ISOLATION OF PIGMENT-PRODUCING BACTERIA FROM SURFACE WATER AND STUDY SUN PROTECTION FACTOR (SPF) OF THE PURIFIED PIGMENTS

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Abstract- During the past two decades research on aquatic bacteria has highlighted because the tremendous potential of these microorganisms as a source of new bioactive secondary metabolites and it is reported that most of the aquatic bacterial pigments exhibited biological activity. In the present study, pigmented bacteria were isolated from surface water samples collected from North West of Iran. The extracted pigments were included: orange pigment (extracted from Exiguobacterium sp), red pigment (extracted from Serratia marcescens) and pink-red pigment (extracted from Serratia marcescens), SPF value of the pigments were found to be 1.785,1.629 and 2.72 respectively The results revealed that bacterial pigments have the ability to absorb UV radiation. Therefore, these agents atoptimum concentrations could produce several beneficial effects to the skin apart from functioning as an uv filters.

Key words- Pigment, Bacteria, Extraction, SPF

I. INTRODUCTION

Pigments produce the colors that we observe at each step of our lives, they are in leaves, fruits, vegetables, and flowers; also, they are present in skin, eyes, and other animal structures; and in bacteria and fungi [1] Pigments are classified as either organic/inorganic or natural/synthetic[1,2,3] . Synthetic pigments are obtained from laboratories. It is well known that synthetic pigments cause adverse toxicological side effects [2],considerably environmental pollution[2,3] and the Carcinogenesis[3,4]. Natural pigments are produced by living organisms such as plants, animals, fungi, and microorganisms[1,4]. Also Natural pigments possess anticancer activity and have some desirable properties like stability to light, heat and pH [2].

There is growing intereste in microbial pigments due to their natural character and safety to use, medicinal property ,nutrients like vitamins, production being independent of season and geographical conditions and conrollableand predictable yield [4].

Bacteria produce pigments for various reasons and it plays an important role [5]. For example Cyanobacteria have two types of sunscreen pigments, scytonemin and mycosporine-like amino acids (MAAs). These secondary metabolites are thought to play multiple roles against several environmental stresses such as UV radiation and desiccation [6]. The role that MAAs play as sunscreen compounds to protect against damage by harmful levels of UV radiation is well established [6,7,8]. Scytonemin is synthesized in response to UV-A exposure and forming a stable, protective layer that absorbs as much as 90% of further incident radiation [7,9,10]. Melanins are usually described as pigments that protect against a number of environmental stress conditions, In addition to its UV absorbing properties, melanin also offers protection as a cellular scavenger against free radicals, ROS, drugs, oxidants and xenobiotics[11]. Marinomonas mediterranea, Vibrio cholerae, Shewanella colwelliana and Alteromonas nigrifaciens were some of the first marine bacterial strains described to produce melanin or melanin like pigments [9,12]. In 2004, the United States issued a patent for compounds having MAAs, in addition to scytonemin and carotenoids, as active sunscreen agents that can be used as solar protection for humans[13]

The UVR that reaches the earth's surface consists mainly of long wavelength ultraviolet A (UVA) (320 – 400 nm) radiation but only a minority (estimated at 5%) of short wavelength ultraviolet B (UVB) (280 - 320 nm)[16,17,19].UVC (200 – 280 nm) is filtered by the ozone layer [15,16]. UVB is higher energy and is responsible for sunburn and direct damage to DNA.More recently identified is the role of lower energy UVA radiation in causing direct and indirect DNA damage by free radical generation, photoageing, immune suppression and photocarcinogenesis[14].

The use of sunscreen products has been advocated by many health care practitioners as a means to reduce skin damage produced by ultraviolet radiation (UVR) from sunlight [17]. in 1974 the term Sun Protection Factor (SPF) was introduced by Greiter [18] The efficacy of a sunscreen is usually expressed by the sun protection factor (SPF), which is defined as the UVenergy required to produce a minimal erythema dose(MED) on protected skin, divided by the UV energy required to produce a MED on unprotected skin[14,16,17,18]. These products were designed to block the ultraviolet B (UVB) rays that cause sunburn but had little effect on ultraviolet A rays (UVA). An SPF of 15 means that if it takes10 minutes for skin to

start to burn without sunscreen it will take 150 minutes with that sunscreen[14].

