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Full Length Research Paper

Evaluation of various functional skin parameters using a topical cream of *Calendula officinalis* extract

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The aim of this study was to determine the effects of newly formulated W/O emulsion (cream) of *Calendula* versus its vehicle (base) as control on skin pH, skin melanin, skin erythema, skin moisture content and transepidermal water loss (TEWL).Hydroalcoholic extract of *Calendula* plant was entrapped in the inner aqueous phase of the W/O emulsion. Base without active and formulation having 3% extarct of calendula in the aqueous phase were prepared. Samples were stored at different storage conditions that is, 8, 25, 40, 40°C+75% RH for four weeks to predict their stability. The newly formulated base and formulation were applied to the cheeks of 21 healthy human volunteers for a period of 8 weeks. The base showed insignificant ($p>0.05$) effects while the formulation decreased TEWL however this decrease was statistically insignificant ($p> 0.05$). Skin moisture content was significantly ($p\leq0.05$) increased by the formulation. The base showed insignificant ($p>0.05$) effects while the formulation showed statistically significant ($p\leq0.05$) decrease in skın melanın content. Skın erythema was significantly reduced by the formulation. Skin sebum was significantly ($p\leq0.05$) incresaed by both creams (base and formulation). Both creams were aesthetic with respect to sensory evaluation. The topical non-invasive application of *Calendula officinalis* cream showed a positive rejuvenating effect on human skin. This study will encourage more attention towards research and more conviction towards utilization of herbal medicines.

Key words: *Calendula* extract, melanin, erythema, skin sebum, TEWL.

INTRODUCTION

Now-a-days herbal extracts are used in the cosmetic preparations for augmenting beauty and attractiveness. Herbal cosmetics are classified on the basis of dosage form like- cream, powder, soaps, solutions etc. and according to part or organ of the body to be applied for like; cosmetics for skin, hair, nail, teeth and mouth etc. The use of cosmetics requires both their efficacy as well as minimal risk of skin irritation/skin sensitization. This is influenced by their formulation, nature of their use and quantity and quality of ingredients (Naveed et al., 2010). The main advantage of applying topical emulsions (creams) is that they increase the solubility and bioavailability of therapeutic drugs as well as the ability to favour the topical transport of hydrophilic solute. Topical emulsions also avoid gastrointestinal environment and first pass effect (Marti-Mestres et al., 2002). Conventional whitening agents, such as hydroquinone and kojic acid are very effective depigmenting agents however they have some safety concerns with long-term exposure. Natural extracts provide an idea to develop new products for hyperpigmentation (Ohguchi et al., 2010). Exposure to UV radiations result in skin damage through several mechanisms such as: collegenase production, thymine

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Abbreviations: TEWL, Transepidermal water loss; **LSD,** least significant difference; **ANOVA,** analysis of variance.

dimer formation and enhancing inflammatory reaction. Antioxidants protect human skin from free radicals produced by UV radiations in the untimely stages of revelation (Bauman, 2003). Morphological change in the skin like wrinkles is directly related to loss of collagen which has strong relation with transepidermal water loss. More the epidermal water loss, less the water is retained by the collagen and results in collagen degeneration (Aburjai and Natsheh 2003). An extract of *Calendula* can be obtained by macerating the peeled plant in hydro alcoholic mixture and then filtering and concentrating it on rotary evaporator. Extract obtained is rich in terpenoids, carotenoids, flavonoids and volatile oils that have appreciable cosmetic benefits for the skin. As a cosmetic, *Calendula* is excellent for antiseptic, healing and anti-irritant properties. *Calendula* extract is often used for skin problems, cuts and bruises etc. (Joanne et al., 2002).

MATERIALS AND METHODS

Identification of plant

The identification of *Calendula officinalis* (Family*: Asteracaea)* was performed by Prof. Dr. Arshad at Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur, Pakistan. The specimen was deposited in the pharmacognosy section of Faculty of Pharmacy and Alternative Medicine. The voucher number is: pharm.23/Feb 2007.

