Pretreatment of Zeaxanthin Extracted from Thermotolerant Strain *Synechocystis pevalekii* Protects 3T3 Fibroblasts against Ultraviolet Radiations.

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Abstract- Carotenoids like Lycopene, β- Carotene, Lutein, Zeaxanthin extracted from various sources are widely studied for their effectivity in sun protection. In our previous studies Zeaxanthin extracted from a thermotolerant algal strain isolated from thermal springs from Western Ghats of Maharashtra *Synechocystis pevalekii* was incorporated in cosmetic formulation which showed a very good boot star rating and proved to be a potential candidate to be used as a sunscreening ingredient. The current report focuses on testing the efficacy of Zeaxanthin on 3T3 fibroblast cell line in vitro. The cytotoxicity of Zeaxanthin was studied by MTT Assay. Maximum proliferation of the cell was observed at 25μg/ml of Zeaxanthin. Concentrations beyond 250μg/ml were found to be cytotoxic. Further Zeaxanthin was tested for its ability to protect the cells from UV radiation. The 3T3 fibroblasts were irradiated by UV A and UV B rays. The dosages of UV A employed was 1.8mW/cm^2 for 600 secs (1.08J/cm^2) and for 1800 secs (3.24J/cm^2) and that of UV B were 5mW/cm^2 for 500 secs (2.5J/cm^2). Zeaxanthin was capable of protecting fibroblasts against UV A radiation upto 1.08J/cm^2 and against UV B radiation upto 2.5J/cm^2.

Key words: Cytotoxicity, Fibroblasts, Proliferation, Sunscreen, Thermal springs Thermotolerant, UV radiation, Zeaxanthin.

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1 INTRODUCTION

In vitro technique refers to performing a given procedure outside a living cell. There is a lot of debate over the use of *in vitro* assays over the *in vivo* testing methods. There is a complete ban on using animal testing for any acute toxic effects of beauty products and toiletries in an amendment to the European Union's cosmetic directive. Groups from the industry, research institutes, universities and organizations of EU have joined hands to initiate a programme called 'Sens-it-iv'. The goal of this project is to develop an in vitro strategy to predict the sensitizing potency of compounds, enabling the full replacement of animals in safety testing **[1].**

There are many advantages involved in the use of *in vitro* assays over *in vivo* testing methods. *In vitro* methods avoid and decrease the involvement of the specialized laboratory personnel that are experienced in animal handling. Also, the use of in vitro methods eliminates the need to submit animal protocols to The Institutional Animal Care and Use Committee (IACUC's). Lastly because of the ease of the technique, ease of handling and because of economic considerations in vitro methods are for frequently used for large scale production in pharmaceutical industry **[2].**
In vitro assays are widely used to study different parameters such as wound healing **[3]**, mechanism and extent of angiogenisis **[4]**, anti diabetic studies **[5]** drug toxixcity, study intestinal permeability, drug metabolism, excretion and toxicity **[6]** as well as cancer studies.
Many studies have been carried out on the effectivity of carotenoids in sun protection by *in vitro* assays. Systemic photoprotection by endogenous supply of carotenoids like β- Carotene and Lycopene may provide a life long protection against UV radiations **[7].** β-Carotene and Lycopene when supplemented with Vitamin E help in prevention of oxidation thus acting as an antioxidant and in turn protecting the skin **[8].** Daily supplementation of Lutein and Zeaxanthin decreased the intensity of erythema caused by UV exposure as compared to the placebo. *In vitro* studies on Lutein and Zeaxanthin suggest that these carotenoids are more resistant to degradation as compared to other Carotenoids **[9].**

In the previous study, Zeaxanthin component of the crude extract of *S pevalekii* showed a very good activity when incorporated in cosmetic formulation. The formulation always showed a 5 star boot star rating along with improved parameters like UV A/ UV B ratio and the Critical wavelength that proved it to be potential sunsccreening agent. Avobenzone; a commercial UV blocker that is unstable individually in any cosmetic formulation and requires various stabilizers to stabilize it and maintain its activity, was also found to be stabilized by Zeaxanthin and also work synergistically with it to improve the quality of sun screen formulation. We wanted to assess if the purified Zeaxanthin abolishes effect of UV radiation *in vitro* in order to establish the algal Zeaxanthin non toxic, skin compatible and a natural sun filter safe to be used in sunscreen formulation.

