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Scavenging of Reactive Oxygen Species by Chlorophyllin: An ESR Study

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The antioxidant effects of chlorophyllin (CHL), a water-soluble analog of the green plant pigment chlorophyll, on different reactive oxygen species (ROS) were investigated by electron spin resonance (ESR) spectroscopy. As a standard, we have used the ability of CHL to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. CHL inhibits the formation of 5,5-dimethyl-1-pyrroline-*N*-oxide adduct with hydroxyl radical (DMPO–$^{\cdot}$OH adduct) generated by γ-radiation in a dose-dependent manner. At a concentration of 1 mM, CHL caused more than 90% inhibition of ESR signal intensity of this adduct. However, the results obtained with the Fenton reaction were different. We also found evidence for the inhibition of $^1O_2$-dependent formation of the 2,2,6,6-tetramethyl-piperidine oxide (TEMPO) radical during photosensitization of methylene blue with visible light. CHL was also able to inhibit hydrogen peroxide induced oxidation of phenol red. The rate constant of the reaction of CHL with H_2O_2 was found to be $2.7 \times 10^6\,M^{-1}\,s^{-1}$. In conclusion, CHL has potent antioxidant ability involving scavenging of various physiologically important ROS.

Keywords: γ-Radiation; Electron spin resonance; Spin trapping; Hydroxyl radical; Singlet oxygen; Hydrogen peroxide; Antioxidant

Abbreviations: ROS, reactive oxygen species; CHL, chlorophyllin; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; TEMP, 2,2,6,6-tetramethyl piperidine; TEMPO, 2,2,6,6-tetramethylpiperdine oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; $^{\cdot}$OH, hydroxyl radical; $^1O_2$, singlet oxygen; MB, methylene blue; HRP, horseradish peroxidase

INTRODUCTION

A wide variety of physicochemical agents from the external environment like visible, UV, X- and

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γ-radiations, toxic chemicals, air pollutants as well as normal and impaired cellular biochemical processes such as mitochondrial metabolism, neutrophil activation and ischemia reperfusion injury can generate reactive oxygen species (ROS).[1,2] Subsequent alterations in crucial cellular components have been related to several degenerative diseases.[3,4] Compounds, especially from natural sources, capable of minimizing such oxidative damage are much sought after to protect against degenerative diseases.[4,5] One such compound, chlorophyllin (CHL), the sodium–copper salt and water-soluble analog of the green plant pigment chlorophyll has been tested in our laboratory and has been shown to have good DNA- and membrane-protective properties against γ-radiation in *in vitro* and *ex vivo* model systems. It inhibited radiation-induced formation of single-strand breaks in plasmid pBR322 DNA.[6] It has also offered protection against membrane damage by photosensitization in rat liver mitochondria.[7]

Earlier studies have shown that CHL exhibits antimutagenic and anticarcinogenic properties against directly and indirectly acting mutagens in different experimental systems.[8–10] It is also radioprotective in Drosophila and laboratory mammals.[11,12] Studies carried out to delineate the mechanism(s) of protection by CHL against radiation, and chemical mutagens/carcinogens have suggested several possibilities.[13,14] CHL may bind to the mutagen and enhance excretion, thus lowering the availability in the cell.[10] Earlier reports showed antioxidant property of CHL in terms of inhibition of lipid peroxidation.[15] It has also been suggested by several investigators that the radioprotective effects of CHL may, at least, in part be due to scavenging of free radicals.[14] However, in such situations and in complex biological systems, several mechanisms may operate including scavenging of ROS, induction of repair enzymes and enhancing cellular defense pathways.[3,4,6]

To the best of our knowledge, there are no reports showing direct scavenging properties of CHL against different ROS except for our earlier report on the hydroxyl radical (·OH) scavenging ability, using pulse radiolysis.[6] Chlorophyllin exhibited reaction with hydroxyl radicals generated by electron pulses at diffusion-controlled rates. In the present study, we attempt to examine the ROS scavenging properties of CHL using electron spin resonance (ESR) with spin traps.[16,17] We have used this technique to trap ·OH and singlet oxygen ($^1O_2$) and the free radicals produced by their reaction with CHL. Radical scavenging studies using ESR and spin traps have been used extensively for estimating antioxidant ability of natural compounds.[18–20] There are also debates regarding the use of spin traps for studying radical scavenging.[21,22] The present study attempts to supplement our earlier reports on the antioxidant ability of chlorophyllin in various model systems (ref).

In the present investigation, ·OH was generated by exposure to γ-radiation as well as by Fenton reaction and $^1O_2$ by photosensitization using methylene blue (MB) as a sensitizer. Another important oxidizing species, hydrogen peroxide, was also tested for its reactivity with CHL.

MATERIALS AND METHODS

Chemicals

Chlorophyllin, 5,5-dimethyl-1-pyrrolidine-*N*-oxide (DMPO), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), phenol red, $FeSO_4$ and MB were obtained from Sigma Chemical, St. Louis, MO. 2,2,6,6-Tetramethylpiperidine (TEMP) was from Aldrich Chemical, USA. Other chemicals like H_2O_2, sodium phosphate, etc. were of analytical grade from reputed local manufacturers. All solutions were stirred with Chelex-100 for several hours to remove traces of metal ions.

