EVALUATION OF *Annona muricata* LEAVES AS POTENTIAL NUTRACEUTIC SUPPLEMENT IN GROWING KIDS †

[EVALUACIÓN DE LAS HOJAS DE *Annona muricata* COMO POTENCIAL SUPLEMENTO NUTRACÉUTICO EN CABRITOS EN CRECIMIENTO]

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SUMMARY

Background. *Annona muricata* has been reported as a potential nutraceutical based mainly on *in vitro* studies. **Objective. t**o determine potential toxicity and nutritional value of *Annona muricata* leaf flour using *in vitro* gas production (IVGP), dry and organic matter digestibility (IVDMD, IVOMD) as well as *in vivo* voluntary intake and apparent digestibility on Criollo kids. **Methodology.** *In vitro* trials were carried out with and without the tannin blocking agent polyethylene glycol (PEG). Kids (n=22) were fed with 0% (control, n=5), 2.5% (T2, n=6), 5% (T3, n=6) and 10% (T4, n=5) of *A. muricata* flour, intake and hepatic function were monitored. **Results.** The IVGP was lower (P<0.05) with increasing *A. muricata* inclusion, but without difference on the IVDMD or IVOMD (P>0.05). PEG inclusion had no effect (P>0.05). The T4 caused 100% feed refusal. Intake was 396a, 379a and 203b g DM (S.E.D. 0.038) for control, T2 and T3 respectively (P<0.05). The IVGP of *A. muricata* was low but not associated to the presence of condensed tannins. **Implications.** Due to reduced feed intake further studies are needed to identify the compound responsible of such effects. **Conclusion.** Although based on chemical composition and IVDMD and IVOMD *A. muricata* leaves can be qualified as medium quality fed, its use can only be recommended up to 2.5% diet inclusion, above such level toxicity might arise in growing goats.

Keywords: *Annona muricata*; *in vitro* gas production; feed intake; digestibility; toxicity.

RESUMEN

Antecedentes. *Annona muricata* ha sido reportada como un potencial nutracéutico basado principalmente en estudios *in vitro*. *Objetivo.* determinar la toxicidad potencial y el valor nutricional de la harina de hoja de *Annona muricata* utilizando la producción de gas *in vitro* (IVGP), la digestibilidad de materia seca y orgánica (IVDMD, IVOMD), así como la ingesta voluntaria *in vivo* y la digestibilidad aparente en cabritos criollos. **Metodología.** Se realizaron ensayos *in vitro* con y sin el agente bloqueador de taninos polietilenglicol (PEG). Los cabritos (n = 22) fueron alimentados con 0% (control, n = 5), 2.5% (T2, n = 6), 5% (T3, n = 6) y 10% (T4, n = 5) de harina de hoja de *A. muricata*. Se registró la ingesta y la función hepática. **Resultados**. La IVGP fue menor (P<0.05) con el aumento de la inclusión de *A. muricata*, pero sin diferencias en el IVDMD o IVOMD (P> 0.05). La inclusión de PEG no tuvo ningún efecto (P>0.05). El T4 causó un rechazo del alimento del 100%. La ingesta fue de 396a, 379a y 203b g de DM (S.E.D. 0.038) para el control, T2 y T3 respectivamente (P <0.05). El IVGP de *A. muricata* fue bajo pero no asociado a la presencia de taninos condensados. **Implicaciones.** Debido a la reducción de la ingesta de alimento, se necesitan más estudios para identificar el compuesto responsable de tales efectos. **Conclusión.** Aunque en base a la composición química y IVDMD e IVOMD las hojas de *A. muricata* pueden ser calificadas como alimento de calidad media, su uso solo puede recomendarse hasta un 2.5% de inclusión en la dieta, por encima de dicho nivel podría presentarse toxicidad en cabras en crecimiento.

Palabras clave: *Annona muricata*; producción de gas in vitro; consumo de alimento; digestibilidad; toxicidad.

