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Scavenging effects of natural phenols on oxidizing intermediates of peroxynitrite

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Most of peroxynitrite ($ONOO^-$/ONOOH) is formed via the diffusion-limited reaction between nitric oxide and superoxide. In biological systems, the decomposition of $ONOO^-$ yields 30–35% of carbonate radical ($CO_3^{\bullet -}$) and nitrogen dioxide ($NO_2^\bullet$), which are strongly oxidizing intermediates and are suggested to take a part of the responsibility for the toxicity of nitric oxide ($NO^\bullet$) or $ONOO^-$. Therefore, the current study focuses on the scavenging activities of phenols toward $CO_3^{\bullet -}$ and $NO_2^\bullet$ to protect biomolecules from damage caused by $NO^\bullet$ or $ONOO^-$ using the technique of pulse radiolysis. From the build-up kinetic of the phenoxyl radicals and the decay kinetic of $CO_3^{\bullet -}$ radical, the rate constants of scavenging reactions were determined to be $1.9–3.4 \times 10^8\ M^{-1} \cdot s^{-1}$ and $0.11–1.9 \times 10^8\ M^{-1} \cdot s^{-1}$ for $CO_3^{\bullet -}$ and $NO_2^\bullet$ respectively. The results indicated that the tested phenols are the efficient scavengers of $CO_3^{\bullet -}$ and $NO_2^\bullet$.

1. Introduction

Oxidative damage to DNA, protein, and other biomolecules is postulated to be a major cause of endogenous damage leading to aging and degenerative diseases. Oxygen species such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), produced by radiation, and by normal or pathological metabolism, have been proposed as the major contributors to endogenous DNA damage [1]. Recently nitric oxide ($NO^\bullet$) has been identified as a cellular generated free radical, which may also contribute to endogenous DNA damage [2]. Exposure to $NO^\bullet$ causes mutations in both bacterial and mammalian cells, and induces various forms of damage to DNA in the presence of O_2 [3–6]. In infection and the inflammatory process, numerous free radicals including $NO^\bullet$ are generated, and these processes have been linked with increased cancer risk [7, 8], as well as with many aging-related diseases [1].

The toxicity of $NO^\bullet$ is believed to be in part due to its reaction with superoxide, and this reaction is currently accepted as the main biological source of $ONOO^-$ [9]. Although $NO^\bullet$ itself is a relatively stable free radical, which may even act as an antioxidant in some circumstances [10], its oxidation products, such as peroxynitrite, are highly reactive cytotoxic oxidants. Correspondingly, the mutagenic effects of $NO^\bullet$ are observed only in the presence of oxygen. However, it is not known which molecular forms of nitrogen oxide are responsible for the damaging effects of $NO^\bullet$. Peroxynitrite, peroxynitrite radical, NO^+ and N_2O_3 may contribute to the mutagenic effects of cellular $NO^\bullet$ synthesis [11].

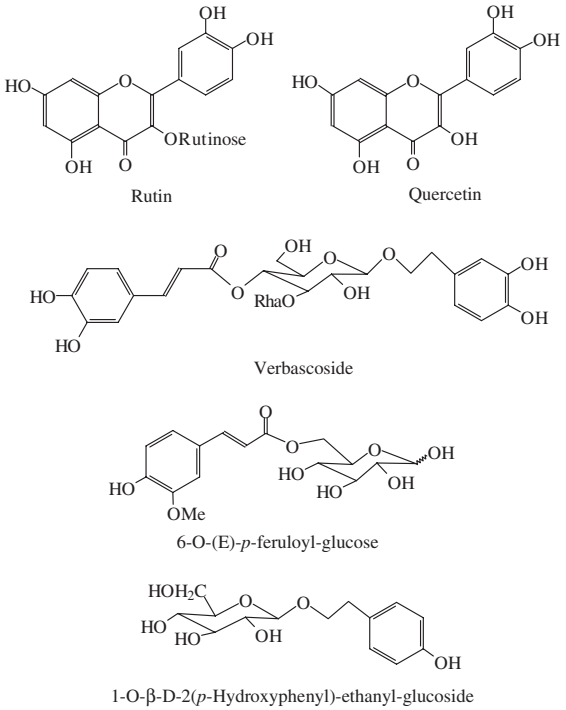
Lymar and Hurst [12] have shown that the peroxynitrite ion reacts very fast with carbon dioxide, apparently forming the $ONOOCO_2^-$ adduct. The decomposition of this adduct generates reactive intermediates capable of oxidizing organic and inorganic compounds [13–15]. These intermediates have been suggested to be the carbonate radical ($CO_3^{\bullet -}$) and nitrogen dioxide ($NO_2^\bullet$), which are strong oxidants formed by the homolytic cleavage of the O–O bond of the $ONOOCO_2^-$ [13–15]. Goldstein et al. [16] have also suggested that in the presence of CO_2 the reaction of $NO^\bullet$ and $O_2^{\bullet -}$ yields 33% $CO_3^{\bullet -}$ and $NO_2^\bullet$ under physiological conditions. Through analysis of the lifetimes of the possible intermediates, it has also been suggested that the free radicals $CO_3^{\bullet -}$ and $NO_2^\bullet$ are responsible for nitration and oxidation reactions with a variety of substrates [17]. Therefore, scavenging both $CO_3^{\bullet -}$ and $NO_2^\bullet$ has some potential bio-significance.

