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Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro

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Abstract

Antioxidant-rich fractions were extracted from grape seeds (*Vitis vinifera*) using various solvents, such as acetone, ethyl acetate, methanol and mixtures of different solvents, such as ethyl acetate (EtOAc) and water in 9:1, 17:3 and 4:1 ratios. The antioxidant activity of the extracts was evaluated using a β-carotene-linoleate model system and linoleic acid peroxidation method. At 100 ppm concentration, various extracts showed 65–90% antioxidant activity. Mixtures of EtOAc and water at different concentrations exhibited more antioxidant activity than other extracts. These extracts also showed good reducing power, at 500 μg/ml concentration, by the potassium ferricyanide reduction method. Grape seed extracts may be exploitable for the preservation of food products as well as for health supplements and nutraceuticals.

Keywords: Antioxidant activity; *Vitis vinifera*; Grape seed extracts; Peroxidation models;

1. Introduction

Lipid peroxidation is one of the major reasons for deterioration of food products during processing and storage. The addition of antioxidants is a method of increasing shelf life, especially of lipids and lipid-containing foods. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytolune, have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic (Madahavi & Salunkhe, 1995). Therefore, the search for natural antioxidants, especially of plant origin, has greatly increased in recent years (Loliger, 1991).

Grape (*Vitis vinifera*) is one of the world's largest fruit crops, which approximates an annual production of 58 million metric tons (FAO, 1997). Phenolics in grapes and red wines have been reported to inhibit human low-density lipoprotein (LDL) oxidation in vitro (Frankel, Waterhouse & Tussedre 1995; Teissedre, Frankel, Waterhouse, Peleg & German, 1996). Grape seeds are a rich source of monomeric phenolic compounds, such as (+)-catechins, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate, and dimeric, trimeric and tetrameric procyanidins, and these compounds act as antimutagenic and antiviral agents (Saito, Hosoyama, Ariga, Kataoka & Yamaji, 1998). There are reports of the possible use of phenolics in grapes in preventing atherosclerosis (Kovac & Pekic, 1991). Recognition of such health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary supplement (Laparra, Michaud & Masquelier, 1979) Phenolic compounds, extracted from twelve different varieties of grapes, showed antioxidant activity toward LDL oxidation in vitro (Mayer, Ock-Sook, Person, Waterhouse & Franke, 1997). Studies have been reported of the procyanidin composition of grape seeds (Lee & Jaworski, 1987). The predominant compounds reported are hexamers, but only the structures of some dimer and trimer procyanidins and their acylated derivatives have been elucidated. All of the acylated procyanidins found in grape seeds are esters of gallic acid (Lee & Jaworski, 1990). Teresa, Yolanda, Julian and Celestino (1992) have reported 17 chemical constituents in *V. vinifera* (Tintal del pais) grape seeds. The major compounds are (+)-catechin (11%), epicatechin-(4β→8)-epicatechin (dimer B2) (6%), (−)-epicatechin (10%), epicatechin 3-*O*-gallate-(4β→8)-catechin (B1-3-*O*-gallate) (7%) and (−)-Epicatechin-3-*O*-gallate (9%). Fuleki and Ricardo da Silva (1997) have reported monomers of (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate, 14 dimeric, 11 trimeri procyanidins and one tetrameric procyanidin from grape seeds. Substantial quantities of highly polymerised procyanidins are also present in the grape seeds. It has been

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found that 55% of the procyanidins in grape seeds consist of more than five monomer units. The objectives of this study were to prepare an antioxidant-rich fraction of grape seed extract and to evaluate its antioxidant activity using a β-carotene-linoleate model system and linoleic acid peroxidation method.

