

**INTERNATIONAL RESEARCH JOURNAL OF PHARMACY**

[www.irjponline.com](http://www.irjponline.com/)

ISSN 2230 – 8407

Research Article

# STUDY OF ANTIOXIDANT ACTIVITY WITH REDUCTION OF DPPH RADICAL AND XANTHINE OXIDASE INHIBITOR OF THE EXTRACT OF *RUELLIA TUBEROSA* LINN LEAF

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Article Received on: 17/09/12 Revised on: 22/10/12 Approved for publication: 02/11/12

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**ABSTRACT**

*Ruellia tuberosa* L belongs to Acanthaceae family. In traditional medicine has been used as diuretic, antidiabetic, antipyretic and antihypertensive, and it also recently been incorporated as a component in a herbal tea in Taiwan. This research aims to determine the antioxidant activity of *R. tuberosa* leaf extract. Extraction of *R. tuberosa* leaf by stratified maceration method using dichloromethana and methanol. Methanol extract was partitioned by using ethyl acetate and n-butanol. Antioxidant activity was tested by reduction of DPPH radical method and inhibition of the xanthine oxidase. The results show n-butanol extract has an antioxidant activity with reduction of DPPH radical and inhibition of the enzyme xanthine oxidase with IC50 value respectively 7.42 and 0.15 µg/mL.

**Keywords**: Acanthaceae, antioxidants, pletekan, *Ruellia tuberosa* Linn, xanthine oxidase.

# INTRODUCTION

Antioxidants are compounds that can reduce, restrain and prevent the oxidation process by donating one or more electrons to free radicals that free radicals can be muted. The human body has an antioxidant called endogenous antioxidants included intracellular enzymes such as superoxida dismutase1. However, some enzymes also producing free radicals in the body, such as xanthine oxidase catalyzing hipoxantin to xanthine and then to uric acid releasing free radicals in the form of superoxide anion and hydrogen peroxide. Consequently, xanthine oxidase contributes to oxidative stress in the body, and the pathology of this condition can lead to inflammation, rheumatoid artitis, Parkinson's, Alzheimer's, cancer and aging2. Study of the mechanism of active structures xanthine oxidase inhibitor and free radical reduction has been appeared in coumarin derivatives3 and phenylpropanoid4. Indonesia has many natural sources that have potential effects to be a traditional medicines. It use based on empirical experience in the pass from one generation to another generations. One of them is from Acanthaceae family. Ruellia are one of genus from Family Acanthaceae5. Ruellia are tropical plants and are found in Southeast Asia. In Traditional medicine uses of *R. tuberosa* as diuresis, antidiabetic, antipyretic, antihypertensive and antidote. In Taiwan, *R. tuberosa* leaf was added in health drinks3. In Trinidad and Tobago used as antihypertensive and relieve fever6. In Indonesia, this plant is not yet widely used, even better known as a weed. To increase the utilization of scientific studies pharmacological activity of *R. tuberosa*.

# MATERIALS AND METHOD

**Material Test**. Leaf of *R. tuberosa* was obtained from forests campus of the University of Indonesia, Depok and has been determined in Bogoriensis Herbarium, LIPI.

**Chemicals**. Distilled demineralized water, allopurinol, hydrochloric acid, DPPH, xanthine oxidase enzyme (Sigma Aldrich), ethyl acetate, methanol, n-butanol, xanthine substrate (Sigma Aldrich).

**Extraction**. Leaf of *R. tuberosa* was dried and pulverized. It was extracted by maceration method using dichloromethane as a solvent, and shaken for 6 hours, allowed to stand for 18

hours, then filtration. Continued extraction using methanol, shaken for 6 hours, let for 18 hours. Then filtrated. The filtrate obtained was concentrated using a rotary evaporator at a temperature of 50o C.

**Partition**. Methanol extract dissolved in 100 mL of distilled water, then added ethyl acetate, shaken and allowed to stand for 12 hours. Water layer separated and the ethyl acetate layer accommodated. Partition followed by the addition of n- butanol in the water layer, shaken and allowed to stand for 12 hours. Water layer separated from the layer of n-butanol.

