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THE PROTECTIVE ABILITY OF THE MEDITERRANEAN PLANT
EXTRACTS AGAINST THE OXIDATIVE DNA DAMAGE.
THE ROLE OF THE RADICAL OXYGEN SPECIES
AND THE POLYPHENOL CONTENT

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The polyphenol plant extracts content seems to be responsible for the scavenging activity of the reactive oxygen species (ROS), resulting in protection against DNA damage induced by the oxidative stress. This assumption was verified analyzing the effect of six Mediterranean plant extracts (*Crepis vesicaria* L, *Origanum heracleoticum*, *Scandix australis* L, *Amaranthus sp.*, *Scolymus hispanicus* L, *Thymus piperella* L) on the oxidative DNA damage induced in lymphocytes by H₂O₂ in relation to the polyphenolic content and the lymphocyte scavenging ability of ROS. The comet assay was used to evaluate oxidative DNA damage and the polyphenol content was analyzed by the Folin-Ciocalteu method. The fluorescence resulting from oxidation of ROS-sensitive dye, dihydrorofluorescein (DHF), was utilized as indicator of the ROS level. Pretreatment with all plant extracts produced the dose-dependent increase in the DNA protection up to the 0.2 µg/ml polyphenol content and the decrease above that dose. Only the *Thymus piperella*, similarly to quercetin, showed a strong positive correlation between the DNA protection and the polyphenol content, but negative correlation with ROS formation. In conclusion, the DNA protective ability of plant extracts seems to be related to the low polyphenol concentration and only to certain extent depends on the polyphenol ROS scavenging activity.

Key words: *Mediterranean plant extracts, oxidative DNA damage, comet assay, polyphenols, reactive oxygen species*

INTRODUCTION

The populations in the Mediterranean region, according to the epidemiological studies, have the lowest prevalence of many degenerative diseases, including cancers (1), which have been ascribed to the reactive oxygen species damage (2). This phenomenon seems to be associated with the healthy plant-based diet comprising complex polyphenols as well as individual flavonoids (3). Polyphenols belong to the most potent anti-oxidants and because of that may protect against cancer through inhibition of oxidative damage, which is likely to be an important cause of mutation (4). Other proposed mechanisms for explaining their action include antiproliferation, estrogenic/antiestrogenic activity, induction of cell-cycle arrest and apoptosis, modulation of activities of many enzymes, induction of detoxification enzymes, and changes in cellular signaling (5-10).

The antioxidative property of polyphenols is a predominant feature of their radical-scavenging capacity (11-13). However, their metal-chelating potential can not be ignored (14). The plant species contain several thousand polyphenols, but most probably only a limited number of them is important for human health. Flavonoids are the best known among them. The estimation of the polyphenol content in plants is very difficult, mainly because of the polyphenol structural diversity (15). In most of the studies, which refer to the polyphenol content, the total phenols were estimated by reduction of the Folin-Ciocalteu reagent (16). The antioxidative power of the individual compound depends on their chemical structure, which is also responsible for the stability of the reactive flavonoid radicals (17). The less stable radicals, formed during the redox-cycle reaction, can propagate the harmful events through the radical attack (18, 19). Thus, plant extracts can act as an anti-oxidant or pro-oxidant, depending on the structure and composition of different classes of polyphenols (19-21). The interaction between individual polyphenols may decide about the final outcome. Because of that, the results obtained when the crude plant extracts are used have to be considered with special precaution.

Since the oxidative DNA damage can play a significant role in mutagenesis, cancer, aging, and other human pathologies (22-24), the decrease of the oxidative stress seems to be the best strategy possible to achieve by eating food rich in antioxidants and/or by taking supplements containing polyphenols, for example, plant extracts (25). Because the nutraceuticals are the most commonly consumed health care products lately, a scientific evaluation of them is a very urgent issue.

The aim of this study was to establish whether the protective ability of the six plant extracts against the oxidative stress induced by the hydrogen peroxide treatment in human lymphocytes is relative to the polyphenol content and their ROS scavenging activity.

