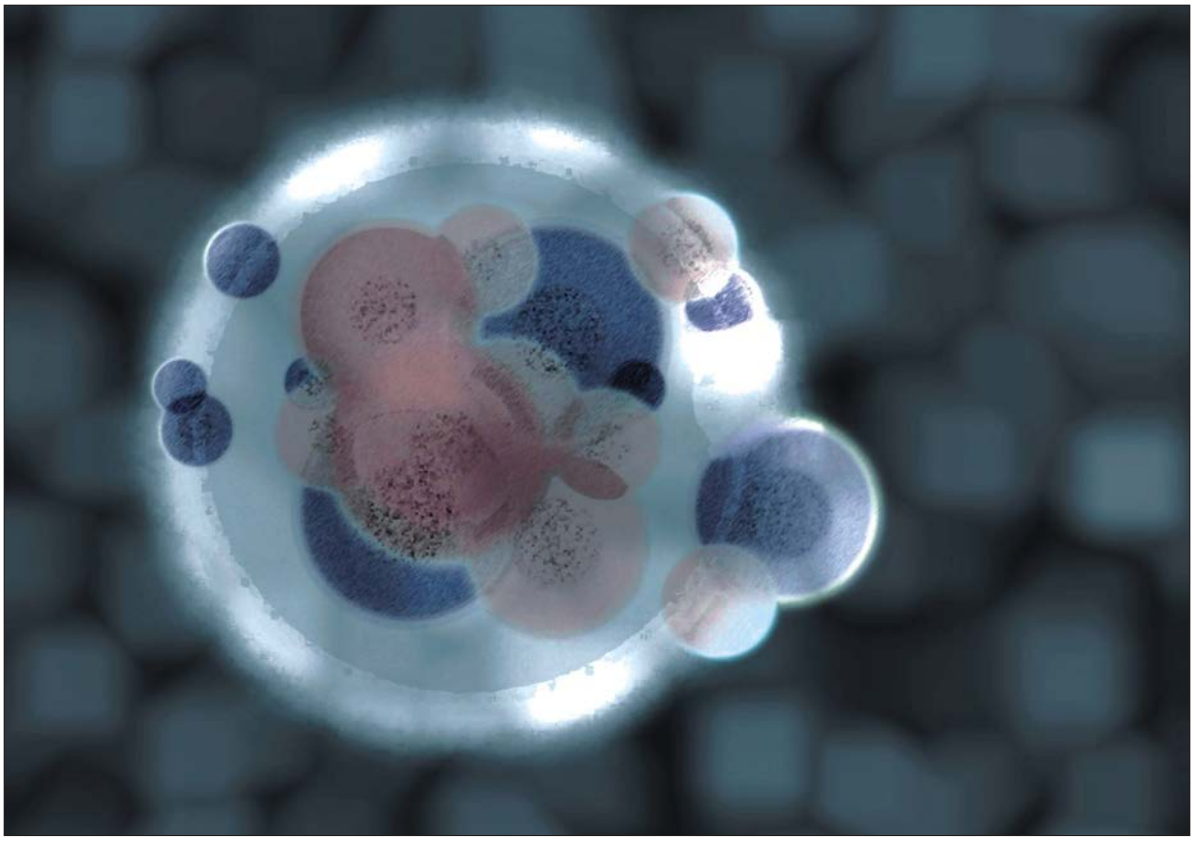

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In vitro Antibacterial Potentials of the Stem Bark of Red Water Tree (*Erythrophleum suaveolens*)

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Abstract: The antibacterial potentials of *Erythrophleum suaveolens* against some selected bacterial isolates were investigated. The aqueous and chloroform fractions of the plant extract exhibited significant inhibitory action against eight of the twelve bacterial isolates tested at a concentration of 10 mg mL^{-1}. The zones of inhibition exhibited by the extract ranged between 8 and 15 mm. The Minimum Inhibitory Concentration (MIC) of the aqueous fraction of extract ranged between 0.625 and 2.5 mg mL^{-1}, while that of chloroform fraction ranged between 0.313 and 5.0 mg mL^{-1}. Also, between 40 and 80% of the test organisms were killed over a period of 2 h in extract concentrations of between 0.078 and 0.625 mg mL^{-1}. Phytochemical analysis of the *Erythrophleum suaveolens* extract revealed the presence of saponins, tannins, steroids and alkaloid. Overall, the two fractions compared favorably with the standard antibiotics, streptomycin and ampicillin at concentration of 1 mg mL^{-1} and 10 μg mL^{-1}, respectively. Cell disruption is proposed as the mechanism of action of the aqueous fraction of the plant extract.

