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## Original Article

# Comparison of free radical formation induced by baicalein and pentamethyl-hydroxychromane in human promyelocytic leukemia cells using electron spin resonance

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## ABSTRACT

Baicalein and pentamethyl-hydroxychromane (PMC) have been investigated for use as antioxidants. However, antioxidants may stimulate free radical formation under certain conditions. The aim of our study was to determine whether PMC and baicalein exhibit both pro-oxidant and antioxidant activities in human promyelocytic leukemia (HL-60) cells. In this study, electron spin resonance spectrometry was used to investigate the effects of baicalein and PMC on free radical formation. In HL-60 cells, baicalein and PMC produced hydroxyl and phenoxyl radicals, respectively, but each inhibited radical formation by the other. The PMC pro-oxidant activity required H<sub>2</sub>O<sub>2</sub>, whereas baicalein produced hydroxyl radicals during the cell resting state only. The antioxidant effect of baicalein on PMC-induced oxidative stress in HL-60 cells may involve myeloperoxidase inhibition, which produces the myeloperoxidase-protein radical. Our investigation of the antioxidant effects of baicalein on arachidonic acid (AA)-induced oxidative stress in HL-60 cells showed that the baicalein-phenoxyl radical was the primary product, and that either carbon-centered or acyl radicals were the secondary products. However, the antioxidant effects of PMC on AA-induced oxidative stress produced only nonradical products. In conclusion, we showed that baicalein displayed both pro-oxidant and antioxidant activities in HL-60 cells. PMC exhibited no pro-oxidant activity during the cells' resting state but produced the PMC-phenoxyl radical in the presence of H<sub>2</sub>O<sub>2</sub>. The reaction of baicalein with AA in HL-60 cells

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produced baicalein-derived phenoxyl radicals that may initiate various pro-oxidative reactions. However, PMC does not produce radicals when it acts as an antioxidant. Thus, PMC is more beneficial as an antioxidant than baicalein.

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## 1. Introduction

Reactive oxygen species (ROS) act as second messengers in cell signaling pathways and are involved in numerous disease states, including inflammation, immune injury, and myocardial infarction [1]. Several age-related diseases and the aging process itself are thought to involve the ROS-mediated degeneration of cell components [2], and ROS may also be involved in the initiation, progression, and metastasis of cancer [3,4]. Paradoxically, ROS production is the mechanism of cell death in chemotherapy, radiotherapy, and photodynamic therapy for cancer [5,6]. Consequently, both pro-oxidant and antioxidant therapies have been proposed for cancer treatment [7,8].

Baicalein (5,6,7-trihydroxyflavone; Fig. 1) is a flavonoid extracted from the traditional Chinese herb *Scutellaria baicalensis* Georgi (Huangqin). It is commonly used in China and Japan to treat chronic hepatitis. In recent years, studies have shown that baicalein may inhibit the growth of cancer cells *in vitro* and may act as a potent inducer of apoptotic cell death [9–15]. However, the precise mechanism of action remains unclear. Several studies have shown that baicalein has antioxidant properties and protects cells from oxidative stress [16–19]. By contrast, the pro-oxidant properties of baicalein have been shown to be cytotoxic [15,20–22]. Our previous study showed that baicalein induced hydroxyl-radical formation through 12-lipoxygenase (12-LOX) in human platelets [23]. In B16F10 cells, baicalein produced hydroxyl radicals and superoxide anion radicals through 12-LOX, which resulted in a reduction in cell viability [24].

A derivative of  $\alpha$ -tocopherol, 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC; Fig. 1), is more hydrophilic than other  $\alpha$ -tocopherol derivatives, and has potent antiplatelet and free radical-scavenging properties [25]. In a previous study, we showed that PMC treatment may produce both preventative and therapeutic effects in ischemia–reperfusion brain injury [26]. We recently demonstrated that PMC inhibits vascular smooth muscle cell proliferation *in vitro* and balloon injury-induced neointimal formation *in vivo*, and we found that the inhibitory mechanism of PMC may involve the

inhibition of hydroxyl radical-mediated PLC $\gamma$ 1–PKC $\delta$  and JAK2–STAT3 activation, which causes cell cycle arrest at the G2/M phase [27]. By contrast, Tyurin et al [28] used myeloperoxidase-containing human promyelocytic leukemia (HL-60) cells to generate phenoxyl radicals from PMC.

Antioxidants function as redox agents, protecting against free radicals under some conditions, and stimulating free radical formation in others [29]. The aim of this study was to determine whether PMC and baicalein exhibit both pro-oxidant and antioxidant activities in HL-60 cells.

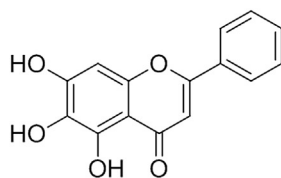
## 2. Materials and methods

### 2.1. Materials

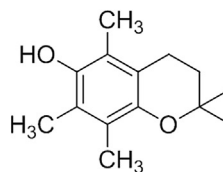
Arachidonic acid (AA); baicalein; dimethyl sulfoxide (DMSO); 5,5-dimethyl-1-pyrroline N-oxide (DMPO); reduced nicotinamide-adenine dinucleotide (NADH); myeloperoxidase (MPO); N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398); PMC; and 4-aminobenzoic acid (ABAH) were purchased from Sigma (St. Louis, MO, USA). Cyclooxygenase-2 (COX-2), AA (sodium salt), hematin, phenol, and 0.1 M Tris buffer (pH 8.3) were obtained from Oxford Biochemical Research (Rochester Hills, MI, USA). Amphotericin B (Fungizone), fetal calf serum (FCS), and Gibco RPMI 1640 medium were purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2. Cell cultivation

The HL-60 cells were provided by Dr. R.T. Wu at the National Yang-Ming University, Taipei, Taiwan, and were cultured at 37°C at a cell density ranging from  $1 \times 10^6$  cells/mL to  $5 \times 10^6$  cells/mL in an RPMI (Roswell Park Memorial Institute) 1640 medium containing 10% FCS in a humidified atmosphere of 5% CO<sub>2</sub>. To ensure optimal growth conditions, the cell culture medium was replaced when necessary by centrifuging the cells and resuspending them in fresh RPMI 1640 supplemented with 10% FCS.



baicalein



2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC)

**Fig. 1** – Structural features of baicalein (5,6,7-trihydroxyflavone), a naturally occurring alkaloid, and 2,2,5,7,8-pentamethyl-6-hydroxychromane, a novel  $\alpha$ -tocopherol derivative.

