Potentials of *Bridelia ferruginea* stem bark extracts in wound care

Ezike AC,*[1] Akah PA,[1] Udegbunam SO,[2] Igboeme S,[3] Ibe C,[1] Ezeike C,[1] and Emedo H[1]

[1]Department of Pharmacology & Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria
[2]Department of Veterinary Surgery, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria
[3]Department of Clinical Pharmacy & Pharmacy Management, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria

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ABSTRACT

The methanol extract of Bridelia ferruginea Benth (Euphorbiaceae) stem bark obtained by 48 h cold maceration (BFME), was fractionated in a silica gel column to yield the petroleum ether (PF), dichloromethane (DF) and methanol (MF) fractions successively. The effects of the extract and fractions on bleeding, wound contraction and rate of epithelialization of excision wounds, as well as carrageenan-induced leucocyte migration in vivo were evaluated in rats. They were also subjected to antimicrobial assay. The extract and fractions significantly (P<0.05) reduced bleeding time and also reduced the coagulation time of whole rat blood. They significantly (P<0.05) increased the rate of wound contraction and epithelialization of excision wounds; on day 17, extract treated rats showed 99.5 – 100% wound contraction. The extracts reduced leucocyte migration on acute oral administration, and also exhibited in vitro antibacterial activity against Bacillus subtilis, Staphylococcus aureus, Eschericia coli, Pseudomonas aeruginosa, Salmonella typhi and Klebsiella pneumonia with minimum inhibitory concentrations of 12.7, 6.3, 6.3, 12.3, 12.2 and 12.1 mg/ml respectively for BFME, and 25.0, 6.3, 25.3, 12.6, 12.6, 12.6 mg/ml respectively for MF. The results demonstrate that B. ferruginea stem bark exerts beneficial effects in wound management through hemostatic, wound contraction and antimicrobial activities.

Keywords: Hemostasis, wound contraction, epithelialization, excision wound, leucocyte migration, antibacterial.
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INTRODUCTION

Bridelia ferruginea Benth. (Euphorbiaceae) is variously employed as an embrocation for the treatment of bruises, boils, dislocation and burns, an antidote for arrow poison, in the treatment of arthritis, intestinal and bladder disorders, and externally for skin infections and eruptions [1-3]. A decoction of the leaves is used to treat diabetes, as a purgative and vermifuge [4]. The morphology has been described [2,5]. *B. ferruginea* is variously known as "Kirni", "Kizni" (Hausa), "Marehi" (Fulani), "Iralodan" (Yoruba), "Ola" (Igbo), "Ede" (Nsukka), and "Kensange abia" (Boki) [6].

The hypoglycemic [7,8], antithrombotic [9], anti-inflammatory [10-12], erythrocyte membrane stabilizing, vascular permeability inhibitory, antipyretic, analgesic [11], antibacterial, antioxidant and fibroblast growth stimulatory [13], gastroprotective and spasmolytic [5] activities of *B. ferruginea* have been reported.

Rutin - a flavonoid glycoside shown to reduce blood sugar level of fasted rabbits [7], two coumestan flavonoids-bridelilactone and bridelilactoside, as well as aesculetin and scopoletin were isolated from the leaves [14]. Terpenoids, steroids, tannins, saponins, and the flavonoids quercetin-3-glucoside and myricetin-3-glucoside [4,7,

15], β-peltatin, gallocatechin-(4'-O-7)-epigallocatechin, podophyllotoxin and β-peltatin-5-O-β-D-glucopyranoside [16] have been isolated from the bark.

The stem bark of *B. ferruginea* is used to arrest bleeding and manage wounds in South Eastern Nigeria. This study evaluated the potentials of the stem bark in wound care.

EXPERIMENTAL SECTION

Drugs and Chemicals
Methanol, petroleum ether, dichloromethane, dimethylsulphoxide (DMSO), carrageenan (Sigma Aldrich, Germany) and penicillin skin ointment (Drug Field Pharmaceuticals, Nigeria) were purchased from the dealers.

Animals
Adult Sprague-Dawley rats (150-200 g) of either sex bred in the laboratory animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used for the study. The animals were maintained freely on standard pellets and water. All animal experiments were done in compliance with National Institute of Health Guide for Care and Use of Laboratory Animals (Pub No. 85–23, revised 1985).

