

1 *Rosmarinus officinalis* polyphenols activate cholinergic activities in PC12
2 cells through phosphorylation of ERK1/2

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6 **Abstract**

7 *Aim of the study:* This paper aimed to elucidate the traditional use of *Rosmarinus*
8 *officinalis* (*R. officinalis*) through the investigation of cholinergic activities and neuronal
9 differentiation in rat pheochromocytoma PC12 cells. These effects were examined in
10 relation to the plant's habitat, the extraction procedure, and the major active compounds
11 of *R. officinalis*.

12 *Materials and Methods:* Cell viability, cell differentiation, acetylcholinesterase (AChE)
13 activity, total choline, acetylcholine (ACh) and extracellular signal-regulated kinases
14 (ERK1/2) were determined in PC12 cells treated with extracts and HPLC-identified
15 polyphenols of *R. officinalis* originated from Tunisian semi-arid and subhumid area in
16 comparison with nerve growth factor (NGF).

17 *Results:* *R. officinalis* extracts potentiated cell differentiation and significantly enhanced
18 AChE activity in PC12 cells. The highest AChE activity was induced by semi-arid
19 hydro-ethanolic extract (137% of control). Among HPLC-identified and screened

1 polyphenols, carnosic acid (CA) and rosmarinic acid (RA) significantly induced cell
2 differentiation, increased ACh level, and enhanced AChE activity in PC12 cells. U0126,
3 inhibitor of ERK1/2, significantly reduced CA and RA effects on cell differentiation
4 and AChE activity.

5 *Conclusions:* *R. officinalis*' CA and RA exhibited neurotrophic effects in PC12 cells
6 through cell differentiation induction and cholinergic activities enhancement. These
7 effects could be regulated by mitogen-activated protein kinase (MAPK), ERK1/2
8 signaling pathway.

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11 **Key words**

12 *Rosmarinus officinalis*

13 Carnosic acid

14 Rosmarinic acid

15 PC12 cell

16 Cholinergic activities

17 ERK1/2

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17 **1. Introduction**

18 *R. officinalis*, member of Lamiaceae family, is an herbal spice native to

19 Mediterranean basin. It is an attractive, fragrant and evergreen shrub with pine needle-

20 like leaves. For long time, this plant was used in Mediterranean cuisine as dried leaves

1 not only to improve or modify the flavor of food, but also to avoid its deterioration
2 because of its antimicrobial and antioxidant activities (Fernandez et al., 2005).
3 Numerous studies reported that *R. officinalis* has several medicinal use such as anti-
4 inflammatory, anti-nociceptive (Takaki et al., 2008), anti-ulcerogenic (Dias et al., 2000),
5 hepatoprotective (Amin and Hamza, 2005), diuretic (Haloui et al., 2000),
6 neuroprotective and anti-aging (Adams et al., 2007). In folk medicine, this plant is
7 known for memory improving and treating cognitive decline. It is also used as sedative
8 and relaxant, against headaches, epilepsy, and depression (Heinrich et al., 2006). In
9 Tunisia, *R. officinalis* is growing wild in bioclimatic zones extending from the sub-
10 humid to the semi-arid covering wide area of forest land (Zaouali and Boussaid, 2007).
11 *R. officinalis* is one of the most collected, transformed and traded aromatic plant. It is
12 used by local population for its relaxing propriety, but its active compounds and its
13 molecular mechanism remain to be clarified.

14 It is well known that the cholinergic system is involved in the regulation of several
15 central nerve system (CNS) functions like cognition, memory, conscious arousal,
16 attention and subsequently regulation of mood impairments like depression and anxiety
17 (Dagyté et al., 2010). The modulation of cholinergic activities is presently the most
18 accepted and recognized therapeutic marker for development of cognitive enhancers
19 (Orhan et al., 2008) and to prevent from some lifestyle disease like stress and depression.