### 2.3. Measurement of free radicals by electron spin resonance spectrometry

Electron spin resonance (ESR) spectrometry was performed using a Bruker EMX ESR spectrometer (Bruker BioSpin, Billerica, MA, USA) as described previously [24], with modifications. The culture medium was replaced with phosphate buffered saline (PBS) prior to each experiment. The reagents or enzyme inhibitors were added to a suspension of  $1.5 \times 10^6$  HL-60 cells in 150  $\mu\text{L}$  of 100 mM DMPO in PBS, and incubated for 3 minutes prior to the addition of baicalein or PMC. Baicalein and PMC were dissolved in DMSO, and the final concentration of DMSO in the suspension was 0.1% (v/v). The ESR spectra were recorded at room temperature using a flat quartz cell designed for aqueous solutions. The sample preparation and ESR analysis were performed within a period of exactly 3 minutes. The ESR experimental conditions were as follows: 20 mW power at 9.78 GHz, a scan range of 100 G; a receiver gain of  $5.02 \times 10^4$ ; modulation amplitude, 1 G; time constant, 164 milliseconds; and a scan duration of 42 seconds, with six scans recorded for each sample.

### 2.4. Statistical analysis

The experimental results are expressed as the mean  $\pm$  the standard error of the mean, with the number of observations shown as *n*. The data were analyzed using analysis of variance. If a significant difference among the group means was

noted, the difference between two groups was assessed using the Newman–Keuls method. A *p* value  $<0.05$  was considered statistically significant.

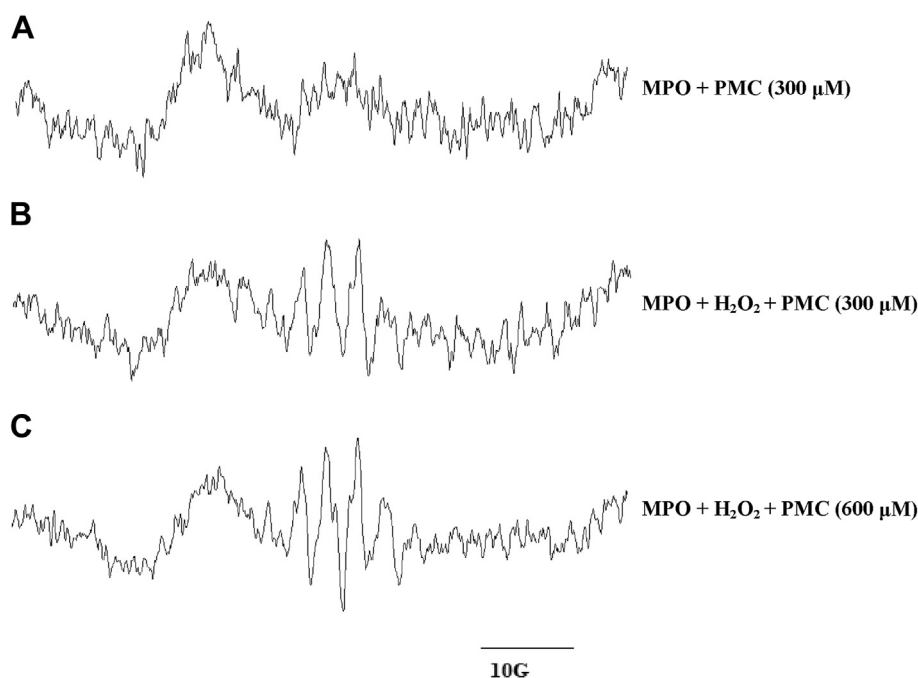
## 3. Results

### 3.1. ESR investigation of free radicals induced by PMC with myeloperoxidase in the presence of $\text{H}_2\text{O}_2$

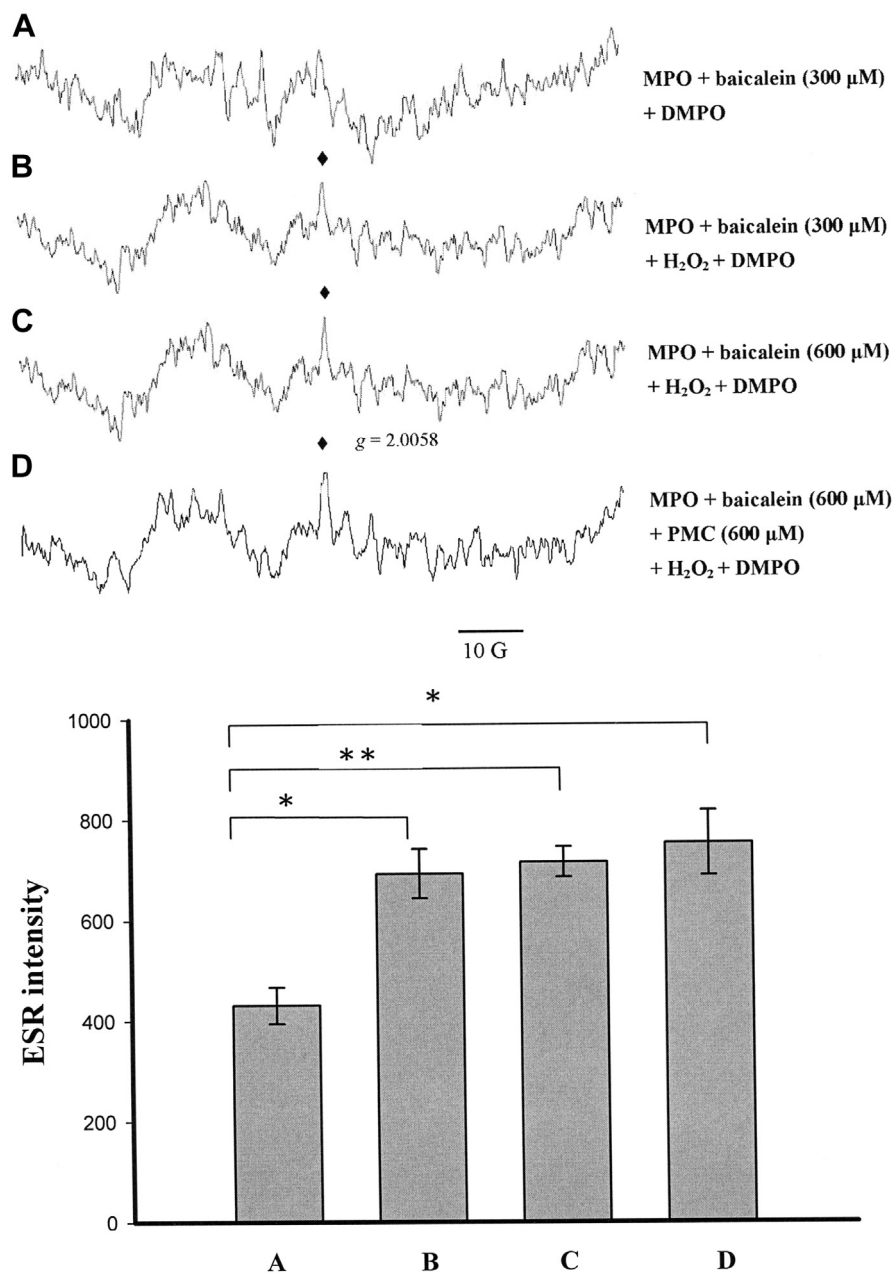
Experiments using MPO/ $\text{H}_2\text{O}_2$  were performed to test the effect of PMC on the formation of phenoxyl radicals. The reaction was conducted in 0.1 M of a sodium phosphate buffer containing 600  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 20  $\mu\text{M}$  MPO, at a pH of 7.4. A typical partially resolved ESR signal of PMC phenoxyl radicals [30] was detected (Fig. 2B and C). No EPR signals were observed for MPO incubated with PMC in the absence of  $\text{H}_2\text{O}_2$  (Fig. 2A) or for PMC incubated with  $\text{H}_2\text{O}_2$  but without MPO (data not shown).

