The effect of *Medicago sativa* extract and light on skin hypopigmentation disorders in C57/BL6 mice

Azal Hussein Najm Ubaydee MSc\(^1\) | Reem Issa PhD\(^1\) | Maha N. Abu Hajleh PhD\(^2\) | Bayan Y. Ghanim MSc\(^3\) | Faisal Al-Akayleh PhD\(^4\) | Nidal A. Qinna PhD\(^3,5\)

\(^1\)Department of Pharmaceutical Sciences, Pharmacological and Diagnostic Research Center (PDRC), Faculty of Pharmacy, Al-Ahliyya Amman University, Amman, Jordan
\(^2\)Department of Cosmetic Science, Pharmacological and Diagnostic Research Centre, Faculty of Allied Medical Sciences, Al-Ahliyya Amman University, Amman, Jordan
\(^3\)University of Petra Pharmaceutical Center (UPPC), University of Petra, Amman, Jordan
\(^4\)Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan
\(^5\)Department of Pharmacology and Biomedical Sciences, Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan

**Correspondence**
Nidal A. Qinna, Department of Pharmacology and Biomedical Sciences, Faculty of Pharmacy and Medical Sciences, University of Petra, PO Box 961343, Amman, Jordan.
Email: nqinna@uop.edu.jo

**Funding information**
Al-Ahliyya Amman University

**Abstract**

**Background:** Vitiligo is a common depigmentation skin disease that affects the quality of life in many patients.

**Aims:** This study aims to investigate the effect of *Medicago sativa* methanol extract on the treatment of skin hypopigmentation disorders.

**Methods:** Antioxidant activity and phytochemical constituents of the extract were determined using DPPH assay, Folin–Ciocalteu, AlCl\(_3\), and HPLC-MS/MS analysis. Oil in water (o/w) creams were prepared to contain the methanolic extract, and applied to hydroquinone-induced depigmentation in vivo model and further challenged in combination with UVA light exposure. Skin and hair colors were visually scored and evaluated at different time intervals, and histopathological examinations of skin layers and hair follicles were performed.

**Results:** A total phenolic content of 187.70 mg/g, equivalent to gallic acid, and total flavonoid content of 21.97 mg/g, equivalent to quercetin, were recorded. Extract showed 71% antioxidant activity. Moreover, the HPLC-MS/MS detection revealed the presence of 18 compounds including P-coumaric acid and antioxidants flavonoids, of those are seven compounds not previously detected in this species. The in vivo study showed a remarkable skin and hair pigmentation effect on plant extract-treated groups, compared to the reference, placebo, and control groups. Histopathological examinations showed the growth of colored hair follicles in the dermis and epidermis layers of the extract-treated mice.

**Conclusion:** The study suggests the use of *M. sativa* extract in enhancing the pigmentation process in hypopigmented skin and hair if combined with UVA light. Therefore, *M. sativa* extract can be considered a potential treatment for vitiligo.

**KEYWORDS**

flavonoids, hyperpigmentation, phenols, UVA light, vitiligo

1 | **INTRODUCTION**

Loss of pigment can be either partial (hypopigmentation), complete (depigmentation), congenital, or acquired. Congenital hypopigmentation usually results from mutations of various genes that are responsible for the processes of development of melanoblasts which differentiate into mature pigment-producing melanocytes.\(^1\) Although the majority of hypopigmented disorders are not infectious and serious, hypopigmentation and depigmentation cause psychological issues for the patients.\(^3\) Various skin diseases are
accompanied by loss of pigments such as Vitiligo which is a skin disorder characterized by a lack of melanocytes, and leads to pigment dilution in the affected areas of the skin. The affected area is a non-scaly, amelanotic, chalky-white with distinct margins.²

Vitiligo is not a curable disease, different treatment choices with different mechanisms for enhancing the pigmentation process or inhibiting the depigmentation process include topical or systemic corticosteroids or immunomodulators, phototherapy, and surgery.² It has also been reported that psoralens or linear furanocoumarin are photosensitizing agents that improve skin sensibility by enhancing melanin production. In conjugation with phototherapy, the former has been reported therapeutically effective in treating vitiligo.³ In addition, several studies reported that coumarins have effective photosensitising properties which may positively influence the treatment of vitiligo when combined with ultraviolet radiation exposure.⁴

