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Safety evaluation of *Eugenia jambolana* seed extract

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ABSTRACT

Objective: To evaluate the safety of ethanolic seed extract of *Eugenia jambolana* (EJSE) using acute and sub-chronic toxicity assays in Swiss albino mice as per Organisation for Economic Co-operation and Development (OECD) guidelines. **Methods:** Possible behavioral changes and lethality were observed in mice administered a single dose [1 000, 2 000, 3 000, 4 000 or 5 000mg/kg body weight (BW)] of EJSE. Plasma levels of metabolic, hepatic, cardiac and renal function markers, electrolytes, blood count and histopathology of major organs were monitored in mice chronically treated with EJSE (1 000, 2 000 or 3 000 mg/kg BW) for 28 days. **Results:** Since no mortality was recorded in the acute toxicity evaluation up to a dose of 5 000 mg/kg bodyweight of EJSE, 50% lethal dose (LD_{50}) was assumed to be >5 000 mg/kg BW. In the sub-chronic toxicity evaluation, no adverse observations were recorded in mice administered with 2 000 mg/kg BW of EJSE; however at 3 000 mg/kg BW dose, moderately significant increase in the plasma levels of urea and creatinine was observed. Hence, the lowest observable adverse effect level (LOAEL) for EJSE was found to be 3 000 mg/kg BW and the no observable adverse effect level (NOAEL) was adjudged as 2 000 mg/kg BW. **Conclusions:** It can be concluded from this study that, orally administered EJSE is safe up to a10 fold higher dose than its reported therapeutic dose.

1. Introduction

India is considered to be the largest producer of medicinal herbs and is rightly called the botanical garden of the world[1]. Use of herbal medicines is a traditional practice in India and recent studies have documented a rise in usage of alternative therapies not only in the developing countries like India but also in other developed countries worldwide[2]. In 2005, Tindle *et al* reported that, 72 million US adult population uses complementary and alternative medicines, of which 12%-19% people were using herbal drugs for countering various ailments[3]. The major hurdle in the use of traditional herbal preparations is the lack of scientific and clinical data related to the efficacy and safety of these drugs. A myth prevails that, drugs of herbal origin are always safe[4] but, studies have reported hepatotoxicity[5], nephrotoxicity[2,6] or other side effects caused due to widely used herbal drugs. According to United States Food and Drug Administration act, herbal drugs do not fall in the category of medicine and hence there are no rigorous safety evaluations. This necessitates determinations of toxicity dosage of any herbal preparation through preclinical acute and sub chronic toxicity evaluations[4].

Eugenia jambolana (EJSE) is of common occurrence in oriental forests ranging from the sub-Himalayan parts to extreme southern region, Thailand and Philippines. Anti-hyperglycemic/anti-diabetic potential of EJSE has been extensively studied by various research groups[7-13]. Also, its hypolipidemic property has been demonstrated in alloxan induced diabetic rabbits[14] and streptozotocin induced diabetic rats[15, 16]. Recently in 2010, Sharma *et al*[17] have reported that, the active principle isolated from EJSE modulates the activity levels of carbohydrate metabolizing enzymes. It has been reported to be hepatoprotective and nephroprotective in experimental diabetes[18] and has also been shown to exhibit anti-ulcer[19] and free radical scavenging properties[20].

There is no dearth of literature available on beneficial and medicinal uses of EJSE but, the available data lacks a systematic safety/toxicity evaluation of the same. Hence, it was thought pertinent to evaluate the same in acute and chronic toxicity model as per the Organisation for Economic Co-operation and Development (OECD) guidelines.

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2. Materials and methods

2.1. Preparation of extract

Fruits of *Eugenia jambolana* were handpicked from fruit shop. The pulp was removed and the seeds were washed several times with Milli-Q water and shade dried at room temperature. Kernel of the seeds was separated from the seed coat, powdered in an electrical grinder. Hundred gm of kernel powder was suspended in 1 000 mL of Milli-Q water overnight and then sieved through several layers of sterile muslin cloth. The resultant residual paste was extracted with 70% ethanol at room temperature and later, was evaporated in a rotatory evaporator at 40-50 ℃[11,12,14,15]. The yield of ethanolic extract was 5 g/100 g of seed powder.

