# Improvements in human skin texture and surface with the use of emulgels containing *Annona squamosa* L. fruit extract along with penetration enhancer

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**Abstract-** This investigational study was designed to characterize anti-aging effects of o/w emulgels containing *Annona squamosa* L. fruit extract by comparing it with its control and the variation in these effects with the addition of penetration enhancer. The control (without extract and penetration enhancer i.e. clove oil 8%) and the two test formulations with 4% fruit extract (one without clove oil and other with clove oil) were formulated and applied on the cheeks of 26 healthy female human volunteers (n=26, divided into two equal groups) for a period of 12 weeks. All the formulation was evaluated for skin texture parameters (energy, contrast and variance) and surface evaluation of the living skin (SELS parameters) using Visioscan ® VC 98. There was a visible improvement of the overall skin appearance and reduced number of fine lines by both of the test formulations. Moreover, skin texture (variance and contrast) and SELS (SEr, SEsc and SEw) parameters showed significant decline ($p < 0.05$) and the texture parameter of energy and SEsm showed significant increase ($p < 0.05$). All our findings indicate that the emulgel containing 4% *Annona squamosa* L. fruit extract improves skin texture and SELS parameters ultimately possesses anti-aging effects and these effects can be increased by the addition of penetration enhancer.

**Keywords:** *Annona squamosa* L, Emulgel, Fruit extraction, Penetration enhancer, Visioscan VC98, Anti-aging, Skin texture,

## 1 Introduction

Aging of skin is a complex natural process resulting from both intrinsic (genetically programmed) aging that occurs with time and extrinsic aging occurring by environmental factors (1). The morphological changes occurring during aging are characterized by loss of elasticity, increased wrinkling, irregular pigmentation, dryness and roughness (2). A primary cause of aging is the imbalance between reactive oxygen species (ROS) production and their neutralization by natural antioxidant systems, which generates oxidative stress (3). In recent years, topical formulations have attained considerable interest as a vehicle for the drug delivery to the human skin (4). One approach in skin protection against aging is the use of plant phenolics and antioxidants topically (5). The phenolics may be helpful in preventing UV-induced oxygen free radical generation and lipid peroxidation, occurring during pathological states such as photoaging and skin cancer (6).

*Annona squamosa* L. (Annonaceae) is native of West Indies and its fruit is now also cultivated in India and other tropical countries like Thailand. The 30% of its fruit weight consists of seeds (7). All parts of the fruit comprise different phytochemicals, possessing medicinal properties (8). These biological chemicals include alkaloids, glycosides, flavonoids, ascorbic acid and phenolic compounds(9). The present investigational study was aimed to evaluate the anti-aging effects of emulgels containing *Annona squamosa* L. fruit extract (4%) along with the penetration enhancer (clove oil 8%) on skin texture and surface parameters.

## 2 Materials And Methods

### 2.1 Chemicals and Reagents

Acetone (Merck KGaA Darmstadt, Germany), DPPH (Sigma, USA), Distilled water (Department of Pharmacy, IUB, Pakistan), Carbopol 940 (Sigma, USA), Triethanolamine (Merck KGaA Darmstadt, Germany), Liquid paraffin (Merck KGaA Darmstadt, Germany), Span 80 (Sigma, USA), Tween 20 (Sigma, USA), Propylene glycol (Merck KGaA Darmstadt, Germany), Methyl paraben (Acros Organics, USA), *Annona squamosa* L. (local market Bahawalpur, Pakistan)

### 2.2 Apparatus

Homogenizer (Euro-Star, IKA D 230, Germany), pH-meter (WTW pH-197i, Germany), rotary evaporator (Eyela, Co. Ltd. Japan), water bath (HH. S 21 4. China), electrical balance (Precisa BJ-210 Switzerland), Mexameter MPA-5 (Courage + Khazaka, Germany), visioscan® VC98 (Courage + Khazaka, Germany).

### 2.3 Plant material

*Annona squamosa* L. was purchased from local market Bahawalpur and then identified from the department of life sciences, The Islamia University of Bahwalpur, Pakistan with voucher number 7687/LS.

