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HPLC profiling of *Mimosa pudica* polyphenols and their non-invasive biophysical investigations for anti-dermatoheliotic and skin reinstating potential

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ABSTRACT

Plant-derived polyphenols are known to have promising biological activities including antioxidant and anti-tyrosinase and may be a potential candidate for anti-dermatoheliotic remedy. The present study was performed to investigate the polyphenolic contents of *Mimosa pudica* (MP) seed extract and its anti-dermatoheliotic potential using *non-invasive* biophysical techniques after developing a stable topical emulgel formulation. Moreover, its *in-vitro* cytotoxicity was also evaluated using normal Human keratinocytes (HaCat cells) to rule out any cellular incompatibility. The results revealed that MP seed extract, constituted with a number of polyphenolic compounds, has very good antioxidant and anti-tyrosinase potential. There were significant positive effects ($p \leq 0.05$) invoked by its topical emulgel formulation on various dermatoheliotic associated skin parameters like erythema, melanin, elasticity, hydration, and sebum as compared to placebo. In the meantime, it was also found to be biocompatible and did not show any effect on HaCat cell viability and structure. In conclusion, the topical emulgel preparation loaded with MP seed extract could be a great strategy to deal with dematoheliosis.

1. Introduction

Skin is constantly exposed to the deleterious effects of the external environment, most important of which is ultraviolet radiations (UVR) of the sun. Chronic and repeated UVR exposure results into pathophysiological photo-damage generally manifested by inflammation, coarseness, dryness, hyper and dyspigmentation, wrinkles, telangiectases, laxity, and a leathery appearance. Dermatoheliosis is a complex and multistep process that effect the skin and its support system [1]. UVR exposure triggers the free radical generation including singlet oxygen, hydrogen peroxide, hydroxyl radical and peroxyl radicals. These free radicals activate the nuclear transcription factor kappa B (NF-κB) and transcription factor (AP-1), responsible for the upregulation of pro-inflammatory cytokines, Interleukins (IL-1, IL-6, IL-10, and IL-12) and also caused the production of inflammatory mediators COX-2, PGE2 from the arachidonic acid of cell membrane phospholipids. All these events result in the acute erythema and inflammation of the skin [2]. This inflammation and erythema affect skin protection, which leads to an increased trans-epidermal water loss and end at the dryness of skin in photo-aging [3]. Moreover, UVR exposure and subsequent free radical generation cause the upregulation of matrix metalloproteinases (MMPs) by NF-κB, AP-1, and AP-2 activation. That causes the degradation of elastin and collagen fibers, which are the key components responsible for the integrity, support and resilience of the skin. In addition, AP-1 also apprehends the collagen synthesis in the skin. This degradation and reduced collagen synthesis lead to coarse, rigid and wrinkled skin, which are the classical signs of photo-aging [4]. The UVR exposure also causes melanocytes to produce more melanin resulting in hyperpigmentation [5].

There are many treatment choices for dermatoheliosis available nowadays including both synthetic as well as natural. The global market for anti-aging products was of worth $140.3 billion in 2015, which will be forecasted to reach $ 216.52 billion in 2021 [6]. Thus, there is a growing trend to use plant-derived natural agents for the prevention and cure of environmental associated skin disorders such as skin allergies, microbial infections as well as photo-aging. The polyphenolic compounds are the most important and widely discussed phytoconstituents in this regard. They have diverse pharmacological effects like photo preventive, antioxidant, anti-carcinogenic, antimicrobial [7]. Owing to their abundance and inexpensiveness with

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lesser side effects, they are highly attractive to be used as an alternative option for the current pharmacological treatments [8]. In this respect, more than 8000 polyphenols, out of which 4000 are flavonoids has been discovered and identified from various plant species [9]. The main classes are phenolic acids, flavonoids, lignans, stilbenes, and ellagic acids. *Mimosa pudica*, (MP) commonly known as sensitive plant (touch-me-not) is an annual or perennial herb belonging to family Fabaceae. Phytochemical investigations of the plant showed the presence of alkaloids, C-glycosides, flavonoids, tannins, non-protein amino acids like mimosine, sterols, terpenoids, and fatty acids. Traditionally, various plant parts have been used in many diseases such as amoebic dysentery, diarrhea, and hypertension, respiratory and gynecological disorders. It has a vast usage in nervous system related disorders like depression, headache, migraine, and insomnia. Topically, the plant parts not only used to treat snake and scorpion bite but also for several skin conditions like leukoderma, inflammation, burning sensation, small pox and in wound management [10–12]. The oral bioavailability of dietary polyphenols is limited as compared to their dietary intake because they undergo extensive metabolic processes like conjugation, methylation, sulfation, and glucuronidation before they reach the systemic circulation and exert their effects [13]. So, the topical dosage forms like emulgel have been the first choice for localized skin disorders as they offer many advantages, importantly the targeted delivery to the affected site as well as increased contact time of drug with the diseased area [14]. Other advantages of the topical route include the avoidance of first-pass effect and gastric degradation of the drugs as well as less frequent dosing [15]. Furthermore, owing to non-destructive sampling and reduced ethical concerns, the trend of the use of *non-invasive* instrumentation to asses skin parameters for diagnostic and treatment purposes constantly increasing these days [16]. Hence, the present study was designed to characterize the polyphenolic and flavonoid contents of MP seed extract by chemical and HPLC profiling methods and its anti-dermatoheliotic and skin reinstating effects were investigated using *non-invasive* biophysical techniques by formulating its stable topical emulgel formulation.

2. Material and method

2.1. Chemicals and equipment

Ethanol, n-hexane, Chloroform, n-Butanol, Liquid paraffin, Triethanolamine, and Propylene glycol were purchased from Merck, Darmstadt, Germany. Carbopol 940, Span 20, Tween 20, DPPH, L-DOPA, Mushroom Tyrosinase, Kojic acid, Ascorbic acid, and all HPLC grade standards (Quercitin, Gallic acid, Apigenin, Luteolin, Epicatechin, Chlorogenic acid, Ferulic acid, Hyperoside, Fisetin, Rutin, Naringenin, Apigenin-7-O-glucoside, Chrysin and Benzenetriol) and MTT (98% Purity) were purchased from Sigma Aldrich (USA). Human keratinocyte (HaCat cells) cell line was purchased from American Type Culture Collection. Fetal-bovine serum, Dulbecco's Modified Eagle's Medium, PBS, Trypsin-EDTA (more than 6000 units/mg) and 100 units/ml penicillin + 100 µg/ml streptomycin were obtained from Gibco (USA). Fluorescein diacetate (FDA) was purchased from ThermoScientific (USA).

Rotary evaporator (Heidolph, Co. Ltd., Japan), UV spectrophotometer (UV 4000 ORI, Germany), microplate reader Synergy HT (BioTek Instrument, USA), Refrigerator (Dawlance, Pakistan), Homogenizer (Germany), Hot Incubator (Sanyo MIR-162, Japan), Digital Humidity Meter (TES Electronic Corp, Taiwan), pH meter (WTW pH-197i, Germany), optical microscope (Eclipse E200, Nikon, Japan), Mexameter®, Corneometer®, Sebumeter® and Elastometer® (Courage + Khazaka Electronic GmbH, Germany)

2.2. Plant material

Mimosa pudica seeds were purchased from a drug store in Bahawalpur, Pakistan, and identified by an expert botanist from the Department of Life Sciences, The Islamia University of Bahawalpur, Pakistan. A voucher specimen (7436/LS) was submitted for future reference.

