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Safety profile of synthetic versus natural Psoralen

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Abstract

Psoralen is used widely in the treatment of various skin diseases such as Psoriasis, Vitiligo etc. The Psoralen used for the above purpose is in oral dosage form and the topical dosage form is likely to reduce the severe side effects of oral Psoralen. However the phototoxic effect of Psoralen in topical form limit the medical fraternity from accepting it when compared to oral Psoralen. The safety profile of synthetic Psoralen versus natural Psoralen was done and natural Psoralen was found to be non-genotoxic and exerted least toxicity to keratinocytes and melanocytes when the cells were exposerd to UV exposure. The present study clearly conclude that natural Psoralen is extremely safe over synthetic Psoralen and the topical dosage form can bring down the side effects and enhance the treatment success. Details are discussed in the paper.

Keywords: Natural Psoralen, Topical Psoralen, Photosensitivity, Methoxsalen, Vitiligo.

INTRODUCTION

Psoralen is one of the well -known phototoxic constituents present in wide variety of plants such as Curcuma species, Psoralea corylifolia etc. Further Psoralen is used extensively in the treatment of several skin diseases such as Psoriasis, Vitiligo, and Eczema and wherever the phototherapy is required, Psoralen is used to enhance the effect of phototherapy [1, 2, 3, 4, 5].

Due to the phototoxic effect of Psoralen, extreme care and caution is being exerted while using Psoralen. Oral Psoralen is used widely for the phototherapy treatment of Vitiligo and Psoriasis however the oral use of Psoralen is known to cause several side effects such as nausea, cramps, headache, vomiting etc. [6, 7, 8].

Topical Psoralen may curtail much of the side effects of Psoralen however the topical preparation is always viewed through the optics of the preexisting oral toxicity of Psoralen even during its topical usage.

If Psoralen extracted from the natural sources is used in preparing topical photosensitizing agent for the treatment of Psoriasis and Vitiligo, the side effects of oral Psoralen can be minimized where synthetic Psoralen is used in the oral therapy. However, the above assumption warrant detailed scientific evidence.

Our earlier studies have clearly demonstrated that Psoralen in the natural form is phototoxic to the skin only for a very short while whereas the synthetic Psoralen is quite stable in its photo toxicity and exhibited prolonged activity until its complete neutralization [9].

In order to establish the safety of synthetic Psoralen versus Psoralen in native form extracted from the herbal source, we have performed genotoxicity assay and toxicity to melanocyte and keratinocyte on 'as is' basis as well as the cells were when exposed to UV radiation in the presence of Psoralen. Details are presented in the paper.

MATERIALS AND METHODS

Details of Psoralen

Methoxsalen was procured from local market.

Extraction of natural Psoralen in native form was achieved through alcoholic extraction of the seeds of Psoralea corylifolia. In brief the shade dried seeds of *Psoralea corylifolia* were ground to powder and then

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treated with alcohol. The proportion of seeds used for the extraction with reference to the solvent was 10%. The resultant extract was filtered, and then distilled to separate the alcohol. The pasty extract thus obtained from *Psoralea corylifolia* seeds were used for further study. TLC confirmation was done to establish the presence of Psoralen and other furocoumarin derivatives in the extract qualitatively.

Genotoxic assay

The bacterial genotoxicity assay was performed wherein the effect of natural and synthetic Psoralen on the DNA of the organism was studied.

The above test utilizes the bacterial SOS DNA-repair system induced by genotoxic compounds. Two genetically engineered *Salmonella typhimurium* strains were used such as TA104 *recN*2-4 (Genox strain) and TA104 *pr1* (Cytox strain)

The plasmid of Genox strain carries the bacterial luciferase operon (*luxCDABE*) of the luminous bacteria *Vibrio fischeri which is fixed under* transcriptional control by a mutated *recN* promoter (*recN*2-4) that is part of the SOS-system.

In the Genox strain, the genotoxic compounds would activate the *recN* promoter and which results in transcriptional induction of the *lux* operon followed by the enhancement of light emission.

The Cytox strain possesses a plasmid containing the *Vibrio fischeri lux* operon, which is transcriptionally controlled by the strong *pr1* promoter and thus constitutively produces light. This strain is used as an internal control system as some compounds appear to have a direct influence on the light production or enhance the metabolism of the bacteria creating false-positive results.