Key words: *Erythrophleum suaveolens*, antibacterial activity, rate of killing, protein leakage

INTRODUCTION

Medicinal plants have become an important element in indigenous medicinal systems (Heinrich, 2000) and a number of scientific investigations have highlighted the importance and contribution of many plant families (Kuete *et al*., 2006). As part of our search for medicinal plants of antimicrobial importance used in Southwestern Nigeria we selected some prospective stem barks, one of which was that of *Erythrophleum suaveolens*, a popular plant used in this region for ameliorating several ailments.

The genus *Erythrophleum suaveolens* known as Obo among the Yorubas of Nigeria belongs to the family Dimorphandreaceae, which consist of about 10 species that are mainly native to Tropical Africa (Dongmo *et al*., 2001). The British colonialists referred to *E. suaveolens* as red water tree (Adeoye and Oyedapo, 2004). Traditionally the plant is used as an ordeal poisons to determine innocence or guilt for witches (Robert and Wink, 1998). Phytochemical analysis of extracts of the bark of this plant revealed the presence of 3-β-etoxy-7-alpha-hydroxy-N-(2-Hydroxyphenyl)-N-methyl-(E)-cass-13-EN-16-Amide (Duke, 1992). The stem bark of *E. suaveolens* has been reported to show anesthetic (Mgbenka and Ejiofor, 1999) and convulsant effects (Harborne and Baxter, 1993); anti-inflammatory and analgesic properties (Dongmo *et al*., 2001) and cardiotoxic effects (Colling *et al*., 1990). Onuorah (2000) reported the presence of Erythrophleguine, which has been confirmed to have anti-fungi activities. Cassaidine, Cassaine and Cassamine from the stem bark of the plant (Duke, 1992), has analgesic, cardiotonic, antiseptic, cytotoxic and anesthetic effects (Harborne and Baxter, 1983; Collings *et al*., 1990). Erythrophlamine, Erythrophleguine, Erythrophleine and Homophleine (Duke, 1992) have cardiotonic, digitalic and diuretic effects (Harborne and Baxter, 1983). In this study, we report the *in vitro* antibacterial potentials of the stem bark extracts of the plant.

MATERIALS AND METHODS

Plants material: Fresh stem bark of *E. suaveolens* was collected from Abeokuta in Ogun state, Nigeria, in the month of September, 2006. Its identity was confirmed by the herbarium unit of the Botany Department, Obafemi Awolowo University, Ile-Ife, Nigeria and deposited therein for reference purpose. The bark was later air-dried, powdered and stored in an air-tight container until ready for use.

Preparation of extract: Exactly 350 g of the powdered bark of the plant was cold-extracted using 60% methanol

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for 4 days. The mixture was then filtered through Whatman filter paper No. 1 and the filtrate was concentrated to dryness *in vacuo* using a rotary evaporator. This gave a yield of 15.5 g.

Preparation of microorganism for the experiment: The following test microorganisms were used for the assessment: *Bacillus subtilis* (NCIB 3610), *Staphylococcus aureus* (NCIB 8588), *Staphylococcus aureus* (ATCC 5538), *Staphylococcus aureus* (LIO), *Streptococcus faecalis* (ATCC 6569), *Micrococcus luteus* (NCIB 196), *Escherichia coli* (ATCC 1175), *Pseudomonas putida* (ATCC 77483), *Pseudomonas fluorescens* (ATCC 3756), *Proteus vulgaris* (NCIB 67), *Serratia marcescens* (NCIB 1377) and *Klebsiella pneumoniae* (ATCC 6380). The organisms were sub-cultured in nutrient broth and nutrient agar (Oxoid.) while Mueller Hinton II Agar was used in antibiotic sensitivity testing.

