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Chromatographic separation of the free radical scavenging components of the leaf extracts of *Leea philippinensis* Merr.

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Abstract

The ability of *Leea philippinensis*, an endemic tree in the Philippines, as *in vitro* free radical scavenger was investigated. Soxhlet extraction using solvents in increasing order of polarity (hexane<chloroform<ethyl acetate<acetone<methanol) yielded acetone and methanol extracts with the highest Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). These crude extracts were further separated using silica gel column chromatography with eluents hexane, hexane:acetone (1:1), acetone, acetone:methanol (1:1) and methanol. Fractions with the most TFC and TPC exhibited concentration-dependent activity with an IC_{50} value range of 0.10 - 0.29, 0.17 - 0.80, 0.29 - 0.84 and 3.40 - 9.75 mg/mL, respectively, against DPPH, hydroxyl, nitric oxide and hydrogen peroxide inhibition assays. In addition, these fractions demonstrated reducing power by transforming the Fe^{3+} to Fe^{2+}. Infrared spectra of fractions at 4000–500 cm^{-1} using FTIR revealed presence of phenolic group.

Introduction

Efficacy of free radical scavengers to prevent oxidative cell damage is gaining substantial interest from many researchers because of inadequacy of effective treatments for most chronic diseases (Ammar *et al*., 2009). Oxidative cell damage is a critical etiological factor implicated to a number of human diseases; namely, cardiovascular and neurodegenerative disorders, rheumatism, diabetes mellitus, mutagenesis, cancer, and other related illnesses (Pong, 2003). Copious scientific studies have suggested that the chief source of compounds with free radical scavenging activity is phytochemical found in fruits, herbs, vegetables, and natural products (Rachh *et al*., 2009). Among the phytochemicals, phenolic constituents are well-recognized free radical scavengers. They have the ability to halt or prevent cell oxidation, scavenge free radicals, inhibit lipid peroxidation and other free radical-mediated processes (Hotta *et al*., 2002; Mathew and Abraham, 2006; Ningappa *et al*., 2008).

Leea philippinensis Merr. is an endemic Philippine tree and has leaves pinnate with 5-13 leaflets; petiole 5-7 cm; lateral petioles 1.0-1.3 cm and leaflets narrowly ovate to lanceolate (Merrill, 1906). There are scant data available about its biological uses. However, other members of the genus have been used traditionally to treat several illnesses. In India, *L. asiatica* is used to heal bone fractures in 15 days (Bhandary *et al.*, 1995); *L. indica* (N. Bum) is given to children to alleviate chest pain (Swarnkar and Katewa, 2008) and cancer (Choudhary, 2008); *L. macrophylla* is administered to cure cancer, sexual debility in male and body ache (Choudhary, 2008, Swarnkar and Katewa, 2008). *L. sambucina* is mixed with coconut oil for treatment of dysentery with blood discharge (Prakash *et al*., 2008). In Nigeria, *L. guineensis* is used to treat enlarged spleen in children (Falodun *et al.*, 2007). In Malaysia, *L. indica* (N. Bum) is used to treat boils (Wiart *et al*., 2004).

The methanolic extract of *L. indica* exhibited enhanced nitric oxide production, and inhibited 1,1-diphenyl,2-picrylhydrazyl (DPPH) radical and thiobarbituric acid (TBA) (Saha *et al.*, 2004) whereas its ethanolic extract inhibited phosphodiesterase activity (Rahimi *et al*., 2010). The aqueous leaf extract of *L. guineensis* has antioxidant activity which is linked to its flavonoid and phenolic acids (Falodun *et al.*, 2007). The methanolic extract of leaves, stems, and root barks of *L. tetramera* has antibacterial property (Khan *et al.*, 2003).

