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ANTIARTHRITIS AND ANTI-INFLAMMATORY ACTIVITY OF *Acalypha indica* L. HERBS ON RATS

Rina Herowati[1,*], Matias N. Kolobani[2], Maria E. Klau[1], Gunawan P. Widodo[1]
[1]Faculty of Pharmacy, Setia Budi University, Indonesia
[2]Pharmacy Departement, Health Science High School of Citra Husada Mandiri, Indonesia

*Corresponding author: R. Herowati Tel: +62-81572687689 Fax: +62-271853275 E-mail: rinagunawan53@gmail.com

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ANTIARTHRITIS AND ANTI-INFLAMMATORY ACTIVITY OF *Acalypha indica* L. HERBS ON RATS

Abstract

Acalypha indica L. has been used as traditional medicine in arthritis therapy due to its anti-inflammatory activity. This research investigated the antiarthritis as well as anti-inflammatory activity of *A. indicia* herbs extract and fractions. Arthritis model rats were induced by 0.2 ml intraplantar Collagen Freund Adjuvant. Ethanol extract of *A. indica* herbs doses of 50, 100 and 200 mg/kg daily were administered for 18 days. The paw volume was recorded using plesthymometer every 3 days. The extract then was fractionated by vacuum liquid chromatography, resulted 5 fractions. Anti-inflammatory test by carrageenan-induced rat paw oedema method, membrane stabilization test as well as protein denaturation inhibition test were conducted on these 5 fractions. Anti-arthritis activity test showed that the *A. indica* ethanol extract dose of 200 mg/kg bw reduced oedema volume and enhanced histopathological profile comparable to triamcinolone. Anti-inflammatory test showed that anti-inflammatory effect of fraction B was comparable to Diclofenac Na. Fraction B also showed highest membrane stabilization and protein denaturation inihibition activites among other fractions. The chemical compounds in fraction B were triterpenoids and steroids, based on the thin layer chromatography profile.

Keywords: *Acalypha indica* L., anti-arthritis, anti-inflammatory, membrane stabilization, protein denaturation inhibition

INTRODUCTION

Arthritis is a slow progressive joint disease, affecting the cartilages, and most occur in the joints of the spine, hips, knees and ankles. The main symptom of this disease is inflammation, characterized by pain, heat, swelling and redness in the joints. Treatment of arthritis is symptomatic, with oral analgesic such as paracetamol and tramadol and nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin, diclofenac, ibuprofen and others. Despite are targeted to relieve pain and decrease the joint inflammation, these drugs are known to produce various side effects, including gastrointestinal disorders, renal toxicity as well as hepatotoxicity. Reducing side effects should be considered while screening, improved therapeutics for arthritis, besides enhancing medicinal effectiveness. Traditional treatment is being increasingly recognized as an alternate approach to arthritis treatment(1). *A. calypha* indica (belongs to Euphorbiaceae family) can be easily found in Indonesia. It has been traditionally used as anthelmintic, cathartic, scabies, and rheumatism. The extract of *A. indica* has been reported to have anti-inflammatory, analgesic and antimicrobial properties[2]. *A. indica n*-hexane extracts show inhibitory effect to 5-lypooxygenase (5-LOX) and cyclooxygenase (COX) enzyme. Methanol extract of *A. indica* dose of 250 mg/kg bw inhibited oedema in the hind paw of mice induced by carrageenan at 30.6%, which was comparable to phenylbutazone[3]. Ethanol extract of the *A. indica* herb dose of 200 mg/kg bw was reported to provide anti-arthritis effect in collagen type 2-induced arthritis rat(2).

The anti-arthritis activity of *A. indica* in Complete Freund's adjuvant (CFA) induced arthritis has not been reported previously. Investigation of chemical constituents which contributed to the anti-arthritis and anti-inflammmatory also important to be conducted. The objectives of our study were to determine the antiarthtritis activity of ethanolic extract of *A. indica* herb (EEA) on CFA-induced arthtritis and anti-inflammatory activity of the fractions by carrageenan-induced rat paw oedema method. Membrane stabilization test and protein denaturation inhibition were used to determine the in vitro anti-inflammatory as well as anti-arthritis activity of the fraction.

