Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Photochemistry and Photobiology A: Chemistry 163 (2004) 141–148
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Photosensitisation with naphthoquinones and binaphthoquinones: EPR spin trapping and optical studies-formation of semiquinone radical and reactive oxygen species on photoillumination

K.K. Mothilal [a], J. Johnson Inbaraj [b], Colin F. Chignell [b], R. Gandhidasan [c], R. Murugesan [c,*]

[a] *Department of Chemistry, Saraswathi Narayanan College, Madurai 625 022, India*
[b] *Laboratory of Pharmacology and Chemistry, National Institute of Environmental, Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA*
[c] *School of Chemistry, Madurai Kamaraj University, Madurai 625 021, India*

Received 30 June 2003; received in revised form 28 October 2003; accepted 19 November 2003

Abstract

Photodynamic action of two naphthoquinones, 7-methyljuglone (MJ) and plumbagin (PG) and two binaphthoquinones, isodiospyrin (IDP) and diospyrin (DP) extracted from the stem of *Diospyros paniculata* and roots and root barks of *Diospyros montana*, are studied by using optical and EPR spin trapping techniques. Efficiency of singlet oxygen generation (by RNO bleaching and EPR spectroscopy) and rate of superoxide generation (by SOD inhibitable ferricytochrome c reduction and EPR spin trapping assays) are determined. In anaerobic medium semiquinone anion of IDP was produced both in dark and in light. On photoillumination of aerobic solution, superoxide radical was generated by all the four quinones via electron transfer to O_2. In aqueous solution, the superoxide radical anion formed, rapidly changes to −OH radical via Fenton's reaction. These findings reveal that the photosensitisation of naphthoquinones and binaphthoquinones may proceed via Type I and Type II mechanisms.

Keywords: ROS; Spin trapping; DMPO; Anthraquinone; Superoxide generation

1. Introduction

Photosensitisation reactions usually involving reactive oxygen species (ROS), are important in many biological systems [1,2]. Quinones, naphthoquinones and their derivatives possess potent photodynamic action [3]. Quinones derived molecules are highly redox active which can undergo redox cycle with their semiquinone radicals, leading to formation of ROS, including superoxide, H_2O_2 and ultimately the hydroxyl radicals [4]. Photodynamic action of aminonaphthoquinone, perylene quinones and their derivatives have been investigated [2,5–7]. Photosensitisation of aminoanthraquinones and water soluble naphthoquinones have been studied for their efficiency to generate reactive oxygen species [2,8]. Photoinduced production of superoxide anion radical by adriamycin and daunorubicin is also well known [9]. Photogeneration of ROS by anticancer agents is of current interest due to its potential in photodynamic therapy [9].

Recently we have investigated some naturally occurring naphthoquinones and binaphthoquinones for their cytotoxic action against human breast cancer MCF-7 cell [10]. Incubation of these quinones with MCF-7 cells produces superoxide anion radicals. The present study reports the photogeneration of $^1O_2$ and superoxide from these quinones. Optical and EPR spin trappings techniques are used to investigate the transient products ($^1O_2$, $O_2^{\bullet -}$, –OH, semiquinone radical anion). In addition, the effect of EDTA and NADH on the efficiency of photogeneration of superoxide anion is also presented here.

Abbreviations: EPR, electron paramagnetic resonance; ROS, reactive oxygen species; SOD, superoxide dismutase; RNO, *N*,*N*-dimethyl-4-nitrosoaniline; TEMPL, 2,2,6,6-tetramethyl-4-piperidinol; TEMPOL, 2,2,6,6-tetramethyl-4-piperidinol-*N*-oxyl; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DABCO, 1,4-diazabicyclo[2,2,2]-octane; RB, rose bengal; NADH, reduced nicotinamide adenine dinucleotide; DETAPAC, diethyltriaminopentaacetic acid; DMSO, dimethyl sulphoxide; $^1O_2$, singlet oxygen; $O_2^{\bullet -}$, superoxide anion radical; EDTA, ethylenediaminetetraacetic acid

* Corresponding author. Tel.: +91-452-858246; fax: +91-452-2459105.
E-mail address: rammurugesan@yahoo.com (R. Murugesan).

1010-6030/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.jphotochem.2003.11.007

2. Experimental

2.1. Chemicals

N,*N*-Dimethyl-4-nitrosoaniline (RNO), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 1,4-diazabicyclo[2,2,2]-octane (DABCO), diethyltriaminopentaacetic acid (DETAPAC), glutathione (GSH) and rose bengal (RB) were obtained from Aldrich. Imidazole, ethylenediaminetetraacetic acid and (EDTA) and sodium azide were purchased from S.D. Fine Chemicals. 2,2,6,6-Tetramethyl-4-piperidinol (TEMPL) was obtained from Merck. Superoxide dismutase (SOD), cytochrome c reductase and catalase were purchased from Sigma while reduced nicotinamide adenine dinucleotide (NADH) was obtained from Boehringer Mannheim. MCF-7, a human tumour cell lines was obtained from American Type Culture Collection, Rockville, MD. Human breast cancer MCF-7 cell were grown in improved minimum essential media, supplemented with 5% (v/v) fetal bovine serum, L-glutamine 292 μg/ml) and 1% (v/v) each of penicillin G (100 μg/ml) and streptomycin (100 μg/ml). Dimethyl sulfoxide (HPLC grade) from Qualigens Fine Chemicals, India, was used. DMPO was purified [11] before use-briefly: 1 g of DMPO was shaken well with 5 ml of water and 5 ml toluene and the supernatant removed (repeated for three times). Excess toluene was removed by bubbling nitrogen through the solution. Then wet charcoal was added to the DMPO solution and shaken well. The solution was centrifuged, the supernatant isolated and filtered. The concentration of the spin trap DMPO was determined spectrophotometrically using $\varepsilon_{227} = 8000\,M^{-1}\,cm^{-1}$. Doubly distilled water was used for all the experiments. Imidazole was used after repeated crystallisation from doubly distilled water. All other compounds were used as received.

