International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2017; 5(4): 92-105
Received: 05-05-2017
Accepted: 06-06-2017

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# Herbal preparations prevent beta-amyloid peptide induced hippocampal cell damage

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**Abstract**
The aggregates of Aβ(1-40) and Aβ(1-42) peptides are considered as the pathological hallmarks of Alzheimer's disease. The present work examined the ability of several plant preparations to protect the viability of hippocampal cells in the presence of aggregated Aβ peptides. The inhibition of the viability of rat hippocampal cells by the preliminarily aggregated peptides down to 20±2.5% of control was proved. In the presence of aggregated Aβ peptides, the cell viability was saved at 51-114% of control in the medium containing the ethanol extracts from grape and sorrel leaves, rose petals, melilot, phenol glycoside and flavonoid fractions from these extracts. The DNA-comet analysis evidenced the cell integrity conserving by the extracts, but not by the phenol glycoside fraction from rose petals, which significantly protected the cell viability from aggregated Aβ(1-40) and Aβ(1-42) ($IC_{50}$ = 0.2 ± 0.02 and 2.1 ± 0.7 μg/ml, respectively). Conclusion: the studied plants can be considered as sources for neuroprotecting agents.

**Keywords:** amyloid beta peptides; DNA-comet analysis; hippocampal cell culture; plant extracts, plant fractions

## 1. Introduction
Misfolding of some proteins and their self-assembly into insoluble amyloid fibrillar structure underlies more than 30 diseases constituting a group of amyloid-related diseases [1, 2]. Alzheimer's disease (AD) is one of them. AD is a severe neurodegenerative disease, characterized by progressive impairment in memory, cognition and behavioral functions, affecting aging population. It is shown that the main reason for neurotoxic fibrillation in AD is accumulation of Aβ(1-40) (Aβ40) and Aβ(1-42) (Aβ42) amyloid peptides, formed at proteolytic cleavage of the amyloid precursor protein [3]. Oligomeric and fibrillar aggregates of Aβ40 and Aβ42 peptides are among the principal components of amyloid plaques found post mortem in AD brain [4]. Currently, the therapeutic agents, successfully preventing the formation of fibril aggregates, are not found yet [5, 6]. The high cost, side effects of synthetic drugs and incomplete recovery of patients became the reasons for the development of natural therapeutics.
Herbal medicines have potential of optimum pharmaceutical effects in AD. There is considerable evidence of beneficial effects of herbal medicines on the processes associated with pathological fibrilogenesis [7, 8]. A number of compounds from medicinal plants efficiently inhibited aggregation of amyloid peptides, disaggregated preformed fibrils and protected neuronal cells against cytotoxic peptides [9]. The results of *in vitro* researches indicated that resveratrol, an active polyphenol in many plants, may directly bind to Aβ42 interfering with its aggregation, changing oligomer conformation and attenuating cytotoxicity [10]. *Rosa damascena* is rich with flavonoids and its extract reversed behavioral deficit in a rat model of Aβ-induced AD, manifesting a potential for prevention and treatment of cognitive dysfunction [11]. The capability of puer0arin, an isoflavone glycoside from the root of Pueraria lobata, to protect *in vivo* against cognitive deficits, oxidative stress, and neurodegeneration induced by Aβ42 in mice was demonstrated [12]. Kim and Oh [6] concluded that herbal medicines can be useful AD-preventive agents, and "synergistic therapeutics, combining conventional medicine and herbal medicine for AD, may emerge in the not-so-distant future".
We demonstrated the anticancer properties of several medical and edible plants of Armenian highland [13]; shown the ability of ethanol extracts of several plants and their fractions to inhibit the aggregation of islet peptide hormone amylin, to disaggregate its preformed aggregates, and to protect the cultured mouse pancreatic β-cells against cytotoxicity of aggregated amylin [14, 15]. Then, the *in vitro* inhibition by plant preparations (PPs) of self-aggregation of Aβ42 and Aβ40 and disaggregation of their preformed aggregates was

demonstrated using Thioflavin-T fluorescence technique [16]. For some PPs, the $IC_{50}$ values in these processes were evaluated [14-16].