ESR Spin Trapping Measurement of ·OH

The Fenton reaction, a well-known source of ·OH, was utilized to examine whether CHL could scavenge this radical. The preparation and maintenance of the stock solutions of the reagents used were essentially the same as described by Perricone *et al.*[23] In the standard reaction (total volume of 1 ml), the components were added in the following order: ADP (20 mM), $Fe(NH_2)(SO_4)_2$ (1 mM in 0.0012 N HCl), CHL solution of different concentrations, buffer (100 mM NaCl + 25 mM $NaHCO_3$, pH 7.1), spin trap (DMPO, 50 mM), and H_2O_2 (0.3% in water). In our experiments, we did not observe any change in pH.

The ESR experiments were carried out using a Bruker type EMX 6/1, X-band spectrometer operating in the first derivative mode. The measurements were performed at 23°C using an aqueous quartz flat cell. The spectrometer was operated at a microwave frequency of 9.736 GHz, with a microwave power of 2 mW, modulation frequency of 100 kHz and amplitude of 2 G. For the spectra, center field was 3467 G, sweep width 65 G, time 84 s and time constant 20.5 ms.

Exposure to Radiation

We have also used another way of generating ·OH, by exposure to γ-radiation from a ^{60}Co source. The reaction mixture for this contained 50 mM DMPO in 50 mM potassium phosphate buffer, pH 7.4, in the presence or absence of required concentrations of CHL. Samples were exposed to 5, 10 or 20 Gy, in the presence of air, at a dose rate of 16 Gy min^{-1}. ESR spectrum was recorded after 2 min.

ESR Spin Trapping of Singlet Oxygen

MB in combination with photo-irradiation was used as a source of $^1O_2$.[24,25] The detection of $^1O_2$ was based on its specific reaction with TEMP which forms a stable and ESR detectable nitroxide TEMPO radical.[26,27] Briefly, the system consisted of 100 W tungsten light source placed 15 cm away from a cuvette kept in a water bath, maintained at 37°C.[28] Intensity of the light was estimated to be 32.5 W m^{-2}. During photo-irradiation, the samples were continuously bubbled with a stream of oxygen at a constant flow rate. Samples containing MB (25 μM) and TEMP (50 mM) in either the absence or presence of CHL were photo-irradiated and kinetic spectra were obtained by fixing the magnetic field at 3477 G. The spectrometer was operated at a microwave frequency of 9.751 GHz, power 8 mW, modulation frequency of 100 kHz and amplitude of 4 G. For the spectra, sweep width was 100 G, time 41.9 s and time constant 163.8 ms.

Assay of H_2O_2

H_2O_2 was determined according to Zang *et al.*[29] This method is based on the horseradish peroxidase (HRP)-mediated oxidation of phenol red by H_2O_2, resulting in the formation of a chromogenic compound showing increased absorbance at 600 nm. The reaction mixture consisted of 0.1 mg ml^{-1} of phenol red, 8.5 U ml^{-1} of HRP and 20 μM H_2O_2 in the presence or absence of CHL, respectively. The reaction was initiated by the addition of HRP.

DPPH Radical Assay

DPPH, used as a standard radical for comparison while estimating antioxidant abilities of natural compounds[18] was dissolved in ethanol to give a 250 μM solution, and mixed with different concentrations of CHL. Samples were transferred to the cavity of ESR spectrometer and spectra were recorded exactly 60 s after additions. Conditions were: center field, 3477; sweep width, 200 G; microwave frequency, 9.751 GHz; power, 20 mW; time constant, 327.7 ms; sweep time, 41.9 s.