2.2. Experimental animals

Swiss albino mice of either sex (Obtained from Zydus research centre, Ahmedabad, India) were housed and maintained in clean polypropylene cages and fed with laboratory chow (M/S Pranav agro, Ltd Baroda, India) and water ad libitum. The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and, approved by the animal ethical committee of The Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

2.3. Acute oral toxicity in mice

The acute oral toxicity study was conducted using the limit test procedure as per OECD test guidelines on acute oral toxicity test 401[21]. Thirty two Swiss albino mice of either sex were divided into four groups (n=8) and were orally administered with a single dose of 1 000, 2 000, 3 000, 4 000 or 5 000 mg/kg body weight (BW) of EJSE. Animals were observed for possible behavioural changes such as tremors, convulsions, sleep, altered feeding, salivation, altered somato-motor activities and diarrhoea till 72 hr post treatment. LD_{50} (50% lethal dose) was calculated from the above table as follows:

$$LD_{50}=LD_{100}-\frac{DD\times MD}{N}$$

$$\text{Therefore, } LD_{50} \text{ of EJSE} = (>5\,000)-\frac{3\,000\times 0}{8} = >5\,000\ (\text{mg/kg BW})$$

2.4. Sub chronic oral toxicity in mice

The sub chronic oral toxicity study was conducted according to OECD Test 407[22]. Thirty two Swiss albino mice of either sex were divided into four groups (n=8) and maintained for 28 days for this experiment. Group 1 was orally fed with 0.5% carboxy methyl cellulose (CMC) that served as control whereas, Groups 2, 3 and 4 were orally administered with 1 000, 2 000 or 3 000 mg/kg BW of EJSE respectively. After 28 days of treatment, blood was collected from overnight fasted mice via retro-orbital sinus under mild anaesthesia and plasma was separated for further biochemical analysis. Thereafter, the animals were sacrificed by cervical dislocation under mild ether anaesthesia, and brain, heart, lungs, liver, spleen, kidney and adrenal were excised and weighed.

2.4.1. Cage side observations

After treatment with EJSE, the mice were observed daily for possible changes in appearance of skin, fur and eyes. Also, the animals were closely observed for somatomotor activity, respiratory and behaviour changes, tremors, convulsions, salivation, diarrhoea etc.

2.4.2. Body weight and food and water intake

Body weight of each experimental animal was recorded using a weighing balance (CITIZEN Model 1621K, Taiwan) at the end of every week during the entire study. Food and water intake in all the experimental groups were monitored daily at 09:00 hrs. Known quantity of food was given to the respective experimental groups and the leftover food was weighed and the quantity was subtracted from the total to record food intake per mice. Also, mice were provided with known volume of reverse osmosis grade water in a 500 mL measuring cylinder and leftover volume of water was measured every 24 hr interval to calculate water intake.

2.4.3. Plasma biochemical analysis

Plasma sodium, potassium (Tulip Diagnostics Pvt Ltd), calcium (Lab Care Diagnostics India Pvt. Ltd), Creatine Kinase-MB (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, cholesterol, HDL (Reckon diagnostics, Baroda, India), triglyceride (Beckon Diagnostics, Baroda, India) and fasting blood glucose (Bayer diagnostics India Ltd) were assayed using commercially available kits as per the instruction of the manufacturer.

2.4.4. Hematological analysis

At the end of experimental protocol blood was collected in K_3-EDTA coated tubes and haemoglobin content, total red and white blood cell counts (RBC and WBC), hematocrit (HCT) value, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution of width-coefficient of variance (RD-WCV), red cell distribution width standard deviation (RD-WSD), platelets count (PLT), mean platelet volume (MPV), red cell distribution width (RDW) and platelet crit (PCT) of blood samples were measured using BC-2300 Haematology Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd).

2.4.5. Pathological examination

At the end of experimental period, all animals were subjected to a detailed gross necropsy with examination of external surface of the body, all orifices and the content in cranial, thoracic and abdominal cavities. Autopsy of brain, heart, lungs, liver, kidney and spleen was done and their wet weights were recorded immediately.

