ORIGINAL ARTICLE

Expression of fibronection and fibroblast growth factor in rats cutaneous wound given green coffee bean extract

Humaryanto, MD[1], Ave Olivia Rahman, M.Sc[2], Fairuz Quzwain, MD[3]

[1]Department of Surgery, Faculty of Medicine and Health Sciences, University of Jambi, Jambi, Indonesia, [2]Department of Pharmacology, Faculty of Medicine and Health Sciences, University of Jambi, Jambi, Indonesia, [3]Department of Pathology, Faculty of Medicine and Health Sciences, University of Jambi, Jambi, Indonesia

ABSTRACT

Background: The application of green coffee bean extract is known to accelerate cutaneous wound healing. Fibronectin and fibroblast growth factors (FGFs) are essential in the wound healing process. However, data on the effect of the green coffee bean extract on fibronectin and FGFs are still limited. Objective: This study aimed to determine the effect of the green coffee extract on the expression of fibronectin dan FGFs in rats' cutaneous wounds.

Materials and Methods: Forty male Sprague Dawney rats, aged 2–3 months, weighing 150–200 grams, were randomly divided into four groups. Cutaneous wounds were made 1.5 cm in diameter and under lidocaine anaesthesia. Group I without treatment was the control group, group II was given a green coffee extract dose of 15%, group III was given a green coffee extract dose of 30%, and group IV was given a green coffee extract dose of 100%. The treatment was applied every day without wound debridement. In each group, five rats were sacrificed after 7 days of treatment (proliferative phase), and the rest were sacrificed after 16 days of treatment (remodelling phase). An anatomical pathologist carried out the immunohistochemical examination to assess fibronectin and FGF expression using a blind method.

Results: The expressions of fibronectin and FGF in the treatment groups were slightly higher than those in the control group, both in the proliferative and remodelling phases. Only, fibronectin expression of the green coffee dose of 100% was significantly higher than the control group in the remodelling phase.

Conclusion: The application of green coffee bean extract in cutaneous wounds could increase fibronectin expression.

KEYWORDS:
fibroblast growth factor, fibronectin, green coffee bean extract, FGF, wound healing

INTRODUCTION

A surgical dressing is a vital step in the post-operative care of surgery patients, including in orthopaedic surgery. As orthopaedic wounds are inherently complex, it is imperative that surgical dressing fulfil the needs of wound care management and accelerate wound healing. Some surgical dressings have been used in orthopaedic surgery, such as antimicrobial dressings, silver, zinc oxide, titanium oxide, and iodine. However, there are reports of bacterial resistance to antimicrobial dressings or other surgical dressings, which increase the risk of wound infection and slow wound healing.[1] Therefore, an alternative wound dressing is needed. One of the traditional treatments for open wounds is applying coffee grounds.[2,3] Coffee beans contain antioxidants, such as phenolic acid, polyphenol, and chlorogenic acid.[4] In a previous study, green coffee bean extract (Coffea canephora) could increase wound contraction and the number of fibroblast and blood vessels.[5,6]

Wound healing is a complex and dynamic process that involves the interaction of various cells and molecules. Given that fibronectin, an adhesive molecule, plays an essential role in wound healing, it mediates various cellular interactions with the extracellular matrix.[7] Fibronectin facilitates fibroblast and other cells migration from periwound to the wound bed and epithelial cells over the new basement membrane. A study has shown that applying fibronectin on rats' wounds effectively speeds healing.[8] Fibronectin creates scaffolding that facilitates the fibrogenesis of collagen. The fibronectin cross-links the collagen fibre and contributes to matrix stability. Fibronectin also serves as the anchor points for myofibroblast involved in wound contraction. In normal wound healing, fibronectin plays a role in all phases of wound healing.[9] Another essential molecule in wound healing is fibroblast growth factor (FGF). FGFs stimulate migration and proliferation of fibroblast to wound area. Fibroblast begins to proliferate and produce fibronectin along with collagen.[10]

However, the effect of green coffee bean extract on the expression of fibronectin and FGF of cutaneous wound healing has not been determined. Therefore, this study aimed to assess the effect of green coffee bean extract on the expression of fibronectin and FGFs on the proliferative and remodelling phase of the extracellular matrix in cutaneous wound healing.