1 AChE is one of the most biological catalysts that play a key role in cholinergic
2 neurotransmission by rapidly hydrolyzing the neurotransmitter ACh at cholinergic
3 synapses and neuromuscular junctions (Taylor and Radic, 1994; Soreq and Seidman,
4 2001). Particularly in hippocampus, it plays an undoubted key role in regulation of
5 learning and memory (Das et al., 2005), and it was demonstrated to be affected by
6 stressful situations in animal model (Nijholt et al., 2004; Mark et al., 1996; Aloisi et al.,
7 1996). However, the biological role of AChE is not limited to cholinergic transmission.
8 It has been implicated in several non-cholinergic actions including cell proliferation,
9 neurite outgrowth (Mazzanti et al., 2009), cell adhesion, hematopoiesis and
10 tumorigenesis (Zhu et al., 2007).

11 PC12 cells are well known model for neuronal differentiation (Shibahara et al.,
12 1998; Isoda et al., 2002). When treated with NGF or NGF-like compounds, PC12 cells
13 cease proliferation and take a number of differentiated phenotypic properties of
14 sympathetic neurons, including neurite outgrowth and increase in AChE activity (Liu et
15 al., 2003) with correlated enhancement of ACh synthesis.

16 The use of NGF seems to be an interesting prevention alternative for cholinergic
17 impairments and its related neuronal diseases. However, their therapeutic use is limited
18 because they do not cross the blood-brain barrier. Epidemiological studies have shown

1 that antioxidant rich plants have several beneficial effects on neurological disease
2 (Blasina et al., 2009). In this sense, *R. officinalis* might be useful plant.

3 In order to elucidate the traditional knowledge of this plant, and as part of our
4 search for substances that have NGF-like effects. PC12 cells were treated with different
5 extracts of Tunisian *R. officinalis* originating from semi-arid and subhumid area to
6 investigate their effects on neurite outgrowth and AChE activity. The main active
7 compounds CA and RA were identified. In this study, CA and RA significantly
8 induced cell differentiation, enhanced cholinergic activities and activated ERK1/2 in
9 PC12 cells.

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11 **2. Materials and methods**

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13 *2.1. Sample preparation*

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15 *R. officinalis*, growing wild in Tunisia, was collected from Sammama-Mount
16 Kasserine (semi-arid area) and Zaghouan-Mount (subhumid area) during the period
17 April-May 2007. Leaves were collected from wild population, from 5 sites/field of 10
18 random fields of 100 m². Fresh leaves were crushed in a mortar and extracted with
19 different solvent 10 % (w/v). The extraction with water (WE) was carried out in

1 autoclave at 105°C for 15 min. 70% ethanol extract (EE) and 70% methanol extract
2 (ME) were prepared at 25°C for 1 week with shaking at least once a day, in the dark.
3 The liquid fraction was then collected and filtered through 0.22 µm filter. EEA, EES,
4 MEA, MES, WEA, and WES are respectively the EE, ME and WE from plants growing
5 in semi-arid area and sub-humid area.

6

7 2.2. Chemicals

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9 CA was purchased from Alexis biochemicals, RA and p-coumaric acid were from
10 MP Biomedicals LLC (Germany). Quercetin dehydrate, ferulic acid, phosphoric acid,
11 acetonitrile (HPLC grade) and acetylcholine iodide were from Wako (Japan). Caffeic
12 acid was from Extrasynthese Genay (France). NGF 7s, Radioimmunoprecipitation assay
13 (RIPA) buffer and p-ERK1/2 antibody were from Sigma Aldrich Co., Ltd. (St Louis,
14 USA). Dulbecco's modified Eagle medium (DMEM) was from Sigma Aldrich Co., Ltd.
15 (Irvine, UK). ERK1/2 antibody and goat anti-rabbit IgG-HRP were from Santa Cruz
16 Biotechnology Inc. (Santa Cruz, USA). U0126 was from Promega (USA). Goat anti-
17 mouse IgG-HRP was from Bethyl laboratories Inc. (Montgomery, USA). Fetal bovine
18 serum (FBS) was from Biowest SAS (France). Heat inactivated horse serum (HS) was
19 from Invitrogen (New Zealand). Penicillin-streptomycin was from Lonza Inc.