2.3. Extraction and fractionation

The coarse powder of ground seeds (250 g) was subjected to 3 cycles of 800 ml ethanol-water (80:20; v/v) extraction at room temperature (each with 3 days soaking). The resultant extract was filtered followed by concentrated using a vacuum rotary evaporator. Final concentrated extract upon drying was weighed and calculated percentage yield was 51 g (20%; w/w). Out of this, 16 g was retained as HAMP (Hydro-alcoholic fraction) and the remaining was suspended in deionized water and then successively extracted to fractionation with n-hexane, chloroform and n-butanol in increasing order of polarity. The resulting fractions were named as NHMP (6 g), CLMP (14 g), and NBMP (12 g) denoting n-hexane, chloroform and n-butanol fractions of MP respectively. All the extracts in sealed containers were stored in the refrigerator for further evaluation.

2.4. Total phenolic content (TPC)

Kim's method with slight modifications was adopted to quantify the total phenolic content (TPC) in the plant samples. [17] 1.0 ml of Folin-Ciocalteu reagent was added to a mixture containing 1 mg of each sample in 9 ml water and was mixed thoroughly for 5 min. Then 10 ml of Na_2CO_3 (7%) was added and the final volume of 25 ml was made with water. The mixture was incubated at room temperature for 90 min and absorbance was recorded at 750 nm. Total phenolic content (TPC) was expressed as µg of Gallic acid equivalents (GAE)/mg of the dry weight of the plant sample.

2.5. Total flavonoid content (TFC)

Park's method with slight modifications was adopted to quantify the total flavonoid content (TFC) in the plant samples [18]. 0.1 ml of 0.3 mol/ L $AlCl_3.6H_2O$ was added to a mixture containing 0.3 ml plant sample and 0.5 mol/L $NaNO_2$. Then 3.4 ml of 30% methanol was added to the above mixture and absorbance was recorded at 506 nm. Total flavonoid content (TFC) was expressed as µg Quercetin equivalents (QE)/mg of the dry weight of the plant sample.

2.6. Antioxidant activity

Majid's method with slight modifications was adopted to perform the antioxidant activity by DPPH radical scavenging with Ascorbic acid as a positive control [19]. 24 mg of DPPH was dissolved in 100 ml of methanol to make a stock solution which was stored at 20 °C. About 3 ml of this stock solution was added to plant samples of 1 mg/ml in test tubes and was incubated at room temperature for 15 min. Absorbance was recorded at 517 nm and the percent inhibition was calculated by the following relation.

$$\text{Percent inhibition} = (\text{Abs. of control} - \text{Abs.of sample})/(\text{Abs. of control}) \times 100$$

2.7. Tyrosinase inhibition assay

Kim's method with slight modifications was adopted to perform the Tyrosinase inhibition assay of the plant samples with Kojic acid as a positive control [20]. In this method 60 units of enzyme were added to 150 µl of buffer (50 mM of pH 6.8) and 10 µl of test compound in each well were incubated at 30 °C for 15 min. After incubation pre-read was recorded at 480 nm. Then 1 mM of substrate per well was added and re-

incubated at same condition for 30 min. After re incubation absorbance was again measured at the same frequency. The result was measured by the following formula. $IC_{50}$was calculated by making a serial dilution of the original concentration.

Percent inhibition = 100 − (absorbance of sample/absorbance of control) × 100

2.8. HPLC analysis

HPLC analysis for Phenolic and flavonoid compounds was performed using Waters Breeze™ HPLC system with a Zorbax SBC-18 (150 × 3 mm, 3.5 µm) column and 1525 Binary Pump. The system was equipped with 717 plus Autosampler (injection volume of 10 µl), in-line Degasser and 2487 UV Detector. Column oven temperature was set to 60 °C and flow rate to 1 ml/min. Sample run time was 70 min each and all the detection was made at 330 nm.

Stock solutions of all flavonoid standards and fractions of MP seed extract were prepared in Methanol (1 mg/ml stock) in amber Eppendorf tubes to protect from light and filtered through micro-filters. Gradient elution system was used with two mobile phases. Mobile phase 1 was 40 mM potassium dihydrogen phosphate (pH 2.3 with 85% Orthophosphoric acid) whereas mobile phase 2 was Methanol. The gradient elution was 95% A to 58% A over 52.5 min., 58:42 (52–57 min) then again to initial composition (57–60 min) at a flow rate of 1 ml/min and allowed to run for another 10 min before injecting the next sample. In present study 13 standards of phenolic and flavonoid compounds were used including Gallic acid, Chlorogenic acid, Epicatechin, Ferulic acid, Hyperoside, Luteolin, Rutin, Fisetin, Apigenin-7-*O*-glucoside, Naringenin, Benzenetriol, Apigenin and Chrysin.

2.9. In-vitro cytocompatibility assay

2.9.1. Cell culture

Normal Human keratinocyte (HaCat cells) were cultured in Dulbecco's Modified Eagle's Medium supplemented with a mixture of FBS (10%) and penicillin + streptomycin (100U/ml + 100 µg/ml) at 37 °C in a 5% CO_2 humidified incubator (Thermo Scientific, Waltham, MA, USA). The stock cultures were grown in 25 cm^2 culture flasks and all the experiments were carried out in 60 mm Petri-plates.

2.9.2. MTT assay

The assessment of HaCat cells cytotoxicity was done using the MTT assay. Briefly, cells were seeded in 96-well plate (1×10^4 cells/well) and cultured for 24 h to allow them to attach to the surface. Next, were incubated with varying concentrations (1, 10, 100, 500, 1000, 2000 and 3000 µg/ml) of HAMP fraction intended to be used to formulate emulgel for 48 h. Untreated cells were used as a control in the experiment. Next, 20 µl of MTT solution in PBS (5 mg/ml) was added to each well and after incubation for 4 h at 37 °C, 150 µl of DMSO was added. Finally, the absorbance was measured at 490 nm using a spectrophotometer.

2.9.3. Cellular morphology

HaCat cells were seeded in 6-well plate (1×10^4 cells/well) and incubated at 37 °C for 24 h. Then cells were incubated with the different concentrations (1, 10, 100, 500, 1000, 2000 and 3000 µg/ml) of HAMP fraction for 24 h. Next, cells were incubated at 37 °C for 5 min with FDA, the FDA enters normal cells where it is cleaved by esterases and emits a green fluorescence. Next, the cells were washed three times with PBS. Finally, the cells were observed under a fluorescence microscope.

Table 1
Composition of placebo and emulgel formulation (100g).