MATERIALS AND METHODS

Reagents

The agaroses [normal and low melting point (LMPA), propidium iodide (PI), phosphate-buffered saline (PBS), hydrogen peroxide (H₂O₂), dimethyl sulphoxide (DMSO), quercetin, dihydrofluorescein diacetate (HFLUOR-DA)] and other chemicals (Tris, sodium chloride, sodium hydroxide, Triton X-100, EDTANa₂H₂) were acquired from Sigma-Aldrich; the medium (RPMI 1640), serum (FBS), phosphate buffered saline (PBS) and antibiotics, from Gibco; microscope slides and cover slides from DHN, Poland. The six Mediterranean plant extracts were obtained by ethanol extraction of dried plants from cultivated and non-cultivated food plants traditionally consumed in the selected areas of Spain, Greece and Italy, and provided by the group of Drs. Diego Rivera, Universidad de Murcia, Spain, and Michael Heinrich, the School of Pharmacy, University of London, UK, partners from the Local Food Nutraceuticals EU FP-5 project. The plant extracts from the following plants were used: *Amaranthus sp.* (from Greece; *Amaranthaceae*, leave;), *Crepis vesicaria* L (from Spain; *Asteraceae tender*, leaves), *Origanum heracleoticum* (from Italy; *Lamiaceae*, flowering tops), *Scandix australis* L (from Spain; *Apiaceae*, aerial parts), *Scolymus hispanicus* L (from Spain; *Asteraceae*, raquis); *Thymus piperella* L (from Spain; *Lamiaceae*, aerial parts). The extraction procedure will be reported elsewhere by that group.

Plant extracts

Each plant extract was dissolved in DMSO at the concentration of 50 mg/ml, aliquoted and stored at -80°C until needed. The solvent, at the same concentration as for the experiment with the plant extracts was used as control. The concentration of DMSO never exceeded 1%. The total polyphenolic content of extracts was determined by the Folin-Ciocalteu method, using gallic acid as the reference compound; it was provided by Dr. Francesco Visioli from the Department of Pharmacological Sciences, University of Milan, Italy (26).

Mononuclear blood cell preparation

Experiments were performed on lymphocytes isolated from the male blood donors using the Histopaque technique, according to the manufacturer's instructions and frozen at -80°C in "freezing medium", consisting of 65% RPMI-1640, 25% fetal bovine serum and 10% DMSO.

Experimental procedure

Lymphocytes were thawed, washed in PBS, resuspended in RPMI 1640 supplemented with 10% of fetal bovine serum, and incubated with the plant extracts at the concentrations of 0.1, 1, 10, and 100 µg/ml for 1 h at 37 °C in the CO₂ humidified incubator.

Following pretreatment, lymphocytes were washed twice with PBS to prevent direct extracellular interactions between compounds and hydrogen peroxide. Then, they were mixed with 0.5 % LMPA and spread on slides pre-coated with 0.5% agarose. Those which had to be treated with hydrogen peroxide were immersed in cold 25 µM hydrogen peroxide for 5 min. After quick washing, the comet assay was proceeded.

Quercetin was used as the internal standard at the same range of the concentrations.

Alkaline Single Cell Gel (SCG) Electrophoresis - Comet Assay

The microgel electrophoretic technique was performed according to the original laboratory protocol for the application of the pH>13 alkaline single cell gel assay. The endonuclease III (0.1

unit/slide) in HEPES buffer was used to disclose the oxidized pyrimidine bases in DNA. The electrophoresis was conducted at 0.74 V/cm for 20 min. The details of the procedure are published in this issue (27).

For visualization of the DNA damage, observations were made on the epifluorescence microscope (Olympus IX-50), equipped with the appropriate filters. Images were transported through a CCD camera to the computer software (CometPlus from Theta System GmbH, Germany). The percentage of tail DNA was automatically generated by the image analysis system. At least two slides per one experimental point, with 50 randomly selected cells per slide, were analyzed. Each experimental point was run in duplicate.

Viability Assay

Fluorescein diacetate (10 μ M) and ethidium bromide (25 μ M) were used to determine the cell viability. The viability in control, and immediately after hydrogen peroxide treatment, was constantly found to be over 95%.

