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Test of anti-inflammatory activity of methanol extract of nyamplung fruit(*calophyllum inophyllum* l.) on male white rats of the wistar strain

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Abstract. The purpose of this study was to evaluate and compare the anti-inflammatory efficacy of the methanol extract of *Calophyllum inophyllum* L. with a standard anti-inflammatory agent in Wistar rats. The research method used is randomized controlled laboratory experiment. The time and location of the research was carried out in May–August 2024 at the Biological Laboratory of Ahmad Dahlan University and the Research Laboratory of HarapanBangsa University. The samples used were 25 mice divided into 5 groups. Group 1 was negative control, group 2 was positive control, groups 3, 4 and 5 given a salution of nyamplung fruit extract at respective doses 50 mg/kgBB, 75 mg/kgBB and 100 mg/kgBB. The results of the study showed that nyamplung fruit extract from groups 3,4 and 5 had a percent anti-inflammatory power value of 27.92% ± 0.1607% (significant value 0.007), 36.38% ± 0.2982 (significant value 0.152)and 39.78% ± 0.0387 (significant value 0.152) respectively. The results of the Post-Hoc LSD analysis showed a p value of 0.525, meaning there was the methanol extract of *Calophylluminophyllum* L. exhibited anti-inflammatory properties at doses of 50 mg/kgBW, 75 mg/kgBW, and 100 mg/kgBW, with optimal efficacy at 75 mg/kgBW, comparable to diclofenac sodium. The conclusion of this research is that groups 3,4 and 5 of the methanol extract of nyamplung fruit have anti-inflammatory activity, and the optimal dose observed was 75 mg/kgBW.

1 Introduction

The nyamplung plant (*Calophyllum inophyllum* L.) is often found along the coast. It can grow up to 100 to 350 meters above sea level (1). Nyamplung plants have many benefits for human life which are used by the community to treat eye pain, rheumatism, inflammation, and wounds. Nyamplung is also a promising alternative, with lower levels of side effects and often comparable efficiency to conventional drugs (2).

Phytochemical studies of the genus Calophyllum show that nyamplung plants contain many secondary metabolite compounds such as flavonoids, steroids, tannins, phenol

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hydrocarbons, saponins, and triterpenoids. However, most components of the nyamplung tree can produce oil (3). Flavonoids have anti-inflammatory properties because they inhibit cyclooxygenase or lipoxygenase, thus inhibiting the accumulation of white blood cells in the area (4).

Isolation and characterization of compounds in nyamplung fruit found inophyllum compounds, xanthone and other compounds. In addition, coumarin compounds were also found which were more obtained from nyamplung fruit than the leaves (5). One class of plant secondary metabolites is coumarin, this organic compound has anti-inflammatory, antioxidant, antiallergic, antithrombotic, antiviral, and anticancer properties (6). Coumarin inhibits the activity of the COX-2 enzyme, which is responsible for converting arachidonic acid into prostaglandins, which are important mediators of the inflammatory response. By inhibiting COX-2, it can reduce prostaglandin production and suppress the inflammatory response (7).

Inflammation is a natural response of body tissues to various damaging agents such as bacteria, chemicals, or physical injury, which can result in cell damage (8). Anti-inflammatory drugs work to relieve or suppress this inflammatory process. Based on their mechanism of action, anti-inflammatory drugs can be classified into two main groups: steroids and non-steroids (9).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most popularly used drugs due to their efficacy in reducing pain and inflammation. However, despite the efficacy, data from several placebo-controlled trials and meta-analysis studies alarmingly show adverse effects of NSAIDs on gastrointestinal, cardiovascular, hepatic, renal, cerebral, and pulmonary complications. Hence, there is a need for relatively safer treatment alternatives especially from herbal ingredients (10).

Wistar male white rats were chosen as the animal model in this study because their genetic and physiological characteristics are very similar to humans. As well as the resulting inflammatory response, it is suitable for use in research and can provide insights relevant to the human inflammatory response.The use of male animals in this study aims to avoid hormonal influences that can affect the results of the study (11).

