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Spin trapping studies of essential oils in lipid systems

Katerina Makarova,
Kinga Drązikowska,
Beata Suska,
Katarzyna Zawada,
Iwona Wawer

Abstract. In the present work, we report the results of a spin trapping ESR study of four essential oils widely used for skin care products such as creams and bath salts. The studied essential oils are *Rosmarini aetheroleum* (rosemary), *Menthae piperitae aetheroleum* (mint), *Lavandulae aetheroleum* (lavender), and *Thymi aetheroleum* (thyme). Fenton reaction in the presence of ethanol was used to generate free radicals. The *N-tert*-butyl-α-phenylnitrone (PBN) was used as a spin trap. In the Fenton reaction, the rosemary oil had the lowest effect on radical adduct formation as compared to the reference Fenton system. Since essential oils are known to be lipid soluble, we also conducted studies of essential oils in Fenton reaction in the presence of lipids. Two model lipids were used, namely 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). The obtained results suggested that in the presence of DOPC lipids, the ˙OH and PBN/˙CHCH$_3$(OH) radicals are formed in both phases, that is, water and lipids, and all the studied essential oils affected the Fenton reaction in a similar way. Whereas, in the DPPC system, the additional type of PBN/X (a_N = 16.1 G, a_H = 2.9 G) radical adduct was generated. DFT calculations of hyperfine splittings were performed at B3LYP/6-311+G(d,p)/EPR-II level of theory for the set of *c*-centered PBN adducts in order to identify PBN/X radical.

Key words: spin trapping ESR • essential oils • DFT calculations • lipids

K. Makarova✉, K. Drązikowska, B. Suska, K. Zawada,
I. Wawer
Department of Physical Chemistry,
Faculty of Pharmacy,
Medical University of Warsaw,
1 Banacha Str., 02-097 Warsaw, Poland,
Tel./Fax: +48 22 572 09 51,
E-mail: kmakarova@wum.edu.pl

Introduction

Since the middle ages, essential oils have been widely used for virucidal, fungicidal, bactericidal, antiparasitical, insecticidal, medicinal, and cosmetic applications [1]. Nowadays, the use of essential oils as functional ingredients in pharmaceutical, sanitary, cosmetic, agricultural, and food industries is gaining wide interest because of increasing concern about potentially harmful synthetic additives [2]. Within the wide range of the above-mentioned products, there is a common need for natural substances with not only a pleasant smell but also with a preservative action, aimed to avoid lipid deterioration, oxidation, and spoilage by microorganisms. In this case, the essential oils and their components are constantly gaining increasing attention because of their relatively safe status, their wide acceptance by consumers, and their application for potential multipurpose functional use [3].

Essential oils are extracted from various aromatic plants. Because of the mode of extraction, they contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components, and aliphatic components [4].

Generally, essential oils have been widely studied mostly from the viewpoint of their flavor and fra-

grance chemistry as they are used for flavoring foods, drinks, and cosmetics [5]. However, the essential oils are natural sources of phenolic components, so the investigators are attracted to evaluate their activity as antioxidants or free radical scavengers [6, 7].

In the present work, we report the results of a study aimed at comparing radical scavenging properties of four essential oils widely used for skin care products such as creams and bath salts. The studied essential oils are *Rosmarini aetheroleum* (rosemary), *Menthae piperitae aetheroleum* (mint), *Lavandulae aetheroleum* (lavender), and *Thymi aetheroleum* (thyme). Spin trapping ESR was performed utilizing *N-tert*-butyl-α-phenylnitrone (PBN) spin probe. Fenton reaction in the presence of ethanol was used to generate free radicals. Since essential oils are known to be lipid soluble, we also conducted studies of essential oils in Fenton reaction in the presence of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) as model lipids. The influence of essential oils on the model lipid bilayer structure was also studied.

Materials and methods

Electron spin resonance

The ESR measurements were performed on a MiniScope MS 200 spectrometer from Magnettech at room temperature ($24 \pm 1°C$) using 50 μl capillary tubes. Typical instrument settings were: sweep width 69 G, microwave attenuation 10 dB, modulation amplitude of 0.5 G, sweep time 20 s.

Spin trapping ESR

Fenton reaction was used to generate free radicals. PBN spin trap and DTBN (di-*tert*-butyl nitroxide) spin probe were obtained from Sigma-Aldrich. Hydrogen peroxide (30%) and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were obtained from POCH (Gliwice, Poland). DOPC and DPPC lipids were obtained from Avanti Polar Lipids as chloroform solution (25 mg/ml). PBN solution was prepared using PBS buffer (pH 7.5), 115 μl of 20 mM PBN solution was mixed with 3 μl of selected essential oil or of 96% ethanol (for reference measurements). Then, 3 μl of water, 7.5 μl of 100 mM $FeSO_4$ solution, and 55 μl of 25 mM H_2O_2 solution were added. The sample was vortexed for 30 s.

