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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Antioxidant phenolic compounds and flavonoids of *Mitragyna rotundifolia* (Roxb.) Kuntze in vitro

Wen-Yi Kang · Cai-Fang Li · Yu-Xin Liu

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Abstract Different solvent extracts of leaves and bark of *Mitragyna rotundifolia* (Roxb.) Kuntze were evaluated by DPPH, ABTS, and FRAP assays, respectively, for antioxidant properties. Total phenolic and flavonoid content was determined as pyrocatechol and rutin equivalents, respectively, and correlated with antioxidant activities. More polar solvent extracts (*n*-butanol and ethyl acetate) had relatively higher antioxidant activity than nonpolar solvent extracts (petroleum ether). The *n*-butanol extract also exhibited a higher phenolic and flavonoid content than the other solvent extracts did. The DPPH assay was highly correlated with the ABTS assay ($R^2 = 0.9628$, $P < 0.0001$). Two phenolic and four flavonoid compounds were isolated from the ethyl acetate leaf extracts. Compounds **3–6** were isolated for the first time from the genus of *Mitragyna* and compound **5** showed the highest antioxidant activity.

Keywords *Mitragyna rotundifolia* (Roxb.) Kuntze · Antioxidant activity · DPPH · ABTS · FRAP · Phenolic · Flavonoids

Introduction

There is increasing interest in the role of free-radical-mediated damage in the etiology of human diseases. Oxidative damage to body cells and molecules has been widely postulated to be involved in the causation and progression of a range of

W.-Y. Kang (✉) · C.-F. Li · Y.-X. Liu
Institute of Natural Products, Henan University, Kaifeng, Henan 475004, People's Republic of China
e-mail: kangweny@hotmail.com

W.-Y. Kang · C.-F. Li · Y.-X. Liu
Institute of Chinese Materia Medica of Pharmaceutical College, Henan University, Kaifeng, Henan 475004, People's Republic of China

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chronic diseases, such as cardiovascular disease, neuronal disease, cataracts, and several forms of cancer (Halliwell, 1997). Human metabolism counts on an antioxidant defensive system involving enzymes and proteins to prevent these effects. However, the defenses can be overwhelmed in certain circumstances so that harmful effects occur. It is accepted that the intake of antioxidant substances reinforces defenses against free radicals. Plants are a rich source of natural antioxidants, and some of them, such as the tocopherols (vitamin E), ascorbic acid (vitamin C), and carotenoids, are substances of major significance in human physiology. In addition, the use of synthetic antioxidants has been limited because of their toxicity (Valentao *et al.*, 2002). Therefore, it is of great significant and necessary that research focuses on discovering potential natural, effective antioxidants to replace the synthetic ones. And a considerable market awaits these compounds.

The genus *Mitragyna* (Rubiaceae) is found in swamps in the tropical and subtropical regions of Africa and Asia, including four species in Africa and six species in India and Asia (Shellard *et al.*, 1978). Several have been used in local folklore medicine for a wide variety of diseases such as fever, colic, and muscular pains and for expulsion of worms (Shellard and Phillipson, 1964). Shellard and his coworkers have contributed significantly to the chemotaxonomic literature (Shellard *et al.*, 1969; Shellard and Houghton, 1971, 1974). *Mitragyna* is very rich in alkaloids and many have been isolated from leaves, stems, and roots (Shellard, 1983). *M. rotundifolia* (Roxb.) Kuntze is a shrub growing in southern Yunnan province, China. As part of our continuing search for natural antioxidants from *M. rotundifolia*, triterpenoid saponins and other compounds were isolated from the bark of *M. rotundifolia* (Kang and Hao, 2006; Kang *et al.*, 2006). The present paper describes the antioxidant activity of different solvent extracts from *M. rotundifolia* and active compounds from leaf extracts. This is the first report of the antioxidant activity of *M. rotundifolia*.

