ORIGINAL ARTICLE

In vitro studies on the effect of methanolic extract of *Elytraria acaulis* on the glucose metabolism of *Cotylophoron cotylophorum*

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Abstract

Introduction: In the present study, the *in vitro* effect of methanolic extract of leaves of *E. acaulis* (*Ea*ME) on the key enzymes, namely pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK) and lactate dehydrogenase (LDH) of *Cotylophoron cotylophorum* have been investigated. Furthermore, the *Ea*ME was subjected to preliminary structural analysis using gas GC-MS. **Materials and Methods:** Adult live *C. cotylophorum* was collected from the rumen of slaughtered sheep and maintained in Hedon-Fleig solution. The flukes were incubated in five different sub-lethal concentrations of *Ea*ME for 2, 4, and 8 h. Control flukes without the plant extract were also maintained simultaneously. The enzymes were assayed in the sample following the standard protocols and were expressed as n moles mg protein^{-1}. The effect of plant extract on the enzyme activity was calculated as percentage inhibition or acceleration. The structural elucidation of components of *Ea*ME was done using a quadrupole mass spectrometer and electron ionization method. **Results:** *Ea*ME significantly inhibited the PEPCK and PK activities of *C. cotylophorum,* and the inhibition was dose- and time-dependent. Interestingly, *Ea*ME accelerated the LDH activity catalyzing the reduction of pyruvate. The GC-MS analysis of *Ea*ME showed the presence of seven major compounds. **Conclusion:** The present study clearly revealed that the anthelmintic potential of *Elytraria acaulis* in impeding the energy-generating mechanism of *C. cotylophoroum* and hence, could be a potential source of natural anthelmintic.

Key words: *Cotylophoron cotylophorum*, lactate dehydrogenase, phosphoenolpyruvate carboxykinase, pyruvate kinase

INTRODUCTION

Livestock is an important sub-sector of Indian agriculture and plays a key role in improving the socioeconomic conditions of rural masses. Small ruminants, namely sheep and goats are principally maintained by a poorer section of the rural community and are a source of livelihood for large proportion of landless as well as small and marginal farmers. In developing countries, the share of livestock in agricultural gross domestic product is estimated to be 33%.[1] Nonetheless, there are a number of socioeconomic and environmental challenges that need to overcome to harness the pro-poor potential of livestock.

Among these, effective control of endoparasites in ruminants has always been the biggest challenge. At some point of time in their life, confined as well as pasture-based animals are certain to be exposed to worms. Paramphistomosis (Stomach fluke disease) is a parasitic infection caused by digenetic trematodes and is considered to be one of the most important parasitic diseases affecting livestock worldwide.[2-4] Several species of paramphistomes, namely *Cotylophoron cotylophorum*, *Fischoederius elongatus*, *Gastrothylax crumenifer*, *Gigantocotyle explanatum*, *Paramphistomum cervi,* and *Paramphistomum epiclitum* in domestic ruminants have been reported from different states of India.[5-7]

Control of helminth infections relies mainly on a limited number of drugs such as the derivatives of benzimidazoles,

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macrocyclic lactones, and imidazothiazoles.[8] The compulsory and often excessive use of chemotherapeutics in combination with poor management practices has resulted in parasites resistant to chemical drugs.[9]

Phytotherapy has, hence, become an alternative solution to control gastrointestinal helminths. *Elytraria acaulis* a stem-less perennial herb belonging to family Acanthaceae is commonly used for wound infection by traditional healers.[10,11] It has also been reported that root paste of *E. acaulis* is used among the tribes of north Gujarat to treat colic pain.[12] Furthermore, in traditional practice, the leaves were used as an antidote and to cure respiratory ailments.[13] The decoction prepared from the leaves of *E. acaulis* is used for the treatment of fever, venereal diseases and root is used in the mammary tumor, abscesses, pneumonia, and infantile diarrhea.[14] However, the anthelmintic potential of *E. acaulis* have been investigated.