### 3.2. ESR investigation of free radicals induced by baicalein and myeloperoxidase

Baicalein was converted by MPO to produce a  $g = 2.0058$  radical (Fig. 3B and C), whereas the formation of this radical was decreased in the controls without  $\text{H}_2\text{O}_2$  (Fig. 3A). Baicalein (600  $\mu\text{M}$ ) inhibited PMC-phenoxyl radical formation completely (Fig. 3D). The intensity of the ESR signal was not



**Fig. 2** – Electron spin resonance spectra of free radicals produced during myeloperoxidase (MPO)-induced oxidation of pentamethyl-hydroxychromane (PMC) in the presence or absence of  $\text{H}_2\text{O}_2$ . (A) MPO (20  $\mu\text{M}$ ) was incubated with 300  $\mu\text{M}$  PMC in the absence of  $\text{H}_2\text{O}_2$ . (B) Oxidation was initiated for the reaction conditions in (A) by the addition of 600  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . (C) MPO (20  $\mu\text{M}$ ) was incubated with 600  $\mu\text{M}$  PMC and 600  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . All experiments were conducted in a 0.1 M phosphate buffer at pH 7.4, and spectra were recorded after a 3-minute incubation period after the addition of the final reactant (A, PMC; B and C,  $\text{H}_2\text{O}_2$ ).



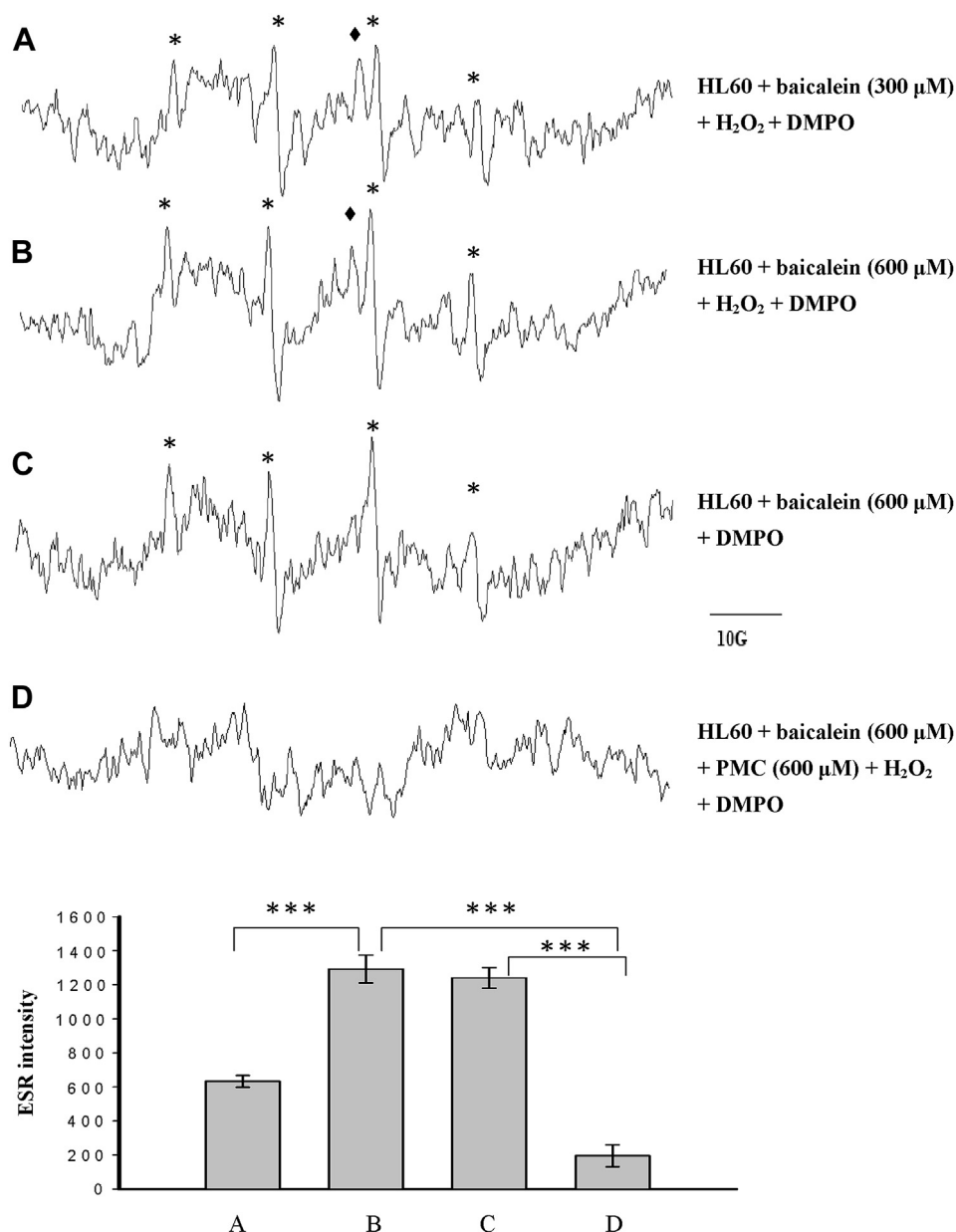
**Fig. 3** – Electron spin resonance spectra obtained from the reaction of myeloperoxidase (MPO) with baicalein and pentamethyl-hydroxychromane (PMC) in the presence or absence of  $\text{H}_2\text{O}_2$ . In all groups, DMPO (5,5-dimethyl-1-pyrroline N-oxide; 100 mM) was added. (A) MPO (20  $\mu\text{M}$ ) in phosphate-buffered saline (PBS) was incubated with 300  $\mu\text{M}$  baicalein in the absence of  $\text{H}_2\text{O}_2$ . (B) MPO (20  $\mu\text{M}$ ) in PBS was incubated with 300  $\mu\text{M}$  baicalein. (C) MPO (20  $\mu\text{M}$ ) in PBS was incubated with 600  $\mu\text{M}$  baicalein. (D) MPO (20  $\mu\text{M}$ ) in PBS was incubated with 600  $\mu\text{M}$  baicalein and 600  $\mu\text{M}$  PMC, with PMC and baicalein added simultaneously. The reactions in (B–D) were initiated by the addition of 600  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The spectra are labeled to indicate the components: protein radical ( $\blacklozenge$ ). The data are shown as the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  compared with the control (A).

significantly altered for 600  $\mu\text{M}$  baicalein with or without PMC, relative to that of 300  $\mu\text{M}$  baicalein (Figs. 3B–D, 4A and B). Previous studies have shown that amino-glutethimide produced protein radicals with MPO in HL-60 cells [31]. Thus, we concluded that the  $g = 2.0058$  radical might be a protein radical formed by MPO interacting with baicalein.

