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Study on *Mentha pulegium* L. from M'rirt (Morocco): Antibacterial and antifungal activities of a pulegone-rich essential oil

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ABSTRACT

Pennyroyal or Mentha pulegium L. is a species from Lamiaceae family which is extremely rich in aromatic essence. Thanks to its medicinal properties, this species is used for various purposes. In the present work, we have carried out a study on chemical composition and evaluation of antimicrobial activity and antifungal activity of its essential oil. To achieve this aim, essential oil was first extracted by hydrodistillation from bloomy tops of Mentha pulegium L. harvested in the wild during the month of July 2014 in M'rirt. This extraction yielded about 5.2 ± 0.25% of essential oils (EOs). Then, EO was analyzed by gas chromatography coupled with mass spectrometry (GC-MS). Results of this analysis showed that it contains two major compounds: the first one is " pulegone" and the second is "piperitenone". Both, these molecules represent 98.01% of the whole identified compounds. Antibacterial activity of this EO was assessed against four bacterial strains by disc-diffusion method on agar medium and macrodilution method in liquid medium. Results showed that EO of Mentha pulegium L. has a very significant antibacterial activity in a liquid medium towards Escherichla coli and Staphylococcus aureus with respectively 1.4 µl/ml and 2.8µl/ml as minimal inhibitory concentrations. Evaluation of antifungal activity revealed that this essential oil is able inhibit mycelial growth as well as sporulation of the three fungal species tested (Aspergillus sp., Penicillium sp. and Rhizopus sp.) at low concentrations. In conclusion, essential oil of pennyroyal was very active. Indeed, it was endowed with a relatively interesting antibacterial activity and an excellent inhibitory potency on mycelial growth and sporulation of the tested fungi. Inhibitory effect of this essential oil suggests prospects of application in the field of foodstuffs conservation.

Keywords: *Mentha pulegium*, essential oil, antibacterial activity, antifungal activity.

INTRODUCTION

Therapeutic use of extraordinary virtues of medicinal and aromatic plants (MAP) to treat human diseases is a very old habit which evolves with mankind history. It has been now recognized that MAP constitute an important source of bioactive molecules. Many researches had proved that plants contain a wide variety of bioactive molecules that belong to different chemical classes. These bioactive agents characterized by very different physical and chemical properties [1] are endowed with a wide variety of biological activities. Despite evolution of science, diversity of these natural molecules that are not essential to plants viability remains a puzzle for researchers who keep trying to decipher their role in nature.

Recently, emergence of antibiotic-resistant bacteria has dramatically increased. Thus, to control these organisms, research for natural substances is of paramount importance [2].

Mentha genus gathers a group of aromatic and medicinal plants from Lamiaceae family. This genus consists of 20 worldwide-spread species including *Mentha pulegium* L.

Mentha pulegium L. which is originated from Asia, Middle East, Europe and North Africa [3], is found everywhere in Morocco and especially in humid places [4]. This plant is known by its english vernacular name "Pennyroyal" and arabic vernacular name 'Fliyou'. It is one of the main medicinal and aromatic plants used and marketed in Morocco in a form of essential oil whose production fluctuates significantly from year to year [5] According to several studies that have been conducted, *Mentha pulegium* L. is used to treat colds, sore throats, cough, bronchitis, lung infections and all kinds of cold. It is also an excellent digestive agent [6, 4, 7]. Dried leaves of this species rolled into cigarettes are smoked to relieve asthma [8]. The species has analgesic, antibacterial and antifungal effects due to its essential oil [9]. In addition, we have shown in our former work insecticidal power of EO from this species against Sitophilus oryzae (L.) [10].

This work is then proposed to study antibacterial of *Mentha pulegium* L.'s essential oil towards four bacterial strains that cause a major problem in hospitals. Antifungal activity of three molds responsible for rot of postharvest apples is also studied.

EXPERIMENTAL SECTION

2.1. Plant Material

Plant material consists of flowering tops of *Mentha pulegium* L. that had been harvested in M'rirt (Middle Atlas) in the month of July 2014. Aerial parts in bloom were dried at room temperature as they were sheltered from sunlight and humidity.