2.2. Extraction of naphthoquinones and binaphthoquinones

Naphthoquinones and binaphthoquinones used in this study were extracted, following the reported procedure [10,12,13] from the stem of *Diospyros paniculata* and roots and root barks of *Diospyros montana*. Quinones MJ and DP were obtained from the air-dried roots and root barks of *D. montana*. Detailed procedures for the extraction of naphthoquinones are described elsewhere [10,12,13]. Recording the ^{1}H NMR on a JEOL GSX 400 spectrometer with tetramethylsilane as internal standard checked the purity of the compounds. The solid MJ obtained from the petroleum ether–benzene eluate of the column, on crystallisation from petroleum ether yielded 7-methyljuglone, MJ as orange red crystalline solid was found to be homogeneous on TLC in various solvent systems. A comparison with literature values confirmed its identity. ^{1}H NMR ($CDCl_3$): δ 2.45 (s, 3H, Me-7) 6.90 (s, 2H, H-2 and H-3) 7.10 (d, 1H, H-6, $J = 1.5$) 7.45 (d, 1H, H-8, $J = 1.5$) 11.90 (s, 1H, HO-5). IR (ν, cm^{-1}) (KBr) 1670, 1638 (C=O), 3442 (–OH), 1590, 1521, 1480,1384. UV (λ_{max}, $CHCl_3$) (nm) 217, 252, 430 (corresponds to naphthoquinone moiety). Pure benzene eluate gave an orange solid diospyrin DP and was crystallised from petroleum ether afforded an orange red crystalline solid. ^{1}H NMR ($CDCl_3$): δ 2.30 (s, 3H, Me-7′) 2.45 (s, 3H, Me-7) 6.90 (s, 1H, H-3) 6.95 (s, 2H, H-2′; H-3′) 7.15 (s, 1H, H-6) 7.55 (s, 1H, H-8) 7.60 (s, 1H, H-8′) 11.90 (s, 1H, HO-5) 12.15 (s, 1H, HO-5′). IR (ν, cm^{-1}) (KBr) 1670, 1644 (C=O), 3440 (–OH), 1609, 1562, 1484,1442. UV (λ_{max}, $CHCl_3$) (nm) 255, 430 (corresponds to naphthoquinone moiety). Quinones IDP and PG were extracted from stem of *D. peniculata*. PG was crystallised from petroleum ether and gave orange yellow needles. ^{1}H NMR ($CDCl_3$): δ 2.18 (d, 3H, C-2, OMe) 6.79 (q, 1H, H-3) 7.24–7.58 (m, 3H, H-6, 7, 8) 11.95 (s, C–H, –HO). IR (ν, cm^{-1}) (KBr) 1663, 1644 (C=O), 3454 (–OH), 902, 835, 753, 671. UV (λ_{max}, $CHCl_3$) (nm) 210, 250, 414 (corresponds to naphthoquinone moiety). IDP was crystallised from benzene yielded red prism crystals. ^{1}H NMR ($CDCl_3$): δ 2.02 and 2.05 (2s, 6H, C-7 & 7′ 2*x*-CH_3) 6.70 (d, 1H, H-2′) 6.90 (d, 1H, H-3′) 6.935 (s, 1H, H-2) 6.939 (s, 1H, H-3) 7.29 (s, 1H, H-6′) 7.59 (s, 1H, H-8) 12.12 and 12.50 (2s, 2H, C-5 & 5′, 2*x*-OH). IR (ν, cm^{-1}) (KBr) 1671, 1639 (C=O), 3461 (–OH), 1278, 1101, 1046, 849, 751, 668. UV (λ_{max}, $CHCl_3$) (nm) 215, 253, 430 (corresponds to naphthoquinone moiety). The chemical structures of these quinones are given in Fig. 1.

2.3. Light source

A 150 W xenon lamp was used for photolysis. A filter combination of 10 cm of potassium iodide solution (1 g in

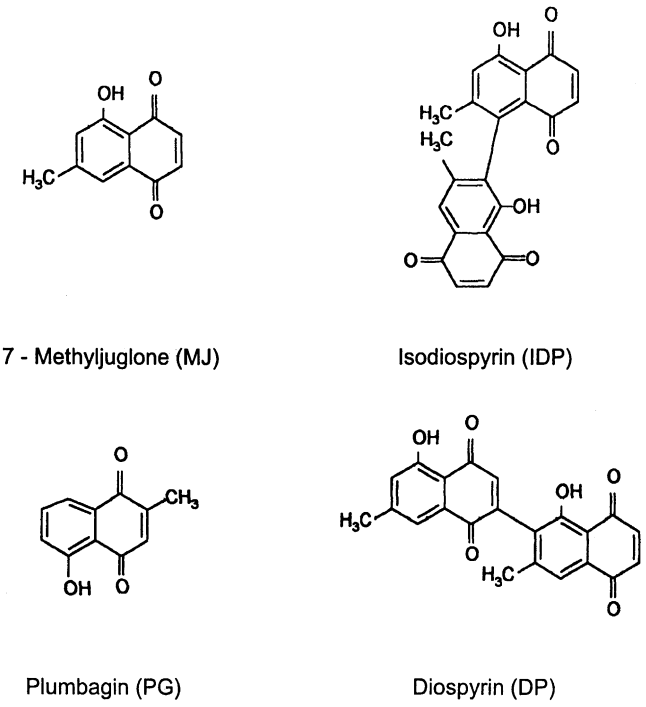


Fig. 1. Chemical structures of MJ, IDP, DP and PG.

100 ml of water) plus 1 cm of pyridine was used to cut off below 300 nm to get a spectral window of 300–700 nm. The reaction mixture in a quartz cuvette placed at a distance of 12 cm from the light source was continuously stirred during irradiation. The irradiation was generally carried out in an open cuvette in equilibrium with the atmosphere.

2.4. EPR measurements

A JEOL JES-TE100 ESR spectrometer was used for EPR measurements. The following parameters were set for the measurements: microwave power, 10 mW; modulation amplitude, 0.01 mT; modulation frequency, 100 kHz.