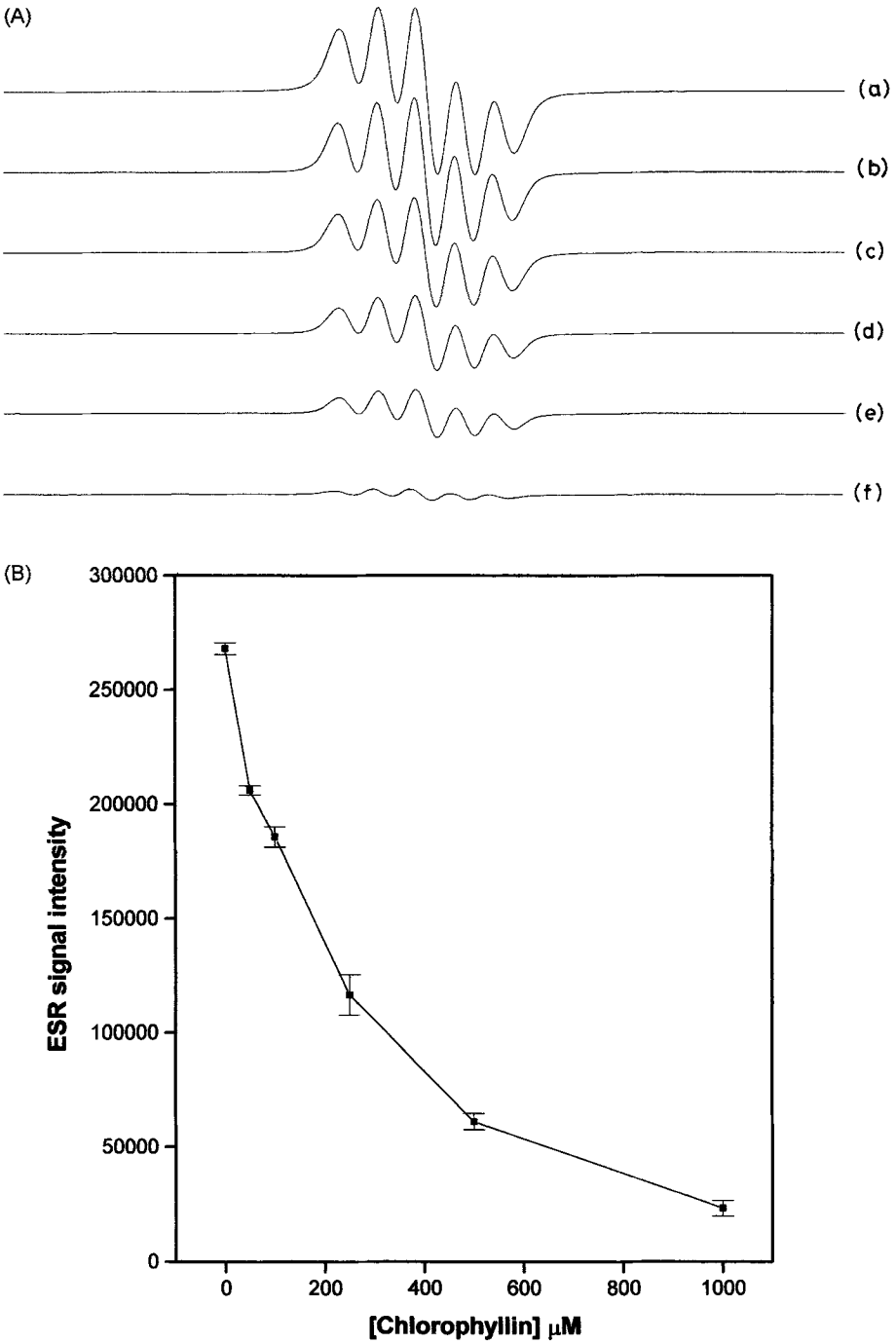


FIGURE 1 (A) ESR spectra of DPPH radical dissolved in ethanol solution. The spectra were obtained in the presence or absence of various concentrations of chlorophyllin (CHL). (a) DPPH radical (DR), (b) DR + 50 μM CHL, (c) DR + 100 μM CHL, (d) DR + 250 μM CHL, (e) DR + 500 μM CHL, and (f) DR + 1000 μM CHL. ESR measurement conditions are listed in "Materials and Methods". (B) Scavenging effect of chlorophyllin on long-lived DPPH radical, expressed as signal intensity. Values are mean ±S.E. of three replicate experiments.

Calculation of IC_{50} Value

The scavenging activity of chlorophyllin towards different ROS was represented in terms of IC_{50}. It was calculated using the following equation[30]

$$V = (I_{control}/I_{test}) - 1$$

where $I_{control}$ and I_{test} are the spin adduct signal intensities observed in the absence and presence of the chlorophyllin, respectively. The plot of V against the concentration of chlorophyllin was linearized using the least-squares method. IC_{50} is the concentration of the chlorohphyllin at which $V = 1$.

RESULTS

The typical ESR spectrum of DPPH radical is shown in Fig. 1A and the scavenging effects of CHL in Fig. 1B. Percent inhibition of DPPH radical increased with higher concentrations of CHL. Approximately, 90% inhibition was observed at 1000 μM. The IC_{50} value of chlorophyllin for DPPH radical was 100 μM (Table I).

Figure 2A shows the ESR spectrum of DMPO–·OH adduct formed during Fenton reaction. The hydroxylation of DMPO by ·OH (Eq. (1)) was started by the addition of H_2O_2 to the incubation medium containing ADP, $Fe(NH_2)(SO_4)_2$ and DMPO:

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + {}^{-}OH + {}^{\cdot}OH \quad (1)$$

$$DMPO + {}^{\cdot}OH \rightarrow DMPO - {}^{\cdot}OH \quad (2)$$

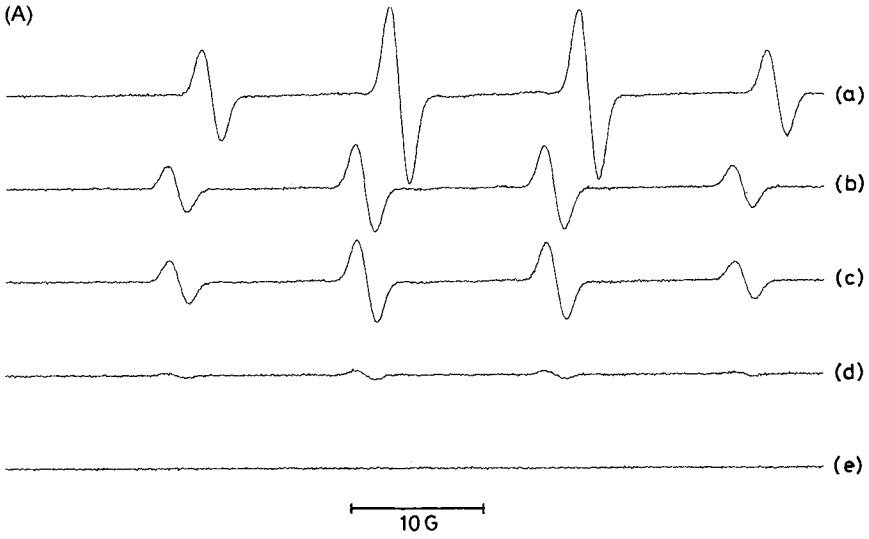
The ESR spectrum was characterized by hyperfine coupling constants of $a^N = a^H = 14.9$ G showing that this radical species represents DMPO–·OH adduct.[27] There is a decrease in the intensity of the signal with increasing concentrations of CHL. Considerable inhibition was exhibited by CHL at a low concentration of 25 μM (43%, Fig. 2B). There was a sharp rise in the protection and the formation of adduct was almost completely inhibited at 500 μM. The IC_{50} value of chlorophyllin for ·OH radical generated by Fenton reaction was 4 μM as shown in Table I. However, inhibition was less when radiation was used as a source for generating ·OH. Figure 2C shows ESR spectra of DMPO–·OH adduct formed by exposure to radiation and the pattern was found to be similar to that shown in Fig. 2A. Higher concentration of CHL was required to inhibit the formation of DMPO–·OH adduct during radiation exposure (20 Gy). About 70% protection was seen at 500 μM concentration (Fig. 2D). It is likely that the amount of ·OH generated differs in these two systems. The IC_{50} value of chlorophyllin for ·OH radical produced by radiation was 83 μM (Table I).

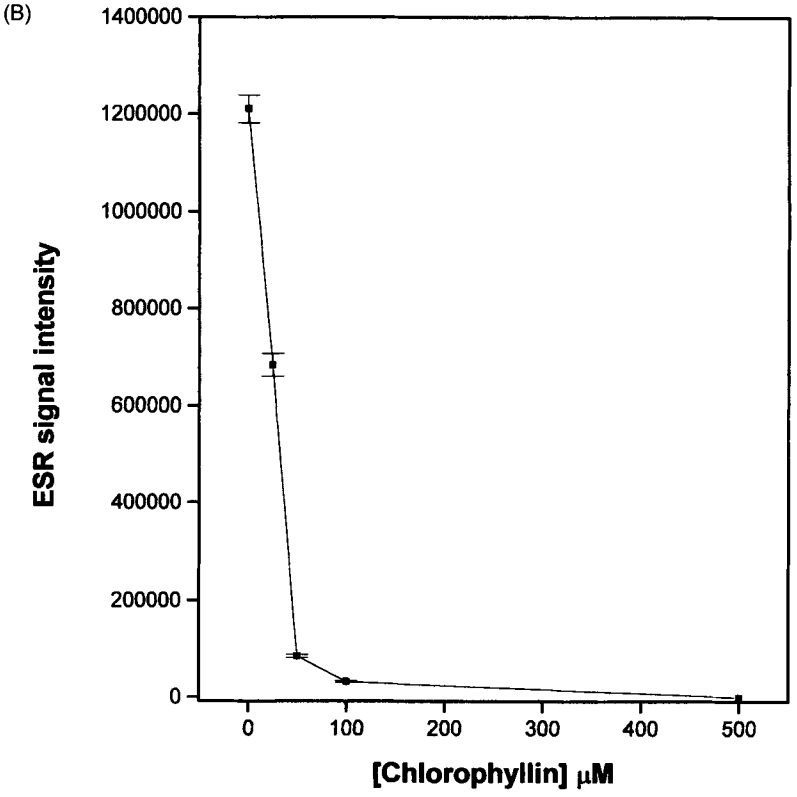
TABLE I Scavenging effect of chlorophyllin calculated in terms of IC_{50} obtained by the spin trapping method (values are obtained from three replicate experiments)

Treatment	IC_{50} (μM)
·OH radicals (γ-radiation)	83
·OH radicals (Fenton reaction)	4
Singlet oxygen	66
DPPH	100

Irradiation of a mixture of MB and TEMP, with visible light, in oxygen-saturated buffer gave ESR signals characteristic of nitroxide radical (Fig. 3A). This was compared with that of the radical from commercial TEMPO. The hyperfine splitting constants were identical ($a^N = 16.3$ G). Under similar conditions, but in the absence of MB, oxygen or light, TEMPO formation did not occur. This demonstrated that the formation of nitroxide radical involves a photosensitization process and it is $^1O_2$ which is involved in the formation of TEMPO. This species is being generated in the system by energy transfer from the excited triplet state of MB to ground state molecular oxygen. Addition of CHL decreased the signal intensity of TEMPO. As shown in Fig. 3B, there was a rise in protection up to 100 μM, with saturation at higher concentrations. The IC_{50} value of chlorophyllin for $^1O_2$ was 66 μM as shown in Table I.