**Antioxidant Activity.** *R. tuberosa* extract (dichloromethane, methanol, ethyl acetate, n-butanol, water) were tested antioxidant activity by DPPH radical reduction methods refer to procedures by Brand-Williams7 with some modifications. IC50 values calculated using the regression equation. Extract solution 0.1 mL added 3.9 mL metanol solution of DPPH, shaken until homogenous, incubated at 37° C for 30 minutes and measured absorption at a wavelength of 517 nm. The same treatment was done BHT and quercetin as a standard. The inhibition percentage of the DPPH radical was calculated from the following equation:

% inhibition = ((A-B)/A) x 100% Where :

A = absorbance of blank, B = absorbance of sample test **Inhibition of Xantin Oxidase Activity**. Inhibition of the xanthine oxidase testing performed on all extracts are dichloromethane, methanol, ethyl acetate, n-butanol and water. Research procedure refers to methods8,9 of Owen et al. and Umamaheswari et al.

**Determination of Maximum Wavelength**. Based on the procedure Sigma Aldrich, to determine the maximum wavelength performed at pH 7.5 and a temperature of 25° C with 0.15 mM substrate concentration. Solution of 0.05 M phosphate buffer pH 7.5 a 2.9 mL, add 2.0 mL of substrate solution with a concentration of 0.15 mM xanthine and then pre-incubation at 25oC temperature for 10 minutes. Further

0.1 mL xanthine oxidase is added and homogenized using a vortex mixer. Mixture was incubated at 25° C for 30 minutes. After incubation, the reaction was stopped by adding 1.0 ml of HCl 1 N. Measured using UV-Vis spectrophotometer to obtain the maximum wavelength. The maximum wavelength of 284 nm is obtained and used to measure next.

**Determination of Xantin Substrate Concentration**. Phosphate buffer solution pH 7.5 a 2.9 mL, add 2.0 mL of xanthine substrate solution of different concentration (0.05, 0.10, 0.15; 0.020 and 0.25 mM) then pre-incubation at 25oC for 10 minutes. After incubation was added 0.1 mL xanthine oxidase solution and homogenized using a vortex mixer. The mixture was incubated at 25°C for 30 minutes. After incubation is complete, the reaction was stopped by adding

1.0 ml of HCl 1 N. Measured using UV-Vis spectrophotometer at maximum wavelength of 284 nm. **Temperature Optimization**. Phosphate buffer solution pH

7.5 a 2.9 mL, add 2.0 mL xanthine solution and pre- incubation at 20, 25, 30, 35 and 40° C for 10 minutes. After pre-incubation, added 0.1 mL xanthine oxidase solution and homogenized using a vortex mixer. The mixture was incubated at 20, 25, 30, 35 and 40° C for 30 min. After incubation, the reaction was stopped by adding 1.0 ml of HCl

1 N. Measured using UV-Vis spectrophotometer at a wavelength of 284 nm.

**Optimization pH**. Phosphate buffer solution (pH 7.5; 7.8; 8.0; 8.3, and 8.5) respectively a 2.9 mL, add 2.0 mL xanthine substrate solution (0,15 mM) and pre-incubation at 30oC for 10 minutes. After preincubation, 0.1 mL xanthine oxidase solution added and homogenized using a vortex mixer. The mixture was incubated at 30oC for 30 minutes. After incubation, the reaction was stopped by adding 1.0 ml of HCl

1 N. Measured using UV-Vis spectrophotometer at a wavelength of 284 nm.

**Calculation of Xantin Oxidase Activity**. The optimum conditions was calculated from the following equation:

Activity = ((Abs. blank – Abs. blank control) x vol x df) / (12.2 x 0.1) Vol

**Where**: vol : Total volume during the test

df: dilution factor

12.2: Coefficient of uric acid (mM)

0.1: Volume xanthine oxidase enzyme

One unit of xanthine oxidase will convert 1.0 μmol xanthine into uric acid per minute (Sigma Aldrich).

