

Down regulation effect of Rosmarinus officinalis polyphenols on cellular stress proteins in rat pheochromocytoma PC12 cells

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1 **Abstract**

2 Polyphenols are known to exhibit wide spectrum of benefit for brain health and to protect from
3 several neurodegenerative diseases. The present study was sought to determine the
4 neuroprotective effects of *Rosmarinus officinalis*' polyphenols (luteolin, carnosic acid, and
5 rosmarinic acid) through the investigation of stress-related proteins. We carried out measurement
6 of the expression of heat-shock protein (Hsp) 47 promoter in heat stressed Chinese hamster
7 ovary transfected cells. We performed proteomic and confirmed gene expression by real time
8 PCR analysis in PC12 cells. Results showed that these compounds modulated significant and
9 different effects on the expression of 4 stress-related proteins: heat shock protein 90 α (*Hsp90*),
10 Transitional endoplasmic reticulum ATPase (*VCP/p97*), Nucleoside diphosphate kinase (*NDK*),
11 and Hypoxia up-regulated protein 1 (*HYOUI*)) at translational and post translational levels in
12 PC12 cells and they downregulated the expression of *Hsp47* activity in Chinese hamster
13 transformed cells. These findings suggest that luteolin, carnosic acid, and rosmarinic acid may
14 modulate the neuroprotective defense system against cellular stress insults and increase neuro-
15 thermotolerance.

16 Key words

17 *Rosmarinus officinalis*

18 *Hsp90 α*

19 *VCP/p97*

20 *NDK*

21 *HYOUI*

22 *Hsp47*

23

24

1 **INTRODUCTION**

2 Neurodegenerative disease manifests in elderly people and most commonly in developed
3 countries where life span is long. However, the World Health Organization has recognized it as a
4 global problem since it is the fourth most common source of death. Neurodegenerative diseases
5 are expected to impose severe impact on our society emotionally, socially and financially by the
6 next coming decades (Park et al. 2010). In this respect understanding, the pathogenesis
7 mechanism and finding potential therapeutic targets for such diseases is becoming a focus point
8 for the scientific community.

9 In neuronal cells, heat shock proteins (Hsps), provide a fundamental mechanism to
10 defend the cell against external diverse physiological stress (Luo et al. 2007). Induced by several
11 stressors like temperature, hypoxia, inflammation, infections and environmental pollutants, stress
12 proteins (Hsps) play key roles in living systems (Taguchi et al. 2007). It was postulated by
13 several studies that Hsps are working as chaperones together with ubiquitin-proteasome system
14 (UPS) to assist folding/refolding of nonnative protein, to help in the degradation of irreversibly
15 damaged proteins, and other proteins essential for the protection and recovery from cell damages
16 associated with perturbation of protein homeostasis. In neuronal cells, Hsps may have anti-
17 apoptotic effects and keep the homeostasis against stress conditions (Chen and Brown 2007; Luo
18 et al. 2007; Winklhofer et al. 2008; Oza et al. 2008). Recently, Hsps became a research
19 therapeutic target in neurodegenerative disorder and aging because the pathogenesis mechanism
20 of these diseases is thought to be related to an abnormal increase of Unfolded Protein Response
21 (UPR), failure of UPS and protein misfolding and/or aggregation (Zhao et al. 2010).

22 Numerous studies in the last decade have shown that dietary polyphenols may have, *in*
23 *vitro* and *in vivo*, a neurorescue impact in aging and neurodegenerative diseases to retard or even
24 reverse the accelerated rate of neuronal degeneration (Rasmassy 2006, Sun et al. 2010, Rajeswari

1 and Sabesan 201; Ortega 2006; Spencer 2009) and aging (Queen and ; Wilson et al. 2006).
2 However, little is done about their effect on Hsps in relation with neurodegenerative disease.

3 *R. officinalis* is traditionally used to improve memory, in connection with AD and
4 dementia, for general symptoms of old age, debility and fatigue (El Omri et al. 2010). Recently
5 several studies showed that *R. officinalis* or its main compounds like carnosic acid (CA),
6 rosmarinic acid (RA) and luteolin (Lut) (Kosaka et al. 2010, El Omri et al. 2010 and Lin et al.
7 2010) can be good candidates to substitute nerve growth factor (NGF). Moreover, this plant and
8 its main active compounds have been reported to be anti Alzheimer's disease AD (Liu et al.
9 2010, Lin et al. 2010 and El omri et al. 2010) anti Parkinson's disease (PD) (Park et al. 2010)
10 and anti amyotrophic lateral sclerosis (ALS) (Shimojo et al. 2009).

11 This study is the first conducted to determine the effects of *R.officinalis*' polyphenols: Lut, CA,
12 and RA on stress-related proteins expression in PC12 cells and to confirm their expression using
13 RT-PCR.

14

15 **MATERIALS AND METHOD**

16

17 **Chemicals**

18

19 The following reagents were purchased from several manufacturers and were used to prepare the
20 culture medium and the required solutions: Dulbecco's modified Eagle medium (DMEM) was
21 purchased from Sigma Aldrich (United kingdom), fetal bovine serum (FBS) was purchased from
22 Bio west (France), horse serum (HS) and Geneticin (G418) were purchased from Invitrogen
23 (Carlsbad, CA, USA), F12 Medium was from Invitrogen (Tokyo, Japan). Penicillin-streptomycin
24 was purchased from Lonza,Walkersville Inc., (MD, USA), DTT and TEMED were purchased

1 from Amersham Bioscience (Sweden). Luteolin, carnosic acid, NGF 7s, Trizma base, kanamycin
2 solution, trypsin (ethylenediaminetetra-acetic acid [EDTA]), $MgCl_2 \cdot 6H_2O$, 4-methylumbelliferyl-
3 β -galactose (MUG), protease inhibitor cocktail, and Ribonuclease A, all were purchased from
4 Sigma-Aldrich (USA). Rosmarinic acid, and $Na_2HPO_4 \cdot 7H_2O$ were purchased from MP
5 Biomedicals LLC (France). Spemine base and acetonitrile were purchased from Sigma-Aldrich
6 (Germany). Thiourea, APS and protein rainbow marker were purchased from GE Healthcare
7 (United Kingdom), urea, acrylamide, Bis, Bromophenol blue, CHAPS, CBB G-250, Glycol,
8 glycine, iodoacetamide, SDS, Tris, urea, IPG buffer and IPG strips were purchased from GE
9 Healthcare (Sweden). Lysis buffer was from (Promega). NaCl, KH_2PO_4 , KCl, $NaH_2PO_4 \cdot 2H_2O$,
10 bovine serum albumin, NaN_3 , dimethyl sulfoxide, glycine, NaOH, and Deoxyribonuclease
11 DNase A were purchased from Wako (Japan).