1 (Walkersville, MD, USA). MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-
2 diphenyltetrazolium bromide) was from (Dojindo, Japan).

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4 2.3. HPLC analysis of *R. officinalis*' extracts

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6 HPLC analysis were performed on a double plunger pump Gilson HPLC using a
7 Beta max Neutral column (150 x 4.6mm i.d. particle size 5 µm) (Tosoh Corporation,
8 Tokyo Japan) heated at 40°C. A binary phase acetonitrile 100% (solvent A) and
9 distilled water-0.1% H₃PO₄ (solvent B) was used as reported by Almela et al., (2006).
10 In these conditions, analytes were eluted from the column in increasing order of polarity.
11 With a flow rate of 3 ml/min, several linear gradients were assayed. *R. officinalis*'
12 extracts were injected at 100 µl. Peak spectra were recorded at 230 and 300 nm using
13 UV detector. Among the main compounds present in rosemary extracts, CA and RA
14 were quantified with regard to pure standards and reported in (mg/g) of fresh leaves.
15 Between the gradients tested, good separation was achieved starting with 23% A and
16 77% B for 8 min, followed by a gradient to 60% A and 40% B in 12 min, kept isocratic
17 for 5 min then 75% A and 25% B in 10 min. All steps of the gradient were linear.

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19 2.4. Cell culture

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2 PC12 cells (Riken, Japan) were maintained in DMEM supplemented with 5% FBS,
3 10% HS and 50 U/ml penicillin, and 50 µg/ml streptomycin in a humidified incubator at
4 37°C, 5% CO₂. For protein extraction and ACh quantification, cell passages were
5 carried out in poly-L-lysine dishes (Wako) using Trypsin 0.25% EDTA to detach cells.
6 For AChE activity and cell viability assay, cell passages were carried out in flasks and
7 cells were detached by pipetting. Prior to each experiment, cells were washed once with
8 medium. Cells used in this study were between passages 3 and 8. Cells treated with
9 NGF 7s at 50 ng/ml were used as positive control.

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11 *2.5. Assessment of cell viability*

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13 PC12 cells were cultured in 100 µl test medium at a density of 1.0×10^4 cell/well in
14 poly-L-lysine coated 96 well plate. After 24 h incubation, cells were treated with
15 different concentrations of sample for another 24 h and 48 h. Then, 100 µl of fresh
16 culture medium was added with 10% of MTT at 5 mg/ml in PBS (-). After overnight
17 incubation, the colored formazan was dissolved in 100 µl of 10% SDS and the
18 absorbance at 570 nm was determined using a multi-detection microplate reader

1 (Powerscan[®], HT, Dainippon Pharmaceutical, Japan). The viability of PC12 cells was
2 presented as percentage of control.

3

4 *2.6. Cell differentiation assay*

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6 PC12 cells were cultured in 100 μ l test medium at a density of 1.0×10^4 cell/well in
7 poly-L-lysine coated 96 well plate. After 24 h incubation, cells were treated with
8 different dilutions of EEA (1/20000, 1/10000, and 1/5000 (v/v)), CA (6.8 μ g/ml) and
9 RA (4.8 μ g/ml) for another 48 h. In case of CA and RA treatments, PC12 cells were
10 also pretreated with 10 μ M U0126 for 30 min.

11 The proportions of neurite-bearing cells and flat phenotype-like cells were counted
12 using a phase-contrast microscope equipped with DFC 290HD camera (Leica).
13 Synapses, longer than one diameter of the respective cell body, were counted as neuritis
14 and considered as differentiated. Cells with short synapses or with fusiform morphology
15 were considered as flat phenotype-like cells (Blasina et al., 2009). The mean value was
16 calculated from 3 random field observations from 3 independent experiments, and a
17 minimum of 100 to 120 cells per field were counted.

