Comparative Clinical Pathology (2019) 28:455–466
https://doi.org/10.1007/s00580-018-2873-4

ORIGINAL ARTICLE

Pathological and immunohistochemical evaluation of wound healing potential of Nigerian bee propolis in albino rats

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Received: 20 July 2018 / Accepted: 26 November 2018 / Published online: 19 December 2018

Abstract

This study evaluated the efficacy of the Nigerian bee propolis as a wound healing agent, on full thickness skin wounds of healthy adult male (150.0 ± 0.5 g) albino rats randomly divided into three treated groups (propolis extract (PE), propylene glycol (PG), silver sulfadiazine) and an untreated group. Each rat had three circular full thickness skin wounds created on the cranial, middle, and caudal surface of the rat's dorsum. The wounds in each group were topically treated with bee propolis extract (PE, 0.1 ml), propylene glycol (PG, 0.1 ml) and silver sulfadiazine (SS, 0.1 ml) twice daily for 21 days, except the untreated group (UT). The wounds were evaluated for gross (exudation, edema, hyperemia, wound contraction), histologic (granulation, angiogenesis, fibroplasia, epithelialization), and expression of epidermal growth factor (EGF) using standard techniques. Data was descriptively summarized as percentages, mean and analyzed using Chi-square and analysis of variance at $\alpha = 0.05$. Wound edge edema (WEE), hyperemia, and exudation were prominent in all the groups between days 0–2. WEE was significantly less on day 3 in the PE (14%) and SS (14%) groups, compared with the PG (35%) and UT (66%) groups. Wounds treated with PG and UT were significantly more hyperemic than those with PE and SS. Wound contraction was significantly less on day 2 in the UT wounds when compared to the treated wounds (PE = 12.63%, SS = 2.22%, PG = 4.94%, and UT = − 2.82%). The wound contraction was remarkable between days 4–8 in the PE- and SS-treated wounds (PE > SS > PG > UT). The microscopic changes at days 4, 8, 12, 16, and 21 showed significant evidence of epithelial proliferation, improved angiogenesis, granulation, and fibrous connective tissue in the PE- and SS-treated rats compared with the negative controls. The inflammatory response showed that the PE group had the highest amount of macrophages and leucocytes on day 4 with the trend being PE > SS > PG > UT. Neutrophils regressed in the treated wounds on day 8 but were consistently high in the untreated group from days 4–16. The immunohistochemical evaluation showed that the intensity of EGF was consistently high in the SS- and PE-treated wounds. Nigerian bee propolis extract accelerated wound healing similar to that of silver sulfadiazine based on wound healing indices and is therefore recommended for the management of wounds especially in low-income communities where propolis is available and affordable.

Keywords Propolis extract · Wound contraction · Management · Stages

Introduction

A wound is an injury to living tissue (skin, muscles, nerves, tendons, and bones) caused by a cut, blow, or other impact and characterized by disruption of the normal tissue integrity and functions (Patrick 2016). Wound healing is a biologic process following injury. geared towards restoration of tissue integrity and functions (Cooper et al. 2001; Eyarefe et al. 2017). It is a surgery cornerstone and the ultimate factor in predicting a patient's surgical outcome. It consists of simultaneous, interwoven, and dynamic phases with measurable indices, and a complex array of chemical and cellular mediators that culminate in repair process characterized by fibroplasia, angiogenesis, and remodeling (Fossum et al. 2007; Oguwike et al. 2013; Eyarefe et al. 2017). Wound healing is influenced by various factors including the following: host factors (Wall et al. 2002), wound characteristics (Rosique et al. 2015), closure methods (Landen et al. 2016), and applied agents (Oguwike et al. 2013). Investigation into agents with