MATERIALS AND METHODS

Extraction and preparation of ointment

This study used green Robusta coffee beans Coffea canephora Pierre ex A. Froehner, as identified by the Biotechnology and Engineering Laboratory, University of Jambi. The beans were dried in an oven at 40°C for 24 hours and then crushed using a blender. The maceration extraction method was done using

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Corresponding Author: Ave Olivia Rahman
Email: aveoliviarahman@unja.ac.id

70% ethanol as a solvent with a ratio of 1:3, 500 grams of coffee bean powder, and 1500 mL of 70% ethanol. The solution was macerated for 24 hours and stirred occasionally. The solution was filtered using Whatman paper size 41 to get the filtrate, which was then concentrated with a rotary evaporator. Vaseline was used as a base for making 15% and 30% ointment of green coffee extract. The 15% ointment was made by mixing 15 grams of extract homogeneously with Vaseline up to 100 grams, the 30% ointment was made by mixing 30 grams of extract homogeneously with Vaseline up to 100 grams, and the 100% ointment was the extract without Vaseline addition. The ointment manufacture was started by completely melting the Vaseline and adding a certain extract weight as mentioned above.

Wounding model

This study has been approved by the Ethics Committee of Medical and Health Science Faculty, University of Jambi, under the ethical clearance number 713/UN21.6/LT/2018. This study used healthy male Sprague Dawley strain rats, aged 2–3 months, weighing 150–200 grams. The fur on each left back of the rat was shaven, and a full-thickness incision was made with a diameter of 1.5 cm using a scalpel and scissors under subcutaneous anaesthesia using lidocaine around the incision area. The topical ointment was immediately applied to the wound according to the treatment of each group. A total of 10 rats were used for each group. In group I as control, no dressing was applied to the wound. Group II was given ointment of green coffee extract 15%, group III was given ointment of green coffee extract 30%, and group IV was given green coffee extract 100% on the wound. The wounds were left open during the experiment. The treatments were held once a day without wound debridement. However, every time the extract would be applied, the wound was carefully cleaned from the remaining ointment previously given without disturbing the wound. All rats were housed in plastic cages containing two rats each at room temperature (25°C) and 50–80% humidity with a 12-hour cycle variation between light and dark. All rats were given a standard diet and water ad libitum.

Wound contraction

Wound areas were measured daily by tracing the wound area using transparent graph paper and determining its size. The evaluated wound surfaces were used to calculate the percentage of wound contraction by taking the initial size of the wound as 100%. Percentage wound retraction was determined by an equation, (wound area on day 0 – wound area on Nth day)/(wound area on day 0) × 100%.[11]

Sample preparation

Five rats from each group were sacrificed using anaesthesia on the 7th day (proliferation phase) and the 16th day (remodelling phase). Wounded skin samples were taken. Samples were collected in formalin fixative, embedded in paraffin, and cut into free-floating sections of 10 to 15 µm thickness. Paraffin sections were deparaffinised, rehydrated, and then placed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 0.3% Triton X-100.

Antibodies

Anti-Fibronectin (Medaysis, F14) was used as the primary antibody for fibronectin and anti-FGF2/BFGF (Medaysis, C2) as the primary antibody for FGF.

Immunohistochemical procedures

Immunohistochemical staining was manually done to determine selected biomarker expression in the stroma using DAB horseradish chromogen. Thick sections (4 µm) were placed on a positively charged slide and heated at 60°C for 30 minutes. Deparaffinisation in xylene and rehydration was done using alcohol and followed by treatment by administering antigens for 40 minutes at 98°C. The sections were then cooled at room temperature and incubated with a blocking agent, hydrogen peroxide, for 10 minutes, followed by primary antibody incubation for 30–60 minutes. The immunolabelling assessment was carried out by one anatomical pathologist using a blind method. Fibronectin and FGF stainings were interpreted as positive when extracellular matrix, cytoplasm, or membrane staining were detected. Positive cells were evaluated in 10 high-power (40x) for each histology section and counted as positive cell percentage. The scoring was as follows: negative or clear immunoexpression = 0–25% positive cells, weak immunoexpression = 26–50% positive cells, strong immunoexpression = >50% positive cells. Positive control of fibronectin was the placenta, and for FGF2 was the brain.