Reactive oxygen species detection

To detect a broad spectrum of ROS, lymphocytes were loaded with the dihydrofluorescein diacetate (HFLUOR-DA) for 30 min. at 37°C. The final concentration of 20 μ M was prepared from the stock solution, which contained 1mg/ml substrate dissolved in ethanol. Following centrifugation, lymphocytes were incubated for 1 h with the plant extracts at the final concentrations 0.1, 1, 10, 100 μ g/ml in PBS. Next, after the plant extracts removal by the centrifugation, the cells were treated with hydrogen peroxide (25 μ M at 4°C for 5 min.). The untreated cells, the cells incubated with the DMSO at same concentrations as used in the experiments with the plant extracts, lymphocytes treated only with hydrogen peroxide were also included for the fluorescence measurement (DHF). The extracellular supernatant, as well as lysed pellets (50 mM potassium phosphate, 1% Triton X-100 and 0.1 mM EDTA), were measured using SPECTREAFleurPlus (Tecan, Austria) for 4 min. in 10 kinetic cycles at λ_{em} 485 and λ_{ex} 535 nm and analyzed by the software Magellan v3.0. The results were calculated according to the following equation: $[(DHF \text{ of the sample treated by the plant extracts and followed by the hydrogen treatment} / DHF \text{ of the sample treated only by hydrogen peroxide}) * 100] - 100$. The obtained values reflect changes caused by the plant extracts pretreatment in relation to the fluorescence induced by the hydrogen peroxide treatment taken as 100%. These values are presented in the graph as "% of ROS production", where values above zero correspond to enhancement of the ROS production, while those below zero represent the lowered level of ROS formation, as compared to ROS produced after hydrogen peroxide treatment when it was used alone. The experiments were repeated at least twice.

Statistical analyses

Statistical analyses were conducted using STATISTICA software, version 6. Data obtained from the comet assay (percentage of DNA in the comet tail, "TDC") were analyzed by t-test (two-sided) to evaluate the differences in means between TDC values obtained for the hydrogen peroxide treated lymphocytes and when the plant extracts preceded the oxidative stress. The TDC values followed log transformation due to heterogeneity of variance. The modifying ability of the plant extracts toward the extent of oxidative DNA damage induced by hydrogen peroxide was determined as follows: $100\% - [TDC \text{ (plant extracts + H}_2\text{O}_2\text{)} / TDC \text{ (H}_2\text{O}_2\text{)} * 100]$ and presented on the graph as the "% protection". Differences were considered significant if $P < 0.05$.

The linear regression analysis of the log polyphenol concentrations of plant extracts versus the percentage of DNA protection, as well as the mean values of the percentage of ROS net production were calculated from two experiments to assess the dose dependency between these three factors.

RESULTS

The 124 extracts from the wild growing plants from the Mediterranean regions were analyzed for their protective ability against the oxidative DNA damage induced in lymphocytes by hydrogen peroxide (25 μ M, 5 min., at 4°C). Extracts were provided by the partner from the Local Food Nutraceuticals EU FP-5. Three of those extracts (*Amaranthus sp.*, *Scolymus hispanicus*, *Thymus piperella*), when used at the concentration of 10 μ g/ml, showed over 20% anti-oxidant ability, and three extracts (*Crepis vesicaria*, *Origanum heracleoticum*, *Scandix australis*) less than 20% ability while being evaluated by the comet assay. The endonuclease III