Here the antioxidant activity of *M. rotundifolia* was measured using three different analytical methods: DPPH radical scavenging activity, ABTS radical scavenging activity, and ferric reducing/antioxidant power (FRAP) for evaluation of reduction. PG, BHA, and BHT were used as positive controls. All determinations were made in triplicate.

Phenolic compounds, or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8,000 phenolic structures currently known (Harbone, 1980). The most widely distributed in plants are the hydroxycinnamic, *p*-coumaric, caffeic, and ferulic acids. They are secondary metabolites and their reactivity is due to the acidic character of the phenolic function and the nucleophilic character of the benzene ring. Based on their carbon skeleton, polyphenols are classified as nonflavonoid compounds (stilbenes, hydroxycinnamic acids, benzoic acids) and flavonoid compounds (flavanones, flavonols, flavones, isoflavonoids, flavanols, anthocyanidins). It was reported that the antioxidant activity of plant materials was well correlated with their phenolic content (Velioglu *et al.*, 1998). Thus, it is important to consider the effect of total phenolic content on the antioxidant activity of *M. rotundifolia* extracts and to

correlate the antioxidant activities with the total phenolic content determined with Folin–Ciocalteu reagent, using pyrocatechol as a standard.

Flavonoids are natural phenolic compounds and have been proven to display a wide range of pharmacological and biochemical properties. In various studies, the antioxidant activity of plant extracts was correlated with the concentration of flavonoids (Cakir *et al.*, 2003). Therefore, we also correlated the antioxidant activity of *M. rotundifolia* extracts with the total flavonoid content by the $Al(NO_3)_3$ method, using rutin as a standard.

Materials and methods

General procedures and reagents

^{1}H-NMR (400 MHz) and ^{13}C-NMR spectra (100 MHz) were recorded on a Bruker Avance DRX400 spectrometer. Chemical shifts are expressed as parts per million referenced to TMS. Coupling constants (J) are reported as hertz. Mass spectra were obtained with an Autospec-Q VG-Analytical instrument. The following were used to determine antioxidant activity: 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Tokyo Kasei; 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), propyl gallate (PG), butylated hydroxyanisole (BHA), and 2,6-di-tert-butyl-4-methylphenol (BHT) from Acros Organics; 6- hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Aldrich; and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) from Fluka. Folin-Ciocalteu reagent was from Merck. All other reagents were analytical grade from China. Spectrophotometric measurements were performed on a UV–VIS spectrometer (Unico UV-2000; China).

Plant material and extraction

The bark and leaves of *M. rotundifolia* were collected in May 2006 in Xishuangbanna, Yunnan province, China. A voucher specimen was deposited at the Institute of Natural Products, Henan University. Plant materials were air-dried at room temperature and ground to a fine powder, then extracted three times with 70% acetone at room temperature. After solvent acetone evaporation in vacuo, the concentrated water extract was sequentially extracted with petroleum ether, ethyl acetate extract, and *n*-butanol. All samples were prepared by dissolving the dried extract in MeOH at different concentrations.

Purification of compounds

Dried powder of the bark (1 kg) and leaves (700 g) was extracted three times with acetone (70%) at room temperature. After solvent evaporation in vacuo, the concentrate was suspended in water and extracted with petroleum ether, ethyl acetate, and *n*-butanol. Dried leaf powder used the same procedure as for bark. The ethyl acetate extract (12 g) was subjected to CC over silica gel (200–300 mesh) developed with petroleum ether–acetone (95:5–6:4). The petroleum ether–acetone

