

Full Length Research Paper

Anti-aging potential of a cream containing milk thistle extract: Formulation and *in vivo* evaluation

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This study was designed to formulate and evaluate anti-aging effects of a topical cream (water in oil (w/o) emulsion) containing extract of milk thistle (*Silybum marianum*) against its vehicle (Base) as control. Base containing no extract and a formulation containing 4% concentrated extract of *Silybum marianum* was developed by entrapping in the inner aqueous phase of w/o emulsion. Both the base and formulation were stored at $8^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ (in refrigerator), $25^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ with 75% RH (in incubator) for a period of 8 weeks to predict their stability. The evaluation parameters consisted of colour, smell, type of emulsion, electrical conductivity, liquefaction and pH. The expected organoleptic stability of creams was achieved from 8 weeks *in-vitro* study period. The formulation and base were evaluated for effects on skin moisture and transepidermal water loss (TEWL). The base showed insignificant ($p \geq 0.05$) while formulation showed significant effects on skin moisture and TEWL. The surface evaluation of living skin (SELS) parameters SE_r , SE_{sc} , SE_{sm} and SE_w were also evaluated which showed significant decline proving that the formulation possesses potential antiaging effects.

Key words: *Silybum marianum*, W/O emulsion, emulsion stability, transepidermal water loss (TEWL), surface evaluation of living skin (SELS).

INTRODUCTION

Formulating emulsions is a common practice in food and pharmaceutical industries. Emulsion containing plant extracts are more promising in the field of research due to their therapeutic importance and reliability of people on natural sources. Both oil-in-water and water-in-oil emulsions are widely used for their medicinal properties and as vehicles of various drugs to skin (Khan et al., 2010). Water-in-oil emulsions are widely used for dry skin treatment and as emollient (Magdy, 2004). Abil[®] EM 90 is a clear, colorless liquid, available in various viscosities, functions as an emulsifying agent, antifoaming agent and emollient. It is a non-ionic surfactant and makes possible

a dispersion of aqueous droplets within an oil phase (Raymond et al., 2003).

Milk thistle (*Silybum marianum* L.) from Asteraceae plant family, is a well known medicinal plant, native to the Mediterranean region of Europe but also widely dispersed to many countries throughout the world. Its medicinal effects are documented among the alternative medicines referred to as liver and bile-related diseases remedy (Fraschini et al., 2002; Kurkin, 2003). The dried seeds contain silymarin flavonoids. Silymarin is a mixture of three flavonolignans, including silybin (silibinin), silidianin, and silichrystin which act as potent antioxidants (Kshirsagar et al., 2009).

Skin aging is a complex process involving several environmental factors, most important of which is UV light from sun. Along with other factors about 80% of the facial wrinkling is considered due to the UV light. UV generates reactive oxygen species, and consequently triggers several mechanisms leading to collagen deficiency and eventually skin wrinkling (Fischer et al., 1997). The plant 'Milk

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Abbreviations: TWEL, Transepidermal water loss; SELS, surface evaluation of living skin.

thistle' is well known for its antioxidative activity on liver associated diseases but to assess possible anti-aging effects on skin, we formulated a w/o emulsion containing extract of Milk thistle and studied its stability over a study period of 8 weeks.

MATERIALS AND METHODS

Plant material

Milk thistle seeds were donated by World Homeo and Herbal Pharma[®], Islamabad, Pakistan. The identification of the seeds was performed by Prof. Dr. Muhammad Arshad at Cholistan Institute of Desert studies (CIDS), The Islamia University of Bahawalpur and a voucher specimen was preserved (voucher # MT-SD-4-11-19) at the herbarium for future reference.

Preparation of plant extract

200 g of finely ground seeds of Milk thistle were extracted at room temperature with 1000 ml of 95% ethanol for 48 h. Glass beaker was sealed with aluminium foil and kept in the laboratory. The beaker was shaken for 10 min after every 12 h.