Major secondary metabolites from *L. indica* leaf include phthalic acid, gallic acid, lupeol, β-sitosterol, ursolic acid (Srinivasan *et al.*, 2008) while its flower contains ester of phthalic acid, di-isobutylphthalate, di-n-butylphthalate, n-butylisophthalate, and 3,5 butylisohexylphthalate (Srinivasan *et al.*, 2009). Similarly three hydrophilic flavonoids were isolated

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and identified from leaves of *L. guineensis* as quercetin-3-sulphate-3-O-α-L-rhamnopyranoside, quercetin-3,3-disulphate and a new compound quercetin-3,3,4-trisulphate, together with kaemferol, quercetin, gallic acid, ethylgallate, mearnsitrin. Alkanes, alcohols, aldehydes, ketones, fatty acids, esters, terpenoids, phenylpropanoids were also identified from the leaves and roots of *L. guineensis* (Op de Beck *et al.*, 1998; Op de Beck *et al*., 2000: Op de Beck *et al.*, 2003).

To date, there are no published data about the ethnomedicinal uses, phytochemical studies nor biological activities of *Leea philippinensis*. This is the first study to assess the free radical scavenging activity of *L. philippinensis*, which is thought to occur due to high concentration of polyphenols it contains.

Materials and Methods

About six (6) kilograms of fresh leaves of *Leea philippinensis* were collected from San Ramon, Buhi, Camarines Sur, Philippines. The leaves were air-dried and protected from sunlight for 14 days and grind to fine powder using Willey mill. The powdered leaves were extracted twice for six hours using Soxhlet apparatus with a series of solvents in increasing polarity (hexane<chloroform<ethylacetate<acetone <methanol). The filtrate obtained from each solvent was concentrated using the rotary evaporator (Buchi R200) until syrup consistency was achieved. Crude extracts (hexane, chloroform, ethyl acetate, acetone and methanol) were labeled accordingly and were weighed to obtain the percentage yield. Extracts were kept under refrigeration until further testing.

Determination of total phenolic content

The concentration of total phenolic of the crude extracts of *L. philippinensis* was determined using the method described by Ruan *et al.*, (2008) with minor modifications. The test was performed in triplicates and expressed as mg of gallic acid equivalent per gram of plant sample (GAE mg/g).

Determination of total flavonoid content

The total flavonoid content of crude extracts was estimated using the method described by Adedapo *et al.*, (2008) and Zovko Koncic´*et al*., (2010) with some modifications. The assay was performed in triplicates and expressed as milligram of quercetin equivalent per gram (QE mg/g) plant extract.

Determination of DPPH radical scavenging sctivity of crude extracts

The DPPH scavenging capacity of *L. philippinensis* crude extract was determined using DPPH reagent (Chaulya *et al*., 2010). Solutions containing varied concentrations of crude extracts (0.10 mg/mL - 3.13 mg/mL in 95% ethanol) were prepared. Freshly prepared methanolic DPPH (0.5 mL, 100 μM) was added to 0.5 mL of different dilutions of the crude extracts and reference standard ascorbic acid. The reaction mixtures were incubated for 15 minutes at room temperature. The absorbance was recorded at 517 nm (Genesis 10 Spectrophotometer). Control sample was prepared containing the same volume of DPPH methanolic solution without any extracts and reference ascorbic acid. The test was carried out in triplicates. The radical scavenging activity of crude extracts was expressed as IC_{50} (median inhibition concentration in mg/mL) of the crude extracts necessary to inhibit the formation of DPPH radicals by 50%.

Column chromatography of crude extracts

Among five crude extracts, two crude extracts with most high concentration of TPC and TFC were subjected to column chromatography to separate further the individual compounds. The column separation of extracts was carried out with a glass column of 150 mm internal diameter and 30 cm in length. The diluted extract was loaded onto the wet packed column with silica gel and was eluted with the mobile phase solvent systems composed of hexane, hexane:acetone (1:1), acetone, acetone:methanol (1:1), and methanol, respectively. Two milliliters (2mL) were collected for each fraction. Subsequently, the fractions were assayed for their TPC and TFC.

Determination of total phenolic content (TPC) and total flavonoid content of fractions

Determinations of TPC and TFC of the fractions were estimated using the same assay procedures for crude extracts.

Determination of free radical scavenging activities of fractions

The four fractions with the highest gallic acid equivalent (GAE mg/g) and quercetin equivalent (QE mg/g) were subjected to free radical scavenging tests. The scavenging action was expressed as median inhibitory concentration (IC_{50}) of the fractions required to produce 50% inhibition of radicals generated *in vitro*. All assays were performed in triplicates and hydrophilic ascorbic acid was used as a reference standard.