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INTRODUCTION

Nutraceutics plants has emerged as a viable strategy to control gastrointestinal nematodes infections in ruminants. The methodology used for scan and testing potential plants to evidence anthelmintic (AH) effect on parasitology veterinary includes *in vitro* procedures, but also the determination of its nutritional value generally assessed by chemical composition, dry matter and organic matter digestibility (IVDMD and IVOMD) (Torres-Fajardo *et al*., 2020). *In vivo* studies are necessary to evaluate the preference and consumptions of foliages and to determining its impact on animal health and production, as well as any pharmacological effect (such as the anthelmintic effect) or toxicity before any general recommendation for usage is given. In the tropics many species of the *Fabacea* family containing condensed tannins have been used as models on this exploration (Alonso-Díaz *et al*., 2010; Hoste *et al*., 2006), but plants of other families as the annonaceae are also endemic of these regions and some evidence of its ethnobotany and ethnoveterinary use has been highlighted (Flores *et al*., 2004; Gajalakshmi *et al.*, 2011; Pandey and Barve, 2011). Annonaceae contains several plant tree species which produce edible fruits such as *Annona glabra* L., *A. muricata* L., and *A. reticulata* L. (Ubiergo-Corvalan et al., 2020). In addition to its edible fruits, *in vitro* AH effects of this plant family have been reported (Ferreira *et al*., 2013; Kamaraj *et al*., 2011; Souza *et al*., 2008). Methanolic extracts obtained from *Annona muricata* tested *in vitro* displayed lethal effective doses of 382.9 ug/mL on *Haemonchus contortus* eggs while a lethal dose of 211.6 ug/mL on L_3 was found for the acetonic extract (Castañeda-Ramírez *et al*., 2020). Based on solely on such *in vitro* results this plant could be considered a potentially useful nutraceutic, however further studies are needed to determine absence of *in vivo* toxic effects due to its secondary compounds suchs as annonacin (Moghadamtousi *et al*., 2015) and swainsonine (Ralphs and Stegelmeier, 2011; Rodríguez *et al.*, 2005) before widespread use is recommended. On this respect, previous *in vitro* tests performed with the *Artemia saline* technique suggests that a possible toxic effect of secondary compounds from *A. muricat*a can be expected (Castañeda-Ramírez, 2014). Reports about the nutritional quality of *A. muricata* leaves fed to small ruminant and their *in vitro/in vivo* toxicity are scarce. Moreover, some plants known to be potentially toxic such as *Lantana camara* can be normally consumed (within limits) by cattle without apparent toxic effect (Gusha *et al*., 2016). Thus, the aim of the present study was to evaluate under *in vitro* and *in vivo* conditions the nutritional quality of *A. muricata* dry leaves and to assess possible toxic effects arising from their consumption by growing kids.

MATERIAL AND METHODS

Experimental site and plant and feed materials

The study was performed at the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma de Yucatán, located 15.1 km from Mérida city, México. Leaves of *A. muricata* were directly collected from trees of the west zone of Mérida, Yucatán, México. Leaves were dried by sun exposition for 24 hours, milled with a 3 mm screen and stored on plastic containers until further use. Crude protein content (CP), ether extract (EE), ash and organic matter (OM) were determined according to the techniques described by the Association of Official Analytical Chemists (AOAC, 1980). Neutral and acid detergent fiber (NDF, ADF respectively) and lignin were determined (Robertson and Van Soest, 1981). The total phenol (TP) content equivalent to gallic acid and condensed tannins (CT) were estimated (Price *et al*., 1978). The same analyses were carried out on the mixture of *Pennisetm purpureum* and concentrate feed used on the *in vivo* trial of consumption.

Experimental animals

Animal management was carried out considering national and university regulations regarding animal welfare. For the *in vivo* test, 22 Criollo goats of 15 ± 5 kg, between three to eight months of age were used. Animals were treated with 12 mg/kg of levamizole via subcutaneous and 10 mg/kg of albendazole via oral (Chartier and Hoste, 1997) and the suppression of gastrointestinal nematodes was confirmed with daily sample of feces during the following week by respective parasitoscopic examen by flotation and modified McMaster techniques. Afterwards all animals were kept on metabolic cages during the whole experimental period.