### 2.4 Preparation of fruit extract and determination of antioxidant activity

*Annona squamosa* L. extract was prepared by using cold maceration technique. 100 g of sliced whole fruit (peel, pulp and seed) was macerated in 500ml of acetone (70%) for 72hr at room temperature. The residues were collected by first passing the extract from different layers of muslin cloth and then by filtering through whatman filter no. 1. The volume of filtrate was reduced to the 1/3 of the initial volume by evaporating it under reduced pressure by using rotary evaporator at $45^0$C.

2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical was used to find out the antioxidant activity with slight modification of method as described by(10). The DPPH was used in 100 μM concentration in methanol. The total volume used for assay was 100ul, including 10 μl of the test solution and 90 μl of DPPH solution in a 96 wellplate.The solutions were mixed, incubated at $37^o$C for the period of 30 minutes and absorbance was taken at 517nm. Ascorbic acid was used as a reference standard antioxidant.

### 2.5 Preparation of emulgel

The emulgels were prepeared by the method reported by Muhammad *et al* (11). This method involves three steps for the formulation of emulgel: first the formulation of emulsion, then the formulation of gel base, then finally incorporating the emulsion into the gel base to develop the final formulation.

One control emulgel (without extract) and two test formulation emulgels were prepared one with extract (FA) and second with extract along with penetration enhancer (FB). The aqueous phase of the emulsion contains Tween 20, Propylene glycol, Methyl Paraben and Distilled water while in oily phase liquid paraffin and span 20 was used. The gelling agent used was carbapol-940(2%) and the penetration enhancer was clove oil (8%).

The *in vitro* characterization of the prepared emulgels was done including pH, conductivity, phase separation, rheology and mean droplet size for a period of 90 days.

### 2.6 Instrumental Assessment:

Visioscan VC 98 is a special UVA-light video camera with high resolution for the direct study of skin parameters. The image output shows the structure of the skin and the level of dryness. The camera was connected to a computer via a digitalization unit (Video Digitizer VD 300) through

FireWire port. All measurements were made at controlled temperature 25±1$^0$C and 45 ±2%relative humidity.

#### 2.7 Study protocol

A total of 26 healthy female volunteers with a mean age of 25 y were selected and divided into two groups each comprising 13 volunteers (one for control and formulation A and second for formulation A and B). A single blinded study was designed to compare the effects of different formulations and a consent form containing the terms and conditions of testing was signed from all volunteers before beginning of a study. Volunteers were examined by a dermatologist for any skin disease especially on cheeks and forearms. Every volunteer was instructed to apply creams on cheeks twice daily for the period of 12 weeks and come for measurement of effects on 4th, 8th and 12th week. Measurements of skin parameters, which included texture parameters (energy, contrast and variance) and for skin roughness (Ser), skin scaliness (SEsc), skin smoothness (SEsm) and skin wrinkles (Sew) were made using visioscan® VC98 (Courage + Khazaka, Germany). Volunteers were instructed to wash their faces with water and sit, to become normal with the environment for 30 min before any measurement. Values for skin parameters were taken at controlled conditions of 25±1$^0$C and 45 ±2% relative humidity.

#### 2.8 Skin irritation assessment

A patch test was performed on both forearms of each volunteer on the first day of skin testing to evaluate primary irritation potential of formulations. A 5 × 4 cm region was marked on forearms of volunteers. For 1$^{st}$ group Patch for left forearm was saturated with 1.0 g of formulation A while that of right forearm was saturated with 1.0 g of the control after application on marked areas. Same was done with 2$^{st}$ group by choosing left forearm for formulation B and right forearm for formulation A. The regions were covered with surgical dressing after application. The patches were removed after 48 hrs and regions of forearms were washed with physiological saline (12). The skin was observed for any irritation by using Mexameter.

#### 2.9 Ethical standards

The approval of human and animal study (Reference no. 33/S-2018-/PREC) was taken from the Board of the Advanced Study and Research (BASAR), the Islamia University of Bahawalpur and the Institutional Ethical Committee, Faculty of Pharmacy and Alternative medicines, The Islamia University of Bahawalpur, Pakistan.

#### 2.10 Mathematical and statistical analysis

The percentage changes for each value of different parameters of volunteers were determined by the following formula:

$$\text{Percentage change} = [(A-B)/B] \times 100 \quad \text{(Equation 1)}$$

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

Paired samples t-test for variation between the two preparations and one-way ANOVA for variation between different times intervals were analysed using SPSS 15.0 using a 5% level of significance.



