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RESEARCH ARTICLE

Evaluation of L929 cell morphology on anthocyanin-containing gelatin-based hydrogel for early detection of infection

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Abstract

Wounds heal through a complex process including four phases. Any interruption or interference in healing process can prevent wound healing. Infection is one of the most common complications preventing wounds from healing. In this study, we investigated the fibroblast cell morphology and behavior of a gelatin-based hydrogel containing anthocyanin to determine whether it can be used for early detection of infection. The experimental results ascertained that 6 h after culturing the fibroblasts, they could be well attached to the surface of the hydrogel, and 48 h after seeding, they were spread over the surface and connected with each other. After the addition of a low dose of anthocyanin, the vitality of the cell increased, and the results of the ATR-FTIR analysis showed that anthocyanin could eliminate remaining glutaraldehyde free radicals. Anthocyanin also could change its color after the addition of bacterial supernatant. Thus, hydrogels containing black carrot anthocyanin may be a potential therapeutic and diagnostic strategy to promote wound healing and early detection of infection.

Keywords Wound healing · Hydrogel · Gelatin · Infection · Anthocyanin · pH

Introduction

Skin, the largest organ of human body, is the first line of defense from pathogens and microorganisms and provides a physical barrier against external physical and chemical attacks [1, 2]. It consists of two distinct layers, namely the epidermis and dermis, which overlay the hypodermis (the subcutaneous fat). Epidermis is the superficial layer of the skin, and the top layer of epidermis, stratum corneum, which is composed of layers of dead keratinocytes, provides resistance to the penetration of microorganisms, and at the same time it retains moisture and nutrients under the skin [2–5]. Skin is considered as an ecosystem, and each gram of this tissue is colonized by over 103 microorganisms such as bacteria, viruses and fungi. Besides, most of them are harmless or beneficial for their hosts [6]. Wounds heal through four well-orchestrated, integrated and overlapping phases: hemostasis, inflammation, proliferation and remodeling [7]. Any interruption or interference in these pathways acts as an obstacle and leads to a non-healing or “stalled” wound. One of the inhibitors of the wound healing process is infection [8]. After a breach on the skin, the wound environment acts as a rich medium and skin flora and pathogenic organisms contaminate the wound immediately. This is beneficial because the presence of bacteria in that environment initiates the inflammatory stage and the second stage of healing, but replicating bacteria lead to the formation of bacterial colonization and colonized wounds do not show invasion or damage to nearby tissue and do not stimulate a host immune response, and it gradually leads to biofilm formation [9]. After biofilm formation, bacteria are placed in a new microbial community, and by producing gelatin matrix they resist antibiotics, antiseptic, macrophages, phagocytic neutrophils and displacement [10]. It seems that a quick diagnosis of infection can be helpful in improving wound healing, so antibiotics can be used immediately. In addition to improving wound healing as an advantage of early detection of infection, its other benefits are avoiding the bacterial resistance problem and unnecessary use of antibiotics, as well as lowering the cost [8]. Infection in advanced stage has cardinal symptoms such as heat, swelling, increasing exudate, smell, pain, systemic illness, loss of function and tissue breakdown [11]. However, it is important to diagnose an infection before it reaches this stage.

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There are a variety of markers that can be used for monitoring wound healing and infection. One of the markers that can be used to predict infection is pH [8]. The skin pH is slightly acidic and varies from 4 to 6 [12]. Researchers have found that bacterial enzymes activity can lead to an increase in the alkalinity of the wound environment. Correspondingly, an increase in pH can increase the chance of biofilm formation [13]. Until now, many pH sensors have been designed; however, they have some drawbacks, including high cost, need for an advanced technology, being harmful for wound healing and difficulties in use [8]. Anthocyanins are polyphenols which are responsible for the colors of innumerable fruits and flowers, and their chemical structures consist of a basic skeleton of 2-phenylbenzopyrylium or flavylium glycoside [14]. Anthocyanins appear in different colors at different pH levels. In acidic condition, they appear red, while by increasing the pH, their color gradually changes to purple and blue [15]. The therapeutic benefits of anthocyanin are due to their antioxidation property [16]. They are well known as strong antioxidants [17]. Due to the health-promoting benefits of anthocyanins, including reduction in the risk of cancer, coronary heart disease, diabetes, neurodegenerative disorders, inflammation, as well as bacterial infections, interest in them has been intensified [18–20]. In particular, the effect of anthocyanins on improving wound healing has been explored in various studies [21, 22]. Black or purple carrots originate from Turkey and the Middle and Far East, where they have been cultivated for at least 3000 years. They mainly consist of cyanidin-based pigments, and their anthocyanin profile has been analyzed by several investigations [23].

