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Study of the *in vitro* anticancer activity of Moroccan phenolic olive cake extracts

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ABSTRACT

The present work aimed to investigate the phenolic composition and the *in vitro* anticancer activity of ethanol olive cake extracts (OCE) originating in different areas of Tadla-Azilal region in Morocco. The HPLC-MS analysis showed that OCE were rich in biophenols mainly composed of secoiridoids and their derivatives, phenolic acids and flavonoids. The bioactivity of these phenolic extracts was evaluated by testing their cytotoxic effect against P815 mastocytoma murine cell line using the MTT assay. The results showed that the OCE exerted an *in vitro* cytotoxic activity in a dose-dependent manner, and the IC_{50} values were ranging from 20 to 40 µg/mL. Interestingly, compared to the conventional antitumor drug methotrexate, no cytotoxic effect of OCE was observed on the normal human peripheral blood mononuclear cells (PBMC). Furthermore, the extracts showed an apoptotic effect against P815 tumor cell line depending on the dose and the extract's phenolic content.

Keywords: Phenolic compounds, Olive cake, HPLC-ESI-MS, Cytotoxic activity, Apoptotic effect.

Abbreviations used in this paper: ACN: Acetonitrile, DMSO: Dimethyl Sulfoxide, FCS: Fetal Calf Serum, PBS: Phosphate Buffer Salin, TFA: Trifluoroacetic acid, OCE: Olive Cake Extract

1. Introduction

Olive oil industry, though ensuring the production of virgin olive oil as food that has different virtues for human health, is a source of pollution because of the generation of large quantities of olive mill wastes (OMW). This has become a major environmental problem in the Mediterranean olive oil producing countries such as Morocco because of their release of high polluting organic compounds. OMW are composed of two by-products: an aqueous liquid known as olive mill wastewaters and a solid waste named olive cake [1]. This solid residue, also called pomace or alperujo, is the most important waste generated in the virgin olive oil extraction process [2, 3]. It consists of skin, pulp and stone pieces of olive fruit. Olive cake is substantially composed of carbohydrates (polysaccharides, cellulose and hemicellulose), proteins, minerals and phenols (simple, acid and glycosilated phenols), [4]. Therefore, the phenolic compounds are present in olives, olive oil and olive by-products. Olive oil contains 2% of the total phenolic content of the olive fruits, while the remaining 98% is present in olive mill waste, confirming the interest of their useful valorization methods [5]. Nevertheless, their concentration and composition vary from a region to another depending on different factors and parameters [6].

Furthermore, optimized methods are expected for the valorization and environmental impact reduction of these by-products mainly as fuel, fertilizer, animal food or energy recovery [7, 8, 9, 10]. Olive cake can be effectively used as a natural and inexpensive source of cytotoxic, antioxidant and antimicrobial phenolic compounds with a wide range of bio-applications [11, 7, 12, 13].
The present work aimed to investigate the phenolic composition of OCE originating from different areas of Tadla Azilal region (central Morocco), and to evaluate the *in vitro* cytotoxic effect of these phenolic extracts as well as the apoptosis induction potential against P815 tumor cell line.

2. Materials and Methods

Chemicals

All solvents and chemicals were of HPLC grade and were obtained from Sigma Chemical Co., Saint Quentin (France).

2.1 Plant material

*Moroccan Picholin*e olives variety was identified and authenticated by Pr. A. Boulli, Department of life science, Sultan Moulay Slimane University, and stored as a voucher specimen in the Faculty of Science and Technologies, Beni Mellal, Morocco. Samples of olive cake were collected in mills from four areas of Tadla-Azilal region, namely: Beni Mellal and Krazza (plain areas), Azilal and Awrir (mountainous areas) during the winter of 2012 (January to March period). These samples were from the three-phase centrifugation oil extraction process of red-black olives maturation stage.

2.2 Phenolic compounds extraction

Dry olive cake samples (60 g each) were grounded in a coffee grinder, sifted and then defatted with 500 ml hexane in a Soxhlet apparatus for four hours. Defatted olive cake samples were subjected to soxhlet extractions using ethanol solvent. Olive cake samples (60 g) were placed in extraction thimbles into the soxhlet apparatus. 500 ml of ethanol were placed in a round flask (500 ml capacity) and then the flask was connected to the soxhlet extractor for 12 h at 70 °C of continuous extraction [11]. The resulting olive cake extracts (OCE) were concentrated by rotary evaporator and stored at – 18 °C for further analysis.

2.3 Evaluation of total phenolic compounds content

The total phenolic compounds content in each extract was evaluated by spectrophotometry using the Folin-Ciocalteu method [14, 15] with some modifications. Briefly, 2.5 ml portion of Folin-Ciocalteu reagent 0.2 N was mixed with 0.5 ml of the sample. The reaction was kept in the dark for 5 min. Then, 2 ml of a sodium carbonate solution (75 g/l) was added to the mixture and the reaction was kept in the dark for 1 h. The absorbance was measured at 760 nm in Jasco V-630 spectrophotometer. Gallic acid was used as phenolic compound standard for calibration curve (10–90 mg/mL; $y = 0.0008x - 0.0002$, where x and y represent gallic acid concentration (mg/mL) and absorbance at 765 nm, respectively; $r^2 = 0,9981$). Contents of total phenolic compounds in OCE were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.4 High performance liquid chromatography/Electro-Spray Ionization Mass Spectrometry (HPLC/ ESI-MS) analysis

HPLC–MS analysis were performed at 279 nm and 30 °C using a RP C18 column (150 x 4.6) x 5 µm with a Thermo Fisher apparatus equipped with a Surveyor quaternary pump coupled to PDA detector (diode array detector: 200-600 nm) and an LCQ Advantage (ESI) ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The injected volume was 20 µL. The mobile phase (0.5 mL/min) consisted of solvent A: TFA 0.05% in water and solvent B: TFA 0.05% in ACN. A Six-steps gradient was applied, for a total run time of 76 min, as follows: Starting from 80% solvent A and 20% solvent B increasing to 30% solvent B over 30 min, then isocratic for 10 min, increased to 30% solvent B over 10 min, to 40% over 30 min and to 20% solvent B over 2 min, and finally isocratic for 4 min. ESI ionization conditions were spray voltage 4 KV, capillary 350 °C, 14 V. Pure nitrogen was the sheath gas and pure helium was the collusion gas. The full scan mass data m/z was obtained in positive mode and ranged from 100 to 2000 Da.