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perceived healing potentials, especially natural products, has been intense in recent times, due to the increasing challenge of bacteria resistance to antimicrobials (Eyarefe et al. 2014). Substances, like Potash salt (Oguwike et al. 2013), *Aloe vera* (Duansak et al. 2003), *Moringa oleifera* (Eyarefe et al. 2015), aqueous Pineapple juice, and honey (Dunford et al. 2000; Molan 2011; Eyarefe and Fabiyi 2016) have been reported to have some wound healing effects on acute and chronic traumatic wounds, as well as burn wounds (Eyarefe and Oguntoye 2016). Success in wound management, therefore, depend to a large extent on the understanding of wound healing mechanism associated with wound types, properties of wound healing agents, and the ability to decide on the most appropriate and cost-effective method of management (Eyarefe et al. 2014). Measurable indices of wound healing, such as gross, histologic, immunologic, and immunohistochemical examination, have been used to evaluate the effectiveness of a healing agent (Prockop and Kivirikko 1995; Gupta and Kumar 2015).

Wound healing agents and methods such as foam dressings, alginates, hydrocolloids, hydrofibers, hydrogels, transparent films, negative-pressure wound therapy, growth factors, skin substitutes, high-pressure water irrigation, topical collagen products, topical insulin, topical antioxidants, stem cells, and gene therapy have been developed and their efficacies investigated with varying results (Dealey 1995; Devasagayam et al. 2004; Atiyeh et al. 2009; Martinotti et al. 2013; Rosique et al. 2015; Patrick 2016). Currently, there is no single optimal treatment method that enhances the resolution of problem wounds (Sell 2012). Besides, the more advanced wound management methods have been proven to be effective but expensive (Patrick 2016).

Propolis or bee glue is a resinous material produced by honeybees by mixing plant exudates with wax, pollen, salivary secretions, and bee enzymes (De Castro 2001). Propolis is used by bees to maintain the stability of the hive; it also makes it more defensible by sealing alternative entrances and prevents diseases and parasites from entering the hive (Simone-Finstrom and Spivak 2010). Propolis is as old as honey, and man has used it for ages as a medicine (Krell 1994). Propolis has been documented to possess several pharmacologic properties (De Groot 2013) that include the following: acceleration of regenerative processes in damaged cartilages and bones (Park et al. 2002; Boudra et al. 2014), immunomodulation (Qiao and Chen 1991; Damov et al. 1991), antimicrobial (Silva et al. 2008), antioxidant (Cushnie and Lamb 2005), analgesic (Demestre 2008), anti-inflammatory (Fearnely 2001), and antitumor properties (Gavanji and Larki 2015). The precise composition of propolis varies with the geographical location, botanical origin, and bee species (De Castro 2001). Crude hives' propolis contains 50% balsam resin, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% other substances, including wood fragments (Aga et al. 1994). Over 300 chemical components belonging to the flavonoids, terpenes, and phenolic acids group have been identified in propolis ((Martinotti and Ranzato 2015). Though bee propolis has been reported to offer numerous health benefits (Walker 2009), there is, however, scarcity of information on the use of Nigerian bee propolis as a healing agent.

The incidence of wounds remains the highest amongst surgical cases presented in Veterinary clinics and hospitals in Nigeria (Eyarefe et al. 2011). The limitations of modern methods of wound management which includes high cost, antibiotic resistance (in antibiotic based wound healing agents), and tissues biocompatibility have led to an increasing interest in the healing potentials of natural products. This study evaluated the wound healing effects of Nigerian bee propolis, on full thickness skin wounds in male albino rats (Wistar strain) using established wound healing indices of gross (wound exudation, wound edge edema, wound hyperemia, and wound contraction), histologic (granulation tissues, fibro-elastic tissue, angiogenesis, and epithelialization), and expression of epidermal growth factors (EGF).

Materials and methods

Ethical approval

Approval of the institutional Animal use and care committee was obtained: (UI-ACUREC/17/0047) before commencement of the study and all applicable guidelines (international, national and/or institutional) were followed.

Animals and management

Twenty (20) healthy adult male Albino rats (Wistar strain) weighing 150.0 ± 5.6 g were used for the study. The rats were sourced from a local breeding unit, housed at the Experimental Animal Housing Unit of the Faculty of Veterinary Medicine, University of Ibadan, in well-aerated individual cages and exposed to a 12-h light/dark cycle, 23 ± 1 °C temperature, and 30 ± 5% relative humidity. They were fed rat pellet feed (vital feed® Nigeria) and clean water ad-libitum and stabilized for 2 weeks before the commencement of the study.