In the present study, a gelatinous hydrogel containing black carrot anthocyanin was synthesized and evaluated whether it can be used for early detection of infection. Also, the effects of anthocyanin on the surface chemical groups, cell viability and morphology have been investigated.

Experimental procedures

Hydrogel preparation

At first, a solution of 2% W/V gelatin (Sigma-Aldrich) and 35% W/W propylene glycol (Sigma-Aldrich) was prepared at 50 °C and magnetically stirred for 1 h. Then, 15 ml of the solution was poured into a 6-cm petri dish and dried for 48 h at a temperature of 40 °C in an oven, in order to prepare film specimens. After drying, the prepared films were detached from the petri dish and immersed in PBS solution containing 0.02% glutaraldehyde (Sigma-Aldrich) for 2 h. The films were washed to remove excessive glutaraldehyde and then soaked into a solution with different concentrations of anthocyanin (Proquimac Food & Pharma) (0.01%, 0.03% and 0.05% W/V %). After 4 h, anthocyanin completely penetrated into the films.

The summary of the concentration and code name of hydrogel samples is presented in Table 1.

Table 1 Various concentrations of anthocyanin were used to study the hydrogels properties

Code	Anthocyanin solution (%v/v)
GA0	0
GA1	0.01
GA2	0.03

Characterization

ATR-FTIR analysis (Fourier Transform Infrared-Attenuated Total Reflection, Nicolet Nexus 670) was used to demonstrate the functional groups present in the scaffolds and to evaluate the effect of anthocyanin on hydrogel surface chemistry.

SEM (scanning electron microscope, Philips XL30, the Netherlands) was used to visualize the morphology of cells seeded on the hydrogel with or without anthocyanin. Finally, cell-containing hydrogels were sputter-coated with gold and then observed under SEM.

The surface wettability was evaluated by measuring contact angle. The sessile drop technique was performed to measure the contact angle using a KSV CAM 200 optical contact angle meter at room temperature. Measurements were performed at room temperature (20 °C).

Biological evaluation

MTT assay

To evaluate the influence of samples on cell viability, reduction of the MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was assessed as an assay using L929 NCBI C161 cell lines. In this study, 1×10^5 cells and 100 μl of culture medium DMEM (Dulbecco's Modified Eagle's Medium, Sigma Chemical Company, St. Louis, USA) were poured into each well in a 24-well cell culture plate and then placed in a 37 °C incubator for 24 h to allow the cells to adhere to the plate. After ensuring cell adhesion, the culture medium was removed from the cells as much as possible and the specimens plus 10 μl of FBS were added to each well and incubated for 1, 3 and 7 days at 37 °C in a humidified 5% CO_2 incubator. After the incubation period, the culture mediums were removed from each well and cell viabilities were tested by MTT assay. 400 μl of MTT at 0.5 mg/ml was poured into each well and placed in an incubator for 4 h. After 4 h, the solution was removed from the cells and isopropanol was added to dissolve the resulting purple crystals.

To better dissolve the MTT sediment, the plate was placed on a shaker machine for 15 min. Then, the concentration of dissolved material in isopropanol was calculated using a laser device (STAT FAX 2100, USA) at a wavelength of 570 nm. The wells with more cells show higher photodynamic (OD) than the wells with less cells.