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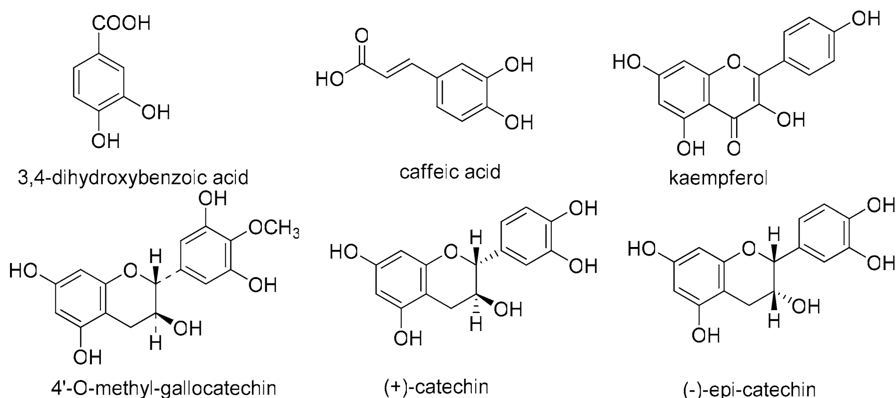
(10:1) fraction was chromatographed repeatedly by CC over silica gel to give 3,4-dihydroxybenzoic acid (60 mg; **1**), caffeic acid (102 mg; **2**), kaempferol (41 mg; **3**), 4′-*O*-methyl-gallocatechin (76 mg; **4**), (+)-catechin (305 mg; **5**), and (−)-*epi*-catechin (32 mg; **6**).



DPPH radical scavenging assay

The stable free radical DPPH·was dissolved in MeOH to give a 60 μM solution; 0.1 ml of a test compound in MeOH (or MeOH itself as control) was added to 3.5 ml of the methanol DPPH· solution. Different concentrations were tested for each compound. After further mixing, the decrease in absorbance was measured at 515 nm after 30 min. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The antioxidant activity is expressed as an IC_{50} value, i.e., the concentration (μg/ml) that inhibits DPPH· absorption by 50%, and was calculated from the concentration-effect linear regression curve (Liu *et al.*, 2004; Li *et al.*, 2008).

ABTS radical scavenging assay

The ABTS radical cation ($ABTS^{+}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark, at room temperature, for 12 h before use. The $ABTS^{+}$ solution was diluted with methanol to an absorbance of 0.800 ± 0.05 at 734 nm. Next, 2.85 ml of this $ABTS^{+}$ solution was added to 0.15 ml of different concentrations of the methanolic samples and the decrease in absorbance at 734 nm was observed after mixing up to 10 min. The radical scavenging activity of the tested samples is also expressed as the IC_{50} value. The percentage inhibition of $ABTS^{+}$ was calculated using the formula: %inhibition = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the sample and the standard compound (Re *et al.*, 1999; Li *et al.*, 2008).

FRAP assay

Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working fresh solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3 \cdot 6H_2O$ solution. Samples (200 μl) were allowed to react with 3800 μl of FRAP solution for 30 min at 37°C. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Trolox was used as a reference standard. The standard curve was linear between 25 and 400 μM Trolox. Results are expressed as micromoles Trolox equivalents (TE) per gram sample. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve (Benzie and Strain 1996; Li *et al.*, 2008).

Determination of total phenolic content

Total phenolic content was determined with Folin-Ciocalteu reagent (FCR) with some modifications (Mohammadzadeh *et al.*, 2007). Methanol extracts (0.1 ml) were diluted with distilled water (0.4 ml) and mixed with 2.5 ml of a 10-fold diluted solution of 2 N Folin–Ciocalteu reagent. Two milliliters of saturated sodium carbonate solution was added to the mixtures, followed by shaking. The absorbance of the reaction mixtures was measured at 765 nm after 2 h. Pyrocatechol was used as a standard in the range of 0.025–0.3 mg/ml to construct a standard curve. Results are expressed as micrograms of pyrocatechol equivalents (PEs) per microgram of extract.

Determination of total flavonoid content

Total flavonoid content was determined by a colorimetric method (Zieliński *et al.*, 2007). Briefly, 0.5 ml of the extract was diluted with 2.5 ml of distilled water. Then 150 μl of a 5% $NaNO_2$ solution was added, and the mixture kept at room temperature. After 6 min, 300 μl of a 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for a further 5 min. After that, 1.0 ml of 1 M NaOH was added. Finally, 0.55 ml distilled water was added to the mixed solution. The final solution was well mixed, and absorbance was immediately measured against the prepared blank at 510 nm. Rutin was used as a standard in the concentration range of 0.025–0.2 mg/ml to construct a standard curve. Results are expressed as micrograms of rutin equivalents (REs) per microgram of extract.

