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Antimicrobial activity of seaweeds of Pernambuco, northeastern coast of Brazil

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The antibacterial efficacy of various solvent extracts of marine algae *Caulerpa racemosa*, *Ulva lactuca* (Chlorophyta), *Jania adhaerens* (Rhodophyta), *Padina gymnospora* and *Sargassum polyceratium* (Phaeophyta) against some selected gram-positive and gram-negative human pathogenic bacteria was screened. Crude extracts were prepared from the selected marine algae using different solvents namely, hexane, chloroform, ethyl acetate and methanol and were tested for their antibacterial activity against human pathogenic bacteria using disc diffusion method. Minimum inhibitory concentration (MIC) was also determined for selected solvent extracts for all the bacterial species. A suitable positive control was also maintained. Among the five marine algae screened *C. racemosa* and *U. lactuca* were found to be more active. It was observed that the ethyl acetate extracts of all the five marine algae showed higher inhibitory activity for the selected bacterial species than other solvent extracts. The results revealed that the crude ethyl acetate extracts seem to be a good source material in identifying the effective pure antibacterial compound(s) in all the five marine algae and particularly, *C. racemosa* and *U. lactuca*. The present study showed that the ethyl acetate extracts of marine algae such as *C. racemosa*, *J. adhaerens*, *P. gymnospora*, *S. polyceratium* and *Ulva lactuca* exhibited good antimicrobial activity. But the ethyl acetate extracts of *C. racemosa* and *U. lactuca* possessed highest antibacterial activity than others and so it could be useful in seeking active principles against human pathogenic bacteria.

Key words: Seaweeds, antimicrobial activity, marine macroalgae, human bacterial pathogens.

INTRODUCTION

Bacterial infection causes high rate of mortality in human population and aquaculture organisms. Preventing disease outbreaks or treating the disease with drugs or chemicals tackles these problems. Nowadays, the use of antibiotics increased significantly due to heavy infections and the pathogenic bacteria becoming resistant to drugs

is common due to indiscriminate use of antibiotics. It becomes a greater problem of giving treatment against resistant pathogenic bacteria (Mahida and Mohan, 2007). The search of new antimicrobial drugs from natural source became an obligation.

There are reports of macroalgae derived compounds that have a broad range of biological activities, such as antibiotic, antiviral, antineoplastic, antifouling, anti-inflammatory, cytotoxic and antimitotic (Jones et al., 2008; Maleki et al., 2008; Tambekar and Dahikar, 2011). The first to observe antimicrobial substances secreted by algae was Harder (Harder, 1917). However, it was not until the 1970s that large-scale screening of antimicrobial activity was carried out (Mahida and Mohan, 2007; Jones et al., 2008) and in the past few decades, macroalgae are attracting increasing attention as a new source for bioactive compounds (Arvinda Swamy, 2011).

Nowadays, infectious diseases are responsible for a high morbidity and mortality rate and are consider as a public health problem because of their frequency and their severity. For the treatment of these diseases, people often use synthetic drug. But, bacteria developed a resistance mechanism to fight against most of the synthetic family of antibiotics. The resistant of microbes is due to indiscriminate utilization of commercial antimicrobial medicines supported by many scientists investigation for modern antimicrobial substances from several medicinal plants and seaweeds (Alagesaboopathi and Kalaiselvi, 2012). There are several bioactive compounds which are produced by seaweeds and they also possess the ability to prevent the disease caused by some gram negative and gram positive pathogenic bacteria (Kolanjinathan et al., 2009).

In the present study, antibacterial efficacy of various organic solvent extracts of the seaweeds *Caulerpa racemosa*, *Ulva lactuca* (Chlorophyta), *Jania adhaerens* (Rhodophyta), *Padina gymnospora* and *Sargassum polyceratium* (Phaeophyta) against some clinically important gram-positive and gram-negative human pathogenic bacteria species is reported.

