Research Article

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Antioxidant activity of ethanol extract and fractions of *Piper crocatum* with Rancimat and cuprac methods

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Abstract

Objectives: Bioactive compounds of *Piper crocatum* Ruiz & Pav, which have acted as antioxidants can be used to prevent and treat degenerative diseases such as hyperglycemia, cancer, gout and hypertension. This research aimed to determine the highest antioxidant activity from extract and fractions of *P. crocatum* leaves and to identify the active compounds such as antioxidants.

Methods: The extraction was performed by maceration with 70% ethanol and then the crude extract was fractionated with three solvents, namely n-hexane, ethyl acetate and water. The identification of antioxidant activity was carried out using Rancimat and CUPRAC. The active compounds was identified using LC-MS (liquid chromatography-mass spectrometry).

Results: The highest of the Rancimat method was obtained from the ethyl acetate fraction with a protective factor value of 1.38. Ten compounds were identified in the ethyl acetate fraction of *P. crocatum* leaves. An antioxidant activity according to the CUPRAC method showed the highest antioxidant activity in the sample of the n-hexane fraction with a value of 31.9 µmol Trolox/g extract. Thirteen compounds were identified in the n-hexane fraction of *P. crocatum* leaves.

Conclusions: The highest antioxidant activity was obtained from ethyl acetate and n-hexane fraction. Various active compounds was identified in the highest value sample.

Keyword: bioactive compounds; ethanol extract; ethyl acetate fraction; LC-MS; n-hexane fraction; water fraction.

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Introduction

Free radicals are molecules with an unpaired electron in its outer shell. Free radicals are considerably known as reactive oxygen species (ROS). ROS are unstable and highly reactive chemically, they will bind to the electrons from other molecules to obtain their electron pairs. The increase in ROS can modify cell signaling proteins that mediate pathological processes such as atherosclerosis, diabetes, inhibited growth, neurodegenerative diseases, intumescence and ageing [1]. Antioxidants are oxidation-preventing compounds that can prevent the activity of free radicals in the body. Antioxidants inhibited the oxidant compounds by donating an electron to the oxidants compounds. Antioxidants are divided into enzymatic antioxidants and non-enzymatic antioxidants. The example of enzymatic antioxidants are superoxide dismutase enzymes (SOD), catalase (CAT) and glutathione peroxidase (GPx). Meanwhile, non-enzymatic antioxidants are also found in plants [2].

Antioxidants come from plants that have proven to inhibit free radicals and break down peroxides [3]. One of the plants that can be used as a source of natural antioxidants is the leaves of the red betel. Flavonoids, tannins and alkaloids are the most important secondary metabolite component from red betel leaves [4]. Ethanol extract from red betel leaves contains polyphenolic compounds and essential oil that may contribute to antioxidant activity [5]. Based on the results of the *in vivo* study, red betel leaf extract at a dose of 1350 mg/kg bb during 14 days can decrease blood glucose levels of 39%, increase insulin levels of 41.5%, significantly increase the activity of SOD

enzymes and red blood cells catalase ($p<0.05$), maintain the normal blood lipid levels and improve rat's pancreas [6]. The 70% ethanol extract of red betel leaves has antioxidant activity in inhibiting the oxidation of fatty acids using malondialdehyde (MDA) and thiobarbituric (TBA) methods with inhibitory power 80.40%, and it can reduce the value of DPPH (diphenyl picrylhydrazin) radicals, that is IC_{50} 85.82 μg/mL [7].

Many studies on the potential of 70% ethanol extract as an antioxidant using DPPH and MDA-TBA methods have been performed with different topics and titles. Nevertheless, it is still difficult to find a study of the potential of the red betel leaf fraction and the component of its active compounds that acts as an antioxidant. Therefore, this study was conducted to evaluate the antioxidant activities from either an extract or fraction using Rancimat and CUPRAC (cupric reducing antioxidant capacity) methods to find the highest antioxidant activity and identify the active compounds that act as antioxidant in the red betel leaf extract and fraction. This study aimed to determine the highest antioxidant activity of 70% ethanol extract, water, ethyl acetate, and n-hexane fractions of red betel leaves using Rancimat and CUPRAC methods. In addition, the active compounds contained in samples of red betel leaves, which have the highest antioxidant activity, should be determined by LC-MS (Liquid Chromatography-Mass Spectrometry) analysis.