Today many sunscreen/cosmetic compositions have been discovered from bacteria, which have been adopted for the photoprotection of human skin and/or hair. Since some bacterial pigments are able to UVR absorption therefore can be estimate sun protection factor of desired pigment by in vitro methods The purpose of this research is isolation of pigment-producing bacteria from surface waters and study Sun protection factor (SPF) of the extracted pigments.

II. MATERIAL AND METHODS

Sampling

Ten surface water samples were obtained from different area of Northwestern Iran. All sampling procedures were carried out according to standard methods for the examination of water. Pre-sterilized 100 mL Schott bottles were filled with water samples (for liquid samples), about 2.5 cm, were left to facilitate mixing, aeration encountered during handling and transportation. Water samples were transferred immediately to laboratory for further experiment.

Isolation of pigmented bacteria

Each of the liquid samples (25 mL) was aseptically transferred into a series of 250 mL Erlenmeyer flasks containing 125 mL Zobell Marine Broth2216 medium (HiMedia-india) followed by incubation at 30 ^{0}C, 180 rpm for 24 -48h. A 10-fold serial dilution of each flask was prepared while 100 µL of $10^{-5}$and 10^{-6} dilutions was plated onto marine agar and incubated at 30°C for 24h. Serial sub-culturings were carried out until single pigmented bacterial colonies were obtained.

Phenotypic characterization

The phenotypic and biochemical characterization of the selected strain was done by using standard techniques including gram staining, API20E, oxidase and catalase tests.

DNA Extraction Procedures

The colonies have solved 1cc sterile distilled water at 1/5 micro tubeand were centrifuged 10 min at 3000 rpm.The supernatant was discarded and dry sedimentand 100 µl lysozyme and 75 µl SDS (10%) added after mixing with Vertex.The samples were incubated for 15 min at room temperature.Then, 150 µl phenol equilibrium and 300 µl chloroform added to it then mixture to become milky.Samples were centrifuged for 15 min at 10,000 rpm.Aqueous phase transferred to a new vial and the same volume was added chloroform to the mixture. Then Samples were centrifuged for 15 min at 10,000 rpm.Again, the aqueous phase transferred to a new vial and 2/3 volume was added of cold isopropanol and mixed.The samples for one hour at -20 °C was placed.After that, the samples were centrifuged for 15 min at 14,000 rpm. The supernatant discarded and 200 ml ethanol (70%) was added to sediment, and 1 min centrifuged at 14,000 rpm.Supernatant discarded incubated for 15 min at 37 °C Dry plate.The sediment added was 30 ml sterile distilled water and kept in the freezer -20 °C [10].

PCR and gel electrophoresis

The genomic DNA thus obtained was amplified using universal primers. PCR primers was 27f (5'-AGA GTT TGA TCCTGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3'). PCRs were performed with a thermocycler (SensoQuest, Germany) by using 25µl (final volume). The content of reaction mixtures indicated in Table 1. We used 30 PCR cycles consisting of 95°C for 5 min, 58.5°C for 1 min, and 72°C for 1.15 min, preceded by 5 min of denaturation at 95°C and followed by a final extension for 10 min at 72°C. Negative controls containing all of the components of the PCR mixture except DNA templates were included. The PCR products were examined on a 1% agarose gel in Tris-borate-EDTA buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) containing ethidium bromide for DNA staining and visualization.

To assertion the phylogenetic position of obtained from GenBank (National Center for Biotechnological Information; http://www.ncbi.nlm.nih.gov). Multiple alignments of the sequence were performed by BioEdit software. A phylogenetic tree was constructed with the evolutionary distances using the Neighbor-joining method. Tree topologies were evaluated by performing bootstrap analysis of 100 data sets with the CLUSTALW software.

Table 1: Reaction mixtures for PCR

material	Mount
Master mix	12.5 µl
Forvard primer	1 µl(10 pmol)
Revers primer	1 µl(10 pmol)
DNA tamplate	5µ l
ddw	55 µl
Total	25 µl

Extraction of pigments

The bacterial cells were first grown for 48 h in marine broth medium followed by centrifugation at 9000 rpm for 20 min. The bacterial cell pellet was then discarded while the supernatant was extracted using ethanol. The concentrated pigment was then transferred onto glass beaker prior to drying in oven at 78C.

