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Cytotoxic effect of *Berberis libanotica* roots extracts on human cancer cells and antioxidant activities

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ABSTRACT

In the present study, we evaluated the cytotoxic effect and the antioxidant activity of methanolic and ethanolic roots extracts obtained from *Berberis libanotica* a Lebanese medicinal tree. Cytotoxic activity was assessed on the colon cancer HT 29, HCT 116, Caco-2 and breast cancer MCF-7 and MDA-MB-231 cell lines, using the MTT viability assay. Both extracts inhibited cancer cells proliferation in a dose and time depending manner without being cytotoxic against the normal MCF-10 cell line. Our results suggest that methanolic extract could induce a caspase-independent cell death in the colon and breast tumor cells HT 29 and MCF-7, respectively. DPPH and FRAP assays showed a moderate to strong antioxidant activity of the methanolic and ethanolic extracts with EC₅₀ values of 0.13 ± 0.001 and 0.1 ± 0.002 mg/ml, respectively. Collectively, these findings suggest that *Berberis libanotica* roots could serve as a promising source of antioxidant and anticancer bioactive compounds.

Keywords: *Berberis libanotica*, Methanolic and ethanolic extracts, Cytotoxic and antioxidant activities.

INTRODUCTION

Free radicals, including highly reactive oxygen species (ROS) such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), can be both helpful and harmful to human health. When found in moderate levels, ROS mediates a beneficial effect on a broad range of physiological processes including cell growth and immune responses. However, excessive ROS amounts can lead to oxidative stress, a toxic process that can damage cellular lipids, proteins and DNA, thus causing cell death^[1,2]. Oxidative stress is reported to be strongly associated with the pathogenic processes underlying several diseases including: cancer, Alzheimer's, autoimmune disorders and coronary vascular diseases^[3-7]. To avoid oxidative stress, human body relies on both endogenous and exogenous antioxidant systems. Endogenous antioxidants, synthesized by the body, include different enzymes such as Superoxide dismutase, catalase and glutathione peroxidase^[8]. Exogenous ones are usually obtained from dietary natural sources such as plants and fruits^[9]. Plants have long been known for their pharmacological and therapeutic potential^[10]. This is, mainly, attributed to their biologically active chemical components including flavonoids and phenolic acids that exhibit a significant antioxidant activity^[11]. Interestingly, a diet rich in plants polyphenols has been associated with efficient antioxidant protection against different diseases including cancer^[12,13].

Lebanon is endowed with a remarkable and very rich flora. *Berberis libanotica* or Lebanese berberry, belonging to the Berberidaceae family, has been widely used in the Lebanese traditional medicine^[14]. Phytochemical analysis of *Berberis libanotica* roots extracts identified a major presence of alkaloids^[15]. *Berberis libanotica* roots extracts have been reported to exert important biological activities. For instance,^[16] showed that the ammonia-dichloromethane *Berberis libanotica* roots extract can induce a significant reduction in cell viability and inhibit the proliferation of different human prostate cancer cell lines. In addition, a recent study^[17] showed that ethanol *Berberis libanotica* roots extract exert a potent anti-proliferative and pro-apoptotic effects in human erythroleukemia cells (HEL and K562 cells). Yet, little is known about the effect of alcoholic *Berberis libanotica* roots extracts on the proliferation and viability of colon and breast cancer cell lines. In the present study, we investigated the cytotoxic effect and the antioxidant activity of two *Berberis libanotica* root extracts. We also checked their efficacy in reducing the viability of different human colon and breast cancer cell lines and the molecular mechanism(s) mediating the cytotoxic effect of the methanolic root extract.

MATERIALS AND METHODS

Plant material

The roots of *Berberis libanotica* were collected from Ehden, Bsharri (north of Lebanon at an altitude of 1400m) in September 2015. Botanical identification and authentication were performed and voucher sample (AMB14) were deposited in the herbarium of Faculty of Pharmacy, Lebanese University, Beirut, Lebanon. The plant roots were open air dried under the shade at room temperature, then pulverized using an electric blender and stored in amber airtight bottles till further use.

