Olive Leaves as a Potential Source of Apigetrin; Relevance for Their Anti-Leukemia Effects and Mechanisms

A Dissertation Submitted to

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Chapter 1

General introduction

1. 1 Chemoprevention by natural components

Currently available research shows that Mediterranean diet high in fiber, fruits, vegetables, and grain products, is associated with reduced risk of many cancers. This diet is not only rich in fruits and vegetables but the consumption of olive oil and olive products occupies a central position in the dietary tradition which guarantee a high intake of various vitamins and minerals and beneficial non-nutrient substances such as flavonoids (Visioli *et al*., 2000). Flavonoids, which are a family of polyphenolic compounds, comprise approximately 5000 compounds that are defined chemically as substances composed of a common phenylchromanone structure (C6-C3-C6), with one or more hydroxyl substitutes. Flavonoids are considered as one of the main bioactive constituents of plants. Many of them have shown to possess anticarcinogenic effects by interfering with the initiation, development and progression of tumors. This has led to an increased emphasis on cancer prevention strategies in which these dietary factors are utilized. There are two major diet based chemoprevention, namely cancer chemoprevention and dietary cancer prevention, with appreciable overlap between them. Cancer chemoprevention involves pharmacologic intervention with naturally occurring compounds to prevent, inhibit or reverse cancer. In contrast, plant-derived agents containing in diet are protective against a variety of diseases including cancer. The development of diet based chemopreventive approaches is very important in many incurable diseases such as cancer. Even though epidemiological studies provide us valuable suggestions, the molecular events on cellular and tissue levels present the solid confirmation of beneficial effects. Valuable research shows that plant based diets do not only reduce the incidence of cancer but in some cases inhibit its progression (Boggs *et*

al., 2010; Büchner *et al*., 2010; Kirsh *et al*.,2007).

1. 2 Cancer and differentiation therapy

Cancer is a major public health burden in Japan and in the world causing the diagnose of more than 11 million people and the death of about 8 million people every year worldwide, according to the World Health Organization (WHO). In Japan alone, a total of 364,872 people died of cancer, which accounts for 28.8% of all deaths and the main cause of all deaths in Japan, according to statistics compiled by the Ministry of Health, Labor and Welfare [http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/kakutei13/dl/10_h6.pdf (accessed 12 January 2015)].

Cancer generally develops over long period of time with the initiation occurring by the change of some genes in normal cells by chemicals or other agents. The body generally repairs most such damage but if the cell reproduces itself before it is repaired, the daughter cell retains this genetic damage becoming the cancer cell. These cells continue to replicate themselves and grow into cell masses thus, promoting cancer. Nutritional factors might be the most important regulators in this step: some dietary elements may promote the growth of cancer but others such as plant derived compounds may reverse this promotion process (Galeone *et al*., 2006). Recent cancer cell research mainly focused on the death of malignant cells through apoptosis. However, inducing cancer cell apoptosis is usually achieved when cells cannot resist the high enough cytotoxic concentration of chemicals (Tsiftsoglou *et al*., 2003). A novel and potentially less toxic form of cancer therapy involves the application of agents that can induce cancer cell differentiation, which is the so-called differentiation therapy. This therapy is

treatment with agents inducing differentiation of neoplastic cells and is specific to only malignant cells instead of killing them through cytotoxicity. With respect to dietary factors the question is always aroused: can we achieve such a high concentration through the diet? There is another mild approach when we can induce cancer cells to differentiate and eliminate them from the body in natural way (Leszczyniecka *et al*., 2001). According to a systematic assessment, tobacco smoking (19.5%) and virus infections (20.6%) are major causes of cancer in Japan. Low intake of fruits and vegetables accounts for a percentage of 1.3%, the fifth causes of cancer (Inoue, *et al.* 2012).With regular consumption of plant-based diet phytochemicals may reverse cancer promotion process by inducing cancer cell differentiation (Visioli *et al*., 2000).

1. 3 Functionality of Olive (*Olea europaea L.*) leaves

Olive tree *Olea europaea L*. is an evergreen tree growing in several areas in the world including Mediterranean countries, Arabian Peninsula, India and Asia. However, it is famous to be the emblematic tree of Mediterranean basin where 11 million tons of olive oil is produced every year which corresponds to 98% of the global production (El and Karakaya, 2009).

All parts of olive tree are an integral part of Mediterranean civilizations, habits, culture and economy. Since the old time, olive tree has been an imminent part of Mediterranean people life. The tree has been cultivated through civilizations for its fruit, oil, leaves, and wood. In fact, the fruit can be used either pickled or for olive oil extraction or for both purposes, depending on the tree variety. Olive fruits and olive oil are well studied and well documented for their alimentary uses and health benefits,

whereas other by-products like olive leaves are less studied.

Olive leaves were used since old time for their medicinal and health benefits as tea or incenses. In fact, olive leaves are an interesting reservoir containing big amount of several secondary metabolites mainly polyphenols and flavonoids. In recent years, there has been high interest in the health benefit of olive-leaf tea (El and Karakaya, 2009). Historically, olive leaves have been used as folk remedy for combating fevers and malaria (Benavente-García *et al*., 2000).

Accumulating evidences from our laboratory's studies and others demonstrated that olive leaves extracts and their active compounds are endowed with several health benefit biological and pharmacological activities including anti-cancer (Bouallagui *et al*., 2011; Han *et al*., 2009), anti-atherosclerosis, cardiovascular diseases and hypertension (Zrelli *et al*., 2011), anti-diabetic (Drira *et al*., 2011), anti-allergy (Yamada *et al*., 2008), anti-oxidant (Park *et al*., 2013), improving immune function (Park *et al*., 2013), and several other activities (El and Karakaya, 2009). In our previous study Abaza *et al*. (2007) reported that olive leaves extract induced differentiation in human leukemia cells.

1. 4 Objective and composition of the thesis

In this study the author focused on apigenin flavone which is abundantly present in common fruits and vegetables, such as parsley, onions as well as in olive products (oil, fruits and leaves). Our previous research showed that olive leaf extract induced granulocytic differentiation and one of the main constituent of the extract was apigenin (Abaza *et al*., 2007). Apigenin has shown remarkable promise as a potent

chemopreventive agent. It received much attention in recent years for its low toxicity in normal cells, whereas numerous studies with different human cancer cell lines have shown that apigenin inhibits cancer cell growth via the promotion of cell cycle arrest and apoptosis (Patel *et al*., 2007; Ruela-de-Sousa *et al*., 2010; Vargo *et al*., 2006). The natural glucosylated form of apigenin was used to elucidate its cancer cell differentiation capacity. Apigetrin (Fig. 1. 1), the natural form of apigenin, is more stable and has better solubility to compare with aglycone (Patel *et al*., 2007).

Leukemia cell lines are widely used as *in vitro* model to investigate the effect of different chemicals on cancer cells differentiation. A characteristic feature of leukemia cell is a blockage of differentiation at a distinct stage of cellular maturation leading to the accumulation of immature cells. HL-60 cells (Fig. 1. 2), which were derived from the peripheral blood of a patient with acute promyelocytic leukemia (APL) and grown in culture as an established cell line, were used as the *in vitro* model for leukemia.

The outline of this study is described as follows. Briefly in chapter 2, the author observes the differentiation inducing effect of apigetrin on HL-60 cells using animal cell culture assays. In chapter 3, it focuses on molecular mechanisms utilized proteomics approach to explain this phenomenon, which allows for the discovery of novel molecular mechanism. In chapter 4, the author targeted 4 Tunisian olive varieties' leaves extracts grown in 5 sites in the northern part of Tunisia, for their total polyphenol, total flavonoid, and apigenin content and their anti-oxidant capacity using analytical micro-methods for possible valorization of apigenin as anti-cancer functional food.

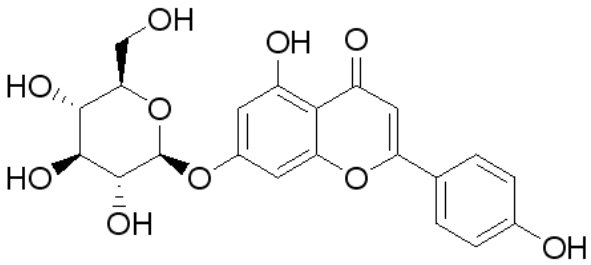


Fig. 1. 1 Chemical structure of apigetrin

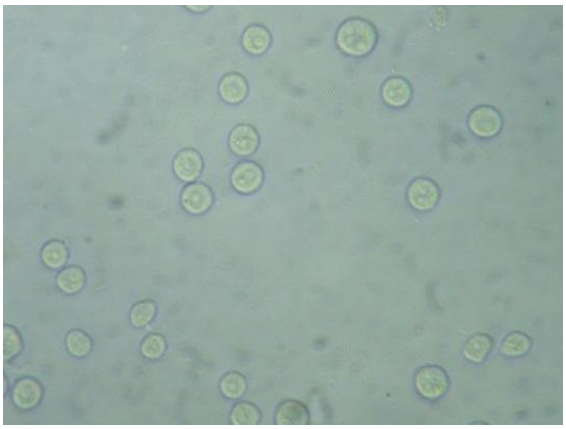


Fig. 1. 2 HL-60 cells. The cells were observed by light microscopy (magnification × 200)

Chapter 2

Induction of differentiation of HL-60 cells by apigetrin

2. 1 Introduction

Apigenin is a flavonoid belonging to the flavone structural class and chemically known as 4', 5, 7,-trihydroxyflavone, with molecular formula $C_{15}H_{10}O_5$ whose molecular weight is MW 270.24. Apigenin is structurally forming yellow needles in pure form. Apigenin is abundantly present in common fruits, such as oranges and grapefruit; plant-derived beverages, such as tea; vegetables, such as parsley and onions; as well as in olive, wheat sprouts and some seasonings (Shukla *et al*., 2010). In natural sources, apigenin is present as apigetrin (Patel *et al*., 2007). Apigenin have received much attention in recent years for its low toxicity in normal cells compared to other structurally related flavonoids. Studies of several human cancer cell lines have shown that apigenin inhibits cancer cell growth via the promotion of cell cycle arrest and apoptosis (Patel *et al*., 2007; Ruela-de-Sousa *et al*., 2010; Vargo *et al*., 2006). However, few studies have reported on the cell differentiation effects induced by apigenin.

HL-60 cells are human promyelocytic leukemia cell line that has a potential to differentiate into granulocytes or monocytes. Many compounds have been reported to induce differentiation of these cells; all-*trans* retinoic acid (ATRA) and dimethylsulfoxide (DMSO) are well known inducers for granulocytes while 1α,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induce HL-60 cells to monocytes (Tsiftsoglou *et al*., 2003). In particular, ATRA which was initially a dietary factor have been used clinically for treatment of acute leukemia (Nowak *et al*., 2009). HL-60 cells committed to cellular maturation undergo an irreversible differentiation and loss of proliferation capacity (Tsiftsoglou *et al*., 2003).

In this chapter, the author tried to investigate to the differentiation inducing effect of apigetrin on HL-60 cells.

2. 2 Materials and Methods

2. 2. 1 Cell culture

Human promyelocytic leukemia cell line HL-60 was obtained from the Riken Cell Bank (Tsukuba, Ibaraki, Japan) and grown in RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioWest, Nuaillé, France) and 1% penicillin (5000 IU/ml)-streptomycin (5000 IU/ml) solution (ICN Biomedicals, Irvine, CA, USA) at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured every 3 days.

2. 2. 2 Cell proliferation assay

At approximately 80% confluence, HL-60 cells were harvested and seeded in 96-well plates at 2.0×10^4 cells per ml in medium. After overnight incubation, apigetrin (Sigma-Aldrich, St. Louis, MO, USA) in dilutions with medium was added to obtain final concentrations of 5, 10, 25 and 50 μM. The cells were cultured for 24, 48 and 72 h, followed by the addition of 10 μl of 5 mg/ml of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Dojindo, Kumamoto, Japan). After 24 h of incubation, 100 μl of 10% sodium dodecyl sulfate (SDS) was added and the cells were incubated for another 24 h to completely dissolve the formazan produced by the cells. The absorbance was spectrophotometrically determined at 570 nm using a multidetection microplate reader (Powerscan HT,

Dainippon Pharmaceutical, NJ, USA). Blanks were prepared at the same time to correct for the absorbance caused by sample color and by the inherent ability of the samples to reduce MTT in the absence of cells.

2. 2. 3 Cell viability assay

Cell viability was assessed using flow cytometry according to the manufacturer's instructions. HL-60 cells were seeded in 100 mm dish at 2.0×10^4 cells per ml in 10 ml of medium. After overnight incubation, cells were treated with 25 and 50 μM of apigetrin for 24, 48 and 72 h. After treatment, cells were suspended in Guava ViaCount reagent (Guava technologies, CA, USA), and incubated for 30 min in darkness at room temperature. The cell number and viability were measured by Guava PCA flow cytometry (Guava technologies, CA, USA).

2. 2. 4 Cell cycle analysis

To determine cell cycle phase distribution, flow cytometric analysis of cellular DNA content was performed. HL-60 cells were seeded in 100 mm dish at 2.0×10^4 cells per ml in 10 ml of medium. After overnight incubation, cells were treated with 25 μM of apigetrin for 24 and 48 h. After treatment, cells were collected, washed with phosphate buffered saline (PBS) twice and fixed in 70% of ice cold ethanol at 4 °C for more than 12 h. The fixed cells were centrifuged at 500 × g for 5 min, the supernatant was removed, and the pellets were washed with PBS twice. The cells were suspended in Cell cycle reagent (Guava technologies, CA, USA), incubated for 30 min in darkness at room temperature, and measured by a Guava PCA flow cytometry (Guava technologies, CA, USA).

2. 2. 5 Cell morphology

The cells were examined by light microscopy (Leica Microsystems, Wetzlar, Germany) to observe cell morphology. HL-60 cells were seeded in 100 mm dish at a density of 2.0×10^4 cells per ml. After overnight incubation, cells were treated with 25 μM of apigetrin for 24, 48 and 72 h and the pictures were taken accordingly.

2. 2. 6 Nuclear Staining

The cells were examined by Giemza staining to observe the morphology of nucleus for distinguishing the degree of differentiation in blood immature cells. HL-60 cells were seeded at a density of 2.0×10^4 cells per ml. After overnight incubation, cells were treated with 25 μM of apigetrin for 48 h. After treatment, culture medium was removed and cells were fixed in methanol 37 °C for 3 min. The fixed cells were washed with MilliQ twice and stained with Giemza Stain Solution (Wako, Osaka, Japan). The cells were incubated for 30 min at 37 °C in darkness. The cells were washed with PBS over 5 times, and air dry for observation under light microscopy (Leica Microsystems, Wetzlar, Germany).

2. 2. 7 Cell differentiation assay

HL-60 cell differentiation was determined by the expression of cell surface markers CD11b and CD14 measured by flow cytometry. 2.0×10^4 cells per ml in 10 ml of medium were seeded in 100 mm dish. After overnight incubation, cells were treated with 25 μM of apigetrin or 25 μM of oleuropein for 48 h. 100 nM ATRA (Sigma-Aldrich, St. Louis, MO, USA) and 100 nM $1,25(OH)_2D_3$ (Sigma-Aldrich, St.

Louis, MO, USA) treated cells were used as positive controls. After washing with cold PBS twice, 1.0×10^5 cells were labeled with R-phycoerythrin-cyanine 5 (PC5) conjugated anti-CD11b antibody and phycoerythrin (PE) conjugated anti-CD14 antibody (Beckman Coulter, CA, USA) for 30 min on ice in darkness. The cells were washed with cold PBS twice, and finally resuspended in 500 μl of PBS for measurement.

2. 2. 8 Functional activity assay

To evaluate the functionality of differentiated HL-60 cells, Nitro blue tetrazolium chloride (NBT) reduction assay was performed. HL-60 cells were seeded into 100 mm dish at a density of 2.0×10^4 cells per ml. After overnight incubation, cells were treated with 25 μM of apigetrin followed by incubation for 48 and 72 h. 100 nM ATRA (Sigma-Aldrich, St. Louis, MO, USA) treated cells were used as positive controls. After washing with growth medium, 1.0×10^6 cells were incubated with 1 ml of 10% NBT in growth medium containing 10 ng/ml of Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C in darkness. The cells were washed with PBS twice, and finally resuspended in 1 ml of PBS and seeded into 96-well plates for measurement. 10% SDS was added and the cells were incubated for another 24 h to completely dissolve the formazan produced by the cells. The absorbance was spectrophotometrically determined at 570 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, NJ, USA).

2. 2. 9 Statistical analyses

Results are expressed as mean ± S.D. of triplicate experiments. Statistical

analysis was performed using Student's t-test. The $p<0.05$ was considered statistically significant.

2. 3 Results

2. 3. 1 Apigetrin inhibits cell proliferation of HL-60 cells

First, in order to elucidate whether apigetrin has biochemical effects in a physiologically relevant dose, the cell proliferation with apigetrin was investigated using a MTT assay. MTT is reduced to formazan by mitochondrial enzyme in living cells. The reduction of MTT can depend on the living cells. HL-60 cells were treated with different doses from 0 to 50 μM of apigetrin up to 72 h. In this experimental condition, apigetrin inhibited HL-60 cell proliferation in a time- and dose-dependant manner (Fig. 2. 1). After 48 h treatment with 25 μM of apigetrin, the cell proliferation was inhibited to 35% compared with the control.

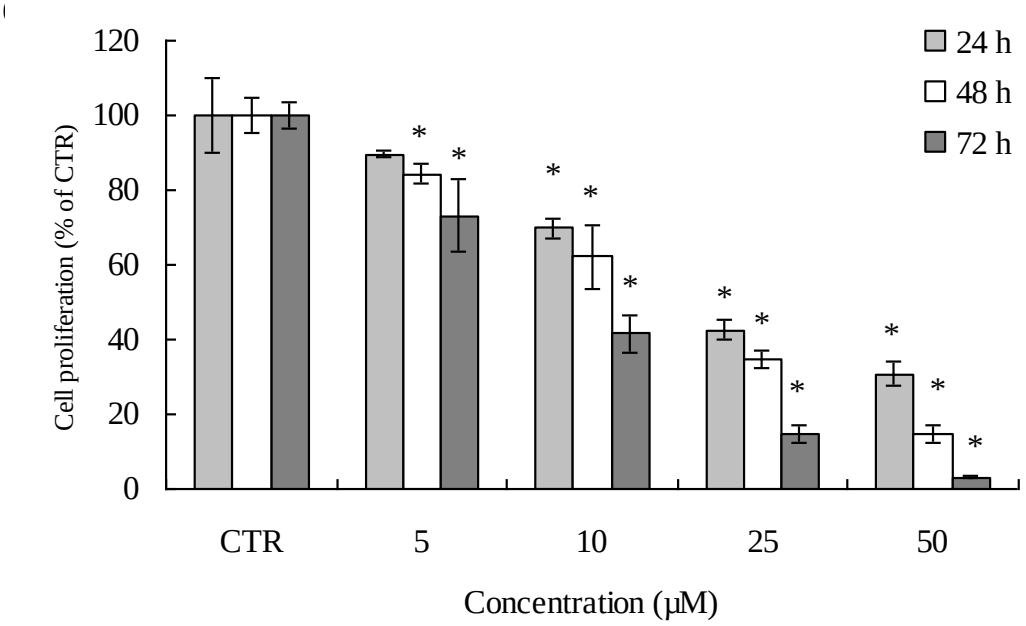
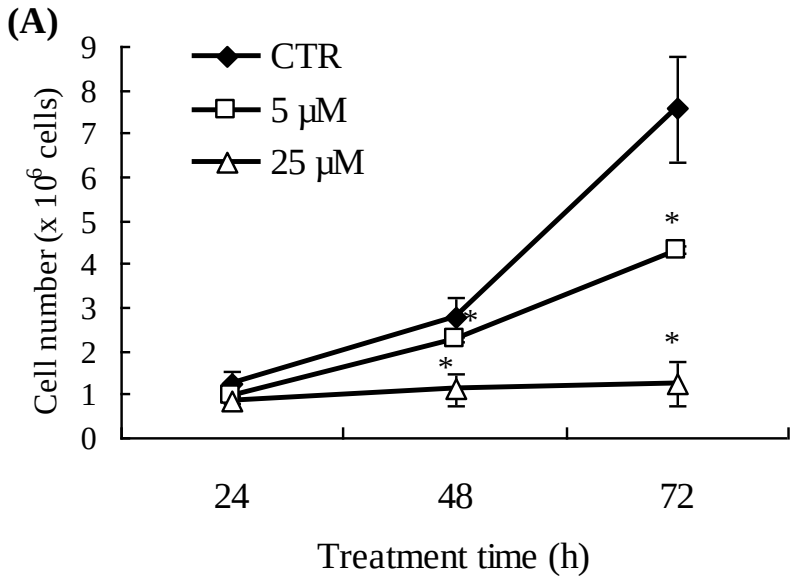
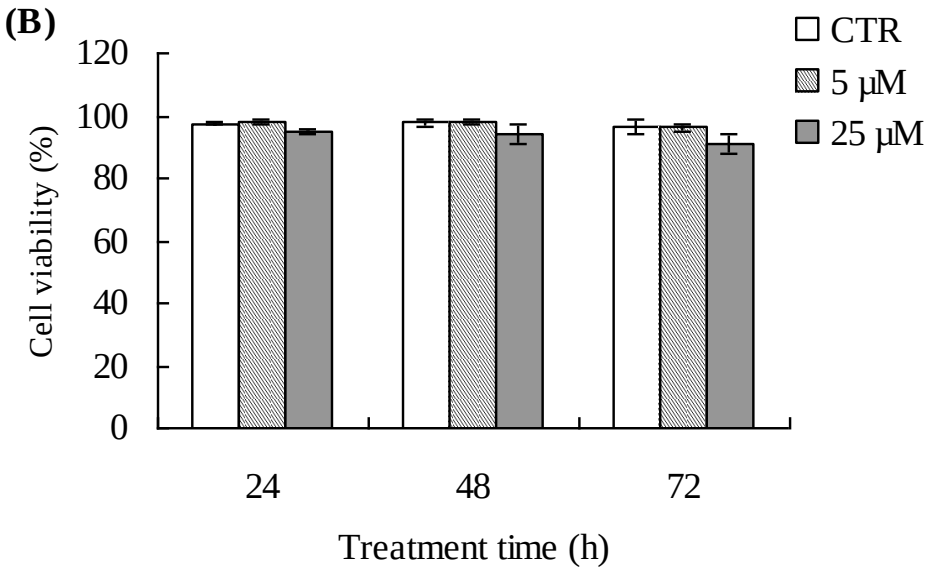


Fig. 2. 1 Effect of apigetrin on the proliferation of HL-60 cells.
The cells were treated with various concentrations (5-50 μM) of apigetrin for 24, 48 and 72 h. The cell proliferaion was measured by MTT assay. CTR represents control cells treated with 1.0% ethanol in medium at the final concentration. Data are presented as a percentage of the control, and as the mean ± S.D. of three independent experiments. *$p<0.05$ significantly different from the control.