Detection of SPF

The in vitro SPF number was determined according to the spectrophotometric method described by Mansur et al that calculated by using the following equation [4]:

$SPF=CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times ABS(\lambda)$

Where: EE (l) – erythemal effect spectrum; I (l) – solar intensity spectrum; Abs (l)- absorbance of sunscreen product ; CF – correction factor (= 10). It was determined so that a standard sunscreen formulation containg 8% homosalate presented a SPF value of 4, determined by UV spectrophotometry (Mansur et al., 1986). The values of EE x I are constants. They were determined by Sayre et al. (1979), and are showed in Table 2.

exact weight of pigments must be specified for determine the SPF . To do this, a weight of beaker was measured before and after the addition of pigment, weight difference was represents the pigment weight. The absorption spectra of samples with 200µg/ml concentration in solution were obtained in the range of 290 to 450 nm using quartz cell, and ethanol as a blank. The absorption data were obtained in the range of 290 to 320, every 5 nm.

Table 2: Wavelength (λ nm) EE x I (normalized)

Wavelength (λ nm)	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
	Total 1

Results

In this study seventeen numbers of pigmented bacterial strains were isolated from water samples. Among them three isolates (S_2-A_1, S_3, S_8-$_1$) was selected for further studies. The biochemical characterizations of the three pigment producing bacterial strains were discussed in Table 3. All of the isolates were grown at the temperature of 25°C to 42°C and the optimum temperature was 37°C but color intensity of bacterial colony was more in 30°C. Samples were incubated in a shaking and without shaking. The amount of pigment producing by bacteria in the absence shaker was considerable.

The genomic DNA of the strains was isolated and subjected to 16s rRNA gene amplification and sequencing.. The size of amplified product was around 1.5kb (Fig.2). The BLAST search with homologous sequences in NCBI database revealed that S2-A1 strain showed maximum (100%) identity with Exiguobacterium sp MB239(Accession number : KJ833797.1), S8-1 strain showed maximum (100%) identity with Serratia marcescens(Accession number: JQ00772801) and S3 strain showed 97% similarity to Serratia marcescens strain MUGA231(Accession number: KJ672384.1). The phylogenetic tree was presented in Fig. 1.

After determination of the weight of pigments and preparation of 200µl dilution by ethanol solvent (Table4), SPF was calculated by applying Mansur mathematical equation. The absorbance and SPF values of the samples calculated through UV-Spectrophotometric method are shown in Tables 5 and Table 6 respectively

Table 3: Morphological and Biochemical characterization of pigment producing isolates

No	Characteristic	S2-A1	S3	S8-1
1	Morphological characteristic			
	Gram reaction	+	-	-
	Cell shape	Coccobacilli	Bacilli	Bacilli
	Colony Morphology In MB	Convex and Orange	Convex and Red-Pink	Convex and Red
	Optimum growth temperature (°C)	37	37	37
	Optimum prduce pigment temperature (°C)	30	30	30
2	Biochemical characteristic			
	Catalase	+	+	+
	Oxidase	+	-	-
	Indole	-	-	-
	VP	-	+	+
	Citrate	-	+	+
	Gelatinase	+	+	+
	H_2S	-	-	-