MATERIALS AND METHODS

Animals

Healthy male Wistar albino rats, weighing 150-200 g, 12-16 weeks old were utilized in this study. They were housed in cages under standard laboratory conditions (12 hours of light period, 27±3°C). The animals were given standard rat pellets and tap water ad libitum. The Institutional Ethical Clearance Committee has approved the protocol adopted for the experimentation of animals. The animals were randomly grouped, each group consisted of 6 animals.

Materials

A. indica herb was collected from Karanganyar, Indonesia and has been identified taxonomically. CFA, bovine serum albumin, and λ-carrageenan were purchased from Sigma-Aldrich. Other chemicals were purchased from Merck. Diclofenac Sodium and Sodium CMC were pharmaceutical grade.

Preparation of extract and fractions

Dried herbs of *A. indica* were crushed into powder and extracted by maceration in 90% ethanol for 5 days at room temperature. The whole extract was collected in conical flasks,

filtered and the solvent were evaporated under reduced pressure. The fractionation was conducted by vacuum column chromatography with gradually eluent consist of *n*-hexane, ethyl acetate and ethanol (from 100:0:0 to 0:0:100). The fractions obtained then were collected based on similarity of TLC profiles, resulted 5 main factions, mentioned as fraction A, B, C, D and E.

Freund's adjuvant induced arthritis test

Thirty rats were divided into 5 groups: group I was treated with CMC Na solution as negative control, group II-IV with EEAA doses of 50, 100 and 200 mg/kg bw respectively, and group V was given the suspension of Triamcinolone. Arthritis condition was induced by injection of 0.2 ml of CFA into the left hind paw. Drug treatments were started from 5th day after adjuvant injection and continued till 27 days. Paw volumes were measured daily from first day after adjuvant induction using plethysmometer. The mean of changes in paw oedema volume nwith respect to initial paw volume, were calculated on respective days and % inhibition of paw oedema with respect to untreated group was calculated(4). Histopatology study of the ankle joint was conducted at the end of the treatment.

Anti-Inflammatory Activity Test

The fractions were evaluated for their anti-inflammatory activity in Wistar rats by carragenan-induced rat paw oedema method (5). Test compounds and standard drugs were orally administered as a suspension (1% carboxymethyl cellulose as a vehicle). Anti-inflammatory activity was determined as oedema volume inhibitory activity.

Membrane Stabilization Study

Fresh human blood was collected in heparin tube from healthy subjects who were not taking any NSAIDS for two weeks prior to the experiment. The blood was mixed and then centrifuged at 3000 rpm for ten minutes. The packed cells were washed with 10 ml of normal saline for three times followed by centrifugation, and 10% (v/v) suspension was made in normal saline. The assay mixtures (contain 0.5 ml of cell suspension, 1 ml of 0.36% hyposaline, and 1 ml of sample in various concentrations) were incubated at 37°C for 30 min and centrifuged. The hemoglobin content in the supernatant solution was estimated by using a spectrophotometer at 560 nm. Distilled water was used as blank solution. All determinations were done in triplicate(6). The percentage of hemolysis inhibition was calculated by equation:

$$\% \text{ inhibition of haemolysis} = 100 \times (A_o - A_1) / A_0$$

A_0 = Absorbance of hyposaline solution, A1 = Absorbance of test sample in hypotonic solution

Protein denaturation inhibition study

The reaction mixture consisted of 0.5 ml of a 5 % aqueous solution of bovine serum albumin and 0.05 ml of various concentrations (25-100 µg/ml) of the fractions. The pH of the reaction mixture was adjusted to 6.3 using 1 N hydrochloric acid (HCl) and it was then incubated at 37°C for 20 minutes and then heated at 57°C for 3 minutes. The reaction mixture was allowed to cool and added 2.5 ml of phosphate buffer saline. Turbidity was measured at 340 nm. In control solution, 0.05 ml distilled water was used instead of test fraction, while sample control was lacked bovine serum albumin. Diclofenac sodium was used as the standard. The percentage inhibition of protein denaturation was calculated. The control represents 100% protein denaturation. All determinations were done in triplicate(6).