### 3.3. Effect of PMC on phenoxyl radical formation in HL-60 cells

We investigated whether the endogenous MPO in HL-60 cells catalyzes the single-electron oxidation of PMC. The results showed that HL-60 cells/ $\text{H}_2\text{O}_2$  catalyzed PMC to produce a phenoxyl radical (Fig. 5A). Controls without  $\text{H}_2\text{O}_2$  or with ABAH (an





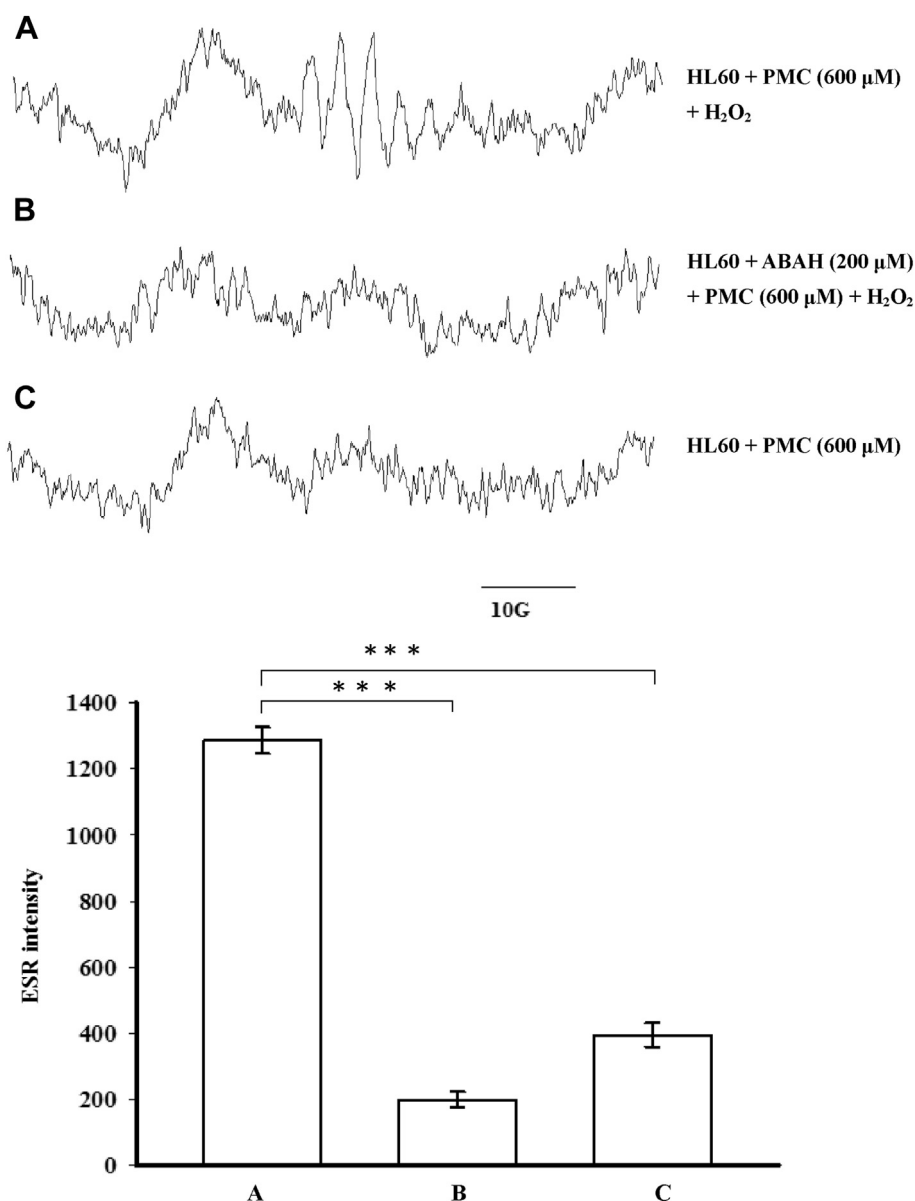
**Fig. 4** – Electron spin resonance spectra of free radicals generated by a suspension of human promyelocytic leukemia cells ( $1.5 \times 10^6$  in 150  $\mu$ L) that were (A) incubated with 300  $\mu$ M baicalein in the presence of 100 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO), with the reaction initiated by the addition of 600  $\mu$ M  $H_2O_2$ ; (B) incubated with 600  $\mu$ M baicalein, 100 mM DMPO and 600  $\mu$ M  $H_2O_2$ ; (C) incubated with 600  $\mu$ M baicalein, 100 mM DMPO in the absence of  $H_2O_2$ ; or (D) incubated with 600  $\mu$ M PMC, 100 mM DMPO, 600  $\mu$ M PMC, and 600  $\mu$ M  $H_2O_2$ . The spectra are labeled to indicate the components: protein radical (♦); DMPO-hydroxyl radical adduct (\*). The data are shown as the mean  $\pm$  SEM for the hydroxyl radical signals of four independent experiments. \*\*\* $p < 0.001$ .

MPO inhibitor) failed to form this radical adduct (Fig. 5B and C). These results suggest that the phenoxyl radical formation resulted from the MPO-mediated catalysis of  $H_2O_2$  in the HL-60 cells.