Finally, the macerated material of plant was filtered through several layers of muslin cloth for coarse filtration. Approximately 800 ml coarse filtrates were then filtered through a Whatman No. 1 filter paper. The filtrates so obtained were evaporated under reduced pressure at 40°C in a Rotary evaporator. The process of evaporation was continued till concentrate reduced to one third of the initial volume of the solvent used. The extract so obtained was stored in freezer at 8°C.

Antioxidant activity

The free radical scavenging activity of Milk thistle was determined according to Marsden S. Blois method using DPPH (1, 1-diphenyl-2-picrylhydrazyl) which is a stable free radical (Khan et al., 2010). Equal volumes of diluted extract were mixed with an equal volume of DPPH 0.5 µl in analytical grade ethanol, and the obtained mixtures were kept at room temperature for 30 min. Then, the absorption of the mixtures at 517 nm was taken, in comparison with the control solution (maximum absorption). Vitamin C was used as standard. The activity of free radicals was calculated in percentage inhibition according to the following relation:

$$\% \text{ Inhibition} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100$$

The free radical scavenging activity of Milk thistle was 89% in comparison to the standard.

Preparation of formulations

In this study, W/O emulsion were prepared by adding up of aqueous phase to the oily phase with continuous agitation. Oil phase comprised of paraffin oil and surfactant (ABI[®] EM 90) heated up to 75±1°C. Aqueous phase comprising of water heated to the same temperature and then extract was added in it. In case of base, no extract was added in the aqueous phase. After that

aqueous phase was added to the oily phase drop by drop with constant stirring at 2000 rpm by the mechanical mixer for 15 min until complete aqueous phase was added. After complete addition of aqueous phase, the mixer speed was reduced to 1000 rpm for homogenization, for 5 min, and then the mixer speed further reduced to 500 rpm for a period of 5 min for complete homogenization until the emulsion cooled to room temperature. The formula found to be most stable among different formulations tested was selected for further stability testing and is given here:

Formula of base:

Paraffin oil: 14%
Abil[®] EM 90: 2%
Distilled water: q.s 100%.

Formula of active formulation:

Paraffin oil: 14%
Abil[®] EM 90: 2%
Plant extract: 4%
Distilled water: q.s 100%.

Properties of formulations

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

Product evaluation on skin

The study was conducted recruiting 11 male un-diseased volunteers with mean age of 46 years after getting consent forms. 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 with two creams. One cream was the base and other was the active formulations.

Various skin parameters were evaluated using non invasive instruments. Skin micro-relief parameters were evaluated using Visio Scan[®] VC98 which consists of special UV light video camera with high resolution and the surface evaluation of the living skin (SELS) method developed using software SELS 2000 especially to study directly the skin surface (Gaspar et al., 2008). The skin moisture was determined with a skin capacitance meter (Corneometer[®] MPA 5) and transepidermal water loss (TEWL) was determined by an evaporimeter (Tewameter[®] MPA 5). Each volunteer applied cream on cheeks for the period of 12 weeks and were instructed to come for measurement on 2nd, 4th, 6th, 8th, 10th and 12th week.

Study design

A single blinded study was designed for the comparison of two creams that is, the active formulation containing Milk thistle extract and base. The 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 25±1°C and 40±2% relative humidity.

Ethical standards

This study was approved by the Board of advance studies and

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

Parameter		*0	*1	*2	*3
No of volunteer	Base	7	3	1	0
	Formulation	8	1	2	0

* No severe erythema occurred in any of volunteer, mild erythema occurred in one and two volunteers, moderate erythema occurred in three and one volunteers, whereas no erythema occurred at all in seven and eight volunteers for both base and formulation, respectively. * Score

Research (BASR), The Islamia University of Bahawalpur and Institutional Ethical Committee (Reference No is 3715/Acad) in compliance with Helsinki declaration.

Burchard (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 the 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

Mathematical analysis

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

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

Where, A is the Individual value of any parameter of 2nd, 4th, 6th, 8th, 10th or 12th week; B is the Zero hour value of that parameter.

