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## Anti-gout effect of *Cardiospermum halicacabum* through antioxidant and xanthine oxidase inhibition property

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### Abstract

The medicinal plant *Cardiospermum halicacabum* (*C. halicacabum*) has multiple beneficial effects known for its rheumatism. The positive effect of aqueous leaf *C. halicacabum* extract (ALCHE) against xanthine oxidase (XO) inhibition has not been studied. The present study aims to depict the effect of ALCHE: (1) antioxidant effect; (2) *in vitro* XO enzyme inhibition and (3) *in vivo* anti-hyperuricemic effect using *Bombyx mori* L. (*B.mori*) model. *In vitro* antioxidant scavenging and XO enzyme inhibition was determined using ALCHE. *In vivo B.mori* model was used to evaluate the effect of ALCHE against anti-hyperuricemia for 5 days. *B.mori* was treated with ALCHE and allopurinol (5 mg/ml) coated fresh mulberry leaves. After the treatment, XO activity and uric acid were determined in the hemolymph of the *B.mori*. ALCHE showed higher antioxidant activity in comparison to ascorbic acid. ALCHE inhibited the XO enzyme (IC<sub>50</sub> value, 28 µg/ml) in a competitively with a *Ki* value of 2.8 µg/ml. The XO enzyme inhibition by ALCHE was further confirmed by *in vivo B.mori* model, it shows the better reduction of uric acid, due to the presence of phenolic antioxidants which inhibits the pro-oxidant enzyme such as XO.

**Keywords:** Xanthine oxidase, *C. halicacabum*, gout, antioxidant.

### 1. Introduction

Gout is an inflammatory disorder caused by a self-limiting innate immune response to the deposition of monosodium urate crystal (MSU) in the synovium of the joints and tissues [1]. Deposition of MSU crystals is directly associated with hyperuricemia (above 7 mg/dl). Xanthine oxidase (XO) is a complex molybdo (Mo) -flavoenzyme which catalyzes the oxidation of hypoxanthine to xanthine and finally to uric acid. In the active site of XO, the two electrons are transferred to FAD cofactor through the reduction of MO<sup>VI</sup> to MO<sup>IV</sup>. Subsequently, the oxidation of flavin moiety transfer the electrons to oxygen and results in the generation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or superoxide (O<sup>2-</sup>). Reactive oxygen species (ROS) generation is the major risk factor for the development of gout. Accordingly, XO is considered as the potential target for gout and various pathological conditions including metabolic disorders, inflammation, atherosclerosis and cancer [2]. Allopurinol is a classical XO inhibitor and has been used in the treatment of hyperuricemia and gout for several years. The numerous side effects such as skin rashes and gastrointestinal disturbances are reported for allopurinol. In addition, febuxostat (non-purine XO inhibitor that bears a thiazole moiety) with acceptable side effects was approved by the US Food and Drug Administration (FDA) [3]. Hence, identifying natural non-purine XO inhibitors with fewer side effects have always been a hotspot.

Medicinal plants are rich in bioactive molecules such as phenols, flavonoids, alkaloids, quinones, coumarins, terpenoids and other metabolites. *Cardiospermum halicacabum* Linn (Sapindaceae) is commonly considered as a weed and widely distributed in tropical/subtropical Africa and Asia. *C. halicacabum* was used in the treatment of rheumatism, nervous diseases, skeletal fractures, the stiffness of the limbs, snakebite and tumors [4]. An earlier report by Reddy *et al.*, [5] stated that the leaf of *C. halicacabum* plant mixed with castor oil was administered internally to treat rheumatism and lumbago. *Bombyx mori* (*B. mori*-silkworm) was used for the screening of XO inhibition for gout treatment. The end product of purine metabolism in *B. mori* is a uric acid which is similar to that of humans. Hence, *B. mori* is used as a model system to screen the active compounds as XO inhibitor. The *B. mori* model system is cost effective and advantageous to overcome ethics in using animal models [6].