Heart, liver and kidney of experimental mice were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using

automated tissue processor. Five μm sections were cut and stained with hematoxyline and eosin and examined under Leica DMRB microscope. Photographs were taken with Canon power shot S70 digital Camera at 100× magnification.

2.6. Statistical analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison test. The results were expressed as mean±SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

3. Results

3.1. Acute oral toxicity

There were no noticeable behavioural changes due to acute toxicity. Since there was no mortality, 50% lethal dose (LD_{50}) could not be determined. As per the arithmetic calculation, it was concluded that LD_{50} of the EJSE is >5 000 mg/kg body weight.

3.2. Sub-chronic oral toxicity

3.2.1. Plasma metabolites and electrolytes

Table 1 represents the effect of EJSE administration on plasma metabolites and electrolytes. EJSE caused significant decrement in plasma TC, TG and LDL levels without any alteration in plasma HDL and VLDL levels. The plasma Na^+, K^+ and Ca^{2+} levels recorded non significant alterations in the experimental groups.

3.2.2. Plasma markers of cardiac, hepatic and renal damage

As shown in Table 2, EJSE administration did not alter plasma markers of cardiac damage (LDH and CK-MB). However, plasma urea, creatinine and ALP levels were significantly increased after administration of high dose of EJSE (3 000mg/kg BW) while, plasma AST, ALT, and billirubin levels were unaltered.

3.2.3. Hematological analysis

Sub chronic administration of EJSE did not alter any of the haematological parameters evaluated (Table 3).

3.2.4. Body weight gain, food and fluid intake and, organ weight

As depicted in Table 4, there was a dose dependent decrement in the body weight and food intake however, non significant alteration recorded in water intake of experimental groups. Organ weights and their necropsy analysis revealed no major changes in experimental groups administered three doses of EJSE (Table 5).

3.2.5. Pathological evaluations

There were no gross aberrations in the structural integrity of heart, liver and kidney after administration of EJSE with dose of 1 000, 2 000 or 3 000mg/kg BW (Figure 1).

