ANTIRADICAL ACTIVITY OF NEW STRAWBERRY CULTIVAR EXTRACTS INCORPORATED INTO LIPOSOMES – AN EPR STUDY

Đ. Nakarada [1], D. Milosavljević [2], J. Milivojević [3], J. Dragišić Maksimović [2] and M. Mojović[1]

[1]University of Belgrade, Faculty of Physical Chemistry, Studentski trg 12-16, 11000 Belgrade, Serbia. (djura@ffh.bg.ac.rs)
[2]University of Belgrade, Institute for Multidisciplinary Research, Kneza Viseslava 1, 11030 Belgrade, Serbia,
[3]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade, Serbia

ABSTRACT
Strawberries are rich in various health-promoting compounds. A particularly high yield of these molecules could be extracted using methanol which is unfortunately harmful to living beings. To surpass the problem of solvent toxicity, in this paper, we have incorporated methanol extracts of two novel strawberry cultivars into liposomes. Our results show that both extracts possess significant antiradical activity towards hydroxyl and DPPH radicals, unaltered by lipids from liposome membranes. These results indicate that otherwise toxic extracts could easily be turned into a promising pharmaceutical product.

INTRODUCTION
Strawberries are among the most consumed berry fruits in the world. They are rich in antioxidant compounds such as vitamins, melatonin, polyphenols, and bioactive sugars. The most abundant phenolic compounds found in strawberries are anthocyanins, flavan-3-ols, ellagitannins, glycosides of quercetin, and kaempferol. These compounds contribute to the sensorial and organoleptic attributes of strawberries, and most importantly, to their health-promoting properties. With large-scale production, several new cultivars of strawberries are introduced each year. 'Tea' and 'Aprika' are two new strawberry cultivars that are in the testing phase with still unexplored antioxidant potential. Extraction is the crucial step in studying biologically active compounds of plants, and it plays an important role in the results and conclusions. Various techniques as well as various solvents and their mixtures have been used to obtain strawberry extracts with high concentration and high variety of biologically active compounds, thus having high antioxidative activity. Previous studies indicate that extracts prepared using 80% methanol (MeOH) possess a plethora of compounds responsible for the high antioxidative activity. However, due to its harmful health effects, MeOH extracts are not suitable for pharmaceutical applications. On the other hand, many of the antioxidant molecules which are present in MeOH extracts have low solubility in water, making their antioxidative activity towards biologically relevant radicals difficult to study. Electron paramagnetic resonant (EPR) spectroscopy is the only experimental technique capable of direct radical detection. EPR technique has high selectivity and detection limit and obtained results do not depend on the optical characteristics of substances. During redox analysis, biologically relevant free radicals, like $^{\bullet}OH$, $^{\bullet}O_2^-$, and $NO^{\bullet}$, have to be generated using water as a solvent. Using other organic solvents give rise to carbon-based radicals as artifacts during their interaction with reactive oxygen species, seriously bringing into question the usability of the obtained results.

To overcome an issue of pharmaceutical applicability of MeOH strawberry extracts, as well as the problem of studying their antiradical activity, the extracts of two novel strawberry cultivars have been incorporated into the membrane of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposomes. The antiradical activity of these liposomes towards hydroxyl ($^{\bullet}OH$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was studied to determine the radical scavenging potential of

strawberry extracts and to evaluate whether lipids from the liposome membranes affect the obtained results.

METHODS

Extracts of two strawberry cultivars ('Tea' and 'Aprika') have been prepared by homogenization of 1 g of frozen fruit with 3 ml of 80% methanol. The mixture was transferred into the microcentrifuge tube and centrifuged for 10 min at 10 000 rpm. The obtained extracts were further filtered through the 0.22 µm HPLC filter. Extracts were stored at -80 °C until analysis. Multilamellar liposomes were prepared from strawberry extracts and DPPC by the modified thin-film method [1]. This was done by dissolving 2.5 mg of DPPC in the mixture of chloroform and methanol (4:1 v/v) in a rotary flask, followed by the addition of 250 µl of the strawberry extract. The solvent was slowly evaporated at room temperature using a rotary vacuum evaporator. The remaining thin film was hydrated with 2 ml of 18 MΩ water in increments of 250 µl, each followed by 3 min of vigorous vortexing and 3 min of sonication using an ultrasound ice bath. The suspension was further sonicated for 20 min. In order to obtain the uniform size of liposomes, the suspension was extruded through a 100 nm membrane. Finally, liposomes were concentrated to the volume of 250 µl using a vacuum concentrator. Control liposomes were prepared following the same procedure, without the addition of strawberry extracts. To determine the size and stability of liposomes, 750 µl of the suspension was placed into the disposable cuvette and size/zeta-potential was measured using Malvern Zetasizer Nano ZS90 dynamic light scattering (DLS) analyzer.