The present work describes the *in vitro* protection of the rat hippocampal cells against toxic action of aggregated Aβ40 and Aβ42 by several plant extracts and their fractions. Using different experimental designs, a dual mechanism of beneficial effects of tested PPs was manifested. The findings suggested that the protection by PPs of hippocampal cells is based both on the lowering of the aggregation state of Aβ40 and Aβ42 peptides and on the correction of induced disorders in the cells.

## 2. Materials and methods

Thioflavin-T (ThT), RPMI-1640 and supplements were purchased from Sigma Ltd, USA; G-25 and LH-20 Sephadex – from Pharmacia Biotech, Upsala, Sweden, Aβ40 and Aβ42 peptides were purchased in "China peptide" (China). Some amounts of Aβs were gifted by Prof. H.-U. Demuth, Head of Department of Drug Design and Target Validation, Fraunhofer Institute of Cell Therapy and Immunology, Germany. All other chemicals were of the highest purity.

Spectral measurements were performed on Specord M-40 UV-VIS spectrophotometer (Germany) and Perkin-Elmer MPF-44A spectrofluorometer (USA). The microscope model BM-800 equipped with epi-fluorescent attachment and digital camera B-CAM3 (Boeco, Germany) were used.

### 2.1 Plant preparations

The oregano (*Origanum vulgare),* roots of bryonia white *(Bryonia alba*) and seeds of thistle (*Silybum marianum*) were purchased from Phytotherapeutic Center "Artemisia" (Armenia). The leaves of grape *(Vitis vinifera)*, sorrel (*Rumex Confertus)* and blackberry (*Rubus Caesius*), rose petals *(Rosa damascena*) and underground part of melilot (*Melilotus officinalis)* were collected in Armenian highland and dried in the shade. A voucher specimen has been deposited in the herbarium of the Botanical Department of Yerevan State University (Dr. Narine Zaqaryan). In previous work [13] we have described the procedures of preparation of extracts of dried materials in 70% ethanol/water, separation of the constituents on LH-20 and G-25 Sephadex columns, examination of all these preparations by qualitative chemical analysis, thin layer chromatography, optical absorbance, etc. The evaporated extracts and fractions stored at refrigerator (-18°C). In the experiments, their stock solutions in 70% ethanol/water were prepared, and the aliquots were added to the cell incubation medium up to the desired concentrations.

### 2.2 Peptides

1 mg of Aβ40 was dissolved in distilled water. Due to low solubility in water, 1 mg of Aβ42 was dissolved in 0.1 M NaOH, immediately neutralized with diluted HCl and 20 mM phosphate buffer, pH 7.2. The solutions were centrifuged, their absorption spectra were recorded and the molar concentrations were evaluated, using the extinction coefficient of tyrosine at 276 nm, 1.39 $mM^{-1}cm^{-1}$.

The aggregates of Aβ40 and Aβ42 were obtained by incubating for 3-7 days at 37°C in 40 mM phosphate buffer, pH 7.4, containing 0.02% $NaN_3$ (w/v) - the conditions ensuring the formation of aggregates, demonstrated earlier by TEM analysis [17]. The aggregation state of the peptides was estimated by elaborated by LeVine ThT staining [18], as described in our works [14, 16, 17].