2. Materials and methods

2.1. Materials

All solvents/chemicals used were of analytical grade and obtained from Merck, Mumbai, India. β-Carotene, catechin, vanillin, epicatechin, linoleic acid and BHA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Extraction

V. vinifera variety Bangalore blue grapes are widely grown in the States of Karnataka and Tamil Nadu in India (The Wealth of lndia, 1976). Grape seeds were collected from local juice-processing industries. Dried grape seeds were powdered and extracted in a Soxhlet extractor with hexane for 6 h for the removal of fatty matter. The defatted seed powder (50 g) was extracted in a Soxhlet extractor for 10 h with 150 ml of extractants, such as acetone, ethyl acetate and methanol at 60–70°C. The extracts were concentrated in a vacuum evaporator (Buchi, Switzerland) to get a viscous liquid. The procyanidins were precipitated by adding a double volume of hexane to the viscous liquid. The precipitate was collected by filtration under vacuum. The extract obtained was stored in a desiccator after noting the yield. In another method of extraction, the defatted grape seed powder (50 g) was twice extracted by stirring with 150 ml of mixtures of EtOAc and water in varying ratios of 9:1, 17:3 and 4:1, respectively, for 5 h at 60–70°C. The extracts were pooled, dried with anhydrous sodium sulphate and concentrated under vacuum to yield a viscous liquid. Procyanidins were precipitated by adding a double volume of hexane to viscous liquid. The precipitate was collected by filtration under vacuum. The extract obtained was stored in a desiccator after noting the yield.

2.3. Antioxidant assay using β-carotene linoleate model system

The antioxidant activity of grape seed extracts was evaluated by the β-carotene-linoleate model system (Hidalgo, Fernandez, Quilhot & Lissi, 1994) with slight modification. β-Carotene (0.2 mg), 20 mg of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed in 0.5 ml of chloroform. Chloroform was removed at 40°C under vacuum using a rotary evaporator. The resulting mixture was immediately diluted with 10 ml of triple-distilled water and was mixed well for 1–2 min. The emulsion was further made up to 50 ml with oxygenated water. Aliquots (4 ml) of this emulsion were transferred into different test tubes containing 0.2 ml of test samples in ethanol. BHA was used for comparative purposes. A control, containing 0.2 ml of ethanol and 4 ml of the above emulsion, was prepared. The tubes were placed at 50°C in a water bath. Absorbances of all the samples at 470 nm were taken at zero time ($t=0$). Measurement of absorbance was continued until the colour of β-carotene disappeared in the control reaction ($t=180$ min) at 15 min intervals (Fig. 1). A mixture prepared as above without β-carotene served as blank. All determinations were carried out in triplicate. Dose–response relationships of antioxidant activity for grape seed extracts were determined at different concentrations. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula (Hidalgo et al., 1994).

$AA = 100[1-(A_o - A_t)/(A_o^\circ - A_t^\circ)]$ where A_o and A_o° are the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t° are the absorbance measured in the test sample and control, respectively, after incubation for 180 min.

2.4. Antioxidant activity using linoleic acid peroxidation method

Antioxidant activity of grape seed extracts was determined using the thiocyanate method (Yen & Hsieh, 1998). The linoleic acid emulsion was prepared by homogenizing 0.28 g of linoleic acid, 0.28 g of Tween-40 as emulsifier and 50 ml of phosphate buffer (0.2 M, pH 7.0). Test samples were prepared in MeOH: water mix-

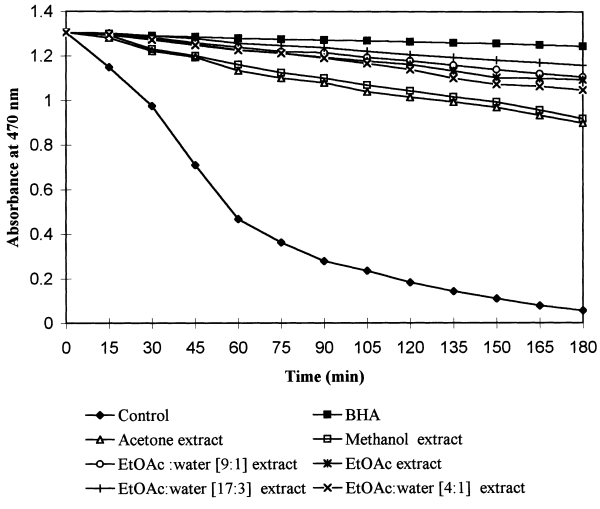

Fig. 1. Antioxidant activity of grape seed extracts and BHA at 100 ppm in β-carotene-linoleate model system.