Interference with the energy generating mechanism of the parasites has been considered as an important target for the pharmacological action of any anthelmintic drug. The metabolic pathway for carbohydrate catabolism in trematodes is essentially anaerobic and involves the glycolytic and part of the reversed tricarboxylic acid cycle.[15] Glycogen or glucose is broken down by normal glycolytic sequence to phosphoenolpyruvate (PEP). PEP can either be carboxylated by phosphoenolpyruvate carboxykinase (PEPCK) to oxaloacetate (OAA) or dephosphorylated by pyruvate kinase (PK) to pyruvate, which is further reduced to lactate by lactate dehydrogenase (LDH). OAA formed is reduced in the cytoplasm to malate by malate dehydrogenase (MDH). The malate so formed permeates into the mitochondrion where dismutation takes place - part is oxidized to pyruvate and part is reduced to succinate through fumarate. *C. cotylophorum* is a facultative anaerobe producing succinate as the end product.[16]

In the view of the above, an attempt has been made to study the anthelmintic efficacy of *E. acaulis* and its mode of action on the key enzymes of carbohydrate metabolism, namely PK, PEPCK, and LDH of *C. cotylophorum*.

MATERIALS AND METHODS

Collection and *In vitro* Maintenance of *C. cotylophorum*

Adult live parasites were collected from the rumen of sheep slaughtered at the abattoir in Chennai, Tamil Nadu, India. The parasites were then rinsed in physiological saline and maintained in Hedon-Fleig solution.[17]

Preparation of Extract from Leaves of *E. acaulis*

Leaves of *E. acaulis* were collected in the month of September 2017, from the Madavilagam village, Sadupperi in Tiruvannamalai district, Tamil Nadu (Lat. 12° 35' 9.834'' N, Long. 79° 13' 58.89'' E). The leaves were washed thoroughly in water and shade dried for 21 days. The dried leaves were crushed, using a pestle and mortar and filled in the porous cellulose thimble of size 33 mm × 94 mm. The thimble loaded with plant material was subjected to Soxhlet extraction.

Extraction was done with solvents based on their increasing polarity, namely n-hexane, ethylacetate, chloroform, acetone, and methanol. Successive solvent extracts were concentrated using a rotary vacuum evaporator (Brand: Equitron and Model: Evator). The crude extracts were stored in the refrigerator at 2–3°C for further analysis.

Selection of Potent Solvent Extract

The flukes were incubated in 25 ml of a Hedon-Fleig solution containing various concentrations (1, 3, and 5%) of each solvent extracts and the motility was visualized at regular interval of time. The effective plant extract was identified by the motility of the flukes in each of the solvent extracts. The live parasites were incubated for 2, 4, and 8 h in the various sub-lethal concentrations of the effective solvent extract for further enzymatic studies. Suitable controls were maintained simultaneously without the plant extract in the medium.

Sample Preparation and Enzyme Assay

The flukes were weighed wet and homogenized in 0.25 M sucrose solution containing 0.15 M Tris-HCl (pH 7.5) in an ice bath to obtain 10% (W/V) homogenate. It was centrifuged at 1000 rpm for 10 min, and the supernatant was used as the enzyme source. This supernatant was used to prepare the cytosolic and mitochondrial fractions of enzyme samples by following the standard procedure.[18]

The activities of the enzyme PK, EC 2.7.1.4 and PEPCK, EC 4.1.1.32 were assayed in the cytosolic fraction.[19] For estimation of PK activity, the reaction mixture contained 1 ml of 300 mM Tris-HCl buffer (pH 7.8), 0.5 ml of 42 mM magnesium sulfate ($MgSO_4$), 0.5 ml of 450 mM potassium chloride (KCl), 0.3 ml of 50 mM adenosine diphosphate (ADP), 0.3 ml of 50 mM PEP, 0.3 ml of 2 mM nicotinamide adenine dinucleotide reduced (NADH), 0.025 ml of 48 mM fructose biphosphate (FBP), 0.025 ml of 15 units of LDH, and 0.05 ml of enzyme sample.

