Spin Trapping Radicals from Lipid Oxidation in Liposomes in the Presence of Flavonoids

[1]NASIMA ARSHAD*, [2]NAVEED KAUSAR. JANJUA, [3]LEIF HORSFELT SKIBSTED AND [3]MOGENS LARSEN ANDERSEN

[1]*Department of Chemistry, Faculty of Sciences, Allama Iqbal Open University, Islamabad, Pakistan.*
[2]*Department of Chemistry, Faculty of Sciences, Quaid-i-Azam University, Islamabad, Pakistan.*
[3]*Food Chemistry, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Denmark*
*nasimaa2006@yahoo.com**

(Received on 13th July 2012, accepted in revised form 19th December 2012)

Summary: Interactions of four structurally related flavonoids – quercetin, rutin, morin and catechin with peroxyl radicals using liposome/N-tert-butyl-α-phenylnitrone (PBN) and liposome/α-(4-pyridyl-N-oxide)-N-tert-butylnitrone (POBN)-spin trap systems have been studied through spin trapping ESR. Results obtained were different from that of conjugated diene analysis experiments, where lag phases indicated radical scavenging activity of all the flavonoids. No clear lag phase was observed in ESR experiments under same conditions. In the presence of flavonoids decreasing ESR signals of spin adducts in PBN, while no or negligibly smaller spin adducts with POBN system were observed which may be attributed to the possibility that spin traps interacted with free radicals. Experiments with buffer/spin trap systems without liposome revealed that spin adducts were only stable with catechin and destroyed by quercetin, rutin and morin in buffer/spin trap systems. These results further assured that quercetin, rutin and morin not only interacted with peroxyl radicals but also with spin adducts.

Key words: Antioxidants, Liposomes, ESR spin trapping, Conjugated diene analysis, O_2-consumption.

Introduction

Damaging of cell membranes and other structures including cellular proteins, lipids and DNA is often related to metabolic processes in human body. Oxidation is a continuous process in cell metabolism and oxidative stress causes functional, structural and physiological disorders and finally leads to cell death and tissue damage [1-3]. Among biological membranous systems, phospholipids are the most unstable biomolecules of a living cell and are mainly affected by uncontrolled production of oxygen radicals. Lipid peroxidation chain reactions in biological systems are propagated by oxidation of lipid radical [4-6]. Peroxyl radical, alkoxyl radicals and lipid hydroperoxides as end products of lipid peroxidation possess potential activities towards carcinogenesis and DNA damages [7].

Antioxidants have an ability to terminate propagating lipid peroxidation chain reactions by removing radical intermediates and can inhibit other oxidation reactions, hence giving oxidative stability to a biological system [1]. Prevention from the diseases has often been associated with a balance between antioxidants and reactive oxygen species [8]. Flavonoids and vitamins have been reported to possess strong antioxidant activity [9] and antioxidant effects have been examined with regard to their interactions with the membranes [10].

In vitro studies on simplified model systems often help to understand the complicated phenomena occurring in the biological systems (i.e., free radical mediated chain reactions). Due to close resemblance of liposome and biological bilayer core, it becomes easy to quantify the antioxidant activity as regard of antioxidant-lipid interaction [11].

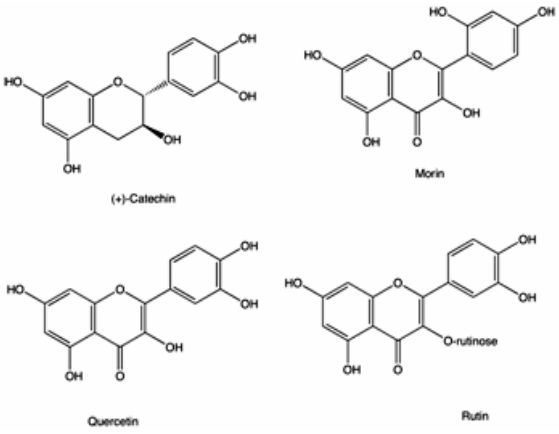
Electron spin resonance (ESR) spectroscopy together with ESR spin trapping technique has been successfully applied to investigate early events in lipid oxidation prior to formation of end products for various food products and to determine the oxidative stability of food lipids under relatively mild conditions [12-14]. The degree of unsaturation of the lipid in various complex foods has been correlated with the formation of a lag phase and determined by spin trapping ESR [15]. Also spin trapping ESR technique is applied to study the antioxidant activities and their efficiencies in biological model membranes in term of the ability of antioxidant to reduce the ESR signal intensities of the stabilized radicals [16-18]. Stabilized radicals (spin adducts) are formed as a result of interaction of the free radicals with spin traps, which are diamagnetic species and their detection is considered as detection of the radicals involved in lipid oxidation [19, 20].

Lipid oxidation in heterogeneous systems is an important phenomenon in many biological

*To whom all correspondence should be addressed.

systems. The spin trapping ESR technique has proven to be a powerful tool for the detection of radical species that are involved in biological phenomena [21]. The use of N-tert-butyl-α-phenylnitrone (PBN) and α-(4-pyridyl-N-oxide)-N-tert-butylnitrone (POBN) as spin traps in biological systems has attracted interest [14, 22].

In present studies, an attempt is made to investigate the effects of antioxidants in preventing lipid oxidation using liposome/PBN and liposome/POBN model systems. We have examined the possibility of using the spin trapping ESR technique based on the commonly applied spin traps (i.e., PBN and POBN) to follow the course of oxidation in a heterogeneous system when initiated by an azo-initiator. Additionally, oxidation in our liposome systems was followed by analysis of the formation of conjugated dienes and/or loss of antioxidants and by analysis of oxygen consumption upon addition of PBN/POBN/antioxidants. Antioxidants – quercetin, rutin, morin and catechin were selected which are structurally related flavonoids (Scheme-1).



