



EPR INVESTIGATIONS ON SCAVENGING CAPACITY AND ANTIOXIDANT ACTIVITY OF IBG-20 A FRACTION ISOLATED FROM *BACILLUS* sp. INM-1

Y. Karamalakova¹, R. Kumar², V. Gadjeva¹, R. Tripathi², R. K. Sharma², A. Zheleva^{1*}

¹Department Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora, Bulgaria

²Institute of Nuclear Medicine and Allied Sciences, DRDO, Brig S.K. Mazumdar Marg, Delhi, India

ABSTRACT

From the fermentation broth of a novel radioresistant bacterium *Bacillus* sp. INM-1 have been isolated several fractions and their antioxidant and pro-oxidant activities were studied. By the present study using Electron Paramagnetic Resonance (EPR) spectroscopy methods we report our investigations on antioxidant and free radicals scavenging abilities of the fraction IBG-20 isolated from the bacterium *Bacillus* sp. INM-1. By direct EPR spectroscopy in both, solid and aqueous solution forms of IBG-20, was registered a single strong symmetrical EPR signal. Based on the stability of the EPR signal registered and the value of its g factor (2.0044 ± 0.0002 G) we accept that the radical recorded can be safely ascribed to a semiquinone radical. It was established that IBG-20 (6,4% aqueous solution) possessed high radical scavenging capacity towards stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Effect of the incubation time (10-30 minutes) on the DPPH radical scavenging capacity of IBG-20 fraction was also evaluated. These first EPR spectroscopy studies characterize IBG-20 fraction as an excellent free radical scavenger and as a new potential natural antioxidant that might find application as a radio protector in biological systems.

Keywords: IBG-20; DPPH radical; EPR spectroscopy; Antioxidant activity.

INTRODUCTION

Several natural products from plants, fungal, bacterial or other microbial sources have been evaluated for their pharmacological activities. Natural pharmaceutical products have application in medicine for the treatment of different diseases, including cancer, heart and some neurodegenerative disease and radiation induced oxidative damages to biological systems (1, 2, 3, 4). The production of metabolites by microbes and particularly by bacteria are of great interest for pharmacological applications (2). Some of the bacterial metabolites are excellent source of antioxidants (5) and exhibit antimicrobial and cytotoxic activities (2). Microbial metabolites also possessed antagonistic activities against

many soil born fungal and bacterial plant pathogens (6). Earlier reports showed that the normal cells possessed an optimized level of ROS (2, 3 and 4) which was maintained by constant cellular free radical scavenging activities of the cells. However, disbalance in the redox status of the cells leads to oxidative stress. Therefore, antioxidant therapy could be an effective strategy to prevent cells from abnormal levels of reactive oxygen species (ROS) production and to reduce harmful effects of that production in biological systems (2, 4, 5 and 7). In the recent years, several reports on the effects of the treatment of many diseases with the combinations of novel natural therapeutic compounds and other radical scavengers has been published. Therapeutic application of appropriate natural antioxidant substances efficiently reduces the level of lipid peroxidation and increases the activity of antioxidant enzyme defense system in the cellular environment and thus ultimately reduces negative consequences of chemotherapy- and or radiotherapy-induced

***Correspondence to:** Antoaneta Zheleva,
Department of Chemistry and Biochemistry,
Medical Faculty, Trakia University, 11 Armeiska
Str., 6000 Stara Zagora, Bulgaria, E-mail:
azheleva@mf.uni-sz.bg

oxidative stress (1, 2, 3, 4) in the cancer patients. To overcome the high levels of reactive oxygen species (ROS) production, external supplementation of antioxidants with natural origin (1-8) is used. Moreover, EPR spectroscopy is a highly sensitive technique utilized for investigation of free radical scavenging capacity, radical structures and antioxidant activity of naturally/microbial isolated products (9, 10).

