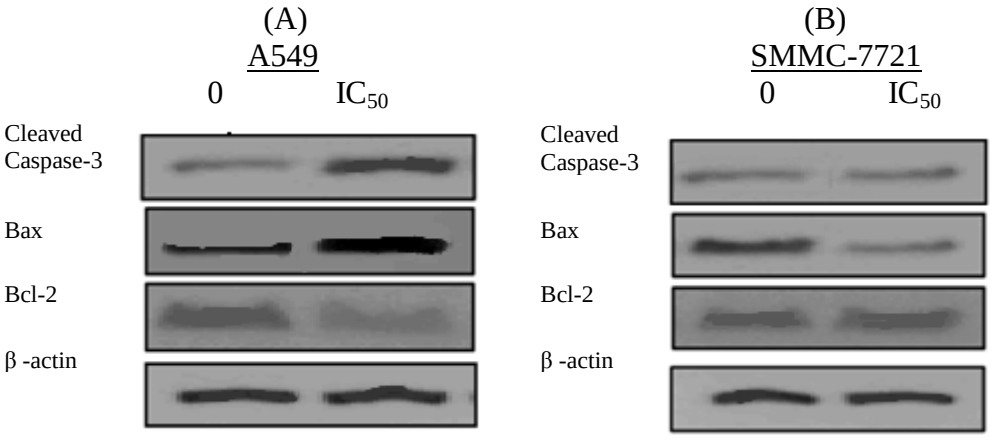

Figure. 4: Bax, Bcl-2 and caspase-3 analysis expression in A549 and SMMC-7721 cells.
(A and B), Western blot analysis. A549 and SMMC-7721 cells were treated or not for 48 h with IC_{50} values of ethyl acetate extract of *O. compactum*. Total protein was extracted from the cells and separated on 15% 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.

4. Discussion

Cancer cells evolve to avoid apoptosis-inducing signaling pathway in order to survive (Hanahan and Weinberg Robert 2011). Hence, induction of apoptosis in cancer cells can be a promising treatment way against malignancies. Natural-derived products, regardless of crude extracts or isolated active compounds, had drawn growing attention as agent in cancer therapy, due to their ability to modulate apoptosis (Fulda 2010; Chaouki et al. 2009).

Recently, we have shown that ethyl acetate extract of *O. compactum* had antiproliferative potential on human breast tumor MCF-7 cells (Chaouki et al. 2010). In order to know if results obtained with extract of *O. compactum* 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. Our results showed that this extract strongly reduced proliferation of A549 and SMMC-7721 cells as previously described in MCF-7 cells (Chaouki et al. 2010) and induced apoptosis. Our findings were in accord with a previous study reported the *in vitro* anti-hepatoma activity of an herb of the same botanical family, named *O. marjorana* L. (Liang et al., 2002). According to the comparison of IC_{50} values, the degree of antiproliferative activity and apoptosis on A549 cell line is higher than on SMMC-7721 cell line. This selectivity could be associated to the sensitivity of the cell line to the bioactive compounds in the extract.

The purpose of this study was also to explore whether the ethyl acetate extract of *O. compactum* 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, included two major types of pathways, namely, the death-receptor-mediated extrinsic pathway and the mitochondria-dependent intrinsic pathway (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, Bcl-xL) and pro-apoptosis proteins (such as Bax, Bak) (Antonsson and Martinou 2000). Bcl-2 and Bax proteins, as two major members of the Bcl-2 family, may form heterodimer complex to cause mutual neutralization of their functions which resulting in apoptosis triggering (Nascimento Pde et al. 2004). Thus, 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, downregulated the protein level of Bcl-2 and upregulated Bax in A549 cells. Hence, increasing Bcl-2 family protein expression (Bax and Bcl-2 ratio) might indicate that ethyl acetate extract of *O. compactum* induced apoptosis in A549 cells, strongly correlates with the intrinsic mitochondrial apoptotic signaling pathway on the contrary to SMMC-7721 cells. The detail pathways and mechanisms of induction of *O. compactum* extract in A549 and SMMC-7721 cells warrant further research.

In cancer biology, it is now evident that 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 an essential role in the terminal and execution phases of apoptosis induced by various 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 degradation and apoptotic death (Cain et al. 2002). In our work, treatment of A549 cells to extract of *O. compactum* resulted in a remarkable increase in the proteolytic activation of caspase-3 (cleaved caspase-3), which is the main executioner of apoptosis, in contrast to SMMC-7721 cells. As expected in A549 cells, caspase-3 degraded clearly which correlated with a caspase signaling pathway and apoptosis that finally fragment DNA.

As a conclusion, we demonstrated that ethyl acetate extract of *O. compactum* caused an inhibition of cell growth with apoptosis 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 A549 cells. These findings suggest that *O. compactum* extracts can have high potential for the development of chemotherapeutic compounds. Obviously, our works show that further research needs towards isolating the active compounds present in this plant, which may be an encouraging step towards development of novel anti-tumors from the extract.

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