CHL was found to react with H_2O_2 in a dose-dependent manner. As shown in Fig. 4A, the percent inhibition of H_2O_2 induced oxidation of phenol red increased with higher CHL concentrations. Even with a low concentration of 25 μM of CHL, 50% inhibition was seen. At 100 μM, it caused about 90% protection. Figure 4B shows the Stern–Volmer plot for the effect of CHL on





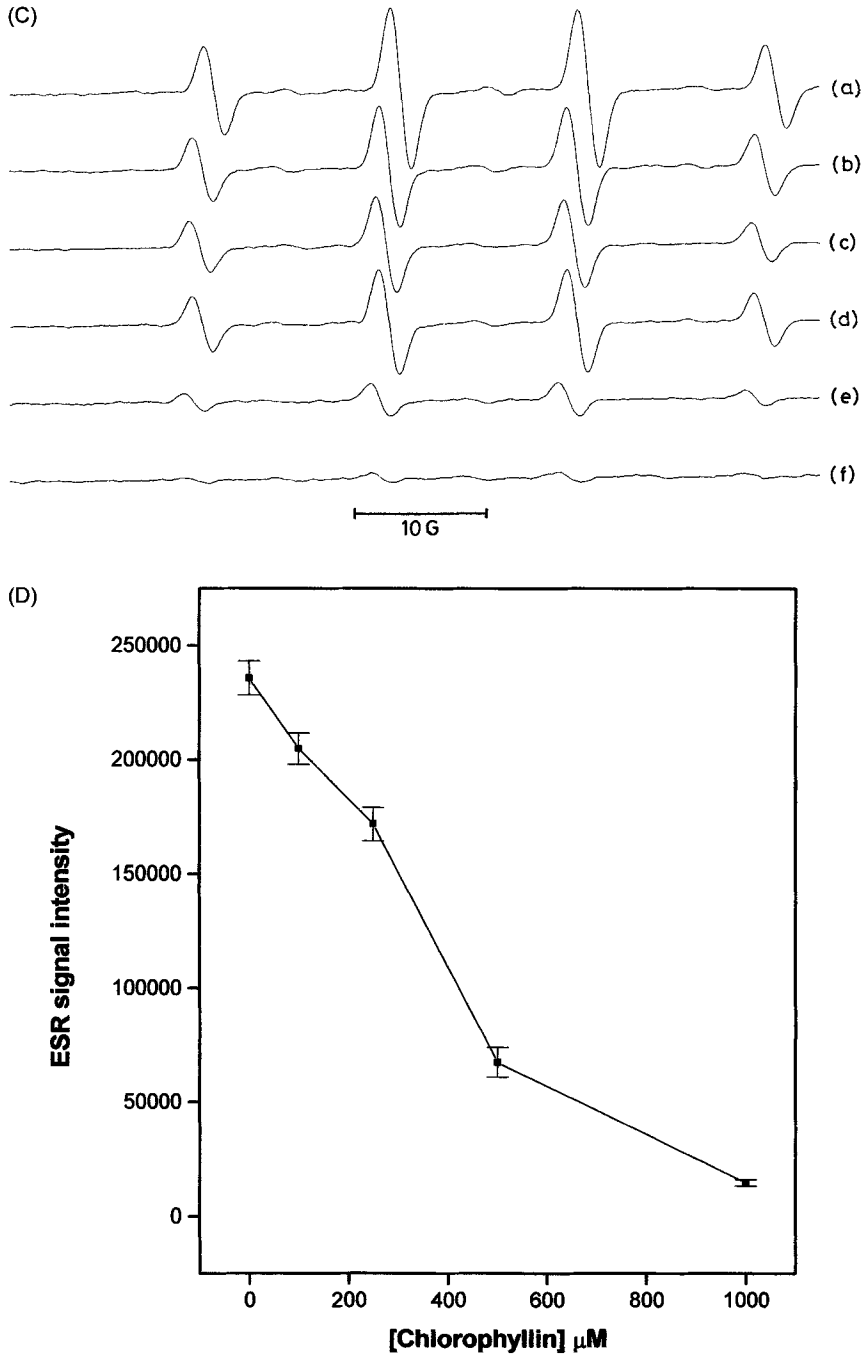


FIGURE 2 (A) ESR spectra of DMPO–·OH adduct formed during Fenton reaction in the presence or absence of various concentrations of chlorophyllin (CHL). (a) Fenton reaction (FR), (b) FR + 25 μM CHL, (c) FR + 50 μM CHL, (d) FR + 100 μM CHL, and (e) FR + 500 μM CHL. ESR measurement conditions are as given in "Materials and methods". (B) Scavenging effect of chlorophyllin on DMPO–·OH adduct during Fenton reaction, expressed as signal intensity. Values are mean ±S.E. of three replicate experiments. (C) ESR spectra of DMPO radical adduct with hydroxyl radical obtained after exposure to γ-radiation, at a dose of 20 Gy, in the presence or absence of various concentrations of