Cellular Membrane Protection Against Reactive Oxygen Species by Terminalia Arjuna and Its Active Component Baicalein

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Summary Terminalia arjuna (T. arjuna) is an Indian medicinal plant credited with cardiotonic and cardioprotective properties; and baicalein is the active ingredient. To determine the possible mechanisms of protective action of T. arjuna and baicalein, we studied the reactions of T. arjuna extracts and baicalein with the biologically important superoxide (O2•−) and singlet oxygen (1O2) by measuring O2•− or 1O2 induced damage of lipids, lipid preoxidation, in the reaction mixture containing rat liver mitochondria and cardiac homogenate. Absorption of T. arjuna and baicalein in rat small intestine was also assessed. In addition, inhibitory effect of T. arjuna and baicalein on radiation-induced ROS generation in NIH3T3 cells and a cell-free chemical system, as well as on whole plasma oxidation were studied. Significant absorption of T. arjuna and baicalein from the intestine was observed. Baicalein is highly effective in inhibiting lipid peroxidation, reactive oxygen species generation, and plasma oxidation, even at low concentrations of 10–25 µM. To determine the possible mechanisms in this phenomena, we studied the reaction of T. arjuna extracts and baicalein with O2•− and 1O2 using electron spin resonance and found that T. arjuna extracts and

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Abbreviations: OH, hydroxyl radical; µs, microseconds; O2•−, singlet oxygen; 4-OH TEMP, 2,2,6,6-tetramethyl-4-hydroxypiperidine; 4-OH TEMPO•, 2,2,6,6-tetramethyl-4-hydroxypiperidine-N-oxide radical; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; CD, conjugated diene; CO•−, formate radical anion; CO2, carbon dioxide; DCF, dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescein; DCFH/DA, 2′,7′-dichlorodihydrofluorescein diacetate; DETAPAC, diethylenetriaminepentaacetic acid; DMEM, Dulbecco’s modified eagle’s medium; DMF, N,N′-dimethyl formamide; DMPO, 5,5-dimethyl-1-pyrorline-N-oxide; eaq−, hydrated electrons; EDTA, ethylene diamine tetra acetic acid; ESR, electron spin resonance; H•, hydrogen atom; H2O2, hydrogen peroxide; HCOO–, formate anion; IC50, concentration at which 50% inhibition is achieved; KSCN, potassium thiocyanate; LDL, low density lipoprotein; LOOH, lipid hydroperoxides; MeCN, acetonitrile; ms, milliseconds; N2, nitrogen; N2O, nitrous oxide; ns, nanoseconds; O2•−, superoxide radical; OH, hydroxyl anion; PBS, phosphate-buffered saline; PUF A, polyunsaturated fatty acid; RB, Rose Bengal; RFI, relative fluorescence intensity; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAQA, singlet oxygen quenching activity; SSA, superoxide scavenging activity; TBARS, thiobarbituric acid reactive substances; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.
baicalein possess higher scavenging activities than that of standard antioxidants; 50% Inhibitory concentration (IC$_{50}$) of the aqueous extract of $T$. arjuna is $0.6 \times 10^{-3}$ and $22.5 \times 10^{-3}\%$ (w/v) and IC$_{50}$ of baicalein for O$_2^-$ is 18 $\mu$M, while IC$_{50}$ for $^{1}$O$_2$ is 129 $\mu$M. Pulse radiolysis study with baicalein shows that the bimolecular rate constant between baicalein and O$_2^-$ is $1 \times 10^8$ M$^{-1}$ s$^{-1}$. In conclusion, our results show that $T$. arjuna and baicalein are very potent in membrane protection against O$_2^-$ and $^{1}$O$_2$ in vitro, and their ability to react with these species may explain previously observed effects of this plant and its active ingredient.

Key Words: electron spin resonance, superoxide radical, singlet oxygen, pulse radiolysis, lipid peroxidation

Introduction

Oriental systems of alternative medicine such as those of India, China, and Japan contain many herbs for their beneficial therapeutic effects. *Terminalia arjuna* (T. arjuna) is a well-known Indian medicinal plant widely used for its cardiotoxic, cardioprotective [7], anti-mutagenic [2], anti-tumor, anti-radical, and hypocholesterolemic [3] effects. It is also known as an “Indian alternative to ginseng”. Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is a major active ingredient present in some medicinal plants such as T. arjuna, Scutellaria baicalensis, Scutellaria rivularis, etc. Baicalein is a naturally occurring flavone found in the bark of *T.* arjuna [4]. It is credited with anti-proliferative [5], anti-tumor [6], anti-genotoxic [7], and anti-oxidant properties [8–10]. Being a potent free radical scavenger and xanthine oxidase inhibitor [11], baicalein improves endothelial dysfunction and confers cardioprotective action against oxidative stress-mediated cell injury induced by ischemia-reperfusion [12]. However, the mechanism involved in the membrane-protective and other antioxidant effects of *T. arjuna* and baicalein have not previously been examined in detail.