Various kinds of both natural and synthetic scavengers, especially phenolic antioxidants, have been studied and used to protect biological molecules from oxidiging radical attack [18]. Cooney et al. [19] showed that γ-tocopherol, which is found in many seed oils, reacts with NO_2 in the dark to form NO. By reducing NO_2 to NO, cellular damage is prevented. As NO possesses some antioxidant character [11, 20], levels of the more reactive nitrogen oxides are lowered. This is consistent with the observation that γ-tocopherol is superior to other tocopherols and β-carotene at preventing DNA single strand breaks in cells exposed to NO_2 [21]. The reaction chemistry between NO_2 and γ-tocopherol suggests that it may play a unique role as an antioxidant in preventing NO_2-mediated cellular damage [11].

Flavonoids are phenol derivatives present in substantial amounts (0.5–1.5%) in plants. The ability of flavonoids to act as antioxidants *in vivo* and *in vitro* has been extensively studied [18, 22]. Various structure-activity relationships have been derived, some of them rather contradictory. The consensus appears to be, however, that these polyphenols act as electron donors in aqueous media and H-atom donors in nonpolar systems [22].

Verbascoside (VER), one type of phenylpropanoid glycoside (PPG), and 1-*O*-β-D-2-(*p*-hydroxyphenyl)-ethanyl-glucoside (salidroside) have been isolated from *Pedicularis* species. *Pedicularis* is a herb used as a tonic in Chinese folk medicine. Some PPGs isolated from other plants have been reported to have antiviral [23], antitumor [24], antioxidation [25, 26], antiplatelet and antibiotic [27], and immunosuppressive activities [28], and to inhibit formation of leukotriene B_4 [29]. 6-*O*-(*E*)-feruloyl-glucose has been isolated and purified from *Aristolochia manshuriensis* Kom. The *Aristolochia L.* genus are herbs used in folk medicine as emmenagogues, lactogogues, diuretics, and painkillers, and have been used to treat stomatitis, glossitis, upset of mind, oedema, amenorrhoea, arthrititis, and pruritus. No reports of the biological activities of salidroside and 6-*O*-(*E*)-feruloyl-glucose have yet been found. Detailed descriptions of PPGs and their activities *in vivo* and *in vitro* have been reported elsewhere [30–32].

In the present study, the scavenging activities of two kinds of flavonoid, rutin and quercetin, one phenylpropanoid (PPG), verbascoside, and its two analogs, salidroside and 6-*O*-(*E*)-feruloyl-glucoside, on $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ were investigated.



2. Investigations and results

2.1. Specific transient absorption spectra of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$

On pulse radiolysis of a 0.2 M aqueous solution of $NaCO_3$ saturated with N_2O, a strong transient absorption spectrum with λ_{max} at 600 nm arising from reaction of $OH^{\bullet}$ with CO_3^{2-} was observed (Fig. 1A). The transient

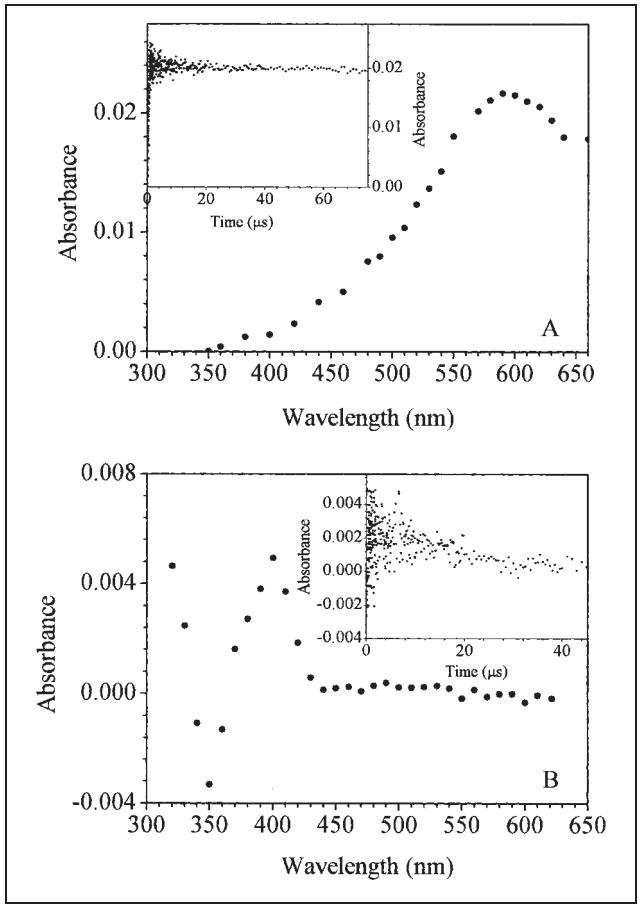


Fig. 1: Transient absorption spectrum on pulse radiolysis of 0.2 M Na_2CO_3 (A) and 50 mM $NaNO_2$ (B) aqueous solution saturated with N_2O. Inset: the buildup trace of absorption at 600 nm (A) and 400 nm (B)

absorption reached a maximum at 1 μs after electron pulse irradiation and decayed with second-order kinetics (Fig. 1A, inset). This transient absorption spectrum may be assigned to $CO_3^{\bullet-}$ [33]. A specific transient absorption spectrum of $NO_2^{\bullet}$ with $\lambda_{max} = 400$ nm (Fig. 1B) was also observed in a similar process with a concentration of $NaNO_2$ of 50 mM. The transient absorption reached its maximum at 1 μs after electron pulse irradiation (Fig. 1B, inset).