For Fenton reaction in lipids: 236 μl (DOPC) or 220 μl (DPPC) of chloroform solutions of lipids were dried for 24 hours. Then 115 μl of 20 mM PBN solution added and vortexed (2 min). The sample was then sonicated for 3 min. Then, 3 μl of ethanol solution of selected essential oil, 3 μl of deionized water (Millipore), 7.5 μl of 100 mM $FeSO_4$, and 55 μl of 25 mM H_2O_2 were added and vortexed for 30 s. The first measurement was performed 105 s after the addition of H_2O_2. The next 20 measurements were performed every 3 min. The following concentrations of ethanol solutions of essential oils were used: lavender (0.033% v/v), rosemary (0.1%

v/v – Fenton, 0.013% v/v for Fenton in lipids), thyme (0.001% v/v), and mint (0.01% v/v). The concentration of PBN radical adducts were estimated from the reference measurements of 0.1111 mM (for Fenton reaction) and of 0.0111 mM (for Fenton reaction in the presence of lipids) solutions of DTBN spin probe at the same conditions and spectrometer settings.

Spin probe ESR

236 μl (DOPC) or 220 μl (DPPC) of chloroform solutions of lipids were dried for 24 h. Then 135 μl of PBS buffer (10 mM, pH = 7.5), 10 μl of ethanol solution of essential oil, and 15 μl of 7 mM DTBN solution were added, vortexed for 2 min, and then placed into an ultrasound bath for 3 min. Samples were measured immediately after preparation, 1 h, and 24 h.

ESR spectra simulations

ESR spectra were simulated using EasySpin Toolbox for Matlab utilizing 'garlic' function [8].

DFT calculations

DFT calculation were performed in Gaussian 09 at Interdisciplinary Centre for Mathematical and Computational Modeling (Warsaw, Poland) under the computational Grant G14-6 [9]. Geometry optimization of PBN/˙OH, PBN/˙$(CH_3)_3$, PBN/˙$(CH_2)_n CH_3$ (n = 0:4), and PBN/˙$CHCH_3(OH)$ were performed at B3LYP/6-31G level of theory using PCM model for water solvent and two solvent molecules. Hyperfine splittings were calculated at B3LYP//6-311G+(d,p)/EPR-II level of theory.