The species has been identified in the laboratory of botany and plant ecology at the Scientific Institute of Rabat (Morocco) by Professor M. IBN TATOU.

2.2. Extraction of essential oils

Extractions of essential oils were conducted by hydrodistillation using a Clevenger-type apparatus. These extractions were repeated three times to confirm the yield obtained by the used mode. Essential oils collected at the end of distillations were quantified (in ml per 100g of dry plant) and then introduced into dark bottles that were tightly closed to preserve EOs from heat and light [11]. Bottles were then kept in a refrigerator at 4 °C.

2.3. Analyses and identification of EOs chemical composition

Chromatographic analysis of EOs from aerial parts of *Mentha pulegium* L was performed using a Thermo Electron-type gas chromatograph (Trace GC Ultra) coupled with a Thermo Electron-type mass spectrometer (Trace MS system, Thermo Electron: Trace GC Ultra, Polaris Q MS). Fragmentation was performed by electron impact at 70 eV. Chromatograph is equipped with a DB-5 column (5% phenyl-methyl-siloxane) (30m x 0.25mm x 0,25µm film thickness), a flame ionization detector (FID) supplied by a gas mixture composed of H_2 and Air (H_2/Air). Column temperature is programmed to raise from 50 to 200 ° C for 5 min at 4 °C / min. Injection mode was split (leak report: 1/70, flow ml / min). Nitrogen was used as a carrier gas at a flow rate of 1ml / min.

Identification of chemical composition of *Mentha pulegium* L.'s EO was performed based on the comparison of their Kovats indices with those of standards compounds mentioned in literature [12, 13]. This task was supplemented by a comparison of indices and mass spectra with different references [13]. Kovats indices compare retention time of a compound with retention time of a linear alkane formed with the same carbon number. They are determined by injecting a mixture of alkanes (standard C_7-C_{40}) in the same operating conditions.

2.4. Evaluation of antimicrobial activity

2.4.1 Microorganisms

Microbial strains are composed of:

✓ Four bacterial strains:

Gram- bacteria: Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa) and Klebsiella pneumoniae (K. pneumoniae).

Gram+ bacterium: Staphylococcus aureus(S. aureus).

They come from the university hospital Hassan II of Fez and were stored at 4 °C until use in test tubes containing Mueller Hinton agar medium.

✓ Three fungal strains: Rhizopus sp., Penicillium sp. and Aspergillus sp.

These three fungal strains were chosen because of the considerable damage that they cause to stored apples. These strains are collected from rotten apples purchased in the market. Strains are regularly maintained onto nutrient PDA medium (Potato Dextrose Agar), and then stored in agar at 4 °C.

2.4. 2. Antibacterial process

2.4.2.1. Test of effectiveness

These tests were performed in vitro using 90 mm Petri dishes and disc-diffusion method on Mueller-Hinton agar [14]. To determine inhibition zone diameters, discs of 6 mm-diameter Whatman paper N°1 were cut and sterilized and then gently layered on the surface of inoculated MHA medium. Discs were then soaked with 2µl of EO. Inhibition diameters were measured around the disc after incubation at 37 °C for 24 hours. Each test was repeated three times to minimize experimental error. Antibiotic discs used for disc-diffusion tests are amoxicillin and imipenem.

2.4.2.2. Antibacterial tests: macrodilution in liquid medium

Macrodilution method in liquid medium was used to determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of essential oil [15]. An amount of essential oil was added to dimethylsulfoxide solution (DMSO) with 30/70 ratio (EO/DMSO: v/v). Then the whole solution was waved for a sufficient period of time to obtain a homogeneous solution, called mother solution: "S".