2.2. Reactions of $CO_3^{\bullet-}$ with the tested phenols

At 1 μs after pulse radiolysis of a 0.2 M Na_2CO_3 aqueous solution containing 0.1 mM rutin and saturated with nitrous oxide at pH 8.6, a transient absorption spectrum at 600 nm was first observed (Fig. 2A a). This spectrum is same as that of $CO_3^{\bullet-}$, therefore, is assigned to $CO_3^{\bullet-}$. At 75 μs after electron pulse irradiation, a new optical absorption with $\lambda_{max} = 480$ nm grew concomitant with the disappearance of that of $CO_3^{\bullet-}$ (Fig. 2A b) reaching its maximum at around 70 μs (Fig. 3A). This change of transient absorption spectrum is due to the reaction of $CO_3^{\bullet-}$ with rutin. By the same process, the reactions of $CO_3^{\bullet-}$ with the other phenols tested were also observed (Figs. 2, 3).

2.3. Reactions of $NO_2^{\bullet}$ with the tested compounds

At 1 μs after pulse radiolysis of a 50 mM aqueous solution of $NaNO_2$ containing 0.175 mM rutin and saturated with nitrous oxide at pH 7.0, a transient absorption spec-

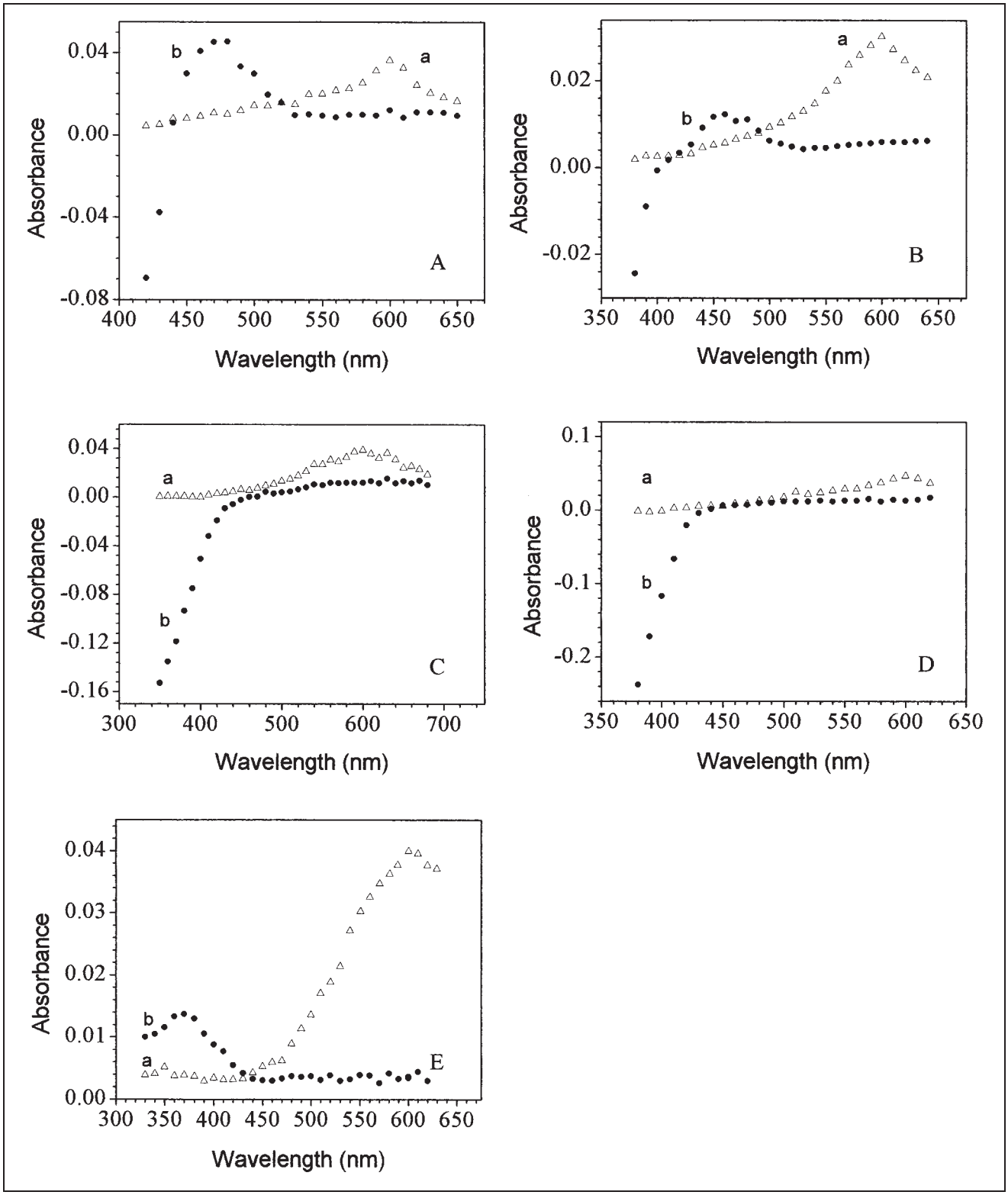


Fig. 2: Transient absorption spectra on pulse radiolysis of 0.2 M Na_2CO_3 aqueous solution containing tested phenolic antioxidants saturated with N_2O. (A) 0.1 mM rutin: a, 1 μs, b, 75 μs; (B) 0.1 mM quercetin: a, 1 μs, b, 75 μs; (C) 0.1 mM verbascoside: a, 1 μs , b, 75 μs ; (D) 0.1 mM 6-*O*-(*E*)-feruloyl-glucose: a, 1 μs, b, 25 μs; (E) 0.1 mM salidroside: a, 1 μs, b, 75 μs

trum at 400 nm was observed (Fig. 4A a). This spectrum is same as that of $NO_2^{\bullet}$, therefore, is assigned to $NO_2^{\bullet}$ [33]. Then a new optical absorption with $\lambda_{max} = 470$ nm grew concomitant with the disappearance of that of $NO_2^{\bullet}$ (Fig. 4A b) and reaching its maximum at around 320 μs (Fig. 5A). The reason for this change of transient absorption spectrum is the reaction of $NO_2^{\bullet}$ with rutin. The reactions of $NO_2^{\bullet}$ with the other phenols tested were also observed by the same process (Figs. 4, 5).

2.4. Rate constants

The curve shown in Fig. 3B represents the change of absorption of the product of reaction between quercetin and

