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Anti-Inflammatory Activity Test of Combination Ethanol Extract of Papaya Leaves (*Carica papaya L.*) and Celery Leaves (*Apium Graveolens L.*) in Male White Rats

Muhammad Athfal Nuransyah[1*], Neli Syahida Ni'ma[1]

[1]Department of Pharmacy, Faculty of Medicine, Universitas Negeri Semarang, Indonesia

***Correspondence to:** *athfal.nuransyah@gmail.com*

Abstract: **Background:** Papaya leaves (*Carica papaya L.*) and celery leaves (*Apium graveolens L.*) are suspected to have anti-inflammatory activity due to their secondary metabolites such as flavonoids, tannins, saponins, alkaloids, steroids, and terpenoids. **Aim:** This study aims to determine the anti-inflammatory activity of a combination of ethanol extracts of papaya leaves and celery leaves compared to the use of single extracts and standard anti-inflammatory drugs. **Material and Methods:** The method used is the Rat Hit Paw Edema, which induces inflammation in the rat paw using carrageenan. Twenty-five rats were divided into 5 treatment groups: negative control (CMC-Na 1%), positive control (Na Diclofenac 4.5 mg/kgBW), papaya leaf ethanol extract 50 mg/kgBW, celery leaf ethanol extract 200 mg/kgBW, and a combination of papaya leaf ethanol extract 25 mg/kgBW and celery leaf ethanol extract 100 mg/kgBW. Data collection was done by measuring the paw thickness using calipers at 30, 60, 120, 180, 240, 360, and 480 minutes after carrageenan injection. Data were analyzed by calculating the percentage of inflammation, percentage inhibition, anti-inflammatory percentage, and statistical tests to determine the effectiveness of the extract combination. **Results:** Phytochemical screening results showed that the ethanol extract of papaya leaves contains flavonoids, tannins, saponins, alkaloids, and steroids, while the ethanol extract of celery leaves contains flavonoids, tannins, saponins, and steroids. The combination of ethanol extracts of papaya and celery leaves showed the highest anti-inflammatory effect at 20.80%, compared to papaya leaf extract at 16.40%, celery leaf extract at 16.93%, and the positive control at 18.75%. **Conclusion:** Statistical analysis showed that the combination of extracts had a synergistic effect, with One Way Anova and LSD results being significantly different compared to the use of single extracts.

Keywords: Anti-inflammatory, carrageenan, celery leaves (*Apium graveolens L.*), combination of extracts, papaya leaves (*Carica papaya L.*)

INTRODUCTION

Inflammation is the body's protective response to tissue damage caused by physical trauma, chemicals, and microorganisms (Aruselvan *et al*., 2016). Inflammation plays an important role in the development of chronic diseases such as diabetes, asthma, autoimmune, cardiovascular, cancer, arthritis and infections (Germolec *et al*., 2018). In inflammatory conditions, the body releases inflammatory mediators such as histamine, serotonin, and prostaglandins that cause symptoms of redness (*rubor*), heat (*kalor*), swelling (*tumor*), pain (*dolor*), and loss of function (*function laesa*) (Soenarto, 2014: Kumar *et al*., 2019). Untreated chronic inflammation can cause tissue damage and trigger serious diseases, making it important to control inflammation effectively (Susanti *et al*., 2015).

Treatment of inflammation often involves the use of anti-inflammatory drugs, either steroids or Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) (Setia & Tjitiaresmi, 2019). Although effective, long-term use of these drugs can cause serious side effects such as renal impairment, hypertension, gastrointestinal bleeding, and hormonal disruption (Idacahyati, 2020). These side effects encourage the development of anti-inflammatory drugs from natural ingredients. In Indonesia, plants such as papaya leaves and celery leaves are known to have anti-inflammatory activity due to flavonoids and other active compounds that work similar to NSAIDs in inhibiting the cyclooxygenase enzyme (A'yun & Laily, 2020).

Research on the combination of papaya and celery leaf extracts shows great potential in anti-inflammatory therapy. Both extracts contain flavonoids that reduce inflammation by inhibiting the cyclooxygenase enzyme (Wulandari *et al*., 2016). Research shows that the combination of these extracts can provide significant anti-inflammatory effects in carrageenan-induced rats, potentially increasing clinical effectiveness and reducing the dose of a single drug, thereby reducing the risk of side effects (Cheng et al., 2019). This study aims to evaluate the effectiveness of a combination of papaya and celery leaf extracts in inflammatory therapy compared with each extract and standard anti-inflammatory drugs.