By the present study using direct EPR spectroscopy we report our first investigations on free radical scavenging and antioxidant abilities of IBG-20 - a natural fraction formerly isolated from the novel radioresistant bacterium *Bacillus* sp. INM-1 at INMAS, Delhi, India.

MATERIAL AND METHODS

The natural fraction IBG-20 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.

Chemicals

1, 1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals (St. Louis, MO, USA). Deionized and distilled water was used for all experiments. Other chemicals used were analytical or HPLC grade.

Electron Paramagnetic Resonance measurements

For all EPR measurements an X-band EMXmicro, EPR spectrometer (Bruker, Germany) equipped with standard Resonator was used. Quartz capillaries were used as sample tubes. The capillary tubes were sealed and placed inside a standard EPR quartz tube (i.d. 3mm) that was placed in the EPR cavity. All EPR experiments were performed in triplicate at room temperature (18-23°C) and relative humidity 40 %. Spectral processing (g-value calculation) was performed using Bruker WIN-EPR and SimFonia software.

EPR spectroscopy study of IBG-20 in powdered form and in aqueous solution

EPR spectra of IBG-20 in powder and aqueous solution form (6.4 %) were recorded at

following EPR settings: for the powdered form – gain 2×10^3 , microwave power 0.645 mW, center field 3514 G, time constant 327.680 ms, sweep time 61.440 s, modulation amplitude, 10.00 G, 1 scan per sample and for the solution form – gain 1×10^5 , 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 IBG-20 in aqueous solution form

DPPH radical scavenging capacity of IBG-20 was determined according to Bernardo dos Santos et al., 2009 (11) with modifications described by Zheleva et al., 2011 (12). Briefly, to 250 µl ethanol solution of DPPH (80 µM) were added 10 µl of IBG-20 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 IBG-20 was calculated according to the equation:

$$\text{Scavenged DPPH radical (\%)} = [(I_0 - I) / I_0] \times 100 \%$$

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 IBG-20 to the control sample.

Effect of the incubation time and high energy ultrasonic waves on the DPPH radical scavenging capacity of IBG-20 in aqueous solution

After addition of 10 µl of IBG-20 (0.8 %) to 250 µl ethanol solution of DPPH the mixtures were incubated in the dark for 10, 20, and 30 min at room temperature.

Solutions with different concentrations of IBG-20 (0.5% - 6, 5%) were sonicated for 5 min and after that were added to the ethanol solution of DPPH and their free radical scavenging capacities were determined and compared.

Statistical analysis

Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means $\pm$ standard error (SE). Statistical significance was determined by the Student's t-test. A value of $p = 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Results from our *in vitro* EPR spectroscopy studies on IBG-20 in powdered and solution forms are presented on Figures 1-4.

In powdered form of IBG-20 was registered a strong symmetrical single EPR spectral line with a g value of 2.0044 ± 0.0002 at 3513 G

magnetic field (**Figure 1**). Almost the same EPR spectrum was recorded in aqueous solution of IBG-20 (result is not shown). Based on the other author's reports (13, 14) and the calculated g value we accept that a semiquinone structure is presenting in IBG-20 structure.