## 3 Results And Discussions

#### 3.1 Antioxidant activity

The studied fruit extract showed a high free radical scavenging activity (90%) against DPPH when compared to the free radical scavenging activity of the ascorbic acid as standard (92%).

#### 3.2 Patch test

Skin erythema contents were measured before application of creams (0 hour readings) and then after 48 hours by Mexameter MPA 5 (Courage and Khazaka GmbH). It was found out that the erythema level after application of the control was slightly decreased while that after application of the formulations was pronouncedly decreased after 48 h. But with a paired sample t-test it was evident that the effects of the formulations and control were insignificant. The percent changes occurred were calculated by using the equation 1 and results are presented in figure **1.**

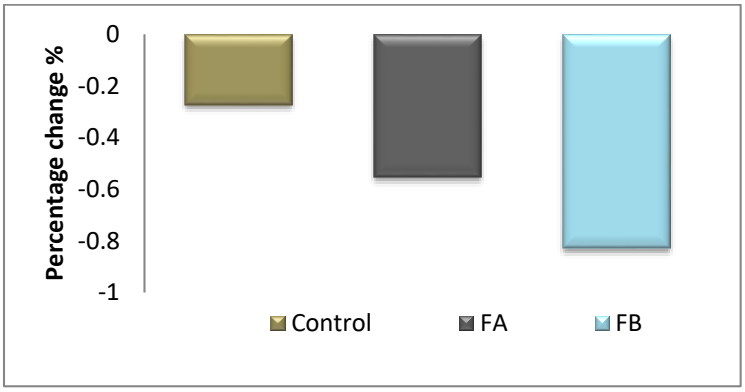



**Fig. 1. Patch test showing the percentage change in skin erythema value after 48 hours with the use of control, formulations FA and FB.**

### 3.3 Skin texture measurement:

Percentage changes in skin texture parameters (energy, contrast and variance) are shown in the figure **2**. The increase in energy while decrease in contrast and variance after the use of formulation A and B was statistically significant ($p < 0.05$) at all the time intervals and same values with the use of control was statistically insignificant ($p > 0.05$). Significant differences between control and formulations were also found.

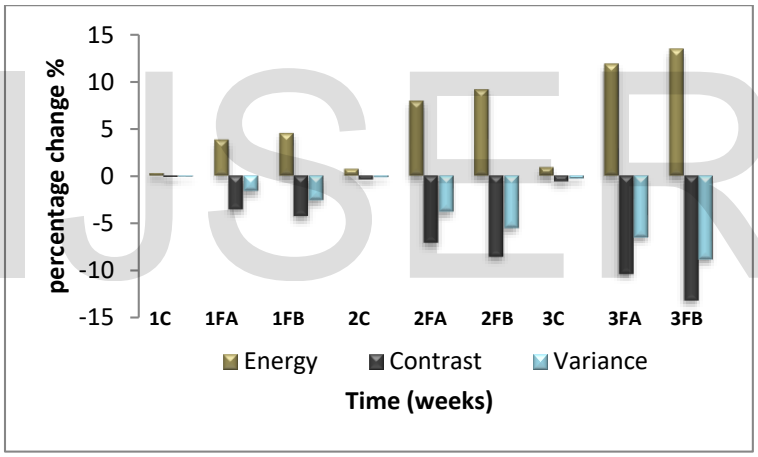



**Fig. 2. Percentage change in skin texture parameters (Energy, Contrast and Variance) after 12 weeks with the use of control, formulation A(FA) and formulation B(FB).**

**Key:** 1C = application of control after 4 weeks, 1FA = application of formulation A after 4 weeks, 1FB = application of formulation B after 4 weeks, 2C = application of control after 8 weeks, 2FA = application of formulation A after 8 weeks, 2FB = application of formulation B after 8 weeks, 3C = application of control after 12 weeks, 3FA = application of formulation A after 12 weeks, 3FB = application of formulation B after 12 weeks.

### 3.4 Surface evaluation of living skin (SELS) Measurement:

Percentage changes in the surface evaluation of skin roughness (SEr), surface evaluation of skin smoothness (SEsm), surface evaluation of skin scaliness (SEsc) and surface evaluation of skin wrinkles (SEw) were shown in the figure **3**.