Recent clinical and experimental data showed the possible involvement of reactive oxygen species/reactive nitrogen species (ROS/RNS) in many human ailments such as cardiovascular diseases, neurodegenerative disorders, and carcinogenesis [13]. These ROS, including superoxide radical (O$_2^-$) and singlet oxygen ($^1$O$_2$), are capable of inducing alterations in the cellular molecules leading to cytotoxicity and cell death. Membrane lipid peroxides and other byproducts formed by ROS action also react with cellular genome, resulting in a loss of cell function, and inactivation of cell-signaling molecules [14]. Hence, antioxidant-rich supplements such as phytoneutrients, functional foods, or medicinal plants rich, are very useful. A number of plants with antioxidant potential have been shown to help in curing free radical-related human diseases. The active and pure ingredients from these plants are also good sources of natural antioxidants due to their low toxicity as compared to synthetic compounds.

In this context, we have studied extracts from *T. arjuna* and baicalein for their lipid protective and antioxidant effects. Earlier pharmacokinetic studies of baicalein in rats have shown that plasma levels of baicalein are low in cases of oral administration as compared to intravenous administration [15]. It is also known that baicalein is absorbed faster and to a greater extent in the intestine [16]. In the rat jejunal loop model, baicalein has also been shown to be readily absorbed [17]. We have studied the intestinal absorption of *T. arjuna* and baicalein in vitro using the ‘inverted loop model’ of rat intestine.

Herein, we reported on the antioxidant effects of *T. arjuna* and baicalein in vitro with regard to the inhibition of different intermediate products of lipid peroxidation by using rat liver mitochondria and cardiac homogenate. We have also investigated ROS scavenging effect of *T. arjuna* and baicalein in γ-irradiated cellular as well as cell-free chemical systems. To examine the mechanism underlying the known cardioprotective properties of *T. arjuna* and baicalein, in vitro studies of lipoprotein oxidation in whole human plasma were carried out. This seems to be a more relevant model of the lipoprotein oxidation in the arterial wall than that of a single isolated lipoprotein such as LDL [18]. To further reveal the more detailed mechanisms of baicalein responsible for the observed antioxidant effects, we allowed baicalein to react with some biologically relevant ROS as studied by different physicochemical methods such as spin trapping by electron spin resonance (ESR), and pulse radiolysis.

Materials and Methods

Materials

Acetonitrile, 2,2,6,6-tetramethyl-4-hydroxypiperidine (4-OH TEMP), 5,5-dimethyl-1-pyroline-N-oxide (DMPO), ammonium ferrous sulfate, butylated hydroxy toluene, chloroform, cyclohexane, 2',7'-dichlorodihydrofluorescein diacetate (DCFH/DA), diethylenetriaminepentaacetic acid (DETAPAC), N,N'-dimethyl formamide (DMF), Dulbecco’s modified eagle’s medium (DMEM), ethanol, ethyl acetate, ethylene diamine tetra acetic acid (EDTA), fetal calf serum (FCS), J.C. Tilak et al.
Preparation of the extracts from Terminalia arjuna bark

The Soxhlet extracts of *T. arjuna* were prepared by using the bark, free of dust and other impurities, sun-dried and finely powdered. Sequential extraction was performed using methanol and methanol-HCl (99:1, v/v) using a Soxhlet apparatus. The bark powder was extracted for 8–10 hours with each solvent to remove the soluble matter. The aqueous extract was prepared by stirring the bark powder in distilled water for 4 hours followed by filtration.

Uptake of baicalein and *T. arjuna* extracts in the ‘inverted intestinal loop’

Three-month-old female Wistar rats (weighing about 250 ± 20 g) were fasted for 24 hours and then sacrificed, and a length of 10 cm of jejunum was removed. The segment was washed with ice-cold saline and then inverted. This sac was filled with 2.0 ml saline, ligated at both ends, and incubated for 1 hr in 50 ml of 10 mg/ml *T. arjuna* extracts or 4 mg/ml baicalein at 37°C. The solution from the intestinal loop was collected and centrifuged at 5000 rpm for 10 minutes. Qualitative analysis of the absorbed components was performed using a Waters HPLC system (Waters/Millipore, Milford, MA, USA) consisting of a model 515 pump, a model 2487 dual wavelength absorbance detector, and a model 717 autosampler. Separation of the components was carried out in a C18 column (Delta Pak), 5 µm, 3.9 × 150 mm, 300 Å. The flow rate was set to 0.5 ml/min. A sample volume of 20 µl was injected to the column using a Waters 717 auto sampler. The mobile phase consisted of aqueous methanol (20%) in hydrochloric acid (0.1%) and acetonitrile (MeCN), and the wavelength used to detect the eluent was 280 nm. Calibration curves of baicalein (0–10 mg/ml concentration range) were constructed with a correlation coefficient of >0.995 [19].

Preparation of rat liver mitochondrial fraction/cardiac homogenate and an assay of lipid peroxidation

Three-month-old female Wistar rats (weighing about 250 ± 20 g) were sacrificed. In brief, rat livers were excised and homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 3,000 × g for 10 min to remove cell debris and the nuclear fraction. The resulting supernatant was centrifuged at 10,000 × g for 10 minutes to sediment the mitochondria. This pellet was washed three times with 5 mM potassium phosphate buffer, pH 7.4, to remove the sucrose. For preparation of rat heart homogenate, heart tissue was homogenized in 5 mM potassium phosphate buffer, pH 7.4. The mitochondria and homogenate were aliquoted and stored at 4°C until use [20]. Protein was estimated and pellets were suspended in the aforesaid buffer at a concentration of 10 mg protein/ml [21].

