LARVICIDAL ACTIVITY OF *PERESKIA BLEO* (KUNTH) DC. (CACTACEAE) FRUIT ENDOCARP CRUDE AND FRACTIONATED EXTRACTS AGAINST *AEDES AEGYPTI* (L.) (DIPTERA: CULICIDAE)

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Abstract. The use of insecticides can cause adverse effects in vector control, a plant bio-insecticide is an advantageous substitute. Currently, the promising mosquito larvicidal activity from plant extracts has been reported worldwide, including Thailand. In this study, the endocarp of *Pereskia bleo* (Kunth) DC. fruit was extracted with distilled water and ethanol. Crudes and fractionated groups of the extracts were evaluated for their larvicidal efficacy against the 3rd instar larvae of *Aedes aegypti*. At 48 hours of exposure, it was found that the activities of the extracts were higher than 24-hour's. The ethanolic extracts showed stronger activities than the aqueous ones, indicating the lower LC_{50} values of both crude and fractionated group extracts. The most toxic activity was found in a fractionated group of the ethanolic extract, E-Gr3, with significantly lowest LC_{50} values of 707.94 and 223.12 ppm for 24- and 48-hour detection times, respectively. The bioassay results indicated the larvicidal property against the *Ae. aegypti* mosquito of the *P. bleo* plant extracts. A safety for non-target organisms or an action on other mosquito vectors of this plant, should be further investigated.

Keywords: *Aedes aegypti*, larvicide, *Pereskia bleo*, plant extract

INTRODUCTION

Aedes aegypti (L.) (Diptera: Culicidae) is the main vector of dengue virus causing dengue and dengue hemorrhagic fevers worldwide. In Thailand, the first dengue case and the first outbreak were reported in 1949 and 1958, respectively (Prasittisuk *et al*, 1998). Subsequently, the outbreaks of dengue fever have been regularly reported every year until the present time. Because there is no effective vaccine to prevent the disease, the preventions are to control the vector and avoid being bitten (WHO, 2009). In Thailand, temephos has widely been used as a larvicide to control *Aedes* larvae for a long time (Chareonviriyaphap *et al*, 1999). Although temephos has a very low toxicity to humans and can be added into a drinking water container (Rozendaal, 1997), high-dose usage might negatively effect humans and other non-targeted organisms. In addition, its

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continuous application might lead to an insecticide resistance of the mosquito vectors (Jirakanjanakit *et al*, 2007; Sornpeng *et al*, 2009). These adverse effects create a significant problem for *Ae. aegypti* control. Therefore, there is a need to find alternative substances for replacement or substitution of chemical use that are less toxic to humans and other organisms.

Currently, bio-insecticide alternatives are focused on plant extracts. The larvicide-effective plant extracts against *Ae. aegypti* vector have been reported worldwide. For example, crude extracts of *Albizia polyantha*, *Ocotea velloziana*, *Tabebuia avellanedae*, *Terminalia fagifolia*, *Vitex cymosa* and *Zanthoxylum* sp (Garcez *et al*, 2009), *Acalypha indica* and *Achyranthes aspera* (Kamalakannan *et al*, 2011) and *Citrus paradisi* (Ivoke *et al*, 2013) were reported for their larvicidal activity, demonstrating LC_{50} values ranging between 210.94 and 875.38 ppm. In Thailand, the finding of high larvicidal activity against *Ae. aegypti* was reported in 2006 in a study of ethanolic extract screening of 112 medical plant species of southern Thailand. Fourteen plants were found to have high activities with LC_{50} values less than 100 ppm, and two of them, *Mammea siamensis* and *Anacardium occidentale,* provided very high efficacy, with 5.9 and 9.1 ppm of LC_{50} values after 24-hour exposure time, respectively (Promsiri *et al*, 2006). Sutthanont *et al* (2010) reported larvicidal activity of the essential oils of 5 edible plants: *Citrus hystrix*, *Citrus reticulata*, *Kaempferia galanga*, *Syzygium aromaticum* and *Zingiber zerumbet* with the LC_{50} values of 30.07, 15.42, 53.64, 124.69, and 48.88 ppm, respectively. Recently, *Amomum biflorum* and *Zingiber cassumunar* oils were proved to have the LT_{50} values of 1.4 minutes and 100% mortality rate at 5 and 10 minutes, respectively (Phukerd and Soonwera, 2013). From previous studies, Thailand has the opportunity to provide a source of plants containing mosquito larvicides.