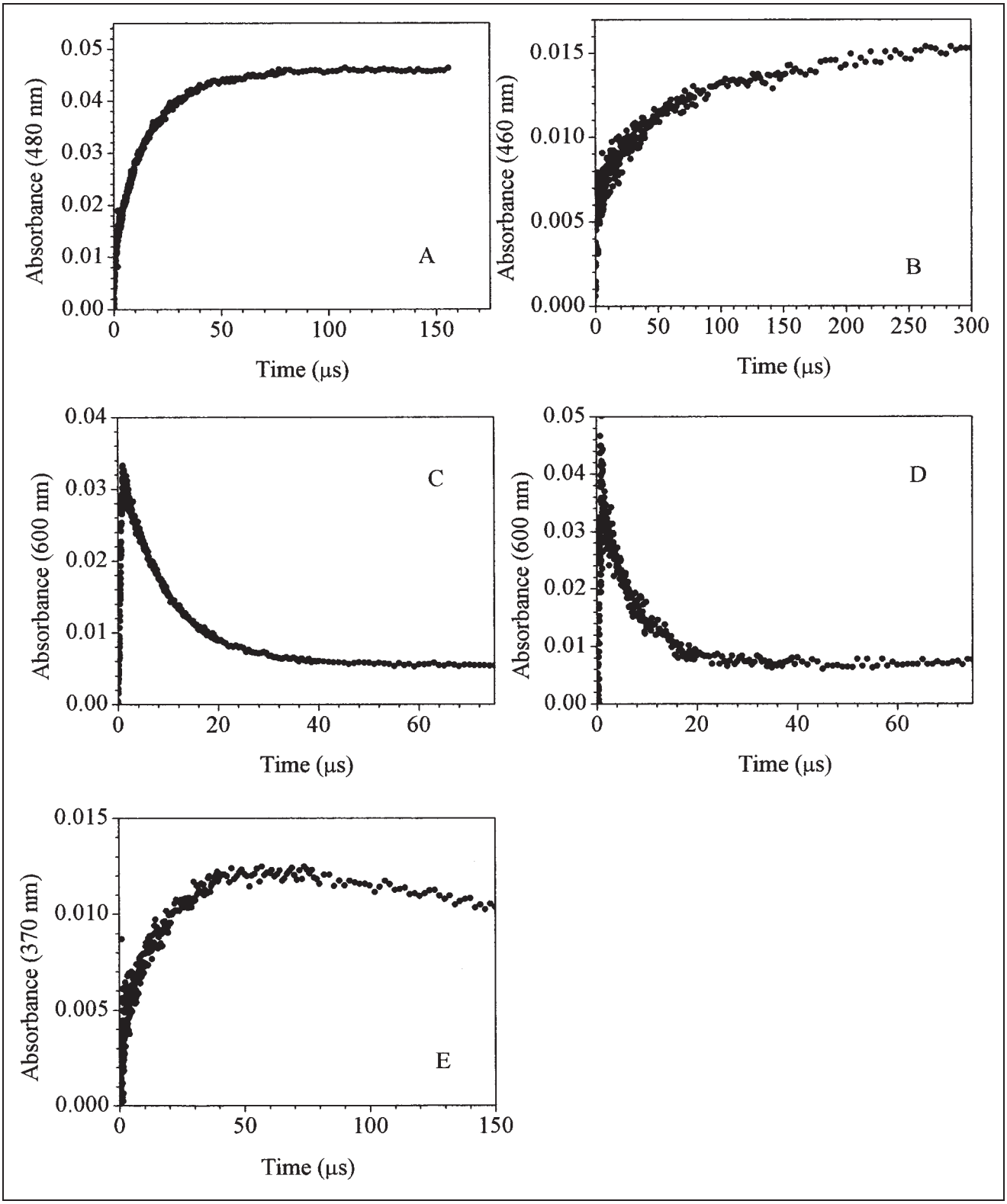


Fig. 3: Absorption trace on pulse irradiation of 0.2 M Na_2CO_3 aqueous solution containing tested phenolic antioxidants saturated with N_2O. (A) 0.1 mM rutin: 480 nm; (B) 0.1 mM quercetin: 460 nm; (C) 0.1 mM verbascoside: 600 nm; (D) 0.1 mM 6-*O*-(*E*)-feruloyl-glucose: 600 nm; (E) 0.1 mM salidroside: 370 nm

$CO_3^{\bullet-}$ with time after pulse irradiation of a 0.2 M aqueous solution of Na_2CO_3 containing 0.1 mM rutin and saturated with nitrous oxide. The growth of absorbance follows first order kinetics. The value of the slope $\varkappa_{app}$ is the apparent rate constant of the reaction of $CO_3^{\bullet-}$ with quercetin. Varying the concentration of quercetin (0.02–0.12 mM), a series of $\varkappa_{app}$ are obtained. The dependence of $\varkappa_{app}$ on [quercetin] is a straight line (Fig. 6A). The slope yields the rate constant ($\varkappa$) for the reaction of $CO_3^{\bullet-}$ and quercetin.

The curve shown in Fig. 3C represents the decay of absorption of $CO_3^{\bullet-}$ at 600 nm, demonstrating a decrease of $[CO_3^{\bullet-}]$, because of the reaction between $CO_3^{\bullet-}$ and VER with time after pulse irradiation of a 0.2 M aqueous solu-