2 MATERIALS AND METHODS

2.1 EXTRACTION OF ZEAXANTHIN

1g of wet biomass was used for extraction of Zeaxanthin. The biomass was soaked in10mL of 100% methanol and incubated at 4°C overnight. After extraction, the mixture was centrifuged for 5 min at 4000 rpm and supernatant (Crude extract) was collected, dried and purified by silica gel open column chromatography. The dried extract was loaded onto the packed silica gel column and then

eluted with a gradient of n- hexane: acetone. Zeaxanthin was extracted as one of the bands. The separated fraction was analyzed spectrophotometrically and analysed by HPLC. This dried extract was used for *in vitro* experiments.

2.2 CELL CULTURE

The 3T3 embryonic mouse fibroblast cells were procured from National Center for Cell Science, Pune. The cells were maintained in Dulbeccos modified Eagles medium (DMEM) (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, Genetix Biotech) at 37°C in 5% CO_2. No antibiotic supplementation was done to the medium.

2.2 CYTOTOXICITY ASSAY

Toxicity of the extract was studied by the MTT colorimetric assay. 3T3 Mouse Embryonic fibroblasts cells were seeded in 96 well plate (1 X 10^3 cells/well) and were treated with various concentrations of the partially purified extract (ranging from 10,000µg/ml to 50µg/ml) for 24 hours. Cells were then incubated with 5mg/mL MTT (Hi-Media) for 4h at 37°C. 100µl dimethyl sulphoxide was added to each well and absorbance was read on enzyme-linked immuno sorbent assay reader at 570 nm.

2.3 UV EXPOSURE

A UV-A tube (Philips, India) was used as a UV-A source with a wavelength range of 340- 400 nm and peaked at 365 nm, whereas a UV –B Bulb (Philips, India) emitting a radiation from 280- 320 nm and peaked at 312 nm served as a UV-B source. For UV –A related study the cells were irradiated at an intensity of 1.8 mW/cm² for 10 mins and 30 mins with a total UV –A radiation of 1.08 J/cm². For UV – B related study the cells were irradiated at an intensity of 5 mW/cm² for 500 s with total UV-B radiation of 2.5 J/cm². After irradiation, cells were incubated at room temperature for 30 mins and studied further.

2.4 CELL VIABILITY

Viability and the effect of Zeaxanthin were also studied by MTT colourimetric assay. 25µg/ml of the extract was added to the test wells at the time of seeding. The fibroblasts were pretreated with the extract for 24hours. After incubation the cells were irradiated with the respective intensity of UV radiation. After irradiation the cells were incubated in serum free medium for 30mins at room temperature and the tested for viability by MTT Assay.

3 RESULTS

3.1 CYTOTOXICITY STUDY OF ZEAXANHIN ON 3T3 FIBROBLAST CELL LINE

This study was conducted to determine the effect of various concentrations of the partially purified extract on the viability of the 3T3 fibroblast cell line. The cells were seeded in 96 well plate at a density of 1 X 10^3 cells/well in DMEM supplemented with 10% FBS. On the second day of the assay,10µl of different concentration of the extract ranging from 5µg/ml to 1000µg/ml was added to the seeded fibroblasts and incubated for 24 hours. The assay was terminated on the third day by addition of the MTT reagent to study the number of viable and non viable cells. Table 4.1 shows the effect of these concentrations on the growth of the fibroblast cells.

The viability observed in the PBSA control was 0.774 ± 0.091. When 5µg/ml of extract was added the wells, the viability was 0.777 ± 0.076 and the percentage proliferation was 0.39%. As the concentration of the extract was further increased, to 10µg/ml the viability of the cells also increased to 0.871 ± 0.047which showed a corresponding increase in the percentage proliferation i.e. 12.53%. However, it was when 25µg/ml of extract was added to the cells in terms of optical density was found to be the maximum i.e. 1.077 ± 0.040 with a maximum proliferation in the percentage of viable cells i.e. 39.14%.

On addition of a still higher concentration of extract i.e. 50µg/ml the viability was found to drop from 1.077 ± 0.040 (for 25µg/ml) to 1.026 ± 0.004showing a corresponding decrease in the percentage proliferation to 32.55%. After 25µg/ml concentration of extract the viability of the cells was found to decrease. In case of 100µg/ml the viability further dropped to 0.948 ± 0.052with percentage proliferation of 22.48%. And on addition of 250µg/ml of extract the percentage proliferation drastically dropped to 2.84%. The concentration of the extract till 250µg/ml showed viability more than the growth in PBSA. Thus there was proliferation till 250µg/ml. the extract was not cytotoxic upto 250µg/ml.