Pereskia bleo (Kunth) DC. belongs to the Cactaceae botanical family. The plant is a spiny shrub that can range from 2 to 8 meters. It has orange-red flowers and waxy hemispherical yellow fruits (Sim *et al*, 2010). In Malaysia, *P. bleo* has been used traditionally for the treatment of various ailments, including cancer, high blood pressure, diabetes, gastric pain and ulcers (Malek *et al*, 2009). The leaves were eaten raw or taken as a concoction brewed from the dried plant (Tan *et al*, 2005). The biological activities of *P. bleo* leaf extracts were reported. The cytotoxicity against various cancer cell lines has been reported (Tan *et al*, 2005; Malek *et al*, 2009). The anti-proliferative effect against mouse mammary cancer cell line and normal mouse fibroblast cell line has also been described (Er *et al*, 2007). Wahab *et al* (2009) reported antibacterial activity against gram-negative bacteria. Recently, the antioxidant activity from this plant was also described (Wahab *et al*, 2009; Sim *et al*, 2010). From this literature, several biological activities of *P. bleo* have been studied, but the insecticidal effect against any vectors, especially mosquito, has not been reported yet, to our knowledge.

Our preliminary study suggested that the aqueous leaf extract did not show any larvicidal activity against *Ae. aegypti* mosquito, while the fruit endocarp likely showed promising results. Therefore, the purpose of this study was to evaluate the larvicidal efficacy against *Ae. aegypti* mosquito vector of aqueous and ethanolic of crude and fractionated extracts of *P. bleo* fruit endocarp. In this study, the finding of valuable alternative biological activity of *P. bleo* was reported.

MATERIALS AND METHODS

Plant material collection and crude extracts preparation

Fresh fruits of *P. bleo* were obtained from an area of Mueang District of Phitsanulok Province, Thailand. A voucher specimen was deposited at Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand. The fruits were cleaned with tap water and then air-dried at room temperature. The seeds were removed, leaving only endocarps. The *P. bleo* fruit endocarps (5 kg) were air-dried in a hot air oven at 45°C for 3 days. After entirely being dried, they were ground into powder by using an electric blender (Single Speed Blender 800G; MRC, Holon, Israel) at 22,000 rpm. The dried powder (295.45 g) was sequentially extracted with ethanol and distilled water in a ratio of 1:10 (powder:solvent). Ten grams of powder were suspended into 100 ml of the solvent in 250-ml Erlenmeyer flask, and then continuously stirred at 180 rpm for 24 hours on a rotary shaker (Thermo Fisher Scientific, New Brunswick Scientific Innova 2300™; Waltham, MA) at room temperature. After that, the extracted suspension was filtered with a Whatman N° 1 filter paper. The residue will be then extracted with the next solvent as described above. An ethanolic crude extract was concentrated by using a rotary evaporator (Büchi® Rotavapor R-205 with Büchi® Vac V-500; Büchi Labortechnik AG, Flawil, Switzerland), while an aqueous crude extract was concentrated by using a rotary evaporator, and then completely dried by a lyophilizer (LTE Scientific Lyotrap LF/LYO/01/1; Fisher Scientific Loughborough, UK). The crude extract yields were 74.67 and 85.26 g for aqueous and ethanolic extraction, respectively. The resulting crude extract was kept in a desiccator (Schott Duran® Desiccator; DURAN Group, Mainz, Germany), until it was required for a bioassay.