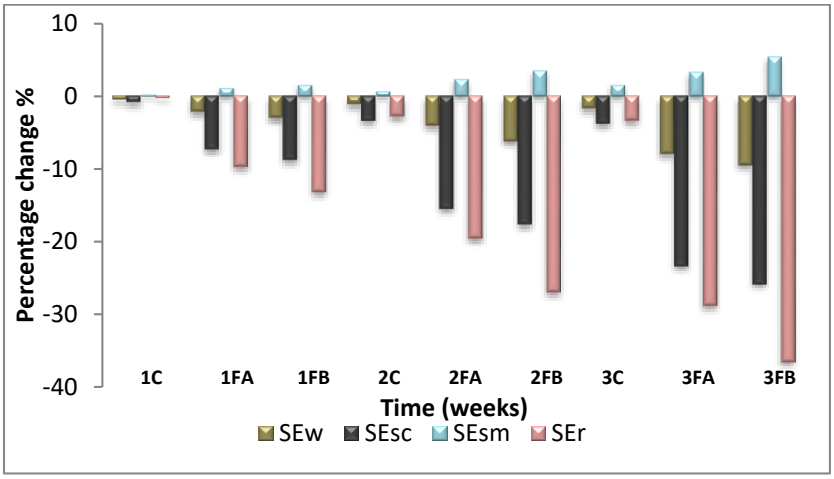
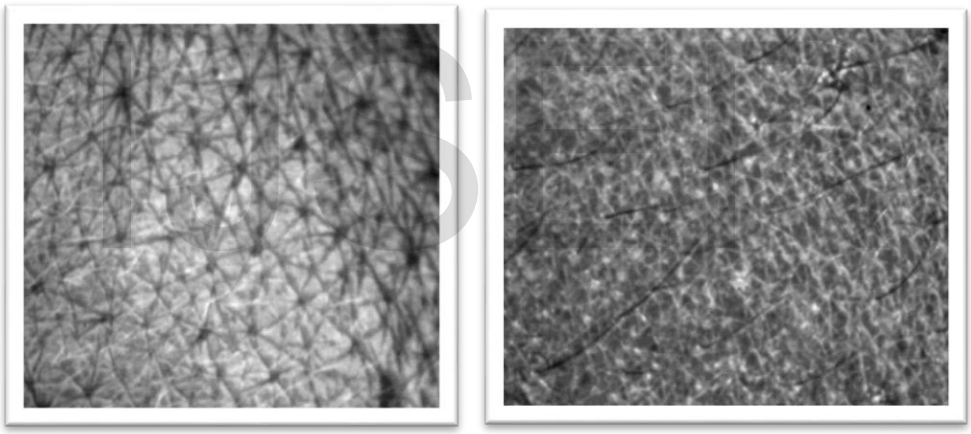



**Fig. 3: Percentage change in SELS parameters after 12 weeks with the use of control, formulation A(FA) and formulation B(FB).**

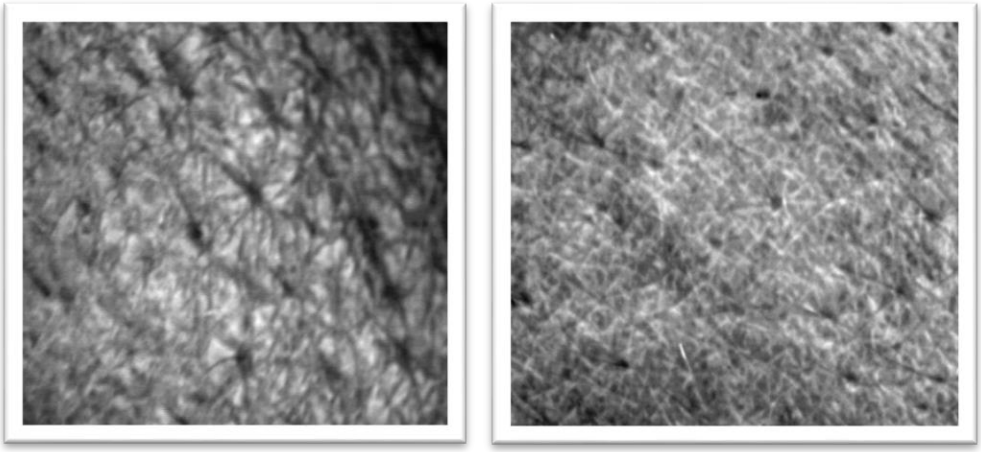
**Key:** 1C = application of control after 4 weeks, 1FA = application of formulation A after 4 weeks, 1FB = application of formulation B after 4 weeks, 2C = application of control after 8 weeks, 2FA = application of formulation A after 8 weeks, 2FB = application of formulation B after 8 weeks, 3C = application of control after 12 weeks, 3FA = application of formulation A after 12 weeks, 3FB = application of formulation B after 12 weeks.