12

13 Cell culture

14

15 Chinese hamster ovary (CHO) cells stably transfected with (+) or without (-) an Hsp 47 promoter
16 were used for this experiment (Isoda et al. 2004). The cells were provided by S. Yokota
17 (Kaneka), and were grown as adherent monolayer in 75 cm² tissue culture flasks using F12
18 Medium supplemented with 10% Fetal Bovine Serum, 200 μ g/mL of G418 (Gibco BRL 13075-
19 015) and 0.1 g/L kanamycin solution. The cultures were maintained in a 5% CO₂ incubator at 37
20 °C. Cell passage was carried out at 80% confluence at 1:2 ratio using 0.25% trypsin with 1 mM
21 EDTA.

22 *Hsp47*-transformed cells were grown as adherent monolayer in 75 cm² tissue culture flasks using
23 F12 Medium supplemented with 10% Fetal Bovine Serum, 200 μ g/ml of and 0.1 g/L kanamycin

1 solution. The cultures were maintained in a 5% CO₂ incubator at 37 °C. Cell passage was carried
2 out at 80% confluence at 1:2 ratio using 0.25% trypsin with 1 mM EDTA. The cells were used
3 between passage 3 and 8 for the reported experiments.

4 PC12 cells (Riken Tsukuba, Japan) were cultured in 75 cm² flask (BD Falcon, USA) and
5 maintained in DMEM containing 10% heat inactivated horse serum and 5% fetal bovine serum
6 supplemented with 100 U/mL penicillin and 100 µg/ml streptomycin, in a water-saturated 5%
7 CO₂ atmosphere at 37°C. The cells were used between passage 3 and 8 for the reported
8 experiments.

9 Heat shock protein 47 assay

10 *Hsp47*-transformed cells were trypsinized and plated onto 96-well plates at initial concentrations
11 of 1×10^4 cells per well in 100 µL of culture medium. The cells were allowed to attach for 48 h at
12 37 °C supplemented with 5% CO₂, heat-shocked for 90 min at 42°C, 5% CO₂ and recovered for
13 2 h in a 5% CO₂ incubator. Then, medium was removed and changed by 100 µL of samples
14 diluted with medium at desired concentrations and incubated with cells for 3h in 5% CO₂
15 incubator at 37 °C.

16 After incubating cells with samples, the medium was removed and the cells washed twice
17 with PBS. 50 µL lysis buffer (Promega) was then added and the plates incubated for 30 min at
18 room temperature. 20 µL of cell lysate was transferred to a new plate, to which 100 µL of
19 substrate solution (10 mM NaH₂PO₄•2H₂O, 100 mM NaCl, 1% BSA, 0.005% NaN₃, 1 mM
20 MgCl₂•6H₂O, 1% 4-methylumbelliferyl-β galactosidase (MUG), pH 7) was added in order to
21 trigger the conversion of MUG into galactose and methylumbelliferyl by galactosidase. After
22 allowing the reaction to occur in the dark for 30 min at room temperature, 60 µl of reaction stop

1 buffer (1 M glycine-NaOH, pH 10.3) was added and the fluorescence at 365 nm excitation/ 450
2 nm emission was then determined using a multi-detection microplate.

3 PC12 cells treatment and protein extraction

4

5 PC12 cells were seeded 2×10^6 cells/100-mm poly-L-lysine coated dishes (Wako, Japan).
6 Following overnight incubation in a 5% CO₂ humidified incubator at 37°C. Cells were treated
7 with 50 μM luteolin, 15 μM rosmarinic acid, 20 μM canosic acid and 50 ng NGF for 48 h. The
8 cells were rinsed three times with ice-cold PBS, scraped gently and collected in PBS. Then, the
9 cell pellet was lysed in 1 mL of lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS,
10 1 mM EDTA, 100 mM DTT, 25 mM spermine base, 1% protease inhibitor cocktail (Han et al.
11 2010) and 0.1 volume of DNase I (1mg/mL)/RNase (0.25 mg/mL) mixture . DNase I, RNase,
12 DTT and Protease inhibitor cocktail were immediately added to the extraction-lysis buffer. The
13 extraction was carried out firstly at 4°C for 45 min to degrade nucleic acid then followed by 1 h
14 at room temperature with rotation (Yang et al. 2006). Then the lysate was clarified by
15 ultracentrifugation at 46.000 rpm at 15°C for 60 min. The final protein amount was determined
16 using 2-D Quant kit.

17 Two-dimensional gel electrophoresis (2-DE)

18 The first dimension electrophoresis was carried out on an Ettan IPGphor II (GE Healthcare)
19 apparatus. Immobilized pH gradient (IPG) strips (pH 3-10, 24 cm, GE Healthcare) were
20 rehydrated (7 M Urea, 2 M Thiourea, 2 % CHAPS, traces of Bromophenol blue, 50 mM DTT
21 and 0.5 % IPG buffer, IPG buffer and DTT were added immediately before use) with 350 μg of
22 sample solution. The total volume loaded by strip was 450 μL. the rehydration and separation
23 programs were processed using the following parameters: step 1: 500 Vhr, step 2: 750Vhr, step

1 3: 16.5 KVhr, step 4: 27.5 KVhr and step 5 was 500 v for 24 h. The proteins were separated
2 according to their isoelectric points. The isoelectrically focused IPG strips were immediately
3 equilibrated for 2x 15 min using equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30 %
4 glycerol (w/w), 2 % (w/v) SDS, traces of bromophenol blue). The first equilibration was with
5 1.0% w/v DTT followed by a second equilibration with 2.5% w/v iodacetamide. Then the strips
6 were immersed in 10 ml of electrophoresis buffer for 5 min.

7 The strips were subsequently subjected to a second dimension SDS gel (255 mm x 200 mm x 1
8 mm), the protein were separated using 12% SDS PAGE, using Ettan DALTSix™ electrophoresis
9 unit (GE Healthcare). The SDS-PAGE was performed at 2 w/gel for 40 min, then 15 w/gel until
10 the dye front reached the bottom of gels. After being fixed with 3% ethanol, 0.5% acetate
11 solution, gels were stained by CBB for 8 h. After being destained by rinsing with fixing solution,
12 gels were scanned at 300 dpi resolution and the image were analyzed with Image Master™ 2D
13 software (ver. 4.9: GE Healthcare). For statistical quantification of expression difference with
14 software, three experiments were performed for each treatment. Coomassie blue stained 2-DE
15 gel images were acquired with image scanner and subsequently subjected to visual assessment to
16 detect changes in protein expression level between different treatments. Spots were expressed as
17 percentages (% vol) of relative volumes by integrating the value of each pixel in the spot area as
18 described previously in our study (Han et al. 2010).

