Defence Life Science Journal, Vol. 2, No. 3, July 2017, pp. 301-309, DOI : 10.14429/dlsj.2.11676
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RESEARCH PAPER

Comparative Real Time EPR Investigation of Natural and Synthetic Antioxidants: As Potential Anticancer Agents and Radio-protectors

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ABSTRACT

The purpose of this study was to evaluate and compare the free-radical scavenging activity against DPPH stable radical and protective properties of the natural products SQGD and *P. corylifolia* and synthetic nitroxyl- free radical containing nitrosoureas SLENU and SLCNUgly against in vivo oxidative toxicity of antitumor drug CCNU. It was found statistically significant increase in the DPPH radical-scavenging capacity of both extracts with increase of radiation. The natural antioxidants were localised mainly in the organs and blood after EPR biodistribution study. All combinations of natural extracts/ synthetics agents exhibited considerably lower levels of Asc. radicals as compared to controls. It should be mentioned that the natural antioxidants possess higher oxidative protection in comparison with the synthetic antioxidants. Considerable decrease in ROS production in livers of mice was found after treatment with SQGD, *P. corylifolia* and SLENU, alone, compared to controls.In conclusion, because of well-expressed antioxidant activities of natural and synthetic antioxidants they might be used in the combination anticancer chemotherapy for reducing toxicity caused by anticancer drugs and/or low levels radiation therapy.

Keywords: DPPH-activity; EPR; Distribution; Radicals; ROS-productionproperties

1. INTRODUCTION

Investigation and development of effective antioxidants as potential anticancer agents and radio-protectors is of a great importance in view of their possible application during radiation exposure[1,2] and medicinal use[3]. Investigations of radio-protectors have been focused on screening of different chemical and biological compounds[4] of synthetic and natural origin. Natural substances[5], immune-modulators[6], sulphydryl compounds[7], vitamin A, C, E[8], DNA- binding ligands[9], nitrosoureas and triazens[10] have been studied in *in vitro*/ *in vivo* models and used for neutralizing of radiation exposure[11]. Searching for potential combination of microbial/ herbal compounds and synthetic drugs at pharmacological processes, in the field of radical-scavenging biology and reduction of ROS/RNS levels, considerably has increased during the past 10 years[12, 3].

Several spin-labelled nitrosoureas, analogues of the anticancer drug CCNU, were tested for their protective and anticancer activity and as radio-modulating agents with low toxicity[13,14]. Nitroxyl- containing nitrosourea compounds showed *in vitro* good antioxidant activity[15] and higher alkylating[15,16], and twice lower carbamoylating activity[16] comparing to their analogue, CCNU. Moreover, some of these nitrosoureas showed higher anti-leukemic and anti-melanomic effect and better immune-modulatory properties, as compared to non-labeled CCNU[17]. To reduce oxidative stress negative consequences, induced by radiation or chemotherapy, a number of microbial/ phyto materials and synthetic antioxidants have been successfully utilised[18-21].

Various combinations can reduce the levels of oxidative stress biomarkers and cause protective efficacy[22,] due to their multi-plant content or chemically-isolated constituents. Arora[23-24], *et al.*, report for presence of plant polyphenols, immune-stimulants, cell-proliferation stimulators, anti-inflammatory agents which can provide protection against radiation exposure and cancer- induced damages[23,24].

Direct and indirect (spin-trapping) electron paramagnetic resonance (EPR) spectroscopy is the most convenient technique for *in vitro* investigation of free radicals and paramagnetic structures, as well as their *ex vivo* distribution in organs[25-27]. EPR spin trapping technique allows be monitored processes related to generating unstable radicals (ROS and RNS), the mechanisms of their transformations and changes in membrane-oxidation levels[27]. Moreover, EPR spectra provide information about chemical structure of the studied radicals and as well as, evaluation of antioxidant activity and radical-scavenging

Received : 21 February 2017, Revised : 12 June 2017
Accepted : 15 June 2017, Online published : 02 August 2017

capacity[27] of different compounds[27,28]. All advantages of EPR spectroscopy led us to conduct current research.

The purpose of the present study was to investigate and compare the changes in radical-scavenging capacity, antioxidant and radio-modulatory properties of antioxidants with natural and synthetic origin, as well as their combinations, by use of EPR methods.