### 2.3 The primary culture of rat hippocampal cells

Brains of adult outbreed rats were dissected and the meninges were removed. The hippocampus was isolated, transferred to 15-ml tubes, adjusted to 2 ml with phosphate-buffered saline (pH 7.4) supplemented with 0.6% glucose and 2 mg/ml sterilized papain. The tissue was digested at 30°C for 30 min, dissociated by triturating and centrifuged. The precipitated cells were suspended in the cultivation medium [19]. The concentration of cell suspension was adjusted to $5\times10^5$ cells/ml and added by 0.1 ml ($5\times10^4$ cells) per plate. Cells were cultured in poly-D-lysine coated 96-wells plate (Nunc EasYFlasks) in the $CO_2$ incubator (37°C, 5% $CO_2$). In the the experiments with PPs, their solutions in 20% ethanol/water were added to the culture medium up to a definite final concentration. The final concentration of ethanol in the assay mixture was below of non-toxic 1% level.

### 2.4 Cell count and viability

After washing out non-attached cells by PBS, the attached cell number was determined by counting the cell number per field under ×10 objective in 96-well plates. To evaluate the viability of cells, the trypan blue (TB) exclusion method was used [20]. the attached cells were covered by TB solution (0.4%), washed out in 1 min and the attached cells were counted. At least 5 randomly selected fields of view are used for each sample. The percentage of TB-negative cells was used as an index of cell viability.

### 2.5 DNA-comet test

The Alkaline Comet assay was conducted as described by Venturi *et al*. [21] with minor modifications. The cell suspension was centrifuged at 100×g for 3 min. The cell pellet was suspended in 75 µl of 0.5% low melting point agarose in PBS, embedded on a microscope slide, lysed for 2 h at 4°C in the solution, containing 2.5 M NaCl; 100 mM $Na_2EDTA$; 10 mM TRIS; 1% Triton X-100. Then the slide was put in a horizontal gel electrophoresis tank, containing electrophoresis solution (1 mM $Na_2EDTA$, 300 mM NaOH, pH>13). After unwinding DNA for 20 min, electrophoresis was conducted for 30 min at 26 V, 300 mA. The slide was washed in 0.4 M TRIS buffer, pH 7.5, stained with 2 µg/ml ethidium bromide and analyzed with a fluorescence microscope at magnification of ×80. One hundred randomly selected cells from each slide were scored using Comet Assay Software Project image analysis (CASP, http://www.casp.sourceforge.net). The parameters: tail length (TL, µm), tail DNA % (TD, %) and extent tail moment (TM, defined as: TM = TL×TD/100 arbitrary units) were used as metrics of DNA damage [22].

### 2.6 Statistical analysis

$IC_{50}$ values were calculated using GraFit (Leatherbarrow, 2001) software. The statistical analyses of data, obtained at least in three independent experiments, were performed using the InStat software, version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The ANOVA variance or unpaired t test with Welch correction was applied. The Gaussian distribution of samples was tested using the method of Kolmogorov-Smirnov. Statistical significance was accepted for one-tailed p-value <0.05. The results are expressed as means ± standard errors of means (SEM).

## 3. Results

### 3.1 Influence of amyloid peptides on the viability of hippocampal cells

Figs. 1A and 1B present the dependences of the number of

TB-negative (viable) cells on the concentrations of non-aggregated and aggregated peptides after a 3-day cultivation. The curves demonstrate the higher toxicity of the aggregated peptides compared with their non-aggregated solutions. This difference was more pronounced for Aβ42 and the toxicity of its aggregates was significantly higher. Based on these results, the concentrations of 2 μM and 0.2-0.4 μM for Aβ40 and Aβ42, respectively, were used in the following experiments.

