

Evaluation of Depigmenting Activity of AHPL/AYTOP/1914 in Mice

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Abstract

Background: Current treatment options for hyperpigmentary disorders include topical application of hydroquinone; combination of hydroquinone, tretinoine corticosteroid; azeleic acid or Kojic acid. These drugs have shown limited efficacy in depigmentation and are also associated with adverse effects like skin irritation, increased erythema, etc. This arouses increased interest in the search of herbal alternatives with de-pigmenting activity.

Aim: To evaluate de-pigmenting activity of AHPL/AYTOP/1914 (Test item-01) in mice in comparison with Hydroquinone and Kanaka oil.

Methods: 40 mice were grouped into 4 groups viz vehicle control, standard control, test item 1 and test item 2. The mice were shaved and the test items were applied topically daily twice on the same skin area for a consecutive period of 28 d. Parameters such as skin irritation, skin whitening index and histopathology of skin were assessed.

Results: No treatment related clinical signs or mortality was observed in mice. Test items were found non-irritant. In male and female mice, after 7, 14, 21, 28 d of topical application of test item no. 1 (Group 3), the Von Luschan chromatic scale readings were 11.80 ± 3.70 , 8.60 ± 1.52 , 6.80 ± 1.30 , 6.60 ± 1.52 and 8.40 ± 1.67 , 6.80 ± 1.30 , 6.40 ± 1.14 , 6.60 ± 1.14 , respectively, which were significantly better than test item no. 2 Kanak oil (Group 4) for which the Von Luschan chromatic scale reading after 7, 14, 21, 28 d of topical application were 17.20 ± 3.49 , 12.40 ± 2.88 , 9.80 ± 1.30 , 9.20 ± 1.64 and 13.20 ± 2.78 , 10.20 ± 2.49 , 9.80 ± 1.79 , 9.20 ± 1.48 , respectively. In male and female mice, after 7, 14, 21, 28 d of topical application of test item no. 1 (Group 3), the Von Luschan chromatic scale readings were 11.80 ± 3.70 , 8.60 ± 1.52 , 6.80 ± 1.30 , 6.60 ± 1.52 and 8.40 ± 1.67 , 6.80 ± 1.30 , 6.40 ± 1.14 , 6.60 ± 1.14 , respectively, which were significantly better than hydroquinone (Group 2) for which the Von Luschan chromatic scale reading after 7, 14, 21, 28 d is of topical application were 12.80 ± 3.27 , 9.20 ± 2.77 , 8.60 ± 0.55 , 6.80 ± 1.30 and 16.80 ± 4.66 , 10.80 ± 3.90 , 6.80 ± 3.83 , 7.20 ± 1.92 , respectively. None of the animals in vehicle control and different treatment groups showed any external or internal gross pathological lesions during necropsy indicating that the test items did not cause any lesions systemically.

Conclusion: Test item no. 1, test item no. 2 and standard hydroquinone are safe and possess de-pigmenting activity. The skin whitening/de-pigmenting activity of test item no. 1 is significantly superior to test item no. 2 and standard hydroquinone. Thus, test item no. 1 is safe and can be effectively used for the treatment of hyperpigmentation.

Keywords: Hyperpigmentation; AHPL/AYTOP/1914; Hydroquinone; Depigmentation; Melasma; Cholasma; Skin whitening

Introduction

There are many hyperpigmentary disorders affecting skin for which patients seek medical help. The most common form of acquired hyperpigmentation is melasma which affects face and commonly found in women with dark complexion. Its onset and exacerbation is also associated with pregnancy and then it is termed as chloasma. Hyperpigmentation, in general, is developed because of excess melanin secretion by melanocytes or proliferation of active melanocytes [1,2]. Genetic factors, UV light exposure and female hormonal activity are thought to be some of the etiological factors for hyperpigmentary disorders [3].