2. 3. 2 Apigetrin induces growth inhibition of HL-60 cells

Since MTT assay is based on mitochondrial activity of cells, the author cannot conclude whether the anti-proliferative effect was derived from apoptosis or growth inhibition only. To precisely define the cell growth inhibition effect by apigetrin, the author performed more specific cytotoxicity assay by flow cytometric analysis. This assay is based on a proprietary mixture of two DNA-binding dyes. The first, a membrane-permeant dye, stains all nucleated cells, helping to eliminate cellular debris. The second, a membrane-impermeant dye, stains only damaged cells and thus indicates compromised cell health in cells that it stains. HL-60 cells treated with apigetrin exhibited slow cell growth compared to control cells in a dose-dependent manner (Fig. 2. 2A); at concentration of 25 μM, the cell number had little increase. The viability of both control and treated cells at 5 μM was nearly 97% during all incubation periods (24-72 h), while at 25 μM treatment it was slightly decreased time-dependently (Fig. 2. 2B). Nevertheless, the cell viability was over 90% throughout the experimental conditions, suggesting apigetrin did not cause apoptosis in HL-60 cells at selected concentrations (Fig 2. 2B, C). The results of cell number correlates well with the results of MTT assay (Fig. 2. 1).





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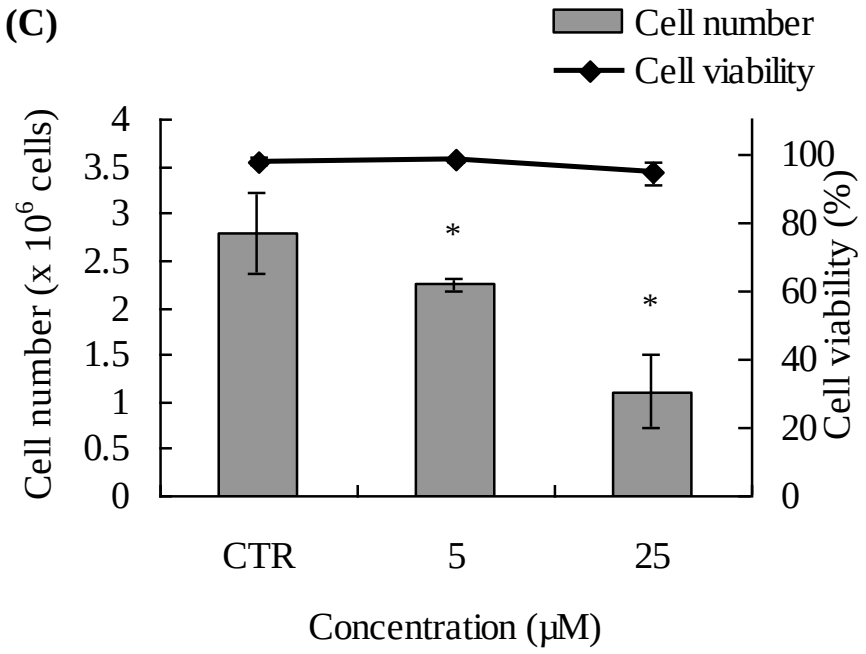
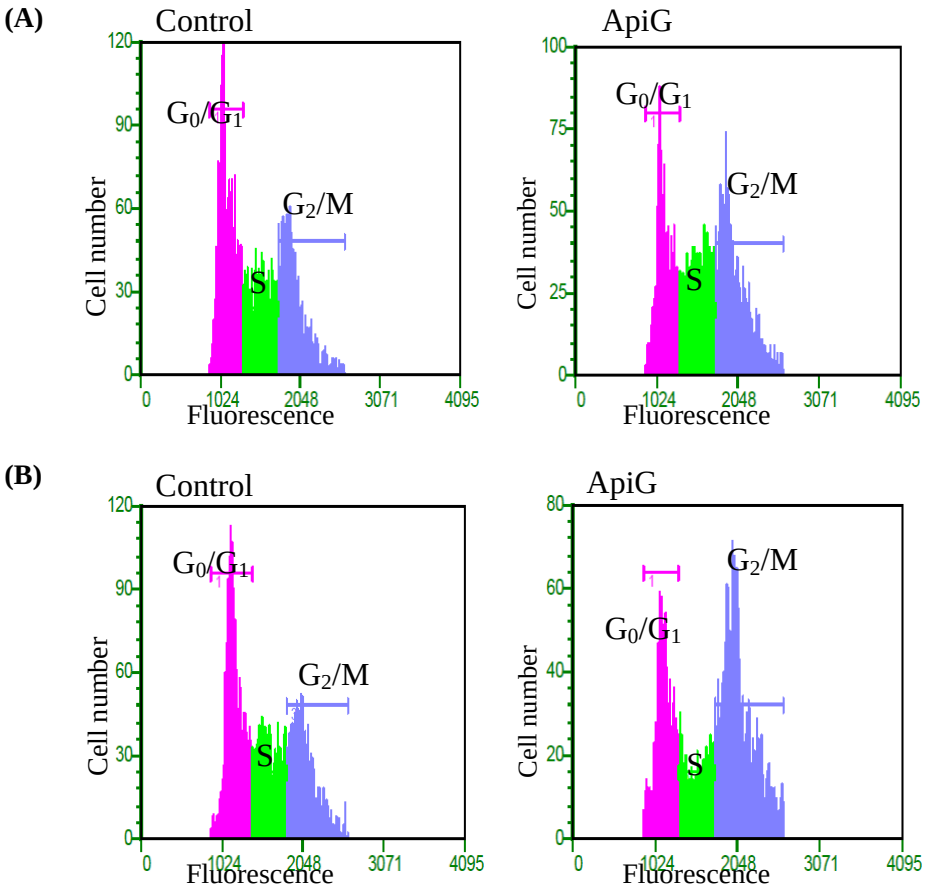


Fig. 2. 2 Effect of apigetrin on the cell number and viability of HL-60 cells. The cells were treated with 5 and 25 μM of apigetrin for 24, 48 and 72 h. The time-dependant effect of apigetrin on cell number (A) and viability (B) were measured by flow cytometry. (C) The dose-dependant effect of apigetrin (5, 25 μM) on cell number and viability after 48 h incubation. CTR represents control cells treated with 0.5% ethanol in medium. Data are presented as the mean ± S.D. of three independent experiments. *$p<0.05$ significantly different from the control.

2. 3. 3 Apigetrin modulates cell cycle progression in HL-60 cells

As apigetrin induced significant growth inhibition of HL-60 cells, the author further analyzed its effect on the cell cycle distribution by measuring DNA content after staining with Propidium iodide (PI). Cells treated with apigetrin, showed accumulation of the cells in G_2/M phase compared with the control (Fig. 2. 3A, B). The number of cells in G_2/M phase with apigetrin treatment was increased to 37.8% and 56.7% after 24 and 48 h of treatment respectively, while it was 30.7% and 29.7% in control cells respectively (Fig. 2. 3C, D). The accumulation of cells in G_2/M phase was time-dependent. This result suggests that the growth inhibition driven by apigetrin was due to a block of cell cycle at G_2/M phase.



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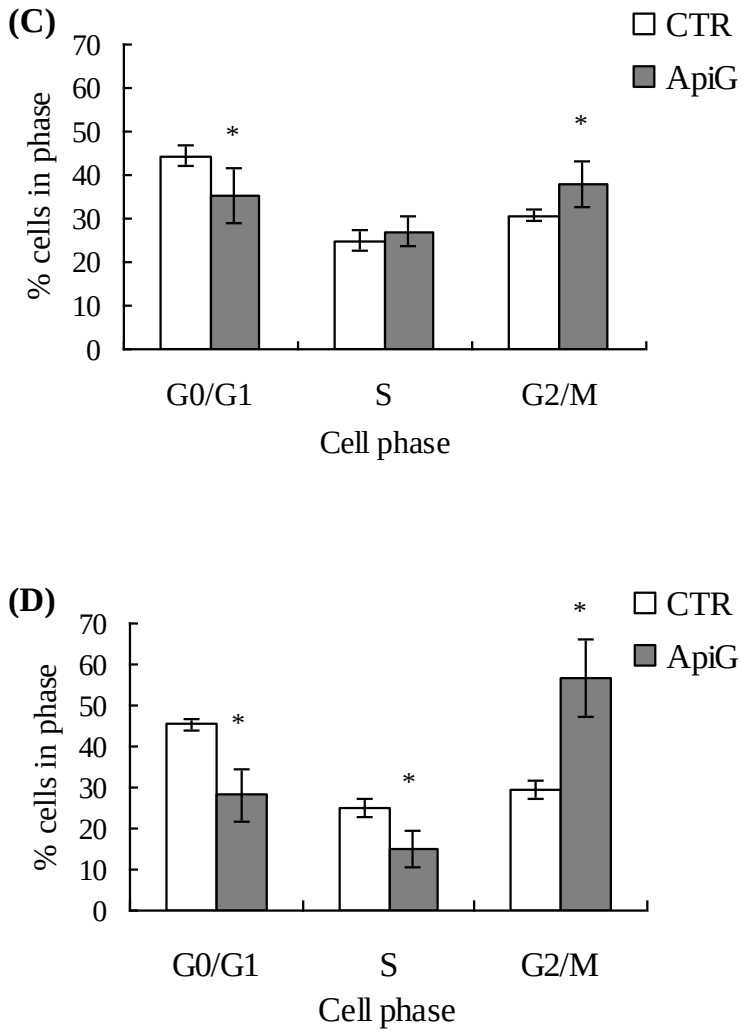


Fig. 2. 3 Effect of apigetrin on the cell cycle distribution of HL-60 cells. The cell cycle distribution according to their DNA content revealed by propidium iodide-derived fluorescence was determined by flow cytometry. An example of the obtained flow cytometric profiles is shown as (A) treated for 24 h and (B) treated for 48 h. CTR represents control cells treated with 0.5% ethanol in medium and ApiG represents apigetrin-treated cells. The left peak indicates that the cells in G_0/G_1 phase while the right peak indicates the cells in G_2/M phase, and the region between two peaks defines the cells at S phase. The cell cycle distribution of HL-60 cells treated with 25 μM of apigetrin for 24 h and 48 h are shown in (C) and (D) respectively. Data are presented as the mean ± S.D. of three independent experiments. *$p<0.05$ significantly different from the control.

2. 3. 4 Apigetrin induced the morphological changes in HL-60 cells

The morphological changes observed in apigetrin-treated HL-60 cells were compared to control cells (Fig. 2. 4A). The control cells had the same morphology with round shape and smooth cell surface throughout treatment times (24-72 h), while at 24 h of treatment, treated cells became bigger with rough surface and were attached weakly to the bottom of the dish. To verify that the morphological changes of appearance could cause the intercellular changes, the cells stained with Giemsa solution to observe the morphology of nucleus. As shown in Fig. 2. 4B, the nucleus of almost all cells in control occupied the cells. Treated cells increased the number of cells with lobed nucleus compared to control cells.

2. 3. 5 Apigetrin induced granulocytic differentiation in HL-60 cells

In order to determine whether growth inhibition via cell cycle arrest by apigetrin is associated with differentiation, the expression of cell surface makers in HL-60 cells was detected by flow cytometry. CD11b and CD14 are both expressed in monocytes while the expression of CD11b only indicates granulocytic differentiation of the cells. As shown in Fig. 2. 5A, the control cells didn't express both markers of CD11b and CD14 antigens. ATRA-treated cells which is the positive control of granulocytic differentiation showed increase in the number of CD11b-positive cells only, while $1,25(OH)_2D_3$-treated cells used for the positive control of monocytic differentiation showed increase in the percentage of both CD11b- and CD14-positive cells. After treatment with apigetrin, HL-60 cells expressed only CD11b marker similar with ATRA treatment and the population of CD11b positive cells was 39.1% while the control was 2.9% (Fig. 2. 5B). On the other hand, oleuropein, which is also abundantly

present in olive leaves, didn't show differentiation effect in HL-60 cells. These results indicate that apigetrin induced differentiation of HL-60 cells to granulocytes, and the active compound in olive leaves for leukemia differentiation .

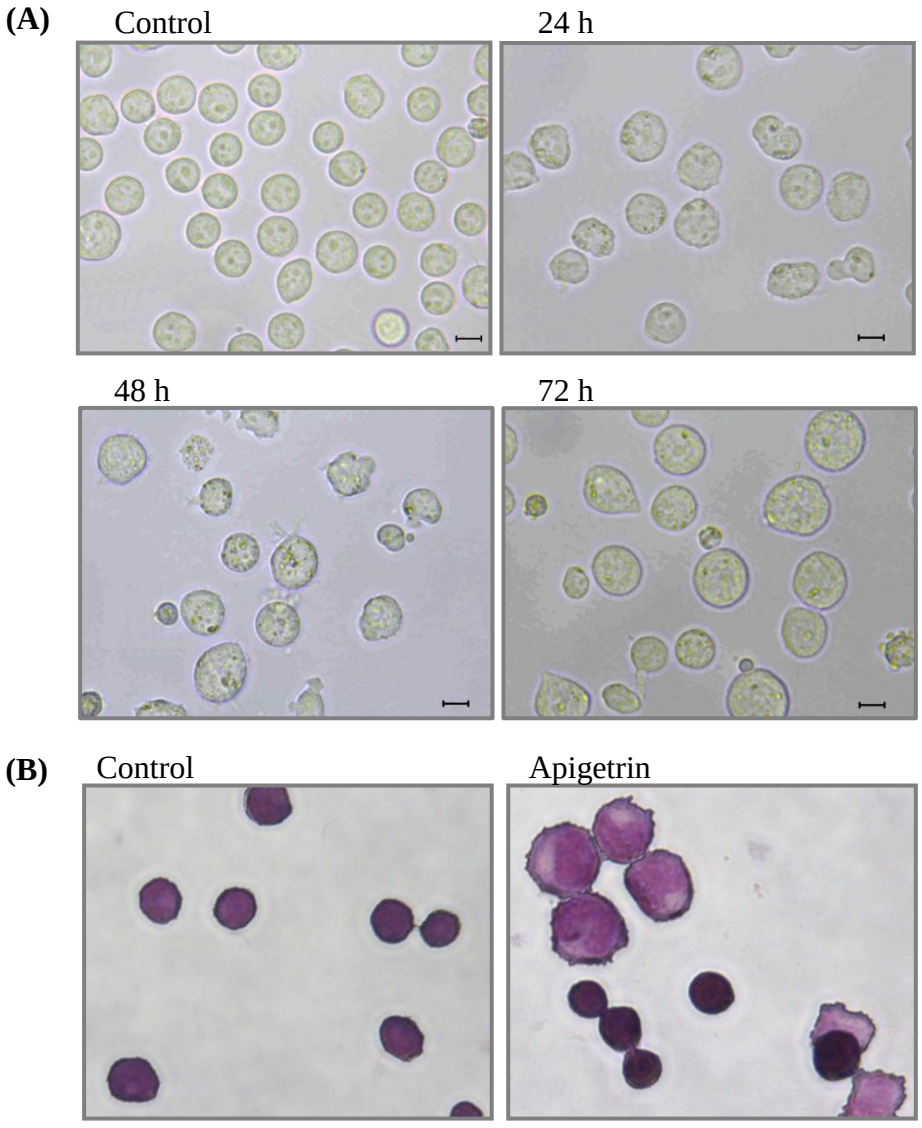
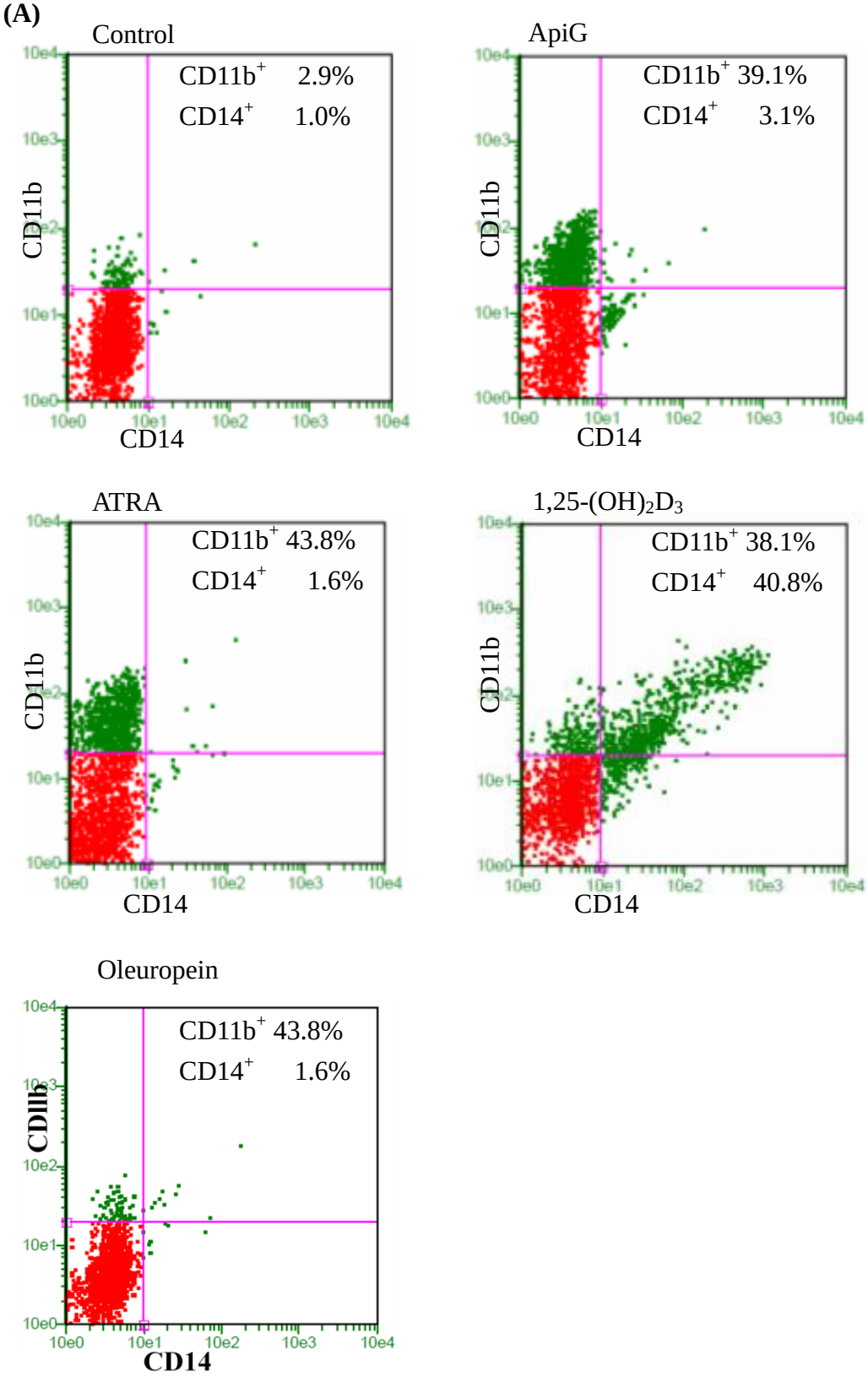


Fig. 2. 4 Effect of apigetrin on the morphology of HL-60 cells
(A) The cells were treated for 24, 48 and 72 h with 25 μM of apigetrin. The cells were observed by light microscopy (magnification × 400). Scale bar represents 10 μm. The photos are representative of three independent experiments. (B) The cells were treated for 48 h with 25 μM of apigetrin. The cells were stained with Giemsa solution and observed by light microscopy (magnification × 400). The photos are representative of two independent experiments.

(A)



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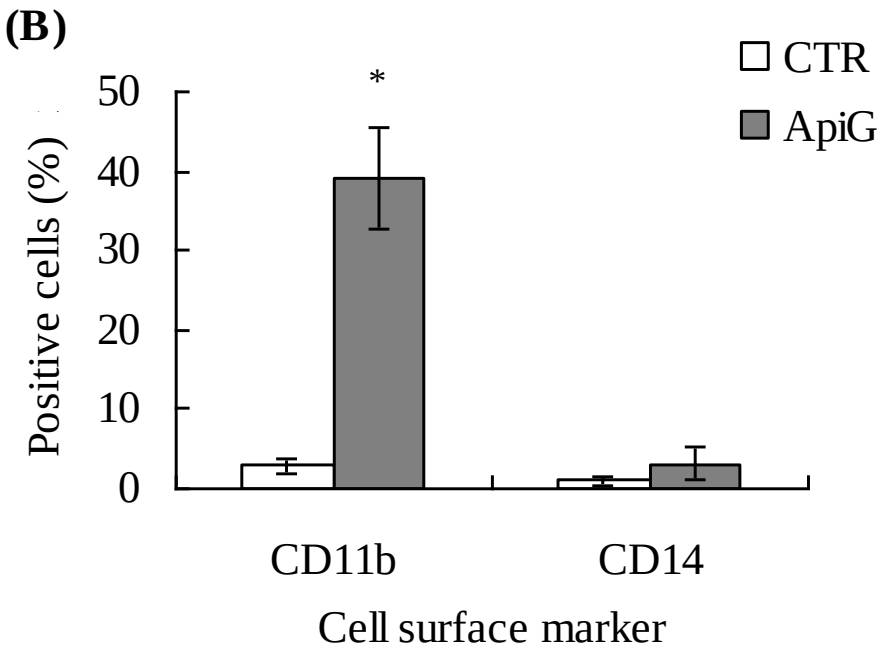


Fig. 2. 5 Effect of apigetrin on induction of differentiation of HL-60 cells. The cells were treated for 48 h with 0.5% ethanol (CTR), 25 μM of apigetrin (ApiG), 25 μM of oleuropein, 100 nM of ATRA, and 100 nM of 1,25-$(OH)_2D_3$. The differentiation analysis according to the expression of cell surface markers CD11b (granulocyte and monocyte specific) and CD14 (only monocyte specific) were determined by flow cytometry. Representative flow cytometric profiles are shown (A). The lines delimit the region of positive cells. The total percentage of positive cells (CD11b – two upper quadrants; CD14 – two right–side quadrants) is showed within each panel. (B) The differentiation data are expressed as a percentage of differentiated cells with the mean ± S.D. of three independent experiments. *$p<0.05$ significantly different from the control.