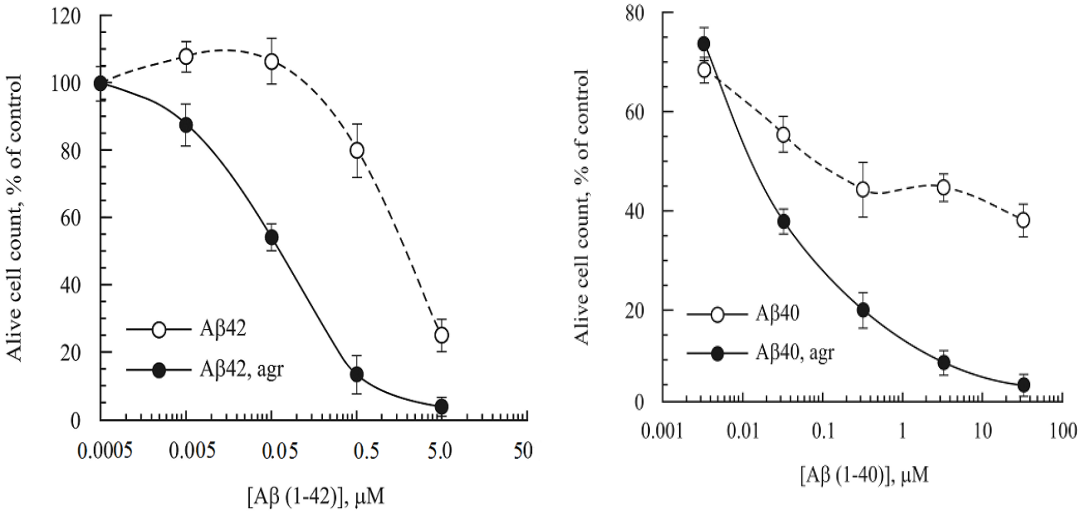


**Fig 1:** The cell viability dependences from the concentrations of aggregated (—●—) and non-aggregated (- - o - -) Aβ42 (A) and Aβ40 (B) peptides after a 3-day cultivation

The effectiveness of chosen concentrations was confirmed in several independent experiments. The number of viable cells in the control samples (59.8±4.0, n=7), after a 3-day cultivation in the presence of 2 μM aggregated Aβ40, decreased down to 22.7% (13.6±1.9, n=5) ($p<0.0001$). In the presence of 0.2 μM Aβ42 it decreased down to 20.2% (12.1±1.5, n=6) ($p<0.0001$).

### 3.2 The viability of hippocampal cells in the presence of plant preparations

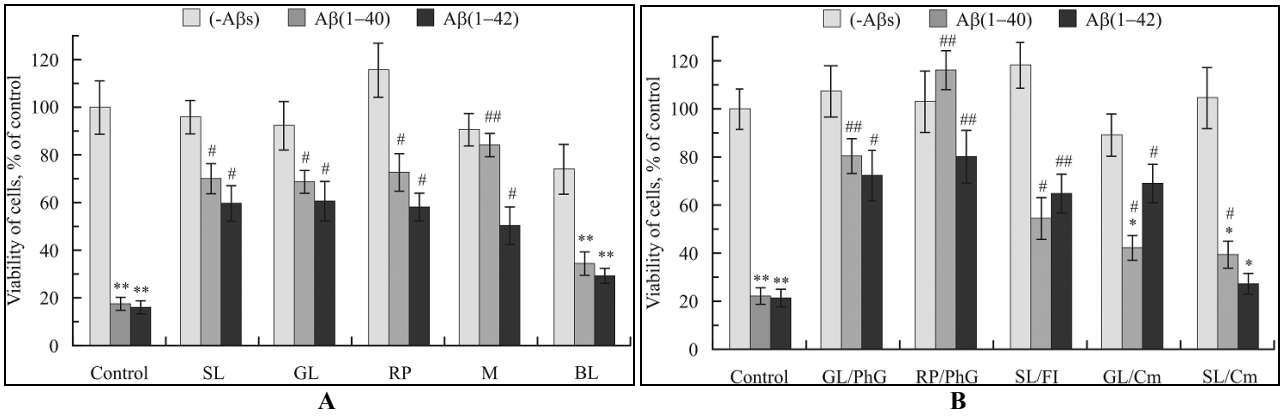


**Fig 2** Protection of hippocampal cells against toxic action of aggregated Aβ-peptides by plant extracts (A) and their fractions (B). The viability of cells after a 3-day cultivation in the presence of 30 μg/ml PPs in the absence (light columns) and presence of aggregated Aβ40 (2 μM, gray columns) and Aβ42 (0.2 μM, black columns) is expressed as percentage of untreated control. The bars indicate ± SEM as % of appropriate values. **P<0.01; *P<0.05 different from the non-treated control; ##P<0.01; #P<0.05 different from Aβ-controls.

