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Antioxidative and UV Light Protective Assessment of Microbial Carotenoids Isolated from Fresh Water Environment

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Abstract Fresh water Ulhas river, flowing through different regions of Thane district was the source used to isolate carotenoid producing bacteria. A total of 14 isolates producing yellow to orange pigmentation were screened. Cultural and morphological studies of the carotenoid producing isolates were examined followed by various chemical confirmatory tests for pigment verification. Antioxidant activity using DPPH method was used as a screening tool for determining significant and value added carotenoid. Potent carotenoid extracts IC_{50} value for antioxidant activity was found to be 51.56 ug/ml and 11.62 ug/ml by DPPH method for FBC2 and ascorbic acid respectively. VITEK 2 mediated process led to identification of *Sphingomomnas paucimobilis,* whose purified extract was used for exploring antibacterial, anticancer nature of the carotenoid. Carotenoid extract demonstrated significant photoprotection to UV light induced cell damage, as performed by cling film assay and was comparable to herbal sunscreens. Thus this extract could be a potent alternative to chemical antioxidants and UV light protective agents, also serving as non-toxic additions in sunscreens and ointments.

Keywords Carotenoid, Antioxidant , Photoprotection, Anticancer, Fresh water.

Introduction

Secondary metabolites have catered to innumerable basic human necessities, Microbial secondary metabolites include a vast array of compounds like antibiotic, alkaloids, pigments and toxins (Cragg and Newman 2013, Malik 1980). Color which is the first sensory attributes acknowledged and perceived by humans is imparted by pigments. Toxicity problems have restricted the use of inorganic pigments in food and beverage industry. Thus transition to biological organic pigments produced by natural producers like plants, animals and microbes over synthetic organic counterparts is the current demand. Their biosafety, stability, and biocompatibility, nature has generated applications in food and beverage, cosmetic, pharmaceutical and therapeutic fields (Manivasagan et al. 2018, Encyclop Britan 2018).

Carotenoids are a class of more than 750 different types making them the most diverse pigments observed in nature. Manivasagan et al. (2018) despite their diversity synthesis is restricted to plants, and a small niche of microorganisms. Structurally carotenoids are tetraterpenoids consisting of isoprene

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units containing 40 to 50 carbon atoms with 9-11 double bonds which may or may not terminate in ring. Extensive conjugated double-bond system, is a distinctive feature of carotenoid which contributes to its light- absorbing chromophore component. This attribute is responsible for the yellow, orange, or red color which we perceive. Hydroxylation, oxidation and hydrogenation of isoprene units results in numerous carotenoid derivatives having in extensive diversification structurally and functionally (Encyclop Britan, Nisar et al. 2015).

Reactive Oxygen Species (ROS) is a term used for molecular oxygen derived, reactive molecules and free radicals. In appropriate diet, lifestyle and radiations are significantly related to elevate levels of ROS. Oxidative stress is the term coined when ROS exceeds the defense mechanisms scavenging capacity. Excess ROS can cause oxidation of biomolecules like lipids, proteins, and DNA. It also affects membrane fluidity, ion transport, protein crosslinking, loss of enzymatic activity and irreparable DNA damage leading to cell death (Kiokias et al. 2016, Rodriguez-Amaya 2001). Oxidative stress is also a pre-disposing condition leading to cancer (https://dtp.cancer 2017). According to WHO cancer accounted for 18.1 million new cases and 9.6 million deaths in 2018. Considering the above prevalence and mortality rate the significance of antioxidants is gaining importance.

Carotenoids are highly efficient ROS quenchers *in vitro* and *in vivo*. Carotenoids can use physical (energy liberated in form of heat) and chemical (oxidation and oxygenation) mechanisms for deactivation of ROS. Oxidative stress which is a leading etiology of cancer can thus be reduced by efficient use of antioxidant carotenoids (Sharma et al. 2012). Established literature also suggests the role of various carotenoids in stimulation of immune response. Increasing blastogensis, enhancing NK cell cytotoxicity, and mitogen mediated lymphocyte proliferation are few of the mechanisms used by them to activate immune system, which can be effective against cancer cells. Prolonged exposure to both pro and non-pro Vitamin A carotenoids has shown resistance to tumor transplants in many animal models (Held 2012, Fiedor and Burda 2014, Seifter et al. 2012). Also customary photoprotection property of carotenoids can be harnessed to reduce UV lightinduced photo oxidation which is prominent cause of skin erythema (Watson et al. 1991).

