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Pedalitin from *Isodon japonica*, an inactivation of soybean lipoxygenase-1

[Pedalitina de *Isodon japonica*, un inactivador de la lipo oxigenasa-1 de poroto de soya]

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Abstract: Pedalitin, isolated from the aerial part of *Rabdosia japonica* (Labiatae), inhibited soybean lipoxygenase-1 (EC 1.13.11.12, Type I) with an IC₅₀ of 152.5 µM. The progress curves for an enzyme reaction, pedalitin inactivate the lipoxygenase-1 in a time dependent, irreversible manner, exhibiting kinetics with a kinact/KI of 59.6 ± 10 mM⁻¹min⁻¹. In the pseudoperoxidase activity, pedalitin is very slowly oxidized by the soybean lipoxygenase-1 catalyzed decomposition of lipid hydroperoxides.

Keywords: *Rabdosia japonica*, Pedalitin, Lipoxygenases inhibitor.

Resumen: Pedalitina, aislada de las partes aereas de *Rabdosia japonica* (Labiatae), inhibió a la lipooxigenasa-1 (EC 1.13.11.12 tipo I) con un IC₅₀ de 152.5 uM. La curva de progreso para una acción enzimática, pedalitina inactivó a la lipooxigenasa-1 de una manera dependiente del tiempo, de una manera irreversible, exhibiendo una cinética con una kinact/KI de 59.6 ± 10 mM⁻¹min⁻¹. En la actividad pseudoperoxidasa, pedalitina es oxidada lentamente por la descomposición de la lípido hidroperóxido de la lipooxigenasa-1 de poroto de soya

Palabras clave: *Rabdosia japonica*, Pedalitina, inhibidor de Lipooxigenasas.

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INTRODUCTION

Lipoxygenase (EC 1.13.11.12) is a non-heme ferric iron-containing enzyme, suggested to be involved in the early event of atherosclerosis by inducing plasma low-density lipoprotein (LDL) oxidation (Cornicelli & Trivedi, 1999; Kris-Etherton & Keen, 2002). Lipoxygenases are a class of enzymes that are widely distributed in both animals and plants. They catalyze the dioxygenation of unsaturated fatty acids containing at least one 1,4-cis, cis-pentadiene unit (Zheng & Brash 2010). An example of this is the peroxidation of linoleic acid by soybean lipoxygenase, to produce 13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD) (Scheller et al., 1995). The lipoxygenase products are mediate a number of biological processes not only to human implicated in an inflammatory diseases (Samuelsson et al., 1987; Sigal, 1991) and cancer growth regulation (Rioux, 1998; Nie et al., 1998) but also in plant involved in immunology, growth regulating, and germination (Suzuki & Matsukura, 1997; Kolomiets et al., 2001). Hence, lipoxygenase inhibitors should have broad applications (Richard-Forget et al., 1995).

Members of the genus Rabdosia grow on lands of eastern Asia, but only in China and Japan have been investigated chemically. In Japan, the leaves are used as a common household medicine for gastrointestinal disorders. In China are used as antitumor and antiphlogistic agents. The genus of Rabdosia (formerly known as Isodon) belonging to Labiatae (Hara, 1985) is rich source of diterpenoids, especially highly oxygenated ent-kaurenoids (Fujita & Node, 1984; Takeda & Otsuka, 1995). For example, a number of diterpenoids, glaucocalyxin A, B, C, D, and E, rabdosin A, and B, rabdophyllin G, acetylexidonin, and rabdosinate, were isolated from the aerial parts of R. japonica (Chen et al., 1989; Wang et al., 1990; Kim et al., 1992). Previous studies on R. japonica led to the isolation of more than 16 diterpenoids (Li & Tian, 2001). This study report about minor diterpenoid constituents of this species was aimed at finding substances with even higher biological activities. Li and Tian, 2001, have isolated two new enmein type diterpenoids, taibaijaponicains A and B, together with six known diterpenoids, norhendosin A, rabdophyllin G, dihydroenmein, nodosin, enmein, rabdosin A, and two other known compounds, β-sitosterol and oleanolic acid (Li & Tian, 2001).