For concentrations beyond 250µg/ml i.e. 500µg/ml, 800µg/ml and 1000µg/ml the viability obtained was 0.433 ± 0.006, 0.434 ± 0.005 and 0.143 ± 0.004 respectively. Which was lesser than the viability observed in PBSA. Thus we can infer that the higher concentrations of the extract are cytotoxic.

For further experiments with UV exposure 25µg/ml which gave the optimum proliferation was chosen.

Concentrations of Extract (µg/ml)	OD Mean ±SD	% Proliferation
PBSA Control	0.774 ± 0.091	0.00%
5	0.777 ± 0.076	0.39%
10	0.871 ± 0.047	12.53%
25	1.077 ± 0.040	39.14%
50	1.026 ± 0.004	32.55%
100	0.948 ± 0.052	22.48%
250	0.796 ± 0.021	2.84%
500	0.433 ± 0.006	-44.06% No proliferation
800	0.434 ± 0.005	-43.00%

		No proliferation
1000	0.143 ± 0.004	-81.52% No proliferation

Table 1: Effect of various concentrations of Zeaxanthin extract on viability of fibroblast cells.

3.2 ROLE OF ZEAXANTHIN IN PROTECTION OF 3T3 FIBROBLASTS WHEN EXPOSEDTO TO UV RADIATIONS

3.2.1 EXPOSURE TO UV RADIATIONS

The cytotoxicity experiments suggested that the extract is nontoxic till a concentration of 25µg/mL. Taking into consideration the effective concentration of 25µg/mL, assays to determine the UV protective capacity of the extract was studied using the two different UV lamps (UV-A and UV-B).

For UV A exposure the cells were irradiated to UV A Philips tube (wavelength maxima 340nm to 400nm) of intensity of 1.8 mW/cm² for variable time intervals. When irradiated for 600 secs the cells were exposed to 1.08 J/ cm² and when it was exposed to 1800 secs the cells were exposed to 3.24 J/ cm². Similarly for the cells were irradiated to UV- B bulb (Philips, wavelength maxima 280- 300nm) of intensity, of 5 mW/cm² was irradiated for period of 500 secs with an effective power of 2.5 J/ cm².

The UV irradiations dosages used are elaborated in table 2

UV Radiation	Intensity (P)(mW/cm²)	Time (secs)	Energy (J/ cm²)
UV- A	1.8	600	1.08
UV- A	1.8	1800	3.24
UV- B	5	500	2.5

Table 2: UV radiation doses used for irradiation of fibroblasts.

3.2.3 ROLE OF ZEAXANTHIN IN PROTECTING 3T3 FIBROBLASTS AGAINST UV A RADIATION

Table 3 shows the effect of UV radiation on 3T3 fibroblasts with and without Zeaxanthin.

The cells that were not exposed to any UV radiation were treated as control cells in these cells the viability was 0.95 ± 0.04 whereras in cells treated with 25µg/ml of Zeaxanthin the viability count was 1.02 ± 0.06. thus confirming that 7.36% proliferation was observed when 3T3 cells were supplemented with Zeaxanthin.

When the cells were exposed to 1.08 J/ cm² of UV A radiation the viability of the cells was found to decrease to 0.76 ± 0.05 which means that there was abiut 20% decrease in viability when exposed to UV A radiation.

But zeaxanthin treated cells when exposed to 1.08 J/ cm² the decrease in viability of the cells was only 4%proving the effectivity of Zeaxanthin to protect 3T3 cells againstUV radiation.

But exposure to 3.24 J/ cm² was found to be deleterious at this exposure and Zeaxanthin was not much effective ion protecting fibroblast degeneration.

	No Exposure (Control)		**UV- A (1.08 J/ cm²)**		**UV- A (3.24 J/ cm²)**	
	Cytotoxicity (OD)	**% Proliferation**	**Cytotoxicity (OD)**	**% Proliferation**	**Cytotoxicity (OD)**	**% Proliferation**
Without extract	0.95 ± 0.04	0%	0.76 ± 0.05	-20%	0.70 ± 0.05	-26.31%
With extract	1.02 ± 0.06	7.36%	0.91 ± 0.09	-4.21%	0.73 ± 0.05	-23.15%

Table 3: Effect of Zeaxanthin extract on cell viability of fibroblasts exposed to various doses of UV A irradiation.