2.4.1. Detection of singlet oxygen

The photoproduction of $^1O_2$ by quinones can be readily studied by EPR spectroscopy using TEMPL (2,2,6,6-tetramethyl-4-piperidinol) as singlet oxygen trap [13,14]. Reaction mixture (1 ml) containing 10 mM TEMPL and quinones (100 μM) in DMSO was irradiated. Reaction mixture (150 μM) was drawn into gas permeable Teflon capillary tube (0.8 mm side diameter, 0.5 mm wall thickness) which was folded and inserted into a narrow quartz tube and placed in the EPR cavity for measurements. The increase in EPR signal intensity of the TEMPOL (2,2,6,6-tetramethyl-4-piperidinol-*N*-oxyl) radical produced with irradiation time was followed.

2.4.2. Detection of superoxide anion

Spin trapping experiments were used to determine the production of superoxide anion on irradiation of sensitisers. Solutions of sensitisers (100 μM) were irradiated in the presence of 40 mM DMPO in DMSO. Experiments were repeated to monitor the signal intensity at different intervals of irradiation time. The transient radical species were trapped by DMPO to form DMPO-adducts. The spectral identification of the spin adducts was confirmed by simulating the spectra with known hyperfine coupling constants (hfc) and comparing them with the experimentally observed one. A BASIC program was used to stimulate the EPR spectra.

2.4.3. Detection of semiquinone radical

EPR spectra of the semiquinone radical of IDP was obtained when the solution containing IDP (1 mM), Tris–HCl buffer (pH = 11.5) was irradiated under nitrogen atmosphere for 10 min. For radical evaluation, the MCF-7 cells are washed twice with phosphate buffered solution (pH = 7.4) and suspended in phosphate buffered solution. A typical incubation for the determination of radical contained in 700 μl, NADH (1 mM), MCF-7 cells (500 μl, 3.75×10^7 cells), IDP (100 μM) and the mixture was purged with N_2 gas for 5 min. The mixture was quickly transferred to a nitrogen flushed flat cell for continuos EPR spectral changes at 25 °C. The spectrum was recorded on a varian E-109 spectrometer (9.15 GHz) with a field modulation of 100 kHz.

2.5. Optical measurements

2.5.1. Singlet oxygen detection

Generation of $^1O_2$ by photosensitisation under aerobic condition was measured in an aqueous solution by RNO bleaching method proposed by Kraljic and El Mohsni [15,16]. The rate of bleaching of RNO absorbance at 440 nm of various sensitisers (Fig. 3) as a function of irradiation time is determined by taking the corrections of molar absorption and photon energy. The rate of disappearance of quencher (*A*) obeys the following equation:

$$-\frac{d[A]}{dt} = \frac{(I_{ab}\Phi^1O_2)k_r[A]}{k_d}$$

where k_r is the rate constant for chemical quenching of $^1O_2$ by *A*, k_d the rate constant for deactivation of $^1O_2$ by the solvent and I_{ab} the intensity of light absorbed by the sensitiser. Taking the $^1O_2$ quantum yield of RB as reference as 0.76, the relative singlet oxygen efficiency of IDP, DP, MJ and PG were determined from the relative ratio of the slopes. From the above ratio it is concluded that IDP and DP generate more singlet oxygen than MJ and PG.

2.5.2. Superoxide detection—SOD inhibitable cytochrome c reduction assay

The generation of superoxide was detected by the reduction of Fe(III)-Cyt.c with $O_2^{\bullet -}$. Solution of quinones (100 μm) were photolysed in the presence of ferricytochrome c (50 μm) in 50 mM phosphate buffer solution (pH, 7.4). The reduction was monitored spectrophotometrically at 550 nm, where ε_{max} for ferricytochrome c is $0.99 \times 10^4\,M^{-1}\,cm^{-1}$ and ε_{max} for ferrocytochrome c is $2.99 \times 10^4\,M^{-1}\,cm^{-1}$. Hence $\Delta\varepsilon_{550} = 20{,}000\,M^{-1}\,cm^{-1}$ was used for reduced–oxidised cytochrome c [17,18].

3. Results and discussion

The absorption spectra of the quinones in DMSO, showed strong bands around 250 and 430 nm. The absorption spectra of MJ, IDP, DP and PG are shown in Fig. 2.

3.1. Singlet oxygen generation

ROS involved in photodynamic therapy may be due to either Type I or Type II reaction. The Type I reaction involves electron transfer from the sensitiser leading to the formation of superoxide anion. Type II reaction involves transfer of excitation energy from the triplet excited photosensitiser to ground state triplet oxygen, forming singlet oxygen. Numerous photosensitised reactions of quinones of biological important molecules have been ascribed to Type II processes. The production of singlet oxygen during photosensitisation of quinones was studied by optical and EPR spectroscopy. The rate of bleaching of RNO absorbance at

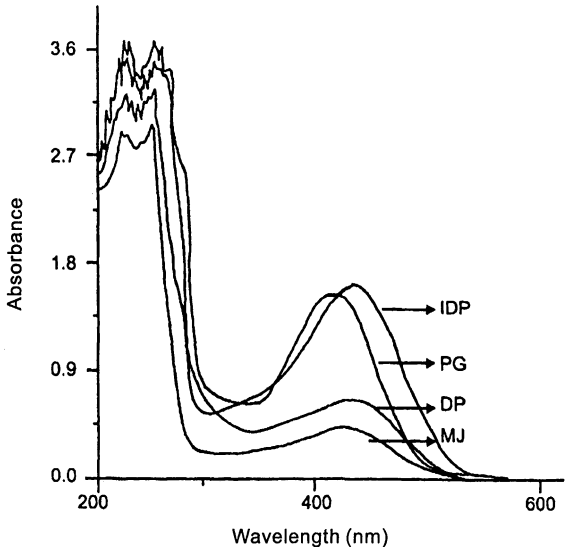
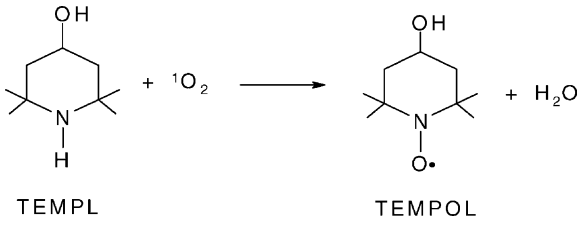


Fig. 2. The absorption spectra of MJ, IDP, DP and PG.

440 nm of various sensitisers as a function of irradiation time is shown in Fig. 3.

