Annona muricata extract containing pharmaceutical emulgels with and without penetration enhancer for depigmenting and antierythmic effects

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Abstract: The basic purpose of this research work was to investigate the skin depigmenting and antierythmetic effects of emulgel containing Annona muricata 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 FA and FB (without clove oil and with clove oil) were formulated and evaluated for in vitro characteristics (pH, conductivity and in vitro release). The emulgels were then applied on the cheeks of 26 healthy female human volunteers (n=26) for a study period of 12 weeks. Skin melanin and erythema contents were measured by Mexameter at base line and then after every 2 weeks. Both the test formulations showed significant decrease in melanin and erythema contents when compared to control but FB showed marked decrease in skin melanin when compared to the FA. While in case of skin erythema, the effects of FA were greater as compared to other formulation. When paired sample t test (5% level of significance) was applied, the test formulations showed significant results. This study reveals that the Annona muricata L. fruit extract naturally contains some important phenolic compounds and can be effectively used in topical preparations for the treatment of skin hyperpigmentation and dermatitis. Skin whitening effects can be increased by the addition of penetration enhancer.

Keywords: Annona muricata L., emulgel, penetration enhancer, depigmentation.

INTRODUCTION

There is an extensive range of colours (white to black) and gradations in human skin. This variation is due to the occurrence of melanin (chemically inert and stable pigment) which is produced within the skin (Costin and Hearing, 2007). Increased production of melanin can cause different hyperpigmented conditions like melasma, post-inflammatory hyperpigmentation, drug-induced hyperpigmentation and erythema dyschromicum which become prominent with age. To treat such disorders, topical formulations containing tretinoin, hydroquinone, azelaic acid, kojic acid etc are being employed (Stratigos and Katsambhas, 2004).

Herbal ingredients are widely being used in cosmetics because of the poor image of animal-derived products (Patil et al., 2014). Plant-derived extracts having compounds which inhibit melanin synthesis may be a good choice for skin whitening and protection against skin darkening (Baurin et al., 2002).

Annona muricata L. family Annonaceae is one of the tropical fruits possesses strong antioxidant properties. These properties are associated with the presence of natural antioxidants like phenolic acids, vitamin C and E, carotenoids and flavonoids, which prevent free radical damage (Akomolafe and Ajayi, 2015). Epidemiological studies have always shown a significant positive correlation between consumption of fruits and vegetables containing antioxidant phytochemicals and reduced chances of heart diseases mortality, other degenerative diseases, common cancers and ageing (Kaur and Kapoor, 2001).

Emulgel, a novel drug delivery system has better patient acceptability due to possessing the activities of both emulsions and gels (Kumar et al., 2015). They have the advantages of being transparent, thixotropic, easily spreadable & having longer shelf life (Haneefa et al., 2013). The presence of penetration enhancing ingredients in the topical formulation temporarily changes the skin barrier, modifies the partitioning of the drug into skin structures and ultimately enhances drug penetration into skin (Hardenia et al., 2014).

This study depicts the depigmenting and anti inflammatory effects of Annona muricata L. extract in a topical preparation and the change in these effects with the addition of penetration enhancer.

MATERIALS AND METHODS

Chemicals and apparatus

Acetone (Merck KGaA Darmstadt, Germany), DPPH(Sigma, USA), Carbopol 940 (Sigma, USA), Triethanolamine (Merck KGaA Darmstadt, Germany), Span 80 (Sigma, USA), Tween 20 (Sigma, USA),...
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Propylene glycol (Merck KGaA Darmstadt, Germany), Liquid paraffin (Merck KGaA Darmstadt, Germany), Methyl paraben (Acros Organics, USA), Annona muricata L. (imported from Malaysia), Rotary evaporator (Eyela, Co. Ltd. Japan), homogenizer (Euro-Star, IKA D 230, Germany), pH-meter (WTW pH-197i, Germany), water bath (HH. S214. China), Mexameter MPA-5 (Courage & Khazaka, Germany)

Plant material
Annona muricata L. was imported from Malaysia and then identified from the department of life sciences, The Islamia University of Bahwalpur, Pakistan with voucher number 7686/LS.

Preparation of fruit extract and determination of antioxidant activity
Annona muricata 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 macerate was stirred daily for 30 minutes. 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°C. The concentrated extract was stored at 4°C for further studies.

2, 2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical was used to quantify the antioxidant activity with slight modification of method as described by Ratshilvha et al. Ascorbic acid was a reference standard antioxidant used in this measurement.

Preparation of emulgels
The emulgels were prepared by the method reported by Muhammad et al. 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 FA and FB were prepared one with extract and second with extract along with penetration enhancer. The aqueous phase of the emulsion contains Tween 20, Methyl Paraben, Propylene glycol 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%).