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## 2. Materials and Methods

### 2.1 Chemicals

Bovine xanthine oxidase (Grade IV, ammonium sulphate suspension, 0.3 U/mg protein) 2,2- azinobis - 3- ethyl benzo thiozoline - 6- sulfonic acid (ABTS), 2, 2-diphenyl-1-picryl hydrazyl (DPPH), 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protein and uric acid estimation kit were purchased from Coral Clinical systems, Goa, India. All other chemicals were of an analytical grade.

### 2.2 Plant material and Preparation of the extract

Fresh leaves of *C. halicacabum* were collected from Omalur, Salem district, Tamil Nadu, India. The voucher specimen was identified (AUT/PUS/091) at ABS Botanical Conservation Research and Training Centre, Kaaripatti, Salem (Dt), Tamil Nadu, India. *C. halicacabum* leaf was shade dried for a week and powdered. The powder was extracted with water using soxhlet apparatus for 6 h. The aqueous leaf *C. halicacabum* extract (ALCHE) was concentrated using lyophilizer and stored at -20 °C until further study.

### 2.3 Qualitative and quantitative phytochemical screening of ALCHE

The presence of various phytoconstituents such as terpenoids, steroids, glycosides, saponins, flavonoids, tannins, lignins and quinones in ALCHE was determined according to the method of Harborne, [7]. The total phenol content (TPC) and total flavonoid content (TFC) were quantitatively determined by the standard method of Singleton *et al.*, [8] and Yao *et al.*, [9], respectively.

### 2.4 Antioxidant scavenging activity

The scavenging activity of various radicals (DPPH, ABTS, hydrogen peroxide) and ferric reducing antioxidant power (FRAP) of ALCHE determined as described earlier [10-11]. Different concentration of ALCHE (20-100 µg/ml) was used for all assays. Ascorbic acid was used as a standard.

### 2.5 In vitro determination of XO inhibitory activity

XO enzyme activity was determined by the method of Sweeney *et al.*, [12] with slight modifications. The enzyme and substrate solutions were prepared instantly before use. The reaction mixture consisted of 25 mM phosphate buffer (pH 7.5), 75 µM xanthine and 50 µl of xanthine oxidase (0.28 units/ml in the buffer). The reaction was measured at 295 nm after 2 min using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan).

The XO enzyme inhibition was determined by various concentrations (10-60 µg/ml) of ALCHE. XO enzyme was incubated with ALCHE at room temperature for 15 min. After the incubation period, XO enzyme activity was measured as given above. Allopurinol was used as a positive control.

### 2.6 Kinetic analysis of XO enzyme inhibition

The Lineweaver-Burk (LB) and Dixon plot analysis were performed to determine the mode of inhibition and  $K_i$  value of ALCHE [13-14]. The kinetic interaction assay was carried out in the presence and absence of ALCHE (25-60 µg/ml) with different concentrations of xanthine (0.025 mM- 0.100 mM) as substrate.

### 2.7 In vivo Bombyx mori experimental study

Age matched (from the 2nd to the 8th day of the fifth instar) *B. mori* was used in the present study. *B. mori* was fed with

fresh mulberry leaves at 25 °C. The *B. mori* model for XO inhibition was carried out according to the method of Zhang *et al.*, [6]. *B. mori* was divided into three groups consisted of 10 in each group. The control group (Group-I) was fed with fresh mulberry leaves coated with water. The group II (ALCHE group) and group III (Allopurinol group) were fed with mulberry leaves coated with 5 mg/ml ALCHE and allopurinol, respectively. Hemolymph was harvested at every 24 h intervals after start of the treatment and stored at -20 °C. XO activity and UA content in hemolymph were determined [15].

### 2.8 Statistical analysis

All the assays were carried out in triplicate and data were presented as mean ± SD. The results of *in vivo* study were statistically analyzed using one-way ANOVA.

