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Silicone based water-in-oil emulsion fortified with anthocyanin: *In-vitro, in-vivo* study

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Abstract: Skin care formulations with antioxidants are being widely explored for their benefits to human skin. The purpose of this study was to formulate a stable w/o emulsion containing anthocyanin derived from *Malus dosmestica* fruit extract and to further explore its beneficial effects on normal human skin. Anthocyanin was extracted using various solvents from the peel of *Malus dosmestica* fruit. w/o creams containing anthocyanin has been prepared and systematically characterized for various physiochemical properties in terms of stability at varying conditions of storage. An efficacy study has been carried out on 12 male healthy Asian subjects to determine effects of anthocyanin on skin melanin, erythema, skin moisture, trans-epidermal water loss (TEWL) and on skin sebum. Solvent system containing methanol/acetone/water (3.5: 3.5: 3 v/v/v) including 1% formic acid established a best recovery of anthocyanin from fruit peel. W/O emulsions presented promising stability profile when kept at different storage conditions over 90 days period. All skin parameters studied, anthocyanin has been found more efficacious ($p<0.05$) for its effects on skin melanin and erythema content of skin. It has been shown that a topical application of anthocyanin derived from *Malus domestica* has substantial potential for human skin system and needs some patient oriented studies could warrant its potential for damaged skin.

Keywords: *Malus domestic*, skin melanin, skin moisture, skin sebum, anthocyanin.

INTRODUCTİON

Creams are necessarily emulsions and have good patient acceptability. The stable creams formulated from W/O (water-in-oil) emulsion system with a high aqueous phase contented has extended period for use in cosmeceutics and pharmaceutics. They are have applications such as night creams, barrier creams and lotions, moisturizing lotions and creams, anti-aging creams, skin cleansers and make-up removers. Such system provides high water content to the skin so they are useful for applications in cosmetics. Water-in-oil emulsions are applied as barrier preparations or pore-occluding products for the provision of a thin oily layer over the skin (France *et al*., 1980).
Chemically anthocyanins are acylatedor glycosylated or antho-cyanidins. Glucose, Galactose, Rhamnose, Arabinose and Xylosemostly exist as 3-biosides,3-monosides and 3-triosides. Anthocyanins can be acylated with some ali-phatic di-carboxylic acids likemalonic, malic, oxalic and succinic acids. Anthocyanins have high water solublility (Strack and Wray, 1994). Anthocyanins have high pH dependent colors. Anthocyanins show instability during chemical and physical processing. Cyanidin galactosides are the most common anthocyanins present in apple. They are not substrates for poly-phenoloxidase (Clifford, 2000).

Several studies show that, anti-aging effects of anthocyanins are due to their control on the activities of inflammation-causing cells. A study shows that anthocyanins reduced inflammation pre-cursor cell activation by 27.0%. 120 male and female taking 300 mg of an anthocyanin extract daily for 3 weeks showed a reduction of 25-60% in inflammation.

Inflammation is the key problem ingeriatics. Their immune system is weak which makes them all the more susceptible to infections and eliciting of inflammations in the body. Inflammation is the preliminary point for a number of diseases like osteoporosis, cardiac disease, diabetes, alzheimer. Thus, the anti-aging benefits of anthocyanins hold a lot of hopes for the aging people. Anthocyanidins and their derivatives are present in many common foods, protect against oxidants by various mechanisms. For example, anthocyanins present in red cabbage protect animals against oxidative stress from the toxin.Cyanidins, present in common fruits have been found to "function as a strong anti-oxidant *in-vivo*" in current Japanese animal studies (Tsuda, 2000). In recent years, there is increasing effort to develop real natural antioxidants in order to retard lipid oxidation. The demand for natural antioxidants has been increased due to consumer concerns with the safety than using of synthetic antioxidants (Hudson, 1990).

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Anthoycyanins also help to stabilize the collagen matrix by cross linkage. It has been studied that anthocyanins provide protection against cell damage and mutagenesis triggered by ultraviolet light light (Fuleki and Francis, 1968).