Statistical analysis

The measured values obtained for different parameters (skin moisture, TEWL and SELS) 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 significance was applied.

RESULTS AND DISCUSSION

Organoleptic tests (color, liquefaction and phase separation)

In this study, base and formulation were divided into four samples separately and these samples were kept at 8°C in refrigerator at 25, 40 and at 40°C + 75% RH (relative humidity) in stability chambers. They were observed

organoleptically with respect to change in color, liquefaction and phase separation for a period of 56 days at definite time intervals. The freshly prepared base and formulation were creamy white in color. There was no change in color of any sample of base and formulation at different storage condition; that is, 8, 25, 40 and at 40°C+ 75% relative humidity up to the observation period of 56 days. This shows that both base and formulation were stable at different storage conditions up to 56 days. No change in color of base and formulation can be due to the components of oil phase which are clear, colorless, transparent and non toxic liquids, that is, paraffin oil and Abil®-EM90 (Raymond et al., 2003).

The samples of base and formulation were observed for phase separation and were found stable at 8 and 25°C but slight phase separation in the sample of base occurred at 40 and 40°C+ 75% RH on the 56th day. There was no liquefaction observed in any of the sample of base and formulation kept at 8 and 25°C during whole observation period of 56 days. A slight liquefaction was observed in the sample of base and formulation kept at 40 and 40°C + 75% RH on 48th and 56th day of observation. It can be considered due to creaming which is a constant phenomenon that occurs in emulsions. As creaming increases, the viscosity of the base and formulation steadily decreases with increasing temperature resulting in liquefaction because the rate of creaming is inversely proportional to the viscosity of the dispersion medium according to the Stokes' law. This liquefaction at higher temperature is responsible for slight phase separation as the time progresses (James and James, 2009).

Electrical conductivity test

In this study, conductivity test was performed for all the samples of base and formulation kept at different storage conditions up to a period of 56 days at definite time intervals. No electrical conductivity was seen in any of the samples of base and formulation kept at different storage conditions, that is, 8, 25, 40 and 40°C+ 75%RH up to the 56th day of observation. The reason behind it can be attributed to w/o type of emulsion in which oil being the continuous phase contributes to no passage of current indicating that creams were stable at different storage conditions.

Table 2. Average pH values of base and formulation kept at 8, 25, 40 and 40°C + 75% RH for a period of 8 weeks.

Cream	*Storage condition			
	8°C	25°C	40°C	40°C+ 75%RH
Base	5.48 ±0.076	5.61 ±0.066	5.47 ±0.068	5.62 ± 0.063
Formulation	5.62 ±0.071	5.61 ±0.077	5.54 ±0.072	5.67 ± 0.066

*Values of pH (mean ± SD).