Cell morphology

Gelatin films were cut into circular pieces (1.5 cm diameter) and sterilized by 30-min UV irradiation before being introduced to the culture media and were placed into the 24-well plates. The cells were seeded (with or without gelatin films) at a density of 2×10^5 cells per well with DMEM supplemented with 10% fetal bovine serum (Gibco, BRL), 1 mm l-glutamine, penicillin (20,000 U/ml) and streptomycin (20,000 mg/ml). 0.5 ml of the DMEM medium was added to each well, and the culture dishes were incubated for 1, 3 and 7 days at 37 °C under an atmosphere of 5% CO_2 (each group had eight duplicate samples). Next, they were washed with sterile phosphate-buffered saline (PBS), and 3.5% glutaraldehyde solution was used for fixation of the cell. Finally, they were washed with graded ethanol series and then gold-coated and examined using a SEM.

Visual inspection of color change of anthocyanin-containing hydrogel

Supernatants from the Staphylococcus aureus with Pseudomonas aeruginosa bacterial co-culture were collected after 8, 16 and 24 h and placed on the films containing anthocyanin. After 5 min, the ability of hydrogel to response to pH changes due to bacterial activity was visually assessed.

Results

ATR-FTIR analysis

The ATR-FTIR analysis results presented in Fig. 1 show the change in the surface of samples before and after immersion in the anthocyanin solutions.

As seen in the ATR-FTIR spectra, the intense peak at 1630 cm^{-1} can be assigned to the carbonyl group of AG0, which was not immersed in the anthocyanin solution. On the other hand, for AG1 and AG2, a decrease in the intensity of carbonyl band can be obviously observed.

MTT assay

Cell viability was examined on cross-linked films by glutaraldehyde, before and after immersion in anthocyanin solutions of different concentrations. Figure 2 indicates the results of MTT assay after different periods of time of cell culture by using L929 cells. It was observed that the cell density value for a sample without anthocyanin after 3 days was 96%, while it increased by 99% with the immersion of the films in 0.01% anthocyanin solution. In contrast, with increasing the anthocyanin concentration, the cell density values of samples were significantly dropped down in comparison with the control value ($P < 0.05$, $n = 8$). Anthocyanin at a concentration of 0.01% could even eliminate the reduction in viability after 7 days, which was observed in GA0 and GA2.

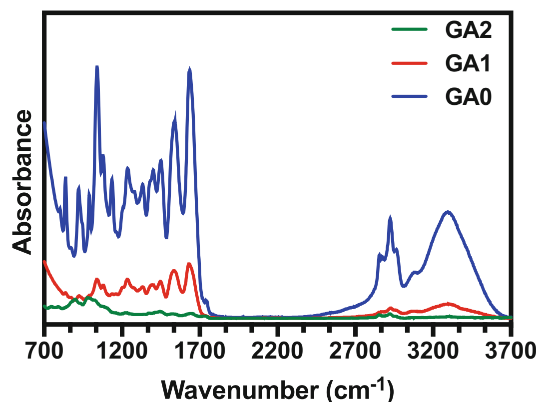

Fig. 1 ATR-FTIR spectra of hydrogels with different concentrations of anthocyanin

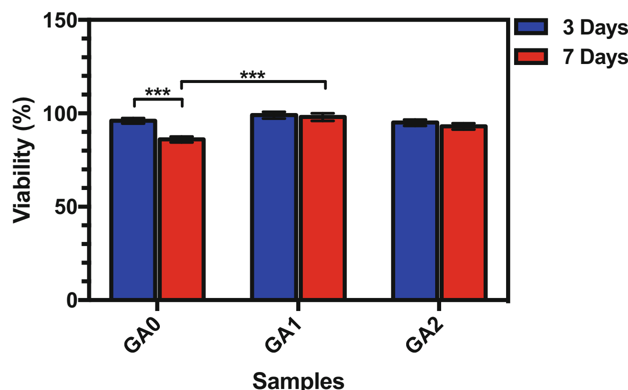

Fig. 2 L929 fibroblasts viability evaluated by the MTT assay

The actual viability of cell was increased by the addition of anthocyanin, but the presence of excessive anthocyanin resulted in reducing cell viability and spreading. Despite the fact that immersion of the films cross-linked by glutaraldehyde into the anthocyanin solution could increase cell viability, it was also observed that with increasing the anthocyanin dosage, the cell viability decreased; therefore, it could be concluded that there was an optimum dosage for anthocyanin.