Berberis libanotica roots extracts preparation

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Briefly, ethanol 80% and methanol 80% extraction methods were inspired from the protocol described in *Berberis vulgaris* monograph of the French Pharmacopoeia. Briefly, 20 g of the plant root powder was extracted using either ethanol/water 160:40 (v/v) or methanol/water 160:40(v/v) as extracting solvents mixtures. The mixture was macerated for 24 h under magnetic stirring, filtered using Whatman paper, then the supernatant was collected and evaporated under reduced pressure at a temperature of 40°C using a rotary evaporator (Heidolph, Schwabach, Germany), and then subjected to freeze drying. The obtained dried extracts were kept in amber airtight bottles at room temperature until required and reconstituted for further analysis.

Evaluation of antioxidant activity

Diphenyl -1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of the methanolic and ethanolic extracts (80/20) of the roots of *Berberis libanotica* were determined via 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay. Firstly, 5 different concentrations of each sample were prepared (0.5, 0.4, 0.2, 0.1, 0.05 mg/mL), the stock solution of each sample was diluted in the suitable solvent and then the other concentrations were prepared by dilution with water. The DPPH solution was prepared at a concentration of 5.2 mg / 100 mL. In separate tubes, we mixed vigorously 1mL of each solution and 1 mL of the DPPH solution. The tubes were then left in the dark for 30 min at room temperature. The absorbance was measured at 517 nm. For each sample measurement, a blank solution containing the same solvent was used for each preparation, and was run in parallel and measured. The DPPH solution was used as a negative control. Ascorbic acid solutions, having the same concentrations as the sample preparations, were used as antioxidant molecule reference. Percent activity was determined using the following equation:

$$\% \text{ Activity} = [1 - (A_{\text{sample}} / A_{\text{Blank}})] \times 100$$

Two replicate absorbance data were recorded. The EC₅₀ value is the extract concentration required to obtain a 50% antioxidant effect.

Ferric Reducing Antioxidant Power (FRAP) assay

The ferric reducing power of the extracts was performed. This assay is normally based on the blue coloration that develops due to the

reduction of ferric iron to the ferrous. A serial dilution of solutions of aqueous methanolic extract (80%), aqueous ethanolic extract (80%) and ascorbic acid (0.5, 0.4, 0.2, 0.1, 0.05 mg / mL), were prepared and diluted in water. An aliquot (200 µL) of each extract solution was mixed with 200 µL of 0.2M phosphate buffer (pH 6.6) and 200 µL of potassium ferrocyanate [K₃Fe (CN)₆](1%). The mixture was incubated at 50°C for 20 min. After cooling, 200 µL of 10% trichloroacetic acid (w/v) were added and the mixture was centrifuged at 1000 rpm for 8 min. The upper layer (800 µL) was mixed with 800 µL of distilled water and 160µL of 0.1% ferric chloride. After a 10 min reaction time, the spectrometric absorbance was recorded at 700 nm and compared with ascorbic acid as positive control (higher absorbance readings indicate higher reducing power). The data are reported as the average of two measurements given as ± SEM.

In vitro cytotoxicity assay

Cell culture

A panel of five human cancer cell lines: estrogen receptor-positive (MCF-7),estrogen receptor-negative (MDA-MB-231) human breast adenocarcinoma cells, human colorectal adenocarcinoma HT 29, human colon carcinoma HCT 116, and human epithelial colorectal adenocarcinoma cancer cells (Caco-2) were used. The MCF-10 cells were used as a non-tumorigenesis breast epithelial cell line model for a comparison to the cancer cell models. For MCF-7, MDA-MB-231 and Caco-2, cells were maintained in dulbecco's modified eagle's medium (DMEM, LONZA) where HT 29 and HCT 116 cells were grown in RPMI-1640 medium (LONZA). Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (LONZA). MCF-10 cells were grown in a 1:1 mixture of DMEM and Ham's F12 medium containing 5% horse serum with 20 ng/mL human epidermal growth factor,100 ng/ mL cholera toxin,0.01 mg/mL bovine insulin and 500 ng/mL hydrocortisone, 95% (Sigma-Aldrich). Cells were harvested by trypsin-EDTA at 37 °C, pelleted, re-suspended and grown in 75 cm² culture flasks, under standard cell culture conditions at 37°C and 5% CO₂ in a humidified incubator. The cell count was determined by trypan blue exclusion.

