ORIGINAL ARTICLE

Flavonoids as Adjuvant in Psoralen-based Phytochemotherapy in the Management of Vitiligo/Leukoderma

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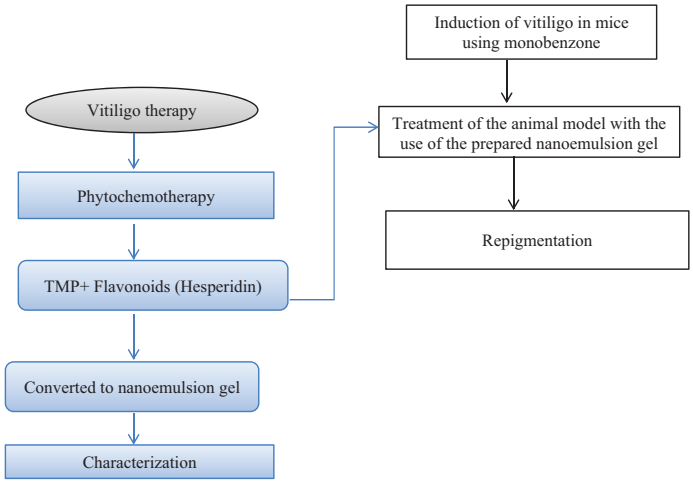
Abstract

Aim: Vitiligo is a skin disorder due to the decrease in the concentration of melanin pigment on our skin. In this work, we propose to utilize the flavonoids to augment the phytochemotherapy with vitiligo. **Materials and Methods:** Hesperidin is used as a natural ingredient in this formulation. This work is to develop a novel drug combination loaded lipid-based nanoemulsion gel. This is a novel strategy to reduce the duration of ultraviolet (UV) radiation in psoralen plus UVA therapy. Induction of the vitiligo in C57BL/6 mice using monobenzone and treating the induced animal with the drug-loaded nanoemulsion gel. **Results and Discussion:** Nanoemulsion gel is prepared and characterization studies were carried out. *In vivo* studies were carried out in the animals immediately after the induction of vitiligo. It is proved that the treatment is supporting with the repigmentation. **Conclusion:** For this study, it is proven that the use of the hesperidin as a flavonoid with trimethylpsoralen combination acts as an effective phytochemotherapy agent for psoralen treatment.

Key words: Flavonoids, hesperidin, nanoemulsion gel, psoralen treatment

Graphical abstract



Note: Flavonoids and TMP loaded into nanoemulsion gel and its characterization aspects

INTRODUCTION

Vitiligo is a skin disorder which is characterized by loss of melanin pigment on the skin.[1] The progression of disease mainly depends on the loss of melanin pigment due to the influence of various causative factors which includes genetic, hormonal, stress, family history, nutritional deficiency, and other neurochemical mediators.[2-4] Usually,