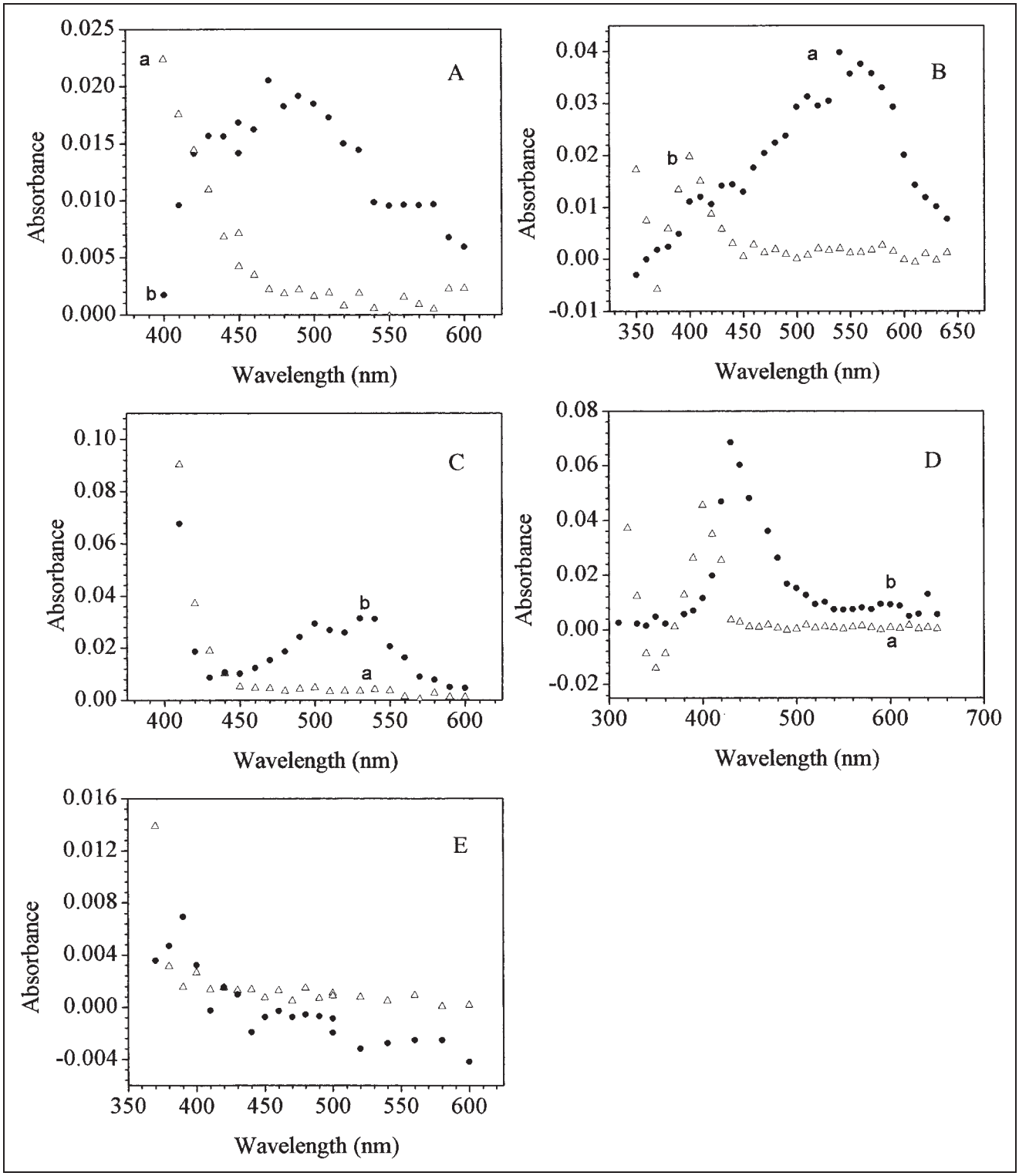


Fig. 4: Transient absorption spectra on pulse irradiation of 50 mM $NaNO_2$ aqueous solution containing tested phenolic antioxidants saturated with N_2O. (A) 0.175 mM rutin: a, 1 µs, b, 350 µs; (B) 0.35 mM quercetin: a, 1 µs, b, 75 µs; (C) 0.9 mM verbascoside: a, 2 µs , b, 55 µs ; (D) 0.7 mM 6-*O*-(*E*)-feruloyl-glucose: a, 1 µs, b, 150 µs; (E) 0.75 mM salidroside: a, 1 µs, b, 300 µs

tion of Na_2CO_3 containing 0.1 mM VER and saturated with nitrous oxide. The decay of absorbance follows first-order kinetics. The apparent rate constant for the decay of $CO_3^{\bullet-}$ can be obtained by kinetic analysis of the curve. This apparent rate constant can be regarded as that of the reaction between $CO_3^{\bullet-}$ and VER, and hence the rate constant of the scavenging reaction of VER of $CO_3^{\bullet-}$ was deduced (Fig. 6B).

The rate constants for the scavenging reactions of the phenols tested with $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ were determined and are shown in the Table.

3. Discussion

In biological environments capable of simultaneous generation of $O_2^{\bullet-}$ and NO, uncontrolled formation of perox-