19 In-gel digestion and mass spectrometry

20 Protein spots of interest were excised from the CBB-stained gel; the excised spots were
21 transferred to Eppendorf tube loaded with 100 µL of 50% ACN/25 mM ammonium bicarbonate
22 solution (1:1). After being destained, gel sample were rehydrated with 100 µL of 100% ACN for

1 5 min and then thoroughly dried in the speedVac concentrator (miVac, England) for 5 min. Then,
2 the dried gel were reduced in 100 μ L 10 mM DTT/25 mM ammonium bicarbonate with shaking
3 at 56°C for 1 h, and washed with 100 μ L of 25 mM ammonium bicarbonate with shaking at
4 room temperature for 10 min. Afterward gels particles were alkylated in 100 μ L of 55 mM
5 Iocetamide/25 mM ammonium bicarbonate and incubated on dark for 45 min at room
6 temperature and washed as described previously. After that, gel sample were dehydrated with
7 100 μ L of 100% ACN for 10 min and then thoroughly dried in the speedVac concentrator for 5
8 min. Subsequently the dried were rehydrated with 2 μ L/ sample trypsin in 25 mM ammonium
9 bicarbonate with about 1:50 enzyme amount ratio to protein after staying at 4°C for 30 min, and
10 incubated at 37°C for 15 h. After trypsin digestion, the supernatant was transferred to another
11 tube. Then, the remained peptide mixture was extracted twice with 50% ACN/5% formic acid at
12 37°C for 30 min using 50 μ L of the extraction solution for the first time and 25 μ L for the second
13 time. Subsequently the combined solution was concentrated in the speedVac to 10 μ L and
14 analyzed using MALDI TOF as described in our previous study (Han et al. 2010).

15

16 Analysis of gene expression by quantitative Real-time PCR

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18

19

20 To confirm the activation of stress-related proteins in *R.officinalis*' polyphenols-treated PC12
21 cells, the expression of *Hsp90 α* , *HYOU1*, *VCP/p97*, and *NDK* were determined by real-time PCR
22 using glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as an internal positive control.
23 After incubating seeded plates for 6 h and 12 h, total RNA was purified using the ISOGEN kit
24 (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions. Total RNA was
25 quantified by measurement with Thermo scientific nanodrop 2000 (USA). Reverse transcription

1 reactions were carried out with the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad,
2 CA, USA, using 1 µg of total RNA. Briefly, RNA was denatured at 65 °C for 5 min. and
3 incubated with 1 µL oligo (dT)₁₂₋₁₅ primers and chilled at 4 °C. After adding SuperScript II
4 reverse transcriptase (200 U) the reaction mix was incubated at 42 °C for 60 min, then 10 min at
5 70 °C (Han et al., 2010). All Primer sets and TaqMan probes for experimental genes were from
6 Applied Biosystems *Hsp90α* (Rn00822023), *GAPDH* (Rn99999916_s1), were inventoried gene
7 expression assays, *VCP/p97* (Rn01439521_m1), *NDK* (Rn01465378-gH), and *HYOUI*
8 (Rn02586251_m1) were obtained as ‘Assays-on-demand’ kits.

9 For the quantification of mRNA, TaqMan real-time quantitative PCR amplification reactions
10 were carried out in an AB 7500 fast real-time system (Applied Biosystems). Amplifications were
11 performed in 20 µL final volume, using 10 µL of TaqMan Universal PCR Master Mix UNG
12 (2X), 1 µL of the corresponding primer/probe mix and 9 µL of template cDNA (70 ng µL⁻¹).
13 Cycling conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s followed
14 by 60°C for 1 min.

15
16

17 **RESULTS**

18

19 Luteolin, carnosic acid and rosmarinic acid decrease the expression of Heat shock protein 47.

20

21 *Hsp47*-transformed cells were heat-shocked for 90 min at 42°C, 5%CO₂ and recovered for 2 h,
22 and then we proceed to the screening of *R. officinalis*’EtOH extract and its main polyphenols
23 (lut, CA, and RA) effect on the recovery of *Hsp47*. As shown in Fig.1A, 70% EtOH extract of *R.*
24 *officinalis* reduced significantly the expression of *Hsp47* at 1/100 dilution v/v, by 25 %.
25 Additionally, Lut, CA, and RA expressed the same activities. As indicated in Fig.1B, all 3

1 compounds significantly reduced the expression of *Hsp47* in a dose dependent manner. At high
2 doses of Lut, CA, and RA there were a recovery of 20% of control.

3

4 Effect of luteolin, carnosic acid and rosmarinic acid on stress related protein expression

5

6 Since the major phenolic compounds of *R. officinalis* are Lut, CA, and RA, in subsequent
7 analysis we assumed that this anti-stress activity could be mainly due to the presence of these
8 compounds and tried to elucidate their neuroprotective effect in PC12 cells using proteomics
9 analysis.

10 PC12 cells were treated with Lut (50 μ M), CA (20 μ M), and RA (15 μ M) for 48 h, then total
11 proteins were separated by 2D-gel electrophoresis. A protein pattern of PC12 cells is shown in
12 Fig. 2A. Approximately 200 well-resolved spots were detected in each coomassie blue-stained
13 gel, with molecular-mass ranges of 15–225 kDa and a *pI* ranging from 3 to 10. Several spot
14 volumes from treated-PC12 cells were changed. As shown in Fig.2B, treatment with *R.*
15 *officinalis* polyphenols caused substantial change in 4 particular spots ($P < 0.05$). Protein spots
16 were selected and analyzed with MALDI-TOF. The peptide mass fingerprinting (PMF) spectra
17 was used to search protein database using Mascot engine versus Swiss prot database as reported
18 in our previous study (Han et al. 2010). These spots were identified to be stress-related proteins:
19 *Hsp90*, *VCP/p97*, *HYOU1*, and *NDK* (Table 1). Lut and CA significantly down regulated all 4
20 proteins: Lut treatment induced a decrease by around 40 % of studied proteins, CA induced
21 severe down regulation of *Hsp90* and *VCP/p97* However, RA decreased significantly only the
22 expression of *HYOU1* and *VCP/p97* to 0.4 and 0.7 as % of Ctrl. Spot volume.