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vitiligo progression occurs almost all over the body portion which is so called as non-segmental vitiligo, but when the progression of the disease is restricted to particular segment of human body, then it is called as segmental vitiligo. The prevalence of non-segmental vitiligo is more compared to that of segmental vitiligo among various population rationales.[5,6] Many research works have been performed around various continents of the world to determine the prevalence and also progression of vitiligo worldwide. Various community-based research works have been performed and the work reveals that Europe and Africa show more prevalence to vitiligo followed by America and Asia. Many surveys have been performed to determine the progression of disease among various age groups. The outcome of survey gave us an idea that young children (0–19) and also geriatric people were susceptible to the disease. Another work on worldwide prevalence of vitiligo reveals that China shows least prevalence to vitiligo compared to Romania showing the highest prevalence rate for vitiligo, whereas India shows a moderate prevalence rate of 0.46% among all age groups.[7,8] Various studies have been performed to determine the epidemiology of childhood vitiligo in Indian continent. These studies have performed among 625 children for the time period of 10 years and it reveals that 57.1% of girls were affected by the disease compared to boys (42.9%). They also reported that among 112,785 patients with skin diseases, 2672 patients developed vitiligo.[9,10] Melanin pathway is the main mechanism involved in the production of melanin. L-phenylalanine and L-tyrosine are the key parameters involved in the melanin pathway for the production of melanin.[11] The main cause of the disease usually starts in the melanin pathway by various key parameters such as stress, nutrient deficiency, and hormonal deficiency.[12] Among the various factors, stress plays a major role in the progression of disease. Increase in these stress factors results in self-destruction of melanocytes.[13] Melanocyte production is also governed by the release of melanocyte-stimulating hormone. Therefore, progression of vitiligo is also due to deficiency of such hormones.[14] There are various nutrients in our body, each having their precise role. In the melanin pathway, tyrosinase enzyme plays a major role in the production of melanocytes. Deficiency of certain nutrients in our body leads to the reduction of tyrosinase enzyme. This leads to reduction in the melanin content of the body.[15] In certain condition, stress factors cause blockage of entire melanin pathway by inhibiting L-phenylalanine and L-tyrosine. There are various treatment modalities available for the management of vitiligo. Among the various treatment methods, photochemotherapy is most commonly used in all cases of vitiligo. The word photochemotherapy itself reveals the information that after the application of medicament, the therapy involves exposure to ultraviolet (UV) radiation.[16,17] Psoralen is the main drug which has been used for the management of vitiligo in the past decades. Then, various research works have been performed on the derivatives of psoralen which reveals that trimethylpsoralen (TMP) shows better activity compared to that of other psoralen derivatives.[18] Many works have been done in ethanomedicinal trends in Indian tradition and found that many herbal constituents from various plants have potential effect in the management of vitiligo.[19,20] From our pipeline of review work on vitiligo, we could able to find the treatment based on psoralen plus UVA therapy involving high exposure to UV rays which resulted in skin lesions.[21] The main aim of the study was to develop the novel lipid-based combination strategy for the treatment of vitiligo. In this work, we hypothesized to use flavonoids as an adjuvant along with TMP for the treatment of vitiligo. We also hypothesized this combination strategy to reduce the time duration of treatment and increasing the effectiveness of treatment. Along with this combination therapy, we incorporated nanotechnology to improve permeation of drug and to provide better effectiveness of therapy.[22]

MATERIALS AND METHODS

Materials

TMP was purchased from Sigma-Aldrich. Hesperidin and propylene glycol were obtained from Avra Synthesis. Polyethylene glycol (PEG) 400, Tween 80, and triethanolamine were obtained from HiMedia Laboratory. Capryol 90 and Capmul MCM were obtained from Gattefosse. Monobenzone cream was purchased from Silverline Medicare Pvt., Ltd. Carbopol 934 LR was supplied by SD Fine Chemicals. Kolliphor was supplied by Sigma-Aldrich as gift sample.

Preformulation studies

Solubility studies

The solubility of TMP and hesperidin in various oils (Capmul MCM, Oleic acid, Capryol 90, castor oil, isopropyl myristate, olive oil, coconut oil, and soybean oil), surfactants (Kolliphor), and cosurfactants (PEG) was determined by the shake flask method. To the cap vials each containing 1 ml of the oils, desired amount of TMP and hesperidin was added. Then, mixture was vortexed using a vortex mixer at a maximum speed for 10 min. This has been done to facilitate proper mixing of TMP and hesperidin with the oils. Mixtures were then placed in an orbitary shaker (24 h) until equilibrium is achieved. The equilibrated sample was removed from shaker and extracted using dimethyl sulfoxide (DMSO) by centrifugation at 4000 rpm for 20 min. The supernatant was taken and filtered through a 0.45 μm membrane filter. Standard calibration curve was made by dissolving 10 mg of drug in 10 ml of DMSO. The concentration of hesperidin and TMP was determined using UV spectroscopy at 283 nm and 287 nm.