EPR spin-trapping technique was employed in order to trap short-lived free radical species generated directly in the liposome suspension and to quantify the amount of their interaction with liposomes. To study the interaction of hydroxyl radicals with strawberry-derived antioxidant compounds from the liposomes, the intensity of the hydroxyl spin-adduct EPR signal was measured. 26 µl of the liposome suspension was mixed together with 2 µl of 5 mM H_2O_2 and 1 µl of 100 mM 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO). After the addition of 1 µl of 5 mM $FeSO_4$, the mixture was transferred into a 1 mm diameter Teflon tube, and EPR spectra were recorded using Bruker ELEXSYS-II E540 EPR spectrometer [2].

The interaction of DPPH free radicals with strawberry-derived antioxidant compounds from the liposomes was studied by measuring the intensity of the DPPH EPR signal. 26 µl of the liposome suspension was mixed with 3 µl of deionized water and 1µl of 3.2 mM DPPH, prior to transferring the mixture into a 1 mm diameter Teflon tube and recording the EPR spectra.

All EPR spectra were recorded using the following parameters: center field 3500 G, microwave power 10 mW, microwave frequency 9.85 GHz, modulation frequency 100 kHz, and modulation amplitude 1 G. Control experiments have been made following the same procedures, using water instead of liposome solution.

RESULTS AND DISCUSSION

The results of the DLS measurements showed that the size of liposomes containing strawberry extracts ranged between 65 and 69 nm, while their mean zeta potential was -6 mV. This indicates the overall uniform size distribution and relative stability of the liposomes. The decrease of the intensities of DEPMPO/OH adduct and DPPH EPR signals were measured 2 min after mixing experimental solution in the suspension of liposomes containing strawberry extracts (Figure 1). The reduction of the EPR signal was calculated using the formula:

$$\Delta h = \frac{h_i - h_2}{h_i} \times 100\ (\%)$$

where h_i and h_2 refer to the intensities of the control free radical signal and the signal after 2 min (liposomes containing strawberry extracts). The calculated percentage of the DEPMPO/OH adduct

EPR signal reduction was 92.65% for liposomes containing extract of 'Tea', and 78.00% for those containing extract of 'Aprika' strawberry cultivar. Regarding DPPH$^{\bullet}$ free radical, the calculated EPR signal reduction amounts to 30.10% for 'Tea' and 16.26% for 'Aprika' cultivar. These results indicate that liposomes containing strawberry extracts have a rather strong antioxidant capacity and that they react more selectively with $^{\bullet}$OH than with DPPH radicals. Liposomes containing extracts of 'Tea' strawberry cultivar showed stronger antiradical activity compared to 'Aprika' cultivar. These findings are very interesting having in mind that DPPH radicals are routinely used in antioxidant scavenging assays despite that they have no biological relevance, while $^{\bullet}$OH radicals are of great importance considering their role in many physiological processes. To confirm that obtained results arise only from strawberry-derived antioxidant compounds from the liposomes, and not from DPPC, control experiments were performed using 100% DPPC liposomes (prepared using the same procedure previously described). These experiments showed no EPR signal reduction (data not shown). Altogether, the results presented in this paper strongly indicate that strawberry-derived antioxidant compounds incorporated into the membrane of the DPPC liposomes have rather significant antioxidant potential, which is not affected by the DPPC.