METHODS

Material and Methods

This study is a laboratory experimental study using the Rat Hind Paw Edema method, which is to create artificial inflammation on the sole of the left paw of rats with carrageenan induction (Amirah & Herman, 2015). The research design was Pretest-Posttest with Control Group Design, which means the dependent variable was measured before and after the intervention in the experimental and control groups (Sugiono, 2019).

Time and Location

This research will be conducted in Chemistry Laboratory for phytochemical screening test and Biology Laboratory for anti-inflammatory activity test, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang from February to March 2024.

Equipment and Materials

The equipment used in the test were gastric sonde, syringe, stopwatch, analytical balance, caliper, beaker glass, porcelain dish, measuring glass, reaction tube, dropper pipette, volume pipette, stirring stick, mortar and stamper, water bath, and marker pen.

The materials used for this study were male white rats of wistar strain, papaya leaf powder, celery leaf powder, ethanol 96%, diclofenac sodium 50 mg, carrageenan, CMC-Na, filter paper, NaCl, magnesium, hydrochloric acid, $FeCl_3$, ethyl acetate, sulfuric acid, dragendorff reagent, mayer reagent, and distilled water.

Extraction Methods

Papaya leaf simplisia powder will be obtained from the Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (BBPPTOOT) RSUP dr. Sardjito. Celery leaf simplisia powder was obtained from Indoplant Herbal Shop Bangunjiwo Yogyakarta. Ethanol extracts of papaya leaves and celery leaves were made by maceration method using 96% ethanol solvent. First, 500 grams of papaya leaf and celery leaf simplisia powder were each put into two different containers. The containers were sealed and left for 24 hours, protected from direct sunlight, with stirring every 6 hours to avoid saturation of the solution. Afterwards, the mixture was filtered using filter paper to separate the macerate from powder or large particles. This procedure was repeated twice with a solvent ratio of 1: 4 and 1:3 The collected filtrate was then evaporated using a rotary evaporator to obtain thick extracts of papaya leaves and celery leaves. The extract yield was then calculated.

Phytochemical Screening

In this phytochemical screening test, each ethanol extract of papaya leaves and celery leaves required is 5 grams. Phytochemical screening test according to the method conducted by Shalsyabillah and Sari (2023) as follows: Flavonoid test was carried out by putting 1 gram of extract into a porcelain cup, adding 100 mL of hot water, boiling for 5 minutes, filtered, then 5 mL of filtrate added 50 mg of magnesium and 1 mL of concentrated HCl, and observed the colour change. A positive reaction is indicated by the formation of red, yellow, or orange colour in the sample solution.

Tannin test with 0.5 grams of extract added 10 mL of hot water, boiled 15 minutes, filtered, and added 2-4 drops of $FeCl_3$ to observe the colour change. A positive reaction is indicated by the formation of a green-blue or green-black colour in the sample solution.

Saponin test with 1 gram of extract mixed with 10 mL of hot water, shaken, and added 1 drop of HCl 2N, then observed the formation of foam. A positive reaction is characterised by the formation of foam as high as 1-10 cm and does not disappear after the addition of 1 drop of HCl 2N.

Alkaloid test with 500 mg of extract dissolved in 1 mL of HCl 2N and distilled water up to 10 mL, heated, filtered, and divided into 5 test tubes, each added HCl 2N reagent (blank), Dragendorff, and Mayer to observe the precipitate. The formation of an orange precipitate in the second tube and a white precipitate in the third tube indicates the presence of alkaloids. Test solution containing alkaloids should show positive reaction in at least 2 tubes.

Steroid/terpenoid test with 2 grams of extract mixed with 2 mL of ethyl acetate, taken ethyl acetate layer then dried, and added 2 drops of concentrated sulfuric acid to see the colour change. A positive reaction is indicated by the formation of a red or yellow colour which means positive for terpenoids and green colour which means positive for steroids.