Statistical analysis

Statistical analysis for each parameter was described as the mean value ± standard deviation (SD). The parametric data were analysed using one-way ANOVA and continued with the Least Significant Difference (LSD) test. The non-parametric data were analysed by the Kruskal Wallis test and continued with the Mann Whitney test. The significance level was set at $p < 0.05$.

RESULTS

The group given coffee bean extract 15% had the highest percentage of wound contraction on the 7th day. LSD test analysis showed a significant difference between the groups given coffee extract 15% and coffee extract 30%, also between the groups given coffee extract 15% and coffee extract 100%, but not significant between the groups given coffee extract 15% and the control group. However, the percentage of wound contraction on day 16 in the group without treatment had not reached 100%, because there were wounds not closed completely. Daily observations of wound contraction in each group are shown in Figure 1.

The expressions of fibronectin and FGF in each group on day 7 and day 16 are described in Figure 2.

Table I describes the expressions of fibronectin and FGFs on the 7th day, where the proliferative phase of wound healing took place. Fibronectin expression in the group obtaining coffee extract 15% did not differ from the control group. In contrast, fibronectin expressions in groups receiving coffee extracts 30% and 100% were higher than in the control group. An increased extract concentration did not cause increasing fibronectin expression. FGF expression in the

Table I: Distribution of fibronectin and FGF expressions on cutaneous wound healing on the 7th and 16th days of each group

Group	Day	Fibronectin expression			Mean score ± SD	P-value	FGF expression			Mean score ± SD	P-value
		Weak	Clear	Strong			Weak	Clear	Strong		
Without treatment	7	3 (60%)	2 (40%)	0	1.40 ± 0,54	-	3 (60%)	2 (40%)	0	1.40 ± 0.54	-
	16	3 (60%)	2 (40%)	0	1.40 ± 0.54	-	3 (60%)	2 (40%)	0	1.40 ± 0.54	-
Coffee extract 15%	7	3 (60%)	2 (40%)	0	1.40 ± 0.54	1.000	2 (40%)	3 (60%)	0	1.60 ± 0.54	0.549
	16	1 (40%)	4 (60%)	0	1.80 ± 0.44	0.221	2 (40%)	3 (60%)	0	1.60 ± 0.54	0.549
Coffee extract 30%	7	0	5 (100%)	0	2.00	0.050	0	5 (100%)	0	2.00	0.050
	16	0	5 (100%)	0	2.00	0.050	1 (20%)	2 (40%)	2 (40%)	2.20 ± 0.83	0.118
Coffee extract 100%	7	0	5 (100%)	0	2.00	0.050	0	5 (100%)	0	2.00	0.050
	16	0	2 (40%)	3 (60%)	2.60 ± 0.54	0.020*	1 (20%)`	4 (80%)	0	1.80 ± 0.44	0.221

P-value by the Mann Whitney Test compared to the control group (without treatment); * significant at $p < 0.05$

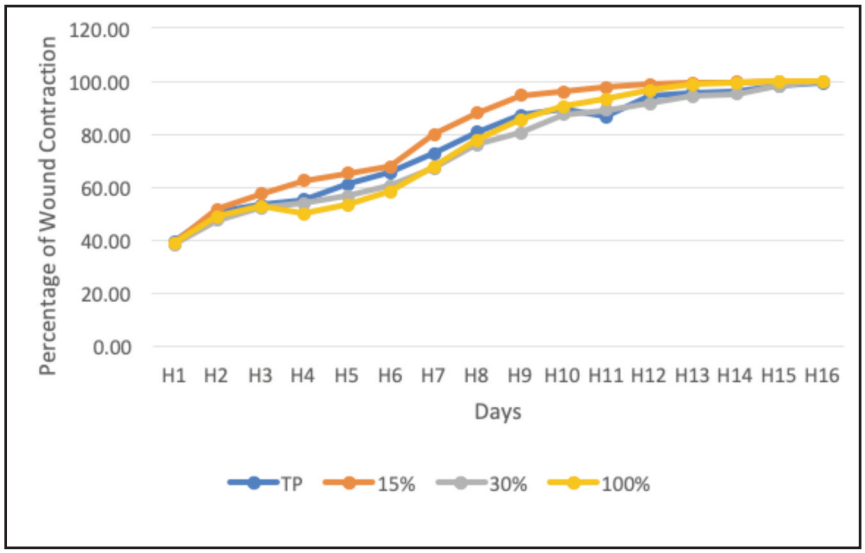


Fig. 1: Mean of wound contraction percentage in each group for 16 days of observation. TP= without treatment.