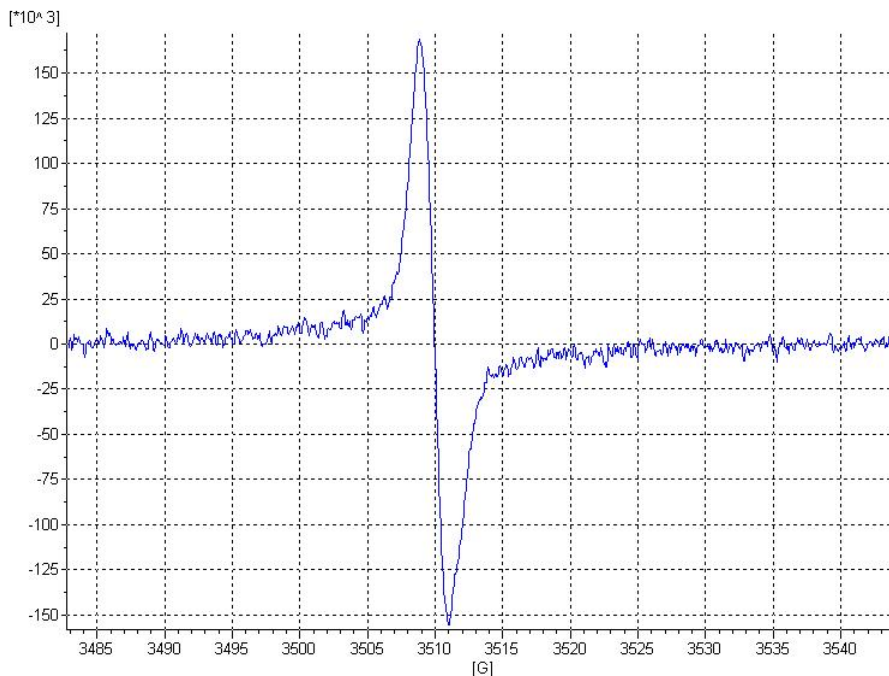


Figure 1. EPR spectrum of IBG-20 in powdered form.

The fact that stable free radical DPPH is widely used as a colorimetric reagent for determination of antioxidant and free radicals scavenging activity of natural compounds (15, 16, 17, 18) led us to study interaction between DPPH and IBG-20. Result from determination of DPPH radical scavenging capacity of aqueous solutions of IBG-20 (0.2 % - 6.4 %) is shown on **Figure 2**.

When the concentration of IBG-20 increased, the percent of the scavenged DPPH radicals also increased in a linear dependence (22.01 ± 0.02 % for 0.2 % concentration and 82 ± 0.01 % for 6.4 % concentration respectively).

In a number of studies was found that the incubation time might affect the radical scavenging abilities of the naturally isolated antioxidant products (19, 20). To check the effect on the incubation time, mixtures of different concentrations of IBG-20 plus DPPH were incubated 10, 20 or 30 min in the dark and after 5 min sonication their EPR spectra

were recorded and compared to those of the control samples (**Figure 3** and **Figure 4**, respectively).

As is seen only slight differences were found for the percent of the scavenged DPPH radicals for the highest (6.4%) concentration of IBG-20 (43.36 ± 0.031 % on 10 min; 39.5 ± 0.012 % on 20 min; 38.8 ± 0.01 % after 30 min incubation) ($p < 0.05$) on the IBG-20 samples (**Figure 3**).

This result indicated that the reaction between DPPH radicals and IBG-20 was quite fast and completed about 10 minutes. According to the findings of the other authors (15) we accepted that IBG-20 was a low molecular fraction isolated from the fermentation broth of the radio resistant bacterium *Bacillus* sp. INM-1.

For every studied concentration of IBG-20, before and after 5 min high energy ultrasonic waves-sonication, was found statistical significant difference for the percent of the

scavenged DPPH radicals (40.71 ± 0.011 % before vs. 50.19 ± 0.023 % after sonication at a concentration of 3, 5% and 79.01 ± 0.041 % before vs. 93.84 ± 0.023 % after sonication at a concentration of 6,5%) (**Figure 4**).

Increasing in the DPPH scavenging capacity of IBG-20 after sonication might be explained by

possible destroying the aggregates formed between its molecules which results in increase of the numbers of active structures with free radical scavenging abilities comparing to those presenting in non-sonicated IBG-20 fraction.