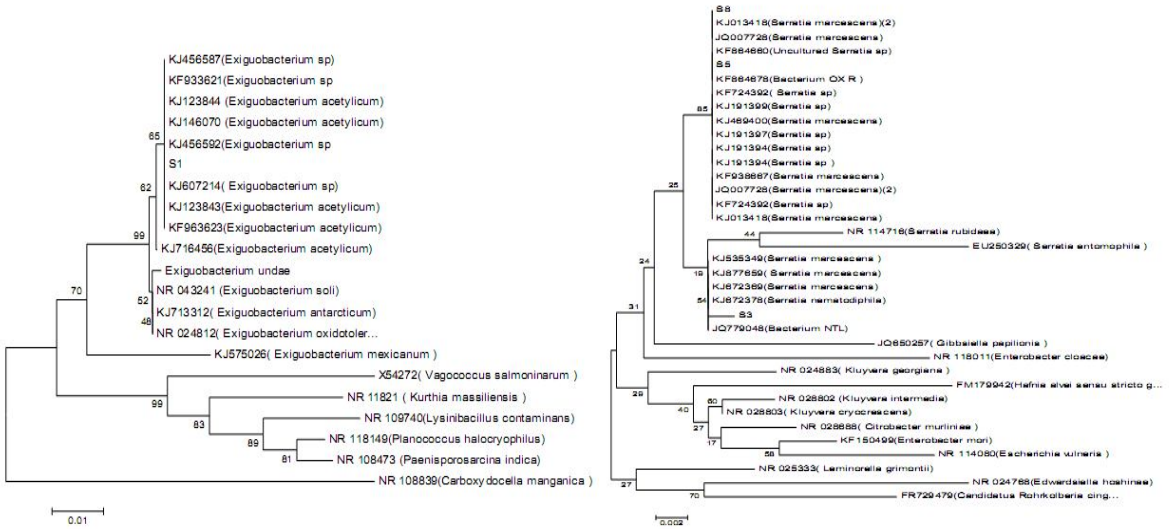

Figure.1 Phylogenetic relationships among representative experimental isolate and the most related bacteria based on 16S rRNA sequences

Table 4:Pigment weight and preparation dilutions

			Solution I		Solution II		Solution III	
Sample	Weight of beaker (gr) with pigment	Weight of beaker without pigment (gr)	Pigment (gr)	Total volume (cc)	Solution I (µl)	Total volume (cc)	Solution II (µl)	Total volume (cc)
S2-A1	56.84	56.89	0.05	5	250	2.5	250	1.25
S3	58.42	58.45	0.03	3	150	1.5	150	0.75
S8-1	56.84	56.90	0.06	6	300	3	300	1.5

Table 5: Absorbance of bacterial pigment

Wavelength	EE(λ) × I(λ)	S_2-A_1	S_3	S_{8-1}
290	0.0150	0.242	0.414	0.269
295	0.0817	0.210	0.328	0.204
300	0.2874	0.190	0.288	0.173
305	0.3278	0.175	0.265	0.151
310	0.1864	0.163	0.248	0.133
315	0.0839	0.155	0.233	0.199
320	0.0180	0.147	0.217	0.108

Table 6: SPF values of bacterial pigment

Sample	SPF
S2-A1	1.785
S3	2.72

CONCLUSION

Microbial pigments are a promising alternative to other pigments that extracted from vegetables or animals because they are considered as natural, pose no seasonal production problems and show high productivity.Pigment producing microorganisms are yeast, fungi,bacteria, micro algae and are quite common in nature.

The secondary metabolites from marine bacteria, especially those with unique color pigments, not only play an important role in bacterial life, but also have diverse biological properties such as antibiotic , anticancer activities and protection from UV irradiation.

Bacterial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications .work on the bacterial pigments should be intensified especially in finding cheap and suitable growth medium which can reduce the cost and increase its applicability for industrial production.There are many studies in the literature on bacterial pigments which focus production and application of specified pigment in each case.

In this study orang pigment extracted from Exigobactrerium sp. it can grow at the temperature of 25°C to 42°C and the optimum temperature is 37°C. Exiguobacterium is a genus of bacilli and a member of the low GC phyla of Firmicutes in another study Balraj et al [21] isolate marine pigmented marin bacteria Exiguobacterium whit orange pigment from Peninsular Region Of India .Purified pigment exhibited antagonism towards Escherichia coli, Staphylococcus aureus, Staphylococcus epidermis, Pseudomonas sp, Bacillus sp, Proteus sp, Klebseialla sp, Shigella sp, and Salmonella sp. In case of DPPH radical scavenging assay, the pigment showed higher radical scavenging activity with IC50 value 51.38µg/mL. The UV absorbance profile indicated that the pigment was probably a derivative of carotenoids. GC-MS analysis revealed that the pigment may be interlinked with methyl ester group.