Simultaneously the cytotoxicity of the compounds is assayed with reference to the Cytox strain to identify the non-specific enhancement of light emission by the test substance.

The Genox and the Cytox strains allows identifying false positive results if any caused by the non-specific light emission induced by other mechanisms, and not by the genotoxic effect.

Operative part of the experiment

Salmonella typhimurium lacks the essential oxidative enzymes for metabolizing foreign substances to electrophilic metabolites and which is capable of reacting with DNA, and therefore the *Salmonella typhimurium* should be treated with the test compound(s) in the presence and absence of a post-mitochondrial supernatant (S9).

After overnight incubation of *Salmonella typhimurium* in enriched growth medium, a dilution of the bacterial suspension was again re-incubated for one more hour in nutrient deficient growth medium on the following day and kept in orbital shaker for one hour.

The different concentrations of the test samples- extract of Psoralea corylifolia, Methoxsalen were made and transferred on to a 96 multi-well plate. In the dilution series and a blank (without test substances) of the solvent and a positive control were added (e.g. in which S9 and 4-Nitroquinoline-oxide without S9).

The microplate luminometer was used for the genotoxicity measurements to calculate signal-to-noise ratio (S/N), i.e. the light production of exposed bacteria divided by the light production of non-exposed bacteria is calculated for each measurement.

Separately the signal-to-noise ratio (S/N) was calculated for Genox and Cytox strain. Based on the above experimental findings, test substance was categorized as genotoxic, cytotoxic or none [10].

Interpretation of the result

Genotoxicity

A test substance is considered to be genotoxic by following criteria are met:

- The Genox strain must give a dose-response correlation over a concentration range of at least 3 dilutions with maximum S/N ratio.
- The Genox/Cytox ratio is > 1.5 and a dose-response correlation over a concentration range of at least 3 dilutions.
- If the expression of both Genox strain and the Cytox strain have happened, one may not conclude the test material to be genotoxic.
- If maximum S/N ratio for the Genox strain is ≤ 1 the result is negative even when the Genox/Cytox ratio is > 1.5

Cytotoxicity

A product is considered to be cytotoxic when the following criterion is met:

- If the S/N ratio rapidly decreases below 0.8 there is a cytotoxic effect.

Cell culture assay

Keratinocytes and melanocytes were used for the study. The cytotoxicity in response to treatment was assayed by MTT. The principle of the assay is based on the ability of the mitochondrial succinate-terazolium reductase system in live cells to convert 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) to a blue colored formazan. The test denotes the survival cells after exposure to the test samples [11].

The malignant keratinocyte cell lines were used for the above study. The cell lines was maintained and sub cultured in 25mm^2 tissue culture flasks using 5 ml of minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 3% L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), Amphotericin B (20 µg/ml), phenol red. The pH of the medium was adjusted to 7.2-7.4 with 7.5% sodium bicarbonate and all flasks were incubated at 37˚C in a humidified 5% CO2 /95% O2 incubator.

Cell were grown in appropriate growth media and seeded at concentrations of 5 × 10^4 cells per ml of media in a 24 well plate. Cells were allowed to adhere for 12 hours followed by treatment with different concentrations of the test samples and incubated for 48 hours at 37 °C with 5 % CO2.

After treatment of 48 hours, cells were treated with 10% MTT in media for 4 hours in 37 °C and 5 % CO2. Media was then aspirated and the adherent cells with formazon product was dissolved in DMSO, centrifuged at an RPM of 5000 for 15 minutes to remove debris and the spectrophotometric absorbance of the sample was measured using a microplate reader. The wavelength used to measure the absorbance of formazan product was 570 nm and the percentage of reduction in cell proliferation was determined.

Concentration of synthetic Psoralen used in the study

Four different concentrations of synthetic Psoralen were arrived based on our initial toxicity assay and the concentrations used in the study were 1, 2, 3 and 5 µg/ml. The above concentration was found to be safe on both keratinocytes and melanocytes in vitro.

Concentration of natural Psoralen from *Psoralea corylifolia*

Based on the toxicity assay done earlier 3, 5, 7 and 10 µg/ml of the extract was used for the study.