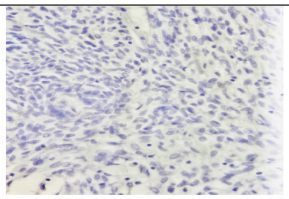
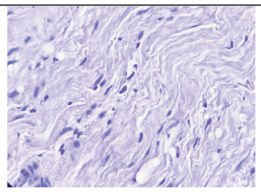
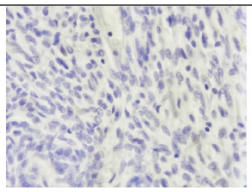
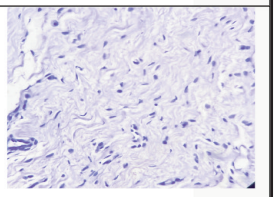
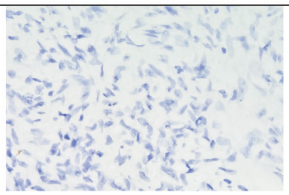
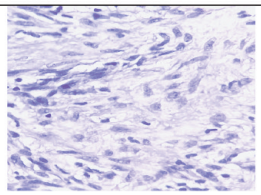
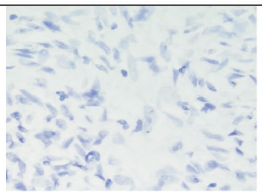
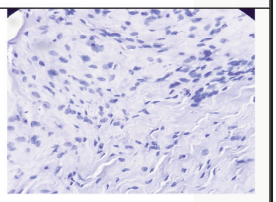
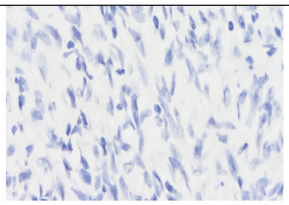
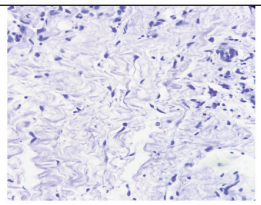
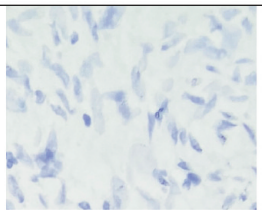
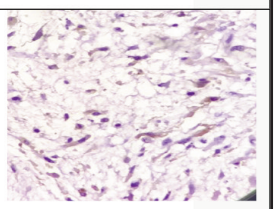
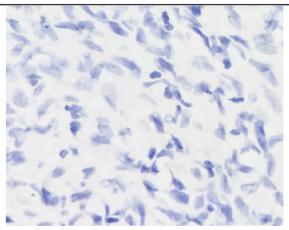
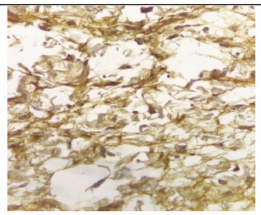
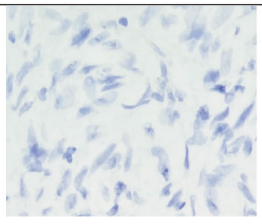
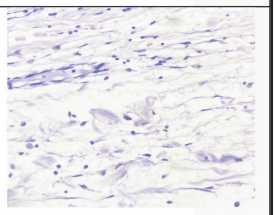
Group	Fibronectin Expression		FGF Expression	
	Day 7	Day 16	Day 7	Day 16
Without treatment				
Coffee extract 15%				
Coffee extract 30%				
Coffee extract 100%				

Fig. 2: Expressions of fibronectin and FGF in each group at day 7 and day 16 with 100x magnification.