Cell viability assay

Cell viability of methanolic and ethanolic *Berberis libanotica* roots extracts was assessed by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay from Sigma-Aldrich (St. Louis, MO, USA).Cells were seeded in triplicates, in 100 µL complete medium, into 96-well plates at a density of 7000 cells/well for HT 29, HCT 116 Caco-2 and MDA-MB-231, 6500 cells/well for MCF-7 and 10 000 cells/well for the MCF-10 cell lines. After 24 h, cells were treated with various extracts (methanol 80% and ethanol 80%) at different concentrations (0, 5, 10, 20, 30, 50, 70 and 100 µg/mL) for 24, 48 and 72 h. MTT reagent was added to each well and incubated for 3h at 37 °C. Finally, the reaction was stopped by adding 100 µL of SDS 10% with 0.01 N HCl into each well. The reduced MTT optical density (OD) was measured by ELISA reader at 595 nm. The percentage of cell viability was presented as an OD ratio between the treated and untreated cells, at the indicated concentrations. Data are expressed as IC₅₀ ± SD obtained from triplicate determinations of three independent experiments (n=3) for each cell line

Statistical analysis

Values presented correspond to Mean ± SD. The IC50 and EC50 values were determined appropriately with graphPad prism software (San Diego, USA).

Immunoblotting

HT 29 and MCF-7 cells were seeded in 6-well plates and treated for 48 h with various concentrations of methanol 80% extract at around IC50. Treated cells were washed with PBS 1X and lysed by Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.01% bromophenol blue). Protein lysates were centrifuged at 14000 rpm for 5 min at 4°C, to remove debris. Proteins quantification was performed using lowry assay. Protein extracts (60 µg for each sample) were prepared, denatured and separated (80V, 120 min) by SDS-polyacrylamide gels electrophoresis and electro transferred to nitrocellulose membranes (100 V, 120 min). Membranes were blocked for 1h with 5% skimmed milk in Tris-buffered saline/0.05% Tween-20 and incubated overnight under shaking at 4°C with primary antibody: PARP (ab6079), p53(ab26), Bax(ab32503), Bcl-2(ab7973) and GAPDH (ab8245). After washing twice, the membranes were probed with appropriate secondary antibodies anti-rabbit (ab6721) and anti-mouse (cell signaling, ref 02/2015) conjugated to horseradish peroxidase. GAPDH was used as loading control to ensure equal loading of proteins for each sample. The signal was detected using an enhanced chemiluminescence system ECL (Luminata Crescendo Western HRP substrate (Millipore). Images were captured using an X-RAY film (MMXBE Film, Care Stream, USA).

RESULTS AND DISCUSSION

Extraction yields of *Berberis libanotica* root extracts

The choice of the extraction procedure depends primarily on the class of targeted compounds to be isolated as well as on the type of solvents used with varying polarities [18]. The extraction yields of aqueous methanolic and ethanolic roots extracts were determined to be 5 and 5.6%, respectively (Table1).

Table 1: Extraction mass and yield of extracts obtained from *Berberis libanotica* roots extracts.

Extract-Solvent	Initial mass used (g)	Mass after extraction (g)	Yield (%)
Methanolic extract	20.0	0.92	5.0
Ethanolic extract	20.0	1.12	5.6

Antioxidant activity of *Berberis libanotica* root extracts

An antioxidant is defined as a substance that inhibits the oxidation process, a reaction that can produce harmful free radicals [19]. Both DPPH radical scavenging activity and the ferric reducing power (FRAP) assays were carried out to evaluate the antioxidant activities of methanolic and ethanolic *Berberis libanotica* roots extracts. Ascorbic acid, a pure antioxidant compound, was used as reference compound and showed the highest antioxidant activity. Interestingly, both extracts exhibited substantial DPPH scavenging capacity after 30 min of the incubation period. This activity increased further upon augmenting the extracts concentrations where they exhibited 90% of activity at a concentration of 0.5mg/mL (Fig. 1). Remarkably, no

significant difference in the antioxidant activity displayed by each of the two extracts in comparison to that of ascorbic acid was noted at concentration of 0.5 mg/mL. The EC50 values are provided in Table 2.

