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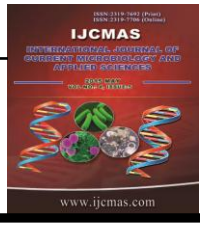

Original Research Article

Study of Phytoconstituents and antibacterial activity of *Kappaphycus alvarezii*

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Keywords

Antibacterial Activity, bioactive , quinones, sterols and isoflavonols

A B S T R A C T

The aim of this investigation was to analyse the antimicrobial activity of *Kappaphycus alvarezii* and to study its bioactive compounds. The crude extracts of *Kappaphycus alvarezii* extracted in ethanol and chloroform was subjected to preliminary phytochemical estimation. Its activity against gram positive and negative bacterial strains of *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Bacillus subtilis* was also investigated. The compounds present were confirmed using HPLC and the functional groups were identified using FT-IR. Phytochemical analysis tested positive for the presence of flavonoids, cardiac glycosides, sterols and quinones. FTIR analysis of the EtOH and $CHCl_3$ extracts of *Kappaphycus alvarezii* showed similar peaks corresponding to functional groups such as amines ($-NH_2$), alcohols (-OH) and carboxyl (-C=O) groups. The qualitative HPLC fingerprint profile of both extracts showed prospective peaks at lower R_f values indicating the major presence of quinones.The results show that, the presence of bioactive quinones, sterols and isoflavonols, lead to active inhibition of microbial growth in a dose-dependent manner.

Introduction

According to WHO report and fact sheet (last updated on April 2014), the causes of antimicrobial resistance (AMR) are several, namely selective pressure, mutation, gene transfer, societal pressures, inappropriate drug use, inadequate diagnostics, hospital use and agricultural use of drugs. The impact of AMR on society and economy is sky-high. It raises the death rate due to common infections, twice than normal and the low awareness among general public is not helping the already deteriorating situation.

Due to unavailability of treatment to resistant forms of microbes, the health care costs and the economic burden on public will further spike specifically in third world countries. In 2050, about 10 million deaths are expected to be due to AMR, which will dominate every other cause of mortality like cancer, road accidents and diabetes. The draft global action plan developed by the World Health Organization to combat antimicrobial resistance (to be submitted to the Sixty-eighth World Health Assembly in May 2015) sets out five strategic objectives.

It includes "strengthening of knowledge through surveillance and research and increasing investment in new medicines, diagnostic tools, vaccines and other interventions." [World Health Organization, 2014].

New antimicrobial compounds with increasing efficiency and capacity habitual intake are being currently investigated to control the increasing incidence of AMR.In fact, the search for the avant-garde avenue has led the countries across world to look into their own treasure house of natural medicine, unfurling the field of complementary and alternative medicine (CAM).

Despite India being a peninsular country, we have not explored our marine resources like countries such as South Korea (Se-Kwon, and Eresha Mendis, 2006), China (Jia-Hui *et al.*, 2008), Japan (Yamaguchi, Katsumi, 1996)or Taiwan (Yan, 2004). Current research in the field of drug discovery and development involve the exploration of "secondary metabolites" for small molecule therapeutic candidates.

Marine algae are such secondary metabolites which are an inclusive component of traditional medicine in several Asian countries. They provide a wide range of bioactive compounds which has been shown to exhibit activity against bacteria, virus, inflammation (Rinehart *et al.*, 1981) and cancer (Li *et al.*, 2015).

Kappaphycus alvarezii is one of the most commercially important species of red algae in India.The algae are morphologically tough, fleshy, firm and coarse thalli, with axes and branches 1 – 2 cm diameter, and grow up to 2 m tall. It can be found asflat reef 1 to 17 m deep underwater, or loosely attached to broken coral, or as unattached fragments floating in shallow and deep waters. Sometimes it can found as large, moving mats of unattached thallus (Abbott, 1999). The algae are economically important due to the extraction of kappa carrageenan for the production of agar, gelatin and other nutraceutical and pharmaceutical products(Stanley, 1987).Antimicrobial, antitumor and antioxidant activity has been reported against various pathogens and cell lines respectively.