2. 3. 6 Apigetrin increases NADPH oxidase activity in HL-60 cells

Since the cell surface marker expression is the early stage of cellular maturation, the author cannot conclude whether the differentiation process was finished or stopped in the way of maturation. To further confirm the ability of differentiated HL-60 cells induced by apigetrin as the mature granulocytes, NBT reduction assay was performed. This method is based on the reduction activity of NADPH oxidase which is activated by PMA-stimulation and forms of superoxide anions to kill microbes. Apigetrin increased the NBT reductive activity in a time-dependent manner (Fig. 2. 6) similar with ATRA treatment. These results indicate that differentiated HL-60 cells by apigetrin have a bactericidal activity and may act as mature granulocytes.

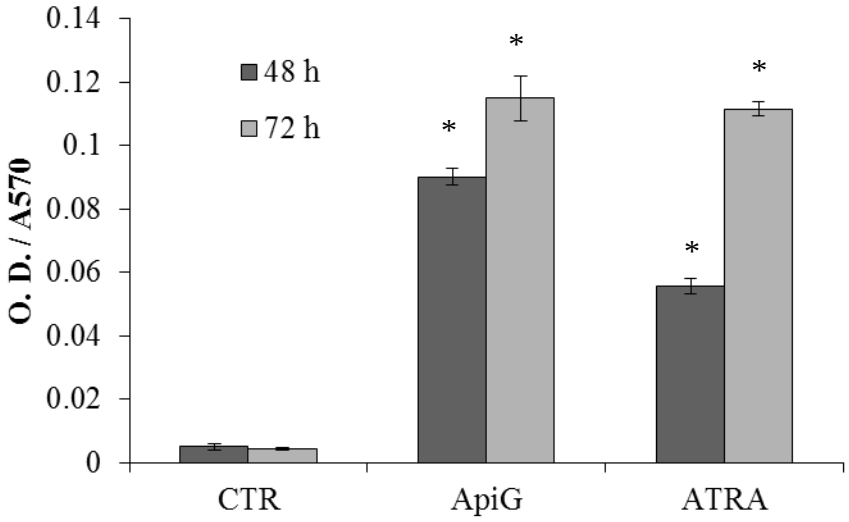


Fig. 2. 6 Effect of apigetrin on NADPH oxidasse activity of HL-60 cells. The cells were treated for 48 h with 0.5% ethanol (CTR), 25 μM of apigetrin (ApiG), 100 nM of ATRA as positive control. The NADPH oxidase activity was measured by NBT reduction assay. CTR represents control cells treated with 0.5% ethanol in medium at the final concentration. Representative data of two independent experiments are shown as the mean ± S.D. *$p<0.05$ significantly different from the control.

2. 4 Discussion

Induction of differentiation is generally associated with a loss of proliferative capacity of cells. Several studies have been reported that the differentiation inducing agents exhibit anti-proliferative effect (Kim *et al*., 2009; Lee *et al*., 2007; Sánchez *et al*., 2009; Tsolmon *et al*., 2009). The results indicate that apigetrin inhibited the cell proliferation in time- and dose-dependant manner (Fig. 2. 1). However, the cell viability was not decreased by treatment with apigetrin (Fig. 2. 2B), indicating apoptosis was not induced. Previous reports have presented anti-proliferative effect of apigenin via apoptosis in breast cancer, prostatic stromal, colon carcinoma as well as leukemia cells but the treatment concentrations were high enough for cytotoxic effect to induce apoptosis (Bektic *et al*., 2006; Choi *et al*., 2009; Vargo *et al*., 2006; Wang *et al*., 1999). Wang *et al.* (1999) demonstrated that 60 μM apigenin induced apoptosis in HL-60 cells. In this experimental condition, the lower dose of apigetrin (25 μM) contributes to the effect of anti-proliferation but not cytotoxicity on HL-60 cells.

It was demonstrated that apigenin arrested the cell cycle at G_2/M phase in various types of cancer cells (Choi *et al*., 2009; Ruela-de-Sousa *et al*., 2010; Wang *et al*., 2000a). Results in this chapter also show that apigetrin induced G_2/M phase arrest on HL-60 cells which is the possible explanation of growth inhibition by apigetrin (Fig. 2. 3). The cell cycle is mediated by many protein complexes such as CDKs, cyclins and CKIs through the cell cycle checkpoints. Cdc2 (CDK1) and cyclinB complex is the master switch for the G2/M phase transition (Kawabe, 2004). Previous studies showed that apigenin inhibits cdc2 kinase activity though exposure of a wide array of malignant cells (Shukla *et al*., 2010). Other potential regulatory effects of apigenin may increase

the expression of p21 which is the negative cell cycle regulatory molecule (Kawabe, 2004). Apigenin has also been reported to up-regulate the expression of p21 in prostate, cervical and breast cancer cells (Choi *et al*., 2009; Ruela-de-Sousa *et al*., 2010). All these studies tried to explain the possible G2/M arrest mechanisms by apigenin treatment in different cell lines with connection to apoptosis. Normally, cells rely on the G_1 checkpoint to protect against DNA damage leading to cell cycle arrest at G_0/G_1 phase, however cancer cells have defective G_1 checkpoint function (Kawabe, 2004). Thus, the G_2 checkpoint could be a potential target for cancer therapy and the compounds modulating G_2 checkpoint can be the attractive agents in cancer treatment. On the other hand, the cell cycle arrest in the common event observed during cell differentiation.

The author studied the cell morphological changes during apigetrin treatment. In the first 24 h, the surface of treated cells lost their smoothness and their size became bigger compared to control cells; later these cells developed small protrusions and were attached weakly to the bottom of the dish (Fig. 2. 4A). Granulocytes have functional characteristics including cell adhesion, migration, chemotaxis and phagocytic activity. Besides HL-60 cells which were differentiated by DMSO also exhibited adhesion (Mansfield *et al*., 1993). These results demonstrate that apigetrin increased a number of cells with lobed nucleus (Fig. 2. 4B). According to the proceeds of differentiation of leukocytes, their nucleus is divided into 2-5 lobes connected by a fine nuclear strand or filament. The mature cells with lobed nucleus can be characterized as granulocytes. On the other hand, apigetrin reduced cell proliferation with no cytotoxic effect. Therefore the author hypothesized that apigetrin may have a potential of inducing differentiation of HL-60 cells accompanied with cell cycle arrest, and performed further studies with

differentiation marker analysis and NBT reduction assay. The results support my hypothesis and reveal that apigetrin has a potential to be not only anticancer agent by induction of cell cycle arrest but also inducer of cellular differentiation (Fig. 2. 4 and 2. 5).

Although the study did not take in consideration that two phenomena, differentiation induction and G_2/M phase cell cycle arrest, occurred in same cells, isoflavon genistein inducing G_2/M phase cell cycle arrest was reported to induce differentiation in HL-60 cells (Sánchez *et al*., 2009). It is believed that ATRA interacts with nuclear receptors to regulator gene transcription for differentiation and with cell membrane receptors to generate rapid nongenomic effects, including the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K), that signaling also mediate the cell cycle (Kandel *et al.*, 2002). Sánchez *et al*. (2009) examined co-treatment of genistein and ATRA for detail evaluation of their mechanism, and they showed that MAPK and PI3-K inhibitors prevented both cell differentiation and G_2/M phase cell cycle arrest. The mechanism by which genistein potentiates ATRA-induced HL-60 cell differentiation coincident with differentiation induction was suggested via MAPK and PI3-K signaling pathways. Moreover co-treatment with ATRA and nargenicin as the inhibitor of cell proliferation in different co-treatment time revealed pre-treatment of nargenicin was more efficient for their combination effect (Kim *et al.,* 2009). Taken together, compounds which potentiate ATRA-induced HL-60 cell differentiation could trigger terminal differentiation. More extensive experiments are needed to elucidate apigetrin effect on the relationship between cell cycle arrest and differentiation by co-treatment with ATRA.

Our previous study reported that apigetrin demonstrates the differentiation inducing effect on human chronic leukemia K562 cells toward erythrocytes (Tsolmon *et al*., 2011). A number of agents have been studied over years for cancer cell differentiation and some of them such as ATRA are used in clinical practice to treat leukemias. But none of them is ideal; they can be effective only in certain types of leukemia and their long term use possesses toxic effects. HL-60 and K562 cells are the models of acute and chronic myeloid leukemias respectively. The development of these leukemias is thought to be different in terms of origin and stages of development. The current studies suggest that apigetrin is capable of triggering terminal differentiation of different types of leukemia and has the good potential for the differentiation inducer.

In this chapter, the author showed that apigetrin inhibited HL-60 cell growth, dose- and time-dependently, but did not cause apoptosis. The distribution of cells at different stages in the cell cycle indicated an accumulation of treated cells in G_2/M phase. Moreover, apigetrin induced granulocytic differentiation of HL-60 cells. The cell cycle arrest is prerequisite for hematopoietic cell differentiation, generally at the G_0/G_1 phase. Differentiation initiates within the G_0/G_1 phase and terminally differentiated cells are arrested at the G_0/G_1 phase cells because cells don't divide anymore and exit from the cell cycle. Although many studies attempt to clarify the correlation between cell cycle arrest and differentiation, the precise mechanisms are largely unidentified. The common view that the initiation of cell differentiation is most likely to occur at the G_0/G_1 phase was not followed in the case of apigetrin. Therefore it might be interesting to investigate the differentiation pathway with apigetrin treatment and explore novel players in the complex process of cellular signaling. With this purpose the author will perform proteomic analysis in the next chapter.

Chapter 3

Molecular approach of differentiation induced by apigetrin

3. 1 Introduction

The proteome of a cell provides information about the protein isoforms and ensemble of proteins expressed in that cell under specific physiological conditions and at a specific time (Vercauteren *et al*., 2007). Proteomic approaches provide several novel possibilities to address biological questions. The large-scale screening approach of proteomics enables protein expression studies that are impossible to perform using classical molecular biology techniques, in which the expression of only one or a few proteins is studied at a time for example like as western blot analysis. In fact, proteomics allow for the analysis of up to thousands of proteins simultaneously, in any tissue or organelle, under any given physiological condition. Moreover there is no limitation of proteins which have already been characterized by cellular functions or role in specific signal transduction pathways.

Cell cycle checkpoints are signal transduction pathways that ensure the timing, sequence, and fidelity of critical cell cycle events and assemble cellular responses to environmentally induced stress. Chk1 and Chk2, which relays the checkpoint activation signal and mediate cell cycle arrest, are structurally unrelated yet functionally overlapping serine/threonine kinases activated in response to various insults. Most of the stress signals from the sensor complexes are conducted via Chk1 and/or Chk2 to Cdc25C, which a main activator of the Cdc2/CyclinB complex. In briefly, activated Chk1 and/or Chk2 also phosphorylate Cdc25C, either leading to its inactivation (O'Connell *et al*., 2000). Taken together, the G_2/M transition relies on the activation of Chk1 and/or Chk2 followed by unable activation of Cdc2/CyclinB complex.

In this chapter, the author tried to investigate molecular mechanisms of

granulocytic differentiation coupled with G2/M phase cell cycle arrest by apigetrin. Proteomic analysis allows for the discovery of novel molecular mechanisms and, opening novel research avenues. The relevant of Chk1 and Chk2 is as the candidate target for apigetrin with the object of cell cycle arrest.

3. 2 Materials and Methods

3. 2. 1 Cell culture

Human promyelocytic leukemia cell line HL-60 was obtained from the Riken Cell Bank (Tsukuba, Ibaraki, Japan) and grown in RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioWest, Nuaillé, France) and 1% penicillin (5000 IU/ml)-streptomycin (5000 IU/ml) solution (ICN Biomedicals, Irvine, CA, USA) at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured every 3 days.

3. 2. 2 Two-dimensional gel electrophoresis and image analysis

HL-60 cells were seeded in 100 mm dish at a density of 2.0×10^4 cells per ml. After overnight incubation, cells were treated with or without 25 μM of apigetrin followed by incubation for 48 h. The medium was then removed and the cells were washed twice with PBS before the total protein was extracted using lysis buffer [7 M Urea, 2 M Thiourea, 4% CHAPS, 40 mM dithiothreitol (DTT), 1 mM EDTA, 25 mM Spermine base] containing protease inhibitor according to the manufacturer's instructions. Samples were stayed for 1 h at room temperature and centrifuged at 17000 × g for 100 min at 15 °C, and then supernatants were carefully collected. The protein

concentration was determined by 2D Quant Kit (GE Healthcare, Little Chalfont, UK). The protein samples (350 μg) were rehydrated with rehydration buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 15 mM DTT) and isoelectrically focused in IPG Buffer (0.5%) using Immobiline IPG DryStrips (240 mm, pH 3–10, GE Healthcare, Little Chalfont, UK) for 12 h at 45 kVh on Ettan IPGphor system (GE Healthcare, Little Chalfont, UK). Focused strips were equilibrated and SDS–PAGE was performed using 12.5% polyacrylamide gels in Ettan DALT *six* Large Electrophoresis System (GE Healthcare, Little Chalfont, UK). Following electrophoresis, gels were fixed and stained with 0.1% Coomassie Brilliant Blue G-350 in 30% methanol and 10% acetic acid. Gel image analysis was performed using software ImageMaster 2D Platinum ver. 5.0 (GE Healthcare, Little Chalfont, UK). Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel. Protein spots with normalized volume of over two fold differences were excised.

3. 2. 3 In gel digestion

Excised spots were destained with 25 mM ammonium bicarbonate for 30 min. The spot pieces were washed with 100% acetonitrile (ACN) and dried completely followed by reduction and alkylation with 10 mM DTT and 25 mM iodoacetamide respectively. After washing with ACN and drying in a speedVac (Genevac, Ipswich, UK), gels were kept at 4 °C for 30 min in trypsin solution (10 μg/ml modified sequence-grade trypsin, 50 mM ammonium bicarbonate), then 50 mM ammonium bicarbonate was added to prevent gel pieces from drying during the digestion at 37 °C overnight. After digestion, the resulted peptides were extracted twice with 5% formic acid in 50% ACN.

3. 2. 4 Liquid chromatography-tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis was performed for protein identification using 3200 QTRAP MS/MS system (Applied Biosystems, Waltham, MA, USA) coupled with Ultimate 3000 LC (Dionex, Sunnyvale, CA, USA). After sample injection by autosampler, the column was washed for 5 min with 98% mobile phase A (0.1% formic acid in water) at a flow rate of 0.3 μL/min. Peptides were eluted from the column using a linear gradient of 2% mobile phase B (0.1% formic acid in ACN) to 40% mobile phase B in 10 min at a flow rate of 0.3 μL/min, then to 90% mobile phase B for an additional 5 min. The column effluent was directed into the electrospray source. The electrospray voltage was set at 2.3 kV. Full MS scan range was 400-1200 m/z, spectra were acquired automatically in Information Dependent Acquisition (IDA) analysis. Protein identification was performed using Mascot software (www.matrixscience.com) and searched against swiss prot database. Search criteria were defined as follows: enzyme as trypsin, taxonomy as *Homo sapiens*, missed cleavage of 1, fixed modifications as carbamidomethylation of cystein and variable modifications as oxidation of methionine. MASCOT protein score >34 was considered to be significantly different ($p<0.05$).

3. 2. 5 Real Time-PCR (RT-PCR)

HL-60 cells were seeded into 100 mm dish at a density of 2.0×10^4 cells per ml. After overnight incubation, cells were treated with 25 μM of apigetrin followed by incubation for 3, 6, and 12 h. The medium was then removed and the cells washed twice with PBS. Total RNA was isolated using the ISOGEN kit (Nippon Gene Co. Ltd.,

Tokyo, Japan) following the manufacturer's instructions. Total RNA was quantified and their quality measured by Nanodrop 2000 (Thermo scientific, Waltham, MA, USA). Reverse transcription reactions were carried out with the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) using 1 μg of total RNA. RT-PCR was performed using 100 ng of cDNA, Taqman Fast Universal PCR Master Mix, and the following Taqman Gene Expression Assays; 14-3-3 beta (Hs00793604_m1), 14-3-3 epsilon (Hs00356749_g1), 14-3-3 gamma (Hs00705917_s1), Glycosidase 2 subunit beta (Hs00160457_m1), Histone-binding protein RBBP4 (Hs001568507_g1), and Actin-beta (Hs03023880_g1). The cDNA amplification reactions were run on the 7500 fast RT-PCR system (Applied Biosystems, Waltham, MA, USA) and normalized to the endogenous control.

3. 2. 6 Western blotting

HL-60 cells were seeded into 100 mm dish at a density of 2.0×10^4 cells per ml. After overnight incubation, cells were treated with 25 μM of apigetrin followed by incubation for 6, 12, 24, and 48 h. The medium was then removed and the cells washed twice with PBS before the total protein was extracted using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitor according to the manufacturer's instructions. Samples were centrifuged at 100,000 rpm for 20 min at 4 °C, and then supernatants were carefully collected. The protein concentration was determined by 2D Quant Kit (GE Healthcare, Little Chalfont, UK). Protein samples (15 μg /well) were mixed with sample buffer [5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue] and then protein samples were electrophoresed on 10% SDS-polyacrylamide gel. Proteins were transferred to

nitrocellulose membrane, and blocked with block ace for 1 h and incubated with primary antibodies for 2 h at room temperature then washed three times in TBST (TBS ＋0.01% Tween-20). Appropriate conjugated secondary antibodies were used for 1.5 h in room temperature then washed three times in TBST. The specific protein bands were detected with ECL prime (GE Healthcare, Little Chalfont, UK). Antibodies against Chk1 and Chk2 were purchased from Abcam (Cambridge, UK), p-chk2 and p-Chk2 were purchased from Cell Signaling Technology (Danvers, MA, USA) and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

3. 2. 7 Statistical analyses

Results are expressed as mean ± S.D. of triplicate experiments. Statistical analysis was performed using Student's t-test. The $p<0.05$ was considered statistically significant.

3. 3 Results

3. 3. 1 Identification of differential expression of proteins in HL-60 cells with or without apigetrin treatment

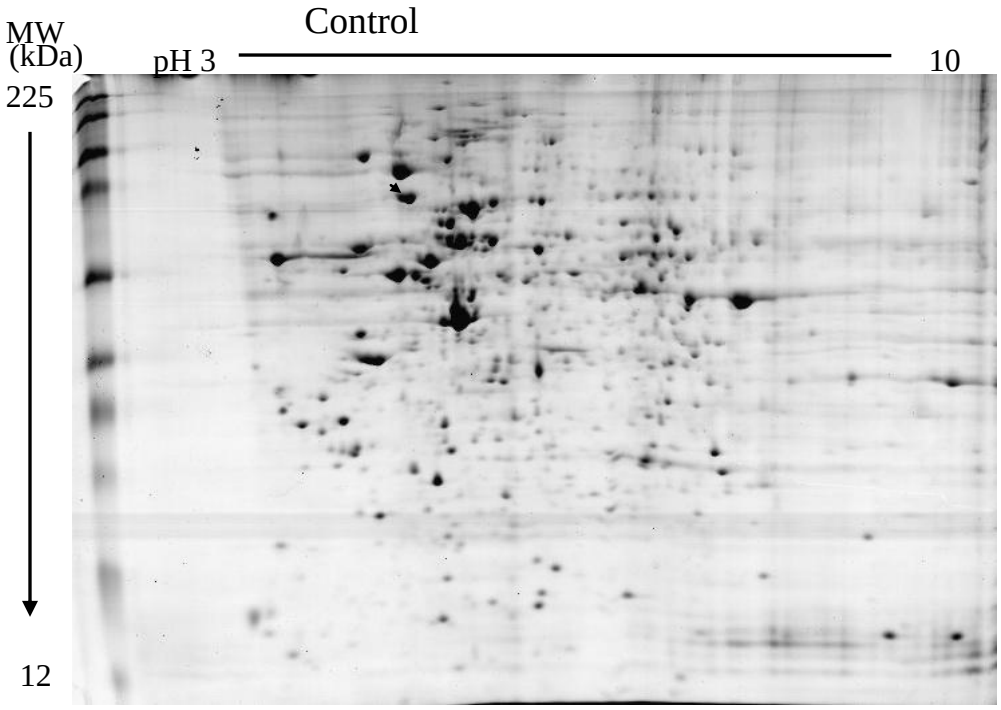
To further characterize the mechanism by which apigetrin induced granulocytic differentiation of HL-60 cells, the protein expression changes in treated HL-60 cells compared to control cells were analyzed using proteomics. 330 spots were detected in each gel after CBB staining and more than 80% of protein spots were matched (Fig. 3. 1A). From the gels, the author selected 10 spots, which were found to be differentially expressed and showed >2-fold change (Fig. 3. 1B). These spots were excised and digested with trypsin and the proteins were identified by LC/MS/MS. In Table. 3. 1,

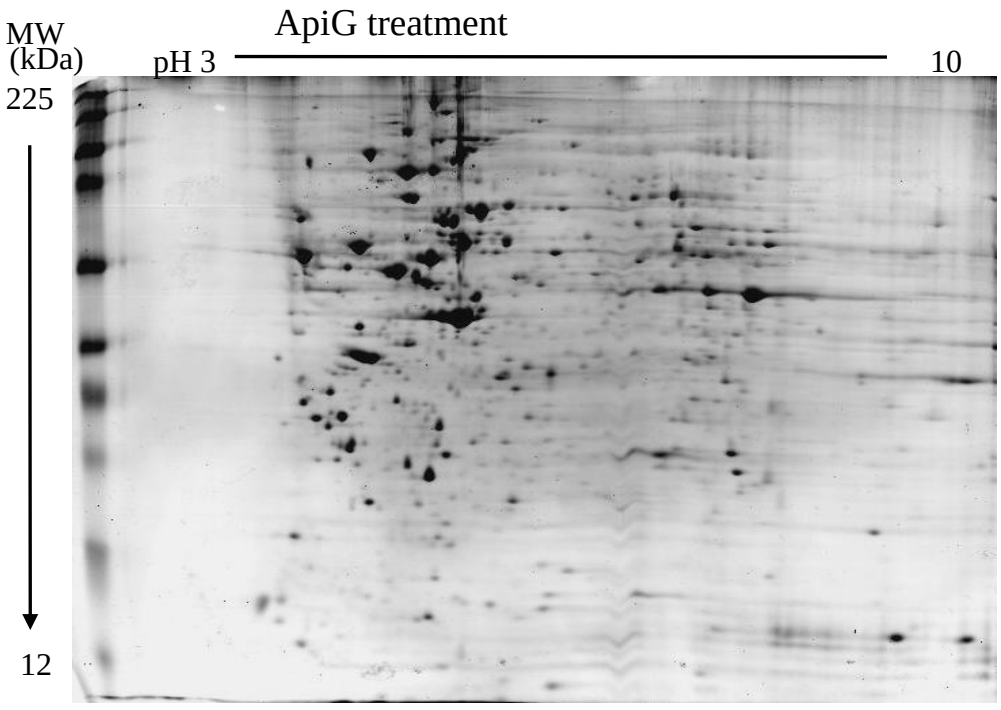
identified protein names, their mascot score, pI/MW and value of fold change compared to control are listed. All identified proteins were up-regulated by apigetrin and associated with the process of differentiation. The up-regulation of proteins was confirmed by the expression of mRNAs of selected proteins by RT-PCR experiments (Fig. 3. 3). As the result, three proteins, 14-3-3 proteins (beta, epsilon and gamma) observed no significant changes at the mRNA level, while other 2 proteins demonstrated similar expression patterns to what the proteomic analysis showed.