MATERIALS

Paraffin oil with dynamic viscosity of 110-230 mPas at 20°C and kinametic viscosity of 34.5 mm^2/s at 40°C, was obtained from Merck (Germany). Abil- EM 90 (Cetyl Dimethicone copolyol with HLB 5) was purchased from Franken Chemical (Germany), Lemon oil (Pakistan) while distilled water was an extract of *C. officinalis* (ethanolic) was prepared in laboratory of Pharmacy department, the Islamia University of Bahawalpur, Pakistan.

Apparatus

Centrifuge Machine (Hettich EBA 20, Germany), Cold Incubator (Sanyo MIR-153, Japan), conductivity-Meter (WTW COND-197i, Germany), Corneometer MPA 5, Mexameter MPA 5, sebumeter MPA 5, TEWA meter MPA 5 (Courage + Khazaka, Germany), digital humidity meter (TES Electronic Corp, Taiwan), electrical balance (Precisa BJ-210, Switzerland), homogenizer (Euro-Star, IKA D 230, Germany), hot incubator (Sanyo MIR-162, Japan), pH-Meter (WTW pH-197i, Germany), refrigerator (Dawlance, Pakistan), rotary evaporator (Eyela, Co. Ltd. Japan), HPLC 20A (Shimadzo Japan) and UV spectrophotometer-16 (Shimadzo Japan).

Determination of flavonoids

Method described by Zu Y et al. (2006) with slight modification was applied for the determination of flavonoids (quercetin, kaempferol and isorhamnetin) in *C. officinalis*. The sample was ultrasonified at high frequency. 75% ethanol was used as extraction solvent at a temperature of 30°C for 1 hour. Water -methanol – acetonitrile with 1% acetic acid was used as mobile phase. Diode array detection was carried out at 368 nm wavelength for the detection of flavonoids. Flavonoids were determined with successful recoveries of 88, 81 and 78% of quercetin, kaempferol and isorhamnetin respectively.

Antioxidant activity of *Calendula officinalis*

The free radical scavenging activity of *C. officinalis* was determined in accordance to Marsden S. Blois method using DPPH (1, 1-diphenil-2-picrylhydrazyl) which is a stable free radical (Marsden 1958). Equal volumes of diluted extract were mixed with an equal volume of DPPH 0.5 µl in absolute ethanol, and the obtained mixtures were kept at room temperature for 15 min. Then, the absorption of the mixtures at 517 nm was taken, in comparison with the control solution (maximum absorption). Quercetine was used as standard. The activity of free radicals was calculated in % inhibition according to the following relation:

$$\% \text{ Inhibition} = \frac{(\text{A control} - \text{A test})}{\text{A control}} x100$$

The free radical scavenging Activity of *Calendula officinalis* was 85% in comparison to the standard after.

Formulation development

In this study, W/O emulsions were prepared by the addition of aqueous phase to the oily phase with continuous agitation (Naveed et al., 2010). Oily phase consisted of paraffin oil (16%) and surfactant ABIL- EM 90 (3.5%) was heated up to 75 ± 5°C. At the same time, aqueous phase consisting of water (q.s) was heated to the same temperature and then the *C. officinalis* extract (3%) was added in it. After that, aqueous phase was added to the oil phase drop by drop. Stirring was continued at 2000 rpm by the mechanical mixer for about 15 min until complete aqueous phase was added. At 50°C, 2 to 3 drops of lemon oil were added when the temperature was to give good fragrance to the formulation. After the complete addition of the aqueous phase, the speed of the mixer was reduced to 1000 rpm for homogenization, for a period of 5 min, and then the speed of the mixer was further reduced to 500 rpm for 5 min for complete homogenization, until the emulsion cooled to room temperature.

Base was also prepared by the same above method and with same ingredients but without *C. officinalis* extracts.

Properties of formulation

Stability tests were performed at 8±0.1°C (in refrigerator), 25±0.1, 40±0.1 and 40±0.1°C (in incubator) with 75% relative humidity (RH). Physical characteristic (colour, creaming and liquefaction), electrical conductivity and pH of formulations were noted at various intervals for 28 days.

