

**EVALUATION OF MELANOGENIC AND ANTI-VITILIGO ACTIVITIES OF  
HOMEOPATHIC PREPARATIONS ON MURINE B16F10 MELANOMA CELLS**Joshi Samidha<sup>1</sup>, Munshi Renuka<sup>2</sup>, Talele Gitanjali<sup>3</sup> and Shah Rajesh<sup>4\*</sup><sup>1</sup>Senior Research Officer, Dept. of Clinical Pharmacology, TNMC & BYL Nair Ch. Hospital, Mumbai.<sup>2</sup>Head In-charge, Dept. of Clinical Pharmacology, TNMC & BYL Nair Ch. Hospital, Mumbai.<sup>3</sup>Research Associate, Life Force Foundation Trust, Mumbai.<sup>4</sup>Principal Investigator, Life Force Foundation Trust, Mumbai.**\*Corresponding Author: Shah Rajesh**

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**ABSTRACT****Background:** Vitiligo is a multi-factorial disorder where the skin loses pigments. Homeopathic medicines are known to be effective for vitiligo. The melanogenic activity of homeopathy has not been evaluated in lab models.**Materials and Methods:** Cell viability assay was performed to observe the cytotoxic effect of homeopathy preparations on melanoma cells. The effect of homeopathy preparations on the production of melanin in melanocytes was determined using B16F10 melanoma cells. The activity was checked at the end of 48 and 96 hours using 30c potency and 1:1 dilutions of homeopathy preparations. **Result:** Cell viability assay results showed no cytotoxic effect on the cells. Hydroquinone, Arsenicum sulphuratum flavum (ASF) and Phosphorous (30C) potency showed increase in melanin content as compared to cell and vehicle controls. However, statistically significant increase was seen with Hydroquinone and ASF. The diluted form (1:1) of all the three homeopathy preparations exhibited slight increase in the melanin content as compared to both the controls but was less as compared to cell control. At the end of 96 hours, ASF and Phosphorus exhibited a slight increase in melanin content as compared to potentized control. There was no inhibition of Tyrosinase activity seen during the assay with the homeopathy preparations. **Discussion and Conclusion:** The experiment has thrown important finding supporting the very fundamental homeopathy principle, by stimulating melanogenesis in the small (potentized) dose of the substance which in the crude form is known to be induce vitiligo.**KEYWORDS:** Vitiligo, Hydroquinone, Arsenicum sulphuratum flavum, Phosphorus, homeopathy.**BACKGROUND**

Vitiligo (vit-ill-EYE-go) is a multi-factorial disorder in which the skin loses melanocyte pigments, which leads to depigmented patches. Vitiligo is a medico-social disorder affecting estimated 1% of world and 8% of the Indian population.<sup>[1]</sup>

The core objective behind treating vitiligo is to control the spread and to achieve re-pigmentation. Generally, vitiligo treatment takes a long time to control the spread. Since the exact cause of vitiligo may not be clearly understood in every case, it is difficult to treat it with desired success. The currently available treatment options for vitiligo include conventional medicine, homeopathy, Ayurveda, surgery, and adjunctive therapies. There is great scope for newer and better treatment modalities for vitiligo.

One of the functions of the melanocytes is synthesis of melanin. Melanin formation (melanogenesis) is quantifiable using murine B16 melanoma cell line model.<sup>[2]</sup> These markers are characteristic of normal

differentiating melanocytes, so B16 melanoma cells represent a useful model for investigating melanogenesis.<sup>[3]</sup>

Homeopathic medicines are known to be clinically effective for vitiligo. The author has significant clinical experience in the treatment of vitiligo using homeopathic medicines. The evidence of efficacy of homeopathic medicines in *in-vitro* model is not found on literature review. Also, the mode of action of homeopathic medicines against vitiligo is not understood.