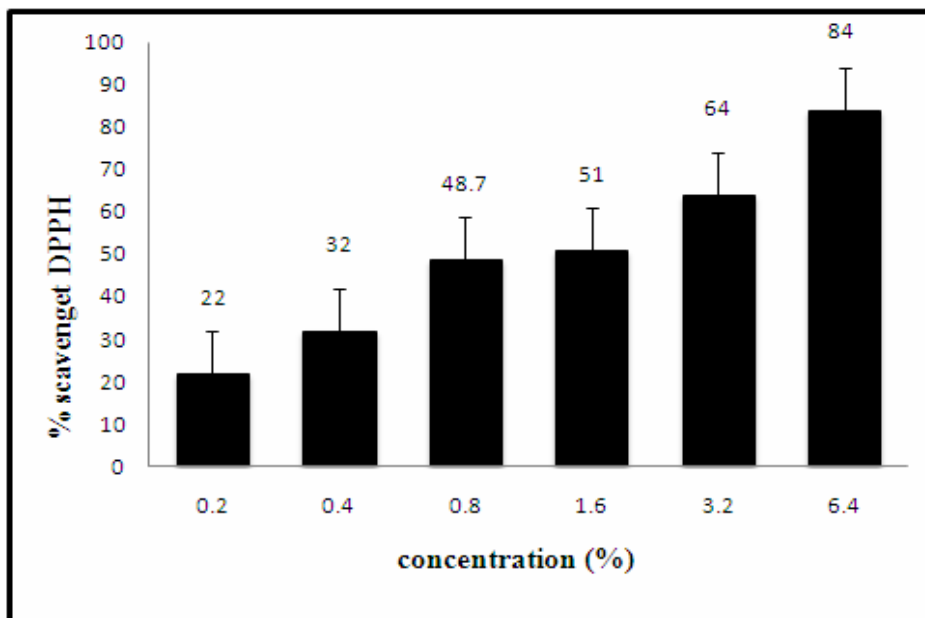


Figure 2. Percent of DPPH radicals scavenged by aqueous solutions of IBG-20 at different concentrations.

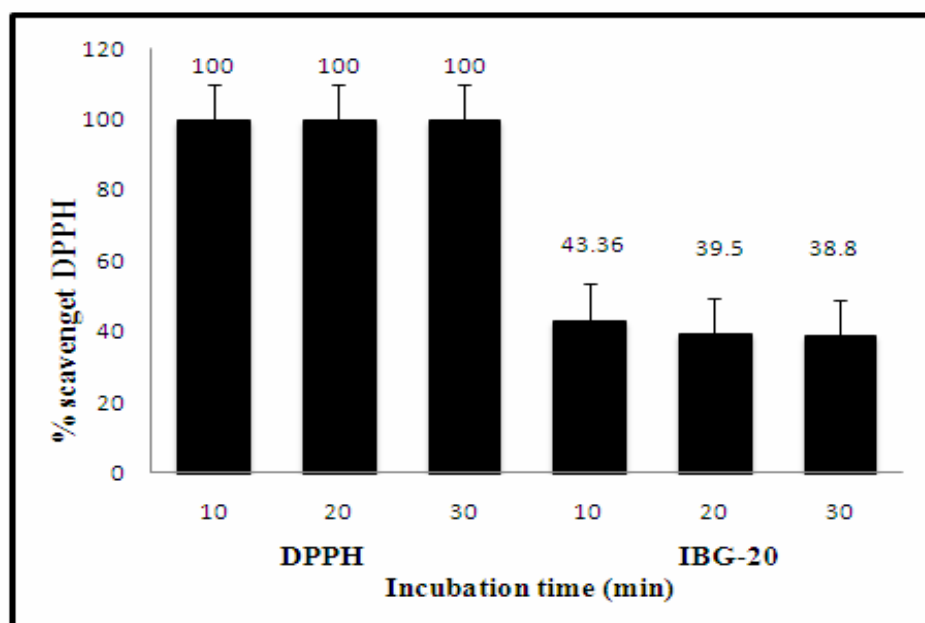


Figure 3. Percent of DPPH radicals scavenged by 0.8 % aqueous solution of IBG-20 after different periods of incubation time