3. 3. 2 Apigetrin induces the phosphorylation of Chk1 and Chk2

To examine the involvement of checkpoint kinases in apigetrin -induced HL-60 cell differentiation, Chk1 and Chk2 phosphorylation were investigated by western blotting with their phosphor-specific antibodies, which recognize activated Chk1 at Ser345 and activated Chk2 at Thr68, respectively. When HL-60 cells were treated with apigetrin for 24 and 48 h, the phosphorylation levels of Chk1 and Chk2 were increased (Fig. 3. 4). The expressions of Chk1 and Chk2 proteins were not affected by the treatment of apigetrin though treatment time. These data suggest that apigetrin activates both Chk1 and Chk2 proteins, which is relevant not only to cell cycle arrest but also granulocytic differentiation.

(A)





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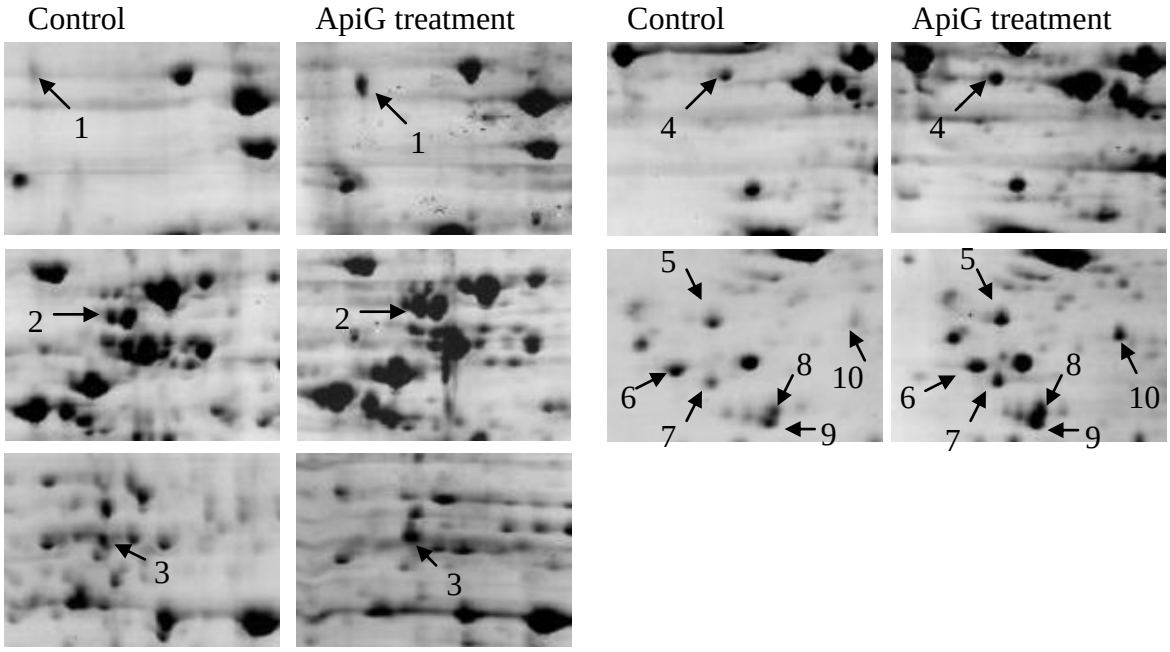
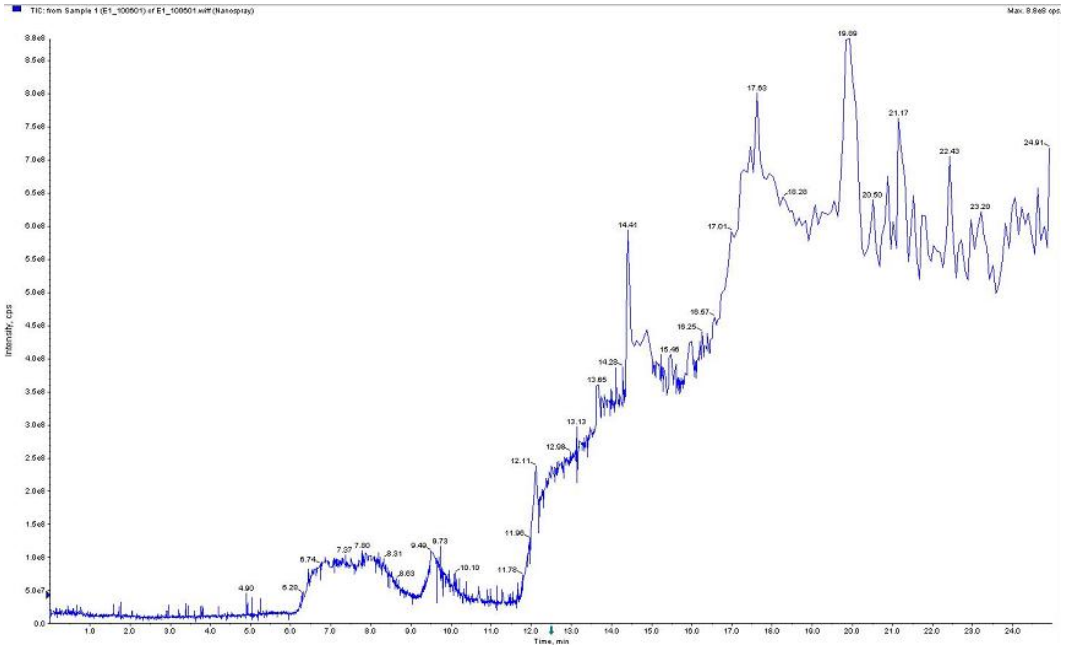
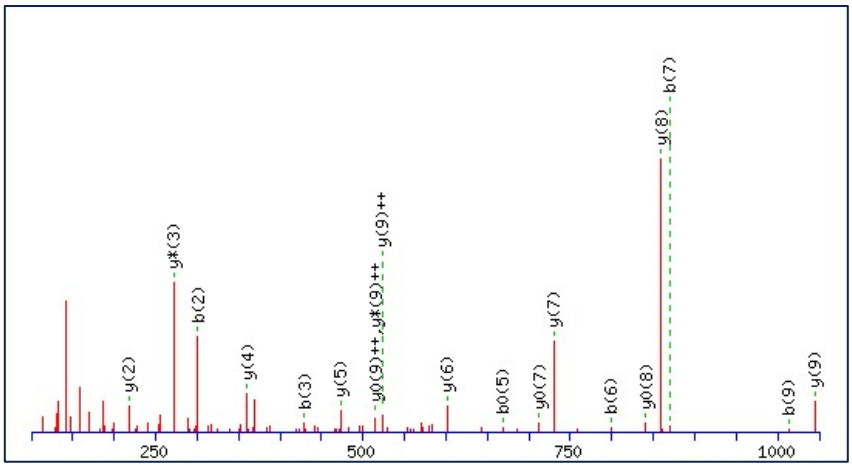


Fig. 3. 1 (A) An overview of representative 2D gel images obtained from control HL-60 cells and treated HL-60 cells with apigetrin (ApiG) for 48 h. 2DE gels were stained with CBB. Proteins differentially expressed were marked by arrow. Data presented is representative of three independent experiments. (B) Specific regions of 2D gel image indicating protein spots with altered expression compared to control. Information on each numbered spot is summarized in Table. 3. 1 ApiG represents apigetrin-treated cells.

(A)



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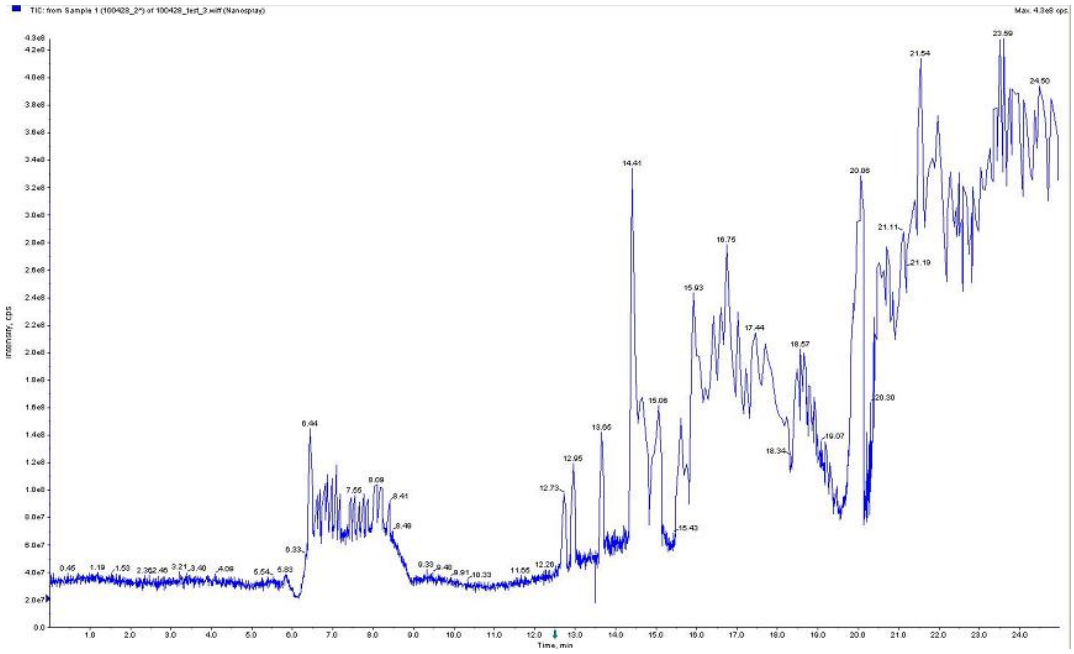

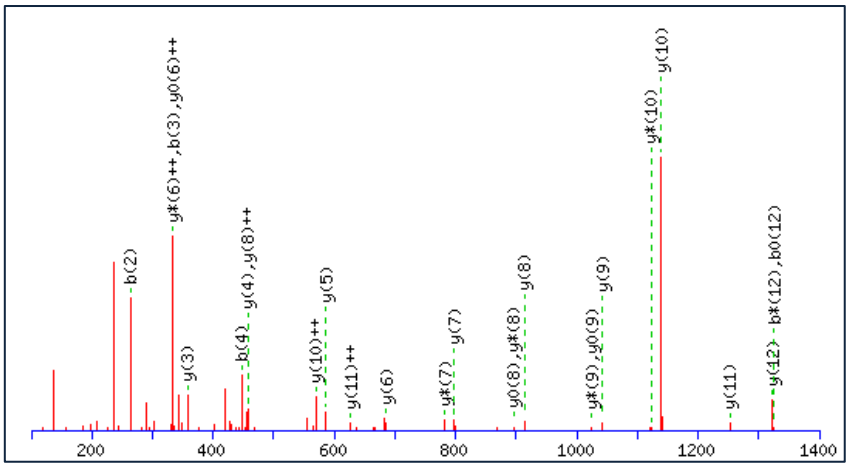


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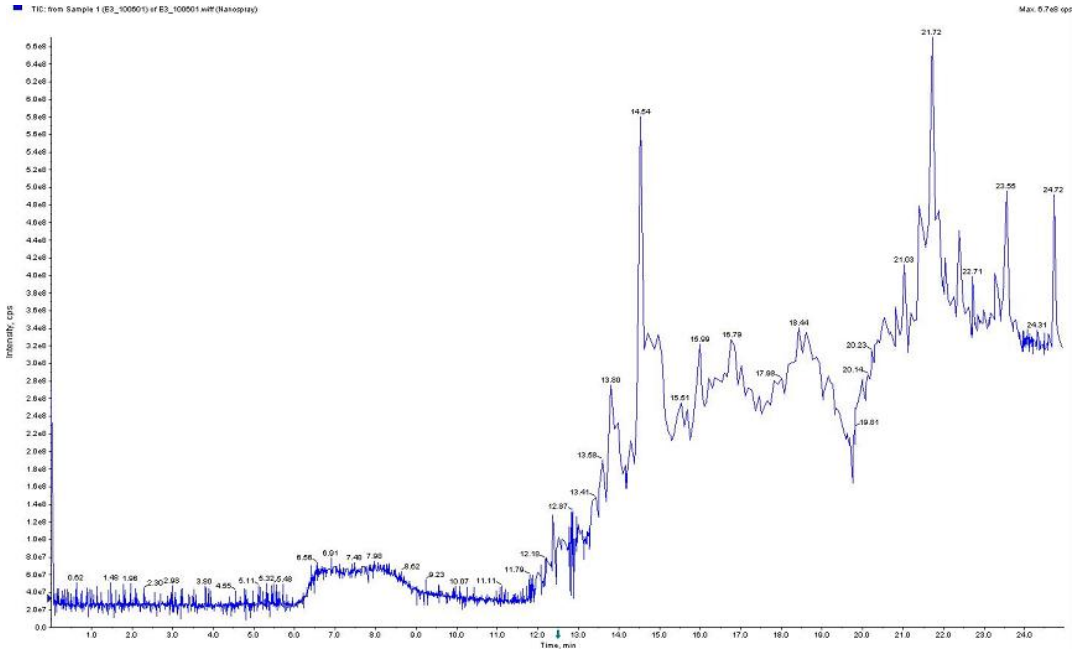

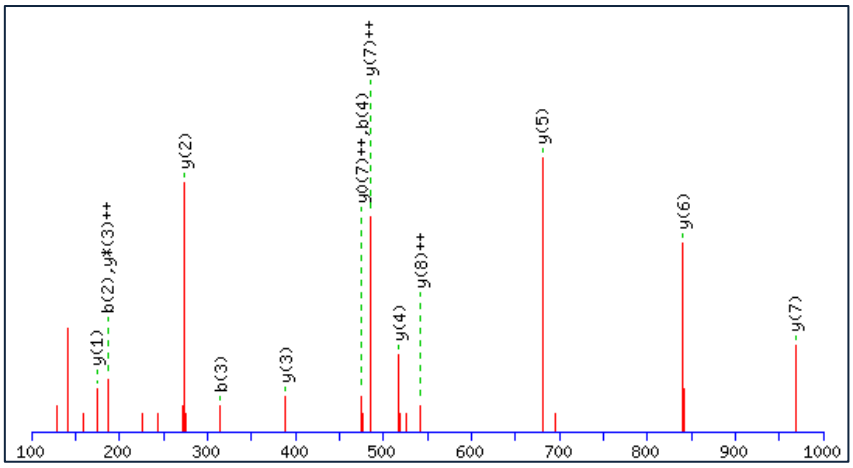


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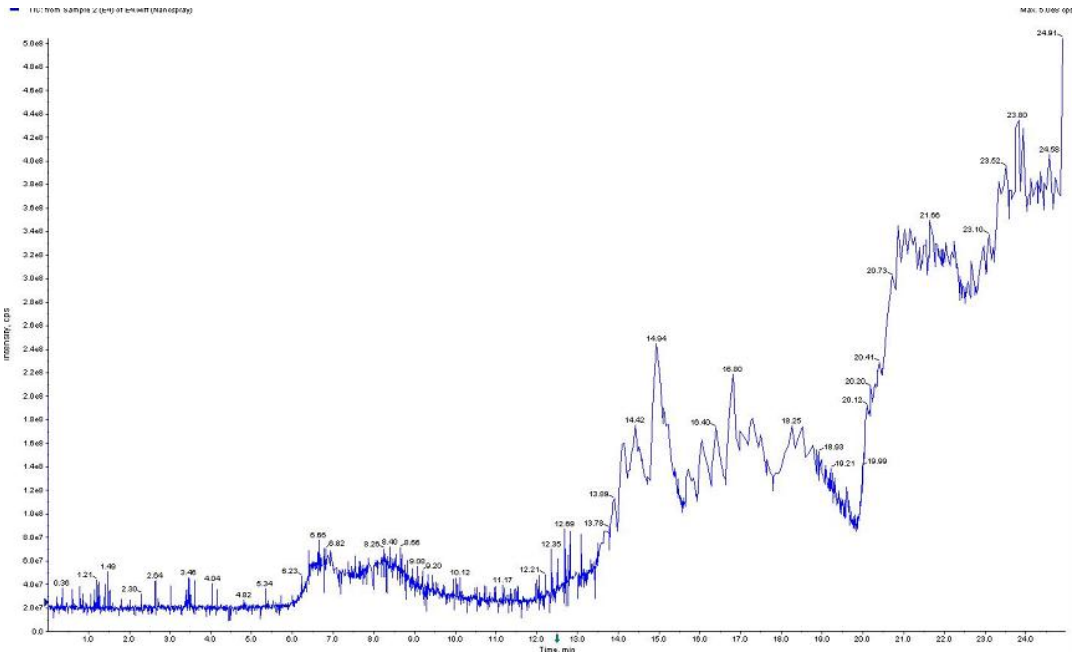

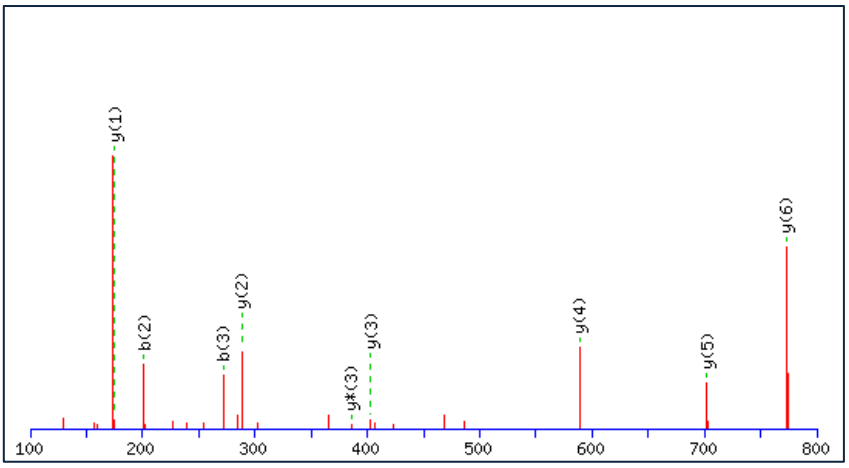


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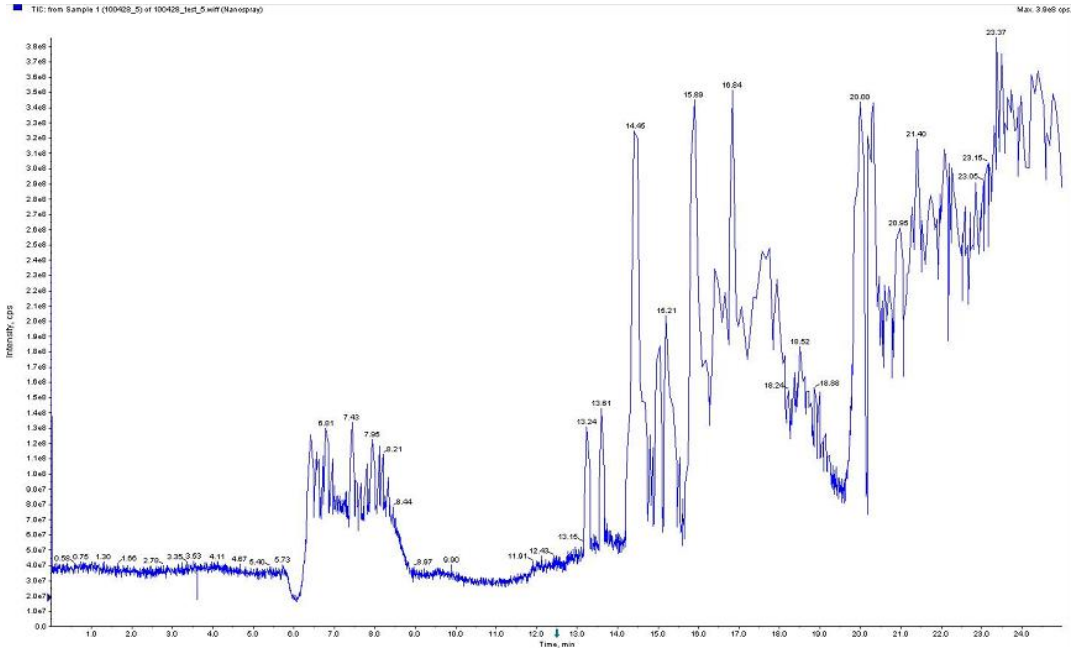