Results and discussion

Active compounds

The secondary metabolites present in the leaves, namely, 3,4-dihydroxybenzoic acid (**1**) (Kang *et al.*, 2006), caffeic acid (**2**) (Kang *et al.*, 2006), kaempferol (**3**) (Ren *et al.*, 2005), 4′-*O*-methyl-gallocatechin (**4**) (Mello *et al.*, 1996), catechin (**5**) (Cui

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et al., 2005), and *epi*-catechin (**6**) (Cui *et al.*, 2005). Compounds **1** and **2** were phenolic compounds; compounds **3–6** were flavonoids. The structures of all compounds were identified by ^{1}H- and ^{13}C-NMR and mass spectroscopic techniques, and their spectral data were confirmed by comparison reported data.

Antioxidant activity of extracts evaluated by DPPH, ABTS, and FRAP assays

Antioxidant activity results for different extracts of leaves and bark of *M. rotundifolia* determined by DPPH, ABTS, and FRAP assays are reported in Table 1. The ethyl acetate extract of leaves was found to be the most active extract in DPPH assay; with a DPPH radical scavenging activity higher than that of BHA and BHT but lower than that of PG. The *n*-butanol extract of bark of *M. rotundifolia* had activity similar to that of the leaf ethyl acetate and had DPPH radical scavenging activity higher than that of BHA and BHT but lower than that of PG. The *n*-butanol extract of leaves and the bark ethyl acetate extract had activity higher than that of BHT but lower than that of BHA and PG. The petroleum ether leaf and bark had no activity in the DPPH assay.

The leaf ethyl acetate extract was also found to be the most active in the ABTS assay; it showed higher ABTS scavenging activity than BHA and BHT but lower activity than PG. The leaf *n*-butanol extract showed activity similar to that of the bark ethyl acetate extract. The *n*-butanol and bark ethyl acetate extracts had higher activity than BHT did but lower than BHA and PG. The petroleum ether extract of leaves and bark had the lowest activity in ABTS assays, lower than that of BHT, BHA, and PG.

Leaf *n*-butanol extracts demonstrated the highest activity in the FRAP assay; it was lower than that of PG and BHA and higher than that of BHT. Bark *n*-butanol extracts, and the ethyl acetate extracts of leaves and bark showed higher activity

Table 1 Antioxidant activity of various extracts of leaves and bark of *M. rotundifolia*

Plant organ	Extract	DPPH assay: IC_{50} (μg/ml)	ABTS assay: IC_{50} (μg/ml)	FRAP value (μmol TE/g extract)
Leaves	70% acetone	NT	NT	NT
	Petroleum ether	NA	75.8	139.69
	Ethyl acetate	2.24	1.165	801.61
	n-Butanol	4.00	1.61	1395.94
Bark	70% acetone	NT	NT	NT
	Petroleum ether	NA	91.73	159.81
	Ethyl acetate	4.90	2.33	784.74
	n-Butanol	3.02	1.685	1275.43
	PG[a]	0.94	0.885	5159.97
	BHA[a]	3.43	1.675	2573.96
	BHT[a]	18.79	4.555	238.11

NT not tested because of solubility, *NA* not active

[a] Used as positive control

than that of BHT. While petroleum ether extracts of leaves and bark had the lowest activity in the FRAP assay, and was lower than that of BHT, BHA and PG.

The results indicate that *n*-butanol and ethyl acetate extracts showed higher antioxidant activity than the petroleum ether extract, and the leaves and bark extract of the same solvent had similar antioxidant activity.