In addition to the diterpenoids, together with monoterpane phenolic acids (Gawlik-Dziki et al., 2014), various phenolic compounds, quercetin (1), rutin (2), pedalitin (3), ursolic acid (4), and rosmarinic acid (5) were also characterized from the same source. In the previous reports, the study was emphasized on diterpenoids, and phenolic compounds were generally overlooked, especially their biological activity (Yoshimoto et al., 1983; Abdel-Mageed et al., 2014; Gawlik-Dziki et al., 2014). It should be noted, however, the phenolic compounds isolated from R. japonica can be expected to possess various biological activities. For example, quercetin is known as a highly efficient hydroxyl radical scavenger, and inhibits the development of stomach, colon, and oral cancer. Therefore, the aim of this study was to reexamine various R. japonica biological activities which were not previously tested. Flavonoids demonstrate various pharmacological activities in vitro and in vivo including anticancer, anti-inflammatory, and antiallergic activities.

Our effort is focuses in the general effect of several compound obtained from plants in experimental models of cell growth, we are working in dissect the mechanism of lipoxygenase inhibition in which this effect is associated to mammalian lipoxygenase and tumor models. Additionally, we are looking for to define whether this model is comparative or have a similar mechanism for several lipoxygenases.

MATERIALS AND METHODS

Chemicals

Pedalitin, quercetin, rutin and ursolic acid were available as well as from extraction of Isodon japonica as from our previous work on Heterotheca inuloides (Kubo et al., 1994). Rosmarinic acid was purchased from Cayman Chemical Co. (Ann Arbor, MI). Nordihydroguaiaretic acid (NDGA) and trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethyl sulfoxide (DMSO), soybean lipoxygenase-1 (EC 1.13.11.12, Type I) and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Tris was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL). 13-Hydroperoxyoctadecadienoic acid (13-HPOD: \( \lambda_{\text{max}} = 234 \text{ nm, } \epsilon = 25 \text{ mM}^{-1} \text{cm}^{-1} \)) was prepared enzymatically by described procedure (Gibian & Galaway, 1976) and stored in ethanol at -18°C.
Inhibition Experiments on lipoxygenase-1

These experiments were performed by measurement of the initial rate of soybean lipoxygenase-1 with a Clark type oxygen electrode (YSI 53, Yellow Springs Instrument Co., Yellow Springs, OH) at 25ºC. The reaction mixture (3.0 mL) contained 80 µM linoleic acid, inhibitor and 0.1 M Tris-HCl (pH 8.0). After 2 min pre-incubation, the reaction was started by addition of lipoxygenase-1 (4.35 nM). In spectrophotometric experiment, the oxygenase activity of the soybean lipoxygenase was monitored at 234 nm by Spectra MAX plus spectrophotometer (Molecular device, Sunnyvale, CA) under conditions similar to those used to follow O2 uptake except for the concentrations of linoleic acid and lipoxygenase. A plot of the natural log of the residual enzyme activity versus pre-incubation time gave a straight line with a slope of -kobs. The values of KI and kinact were calculated from the double reciprocal plot of the kobs versus concentration of inhibitors according to the method of Kitz-Wilson (Kitz & Wilson, 1962; Parsons & Gates, 2013).

Pre-incubation Experiments

Most experiments were performed that lipoxygenase-1 (4.35 nM) and 0.5 to 10 µM for pedalitin were incubated with 0.1 M Tris-HCl (pH = 8.0) in the oxicgraphic measuring chamber at 25ºC. At timed intervals, reactions were started by addition of 80 µM linoleic acid. Control samples were incubated under identical conditions except for the absence of inhibitor. The oxygen consumption was monitored for the first 5 min of the reaction immediately.

Measurement of Pseudoperoxidase Activity

The pseudoperoxidase activity was determined from the inhibitor dependent consumption of 13-HPOD catalyzed by the soybean lipoxygenase-1 using the variation in A234 as previously described (Falgueryet et al., 1993). The assay mixture contained 15 µM 13-HPOD, various concentrations of inhibitors, and soybean lipoxygenase-1 (320 nM) in 0.1 M Tris-HCl, pH = 8.0.