MATERIALS AND METHODS

Collection of algae

In this study, a total of five seaweed species (Table 1) were collected by hand picking from the submerged marine rocks at Paiva Beach (08º 15'10.50" S e 34º 56'51.80" W) and Pedra do Xareu Beach (08º 18'00.30" S e 34º 56'34.86" W), Cabo de Santo Agostinho municipality, Pernambuco State, Brazil (Figure 1) during low tide in December 2010 and January 2011. All samples were brought to laboratory in plastic bags containing sea water to prevent evaporation. Some of the collected seaweeds were preserved for identification. Seaweeds were identified by Dra. Paula Regina Fortunato do Nascimento, expert in macroalgae, Universidade Federal Rural de Pernambuco, Brazil. Voucher specimens of each species have been deposited at Instituto Agronômico de Pernambuco Herbarium (IPA) (Table 1).

Extract preparation

Algal samples were cleaned of epiphytes and extraneous matter, and necrotic parts were removed. Plants were washed with seawater and then in fresh water. The seaweeds were transported to the laboratory in sterile polythene bags at 0°C temperature. In the laboratory, samples were rinsed with sterile distilled water and were shade dried, cut into small pieces and powdered in a mixer grinder. The algal powdered samples were extracted using four different solvents hexane, chloroform, etyl acetate and methanol. 100 g of powdered algal material were extracted in Soxhlet extractor at 40°C containing 1000 mL of solvent separately using all the four solvents. The material was refluxed for about 36 to 48 h until saturation and the resulting extracts were evaporated in a rotary flash evaporator. The obtained extracts were collected in a clean Petri dish and weighed.

Test organisms

The antimicrobial activity of the seaweeds extracts were tested against the following microorganisms: three Gram-positive bacteria *Bacillus subtilis* (UFPEDA 82), *Micrococcus luteus* (UFPEDA 100) and *Staphylococcus aureus* (UFPEDA 02), the two Gram-negative bacteria *Escherichia coli* (UFPEDA 224) and *Klebsiella pneumoniae* (UFPEDA 396). All strains were provided by Departamento de Antibioticos, Universidade Federal de Pernambuco (UFPEDA) (Table 1) and maintained in Nutrient Agar (NA) and stored at 4□C.

Antibacterial assay

Antibacterial activity was evaluated by agar diffusion method (Bauer et al., 1966). Twenty milligrams of crude extract was dissolved in 1 mL of 10% of Di Methyl Sulphoxide (DMSO). From this stock solution, 10 µL of each extract was loaded on sterile antibiotic discs (6 mm diameter) (Hi-media company) and air-dried. After drying, discs were placed on the Tryptic soy agar. Chloramphenical antibiotics disc and disc loaded with 10 µL of respective solvent were used as positive and negative control respectively. Each sample was used in triplicate for the determination of antibacterial activity.

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The minimal inhibitory concentrations (MICs) of all extracts and reference antibiotic (Chloramphenicol) were determined by microdilution techniques in Mueller-Hinton broth (Merck) following the protocol established by the CLSI (NCCLS, 2009) for bacteria. Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [10^8 colony-forming units (CFU)/mL] and diluted 1:10 for the broth microdilution procedure. Microtiter plates were incubated at 37°C and the MICs were recorded after 24 h of incubation. Minimum inhibitory concentration

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Table 1. Selected seaweeds from Brazilian coast.

Order	Species	Local	Date	Voucher
Rhodophyceae	*Jania adhaerens* J.V. Lamour.	Paiva Beach, Cabo de Santo Agostinho, Pernambuco State	January 2011	IPA 91010
Ulvophyceae	*Caulerpa racemosa* (Forssk.) J.Agardh	Pedra do Xaréu Beach, Cabo de Santo Agostinho, Pernambuco State	December 2010	IPA 91011
Phaeophyceae	*Padina gymnospora* (Kütz.) Sonder	Pedra do Xaréu Beach, Cabo de Santo Agostinho, Pernambuco State	December 2010	IPA 91012
Phaeophyceae	*Sargassum polyceratium* Mont.	Pedra do Xaréu Beach, Cabo de Santo Agostinho, Pernambuco State	December 2010	IPA 91013
Ulvophyceae	*Ulva lactuca* L.	Gaibu Beach, Cabo de Santo Agostinho, Pernambuco State	January 2011	IPA 91014