From this stock solution “S”, specific volumes were taken in order to have the following final concentrations: 0.18; 0.35; 0.70; 1.40; 2.80; 5.60; 11.20; 22.40 and 44.80 (µl/ml). Volumes from stock solution were aseptically added, in a series of test tubes containing each an initial volume in ml of Mueller Hinton sterile broth. Then, the set of tubes containing each 3.96 ml of the mixture “broth + EO”, were inoculated with 40 µl of a standardized inoculum of 10^8 CFU/ml. The final volume of the solution in each tube was equal to 4 ml. This inoculum was a suspension prepared in a sterile saline solution (0.9% NaCl). After incubation for 18 to 24 hours at 37 °C, MICs of EOs were determined. MIC corresponds to concentration of the first tube in which there is no growth of the tested germ visible with the naked eye. Then, in order to determine MBC, Mueller-Hinton agar (MHA) plates were inoculated with 100 µl of tubes content whose concentrations are superior or equal to MIC. MBC is the lowest concentration that completely inhibits bacterial growth in 24 hours. Moreover, MBC/MIC ratio of each bacterial strain was calculated to assess antibacterial power of this EO.

2.4.3. Evaluation of antifungal activity

2.4.3.1. Preparation of EOs dilutions

Antifungal screening is carried out using direct contact method described by Remmal et al. [16] and Satrani et al. [17].

A 0.2% agar solution in distilled water was sterilized at 120 °C for 15 min. An amount of essential oil was aseptically added to this agar solution that promotes contact between strains and compounds at 1/9 (v/v) ratio (EO/agar 0.2%). After that, the whole solution was waved for enough time to disperse essential oil compounds in the agar solution. Dilutions were prepared from this solution to obtain the following range: 1/10, 1/25, 1/50, 1/100, 1/200, 1/300 and 1/500 in agar solution.

Then, a volume of 1.5 ml from each dilution was aseptically added in test tubes containing each 13.5 ml of sterilized (and hot) PDA medium to obtain the following final concentrations of essential oil: 1/100, 1/250, 1/500, 1/1000, 1/2000, 1/3000 and 1/5000 (v / v) in PDA medium before cooling. Then, we have properly waved tubes before pouring their content into 90 mm-Petri dishes for solidification. Controls, containing culture medium and agar solution alone at 0.2% are also prepared.

2.4.3.2. Incoculation

After solidification of PDA medium with increasing concentrations of essential oil, wells were dug in the center of the Petri dishes. Then, ten microliters (10µl) from a suspension prepared with spores of seven-day fungal culture were added in wells. The whole was incubated at 25 °C in an incubator for seven days except Rhizopus sp. whose hyphae reached the edge of the control box in three days. All tests were repeated three times.

2.4.3.3. Evaluation of mycelial growth

Mycelial growth was monitored by measuring colony diameter along two perpendicular lines. Inhibition percentage of mycelial growth is calculated by the following formula:

$$\text{Inhibition percentage (I\%)} = \frac{Dt-De}{Dt} \times 100$$

Dt: Diametrical growth of the control
De: Diametrical growth of the fungus in presence of a precise concentration (c) of EO.

2.4.3.4. Evaluation of sporulation
To assess sporulation, a 1.3 mm-diameter piece is taken from a ten-day culture which was used to assess mycelial growth. This piece is placed in 20 ml of sterile distilled water and stirred a few minutes to release spores. Spores' counting is carried out using Malassez's cell and 10 counts were carried out per suspension. Only strains which induce lesions with diameter greater or equal to 1cm are considered pathogenic, the other strains are released and their sporulation potential is not considered.

Sporulation is expressed as the number of spores per surface unit (spores/cm^2). For each treatment, three repetitions were performed.

Inhibition percentage of sporulation in comparison to the control is calculated according to the following formula: $(\text{Is}\%) = \frac{\text{Nt}-\text{Nf}}{\text{Nt}} \times 100$

Nt: Estimated number of conidia in the control.
Nf: Estimated number of conidia in the presence of EO.

RESULTS AND DISCUSSION

3.1. Extraction yield and chemical composition of Mentha pulegium L.'s EO
Average yield of EOs extracted from *Mentha pulegium* L. (harvested in July 2014) is around 5.2%. This yield is expressed in milliliters for 100g of dry matter. This yield is higher than EOs extracted from the same species collected in November 2012 in Lavras, Brazil (2.54%) [18] and the species harvested in August 2014 in Reguiba, El-Oued in Algeria south-east (2.34%) [19]. Compared to the yield of pennyroyal harvested in Moroccan north (1.9%) [20] and the one obtained by Boughdad et al., 2011 [21] the yield obtained in our study is also higher . Variation observed concerning yield may depend on ecological, genetic and environmental factors.