2.5 Cytotoxicity assay

The cytotoxic activity was studied against the murine mastocytoma cell line (P815) using the colorimetric (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) MTT test (Sigma Aldrish) as described and modified by Tim Mosmann [15]. The cells were washed twice and placed in 96-well microtiter plates (Bioster, Italy) at a density of 1.5 10^4 cell/mL in 100 µL/well of culture medium (DMEM supplemented with 5% FCS and 1% of penicillin and streptomycin) (Sigma Aldrish). Then, 100 µL of culture medium containing the specified concentration of the tested compounds (solubilised in DMSO) were added in each well at the starting concentration of 50 µg/mL. After exposure of cells to serial concentrations of tested extracts for 48 h at 37 °C and 5% CO_2, 100 µL of medium were carefully aspirated from each well and replaced by 20 µL of MTT solution (5 mg/mL of PBS). After incubation in the same conditions for 4 hours, the plates were treated with a solution of HCL/isopropanol (24:1) to dissolve the blue intracellular formazan product. One hour later, the plates were read in a MicroELISA reader at two wavelengths (540 and 630 nm). DMSO was used as a negative control while methotrexate as a positive one.

2.6 Apoptotic cell death assay

2.6.1 Annexin V Biotin-Streptavidin FITC Test

The apoptosis analysis was performed using the Annexin V Biotin-Streptavidin FITC test. Briefly, a dense culture of P815 tumor cells in 25 cm^2 flasks ($2x10^6$ cells in 10 mL DMEM) was treated with 25 µg/mL of OCE. After 24 h incubation in the same culture conditions as above, cell pellets were washed with PBS, stained with Annexin V-Biotin, and treated with streptavidin conjugated to fluorescein isothiocyanate (FITC) for 30 min. The fluorescence was visualized using an Olympus BX50 microscope equipped with an appropriate fluorescence filter in order to detect apoptosis induction. The assay is based on the ability of Annexin V (green fluorescence) to be bound to the phosphatidylserine exposed on the surface of cells undergoing apoptosis [16].

2.6.2 DNA fragmentation test

P815 tumor cells were incubated with OCE (IC_{50} corresponding concentrations) for 24 h at 37 °C and 5% CO_2. The cells were recovered and lysed by the lysis buffer (100 mM tris HCl, 0.5M EDTA, 10% SDS (sodium

dodecyl sulfate), 5 M Nacl and 20 mg/ml proteinase k). Digestion was complete within 2-3 hours at 37 °C with constant agitation. About 500 µl of isopropanol were then added to the lysate to complete DNA precipitation (10-20 min). After precipitation, DNA was dissolved in 60 µL of a solution (7.5 pH) containing 10 mM tris HCl, 0.1 mM EDTA. Complete dissolution of DNA required several hours with constant agitation at 37 °C. Finally, 10 µl of the loading buffer (glycerol, xylene cyanol, bromophenol blue, ficcol, EDTA) were added and DNA samples were loaded onto a 2% agarose gel. DNA fragments were separated by horizontal electrophoresis (50 V, 30 mA for 4 h). The gel was stained with ethidium bromide and visualized under UV light (310 nm). Molecular weight marker and DNA extracted from cells cultures in serum starvation condition were used as a positive control.

2.7 Effect of OCE on human peripheral blood mononuclear cells (PBMC)

This test was realized in order to evaluate the effect of the extracts on human normal cells using the MTT colorimetric assay described above. To isolate the PBMC, blood samples were collected, under medical control, from healthy volunteer donors in heparinised tubes. The peripheral blood mononuclear cells were isolated using standard Ficoll-hypaque density centrifugation (Amersham Pharmacia Biotech AB). Interface lymphocytes were washed twice with PBS. OCE and methotrexate cytotoxic effect was measured in the same conditions as detailed above for tumor cell lines.

2.8 Statistical analysis

The experimental results were performed in triplicate and the data are expressed as means ± standard deviation. The comparison of the averages is made by Student test (STATISCA software). Differences are considered significant at $p<5\%$.

3. Results and Discussion

3.1 Total phenolic compounds content

The total phenolic compounds content was found to be higher in extracts from mountainous areas, 2.35 mg/g (DW) and 0.92 mg/g (DW), compared to plain areas extracts, 0.80 mg/g (DW) and 0.65 mg/g (DW) (Table 1).

Table 1: Total phenolic content expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) in olive cake extracts

Area	Total phenolic content (mg GAE/g DW)
Awrir	2.354±0.045[a]
Azilal	0.92±0.047[b]
Beni Mellal	0.80±0.031[b]
Krazza	0.65±0.009[b]

Samples were collected from Tadla-Azilal region; phenolic compounds were extracted and determined for each area as described in materials and methods. Each value is expressed as mean ± standard deviation. Different letters mean significant differences (P<0.05) (Student's test).

The content variation seems to be related to the impact of climate, soil composition, and geographical conditions [17, 18]. The plain areas of Tadla-Azilal region with irrigated rich deep clay soils, is characterized by a continental climate with an intense cold winter and a very hot summer. Mountainous areas are characterized with poor rocky soils and large periods of water shortage. The temperature varies from 3 - 4 °C to 48 - 50 °C with large variations of rainfall [19]. These conditions suggest an important degree of environmental stress mainly in the mountainous areas.

In this perspective, Boscaiu *et al*. [20] have found a positive correlation between the degree of environmental stress (soil composition, temperature variations and drought) and the level of phenolic compounds accumulated in plants. This correlation indicates the importance of the secondary metabolites in the defence mechanisms against stress.

3.2 Identification of phenolic compounds present in OCE by HPLC/ ESI-MS analysis

The screening of OCE by HPLC/ESI-MS provided the data for representative samples in figure 1 (a, b, c, d).

The identification of phenolic compounds was achieved by HPLC-MS analysis according to literature relative to previous HPLC/MS studies on olive cake phenolic extracts as indicated in Table 2 (A; B; C; D). The examination of the mass spectra of the investigated OCE revealed the presence of the most representative phenolic compounds of olive mill waste, especially glucosilated phenols (secoiridoids): oleuropein, ligstroside and secoiridoid derivatives; phenolic acids: sinapic acid, caffeic acid and vanillic acid; simple phenols: tyrosol and hydroxytyrosol, as well as flavonoids: apigenin, luteolin and their derivatives [4, 7, 13, 18, 21, 22].