In-vitro characterization
pH determination
The pH of freshly prepared emulgels and emulgels kept at different storage temperatures (8°C, 25°C, 40°C and 40 ºC & ± 75 % relative humidity) was measured with the help of a digital pH meter Ino-Lab pH7110 pH meter (WTW, Germany) at regular intervals of 15 days, 30 days, 45 days, 60 days, 75 days and 90 days of investigation. All the measurements were performed in triplicate.

In-vitro release study
Ascorbic acid release across rabbit’s skin was determined using Franz diffusion cell with an effective diffusion area of 1.72cm². Prior to the use the rabbit’s skin was rinsed with distilled water and soaked in receptor liquid (glycerine: water pH4) for at least 1 hr. The skin was then placed horizontally dividing the cell into receptor and donor compartments.

0.5g emulgels was applied homogeneously on skin surface. The experiment was done on thermostatically controlled water bath at 25±2°C with agitation. The aliquots of 1ml at regular intervals of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5hr were removed and ascorbic acid amount was determined by UV at 268nm (Farahmand et al., 2006)

In-vivo studies
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 contrast 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 specialist for any type of skin disease particularly on cheeks and forearms. Each volunteer was instructed to apply creams on cheeks twice daily during the entire study period and appear for measurement of effects on 2nd, 4th, 6th, 8th, 10th and 12th week. The measurements were taken by using Mexameter® MPA 5 (Courage + Khazaka, Germany) at controlled conditions of 25±1°C and 45±2% relative humidity.

Skin irritation assessment
A patch test was done on both forearms of every volunteer on the first day of in vivo study to evaluate primary irritation potential of formulations. A 5 × 4 cm region was marked on forearms of volunteers. For 1st group Patch for left forearm was applied with 1.0 g of formulation A while that of right forearm was applied with 1.0 g of the control after application on marked areas. Same was done with 2nd group by choosing left forearm for formulation B and right forearm for formulation A. A surgical dressing was applied on the marked regions for patch test after application of formulations. The dressings were removed after 48 hrs and regions of forearms were rinsed with physiological saline. The skin was observed for any irritation by using Mexameter.
**Ethical standards**
The human and animal study was approved (Reference no. 33/S-2018/-PREC) by 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.

**Fig. 1:** Graph showing the pH changes in the control emulgel. Key: (d=day, °C =degree Celsius, RH = relative humidity)

**Fig. 2:** Graph showing the pH changes in the Formulation A. Key: (d=day, °C =degree Celsius, RH = relative humidity)

**Fig. 3:** Graph showing the pH changes in the Formulation B. Key: (d=day, °C =degree Celsius, RH = relative humidity)

**Mathematical and statistical analysis**
The percentage changes for the each value of different parameters of volunteers were determined by the following formula:
Percentage change = [(A−B)/B] ×100  
(Equation 1)
Where; A = Individual value of any parameter specific week, B = Zero hour value of that parameter.

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

**Fig. 4:** Graph showing the release profile of control, formulation A (FA) and formulation B (FB)

**Fig. 5:** Patch test showing the percentage change in skin erythema value after 48 hours with the use of control, formulation A (FA) and formulation B(FB).

**Fig. 6:** Percentage change in skin erythema value after 12 weeks with the use of control, formulation A (FA) and formulation B (FB).

**RESULTS**

**Antioxidant activity**
The studied fruit extract showed a remarkable free radical scavenging activity (88%) against DPPH when compared to the free radical scavenging activity of the ascorbic acid as standard (92%).

**pH determination**
The pH of the freshly formulated emulgels was 6.02, 5.53 and 5.67 of control, formulation A and formulation B
respectively which is within the skin pH range. The changes occurred in the pH values of control and test formulations at different storage conditions are noted in figs. 1-3. The pH values of all the formulations kept on decreasing with the passage of time but that change was within the acceptable range except at accelerated storage condition of 40 °C & ± 75 % relative humidity.

**Skin melanin contents**

Skin melanin content were measured prior to the application of emulgel (0 hour readings) and then at 2nd, 4th, 6th, 8th, 10th and 12th week of study period by Mexameter MPA 5 (Courage and Khazaka GmbH). After the application of control, there was a slight increase observed in skin melanin contents, but in the case of the formulation A there was a continuous decrease in skin melanin content, and that decrease was more pronounced in case of formulation B throughout the study period. The percent changes occurred were calculated by using the equation 1 and result is presented in fig. 7.

When the ANOVA test was applied, it was found that changes in skin melanin values produced by the application of control were insignificant and by the test formulations were significant over time. After the application of paired sample t-test it was found that the control and formulations showed significant variations as regards to erythema values over time.

**DISCUSSION**

The presence of antioxidant phytochemicals like polyphenols and carotenoids contributes to the antioxidant properties of plants (Zhang et al., 2015). Polyphenols are powerful antioxidants and helpful in the prevention of certain neurodegenerative diseases, cardiovascular diseases and cancers etc. (Florence et al., 2014). The presence of such ingredients is responsible for its antioxidant activity and thus makes the extract a potential ingredient for skin care preparations.