The assay mixture for estimation of PEPCK contained 1 ml of 300 mM imidazole buffer (pH 7.4), 0.4 ml of 300 mM $MgSO_4$, 0.3 ml of 400 mM KCl, 0.3 ml 70 mM sodium bicarbonate ($NaHCO_3$), 0.3 ml of 20 mM ADP, 0.3 ml of 40 mM PEP, 0.3 ml of 2 mM NADH, 0.05 ml of 15 units of MDH, and 0.05 ml of enzyme sample. For both PK and PEPCK activities, the decrease in absorbance at 340 nm was recorded for 3 min at an interval of 15 s. The enzyme

activities were calculated from the millimolar coefficient of 6.22 for NADH and expressed was expressed in nmoles NADH oxidized/min/mg protein.

LDH, EC 1.1.1.27 catalyzing the oxidation of lactate and reduction of pyruvate was assayed as described by standard protocol.[20] For the oxidation of lithium lactate, 0.8 ml of 60 mM phosphate buffer (pH 7.5), 0.1 ml of 0.5 M lithium lactate, 0.05 ml of enzyme sample, and 0.05 ml of 20 mM NAD were placed in a 1 ml cuvette. The increase of absorbance at 340 nm was recorded for 3 min at an interval of 15 s.

For the reduction of pyruvate, 0.05 ml of enzyme sample was added to 0.8 ml of 60 mM phosphate buffer (pH 6.5), 0.01 ml of 1 mM NADH, and 0.01 ml 10 mM sodium pyruvate, and final volume was adjusted to 1 ml by the addition of distilled water in 1 ml cuvette. The decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 s.

Activities of LDH catalyzing both oxidation and reduction reactions were calculated from the millimolar coefficient of 6.22 for NAD and NADH and were expressed in n moles NAD reduced or NADH oxidized/min/mg protein.

The enzyme activities were expressed in terms of protein, and the protein was estimated following the procedure of Lowry *et al*.[21]

Statistical Analysis

Data were expressed as the mean and standard deviation of the means. The significance of the plant extract induced inhibition among different concentrations of plant extract for each duration of incubation was assessed using one-way analysis of variance.

Preliminary Structural Analysis using Gas Chromatograph Coupled with Mass Spectrometer (GC-MS)

The GC-MS of an effective solvent extract of *E. acaulis* was recorded using Agilent mass hunter 7000 GC coupled with a triple quadrupole mass spectrometer. The recorded gas chromatogram and mass spectra have been analyzed using Agilent mass hunter workstation version DSP:7000.3000. The molecular ion peak, base peak and other fragments from the compounds have been recorded, and the fragmentation pattern has been correlated with the library search. The compounds listed from the library with maximum (more than 95%) overlap with the mass spectra of the component have been given as the speculative chemical structure of the component.

RESULTS

It is evident from Table 1 that methanolic extract of leaves of *E. acaulis* (*Ea*ME) manifested potent efficacy against *C. cotylophorum*. In 3 and 5% concentration of *Ea*ME 100% mortality of flukes was observed within 6 h of exposure whereas, in 1 % *Ea*ME 100% mortality was observed within 10 h. Based on the motility and survival of the parasite for 8 h of exposure to *Ea*ME five different sub-lethal concentrations (0.5, 1, 5, 10 and 15 mg ml^{-1}) were selected for the subsequent

Table 1: Gross visual observation on the motility of the parasites incubated in various solvent extract of leaves of *Elytraria acaulis*

Medium of extract	Conc. (%)	30 min	2 h	4 h	6 h	8 h	10 h	12 h	24 h
Hexane	1	+ + + +	+ + + +	+ + + +	+ + + +	++++	+ + +	+ +	+ +
	3	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ +	+ +	+
	5	+ + + +	+ + + +	+ + + +	+ + +	+ +	+	+	-
Chloroform	1	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ +
	3	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ +	+ +	+
	5	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ +	+	-
Ethyl Acetate	1	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ +	+ +	+
	3	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	-
	5	+ + +	+ +	+ +	+ +	+	+	-	-
Acetone	1	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ +	+	-
	3	+ + + +	+ + +	+ + +	+ +	+ +	+ +	-	-
	5	+ + + +	+ ++	+ +	+	-	-	-	-
Methanol	1	+ + + +	+ + +	+ +	+ +	+	+	-	-
	3	+ + + +	+ + +	+	-	-	-	-	-
	5	+ + +	+ +	-	-	-	-	-	-
Control		+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Very active (+ + + +), moderately active (+ + +), slightly active (+ +), sluggish (+), dead (-). *E. acaulis: Elytraria acaulis*