2. MATERIAL AND METHODS

2.1 Natural Compounds

Semiquinone glucoside derivative (SQGD), a bacterial metabolite, was isolated from radio-resistant *Bacillus sp INM*-1*,* using solvent fractionation and extraction procedures (i.e. column chomatography, UV- VIS, fluorescence, FTIR, H-NMR, LC-MS, and EPR) as described in[29].

Psoralea corylifolia Linn (*P. corylifolia*) was collected from plants growing in Bhopal, Madhya Pradesh, India and made from fresh seeds/ coarse powder dissolved in 2l of distilled water and subjected to cold maceration process for 24h and characterised by EPR[30].

2.2 Synthetic Agents

Nitroxyl- labeled agents1-ethyl-1-nitroso-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-urea (SLENU) and N-[N'-(2-chloroethyl)-N'-nitroso carbamoyl-glycine amide of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (SLCNUgly) analogues of the anticancer drug Lomustine (CCNU), were synthesised as described previously[10, 31].

3. CHEMICALS

Stannous chloride dehydrated ($SnCl_2.2H_2O$), 2,2-dipheniyl-1-picrylhydrazyl (DPPH), the spin-trapping agents, naphthylethylene diamine (PBN), phenilbuthylsufoxide (PBS), $K_3[Fe(CN_6)]$and Lomustine (CCNU) were purchased from Sigma-Aldrich Chemical Co, St. Louis, USA. Deionised and distillated water was used for all experiments. Other chemicals used were analytical or HPLC grade.

3.2 Instrumentation

EPR measurements were performed on an X-band EMX^{micro}, spectrometer (Bruker, Germany), equipped with standard Resonator. Experiments were carried out in triplicate.

3.3 Radiation Exposure of Natural Compounds

Natural samples were irradiated directly:

1 group: Solution samples were irradiated with UV-light (UV–VIS Transilluminator – 4000, Stratagene, USA) in the range of 290 nm -3 20 nm light-waves/ 2 h, relative humidity of 43 per cent.

2 group: Solution samples were irradiated with ^{60}Co source at doses of 5, 10, 20, and 30 Gy in a γ-chamber (Gamma cell 5000, Board of Irradiation and Isotope Technology, BRIT, Mumbai, India).

4. EXPERIMENTAL ANIMALS

Male albino mice (specific pathogen-free, second line, non- inbred, weighted 35 [±3] g, 5-6 weeks old) were purchased at two-weeks of age; housed in polycarbonate wire floor cages in controlled conditions (12 h light/ dark cycles); 18 °C - 24 °C and humidity of 40 per cent – 60 per cent; free access to tap water and standard laboratory chow were maintained. Mice were divided into 11 experimental groups and 1control group (x 6 animals) and fed, respectively:

Control group - standard diet;
1 group –SQGD (40mg/kg);
2 group –*P. corylifolia*(40mg/kg),
3 group –SLENU (40mg/kg);
4 group –SLCNUgly (40mg/kg);
5 group –SQGD + SLENU (40/ 40mg/kg);
6 group–SQGD + SLCNUgly (40/ 40mg/kg);
7 group –*P. corylifolia*+ SLENU (40/ 40mg/kg);
8 group –*P. corylifolia* + SLCNUgly (40/ 40mg/kg);
9 group –SQGD + CCNU (40/ 40mg/kg);
10 group –*P. corylifolia* + CCNU (40/ 40 mg/kg).
11 group –CCNU (40mg/kg);

Experiments were carried out in accordance with national regulations (approval from Research Ethics Commission of the Medical Faculty, Trakia University, Stara Zagora) and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes.

4.1 *Ex vivo* EPR Study on Blood Clearance of the Drugs

After i.p. administration (40 mg/kg), blood sample were taken from the free streaming blood of mice at the indicated periods of time and were collected into heparinised tubes containing cold PBS (pH=7-7.4).