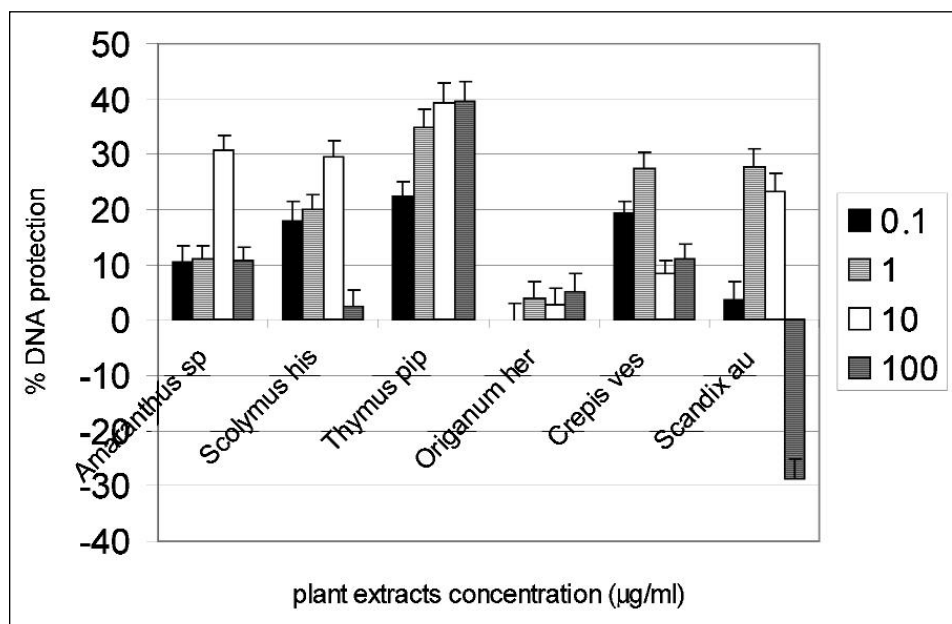


Fig. 1. The DNA protective ability of six plant extracts (*Amaranthus sp.*; *Scolymus hispanicus*; *Thymus piperella*; *Origanum heracleoticum*; *Crepis vesicaria*; *Scandix australis*). The percentage of oxidative DNA damage-induced by hydrogen peroxide (25 μ M for 5 min. at 4°C) in lymphocytes pre-treated for 1 h with four doses of plant extracts (0.1; 1; 10 and 100 μ g/ml). The percentage by which the plant extracts exerted the protective ability was calculated according to the equation: $100 - [(TDC \text{ for extracts} + H_2O_2) / TDC \text{ } H_2O_2] * 100$, where TDC is the percentage of DNA in the comet tail. The difference between the mean of TDC values for H_2O_2 and the combined treatment following log transformation was evaluated by t-test (two-sided) and were considered significant if $P < 0.05$. Only *Origanum heracleoticum* did not show significant difference, as compared to hydrogen peroxide treatment given alone.

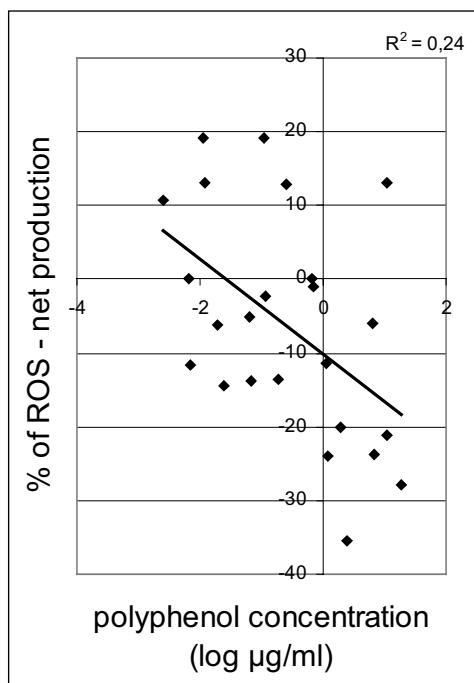


Fig. 2. Positive correlation between polyphenol concentration, expressed as log values, and the percentage of changes in the reactive oxygen species intracellular formation calculated according to the equation: [(DHF fluorescence of the sample treated by the plant extracts followed by hydrogen peroxide treatment/ DHF fluorescence of the sample treated only by hydrogen peroxide)*100] –100. The values above zero correspond to the enhancement of ROS production while those below zero represent the lowered level of ROS formation, as compared to ROS produced after hydrogen peroxide treatment when it was used alone. There was a significant dose-response relation, with a significant decrease of the % of ROS-net formation estimated by linear regression analysis ($P = 0.014$).