## 3. Results and Discussion

### 3.1 Antioxidant activity parallels with the phytochemical constituents present in ALCHE

The presence of various biological activities such as antioxidants, anti-inflammatory, anti-bacterial, anti-diabetic, anti-carcinogenic and anti-leprosy in a medicinal plant is dictated by the presence of different phyto-constituents in particular secondary metabolites [16]. The screening for phytochemicals showed that a broad spectrum of secondary metabolites such as phenols, flavonoids, terpenoids, saponins, tannins, lignins, quinines and glycosides are present in ALCHE (Table 1). The qualitative analysis revealed that the concentration of TPC and TFC was found to be 56.7±3.06 mg/g and 38.8±4.28 mg/g, respectively. As in an expected line of results such high content of TPC and TFC paralleled with the significantly higher scavenging activity of ALCHE against various radicals (DPPH, ABTS and hydrogen peroxide) and FRAP activity. The percentage scavenging activity of ALCHE was found to increase in a dose-dependent manner (Figure 1). Approximately, 75% scavenging activity of ALCHE was observed at 100 µg/ml concentration. The half maximal inhibitory concentration ( $IC_{50}$ ) of DPPH, ABTS, hydrogen peroxide and FRAP activity was found to be 47 µg/ml, 53 µg/ml, 61 µg/ml and 69 µg/ml, respectively. The qualitative presence of various phyto-constituents in the plant material used in present study differed from previous reports [17]. This variation might be due to the influence of geological and environmental factors from which plant material was detected. The presence of bioactive compounds as reported by the previous studies such as saponins and tannins were synergistically correlate with the antioxidant activity of ALCHE [17]. In particular, the abundant presence of gallic acid (polyphenol) in *C. halicacabum* might have resulted in antioxidant activity as observed in the present study [18]. The hydrogen and electron donating property of polyphenols are considered important in chain-breaking of oxidant production and metal reduction property, respectively [19].

### 3.2 ALCHE inhibited XO activity in in vitro and in vivo

Increased XO activity results in hyperuricemia and contributes to the development of gout. It is important to inhibit XO activity to prevent the progression of gout. In this context, it is worth noting that ALCHE showed a dose-dependent inhibition of XO activity and  $IC_{50}$  value was found to be 28 µg/ml. LB plot revealed the competitive mode of inhibition by ALCHE with  $K_i$  value of 2.8 µg/ml. The presence of higher concentration of TPC and TFC in ALCHE might have contributed to its inhibitory activity against XO