Silicon is considered for optimal synthesis of collagen of the human skin as well as for activating the hydroxylation enzymes thus improving the skin strength and skin elasticity. It has been shown that physiological concentrations of silicon stimulates fibroblasts to emit collagen type-I **(**Lidiane *et al.,* 2016).

MATERIALS AND METHODS

Identification of Plant Material

Malus domestica (kalakolo) fruit was purchased from local market of Bahawalpur, Pakistan. Fruit identification was done by Cholistan Institute of Desert studies, The Islamia University of Bahawalpur, Pakistan.

Extraction and antioxidant activity of extract

Anthocyanins were extracted from crushed fresh peel and pulp tissues (20 g) of *Malus dometica* fruit, according to the method of (Giustiand Wrostad, 1996). A stable free radical, DPPH was used for the purpose of free radical scavenging activity determination of extract (Barkat et al. 2011). In 5µL of each ethanolic plant extract, mixed DPPH to make the volume up-to 100µL in 96 well plates.The contents incubated at 37°C for 30min. The optical density (OD) was measured at 517nm. Quercetin was used as standard because of its strong antioxidant property to evaluate the antioxidant activity of extract. Experiment was performed in triplicates. Result was taken as mean and standard error of mean of three independent experiments.

% DPPH scavenging activity = (100-OD of test sample/ OD of controlled x100)

Determination of total anthocyanin

A slight liquate of the extract was diluted with the extracting solvent to yield an optical density within the optimum range of the instrument. The diluted extract was stored in the dark for 4 hours and absorbance was measured at λmax 520 nm.

The total anthocyanin content was calculated using the equation described by (Du and Francis, 1973) as follows:

$$Total\ anthocyanin\ content\ (mg/100g) = \frac{OD\ X\ DV\ X\ TEV\ X\ 100}{SV\ X\ SW\ X\ 51.56}$$

Whereas
OD = optical density
DV = diluted volume for the OD measurement
TEV = total extract volume
SV = sample volume
SW = sample weigh in grams
51.56 = E. value for which the major constituent (Cyanidin).

Identification of anthocyanins pigments by High-Performance Liquid Chromatography (HPLC)

The purified anthocyanin of fresh peel and pulp tissues of *Malus dometica* was identified by HPLC according to the method reported by Andersen (1989). Two solvents were used for elution: (1) formic acid, water (1:9) and (2) formic acid, water, methanol (1:4:5). The flow rate was 1.5ml/min. The elutes were monitored by visible spectrometry at maximum wavelength 520nm

Preparation of formulation

Creams were prepared by homogenization and ingredients used for the preparation of emulsions (Active & Base) with varying concentration of oil and surfactant as mentioned in table 1.

Characterization of creams

Organoleptic control involving odor, color, liquefaction and phase separation were determined for the period of 90 days. pH, viscosity and centrifugation of creams were observed to get the stable formulation.

In vivo studies

A single blinded study was designed for the comparisons of two creams (Akhtar, 2010b). Twelve male volunteers whose ages were in between 25 and 35 years having no skin or other diseases were selected for the study and consent was taken. The volunteers were not informed about the contents of the creams. One cream was the base and other was the active formulation. Each volunteer applied the cream for the period of 12 weeks on cheeks. Every individual was instructed to come on week 1, 2, 3, 4, 6, 8, 10 and 12 for the skin measurements. All the skin tests were done at 25^0C and 40% relative humidity condition.

Patch test

Patch tests were accomplished on the fore-arms of volunteers. A 5X4cm area was marked on both the fore-arms. Mexameter was used for the basic values for erythema measurement. 1.0 gm of base and formulation, each were applied to the marked regions separately on each forearm. The regions were covered with the surgical dressing. Dressings were detached after 48 hours and forearms were washed with physiological saline. Scores were recorded for the presence of erythema by using a scale with 4 points from 0 to 3. The scores given by the volunteers are shown in table 2.