Table 2: DPPH antioxidant capacities of the *Berberis libanotica* roots extracts.

Samples	DPPH scavenging capacity EC ₅₀ value (mg/ml)
Methanolic extract (80%)	0.1066 ± 0.001
Ethanolic extract (80%)	0.1309 ± 0.002

The EC₅₀ value is defined as the inhibitory concentration of the roots extracts necessary to decrease the initial DPPH radicals concentration by 50%. The EC₅₀ values were obtained by interpolation from linear regression. Analysis. Each EC₅₀ value is determine as mean ± standard deviation (n=3).

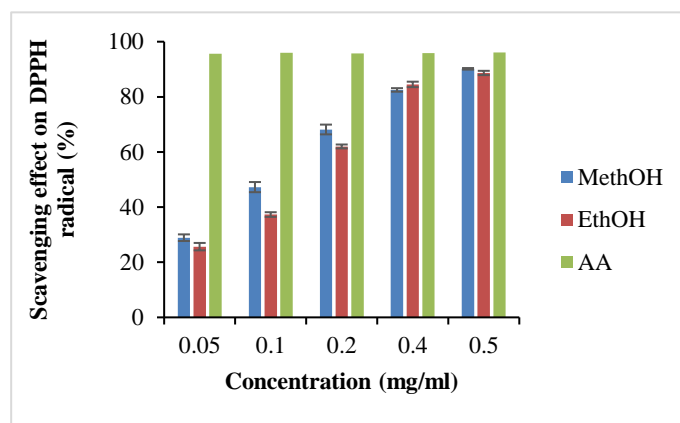


Figure 1: DPPH free radical scavenging activity of ethanol and methanol *Berberis libanotica* roots extracts. Ascorbic acid (Vitamin C) was used as a positive control. Each value is the average ±SD of two separate experiments each done in duplicate (n=2). AA = ascorbic acid.

The FRAP assay was applied on the basis of evaluating the capacity of both extracts to reduce ferric tripyridyltriazine (Fe⁺⁺⁺) to ferrous tripyridyltriazine (Fe⁺⁺). The reducing power of the methanolic and ethanolic extracts is shown in figure 2. Apparently, both extracts showed a moderate reducing activity in comparison to that displayed by the ascorbic acid. This activity increased in a manner dependent on the extract concentration where it reached about half that of ascorbic acid when the used concentration was augmented to 0.5mg/mL (Fig. 2).

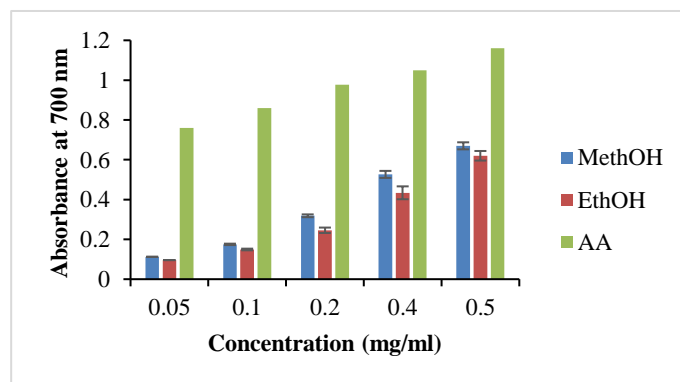


Figure 2: Ferric reducing power of ethanol and methanol *Berberis libanotica* roots extracts at various concentrations. Ascorbic acid (Vitamin C) was used as a standard control. Each value is the average ± SD of two separate experiments each done in duplicate (n=2). AA = ascorbic acid.

Generally, *Berberis libanotica* extracts have been reported to protect against oxidative stress [20]. This substantial antioxidant potential may be attributed to its chemical composition. Here, two different solvents with different polarities were used, and probably extracted different classes of chemical compounds. Polyphenolic compounds divided into several classes, may react with free radicals in different ways, depending on their chemical structure [21]. This could underline the different behaviors observed for the same extraction a manner depending on the antioxidant evaluation methodology.