**Xantin Oxidase Inhibition Assay**. *R. tuberosa* extract (dichloromethane, methanol, ethyl acetate, n-butanol and water) and allopurinol was tested xanthine oxidase enzyme inhibitory activity. Solution test of 1.0 mL and 2.9 mL of phosphate buffer solution pH 7.8 and 2.0 mL of 0.15 mM xanthine substrate solution were mixed and pre-incubation at 30oC temperature for 10 min, the reaction was initiated by adding 0.1 mL of 0.1 U/mL of xanthin oxidase enzyme. The mixtures was incubated at 30oC for 30 minutes. The reaction was stopped by adding 1.0 ml of HCl 1 N, measured absorption at a wavelength of 284 nm using a UV-Vis spectrophotometer. Control samples. Solution test of 1.0 mL dan 3.0 mL of phosphate buffer pH 7.8 and 2.0 mL of 0.15 mM of substrate xanthine solution and pre-incubation at 30oC for 10 minutes. The reaction was stopped by adding 1.0 ml of HCl 1 N. The mixtures was incubation at 30oC for 30 minutes. After incubation, absorption of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 284 nm. The same test was done allopurino as a standard. Perform a calculation of IC50 value Xantin oxidase enzyme inhibition. The inhibition percentage of xanthine oxidase enzyme inhibitory was calculated from the following equation:

% Inhibition = (1-B / A) x 100%

**Where** A: Changes in the absorbance of the blank test solution

B: Changes in the absorbance of the extract test

solution

IC50 values were calculated using the formula to determine the regression equation y = a + bx. Inhibitory activity expressed by 50% Inhibition Concentration (IC50) is the concentration of sample that can inhibit the xanthine oxidase by 50%.

# RESULTS

*R. tuberosa* extract was tested of antioxidant activity by reduction of DPPH radical and inhibition of xanthin oxidase enzyme. Table 1 describes the results of antioxidant activity by reduction of DPPH radical on the dicloromethana, etyl acetate, methanol and n-butanol with range of IC50 value 21.69-7.42 µg/mL. Highest antioxidant by n-butanol extract and weak antioxidant is water extract. BHT and quercetin as a standard with IC50 value 3.17 and 2.87 µg/mL.

Inhibitory activity assay of xantin oxidase enzyme on optimum conditions (Table 2-4), with using xanthin as substrate. The assay result of inhibitory XOD (Table 5) on dichlorometana, etyl acetat, methanol, n-butanol and water extracts of *R. tuberosa* leaf exhibit of IC50 value respectively 0.32; 0.18; 0.16; 0.15; 0.43 µg/mL, and allopurinol as a standard IC50 value 0.02 µg/mL.

# DISCUSSION

*R. tuberosa* leaf extracted by maceration method, this method includes the simple extraction and not damage chemical compounds on botanicals sample, and the process using so easy to do. The first extraction using dichloromethane solvent with purpose to attract semipolar compounds and there is lots of chlorophyll in the leaf. Further, residue extracted again using methanol solvent. In according to the laws of solubility like disolves like, that will occur when the solubility properties of the same polarity. As with ethanol, methanol is a universal solvent, which can dissolve secondary metabolites in plants10.

Partition also known as liquid-liquid extraction is the process of separation compounds in the two kinds of solvents that do not mix with each other. Partitions is done in stages of semi- polar solvent (ethyl acetate) until polar solvent (n-butanol, it aims to separate the compounds according to the polarity. Antioxidant Activity assay**.** Reactive oxygen species (ROS) or free radicals can be inhibited in several ways that prevent or inhibit the formation of ROS, reduction of free radicals by electron donation and repair damage caused by radical reactivity1. Reduction methods can be performed using DPPH radicals and preventing the formation of ROS by inhibiting ROS-producing enzymes, one of which is the xanthine oxidase enzyme. Extract of *R. tuberosa* (dichloromethane, methanol, ethyl acetate, n-butanol and water) and standard (quercetin and BHT) tested with DPPH radical reduction method. Quantitative testing, the extract was dissolved using methanol pa solvent with reaction time (incubation) at 37°C for 30 minute,11,12 measurements using UV-Vis spectrophotometer at maximum wavelength of 517 nm. In this assay, quercetin and BHT are used as a standard with IC50 values 3.17 and 2.87 µg /Ml .