Table 2: Phenolic compounds identified by HPLC-MS analysis in Moroccan olive cake extracts originate from different areas of Tadla-Azilal region.

(A). Beni-Mellal's OCE

Comp. no.	RT (min)[a]	Compounds	$[M+H]^+$ /$[M-H]^-$ (m/z)[b]	Main fragments by ESI-MS	References
1	6.22	Oleuropein derivatives	309/307	185/183, 155/153	(22, 7)
3	6.22	Unknown	229/227	213/211, 187/185	
2	6.38	Hydroxytyrosol	155/153		(13, 22, 4, 3, 7, 25, 2)
4	7.03	Dihydroxymandelic acid	185/183		(4)
6	7.03	Hydroxytyrosol glucoside	317/315	137/135	(4, 7, 24)
5	7.26	Vanillic acid	169/167		(22, 4, 17, 7, 24, 1, 2, 29)
7	11,5	Unknown	423/421	391/389, 239/237	(24)
8	11.5	Sinapic acid	225/223		(17,1)
9	22.32	3,4-DHPEA-EDA	321/319	185/183, 167/165, 141/139	(22, 7)
10	22.32	Tyrosol	139/137		(13, 22, 17, 3, 23, 26, 1, 2)
11	22.32	3,4,5 trimethoxybenzoic acid	213/211		(30)
12	28.29	Elenolic acid	243/241	225/223, 197/195, 179/177	(22, 4, 3, 7, 1, 2)
13	28.29	Luteolin	287/285	153/151	(13, 22, 4, 3, 24, 1, 2)
14	28.29	Apigenin	271/269		(13, 22, 17, 3, 1, 2)
15	29.18	Oleuropein aglycone (3, 4-DHPEA-EA)	379/377	303/301, 243/241, 225/223	(22, 3, 7, 23, 2)
16	42.59	Ligstroside derivatives	337/335	217/215, 155/153	(22, 23)
17	48.77	Unknown	790/788	773/771	
18	57.92	Ligstroside	525/523	395/393	(13, 22, 4, 17, 23)
19	57.92	Oleuropein	541/539	227/225, 303/301	(13, 22, 4, 7, 24, 27, 28, 1, 2)
20	62.78	Ligstroside derivatives	293/291		(23)

Table 2 (continued)

(B). Azilal's OCE

Comp. no.	RT (min)[a]	Compounds	$[M+H]^+$ /$[M-H]^-$ (m/z)[b]	Main fragments by ESI-MS	References
1	5.37	Oleuropein	541/539	241/139	(13, 22, 4, 7, 24, 27, 28, 1, 2)
2	7.56	Vanillic acid	169/167		(22, 4, 17)
3	7.56	Unknown	229/227	213/211, 185/183	
4	9.51	Dihydroxymandelic acid	185/183		(4)
5	9.51	Oleuropein derivatives	369/367	225/223, 141/139	(7, 22)
6	25.65	Apigenin	269/267		(13, 22, 17, 3, 2)
7	25.65	Ligstroside derivatives	395/393	271/269	(23)
8	27.76	Luteolin-7-glucoside	449/447	287/285	(22, 4, 17, 3, 24, 2)
9	27.76	Elenolic acid	243/241	225/223, 167/165, 141/139	(22, 4, 3, 7, 1, 2)
10	29.62	Oleuropein aglycone (3, 4-DHPEA-EA)	379/377	243/241, 225/223, 185/183	(22, 3, 7, 2)
11	29.62	Luteolin	287/285		(13, 22, 4, 17, 23, 24)
12	31.89	Ligstroside	525/523	243/241, 139/137	(13, 22, 4, 17)
13	31.89	Quercetin	303/301		(13, 17, 1)
14	32.62	Unknown	703/701	540/538	(24)
15	42.06	Ligstroside derivatives	337/335		(22, 24)
16	42.06	Nüzhenide	685/683		(2)
17	45.48	Unknown	790/788	773/771, 755/753, 737/735	
18	50.77	Naringenin	273/271		(29)
19	57.90	Verbascoside derivatives	463/461	299/297	(22, 7, 24)
20	57.90	Oleoside	391/389		(13, 4, 17, 24)
21	64.96	Unknown	730/728	713/711, 695/693	
22	69.20	Unknown	559/557	539/537, 379/377	(24)

Table 2 (continued)

(C). Awrir's OCE

Comp. no.	RT (min)[a]	Compounds	$[M+H]^+$ /$[M-H]^-$ (m/z)[b]	Main fragments by ESI-MS	References
1	3.51	Unknown	409/407	391/389	(24)
2	4.11	Luteolin-7-glucoside	449/447	287/285, 258/256	(22, 17, 3, 24, 2)
3	7.16	Vanillic acid	169/167	154/152	(22, 4, 17, 7, 24, 1, 2, 29)
4	7.16	Vanillin	153/151		(22, 3, 2)
5	7.89	Oleoside	391/389	247/245, 211/209	(13, 4, 17, 24)
6	7.89	Unknown	282/280	267/265	
7	10.06	Unknown	423/421	391/389, 197/195	(24)
8	13.24	Hydroxytyrosol	155/153		(13, 22, 4, 3, 7, 25, 2)
9	13.24	Unknown	229/227	211/209	
10	15.25	Oleuropein aglycone (3, 4-DHPEA-EA)	379/377	197/195	(22, 3, 7, 23, 2)
11	15.25	Ligstroside derivatives	395/393	139/137	(23)
12	24.69	Elenolic acid	243/241	225/223, 181/179, 141/139	(22, 4, 3, 7, 1, 2)
13	24.69	Hydroxytyrosol acetate (3, 4-DHPEA-AC)	197/195		(22, 4, 3)
14	25.50	Luteolin	287/285	153/151	(13, 22, 4, 17, 23, 24)
15	25.50	Rutin	611/609		(13, 22, 17, 3, 11, 24, 1)
16	26.64	Ligstroside	525/523	139/137	(13, 22, 4, 17)
17	29.40	Tyrosol	139/137		(13, 22, 4, 3, 23, 26, 1, 2)
18	29.40	Caffeic acid	181/179	135/133, 117/115	(13,22, 17, 3, 11, 27, 2, 29)
19	37.22	Verbascoside derivatives	571/569	299/297	(7)
20	41.23	Oleuropein	541/539	151/149	(13, 22, 4, 7, 24, 27, 28, 1, 2)
21	41.23	Methoxyluteolin	301/299		(23)
22	60.49	Pinoresinol	359/357		(22, 3, 1, 2)
23	60.49	Ligstroside derivatives	293/291	141/139	(23)
24	62.94	Apigenin	271/269	153/151	(13, 22, 17, 3, 2)
25	64.31	Unknown	730/728	713/711, 695/693	

