沙漠研究 **19**-1, 117- 120 (2009) "Desert Technology IX "
Journal of Arid Land Studies Refereed Paper

Anti-Neuronal Stress Effect of Tunisian *Rosmarinus officinalis* Extract

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Abstract: The present study is aimed to the effect of a Tunisian aromatic plant *Rosmarinus officinalis* extract on - a neuronal cell model - PC12 cells and on HSP47. The extract of *R. officinalis* and two pure compounds rosmarinic acid and carnosic acid promote markedly the neurite outgrowth of PC12 cells and enhanced the acetylcholinesterase activity same as NGF (neurite outgrowth factor). The investigation of phosphorylation marker illustrates that Rosemary extract treatment showed ERK1/2 phosphorylation same as NGF. Moreover, *Rosmarinus* extracts and its pure compounds have shown a stress recovery activity on HSP47.

Keywords: Acetylcholine, Neurite outgrowth, *Rosmarinus officinalis*, Stress recovery

1. Introduction

Stress is a widely prevalent phenomenon that exacts a heavy toll on the quality of human life. During times of stress, the body responds by releasing hormones that trigger physical changes. Chronic stress can have negative effects on our well being, leading to many physiological changes. The effects of stress are supposed to be an outcome of altered activity of different mechanism such as central neurotransmitters, neurohormonal factors, and free radical generation (Herman *et al*., 1997). There are some studies linking stress with cholinergic activity. Moreover, motor neurons have a high threshold for induction of heat shock proteins (Hsps) in response to stress. Numerous plants have been used to treat cognitive disorders, over 150 plant species in various preparations and mixtures were cited by (Das *et al*., 2007) as relevant to dementia, Alzheimer disease and cognitive aging disease. The present study is aimed to the effect of a Tunisian aromatic plant "*R. officinalis*" extracts on neuronal stress and its mechanism using PC12 cells and Heat shock protein HSP47 as a model.

2. Materials and Methods

2.1. Sample preparation and HPLC analysis

Rosemary was collected from Tunisia, from Sammama-Mount kasserine and Zaghouan-Mount during the period April-May 2007 corresponding to the flowering season of this plant. Fresh leaves were crushed in a mortar and extracted with different solvent 10% w/v. The extraction with water was carried out in autoclave at 105 ˚C for 15 min. For ethanol (70%) and methanol (70%), the extraction was done in dark in an Erlenmeyer at 25 ˚C, for 1 week, with shaking at least one time a day. HPLC analysis were done by Gilson apparatus using a binary acetonitrile/water gradient, Column: Beta max Neutral (150 × 4.6 mm id; particle size 5 μm), a flow rate of 3 ml/min and detection was recorded using UV detector at 300 and 230 nm.

2.2. Acetylcholinesterase activity

The rat pheochromocytoma PC12 cell line was routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 5% heat inactivated fetal bovine serum, 10% heat inactivated horse serum (Gibco®, Invitrogen), and 1% streptomycin (5000 I.U. ml^{-1})-penicillin (5000 μg ml^{-1}) solution in tissue culture flasks and incubated at 37 ˚C in a 95% air-5% CO_2 incubator. The cells were plated onto 96-well plates at initial concentrations of 1×10^4 cells per well in 100 μL of culture medium, incubated and allowed to attach for at least 6 h before the different extract of Rosemary were added to obtain the required final concentrations. After incubating for 24 hr, the medium was carefully removed and discarded and the cells carefully washed twice with 200 μl of PBS(-). After washing, 20 μl of 5.6

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mM acetylcholine iodide and 180 µl of lysis solution, pH 7.5 (0.12 M NaCl, 0.2% TritonX-100, 1 mM EDTA, 50 mM Hepes) were added into each well. After incubating for 2 h in room temperature, 20 µl of the cell lysates were transferred to a fluorescence reading multiwell plate and incubated for 1 h with 160 µl buffer solution, pH 5.0 (1 mM EDTA, 0.2% TritonX-100, 50 mM acetate buffer) and 20 µl of 0.4 mM 7-diethylamino-3-(4-maleimidyl-phenyl)- 4-methylcoumarin in acetonitrile. The fluorescence in each well was then measured using a multi-detection microplate reader (Powerscan® HT, Dainippon Pharmaceutical, Japan) at 360 nm/ 460 nm.