Total phenolic and flavonoid content

The total phenolic and flavonoid content extracts were examined and the results are presented in Table 2. All of the solvent extraction systems used showed a wide range of concentrations, from 0.019 to 0.333 mg PE/mg extract for total phenolic content, and from 0.040 to 1.835 mg RE/mg extract for total flavonoid content. The extraction yields also varied, from 0.20 to 21.13 g/100 g plant material.

The leaf *n*-butanol extract had the highest total phenolic content, followed by *n*-butanol bark extract, ethyl acetate extract of leaves, 70% acetone extract of leaves, ethyl acetate extract of bark, 70% acetone extract of bark, petroleum ether extract of leaves, and petroleum ether extract of bark, respectively.

The leaf ethyl acetate extract had the highest total flavonoid content, followed by *n*-butanol extract of bark, *n*-butanol extract of leaves, 70% acetone extract of leaves, ethyl acetate extract of bark, 70% acetone extract of bark, petroleum ether extract of leaves, and petroleum ether extract of bark, respectively.

Leaves had higher total phenolic and flavonoid contents than bark did for the same solvent extract, significantly different for the 70% acetone and ethyl acetate extract in total flavonoid content, and the leaves had a higher total extract yield than the bark. The *n*-butanol extract showed higher phenolic and flavonoid contents than other extracts did. Phenolic and flavonoid compounds existing in plants are known as powerful chain-breaking antioxidants and may contribute directly to antioxidative action. This may explain the stronger antioxidant activity of *n*-butanol and ethyl acetate extracts than of petroleum ether extracts.

Table 2 Percentage yield and total phenolic and flavonoid content of *M. rotundifolia* extracts

Plant organ	Extract	Yield (w/w, g/100 g)	Phenolic content (mg PE/mg extract)	Flavonoid content (mg RE/mg extract)
Leaves	70% acetone	17.79	0.275 ± 0.003	1.211 ± 0.027
	Petroleum ether	0.65	0.021 ± 0.001	0.078 ± 0.006
	Ethyl acetate	3.53	0.284 ± 0.002	1.835 ± 0.034
	n-Butanol	11.69	0.333 ± 0.006	1.470 ± 0.028
Bark	70% acetone	21.13	0.167 ± 0.005	0.598 ± 0.025
	Petroleum ether	0.20	0.019 ± 0.001	0.040 ± 0.004
	Ethyl acetate	1.54	0.264 ± 0.002	0.907 ± 0.066
	n-Butanol	7.97	0.313 ± 0.008	1.718 ± 0.060

PE pyrocatechol equivalent, *RE* rutin equivalents. Values are the mean ± SD of three parallel measurements ($P < 0.05$)

Correlation between total phenolic and flavonoid content and antioxidant activity

In both DPPH and ABTS assays, the lower the IC_{50} value of a sample, the stronger its antioxidant activity. In FRAP assays, samples with a higher FRAP value have greater antioxidant activity. Thus, when comparing the FRAP assays with the DPPH and ABTS assays, we correlated the $1/IC_{50}$ values in DPPH and ABTS assays with the FRAP values.

As reported in Table 3, the DPPH assay correlated highly with the ABTS assay ($R^2 = 0.9628$, $P < 0.0001$), and the $1/IC_{50}$ in the DPPH assay correlated reasonably well with the FRAP values ($R^2 = 0.8031$, $P < 0.01$). The correlation between the $1/IC_{50}$ in the ABTS assays and FRAP values was less significant ($R^2 = 0.6376$, $P < 0.01$). The results suggest that the three methods have a similar predictive capacity for antioxidant activity.

The antioxidant activity data from ABTS assays ($1/IC_{50}$) were highly correlated with the total flavonoid content ($R^2 = 0.955$, $P < 0.001$) and reasonably well correlated with the total phenolic content ($R^2 = 0.8404$, $P < 0.05$). The total phenolic content also correlated well with the FRAP values ($R^2 = 0.8975$, $P < 0.01$). The correlation between the total flavonoid contents and the FRAP values were relatively higher ($R^2 = 0.751$, $P < 0.05$). The antioxidant activity data from DPPH ($1/IC_{50}$) and the total flavonoid contents had a relatively higher correlation coefficient but a higher p value ($R^2 = 0.7612$, $P > 0.1$). The antioxidant activity data from DPPH ($1/IC_{50}$) and the total phenolic contents had a very low correlation coefficient ($R^2 = 0.00008$).