Differential scanning calorimeter (DSC)

DSC was used to characterize the thermal behavior and compatibility of TMP, hesperidin, and their combination. Samples were weighed and taken in a standard aluminum pan. The aluminum pan was heated slowly from 20°C to 400°C

at heating rate of 10°C/min. The dry nitrogen was purged constantly at the rate of 50 ml/min. A sealed empty aluminum pan was used as a reference; DSC thermograms were obtained using an automatic thermal analyzer system. Indium calibration reference standard was used for temperature calibration. The thermograms obtained from the DSC were used for comparing and finding the compatibility in nanoparticles.

Preparation of nanoemulsion

Nanoemulsions were prepared by the spontaneous emulsification method. Nanoemulsion was prepared by mixing of oil, water, surfactant, and cosurfactant (SCoS), in the right proportion, with mild agitation. Emulsification is affected by the addition of surfactant and cosurfactant. The energy required for emulsification is supplied by the surfactant mixture. The composition of drug-loaded batches is given in Table 1.

Formulation of TMP nanoemulsion

About 50 mg of TMP was added to 3 ml of Capryol 90, 2 ml of Capmul MCM, and 1 ml of Tween 80. To this mixture SCoS (Smix) was added in the ratio of 1:1. The aqueous phase which is nothing, but distilled water is added drop by drop till transparent to translucent nanoemulsion is formed.

Formulation of TMP and hesperidin nanoemulsion

About 50 mg of TMP and 50 mg of hesperidin were added to 6 ml of Capryol 90, 4 ml of Capmul MCM, and 2 ml of Tween 80. To this Smix was added in the ratio of 1:1. The aqueous phase which is nothing but distilled water is added drop by drop till transparent to translucent nanoemulsion is formed.

Characterization of obtained nanoemulsion

Particle/globule size, polydispersity index (PI), and zeta potential analysis

The mean particle/globule size and zeta potential of TMP and hesperidin-loaded nanoemulsion were determined using a zetasizer (Malvern Instruments, UK). The average particle size was measured based on light scattering mechanism. The movement of particles results in scattering of dynamic light which is analyzed to give particle size. The zeta potential of nanoemulsion was found by placing samples in zeta cells after dilution. The main mechanism involved in the analysis of zeta potential was electrophoretic light scattering. The PI was determined to find the nature of distribution of particles. Narrow distribution of particles indicates that particles size uniformity.

Table 1: Composition of drug-loaded batches

Ingredients	TMP nanoemulsion	TMP and hesperidin nanoemulsion
TMP (mg)	50	50
Hesperidin (mg)	–	50
Capryol 90 (ml)	3	6
Capmul MCM (ml)	2	4
Tween 80 (ml)	1	2
Cremophor (ml)	1	2
PEG 400 (ml)	1	2

PEG: Polyethylene glycol, TMP: Trimethylpsoralen

Formulation of nanogel

Selection of gelling agent

Usually, Carbopol is acidic in condition when dissolved in water. When pH is raised using neutralizing agents, there occurs transition from solution to gel. Gel was prepared by dispersing various concentrations of Carbopol in water and then neutralizing it with triethanolamine to pH 6.8–7.4. After preparing various concentrations of Carbopol gel, the suitable concentration was found out by the nature and appearance of the gel.

Formulation of gel

About 0.5% Carbopol was dispersed in water under continuous stirring at 50 rpm and kept overnight for the polymer to get expand. To the Carbopol solution, the prepared drug-loaded nanoemulsion was added. Then, this dispersion was neutralized using triethanolamine and the pH of gel was adjusted to 6.8–7.4.

Characterization of drug-loaded nanoemulsion gel

Measurement of pH

Digital pH meter was used to determine the pH of the developed drug-loaded nanoemulsion gel. In 100 ml of distilled water, about 1 g of drug-loaded nanoemulsion gel was added. After the addition of gel, the beaker was kept aside for 2 h. Then, pH of drug-loaded emulsion gel was measured 3 times and the average pH value was calculated.