Materials and methods

Samples preparation

Red betel leaves collected from Tropical Biopharmaca Research Center (Trop BRC) were sorted and washed with the running water and drained. The leaves were chopped and dried in an oven at 50 °C for three days, then blended and filtered to obtain simplicia with a size of 60 mesh. Simplicia was stored in plastic at room temperature [4].

Measurement of simplicia's moisture content

This step was determined using AOAC method [8]. Ceramic bowls were rinsed and dried in an oven at 105 °C for 30 min and then put in the desiccator for 30 min, the empty weight was weighed. One gram of samples was dried in an oven at 105 °C until a constant weight was obtained. Bowls containing the sample were removed from the oven, stored in a desiccator for 30 min and were reweighed. The measurement was conducted three times. As stated in Eq. (1), moisture content is calculated by subtracting weight before drying (W1) with weight after drying (W2) in gram

$$\text{Moisture content} = \frac{\text{WI} - \text{W2}}{\text{W1}} \times 100\% \quad (1)$$

Red betel leaves extraction was performed with the maceration method with modification [7]. Simplicia was immersed in a solution with a ratio of 1:4. The simplicia were extracted up to 25 g with 10 mL 70% ethanol (CAS 64-17-5, Merck) at 130 rpm and room temperature for 24 h. The extract was filtered to obtain the filtrate and concentrated using a rotary evaporator (the 1110S-WD eyela) at 50 °C to obtain a crude extract. The extraction was performed twice. The yield extract was presented in percent and calculated by the Eq. (2).

$$\text{Yield extract} = \frac{\text{final sample weights}}{\text{initial weights} \times (1 - \text{water content})} \times 100\% \quad (2)$$

The fractionation was carried out using three solvents [9] depending on the polarity degree, which was n-hexane (non-polar), ethyl acetate (semi polar) and water (polar) as solvents. The fractionations was performed with a volume ratio of 1:1 for each solvent. One gram of 70% ethanol extract from red betel leaves was dissolved in 75 mL distilled water and poured into a Pyrex 250 mL separating funnel. The fractionation was then mixed with 75 mL n-hexane (CAS 110-54-3, EMSURE®) and homogenized by horizontal rotation within 5 min using the separating funnel. Subsequently, the funnel tap was opened occasionally to release the air and decrease the air pressure. The mixture was put aside for a few moments until a separate layer of n-hexane and water was clearly visible. The n-hexane and water layers were separated in different bottles. The fractionation was repeated up to three times, and it was repeated with the same steps on ethyl acetate (CAS 141-78-6, Merck) and water. In addition, the obtained n-hexane, ethyl acetate, and water fractions from the fractionation results were concentrated with a rotary evaporator (the 1110S- WD eyela) at 50 °C.

Determination of the total phenolic content

The total phenol content was detected with the Follin-Ciocalteu reagent [10]. The 0.2 mL of extracts and each fraction of (n-hexane, ethyl acetate, and water), red betel leaves (with three replications), concentrated using 200 mg/L, 2.5 mL of Follin-Ciocalteu 10% reagents (Merck) and 2 mL of Na_2CO_3 7.5% (CAS 497-19-8, Merck), were mixed and incubated for 30 min. Measured the absorbance with a spectrophotometer (Genesis 10 UV Thermo Scientific) at a wavelength of 765 nm. The total phenolic extract of red betel leaves was demonstrated as milligram (mg) equivalent gallic acid per gram of dry extract weight (mg/GAE/g red betel leaves). Gallic acid was used at various concentrations as the basis which was 0, 25, 50, 75, 100, 125 mg/L.

