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# A pilot study on wound healing using an antibacterial steroidal saponin

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## ABSTRACT

Cost effective primary and secondary wound care is needed and have significant importance in current medicine. The steroidal saponin Flabelliferin B with a UV active binder attached ($F_B$), isolated from palmyrah (*Borassus flabellifer* L.) has a known structure and proven antibacterial activity. The objectives of the study were to (i) study the effects of $F_B$ on male Wistar rats in relation to toxicity by wound healing (ii) study the toxicity of $F_B$ by the eye tests on rats and rabbits (iii) test the allergenic reactions by topical application of $F_B$ on healthy human skin by the "patch test" (iv) evaluate the feasibility of full scale clinical trial of the $F_B$ on infected wounds of humans. Toxic or other adverse effects on animals were not observed. Allergic reactions were also not observed on normal healthy human skin by the patch test. Having obtained ethical approval a prospective clinical trial was carried out in Colombo South Teaching Hospital. An ointment of 2% $F_B$ was tested on wounds and ulcers with no subcutaneous tissue involvements. Human volunteers (n = 14) were employed in this study. Wound healing rates after treatment of $F_B$ ointment were monitored by measuring the percentage decline in wound area with time using normal standard hospital treatment as control. Microbiological tests by swabbing were carried throughout these experiments. The rate of wound healing was not significantly different from current hospital treatment ($p = 0.512$) and no allergic or other adverse symptoms were shown. It is concluded that it may be possible to use $F_B$ in treatment of wounds and ulcers with no subcutaneous involvement.


**Keywords:** Palmyrah, Flabelliferin B, Ulcers, Toxicity, Antibiotic.

## INTRODUCTION

Cost effective primary and secondary wound care is needed and have significant importance in current medicine. Chronic wounds are a major problem encountered in the day to day medical practice (Simon et al., 1996). Wound healing is a complex process which is interpreted by many underlying factors such as vascular diseases, infections, metabolic diseases including diabetes mellitus, immunosuppression, unrelieved pressure and malnutrition (Harding et al., 2002; Usui et al., 2005). Infection is one that could be easily controlled with appropriate local antibiotic applications provided. They are safe and effective (Howell-Jones et al., 2005). This paper describes the search into the preparation, use and application and effectiveness of a local application made from an isolate of palmyrah fruit pulp known to contain antibacterial properties.

Flabelliferin B isolation was reported from palmyrah (*Borassus flabellifer* L.) fruit pulp (Nikawala et al., 1998a). Flabelliferin B has a steroidal saponin structure with a molecular weight of 868 which was elucidated as β-sitosterol with a β-glucosyl group and two rhamnosyl groups (α 1, 2 and α 1, 4)

attached to the OH at C-3 of the aglycone (Ariyasena et al., 2002). Flabelliferin ($F_B$) has proven activity against bacterial species such as *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeroginosa, Proteus rettigeri and Acinetobacter calcoacetius* (Nikawala et al., 1998b). Furthermore $F_B$ can inhibit the activity of yeast (Nikawala et al., 1998b). A naturally occurring UV active carotenoid is complexed to flabelliferin and enhances the antimicrobial activity of this molecule against *E. coli* (Uluwaduge et al., 2005). The evidence from computational chemistry (Jayaweera et al., 2004) has supported the molecular association between flabelliferins and the UV active carotenoid. Thus the objectives of the study were to (i) study the effects of $F_B$ on male Wistar rats in relation to toxicity by wound healing (ii) study the toxicity of $F_B$ by the eye tests on rats and rabbits (iii) test the allergenic reactions by topical application of $F_B$ on healthy human skin by the "patch test" (iv) evaluate the feasibility of full scale clinical trial of the $F_B$ on infected wounds of humans.

## MATERIALS AND METHODS

### Separation of flabelliferin B

Animal and human experimental trials were carried out to determine the efficacy of a local topical application based on $F_B$. Depending on the geographical location of the palmyrah tree the $F_B$ content of the fruit is known to vary. Kalpitiya and the Mannar plants which are located in the North-West of Sri Lanka and are known to be rich in $F_B$, were used in this study for the preparation of an ointment. $F_B$ containing the binder was extracted according to methods described previously (Ariyasena et al., 2000). Pure preparations were stored in a freezer until used in experiments.