Herbal medicine, in addition to other treatment options, was used for the treatment of vitiligo, especially using herbs that contain furanocoumarins such as Khellin, Green Tea Polyphenols, St. John’s wort naphthodianthrones, and others of high Coumarin composition.⁵ These remedies seem to be related to their anti-inflammatory, immunomodulatory, and antioxidant characteristics as well as photosensitizing properties.⁶ Moreover, several agents have been found to act as tyrosinase inducers to increase pigmentation. For example, Daphne gnidium extracts are essential oils that contain fatty acids, coumarins, flavonoids, terpenoids, and alkanes. Moricandia arvensis leaves contain saponins, tannins, flavonoids, cardenolides, and alkaloids.⁷ Vernonia anthelmintica contains steroids, flavonoids, carboydrates, fatty acids, sesquiterpene lactones, and terpenes.⁸ Medicago sativa L. is a large genus of the Leguminosae tribe, with around 83 distinct species. M. sativa has long been used in China, Turkey, and America as a popular herbal remedy for a range of ailments.⁹ M. sativa contain a variety of phytochemicals including flavonoids, alkaloids, phytoestrogens, digestive enzymes, triterpene, saponins, and coumarins. Several research attempts concerning coumarin use for skin hypopigmentation disorders and vitiligo treatment have been reported Bora & Sharma.¹⁰ Bergapten has also been successful in the treatment of vitiligo.¹¹ Thus, this study will focus on M. sativa role in the treatment of hypopigmentation disorders by its component of coumarin phytochemical.

Up to our knowledge, there is no previous studies have been performed on the species M. sativa investigating its effect on the treatment of hypopigmentation disorders on hydroquinone-induced hypopigmented skin animal models. This plant species is known to contain phenols and flavonoids and their derivatives. Previously, other plant species with similar content have shown hyperpigmentation activity with potential use for the treatment of vitiligo in addition to other skin pigmentation disorders.

2 | METHODS

2.1 | Plant material collection and extraction

Medicago Sativa plant samples were collected from commercial farms at Al-Azraq, south of Jordan. Samples were sent to the Royal Society for the Conservation of Nature (Amman, Jordan) for identification and authentication. The plant was dried under the sun for 3 days then grinded using a blender and stocked in a dark, dry place at room temperature. A 50g of dry plant material was soaked in methanol for 24h. The prepared extract was filtered and evaporated until completely dried. The extract was stored in a dark place at room temperature until used.

2.2 | Determination of total phenols content

Total phenol was determined using Folin–Ciocalteu method.¹² Briefly, 1 ml of serial dilutions of the plant extract was added into 4 ml of 7.5% Na₂CO₃ and 5 ml Folin–Ciocalteu reagent. These mixtures were covered and kept at room temperature for 1 h, and absorbance was recorded for each extract concentration using UV-V spectrophotometer at 765 nm. All concentrations were expressed as mg equivalents to gallic acid per gram of plant extract (mg/g) based on Equation 1:

\[
\text{Total phenol (mg equivalents to gallic acid / g of plant extract)} = \frac{y - 0.0042}{0.0004} + \frac{100 \text{ mg}}{1000 \text{ ml}}
\]

2.3 | Determination of total flavonoids content

Total flavonoid content was determined by the colorimetric method described by Cosmulescu et al.¹³ Briefly, 1 ml of serial dilution from each sample was added into 0.5 ml AlCl₃, 0.5 ml NaNO₂, 2 ml NaOH, and 4 ml distilled water. After 15 min, the absorbance was recorded using a UV-V spectrophotometer at 510nm (Shimadzu, Japan). All concentrations were expressed as mg equivalents to quercetin per gram of plant extract (mg/g) based on Equation 2:

\[
\text{Total flavonoid (mg equivalents to quercetin / g of plant extract)} (\text{mg/g}) = \frac{y + 0.0035}{0.0037} \times \frac{100 \text{ mg}}{1000 \text{ ml}}
\]

2.4 | Determination of antioxidant

The principle of the antioxidant assay was based on the method described by Pramod et al.¹⁴ Briefly, 1 ml of serial dilutions of the plant extract was added into 3 ml of prepared DPPH and 6 ml of methanol. The mixtures were placed for 30min in a dark place; then,
the absorbances were recorded using UV-V spectrophotometer at 517 nm. Trolox was used as a control to prepare the calibration curve. Inhibition percent was calculated using Equation 3:

\[
\text{Inhibition\%} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100\%
\]

2.5 | HPLC/MS–MS analysis for plant extracts

Stock solutions for the plant extract were prepared by dissolving a 2 ml of crude extract in dimethyl sulfoxide (DMSO), then diluted with Acetonitrile and used for injection into the II ESI-Q-TOF System equipped with Bruker Daltonik Elute UPLC system (Bremen, Germany) for screening of compounds of interest. Standards were used for the identification of m/z with high-resolution Bruker TOF MS and the exact retention time of each analyte after chromatographic separation. This instrument was operated using the Ion Source Apollo II ion Funnel electrospray source. Chromatographic separation was performed using Bruker solo 2.0 C-18 UHPLC column (100 mm × 2.1 mm × 2.0 μm) at a flow rate of 0.51 ml/min and a column temperature of 40°C. The mobile phase was composed of water with 0.05% formic acid and acetonitrile. Gradient: 0-27 min linear gradient from 5% to 80% B; 27-29 min 95% B; 29.1 min 5% B, total analysis time was 35 min on positive and 35 min on negative mode, injection volume 3 μl.