The ethanol extracts of sorrel leaves (SL), grape leaves (GL), rose petals (RP), melilot (M) and blackberry leaves (BL) as well as the fractions of phenol glycosides (PhG) from GL and RP, flavonoids (Fl) from SL, coumarins (Cm) from GL and SL in the concentration range 3-300 μg/ml had no viability-compromising effects on the hippocampal cells. Light columns in Figs. 2A and 2B show the viability of cells in the presence of the named preparations at concentration of 30 μg/ml. The diagrams in these Figures demonstrate that the aggregated Aβ-peptides (Aβ-controls) reduced the viability of cells down to nearly 20% of the non-treated control ($p<0.001$).

The plant extracts, except BL, significantly improved the viability of hippocampal cells, disturbed by Aβ-peptides (Fig. 2A, gray and black columns, $p<0.05$). The gray and black columns in Fig. 2B show that phenol glycoside (PhG) and flavonoid (Fl) fractions were rather effective in protection of the viability of the cells ($p<0.05$), while the coumarins (Cm) were less effective in attenuation of Aβ-peptides-induced death of the cells.

**Table 1:** $IC_{50}$ values of plant preparations in prevention of Aβs-induced death of hippocampal cells ($IC_{50}$ ± SEM, μg/ml)

| Plant Preparation | | Aβ40 | Aβ42 |
|---|---|---|---|
| Extract | SL | 2.6±0.8 | 14.0±0.1 |
| | GL | 10.5±0.9 | 15.8±2 |
| | RP | 27.1±0.7 | 24.2± 2.1 |
| | M | 0.5±0.1 | 26.2±2.9 |
| Fraction | RP/PhG | 0.2±0.02 | 2.1±0.7 |
| | GL/PhG | 0.8±0.3 | 6.1±1.3 |
| | SL/Fl | 19.9±5.6 | 7.99±1.6 |
| | GL/Cm | 47.9±6.9 | 4.1±1.5 |
| | SL/Cm | 60.7±7.2 | 162.2±18.4 |

In Table 1, the $IC_{50}$ values are listed for plant preparations in protection of hippocampal cells from toxic action of amyloid peptides. Interestingly, in the case of the ethanol extracts, the orders of these values for two peptides correlate, except the very low value for melilot extract in the case of Aβ40-induced death, contrary to the highest value in the case of Aβ42. This discrepancy may reflect the molecular peculiarities of Aβ40, ensuring its specific interaction with coumarins, highly presented in melilot [13]. For plant fractions, the conformity between $IC_{50}$ values for the amyloid peptides is observed for two PhG fractions: in the case of Aβ40 they are by an order lower than in the case of Aβ42. For Cm fractions from GL and SL, the $IC_{50}$ values are opposite in the cases of two Aβ-peptides.

Two possible reasons of the observed cell-protecting effects of PPs can be considered. As the one of them, we can suggest the lower aggregation state of Aβ-peptides in the presence of PPs, demonstrated in our earlier work [16]. In the analogous study of protection of pancreas β-cells by plant preparations from toxicity of the aggregated peptide hormone amylin, the strong correlation (R=0.99) between the aggregation state of the amylin and the viability of cells in the presence of herbal materials was manifested [14, 15]. Despite the spectral properties of plant preparations limited the application of ThT430/485 fluorescence in a wide range of their concentrations, we obtained the low but positive correlation between the aggregation states of peptides and protection of hippocampal cells by several plant preparations.