Measurement of spreadability

Spreadability of the gel was one of the typical parameters which are related with therapeutic efficacy of formulation. About 3 g of sample (drug-loaded nanoemulsion gel) was weighed and placed in between two parallel glass slides. Then, 1000 g weight was placed on the glass slides for 5 min to compress the gel uniformly. Then, about 20 g weight was tied to the upper slide with the help of hook. The time taken for the upper slide to move a total distance of 15 cm is noted. The spreadability of drug-loaded emulsion gel was measured 3 times and the average spreadability value was calculated. The formulation will have better spreadability when the upper slide takes minimum time to cover the entire distance.

The spreadability (S) can be calculated using the following formula:

$$\text{Spreadability} = \frac{\text{weight tied to upper slide(g)} \times \text{Length (cm)}}{\text{Time taken(s)}}$$

Viscosity

The viscosity of the formulated gel (0.5 g) was determined without dilution using a Brookfield cone and plate rheometer at 25 ± 0.5°C. The viscosity of the drug-loaded nanoemulsion gel was determined by viscometer with the help of spindle 4 (Brookfield Engineering Laboratories). Viscosity of the formulation was determined by allowing the spindle to come in contact with the formulation. The viscosity of drug-loaded emulsion gel was measured 3 times by rotating the spindle with desired rpm and the average value was calculated.

Extrudability

Extrudability of gel was also typical parameter which is correlated to viscosity. About 20 g of gel was weighed and filled in collapsible tube. After filling the gel into the collapsible tube, the open end was sealed. Then, the tube was pressed from the sealed crimp end with the desired weight (g), but the cap should be closed. When the cap was removed, the gel from the collapsible tube extrudes with the applied pressure. The extrudability of drug-loaded emulsion gel was measured 3 times and the average value was calculated.

The extrudability was than calculated using the following formula:

$$\text{Extrudability} = \frac{\text{Applied weight to extrude gel from tube (gm)}}{\text{Area (in cm}^2\text{)}}$$

In vivo studies

An 8–9-week-old male C57BL/6 mice weighing 25–30 g were obtained from the Liveon Biolabs Pvt., Ltd., Tumakuru - 572106, Karnataka (Reg no: 1610/ROBiBt/S/2012/CPCSEA). Before initiation of the experiment, the animals were habituated to laboratory conditions. This is done so that animals will adjust to the new environmental conditions. Polypropylene cages with paddy husk bedding were used to house the animals. Throughout the experimental period, the animals were fed with pellets and water. All the experiments were carried out with prior approval of the Institutional Animal Ethical Committee, J. S. S. College of Pharmacy, Udhagamandalam, India (Reg no: 118/Po/Rebi/s/1999 CPCSEA 19/05/1999).

Induction of vitiligo

Induction of vitiligo in animals is carried out using monobenzone cream USP 20% w/w. Monobenzone cream is 20% purified monobenzyl ether of hydroquinone in a special base. Application of cream results in reduction of melanin pigment formation by inhibiting the mechanism of melanin pathway. The skin of animals containing hairs is trimmed using scissors. Initially, the cream was applied once daily on the trimmed areas of mice to check any appearance of erythema's and skin lesions. If there is no appearance of skin lesions, rashes or redness the cream was applied twice daily.

Treatment of the vitiligo-induced subject

After the induction, the preparation under study was used for the treatment. Animals were divided into three groups each group containing three animals as shown in Table 2. Animals are fed *ad libitum*. Treatment was initiated form the day of complete induction (depigmentation) of vitiligo. Formulation under study was divided into three which includes TMP nanogel, marketed formulation, and combinational (TMP + hesperidin) nanogel. Based on literature, the formulations were applied once daily for 1 month followed by exposure to UVA light on each application for 15–20 min. Based on the appearance of erythema, the application may be increased or decreased accordingly.