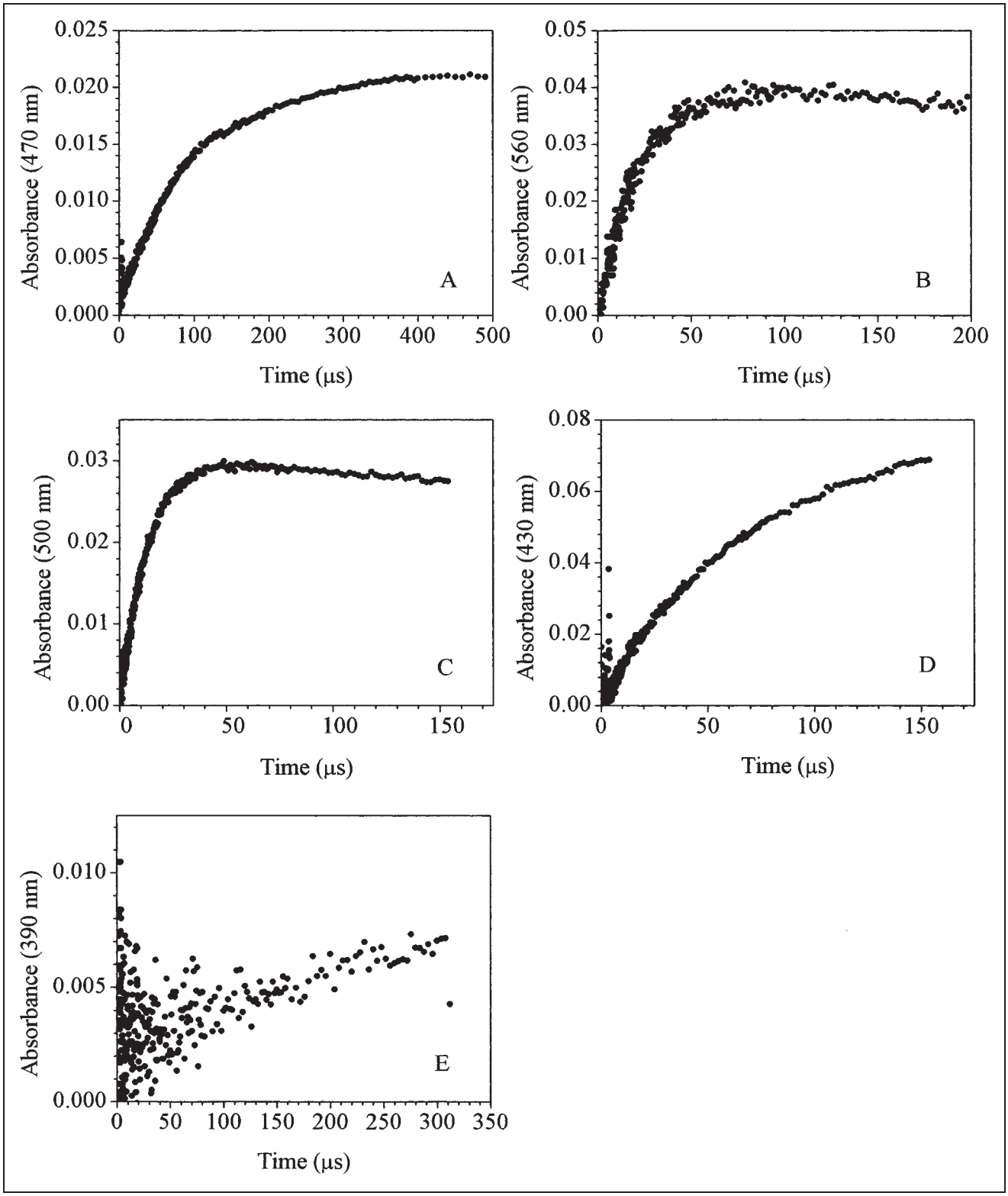


Fig. 5: Absorption trace on pulse irradiation of 0.2 M $NaNO_2$ aqueous solution containing tested phenolic antioxidants saturated with N_2O. (A) 0.175 mM rutin: 470 nm; (B) 0.35 mM quercetin: 560 nm; (C) 0.9 mM verbascoside: 500 nm; (D) 0.7 mM 6-*O*-(*E*)-feruloyl-glucose: 430 nm; (E) 0.75 mM salidroside: 370 nm

ynitrite ($ONOO^-$) has been proposed [34] to cause oxidative damage of biological tissue, giving rise to a variety of pathogenic conditions that may include pulmonary [35] and coronary [12] diseases, impairment of central motor nervous system function [36, 37], and injury to ischemic tissue accompanying reperfusion with aerobic fluids [12]. However, in carbonate-containing media $ONOO^-$ is also unstable and reacts rapidly with CO_2, forming an adduct whose composition is $ONOOCO_2^-$ (eq. 1) [12].

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad (1)$$

The rate constant of this reaction is $3 \times 10^4\ M^{-1}\ s^{-1}$ [12], which is sufficiently large, together with the high biological concentration of CO_2 (about 1 mM CO_2), to make the reaction of $ONOO^-$ with CO_2 the predominant reaction of peroxynitrite (except at localized cellular sites with high

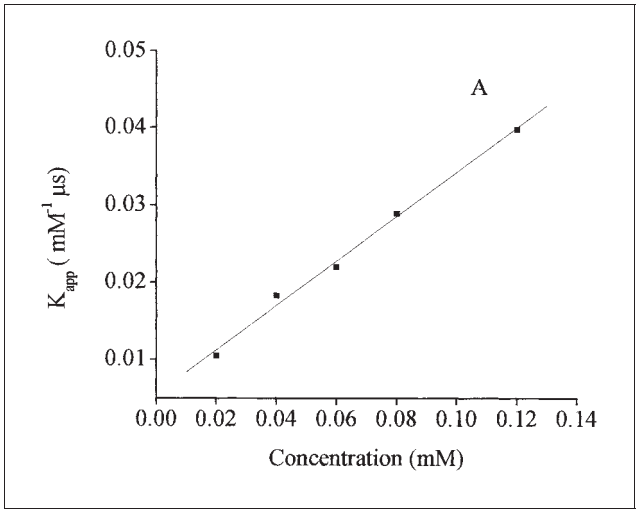