2.6 | Formulation of creams

A 10% and 20% M. sativa extract containing creams, and 0.002% bergapten cream were prepared as oil in water emulsion as shown in Table 1. The oil phase was prepared by melting the stearic acid, white soft Paraffin, and emulsifying wax at 70°C and mixing the ingredients uniformly. The aqueous phase was prepared by dissolving the water-soluble ingredients (glycerol, propylene glycol, triethanolamine, Methylparaben, and Medico Sativa extract) in deionized water.

For bergapten cream, 0.002 g bergapten was dissolved in propylene glycol and then added to the aqueous phase. The water phase was warmed to 70°C until all ingredients were dissolved. When the water and oil phases were at the same temperature, the aqueous phase was slowly added to the oil phase with moderate agitation and was kept stirred until the temperature dropped to 40°C. The emulsion was cooled to room temperature to form a semisolid cream base. The mixture was stirred for 15 min until the formulation became uniform.

2.6.1 | Physical stability test for cream

A stability test was performed throughout the study (4 weeks) for the 10% and 20% M. sativa extract cream. Three samples of each plant extract cream were taken. The first sample was stored at 4°C, the second sample was stored at 25°C, and the last sample was stored at 40°C. The physical appearance (separation, homogeneity, odor, color, and pH) of the samples was observed and recorded each week for 1 month.

2.6.2 | Rheological testing

The viscosity and rheological behavior of the prepared creams were determined using a cone and plate viscometer (Anton Paar, Rheometer Germany GmbH, Model MCR 101). All measurements were carried out at a temperature of 25 ± 1°C, using spindle CP 35. The formulations were loaded on the plate at an amount of 0.5 g and allowed to reach the stable temperature for 5 min. To assure accuracy, the rheometer was calibrated and programmed via a computer-controlled RheoCompass software (Anton Paar).

2.6.3 | Flow curve

Flow curves are measured by either controlled shear rate or controlled shear stress, and the viscosity function of the samples is shown at a shear range of 0.01-100 1/s with a logarithmic increase and with a decreasing measuring point duration from 30 to 2 s to avoid transient effects.

2.6.4 | Temperature sweep test

The heating range is set as 14-37°C, the heating rate is 5°C/min, the strain is fixed at 1%, and the oscillation frequency is 1.0 rad/s. All measurements were carried out in triplicate, and average values were reported.

2.7 | Animal model and experimental design

Forty-eight female C57/BL6 mice weighing 22 ± 2 g were housed and acclimatized. Mice were maintained under controlled conditions
of temperature (22–24°C), humidity (55–65%), and photoperiod cycles (12 light/12 h dark) with a standard diet and free access to water.

After acclimatization, mice were randomized into six groups (n = 8) as follows: negative control treated with neither formulas nor exposed to UVA light, control receiving vehicles only, placebo receiving vehicles and exposed to artificial UVA light (Philaquin 4%, Philadelphia Pharmaceuticals, Amman, Jordan), a group receiving Bergaptin (0.002%) as a positive control to depigmentation models, and two groups receiving different concentration of the study plant extract (10% and 20%). On the first day of the study, animals’ hair was shaved off the dorsal region using an electronic trimmer. Thereafter, animals were treated with either vehicle or 4% hydroquinone (HQ) for 15 consecutive days for induction of skin depigmentation “vitiligo model.” The treatment continued even after confirmation of depigmentation in the animal models on Day 9; thus, they were treated for another 6 days. On Day 9 from the initiation of the study, signs of vitiligo were noted; therefore, animals were prepared for hair shaving and were treated with corresponding study treatments. Thereafter, animals were exposed to artificial UVA light every second day and 30 min after the application of treatments. Application of treatments was 2 h from the application of HQ and was consistent until the termination of the study (study day 28) while HQ was discontinued after Day 15. Mice were observed daily for any signs of irritation, distress, or unusual behavior, and photographed every 3 days to monitor hair cycle and changes in skin color. On Day 28, mice were subjected to a physical examination of the skin and hair nature. Skin biopsies from each group were collected on Day 24 and preserved for further genetic analysis.

2.7.1 | Histopathology

Skin biopsies for histology were dissected and kept in 10% formalin and refrigerated at 4°C. Before 24 h of reading, all samples were submerged in absolute ethanol 70% for 24 h for dehydration. Specimens were prepared, sectioned, and stained with hematoxylin and eosin; then, slides were compared under a light microscope (Optica B-190).