In vitro evaluation of gas production and digestibility

Experimental design

For both gas production and digestibility evaluations a maximum of 0.5 g (100%) of one or various substrate previously dried and milled with 1 mm screen were used. For each substrate two replicates using rumen liquors from three different animal were carried out. The samples tested were based on the substrates alone or their combinations simulating

potential feed formulation or supplementation levels. Treatments were:
1) 0.5 g of *Annona muricata (Am),*
2) 0.5 g of *Pennisetum purpureum (Pp),*
3) 0.5 g of concentrate feed (C),
4) 0.15 g (*Pp*) + 0.35 g (C),
5) 0.15 g (*Am*) + 0.35 g (C),
6) 0.1 g (*Am*) + 0.05 g (*Pp*) + 0.35 g (C),
7) 0.05 g (*Am*) + 0.1 g (*Pp*) + 0.35 g (C),
8) Blank: only ruminal liquor + medium

In vitro gas production (IVGP)

An ANKOM^{RF} gas production system was used (Ankom Technology, Macedon, NY) for the IVGP. The medium was prepared according to Menke and Steingass (1988). Samples of ruminal liquor were directly collected from three cows (Bos indicus x *Bos taurus*) previously fastened during 12 h. Cow were routinely fed with 7 kg DM of fresh *P. purpureum* foliage and 3 kg DM of concentrate (18% CP, Lorgam®, Merida, Mexico). Rumen digesta sample were kept on hermetic plastic bags, after the contents were sieved to recover the liquid phase under a continuous flush with CO_2. The solids obtained from the sieve were blended and incorporated to the initial sample, this mixture conformed the inoculum maintained with constant CO_2 flux and kept in warm plaque at 39 °C. For the incubation, 250 ml bottles with 0.5 of substratum were considered as the base (100%) to prepare the double or triple substrate combination as described above. Every bottle was added with 100 ml of buffer solution and 25 ml of inoculum, the CO_2 was added immediately before the closure of bottles and were place into incubator at 39 °C for 48 h. Fermentation kinetics was measured using the ANKOM^{RF} equipment with readings every 5 minutes for 48 hours.

In vitro digestibility of the dry matter (IVDMD) and organic matter (IVOMD)

The IVDMD was evaluated using 100 ml bottles containing 0.5 g of substrate or the respective combinations (0.5 g) with 42 ml of buffer solution and 18 ml of inoculum. Bottles were equipped with a needle for gas liberation and incubated during 24 h at 39 °C. Then, the incubated samples were filtered using filter paper and dried to 60 °C for 24 h. The IVDMD and IVOMD were obtained subtracting the incubated DM or OM weight from the DM or OM weight of the residues at end of incubation (retained in filter paper). For the determination of the IVOMD, the residues were incinerated to 550 °C during 4 h to obtain estimate OM content. Both IVDMD and IVOMD were evaluated with and without the addition of 0.5 g polyethylene glycol (PEG) to assess the effect of condensed tannins on the fermentation and digestibility and correcting with the residual DM or OM in the blanks.

In vivo consumption and apparent digestibility

Animals and experimental diets

Twenty-two kids were used to conform four treatment groups balanced according to their weight, age and sex. They received feeds which included commercial grain-based concentrate (18% CP, Lorgam®, Merida, Mexico) mixed with *A. muricata* at 0% (n=5, control), 2.5% (n=6, T2), 5% (n=6, T3) or 10% (n=5, T4) level. The amount of feed offered included *P. purpureum* grass (1% BW on DM base). Total amount of feed offered was fixed at 3% BW on DM base. Experimental period consisted of 11 days of adaptation to the diet and 6 days of measurements.

Consumption of *A. muricata*

Feed offered and refused was weighted daily for each animal. After the adaptation period, consumption of *A. muricata* was registered. Animals were monitored for any clinical sign of toxicity such as: general incoordination, paralysis, hind-limbs incoordination, icterus, absence of rumination, difficulty of mastication and diarrhea; both immediately after the feed offering, and four hours after. In addition, during days 0, 7 and 17, samples were taken (2 ml) directly from the jugular vein to perform hematic analysis (white and red cells), alanine aminotranspherase (ALT) activity and creatinine. Animals were weighted on days 0, 7, 14 and 21 to estimate weight gain. Animals displaying diarrhea or total avoidance of the feed were withdraw from the study.