chlorophyllin (CHL). (a) 20 Gy, (b) 20 Gy + 100 μM CHL, (c) 20 Gy + 250 μM CHL, (d) 20 Gy + 500 μM CHL, and (e) 20 Gy + 1 mM CHL. ESR measurement conditions are given in "Materials and Methods". (D) Scavenging effect of chlorophyllin on hydroxyl radicals generated by γ-radiation, at a dose of 20 Gy (dose rate: 16.0 Gy min^{-1}) expressed as signal intensity, calculated from the intensity of peaks. Values are mean ±S.E. of three replicate experiments.

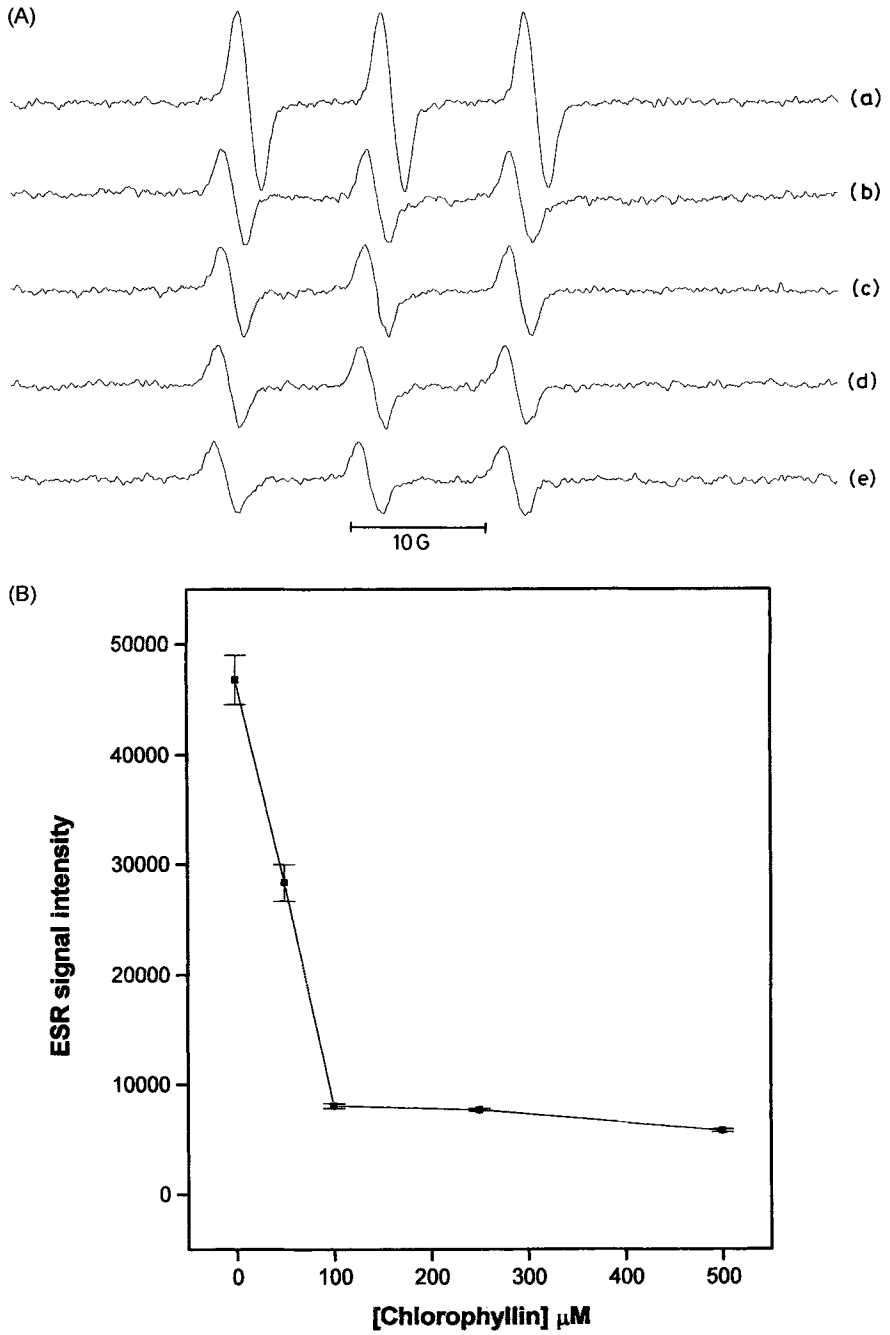


FIGURE 3 (A) ESR spectra of TEMPO formed by the reaction of TEMP with singlet oxygen in the presence or absence of chlorophyllin. Singlet oxygen was generated by methylene blue photosensitization (MB, 25 μM) in the presence of oxygen. (a) MB, (b) MB + 50 μM CHL, (c) MB + 100 μM CHL, (d) MB + 250 μM CHL, and (e) MB + 500 μM CHL. ESR measurement conditions were as described in "Materials and Methods". (B) Effect of chlorophyllin on singlet oxygen generated by methylene blue plus light in the presence of oxygen, expressed as signal intensity. Values are mean ±S.E. of three replicate experiments.

