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REVIEW ARTICLE

ANTITUMOUR-PROMOTING AND CYTOTOXIC CONSTITUENTS OF *ETLINGERA ELATIOR*

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Phytochemical studies on rhizome of *Etlingera elatior* have resulted in the isolation of 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone (1), demethoxycurcumin (2), 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (3), 16-hydroxylabda-8(17),11,13-trien-16,15-olide (4), stigmast-4-en-3-one (5), stigmast-4-ene-3,6-dione (6), stigmast-4-en-6b-ol-3-one (7), 5α,8α-epidioxyergosta-6,22-dien-3β-ol (8). 1 and 4 were new compounds. Compounds 5 and 7 displayed high antitumour-promoting activity. Ethyl acetate extract showed a very significant cytotoxic activity against CEM-SS and MCF-7 cell lines (4 μg/ml and 6.25 μg/ml respectively). The antitumour-promoting activity was determined by EBV-EA assay and cytotoxic activity was determined by MTT assay.

Key words *: Etlingera elatior, antitumour-promoting, EBV-EA, cytotoxic, CEM-SS, MCF-7*

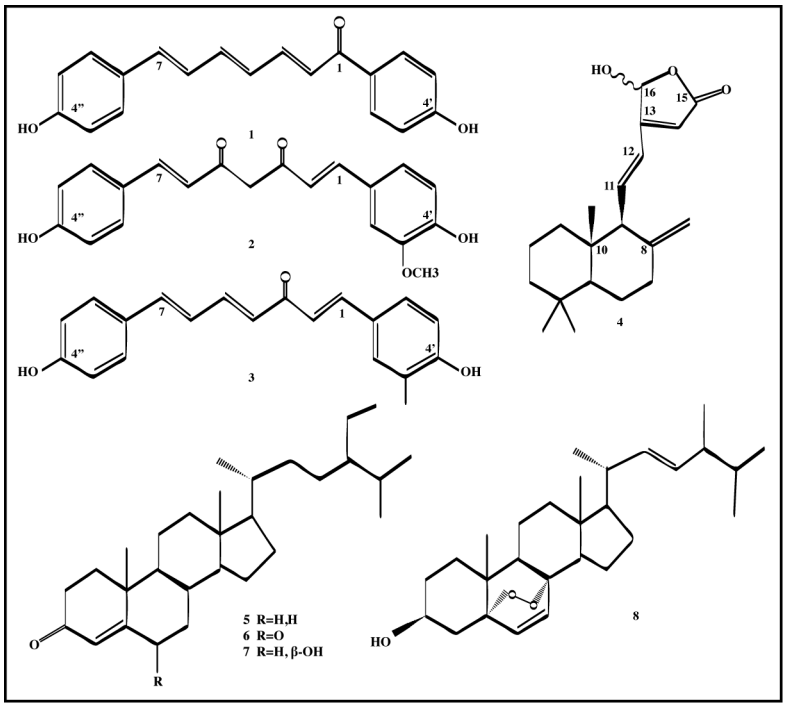
Introduction

Etlingera elatior belongs to the Zingiberacea family, and is classified under the genera of *Etlingera*. This plant is * Locally known as kantan. The mature fruits of *Etlingera elatior* are edible but sour, and are reputed for their antihypertensive activity. In many parts of Southeast Asia, the young inflorescence is used as "ulam" or as ingredients in laksa, curry, and mixed vegetables. A decoction of the fruits may be dropped into the ear to treat earache, and a decoction of leaves may be used to clean wounds (1). The decoction of young shoots is used to reduce body odour after giving birth. Mackeen *et al*. (1997) reported that the aqueous ethanol extract of the flower shoots of *E. elatior* possessed antimicrobial activity and was cytotoxic to HeLa cell line (2). Habsah *et al.* in 2003, reported the antioxidant activity and antitumour promoting activity of the crude dichloromethane and methanol extracts of *E. elatior* (3). The diarylheptanoids **1**-**3** from the ethyl acetate extract was reported to have high antioxidant activity (4). The previous screening of its flower shoot extract showed promising antitumour promoting activity (5). No phytochemical study has been done on this species except their essential oil of it young flower shoots (6), thus the objective of this study was to isolate cytotoxic and antitumour promoting compounds from the rhizome of *E. elatior* .