Research design

The design was simply randomized into three treatment groups: propolis extract (PE), propylene glycol (PG), silver sulfadiazine (SS) and an untreated group UT/ control. In all, there were four groups comprising 5 rats each.

Propylene glycol was used as the carrier agent for bee propolis, due to its neutrality and stability in various preparations (Couto 2001). It was also used as negative control to ascertain that the effects observed are those of bee propolis

activities. Silver sulfadiazine was used as a positive control for its previously confirmed wound healing properties as established in previous studies (Miller et al. 2012).

Anesthesia and wound creation

Each rat was anesthetized with an intramuscular injection of 2% xylazine HCL at a dose of 5 mg/kg body weight and 5% ketamine at a dose of 35 mg/kg via the quadriceps group of muscles as earlier described (Eyarefe and Amid 2010). Following anesthesia, the dorsum (back) of each rat was prepared for aseptic surgery by shaving and sterilization with chlorhexidine and alcohol (Khoo et al. 2010). Three (3) circular full thickness skin wounds of 6.5 ± 0.5 mm in diameter were created on the dorsum of each rat to serve for replicate for each treatment. The untreated group ($n = 5$) had two (2) circular skin wounds created on their dorsum. The wound diameter was measured immediately after wound creation as baseline values.

Preparation of propolis extract

Crude bee propolis was obtained from the Apiary unit, Department of Crop Science and Protection, University of Ibadan, and stored in a dark waterproof away from light. The propolis source location was at Iwo, in the Osun state of Nigeria, and collection was done during the rainy season (June/July). The extraction was done using a standard technique (Couto 2001).

Phytochemical analysis Phytochemical qualitative analysis of bee propolis sample was carried out as earlier described (Ejikeme et al. 2014).

Treatment application and evaluation with gross wound healing indices Two drops (0.1 ml) of each agent was applied to wounds twice daily in line with experimental design. Each wound was evaluated and scored from day 0 to 21 for wound surface exudation, wound edge edema, wound surface hyperemia, and rate of wound contraction as earlier described (Eyarefe et al. 2014). Wound size (surface area of the wound in (cm^3) was measured on alternate days using a transparent graph sheet. The evaluated wound size was used to calculate the percentage of wound contraction, taking initial wound size as 100%, and employing the following formula:

$$\%\text{Wound contraction} = \text{initial wound size} - \text{specific day wound size} / \text{initial wound size} \times 100.$$

A pictorial capture of each wound was also made to corroborate the measurements.

Histologic parameters Excisional biopsies of the rats' wounds were obtained at 4-day intervals (4, 8, 12, 16, 21) for histopathological evaluation of healing quality following animals' euthanasia with ketamine (70 mg/kg) and xylazine (15 mg/kg) injections. The entire wound region was excised in-depth with liberal margins of the surrounding skin and the underlying connective tissues above the external fascia of the dorsal muscles.

The excised skin tissues were fixed in 10% formalin and cut in serial sections of 5-μm thickness after processing and embedding the tissue. The histological hematoxylin-eosin staining method was used for morphological and pathological examinations. Sections were qualitatively assessed under the light microscope and the histologic parameters including leukocyte count and fibroblast counts were quantified, while other histomorphologic parameters (granulation tissue, vascularization, fibro-elastic tissue, and epithelialization) were estimated and scored, as described by Gupta and Kumar (2015), using the semi-quantitative four-point scale scoring system.

Immunohistochemical assessment of epidermal growth factor The immunochemical staining procedure was done as follows: tissue sections were deparaffinized and rehydrated for antigen retrieval before blocking steps, addition of primary and secondary antibodies, and the application of streptavidin-biotin peroxidase complex (IHC Select Detection System, Merck, Germany® LOT: 2775482) to determine the intensity of EGF in wounds. The expression of epidermal growth factor in the wounds was determined on days 4, 8, 12, 16, and 21. The positive wound tissues were graded as weak, moderate, and strong following reciprocal intensity quantification (Nguyen et al. 2013).