Scheme-1: Structures of (+)-catechin, morin, quercetin and rutin.

Results and Discussion

Flavonoids as antioxidants in the liposome system by UV-Spectroscopy

Lipid oxidation was initiated by the hydrophilic azo radical initiator, AAPH, in liposome suspension made from phosphatidylcholine (PC) from soybean. The formation of conjugated dienes (primary oxidation products of polyunsaturated fatty acids) began immediately after the addition of AAPH initiator to a pure liposome suspension, Fig. 1. However, in the presence of flavonoids; quercetin, rutin, morin, and catechin, clear lag phases were observed before the formation of conjugated dienes, Fig. 1(only shown for morin and rutin). Formation of lag phases indicated that the four flavonoids efficiently scavenge the radicals [23] involved in the initiation and propagation steps. At the end of the lag phases, the antioxidants are depleted and the system is characterized by a change to uninhibited lipid oxidation. Among the four flavonoids applied at the same molar concentrations, the lag phases were evaluated as; 890 min, 610 min, 160 min and 90 min for the samples with catechin, rutin, quercetin and morin, respectively. The decreasing order of antioxidative efficiency of four flavonoids is as follows:

catechin > rutin > quercetin> morin.

On the basis of lag phase time, the three flavonoids (catechin, rutin and quercetin) with a catechol structure in the B-ring may be attributed better antioxidants than morin, which has two hydroxyl groups placed in meta positions in the B-ring. The order of catechin, rutin and quercetin correlates with their lipophilicity, where the octanol-water partition coefficients (log *P*) are 1.04, 1.53 and 2.29 for the three flavonoids respectively [24]. Thus hydrophilic antioxidants generally produce the longest lag phases, as described by Robert and Gordon [25] and Wang *et al*. [26].

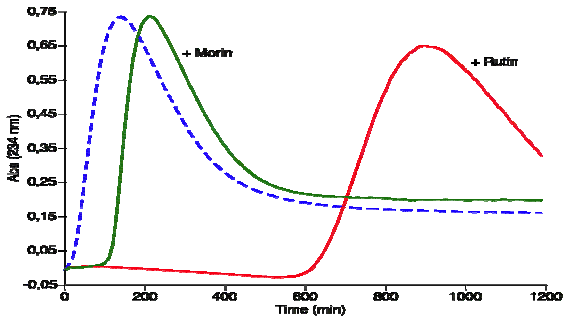


Fig. 1: Formation of conjugated dienes at 37°C in peroxidizing liposomes measured as absorbance changes at 234 nm. Oxidation in the liposome solutions (0.075 mM phosphatidyl choline) with 0.30 μM flavonoids (only shown for morin and rutin) was initiated by AAPH (0.75 mM). Control without flavonids, dashed line.

Effect of Spin Traps on Lipid Oxidation in the Liposome Eystem

The effect of PBN and POBN on lipid oxidation was studied by measuring the rate of

oxygen consumption in the PC liposome systems containing either PBN or POBN and in control liposome systems without spin traps. The detection of lipid oxidation by the measurement of oxygen consumption was chosen since the measurements of conjugated dienes were not possible due to the high UV- absorbance of the two spin traps at 234 nm. The rate of oxygen consumption for the liposome system without spin traps was 0.022 ± 0.004 M $O_2 \cdot s^{-1}$. The rate of oxygen consumption decreased by almost a factor of two to 0.012 ± 0.002 M $O_2 \cdot s^{-1}$ when the liposome system contained POBN; while for the liposome system with PBN it decreased by more than a factor of three to 0.00063 ± 0.00005 M $O_2 \cdot s^{-1}$. This indicated PBN inhibit lipid oxidation more efficiently than POBN (Fig. 2). However, the two spin traps did not completely inhibit lipid oxidation and only lowered the rates of oxygen consumption, without forming a lag phase where the oxygen consumption was efficiently stopped [27]. Hence PBN and POBN both retard lipid oxidation.

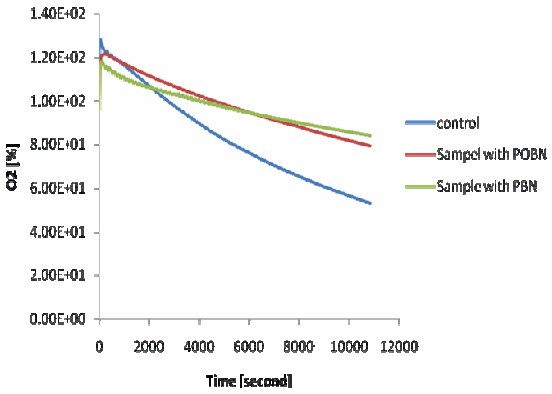
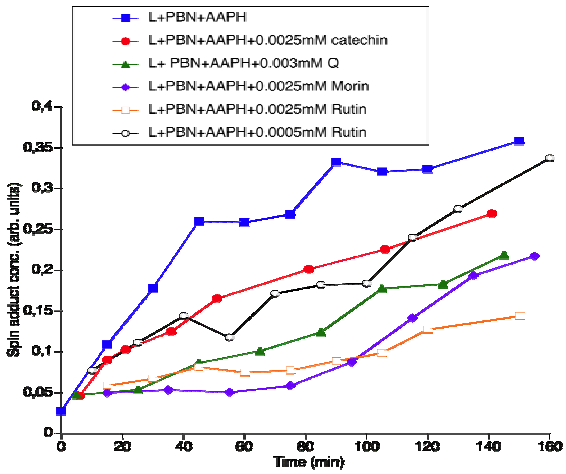
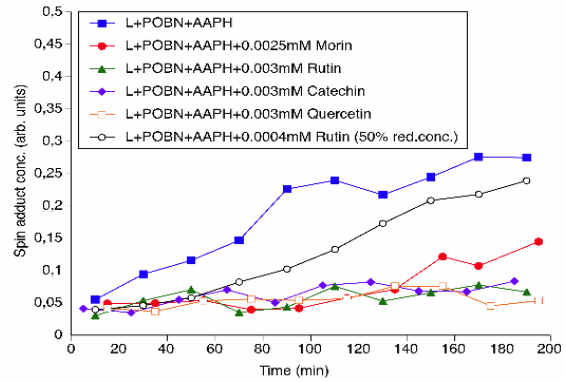


Fig. 2: Oxygen consumption measurements (blue) control ; 1.5 ml liposome + 1.5 ml buffer + 30 ul of 75 mM AAPH, (red) sample; 1.5 ml liposome + 1.5 ml PBN + 30 ul of 75 mM AAPH, (green) sample ; 1.5 ml liposome + 1.5 ml POBN + 30 ul of 75 mM AAPH.