Cytotoxic activity of *Berberis libanotica* root extracts

To assess the cytotoxic capacity of *Berberis libanotica* roots extracts,

MTT assay was carried out. Both extracts negatively affected the proliferation of three colon cancer cell lines (HCT 116, HT 29, and Caco 2) and two breast cancer cell lines MCF-7 and MDA-MB-231 in a dose- and time-dependent manner (Fig. 3 and Fig. 4).

Methanolic extract exhibited the highest cytotoxic activity against HT 29 with an IC₅₀=26.09µg/mL at 48 hours. It also displayed a remarkable cytotoxic effect, after 48 hours, towards HCT 116, Caco 2 and MCF-7 with IC₅₀ values of 35.08, 32.33 and 32.79 µg/mL, respectively. On the other hand, methanolic extract was less efficient in inhibiting MDA-MB-231 cell proliferation as it exhibited higher IC₅₀ values (IC₅₀= 61.48 ± 0.69 µg/mL) (Fig. 3 and Table 3).

Table 3: The growth inhibitory effects of the methanol and ethanol *Berberis libanotica* extracts against cancer selected cell lines expressed as IC₅₀ values (µg/mL).

	Cancer cell line				
	MCF-7	MDA-MB-231	HCT 116	HT 29	Caco-2
	IC ₅₀ ^a (µg/ml)				
Methanolic extract	32.79 ± 0.96	1.48 ± 0.6	35.08 ± 0.9	26.09 ± 0.7	32.33 ± 0.8
Ethanoliceextract	79.97 ± 0.93	2.61 ± 0.93	34.16 ± 0.7	48.94 ± 0.94	76.66 ± 0.6

^a Each IC₅₀ value is determine as mean ± standard deviation(n=3)