was used as the repair enzyme, specifically recognizing the oxidized pyrimidine bases. An increase of the fluorescence level in comet tail was observed, in comparison to the level detected in the no-enzyme-treated comets. All six extracts were chosen for further analysis. As shown in *Fig. 1*, the pre-treatment of lymphocytes with the plant extracts within the range of the concentrations from 0.1 to 10 µg/ml resulted in the different degree of the DNA protection against the hydrogen peroxide-induced damage. The plant extracts, when used at 100 µg/ml concentration, showed much lower protection than at other concentrations, with significant enhancement of the DNA damage exerted for *Scandix australis*. The dose-related DNA protective ability, within the whole range of studied concentrations, was only observed for *Thymus piperella*. Other plant extracts, when used at lower concentrations, showed the significant protection (except *Origanum heracleoticum*). The extent of protection was, however, different for each plant extract. Since the polyphenol content was different for each plant extract (mean value was 97 mg/g ± 57.4), the concentration of polyphenols was used for further analysis instead of the amount of plant extracts. The mean polyphenol concentration calculated from the plant extract doses used for the experiments was 2.7 µg/ml ± 4.9. No association was found between the polyphenol content and the percentage of DNA protection. However, the significant correlation ($P=0.014$) was observed between the polyphenol content and the level of the intracellular ROS formed within lymphocytes pretreated with

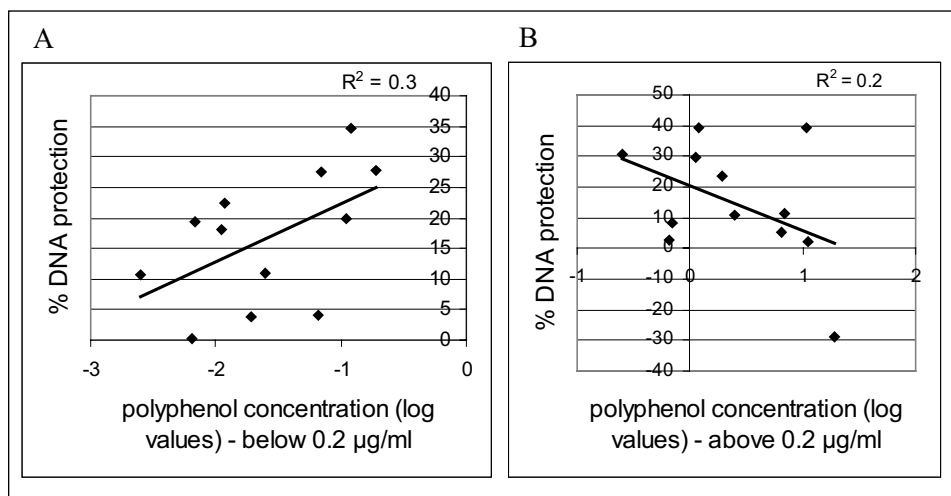


Fig. 3. The DNA protective effect grouped according to the median polyphenol concentration in all six investigated plant extracts. The relationship between the % of DNA protection against oxidative DNA damage induced by hydrogen peroxide (25 µM for 5 min. at 4°C) in lymphocytes pre-treated with the plant extracts and the log values of polyphenol concentrations below 0.2 µg/ml (A) and above 0.2 µg/ml (B) within each of those plant extracts. P values were 0.08 and 0.1, respectively.

the plant extracts before the hydrogen peroxide treatment (Fig. 2). The percentage of the ROS net values above zero on the figure axis indicates the heightened ROS production; when the experimental points appear below zero, they reflect the decrease of the ROS formation, as compared to that induced by the H₂O₂ treatment alone. Despite the fact that correlation between these variables was significant, the model explains only 24% of the original variability between the ROS production and polyphenol concentrations.

In further analysis, the polyphenol content in all plant extracts was grouped according to the median value (below and above 0.2 µg/ml). As shown in Fig. 3A and 3B, the DNA protection presented a two phase response. For concentrations of polyphenols up to 0.2 µg/ml, the percentage of protection against oxidative DNA damage induced by hydrogen peroxide increased (P= 0.08), whereas above that dose the attenuation of DNA protection was observed (P=0.1). There was also a substantial variation of the observed points around the fitted regression line. Moreover, the intracellular level of ROS was not significantly related to the polyphenol content within these groups.