Column chromatography fractionated extraction

The aqueous (60.0 g) and ethanolic (70.0 g) extracts were fractionated by quick column chromatography (60 mm Pyrex® Buchner Filter Funnel; Sigma-Aldrich, St Louis, MO) with 250 g Silica gel 60, P/N 1.07729.5000 <0.063 mm, 250 g; Merck Chemicals, Hessen, Germany). For the aqueous extract, a gradient solvent system of CH_2Cl_2, CH_2Cl_2-MeOH and MeOH-H_2O with increasing amounts of the more polar solvent was used for fractionation. The eluted substances were examined using Thin Layer Chromatography (TLC Silica gel 60 F_{254}, P/N 1.05554.0001; Merck Chemicals, Hessen, Germany). Fifty fractions were obtained and clustered into four groups of eluting fractions: A-Gr1, fractions 1-20 (10.42 g), A-Gr2, fractions 21-34 (4.71 g), A-Gr3, fractions 35-43 (3.75 g) and A-Gr4, fractions 44-50 (23.22 g). For the ethanolic extract, a gradient solvent system of CH_2Cl_2, CH_2Cl_2-MeOH, and MeOH with increasing amounts of the more polar solvent was used. After the TLC examination, 87 fractions were obtained. They were clustered into 5 groups of eluting fractions: E-Gr1, fractions 1-4 (2.23 g); E-Gr2, fractions 5-19 (10.96 g); E-Gr3, fractions 20-32 (1.73 g); E-Gr4, fractions 33-40 (16.20 g) and E-Gr5, fractions 41-87 (11.22 g).

Mosquito collection and colonization

Aedes larvae were collected from breeding places in Mueang District of Phitsanulok Province, Thailand using a plastic pipette and then kept in a plastic bottle. The collected larvae were transferred to the same laboratory as mentioned above. They were reared in a plastic tray filled with 2 liters of tap water. During the rearing pro-

cess, a laboratory condition was set up to 25±2°C with 10:14 (L:D) photoperiod. The dog biscuits (Pedigree® Adult Complete Nutrition; Mars Petcare, Melton Mowbray, UK) were crushed into powder, and then fed to the larval rearing tray as food. The larvae were reared until they pupated. The pupae were transferred into a small plastic cup filled with tap water and covered with a net. They were reared until turn to adults. The adults were individually transferred into a clean and dry plastic cups covered with a net. After that, 2 to 3-day-old adults were morphologically identified following the illustrated keys of the mosquitoes in Thailand (Rattanarithikul *et al*, 2010).

The identified *Ae. aegypti* mosquitoes were transferred into the same mosquito cage (30x30x30 cm) and provided with 5% sugar mixed with 5% multivitamin syrup solution [Seven Seas® Multi-Vitamin Syrup; Olic (Thailand), Ayutthaya, Thailand]. Five to 7-day-old females were permitted to feed on a blood meal by using an artificial membrane feeding method (Rutledge *et al*, 1964). After the blood fed females became gravid (3-4 days after blood meal), they were allowed to lay eggs on a wet filter paper (Whatman Nº 1). Those eggs were air-dried for 3 days, and then kept in a humidity controlling glass jar until being required. A colony of *Ae. aegypti* was established for producing the larval material for the further experiments.

Larvicidal bioassay

Ae. aegypti larvae were tested for a larvicidal activity of the *P. bleo* extracts following the protocol of WHO (2005). Briefly, for aqueous extracts, a stock solution (1% w/v) was prepared by weighing 200 mg of the extract and adding 20 ml of distilled water. For ethanolic extracts, the distilled water was replaced by a dimethylsulphoxide (DMSO). The stock solutions were kept in screw-cap vials in a refrigerator (4ºC). Series of concentration (100-1,000 ppm) were prepared for the larvicidal activity testing. After that, 200 ml of various concentrations of each extract was put into a plastic bowl, and then twenty-five 3rd instar larvae of *Ae. aegypti* were transferred into the solutions. Mortality rates were determined after 24- and 48-hour exposure times. The experiments were performed in four replicates, with 100 larvae for each concentration of each crude or fractionated group extract. An equal number of controls was set up simultaneously with 200 ml of distilled water alone for the aqueous extract, and 2 ml of DMSO in 198 ml distilled water for the ethanolic extract.

Data analysis

Data from all replicates of each crude and fractionated group extracts were pooled for analysis. The mortality data was subjected to the Probit analysis for the 50% lethal concentration (LC_{50}) value determination (Finney, 1971) using the commercial LdP Line® software (Plant Protection Research Institute, Cairo, Egypt). The 95% confidence intervals (CI) of upper and lower fiducial limits were also determined.