Results and discussion

Spin trapping ESR

The Fenton reaction in the presence of ethanol was used as a reference system. In this system, two types of PBN radical adducts were identified, namely PBN/˙OH and PBN/˙$CHCH_3OH$ (Fig. 1a), which is in agreement with previously published data [10]. Moreover, PBN/˙$CHCH_3OH$ is responsible for 90–95% of the total ESR signal. With time, the decrease of PBN/˙$CHCH_3OH$ radical adduct signal was responsible for the decrease of the total ESR signal (Fig. 1c). All studied essential oils show high antioxidant activity (Fig. 1b). As the ESR signal decreases too fast to be registered after 105 s (Fig. 1b), the essential oils were appropriately diluted to slow down the reaction and get comparable results (Fig. 1d). In the systems with essential oils, the same radical adducts (90–95% of PBN/˙$CHCH_3OH$ and 5–10% of PBN/˙OH) were identified as the ones in the reference system. The concentration of PBN radical adducts (calculated from the reference DTBN signal) at 240 s after adding H_2O_2 was compared to
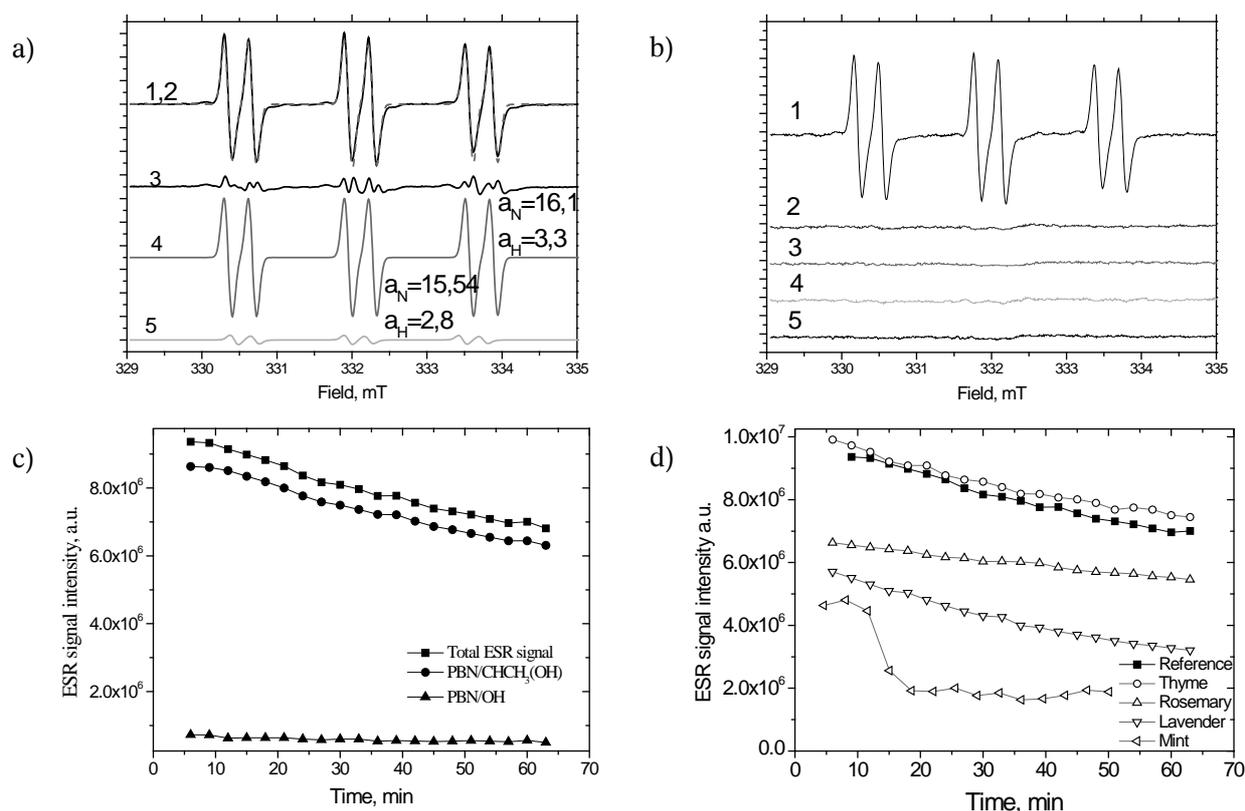
Fig. 1. (a) Experimental (1) and simulated (2) spectra of PBN spin adducts, residuals (3), PBN/$^\bullet$OH radical adduct component (4), and PBN/$^\bullet$CHCH$_3$OH radical adduct component (5). (b) Experimental ESR signal of the spin adducts produced upon the Fenton reaction in the presence of ethanol in reference system (1) and in the presence of essential oils: lavender (2), mint (3), rosemary (4), and thyme (5) after 3 min of the reaction. (c) Kinetics of ESR signal – integrated intensity of spin adducts of PBN that resulted from radicals formed upon the Fenton reaction in the presence of ethanol together with PBN/$^\bullet$OH and PBN/$^\bullet$CHCH$_3$OH components obtained from simulations. (d) Kinetics of ESR signal of radicals formed upon the Fenton reaction in the presence of ethanol and essential oils.

the reference system ($c_{PBN\_adducts}$ = 0.09 mM). Even though the lavender essential oil has lower concentration (0.033% v/v) than that of rosemary (0.1% v/v), it shows slightly higher antioxidant activity ($c_{PBN\_adducts}$ = 0.0555 mM) in Fenton reaction than that of rosemary oil ($c_{PBN\_adducts}$ = 0.0648 mM). Thyme essential oil at 0.05% v/v concentration shows strong antioxidant effect ($c_{PBN\_adducts}$ = 0), whereas its 0.001% v/v concentration leads to slight prooxidant effect ($c_{PBN\_adducts}$ = 0.098 mM). The mint essential oil (0.01% v/v) shows strong antioxidant effect ($c_{PBN\_adducts}$ = 0.0485 mM) as compared to the reference systems.

Taking into account the dilutions of the essential oils, we could conclude that the strongest effect on Fenton reaction was induced by thyme essential oil (0.05% v/v), followed by mint and lavender oils. Rosemary oil has the weakest antioxidant properties. Since essential oils are known to be lipid soluble, we also conducted studies of essential oils in Fenton reaction in the presence of lipids.

Effect of essential oils on lipids

Prior to Fenton in lipids studies, the effect of essential oils on lipid structure was studied using free spin probe DTBN, which can easily partition into both phases, that is, lipid and water. In this study two model lipids, DOPC and DPPC, were used.

In the reference, DOPC system ratio of DTBN in water and lipid phase was 0.75:0.25 (Fig. 2-IA,B). At first, it is the mint essential oil that has the largest effect on DOPC. However, after 24 h, the strongest effect (larger ratio of DTBN in lipid phase) was observed in DOPC systems with thyme and rosemary essential oils (0.63:0.37 water to lipid ratio) (Fig. 2-IA).

In the case of the second lipid system, DPPC lipids organize into a more rigid structure at room temperature, thus the DTBN is not partitioning into lipid phase in the reference system (Fig. 2-IIA,B). The same situation was observed in the presence of lavender essential oil. Mint and thyme essential oils induce some change in the lipid structure as 30% of the DTBN spin probes were in lipid phase immediately after sample preparation. After 24 h, that ratio decreased to only 20% of the spin probes in DPPC lipids (Fig. 2-IIA). The effect of rosemary essential oil was similar to those of mint and thyme oils, but weaker (15% of DTBN in lipid phase, decreasing to 3%) (Fig. 2-IIA).