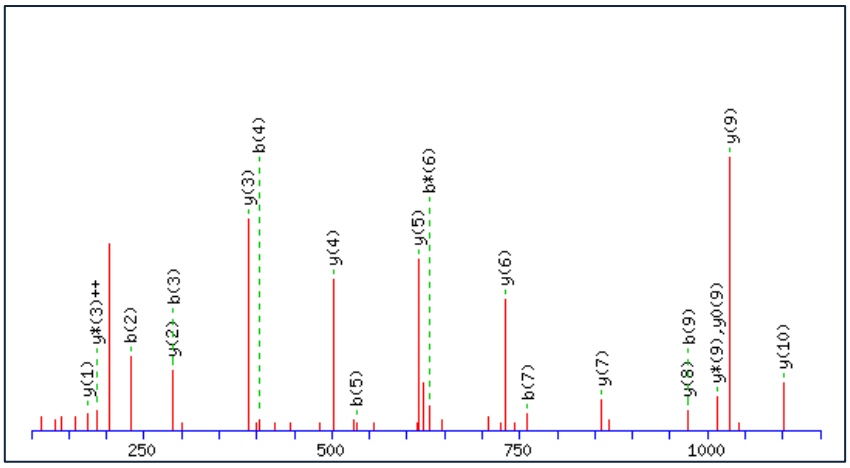


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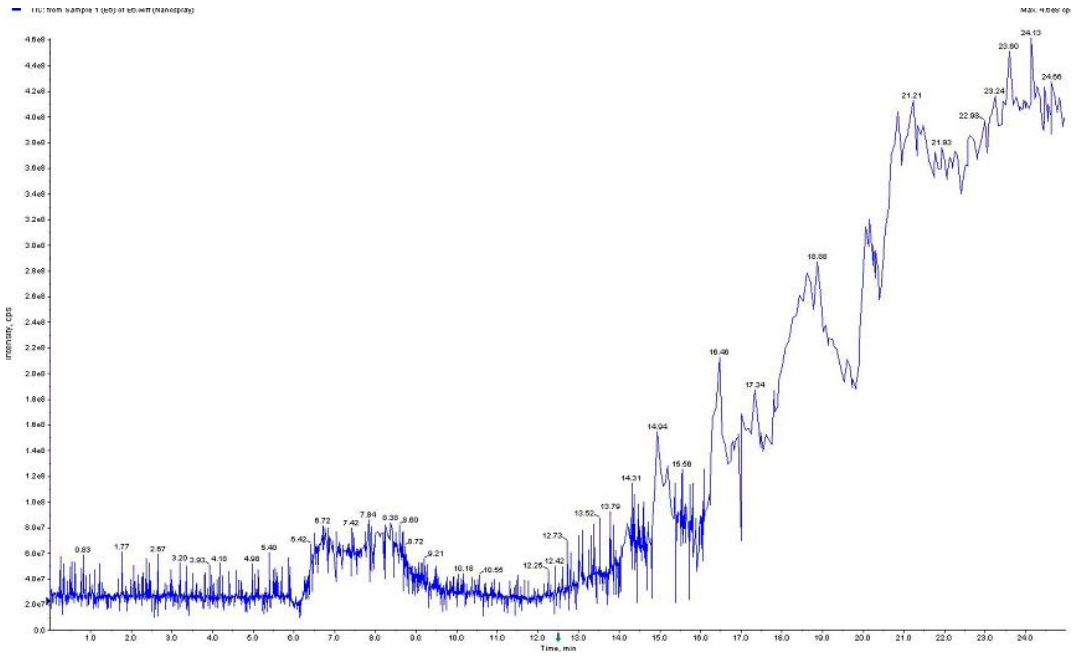
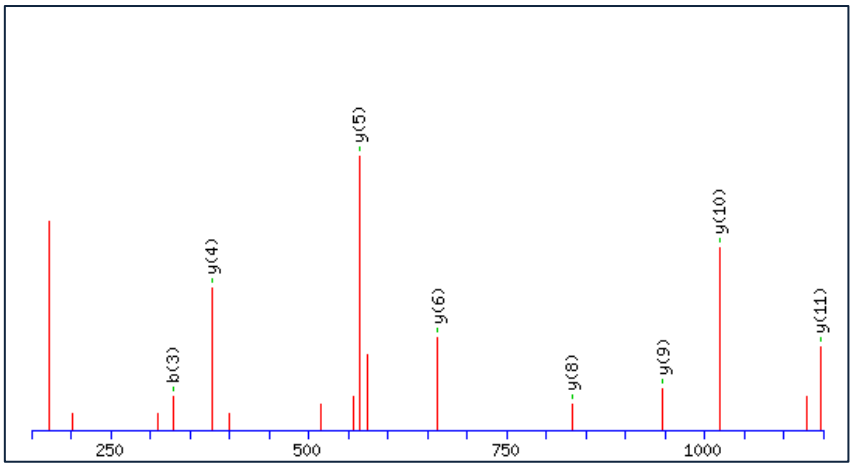
(F)



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101 IDLFGSDDEE ESEEAKRLRE ERLAQYESKK AKKPALVAKS SILLDVKPWD
151 DETDMAKLEE CVRSIQADGL VWGSSKLVPV GYGIKKLQIQ CVVEDDKVGT
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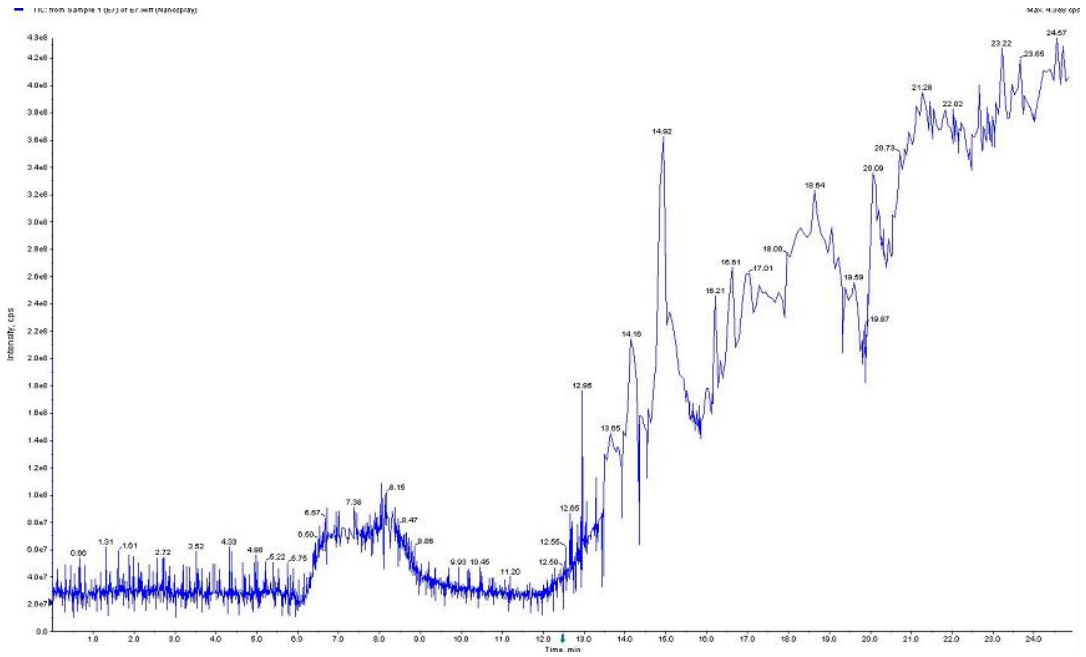
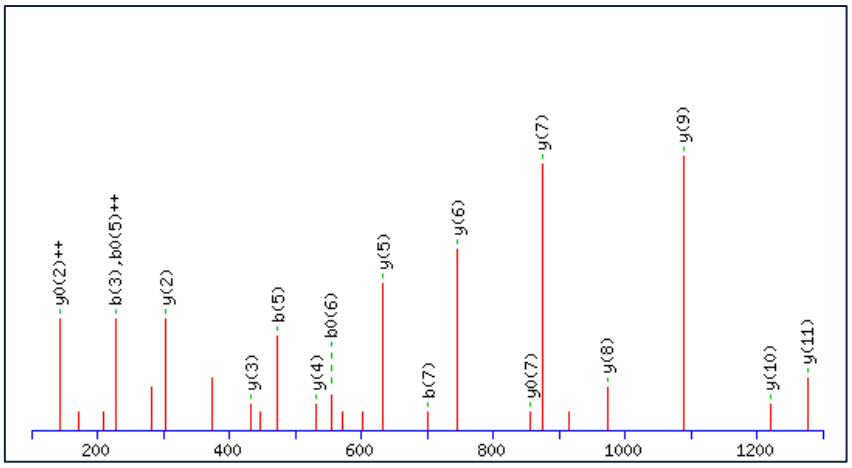
(G)



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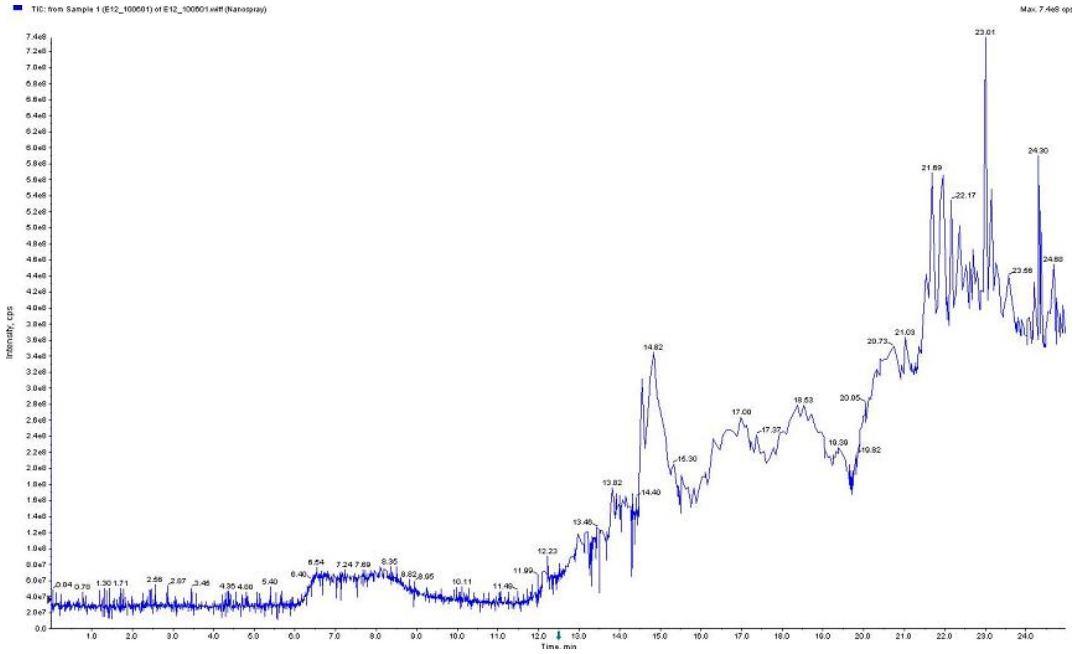
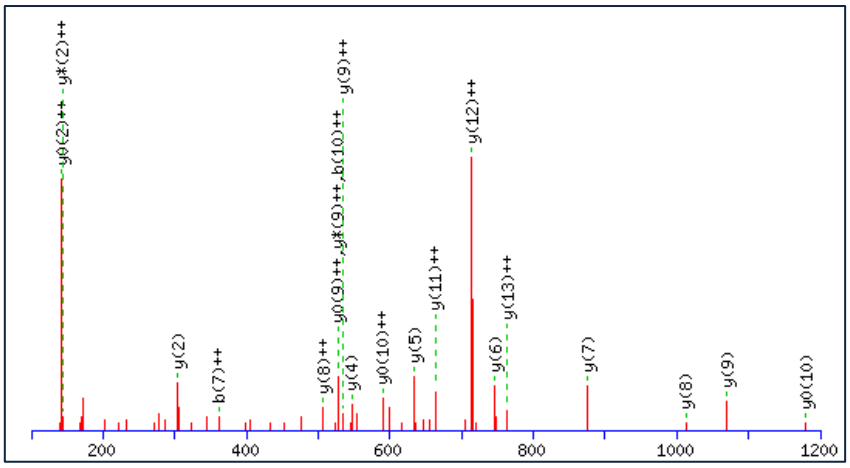
(H)



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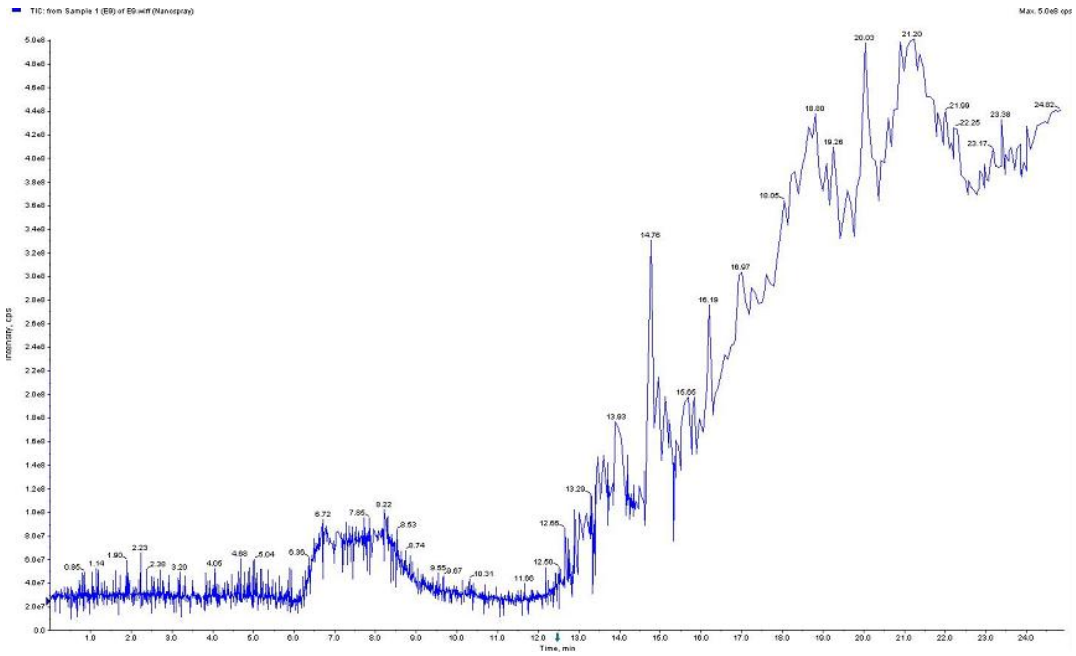

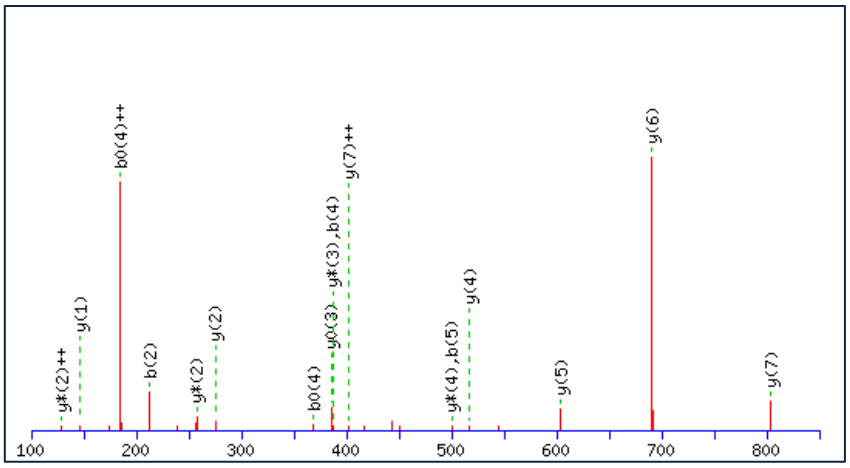


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101 LSLLDNYLIK NCSETQYESK VFYLKMKGDY YRYLAEVATG EKRATVVESS
151 EKAYSEAHEI SKEHMQPTHP IRLGLALNYS VFYYEIQNAP EQACHLAKTA
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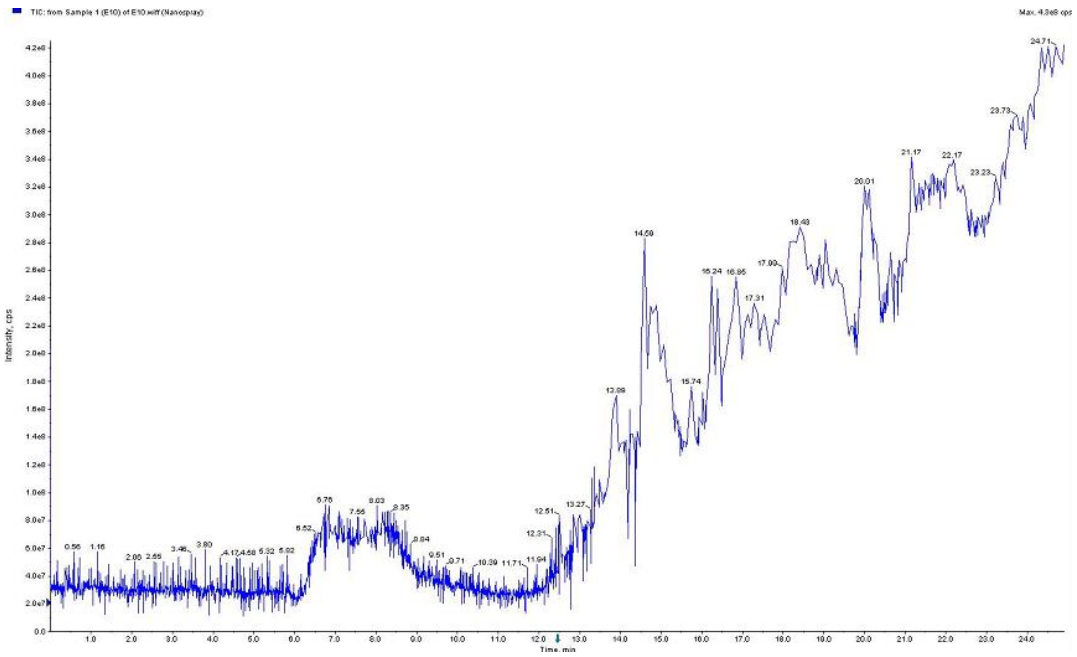
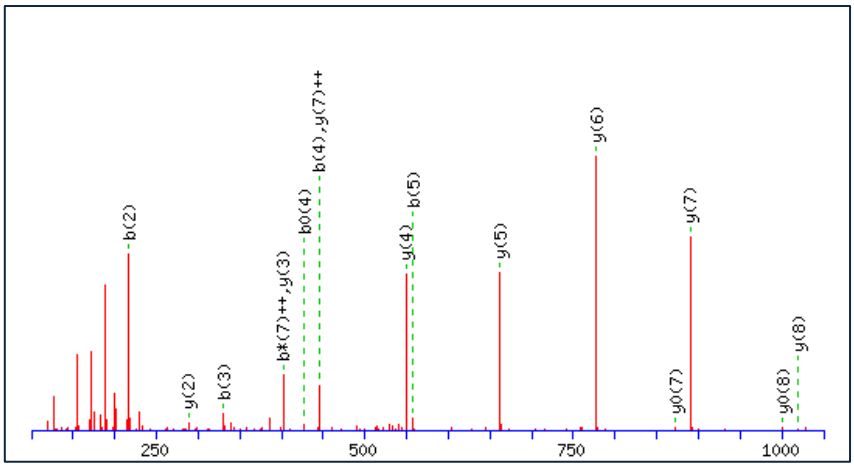
(J)



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101 KGAGTNEKVL TEIIASRTPE ELRAIKQVYE EEYGSSLEDD VVGDTSGYYQ
151 RMLVVLLQAN RDPDAGIDEA QVEQDAQALF QAGELKWGTD EEKFITIFGT
201 RSVSHLRKVF DKYMTISGFQ IEETIDRETS GNLEQLLLAV VKSIRSIPAY
251 LAETLYYAMK GAGTDDHTLI RVMVSRSEID LFNIRKEFRK NFATSLYSMI
301 KGDTSGDYKK ALLLLCGEDD
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Fig. 3. 2 Total ion count chromatogram of peptides from excised protein gel spots detected by nano-HPLC (upper side). Example of MS/MS fragment of one product ion analyzed by Mascot software (middle side). Matched peptides showed in bold red (lower side). (A)Spot1. Glucosidase 2 subunit beta (LWEEQLAAAK). (B)Spot2. Plastin-2 (VYALPEDLVEVNPK). (C)Spot3. Coronin-1A (ADQCYEDVR). (D)Spot4. Histone-binding protein RBBP4 (TVALWDLR). (E)Spot5. Proliferating cell nuclear antigen (CAGNEDIITLR). (F)Spot6. Elongation factor 1-beta (SIQADGLVWGSSK). (G)Spot7. 14-3-3 protein epsilon (VAGMDVELTVEER). (H)Spot8. 14-3-3 protein beta/alpha (AVTEQGHELSNEER). (I)Spot9. 14-3-3protein gamma (VISSIEQK). (J)Spot10. Annexin A5 (SEIDLFNIR).

Table. 3. 1 List of proteins affected by apigetrin and identified by LC/MS/MS.

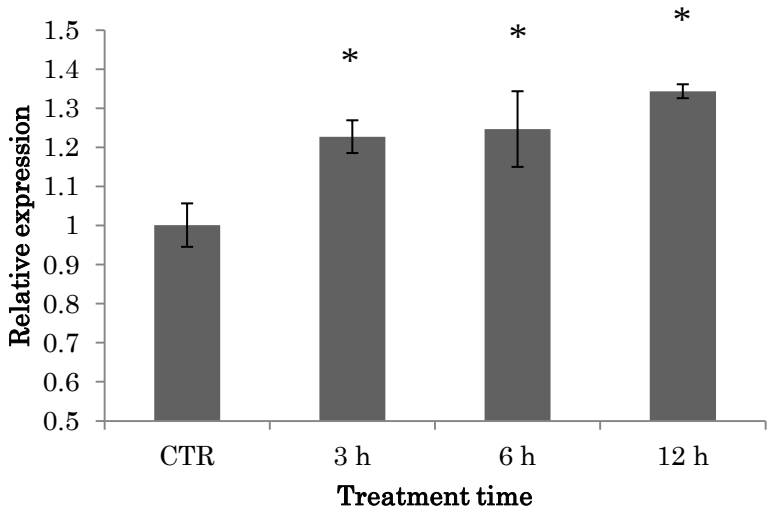
Spot no.	Protein name	Score[a]	Theoretical[b]		Observed		Fold change[c]
			pI	MW	pI	MW	
1	Glucosidase 2 subunit beta	71	4.33	60.4	4.43	90.5	2.25
2	Plastin-2	221	5.20	70.8	5.80	67.0	2.35
3	Coronin-1A	83	6.25	51.7	7.63	58.0	2.25
4	Histone-binding protein RBBP4	45	4.74	47.9	4.97	53.0	2.12
5	Proliferating cell nuclear antigen	261	4.57	29.1	4.78	33.0	2.03
6	Elongation factor 1-beta	67	4.50	24.9	4.60	29.5	2.22
7	14-3-3 protein epsilon	122	4.63	29.3	4.77	28.5	2.29
8	14-3-3 protein beta/alpha	138	4.76	28.2	5.10	27.0	2.77
9	14-3-3 protein gamma	79	4.80	28.5	5.07	26.5	4.09
10	Annexin A5	132	4.94	36.0	5.63	30.5	2.74

[a] Mascot score of >34 was considered as significant ($p<0.05$).

[b] Theoretical pI and MW were derived from the amino acid sequence in swiss prot.

[c] Fold-changes of spot volume (Apigetrin vs. control).

