Herbal Medicines Journal **2020; 5(4):145-52**
DOI: 10.22087/herb%20med%20j.v5i4 **ISSN:** 2538-2144

Original Article

Subchronic Toxicity of the Ethanolic Extract of *Lecaniodiscus cupanioides* on Albino Wistar Mice (*Mus musculus*)

Oloyede AM[1]*, Ottu B[1], Ogunsanwo A[1], Sobiye S[1], Bolarinwa K[2], Aromolaran C[1], Ogidi E[1], Okafor E[1]

[1] Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos-Nigeria
[2] Distance Learning Institute, University of Lagos, Nigeria

Received: 15.05.2020; Accepted: 31.03.2021

Abstract

Background and Aim: *Lecaniodiscus cupanioides* has been used in traditional therapies in Nigeria for the management of several ailments. This study investigated its subchronic toxicity.

Materials and Methods: 30 mice (20–25g) were placed in three groups of 10 mice. Groups A and B were orally administered 100 and 400 mg/kg of ethanolic extract respectively for 49 days, while group C received distilled water as the control. At expiration of the treatment, the animals were sacrificed to harvest blood and internal organs for hematologic, biochemical, antioxidant and histologic analyses.

Results: Hematologic analysis revealed increased monocytes in all the groups. Biochemical and Electrolytes analytes did not show any significant difference. Antioxidant results showed a dose-dependent significant decrease in super oxide dismutase (SOD). Glutathione (GSH) revealed a significant increase in 400mg/kg. No pathology was observed in the heart, but mild toxicity in the kidneys, loss of alveoli architecture in the lungs, inflammation, and congestion of portal triads and central vein in the liver were observed. Increased monocytes observed may depict antibody and anti-infection potentials of the extract. Decreased value of RBC, Hb at higher doses, and mean corpuscular hemoglobin (MCH) at lower doses suggest hemolytic anaemia. Insignificant difference in liver enzymes may suggest extract is not hepatotoxic. Insignificant increased creatinine and urea implies that the extract is not nephrotoxic.

Conclusion: The extract displayed a great potential to inhibit free radicals. It increased the antioxidant defense system and GSH at 400mg/kg. Increased SOD and catalase suggest the upregulation of protein which culminates in the clean-up of free radicals and decreasing oxidative stress, thus protecting tissues from extremely reactive radicals.

Keywords: *L. cupanioides,* Toxicity, Antioxidants, Hematology

***Corresponding Author:** Oloyede, A.M, Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos-Nigeria. Email: moloyede@unilag.edu.ng

Please cite this article as: Oloyede AM, Ottu B, Ogunsanwo A, Sobiye S, Bolarinwa K, Aromolaran C, et al. Subchronic Toxicity of the Ethanolic Extract of *Lecaniodiscus cupanioides* on Albino Wister Mice (*Mus musculus*). Herb. Med. J. 2020; 5(4):145-52

Introduction

Medicinal plants are generally recognized as indisputable synthesizers of bioactive compounds possessing therapeutic potentials. They are accessible, safe and effective, and can serve as veritable alternative to expensive pharmaceutical drugs (1). These therapeutic interests are so tremendous that roughly 85% of primary health remedies are derived from natural resources globally