2.8 | Skin irritation/corrosive potential test

The safety of *M. sativa* on the skin of young albino female rabbits weighing 2250 ± 150 g was housed and acclimatized. Rabbits were housed and maintained under controlled temperatures (20 ± 3°C), humidity (50 ± 15%), and 12 light/dark cycles with free access to diet and drinking water.

According to the OECD Guideline for Testing of Chemicals, Guideline 404 for Acute Dermal Irritation/Corrosion, initial and confirmatory studies were conducted. One animal was prepared for the initial testing of the formula. First, fur at the dorsal area of the rabbit trunk was clipped and skin was observed for the next day to assure intact skin. Thereafter, 0.5 ml of both *M. sativa* formulations was applied sequentially as three patches to an area of approximately 6 cm² and covered with a gauze patch, the first patch was left on the skin for 3 min, the second patch for 1 h and the third for 4 h only. Animals were examined immediately after patch removal for signs of erythema and edema, and dermal reactions were scored. Proceeding the initial test, a confirmatory test was conducted on another animal to confirm the response. A patch was applied to previously prepared skin, described earlier, and left for a dermal exposure period of 4 h.

2.9 | Analysis

2.9.1 | Skin and hair depigmentation model

The depigmentation of skin was evaluated using a scoring method (Table 2). Observations included skin color, hair color, and growth.

2.9.2 | Skin irritation/corrosive model

The dermal response was evaluated according to Draize’s dermal irritation scoring model (Tables 3). Scoring was performed immediately at the removal of the patch, after 60 min, 24, 48, and 72 h.

3 | RESULTS

3.1 | Total phenol and flavonoid content

Methanol extract of *M. sativa* was examined for phenol and flavonoid content. The extract presented a yield of 187.7 ± 0.43 mg/g phenolic content in dry extract and calculated as Gallic acid equivalence. As

| Parameter | Scoring (in comparison with healthy subjects) |
|-----------|---------------------------------------------|
| Skin color | Normal |
|           | Gray |
|           | White (positive for depigmentation) |
|           | 1:1 mixture of black and white skin color |
|           | Black (positive for UV-induced pigmentation) |
| Hair color | Black (normal) |
|           | White (positive for depigmentation) |
|           | 1:1 mixture of black and white hair color |
|           | More black |
|           | More white |
| Hair growth | Normal hair growth |
|           | ↓ (once less than healthy) |
|           | ↓↓ (twice less than healthy) |
|           | ↓↓↓ (three-times less than healthy) |
3.2 | Antioxidant activity

*Medicago sativa* methanol extract exhibited an antioxidant inhibition activity of 71% when compared to Trolox.

3.3 | HPLC/MS–MS analysis for plant extracts

A total of 18 compounds were determined in the methanolic extract of *M. sativa* (Shown in Supplementary Table 1), seven of which were not reported previously, specifically, 7-Methoxy-4′-hydroxyflavone, Linoelaidic acid, 3,6,2′,4′-Tetrahydroxyflavone, 3′,4′-Dimethoxy-7-hydroxyflavanone, ISO-Orientin, 7-Glu Chrysoeriol, and Saponarin. The former compounds are flavonoid derivatives except for linoelaidic acid which is mainly a fatty acid.

3.4 | Cream physical stability study

Stability tests were performed for *M. sativa* extract containing creams. According to the study period and conditions, the 10% and 20% plant extract cream samples appeared to stay homogeneous, odorless, light or dark green, with slight pH changes at all temperatures, thus indicating the physical stability of the prepared samples.

3.5 | Rheological test and flow curve

All tested formulations demonstrated a typical shear-thinning behavior of decreased viscosity with increased shear rate, as shown in Figure 1.
3.6 | Temperature sweep test

Prepared formulations showed thermostability when tested at temperatures 15–32°C. Moreover, formulation viscosities were 2520, 1360, 1040, and 642.4 mPa·s for Bergapten, vehicle, 10% extract, and 20% extract formulations, respectively (as shown in Figure 2).

3.7 | In vivo pigmentation activity of M. sativa

At the termination of the dosing period, animals were examined for overall skin color as well as hair color and growth. Skin color was assessed after shaving the inspected area, and results were scored and interpreted as presented in Figure 3. About 70% of the placebo group showed induction of vitiligo (white skin) and only 17% of mice got a gray skin color, while 15% were with normal skin color, in the bergapten-treated group, a low percent of the mice showed white color skin, while a high percentage of the mice showed normal skin color after treatment. Twenty-eight percent of mice had black skin color, and 15% of them had mixed skin color. The plant extract-treated group results showed that in the 10% extract cream receiving group, 15% of the mice kept vitiligo but most of them showed pigmentation, where 28% had mixed color skin (75% gray and 25% white). For the 20% plant extract cream-treated group, no mice showed vitiligo at the termination of the experiment. The total skin pigmentation ratio for groups receiving creams of 10% and 20% plant extract was better than other groups of which Bergapten-containing cream (Figure 3).