Dry matter (DMD) and organic matter (OMD) digestibility

The DMD was measured from the days 12 and 17 using total fecal collection technique (Rymer, 2000). Digestibility was calculated as: %D= (consumed-excreted)/consumed *100

Statistical analyses

In vitro: Gas production was used to analyze the kinetic of *in vitro* fermentation by fitting data with a monophasic model as described by Groot *et al.* (1996). Data fitting and equation parameter comparisons were performed with GraphPad Prism (2007). A mixture design was used to analyze IVDMD and IVOMD, the model included single substrates and their combinations (Minitab, 1997).

In vivo: DM intake, live weight change (g/d and total LW gain), hematic values (white and red cells), alanine aminotransferase (ALT) and creatinine were analyzed in complete randomized design (ANOVA) using Minitab (1997).

RESULTS

Chemical composition

As commonly reported for tropical fodders, *A. muricata* had a medium-high crude protein (15.5%) and tannin (4.7%) contents. Fiber level were within normal range (NDF = 44.8% and ADF = 32.5%) (Table 1).

In vitro gas production, dry matter and organic matter digestibility

The cumulative gas production values for substrates and their combinations are presented on Table 2. The lowest IVGP value was for *A. muricata* and the highest for the concentrate feed (P<0.05) IVDMD and IVOMD with and without PEG are presented on the Table 3. The addition of PEG did not cause any effect on digestibility (P>0.05).

Annona muricata intake digestibility and physiological variables

The experimental group T4 was removed from the experiment according to the elimination criteria indicated on the methodology as all animals refused the fed (withdrawn to avoid starvation) and one of the kids presented diarrhea.

For the remaining groups, intake was 396, 379 and 203 g DM/d (s.e. 0.038) and 52, 40 and 29 gDM/kg $LW^{0.75}$ (s.e 0.34) for control, T2 and T3, respectively. Goats that received 5% of *A. muricata* displayed a lower intake (P<0.05) when compared with the control (0%) and T2 (2.5%) groups.

Table 1. Chemical composition (g/kg DM except where stated) of substrates and experimental feed containing different levels of inclusion of *Annona muricata* used during the *in vitro* gas production and *in vivo* experiments.

Substrate	DM†	CP	EE	NDF	ADF	LIG	Ash	TPh	TT	CT
In vitro										
Annona muricata	300	155.6	42.3	476.3	324.9	76.1	79.2	20.1	13.6	47.6
Pennisetum purpureum	330	79	17.7	736.5	448.8	87.4	67.4	8.7	8.8	9.6
Concentrate feed	940	171.4	15.1	283.9	92.8	32.2	61.4	6.5	7.4	15.5
In vivo										
Control	880	162.2	15.4	329.2	128.4	37.7	62.0	--	--	--
T2 -2.5%	860	157.9	15.0	322.1	126.1	36.9	60.5	--	--	--
T3- 5%	850	153.6	14.6	315.0	123.8	36.1	58.9	--	--	--
T4- 10%	820	145.1	13.9	300.8	119.1	34.5	55.9	--	--	--

† g/kg fresh matter; CP: crude protein; EE: ether extract; NDF and ADF: Neutral and acid detergent fiber; LIG: lignin; Ash; TPh: total phenols; TT: total tannins and CT: condensed tannins. (Dietary inclusion levels of *A. muricata*, T2 2.5%, T3 5% and T4 10%).

Table 2. Cumulative gas production (ml gas/g DM ± standard deviation) for substrates and their combinations.

Treatment	GV	B	C	R^2	Sy.x	IVDDM ±S.D.
Annona muricata (Am)	$47.6 \pm 0.9_a$	$9.6 \pm 0.3_a$	$1.586 \pm 0.08_a$	0.905	3.886	$37.3 \pm 1.1_a$
Penisetum purpureum (Pp)	$86.8 \pm 1.5_b$	$13.2 \pm 0.3_b$	$1.800 \pm 0.07_a$	0.948	5.578	$53.3 \pm 4.6_b$
Concentrate fed (C)	$114.8 \pm 0.3_c$	$9.3 \pm 0.05_a$	$2.632 \pm 0.03_b$	0.992	3.141	$81.3 \pm 7_c$
30% Pp: 70% C	$105.0 \pm 0.6_c$	$9.6 \pm 0.1_a$	$2.200 \pm 0.05_c$	0.978	4.621	$70.6 \pm 5_d$
30% Am: 70% C	$92.7 \pm 0.6_e$	$8.3 \pm 0.1_c$	$2.498 \pm 0.08_b$	0.953	5.972	$62 \pm 9.1_d$
20% Am :10% Pp: 70% C	$92.8 \pm 0.3_e$	$9.2 \pm 0.06_a$	$2.499 \pm 0.04_b$	0.989	2.955	$65.3 \pm 6.1_d$
10%Am-20%P-70% C	$96.02 \pm 0.6_f$	$9.059 \pm 0.1_a$	$2.249 \pm 0.06_c$	0.978	4.285	$70 \pm 2.0_d$

