UV Irradiation Affects Melanocyte Stimulatory Activity and Protein Binding of Piperine

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Received 14 April 2006; accepted 4 July 2006; published online 14 July 2006 DOI: 10.1562/2006-04-21-RA-882

ABSTRACT

Piperine, the major alkaloid of black pepper (Piper nigrum L.; Piperaceae), stimulates melanocyte proliferation and dendrite formation in vitro. This property renders it a potential treatment for the skin depigmentation disorder vitiligo. However, piperine does not stimulate melanin synthesis in vitro, and treatments based on this compound may therefore be more effective with concomitant exposure of the skin to ultraviolet (UV) radiation or sunlight. The present study investigated the effect of UVA and simulated solar radiation (SSR) on the chemical stability of piperine, its melanocyte stimulatory effects and its ability to bind protein and DNA. Chromatographic and spectroscopic analysis confirmed the anticipated photoisomerization of irradiated piperine and showed the absence of any hydrolysis to piperinic acid. Isomerization resulted in the loss of ability to stimulate proliferation of a mouse melanocyte cell line, and to bind to human serum albumin. There was no evidence of DNA binding by piperine either before or after irradiation, showing the absence of photoduct formation by either piperine or its geometric isomers. This is unlike the situation with psoralens, which form DNA adducts when administered with UVA in treating skin diseases. The present study suggests that exposure to bright sunlight should be avoided before or after application of piperine to the skin and in the storage of piperine products. If UVA radiation is used with piperine in the treatment of vitiligo, application of the compound and irradiation should be staggered to minimize photoisomerization. This approach is shown to effectively induce pigmentation in a sparsely pigmented mouse strain.

INTRODUCTION

We have previously reported that piperine (Fig. 1), the major alkaloid found in the fruit of black pepper (Piper nigrum L.; Piperaceae) (1), stimulates the replication of melanocytes and induces the formation of melanocytic dendrites in vitro (2,3). Melanocytes are pigment-producing dendritic cells located within the basal layer of the epidermis and in the matrix of hair follicles. Melanin is synthesized within organelles known as melanosomes, which are transferred through melanocytic dendrites to epidermal keratinocytes (4), resulting in the observed pigmentation of mammalian skin. The stimulatory effects of piperine on melanocyte proliferation and dendriticity render it a potential treatment for vitiligo, a skin disorder characterized by depigmented lesions (4). Melanocytes have been shown to be absent, or present in very small numbers, in vitiligo lesions apart from reservoirs found in the hair follicles (5–7). Piperine is expected to cause the repopulation of vitiligo patches through a stimulatory effect on perilesional and follicular melanocytes. However, piperine does not stimulate melanin synthesis in melanocytes in vitro (2,3). A potential treatment option is to expose the skin to ultraviolet radiation (UVR) along with piperine in order to induce pigmentation in the new melanocytes. UVR can act directly on melanocytes to increase skin pigmentation or indirectly through the release of keratinocyte-derived factors such as cytokines, eicosanoids, growth factors, nitric oxide or melanotropic hormones (8–10).

UVA irradiation (320–400 nm) is already used in the treatment of vitiligo, in conjunction with orally or topically administered psoralens (PUVA therapy—psoralens plus UVA) (11). Psoralens form monofunctional and bifunctional photodadducts with cellular DNA upon exposure to UVA; this process stimulates melanocyte replication and melanogenesis (12,13). Solar simulated radiation (SSR) plus 5-methoxypsoralen (5-MOP) has been used to induce human pigmentation (12). 8-MOP, which is more commonly used in clinical practice, has been shown to induce epidermal melanogenesis in human (14) and murine (15) skin when administered with UVA.

Unlike psoralens (12–15), piperine is able to induce melanocyte proliferation in vitro even in the absence of UVR, possibly by a mechanism involving stimulation of protein kinase C (2). A potential complicating factor in the concomitant use of UVR to induce melanogenesis in these cells is that piperine is known to undergo photoisomerization at the double bonds on exposure to UVR (350 nm) (16) and sunlight (17). Although a number of structural analogs of piperine have been found to share its melanocyte stimulatory effects (3), the activity of its geometric isomers has not previously been examined.

The present study investigates the effect of UVR on the in vitro and in vivo stimulatory effects of piperine on melanocytes and on...
MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK) unless stated otherwise. Piperinic acid was prepared in our laboratory by hydrolysis of piperine (24).

Radiation sources and dosimetry for in vitro experiment simulated solar radiation (SSR). SSR was obtained from a Solar Simulator (Oriel, Stratford, CT) with the use of a 1-kW xenon arc lamp (ORC Lighting Products, Azusa, CA) in conjunction with a quartz collimator, quartz lens and a Schott WG320 filter (1 mm thick). Irradiance was routinely measured with the use of an IL 442 radiometer (International Light, Newburyport, MA) and calibrated in the same way as the thermopile used for SSR. Irradiance was 74 mW cm\(^{-2}\) (over the spectral range 340-400 nm) at the test surface (a distance of 10 cm); of the total output, 98.88% was UVA-I (340-400 nm), 1.11% was UVA-II (320-340 nm) and 0.01% was UVB (280-320 nm). The emission spectrum of this source has been previously reported (25).

Broad-spectrum UVA. Broad spectrum UVA (340-400 nm) was obtained from a UVASUN2000 (Mutzhas, Munich, Germany), which was housed on a custom-built trolley allowing irradiation from above. Two fans blew across the irradiation area to provide cooling. Routine irradiance was measured with an IL 442 radiometer (International Light, Newburyport, MA) and calibrated in the same way as the thermopile used for SSR. Irradiance was 74 mW cm\(^{-2}\) (over the spectral range 340-400 nm) at the test surface (a distance of 10 cm); of the total output, 98.88% was UVA-I (340-400 nm), 1.11% was UVA-II (320-340 nm) and 0.01% was UVB (280-320 nm). The emission spectrum of this source has been previously reported (25).

Irradiation of piperine solutions. Piperine was dissolved in CD\(_3\)OD (40 mg/mL). This solution (2 mL) was carefully pipetted into petri dishes placed on ice to minimize evaporation. Petri-dish lids were removed prior to irradiation with either UVA or SSR. After irradiation, solutions were collected and any volume lost by evaporation made up to 2 mL with CD\(_3\)OD. The control solutions (nonirradiated) were covered with aluminum foil and kept at 4°C.

Analysis of irradiated solutions. \(^1\)H NMR was recorded using a Bruker (360 MHz) NMR spectrometer. Reversed-phase HPLC analysis of irradiated and unexposed piperine was performed with the use of an Alttech Adsorbosphere 4.6 mm \(\times\) 25 cm RP-C18 (10 \(\mu\)m) column with a 10 mm C18 guard column with a LDC Analytical 3100 pump and a HP 3390A Integrator connected to a spectrometer 3100 UV detector (LDC Analytical, Riviera Beach, FL). Analyses were performed isocratically with a mobile phase containing 50% water, 40% acetonitrile; 1 mL/min. An injection volume of 10 \(\mu\)L was used; the eluent was monitored at 348 nm. The retention time of piperine in this system was 12.7 min. LC-MS analysis was undertaken with the use of an Alttech Adsorbosphere 4.6 mm \(\times\) 25 cm RP-C18 (10 \(\mu\)m) column in a ThermoFinnigan Surveyor LC system directly coupled to ThermoFinnigan LCQ DECA XP ion-trap mass spectrometer operating in the electrospray positive mode. UV spectra were obtained from the ThermoFinnigan Surveyor Photo Diode Array (PDA) detector associated with the LC-MS system. Analyses were performed isocratically with a mobile phase comprising 85% acetonitrile, 15% water. An injection volume of 10 \(\mu\)L was used; the samples were eluted at a flow rate of 0.2 mL/min and monitored at 348 nm on the PDA for piperine. The retention time of piperine in this system was 11.7 min.

Cell culture experiments

Stock cultures. Melan-a cells are an immortal pigmented mouse cell line, cultured from epidermal melanoblasts from embryos of inbred C57BL mice (26). Subconfluent to nearly confluent melan-a cells (passage number 26-31) were used in this study. Cell cultures were maintained in culture flasks in supplemented RPMI 1640 growth medium with 200 nM TPA, trypsinized, harvested and resuspended for experiments in the same medium without TPA, as described earlier (25).

Preparation of microplates with melan-a cell suspension. Melan-a cells were inoculated (100 \(\mu\)L, \(6 \times 10^5\) cells per well) with a repeater pipetter into 96-well microtiter plates (Nunc, Cambridge) and incubated at 37°C in a 10% CO\(_2\), 90% air humidified atmosphere for 4 h. Piperine was dissolved in methanol and the solutions were sterilized by filtration (pore size 0.2 \(\mu\)m) and then diluted with the cell culture medium to give a final stock solution of 30 \(\mu\)M piperine and a nontoxic concentration of methanol. Each plate was subdivided into sections each consisting of two adjacent columns of 6 wells each for piperine (10 \(\mu\)M, 50 \(\mu\)L of stock solution) or control (50 \(\mu\)L medium only), with a gap of 2 empty columns in between each section.

Irradiation and culture of melan-a cells. The plates, placed on ice, were positioned under the UVA and SSR irradiation sources. In order to maintain sterility of the cell cultures, irradiations needed to be carried out with the microplate lid in place. The lid reduced the UVB and UVA irradiances of the SSR source by about 45 and 30%, respectively, and the UVA irradiance of the UVA source by about 20%. The doses given below and in Figs. 6 and 7 are not corrected for the effects of the lid, so that comparisons can be made with Fig. 3B. Microplates were irradiated with UVA doses ranging from 0 to 124 \(\mu\)J cm\(^{-2}\) with the use of the Mutzhas UVASUN2000 broadband UVA source. This represents a dose of less than 2 minimal erythema doses (MED) (27) for sun-sensitive skin Type II after correction for absorption by the 96-well-plate lid. Different doses were achieved by exposing particular sections of the plate (each consisting of one row of...
piperine exposed cells and one row of control cells) for different time periods (0, 7, 13, 22 or 28 minutes; n = 6). Unexposed areas were covered with a piece of cardboard. Another set of microplates was irradiated with SSR doses ranging from 0 to 15 J cm\(^{-2}\) with the use of the solar simulator for 0, 5, 7, 15 or 21 min (n = 6). This represents doses of about 1.5 MEDs for skin Type II (filter 2 data [27]) after corrections for the effect of the microplate lid. All plates were incubated at 37°C in a 10% humidity controls. The irradiance was monitored daily immediately before application of SSR. Irradiations with International Light radiometer (IL 422A; Newburyport, MA) were applied with a micropipette (100 μl) on dorsal skin twice a day out every Monday, Wednesday and Friday immediately prior to the first administration. For group (C) the irradiations were carried out 5-6 days prior to circular dichroism, CD (Jasco J-600 spectropolarimeter) and linear dichroism, LD (Jasco J-720 spectropolarimeter) analysis immediately after irradiation and after 3 days' storage in a refrigerator. No changes were found to have occurred during the storage period (results not shown). CD spectroscopy was performed using a CD cell of 1 cm path length. LD analysis was performed to validate CD experiments on pipenone binding to DNA.