18

19 *2.7. Measurement of AChE activity*

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2 PC12 cells were seeded into 96-well plates as mentioned above. After 24 h
3 incubation, cells were treated with different dilutions of Rosemary extracts (1/20000,
4 1/10000 and 1/5000 (v/v)). CA, RA, ferulic acid, caffeic acid, p-coumaric acid and
5 quercetin were also added at required concentrations as indicated in figure legends.
6 AChE activity was conducted as reported in our previous study (Isoda et al., 2002).
7 After 24 h treatment, medium was removed and the cells carefully washed twice with
8 200 μ l of PBS (-). Then 20 μ l of 5.6 mM acetylcholine iodide and 180 μ l of buffer
9 solution (0.12 M NaCl, 0.2% TritonX-100, 1 mM EDTA, 50 mM Hepes, pH 7.5) were
10 added into each well. After 2 h incubation at room temperature, 20 μ l of the cell lysates
11 were transferred to a fluorescence reading multiwell plate and incubated for 1 h with
12 160 μ l buffer solution (1 mM EDTA, 0.2% TritonX-100, 50 mM acetate buffer, pH 5.0)
13 and 20 μ l of 0.4 mM 7-diethylamino-3(4-maleimidyl-phenyl)-4-methylcoumarin in
14 acetonitrile at room temperature. The fluorescence in each well was then measured
15 using a multi-detection microplate reader at 360 nm/ 460 nm and the activity was
16 reported as percentage of control.

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18 *2.8. Effect of U0126 on CA and RA activities*

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1 U0126 is a selective and potent inhibitor of MEK1 activity *in vitro*, as well as
2 activation of ERK1/2 by MEK1/2. It is capable of directly inhibiting activated MEK1
3 and preventing endogenously active MEK1/2 from phosphorylating and activating
4 ERK1/2 (Said et al., 1998). PC12 cells were pretreated with 10 μ M U0126 for 30 min
5 then washed with DMEM and treated with CA (2.4, 4.8 and 6.8 μ g/ml) and RA (2.4, 3.6,
6 and 4.8 μ g/ml) for AChE activity and cell differentiation observation. The effect of
7 U0126 on cell viability was carried out using MTT assay in preliminary experiments.
8

9 2.9. Measurement of ACh

10

11 PC12 cells were seeded at a density of 2.0×10^6 cells in 10 cm poly-L-Lysine coated
12 dish. After 24 h incubation, cells were treated with CA (6.8 μ g/ml) and RA (4.8 μ g/ml)
13 for another 24 h, then washed twice with 20 ml cold PBS(-). Total choline, free choline
14 and ACh were quantified by choline/Acetylcholine kit (Biovision, USA) according to
15 manufacturer's instructions. Briefly, cells were lysed in choline assay buffer on ice for
16 10 min and sonicated, the supernatant was collected after centrifugation at 600 x g for
17 30 min. In the assay free choline is oxidized to betaine, via the intermediate betaine
18 aldehyde. The reaction generates products which react with the choline Probe to
19 generate fluorescence (Ex/Em 535/587 nm). ACh can be converted to choline by adding

1 AChE enzyme to the reaction. Without adding the enzyme, the assay detects free
2 choline. The quantification was determined according to standard curves of choline with
3 and without adding AChE enzyme.

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5 *2.10. Western blotting*

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7 After treatment with CA, RA and NGF as indicated in figure legends, cells were
8 washed twice with cold PBS (-) and total protein was extracted using RIPA buffer
9 according to the manufacturer's instructions. 15 µg of extracted protein sample was
10 resolved by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-
11 PAGE) and blotted using Invitrogen IBlot system. The blots were probed with anti-
12 activated ERK1/2 monoclonal antibody (1:3500 dilution), anti-ERK1/2 monoclonal
13 antibody (1/200 dilution) using Snap Id system (Millipore). The signal was visualized
14 using Western blotting Chemiluminescent HRP Substrate (GE Healthcare) Amersham
15 ECL system, after reaction with labelled goat anti-mouse IgG antibody for activated
16 ERK1/2 (1:4000 dilution) and goat anti-rabbit IgG for activated ERK1/2 (1:4000
17 dilution).