In Vivo Anti-Inflammatory Activity Test

The in vivo anti-inflammatory activity test was conducted after obtaining ethical clearance from the Health Research Ethics Commission of the Faculty of Medicine, Universitas Negeri Semarang. The experiment began with

the preparation stage, namely by selecting 25 samples of experimental animals in the form of white rats (*Rattus norvegicus*) wistar strain which weighed 150 - 200 mg, 2-3 months old, healthy body marked by active movement, and male sex. The experimental animals were grouped into 5 groups (each group consisted of 5 rats) and put into separate cages. Each rat was marked using a marker and the diameter of its foot was measured. The study began with the preparation of test solutions, namely 1% carrageenan solution, CMC-Na suspension, Diclofenac Sodium suspension, and a suspension of ethanol extracts of papaya and celery leaves.

This test procedure involves several stages. First, acclimatisation of the test animal samples was carried out for one week. Secondly, the rats were fasted for 8-12 hours, but given drinks ad libitum to reduce variations that could affect the drug absorption process. Thirdly, the rat's paw was marked and its initial diameter was measured using a caliper. Fourth, the rats were divided into five treatment groups:

1. Negative control was given 1% CMC-Na as much as 0.5 mL using a gastric sonde and after 30 minutes, 1% carrageenan (0.1 mL) was injected intraplantar on the hind paw;
2. Positive control was given Na diclofenac 4.5 mg/kgBB rats and after 30 minutes, 1% carrageenan (0.1 mL) was injected intraplantarly on the hind paw;
3. Treatment group 1 was given ethanol extract of papaya leaves (EEPL) 50 mg/kgBB and after 30 minutes, 1% carrageenan (0.1 mL) was injected intraplantarly on the hind paw;
4. Treatment group 2 was given ethanol extract of celery leaves (EECL) 200 mg/kgBB and after 30 minutes, 1% carrageenan (0.1 mL) was injected intraplantar on the hind paw;
5. Treatment group 3 was given a combination of papaya leaf ethanol extract 25 mg/kgBB and celery leaf 100 mg/kgBB and after 30 minutes, 1% carrageenan (0.1 mL) was injected intraplantarly on the hind paw.

Edema thickness was measured at 30, 60, 120, 180, 240, 360, and 480 minutes by measuring the diameter of the rat's paw using a caliper.

Data Analysis

Data obtained in the form of thickness or diameter of the rat's paw measured using a caliper. Data were processed by calculating the percentage of oedema inhibition and the percentage of anti-inflammatory power grouped at each observation time. Calculated the percentage of inhibition in the form of percent inflammation, percent inhibition, and percent anti-inflammatory power. Observation data in the form of rat paw diameter will then be analysed using the SPSS application. The first test is normality and homogeneity of Kolmogorov-Smirnov to determine the distribution of data and homogeneity of variance. Data that has homogeneous variance and normally distributed has a significance value greater than 0.05 (P value > 0.05). Data that are not homogeneous and not normally distributed have a significance value smaller than 0.05 (P value < 0.05).

Normally distributed and homogeneous data will be tested parametric statistics, namely One Way Anova with a 95% confidence level and Post Hoc test (LSD test) to determine significant differences between groups. The results of the One Way Anova test can show whether there is a difference between groups, if the significance value is <0.05, it shows that there is a difference between groups and vice versa. The LSD test will provide an overview of the location of significant differences in the test sample. Data that are not normally distributed are subjected to the Kruskall Wallis non-parametric statistical test to determine differences between groups and the location of significant differences. In the Kruskall Wallis test, if the significance value <0.05 will indicate that there are differences between groups and vice versa.

RESULT AND DISCUSSION

The results of the phytochemical screening test can be seen in Table 1 below:

Table 1. Result of Phytochemical Screening

No	Compounds	Parameters	Result		Output
			Papaya	Celery	
1.	Flavonoids	Formation of red, yellow or orange colour in the solution	(+)	(+)	Formation of yellow colour in both extract.
2.	Tannins	Formation of blue green/black green colour in the solution	(+)	(+)	Formation of black green colour in both extracts
3.	Saponins	Formation of stable foam 1-10 cm high	(+)	(+)	Formation of stable foam ± 1 cm in both extract
4.	Alkaloids	Formation of orange precipitate in the solution	(+)	(+)	Formation of orange precipitate in the both extract

