MICROBIOLOGICAL CONTENTS AND ANTIBACTERIAL POTENTIAL OF *ORTHOSIPHON STAMINEUS BENTH* AT DIFFERENT POSTHARVEST MATURITY STAGES GROWN ON PEAT SOIL

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ABSTRACT

Orthosiphon stamineus Benth or misai kucing is widely used in Malaysia and the Southeast Asia as medicinal herb known for its health benefits. In this study, O. stamineus was planted and harvested by following Malaysian Good Agricultural Practices (MyGAP) at peat soil field plot at MARDI Station Pontian, Johor. O. stamineus was harvested at different maturity stages of 8, 10, 12 and 14 weeks after planting. In the preliminary studies, a total of 24 samples of stems and leaves parts of O. stamineus were dried and subjected to microbiological analysis to evaluate possible source of contamination. Subsequently, 56 samples of O. stamineus methanolic extracts (stems and leaves parts) were investigated by using the disc diffusion method and Minimum Inhibitory Concentration (MIC) against Escherichia coli, Vibrio parahaemolyticus, Salmonella Typhimurium, Staphylococcus aureus, Bacillus cereus and Listeria monocytogenes, respectively. Satisfactory microbiological results have been shown during all postharvest maturity stages at the Pontian plot, based on national and international herbal standards. Findings also exhibited greater inhibitory effects of O. stamineus extracts against the Gram-positive bacteria, Staphylococcus aureus and Gram negative bacteria, Vibrio parahaemolyticus. Interestingly, the leaves methanolic extracts showed better microbiological contents and antibacterial properties in week 10 of maturity stages, respectively. The strongest antibacterial activity was recorded by the leaves extracts of O. stamineus with the highest zone of inhibition of 13.4 mm and MIC value 1.56 mg/mL against S. aureus. The presence of rosmarinic acid compound found in the leaves extracts was believed to be the key predominant factor. The study indicated that O. stamineus Benth has the economic potential to be developed into natural antibacterial agents towards shelf life extension and food safety.

Key words: O. stamineus, misai kucing, microbiology, antibacterial, postharvest.

INTRODUCTION

There has been increasing interest for developing effective and natural preservation methods using plant extracts due to the human health concerns on the food containing synthetic preservatives (Shan *et al*., 2007). Numerous screening studies have reported medicinal herbs containing phenolic secondary metabolites and their antimicrobial activity i.e. cinnamon (Burt, 2004), coriander (Delaquis *et al*. 2002), clove (Daferera *et al*., 2000) and oregano (Marino *et al*., 2001). This unique molecular structures and mode of action between microbes and bioactive compounds may discover new effective substitutes for consumer health.

In Malaysia, *O. stamineus* Benth is also called as *misai kucing* or "cat whisker". *O. stamineus* was commonly used in the form of dried leaves as a beverage to treat several health treatments (Malahubban *et al*., 2013). It has huge potential as food preservative due to over than 20 chemically active phytochemicals that have been detected in this herb (Akowuah *et al*., 2004). Moreover, these phytochemical compounds have the ability to inhibit food pathogens and food spoilage microorganisms (Ray, 2001). Thus, the screening of microbiological properties and quality assessment of raw herbal *O. stamineus* is a priority towards the development of Standard Operating Procedure (SOP) for *O. stamineus* plantation as a key reference for target community of local agricultural entrepreneur.

More evaluation should be adopted in continuation efforts towards *O. stamineus* potential as antibacterial against multi-resistant bacteria. Noteworthy, several researchers have described *O. stamineus* in their studies (Malahubban *et al*., 2013; Himani *et al*., 2013: Jamal *et al*., 2011). Nevertheless, there is little published manuscript of *O. stamineus* as antibacterial agents in Malaysia from different postharvest maturity stages especially grown on peat soil condition. Hence, the main objectives of this study were to determine the microbiological contents of *O. stamineus* Benth stems and leaves at different postharvest maturity stages grown on peat soil and to determine their antibacterial potential against six different types of pathogens.