Extraction of Plant Material
Fresh stem bark of *B. ferruginea* was collected in November, from Adoka in Adoka L.G.A. of Benue State, Nigeria. The plant material was identified and authenticated at the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State.

The stem bark was dried under the shade for 7 days and milled to coarse powder using an electric grinder. The powdered bark (4 kg) was extracted with methanol by maceration at room temperature ($28 \pm 1°C$). Concentration of filtrate in a rotary evaporator at 40°C yielded the methanol extract (BFME; 650 g; 16.25% w/w).

Fractionation of Extract
The BFME (600 g) was fractionated in a silica gel column successively eluted with petroleum ether, dichloromethane and methanol to yield the petroleum ether (PF; 0.598 g; 0.10% w/w), dichloromethane (DF; 1.449 g; 0.24% w/w) and methanol (MF; 545 g; 90.83% w/w) fractions. The PF was not studied further because of its very low yield.

Bleeding Time
Rats of either gender were randomly divided into five groups (n=5). The tail of each rat was cut with a sharp pair of scissors and immediately, a drop of the test substance was applied on the cut simultaneously with the start of the stopwatch. Each group received one drop (0.01 ml) of one of 0.1, 10 and 100 mg/ml of BFME, DF or MF. The control group received either the vehicle (distilled water) or normal saline. The cut was dabbed with a piece of filter paper every 15 s until the paper no longer stained red with blood oozing from the cut. Bleeding time was taken as the time for the first drop of blood to show to the time when the filter paper stopped showing blood stain [17,18].

Coagulation time of whole rat blood
Rats of either gender were randomly divided into five groups (n=5). Each animal was anaesthesized with ether and the thoracic cavity opened to expose the aorta. The aorta was severed and 1 ml of blood quickly withdrawn using a plastic disposable syringe and transferred into clean, uniform sized paraffin coated plastic tubes (2.5 cm diameter) containing 0.5 ml of one of 10, 50 and 100 μg/ml of BFME, DF or MF in distilled water [17,18]. The vehicle and normal saline served as control. The plastic tubes were swirled every 15 s to check the fluidity of the contents. The interval between the introduction of the blood and the time of clot formation was taken as the coagulation time [17].

Excision wound
The excision wound model is usually employed to study the rate of wound contraction and epithelialization. Each of the rats randomly grouped (n=5) was anaesthetized with ether and held in a standard crouch position. A circular seal of 2.5 cm uniform diameter was impressed on the shaved dorsal thoracic central region, and the entire thickness of the skin from the marked area excised [19] to obtain a wound of about 500 mm^2. The wounded animals were topically treated with one of BFME (5, 10 or 20%), or MF (5, 10 or 20%) ointment (using soft paraffin as ointment base). Control animals were also topically treated with penicillin ointment or soft paraffin. The animals were housed individually in metal cages and treated once daily from day zero till the wound healed or up to day 21 post wounding, whichever was earlier. The wound area was measured by tracing the wound margins on a transparent paper on alternate days and compared with the wound area on day 0. The level of wound contraction (%) was calculated using the relation; wound contraction (%) = $[(WA_O - WA_T)/(WA_O)] \times 100$, where WA_O = wound area on

day 0; WA_T = wound area on day t. Epithelialization time was measured as the time for fall of eschar leaving no raw wound [19,20].

Histopathological evaluation of healing wound
On the 10th day a specimen sample of the healing wound was excised from rats for histopathological examination. Briefly, the animals were sacrificed by thiopental anesthesia, and the healing wounds were excised, leaving a 5 mm margin of normal skin around the edges of the wound. The tissue samples were fixed in 10% formol saline and dehydrated in ascending grades of ethanol. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5-6 µm. The sections were de-paraffinized in xylene, taken to water and subsequently stained with haematoxylin - eosin (H and E) and haematoxylin–Van gieson (HVG) for light microscopy respectively [21]. Photomicrographs of the sections were taken using a motic camera fixed onto a light microscope.

Carrageenan induced leucocyte migration
This was done as previously described [22]. Rats of either gender were divided into six groups to receive oral administration of BFME (200 and 400 mg/kg) or MF (200 and 400 mg/kg). Control groups received dexamethasone (2mg/kg) or distilled water. One hour later, the animals received intraperitoneal injection of 0.5 ml of 1% (w/v) carrageenan in normal saline. Four hours later the animals were sacrificed and the peritoneal cavities washed with 5 ml of a 5% solution of EDTA in phosphate buffered saline (PBS) to recover the peritoneal fluid. Total (TLC) and differential (DLC) leucocyte counts were carried out on the perfusate.