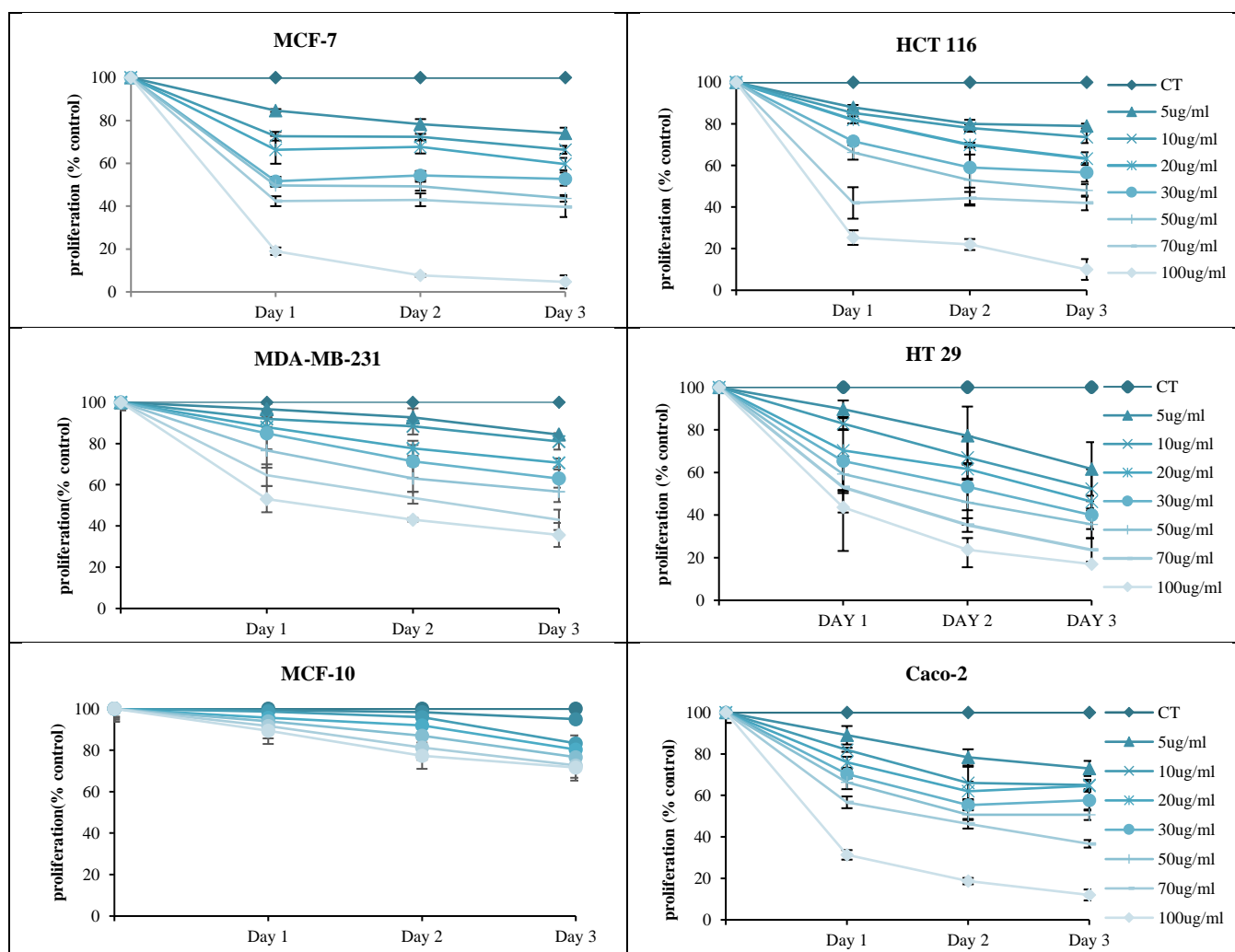


Figure 3: Methanol roots extracts of *Berberis libanotica* treatment induced a time and dose dependent inhibition of proliferation in HCT 116, HT 29, Caco-2, MCF-7 and MDA-MB-231 cancer cell lines, with a low cytotoxic activity on the normal breast cancer cell MCF-10. Cells were treated at 60% confluency with different concentrations (5, 10, 20, 30, 50, 70 and 100 µg /mL) for 24, 48 and 72 h. Cell proliferation was assessed by MTT assay as described in materials and methods. Results are expressed as percentage of control non treated cells. Each value is the average ± SD of three separate experiments each done in triplicates (n=3).

The ethanolic extract also exerted a time and dose-dependent inhibitory effect. The different tested cell lines exhibited differential sensitivities to the ethanolic extract with MCF-7 and Caco-2 being the least sensitive with IC₅₀ values of 76.66 and 79.97 μg/mL,

respectively, followed by HT 29 (IC₅₀=48.9 μg/mL), HCT 116 (IC₅₀=34.16 μg/mL) and finally MDA-MB-231 (IC₅₀=32.61 μg/mL). It is noteworthy that the highest sensitivities were detected following 48 hours of ethanolic extract addition (Fig. 4).