Nonenzymatic generation of O₂⁻ was carried out using a reaction mixture consisting of 50 µM riboflavin, 10 mM L-methionine, 50 µM EDTA, and rat liver mitochondria/heart homogenate (final concentration 250 µg/ml) in the presence and absence of *T. arjuna* or baicalein. Riboflavin acts as a sensitizer, whereas methionine is used as an electron donor and EDTA as a chelating agent. The system was photo-irradiated for 60 min to generate O₂⁻ [22]. Photosensitization using Rose Bengal is a very good source of O₂⁻ under aerobic conditions. The system consisted of 20 µM Rose Bengal and rat liver mitochondria/heart homogenate (final concentration 250 µg/ml) in the presence and absence of *T. arjuna* or baicalein in 50 mM potassium phosphate buffer (pH 7.4), photo-irradiated by a 300-W light source for 10 min with continuous oxygen bubbling. The distance between the light source and the trap was 15 cm [23]. After the incubations, conjugated dienes (CD) [24], lipid hydroperoxides (LOOH) [25], and thiobarbituric acid reactive substances (TBARS) [20] were estimated.

Whole plasma oxidation assay

Blood was taken from normal, healthy, nonsmoking males 35–40 years of age who were not submitted to any drug treatment, collected in heparin-containing tubes (15 I.U. heparin) after an overnight fast, and was centrifuged at 4°C for 10 min to obtain plasma. Prior consent in the prescribed format was obtained from the recruited individuals. Plasma samples were diluted 100-fold with phosphate-buffered saline (PBS), pH 7.4, containing 0.16 M NaCl and oxidized in the presence of AAPH at 37°C. The change in sample absorbance was monitored spectrophotometrically at 234 nm at 30-min intervals for 20 hours [18]. The plasma oxidation was studied in the presence and absence of *T. arjuna* extracts as well as baicalein.

Determination of ROS scavenging in NIH3T3 cells

The fluorescent probe DCFH/DA was used to monitor net
intracellular generation of ROS induced by γ-radiation. DCFH/DA is a cell-permeable, nonfluorescent probe that is cleaved by nonspecific cellular esterases inside cells to nonfluorescent DCFH and oxidized by ROS to a fluorescent product DCF, (excitation 490 nm and emission 520 nm). DCF fluorescence is therefore directly proportional to the ROS concentration [26].

A murine normal fibroblast cell line (NIH3T3) was maintained in DMEM media supplemented with 10% fetal calf serum in 95% air/5% CO2 atmosphere at 37°C. Cultures were passaged every third day after trypsinization (0.05% trypsin, 0.2% EDTA) with fresh medium. Cells were washed with phosphate-buffered saline (PBS), pH 7.4, and incubated with 100 µM DCFH/DA for 20 min at 37°C. They were then irradiated with a 2-Gy dose (Gy, dose rate 0.36 cGy/min), and fluorescence was immediately recorded at an excitation wavelength of 490 nm and an emission wavelength of 520 nm in the presence or absence of T. arjuna extracts as well as baicalein [27].

**Determination of ROS generation in a cell-free chemical system**

ROS were generated by γ-radiation (2 Gy at the dose rate 0.36 cGy/min) and was measured in reaction mixture containing DCFH/DA in a balanced salt solution. Either different T. arjuna extracts or different concentrations of baicalein were added, and fluorescence was measured by spectrofluorometer. A decrease in fluorescence (expressed as relative fluorescence increase, RFI) would be indicative of the free radical scavenging activity of the compounds [26].

**ESR spin-trapping measurements of singlet oxygen**

The hypoxanthine and xanthine oxidase system was used as a source of O2− [28]. Thus formed, O2− was studied by an ESR spin-trapping experiment using DMPO as a spin trap. The standard reaction mixture consisted of hypoxanthine (0.5 mM), xanthine oxidase (20 mU/ml), DETAPAC (0.1 mM), spin trap (DMPO, 625 mM), test sample, buffer (100 mM phosphate-buffered saline, pH 7.4, containing 1%, v/v DMF). T. arjuna aqueous, methanolic, and acidic methanolic extracts as well as baicalein at different concentrations (70–250 µM) were tested for superoxide quenching activities using an ESR technique. A JEOL-JES-TE200 spectrophotometer was operated for determination of O2− scavenging signals. The measurements were performed at 25°C using an aqueous quartz flat cell. The spectrometer was operated at a microwave frequency of 9.432 GHz with microwave power of 4 mW, amplitude 100, and a modulation width of 0.1 mT. For the spectra, center field was 338.5 mT, the sweep width 5 mT, the time 1 min, and the time constant 0.1 sec.