4.2 *Ex vivo* EPR Study on Organ and Blood Bio Distribution of Tested Drugs

Study of drugs biodistribution in organ homogenates (liver, lungs, spleen, pancreas, brain, kidneys) and serum was evaluated and compared as previously described by[15]. Agents were i.p. administrated at a single dose of 40 mg/kg. Animals were decapitated at the time points following injection (10, 30, 60, 90 min and on the 4h, 24h) and dissected. Tissues and serum were collected and processed immediately. For drugs extraction, samples were weighed, homogenised in cold PBS (10 per cent w/v) and centrifuged at 2000 g for 15 min. Supernatants were collected and concentration of each tested natural/synthetic antioxidant was evaluated by double integration of plots under EPR spectra registered in the sample containing the respective drug. In view, fast *in vivo* reduction of the nitrosourea nitroxyl function before to measure concentration of SLENU or SLCNUgly the samples were reoxidised by $K_3[Fe(CN_6)]$.

4.3 *In vitro* EPR Study on DPPH Radical Scavenging Capacity

Scavenging abilities towards DPPH radical was studied according to[32] with slight modifications[27].SQGD and *P. corylifolia* samples (40μg/ml) were dissolved in d.water and after UV/γ-irradiation was added to 250 μl ethanol solution

of DPPH (80 µmol/l). After stirring, the mixtures were incubated for 10 min/in the dark and immediately transferred and placed in the EPR cavity. Control sample containes 250 µl of ethanol solution of DPPH + 50 µl of ethanol. DPPH radical scavenging capacity of the tested samples was calculated according to the equation:

Scavenged DPPH radicals (%) = $[(I_0 - I)/I_0] \times 100$

where I_0 is integral intensity of the DPPH signal of control sample and I is the integral intensity of the DPPH signal after addition of the tested drugs to control sample.

EPR settings were as follows: center field 3516.00 G, sweep width 200.00 G, microwave power 3.232 mW, modulation amplitude 5.00 G, receiver gain 5.02×10^3, time constant 163.84 ms, 1 scan per sample.

4.4 *Ex vivo* EPR Study on Ascorbate Radical Levels

Ascorbate radical (Asc·) levels in liver and blood were studied according to[33] with slight modifications. Liver tissues and blood were isolated at 4th h after i.p. administration and collected immediately in cold saline and processed. Tissue were weighed, homogenised in DMSO (10 per cent w/v) and centrifuged at 4000 g for 10 min, at 4 °C. Supernatants were collected and their EPR spectra were recorded. The levels of Asc· were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units. EPR settings were as follows: centre field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1 x 10^4; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1 scans per sample.

4.5 *Ex vivo* EPR Study on Levels of ROS Production

The level of ROS production was studied according to[34] with some modifications by[27]. Briefly, about 0.1 g of liver and serum (4h after i.p. administration) were homogenised after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. After centrifugation at 4000 g, at 4^0C/ 10 min supernatants were collected and their EPR spectra were recorded. The levels of ROS products were calculated after double integrating of EPR spectra of the registered PBN spin-adducts, and results were expressed in arbitrary units. EPR settings were as follow: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain 1 x 10^6; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92 s, 5 scans per sample.

5. STATISTICAL ANALYSIS

Statistical analysis was performed with Statistica 6.1, Sta-Soft, Inc. and results were expressed as mean ± standard error (SE) or standard deviation (SD). Statistical significance was determined by the Student's t-test.

6. RESULTS

6.1 EPR Study on DPPH Radical Scavenging Capacity

DPPH radical scavenging capacity of SQGD and *P. corylifolia* before and after UV, and γ- irradiation is shown in Fig. 1. For SQGD and *P. corylifolia* before irradiation, maximal

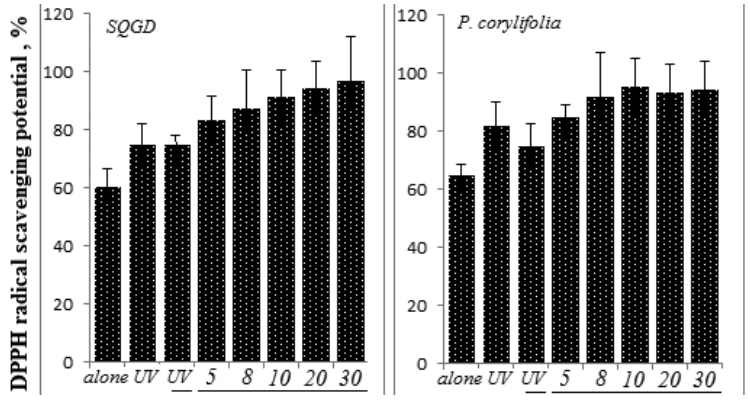

Figure 1. EPR determination of DPPH radical scavenging potential of SQGD and *P. corylifolia* (40 µg/ml) alone and after UV and γ- irradiation.