GV, B and C correspond to the parameters of the equation. Total ml gas = $GV\ (1+ (B/t)^C)^{-1}$ (Groot *et al.*, 1996). R^2: regression value, Sy.X: residuals. IVDDM= *In vitro* digestibility of the dry matter, S.D.= Standard deviation.

Table 3. *In vitro* digestibility (%) of the dry matter (IVDMD) and organic matter (IVOMD) of the experimental substrates and their combinations with and without adding polyethylene glycol (PEG).

Treatment	IVDMD		IVOMD	
	Without PEG	With PEG	Without PEG	With PEG
Annona muricata (Am)	40 ± 5.51[a]	39.3 ± 7.11[a]	42.44 ± 5.62[a]	39.5 ± 8.40[a]
Pennisetum purpureum (Pp)	54.6 ± 2.07[b]	53.6 ± 3.44[b]	54.96 ± 3.59[b]	48.2 ± 5.80[b]
Concentrate feed (C)	81 ± 2.76[c]	77 ± 4.69[c]	82.60 ± 2.10[c]	77.2 ± 4.40[c]
30% Pp – 70% C	74.6 ± 5.61[d]	70 ± 1.78[d]	75 ± 5.68[d]	71. 18 ± 2.61[d]
30%Am -70% C	67 ± 5.02[d]	64.3 ± 4.08[d]	67.8 ± 6.06[d]	64.4 ± 4.99[d]
20%Am-10%P-70% C	72.6 ± 5. 75[d]	67.6 ± 1.96[d]	74.5 ± 5.70[d]	68.1 ± 2.32[d]
10%Am-20%P-70% C	75 ± 8. 46[d]	70 ± 2.19[d]	76.8 ± 9.88[d]	71.5 ± 2.22[d]

PEG: polyethylene glycol.
Within columns, different letter indicates significant difference (P<0.05)
Within IVDMD or IVOMD, no significant differences were observed due to PEG (P>0.05).

Similar DMD and OMD were observed between treatments (*P*<0.05). DMD was 77.2 ± 4, 78.7 ± 2 and 77.6 ± 0.9% for control, T2 and T3 respectively, while OMDwas 77.5 ± 2.7, 77.9 ± 2.1 and 75.1 ± 2.2% for the same treatments, respectively.

No neurological signs were observed and hematic values (white and red cells) remained within the normal range for all animals (*P*>0.05). Similarly, alanine aminotransferase values were similar between the control (13.2 ± 4.6), T2 (12.3 ± 2.7) and T3 (12.5 ± 5.9) (*P*>0.05) groups. A similar result was found for the creatinine values without significant differences between groups, (control = 1.06 ± 0.52, T2 = 0.99 ± 0.58 and T3 = 0.96 ± 0.40).

DISCUSSION

Chemical composition of *A. muricata*

In the present study, macronutrient values obtained from the leaves of *A. muricata* resulted very similar to those reported by Vit *et al*. (2014) (CP 13.9%, Ash 7.17% and EE 2.94%). The chemical composition of *A. muricata* also resulted similar to those of tropical trees considered as important forages for example *Brosimum alicastrum* (CP 16.9%, NDF 42% and ADF 16.9%) (Monforte-Briceño *et al.*, 2005; Sánchez *et al.*, 2001) and other tropical forages (Landa-Becerra *et al*., 2016). Thus, according to the content on macronutrients, *A. muricata* could be considered as a medium quality feed.

