A NEW ANTIOXIDANT WITH NATURAL ORIGIN
CHARACTERIZED BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY METHODS

A. Zheleva1, Y. Karamalakova1, G. Nikolova1, R. Kumar2, R. Sharma2 and V. Gadjeva1
1Trakia University, Medical Faculty, Department of Chemistry and Biochemistry, Stara Zagora, Bulgaria
2Institute of Nuclear Medicine and Allied Sciences, Delhi, India
Correspondence to: Antoaneta Zheleva
E-mail: azheleva@mf.uni-sz.bg

ABSTRACT
Formerly, naturally isolated SQGD exhibited good in vitro radical scavenging capacity towards 1,1-diphenyl-2-picrylhydrazyl (DPPH). By the present research using EPR in vitro and ex vivo methods we report our further studies on the antioxidant and free radical properties of SQGD. SQGD in powder or in solution form was studied before and after 2 h of UV irradiation by direct EPR in vitro spectroscopy. A single almost symmetrical EPR signal with a g value of 2.0056±0.0002 was registered for the powder form and g=2.0044±0.0002 for the solution form. Based on the calculated g values and the strong EPR signal stability we accept that the radical recorded can be safely ascribed to a semiquinone radical. To study in vivo antioxidant properties of SQGD, white laboratory mice were inoculated i.p.: first group with SQGD (20 mg/kg), second with anticancer drug N’-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea (CCNU, 80 mg/kg), third with SQGD plus CCNU and the controls were inoculated with the solvent only. At the 3rd h after treatment mice livers were isolated and homogenates in DMSO solution of the spin trap n-tert-butyl-alpha-phenylnitrone (PBN) were prepared and their EPR spectra were recorded. Statistical significant increased level of ROS production was found in liver homogenates of mice treated by CCNU comparing to those of the controls. ROS production in livers of mice treated by SQGD, or by the combination of SQGD plus CCNU was slightly decreased comparing to the controls.

In conclusion, obviously SQGD does not cause oxidative stress in the livers of mice for the followed period and behaves in vivo as an excellent antioxidant and hepatoprotector.

Keywords: EPR spin trapping technique, anticancer drug, semiquinone radical, antioxidants

Introduction
The cellular injury caused by oxidative stress and excess of free radicals such as reactive oxygen species (ROS) has been associated with aging and over 200 clinical disorders, that include cancer, heart disease, liver damage, neurodegenerative diseases and other degenerative diseases related to inflammation (11). ROS and oxidative stress may contribute to radiation-induced cytotoxicity and to metabolic and morphologic changes in animals and humans during radiotherapy, experimentation, or even space flight (4). The deleterious effects of ionizing radiation in biological systems are mainly mediated through the generation of ROS in cells as a result of water radiolysis (9). Among them, particularly, the highly damaging hydroxyl radicals (OH) can cause injury by reacting with biomolecules (2, 8). These free radicals cause, directly or indirectly, a variety of DNA damage products, such as base modifications, DNA single-strand and double-strand breaks. (6). Extreme environments are a permanent challenge for living organisms, in spite of that from harsh environments such as hydrothermal vents, surface sands of hot arid deserts exposed to intense ultraviolet (UV) radiation, cycles of extreme temperatures, and desiccation (17) a large diversity of microorganisms belonging to the bacteria domain of life were isolated and characterized (3). Extremely high level of resistance either to gamma radiation or UV radiation or both, was found for the members of the genus Deinococcus and Rubrobacter (5, 7, 20). The production of metabolites by bacteria is of great interest for pharmacological applications (12). Many of the bacterial metabolites may exhibit antibiotic and cytotoxic activities (12) and are excellent source of antioxidants (14). The aim of the present research was by EPR spectroscopy methods to investigate the radical scavenging abilities of SQGD, formerly isolated from the fermentation broth of a novel radio resistant bacterium Bacillus sp. INM-1.