Determination DPPH radical scavenging activity of

fractions

The DPPH scavenging capacity of the fractions was determined using DPPH reagent (Chaulya *et al.*, 2010). Freshly prepared methanolic DPPH (0.5 mL, 100 µM) was added to 0.5 mL of different dilutions of the fractions and reference ascorbic acid. The reaction mixtures were incubated for 15 minutes at room temperature. The absorbance was recorded at 517 nm using Genesys 10 Spectrophotometer. Control sample was prepared containing the same volume of DPPH methanolic solution without any extracts and ascorbic acid.

Determination of hydroxyl radical scavenging activity of fractions

Hydroxyl radical scavenging activity of fractions was measured by Ferric-ascorbate-EDTA–H_2O_2 (Fenton reaction) used by Samak *et al*., (2009). The assay was performed by adding EDTA (0.1 mL), $FeCl_3$ (0.01 mL), H_2O_2 (0.1 mL), deoxyribose (0.36 mL), fractions (1.0 mL, different concentrations 63 µg/mL – 1000 µg/mL), phosphate buffer (0.33 mL) and ascorbic acid (0.1 mL) in sequence. The reaction mixture was incubated for 30 minutes at 37°C then was added with 5% trichloroacetic acid (0.5mL) and 1% thiobarbituric acid (0.5 mL). The reaction mixture was kept in boiling water bath for 30 minutes and cooled. Pink chromogen was measured at 532 nm (Genesys 10 Spectrophotometer).

Determination of nitric oxide radical scavenging activity of fractions

The nitric oxide radical scavenging capacity of the fractions was measured by Griess reaction (Sangameswaran *et al*., 2009). Various concentrations of fractions (63 µg/mL – 1000 µg/mL) were prepared. Sodium nitroprusside (1.5 mL, 10 mM) in phosphate buffer was added to 0.5 mL different concentrations of fractions. The reaction mixture was incubated at 25°C for 150 minutes. After incubation, 0.5 mL aliquot was removed and 0.5 mL of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H_3PO_4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance was measured at 546 nm (Genesys 10 Spectrophotometer). The standard was treated the same way as that of fractions. Sodium nitroprusside in phosphate buffer solution (2 mL) was used as control.

Determination of hydrogen peroxide radical scavenging activity of fractions

The hydrogen peroxide (H_2O_2) radical scavenging ability was determined according to the method described by Ebrahimzadeh *et al.*, (2010). One milliliter of different concentrations of fractions (63 µg/mL – 1000 µg/mL) was added to solution of H_2O_2 (0.6 mL, 40 mM) in phosphate buffer pH 7.4. After 10 minutes of incubation, the absorbance of H_2O_2 was recorded at 230 nm against blank solution with phosphate buffer only.

Infrared Spectroscopy

Infrared spectroscopic (IR) analysis was used to determine the chemical functional groups in the fractions. Different functional groups absorb characteristic frequencies of IR radiation. The IR spectra of fractions were recorded in KBr discs in the range 4000–500 cm^{-1} on Fourier Transform Infrared Spectroscopy (FTIR-6700 Nicolet).

Data interpretation and calculations

The data were analyzed by an analysis of variance one way ANOVA. The Median Inhibitory Concentration (IC_{50}) values were obtained from plotted graph of % scavenging activity and calculated from probit regression analysis. Locatelli *et al*., (2010) used the same method of analysis due to restricted linearity range between concentration of fractions and radical inhibition. The data were expressed as means standard error (SE) and analyzed using SPSS (version 13.0). One-way analysis of variance (ANOVA) and Turkey multiple comparisons were carried out to test any significant differences between the means. Differences between the means at the 5% confidence level were considered significant.

Results

Extraction yield

Different crude extracts were obtained by sequential extraction using solvents with increasing polarity (hexane<chloroform<ethyl acetate<acetone<methanol). Hexane yielded highest amount of extract (3.06 ± 0.05 %). Chloroform, ethyl acetate, acetone and methanol had extracted 2.28 ± 0.04%, 1.39 ± 0.02%, 1.26 ± 0.02% and 2.03 ± 0.03% respectively. These results were average percentage yield of each extracting solvent based on triplicate analysis of 500-gram.