The retardation of lipid oxidation by PBN and POBN was most likely caused by trapping of radicals. Accordingly, formation of spin adducts in the liposome system were observed by ESR with both spin traps immediately from the beginning of experiments in the absence of flavonoids. The intensity of the ESR signals increased linearly with time, and with PBN and POBN the intensity of the signals began to level off after 1 hr (Fig. 3). The results obtained with the spin trapping technique and the measurement of oxygen consumption were thus in good agreement.



(a)



(b)

Fig. 3: Effect of the four flavonoids (3.0 μM quercetin, rutin, morin or catechin) on the inhibition of PBN-spin adducts (a) and POBN-spin adducts (b) in liposome solutions (0.075 mM phosphatidyl choline) at 37°C and added AAPH (0.75 mM) as initiator of oxidation. Relative ESR intensities were obtained by comparing the peak intensities of spin adducts and an internal manganese standard. POBN/PBN = 5.0 mM. Fig. 3a also includes a liposome sample with 0.50 μM rutin. Fig. 3b also includes a liposome sample with 0.40 μM rutin (50% reduction concentrations).

Spin Trapping in Presence of Flavonoids

Experiments with spin trapping in the PC liposome systems were conducted with similar concentrations of flavonoids as used in conjugated diene experiment (0.003 mM). ESR detection of spin adduct formation showed that PBN and POBN spin traps behave differently towards lipid oxidation in the

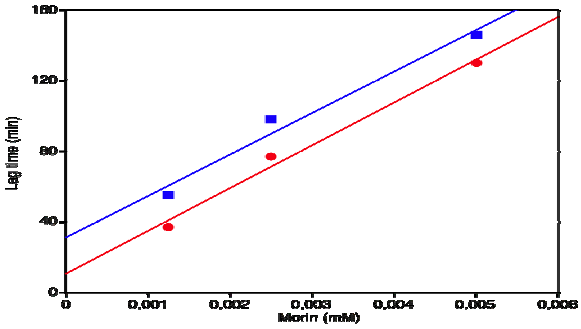
presence of the four flavonoids (Fig. 3). In the experiments with POBN as spin trap (Fig. 3b), no spin adduct formation was observed with quercetin, catechin and rutin, while with morin, ESR signals with a low intensity from spin adducts were seen after a lag phase of 98 min.

In experiments with PBN it was observed that catechin and quercetin reduced the rate of formation of spin adducts, but without giving rise to a lag phase (Fig. 3a). A clear lag phase (77 min) was observed with morin, whereas rutin only gave ESR signals with increasing intensity at the end of the experiment. Form these results it appeared that morin and rutin were efficiently preventing radical formation, whereas catechin and quercetin only partially inhibited the radical formation. These conclusions were in contrast to the results from the conjugated diene measurements and suggested that the presence of spin traps may significantly perturb the oxidation mechanisms in the liposome system.

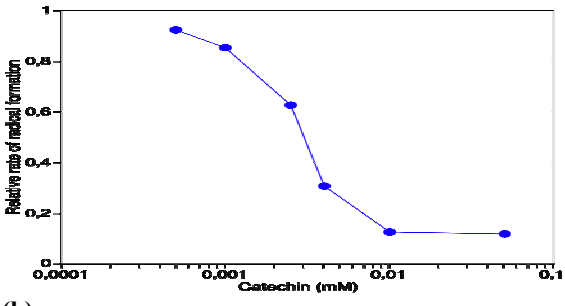
The lengths of the lag phases were linearly correlated to the concentrations of morin in both spin trap systems (Fig. 4a). The lag phases observed with POBN were slightly longer as compared to lag phases for the PBN spin trap system; however, the slopes of the two linear correlations were similar (2.35×10^4 min. mM^{-1} and 2.43×10^4 min. mM^{-1} for POBN and PBN respectively).

Spin trapping ESR experiments were also carried out for various concentrations of quercetin, rutin and catechin, but clear lag phases were not observed. Hence, from experiments with various concentrations of the flavonoids in the liposome/PBN system (as shown for catechin in Fig. 4b), 50% reduction concentrations (the concentrations of flavonoids at which the rate of PBN spin adduct formation is 50 % of the rate in the pure liposome system) were calculated. Linear regressions of plots of relative slopes vs. logarithm of antioxidant concentrations have given 50% reduction concentrations of quercetin and catechin as 0.003 mM each and rutin as 0.004 mM by interpolation. While for morin lag phase was observed at 0.0025 mM. Based on this type of evaluation rutin appeared to be almost ten times more efficient antioxidant as compared to catechin and quercetin.

ESR experiments were also carried out with 50 % reduction concentrations of the four flavonoids in the liposome system with POBN as spin trap (Fig. 3). However, in the liposome/POBN-system, formation of spin adducts with quercetin, and catechin were negligibly small, while a lag phase was observed for rutin, the reason being the concentration of rutin which was 10 times smaller than that of other three flavonoids.