Materials and Methods

Plant material. One hundred kg of the fresh *E. elatior* rhizomes were collected in Klang and Banting, Selangor in October 1999. The rhizomes were cleaned, chopped into smaller pieces (3-5 mm thickness) and dried under the shade. A voucher specimen (No. SK 80/01) was deposited at the Herbarium of Laboratory of Phytomedicines (LF),

Figure 1: Compounds isolated from Etlingera elatior



University Putra Malaysia.

Extraction and isolation. Sixteen kg of the dried powdered rhizomes (16% w/w of fresh rhizomes) were extracted three times each, first with $CHCl_3$, then with acetone, and finally with MeOH, to give 120 g, 50 g and 8 g of extracts, respectively. The $CHCl_3$ extract was triturated with hexane and filtered to give hexane (60 g) and $CHCl_3$ soluble extracts (60 g). The acetone extract was triturated with ethyl acetate to give 8 g of ethyl acetate soluble extract. Column chromatography (CC) of $CHCl_3$ extract (40 g) on silica gel (5 x 40 cm) eluted with hexane/diethyl ether, diethyl eter/ethyl acetate, ethyl acetate/MeOH, gave combine fractions A-J respectively. Repeated CC of fraction C (3 g) on silics gel using diethyl ether in hexane (1:9) gave **7** (20 mg) and **8** (8 mg). Repeated CC of hexane extract (20 g) on silica gel (5 x 40 cm), eluted with hexane/diethyl ether, afforded eight fractions (A-H). Repeated column of fraction F (3 g) afforded four fractions (F1-F4), from which **5** (65 mg) was isolated from fraction F2 (138 mg) after recrystalisation with MeOH. Compound **6** (50 mg) was isolated from fraction F4 (77.3 mg) after preparative TLC (20% diethyl ether in hexane). Compound **4** (11.9 mg). was isolated from fraction H (80 mg) after repeated column chromatography on silica gel eluted with 10% ethyl acetate in $CHCl_3$. Repeated CC of the ethyl acetate soluble extract (8 g) on sephadex LH 20 (2.5 x 40 cm), eluted with MeOH, afforded 14 fractions (fractions EA-EN) Repeated CC of fraction EK (160 mg) on silica gel, with 10% ethyl acetate in $CHCl_3$ as the eluent, followed by CC on sephadex LH-20, using MeOH as eluent, gave **2** (5 mg). Compound **1** (4 mg) and **3** (5 mg) were afforded after reversed phase HPLC of fraction EI (50 mg) (Waters PrepPak Cartridge C_{18} HPLC column (25 x 10 cm), 30% methanol in water as a solvent system, flow rate 5 ml/min, PDA detector, wavelength 254 nm).

1,7-Bis(4-hydroxyphenyl)-2,4,6-heptatrienone (1):

Yellow powder; UV (CH_3OH) λ_{max} (log e) 395 (4.51); IR (KBR) ν_{max} 3300, 1653, 1578, 1511 cm^{-1}; ^1H NMR (CD_3COCD_3, 500 MHz) δ 7.96 (2H, d, *J* = 8.8 Hz, H-2',6'), 6.95 (2H, d, *J* = 8.8 Hz, H-3', 5'), 7.41 (2H, d, *J* = 8.5 Hz, H-2",6"), 6.85 (2H, d, *J* = 8.5 Hz, H-3",5"), 7.20 (1H, d, *J* = 15.0 Hz, H-2), 7.46 (1H, dd, *J* = 15.0 Hz, *J* = 11.0 Hz, H-3), 6.64 (1H, dd, *J* = 15.0 Hz, *J* = 11.0 Hz, H-4), 6.94 (1H, dd, *J* =15.0 Hz , *J* = 11.0 Hz, H-5), 6.92 (1H, dd, *J* = 15.0 Hz, *J* = 11.0 Hz, H-6), 6.80 (1H, δ, *J* = 15.0 Hz, H-7); ^{13}C NMR (CD_3COCD_3, 125 MHz) δ 131.3 (C-1'), 131.5 (C-2', 6'), 116.1 (C-3',5'), 162.5 (C-4'), 129.5 (C-1"), 129.3 (C-2",6"), 116.5 (C-3",5"), 158.9 (C-4"), 187.9 (C-1), 124.9 (C-2), 144.1 (C-3), 130.8 (C-4), 143.1 (C-5), 126.5 (C-6), 137.6 (C-7); EIMS *m/z* 292 $[M]^+$ (94), 171 (38), 121 (100); HREIMS *m/z* 292.1113 (calcd for $C_{19}H_{16}O_3$, 292.1099)