**RESULTS AND DISCUSSION**

Piperine is 1-2E,4E-pipenoyl-piperidine (Fig. 1) with two trans double bonds in the chain connecting the methylenedioxyphenyl and piperidine groups. Figure 2 shows the UV spectrum of this compound. Possible geometric isomers (Fig. 1) are chavicine (2Z, 4Z; cis-cis), isopiperine (2Z, 4E; cis-trans) and isochavicine (2E, 4Z; trans-cis). Conversion of piperine to these geometric isomers has been reported following exposure to UVR (λmax 350 nm [16,18] or 366 nm [19]) and sunlight (17). In early work (16), chavicine, and later isochavicine, were noted as being the major product of piperine’s photoisomerization. However, later studies (17-19) have shown that all three isomers form from piperine, their ratio being dependent on the length of exposure to irradiation. Chavicine appears to be the last isomer to be produced (17,18) and was the dominant isomer after 24 h exposure to sunlight (17).

In the present study, irradiated and unirradiated solutions of piperine in methanol were compared by high-performance liquid chromatography (HPLC), liquid chromatography coupled to mass spectrometry (LC-MS) and UV and \(^1\)H NMR (nuclear magnetic resonance) spectroscopy. An HPLC chromatogram of piperine irradiated with UVA (124 J cm\(^{-2}\)) is shown in Fig. 3A. The main peak at \(R_t 12.7 \text{ min}\) corresponds to piperine, whereas the peak eluting just before piperine (\(R_t 12.0 \text{ min}\)) represents one or more photoproducts of piperine. Figure 3B shows that the relative area of the photoproducts at 12.0 min increased with increasing UVR dose. No peak for piperincic acid (retention time of standard = 6.3 min) was observed in HPLC analysis, showing that no hydrolysis had taken place at the amide function. Individual isomers were not resolved on this HPLC system, but methods for their baseline separation have been reported elsewhere (17).

Tandem LC-MS (LC-MS\(^2\)) analysis of the two HPLC peaks revealed that both were comprised of substances with identical mass spectra. ESI-MS \(m/z\) in positive-ion mode for the piperine peak gave a quasimolecular ion at \(m/z\) 286 (100%) [M + H]\(^+\). Further fragmentation (MS\(^2\)) of this ion gave \(m/z\) 201, which was then further fragmented (MS\(^3\)) to give \(m/z\) 173, 171, 143 and 115. The photoprodut HPLC peak gave virtually identical ions in terms of the following scoring system: 0 = no pigmentation; 1 = first signs of pigmentation (spots); 2 = light brown; 3 = medium brown; 4 = dark brown; 5 = black. Scores obtained at the end of each week (Friday) are shown in Fig. 11.

**Statistical analysis.** Differences between treatment groups across the entire treatment period were compared by the Mann-Whitney U-test.
of both m/z value and relative abundance. Mass spectra are reported to be identical for the four isomeric compounds (19,20). The MS fragments obtained in this study were in excellent agreement with the literature for piperine and its isomers (19).

Figure 4. UV spectra of HPLC peaks corresponding to piperine (A) and photoproduct (B) after exposure to UVA (124 J cm⁻²). Spectra were obtained using a photodiode array detector attached to the HPLC system.

LC-MS data therefore confirm that the photomodified product consists of one or more geometric isomers of piperine.

Piperine isomers vary in their UV absorption $\lambda_{\text{max}}$ values. Reported values (17,19,20) range as follows: piperine 340–343 nm, isochavicine 330–336 nm, isopiperine 332–335 nm and chavicine 317–321 nm. UV analysis of the peaks observed in HPLC, using diode array detection coupled to the LC-MS instrument, showed a $\lambda_{\text{max}} = 326$ nm for the photomodified product peak as compared to 340 nm for piperine (Fig. 4). This clearly shows the presence of chavicine, which is the only isomer with a $\lambda_{\text{max}}$ value lower than 330 nm. The presence of other isomers is likely to have caused the $\lambda_{\text{max}}$ to shift to a slightly higher wavelength than the range reported for chavicine (317–321 nm).

The $^1$H-NMR spectra of piperine in CD₃OD (deuterated methanol) before and after irradiation are shown in Fig. 5A–C.
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Figure 6. Effect of UVA irradiation on cell growth in the presence or absence (control) of 10 μM piperine. Cell growth is expressed as a percentage of cell growth in cultures unexposed to radiation or piperine. **P < 0.01 when compared to control incubation at the same radiation dose (one-way ANOVA followed by Dunnett’s t-test). Note that doses are not corrected for the absorption of the 96-well-plate lid, which absorbs about 20% of the UVA.

The $^1$H NMR (CD$_3$OD, 360MHz) signals for piperine are at δ 7.31 (1H, ddd, $J_{3.2} = 14.7$ Hz, $J_{3.4} = 8.2$ Hz, $J_{3.5} = 2.0$ Hz, H-3), 7.07 (1H, d, $J_{7.11} = 1.6$ Hz, H-7), 6.95 (1H, dd, $J_{11.10} = 8.0$ Hz, $J_{11.7} = 1.6$ Hz, H-11), 6.88 (1H, dd, $J_{4.5} = 15.2$Hz, $J_{4.3} = 8.2$ Hz, H-4), 6.80 (1H, d, $J_{5.4} = 15.2$ Hz, H-5), 6.78 (1H, d, $J_{10.11} = 8.0$ Hz, H-10), 6.60 (1H, d, $J_{2.3} = 14.7$ Hz, H-2), 5.95 (2H, s, OCH$_2$O), 4.90 (solvent), 3.60 (4H, br, H-1', H-5'), 1.70 (2H, m, H-3'), 1.60 (4H, m, H-2,4'). This spectrum is in agreement with the literature (17,19,20), minor differences in δ values being due to the use of CDC$_1$ (deuterated chloroform) as solvent in the earlier studies.

The $^1$H-NMR spectra of irradiated piperine solutions (Fig. 5B,C) showed significant differences from that of piperine, which were more pronounced in the UVA- than in the SSR-irradiated sample. The spectra were very similar to that previously reported for piperine samples exposed to sunlight (17). In both cases (Fig. 5B,C), the most significant changes are observed in the 6–8 ppm region, representing the double-bond and phenyl protons, indicative of changes in the configuration of the double bond. In both irradiated samples, the strong signal at about 5.98 ppm is still apparent, indicating that the methylenedioxyphenyl group is intact, although there is now some overlap with the double-bond signals. Three previous studies (17,19,20) discuss comparative $^1$H-NMR spectra of the four isomers. A doublet assignable to H-2 is found at around δ 6.0 in the $^1$H-NMR spectra of isopiperine and chavicine, both of which have a cis arrangement of the H-2, H-3 double bond, whereas this proton is seen at around δ 6.5 for piperine or isochavicine. Isochavicine is the only isomer reported to have a signal (H-3) downfield of δ 7.5 (15,17,18), and the H-4 signal appears uniquely in the δ 6.3–6.4 region for this isomer. New signals around δ 6 were seen in the two irradiated samples of piperine (Fig. 5B,C), showing that either chavicine or isopiperine, or both, are present in the mixture. The irradiated solutions also show a new double doublet at δ 6.4 and multiplet at around δ 7.75, confirming the presence of isochavicine. $^1$H NMR analysis therefore confirms that UVA and, to a lesser extent, SSR cause the photoisomerization of piperine in the present study. The proportion of isochavicine in the mixture can be obtained from a comparison of the integration value for the isochavicine H-3 signal at δ 7.75 (1.08 SSR; 0.42 UVR) with the value obtained for the six piperidine protons at H-2', 3', 4' appearing at δ < 2.0 (29.84 SSR; 23.09 UVR). Interestingly, there is less isochavicine in the UVR-treated (11%) than the SSR-treated (22%) sample.

In cell cultures unexposed to irradiation (Figs. 6 and 7), piperine stimulated melanocyte proliferation and dendrite formation (Fig. 8) as expected from earlier studies (2,3). However this effect was lost on exposure to UVA (Fig. 6) or SSR (Fig. 7). For SSR, the effect

Figure 7. Effect of SSR irradiation on cell growth in the presence or absence (control) of 10 μM piperine. Cell growth is expressed as a percentage of cell growth in cultures unexposed to radiation or piperine. **P < 0.01 when compared to control incubation at the same radiation dose (one-way ANOVA followed by Dunnett’s t-test). Note that doses are not corrected for the absorption of the 96-well-plate lid, which absorbs about 30% of the UVA and 45% of the UVB.

Figure 8. Melan-a cells grown in control culture medium show a bipolar morphology (A). Exposure to piperine (10 μM) induces dendricity in these cells (B).
was radiation dose dependent, probably due to lower levels of isomerization with this form of radiation as compared to the doses used with UVA alone. UVA has previously been shown to cause DNA damage in cultured human melanocytes in vitro (21–23). However, in the present study, this does not account for the observed decline in cell growth in irradiated, piperine-treated cultures. Control cells exposed to SSR radiation at all test doses grew as well as unexposed cells (Fig. 7), whereas UVA-exposed cells showed a small decline in growth only at radiation doses of 98 J cm\(^{-2}\) and above, whereas the stimulatory effect of piperine was lost even at 31 J cm\(^{-2}\) of UVA (Fig. 6). We have previously reported that a large number of analogues of piperine can stimulate melanocyte proliferation (3), implying that the structural requirements for activity may not be very stringent. However, the present study shows that the configuration around the double bonds in the 1-piperinoyl-piperidine molecule may have a profound effect on activity. The isomerization induced by UVA and SSR radiation results in a loss of activity of the molecule in proportion to the radiation dose received. A change from a trans, trans configuration to one involving cis bonds would lead to a considerable alteration in the overall shape of the molecule. Piperine would be expected to have a relatively linear structure, whereas the other three isomers, each containing at least one cis bond, are more folded (Fig. 1). It is of note that tetrahydropiperine, in which the two double bonds are replaced by a saturated 4-carbon aliphatic chain is also active (3), presumably because it can adopt a linear conformation similar to piperine.

Potential binding of piperine to protein and DNA before and after irradiation was investigated using circular dichroism (CD). Piperine is not optically active, but if bound to a chiral substance such as human serum albumin (HSA) or DNA, optical activity is induced and a signal will be observed in the CD spectrum associated with piperine’s UV absorption \(\lambda_{\text{max}}\) (about 340 nm). Piperine was found to bind to HSA (Fig. 9A) but on exposure to UVA (Fig. 9B) or SSR (Fig. 9C), this was abolished, suggesting that the structural changes induced by radiation were detrimental to protein binding of the molecule. No binding to DNA was observed by CD either before or after irradiation (Fig. 10A–C); the increase in absorbance at 275 nm (Fig. 10B,C) is due to a concentration effect (solvent evaporation). Absence of binding to DNA was confirmed with the use of linear dichroism (data not shown). This suggests that unlike the situation with psoralens (13), piperine and its isomers do not bind DNA and would not form photoadducts in vivo.