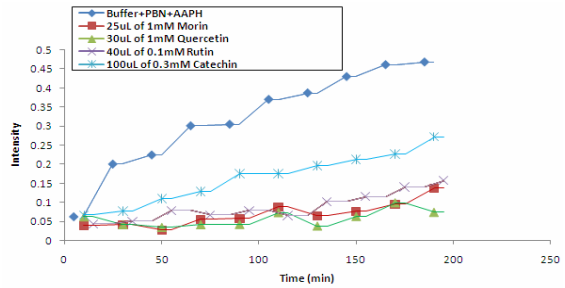
(a)



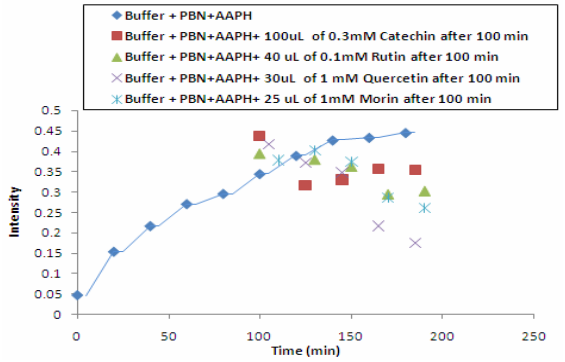
(b)

Fig. 4: Effect of the morin concentration on the lag time for formation of spin adducts (a) and effect of catechin concentrations on the relative rate of PBN-spin adduct formation (b) in liposome solutions at 37°C. Liposomes (0.075 mM phoshatidyl choline) with AAPH (0.75 mM) as radical initiator, containing PBN (5 mM) or POBN (5 mM). For Fig. 4a; PBN (circles), POBN (squares).

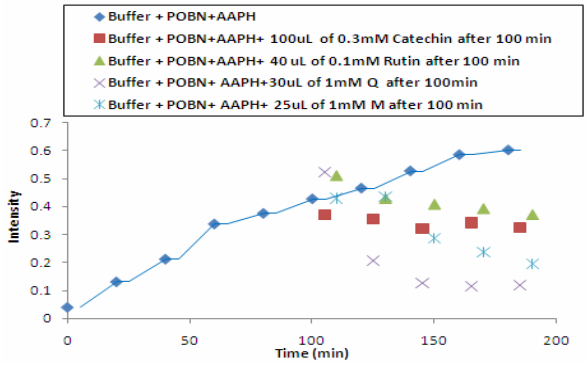
The interaction between the flavonoids and the spin traps were studied by experiments performed with a buffer/PBN-spin trap systems without liposomes (Fig. 5a). In this system the dominating radicals are assumed to be peroxyl radicals derived from the alkyl groups in AAPH. It was observed that in buffer/PBN spin trap system, spin adduct formation was efficiently quenched by quercetin, rutin and morin, showing lag phases of greater than 100 min. However, catechin partially reduced the rate of spin adduct formation and no lag phase was observed. From ESR spin trapping data for buffer/spin trap systems, quercetin, rutin and morin were looking efficient scavengers of peroxyl radicals. However, higher intensities of spin adducts with catechin in buffer/PBN spin trap system showed that catechin did not efficiently scavenge peroxyl radicals.



(a)



(b)



(c)

Fig. 5: Effect of 50 % reduction concentrations of the four flavonoids (quercetin 0.003mM, rutin 0.0004 mM, morin 0.025 mM and catechin 0.003 mM) on the inhibition of PBN-spin adducts in 0.01 mM phosphate buffer (pH 7.4) at 37°C and added AAPH (0.75 mM) as initiator of oxidation (a); interaction of quercetin, rutin, morin and catechin with PBN-spin adducts (b) and POBN-spin adducts (c) as formed at 37°C in 0.01 mM phosphate buffer (pH 7.4) upon addition of AAPH (0.75 mM). The four flavonoids were added at 50 % reduction concentrations after 100 min of incubation.

The effect of catechin in the buffer/PBN-spin trap system was further investigated by studying the interaction between spin adducts and the flavonoids. This was carried out by adding the flavonoid to a buffer system with preformed spin adducts (Fig. 5b and c). The flavonoids were added to a buffer system containing AAPH and a spin trap, and where a substantial concentration of spin adducts had been generated by heating at 37°C for 100 min. Catechin stopped the formation of both the PBN and the POBN spin adducts, and the preformed spin adducts were not destroyed. This demonstrated that catechin trapped the initial radicals (peroxyl radical) and that it did not interact with spin adducts. In the case of three other flavonoids, the concentrations of spin adducts decreased after the addition of the flavonoid, indicating that both PBN and POBN spin adducts were not stable in the presence of rutin, quercetin and morin.

Relative ESR intensities were obtained by comparing the peak intensities of spin adducts and an internal manganese standard. PBN and POBN = 5.0 mM.

The ESR signals and hyperfine coupling constants (a_H and a_N) calculated for liposome/spin trap and buffer/spin trap systems, with and without antioxidants, have more or less similar values, Fig. 6. Only the intensity of the ESR spectra of the spin adducts were affected by the addition of the flavonoids. The hyperfine coupling constants; a_H = 4.0 G and a_N = 15.5 G for the PBN spin adducts suggested that alkyl radicals were trapped [28]. Similarly, the hyperfine coupling constants a_H = 2.5 G and a_N = 15.0 G for the POBN spin adducts were in agreement with trapping of alkyl radicals [29]. No additional signal was observed, showing that identical radical species were trapped within liposome/spin trap and buffer/spin trap systems; hence must be present in the aqueous phase and most likely derived only from the AAPH initiator. The results also indicated that PBN, being partially lipophilic, was not able to generate spin adducts derived from trapping of lipid-derived radicals in the liposomes.