Lethal dose of UV on kerationctyes and melanocytes

The cell lines were exposed to UVA and UVB separately to determine the IC90 value. The dosage of UVA used was 2, 4, 6 and 10 joules per minute for keratinocytes. Similarly, the dosage of UV B used in the study was 1, 2, 3, 4, 5 milli joules per minute. In the case of melanocyte the dosage of UV A was 1, 3, 5, 7 and 9 milli joules per minute and dosage of UV B was 1, 2, 3, 4, 5, 6 milli joules per minute.

Co-treatment of cells with Psoralen and UV

After determining the IC90 value of both UV A and UV B as well as synthetic and natural Psoralen, the cells (keratinocytes and Melanocytes) were exposed to UV A and UV B at sub-lethal dose along with sub-lethal concentration of test compounds.

RESULTS

Genotoxic assay

Extracts of *Psorolea corylifolia* did not show any DNA damaging effect up to a concentration of 100 microgram per milliliter whereas the synthetic Psoralen caused DNA damage at 20 micro gram per milliliter.

Cell culture assay

Up to 5 microgram per ml concentration the synthetic Psoralen did not induce cytotoxic effect on keratinocyte

Table 1: The cytotoxic effect of synthetic Psoralen on keratinocytes

Concentration (µg/ml)	OD Value	% inhibition
Control	0.6892	
1	0.6799	1.349
2	0.6880	0.174
3	0.6677	3.119
5	0.6580	4.526

Natural Psoralen extracted from Psoralea corylifolia did not show any cytotoxic effect on keratinocytes up to a concentration of 10 microgram per ml. Table- 2

Table 2: The cytotoxic effect of natural Psoralen on keratinocytes

Concentration (µg/ml)	OD Value	% inhibition
Control	0.6944	
3	0.6932	0.172
5	0.6930	0.201
7	0.6920	0.346
10	0.6917	0.388

The synthetic Psoralen exhibited mild level of toxicity to melanocytes from 2 to 5 microgram per ml concentration. Table- 3

Table 3: The cytotoxic effect of synthetic Psoralen on Melanocytes

Concentration (µg/ml)	OD Value	% inhibition
Control	0.5534	
1	0.5121	7.462
2	0.4980	10.01
3	0.4567	17.47
5	0.4544	17.88

Natural Psoralen up to 10 microgram per ml did not produce cytotoxicity to melanocytes. Table – 4

Table 4: The cytotoxic effect of natural Psoralen on melanocytes

Concentration (µg/ml)	OD Value	% inhibition
Control	0.5980	
3	0.5899	1.354
5	0.5871	1.822
7	0.5788	3.210
10	0.5767	3.561

Lethal dose of UV on kerationctyes and melanocytes

UV A at 6 milli joules per minute caused 90% death of keratinocytes and 7 milli joules per minute caused the above effect (IC90) in melanocytes. UV B at 4 milli joules per minute caused 90 % death of keratinocytes and UV B at 5 milli joules per minute produced the same effect in melanocytes.

Co-treatment of cells with Psoralen and UV

The co-treatment of UV A and synthetic Psoralen on keratinocytes showed great lethality when either increase in concentration of Psoralen to 5 microgram per ml or increase in the milli joules of UV A per minute were effected. Synthetic Psoralen produced 91% of toxic effect at 5 microgram per ml concentration when keratinocytes were exposed to 2 milli joules per minutes of UV A. Table-5.

Natural Psoralen up to a concentration of 10 microgram per ml concentration with 5 milli joules per minute of UV A did not produce toxic effect to the cells. Table- 5.

UV B was found to be quite toxic to keratinocytes even when cells were treated with synthetic Psoralen as low as 3 microgram per ml. on the contrary UV B showed less toxic to keratinocytes when natural Psoralen was used. However, when the dosage of UV B was increased to 3 milli joules per minutes and when the cells treated with 10 microgram per ml of natural Psoralen 100% cell death was observed. Table- 5.