ture (6:4 v/v). Different test samples (0.5 ml) were mixed with 2.5 ml of linoleic acid emulsion and 2.5 ml of phosphate buffer (0.2 M, pH 7.0) and incubated at 37°C for 120 h. The mixture prepared as above without test sample served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at an interval of 20 h and mixed with 5.0 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM in ferrous chloride in 3.5% HCl and allowed to stand at room temperature for 3 min. The colour developed was measured at 500 nm. The degree of linoleic acid peroxidation was calculated at 100 h using the following formula (Pin-Der Duh, 1998).

$$\text{Antioxidant activity} = 100 - \left(\frac{\text{Increase in absorbance of sample}}{\text{Increse in the absorbance of control}}\right) \times 100$$

Ascorbic acid and BHA were included as standard antioxidants for the comparison. All tests and analyses were carried out in triplicate.

2.5. Determination of reducing power

The reducing power of the test samples was determined by the method of Yen and Duh (1993). Different concentrations of grape seed extracts (167, 334 and 500 μg/ml) in 1 ml methanol were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide in 10-ml test tubes. The mixtures were incubated for 20 min at 50°C. At the end of the incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixtures, followed by centrifuing at 5000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm. The reducing power tests were run in triplicate. Increase in absorbance of the reaction mixture indicated the reducing power of the samples.

2.6. HPLC analyses

Quantification of monomeric flavonols was done by HPLC using catechin and epicatechin as external standards. The chromatographic system consisted of a Shimadzu LC-6A model (Shimadzu, Tokyo, Japan), fitted with a Waters μ-Bondapack™ (Waters Corporation, Milford, MA, USA) C_{18} column (300×3.9 mm I.D.) and a System Controller SCL-6A. The injection system used was a 20-μl sample loop. Detection was done by an UV-visible Spectrophotometer SPD-6AV set at a sensitivity of 0.04 AUFS and a wavelength of 280 nm. Elution was carried out at a flow rate of 1.0 ml/min. The binary mobile phase consists of (A) acetonitrile: 4.5% formic acid (1:9) and (B) 4.5% formic acid. The elution was carried out as by Teresa et al. (1992). The compounds were quantified using a Shimadzu C-R4A Chromatopak data processor at chart speed of 2.5 mm/min. The content of monomeric flavanols was estimated as the sum of each of the monomeric flavanols, such as catechin and epicatechin.

2.7. Estimation of total flavanols

The amount of total flavanols was assayed colorimetrically by the vanillin method using catechin as a standard (Price, Scoyoc & Butler 1978). 5.0 ml of 0.5% in vanillin in MeOH was added to 1.0 ml of methanolic grape seed extracts and mixed well. Similarly, a blank was prepared by adding 5.0 ml of 4% HCl in methanol to 5.0 ml of 0.5% vanillin in MeOH. The absorbances of sample and blank were measured at 500 nm after 20 min in the dark at room temperature. The absorbance of the blank was subtracted from the absorbance of the sample. The content of total flavonols in the grape seed extracts was expressed as catechin equivalents per 100 g of extracts. The content of procyanidins was calculated as the difference between total flavanols and monomeric flavanols and is shown in Table 2 (Saito et al., 1998). The estimation of procyanidins and monomeric flavanols in these fractions was carried out in triplicate and the results were averaged.

2.8. Statistical analysis

Student *t*-test has been done to compare the data and all tests were considered statistically significant at $P < 0.05$.