RESULTS

Solubility studies

Shake flask method was used for the determination of solubility of drug in different oils such as (Capmul MCM, oleic acid, Capryol 90, castor oil, isopropyl myristate, olive oil, coconut oil, and soybean oil), surfactants (Kolliphor), and cosurfactants (PEG). Since the solubility of drugs in certain oils was found to be less (1–3.21 mg/ml), it was decided to use combination of oils. The solubility of the drug in oil is considered as the most important and preliminary criteria for the formulation into a nanoemulsion. TMP solubility in individual oils was very less ranging from 0.1 to 0.5 mg/ml. Similarly, for hesperidin, it was found to be in the range of 2–4 mg/ml. Hence, it was decided to use combination of oils to increase the solubility. Combination of Capryol 90, Capmul MCM, Kolliphor, and PEG 400 was tried and we arrived at better solubility of both drugs in the latter case. The possible reason for the increased solubility of drugs could be due to the combined solvent action of the oils and surfactants and also the affinity of drugs to these combinations. Solubility studies of the drug in different oils is represented in Table 3.

Table 2: Grouping of animals

Group	Treatment	Number of animals
I	TMP nanogel	3
II	TMP+hesperidin nanogel	3
III	Marketed formulation	3

TMP: Trimethylpsoralen

Table 3: Solubility studies

Oils	Hesperidin (mg/ml)	TMP (mg/ml)
Olive oil	2.5	0.15
oleic acid	2.7	0.17
IPM	2.4	0.16
Coconut oil	2.42	0.15
Soya oil	2.52	0.18
Castor oil	3.2	0.32
Capryol 90	4.2	4.5
Capryol 90+Capmul MCM	5.2	4.7
Kolliphor	4.8	4.2
PEG 400	4.3	4.1
Capryol 90+Capmul MCM+Kolliphor+PEG 400	6.12	6.15

PEG: Polyethylene glycol, TMP: Trimethylpsoralen

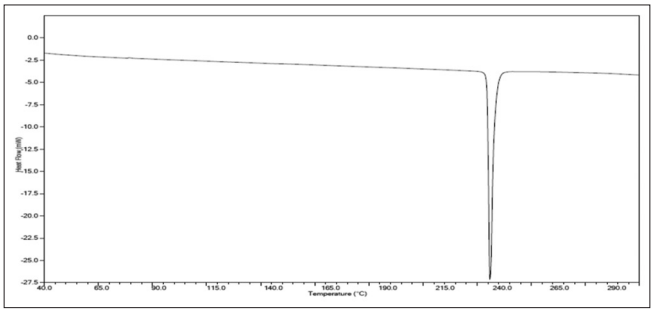

Figure 1: Differential scanning calorimeter thermogram of trimethylpsoralen

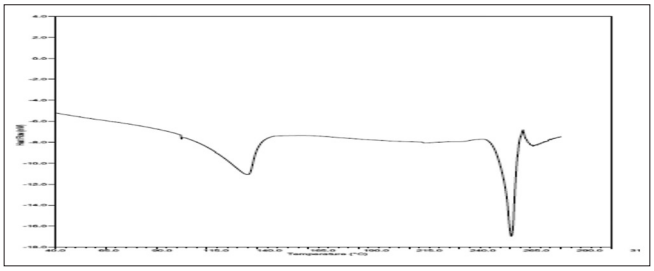

Figure 2: Differential scanning calorimeter thermogram of hesperidin

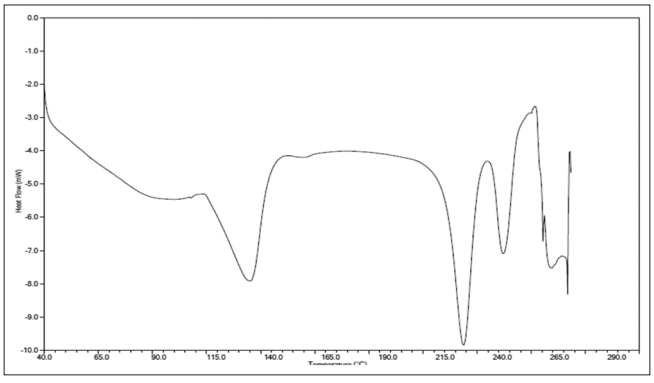

Figure 3: Differential scanning calorimeter thermogram of hesperidin+trimethylpsoralen