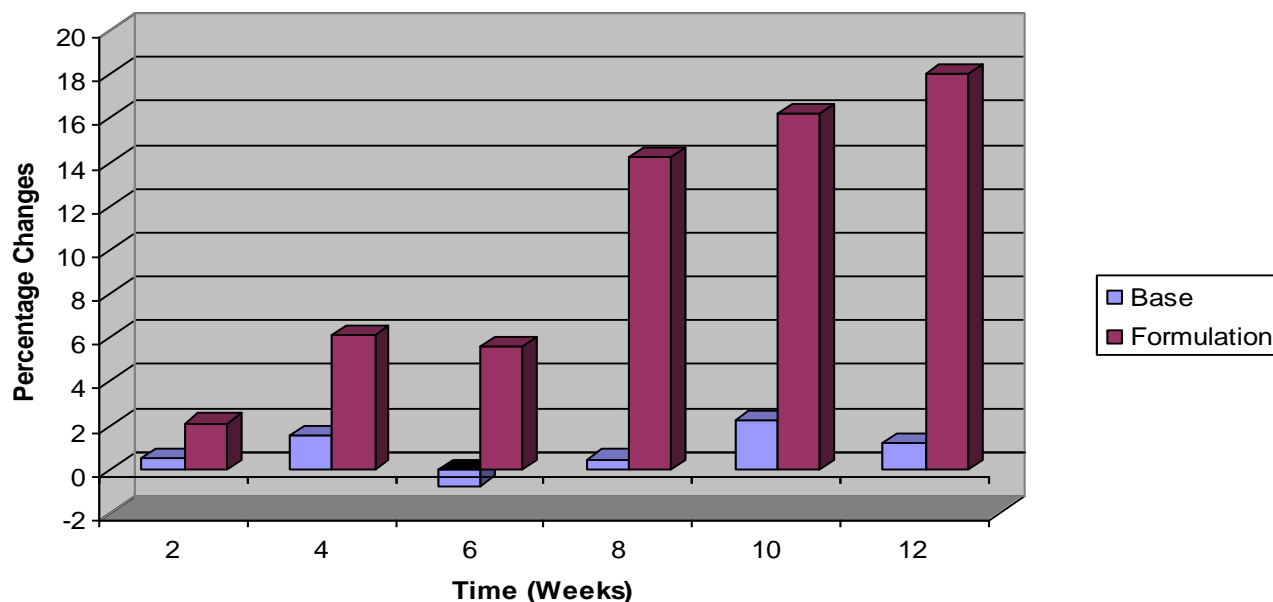


Figure 1. Percentage of change in skin moisture content after application of base and formulation.

pH tests

The pH of freshly prepared base and formulation was 5.62 and 5.94 respectively. Average changes in pH values of both base and formulation occurred from the time of preparation up to 8th week of study period kept at various storage conditions; and have been determined and shown in Table 2. The pH values were noted immediately after preparation and then after 24 h, 1st, 2nd, 3rd, 4th, 5th, 6th, 7th and 8th week.

The pH of human skin ranges from 4.5 to 6.0 (Jennifer, 2006) and 5.5 is considered to be average pH of the skin. Therefore, the formulations intended for application to skin should have pH closer to this range. In this study, the pH of freshly prepared base and formulation was 5.62 and 5.94 respectively which is within the range of skin pH. The average pH values of the samples of base and formulation kept at different storage conditions, that is, 8, 25, 40 and 40°C+ 75% RH was found to be increasing gradually with increasing temperature.

By using two-way analysis of variance (ANOVA) technique at 5% level of significance, it was found that the change in pH of different samples of base was significant

at different levels of time and temperature and there was also significant difference in change of pH of different samples of formulation at different levels of time and temperature. pH has an imperative character in the stability of pharmaceuticals and in case of products intended for skin application, these must be compatible with the skin. As the pH determined at various intervals was within the skin pH range, so the formulations can be used safely on human skin.

Skin moisture

Skin moisture content was measured before application of creams (0 h readings) and then at 2nd, 4th, 6th, 8th, 10th and 12th week of study period by Corneometer® MPA 5. The percent changes occurred in the values for 11 volunteers were calculated by using the Equation 1, and are represented in Figure 1.

In this study, base improved the moisture content of the skin to some extent but there was regular increase in the skin moisture contents after the application of formulation throughout the study period. With the help of ANOVA two