RESULTS

To gain some insight of the activity of the crude and fractionated *P. bleo* extracts against the 3rd instar larvae of *Ae. aegypti*, the mortality rate observation was extended to 48 hours after exposure. We found that the 48-hour activity was higher than the 24-hour in both crude and fractionated of aqueous and ethanolic extracts tested in the experiments (Table 1), and then the results indicated a slow action of the *P. bleo* extracts. Based on the crude extract activity analysis, the ethanolic extract showed stronger activity than the aqueous one

Table 1
24- and 48-hour larvicidal activities of crude and fractionated group extracts of *P. bleo* against the 3rd instar larvae *Ae. aegypti*.

P. bleo extracts	24-hours			48-hours		
	LC_{50} with fiducial limits (ppm)	Parameters		LC_{50} with fiducial limits (ppm)	Parameters	
		χ^2	Slope (±SE)		χ^2	Slope (±SE)
Aqueous extract[a]						
Crude extract	>2,000[b]	-	-	484.4 (426.36 - 551.17)	15.17	1.4706 ± 0.1457
A-Gr1	1,453.47[c] (1,111.66 - 2,470.98)	9.85	1.9427 ± 0.3602	704.03 (606.16 - 850.56)	13.09	1.8668 ± 0.2364
A-Gr2	1,150.15[c] (929.44 - 1,622.50)	12.99	1.8444 ± 0.2726	561.67 (433.71 - 762.96)	19.03	1.9155 ± 0.2271
A-Gr3	1,122.53[c] (940.84 - 1,505.32)	6.48	2.4147 ± 0.3710	656.02 (593.56 - 734.11)	13.16	2.8546 ± 0.2982
A-Gr4	>2,000[b]	-	-	>2,000[b]	-	-
Ethanolic extract[a]						
Crude extract	1,094.84[c] (1,004.09 - 1,682.03)	33.41	3.4789 ± 0.3498	416.50 (381.55 - 452.15)	3.17	2.3903 ± 0.1657
E-Gr1	>2,000[b]	-	-	905.00 (849.45 - 985.72)	5.15	6.2675 ± 0.7913
E-Gr2	1,759.12[c] (1,286.40 - 6,449.59)	0.69	4.0386 ± 1.2859	1,0042.99[c] (921.64 - 1,260.64)	3.79	3.4973 ± 0.4786
E-Gr3	707.94 (593.56 - 893.48)	5.07	1.5625 ± 0.2206	223.12 (178.64 - 263.87)	9.97	2.0312 ± 0.2285
E-Gr4	878.07 (777.85 - 1,297.90)	36.08	4.9724 ± 0.5827	449.81 (311.43 - 613.71)	35.70	2.3141 ± 0.2345
E-Gr5	830.89 (772.02 - 911.72)	5.38	4.6803 ± 0.5440	343.59 (294.13 - 392.06)	9.65	2.1403 ± 0.2191

[a] Control groups of all extracts testing were resulted with nil mortality.
[b] The LC_{50} was higher than 2,000 ppm, and then the parameters could not be calculated.
[c] The LC_{50} is estimated by the Probit analysis of *Ldp Line* software.

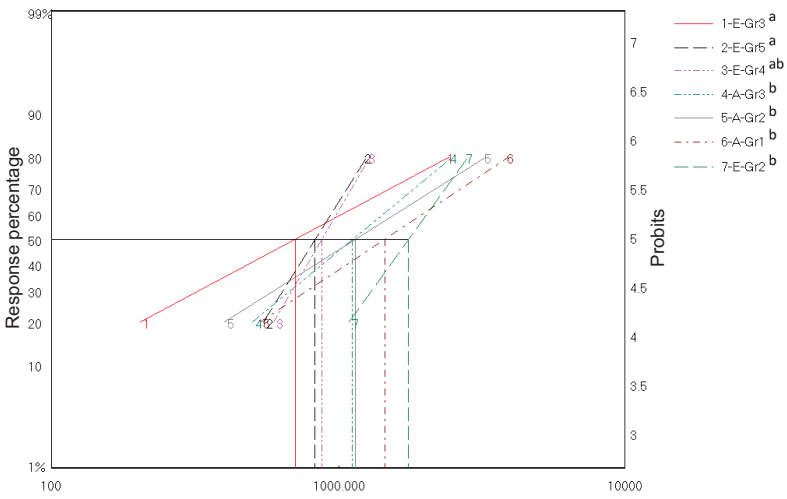

Fig 1–Graph showing the LC_{50} values of the aqueous and ethanolic fractionated group extracts on the 3rd instar *Ae. aegypti* at 24-hour detection time.