Ingredients	Placebo(g)	Formulation(g)
Liquid Paraffin	7.5	7.5
Span 20	1	1
Tween 20	0.5	0.5
Propylene glycol	5	5
Methyl paraben	0.03	0.03
Propyl paraben	0.01	0.01
Carbopol 940	2	2
Extract	–	2
Distilled Water	qs	qs

2.10. Formulation development

A topical emulgel formulation was developed using Carbopol 940 as a gelling agent. Firstly, the gel phase was prepared by dissolving 2% Carbopol 940 in double distilled water with constant stirring with the help of a mechanical stirrer. Then pH was adjusted to 6–6.5 using Triethanolamine and a clear gel was obtained at this pH. Then oil in water emulsion was prepared using ingredients described in Table 1. The oil phase was prepared by dissolving Span 20 in paraffin oil while aqueous phase was prepared by dissolving Tween 20 in double distilled water. A mixture of methyl and propyl parabens in propylene glycol and 2 g HAMP fraction (1 mg/ml) was also prepared and added to the aqueous phase. Both the phases (oil and aqueous) were separately heated to 70–80 °C and then the oil phase was incorporated into the aqueous phase with constant stirring. The emulsion was continued to stirring until it was cooled to room temperature. After this, the emulsion was mixed with pre-prepared gel phase in 1:1 ratio to obtain emulgel. The only difference between placebo and active emulgel formulation was the addition of MP seed extract was not added to the placebo formulation.

2.11. Stability studies and microscopic evaluation

The prepared placebo and formulation were subjected to stability studies for 12 weeks period under varying conditions of temperature and humidity. The conditions used were 8 °C ± 1, 25 °C ± 1, 40 °C ± 1 and 40 °C ± 1 with 75% ± 1 relative humidity. During this period, placebo and formulation were evaluated for any change in physical stability (color, liquefaction, and phase separation. Moreover, Microscopic evaluation was carried out to assess the mean droplet size and morphology of both placebo and formulation, with the help of CCD camera loaded optical microscope (Eclipse E200, Nikon, Japan) with 100× oil immersion lens. Very small amount of the sample was taken on a glass slide and diluted with external phase, then covered with a glass coverslip observed under oil immersion lens. The images were processed with the help of image analysis software, Digimizer V. 4.3.1. Morphology and the mean droplet size of the internal phase were measured.

2.12. In-vitro sun protection factor

In-vitro sun protection factor of the formulation was calculated by using a spectrophotometer by the method described previously by Dutra et al., About 1 g of sample was made to 100 ml with ethanol in a volumetric flask and sonicated for 5 min. After sonication, it is filtered through a cotton plug and first 10 ml of this solution is discarded. Next 5 ml of this was again made to 50 ml with ethanol and 5 ml of which was made to 25 ml with ethanol. Then by taking ethanol as blank, the absorption of the sample was measured in the range of 290–320 nm every 5 nm [21] and Mansur's equation was applied to calculate the SPF [22].

2.13. Non-invasive in-vivo investigations

2.13.1. Study design and ethical approval

A single-blinded, randomized study was designed for the non-invasive investigation of polyphenol-containing emulgel formulation on human skin for 12 weeks. The study was approved by the Advance Studies and Research Board, The Islamia University of Bahawalpur (No: 496/AS&RB) and Institutional Ethical Review Committee of Faculty of Pharmacy and Alternative Medicines, The Islamia University of Bahawalpur (No: 45/S-2017/PREC). This study was also in accordance with the International guidelines of Helsinki Declaration, International Ethical Guidelines for Biomedical Research Involving Human Subjects by The Council for International Organizations of Medical Sciences (CIOMS) [23] All the measurements of skin parameters were conducted at the Cosmetic lab, Department of Pharmacy, Faculty of Pharmacy and Alternative Medicines, The Islamia University of Bahawalpur. A total of 13 volunteers (male, healthy, insensitive and non-smoker) of age 20–40 years were selected. Prior to the study, all the volunteers were educated about the importance, possible risks and protocol for the study and they were asked to sign a written consent form as a document of evidence for terms and conditions of the study. A dermatologist checked all the volunteers for any type of skin disease or damage especially on the skin areas being involved in the study (forearms and cheeks). The volunteers were asked to continue their routine diet and to strictly to avoid the use of any sort of multivitamin/ antioxidant or skin preparation and so as to nullify the chances of potentiation to the research product. All the skin tests were carried out at controlled conditions of temperature and humidity (25 ± 1 °C and 40% Relative Humidity). Prior to each measurement of skin parameters, volunteers were asked to stay in the normal sitting position in the cosmetic lab for 15 min so as they would imbibe the inside conditions of temperature and humidity. Each volunteer was provided with the two formulations, placebo, and active formulation indicated as “Left” and “Right” depicting their application to the respective cheek. They were instructed to use both the formulations to the respective cheek twice a day. The measurements were taken at zero time, and on 2nd, 4th, 6th, 8th, 10th and 12th week.

2.13.2. Assessment of primary skin irritation by patch test

Prior to the study, to rule out any possible skin irritation by any ingredient of the formulations (placebo and active formulation), each volunteer was examined by performing a patch test. For this purpose, an area of 5 × 4 cm was marked on each forearm of the volunteers. Baseline values for skin erythema index were recorded with Mexameter®. Then about 1 g of placebo and active formulation was applied to the marked area of the left and right forearm respectively and covered with a hypo-allergic surgical dressing. After 48 h, skin melanin and erythema indexes were recorded again.

2.13.3. Evaluation of skin erythema and melanin indexes

Mexameter® was used to quantify the skin erythema and melanin indexes. This instrument works on the principle of light emission and reflectance. Its probe contains an elastic spring to ensure a constant pressure on the skin. On touch with skin it emits light of three specific wavelengths; green, red and infrared with 568, 660 and 880 nm respectively. This emitted light gets reflected from the skin and the amount of absorbed light is quantified by the receiver. Erythema index is quantified from the intensity of absorbed and reflected light of 568 and 660 nm whereas Melanin index from 660 and 880 nm respectively. [24] Three consecutive measurements were performed on the tested skin area and their mean was taken to avoid chances of error.

2.13.4. Evaluation of skin hydration level index

Corneometer® was employed to measure the skin hydration level in this study. The probe comprises of gold electrodes overlaid by a thin layered insulation material with low dielectric constant. When the probe is placed vertically on the skin, an electric field penetrates the stratum corneum and the hydration level is measured as dielectric constant dependent capacitance changes of water. The measured value is displaced as arbitrary units [25]. Three consecutive readings were recorded and their mean was taken as final reading to minimize the chances of error.

2.13.5. Evaluation of skin elasticity index

Elastometer® is a widely used elasticity measuring probe. It allows quick and convenient quantification of elastic properties of the skin with the easy user interface. It works on the principle of the resilient ability of the skin when disturbed (suction and stretching). The measured value is displayed in the LED display present on the instrument in terms of percentage of elasticity [26]. Three consecutive readings were recorded and their mean was taken as final reading to minimize the chances of error.

2.13.6. Evaluation of skin sebum index

Sebumeter® is a widely used method to quantify the sebum production on the skin surface. It works on the principle of grease spot photometry and uses the light intensity difference through a plastic strip which is termed as sebumeter tape. When this tape is brought into contact with skin sebum, it becomes transparent. After inserting it into the device aperture, its transparency is measured by photocell by the emission of light [27]. Three consecutive measurements were taken and their mean was taken for analysis.

2.14. Mathematical and statistical analysis

The percentage change of individual values of each skin parameter was calculated by the following formula:

$$\text{Percentage change} = [(A - B) / B] \times 100$$

Where A indicates the individual value of each parameter at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} week and B indicates the baseline value of that parameter at zero time.