### 3.4. Free radicals induced by baicalein in the presence of DMPO in HL-60 cells

The addition of baicalein to the HL-60 cells in the presence of 600  $\mu$ M of  $H_2O_2$  and the spin trapper DMPO produced a four-line

ESR signal, which is typical for the hydroxyl radical ( $a_N = a_H = 14.8$  G), which was baicalein-dose-dependent (Fig. 4A, control; Fig. 4B). In controls without  $H_2O_2$ , the formation of a hydroxyl radical was not affected (Fig. 4C), suggesting that MPO was not involved in this reaction. Our previous studies showed that baicalein induced hydroxyl radical formation through 12-LOX in human platelets [23] and B16F10 cells [24]. Therefore, baicalein might induce hydroxyl radical formation through 12-LOX in HL-60 cells as well. However, baicalein-induced MPO-protein radical formation was also



**Fig. 5** – Electron spin resonance spectra of free radicals produced in a suspension of HL-60 cells ( $1.5 \times 10^6$  in 150  $\mu\text{L}$ ) that were (A) incubated with 600  $\mu\text{M}$  pentamethyl-hydroxychromane (PMC) in the presence of 600  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>; (B) pre-incubated in 200  $\mu\text{M}$  4-aminobenzoic acid (ABAH) for 5 minutes prior to the addition of PMC; or (C) incubated with 600  $\mu\text{M}$  PMC in the absence of H<sub>2</sub>O<sub>2</sub>. The data are shown as the mean  $\pm$  SEM of four independent experiments. \*\*\* $p < 0.001$  compared with the control.

detectable in HL-60 cells (Fig. 4A and B). The formation of this radical was decreased in controls without H<sub>2</sub>O<sub>2</sub> (Fig. 4C). Furthermore, PMC (600  $\mu\text{M}$ ) significantly reduced the baicalein-induced hydroxyl radical signals, suggesting that PMC acted as an antioxidant under this condition (Fig. 4D).

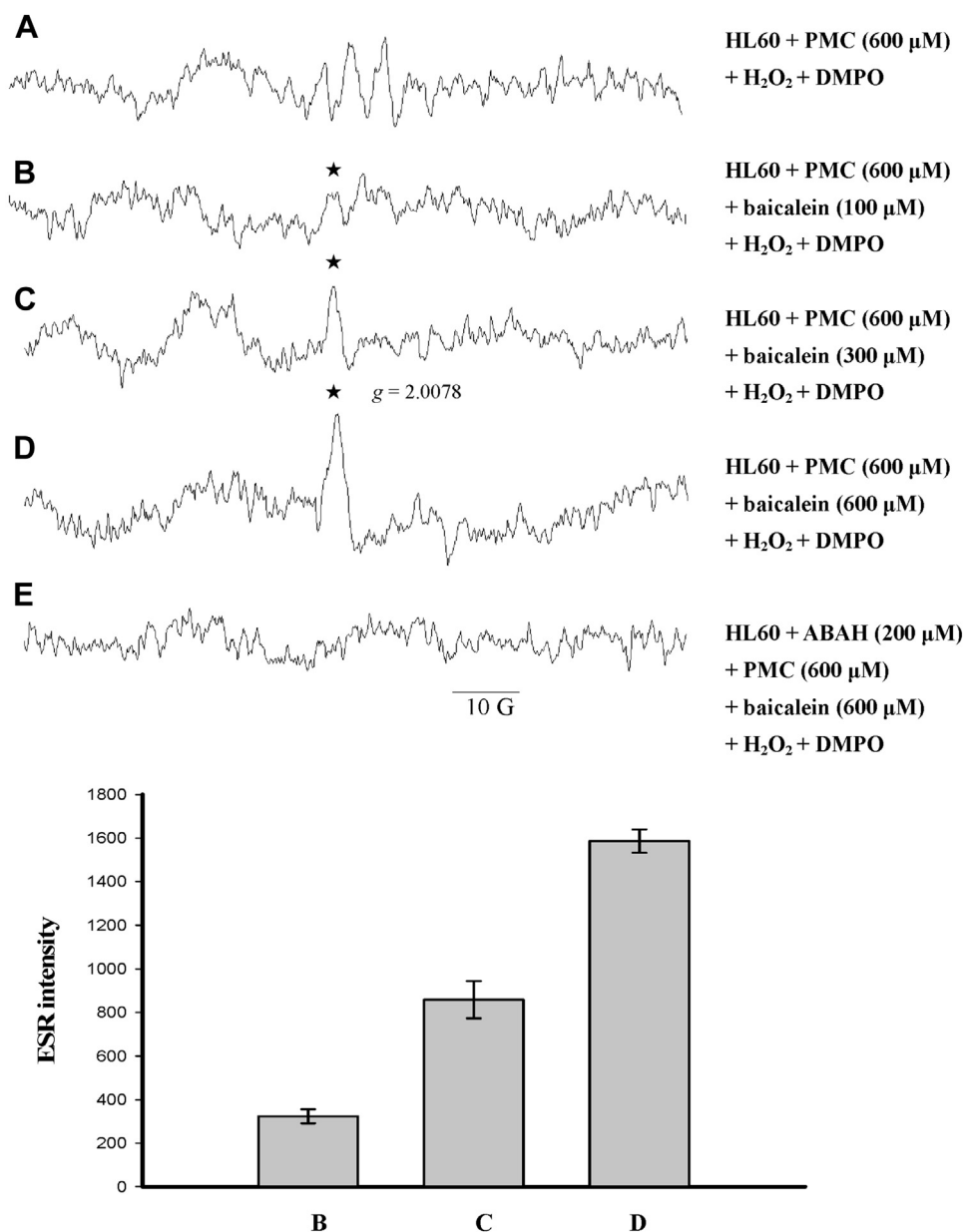
### 3.5. Effect of baicalein on PMC-phenoxyl radical formation in HL-60 cells

We investigated whether baicalein inhibits the MPO-mediated single-electron oxidation of PMC in HL-60 cells in the presence of 600  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>. The results showed that baicalein produced a radical with a  $g$  value of 2.0078, and completely inhibited PMC-

induced phenoxyl radical formation in the presence of H<sub>2</sub>O<sub>2</sub> in HL-60 cells (Fig. 6C and D). The radical produced from baicalein under these conditions differed from the radical ( $g = 2.0058$ ) for which formation was inhibited by ABAH, indicating that endogenous MPO catalyzed the reaction (Fig. 6E). The intensity of the  $g = 2.0078$  radical signal was baicalein-dose-dependent, suggesting that it was a baicalein-phenoxyl radical.

### 3.6. Effect of baicalein on AA-induced free radical formation in HL-60 cells

Previous studies have shown that AA-induced free radical generation might occur through an oxidative metabolic



**Fig. 6** – Electron spin resonance spectra of free radicals generated by a suspension of human promyelocytic leukemia (HL-60) cells ( $1.5 \times 10^6$  in 150  $\mu$ L) that were (A) incubated with 600  $\mu$ M pentamethyl-hydroxychromane (PMC), 100 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO), and 600  $\mu$ M H<sub>2</sub>O<sub>2</sub>. After 1 minute, (B) 100  $\mu$ M, (C) 300  $\mu$ M, and (D) 600  $\mu$ M baicalein were added. (E) The HL-60 cells were treated identically to sample (D), except that they were pre-incubated for 5 minutes in 200  $\mu$ M 4-aminobenzoic acid (ABAH) before PMC was added. The spectra are labeled to indicate the components: phenoxyl radical (★). The data are shown as the mean  $\pm$  SEM of protein radical signals for four independent experiments.