3. Results and discussion

The yields obtained by using various extractants and their composition of total flavanols and monomeric flavanols are shown in Tables 1 and 2. Of the various sol-

Table 1
Yield of grape seed extracts[a]

Solvents used for extraction	Extract yield (% dry grape seeds)
Acetone	6.7±0.12[b]
Methanol	8.1±0.14[b]
EtOAc	4.5±0.13[b]
EtOAc: water (9:1)	2.0±0.08[c]
EtOAc: water (17:3)	2.5±0.09
EtOAc: water (4:1)	3.1±0.08[c]

[a] Values expressed are mean ± S.D. of five experiments
[b] Significant when EtOAc:water (17:3) was compared to other extracts.
[c] Not significant when EtOAc:water (17:3) was compared to other extracts.

vents and solvent mixtures used for the extraction of total flavanols, the mixture of EtOAC and water (17:3 v/v) was found to be the most effective. Extraction with MeOH gave maximum yield of the extract but with a lower content of total flavanols. Yields of extracts in EtOAc:water (17:3) are significantly less than in acetone, methanol, EtOAc and not significantly different from EtOAc:water (9:1) or EtOAc:water (4:1).

The antioxidant activity of grape seed extracts and BHA at 100 ppm concentration as measured by the bleaching of β-carotene, is presented in Fig. 1. It can be seen that grape seed extracts prepared by different solvents exhibited varying degrees of antioxidant activity. EtOAc: water mixture (17:3) was found to give the maximum antioxidant activity. The mechanism of bleaching of β-carotene is a free-radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene, in this model system, undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. The presence of different extracts can hinder the extent of β-carotene-bleaching by neutralising the linoleate-free radical and other free radicals formed in the system.

The antioxidant effect of various extracts from grape seeds, in preventing the peroxidation of linoleic acid, as measured by thiocyanate method, is shown in Fig. 2. In this control, the OD has increased up to 1.17 at 100 h, then it has decreased. This is due to oxidation of linoleic acid generating linoleic acid hydroperoxides, which decompose to many secondary oxidation products (Hua-Ming, Koji, Fumio & Nokihara 1996). The oxidised products react with ferrous sulphate to form ferric sulphate, then to ferric thiocyanate of blood-red colour. After the incubation period (120 h), the formation of peroxides will be stopped, due to non-availability of linoleic acid. Also, the intermediate products may be converted to stable end-products. The non-availability of hydroperoxides, results in the stoppage of oxidation of ferrous sulphate. Hence, the OD will be reduced. In the presence of antioxidants, oxidation of linoleic acid will be slow. Hence, the colour development, due to formation of thiocyanate, will be slow. Of the six extracts, the highest antioxidant activity was observed with the extract prepared by the EtOAc water-mixture (17:3), which exhibited 86% inhibition of linoleic acid peroxidation at 100 h.

The antioxidant activity has been reported to be concomitant with the reducing power (Tanaka, Kuie, Nagashima & Tuguchi, 1988). Fig. 3 shows the reducing powers of different grape seed extracts using the potassium ferricyanide reduction method. At 0.5 mg/ml concentration, the extract obtained using acetone, ethyl acetate, methanol or EtOAc: water (9:1), (17:3) and (4:1) showed absorbances of 0.52, 1.737, 0.903, 1.95, 2.59 and 1.78, respectively. Thus, the highest reducing activity was observed for the extract obtained by ethyl acetate water mixture (17:3). The reducing properties are generally associated with the presence of reductones (Pin-Der Duh, 1998). Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of the free-radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The data presented here indicate that the marked antioxidant