The composition of carotenoid in plants depends upon cultivation practices, post-harvest handling, processing, and storage. Factors like stage of maturity, climate, cultivar, and infestations all of which create carotenoid inconsistency in plants (Kim et al. 2000, Stahl and Sies 2002). Greatest diversity of carotenoids is observed in microorganisms, which combines with microbial diversity to produce novel carotenoids. Microorganisms also can be tapped in using biotechnological, molecular techniques to give consistent and high yield of beneficial carotenoids (Namitha and Neg 2010). The present investigation deals with isolation of carotenoid producing organisms from fresh water environments and study of their many potential health benefits with major consideration on its antioxidative property.

Materials and Methods

Chemicals and bacterial media

All chemicals, solvents and bacteriological media are of analytical and microbiological grade, which were procured from Himedia and Loba chemicals, Mumbai.

Sample collection

Ulhas river flowing from Karjat to Thane was selected as fresh water source for isolation of carotenoid producers. Samples were collected from four regions namely Karjat, Badlapur, Ulhasnagar and Kalyan through which the river flows (Sadekar Environ Engineers 2017). Waters samples were collected from a distance of two meters from the soil boundary in pre-sterilized bottles. Isolation was done post transportation within a period of 4 h.

Isolation

Samples were first streaked on sterile Nutrient agar plates (peptone : 0.5 g, meat extract: 1 g, Nacl 0.5 g, Agar 2% pH 7.0) and incubated at 25^0C, observations were done every 24 h for five days. Pigmented

colonies from yellow to orange were selected and re-isolated on sterile Nutrient Agar plates.The transfer was repeated to affirm consistency in pigment production. Colony characteristics and Gram nature of the consistent pigment producing isolates was recorded.

Pigment extraction

For each isolate, cultivation was done to get cell mass of 10 g, which was then suspended in 50 ml of methanol and refrigerated for 24 h to allow extraction. Re-extraction was done if retention of pigment was observed in cell pellet. Post centrifugation, the supernatant was concentrated to 20 ml, which was used as crude extract. These crude extracts were used for screening of antioxidant activity. Isolates showing high antioxidant activity only were used for further purification as described by Marshall and Wilmoth method. In this process re-extraction was done with ethyl acetate and 1.7 M aq NaCl (1:1). The colored ethyl acetate was dried over di sodium sulfate. Dried residue was dissolved in methanol/ethyl acetate/ DMSO. Purified dry pigment dissolved in methanol was used for antioxidant study at different concentrations in triplicates. Purified dry pigment dissolved in DMSO was used for antibacterial and anticancer studies.

Chemical tests for carotenoids

Chemical tests like acidified pyridine test, hexane TBME test, sulfuric acid -petroleum ether test (Prado et al. 2014, Mijts et al. 2004) to confirm the presence of carotenoids. Each test is specific for carotenoid and positive result confirms the presence of carotenoid (Mark etal. 1949, McGraw et al. 2005).

Antioxidant activity test by DPPH and ABTS method (Mark et al. 1949)

The crude extracted pigment was used for detection of antioxidant activity as described by Umamaheswari and Chatterjee (2008), Mark et al. (1949). Anti oxidant activity was determined using standard oxidant DPPH (2.5 mg/l). Test : 0.2 ml of extract + 1.8 ml of DPPH. Control : 0.2 ml of methanol + 1.8 ml of DPPH. Measure discoloration after 30 minutes at 515 nm % antioxidant activity was calculated as follows :

$$\frac{(\text{ABs of C} - \text{Abs of T})}{(\text{Abs of C})} \times 100$$

Isolate showing more than 50% antioxidant activity was used for further studies. 0.1 mg/ml, purified extract of selected isolate was prepared in methanol for antioxidant activity confirmation.The study was done in triplicates by using two oxidants namely, DPPH and ABTS and ascorbic acid was used as standard antioxidant. ABTS method was followed as described by Re et al. (1999). 7 mM ABTS was added to 2.45 mM ammonium persulfate and kept in dark for 16 h for production of active ABTS radical cation. 500 ul of pigment was mixed with 3000 ul of ABTS and percent activity was determined by measuring decrease in coloration at 745 nm.