Fig. 6: Dpendence of first-order rate constant for the growth of absorbance at 480 nm on [quercetin] on pulse irradiation of 0.2 M Na_2CO_3 aqueous solution containing quercetin saturated with N_2O

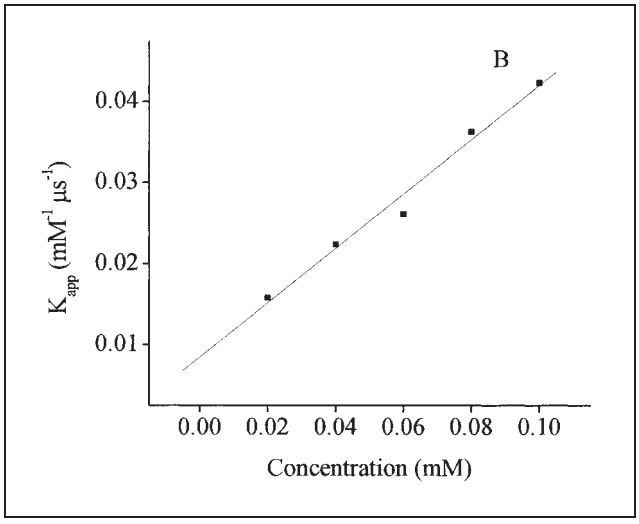


Fig. 7: Dependence of first-order rate constant for the decay of absorbance at 600 nm on [verbascoside] on pulse irradiation of 0.2 M Na_2CO_3 aqueous solution containing verbascoside saturated with N_2O

Table: Rate constants ($10^8\ M^{-1}\ s^{-1}$) of scavenging reactions of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ by tested phenols

	$CO_3^{\bullet-}$	$NO_2^{\bullet}$
Rutin	2.87	0.66
Quercetin	2.80	1.91
Verbascoside	3.36	1.49
Salidroside	1.93	0.11
6-*O*-(*E*)-Feruloyl-glucoside	2.03	0.12

concentrations of other reactive biotargets). Therefore, the proposition that $ONOO^-$ itself is damaging to cells is inferred to be highly unlikely, although it might be an obligatory intermediate for forming destructive cellular oxidants. Based on this consideration, the formation of $ONOO^-$ might be regarded as serving the unique function of allowing entry into a pathway for generating peroxide-based toxins that is not accessible by H_2O_2 itself in physiological environments [12]. In this respect, attention should focus on the scavenging of $ONOOCO_2^-$ or the free radicals derived from $ONOOCO_2^-$ to protect biomolecules from damage caused by NO or $ONOO^-$.