Based on the background, this study aims to determine the anti-inflammatory effect of nyamplung fruit extract *(Calophyllum inophyllum* L.) as an alternative anti-inflammatory treatment. Therefore, the findings of this study will provide additional information on the benefits of nyamplung fruit extract as an alternative anti-inflammatory treatment.This study hypothesizes that the methanol extract of nyamplung fruit (*Calophylluminophyllum* L.) will demonstrate comparable anti-inflammatory effects to standard NSAIDs in a rat model.

2 Material and Methods

2.1 Material

The tools used in this study are rat cages, rat food and drink containers, cleaning equipment, plestismometer, rotary evaporator (biobased), analytical balance (Kenko KK-LAB), refrigerator (Sharp), porcelain cup, ointment pot, plastic wrap, aluminum foil, laboratory glassware (Pyrex®), syringe, sonde, stirring rod, label, marker and watch.

The materials used in this study include nyamplung fruit extract, methanol5 M, NaCl 0.9%, CMC-Na0.5% solution, in this study also used 1% keragenan material induced on the soles of rat feet and diclofenac sodium used as treatment for experimental animals. The experimental animals used in this study were male white wistar rats.

2.2 Methods

The location of this research was carried out at the Biology Learning Laboratory of the Faculty of Applied Science and Technology, Ahmad Dahlan University, Research Laboratory and Harapan Bangsa University Research Laboratory for anti-inflammatory testing. While the research time will be carried out in May-August 2024. This study used the One Way ANOVA test followed by the Least Significant Different (LSD) method test to see differences between treatment groups. The software used for data analysis is Statistical Program for Social Science (SPSS). This research has received research ethics approval issued by the HarapanBangsa University Health Research Ethics Commission with a letter of ethical eligibility No.B.LPPM-UHB/698/07/2024. This research ethic ensures that everything goes according to the rules that apply to animal testing.

Preparation of simplisia
The 7 kg nyamplung fruits were then wet-sorted to remove dirt (dust, insects, and twigs) and washed under clean running water. After that, the seeds are removed and the fruit is dried by drying in the sun. Drying is done using a drying cabinet with a temperature of 40°C. The main purpose of drying is to reduce the moisture content of the material so that it can inhibit unwanted microbial growth (12). Materials can generally be dried at a temperature of 40-60 ° C, because if the temperature is too low the drying takes longer and if the temperature is too high it will affect the bioactive compounds contained therein, thus affecting the quality of the simplisia (13). Then, the simplisia was pulverized using a blender to reduce particle size and obtained 500 grams of nyamplung fruit simplisia. The purpose of reducing particle size is to expand the surface of the particles so as to accelerate the penetration of solvents into the extracted particles so that the extract process takes place properly.

Preparation of extract
500 grams of nyamplung fruit simplisia powder was put into a vessel, then soaked using 5 liters of methanol solvent. The maceration process was carried out for 3 x 24 hours with occasional stirring. The results of the maceration process were then filtered. The filtrate obtained was then evaporated using a vacuum rotary evaporator at 40 ℃ and thickened using a waterbath at 70 ℃ to obtain a thick extract.

Phytochemical screening
Phytochemical screening testing was carried out because it was to determine whether the methanol extract of nyamplung fruit really contained bioactive compounds of flavonoids, coumarins, tannins, saponins and steroids. Where flavonoids and coumarins are bioactive compounds that have anti-inflammatory activity.

Coumarin
Methanol extract of nyamplung fruit as much as 0.05 grams is evaporated to dryness add hot water and cool. After cooling divide into two tubes. Tube I was given 10% ammonia and tube II as a comparison. And seen under UV light, if there is yellow, green and blue color, it means positive for coumarin (14).

Flavonoids
A total of 2 mL of nyamplung seed extract sample was heated for about 5 minutes and then added with 0.1 gram of Mg metal and 5 drops of concentrated HCl. If an orange yellow to red color solution is formed, it is positive for flavonoids (15).

Tannins
The tannin compound test was carried out using FeCl3 1%. If a blackish green solution appears, it means that the sample is positive for tannins (16).

Saponin
Testing for saponin compounds using distilled water and then shaken for 30 seconds if foam forms for 10 minutes with the addition of 2 N HCl means the sample is positive for saponins (17).
Steroids
A total of 2 mL of nyamplung fruit extract sample was added with 3 drops of concentrated HCl and 1 drop of concentrated H2SO4. If the solution forms a green color, it is positive for steroids (18).