DPPH scavenging capacity was found 60.02 ± 0.074 % and 66.52 ± 0.089 %, respectively while for irradiated samples up to 20 Gy was established statistically significant higher scavenging activity (81.11 ± 2.94 % for SQGD and 73.35 ± 1.67 %, for *P. corylifolia*, respectively). Statistically significant increase in radical-scavenging ability of both extracts was observed with increasing radiation.

6.2 EPR Blood Clearance Study

Results from blood clearance of SLCNUgly, SLENU, SQGD and SLCNUgly are showed in Fig. 2. Maximal concentration for SLCNUgly and SLENU was reached at 10th and 60th min, respectively and was completely reduced to 6th hrs. Maximal concentration of SQGD and *P. corylifolia* was reached at 30th and 90th min, respectively p. i. and then declined gradually.

6.3 EPR Study on Organ and Blood Bio Distribution of the Tested Drugs

EPR biodistribution of natural and synthetic agents monitored in organ homogenates and in whole blood at different time intervals is shown in Fig. 3. As is seen within 4th h the intensity of EPR spectra registered in the four drugs samples was almost completely disappeared in all studied tissues and blood. The main localisation of natural compounds was in the liver and lungs for *SQGD* and pancreas and spleen for *P. corylifolia*. For SLENU and SLCNUgly maximum concentrations were detected in liver and in most of the other organs at 60th min and at 10th min, after administration. Following 90th min in all tested organs was registered a triplet signal with extremely feeble intensity.

6.4 *Ex vivo* EPR study on ascorbate radicals levels

Figure 4 presents the results of Asc· levels measured in liver and blood at 4th h. Asc· levels in the livers after SQGD or *P. corylifolia* administration (Fig. 4(a)) was close or considerably lower than the controls (average 0.22 ± 0.007 vs. mean 0.208 ± 0.01, SQGD and 0.13 ± 0.00 vs. mean 0.208 ± 0.01, *P. corylifolia*, *p<0.00001). The combinations of SQGD + CCNU and *P. corylifolia* + CCNU showed approximately 4.5-fold decrease in the level of Asc· compared to CCNU treated animals (**p <0.00003). In serum natural antioxidants

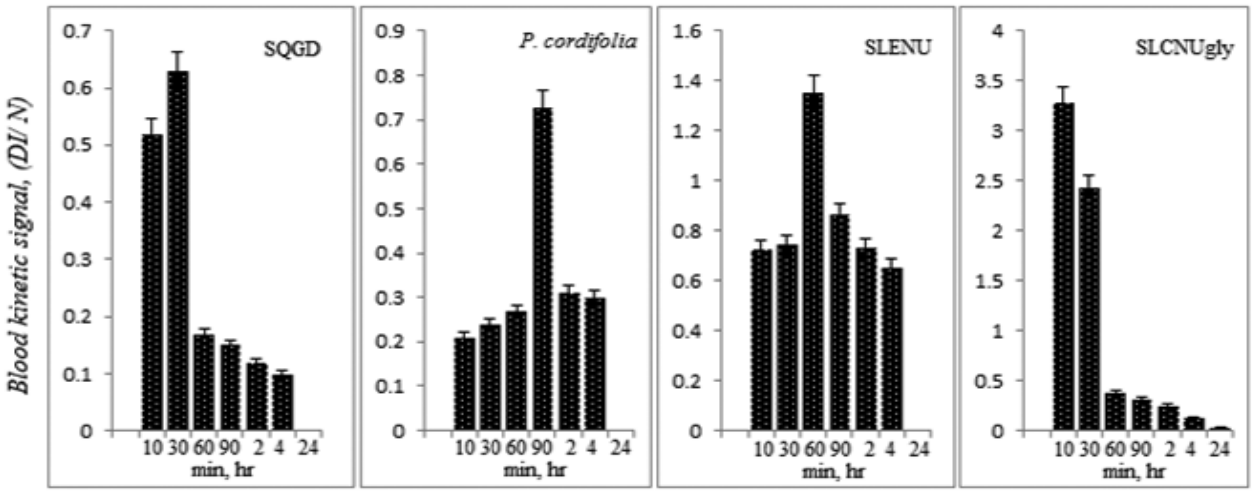

Figure 2. EPR measurements of blood kinetic signal (arbit. units) of SQGD, *P. corylifolia,* SLENU and SLCNUgly in mice (n=6). Concentration was calculated by double integration of the registered EPR spectrum.