Data were analyzed using Statistical Package for Social Sciences (SPSS, V. 17.0) software. The Analysis of Variance (ANOVA) was applied to assess eventual variation between different time intervals and paired sample *t*-test to describe any difference between two formulations (placebo and active), and *Post-hoc* analysis through Least Significant Difference (LSD), which computes “pairwise comparisons”, i.e. the smallest significant difference between two means variation. A difference was considered significant at a P-value inferior to 5% ($P < 0.05$)

3. Results

3.1. Total phenolic contents (TPC)

According to Kim’s method and using Folin-Ciocalteu’s reagent, a hydro-alcoholic crude fraction of MP seeds showed the highest quantity of phenolic compounds as compared to other fractions as indicated in Figs.1 and 2(B) The total phenolic contents in HAMP, NHMP, CLMP, and NBMP were 224.38 µg, 171.16 µg, 193.74 µg and 221.16 µg GAE/mg respectively.

3.2. Total flavonoid contents (TFC)

According to Park’s method and using aluminum chloride, a hydro-alcoholic crude fraction of MP seeds showed the highest quantity of flavonoid compounds as compared to other fractions as indicated in Fig. 2(B) The total flavonoid contents in HAMP, NHMP, CLMP, and NBMP were 186 µg, 126.74 µg, 156.97 µg and 166.27 µg QE/mg respectively.

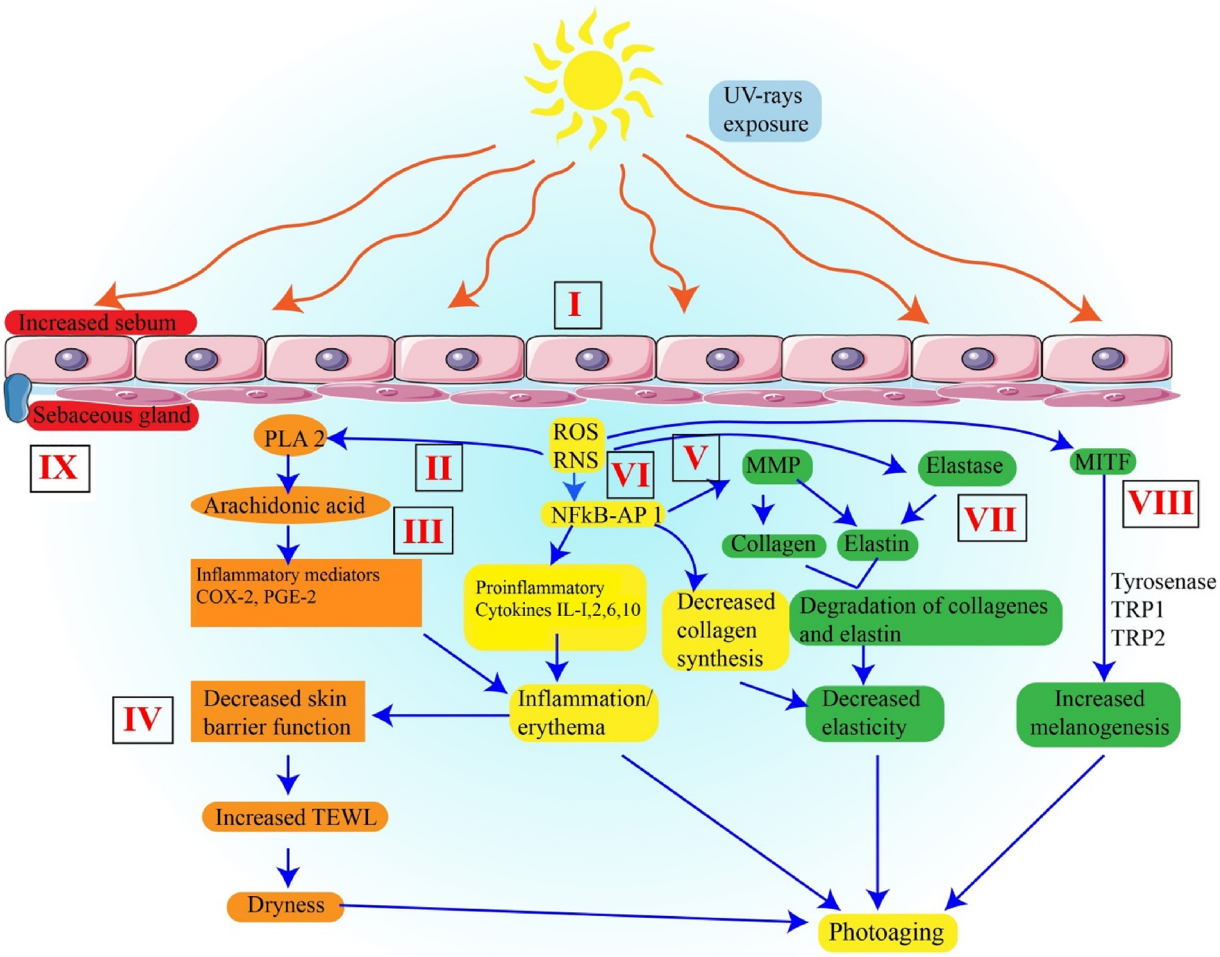

Fig. 1. Mechanism of Dermatoheliosis and Polyphenol activity at different steps.I)Photo-protection by absorbing harmful sunrays topically. II, III) Inhibition of inflammatory mediators production. IV) Ameliorated skin hydration by improved skin barrier function. V) Downregulation of MMPs by inhibiting NF-κB and AP-1. VI) Inhibition of pro-inflammatory cytokine production by the suppression of NF-κB and AP-1. VII) Decreased elastin degradation by the inhibition of elastase activity. VIII) Decreased melanogenesis by the inhibition of tyrosinase, TRP-1, and TRP-2. IX) Decreased sebum production by the inhibition of 5α-reductase 1 activity.

3.3. Antioxidant activity

According to the DPPH method, various fractions of MP seed extract were found to have very good antioxidant activities when compared with Ascorbic Acid (control). The antioxidant activity at a concentration of 1 mg/ml of HAMP, NHMP, CLMP, and NBMP was 80%, 66.66%, 68.88%, and 77.77% respectively in comparison with Ascorbic Acid (93%) as indicated in Fig. 2(A). From these results, it is evident that crude hydro-alcoholic fraction had the highest antioxidant potential than other fractions.

3.4. Tyrosinase inhibition assay

Tyrosinase inhibition assay was done on all the fractions of the MP seed extracts. Results indicated that, among all the four fractions, crude ethanolic fraction showed highest Tyrosinase inhibition activity. As indicated in Fig. 2(A) at a concentration of 1 mg/ml, HAMP, NHMP, CLMP, and NBMP had 90%, 50%, 88% and 70% mushroom tyrosinase

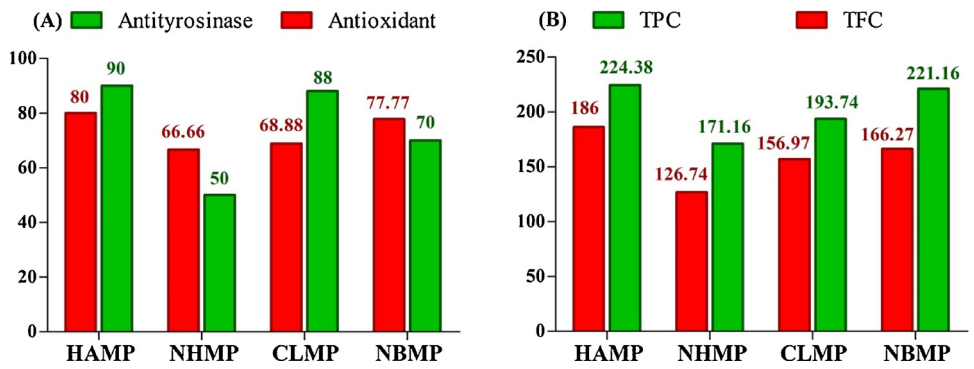

Fig. 2. Antioxidant, Antityrosinase activities and total phenolic and total flavonoid contents of fractions (TPC-TFC).