studies. The results of the inhibitory effect of *Ea*ME on the PK and PEPCK activity of *C. cotylophorum* is depicted in Tables 2 and 3. Maximum inhibition of PK (93.38 %) and PEPCK (97.82 %) activities was observed at 15 mg ml^{-1} concentration after 8 h exposure. LDH catalyzing oxidation reaction was inhibited by 57.19% in 15 mg ml^{-1}concentration of *Ea*ME after 2 h exposure; however, after 4 and 8 h exposure inhibition in the LDH activity under the same condition was 70.89 and 97.39%, respectively [Table 4].

Table 5 shows the effect of *Ea*ME treated flukes on the LDH activity catalyzing the reduction of pyruvate. Inhibition (46.74 %) of LDH activity catalyzing reduction reaction was observed in 0.05% concentration of *Ea*ME after 2 h exposure; however, the inhibition in the activity of the same decreased to 34.24 and 22.59% after 4 and 8 h exposure, respectively. Inhibitory effect of *Ea*ME on the LDH catalyzing reduction of pyruvate was inversely proportional to the concentration of the extract and period of exposure.

Interestingly, an increase of 5.73 and 18.11% in the activity of LDH catalyzing reduction of pyruvate was observed after 2 and 4 h exposure of the parasite to 1.5% concentration of *Ea*ME; whereas, the activity of the enzyme stimulated to 30.62% after 8 h exposure. The acceleration in the enzyme activity has been reported in negative percent inhibition. Statistical analysis revealed that the data were significantly different at various concentrations and different periods of incubation ($P < 0.01$)

Statistical analysis showed that the inhibitory effect of *Ea*ME among the different concentrations is significantly different for each duration of incubation ($P < 0.01$).

GC-MS analysis of *Ea*ME showed the presence of seven major compounds [Figure 1]. The components with their retention time, peak height, peak area (%), molecular formula, and molecular weight are presented in Table 6.

DISCUSSION

The current *in vitro* investigation undoubtedly elicited the anthelmintic potential of *E. acaulis* against the digenetic trematode *C. cotylophorum*. The *Ea*ME was effective in altering the metabolic pathway of *C. cotylophorum*. The primary energy source which trematodes are capable of utilizing at the adult parasitic stage is carbohydrate, and the metabolism is predominantly anaerobic. Glycogen is the major polysaccharide in most helminths and digeneans store between 2 and 30% of their dry weight as glycogen.[15]

PEP occupies a central position in the metabolic pathway of trematodes. The two enzymes PK and PEPCK are likely to compete for the common substrate PEP, channeling it to aerobic (PK) or anaerobic (PEPCK) pathways[22,23] so that low PK/PEPCK activity ratio is indicative of relatively higher anaerobic capacity. PEPCK plays a key role in invertebrate

Table 2: *In vitro* effect of *Ea*ME on Pyruvate kinase activity (% inhibition) of *C. cotylophorum*

Concentration (mg ml^{-1})	% Inhibition (mean±SD of *n*=5) at various periods of incubation		
	2 h	4 h	8 h
0.5	31.96±0.62	49.57±0.99	76.29±1.60
1	39.56±0.87	52.59±0.40	79.78±0.41
5	44.93±0.20	57.58±0.46	83.49±0.70
10	50.41±0.50	66.12±0.28	85.85±0.84
15	59.40±0.80	75.03±0.71	93.38±0.39

*Inhibitory effect of *Ea*ME among different concentrations for each period of incubation is significantly different ($P<0.01$). **Inhibitory effect of *Ea*ME among different hours of incubation for each concentration is significantly different ($P<0.01$). *C. cotylophorum: Cotylophoron cotylophorum*. *Ea*ME: Methanolic extract of leaves of *Elytraria acaulis*, SD: Standard deviation

Table 3: *In vitro* effect of *Ea*ME on Phosphoenolpyruvate carboxykinase activity (% inhibition) of *C. cotylophorum*