On the basis of location of the pigments, hyperpigmentation disorders can be broadly divided into 2 types viz. dermal and

epidermal. Various factors affect specific layer and impart different color to the skin, e.g. brownish discoloration in epidermal whereas blue discoloration in dermal hyperpigmentation [4]. Moreover, overall skin complexion is also influenced by amount and type of melanin, its transfer to keratinocytes and succeeding expression [3]. Since the freckles, naevi, age spots or melasma do not appear aesthetically favorable to anybody, there is increased tendency of individuals suffering from hyperpigmentary disorders to seek treatment for it.

Conventional treatment options used in hyperpigmentary disorders include topical application of hydroquinone, combination of hydroquinone, tretinoine, corticosteroid; azeleic acid or Kojic acid. These drugs have shown limited efficacy in depigmentation and most of these are associated with adverse effects like skin irritation, increased erythema, scaling, sensitization and risk of permanent depigmentation following long term use [5]. Hence research is focused on the development of safe and effective anti-hyperpigmentation drugs

from alternative systems of medicine. Ari Healthcare Pvt. Ltd. has conceptualized and developed test item no. 1 AHPL/AYTOP/1914 in cream dosage form which is intended to be used in hyperpigmentation as a topical application. To test safety and depigmentation activity of test item no. 1 (AHPL/AYTOP/1914), a study titled "Evaluation of depigmenting activity of AHPL/AYTOP/1914 in mice" was planned.

Materials and Methods

Animal description

Animal species and sex: Mice, male and female.

Strain: C57BL/6J.

Justification for selection of species: Mice were used for testing because it has been used extensively for safety, efficacy and toxicity studies because of availability of huge historical and reference data. Mice are the preferred species of animal as per the literature. C57BL/6J was used as test system for the study.

Source: Mice for the study were procured from Liveon Bio Labs, Tumkur, India.

Animal husbandry

40 mice (20 males and 20 females) were selected for the study. Animals were housed under standard laboratory conditions in air-conditioned house with adequate fresh air supply (12-15 air changes per hour), room temperature $20^{\circ}\text{C} \pm 3^{\circ}\text{C}$, relative humidity 30%-70%, with 12 h light and 12 h dark cycle. The animal husbandry conditions such as temperature and relative humidity were recorded once daily. The animals were acclimatized minimum for 5 d to laboratory conditions and observed for clinical signs daily. Veterinary examination for all the animals was performed on the day of receipt. The pellet feed and water were provided ad libitum throughout the acclimatization and experimental period.

Animal welfare

This protocol was approved by the Institutional Animal Ethics Committee (IAEC) vide protocol number VIP-IAEC-66-2015 dated 25th November 2015. The experiments were conducted as per the recommendation of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facility published in the gazette of India, 15th December 1998.

Grouping and study design

Animals were grouped by body weight stratification and randomization. The grouping was done one day prior to the experiment initiation. 40 mice were grouped into 4 groups with 10 mice in each group (5 males and 5 females). Details are presented in Table 1.

Group	Group type	Dose (mg/1.5 cm ² area)	Sex		Number of animals per group
			Male	Female	
G1	Control+vehicle administration	200 (w/w)	5	5	10

G2	Standard drug (hydroquinone)	200 (w/w)	5	5	10
G3	Test item-01 (AHPL/AYTOP/1914)	200 (w/w)	5	5	10
G4	Test item-02 Kanak oil (AHPL/AYTOP/1914A)	80 (v/w)	5	5	10
Total number of animals					40

Table 1: Study design.

Formulation preparation

2 test items and 1 standard drug were used for the study. Test item 1 was cream based formulation AHPL/AYTOP/1914; Test item 2 was AHPL/AYTOP/1914A and 4% hydroquinone was used as reference or standard control.

Application of test items

Animals were shaved; Vaseline wax was applied and kept overnight prior to first day treatment. Vaseline wax was later washed with 0.9% warm saline. Test items were applied topically daily twice on the same skin area for a consecutive period of 28 d. If the hair grows back, it was shaved at regular intervals.