Statistical Analysis

The data are expressed as mean ± S.E.M. The differences between the positive control and treatment groups were tested by ANOVA statistical method, followed by the Tukey Post Hoc test. The *P* value < 0.05 was considered to show significant differences for all comparisons made.

RESULTS

Freund's adjuvant induced arthritis

Extract of *A. indica* dose of 200 mg/kg bw produced comparable effect to triamcinolone (Figure 1).

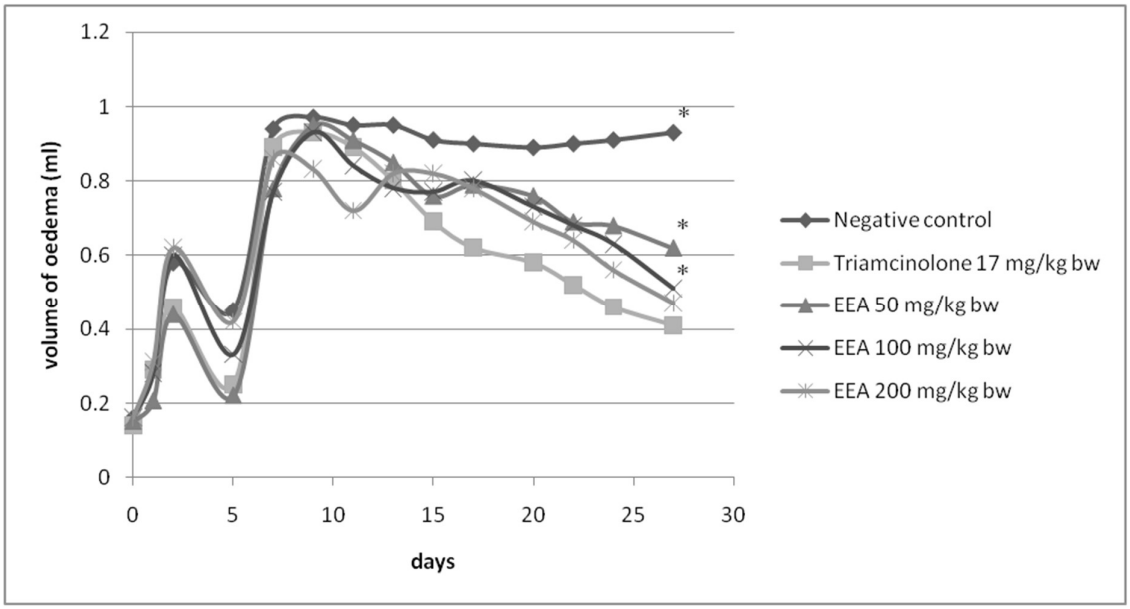

Figure 1. Reduction of oedema volume on anti-arthritis test

* *P* < 0.05, in comparison with the Triamcinolone group, one-way ANOVA followed by Tukey test

Histopatology study ankle joints results were presented in figure 2. Arthritis rat's ankle showed narrow joint space, while in group which was treated with ethanol extract of *A. indica* herb (EEA) dose of 200 mg/kg bw, showed clear and wide joint space, similarly to triamcinolon group. Histopathology of ankle joint in negative control rats, revealed enhanced neutrophil infiltration, whereas in triamcinolone as well as EEA 20 mg/kg bw treated rats there were significant reduction in neutrophil infiltration. Our phytochemical investigation showed the presence of alkaloids, flavonoids, terpenes, and saponin in the *A. indica* extract, that could be suggested to contribute to the antiarthritis activity of the extract.