**Pulse radiolysis studies**

The pulse radiolysis system using 7-MeV electrons has been described previously [32]. The dosimetry was carried out using an air-saturated aqueous solution containing 50 mM KSCN (Gy = 23,889 M−1 cm−1 per 100 eV at 500 nm) [33]. The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. The optical path length of the cell was 1.0 cm. The width of the electron pulse was 50 ns. Time-dependent absorbance differences were recorded on a digital oscilloscope. High purity (>99.9%) N2O, from British Oxygen Corporation India Pvt. Ltd. was used. Superoxide radicals were generated during the scavenging of radiolytically generated HO− free radicals by 50 mM formate, in air-saturated solution. This radical is obtained in pure form in less than 1 µs. Samples to be irradiated were prepared in 50 mM sodium formate (pH 8.0) and saturated with air. The doses per pulse were 15 Gy (O2− ≈ 9 µM). The reactions occurring in the working medium are as follows:

\[
\begin{align*}
\text{H}_2\text{O} & \rightarrow \epsilon_{\text{aq}}^{-}, \text{H}^{+}, \cdot \text{OH}, \text{H}_2\text{O}_2, \text{etc.} \\
\epsilon_{\text{aq}}^{-} + \text{N}_2\text{O} + \text{H}_2\text{O} & \rightarrow \cdot \text{OH} + \text{OH}^{+} + \text{N}_2 \\
\cdot \text{OH} + \text{HCOO}^{-} & \rightarrow \text{CO}_2^{-} + \text{H}_2\text{O} \\
\text{CO}_2^{-} + \text{O}_2 & \rightarrow \text{O}_2^{-} + \text{CO}_2
\end{align*}
\]

The bimolecular rate constants were calculated by plotting the pseudo-first order rate of formation of the transient against the concerned solute concentration. The uncertainty in the measurement for bimolecular rate constant is <10%. The transients obtained in the pulse radiolysis study were
used to characterize the product radical. The rate constants determined and presented in the text are not mere radiation chemical parameters, but they reflect on the efficiency of scavenging free radicals and the ease with which competing reactions occur.

Calculation of the IC₅₀ Value

The scavenging activity of baicalein towards different ROS is represented in terms of 50% inhibitory concentration (IC₅₀). The IC₅₀ values were calculated using the following relation:

\[ F = \frac{Q}{100} \]

where \( Q \) = % quenching activity of baicalein. The graph of (F/1-F) against the concentration of baicalein was plotted. IC₅₀ is the concentration of baicalein at which \((F/1-F) = 1\). The experiments were repeated and reproduced on independent days. Results are expressed as mean values with corresponding standard errors.

Statistics

Data were analyzed by using a two-way ANOVA. If the ANOVA indicated a significant difference, student’s t test was performed to determine the significance between means. A value of \( p<0.05 \) was considered to indicate statistical significance.

Results

Effects of T. arjuna and baicalein on \( O_2^− \)-induced lipid peroxidation

Inhibitory effects of \( T. \) arjuna and baicalein against superoxide-induced lipid damage as measured by different intermediates of lipid peroxidation such as conjugated dienes, lipid hydroperoxides, and TBARS are presented in Table 1. Baicalein inhibited lipid peroxidation in a concentration-dependent manner. Concentrations as low as 10 \( \mu \)M baicalein were effective in inhibiting the formation of CD by 66%. In the case of LOOH and TBARS formation, 10 \( \mu \)M baicalein showed 40 and 45% inhibition, respectively, in rat liver mitochondria. Baicalein concentrations of 10 \( \mu \)M showed the highest inhibition of LOOH and TBARS formation in rat cardiac homogenate by 45 and 75%, respectively. In the case of \( T. \) arjuna extracts, aqueous extract was the most effective in protecting LOOH (84%), while methanolic extract was the most effective in inhibiting TBARS formation (76%). In rat cardiac homogenate, aqueous extract was the most effective, giving inhibitions of 69 and 37% in LOOH and TBARS formation. Methanolic and methanolic-HCl extracts were less effective compared to the aqueous extract.

<p>| Table 1. Effect of baicalein and ( T. ) arjuna extracts on superoxide-induced lipid peroxidation in rat liver mitochondria and rat heart homogenate |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>( CD )</th>
<th>( LOOH )</th>
<th>( TBARS )</th>
<th>( LOOH )</th>
<th>( TBARS )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.3 ± 0.7</td>
<td>19.6 ± 0.5</td>
<td>2.7 ± 0.2</td>
<td>2.08 ± 0.44</td>
<td>2.72 ± 0.25</td>
</tr>
<tr>
<td>Damage</td>
<td>88.6 ± 3.5</td>
<td>70.1 ± 2.2</td>
<td>12.0 ± 0.3</td>
<td>13.46 ± 1.09</td>
<td>7.49 ± 0.38</td>
</tr>
<tr>
<td>1 ( \mu )M baicalein</td>
<td>75.7 ± 2.1</td>
<td>57.0 ± 4.3</td>
<td>9.6 ± 0.6*</td>
<td>13.45 ± 1.37</td>
<td>5.85 ± 0.33*</td>
</tr>
<tr>
<td>5 ( \mu )M baicalein</td>
<td>68.7 ± 2.2*</td>
<td>53.8 ± 3.8*</td>
<td>8.5 ± 0.2**</td>
<td>9.28 ± 1.71</td>
<td>5.29 ± 0.16**</td>
</tr>
<tr>
<td>10 ( \mu )M baicalein</td>
<td>33.4 ± 1.0***</td>
<td>49.9 ± 2.1**</td>
<td>7.8 ± 0.4**</td>
<td>8.35 ± 0.33*</td>
<td>3.90 ± 0.40**</td>
</tr>
<tr>
<td>( T. ) arjuna Aqueous extract</td>
<td>ND</td>
<td>27.8 ± 1.8***</td>
<td>7.38 ± 0.71**</td>
<td>5.57 ± 0.61**</td>
<td>5.71 ± 0.11**</td>
</tr>
<tr>
<td>( T. ) arjuna MeOH extract</td>
<td>ND</td>
<td>36.5 ± 3.8**</td>
<td>4.95 ± 0.29***</td>
<td>7.89 ± 0.76*</td>
<td>6.20 ± 0.80</td>
</tr>
<tr>
<td>( T. ) arjuna MeOH-HCl extract</td>
<td>ND</td>
<td>59.2 ± 0.5*</td>
<td>11.28 ± 0.33</td>
<td>12.88 ± 0.29</td>
<td>7.42 ± 0.17</td>
</tr>
</tbody>
</table>