Recently preventive effect of Ferulic acid on Human dermal fibroblasts against UV A radiation has been studied by Hahn et al 2016. Initially Proliferative/ Cytotoxic effect of various concentrations of Ferulic acid ranging from 0- 50µM was studied on Human dermal fibroblasts. 20µM of ferulic acid produced optimum proliferation. 20µM ferulic acid brought about 5% increase in cell viability of fibroblasts. Hence 20µM of ferulic acid further used for protecting human dermalfibroblasts from UV A radiation.

Further Human dermal Fibroblasts were exposed to UV A radiation ranging fro 5- 20 J/ cm². When fibroblasts were exposed to 5 J/ cm² of UV A radiation the viability was decreased by 20% whereas fibroblasts previously treated with 20µM of ferulic acid for 6hours prior to UV radiation, the decrease in the viability was only 5%.

When fibroblasts were exposed to 10 J/ cm² of UV A radiation the decrease in cell viability was about 30% whereas fibroblasts pretreatred for 6 hours with 20µM of ferulic acid the viability decreased by about 10%. Hence ferulic acidwas protecting fibroblasts upto 10 .08 J/ cm²

Many other studies have been carried out to study the effect of UV A radiations on fibroblasts. A dose of 15J/ cm² was also provided to 3T3 fibroblasts to study the protective effect of Melatonin on it. It was found that melatonin acted as an antioxidant and suppressed UV A induced photo damage **[10].** *Vaccinium myrtillus* has many polyphenols and anthocyanins with multiple pharmacological properties. The water extract of *V. myrtillus* was studied for its UV protective ability in Human keratinocyte cell line. These cells were

pretreated with extract for 1 hour in serum free medium followed by UV A irradiation (8- 40mJ/cm²). The extract was able to reduce genotoxicity, unbalance of redox intracellular status and reduced apoptosis. Thus *V. myrtillus* extract showed free radical scavenging properties reducing oxidative stress and apoptotic markers in UV A irradiated cells **[11].** Modulatory effect of Astaxanthin on UV A irradiated cells was studied by **Lyon and O'Brien [12].** Different cell lines namely Human skin fibroblasts (BA3), human dermal melanocytes and colonic adenocarcinoma cell line were exposed to a fixed dose of UV A radiation for definite time period either in presence or absence of Ataxanthin. Following exposure to UV A radiation DNA damage was studied using single cell gel electrophoresis assay (comet assay). The cells were incubated with 10nM, 100nM and 10µM of Astaxanthin extract for 18 hours and subsequently irradiated with UV A. in 1BA3 human fibroblast cell line Astaxanthin prevented UV A induced DNA damage in all three concentrations viz, 10nM, 100nM and 10µM. For human dermal melanocytes and colonic adenocarcinoma cell line lower concentrations i.e. 10nM, 100nM were not enough to significantly prevent the DNA damage. 10µMof Astaxanthin significantly protected cells from DNA damage.