2.3. Stress recovery activity

The Chinese hamster ovary (CHO) cells with (HSP(+)) or without (HSP(-)) a heat shock protein promoter were used for this experiment. Cells were grown as adherent monolayer in 75-cm^2 tissue culture flasks using F12 Medium (Gibco®, Invitrogen, Tokyo, Japan) supplemented with 10% Fetal Bovine Serum, 200 µg/mL of G418 (Gibco BRL 13075-015) and 0.1 g/L kanamycin solution (Sigma). HSP47-transformed cells were trypsinized and plated onto 96-well plates at initial concentrations of 1×10^4 cells per well in 100 µL of culture medium. The cells were allowed to attach for 48h at 37 ˚C supplemented with 5% CO_2, shocked for 90 min at 42 ˚C, 5% CO_2 and recovered at 37 ˚C for 2 h in a 5% CO_2 incubator. Then, the medium was removed and 100 µL of samples diluted with medium followed by incubation for 3 h in a 5% CO_2 incubator at 37 ˚C. The medium was then carefully removed and the cells washed twice with PBS(-). 50 µl of lysis buffer (Promega) was then added and the plates incubated for 30 min at room temperature. 20 µl of cell lysates were transferred to a new plate, to which 100 µL of substrate solution (10 mM NaH_2PO_4•$2H_2O$, 100 mM NaCl, 1% BSA, 0.005% NaN_3, 1 mM $MgCl_2$•$6H_2O$, 1% 4-methylumbelliferyl-*β*-D-glactoside (MUG), pH 7) was added in order to trigger the conversion of MUG into galactose and methylumbelliferyl by galactosidase. After allowing the reaction to occur in the dark for 30 min at Room temperature, 60 µl of reaction stop buffer (1 M glycine-NaOH, pH 10.3) was added and the fluorescence at 365 nm excitation/ 450 nm emission was then determined using a multi-detection microplate reader (Powerscan® HT, Dainippon Pharmaceutical, Japan).

3. Results and Discussion

3.1. Tunisian *Rosmarinus officinalis*

R. officinalis (**Fig. 1**) is a woody shrub with a pine needle like leaves. Its trusses of blue flowers last through spring and summer. It will grow to a height of 1.5 m. In Tunisia, *R. officinalis* is growing wild in bioclimatic zones extending from the sub-humid to the arid, with a rain fall level of 200 to 600 mm/year (Rejeb *et al*., 2006) on sandy, calcareous or marno-calcareous soils.

Main populations developed in garrigues resulting from the degradation of *Pinus halepensis* L., *Juniperus phoenicea* L. and *Tetraclinis articulata* L., (Zaouali and Boussaid, 2007). In Tunisia, this plant covers an area of about 340000 ha (Rejeb *et al*., 2006). Leaves of this plant have been used for a long time in Mediterranean cuisine, not only to improve or modify the flavor of foods, but also to avoid its deterioration. Also this plant is a symbol of remembrance and has been used since many centuries to improve memory. The infusion of leaves is used as antispasmodic, antiseptic, diuretic and relaxing. Dried leaves are used as additives to prevent fat oxidation and as flavoring agent in some meal. In the present study, we identified by HPLC analysis from different solvent extract of *R. Officinalis* leaves about 8 compounds: Ferulic acid, Caffeic acid, *p*-Coumaric acid, Rosmarinic acid, Apigenin, Luteolin, Carnosol and Carnosic acid. It was reported by (Kosaka and Yokoi, 2003) that Carnosic acid (**Fig. 2a**), was able to promote markedly the synthesis of NGF (nerve growth factor) in T98 glioblastoma cells. NGF is a factor vital for the growth and functional maintenance of nerve tissue growth factor. Furthermore, Rosmarinic acid (**Fig. 2b**) and Caffeic acid may produce antidepressive-like effect in the forced swimming test in mice (Takeda *et al*., 2002) i.e. that these compounds have a positive effect on memory and have relaxant effect.

3.2. Acetylcholinesterase activity

AChE is a glycoprotein, responsible of the breakdown of acetylcholine (ACh) (**Fig. 3**). In fact, Ach is cleaved by AChE into choline and acetate. The choline group was quantified at 360 nm excitation and 460 nm emission by adding MDCC (7-diethyl-3(4-maleimidyl-phenyl)-4-methylcoumarin

Fig. 1. ***Rosmarinus officinalis***

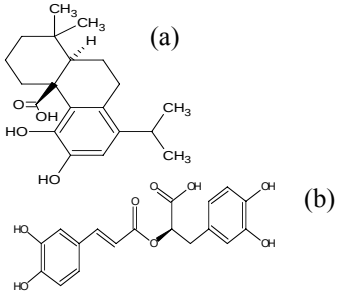

Fig. 2. Chemical structure of carnosic acid (a) and rosmarinic acid (b)