The clinical use of homeopathic medicines is based on the fundamental principle of the law of similar and the phenomenon of hormesis, which state that the substance known to produce certain symptoms, may also have built-in capacity to relieve the same, if administered in small dose.<sup>4</sup> Arsenic toxicity is known to induce loss of melanin and vitiligo,<sup>[5, 6]</sup> so as Phosphorus.<sup>[7]</sup> On the basis of the homeopathic principle, Arsenic, its related compound Arsenicum sulphuratum flavum and Phosphorus in ultra-dilute, potentized form are

empirically used in homeopathy to treat vitiligo.<sup>[8,9]</sup> Hydroquinone (C<sub>6</sub>H<sub>4</sub>(OH)<sub>2</sub>) is conventionally a skin whitening agent. It is used as a homeopathically potentized preparation, logically and clinically used by the author as a therapeutic measure to treat vitiligo.<sup>[10]</sup>

Thus the aim of this work was to investigate the melanogenic and anti-vitiligo activities of the homeopathic preparations viz., Hydroquinone 30C, Arsenicum sulphuratum flavum 30C (SBL) and Phosphorus 30C (SBL) on murine B16F10 melanoma cells, in order to support its popular use in vitiligo treatment.

## MATERIALS AND METHODS

### Chemicals and reagents

Mushroom tyrosinase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-tyrosine and dimethyl sulfoxide and Kojic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum and Penicillin/streptomycin were purchased from Invitrogen.

### Cell culture

The Murine B16F10 melanoma cell line procured from ATCC was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (10,000 U/100µg/mL) at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere.

Homeopathic preparations details: The homeopathic preparations viz., Hydroquinone 30C, Arsenicum sulphuratum flavum 30C (SBL) and Phosphorus 30C (SBL) were used in concentrated form i.e., 30 potency and 1:1 diluted form of the same potency. The homeopathic preparations were made in alcohol and potentized alcohol (sourced from Merck), which acted as vehicle controls. Standard used was Kojic acid (Sigma Aldrich).

### 1. Cell viability assay

Cell viability after treatment with the study samples was determined using 3-(4, 5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, USA).<sup>[11]</sup> Briefly, 1×10<sup>4</sup> cells were added in each well of a 96-well plate. After 24 h, cells were exposed to the given samples for 48 and 96 hours. Untreated cells were kept as cell control group and cells treated with the alcohol and potentized alcohol were kept as Vehicle control group. MTT solution was added and cell viability was then assessed in a colorimetric assay through mitochondrial dehydrogenase activity in active mitochondria to form purple formazan. Absorbance of each well was read at 570 nm using a plate reader.

### 2. Determination of melanin content in melanocytes

The influence of the given samples on the production of melanin in melanocytes was determined using the modified method of Tsuboi et al. (1998)<sup>[12]</sup>. Murine B16F10 melanoma cells were added into the wells of a 24-well plate (1 ×10<sup>4</sup> cells per well). After 24 hours, different concentrations of the given samples and Kojic acid (100 µM, as a reference drug) was added to the cells and incubated at 37°C in 5% CO<sub>2</sub>, humidified atmosphere for 48 hours and 96 hours respectively. Control group was incubated only with DMEM and the Vehicle control group was incubated with the alcohol and potentized alcohol. Then, the medium was removed and cells were lysed with 500 µL of NaOH 1 N in 10% DMSO at 80°C for 1 hour. The relative melanin content was determined by measuring the absorbance at 405 nm in a plate reader.

### 3. Mushroom tyrosinase activity assay

Tyrosinase activity was determined according to the method of Hyun et al. (2008)<sup>[13]</sup> with modifications. In a 96-well microplate different concentrations of the given samples, 20 µL of mushroom tyrosinase (500 U/mL in phosphate buffer, pH 6.5), 170 µL of a mixture [L-tyrosine solution 1 mM, potassium phosphate buffer 50 mM/pH 6.5, and distilled water (10:10:9, v/v/v), respectively] was added. Microplate was incubated at 37°C for 40 minutes and the absorbance of the mixture was measured at 490 nm using a microplate reader. One unit (U) of enzymatic activity is defined as the amount of enzyme needed to increase the absorbance at 490 nm by 0.001 per min, in a 3-mL reaction mixture containing L-tyrosine at pH 6.5 and 25°C. The value of each measurement was expressed as percentage changes from the control (reaction mixture without the test drug).