Fig. 3 shows the correlation (R=0.445) between $IC_{50}$ values of three extracts (from SL, M and GL) and of four fractions (GL/Cm, RP/PhG, SL/Cm and SL/Fl) in the protection of hippocampal cells from Aβ(1-40)-induced death (Table 1), and the aggregation of Aβ(1-40) in the presence of the same PPs in concentration of 7.5 μg/ml. The similar correlation (R=0.559) was obtained between the peptide aggregation in the presence of the same three extracts but only two isolated fractions (RP/PhG, and SL/Fl), and $IC_{50}$ values of PPs in the cell protection against the Aβ(1-42)-induced death.

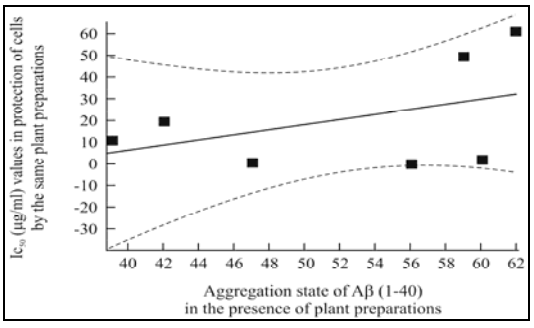


**Fig 3:** Correlation between $IC_{50}$ values in protection of hippocampal cells from Aβ(1-40)-induced death and the inhibition of the peptide aggregation by some of the studied plant preparations.

The second possible mechanism of the curative action of PPs might be their ability to correct the cell damage, induced by aggregated Aβ-peptides. Although precise mechanism of neurotoxicity of Aβ aggregate forms remains unclear, there are reports suggesting that they induce apoptosis and initiate oxidative stress and free-radical degeneration in neuronal cells [23]. At this, the anti-oxidative and anti-apoptotic capacity of different natural compartments of plants are documented [24]. These capacities might condition the realization of the second mechanism of the cell protection.

### 3.3 DNA-comet assay

The above presented researches do not allow deciding which mechanism prevails in the observed beneficial effects of PPs. To check, if the second way is realized, we applied another strategy. In the next pack of the study, concurrently with the non-treated control, two hippocampal cell samples, containing aggregated Aβ40 (2 μM) and Aβ42 (0.4 μM), were prepared. After a 2-day cultivation, each of the Aβ-containing samples was divided to six aliquots, and to five of them ethanol extracts of SL, GL, RP, M and PhG fraction of RP were added up to the concentration of 200 μg/ml. The cultivation was continued for two more days.

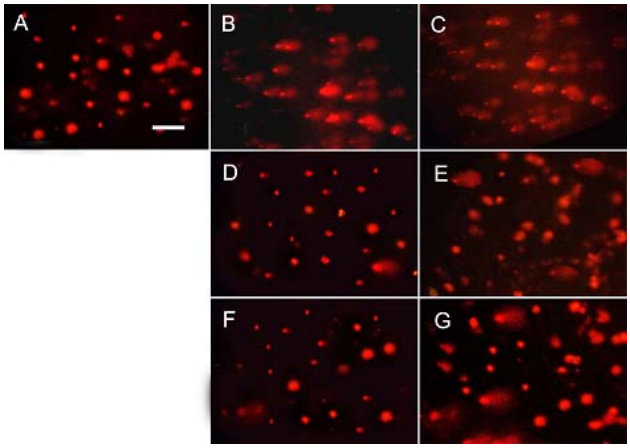


**Fig 4:** DNA-comet pictures of hippocampal cells cultivated four days in the absence (A, non-treated control) and presence of aggregated Aβ40 (2 μM, B, D, F) and Aβ42 (0.4 μM, C, E, G). B and C are Aβ-peptides containing controls. The last two days D, E and F, G contained 200 μg/ml RP and M extracts, respectively; scale bar in (A) equals 40 μm and applies to all frames.