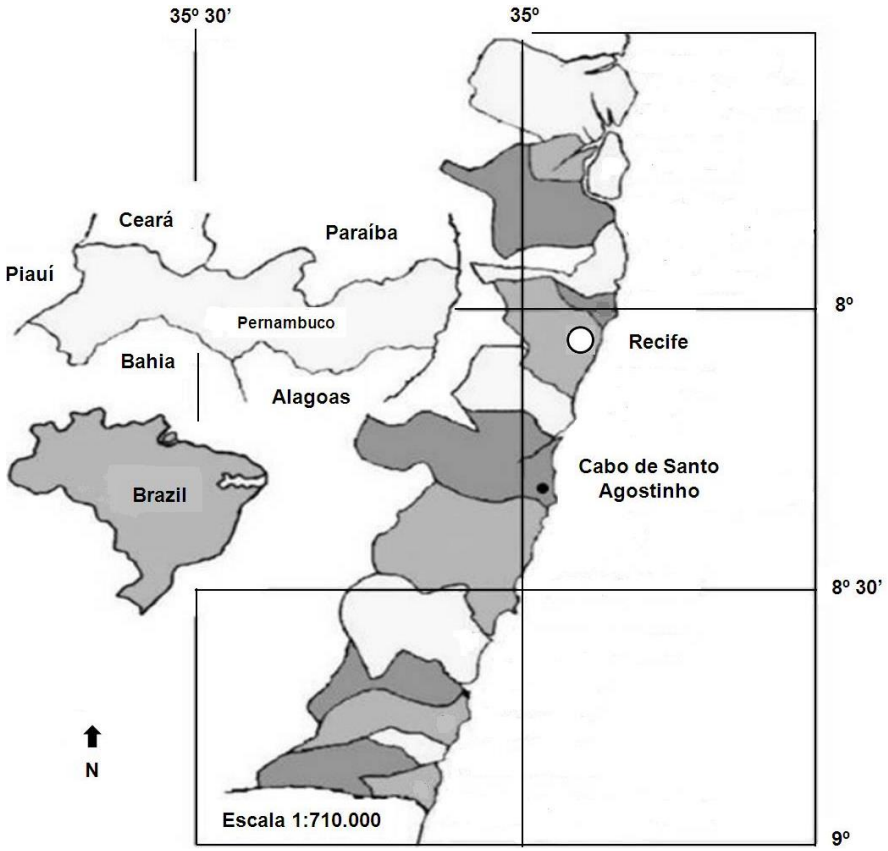

Figure 1. Geographical localization of Pedra do Xaréu Beach and Gaibu Beach, Cabo de Santo Agostinho municipality, Pernambuco state, Brazil.

Table 2. Results of antimicrobial activity of 18 extract crude of five seaweeds of Brazilian coast, determined by agar disc diffusion method (inhibition zone in mm).

Seaweeds	Solvents	Zone of Inhibition (mm)				
		Bacilus subtilis	***Micrococcus luteus***	***Staphylococcus aureus***	***Escherichia coli***	***Kleibsiela pneumoniae***
Jania adhaerens J.V. Lamour.	Hexane	14.33±0.58	10±0.00	-	-	-
	Chloroform	15.33±0.58	8.67±0.00	-	-	-
	Ethyl acetate	15±0.00	14.33±0.58	-	-	-
	Methanol	13±0.00	9.33±0.58	-	-	-
Caulerpa racemosa (Forssk.) J.Agardh	Hexane	16±0.00	16±0.00	16.33±0.58	15.33±0.58	10±0.00
	Chloroform	17±0.00	17±0.00	17±0.00	15±0.00	10.67±0.58
	Ethyl acetate	17±0.00	19±0.00	17±0.00	15.33±0.58	12.33±0.58
	Methanol	15.33±0.58	18±0.00	18±0.00	16.33±0.58	13±0.00
Padina gymnospora (Kütz.) Sonder	Hexane	16±0.00	16.33±0.58	-	-	-
	Chloroform	16±0.00	15.33±0.58	-	-	-
	Ethyl acetate	17±0.00	15±0.00	-	-	-
	Methanol	15±0.00	13±0.00	-	-	-
Sargassum polyceratium Mont.	Hexane	17±0.00	12±0.00	10±0.00	16.33±0.58	6.67±0.58
	Chloroform	15±0.00	15.33±0.58	8.67±0.00	11.33±0.58	8.67±0.58
	Ethyl acetate	17±0.00	16.33±0.58	15.33±0.58	12.33±0.58	10.67±0.58
	Methanol	17±0.00	11±0.00	12±0.00	15±0.00	8.67±0.58
Ulva lactuca L.	Hexane	18±0.00	15±0.00	14±0.00	12±0.00	10.67±0.58
	Chloroform	19±0.00	18±0.00	15±0.00	10±0.00	8.67±0.58
	Ethyl acetate	20±0.00	23±0.00	18±0.00	15±0.00	10.67±0.58
	Methanol	20±0.00	20±0.00	17±0.00	9.33±0.58	10±0.00
Chloramphenicol	-	20±0.00	19±0.00	21±0.00	17±0.00	17±0.00