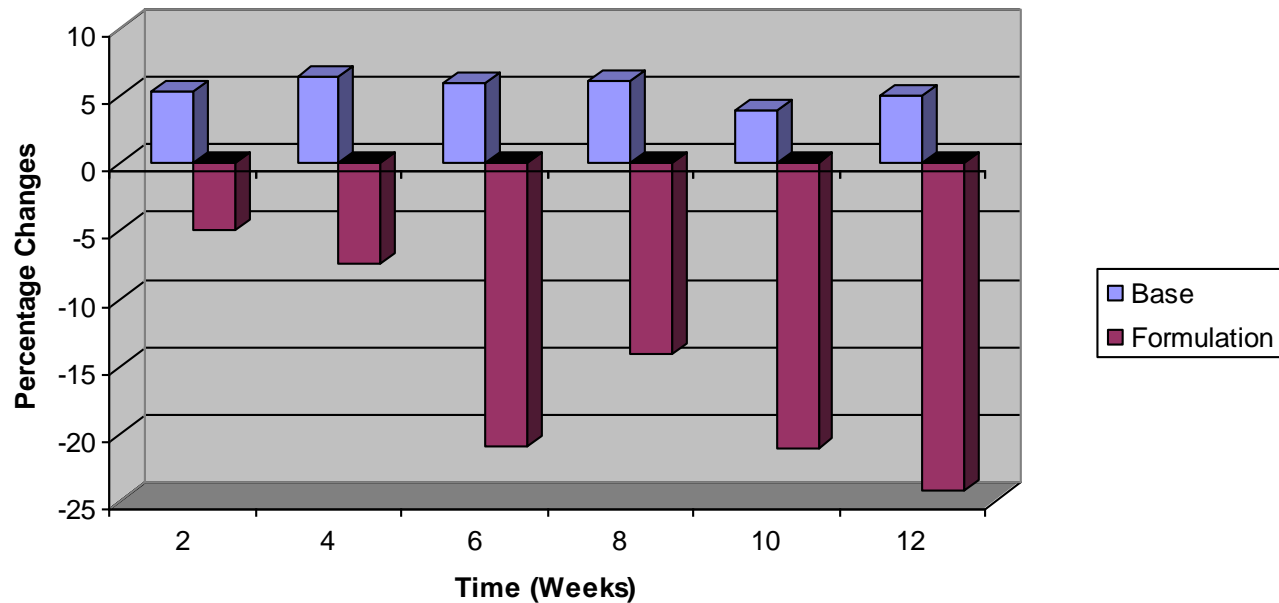


Figure 2. Percentage of change in TEWL content after application of base and formulation.

way analysis, it was found that the base produced insignificant effects and the formulation produced significant ($p \leq 0.05$) effects on moisture contents with respect to time. With the help of paired sample t-test it was evident that significant differences in the moisture values were observed except 1st and 4th week after application of base and formulation throughout the study period. The improvement in the skin moisture content after the application of formulation can be attributed to flavonoids of Milk thistle as flavonoids increase the moisture content of skin due to swelling of corneocytes on surface of skin (Froscler et al., 2004).

Transepidermal water loss (TEWL)

Transepidermal water loss (TEWL) was measured before application of creams (0 h readings) and then at 2nd, 4th, 6th, 8th, 10th and 12th week of study period by Tewameter® MPA 5. The percent changes that occurred in the values for 11 volunteers were calculated by using the Equation 1, and are given in Figure 2.

In this study, base showed irregular pattern in the values of TEWL of skin but there was regular decrease in the skin transepidermal water loss after the application of formulation throughout the study period. Transepidermal water loss (TEWL) is the outward transmission of water through skin. An increase in TEWL reveals an impairment of the water barrier. With the help of ANOVA test, it was found that changes in TEWL produced by base and formulation were insignificant with respect to time. With the help of paired sample t-test, it was found that there was significant variation in TEWL with respect to base and formulation. The underlying mechanism of TEWL decrease

is not known but it is proved scientifically that flavonoids mediated increase in cutaneous blood flow may contribute towards an improved skin appearance (Khan et al., 2010).