Antioxidant activity analysis using the Rancimat method

The antioxidant activity analysis using the Rancimat method [11]. To each 1 mL extract and fraction of red betel leaves, added 10 mL of soybean oil at a concentration 200 mg/L and tween 80 (CAS 9005-65-6 Merck) up to 3 mL. The negative control was performed without addition of extract and fraction. Meanwhile, the positive control was invented with BHT addition (CAS 128-37-0 Merck) 200 mg/L. The mixed solution was weighed up to 2.5 g in a test tube and placed in a heating block. The air velocity was set to 18–20 L/h at a temperature of 110 °C. The measurement was determined during the calculation of the incubation time, i.e., when the conductivity of electricity was rapidly increased. Antioxidant activity was denoted as a protection factor (FP) and accumulated by the Eq. (3).

$$\text{The FP} = \frac{\text{Incubation time of soybean oil + sample (hour)}}{\text{Induction time of soybean oil (hour)}} \quad (3)$$

Antioxidant activity analysis tested using the CUPRAC method with modification [12]. The 0.5 mL of the sample was dissolved in 99.9% (Sigma Aldrich). The 50 µL sample solution, 50 µL $CuCl_2.2H_2O$ 0.01 M (Sigma Aldrich), 50 µL ethanolic neucoprine 0.0075 M (Sigma Aldrich) and 50 µL ammonium acetate buffer 1 M with a pH of 7 (Sigma Aldrich) were mixed and placed into a micro plate. The total volume of each well was 200 µL. The sample solution was made with concentrations of 100, 200 and 300 ppm. The sample mixture and reagents were homogenized and then incubated at darkroom temperature for 30 min. The absorbance was measured with an ELISA micro plate reader (Epoch) at a wavelength of 450. Standard curves were generated using Trolox solution (Sigma Aldrich) with concentrations of 10, 20, 30, 40 and 50 µL. The antioxidant capacity was expressed in µmol Trolox/g extract.

Red betel leaves active compound analysis

The most active samples in the total tannin and antioxidant assays were analyzed using the LC-MS (Liquid Chromatography-Mass Spectrometry) QMicro QAA 842 analytical technique and the MS-MS Waters Quatro Micro detector [13]. The sample stock, which has the highest total tannin and antioxidant activity, were dissolved in 5 mL of solution, and up to 5 µL was poured into the LC column with a flow rate 0.2 mL/min. The sample was converted into a gas phase, which would be ionized under vacuum. The ions were accelerated by an electric or magnetic field, which was detected as a mass-to-charge ratio (*m/z*). The column temperature used was 50 °C and the final time was 35 min. The separation of chemical components was performed inside the column using a pump pressure of 300 Bar. The result obtained by LC followed by MS was to identify chemical components in the solution-based mixture. A quadrupole analyzer with a scanning time of 5 s was used. The obtained spectrum indicated a mass-to-charge ratio (*m/z*).

Data analysis

The statistical technique against antioxidant activities was completely randomized, namely the Analysis of Variance (ANOVA) at 95% accuracy and the α value (0.05). The data were analyzed using PASW 18.0 SPSS Statistical Programed for Social Science software program.

Results

The average yield obtained by the simplicia's moisture content was 6.85%, and 81.16% of fresh red betel leaves. The yield from the extraction of red betel leaves macerated with 70% ethanol as solvent was 10.85%. The most abundant yield was detected within the water fraction of 48.50%. Meanwhile, the lowest yield was found within the ethyl acetate fraction of 1.42% (Table 1).

The total phenolic measurement within 70% ethanol extract and the three red betel leaves fraction (n-hexane, ethyl acetate, and water) gave a contradictory result ($p<0.05$). The highest total phenolic content obtained by the ethyl acetate fraction with a value of.198.372 mg GAE/g, whereas the water fraction had a total value of the lowest phenolic content, which was 134.88 mg GAE/g (Table 2).

The Rancimat method resulted that the highest antioxidant activity obtained by the ethyl acetate fraction with a protection factor (FP) was 1.388, while the lowest FP value obtained was 70% ethanol extract with 0.811. The calculated FP BHT value was lower than the FP value of the ethyl acetate and n-hexane fraction, which was 1.153 (Table 3). However, the statistical test not significantly indicated a different result ($p<0.05$).

An antioxidant activity according to the CUPRAC method showed that the highest antioxidant activity was found in the n-hexane fraction sample, followed by the

Table 1: Moisture content, soaked extract, and soaked of red betel leaves.