Antimicrobial assay
Following preliminary antibacterial sensitivity tests, the minimum inhibitory concentration (MIC) of BFME and MF against laboratory strains of *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi, Pseudomonas aeruginosa* and *Bacillus subtilis* were evaluated using the agar well diffusion method [23]. Briefly, sterile Muller Hinton agar plates were flooded with the microorganisms (1×10^6 cfu/ml). Using a sterile cork borer (7 mm diameter), 6 wells were bored on the agar and 3 drops of different concentrations of BFME or MF (100, 50, 25, 12.5, 6.25 mg/ml) dissolved in 10% dimethylsulfoxide (DMSO) in water were placed in the appropriate well respectively. The plates were allowed 30 min for diffusion and incubated in an inverted position for 24 h at 37°C. Tests were done in triplicate. After incubation, the inhibition zone diameter (IZD) for each well was measured horizontally and vertically and the mean obtained. The MIC was determined as intercept on the concentration axis of log concentration against mean IZD^2 plot.

Statistical analysis
Data was analyzed using One way Analysis of Variance (ANOVA) and the results expressed as Mean ± SEM. Data was further subjected to LSD post hoc test for multiple comparisons and differences between means accepted significant at $P<0.05$.

RESULTS

Effects of extract and fractions on bleeding time
The extract and fractions significantly ($P<0.05$) reduced bleeding time with order of magnitude DF>BFME>MF (Table 1).

Table 1: Effect of extract and fractions on bleeding time in rats

Treatment	Concentration (mg/ml)	Bleeding time (s)	Reduction in bleeding time (%)
BFME	0.10	213±40.64*	56.71
	10	174±18.73*	64.63
	100	143±26.58*	70.93
DF	0.10	113±11.79*	77.03
	10	152±17.49*	69.11
	100	231±13.91*	53.05
MF	0.10	378±37.17*	23.17
	10	186±6.0*	62.20
	100	192±13.91*	60.98
Normal saline	-	428±40.39*	13.01
Control	-	492±66.31	-

*n=5; *P<0.05 compared to control (One Way ANOVA; LSD post hoc); Reduction (%) in bleeding time was calculated relative to control*

Effects of extract and fractions on coagulation time
The extract and fractions significantly ($P<0.05$) reduced blood coagulation time with order of magnitude MF>DF>BFME (Table 2).

Table 2: Effect of extract and fractions on blood coagulation time

Treatment	Concentration (µg/ml)	Coagulation time (s)	Reduction in coagulation time (%)
BFME	10	32.0±3.65*	23.08
	50	19.6±0.51*	52.88
	100	15.8±0.51*	62.02
DF	10	29.2±1.24*	29.81
	50	18.0±0.84*	56.73
	100	13.6±0.6*	67.31
MF	10	38.2±1.93*	8.17
	50	12.6±1.36*	69.71
	100	9.8±1.59*	76.44
Normal saline	-	15.8±0.97*	62.02
Control	-	41.6±3.47	-

n=5; *P<0.05 compared to control (One Way ANOVA; LSD post hoc); Reduction (%) in coagulation time was calculated relative to control.

Effects of extract and fraction on excision wound
The extract and fractions significantly (P<0.05) increased the rate of contraction of excision wounds and also reduced epithelialization time compared to control rats (Table 3).

Histopathology of healing wounds
The H&E stain revealed fibroplasia in all the tissues. The extract treated wounds expressed equal or less amount of fibroblasts (5 and 10% MF) compared to control. Also BFME (5%) and MF (5%) had adipocytes lined with lipofuscin granules. The BFME (5 and 10%) and MF (5%) had large number of macrophages (4-6) per HPF. Except for MF (20%) there were more leucocytes in the extract treated rats compared to control (Figure 1). The extract treated rats had varying amounts of neutrophils, macrophages and eosinoplils, while control rats expressed mainly lymphocytes and eosinophils (Figure 1).

The HVG stain showed that extract treated rats had more Type I collagen fibres than control; the collagen fibre in control rats were thinly spread and highly cellular. The BFME (5%) had the highest amount of Type I collagen fibres (Figure 2).