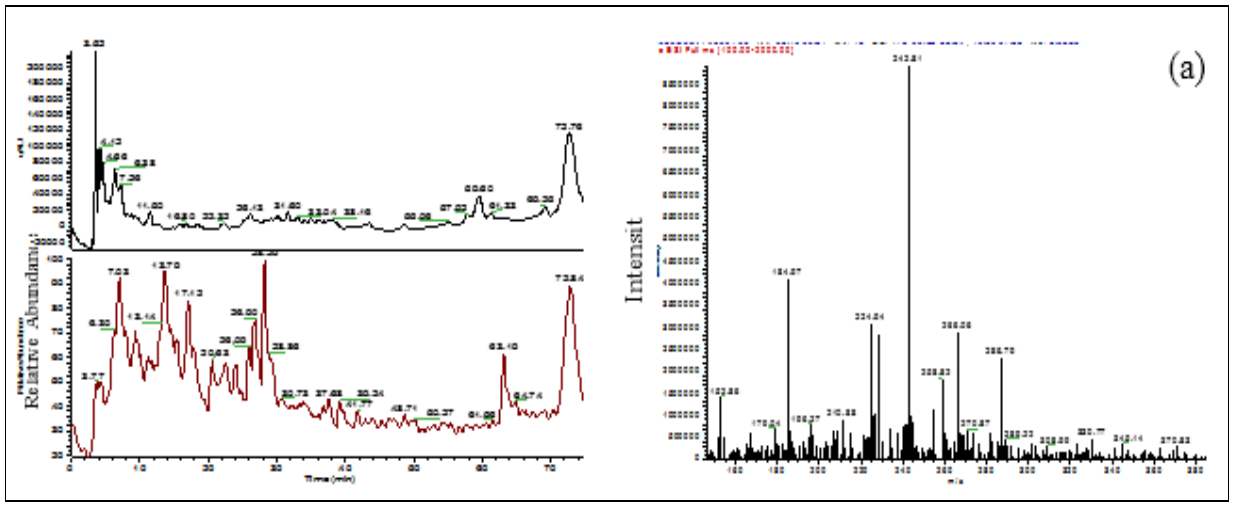
Table 2 (continued)

(D). Krazza's OCE

Comp. no.	RT (min)[a]	Compounds	$[M+H]^+$ / $[M-H]^-$ (m/z)[b]	Main fragments by ESI-MS	References
1	6.04	ME. 3,4- DHPEA-EA	409/407	379/377, 277/275	(22, 7, 23)
2	7.18	Unknown	229/227	213/211	
3	8.83	Apigenin-7-rutinoside	579/577	433/431, 379/268	(22, 17, 1)
4	12.93	11-Methyl-oleoside	405/403		(24)
5	22.26	Oleuropein derivatives	309/307	275/273, 231/229	(22, 7, 23)
6	28.67	Ligstroside aglycone (p-DHPA-EA)	363/361	243/241, 259/257, 141/139	(22, 3, 23, 2)
7	28.67	Luteolin	287/285		(13, 22, 4, 17, 23, 24)
8	32.74	3,4,5 trimethoxybenzoic acid	213/211		(30)
9	40.55	Unknown	537/535	493/491	(24)
10	40.55	Oleuropein	541/539	243/241, 185/183, 141/139	(13, 22, 4, 7, 24, 27, 28, 1, 2)
11	41.77	Elenolic acid	243/241	225/223, 167/165, 141/139	(22, 4, 3, 7, 1, 2)
12	41.77	Methoxyluteolin	301/299	287/385	(23)
13	42.37	Dihydroxymandelic acid	185/183		(4)
14	47.54	Unknown	790/788	773/771, 755/753, 737/735	
15	58.48	Ligstroside derivatives	395/393	259/257, 139/137	(23)
16	58.71	Unknown	423/421	243/241, 167/165	(24)
17	58.71	Caffeic acid	181/179		(13, 22, 17, 3, 11, 27, 2, 29)

[a] Retention time (min). [b] Mass charge value

Samples were collected from different areas of Tadla-Azilal region and phenolic compounds were extracted and analyzed using HPLC-ESI-MS technique as described in materials and methods