corresponded to the minimum extract concentration that inhibited visible bacterial growth. Afterwards, cultures were seeded onto MHA and incubated for 24 h at 37°C to determine the minimum bactericidal concentration (MBC) which corresponded to the minimum concentration of extract that caused the bacteria elimination. The antibiosis (bacteriostatic or bactericidal) activity is determined by the ratio of MBC/MIC. When the ratio of MBC/MIC is ≤ 2, the active fractions were considered as bactericidal, and when the ratio was higher ≥ 2 was considered bacteriostatic. Finally if the ratio is ≥ 16 the fractions were considered as ineffective (Traczewski et al., 2009; Sader et al., 2009).

RESULTS

The antibacterial activity of various solvent extracts of *C. racemosa*, *U. lactuca* (Chlorophyta), *J. adhaerens* (Rhodophyta), *P. gymnospora* and *S. polyceratium* (Phaeophyta) on five different human bacterial pathogens are presented in Tables 2 and 3. Seaweeds extract of *C. racemosa*, *S. polyceratium* and *U. lactuca* demonstrated good antimicrobial activity against all gram-positive and gram-negative pathogenic bacteria. *J. adhaerens* and *P. gymnospora*

Table 3. Results of antimicrobial activity of 20 extract crude of five seaweeds of Brazilian coast, determined by the agar-dilution methods (Minimum Inhibitory Concentration, MIC, in mg/mL).

Seaweeds	Solvent	Microorganisms														
		MIC					MBC					MBC/MIC				
		B.s.	***M.l.***	***S.a.***	***E.c.***	***K.p.***	***B.s.***	***M.l.***	***S.a.***	***E.c.***	***K.p.***	***B.s.***	***M.l.***	***S.a.***	***E.c.***	***K.p.***
Jania adhaerens J.V. Lamour.	Hexane	12.5	12.5	25	12.5	25	12.5	25	12.5	12.5	12.5	1	2	2	1	1
	Chloroform	6.25	12.5	25	12.5	25	6.25	25	12.5	12.5	12.5	2	2	2	1	1
	Ethyl Acetate	6.25	6.25	12.5	12.5	12.5	6.25	12.5	12.5	12.5	12.5	1	2	1	1	1
	Methanol	6.25	6.25	12.5	12.5	12.5	6.25	12.5	12.5	12.5	12.5	1	2	1	1	1
Caulerpa racemosa (Forssk.) J.Agardh	Hexane	1.56	3.12	1.56	3.12	6.25	1.56	3.12	3.12	3.12	6.25	1	1	2	1	1
	Chloroform	3.12	3.12	1.56	3.12	6.25	3.12	3.12	3.12	3.12	6.25	1	1	2	1	1
	Ethyl Acetate	1.56	6.25	0.78	3.12	3.12	1.56	6.25	0.78	3.12	6.25	1	1	1	1	2
	Methanol	1.56	6.25	0.78	3.12	3.12	1.56	6.25	0.78	3.12	6.25	1	1	1	1	2
Padina gymnospora (Kütz.) Sonder	Hexane	6.25	12.5	12.5	25	50	6.25	12.5	25	50	50	1	1	2	2	1
	Chloroform	6.25	12.5	12.5	25	50	6.25	12.5	25	50	50	1	1	2	2	1
	Ethyl Acetate	3.12	6.25	12.5	50	50	3.12	6.25	12.5	50	50	1	1	1	1	1
	Methanol	6.25	6.25	12.5	50	50	6.25	6.25	12.5	50	50	1	1	1	1	1
Sargassum polyceratium Mont.	Hexane	12.5	6.25	12.5	12.5	25	12.5	6.25	12.5	25	25	1	1	1	2	1
	Chloroform	12.5	6.25	12.5	12.5	25	12.5	6.25	12.5	25	25	1	1	1	2	1
	Ethyl Acetate	6.25	3.12	6.25	12.5	12.5	6.25	6.25	6.25	50	12.5	1	2	1	4	1
	Methanol	6.25	6.25	6.25	12.5	12.5	6.25	6.25	6.25	50	12.5	1	1	1	4	1
Ulva lactuca L.	Hexane	0.39	0.78	1.56	3.12	6.25	0.39	3.12	3.12	6.25	12.5	1	4	2	2	2
	Chloroform	0.39	0.78	1.56	3.12	6.25	0.39	3.12	3.12	6.25	12.5	1	4	2	2	2
	Ethyl Acetate	0.39	0.39	0.78	1.56	3.12	0.39	1.56	1.56	3.12	3.12	1	4	2	2	1
	Methanol	0.39	0.39	0.78	1.56	6.25	0.39	1.56	1.56	6.25	6.25	1	4	2	4	1
Choramphenicol	-	0.09	0.04	0.09	0.19	0.19	0.09	0.04	0.09	0.39	0.39	1	1	1	2	2