Effects of extract and fraction on leucocyte migration
The BFME and MF significantly ($P<0.05$) reduced total and differential leucocyte counts compared to control (Table 4).

Antimicrobial effects of extract and fraction
The BFME and MF exhibited varying degrees of antibacterial effect against the tested microorganisms with MIC values shown in Table 5.

Table 3. Effect of extract and fractions on excision wounds in rats

Treatment	Dose (%w/w)	Epithelialization (days)	Wound contraction (%)								
			Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17
BFME	5	18.6±0.98	22.19±1.65*	49.78±5.37*	77.91±5.29*	85.88±3.00*	93.58±1.64*	95.92±0.58*	98.05±0.55*	99.42±0.58*	100.00±0.00*
	10	15.2±0.92	9.61±2.80	28.18±2.73	73.00±3.65	91.22±0.84*	95.97±1.04*	96.36±0.98*	98.47±0.10*	99.64±0.16*	100.00±0.00*
	20	17.8±0.49	-1.86±0.65	11.21±5.57	62.36±3.90	82.65±2.28*	88.06±1.43*	92.05±0.85*	93.26±1.38	98.29±0.54*	99.55±0.45*
MF	5	15.6±0.87	3.41±3.04	29.14±2.01	63.15±4.08	86.80±5.70*	91.44±5.55*	93.67±3.58*	95.66±3.46*	99.82±0.18*	100.00±0.00*
	10	16.2±0.49	4.71±1.62	16.93±2.41	57.84±1.40	82.19±1.87*	88.74±2.50*	95.36±0.54*	97.20±0.54*	97.98±0.83*	98.80±0.49*
	20	15.4±0.40	6.45±2.51	23.54±5.98	63.97±4.80	84.06±1.30*	92.45±1.60*	97.98±0.35*	99.01±0.60*	100.00±0.00*	100.00±0.00*
Penicillin	-	19.0±0.63	14.28±3.98*	37.22±3.36	64.12±2.83	80.73±2.00*	82.55±2.85*	85.33±2.71*	89.27±2.65*	93.02±2.15*	96.50±1.77*
Control	-	21.0±0.00	2.85±0.78	32.16±3.66	65.23±0.35	73.94±1.54*	80.04±1.04*	86.87±0.20*	89.54±0.75*	92.47±0.64*	95.47±1.40*

*n=5; *P<0.05 compared to control (One Way ANOVA; LSD post hoc); wound contraction (%) was calculated relative to wound area on day 0.*
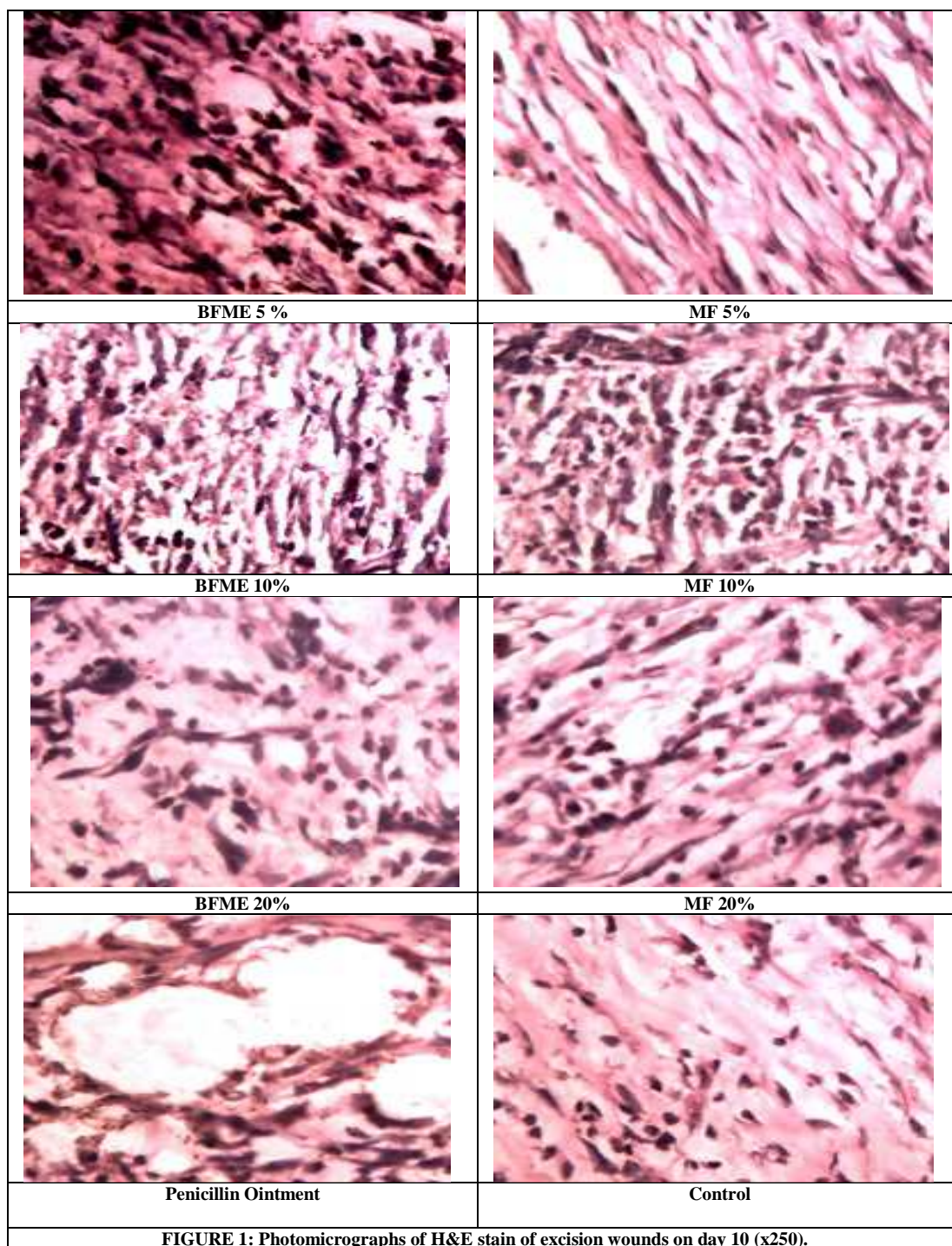