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19 *2.11. Statistical analysis*

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2 The results were expressed as mean \pm standard deviation (S.D.). Differences
3 between treatments were assessed by T test at p-values<0.05 versus control (vs. Ctrl.),
4 and the least significance difference (LSD) test at 95% using Statgraphics® package for
5 windows.

6

7 **3. Results**

8

9 *3.1. Effect of R. officinalis' extracts on PC12 cells differentiation and AChE activity*

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11 In preliminary experiments using MTT assay, no cytotoxic effect of *R. officinalis*
12 extract dilutions (1/20000, 1/10000, and 1/5000 (v/v)) was detected in PC12 cells (data
13 not shown).

14 *R. officinalis* extracts were screened for their effect on cell morphology and AChE
15 activity in PC12 cells. Among these extracts, EEA showed a clear morphological
16 change in PC12 cells (Fig. 1A). At 1/5000 (v/v) fold dilution, 12% of cells were
17 differentiated and 50% changed to flat phenotype-like cells (Fig. 1B).

18 AChE activity was carried out after 24 h incubation. All extracts displayed a
19 significant dose dependent increase in AChE activity (p<0.05) except for EES (Fig. 1C

1 and Fig. 1D). Extracts from Semi-arid plants were more effective in increasing AChE
2 activity than those from plants growing in sub-humid area. The highest AChE activity
3 137% was found after treatment with EEA at 1/5000 (v/v) (Fig. 1C). While, extracts
4 from *R. officinalis* growing in sub-humid area showed low AChE activity (Fig.1D).

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6 3.2. Quantification of CA and RA compounds in *R. officinalis* extracts

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8 It is recognized that the concentration of active compounds is dependent on plants
9 habitat (Munné-Bosch et al., 2000), the processing methods and solvents used for
10 extraction (Yu et al., 2005). We demonstrated that the highest concentrations of CA and
11 RA were found in the extracts of plant leaves growing in semi-arid area extracted using
12 ethanol and methanol solvent (Table 1). EEA presented the highest amount of CA
13 (13.29 mg/g of fresh leaves) and RA (39.9 mg/g of fresh leaves). Concerning, subhumid
14 area plant extracts, MES presented the highest concentration of CA (10.67 mg/g of fresh
15 leaves) and RA (18.1 mg/g of fresh leaves). Also, we noticed that CA, a water insoluble
16 compound, was absent in water extracts from both growing sites as reported by Kosaka
17 and Yokoi (2003). Other compounds like ferulic acid, caffeic acid, p-coumaric acid, and
18 quercetin were detected (data not shown).

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1 3.3. *Effect of U0126 on morphological differentiation and AChE activity induced by CA*
2 *and RA in PC12 cells*

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4 Treatment of PC12 cells with NGF triggers their differentiation into cells that
5 resemble sympathetic neurons, which are characterized by neurite outgrowth and
6 enhancement of AChE activity (Liu et al., 2003). Neurite-like process outgrowth was
7 estimated after 48 h treatment with CA and RA. CA significantly promoted
8 morphological change of PC12 cells compared to RA. As shown in Fig. 2A and Fig. 2C,
9 16% of CA treated-PC12 cells were differentiated and 55% changed to flat phenotype-
10 like cells. These proportions were 5% differentiated cells and 25% flat phenotype-like
11 cells in RA treated-cells. The neurite-like process extension response was more robust
12 in NGF-treated cells (27% differentiated cells, and 68% flat phenotype-like cells).