Thymus piperella was the only extract which exhibited not only the positive correlation (P<0.1) between the DNA protective ability and the polyphenol content (Fig. 4A), but also the strong negative correlation between the polyphenol content and the intracellular (P=0.06) and extracellular (P= 0.03) ROS formations (Fig. 4B). The level of the intracellular ROS had decreased with the increasing polyphenol concentrations. As the ROS-net intracellular production values were

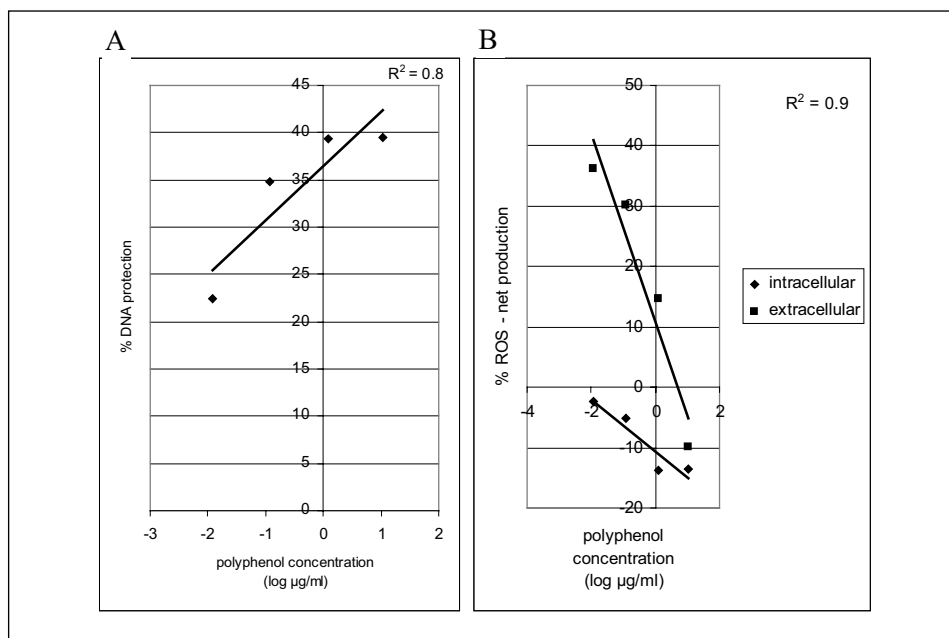


Fig. 4. The relationship between the log values of polyphenol concentrations ($\mu\text{g/ml}$) in *Thymus piperella* extract and A) the % of protection against the DNA oxidative damage induced by hydrogen peroxide (25 μM for 5 min. at 4°C) in lymphocytes pre-treated with this plant extract, and B) the % of changes in the reactive oxygen species intracellular and extracellular formation calculated according to the equation: [(DHF fluorescence of the sample treated by the plant extracts followed by hydrogen peroxide treatment/ DHF fluorescence of the sample treated only by hydrogen peroxide)*100] -100. The values above zero correspond to the enhancement of ROS production, while those below zero represent the lowered level of ROS formation, as compared to ROS produced after the hydrogen peroxide treatment when it was used alone. There was a significant dose-response relation with a significant decrease of the % of the intracellular and extracellular ROS-net formation estimated by linear regression analysis ($P = 0.06$ and 0.03 , respectively).

located below zero, they reflected the ability of polyphenols within the appropriate concentrations to lower the level of ROS induced by hydrogen peroxide. At the same time, when the ROS production was lowered within the cell pellets, the extracellular ROS, released into the surrounding medium by lymphocytes pretreated with the *Thymus piperella* and exposed to H_2O_2 , was enhanced above the value obtained for the H_2O_2 treatment given alone. The extracellular ROS production reached the maximal value for the lowest polyphenol concentration and was linearly decreasing when the concentration was rising. The level of extracellular ROS reached the comparable value with the intracellular ROS at the highest concentration (11 $\mu\text{g/ml}$). Quercetin, as the positive standard, was also employed within the range of concentrations (0.03 to 16 $\mu\text{g/ml}$), comparable to those of polyphenols in the plant extracts used for our experiments. Pretreatment of lymphocytes with quercetin for 1 h significantly