The bleaching of RNO is caused by the transannular peroxide intermediate formed as a result of reaction between photogenerated $^1O_2$ and imidazole. SOD (50 μg/ml) and catalase (40 μg/ml), which remove $O_2^{\bullet -}$ and H_2O_2 from the reaction mixture respectively were added to permit the detection and quantification of $^1O_2$ production, without interference from OH radical. IDP and PG are found to be good $^1O_2$ generator. To confirm the production of $^1O_2$, experiments were carried out in the presence of specific $^1O_2$ quenchers such as DABCO and sodium azide. Both DABCO and imidazole are known to have almost comparable RNO quenching rate constant [19]. Since the quenching rate constants of equimolar amounts (10 mM) of imidazole and DABCO are similar, both of these compounds would decrease the observed rate by about half, when compared to the rate constant of RNO bleaching in the absence of DABCO [20]. Similarly, sodium azide reacts with $^1O_2$ about 100 times faster than imidazole. In our experiments, the concentrations of sodium azide and imidazole used are 0.1 and 10 mM, respectively. Hence at these concentrations, it was found that the RNO bleaching was inhibited by about 50%. These results confirm the generation of singlet oxygen during the photosensitisation process.

Since the absorption bands of the naphthoquinone (sensitisers) can overlap the absorption bands of RNO, the RNO bleaching method could not be used for the quantification of singlet oxygen yield. EPR spectroscopy was used to determine the generation of singlet oxygen during photoillumination of quinones using TEMPL as a singlet oxygen trap. Irradiation of oxygen saturated solution containing sensitisers (100 μM) and TEMPL (10 mM) afforded as typical three-line EPR spectrum of nitroxide at room temperature. The formation of TEMPOL from TEMPL is due to the oxidation by $^1O_2$ as shown in Eq. (1). The hfc observed (1.58 mT, $g = 2.0051$) was in agreement with literature [20] and



(1)

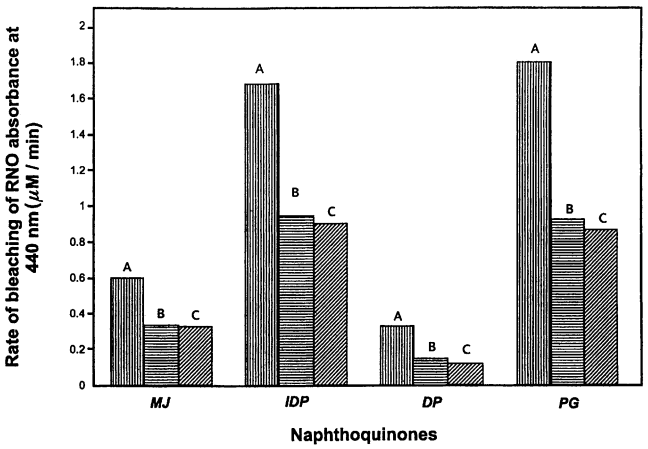


Fig. 3. Generation of singlet oxygen by photosensitised reaction system: RNO (50 μM) + imidazole (10 mM) + quinones (100 μM) + SOD (50 μg/ml) + catalase (40 μg/ml) in 50 mM phosphate buffer (pH = 7.4). Singlet oxygen was monitored by measurement of the decrease in absorbance at 440 nm. (A) Quinone alone; (B) quinone+DABCO (10 mM); (C) quinone + azide (0.1 mM).

ascribed to that of TEMPOL. The intensity of EPR signal was found to increase with increase of irradiation time as shown in Fig. 4 under similar conditions and in the absence of quinones, negligible amount of nitroxide free radical was formed. Control experiment indicated that, oxygen and light were all essential, for the generation of TEMPOL, revealing that the formation of TEMPOL radical is a photodynamic process. The addition of DABCO (10 mM), a typical $^1O_2$ quencher, inhibited the EPR signal (Fig. 4) significantly. These observations verified that the TEMPOL signal come from the reaction of TEMPL with $^1O_2$ during IDP photosensitisation. Under similar conditions irradiation of TEMPL with RB, a good $^1O_2$ generator, also showed the formation of the three-line EPR spectrum. The singlet oxygen generating efficiency ratio of RB, MJ, IDP, DP and PG was found to be 1:0.18:0.45:0.23:0.41, respectively, comparing with $\Phi^1O_2 = 0.76$ for RB [15]. These results suggest that quinones generate singlet oxygen based on energy transfer from the excited states of quinones to oxygen molecules, i.e., by Type II mechanism.

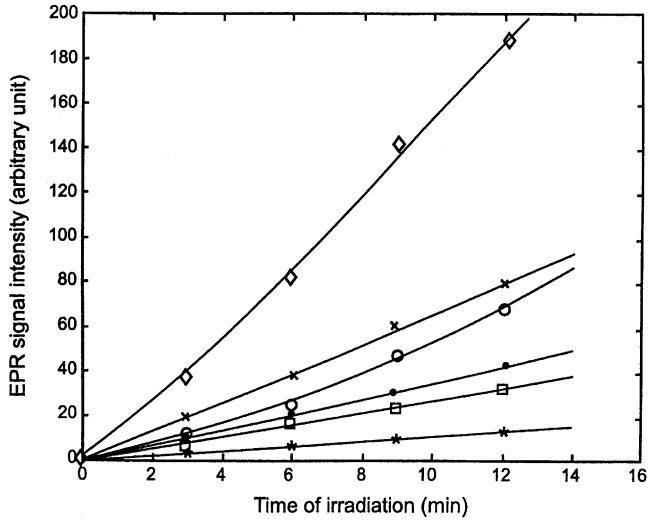


Fig. 4. Production of TEMPOL as a function of irradiation time for different sensitisers (100 μM), RB (◇), PG (×), IDP (○), MJ (●), DP (□) and IDP + DABCO (∗) in the presence of TEMPL (10 mM) at room temperature in DMSO. Spectrometer settings: microwave power: 2 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; gain level: 6.3×10^3; time constant: 0.1 s; scan rate: 4 min.