Concentration (mg ml^{-1})	% Inhibition (mean±SD of *n*=5) at various periods of incubation		
	2 h	4 h	8 h
0.5	28.73±0.92	52.60±1.07	79.43±0.41
1	36.71±0.49	59.63±0.16	84.72±0.46
5	42.93±0.48	63.34±0.80	89.92±0.92
10	45.30±0.95	70.59±0.35	91.96±1.06
15	61.02±0.94	76.64±0.52	97.82±0.37

*Inhibitory effect of *Ea*ME among different concentrations for each period of incubation is significantly different ($P<0.01$). **Inhibitory effect of *Ea*ME among different hours of incubation for each concentration is significantly different ($P<0.01$). *C. cotylophorum: Cotylophoron cotylophorum*. *Ea*ME: Methanolic extract of leaves of *Elytraria acaulis*, SD: Standard deviation

Table 4: *In vitro* effect of *Ea*ME on lactate dehydrogenase activity (% inhibition) catalyzing oxidation reaction of *C. cotylophorum*

Concentration (mg ml^{-1})	% Inhibition (mean±SD of *n*=5) at various periods of incubation		
	2 h	4 h	8 h
0.5	30.87±1.08	49.02±1.50	74.86±1.30
1	39.93±0.23	51.06±0.76	83.19±1.40
5	43.75±0.28	55.41±1.30	87.67±0.03
10	47.33±0.37	63.86±0.54	95.22±0.38
15	57.19±0.63	70.98±1.12	97.39±0.36

*Inhibitory effect of *Ea*ME among different concentrations for each period of incubation is significantly different (P<0.01). **Inhibitory effect of *Ea*ME among different hours of incubation for each concentration is significantly different (P<0.01). *C. cotylophorum: Cotylophoron cotylophorum*. *Ea*ME: Methanolic extract of leaves of *Elytraria acaulis,* SD: Standard deviation

Table 5: *In vitro* effect of *Ea*ME on lactate dehydrogenase activity (% change) catalyzing reduction reaction of *C. cotylophorum*

Concentration (mg ml^{-1})	% inhibition (mean±SD of *n*=5) at various periods of incubation		
	2 h	4 h	8 h
0.5	46.74±0.20	34.24±0.75	22.59±0.47
1	38.34±0.52	29.68±0.61	13.67±0.71
5	33.81±0.52	25.74±0.33	06.82±0.84
10	19.38±0.69	05.05±0.32	−15.01±0.24
15	−05.73±0.27	−18.11±0.65	−30.62±0.98

*Inhibitory/stimulatory effect of *Ea*ME among different concentrations for each period of incubation is significantly different (P<0.01).
**Inhibitory/stimulatory effect of *Ea*ME among different hours of incubation for each concentration is significantly different (P<0.01).
C. cotylophorum: Cotylophoron cotylophorum. *Ea*ME: Methanolic extract of leaves of *Elytraria acaulis*, SD: Standard deviation

Table 6: List of compounds identified from GC-MS analysis of *Ea*ME

Compounds	Rt (min)	Peak height	Area%	Molecular Weight	Molecular formula
(*Z*)-7-methyl tetradec-8-enyl acetate	4.204	8.23×10^6	12.00	268.43 Da	$C_{17}H_{32}O_2$
1-(2-(2-norborn-5-enyl methylamino) ethyl) aziridine	5.412	1.42×10^7	13.18	192.16 Da	$C_{12}H_{20}N_2$
9-amino-1-methyl-3,6-diazahomoadamantane	5.760	1.98×10^7	12.16	181.28 Da	$C_{10}H_{19}N_3$
N-methyl-N-[4-(3-hydroxy pyrrolidinyl)-2-butynyl]	6.461	4.44×10^6	9.33	210.27 Da	$C_{11}H_{18}N_2O_2$
3-hydroxy dodecanoic acid	6.979	1.30×10^7	28.74	216.32 Da	$C_{12}H_{24}O_3$
Diphenyl methane derivative	7.745	1.63×10^7	16.74	293.40 Da	$C_{20}H_{23}NO$
Pyrazine derivative	9.845	7.67×10^6	7.63	250.34 Da	$C_{14}H_{22}N_2O_2$