*All values are expressed as nmoles products/mg protein. Data expressed as Mean ± S.E. from four different experiments. \( \ast P<0.05 \), \( \ast\ast P<0.01 \) and \( \ast\ast\ast P<0.001 \), as compared to superoxide-induced damage. (CD: conjugated diene; LOOH: lipid hydroperoxides; TBARS: thiobarbituric acid reactive substances; ND- not determined)
monitored by HPLC. Fig. 1a, represents the chromatogram concentrations of 50 µM the extent of plasma oxidizability followed by 10 and 5 µM baicalein monitored spectrophotometrically at 234 nm. Baicalein showed a concentration-dependent protection pattern. Baicalein inhibited LOOH and TBARS formation by 58 and 42%, respectively, followed by methanolic and acidic methanolic extracts, as shown in Fig. 2a, at 50 µM showed almost 40% inhibition of ROS generation, while aqueous extract of T. arjuna was more potent in inhibiting ROS production in cells than the methanolic extract. In the case of the cell-free chemical system, 50 µM Trolox, a standard antioxidant. In the case of T. arjuna extracts, as shown in Fig. 2b, at 50 µg/ml, the aqueous extract was better than the methanolic extract in decreasing plasma lipoprotein oxidation.

Intestinal absorption of T. arjuna and baicalein

To examine the intestinal absorption of T. arjuna and baicalein extracts, we used an isolated preparation of inverted loop of rat intestine, and the absorbed compounds were monitored by HPLC. Fig. 1a, represents the chromatogram of 4 mg/ml baicalein solution before absorption, compared to the absorbed baicalein sample (around 1 mg/ml). Almost 25% baicalein was recovered through intestinal cells in the serosal cavity formed by the inverted intestine loop model. Both aqueous and methanolic extracts of T. arjuna were found to be absorbed in significant amounts in the intestine, as shown in Figs. 1b and 1c, respectively.

Effects of T. arjuna and baicalein on AAPH-induced plasma oxidation

To further characterize the antioxidant role of T. arjuna extracts and baicalein in peroxyl radical-induced whole plasma oxidation, inhibition of lipoprotein oxidation by spectrophotometric analysis was studied. AAPH-treated human plasma samples were incubated in the presence and absence of different concentrations of baicalein and monitored spectrophotometrically at 234 nm. Baicalein showed a concentration-dependent protection pattern. Baicalein concentrations of 50 µM were the most effective in controlling the extent of plasma oxidizability followed by 10 and 5 µM baicalein (Fig. 2a). Baicalein concentrations of 10 and 50 µM showed higher protection than that induced by 50 µM Trolox, a standard antioxidant. In the case of T. arjuna extracts, as shown in Fig. 2b, at 50 µg/ml, the aqueous extract was better than the methanolic extract in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver Mitochondria</th>
<th>Heart homogenate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD</td>
<td>LOOH</td>
</tr>
<tr>
<td>Control</td>
<td>2.89 ± 0.82</td>
<td>13.22 ± 1.36</td>
</tr>
<tr>
<td>Damage</td>
<td>8.87 ± 0.65</td>
<td>37.58 ± 1.21</td>
</tr>
<tr>
<td>1 µM baicalein</td>
<td>8.47 ± 0.52</td>
<td>32.71 ± 1.10</td>
</tr>
<tr>
<td>5 µM baicalein</td>
<td>7.24 ± 0.76</td>
<td>31.49 ± 0.82*</td>
</tr>
<tr>
<td>10 µM baicalein</td>
<td>5.48 ± 0.59*</td>
<td>28.19 ± 0.72**</td>
</tr>
<tr>
<td>T. arjuna Aqueous extract</td>
<td>ND</td>
<td>22.68 ± 0.74**</td>
</tr>
<tr>
<td>T. arjuna MeOH extract</td>
<td>ND</td>
<td>21.11 ± 2.66*</td>
</tr>
<tr>
<td>T. arjuna MeOH-HCl extract</td>
<td>ND</td>
<td>32.48 ± 0.69*</td>
</tr>
</tbody>
</table>

All values are expressed as nmoles products/mg protein. Data expressed as Mean ± S.E. from four different experiments.

*P<0.05, **P<0.01 and ***P<0.001, as compared to singlet oxygen-induced damage. (CD: conjugated diene; LOOH: lipid hydroperoxides; TBARS: thiobarbituric acid reactive substances; ND- not determined)

Effects of T. arjuna and baicalein on ROS generation in NIH 3T3 cells

Inhibitory effects of T. arjuna extracts and different concentrations of baicalein on γ-radiation-induced ROS generation in NIH3T3 cells are shown in Table 3. Baicalein inhibited intracellular γ-radiation-induced ROS formation in a concentration-dependent manner. Baicalein concentrations of 50 µM showed almost 40% inhibition of ROS generation, while aqueous extract of T. arjuna was more potent in inhibiting ROS production in cells than the methanolic extract. In the case of the cell-free chemical system, 50 µM baicalein inhibited ROS formation by 60%. The inhibition by aqueous extract of T. arjuna was more than that by methanolic extract.

