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Antioxidant activities of polyphenols from sage (*Salvia officinalis*)

Yinrong Lu*, L. Yeap Foo

Industrial Research Limited, PO Box 31310, Lower Hutt, New Zealand

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Abstract

The antioxidant activities of the sage polyphenols, consisting of flavone glycosides and a range of rosmarinic acid derivatives, were evaluated for their capacity to scavenge DPPH and superoxide anion radicals and also to reduce Mo(VI) to Mo(V). The rosmarinic acid derivatives all showed potent antioxidant activity in three test systems and their capacity to reduce Mo(VI) to Mo(V) and their superoxide radical scavenging activities, with values ranging from 220 to 300 SOD units/mg, in particular, were 4–6 and 15–20 times greater than trolox, respectively. The high SOD activity of rosmarinic acids could be attributed to the radical-scavenging catechols and the xanthine oxidase-inhibiting caffeic acid moieties contained in them. The antioxidant activity of the flavonoids was variable and those with a catechol B-ring (luteolin glycosides) were more active than those without (apigenin glycosides).

Keywords: *Salvia officinalis*; Phenolic acids; Flavonoids; Antioxidant activities; DPPH; Superoxide radical; Phosphomolybdenum complex

1. Introduction

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. Spices used in different types of food to improve flavours, since ancient times, are well known for their antioxidant properties (Madsen & Bertelsen, 1995). In various studies, rosemary and sage, both belonging to the mint family (*labiatae*), have been shown to be the most potent natural antioxidants of the common spices (Chipault, Mizuno, & Lundberg, 1956; Herrmann, 1981).

In earlier studies, sage and rosemary were shown to have similar patterns of phenolic compounds and the antioxidative activity had been attributed mainly to carnosic acid and rosmarinic acid (Brieskorn & Dömling, 1969; Cuvelier, Richard, & Berset, 1996). However, more studies on sage have revealed the presence of additional classes of active compounds, including terpenoids, flavonoids (Bisio, Romussi, Ciarallo, & De Tommasi, 1997; Gökdil, Topcu, Sönmez, & Ulubelen, 1997) and phenolic acids. The latter-mentioned consist of an impressive array of biologically active caffeic acid oligomers, ranging from trimers, tetramers and higher oligomers, such as salvianolic acids and yunnaneic acids (Li, 1998; Tanaka, Nishimura, Kouno, Nonaka, & Young, 1996; Tanaka, Nishimura, Kouno, Nonaka, & Yang, 1997). According to some workers, these compounds may contribute to the health property of sage used as a popular folk medicine for the treatment of various ailments (Keller, 1978). There is increasing evidence to suggest that many degenerative diseases, such as brain dysfunction, cancer, heart diseases and immune system decline, could be the result of cellular damage caused by free radicals, and antioxidants present in human diet may play an important role in disease prevention (Aruoma, 1998; Nees & Powles, 1997; Steinmetz & Potter, 1996). The reported high antioxidant activity of sage and its traditional medicinal uses prompted us to investigate this herb further to provide a better understanding of the chemistry involved.

Earlier studies on the antioxidant activity of sage had been limited to the diterpenoid compounds (Cuvelier, Berset, & Richard, 1994; Zhang, Bao, Wu, Rosen, & Ho, 1990). It was only recently that Wang et al. (1998, 1999) reported two sage phenolic glycosides that showed moderate antioxidant activities. We previously characterised a number of flavonoids and phenolic acids, including the novel rosmarinic acid derivatives, sagecoumarin and sagerinic acid, in sage (Lu & Foo,

* Corresponding author. Fax: +64-4-5690055.
E-mail address: y.lu@irl.cri.nz (Y. Lu).

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1999, 2000b; Lu, Foo, & Wong, 1999). This report deals with the antioxidant activity of these compounds using three different test methods, namely DPPH free radical (Brand-Williams, Cuvelier, & Berset, 1995), superoxide anion radical generated from the xanthine/xanthine oxidase system (Lu & Foo, 2000a) and reduction of phosphomolybdenum complex (Prieto, Pineda, & Aguilar, 1999).