The effects of PPs on the integrity of hippocampal cells, preliminarily disturbed by Aβ-peptides, were examined using DNA-comet test (see Materials and methods). Fig. 4 shows some of the comet images captured on the fourth day of cell cultivation. In Table 2, the TD and TM parameters obtained in CASP analysis of DNA-comet test of all the samples are listed. Both the Fig. 4 (B, C) and Table 2 (line 2) data confirm that on the 4th day of cultivation, the aggregated Aβ-peptides induced substantial DNA-damage in hippocampal cells. TD and TM parameters in Aβ40/42-controls (Table 2, line 2) increased with high statistical significance compared with the values in non-treated control (Table 2, line 1).

Both of the parameters for the samples, incubated during the last two days in the presence of plant extracts (Table 2, rows 3-6), differ from the parameters for Aβ-controls significantly but from those for the non-treated control – non-significantly, manifesting that plant extracts almost completely attenuated the induced by Aβ-peptides DNA damage. This result is in good accordance with the data obtained above in the living

cell-count test.

**Table 2:** Parameters of DNA-comet assay on hippocampal cells cultivated in the presence of aggregated Aβs and plant preparations, estimated by CASP analysis

| Sample | TD | | TM | |
|---|---|---|---|---|
| | Aβ40, 2μM | Aβ42, 0.4μM | Aβ40, 2μM | Aβ42, 0.4μM |
| **Non-treated control** | **15.3 ± 1.3** | | **2.2 ± 0.4** | |
| Aβ-controls | 50.4 ± 4.0 ** | 55.6 ± 2.0 *** | 18.0 ± 2.5 ** | 20.4 ± 1.1 *** |
| SL | 23.3 ± 3.6 ### | 23.2 ± 5.1 ### | 4.1 ± 0.7 # | 5.0 ± 1.9 # |
| GL | 20.5 ± 3.3 ### | 21.3 ± 4.3 ### | 2.6 ± 0.8 ## | 4.5 ± 1.4 ## |
| RP | 23.5 ± 2.4 **/ ### | 21.3 ± 3.6 ### | 3.5 ± 0.6 ## | 5.4 ± 1.5 ## |
| M | 18.6 ± 3.0 ### | 22.8 ± 4.3 ### | 2.8 ± 0.7 ## | 6.3 ±1.9 # |
| PhG from RP | 34.7 ± 3.2 ***/ ## | 35.4 ± 6.3 **/ ## | 6.1 ± 0.8 | 11.3 ± 2.9 |

***P<0.001; **P<0.01 – statistical significance compared to the non-treated control;
###P<0.001; ##P<0.01; #P<0.05 – statistical significance compared to the Aβ-controls.

On the other hand, PhG fraction obtained from RP appeared not so effective in the last experiment. 103 For the samples, containing RP PhG (Table 2, line 7, columns 4 and 5), the TM values are less than the values for Aβ40/Aβ42-controls, however, these differences are not statistically significant (P>0.05). Let's remind that in the living cell-count test (Fig. 2, B) the RP PhG fraction was the most effective in protection of cells from toxic actions of the amyloid aggregations. This discrepancy might mean that the cell protection by this fraction is mainly based on its ability to shield the cells. This discrepancy might be explained by the shielding of cells by PhG.

## 4. Discussion

AD prevention and treatment are primary tasks of modern neurodegenerative diseases' research. Unfortunately, at present there are no drugs available to cure, hinder the progression and arrest the AD development effectively [25], while a wide range of antipsychotic drugs is used to reduce the severity and frequency of the disease symptoms [26].