The hair color of control group mice was black and comparable to comparable to normal non-treated animals, thus indicating the vehicle did not affect hair color. As for the placebo group, all animals developed white hair and some had a mixture of white and black hair growth. The suppression of the depigmentation model through either bergapten or the plant extract was evident. Animals baring a combination of color, most of which is white, was the highest among the mice group receiving bergapten cream. However, animals treated with the plant extracts cream showed an increase in the density of black color hair in mice. The percent of mice that had mixed color hair was the highest in Bergaptin-treated group and declined among the groups treated with creams containing plant extract at 10% and 20%, while the percent of mice had more black than white hair was the opposite. In addition, black hair was observed only in groups treated with cream containing the plant extract at 10% and 20% (Figures 3 and 4).

Observing the hair growth among groups (Figure 3), some mice of the control group presented a slower growth rate in comparison
with normal non-treated animals, thus, indicating either an impact of UV or the vehicle in hair growth rate. Nevertheless, mice of the placebo group showed three times less hair growth which might be attributed to the vitiligo model, UV or vehicle. However, animals treated with bergapten or 10% extract creams showed a reversal in such effect. While most of the animals had a 1-time or 2-time less hair growth rate, such effect was mildly observed on mice treated with 20% extract cream.

Figure 5 illustrates pictures of mice from all tested groups on Days 0, 8, 16, and 29 of the experiment periods. For the negative control group, pictures show that there is no change in skin or hair color. The same result was observed for the control group. Changes had occurred for the placebo group; the skin started to convert to gray color after 8 days of receiving hydroquinone and exposure to UVA light. On Day 16, vitiligo was noticed and most of the hair turned white. On Day 15 of the experiment, hydroquinone treatment was terminated, which resulted in the recovery of the skin color to its normal color on Day 29.

Regarding the Bergapten-treated group, on Day 16, most of the hair turned white with a small fraction that remained black in color. On Day 29, hair color started to recover back to normal and a mild improvement in skin color was observed. Animals treated with plant extract reduced the development of hydroquinone-induced depigmentation. On Day 16, both groups treated with the extract developed skin vitiligo with a higher presence of white hair. On Day 29, remarkable improvement in hair color and total skin vitiligo recovery of mice treated with either 10% and 20% plant extract was noted.

3.8 | Skin and hair morphology

Figure 6 shows the skin histopathology of mice exposed to UVA, the layers of skin with magnification of 10×, specifically; Figure 6A the stratum corneum (SC) followed by the stratum spinosum (SS). Figure 6B shows the papillary dermis (PD) is thinner than the reticular dermis (RD) and both contain hair follicles (HF) and eccrine sweat glands (SG). Below, are layers of the adipose white dermis (WD) preceding the panniculus carnosus (PCM). Figure 6C,D shows the melanocytes are normally restricted to hair follicles, and after exposure to UVA light, the epithelial matrix around the hair follicles increases and causes darker hair to grow.

For the Bergapten-treated group (Figure 7), the hair shaft was observed as black and white, while the root was white. For the 10% plant extract cream-treated group, the hair shaft and roots were observed black. The effect of herbal cream treatments against hydroquinone-induced vitiligo can be observed in both Figure 7A,B, in which the 10% plant extract cream-treated group showed a protective effect against the growth of white hair. The same result was seen in the 20% plant extract cream-treated group.

3.9 | Skin irritation/corrosive potential

Following the OECD guideline and recommendation of conducting skin irritation/corrosive potential for dermal exposure of animals to study formulation, the M. sativa containing formulas were concluded safely on skin, described in Tables 3 and 4.

4 | DISCUSSION

Plants that contain coumarins and their derivatives have been used for re-pigmentation of the skin in Egypt, India, and other countries, including Ammi majus L., Psoralen corylifolia L., and Ficus carica L. The presented data showed high phenol and flavonoids content, as well as antioxidant activity in the methanolic extract of M. sativa.
Similarly, previous research found that methanol was the best solvent to extract phenolic compounds from *M. sativa* flower, which has also been reported to pose anti-inflammatory and antioxidant activities.\(^{16}\)