Product evaluation on skin

21 volunteers (male) were selected for study whose ages were in between 25 - 35 years. Patch test was performed to determine any possible reactions of creams, on forearms of each volunteer on first day of sampling. After 48 h, each volunteer was provided two

Table 1. Score given by volunteers to base and formulation on the basis of itching/irritation*.

Score		0	1	2	3
No. of volunteer	Base	16	4	1	0
	Formulation	14	3	4	0

*No severe erythema occurred in any of volunteer, mild erythema occurred in one and four volunteers, moderate erythema occurred in four and three volunteers, whereas no erythema (absence) occurred in sixteen and fourteen volunteers for base and formulation, respectively.

creams. One cream was the base and other was the active formulations.

Each volunteer applied cream for the period of 8 weeks. Every volunteer was instructed to come for measurement on 1, 2, 3, 4,5,6,7 and 8 week.

Study design

A single blinded study was designed for the comparisons of two creams that is, the active formulation containing *Calendula extracts* and base. Two formulations were named A (active formulation) and B (base formulation) and given to the volunteers with instructions of application. Results were measured in controlled room at 20 ± 1°C and 40±2% relative humidity (Naveed et al., 2010).

Ethical standards

This study was approved by the board of advance study and research (BASR), The Islamia University of Bahawalpur and institutional ethical committee in compliance with NIH Principles of Laboratory Animal Care 1985. The reference no. is COSM - 3986/07.

Burchard tests (patch tests)

Patch tests were performed on the forearms of each volunteer. The patch (Bandage disc) for the right forearm was saturated with 1.0 g of base while the patch for left forearm was saturated with 1.0 g of formulation. Each was applied to the 5 × 4 cm marked regions separately on each forearm. The regions were covered with the surgical dressing after application. The patches were removed after 48 h and the forearms were washed with physiological saline. After 48 h, scores were recorded for the presence of erythema (skin redness) using a scale with 4 points from 0 to 3, where 0 stands for absence of erythema, 1 for mild erythema, 2 for moderate erythema while 3 stands for severe erythema. Each volunteer was asked to note their irritation/itching towards the patches and then assign a score from the same scale. Average score with respect to volunteers is given in Table 1 (Barkat et al., 2010).

Panel test

Every individual was provided with a form prepared previously to test the sensory values of cream. This form consisted of seven parameters to be evaluated and every parameter was assigned 11 values from –5 to +5 indicating very bad to very good, respectively. This form was asked to be completed independently by each individual on day 28. Average points were calculated from the points assigned by each volunteer for each question for both of the creams, base and formulation.

It was concluded that there was no variation between base and formulation regarding the sensory evaluation. Both of the creams behaved similarly from the sensory point of view.

Mathematical analysis

The percentage changes for the individual values of different parameters, taken every week of volunteers were calculated by the following formula;

$$\text{Percentage Change} = [(A - B) / B]*100 \quad (1)$$

Where;
A = Individual value of any parameter of 1st, 2nd, 3rd, or 4th week
B = Zero hour value of that parameter

Statistical analysis

The measured values obtained for different parameters (skin moisture, sebum, melanin, erythema and pH) were analyzed using SPSS 12.0 on computer (paired samples t-test for variation between the two preparations; two-way ANOVA for variation between different time intervals). 5% level of significant was applied.

RESULTS AND DISCUSSION

Evaluation of creams

Stability of creams (base and formulation) was evaluated on different storage conditions that is, 8, 25, 40 and 40°C + 75% RH for 28 days. Centrifugation and accelerated temperature conditions are very important parameters for stability of creams. No phase separation was observed during the stability study of creams. As elevated temperature cause change in viscosity, partition and solubility of molecules between two phase. But it has found that lipophilic surfactant is more stable at elevated temperature (Rowe et al., 2003). No liquefaction is observed through out the study period of 28 days.

Table 2. pH Values of base and formulation kept at 8, 25, 40 and 40°C + 75% RH.