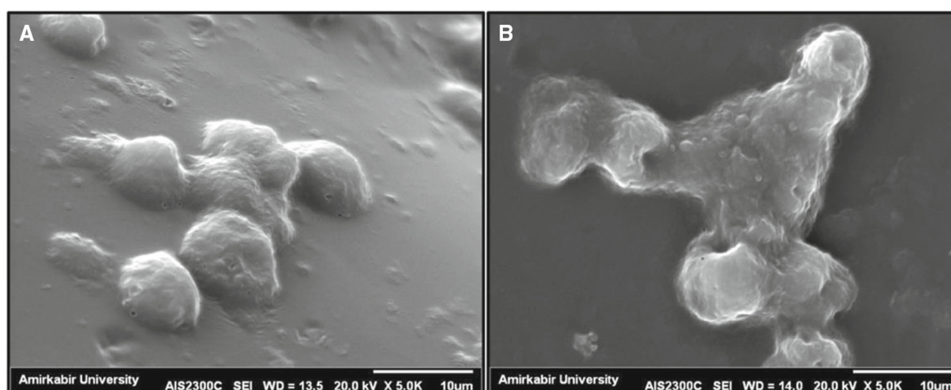

Fig. 3 Morphology evaluation of L929 fibroblasts on hydrogels by SEM. **a** GA1 and **b** GA2. Cells were cultured for 6 h

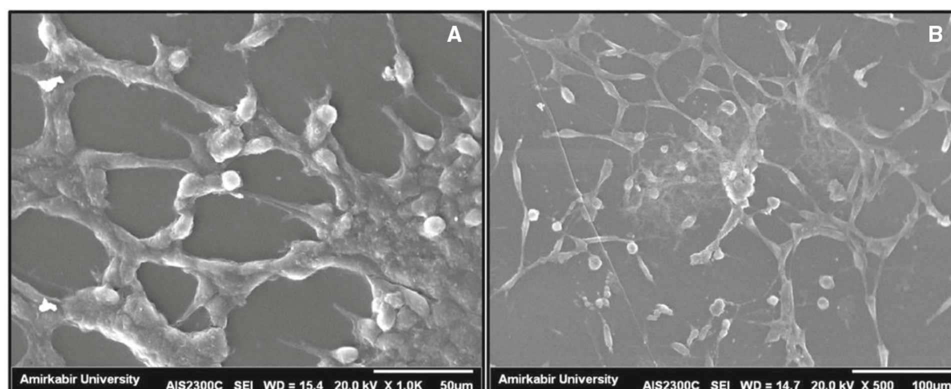

Fig. 4 Morphology evaluation of L929 fibroblasts on hydrogels by SEM. **a** GA1 and **b** GA2. Cells were cultured for 48 h