Hyperchem PM3 Semi-Emperical Studies of Flavonoids

Hyperchem version 5.0 package was used to find the charges on reactive sites of structurally related flavonoids, i.e., quercetin, rutin, morin, Fig. 7. Structures of flavonoids were drawn and optimized using the Restricted Hartree-Fock (RHF) method with PM3 parameterization. From the charge data [9],

it was indicated that oxygen atoms of hydroxyl groups present on ring-B moiety have low negative charges as compared to other atoms of flavonoid structure {quercetin: 3′-OH(-0.218); 4′-OH(-0.214), rutin: 3′-OH (-0.081); 4′-OH(-0.227), morin: 2′-OH(-0.099); 4′-OH(-0.226)}, Fig.7. Hence the possibility of proton/hydrogen-atom transfer from the hydroxyl groups of B-ring was comparatively larger. The total charge on oxygen atoms of reactive entity (ring-B) of rutin was calculated lesser (-0.308) than that on morin (-0.325) and quercetin (-0.432).

Spin trapping ESR studies of the structurally related flavonoids – quercetin, rutin, morin and catechin indicated that these compounds may scavenge free radicals effectively upto 50% reduction concentration. The effective concentration (50% reduction concentration) of rutin was about 10 times lesser than that of quercetin, morin and catechin. The greater efficiency of rutin may also be related with its more stable radical formed while scavenging free radicals. Formation of stable radical of rutin may further be correlated to its bulky nature as compared to other flavonoids. Hyperchem PM3 semi-emperical calculations of charges on quercetin, morin and rutin also supported this evidence. Thus rutin has the greater chance to lose its proton/hydrogen-atom even at its low concentration while interacting with a free radical as evident by comparatively low total charge on oxygen atoms of reactive entity (ring-B) of rutin(-0.308) than that on morin (-0.325)and quercetin (-0.432).

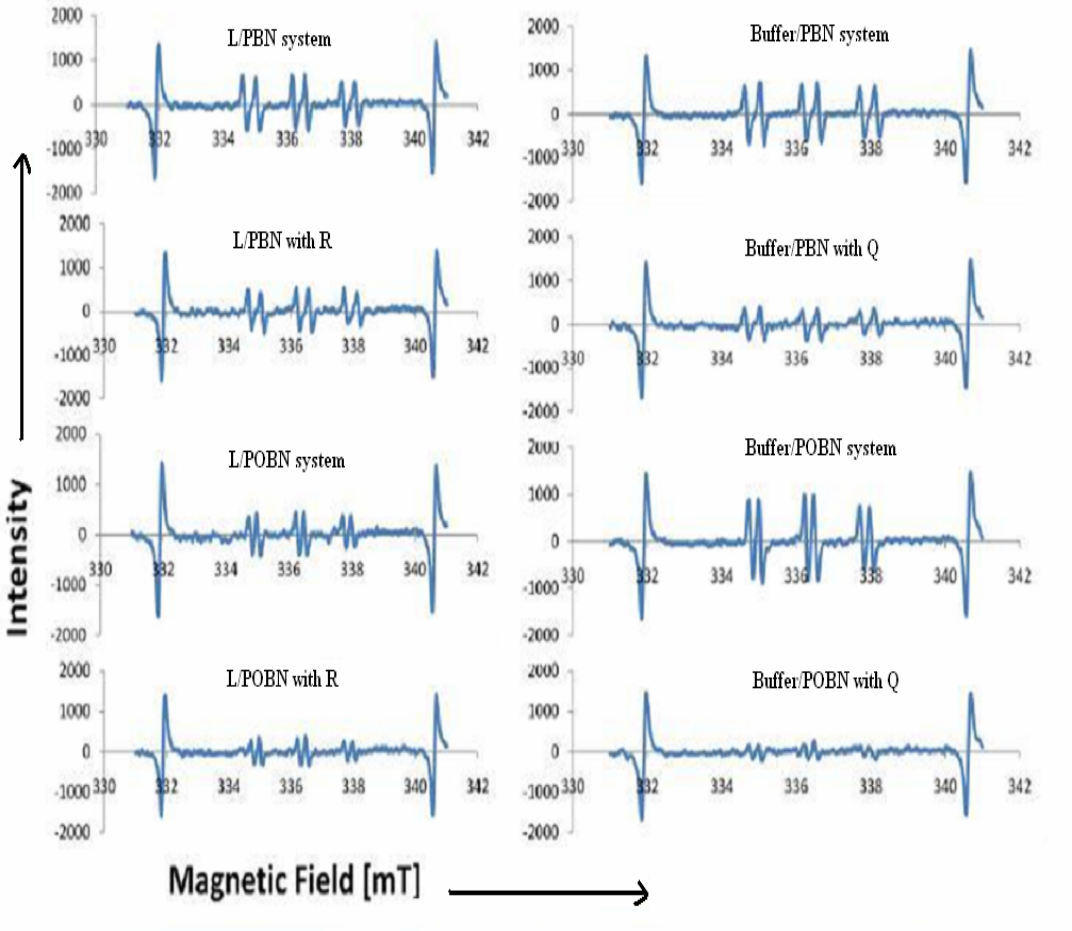


Fig. 6: ESR intensity signals of liposome (L)/spin trap and buffer/spin trap systems with and without antioxidants. Only few spectra are presented.