Identification of the isolate

VITEK 2 is a fast, easy and reliable automated method for identification of bacteria and yeast. It is highly reproducible and provides identification upto species level with accuracy greater than 95% (spanu et al. 2003). This system has well advanced hardware and software with very high safety standars for users. The instrument makes use of ID cards (identification cards), which has 96 wells, containing different substrates. Suitable card has to be selected depending upon the type of organism, GP (Gram positive card), GN (Gram negative card), YST (yeast card), ANC (anaerobic card) (Pincus et al. 2006). In the present study carotenoid producing isolate with high antioxidant activity was identified using Biomerieux VITEK2 (V2C13764) GN card.

Antibacterial and anticancer study

One percent, 5% and 10% of the purified extract prepared in DMSO was used for antibacterial study using agar cup method described by Miller and Rose (1939). Activity was checked against standard pathogenic cultures procured from IMTECH Chandigarh namely, *Escherichia coli* 1885, *Staphylococcus aureus* 3160, and *Pseudomonas* 1688. All the three cultures were maintained on Mueller-Hinton agar (MHA agar) and 24 h old culture was used for detection of

Table 1. Sampling location and their co-ordinates are as follows.

Site	Latitude	Longitude	Pollution
Ulhas River Bridge, Rameshwadi, Badlapur	19.1628^0 N,	73.2481^0 E	High
Ulhas River Bridge, Mohane Rd, Ulhasnagar	19.2559^0 N,	73.1638^0 E	High
Shree Ram Bridge, Ulhas River Karjat	$18.54'37^0$ N	$73.19'45^0$ E	Low
Ganesh Ghat, Durgadi Killa, Kalyan	19.2470^0 N	73.1183^0 E	High

activity; 1 ml of 0.5 OD of the above cultures were added to 20 ml molten MHA butt. 0.1ml of purified pigment extract was added in wells punched in agar by gel punchers of 6 mm diameters. Post incubation at 37^0C for 24 h, presence and measurement of zone of inhibition was done.

In vitro anticancer study of the pigment was done on human skin carcinoma cell line SK-MEL-2, selection of the cell line was a attribute to the feature of photoprotection of carotenoids. SRB assay was used for *in vitro* cytotoxicity and by protocols recommended by NCI USA (https://dtp.cancer.gov/discovery_development_nei-60/methodology.htm). Human SK-MEL-2 cell line was cultivated in RPMI media supplemented with 5% fetal bovine serum and incubated at 37^0C with 5% CO_2. Adriamycin and DMSO were used as positive control drug and negative control respectively. Shortly, four different concentrations (10-80 ug/ml prepared in DMSO) were added in triplicates to adherent cells in a 96-well format. Post incubation period, fixation of monolayers with 10% (w/v) trichloroacetic acid, followed by staining for 30 min. The excess dye was removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for absorbance determination at 510 nm using a microplate reader.

UV light protection activity by cling film assay

Cling film assay is a quick and easy method to screen potential ultra violet (UV) protecting compounds. This assay makes use of UV transparent film and a biological agent which acts an indicator organism for determining affectivity of the UV protective compound. The biological agent could be either *Escherichia coli* or any other ultra violet light sensitive organism. Compounds showing protective nature by this assay can get easy access and acceptibility in sunscreen and oinments.

In the present study, qualitative cling film assay was performed using *Escherichia coli* as the indicator organism. Sensitivity of the biological agent was determined by exposure to UV light prior to assay by exposing the culture to UV light (1 min to 10 mins, 254 nm). For the assay 0.5 ml of logarithmic phase *Escherichia coli* of 0.2 OD was spread on sterile Nutrient agar. This was followed by covering the surface of plate (without lid) with cling film. The surface of the film was coated by extracts and exposed to ultra violet light for 10 mins. After exposure incubation was done (with lid) at 37^0C for 24 h. Various controls were maintained with the above mentioned test, with similar incubations, and exposure conditions. Growth only belong the region of coating is considered as positive test.