Spin trapping in the presence of lipids

Based on spin probe ESR results in DOPC lipid systems, we could expect partitioning of Fenton generated free radicals as well as of PBN into lipid bilayer. Here one could expect that PBN would also
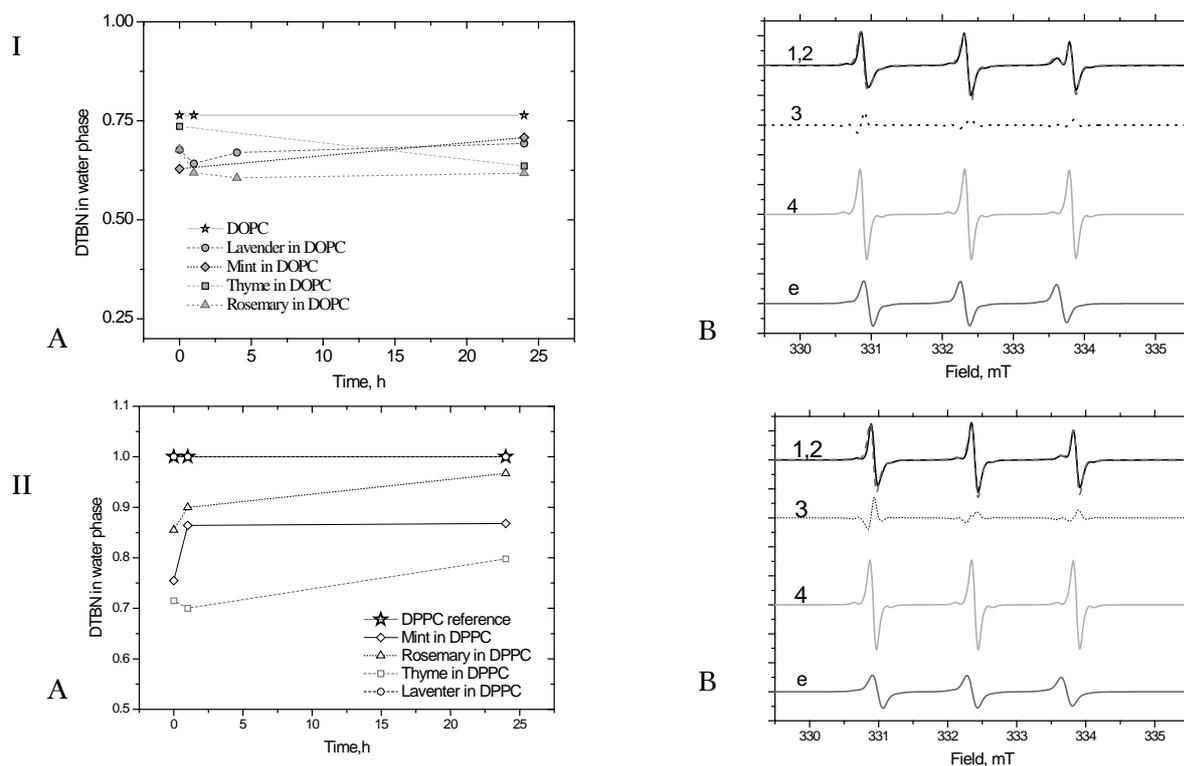
Fig. 2. (A) Ratio of DTBN spin probes in water phase in the reference system and in the presence of essential oils. (B) Experimental (1) and simulated (2) spectra of DTBN spin probe in lipid system with mint essential oil, residuals (3), DTBN in water phase component (4), DTBN in lipid phase component (5). (I) DOPC lipids, (II) DPPC lipids.

trap radicals inside the lipid bilayer. The obtained results suggested that in the presence of DOPC lipids, the PBN/$^{\bullet}$OH and PBN/$^{\bullet}$CHCH$_3$(OH) radicals are formed in both phases, that is, water and lipids (Table 1, Fig. 3A). Lavender (0.033% v/v), mint (0.01% v/v), and thyme (0.001% v/v) essential oils show similar antioxidant activity (Fig. 3B,D,E,F). The rosemary essential oil (0.013% v/v) reveals prooxidant properties in the presence of DOPC lipids (Fig. 3C). When comparing the systems at the moment of first measurement (240 s), in contrast to the reference system (Fig. 3B), where most radicals (*c*-centered (0.0309 mM) and $^{\bullet}$OH (0.0023 mM)) are formed in the water phase, in these systems PBN/$^{\bullet}$OH radical adduct concentration in water is much lower (0.0001–0.001 mM) (Table 2). At the same time, there is noticeable increase in PBN/$^{\bullet}$CHCH$_3$OH concentration in lipid phase, that is, 0.0103 to 0.0239 mM as compared to reference 0.0055 mM (Table 2). The increased concentration of spin adducts in lipid phase in the presence of essential oils correlates with the increase of DTBN partitioning in the same systems, that is, it could be explained by more fluid DOPC lipid structure.