The high degree of correlation in the simple spectrophotometric assay between total phenolic and flavonoid contents and antioxidant activity of the extracts, as determined by ABTS and FRAP assays, showed that the assay for total phenolic and flavonoid content would be a useful technique for rapid evaluation of antioxidant activity in this plant.

Antioxidant activity of the compounds isolated

Antioxidant activity of compounds **1–6** isolated from leaf ethyl acetate extract, evaluated by DPPH and ABTS assays (Table 4).

Table 3 R^2 correlation values between antioxidant activities and total phenolic and flavonoid contents of extracts of *M. rotundifolia*

	DPPH	ABTS	FRAP
ABTS	0.9628 (7)[a]		
FRAP	0.8031 (7)	0.6376 (9)	
Phenolic content	0.00008 (4)	0.8404 (6)	0.8975 (6)
Flavonoid content	0.7612 (4)	0.9550 (6)	0.7510 (6)

[a] Number of samples in parentheses

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Table 4 Antioxidant activity of compounds **1–6** isolated from *M. rotundifolia* in DPPH and ABTS assays

Compound	Test compound	DPPH assay: IC_5 (μg/ml)	ABTS assay: IC_5 (μg/ml)
1	3,4-Dihydroxybenzoic acid	NT	2.47
2	Caffeic acid	1.80	1.54
3	Kempferol	2.51	1.03
4	4′-*O*-Methyl-gallocatechin	2.57	0.78
5	Catechin	1.40	0.56
6	*epi*-Catechin	1.61	1.83
	PG[a]	0.94	0.885
	BHA[a]	3.43	1.675
	BHT[a]	18.79	4.555

NT not tested because of solubility

[a] Used as positive control

In the DPPH assay, the activity of compound **5** was the highest, with a higher antioxidant activity than the synthetic antioxidants BHA and BHT, and less antioxidant activity than PG. Compounds **2**, **3**, **4**, and **6** also had higher activity than that of BHA and BHT.

In ABTS assays, compound **5** also showed the highest activity, with higher antioxidant activity than PG, BHA, and BHT. Compounds **2** and **3** had higher antioxidant activity than BHA and BHT and lower than PG. Compounds **1** and **6** had higher antioxidant activity than BHT but lower than PG and BHA.

The results show that compound **5** is the highest-activity compound in both DPPH and ABTS assays. Compounds **1–4** and **6** are less active but also display higher antioxidant activities compared with the positive control, BHT.

Conclusion

This is the first time that the antioxidant activity of *M. rotundifolia* has been reported. The antioxidant activity in vitro and total phenolic and flavonoid contents were assessed in 70% acetone, petroleum ether, ethyl acetate, and *n*-butanol bark and leaf extracts. The antioxidant activities of polar solvent extracts (*n*-butanol, ethyl acetate) were in general relatively higher than those of nonpolar solvent extracts (petroleum ether), and the leaf and bark extracts of the same solvent had similar antioxidant activity in the same antioxidant activity determination. In determinations of the total phenolic and flavonoid contents, the leaves had higher total phenolic and flavonoid contents than bark did for the same solvent extract. The *n*-butanol extracts showed higher phenolic and flavonoid contents than the other solvent extracts. The antioxidant activity of plant material is dependent on the type and polarity of the extracting solvent, as well as on the test system and the substrate protected by the antioxidant (Heinonen *et al.*, 1998; Moure *et al.*, 2000; Kang and

Lee, 2001). Six compounds were isolated, including two phenolic and four flavonoids from the ethyl acetate extract from leaves, and compound **5** had the highest antioxidant activity. Thus, *M. rotundifolia* is a good source of natural antioxidants.

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