2. Materials and methods

2.1. Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), xanthine (99%), xanthine oxidase (25 units), superoxide dismutase (75 000 units) and sodium phosphate were purchased from Sigma Chemical Co, PO Box 14508, St. Louis, MO 63178, USA; nitro blue tetrazolium chloride (NBT) and sodium dodecylsulphate (SDS) were from Serva Feinbiochemjca GmbH & Co. KG, Carl-Benz-Str. 7, D-69 115 Heidelberg, Germany, and ammonium molybdate from AppliChem, Ottoweg 10b, D-64291 Darmstadt, Germany. All solvents were of analytical grade. The sage polyphenols used were those isolated and purified, as previously described (Lu & Foo, 1999, 2000b; Lu et al., 1999).

2.2. DPPH radical-scavenging activities

A 2.0 ml methanolic solution of DPPH (0.1 mM) was mixed with 0.1 ml of a sage polyphenolic solution (0.1 mg/ml) in methanol and, after 60 min standing, the absorbance of the mixture was measured at 517 nm against methanol as the blank. Triplicate measurements were made and the antioxidant activity was calculated by the percentage of DPPH that was scavenged.

2.3. Superoxide anion radical-scavenging activities

0.1 ml of aqueous superoxide dismutase (SOD) standard solutions (5, 10, 25, 50, 100 units/ml) and a sage polyphenolic solution in DMSO (0.2 mg/ml) were separately added to a 1.0 ml mixture of 0.4 mM xanthine and 0.24 mM nitro blue tetrazolium chloride (NBT) in 0.1 M phosphate buffer (pH 8.0). A 1.0 ml solution of xanthine oxidase (0.049 units/ml), diluted in 0.1 M phosphate buffer (pH 8.0), was added and the resulting mixture incubated in a water bath at 37°C for 20 min. The reaction was terminated by adding 2.0 ml of an aqueous solution of 69 mM sodium dodecylsulphate (SDS) and the absorbance of NBT was measured at 560 nm. The superoxide radical-scavenging activity of the polyphenol was calculated as superoxide dismutase equivalents (SOD units/mg) from the SOD standard curve.

2.4. Evaluation of antioxidant activity

The antioxidant activity of sage polyphenols was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al. (1999). An aliquot of 0.1 ml of sample solution (1 mM in DMSO) was combined in a 4-ml vial with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expressed relative to that of trolox, the synthetic water-soluble vitamin E (α-tocopherol) equivalent.

3. Results and discussion

3.1. DPPH radical-scavenging activities of sage polyphenols

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a stable radical with a maximum absorption at 517 mm that can readily undergo reduction by an antioxidant. Because of the ease and convenience of this reaction it now has widespread use in the free radical-scavenging activity assessment (Brand-Williams et al., 1995; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998). The DPPH radical-scavenging activity of sage polyphenols (see Fig. 1 for chemical structures) is shown in Fig. 2 and expressed as the percentage reduction of the initial DPPH absorption by the test compound. The Figure shows that rosmarinic acid and its oligomeric derivatives (**1**–**5**) are excellent DPPH radical-scavengers, with about 90% of DPPH scavenged under the experimental conditions. The flavonoids, luteolin and apigenin glycosides (**6**–**10**), on the other hand, show only weak to moderate activities. The rosmarinic acid derivatives are as effective as caffeic acid on a weight basis and they are all significantly better than trolox. Caffeic acid and rosmarinic acid, being common in many plants and often present in our diet, are both strong radical-scavengers with caffeic acid being slightly superior to rosmarinic acid (Brand-Williams et al., 1995; Cuvelier, Richard, & Berset, 1992). Chemical structure-activity studies of the phenolic acids by various methods have consistently shown that cinnamic acid derivatives have superior antioxidant activity to the benzoic acid analogues, which may be due to the presence of the conjugated unsaturation that facilitates the delocalization of the resulting free radicals. Among the cinnamic acids, caffeic acids are much better than ferulic acids, which are in turn better than *p*-coumaric acids (Cuvelier et al., 1992; Herrmann, 1993; Natella, Nardini, Di Felice, & Scaccini, 1999). This observation suggests that an ortho-

