PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2020, **54**(3), pp. 216–222

Biology

INCREASING OF THE SUPEROXIDE DISMUTASE TOTAL ACTIVITY IN MICROGLIAL CELLS UNDER THE TREATMENT BY *RIBES NIGRUM* L. ALCOHOL EXTRACT

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Cell redox homeostasis is formed as a result of the balance between the accumulation of reactive oxygen species and functioning of the antioxidant enzymes or non-enzymatic antioxidants. The alcohol extracts from the leaves of *Ribes nigrum* L. were prepared to investigate the antioxidant activity and protective effects against both BV-2 microglial wild type (WT) cells and acyl-CoA oxidase type 1 (ACOX1) deficient cell ($Acox1^{-/-}$) lines. Our investigations showed that *R. nigrum* extracts possess remarkable antiradical activity in 1-diphenyl-2-picryl-hydrazyl (DPPH) with IC_{50} value of 32.7±0.8 $\mu g\ mL^{-1}$. 3-(4,5-dimethyltrazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that the sub-cytotoxic concentration of *R. nigrum* extract in both cases was 1 $mg\ mL^{-1}$ The further treatment of microglial cells with the mentioned concentration of extract brought the remarkable increasing (up to 350%) of activity of the total superoxide dismutase (SOD) – the core antioxidant enzymes, under the treatment during the 48–72 *h*. So, relaying on the obtained data it is possible to insist that the lack of ACOX1 can be compensated by the activation of cell enzymatic antioxidant defense mechanisms.

https://doi.org/10.46991/PYSU:B/2020.54.3.216

***Keywords*:** *Ribes nigrum*, antiradical activity, microglial cells, superoxide dismutase.

Introduction. Plant origin antioxidant substances are of great importance in the treatment of different age-related and metabolic disorders due to their therapeutic value without visible side effects [1–3]. Antioxidants are used as food additives to preserve the lipid components from oxidation and are also engage in the process of neutralization of the oxidative stress negative consequences, so they can be used in medicine as well. Synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) or tert-butyl hydroquinone (TBHQ) are among the most frequently used [4]. But due to the toxicity and supposed action of these substances as carcinogenesis possible promoters, the growing demand for searching of natural origin substances with antioxidant properties is still exist.

Plant origin antioxidants act *via* different chemical and molecular mechanisms: they can decrease the level of oxidative damage in cells directly

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reacting with free radicals or indirectly – by inhibition the activity and expression of free radical generating enzymes or enhancing the activity or expression of intracellular antioxidant defense enzymes among which the superoxide dismutases (SOD) possess the core state [5]. According to some literature data, the Mn-SOD deficiency may result in several neurodegenerative disorders in mice [6]. The expression of Mn-SOD (in contrast to Cu,Zn-SOD) is induced mainly by oxidative stress [7]. The amyotrophic lateral sclerosis, which is considered the ultimately lethal neurodegenerative disease, can be connected with the mutations in gene coding for cytosolic SOD [8].

Microglial cells play a central role in the degeneration of neurons during the age-related diseases. So, investigation of antioxidant and anti-inflammatory activity of plant origin metabolites using BV-2 wild type microglial cells as well as deficient cells for acetyl-CoA oxidase 1 (ACOX1) – the rate-limiting enzyme of peroxisomal beta-oxidation of very long chain fatty acids (VLCFA), is of scientific interest in order to reveal the neuroprotective effect of plant extracts, as well as suggest some mechanisms of their action. In this regard, microglial cells can serve also as tools for our investigations [9].

The leaves of *Ribes nigrum* L. are used in European traditional medicine for treatment and prophylactics of various deceases of inflammatory, rheumatic and other nature [10, 11]. For these, different *in vitro*, *ex vivo* and *in vivo* experimental models have been used. The most useful preparations on the basis of *Ribes nigrum* leaves were alcohol liquid extracts, which were then lyophilized in order to determine the exact concentrations of orally or intraperitoneally administered portions [11]. According to the recent year literature data, the extracts, obtained from the leaves of *R. nigrum*, possess remarkable antioxidant activity [12]. No data on acute toxicity, genotoxicity, reproductive and developmental toxicity or carcinogenicity [11].

The aim of this investigation was to evaluate the antioxidant activity of *R. nigrum* leave extract on microglial cell lines.

Materials and Methods.

Plant Material. The investigated plant material was collected from Lori region (Spitak, 1600–1650 m a. s. l., July, 2019). Harvested plant leaves had been dried in ventilated room at 25–30°C until steady weight. Extract preparation was carried out using methanol as solvent as described before [13].