Table 1. Experimental hyperfine splittings of PBN adducts in DOPC and DPPC systems

Spin adduct	Hyperfine splitting, G					
System	DOPC				DPPC	
Phase	water		lipid		water	
	a_N	a_H	a_N	a_H	a_N	a_H
PBN/$^{\bullet}$CHCH$_3$(OH)	16.10	3.3	14.10	3.35	16.10	3.3
PBN/$^{\bullet}$OH	15.54	2.8	14.46	2.74	15.54	2.8
PBN/X					16.10	2.9

Table 2. Concentrations of PBN radical adducts at 240 s of the Fenton reaction in water and lipid (DOPC) phases in the presence of essential oils

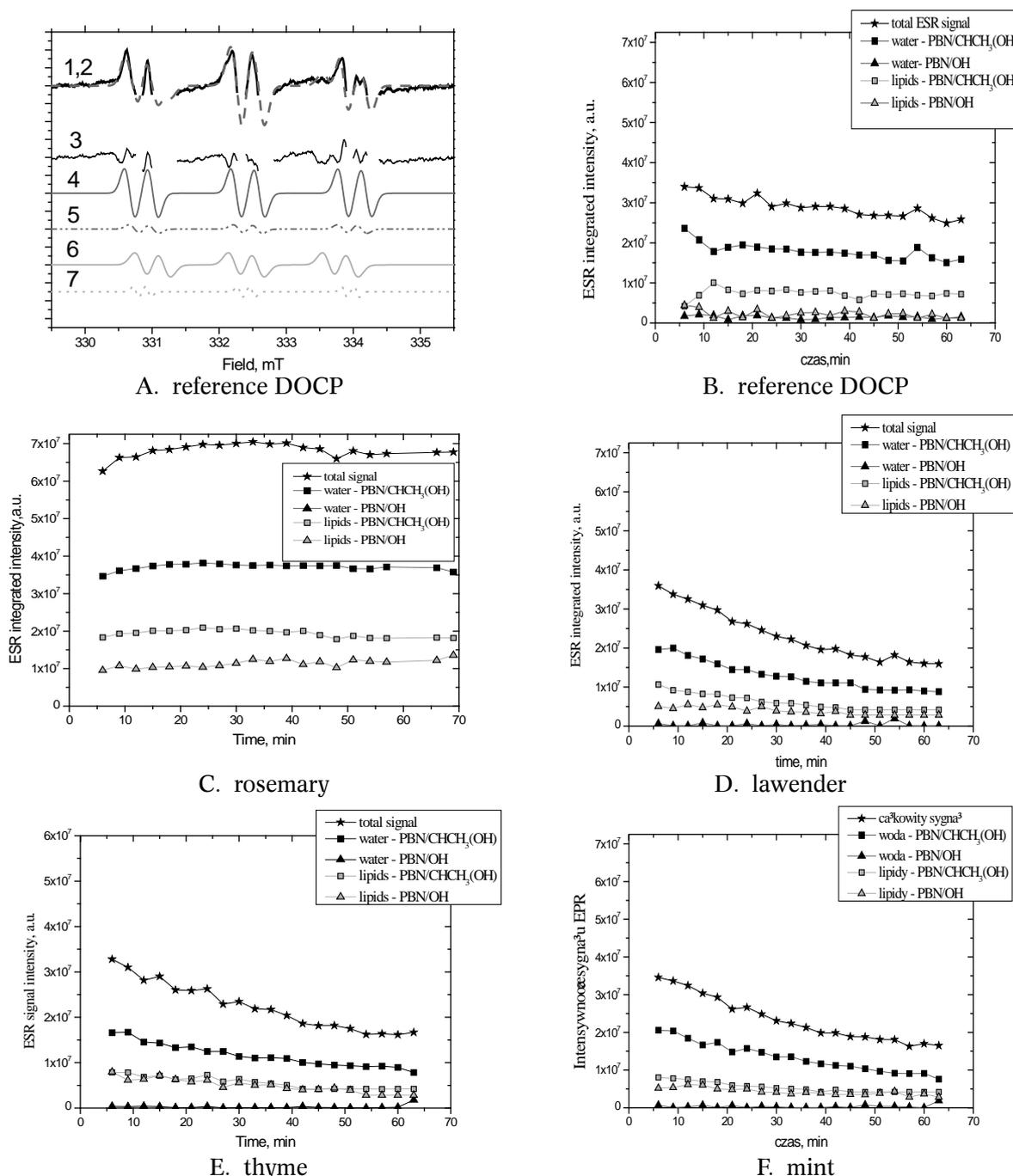| System of study	Total concentration of PBN radical adducts [mM]	Water phase		DOPC lipid phase	
		PBN/$^{\bullet}$CHCH$_3$(OH)	PBN/$^{\bullet}$OH	PBN/$^{\bullet}$CHCH$_3$(OH)	PBN/$^{\bullet}$OH
Reference	0.0444	0.03090	0.0023	0.0055	0.0057
Lavender	0.0469	0.02256	0.0007	0.0139	0.0066
Mint	0.0452	0.02690	0.0009	0.0105	0.0068
Thyme	0.0428	0.02170	0.0004	0.0103	0.0104
Rosemary	0.0818	0.04530	0.0001	0.0239	0.0125