3.2. Superoxide anion generation

3.2.1. SOD inhibitable cytochrome c reduction assay

Photoillumination of quinones in aerated solution generate superoxide that could be readily studied by using ferricytochrome c reduction assay according to Eq. (2) [23].

$$\text{Fe(III)-Cyt.c} + O_2^{\bullet -} \rightarrow \text{Fe(II)-Cyt.c} + O_2 \quad (2)$$

Fig. 5 shows the reduction of cytochrome c as a function of irradiation time when air saturated solutions of the

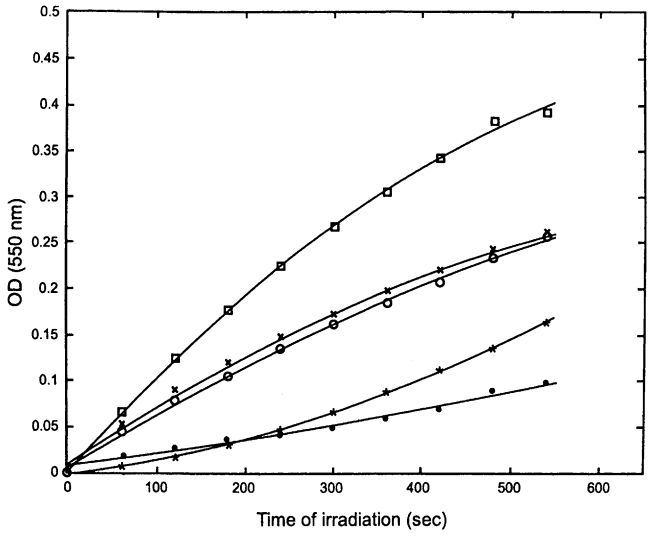


Fig. 5. Photosensitised superoxide generation measured as the rate of cytochrome c reduction in the presence of ferricytochrome c (50 μM) in 50 mM phosphate buffer pH = 7.4, with DP (×), IDP (○), MJ (●), PG (∗) and IDP+SOD (□).

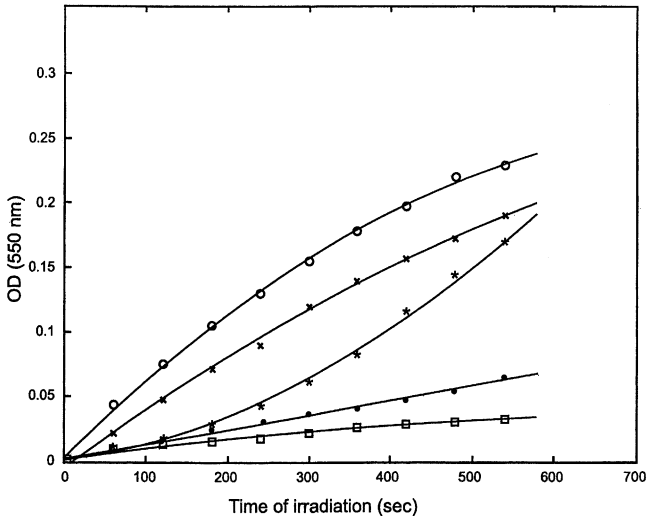
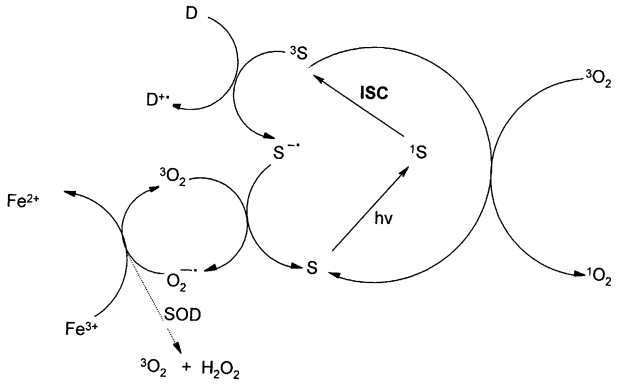


Fig. 6. Photosensitised cytochrome c reduction in 50 mM phosphate buffer (pH = 7.4) in the presence of EDTA by IDP (×), DP (○), PG (∗), MJ (●), and in the presence of NADH by IDP (□) as a function of irradiation time.

sensitisers were photolysed in the presence of 50 μM cytochrome c in phosphate buffer (50 mM and pH = 7.4). The rate of superoxide generation was arrived to be 0.045, 0.124, 0.11 and 0.095 μM/s for MJ, IDP, DP and PG, respectively. Addition of SOD was found to inhibit the cytochrome c reduction. Control experiments indicated that sensitiser, oxygen and light were all essential for the reduction of ferricytochrome c. The rate of cytochrome c reduction, when air saturated solution of quinones were photolysed in the presence of EDTA (10 mM) and cytochrome c (40 μM) in phosphate buffer (pH = 7.4) enhance the superoxide production (Fig. 6). The rate of reduction in the presence of EDTA were found to be 0.155, 0.315, 0.291 and 0.210 μM/s for MJ, IDP, DP and PG, respectively. IDP and DP are the two different dimers of MJ and they have extensive conjugation. In IDP, arene ring of one MJ is attached to arene ring of another MJ. In DP, arene ring of one MJ is attached to quinone ring of another MJ. The semiquinone radicals of IDP and DP may be more stable than that of MJ and PG. This is also revealed by the fact that we have succeeded to obtain the semiquinone radical of IDP and DP on photoillumination under aerobic condition. There may be a correlation between the rate of superoxide production and stability of semiquinone radical formation. Since IDP and DP having more quionone structures they generate more superoxide than MJ and PG. Factors such as the nature of quinones, oxygen concentration in the cell, stability of semiquinone radical may affect superoxide generation. Depending upon these factors, different time profile curves may be obtained. PG may undergo photodegradation and the photodegrading product may generate ROS, thus resulting in an accelerated time profile curve. Due to extensive conjugation the sensitisers IDP and DP may generate larger amount of semiquinone radical anion, which on reaction with oxygen leads to superoxide radical formation. Since the



Scheme 1.

dissolved oxygen in the cell may be depleted, saturating time profile curves are obtained for IDP and DP. Enhancement of generation of superoxide by the sensitisers in the presence of electron donor is indicative of formation of an anionic intermediate ($S^{\bullet-}$) due to the interaction of electron donor with the triplet state of the sensitiser [24], as shown in Scheme 1. In the presence of oxygen the intermediate $S^{\bullet-}$, can give superoxide ($O_2^{\bullet-}$) radical. The addition of another electron donor, NADH also enhanced the rate of superoxide production which was estimated to be 0.414 μM/s for IDP (Fig. 6).