### Animal experiments

These are carried out to assess the effect of the test preparation on the rat wound healing and to ascertain the presence of any local or systemic toxic effects. Ethical clearance was obtained from the Ethical Review Committee, University of Sri Jayewardenepura, before commencement of the study.

***Effects on rats***

Male Wistar rats (n = 8) aged between 5 - 7 months were obtained from Medical Research Institute (MRI), Colombo 08 and maintained under standard conditions (RIVM & WHO, 1999). Commercially available animal feed and water were provided *ad libitum*. A piece of skin of about 9 mm diameter was removed from either side of the vertebral column of each rat using surgical scissors and scalpel under mild anesthesia in order to create wounds under experimental conditions. The animals with the above wounds were left for 24 hours to allow for natural infection. One wound was used as the test and the other as the control. Test wounds were treated with 25 µL from 18 mg/mL concentration of $F_B$ dissolved in saline twice a day for four days. The control wound was treated using equal amount of saline. Two animals without wounds were observed for systemic effects. Wound measurements, photographs, wound swabs for micro-organisms and independent observations for wound healing were obtained.

***Toxicity on rat eye***

After a flush-out period of one week the same animals were subjected to the "eye test" to evaluate ocular toxicity of isolated $F_B$. 25 µL from 18 mg/mL concentration of $F_B$ dissolved in saline was introduced in to one eye (test) daily while the other eye (control) was treated with saline for two days. $F_B$ treated animals were observed for eye color, increased tearing, blinking of eyes, wiping and washing of the test eye in comparison to the control for three consecutive days. In addition, for evaluation of possible systemic toxic effects, two untreated rats were observed for any behavioral changes.

***Toxicity on rabbit eye***

New-Zealand white rabbits (n = 6, 3 pairs) aged between 6- 10 months were obtained from MRI, Colombo 08. Animals were fed with commercial animal feed together with green leaves and had free access to water *ad libitum*. A 12 hours light/dark cycle was maintained to maintain the metabolic rates (RIVM & WHO, 1999). Single blind animal trials were carried out to evaluate toxicity by a modified method similar to that of Clark et al. (2004). A solution of 50 µL (from ~ 60 mg/mL concentration of $F_B$) dissolved in isotonic

saline was introduced to the test eye twice a day for 3 days. In each animal the other eye was its control and controls were treated using only isotonic saline. Ophthalmoscopic observations which were directed to identify any conjunctivitis, inflammation, purulent discharges, hyperemia, and sub-conjunctival hemorrhages, were independently carried out by a panel of four medically qualified personnel and two qualified veterinarians. Animals were observed for behavioral changes for more than 6 hours daily.

**Patch test**

A preliminary study was carried out before application of ointment on wounds using human volunteers. A panel of human volunteers (n = 7) was selected for patch testing with $F_B$ on normal skin. Volunteers were given two plasters (1 × 1 $cm^2$) one soaked with 50 µL of 74 mg/mL of $F_B$ in distilled water as test and another soaked with water for control. The volunteers were unaware of the test and the control patches. Plasters were placed on the skin of the biceps muscles and independent comments on sensation were elicited.

**Preparation of ointment**

Commercially available white soft paraffin was obtained from the local pharmacy. $F_B$ ointment (50 g of 2% $F_B$) was prepared by gradual mixing of the purified flabelliferins with white soft paraffin under hygienic conditions in a research laboratory. About 2-3 g of prepared ointment were impregnated on 4 cm × 4 cm piece of gauze and covered by aluminum foil until use to avoid any possible destruction of attached UV molecule. Povidone-iodine, Metronidazole and Framycetin sulphate cream (the hospital treatment) obtained from local pharmacy were impregnated on gauze in the same manner and were used as the controls.

**Clinical trial**

Ethical clearance for clinical trial was obtained from Ethical Review Committee, University of Sri Jayewardenepura (Application No.196) and Colombo South Teaching Hospital Ethical Committee (Application No. 023). A prospective clinical trial was carried out in the Colombo South Teaching hospital for 3 months from July 2005. Patients were selected from dermatology clinic, ulcer clinic and the out-patients department. Patients with superficial ulcers without any subcutaneous tissue involvement were selected by physicians. Patients with ulcers due to diabetes, varicose veins and patients with other underlying pathology were not included in the study. Patients were not on any systemic antibiotic therapy throughout the study as well as one-week prior to start of experimental treatment. After detailed explanations of the study, written informed consent was obtained from patients. Patients were instructed to return to the hospital immediately, in case of any adverse effects.