Scavenging activities of T. arjuna and baicalein for O2− and H2O2

Direct scavenging abilities of baicalein for O2− were determined using ESR spectroscopy. Xanthine oxidase converts hypoxanthine to xanthine on oxidation, producing O2−. This O2− in turn combines with the spin trap, DMPO, forming DMPO-OOH adduct, which later decomposes to form DMPO-OH adduct. A control study showed a multiline spectrum, which can be analyzed in terms of a mixture of both hydroxyl- and superoxide-DMPO adducts. In the presence of baicalein (10–100 µM), signal intensities of the

DMPO-OOH adduct were markedly attenuated in a concentration-dependent manner (Fig. 3). The IC$_{50}$ value of baicalein for $\text{O}_2^\cdot$ radical-induced damage generated in the system was 18 $\mu$M, as shown in Table 4. However, the inhibition by baicalein as reported here might not only be due to the $\text{O}_2^\cdot$ scavenging ability but also due to an inhibition of $\text{O}_2^\cdot$ formation, since baicalein is a known xanthine oxidase inhibitor [11].

The photo-irradiated Rose Bengal can generate $^1\text{O}_2$, and in the presence of 4-OH TEMP gives a three-line spectrum, which is characteristic of a stable nitroxide-like radical, 4-OH TEMPO$, which generated in the reaction of $^1\text{O}_2$ with TEMPO, as shown in Fig. 4. The addition of baicalein decreased the signal intensity of 4-OH TEMPO$^\cdot$ in a dose-dependent manner. The IC$_{50}$ value of baicalein for $^1\text{O}_2$ as calculated is 128.1 $\mu$M and is shown in Table 4. The value is lower than that for other standard antioxidants such as caffeic acid, catechin, and curcumin.

Data on the scavenging activities of $T$. arjuna extracts against $\text{O}_2^\cdot$ and $^1\text{O}_2$ are shown in Table 5. The values are expressed as IC$_{50}$ (%, w/v of the extracts). In both cases,
aqueous extract possessed the highest antioxidant potential, followed by that of methanolic and then acidic methanolic extracts. The IC$_{50}$ of the aqueous extract for O$_2^-$ is $0.6 \times 10^{-3}$%, while that for singlet oxygen it is $22.5 \times 10^{-3}$%.

**Pulse radiolysis analysis**

Fig. 5 depicts the transient absorption spectra recorded when 0.5 mM baicalein was allowed to react with ~10 µM O$_2^-$, O$_2^-$ radical reacts very fast to produce a transient species that absorbs at 300–340 nm region with an absorption maximum at 320 nm at 30 µs after the electron pulse. At the same time, as the parent molecules get depleted due to this reaction, a bleaching in absorption is evident in the 350–425 nm wavelength region. This shows that all the superoxide radical anions generated have reacted with the substrates within 30 µs and the bimolecular rate constant as

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ROS generation (fluorescence intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIH3T3 cell system</td>
</tr>
<tr>
<td>Damage</td>
<td>24.90 ± 1.22</td>
</tr>
<tr>
<td>1 µM baicalein</td>
<td>23.83 ± 1.81</td>
</tr>
<tr>
<td>5 µM baicalein</td>
<td>22.68 ± 1.06</td>
</tr>
<tr>
<td>10 µM baicalein</td>
<td>19.96 ± 0.59</td>
</tr>
<tr>
<td>50 µM baicalein</td>
<td>14.37 ± 2.10*</td>
</tr>
<tr>
<td>50 mg/ml T. arjuna Aqueous extract</td>
<td>7.68 ± 0.82*</td>
</tr>
<tr>
<td>50 mg/ml T. arjuna Methanolic extract</td>
<td>8.25 ± 0.93*</td>
</tr>
</tbody>
</table>

All values are expressed as nmols products/mg protein. Data expressed as Mean ± S.E. from four different experiments. *P<0.05 and **P<0.01 as compared to γ-radiation-induced damage. (ROS: reactive oxygen species)
measured at 330 nm is $1 \times 10^8$ M$^{-1}$s$^{-1}$. With progress in time, the initial absorption peak at 320 nm decays and shows negative absorption, while the bleaching recovers and an intense absorption peak develops at 375 nm at 1.6 ms. In this time-evolved spectra, one can observe a clear isobestic point at 340 nm. In Inset of Fig. 5, the absorbances at two different wavelengths have been plotted as a function of time. It is evident that the decay of absorbance at 330 nm is concomitant with the increase in absorbance at 380 nm.

Fig. 6 shows the transient absorption spectra recorded when 0.5 mM baicalein was allowed to react with 10 µM CO$_2$· radical. The bimolecular rate constant for this reaction as measured at 330 nm is $5 \times 10^8$ M$^{-1}$s$^{-1}$. Here again as the time progresses the absorption at 320 nm due to the initial radical decreases, while another absorption at 380 nm is developed.