23

1 Validation of differentially expressed stress related protein by quantitative RT-PCR.

2

3 To confirm the protein expression of *Hsp90*, *HYOUI*, *VCP/p97*, and *NDK*, we evaluated
4 mRNA expression of previously cited proteins after 6 h and 12 h treatment. As shown in Fig. 3A,
5 CA, Lut and NGF treatments respectively significantly reduced *Hsp90* mRNA expression to 0.2,
6 0.84 and 0.25. Lut decreased *Hsp90* expression from 1.5 (6 h treatment) to 0.81 (12 h treatment).
7 Meanwhile, CA and NGF increased *Hsp90* after 12 h to 0.80 and 0.88, respectively. RA showed
8 significant high expression of *Hsp90* at 6 h which was decreased to control level after 12 h
9 treatment. *VCP/p97* was significantly down regulated by CA, RA, and NGF to 0.23, 0.64, and
10 0.15 respectively after 6 h treatment. However Lut treatment only showed decrease in mRNA
11 expression after 12 h (Fig. 3B). Similarly, *HYOUI* was down regulated by CA, RA, and NGF to
12 0.16, 0.65, and 0.31 respectively. Lut treatment showed an increase after 6 h to 1.4 fold and a
13 decrease to 0.73 fold after 12 h (Fig. 3C).

14 *NDPK* mRNA expression was decreased by all treatments. Lut, CA, RA and NGF
15 showed a significant decrease in *NDK* expression after 6 h treatment to 0.77, 0.58, 0.47, and
16 0.13. This effect was maintained in case of Lut and CA and it was increased to 1.17 and 1.11
17 respectively for NGF and RA after 12 h treatment.

18

19

20 **Discussion**

21 Previously, we and others demonstrated that the main polyphenols of *R.officinalis*: Lut, CA, and
22 RA are able to induce PC12 cells differentiation (El Omri et al. 2010; Lin et al. 2010). In the
23 present study we observed for the first time that Lut, CA, and RA reduced significantly the

1 expression of *Hsp47* in stress- heated Chinese hamster ovary transfected cells (Fig. 1). Proteomic
2 analysis and qRT-PCR showed that differentiation of PC12 cells into neuron-like cells is
3 associated with an attenuated stress related proteins. Our result is consistent with previous
4 observations of a reduced induction of Hsp70 (Dwyer et al. 1996), NDPK (Kim et al. 2007), and
5 *VCP/p97* (Kobayashi et al. 2002) during neuronal differentiation of PC12 cells (Dwyer et al.
6 1996).

7 *Hsp47* is a heat stress protein that interacts with procollagen in the lumen of the
8 endoplasmic reticulum (ER) (Taguchi et al. 2007). It is the main chaperone involved in collagen
9 elaboration and maturation (Rocnik et al. 2002). Reducing the activity of *Hsp47* means that *R.*
10 *officinalis* polyphenols could protect mammalian cells against heat stress and increase
11 thermotolerance. *Hsp90* is a molecular chaperone. In neurodegenerative diseases, it is involved
12 in the protection of neuronal cells against the accumulation of toxic aggregates (Luo et al.
13 2010). *VCP/p97* is cytosolic chaperone required for Endoplasmic Reticulum-Associated Protein
14 Degradation (ERAD). It is involved in a variety of cellular processes, including membrane
15 fusion and ubiquitin-dependent protein degradation and it is chaperone-like protein (Nagahama
16 et al. 2002). Recent studies showed that inhibition or stable complexing of *Hsp90* may alleviate
17 and prevent from some neurological disease with motor impairments and Taupathie (Ali et al.
18 2010). *HYOU1* belongs to Hsp70 superfamily. It has been suggested to be a neuroprotective
19 factor against ischemia and excitotoxicity. It was reported to be upregulated under hypoxic or
20 excitotoxicity conditions, that potentially induce ER stress in neurons (Zhao et al. 2010). In our
21 study, we demonstrated that *R.officinalis* polyphenols downregulated the expression of
22 *HYOU1*. Subsequently they may alleviate stress insults in PC12 cells through their antioxidant
23 activity. *NDK* is involved in the proteolytic functions of the proteasome. It may act by

1 catalyzing the activities of ATP hydrolysis when Hsp70 and *VCP/p97* are activated (Yano et al.
2 1999). Meanwhile, (Kimura et al. 2002) reported the involvement of NDP kinases in the
3 regulation of cell growth and differentiation. Particularly in PC12 cells, *NDK* may control the
4 molecular switch to determine the cell fate toward proliferation or differentiation in response to
5 environmental signals.

6 In neuron cells, physiological and pathological processes that disturb protein folding in
7 the ER cause ER stress and activate a set of signaling pathways termed UPR (Samali et al. 2009).
8 Particularly, in neurons misfolded and/or aggregated proteins cannot be diluted, and accumulate
9 with aging (Chen and Brown 2007), leading to several neurodegenerative disorders, like AD,
10 PD, ALS, Huntington's disease (HD), and other polyglutamine expansion disorders (Luo et al.
11 2007, Oza et al., 2008). It was suggested by Taguchi et al. (2007) that targeting Hsp is a
12 promising alternative in the area of neurodegenerative disorder, where protein aggregation and
13 neuron degeneration are the common pathological features. In this respect, it was demonstrated
14 by Wilson et al. (2006) that blue berry polyphenol uptake increased life span of *C. elegans* by
15 promoting stress resistance. Melatonin was demonstrated by Ozacmak et al. (2006) to protect
16 rats by reducing Hsp70 expression during chronic cerebral hypoperfusion. Resveratrol was
17 reported to protect cells against heat stress through chaperone activation (Putik et al. 2005).
18 Curcumin consumption by Indian reduced AD incidence in comparison to American people (Ali
19 et al. 2010).

20 As a part of this study, we examined possible mechanisms for the beneficial effects of
21 *R.officinalis*' polyphenols treatment in a neuronal cell-like model. As shown stress induced-
22 protein expression by Lut, CA, and RA was correlated with qRT-PCR. However at mRNA level
23 we observed a decrease after 6 h followed by an increase of mRNA of different gene in case of

1 CA and RA treatment, and the opposite was observed for Lut treatment. This observable fact
2 could be correlating with structure-functions of these 3 different polyphenols. Apart from being
3 great scavengers of free radicals, *R. officinalis*' polyphenols may directly stimulate the cell
4 defense against stress response through cellular chaperone in early time treatment. As it is
5 known, that antioxidant after scavenging free radical, they became themselves pro-oxidant after
6 being oxidized in cell culture media (Balliwell 2008). Oxidation of polyphenols produces
7 peroxide, hydroperoxide a complex mixture of semiquinones and quinones, all of which are
8 potentially cytotoxic (Balliwell 2008). In response to these pro-oxidant, the cell may act to
9 regulate and conserve its stress defense system to maintain ER function and thus protect cells
10 from toxic insults (Zhang et al. 2007).