HPLC Analysis

HPLC analysis was performed on LPG-1000 with an UV-7000 detector (Tokyo Rikakikai, Tokyo, Japan) and a 4.6mm × 250 mm i.d., 5 µm, Capcell Pack C-18 column (Shiseido, Tokyo, Japan) as previously reported with some modifications (Ha et al., 2012). The operating conditions were as follows: solvent; 30% MeCN/H2O containing 0.1% TFA, flow rate; 0.5 mL/min, detection; UV at 254nm, injected amount; 20 µL from 3 mL assay system containing 100 µM pedalitin (1) at the same conditions of pseudoperoxidase measurement. Sampling time was chosen at 0, 30, 60, 90, and 120 min, respectively.

Data Analysis and Curve Fitting

The assay were conducted in triplicate of separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc, Chicago, IL). The inhibitory concentration leading to 50% activity loss (IC50) was obtained by fitting experimental data to the logistic curve by the equation as follows (Copeland, 2000):

$$\text{Activity} \% = 100\left[1/(1-([I]/IC_{50}))\right]$$

The time course of inactivation of soybean lipoxygenase-1 by pedalitin was analyzed by fitting the residual activity (v/v0) at a given pre-incubation time (t) to eq 1, where kobs is the observed rate of inactivation. The rate of inactivation of the enzyme were fit to eq 2, where k_inact is the first-order rate constant for the conversion of the reversibly formed enzyme-inhibitor complex to irreversibly inactivated enzyme, K1 is the dissociation constant for the complex, and [I] is the concentration of pedalitin.

$$v/v_0 = \exp(-k_{obs}t) \quad (1)$$

$$1/k_{obs} = [(K_1/k_{inact})(1/[I])] + 1/k_{inact} \quad (2)$$

In the time-dependent inhibition of soybean lipoxygenase-1 by different concentrations of quercetin were fitted by nonlinear regression analysis to the integrated rate eq 3 for slow-binding inhibitors as described previously (Copeland, 2000).

$$P = v_i t + (v_i-v_s)\left(1-\exp(-k_{obs}t)\right)/k_{obs} \quad (3)$$

$$k_{obs} = k_d[I]/K_{app} + k_4 \quad (4)$$

Where P is the oxygen consumption concentrations at time t, v_i and v_s are the initial and final steady-state velocity, respectively, k_obs is the apparent first-order rate constant.
RESULTS AND DISCUSSION

Inhibition activity of soybean lipoxygenase-1

In previous work we isolated flavonoids and terpenoids from aerial parts of *R. japonica* and tested their antioxidant properties and inhibitory melanogenesis in tumor cells (Masuoka *et al.*, 2006; Satooka *et al.*, 2012).

In 2006, Sadik *et al.*, reported the inhibition of mammalian lipoxygenases by flavonoids; in our preliminary assay, the methanol extract of the aerial parts of *R. japonica* was found to inhibit the oxidation of linoleic acid by soybean lipoxygenase (EC 1.13.11.12, Type I) at 800 μg/mL; when we tested the major diterpenoids, represented by ent-kaurenes isolated from the same source, did not show any inhibitory activity. However the flavonoids quercetin (1), rutin (2) and pedalitin (3) were actives. In addition, an ursane type triterpene, ursolic acid (4) isolated from the same plant in quantity and rosmarinic acid (5) (Figure 1), was also assayed since it was previously reported to inhibit 15-lipoxygenase (Najid *et al.*, 1992). Additionally, cirsiliol together pedalitin obtained synthetically showed a high selectivity inhibition activity of arachidonate 5-lipoxygenase a mammalian lipoxygenase (Yoshimoto *et al.*, 1983), also a ethanolic extract of *Simmondsia chinensis* (Jojoba) leaves showed similar effect (Abdel-Mageed *et al.*, 2014), and in similar form green coffee beans rich in 5-caffeoylquinic, 4-caffeoylquinic, 3-feruoylquinic and 5-feruoylquinic acids were potent inhibitors of lipoxygenase (Gawlik-Dziki *et al.*, 2014).