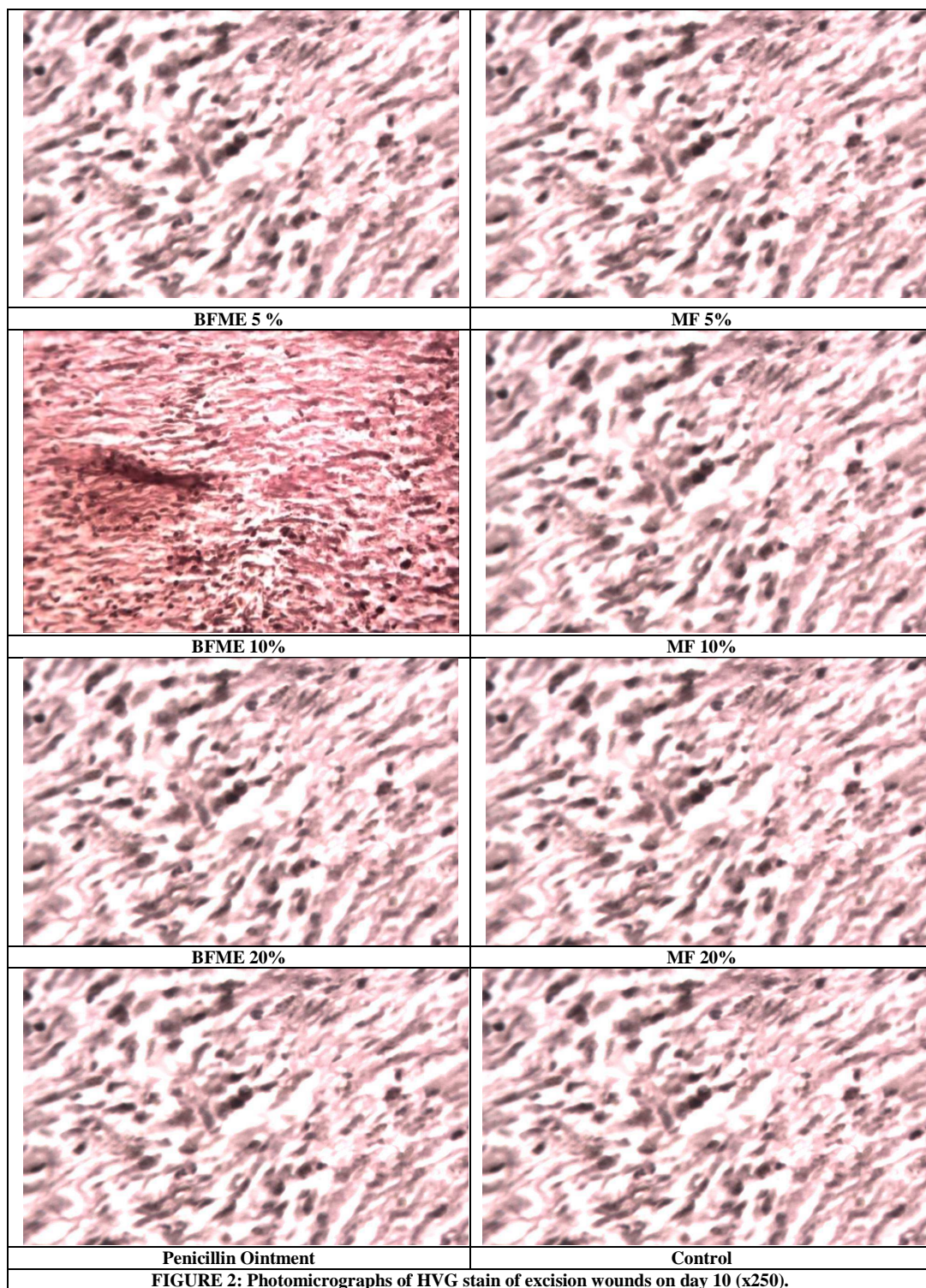**FIGURE 1: Photomicrographs of H&E stain of excision wounds on day 10 (x250).**