Here we attempt to provide a comprehensive study of the phytochemical components of *Kappaphycus alvarezii* by subjecting the ethanolic and chloroform extract to phytochemical screening, FT-IR and HPLC studies. Here, the antibacterial activity of the algae is evaluated against both strains of gram negative and gram positive bacteria using different methods for comparative analysis.

Materials and methods

Collection of samples

Based on pilot study, seaweeds were obtained from the Gulf of Mannar coastal region. The samples of *Kappaphycus alvarezii* were collected by handpicking at Mandapam coastal waters (Gulf of Mannar Coast). The samples were washed with distilled water thoroughly (any impurities, cleaned), spread out and shade dried. The powdered samples were then stored in refrigerator for further use.

Preparation of extracts

Preparation of Extracts: The coarsely powdered dried seaweeds were packed in Soxhlet apparatus and extracted with water, ethanoland chloroform for 8 h. The crude extracts were weighed and deep frozen (-20℃) until tested.

Phytochemical analysis

Phytochemical Screening

Preliminary qualitative phytochemical analyses of crude ethanol (EtOH) and Chloroform (CHCl3) extracts and their fractions were carried out for the detection of tannins, saponins, flavonoids, cardiac glycosides, coumarins, alkaloids, phenols, photobatannins, sterols, quinones, oxalate, carboxylic acidwas done using the methods reported by Harborne *et al.*, (1984).

FT-IR analysis

The FT-IR studies have been followed by the method described by Jagmohan (2005). The ethanolic and chloroform crude extract were mixed with dry potassium bromide pellet (KBr) (Sigma) and subjected to a pressure of about 5x106 Pa in an evacuated die to produce a clear transparent disc of diameter 13 mm and thickness 1mm. IR spectra region 4000-1000 cm-1 were recorded at room temperature on a Fourier Transform Spectrometer (Nicolet iS5, Thermo Fisher Scientific, (USA).

HPLC analysis

The ethanolic and chloroform crude extract was mixed with 100% HPLC grade methanol (Sigma) and injected 25µl in UPLC-Accela 1250 model (Thermo Fisher Scientific, (USA) volume of sample and 100% methanol was used as an eluent (Mobile phase). C18 column (250x 4.6 µm) - (Waters) was used and the flow rate is 1ml/min, the extract was read in photo diode array (PDA) as a detector.

Microbial strains

Escherichia coli, *Staphylococcus aureus*, *Proteus mirabilis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Bacillus subtilis*were isolated from samples of clinical significance. The strains were identified and isolated by repetitive culturing, staining and subjected to biochemical tests for confirmation. The bacterial stock cultures were maintained on Mueller Hinton Agar medium at 4ºC.

Antibacterial assay

The antimicrobial activity of ethanolic and chloroform extracts of *Kappaphycus alvarezii* were determined by modified Kirby-Bauer method, through both disk diffusion and well diffusion method(Bauer,1966).

Agar disc diffusion method: Sterilised paper discs madeof Whatman No.1 filter paper was soaked and dried repeatedly in respective concentrations of the seaweed extracts. The bacterial strains, grown overnight, were plated on sterile Mueller Hinton Agar plates along with controls. The paper discs were placed strategically and incubated at room temperature for 24 hrs. The zone of inhibition was measured after incubation.

Agar well diffusion assay: Similarly, overnight incubated bacterial strains were plated onto sterile Mueller Hinton plates. 4wells per plate were made in the plates. Equal amounts of the different concentrations of the extract was loaded in the plates and incubated at room temperature for 24hrs, along with controls. The zone of inhibition was measured after incubation.

Results and Discussion

The phytochemical constituents of the red algae *K.alvarezii* was extracted using two solvents chloroform and ethanol.