11 Regardless of their effect on downregulation of *Hsp47*, *Hsp90*, *VCP/p97*, *HYOU1*, and *NDK*, it
12 is clear from these experiments that natural polyphenols available in rosemary leaves can reduce
13 neuronal stress, and increase thermotolerance. This is a significant finding that lends support to
14 previous experiments on cultured neuronal cells or *in vivo* studies showing beneficial effects
15 against neurodegenerative-related declines.

16

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19

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List of table and figure

Table 1 Proteins of PC12 cells changed by *R. officinalis*' polyphenols and identified by TOF analysis

Fig. 1 Effect of *R. officinalis* polyphenols on *Hsp47* expression in Chinese Hamster ovary transfected cells. **(A)** The effect of *R. officinalis* EtOH extract on *Hsp47* expression, cells were treated with *R. officinalis* 70% EtOH at 1/10000, 1/1000, 1/100 v/v dilution. **(B)** the effect of *R. officinalis* polyphenols on *Hsp47* expression, cells were treated with luteolin (10, 30, 50 μ M), carnosic acid (5, 10, 20 μ M), rosmarinic acid (5, 10, 15 μ M) and NGF 50 ng/ml. The region plasmid containing the plasmid used in this work was constructed by connecting the restriction enzyme avall (-197 to +38 KDa) fragment containing the heat shock factor binding DNA sequence of the mouse *Hsp47* promoter to a 3.1 -Kb HindIII containing the structural gene for β -galactosidase on the upstream side of HindIII. *Hsp47* expression was determined as described in Materials and Methods. Results are expressed as the mean of 12 wells from three independent experiments \pm S.D. * $P < 0.05$ treatment vs. control (Student's t-test).

Fig. 2 Two-dimensional gel electrophoresis of PC12 cells (A), the magnified images of the boxed regions (B) and spot volume (C). PC12 cells were treated with 50 μ M luteolin, 20 μ M carnosic acid, 15 μ M rosmarinic acid and 50 ng/ml NGF for 48 h. The 2-DE gel was stained with coomassie brilliant blue. Spot volume was measured by ImageMaster 2D Platinum software. These spots were identified as *Hsp90*, *VCP/p97*, *HYOU1*, and *NDK* by MALDI-TOF mass spectrometry. Each bar represents the mean \pm SD of three independent experiments. * $P < 0.05$ treatment vs. control (Student's t-test).

1 **Fig. 3** Effect of *R. officinalis* polyphenols on the expressions of *Hsp90*, *Vcp*, *HYOU1*, *NDK*
2 mRNAs in PC12 cells. GAPDH was used as a housekeeping gene. The mRNA expression of all
3 gene was normalized by GAPDH mRNA expression and expressed as ratio of Ctrl. PC12 cells
4 were treated with 30 μ M luteolin, 20 μ M carnosic acid, 15 μ M rosmarinic acid and 50 ng/ml
5 NGF for 6 h and 12 h. Each bar represents the mean \pm SD of three independent experiments. **P*
6 < 0.05 treatment vs. control (Student's t-test).

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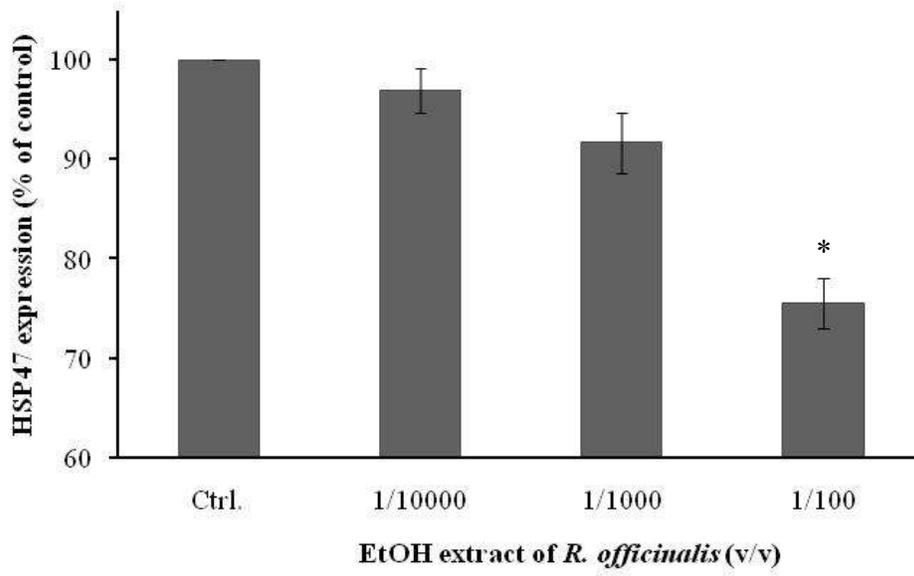
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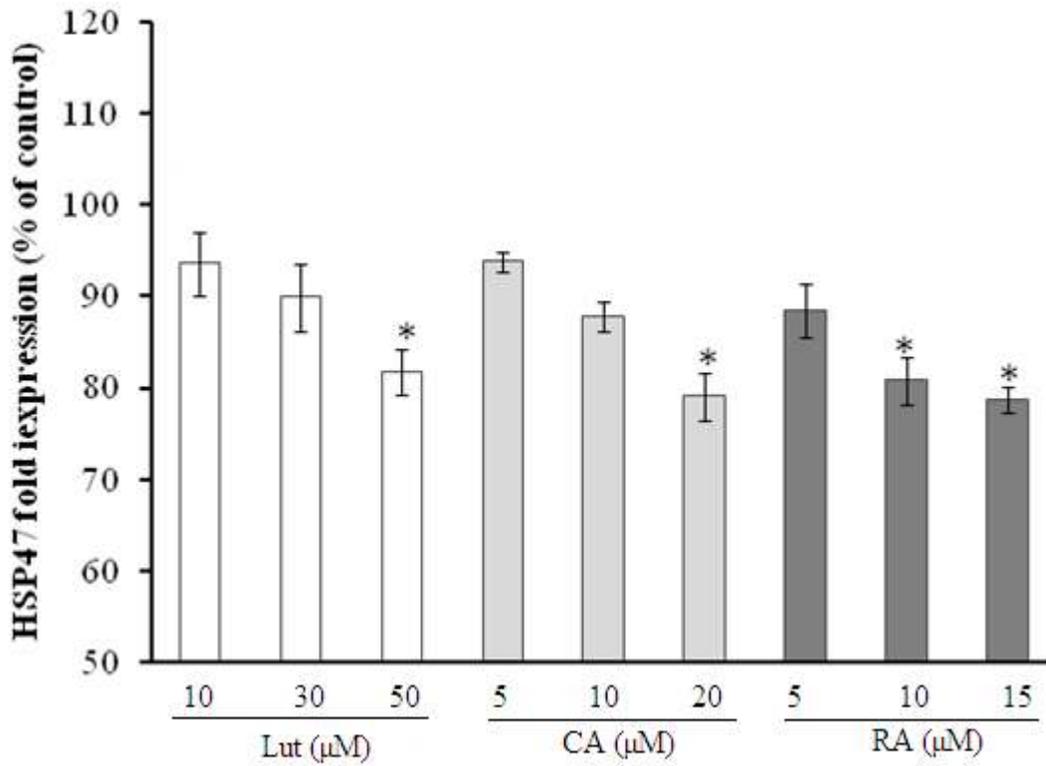
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1 A