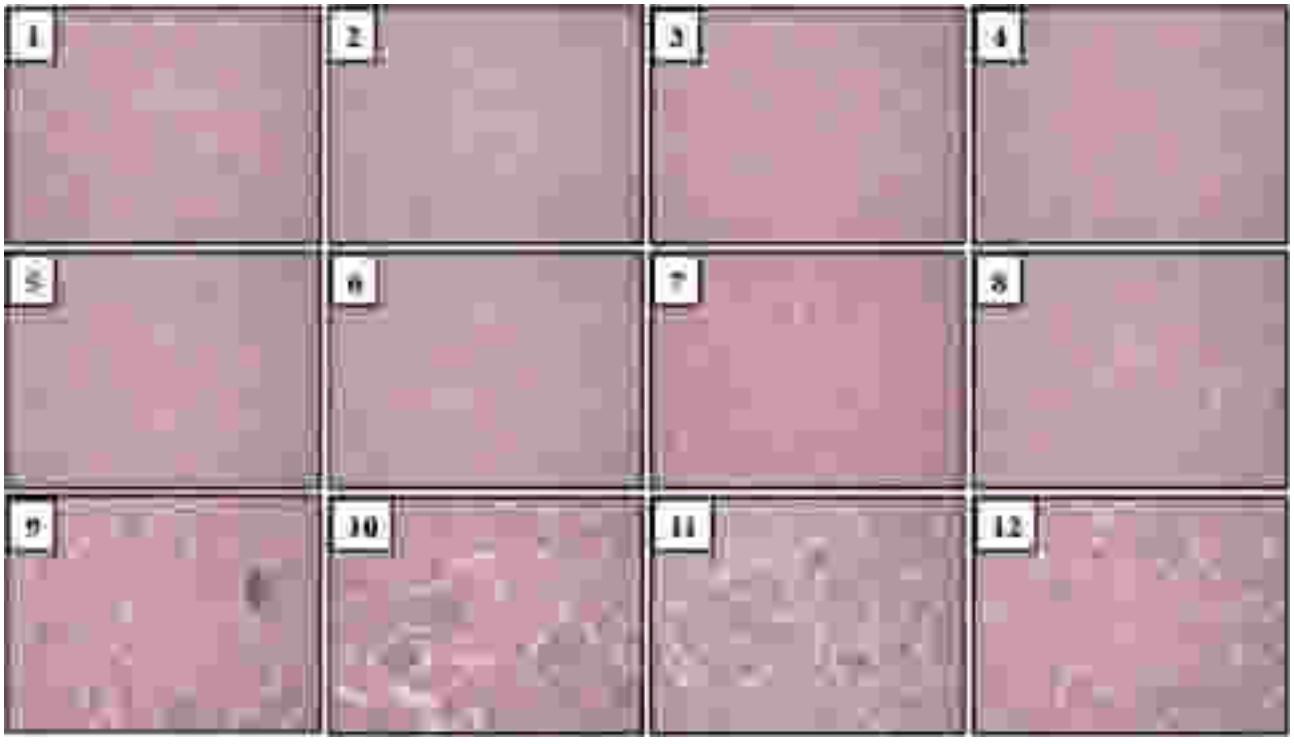

Figure 1. Photomicrographs of heart, liver and kidney.
Photomicrographs of heart of control (1) and 1 000, 2 000 and 3 000 mg/kg BW of EJSE treated mice (2, 3, 4), liver of control (5) and 1 000, 2 000 and 3 000 mg/kg BW of EJSE treated mice (6, 7, 8) and kidney of control (9) and 1 000, 2 000 and 3 000 mg/kg BW of EJSE treated mice (10, 11, 12) stained with hematoxyline and eosin (100×).

Table 1

Effect of chronic administration of EJSE on plasma metabolic indices in Swiss albino mice (Mean ± SEM) (n=8).

Plasma metabolic indices	Control	EJ1 000	EJ2 000	EJ3 000
Cholesterol (mg/dL)	45.33±2.55	40.00±4.51	41.00±3.05	33.33±4.80
Triglycerides (mg/dL)	46.95±3.05	36.22±2.42	36.59±1.79	34.43±1.29*
High density lipoprotein (mg/dL)	24.30±0.35	23.83±0.36	24.00±0.56	23.78±0.46
Low density lipoprotein (mg/dL)	32.00±1.33	30.50±2.36	26.56±2.04	12.81±0.93*
Very low density lipoprotein (mg/dL)	8.98±0.48	7.42±0.23	7.81±0.56	7.02±0.32
Fasting plasma glucose (mg/dL)	93.83±7.52	86.28±1.92	76.76±4.69	73.50±3.03
Sodium (mmol/L)	135.60±1.47	135.40±1.63	137.40±1.50	137.20±2.71
Potassium(mmol/L)	4.22±0.06	4.26±0.07	4.44±0.09	4.44±0.11
Calcium (mmol/L)	9.38±0.18	9.73±0.08	9.33±0.12	9.51±0.14

*P<0.05 compared to control.

Table 2

Effect of chronic administration of EJES on plasma markers of cardiac, hepatic and renal function in Swiss albino mice(Mean ± SEM) (n=8).

Plasma markers		Control	EJ1 000	EJ2 000	EJ3 000
Cardiac injury markers	Lactate dehydrogenase (U/L)	113.60±15.41	123.2±7.44	117.00±8.86	143.90±17.45
	Creatine Kinase-MB (U/L)	36.40±1.03	53.00±7.21	55.60±8.30	50.60±3.58
Hepatic injury markers	Aspartate transaminase (U/L)	35.87±1.16	34.00±3.31	40.60±3.47	43.00±1.51
	Alanine transaminase (U/L)	32.50±2.99	24.83±2.40	27.00±1.53	26.67±1.66
	Alkaline phosphatase (U/L)	10.23±1.09	11.34±1.21	14.53±1.67	15.32±1.99
	Billirubin (mg/dL)	0.33±0.02	0.40±0.03	0.47±0.05	0.49±0.01*
Renal injury markers	Urea (mg/dL)	54.72±3.28	60.66±2.87	64.72±4.97	74.13±2.78**
	Creatinine (mg/dL)	0.42±0.01	0.43±0.02	0.43±0.01	0.52±0.01*

*P<0.05 and **P<0.01 compared to control.