Dosage volume

The dosage volume of test item no.1 was 200 mg/1.5 cm² area and test item no. 2 was 80 mg/1.5 cm² area. The above dose volume were chosen since, test item 1 was a cream base that required 200 mg (w/w) (i.e. 200 mg/1.5 cm²) to cover the dorsal side of the mice skin. Whereas since test item 2 was oil that required 80 mg (v/w) (i.e. 80 mg/1.5 cm²) to cover the dorsal side of the mice skin also dose volume less than 80 mg/1.5 cm² not have covered the treatment area and the higher dose volume (more than 80 mg/1.5 cm²) could have covered the unshaved area. The dosage volume of hydroquinone was 200 mg/1.5 cm² area.

Duration of the study

The total duration of the study was 28 d (4 weeks). The assignment of animal is presented in Table 1.

In-life observations

The following in-life observations and clinical, pathological parameters were assessed during the study period:

Clinical signs and mortality observations: The animals were observed for general clinical signs once daily and mortality check was carried out twice daily and observations were recorded.

Skin irritation: Assessments for skin irritation was done on Draize scale immediately after application of test items, then every week till the completion of the study. The skin irritation index (Draize scale) is presented as Table 2.

Score	Observed effect
0	Non-irritant
0-0.5	Minimal irritant
0.5-2	Mild irritant

2-5	Moderate irritant
5-8	Severe irritant

Table 2: Skin irritation index (Draize scale).

Body weights: All the animals were weighed individually and the animal body weights were recorded. The body weights were measured during receipt, after grouping and before test items application. Body weights were also recorded on first day of treatment, weekly thereafter and at termination. The animals were weighed using calibrated electronic balance and the weights were recorded.

Skin whitening index by Von Luschan’s chromatic scale method

The skin whitening index was measured on the same skin area using the skin strip method using Von Luschan’s chromatic scale. Scoring for test items was recorded on day 1 and thereafter weekly once for 28 d period.

Pathology

The following pathological observations were made during the study period:

Gross necropsy: At termination i.e., after the completion of 28 d of the study, on day 29, all the animals were individually weighed and the body weights were recorded. The animals were then sacrificed by carbon-dioxide asphyxiation and subjected to external and internal gross necropsy observations. The gross pathological examinations made were recorded.

Histopathology of skin: Skin (where the test items were applied) of animals from all the groups was collected and preserved in 10% neutral buffered formalin. Histopathology of skin was evaluated by using Fontana-Masson silver stain. Melanocytes were counted at different fields in the skin sections by using micrometric method. Mean percent (%) melanocytes were calculated in each group and subjected to statistical analysis.

Statistical analysis

The raw data obtained from the study was subjected to GraphPad Prism Version 5.01 computer statistical analysis processing. The data on body weight was statistically analyzed by One-Way Analyses Of Variance (One-Way ANOVA) with Dunnett’s pair-wise comparison of test groups mean values with control groups mean values. All analyses and comparisons were evaluated at the 95% level of confidence (P ≤ 0.05).

Groups	Dose (mg/1.5 cm ² area)	Days				
		Day 1	Day 7	Day 14	Day 21	Day 28
Group-1 Vehicle control	200 (w/w)	25.22	26.15	26.76	27.8	28.98
		± 3.17	± 2.92	± 3.05	± 3.03	± 2.89
Group-2 Standard drug (hydroquinone)	200 (w/w)	23.76	24.27	24.43	25.9	26.93
		± 3.19	± 3.35	± 3.32	± 3.28	± 3.04

Results

The results of the statistical analyses were presented in tabular form with values as Mean ± Standard Deviation (SD). The statistical significance values were designated by the “*”, “a”, “b” and “c” superscript in the results which indicates significant changes between the groups.

Clinical signs and mortality check

There were no treatment related clinical signs observed in males and females in all the treated groups including vehicle control either during the treatment period or after the treatment period. The summarized clinical signs of male and female animals are presented in Table 3.