(a)
Relative Abundance
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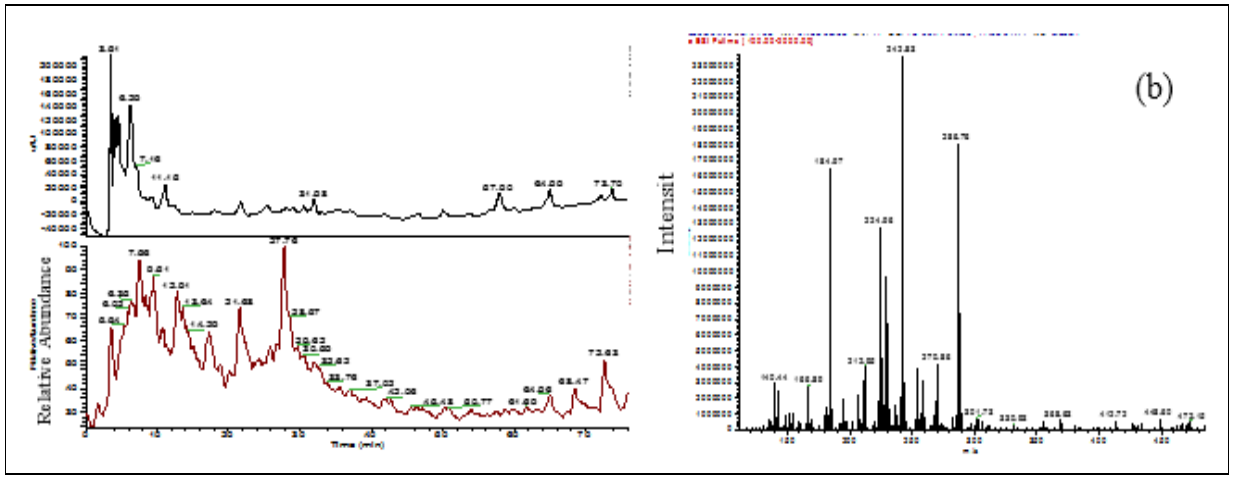
(b)
Relative Abundance
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Time (min)
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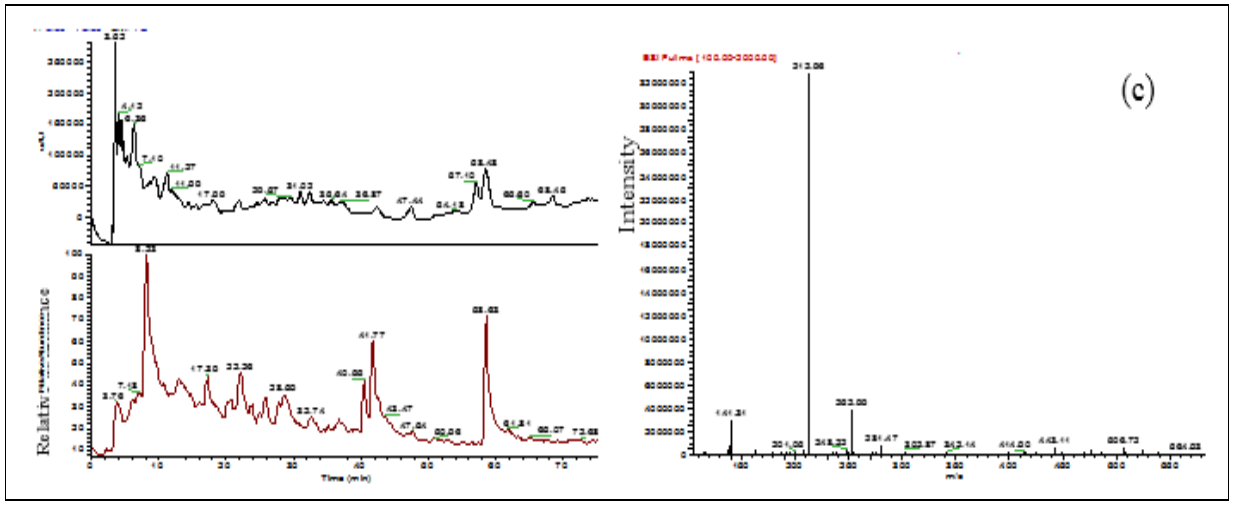

(c)
Relative Fluorescence
Intensity
Time (min)
m/z

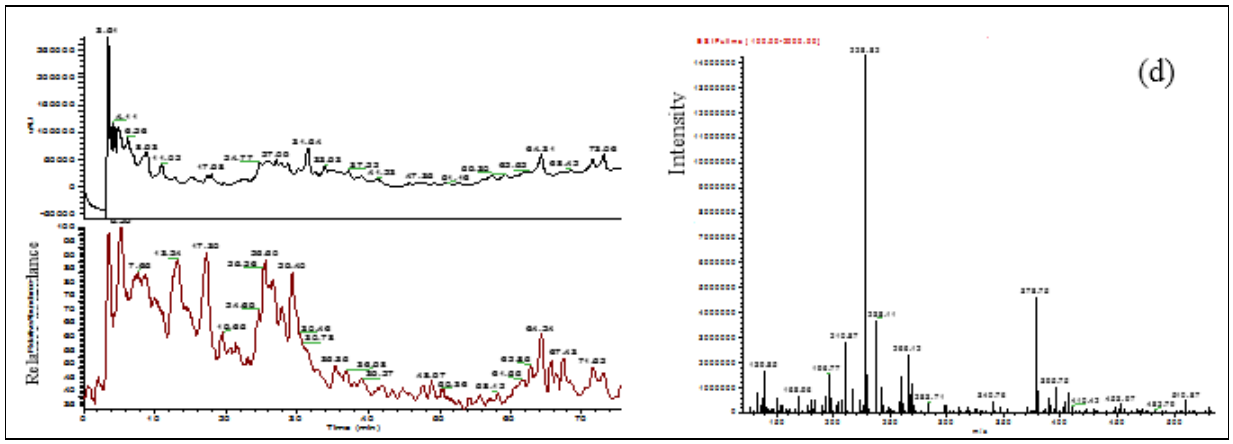


Fig 1: Representative HPLC/ESI-MS analysis: Chromatograms/TIC MS scan and full scan MS) of OCE derived from different areas: (a) Beni Mellal's OCE; (b) Krazza's OCE; (c) Azilal's OCE; (d) Awrir's OCE.
Olive cake samples were collected from Tadla-Azilal region; the HPLC-ESI-MS was performed for each OCE as described in materials and methods.

Furthermore, important levels of molecules with molecular mass up to 1000 Da corresponding to dimeric or polymeric phenols (800- 2000 Da) were detected in all the studied samples (figure 2). These compounds could be attributed to the polysaccharides, tannins and catechins [17, 31]. These results are in agreement with those reporting that olive mill wastes, in addition to the phenols (simple, acids and glycosilated phenols), contain carbohydrates and polysaccharides substantially composed of polymerin [4].