Treatment of test animals
Test animals totaling 25 rats were divided into 5 groups (each group consisted of 5 rats), acclimatized for 7 days to adapt to the experimental environment. Rats were given standard food and drinks were given ad libitum. The cage used was a single cage with a size of 41 x 31 x 13 cm with husks as a base in the cage(19).Then the day before testing the rats, each group of rats was first fed for 12-18 hours (20). Measurements were taken on the left leg that had been marked at the hind ankle joint using a marker (21). The treatment of the test animal groups is as shown in the table below:

Table 1. Treatment group (Oral administration volume: 1 mL)

Group	Treatment
Group I (Negative control)	Na-CMC 0.5%
Group II (Positive control)	Diclofenac sodium suspension
Group III	Methanol extract of nyamplung fruit dose 50 mg/kgBB
Group IV	Methanol extract of nyamplung fruit dose of 75 mg/kgBB
Group V	Methanol extract of nyamplung fruit at a dose of 100 mg/kgBB

Thedose was chosen based on the research of Zakaria et al., tahun2014 which stated that nyamplung in in-vitro testing showed that the crude fruit extract at a concentration of 50 µg/mL inhibited cyclooxygenase and lipooxygenase activity by 77% and 88%, respectively, thus showing its potential as an anti-inflammatory agent (5). Determination of dose consistency was ensured by normalizing the body weight of the rats.

3 Result and discussion

Preparation of simplisia
The results of making simplisia obtained fresh nyamplung fruit as much as 7 kg. Then the stage of peeling and separating the fruit from the seeds is carried out for the next drying process.In the process of processing simplisia, the drying stage is one of the activities that can affect the quality of the resulting product. The main purpose of drying is to reduce the moisture content of the material so that it can inhibit unwanted microbial growth. In addition, drying also aims to obtain non-perishable simplisia so that it can be stored for a longer time, because by reducing the water content and stopping enzymatic reactions, the deterioration of quality or damage to simplisia will be prevented so that the resulting extract is of good quality (22). Drying with high temperatures can cause the content of phytochemical compounds in nyamplung fruit to be lost, thus affecting the extraction results (23).

The dried nyamplung fruit is then crushed using a cooper to get smaller particles, this aims to reduce the particle size will increase the penetration of solvents into the extracted

particles so that the extract process takes place properly.The drying percentage of nyamplung fruit powder is 16.66%. These results were obtained from comparing the weight of wet nyamplung fruit simplisia with dry nyamplung fruit simplisia.

Preparation of extract

The extraction process of nyamplung fruit was carried out using the maceration method using methanol solvent. The use of methanol solvent because it is considered a polar solvent in this study, so it can dissolve coumarin and phenol groups such as phenolic acids, flavonoids, alkaloids, tannins and lignin. Methanol solvent has a high polarity so that it can maximize the extraction of flavonoids and coumarins which are polar in nature. The chemical structure of flavonoids and coumarins has hydroxy groups (-OH) and double bonds that allow interaction with polar solvents such as methanol, so that these compounds can dissolve optimally (24). The extraction process is carried out at room temperature or room temperature in a dark bottle, this is done to prevent the destruction of secondary metabolites by direct sunlight (25). Other factors or conditions that can affect extraction are temperature, stirring speed, size, shape, and condition of solid particles, type, and amount of solvent (26).

The methanol liquid extract of nyamplung fruit obtained was concentrated using a rotary evaporator and waterbath to obtain a thick extract. The thick extract was then weighed to calculate the % yield. The thick extract of nyamplung fruit obtained from the thickening process is 82.26 g with a yield of 16.45%. The yield of nyamplung fruit extract meets the requirements of a good viscous extract yield, which is not less than 10% (27). The yield value indicates the amount of bioactive compounds contained in the extract. The greater the yield value, the greater the value of the extract produced (28). The results of this extraction are different from the results of the extraction of nyamplung fruit seeds in the research of Khery et al., 2023 by maceration method and using alcohol solvents which produced an extract of 27.55% (18).

Phytochemical screening

Phytochemical screening is carried out with the aim of knowing the content of secondary metabolites contained in methanol extract of nyamplung fruit. Data from phytochemical screening of methanol extract of nyamplung fruit can be seen in Table 2.