Material and Methods
SQGD was isolated from the fermentation broth of a novel radio resistant bacterium. This bacterium was isolated and characterized by 16S ribosomal RNA analysis, fatty acid methyl ester analysis, and biochemical analysis as Bacillus sp. INM-1. The type strain (Bacillus sp. INM-1, MTCC No. 1026) was deposited at Microbial Type Culture Collection, Institute of Microbial Technology, and Chandigarh, India as reference.

Spin-trap N-tert-butyl-alpha-phenylnitrone (PBN), dimethylsulfoxide (DMSO) and anticancer drug N’-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea (CCNU) were purchased by Sigma Chemical Co,
St. Louis, USA. All other chemicals used in this study were HPLC grade.

Animals and treatment
White laboratory mice with weight 20-40 g were used. The mice were housed in polycarbonate cages in controlled conditions (12h light/dark cycles), temperature of 18-23°C and humidity of 40-70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for protection of animals used in scientific and experimental purposes. Mice were divided in groups (5 mice in each group) and inoculated i.p. with the drugs dissolved in 10% solution of Tween 20 in saline. First group was treated by CCNU (80 mg/kg), second by SQGD (20mg/kg); third group was inoculated by SQGD and after 1h with CCNU at the same doses of the drugs. The control group was inoculated with the solvent only. At the 3rd h all animals in the tested and control group were exsanguinized under light ether anesthesia and the livers were immediately collected, washed in cool saline and start to prepare tissues homogenates.

Electron paramagnetic resonance (EPR) studies
EPR measurements were performed at room temperature on an X-band EMX™ instrument, spectrometer Bruker, Germany, equipped with standard Resonator. All EPR experiments were carried out in triplicate and repeated. Spectral processing was performed using Bruker WIN-EPR and Simfonia software.

EPR spectroscopy study on SQGD in powdered and aqueous solution form before and after UV irradiation
EPR spectra of SQGD in powder and aqueous solution form (6.4%) were recorded before and after 2 h of UV irradiation in the range of light-waves from 290 nm to 320 nm using Transilluminator - 4000 (Stratagene, USA). EPR settings were as follows: for the powdered form - gain 2 x 10^3, microwave power 0.645 mW, center field 3514 G, time constant 327.68 ms, sweep time 61.440 s, modulation amplitude, 10.00 G, 1 scan per sample and for the solution form - gain 1 x 10^5, microwave power 6.494 mW, center field 3514 G, time constant 163.840 ms, sweep time 16.384 s, modulation amplitude 10.00 G, 10 scans per sample.

DPPH radical scavenging capacity of SQGD aqueous solution before and after 2h of UV irradiation
DPPH radical scavenging capacity of SQGD was determined according to Bernardo dos Santos et al., 2009 with slight modifications (1). Briefly, to 250 µl ethanol solution of DPPH (80 µM) were added 10 µl of SQGD dissolved in water. After 10 min incubation the mixture was transferred to a capillary tube. The capillary tube was sealed and placed inside a standard EPR quartz tube (i.d. 3mm) that was placed in the EPR cavity. The control sample contained 250 µl ethanol solution of DPPH plus 10 µl of distillate water. The percent of the DPPH radicals scavenged by SQGD was calculated according to the equation:

Scavenged DPPH radical (%)=[(I_0-I)/I_0]x100%

where - I_0 was integral intensity of the DPPH signal of the control sample and I was integral intensity of the DPPH signal after addition of SQGD to the control sample.

EPR ex vivo ROS production in liver tissues of mice
Preparation of the homogenates and EPR study of the ROS production was performed according to Shi et al., 2005 with minor modifications (18). Briefly, 0.1 g of liver tissue was homogenized for 2 min after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. After centrifugation, 0.4 ml supernatant of homogenized tissue was taken in quartz tube and stored in liquid nitrogen for EPR measurement. EPR spectra were recorded at room temperature. EPR settings were as follows: center field 3503.74 G, microwave power 20.42 mW, modulation amplitude 0.50 G, sweep width 100 G, gain 1x10^6, time constant 327.68 ms, sweep time 81.92 s, 5 scans per sample.