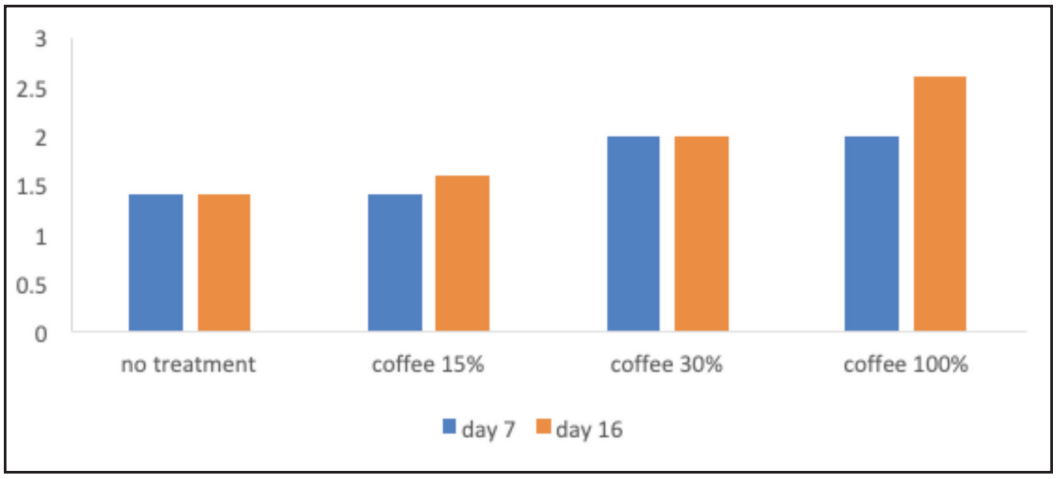


Fig. 3: Mean score of fibronectin expression in each group. Fibronectin expression tends to increase on the 16th day than on the 7th day. On the 16th day in the group obtaining coffee extract 100%, the fibronectin expression was the highest.

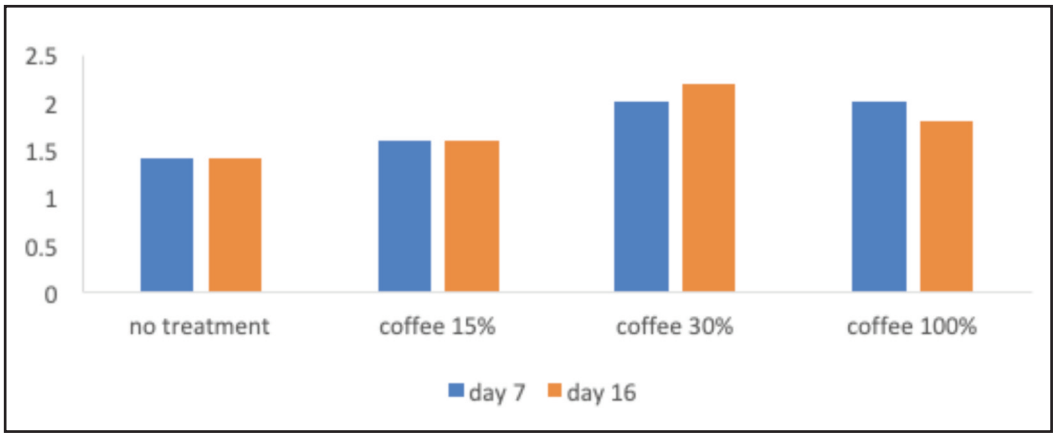


Fig. 4: Mean score of FGF expression in each group. FGF expression tends to be stable on the 16th day compared to the 7th day. There was an increase in FGF expression in the group obtaining coffee extract 30% on the 16th day compared to that on the 7th day, while there was a decrease in the group receiving coffee extract 100%.

treatment group was higher than that in the control group. When the extract was increased from 15% to 30%, FGF expression increased, but when the extract was increased to 100%, FGF expression did not increase. Table 1 also describes fibronectin and FGF expressions on the 16th day, where the remodelling phase of wound healing took place. Fibronectin expression in treatment groups was higher than in the control group. Fibronectin expression in the group given coffee extract 100% was significantly higher than in the control group (Figure 3). FGF expressions in treatment groups were higher than in the control group (Figure 4).