MATERIALS AND METHODS

Plant materials

O. stamineus plant parts were collected from MARDI Station Pontian, Johor plantation plot. The powder of *O. stamineus* stems and leaves, respectively was the plant materials used. The experimental design was Randomized Complete Block. Harvesting of the aerial plants at four different maturity stages, that was 8, 10, 12 and 14 weeks. Harvested plants were weighed, washed and air–dried, before being placed in an oven at 50ºC until the moisture content dropped to 10%. Then, the powder was packed in air-tight opaque bottle, each holding 50 grams of powder, for further use.

Microbiology analysis

The preliminary work was focused on the microbiology analysis on dried stems and leaves at four different maturity stages grown on peat soil to evaluate possible source of contamination and quality control screening. For preparation of homogenized samples, 10 grams of samples were aseptically weighed and diluted with 90 ml Ringers (Oxoid, UK) solution. Then, samples were homogenized for 120 sec in a stomacher (Seward, UK) and serial dilutions 10^1 - 10^5 were prepared in sterile Ringers. The method from Wallace & Thomas (2001) was used with the following parameter; Total Plate Counts on Plate Count Agar (PCA) (Oxoid, UK), incubation at 37ºC for 48 h ± 2 h, Yeast & Mould Counts on Malt Extract Agar (MEA) (Oxoid, UK), incubation at 32ºC for 72 h ± 2 h, Coliform and *Escherichia coli* on Petrifilm (3M, USA), incubation at 37ºC for 24 - 48 h ± 2 h and *Staphylococcus aureus* using Baird Parker Agar (BPA) (Oxoid, UK), incubation at 37ºC for 48 h ± 2 h.

For *Salmonella*, 25 grams of samples were aseptically weighed and enriched with 225 ml of sterile Buffered Peptone Water (BPW) (Merck, Germany) and homogenized for 120 sec. The mixed solution was incubated at 37ºC for 24 h ± 2 h. One ml and 0.1 ml of enriched samples were transferred to 9 ml and 9.9 ml of Selenite Cystine (SC) (Merck, Germany) and Rappaport-Vassiliadis (RV) (Merck, Germany) broth, respectively before being incubated at 37ºC and 42ºC for 24 h ± 2 h, respectively. Subsequently, one loop from each enriched broth was streaked to Xylose Lysine Desoxycholate (XLD) (Merck, Germany), Xylose Lysine Tergitol-4 (XLT-4) (Oxoid, UK) and Rambach (RB) (Merck, Germany) before being incubated at 37ºC for 24 h ± 2 h. Typical colonies were considered as presumptive *Salmonella* according to BAM (2001) before purified to Nutrient Agar (NA) (Merck, Germany). Isolated colonies were confirmed via biochemical tests i.e. triple sugar iron, lysine iron, catalase, cytochrome oxidase, indole, motility (Merck, Germany).

Preparation of crude extracts

Five grams of dried *O. stamineus* were coarsely grounded with a grinder (IKA MF10 micro fine grinder, UK) and sieved with 0.25 mm mesh and then mixed with 150 ml of 70% methanol. The mixture was continuously shaken for 24 h at room temperature using an orbital shaker (Protech Model 720, Malaysia). The extract was then filtered under suction and evaporated using a rotary evaporator (Buchi R-215, Switzerland), later diluted to 10 ml final volume with 70% methyl alcohol and stored at 4ºC for further analysis.

Bacterial cultures

The bacterial species used in the experiment were *Escherichia coli* (ATCC 25922), *Vibrio parahaemolyticus* (NCTC 10885), *Salmonella* Typhimurium (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876) and *Listeria monocytogenes* (ATCC 7644). Each bacterial species were cultured in a Trypton Soy Broth (TSB) (OXOID, England) for 24 h ± 2 h.