Following controls were maintained. Control 1 : NA+Culture (Unexposed to UV), Control 2 : NA + Culture (Exposed in UV), Control 3 : NA+ Culture+ Film (Exposed to UV), Control 4 : NA + Culture + Film+Methanol (Exposed to UV), Control 5 : NA+Culture+Film+ Sunscreen Cream (Exposed to UV), Test : NA + Culture + Film + Pigment extract (Exposed to UV).

Results and Discussion

Isolation and study of isolates

Fresh water environments are characterized by having low concentrations of dissolved salts and dissolved organic matter, and are a major source of portable water. Ulhas river which stretches from Karjat to Thane has been significantly affected by pollution

Table 2. Potential pigmented isolates from various sampling locations.

Site	Subsite	Isolates	Number	Site	Subsite	Isolates	Number	Site	Subsite	Isolates	Number	Site	Subsite	Isolates	Number
Badlapur	L1	2	FBA1, FBA2	Karjat	L1	0	-	Ulhasnagar bridge	L1	0	-	Kalyan	L1		
	L2	0	-		L2	2	FKB, FKB2		L2	0	-		L2		
	L3	4	FBC1, FBC2, FBC3 FBC4		L3	1	FKC1		L3	0	-		L3		
	L4	2	FBD1,FBD2		L4	0	-		L4	2	FUD1 FUD2		L4	1	FKD1

which is having a negative effect and high variance in microbial flora. Though a high load of microbial flora were observed in all sites, our preliminary screening for pigmentation led to only14 isolates (Tables1, 2).Very few pigmented isolates were obtained from Ulhasnagar, Kalyan sites; pollution could be a probable reason as decrease or loss of pigmentation in bacteria has been found to be bioindicator of various inorganic pollutants (Mulik et al. 2017). All the 14 isolates were macroscopically different from each other and easy to cultivate and maintain on Nutrient Agar medium. Pigment loss or change can be observed due to variance in environmental parameters. Thus, consistent pigment production on synthetic media was checked by repeated sub-culture, all the isolates of varying color intensities from yellow to orange were found to maintain consistency.

From Table 3 it is clearly evident that majority of the isolates produce yellow color of varying intensities while only four isolates generate different color intensities of orange. This indicates a higher prevalence of xanthophyll group of carotenoids over the carotene type. Further in parallel with literature most of the pigment producers belonged to Gram positive cocci cluster while only two were Gram negative rods, suggesting prominence of pigment production in Gram positive cluster (Leiva et al. 2015). All the isolates showed positive results for confirmatory tests except FBD1 which is, the only isolate giving the sulfuric acid test negative. As these tests provide fruitful evidence for presence of carotenoids all were considered for antioxidant assay.

Antioxidant activity

Crude extracts (Fig. 1) of each isolate were successfully prepared to determine antioxidant activity using

Table 3. Isolates characteristics and their pigment confirmation.

Isolate	Number	Color	Gram nature	Pyridine	Hexane + TBME	Sulfuric acid test
FBA1	46	Yellow	Positive cocci	+	+	+
FBA2	47	DYellow	Positive short rod	+	+	+
FBC1	48	Yellow	Positive cocci	+	+	+
FBC2	49	Porange	Negative rods	+	+	+
FBC3	50	Dyellow	Positive cocci	+	+	+
FBC4	51	Orange	Positive cocci	+	+	+
FBD1	52	Yellow	Positive cocci	+	+	-
FBD2	53	Yellow	Positive cocci	+	+	+
FKB1	54	Orange	Positive cocci	+	+	+
FKB2	55	Porange	Positive cocci	+	+	+
FKC1	56	Pyellow	Positive cocci	+	+	+
FUD1	58	Yellow	Negative rods	+	+	+
FUD2	59	Yellow	Positive cocci	+	+	+
FKD1	57	Yellow	Positive cocci	+	+	+