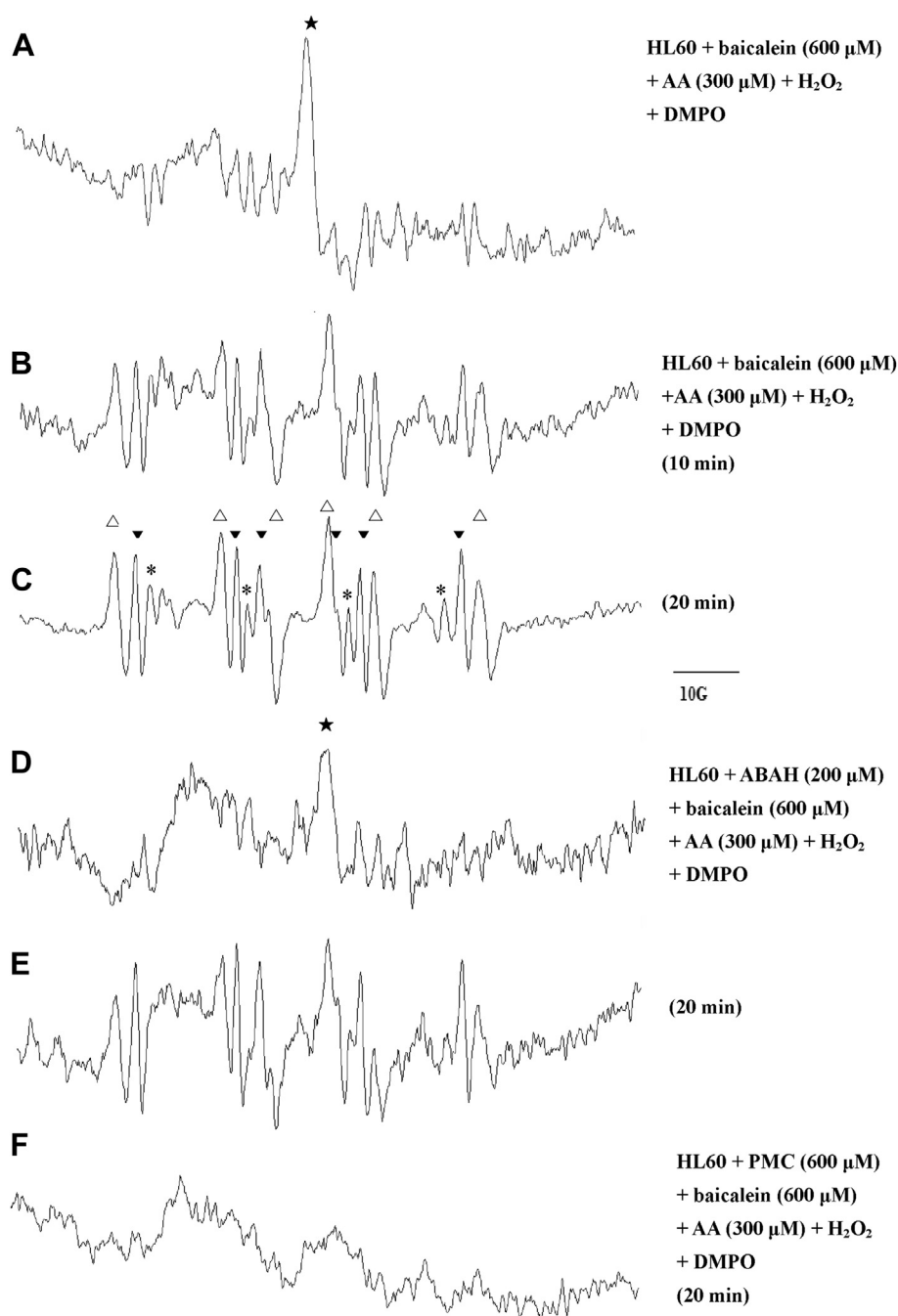
process involving cyclooxygenase (COX), lipoxygenase (LOX) [32], and NADPH oxidase [33,34]. We investigated whether baicalein or PMC inhibits AA-induced free radical formation in HL-60 cells. Our ESR analysis showed that the incubation of HL-60 cells in 300  $\mu$ M of AA, 600  $\mu$ M of H<sub>2</sub>O<sub>2</sub>, and 600  $\mu$ M baicalein produced a narrow signal with a  $g$  value of 2.0078 that corresponded to that of the baicalein-phenoxyl radical. After 20 minutes, the following three signals were detected (Fig. 7C): a hydroxyl radical ( $a_N = a_H = 14.8$  G), a carbon-centered radical ( $a_N = 16.0$  G,  $a_H = 22.8$  G), and an acyl radical ( $a_N = 14.9$  G,  $a_H = 18.3$  G). These radicals persisted in the presence of ABAH,

suggesting that endogenous MPO in the HL-60 cells was not involved in this reaction (Fig. 7E). By contrast, the formation of these radicals was almost completely inhibited by pre-incubation with 600  $\mu$ M PMC (Fig. 7F), indicating that PMC acted as an antioxidant in that reaction.

### 3.7. Free radicals induced by AA and baicalein with COX-2 in the presence of DMPO

We hypothesized that baicalein and AA would produce a hydroxyl radical, a carbon-centered radical, and an acyl radical