16-Hydroxylabda-8(17),11,13-trien-15,16-olide (4):

Gummy solid; UV (CH_3OH) λ_{max} (log e) 260

Table 1: Antitumour promoting activity of crude extracts of Etlingera elatior using EBV-EA assay in Raji cell line.

Extract	Cell Viability (%)	Inhibition Rate (%)
Hexane[a,d]	83.3 ± 1.3	18.3 ± 1.3
$CHCl_3$[b]	54.16 ± 1.5	92.18 ± 1.5
EtOAc[c,d]	19.05 ± 0.9	-
MeOH[b]	92.5 ± 1.6	85.9 ± 1.6

[a]50 µg/ml, [b]200 µgm/ml, [c]25 µgm/ml,
[d]cytotoxic at 200 µg/ml

(4.51); IR ν_{max} (KBr) cm^{-1}: 1750 (α,β-unsaturated δ -lactone), 3090, 892 cm^{-1} (exo-methylene). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.04 (1H, ddd, *J* = 13.2,13.2, 3.7 Hz, H-1a, ax), 1.38 (1H, m, H-1b), 1.40 (1H, m, H-2a), 1.52 (1H, m, H-2b), 1.18 (1H, br dd,*J* = 13.2, 13.2 Hz, H-3a, ax), 1.42 (1H, m, H-3b), 1.10 (1H, dd, *J* = 2.7 Hz, *J* = 12.5 Hz, H-5), 1.39 (1H, m, H-6a), 1.72 (1H, ddddd,*J* = 12.9, 2.7, 2.7, 2.7, 2.7 Hz, H-6b, eq), 2.09 (1H, ddd,*J* = 13.4, 13.4, 5.6 Hz, H-7a, ax), 2.44 (1H, m, H-7b, eq), 2.47 (1H, d, *J* = 10.0 Hz, H-9), 6.58 (dd, *J* = 16.0 Hz, *J* = 10.0 Hz, H-11a), 6.59 (dd, *J* =16.0 Hz, *J* = 10.0 Hz, H-11b), 6.31 (1H, d,*J* = 16.0 Hz, H-12), 5.85 (1H, s, H-14), 6.25 (s, H-16a), 6.27 (s, H-16b), 4.38 (d,*J* = 1.5 Hz, H-17aa), 4.79 (2H, brs, H-17ab, H-17ba), 4.47 (d,*J* = 1.5 Hz, H-17bb), 0.90 (3H, *s,* H-18), 0.85 (3H, *s,* H-19), 0.87 (3H, *s,* H-20); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 40.9 (C-1a), 39.6 (C-1b), 19.0 (C-2a), 19.0 (C-2a), 42.1 (C-3), 33.5 (C-4), 54.5 (C-5a), 54.5 (C-5b), 23.2 (C-6), 36.6 (C-7), 148.7 (C-8a), 148.9 (C-8b), 62.1 (C-9a), 62.1 (C-9b), 39.5 (C-10a), 39.6 (C-10b), 144.0 (C-11a), 144.1 (C-11b), 122.6 (C-12a), 122.7 (C-12b), 161.0 (C-13a), 161.0 (C-13b), 115.5 (C-14), 171.2 (C-15), 97.5 (C-16a), 97.6 (C-16b), 108.5 (C-17a), 108.9 (C-17b), 21.9 (C-18), 33.6 (C-19), 15.1 (C-20a), 15.2 (C-20b); EIMS *m/z* 316 [M^+] (13), 180 (30), 162(14), 137(100), 123(25); HREIMS *m/z* 316.2030 (calcd for $C_{20}H_{28}O_3$, 316.2038)

Demethoxycurcumin (2):

Yellow powder, m.p. 170-172 ^{0}C; EIMS *m/z* 337.8 (M^+, $C_{19}H_{18}O_5$); ^{1}H-NMR and ^{13}C-NMR are in agreement with (7,8).