Table 5: Co-treatment of UBA or UVB + Psoralen - % inhibition of Keratinocytes

Details	Jules / minutes	Synthetic Psoralen / concentrations and % inhibition				Natural Psoralen / concentrations and % inhibition			
		1	2	3	5	3	5	7	10
UVA	2	20	44	60	91	6	8	13	21
	4	29	58	80	100	10	11	19	28
	5	40	60	100	100	13	29	34	41
UVB	1	44	56	88	100	18	22	30	44
	2	60	90	100	100	24	45	78	100
	3	100	100	100	100	36	60	90	100

The synthetic Psoralen was more toxic to melanocytes than natural Psoralen. The concentration difference between synthetic to natural was 50% when UV A exposure was administered. Psoralen exhibited toxic effect on melanocytes even at low concentration and least time of UV B exposure suggesting toxic effect of UV B than UV A. Table- 6

Table 6: Co-treatment of UVA or UVB + Psoralen - % inhibition of Melanocytes

Details	Joules / minutes	Synthetic Psoralen / concentrations and % inhibition				Natural Psoralen / concentrations and % inhibition			
		1	2	3	5	3	5	7	10
UVA	2	40	69	100	100	33	50	88	100
	4	59	88	100	100	39	68	98	100
	6	62	96	100	100	49	74	100	100
UVB	2	88	100	100	100	100	100	100	100
	3	99	100	100	100	100	100	100	100
	4	100	100	100	100	100	100	100	100

DISCUSSION

The phototoxic effect of Psoralen is well known. Therefore, Psoralen is being used widely for the treatment of Psoriasis, vitiligo, eczema etc. during phototherapy. Conventionally Psoralen is administered orally to induce photosensitivity of the skin prior to UV therapy. The severe side effects of Psoralen oral dosage have limited its usage significantly.

The topical Psoralen preparations may overcome the side effects of Psoralen oral administration. The topical Psoralen is expected to be less toxic than oral use but however sufficient scientific evidence is lacking for the above hypothesis. The larger stigma among dermatologist is that the topical Psoralen may be more harmful than oral Psoralen as it may make the skin photosensitive and susceptible to sun damage and such problem may manifold several times during inadvertent/accidental exposure to sun.

In order to establish the safety of topical Psoralen and its role in the treatment and Psoriasis and Vitiligo we have undertaken the present study. The genotoxicity assay of synthetic Psoralen versus natural psoralen revealed that up to 100 microgram per ml concentration, the natural Psoralen did not induce DNA damage in S.typhimurium strain - TA104 *recN*2-4 (Genox strain). The synthetic Psoralen however produced DNA damage even at 20 microgram per ml concentration. Our subsequent studies have revealed that synthetic Psoralen was more toxic to keratinocyte and melanocyte than natural Psoralen.

The UV exposure cells treated with natural or synthetic Psoralen, the cells treated with synthetic Psoralen showed greater cytotoxic effect than the cells treated with natural Psoralen. The above finding clearly show that natural psoralen is extremely safe when compare to synthetic psoralen. The question of why the natural Psoralen is more safe than synthetic Psoralen may be due to its existence in the native form. The Psoralea corylifolia extract besides having Psoralen also have many other metabolites. Therefore, several counteractions, counterbalances and counter corrections may be happening when the natural psoralen extract is used whereas in the case of synthetic Psoralen such possibility is remote.

Our earlier study has clearly demonstrated that the photosensitive effect of natural Psoralen was very short when compared to synthetic Psoralen. This suggest that a natural Psoralen may make the skin photosensitive only for a very short while therefore the sustained photo susceptibility of the skin and associated photo damages due to subsequent sun exposure or UV exposure is minimal.

The above finding clearly confirms the advantages of topical natural Psoralen over synthetic Psoralen for the treatment of Psoriasis and Vitiligo as well as its safety profile.

The topical natural Psoralen can be used in 3 ways such as

1. Pre-medicament for phototherapy
2. Replacement for phototherapy if the lesion is localized
3. An agent to advance the benefit of sun (natural healing)

The photosensitivity of the skin during UV/sun exposure would lead to an array of biochemical and immuno- pathological reactions starting from langerahan cells to release of several cytokine and lymphokines resulting in cell suppression as well as pigmentogenesis. Considering the role of topical Psoralen in aiding healing from within must be included in the modern therapy to manage Psoriasis and Vitiligo.

Psoralea corylifolia being part of traditional healing practices, the present study has revalidated and revitalized the enormous scope and science of ancient healing practices. No doubt the topical Psoralen preparation with the natural extract of *Psoralea corylifolia* can indeed offer great and safe treatment success to many diseases.

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