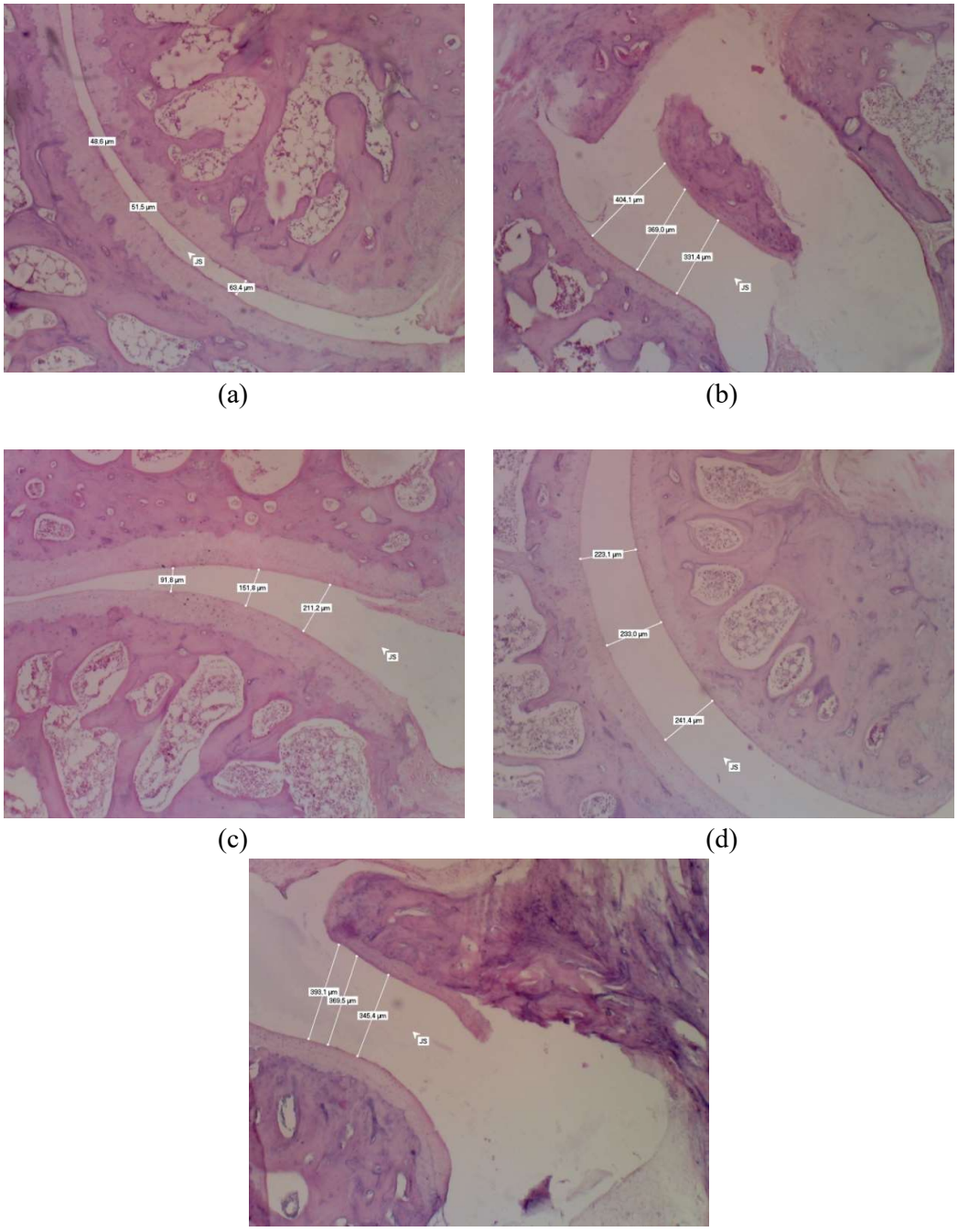

Figure 2 Histopatology profiles of ankle joints after the treatment (a) negative control, (b) Triamcinolone 17 mg/kg bw, (c) EEA 50 mg/kg bw, (d) EEA 100 mg/kg bw, (e) EEA 200 mg/kg bw

Anti-inflammatory activities of the extract and fractions

A. indica extract doses of 100 and 200 mg/kg bw were tested the anti-inflammatory effect by carrageenan-induced rat paw oedema. Carrageenan-induced rat paw oedema is a widely used test to determine the anti-inflammatory activity. Inflammation induced by carrageenan is acute, nonimmune, well-researched, and highly reproducible(7). The inhibitories of oedema volume were presented in figure 3. Both doses of *A. indica* extract showed anti-inflammatory effect. This anti-inflammatory activity was suggested to be mediated by terpenoids and steroids compounds(8). However, only the effect of EEA dose of 200 mg/kg bw was comparable to that's of Diclofenac Sodium as positive control.