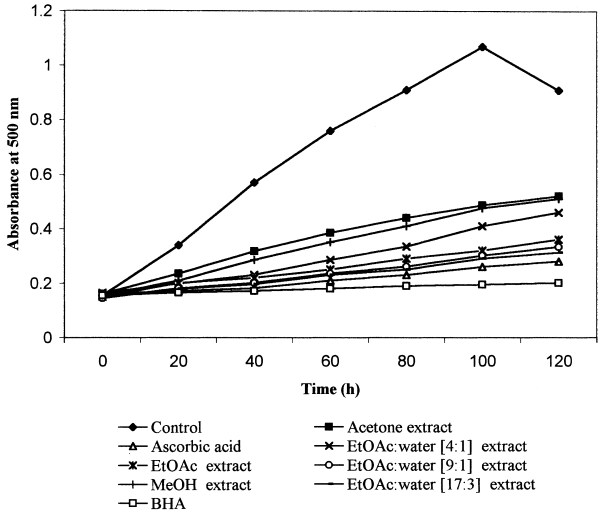

Fig. 2. Antioxidant activity of grape seed extracts, BHA and ascorbic acid at 100 ppm by thiocyanate method.

Table 2
Composition of grape seed extracts (%) (in catechin equivalents/100 g extract)[a]

Component	Acetone extract	EtOAc extract	MeOH extract	EtOAc:water (9:1) extract	EtOAc:water (17:3) extract	EtOAc:water (4:1) extract
Monomeric flavanols	11.5±0.24	22.5±1.22	10.5±1.82	24.8±2.40	30.2±2.26	29.7±2.21
Procyanidins	3.5±1.11	12.5±0.98	5.5±0.89	18.2±0.94	23.8±2.16	20.8±2.14
Total flavanols	15.0±1.35[b]	35.0±2.10[b]	16.0±2.71[b]	43.0±3.34[b]	54.0±4.86	50.5±4.35[c]

[a] Values expressed are mean S.D. of three experiments
[b] Significant when EtOAc:water (17:3) was compared to other extracts.
[c] Not significant when EtOAc:water (17:3) was compared to other extracts.

activity of grape seed extracts seems to be the result of their reducing power. The grape seed flavanol/procyanidin compounds may act in a similar fashion as reductones by donating electrons and reacting with free-radicals to convert them to more stable products and terminating the free-radical chain reaction.

The acetone, methanol, EtOAc and EtOAc: water (9:1), (17:3) and (4:1) extracts showed 63, 66, 70, 81, 86 and 78% antioxidant activities respectively, by the thiocyanate method at 100 ppm concentration. The dose-response of antioxidant activity was obtained by the β-carotene bleaching method. The percentage of antioxidant activity by all the extracts showed positive correlation [acetone, $r = +0.88$; MeOH, $r = +0.93$; EtOAc, $r = +0.92$; EtOAc:water (9:1), $r = +0.96$; EtOAc:water (17:3), $r = +0.98$ and EtOAc:water (4:1), $r + 0.96$] with increasing dose. However, the degree of antioxidant activity by the EtOAc:water (17:3) extract was higher than any fraction at any dose. At 75 ppm for all the extracts taken into consideration, the percentage of antioxidant activity of the EtOAc:water (17:3) was significantly higher than all other extracts (Table 3).