**Table 1. Data of DPPH Radical Reduction**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample | | Concentration  (µg/mL) | Absorbance(λ 517 nm) | | % Inhibition | IC50  (µg/mL) |
| Blank | Sample |
| Dichlorometana | | 5 |  | 0,817±0,0017 | 3,02 |  |
|  | | 10 |  | 0,815±0,0020 | 3,26 | 14,57 |
|  | | 20 | 0,843 | 0,812±0,0018 | 3,62 |  |
|  | | 30 |  | 0,804±0,0032 | 4,57 |  |
|  | | 40 |  | 0,802±0,0009 | 4,77 |  |
|  | | 50 |  | 0,782±0,0009 | 7,19 |  |
|  |  | Regression equation | | y = 2,289 + 3,274x |  |  |
|  | | 5 |  | 0,802±0,0024 | 4,83 |  |
|  | | 10 |  | 0,799±0,0005 | 5,23 |  |
| Ethyl acetate | | 20 | 0,843 | 0,787±0,0020 | 6,63 | 8,79 |
|  | | 30 |  | 0,769±0,0011 | 8,68 |  |
|  | | 40 |  | 0,762±0,0007 | 9,61 |  |
|  | | 50 |  | 0,756±0,0011 | 10,26 |  |
|  |  | Regression equation | | y = 4,173 + 5,211x |  |  |
| *n*-butanol | | 5 | 0,843 | 0,806±0,0003 | 4,34 |  |
|  | | 10 |  | 0,805±0,0014 | 4,46 | 7,42 |
|  | | 20 |  | 0,775±0,0008 | 8,04 |  |
|  | | 30 |  | 0,772±0,0006 | 8,42 |  |
|  | | 40 |  | 0,761±0,0007 | 9,65 |  |
|  | | 50 |  | 0,747±0,0016 | 11,31 |  |
|  |  | Regression equation | | y = 3,673+ 6,242x |  |  |
| Water | | 5 | 0,720 | 0,716±0,0009 | 0,54 | 21,69 |
|  | | 10 |  | 0,711±0,0001 | 1,26 |  |
|  | | 20 |  | 0,706±0,0021 | 1,86 |  |
|  | | 30 |  | 0,705±0,0011 | 1,99 |  |
|  | | 40 |  | 0,702±0,0012 | 2,43 |  |
|  | | 50 |  | 0,689±0,0001 | 4,22 |  |
|  |  | Regression equation | | y = 0,712 + 2,272x |  |  |
| Methanol | 5 | |  | 0,700±0,0006 | 2,75 | 11,55 |
|  | 10 | |  | 0,693±0,0001 | 3,79 |  |
|  | 20 | |  | 0,692±0,0002 | 3,92 |  |
|  | 30 | | 0.720 | 0,680±0,0005 | 5,57 |  |
|  | 40 | |  | 0,678±0,0012 | 5,87 |  |
|  | 50 | |  | 0,663±0,0013 | 7,87 |  |
|  |  | Regression equation | | y = 2,289 4,129x |  |  |
| Quercetin | 5 | | 0.653 | 0,647±0,0007 | 0,92 | 2,87 |
|  | 10 | |  | 0,626±0,0003 | 4,13 |  |
|  | 15 | |  | 0,618±0,0011 | 5,36 |  |
|  | 20 | |  | 0,602±0,0014 | 7,81 |  |
|  | 25 | |  | 0,587±0,0001 | 10,11 |  |
| Linear equation y = -0,952 + 17,684x | | | | | |  |
| BHT | 5 | | 0,903 | 0,869±0,0005 | 3,69 | 3.17 |
|  | 10 | |  | 0,839±0,0002 | 7,02 |  |
|  | 15 | |  | 0,822±0,0002 | 8,90 |  |
|  | 20 | |  | 0,808±0,0002 | 10,43 |  |
|  | 25 | |  | 0,797±0,0002 | 11,74 |  |
| Linear equation y = 2,501 + 15,624x | | | | | | |