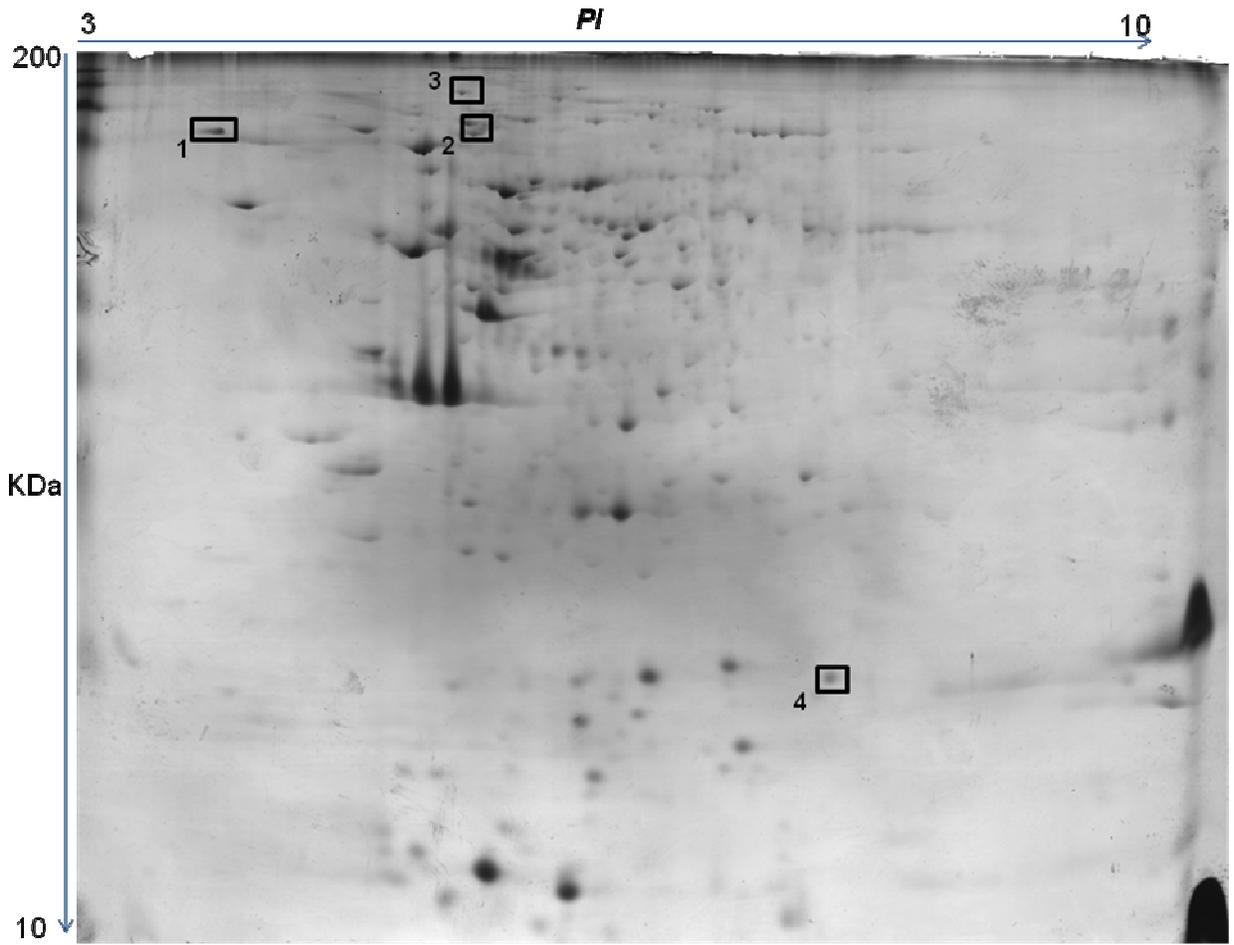
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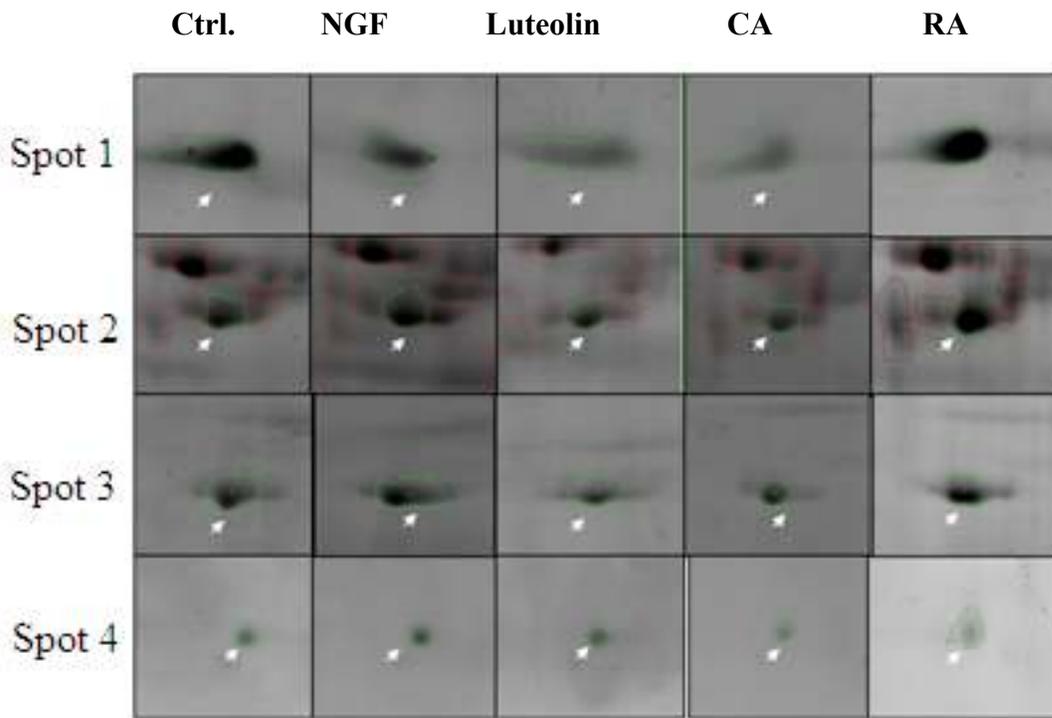
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Fig. 2 A