The present results show that UVR-induced photoisomerization of the piperine molecule results in the loss of its protein binding and melanocyte stimulatory activity. These results do not, however, preclude the use of UVR in conjunction with piperine in the treatment of vitiligo. We have conducted in vivo experiments with a hairless, sparsely pigmented mouse strain (HRA.HRII-c+/-Skh) in which piperine solution was applied topically twice every weekday for 9 weeks and UV irradiation was administered three times a week from Weeks 5–9, just prior to application of piperine. Using this protocol, the melanocyte stimulatory effect of piperine was retained. Pigmentation was better in mice receiving both piperine and UVR than in mice treated with either agent alone (Fig. 11), verifying the usefulness of their concomitant use. It is important that administration of UV irradiation to patients receiving piperine must be appropriately timed and that piperine-containing preparations are protected from light.

CONCLUSIONS

When exposed to physiologically relevant doses of UVA and SSR, piperine photoisomerizes at the conjugated double bond to give a mixture of isomeric products. UV and \(^1\)H NMR data provided good evidence for the presence of chavicine and isochavicine in the mixture, although the presence of isopiperine could not be ruled out. Piperinic acid, a potential hydrolysis product of piperine, was not detected. Conversion to its geometric isomers led to a loss of piperine’s ability to stimulate melanocyte proliferation and to bind to HSA in vitro. No in vitro binding to DNA was observed either before or after irradiation. These results are not surprising, given
that piperine absorbs UVR across the whole solar range (Fig. 2). Thus both UVB and UVA are likely to have contributed to the photoisomerization and loss of melanocyte stimulatory activity observed here with environmentally and physiologically relevant doses of SSR. If UVR is used with piperine in the treatment of vitiligo, application of the compound and irradiation should take place at different times, to minimize photoisomerization of piperine. This protocol was more effective than piperine or radiation alone in inducing pigmentation in a sparsely pigmented mouse strain. Bright sunlight should be avoided both during active application of piperine to the skin and in the storage of piperine products.

Acknowledgements—Dr. R. Venkatasamy was funded by Overseas Research Students Award Scheme (ORS) and the project was funded by British Technology Group (BTG), UK. The authors thank Professor D. C. Bennett of St. Georges Medical School, UK for the gift of the melan-a cell line and Mr. Graham Harrison for assistance with the UV radiations and dosimetry. Some data reported here were presented in posters at the following conferences: 42nd Annual Meeting of the American Society of Pharmacognosy, Oaxaca, Mexico, July 14–18, 2001; International Symposium of the Phytochemical Society of Europe (PSE), 2001, Lausanne, Switzerland, September 12–14, 2001; British Pharmaceutical Conference, 2001, Glasgow, Scotland, September 23–26, 2001, and in one published abstract (32).

REFERENCES
In vivo evaluation of piperine and synthetic analogues as potential treatments for vitiligo using a sparsely pigmented mouse model

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Accepted for publication
4 September 2007

Key words
HRA/Skh-II, melanocyte, pigmentation, piperine, tetrahydropiperine, vitiligo

Conflicts of interest
None declared.

Summary

Background Piperine and its analogues have been reported to stimulate melanocyte replication in vitro and may be useful in treating the depigmenting disease, vitiligo.

Objective To investigate the ability of piperine (PIP) and three analogues to stimulate pigmentation in a strain of sparsely pigmented mice.

Methods The test compounds were PIP [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylpiperidine], tetrahydropiperine [THP, 5-(3,4-methylenedioxyphenyl)-pentanoylpiperidine], a cyclohexyl analogue of piperine [CHP, 5-(3,4-methylenedioxophenyl)-2,4-pentadienoylcyclohexylamine], and reduced CHP [rCHP, 5-(3,4-methylenedioxyphenyl)-2,4-pentanoylcyclohexylamine]. Sparsely pigmented, HRA/Skh-II mice were randomized to receive topical treatment with test compounds or vehicle twice a day for five days a week, with or without ultraviolet (UV) irradiation on 3 days a week. Treatment was either continuous or interrupted to evaluate fading and repigmentation. Skin inflammation and pigmentation were evaluated regularly during treatment. DOPA+ melanocytes were determined histologically at the termination of treatment.

Results Four weeks of treatment with one of the compounds PIP, THP or rCHP, but not CHP, induced greater pigmentation than vehicle with low levels of inflammation. Additional exposure to UVR led to darker pigmentation than did the compound or UVR alone, and greater numbers of DOPA+ melanocytes were found. The combination produced an even pigmentation pattern, contrasting with the speckled, perifollicular pattern produced by UVR alone. Treatment interruption led to a decrease in pigmentation but not its loss. Repigmentation was achieved by administering one of the compounds, UVR or both, and occurred faster than in naïve mice.

Conclusions Treatment with PIP, THP or rCHP and UVR induced a marked pigmentation response in HRA/Skh-II mice, with clinically better results than UVR alone. This result supports the potential use of these compounds in treating vitiligo.

The skin disorder vitiligo is the most common acquired hypomelanosis, affecting approximately 1% of the world’s population, with serious cosmetic and psychological effects.1 The characteristic depigmentation can be restricted to a limited skin area (segmental vitiligo) or generalized in symmetrical patches (nonsegmental vitiligo). In most cases, loss of skin colour corresponds with melanocyte loss, first in the epidermal compartment, and later in the follicular reservoir where most melanocytic stem cells are probably situated.2

Treatment of vitiligo is often difficult and disappointing. This is most probably because the aetio-pathogenesis is unknown, and a treatment directed to the cause has not
been established. Several treatment modalities, such as PUVA [psoralen + UVA (320–400 nm) radiation], broad-band (280–320 nm) and narrow-band (311 nm) UVB, and local corticosteroids are currently used. However, it has been reported that these standard treatments result in limited success; less than 25% of patients responded successfully to topical corticosteroids. Moreover, corticosteroids applied either systemically or topically carry the risk of significant side effects in long-term therapy. Alternatively, PUVA therapy seldom achieves extensive repigmentation that is cosmetically acceptable, and treatment response is often followed by relapse. A recent Cochrane review highlights the lack of research on current treatments as well as the need to identify novel clinical approaches for vitiligo.

Several clinical studies strongly suggest that reservoirs in hair follicles are the source of melanocytes in skin repigmented by standard therapies. Small circular areas of repigmentation centred around hair follicles enlarge and eventually coalesce. Consequently, the identification of stimuli that activate outer root sheath melanocytes is a prospective means of developing new treatments for vitiligo.

Recent evidence from our laboratory indicates that piperine [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylpiperidine; PIP] has a potent stimulatory effect on mouse melanocytes in vitro. Culture media supplemented with Piper nigrum (black pepper) fruit extract or its main alkaloid, PIP, induced nearly 300% stimulation of melan-a mouse melanocyte proliferation after 8 days of treatment in vitro. The increase of growth was effectively inhibited by RO-31-8220, a broad-spectrum protein-kinase C (PKC) inhibitor, suggesting that PKC signalling is involved in its activity. Both Piper nigrum extract and PIP also induced an increase in the number and length of cell dendrites. Melanin synthesis, however, was not stimulated. We have also shown that several synthetic derivatives of piperine share these in vitro effects.

The aim of the present study was to evaluate the melanocyte-stimulatory activity of PIP and three of its synthetic derivatives (Fig. 1) in vivo as a putative new chemical group for the treatment of vitiligo, alone or in association with UVR. Studies were performed in HRA.HRII-c/+ Skh mice, a hairless, sparsely-pigmented mouse model that has white skin except for the ears and tails. This line, congenic with albino inbred HRA/Skh mice, segregates into albino and pigmented phenotypes and was developed by Dr P. Forbes, Temple University Centre for Photobiology, Philadelphia, PA, U.S.A. These mice have melanocytes in the epidermal layer (as in human skin), whereas in many other pigmented mouse strains, melanocytes are found only in the dermis. The numbers of epidermal melanocytes in this model are small – two or three DOPA+ melanocytes mm⁻². However, unlike albino mice, pigmentation in HRA.HRII-c/+ Skh mice is inducible with melanocyte numbers reaching close to 600 mm⁻². As with vitiligo, perifollicular pigmentation is evident after exposure to UVR with and without photosensitizers. We therefore advocate the induction of pigmentation in this strain as an in vivo model for repigmentation in vitiligo.

![Chemical structures of piperine and analogues](image)

**Fig 1.** Piperine (PIP) and structural analogues tetrahydrodopiperine (THP), a cyclohexyl analogue of piperine (CHP) and reduced CHP (rCHP).

### Materials and methods

#### Animals

Male and female inbred HRA.HRII-c/+ Skh hairless pigmented mice, age-matched (8–16 weeks old), were used. Animals were bred by the Biological Services Division, KCL, University of London, U.K. and the Rayne Institute, St Thomas's Hospital, London, U.K. Animals were killed by cervical dislocation and skin samples removed surgically when required.

#### Chemicals

PIP [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylpiperidine] was purchased from Sigma-Aldrich Ltd (Dorset, U.K.). PIP derivatives, i.e. tetrahydrodopiperine [5-(3,4-methylenedioxyphenyl)-pentanoylpiperidine; THP], a cyclohexyl analogue of piperine [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylcyclohexylamine; CHP] and reduced CHP [5-(3,4-methylenedioxyphenyl)-2,4-pentanoylcyclohexylamine; rCHP] were synthesised in our laboratory.

#### Selection of vehicles by **ex vivo** skin assays

To determine the optimum vehicle for delivery of test agents, **ex vivo** permeation studies were conducted with vertical Franz diffusion cells using a modification of reported methods.

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Each cell consists of a chamber with upper (donor) and lower (receiver) compartments divided by the mounted skin sample. The skin acts as a seal between the two half-cells when they are clamped together. The upper, stratum corneum side is filled with the drug formulation and the lower one (dermal side) with receiving fluid. Samples of the receiving fluid are taken at intervals to quantify the amount of the drug passing through the skin. The cells used in this study had a 10 mL capacity receptor compartment and a 1.75 cm² diffusion area. A circular piece of full-thickness dorsal skin from HRA.HRII-c+/Dk mice was carefully mounted onto the receiver compartment of the diffusion cells with the stratum corneum facing the donor compartment. The receptor compartment was filled with phosphate buffered saline; PBS (pH 7.4) which was continuously stirred with a magnetic bar. Test solutions [175 mmol L⁻¹ PIP in ethanol, diethylene glycol monoethyl ether (Transcutol®), Gattefossé, Saint-Priest Cedex, France), dimethyl sulfoxide (DMSO), polyethylene glycol (PEG) or 5% oleic acid (OA) in PEG] were added into the donor compartment of each cell (n = 4 for each formulation). Samples of fluid from the receiver cell were taken at 3, 19 and 22 h and the concentration of PIP was determined by high performance liquid chromatography (HPLC) using a model 3100 pump (LDC Analytical, Riviera Beach, FL, U.S.A.) with a Spectro- monitor 3100 UV detector (LDC Analytical) and Hewlett Packard 3390 A integrator. A 4.6 × 25 cm, 10 μm, C18 Econosil reverse phased column (Alltech U.K., Stamford, U.K.) was used, eluting with methanol : water (60 : 40; HPLC grade, 1 mL min⁻¹). The detector wavelength was set at 348 nm. Under these conditions, PIP eluted at 10.59 min. Results were expressed as mg mL⁻¹ according to a previously determined calibration curve (0.003–0.1 mg mL⁻¹ PIP in PBS).