$ONOOCO_2^-$ is highly reactive, and very unstable, e.g., it decomposes before diffusing to cellular target sites [33] to generate strongly oxidizing intermediates, which are suggested to be $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ formed by the homolytic cleavage of the peroxy O–O bond of the adduct [13–15] (eq. 2).

$$ONOOCO_2^{\bullet-} \rightarrow CO_3^{\bullet-} + NO_2^{\bullet} \quad (2)$$

In the presence of CO_2, the decomposition of peroxynitrite yields 30–35% of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ [13, 14, 16]. With a redox potential of 1.5 V, $CO_3^{\bullet-}$ is a highly oxidizing intermediate whose reactivity is similar to that of the hydroxyl radical, and it is capable of oxidizing organic and inorganic compounds including biomolecules [33]. In the case of the absent reactants, the decay of $CO_3^{\bullet-}$ is second order (eq. 3) (Fig. 1A, inset).

$$CO_3^{\bullet-} + CO_3^{\bullet-} \rightarrow \text{products} \quad (3)$$

In the current study, the decay rate of $CO_3^{\bullet-}$ was enhanced in the presence of phenolic compounds, while second-order kinetics became first-order kinetics and the transient absorption spectrum of $CO_3^{\bullet-}$, which was initially observed, was substituted by a new spectrum. All these results indicated that the phenolic compounds tested can scavenge $CO_3^{\bullet-}$.

In the case of $NO_2^{\bullet}$, situation was similar to that of $CO_3^{\bullet-}$, while the rate constants of the scavenging reactions of $NO_2^{\cdot}$ by the compounds tested are lower by almost one order than those of $CO_3^{\bullet-}$ because the redox potential of $NO_2^{\cdot}$ is 1.04 V, far lower than those of $OH^{\bullet}$ and $CO_3^{\bullet-}$ [33].

The present results indicate that flavonoids, PPG and its analogs may act as effective scavengers of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$, which are generated in biological systems in the pathway shown in reactions (1) and (2), especially in the case of infection and inflammatory reactions, and therefore the compounds tested can protect biomolecules from damage induced by $CO_3^{\bullet-}$ and $NO_2^{\bullet}$. Because flavonoids are present in substantial amounts (0.5–1.5%) in epidermal plant cells, and in plant derived foods and beverages, it is inferred that human beings benefit from intake of fruits and beverages. The $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ scavenging activities of PPGs and their analogs may related to the pharmacological effects of the Chinese medicinal herbs from which these compounds were isolated and purified.

4. Experimental

4.1. Materials

Rutin and quercetin were purchased from Sigma, and verbascoside (VER) and salidroside were isolated from *Pedicularis* species [33–35]. 6-*O*-(*E*)-Feruloyl-glucose was isolated and purified from *Aristolochia manshuriensis* Kom. All other chemicals were purchased from Shanghai Biochemical Co. (Shanghai, China). All solutions were prepared with triple distilled water, saturated with high purity nitrous oxide. All experiments were carried out at room temperature.

4.2. Pulse radiolysis

Pulse radiolysis experiments were conducted using a linear accelerator providing an 8 MeV electron pulse with a duration of 8 ns. The dosimetry of the electron pulse was determined by a thiocyanate dosimeter containing a 10 mM aqueous solution of KSCN saturated with nitrous oxide, by taking $\varepsilon_{(SCN)^-} = 7600\ M^{-1}\ cm^{-1}$ at 480 nm. A detailed description of the pulse radiolysis equipment and experimental conditions has been given elsewhere [38]. In the present work, the average pulse dose is 10 Gy.

4.3. Generations of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$

On pulse radiolysis of a 0.2 M Na_2CO_3 or 50 mM $NaNO_2$ aqueous solution saturated with nitrous oxide (N_2O) and buffered with phosphate (2 mM) at pH 8.6 and 7.0, respectively, water produces hydrated electrons (e_{aq}^-) and $OH^{\bullet}$, and then $OH^{\bullet}$ reacts with CO_3^{2-} or NO_2^- to form $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ respectively (eqs. 4–7).

$$H_2O \rightarrow OH^{\bullet} + eaq^- + H^{\bullet} + H_3O^+ \quad (4)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow OH^{\bullet} + OH^- + N_2 \quad (5)$$

$$CO_3^{2-} + OH^{\bullet} \rightarrow CO_3^{\bullet-} + OH^- \quad (6)$$

$$NO_2^- + OH^{\bullet} \rightarrow NO_2^{\bullet} + OH^- \quad (7)$$

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