(2). Medicinal plants are leading sources for drug synthesis (3). *Lecaniodiscus cupanioides* Planch. Ex Bth., is categorized as a member of the Sapindacaeae family. It is a low-branching tree with spreading crown whose height can reach 12 m or more. It is primarily celebrated for its traditional medicinal purposes, and is economical as it also provides food and timber (4). Its folkloric uses are common in countries like Nigeria, Angola, Sierra Leone, Uganda, and Zaire (5). It is known as aaka or akika, okpu, kafi-nama-zaki and utantan in Yoruba, Igbo, Hausa and Edo respectively (6). Lecanodiscus cupanioides has been shown to have aphrodisiac (7), antimicrobial (8), antimalarial (9), anti-inflammatory and anti-hepatomegaly properties (10). It is also effective against measles, fever, certain liver diseases and wounds (11). Researchers have reported the presence of Saponins such as 3-O-[-l-arabinopyranosyl-(1-3)--l-rhamnopyranosyl-(12)--l-arabinopyranosyl]-hederagenin; and :3-O- [-l-arabinopyranosyl-(13)--l-rhamnopyranosyl-(1- 2)--l-arabinopyranosyl]-hederagenin (12), two triterpenoid saponins identified as 3-O-(alpha -L-arabinofuranosyl-(1-3)- alpha -L-rhamnopyranosyl-(1-2)- alpha -L-arabinopyranosyl-)-hederagenin and 3-O-(alpha -L-arabinopyranosyl- (1-3)- alpha -L-rhamnopyranosyl (1-2)- alpha -L-arabinopyranosyl-)-hederagenin (13) and also alkaloids , tannin, phenol, and anthraquinone (9). Apart from the synthesis of useful phytochemicals, medicinal plants also generate toxic agents, which naturally serve as defense mechanisms against infections and some consumers, including humans (14). The aqueous extract of Lecaniodiscus cupanioides had been evaluated to be non-toxic in acute toxicity assays (15). This study investigated the subchronic toxicity using ethanolic extract, as some phytochemicals are possible, best extracted with ethanol. In view of its reliable ethnomedicinal uses, it is pertinent to probe its toxicological effects for safety purposes. The research project was approved by the ethical committee of College of Medicine University of Lagos (CMULHREC Number: CMUL/ACUREC/01/21/803).

Materials and Methods

Collection of the Plant

The leaves of *Lecanodiscus cupaniodes* were collected in December 2018 in Oyo State, Nigeria. The plant was verified at the Forest Research Institute of Nigeria (FRIN), Ibadan by Mr. Odewo S.A. Voucher specimen was prepared and deposited with identification FHI111668 at the Herbarium of the Forestry Research Institute of Nigeria (FRIN).

Plant Extraction

Freshly collected or harvested leaves were washed, cut into smaller pieces and air-dried at room temperature (40°C) for 21 days. It was pulverized into powdery texture. The powdered leave (1039g) sample was weighed and soaked in 2700mls ethanol for 48hours. The extract was filtered and the filtrate was evaporated into paste using regulated hotplate at $40 \pm 10°C$. The concentration was collected into a suitable container and weighed 36.3g.

Experimental Animal

Thirty *Mus musculus* (mice) weighing between 20-25g were purchased from BioVaccine Centre, NAFDAC, Yaba, Lagos. The animals were grouped into three cages of 10 mice and allowed to acclimatize for at least 7 days to laboratory condition (29 ± 2˚C and Relative Humidity 70 ± 2%) before the beginning of the bioassay. They were adequately supplied with water and were fed with ad-libitum. After acclimatization, groups A and B were orally administered 100 mg kg^{-1} and 400 mg kg^{-1} of the extract respectively, while group C received distilled water. The treatment was administered daily for 49 days after which the mice were sacrificed via jugular puncture. The blood, internal organs (hearts, lungs, livers and kidneys) and femur were harvested for hematologic, biochemical, antioxidants, histologic and bone marrow assays.

Haematology

Haematology test was carried out to determine parameters such as the haemoglobin, mean cell haemoglobin, mean cell haemoglobin concentration, mean cell volume and platelets.

Biochemical Evaluation

Liver samples were harvested separately, and washed in ice-cold saline to ensure it is blood-free and homogenated. Different homogenates (5%w/v) were prepared in cold 50 mmol/L potassium phosphate buffer at pH 7.4 in ice homogenizer. They were centrifuged at 5000 r/min for 15min at 4 ^{o}C to remove

cell debris. Some supernatants were used to estimate catalase (CAT), glutathione peroxidase (GSH), superoxide dismutase (SOD)' malondialdehyde (MDA), lactate dehydrogenase (LDH) and protein concentration. The remaining supernatant was used to estimate aspartate transaminase (AST), alanine transaminase (ALT), and alanine phosphatase (ALP) using Randox diagnostic test following the manufacturer's instruction.

Lipid Peroxidation Level

Estimation of malondialdehyde (MDA) is often used to measure lipid peroxidation. Tissue MDA was measured by the use of the assay method of measuring thiobarbituric reacting substances (TBARs) by Nagababu *et al*., (16). We blended twenty-fold dilution of supernatant (100 µL) in 0.15M Tris-KCl buffer with 30% trichloroacetic acid (500 µL) and 0.75% TBA (500 µL). Subsequently, we heated the mixture at 80 °C for 1 h and extracted it with 1 mL butanol. The separation of the organic phase was conducted using centrifugation for 5 min at 3000 g, and it was measured at 532 nm. The molar extinction coefficient 1.56×10^5/M/cm was used in calculating the amount of MDA formed. The concentration of TBARS in the liver tissues was expressed as nmol MDA/mg protein.