Ethical approval

This study was approved from the institutional ethical review (IERB) and all protocols oh Helsinki's declaration were followed. Ref. No.233/ERB-IUB/2018.

STATISTICAL ANALYSIS

GraphPad Prism 7.0 software (San Diego, CA, USA) was used for statistical analysis. All experiments were performed in triplicate. Data was presented as mean ± SD. One-way analysis of variance (ANOVA) LSD and t-test were applied for the comparison of mean values. $P< 0.05$ was regarded as statistically significant.

RESULTS

Total anthocyanins content

Results showed that the extracted *Malus dometica* contained about 181.30 mg/100 g on fresh weight of anthocyanins pigments; the results declared that *Malus dometica* contained high concentration of total anthocyanins. These results are in agreement with that obtained by Mazza and Miniati (1993).

Identification of anthocyanins

Anthocyanins pigments extracted from *Malus dometica* were separated and identified by HPLC are shown in fig. 1.The major anthocyanins present in extraction of *Malus domestia* were Cyanidin-3-xylosyl-glucosyl-galactoside as presented by peak 3which represented 30.31% of the total area.

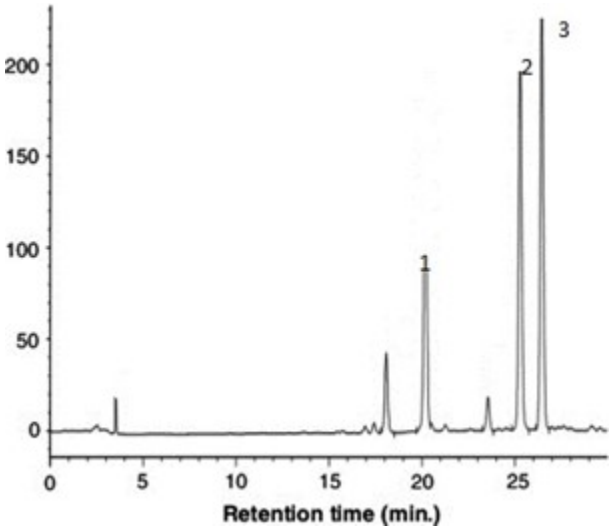

Fig. 1: Identification of compounds for anthocyanin pigments extracted from *Malus domestica*

Melanin and erythema

The percent change with respect to time in melanin and erythema following the applications of the base and formulation on the cheeks of human volunteers were measured before application of creams and after 1st , 2nd ,3rd, 4th, 6th, 8th, 10th and 12th week of study period by mexameter and given in table 3 and 4.

Skin moisture content

The percent change with respect to time in skin moisture content following the applications of the base and formulation on the cheeks of human volunteers were measured before application of creams and after 1st, 2nd, 3rd, 4th, 6th, 8th, 10th and 12th week of study period by corneometer and given in table5.

Transepidermal water loss (TEWL)

The percent change with respect to time in Trans epidermal water loss following the applications of the base and formulation on the cheeks of human volunteers were measured before application of creams and after 1st, 2nd ,3rd, 4th, 6th, 8th, 10th and 12th week of study period by mexameter and given in table 6.

Skin sebum content

The percent change with respect to time in skin sebum contents following the applications of the base and formulation on the cheeks of human volunteers were measured before application of creams and after 1st, 2nd ,3rd, 4th, 6th, 8th, 10th and 12th week of study period by sebumeter and given in table 7.

DISCUSSION

Melanin

The effects of the base and formulation containing anthocyanin extract on skin melanin were examined. It was found that, after application of base there was variation in the melanin contents of the skin during the study period, whereas in case of formulation there was continuous decline in the melanin contents of the skin throughout the study period.

ANOVA test showed that the base produced in-significant possessions while formulation produced significant effects with respect to time. With the help of students paired sample t-test, significant differences were observed between the melanin effects of base and the throughout the study period.