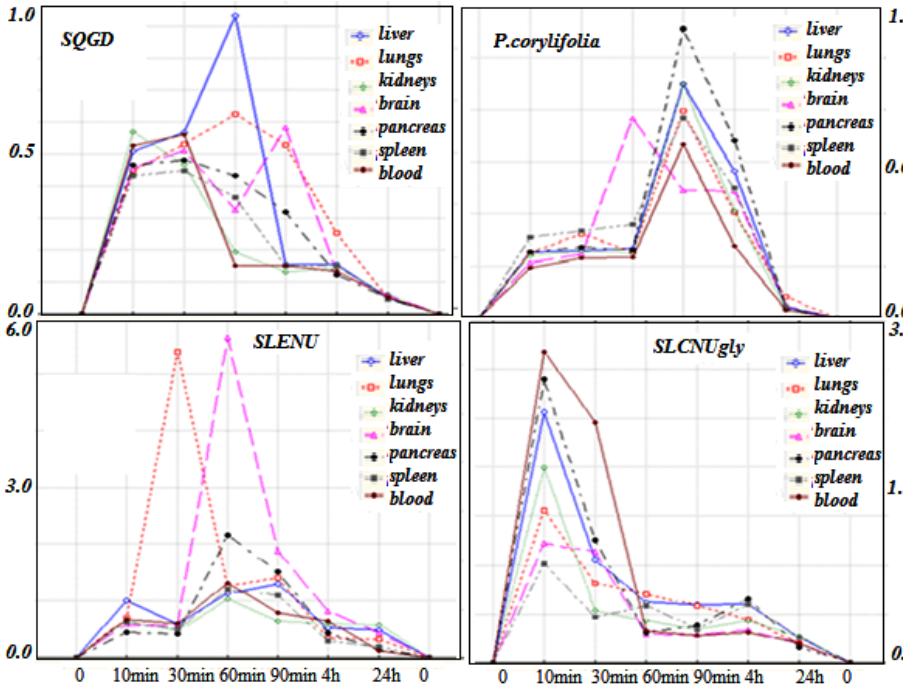

Figure 3. Tissue bio-distribution of SQGD, *P. corylifolia,* SLENU and SLCNUgly in organ homogenates (liver, spleen, lung, kidneys, pancreas, brain) and blood (n=6). Concentration was calculated by double integration and calculated g-value (arbit. units) of the registered EPR spectrum.

(Fig. 4(b)) exhibited twice reduced levels of Asc·, as compared to controls (average of 0.093 ± 0.0008 for SQGD and 0.0664 ± 0.018 for *P. corylifolia* vs 0.145 ± 0.003 for controls, au, *p <0.001). The groups treated with the synthetic agents only, showed high but not statistically significant Asc· levels, compared to controls. The four drugs combinations (Fig. 4(b)) exhibited considerably lower levels of Asc· as compared to controls.

In liver (*p<0.05) and blood serum (*p<0.005) (Fig. 4(a), (b)) of CCNU- treated mice was found statistically significant increase in Asc· compared to controls; and also statistically significant (*almost four-fold*) higher levels, compared to groups treated with natural and synthetic antioxidants, alone or in combinations (*p<0.005).

6.5 *Ex vivo* EPR study on levels of ROS production

ROS production in liver and blood plasma are shown in Fig. 5. After treatment with *P. corylifolia*, SLENU or SLCNUgly (Fig. 5(a)) in the livers was found statistically significant decrease in ROS production, comparing to controls (*p <0.002).