In the current study, HPLC-MS/MS analysis revealed the presence of 18 different phytochemical compounds detected in the methanol extract, of these seven compounds were not previously detected in the study plant species. In that regard, succinic acid, 3',4'-Dimethoxy-7-hydroxyflavanone, and isoferulic acid were detected and are known for their antioxidant effects. In addition, benzoic acid and 4-Hydroxybenzoic acid were also observed which are usually used in pharmaceutics and cosmetic products due to their antimicrobial activity.\(^{17,18}\) Nevertheless, salicylic acid was among the constituents, and well-documented for its capacity in exfoliation, therefore, is often used in photodamage, melasma, freckles, and lentigines treatment.\(^{19}\) In addition, vanillin was also detected and has been reported to enhance psoriatic skin inflammation was detected\(^ {20}\) and 2,5 dihydroxybenzoic acid, which is widely used in cosmetics to treat pigmentary disorders.\(^ {21}\) As well, rutin which has the ability to increase skin elasticity and decrease wrinkles,\(^ {22}\) and saponarin, 7-Glu Chrysoeriol, ISO-Orientin were also detected and are reported to have anti-inflammatory activity and are used for inflammatory skin diseases.\(^ {23}\) ISO-Orientin may be accounted to the inhibition of UVB-light induced skin injury in mice, and genistin might be the mediator in treating skin keloid scars.\(^ {24}\)

Of the most important is the detection of P-Coumaric acid in the studied plant extract, which poses an antioxidant effect, reducing UV-induced erythema formation and subsequent pigmentation in human
skin,\textsuperscript{25,26} and is considered a concentration-dependent tyrosinase inducer.\textsuperscript{27} Tyrosinase activators, which have stimulating effect on melanin formation, are beneficial in the treatment of depigmentation diseases.\textsuperscript{28} Plants that have compounds that act as tyrosinase inducers such as Daphne gniidium, Moricandia arvensis, and Vernonia anthelmintica were previously used to increase skin pigmentation.\textsuperscript{29} The prepared creams containing M. sativa plant extract showed good physical stability when tested at different conditions, in comparison with commercial bergapten-containing formula. The flow behavior of skin formulations was evaluated using viscosity measurements. Thereby, it is assumed that the sample can be spread easier at low viscosity values. Skin formulations including cosmetic or therapeutic generally show shear-thinning behavior which indicates that the viscosity is not a constant value but depends on the shear intensity. The former measures are useful for optimizing the manufacturing process and the long-term stability of the dispersion. The findings of the presented study show that all tested formulations demonstrated a typical shear-thinning behavior of decreased viscosity and increased shear rate.
Melanin synthesis has several critical physiological functions, including protecting human skin from ultraviolet (UV) radiation. Melanogenesis is a complex pathway that involves melanin synthesis, transport, and release. Melanin synthesis is stimulated by variable factors, including α-melanocyte-stimulating hormone and cyclic AMP enhancers. Loss of hair shaft melanin is associated with decreased tyrosinase activity in melanocyte bulbs. Therefore, the black color of the skin which was observed in bergapten-treated mice can be explained by the ability of Bergapten to increase skin pigmentation by stimulating melanin synthesis. Restoration of black skin color was also observed in the 20% plant extract cream treated mice, which may be due to the ability of M. sativa extract to increase the pigmentation by inducing the tyrosine enzyme activity. In addition, reported that UVA treatment would enhance epidermal melanocyte activity and reduces melanocyte degeneration. In melanocytes, the photo conjugation of psoralens and DNA promotes the differentiation and proliferation of melanocytes and enhances the synthesis of tyrosinase by regulating the activity of cAMP, thus increasing the formation of melanosomes. In addition, UVA exposure results in increased melanin content in the deeper layer of the epidermis. The latter could be another proposed mechanism, suggesting a photosynthesizing effect for the plant extract similar to psoralens.

The adopted model is a classical in vivo model for mimicking vitiligo induced in C57BL/6 mice through the topical application of hydroquinone daily for several consecutive days, which might in some studies reach up to 60 days. The use of hydroquinone-induced hypopigmentation is a practical, efficient, and simple method to mimic the phenotypic characteristics of vitiligo, yet is considered limited to the phenotypic changes of vitiligo but not the actual etiology. However, more sophisticated and expensive models like the utilization of spontaneous animal models are considered the ideal approach to studying vitiligo. Nevertheless, several chemical compounds were studied using chemically induced models before translating them into clinical settings.

In the current study, hair color of mice observed at the end of experiments indicated that plant extract had a concentration-dependent activity of restoring black hair color in hydroquinone depigmentation model, in comparison with bergapten-treated animals. Hydroquinone has been reported capable of reducing the number of basal melanocytes, melanin-containing follicles, and melanin-containing epidermal cells. The melanocytes that cause hair pigmentation are located in the bulb of the hair follicles, where they transfer melanin to the cortical keratinocytes of the hair shaft and the melanin-containing hair follicles determines the color of the hair. Nevertheless, melanin-containing hair follicles were less due to the activity of hydroquinone on tyrosine.

