Evaluation of Antioxidant Capacity of Plant Extracts Using Flow Cytometry

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This study is aimed to evaluate the inhibitory effect of natural extracts on reactive oxygen species (ROS) in human red cells (RBCs) under oxidative stress and to demonstrate the importance of using natural extracts from plants in the fight against oxidative stress. Therefore, seven nonalcoholic extracts were tested at several dilutions, in order to prove their antioxidant activity using flow cytometry. The level of intracellular hydrogen peroxide significantly decreased when the cells were coincubated with different extracts, the values of the Mean Fluorescence Intensity (MFI) ranging from 206 to 3702, compared with 3872, the value obtained in case of H_2O_2-treated cells (RBCs + H_2O_2). All the tested extracts demonstrated a good inhibition, Melissa officinalis L. extract showed a greater percentage of hydrogen peroxide inhibition even at the last dilution (94.70% (D-a)….85.76% (D-e)). The results suggest the importance of the investigated natural extracts to combat oxidative stress.

Keywords: plant extracts, antioxidant capacity, MFI, ROS inhibition

In recent decades significant progress has been made in understanding the disease causing alarming morbidity and mortality in humans (*e.g.* cancer and coronary heart disease): their processes, possible prevention and therapies. Due to various research, was proven that major causes of cancer are diverse and countless but changing food manners and lifestyle can reduce in many cases risk of disease. Studies have shown that many common foods have nonnutritive components, commonly known as chemopreventive agents, which can provide protection against a variety of diseases, including cancer [1]. Besides food, a particular interest is represented by medicinal/ aromatic plants. Plants, plants derivatives, plants extract and other forms are involved in preventive treatment of different diseases based on their bioactive principles. One major problem regarding plant material is their content, because can contain both type of compounds, bioactive molecules and toxic elements (*e.g.* tests conducted on Cd content from different plants selected from a specific region in West Romania proved that a half of them are in according with the upper limit established by the WHO) [2].

The most studied class of chemopreventive agents, from food and plants, is represented by polyphenolic antioxidants. The bioavailability of polyphenols varies, but there is no link between the amount of polyphenols founded in food/plants and their bioavailability in the human body. Polyphenols forms arriving in blood and tissues are different from those founded in food and it is very difficult to identify all metabolites and to evaluate their biological activity [3-6]. Important is the chemical structure of polyphenols and not their concentration, that determines the rate and extent of absorption and the nature of the metabolites circulating in plasma. Most abundant polyphenols in our diet are not necessarily those with the highest concentration of active metabolites in target tissues, consequently, biological properties of polyphenols differ greatly from one to another polyphenol. Their absorption by the intestinal barrier is demonstrated by the increase in antioxidant capacity of plasma after consumption of foods rich in polyphenols [7,8].

Reactive oxygen species (ROS), like peroxides, superoxides, nitric oxides etc. that possess an unpaired electron are produced continuously in the cell as by-products of metabolism and various molecules can be oxidized in order to cause cell death and tissue damage. All these reactions take place as protection mechanism against various stimuli and exceeding production may produce some alterations in the DNA, and other cells, conducting to mutations which lead to multiple diseases [9]. Polyphenols act as chemopreventice agents due to their high antipxidant properties, being capable to bind metals, to stimulate enzymes, and other processess. It is also known that reactive oxygen species in many cases may contribute to the pathogenesis of hereditary diseases [10-14].

ROS measurement in general is extremely difficult because they have a short lifetime, and current methods used are complicated, insufficiently sensitive, may produce incorrect results when studied cell population is heterogeneous. Flow cytometry offers numerous advantages, including the ability to quantitatively examine the characteristics of a large number of individual cells, of the subpopulations, but is not limited to the measurement of the mean of the total population and it was used to measure oxidative stress in many cell types [15].

The aim of the present study was to evaluate the inhibitory effect of seven nonalcoholic extracts (tested at several dilutions) on reactive oxygen species (ROS) in human red cells (RBCs) under oxidative stress and to demonstrate the importance of using natural extracts from plants in the fight against oxidative stress.

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Extract no.	Plant name	Tested dilutions (% v/v)				
		D-a	D-b	D-c	D-d	D-e
E1	*Melissa officinalis* L.	100	50	25	12.5	6.25
E2	*Salvia officinalis* L.					
E3	*Viscum album* L.					
E4	*Salix alba* L.					
E5	*Vaccinium myrtillus* L.					
E6	*Tilia tomentosa* M.					
E7	*Hypericum perforatum* L.					