CAT Assay

This was estimated in triplicate according to Aebi (17). The disappearance of H_2O_2 was monitored at 240nm. 30 µL liver homogenate was suspended in 2.5 mL of 50 mmol/L phosphate buffer (pH 7.0) (17). CAT assay commenced by adding 0.5 mL of 0.1 mol/L hydrogen peroxide solution and absorbance at 240 nm and was recorded every 10 seconds during 2 min and used to calculate CAT activity. Phosphate buffer was used to substitute H_2O_2 solution, serving as negative control. Molar extinction coefficient 39.4 M^{-1} cm^{-1} for H_2O_2 was used to determine the activity of CAT, and expressed as nmol of hydrogen peroxide converted per min per mg total protein where 1 IU activity=1 µmoL H_2O_2 converted to H_2O per min.

SOD Assay

SOD activity was estimated according to Thanh *et al.,* (18). This method is predicated upon the ability of SOD to inhibit the autoxidation of pyrogallol. In 970 µL of buffer (1 mmol/L EDTA, 100 mmol/L Tris-HCl, pH 8.2), 20 µL pyrogallol 13 mmol/L and 10 µL of liver homogenates were mixed. The assay was carried out in thermostated cuvettes at 25 °C, and alterations of absorption were recorded by a spectrophotometer in triplicate at 420 nm. One unit of SOD activity was defined as the amount of enzyme that can inhibit the auto-oxidation of 50% of the total pyrogallol in the reaction.

GSH Assay

GSH was measured according to Thanh *et al.,* (18). 1 mL of the assay mixture contained 100 µL of 10 mmol/L GSH, 770 µL of 50 mmol/L sodium phosphate (pH 7.0), 10 µL of 1.125 mol/L sodium azide,100 µL of 2 mmol/ L nicotinamide adenine dinucleotide phosphate (NADPH), 10 µL 100 IU/mL glutathione reductase and 10 µL deproteinized liver supernatant. The mixture was equilibrated for 10 min. The assay began by adding 50 µL of 5 mmol/ L H_2O_2 to the mixture, NADPH oxidation was measured during 5 min at 340 nm. One unit of glutathione peroxidase was defined as the amount of enzyme able to produce 1 µmol NADP+ from NADPH per min. GSH activity was determined using the molar extinction coefficient 6 220 M^{-1} cm^{-1} for NADPH at 340 nm and reported as IU per mg total protein.

Histologic Preparation

We inserted the vital organs from every animal in 10% formaldehyde and processed them for hematoxylin-eosin staining. A camera attached to the compound light microscope in the department of Morbid Anatomy, Lagos State University Teaching Hospital, was used to take photographs of the prepared slides hematoxylin-eosin stained tissues sections. The Microtone model that was used for the histopathological preparation is LEICA.

Statistics

The statistical analyses were performed using Microsoft Excel. The data expressed as the Mean ± SEM was subjected to sample t-test, evaluating the statistical significance of the difference between two means of various parameters between the control and experimental groups. The P value was found by means of Microsoft Excel.

Results and Discussion

Plants have been used for the management of many health challenges as they are natural sources of numerous bioactive compounds (secondary metabolites) possessing vital bioactivities. These metabolites are chemical constituents and have been used in traditional folks as drugs (19). Some of the

Table 1: Weight of the Mice during the Treatment with *L. cupanioides* for 49 Days.

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week7
Control	22.65±0.86	20.57±0.61	22.25±0.53	20.60±0.53	20.41±0.98	19.80±0.52	21.55±0.52
100mg/kg	24.45±0.65	23.33±0.90	22.71±0.83	22.60±0.60	21.24±0.30	20.54±0.60	21.48±0.70
400mg/kg	22.66±0.78	22.26±0.74	20.48±1.20	22.20±0.63	22.80±0.60	20.62±0.54	20.70±0.87

Values are mean ± SEM. Where N=10 value a =<0.05, b=<0.01, c=<0.001. There is no significant increase in the values obtained from the weekly weights of the mice at 400mg/kg-1 and 100mg/kg-1 when compared to the control

Table 2: Hematological Profile of the Mice after the Administration of *L cupanioides* for 49 Days.