Table 2. Phytochemical screening test results of methanol extract of nyamplung fruit

Compound	Result		Conclusion
	Positive as per literature	Research Results	
Coumarin	Yellow, green, blue solution.	Green solution	+
Flavonoids	Orange-yellow solution.	Orange solution	+
Tannins	Green-black solution.	Green-black solution	+
Saponins	Foam formed on top of the solution.	Foam formed on top of the solution	+
Steroids	Solution is green in color.	Green solution	+

Based on the results of phytochemical screening, the methanol extract of nyamplung fruit showed positive results for coumarin, flavonoids, tannins, saponins and steroids.The results of this study are in line with the research of Khery et al., 2023 which states that nyamplung fruit seed extract contains coumarins,alkaloid, flavonoid, polyphenol/tannin, saponin, terpenoid and steroid compounds (18).

Coumarins, flavonoids, tannins, saponins and steroids are closely associated with anti-inflammatory activity. Where coumarin inhibits the activity of the COX-2 enzyme, which is

responsible for converting arachidonic acid into prostaglandins, which are important mediators of the inflammatory response. By inhibiting COX-2, it can reduce prostaglandin production and suppress the inflammatory response (7). Flavonoids have anti-inflammatory properties because they inhibit cyclooxygenase or lipoxygenase, thus inhibiting the accumulation of white blood cells in the area (4). Furthermore, tannins, saponins and steroids are responsible for anti-inflammatory by the mechanism of stimulating lipomodulin protein biosynthesis which inhibits the enzymatic work of phospholipase which affects the metabolic activity of arachidonic acid enzymes (29).

Anti-Inflammatory Activity

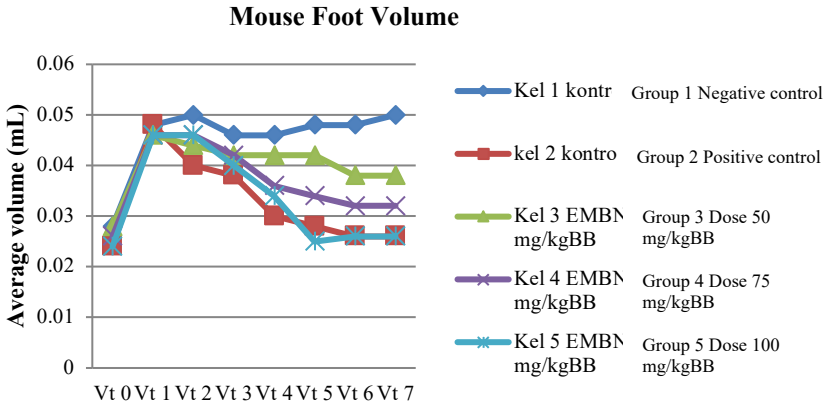
The method used in testing anti-inflammatory activity is by forming edema on the soles of rat feet using carrageenan induction. The choice of carrageenan as an inflammatory induction material in this study is because carrageenan does not cause systemic effects. Carrageenan is a potent inflammatory agent used to release inflammatory and proinflammatory mediators such as prostaglandins, leukotreins, histamine, bradykinin, TNF-a and others (30). This study used the induction of 0.1 mL of 1% carageenan suspension on the left hind paw of rats subplantarly.

Measurement of edema volume using a plestismometer. Measuring the volume of edema of the feet of rats using a mercury plestismometer is the best choice because researchers do not need to sacrifice rats every time they take measurements, besides measuring edema volume can be done at a certain time interval, and can determine the maximum inhibition of the test material used (31). In this study, edema volume measurements were taken at 1 hour intervals for 6 hours. This is because edema develops rapidly and stays at its maximum volume for about 6 hours after carrageenan induction (32).

In this study, the animals used were male white rats of the wistar strain with a body weight of 150-250 grams. The use of white rats as test animals because white rats are considered a good experimental animal model, because they are easy to care for, can be obtained in large quantities and have reliable replication values (33). The selection of wistar strains is because wistar rats have a relatively fast metabolic ability so that they are more sensitive when used in research related to body metabolism. Male white rats also have a faster speed of drug metabolism and a more stable biological condition than female rats (34).