1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one (3):

Yellow powder, m.p 168-170 ^{0}C; EIMS *m/z* 291.9 (M^+, $C_{19}H_{16}O_3$); ^{1}H-NMR and ^{13}C-NMR are in agreement with (9).

Stigmast-4-en-3-one (5):

White needles, m.p 80-82 ^{0}C; EIMS *m/z* 412 (M^+, $C_{29}H_{48}O$); ^{1}H-NMR and ^{13}C-NMR are in agreement with (10).

Stigmast-4-ene-3,6-dione (6):

White needles, m.p 75-76 ^{0}C; EIMS *m/z* 426 (M^+, $C_{29}H_{46}O_2$); ^{1}H-NMR and ^{13}C-NMR are in agreement with (10).

Stigmast-4-en-6β-ol-3-one (7):

White needles, m.p 217-218 ^{0}C; EIMS *m/z* 428 (M^+, $C_{29}H_{48}O_2$); ^{1}H-NMR and ^{13}C-NMR are in agreement with (10).

5α,8α-Epidioxyergosta-6,22-dien-3β-ol (8):

Off-white amorphous solid, m.p 176-178 ^{0}C; EIMS *m/z* 428 (M^+, $C_{29}H_{48}O_2$); ^{1}H-NMR and ^{13}C-NMR are in agreement with (11).

Antitumour Promoting Activity

Stock solution of pure compounds.

The extract was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, with concentrations and 10 mg/ml for crude extract and 4 mg/ml for pure compound.

Cell Lines

The Raji cells were maintained in medium RPMI 1640 (Flow Lab., UK) supplemented with 10% foetal calf serum (Gibco, UK), 100 IU/ml penicillin/streptomycin, 50 mg/mL Amphostat B and

Table 2: Antitumour promoting activity of the fractions from crude $CHCl_3$ extracts of Etlingera elatior using EBV-EA assay in Raji cell line.

Fraction	Cell viability (%)	Inhibition Rate (%)
A[a,d]	100.0 ± 0.2	69.7 ± 0.2
B[b]	89.8 ± 0.5	85.6 ± 0.5
C[b]	80.3 ± 1.2	97.9 ± 1.2
D[a,d]	100.0 ± 1.4	69.1 ± 1.4
E[a,d]	80.0 ± 1.7	74.4 ± 1.7
F[a,d]	87.5 ± 0.8	2.7 ± 0.8
G[c,d]	50.0 ± 0.5	17.6 ± 0.5
H[c,d]	4.0 ± 0.7	-
I[c,d]	94.4 ± 0.8	43.8 ± 0.8
J[b,d]	100 ± 1.2	73.4 ± 1.2

[a]50 µg/ml, [b]200 µgm/ml, [c]25 µgm/ml,
[d]cytotoxic at 200 µg/ml

120 mg/mL *L*-glutamine as a static suspension culture at 37^{0}C in a humidified atmosphere of 50 % CO_2 in air.

Antitumour-promoting Activity in Raji Cells Assay

The inhibitory activity of Eipstein-Barr virus (EBV) activation assay was performed as previously described (4). Raji cells were activated with 20 ng/ml of TPA (Sigma, USA) and 4 mM/ml of sodium-n-butyrate (Nacarai Tesque, Japan) to induce the expression EBV EA. The plant extracts at the concentration of 200 mg/ml were added immediately after the addition of TPA as tumour promoter. The cells were incubated at 37^{0}C for 72 hours, after which they were subjected to indirect immunoflourescence assay using EBV EA positive nasopharyngeal carcinoma serum and FITC-conjugated anti-human IgG (Sigma, USA). The inhibitory rate (IR) of each test sample against the EBV activation was classified into four ranks as follows: +++, strongly active (IR ≥ 70%); ++, moderately active (70% > IR ≥ 50%); +, weakly active (50% > IR ≥ 30%); -, inactive (30% > IR) (5). All tests and analyses were run in triplicate and averaged.