13 Pretreatment of PC12 cells with U0126 resulted in significant change in cell
14 morphology. U0126 treatment prevented CA, and RA effect on PC12 cells
15 differentiation. However, the proportion of flat phenotype-like cells was only reduced
16 by 5% for CA, 10% for RA, and by 10% for NGF. This effect was more severe for RA
17 treated cells (Fig. 2B and Fig. 2C).

18 After 24 h treatment with CA and RA, both compounds dose dependently increased
19 AChE activity to 130% for CA (Fig. 2D) and 129% for RA (Fig. 2E). Pretreatment with

1 U0126 totally inhibited CA (2.4 $\mu\text{g/ml}$, 4.8 $\mu\text{g/ml}$) (Fig. 2D) and RA (2.4 $\mu\text{g/ml}$, 3.6
2 $\mu\text{g/ml}$) (Fig. 2E) induced AChE activity. However, U0126 pretreatment decreased CA
3 (6.8 $\mu\text{g/ml}$) and RA (4.8 $\mu\text{g/ml}$) AChE activity to 121 % and 115%, respectively (Fig.
4 2D and Fig. 2E).

5

6 *3.4. Effect of CA and RA on ACh release.*

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8 ACh is a neurotransmitter synthesized in PC12 cells by choline acetyltransferase
9 (ChAT) from the precursors choline and acetyl-CoA (Pedersen et al., 1997), and
10 degraded by AChE in synapses cleft. As shown in Fig. 3, after 24 h treatment, CA and
11 RA significantly increased total choline to 7.14 nmole/ml and 6.8 nmole/ml. Also, they
12 significantly enhanced ACh level to 2.5 nmole/ml and 2.8 nmole/ml, respectively.
13 Meanwhile, NGF-treated cells showed same level of total choline and no significant
14 effect on ACh level compared to CA and RA-treated cells.

15

16 *3.5. CA and RA induce PC12 cells differentiation and enhance AChE through ERK1/2*

17

18 The MAPK/ERK is a signal transduction pathway that couples intracellular
19 responses to the binding of growth factors to cell surface receptors (Kawano et al.,

1 2007). Activation of ERK1/2 plays an important role in neuronal differentiation, cell
2 survival and maintenance of distinct populations of neurons (Liu et al., 2003).

3 Western blotting results shows that both CA and RA significantly activated ERK1/2
4 (Fig. 4A). However, CA was more effective than RA to induce ERK1/2
5 phosphorylation. Treatment with U0126 inhibited CA and RA-induced ERK1/2
6 phosphorylation (Fig. 4B), and significantly reduced their correspondent AChE activity
7 and cell differentiation, suggesting that such activities could be related to ERK1/2
8 pathway.

9

10 **4. Discussion**

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12 Here, we demonstrated that Tunisian *R. officinalis* extracts potentiated PC12 cells
13 differentiation and significantly increased AChE activity. Such effects were strongly
14 related to the plant habitat (Wellwood and Coler, 2006) and the extraction method.
15 Extracts of plants growing in semi-arid area have shown higher activities than sub-
16 humid area. These neurotrophic capacities of *R. officinalis* could be related to the
17 presence of pro-electrophilic compounds reported to be good candidate for treating
18 neurodegenerative disease (Sato et al., 2008). HPLC results supported this finding,

1 showing that *R. officinalis* growing in semi-arid environment present higher amounts of
2 CA and RA.

3 *R. officinalis* and its main polyphenols have been the topic of many studies related
4 to neurotrophic activity, neurobehavioral and neurodegenerative disease. In fact, CA has
5 been reported to promote synthesis of NGF in T98G human glioblastoma cells (Kosaka
6 and Yokoi, 2003). As well, RA has been demonstrated to have antidepressive effects in
7 the forced swimming test in mice (Takeda et al., 2002). More recently, it has been
8 shown that *R. officinalis* produces a specific antidepressant-like effect in animal
9 experiment (Machado et al., 2009).