Groups	Dose (mg/1.5 cm ² area)	Male mice clinical signs	Female mice clinical signs	Male animal mortality
Group-1 Vehicle control	200 (w/w)	NAD	NAD	Nil
Group-2 Standard drug (Hydroquinone)	200 (w/w)	NAD	NAD	Nil
Group-3 Test item-01 (AHPL/AYTOP/1914)	200 (w/w)	NAD	NAD	Nil
Group-4 Test item-02 Kanak oil (AHPL/AYTOP/1914A)	80 (v/w)	NAD	NAD	Nil
n=5; NAD: No Abnormalities Detected				

Table 3: Summary of clinical signs, mortality of male and female mice.

Skin irritation: The observed skin irritation scores for male and female animals treated with vehicle, test item-01 and test item-02 were found non-irritant.

Body weights: The summarized weekly body weights of male and female animals are presented in Tables 4 and 5 respectively.

The body weights of male and female animals treated with test item-01 and test item-02 were found to be statistically non-significant when compared to vehicle control animals.

Group-3 Test item-01 (AHPL/AYTOP/1914)	200 (w/w)	25.18 ± 3.49	25.9 ± 3.91	26.56 ± 4.35	27.8 ± 3.79	28.89 ± 3.34
Group-4 Test item-02 Kanak oil (AHPL/AYTOP/1914A)	80 (v/w)	23.79 ± 1.82	24.72 ± 1.96	26.07 ± 2.82	27.19 ± 2.77	28.02 ± 2.96

n=5; Values are in Mean ± SD; p>0.05.

Table 4: Summary of body weights of male mice.

Groups	Dose (mg/1.5 cm ² area)	Days				
		Day 1	Day 7	Day 14	Day 21	Day 28
Group-1 Vehicle control	200 (w/w)	24.35 ± 2.74	25.23 ± 3.21	25.99 ± 2.94	27.41 ± 2.89	28.31 ± 3.10
Group-2 Standard drug (Hydroquinone)	200 (w/w)	23.81 ± 1.28	24.36 ± 1.37	24.48 ± 1.41	25.33 ± 1.63	26.56 ± 1.54
Group-3 Test item-01 (AHPL/AYTOP/1914)	200 (w/w)	23.91 ± 1.97	24.77 ± 1.78	25.19 ± 1.95	26.13 ± 2.23	27.32 ± 2.15
Group-4 Test item-02 Kanak oil (AHPL/AYTOP/1914A)	80 (v/w)	25 ± 3.55	25.61 ± 3.67	26.26 ± 3.91	27.61 ± 4.01	28.51 ± 3.70

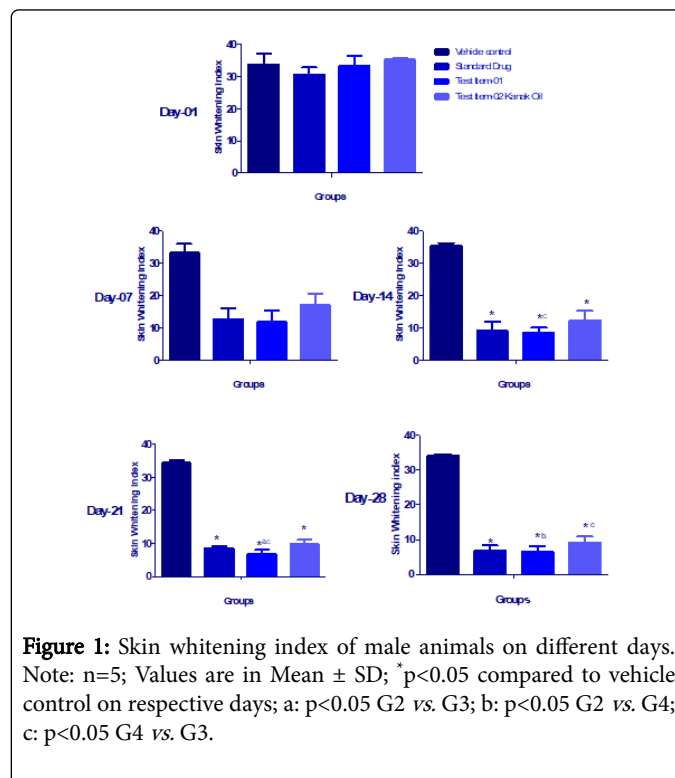
n=5; Values are in Mean ± SD; p>0.05.