Total phenolic content and total flavonoid content of crude extracts

Among the crude extracts, the acetone extracts yielded the highest TPC and TFC with 83.48 ± 0.04 GAE mg/g, and 87.13 ± 0.03 QE mg/g, respectively. Meanwhile, methanol extracts obtained 41.71 ± 0.06 GAE mg/g and 83.28 ± 0.03 QE mg/g.

DPPH scavenging activity of crude extracts

Table 1. Inhibitory concentration of fractions against free radicals

Fractions	Inhibitory Concentration (IC_{50}) mg/mL			
	DPPH	NO	OH^-	H_2O_2
A9	0.29± 0.002	0.80± 0.015	0.84± 0.008	9.75± 0.02
A10	0.10± 0.002	0.17± 0.005	0.29± 0.008	3.40± 0.03
M7	0.24± 0.002	0.22± 0.006	0.44± 0.005	5.97± 0.01
M8	0.18± 0.004	0.24± 0.001	0.33± 0.004	5.81± 0.04
Ascorbic acid	0.06± 0.001	0.10± 0.003	0.13± 0.002	1.65± 0.00

A higher DPPH radical scavenging activity is associated with lower IC_{50} values. Acetone and methanol extracts yielded lowest IC_{50} values 0.66 ± 0.12 and 1.17 ± 0.26 mg/mL respectively. Whereas, 0.66 ± 0.03 mg/mL for standard ascorbic acid was observed.

Total phenolic content and total flavonoid content of fractions

Fifteen (15) fractions were collected from acetone and methanol crude extracts through column chromatography. Among acetone fractions, highest TPC (mg GAE/g) was found in fractions no.9 (196.89 ± 0.12) and no.10 (291.35 ± 1.27). Meanwhile, for methanol fractions, fraction no.7 (216.13 ± 0.43) and no. 8 (255.80 ± 0.44) yielded high concentration of polyphenols

Similarly, highest total flavonoid content (mg of QE/g) were found in A9 (85.45 ± 0.08), A10 (109.90 ± 0.09), M7 (163.38 ± 0.18), and M8 (145.32 ± 0.17). Thus, these fractions (A9, A10, M7, and M8) were evaluated for their free radical scavenging capacities and qualitative phenolic content.

Free-radical scavenging activities of fractions

The fractions (A9, A10, M7 and M8) demonstrated concentration-dependent scavenging activity against DPPH, nitric oxide, hydroxyl, H_2O_2 radicals. Among the four fractions, A10 had highest total phenolic and flavonoid content and showed strongest scavenging activity against all radicals as summarized in Table 1.

Infrared spectroscopy

FT-IR spectroscopy was used to predict some functional groups regarding the compounds in the fractions of *Leea philippinensis*. Several indicator bands that are pertained to functional groups represent chemical components. Representative spectral feature of fraction A10 is shown in Figure 1.

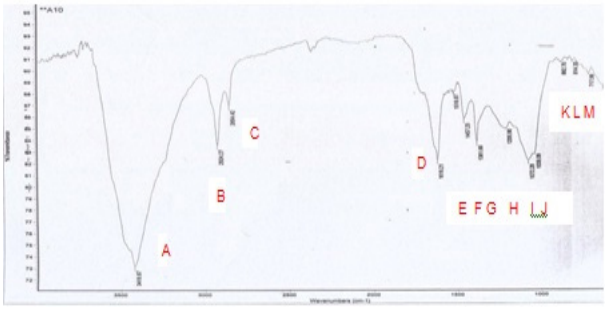

Figure 1. IR Spectrum of A10

Discussion

Sequential solvent extraction was employed to resolve all types of compounds with different polarity effectively and completely. Sarker *et al.* (2006) mentioned that nonpolar solvents are used to solubilize mostly lipophilic compounds (e.g., alkanes, fatty acids, pigments, waxes, sterols, some terpenoids, alkaloids, and coumarins). Medium-polarity solvents are used to extract compounds of intermediate polarity (e.g., some alkaloids, flavonoids), while more polar ones are used for more polar compounds (e.g., flavonoid glycosides, tannins, some alkaloids). Hexane yielded highest amount of extracts thus the leaves of *Leea philippinensis* contain mostly of lipophilic compounds such waxes, chlorophyll and fatty acids.