Results of Gas chromatography coupled with mass spectrometry of EO are shown in **Table 1**. Separation technique based on GC-MS in combination with Kovats's Indice allowed us to identify 12 chemical structures equivalent to 99.787% of EO's compounds.

Table 1: Chemical composition of essential oil obtained from flowering parts of *Mentha pulegium* L

Monoterpenes		Sesquiterpenes	
Hydrocarbon (0 ,27%)	Oxygenated (99,39%)	Hydrocarbon (0,06%)	Oxygenated (0,06%)
α-pinène (0, 14%) β-pinène (0, 13%)	1,8- cinéole (0,10%) Menth-2-en-1-ol (trans-p-) (0,28%) Chrysanthenol(Cis) (0,80%) α –Terpinéol (0,10%) Trans-pulegol (0,06%) Pulegone (71,97%) Thymol (0,04%) Piperitenone (26,04%)	α -Guaiene (0,06%)	Himachal-4-en-1- β-ol (11- α H-) (0,06%)
Total	99,66%	0.12%	

These results indicate that essential oil of *Mentha pulegium* L. is mostly composed of monoterpenes (99.66%); abundance of oxygenated monoterpenes (99.39%) is marked by high percentages of pulegone (71.97%) and piperitenone (26.04%). Hydrocarbon monoterpenes (0.27%) in particular and sesquiterpenes (0.12%) in general are both in the minority in this essence. Essential oil of the same species *Mentha pulegium* L, analyzed by Silva et al. [18], is characterized by high percentages of pulegone (50.01%), menthol (31.90%) and menthone (16.56%). Ouakouak et al. [19] found that the species is dominated by pulegone (46.31%), piperitenone (23.3%) and menthone (6, 2%). We note that menthone is totally absent in our EO sample.

Moreover, Derwich et al. [22] highlighted other chemotypes whose major compounds were piperitone (35.56%) and piperitenone (21.18%). A low level of pulegone (6.42%) was found in these researches.

Generally, difference in chemical composition of essential oils depends on several factors, such as the species and its genetic material, the origin, phenological stage and environmental influences [23, 24, 25].