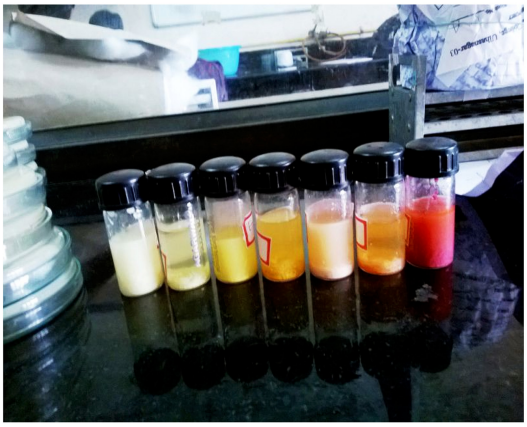


Fig. 1. Extracts prepared.

DPPH. One extract FBC2 (Fig. 2) showed highest antioxidant activity >50%, followed by FUD1, while most others demonstrated in range of 30% to 40%. Antioxidant activity owing to its significance was used as screening criteria and accordingly FBC2 isolate was used in further studies.

Concentrated FBC2 crude extract was purified and the yield obtained was found to be 500 mg /20 g of cell mass. This dried extract was dissolved in different solvents for respective activities.

Reconfirmation of antioxidant activity with different concentrations of purified FBC2 extract was done by DPPH and ABTS method in triplicates. Ascorbic acid was used as positive control. Antioxidant activity of FBC2 was found to increase with concentration of extract. IC_{50} values determined for FBC2 and ascorbic acid are 51.56 ug/ml and 11.62 ug/

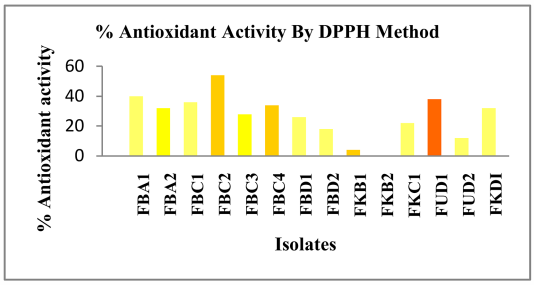


Fig. 2. Antioxidant activity.

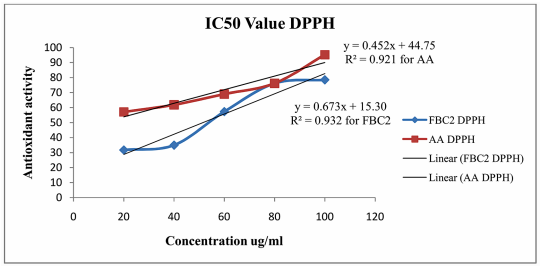


Fig. 3. IC 50 value for antioxidative activity by DPPH method.

ml respectively by graph calculation (Fig. 3). Since by ABTS antioxidant method the activity was substantially higher even at lowest concentration used, IC_{100} was used to express activity. IC_{100} activity of FBC2 and ascorbic acid was found to be 110 ug/ml and 80 ug/ml respectively as calculated by using trend analysis least square method (Fig. 4). Not much variance in activity is observed between standard antioxidant and FBC2 extract by ABTS. Thus FBC2 pigment extract could prove a potential antioxidant *in vivo.*

Identification

Gram negative card was used for identification by VITECK method. With the biochemical results (Table 4) the isolate was identified to be *Sphingomomnas paucimobilis* with 91% probability. VITEK 2 GN correctly identifies 96.8% of the isolates, misidentifications occurs at 3.0% and no identifications occur at 0.2%. Major literature suggests that *Sphingomomnas paucimobilis* produces a yellow pigment and our isolate demonstrates orange pigmentation which could be contradictory. However Busse, has isolated orange pigmented, novel species of *Sphingomomnas.*