The inhibition activity of soybean lipoxygenase-1 was measured by two methods for comparison, because this enzyme seems to be sensitive to assay conditions. Soybean lipoxygenase-1 is known to catalyze the reaction of oxygen with unsaturated fatty acids containing 1,4-cis,cis-diene units to yield 1,3-cis-trans-diene-5-hydroperoxides. Hence, the enzyme assay was usually performed using a Clark type oxygen electrode at 25°C. And also this enzyme is catalyze the dioxygenation of (Z, Z)-diene moiety of linoleic acid. In plants, the primary dioxygenation product is 13S linoleic acid hydroperoxide (13-HPOD) (Grechkin, 1998). Hence, UV spectrophotometer to detect the increase at 234 nm associated with the (Z, E)-conjugated double bonds newly formed in the product but not the substrate. As a result, in the inhibition of soybean lipoxygenase-1 by pedalitin (3), the formation of conjugated diene was recorded spectrophotometrically at 234 nm, and oxygraphic measurement of oxygen consumption yielded similar curves as shown in Figure 2. This inhibition pattern suggests that pedalitin (3) inactivates soybean lipoxygenase-1 by a slow binding mechanism and irreversibly. The effect of pedalitin on the rate of enzyme inactivation can be readily observed at low concentrations (e.g. 10 μM) where a 78% decrease in product accumulation. At higher pedalitin concentrations, the rate of the reaction was also inhibited, together with the increase in enzyme inactivation (Figure 2A).

![Chemical structures of compounds 1-5 isolated from R. japonica.](image-url)
Pedalitin showed a dose-dependent oxygen consumption inhibitory effect on this oxidation as shown in Figure 3. As pedalitin increase, oxygen consumption was rapidly decreased. The inhibitory concentration leading to 50% activity lost (IC₅₀) was estimated to be 152.5 µM. And also the IC₅₀ value of quercetin, rutin, ursolic acid, rosmarinic acid, and NDGA obtained are listed in Table 1. The enzyme pre-incubation for 5 minutes with pedalitin, resulted in about a 40-fold decrease in IC₅₀ (3.7 µM).

Possible mechanism of lipoxygenase inhibition by pedalitin

Subsequently, pedalitin inhibited soybean lipoxygenase-1 in a time-dependent inactivation fashion as shown in Figure 4A to suggest that this inhibit the enzyme in an irreversible manner, the kinetic parameters for the irreversible inhibitions, $K_i$ and $k_{inact}$ were estimated from the respective double reciprocal plot $1/k_{\text{obs}}$ as a function of $1/[$pedalitin$]$ according to the method of Kitz and Wilson (Kitz & Wilson, 1962; Parsons & Gates, 2013) for kinetic analysis of covalent enzyme inactivation, as exemplified by Figure 4B, and are listed in Table 2. These values were found to be equal to 5.37 µM and 0.32 min⁻¹, respectively.

Figure 2

Progress curves for the inactivation of soybean lipoxygenase-1 by pedalitin by using a spectrophotometer (A) and a Clark-type oxygen electrode (B). A: 40 µM linoleic acid, 1.3 nM SLO-1 and concentrations of pedalitin for curves 0-8 were 0, 5, 10, 15, 20, 25, 30, 35, and 40 µM. B: 80 µM linoleic acid, 6.54 nM SLO-1 and concentrations of pedalitin for curves 0-6 were 0, 5, 10, 20, 40, 60, and 80 µM.

Figure 3

Effects of pedalitin on the activity of soybean lipoxygenase-1 for the catalysis of linoleic acid at 25º C. A: pedalitin was mixed with 80 µM linoleic acid in 0.1m tris-hcl buffer (ph8.0) at 25º C and the reactions were started by addition of soybean lipoxygenase-1 (6.54 nM). B: pedalitin was pre-incubated for 5 min with enzyme and the reactions were started by addition of 80 µM linoleic acid at same conditions.
On the other hand, progress curves of quercetin (1) display in Figure 5, show soybean lipoygenase-1 inhibition by reversible slow-binding mechanism. The $k_{obs}$ values for the quercetin inhibition of lipoygenase-1 at different concentrations of quercetin were determined by fitting data to the slow-binding equation (eq 3). The $k_{obs}$ values were plotted as a function of quercetin concentration. The results indicated that quercetin inhibits soybean lipoygenase-1 by simple reversible slow binding. This was evidenced by the observation that the $k_{obs}$ values exhibited a liner dependence on the inhibitor concentration as shown in Figure 5 inset. Thus, analysis of data according to eq 3 and 4 yielded the following values: $k_3 = 0.0325 \pm 0.01 \text{ µM}^{-1} \text{min}^{-1}$, $k_4 = 0.0345 \pm 0.02 \text{ min}^{-1}$, $K_{pp} = 1.06 \pm 0.2 \text{ µM}$.