Phytochemical analysis of the plant extract: A small portion of the dry extract was used for presumptive phytochemical screening. Test for alkaloids, tannins, saponins and steroids were carried out as described by Igwe *et al.* (2007) with modifications.

Test for alkaloids: One half of a gram of the plant extract was dissolved in 5 mL of 1% HCl on steam bath. A milliliter of the filtrate was treated with few drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids.

Test for tannin: About 1.0 g of plant extract was stirred with 10 mL sterile distilled water and filtered (using Whatman No. 1 filter paper). A blue colouration resulting from the addition of $FeCl_3$ reagent to the filtrate indicated that tannin was present.

Test for steroids: A small sample of chloroform extract was dissolved in methanol, spotted on the TLC plate and developed in chloroform: Methanol (4:1) solvent system. The plate was air-dried and sprayed with concentrated sulphuric acid before heating and the development of black spots was recorded as positive for steroids.

Test for saponins: About 2 g of the powdered sample was boiled in 20 mL distilled water in a water bath and filtered. 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Fractionation of the crude extracts with organic solvents of different polarity: The method of Harborne (1998) with modification was adopted for the preparation of fractions of the crude extracts of the plant. Exactly 50 g of the crude extract of *E. suaveolens* was resolved in 100 mL sterile distilled water in a separatory funnel and later fractionated using different organic solvents in order of their polarity. First, about 500 mL of n-hexane was used to extract part of the resolved extract. The extraction was done thrice until the n-hexane layer became colourless. The n-hexane layer was later separated from the aqueous layer and was concentrated to dryness and labeled HX fraction. The aqueous layer left was re-concentrated to eliminate the n-hexane left over. Five hundred of chloroform was then added to the aqueous layer for further extraction. The extraction followed the same procedure with n-hexane. The chloroform layer separated from the aqueous layer was concentrated to dryness and labeled CL fraction. Lastly, 500 mL of butanol was used for the extraction and the part collected was also concentrated to dryness and labeled BL fraction. The aqueous fraction was concentrated to dryness and labeled AQ fraction. The four fractions collected were kept under freezing condition until ready for use.

Antibacterial tests

Susceptibility of bacteria to plant extracts and standard antibiotics: Susceptibility of bacterial strains to the plant extracts and standard antibiotics was carried out following a modified bioassay method of Brenholt *et al.* (1997) and Chen *et al.* (1997). The standard antibiotics used were streptomycin (1 mg mL^{-1}) and ampicilin (10 μg mL^{-1}). Mueller Hinton sterile agar plates were seeded with 100 μL of suspension of indicator bacterial strains containing approximately 10^5 cells and allowed to stand for a while at room temperature. Using a sterile cork borer, wells were made on the seeded plates and these were filled with plant extracts of known concentrations and to the second set of seeded plates, standard antibiotics multodisc was added. The two sets of plates were incubated at 37°C for 30 h and the zones of inhibition were measured at the end of the incubation period.

Minimum inhibitory concentration: The Minimum Inhibitory Concentration (MIC) of the plant extracts was determined by broth dilution technique as described by Irobi *et al.* (1996). The reconstituted plant extracts were diluted to give 5.00, 2.50, 1.25, 0.625, 0.313, 0.157, 0.078 and 0.039 mg mL^{-1} final concentration in nutrient broth. Using a standard micropipette, 0.05 mL of 18 h old bacterial broth (10^6 cfu mL^{-1}) culture was introduced into appropriately

labeled test tubes. A set of tubes containing only growth medium plus each of the test bacteria was set up separately to serve as controls. All the tubes were incubated at 35°C for 30 h. The minimum inhibitory concentration was taken as lowest concentration that prevents growth of the bacterial strains. The same test was repeated for standard antibiotics.

Minimum bactericidal concentration: The Minimum Bactericidal Concentration (MBC) of the plant extracts was determined by a modification of the method of Olorundare *et al*. (1992). Samples were taken from test tubes with no visible turbidity (growth) in the MIC assay and subcultured onto freshly prepared Mueller Hinton 11 agar plates. After incubating for 48 h at 35°C, the minimum bactericidal concentration was taken as the lowest concentration of the extract that did not allow any bacterial growth on the surface of the agar plates.