**Topical application of test compounds in vivo**

Test agents were dissolved in vehicle (either OA/PEG or in DMSO) to a final concentration of 175 mmol L⁻¹ and 100 μL (17.5 μmol) applied with a micropipette on the central area of mouse dorsal skin (2–3 cm²), twice a day (weekdays only) with an interval of 5–6 h between applications. In protocols with UVR exposure, the irradiations were carried out every Monday, Wednesday and Friday immediately prior to the first daily application, to avoid a possible photosensitizing effect of UVR exposure. The irradiance measured at mouse level was typically about 0.16 mW cm⁻². Animals were irradiated unrestrained in metal cages with a dose of 354 mL cm⁻², confirmed to be sub-inflammatory from a single exposure (increase in skin fold thickness (SFT) < 10%; data not shown). Irradiations lasted for a maximum of 1 h. The position of cages was systematically rotated to ensure even UVR exposure.

**Experimental groups**

In initial experiments, animals were treated topically with PIP, THP and CHP dissolved in either OA/PEG or in DMSO or with vehicle alone for 9 weeks with concomitant exposure to UVR. Further experiments (summarized in Fig. 2) were conducted using compounds dissolved in DMSO, with DMSO as control. For continuous treatment, animals were irradiated unrestrained in metal cages with a dose of 354 mL cm⁻² for up to 13 weeks (Fig. 2, Group A). A second group (Fig. 2, Group B) received the same treatment, but was additionally exposed to UVR from week 5 to 13. For studies on discontinuous treatment, animals were treated as in Group B up to week 7. All treatment was then suspended for 3 weeks (weeks 8–10) and re-started as topical application only (Fig. 2, Group C). UVR only (Fig. 2, Group D), or topical application plus UVR (Fig. 2, Group E) for weeks 11–13. Mice exposed only to UVR (i.e. no vehicle treatment) from week 5 onwards (Fig. 2, Group F) were used as controls for all groups treated with UVR.

<table>
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<tr>
<th>Group</th>
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Fig 2. Treatments. Mice were treated for 13 weeks with topical compounds alone (A) or with addition of ultraviolet radiation (UVR) exposure (B). For other groups, treatment was interrupted and restarted as compounds only (C), UVR only (D), or compounds + UVR (E). Group F received UVR alone.

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Assessment of inflammation and pigmentation

Dorsal SFT was recorded to evaluate potential inflammatory effects of treatments. Measurements were taken every day during the first week of treatment and twice a week thereafter with a spring-loaded micrometer (Mitutoyo, Kawasaki, Japan). Pigmentation was assessed independently by two investigators and the average score calculated. The first type of assessment was conducted visually, every day, and pigmentation scored from 0 to 5 according to the following scheme: 0 = no pigmentation; 1 = first signs of pigmentation (freckles); 2 = light brown; 3 = medium brown; 4 = dark brown; 5 = black. Pigmentation was also assessed histologically by DOPA staining at the end of the experiment. Animals were killed and skin samples from representative dorsal areas (1 cm²) were removed surgically and incubated in 2 mol L⁻¹ NaBr in PBS for 2 h at 37°C. The epidermis was carefully removed with tweezers and further incubated in 0-1% L-DOPA in PBS (pH 7-2) for 4 h at 37°C. The DOPA solution was changed periodically to prevent auto-oxidation. Finally, epidermal sheets were fixed in 4% paraformaldehyde in PBS (pH 7-4) for 15 min, dehydrated through a graded series of alcohol concentrations and mounted on glass microscope slides for examination. The number of DOPA+ cells per mm² was calculated from at least 30 fields per sample (n = 4 animals). DOPA+ cells were also classified as highly or poorly melanized according to their melanin granule content. The percentage of cells in each category per mm² was calculated.

Results

Selection of vehicles based on skin penetration measured with Franz cells

Skin penetration of PIP when dissolved in five vehicles was compared using Franz cells. At 22 h after application, the concentration of PIP in the receiver compartment was highest with DMSO followed by OA/PEG in both male and female skin (Fig. 3). PIP was undetectable when delivered in other vehicles (ethanol, diethylene glycol monoethyl ether and PEG). No PIP was detected at shorter time periods (3 h and 19 h) with any vehicle. DMSO and OA/PEG were therefore chosen as vehicles for the in vivo studies.

Inflammatory and irritant effects

Differences in inflammatory response were seen depending on the vehicle, test compound and sex of animal. OA/PEG based formulations induced stronger adverse effects than those with DMSO in both male and female mice (Figs 4 and 5). However, in males, PIP and THP solutions in OA/PEG induced a stronger inflammatory response (more than 30% increase in SFT; Fig. 4a), than in females where THP had only a mild inflammatory effect (20% increase in SFT, Fig. 5a). The inflammatory effect of CHP was comparable to vehicle alone in both males and females (around a 20% increase in SFT, Figs 4a and 5a). The inflammatory response induced by formulations...
four weeks of topical treatment with PIP or THP, in either DMSO or OA. For PIP (Fig. 6b) and THP, subsequent suberythemal exposure to two UVR exposures alone significantly enhanced pigmentation induced by the test compounds compared with controls treated with vehicle and UVR, or UVR alone. Pigmentation was observed as a dark, even pattern after 6–8 exposures (Fig. 6c,d). The pigmentation induced by topical treatment with vehicle was lighter and uneven (DMSO, Fig. 6a–d). The pigmentation induced by UVR alone (Fig. 6c) was observed to be perifollicular and therefore speckled, in contrast to the even pigmentation of PIP and THP alone (Fig. 6a), or in combination with UVR (Fig. 6b–d).

Different pigmentation responses observed in vivo corresponded with changes in the number of DOPA+ cells mm⁻² in the skin (Fig. 7). Pigmentation responses were slower and less evident in females (scores not shown) than in males. In male mice, treatment with PIP and THP in either DMSO or OA/PEG significantly (P < 0.05) increased the number of DOPA+ cells compared with vehicles. The lower pigmentation responses in female mice corresponded with a smaller mean number of DOPA+ cells mm⁻² under all treatment conditions compared with males receiving equivalent treatments (Fig. 7). In females the stimulatory effects of THP and PIP on pigmentation reached statistical significance only with PIP in OA/PEG and THP in DMSO (Fig. 7) although a trend towards an increase was apparent with both compounds in either vehicle. CHP, in contrast, did not show any effect on the number of DOPA+ cells compared with vehicles in either males and females, in agreement with the low pigmentation levels observed on visual examination of the animals (Fig. 6).

Further experiments were carried out in order to determine the persistence of the pigmentation effect after the cessation of treatment and the stimuli needed to restore pigmentation if lost, according to protocols summarized in Fig. 8. Different pigmentation responses observed in vivo corresponded with changes in the number of DOPA+ cells mm⁻² in the skin (Fig. 7). Pigmentation responses were slower and less evident in females (scores not shown) than in males. In male mice, treatment with PIP and THP in either DMSO or OA/PEG significantly (P < 0.05) increased the number of DOPA+ cells compared with vehicles. The lower pigmentation responses in female mice corresponded with a smaller mean number of DOPA+ cells mm⁻² under all treatment conditions compared with males receiving equivalent treatments (Fig. 7). In females the stimulatory effects of THP and PIP on pigmentation reached statistical significance only with PIP in OA/PEG and THP in DMSO (Fig. 7) although a trend towards an increase was apparent with both compounds in either vehicle. CHP, in contrast, did not show any effect on the number of DOPA+ cells compared with vehicles in either males and females, in agreement with the low pigmentation levels observed on visual examination of the animals (Fig. 6).

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Pigmentation

Four weeks of topical treatment with PIP or THP, in either DMSO (Fig. 6a) or OA/PEG (not shown), induced a light, even pigmentation of the treated area compared with vehicle control whereas CHP had virtually no effect. The vehicles used also showed some effect, as previously reported for DMSO and OA. For PIP (Fig. 6b) and THP, subsequent suberythemal exposure to two UVR exposures alone significantly enhanced pigmentation induced by the test compounds compared with controls treated with vehicle and UVR, or UVR alone. Pigmentation was observed as a dark, even pattern after 6–8 exposures (Fig. 6c,d). The pigmentation induced by topical treatment with vehicle was lighter and uneven (DMSO, Fig. 6a–d). The pigmentation induced by UVR alone (Fig. 6c) was observed to be perifollicular and therefore speckled, in contrast to the even pigmentation of PIP and THP alone (Fig. 6a), or in combination with UVR (Fig. 6b–d).

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to obtain a similar, but less even, response in naïve mice (Group F). This data clearly shows a combined pigmentation enhancing effect of PIP, THP and rCHP with UVR. Pigmentation in Group B was maintained up to week 13 with continued treatment with the compounds plus UVR. After three weeks without treatment (Week 8–10), the degree of pigmentation decreased in animals treated with a compound and UVR (Fig. 8, Groups C, D and E) compared with week 7 pigmentation levels, but did not disappear completely. By contrast, there was no remaining detectable pigmentation after week 9 in animals treated with only DMSO and UVR in both male (Fig. 8, Group C, D and E) and female (data not shown) mice. Retreatment with topical solutions, UVR, or a combination of both, all resulted in re-pigmentation after 3 weeks (Week 11–13; Fig. 8, Group C, D and E). The rate of increase in pigmentation was faster than the initial pigmentary response (weeks 1–4), reaching scores of 2 or more within 2 weeks of retreatment (Week 12; Group C, D and E). Retreatment with the compounds alone (Fig. 8, Group C) increased pigmentation to levels comparable with those obtained by continuous topical treatment alone (Fig. 8, Group A). Retreatment with UVR alone (Fig. 8, Group D) or combined topical applications plus UVR (Fig. 8, Group E) resulted in higher pigmentation levels (score 3) than after retreatment with the compounds alone (score about 2), but comparable with UVR alone (Fig. 8, Group F). The pigmentation patterns resulting from a compound alone (Group C) or a compound plus UVR (Group E) were both even (Fig 6c and data not shown). In contrast, pigmentation induced by retreatment with UVR (Fig. 8, Group D) resembled the spotted pattern obtained with continuous UVR exposure (Fig. 6c).

Histological analysis of skin melanocyte numbers (Fig. 9) again showed a good correlation with visually observable differences in pigmentation for both male and female mice. Group B animals (compound plus UVR) showed significantly more melanocytes mm$^{-2}$ than those receiving compounds (Group A) or UVR (Group F) alone. Based on the fading of pigmentation, treatment withdrawal (Groups C–E, weeks 8–10) is assumed to have caused a decrease in the activity of melanocytes. In males (Fig. 9a), retreatment with UVR alone (Group D) or with topical compounds plus UVR (Group E) increased the number of DOPA+ cells mm$^{-2}$ at the end of Week 13 to levels comparable with those in Group B which...
had received continuous treatment with UVR and a compound. The number of cells mm\(^{-2}\) in Group D animals was significantly higher than in the group that received continuous UVR (Group F) or continuous topical treatment (Group A).

Retreatment with a compound alone significantly increased the number of DOPA+ cells mm\(^{-2}\) compared with vehicle control (Group C), reaching comparable levels with those in animals treated continuously with a compound alone (Group A). However, the number of cells mm\(^{-2}\) was lower than for groups that were retreated with UVR alone (Group D) or with a compound and UVR (Group E). The results obtained in female mice (Fig. 9b) showed the same trends as in males, except that in animals retreated with UVR alone (Group D) or UVR with a compound (Group E) no significant differences were observed compared with vehicle controls.