Meanwhile, the $AlCl_3$ assay is a widely adopted method in almost all published works to measure the total flavonoid content. The principle of this colorimetric method is based on the acid-stable complex between $AlCl_3$, and the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Chang *et al*., 2002). The TPC and TFC results clearly revealed that polar solvents had extracted more phenolic constituents than non-polar solvents. Hayouni *et al.*, (2007) noted similar results that the lowest amount of phenolic was recorded in less polar solvents.

The DPPH radical scavenging activity of crude extracts were measured based on their ability to reduce 1, 1,-diphenly-2-picrylhydrazyl to 1, 1,-diphenyl-2-picrylhydrazine by donating electron or hydrogen. The changes in color from deep violet to light yellow were measured spectrophotometrically at 517 nm. Decreased absorbance is associated with DPPH radical scavenging activity, which is expressed in percentage, caused by different concentrations of crude extracts and standard ascorbic acid and measured at 517 nm. The strong scavenging activity of acetone and methanol crude extracts against DPPH radical was linearly correlated to the TPC and TFC.

Ghasemzadeh *et al.* (2010) emphasized that

Table 2. The wavenumbers (cm^{-1}) of fractions and corresponding functional groups

	Wave number (cm^{-1})				FUNCTIONAL GROUP
	A9	A10	M7	M8	
A	3413	3417	3414	3402	OH
B	2925	2924	2926	2930	Aliphatic $-CH_3$ and CH_2 stretching
C	2855	2854	2852	2856	Aliphatic $-CH_3$ and CH_2 stretching
D	1617	1619	1637	1639	C-C stretching in benzene ring, aromatic ring
E		1516	1512	1509	C-H bending in benzene ring
F	1450	1457	1456	1404	Deformations of –CH2 or –CH3
					C=C stretching in benzene ring
G	1380	1381	1382		Deformations of –CH2 or –CH3
H	1212	1205	1250	1219	Ester carbonyl, phenol
I	1099	1072	1074	1076	CH_2OH of carbohydrates
J		1038	1042	1048	CH_2OH of carbohydrates
K	874	882			Benzene ring substitution
L	829	814			Benzene ring substitution
M	766	717			Benzene ring substitution

application of different assay methods is necessary to evaluate the free radical scavenging activity of various sample because of the following reasons; complexity of the oxidation process, the diversity of the substrates and the specific active radical species involved. Thus, the present study performed several antioxidant assay such as DPPH, nitric oxide, hydroxyl and hydrogen peroxide and reducing power.

The nitric oxide produced in the body has vital function in inflammatory processes. In the present study, the nitric oxide was generated from sodium nitroprusside at physiological pH. The fractions were added to compete with oxygen leading to reduce production of nitric oxide that can be measured by Griess reagent. The absorbance of the pink chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine was measured at 546 nm (Shukla *et al.*, 2009). The fractions and standard ascorbic acid demonstrated inhibitory effect in a concentration-dependent manner.

The hydroxyl radical, on the other hand, is a highly reactive free radical formed in biological systems and capable of damaging almost every molecule in living cells (Yasuda *et al*., 2000). This radical has a capacity to join nucleotides in DNA and cause strand breakage which may contribute to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz *et al*., 2002; Duan *et al.*, 2007; Manian *et al.*, 2008). It is also considered to be one of the quick initiators of the lipid peroxidation process (Duan *et al.*, 2007). The hydroxyl radical scavenging activity of fractions was determined by Fenton assay. The hydroxyl radicals were generated from reaction of Ferric-ascorbate–EDTA–H_2O_2 (Fenton reaction): $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + \cdot OH$. The highly reactive ▪OH radicals degrade the DNA deoxyribose, using Fe^{2+} salts as catalytic components (Duan *et al*., 2007; Shukla *et al*., 2009; Motamed *et al*., 2010). Thus any other molecule that is capable of reacting with ▪OH, competes with deoxyribose for ▪OH to decrease the degradation of deoxyribose (Motamed *et al*., 2010). The capacity of fractions to reduce hydroxyl radical generation either by chelating metal ions or by directly scavenging the hydroxyl radicals was analyzed by measuring the degree of deoxyribose degradation. Fractions and ascorbic acid exhibited statistically significant ($p<0.05$) scavenging activity against hydroxyl radical in concentration-dependent manner (Table 1).