Determination of rate of killing: For the determination of rate of killing and protein leakage by the aqueous extracts of the plant, *Staphylococcus aureus* (NCIB 8588) and *Pseudomonas putida* (ATCC 77483) were selected to represent susceptible gram positive and gram negative bacterial strains respectively. The rates of killing experiments were done in accordance with the method of Odenholt *et al*. (2001) with modifications. Viable counts of the test organisms were initially determined. A 0.5 mL volume of known cell density (by viable counts) from each organism suspension was added to 4.5 mL of different concentrations of the aqueous fraction. These were held at room temperature and the killing rate was determined over a period of 2 h. Exactly 0.5 mL volume of each suspension was withdrawn at time intervals and transferred to 4.5 mL of nutrient broth recovery medium containing 3% Tween-80 to neutralize the effects of the antimicrobial compounds carry-overs from the test suspensions. The suspension was then serially diluted and plated out for viable counts.

Determination of protein leakage: Protein leakage was determined by measuring the protein concentrations of the cell free culture broth as described by Kruger (1994). Washed suspensions of test organisms were treated with various concentrations of the extracts prepared relative to the MIC concentration. At various time intervals, each suspension was centrifuged at 7000 rpm and the supernatant was assayed for protein using Bradford reagents. The absorbance of the mixture at 595 nm was measured in duplicates after 2 min and before 1 h against a blank prepared from 0.1 mL sterile distilled water and 5 mL Bradford reagent. Concentration of protein was estimated from a Bovine Serum Albumin standard curve.

RESULTS AND DISCUSSION

The investigations done on the four fractions from *E. suaveolens* extracts revealed the plant to possess antimicrobial activities against some of the tested bacterial isolates at a concentration of 10 mg mL^{-1} (Table 1). Two of the fractions, that is AQ and CL fractions compared favorably with two standard antibiotics streptomycin and ampicillin, while the other two fractions (HX and BL) showed low antimicrobial activities. Since the antimicrobial activities exhibited by both AQ and CL fractions cut across both Gram positive and Gram negative bacteria, the fractions were proposed to exhibit broad spectrum activities.

The Minimum Inhibitory Concentration (MIC) of the aqueous fraction of the plant ranged between 0.625 and 2.5 mg mL^{-1} while that of chloroform fraction ranged between 0.313 and 5.0 mg mL^{-1}. The standard streptomycin had MIC values varying between 0.0313 and 0.500 mg mL^{-1} (Table 2). Also, different concentrations of the aqueous fractions of the plant extract exhibited significant bactericidal effects on *Staphylococcus aureus* and *Pseudomonas putida*. With increasing concentrations of the extract from 0.0785 to 0.625 mg mL^{-1}, between 63 and 80% of the test

Table 1: The antimicrobial activities of four fractions obtained from *E. suaveolens*

	Zone of inhibition (mm)±SE*					
Test organisms	AQ (10 mg mL^{-1})	BL (10 mg mL^{-1})	CL (10 mg mL^{-1})	HX (10 mg mL^{-1})	ST (1 mg mL^{-1})	AMP (10 µg mL^{-1})
Bacillus subtilis (NCIB 3610)	15±1.00	8±2.00	13±1.00	8±1.55	20±2.00	0±0.00
Staphylococcus aureus (NCIB 8588)	13±0.55	0±0.00	11±2.00	0±0.00	20±0.55	0±0.00
Staphylococcus aureus (ATCC 5538)	11±1.55	0±0.00	8±1.05	0±0.00	20±0.00	0±0.00
Staphylococcus aureus (LIO)	14±2.00	0±0.00	12±2.00	0±0.00	22±0.55	0±0.00
Streptococcus faecalis (ATCC 6569)	9±1.05	0±0.00	14±0.00	0±0.00	23±1.00	16±0.05
Micrococcus luteus (NCIB 196)	12±0.00	9±1.55	11±1.05	0±0.00	18±0.00	12±2.00
Escherichia coli (ATCC 1175)	10±1.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Pseudomonas fluoresceus (ATCC 3756)	0±0.00	0±0.00	0±0.00	0±0.00	30±0.55	22±0.55
Pseudomonas putida (ATCC 77483)	9±0.55	0±0.00	8±2.00	0±0.00	21±2.00	0±0.00
Proteus vulgaris (NCIB 67)	0±0.00	0±0.00	0±0.00	0±0.00	19±1.00	20±1.55
Serratia marcesceus (NCIB 1377)	0±0.00	0±0.00	0±0.00	0±0.00	15±2.50	16±1.00
Klebsiella pneumoniae (ATCC 6380)	10±2.00	8±0.55	11±0.55	10±2.55	0±0.00	0±0.00