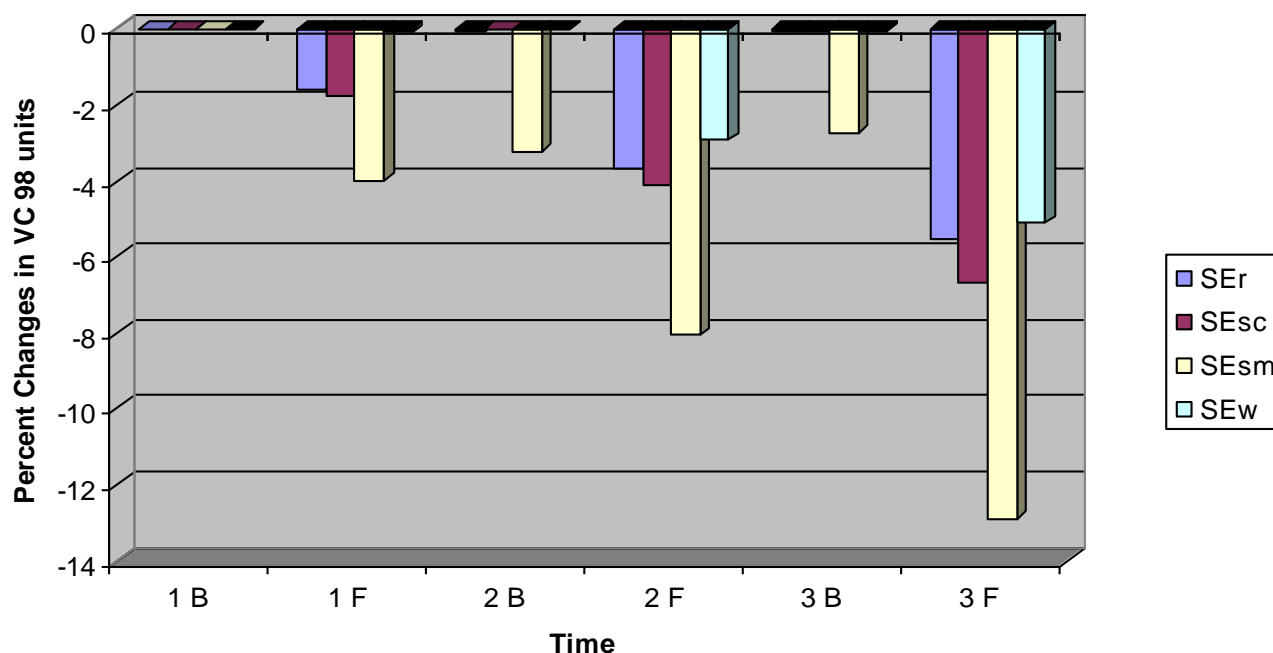
Surface evaluation of living skin (SELS)

Visioscan® VC 98 take images that illustrate the structure of the skin and the level of dryness and the grey level distribution of the image is used to evaluate the following SELS (surface evaluation of the living skin) parameters: Skin roughness (SEr), skin smoothness ($SEsm$), skin scaliness ($SEsc$), skin wrinkles (SEw). The parameters were measured before application of creams (0 h readings) and then at 1st, 2nd and 3rd month of study period by software SELS 2000 (Courage and Khazaka GmbH). The mean values of different SELS parameters are given in Table 3 and the percent changes that occurred in the values for 11 volunteers and calculated by using the Equation 1 are represented in Figure 3.

SEr is the roughness parameter which depicts the asperity of the skin and calculates the gray levels above the threshold in contrast with the whole image. It calculates the fraction of dark pixels (Ferreira et al., 2010). In this study, it was found that the base produced statistically insignificant ($p \geq 0.05$) effects on the roughness parameter of skin and the formulation produced significant effects when ANOVA two way analysis was performed. Gradual decrease in the values of roughness was observed for the formulation. When paired sample t-test was applied, significant effects were obtained. Lesser values indicate that the skin is less rough (Khazaka, 2000). $SEsc$ is the indicator representing scaliness of skin

Table 3. SELS parameter values (mean \pm SD).

Parameter	Cream	0 h	4th week	8th week	12th week
<i>SEr</i>	Base	2.66 \pm 0.086	2.66 \pm 0.086	2.65 \pm 0.084	2.66 \pm 0.084
	Formulation	2.66 \pm 0.086	2.62 \pm 0.084	2.56 \pm 0.082	2.51 \pm 0.081
<i>SEsc</i>	Base	1.73 \pm 0.053	1.73 \pm 0.054	1.73 \pm 0.053	1.73 \pm 0.053
	Formulation	1.68 \pm 0.031	1.65 \pm 0.030	1.61 \pm 0.027	1.57 \pm 0.024
<i>SEsm</i>	Base	82.74 \pm 5.22	82.74 \pm 5.22	80.11 \pm 5.10	80.52 \pm 5.17
	Formulation	83.24 \pm 3.95	79.87 \pm 3.75	76.59 \pm 3.79	72.65 \pm 3.82
<i>SEw</i>	Base	42.08 \pm 2.20	42.08 \pm 2.23	42.08 \pm 2.22	42.06 \pm 2.20
	Formulation	41.81 \pm 1.99	41.77 \pm 1.98	40.65 \pm 2.05	39.73 \pm 2.01