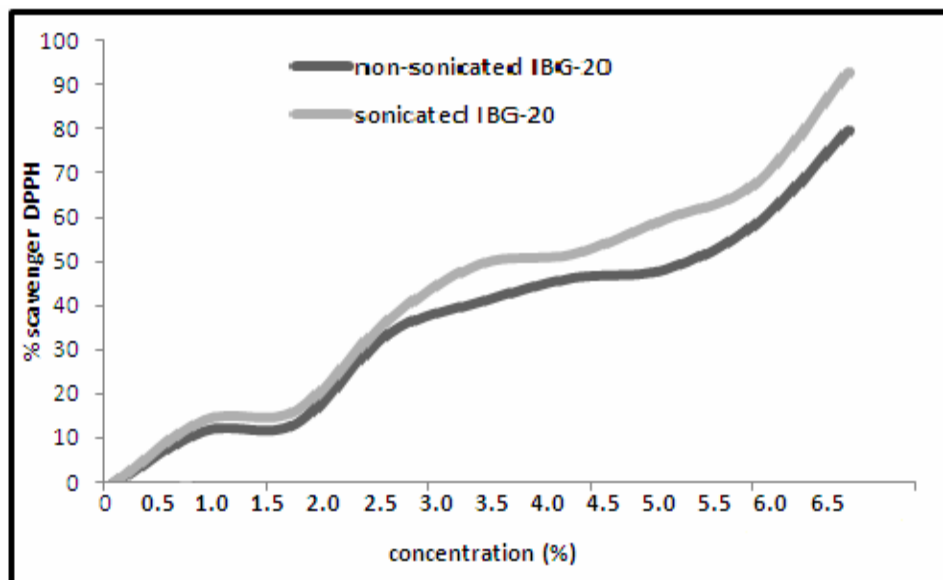


Figure 4. Percent of DPPH radicals scavenged before and after 5 min microwave sonication of aqueous solution IBG-20 at different concentrations.

CONCLUSION

By the present EPR *in vitro* spectroscopy studies we have demonstrated that the naturally isolated fraction IBG-20, either in solid or in solution form exhibits a strong EPR signal and *in vitro* behaves as an excellent antioxidant. We also hypothesis that IBG-20 might be played an important role in *Bacillus sp.* INM-1 defense system against possible oxidative stress caused by inimical environment.

ACKNOWLEDGMENT

This study was supported by grants of Ministry of Education, Youth and Science – Indo-Bulgarian collaborative project (BIn-7/2008) and project BG051PO001-3.3.06- 0006/ 2012. The authors are thankful to Dr. Raj Kumar, INMAS for helpful suggestions and discussions.

REFERENCES

1. Arora, R., Gupta, D., Chawla, R., Sagar, R., Sharma, A., Kumar, R., Prasad, J., Singh, S., Samanta, N., Sharma, R. K., Radioprotection by plant products: present status and future prospects. *Phytother. Res.*, 71: 425–435, 2005.
2. Kumar, R., Bansal, D. D., Patel, D. D., Mishra, S., Karamalakova, Y., Zheleva, A., Gadjeva, V., Sharma, R.K., Antioxidant and radioprotective activities of semiquinone glucoside derivative (SQGD) isolated from *Bacillus sp.* INM-1. *Mol Cell Biochem*, DOI 10.1007, 2010.
3. Arora, R., Chawla, R., Puri, S. C., Sagar, R., Singh, S., Kumar, R., Sharma, A., Prasad, J., Kaur, G., Chaudhary, P., Qazi, G., Sharma, R.K. Radioprotective and antioxidant properties of low altitude *Podophyllum Hexandrum* (LAPH). *J Environ Pathol Toxicol Oncol* 25: 299-314, 2005.
4. Arora, R., Herbal radiomodulators: Applications in medicine, Homeland Defence and Space. CABI Publishing, Wallingford, Oxon, UK, 332, 2008.
5. Konzen, M., De Marco, D., Cordova, C. A.S., Vieira, T.O., Antonio, R.V. and Creczynski-Pasa T.B. *Bioorganic Med. Chem.*, 14: 8307-8313, 2006.
5. Clanway, C.P.: Plant growth Promotion by *Bacillus* and Relatives. [In:] R. Berkeley, M. Heyndriclex, N. Logen, P. De Vos: Applications an Systematic of *Bacillus* and Relatives. Blackwell Publishing, 219-236, 2002.
6. Datta, K., Sinha, S., Chattopadhyay, P., Reactive oxygen species in health and disease. *Nat. Med. J. of India*, 13: 304-310, 2000.
7. Sen, S., Chakraborty, R., Sridhar, C., Reddy R., Biplab De Free Radicals, Antioxidants, Diseases And Phytomedicines: Current Status And Future Prospect. *Int. J. PSR. Res.*, 3: 976-984, 2010.