Anti-inflammatory testing was carried out using 5 treatment groups, each group consisting of 5 test animals. The first group is a negative control only given Na-CMC. The second group as a positive control was given diclofenac sodium. The third, fourth and fifth groups were consecutively given methanol extract of nyamplung fruit at a dose of 50, 75 and 100 mg/KgBB. All groups of test animals were induced with 1% carrageenan. Measurement of leg edema volume was done every 1 hour for 6 hours. Measurement of edema volume using a mercury plestismometer. The data obtained from the observations were the edema volume data of each treatment.

The volume curve above shows that the negative group data increased until the 1st hour which occurred after the administration of 1% carrageenan induction, then decreased at the 2nd hour and increased until the 6th hour, this indicates that the test animals were only given Na-CMC which could not inhibit edema formation so that the volume of rat feet tended to increase gradually. Group 1 positive control showed an increase after 1% carrageenan induction and continued to decrease from the 1st hour to the 6th hour with faster results in reducing the volume of rat feet compared to the other dose extract treatment groups.



Description:
Vt 0: Before 1% carrageenan induction (mL)
Vt 1: After 1% carrageenan induction (mL)
Vt 2: Measurement of edema volume after treatment (mL) 1st hour
Vt 3: Measurement of edema volume after treatment (mL) 2nd hour
Vt 4: Measurement of edema volume after treatment (mL) 3rd hour
Vt 5: Measurement of edema volume after treatment (mL) 4th hour
Vt 6: Measurement of edema volume after treatment (mL) 5th hour
Vt 7: Measurement of edema volume after treatment (mL) 6th hour

Fig.1. Mouse paw volume curve

Group 3 administration of EMBN at a dose of 50 mg / kgBB experienced an increase after 1% carrageenan induction, at hours 1-3 began to decline and at hours 4-6 the decline in the volume of rat feet continued. Group 4 administration of EMBN at a dose of 75 mg / kgBB increased after 1% carrageenan induction, then showed a decrease in the volume of rat feet at hours 1-6 which was faster than group 3. Group 5 administration of EMBN at a dose of 100 mg / kgBB began to work as an anti-inflammatory at hour 1 and still worked to inhibit edema formation until hour 6. So that the administration of EMBN doses of 50, 75 and 100 mg / kgBB has anti-inflammatory activity.

The results of statistical tests on the volume of edema of rat feet in normality and homogeneity tests obtained consecutive sig values. 0.065 ($p>0.05$) and 0.069 ($p>0.05$). These results indicate that the data is normally distributed and homogeneous. Furthermore, the One Way ANOVA test obtained a sig value. 0.014 ($p<0.05$) which means there is a significant difference between treatment groups. The test was continued with the post hoc test with Least Significant Different (LSD).

Table 3. Post Hoc LSD test results of edema volume

Group	Significant Value			
	K+	K1	K2	K3
K-	0,000*	0,000*	0,000*	0,000*
K+		0,007*	0,152	1,000
K1			0,152	0,007*
K2				0,152

Based on the data from the Post Hoc LSD test, the edema volume of the treatment group using methanol extract of nyamplung fruit shows that the doses of 75 mg/kgBB and 100 mg/kgBB have similarities with the positive control group. This shows that the treatment

group doses of 75 mg / kgBB and 100 mg / kgBB have anti-inflammatory activity with a significant value> 0.05, namely 0.152 and 1.000.

Calculation of the average AUC (Area Under the Curve) and percent anti-inflammatory power of methanol extract of nyamplung fruit

The following are the results of the average AUC and percent anti-inflammatory power (DAI) of each treatment group.

Table 4. Average AUC and %DAI

Group	Average AUC	Average %DAI
Group 1 Negative control (Na-CMC)	0,0204± 0,0024	-
Group 2 Positive control (Na diclofenac)	0,0111 ± 0,0052	49,21% ± 0,3181
Group 3 Dose 50 mg/kgBB	0,015 ± 0,0021	27,92% ± 0,1607
Group 4 Dose 75 mg/kgBB	0,0137 ± 0,0042	36,38% ± 0,2982
Group 5 Dose 100 mg/kgBB	0,0127 ± 0,0015	39,78% ± 0,0387

The AUC value in the negative control group has the largest value compared to the other test treatment groups, the AUC value of the negative control group is large enough to have the least edema reducing effect. The AUC value of the positive control group is smaller than the three extract dose groups, this shows that the positive control AUC is the smallest value, indicating that the positive control group has the greatest edema reducing effect (35).