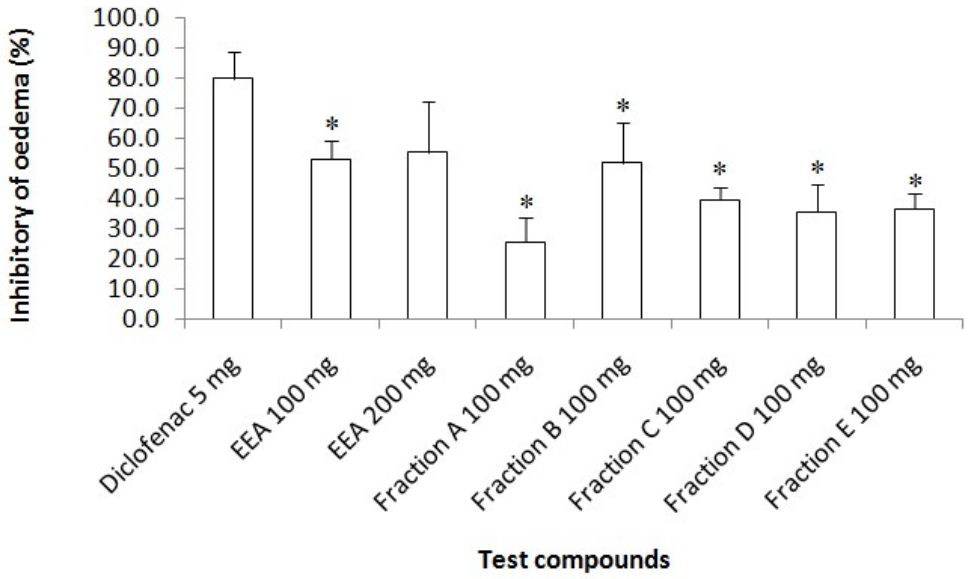

Figure 1. Oedema of inhibitory activities of extract and fractions on carrageenan induced paw oedema

The doses were in mg/kg bw. * $P < 0.05$, in comparison with the Diclofenac group, one-way ANOVA followed by Tukey test.

The fractions of the extract also were tested their anti-inflammatory activity. Fraction B showed highest anti-inflammatory activity, however, still lower than that's of positive control.

Membrane stabilization test

The membrane stabilization test was conducted to all fractions to obtain the most active fraction. Table 1 featured the percentage of membrane stabilization of fractions A-E. Fraction B and E showed the highest stabilization activity.

Table 1. Percentage of membrane stabilization of fractions A-E

Group	Percentage of membrane stabilization (%) of fractions in various concentration (µg/ml)					
	1	3	6	9	12	15
Asetosal	19.149	22.340	35.106	43.085	45.213	48.032
Fraction A	3.191	23.404	45.745	46.809	76.064	80.053*
Fraction B	54.521	69.149	70.745	75.532	83.245	84.574*

Fraction C	6.755	8.777	36.968	58.777	48.936	40.691
Fraction D	24.468	38.032	55.053	60.904	70.745	72.074*
Fraction E	48.138	63.564	67.021	73.138	84.309	87.234*

* P < 0.05, in comparison with the Diclofenac group

3.4 Inhibition of protein denaturation

Protein denaturation is a process characterized by the alterations or modifications of the protein structure. Denaturation of proteins is one cause of autoantigen production in inflammation process. The mechanism may involve is the electrostatic, hydrogen, hydrophobic, as well as disulphide bonding alteration. The inhibitories activity of protein denaturation were presented as percentage of inhibition(9). Table 2 presented the percentage of protein denaturation inhibition of fraction A-E. Fraction B and E showed the inhibition of protein denaturation activities comparable to Diclofenac sodium.