Table 5: Summary of body weights of female mice.

Skin whitening index by skin strip method

Males: After the topical application of the vehicle/standard/test items on day 7, day 14, day 21 and day 28 the skin whitening index was noted by using Von Luschan's chromatic scale. Standard drug hydroquinone (Group 2), test item-01 (Group 3) and test item-02 (Group 4) were found significantly better than vehicle control (Group-1) in terms of skin whitening effect (Figure 1). After 21 d of topical application, test item-01 (Group 3) showed significantly better results than standard drug hydroquinone (Group 2) in terms of skin whitening effect. After 28 d of topical application, standard drug hydroquinone (Group 2) showed significantly better results than test item-02 Kanak oil (Group 4) in terms of skin whitening effect. After 7, 14, 21 and 28 d of topical application, test item no. 1 (Group 3) showed significantly better results than test item-02 Kanak oil (Group 4) in terms of skin whitening effect (Figures 1 and 2).

Females: After similar assessment for female group, it was observed that standard drug hydroquinone (Group 2), test item no. 1 (Group 3) and test item-02-Kanak oil (Group 4) were significantly better than vehicle control (Group-1) in terms of skin whitening effect after 7, 14, 21 and 28 d of topical application (Figure 2). After 7 d of topical application, test item no. 1 (Group 3) was significantly better than standard drug hydroquinone (Group 2) in terms of skin whitening effect. Test item-02 Kanak oil (Group 4) also demonstrated significantly better skin whitening activity than standard drug hydroquinone (Group 2) on day 7.



After 7, 14, 21 and 28 d of topical application, test item no. 1 (Group 3) showed significantly better activity than test item-02 Kanak oil (Group 4) in terms of skin whitening effect.

Pathology

The following pathological observations were made during the study period.

Gross necropsy: The summarized external and internal necropsy findings on animals belonging to control and different treatment groups are presented in Table 6. None of the animals in vehicle control and different treatment groups showed any external or internal gross pathological lesions during necropsy indicating that the test items did not cause any systemic lesions.

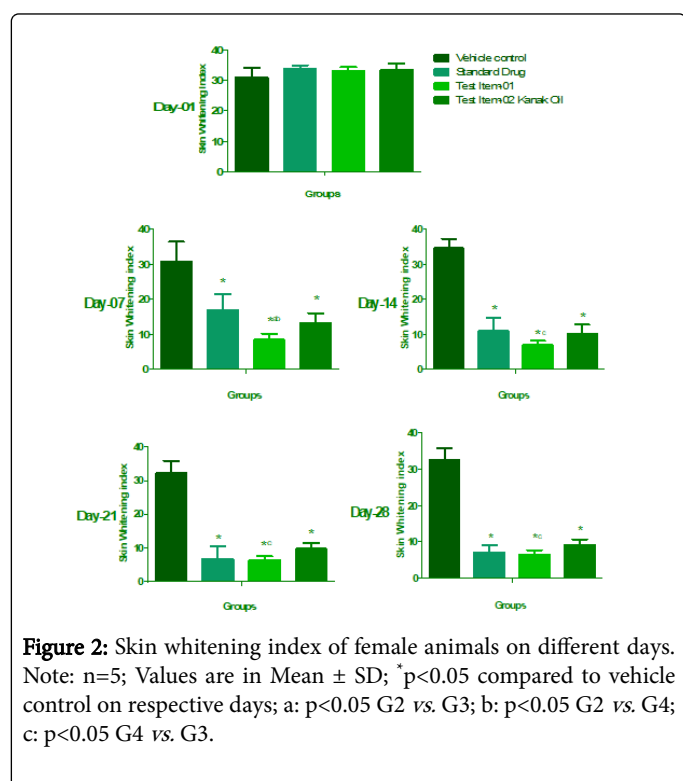


Figure 2: Skin whitening index of female animals on different days. Note: n=5; Values are in Mean ± SD; *p<0.05 compared to vehicle control on respective days; a: p<0.05 G2 vs. G3; b: p<0.05 G2 vs. G4; c: p<0.05 G4 vs. G3.