BV-2 Microglia Cell Culture. Murine microglial BV-2 cell lines (BV-2, Acyl-CoA oxidase type 1 (ACOX1) deficient mutants ($Acox1^{-/-}$) and WT cells) were grown in a 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (*v*/*v*) heat inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin); culture medium was changed every 2 days. BV-2 cells were seeded on 96-well microplates at 25×10^4 cells per well for viability assay, 6-well microplates at 5×10^5 cells per well – for enzymatic activity determination.

Radical Scavenging Activity Determination. Scavenging free radical potentials were determined in ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [14]. Catechin was used as standard. Sample solution contained 125 μL (1 mM) DPPH, 375 μL ethanol and 500 μL of test-solution (plant extract and catechin with

different concentrations (1.25 to 150 $\mu g\ mL^{-1}$ and 1.95 to 1000 $\mu g\ mL^{-1}$, respectively)). Test-solution was replaced by ethanol in the control sample. The absorbance was measured at the wavelength of 517 *nm* using spectrophotometer Genesys 10S UV-Vis (Thermo Scientific USA).

The radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100,$$

where A_c is absorbance of control (DPPH without the addition of test solution), A_s is sample absorbance.

MTT Assay. The cell proliferation and/or mitochondrial activity were measured using 3-(4,5-dimethyltrazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells, plated in 96-wells plates, were treated for 24 to 72 *h* with different concentrations of plant extracts (50–5.10^{-4} $\mu L\ mL^{-1}$) in dimethylsulfoxide. Cells were incubated for 2 *h* with MTT dye followed by the absorbance (Abs) measurement at the 570 *nm* with a microplate reader and the sub-cytotoxic concentration was selected for further investigations [15].

Preparation of BV-2 Cell Lysate. After treatment of BV-2 microglia cells with *R. nigrum* extract, cells were washed with *phosphate-buffered* saline (PBS) and were lysed in 50 μL of radioimmunoprecipitation (RIPA) buffer: 50 *mM* Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecylsulfate, 150 *mM* NaCl, 2 *mM* Ethylenediamine tetraacetic acid (EDTA), 50 *mM* NaF. Cells were placed in ice for 30 *min* and the lysate was cleared by centrifugation at 20,000 × *g* for 20 *min* (at –4°C).

Protein content was assessed by a bicinchoninic acid assay [16]. The supernatant was stored at –80°C until further use, if needed.

Enzymatic Activity Measurement. Total SOD activity was measured according to the Beauchamp and Fridovich [17].

Data Processing. A statistical analysis was done with the Student-t test (Excel-2013 software) for calculating the probability values; and data were considered statistically different at a p-value of 0.05 or less.

Results and Discussion. Our results suggested that IC_{50} value in DPPH-assay of standard (catechin) and *R. nigrum* extracts were: catechin – IC_{50} 12.623±0.5 $\mu g\ mL^{-1}$, extract – 32.7±0.8 $\mu g\ mL^{-1}$ (Fig. 1).

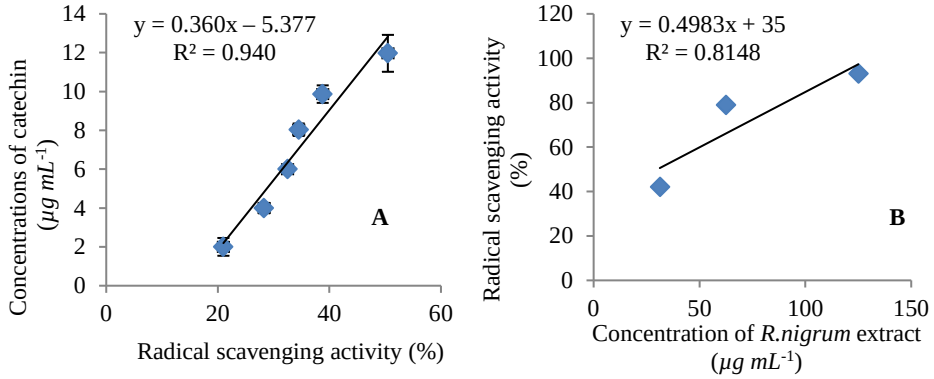

Fig. 1. Antiradical activity of A) catechin (positive control) and B) *R. nigrum* extract.

These data suggesting that *R. nigrum* extract possess remarkable antiradical activity and can be processed for further investigations for revealing some mechanisms of action.

The MTT test was applied to clarify the influence of investigated extracts on cell viability and mitochondrial function. In this test tetrazolium ring of 3-4,5-dimethyl-2-thiazolium bromide reduce by the mitochondrial succinate dehydrogenase of active living cells into formazan. The quantity of produced formazan is proportional to the quantity of living cells. The determination of sub-cytotoxic concentration allowed excluding the toxic influence of investigated acting agent and consider only its activity on cellular enzymatic antioxidant system.