DISCUSSION

Fibronectin emerges in various phases of wound healing. Fibronectin plays an essential role in wound healing, so the lack of fibronectin can cause poor wound healing. Fibronectin is active through all stages of wound healing. In the first phase, plasma fibronectin helps form the clot and assembles an extracellular matrix. Then, platelets help to change plasma fibronectin into fibrillar form.[12] In the inflammatory phase, fibronectin is degraded by proteolytic enzymes and may be oxidatively cross-linked. Fibronectin has a collagen-binding domain that mediates various cellular interactions with the extracellular matrix. However, the synthesis of new fibronectin is activated at the bottom of the wound. The fibronectin serves as an attachment site for the movement of fibroblast and epithelial cells. Fibronectin also serves as the anchor points for myofibroblast involved in wound contraction. The fibronectin cross-links the collagen fibre and contributes to the stability of the matrix.[12,13] Applying fibronectin on the wound of rats effectively speeds the healing.[8] Fibronectin matrix deposition in wound stimulates collagen deposition and contributes to wound contraction. During the proliferation phase, fibronectin is associated with the type III collagen matrix, but collagen type III is then remodelled into type I collagen. Fibronectin creates scaffolding that facilitates the fibrogenesis of collagen.[14] Cellular fibronectin expression in skin wound is induced by TGF-β (transforming growth factor β) and is mediated by CCN2 (cellular communication network factor 2).[12] Fibronectin can bind to many growth factors, such as

TGF β, PDGF, VEGF, and FGF. Therefore, fibronectin can act as a reservoir for growth factors.[15,16] Fibronectin also regulates the lysyl oxidase (LOX), a proteolytic enzyme responsible for covalent cross-linking of collagen fibrils into mature collagen.[10] However, excessive fibronectin can cause abnormal wound healing.[12] In the remodelling phase, the fibronectin matrix will be turned by a disintegrin and metalloproteinase (MMP).[17] Matrix remodelling at the wound depends on MMP and MMP inhibitors. Fibronectin binding to integrin will mediate re-epithelialisation of the keratinocytes.[12]

FGFs have a role in cell migration for tissue formation during wound healing. FGFs play essential roles in the migration of cells since FGFs stimulate the proliferation of fibroblast and angiogenesis. Fibroblast expresses a dermatan sulphate. It seems that FGFs bind to an iduronic acid part of dermatan sulphate.[10] FGF1 and FGF2, which are the subtypes of FGFs, are known to be highly released by damaged endothelial cells and macrophages at wound sites. Syndecans are heparin sulphate proteoglycans in the cell membrane that acts as a cofactor for FGF2 to bind their receptors. Heparin sulphate also protects FGFs from proteolysis, prolonging FGF activity.[16] Dermatan sulphate is responsible for mediating FGF responsiveness in the fibroblast.[10] CCN2 that is upregulated during tissue injury increases fibroblast expression of collagen, MMP, and FGFs.[10] FGF7 and FGF10 play a role in stimulating the migration and proliferation of keratinocytes.[18] Applying FGF2 and FGF10 on a wound could accelerate wound healing.[19]

Continuous applications of green coffee bean extract doses of 30% and 100% in this study could increase the expression of fibronectin on the 7th day; and on the 16th day, the fibronectin expressions on groups treated with green coffee bean extract dose of 100% were significantly higher than the control group ($p < 0.05$). It appears that components in green coffee beans extract could increase wound healing by upregulating fibronectin expression. Two possibilities can occur, either increased synthesis stimulation by TGF-β or inhibition of MMP activity. Study of chlorogenic acid, one of the major components in coffee beans, indicated that chlorogenic acid increased the expression of MMP in bone cells.[20] However, fibronectin degradation can last up to day 21.[14] The effect of green coffee bean extract on FGF expression was not statistically different compared to the control group on the 7th and 16th days. FGF is a paracrine growth factor secreted highly by damaged endothelial cells and macrophages at wound sites, and FGF has a short life.[18]

Vaseline was used for composing 15% and 30% extract concentrations because Vaseline is one of the safe and cost-effective moisturizers. The selection of a semisolid base influences the transdermal delivery of active substances.[21,22] The differences in the effect of each dose of green coffee bean extract were probably due to the delivery factor of the active compound throughout the Vaseline into the wound area. Percentages of wound contraction in the treatment groups were higher than in the control group. The limitation of this study was that the number of samples was small.

CONCLUSION

Coffee beans are used as a traditional wound medicine. Fibronectin and FGF are two important components in the wound healing process. Here, we assessed the effect of green coffee beans in vaseline base on the expression of these two components in cutaneous wounds by immunohistochemistry. This research concludes that the application of green coffee bean extract to cutaneous wounds could modulate fibronectin expressions.

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CONFLICT OF INTEREST DECLARATION

The authors state that there is no conflict of interest to declare.

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