Statistics The wound contraction, and leucocyte and fibroblast counts were expressed as mean, standard deviation and analyzed using analysis of variance (ANOVA) while the other gross wound indices (exudation, edema, hyperemia) and histomorphologic parameters (granulation tissue, vascularization, fibroelastic, tissue and epithelialization) were analyzed using Chi-square at $\alpha = 0.05$.

Results

Qualitative phytochemical analysis of bee propolis The results showed the presence of flavonoids (+), anthraquinones (++), cardiac glycosides (+), steroids (+), terpenoids (+), and alkaloids (+).

Wound surface exudation evaluation Wound surface exudation was prominent in all the groups up to day 2 post-wound creation. It was significantly less in the PE (14) and SS (14) groups on day 3 compared to the PG (35) and UT (66) groups with the trend being PE = SS < PG < UT (Table 1). A

significantly high percentage (33%) of wounds in the untreated group had wound surface exudation from days 7–12.

Wound edge edema Wound edge edema was prominent in all the groups up to day 2 post-wound creation. It was absent in the PE and SS groups on day 3 but present in the PG (21%) and UT (50%) groups. Wound edge edema was severe in the untreated group ($p = 0.001$) between days 8 and 11.

Wound surface hyperemia Wound surface hyperemia was prominent in all the groups up to day 2 post-wound creation but significantly high ($p = 0.003$) on days 3 and 4 in the untreated group. Wound hyperemia was absent on day 5 in the treated wounds while the untreated wound continued to show surface hyperemia at days 5–10.

Wounds contraction evaluation Wound contraction was gradual in all the groups from days 0–21. It was significantly higher ($p = 0.002$) between days 0 and 2 in the PE group compared with the other groups (Table 2). Marked contraction ($p < 0.05$) was observed between days 6 and 8 in the PE and SS groups with the trend being (PE > SS > PG > UT). Complete wound closure occurred on day 16 in the PE group, SS (day 18), PG (day 21), and UT (day 21).

Inflammatory cells and fibroblast response Inflammatory cellular response was significantly higher ($p = 0.002$) in the PE and SS groups when compared with the PG and UT groups on day 4. The number of macrophages and monocytes were significantly high ($p = 0.003$) in the PE group on day 4, with the trend being (PE > SS > PG UT). Other inflammatory cells such as eosinophils, mast cells, and platelets were significantly high ($p = 0.001$) in the PE group on day 4 but was completely absent in the other groups. The untreated wound had neutrophilia from days 4–16 (Table 3, Figs. 1, 2, 3). Fibroplasia was significantly high ($p > 0.05$) in the PE group on day 4, but was more sustained in the SS group from days 4–21 (Fig. 4).

Granulation tissue Wound granulation tissues comprising of budding capillaries and type III collagen fibers first appeared in the PE and SS groups on day 8, but on day 12 in the PG and UT groups. Conversion of type III collagen granulation tissues to type I occurred faster in the PE and SS groups when compared to the other groups leading to increased collagen lay down and wound closure. The amount of granulation tissue at day 21 was not significant ($p = 0.000$) in all the groups (Table 4).

Vascularization/angiogenesis Capillary budding was significantly high ($p = 0.004$) in the PE and SS groups on days 4 and 8 when compared to the PG and UT groups. Blood vessel regression also occurred faster in the PE and SS groups. The level of vascularization on day 21 was not significant ($p = 0.082$) across the groups.

Table 1 Wound wetness, edge edema, and surface exudation across the treatments in the first 14 days on the rats

Day	Propolis extract (PE)				Propylene glycol (PG)				Silver sulfadiazine (SS)				Untreated (UT)			
	WW (%)	WEE (%)	WSE (%)	S.HYP (%)	M.HYP (%)	WW (%)	WEE (%)	WSE (%)	S.HYP (%)	M.HYP (%)	WW (%)	WEE (%)	WSE (%)	S.HYP (%)	M.HYP (%)	WW (%)	WEE (%)	WSE (%)	S.HYP (%)	M.HYP (%)
0	14	14	14	14	0	14	14	14	14	0	14	14	14	14	0	6	6	6	6	0
1	14	14	14	0	14	14	14	14	0	14	14	14	14	0	14	6	6	6	6	0
2	7	2	2	0	5	11	10	5	0	11	8	7	4	0	6	6	6	5	0	6
3	2	0	0	0	0	5	3	0	0	7	2	0	0	0	2	4	3	2	0	2
4	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	1	0	0	0	1
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	1	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	1	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	1	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	1	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