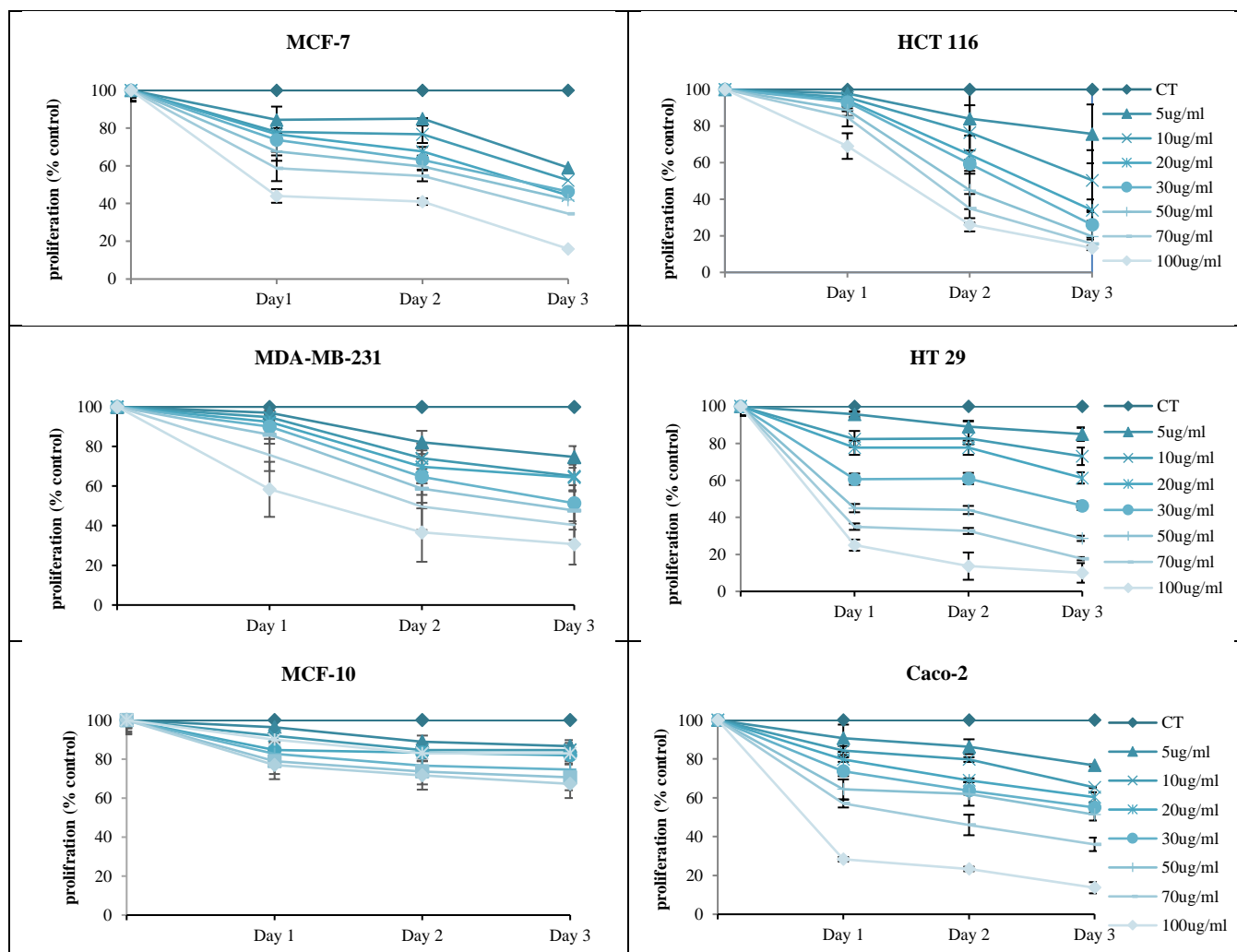


Figure 4: Ethanol roots extracts of *Berberis libanotica* treatment induced a time and dose dependent inhibition of proliferation in HCT 116, HT 29, Caco-2, MCF-7 and MDA-MB-231 cancer cell lines, with a low cytotoxic activity on the normal breast cancer cell MCF-10. Cells were treated at 60% confluency with different concentrations (5, 10, 20, 30, 50, 70 and 100 μg/mL) for 24, 48 and 72 h. Cell proliferation was assessed by MTT assay as described in materials and methods. Results are expressed as percentage of control non treated cells. Each value is the average ±SD of three separate experiments each done in triplicates (n=3)

Clearly, active compounds in both methanol and ethanol extracts from *Berberis libanotica* root exhibit cytotoxic activity with variable efficacy against colon and breast cancer cell lines. It is noteworthy that only HCT 116 cell line showed similar sensitivity to both extract.

For a plant extract to act as successful anti-cancer drug, it should kill cancer cells without causing excessive damage to normal cells. Accordingly, we assessed the cytotoxic effect of *Berberis libanotica* roots extracts on the non-tumorigenic breast epithelial cell line, MCF-10. Interestingly, MCF-10 showed higher resistance to *Berberis libanotica* root extracts compared to the cancer cell lines. In fact, treating MCF-10 cells, for 72 hours, with a concentration up to 100 μg/mL, of either extract, reduced the growth rate by less than 30% (Fig. 3 and Fig. 4). These observations indicate that both extracts were

more selective for cancer than normal cells.