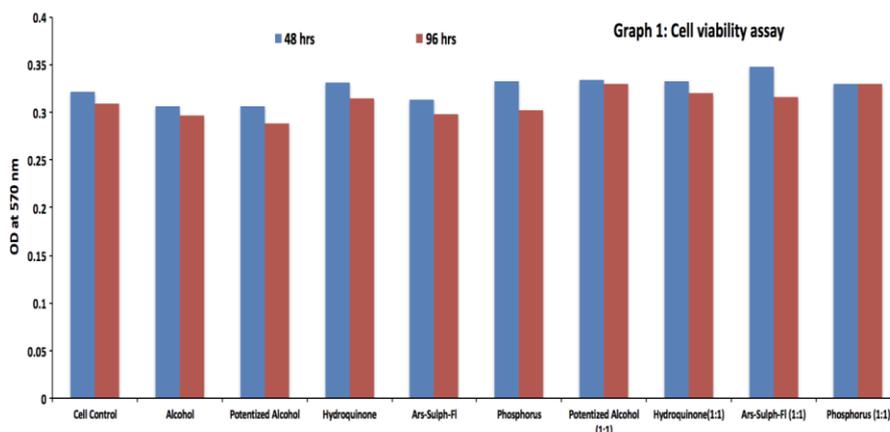
### Statistical analysis

between the groups was assessed by one-way analysis of variance (ANOVA) followed by a post hoc test. The accepted level of significance for the test was P < 0.05. All tests were carried out using Graph Pad Software (Version 3.06).

## RESULTS

### Cell viability assay

The effect of the different homeopathic preparations on cell viability was measured by MTT assay. Although the vehicle used exhibited a decrease in cell viability, the preparations did not show any additional cytotoxic effect on cells when compared with vehicle group at both 48 and 96 hours (Graph 1).



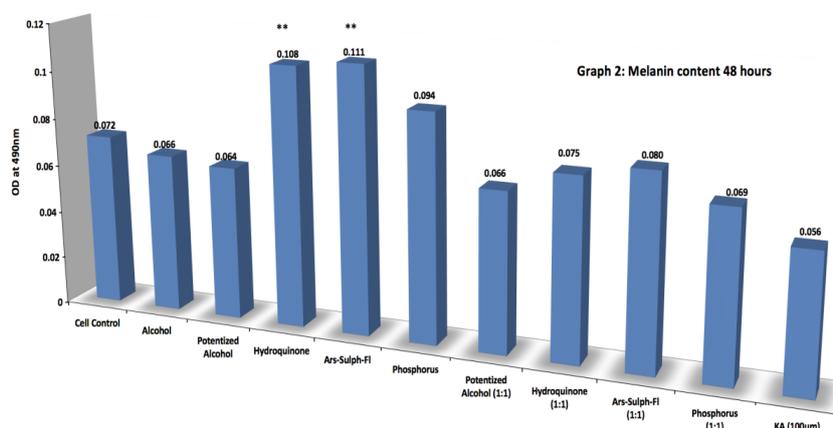
**Graph 1: Effects of homeopathic preparations on cellular viability in cultured B16 murine melanoma cells. B16 cells were incubated for 48 h and 96 h at different concentrations. Vehicle (Alcohol and Potentized alcohol). Data are mean  $\pm$ SD from three separate experiments. \*P < 0.05: statistically significant vs. control group (untreated cells).**