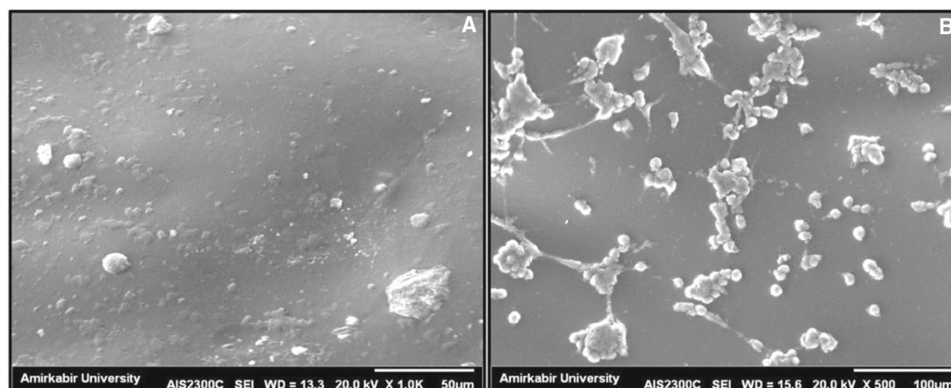

Fig. 5 Morphology evaluation of L929 fibroblasts on hydrogel, immersed in 0.05% anthocyanin solution by SEM. **a** Cells were cultured for 6 h, and **b** cells were cultured for 48 h

Cell morphology observations

The mouse fibroblast cells (L929) were seeded on the surface. Adhesion, spreading and morphology of cells on the surface of samples were evaluated using SEM image. After seeding the cells on the samples, cell/material interactions could indicate the biocompatibility of the materials. Cell adhesion is an essential factor in cellular process because of its direct influence on cell growth, differentiation and migration [24, 25]. Figure 3a, b shows SEM images of cells, respectively, on GA1 and GA2 after 6 h, and Fig. 4a, b depicts their SEM images after 48 h. Cells on GA1 and GA2 after 6 h showed good attachment of cells to these samples, and after 48 h, cells displayed a typical morphology, with a spread shape and cytoplasmic extensions. Cell morphology on the surface of the film, immersed in 0.05% anthocyanin solution, was also investigated. As can be seen in Fig. 5, after 6 h, only a few amount of cells are on the surface as a result of rapid release of anthocyanin, confirming that the number of attached cells is significantly lower than the number on the GA1 and GA2. Also, cell spreading after 48 h is not satisfying.

Bacterial culture and color changing

After co-culturing of gram-positive bacteria and gram-negative bacteria, the resultant supernatant was collected after 8, 16 and 24 h and added to colored GA1 hydrogels. It was observed that with increasing bacterial culturing time,

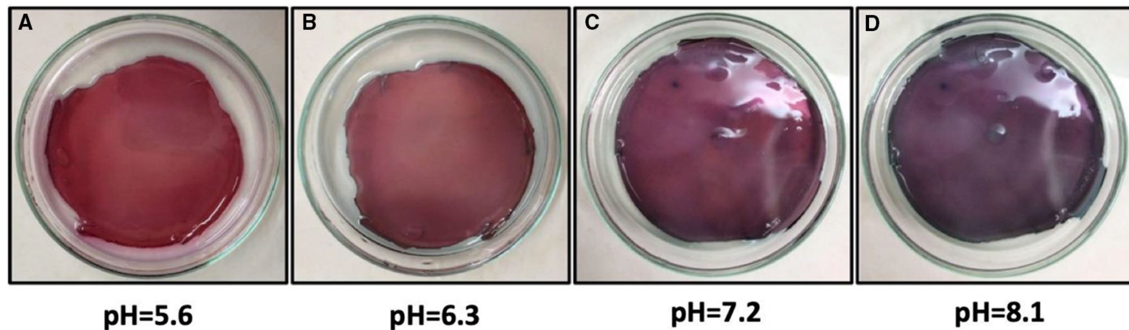

Fig. 6 In vitro colorimetric detection of bacterial infection through exposing the hydrogels to bacterial supernatant. **a** Before supernatant addition, **b–d** after addition of the supernatant collected after 8 h, 16 h and 24 h, respectively

the supernatant was able to make more color changes. Figure 6a exhibits hydrogel color before being introduced to the supernatant. Figure 6b shows change in the hydrogel color after addition of the supernatant collected after 8 h of bacterial activity. Also, Fig. 6c, d, respectively, displays the color change after 16 and 24 h.

Discussion

The gelatinous hydrogel introduced here is a multifunctional scaffold that can be used for detecting infections and measuring progress in wound healing. It has both diagnostic and therapeutic effects.