Table 3

Effect of chronic administration of EJES haematological parameters of Swiss albino mice(Mean ± SEM) (n=8).

Haematological parameters	Control	EJ1 000	EJ2 000	EJ3 000
White blood count (10^3/μL)	6.76±1.26	5.57±1.67	4.16±0.88	4.20±0.85
Hemoglobin (g/dL)	14.30±0.25	15.17±0.16	14.07±0.29	14.30±0.11
Red blood count (10^{12}/L)	8.20±0.17	8.73±0.03	8.06±0.16	8.35±0.04
Hematocrite count (%)	35.80±0.49	36.83±0.46	34.60±0.60	35.03±0.10
Mean corpuscular volume (fl)	43.70±0.37	42.20±0.45	43.00±0.49	42.03±0.40
Mean corpuscular Hemoglobin (pg)	17.40±0.05	17.34±0.20	17.40±0.10	17.07±0.12
Mean corpuscular hemoglobin concentration (g/dL)	39.90±0.26	41.13±0.20	40.60±0.25	40.77±0.38
Red cell distribution of width-coefficient of Variance (%)	16.10±1.66	16.60±1.84	16.60±1.72	16.60±1.72
Red cell distribution width standard deviation (fl)	19.87±0.23	19.40±0.09	19.87±0.46	19.17±0.23
Platelets (10^3/μL)	5.88±0.09	5.22±0.26	5.26±0.38	5.44±0.12
Mean platelet volume (fl)	10.17±0.03	9.70±0.05	9.80±0.15	9.90±0.05
Red cell distribution width	14.80±0.05	14.50±0.11	14.63±0.06	14.60±0.05
Plateletcrit (%)	0.59±0.01	0.50±0.02	0.51±0.04	0.53±0.01

P values all>0.05 compared to control.

Table 4

Effect of chronic administration of EJES on bodyweight, food and fluid intake of Swiss albino mice(Mean ± SEM) (n=8).

Index		Control	EJ1 000	EJ2 000	EJ3 000
Body weight	Initial (g)	22.45±1.33	23.57±1.12	24.02±0.51	23.50±0.34
	Final (g)	26.85±0.59	25.40±0.41	25.67±0.66	24.55±0.72
Weight gain (g)		4.40±0.02	1.82±0.04*	1.65±0.05**	1.05±0.03***
Food intake (g/day)		4.33±0.13	4.03±0.11	3.87±0.24	3.55±0.31
Fluid intake (mL/day)		8.99±0.63	10.01±0.50	9.08±0.39	9.88±0.41

*P<0.05, **P<0.01 and ***P<0.001 compared to control.

Table 5
Effect of chronic administration of EJES organ weights of Swiss albino mice(Mean ± SEM) (n=8).

Organ weights (g)	Control	EJ1 000	EJ2 000	EJ3 000
Brain	0.400±0.010	0.390±0.010	0.410±0.020	0.420±0.030
Heart	0.120±0.004	0.120±0.005	0.120±0.003	0.110±0.005
Lungs	0.160±0.014	0.180±0.008	0.180±0.010	0.170±0.009
Liver	0.980±0.020	0.960±0.040	1.000±0.020	0.980±0.020
Spleen	0.120±0.010	0.120±0.009	0.130±0.012	0.260±0.014
Adrenal	0.030±0.001	0.030±0.001	0.040±0.002	0.037±0.002
Kidney	0.280±0.006	0.270±0.008	0.270±0.005	0.260±0.008

P values all>0.05 compared to control.

4. Discussion

Since no mortality was recorded following a single acute dose of EJSE (5 000 mg/kg BW), LD_{50} was arithmetically calculated to be >5 000 mg/kg BW[23,24]. There were no observable symptoms of any behavioural alterations post 72 hours, indicating that EJSE has no major side effects even at high dose (5 000 mg/kg BW).