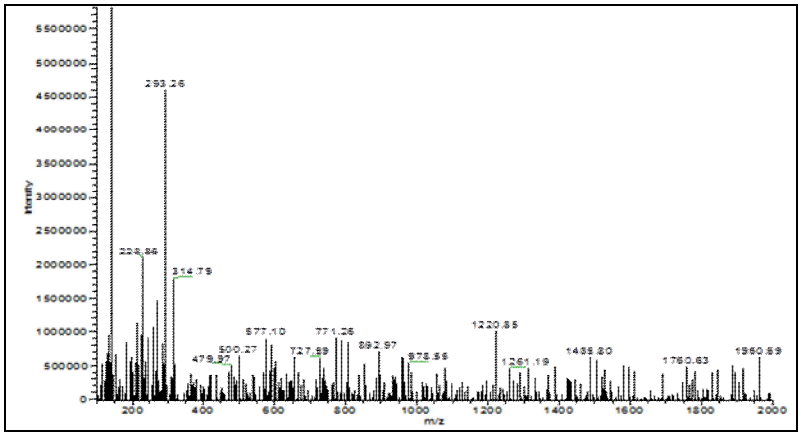


Fig 2: Representative MS spectrum of OCE with m/z > 1000 Da

However, the observed difference on phenolic composition OCE may be ascribed to any or all of agricultural, varietal and seasonal practices in addition to the olive oil extraction technique and the fruit variety and maturity stage [13, 18]. Excluding fruit variety and maturity and olive oil extraction system in the present study, the remaining factors may affect the phenolic profile in olive fruit, leading to an effect on the phenolic profile in OMW [1]. The molecular analysis of Moroccan OCE by modern hyphened techniques, HPLC-ESI-MS, is reported for the first time. A detailed characterization of individual compounds was not attempted and only the major compounds were identified to assist understanding of the relation between the phenolic composition and the observed cytotoxic activity. The careful determination of the molecular composition requires further chromatographic and spectral studies.

3.3 *In vitro* cytotoxic effect of OCE

The *In vitro* cytotoxic effect of the OCE was evaluated using MTT essay (Figure 3). The phenolic extracts of olive cake present an important dose-dependent cytotoxic effect starting at small concentrations (less than 0.05 µg/mL). Indeed, at high concentration (50 µg/mL), the percentage of cell lysis was about 60%. The IC_{50} ranged from 20 to 40 µg/mL.

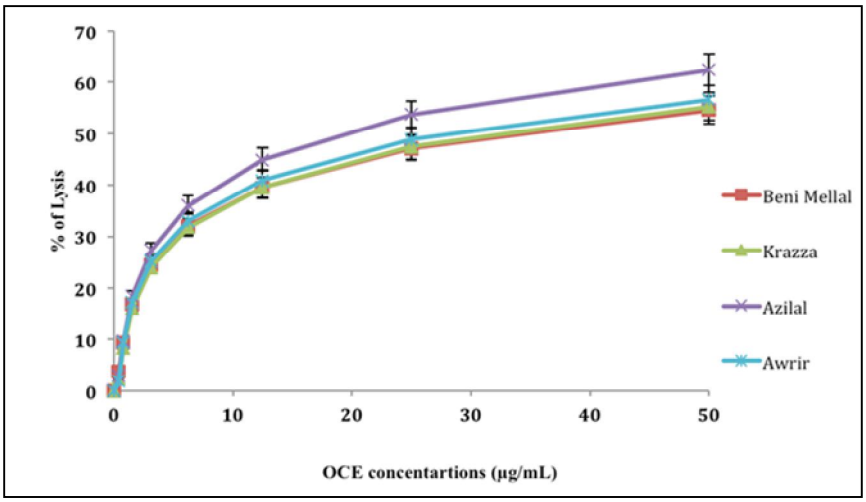
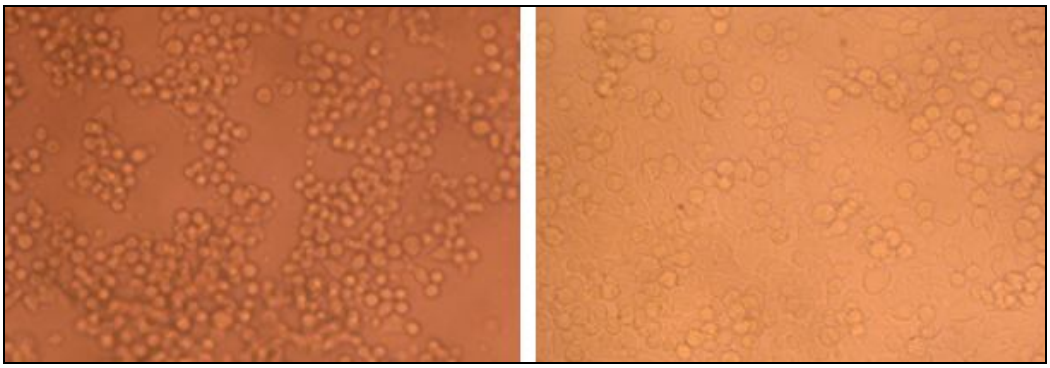


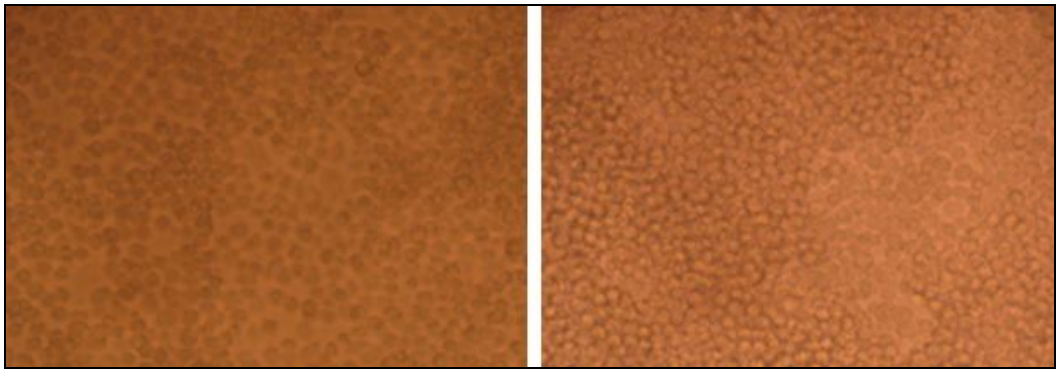
Fig 3: *In vitro* cytotoxic activity of phenolic compounds extracted from olive cake against P815 tumor cell line. The cytotoxic activity of these compounds was evaluated using the MTT test as described in materials and methods. Results are the mean ± SD of three independent experiments.

Moreover, the highest cytotoxic activity was detected in the mountainous areas of Azilal and Awrir (20 and 28.5 µg/mL, respectively), compared to the plain areas of Beni Mellal and Krazza which indicate the same IC_{50} value (34 µg/mL). These differences are statistically significant ($p<0.05$). Mountainous areas OCE showing the higher phenolic compounds content, were significantly more cytotoxic than plain areas OCE.