SQGD treated group showed considerably higher levels of ROS both in liver and blood plasma in comparison with controls, (**p <0.001), but exhibited statistically significant decrease compared to CCNU-treated mice (**p <0.005). SQGD + CCNU combination showed a statistically significant reduction in ROS products, compared to those registered in CCNU and SQGD, alone (**p<0.05). Levels of ROS in

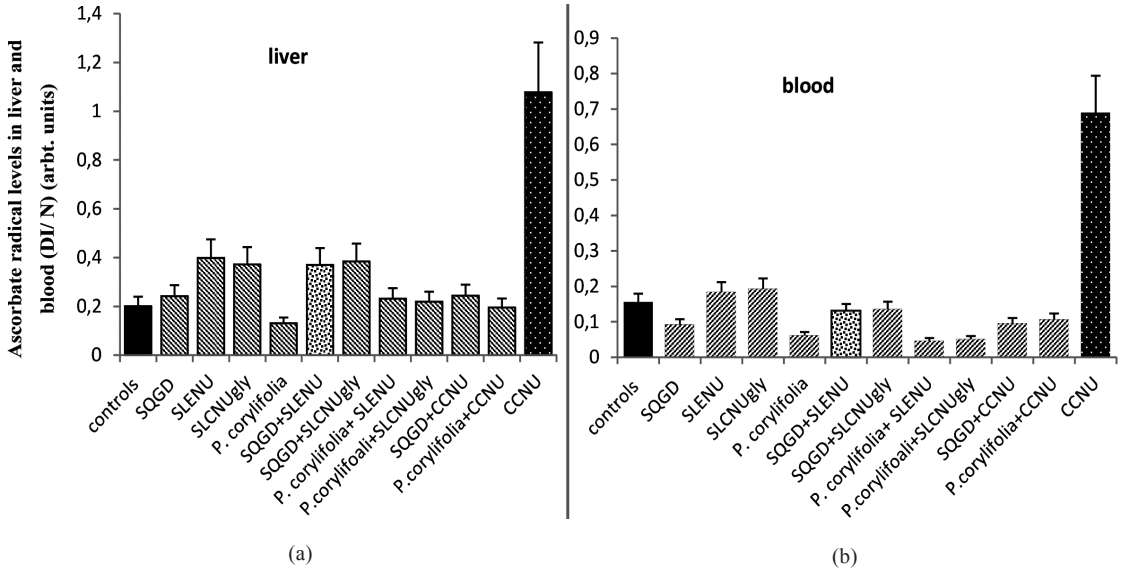

Figure 4. The levels of ascorbate radicals (arbt. units) of healthy animals treated with SQGD, *P. corylifolia*, SLENU and SLCNUgly, CCNU and combinations in liver homogenates (a) and blood (b) after 2 h p.i. (12 groups; n= 6). In an aqueous solution at pH=7.4 the ascorbate radical expresses a characteristic doublet spectrum, with the g-factor 2.0052 ± 0.002 G and spliting constant aN=1.8 G. The values are presented as mean ± SE. *p to healthy controls; **p compared to the group treated with CCNU alone.