**Discussion**

*T. arjuna* has been credited with cardiotonic and cardioprotective abilities and other beneficial therapeutic effects in the Indian medicinal system, Ayurveda. Baicalein is one of the flavonoids found in many Indian and Chinese medicinal plants including *T. arjuna*. Besides its anti-inflammatory [34] and chemopreventive [35] properties, baicalein has anti-proliferative and lipoxygenase inhibitory activities and hence is helpful in the prevention of atherosclerosis [5]. It can also scavenge ROS generated in cardiomyocytes [12].

In the present study, we examined the extent of absorption of *T. arjuna* extracts and baicalein through the isolated jejunum loop of rat intestine. Flavones and flavonol glucosides and their corresponding aglycones were transferred
across the rat jejunum either after glucuronidation or unmetabolised [19]. Our results showed significant recovery of baicalein (about 25%) in rat jejunum in its native form, with aglycone. *T. arjuna* extracts also being shown to be absorbed in the rat intestine in vitro, suggesting their bioavailability in the intestine.

We have examined the inhibitory effects of baicalein against O$_2^-$ and 1O$_2$-induced oxidative damage. In order to evaluate antioxidant potential of new free radical scavengers from natural resources, we have adopted biochemical as well as steady-state and kinetic spectroscopic techniques. The former measures modification of a biological marker, i.e. lipid peroxidation, in a model system of rat liver mitochondria. It is well known that abstraction of a hydrogen atom from polyunsaturated fatty acid (PUFA) leads to the formation of lipid radicals and lipid radicals are stabilized by molecular rearrangement forming CD, which then reacts with O$_2^-$, producing LOOH. The end product of lipid peroxidation is aldehyde that can be measured by TBARS formation. We estimated lipid peroxidation by three indices, namely CD, LOOH, and aldehydes as TBARS.

Superoxide radical can do direct damage, but its reactivity is limited. For the assessment of lipid peroxidation, we have used nonenzymatic superoxide generator, i.e. a system containing riboflavin, methionine, and EDTA. In this system riboflavin acts as a sensitizer, which upon absorption of light, generates superoxide radicals in the presence of oxygen. Methionine acts as an electron donor and EDTA as a chelating agent. Damage is caused by auto-oxidation of lipid substrate. Lipid peroxidation in terms of CD was found to be the highest in superoxide-induced damage.

Among the ROS, singlet oxygen is of particular physiological importance because of its relatively long half-life (µs) [36]. It can be generated in mammalian cells under both normal as well as pathophysiological conditions [37]. It combines directly with unreactive molecules, oxidizes them and causes cell death or mutations. When we studied 1O$_2$-induced lipid peroxidation in rat liver mitochondria, LOOH formation was the highest as compared to CD and TBARS formation. This difference could be due to the addition of 1O$_2$ only to the end carbon atom of a double bond and then taking the trans configuration, forming more LOOH than CD. In this case, 50% protection was offered by 50 µM baicalein. In this case, the lipid peroxidation process is much faster than the auto-oxidation, as the time for optimum damage was found to be 10–20 minutes. In the case of O$_2^-$-induced lipid peroxidation, the time required for the optimum level of damage was 60 min. Baicalein concentrations of 10 µM, however, were able to inhibit O$_2^-$ and 1O$_2$-induced LOOH formation to a similar extent.

Ionizing radiations such as γ-rays cause damage to cellular components through ROS formed upon radiolysis of water. The primary and secondary free radicals that are formed cause alterations in various biomolecules, which has been linked to many human diseases [13]. In this study, the fluorogenic probe DCFH/DA was used to detect oxidative stress in NIH3T3 cells. DCFH/DA diffuses across cell membranes and generates the product that gives fluorescence upon reaction with ROS. In this study, total ROS generation after exposure to 2 Gγ radiation was decreased in the presence of *T. arjuna* extracts as well as baicalein, suggesting the potent ROS-scavenging abilities of *T. arjuna* extracts and baicalein.

Lipoprotein oxidation has been known to have relevance in a major human health problem, atherosclerosis. To evaluate the antioxidant properties of *T. arjuna* and baicalein, lipoprotein oxidation induced in whole plasma was studied in vitro. The composition of human plasma is similar to the composition of interstitial fluid in arterial walls containing various lipoproteins such as HDL, LDL, and VLDL along with water-soluble antioxidants. This method of lipoprotein oxidation in the arterial wall is therefore more relevant than the in vitro oxidation of single isolated lipoprotein such as LDL. Measurement of absorbance at 234 nm reflects the lipoprotein oxidation induced in vitro in whole plasma [18]. It is also known that dietary flavonoids reduce the oxidizability of LDL in vitro [38]. Although the above-mentioned assays directly determine the antioxidant effect, it is impossible to obtain information on all the reactions occurring in a complex system. The spectroscopic methods measure the extent and rate of reaction of free radicals with the compound by using highly sensitive techniques such as ESR and pulse radiolysis. ESR spin-trapping provides an accurate and direct means of monitoring ROS at room temperature. *T. arjuna* extracts as well as baicalein were found to inhibit the formation of DMPO–OOH adduct. Micromolar concentrations of baicalein (IC$_{50}$ = 18 µM) were sufficient to completely inhibit the formation of the adduct as compared to other antioxidants such as caffeic acid, catechin, rosmarinic acid, and curcumin. This ability could be due to the O$_2^-$-scavenging activity as well as the inhibition of O$_2^-$ formation, as baicalein is also known as an inhibitor of xanthine oxidase [13]. As shown in ESR studies, the aqueous *T. arjuna* extracts and baicalein (IC$_{50}$ = 129 µM) can effectively quench O$_2^-$, even at very low concentrations, by reducing the TEMPO' signal intensities.