Disc diffusion method

The antibacterial property of *O. stamineus* extracts was determined against six bacterial pathogens using disc diffusion method (Bauer *et al*., 1966) and according to National Committee for Clinical Technique Laboratory Standards (NCCLS, 2003). Fresh bacterial suspension was uniformly inoculated using sterile cotton swab, respectively on the surface of Mueller-Hinton agar (MHA) (Oxoid, England). The turbidity of bacterial suspension was adjusted having equivalent turbidity to 0.5 McFarland standard (approximately 1 x 10^6 cfu/ml).

Six mm diameter sterile blank paper discs (Oxoid, UK) were wetted with 20 ul methanolic extract of *O. stamineus* (20 mg/ml) and left to dry before being placed on the surface of inoculated agar plates. Lactic acid (5%), tetracycline (10 ug/disc) and chloramphenicol (30 ug/disc), respectively served as positive controls. The extract samples and standard control discs were placed on MHA and incubated at 37ºC for 24 h ± 2 h. The diameters of inhibition zone (mm) were recorded after 24 hrs. The test was done in thrice and the average diameter of the inhibition zones was calculated.

All samples for antibacterial test were measured according to the four different maturity stages and run in two batches (Batch 1 as harvested in 2016 and Batch 2 as harvested in 2017) from *O. stamineus* MARDI Station Pontian plot, respectively.

Minimum Inhibitory Concentration (MIC)

The MIC assay was performed only with the *O. stamineus* extracts that demonstrated strong inhibition against test bacteria in the disk diffusion method (inhibition zone ≥ 10 mm). The MIC values of the 70% methanolic extracts against test bacterial were based on a micro-well dilution method (Sahin *et al*., 2003) and NCCLS (2003). All tests were also performed in triplicate.

Each *O. stamineus* extracts was subjected to a serial twofold dilution via sterile nutrient broth to the sterile 96-well microplate. The antibacterial activity of *O. stamineus* extracts was tested using seven different concentrations (50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 mg/mL). Ten µL of bacterial suspension, approximately, corresponding to 0.5 McFarland standard, was added to each well and incubated at 37ºC for 18 - 20 h ± 2 h. Controls for bacterial growth were also included in the control wells. The higher dilution of the *O. stamineus* extracts that produced no bacterial growth was considered as the MIC of the extract.

RESULTS AND DISCUSSION

Microbiology contents

Twenty-four *O. stamineus* samples (3 replicate) from different postharvest maturity stages in MARDI Pontian, Johor have been randomly collected and analysed for microbiological contents. In this study, the results of all samples were compared with Malaysian Herbal Monograph (MHM, 2015) and European Herbal Infusion Association (EHIA, 2011) (Table 1). These are national and international standard of microbiological specification for herbs (raw material), respectively that being established to assist the quality and safety testing for herbal industrial sector in product preparations. For Total Plate Counts, MHM and EHIA stated bacterial contamination must be $<10^5$ cfu/g and $\leq 10^8$ cfu/g, respectively. In our study, all samples showed satisfactory Total Plate Counts results during postharvest maturity stages (Table 2) whereby lower than MHM and EHIA guidelines.

Table 1: Microbiology specification for herbs (raw material) based on Malaysian Herbal Monograph (MHM) and European Herbal Infusions Association (EHIA) standards

Standards	Total Plate Counts, cfu/g	Yeast and Mould Counts, cfu/g	Coliform, cfu/g	*Escherichia coli*, cfu/g	*Staphylococcus aureus*, cfu/g	*Salmonella* (presumptive in 25g)
MHM	$<10^5$	$<10^4$	Nil	Absent in 1g	Absent 1g	Absent in 25g
EHIA	$\leq10^8$	$\leq10^6$	Nil	$\leq10^4$	Nil	Absent in 25g

Table 2: Microbiology contents for O. stamineus at different postharvest maturity stages at MARDI Pontian plot