[1]A-Gr4 and E-Gr1 having >2,000 ppm of LC_{50} values are excluded. [2]Statistically significant differences are indicated by different letters on the fractionated groups (upper right).

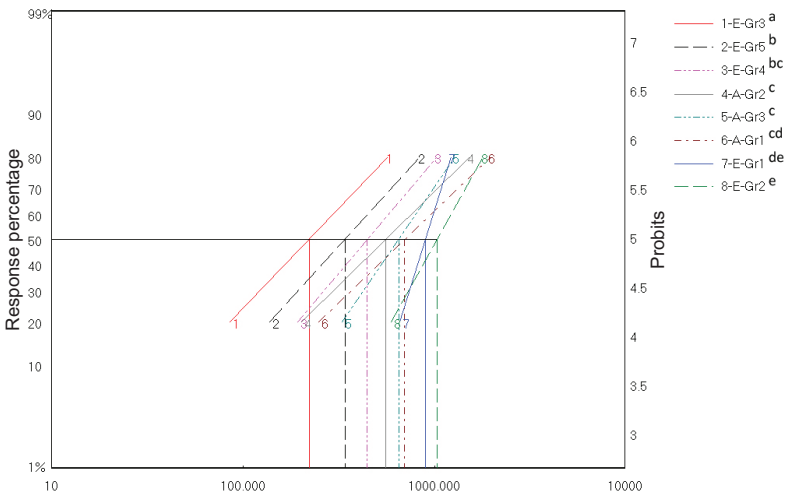

Fig 2–Graph showing the LC_{50} values of the aqueous and ethanolic fractionated group extracts on the 3rd instar *Ae. aegypti* at 48-hour detection time.

[1]A-Gr4 having >2,000 ppm of LC_{50} value is excluded. [2]Statistically significant differences are indicated by different letters on the fractionated groups (upper right).

did. Particularly in the 24-hour detection time, the LC_{50} value of ethanolic extract (1,094.84 ppm) was significantly lower than the aqueous one (>2,000 ppm), while the activity of 48-hour detection showed insignificant differences with 416.50 and 484.40 ppm, of ethanolic and aqueous extracts, respectively.

After the column chromatography fractionated extraction, 50 and 87 eluted fractions were obtained from the crude *P. bleo* aqueous and ethanolic extracts, respectively. For this experiment, the aqueous fractions were clustered into four groups (A-Gr1 to A-Gr4), while the ethanolic fractions were clustered into five groups (E-Gr1 to E-Gr5). After the bioassay, for 24-hour activity detection, three fractionated groups of the ethanolic extract, including E-Gr E-Gr3 (707.94 ppm), E-Gr5 (830.89 ppm) and E-Gr4 (878.07 ppm), showed higher larvicidal activity than the aqueous fractions (1,122.53 - 1,150.15 ppm). Moreover, both E-Gr3 and E-Gr5 exhibited significantly lower LC_{50} values than the aqueous extract fractions (Fig 1). For the 48-hour bioassay,

the ethanolic fractions still exhibited the higher activity than the aqueous extracts (Table 1). The most toxic activity against 3rd instar *Ae. aegypti* was found from the E-Gr3 fractionated group. The LC_{50} value of E-Gr3 (223.12 ppm) was significantly lower than the LC_{50} values of all aqueous or ethanolic fractionated groups (Fig 2). The 24- and 48-hour bioassays revealed that the most active larvicidal compounds of *P. bleo* extract should be contained in the E-Gr3 ethanolic fractionated group.

DISCUSSION

From the previous reports, the bioassay studies of *P. bleo* were mainly on the medical purposes including cytotoxicity, anti-proliferative, antibacterial, or antioxidant activities (Tan *et al*, 2005; Er *et al*, 2007; Malek *et al*, 2009; Wahab *et al*, 2009; Sim *et al*, 2010). All of the reports from Malaysia were studies on the leave or stem extract of the plant. Other parts of *P. bleo* have never been studied and the mosquito larvicidal activity has never been reported. Our study revealed the promising larvicidal ability of the ethanolic *P. bleo* extract that showed the higher activity than the aqueous extracts (crude or fractionated groups). The bioassay results of the ethanolic extracts indicated that the larvicidal activity was not a synergistic action of all compounds of the extract. The active substances of the ethanolic extract were found in the E-Gr3 fractionated group, which indicated the significantly lowest LC_{50} value, especially at the 48-hour detection time.