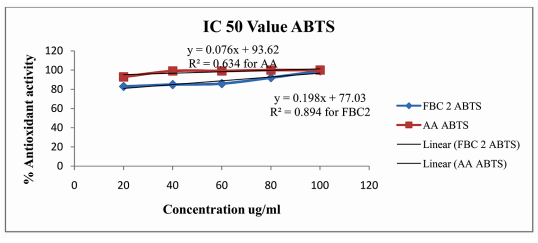
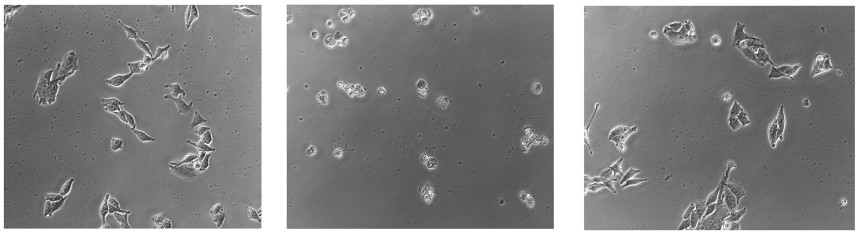


Fig. 4. IC 50 value for antioxidative activity by DPPH method.

Table 4. Biochemical characteristics of FBC2.

Biochemical details																
2	APPA	(-)	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCLE	-	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	-	14	GGT	-	15	OFF	-
17	BGLU	+	18	dMAL	-	19	dMAN	-	20	dMNE	(-)	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	+	42	SUCT	-	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	+	64	ILATa	-			



Figs. 5 - 7. UV light protection activity by Cling film assay : **Fig. 5.** FBC2 extract. **Fig. 6.** Adriamycin (positive cont). **Fig. 7**. Negative control.

Similarly, Asker isolated dark orange pigmented astaxanthin producing species of *Sphingomomnas* thus confirming presence of orange pigmentation (Pincus 2006, Asker et al. 2007, Busse et al. 2003). Thus, species confirmation should be followed up by 16s RNA identification.

Antibacterial and anticancer activity

No antibacterial activity of the extract was detected (1%, 5% and 10%) against standard pathogens by agar cup method. Similarly, no anticancer activity was demonstrated by the extract against human skin carcinoma cell line SK-MEL-2 at concentration upto 80 ug/ml (Figs. 5–8). Role of carotenoids in restraining cancer depends on its ability to activate immune cells as cited in literature. Another established role involves reducing oxidative stress, thus preventing the etiological agent in developing cancer. The above study confirms that *in vitro* carotenoids do not directly damage cancer cells. Effect of the extract against cancer cell line should be explored in the presence of immune cells before claiming its negative activity against cancer.

Ultra violet light protective activity

Cling film assay (Fig. 2) proved an effective tool to demonstrate UV light protection activity by FBC2. The indicator organism *Escherichia coli* was found to be very susceptible to UV light and complete growth inhibition was observed for exposure of 1 min at distance from 20 cm form the UV source. In case of FBC2 pure extract and sunscreen coatings on the thin film supported growth of *Escherichia coli* even after exposure to UV light for 10 mins. Owing to its natural origin, this photoprotection attribute of carotenoids

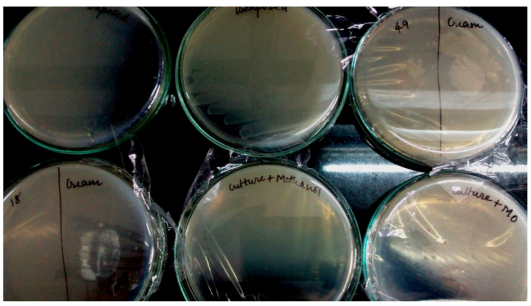


Fig. 8. UV light protective activity.

can be explored in various cosmetic products and sunscreens. Further its additional antioxidant nature can aid in preparations of various topical ointments to prevent UV light induced skin erythema (Dreher et al. 1999).

Conclusion

Fresh water source led to various carotenoid producing isolates. One isolate showing significant antioxidant activity by multiple methods was identified to be *Sphingomomnas paucimobilis* . Antibacterial, and anticancer activity was not demonstrated by the extract. UV light induced protective feature of the extract could thus be a safe alternative to chemical counterparts used in sunscreens and ointments.

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