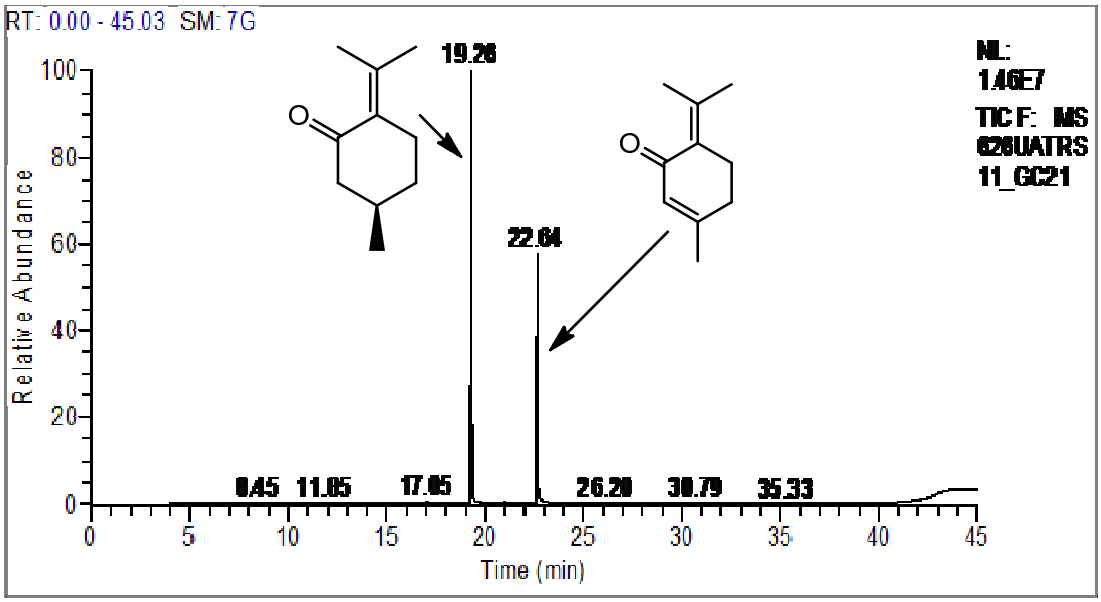

Figure 1: Chromatogram of *Mentha pulegium* L.'s EO

3.2. Antibacterial activity of the essential oil

3.2.1. Disc-diffusion method on agar

During our investigations, antimicrobial activity was evaluated using two methods: Disc-diffusion method on agar and dilution method in liquid medium. These methods were used to demonstrate inhibitory power of our essential oil sample towards selected bacteria after 24 hours of incubation at a suitable temperature of 37 °C. **Table 2** summarizes results of disc- diffusion method tests.

Table 2: Diameters of inhibition zones of four pathogenic strains by diffusion method on Mueller-Hinton Agar (MHA) after 24 hours of incubation at 37 °C

Bacterial strains	Diameter of inhibition zone in mm		
	M. pulegium 's EO	Imipenem	Amoxicillin
E. coli	18,00±0,40	26,00±0,00	6,00±0,00
P. aeruginosa	12,70±3,10	25,00±0,00	6,00±0,00
S. aureus	10,00±0,33	61,00±1,00	19,50±0,50
K. pneumoniae	10,00±0,50	28,00±1,00	6,00±0,00

With disc-diffusion method, results demonstrate that pennyroyal's essential oil is endowed with a relatively interesting antibacterial activity, despite the small concentration used (2µl), against the tested strains. Comparison of inhibition diameters due to essential oil and those of antibiotics (amoxicillin and imipenem) shows that essential oil caused inhibition zones smaller than imipenem. However, inhibition zones of essential oil are superior to amoxicillin inhibition zones except for S. aureus. The latter has a very noticeable susceptibility towards amoxicillin with an inhibition area around 19.50 ± 0.5 mm. this diameter is greater than inhibition zone caused by essential oil which is about 10.00 ± 0.5 mm.

E. coli strain was very susceptible to this essential oil with an inhibition zone around 18.00 ± 0.40 mm, it is followed by P. aeruginosa strain which was characterized by an inhibition zone of 12.70 ± 3.10 mm. For bacterial strains: S. aureus and K. pneumoniae, inhibition zones generated by essential oil are the same (10.00 ± 0.50 mm). In our conditions, these bacterial strains seem to be the most resistant ones to Mentha pulegium's EO.

As a conclusion, all tested bacterial strains are highly susceptible to imipenem, they are susceptible to Mentha pulegium's EO and very resistant to amoxicillin excluding S. aureus.

3.2.2. Dilution method in liquid medium

Results related to minimal inhibitory concentrations (MIC) determination, summarized in **Table 3**, show that MIC values vary according to the tested strain.

Table 3: Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of pulegium Mentha L.'s EO (in µl/ml)

Bacterial strains	*Mentha pulegium* L.		
	MIC	MBC	MBC/MIC
E. coli	1.40	2.80	2
P. aeruginosa	11.20	>44.80	-
S. aureus	2.80	5.60	2
K. pneumoniae	5.60	11.20	2

In view of the obtained results, it seems that EO of *Mentha pulegium* L. acts differently according to the tested strain. Minimum inhibitory concentrations (MIC) towards bacterial strains are between 1.40 and 11.20 µl/ml. E. coli was the most susceptible strain with the lowest MIC value (1.40 µl/ml). Bacterial strains P. aeruginosa, S. aureus and K. pneumoniae, were inhibited respectively by the following concentrations: 11.20 µl/ml, 2.80 µl/ml and 11.20 µl/ml.

Using two methods, we find that there is coherence between essential oil MIC values and inhibition diameters except for P. aeruginosa strain. This strain seems to be more resistant in liquid medium than in agar medium comparatively to the other tested strains. Concerning MBC/MIC ratio, we found that it is equal to 2 for E. coli, S. aureus and K. pneumoniae. According to Berche et al. [26], this value allows us to affirm that pennyroyal essential oil exerts a bactericidal effect. The substance is still considered bactericidal when the value of MBC/MIC ratio is less or equal to 4, while for a ratio value greater than 4, the substance is known as a bacteriostatic one.