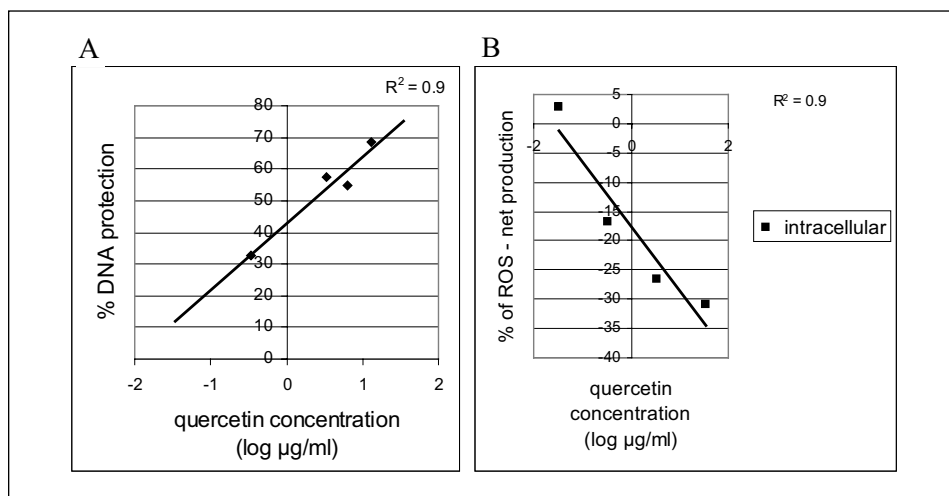


Fig. 5. The relationship between the log values of quercetin concentrations ($\mu\text{g/ml}$) and % by which quercetin protects lymphocytes against the hydrogen peroxide-induced DNA oxidative damage (25 μM , 5 min., at 4°C), and A) the % of DNA protection, and B) the % of changes in the reactive oxygen species intracellular formation calculated according to the equation: $[(\text{DHF fluorescence of the sample treated by the plant extracts followed by the hydrogen peroxide treatment} / \text{DHF fluorescence of the sample treated only by hydrogen peroxide}) * 100] - 100$. Quercetin was present for 1 h before oxidative stress and removed before hydrogen peroxide treatment.

reduced the oxidative DNA damage induced by hydrogen peroxide in a dose-related fashion (*Fig. 5A*). The intracellular ROS formation induced by hydrogen peroxide had decreased also in the dose-dependent manner when lymphocytes were pretreated with quercetin.

DISCUSSION

The protective effect of the Mediterranean diet against cardiovascular, as well as other degenerative diseases has been attributed to the significantly higher consumption of foodstuff of plant origin (28). Fruits and vegetables guarantee a high intake of phytochemicals, which seems to be involved in many cellular processes preventing the harmful effect of the elevated level of the oxidative stress (29-31). Enriching the daily nutrition in anti-oxidant compounds seems to be the best possible preventive strategy. The naturally occurring antioxidants among wild edible growing plants across the Mediterranean region, which are able to exert positive effect upon cellular protection, and possible upon human health, were often studied recently (28, 32-34). The first screening of any compounds, drugs or potential nutraceuticals starts with the genotoxicity test. The comet assay, broadly used in recent years, was also applied in our study to analyze the effect of plant extracts on the DNA level (35-38). Endonuclease III

was introduced to examine the level of oxidized pyrimidines in DNA (39). Over 120 plants, adopted by local population as part of its daily foodstuff, were collected in remote areas of Spain, Greece and Italy. None of these ethanolic extracts (prepared by the partners for the project: Local Food and Nutraceuticals, EU FP-5), when used at the concentration of 10 $\mu\text{g/ml}$, were itself cytotoxic (fluorescence/ EtBr test) or genotoxic to lymphocytes, when treated for 1 h. The association between the doses (0.1 to 100 $\mu\text{g/ml}$) of the six chosen extracts (*Amaranthus sp.*, *Scolymus hispanicus*, *Thymus piperella*, *Crepis vesicaria*, *Origanum heracleoticum*, *Scandix australis*), and their DNA protective ability against the oxidative DNA damage induced by hydrogen peroxide, revealed a very broad spectrum of response, confirming our expectation, that the constituent of the plant extracts is much more significant than the dose used. In some cases, 100 $\mu\text{g/ml}$ concentration was very protective (for *Thymus piperella*), whereas for *Scandix australis* it enhanced the DNA damage induced by hydrogen peroxide. As the phenolic compounds contribute significantly to the antioxidant capacity (40), we attempted to correlate the polyphenol content in the plant extract doses used in the experiments with their ability to influence the extent of the oxidative DNA damage induced by hydrogen peroxide. The correlation between the logarithmic values of polyphenol concentrations (from 0.0025 to 19.2 $\mu\text{g/ml}$) was not found. When the analysis was performed within the group containing polyphenols in the amount below and above the median value, the protective tendency was visible only when the concentration was lower than 0.2 $\mu\text{g/ml}$. Higher amount of polyphenols decreased the protective ability, which could result from outweighing their oxidative potential over their antioxidant potential in a cellular environment. The observed enhancement of the DNA damage could be caused by the autooxidation of polyphenols and accumulation of damaging intermediates, or by the presence of the trace amount of transient metal ions, which were able to catalyze the formation of hydroxyl radicals and induce additional damage to DNA (41-43).