Infection and wound colonization have the same signs and symptoms, so distinguishing them from each other is difficult. For instance, inflammation which is a common sign for both infection and wound colonization is a vital immune response and necessary to remove pathogens. On the other hand, immunocompromised patients may not have signs of inflammation or infection [26]. Confirmation of an infection is carried out by colony count which is costly, and it must be done by trained personnel [27]. However, the use of this hydrogel as wound dressing is easy and comfortable for patient to change the wound dress by observing the color changes and infection. Wound pH is a marker for monitoring infection, and this hydrogel uses pH as a marker for detection of infection. The bacterial co-culture supernatant (*P. aeruginosa* and *S. aureus* are the two most prevalent bacteria in infected wounds) was added to hydrogel, and the supernatant pH was measured every 8 h with a pH probe. The pH increased with time and led to a more intense color change. The supernatant of bacterial culture could make a clear change in the color of hydrogel. These dressings also showed no toxicity in contact with mouse fibroblast cells (L929), making them a suitable candidate for the treatment of dermal injuries. It was observed that adding anthocyanin could reduce the negative effects of glutaraldehyde and increase cell viability. However, the excessive addition of anthocyanin reduced the cell viability, indicating a maximal tolerable dose for anthocyanin, like any other substance. Anthocyanins are strong antioxidants since they can give their electrons to free radicals and act as free radical scavengers [17]. Investigations have shown that anthocyanins from black soybean seed coats could promote wound healing by increasing the production of VEGF, improving cell migration, preventing excessive and persistent inflammation and inhibiting ROS generation [21, 28].

In this study, it was shown that anthocyanins are capable of eliminating the remaining free radicals of aldehydes in biomaterials. So, the dressing introduced here promotes the healing process for the following reasons: (1) It helps to maintain a moist environment for the wound; (2) it reduces the health care costs caused by patient's examination and replacement of wound dressings; and (3) it can be integrated with antiseptics and antibiotics for localized drug delivery.

Conclusion

The present article studies the ability of gelatinous hydrogel integrated with anthocyanin for early detection of infection and L929 cell behavior. The need for detection of infection stems from the complexity of not just the dynamic wound infection and healing biochemistry but also the distinguishing normal wound drainage from initial inflammatory response and from infectious process. The obtained results revealed that low doses of anthocyanin increased the viability and cell adhesion by eliminating the aldehyde free radicals. Apart from being able to detect the infection, anthocyanin in hydrogel could be effective in wound healing. The ability of early detection of infection reduces cost and unnecessary use of antibiotics. Our efforts are now focused on trying to increase the sensitivity of this system by integrating more sensing elements and improving the performance of wound dressing by the addition of antiseptics.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

Ethical approval This paper does not contain any studies with human or animal subjects.