Materials	(%)
Red betel leaves' moisture content	81.16 ± 0.47
Simplicia's moisture content	6.85 ± 0.19
70% ethanol extract yield	10.85 ± 1.86
Fraction yield	
n-Hexane	3.20
Ethyl acetate	1.42
Water	48.50

Table 2: Total phenolic content of extract and fraction of *P. crocatum*.

Sample	Total phenolic, mg GAE/g
70% ethanol extract	162.65 ± 4.18[a]
n-Hexane fraction	160.67 ± 4.50[ac]
Ethyl acetate fraction	198.37 ± 3.82[d]
Water fraction	134.88 ± 1.19[b]

Different letters in the same column indicate significant differences ($p<0.05$) after analysis of variance and a Duncan comparison test.

Table 3: Rancimat method antioxidant activity of extract and fraction of *P. crocatum*.

Sample	Protection factor value
70% ethanol extract	0.81 ± 0.19[a]
n-Hexane fraction	1.30 ± 0.29[a]
Ethyl acetate fraction	1.38 ± 0.17[a]
Water fraction	0.84 ± 0.09[a]
BHT	1.15 ± 0.92[a]

Different letters in the same column indicate significant differences ($p<0.05$) after analysis of variance and a Duncan comparison test.

Table 4: CUPRAC method antioxidant activity of extract and fraction of *P. crocatum*.

Sample	Antioxidant activity, µmol Tr/g extract
70% ethanol extract	21.33 ± 4.26[a]
n-Hexane fraction	26.23 ± 5.86[ab]
Ethyl acetate fraction	23.00 ± 5.00[ab]
Water fraction	17.33 ± 2.10[a]

Different letters in the same column indicate significant differences ($p<0.05$) after analysis of variance and a Duncan comparison test.

ethyl acetate fraction and 70% ethanol extract. The lowest antioxidant activity was found in the water fraction. Antioxidant activity between the extract and the red betel leaf fraction did not show significantly different result ($p<0.05$) (Table 4).

The most active sample analyzed by the bioactive compound using the Rancimat method was the ethyl acetate fraction. Ten compounds were identified as having the on the highest abundance (Table 5). Schisandrin B, columbine and N-1, N-9-Bis [€-(2-nitrophenyl) methylene] nonanedihydrazide compounds had the highest abundance, that was 25.65% with retention time 7.54.

The most active sample analyzed by the bioactive compound using the CUPRAC method has been found within the n-hexane fraction. The data obtained by sample analysis in the form of a chromatogram spectrum indicated a mass-to-charge ratio (*m/z*). Thirteen compounds were detected based on the highest abundance through chromatogram analysis results (Table 3).

Discussion

The moisture content of red betel leaves average value obtained in this study was 6.75% (Table 1). It was lower compared to the previous study, which was 8.22% [4], This result showed that the simplicia of red betel leaves was good since it was under 10%, based on the stated that the maximum standard moisture content of simplicia is 10% [14]. The moisture content below 10% could inhibit enzymatic processes and damage from microbes such as bacteria, mold and yeast [15]. Therefore, the simplicia of red betel leaves were safely stored before being used for maceration.

The average yield of 70% ethanol extract of red betel leaves was 9.211% (Table 1). The yield value obtained in this study was higher than the previous study which was only 4.42% [7]. Based on the yield value of the fraction obtained by the secondary metabolite compound, the red betel leaves extract was extracted more strongly in polar solvents, which was the water fraction (48.5%) (Table 1). It was because the 70% solvent used in the fractionation process was polar so that it would be easily mixed with water that was also polar [16]. This results was higher than the previous fractionation results study, which was 0.42% for ethyl acetate, but it dropped to 1.03% n-hexane fraction and water fraction was 4.56% [17].

The total phenolic measurement of the red betel leaves sample obtained from the ethyl acetate fraction possessed the highest value of 198.372 mg/g GAE, so that this fraction had the most active compounds compared to other

Table 5: Bioactive compounds of ethyl acetate fraction by LC-MS analysis.