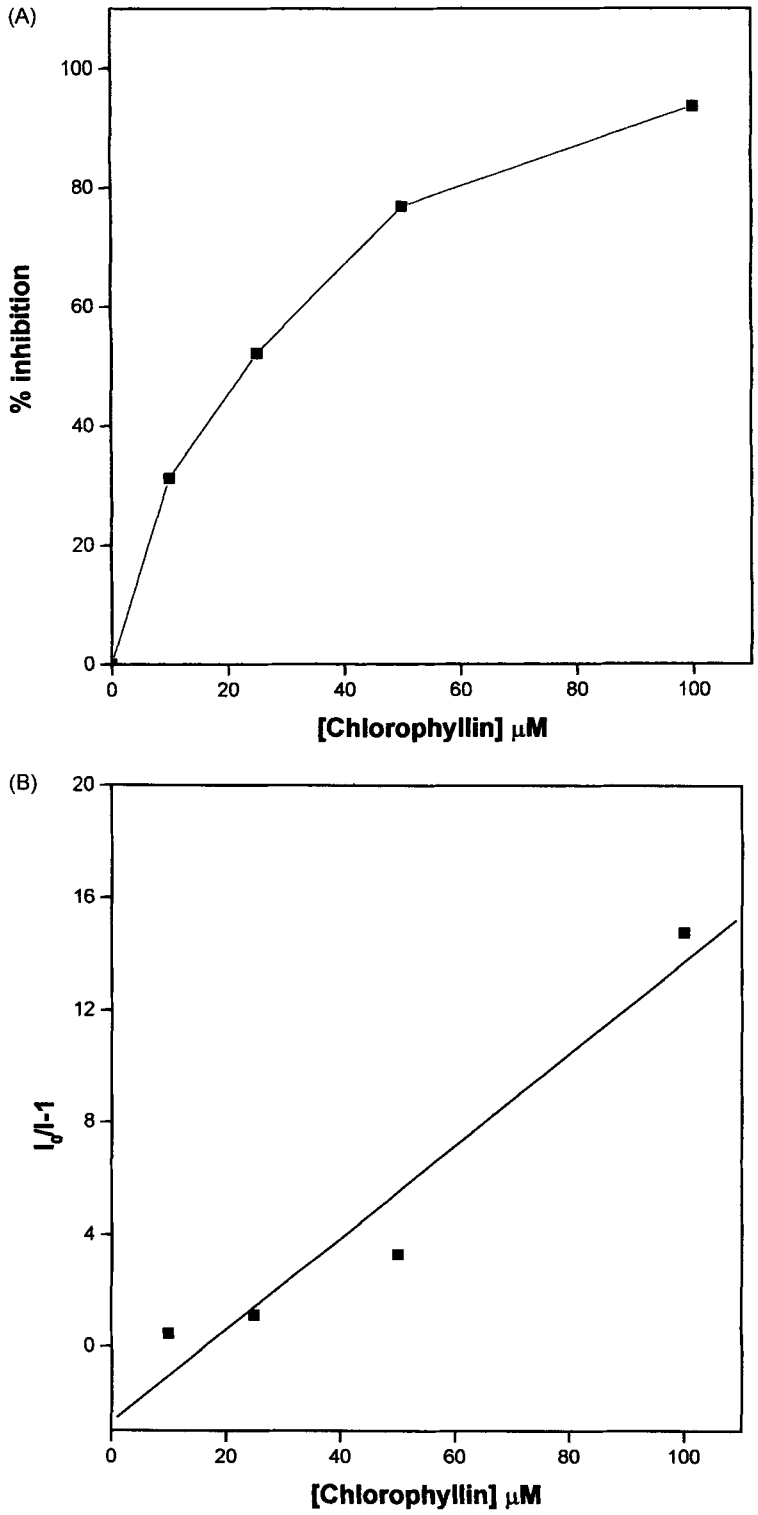


FIGURE 4 (A) Scavenging ability of chlorophyllin (CHL) on hydrogen peroxide. The assay mixture consisted of 20 μM H_2O_2, 0.1 mg ml^{-1} phenol red and 8.5 U ml^{-1} horseradish peroxidase in buffered solution in the presence of indicated amounts of CHL. Values are mean ±S.E. of three independent experiments. (B) Stern–Volmer plot for the determination of rate constant of chlorophyllin (CHL) with H_2O_2. The $I_0/I - 1$ is the ratio of the absorbance of oxidized phenol red at 600 nm in the presence or absence of CHL. Slope of the line was used to calculate the rate constant of CHL with hydrogen peroxide.