(A)



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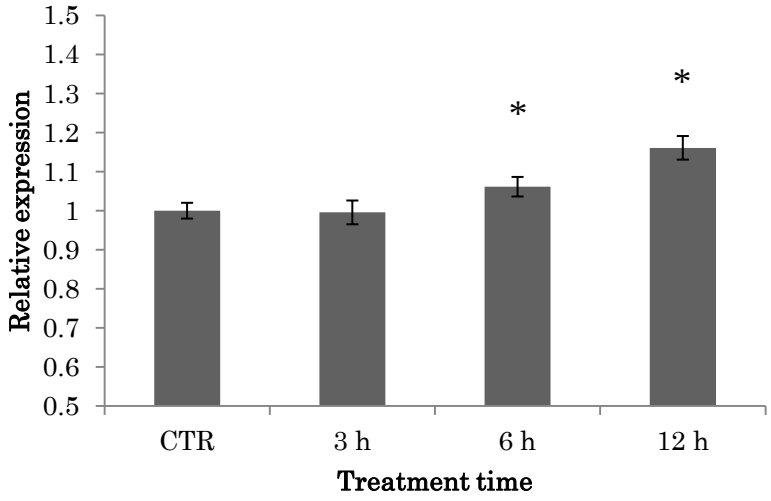

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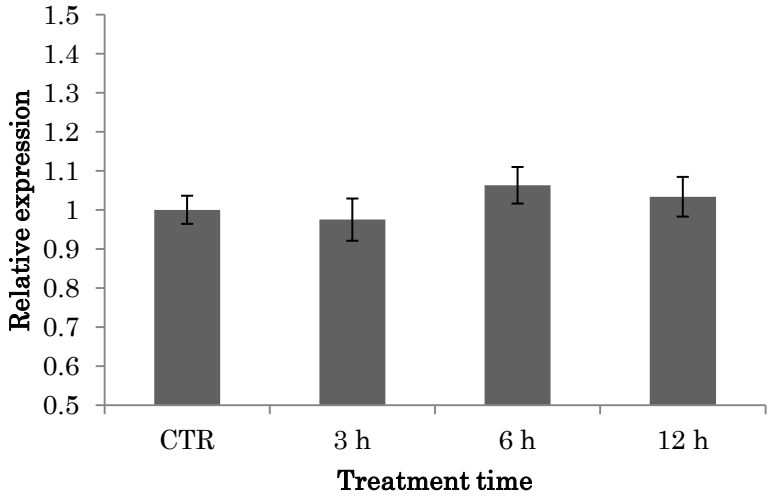

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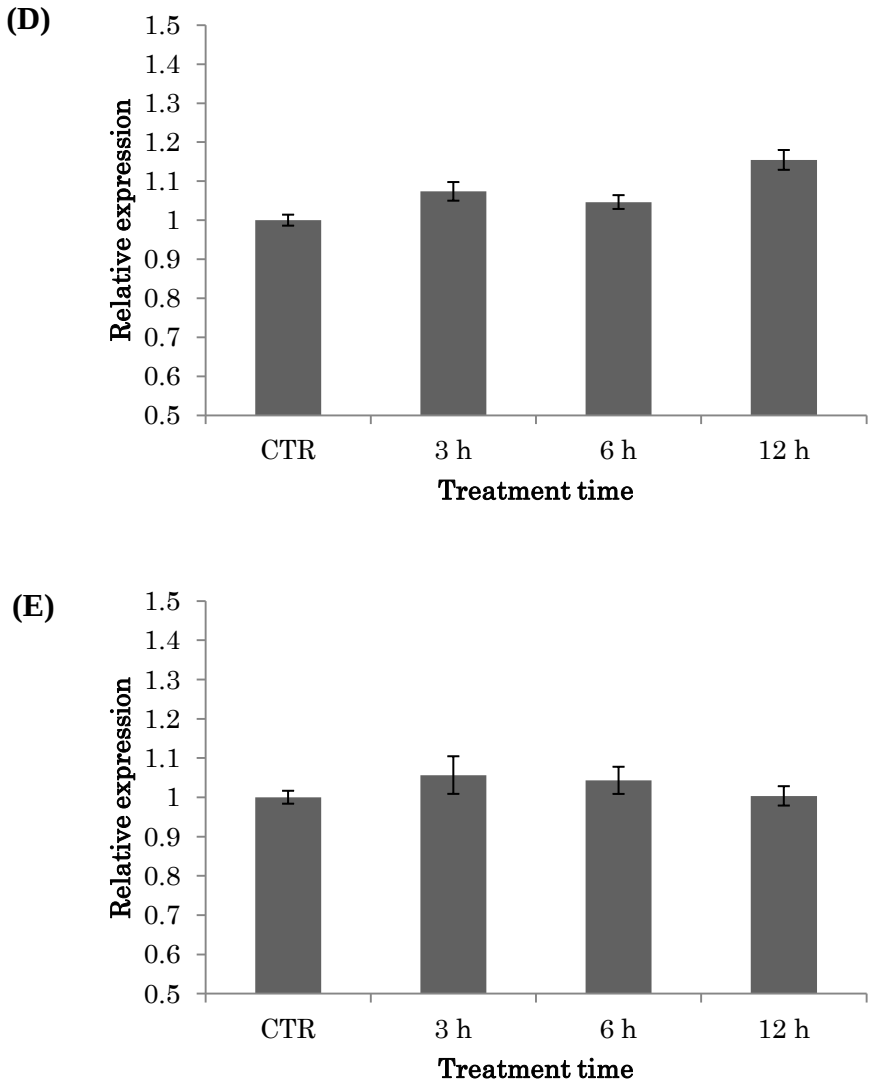


Fig. 3. 3 Effect of apigetrin on the expression of Histone-binding protein RBBP4 (A), Glucosidase 2 subunit beta (B), 14-3-3 beta (C), 14-3-3 epsilon (D) and 14-3-3 gamma (E) mRNAs of HL-60 cells. The cells were treated with 25 μM of apigetrin for 3, 6 and 12 h. CTR represents control cells treated with 0.5% ethanol in medium. RT-PCR was performed using the following Taqman Gene Expression Assays; Histone-binding protein RBBP4 (Hs01568507_g1) and Glucosidase 2 subunit beta (Hs00160457_m1) on the 7500 fast RT-PCR system (Applied Biosystems) and normalized to the endogenous control β-actin (Hs03023880_g1). Representative data of two independent experiments are shown as the mean ± S.D. *$p<0.05$ significantly different from the control.

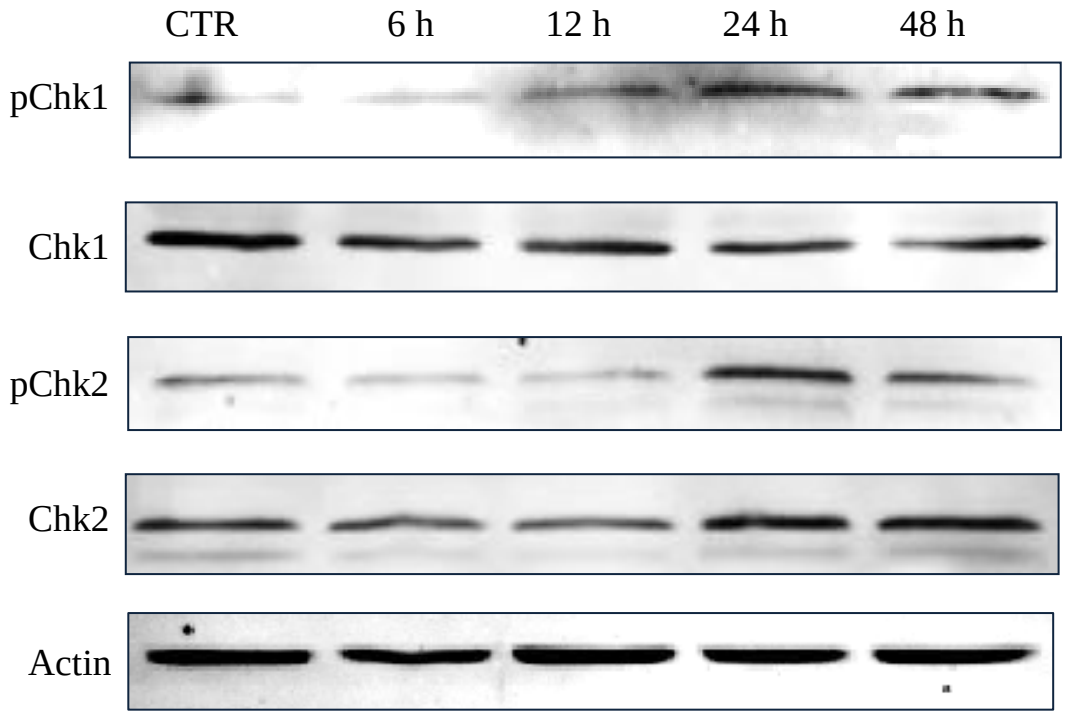


Fig. 3. 4 Effect of apigetrin on phosphorylation status of checkpoint kinases in HL-60 cells. The cells were treated with 25 μM of apigetrin for 6, 12, 24 and 48 h. CTR represents control cells treated with 0.5% ethanol in medium. The cells were lysed and immunoblotted with antibodies against Chk1, phospho-Chk1, Chk2, phospho-Chk2 and actin. Representative data of two independent experiments are shown.

3. 4 Discussion

Proteomics is one of the most valuable tools to analyze the poorly unknown mechanisms. Traditional techniques of molecular biology were usually highly focused, targeting one or few molecules at a time, whereas proteomics provide possible overview of large-scale protein expression changes. The author identified 10 proteins consistently modulated in response to apigetrin treatment by proteomic approach (Table. 3. 1). Apigenin has been reported to inhibit the proteasome activity in human leukemia cells and it gives the possible explanation the up-regulation of all selected proteins (Chen *et al*., 2005).

First, the expression of glucosidase 2 subunit beta protein was increased in apigetrin treated-cells. This protein is regulatory subunit of β-glucosidases which hydrolyses flavonoid glycosides. In human small intestine two of the β-glucosidase, one of which bound in membrane and the other distributed in cytosol, hydrolyze flavonoid glucosides in distinct pathway (Németh *et al*., 2003), for instance quercetin glucoside has been reported to be absorbed via sugar transporters and to be hydrolysed by β-glucosidase (Heim *et al*., 2002). These previous reports provide a possible explanation for better bioavailability of glucosides. Expression of glucosidase 2 subunit beta suggests that apigetrin might be hydrolyzed by β-glucosidase and released aglycone could affect HL-60 cells.

When apigenin is taken orally, it reaches the gut and is extensively metabolized and converted by phase I and phase II enzymes. *In vitro* study has demonstrated that apigenin was converted to the three mono-hydroxylated derivatives via phase I metabolism (Gradolatto *et al*., 2004). Apigenin and luteolin which are the major phase

metabolite of these derivatives, were conjugated to three mono-glucuronated and one mono-sulfated compounds, and four mono-glucuronated, two sulfated and one methylated compounds respectively via phase II metabolism *in vitro* (Gradolatto *et al*., 2004). These observations suggest that the bioavailability of apigenin is limited, but Gradolatto *et al.* (2005) have shown that a single oral administration of radio-labeled apigenin in rats resulted in 24.8% in the rest of the body within 10 days and also its radioactivity appeared in blood after 24 h but eliminated slowly. These results suggest slow metabolism of apigenin and give rise to the possibility of an accumulation of apigenin in the body and tissue for its effective chemopreventive properties. Furthermore apigenin was shown to have no mutagenic activity and effects to normal cells such as liver and prostate cells (Chiang *et al*., 2006; Gupta *et al*., 2001); normal human peripheral blood lymphocytes were not affected by apigenin even at high concentrations: the viability of cells was over 80% with apigenin treatment at 200 μM (Ruela-de-Sousa *et al*., 2010). These reports indicate that apigenin has low intrinsic toxicity even though flavonoids are present in systemic circulation at low micro-molar concentration and differential effects in normal versus cancer cells. However, further studies about the metabolism of apigenin and the usage of administration are needed to develop apigetrin as a food-based anti-leukemia compound.

Next identified proteins were 3 isoforms of 14-3-3 proteins. 14-3-3 proteins are a family of highly conserved molecules that play important roles in a wide range of cellular processes within all eukaryotic cells and seven isoforms are identified in mammals (Tzivion *et al*., 2002). 14-3-3ε and 14-3-3γ were reported to make a complex with tyrosine phosphatase cdc25C and inhibit its entry into the nucleus (Dalal *et al*., 2004). In the cell cycle progression, the cytoplasmic localization of cdc25C does not

activate the cyclinB/cdc2 complex and induce G_2/M arrest (O'Connell *et al*., 2000). 14-3-3β expression is also related to G_2/M cell cycle arrest by indirect inhibition of cell division control protein cdc2 activity (Wang *et al*., 2000b). Overall observation of this study supports the involvement of 14-3-3 proteins in G_2/M arrest particularly with apigetrin treatment of cancer cells.

Other regulatory proteins up-regulated during the HL-60 cells differentiation were Proliferation cell nuclear antigen (PCNA) and Elongation factor 1-β (EF-1β). Although all of the PCNA functions described to date reflect its crucial role in DNA synthesis and repair, recent studies of Witko-Sarsat *et al*., demonstrated its specific function in neutrophils exclusively (Naryzhny, 2008; Witko-Sarsat *et al*., 2010). Unlike macrophages and other cells which showed low-level expression of PCNA during off-proliferation, differentiated neutrophils do not proliferate but highly expressed PCNA protein, which was shown to be associated with procaspases protecting neutrophils from apoptosis. The observations with less apoptotic feature but cell differentiation with apigetrin in the chapter 2 supports this evidence. Interestingly, the expression of PCNA protein increases steadily though the whole cell cycle term and remains high at G_2/M phase (Zeng *et al*., 1994), further interacting with cdc25C at G_2/M transition (Kawabe *et al*., 2002) and with EF-1β (Naryzhny, 2008). EF-1β exchanges GDP for GTP to regenerate active EF-1α. This active EF-1α is then able to perform another round of elongation of polypeptide chain (Le Sourd *et al*., 2006). Increase in size of differentiated cells (Fig. 1B) may implicate the active protein synthesis, which could lead to the increase of EF-1β in protein level.

Histone acetylation is important chromatin modification reaction controlling gene transcription. In this process, histone deacetylase 1 (HDAC1) was reported to form

a complex with PU.1 to act as transcriptional repression (Nicolas *et al*., 2001; Suzuki *et al*., 2003) and PU.1 is known to be suppressed in human leukemic cells but in the process of granulocytic differentiation its expression is increased (Mueller *et al*., 2006). The observed up-regulation of plastin-2, Annexin A5 and Coronin-1A with apigetrin treatment confirms granulocytic differentiation in terms of granulocytes characteristic features. Plastin-2 and Annexin A5 are reported to be the components of NADPH oxidase system (Babior, 1999; El Btaouri *et al*., 1996; Karlsson *et al*., 2005; Kim *et al*., 1994; Mira *et al*., 1997; Munshi *et al*., 2002; Oshizawa *et al*., 2003) which is the basis of NBT reduction assay to identify the differentiated granulocytes and also I reported that apigetrin increased the NBT reductive activity in chapter 2. Coronin-1A is a member of actin-associated proteins that have been implicated in a variety of cellular processes dependant on actin rearrangements. In primary human neutrophils, the expression of coronins is required for actin-dependant changes in cell morphology that lead to migration and chemotaxis (Yan *et al*., 2007). Morphological observations of the differentiated cells with apigetrin also showed the protrusions and pseudopod developments in HL-60 cells with weak attachment to the dish. These 3 proteins support these results of apigetrin induced granulocytic differentiation in HL-60 cells.

The RT-PCR results revealed that of the 5 differentially expressed proteins identified by proteomic analysis, two showed similar changes at the mRNA and protein levels. However, there were no clear changes at the mRNA levels for 14-3-3 proteins after apigetrin treatment, although their expression increased at the protein level. A study of the myeloid differentiation in MPRO murine cells by Lian *et al.* (2002) in which both mRNA and protein have been compared in the same sample have also shown that there is not a good correlation between mRNA and protein levels. The

discrepancy between mRNA and protein level may indicate the importance of post-transcriptional and post-translational processes during cell differentiation.

One of proteins which were identified by proteomic analysis is the signal transduction molecules of 14-3-3 proteins. 14-3-3 proteins are phosphorylated to bind protein ligands. Hence the author focused in Chk1 and Chk2 protein, which is the up-stream protein for 14-3-3 proteins and regulated the phosphorylation of 14-3-3 proteins to transduce cell signaling (Okada *et al*., 2010). Chk1 and Chk2 are functionally related kinases that phosphorylate overlapping pools of cellular substrates, although these proteins have distinct regulatory domains. To directly investigate the role of Chk1 and Chk2 in apigetrin-induced the differentiation of HL-60 cell, western blot analysis was performed. Results support my hypothesis the activation of Chk1 and Chk2 is involved the effect of apigetrin. Chk1 has been reported to be mainly responsible for phosphorylation of substrates while Chk2 plying a supplementary role, and it gives the possible explanation the early activation of Chk1 compared with Chk2 (Okada *et al*., 2010). Generally Chk1 and Chk2 play a central role in implementing many aspects of cell cycle check point response; cell cycle arrest or induction of apoptosis. Recent study of Carrassa *et al*., demonstrated a possible role of Chk1 in hematopoietic stem cell differentiation isolated from umbilical cord blood (Carrassa *et al*., 2010). Moreover the activation of both Chk1 and Chk2 is critical for Ara-C-induced erythroid differentiation, which is famous inducer of erythroid differentiation (Takagaki *et al*., 2005). Taken together, Chk1 and Chk2 are at a key cross-road where many biological processes meet; cell cycle progression, apoptosis, DNA repair and differentiation. Results of this study suggest that apigetrin- induced granulocytic differentiation of HL-60 cells is mediated by both Chk1 and Chk2 pathways. However it

remains to be seen which effect is dominant in the activation of Chk1 and Chk2.

It has been unknown what the molecular target of apigetrin is, however recent study has revealed apigenin binding target proteins by Phage Display sequencing for comprehensive identification (Arango *et al*. 2014). Apigenin (and likely, other flavones) directly interacts with heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2), inhibiting its dimerization/activation and altering the alternative splicing patterns of hnRNPA2 substrates. Choi *et.al.* reported (2013) hnRNP A2/B1 is essential for human ES Cells self-renewal and pluripotency. Moreover hnRNP A2/B1 knockdown inhibited hESC proliferation by inducing phosphorylation of Chk1 though Akt/phosphatidylinositol 3-kinase (PI3K) pathway. This pathway has been reported to phosphorylate Chk1 (King *et al*., 2004). hnRNP A2 expressed even in HL-60 cells (Gallo *et al.*, 2014). To the best of knowledge, apigetrin is proposed that a possibility of molecular target is hnRNPA2 in HL-60 cells. Further studies are required in order to clarify downstream events in Chk1 and Chk2-mediated signaling pathways during differentiation.

In this chapter, the author showed that ten proteins that might play essential role in granulocytic differentiation which is previously-unreported as proteins related to differentiation, and a possible role for Chk1 and Chk2 in the granulocytic differentiation on HL-60 cells. On the basis of these results and reports, the author proposes a possible mechanism for apigetrin- induced granulocytic differentiation of HL-60 cells (Fig. 3. 5). Apigenin, which is hydrolyzed by glucosidase from apigetrin, inactivates hnRNPA2, and each kinases phosphorylate Chk1 and Chk2, which might lead to signal transduction toward G2/M cell cycle arrest through 14-3-3 proteins and granulocytic differentiation though transcriptional repression by PU.1.

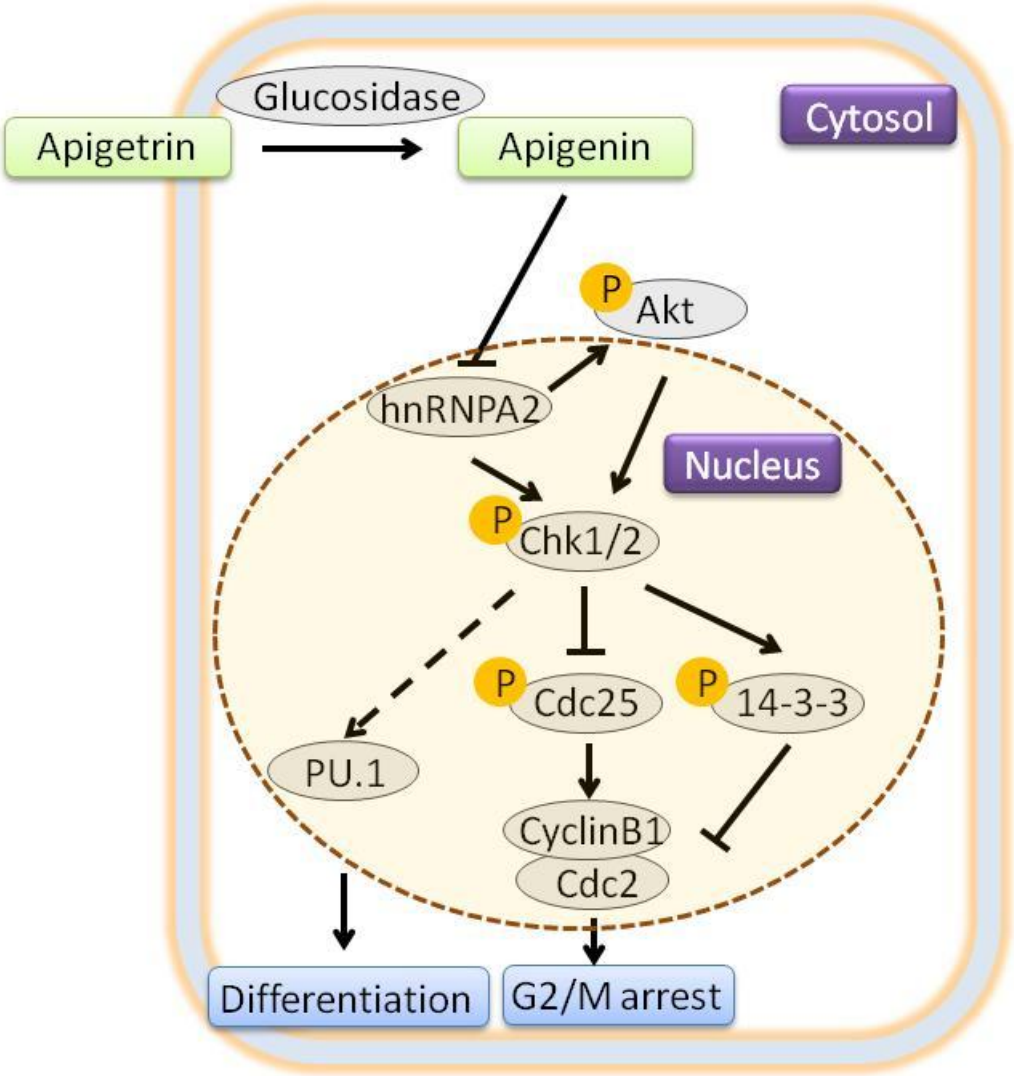


Fig. 3. 5 Proposed pathways of apigetrin- induced granulocytic differentiation in HL-60 cells.

