ORIGINAL RESEARCH ARTICLE EXPERIMENTAL

Formulation and *in vitro* evaluation of the topical antiageing preparation of the fruit of *Benincasa hispida*

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ABSTRACT

Ageing is the phase of gradual decline of body efficiency and metabolic activities after reaching a maturity stage. Free radicals cause oxidative alterations in collagen, elastin material and changes in membrane characteristics and induce polymerization reactions. Use of topical antioxidants can overcome some of these effects and retard actinic ageing. Herbal products are popular due to their minimum risk of side-effects with maximum efficacy. The present study was undertaken to evaluate the antiageing potential of *Benincasa hispida* fruit extract as not many scientific studies have been carried out to explore its utility as skin renewal enhancer and as an antioxidant. After removing the outer layer and the seeds, the fruit pulp was dried. The dried fruit pulp was extracted successively with petroleum ether, chloroform, ethyl acetate and methanol by Soxhlation for 2 days. Methanol was recovered under vacuum and a dry extract was obtained (yield 4.2% w/w), which was stored in a desiccator. Suitable topical cream base for effective carriage of fruit extract was developed and its *in vitro* evaluation for skin renewal activity was tested by application to the stratum corneum of human cadaver skin and by dansyl chloride fluorescence method. The results show that the cream prepared from Benincasa fruit extract may prove as an antiageing preparation and can be used for retarding the symptoms of ageing.

Key words: Antioxidants, antiageing, *Benincasa hispida*, dansyl chloride, wax gourd

INTRODUCTION

Skin is a flexible, self-repairing capsule that separates the internal environment of the body from the external environment.[1] Ageing is the phase of gradual decline of body efficiency and metabolic activities after reaching a maturity stage. The body changes that lead to decrease in life expectancy with age are known as senescent, which is the characteristics of living. Cutaneous ageing results due to exposure to chemicals, radiation and temperature in the surroundings. Ageing can be classified as intrinsic ageing, which is a chronological and inevitable event, and actinic ageing, which is dependant on an individual's exposure to UV radiation.[2] This ageing is enhanced due to free radicals that are generated in the body through various metabolic pathways due to oxidation of fatty food and constant exposure to UV radiations. The visible effects of ageing, such as dry, leathery skin with less elasticity, are a combination of dermal and epidermal changes taking place in the skin. Dermal changes are disturbances in the collagen and elastin network, decreased water retention capacity and shrinkage of dermis.[2,3] Epidermis is a proliferating layer of the skin. Normal skin renewal or epidermal turnover time is 56–72 days.[4] This time is increased during ageing due to a decline in cell metabolism and mitotic rates. The Dansyl chloride fluorescence method by Jansen *et al*.[5] was chosen for determining the stratum corneum renewal time.

The fruit of *Benincasa hispida* (Thunb.) Cogn., commonly called as ash gourd, belonging to the family Cucurbitaceae, is employed as a main ingredient in kusmanda lehyam, which is used in numerous nervous disorders in the Ayurvedic system of medicine.[6] According to Raja Nirghantu (an

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ancient work on therapeutics), medicine from *Benincasa hispida* was prepared from old, ripe fruits. The pulp was scraped into thin strips and the water juice that oozes out abundantly was collected and preserved.[7] The major constituents of this fruit are triterpenoids, flavanoids, glycosides, saccharides, carotenes, vitamins, β sitosterin and uronic acid.[6,8-14] Many applications of Benincasa fruit have been reported for various ailments such as dyspepsia, burning sensation, heart disease, vermifuge, diabetes, anti-inflammatory activity, diuretic activity and as an anticancer agent.[15] But, not many scientific studies have been carried out to explore its utility as a skin-renewal enhancer and as an antioxidant. In the light of this information, the present study was carried out to formulate a cream and evaluate the antiageing potential of *Benincasa hispida* fruit extract.

MATERIALS AND METHODS

The matured fruits of *Benincasa hispida* were collected from the local market of Nagpur city in the month of September and were identified and authenticated by the Botanist of Shri M. M. Science College, Nagpur. A voucher specimen (MMSC-8127) was deposited in the Department of Botany.