**Effect on pseudoperoxidase activity of soybean lipoygenase**

Many of lipoygenase inhibitors such as flavonoids, catechols, benzoefuranol, and aminopyrazolines have been found (Yoshimoto *et al.*, 1983; Summer *et al.*, 1987; Jackson *et al.*, 1988) and there were act by a chelating the iron of the active site of enzyme (Clapp *et al.*, 1985; Nelson, 1988) and/or by reducing the ferric form of the enzyme to an inactive ferrous form (Kemal *et al.*, 1987; Mansuy *et al.*, 1988). Indirect evidence that N-alkylhydroxylamines, benzo-furanol and NDGA (norhydroguauretic acid) inhibitors also reduce the catalytically active ferric enzyme to the catalytically inactive ferrous enzyme lipoygenase has been obtained from the observations that the inhibitors stimulate the lipoygenase-catalyzed degradation of hydroperoxides (pseudoperoxidase activity). Figure 6 demonstrates a fast decrease in A234 with the NDGA addition in this pseudoperoxidase assay. Soybean lipoygenase-1 also exhibited a pseudoperoxidase activity when is incubated with 15 µM 13-HPOD and 10 µM of quercetin (1) also showing a time-dependent decrease in the hydroperoxide levels. Pedalitin (3), however, did not cause any significant stimulation of the pseudoperoxidase activity, because observed changes in A234 represent less than 5% of that observed with NDGA.

<table>
<thead>
<tr>
<th><strong>Table 1</strong></th>
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<tbody>
<tr>
<td><strong>IC$_{50}$ values of soybean lipoygenase-1</strong></td>
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<tr>
<td>Compound</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
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<tr>
<td>NDGA</td>
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Figure 4
Time course of the inactivation of soybean lipoxygenase-1 by pedalitin. The enzyme was preincubated at 25°C with pedalitin in 0.1 M Tris-HCl buffer (pH = 8.0) and the reactions were started by addition of 80 µM linoleic acid. A: Concentrations of pedalitin were as follows: (●) 0 µM; (○) 0.5 µM; (▼) 1 µM; (▽) 2 µM; (■) 3 µM; (□) 5 µM; (◇) 10 µM. B: Double reciprocal plot of the apparent rate of inactivation as a function of pedalitin concentration.

Table 2
Kinetic parameters of soybean lipoxygenase-1 by quercetin (1) and pedalitin (2)

<table>
<thead>
<tr>
<th>Compds</th>
<th>$k_3$ (µM$^{-1}$min$^{-1}$)</th>
<th>$k_4$ (min$^{-1}$)</th>
<th>$K_i^{app}$ (µM)</th>
<th>$k_{inact}$ (min$^{-1}$)</th>
<th>$K_1$ (µM)</th>
<th>$k_{inact}/K_1$ (mM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0325±0.01</td>
<td>0.0345±0.02</td>
<td>1.06±0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.32±0.07</td>
<td>5.37±0.5</td>
<td>59.6±10</td>
<td></td>
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<td></td>
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</tbody>
</table>
**Figure 5**
Time-dependent inhibition of soybean lipoxygenase-1 in the presence of quercetin. Conditions were as follows: 80 µM linoleic acid, concentrations of quercetin for curves 0-5 were 0, 2, 4, 6, 8, and 10 µM. (Inset) Dependence of the values for $k_{obs}$ on the concentration of quercetin.