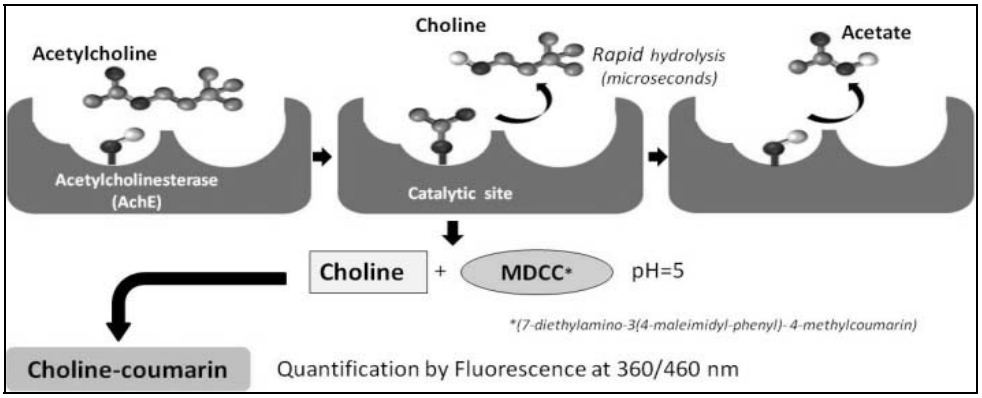

Fig. 3. Fluorescence detection of AchE activity using PC12 cells

after cell lysis. And AChE activity was reported as percentage of control. *R. officinalis* solvent extract enhanced AChE activity same as NGF. Also the microscopic observation of cells treated with Rosemary extract, has shown that this plant promote markedly neurite outgrowth and cell proliferation. In this study, AChE was increased i.e. that the amount of ACh also was improved. Aloisi *et al*. (1996) reported that in animal experimental model, the synthesis and release of ACh in hippocampus is correlated to stress and environment changes. Hippocampal cholinergic system is implicated in several functions such as arousal and attention, in certain aspects of learning and memory and in the neuroendocrine and emotional responsiveness to aversive stimuli. This fact, show clearly that Tunisian *R. officinalis* has a relaxing index effect and can be recommended for memory improvement. Such effect can be due to the presence of some polyphenolic and terpenic compound such as Rosmarinic acid and carnosic acid.

3.3. Molecular mechanism of regulation of AChE

In the present study, mechanism of promoting action of *R. officinalis* extract of PC12 cells differentiation and acetylcholinesterase activity seems to be regulated by MAPK: Mitogen-activated protein (MAP) kinases ERK1/2 that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis.

3.4. Stress recovery activity of *Rosmarinus officinalis*

We evaluate this activity using a transformed cell model: transfected Chinese Hamster cell Ovary (CHO) with HSP 47 (Isoda *et al*., 2003). Heat shock protein HSP 47 is a 47 kDa collagen specific molecular chaperone localized in the endoplasmic reticulum that plays a critical role during the folding, maturation, and secretion of procollagen (Higuchi *et al*., 2006).

As HSP47 is a heat shock (stress) protein, its expression is dramatically enhanced after the cells are treated at high temperature (42-45 ˚C) for short periods (about 30 min). Nuclear run-on assay demonstrated that a more than ten-fold increase in the transcription rate of HSP47 gene occurred after 2 h of the heat treatment of chick embryo fibroblasts at 45 ˚C, which rapidly returned to the basal level during

the recovery period at 37 ˚C (Nagata, 1998). In this case heat stress at 42 ˚C lasts 90 min, the recovery for 2 h at 37 ˚C, and the treatment time took 3 h. Then, the expression of the heat shocked protein was measured according to the amount of *β*-galactosidase that cleave MUG to methylumbelliferyl and galactose that can be measured by fluorescence at 360 nm emission and 460 nm excitation. Rosemary extracts have an interesting stress recovery activity at some specific concentration.

4. Conclusions

In this study we report that Tunisian *R. officinalis* extracts have an index of relaxing effect, through their high antioxidant activity, their capacity of enhancing neurite outgrowth and cell differentiation of PC12 cells and their aptitude of regulation of cholinergic system. The mechanism of PC12 cells differentiation and AChE activity are regulated by MAPK: ERK1/2. In addition, these extracts have a recovery effect on HSP47. These findings, suggest that this plant has a neuroprotective effect and can be used to prevent from some neurodegenerative disease and stress. The promising future treatment of neurodegenerative diseases and aging depends on availability of effective brain permeable, ironchelatable/ radical scavenger neuroprotective drugs that would prevent the progression of neurodegeneration. Further studies are required to test out its outcome on the protection from neurological diseases of a wide array of neuronal cell and animal models.

Acknowledgement

This study was supported in part by funds from the Grant-in-Aid for Scientific Research (Grant No. 17255011).

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