Dansyl chloride was procured from SRL Chemicals, Mumbai, India. Spreadability apparatus, Brookfield viscometer and Spectrofluorometer obtained from Horiba Jobin Yvon, Kyoto, Japan, were employed for the present study. All other chemicals and solvents used were of analytical grade.

Extract preparation

After removing the outer layer and the seeds, the fruit pulp was dried at temperature not exceeding 60°C using a tray dryer. The dried fruit pulp was extracted successively with petroleum ether (60–80°C), chloroform, ethyl acetate and methanol by Soxhlation for 2 days. Methanol was recovered under vacuum and the dry extract was obtained (yield 4.2% w/w), which was stored in a desiccator.[16]

Formulation and preparation of suitable topical cream base

Formula no. 1

Oil phase: Cetyl alcohol 5%, glyceryl monostearate 15%, sorbiton monooleate 0.3%, polysorbate 0.3%.

Aqueous phase

Methyl Cellulose 1%, purified water q.s.

Formula no. 2

Oil phase: Stearic acid 4%, stearyl alcohol 5%, cetyl alcohol 2%, lanolin 5%, isopropyl myristate 8%.

Aqueous phase: Propylene glycol 5%, glycerine 5%, triethanolamine 0.75%, water 66.5%, BHT 0.02%, methyl paraben 0.18%, propyl paraben 0.02%, EDTA 0.05%.

The above cream bases were prepared by accurately weighing the oil phase and the aqueous phase and taking in a beaker separately and heating to about 70°C. The water phase was then mixed with the oil phase by trituration till the cream congealed and cooled.[17]

Comparative evaluation of cream

Both the bases were compared with each other for appearance, spreadability, water number, diffusibility, rheological and stability studies. Washability was determined by rubbing the little amount of base on the hand and washing off with warm water without using soap.[17-20]

Spreadability test

Cream base should spread easily without too much drag and should not produce greater friction in the rubbing process. Spreadability was calculated using the spreadability apparatus made of wooden board with scale and two glass slides having two pans on both sides mounted on a pulley.

Excess sample was placed between the two glass slides and 100 g weight was placed on the glass slide for 5 min to compress the sample to a uniform thickness. Weight (250 g) was added to the pan. The time in seconds required to separate the two slides was taken as a measure of spreadability.

$S = m * l/t$

m – weight tied on upper slide

l – length of glass slide

t – time in s

Determination of water number

Water number is the maximum amount of water that can be added to 100 g of the base at a given temperature. It was determined by continuously stirring the base with the addition of distilled water. When no more water was absorbed into the base, evidenced by droplets of water remaining in the container, this was taken as the end point.

Diffusion of active ingredient

Diffusibility gives the amount of cream base diffused with the body surface. For this, salicylic acid cream was prepared by using Formula no. 1 and Formula no. 2 (salicylic acid 2 g and cream base 98 g).

Nutrient agar medium was prepared using beef extract 10 g, peptone 10 g, sodium chloride 5 g, agar 1.2 g and distilled water 1000 ml. This was poured into a Petri dish and a hole was made in the center of the medium and the cream was then applied to the hole and time for diffusion

was noted, evidenced by pink rings on the agar medium after a particular interval.

Rheological studies

In order to determine the viscosity of Formula no. 2, it was kept at room temperature and at an elevated temperature of 45°C. For this, 50 g of the cream base was kept at 45°C in an oven. Viscosity of this cream was measured after regular intervals of time for 1 month. Changes in viscosity were determined by the Brookefield Viscometer using Spindle number 7.

Another 50 g of base was kept at room temperature and its rheological studies were carried out in the same manner on alternate days.

Stability testing

Stability testing was carried out for Formula no. 2 by keeping 50 g of cream at 45°C and another 50 g at room temperature. It was checked for any visual disturbances and phase separation from time to time over a period of 1 month.

Preparation of investigational cream

To the amount of propylene glycol in the base (Formula no. 2), 5% fruit extract was mixed and the cream was formulated in the same way as that of plain base cream (i.e., devoid of fruit extract).