Fig. 3. (A) Experimental (1) and simulated (2) spectra of PBN spin adducts produced upon the Fenton reaction in DOPC lipids reference system and residuals (3), PBN/˙OH (4) and PBN/˙CHCH₃OH (5) radical adduct component in water phase, PBN/˙OH (6) and PBN/˙CHCH₃OH (7) radical adducts components in lipid phase. (B–F) Kinetics of ESR signal integrated intensity of spin adducts of PBN that resulted from radicals formed upon the Fenton reaction in DOPC lipids system in reference system (B) and in the presence of rosemary (C), lavender (D), thyme (E), and mint (F) essential oils.

Partitioning of PBN into DPPC is highly unlikely at room temperature (below its phase transition), as PBN is a larger molecule than DTBN. In Fenton reaction, the presence of DPPC PBN/˙OH and PBN/˙CHCH₃(OH) radicals were observed in the water phase. However, the additional PBN/X radical adduct was also observed in all systems with DPPC lipids (Fig. 4, Table 3) at different concentrations. It could be located in the water environment as suggested by the large value of a_N splitting (16.1 G) (Table 1). The highest concentration of PBN/X radical adduct

was observed in DPPC system with mint essential oil (Fig. 4C). The lowest concentration (0.0007 mM) of PBN/X was observed in lipid system with thyme essential oil (Table 3, Fig. 4D). Also, in the presence of lavender and rosemary essential oils, the systems appeared to be stabilized as compared with reference DPPC system (Fig. 4E,F).

It seems possible that X is part of the oxidized DPPC lipid. It was shown that during the oxidation process in DPPC lipid molecule, only hydrogen abstraction could occur, leading to formation of

Table 3. PBN adducts concentrations at 240 s of reaction in Fenton reaction in the presence of DPPC lipids

System of study	Total concentration of PBN radical adducts [mM]	PBN/˙OH	PBN/˙CHCH$_3$(OH)	PBN/X
Reference	0.0107	0.0410	0.0013	0.0053
Lavender	0.0057	0.0001	0.0019	0.0030
Mint	0.0060	0.0025	0.0007	0.0029
Thyme	0.0048	0.0018	0.0023	0.0007
Rosemary	0.0050	0.0014	0.0014	0.0022