Table 1
NATURAL EXTRACTS STUDIED BY FLOW CYTOMETRY

Table 2
THE EFFECT OF NATURAL EXTRACTS ON ROS LEVELS IN HUMAN RED BLOOD CELLS SUBJECTED TO OXIDATIVE STRESS INDUCED BY H_2O_2

Extract no.	Plant name	Mean fluorescence intensity (MFI)						
		RBCs	RBCs+H_2O_2	D-a	D-b	D-c	D-d	D-e
		177	3872					
E1	*Melissa officinalis* L.			206	402	513	541	551
E2	*Salvia officinalis* L.			215	373	602	2057	2642
E3	*Viscum album* L.			597	1420	3230	3438	3702
E4	*Salix alba* L.			841	983	2202	2947	3560
E5	*Vaccinium myrtillus* L.			538	809	859	2531	3594
E6	*Tilia tomentosa* M.			571	1301	1667	2040	3382
E7	*Hypericum perforatum* L.			537	1087	1090	1105	1132

Experimental part

Materials and instruments

All the reagents were purchase from Sigma-Aldrich, USA. For the sample preparation, SIGMA 2-16K centrifuge was used. The measurements and data aquisition were achieved using FACScan Becton Dickinson cyto-photometer with CellQuest Pro software.

Biological samples

Blood samples were collected from healthy donors as controls into heparinized tubes and stored at +4 °C for less than 24 h until analyzed. Cells were sedimented by centrifugation (1000 g at 4°C for 5 min). After the removal of plasma, platelets, and leukocytes by pipetting, RBCs were washed three times in Dulbecco's phosphate buffered saline solution (PBS; *p*H 7.4; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4).

Measurement of intracellular reactive oxygen species [15]

A stock solution of 100 mM H_2DCFH-DA (50 mg H_2DCFH-DA in 1 mL DMSO) is prepared and preserved at -20°C. During the analysis a work solution of 500 μM H_2DCFH-DA in distilled H_2O (1μL stock solution in 199 mL of distilled H_2O) was prepared. A cell suspension of erythrocytes of approx. 10^6 cells/mL was prepared and incubated with 20 μL work solution 500 μM H_2DCFH-DA (final concentration: 10 μM), for 1 h at 37 °C, in the dark.

In a similar manner, a sample which will serve as positive control, for the ROS production was prepared and treated as follows: centrifugation for 5 minutes at 2000 rpm to remove the supernatant, addition of 1 mL H_2O_2 2 mM in phosphate buffered saline (PBS) (1μL H_2O_2 30% in 4410μL PBS), incubation 1 h at 37°C, in the dark.

The oxidative conversion of DCFHDA to its fluorescent product was assessed by determining the amount of fluorescent signal detected by flow cytometry using the green fluorescence (FL1).

Results and discussions

Out of 13 extracts obtained by maceration were chosen those with antioxidant activity values over 10 μmol Trolox/mL extract, measured by DPPH, TEAC and FRAP assays [16]. The alcoholic extracts were subjected to evaporation to remove ethanol. Five dilutions were prepared and further

Extract no.	Plant name	Inhibition (%)						
		RBCs	RBCs+H_2O_2	D-a	D-b	D-c	D-d	D-e
		100	0					
E1	*Melissa officinalis* L.			94.70	89.61	86.75	86.02	85.76
E2	*Salvia officinalis* L.			94.44	90.36	84.45	46.87	31.76
E3	*Viscum album* L.			84.58	63.32	16.58	11.20	4.40
E4	*Salix alba* L.			78.27	74.61	43.13	23.88	8.06
E5	*Vaccinium myrtillus* L.			86.11	79.11	77.81	34.63	7.18
E6	*Tilia tomentosa* M.			85.26	66.40	56.95	47.32	12.66
E7	*Hypericum perforatum* L.			86.13	71.93	71.85	71.47	70.77

Table 3
ROS INHIBITION OF NATURAL EXTRACTS IN HUMAN RED BLOOD CELLS SUBJECTED TO OXIDATIVE STRESS INDUCED BY H_2O_2

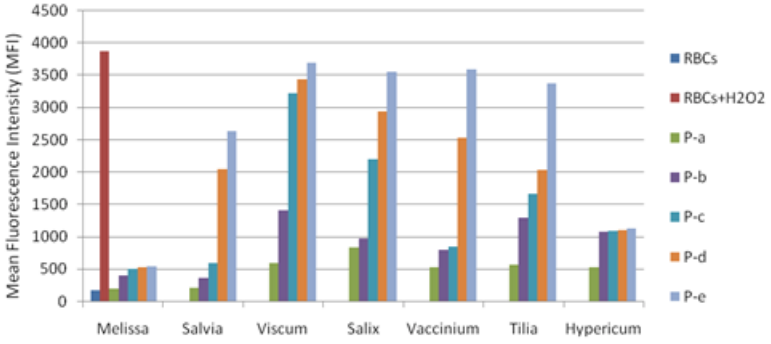

Fig. 1. Average fluorescence intensity obtained for extracts (E1-E7) tested by flow cytometry on human erythrocytes subject to oxidative stress induced by H_2O_2 for 5 serial dilutions of each extract (P-a–>P-e).