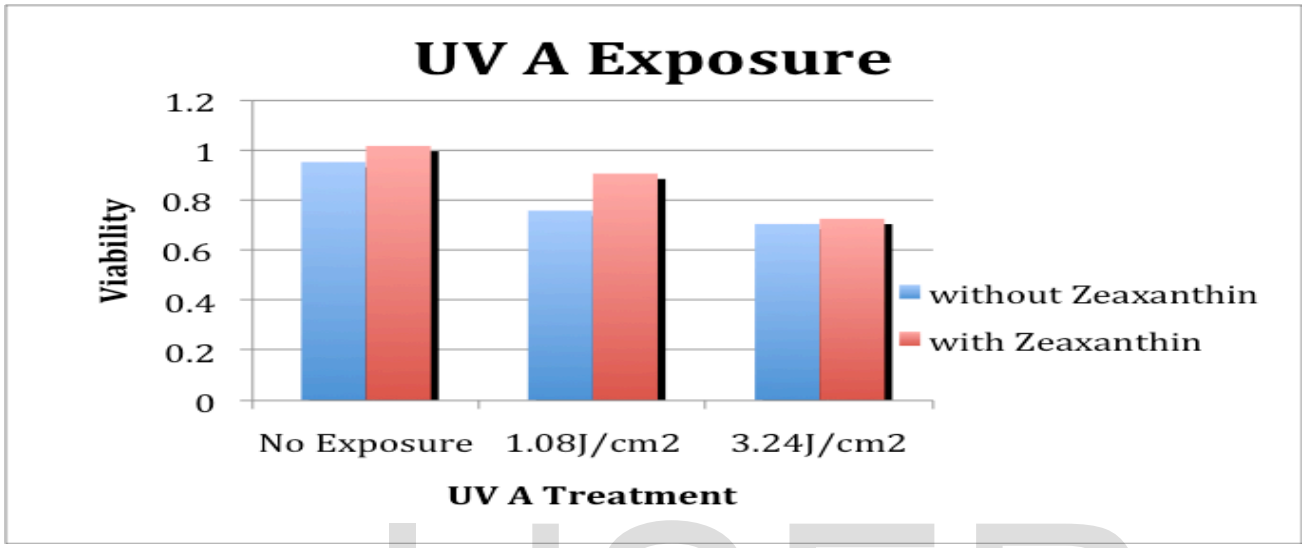

Fig. 1. Viability of fibroblasts exposed to various doses of UV A radiation.

3.2.4 ROLE OF ZEAXANTHIN IN PROTECTING 3T3 FIBROBLASTS AGAINST UV B RADIATION

Same concentration (25µg/mL) was used to study the effect of UV- B radiations on the viability of the fibroblast cells and also to study the effectivity of Zeaxanthin. Radiation intensity 5 mW/cm² for 500 s (2.5J/ cm²) was used to irradiate the cells with and without the extract. To study the effect of Zeaxanthin 25µg/ml of Zeaxanthin was used to pretreat the cells.

The cells that were not exposed to any UV radiation were treated as control cells in these cells the viability was 0.95 ± 0.04 where as in cells treated with 25µg/ml of Zeaxanthin the viability count was 1.02 ± 0.06. This accounts to 7.36% proliferation which was observed when 3T3 cells were supplemented with Zeaxanthin.
When the cells were exposed to 2.5 J/ cm² of UV B radiation the viability of the cells was found to drop down to 0.74 ± 0.05 accounting to about 22% decrease in viability when exposed to UV B radiation 2.5 J/ cm² .

	No Exposure		**UV- B (2.5 J/ cm²)**	
	Cytotoxicity (OD)	**% Proliferation**	**Cytotoxicity (OD)**	**% Proliferation**
Without extract	0.95 ± 0.04	0%	0.74 ± 0.03	-22%
With extract	1.02 ± 0.06	7.36%	0.75 ± 0.04	-21%

Table 4: Ability of Zeaxanthin to protect fibroblasts when exposed to UV B radiation.

Effect of polyscaaharide from *Ganoderma lucidum* in protection against UV B radiation was studied by **Zeng et al [13].** Human dermal fibroblasts were seeded on 96 well plate following attachment to the dish. All media was vacuumed prior to UV B exposure. Fibroblasts were exposed to a range of UV B doses ranging from 0mJ/cm² to 200mJ/cm². After exposure cells were cultured in serum free medium for 24 hours. The cell viability was assayed by MTT Assay. Following UV B exposure the cell viability decreased in dose dependent manner. The lowest dose that significantly reduced cell viability compared to unexposed cells was 60mJ/cm². To study the effect of *Ganoderma* polysaccharide in UV protection following exposure to 60mJ/cm² UV B, the fibroblasts were treated with 10µg, 20µg and 40µg of *Ganoderma* polysaccharide. Increase in cell viability was compared with untreated cells. The aging cells were detected by SA β Gal kit. The aged cells are stained blue with SA β Gal. the percentage of stained cells was 25.95%in the cells not exposed to UV

B whereas in the case of UV B exposed cells the percentage of aged cells was 63.53%. No significant difference in staining of cells was observed. In UV B exposed cells treated with 10 (57.00%) and 20µg/ml (55.98%) polysaccharide. However for UV B exposed cells treated with 40µg/ml of polysaccharide there was a significant decrease in dead cells (50.96%) that were stained.