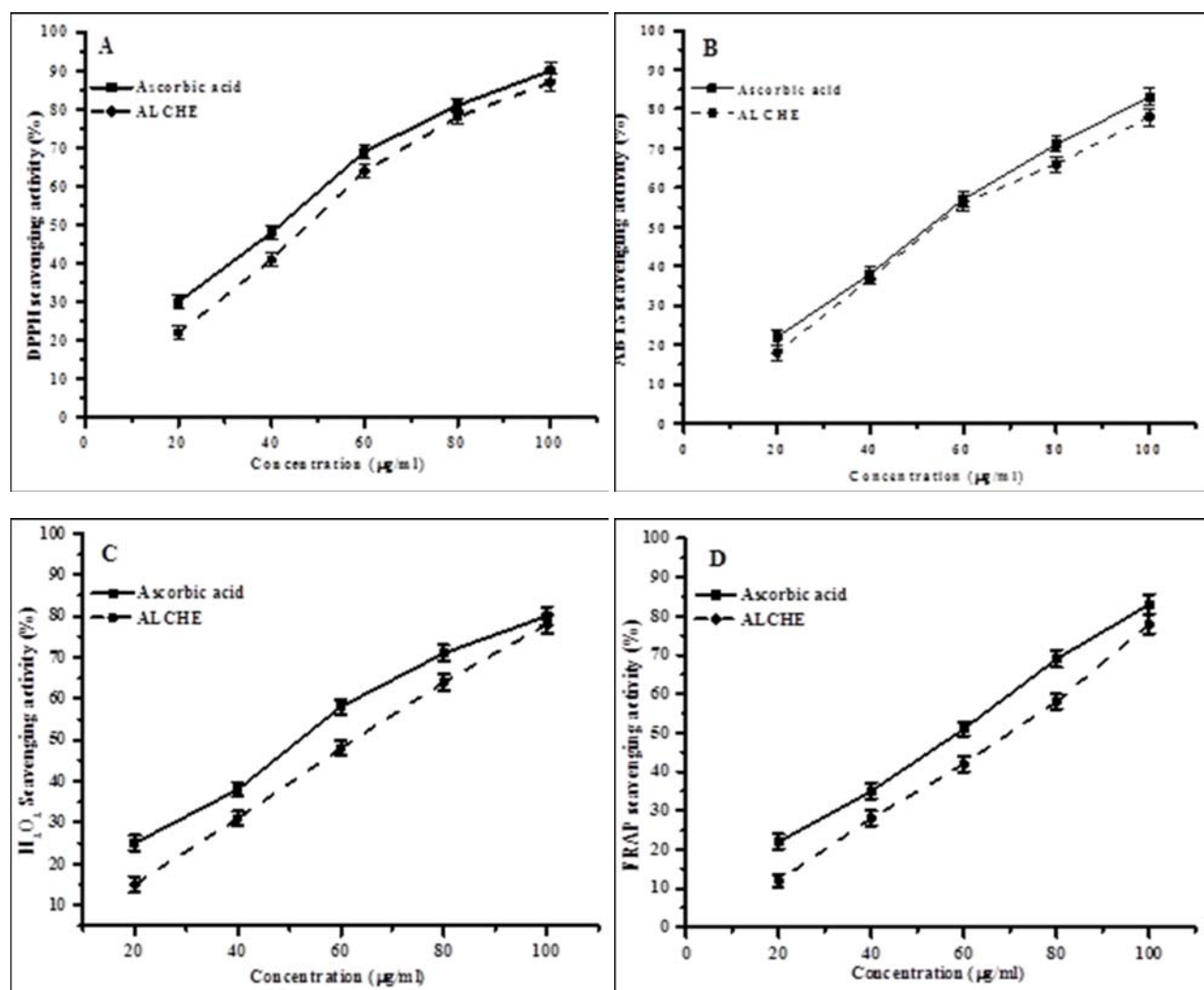
activity. To support this line, various other phenolics or flavonoids such as quercetin, alkyl gallate, caffeic acid, p-coumaric acid, ferulic acid, methyl gallate, secoiridoid oleuropein, vinyl caffeate, rosmarinic acid, methyl rosmarinate have been reported for their XO inhibition activity [20-21]. In particular, the previous study has shown gallic acid which is highly present in *C. halicacabum* inhibited XO activity with an  $IC_{50}$  value of 68  $\mu\text{g/ml}$  [22]. The advantage of ALCHE lies in its dual role; (i) through its binding to XO enzyme might decrease the urate production and (ii) through its antioxidant activity might quench the ROS generated by XO enzyme. The  $IC_{50}$  value of ALCHE was found to be less than gallic acid alone, which shows the synergistic effect of various phyto-constituents present in ALCHE. In addition, natural based medicine such as the use of plant extract (ALCHE) which is rich in various bioactive components is advantageous over the use of purified compounds (phenol / flavonoids) due to cost effectiveness and decreased side effects. The effect of ALCHE on XO enzyme activity was further reflected in *B. mori* *in vivo* model. Oral administration of phenolic acid-rich ALCHE significantly ( $P < 0.05$ ) decreased the activity of XO enzyme when compared to the control *B. mori* (group I) from day 1 to day 5 (Figure. 3A). The percentage of XO enzyme inhibition increased to  $\approx 80\%$  at 5 mg/ml concentration on the 5<sup>th</sup> day.

The concentration of uric acid is significantly ( $P < 0.05$ ) decreased from day 1 to day 5 in ALCHE treated group (group II) (Fig. 3B). The reduction of uric acid production was observed with concomitant XO enzyme inhibition when compared to allopurinol treated group (group III). The positive effect if ALCHE on *B. mori* model is promising which can be further evaluated for its medicinal use. However, further detailed analysis of various phyto-constituents present in ALCHE is necessary to understand its clinical use.