B.s. = Bacilus subtilis; *M.l. = Micrococcus luteus*; *S.a. = Staphylococcus aureus*; *E.c. = Escherichia coli*; *K.p. = Klebsiella pneumoniae.*

demonstrated antimicrobial activity against only *Bacillus subtilis* and *Micrococcus luteus*. Among the four solvents tested, ethyl acetate and methanol extracts exhibited maximum inhibition on the growth of the tested bacterial species. As observed, the ethyl acetate extracts of all the five marine algae showed the highest inhibitory activity for the chosen bacterial strains followed by other solvent extracts.

Maximum activities were recorded in the green marine algae *U. lactuca* ethyl acetate (23 ± 0.00 mm) and methanol (20 ± 0.00 mm) extracts and *C. racemosa* ethyl acetate (19 ± 0.00 mm) and methanol (18 ± 0.00 mm) extracts when compared to other solvent extracts as well as

various solvent extracts of the marine algae *J. adhaerens*, *P. gymnospora* and *S. polyceratium* (Table 2). Less inhibitory effects for all the test organisms were recorded in the *J. adhaerens* and *P. gymnospora*. Among the five groups of marine algae tested, maximum activities were recorded in green marine algae *U. lactuca* and minimum activity was recorded in red marine algae. All the four solvent extracts of the marine algae, *J. adhaerens* and *P. gymnospora* were not revealed any zone of inhibition against *Staphylococcus aureus*, *Escherichia coli* and *K. pneumonia*e.

Of the five marine algae screened in the present study for their antibacterial activity, *U. lactuca* and *C*. *racemosa* were observed to be more active than *J. adhaerens*, *P. gymnospora* and *S. polyceratium* gainst human pathogens in the control of their growth.

There were also specific antibacterial activities with reference to either the known solvent extract effective to a number of bacterial strains or specific effect of marine algae to some bacterial pathogens. The ethyl acetate extract of *C. racemosa* and *U. lactuca* showed excellent antibacterial activity. Specifically hexane extracts of *C. racemosa* and *U. lactuca* indicated inhibition of bacteria such as *B. subtilis* and *S. aureus*. In *P. gymnospora* species hexane extract shows prominent activity against bacteria *B. subtilis*. It was observed that hexane extracts of *J. adhaerens* and *S. policeratium* produced broad spectrum antibacterial activity against *S. aureus* and *B. subtilis*. Ethyl acetate extract of all the five marine algae exhibited activity against *B. subtilis*, *M. luteus* and *S. aureus*.

Table 2 shows the MIC, MBC and MBC/MIC ratio value of the extracts from algae. These MIC and MBC values were demonstrated to range from 0.39-50 mg/mL. A minimum value of MIC as 0.39 mg/mL was observed for *B. subtilis* to all organic extracts of *U. lactuca*. Among various crude solvent extracts tested, ethyl acetate extracts of all the five marine algae performed better than the other solvent extracts.