To investigate the molecular mechanism(s) mediating the antitumor activity of *Berberis libanotica* root extracts, we assessed, using Western blot analysis, the protein levels of several apoptosis-related components in HT 29 and MCF-7 being treated, for 48 hours, with methanolic extract (Fig. 5). Interestingly, we observed an up-regulated expression of tumor-suppressor protein p53 in both cell lines at the indicated concentrations (Fig. 5). We also detected up-regulated levels of the pro-apoptotic protein Bax but down-regulated expression of the anti-apoptotic protein Bcl-2 after 48 hours (Fig. 5). It is noteworthy that this effect was more pronounced in the HT 29 than MCF-7 cells when treated with at low extract concentrations.

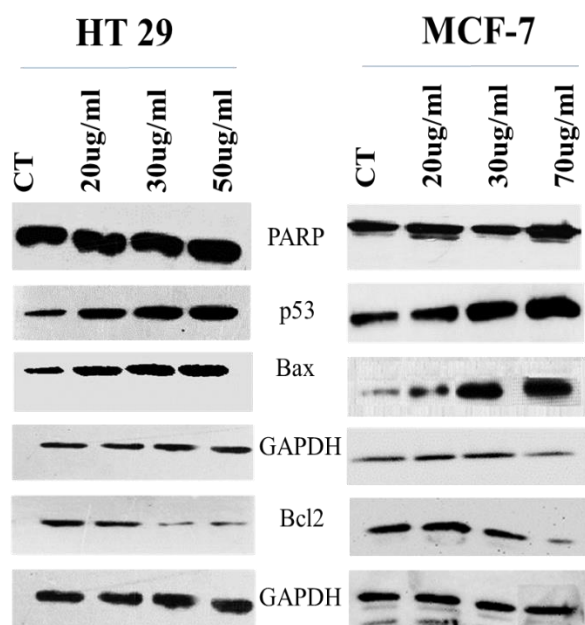


Figure 5: Expression of apoptosis related proteins in HT 29 and MCF-7 treated with increasing concentrations of methanolic 80% *Berberis libanotica* roots extract. Cells were cultured and treated with 20, 30 and 50 µg/mL for HT 29 and 20, 50 and 70 µg/mL for MCF-7, compared to an untreated control for 48 h. Proteins expression were analyzed by western blotting. GAPDH was used as a loading control.

We also analyzed poly-ADP-ribose-polymerase (PARP) cleavage. Our results showed that methanolic *Berberis libanotica* root extract did not stimulate cleavage of PARP in neither HT29 or MCF-7 cells (Fig. 5). Activation of p53 induces the transcription of pro-apoptotic genes such as Bax and represses the transcription of a number of anti-apoptotic genes such as Bcl-2. In most cases, the activation of p53 proceeds through mitochondrial release of respiratory chain component, cytochrome *c*, from the intermembrane space of the mitochondria into the cytoplasm, where cytochrome *c* activates a cascade of caspases [22, 23]. Moreover, poly-ADP-ribose polymerase (PARP) is a substrate for caspases and it is typically cleaved and inactivated during apoptosis, causing DNA fragmentation [24]. Apoptosis can also occur via alternative processes not involving caspases [25]. For instance, AIF (Apoptosis inducing factor) can be cleaved by activated poly (ADP-ribose) polymerase (PARP), and yields truncated AIF (tAIF) which translocates from the mitochondria to the nucleus where it leads to chromatin condensation and DNA fragmentation [26, 27]. Our findings suggest that the *Berberis libanotica* methanolic extract induces non classical features of apoptosis in HT 29 and MCF-7 cell lines due to the intact PARP. These observations suggest that methanolic *Berberis libanotica* root extracts might induce caspase-independent cell death in the colon and breast tumor cells HT 29 and MCF-7, respectively.

CONCLUSION

In conclusion, our results revealed that the methanolic and ethanolic extracts of *Berberis libanotica* roots exhibit considerable antioxidant activity. Moreover, both extracts showed a significant cytotoxic capacity against cancer (colon and breast) cell lines without harming normal cells. In addition, this study suggests that methanolic *Berberis libanotica* root extract triggers a caspase-independent cell death in human colon and breast tumor cells. In future work, we will assess, *in vivo*, the effect of *Berberis libanotica* root extract on colon and breast

tumor cancer development. This, in turn would highlight new therapeutic potential for *Berberis libanotica* plant especially in the field of cancer treatment.

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