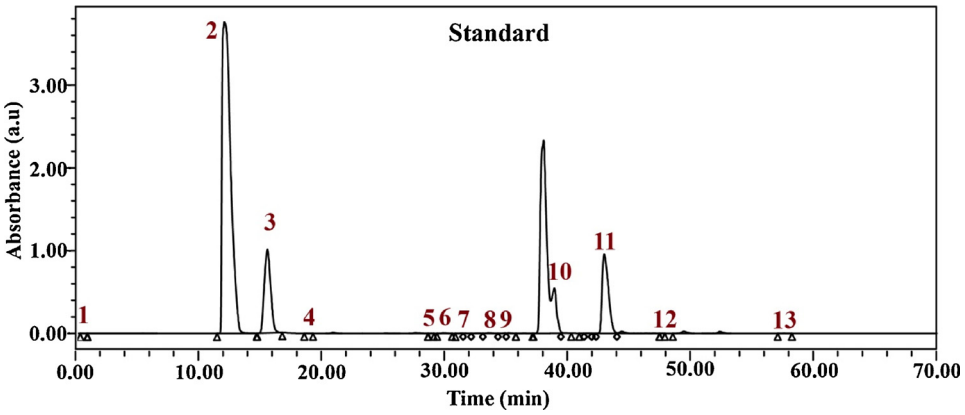

Fig. 3. PLC Chromatogram of reference standards for flavonoids. 1: Gallic acid (1.116 min), 2: Chlorogenic acid (12.091 min), 3: Epicatechin (15.567 min), 4: Ferulic acid (18.828 min), 5: Hyperoside (28.941 min), 6: Luteolin (29.927 min), 7: Rutin (31.338 min), 8: Fisetin (33.593 min), 9: Apigenin-7-glucoside (34.914 min), 10: Naringenin (38.908 min), 11: Benzene-triol (42.954 min), 12: Apigenin (46.996 min) and 13: Chrysin (57.582 min).

inhibition respectively in comparison with Kojic acid (control) which showed 99% activity. These results showed that again hydro-alcoholic crude fraction was come up with highest tyrosinase inhibitory activity than other fractions.

3.5. HPLC analysis

The HPLC assay was performed to determine the presence of phenolic and flavonoid compounds. Therefore, different standard phenolic and flavonoid compounds namely, Gallic acid, Chlorogenic acid, Epicatechin, Ferulic acid, Hyperoside, Luteolin, Rutin, Fisetin, Apigenin-7-glucoside, Naringenin, Benzene-triol, Apigenin and Chrysin were analyzed and thereby eluted at 1.1, 12.0, 15.5, 18.8, 28.9, 29.9, 31.3, 33.5, 34.9, 38.9, 42.9, 46.9 and 57.5 min respectively, as indicated in standard HPLC chromatogram (Fig. 3). The chromatogram of HAMP fraction showed seven peaks and the retention time of these peaks coincided closely with the retention time for Gallic acid, Chlorogenic acid, Hyperoside, Luteolin, Rutin, Fisetin and Chrysin indicating the presence of phenolic compounds, as shown in Fig. 4. Moreover, CLMP demonstrated six obvious peaks coinciding closely with the standard retention times for Gallic acid, Hyperoside, Luteolin, Fisetin, Naringenin and Benzene-triol (Fig. 5). Similarly, the NBMP fraction represented 11 peaks, again with very similar retention times to the standard Gallic acid, Chlorogenic acid, Ferulic acid, Hyperoside, Luteolin, Fisetin, Apigenin-7-*O*-glucoside, Naringenin, Benzene-triol, Apigenin and Chrysin peaks (Fig. 6) again suggesting the presence of phenolic compounds. The order of presence of phenolic and flavonoid compounds is n-butanol > ethanolic extract > chloroform as evident from the HPLC assay. (Figs. 4–6)

3.6. In vitro cytocompatibility assay

To investigate the effect of HAMP fraction of MP seed extract on the cell viability of normal human keratinocyte (HaCat cells) cell line, cytocompatibility assay was performed. The cells were incubated with varying concentrations (1–3000 µg/ml) of the extract. Results of the cytotoxicity assay showed a negligible effect on the cellular viability of HaCat cells. The cell survival rate was above 80% on all concentrations even at a highest concentration of 3000 µg/ml, as compared to control as shown in Fig. 7(A). From cytocompatibility data, it could be concluded that the HAMP fraction does not produce toxicity in HaCat cell line.

Furthermore, to examine the integrity of cell membrane after treatment with varying concentrations HAMP fraction of MP seed extract, the cells were incubated with FDA. After 8 h of incubation, the microscopic examination again revealed no obvious cell membrane damage of HaCat cells indicating its strong biocompatibility, as shown in Fig. 7(B).

3.7. Formulation development

In conclusion, the phytochemical and HPLC analysis revealed that the HAMP fraction of MP seed extract contains the highest amount of TPC and TFC contents as well as highest antioxidant and anti-tyrosinase activity in comparison with other fractions. Moreover, it showed promising cell viability, apoptosis, and membrane integrity proving it to be cytocompatible and safe to be used for topical use. Therefore, we formulated a topical emulgel formulation using 2 g of 1 mg/ml of this fraction and placebo without plant extract. The prepared placebo and formulations, both were white in color.

3.8. Stability studies and microscopic evaluation

Samples of placebo and formulation were kept at varying storage conditions of temperature and humidity and observed for any change in color as well as liquefaction and phase separation. There was no change

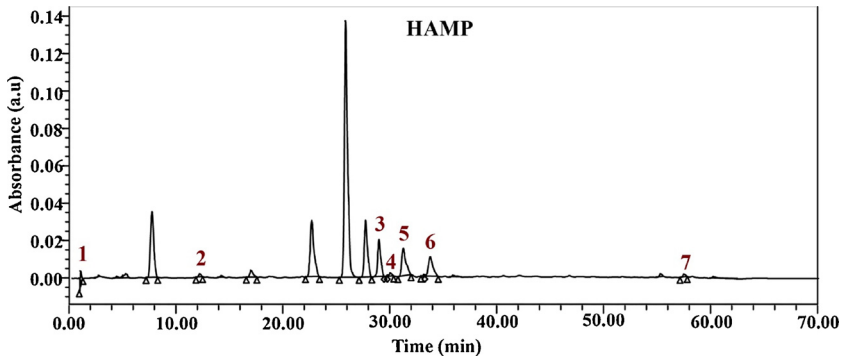

Fig. 4. HPLC Chromatogram of Hydro-alcoholic (HAMP) fraction showing peaks with similar retention time to 1: Gallic acid (1.044 min), 2: Chlorogenic acid (12.184 min), 3: Hyperoside (28.923 min), 4: Luteolin (29.767 min), 5: Rutin (31.201 min), 6: Fisetin (33.719 min) and 7: Chrysin (57.476 min).