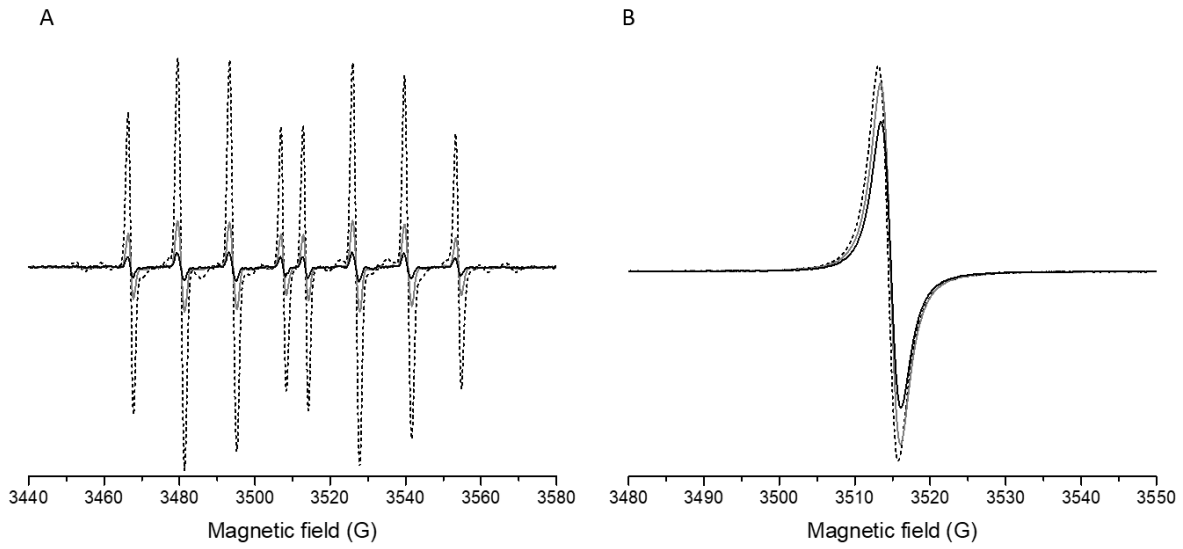

Figure 1. EPR spectra of DEPMPO/OH adduct (A) and DPPH radical (B) recorded in the control sample (dotted) and 2 min after mixing the experimental solution in the suspension of liposomes containing 'Aprika' (grey) and 'Tea' (black) strawberry extracts.

These findings further confirm the validity of the recent approach for measuring the antioxidative activity of water-insoluble molecules towards biologically relevant radicals, developed by our group [3]. To additionally validate selectivity in terms of the antiradical activity of strawberry extracts, experiments on other free radical species (such as NO$^{\bullet}$, $^{\bullet}O_2^-$ and Asc$^{\bullet}$) will be performed. Experiments obtaining strawberry extracts using different solvents should be performed to determine the type of extract bearing the strongest antiradical activity and being the most appropriate for liposomal encapsulation.

CONCLUSION

Liposomes are important nanostructures suitable for studying the antioxidant activity of various hydrophobic and hydrophilic molecules. In this paper, the method of incorporating biologically active compounds into liposomes was applied for MeOH extracts of two novel cultivars of strawberries, assessing their antioxidative activity towards $^{\bullet}$OH and DPPH radicals. Liposomes

obtained using 'Tea' strawberry extract in MeOH have reduced DEPMPO/OH adduct EPR signal by 92.65% while the reduction rate was 78.00% for those obtained using MeOH extract of 'Aprika' strawberry cultivar. The calculated DPPH EPR signal reduction amounts to 30.10% for 'Tea' and 16.26% for 'Aprika' cultivar. These results indicate strong and selective antiradical activity of liposomes containing strawberry-derived antioxidant compounds, unaffected by lipids from the liposomes. Taken altogether, the findings of this study demonstrate the significant potential of the encapsulation of strawberry-derived antioxidant compounds obtained from MeOH extracts into liposomes and their promising use for various pharmaceutical purposes.

Acknowledgment
This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Contracts No 451-03-68/2022-14/200146, 451-03-68/2022-14/200053; 451-03-68/2022-14/200116).

REFERENCES

[1] V. Weissig, Liposomes: Methods and Protocols, Vol. 1: Pharmaceutical Nanocarriers, Humana Press, Mumbai, 2010.
[2] M. Mojović, M. Vuletić, G. Bačić, Detection of Oxygen-Centered Radicals Using EPR Spin-Trap DEPMPO: The Effect of Oxygen, Ann. N. Y. Acad. Sci., 1048 (2006) 471 – 475.
[3] Đ. Nakarada, B. Pejin, G. Tommonaro, M. Mojović, Liposomal integration method for assessing antioxidative activity of water insoluble compounds towards biologically relevant free radicals: example of avarol, J. Liposome Res., 30 (3) (2019) 218–226.