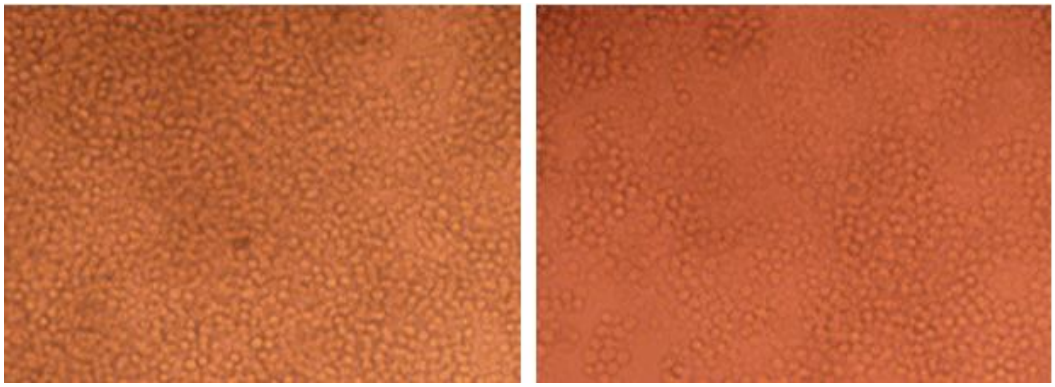
On the other hand, significant changes were observed in the morphological aspect and cell density of murine mastocytoma (P815) cells treated with OCE (IC_{50} concentrations) comparatively to control cells (Fig. 4).



Methotrexate (Positive control) Untreated cells (Negative control)



Beni- Mellal's OCE Krazza's OCE



Azilal's OCE Awrir's OCE

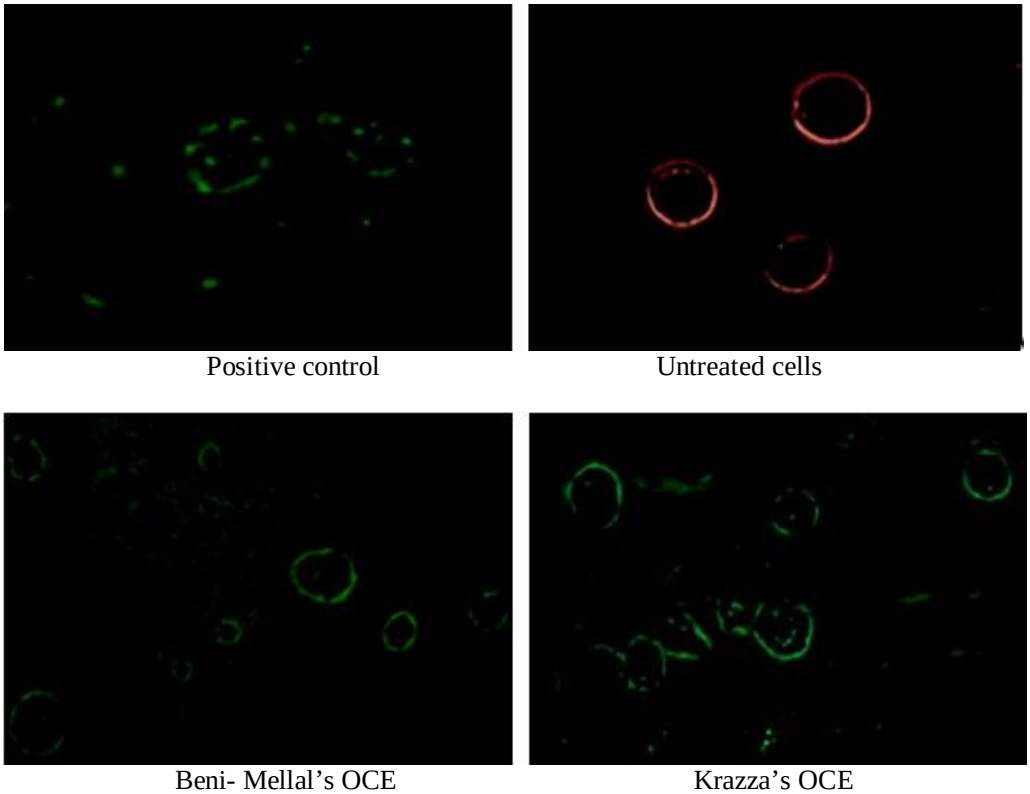
Fig 4: Photographs of P815 cells at x 400 magnifications.
P815 cells (2x106 cells) were treated for 24 h with the IC_{50} concentration of OCE derived from different areas.

According to these data, cytotoxic activity may depend on the phenolic compounds dose and on the phenolic composition of the OCE. These data confirm the interest of olive oil residues as a rich source of natural antiproliferative phenolic compounds [7]. Also, Menendez *et al*. [12] have confirmed important antitumor effect of phenolic compounds isolated from extra-virgin olive oil.

3.4 Apoptosis induction by phenolic OCE

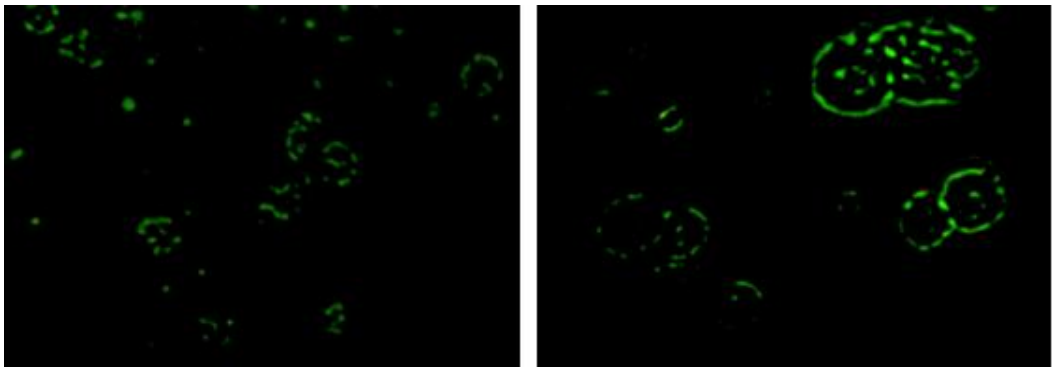
In order to contribute to the understanding of molecular mechanisms involved in the observed cytotoxic activity of OCE, apoptosis induction assay was performed using the murine mastocytoma (P815) tumor cell line. The obtained results are shown in Figure 5.