DSC

Thermograms of hesperidin, TMP, and drug-drug complex were illustrated in the following Figures 1-3. The endotherm peak of the drug remains the same in drug and drug complex, indicating that there is no interaction. The data also indicate that there seems to be no incompatibilities between the drugs. The DSC curve of TMP shows corresponding endothermic peak (T peak = 230.01°C) corresponding to its melting point. It can thus be concluded that the drug is in pure form. The DSC curve of hesperidin shows corresponding endothermic peak (T peak = 252.24°C) corresponding to its melting point. It can thus be concluded that the drug is in pure form. The selection criteria of the gelling agents are represented in the Table 4.

Characterization of nanoemulsion

Particle size and PI

Particle size and PI of the nanoemulsion were measured by photon correlation spectroscopy using a Zetasizer (Malvern Instruments, UK). The Z-average particle size (d.nm) of nanodroplets formulation (F2) was found to be 380 nm. Particle size distribution graph (size distribution by intensity) is shown in Figure 4. From the graph, the intensity of particles with particle size 200 (d.nm) was found to be 10%. The intensity of particles with particle size 300 (d.nm) was found to be 17%. The intensity of particles with particle size 400 (d.nm) was found to be 23%, whereas the intensity of particles with particle size range 500–600 (d.nm) was found to be comparatively less than that of 400 d.nm. From the graph, it is evident that particles with particle size 400 d.nm show better intensity. The particle size distribution in the formulation can be found out by PI which is related to uniformity of particle size. PI was measured using Malvern Zetasizer. PI values range from 0.000 to 1.000, i.e. monodisperse to very broad particle size distribution. The PI of the nanoemulsion was 0.652 which indicates that sample has broad size distribution.

Zeta potential

Zeta potential mainly deals with the surface charge of the particles. The determination of zeta potential is mainly important to point out the stability of the formulation. Usually, the surfaces of particles in dispersion have certain charges which will cause repelling of the nearby particles. When the repelling charges in nanoemulsion are more it result in instability and phase separation. Nanoemulsion will have better stability when the zeta potential value lies between -30 mV and +30 mV. Nanoemulsion with less zeta potential or zeta potential close to zero will face the problem of instability. Zeta potential should be high for smaller molecules or particles to maintain stability. If the zeta potential is low, there occurs breakdown of dispersion and results in instability. The drug-loaded nanoemulsion has a zeta potential of −14.5 mV which is closest to the normal zeta potential values. This indicates that the prepared nanoemulsion was stable in condition. The zeta potential report is given in Figure 5.

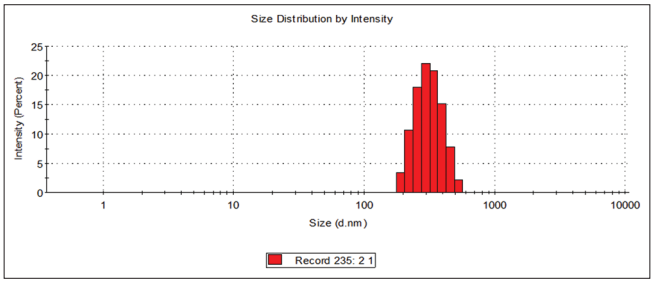

Figure 4: Particle size report (size distribution by intensity)