Physiological production of O$_2^-$ is unavoidable in aerobic cells. Under conditions where the production rate of O$_2^-$ is high and/or scavenging of it is poor, the radical may cause damage to cells through nonspecific interactions. This potential for damage explains why it is indispensable to determine the reactivity of a compound with O$_2^-$ to evaluate its antioxidative capacity for O$_2^-$ scavenging. Because of its relatively short life in aqueous media, O$_2^-$ cannot be handled like other substrates for usual enzyme assays. A direct method is more desirable for accurate determination
of the reactivity of $O_2^{-\cdot}$. Pulse radiolysis is the most powerful and simple technique for analyzing a diffusion-controlled reaction because of its rapid and controlled production of $O_2^{-\cdot}$. Our results showed that the isobestic point at 340 nm clearly provides information regarding the presence of two species, one at the early time scale and transformation of that to some other species at a later stage. The initial radical has a higher absorption coefficient than baicalein at a wavelength of 300–340 nm. At approximately 1 ms, the decay of the initial radical is complete by ~90%, and the bleaching of the parent becomes visible in this range. In the wavelength region from 350–425 nm, baicalein has a higher absorption coefficient than the initial radical, and the bleaching in the absorption spectrum appears at an early stage. At approximately 1 ms, the initial radical decays and is transformed to another radical with a much stronger absorption coefficient at 375 nm and that shows an intense absorption peak at this wavelength. The kinetic traces at different wavelengths of interest represent the aforesaid radical transformation.

The point to be noted here is that the transient characteristics in the superoxide radical reaction do not match those obtained in the reaction of oxidizing radicals like $\cdot OH$, CCl$_2$OO$^\cdot$, LOO$^\cdot$, and N$_2$O$^\cdot$ (not shown here). All these oxidizing radicals generate a phenoxyl radical with broad absorption in the 400–500 nm region of the wavelength without the time evolution, as mentioned in the case of the superoxide radical reaction. Superoxide radical anion is known to cause both oxidation [39] and reduction [40], depending on the nature of the substrates. In order to understand the reaction pathway of superoxide radical anion with baicalein, its reaction with CO$_2^{-\cdot}$ radical has been investigated, since CO$_2^{-\cdot}$ radical is a moderately strong reducing agent. In the present study it was seen that the CO$_2^{-\cdot}$ radical reacts faster to produce a similar transient spectra as compared to that in the $O_2^{-\cdot}$ radical reaction. The time evolution of the initial spectra is also faster in this case and is complete at around 170 $\mu$s. Moreover, it has previously been shown [41] that the CO$_2^{-\cdot}$ radical reaction is also possible by addition in certain cases. Therefore, the initial radical we assign to be an adduct, while the final species is the anion radical. Based on these results it can be concluded that the superoxide radical scavenging mechanism for baicalein is a reduction pathway and not oxidation. Our results substantiate an earlier report [42] proposing that even though baicalein scavenges superoxide radical, the reaction pathway does not mimic the effects of superoxide dismutase. The whole redox reaction mechanism is described in Scheme 1.

The present study provides information on the antilipoperoxidative and radical scavenging effects of baicalein with physiologically relevant radicals such as $O_2^{-\cdot}$ and $O_2^-$. Even at micromolar concentrations, baicalein is able to inhibit the lipid peroxidation process, as examined through the formation of CD, LOOH, and TBARS. Spin trapping in combination with an ESR technique clearly demonstrates the scavenging effects on $O_2^{-\cdot}$ and $O_2^-$. Our other studies (not presented here) have shown that baicalein reacts with hydroxyl, peroxyl, and thyl radicals with fairly high rate constants, eg. $3.7 \times 10^9$ dm$^3$ mol$^{-1}$ s$^{-1}$ for $\cdot OH$, and $8.5 \times 10^7$ dm$^3$ mol$^{-1}$ s$^{-1}$ for CCl$_2$OO$^\cdot$. Baicalein has been shown to decrease the signal intensities of $\cdot OH$, $O_2^{-\cdot}$, tert-butyl peroxyl radical, and H$_2$O$_2$ using ESR [9, 10] and also to protect against AAPH, ascorbate-Fe$^{3+}$-induced lipid damage [8]. Some recent studies have explored the neuroprotective [42, 43], hepatoprotective effects [44] and anti-apoptotic activity in cancer cells [45]. T. arjuna has been used as a cardiac stimulator and tonic in India. The bark helps to reduce the triglycerides and cholesterol content of blood in addition to inhibiting the LDL oxidation that encourages synthesis of HDL cholesterol. The bark also has diuretic properties and is a general tonic for people suffering from liver cirrhosis. Long-term use of the bark does not have any side effects [46]. The present study supplements these findings and establishes the potent antioxidant activity of T. arjuna extracts and baicalein, which might be in part responsible for their known therapeutic efficacy.

References