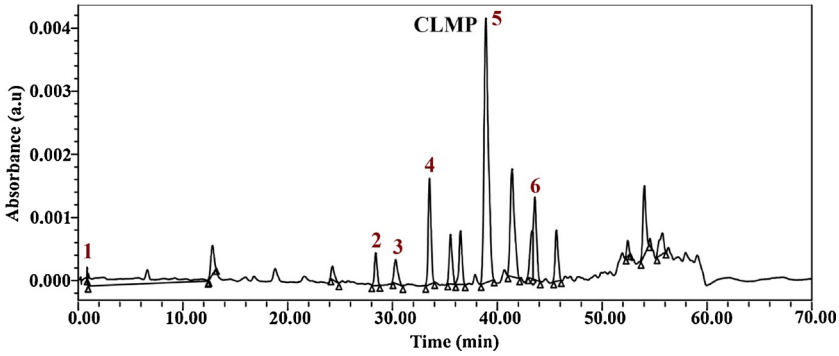

Fig. 5. HPLC Chromatogram of chloroform (CLMP) fraction showing peaks with similar retention time to 1: Gallic acid (0.915 min), 2: Hyperoside (28.454 min), 3: Luteolin (30.353 min), 4: Fisetin (33.586 min), 5: Naringenin (38.963 min) and 6: Benzene-triol (43.367 min).

in the color of placebo and formulation at all conditions of temperatures and humidity. As far as the liquefaction and phase separation is concerned, both placebo and formulation were stable at all conditions except at 40 °C + 75% RH, where a slight change was observed after 12 weeks as indicated in Table 2.

Moreover, the morphology of both placebo and formulation was evaluated from the globule shape and size of the internal phase. The microscopic examination revealed that the globules of freshly prepared placebo and formulation were spherical and about 1.0780 µm and 1.0645 µm of radius respectively at 25 °C. After 12 weeks, there was a slight increase in the globule size was observed and it was found to be 1.231 µm for formulation and 1.3085 µm for placebo as shown in Fig. 8(A). But there was no change in the shape of the globules was seen and they were of same spherical and round in shape as shown in Fig. 8(B).

3.9. In-vitro sun protection factor

The SPF measurement is a quantitative method to evaluate its effectiveness against a wide range of UV- radiations, which makes a formulation to be used as a sunscreen to avoid sunburn and other skin damages. In the present study, the emulgel formulation showed a very good SPF value of 3.8%.

3.10. Non-invasive in-vivo investigations

3.10.1. Assessment of primary skin irritation by patch test

Patch test was performed to assess any chance of primary skin irritation, hypersensitivity or allergy from any ingredient of the placebo or the formulation. Results of Patch test revealed that there was no any kind of irritation or allergy was seen in any volunteer. There was a mild decrease in the erythema levels of the volunteers after 48 h of the application of a patch of both placebo and formulation as mentioned in Fig. 9(A). These observations suggest that both of these can safely be used for further *in-vivo* investigations.

3.10.2. Evaluation of skin erythema index

The erythema index of the skin was measured at regular intervals at zero time and then at 2nd, 4th, 6th, 8th and 12th weeks of the study and change in erythema index by both placebo and formulation was recorded. Results showed that unlike placebo, formulation markedly decreased the erythema index throughout the study period of 12 weeks in a regular manner. Placebo increased the erythema up to 2.87% while formulation decreased it up to 10.77% from the baseline value as shown in Fig. 9(B). Statistical analysis involving ANOVA with 5% level of significance revealed that the change in the erythema index by formulation was significant throughout the study period while by placebo it was insignificant. LSD test showed that formulation produced significant effects at all the weeks i.e. 2nd, 4th, 6th, 8th, 10^{th,} and 12th, while change exerted by placebo was significant only at 6th, 10th and 12th week. Moreover, from pair sample *t*-test, it is evident that there is a significant variance between the effects of placebo and formulation with the passage of time.

3.10.3. Evaluation of skin melanin index

The Melanin index was quantified during the study period at zero time then at 2nd, 4th, 6th, 8th, 10th and 12th weeks. Results demonstrated a slight increase in skin melanin index after the application of placebo till 10th week, but at 12th week there was a slight decrease in the Melanin index. While the formulation markedly decreased the Melanin index in a steady manner and the percent change observed was −17.96% as compared to the baseline zero time value as shown in Fig. 9(C). Two way ANOVA analyses revealed that the decrease in Melanin index was significant while the placebo showed an insignificant increase in it. LSD testing indicated that formulation effect was significant at 4th, 6th, 8th, 10th and 12th week whereas the effect of placebo was not significant at all weeks. Moreover, there was a significant variance observed in the effects of placebo and formulation throughout the study period when pair sample *t*-test was applied.

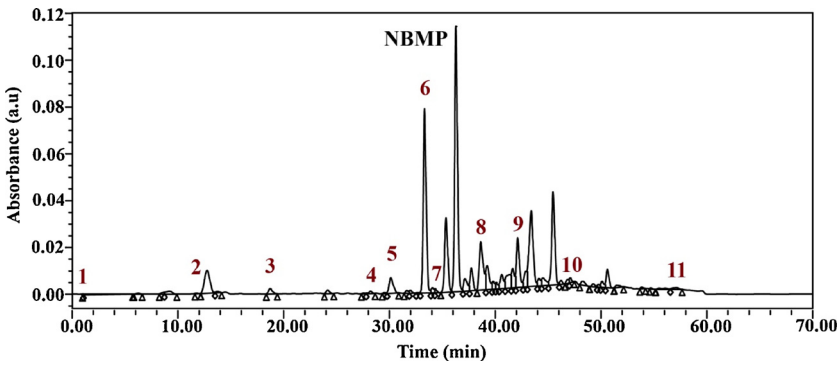

Fig. 6. HPLC Chromatogram of n-butanol (NBMP) fraction showing peaks with similar retention time to 1: Gallic acid (1.116 min), 2: Chlorogenic acid (11.849 min), 3: Ferulic acid (18.805 min), 4: Hyperoside (28.300 min), 5: Luteolin (30.215 min), 6: Fisetin (33.403 min) Apigenin-7-glucoside (34.391 min), 8: Naringenin (38.723 min), 9: Benzene-triol (42.960 min), 10: Apigenin (46.915 min) and 11: Chrysin (57.193 min).