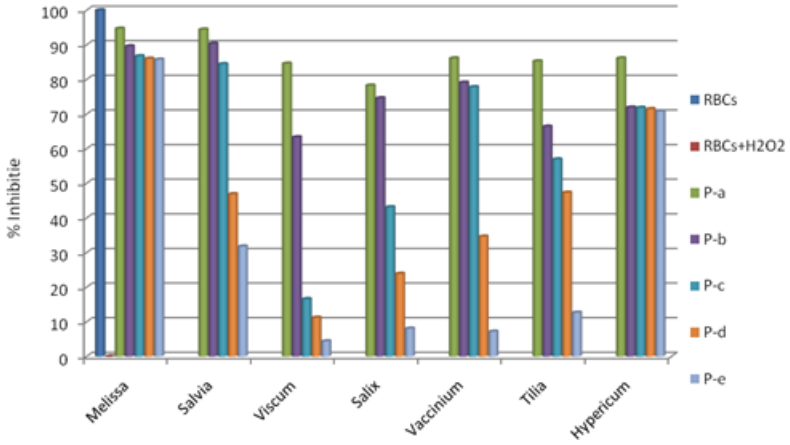

Fig. 2. The percentage of inhibition of natural extracts (E1-E7) tested by flow cytometry on human erythrocytes subject to oxidative stress induced by H_2O_2 for 5 serial dilutions of each extract (P-a–>P-e).

tested in order to establish their inhibitory effect on ROS in RBCs subjected to oxidative stress, using flow cytometry.

The tested dilutions of the natural extracts are presented in table 1.

The behaviour of the extracts was evaluated based on the Mean Fluorescence Intensity (MFI) values of fluorescent product DCF resulted from the cleavage of the non fluorescent substrate DCFH-DA by the cellular esterases and oxidized by the reactive oxygen species (ROS), measured by flow cytometry flow (table 2). The lower MFI values are, the higher the antioxidant activity is.

We found an increase of 22 fold in dichlorofluorescein (DCF) fluorescence intensity in case of human red cells subjected to oxidative stress (RBCs + H_2O_2) compared to the initial value which was 177. Intracellular levels of hydrogen peroxide significantly decreased when the cells were coincubated with different extracts.

For a better overview of the antioxidant activities, the inhibition percentage of the extracts on the ROS, as a ratio between the values of MFI corresponding to the studied extracts and the value of MFI corresponding to the sample of RBCs subjected to oxidative stress, was calculated (table 3).

Thus, at the D-a dilution, the amount of intracellular hydrogen peroxide was reduced up to 5.30% in the case of *Melissa officinalis* L. extract, up to 5.56% in the case of *Salvia officinalis* L. extract, the lowest value was obtained when the extract of *Salix alba* L. was used (21.73%).

Comparing the inhibition percentage of hydrogen peroxide obtained in the same species but at different concentrations over the serial dilutions, a high inhibition, even at the final dilution (D-e), can be observed in the case of *Melissa officinalis* L. (94.70% (D-a) ....85.76 (D-e)) and *Hypericum perforatum* L. (86.13% (D-a)....70.77 (D-e)), unlike the *Salvia officinalis* L., which showed significant inhibition at D-a dilution (94.44%), but at the final dilution (D-e), the inhibitions decreased to 31.76% (figs. 1, 2).

Conclusions

All analyzed extracts showed a significant inhibitory capacity of ROS in human red cells subjected to oxidative stress. The percentage of inhibition for D-a dilution lies between 78.27 and 94.70%, and for D-e dilution between 4.40 and 85.76%.

Melissa officinalis L. and *Hypericum perforatum* L. extracts showed greater inhibition even at the final dilution. *Salvia officinalis* L. extract showed significant inhibition rate for the D-a dilution (94.44%), effect that significantly decreased up to the final dilution (31.76%). The lowest antioxidant activity was obtained for *Salix alba* L. extract, which exhibits an inhibition of 78.27% at the first dilution.

The results suggest the importance of the investigated natural plant extracts to combat oxidative stress in cell cultures.

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Manuscript received:10.11.2016