8. Halliwell, B., Gutteridge, J. M., The importance of free radicals and catalytic metal ions in human diseases. *Mol Aspects Med.*, 8:89-193, 1985
9. Raffi, J., Yordanov, N.D., Chabane, S., Douifi, L., et al. Identification of irradiation treatment of aromatic herbs, spices and fruits by electron paramagnetic resonance and thermoluminescence. *J. Spectrochimica Acta A*, 56: 409–416, 2000.
10. Bernardo Dos Santos A., Silva, D. H. S., Bolzani, V. S., Santos, L. A., Schmidt, T. M., Baffa, O., Antioxidant Properties of Plant Extracts: an EPR and DFT Comparative Study of the Reaction with DPPH, TEMPOL and Spin Trap DMPOJ. *J. Braz. Chem. Soc.*, 20: 1483-1492, 2009.
11. Zheleva, A., Karamalakova, Y., Nikolova, G., Kumar, R., Sharma, R., and Gadjeva, V. A new antioxidant with natural origin characterized by Electron Paramagnetic Resonance spectroscopy methods. *Biotechnology & Biotechnological Equipment*, DOI: 10.5504/50YRTIMB.2011.0027.
12. Takeshita, K., Fujii, K., Anzai, K., Ozawa, T., 2004. *In vivo* monitoring hydroxyl radical generation caused by X-ray irradiation of rats using the spin trapping/EPR technique. *Free Radic. Biol. Med.* 36, 1134-1143.
13. Luo, Yi., Su, Y., Lin, R., Shi, H., Wang, X., 2006. 2-Chlorophenol induced ROS generation in fish *Carassius auratus* based on the EPR method. *Chemosphere*. 65, 1064-1073.
14. Glavind, J. 1963. Antioxidants in animal tissue. *Acta Chem. Scand.* 17,1635-1649.
15. Emaru, K., Askal, H., Saleh, G., 1991. Spectrophotometric determination of tetracycline and oxytetracycline in pharmaceutical preparations. *Talanta*, 38, 1219-1221.
16. Perez, M.B., Calderon, N.L., Croci, C.A., 2007. Radiation-induced enhancement of antioxidant activity in extracts of rosemary (*Rosmarinus officinalis* L.). *Food Chem.* 104: 585-592.
17. Anesini, C., Ferraro, G.E., Filip, R., 1993. Total Polyphenol Content and Antioxidant Capacity of Commercially Available Tea (*Camellia sinensis*) in Argentina. *J. Agric. Food Chem.* 56, 9225–9229.
18. Wolniak M, Tomczykowa M, Tomczyk M, Gudej J, Wawer I., Antioxidant activity of extracts and flavonoids from *Bidens tripartita*. *Acta Pol Pharm.* 64(5):441-447. 2007
19. Zheleva A., Karamalakova Y., Nikolova G., Gadjeva V. New possibilities for treatment of amatoxin intoxications: an EPR spectroscopy study on free radical formation caused by the mushroom toxin alpha amanitin. *Pakis. J. Pharm. Sci.* (in press).