#### Determination of melanin content in melanocytes

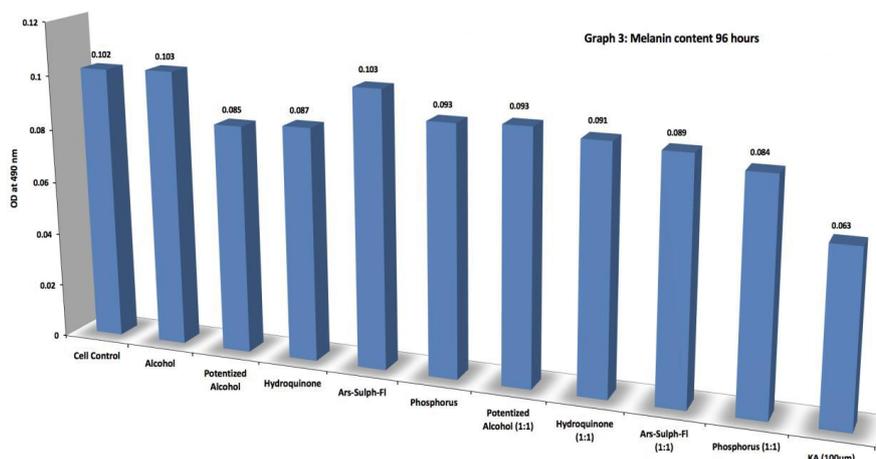
Melanogenic activity in cultured murine B16F10 melanoma cells is directly related to the quantity of produced melanin which is estimated through the amount of melanin retained in the cells (intracellular melanin). Both the vehicles used did not exhibit increase in Melanin content at the end of 48 hours as compared to the cell control. All the three preparations in the 30C potency showed increase in Melanin content as compared to cell control and both the vehicle controls used in the study. However maximum increase was seen with Hydroquinone and Arsenicum sulphuratum flavum which was also statistically significant when compared to cell control, and vehicle controls (Graph 2).

The diluted form of all the three preparations exhibited slight increase in the melanin content as compared to both the controls but was less as compared to cell control. Kojic acid (KA), the standard used did not induce melanin production at the concentration tested.

At the end of 96 hours (Graph 3), all the three samples in both concentrated and diluted form did not induce increase in melanin content as compared to cell control and alcohol control. However, when compared to potentized control, Arsenicum sulphuratum flavum and Phosphorus exhibited a slight increase in melanin content however the effect was not statistically significant.



**Graph 2: Effects of homeopathic preparations on melanin content in cultured B16 murine melanoma cells after 48 hrs. B16 cells were incubated for 48 h in the presence of the preparations in concentrated and diluted form. Kojic acid was used as a drug reference. Vehicle (Alcohol and Potentized alcohol). Data are mean  $\pm$ SD from three separate experiments. \*p<0.05 as compared to the Cell Control group; \* p<0.01 as compared to the Vehicle Controls (alcohol and potentized alcohol).**



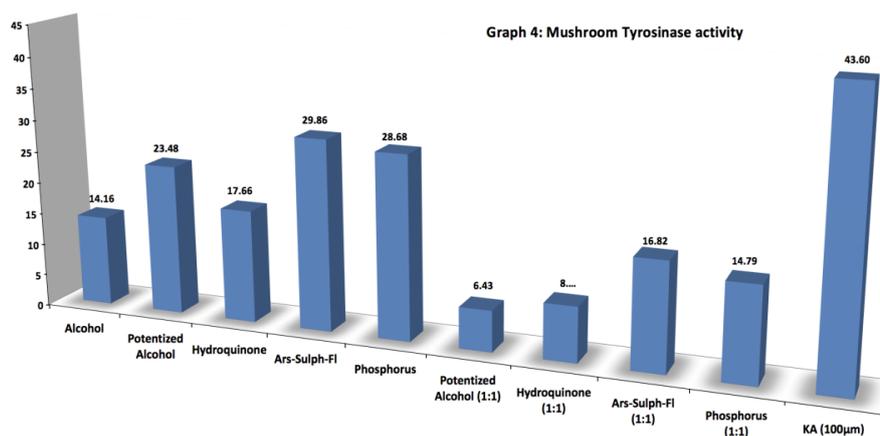
**Graph 3: Effects of homeopathic preparations on melanin content in cultured B16 murine melanoma cells after 96 hrs. B16 cells were incubated for 96 h in the presence of the preparations in concentrated and diluted form. Kojic acid was used as a drug reference. Vehicle (Alcohol and Potentized alcohol). Data are mean  $\pm$ SD from three separate experiments.**