FIGURE 2: Photomicrographs of HVG stain of excision wounds on day 10 (x250).

Table 4: Effect of *B. ferruginea* on carrageenan-induced leucocyte migration

Treatment	Dose (mg/kg)	TLC (Cells/µl)	Differential leucocyte count (Cells/µl)				
			Neutrophil	**Lymphocyte**	**Monocyte**	**Basophil**	**Eosinophil**
BFME	200	1360±92.74* (69.71)	186.00±36.62* (71.52)	1075.2±6.27 (63.77)	104.5±1.72* (87.27)	2.9±0.3* (84.74)	0.00±0.00* (100)
	400	1440±212.96* (67.93)	341.3±46.44* (47.75)	724.2±14.5 (75.59)	361.6±4.18* (55.93)	2.3±0.2* (87.89)	10.6±0.8 (10.17)
MF	200	1200±284.17* (73.27)	298.8±100.44* (54.26)	769.4±14.06* (74.07)	133.0±5.51* (83.79)	2.6±0.26* (86.32)	0.00±0.00* (100)
	400	710±177.06* (84.19)	129.9±35.63* (80.11)	527.1±12.17* (82.24)	44.8±1.64* (94.54)	5.5±0.26* (71.05)	0.00±0.00 (100)
Dex	2	1320±523.59* (70.60)	243.4±93.62* (62.74)	540.69±28.7* (81.80)	212.7±15.07* (74.08)	0.00±0.00* (100)	7.4±0.56 (37.29)
Control	-	4490±477.60	653.20±153.38	2967.4±37.9	820.6±24.53	19.0±1.17	11.8±1.12

n=5; *P<0.05 compared to control (One Way ANOVA; LSD post hoc); values in parenthesis represent reduction (%) calculated relative to control, BFME = methanol extract, MF = methanol fraction, Dex = dexamethasone.

Table 5: Minimum inhibitory concentration (MIC) of extract and fraction

Microorganism	Minimum Inhibitory Concentration (mg/ml)	
	BFME	**MF**
B. subtilis	12.7	25.0
S. aureus	6.3	6.3
E. coli	6.3	25.3
P. aeruginosa	12.3	12.6
S. typhi	12.2	12.6
K. pneumonia	12.1	12.6

DISCUSSION

Assessment of the effects of *B. ferruginea* stem bark extracts on parameters of wound care revealed potent hemostatic and antibacterial activities with acceleration of wound contraction.

Wound healing, a complex, but orderly phenomenon by which a damaged tissue is restored as closely as possible to its normal state, involves hemostasis, epithelialization, granulation tissue formation, remodeling of the extracellular matrix, collagenization and acquisition of wound strength.