Peaks	Retention time	Molecular weight, *m/z*	Suspect compounds	Molecular formula	% abundance
1	4.46	211.0970	6XO32ZSP1D	$C_{11}H_{14}O_4$	1.83
	5.74	170.0584	Ethyl L-serinate hydrochloride	$C_5H_{12}NO_3Cl$	8.21
2	7.54	401.1957	Schisandrin B	$C_{23}H_{28}O_6$	25.65
	7.54	359.1490	Columbin	$C_{20}H_{22}O_6$	25.65
	7.54	483.1979	N-1,N-9-Bis[€-(2-nitrophenyl)methy lene]nonanedihydrazide	$C_{23}H_{26}N_6O_6$	25.65
3	8.01	355.1559	4-(4-Methoxy-phenylamino)-2,3-dihydro-1H-4a,9-diaza-cyclopenta(b)fluorine-10-carbonitrile	$C_{22}H_{18}N_4O$	6.65
	8.01	401.1980	6-Amino-4-[3-(benzylox xny)phenyl]-3-tert-butyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	$C_{24}H_{24}N_4O_2$	6.65
	8.01	461.2200	6-Amino-4-[3-(benzyloxy)phenyl]-3-tert-butyl-2,4-dihydropyrano [2,3-c]pyrazole-5-carbonitrile	$C_4H_{24}N_{22}O_3S$	6.65
	8.01	478.2462	4-({4,6-Bis[(3R,5S)-3,5-diamino-1-piperydinyl]-1,3,5-triazin-2-yl} amino)benzene sulfonamide	$C_{19}H_{31}N_{11}O_2S$	6.65
4	12.21	593.2772	1,1′-(1,4-Butanediyl)bis{2,6-dimethyl-4-[(3-methyl-1,3-benzothiazol-2(3H)-ylidene)methyl]pyridinium	$C_{36}H_{40}N_4S_2$	9.88

samples. The water fraction possessed a total value of the lowest phenol, 134.8805 mg/g GAE. The difference value was influenced by the level of solvent contamination used against compounds of the metabolites found in the sample. Phenolic compounds were readily soluble in polar solvents, however, phenolic compounds were not only polar but also several free aglycones of this compound that were semi polar [18]. The total phenolic content contained in ethyl acetate extract of Indian Plum (*Flacourtia jangomas L.*) was higher than that of methanol and chloroform extracts [19]. If the phenolic content in the sample was high, it indicated that the antioxidant activity was also high [20].

Measurement of antioxidant activities of red betel leaves using the Rancimat method showed different results between fractions and 70% ethanol extract. The highest antioxidant activity obtained by ethyl acetate fraction with a protection power (FP) was 1.38 (Table 3). The results obtained in this study were higher compared to [21], where the ethyl acetat extract of dragon fruit tendrils had an FP value of 1.28. The existence of antioxidant activity in the ethyl acetate fraction was due to the content of phenolic compounds, including the class or its dominant class, namely flavonoid compounds, which had oxidation-preventing effects [22]. Phenolic compounds were classified as primary antioxidants. They acted as free radical scavengers (FRS) to delay or inhibit the initiation phase or to disrupt the lipid oxidation propagation stage, thereby reducing the formation of volatile decomposition products such as aldehydes and ketones that cause rancid odors. The delocalization of unpaired electrons stabilized phenolic radicals throughout the aromatic ring involved in the termination reaction. Phenolic antioxidants could contribute hydrogen atoms for lipid radicals and produce lipid derivatives and antioxidant radicals that were more stable and could not cause autoxidation [23].

Ten compounds were identified based on the highest abundance in the ethyl acetate fraction (Table 2). The second peak with a retention time of 07.54 and abundance of 25.65% was identified as a compound with a molecular weight of 401.1957 *m/z*, namely Schisandrin B ($C_{23}H_{28}O_6$). Sch B (Schisandrin B) was one of the most abundant and most active dibenzocyclooctadiene derivatives found in *Schisandra Chinensis* fruit. Sch B compounds have been proven to prevent or reduce damage to fat molecules (oxidized fats) due to CC14 exposure to liver cells. Sch B had also been shown to reduce liver transaminase levels. The mechanism of hepatoprotective function possessed by this compound by improving liver function as an anti-inflammatory and antioxidant [24].