As shown in this figure, OCE induced significantly the programmed cell death (apoptosis). As observed in the cytotoxic activity, OCE obtained from mountainous areas (Azilal and Awrir) underlay a higher antiproliferative potential and were more effective than OCE obtained from plain areas (Beni Mellal and Krazza) in inducing apoptosis. Therefore, mountainous areas OCE showed an advanced apoptotic state compared to plain areas OCE (Figure 5), confirming the highly important impact of the geographical parameter.



Positive control Untreated cells

Beni- Mellal's OCE Krazza's OCE



Azilal's OCE Awrir's OCE

Fig 5: Apoptosis induction analysis in P815 mastocytoma cell line**.**
P815 tumor cells ($2x10^6$ cells) were treated with 25 µg/mL of OCE. The assay is based on the ability of Annexin V (green fluorescence) to bind to the phosphatidylserine exposed on the surface of cells undergoing apoptosis. Cells cultured in a medium without serum were used as a positive control.

In this way, mountainous areas extracts may contain some phenolic compounds while they are absent in plain areas extracts. In fact, quercetin, for example, a natural phenolic compound found in this study only in Azilal's OCE, showed an antiproliferative effect against U937 cell line especially by inhibiting the cell cycle at the G2/M phase and induced caspase-dependent cell death [32].

3.4.1. Detection of OCE induced apoptosis by DNA fragmentation test: DNA fragmentation was analysed after exposure of P815 cells to the OCE for 24 h (Figure 6). The endonucleolytic DNA cleavage was checked by agarose gel electrophoresis and revealed 180-200 bp characterizing programmed cell death fragments for all the extracts. Taken together, these results permit to conclude that OCE inhibit the proliferation of P815 tumor cells through the induction of apoptosis.

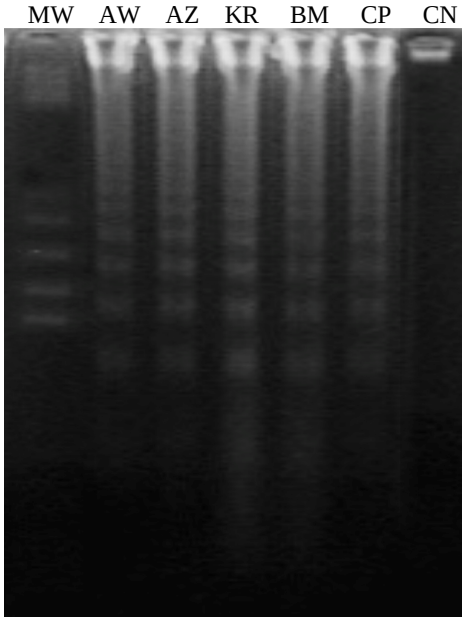


Fig 6: Apoptosis induction effect of OCE.

OCE induced DNA fragmentation was detected by agarose gel electrophoresis of DNA isolated from P815 tumor cells. P815 cells were incubated for 24 h with OCE (corresponding to IC_{50} concentrations).
*BM: Beni mellal, KR: Krazza, AZ: Azilal, AW: Awrir. DNA of untreated cells was used as negative control (NC). Positive control (PC): serum starvation.

Interestingly, this is the first study assessing an effective cytotoxic activity associated with apoptosis induction effect of phenolic compounds derived from olive cake extracts against the P815 cell line.

3.5 Cytotoxic activity of OCE against human normal cells: In order to investigate the effect of OCE on normal cells, their cytotoxicity against PBMC from healthy donors was evaluated. Cells were incubated with increasing concentrations of OCE extracts in the same conditions as those used for tumor cells. Figure 7 represents the percentage of viability percentage of OCE treated PBMC.

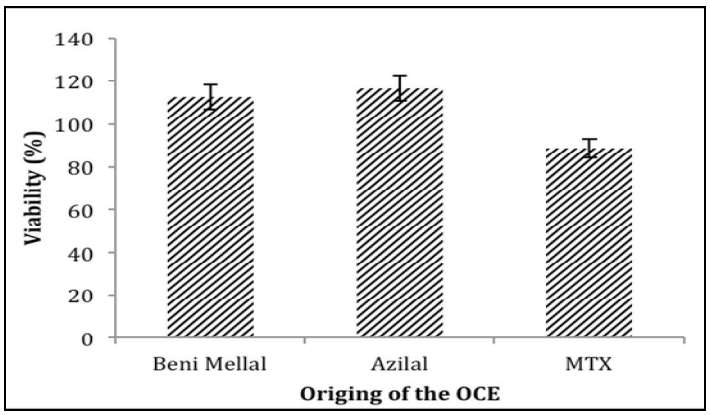


Fig 7: Percent viability of human PBMC treated with OCE.
Cells from healthy human donors are isolated and cultured as detailed in material and methods. Data are the means ± SD of three independent experiments

It is shown in this figure that OCE exhibited a proliferative effect on PBMC enhancing the viability with 12% and 16% in 48 h for the two studied olive cake extracts from Beni Mellal and Azilal, respectively. However, methotrexate suppressed 20% viability of these cells. These data showed the differential and selective effect of OCE. To the best of our knowledge, OCE immune-modulatory potential has never been reported in the literature. Taking together, our results underlined a high potential of natural phenolic extracts as anticancer agents.

4. Conclusion

This study shows a richness and variability of the phenolic compounds in OCE. It also confirms the interest of olive oil residues as a rich source of natural biophenols. The results demonstrate the potential *in vitro* anticancer applications of phenolic extracts from Moroccan olive cake. The differential and selective antiproliferative activity of olive cake derived extracts against P815 cells was demonstrated for the first time. Indeed, mountainous areas extracts, with the highest phenolic compounds content compared to plain areas, showed a good antiproliferative potential against tumor cell line while they exhibited an immuno-stimulating effect on PBMC confirming the selective action of OCE. These related beneficial health-promoting effects of OCE seem to be correlated to the phenolic compounds amount and the phenolic composition. Finally, it is clear that if we promote these natural phenolic compounds and we understand the molecular mechanisms of their biological activities, we will be able to develop new therapeutic modalities whether they are used alone or in combination with other antitumor agents.

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