Chapter 4

Apigenin quantification and phenolic profiling of olive (*Olea europaea L.*) leaves from Tunisia

4. 1 Introduction

Olive tree *Olea europaea L*. is an evergreen tree growing in several areas in the world, and is famous to be the emblematic tree of Mediterranean basin. Olive fruits and olive oil are well studied and well documented for their alimentary uses and health benefits, whereas other by-products like olive leaves are less studied.

Olive leaves were used since old time for their medicinal and health benefits as tea or incenses. In fact, olive leaves are an interesting reservoir containing big amount of several secondary metabolites mainly polyphenols and flavonoids. Accumulating evidences demonstrated that olive leaves extracts and their active compounds are endowed with several health benefit biological and pharmacological activities. In previous chapter, the author demonstrated apigetrin is the most bio-active compound against leukemia using HL-60 cells. Even though these studies provide us valuable suggestions the functionalities of apigenin for human health, however the chemical contents of apigenin in olive leaves is not fully evaluated. On the other hand, oleuropein, which was also thought as the bioactive components in olive leaves with their high anti-oxidant activity, has been reported their content in olive by Bouaziz *et al*. (2004) and Salah *et al*. (2012). The chemical composition of olive products differs depending on several conditions such as the variety, the growing area, the season and the plant part (Luján *et al*., 2009).

Tunisia is an important olive oil producing and exporting country, where olive tree plantation is estimated to around 70 million trees, covering almost 30% of Tunisian arable land (1.7 Million Hectares). It is occupying the fourth position as olive oil exporter in the world. In Tunisia, olive sector is representing 59.8 % of agro-products

exportation, and is offering 34 million working day/year, that is corresponding to 20% of employment capacity in Tunisia (Kashiwagi *et al*., 2012). Despite its small area, there is 70 million olive trees and there are dozens of identified cultivars in the country with great environmental diversity due to its north-south extent.

In this chapter, the author conducted the current research in order to quantify the amounts of apigenin and apigetrin from 4 different varieties of Tunisian olive tree grown in the northern part in Tunisia, to determine total polyphenol and flavonoids content and the antioxidant capacity of olive leave extracts.

4. 2 Materials and Methods

4. 2. 1 Preparation of olive leaves'extracts

Olive leaves were collected from Tunisia at 16 or 17 November 2011. The olive leaves used for this study were collected from 4 varieties; Chetoui, Meski, Chemlali and Sahli, grown in 5 various regions of North part in Tunisia (Table. 4. 1, Fig. 4. 1). Olive leaves were dried at room temperature and protected from the light. and ground to powder with an electrical blender. Then dried powder sample (1 g) was extracted with 10 ml 70% ethanol (EtOH) (w/v) at room temperature in darkness for 2 weeks. The obtained solution was filter-sterilized by 0.22 μm filter (Millipore) then stored -80 °C until use. Dry weight (DW) in each sample was evaluated after solvent evaporation under vacuum at 37°C using Speed Vac (Genevac, Ipswich, UK).

4. 2. 2 High Performance Liquid chromatography (HPLC)

HPLC analysis was performed using Ultimate 3000 LC (Dionex, Sunnyvale,

CA, USA) and Acclaim C18 column (150×1.0 mm i.d.) thermostated at 40 °C. The flow rate was 35 μL/min and the detection was performed at 280 nm. A linear gradient was run from 80% of solvent A (10 mM phosphoric acid in water) and 20% of solvent B (Acetonitrile). Then Solvent B was increased to 55% in 10 min, then up to 85% within 5 min, and kept isocratic for 5 min. The HPLC-grade standards apigetrin was obtained from Sigma-Aldrich (St Louis, MO, USA), and aglycone apigenin was obtained from Merck (Whitehouse Station, NJ, USA). Apigenin and apigetrin in different extracts were identified by comparison of their retention times with the standards.

4. 2. 3 Total phenolic content

Total phenolic content in olive leaves was determined using a Folin–Ciocalteu reagent method (Singleton and Rossi, 1965) with some modification for micro-method. To 70 μl of diluted extract or a standard solution of gallic acid (Sigma-Aldrich, St. Louis, MO, USA), 70 μl Folin–Ciocalteu reagent (1:4 v/v in ddH_2O) were added in 96 well plate and allowed to stand for 3 min at room temperature. Then 70 μl Na_2CO_3 (wako, Japan, 10% w/v in ddH_2O) were added. The plate was incubated for 60 min at room temperature. Then, the absorbance was recorded at 760 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, NJ, USA). The results were expressed as gallic acid equivalents (GAE) in mg/g of olive leaves extract dry weight (mg GAE g^{-1}). All extracts were analyzed in triplicate.

4. 2. 4 Total flavonoid content

Total flavonoid content was determined using a colorimetric assay (Kim *et al.*, 2003) with some modifications for micro-method. To 150 μl of diluted extract or a

standard solution of quercetin (Sigma-Aldrich, St. Louis, MO, USA) in EtOH, 7.5 μl $NaNO_2$ (wako, Japan, 5% w/v in ddH_2O) were added the mixture in 96 well plate and allowed to stand for 5 min at room temperature. Then 15 μl $AlCl_3$ (wako, Japan, 10% w/v in ddH_2O) were added and incubated for an extra 5 min at room temperature. Finally, 50 μl NaOH (1 M) were added, immediately the mixture was diluted by the addition of 27.5 μl of ddH_2O. The absorbance was recorded at 510 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, NJ, USA). The results were expressed as quercetin equivalents (QE) in mg/g of olive leaves extract dry weight (mg QE g^{-1}). All extracts were analyzed in triplicate.

4. 2. 5 DPPH radical scavenging assay

The antioxidant effect of olive leaves was determined using a 1,2-diphenyl-2-picrylhydrazy (DPPH) radical scavenging assay micro-method. The reaction mixture (190 μl) containing 0.2 mM DPPH dissolved in EtOH, 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6.1), and 10 μl of diluted extract or a standard solution of trolox (Sigma-Aldrich, St. Louis, MO, USA) in EtOH were added to each well of 96-well microplate and incubated for 10 min at room temperature in darkness. The absorbance was recorded at 520 nm using a multidetection microplate reader (Powerscan HT, Dainippon Pharmaceutical, NJ, USA). The results were expressed as trolox equivalent antioxidant capacity (TEAC) in mM/g of olive leaves extract dry weight (mM TEAC g^{-1}). All extracts were analyzed in triplicate.

4. 2. 6 Statistical analyses

Results are expressed as means ± SD. Differences at $P < 0.05$ were considered

statistically significant. Statistical analysis was performed using Student's t-test on the data of functional activity assay. Data of individual phenols, flavonoids and anti-oxidant activity were analyzed using one way ANOVA followed by Tukey post hoc test to assess for significant differences between different Tunisian olive leaves. Further, principal component analysis (PCA) was used as multivariate statistical tool in order to interpret the correlations between physico-chemical profiles of olive leaves cultivar and their geographical origin, using StatGraphics 5.0.

Table. 4. 1

Description of different olive leaves sample collected in Tunisia: sampling point location, variety, fruit harvesting period, and abbreviation of each sample.

Location	Latitude N	Longitude E	Variety[a]	Harvesting period	Abbreviation
Borj Ettoumi	36.76764	9.691806	Chetoui (n=5)	Nov – Jan	BC
Borj Ettoumi	36.75344	9.699806	Meski (n=5)	Nov – Jan	BM
Metline	37.24331	10.07	Chetoui (n=5)	Dec – Jan	MC
Oum heni	37.08364	9.82725	Chetoui (n=5)	Nov – Feb	OC
Oum heni	37.08364	9.82725	Chemlali (n=3)	Nov – Feb	OCL
Oum heni	37.08364	9.82725	Meski (n=5)	Nov – Feb	OM
Sidi Hasseine	36.75122	10.12128	Chetoui (n=5)	Jan – Feb	SC
Sidi Hasseine	36.75122	10.12128	Sahli (n=5)	Jan – Feb	SS
Takelsa	36.79881	10.63333	Chetoui (n=5)	Nov – Jan	TC

a) The number of samples was that of olive trees from which were collected leaves.

(A)

(B) ①Metline ②Oum heni ③Borj Ettouni ④Sidi Hassine ⑤Takelsa

Fig. 4. 1 The location of sampling point in Tunisia. (A)Outline map of Tunisia, (B) map of northern part in Tunisia.

4. 3 Results

4. 3. 1 Quantitative analysis of apigetrin and apigenin in olive leaves

HPLC profiles of phenolic compounds present in olive leaves were shown in Fig. 4. 2C. The retention times of the apigetrin and apigenin in olive leaves extract were 13.89 min and18.12 min, respectively (Fig. 4. 2D). Fig. 4. 3 showed the overlay chromatogram of representative for each representative samples. As shown in Table. 4. 2, apigetrin content in olive leaves ranged from 2.52 ± 0.35 to 4.60 ± 1.35 μg/g DW. The order of mean apigetrin content was Meski/Oum heni > Chetoui/Takelsa > Chetoui/Sidi Hasseine, Sahli/Sidi Hasseine > Chemlali/Oum heni > Chetoui/Borj Ettoumi > Meski/Borj Ettoumi > Chetoui/Oum heni > Chetoui/Metline. Apigenin content in olive leaves ranged from 0.45 ± 0.03 to 0.97 ± 0.15 μg/g DW. The order of mean apigenin content was Sahli/Sidi Hasseine > Chetoui/Takelsa > Meski/Oum heni > Chetoui/Metline > Chemlali/Oum heni > Meski/Borj Ettoumi > Chetoui/Sidi Hasseine > Chetoui/Borj Ettoumi > Chetoui/Oum heni. Analysis of variance (ANOVA) showed difference between samples, further Tukey post hoc test showed the difference between high and low apigetrin content. Moreover the results revealed that apigenin was present in olive leaves mostly as glucoside form in contrast the aglycon form was present as trace.

4. 3. 2 Total flavonoids and total phenolics content in olive leaves, and their antioxidant capacity

Total flavonoids and total phenolic contents in olive leaves dtermined by calorimetric assay. This analysis of micro methods developed in our laboratory makes it

possible to improve the high throughput screening. As shown in Table. 4. 2, total flavonoids content in olive leaves ranged from 165.73 ± 57.16 to 447.79 ± 152.06 mg QE/g DW. The order of mean total flavonoids was Chetoui/Takelsa > Chetoui/Sidi Hasseine > Chetoui/Borj Ettoumi > Sahli/Sidi Hasseine > Meski/Borj Ettoumi > Chemlali/Oum heni > Chetoui/Oum heni > Chetoui/Metline > Meski/Oum heni. Total phenolics content in olive leaves ranged from 12.44 ± 2.19 to 20.78 ± 4.05 mg GAE/g DW. The order of mean total phenolics was Chemlali/Oum heni > Chetoui/Borj Ettoumi > Meski/Oum heni > Chetoui/Oum heni > Chetoui/Metline > Sahli/Sidi Hasseine > Chetoui/Taklsa > Chetoui/Sidi Hasseine > Meski/Borj Ettoumi. DPPH radical is well known as a stable organic free radical which has been used for estimation of the antioxidant capacity. DPPH radical is changed to nonradical to react antioxidants. We determined the antioxidant capacity of 9 samples (Table. 4. 2). The antioxidant capacity in olive leaves ranged from 479.64 ± 66.38 to 698.26 ± 24.05 mM TEAC/g DW. The order of mean antioxidant capacity was Chemlali/Oum heni > Chetoui/Borj Ettoumi > Sahli/Sidi Hasseine > Chetoui/Oum heni > Chetoui/Takelsa > Chetoui/Metline > Meski/Oum heni > Meski/Borj Ettoumi > Chetoui/Sidi Hasseine.

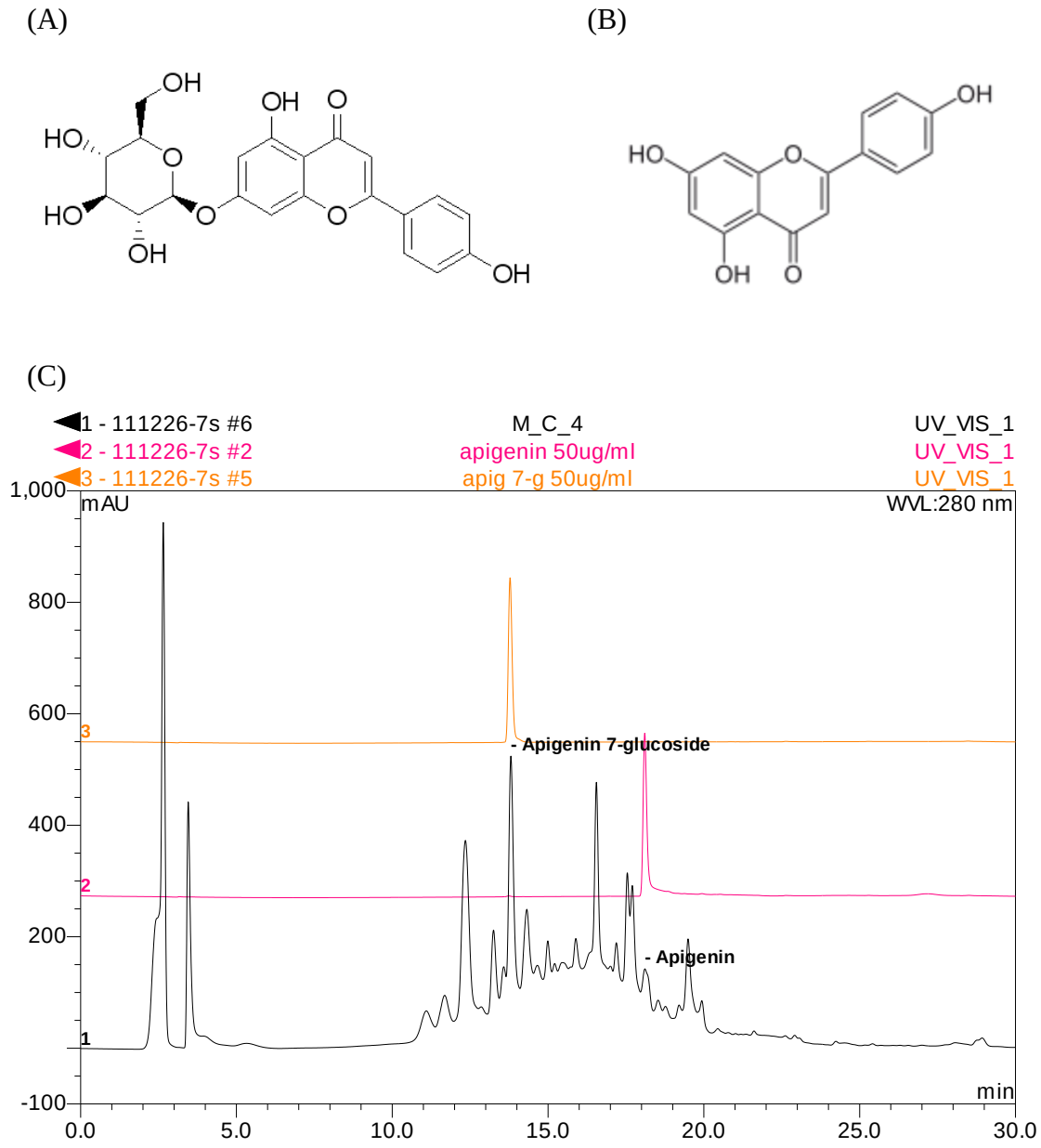


Fig. 4. 2 Chemical structure of apigetrin (A) and apigenin (B). HPLC chromatogram at 280 nm of (1) olive leaves extract, (2) apigenin standard and (3) apigetrin (C). Retention time of apigetrin and apigenin in olive leaves extract were 13.89 min and 18.12 min respectively.

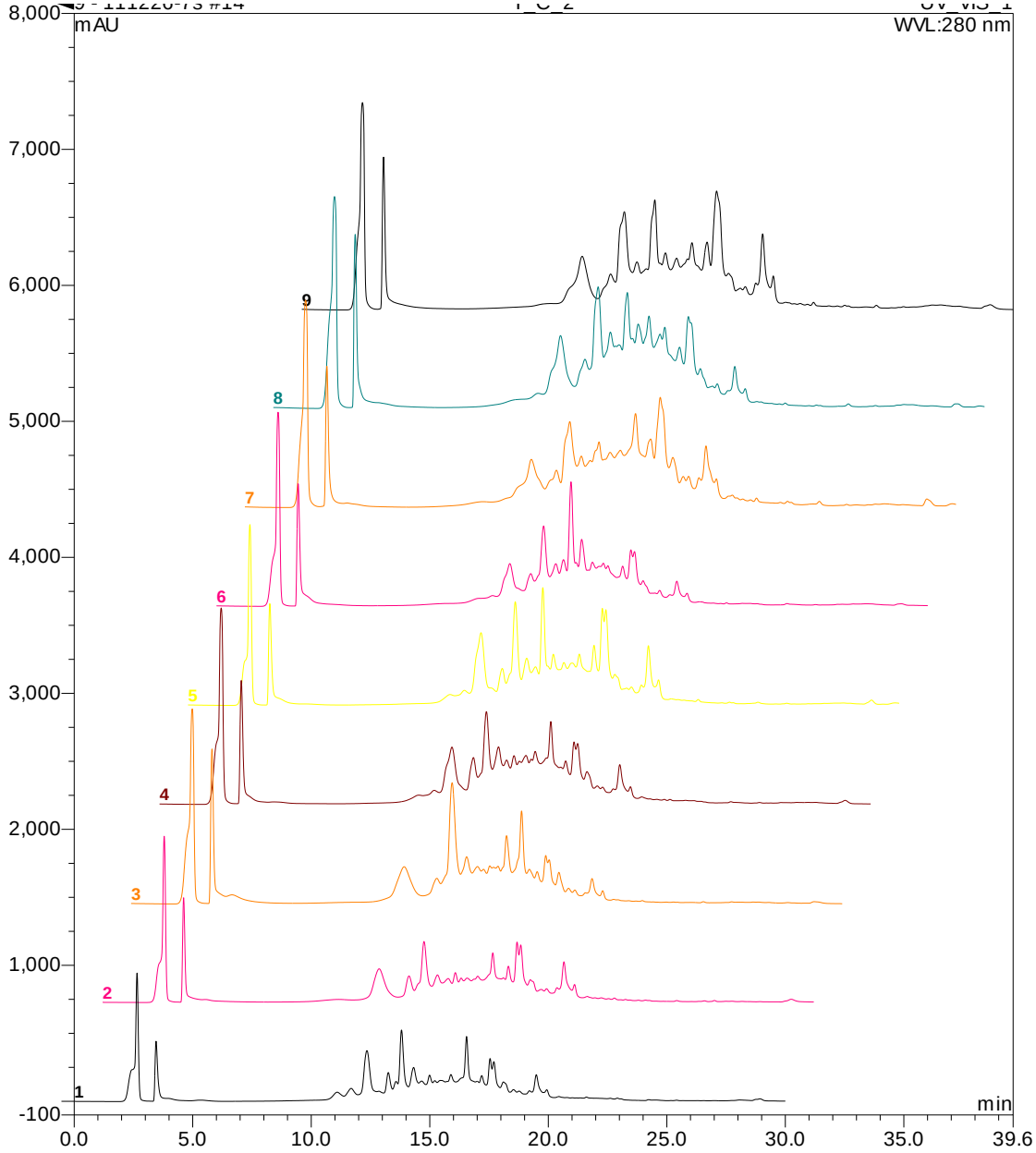


Fig. 4. 3 Representative HPLC chromatogram of each variety's olive leaves extract, comparison at 100 mg of fresh weight leaves equivalent; (1)MC, (2)OC, (3)OM, (4)OCl, (5)BC, (6)BM, (7)SS, (8)SC, (9)TC.

Table. 4. 2

Mean Composition and Standard Deviation of different chemical descriptor of olive leaves extracts: Apigetrin (μg/g DW), Apigenin (μg/g DW), Total flavonoids (mg QE g^{-1} DW), Total phenolics (mg GAE g^{-1} DW), and Antioxidant activity (mM TEAC g^{-1} DW).

Sample	Apigetrin	Apigenin	Total flavonoids	Total phenolics	Antioxidant activity
BC	3.32 ± 0.50^{ab}	0.46 ± 0.18^{a}	391.07 ± 109.62^{acd}	16.58 ± 2.07^{ab}	650.21 ± 28.39^{ab}
BM	3.14 ± 0.44^{ab}	0.52 ± 0.16^{a}	267.91 ± 148.74^{abd}	12.44 ± 2.19^{a}	490.84 ± 141.78^{ac}
MC	2.52 ± 0.35^{a}	0.60 ± 0.37^{ab}	198.94 ± 45.67^{ab}	15.89 ± 0.96^{a}	549.20 ± 54.69^{abc}
OC	2.68 ± 0.12^{a}	0.45 ± 0.03^{a}	236.66 ± 78.90^{abd}	15.91 ± 1.54^{a}	575.81 ± 73.07^{abc}
OCL	3.60 ± 0.20^{ab}	0.59 ± 0.02^{ab}	263.32 ± 73.19^{abcd}	20.78 ± 4.05^{b}	698.26 ± 24.05^{b}
OM	4.60 ± 1.35^{b}	0.62 ± 0.17^{ab}	165.73 ± 57.16^{b}	16.26 ± 2.75^{ab}	504.57 ± 75.41^{ac}
SC	4.52 ± 0.49^{b}	0.50 ± 0.10^{a}	459.79 ± 113.70^{cd}	14.38 ± 0.64^{a}	479.64 ± 66.38^{c}
SS	4.52 ± 0.46^{b}	0.97 ± 0.15^{b}	362.85 ± 91.71^{abcd}	15.56 ± 1.52^{a}	617.35 ± 72.14^{abc}
TC	4.58 ± 1.13^{b}	0.65 ± 0.37^{ab}	447.79 ± 152.06^{d}	15.15 ± 1.69^{a}	563.13 ± 89.76^{abc}

Values are expressed as means ±SD (n=5) for HPLC analysis, with exception for OCL (n=3), and also (N=5) for total flavonoids, total phenolics, and antioxidant activity, with exception for OCL (n=3). Means with different letters in the same column were significantly different at the level $p<0.05$ (One way ANOVA followed by Tukey post hoc test).