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24 **Fig. 2 B**

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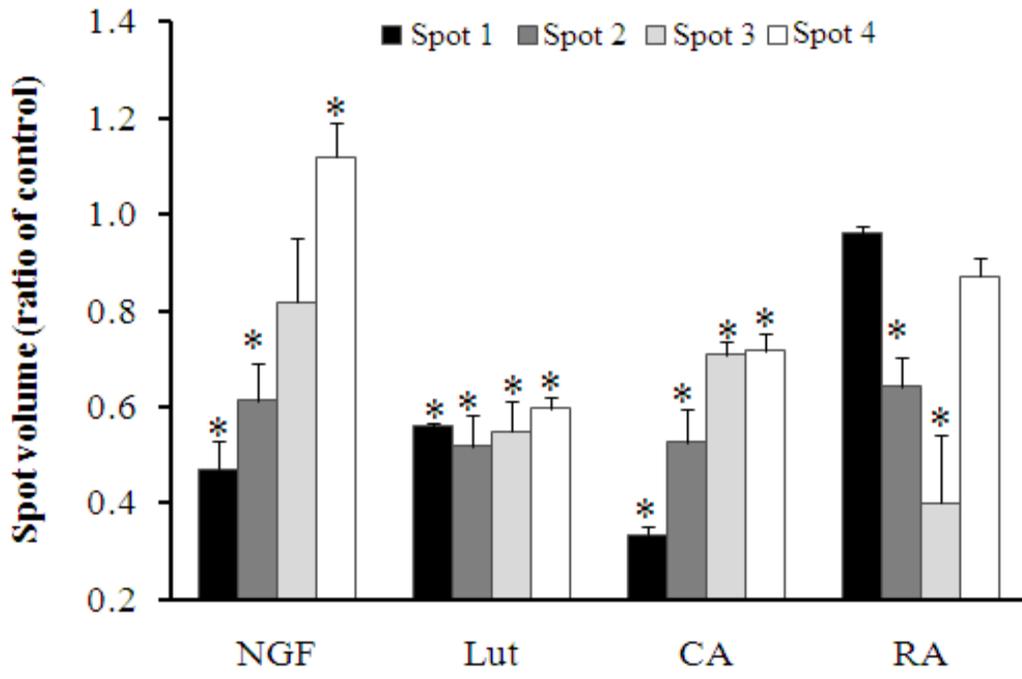
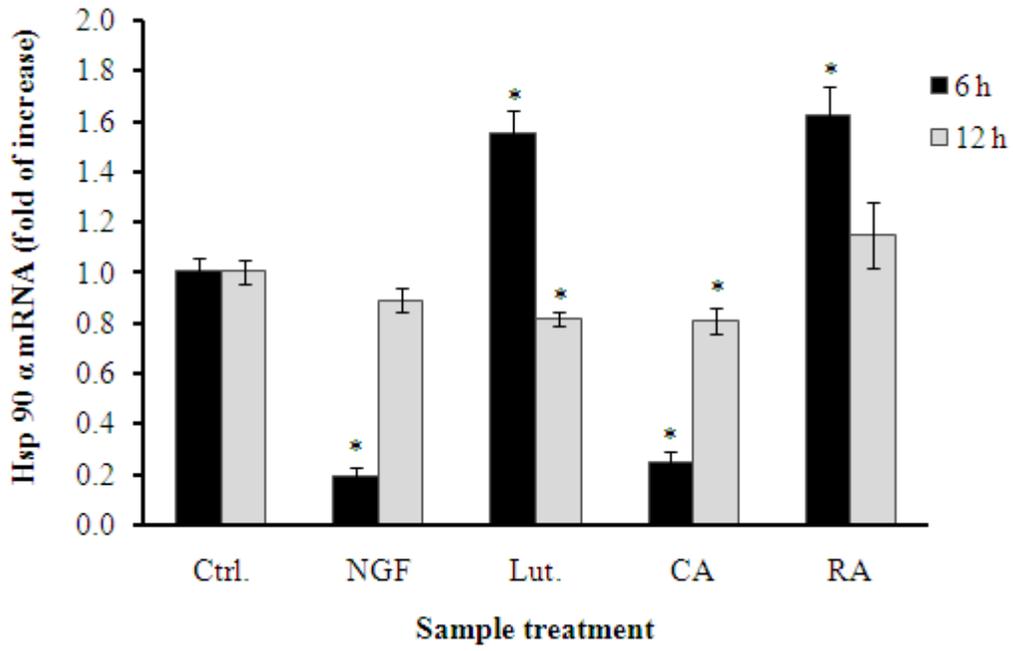
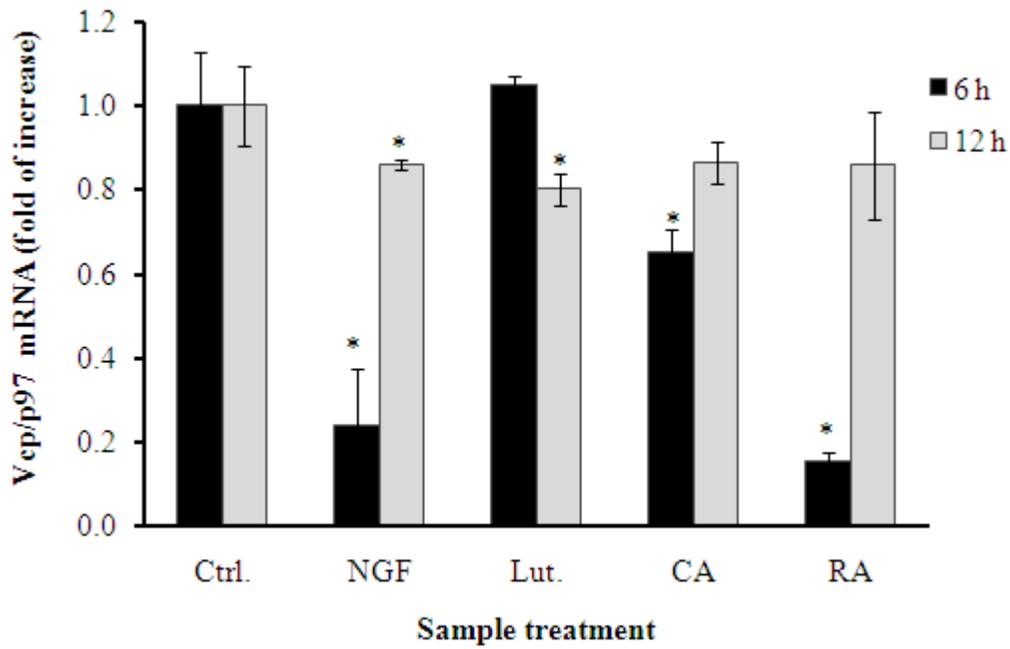