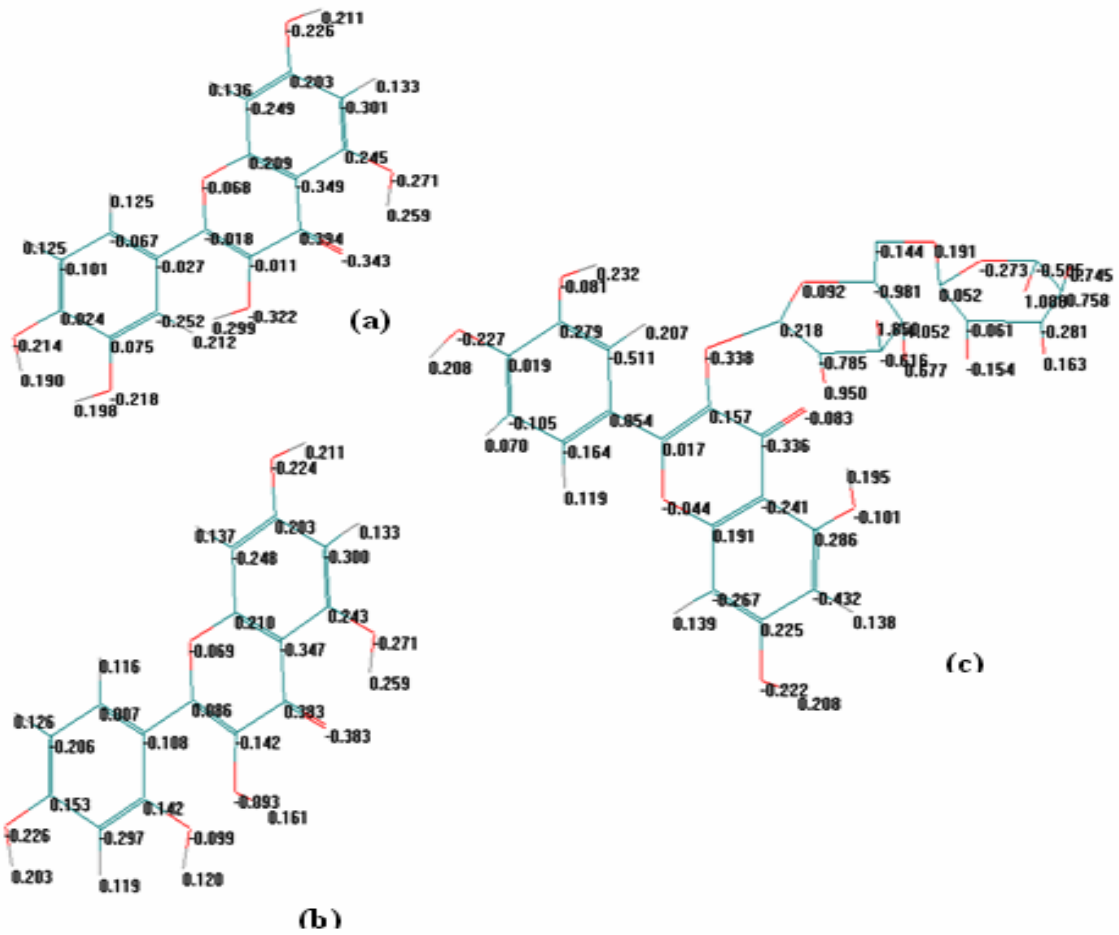


Fig. 7: Charges on optimized structures of three structurally related flavonoids: (a) quercetin, (b) morin and (c) rutin calculated by PM3 method using Hyperchem version 5.

Pertaining to different nature of spin traps, quercetin, rutin and catechin showed greater ability to quench free radicals generated in the lipid system with POBN than those with PBN spin trap, respectively. However, spin trapping ESR studies of these flavonoids with PBN and POBN spin traps to investigate radical scavenging efficiencies within liposome system have raised several questions.

In ESR spin trapping experiments, with liposome/POBN spin trap system, no spin adduct formation was observed with most of the concentrations and even with similar concentrations (3.0 μM) of quercetin, rutin and catechin. It showed that POBN is not interacting with peroxyl radicals and these radicals were only scavenged by antioxidants, hence inhibiting lipid oxidation. However, no clear lag phase was observed. Higher intensity signals were obtained in liposome/PBN-spin trap system, showing that these antioxidants were not very efficient towards stopping lipid peroxidation. Decreasing slopes of intensity signals in the presence of antioxidants in this system revealed that PBN may also be trapping the peroxyl radicals. Delayed lag phases with various concentration of morin in liposome/POBN as compared to liposome/PBN-spin trap system showed that quenching ability of morin is more in former system. Hence, it may be inferred that morin is comparatively more efficient towards inhibiting lipid peroxidation in liposome/POBN instead of liposome/PBN spin trap system.

The destruction of spin adducts with quercetin, rutin and morin in buffer/PBN and buffer/POBN spin trap systems have shown that antioxidants were not only interacting with the free radicals but also with the spin adducts within both systems, hence leading to the longer lag phases with buffer/spin trap systems. Only catechin has shown comparatively good antioxidant activity towards scavenging peroxyl radical in buffer/spin trap systems, as evident by the formation of stable spin

adducts with it. The similar values of hyperfine coupling constants further ensured that only one type of radical species exists whether the system is buffer or liposome. The coupling constant results also showed that these species must exist in the aqueous phase and not in the lipid phase.

Oxygen consumption experiments have shown the possibility of PBN spin trap to contribute more towards stopping lipid oxidation. Within liposome/spin trap systems, the possibility of PBN (being partially lipophlic) to go inside the lipid bilayer is looking more probable as compared to POBN, which is more water soluble than PBN.

Spin trapping ESR along with oxygen consumption experiments have shown that the possibility of PBN spin trap to contribute towards stopping lipid oxidation (outside the lipid bilayer in an aqueous phase) cannot be ignored. On the other hand, decreasing signal intensities of spin adducts in liposome/POBN spin trap system with some concentrations of antioxidants also showed POBN interaction towards peroxyl radical. With these results, the extent of interaction of flavonoids with the peroxyl radicals in an aqueous phase (outside the lipid bilayer) to stop lipid oxidation within liposome/spin traps systems seems relatively more difficult to evaluate for liposome/PBN as compared to liposome/POBN spin trap system.