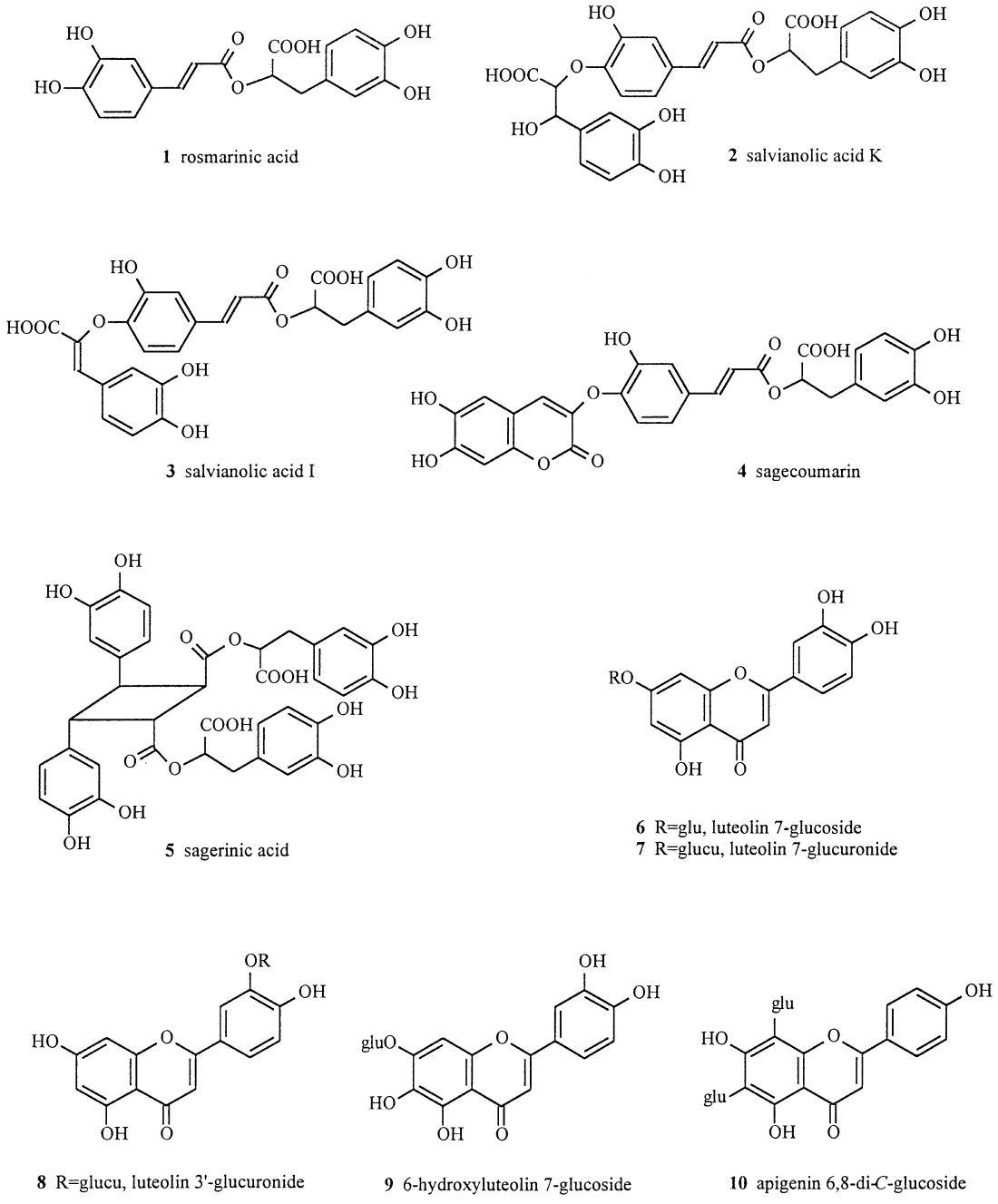

Fig. 1. Chemical structures of polyphenols from *Salvia officinalis*.