Cytotoxicity Assay
Plant Extract

The extract was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, with concentrations 4 mg/ml for pure compounds and fractions.

Microculture Cytotoxicity Screening Using Methyl Thiazole Tetrazolium (MTT) Assay

A 10-fold dilution gradient microtitre cell culture was adopted and modified to 3-fold dilution cell plating. All cells were cultured in sterile RPMI-1640 complete media, supplemented with antibiotic-antimycotic mixture (containing 103 U/mL Penicillin G; 100 mg/mL Streptomycin SO_4; 2.5 mg/L Amphoterin B), 2 mM *L*-Glutamine and 10% FBS (all from Sigma). Exponentially growing cells were pre-determined by trypsinisation (0.25% p.p trypsin) and/or re-suspension, with a 100% confluency and 96% viability (using 0.2% typan blue exclusion cell-count in an Improved Neubauer Haemacytometer). A final cell concentration of 2.5 x 10^4 cells/well was used as inoculation density for all anchorage

Table 3: Antitumour promoting activity of compounds isolated from Etlingera elatior using EBV-EA assay in Raji cell line.

Compounds [20 µg/ml]	Cell Viability (%)	Inhibition Rate (%)
5	97.93 ± 1.5	78.4 ± 1.5
7	77.5 ± 0.7	80.6 ± 0.7
8	80.4 ± 0.3	14.1 ± 0.3
β-Sitosteral and stigmasterol	89.2 ± 0.9	85.1 ± 0.9
6	91.0 ± 1.4	56.9 ± 1.4
Tetracosanoic acid	85.9 ± 0.7	72.4 ± 0.7

dependent cell lines, and 5 x 10^4 cells/well for CEM-SS cell suspensions.

Into a sterile and labeled NUNCLON™ 96 well (180 mL volumes) micro-titre plates (Nunc, Denmark), 180 mL volumes were pipetted appropriately for all cell lines. The plated cells were then incubated overnight, under standard culture conditions of 5% CO_2, 95% air and 100% humidity, to allow cell settling and differentiation.

Stock solutions of all samples were prepared as 10 mg/mL in absolute dimethylsulphoxide, DMSO (HPLC-grade, Sigma, USA). Using the same culture media as diluent, a sub-stock solution of 1000 mM was prepared immediately before addition and serially diluted in sterile sample containers, to give 10x working stock solutions for each of the final test-range concentrations, topping from 100 mM down to the lowest of 0.1 mM. Having prepared the above mentioned dilutions, 20 mL quadruplicates of the corresponding 10x working stock samples were all added up to give the required final concentrations, in total volume of 200 mL. Plates were returned to the incubator for a further 4-day culture period. Cultures were regularly observed for any visual interference(s), with morphological changes and or cell killing effects being monitored using CK2 light microscope (Olympus, Japan).

At the end of each culture successfully attained, devoid of any contaminating and other interfering notifications, cells were aseptically subjected to further analysis, using the MTT biochemical assay (11). A 20 mL volume of MTT (Sigma, USA), prepared at 5 mg/ml in phosphate buffered saline (PBS), was added into each 200 mL culture of the 96 well microtitre plate, wrapped with aluminium foil and incubated for a further 4 hours culture under similar conditions as above. This allowed the activation of mitochondrial dehydrogenases of the CEM-SS cells to reduce the yellowed colour MTT into a crystallized blue-violet

Table 4: Cytotoxic activity of Etlingera elatior extracts

	IC_{50} µg/ml	
Extract	**CEM-SS**	**MCF-7**
Hexane	42.5 ± 0.1	36.0 ± 0.3
$CHCI_3$	14.0 ± 0.5	26.0 ± 0.5
EtOAc	4.0 ± 0.1	6.25 ± 0.1
MeOH	46.0 ± 0.4	47.0 ± 0.9

Note: The standard use was tamoxifen with IC_{50} = 30 µM and IC_{50} = 15 µM against MCF-7 and CEM-SS cell lines respectively.

colour complex product, *formazan*. A total volume aspiration followed by 200 mL addition of pure DMSO (Ajax, Australia) was carried out, with gentle mixing and 5 minute incubation at room temperature, to allow for faster and more enhanced formazan solubility. Optical densities (O.D) of the respective concentrations of sample were then measured using DYNEX MRX ELISA reader (Dynex Instruments, Inc. USA), at a 550 nm test and 630 nm reference wavelengths. Percentage proportions of the control O.D. values were then compacted in a dose-response standard curve to enable a more accurate and standardized determination of the 50% growth inhibitory concentration, $IC_{50.}$ All tests and analyses were run in triplicate and averaged.