Methanol extract showed high total phenols, flavonoids, and antioxidant content. P-coumaric acid which is known to act as a tyrosinase inducer and may have a good effect on depigmentation and hypopigmentation disorder was detected in the study plant extract. Based on the findings of the in vivo study, cream with M. sativa extract had the capacity of restoring pigmentation of both the skin and hair without causing any skin irritation. Therefore, the current knowledge suggests that M. sativa might be a potential treatment for the treatment of vitiligo and other skin hypopigmentation or pigmentation disorder, thus, to be implemented in pharmaceutical formulations.

**AUTHOR CONTRIBUTIONS**

All authors contributed to the study conception and design. All authors contributed to the material preparation and data collection. Azal Hussein Najm Ubaydee, Reem Issa, Bayan Y. Ghanim, and Faisal Al-Akayleh contributed to the data analysis. The first draft of the manuscript was written by Azal Hussein Najm Ubaydee, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**ACKNOWLEDGEMENTS**

The authors acknowledge the Pharmacological and Diagnostic Research Center (PDRC) and the Faculty of Pharmacy at Al-Ahliyya Amman University for supporting the conduct of this research project. The authors acknowledge the Deanship of Scientific Research of the University of Petra (Ref. 9/4/2020) and the University of Petra Pharmaceutical Center (UPPC) for providing the Laboratory Animals Research Unit and facilitating the conduct of parts of the study.

**CONFLICT OF INTEREST**

The authors report no conflicts of interest in this work.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**ETHICAL APPROVAL**

The protocol of the animal testing on mice was reviewed and approved by the Ethical Committee of the University of Petra under the approval number A1, A4/4/2022, University of Petra (Amman, Jordan).

**ORCID**

Nidal A. Qinna https://orcid.org/0000-0002-9628-6036

**REFERENCES**

1. Prćić S, Duran V, Katanić D. Vitiligo and other hypopigmentation disorders in children and adolescents. Acta Med Acad. 2011;40(2):174-181.
2. Bergqvist C, Ezzedine K. Vitiligo: a review. Dermatology. 2020;236(6):571-592.
3. da Silva VB, Kawano DF, Carvalho I, et al. Bergapten: in silico metabolism and toxicophoric analysis of drugs used to treat vitiligo. J Pharm Pharm Sci. 2009;12(3):378-387. doi:10.18433/J3W01D
4. Niu C, Aisa HA. Upregulation of melanogenesis and tyrosinase activity: potential agents for vitiligo. *Molecules*. 2017;22(8):1303. doi:10.3390/MOLECULES22081303

5. Shahkhabazova A, Wu H, Chambers CJ, Sivamani RK. A systematic review of nutrition, supplement, and herbal-based adjunctive therapies for vitiligo. *J Altern Complement Med*. 2021;27(4):294-311.

6. Gianfaldoni S, Wollina U, Tiran M, et al. Herbal compounds for the treatment of vitiligo: a review. *Open Access Maced J Med Sci*. 2018;6(1):203-207. doi:10.3889/OAMJMS.2018.048

7. Berreghioua A, Cheriti A. Phytochemical investigation of the medicinal plant *Moricandia arvensis* L. from Algerian Sahara. *Asian J Pharm Clin Res*. 2018;11(5):450. doi:10.22159/AJP.CHR.2018.V11I5.23822

8. Dogra NK, Kumar S, Kumar D. *Vernonia anthelmintica* (L.) willd.: an ethnomedicinal, phytochemical, pharmacological and toxicological review. *J Ethnopharmacol*. 2020;256:112777. doi:10.1016/J.JEP.2020.112777

9. Li F, Guo S, Zhang S, et al. Bioactive constituents of *F. esculentum* Bee Pollen and quantitative analysis of samples collected from seven areas by HPLC. *Molecules*. 2019;24(15):2705. doi:10.3390/MOLECULES24152705

10. Bora KS, Sharma A. Phytochemical and pharmacological potential of Medicago sativa: a review. *Pharm Biol*. 2011;49(2):211-220.

11. Tomasz Kubrak T, Rafal Podgórski R, Monika SM. Natural and synthetic coumarins and their pharmacological activity. *Eur J Clin Exp Med*. 2017;2:169-175.

12. Kabtni S, Sdouga D, Bettaib Rebey I, et al. Influence of climate variation on phenolic composition and antioxidant capacity of Medicago minima populations. *Sci. Rep.* 2020;10(1):8293. doi:10.1038/S41598-020-65160-4

13. Cosmulescu SN, Trandafir I, Cornescu F. Antioxidant capacity, total phenols, total flavonoids and color component of cornelian cherry (*Cornus mas* L.) wild genotypes. *Not Bot Horti Agrobot Cluj Napoca*. 2019;47(2):390-394.