Maturity Stages	Plant Parts	Total Plate Counts, cfu/g	Yeast and Mould Counts, cfu/g	Coliform, cfu/g	*Escherichia coli*, cfu/g	*Staphylococcus aureus*, cfu/g	*Salmonella* (presumptive in 25g)
Week 8	Leaves	4.2×10^4	$1.6Y \times 10^3$	7.1×10^3	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
	Stems	2.1×10^4	$1.4Y \times 10^3$	8.9×10^3	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
Week 10	Leaves	6.7×10^3	$2.1Y \times 10^2$	6.7×10^2	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
	Stems	4.8×10^4	$3.8Y \times 10^3$	9.1×10^3	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
Week 12	Leaves	7.1×10^4	$3.5Y \times 10^3$	4.2×10^3	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
	Stems	7.0×10^4	$6.7Y \times 10^3$	6.7×10^4	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
Week 14	Leaves	7.6×10^4	$7.1Y \times 10^3$	4.8×10^4	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g
	Stems	9.2×10^4	$8.8Y \times 10^3$	2.6×10^5	$<1 \times 10^2$	$<1 \times 10^2$	Absent in 25g

Note: Y = Yeast Counts

For Yeast & Mould Counts, MHM and EHIA stated yeast & mould contamination must be $<10^4$ cfu/g and $\leq 10^6$ cfu/g respectively. Overall, this study showed good microbiological results for yeast & mould with results $<10^4$ cfu/g in all samples during all *O. stamineus* maturity stages. For Coliforms, our findings indicated results with the range of $10^2 - 10^5$ cfu/g for *O. stamineus* even though there were no apparent criteria for Coliform in MHM and EHIA. It was thought that it was natural for herbal raw material exposed to slight contamination due to environment factor. However, we can conclude that all samples were in good condition with the absence of *Escherichia coli* pathogen along the postharvest maturity stages in all plant parts. Based on MHM and EHIA guidelines, *E. coli* must be absence in 1 g and $\leq 10^4$ cfu/g, respectively.

From other pathogenic perspective, our study showed $<1 \times 10^2$ cfu/g for *Staphylococcus aureus* which in agreement with MHM standard that require absence in 1 g of *S. aureus*, even though EHIA does not state any requirement for this pathogen. For *Salmonella* spp, our results showed absence in 25 g in all samples along week 8, 10, 12 and 14 of maturity stages. This was in line with MHM and EHIA that strictly require absence of *Salmonella* in herbal due to its serious food safety concern. In general, all samples showed satisfactory results with no safety issue and within allowable range for spoilage microorganisms. Traditionally, *O. stamineus* end products are normally involve heat treatment during preparation such as herbal tea making it safe for consumption.

Interestingly, the results for Total Plate Counts and Yeast & Mould Counts including Coliform for *O. stamineus* from leaves part highlighted reduction in 1 log count at postharvest maturity stages of week 10. In contrast, the microbiological range (cfu/g) for stems part showed consistent increase from week 8 to 14, even though still within allowable level of MHM and EHIA guideline. Phytochemical was thought to contribute to this effect when rosmarinic acid was found optimum at week 10 from leaves part of *O. stamineus* by Noor Ismawaty *et al*. (2015) (42 mg/g) whereby support the potentially antibacterial activities of *O. stamineus* medicinal herb.

Antibacterial potential of *O. stamineus* extracts

A total 56 samples of *O. stamineus* methanolic extracts were tested at concentration of 20 mg/disc in disc diffusion and MIC assay for their antibacterial properties against 6 types of bacteria. For disc diffusion test, the diameters of the inhibition zones obtained from *O. stamineus* leaves and stems extracts are presented in Table 3 and 4, respectively. It was shown that *O. stamineus* leaves extracts exhibited antibacterial activity against *S. aureus* and *V. parahaemolyticus* with inhibition zones more than 8 mm, respectively from week 8 to 14 of *O. stamineus* maturity stages (Table 3). The inhibition zones observed with *O. stamineus* extracts were comparable to the inhibition seen with the food preservative lactic acid, while tetracycline and chloramphenicol showed equal strong antibacterial effect.