3.2.2. Spin trapping assay

Irradiation of an aerated DMSO solution of sensitiser (100 μM) and DMPO (40 mM) gave an EPR spectrum which is characteristic of the DMPO-$O_2^{\bullet-}$ adduct. EPR spin trapping studies were carried out in DMSO because of the longer life time of DMPO-$O_2^{\bullet-}$ in DMSO [25,26]. The DMPO-$O_2^{\bullet-}$ signal was not observed on control experiments as oxygen, light or sensitisers was omitted, respectively. Fig. 7a shows the multiline EPR spectrum obtained when MJ containing DMPO was illuminated in air saturated DMSO. The identity of this radical was confirmed by using the computer simulated spectrum (Fig. 7b). The hfc of the spin adduct was analysed as primary nitrogen triplet (a_N = 1.3 mT) split by a proton (a_H^β = 1.05 mT) which in turn is further split by a secondary proton (a_H^γ = 0.139 mT). The hfc are consistant with DMPO-$O_2^{\bullet-}$ adduct in DMSO [27]. Addition of SOD (40 μg/ml) prior to illumination inhibited the generation of spin adducts as shown in Fig. 7c. The EPR signal intensity of the spin adduct of all the quinones increased with irradiation time (data not shown). Addition of electron donors, reduced glutathione and ascorbic acid significantly enhanced the EPR signal intensity of the spin adduct. The addition of electron donor can promote the formation of semiquinone radical which is the precursor for the formation of $O_2^{\bullet-}$ (Scheme 1).

When sensitisers PG and DP were irradiated in the presence of DMPO (40 mM) in air saturated phosphate buffer (50 mM, pH = 7.4), a four-line EPR spectrum (not shown) (a_N = a_H = 1.45 mT) with an intensity of 1:2:2:1 was observed. This spectrum corresponds to the hydroxyl radical spin adduct of DMPO [25]. The EPR signal intensity of DMPO-OH adduct was also found to increase with increase of irradiation time. Control experiments ensured that no signal was obtained without light, oxygen, sensitisers and DMPO. The formation of DMPO-OH adduct may be due to the decomposition of the unstable DMPO-$O_2^{\bullet-}$

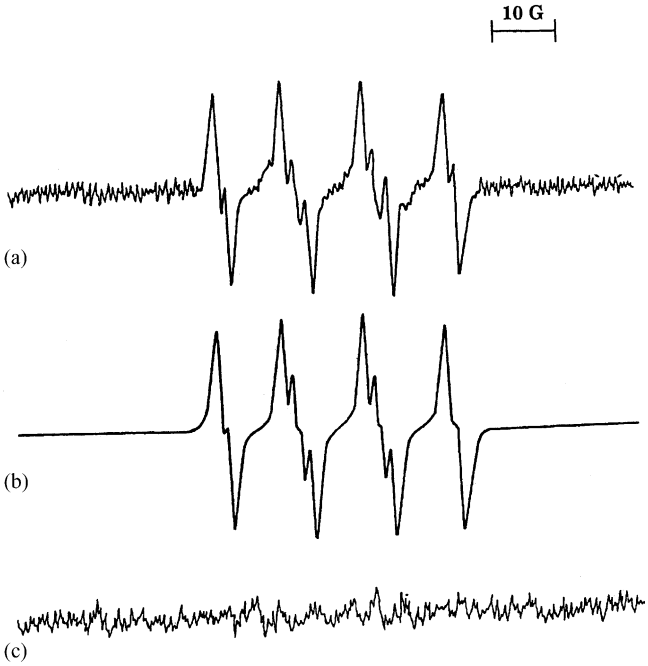
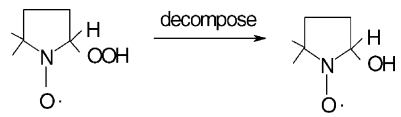


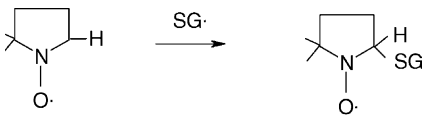
Fig. 7. EPR spectra DMPO-superoxide adduct obtained by photolysis of MJ (100 μM) in air saturated DMSO solution containing DMPO (40 mM). (a) After 4 min irradiation; (b) computer simulated spectrum of DMPO-$O_2^{\bullet-}$ adduct of MJ using hfc a_N = 1.30 mT, a_H^β = 1.05 mT and a_H^γ = 0.139 mT; (c) after 4 min irradiation in the presence of SOD. Spectrometer settings: microwave power: 10 mW; modulation amplitude: 0.1 G; gain level: 1.6×10^4; time constant: 0.25 s; scan rate: 4 min.



DMPO-OOH DMPO-OH (3)

adduct in the protic solvent [25,28] as given in Eq. (3).

To study the effect of electron donor such as reduced glutathione (GSH) was also investigated in aqueous medium. When DP was irradiated in the presence of DMPO (40 mM), GSH (10 mM) in air saturated phosphate buffered solution



DMPO DMPO-SG (4)

(pH = 7.4), a multiline EPR spectrum was obtained (Fig. 8a). This spectrum was identified as a mixture of two types of DMPO-adducts by computer simulation (Fig. 8b).