Antioxidant activities using the CUPRAC method showed that the highest antioxidant activity was found within the non-polar n-hexane fraction sample and the lowest antioxidant activity has been discovered in the polar water fraction (Table 4). The previous research showed that the antioxidant test results using the DPPH method differed from the results obtained by the CUPRAC method [25], possibly due to the differences in solvent and method sensitivity. The CUPRAC method had selective reagents and a low reduction potential value of 0.17 V [26]. Bioactive compounds that acted as antioxidants in the n-hexane fraction were methyl eugenol, protocatechuic acid, L-(+)-arginine hydrochloride (Table 6). People have made beneficial use of methyl eugenol as it plays roles as a stabilizer and an antioxidant, not to mention as a natural

Table 6: Bioactive compounds of n-hexane fraction by LC-MS analysis.

Peaks	Retention time	Molecular weight, *m/z*	Suspect compounds	Molecular formula	% abundance
1	3.76	179.1069	3,4-(Dimethoxyphenil)-1-propene (methyl eugenol)	$C_{11}H_{14}O_2$	1.00
	3.76	148.0756	4-methoxyindole	C_9H_9NO	1.00
2	6.58	280.1796	Leusil leusin amide hydrochloride (1:1)	$C_{12}H_{26}ClN_3O_2$	2.00
3	7.72	209.1171	5-isopropyl-3-pyrazolidine carbohydrazide hydrochloride	$C_7H_{17}ClN_4O$	11.07
	7.72	147.0441	1H-Pyrazole-1-carboxamidine hydrochloride	$C_4H_7ClN_4$	11.07
	7.72	155.0342	Protocatechuic acid	$C_7H_6O_4$	
	7.72	170.0570	N1-(5-metil isosazol-3) ethane diamide	$C_6H_7N_3O_3$	11.07
4	8.77	345.0958	1-amino-3(aminooxy)-2-propanil N-(4,6-diamino-1,3,5-triazine) glycinate ihydrochloride	$C_8H_{18}Cl_2N_8O_3$	2.49
5	9.22	327.1228	2-(4-morpholinyl methyl) aniline sulfate	$C_{11}H_{22}N_2O_7S$	28.24
	9.22	369.1695	2,2,12,12-tetra metil-4,10-diokso-3,11-dioksa-5,9-diazatridekan metana sulfonat	$C_{14}H_{28}N_2O_7S$	28.24
	9.22	211.0960	L-(+)-arginine hydrochlorine	$C_6H1_5ClN_4O_2$	28.24
6	9.65	281.0817	1-(1,4-ditian-2-metil)-3-(3-metoksi propil) tiourea	$C_{10}H_{20}N_2OS_3$	7.86
	9.65	355.1545	2-(3,5-dimethoxyphenyl)-ethoxy-7-methoxy-1-naphtol	$C_{21}H_{22}O_5$	7.86

attractant. Male fruit flies were strongly attracted by methyl eugenol because it was a sex pheromone compound naturally produced by female fruit flies [27]. Protocatechuic acid compounds possessed an antioxidant activity with the IC_{50} stood at 1.32 µg/mL [28], which indicated that this compound had a potent antioxidant activity. Protocatechuic acid participated in DPPH radical inhibitory activity [29]. Protokatekuat acid was one of the type of phenolic acid, which was more effective in the activity of donating hydrogen and radical inhibitory reactions. L-(+)-arginine hydrochloride was one of the complex amino acids that were often found on active sites in proteins and enzymes because of the side chains that contain amine groups. Arginine was found to be able to prevent or treat heart disease and stimulate the immune system. Arginine hydrochloride functioned as a precursor for the synthesis of nitric oxide (NO) and other critical biological compounds involved in cellular homeostasis [30].

The highest antioxidant activity of the Rancimat method was obtained from the ethyl acetate fraction with a protective factor value of 1.38. Ten compounds were identified in the ethyl acetate fraction of *P. crocatum* leaves. An antioxidant activity according to the CUPRAC method showed the highest antioxidant activity in the sample of the n-hexane fraction with a value of 31.9 µmol Trolox/g extract. Thirteen compounds were identified in the n-hexane fraction of *P. crocatum* leaves.

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Author contributions: Mega Safithri conducted the sample preparation, extraction and fractionation process. Didah Nur Faridah conducted Rancimat and LCMS process. Fitri Ramadani assisted in conducting the CUPRAC process. Rahadian Pratama performed the statistical analysis and data visualization.

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Informed consent: Non applicable.

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