Fig. 2 C

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10 **B**



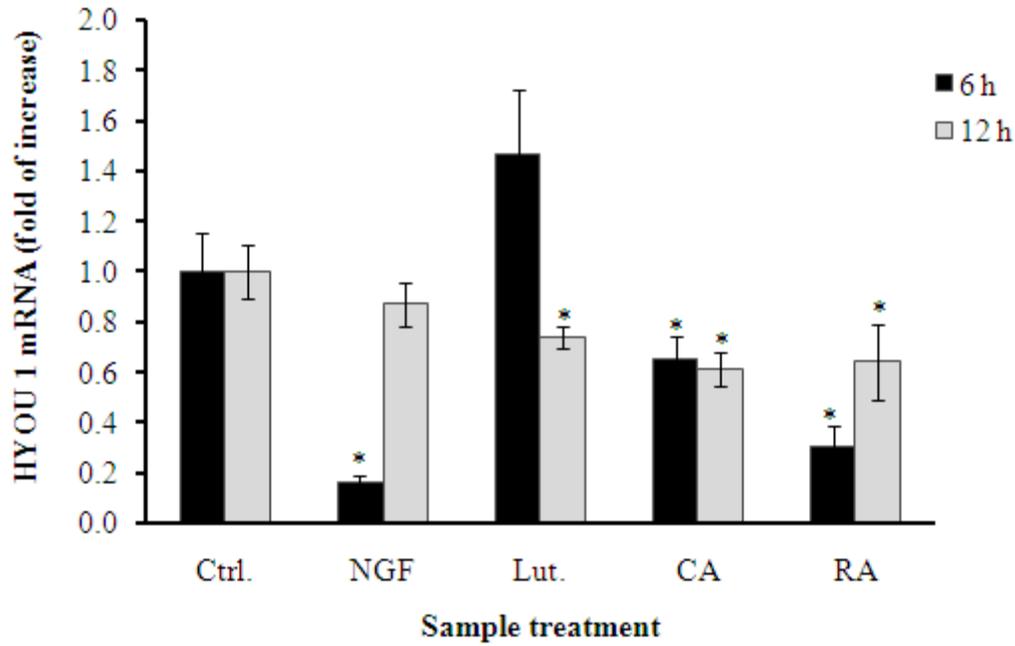
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13 **Fig. 3**

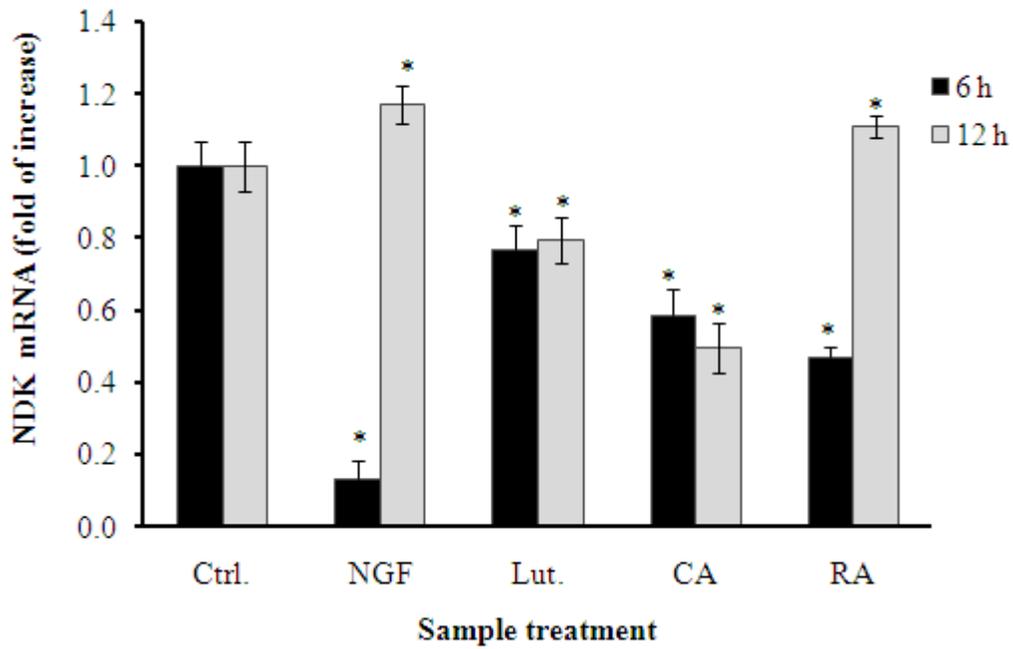
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C



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D



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13 **Fig. 3**

1 **Table 1**

Spot No	Accession No.	Score	Calculated <i>PI</i> value / Observed <i>PI</i> value	Calculated Mw value / Observed Mw value (KDa)	Protein sequence	Name of protein
1	P82995	80	4.93/ 4.0	85.161/ 100	K.VILHLKEDQTEYLEER.R	Heat shock protein alpha (<i>Hsp90α</i>)
2	P46462	454	5.14/ 5.5	89.977/ 105	K.MDELQLFR.G K.EMVELPLRHPALFK.A R.RIVSQLLTLMDGLK.Q R.EVDIGIPDATGRLEILQIHTK.N R.ETVVEVPQVTWEDIGGLEDVKR.E R.ELQELVQYPVEHPDKFLK.F K.GPELLTMWFGESEANVR. R.KYEMFAQTLQQSR.G	Transitional endoplasmic reticulum ATPase Valosin containing protein (<i>Vcp/p97</i>)
3	Q63617	639	5.11/ 5.5	111.448/ 115	R.SRFPEHELNVDPQR.Q R.SLAEDFAEQPIKDAVITVPAFFNQAER.R K.VLQLINDNTATALSYGVR.R R.TLGGLEMELR.L R.DAVIYPILVEFTR.E R.YSHDFNFHINYGDLGFLGPEDLR.V K.LYQPEYQEVSTEEQREEISGK.L K.LCQGLFFR.V	Hypoxia up-regulated protein 1 (<i>HYOUI</i>)
4	P19804	313	6.92/7.2	17.386/ 23	R.TFIAIKPDGVQR.G K.DRPFFPGLVK.Y R.VMLGETNPADSKPGTIR.G R.GDFCIQVGR.N R.NIIHGSDSVESAEKEIGLWFKPEELIDYK.S K.EIGLWFKPEELIDYK.S	Nucleoside diphosphate kinase B (<i>NDK</i>)

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