AQ = Aqueous fraction; BL = Butanol fraction; CL = Chloroform fraction; HX = n-hexane; ST = Streptomycin; AMP = Ampicilin; LIO = Locally Isolated Organism; O = Resistant; *: Standard error

Table 2: The Minimum Inhibitory Concentrations (MICs) of the aqueous and chloroform extracts of the plant and that of the antibiotic Streptomycin

	MIC (mg mL^{-1})		
Test organisms	AQ	CL	ST
Bacillus subtillis (NCIB 3610)	0.625	1.25	0.0313
Staphylococcus aureus (NCIB 8588)	1.250	1.25	0.0625
Staphylococcus aureus (ATCC 6538)	2.500	2.50	0.2500
Staphylococcus aureus (LIO)	1.250	2.50	0.2500
Streptococcus faecalis (ATCC 6569)	2.500	0.31	0.0625
Micrococcus luteus (NCIB 196)	1.250	2.50	0.2500
Escherichia coli (ATCC 1175)	2.500	-	-
Pseudomonas putida (ATCC 77483)	2.500	5.00	0.2500
Klebsiella pneumoniae (ATCC 6380)	2.500	5.00	-

AQ = Aqueous fraction; CL = Chloroform fraction; LIO = Locally Isolated Organism; ST = Streptomycin; MIC = Minimum Inhibitory Concentration

Table 3: The rate of killing of *Staphylococcus aureus* (NCIB 8588) in different concentrations aqueous fraction of *E. suaveoleus*

	Killing rate (%)±SE*			
Time (min)	0.0785	0.157	0.313	0.625
	mg mL^{-1}			
0	0±0.00	0±0.00	0±0.00	0±0.00
15	29±0.50	30±0.55	50±1.50	52±0.50
30	30±0.50	40±0.45	62±1.00	68±0.50
45	32±1.00	50±1.00	65±1.50	69±0.50
60	35±0.50	55±0.50	67±1.00	70±0.55
75	60±0.50	60±1.55	70±0.20	72±1.00
90	60±0.00	60±0.50	72±0.55	78±1.00
105	69±0.45	62±0.50	72±2.00	79±2.00
120	69±0.50	62±1.00	2±1.50	80±1.50*

*Standard Error

Staphylococcus aureus were killed within 120 min of exposure (Table 3). However, the rate of killing study for the Gram negative *Pseudomonas putida* revealed that between 42 and 73% of the bacterium were killed within the 120 min exposure time and increasing with increasing concentration of the extract up to 0.625 mg mL^{-1} (Table 4). It would appear that the aqueous extract of the plant is more active against Gram positive than gram negative bacteria.

Aqueous fraction of *E. suaveolens* used in this study caused the leakage of considerable amount of proteins from *Staphylococcus aureus* and *Pseudomonas putida* cells with increasing concentrations of the extract (Table 5 and 6), thus suggesting a cell wall/membrane disruption mechanism of action of the aqueous fraction. Protein leakage from the test *Staphylococcus aureus* at the end of the 120 min exposure period was between approximately 6.0 and 22 μg mL^{-1} (Table 5). A similar trend was observed for the Gram negative *Pseudomonas putida* (Table 6).