Further treatment of cells was carried out with the sub-cytotoxic concentration of *R. nigrum* extracts. In case of both cell lines this concentration was $1 \cdot 10^{-1}$ $\mu g \cdot mL^{-1}$ (Fig. 2, A, B). The further investigations showed that the optimal treatment period was 48-hour treatment. This period allows gaining the maximal viability of the WT cells (compared to the control) (data not shown).

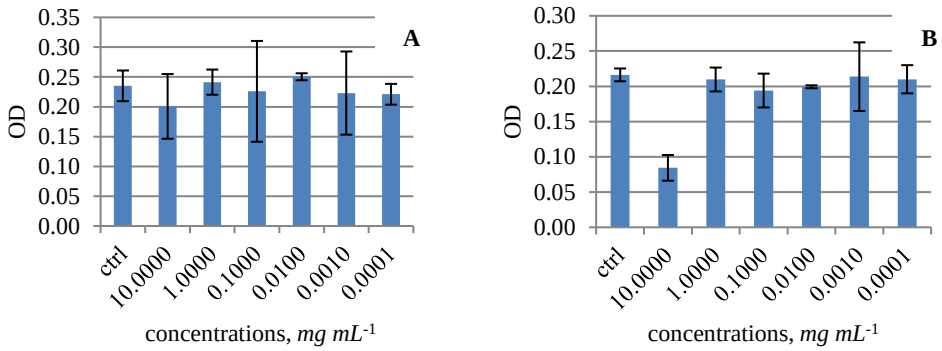

Fig. 2. Effects of *R. nigrum* extracts on viability of BV-2 WT and ACOX1 deficient microglial cells (MTT assay; A and B, respectively). Cells were treated for 24 *h* with EO at different concentrations (10 to $1 \cdot 10^{-4}$ $\mu g\ mL^{-1}$). The results represent the mean ±SD of the three repetitions. Values are given as average of the repetitions. The significance is presented with the Student-t test: $p < 0.05$ for BV-2 both cell lines.

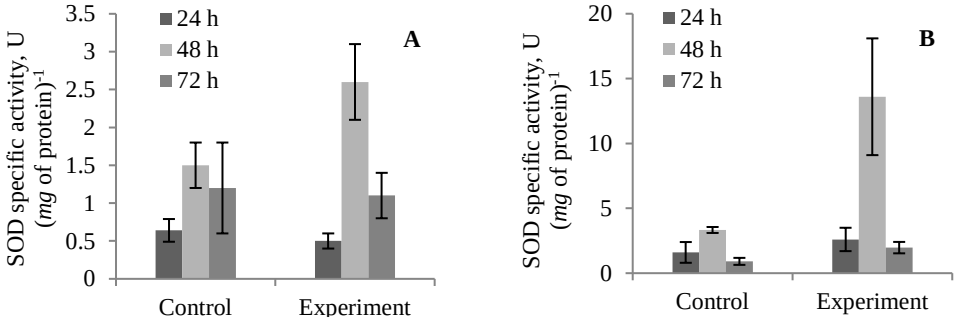

Fig. 3. *R. nigrum* extract influence dynamics on SOD activity of BV-2 WT and Acox1 deficient microglial cells (A and B, respectively). Cells were treated with EO of $1 \cdot 10^{-1}$ $\mu g\ mL^{-1}$ concentration. The results were the mean ±SD of the three repetitions. Values are given as average of the three repetitions and were normalized to the control and the significance is presented with the Student-t test. In case of SOD activity: $p = 0.1$ for 24 *h* treatment; $p < 0.1$ for 48 *h* and $p < 0.05$ for 72 *h* treatment (for WT BV-2 cells); $p < 0.5$ for 24 *h* treatment; $p < 0.1$ for 48 *h* and $p = 0.1$ for 24 *h* treatment (for *Acox1*$^{-/-}$ BV-2 cells).

The activity of total SOD in treated cells was significantly higher in both cell lines during the 48-h treatment. Even the SOD activity of 24-h treated WT cells was decreased, but as a result of 48-h treatment the SOD activity was increased by 60%. Meanwhile the activity of SOD of *Acox1*$^{-/-}$ cells was increased up to 66 and 300%, during the 24 and 48-hour treatment, respectively. In case of both cell lines the activity of SOD decreases at the end of growing cycle (72 *h*) (Fig. 3).

All these data states that the best treatment time period for both investigated cell lines was the 48-h treatment.

Conclusion. Different microglial cell lines, including BV-2 cells, are of great importance in order to study various mechanisms of neuroprotective action of substances possessing antioxidant activity as the microglia have a pivotal role in the immune response of nervous system [18–20]. Our investigation suggests that phytochemicals of *R. nigrum* can promote the activity of SOD in the BV-2 microglial wild type (WT) cells and acyl-CoA oxidase type 1 (ACOX1) deficient cell (Acox1$^{-/-}$) lines and thus can be suggested for further investigation in order to reveal other mechanisms of their action as antioxidant agents.