No	Compounds	Parameters	Result		Output
			Papaya	Celery	
		Formation of white precipitate in the solution	(+)	(-)	Formation of white precipitate in the papaya leaf extract
5	Steroids	Formation of green or blue colour in the solution	(+)	(+)	Formation of green colour in the both extract
6	Terpenoids	Formation of red or yellow colour in the solution	(-)	(-)	No formation of red or yellow colour in the both extract

Phytochemical screening was conducted to qualitatively detect active compound components in ethanol extracts of papaya leaves and celery leaves. The identified compounds include flavonoids, tannins, saponins, alkaloids, and steroids or terpenoids. Changes that occur after applying reagents to the sample, such as colour changes, the formation of deposits, and the appearance of stable foam, indicate a positive or negative reaction[14]. The results of the phytochemical screening test showed (Table 1) that the ethanol extract of papaya leaves contained flavonoids, tannins, saponins, alkaloids, and steroids, while the ethanol extract of celery leaves contained flavonoids, tannins, saponins, and steroids. The results of the calculation of percent inflammation can be seen in Figure 1 below:

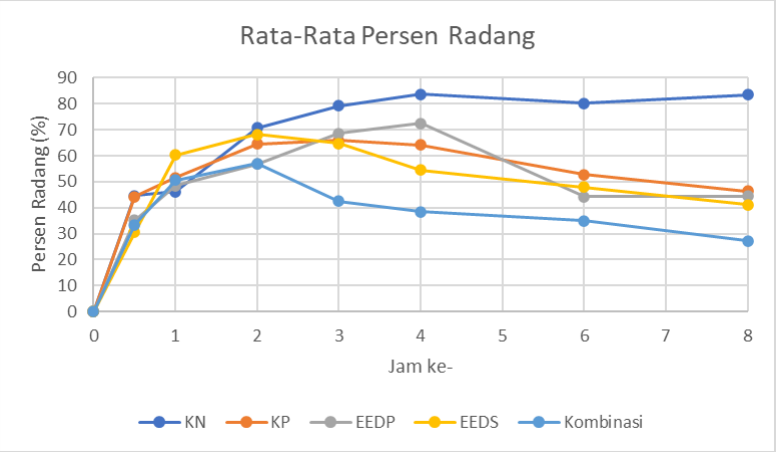

Figure 1. Graph of Average Percent Inflammation Over Time

Based on the average percent inflammation in Figure 1, it shows that edema in the negative control group increased drastically until the 2nd hour. Edema in the negative control group tends to stabilise at the 4th hour until the last measurement, which is the 8th hour. There was no decrease in inflammation in this group. This is in accordance with the theory, where induction with carrageenan will trigger the formation of new edema that will gradually disappear within 12 hours after induction (Sukmawati *et al*., 2015). Meanwhile, the positive control group experienced an increase in edema until the 3rd hour and began to decrease at the 4th hour. The EEPL group experienced an increase in edema until the 4th hour and began to decrease at the 6th hour. The EECL group and the combination group experienced an increase in edema until the 2nd hour and a decrease began to occur at the 3rd hour. This shows that there is an anti-inflammatory effect of EEPL, EECL, the combination of EEPL and EECL, and diclofenac sodium. Judging from the progression of inflammation over time, the fastest onset of anti-inflammation was shown by the single and combined EECL groups which were able to reduce inflammation at the 3rd hour, followed by diclofenac sodium which began to take effect at the 4th hour, and single EEPL which only took effect at the 6th hour.

The average percent inhibition data in Figure 2 shows that the combination group has a much higher average percent inhibition compared to the other groups. Meanwhile, the other three groups showed similar percent inhibition profiles. The difference was in the speed of onset at 2nd hour, the percent inhibition of EEPL was superior to that of EECL and diclofenac sodium. All treatment groups experienced a decrease in percent inhibition at 1st hour because the drugs administered may not have had an optimal effect, so the percent inflammation in the treatment group was still higher than the negative control group. In addition, this was also due to the trendline of inflammation formation in the negative control from hour 0.5 to hour 1 was stagnant and only increased drastically from hour 1 to hour 2. According to Karina *et al*. (2023), the percentage of inhibition can experience an unstable increase and decrease because the extract used contains a variety of active compounds that vary so that it is suspected that there are antagonistic compound interactions that can interfere with and inhibit the performance of anti-inflammatory compounds in the extract. The results of the calculation of percent inhibition can be seen in Figure 2 below:

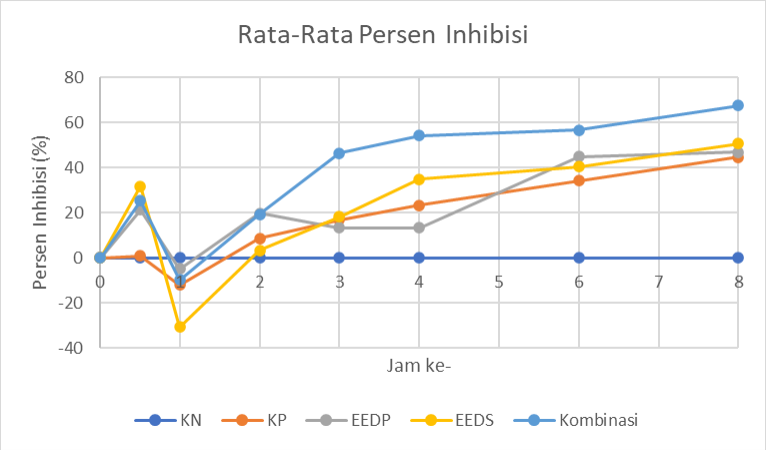

Figure 2. Graph of Average Percent Inhibition Over Time

Percentage of anti-inflammatory power was calculated by comparing the Area Under Curve (AUC) of the treatment group with the AUC of the negative control group. In the calculation of anti-inflammatory power with this method, it has included the variable variation of non-uniform edema measurement time intervals. So, the results of the calculation of anti-inflammatory power will produce only one value for each treatment group and can be used to draw conclusions from the experimental results. Based on the calculation of anti-inflammatory power, the results of the percent of anti-inflammatory power of the positive control group, EEPL, EECL, and the combination of extracts were 18.75%; 16.40%; 16.93%; and 20.80%, respectively. The anti-inflammatory power of EEPL at a dose of 50 mg/kgBB is similiar to EECL at a dose of 200 mg/kgBB, as predicted when designing this study. The anti-inflammatory power of this single extract was still inferior compared to diclofenac sodium. However, the combination of papaya leaf and celery leaf extracts at half the single dose showed the highest anti-inflammatory power, even superior to the standard drug. The combination of the two extracts was able to provide an increase in anti-inflammatory power by ±4% compared to single use. Thus, the combination of the extracts showed a synergistic effect, which is a greater effect than the sum of their individual effects. This analysis was reinforced by the calculation of inferential statistics on the percentage of inflammation to determine the difference in the percentage of inflammation between treatment groups over time. The results of statistical analysis of one way anova and LSD tests can be seen in Table 2 below:

Table 2. Result of One Way Anova and LSD Test

Group	Average percent inflammation at hour-						
	0,5	1	2	3	4	6	8
G1	44,6 ± 16^{d}	46,1 ± 10	70,7 ± 11	79,2 ± 12^{e}	83,7 ± 22^{d,e}	80,3 ± 16^{b,c,d,e}	83,4 ± 12^{b,c,d,e}
G2	44,2 ± 8^{d}	51,6 ± 11	64,6 ± 10	65,9 ± 17^{e}	64,2 ± 19	52,7 ± 21^{a}	46,3 ± 13^{a,e}
G3	35,1 ± 11,1	48,4 ± 23,6	56,7 ± 20,9	68,6 ± 27,6^{e}	72,5 ± 30^{e}	44,3 ± 22^{a}	44,4 ± 17,9^{a,e}
G4	30,4 ± 5,3^{a,b}	60,2 ± 10,9	68,3 ± 12,3	64,8 ± 4,7^{e}	54,4 ± 12^{a}	47,9 ± 12,9^{a}	41,1 ± 6,2^{a}
G5	33,3 ± 5,7	50,6 ± 8,2	56,9 ± 6,5	42,5 ± 10,5^{a,b,c,d}	38,3 ± 13,5^{a,c}	34,9 ± 15,3^{a}	27,1 ± 8,9^{a,b,c}