For the studies of Cactaceae plants, some research relevant to insecticidal activity has been reported. In 2005, methanolic extract of root and aerial parts of *Myrtillocactus geometrizans* was found to have larvicidal activity against *Spodoptera frugiperda* and *Tenebrio molitor* insects, with lethal doses between 100 and 200 ppm (Cespedes *et al*, 2005). Although the orders (Lepidoptera and Coleoptera) of responded insects are not related to the Diptera insect in this study, the positive result indicated the possibility of the Cactaceae plant cluster in providing the insecticidal activity. The activity of Cactaceae plants against the Diptera insects were afterward reported from *Opuntia vulgaris*. Ethanolic extract *O. vulgaris* fruit rinds were tested for the larvicidal activity against the malaria vector of Kenya, *Anopheles pharoensis* (Diptera: Culicidae), and the house flies, *Musca domestica* (Diptera: Muscidae). It was found that 10% mortality rate at 1,000 ppm concentration was found from the testing against the closely related species of the mosquito of this study, *An. pharoensis* (Mansour *et al*, 2010), while non-effective to *M. domestica* larvae with nil mortality was reported (Mansour *et al*, 2011). Compare to Mansour *et al* (2010), the better activity against *Ae. aegypti* in our study was found from *P. bleo* ethanolic extracts with 50% mortality rates at 1,094.84 ppm and 707.94 ppm of crude and E-Gr3 fractionated group extracts, respectively.

Compared to out-group of Cactaceae plant extracts, *P. bleo* showed lower activity of the mosquito larvicide. However, *P. bleo* has been traditionally used for treatment of various ailments for Malaysians (Malek *et al*, 2009), and some Malaysian natives eat the leaves raw as a vegetable (Tan *et al*, 2005). It then could guarantee the edible safety of *P. bleo*. Although the study of the fruit has never been reported, the reports of the leave or stem could assume the edible safety of this fruit. The using of *P. bleo* fruit endocarp extract in high concentration might, therefore, be safer for human than the low concentration from inedible

plant extracts. However, the greatly higher activity of the *P. bleo* ethanolic extracts was found after 48 hours of exposure time. The LC_{50} values of the crude and E-Gr3 extracts were reduced to 416.50 ppm (from 1,094.84 ppm) and 223.12 ppm (from 707.94 ppm), respectively.

Comparable findings that the 48-hour LC_{50} value was lower than the 24-hour were reported from the ethanolic extracts of some Thai medical plants against *Ae. aegypti* larvae (Lapcharoen *et al*, 2005). The LC_{50} values of *Pueraria mirifica*, *Butea superba* and *Thevetia peruviana* extracts were reduced from 889.33, 1,156.68 and 346.42 ppm (24 hours) to 317.63, 496.23 and 31.98 ppm (48 hours), respectively. Moreover, similar results were reported in 2006 from the study of 14 Thai plant extracts (Promsiri *et al*, 2006). A slightly reduction of the LC_{50} values was reported from all extracts after extending the exposure time to 48 hours. From total 14 plant extracts, 5 (*Anethum graveolens*, *Anacardium occidentale*, *Costus speciosus*, *Mammea siamensis* and *Phyllanthus pulcher*) showed significant reduction of the LC_{50} values.

In conclusion, the bioassay result of this study indicated the larvicidal property against *Ae. aegypti* mosquito of the *P. bleo* fruit endocarp ethanolic extracts, especially for the E-Gr3 fractionated group. Although the LC_{50} values at post 24-hour exposure time were slightly high, however, better activities were found at 48-hour extended exposure. From the results, the fractionated groups showed the significantly higher activity than the crude. Future research to extract a pure compound of the active fractionated group should be explored to find a new highly efficient larvicidal substance. The study of effects on non-target organisms including human are also necessary. Additionally, the action on other mosquito vectors including *Culex* sp, the vector of filariasis, and *Anopheles* sp, the vector of malaria, should be further investigated.

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