WW Wound wetness, *WEE* wound edge edema, *WSEE* wound surface exudation, *HYP* wound surface hyperemia

Table 2 Mean wound surface contraction across the treatments and untreated in the rats

Mean wound surface area [± standard deviation] in (cm^3)

Days	PE		SS		PG		UT	
	RWC	PWC	RWC	PWC	RWC	PWC	RWC	PWC
0	6.56 ± 2.30	0.00 ± 0.00	7.77 ± 2.90	0.00 ± 0.00	6.78 ± 1.79	PG	7.93 ± 1.93	0.00 ± 0.00
2	5.54 ± 1.31	12.63 ± 10.58	7.54 ± 2.63	2.22 ± 14.45	6.41 ± 1.66	0.00 ± 0.00	8.15 ± 1.78	− 2.82 ± 12.15
4	4.38 ± 1.24	26.10 ± 7.74	6.22 ± 2.79	18.21 ± 13.61	5.20 ± 2.05	4.94 ± 8.33	7.86 ± 2.07	− 0.30 ± 16.80
6	3.63 ± 1.47	42.35 ± 16.05	5.02 ± 1.85	31.74 ± 15.51	4.72 ± 2.08	22.91 ± 12.43	7.14 ± 2.08	8.97 ± 10.14
8	1.81 ± 1.31	69.68 ± 20.18	2.85 ± 1.19	55.81 ± 23.69	3.40 ± 0.85	29.98 ± 12.30	5.33 ± 2.87	28.63 ± 11.74
10	1.00 ± 0.85	83.10 ± 13.80	1.80 ± 1.28	69.83 ± 26.34	2.46 ± 0.96	43.90 ± 11.89	4.27 ± 1.23	36.33 ± 5.43
12	0.80 ± 0.88	86.08 ± 14.27	1.10 ± 0.87	78.01 ± 22.30	1.75 ± 0.57	61.17 ± 13.85	3.20 ± 1.38	57.47 ± 6.19
14	0.35 ± 0.68	92.97 ± 15.96	0.37 ± 0.37	91.93 ± 11.07	0.90 ± 0.51	71.14 ± 10.83	1.40 ± 0.71	82.77 ± 5.00
16	0.00 ± 0.00	1.00 ± 0.00	0.20 ± 0.30	95.55 ± 10.90	0.37 ± 0.23	87.32 ± 4.68	0.40 ± 0.00	93.05 ± 0.64
18	0.00 ± 0.00	1.00 ± 0.00	0.10 ± 0.24	98.95 ± 1.63	0.13 ± 0.10	96.80 ± 2.92	0.20 ± 0.00	96.50 ± 0.28
21	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	96.61 ± 1.63	0.00 ± 0.00	99.05 ± 1.34

RWC rate of wound contraction, *PWC* rate and percentage of wound contraction, *PE* propolis extract, *PG* propylene glycol, *SS* silver sulfadiazine, *UT* untreated group

Difference of values with superscript a is significantly higher than the differences between values with superscripts b and c

*Days of complete wound closure across the groups

Fibro-elastic tissue The amount of fibro-elastic tissue at day 8 was significantly higher ($p < 0.05$) in the PE and SS groups than in the PG and UT groups with the trend being SS > PE > PG > UT. The treated groups had higher ($p = 0.006$) fibro-elastic tissue on day 21 when compared with the controls.

Epithelialization Epithelialization occurred faster in the treated wounds than in the controls. The SS groups showed a faster rate of epithelialization comparable to the propolis extract group ($p < 0.05$). The untreated group had the lowest rate of epithelialization on day 16. All wounds showed complete epithelialization by day 21.