The AUC data that has been obtained is then used to calculate the percent value of anti-inflammatory power (% DAI). The results obtained in the positive control group of diclofenac sodium had the highest percent anti-inflammatory value of 49.21%. Table 4 shows that the greater the percentage of anti-inflammatory power, the greater the anti-inflammatory effect. This is inversely proportional to the AUC value,where the smaller the AUC value, the greater the %DAI value, indicating the greater the anti-inflammatory activity of the methanol extract of nyamplung fruit.

% DAI or % anti-inflammatory power is the ratio of the percentage value of udem inhibition of the control and treatment groups(36). Meanwhile, AUC is the area under the curve that describes the relationship between the average udem volume and the time of data collection (37). So that the smaller the AUC value, the greater the % DAI, which means that the value of the relationship between the average volume of udem and the time of data collection decreases (the magnitude of inflammation decreases), then the percentage of resistance to udema in the control and treatment groups is greater (the inhibition of inflammation is greater) (36).

The percentage of anti-inflammatory power provides an initial picture of the potential anti-inflammatory effectiveness of nyamplung fruit extracts. A high percentage of DAI indicates that nyamplung fruit extract has a strong anti-inflammatory activity on the test animals. This potential does not rule out the possibility of further research or application through clinical trials on humans.

The % DAI value that has been obtained is then subjected to statistical tests to determine differences between groups. The results of the statistical analysis of % DAI in the data normality test found that the data were normally distributed with sig. 0.225 (>0.05) and homogeneous data with a sig value of 0.302 (>0.05). The One Way Anova test showed that there was no difference between the treatment groups with a significance value of 0.888>0.05, so no LSD Post-Hoc test was performed.The results of the Post Hoc LSD % DAI test showed that there was no significant difference in the anti-inflammatory effect on rat paw edema in the positive control group and the dose group. This is due to the content of flavonoid compounds, coumarin in nyamplung fruit which functions as an anti-inflammatory.

Flavonoids inhibit prostaglandin biosynthesis by inhibiting the COX/cyclooxygenase enzyme (38). This results in a reduction in prostaglandin production in the arachidonic acid pathway which will result in no inflammation being formed, thus reducing the incidence of pain. Apart from inhibiting the cyclooxygenase enzyme, flavonoids also stop neutrophil degranulation thus preventing the release of cytokines, free radicals and enzymes that play a role in the inflammatory process (39).Coumarin inhibits the activity of the COX-2 enzyme, which is responsible for converting arachidonic acid into prostaglandins, which are important mediators of the inflammatory response. By inhibiting COX-2, it can reduce prostaglandin production and suppress the inflammatory response (40).

The mechanism of saponin compounds as anti-inflammatory is by inhibiting exudate formation and inhibiting vascular permeability (41). The anti-inflammatory mechanism of tannin compounds works through several pathways, the first pathway with inhibition of oxidant production by macrophages, neutrophils and monocytes. The second pathway is by direct inhibition of reactive oxidants such as hypochlorous acid and hydroxy radicals (42).

4 Conclusion

Matanol extract of nyamplung fruit in doses of 50, 75 and 100 mg/kgBW has anti-inflammatory activity because it is significantly different from the negative control with a sig value. 0,00 (P<0,05). The level of efficacy can be seen from the % DAI from the lowest to the highest in order, namely doses of 50, 75, and 100 mg/kgBW.

Based on AUC data and edema volume, there are statistically significant differences between these groups. The best recommended dose is 75 mg/kgBW as evidenced by the results of data analysis in the post hoc test which shows a p value of 0.152 which is equivalent to the anti-inflammatory activity of the positive control, namely na. diclofenac.

The limitation in this study is that the anti-inflammatory testing of nyamplung fruit methanol extract was not carried out using other comparative controls to inhibit inflammation. So for future researchers, it is hoped that they can use other comparative controls. In addition, further optimization of the dose of nyamplung fruit extract or testing in additional models can be done. The results of this study can also be used as literature material on the anti-inflammatory activity of nyamplung fruit.

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