dihydroxybenzene (catechol) structure is crucial for enhanced antioxidant activities. As the polyphenols from sage are in many respects caffeic acid oligomers (dimers to tetramers), their strong DPPH radical-scavenging activities may be attributed to the catechol moieties contained in them.

The weaker DPPH-scavenging activities of the flavonoid glycosides on a weight basis, as compared with the phenolic acids, were apparently due to the inclusion of non-participating structures, such as sugars and the weakly active phloroglucinol A-ring in their molecules. Glycosylation has been reported to decrease radical-scavenging activity of the host molecule (Plumb, De Pascual-Teresa, Santos-Buelga, Cheynier, & Williamson, 1998) and the contribution of the phloroglucinol A-ring in flavonoids to the activity is far smaller than the corresponding catechol B-ring (Senba, Nishishita, Saito, Yoshioka, & Yoshioka, 1999).

Among the flavonoids, the antioxidant activities decrease in the following order: 6-hydroxyluteolin 7-glycosides > luteolin-7-glycosides > luteolin-3′-glycosides > apigenin glycosides. This finding is in accordance with the results reported by Bors and co-workers (1990) and confirms that the *o*-dihydroxybenzene (catechol)

structure in the B-ring is important for enhanced radical-scavenging activity. The low DPPH radical-scavenging activities of apigenin 6,8-di-*C*-glucoside (**10**) and luteolin 3′-glucuronide (**8**) (3 and 8% reduction of DPPH, respectively) are apparently due to the lack of an ortho-dihydroxyl moiety in their B rings. The presence of an additional hydroxyl group in 6-hydroxyluteolin-7-glucoside (**9**) significantly boosts the radical-scavenging activity to 75% DPPH reduction, as compared to 34% for luteolin 7-glycosides. Among the flavonoids, 6-hydroxyluteolin-7-glucoside is the only one which shows better DPPH radical-scavenging activity than trolox (45% DPPH reduction).

3.2. Superoxide anion radical-scavenging activity of sage polyphenols

Superoxide anion radical (O_2^-) is generated in vivo by several oxidative enzymes, including xanthine oxidase (XO), which converts hypoxanthine to xanthine and subsequently to uric acid. Superoxide dismutase (SOD) is the cellular antioxidant enzyme, which removes this ubiquitous superoxide metabolic product by converting it into hydrogen peroxide and oxygen in biological systems. Many polyphenols present in our diet have been shown to be effective XO inhibitors and/or superoxide radical scavengers (Aucamp, Gaspar, Hara, & Apostolides, 1997; Chan, Wen, & Chiang, 1995; Cos et al., 1998; Cotelle, Bernier, Catteau, Pommery, Wallet, & Gaydou, 1996; Lin, Chen, Ho, & Lin-Shiau, 2000). In this sense, this assay is a relevant and important way to assess antioxidant activity of dietary compounds.

The XO inhibiting and/or superoxide radical-scavenging activities of the sage polyphenols, determined using the xanthine/xanthine oxidase system, are shown in Table 1. For convenience, these inhibition and/or superoxide scavenging activities are collectively expressed as superoxide dismutase equivalents (SOD units/mg). The results show all the phenolic acids **1**–**5** have potent activity, with values ranging from 220 to 300 SOD units/mg. The activities are some 15–20 times greater than trolox. In contrast, the flavonoid glycosides **6**–**10** all show weak to moderate activities, a1though they are still better than trolox.