In vitro gas production and digestibility (*in vitro* and *in vivo*)

Cumulative gas production was significantly different on single feeds as compared with the mixtures. These results can be attributable to the difference between the type of feed and the resultant microbial consortia which results for each diet. It has been reported for forages containing large amounts of structural carbohydrates (fiber) that they are poorly digested and fermentation develops slowly at 2 - 5% per hour (Bach and Calsamiglia, 2006). In contrast, it was expected that leaves of *A. muricata* due to its chemical composition could result with high values on the IVGP test. However, the gas production was lower than those from *P. purpureum* grass (86.8 vs. 47.5 ml, *P*<0.05). Thus, it seems possible it contains anti-nutritional factors (not identified in the present experiment) which could be causing a reduced microbial activity and hence the low IVGP observed. These anti-nutritional factors released during incubation of *A. muricata*, could affect fermentation proportionally to its inclusion on the mixture resulting in a lower gas production and a trend to reduce the digestibility (Posada and Noguera, 2005). Various studies related to the content of alkaloids, flavonoids and saponins on leaves of *A. muricata*, link these metabolites to an antimicrobial and antiprotozoal activity and a direct effect on the microbial fermentation and a reduction of the *in vitro* gas production and digestibility (Dos Santos *et al*., 2013; Hu *et al*., 2005; Oskoueian *et al*., 2013). Nevertheless, the IVDMD and IVDOM was similar with and without PEG, thus it can be assumed that among the secondary compounds contained in *A. muricata*, the phenols and condensed tannins contained on leaves from *A. muricata* were not related with the reduction of the *in vitro* digestibility.

The similar *in vivo* digestibility found for all treatments was probably due to the feed intake reduction in T2 and T3. Reduced intake is usually associated with increased digestibility (McDonald *et*

al., 2011). However, as nutrient supply is the result of the DMI, nutrient content of feed and its digestibility, the net result of an increased level of *A. muricata* would be a negative effect on nutrient supply due to reduced DMI possibly leading to a reduced performance.

Consumption of *A. muricata* and physiological variables of kids

In the present study, the group with the highest level of *A. muricata* inclusion on fed (10%, T4 group) was withdraw from the experiment due to a fed refusal of 100% and the presence of diarrhea in one animal. Voluntary feed refusal increased as *A. muricata* level increased in the diet explaining differences on the intake between groups (control >T2 > T3 > T4). It seems evident that animals try to avoid the consumption of higher quantities of this material as increased feed refusals were observed above 2.5% of *A. muricata* inclusion level. The presence of saponins on leaves of *A. muricata* has been reported as a probable cause of diarrhea, discomfort, abdominal pain and irritation of the gut mucosa (Díaz-Gonzalo, 2010). However, although not measured, negative effects caused by other metabolites cannot be dismissed. Thus, further phytochemical studies should be conducted to identify any secondary compounds on the *A. muricata* leaves which are linked to the rejection of food and associated to diarrhea.

Concerning the physiological variables in response to the intake of *A. muricata*, the experimental animals on this study consumed two times (from 9 to 11 gDM/kg LW) the safe dose suggested by Arthur *et al*. (2011) (5 gDM/kg LW) which was obtained from a toxicity test performed on mice. Nevertheless, no neurological signs associated with a toxicity problem were observed. Moreover, despite that *A. muricata* is reported to contain the neurotoxin annonacin (Moghadamtousi *et al*., 2015) and the alcaloid swainsonine (Ralphs and Stegelmeier, 2011; Rodríguez *et al*., 2005) no modifications on the normal values for white and red cells, ALT and creatinine were observed on the present study. This result agrees with those previously reported by Mahour *et al*. (2007) with *A. squamosa*. Thus, it could be considered that *A. muricata* up to 5% inclusion has not hepatotoxic effect when used on short trials with goat kids at the doses used on this study. Nevertheless, studies are needed to assess potential renal damage or other toxic effect when administrated for longer time periods (Arthur *et al*., 2011).

CONCLUSION

Although according to their chemical composition, *in vitro* gas production and digestibility, *A. muricata* leaf meal could be considered a medium quality feedstuff, it contains antinutritional factors other than condensed tannins which could cause feed refusal and potential toxicity. If used as supplemental feed a maximum amount of 2.5% dietary level is recommended.

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Conflict of interest. There are no conflicts of interest.

Compliance with ethical standards. Animal management was carried out considering national and university regulations regarding animal welfare.

Data availability. Data is available with the corresponding author upon reasonable request.

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