various length aliphatic radicals [11]. On the other hand, the PBN adduct with similar hyperfine splittings (a_N = 16.1 and a_H = 2.9) was observed in Fenton system together with PBN/˙OH, however it was not identified [10]. We could assume that PBN/X adduct in both cases is a *c*-centered radical originating either from decomposition of PBN spin probe or DPPC lipid oxidation (both reaction could lead to *c*-centered radical formation). However, PBN splitting pattern is not very sensitive to *c*-centered radical structure [10].
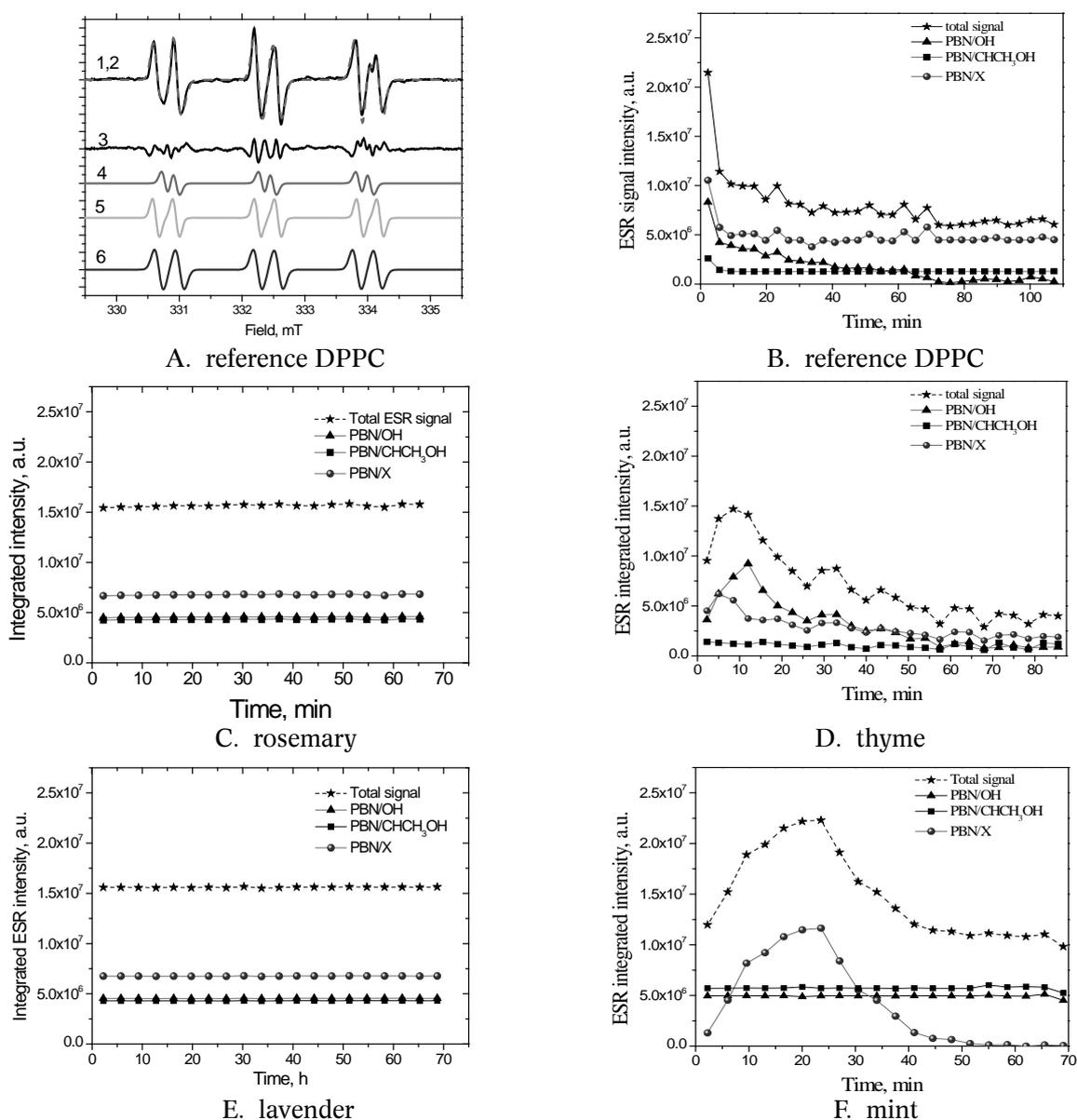

Fig. 4. (A) Experimental (1) and simulated (2) spectra of PBN spin adducts produced upon the Fenton reaction in DPPC lipids system in the presence of ethanol, residuals (3); PBN/˙OH (4); PBN/˙CHCH$_3$OH (5) and PBN/X (6) radical adduct components. (B–F) Kinetics of ESR signal integrated intensity of spin adducts of PBN that resulted from radicals that were formed upon the Fenton reaction in DPPC lipids system in reference system (B) and in the presence of rosemary (C), thyme (D), lavender (E), and mint (F) essential oils.

Table 4. Hyperfine splitting constants of various PBN spin adducts from DFT calculations at the B3LYP//6-311+G(d,p)/ EPR-II level of theory using the PCM model for the water solvent and two water molecules

EPR	EPR-II		6-311+G(d,p)	
	a_N	a_H	a_N	a_H
PBN/˙OH	13.16077	1.13516	11.51572	1.07258
PBN/˙CHCH$_3$OH	12.20840	4.86681	10.47146	4.64074
PBN/˙(CH$_2$)$_4$CH$_3$	13.26373	0.21139	11.43028	0.21751
PBN/˙(CH$_2$)$_3$CH$_3$	13.70019	0.06914	11.87515	0.09204
PBN/˙(CH$_2$)$_2$CH$_3$	13.67142	0.06750	11.84538	0.09108
PBN/˙CH$_2$CH$_3$	13.69387	0.08547	11.86356	0.11929
PBN/˙CH$_3$	13.77089	0.06759	11.94251	0.09022
PBN/˙(CH$_3$)$_3$	12.20162	2.58752	10.43217	2.43723

DFT calculations

Based on the suggested mechanism of DPPC oxidation [11], there is a set of ˙(CH$_2$)$_n$CH$_3$ radicals which could be trapped by PBN. The mechanism of PBN degradation was proposed by [12] and lead to the formation of t-BuNO, which subsequently decomposes to NO˙ and t-Bu˙.