	100mg/kg^{-1}	400mg/kg^{-1}	CONTROL
HB g/dl	12.42±0.47	13.5±0.22	13.13±0.42
PVC %	37.3±1.10	40.2±0.57	38.7±1.40
WBC mm^3	3590.00±306.00	5018.00±426.70	4330.00±320.43
RBC X10 $^{12/L}$	4.56±0.10	4.93±0.15	4.87±0.19
MCV PL	81.87±1.29	81.04±1.86	80.05±1.41
MCH PG	27.37±0.59	27.16±0.75	27.03±0.63
MCHCg/dl	33.31±0.45	33.44±0.38	34.00±0.48
PLT X10 $^{9/L}$	220.00±16.51	278.50±11.21	194.3±10.93
LYMPH%	68.8±0.49	67.8±0.99	60.1±1.03
NEUT %	27.8±0.53	29.5±0.95	36.5±0.62
MONO %	23.00±0.21	16.00±0.27	29.00±0.23
EOS %	8.00±0.25	7.00±0.30	12.00±0.22
BAS %	0.00±0.00	0.00±0.00	0.00±0.00

The values of the table above are expressed as Mean±SEM. Where N=10 a = <0.05, b = <0.01, c =<0.001. There is an increase in the monocytes between 400mg/kg, 100mg/kg and the control while there is no significant increase in the values obtained from the other aematological parameters at 400mg/kg^{-1} and 100mg/kg^{-1} when compared to the control.

Table 3: Biochemical Analytes Indices of the Mice after the Administration of *L. cupanioides* for 49 Days.

	100 mg/kg^{-1}	400 mg/kg^{-1}	CONTROL
AST U/I	19.60±0.94	21.9±0.91	14.6±0.65
ALT U/I	37.05±1.28	32.02±2.11	41.20±1.32
CRT µmol/L	56.8±5.65	65.74±5.11	57.90±1.92
ALP U/I	91.3±1.44	99.6±0.71	83.6±3.17
UREA mg/ml	45.22±2.96	51.56±2.12	37.9±3.08
PRO g/L	24.63±3.00	34.10±1.07	24.50±2.48

The values of the table above are expressed as Mean±SEM. Where N=10 a =<0.05, b = <0.01, c =<0.001. There was no increase between 400mg/kg^{-1}, 100mg/kg^{-1} and the control in all the biochemical parameters when compared to the control.

Table 4: Antioxidant Activities of L. Cupanioides on the Mice Post the 49-Day Treatment.

Enzyme	100mg kg^{-1}	400mg kg^{-1}	Control
PRO g/L	24.98±3.18	33.57±0.68	23.32±2.60
CAT/min/mg protein	0.86±0.15	0.89±0.05	1.72±0.22
SOD/min/mg protein	269.74±8.14[a]	300.59±16.88[a]	321.07±19.04
MDAnmol/ml	9.74±0.62	5.97±0.55	5.03±0.43
GSH nmol/ml	2.52±0.10	3.24±0.16[a]	2.12±0.07

The values of the table above are expressed as the Mean±SEM. Where N=10 a = <0.05, b = <0.01 There was no difference between 400mg/kg^{-1}, 100mg/kg^{-1} and the control in the PRO parameter while there was slightly significant difference between 400mg/kg^{-1}, 100mg/kg^{-1} and the control in the remaining anti-oxidant parameters.

advantages for medicinal plants patronage include cost

effectiveness, broad spectrum activities, little or no side effects, and curative as well as preventive activities (19;20;21). In this study, *L. cupanioides* was evaluated for its subchronic toxicity in mice.

Table 5: Electrolyte activity of *L. cupanioides* on mice after 49days of treatment.

Treatment	Sodium Na^+	Potassium K^+	Bicarbonates HCO_3	Chloride Cl^-
100mg kg^{-1}	134.40±4.68	6.44±0.70	31.90±5.25	110.80±2.28
400mg kg^{-1}	142.70±1.25	5.92±0.22	44.70±1.28	104.20±1.50
CONTROL	134.50±0.58	5.10±0.35	32.95±1.65	106.50±1.04

Values of the table above are expressed as the mean±SEM.