Effect of prepared cream on skin renewal

The Dansyl chloride fluorescence method given by Jansen *et al*.[5] was used to measure the skin-renewal effect of the prepared cream with fruit extract. Dansyl chloride is a fluorescent dye that binds avidly with amino groups and thus is useful for fluorescence tagging of proteins. The time required for the dye to disappear from the fully stained horny layer provides an estimate of its replacement time.

Preparation of dansyl chloride base

A 5% w/w dispersion of dansyl chloride was prepared with white petrolatum in dark with subdued red light as the dye is light-sensitive.

Preparation of human cadaver skin

A fresh piece of forearm skin was obtained from the Govt. Medical College, Nagpur. It was immediately put into ice to avoid deterioration and brought to the Laboratory. Skin was cleaned by removing subcutaneous fats adhering to the skin with a forceps and scissors.

The epidermis was then separated by the heat trypsinization method given by Kligman. The skin was dipped in hot water and the epidermis was peeled off slowly using a forceps. The epidermis was then placed in trypsin solution (5% in water) for 5 min. The treatment of epidermis with trypsin solution caused separation of the stratum corneum. This was then dried overnight in a desiccator by spreading the stratum corneum on a stainless steel wire mesh. After 24 h, the stratum corneum was cut into three pieces of 1 cm x 1 cm and stored in a desiccator, which was then placed in a refrigerator.[21]

Development of fluorescent patches on human cadaver skin

Dansyl chloride base was liberally and uniformly smeared on the pieces of skin with the help of the index finger in the dark. The skin pieces with patches were preserved in a refrigerator below 0°C by sandwiching them between two glass slides. After 24 h, the fluorescence intensity of these stained pieces was measured at 340 nm by a spectrofluoremeter at RSIC, Nagpur. Fluorescence intensity of these pieces was checked over a 2-month period to ascertain that the fluorescence did not decline.

Effect of investigational cream for the skin renewal activity on the developed dkin patches

Of the three skin patches, the first patch was treated with Benincasa fruit extract cream, the second with plain base cream and the third patch was treated as control (treated with standard antiageing preparation). Creams were applied daily and fluorescence intensity was noted on alternate days. The number of days needed for complete disappearance of the patches provided a measure of the stratum corneum renewal time.

Statistical analysis

Statistical analysis was carried out for determining the significance ($P < 0.05$) of the result obtained. t-test was carried out for comparison between the mean number of days for removal of patches treated with the investigational cream and control [Table 4].

RESULTS

Modified Formula no. 1 and Formula no. 2 were prepared by the described method, and their comparative evaluation was carried out for spreadability [Table 1], diffusibility and water number [Table 2]. Table 3 shows the viscosity of the cream base kept at room temperature and at elevated temperature, 45°C. For assessment of the thermal stability of Formula No. 2, half portion of the base was kept at 45°C and the remaining half was kept at room temperature. Table 4 shows the effect of the *Benincasa hispida* fruit extract (5%) cream on skin renewal. Also, the study was designed to ascertain the effect of plain base cream (devoid of fruit extract) on skin renewal using the Dansyl chloride technique. Decline in fluorescence intensities of the cream are given in Table 4, which shows the number of days required for the disappearance.

Table 1: Spreadability test

Formulation	Weight tied to upper slide (m)	Length of glass slide moved (l)*	Time taken (t)	Average spreadabiliy* S = m x l /t
Formula no. 1	240 g	0.36 ± 0.05 cm	10 s	8.8 ± 1.22
Formula no. 2	240 g	0.76 ± 0.05 cm.	10 s	18.4 ± 1.38

*Values represent average of three separate determinations ± SD

Table 2: Diffusion of active ingredients and determination of water number

Formulation	Time interval	Length of ointment diffused (cm)	Amount of water added to 10 g of base
Formula no. 1	20 min	*1.35 ± 0.05	2.0 ml
Formula no. 2	20 min	*2.05 ± 0.07	3.0 ml

*Values represent average of three separate determinations ± SD

Table 3: Viscosity determination

Formula no. 2		
Time in days	Viscosity (cp)	
	Room temperature	45°C
0	29,000	28,000
2	30,000	26,000
7	31,000	25,000
14	31,000	24,000
22	32,000	24,000
30	32,000	24,000