Taraxacum officinale i.e. Dandelion extract was found to be non cytotoxic from 10µg/ml to 300µg/ml to Human dermal fibroblasts (HDFs). When the extracts were added to the HDFs, either before UV exposure (pretreatment) or just immediately after exposure. The UV B dose administered was 200mJ/cm² for 60 seconds. It was found that the leaf and the flower extracts significantly protected the cell from UV B induced cell death. Dandelion leaf and flower extracts also were found to inhibit UV B irradiation stimulated MMP activity and oxidative stress **[14].**

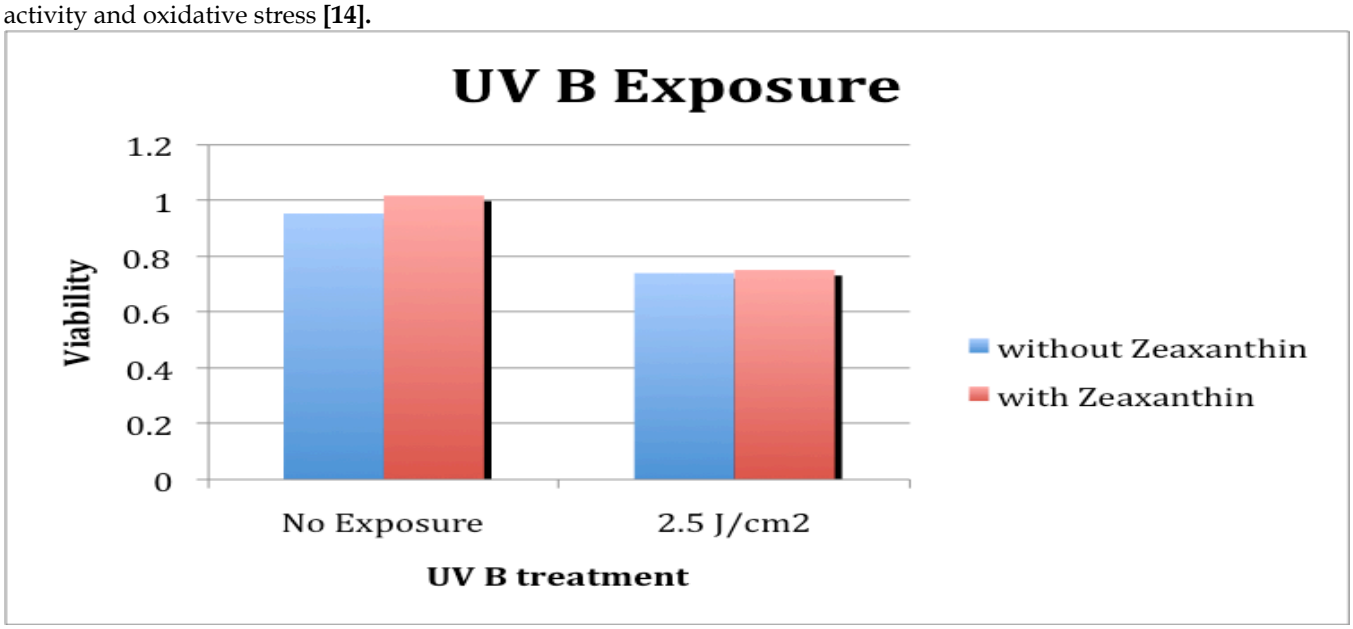

Fig. 2. Effect of Zeaxanthin on dermal fibroblasts exposed to UV B radiation.

It appears that UV B exposure of 2.5J/cm² is very high. UV B exposure of 60mJ/cm² decreases the viability of fibroblast significantly. It may be possible that our extract might work at lower doses of UV B radiation.

CONCLUSION

In our previous studies had been carried out on the effectivity of Zeaxanthin in cosmetic formulation. It was observed that zeaxanthin in a formulation was stable, improved the sun protection parameters related to UV A protection and also was able to stabilize Avobenzone- a chemical UV A protector. These results correlated well with the *in vitro* tests. The extract was not cytotoxic till a concentration of 25µg/ml beyond which it did not show any cell proliferation. Zeaxanthin was found to protect the fibroblasts from the damaging UV A rays but not from the UV B radiation the the dose employed was very high. However it calls for further experiments to fine tune the UV B dosage and study the effect with different concentrations of the extract i.e. Zeaxanthin.

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