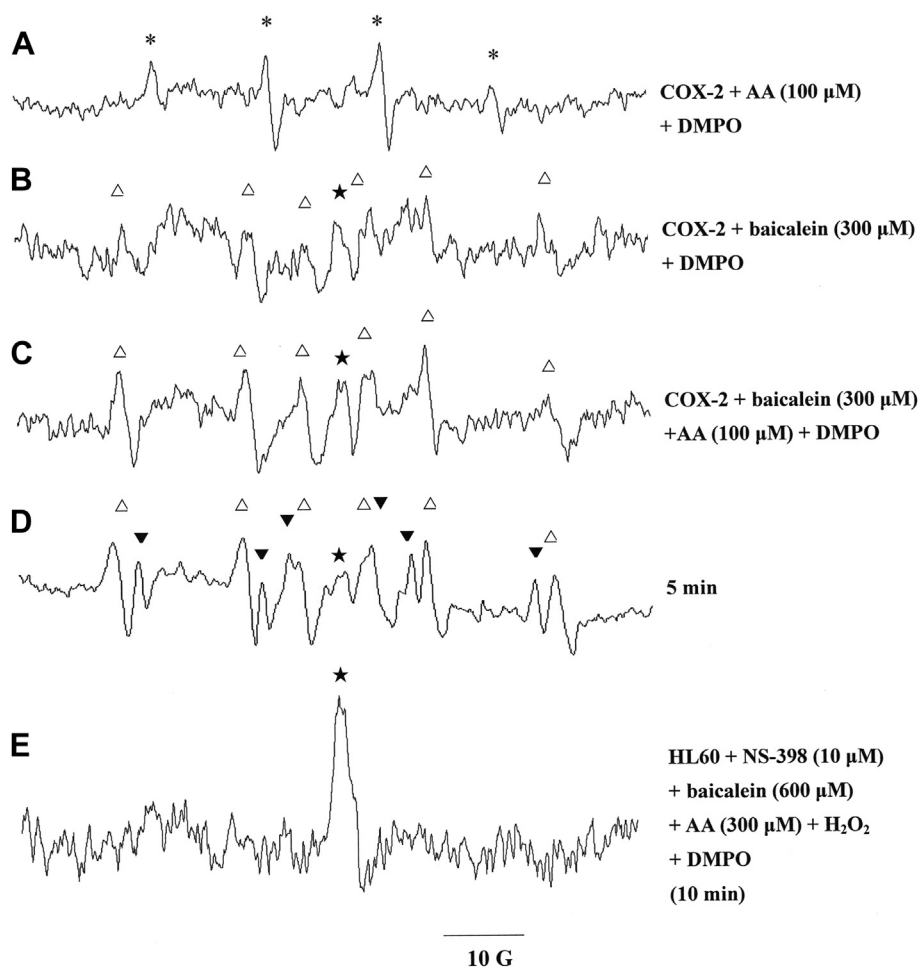




**Fig. 7** – Electron spin resonance spectra of free radicals generated by a suspension of human promyelocytic leukemia cells ( $1.5 \times 10^6$  in  $150 \mu\text{L}$ ). To each group we added  $100 \text{ mM}$  5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and  $600 \mu\text{M}$   $\text{H}_2\text{O}_2$ . The cells were incubated with acid (AA) to trigger free radical formation for (A) 3 minutes, (B) 10 minutes, and (C) 20 minutes. Using these reaction conditions, a (D) 10-minute reaction and (E) a 20-minute reaction were performed in which the cells were pre-incubated in  $200 \mu\text{M}$  4-aminobenzoic acid (ABAH) for 5 minutes prior to the addition of baicalein, and (F) a 20-minute reaction was performed in which the cells were pre-incubated in  $600 \mu\text{M}$  PMC for 5 minutes prior to the addition of baicalein. The spectra are labeled to indicate the components: DMPO-hydroxyl radical adduct (★), DMPO-carbon-centered adduct ( $\Delta$ ), and DMPO-acyl radical adduct ( $\blacktriangledown$ ).

in HL-60 cells, mainly through COX. We tested this hypothesis using a COX-2 system. ESR analysis showed that treatment with  $100 \mu\text{M}$  AA produced a hydroxyl radical signal ( $a_N = a_H = 14.8 \text{ G}$ ) in cells suspended in the  $0.1 \text{ M}$  Tris-HCl buffer, pH 8.3, containing  $500 \mu\text{M}$  phenol,  $100 \mu\text{M}$  NADH,  $1 \mu\text{M}$

hematin, and  $10 \text{ U}$  of COX-2 (Fig. 8A). By contrast, baicalein produced a carbon-centered radical signal ( $a_N = 16.0 \text{ G}$ ,  $a_H = 22.8 \text{ G}$ ) and another radical signal with a  $g$  value of 2.0078 (Fig. 8B) under the same conditions (COX-2 system). The simultaneous addition of  $300 \mu\text{M}$  baicalein and  $100 \mu\text{M}$  AA to



**Fig. 8** – Electron spin resonance spectra detected from the reaction of cyclooxygenase-2 (COX-2) with (A) 100  $\mu\text{M}$  arachidonic acid (AA), (B) 300  $\mu\text{M}$  baicalein, (C) 100  $\mu\text{M}$  AA and 300  $\mu\text{M}$  baicalein, and (D) 1 mM AA and 1 mM baicalein. (E) Electron spin resonance spectra of free radicals generated by a suspension of human promyelocytic leukemia cells ( $1.5 \times 10^6$  in 150  $\mu\text{L}$ ) that were incubated with 10  $\mu\text{M}$  NS-398, 600  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 600  $\mu\text{M}$  baicalein, and 300  $\mu\text{M}$  AA to trigger free radical formation for 10 minutes. The 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 100 mM) was added to all the reactions. The spectra are labeled to indicate the components: phenoxyl radical ( $\star$ ), DMPO-carbon-centered adduct ( $\triangle$ ), and DMPO-acyl radical adduct ( $\blacktriangledown$ ).

HL-60 cells in the COX-2 system produced a distinct carbon-centered radical signal and another radical signal with a  $g$  value of 2.0078 (Fig. 8C). An additional acyl radical signal ( $a_N = 14.9$  G,  $a_H = 18.3$  G) appeared, and the intensity of the original radical signal ( $g = 2.0078$ ) decreased, following an additional 5 minutes of incubation (Fig. 8D). We tested the effect of a selective COX-2 inhibitor, NS-398, on the production of radicals in the presence of baicalein/AA in HL-60 cells. Our ESR analysis showed that the incubation of HL-60 cells in 10  $\mu\text{M}$  of NS-398, 300  $\mu\text{M}$  of AA, 600  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ , and 600  $\mu\text{M}$  baicalein only produced a narrow signal with a  $g$  value of 2.0078 that corresponded to that of the baicalein-phenoxyl radical (Fig. 8E).

#### 4. Discussion

We analyzed the free radical formation induced by PMC and baicalein in HL-60 cells to investigate the pro-oxidant and

antioxidant properties of these substances. The results showed that baicalein produced five radical species in HL-60 cells, whereas PMC produced a phenoxyl radical only, and both baicalein and PMC mutually inhibited radical formation induced by the other. A radical signal with a  $g$  value of 2.0058 was recorded for the reaction of  $\text{H}_2\text{O}_2$ , MPO, and baicalein (Figs. 3 and 4), but its intensity was independent of the concentration of baicalein. We thus interpreted it to represent the formation of an MPO-protein radical. By contrast, a radical signal with a  $g$  value of 2.0078 that was also recorded was found to be baicalein dose-dependent, and did not require the presence of MPO and  $\text{H}_2\text{O}_2$  (Fig. 7). We thus assigned it as a baicalein-phenoxyl radical.