Photographs of human cheeks before and after the application of formulation A and B are shown in the figures **4** and **5** respectively.



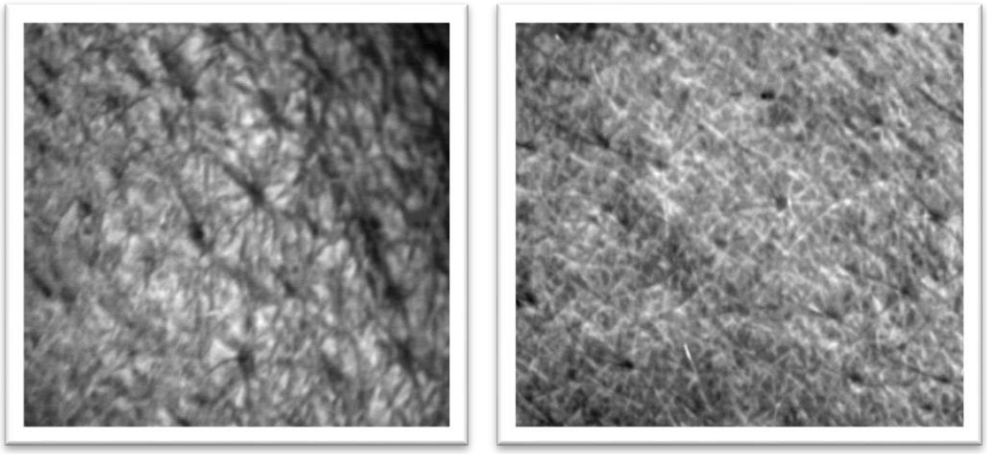
**A** **B**

**Fig 4. Part of right cheek of a human volunteer before application of FA (A) and after application of FA (B)**



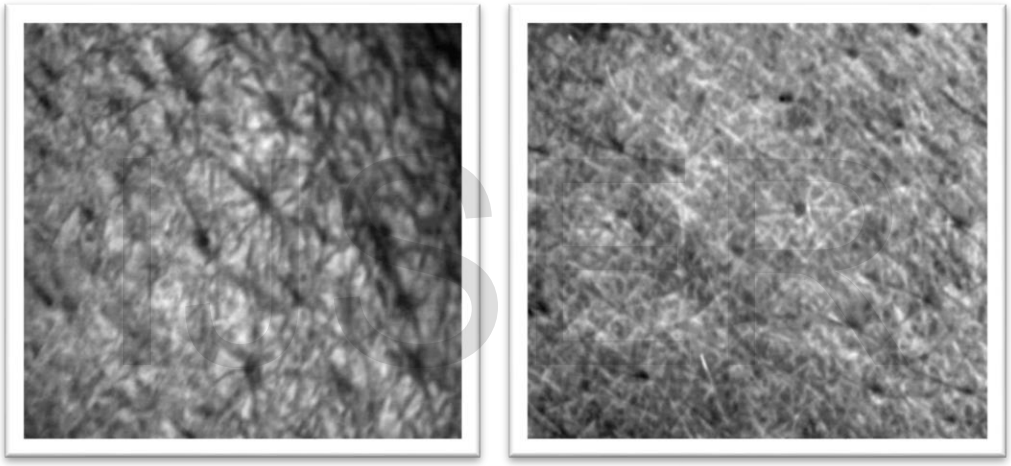
A B

**Fig 4.** Part of right cheek of a human volunteer before application of FA (A) and after application of FA (B)



A B

**Fig 4.** Part of right cheek of a human volunteer before application of FA (A) and after application of FA (B)



A B

**Fig 5. Part of right cheek of a human volunteer before application of FB (A) and after application of FB (B).**

Polyphenols and carotenoids are the two major groups of antioxidant phytochemicals contributing the antioxidant properties of plants(13). There is a direct relationship in total phenolic contents and total antioxidant activity of different fruit's extracts(14). So, the antioxidant activity of the plant is due to the presence of these phytochemicals.