**Figure 3.** Percentage of change in mean VC 98 units of SELS parameters after application of base and formulation. 1B= Base values after one month, 1F= Formulation values after one month, 2B= Base values after two months, 2F= Formulation values after two months, 3B= Base values after three months, 3F= Formulation values after three months.

which shows the intensity of dryness of the stratum corneum, that is, state of dehydration of the skin. It is the number of pixels where the gray level is higher than the threshold of *SEsc* (Hiroshi et al., 2008). The base produced statistically insignificant ($p \geq 0.05$) effects on the skin scaliness while the formulation produced significant effects when ANOVA two way analysis was performed. Gradual decrease in the values of scaliness was observed for the formulation. When paired sample t-test was applied, significant effects were obtained regarding formulation. The smaller *SEsc* value corresponds to higher skin moisture. The formulation increased moisture content which is also supported by the values obtained by

Corneometer[®] MPA 5. *SEsm* is the index of smoothness and is calculated from mean width and depth of wrinkles. In this study, it was found that base produced statistically insignificant ($p \geq 0.05$) effects on the skin smoothness while the formulation produced significant effects when ANOVA two way analysis was performed. A decrease in the values of the parameter *SEsm* was observed for the formulation. When paired sample t-test was applied, significant effects were obtained regarding formulation. By treatment with moisturizing or anti-aging formulations the values for *SEsm* go down. The formulation showed decrease in mean values of skin smoothness which indicates that the formulation possess anti aging

properties (Khazaka, 2000).

SEw identifies aging including wrinkles and is calculated from the proportion of horizontal and vertical wrinkles. The base produced statistically insignificant ($p \geq 0.05$) effects on the skin wrinkles while the formulation produced significant effects when ANOVA two way analysis was performed. A decrease in the values of the parameter SEw was observed for the formulation. The formulation showed significant effects when paired sample t-test was applied. The formulation showed decrease in mean values of skin wrinkles which indicates that the formulation reduces the fine wrinkles and improves the appearance of skin as higher values for the parameter SEw indicates that there are more wrinkles present on the skin (Hiroshi et al., 2008).

Antioxidants protect human skin from free radicals produced by UV radiations, while flavonoids are capable of increasing collagen and have photoprotective properties against UV radiation. The changes in the morphological characters of skin like wrinkles is directly associated to loss of collagen which has strong relation with trans-epidermal water loss. Increasing epidermal water loss leads to fewer water retained by the collagen and results in collagen degeneration (Aburjai et al., 2003). The decrease in TEWL as measured by Tewameter[®] MPA 5 support the development of collagen which ultimately leads to less wrinkles. The improvement in skin surface SELS parameters can be attributed to the polyphenolic flavanoids present in Milk thistle which include silymarin comprising of silibinin, silydianine, silychristine and other flavonolignans such as silandrin, silybinome, silyhermin, silybonol, dehydrosilybin, deoxysilycistin, deoxysilydianin, and neosilyhermin. In addition, milk thistle contains apigenin, taxifolin, myristic, oleic, palmitin, and stearin acids. All these compounds are potent antioxidants contained in Milk thistle and are responsible for anti-aging effects of the formulations (Tumova et al., 2006).

Conclusion

This study reveals that a stable topical cream (W/O emulsion) containing Milk thistle (*S. marianum*) extract can be produced which can be cost effective with no harmful effects. The formulation was observed to have skin moist-turizing effects as it causes an increase in skin moisture content. The decrease in SELS parameters and TEWL shows that the formulation exerts anti-aging affects.

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