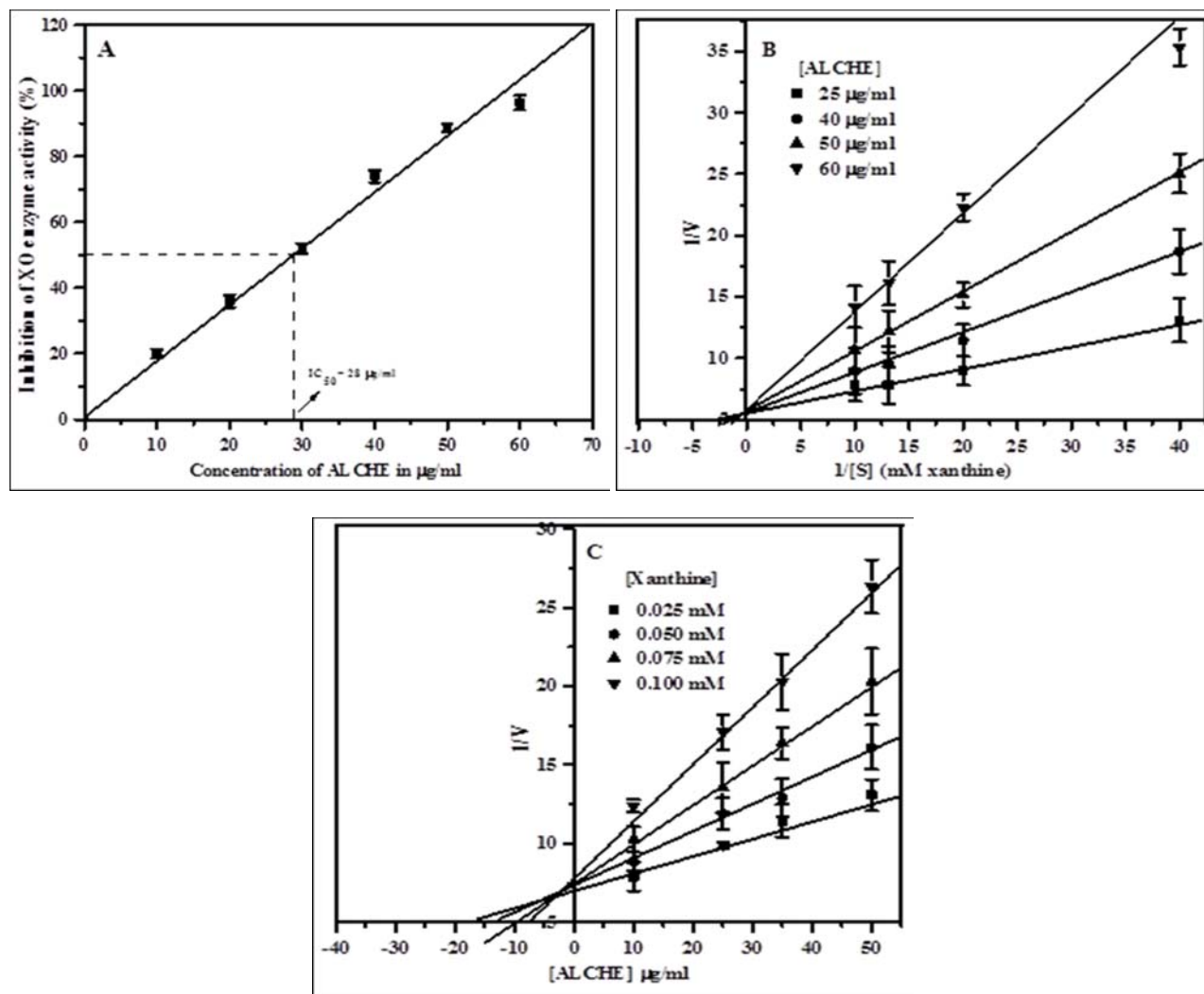
**Table 1:** Qualitative phytochemical analysis in aqueous leaf *C. halicacabum* extract (ALCHE)