Time	8°C		25°C		40°C		40°C+75% RH	
	B	F	B	F	B	F	B	F
0 Hour	5.44	5.96	5.44	5.96	5.44	5.96	5.44	5.96
12 Hours	5.60	6.07	5.69	6.33	5.12	6.48	5.05	5.83
24 Hours	5.88	6.25	5.85	6.35	5.67	6.44	5.88	6.12
36 Hours	5.78	6.18	5.91	6.39	5.64	6.34	5.86	6.05
48 Hours	5.97	6.13	5.94	6.31	5.38	6.28	5.76	6.12
72 Hours	5.78	5.95	5.74	6.27	5.34	6.29	5.63	6.19
7 Days	5.99	6.21	5.94	6.38	5.70	6.06	5.25	6.28
14 Days	6.15	5.80	5.64	5.96	5.10	5.47	4.88	5.45
21 Days	5.76	6.21	5.96	5.88	4.58	5.83	5.13	5.52
28 Days	5.47	6.33	6.08	5.95	4.46	5.76	4.53	5.85

B = Base, F = formulation, RH = relative humidity.

pH test

pH values of base and formulation of fresh creams and samples kept at different storage conditions up to 28 days was determined and is reported in Table 2.

In this study, the pH of freshly prepared base and formulation was 5.44 and 5.96 respectively, which is within the range of skin pH. The pH values of the samples of base kept at 8°C increased up to 14th day and then started decreasing with some variations.

At 25°C, the values continued to increase with some variations. At 40 and 40°C+ 75% RH it was found to be increasing gradually in the 1st week and then it started to decline continuously till 28th day with some variations. At the end of study pH of the samples of base at 8, 25, 40 and 40°C+ 75% RH was 5.47, 6.08, 4.46 and 4.53 respectively.

Whereas pH of the samples of formulation kept at 25, 40 and 40°C + 75% RH showed an initial increase and then gradual reduction in pH values with slight variations with time. At 8°C the pH gradually increased. The pH values of samples of formulation kept at 8, 25, 40 and 40°C+ 75% RH were 6.33, 5.95, 5.76 and 5.85 at the 28th day respectively.

By using two-way analysis of variance (ANOVA) technique, it was found that the change in pH of different samples of base and formulation were significant at different levels of time and temperature. When least significant difference (LSD) test was applied to check the individual average effects of the pH of the samples of base at different temperatures with the passage of time by taking average pH values of zero hour at different temperatures as standard, it gave insignificant changes at 25°C and significant at 40 and 40°C + RH. Again when LSD test was applied to check the individual average effect of the pH of the samples of formulation at different temperatures with the passage of time by taking average pH values of zero hour at different temperatures as standard, it gave significant changes at temperature 40°C + RH 75%.

From LSD test it was concluded that there was insignificant change in pH of the samples of base at different storage conditions but significant changes were observed in pH of the samples of formulation at 24 and 36 h, 7day and 14day, while insignificant changes at other times.

The decrease in pH of the formulation at different storage conditions might be due to the production of any acidic metabolite or decomposition of any ingredient during the heating process, especially paraffin oil (Raymond et al., 2003).

Dermatological test

Melanin

The effect of the base and the formulation on the production of skin melanin was examined in this study. The amount of melanin was measured for 8 weeks at different time intervals in each individual after application of base and formulation. It was found that the formulation decreased the melanin contents throughout the study period. With the help of ANOVA test it was found that the base and formulation produced significant effects on skin melanin content in volunteers (Figure 1). LSD test showed that base produced significant effects at 7th and 8th week while formulation showed significant effects throughout the study. With the help of paired sample t-test it is evident that a significant difference was produced between the melanin effects of base and the formulation from 2nd week and it lasts up to the 8^{th} week of study period. It has been established by a number of studies that flavonoids especially quercetin is a potent tyrosinase inhibitor so the formulation reduced the skin