Statistically in-significance changes were observed throughout the study period when LSD test was applied to observe the average effect of base formulation on skin melanin at different time. While in case of active formulation, it gave significant changes throughout the study period starting from 4th week onwards.

It was detected from the earlier study of Takayoki *et al*. that plant extract comprising anthocyanins could inhibit the tyrosinase activity (Takayoki *et al*., 2008). The results of current study showed that skin lightening effect of *Malus domestica* anthocyanin extract can somewhat be attributed to the suppression of melanogenesis by the inhibition of tyrosinase enzyme in melanocytes.

Skin erythema

Skin irritation was constantly checked every week for both the base and the formulation throughout the study period. It was observed that there was irregular decline in

Table 1: % Contents of active formulation

Oily Phase					
Ingredients	F1	F2	F3	F4	F5
Parrafin oil	16	18	20	22	24
Abil EM90	2	2.5	2	2.5	3
Bees wax	3	3	3	3	3
Water Phase					
Anthocyanin* extract	3	3	3	3	3
Purified water (q.s)	100	100	100	100	100

*Base/placebo contained no extract

Table 2: Score given by volunteers to base and formulation on the basis of itching/irritation

Score		0	1	2	3
No of volunteers	Base	8	3	1	0
	Formulation	9	1	2	0

Table 3: Percentage of change in skin melanin contents after application of formulation and base

Volunteer no.	1st Week	2nd week	3rd Week	4th week	6th week	8th week	10th week	12th week
Formulation								
Mean ±SEM	-0.4±0.70	-0.6±0.63	-0.7±0.76	-1.3±0.88	-1.4±0.54	-2.1±0.52	-2.6±0.58	-3.2±0.69
Base								
Mean± SEM	0.1±0.19	0.1±0.38	0.1±0.41	-0.1±0.54	-0.1±0.55	0.0±0.50	0.1±0.53	0.1±0.51

Table 4: Percentage of change in skin erythema content after application of formulation and base

Volunteer no.	1st week	2nd week	3rd week	4th week	6th week	8th week	10th Week	12th week
Formulation								
Mean± SEM	-0.6±0.36	-2.8±0.65	-4.4±0.78	-6.1±0.55	-6.9±0.86	-7.1±1.10	-8.9±0.99	-9.4±0.87
Base								
Mean± SEM	-0.4±0.14	-1.1±0.27	-2.0±0.17	-2.0±0.42	-1.9±0.49	-1.0±0.33	-1.0±0.25	-0.1±0.35

the skin erythema after the application of base while formulation showed continuous drop throughout the study period. With the help of ANOVA test, it was obvious that there were significant effects of base and formulation on skin erythema throughout the study period. With the help of paired sample t test, it was found that the base and formulation produced in-significant results with respect to time regarding the skin erythema content after 1st, 2nd, 3rd and 4th week while significant variations were shown after 4th week of the study. Different studies defined that antioxidant capacity of anthocyanins have shown the photo protection effect induced by UV radiations which is a major factor of cumulative erythema level in skin (Jack, 1998).

Skin moisture content

The effects of the base and formulation on skin hydration were inspected. The effect on skin hydration was noted for 3 months (12 weeks) at different time intervals in all individuals after application of base and formulation. It was found that, after application of base and formulation there was slight increase in the hydration contents of the skin during the study period with irregular pattern. Statistically (ANOVA test), it was found that the base and formulation produced significant effects with respect to time. With the help of paired sample t-test, insignificant differences were observed between the hydration effects of base and the formulation.

Uneven and dry skin may benefit from creams, lotions or soaps containing bees wax and oils. These ingredients when added to skin care products, acts as emollient and humectant, drawing moisture to the skin and sealing it. Beeswax also contains vitamin A, which may be beneficial in softening and rehydrating dry skin and in cell reconstruction (Sheridan, 2010).