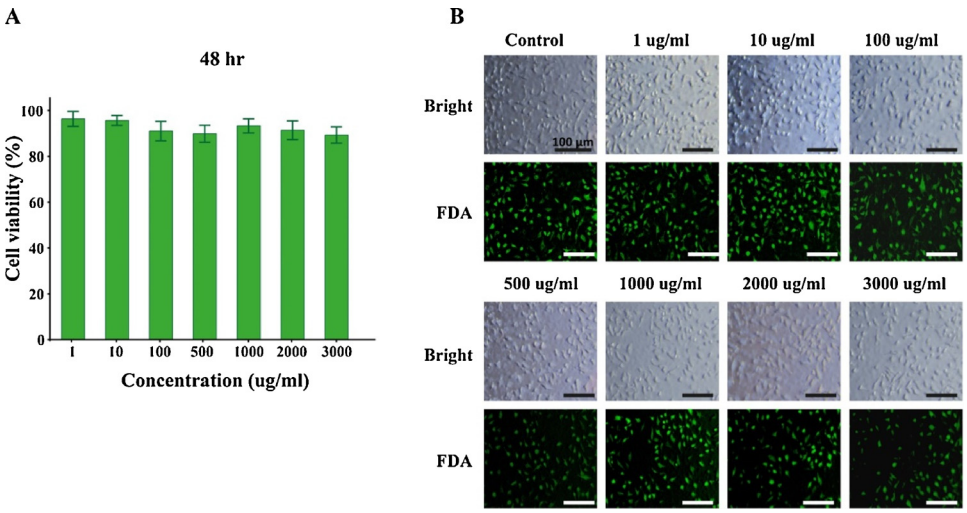

Fig. 7. Biocompatibility examination of plant extract (A) in vitro cytocompatibility assay of HAMP fraction incubated at different concentration with HaCat cells for 48 h,*P < 0.05, **P < 0.01, n = 3, (B) Cellular morphology assessment of HaCat cells after incubation with different concentration of extracts for 8 h, then the cells were incubated with FDA for 5 min and examined under fluorescence microscope.

Table 2
Physical characteristics of placebo and formulation kept at 8 °C, 25 °C, 40 °C and 40 °C + 75% RH.

Observed Parameter	Fresh		12 Weeks							
			8°C		25°C		40°C		40°C+75% RH	
	P	F	P	F	P	F	P	F	P	F
Color	W	W	W	W	W	W	W	W	W	W
Liquefaction	NA	NA	–	–	–	–	–	–	+	+
Phase Separation	NA	NA	–	–	–	–	–	–	+	+

W = White; F = Formulation; P = Placebo; – = No change; + = Slight change; NA = Not Applicable.

3.10.4. Evaluation of skin hydration level index

It was found from the results that both placebo and formulation increased the skin hydration level. Placebo increased it at 2nd, 4th, 8th and 12th week of the study while decreased it at 6th and 10th week. But the effect of formulation on the skin hydration level was more pronounced and regular throughout the study period as it increased it to 3 folds as compared to placebo as indicated in Fig. 9(D). Statistical analysis indicated that the formulation increased the skin hydration level significantly while there was an insignificant change was observed by the application of placebo. Moreover, pair sample *t*-test showed a significant divergence between the effects of formulation and placebo throughout the study period.

3.10.5. Evaluation of skin elasticity index

In the present study, formulation exhibited articulated marked increase (18.01%) in skin elasticity index as compared to placebo which showed an irregular decrease. Placebo decreased the skin elasticity at 2nd, 4th and 12th week and then showed a slight increase in it at 6th, 8th and 10th week as mentioned in Fig. 9(E). The ANOVA analyses revealed that formulation increased the skin elasticity significantly from zero time to 12th week whereas the decrease made by placebo was insignificant. LSD findings revealed that the effect of the formulation was significant at 6th, 8th, 10th and 12th week of the study whereas the placebo's effect was not significant at any week. Pair sample *t*-test showed a significant variance between the effects of placebo and formulation.

3.10.6. Evaluation of Skin Sebum Index

The formulation demonstrated a high and regular reduction in the sebum content of the skin up to -22.38% after application. Whereas, placebo increased the sebum content of the skin but this increase was not regular as at 8th week there was a reduction in the sebum index as shown in Fig. 9(F). Statistical analysis revealed that the increase in sebum content by placebo was not significant while the decrease by the formulation was significant. LSD calculations indicated that formulation exerted a significant effect at 4th, 6th, 8th, 10th and 12th week in continuous while placebo's effect was significant at 12th week only. Pair sample *t*-test showed a significant disagreement between the effects of placebo and formulation throughout the study period of 12 weeks.

4. Discussion

Polyphenolic compounds such as phenolic acids and flavonoids are secondary metabolites and very important constituents of plants.

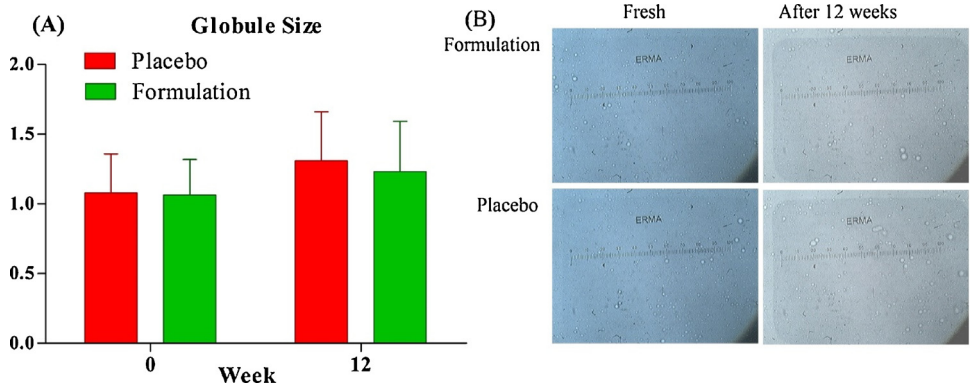

Fig. 8. Globule size of placebo and formulation at the time of preparation and after 12 weeks at 25°C.