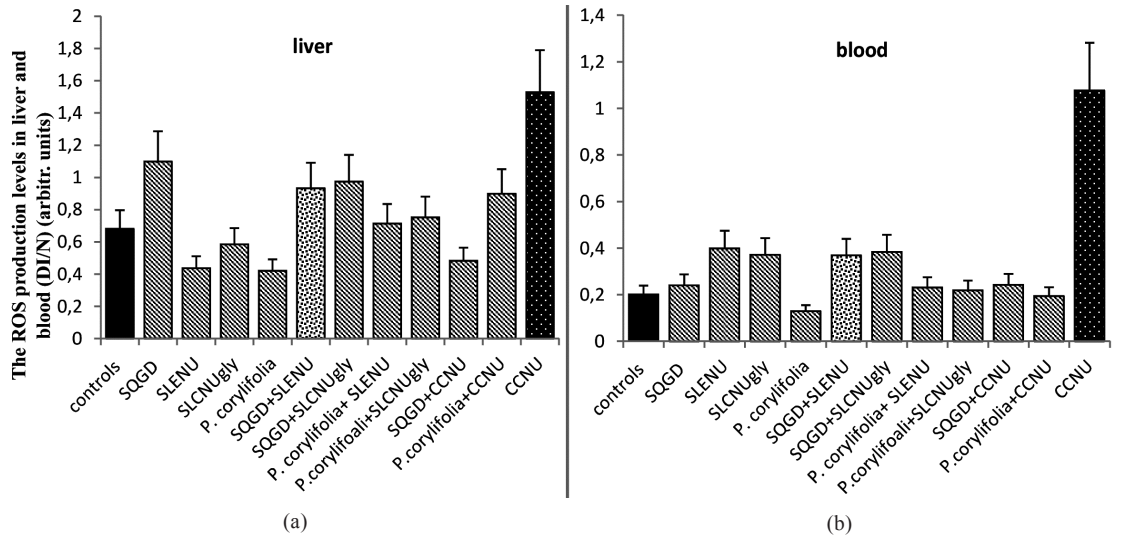

Figure 5. The ROS production levels (arbt. units) of healthy animals treated with SQGD, *P. corylifolia*, SLENU and SLCNUgly, CCNU and combinations in liver homogenates (a) and blood serum (b) 4h post administration (12 groups; n= 6). The values are presented as mean ± SE. *p to healthy controls; **p compared to the group treated with CCNU alone.

blood plasma of SLCNUgly treated mice (Fig. 5(b)) showed statistically significant increase, compared to controls (mean 1.26 ± 0.62 vs, mean 1.066 ± 0.85). A tendency of considerable decrease in ROS production was found after treatment with SQGD, *P.* corylifolia and SLENU, comparing to controls and SLCNUgly treated group.

7. DISCUSSION

The DPPH method is widely recognised method for evaluation the antioxidant activity of natural extracts and fractions. In current study we have used EPR spectroscopy to evaluate DPPH scavenging activity of the tested natural antioxidants. Unlike visible spectrophotometry the advantage of EPR method is that measures only the amount of DPPH radicals in the studied sample[31,35]. Relatively close scavenging activity against DPPH was detected for SQGD and *P. corylifolia,* alone and after UV and γ-radiation. Formerly, in an aqueous solution of SQGD we reported presence of an EPR spectrum consisting of a symmetrical single spectral line which intensity did not change as before and after UV irradiation[27]. The radical registered in SQGD was identified as *o*-semiquinone structure[27, 29]. As a rule scavenging properties of antioxidant compounds like flavonoids and phenolic acids are often associated with their abilities to form stable radicals. In many studies was

shown that flavonoids and phenolic acids scavenging radicals effectively, could form semiquinone free radicals. Having in mind both *P. corylifolia* contain different organic classes, and present result the radical structure registered in *P. corylifolia* sample was also identified as a semiquinone radical[27,30] . Findings that of SQGD and *P. corylifolia* showed close DPPH activity which was not affected after radiation might be partially explained with presence of similar radical structures confirmed formerly and by the present EPR study. In a large number of studies was reported that low doses of ionising radiation did not affect in antioxidant activity and protective effects of natural compounds, which also additionally supported present results[27-30].

Building on evidence demonstrating the selective accumulation of TEMPO[36] and the accumulation of synthetic antioxidants and triazenes in certain organs and blood[15] were studied and compared the biodistribution of spin-labelled nitrosoureas and plant antioxidants. The maximum significant accumulation of SQGD and *P. corylifolia*, predominantly in the liver and lungs cells (*60-90th min post injection*) showed that the majority of natural products are separated by the hepatobiliary pathway. Insignificantly effective utilisation of *P. corylifolia* in brain compared with SQGD could be due to receptor-mediated specificity of *P. corylifolia* in relation to brain tissues, or more probably to the presence of a greater variety of functional groups/entities in this extract[30]. The pharmacokinetic profile of SLENU[15] reveals maximum concentration in lungs, brain and pancreas (30-60 min p.i.) and low accumulation in liver (90 min p.i.). Thus characterised drug as a typical antioxidant with negligible hepatotoxicity[37] and suggesting stable binding with plasma proteins and rapid bloodstream clearance. The nitrosourea SLCNUgly reached the maximum localisation in the blood, liver, pancreas, lungs, kidneys and brain even at 10 min after administration. The result could be explained by the presence of amino acid (*glycine*) residue in the SLCNUgly structure, which is easily recognised by cell receptors, and is a precondition for high and selective organ/ blood accumulation. The low SLCNUgly concentration established 30 min post injection in all tested organs was in accordance with its formerly reported comparatively short half-life[31]. At 60th min the SLENUgly- concentration in bloodstream is about 5 times lower, compared to SLENU- treated group, that corresponds to 2.5 times shorter half-life[10]. The relatively low accumulation of both nitrosoureas in all studied organs 90 min p.i. (Fig. 3) confirmed their low toxicity[10, 15, 31]. The low toxicity is important requirement for the synthetic antioxidants to be included in developing of pharmaceutical schemes for treatment of pathophysiological conditions and to reduce reactive oxygen species.