Shatila et al [22] extracted orange pigment frome Exiguobacterium aurantiacum FH. The analysis of

pigment produced revealed the presence of carotenoids. Both carotenes and xanthophylls were detected in the methanolic extract of carotenoids. The carotenoids produced were characterized by considerable stability and demonstrated antifungal activity against Fusarium sp., Penicilium sp., and Alternatria sp. Two other isolates, including s3 and s8-1 belonging to the genus serratia . Serratia spp are gram negative bacteria, classified in the large family of Enterobacteriaceae, appears to be ubiquitous genus in nature. Ten species of Serratia spp are recognized, occurring naturally in water and soil, on plant, in insects and in man and animals [1]. Serratia marcescens has a historical background that may be described as literally colorful because of their red pigment called prodigiosin . Prodigiosin is of great interest due to its antifungal, antibacterial, antiprotozoal, antimalarial, immunosuppressive, and anticancer activities(jasmine). Montaner and Pérez-Tomás' [23] characterize the apoptotic action of prodigiosin in colon cancer cells. Metastatic SW-620 cells were more sensitive to prodigiosin (IC_{50}: 275 nM) than DLD-1. They did not observe a significant decrease in the viability of NRK cells. They confirmed that prodigiosin induces apoptosis in DLD-1 and SW-620 cancer cell lines by the characteristic DNA laddering pattern and condensed nuclei or apoptotic bodies identified by fluorescence microscopy. These results indicate that prodigiosin induces apoptosis in colon cancer cells.

Montaner et al [23] Found that prodigiosin, an immunosuppressor,induces apoptosis in haematopoietic cancer cells with no marked toxicity in nonmalignant cells, raising the possibility of its therapeutic use as an antineoplastic drug.

The present study indicates that pigment production is influenced by physical factors such as temperature and type of culture medium. There should be many other factors, affecting pigmentation by the bacterium such as shaking. The results show that all of isolates has considerable SPF and can be use as UVB filter agent in cosmotic product. SPF numbers has become a worldwide standard for measuring the effectiveness of sunscreen products. It gives an idea about how long one can stay in the sun without getting burn by the sun rays. Application of sunscreen to the skin changes the way the body reacts to the sun rays (24). Sunscreens and sunblocks are chemicals that absorb or block UV rays and show a variety of immunosuppressive effects of sunlight. There are several agents available from both synthetic and natural sources with UV-filtering properties.

Synthetic UV filters are known to have potential toxicity in humans and also showed ability to interfere only in selected pathways of multistage process of carcinogenesis.

Malsawmtluangi et al.[25] determined the ultraviolet (UV) absorption properties of aqueous herbal extracts of some commonly found vegetable sources by determining the sun protection factor (SPF) number. was calculated by applying Mansur mathematical equation. The absorbance and SPF values of the samples calculated through UV Spectrophotometric method are shown in Tables.

Table. 2: SPF values of aqueous herbal extracts.

Herbal extracts	SPF values
Aloe Vera	1.28±0.02
Carrot	1.34±0.13
Coconut	7.38±0.22
Cucumber	1.45±0.35
Papaya	1.75±0.26
Strawberry	1.63±0.34
Watermelon	0.97±0.41

If we compare SPF of isolates pigments with spf of herbal extract we Concluded that bacterial pigments has higher spf , Except coconut spf. The SPF of bacterial pigments were evaluated. It was found that most of them have the UV protection capabilities. Along with their many beneficial effects and safety, these could become a good, cheap and easily available formulation ingredients in sunscreen products.

REFERENCE

[1] Delgado-Vargas F, Jiménez AR, Paredes-López O.(2000). Natural Pigments: Carotenoids, Anthocyanins, and Betalains — Characteristics, Biosynthesis, Processing, and Stability, Critical Reviews in Food Science and Nutrition, 40(3):173–289.

[2] Malik k, Tokkas J, Goyal S.(2012). Microbial Pigments: A review. International Journal of Microbial Resource Technology,1(4):361-365.

[3] Venil CK, Zakaria ZA, Ahmad WA.(2013). Bacterial pigments and their applications. Process Biochemistry, 48 (2013):1065–1079.

[4] Joshi VK, Attri D, Bala A, Bhushan.(2003).Micbial pigmens.Biotechnology,2:262-369.

[5] Nilam .G P, Chincholkar S B. (2014). Probing natural carbon sources for bioactive pigment production from S. nematodiphila 213 C. International Journal of Advanced Research ,2(2): 838-846.

[6] Wada N, Sakamoto T, Matsugo S.(2013). Multiple Roles of Photosynthetic and Sunscreen Pigments in Cyanobacteria Focusing on the Oxidative Stress. Metabolites, 3:463-483.