The strong superoxide scavenging activities of the rosmarinic acid derivatives **1**–**5**, like their DPPH radical-scavenging activities, may also be attributed to the presence of the caffeic acid moiety which is contained in them. Caffeic acid has a value of 260 SOD units/mg, which is slightly greater than the 230 SOD units/mg found for rosmarinic acid (**1**), which is composed of a caffeic acid and a dihydrocaffeic acid moiety. Caffeic acid has been reported to exert both strong inhibitory activity on xanthine oxidase (Chan et al., 1995) and strong radical-scavenging activity (Brand-Williams et al., 1995; Cuvelier et al., 1992), while dihydrocaffeic acid had no XO inhibition (Chan et al., 1995) but had slightly better radical-scavenging activity than caffeic acid (Silva, Borges, Guimaraes, Lima, Matos, & Reis, 2000). These results indicate that the α,β-unsaturated COOH moiety, has an important effect on XO inhibition, while the catechol group is important for enhanced radical-scavenging activity. From these observations it could be suggested that the strong superoxide scavenging activities of rosmarinic acid derivatives **1**–**5** are mostly attributable to their strong superoxide radical-scavenging capacity and less to XO inhibition. Sagerinic acid (**5**), in which the side chains of both caffeic acid moieties are saturated, therefore would only have superoxide radical-scavenging activity, due to the catechol

Table 1
Superoxide scavenging activities of sage polyphenols as superoxide dismutase equivalents (SOD units/mg)

Compound	SOD	Compound	SOD
Caffeic acid (reference)	260	Luteolin 7-glucoside (**6**)	95
Rosmarinic acid (**1**)	230	Luteolin 7-glucuronide (**7**)	80
Salvianolic acid K (**2**)	280	Luteolin 3′-glucuronide (**8**)	20
Salvianolic acid I (**3**)	290	6-Hydroxyluteolin 7-glucoside (**9**)	176
Sagecoumarin (**4**)	220	Apigenin 6,8-di-*C*-glucoside (**10**)	18
Sagerinic acid (**5**)	242	Trolox (reference)	15

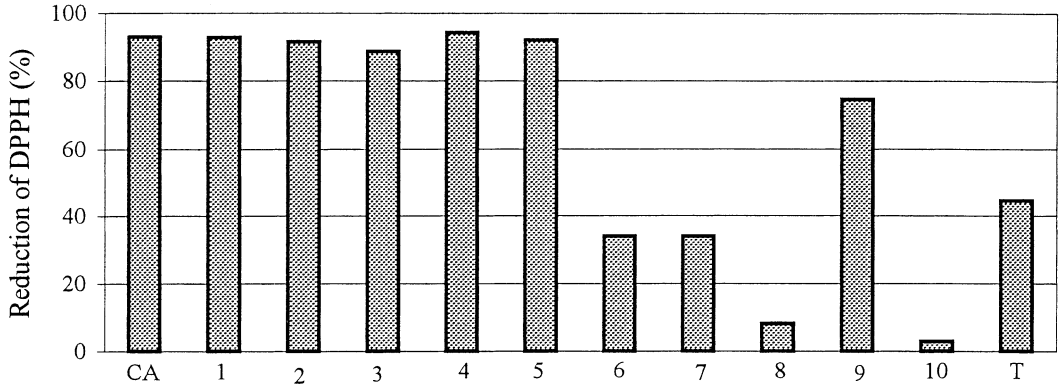

Fig. 2. DPPH radical scavenging activities of sage polyphenols **1**–**10** (CA and T for caffeic acid and trolox, respectively).