Initially, the patch test was performed to evaluate the safety of all the formulations. Patch testing after a single application is a widely used procedure to evaluate acute irritant reactions of the formulations(15). Both the formulations as well as control showed no skin irritation during patch test, when observed after 48 hours. This shows that all the emulgels can safely be used to the human skin for *in vivo* evaluation.

The texture parameters (energy, contrast and variance) are used for analysing differences in colours of neighbouring pixels(16). Changes in the texture parameters after applying the control and formulations are shown in the figure **2** .Energy is increased when the hydration level of the skin is increased and ultimately results in the improved homogeneity of an image (4). Variance is the average of a local variance over a number of pixels. The actual value of the pixel is compared to the average. High variance indicates high roughness of skin surface (17). Contrast indicates the difference between grey levels of the two neighbouring pixels. The higher the value of contrast, the higher is the difference between the values of two neighbours (17).In this study, an increase in the energy values while decrease in the variance and contrast was observed for the active formulation A while active formulation B showed the same effects but with greater intensity. When ANOVA and paired sample t-test was applied, these effects were statistically significant at all reading intervals, but the control produced insignificant effects. A significant effect of energy obtained

by active formulations was indicative of improving the hydration level and elasticity of the skin. A significant effect of variance obtained by active formulations was indicative of decreased roughness of the skin. A significant effect of contrast obtained by active formulations was indicative of better skin condition and smoothness of the skin.

With regard to the surface evaluation of living skin (SELS) parameters, SEr is the roughness parameter which calculates the proportion of dark pixels. SEsm is the key of smoothness and is calculated from the mean width and depth of wrinkles. SEsc is the indicator of scaliness of skin which shows the level of dryness of the skin. SEw identifies aging including wrinkles and is calculated from the proportion of horizontal and vertical wrinkles(18). Changes in the SELS parameters after the application of control and active formulations are showed in the figure **3**. In this study, a gradual increase in the value of SEsm and gradual decrease in the values of SEr, SEsc and SEw was observed after the application of active formulation A while active formulation B showed the same effects but with greater intensity as compared to the formulation A. When ANOVA and paired sample t-test was applied, these effects were statistically significant at all reading intervals, but the control produced insignificant effects.

A gradual increase in the skin SEsm while decrease in the SEsc showed the moisturizing effect of the active formulations while decrease in the SEr and SEw showed the anti-aging effect of the active formulations. It is assumed that the improvement in skin surface evaluation parameters can be linked to the presence of phenolic contents (19), flavonoids (20), and vitamin A, B, C (21) in the *Annona squamosa* L. fruit extract. Phenolics inhibit reactive oxygen species (ROS) and provide protection against collagenase that cause breakdown of elastin and collagen (22). Vitamin C gives photo protection and avoids UVB-induced immunesuppression while Vitamin B acts as a humectants and draw water into the stratum corneum to give soft texture to the skin (23). Polyphenols are antioxidants which scavenge free radicals and contribute to anti-aging effect of formulations (24). These investigational supports that the active formulation A and B containing *Annona squamosa* L. extract possess the anti- aging activities. The increased effects of formulation B may be associated with the penetration enhancement effect of clove oil (25) . Eugenol in the clove oil is reported in increasing the partitioning of the drug to the stratum corneum (26).

## 4 Conclusion And Recommendations

The findings of this study suggest that the emulgels containing *Annona squamosa* L extract (4%) has potential to enhance skin surface and texture parameters and reduce the signs of skin aging. And the effects were increased with the addition of penetration enhancer. Furthermore, the formulation can be used safely as cost effective anti-aging alternative treatment to synthetic commercial formulations. Further studies are required to reveal the anti-aging mechanism of the constituents of the fruit extract.



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