After careful observations by the physician, wound swabs were taken into sterilized containers for microbiological assessments. Hypertonic saline was used to clean the wound (Tierney et al., 2004). Many studies have reported the use of the reduction of the wound size to be a good indicator of healing (Gilman, 2004). Therefore, wound outline was taken on a transparent sheet and photographs were also obtained. Prepared gauze with the $F_B$ preparation/control was placed on the wound and bandaged. The control patients were treated with normal hospital treatment, which was Povidone-iodine solution (out-patient department), Povidone-iodine cream (dermatology clinic) commercial cream containing Metronidazole (ulcer clinic) and Framycetin sulphate (dermatology clinic). Treatments were limited to six double blind cases in the dermatology clinic but due to logistic reasons blinding could not be done in ulcer clinic and out-patient department. Except for admitted patients who were observed daily, others were observed at least twice a week.

All relevant data including, wound swabs, wound measurements and photographs were obtained repeatedly throughout the study period (Gilman, 2004). Wound swabs were transported (2 km) to the University of Sri Jayewardenepura, Nugegoda, Sri Lanka and all microbiological examinations were carried out under standard conditions (Cooper and Lawrence, 1996).

**Statistical analysis**

The significant difference between test and control groups where possible was

evaluated using student t-test at 95% confidence interval (Microsoft® Office 2002–Excel).

## RESULTS

### Animal experiments

***Effects on rats***

Results observed from wound healing study on rats are summarized in Table 1. There was no significant difference between the wound healing rate of the test and control wounds on rats. Four test wound swabs resulted in lower number of colonies compared to controls while others resulted in similar numbers. Microbiological tests carried out indicated that the bacteria were present on all wounds even after the treatment. Adverse effects were not seen when compared to the control animals without injuries and those animals without $F_B$ application, showing no possible systemic effects. Even though there was no significant difference obtained in the rates of wound healing, the absence of any local or systemic adverse effects permitted the clinical use of $F_B$ as an ointment.

***Toxicity on rat and rabbit eye***

This has been confirmed by rabbit eye test which is the gold standard for systemic toxicity, where opthalmoscopic observations were carried out. A panel of six medical practitioners was unable to observe any difference after 3 days of treatment. Behavioral changes were not observed in both cases. Therefore it was concluded that toxicity was not effected by $F_B$.

### Patch test

Patch test carried out confirmed that there are no adverse effects from application of $F_B$ on normal healthy human skin.

### Clinical trial

Results obtained from human trial are given in Table 2. Wound healing rate is expressed as the percentage reduction of the wound area compared to initial state per week. Wound healing rate for test group had an average of 23.7% per week whereas the controls had an average of 17.5%. Wound healing was clinically significant without any adverse effects. However, this difference was statistically not significant ($p = 0.512$). *Staphylococcus, Pseudomonas* species and Gram +ve bacilli were mainly found in the infected wounds. $F_B$ (4 mg/mL) completely inhibited mixed bacterial cultures in liquid medium under laboratory conditions except for one case, which resulted in slight turbidity. This indicated the inhibition of bacterial growth by $F_B$. However there were no remarkable differences between the colony counts of the same wound throughout the study.

**Table 1:** Effect of $F_B$ on wounds of rats.

| Rat No | Wound size/mm | No of colony types from wound swabs | |
|---|---|---|---|
| | | Before | After |
| 1 Test | 10.0 × 5.0 | B&F | 1 B |
| 1 Control | 7.5 × 5.0 | B&F | 4 B |
| 2 Test | 6.0 × 6.0 | B&F | 1 B |
| 2 Control | 7.5 × 7.5 | B&F | 3 B |
| 3 Test | 8.0 × 6.0 | B&F | 2 B |
| 3 Control | 7.5 × 7.0 | B&F | 3 B |
| 4 Test | 11.0 × 8.0 | B&F | 1 B |
| 4 Control | 12.5 × 6.0 | B&F | 1 B |
| 5 Test | 8.0 × 6.0 | B&F | 2 B |
| 5 Control | 10.0 × 7.5 | B&F | 1 B |
| 6 Test | 11.0 × 7.0 | B&F | 3 B |
| 6 Control | 9.0 × 6.0 | B&F | 3 B&F |

B&F – Both bacterial and fungal infections were observed (Difficult to obtain exact number of colony types), B – Bacteria only.