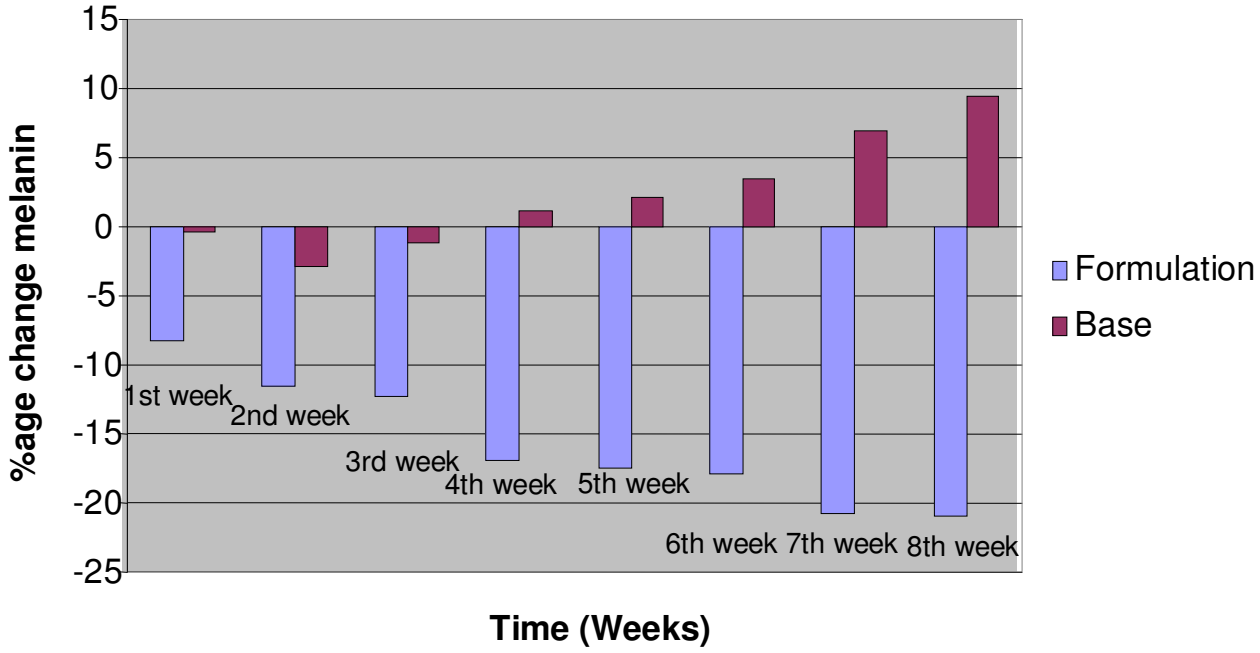

Figure 1. Percentage of change in skin melanin production after application of base and formulation for a period of 8 weeks.

melanin content. The main flavonoids in *C. officinalis* are isorhamnetein, quercetin, myricetin and kaempferol (Zu et al., 2006 and Yuzhen and Fuheng, 1997).

Erythema

In this study, irritation was constantly monitored every week for the base and the formulation throughout the period of application. It was found that erythema contents were slightly increased at 1st week after the application of base and then gradually decreased up to 8th week of study period. Whereas after the application of formulation erythema contents were continuously decreased from first to eighth week (Figure 2).

With the help of ANOVA test it was found that the base and formulation produced significant effects on skin erythema at different time intervals. By applying LSD test it was seen that base produced significant effects at 4th, 5th, 6th, 7th and 8th week. On the other hand formulation showed significant effects throughout the study period. With the help of paired sample t-test it was evident that there was significant variation in irritation with respect to base and formulation at 1st, 3rd and 4th week, it was concluded that the formulation decreases the erythema contents of skin at the end of study period and overall effect of formulation on skin erythema is significant, so it can be used safely without any skin irritation.

The active ingredient in calendula is a good source of Triterpenoids, flavonoids etc. and because of which it shows anti-inflammatory properties (Adela et al., 2003).

Skin moisture content

In this study, it was found that there was an increase in moisture values from 1st to 5th week after the application of the base and then gradually decreased up to the 8th week, however after the application of formulation the increase in skin moisture content is more pronounced from 1st to 5th week and then showed little change onwards from 6th to 8th week (Figure 3). With the help of ANOVA test it was found that the base and formulation showed a significant effect on the skin moisture content. By LSD test for formulation, it was found that significant change in moisture content was observed at 2nd, 3rd and 5th week after application of base and at 2nd, 3rd, 4th, 5th, 6th, 7th and 8th week after application of formulation. With the help of paired sample t-test it was evident that a significant difference in the moisture values was produced at the 2nd week when base was compared with formulation.