The ROS scavenging activity seems to account for the protective ability of polyphenols (44). When the relationship between the polyphenol content and ROS induced by the hydrogen peroxide removal was evaluated, the significant statistical dependence ($P= 0.014$) was observed between these two factors. These results implicate that the DNA protective ability of the investigated plant extracts only to certain extent depends on the ROS scavenging ability of polyphenols. Other factors as: interactions between the plant extract compounds and the cellular milieu, the mixed composition of polyphenolic compounds, the differences in the ratio between the individual compounds, or presence of certain amount of the non-Folin-Ciocalteu reactive substance can play a very important role. Many papers published recently underlined this issue. Certain plant extracts exhibit protective properties against the oxidative stress (45, 46) while other are cytotoxic or genotoxic (37, 47-49). These differences are not necessarily the inner property of some plant extracts but can be also attributed to the variation in the

redox state of cells used in the experiments, the trace amount of the metals in the incubation medium, or even the individual variants in the blood of donors, when the white blood cells are used (47, 50).

To understand the involvement of polyphenols in the antioxidant activity of the dietary non-nutrients, the selected flavonoids were used in most of the studies. Their biological activity usually correlates with the chemical structure (17, 51-53). Quercetin, the most abundant flavonoid in foodstuff, is most frequently used in such studies (54). Nevertheless, the search across the literature revealed that even for such a simple compound the obtained results are very controversial (37, 55-58).

In our studies, quercetin produced the dose-dependent DNA protection against the oxidative DNA damage induced by hydrogen peroxide, and its ROS scavenging activity increased linearly with dose. This proves that quercetin exerts a site specific protection, which is related to the ROS removal ability. *Thymus piperella* extract was the only one, which showed similar linear dependence between the DNA protective ability, polyphenol concentrations and the intracellular ROS reduction. In addition, the interesting observation was made, when the fluorescence of DHF, which reflects the level of the oxidative stress, was measured extracellularly. The extent of the ROS produced and released to the surrounding medium by lymphocytes pre-treated by the *Thymus piperella* extract, followed by incubation with hydrogen peroxide, was higher, as compared to hydrogen peroxide given alone, and was decreasing linearly with the polyphenol concentrations. This phenomenon may explain the medical properties of thyme, well recognized already by traditional medicine (59). Thyme is a very well known remedy for treatment of many respiratory diseases (59). Its chemical composition was extensively studied (60, 61). Some of its compounds showed antimicrobial and antifungal activities (62, 63). They are also very efficient antioxidants (62, 63). Moreover, the flavonoid-mediated immunomodulatory effect (66, 67) is currently studied for development of new flavonoid-based nutraceutical agents.

In conclusion, the plant extracts derived from the Mediterranean region can be very effective antioxidants and can protect lymphocytes against the oxidative stress. This activity is related only to certain range of polyphenol concentrations, which in turn efficiently scavenges the reactive radical species. However, as shown recently, not the overall DNA protective effect of flavonoids can account for their antioxidant ability. For example, quercetin (up to 10 μM) inhibits the H_2O_2 -induced NF- κB transcriptional activation and inhibits DNA strand breaks produced by H_2O_2 (68). Although antioxidant properties are important, the influence of polyphenols on the regulation of gene expression in relation to their protective ability can have a far greater impact than previously thought.

Since in many countries there is a growing interest in consuming various food supplements, particularly plant extracts, more studies are needed to release them safely to the market, especially as concentration of flavonoids in such

supplements greatly exceeds the flavonoid concentration in diets high in plant products, including the vegetarian diets.

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