[7] Ferroni F, Klisch M, Pancaldi S, Häder DP.(2010). Complementary UV-Absorption of Mycosporine-like Amino Acids and Scytonemin is Responsible for the UV-Insensitivity of Photosynthesis in Nostoc flagelliforme. Marine Drugs, 8: 106-121.

[8] Oren A, Gunde-Cimerman N.(2007). Mycosporines andmycosporine-like amino acids: UVprotectants or multipurpose secondarymetabolites?. FEMS Microbiol Lett,269:1–10.

[9] Soliev AB, Hosokawa K, Enomoto K.(2011). Bioactive Pigments fromMarine Bacteria: Applications and Physiological Roles. Evidence-Based Complementary and Alternative Medicine:1-17.

[10] Balskus EP, Case RJ, Walsh CT.(2011). The biosynthesis of cyanobacterial sunscreen scytonemin in intertidalmicrobialmat communities. FEMS Microbiol Ecol, 77 :322–332.

[11] Baozhong Chai, He Wang, and Xiangdong Chen.(2012). Draft Genome Sequence of High-Melanin-Yielding Aeromonas media Strain WS. Genome announcement.194,23: 6693–6694.

[12] Kotob SI, Coon SL, Quintero EJ, Weiner R M.(1995). Homogentisic Acid Is the Primary Precursor of Melanin Synthesis in Vibrio cholerae, a Hyphomonas Strain, and Shewanella colwelliana. Applied And Environmental Microbiology :61(4): 1620–1622.
[13] Dionisio-Sese ML.(2010). Aquatic Microalgae As Potential Sources Of Uv-Screening Compounds. Philippine Journal of Science, 139 (1): 5-16.
[14] Hanrahan JR.(2012). Sunscreens.Australian prescriber.35(5):148-151.
[15] Brenner M, Hearing VJ.(2008). The Protective Role of Melanin Against UV Damage in Human Skin. Photochem Photobiol,84(3): 539–549.
[16] Dutra AB, Oliveira DAGCKedor-Hackmann ERM, Santoro MIRM.(2004) Determination of sun protection factor (SPF) of sunscreens by ultraviolet spectrophotometry. Brazilian Journal of Pharmaceutical Sciences.40(3):381-385.
[17] Gasparro FP, Mitchnick M, Nash JF.(1998). A Review of Sunscreen Safety and Efficacy. Photochemistry and Photobiology, 1998, 68(3): 243-256.
[18] Schalka S, Reis VMS.(2011).Sun protection factor: meaning and controversies. An Bras Dermatol,86(3): 507-515.
[19] Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematic. Stackebrandt, E., and Goodfellow, M., eds., John Wiley and Sons, New York, NY, 115-175.
[20] Mansur, J. S.; Breder, M. N. R.; Mansur, M. C. A.; Azulay, R. D. (1986).Determinação do fator de proteção solar por espectrofotometria. An. Bras. Dermatol., Rio de Janeiro.61: 121-124.
[21] Balraj J, Pannerselvam K, Jayaraman A.(2014). Isolation Of Pigmented Marine Bacteria Exiguobacterium Sp. From Peninsular Region Of India And A Study On Biological Activity Of Purified Pigment.Scientific and technology research,3(3):375-384.
[22] Shatila F, Yusef H, Holail H.(2013). Pigment production by Exiguobacterium aurantiacum FH, a novel Lebanese strain. Internatunal jurnal of current microbiology and applied science, 2(12): 176-191
[23] Montaner B, Navarro S, Piqué M, Vilaseca M, Martinell M et al.(2009). Prodigiosin from the supernatant of Serratia marcescens induces apoptosis in haematopoietic cancer cell lines. British Journal of Pharmacology,131(3): 585–593.
[24] Mishra S, Scarano FJ, Calvert P. 2012. Entrapment of Saccharomyces cerevisiae and 3T3 fibroblast cells into blue light cured hydrogels. J Biomed Mater Res Part A 2012:100A:2829–2838.
[25] Malsawmtluangi C, Nath DK, Jamatia I, Lianhimgthangi, Zarzoliana E, Pachuau L.(2013). Determination of Sun Protection Factor (SPF) number of some aqueous herbal extracts. Applied Pharmaceutical Science .3 (09)150-151.

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