Notes:
a = significantly different from the negative control (K1)
b = significantly different from the positive control (K2)
c = significantly different from the EEPL group (K3)
d = significantly different from the EECL group (K4)
e = significantly different from the combination group (K5)

Statistical test results (Table 2) at hour 0.5 showed a significant difference between the EECL group with the negative control group and the positive control group. This difference indicates that the inhibition of oedema activity in EECL occurs 30 minutes after carrageenan injection, but has not yet given an optimal effect. The results at the 3rd hour showed that the percentage of inflammation in the combination group was significantly lower than all groups. Meanwhile, the other four groups did not show different per cent inflammation. Therefore, the anti-inflammatory onset of the combined extracts proved to be the fastest compared to the single extract or diclofenac sodium. At the 4th hour, EECL started to show anti-inflammatory activity. The percent inflammation of EECL was significantly lower

compared to the negative control. At the 6th hour, all treatment groups showed significantly lower percent inflammation compared to the negative control. At this time, there was no significant difference in percent inflammation between the positive control, EEPL, EECL, and combination groups. This means that at this time, the potential of the four types of treatment as anti-inflammatory agents is equally good. At the last measurement point, the 8th hour, the combination group showed the lowest per cent inflammation. When tested with One Way Anova, the percent inflammation of the combination group was significantly lower than the negative control, positive control, and EEPL. Meanwhile, the percentage of inflammation in the single extract group was not significantly different when compared to the positive control group. This further strengthens that there is an additional anti-inflammatory effect when these two extracts are combined compared to their single use.

The use of a combination of ethanol extracts of papaya leaves and celery leaves provides a clinical advantage because the interaction causes a synergistic effect on anti-inflammatory activity seen from the results of parametric tests on the percentage of inflammation and the results of the calculation of the percentage of anti-inflammatory power. In addition, the combination of extracts also has a faster anti-inflammatory onset compared to single extracts and diclofenac sodium. A synergistic effect is an interaction that occurs between two or more drugs when the combined effect is greater than the single drug effect (Pezzani *et al*., 2019). The existence of synergistic, additive, or antagonistic effects in this extract combination is suspected by the interaction of phytochemical substances contained in each of the two extracts (Wang *et al*., 2011). The synergistic effect on the combination of extracts indicates the use of a combination of extracts is more effective than its use alone. The dose in the combination of extracts that has been reduced to half the dose of a single extract can minimise the toxic effects and unwanted side effects of using a single dose of extracts. According to Zhang *et al*. (2019) the lower the dose used, the toxic and side effects of a drug will be reduced so that the use of drugs is safer.

Ethanol extracts of papaya leaves and celery leaves show anti-inflammatory activity which is thought to be caused by the content of secondary metabolites such as flavonoids and alkaloids. Flavonoids such as quercetin, kaempferol, narigenin, and rutin in papaya leaf extract are known to inhibit the activity of COX and lipooxygenase enzymes, reducing the production of prostaglandins and leukotrienes that trigger inflammation (Solihah *et al*., 2017). Celery leaves, which contain apigenin and luteolin, also have anti-inflammatory effects by suppressing cell migration to the injured area and stimulating immune responses (Silimi & Pourahmad, 2017). Both extracts also contain tannins and saponins that can stimulate phagocytosis, improve resolution of inflammatory tissues, and steroids that inhibit the release of inflammatory mediator (Djuwarno *et al.,* 2022). Papaya leaf extract also contains alkaloids such as carpain which can accelerate cell proliferation and the healing process in injured areas (Menon & Mutusekhar, 2021).

CONCLUSION

The ethanol extract of papaya leaves contains flavonoids, tannins, saponins, alkaloids, and steroids. Ethanol extract of celery leaves contains flavonoids, tannins, saponins, and steroids. The study showed that the percentage of anti-inflammatory power of diclofenac sodium, papaya leaf ethanol extract, celery leaf ethanol extract, and their combination was 18.75%, 16.40%, 16.93%, and 20.80%, respectively. The use of the combination of the two extracts showed a synergistic interaction that increased the anti-inflammatory activity compared to the use of the single extract.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest

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