To investigate whether the differences in pigmentation observed were due to an increase in melanocyte number or in melanin production, DOPA+ cells were classified as highly or poorly melanized according to the content of pigment granules, and the percentage of DOPA+ cells in each category per mm\(^2\) of skin was calculated for each experimental group. As expected, UVR exposure considerably increased the degree of melanization of DOPA+ cells (Fig. 10c and Groups B, D, E and F in Fig. 11a,b) compared with mice treated with a compound alone (Fig. 10b or Groups A and C in Fig. 11a,b), where poorly melanized cells were predominant.

Fig 8. Pigmentation response of male mouse skin (n = 4) to continuous (Groups A, B, F) or discontinuous (Groups C–E) treatment as summarized in Figure 2. Mice were treated for 13 weeks with topical compounds alone (A) or with additional ultraviolet radiation (UVR) exposure (B). For other groups, treatment was interrupted and restarted as compounds only (C), UVR only (D), or compounds + UVR (E).

Group F received UVR alone. Pigmentation scores range from 1 (freckles) to 5 (black). *P < 0.05 compared with vehicle; **P < 0.05 compared with Group B (Mann–Whitney U-test).
Topical treatment of HRA/SkH-II mice with PIP, or two of its synthetic derivatives, THP and rCHP, stimulates the development of even skin pigmentation in vivo after four or more weeks of continuous topical application. The darkening of skin in treated areas corresponds with an increase in the number of DOPA+ melanocytes. This in vivo finding correlates well with our previous studies showing the stimulation of in vitro melanocyte proliferation by PIP and chemically related compounds. Animals treated with PIP or analogues before UVR exposure showed more rapid and darker pigmentation than those treated with UVR exposure or a compound alone (Fig. 8). These findings highlight the potential of these compounds as novel treatments for vitiligo. Notably, supplementing UVR with these compounds may offer a means of reducing UV exposure in vitiligo therapy, thereby reducing the risk of developing skin cancer.

The degree of skin pigmentation is a consequence of both number of melanocytes and their degree of melanization. UVR, for example, stimulates both melanocyte proliferation
and melanin synthesis.21,25 The relatively low pigmentation scores in the absence of UVR (Fig. 8) and the low degree of melanization of DOPA+ cells observed in skin treated with a compound alone (Fig. 11) suggests that these compounds stimulate melanocyte proliferation rather than melanin synthesis. This is in good agreement with in vitro data showing that PIP derivatives do not stimulate melanin production although they stimulate melanocyte proliferation.11,12 Retreatment with a compound alone induced a higher difference in DOPA+ cell numbers between compound and vehicle (Fig. 9a, Group D) than did retreatment with UVR alone (Fig. 9a, Group D). This again suggests that the primary effect of piperine is to stimulate rapid melanocyte proliferation and population of epidermal areas. This phenomenon, as well as effects on melanocyte differentiation by PIP analogues could be further examined through bromodeoxyuridine incorporation experiments and immunohistochemical determination of specific markers involved in melanocytic proliferation and population of epidermal areas. This phenomenon, as well as effects on melanocytic differentiation by PIP analogues could be further examined through bromodeoxyuridine incorporation experiments and immunohistochemical determination of specific markers such as Kit, Mif, TRP-1 and TRP-2, indicative of different developmental stages of melanocytes.26

A gender difference in induced pigmentation was observed in these studies, with males showing a greater response than female mice. However, skin penetration of PIP was the same in both sexes using a Franz cell model (Fig. 3), suggesting equal bioavailability in both sexes. However, the mild inflammatory and irritant effects seen with PIP and its analogues (Figs 4 and 5) may be significant, in explaining the activity of the compounds per se, as well as the differences in pigmentary response of male and female animals. Females showed a lower inflammatory response than males. Gender differences in sensitivity to UVR have also been observed in humans with males showing a greater sensitivity and lower MED.17

An important feature of treatment with PIP and its analogues is the even pigmentation pattern that is obtained with or without additional UVR (Fig. 6). This correlates well with the finding that DOPA+ melanocytes in treated skin (Fig. 10) were distributed in interfollicular areas rather than associated with hair follicles, and suggests an active epidermal distribution of melanocytes after treatment with PIP or its analogues. An examination of the in vivo cutaneous absorption and distribution of PIP and its analogues, particularly the relative roles of the stratum corneum and hair follicles, would be of interest in determining their site of action and understanding the repigmentation patterns seen. Hair follicles are known to play a significant role in the percutaneous absorption of many drugs.18

The use of PIP and its analogues in vitiligo clearly offers potential cosmetic advantages over the use of PUVA or UVR alone (common current treatments for vitiligo) if an even pattern can be obtained in humans. PUVA repigmentation, when successful, progresses from a perifollicular pattern in early stages of therapy, with the circular patches of pigment coalescing after further treatment to a more even pattern in humans5 and in mice.15 A similar progression has been observed using therapies based on UVR.9,27 Mice treated with UVR alone in the present study also showed this speckled pattern (Fig. 6c).

Continuous treatment appears to be needed to maintain pigmentation as shown by the gradual, though not complete, loss of pigmentation when treatment is suspended. Retreatment with either UVR alone, topical compounds alone or the combination of both, restored pigmentation over a shorter period of time than in naïve mice. This indicates the possible presence of poorly melanized melanocytes but in greater numbers than in naïve skin. Consistent with our previous observations, the resulting pigmentation after retreatment with UVR often showed darker perifollicular areas, in contrast to the even pattern produced by retreatment with a compound alone or a compound plus UVR.

Although our results suggest that the melanocyte is the main target for these compounds, no known melanocytic receptor for PIP or its derivatives has been identified to date. Interestingly, the presence of one of the subtypes of vanilloid receptor, the receptor for PIP and PIP-related molecules, has recently been shown in keratinocytes.35 In this respect, it is well known that melanocytes and keratinocytes exhibit a close functional relationship. Keratinocytes are known to produce several factors that regulate melanocyte activity and survival, such as nerve growth factor, granulocyte-monocyte colony stimulating factor, basic fibroblast growth factor, endothelin-1, stem cell factor and other cytokines.11–13 It has recently been shown that some of these molecules are imbalanced in vitiligo skin,38 suggesting that the deregulation of the melanocyte microenvironment could be involved in the selective destruction of melanocytes in vitiligo. Indeed, an impairment of keratinocyte function is observed in perilesional skin.39 It is reasonable to speculate that PIP and PIP analogues could have an effect on modulating cytokine production by keratinocytes in vivo, consequently stimulating melanocyte replication or activity, which could result in an increase in pigmentation. Nevertheless, we have observed an effect on PKC activation by PIP in vitro that is suggestive of a direct effect on melanocytes.11

In summary, we have shown that topical treatment with PIP, and two of its synthetic analogues, THP and rCHP, stimulates even pigmentation in mice. Topical treatment in combination with low dose UVR significantly enhances the pigmentation response with results that are cosmetically better compared with conventional vitiligo therapies when applied to mice. Although fading may occur when the treatment is interrupted, a good pigmentation response is readily achieved again after short periods of retreatment. Side effects, such as irritation and inflammation, were transient and tolerable. These data provide strong support for the future clinical evaluation of PIP and its derivatives as novel treatments for vitiligo.

Acknowledgments
This work was funded by BTG International Ltd and by an Overseas Research Student Award to RV. We thank Dr Marc Brown and Richard Harper of the Pharmacy Department, King’s College London for, respectively, guidance on the Franz cell assay and photography of the mice. At St John’s Institute of Dermatology, we acknowledge the technical support in histology provided by Guy Orchard and thank Dr Susan Walker for helpful discussions and critical reading of the
References

FORMULATION AND EVALUATION OF 1% PIPERINE PHOSPHATIDYL CHOLINE COMPLEX EMULSION SYSTEM INTENDED FOR VITILIGO

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Abstract

Vitiligo is an auto immune disorder where the pigment cells (melanocytes) in various parts of the skin are destroyed. Use of piperine in vitiligo has been proved by many researches quite recently. The aim of the present work is to formulate a topical preparation of piperine for vitiligo. This was achieved by complexing the piperine with an amphiphilic polymer, phosphatidyl choline in 1:1, 1:3, 1:5 ratios in order to improve its localization at dermal layer. The piperine dispersion complexes are incorporated into topical formulations i.e., emulsion systems and a formulation containing pure piperine alone, was prepared and are evaluated for drug content, in vitro diffusion study. By the in vitro study it was noticed that 1:1 type of piperine dispersion complex emulsion system has shown optimum localization of drug in the dermal region when compared to piperine cream alone. The selected formulation was then evaluated for TLC, partition coefficient, FTIR study, X-Ray powder diffraction study, physical stability, accelerated stability studies. The FTIR study shown no interaction and X-ray diffraction study shown reduction in
crystallinity. The globules retained its size more than 4 months and accelerated stability studies suggested that the formulation retained a shelf life of 17.6 months.

**Key words:** Vitiligo, Phospholipid complex, X-ray diffraction, SEM, FTIR

**Introduction**

The corresponding author has reported the herbal initiative of vitiligo therapy\(^1\). This is caused by the loss of pigment, resulting in irregular pale patches of skin. There is some evidence suggesting it is caused by a combination of auto-immune, genetic, and environmental factors. Vitiligo develop patches of de-pigmented skin appearing on extremities. The patches may grow or remain constant in size. It has been reported in recent years about the use of piperine obtained from Piper nigrum in vitiligo. Conventional treatment of vitiligo involves corticosteroids and UV radiation. But these have some side effects like Cushing syndrome, skin carcinoma etc. Use of piperine in vitiligo not only reduces the UV radiation but also avoids side effects.

Phospholipids are an important component of cell membrane and play a major role in drug delivery technology. It is an important carrier for the drug molecules, which require controlled release.

**Materials and methods**

Piperine was extracted and isolated in the laboratory, phosphatidyl choline (LIPOID phospholipin 80 H) was obtained as a gift sample from LIPOID GmbH Germany. All other chemicals were of analytical grade. Extraction and isolation of piperine crystals was done based on the work reported by Carol white, Athens technical college\(^2\). Physicochemical tests of piperine crystals like chemical tests\(^3\), Thin layer chromatography\(^4\), melting point and UV spectral analysis\(^5\) are done.

**FTIR Analysis\(^6\)**

The extracted Piperine was subjected to IR Spectral analysis by using FTIR analysis (Thermo Nicolet Nexus 670 IR Spectrometer), detector-DTGS KBr Beamsplitter KBr, Source IR.
X-Ray Diffraction studies

Powdered X-Ray diffraction studies of Piperine powder is carried out to study crystalline nature of the drug. Powdered piperine was dissolved in ethanol by ultrasonicator and then it was subjected to evaporation, then dried and triturated. D8 Advanced Bruker AXS, instrument was used for this purpose. Type of radiation used was copper radiation. The diffractograms of piperine before and after sonication were studied.

Scanning electron microscopy

Topology studies were carried out for crystals by using scanning electron microscopy. Powders were coated with platinum in a sputter coater (JFC-1100, Jeol, Japan), and their surface morphology was viewed and photographed with a Jeol scanning electron microscope (JSM-5310-LV, Jeol).