On the other hand, H_2O_2 itself is not a very reactive oxygen species but can cause oxidative damage in the cells by giving rise to more toxic hydroxyl radical via metal- catalyzed reaction (Anderson and Philips, 1988). Thus, removing H_2O_2 is extremely necessary. Fraction A10 demonstrated strongest activity against H_2O_2 radical in concentration-dependent manner (Table 1).

The spectral features showed a strong bond absorption band around 3402-3417 cm^{-1} found in all fractions is due to –OH. The bands at 2852-2926 cm^{-1} are attributed to the stretching vibration of –CH3 and $–CH_2$ groups indicative of the chlorophyll groups (Ramamurthy and Kannan, 2007). Lu *et al.* (2011) mentioned that the bands between the wavenumbers of 1800-759 cm^{-1} reflected the biochemical compositions, especially the moieties of carbohydrate, lipid, protein secondary structures and polyphenols in plants.

Phenolic ring structures are usually associated between the wave numbers from 1800 - 1500 cm^{-1} (Lu *et al*., 2011). All fractions yielded distinctive peak at the wavenumbers 1617 - 1639 cm^{-1} which are assigned to ring C-C stretch of phenyl or benzene rings (Schulz and Baranska, 2007; Lu *et al*., 2011). The bands at the 1509 - 1516 cm^{-1} are represented by in-plane CH bending vibration from the benzene rings (Lu *et al.*, 2011).

The absorptions at 1405 – 1457 cm^{-1} correspond to the asymmetric deformations of CH_3 or $-CH_2$ and in the same spectral region phenyl nucleus (C=C) absorbs (Socrates, 1997; Tarantilis *et al*., 2008). According to Ramamurthy and Kannan (2007),

the weak peaks at 1381-1382 cm^{-1} are likely due to asymmetric deformations of CH_3 or -CH_2, whereas, the absorptions at 1205-1250 cm^{-1} are from ester carbonyl and phenol.

The absorption bands at 1038-1099 cm^{-1} in the fingerprint region are brought about by to vibrational frequency of –CH_2OH groups of carbohydrates (Mordechai *et al*., 2001; Tarantilis *et al.*, 2008). Lu *et al.*, (2011) state that the band at around 814 cm^{-1} is caused by ring CH deformation which could also reflect structural information about polyphenols.

Fraction A9 and A10 showed minor differences in spectral features. There was no observable peak at around 1500 cm^{-1} but only one band at 1000 cm^{-1} in the IR spectrum of A9 which are associated with in-plane CH from benzene ring and –CH_2OH groups of carbohydrates respectively. Likewise, M7 and M8 yielded nearly the same absorption peaks. Absence of band was noticed at 1300 cm^{-1} in M8 which related to CH_2 and CH_3 deformations. The acetone fractions (A9 and A10) and methanol fractions (M7 and M8) spectral attributes greatly differ only at IR region 700-900 cm^{-1} which represent the substitution in the benzene ring. No absorption bands occurred in this region for methanol fractions (M7 and M8). To summarize, all fractions contained hydroxyl (-OH), benzene ring, ester carbonyl, phenol, and carbohydrates. These results affirmed the presence of phenolic functional group (-OH and benzene ring) which can be closely associated with free radical scavenging activity of fractions.

Conclusion

The present study provides an additional information on scant database regarding the pharmacologic value of *Leea philippinensis*. Like other *Leea* species, *L. philippinensis* showed potent free radical scavenging action which is directly proportional to its total phenolic and flavonoid content. Furthermore, spectral features affirmed the presence of phenolic compound. Therefore, the data generated from this research could be very useful for *in vivo* biological studies to support further the potential medicinal significance of *Leea philippinensis* in the prevention of free radical-associated diseases.

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