*Ea*ME: Methanolic extract of leaves of *Elytraria acaulis*, GC-MS: Gas chromatograph-mass spectrometer

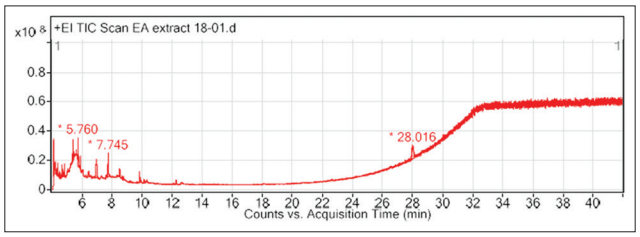

Figure 1: Gas chromatogram of a methanolic extract of *Elytraria acaulis*

energy metabolism because it is regulatory terminal branch point enzyme, which directs the flow of carbon from PEP into end products of anaerobic metabolism through PEP-succinate pathway.[24] Thus, in PEP-succinate pathway 1 mole of glucose provides 2 moles of malate in the cytosol, the net result on the energy balance is that 2 moles of nucleoside triphosphate are formed. 2 moles of NADH are also formed and used again.[25]

Parasites with low ratios (0.05–0.1) are carbon dioxide fixers and those with high ratios (2–10) are lactate fermenters.[15,23] The PK/PEPCK ratio in *C. cotylophorum* was observed to be 0.36 which lies between the two extremes suggesting that it is likely to utilize both pathways for energy production.[26] Such intermediate ratios were observed in *Litomosoides carinii* (1.9), *Hymenolepis diminuta* (0.18),

Schistosoma solidus (1.7), *Fasciola hepatica* (0.25–0.4), *Setaria digitata* (0.6), and *Isoparorchis hypselobagri*.[24,27-31]

In *C. cotylophorum* glucose catabolism is predominantly by PEP-succinate pathway, which produces more ATP rather than pyruvate-lactate pathway. In the current study, PEPCK activity of *C. cotylophorum* treated with *Ea*ME was inhibited to greater degree when compared to PK activity. Inhibition of PEPCK activity may lower the production of OAA which in succession hinder the production of other substrates required for PEP-succinate pathway. Eventually, the parasites now tend to enter PEP-lactate pathway to counterpoise for the energy loss. Similar inhibitory effects on higher inhibition of PEPCK activity of *C. cotylophorum* treated with *A. concinna* and *A. calamus* have been documented.[16,32]

In PEP-lactate pathway, PK converts PEP to pyruvate which is further reduced to lactate by LDH. Furthermore, the same enzyme (LDH) catalyzes the oxidation of lactate to pyruvate. It is evident that *Ea*ME inhibited the LDH activity of *C. cotylphorum* catalyzing the oxidation of lactate to pyruvate. However, LDH activity catalyzing reduction of pyruvate to lactate is stimulated. Acceleration in the LDH activity catalyzing reduction reaction appears to be an important target for drug action.[33,34,16] The greater inhibition of PEPCK activity together with the acceleration of LDH activity catalyzing reduction reaction of *C. cotylophorum* exposed to *Ea*ME suggests the shift in the metabolic fate of PEP and resulted in the higher production of lactate than succinate. Hence, it is clear from the present investigation that *E. acaulis* modified the energy generating process of *C. cotylophorum* with PEPCK and LDH as the viable target of the action. Deprivation of energy may lead to the death of the parasites.

The curative properties of medicinal plants may be attributed to the presence of the various complex chemical substances or bioactive constituents present in them. The most effective extract (*Ea*ME) subjected for further GC-MS analysis revealed the presence of seven major compounds and the structure of which have been tentatively identified.

From the foregoing account, it is very clear that *E. acaulis* contained several chemicals which may contribute to its effectiveness against *C. cotylophorum*. This study has helped in identifying the putative drug target for anthelmintic action. Greater efficacy of *E. acaulis* suggests its use against a variety of parasites of medical and veterinary importance. Future prospects for drug designing relies on the isolation and purification of compounds (active principle) from *E. acaulis*.

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