Body weight is a pertinent criterion in evaluating toxic effects of treatments, and drugs or toxic substances (22). Variation in weights can be one of the first critical evidence of toxicity (23). Evaluation of growth in animals using weight index is a routine in toxicological investigations as it assists in interpreting compound-related effects (24). In this investigation, weight of the mice (Table 1) assessed weekly for 49 days did not show any significant difference, implying that the extract does not seem to impose any threat to the physiognomy of the mice.

Study of hematologic and biochemical parameters is an important way for diagnosing the root causes of diseases. Alteration in blood parameters might be caused by alterations in cellular integrity, membrane permeability and metabolism or even because of being exposed to toxic chemicals (25). The significant increase of leukocytes as observed in the result (table 2) may depict the anti-infection properties of *L. cupanioides* (26). This will develop the animals' ability to generate antibodies and enhance the adaptability to local environmental and disease prevalent conditions (27). The decreased value of RBC, Hb at the higher doses and MCH at the lower dose may indicate blood loss which may be gastrointestinal bleeding, bleeding of internal organs or hemolytic anaemia (28).

Evaluating the activities of marker enzymes is of immense importance in the investigation of plant extracts safety and toxicity. ALT, ALP and AST were the enzymes considered in this study. Liver enzymes ALT, ALP and AST are frequently used as biological markers of liver injury (29). ALP is commonly used in the assessment of plasma membrane integrity of the liver cells. Hyperproduction of ALP within the 49-day treatment could destroy the cells that are dependent on various phosphate esters because ALP hydrolysis phosphate esters metabolites in the hepatocytes (30). Aspartate aminotransferase (AST), a pyridoxical phosphate dependent transaminase enzyme catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and a very vital enzyme in the metabolism of amino acid. AST, though present in the heart, kidney, RBC, liver, skeletal muscles and brain, is popularly evaluated clinically as a marker for liver wellbeing (30). The insignificant increase of the liver enzymes observed in this study (table 3) suggests that *L. cupanioides* extract has no hepatotoxic effect on the liver as observed in the doses implored (31). ALT specifically serves as an indicator of inflammation in the liver rather than AST (30), the low level of AST again suggests that the extract may not be toxic to the liver. Creatinine, urea and serum have been used to detect kidney injury. There was no significant influence of *L. cupanioides* extract on any of the biochemical parameters in comparison with the control which may indicate that the extract did not have any effect on the kidney of the albino mice which is in consonance with the report of Joshua and Timothy (15) on the toxic effect of *L. cupanioides* on albino mice.

Antioxidants are radical scavengers, enzyme inhibitors, hydrogen donors and singlet oxygen quenchers. Our results (table 4) revealed the high potential of *L. cupanioides* to inhibit actions of free radicals which contribute to the oxidative stress that may consequently cause diseases (32). The results exposed the ability of *L. cupanioides* extract to increase antioxidant defense system and GSH, particularly in the 400mg/kg group. It was indicated that there was increased formation of MDA and promoted lipid peroxidation (33). The results also suggest that the extracts can be used as an enhancer due to its increased effects on the anti-oxidant parameters (34). Antioxidant enzymes SOD and catalase decrease oxidative stress via scavenging oxygen radical and changing it to hydrogen peroxide

and oxygen and the subsequent decrease of the hydrogen peroxide to minimize the tissue damage by the radicals. The increase SOD and catalase activities might be related to the upregulation of protein which culminates in effective clean-up of circulating free radicals and reduction in oxidative stress, thus protecting the tissue from highly reactive hydroxyl radicals (34).