Table No. 1 List of phytochemical constituents tested positive in the chloroform and ethanol extracts of *Kappaphycus alvarezii*

S.No	Phytochemical present	*Kappaphycus alvarezii*	
		Chloroform	Ethanol
1	Tannins	--	--
2	Saponins	--	--
3	Flavonoids	+	+
4	Cardiac glycosides	+	+
5	Coumarins	--	--
6	Alkaloids	--	--
7	Phenols	--	--
8	Photobatannins	--	--
9	Sterols	+	+
10	Quinones	--	+
11	Oxalates	--	--
12	Carboxlic acid	--	--

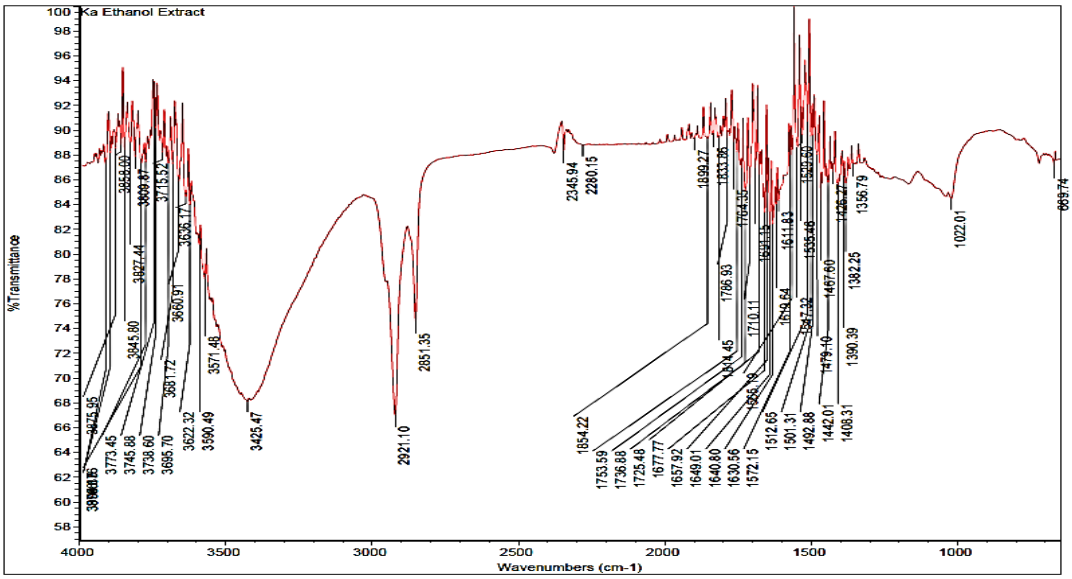

Fig No: 1 FT-IR spectrum of Ethanol (EtOH) extract of *Kappaphycus alvarezii*

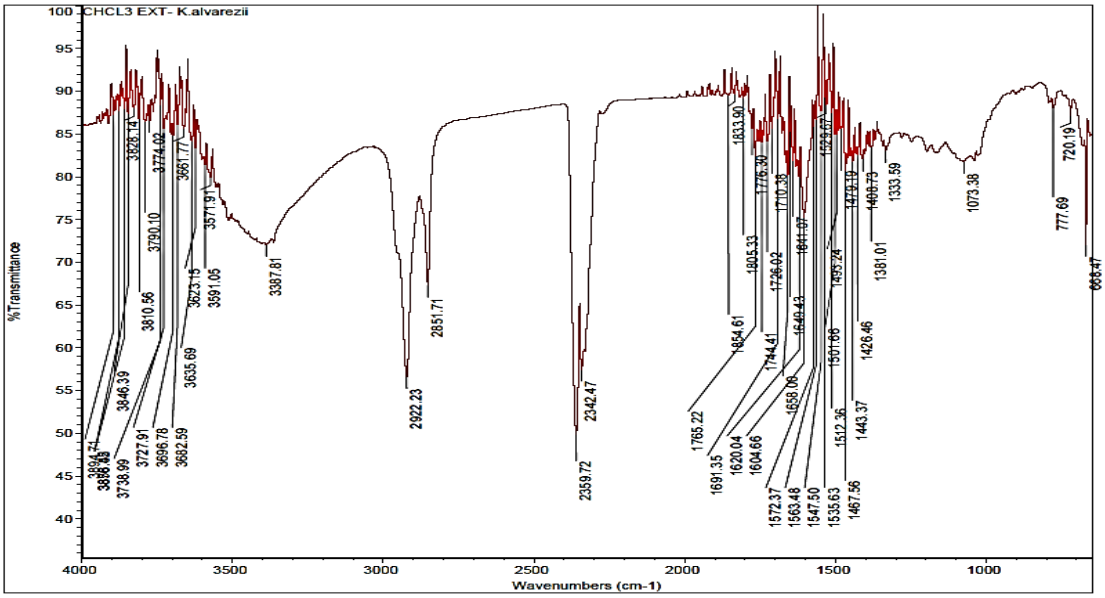

Fig.no.2 FT-IR spectrum of Chloroform (CHCl3) extract of *Kappaphycus alvarezii*

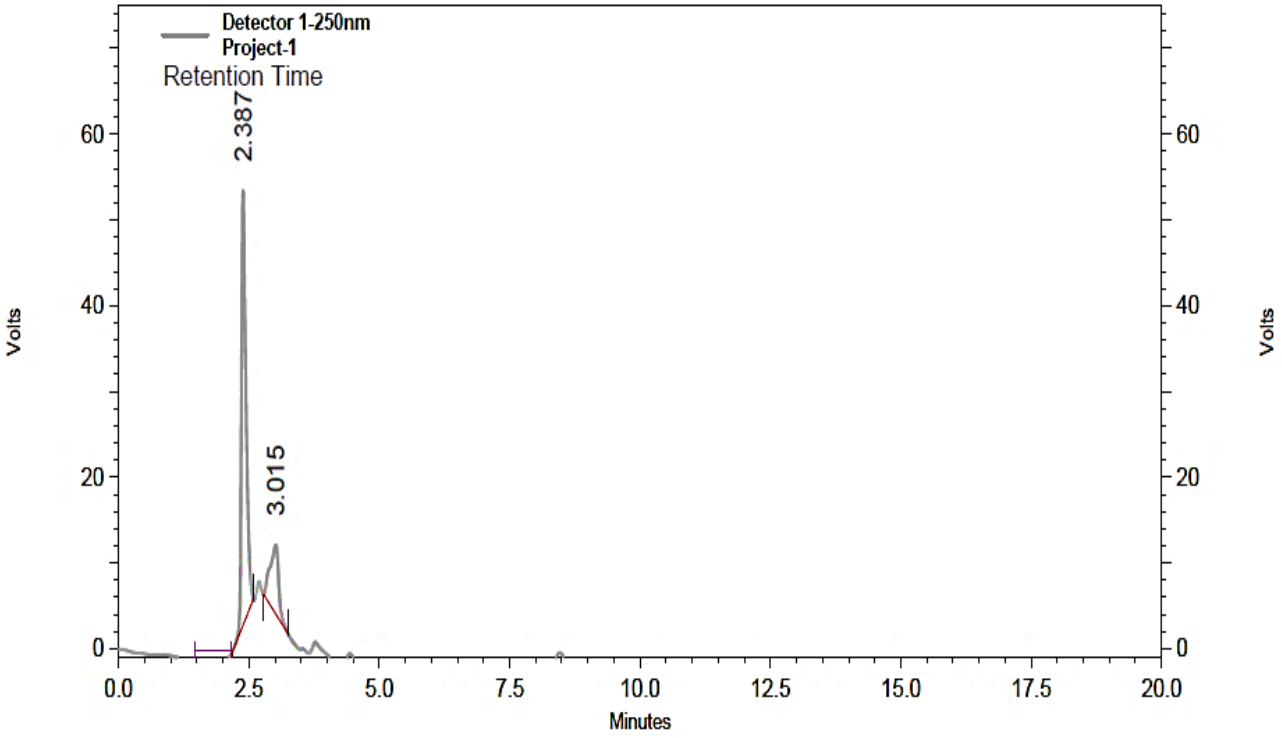

Fig No: 3 HPLC retention spectrum of Ethanol (EtOH) extract of *Kappaphycus alvarezii*