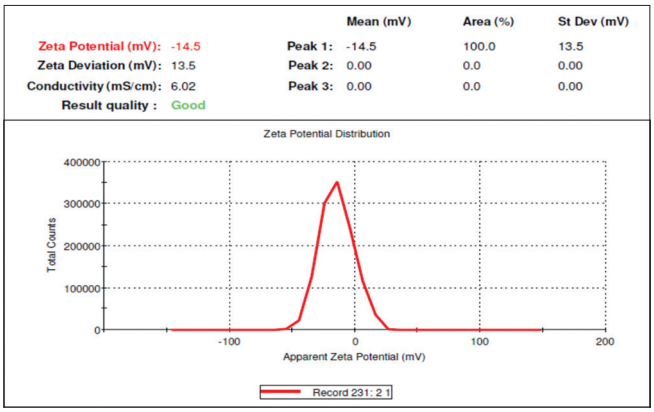

Figure 5: Zeta potential report of nanoemulsion

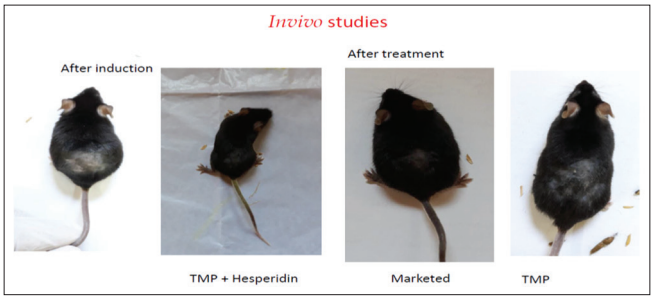

Figure 6: *In vivo* studies

Selection of gelling agent

The gelling agent Carbopol 934 (0.5%) was clear in appearance and has viscosity of 32,400 Cps. Up to the concentration of 0.7%, the gel was clear in appearance. Further, increase in gelling agent concentration (0.8–1%) results in appearance of turbidity as well as increase in viscosity. Carbopol (0.9%) and Carbopol (1%) exhibit more viscosity as well as more turbidity. Increase in viscosity results poor permeation of drug-loaded formulation which will affect the effectiveness of the therapy. Thus, Carbopol 934 (0.5%) was selected as it formed a better gel at low concentration and it would even be more economic. The criteria used for selection of gelling agent is given in Table 4

Characterization of nanogel

The pH of the drug-loaded nanoemulsion gel was done using digital pH meter. The measurement of pH of each formulation was done in triplicate and average values were calculated. The pH of the TMP drug-loaded nanoemulgel was found to be 6.4. The pH of TMP and hesperidin nanogel was found to be 6.5. The pH of the formulation was found to be slightly acidic. The formulation was made slightly acidic because basic pH results in skin irritation. Spreadability of the gel is one of the typical parameters which are related with therapeutic efficacy of formulation. The therapeutic effectiveness of the formulation can be achieved if the prepared gel has good spreadability. This is because when the formulation is having good spreadability, it results in transfer of applied dose to the targeted site. The most commonly used method for determining spreadability was parallel plate method. This is because assembling of the apparatus is easy, less time consumption and the cost was also less. The spreadability of drug-loaded emulsion gel was measured 3 times and the average spreadability value was calculated.

Table 4: Selection of gelling agent

Gelling agent (%)	Viscosity (Cps)	Appearance
Carbopol 934 (0.5)	32400	Clear
Carbopol 934 (0.6)	33625	Clear
Carbopol 934 (0.7)	35250	Clear
Carbopol 934 (0.8)	38920	Slightly turbid
Carbopol 934 (0.9)	40025	Turbid
Carbopol 934 (1)	42575	Turbid

Table 5: Evaluated parameters

Parameters	TMP nanogel	TMP+hesperidin nanogel
pH	6.4	6.5
Spreadability (g. cm/s)	13.65	13.09
Viscosity (Cps)	34424	36398
Extrudability (g/Sq. cm)	6.66	8.33