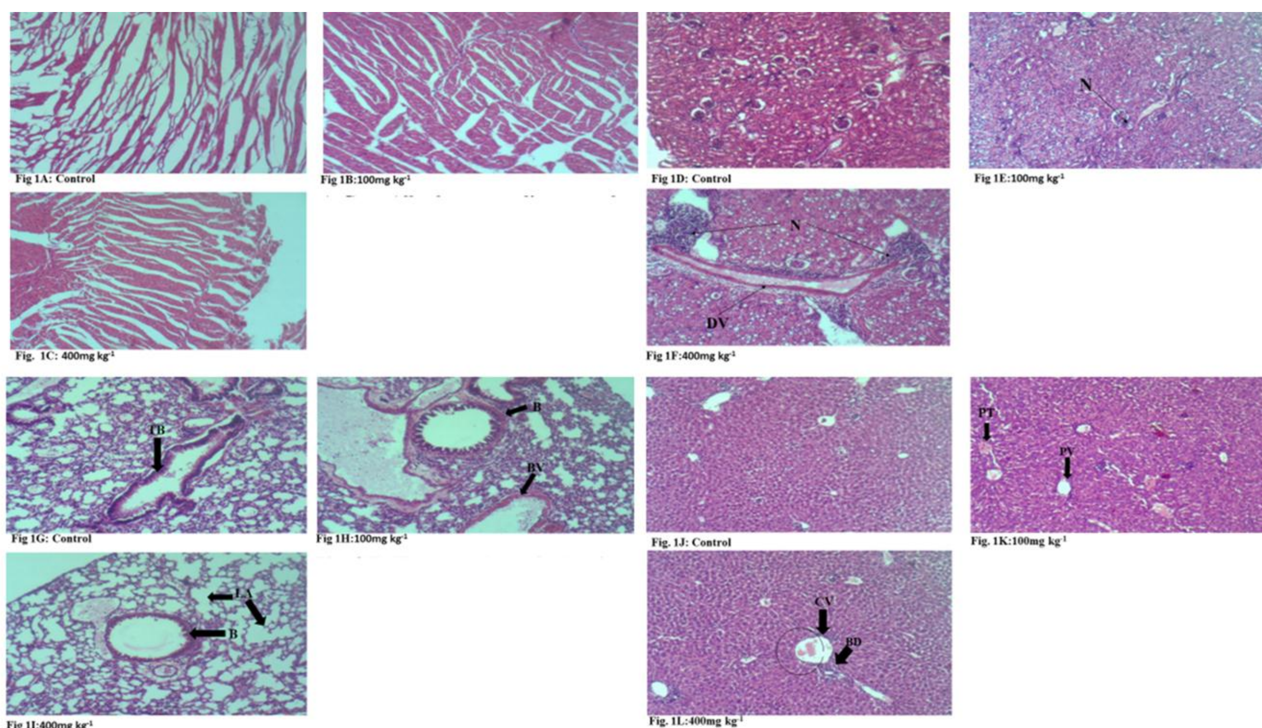

Figure 1. Histology of internal organs of control and treatments

Lecaniodiscus cupanioides at 100 mg/ kg and 400 mg/kg brought about a reduction in the activity of CAT enzyme. There was a reversible reduction in the activity of SOD and treatment of the mice with the oncentration of 400 mg/kg that caused a remarkable decrease in the MDA activity. This also caused an increase in the GSH activity.

Histologic analysis (fig. 1A – L) indicated the availability of mild perivascular mononuclear cell infiltrates and bronchiolitis in the lungs of the mice, particularly at 400mg/kg group which may suggest mild toxicity (35;36). The liver may be the target of organ toxicity, particularly because it can serve as the site of detoxifying chemicals and metabolizes drugs (37). However, this investigation revealed mild toxicity for the treated liver and kidney as compared to the control groups.

Conclusion

Lecaniodiscus cupanioides possesses some anti-infection properties occasioned in increased value of the monocytes. It had no effect on the other hematological and biochemical parameters which suggests that it could be used as a medicinal plant for the treatment of several illnesses. However, it has the potential to increase the activity of antioxidant enzymes. No toxicity was observed in the bone marrow cells and no significant alteration was noticed in the histoarchitecture of the evaluated internal organ, which implies that the doses did not induce toxicity in the animal at subchronic exposure. Nevertheless, further assessment and evaluation of medicinal uses and safety, particularly when administered at higher doses, is required and a longer period of time should be executed.

Acknowledgment

The authors appreciate Mr. A. E. Adeshola for taking care of the mice during acclimatization and the period of treatment in the Animal House of the Department of Cell Biology and Genetics, University of Lagos.

Conflict of Interest

The authors declare that they have no conflict of interest.

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