10 CA and RA showed lower AChE activity in comparison with EEA, suggesting a
11 possible synergetic activity between these 2 compounds or with other constituents of
12 rosemary leaves. For this reason, we explored AChE activity of some other polyphenols
13 present in *R. officinalis* extracts detected by HPLC. We have noticed that p-coumaric
14 acid enhanced AChE activity to 117% of control at 30 μ M, whereas ferulic acid,
15 quercetin and caffeic acid exhibited an AChE activity compared to the control level (Fig.
16 5).

17 To the best of our knowledge, the study reported here could be the first to show that
18 *R. officinalis*' CA and RA induced PC12 cells differentiation, improved total choline
19 level and ACh synthesis in PC12 cells. These compounds may have NGF-like

1 neurotrophic effects in PC12 cell. In CNS, CA and RA may support the survival of
2 existing neurons, and encourage the growth and differentiation of new neurons and
3 synapses (Martin et al., 2010). By improving cholinergic activities, *R. officinalis*' CA
4 and RA may attenuate cholinergic neurons atrophy resulting in an enhancement of
5 memory, attention and impaired behavior like depression and anxiety (Daghypté et al.,
6 2010).

7 PC12 cells differentiation and AChE activity have been previously reported to
8 correlate with a sustained activation of ERK1/2 (Liu et al., 2003; Liu et al., 2006). Our
9 results show that exposure of PC12 cells to CA and RA has activated ERK1/2. Priming
10 with U0126 prevented PC12 cells differentiation and suppressed AChE activity at low
11 concentrations of CA and RA. However, at high concentrations, AChE activity was
12 reduced but not suppressed. Our results correlate with previous finding suggesting that
13 CA and RA could upregulate AChE activity regardless of ERK1/2 MAPK (Madziar et
14 al., 2005). Meanwhile, other pathways like PI3K/Akt (Jiang et al., 2007), protein kinase
15 C (PKC) (Birikh et al., 2002), Nrf2 (Kosaka et al., 2009) may be involved in such
16 signaling. Moreover, PC12 cells have been shown to synthesize nuclear and
17 cytoplasmic isoforms of AChE (Santos et al., 2007). In fact this enzyme is expressed in
18 several molecular forms, with different structural features but identical catalytic sites
19 (Schweitzer, 1993). The exploration of specific pathways and molecular mechanisms of

1 CA and RA toward neuronal differentiation and cholinergic functions are the future
2 targets of our research.

3 In conclusion, our data indicate that treatment of PC12 cells with Tunisian *R.*
4 *officinalis* extracts significantly increased AChE activity and potentiated cell
5 differentiation to flat phenotype-like cells. These effects were dependent from plant
6 environment and solvent used for extraction. This work emphasize the use of solvent
7 extracts of *R. officinalis* in industrial scale to upgrade the valorization of this plant
8 commonly used as dried leaves, water infusion tea or essential oil. Moreover, we
9 demonstrated that the neurotrophic activity of *R. officinalis* is correlated to the presence of CA
10 and RA. Both compounds reproduced AChE activity of the plant extract. CA and RA-induced
11 cholinergic activities in PC12 cells were nearly similar and partially dependent on
12 ERK1/2 signaling pathway.

13

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16

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14

LIST OF FIGURE AND TABLE

15 **Table1.** Amount of CA and RA in *R. officinalis* extracts in mg/g of fresh leaves. Results
16 are the mean \pm S.D. of 3 independent trials.

17

18 **Fig. 1.** Effect of *R. officinalis* ethanol extract (EEA) on PC12 cells differentiation and
19 AChE activity. (A) Shows photo taken at 200 x magnification, of the effect of *R.*

1 *officinalis* EEA in PC12 cells after 48 h incubation, in comparison with control, scale
2 bar represents 50 μm . (B) shows the effect of EEA on the proportion of differentiated
3 cells (Diff. cells), and flat phenotype-like cells as shown in Material and methods.
4 Results are mean \pm S.D. of 3 independent trials (n = 9). (C) Shows AChE activity of *R.*
5 *officinalis* extracts collected from Sammama (Semi-arid area) (D) Shows AChE activity
6 of *R. officinalis* extracts collected from Zaghouan (Subhumid area). AChE activity was
7 determined as described in Materials and methods. Results are mean \pm S.D. of 3
8 independent trials (n =12) (* $p < 0.05$ vs. Ctrl.).