The results shown above indicate that use of a single solvent, such as acetone and methanol give a high yield of the extract with low antioxidant activity and low reducing power, whereas EtOAc and mixtures of EtOAc and water give low yields of extract with high antioxidant activity and high reducing power. Dumon (1990) reported that an acetone-water mixture gives a better extraction of procyanidins from grape seeds in comparison with other extractants. However, the use of these extractants generally results in significant co-extraction of other substances and a decrease in the concentration of procyanidins. Alonso, Bourzeix and Revilla (1991) reported that the extraction of catechins and procyanidins was more efficient when the ethanol content of the mixture of ethanol-water extractant was increased and the extraction time increased from 3 to 72 h. On the other hand, Kalhithraka, Garcia Viguera, Bridle and Bakker (1995) reported that methanol was the best solvent for the qualitative extraction of (+) catechins, (−) epicatechin and epigallocatechin from the grape seeds. It is known that procyanidins are highly soluble in EtOAc and that this solvent exhibits significant selectivity in extracting procyanidins from natural products (Pekic & Stamenkovic, 1972). The present study confirms the extraction of more total flavanol compounds by EtOAc with respect to yield. The EtOAc extract gives more monomers, such as catechin and epicatechin type compounds, than procyanidins. However EtOAc: water (9:1), (17:3) and (4:1) may extract more polar compounds, such as trimers, tetramers and pentamers. The presence of water increases permeability of seed tissue and thus enables a better mass transport by molecular diffusion. Generally, monomers have lower antioxidant activities than dimers and others. On comparing Tables 2 and 3, the EtOAc: water (17:3) gave a 4-times higher level of total flavanols than the acetone extract and the two extracts showed 90 and 65% of antioxidant activity, respectively. This may be due to the different flavanol compositions in the two extracts. It is evident that the level of procyanidins in EtOAc: water (17:3) is 23.8% whereas, in the acetone extract it is only 3.5%. Thus the results of the present work indicate that the selective extraction of antioxidant from natural sources by an

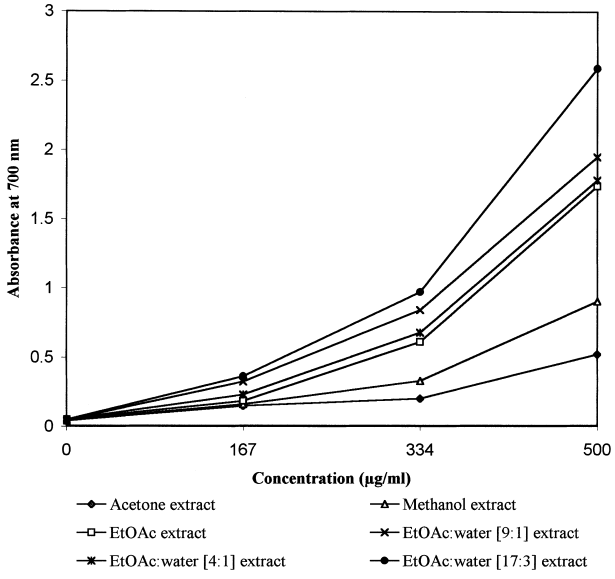

Fig. 3. Reducing power of grape seed extracts.

Table 3
Dose (ppm)-response of antioxidant activity (%) for different grape seed extracts by β-carotene bleaching method[a]

Extracts	10 ppm	25 ppm	50 ppm	75 ppm	100 ppm	150 ppm	200 ppm
Acetone	7.0±1.76	18.7±1.51	25.0±1.04	46.0±1.76[b]	65.0±1.53	76.0±2.81	87.3±3.01
MeOH	7.5±1.50	19.7±1.93	28.0±1.53	49.0±2.76[b]	66.0±2.26	78.7±3.51	88.7±3.85
EtOAc	10.0±1.76	18.0±1.04	38.0±2.52	64.0±3.76 [b]	77.3±2.51	–	–
EtOAc:water (9:1)	12.0±2.04	28.0±2.26	54.7±1.66	71.3±3.56[b]	81.0±1.76	–	–
EtOAc:water (17:3)	15.7±1.85	33.0±2.53	58.3±1.29	80.7±1.50	89.3±2.36	–	–
EtOAc:water (4:1)	10.0±2.50	20.0±1.75	44.0±1.08	69.0±2.32[b]	83.3±2.69	–	–

[a] Values expressed are mean S.D. of three experiments
[b] Significant when EtOAc:water (17:3) was compared to other extracts

appropriate solvent mixture is very important in obtaining a fraction with high antioxidant activity.

4. Conclusion

The results of the present work indicate the presence of compounds possessing high antioxidant activity in grape seeds (*Vitis vinifera*). The different activities of the grape extracts can be ascribed to their different phenolic compositions. Further studies are needed, however, with individual phenolic compounds of grape seeds to elucidate the different antioxidant mechanisms and possible synergism.

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