References

1. Koh A, Kang D, Xue Y, Lee S, Pielak RM, Kim J et al (2016) A soft, wearable microfluidic device for the capture, storage, and colorimetric sensing of sweat. Sci Transl Med 8(366):366ra165
2. Sussman C, Bates-Jensen BM (2007) Wound care: a collaborative practice manual. Lippincott Williams & Wilkins, Philadelphia
3. Hendriks F, Brokken D, Oomens C, Bader D, Baaijens FPT (2006) The relative contributions of different skin layers to the mechanical behavior of human skin in vivo using suction experiments. Med Eng Phys 28(3):259–266
4. Myers BJUSRN (2004) Wound management principles and practice. Prentice Hall, Upper Saddle River
5. Ayati Najafabadi SA, Shirazaki P, Zargar Kharazi A, Varshosaz J, Tahriri M, Tayebi L (2018) Evaluation of sustained ciprofloxacin release of biodegradable electrospun gelatin/poly (glycerol sebacate) mat membranes for wound dressing applications. Asia Pac J Chem Eng 13(6):e2255
6. Grice EA, Segre JA (2011) The skin microbiome. Nat Rev Microbiol 9(4):244
7. Percival SL, McCarty S, Hunt JA, Woods EJJWR (2014) The effects of pH on wound healing, biofilms, and antimicrobial efficacy. Wound Rep Regerer 22(2):174–186
8. Dargaville TR, Farrugia BL, Broadbent JA, Pace S, Upton Z, Voelcker NHJB et al (2013) Sensors and imaging for wound healing: a review. Biosens Bioelectron 41:30–42
9. Cefalu JE, Barrier KM, Davis AHJCCNC (2017) Wound infections in critical care. Crit Care Nurs Clin 29(1):81–96
10. Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JWJWR et al (2012) A review of the scientific evidence for biofilms in wounds. Wound Rep Regerer 20(5):647–657
11. Healy B, Freedman AJBBMJ (2006) ABC of wound healing: infections. BMJ 332(7545):838
12. Schneider LA, Korber A, Grabbe S, Dissemond J (2007) Influence of pH on wound-healing: a new perspective for wound-therapy? Arch Dermatol Res 298(9):413–420
13. Hoštacká A, Čižnár I, Štefkovičová M (2010) Temperature and pH affect the production of bacterial biofilm. Folia Microbiol 55(1):75–78
14. Choung M-G, Baek I-Y, Kang S-T, Han W-Y, Shin D-C, Moon H-P et al (2001) Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). J Agric Food Chem 49(12):5848–5851
15. B'kowska-Barczak A (2005) Acylated anthocyanins as stable, natural food colorants–a review. Pol J Food Nutr Sci 14:55
16. Chandra P, Arora DS, Valentin H, Qi Q, Kumar A, Chattopadhyay S et al (2005) Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Asian J Biotechnol 9(1):436–444
17. Khoo HE, Azlan A, Tang ST, Lim SMJF (2017) Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr Res 61(1):1361779
18. dePascual-Teresa S, Sanchez-Ballesta MT (2008) Anthocyanins: from plant to health. Phytochem Rev 7(2):281–299
19. Pala C, Sevimli-Gur C, Yesil-Celiktas OJFAM (2017) Green extraction processes focusing on maximization of black carrot anthocyanins along with cytotoxic activities. Food Anal Methods 10(2):529–538
20. Olsson ME, Gustavsson K-E, Andersson S, Nilsson Å, Duan RD (2004) Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. J Agric Food Chem 52(24):7264–7271
21. Nizamutdinova IT, Kim YM, Chung JI, Shin SC, Jeong Y-K, Seo HG et al (2009) Anthocyanins from black soybean seed coats stimulate wound healing in fibroblasts and keratinocytes and prevent inflammation in endothelial cells. Food Chem Toxicol 47(11):2806–2812
22. Priprem A, Damrongrungruang T, Limsitthichaikoon S, Khampaenjiraroch B, Nukulkit C, Thapphasaraphong S et al (2018) Topical niosome gel containing an anthocyanin complex: a potential oral wound healing in rats. AAPS PharmSciTech 19(4):1681–1692
23. Kammerer D, Carle R, Schieber AJEFR (2004) Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. sativus var. atrorubens Alef.) and evaluation of their color properties. Eur Food Res Technol 219(5):479–486
24. Matsunaga T, Iyoda T, Fukai F (2014) Adhesion-dependent cell regulation via adhesion molecule, integrin: therapeutic application of integrin activation-modulating factors. In: Colloid interface science in pharmaceutical research and development, Elsevier, pp 243–260
25. Khalili AA, Ahmad M (2015) A review of cell adhesion studies for biomedical and biological applications. Int J Mol Sci 16(8):18149–18184
26. Roupé KM, Nybo M, Sjöbring U, Alberius P, Schmidtchen A, Sørensen OE (2010) Injury is a major inducer of epidermal innate immune responses during wound healing. J Investig Dermatol 130(4):1167–1177
27. Bryant R, Nix D (2015) Acute and chronic wounds-E-book. Elsevier Health Sciences, Amsterdam
28. Xu L, Choi TH, Kim S, Kim S-H, Chang HW, Choe M et al (2013) Anthocyanins from black soybean seed coat enhance wound healing. Ann Plast Surg 71(4):415–420