Groups	Dose (mg/1.5 cm ² area)	Male mice		Female mice	
		External	Internal	External	Internal
Group-1: Vehicle control	200 (w/w)	NAD	NAD	NAD	NAD
Group-2: Standard drug (Hydroquinone)	200 (w/w)	NAD	NAD	NAD	NAD
Group-3: Test Item-01 (AHPL/AYTOP/1914)	200 (w/w)	NAD	NAD	NAD	NAD
Group-4: Test Item-02 Kanak oil (AHPL/AYTOP/1914A)	80 (v/w)	NAD	NAD	NAD	NAD

n=5; NAD: No Abnormalities Detected.

Table 6: Summary of necropsy observation of male and female mice.

Histopathology of skin: The histopathological findings of melanocytes in Fontana-Masson silver stained skin tissues of animals belonging to vehicle control and different treatment groups were presented in Figure 3. Standard drug hydroquinone (Group 2), test item-01 (Group 3) and test item-02 Kanak oil (Group 4) were significantly better than vehicle control (Group-1) in terms of reduction in melanocytes after 28 d of topical application. Test item-01 (Group 3) showed statistically significant reduction of melanocytes compared to standard drug hydroquinone (Group 2) after 28 d of topical application. Though the test item-02 Kanak oil (Group 4) showed reduction in melanocytes but not equal as that of test item-01 (Group 3).

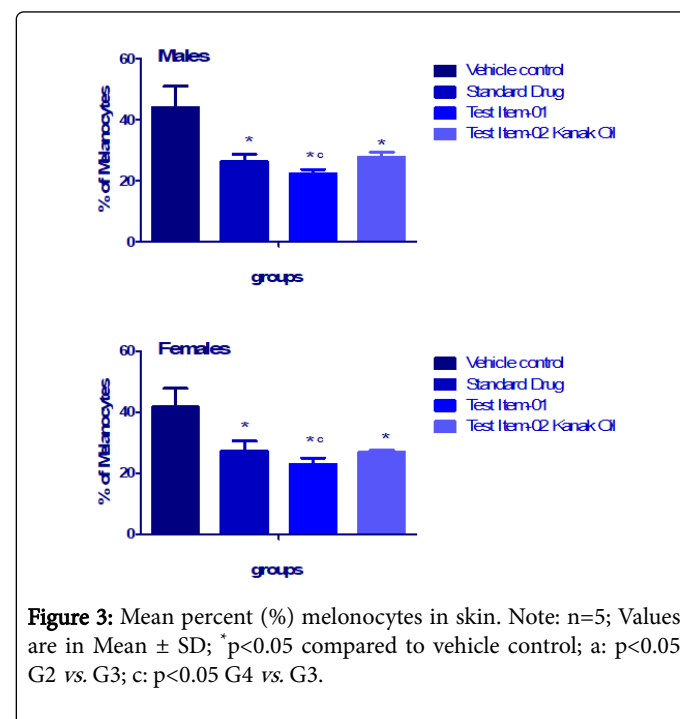


Figure 3: Mean percent (%) melanocytes in skin. Note: n=5; Values are in Mean ± SD; *p<0.05 compared to vehicle control; a: p<0.05 G2 vs. G3; c: p<0.05 G4 vs. G3.