Received 20.12.2019
Reviewed 17.07.2020
Accepted 12.08.2020

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ՍՈՒՊԵՐՕՔՍԻԴ ԴԻՍՄՈՒՏԱԶԻ ԱԿՏԻՎՈՒԹՅԱՆ ԲԱՐՁՐԱՑՈՒՄԸ ՄԻԿՐՈԳԼԻԱՅԻ ԲՋԻՋՆԵՐՈՒՄ *RIBES NIGRUM* L. ԱԼԿՈՀՈԼԱՅԻՆ ԼՈՒԾԱՄՁՎԱԾՔՆԵՐՈՎ ՄՇԱԿՄԱՆ ՊԱՅՄԱՆՆԵՐՈՒՄ

Բջջային հոմեոսթազը ձևավորվում է թթվածնի ակտիվ ձևերի կուտակման և ֆերմենտային ու ոչ ֆերմենտային հակաօքսիդանտների գործունեության միջև հավասարակշռության արդյունքում: Ուսումնասիրվել է *Ribes nigrum* L. տերևներից ստացված ալկոհոլային լուծամզվածքների հակաօքսիդանտային և պաշտպանական ազդեցությանը՝ BV-2 միկրոգլիալ բջիջների երկու բջջային գծերի՝ վայրի տիպի և ացիլ-CoA օքսիդազ ֆերմենտի (ACOX1) սինթեզի բացակայությամբ մուտանտ բջիջների ($Acox1^{-/-}$) կիրառմամբ: Մեր հետազոտությունը ցույց տվեց, որ *R. nigrum*

լուծամզվածքները ցուցաբերում են նշանակալի հակառադիկալային ակտիվություն 1-դիֆենիլ-2-պիկրիլհիդրազիլ (ԴՖՊՀ) թեստով, որի դեպքում IC_{50} արժեքը կազմում է 32,8±0,8 *մկգ/մլ*: 3-(4,5-դիմեթիլթիազոլ-2-իլ)-2,5-դիֆենիլտետրազոլիում բրոմիդ թեստը ցույց տվեց, որ *R. nigrum* լուծամզվածքների ենթա-բջջատոտոքսիկ կոնցենտրացիան կազմում է 1 մգ/մլ՝ ուսումնասիրվող երկու բջջային գծերի դեպքում: Նշված կոնցենտրացիայով լուծամզվածքով միկրոգլիալ բջիջների 48–72-ժամյա մշակումը բերում է հակաօքսիդանտային պաշտպանության հիմնական ֆերմենտի՝ տոտալ սուպերօքսիդ դիսմուտազի (ՍՕԴ), ակտիվության բարձրացման (մինչև 350%): Այսպիսով, ստացված տվյալների հիման վրա կարելի է պնդել, որ ACOX1 բացակայությունը կարող է կոմպենսացվել բջջի ֆերմենտային հակաօքսիդանտային պաշտպանության այլ մեխանիզմներով:

Н. Ж. СААКЯН, М. Т. ПЕТРОСЯН, А. А. ТРЧУНЯН

ПОВЫШЕНИЕ АКТИВНОСТИ СУПЕРОКСИД ДИСМУТАЗЫ В КЛЕТКАХ МИКРОГЛИИ ПРИ ОБРАБОТКЕ АЛКОГОЛЬНЫМ ЭКСТРАКТОМ *RIBES NIGRUM* L.

Редокс гомеостаз формируется в результате баланса между накоплением активных форм кислорода и функционированием антиоксидантных ферментов или неферментативных антиоксидантов. Были получены алкогольные экстракты из листьев *Ribes nigrum* L. для исследования антиоксидантной активности и защитных воздействий на клеточных линиях микроглии дикого типа BV-2 и клеток с дефицитом ацил-КоА-оксидазы типа 1 (ACOX1 ($Acox1^{-/-}$)). Наши исследования показали, что экстракты *R. nigrum* обладают высокой антирадикальной активностью в тесте 1-дифенил-2-пикрилгидразила со значением IC_{50} равном 32,8 ± 0,8 *мкг*/*мл*. 3-(4,5-Диметилтиазол-2-ил)-2,5-дифенилтетразолиум бромидный тест показал, что субцитотоксическая концентрация экстрактов *R. nigrum* для обеих клеточных линий составляет 1 *мг*/*мл*. Дальнейшая обработка микроглиальных клеток данной концентрацией экстракта привела к значительному повышению (до 300%) активности тотальной супероксид дисмутазы (СОД) – стержневого антиоксидантного фермента, при обработке в течение 48–72 *ч*. Таким образом, основываясь на полученных данных, можно утверждать, что недостаток ACOX1 может быть компенсирован активацией клеточного ферментативного механизма защиты.