The moisturizing treatment involves repairing the skin barrier, retaining/increasing water content, reducing TEWL, restoring the lipid barrier's ability to attract, hold and redistribute water, and maintaining skin integrity and appearance. Calendula has been reported to stimulate physiological regeneration and epithelisation, which can

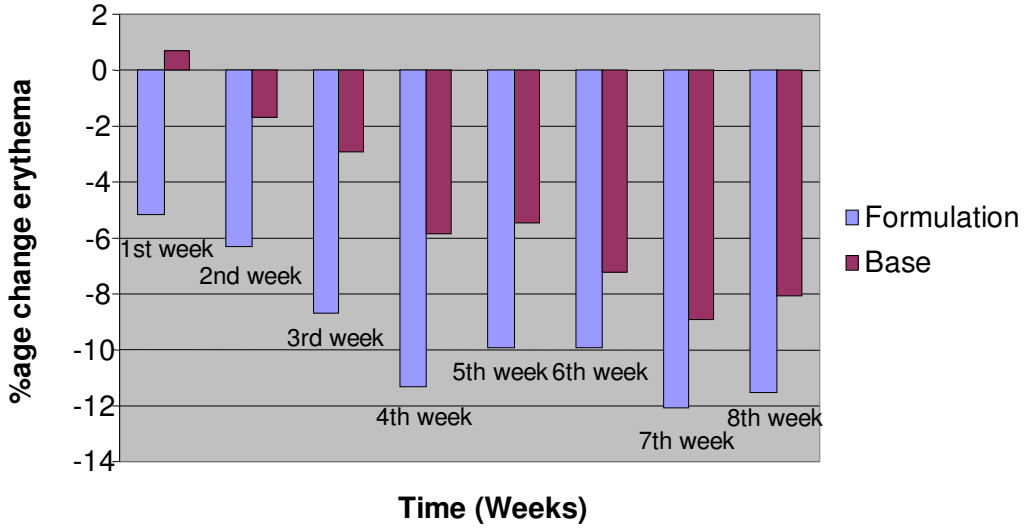

Figure 2. Percentage of change in skin erythema after application of base and formulation for a period of 8 weeks.