Lipid peroxidation in the lipid phase of liposome may be initiated when peroxyl radicals are generated in the aqueous phase of liposome and go inside the lipid bilayers. Here they interact with lipid molecule and convert it into lipid radical which in the presence of oxygen changes into lipid derived peroxyl radical. As no new signals of lipid derived peroxyl radicals were observed in this study, therefore in liposome/PBN spin trap system there could be least possibility of spin adducts formation due to the interaction of peroxyl radicals with PBN within lipid bilayers. Hence to ensure the extent of radicals to be trapped by antioxidants looks quite uncertain and quantification of antioxidants in terms of scavenging radical species in liposome model system in the presence of PBN and POBN spin traps through ESR spectroscopy may needs care.

Experimental

Chemicals

(+)-catechin hydrate, rutin hydrate, quercetin dihydrate, morin, L-α-phosphatidylcholine (PC) from soybean (~99%), N-tert-butyl-α-phenylnitrone (PBN) (98%), α-(4-pyridyl-N-oxide)-N-tert-butylnitrone (POBN) (99%) were purchased from Sigma-Aldrich (Steinheim, Germany), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals Inc. (Richmond, VA, USA). All solvents were of HPLC-grade and supplied by Lab Scan Analytical Sciences (Dublin, Ireland). Water was purified through a Millipore Q-plus purification train (Millipore Crop., Bedford, MA, USA).

Preparation of Liposomes

Liposomes were prepared according to Roberts and Gordon [25] with minor modifications as added by Graversen *et al*. [30]. For experiments with catechin, a solution of 2.0 ml 0.75 mM (1.5 µmol) soybean phosphatidylcholine dissolved in chloroform was mixed with 1.0 ml of hexane. While with the more lipid soluble rutin, quercetin and morin, hexane was replaced by methanol with the actual antioxidant dissolved. The resulting solvent 3.0 ml was subsequently removed on rotary evaporator under reduced pressure of approximately 100 mbar and the water bath was at 30°C. After complete evaporation of solvents, nitrogen was flushed to re-establish atmospheric pressure in the evaporation flask. The lipid residue was subsequently re-hydrated with 10 ml of 0.01 mM phosphate buffer ($NaH_2PO_4.H_2O$, pH = 7.4) and vortex for 10 min. Ultrasonification for 30 sec was carried out after vortex to ensure the complete recovery of white homogeneous suspension of multilamellar liposomes. Multilamellar liposomes were extruded to unilamellar liposomes by passing the suspension 15 times through an Avestin Lipofast Basic small volume (500 µl) extrusion device (Avestin Europe GmbH, Mannheim, Germany) equipped with a double layer of polycarbonate membranes with a pore size of 100 nm. The concentrations of antioxidants in the liposomes preparations were calculated as mol % (antioxidant: lipid).

Kinetic Analysis of the Formation of Conjugated Dienes

Lipid peroxidation in the liposomes was followed by measuring the formation of AAPH initiated conjugated dienes and by monitoring the changes in absorbance at 234 nm (A_{234}) using a HP 8453 UV-Vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with an automatic cell changer. 0.01 mM Phosphate buffer (pH = 7.4) was used as blank. Liposome suspensions (3.0 ml) were taken into quartz cuvettes and thermostated at 37°C for 10 min. Lipid oxidation was then initiated by adding 30 µl of 75 mM AAPH dissolved in phosphate buffer to each cuvette and immediately hereafter the absorbance was measured continuously for 20 hr. After adding AAPH, the

cuvettes were quickly inverted 4 to 5 times and then covered to avoid evaporation. Four samples containing same concentrations of quercetin, rutin, morin and catechin were measured along with two controls. Antioxidant concentration was 0.003 mM (3.0 µM) and liposome suspension without antioxidants was used as control. The absorbance was measured at 234 nm (absorption maximum of conjugated dienes) at every 600 sec (10 min) for 20 hr. Lag phases were measured as the time in minutes corresponding to the intercept between the tangent to the propagation phase and the tangent to the lag phase [25].

ESR Spin Trapping Analysis

All electron spin resonance experiments were performed on an ESR instrument (Jeol, JES-FR30 Free Radical Monitor, Japan). ESR experiments were carried out for various concentrations of antioxidants in liposome solutions, and the samples were prepared in glass tubes by mixing 5.0 ml of extruded unilamellar liposomes and 5.0 ml of the spin-traps PBN or POBN (10.0 mM) in 0.010 mM phosphate buffer (pH = 7.4). In each glass tube was added 100 µl of 75.0 mM AAPH initiator immediately before start and stored in a water bath at 37°C throughout the experiment. Time-profiles of ESR signals for all samples were then recorded by withdrawing samples from the actual glass tubes and into the ESR cavity in regular time intervals. 5.0 ml of unilamellar liposome (without antioxidant) along with 5.0 ml of PBN or POBN (10.0 mM) and 100 µl of 75.0 mM AAPH were used for control samples. The ESR settings were as follows: center field, 336.000 mT; microwave power, 4.0 mW; sweep width, 10.0 mT; sweep time, 2.0 min; accumulation, 1; time constant, 0.3 s; Modulation frequency, 100 kHz, modulation width, 0.125 mT. In the resulting ESR spectra, which showed the presence of PBN spin adducts or POBN spin adducts (triplet of dublets), the signal intensity of the center field line was measured relative to the signal height of an internal manganese standard.

Oxygen Consumption Measurement

The effect of the spin traps PBN and POBN on the oxidation of liposomes was evaluated by measuring the rate of oxygen consumption in the liposomes upon addition of AAPH. Microsensors (Unisense picoammeter, PA 2000 with unisense OX-MR electrodes, Aarhus N, Denmark) equipped with Profix software v.3.05 (Unisense, Denmark) were used to follow the depletion of oxygen at 37°C in the liposome solutions. 1.5 ml liposome solution along with 1.5 ml buffer was used as control while samples contained 1.5 ml liposome solution and 1.5 ml PBN or POBN. 30 µl of 75 mM AAPH was added into control and samples immediately before running the experiment. Oxygen consumption analysis experiment was carried out for the control and the samples for 3 hr at time intervals of 10 sec. Calibration of the microsensors was done with anoxic (0% O_2) and air-saturated water (100% O_2). The rate of oxygen consumption υ (O_2) $M \cdot s^{-1}$ was measured from the slope (α) of the consumption curve by using:

$$\upsilon (O_2) = -\alpha [O_2]_{initial}/100 \quad (1)$$

where water air-saturated at 37°C, $[O_2]_{initial}$ is 2.2×10^{-4} M [31], corresponding to the 100% calibration point.