**Figure 6**
Effects of pedalitin, quercetin and NDGA on soybean lipoxygenase-1 catalyzed consumption of 13-HPOD. The conditions of incubation were as described for pseudoperoxidase assay using 10 µM pedalitin (1), 30 µM pedalitin (2), 10 µM quercetin (3), 30 µM quercetin (4), and 10 µM NDGA (0) in the presence of enzyme (320 nM).
**Inhibitor modification (Pedalitin structure changes) during time course inhibition**

Oxidation of quercetin (1) and pedalitin (3) during pseudoperoxidase activity were studied by repetitive UV-visible spectrum during 2 hrs. The UV-Vis spectra showed the formation of a new product (λ max = 321 nm) indicating the formation of an intermolecular complex between LOX and quercetin. To explain the results obtained, Pinto and Macias (2005) concluded that in the presence of hydroperoxylinoleic acid, lipoxygenase produces a quinoid product as a result of the enzymatic cooxidation (in the presence of linoleic acid) of quercetin. Ha et al. (2010) indicate that quercetin might first be oxidized to the corresponding o-quinone and then isomerized to p-quinone. The pedalitin (3) is very slowly oxidation by soybean lipoxygenase-1 in the presence 13-HPOD, as followed by observing slowly changes in the UV-visible spectrum (Figure 7). Maximal spectral changes in the oxidation medium were observed at 280 and 359 nm (decreases in absorbance) and at 310 and 450 nm (increases in absorbance), and the presence of three isosbestic points at 288, 330 and 415 nm, respectively. In the contrast to the pedalitin, the quercetin (1) was quickly oxidized by soybean lipoxygenase-1 in the presence 13-HPOD (Figure 7 inset). The decrease in the absorbance of the band centered at 270 and 385 nm, the concomitant increase in the absorbance at 330 nm, and the presence of two isosbestic points at 286 and 357 nm, respectively.

Reverse phase high pressure liquid chromatography (RP-HPLC) analysis was performed using reaction mixture with pedalitin (3) (100 μM) and lipoxygenase-1 under the same conditions of pseudoperoxidase experiments (Figure 8). It shows that peak 3 (12.2 min) which came from pedalitin, was slowly decreased and peak a (6.4 min) was mainly increased.

A wide number of biological activities of flavonoids have been reported, which overall are believed to be beneficial to human health like the antioxidant capacity and the inhibitory effects on prooxidant enzymes (Sadik et al., 2003). Our previous paper reported that the catechol moiety is

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Figure 7

Repetitive scans of 30 μM pedalitin (A) and 30 μM quercetin (B) by soybean lipoxygenase-1 in the presence of 13-HPOD. The reaction medium contained 0.1M Tris-HCl buffer, pH = 8.0, 15 μM 13-HPOD, and lipoxygenase-1 (320 nM). A: The elapsed time between scans was 10 min during 2 hrs. B: The elapsed time between scans was 4 min during 48 min.
one of the essential head portions to elicit the lipoxygenase inhibitory activity (Ha et al., 2004). For example, luteolin (7), a common flavone bearing a catechol moiety in B-ring, is known to have this specific activity like a slow binding inhibitor, not oxidized despite the addition of hydroperoxides and remained without any change (Ha et al., 2012). However, in our previous pseudoperoxidase assay, quercetin was slowly oxidized by hydroperoxides to a rather stable intermediate, 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2H)-one, and this oxidized intermediate still inhibited the enzymatic oxidation (Ha et al., 2010).

**Concluding remarks**

The results obtained allow us to infer that pedalitin is very slowly oxidized by the soybean lipoxygenase-1 during a time dependent reaction inactivating the lipoxygenase-1 under irreversible way.

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**Figure 8**

HPLC analysis of the reaction medium with 100 μM of pedalitin and soybean lipoxygenase-1. Sampling time was chosen at 0, 30, 60, 90, and 120 min, respectively. The HPLC operating conditions was as follow: column; Capcell Pak C-18, solvent; 30% MeCN/H₂O containing 0.1% TFA, flow rate; 0.5 mL/min, detection; UV at 254 nm, injected amount; 20 μL from 3 mL assay system.
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REFERENCES