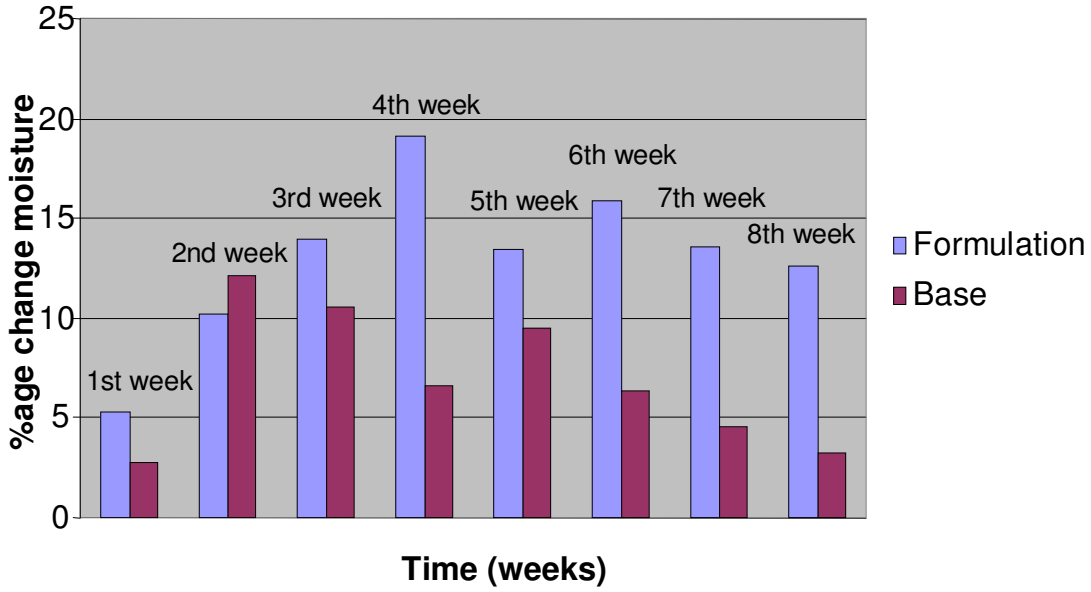

Figure 3. Percentage of change in skin hydration after application of base and formulation for a period of 8 weeks.

reduce TEWL (Joanne et al., 2003).

Skin sebum content

Sebaceous glands, located in each hair follicle, produce an oily substance to lubricate and protect the skin called sebum. Sebum production is measured using a special opalescent plastic film, which becomes transparent when it is in contact with sebum lipids. The device relies on a probe, which presses a piece of special film on the skin for a measured length of time. The sebum is adsorbed on this film like ink on the blotting paper and the film becomes transparent. The probe is then placed into the device which radiates a light beam onto the film. A metal mirror behind the film reflects the beam back again

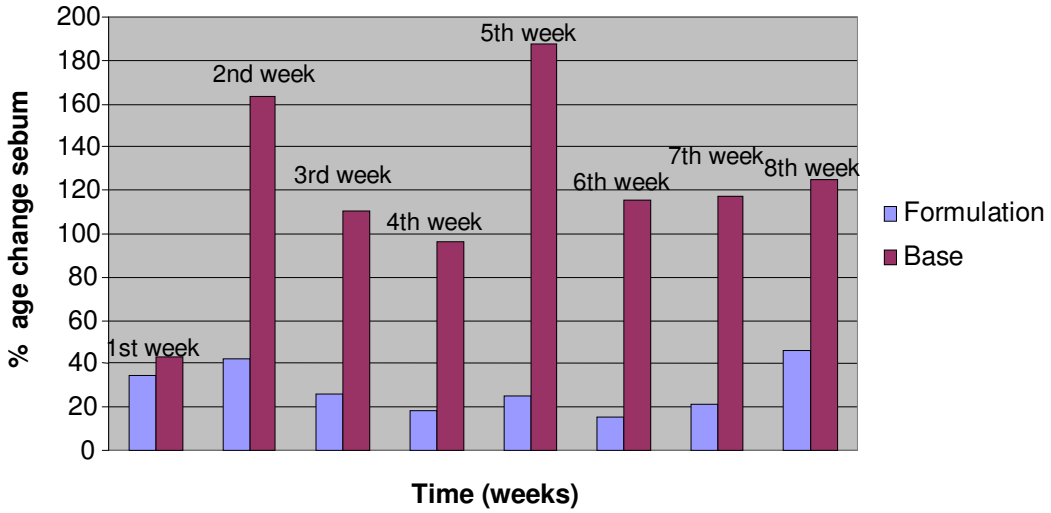

Figure 4. Percentage of change in skin sebum after application of base and formulation for a period of 8 weeks.

through the film and then into an instrument called a photomultiplier, which measures the amount of light in the beam. The more sebum on the skin, the more transparent is the film and greater is the amount of light reflected.

In this study, the effects of the base and formulation on the sebum contents of human cheeks were investigated. Sebum was measured every week in all the individuals. It was found that the base and formulation both increased sebum contents from 1st to 8th week of study period (Figure 4). With the help of ANOVA test it was evident that there was a significant effect of base and formulation on skin sebum throughout the study at volunteer level but insignificant at time level. By applying LSD test it was evident that significant changes in sebum contents were observed at 2nd, 5th and 8th week after application of base and 8th week with formulation. With paired sample t-test it was found that the base and formulation showed insignificant variations regarding the skin sebum content.

It was concluded that increase in sebum contents after the application of base and formulation may be attributed to the oily nature of W/O emulsion having a thick viscous oily liquid that is, the paraffin oil (Rowe et al., 2003).