Herbal remedies worldwide (particularly in Asian countries) have a long history of use in alleviating symptoms of many diseases. They are considered as alternative strategy for AD therapy as well. Several epidemiological studies suggest that diets rich in polyphenols are beneficial for human brain function [27]. However, the mechanism of the beneficial effects of natural plant preparations is not yet fully clear. Antioxidant activity [28], metal chelating [29], modulating the enzyme activity [30], effects on neuronal signaling pathways [31], anti-amyloidogenic [32, 33] and anti-inflammatory [34] properties of natural dietary polyphenol compartments can contribute to the PPs salutary action in neurodegenerative pathologies.

All the properties listed above may be combined into two main groups: 1 – direct effects on cell development; 2 – decreasing the toxic aggregation state of amyloid peptides. Despite the fact that these properties were documented earlier, the mixed action of the studied by us PPs does not allow to differentiate them. To address this issue, we used a specific experimental design, trying to differentiate two capabilities of PPs.

DNA damage and induction of apoptotic cell death is a characteristic feature of many neurodegenerative diseases including Alzheimer's disease [35]. Hence, the results, obtained in our DNA-comet analysis, demonstrated an anti-apoptotic capability of studied plant extracts as one of the mechanisms of their effectiveness in prevention of death of rat hippocampal cells. The similar ability was observed for neroprotective ability of mentioned in the Introduction isoflavone glycoside puerarin, which was realized through prevention of Aβ-induced microglial apoptosis [36].

The beneficial results of the first set of the experiments with PPs presume that the protection of hippocampal cells against death induced by Aβ-peptides, at least partly, can also be explained by a rather high content of flavonoids [13] with a range of beneficial characteristics [27-34].

It's worth mentioning that the effective plants are also rich in PhGs possessing low $IC_{50}$ values in protection of hippocampal cells (Table 1). In our previous research, RP PhG fraction demonstrated essential activity in the prevention of aggregation of Aβ-peptides and in the disaggregation of their preformed aggregates [16]. Earlier we had shown that the PhG fractions from SL, GL and RP are provided with rather high antioxidant properties and possess effective anti-cancer activity, inhibiting the growth of Ehrlich ascites carcinoma cells [13]. The PhG fractions from GL and RP prevented amylin aggregation and protected the islet β-cells against amylin-induced death with very low $IC_{50}$ values [14, 15]. Unfortunately, as the DNA-comet test indicated in the experiments with Aβ-pretreated hippocampal cells, the RP PhG fraction, having many live-supporting activities, appeared not effective in prevention of DNA damage induced by Aβ-peptides (Table 2, last line).

## 5. Conclusion

Our data confirm that the aggregated Aβ40 and Aβ42 are *in vitro* cytotoxic towards the hippocampal cells versus their non-aggregated forms, with Aβ42 being ten-fold more toxic. The ethanol extracts of rose petals, melilot, grape and sorrel leaves, as well as some of their fractions effectively protected the hippocampal cells in the presence of aggregated Aβ-peptides. The beneficial action of plant ethanol extracts is based both on their cell-promoting (anti-apoptotic) and anti-aggregative properties, but the effect of RP PhG fraction is based mainly on its anti-aggregative effect.

The studied plants grow all over the world and are widely used in Eastern cuisine, being beneficial as the neurogenesis supporting agents. Hence, they can be considered as valuable sources for therapeutics with the potential to prevent and treat neurological and other amyloid disorders.

To the best of our knowledge, this is the first report on the neuroprotective efficacy of melilot, grape and sorrel leaf extracts.

## 6. Acknowledgments

The work is realized by financial support of the State Committee of Science MES RA in the framework of the research project № SCS 13-1F186; of a research grant awarded in 2015 to H. Harutyunyan from the Armenian National Science and Education Fund (ANSEF, biochem-

3787). The authors appreciate Prof. H.-U.Demuth for gifted amyloid peptides.

**7. Ethical approval**

The experiments involving the laboratory animals were approved by Ethics Committee of Yerevan State Medical University after M. Heratsi, No 7-26.04.2012: Research is not contrary to the Directive 2001/20/EC of The Legal Aspects of Research Ethics and Science in European Community.

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