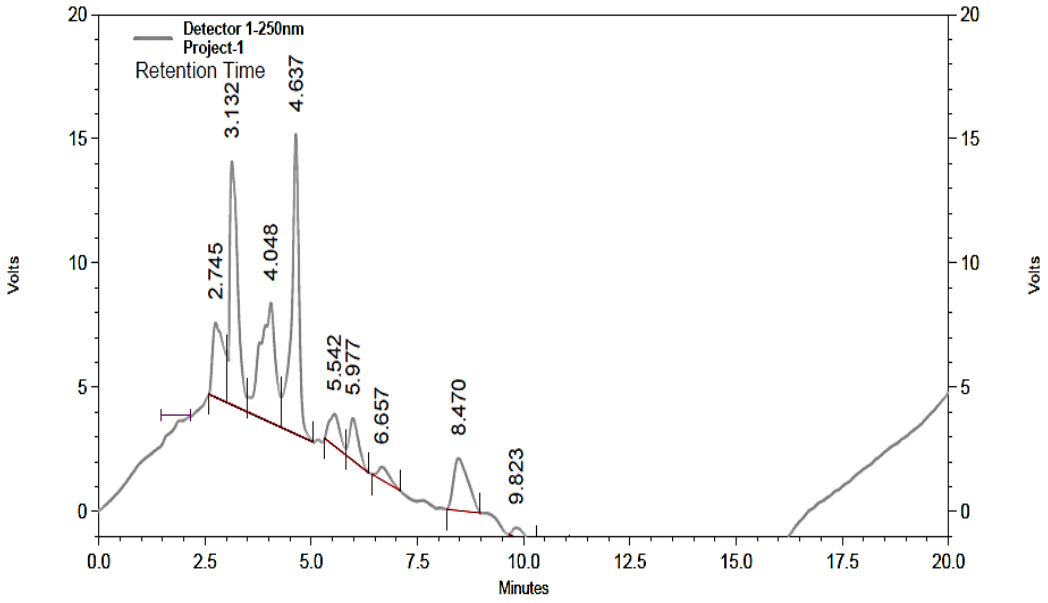

Fig No: 4 HPLC retention spectrum of Chloroform (CHCl3) extract of *Kappaphycus alvarezii*

Table No: 2 Zone of inhibition (measured in mm) of chloroform, ethanol, aqueous extract of *Kappaphycus alvarezii*

Name of the pathogens	Concentration mg/ml	Diameter of Zone of Inhibition (mm)						
		Chloroform extract		Ethanol extract		Aqueous Extract		Gentamicin (cm)
		Disc	Well	Disc	Well	Disc	Well	Disc
Bacillus subtilis	40	4			8	7		3.5
	60	6		6	11	10	8	
	80	10	7	11	13	11	9	
	100	12	9	12	14	11	12	
Staphylococcus aureus	40		5	6			5	2.6
	60			7	11	9	7	
	80	7	8	8	12	11	12	
	100	12	9	14	16	10	15	
Streptococcus pneumonia	40			5		6	8	0.8
	60		3	8	8	7	10	
	80	5	7	10	9	10	9	
	100	9	8	13	12	10	12	
Escherichia coli	40			5	6	5	5	2.4
	60	5		7	7	6	6	
	80	6	7	10	7	9	7	
	100	8	10	12	11	10	10	
Pseudomonas aeruginosa	40					3	4	1.1
	60			5	6	5	8	
	80	7		8	8	8	9	
	100	9		11	10	8	11	
Proteus mirabilis	40				7	5		0.8
	60		3	6	7	6	7	
	80		5	8	9	7	9	
	100	9	8	13	10	11	12	
Vibrio cholerae	40				7.5	4	5	2.6
	60	8	8	5	7.5	6	8	
	80	11	9	10	11	10	9	
	100	10	9	12	10	9	13	

Factors like extraction efficiency, diversity and quantity of phytochemical extracted depends upon the choice of solvent (Pandey, 2014). In the process of extraction, chloroform is known to concentrate phytochemicals of terpenoids and flavonoid in nature, whereas ethanol elutes sterols, alkaloids, flavonols, quinones and polyphenols (Tiwari, 2011).

Qualitative analysis of the ethanolic and chloroform extract of *K. alvarezii* showed the presence of three major groups of phytochemical constituents is summarized in Table 1. Preliminary qualitative phytochemical analyses of crude ethanol (EtOH) and Chloroform (CHCl3) extracts revealed the presence of flavonoids, cardiac glycosides, sterols and quinones.

Previous studies on the phytochemical composition of *Kappaphycusalvarezii* and other red algae reveals the presence of bioactive compounds such flavonoids (Lalopua, 2011), carrageenans (Cardozo, 2007), polyphenols (Sithranga, 2011), sterols, and alkaloids (Koplík, 2010).