Transepidermal water loss (TEWL)

It was found that there was an increase in TEWL values after the application of base having the greatest value after 1st week then gradual reduction. After the formulation there was an increase in TEWL after 1st, 2nd and 3rd week but a decrease in the remaining period of study, as shown in (Figure 5). With the help of ANOVA test, it was found that changes in TEWL produced by both formulation and base were significant.

By applying LSD test, it was found that in both the cases, that is, the base and formulation; the changes in TEWL values became significant after 5th and 6th week of application. With the help of paired sample *t*-test it was found that there was significant variation in TEWL with respect to the base and formulation in 2nd week of study, while insignificant for other periods.

It was concluded that both the base and the formulation prevent the transepidermal water loss, is due to a number of factors, such as both creams containing glycerin, a humectants, which creates a “reservoir” of moisture retaining ability in the skin. It moisturizes the full thickness of stratum corneum (Diepgen 2005). Paraffin oil which has been used in both, the base and the formulation, forms an occlusive covering on the skin thus preventing TEWL. So due to moisture retaining properties; the formulation and the base enhanced the stratum corneum ability to attract, hold and redistribute water thus reducing the TEWL.

Conclusion

From our preliminary study we concluded that; A stable topical cream (W/O emulsion) containing *Calendula* extract can produce a decrease in melanin content of the skin showing that the formulation has skin whitening effects. The formulation was observed to decrease skin erythema significantly which shows that the formulation has anti-inflammatory affects. The cream has skin moisturizing effects as it produced an increase in skin moisture content. The formulation was observed to decrease TEWL significantly which shows that the

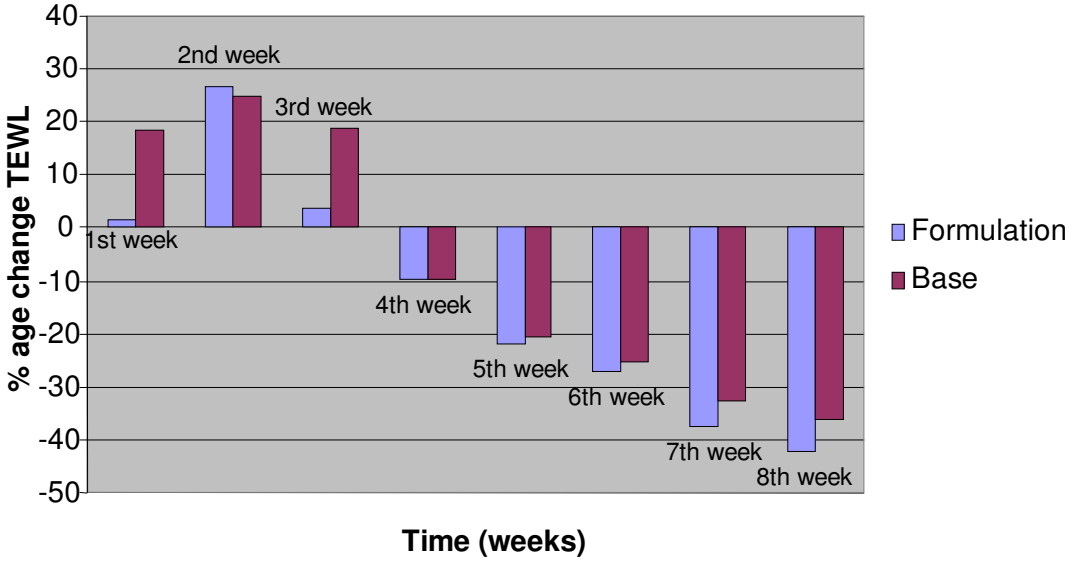

Figure 5. Percentage of change in skin TEWL after application of base and formulation for a period of 8 weeks.

formulation has anti-wrinkle affects. The *Calendula* extract cream is not suitable for people with oily skin as it causes increase in skin sebum content.

A targeted study needs to be conducted in future in patients with freckles/melasma, psoriasis as well in people with dry wrinkled skin so that the actual potential of this very plant against these disorders can be explored scientifically.

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