Statistical analysis
Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means ± standard error (SE). Statistical significance was determined by the Student’s t-test. A value of p < 0.05 was considered statistical significant.

Results and Discussion
The EPR spectra recorded in powder and solution form of SQGD before and after 2h of UV irradiation exhibited almost a symmetrical single EPR spectral line with a g value of 2.0056±0.0002 for the powdered form and g=2.0044±0.0002 for the solution form (Fig. 1A, Fig. 1B) and peak-to-peak line width 9 G. Based on these results and other authors reports (15, 19) the EPR spectra shown on Fig. 1 can be safely ascribed to a semiquinone radical presenting in SQGD structure.

Moreover, the intensities of the EPR signals registered in powdered or in solution form of SQGD were not affected by the UV irradiation (p > 0.05, Fig 2) which means that the radical structure presenting in SQGD is rather stable to the UV rays treatment.
Results from determination of DPPH radical scavenging capacity in aqueous solution of SQGD before and after 2h of UV irradiation are shown on Fig. 3.

It was established that before UV irradiation the percent of the scavenged DPPH radicals statistical significant increased when the concentration of SQGD increased (p<0.05). The same dependence was demonstrated for the UV irradiated samples (Fig. 3). For every studied concentration of SQGD, either before or after UV irradiation, there was not found statistical significant difference in the percent of the scavenged DPPH radicals. This result was in accordance with our finding that the free radical structure presenting in SQGD was not affected by UV treatment for the period of 2 hours and probably the same structure was involved in the reaction with DPPH.
The best way for confirmation of short-lived free radicals at \textit{in vitro} or \textit{in vivo} is to be used proper chemical compounds termed spin-trapping agents. Spin traps react with short-lived free radicals to form more stable free radical products (spin-adducts) that can be detected and studied by EPR spectroscopy (10, 13). In the present study using PBN as a spin trap we have found that at the 3rd h after CCNU treatment in liver homogenates of mice could be detected six-lines EPR spectra of the PBN spin adducts. After calculation of the hyperfine splitting constants of the spin adducts (aN=13.94±0.05 G and aH=2.35±0.04 G) the radicals registered in mice liver homogenates in presence of DMSO solution of PBN at aerobic conditions were identified as PBN/\textit{OCH3} (19). It is accepted that the reaction of DMSO with \textit{OH} produces \textit{CH3}, and the oxidation of \textit{CH3} under aerobic conditions produces \textit{OCH3} (15, 16, 19). Thus, the radical formed in the livers of CCNU treated mice might be identified as \textit{OH}. The fact that the levels of ROS production (calculated as double integrated plots of EPR spectra of the PBN spin adducts) in liver homogenates of mice treated by CCNU comparing to those of the control mice were statistical significant increased (p<0.05, Fig. 4) we can explain by generation of toxic \textit{OH} radicals during liver decomposition of CCNU. As is seen ROS production in liver homogenates of mice treated by the combination of SQGD plus CCNU were slightly decreased (p>0.05) comparing to that of the controls (Fig. 4).

Almost the same level of ROS production was calculated for the mice treated by SQGD, alone (result is not presented). By the present \textit{ex vivo} EPR spin trapping study we demonstrate high levels of ROS (identified as \textit{OH}) in livers of CCNU treated mice. Complete reduction of the oxidative process in the livers of mice pretreated by SQGD we can explain by the well expressed \textit{in vitro} hydroxyl radical scavenging potential of SQGD recently reported by us (14).

**Conclusions**

By the present EPR \textit{in vitro} and \textit{ex vivo} spectroscopy studies we have demonstrated that the naturally isolated molecule SQGD does not cause oxidative stress in the livers of mice for the followed period and behaves \textit{in vivo} as an excellent antioxidant and hepatoprotector. We also hypothesis that SQGD might play an important role in the of \textit{Bacillus} sp. INM-1 defense system against possible oxidative stress caused by inimical environment.

**Acknowledgments**

This study was supported by grants of Ministry of Education, Youth and Science – Indo-Bulgarian collaborative project (Bin-7/2008).

**REFERENCES**