H_2O_2. The relationship of concentration with the scavenging effect was linear. Since there exists a competition between HRP and CHL for H_2O_2 in our assay, the rate constant for CHL reaction with H_2O_2 at the fixed concentration of HRP and phenol red can be obtained using the following equation[29]

$$k_{CHL} = k_{HRP}[HRP][I_0/I - 1]/[CHL] \quad (3)$$

where I_0/I represents the ratio of the rates of phenol red oxidation in the absence and presence of CHL and k_{CHL} the rate constant of CHL with H_2O_2. The constant term $k_{HRP}[HRP]$ is reported to be $16.82\,M^{-1}s^{-1}$ using the rate constant of $3.7 \times 10^6\,M^{-1}s^{-1}$ for the formation of intermediate compound I of HRP with H_2O_2.[27] Thus, the rate constant k_{CHL} was estimated to be $2.7 \times 10^6\,M^{-1}s^{-1}$.

DISCUSSION

Chlorophyllin, a water-soluble derivative of chlorophyll (present in green plants as well as in nutrition supplements such as Spirulina) has shown significantly higher antimutagenicity over well-known antioxidants like β-carotene, vitamin E, ascorbic acid against a spectrum of mutagens.[8] Its anticarcinogenic activity *in situ* has been shown to reduce heterocyclic amine induced mammary carcinogenesis.[31] It is an effective inhibitor of lipid peroxidation.[7,15] It has also been used as a food supplement to reduce the side effects of cancer therapy.[32]

Ionizing radiation is an important source of oxygen-derived free radicals and excited species.[2] During water radiolysis, ·OH, hydrogen atoms and hydrated electrons are produced. Among these, ·OH is highly oxidizing in nature. However, during the Fenton reaction, only ·OH are generated.[33] In the present studies, CHL inhibited formation of DMPO–·OH adduct by irradiation as well as by the Fenton reaction. But, concentrations required to inhibit the DMPO–·OH adduct were higher in case of exposure to γ-radiation in comparison with the Fenton reaction. Micromolar concentrations of CHL was sufficient to completely inhibit the formation of DMPO–·OH adduct. This could be because of different radicals generated during water radiolysis may react with CHL and a part of CHL may be used up by H· and e^-_{aq} and only the remaining amount may be acting on ·OH or the yield of ·OH during radiation exposure and the Fenton reaction are different. It is also likely that CHL may inhibit chelation of ferrous iron responsible for ·OH generation. Alternatively, during Fenton reaction, the reactive species involved is believed to be caged or bound. ·OH, often denoted as $[Fe{-}H_2O_2]^{2+}$.[34] This species may have different reactivity towards CHL than with radiation-derived ·OH and this might also account for the noted differences. Additional studies are needed to resolve this and may be possible only with availability of more techniques.

Singlet oxygen is generated in mammalian cells under both normal as well as pathophysiological conditions,[35] it has a fairly long half life, in the range of μs and is capable of traveling appreciable distances inside cells leading to considerable damage to biomolecules.[36] Photosensitization by MB and light is a commonly used method for generation of $^1O_2$ and has been utilized to investigate cellular and genotoxic effects in a wide variety of organisms.[37–39] CHL effectively quenched the formation of TEMPO by reacting $^1O_2$ at micromolar concentrations. Hydrogen peroxide is a well-known pro-oxidant and is responsible for production of several other reactive oxygen intermediates such as ·OH, in the presence of metal ions. CHL reacted with this physiologically important pro-oxidant at micromolar concentrations and exhibited a rate constant of $2.7 \times 10^6\,M^{-1}s^{-1}$. Since the solutions used in our studies are essentially metal-free, it is protective rather than the damaging effect of CHL we have observed.

The present studies provide information on direct radical-scavenging effects of CHL on different ROS. Spin-trapping in combination

with ESR techniques clearly demonstrated a scavenging effect on the physiologically relevant $^{\cdot}OH$ and $^1O_2$ besides the standard DPPH radical. CHL also reacted with H_2O_2 effectively. Our earlier experiments on chlorophyllin using pulse radiolysis showed a rate constant of $6.1 \times 10^9\,M^{-1}\,s^{-1}$ with $^{\cdot}OH$. The rate constant calculated by the spin-trapping method may not be very accurate as this method neglects the decay of spin adduct after its generation, but it conveniently provides relative values.[40] Hence, we have not calculated rate constant in the present study. ESR was considered to be the least ambiguous method for the detection of free radicals.[18] Recent studies also indicate that results from ESR combined with spin traps like DMPO–$^{\cdot}OH$ or DMPO–superoxide may be subject to debate.[21,22] However, these studies supplement our earlier investigations[6,7,41] as well as those of others[14,15] establishing the potent antioxidant activity of CHL.

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