Viscosity in cp = dial reading of the Brookfield viscometer x factor

Table 4: Decline in fluorescence intensities (%) of Dansyl chloride-stained patches with the application of the investigational cream for the skin-renewal activity

Time in days	Skin patches		
	Test*	Control*	
1	81 ± 2.51	91 ± 1.0 (0.0539#)	
3	62 ± 2.0	90 ± 1.52 (0.0014#)	
5	51 ± 3.21	80 ± 2.51 (0.0011#)	
7	35 ± 2.51	61 ± 1.0 (0.0061#)	
9	25 ± 2.0	42 ± 2.51 (0.0012#)	
11	12 ± 1.52	32 ± 1.0 (0.0013#)	
13	-	29 ± 1.0	
15	-	20 ± 1.52	
17	-	16 ± 0.57	
t-test	Mean diff.	*P*-value	95% CI of diff.
Test and control	-21.67	$P < 0.05$	-29.43 to -13.91

*Data expressed as mean ± SD; #indicates P-value; n = 3; data analyzed by t-test ($P < 0.05$) when compared with control

DISCUSSION

A suitable topical base was developed that could act as an effective carrier for Benincasa fruit extract, showing skin-renewal activity. The generally preferred formulations are creams and lotions. Lotions are less acceptable to the users than creams. Therefore, it was decided to develop a topical cream base that is widely accepted, i.e. an ideal oil-in-water cream base with good consistency, smooth and shining texture, good spreadability, non-greasy nature, easy washability and good stability. Keeping these points in mind, a cream was formulated that fulfills all these characteristics.

The results show that Formula no. 2 had good spreadability (average spreadability 18.4 ± 1.38) as compared with Formula no. 1 (average spreadibility 8.8 ± 1.22). Formula no. 1 showed a decrease in water number as compared with Formula no. 2, which represents its less-water-absorbing capacity (3.0 ml vs. 2.0 ml). Formula no. 2 showed good diffusibility (average diffusibility 2.05 ± 0.07) than Formula no. 1 (average diffusibility 1.35 ± 0.05). Thus, Formula no. 2 was found to satisfy all the desirable properties of oil-in-water cream base and was decided to be the final cream base.

After a definite interval of time, the results of viscosity show that at room temperature and at elevated temperature, the cream does not show any sudden changes in viscosity, which indicates the stability of the developed cream base at higher as well as lower temperatures.

Regular inspection of both the creams over a period of 1 month for thermal stability showed that Formula no. 2 is stable, without any changes of liquefaction and phase separation.

Decline in fluorescence intensity of the investigational cream was studied and noted on alternate days. On Day 1, decline in fluorescence intensity of the investigational cream was found to be 81% as compared with 91% of the control (*P*-value = 0.0539). Similarly, on Days 3, 5, 7, 9 and 11, decline in fluorescence intensities of the investigational cream were found to be 62%, 51%, 35%, 25% and 12% as compared with 90%, 80%, 61%, 42% and 32% of the control (*P*-value = 0.0014, 0.0011, 0.0061, 0.0012 and 0.0013). The mean differences for Days 1, 3, 5, 7, 9 and 11 were found to be -7.667, -27.00, -30.00, -23.07, -17.00 and -18.07, respectively.

The t-test shows that increase in skin renewal by investigational cream is significant at the $P < 0.05$ level of significance.

Control patches took 15–17 days to disappear while the Benincasa fruit extract cream took 11 days to disappear, showing better skin-renewal activity. Plain base cream did not show any disappearance and skin-renewal effect.

CONCLUSIONS

It can be concluded that the cream prepared from Benincasa fruit extract showed statistically significant better antiageing efficacy as compared with the control. Thus, the cream prepared from Benincasa fruit extract may prove to be an antiageing preparation and can be used for retarding the symptoms of ageing. The results obtained in the present investigation are only directional in view and further investigation can be made on this basis to get additional data and information about the Benincasa fruit, and combined effects of various botanical extracts can also be studied on skin renewal.

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