Preparation of piperine – phospholipid complexes

The required amounts of extract and phospholipids were placed in a 100 ml round-bottom flask and dissolved in anhydrous ethanol. After ethanol was evaporated off under vacuum at 40°C, the dried residues were gathered and placed in desiccators overnight, then sieved through a 100 mesh. The resultant extract–phospholipid complex was transferred into a glass bottle and stored in the room temperature.

In order to evaluate the effect of polymer on the drug release, three different types of complexes were prepared, varying the polymer concentration. Complex of drug and polymer in 1:1, 1:3, 1:5 ratios.

Characterization of piperine phospholipid complexes:

Complex prepared were subjected to various characterization parameters like determination of drug content in the complex by dissolving 100 mg equivalent complex in 100 ml methanol, stirred diluted and measured UV spectrophotometrically by Lambda25 Perkin Elmer UV/Visible Spectrophotometer, Interaction studies by FT-IR, topology studies by Scanning Electron Microscopy (SEM) and X–ray powder diffraction study.
Formulation of the complex into topical emulsion system:

Beeswax (12.5 gm), Lanolin (2 gm) and Stearic acid (2.5 gm) were taken in one beaker. Glycerine (6.25 gm), water (7.25 gm), triethanolamine (0.437 gm) was taken in another beaker. Complex was dissolved in ethanol by sonication and maintained at 40°C. Both the beakers were maintained at 60°C and all the ingredients were melted. Then oily phase is added to aqueous phase and stirred continuously. The complex was added to the mixture when the temperature dropped to 40°C. As the temperature goes down, rose oil was added and mixed well until required consistency was obtained. Complexes of piperine in phosphatidyl choline were prepared in the ratio of 1:1, 1:3 and 1:5. Topical preparations were formulated by using Fo (pure piperine), F1 (1:1), F2 (1:3) and F3 (1:5).

Evaluation of the Piperine – phosphatidyl choline complex emulsion system:

Evaluations of the topical preparations were done by performing drug content uniformity, ex-vivo permeation study by using pig ear skin as semipermeable membrane. Drug concentrations on the dorsal skin surface, within the skin and in buffer solution pH 6.6 were calculated.

 Determination of pH, Partition coefficient of cream between pH 7.4 phosphate buffer and n-hexane spectorscopically, Stability Studies by optical microscopy (10 X), and accelerated stability studies by Arrhenius method, phase separation, were also carried out. Ex-Vivo diffusion studies were performed to determine the percentage drug release of the drug in 6.6 pH phosphate buffer at 344 nm using pig skin. Drug concentration with in the skin and on the dorsal side of the skin were also determined.

Stability studies: As a part of stability studies, physical stability studies like microscopical determination of globule size, and an accelerated stability study performed. The formulation was subjected to accelerated stability study by Arrhenius method. Log % retained was calculated and a graph was plotted between log % retained and time in hours. As it is a straight line, it is following first order reaction. From the graph the k values are calculated for 55°C and 45°C.

Results and discussion
Piperine was extracted from black pepper by using Soxhelet and method and the % practical yield was found to be 4.4 %. Test for alkaloids was done by DragonDroff’s test, Hager’s test, Wagner’s test and Mayer’s test and it has given positive reaction for all the reagents confirming the presence of alkaloids. Thin layer chromatography was carried and from the chromatogram, the Rf value was calculated and found to be 0.25 which complies with that of the standard piperine. Melting point of piperine was found to be 129°C which was compared with the standard value 130°C. From UV spectral analysis, the absorption maximum was found to be 344 nm. Where as absorption maximum of standard piperine is 342 nm. As the value of test is near to standard value, it is confirmed that the piperine crystals formed are intact. The FTIR spectrum of the piperine crystals have produced the characteristic peaks at 1634, 2937, 1489, 1442, 1362, 1251, 1027, 3008.2 cm\(^{-1}\) responsible for their respective functional groups. The FTIR spectrum can be seen in figure 1. From the X – ray diffraction study, it is noticed that the crystallinity was reduced and solubility was increased after complexation. The diffractograms can be seen in figure 2. In Scanning electron microscopy (SEM), the surface morphology of the crystals was found to be needle like in the pictures, which are shown in figure 3.

The prepared complex was dispersed in water, and the pictures were observed under 10x. The globules showing drug inserted inside were observed, which can be seen in figure 5.

Drug content uniformity of piperine polymer complex shown the drug concentration present in 1:1, 1:3, 1:5 drug, polymer ratio complex is, 249 mg, 247.3 mg, 247.3 mg respectively. Surface morphology of phospholipid complex as examined by SEM is given in fig.3. Phospholipid complexes are made up of phospholipid and drug and appeared in column shape. In the FTIR spectrum of piperine – phosphatidyl choline complex, the peak responsible for the unsaturation was missing, which indicates that the phospholipid has interacted with piperine through these hydrogen bonding, in process of complexation. On other hand, physical mixture of PC and piperine shows all sharp peaks as that of the piperine and a broad peak at 3424.2cm\(^{-1}\) as that of PC. The spectra can be seen in fig.1.(c), (d).

From the drug content uniformity, the drug concentrations found in F1, F2, F3 formulations are 247.36mg, 239.42 mg and 239.42mg/ml respectively. As the concentrations are near to 250 mg, it is confirmed that the creams formulated are maintaining good drug content.
Table 1: Drug release from different formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% Drug retained within the skin</th>
<th>% Drug remained on the dorsal side of the skin</th>
<th>% Drug retained in the buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>57.7</td>
<td>4.3</td>
<td>37.63</td>
</tr>
<tr>
<td>F2</td>
<td>49.8</td>
<td>20.1</td>
<td>34.88</td>
</tr>
<tr>
<td>F3</td>
<td>32.0</td>
<td>41.9</td>
<td>23.8</td>
</tr>
<tr>
<td>F0</td>
<td>51.8</td>
<td>22.1</td>
<td>20.8</td>
</tr>
</tbody>
</table>

The intention of the formulation was to retain the drug within the epidermis of the skin, so that the drug available for melanocytic proliferation will be more. Phosphatidyl choline as a polymer was used in the complex formation to see the retention efficacy of the drug, piperine, with in the skin. Formulation without phosphatidyl choline was incorporated with piperine alone and the drug retained within the epidermis was found to be 51.8%. But when the complex was prepared, (piperine – phosphatidyl complex), drug concentration was augmented to 57.7%. Almost 6% increase was there in the retention. It shows that when the drug and polymer were taken in ratio 1:1 i.e., formulation F1 enhances the retention power. Ironically, when the polymer concentration was increased, as in F2 and F3, the drug retention within epidermis of skin was proportionally decreasing. Therefore, formulation F1 was optimized. F1 was selected as suitable formulation and allowed for further evaluations.

Curve fitting analysis
The values from the diffusion studies were plotted in different kinetic model graphs, the selected formulation fits the zero order kinetic model best (Table no. 2).

**Table 2: Drug release kinetic model fitting**

<table>
<thead>
<tr>
<th>Model</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero order</td>
<td>0.967</td>
<td>0.932</td>
<td>0.833</td>
<td>0.941</td>
</tr>
<tr>
<td>First order</td>
<td>0.499</td>
<td>0.717</td>
<td>0.606</td>
<td>0.604</td>
</tr>
<tr>
<td>Higuchi</td>
<td>0.933</td>
<td>0.887</td>
<td>0.805</td>
<td>0.891</td>
</tr>
<tr>
<td>Peppas</td>
<td>0.735</td>
<td>0.728</td>
<td>0.428</td>
<td>0.495</td>
</tr>
</tbody>
</table>

The pH of the formulation F1 was found to be 5.9 which denotes the cream is slightly acidic in nature. FTIR Reports of formulation F1, from the graph in fig.1, it is noticed that the peaks responsible for the piperine are present in the graph and hence it is reported that the piperine has not undergone any interaction with the excipients of the topical preparation. By performing the Partition coefficient, the drug concentration found concentration of piperine in organic phase is 0.972mg/ml; concentration of piperine in aqueous phase is 0.02mg/ml. The partition coefficient value of the cream was found to be 1.68. Phase separation was observed carefully every 24 hrs for 30 days. The formulated cream was kept intact in a closed container at 25-30°C not exposed to light. No change in phase separation was observed. A comparative TLC study of piperine and cream was carried out, which has shown the Rf value of cream as 0.24, which is nearer to the Rf value of piperine.
Table 3: Determination of globule size

<table>
<thead>
<tr>
<th>Globule size</th>
<th>1st Month (µm)</th>
<th>2nd Month (µm)</th>
<th>3rd Month (µm)</th>
<th>4th Month (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min size</td>
<td>5.4</td>
<td>5.4</td>
<td>5.42</td>
<td>5.42</td>
</tr>
<tr>
<td>Max size</td>
<td>55.81</td>
<td>55.8</td>
<td>55.75</td>
<td>55.75</td>
</tr>
<tr>
<td>Average size</td>
<td>30.6</td>
<td>30.6</td>
<td>30.58</td>
<td>30.55</td>
</tr>
</tbody>
</table>

The globule size of the Formulation F1 has shown no significant change even after 4 months.

Fig 5: Plots of log % drug concentration VS time for different temperatures

1 / T values of 45°C and 55°C in Kelvin are $3.048 \times 10^{-3}$ and $3.144 \times 10^{-3}$. 
A graph is plotted between the $1/T$ values and log $k$ values and the $k$ value obtained by extrapolating the curve at 27 °C is 0.006 / months.

**Fig 6: Plot of log $k$ Vs $1/T$**

From the above $k$ value, the shelf life $t$ can be calculated from the first order equation.

First order reaction:  
$$\log c = \log C_0 - k t$$

The shelf life of the prepared piperine dispersion complex emulsion system was found to be 17.65 months if stored at 27 °C.

**Fig 1: FTIR reports: (a)piperine (b) phosphatidyl choline (c) physical mixture (d) complex**
Fig 2: X – Ray diffraction reports of Piperine and Piperine polymer complex

(a) Piperine (b) Piperine polymer complex

Fig. 3. SEM pictures of (a) piperine crystals (b) phosphatidyl choline (c) piperine – phosphatidyl Choline complex (d) Microscopical view of piperine- phospholipid complex.
Conclusion

In the present study, piperine phospholipid complex emulsion system was prepared and evaluated for various physicochemical parameters. It was concluded by good formulation results and the evaluation studies of the piperine crystals, piperine polymer complex, and finally the topical preparations F1, F2, F3 gave the satisfactory results. The FTIR, SEM, have shown the formation of complex and the XRPD studies revealed that there is a decrease in crystallinity after sonication and after complexation. The main intention of the work i.e., localization of the drug at the dermal layer was attained by F1 piperine - phosphatidyl choline complex emulsion system. i.e., the less amount of drug is entered into the buffer solution(37.63 %) and more is localized
with in the dermis(57.7%). And the formulation will be safe under preservation at room temperature for 17.65 months.

Acknowledgment

The authors express indebt gratitude to Nalanda College of Pharmacy for providing facilities of the Central library, Formulation research laboratory with regard to the successful completion of the present project.

References


2. Carol White, Athens Technical College, Athens, GA, Infrared Analysis of Piperine in Black Pepper, Developed through the National Science Foundation-funded Partnership for the Advancement of Chemical Technology (PACT).