Trans-epidermal water loss (TEWL)

The effects of base and formulation containing anthocyanins were also evaluated for skin trans-epidermal water loss level (TEWL). TEWL contents were noted in all volunteers for the period of 3 months. It was observed that both the base and formulation incessantly diminished trans-epidermal water loss throughout the study period. With the help of ANOVA test it was found that the base and formulation showed significant properties with respect to time.

Table 5: Percentage of change in values of skin moisture after application of base

Volunteer no.	1st week	2nd week	3rd week	4th week	6th week	8th week	10th week	12th week
Formulation								
Mean± SEM	0.3±0.22	4.9±0.77	8.3±0.90	6.6±1.42	7.7±1.65	9.4±1.78	9.1±1.65	7.5±1.56
Base								
Mean ±SEM	0.4±0.23	4.8±0.67	8.7±0.93	6.9±1.13	8.3±0.65	9.1±0.75	9.1±1.07	8.4±1.36

Table 6: Mean Percentage of change in values of transepidermal water loss (TEWL) after application of formulation and base

Time period	1st week	2nd week	3rd week	4th week	6th week	8th week	10th week	12th week
Formulation								
Mean ±SEM	-6.0±0.72	-10.4±1.48	-16.8±1.71	-21.9±1.59	-25.5±1.57	-30.4±2.08	-32.9±1.67	-35.3±1.74
Base								
Mean ±SEM	-3.4±0.96	-5.2±1.70	-14.7±2.66	-19.8±2.73	-25.5±3.78	-29.3±3.82	-36.0±4.87	-35.3±6.04

Table 7: Mean Percentage of change in values of skin sebum after application of formulation and base

Time period	1st week	2nd week	3rd Week	4th week	6th week	8th week	10th week	12th week
Formulation								
Mean± SEM	2.4±0.37	4.5±0.43	6.4±0.58	9.1±0.80	7.5±0.94	7.3±0.91	3.2±0.98	2.5±0.72
Base								
Mean± SEM	3.1±0.41	5.0±0.60	8.7±0.72	10.8±0.81	7.1±0.75	9.0±0.86	6.8±0.73	3.2±0.51

Insignificant differences were observed in the effects of both the base and formulation when paired sample t test was applied. It has been observed from the study of Marty that the petrolatum based creams decreased the Trans epidermal water loss. It was also observed by Marty that creams containing waxes also improved the conditions of skin when compared between controlled and test groups (Marty, 2009; Vasiljevic *et al.*, 2005).

Bees wax reduces the TEWL by creating a hydrophobic barrier over skin and contributing the matrix between corneocytes and have the most pronounced effect when applied to skin (Loden 1999). It can be concluded that cream containing anthocyanins in this concentration might have no effect on trans epidermal water loss.

Creams having mineral oil and bees wax may contribute to reduce trans-epidermal water loss by generating hydrophobic layer over skin (Marty, 2009; Zhang *et al.,* 2008). Antioxidants subsidize the photo protection and are important in the preservation of skin health.

Skin sebum content

Skin sebum contents were also observed after the application of both active formulation and base (placebo) for 3 months. It was established that active formulation and base showed in constant effects. The results of ANOVA test it was established that both formulations (active and base) had significant possessions with respect to time.

With the help of paired sample t test, in-significant differences were noted by formulations. It can be clinched that cream containing anthocyanins in current concentration might have no effect on sebum contents. However the increase in sebum might be due to the oily nature of creams containing thick viscous oily liquid i.e., paraffin oil which has the potential to rise the sebum content of the skin (Henriette, 1995; Acevedo *et al.* 2005).

CONCLUSION

The w/o emulsions have been found to be stables. These water-in-oil emulsions may find potential applications as a system to entrap anthocyanin and an interesting vehicle for topical uses. The anthocyanin containing emulsions can be used as a natural moisturizing system because it does absorb the UV radiation, and it does increase the SPF value. These emulsions can be used in cosmetic products because of their antioxidant, anti-ageing and anti-inflammatory activities. All formulations containing anthocyanin extract also significantly decreased skin melanin and skin erythema.

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