Acknowledgments

We appreciate Higher Education Commission of Pakistan for providing the financial support under "International Research Support Initiative Program" to pursue this research work at Department of Food Science, Faculty of Life Sciences, and University of Copenhagen, Denmark.

References

1. Y. Yesiloglu and L. Sit, *Spectrochimica Acta Part* A, **95**, 100 (2012).
2. G. Petrosillo, N. D. Venosa, F. M. Ruggiero, M. Pistolese, D. D. Agostino, E. Tiravanti, T. Fiore, G. Paradies, *Biochimica et Biophysica Acta,* **1710**, 78 (2005).
3. A. Wood-Kaczmar, S. Gandhi and N. W. Wood, *Trends in Molecular Medicine,* **12**, 521 (2006).
4. K. F. Azhar and K. Nisa, *Journal of the Chemical Society of Pakistan,* **28**, 3 (2004).
5. E. N. Frankel, Free radical oxidation. In: Lipid Oxidation; 2nd Ed., PJ Barnes 6 Associate, England, the Oily Press (2005) pp. 15–23.
6. A. Kamal-Eldin and J.Pokorný, Lipid oxidation products and methods used for their analysis, In: Analysis of Lipid Oxidation, (eds. A. Kamal-Eldin and J. Pokorný),Champaign, IL, USA, AOCS Press (2005) pp. 1–7.
7. M. H. Yang and K. M. Schaich, *Free Radical Biology and Medicine,* **20**, 225 (1996).
8. A. T. Diplock, *Molecular Aspects of Medicine,* **15**, 293 (1994).
9. N. Arshad, N. K. Janjua, A. Y. Khan, J. H. Zaidi and L. H. Skibsted, *Monatshefte für Chemie-Chemical Monthly,* **143**, 377 (2012).
10. A. Saija, M. Scalese, M. Lanza, D. Marzullo, F. Bonina and F. Castelli, *Free Radical Biology and Medicine.* **19**, 481 (1995).
11. S. M. Elfaramawy and A. R. Rizk, *Journal of American Society,* **7,** 363 (2011).

12. L. L. Yu, Z. Cheng, *Molecular Nutrition and Food Research,* **52**, 62 (2008).
13. J. Velasco, M. L. Andersen and L. H. Skibsted, *Food Chemistry,* **85**, 623 (2004).
14. J. Velasco, M. L. Andersen and L. H. Skibsted, *Journal of Agricultural and Food Chemistry,* **53**, 1328 (2005).
15. M. K. Thomsen, C. Jacobsen and L. H. Skibsted, *European Food Research and Technology,* **211**, 381 (2000).
16. M. Murakami, K. Fukatsu, S. Ohkawa, F. Kasahara and T. Sugawara, *Chemical and Pharmaceutical Bulletin,* **48**, 784 (2000).
17. P. Stocker, J. F. Lesgards, N. Vidal, F. Chalier and M. Prost, *Biochimica. et Biophysica Acta,* **1621**, 1 (2003).
18. M. E. Gutierrez, A. F. García, M. A. de Madariaga, M. L. Sagrista, F. J. Casadó and M. Mora, *Life Sciences*, **72**, 2337 (2003).
19. E. G. Janzen and D. L. Haire, Adv. *Free Radical Chemistry,* **1**, 253 (1990).
20. S. Pou, H. J. Halpern, P. Tsai and G. M. Rosen, *Accounts of Chemical Research,* **32**, 155(1999).
21. M. L. Andersen, J. Velasco and L. H. Skibsted, Analysis of lipid oxidation by ESR spectroscopy In: Analysis of Lipid Oxidation (eds. A. Kamal-Eldin and J. Pokorný), Champaign, IL, USA, AOCS Press (2005) pp. 127-151.
22. F. J. Monahan, L. H. Skibsted and M. L. Andersen, *Journal of Agricultural and Food Chemistry,* **53**, 5734 (2005).
23. S. Jongberg, C. U. Carlsen and L. H. Skibsted, *Free Radical Research,* **43**, 932 (2009).
24. J. Zhang, L. D. Melton, A. Aselle and M. A. Skinner, *European Food Research and Technology,* **228**, 123 (2008).
25. W. G. Robert and M. H. Gordon*, Journal of Agricultural and Food Chemistry,* **51**, 1486 (2003).
26. H. Wang, G. Cao and R. L. Prior, *Journal of Agricultural and Food Chemistry,* **44**, 701 (1996).
27. L. R. C. Barclay and M. R. Vinqvist, *Free Radical Biology and Medicine,* **28**, 1079 (2000).
28. M. P. Murphy, K. S. Echtay, F. H. Blaikie, J. A. Cayuela, H. M. Cochemé, K. Green, J. A. Buckingham, E. R. Taylor, F. Hurrell, G. Hughes, S. Miwa, C. E. Cooper, D. A. Svistunenko, R. A. J. Smith, M. D. Brand, *The Journal of Biological Chemistry,* **278**, 48534 (2003).
29. M. B. Kadiiska, A. J. Ghio and R. P. Mason, *Spectrochimica Acta Part* A*,* **60**, 1371 (2004).
30. H. B. Graversen, E. M. Becker, L. H. Skibsted and M. L. Andersen, *European Food Research and Technology,* **226**, 737 (2008).
31. G. S. Allgood and J. J. Perry, *Journal of Basic Microbiology,* **7**, 379 (1986).