ABSTRACT: Vitiligo also known as leukoderma is a pigmentation disorder in which melanocytes (the cells that make pigment) in the skin are destroyed. As a result, white patches appear on the skin on different parts of the body which affects even the psychology and social status of the patient. In recent years, it has been proved that Piperine, an alkaloid from black pepper has the repigmenting capacity. Use of Piperine in Vitiligo not only reduces UV Radiation but also prevents side effects. The present work is about the extraction of Piperine from Black Pepper and its evaluation followed by formulation and evaluation of cream.

Keywords: Vitiligo, Leukoderma, Pigmentation, Melanocytes, UV Radiation.

INTRODUCTION
Vitiligo is a pigmentation disorder in which melanocytes (the cells that make pigment) in the skin are destroyed. As a result, white patches appear on the skin on different parts of the body. Similar patches also appear on both the mucous membranes (tissues that line the inside of the mouth and nose), and the retina (inner layer of the eyeball). The hair that grows on areas affected by Vitiligo sometimes turns white. Vitiligo is not a fatal disease, but it is chronic and progressive. The most important consequence of the disease is probably social and psychological, as people may feel devastated by their changed appearance.

There are many treatments available for Vitiligo but has some side effects like Cushing's syndrome, Skin Cancer, GI disorders etc. Piperine is used as anti Vitiligo agent by reducing the effects of UV radiation and also in avoiding side effects.

Piperine is extracted and isolated from Black pepper by two different methods. They are Soxhlation and reflux method in which highest %yield is obtained for Soxhlation (89.432%). The following evaluation works has been carried out for Piperine pH, Solubility, thin layer chromatography, X-Ray diffraction studies, IR spectral analysis, chemical tests, Melting point, Partition coefficient, Particle size and UV Spectral analysis.

A 1% Piperine cream was prepared using bees wax as base and it’s evaluation was carried out. Thin layer chromatography, Globule size, Partition coefficient, Scanning electron microscopic studies, pH, Moisture absorption studies, consistency, Irritancy test, Drug content uniformity, phase separation, Organoleptic characters etc.

MATERIALS AND METHODS
A comparative extraction of Piperine and its recrystallization
The piperine was extracted by both Rebuff method and Soxhlation method by using 95% ethanol as solvent. The solution was filtered and concentrated under vacuum in a water bath at 60°C. 50ml alcoholic potassium hydroxide was added to the concentrate and the solution was stirred continuously for 30min. The obtained solution was heated and water was added drop wise until yellow precipitate was formed. Water was added until no more precipitate appeared to form and this was allowed to settle overnight. Needles of Piperine were observed to be separated out. The solid was collected and washed with cold ether 2-3 times. It was recrystallized by using acetone. For this, dissolve solid in acetone and filter it to remove extraneous matter and keep the filtrate aside for 24hrs so that crystals of Piperine are formed. Yellow coloured rod shaped crystals were recrystallized after 24 hrs.

Analytical works on Piperine
UV spectral analysis
5μg/ml of the drug in ethanol was used for complete scan between 190-900nm and the maximum absorption was obtained at 344nm as shown in the fig.1 whereas the λ max for standard Piperine is 342nm.

Moisture absorption studies, consistency, Irritancy test, Drug content uniformity, phase separation, Organoleptic characters etc.

Fig. 1: Absorption maximum of Piperine
Partition coefficient of Piperine

**Procedure:** The partition coefficient of drug between phosphate buffer solution (pH 6.6) & n-hexane was determined at 37 ± 0.2°. An excess amount i.e. 50 mg of Piperine was taken in a separating funnel containing 1:1 ratio of buffer 6.6 & n-hexane & placed in a water bath for 24h. The solution was shaken at regular intervals. Then, both of them were separated & filtered through a 2 μ filter & the drug concentration in each phase was determined by measuring the absorbance using UV spectrophotometer at 344nm.

**Particle size of Piperine using microscope**

A small amount of powdered drug is placed on the slide & mounted using glycerin. By using eye piece micrometer, the diameters of 200 particles are determined randomly. Particle size distribution was expressed as histogram (Fig. 2).

![Fig. 2: Histogram of Particle size distribution of Piperine](image)

X-Ray diffraction studies

Powdered X-Ray diffraction studies of Piperine crystals and powdered Piperine by sonication were carried out to study crystalline nature of the drug. Advanced Bruker AXS instrument was used for this purpose. Type of radiation used was copper radiation. The diffractograms are shown in fig 3. It shows that the crystallinity reduces for Piperine powdered using ultrasonicator i.e., solubility has increased.

**Determination of pH**

A small quantity of Piperine was dissolved in ethanol and its pH was checked by using pH meter and it is found to be 7.9.

**Chemical tests**

10 mg of Piperine crystals were dissolved in 10ml ethanol and this solution is used as sample for chemical tests.

Solubility of Piperine was determined in water, ethyl alcohol, chloroform and Acetone. Temperature was maintained at 37±0.2°. Melting point and TLC studies of piperine were also conducted. The chromatogram was observed under UV lamp (365nm). The alkaloid shows violet colored zone. Rf value was found to be 0.26 (value of standard piperine~0.25)

**Fig. 3: X-ray Diffractograms of Piperine crystals and powder**

![Fig. 3: X-ray Diffractograms of Piperine crystals and powder](image)
<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dragendorff’s test</td>
<td>Orange brown ppt is formed</td>
</tr>
<tr>
<td>Mayer’s test</td>
<td>Cream coloured ppt was observed</td>
</tr>
<tr>
<td>Hager’s test</td>
<td>Yellow ppt was observed</td>
</tr>
<tr>
<td>Wagner’s test</td>
<td>Reddish brown ppt was formed</td>
</tr>
</tbody>
</table>

Table 1

<table>
<thead>
<tr>
<th>Wavenumber</th>
<th>Functional Group</th>
<th>Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3008.18 cm⁻¹</td>
<td>Alkenes</td>
<td>Present</td>
</tr>
<tr>
<td>2932.66 cm⁻¹</td>
<td>CH₂-CH₂-CH₃</td>
<td>Present</td>
</tr>
<tr>
<td>1630.82 cm⁻¹</td>
<td>Amines</td>
<td>Present</td>
</tr>
<tr>
<td>1582.55 cm⁻¹</td>
<td>Ketonic group</td>
<td>Present</td>
</tr>
</tbody>
</table>

Formulation of cream²¹,²²

Procedure: Beeswax, Lanolin and Stearic were taken in one beaker. In another beaker, Piperine was dissolved in ethanol by sonication and introduced into glycerine, water, triethanolamine. Both the beakers were maintained at 60°C and all the ingredients were melted. Then oily phase is added to aqueous phase and stirred continuously. As the temperature goes down peppermint oil was added and mixed well until required consistency was obtained.
Evaluation of cream

Organoleptic characters were studied by visual appearance, colour and odour21. By using eyepiece micrometer, the diameters of 200 particles are determined randomly18, 20, 21. Topology studies were carried out for cream by using scanning electron microscopy Presence of foreign particles/grittiness was observed against diffused light to check for foreign particles22. Drug content uniformity of the cream was also carried out21,23.

Partition coefficient of cream

The partition coefficient of drug between phosphate buffer solution (pH 6.6) & n-hexane was determined at 37±0.2°C. An excess amount i.e. 50 mg of cream was taken in a separating funnel containing 1:1 ratio of buffer 6.6 & hexane & placed in a water bath for 24h. The solution was shaken occasionally. Then, both of them were separated & filtered through a 2 µ filter & the amount solubilized in each phase was determined by measuring the absorbance using UV spectrophotometer at 344nm.

The formulated cream was kept intact in a closed container at 25-30°C not exposed to light. Any change in phase separation was checked every 24 hrs for one month.

Irritancy test

Mark an area (1sq.cm) on the left hand dorsal surface. The cream was applied to the specified area and time was noted. Irritancy, erythma, edema, was checked if any for regular intervals up to 24 hrs and reported.

Rheological studies

The formulated cream was found to be non - newtonian. Take a fixed quantity 10gms of cream in a 10ml beaker. Keep it impact for 1 hr. The beaker was inclined to one side see whether the cream is liquefied or not. beaker is shaken to and fro for continuous 5mins and checked whether consistency has changed or not. The beaker was again tilted and checked for pourability of the cream. The formulation showed no thixotropic (shear thinning) characteristics.

Diffusion studies of Piperine incorporated cream using goat's skin as semi-permeable membrane

Fresh goat's skin was shaved well to remove all the hair and cleaned thoroughly in water and rinsed in isotonic solution and later with 6.6 buffer solution. Fresh skin is used for the study. A student diffusion cell was fabricated and the goat's skin was used as semi permeable membrane. A 200mg of 1% Piperine cream was placed on the outer layer of the skin which was fixed as to face unit I from inside.

At predetermined intervals, samples 2ml from recipient chamber were withdrawn and transferred to amber coloured ampoules. The samples were suitably replaced. The samples were estimated for drug content, analyzed at maximum absorbance 344nm using UV-Spectrophotometer ( Elico SL 196).The above work was repeated 3 times and average was calculated From this, concentration of cream in buffer can be obtained.

After the completion of diffusion studies the skin was taken and dissolved in ethanol and left for 24hrs.Then the absorbance was calculated at 344nm.From this, concentration of cream in skin layer was calculated.

RESULTS AND DISCUSSION

Black pepper which was obtained from the local source was subjected to standardization and the results were found to comply with the standard values. Piperine was extracted from black pepper by using Soxhlet and Reflux method and the % yield found was to be maximum in Soxhlet method i.e., 98.632% whereas with reflux method it is 85%.Yellow coloured rod shaped crystals were found. Melting point determination of Piperine was performed thrice by melting point apparatus and the average was found to be 129°C which was compared with the standard value 130 °C. The alkaloid shows violet colour zone under UV Radiation in TLC studies. The Rf value of test (extracted Piperine) is found to be 0.26 which corresponds to the standard value of Piperine (0.25) and only one spot was obtained indicating that it is pure. From UV Spectral analysis absorption maximum was found to be 344nm which almost matched with the standard. Infra red spectra of Piperine were compared with structure and the corresponding bonds were found to present.

Test for alkaloids was done by Dragendorff's test, Hager's test, Wagner's test and Mayer's test and it has given positive reaction for all the reagents confirming the presence of alkaloids. Assay of Piperine was performed and % purity of Piperine extract was 98.67%. Solubility of piperine was found to be in the orderchloroform>ethanol>Acetone but insoluble in water.

X-Ray diffractograms revealed peak heights was reduced in powder after sonication indicating that crystallinity has reduced by ultrasonication and solubility has increased.

Piperine was formulated into % cream and was subjected to various evaluation works Thin layer chromatographic studies were performed. Clear single spots were obtained and Rf value of Piperine and the formulated cream were found to be correlating with each other. Melting point and Rf values of test(cream) and standard(Piperine) were comparable which indicates there is no change in the physical and chemical nature of the Piperine. This also reflects that the drug is compatible with other excipients like bees wax, lanolin etc. Arithmetic mean particle size of Piperine and globule size was found to 21.6 µ and 28.67µ respectively. Minimum globule size and maximum globule size of cream was found to be 4.72µ and 52.72µ respectively.