Results

Ten compounds were isolated from *E. elatior* after extensive chromatography of the crude extracts. Compouds **1**-**8** (Figure 1), a mixture of stigmasterol and β-sitosterol, and tetracosanoic acid were identified based on spectral data (UV, MS, IR, ^{1}H NMR, ^{13}C NMR, H-H COSY, HMQC and HMBC) and comparison with literature values (7-11, 13-15). The antitumour promoting activity of the crude extracts, fractions and compounds is shown in Table 1-3, respectively. The cytotoxic activity of the crude extracts is shown in Table 4.

Discussion

The preliminary screening showed both $CHCl_3$ and MeOH extracts of *E. elatior* possessed high antitumour promoting activity, with 92.18% and 85.9% inhibition rate, respectively. Both hexane and ethyl acetate were cytotoxic against Raji cell at initial concentration (200 mg/ml) (Table 1). Five fractions (fractions A-C, E and J) of $CHCl_3$ extract showed strong antitumour promoting activity. The less polar fractions showed high antitumour promoting activity compared to the more polar fractions (Table 2). Seven compounds (**5, 6** and tetracosanoic acid from the hexane extract; **7**, **8** and a mixture of stigmasterol and sitosterol from $CHCl_3$ extract) were screened for antitumour promoting activity (Table 3). Among them, **5**, **7**, a mixture of b-sitosterol and stigmasterol, and tetracosanoic acid showed high antitumour promoting activity, with inhibition rate of 78.4%, 80.6%, 85.1% and 72.4% respectively. Compound **6** only showed moderate activity with inhibition rate of 56.9%. Compound **8** and the mixture of stigmast-4-en-6a-ol-3-one and **8** did not show any significant activity. Our finding suggested that the $\Delta^{4(5)}$-3-keto steroids (**5-7**) displayed high antitumour promoting activity. The activity of the $\Delta^{4(5)}$-3-keto steroids increased due to the b-hydroxy group at C-6 as in the case of **7**. The activity decreased when this 6-hydroxy group was in its oxidized form as in the case of **6**. In a related study, a $\Delta^{8(9)}$-11-keto steroid, 5α,14α-dimethyl-ergosta-8,24(28)-dien-11-one from *Euphorbia chamaesyce* also displayed a potent inhibitory effect on EBV-EA (15). This finding suggested that both $\Delta^{4(5)}$-3-keto and $\Delta^{8(9)}$-11-keto steroids could act as potent antitumour promoters. Four extracts of *Etlingera elatior* rhizome were tested for their cytotoxic activity against CEM-SS and MCF-7 cell lines (Table 4). The *in vitro* cytotoxic assay was based on modification of Monsman's method (11). The ethyl acetate extract was found to show a significant cytotoxic to both CEM-SS (IC_{50} 4 mg/ml) and MCF-7 (IC_{50} 6.25 mg/ml). From the ethyl acetate extract, we successfully isolated three diarylheptanoids, **1-3,** which showed strong antioxidant activity (4). However the cytotoxicity of each diarylheptanoid could not be evaluated because of insufficient amount. It was reported that demethoxycurcumin had cytotoxicity effect against ovarian cancer OVCAR-3 cells (7), displayed DPPH free radical scavenging activity and showed significant hepatoprotective effects on tacrine–induced cytotoxicity in human liver–derived Hep G2 cells (17). The other extracts, including the hexane, $CHCl_3$ and MeOH extracts, also showed significant cytotoxicity against both MCF-7 and CEM-SS cell lines. It was also reported that the ethanol aqueous extract of the young flower shoots was cytotoxic against HeLa cell line with IC_{50} value of 10 mg/ml (2). This implied that besides the young flower shoots, the rhizome is also a potential source for cytotoxic compounds.

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