S. No	Phytoconstituent	ALCHE
1	Phenols	+++
2	Flavonoids	+++
3	Terpenoids	+++
4	Saponins	+++
5	Tannins	+++
6	Lignins	++
7	Quinones	++
8	Glycosides	++
9	Steroids	-

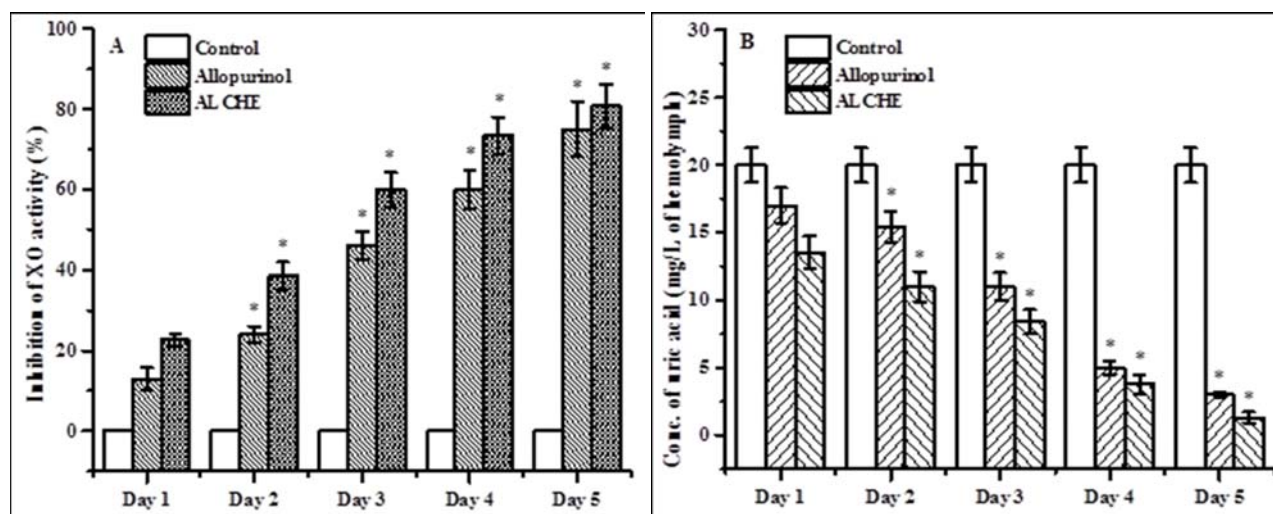
+ (slightly present), ++ (moderately present), +++ (highly present), - (absent)



**Fig 1:** Free radical scavenging activities of ALCHE (A) DPPH radical scavenging activity, (B) ABTS scavenging activity, (C)  $\text{H}_2\text{O}_2$  radical scavenging activity and (D) FRAP activity of ALCHE at various concentrations. ALCHE showed potent antioxidant scavenging effect in comparison to standard ascorbic acid. Results are expressed as mean  $\pm$  SD (n = 6).



**Fig 2:** Effect of ALCHE on XO enzyme activity (A) Inhibition of XO enzyme activity (%) by ALCHE at various concentrations; (b) LB plot shows competitive mode of inhibition of ALCHE on XO enzyme activity; (C) Dixon plot for determining the inhibitor constant of ALCHE. Results are expressed as mean  $\pm$  SD (n = 3).



**Fig 3:** Effect of ALCHE on (A) XO enzyme activity and (B) uric acid content in the hemolymph of 5<sup>th</sup> instar *B. mori*. Results are expressed as mean  $\pm$  SD (n = 10 per group). \* $P < 0.05$  vs. Control.

#### 4. Conclusion

The present study showed the antioxidant activity and XO enzyme activity of ALCHE, which is both required for the prevention and treatment of gout. The presence of secondary

metabolites might contribute to the observed effect which needs detailed evaluation before medicinal use.

## 5. Acknowledgments

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## 6. Conflict of Interest

All the Authors declare no conflict of interest.

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