Alterations in plasma lipid load and fluctuations in electrolyte content following administration of an herbal extract are of pivotal importance and hence, it is mandatory to monitor the same following low as well as higher doses of EJSE. Observed decrement in plasma TC, TG, LDL and VLDL and unchanged HDL levels are in accordance with one of our ongoing studies on experimentally induced atherosclerosis in rats. However, non significant alterations were recorded following administration of low dose of EJSE (1 000 or 2 000 mg/kg BW) suggesting that, hypolipidemic effect of EJSE in normolipidemic mice manifests only at a higher dose. No significant alterations were observed in plasma glucose and electrolyte levels suggesting that EJSE does not manifest any major changes in the glycaemic status or electrolyte balance even at higher doses of 1 000, 2 000 or 3 000 mg/kg BW.

Plasma levels of CK-MB and LDH have been used as a marker of cardiac damage under various toxic manifestations[25,26]; however, our study recorded non significant alterations in the level of plasma CKMB and LDH after sub chronic administration of EJSE indicating no damage to cardiomyocytes. These observations get further substantiated with the microscopic evaluation of cardiac tissue wherein, no observable alterations in their cellular integrity could be noted after administration of different doses of EJSE.

Some herbal extracts can be heptotoxic and their oral administration leads to elevated levels of plasma AST and ALT[27,28]. Oral administration of EJSE has been reported to reduce streptozotocin and carbon tetrachloride induced elevation in plasma AST and ALT at a dose range of 100 to 500 mg/kg BW[18,29,30]. In our study the plasma AST and ALT levels in EJSE administered mice were very much comparable with those of control animals and the same was further corroborated with comparable histoarchitectural details of liver in control and EJSE administered groups. It is inferable from these observations that, EJSE is non toxic to hepatic tissue in the dosage employed herein. EJSE can therefore be considered as safe and non toxic to liver thus, adding further validity to its already reported hepatoprotective potential.

Studies have shown that, use of traditional herbal medicines in treating renal diseases is limited by their adverse effects on renal functions[2,6]. No significant alterations in plasma markers of renal damage (urea and creatinine) could be after sub chronic administration of EJSE in low doses (1 000 and 2 000 mg/kg BW). However, significant elevation in plasma levels of these markers could be were observed after administration of high dose of EJSE (3 000 mg/kg BW). However, no histoarchitecture destruction was observable in the renal tissue of mice administered lower as well as higher doses of EJSE. It is inferable from these observations that, EJSE may manifest moderate levels of renal damage at very high dose (3 000 mg/kg BW) but, is never the less safe at therapeutic dosage (100-500 mg/kg BW).

A complete haemogram is most appropriate to evaluate haemolytic property of the herbal extract in question[27,31]. No significant alterations were seen in the haematological profile of any of the experimental groups studied herein. Also, food and water intake and absolute weight of vital organs were very much comparable in control and EJSE treated groups. However, a dose dependent decrement in body weight gain was recorded at the end of 28 days. Significant decrement in plasma lipid profile and body weight gain observed after EJSE administration is attributable to reduced fat absorption through intestine and effective elimination of lipids through faeces as observed in EJSE treated atherogenic rats.

It can be concluded from the study that, EJSE is non toxic to cardiac and hepatic tissue and moderately nephrotoxic at the dose below 3 000 mg/kg BW. Hence, the Lowest Observable Adverse Effect Level (LOAEL) for EJSE is 3 000 mg/kg BW while, the No Observable Adverse Effect Level (NOAEL) is up to 2 000 mg/kg BW[32]. According to World Health Organization toxicity guidelines for herbal extracts, it can be concluded that the calculated Acceptable Daily Intake (ADI) is equal to NOAEL (2 000 mg/kg BW)/100; wherein, 100 is safety factor. Thus, ADI for EJSE for mice is 20 mg/kg BW. An extrapolation of these results to an average adult human (70 kg) would be 1.4 g of EJSE or 28 g of dried EJ powder (based on final percentage yield of 5% w/w after extraction).

Conflict of interest statement

We declare that we have no conflict of interest.

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