FTIR analysis of the EtOH and $CHCl_3$ extracts of *Kappaphycus alvarezii* showed similar peaks corresponding to functional groups such as amines (-NH_2), alcohols (-OH) and carboxyl (-C=O) groups. The continuous band in the region of 3387.81cm^{-1} indicates the presence of hydroxyl (-OH) at higher concentrations. The presence of doublet peaks at 2922.23 cm^{-1} and 2851.71 cm^{-1} indicates the amine (-NH_2) functional groups in the extracts. The numerous multiple peaks in the region of 1600 cm^{-1} to 1800 cm^{-1} signified functional groups of free ketonic carbon such as –CH, -C=C, -C≡C and various other carboxyl groups at varying concentrations. The peaks corresponding to the region of 2300 cm^{-1} to 2500 cm^{-1} revealed the presence of carboxylic groups at low concentration in EtOH extract and at moderate concentration in $CHCl_3$ extract, which may point towards the influence of solvent used for extraction (Fig 1 & 2).

The qualitative HPLC fingerprint profile of *Kappaphycus alvarezii* ethanol extract showed two peaks- a prominent one at retention time of 2.387 mins and a smaller one at 3.015 mins. [Area=76.86%, 23.14% resp] (Fig 3). Even though repetitive extraction identification process is required to determine the exact phytochemical constituent, at 245-270 nm, 300-350 nm, the bioactive compounds quinones and isoflavonols have made appearances often in plants extracts (Ghosheh, 1999), (Costa, 2004), (Encyclopædia Britannica Inc., 2015).

The qualitative HPLC fingerprint profile of *Kappaphycus alvarezii* chloroform extract showed two prominent peaks at retention time of 3.132 mins and 4.637 mins and moderate peaks of different retention time of 2.745, 4.048, 5.542, 5.977, 6.657, 8.470, 9.823, and 10.527 indicating a very heterogenous mixture (Fig 4). In chloroform extracts, sterols are often detected at 210-260 nm in the space of 8 to 10 minutes.

The antibacterial activity of different solvent extracts (ethanol,water and chloroform) against clinical pathogens (*Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa, Proteus mirabilis, Vibrio cholerae*) at various concentrations showed diverse zone of inhibitions (Table 2). The zone of inhibition was measured in millimeters (mm). The ethanolic extract of *Kappaphycus alvarezii* exhibited the highest and broadest antibacterial activity against *Staphylococcus aureus* (16 mm). On average chloroform extract exhibited minimal activity against both gram positive and gram

negative strains. The Chloroform extract showed maximum inhibition zone of 12 mm at maximum concentration against gram positive strains *Staphylococcus aureus* and *Bacillus subtilis*. A redundant pattern of dose-dependent antibacterial activity has been observed.Primarily, the activity has been directly proportional to concentration in the cases of the ethanolic extracts tested in well diffusion method and aqueous extracts in the disk diffusion method.Any differences in the efficiency of the disk diffusion and well diffusion could not be identified in the study.

Based on the prominent functional groups, HPLC data and primary phytochemical positives obtained, the major components in the extracts that exhibit antimicrobial activity could be quinones, sterol or isoflavonols.

Quinones are members of a class of cyclic organic compounds containing two carbonyl groups, > C = O, either adjacent or separated by a vinylene group, −CH = CH−, in a six-membered unsaturated ring (21). Vitamin K,known for its antihemorrhagic and antioxidant activity, is a complex naphthoquinone(Florkin, 2014). Some quinones inactivate proteins and cause loss of its function, by conjugating with nucleophilic amino acids in proteins in an irreversible reaction (Stern, 1996). This can affect surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes and cause cell lysis in microbes (Cowan, 1999). The quinones isolated from the *Kappaphycus alvarezii* should be subjected to further study as potential antibacterial candidate.

Specific phenolic secondary metabolites compounds have been reported for its antibacterial activity in the biological literature. This study has been one such approach to re-search the antibacterial activity shown by the crude extracts of *Kappaphycus alvarezii* and its bioactive constituents.

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