Increased generation of ROS in the cells can modify some endogenic biomolecules, in particular ascorbic acid to stable radical forms[26]. Asc· radicals possesses comparatively long half-life in aqueous solution and can be detected by direct EPR spectroscopy, a method does not interfere with the biochemical processes. It was documented that Asc· concentration was a reliable, *in vivo* 'real-time' and quantitative biomarker for free radical generation[37,38]. The natural antioxidants showed insignificant increase (lower levels) of Asc· radicals compared to controls (Fig. 4(a), 4(b)) presumably, their administration does not induce oxidative processes in livers/ blood of treated animals. Higher levels of Asc· measured in both nitrosoureas sample might due to their carbamoylating (SLENU) and alkylating (SLCNUgly) abilities[10] and partially involved in additional oxidative processes. Findings that the combinations of SLENU or SLCNUgly with CCNU revealed significantly lower levels of Asc· compared to controls means that these compounds act as antioxidants and are able to reduce substantially (in liver) or completely (in blood) oxidative stress changes. It should be stressed that almost fivefold increase in the Asc· levels after CCNU-treatment, once again confirms its high in vivo oxidative toxicity[10,15,17]. Combinations of SLENU and natural antioxidants reduce oxidative toxicity as compared to the same combinations of SLCNUgly. Our study confirms once again *in vivo* protective abilities of SLENU against oxidative toxicity of clinically used antitumor antibiotics and radiation[38,39].

Gadjeva and co-workers, 1994[40] using *in vitro* spin-trapping EPR method demonstrated that SLENU possessed high superoxide radical scavenging activity (SSA) even higher than antioxidant Trolox. Later on well expressed *in vitro* SSA for SLCNUgly was also found[10]. To investigate the ROS production involved in oxidative processes in mice treated by studied substances alone and in combinations was used spin-trapping EPR spectroscopy. Since, PBN is the most popular nitrone used for evaluation of oxidative status at *ex vivo* and *in vitro* systems[41] in the current study was selected as a spin-trapping agent to evaluate levels of ROS products generated in studied tissues and blood samples of mice treated with the drugs and their combinations. As expected CCNU treated group induced the highest oxidative toxicity confirmed by the highest ROS levels measured in liver and blood (Fig. 5(a), 5(b)). It should be mentioned that SQGD and *P. corylifolia* considerably reduced the levels of ROS products when combined with antitumour drug CCNU. This result indicates that SQGD and *P. corylifolia* are as strong antioxidants and were able to neutralize *in vivo* induced CCNU oxidative toxicity. The highest level of ROS products was observed after single treatment with SLENU (*in liver*) and SLCNUgly (in blood). The fact that SLENU and SLCNUgly treatment (Fig. 4(a)) reduces blood ascorbate radical levels (Fig. 4(b)) bringing to controls showed that in a great extent synthetic antioxidants were able to neutralize ongoing oxidative processes. This finding may be explained by their well expressed scavenging activity against superoxide radicals[40,10]. The lowest ROS levels were observed after *P. corylifolia* treatment in liver and blood of mice. The presence of natural antioxidant ingredients in the extract, such as tocopherols, phenolic acids, ascorbic acid and especially flavonoids, contribute to neutralizing of lipid peroxidation processes. All studied combinations showed close or lower levels of ROS compared to controls indicating a synergy between natural and synthetic antioxidants in overcoming oxidative damages.

9. CONCLUSIONS

In conclusion, the four studied antioxidants alone and in combinations exhibited well –expressed antioxidant and

radical-scavenging properties at the experimental conditions. Present results give us a reason to consider that further *in vivo* experimental studies will help to be developed new pharmaceutical schemes with the new antioxidants, introduced as radio-modulating and anticancer reducing-agents.

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