Table 2
Antioxidant activities of sage polyphenols relative to trolox

Compound	A_m[a]	A_w	Compound	A_m	A_w
Caffeic acid (reference)	3.0	4.1	Luteolin 7-glucoside (**6**)	1.3	0.7
Rosmarinic acid (**1**)	3.7	2.6	Luteolin 7-glucuronide (**7**)	–	–
Salvianolic acid K (**2**)	4.4	2.1	Luteolin 3′-glucuronide (**8**)	1.6	0.9
Salvianolic acid I (**3**)	4.4	2.0	6-Hydroxyluteolin 7-glucoside (**9**)	2.2	1.1
Sagecoumarin (**4**)	5.2	2.4	Apigenin 6,8-di-*C*-glucoside (**10**)	0.7	0.2
Sagerinic acid (**5**)	5.8	2.0	Trolox (reference)	1	1

[a] A_m and A_w, activities relative to trolox on a molar and weight basis, respectively.

groups. The flavonoid glycosides **6**–**10** overall have weaker superoxide scavenging activities than the phenolic acids **1**–**5**, but their activities are still better than trolox. The activities among the flavonoids are highly variable, with a low of 18 SOD units/mg for apigenin 6,8-di-*C* glucoside (**10**) to a high of 176 SOD units/mg for 6-hydroxyluteolin-7-glucoside (**9**). This large difference could be explained by the differences in their chemical structure. Studies on structure-activity relationships of flavonoids consistently showed that a catechol or pyrogallol B-ring was essential for strong radical-scavenging activity (Bors et al., 1990; Cos et al., 1998) and the phloroglucinol A-ring was far less effective (Senba et al., 1999). Although the 7-OH group in the A-ring could inhibit XO competitively, as it may take the place of the C-2 or C-6 OH of xanthine in the enzyme active site, the activity was reduced by substitution on the B-ring (Cos et al., 1998). The flavone glycosides tested in this work therefore exert activity predominantly by scavenging superoxide radical and, to a lesser extent, by XO inhibition. The low SOD value (18 SOD units/mg) for apigenin 6,8-di-*C*-glucoside (**10**) is clearly due to the lack of a catechol B-ring in the molecule, while the activity of luteolin 3′-glucuronide (**8**) decreased dramatically (20 SOD units/mg) with glycosylation on C-3′ as compared with values of 95 and 80 SOD units/mg for luteolin 7-glycosides **6** and **7**, respectively. The significant increase in the superoxide scavenging activity (176 SOD units/mg) for 6-hydroxyluteolin 7-glucoside (**9**) was apparently attributed to the additional hydroxyl group at C-6, which made up for additional catechol or *p*-hydroquinone moiety in the A-ring.

3.3. Antioxidant activity of sage polyphenols evaluated by the phosphomolybdenum method

The phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. The assay was successfully used to quantify vitamin E in seeds (Prieto et al., 1999) and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant polyphenols. Our value for trolox ($A_{695} = 0.4104$), at a concentration of 1 mM, was well comparable with the molar absorption coefficient of vitamin E [$\varepsilon = (4.0 \pm 0.1) \times 10^3$ M^{-1} cm^{-1}] reported by Prieto et al. (1999).

The results (Table 2) show that the sage polyphenols, with the exception of apigenin 6,8-di-*C*-glucoside (**10**), have better antioxidant activities than trolox. The rosmarinic acid derivatives **1**–**5** are particularly potent, being 3.7–5.8 times greater than that of trolox based on a molar basis while, on a weight basis, they are 2.0–2.6 times that of trolox. The flavonoids, on a weight basis, are roughly equivalent to trolox except for apigenin glycoside.

4. Conclusion

The antioxidant activity assessments of sage polyphenols, by measuring their capacity to scavenge the DPPH and superoxide anion radicals and to reduce Mo(VI) to Mo(V), showed that the rosmarinic acid derivatives were potent antioxidants, while the flavonoids, luteolin and apigenin glycosides, possessed comparatively weak to moderate activities. The superoxide scavenging activities of the rosmarinic acid derivatives were 15–20 times stronger than trolox. Being the major polyphenols in sage (or sage residue), the rosmarinic acid derivatives were more likely to be responsible for most of the observed antioxidant activity of the non-diterpenoid component in sage. The potency of these compounds could provide a chemical basis for some of the health benefits claimed for sage in folk medicine and warrant further studies to assess their potential as effective natural remedies.

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