Table 3: Inhibition zone indicating the antibacterial activities of methanolic leaves extracts of *O. stamineus* Benth

O. stamineus Benth /Test Bacteria	Zone of inhibition (mm)																			
	Leaves (Pontian)																			
	Week 8					Week 10					Week 12					Week 14				
	B1	B2	Te	Chl	LA	B1	B2	Te	Chl	LA	B1	B2	Te	Chl	LA	B1	B2	Te	Chl	LA
Gram positive																				
B. cereus	+	+	+++	+++	++	+	+	+++	+++	++	+	+	+++	+++	++	+	+	+++	+++	++
S. aureus	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	++	++	+++	+++	++
L. monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram negative																				
E. coli	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++
S. Typhimurium	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++
V. parahaemolyticus	++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+++	++	++	+++	+++	++

Key: (-): no inhibition; (+): weak inhibition (<8 mm); (++): modest inhibition (8 mm<x<10 mm); (+++): strong inhibition (>10 mm); all readings were inclusive of 6 mm disc diameter.

Legends: B1: Sample extracts Batch 1 (harvested in 2016); B2: Sample extracts from Batch 2 (harvested in 2017); Te: Tetracycline (positive control); Chl: Chloramphenicol (positive control); LA: 5% Lactic Acid (positive control).

Table 4: Inhibition zone indicating the antibacterial activities of methanolic stems extracts of *O. stamineus* Benth

Sample/Bacteria	*O. stamineus* Benth																			
	Stems (Pontian)																			
	Week 8					Week 10					Week 12					Week 14				
	B1	B2	Te	Chl	LA	B1	B2	Te	Chl	LA	B1	B2	Te	Chl	LA	B1	B2	Te	Chl	LA
Gram positive																				
B. cereus	++	++	+++	+++	++	++	++	+++	+++	++	+	+	+++	+++	++	+	+	+++	+++	++
S. aureus	+	+	+++	+++	++	++	++	+++	+++	+++	+	+	+++	+++	++	+	+	+++	+++	++
L. monocytogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram negative																				
E. coli	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++
S. Typhimurium	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++	-	-	+++	+++	++
V. parahaemolyticus	++	++	+++	+++	++	++	++	+++	+++	++	+	+	+++	+++	++	+	+	+++	+++	++

Key: (-): no inhibition; (+): weak inhibition (<8 mm); (++): modest inhibition (8 mm<x<10 mm); (+++): strong inhibition (>10 mm); all readings were inclusive of 6 mm disc diameter.

Legends: B1: Sample extracts Batch 1 (2016); B2: Sample extracts from Batch 2 (2017); Te: Tetracycline (positive control); Chl: Chloramphenicol (positive control); LA: 5% Lactic Acid (positive control).

Strong inhibition against *S. aureus* and *V. parahaemolyticus* with inhibition zones more than 10 mm shown at week 10 was observed from *O. stamineus* leaves extracts. On the other hand, *E. coli*, *S.* Typhimurium and *L. monocytogenes* exhibited strong resistance to *O. stamineus* leaves extracts, while *B. cereus* showed antibacterial activities with inhibition zones less than 8 mm to all leaves extracts. This is confirmed by the findings of Olah *et al* (2003); Chung *et al* (1999), which identified leaves with the main components of pharmacologically active polyphenols and antioxidants, respectively. A report by Malahubban *et al* (2013) similarly indicated *S. aureus* and *B. cereus* were more sensitive to *O. stamineus* methanolic leaves extracts.

Findings also indicated that *O. stamineus* stems extracts shown antibacterial activity against *S. aureus* only at week 10, whereas *V. parahaemolyticus* displayed antibacterial activity at week 8 and 10 with inhibition zones more than 8 mm, respectively (Table 4). In addition, *E. coli*, *S.* Typhimurium and *L. monocytogenes* showed strong resistance to all stems extracts, while *B. cereus* showed antibacterial activities with inhibition zones less than 8 mm. In general, all methanolic extracts of *O. stamineus* exhibited at least some degree of bacterial growth inhibition against *S. aureus*, *V. parahaemolyticus* and *B. cereus* but not *E. coli*, *S. Typhimurium* and *L. monocytogenes*. The bacterial tolerance to *O. stamineus* extracts could be to the presence of chemical components (Cowan, 1999) but the efficacy may varies depends on plant parts and maturity stages.