In order to perform the identification of the observed PBN/X radical adduct, the hyperfine splittings of PBN/˙(CH$_2$)$_n$CH$_3$ (n = 0:4), PBN/˙(CH$_3$)$_3$, PBN/˙CHCH$_3$OH, and PBN/˙OH were calculated (Table 4). The solvent effect was accounted by PCM model and two water molecules [13]. The previous studies showed that B3LYP//6-311+G(d,p)/EPR-II basis are the most accurate for PBN adducts calculations [13, 14], however 1-2G difference is still produced. Previous studies suggest the evaluation of the trend in hyperfine splitting's (correlation plot) rather than direct comparison [14]. The DFT calculations reveal similar values for the whole set of PBN/ ˙(CH$_2$)$_n$CH$_3$ radicals (Table 4) as well as similar values for PBN/˙CHCH$_3$OH and PBN/˙(CH$_3$)$_3$ radical adducts. Since the experimental hyperfine values of PBN/X radical adduct is close to that of PBN/˙CHCH$_3$OH, we could suggest that the observed radical adduct is PBN/˙(CH$_3$)$_3$. However, the additional experiments with another spin trap with more specific splitting pattern for c-centered radicals are necessary.

Conclusions

Spin trapping studies of interactions of essential oils with radicals produced in Fenton reaction showed that the presence of essential oils generally decreased the amount of radical adducts formed, but did not change the type of radicals formed. The weakest effect on radical adducts formation was observed for rosemary essential oil. In the presence of DOPC and DPPC lipids systems, rosemary essential oil stabilized the trapping of radicals, that is, ESR integrated intensity as well as ratio of the radical adduct components remind the same with time. Also, in the presence of DOPC lipids two radical adducts (PBN/˙OH and PBN/˙CHCH$_3$OH) in two phases (water and lipid) were identified. Whereas in the case of the DPPC lipids, all radicals appeared to be in water phase. In addition to PBN/˙OH and

PBN/˙CHCH$_3$OH radical adducts, the new PBN/X radical adduct was observed in the presence of DPPC. Based on DFT calculations, this PBN/X radical could originate from the decomposition of PBN spin trap.

References

1. Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils – a review. *Food Chem. Toxicol.*, *46*, 446–475.
2. Reische, D., Lillard, D., Eitenmiller, R., Akoh, C., & Min, D. (1998). Antioxidants. In C. C. Akoh & D. B. Min (Eds.), *Food lipids: chemistry, nutrition and biotechnology*, 3rd ed. (pp. 409–433). Boca Raton: CRC Press.
3. Ormancey, X., Sisalli, S., & Coutiere, P. (2001). Formulation of essential oils in functional perfumery. *Parfums, Cosmetiques, Actualites*, *157*, 30–40.
4. Edris, A. E. (2007). Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother. Res.*, *21*, 308–323.
5. Smith, R., Cohen, S., Doull, J., Feron, V., Goodman, J., Marnett, L., Portoghese, P., Waddell, W., Wagner, B., & Hall, R. (2005). A procedure for the safety evaluation of natural flavor complexes used as ingredients in food: essential oils. *Food Chem. Toxicol.*, *43*, 345–363.
6. Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods – a review. *Int. J. Food Microbiol.*, *94*, 223–253.
7. Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., & Bruni, R. (2005). Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. *Food Chem.*, *91*, 621–632.
8. Stoll, S., & Schweiger, A. (2006). EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.*, *178*, 42–55.
9. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Montgomery, J. A. Jr, Vreven, T., Kudin, K. N., Burant, J. C., Millam, J. M., Iyengar, S. S., Tomasi, J., Barone, V., Mennucci, B., Cossi, M., Scalmani, G., Rega, N., Petersson, G. A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Li, X., Knox, J. E., Hratchian, H. P., Cross, J. B., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Yazyev, O., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J. W., Ayala, P. Y., Morokuma, K., Voth, G. A., Salvador, P., Dannenberg, J. J., Zakrzewski, V. G., Daniels, A. D., Farkas, O., Rabuck, A. D., Raghavachari, K., & Ortiz, J. V. (2009). *Gaussian 03, Revision C.02.* Wallingford CT: Gaussian, Inc.

10. Buettner, G. R. (1987). Spin trapping: ESR parameters of spin adducts 1474 1528V. *Free Radic. Biol. Med.*, *3*, 259–303.

11. Dilbeck, C. W., & Finlayson-Pitts, B. J. (2013). Hydroxyl radical oxidation of phospholipid-coated NaCl particles. *Phys. Chem. Chem. Phys.*, *15*, 9833–9844.

12. Chamulitrat, W., Parker, C. E., Tomer, K. B., & Mason, R. P. (1995). Phenyl N-tert-butyl nitrone forms nitric oxide as a result of its Fe(III)-catalyzed hydrolysis or hydroxyl radical adduct formation. *Free Radic. Res.*, *23*, 1–14.

13. Jerzykiewicz, M., Ćwieląg-Piasecka, I., Witwicki, M., & Jezierski, A. (2010). EPR spin trapping and DFT studies on structure of active antioxidants in biogycerol. *Chem. Phys. Lett.*, *497*, 135–141.

14. Ucun, F., & Aydın, S. G. (2014). Calculated optimized structures and hyperfine coupling constants of some radical adducts of α-phenyl-N-tert-butyl nitrone in water and benzene solutions. *J. Organomet. Chem.*, *759*, 27–32.