Bleeding due to injury is a direct consequence of vascular damage and must be arrested by hemostatic mechanisms for healing and repair of the wounded tissue to commence. Hemostasis involves a cascade of reactions starting with vascular constriction, platelet adhesion and aggregation to platelet plug formation, blood coagulation and growth of fibrous tissue into the blood clot to close the hole in the vessel permanently [24]. The ability of the extracts to reduce bleeding and coagulation time respectively suggest hemostatic effect. Furthermore, reduction in coagulation time suggests the hemostatic activity may derive partly from acceleration of the coagulation process.

Epithelialization or renewal of epithelial tissues after injury involves the proliferation and migration of epithelial cells towards the center of the wound, while wound contraction is largely due to the action of myofibroblasts [25,26]. Excision wounds heal by secondary union or second intention, and wound contraction is a characteristic of excision wounds [25,26]. The ability of the extracts to increase the rate of excision wound contraction and epithelialization suggests facilitation of the proliferation, migration and action of epithelial cells and myofibroblasts and hence tissue repair. Myofibroblasts, which have the ultrastructural characteristics of smooth muscle cells, are altered fibroblasts involved in healing. Hence ability of extracts to enhance action of myofibroblasts suggests facilitation of healing. In addition, proliferation of fibroblasts observed in the tissue sections is essential for wound filling and healing as they are the most common cells that create collagen, which ultimately provides the tensile strength of healing wounds [26].

The foregoing is also consistent with the observation that the extract treated rats had greater amount of collagen Types I and III compared to control, suggesting higher tensile strength of wounds. Collagen Type I, are bundles of banded fibers with high tensile strength, and constitute about 80% of collagen in skin. It is late collagen, present in scar tissue and is the end product when tissue heals by repair [26]. Collagen III, early collagen, is the main component of reticular fiber and is made up of thin fibrils; they are pliable and make up 10% of collagen in skin [26]. Collagen III which is commonly found alongside type I, is the collagen of granulation tissue, produced quickly by young fibroblasts before the tougher type I collagen is synthesized. The presence of greater amounts of collagen I

in extract treated rats compared to control indicate increased rate of conversion of Type III to the tougher and stronger Type I. In addition, though there was little or no difference in the degree of expression of fibroblasts in extract treated compared to control rats, the former had higher collagen levels. This could be possibly due to faster transformation of fibroblasts to collagen in extract-treated compared to control rats.

Recruitment of leucocytes to the site of tissue injury is an essential feature of the inflammatory response; neutrophils predominate in the early stage, while monocytes predominate later. The ability of the extract to reduce leucocyte migration is consistent with its earlier reported anti-inflammatory activity [10] and indicates inhibition of leucocyte migration as an anti-inflammatory mechanism. This suggests acute modulation of the inflammatory response to injury and facilitation of amelioration of symptoms. However histopathological studies revealed that except for MF 20%, the number of leucocytes in the extract treated rats on day 10 post wounding were slightly more than that of control. The import of this is not clear, but it is apparent that chronic topical application of extract may not hamper migration of leucocytes to areas of injury, where their effects may be beneficial. Furthermore, the presence of neutrophils and macrophages in the 10 day old excision wound indicates active phagocytosis, suggesting that the extractives may ultimately facilitate wound healing by enhancing the clearing of unwanted substances and the salutary aspect of the inflammatory process. Also the presence of lipofuscin granules in adipocytes of BFME and MF (5%) treated rats indicate damage, repair and reconstruction in the excised area as lipofuscin, known as "wear and tear" pigment, occurs in cells/tissues where there is damage and repair.

Despite the deployment of appropriately enhanced tissue growth and repair processes, microbial contamination poses a great threat to timely and successful healing of wounds [27]. Microbial infection of wound delays healing and causes a more pronounced acute inflammatory reaction [25, 28-30] which can lead to further tissue injury and damage. Unfortunately, wounds also provide environment conducive for the growth of microbes. Several organisms such as *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli* have been implicated as wound contaminants. The antibacterial effect of the extracts further contributes to the beneficial effects of *B. ferruginea* in wound healing.

CONCLUSION

The results of this study demonstrated that the beneficial effects of *B. ferruginea* stem bark extracts in wound care derive from ability to arrest bleeding from fresh wounds, inhibit the growth of bacterial wound contaminants, and accelerate wound healing by enhancing epithelialization, wound contraction and tissue repair.

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