Shen et al [35] showed that baicalein inhibited MPO activity in human leukocytes. When baicalein acts as a free radical scavenger, it produces baicalein-derived radicals, such as phenoxyl, carbon-centered, and acyl radicals. However, our ESR results showed that baicalein completely blocked the production of PMC-derived phenoxyl radicals by MPO *in vitro*

and in HL-60 cells. Under both sets of conditions, an MPO-protein radical was produced (Fig. 9, Equation 5). This result provided further evidence that the antioxidative effects of baicalein on PMC-induced lipid peroxidation may be the result of MPO inhibition.

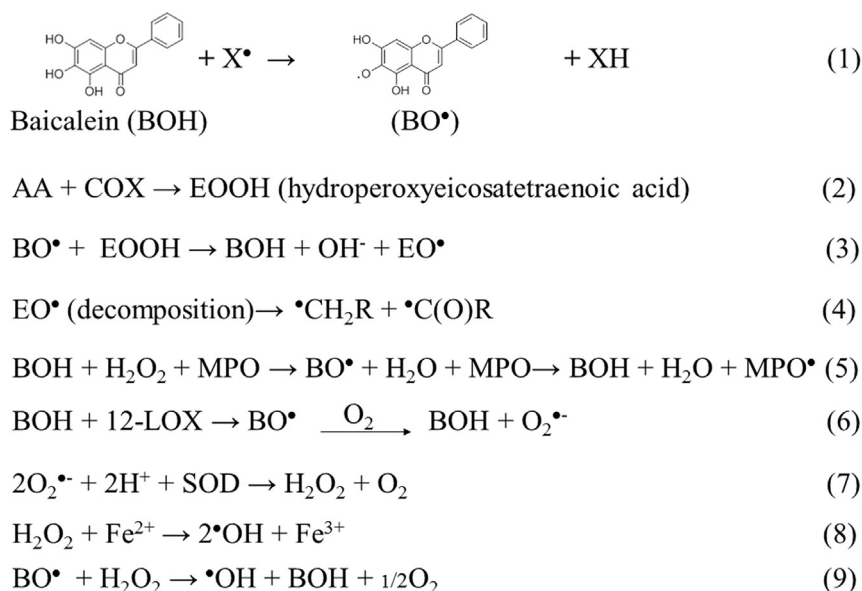
Numerous lipoxygenase (LOX) isoforms exist, including 12-LOX in platelets and leukocytes, 5-LOX in neutrophils, and 1-LOX in soybeans. The LOX inhibitors, phenidone and nordihydroguaiaretic acid, are oxidized to free-radical metabolites during the reduction of the catalytically active ferric LOX to its inactive ferrous form [36]. Baicalein is known to block the activity of 12-LOX that is often overexpressed in tumor tissues, and we showed in a previous study that baicalein produced superoxide anions in B16 F10 cells via a 12-LOX pathway [24]. We suggested that superoxide anions produced by baicalein were promptly converted to hydroxyl radicals through superoxide dismutase and the Fenton reaction in B16 F10 melanoma cells [24]. Similarly, the results of our current study showed that baicalein induced hydroxyl radical formation in HL-60 cells. However, baicalein did not produce hydroxyl radicals through MPO (Fig. 3) or COX-2 (Fig. 8). Therefore, we concluded that baicalein produces hydroxyl radicals through a 12-LOX-mediated pathway in HL-60 cells.

The radical-scavenging activity of flavonoids depends on the availability of phenolic hydrogens and on the stabilization of the resulting phenoxyl radicals [37,38]. Adhikari et al [39] reported that baicalein acted as an effective scavenger of hydroxyl, azidyl, and alkylchloroperoxyl radicals, and produced a baicalein-derived phenoxyl radical. Our study confirmed that baicalein exerted a direct antioxidant effect. Based on theoretical considerations, we expected that baicalein-derived phenoxyl radicals would be the initial intermediates resulting from the interaction between a radical and baicalein (Fig. 9, Equation 1). However, it is difficult to detect phenoxyl radicals *in situ* at room temperature in aqueous solutions [40]. The transient baicalein-derived phenoxyl radicals can be

oxidized by oxygen to produce superoxide radicals (Fig. 9, Equation 6). Hydroxyl radicals may be generated through the reductive homolytic cleavage of  $\text{H}_2\text{O}_2$  at the expense of baicalein-derived phenoxyl radicals [41] (Fig. 9, Equation 9) or by reacting with ferrous ions in the Fenton reaction [24] (Fig. 9, Equation 8).

Buettner [42] reported that an interaction between an antioxidant and lipid radicals leads to various secondary radical products, all of which possess pro-oxidant abilities. We observed that the interaction between baicalein and AA in HL-60 cells led to the production of hydroxyl, carbon-centered, and acyl radicals. Substantial amounts of these products were produced within minutes, indicating that they were not primary radicals. Based on the results of the COX-2 experiments, we concluded that phenoxyl radicals were the primary radicals, and that the carbon-centered and acyl radicals were secondary radicals. Nohl et al [41] previously showed that ubisemiquinone reacted with linoleic acid hydroperoxide to produce carbon-centered and acyl radicals. Similarly, COX can convert AA to hydroperoxide [43] (Fig. 9, Eq. 2). The baicalein-derived phenoxyl radical interacts with AA hydroperoxide to produce carbon-centered and acyl radicals (Fig. 9, Equations 3 and 4). The possible pathways, with or without AA, of baicalein-induced radical formation in HL-60 cells are shown in Figure 9.

An antioxidant can act as a pro-oxidant under certain conditions [29]. In this study, we demonstrated that PMC acted as a pro-oxidant in cells in the absence of other free radicals (Fig. 5A), but acted as an antioxidant under conditions of high oxidative stress (Figs. 4D and 7F). Similarly, baicalein produced hydroxyl radicals in HL-60 cells in the resting state (Fig. 4), and inhibited PMC-derived phenoxyl radical and AA-derived hydroxyl radical formation (Fig. 8). The reaction components were the same as those for the reactions described in Figs. 4D and 6A, but the results in each condition differed according to the degree of oxidative stress.



**Fig. 9** – Equations describing the possible reaction pathways for the formation of the five radical species that were induced by baicalein.

We propose that the sequence in which the reagents were added determined the role of the reagent regarding the redox reaction, with the reagents added first acting as pro-oxidants, and those added later acting as antioxidants.

In conclusion, we showed that baicalein displayed both pro-oxidant and antioxidant activities in HL-60 cells. PMC exhibited no pro-oxidant activity during the cells' resting state but produced the PMC-phenoxy radical in the presence of H<sub>2</sub>O<sub>2</sub>. The reaction of baicalein with AA produced baicalein-derived phenoxy radicals that may initiate various pro-oxidative reactions. However, PMC does not produce radicals when it acts as an antioxidant. Thus, PMC is more beneficial as an antioxidant than baicalein.

## Conflicts of interest

All authors declare no conflicts of interest.

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