Discussion

Current study was carried out to evaluate de-pigmenting activity of test item no. 1 i.e. AHPL/AYTOP/1914 cream which is unique poly-herbal formulation developed by Ari Healthcare Pvt. Ltd. for the treatment of hyperpigmentation. Test item no. 1 was tested for depigmentation activity in C57BL/6J mice in comparison to standard drug i.e. hydroquinone and test item no. 2 i.e. Kanak oil which is a classical Ayurvedic formulation indicated for the treatment of Vyanga and Nilika (black spotting or melasma) [6]. All the test items including vehicle control and hydroquinone were applied locally for 28 d. Skin whitening effect, body weight, clinical signs and symptoms and skin irritation effect were evaluated at the interval of 7 d. Gross necropsy for overall observation and skin histopathology for estimation of melanocytes were done at the end of the treatment.

No abnormal clinical sign and mortality in any of the mice (male and female) was observed during the study period and at the end of the study. No statistically significant increase or decrease in weight of male and female mice was observed during the study period and at the end of the study indicating that the test formulations are safe for topical use. Moreover, skin irritation index was zero for all the test items including hydroquinone, which suggests safety of all the test

items when used as topical application. It was observed that all the test items including hydroquinone possess statistically significant skin whitening/de-pigmenting activity as compared to vehicle control in male and female mice. In male mice, hydroquinone showed significantly better activity than Kanak oil on day 28. Test item no. 1 was significantly better than hydroquinone in terms of de-pigmenting activity on day 21 of treatment. Test item no. 1 showed significantly better de-pigmenting activity than Test item no. 2 (Kanak oil) throughout the treatment period.

In female mice, hydroquinone showed significantly better activity than Kanak oil on day 7. Test item no. 1 was significantly better than hydroquinone in terms of de-pigmenting activity on day 7 of treatment. Test item no. 1 demonstrated significantly better depigmenting activity than Test item no. 2 (Kanak oil) throughout the treatment period. In the necropsy study, no gross pathological changes in any of the organ of male and female mice were observed. Melanocyte count was significantly better in all the test item groups than vehicle control group suggesting all the test items possess depigmentation activity. Test item no. 1 was significantly better than hydroquinone and Test item no. 2 i.e. Kanak oil. It was also observed that test item no. 1 and test item no. 2 were found safe and no toxicity was observed over the study duration. Though there is a risk of side effects associated with the use of hydroquinone [5], it was found to be safe and was not associated with any clinical abnormality or toxicity throughout the study period.

Test item no. 1 contains 6 ingredients [6-16] Kanak oil, Jatiphala (*Myristica fragrans*) extract, Yashtimadhuka (*Glycyrrhiza glabra*) extract, Lodhra (*Symplocos racemosa*) extract, Arjuna (*Terminalia arjuna*) extract and Manjistha (*Rubia cordifolia*) extract. These ingredients have been used in Ayurveda for treatment of various skin disorders such as Vyanga (dark spots or hyperpigmentation) and Nilika. These ingredients help to decrease hyperpigmentation, dark spots, melasma and chloasma. These ingredients also possess skin lightening, skin glowing, anti-microbial and anti-inflammatory activities. These ingredients provide nourishment to the skin and show skin moisturizing effects [6-16]. By virtue of these activities; test item no. 1 could have yielded significant skin whitening/de-pigmenting activity in the current study.

Kanak oil is one of the ingredients of test item no. 1. In the present study, Kanak oil was taken as one of the comparators. The reason behind doing so was to prove a synergistic/significantly enhanced effect of formulation (test item no. 1) rather than just an added effect. It was observed in the results that test item no. 1 is significantly better than test item no. 2 in terms of skin whitening effect and reduction in melanocyte count.

Conclusion

Based on the results of the study, it can be concluded that test item no. 1, test item no. 2 and standard hydroquinone are safe and possess skin whitening/de-pigmenting activity. The skin whitening/de-

pigmenting activity of test item no. 1 is significantly superior to test item no. 2 and standard hydroquinone. Thus, test item no. 1 is safe and can be effectively used for the treatment of hyperpigmentation, melasma, chloasma and various skin pigmentation disorders.

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