The pH determination is an essential parameter for the stability and efficacy of topical formulations. The pH of the topical emulgels should be inside the range of skin pH i.e. 5-7 to avoid any kind of skin irritation (Johnsey Joseph, 2017). The decrease in the pH of the formulations in the present study may be due to the hydrolysis reaction or oxidation of any of the ingredients of the extract while the marked change in formulation B may be due to the production of acidic products by additional presence of clove oil (Mahmood et al., 2013).

In the development of topical formulations, the in vitro release of the drug from artificial membranes is necessary for predicting an suitable vehicle as they measure
drug/vehicle interaction (Özer et al., 2007). In this study, the release of ascorbic acid was increased during first 3.5 hrs and then decreased from both the formulations FA and FB but the release of drug was greater in case of FB. Previous studies also supported such release pattern of ascorbic acid in multiple emulsion (Farahmand et al., 2006). The increased release in case of formulation B is related to the presence of clove oil (penetration enhancer). The occurrence of eugenin (terpene) in clove oil is related to the increased release of the formulation containing clove oil (Kumar et al., 2014). According to lipid partition theory, there are three different mechanisms of penetration enhancers, one of which is increasing the portioning of drug into skin tissue (Fox et al., 2011). Clove oil acts as penetration enhancer by increasing the partition of drug into the stratum corneum.

Initially, the patch test was performed to evaluate the safety of all the formulations. For the evaluation of acute irritation potential of topical formulation, the patch testing following a single application is a extensively used method (Gaspar et al., 2008). Both the formulations as well as control showed no skin irritation during patch test, and decreased the skin erythema contents when observed after 48 hours. This shows that all the emulgels can safely be used to the human skin for cosmetic and therapeutic purposes.

Regarding the skin erythema contents, all the three emulgels decreased the skin erythema contents and the order of decrease was: FA>FB>Control. The inflammatory response subsequent of acute UV irradiation and the degenerative progressions related to chronic skin exposure to UV radiations are largely associated by the overproduction of reactive oxidative species and by destruction of the antioxidant defence system (Ali et al., 2012). The presence of non-enzymatic antioxidants namely ascorbic acid, total carotenoids, lycopene, flavonoids, α-tocopherol, and reduced glutathione are crucial for the cellular systems in decreasing reactive oxygen species (ROS) (Rani et al., 2004). The reduced skin erythema contents with the application of the formulation A and B may be related to the occurrence of antioxidants like carotenones, tocopherols and ascorbic acid in the fruit extract (Muthu and Durairaj, 2015). The decreased effect of formulation B as compare to formulation A may be associated to the components of clove oil i.e. eugenol and β carophyllene which are slightly irritant (Chen et al., 2015).

In case of skin melanin contents, the control emulgel increased the skin melanin contents and the test formulations decreased the skin melanin contents with a order of decrease: FB>FA. The colour of human skin is due to the existence of melanin pigment in skin. It is also present in bacteria, plants and fungi. Tyrosinase is the major enzyme responsible for the biosynthesis of melanin (Nerya et al., 2003). Hyper-activity of this enzyme results in the over production of melanin which leads to hyper-pigmentation of the skin and hypo-activity of this enzyme results in certain disorders like vitiligo (depigmentation spots occurring on the skin) and hair whitening (Mapunya and Lall, 2011). So, tyrosinase enzyme inhibition can lead to decreased melanin production.

The reduction in skin melanin contents can be related to the phenolics and flavonoids present in Annonona muricata L. fruit like quercetin, caffeic acid, cinnamic acid, syringic acid, ferulic acid and p-coumaric acid etc. (Jiménez et al., 2014, Adefegha et al., 2015). The inhibition of tyrosinase enzyme, associated with flavonoids might be due to chelating the active center of tyrosinase enzyme leading to decreased melanin synthesis (Saewan et al., 2011). The skin depigmenting and antioxidant activity of Annonona muricata L. fruit can also be related to presence of ascorbic acid (Singh et al., 2014). The increased effects of formulation B may be associated with the penetration enhancement effect of clove oil (Jiang et al., 2017). Eugenol in the clove oil is reported in increasing the partitioning of the drug to the stratum corneum (Aggarwal et al., 2013).

CONCLUSION

From the present findings, it can be concluded that the Annonona muricata L. extract was successfully incorporated into a topical emulgel and showed its skin depigmenting activity at 4% concentration. The depigmenting effect was increased with the addition of penetration enhancer but that effect is not seen in case of anti inflammatory action. So, this extract can be successfully used as a natural alternative treatment for certain skin diseases. A targeted study is needed in the future to explore the actual potential of this plant in patients with melasma and psoriasis.

REFERENCES


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