**Table 2. Data of Optimize Temperature**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Temperature (oC) | Absorbance(λ 284 nm) | | | Activity (Unit/mL) |
| Control Blank (KB) | Blank  (B) | B-KB |
| 20 | 0,099 | 0,340±0,0007 | 0,241 | 0,98 |
| 25 | 0,099 | 0,490±0,0247 | 0,391 | 1,97 |
| 30 | 0,099 | 0,556±0,0014 | 0,457 | 2,25 |
| 35 | 0,075 | 0,222±0,0021 | 0,147 | 0,72 |
| 40 | 0,071 | 0,200±0,0035 | 0,129 | 0,63 |

**Table 3. Data of Optimize pH**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| pH | Absorbance(λ 284 nm) | | | Activity (Unit/mL) |
| Control Blank (KB) | Blank (B) | B-KB |
| 7,5 | 0,099 | 0,555±0,0042 | 0,456 | 2,24 |
| 7,8 | 0,101 | 0,605±0,0035 | 0,504 | 2,47 |
| 8,0 | 0,094 | 0,320±0,0014 | 0,226 | 1,11 |
| 8,3 | 0,085 | 0,272±0,0049 | 0,187 | 0,92 |
| 8,5 | 0,074 | 0,211±0,0028 | 0,137 | 0,67 |

**Table 4. Data of Optimize Substrat Concentration**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Concentration  Xantin (mM) | Absorbance(λ 284 nm) | | | Activity (Unit/mL) |
| Control Blank(KB) | Control (B) | B-KB |
| 0,05 | 0,034 | 0,188±0,0063 | 0,154 | 0,76  1,40  2,58  2,13  2,06 |
| 0,1 | 0,098 | 0,383±0,0042 | 0,285 |
| 0,15 | 0,113 | 0,637±0,0021 | 0,524 |
| 0,2 | 0,150 | 0,583±0,0056 | 0,433 |
| 0,25 | 0,202 | 0,620±0,0007 | 0,418 |

**Table 5. Data of Xanthine Oxidase Inhibitor**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample | Concentration (µg/mL) | Absorbance(λ 284 nm) | | | %  inhibition | IC50  (µg/mL) |
| Sample  Control | | Sample |
| Dichlorometana | 0,1 | 0,011 | | 0,398±0,0014 | 33,73 | 0,32 |
|  | 0,5 | 0,027 | | 0,393±0,0021 | 37,33 |  |
|  | 1 | 0,068 | | 0,290±0,0035 | 61,98 |  |
|  | 5 | 0,040 | | 0,240±0,0028 | 65,75 |  |
|  | 10 | 0,022 | | 0,220±0,0007 | 66,09 |  |
|  | Regression equation | | y = 43,91 + 19,10x | |  |  |
| Methanol | 0,1 | 0,036 | | 0,386±0,0021 | 40,06 | 0,16 |
|  | 0,5 | 0,040 | | 0,372±0,0056 | 43,15 |  |
|  | 1 | 0,020 | | 0,243±0,0014 | 61,81 |  |
|  | 5 | 0,016 | | 0,236±0,0042 | 62,32 |  |
|  | 10 | 0,071 | | 0,288±0,0014 | 62,84 |  |
|  | Regression equation | | y = 47,95 + 12,82x | |  |  |
| Ethyl acetate | 0,1 | 0,034 | | 0,382±0,0070 | 40,41 | 0,18 |
|  | 0,5 | 0,044 | | 0,367±0,0023 | 44,69 |  |
|  | 1 | 0,052 | | 0,268±0,0328 | 58,73 |  |
|  | 5 | 0,049 | | 0,272±0,0014 | 61,82 |  |
|  | 10 | 0,048 | | 0,241±0,0028 | 66,95 |  |
|  | Regression equation | | y = 47,11 + 15,60x | |  |  |
| *n*-butanol | 0,1 | 0,028 | | 0,333±0,0028 | 47,77 | 0,15 |
|  | 0,5 | 0,032 | | 0,334±0,0007 | 48,28 |  |
|  | 1 | 0,125 | | 0,405±0,0021 | 52,05 |  |
|  | 5 | 0,030 | | 0,288±0,0014 | 55,82 |  |
|  | 10 | 0,145 | | 0,336±0,0014 | 65,14 |  |
|  | Regression equation | | y = 48,3 + 11,62x | |  |  |
| Water | 0,1 | 0,033 | | 0,382±0,0014 | 40,24 | 0,43 |
|  | 0,5 | 0,008 | | 0,332±0,0021 | 44,52 |  |
|  | 1 | 0,040 | | 0,372±0,0035 | 43,15 |  |
|  | 5 | 0,078 | | 0,289±0,0042 | 62,33 |  |
|  | 10 | 0,028 | | 0,238±0,0049 | 64,04 |  |
|  | Regression equation | | y = 42,59 + 17,43x | |  |  |
|  | Blank 0.647 | | Control blank 0.099 | |  |  |
| Allopurinol | 0,1 | 0,024 | | 0,333±0,0028 | 45,11 | 0.02 |
|  | 0,25 | 0,024 | | 0,275±0,0353 | 55,42 |  |
|  | 0,5 | 0,028 | | 0,171±0,0042 | 74,60 |  |
|  | 1 | 0,034 | | 0,104±0,0014 | 87,56 |  |
|  | Regression equation | | y = 44,21 + 325,05x | |  |  |
| Blank : 0,660 | | | | Control blank : 0,097 | | |