TMP: Trimethylpsoralen

Table 6: Overall treatment strategy and its efficacy

Days	Time of exposure (min)	Group I (Marketed)	Group II (TMP)	Group III (TMP+hesperidin)
1	30	+	+	+
2	30	+	+	+
3	30	+	+	+
4	30	+	+	+
5	30	++	+	++
6	30	++	+	++
7	30	++	+	++
8	30	++	+	++
9	30	++	+	++
10	30	++	+	++
11	30	++	+	++
12	30	++	+	++
13	30	++	+	++
14	30	++	+	++
15	30	++	+	++
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21	30	++	++	+++
22	30	++	++	+++
23	30	++	++	+++
24	30	+++	++	+++
25	30	+++	++	+++
26	30	+++	++	+++
27	30	+++	++	+++
28	30	+++	++	+++
29	30	+++	++	+++
30	30	+++	++	+++

TMP: Trimethylpsoralen

The spreadability of the TMP drug-loaded nanoemulsion gel was found to be 13.65 g. cm/s. The spreadability of TMP and hesperidin nanogel was found to be 13.09 g. cm/s. Since the time taken by the upper slide to cover the entire distance of lower slide was less, the formulation had good spreadability. Viscosity of the formulation is one of the main parameters which are linked with all other parameters such as spreadability, extrudability, and penetration of the formulation. Viscosity of the formulation mainly depends on the concentration of gelling agent. Therefore, the viscosity of the formulation will be more with increase in the concentration of Carbopol. The viscosity of the TMP drug-loaded nanoemulsion gel was found to be 34,424 Cps. The viscosity of TMP and hesperidin nanogel was found to be 36,398 Cps. The extrudability of the prepared drug-loaded formulation is associated directly with the viscosity of the formulation. The extrudability of the TMP drug-loaded nanoemulsion gel was found to be 6.66 g/sq. cm. The extrudability of TMP and hesperidin nanogel was found to be 8.33g/sq. cm. The evaluated parameters are shown in Table 5 and 6.

In vivo studies

The treatment was carried out immediately after the induction of vitiligo in animals. The animals were divided into three groups, namely marketed formulation, TMP, and TMP and hesperidin. The drug-loaded nanoemulsion gel was applied daily to two groups (Groups II and III), whereas Group I is treated with marketed formulation. After the application of drug to the induced area, the animals were exposed to natural sunlight for a time period of 30 min. The intensity

Table 7: Score and intensity of pigmentation

Score	Intensity of pigmentation
+	Poor
++	Fair
+++	Excellent

of pigmentation after application was done by scoring as in Table 7. Initially, the intensity of pigmentation in all groups was poor. However, after 1 week of treatment, Groups I and II showed better improvement in pigmentation than Group II. Further, continuation of therapy results in better pigmentation in Group III followed by Group II. However, Group II did not show any improvement in pigmentation even after continuation of therapy. The results of the treatment are well supported with the repigmentation as shown in Figure 6.

CONCLUSION

Results reveal the exposure time to UV rays. Animals received the marketed formulation attributed to exposure of UV rays up to 30 min. Whereas the animals received nanoemulgel, exposure to UV required <30 min. Same case resulted with Group III which received the treatment of TMP + hesperidin in nanoemulgel but with less exposure time. Experiments also reveal the treatment period with TMP and TMP + hesperidin. With Group I required almost 30 days for repigmentation to initiate, whereas the repigmentation is faster with only 20 days with Group III. It could be the hesperidin which triggered the induction melanin synthesis along with TMP. Group II which received only TMP in nanoemulgel could not initiate repigmentation completely with full treatment period. Further, the behavior point of view of the animals, treatment with TMP + hesperidin in nanoemulgel did not result in any untoward reactions/side effects when compared to marketed formulation. Hence, patience compliance could be improved with the above-said combination. Therefore, the novel combination of flavonoid with TMP would be a better combination strategy for the management of leukoderma.

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