9

10 **Fig. 2.** Effect of CA, RA and NGF on PC12 cells differentiation and AChE activity with
11 and without U0126 pretreatment. (A) Shows the photo at (200 x) magnification of
12 PC12 cells treated with CA at 6.8 $\mu\text{g/ml}$, RA at 4.8 $\mu\text{g/ml}$, and NGF at 50 ng/ml after 48
13 h treatment. The scale bar represents 50 μm . (B) shows the photo at (200 x)
14 magnification of PC12 cells pretreated with 10 μM of U0126 for 30 min then treated
15 with CA at 6.8 $\mu\text{g/ml}$, RA at 4.8 $\mu\text{g/ml}$, and NGF at 50 ng/ml for 48 h. The scale bar
16 represents 50 μm . (C) shows the effect of CA at 6.8 $\mu\text{g/ml}$, RA at 4.8 $\mu\text{g/ml}$, and NGF
17 at 50 ng/ml after 48 h treatment on the proportion of differentiated cells (Diff. cells) and
18 flat phenotype-like cells as shown in Material and methods. Results are mean \pm S.D. of
19 3 independent trials (n = 9) (* $P < 0.05$ vs. Ctrl.). (D) shows AChE activity of CA (2.4,

1 4.8, and 6.8 $\mu\text{g/ml}$) in PC12 cells after 24 h treatment with or without 10 μM U0126
2 pretreatment for 30 min. (E) shows AChE activity of RA (2.4, 3.6, and 4.8 $\mu\text{g/ml}$) on
3 PC12 cells after 24 h with and without 10 μM U0126 pretreatment for 30 min. 50 ng/ml
4 of 7s NGF was used as a positive control. AChE activity was determined as percentage
5 of control as described in Materials and methods. Control PC12 cells were grown in test
6 medium. AChE activity was determined as described in Materials and methods. Results
7 are mean \pm S.D. of 3 independent trials (n =12) (* p < 0.05 vs. Ctrl.).

8

9 **Fig. 3.** Effect of CA and RA treatment on total choline and ACh in PC12 cells. Cells
10 were treated with CA at 6.8 $\mu\text{g/ml}$, RA at 4.8 $\mu\text{g/ml}$ and NGF at 50 ng/ml for 24 h.
11 Total choline was quantified according to a standard curve of choline with and without
12 AChE addition. ACh was determined by subtraction of free choline from total choline.
13 Results are mean \pm S.D. of 3 independent trials, (($*p$ < 0.05 vs. Ctrl.), # Least significant
14 difference (LSD at 95%) between treatment).

15

16 **Fig. 4.** Effect of CA and RA on ERK1/2 phosphorylation in PC12 cells. Cells were
17 seeded at a density of 2.0×10^6 cells in 10 cm poly-L- lysine coated dish. Cells were
18 treated with CA at 4.8 $\mu\text{g/ml}$ and 6.8 $\mu\text{g/ml}$, RA at 2.4 $\mu\text{g/ml}$ and 3.6 $\mu\text{g/ml}$, and NGF at

1 50 ng/ml. (A) Without U0126 pretreatment. (B) With 10 μ M U0126 pretreatment.

2 Results are representative of 3 independent trials.

3

4 **Fig. 5.** Effect of some *R. officinalis*' polyphenols on AChE activity in PC12 cells. Cells

5 were treated with ferulic acid, caffeic acid, and p-coumaric acid at 20, 30, and 50 μ M

6 and quercetin at 1, 5, and 10 μ M for 24 h. AChE activity was determined as described in

7 Materials and methods. Results are mean \pm S.D. of 3 independent trials (n =12) (* p <

8 0.05 vs. Ctrl.)

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