**Figure 1. *R. tuberosa* L.**

The research results showed antioxidant activity with IC50 values of each extract the dichloromethane extract 14.57, methanol 11.55, ethyl acetate 8.79, n-butanol 7.42 and water

21.69 µg/mL. The antioxidant activity with IC50<10 µg/mL value is the smallest (good antioxidant) are n-butanol and ethyl acetate extract and included in the category of extremely powerful antioxidants. Extract of dichloromethane, methanol and water show IC50 values were in the range 10-50

free electron of DPPH radical has been paired with electrons from traps compounds (antioxidants) would reduce of DPPH radical (DPPH-H), and form stable compounds are DPP- Hydrazine12.

Inhibition of xantin oxidase enzyme (XOD) assay on all extract of *R. tuberosa* leaf and allopurinol as a standard. Activity of the XOD is indicated by the formation of uric acid15 with measured using UV-Vis spectrophotometry at a

.

µg/mL included in powerful antioxidants class13. The process

wavelength of 295 nm in aerobic conditions16

In this study,

of reduction of free radicals through the mechanism of donation hydrogen from antioxidant compounds. Free radicals used are synthetic DPPH reacts with an antioxidant compound through the donation of electrons from an antioxidant compound to get a pair of electrons. DPPH radical compound deep purple would fade to yellow if it is reduced by antioxidants into non radical DPPH14, when the

testing was conducted at the optimum conditions, so that will be the determination of the optimum conditions and the determination of the maximum wavelength. The optimum condition at temperature of 30oC, pH 7.8 and 0.15 mM of substrate concentration and maximum wavelength at 284 nm. Inhibition of xanthine oxidase. Allopurinol is used as standard with IC50 value 0.02 µg/mL. Extract of *R. tuberosa*

(dichloromethane, methanol, ethyl acetate, n-butanol and water) was tested inhibitor of XOD*.*The test result extract of dichloromethane, methanol, etyl acetat, n-butanol and water with IC50 value respectively 0.32; 0.16; 0.18; 0.15 and 0.43 µg/mL. Inhibition of the enzyme xanthine oxidase with IC50 values 0.15 µg/mL are the smallest by n-butanol extract. Extract of methanol, ethyl acetate and n-butanol have IC50 values are small, because the extract contains chemical compounds such as phenols, flavonoids and tannins. It is potentially inhibits xanthine oxidase8.

This study, was found a correlation of antioxidant activity mechanism between reduction of DPPH radical and xanthine oxidase inhibition of the extract of *R.tuberosa*.

# CONCLUSION

Extract of *R. tuberosa* active as antioxidant with mechanism by reduction of DPPH radical and inhibition of the xanthine oxidase enzyme. The best antioxidant activity is n-butanol extract with mechanism reducing of DPPH radical and inhibition of the xanthine oxidase enzym with IC50 values respectively 7.42 and 0.15 µg/mL.

# ACKNOWLEDGEMENTS

The authors thank Head Office of Bogoriensis Herbarium, Research Center for Biology, Indonesian Institute of Sciences, for botanical identification of *R. tuberosa*.

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Source of support: Nil, Conflict of interest: None Declared

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