Expectation of the topical formulation was that the drug should penetrate the stratum corneum, get into the dermis but should not get into the systemic circulation. The % of Piperine for cream retained in the skin, the site of action was found to be was found to be 69.06% drug released into the buffer was found to be 18.62%. It could be postulated that in addition to transcellular permeation, paracellular permeation also is significant through tight junctions. The final preparation was found to be smooth texture and consistency and free from gritty nature with light yellow colour and peppermint odour. The globules retained its size and no coalescence was found. Accelerated stability studies were done which shows no significant change in the concentration of drug which shows stability of the formulation. Moisture absorption studies showed no significant absorption of moisture. pH of the cream was found to be 6.5. Topology studies by SEM revealed almost smooth globules. In addition irritancy test was also performed on rabbits and found no redness, edema, Inflammation and irritation.

CONCLUSION

The formulation of Piperine cream intended for Vitiligo was successfully done and evaluated. Drug targeting at the skin were the melanocytic proliferation is intended was achieved 69.06%. The topical formulation was physically stable throughout the shelf life. Further novel drug delivery formulations are highly recommended to increase the percentage drug targeting.

ACKNOWLEDGMENT

The authors express their gratitude to Nalanda college of Pharmacy, A.P. for the support throughout the project. The authors wish to express Acharya Nagarjuna University, Guntur for providing technical support for this project.

REFERENCES

Effect of UV radiation in the antivitiligo therapy by piperine topical formulation

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Abstract

Vitiligo also known as Leukoderma is caused by the loss of pigment, resulting in irregular pale patches of skin. Vitiligo develops patches of de-pigmented skin appearing on extremities. A team of scientists at King’s College London have discovered that Piperine and its synthetic derivatives can stimulate pigmentation in the skin. The present study evaluates the pharmacological response of repigmentation to a herbal topical formulation incorporated with piperine with and without UV radiation using New Zealand strain rabbits. Although the group deprived of UV radiation took more time for repigmentation, the pattern was more homogenous.

Keywords: Vitiligo, melanocytes, UV radiation, herbal cream, evaluation.

INTRODUCTION

About 0.5 to 1 percent of the world’s population, or as many as 65 million people, have Vitiligo[1]. Most develop vitiligo before their fortieth birthday! The disorder affects both sexes and all races equally. However, it is more noticeable in people with dark skin[2].

Vitiligo seems to be somewhat more common in people with certain autoimmune diseases. These include hyperthyroidism, adrenocortical insufficiency, alopecia areata and pernicious anemia. In the past two decades, research on the role that melanocytes play in Vitiligo has greatly increased. A variety of technical advances, such as gene mapping and cloning have permitted relatively rapid advances in knowledge of melanocytes at the cellular and molecular levels.

People who develop Vitiligo usually first notice white patches (Depigmentation) on their skin. These patches are more commonly found on sun-exposed areas of the body, including the hands, feet, arms, face, and lips. Other common areas where these white patches appear are the armpits and groin, and around the mouth, eyes, nostrils, navel, genitals, and rectal areas. In addition to white patches on the skin, people with Vitiligo may have premature graying of the scalp hair,
eyelashes, eyebrows, and beard. People with dark skin may notice a loss of color inside their mouths.

Systemic phototherapy induces cosmetically satisfactory repigmentation in up to 70% of patients with early or localized disease.

Narrow-band UV-B phototherapy is widely used and produces good clinical results. Narrow-band fluorescent tubes with an emission spectrum of 310-315 nm and a maximum wavelength of 311 nm are used. Treatment frequency is 2-3 times weekly, but never on consecutive days. This treatment can be safely used in children, pregnant women, and lactating women. Short-term adverse effects include pruritus and xerosis. Several studies have demonstrated the effectiveness of narrow-band UV-B therapy as monotherapy[3].

Groundbreaking new research in the British Journal of Dermatology has revealed that black pepper could provide a new treatment for the skin disease Vitiligo. Current treatments include corticosteroids applied to the skin, and phototherapy using UV radiation (UVR) to re-pigment the skin[4-5]. Both, however, carry possible long-term side effects and are not always effective. In particular, less than a quarter of patients respond successfully to corticosteroids, while UVR causes a re-pigmentation that is spotted and patchy and in the long-term could lead to a higher risk of skin cancer. But now a team of scientists at King’s College London have discovered that Piperine the compound that gives black pepper its spicy, pungent flavour and its synthetic derivatives can stimulate pigmentation in the skin[6].

MATERIALS AND METHODS

Piperine was extracted, purified and recrystallised in our lab. Beeswax, Lanolin and Stearic acid were obtained from S D fine-chem Ltd (Mumbai) were as Triethanolamine Lab company (Hyderabad). 4-Hydroxyanisole was procured from Hychem laboratories. All reagents used in this work is of high quality analytical grade.

Process development of topical herbal cream
Beeswax (12.5 g), Lanolin (2 g) and Stearic acid (2.5 g) were taken in one beaker. In another beaker, Piperine as API (1%) was dissolved in ethanol by sonication and introduced into glycerine (6.25 ml), water (7.26 ml), triethanolamine (0.437 g). Both the beakers were maintained at 60°C and all the ingredients were melted. Then oily phase is added to aqueous phase and stirred continuously. As the temperature goes down peppermint oil was added and mixed well until required consistency was obtained[7-8].

Irritancy
Patch test was carried out by applying cream on their ear pinne and dorsal surface to check for the redness or edema of skin. An area (1sq.cm) on the dorsal surface of the shaved rabbit was marked. The cream was applied to the specified area and time was noted. Irritancy, erythma and edema, was checked if any for regular intervals upto 24 hrs and reported[9].

Penetration
Penetration of topical formulations was performed on (n=3) after testing for irritancy on rabbits[10]. A specific amount of the drug (1g) was spread evenly on the dorsal surface of rabbits. After 12 hrs the topical preparation was scraped off and made sure that there is no left
The quantity scraped off was weighed and subtracted from the initial quantity. The difference is assured to be penetrated and reported.

**Preparation of animals** A protocol hard copy in triplicate was submitted to the institutional animal ethical committee and got approval (code no.2/IAEC). The animals were conditioned to the normal diurnal and nocturnal rhythms. The animals were fed with leafy vegetables and water ad libitum. The average weight of rabbits was 2.5kgs.

Three groups of adult rabbits (Average weight 2.5kgs) (New Zealand strain rabbits) were selected containing 6 animals each. All animals were subjected to depigmentation by using 4-hydroxyanisole ointment. Animals were separated into three groups G1, G2 and G3. G1 group of animals under control, G2 for the group of animals were cream was applied and exposed to UV Radiation (Ultraviolet source lamp 15W, Sankyo Denki, Japan) and G3 for the animals were applied cream alone [11-13].

**RESULTS AND DISCUSSION**

Pharmacological evaluation of the cream was performed to prove the anti Vitiligo efficacy by using adult New Zealand OB Brown rabbits [14-18]. Depigmentation of the animals was successfully carried out. All animals in each group were defoliated and followed by depigmentation by 35 days. Repigmentation was carried out by applying only cream for G2. G3 was subjected to Cream and UV therapy. The group which was subjected to the topical cream therapy was found to commence repigmentation within 59 days.
CONCLUSION

It is proved that the formulated topical piperine cream was effective in the repigmentation. In UV therapy the repigmentation was faster but was found to be patchy pigmentation. When the repigmentation was performed only with the cream the rate was found to be slow but homogenous. Therefore it can be concluded that the topical treatment can be facilitated even without UV radiation.

Acknowledgement
The authors express their heartfelt gratitude to Nalanda College of pharmacy for providing information sources, library, internet and electronic facilities.

REFERENCES

Testing a piperine cream with and without ultraviolet B phototherapy in 75 patients affected by bilateral vitiligo

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Introduction

The bilateral vitiligo vulgaris (UVV) is an autoimmune disease with clinical course somewhat unpredictable. It affects about 1% of the population without significant differences of race and sex.

To date there is no universally accepted effective treatment. Based on more than 10 years of study and treatment of these patients and the observations on pigment in the international literature (1-4), we decided to use a cream with the active principle Piperine to evaluate their effectiveness. The proposed wording for vitamin, the name of the cream has been developed around the pesticide based on observing the lesson at King's College London who had tested on animal model the efficacy of the extract of Piper nigrum as a stimulator of melanocytes (1).

In this work, mice were treated with piperine in health with and without ultraviolet irradiation, which is why we have decided to experiment with and without ultraviolet B irradiation 311nm, now the gold standard of treatment of vitiligo.

Materials & Methods

Patients

75 patients affected by SV underwent topical treatment with piperine cream. Of the 75 patients 39 were males and 36 females aged between 18 and 53 years.

The extension of vitiligo ranged between 5 and 35% of the total skin surface.

32 patients (group A) carry 311nm UVB phototherapy, but the other 43 patients not subjected to any form of phototherapy (group B).

Patients enrolled in this open study should satisfy the following rules:

Inclusion criteria:

1. SV with an extension from 5 to 50% of the total skin surface;
2. Aged between 18 and 65;
3. Compiled of written informed consent.

Exclusion criteria:

1. SV with an area greater than 50% of the total skin surface and other forms of vitiligo (seborrheic, segmental, etc.);
2. Age outside the range between 18 and 65;
3. Serious local infection;
4. Specific therapies carried out continuously in the two months preceding the enrollment phase.
5. Pregnancy.

During the first visit in addition to completing the clinical pictures were made (with a particular flash to fluorescent lighting and ambient lighting) for an objective proof of the initial clinical picture and provided all information relating to therapy. All patients had given informed consent prior to treatment also includes the possible alternative treatments and possible side effects and patients recruited for the written declaration that they were not pregnant. None of the patients had undergone in the two months prior to other drug continuous systemic treatment, topical and/or physical vitiligo. Some patients (3 men and 4 women and 5 men in Group B) were used occasionally in the two months prior to topically treatment. Similarly prohibited was any other medication and/or physically during and/or physically during and after a minimum of two months from the end of the protocol. The patient history is another important feature, it is essential to discuss the expectations of the patient, medication taken on, assessing the presence of scars, keloids, and local infection, the immune status of patients, patient’s habits as exposure to the sun.

The protocol provided for a single daily application of the cream in the morning to the skin for six consecutive months. Patients were visited and photographed at the time of first visit and every month until the end of the treatment protocol.

Patients were visited and photographed in each patch. Through a morphometric evaluation of the spots using a dedicated software (DDAX-MIPS) were then determined the percentage of repigmentation of each individual at intervals of photography.

The percentage of repigmentation obtained remained stable even after 3 and 6 months after the end of the protocol.

Regpigmentation rate at the end of the study

![Image 3](https://example.com/image3)

The cream in Vitiligo monopigmentation daily, has proved highly effective in inducing repigmentation of the vitiligo affected skin areas with and without stimulation ultraviolet B 311 nm.

The patient compliance was excellent and only transient burning sensations were complained by some patients especially during the implementation of the lips and eyelids.

Regpigmentation rate were extremely positive, although as always, the face and chest have responded faster and more complete patches of the limbs.

We believe that both ultraviolet treatment without the cream vitiligo is the real novelty in the treatment of vitiligo.

Results

![Image 4](https://example.com/image4)

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Conclusions

![Image 5](https://example.com/image5)

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References