**Table 2:** Effect of $F_B$ on wound healing in humans.

| Patient No | % Wound healing rate/week * | |
|---|---|---|
| n/n' | Test | Control |
| 1/1' | 58.2(B) | 31.8(B) |
| 2/2' | 26.6(B) | 14.5(B) |
| 3/3' | 12.2 | -3.1(B) |
| 4/4' | 16.7 | 36.2(B) |
| 5/5' | 1.4 | 16.2 |
| 6/6' | 27.2 | 13.9 |
| -/7' | - | 12.7 |
| -/8' | - | 18.3 |
| **Average** | **23.7** | **17.5** |

(B) - Conducted as double blind trials.
* - Wound healing rate is given as the percentage reduction of the wound size compared to its initial size. Test and controls are from different patients and are not statistically significant ($p = 0.512$).
n/n' - Patient No for test/control.

## DISCUSSION

Microbiological colony studies of rat wounds resulted in a lesser number of colonies in the test compared to the controls. However there was no significant difference ($p > 0.05$) in wound healing rates in rats compared to controls. Adverse behavioral effects were not observed in rats. This is possible due to the very high natural healing rate of rats. It has been reported that the wound healing of rats depend on many other factors (Fukao et al., 2000). In addition rats are capable of cleansing the wound, using their fore and hind limbs, even on wounds on either side of the vertebral column.

Rabbit models are widely used in ocular toxicity testing for drugs and cosmetics (Behar-Cohen et al., 2002; Clark et al., 2004). Even though there are few differences between the human and rabbit eye it can be used as a good parameter to evaluate toxicity. Dissolved $F_B$ in saline water on rats and rabbits eyes did not result in any evidence of adverse effects. Application of the prepared ointment on human volunteers confirmed that there is no adverse effect on normal healthy skin.

The $F_B$ ointment prepared also resulted in wound healing without any adverse effects. There was no statistical significance in applying $F_B$ compared to normal hospital treatment and indicates the discovery of a new structure of antibiotic which is important, *vis-à-vis* increasing bacterial resistance to antibiotics. This new compound is a steroidal saponin containing a vital structural moiety for antibacterial activity (Hu et al., 1996; Bacigalupo et al., 2004). The attached UV compound can alter the characteristics of its complex (Uluwaduge et al., 2005). Though there is no supporting scientific data, it is presumed that the binder alters membrane permeability.

The observations made during the trial indicated that the above complex can act in three different ways on the wound. Primarily this can act as a cleanser. This can clean the surface of the wound and the surrounding area to facilitate the wound healing process. After diffusion into the wound, and due to the antibacterial activity, it can inhibit or kill harmful pathogens non-specifically. Inhibition of the mixed cultures obtained from the test wound confirmed the activity of $F_B$ on wound bacteria non-specifically. Any remarkable microbiological differences were not observed in the same wound between visits. A wound contains many micro-environments, and infected pathogens also vary according to these micro-environments. Therefore swabs obtained may not represent the entire pathogen population of the wounds.

It was observed that $F_B$ can act as a cleansing agent as well as a wound debridement agent. This debridement action is similar to the action of proteases or collagenases, which can clean the wound by breakdown of necrotic tissues present in wound (Yaakobi et al., 2004). These three factors act in concert in wound healing. Having all these effects together in one

molecule, increases the potential use of this compound in the treatment of wounds.

This study can only be treated as a pilot study as there is variation of the ulcers in different individuals, since their immunological response varies. Statistical significance is difficult to attain in such studies due to large individual variation in wounds as well as immune system of tests and controls. However the study enhances that the structure of the compound is different to the antibiotics that are currently being used. This, in a situation of increasing antibiotic resistance by bacteria will be of some significance (Palumbi, 2001). Therefore this warrants a full scale population study before implementation.

**Conclusion**

It can be confirmed from the animal and human studies that the application of $F_B$ on infected wounds has no adverse effects. The application of the ointment on wounds appears to be as effective as treatments used in the hospital and warrants a full scale clinical trial.

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