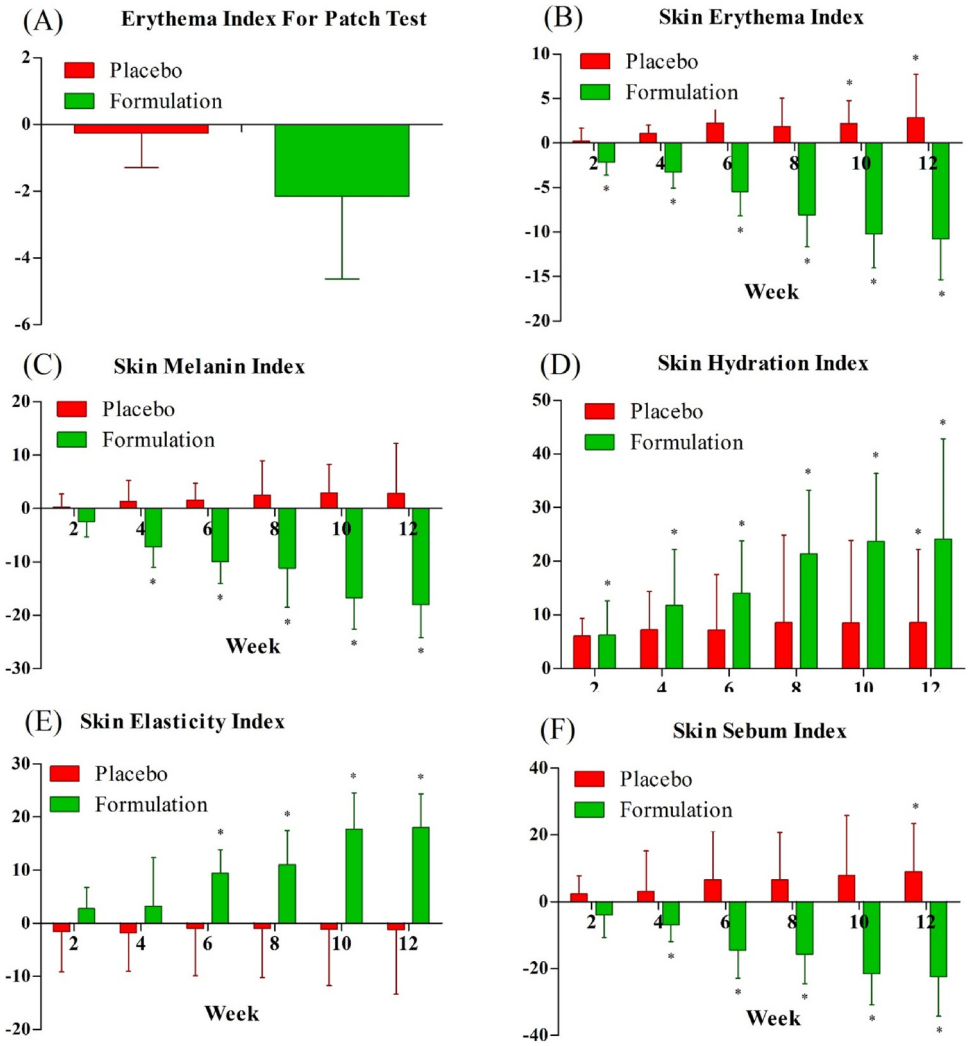

Fig. 9. (A) Patch test and Effect of placebo and formulation on different skin parameters (B) Skin Erythema Index, (C) Skin Melanin Index, (D) Skin Hydration Index, (E) Skin Elasticity Index and (F) Skin Sebum Index applied on human volunteers (n = 13) for 12 weeks. Error bars represented the mean ± SD and *P ≤ 0.05.

Analysis of phenolic compounds from plants has attracted the attention of researchers due to their potential preventive as well as therapeutic effects on human health. T.S. Tunna et al. carried out chemical profiling and characterization of the MP that showed the presence of an abundance of phenolic compounds in it [28]. In our study, HPLC analysis also confirmed the presence of a number of phenols and flavonoids in it. The hydro-alcoholic crude fraction of MP has the highest TPC and TFC contents followed by n-butanol, chloroform and n-hexane fractions respectively. This trend might be due to the higher polarity of a hydro-alcoholic solvent mixture than the other solvents used. In a previous study, similar results were reported by Chavan et al. [29].

Similarly, hydro-alcoholic crude fraction demonstrated the highest antioxidant and anti-tyrosinase activity among all the fractions of MP seed extract. These activities are attributed to the presence of ample amount of phenolic and flavonoids contents because antioxidant and anti-tyrosinase potential of plants are mainly due to the presence of these phenolic compounds in them [30,31]. As this fraction contains the highest amount of TPC and TFC contents, so the reason for its highest activities.

The avoidance of hypersensitivity or skin allergy by any of the ingredients is a major concern in topical formulation development and is dependent on their compatibility with the skin. Paraffin oil, the main ingredient, and vehicle for the current topical formulation is safe and void of any harmful hazardous effects on human health even after the long-term of use [32]. Additionally the biocompatibility assay proved the extract to be bio-safe and cytocompatible with the human skin.

Sun protection in terms of sunscreens that absorbs harmful UV rays is the first and foremost important strategy to avoid photo-damage of the skin [33]. Previous studies revealed that the plant extracts rich in phenolic and flavonoid compounds have the tremendous ability to absorb the UV-radiations and may prove potential natural sunscreens in cosmetic formulations and therefore avoid photo-induced skin damage [34]. Thus, the SPF value of present emulgel formulation could be associated with the presence of these phenolic and flavonoid compounds present in MP seed extract.

Dermatoheliosis is attributed to the photo-oxidative damage to the skin, mainly by high levels of ROS and RNS when the natural anti-oxidative defense system of the body gets compromised [35]. This over production triggers the activation of nuclear transcription factor kappa B (NF-κB) and transcription factor (AP-1) which cause the production and regulation of pro-inflammatory cytokines (IL-1, IL-6, IL-10 and IL-12) and inflammatory mediators (COX-2 and PGE2) and in turn are responsible for the photo-induced erythema and inflammation [36]. In the current study, the marked decrease in the erythema index of the skin may be attributed to the presence of polyphenols in MP seed extract, which are very good scavengers of free radicals and also strongly attenuate the pro-inflammatory cytokines and inflammatory mediators [37].

In humans, melanin is synthesized by melanosomes present in melanocytes in the skin from L-Tyrosine, a phenolic amino acid. This

cascade of biochemical reactions is controlled by the activity of three enzymes; Tyrosinase, Tyrosinase Related Proteins-1 (TRP-1) and Tyrosinase Related Proteins-2 (TRP-2). Polyphenols and flavonoids not only competitively inhibit the tyrosinase enzyme but also TRP-1 and TRP-2 [38]. So, MP seed polyphenols are creditworthy for the evident reduction in the skin melanin index in the current study.

Hydration level of the skin has a prime role in its physiological functioning, maturation, and desquamation. Increased trans-epidermal water loss leads to the improper enzymatic activity which results into the dry, aged and wrinkled skin [39]. There are a number of factors contribute to trans-epidermal water loss and ultimate dryness of the skin and photoinduced inflammation is one of them [40]. The inhibition of cutaneous erythema and inflammation can significantly reduce the trans-epidermal water loss and contribute to the skin's hydration level which is evident from the current study. In addition to this, flavonoids such as gallic acid and fisetin present in MP seed extract cause upregulation of Profilaggrin, a protein responsible for maintaining epidermal barrier and hydration level of the skin [41,42].

Pathophysiological changes in dermatoheliosis adversely affect collagen and elastin which are major constituents of skin extracellular matrix (ECM) and functions for its support, strength, elasticity, and resilience. They are degraded by matrix metalloproteinases (MMPs), and elastase upregulated by activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) activation due to oxidative stress in dermatoheliosis [43]. This enzymatic demolition of elastin and collagen, results in their un-metabolized accumulation in the skin that leads to a complex process of wrinkling [44]. Previous studies have revealed that phenolic acids and flavonoids present in plants like MP significantly inhibit the up-regulation of MMPs [45] and elastase [46] and are responsible for the reduced degradation of these fibers. This eventually results in increased skin elasticity as manifested by our study.

The current study also recorded a marked decrease in skin sebum content which acts as a lubricant and gives waterproofing properties to the stratum corneum. Photo-oxidative stress also contributes to the excessive sebum secretion that involves facial acne development. These results may be due the anti 5α-reductase 1 and antioxidant activity polyphenols like gallic acid and luteolin present in MP seed extract [47,48].

5. Conclusion

The present study revealed that MP seed extract contains a significant number of polyphenols and represents good antioxidant and anti-tyrosinase potential. Moreover, the non-*invasive* biophysical investigations demonstrated that unlike placebo, there were promising positive effects invoked by emulgel formulation of MP seed extract on various skin parameters like erythema, melanin, elasticity, hydration, and sebum. In conclusion, the topical emulgel preparation loaded with MP seeds extract could be a great strategy to deal with dematoheliosis.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

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