14. Pramod HJ, Yadav AV, Raje VN, Mohite M, Wadkar G. Antioxidant activity of *Borassus flabellifer* (linn.) fruits. *Asian J Pharm Technol*. 2013;3(1):16-19.

15. Kabtni S, Sdouga D, Rebeey IB, et al. Influence of climate variation on phenolic composition and antioxidant capacity of Medicago minima populations. *Sci. Rep.* 2020;10(1):1-15.

16. Krakowska A, Rafińska K, Walczak J, Kowalkowski T, Buszewski B. Comparison of various extraction techniques of *Medicago sativa*: yield, antioxidant activity, and content of phytochemical constituents. *J AOAC Int*. 2017;100(6):1681-1693. doi:10.5740/JAOACINT.17-0234

17. Kosová M, Hrádková I, Mátlová V, Kadlec D, Šmidrkal J, Filip V. Antimicrobial effect of 4-hydroxybenzoic acid ester with glycerol. *J Clin Pharm Ther*. 2015;40(4):436-440.

18. del Olmo A, Calzada J, Nuñez M. Benzoic acid and its derivatives as naturally occurring compounds in foods and as additives: uses, exposure, and controversy. *Crit Rev Food Sci Nutr*. 2017;57(14):3084-3103. doi:10.1080/10408398.2015.1087964

19. Arif T. Salicylic acid as a peeling agent: a comprehensive review. *Clin Cosmet Investig Dermatol*. 2015;8:455-461. doi:10.2147/CCID.S84765

20. Cheng HM, Chen FY, Li CC, et al. Oral administration of vanillin improves imiquimod-induced psoriatic skin inflammation in mice. *J Agric Food Chem*. 2017;65(47):10233-10242.

21. Bian S, Doh HJ, Zheng J, Kim JS, Lee CH, Kim DD. In vitro evaluation of patch formulations for topical delivery of gentisic acid in rats. *Eur J Pharm Sci*. 2003;18(2):141-147. 10.1016/S0928-0987(02)00255-5

22. Choi SJ, Lee SN, Kim K, et al. Biological effects of rutin on skin aging. *Int J Mol Med*. 2016;38(1):357-363.

23. Min SY, Park CH, Yu HW, Park YJ. Anti-inflammatory and anti-allergic effects of saponarin and its impact on signaling pathways of RAW 264.7, RBL-2H3, and HaCaT cells. *Int J Mol Sci*. 2021;22(16):8431.

24. Irrera N, Pizzino G, D’Anna R, et al. Dietary management of skin health: the role of genistein. *Nutrients*. 2017;9(6):622.

25. Kilic I, Yesiloğlu Y. Spectroscopic studies on the antioxidant activity of p-coumaric acid. *Spectrochim Acta A Mol Biomol Spectrosc*. 2013;115:719-724.

26. Boo YC. p-Coumaric acid as an active ingredient in cosmetics: a review focusing on its antimelanogenic effects. *Antioxidants*. 2019;8(8):275.

27. Hamed M, El-Sharkawy RM. Evaluation of putative inducers and inhibitors toward Tyrosinase from two Trichoderma species. *Jordan J Biol Sci*. 2020;13(1):7-12.

28. Zaidi KU, Ali S, Ali A, Thawani V. Natural melanogenesis stimulator a potential tool for the treatment of hypopigmentation disease. *Int J Mol Biol Open Access*. 2017;2(1):37-40.

29. Nakajima M, Shiooda I, Fukushima Y, Hayasawa H. Arbutin increases the pigmentation of cultured human melanocytes through mechanisms other than the induction of tyrosinase activity. *Pigment Cell Res*. 1998;11(1):12-17.

30. Wang XB, Kong LL, Du GH. Bergapten. *Natural Small Molecule Drugs from Plants*. Springer; 2018:675-678.

31. Anbar TS, El-Sawy AE, Attia SK, et al. Effect of PUVA therapy on melanocytes and keratinocytes in non-segmental vitiligo: histopathological, immuno-histochemical and ultrastructural study. *Photodermatol Photoimmunol Photomed*. 2012;28(1):17-25.

32. Höningmann H. Erythema and pigmentation. *Photodermatol Photoimmunol Photomed*. 2002;18(2):75-81.

33. Essien KI, Harris JE. Animal models of vitiligo: Matching the model to the question. *Dermatologica Sinica*. 2014;32(4):240-247. doi:10.1016/J.DSI.2014.09.008

34. Huo SX, Liu XM, Ge CH, et al. The effects of galangin on a mouse model of vitiligo induced by hydroquinone. *Phytother Res*. 2014;28(10):1533-1538.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.