Phytochemical analysis revealed the presence of, tannins, steroids, alkaloids and saponins in the extracts. These compounds are known to possess antimicrobial activities. Tannins have been found to form irreversible complexes with proline-rich proteins (Hagerman and Butler, 1989) resulting in the inhibition of the cell protein synthesis. Fractions AQ and CL exhibited this reaction

Table 4: The rate of killing of *Pseudomonas putida* (ATCC 77483) in different concentrations aqueous fraction of *E. suaveoleus*

	Killing rate (%)±SE*			
Time (min)	0.0785	0.157	0.313	0.625
	mg mL^{-1}			
0	0±0.00	0±0.00	0±0.00	0±0.00
15	15±1.50	25±0.55	32±1.50	38±0.50
30	19±0.50	30±1.55	40±1.00	60±0.50
45	25±1.00	40±2.00	45±1.50	62±0.00
60	35±1.50	42±2.50	59±1.00	65±1.55
75	40±0.50	43±1.50	59±0.20	68±2.00
90	40±0.00	47±0.50	59±0.55	69±1.00
105	40±0.00	52±0.50	59±2.00	69±2.00
120	42±0.50	55±0.00	60±1.50	72±1.00

*: Standard Error

Table 5: The leakage of protein from *Staphylococcus aureus* (NCIB 8588) in different concentrations of aqueous fraction of *E. suaveoleus*.

	Protein leaked (μg mL^{-1})±SE*			
Time (min)	0.0785	0.157	0.313	0.625
	mg mL^{-1}			
0	0±0.00	0±0.00	0±0.00	0±0.00
15	5±1.50	8±0.55	10±1.50	12±0.50
30	6±0.50	8±1.55	18±1.00	18±0.50
45	7±1.00	9±2.00	20±1.50	21±0.00
60	7±1.50	9±2.50	20±1.00	30±1.55
75	7±0.50	12±1.50	20±0.20	31±2.00
90	8±0.00	12±0.50	20±0.55	36±1.00
105	8±0.00	12±0.50	20±2.00	38±2.00
120	8±0.50	14±0.00	20±1.50	42±1.00

* Standard Error

Table 6: The leakage of protein from *Pseudomonas putida* (ATCC 77483) in different concentrations aqueous fraction of *E. suaveoleus*

	Protein leaked (μg mL^{-1})±SE*			
Time (min)	0.0785	0.157	0.313	0.625
	mg mL^{-1}			
0	0±0.00	0±0.00	0±0.00	0±0.00
15	5±0.50	7±0.00	12±1.00	17±0.00
30	7±0.00	8±1.00	13±1.00	18±0.00
45	7±1.00	10±2.00	10±1.50	20±0.00
60	7±1.00	10±0.50	10±2.00	22±0.55
75	7±0.50	10±1.50	10±0.20	22±0.00
90	7±0.00	10±0.50	10±0.55	23±1.00
105	7±0.00	10±0.50	10±2.00	23±1.00
120	7±1.50	10±1.00	10±1.50	23±1.00

* Standard Error

against the tested bacterial isolates. Furthermore, tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues (Mota *et al*., 1985). Herbs that have tannins as their main component are astringent in nature and are used for treating intestinal disorder such as diarrhoea and dysentery (Dharmananda, 2003). These facts thus support the use of *E. suaveolens* in herbal cure remedies. Alkaloids have amazing effect on humans and thus had led to the development of powerful pain killer medications (Raffauf, 1996). Mimaki *et al*. (1993) worked on steroidal extract from some medicinal plant which exhibited antibacterial activities on some tested bacterial isolates and they also confirmed the antiviral

property of steroids. Thus, the presence of these compounds in *E. suaveolens* corroborates the antimicrobial properties of the plant.

Generally, the results obtained in this study has shown that the mechanism of antibacterial activities of AQ and CL fractions are by way of physical damage to bacterial cells which led to the protein leakage from the bacterial cells. The antibacterial potency of these fractions is both a function of time and concentration, when a young actively dividing cell is used for investigation. Cellular accumulation of the active components leading to protein leakage and inhibition of cell wall biosynthesis might have accounted for bacterial death as previously reported (Sanati *et al*., 1997). *Erythrophleum suaveolens* has been in use among the Yoruba tribe of southwestern Nigeria in the preparation of decoction, thus suggesting it to be non-toxic. Hence, there is need for the preparation of different formulations towards ensuring acceptable dosing regime pursuant to clinical trials. It is hoped that this study would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin.

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