Almost all organic extracts of the five seaweed showed bactericidal action.

DISCUSSION

There is high expectation that organisms from the marine environment will yield a vast array of new pharmaceutical compounds with novel activities that will provide new drugs in the fight against a number of microbial pathogens currently developing resistance conventional antibiotic therapies.

In this study, green algae had higher inhibition activity than the red and brown algae. But the brown algae exhibited the moderate inhibition growth when compared with green algae. Some previous investigations revealed higher antibacterial activity in the extracts of brown algae than the red algae extract (Reichelt and Borowitzka, 1984).

Reichelt and Borowitzka (1984) and Salvador et al. (2007) screened many species of algae for their antibacterial activity. They reported that the members of the red algae exhibited high antibacterial activity. In contrast the green algae (Clorophyceae) were the most active species. Present results are in accordance with those of Kandhasamy and Arunachalam (2008) who reported that green algae (Chlorophyceae) were the most active division than others.

Antimicrobial activity depends on both algal species and the solvents used for their extraction. In our study it was reported that the green algae (*U. lactuca* and *C. racemosa*) showed antibacterial activity against several Gram-negative and Gram-positive bacteria. Maximum activities were recorded in the green algae *U*. *lactuca* and *C. racemosa* against *S. aureus* in ethyl acetate and metanol extracts when compared to other solvent extracts of the marine algae *J. adhaerens*, *P. gymnospora*, *S. polyceratium*.

Perez et al. (1990) observed that the extract of *U. lactuca* had no antibacterial activity. In contrast, results of our study shows that *U. lactuca* inhibited mostly all the organisms in all the solvents tested. To *C. racemosa,* the literature reports the presence essentially of alkaloids, terpenoids and steroids. It is reported that these compounds possess therapeutical applications (Güven et al., 2010; Ornano et al., 2014).

A few workers tried using different solvents for screening the antimicrobial activity of seaweeds and made comparisons. Martinez–Nadal et al. (1966) mentioned that benzene and diethyl ether were suitable solvents for extracting the antibiotic principle. In another study, acetone was found best among several solvents used for extracting antibacterial substances (Patra et al., 2009).

Some other studies performed in the extraction of seaweeds using chloroform and ethyl acetate also exhibited good antibacterial activity (Vonthron-Sénécheaus et al., 2011). It was reported that methanol extracts of seven different seaweeds tested showed broad spectrum antibacterial activity against human pathogenic bacteria (Jebasingh et al., 2011; Kandhasamy and Arunachalam, 2008; Kannan et al., 2010; Rajasulochana et al., 2009). This kind of less or more activity could also be attributed to the sequential extraction of marine algae using solvents from low polar to high polar.

Differences between the results of the present investigation and results of other studies may be due to the production of bioactive compounds related to the seasons, method, organic solvents used for extraction of bioactive compounds and differences in assay methods. Among the seaweed species screening in this paper, *C. racemosa* is the species most studied. Investigation of phytochemicals of *C. racemosa* led to the isolation of several secondary metabolites related to different categories of natural products: indol derivatives, indan

derivatives, sesquiterpenoid derivatives, diphenyl pentadiene derivatives, terpenoids and fatty acids (Ornano et al., 2014). Liu et al. (2013a) discoveried two rare antifungal prenylated para-xylenes, caulerprenylols A and B. The same authors (Liu et al., 2013b) isolated two bisindole alkaloids, racemosins A and B, and one well-known pigment in the genus *Caulerpa*, caulerpin. The antimicrobial activity of organic extracts of C. racemosa is due to the action of these compounds.

Finally it can be concluded from the study that extracts of algal species used in the present investigation showed better antibacterial activity against pathogens used. In general, the ethyl acetate and methanol extracts of all the five marine algae showed antibacterial activity against both gram positive and gram negative bacteria with very well-known higher levels of antibacterial activity of *U. lactuca* and *C. racemosa*. It is thus concluded from this study that the ethyl acetate extract of marine alga, *U. lactuca* and *C. racemosa* could be used for further investigation to identify actual components against human bacterial pathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

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