This antimicrobial activity of *Mentha pulegium* L.'s essential oil can only be explained by its pulegone-rich (71.97%) and piperitenone-rich (26.04%) chemical profile. However, minor compounds may interact directly or a synergistically or in an antagonistic manner to create a mixture endowed with a biological activity. Indeed, Vincenzo De Feo et al. [27] attributed antibacterial and antiviral activity of Minthostachys verticillata's essential oil to the presence of pulegone whose rate was about 37.8%. But, in any case, it is necessary to take into account integrity of EO constituents [28].

3.3. Antifungal activity of Mentha pulegium L.'s EO

Antifungal potency of *Mentha pulegium* L.'s EO was investigated against three fungi (Rhizopus sp., Aspergillus sp. and Penicillium sp.) that cause rot in post-harvest apples. Average inhibition percentages of growth and sporulation are shown in **Table 4**.

According to Koba et al. [29], inhibitory power of essential oils towards a microbial strain is classified as follows:
• Excellent inhibitory power for: MIC <50 µl/ml;
• Interesting inhibitory power for: 50 µl/ml <MIC <250 µl/ml;
• Low inhibitory power: 250 µl/ml <MIC <500 µl/ml;
• Poor or no inhibitory power for: CMI> 500 µl/ml

Table 4: Average percentages of growth inhibition (% Ic) and sporulation inhibition (% Is) of Rhizopus sp., Penicillium sp. and Aspergillus sp. according to concentrations of *Mentha pulegium* L.'s EO

EO concentration in (V/V)	Rhizopus sp.		Aspergillus sp.		Penicilium sp.	
	% Ic	% Is	% Ic	% Is	% Ic	% Is
1/100	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00
1/250	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00
1/500	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00
1/1000	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00	100±0,00
1/2000	100±0,00	79,63±3,32	90,23±8.82	87,27±9,77	91,07±11,90	74,80±5,84
1/3000	100±0,00	54,04±8,33	72,75±9,49	57,46±9,79	56,04±7,88	68,40±8,47
1/5000	79,44±1,85	22,12±2,44	35,52±5,24	46,52±7,68	37,68±2,50	30,90±2,32
0 (témoin)	0,00	0,00	0,00	0,00	0,00	0,00

From the results of **Table 4**, we clearly observe that essential oil of *Mentha pulegium* L. exerted a significant inhibitory activity towards the three tested fungi. Thus, Rhizopus sp. was the most susceptible; it was inhibited at a minimum concentration of 1/3000 v/v with a inhibition percentage that reach 100%. Sporulation of this fungal strain was inhibited in 10 days at 1/1000 v/v. Aspergillus sp. and Penicillium sp. showed a certain resistance towards the oil until 1/1000 v/v concentration that was sufficient to completely stop their mycelial growth and their sporulation. Based on the observed MIC and classification of Koba et al. [29], essential oil of aerial parts of *Mentha pulegium* L. has an excellent inhibitory power against the tested fungal strains. This antifungal potency of essential oil can be attributed to its chemical composition. Indeed, this plant is dominated by ketonic molecules (pulegone and piperitenone) that are more active against microbial agents thanks to the presence of the oxygen atom [30, 31, 32].

CONCLUSION

During this work, chemical composition, antibacterial and antifungal activity of *Mentha pulegium* L.'s essential oil from M'rirt (Middle Atlas) were studied. Pulegone and piperitenone are the major compounds of this oil sample with 71.97% and 26.04% respectively.

All studied microorganisms have undergone a growth inhibition by contact with essential oil on agar medium. This essential oil possesses an excellent inhibitory potency on mycelial growth and sporulation of the three tested fungal strains. This study has also highlighted a relatively high antimicrobial activity of *Mentha pulegium* L.'s oil essential against all bacterial strains used. In conclusion, this work shows that this plant has a low antimicrobial activity on agar medium compared to imipenem (antibiotic). In addition, results of antibacterial activity of EO in liquid medium are different depending on the tested strains. Results also show that this species possesses a bactericidal activity.

Nevertheless, these in vitro methods are not enough to confirm antibacterial and antifungal activity of EOs. Thus, further in vitro and in vivo tests are required.

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