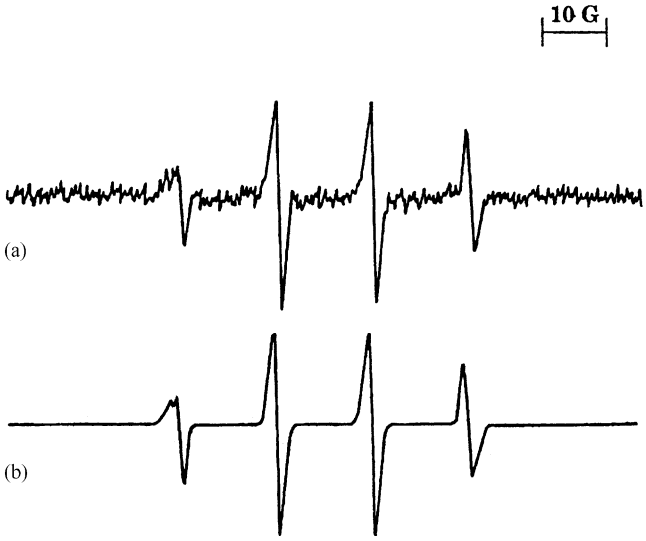


Fig. 8. (a) EPR spectrum obtained by photolysis of aerobic incubation of solution containing DP (100 μM), DMPO (40 mM) and GSH (10 mM). (b) Computer simulated spectrum of (a) obtained by the combination of two spin adducts. The spectrum contains two spin adducts: DMPO-OH ($a_N = 1.52$ and $a_H = 1.47$ mT, LW = 0.55) and DMPO/•SG ($a_N = 1.53$ and $a_H = 1.64$ mT, LW = 0.84) in the ratio of 47:53. Spectrometer settings: microwave power: 10 mW; modulation amplitude: 0.1 G; gain level: 1.6×10^4; time constant: 0.25 s; scan rate: 4 min.

Adduct I was assigned to DMPO-OH, based on its hfc $a_N = 1.52$, $a_H = 1.47$ mT. Adduct II was identified as DMPO/•SG, based on its hfc $a_N = 1.53$ and $a_H = 1.64$ mT. With simulated EPR spectrum the relative contribution of 47% from adduct I and 53% from adduct II matched well with the experimental spectrum [29,30]. No EPR signal was observed in the dark and in the absence of IDP, suggesting that nearly half of the superoxide is converted into DMPO-OH adduct and remaining form DMPO/•SG adduct in aerated buffer solution.

3.3. Formation of semiquinone radical

The presence of semiquinone radical anions upon incubation in anaerobic condition of heart and liver mitrochondrial preparation containing NADH dehydrogenase and cytochrome p-450 reductase, respectively, was evidenced by EPR spectroscopy [21]. When human breast cancer MCF-7 cells in phosphate buffered solution were incubated with IDP in the presence of NADH under anaerobic condition, a single line EPR spectrum of IDP was observed (Fig. 9). However, no free radical signal observed in an aerobic condition. The EPR spectrum of IDP free radical anion has a line width of 4 G; a g value of 2.0011 and a symmetrical line shape. We have further investigated the photogeneration of radicals in both anaerobic and aerobic conditions. Irradiation of IDP (1 mM) (in Tris–HCl buffer (pH = 11.5)) in nitrogen atmosphere led to the generation of 14-line strong EPR signal as shown in Fig. 10. The intensity of EPR signal increases

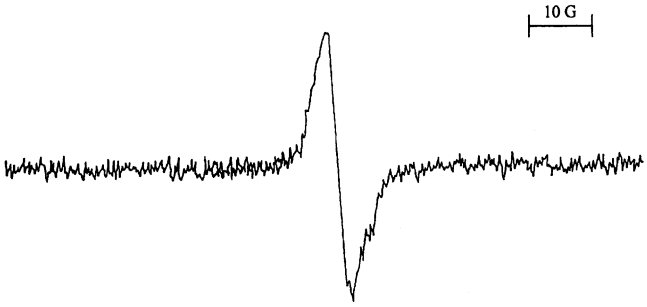


Fig. 9. EPR spectrum of IDP (100 μM) free radical anion generated by human breast cancer MCF-7 cells incubated in phosphate buffered solution (pH = 7.4) and in nitrogen atmosphere. EPR settings: microwave power: 20 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; gain level: 2.5×10^4; time constant: 0.25 s; scan rate: 4 min.

rapidly with increasing irradiation time and decreases very slowly in the dark. As in the case of IDP, DP also produces a semiquinone radical on illumination. However, we have not succeeded to obtain the semiquinone radical for PG and MJ. When the sample solution was exposed to oxygen, the EPR signal is quenched rapidly. Oxygen quenches the EPR signal via single electron transfer to produce the superoxide anion radical, $O_2^{\bullet -}$ [22] as shown in Eq. (5).

$$IDP^{\bullet -} + O_2 \rightarrow IDP + O_2^{\bullet -} \quad (5)$$

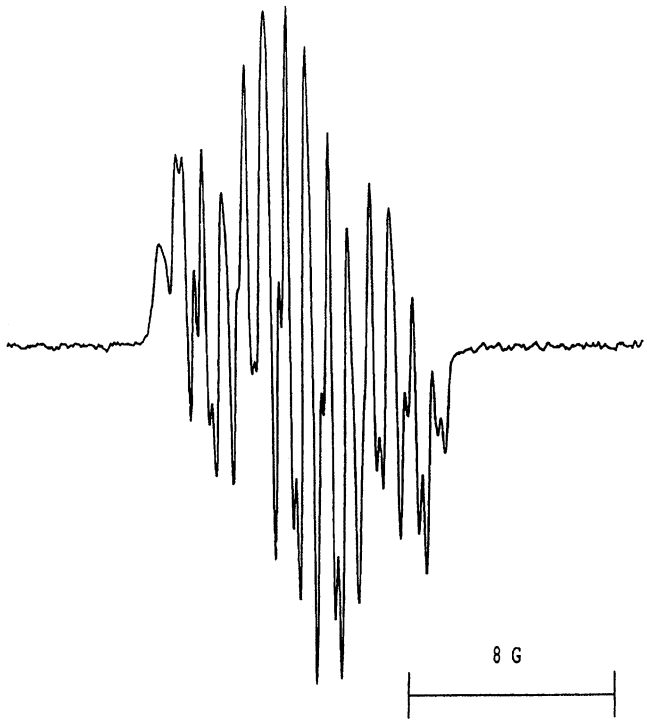


Fig. 10. EPR spectrum of IDP (1 mM) semiquinone radical containing Tris–HCl buffer (pH = 11.5) in nitrogen atmosphere after irradiation for 10 min. EPR settings: microwave power: 2 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; gain level: 1.25×10^4; time constant: 0.25 s; scan range: 25 G; scan rate: 4 min.