4. 3. 4 PCA analysis

PCA was applied to the dataset of apigetrin, apigenin, total falvonoids, total phenolics and antioxidant capacity (TEAC) of four olive cultivar leave sample collected from five sites in Tunisia during November 2011 (Fig. 4. 4). PCA accounted for 72.11% cumulative variance with 37.83% for PC1 and 34.28 %for PC2. PCA plot (Fig. 4. 4) generated 4 distinctive groups :(i) Chemlali cultivar grown in Oum Heni area (OCL) which is characterized by high phenolic content and high anti-oxidant capacity, (ii)

Meski cultivars grown in Oum Heni and Borj Toumi (OM and BM), (iii) Chetoui cultivar grown in Borj Toumi, Oum Heni, and Metline (BC, OC, and MC), and (iv) the last groups is composed by three sample which are Sahli and Chetoui cultivar grown in Sidi Hassine and Takelsa (SS, TC, SC).

PC1 was highly correlated with total phenolic content and antioxidant capacity TEAC while PC2 was highly correlated with apigenin and apigetrin content. Moreover, Fig. 4.5 shows that there is high correlation between total phenolics and TEAC ($p<0.05$) on the one hand apigetrin and total flavonoids ($p<0.05$). This results is concordant with previous findings and demonstrates that phenolics are the main compounds to contribute to total anti-oxidant capacity, and apigenin aglycone or glucoside highly contribute to total flavonoids content.

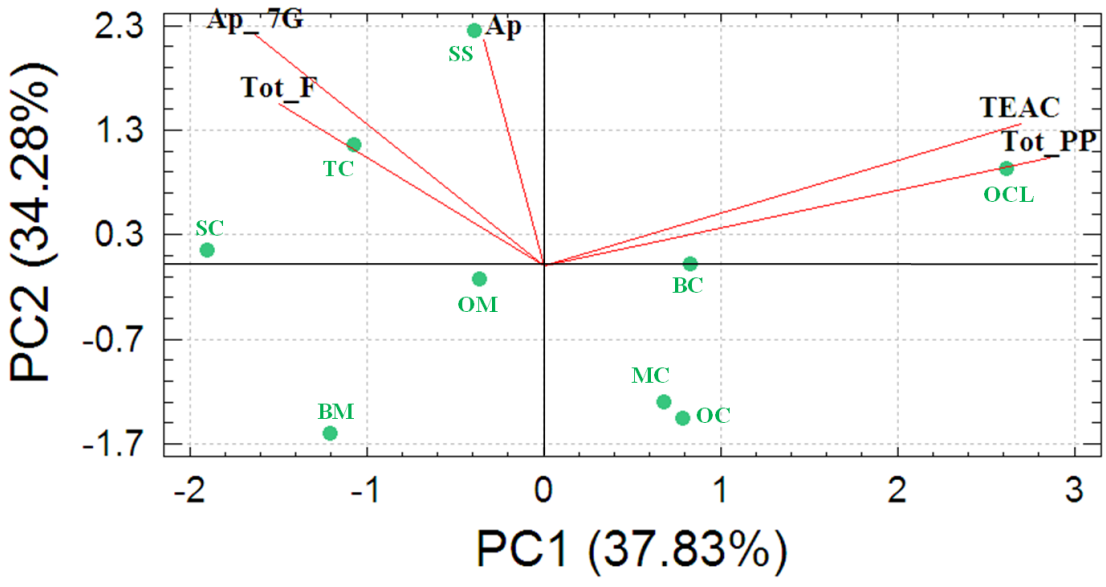
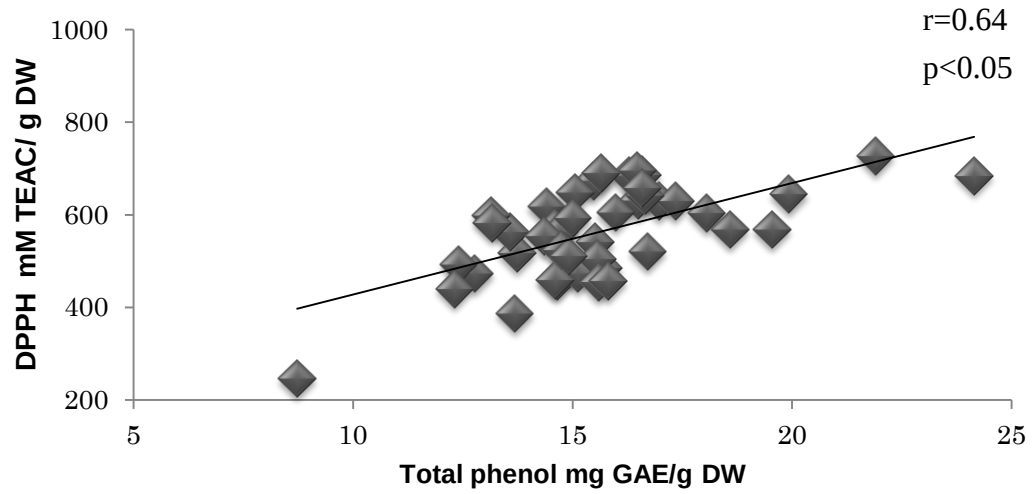


Fig. 4. 4 Principal Component Analysis (PCA) for chemiometric analysis in Tunisian olive leaves extracts. *Abbreviations: Ap_7G: Apigetrin, Ap: Apigenin, Tot_PP: total phenolics, Tot_F: total flavonoids, TEAC: Trolox equivalent antioxidant capacity*.

(A)



(B)

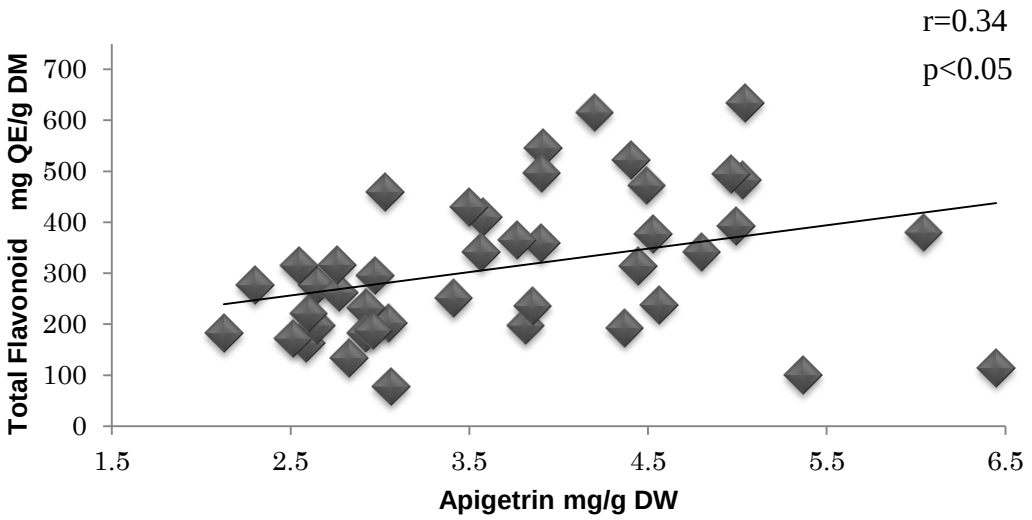


Fig. 4. 4 Correlation Analysis for chemiometric analysis in Tunisian olive leaves extracts (n=43). Relationship between total phenol and anti-oxidant activity is shown (A) and apigetrin and total flavonoid is shown (B).

4. 4 Discussion

Olive phytochemicals are highly available in olive leaves and could be extracted and purified with low cost in order to be used as functional food supplement or food additives. Herein, the author screened 4 Tunisian olive varieties' leaves extract: Chetoui, Chemlali, Meski, and Sahli grown in 5 sites in the northern part of Tunisia, for their total polyphenol, total flavonoid, and apigenin content and their anti-oxidant capacity using analytical micro-methods. Results demonstrated a significant difference between cultivars and growing sites. Although the study lacked some limitations (did not take in consideration climatic stage effect, wider regional disparities, and farming techniques), the PCA revealed that the studied parameters could discriminate between olive cultivars and growing sites, generating 4 different sub-groups with high content of apigenin in Chetoui cultivars and high polyphenol content in Chemlali cultivar. Meski cultivar showed low content of apigenin and polyphenol compared to other cultivars as revealed by PCA sub-group analysis. Meski cultivars are situated in the lower quadrant of PCA biplot and negatively correlated with flavonoids, phenolics, apigenin and Anti-oxidant activity. Even there are thousands of varieties of the *Olea europaea* olive tree, there are two major varieties which are as follows; Chetoui in the north, Chemlali in the middle and south of Tunisia. Various other types of olive may be found such as Meski which is in north and newly irrigated area. According to the Seeds and Plant Genetic Resources team of FAO's Plant Production and Protection Division (AGP), Chetoui has the middle content of oil used for oil, Chemlali has the high content of oil used for oil, and Meski has the low content of oil used for table. This finding is

concordant with the common use of the variety in Tunisia, since it is frequently used for table olive preparation and the other varieties are used for olive oil production.

Meanwhile the biosynthesis of apigenin starts from three molecules of malonyl-CoA and one of 4-coumaroyl- CoA (Petrussa *et al.*, 2013). Indeed, flavonoids including apigenin are often present in the epidermal cell layers of leaves and in tissues that are susceptible to UV light, than pollen and the apical meristem (Winkel-Shirley, 2002). Hahlbrock *et al*. (1971) showed that increase of apigenin was induced by light irradiation via activation of synthesized enzymes in cell suspension cultures of parsley, provided the evidence that UV light induces the synthesis of flavonoids. Several studies have revealed the effect of temperature and also water deficit on the concentration of flavonoids (Ojeda *et al*., 2002, Yamane *et al.*, 2006). There is also interesting evidence that flavonoids accumulates for aluminum-resistant in roots of maize (Kidd *et al.*, 2001). The accumulation of flavonoids is still controversial due to many aspects. On the other hand Meski cultivar, which is characterized by bigger size of fruits, showed low content of apigenin and polyphenol compared to other cultivars as revealed by PCA sub-group analysis. Utilization of malonyl-CoA and 4-coumaroyl- CoA, which are starting molecules of apigenin biosynthesis and also primary metabolite, possibly to be considered as antagonistic to metabolism in the case of growing. The current study focused on olives grown in the northern area of Tunisia, but further studies using samples grown in all Tunisian territory and why not in mediterranean region will be required for the evaluation of climate and environmental effect on chemical descriptors in olive leaves.

The content of apigetrin was highly correlated with apigenin content and total phenolic content with ant-oxidant activity. The anti-oxidant compound was reported as

the potential differentiation inducer in HL-60 cells (Sokoloski *et al*., 1997). The results in this chapter did not demonstrate a correlation between apigenin content and anti-oxidant activity, but the possibility of apigenin as potent strong differentiation inducer in human leukemia cells. The current study highlighted apigenin content as a selecting parameter for olive leave extract (all the extracts available in the market are based on oleuropein and hydroxytyrosol percentages). Several studies have reported that apigenin glucoside is only available in olive leaves and not in olive oils (Brenes *et al*., 1999; Christophoridou and Daris, 2009; Fu *et al*., 2009; García-González, *et al*., 2010; Molina-Alcaide and Yáñez-Ruiz, 2008; Murkovic *et al*., 2004). In contrast the aglycon form is released in olive oil because the hydrolysis reaction occurs during the mechanical process of olive oil production. The current results indicate that olive leaves apigenin is mostly present as glucoside not aglycon form (Table. 4. 1) which is concordant with previous reports (Abaza *et al*., 2007). Apigetrin is more useful compared to apigenin aglycon in the point of stability, solubility, palatability and bioavailability (Patel *et al*., 2007).

There is traditional and widespread use of olive leaves and olive-leaf tea in Mediterranean region. In Tunisia, despite its small area, there is 70 million olive trees covering 1,700,000 ha and there are dozens of identified cultivars in the country. However, Chetoui in the northern part, and Chemlali in the middle and the southern part of the country are the main cultivar. Other cultivars like Meski, Sahli, Chemchali, Oueslati, baldi, Zarrazi, Zalmati, Gerboui, Sayali, etc.... are frequent in some part of the country (Trigui *et al.*, 2002). Moreover, some Spanish and Greek cultivar like Arbequina, Arbosana, and Koroneiki were recently introduced to the country. World widely, there is an increasing interest in olive products phenolics, and this is due to their

high biological and pharamacological properties. In this respect, apigenin-rich olive leaves extract would be a promising issue for industrial valorization of olive leaves in Tunisia and in Mediterranean countries.

In this chapter, the author showed that olive leaves have proved their capacity for storage of phenolic compounds such as apigenin, as glucoside derivatives. This study supports the potential of leaves source for isolation of the target compounds on an industrial scale, and highlighted Tunisian olive leaves' apigenin content and bioactivities and reported that Chetoui would be the variety with the highest concentration. Moreover, we demonstrated that analytical micro-methods which are cost and time effective could be considered as important tool for chemical and biological screenings. Finally, this study although it has some limitations it can be helpful tool for industrial, academic researchers, and decision makers, and may contribute to elaboration of large scale and more comprehensive screening studies

Chapter 5

General conclusion

Cancer is one of the major public health burdens in many countries. Global cancer incidence is increasing dramatically; In fact 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 (Jemal *et al*., 2011). Blood cancers are among the most severe and virulent forms of cancer. In 2010 leukemia caused the death of 281,500 persons (Lozano *et al*., 2012) with a lower incidence in Mediterranean countries (Jemal *et al*., 2011). Physical exercise and healthy diet like the Mediterranean one are among WHO recommendation to reduce the burden and incidence of cancer. Cumulating evidence demonstrated that polyphenol-rich diet reduced the incidence and the prevalence of malignancies (Fresco *et al*., 2010). Epidemiological studies reported a lower incidence of cancer among Mediterranean people and this could be related to the diet rich in fruits, vegetables and olive oil (Filomeno ei al., 2014; Castelló *et al*., 2014). Olive oil minor compounds were reported in our previous studies and by others to be efficient against malignancies (Abaza *et al*., 2007; Tsolmon *et al*., 2011). Cancer prevention aims at blockage or reversal of the initiation phase of carcinogenesis or arrests it at progression stage. In this context, plant derived naturally occurring constituents may be largely involved since increased consumption of fruits and vegetables correlates with lower incidence of cancer (Visioli *et al*., 2000). Here the author tried to investigate yet another approach of cancer prevention such as cancer cell differentiation using a common non-mutagenic flavonoid apigetrin.

In chapter 2, the author showed that apigetrin inhibited HL-60 cell growth, dose- and time-dependently, but did not cause apoptosis. The distribution of cells at different stages in the cell cycle indicated an accumulation of treated cells in G_2/M phase. Moreover, apigetrin induced granulocytic differentiation of HL-60 cells. The

common view that the initiation of cell differentiation is most likely to occur at the G_0/G_1 phase was not followed in the case of apigetrin. Therefore it might be interesting to investigate the differentiation pathway with apigetrin treatment and explore novel players in the complex process of cellular signaling.

To consider these findings in a clinical application, in vivo safety and efficacy studies of apigenin and apigetrin should be reported. Apigenin' pharmacokinetic study was reported by Gradolatto *et al*. (2005) showed that a single oral administration of radio-labeled apigenin in rats appeared in blood plasma after 24 h and resulted in 24.8% in the rest of the body within 10 days. It has been also reported that apigenin is very effective against several cancer animal models except leukemia. Orally administered apigenin (20 and 50 μg/mouse/day, 6 days/week for 20 weeks) resulted in a significant decrease in tumor volumes of prostate in transgenic adenocarcinoma mice model indicating that apigenin inhibits prostate cancer progression (Shukla *et al.*, 2014). Same doses of apigenin following 8 weeks treatment induced apoptosis in prostate tumor xenografts in nude mice (Shukla and Gupta, 2006). Another in vivo study of apigenin (40 mg/kg apigenin for 7 days as intraperitoneally injection), improved glucose tolerance in miR103 transgenic mice (Ohno *et al.*, 2013). Moreover, oral administration of apigenin at 5, 10, and 20 mg/kg/day decreased furan-induced toxicity in liver and kidney on mice (Wang *et al.*, 2014). On the other hand, safety of apigenin was evaluated following acute exposure though intraperitoneal injection of apigenin at doses of 25, 50, 100 and 200 mg/kg in Swiss mice. The liver function makers, serum ALT, AST and ALP, in 25 or 50 mg/kg apigenin doses indicated that it has no toxic effect at these doses. In contrast these markers significantly increased in 100 and 200 mg/kg apigenin doses, indicating a severe hepatotoxicity (Singh *et al.*, 2012). Taken together, previously

reported studies demonstrated that apigenin is expected to be effective against cancer at low dose without any side effects. Further studies are required to evaluate apigein benefit on leukemia in small animal models.

In chapter 3, the author showed that ten proteins that might play essential role in granulocytic differentiation which is previously-unreported as proteins related to differentiation, and a possible role for Chk1 and Chk2 in the granulocytic differentiation on HL-60 cells. To summarize identified 10 proteins by proteomics, apigenin, which is hydrolyzed by glucosidase from apigetrin, inactivates hnRNP A2 by directly binding, and activates phosphorylate Chk1 and Chk2, which might lead to signal transduction toward G2/M cell cycle arrest through 14-3-3 proteins interacted with PCNA. PCNA coordinates multiple functions to bind many proteins especially EF-1β in protein synthesis according to differentiation. Besides RbAp48 is related transcriptional regulation implicated in granulocytic differentiation. Plastin-2, annexin A5 and coronin-1A are involved in granulocytes feature and support the induction of differentiation by apigetrin.

hnRNP A2, apigenin target protein has been reported its higher expression in several human cancers, including breast (Zhou *et al.*, 2001), and hnRNPA2 expression is recognized as a marker of glioblastoma and lung cancer (Golan-Gerstl *et al*., 2011, Sueoka *et al.*, 2005, Wu *et al*., 2003). This study does not only support the potential of Apigenin also for leukemia but also suggest hnRNP A2 as a marker of leukemia. Stem cell study, that hnRNP A2 regulates self-renewal and pluripotency in hESC, supports hnRNP A2 is key regulator of cell differentiation. Further study requires to evaluate the relationship between hnRNP A2 and leukemia differentiaion mechanism. Apigenin reported to have high binding affinity compared with another flavonoid (Arango *et al*,

2013), that lead to strong potential of apigenin for anti-carcinogenesis compared with another flavonoids.

In chapter 4, the author showed that olive leaves have proved their capacity for storage of phenolic compounds such as apigenin, as glucoside derivatives.

In fact, pruning generates 25 kg of leaves/ tree (Molina-Alcaide and Yáñez-Ruiz, 2008). In Tunisia, there is around 70 million trees which means potentially, this country can produce 1.750.000 ton of olive leaves. However, a lot of efforts are required in order to standardize olive leaves extracts, because several conditions: such as variety, growing area, season and plant part may significantly influence its chemical composition (Luján *et al*., 2009). However Damak *et al*. (2008) and Luján *et al*. (2009) reported the content of phenols in olive oil analyzed by LC/MS decreased over the season. As maturing progress olive fruits become darken and the oil content increases, at the same time the photosynthetic activity decreases and the concentration of phenols also decrease progressively. Olive is an evergreen tree, therefore leaves does not modify their chemical composition over a season as far as they are not affected by external factors compared with their oils. Taken together, results in this chapter also indicate the key role of leaves as stable source of phenol apigenin from olive trees.

This study supports the potential of leaves source for isolation of the target compounds on an industrial scale. Although this sector is strategic and promising for Tunisian economy, there are several challenges: (i) irregularity of yield due to traditional farming systems, (ii) the harvesting and pressing methods need to be improved (iii) quasi-absence of Tunisian olive oil brand in the international market. In this respect, olive tree farming and olive oil production by-products should be highly valorized in order to diversify olive sector products, contribute to upgrading the olive

oil sector. This study will address a specific attention to Tunisian olive leaves valorization with a focus on apigenin health benefits and supports the potential of leaves source for isolation of the target compounds on an industrial scale.

In conclusion, the author has demonstrated that plant flavone apigetrin not only induces apoptosis of different cancer cells but also has a potential to induce cell differentiation. This study will contribute not only in the findings of the differentiation inducing effect of apigetrin but also the development of apigetrin as a potential inducer of leukemia cell differentiation and a functional food by molecular mechanism analysis. Moreover the author has demonstrated that olive leaves prospect as low cost apigenin-rich byproducts as a functional material for the industrial application. The author expect that this findings may contribute to understand different approaches of chemopreventive properties of natural compounds against cancer disease and the usage of olive leaves as a stable source.

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Summary

Several epidemiologic studies have reported associations between high consumption of fruits and vegetables and reduced risk of cancer. However these findings are inconsistent and their mechanism of action on cellular and tissue levels are limited. The current study was conducted to investigate cancer prevention using cancer cell differentiation strategy. Herein, the author reports the effect of a common non-mutagenic flavonoid apigetrin on human acute promyelocytic leukemia using HL-60 cell model.

Apigenin is abundantly present in common fruits and vegetables, such as parsley, onions as well as in olive products (oil, fruits and leaves). Several studies reported that apigenin inhibits cancer cell growth via cell cycle arrest and apoptosis. This strategy may be clinically limited because of its cytotoxicity. Recently there is an increase in the usage of another mild approach such as cancer cell differentiation. However, few research studies have been reported about apigenin activity.

In this study, apigetrin inhibits cell growth dose- and time-dependently in HL-60 cells without cytotoxic effects. The cell cycle distribution of apigetrin treated-cells showed G_2/M phase arrest. Moreover, apigetrin treatment induced HL-60 cell morphological changes (big cell size and cells with lobed nucleus). These observations were confirmed by the expression of cell surface differentiation markers CD11b and CD14, and NBT reduction assay, revealing HL-60 cell granulocytic differentiation. The common view that the initiation of cell differentiation is most likely to occur at the G_0/G_1 phase arrest, suggests the potential role of apigetrin as a novel player of cellular signal transduction. To elucidate this molecular mechanism, the author

performed advanced molecular biology approach based on proteomics.

Proteomics results showed significant expression of ten proteins which were relevant to granulocytes function and signaling. Based on these results, the author found that apigetrin induced-signaling was regulated at post-translational level. The author demonstrated that apigetrin activity was triggered by Chk1 and Chk2 phosphorylation. This finding is in concordance with previous report about the key role of Chk1 and Chk2 in the granulocytic differentiation in HL-60 cells.

Since this study was designed for apigetrin as a functional food product. The author was prospecting low cost apigenin-rich byproducts. This study demonstrated that olive leaves could be the target. HPLC analysis of several Tunisian olive cultivars growing in different area demonstrated that olive leaves present high amounts of apigenin as glucoside form. This study supports the potential valorization of olive leaves at industrial scale.

In conclusion, this study will contribute not only in the findings of the differentiation inducing effect of apigetrin but also the development of apigetrin as a potential inducer of leukemia cell differentiation and a functional food by molecular mechanism analysis. The author expects that current findings may contribute to understand different approaches of chemopreventive properties of natural compounds against cancer disease and the usage of olive leaves as a stable source.

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