Among the investigated extracts, *S. aureus* (bacterium that normally causes skin infections and food poisoning in humans) was found to be the most susceptible to *O. stamineus* leaves extracts (at week 10) followed by *V. parahaemolyticus* (bacteria that commonly associated with gastrointestinal illness from consuming undercooked seafood). The primary polyphenol (rosmarinic acid) in *O. stamineus* leaves was likely responsible for the antibacterial activity. The findings of this study are consistent with Ho *et al* (2010); Nissar *et al* (2017) that summarized the antibacterial properties of *O. stamineus* and *O. aristatus* methanolic extracts, respectively against foodborne pathogens. Antibacterial activity of other medicinal plant leaves extracts such as *Barleria lupulina*, has been previously reported (Moin *et al*. 2012).

Lastly, Table 5 revealed the MIC results with special focus on *S. aureus* and *V. parahaemolyticus* antibacterial efficacy at week 10 of postharvest maturity stages of *O. stamineus*. This selection was due to the high inhibition zones showed by *S. aureus* with batch 1 (12.2 mm); batch 2 (14.6 mm); average (13.4 mm) and *V. parahaemolyticus* with batch 1 (11.0 mm); batch 2 (10.5 mm); average (10.8 mm), respectively at week 10. Based on the lowest MIC values given, *S. aureus* shown to be the most susceptible with *O. stamineus* leaves extracts with MIC values of 1.56 mg/mL for batch 1 and batch 2, respectively. Moderate antibacterial activity was shown by same methanolic leaves extracts against *V. parahaemolyticus* with MIC values of 3.13 mg/mL for batch 1 and 2, respectively (Table 5). Observation of the present study was supported by the previous work by Alshawsh *et al* (2012); Ho *et al* (2010). Thus, further work is required to explore more high-value-potential of *O. stamineus* leaves extracts for the development of antimicrobial food preservatives. This is in line with Malaysia National Agro-Food Policy which promotes the exploration of local herbs for nutraceuticals and phytomedicine that will subsequently create more income and business value for agro-food entrepreneurs (Ministry of Agriculture and Food Industries Malaysia, Dasar Agromakanan Negara, 2011-2020).

Table 5: Minimum inhibitory concentration (MIC) of *O. stamineus* Benth leaves extracts on *S. aureus* and *V. parahaemolyticus*

Sample and controls	Notes	*S. aureus*		*V. parahaemolyticus*	
O. stamineus Benth	Methanolic (70%)	MIC (mg/mL)		MIC (mg/mL)	
	Batch 1	B1	B2	B1	B2
	Leaves (week 10)	1.56	1.56	3.13	3.13
Te (10ug)	Control 1	0.78	0.78	0.78	0.78
Chl (30ug)	Control 2	0.78	0.78	0.78	0.78
LA (5%)	Control 3	1.56	1.56	1.56	1.56
O. stamineus Benth	Inhibition zones (mm)	B1 = 12.2, B2 = 14.6, Ave = 13.4		B1 = 11.0, B2 = 10.5, Ave = 10.8	

Legends: B1: Sample extracts Batch 1 (harvested in 2016); B2: Sample extracts from Batch 2 (harvested in 2017);
Te: Tetracycline (positive control); Chl: Chloramphenicol (positive control); LA: 5% Lactic Acid (positive control).

CONCLUSION

In conclusion, this study indicated the microbiology contents and antibacterial potential of *O. stamineus* Benth at different postharvest maturity stages grown on peat soil. Based on the current national and international standard, the microbiological quality of *O. stamineus* was in satisfactory condition with no safety issue at all postharvest maturity stages. Moreover, the *O. stamineus* methanolic leaves extracts showed stronger antibacterial properties than stem extracts. *S. aureus* was found to be the most susceptible to *O. stamineus* leaves extracts based on the highest inhibition zones and lowest MIC values given, followed by *V. parahaemolyticus.* These findings warrant technical database for *O. stamineus* Benth as local economic potential as green antibacterial preservative.

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