#### Mushroom tyrosinase activity assay

Tyrosinase is the rate-limiting enzyme for melanin biosynthesis, the effect of the homeopathy samples on tyrosinase activity was evaluated. All the three samples in concentrated and diluted form showed inhibition of Tyrosinase activity however inhibition was also seen with the both the vehicle controls used in the study

(Graph 4). Hence the inhibition exhibited by the samples may be due to the control used.

Kojic acid a known depigmenting agent used as a reference drug reduced tyrosinase activity to about 44 %.



**Graph 4. Effects of homeopathic preparations on Mushroom Tyrosinase activity assay. Inhibition of homeopathic preparations in concentrated and diluted form. Tyrosinase activity was examined in the presence of l-tyrosine as substrates. It is represented as an inhibition percentage. Each value is given as mean  $\pm$  SD from independent triplicate experiments.**

#### DISCUSSION AND CONCLUSION

In the present study the melanogenic effect of three homeopathic preparations viz., Hydroquinone 30C, Arsenicum sulphuratum flavum 30C (SBL) and Phosphorus 30C (SBL) was evaluated in concentrated form i.e., 30 potency and 1:1 diluted form of the same potency using murine B16F10 melanoma cells. This cell line has been widely used for this purpose once it is relatively easy to be cultured *in vitro*, and has similar melanogenic mechanisms to human melanocyte.<sup>[14]</sup> When the cells were treated with preparations the

cellular melanogenesis was significantly enhanced at the end of 48 hours, although the same concentrations did not influence the activity of tyrosinase enzyme in the cell-free assay using DOPA as a substrate. The preparations did not influence the enhancement of melanogenesis when treated for 96 hours indicating a time-bound effect. Although Arsenicum sulphuratum flavum and Phosphorus exhibited a slight increase in melanin content but the effect was not statistically significant.

Melanin synthesis is developed by a large number of effectors, but the tyrosinase is the rate-limiting enzyme. Furthermore, other enzymes in the pathway of melanin synthesis have been reported including tyrosinase related proteins 1 (TRP-1) and 2 (TRP-2) which also regulate melanogenesis. Other protein kinases such as cAMP-dependent protein kinase, protein kinase C and protein kinase C- $\beta$  have been shown to participate in the regulation of melanin synthesis in pigment cells. Furthermore, mitogen-activated protein kinase has been shown to be involved in cAMP-induced melanogenesis and c-kit-mediated activation of the microphthalmia-associated transcription factor (MITF), a trans-acting factor that regulates the gene transcription of tyrosinase.<sup>[15]</sup> Thus, a probable mechanism through which the given samples stimulate melanogenesis might be related to other enzymes involved in the process of melanogenesis, other than tyrosinase. Moreover, it is also possible that the samples could be inducing an increase in the expression of tyrosinase enzyme, without affecting its activity, which would also result in a raise in melanogenesis. Hence to confirm its effect on melanin content gene expression studies need to be carried out.

The homeopathic medicines in high dilution have shown to retain the nano particles of the original source material in certain studies.<sup>[16]</sup> The disease modifying effects of such medicines in *in-vitro* model have been evaluated by some researchers.<sup>[17]</sup> By demonstrating the melanogenetic efficacy of high dilution medicine, the results of the experiment are likely to help more research establishing the therapeutic effects of homeopathy.

The experiment has thrown interesting and important findings supporting the very fundamental homeopathy principle, by stimulating melanogenesis in the small (potentized) dose of the substance which in the crude form is known to be induce vitiligo. It also created an evidence that the ultra-dilute, potentized preparations maintain certain relevant, predictable therapeutic efficacy.

#### CONFLICT OF INTEREST

One of the authors has a patent for one of the homeopathic preparations used in the study; however, he had no control over the conduct or the results of the experiment.

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