Table 2. Percentage of protein denaturation inhibition of fraction A-E

Group	Percentage of protein denaturation nhibition (%) of fractions in various concentration (µg/ml)			
	25	**50**	**75**	**100**
Diclofenac Na	6.110	6.917	12.219	24.017
Fraction A	4.806	4.840	5.716	12.697*
Fraction B	5.295	8.869	13.490	19.50
Fraction C	3.711	5.077	9.143	11.271*
Fraction D	3.131	5.042	5.920	11.847*
Fraction E	3.466	7.760	11.854	18.841

* P < 0.05, in comparison to the Diclofenac Na group

DISCUSSION

CFA was widely used to initiate induction of arthritis. This was the original model of Rheumatoid Arthritis (RA), has been extensively used for preclinical screening of new antiarthritis compounds[5]. After a single injection of the adjuvant, a rapid, reliable, robust, and easily measurable polyarthritis develops. The joint pathology seen in this animal model shares the cartilage degradation, bone resorption, and cellular influx seen in human RA(4). CFA‑induced arthritic animals produced an increase in paw volume and paw thickness in the disease control group. Treatment with *A. indica* extracts and triamcinolone showed statistically significant (P < 0.05) reduction in oedema volume compared to the negative control group.

The development of the CFA-induced arthritis because of the reactivity to the cartilage proteoglycan and the interaction with the intestinal flora(4). Arthritis occurs in CFA induced rat caused by the molecular similarity of *Mycobacterium* and joint cartilage molecules, causing cross-reactivity between the antigens of Mycobacterium and the animal model. This cross-reactivity trigger antibodies attack to to the joints. CFA-induced arthritis effects with symptoms of oedema and erythema began to develop on the first day after the induction to the day-15. After 15 days, symptoms of arthritis were increased, characterized by joint space

narrowing(10). Triamcinolone inhibited the migration of polymorphonuclear leukocytes and fibroblasts as well as lowered the permeability of the capillaries thus prevented the high anti-inflammatory effect. In EEA, chemical compounds responsible for the decreasing oedema volume are alkaloids, flavonoids, steroids and terpenoids(2). Steroid compound has a similar structure with triamcinolone so is suggested to have the same mechanism of action.

Stabilization of red blood cell membrane has been used as a method to determine *in vitro* anti-inflammatory activity, because the red blood cell membrane similar to the lysosomal membrane which may affect the inflammatory process(11). Lysosomal membrane stabilization is important in limiting the inflammatory response, by preventing the release of the lysosomal enzyme during the inflammatory process. The enzymes in the lysosome apart during inflammation (due to the activation of neutrophils) produce a variety of disorders that may be associated with the occurrence of acute or chronic inflammation. Red blood cell membrane stability against induction of hypotonic solution and high temperature (57^{o}C), can be used as a measure of stabilization of the lysosomal membrane, where an oxidative stress that can disrupt the stability of cell membrane. Inhibition of hemolysis of red blood cell membrane was characterized by the decrease of spectrophotometric absorption at wavelength of 560 nm. Aspirin was used as a positive control because it was a nonsteroidal anti-inflammatory drug that works by preventing the release of inflammatory mediators that can inhibit prostaglandin synthesis or cyclooxygenase(12).

Protein denaturation inhibition assay used Diclofenac sodium a positive control because it has been proven to inhibit protein denaturation(12). Proteins in the body vulnerable to denaturation caused by the formation of free radicals that cause inflammatory mechanisms by stimulating the release of inflammatory mediators. Denaturation of proteins is a process in which the protein loses tertiary structure and secondary structure due to external substances such as strong acids or strong bases, inorganic salts, organic solvents, and heating(14). The possibility of interaction or bonding between the molecules contained in the BSA molecule contained in the extract that inhibit the protein denaturation.

Phytochemistry identification of fraction B by thin layer chromatography revealed that this fraction contained steroids and polyphenol. The steroids contained in *A.indica* herb were stigmasterol and β-sitosterol. Both of these compounds have anti-inflammatory and antiarthritis activity(15).

CONCLUSIONS

Acalypha indica herb ethanol extract showed antiarthritis as well as anti-inflammatory activity. Fraction B of the extract, which contain polyphenol and steroids had highest anti-inflammatory, membrane stabilization as well as protein denaturation inhibition activity.

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