These suggest that the semiquinone radical anion is the precursor to the formation of $O_2^{\bullet -}$. Both electron transfer (superoxide generation—Type I) and energy transfer (singlet oxygen generation—Type II) paths are involved in photosensitisation of quinones to different extent depending on oxygen concentration, sensitiser concentration and other environmental factors. The pathways for the photodynamic action of sensitiser can be reasonably as shown in the given Scheme 1. When the excited state triplet is stabilised it leads to energy transfer, i.e., electronic excitation energy is transferred from ^3S to ground state (triplet) oxygen to give singlet oxygen

$$^3S + {}^3O_2 \rightarrow S_o + {}^1O_2$$

However, in the presence of electron donor, the pathway of singlet oxygen generation can be effectively switched over into the production of species $S^{\bullet -}$ due to the interaction of electron donor with the triplet state of the sensitiser, as shown in Scheme 1. In the presence of O_2, $S^{\bullet -}$ can yield $O_2^{\bullet -}$ radical.

4. Conclusion

In this work we have demonstrated that photosensitisation of four naturally occurring naphthoquinones and binaphthoquinones, involves both Types I and II process. In anaerobic condition, the semiquinone radical, arising from Type I process, are largely responsible for the quinones photosensitisation. RNO bleaching method, EPR spectroscopy, SOD inhibitable ferricytochrome c reduction assay and EPR spin trapping experiments show that both Type I and Type II mechanisms are involved in the photosensitisation of naphthoquinones derivatives.

Acknowledgements

One of us (K.K. Mothilal) thanks UGC, New Delhi for a Teacher Fellowship (FIP) and the management of Saraswathi Narayanan College, Madurai, India for encouragement and permission to carry out this work. We thank the UGC, India for the special assistance under DRS scheme.

References

[1] M.A. Pathak, P.C. Joshi, Biochim. Biophy. Acta 798 (1984) 115.
[2] L.C. Pennings, T.M.A.R. Dubbelman, Fundamentals of photodynamic therapy: cellular and biochemical aspects, Anticancer Drugs 5 (1994) 139.
[3] Z. Diwu, J.W. Lown, J. Photochem. Photobiol. B: Biol. 18 (1993) 131;
J.L. Bolton, M.A. Trush, T.M. Penning, G. Dry hurst, T.J. Monks, Role of quinones in toxicology, a review, Chem. Res. Toxicol. 13 (2000) 135.
[4] B.K. Sinha, E.G. Mimnaugh, Free Radic. Biol. Med. 8 (1990) 561.
[5] Z.J. Diwu, R.P. Haulgland, J. Liu, J.W. Lown, G.G. Miler, R.B. Moore, K. Brown, J. Tulip, M.S. Mcphee, Free Radic. Biol. Med. 20 (1996) 589.
[6] Z. Diwu, J.W. Lown, J. Photochem. Photobiol. A: Chem. 69 (1992) 191.
[7] T. Wu, J. Shen, A. Song, S. Chen, M. Zhang, T. Shen, J. Photochem. Photobiol. B: Biol. 57 (2000) 14.
[8] K.J. Reszka, P. Bilski, C.F. Chignell, J.A. Hartley, N. Khan, R.L. Souhami, A.J. Mendoca, J.W. Lown, J. Photochem. Photobiol. 15 (1992) 317.
[9] K.J. Reszka, P. Kolodziejczyk, J.W. Lown, Free Radic. Biol. Med. 5 (1986) 63.
[10] K.K. Mothilal, J. Johnson Inbaraj, C.F. Chignell, R. Murugesan, Cytotoxicity, redox cycling and reactive oxygen species (ROS) generation by some naturally occurring hydroxynaphthoquinones and binaphthoquinones, submitted for publication.
[11] B. Kalyanaraman, R.C. Felix, R.C. Sealy, Photochem. Photobiol. 36 (1982) 5.
[12] S.A. Noor Mohamed, Phytochemical studies (phytochemical investigation of *Diospyros paniculata*, Ph.D. Thesis, Madurai Kamaraj University, Madurai, India, 2001, unpublished results.
[13] J.A. Charles, Studies in natural products, Ph.D. Thesis, Madurai Kamaraj University, Madurai, India, 2002, unpublished results.
[14] J. Moan, Nature 279 (1979) 450.
[15] I. Kraljic, A. El Mohsni, Photochem. Photobiol. 28 (1978) 577.
[16] J. Johnson Inbaraj, R. Gandhidasan, S. Subramanian, R. Murugesan, J. Photochem. Photobiol. A: Chem. 117 (1998) 21.
[17] W.H. Koppenol, J. Butler, Isr. J. Chem. 24 (1984) 11.
[18] M.C. Krishna, S. Uppuluri, P. Riesz, J.S. Ziglet Jr., J.S.D. Balasubramanian, Photochem. Photobiol. 54 (1991) 51.
[19] E. Wilkinson, J.G. Brummer, J. Phys. Chem. Ref. Data 10 (1981) 809.
[20] Z. Diwu, J.W. Lown, Free Radic. Biol. Med. 14 (1993) 209.
[21] K.J.A. Davies, J.H. Doroshow, J. Biol. Chem. 261 (1986) 3068.
[22] J. Johnson Inbaraj, R. Gandhidasan, R. Murugesan, Free Radic. Biol. Med. 26 (1999) 1072.
[23] J. Johnson Inbaraj, R. Gandhidasan, R. Murugesan, J. Photochem. Photobiol. A: Chem. 124 (1999) 95.
[24] Z. Diwu, Free Radic. Biol. Med. 14 (1993) 209.
[25] E. Ben-Hur, A. Carmichael, P. Riesz, I. Rosenthal, Int. J. Radiat. Biol. 48 (1985) 837.
[26] J.V. Bannister, in: R.A. Greenwald (Ed.), CRC Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, p. 153.
[27] Z. Zhiyi, M. Nenghui, W. Qian, L. Meifan, Free Radic. Biol. Med. 14 (1993) 1.
[28] J.W. Lown, Anthracycline and Anthracyclinedione-Based Anticancer Drugs, Elsevier, Amsterdam, 1988.
[29] J. Dilon, B.J. Drtwerth, C.F. Chignell, K.J. Reszka, Photochem. Photobiol. 69 (1999) 259.
[30] L.S. Harman, D.K. Carver, J. Schreiber, R.P. Mason, J. Biol. Chem. 261 (1986) 1642.