Expression of epidermal growth factor Immunohistochemical evaluation of the intensity of epidermal growth factor (EGF) was significantly high ($p = 0.001$) in the SS and PE groups from days 4–12 when compared to the PG and UT groups with the trend being SS > PE > PG > UT. The high intensity of EGF was recorded across the groups on day 16 (Table 5). The intensity of EGF was significantly low ($p = 0.001$) in the untreated wounds from days 4–21 when compared to the other groups.

Discussion

The study evaluated the gross, histologic, and immunohistochemical expression of epidermal growth factor. Results showed that Nigerian bee propolis accelerated the healing of full thickness skin wounds in rats at a rate that is similar to silver sulfadiazine, a known wound healing agent. Propylene glycol was used as the carrier agent for bee propolis because of its neutrality and stability in various preparations (Couto 2001). It was used as a negative control in this study to prove that the effects observed are those of bee propolis activities. Silver sulfadiazine was used as a positive control for its previously confirmed wound healing properties as established in previous studies (Miller et al. 2012).

The wound healing indices which includes gross (wound exudation, wound edge edema, and wound color/hyperemia), immunologic, histologic (granulation tissue, angiogenesis, fibro-elastic tissue, and epithelialization), and immunohistochemistry assessment adopted for this study are conventional assessment parameters in wound healing studies (Eyarefe et al. 2014; Gupta and Kumar 2015).

Wound surface exudation, wound edge edema, and surface hyperemia observed in this study were severe on days 0–2. These indices are indications of tissue response to injury (Rosique et al. 2015). Acute wound exudation, wound edge edema, and wound surface hyperemia were significantly less on day 3 in the PE and SS group compared to the PG and UT groups. The qualitative phytochemical analysis of this propolis sample showed the presence of flavonoids which may account for the anti-edema and anti-hyperemic effects (Mirzoeva and Calder 1996; De Moura et al. 2011). Terpenoids are phytochemicals that have been shown to possess antibacterial and antifungal properties (Mirzoeva and Calder 1996) while anthraquinones and cardiac glycosides which are also present in this propolis sample have been used to treat inflammation and edema (Khan et al. 2011).

Table 3 Mean inflammatory cell counts across the treatments and untreated in the rats

Histologic parameters	PE	SS	PG	UT
Day 4				
Macrophage	74.00 ± 14.14	54.00 ± 36.77	28.00 ± 11.31	20.00 ± 28.28
Lymphocyte	44.00 ± 16.97	32.00 ± 16.97	20.00 ± 0.00	16.00 ± 22.62
Fibroblast	112.00 ± 11.31	58.00 ± 42.43	30.00 ± 8.49	56.00 ± 79.19
Neutrophil	84.00 ± 73.54	10.00 ± 14.14	4.00 ± 5.66	92.00 ± 130.12
Mast cell	2.00 ± 2.82	4.00 ± 5.66	0.00 ± 0.00	2.00 ± 2.83
Eosinophil	12.00 ± 16.97	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelet	14.00 ± 19.79	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 8				
Macrophage	24.00 ± 33.94	40.00 ± 56.57	32.00 ± 45.25	136.00 ± 79.19
Lymphocyte	10.00 ± 14.14	30.00 ± 36.77	16.00 ± 22.63	46.00 ± 2.83
Fibroblast	70.00 ± 0.00	62.00 ± 70.71	66.00 ± 82.03	64.00 ± 45.25
Neutrophil	156.00 ± 220.61	18.00 ± 25.46	8.00 ± 11.31	84.00 ± 50.91
Mast cell	0.00 ± 0.00	2.00 ± 2.82	6.00 ± 8.49	4.00 ± 5.66
Eosinophil	4.00 ± 5.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelet	6.00 ± 8.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 12				
Macrophage	40.00 ± 11.31	80.00 ± 5.66	16.00 ± 22.63	14.00 ± 8.46
Lymphocyte	16.00 ± 22.63	44.00 ± 5.66	0.00 ± 0.00	14.00 ± 14.14
Fibroblast	40.00 ± 11.31	146.00 ± 31.11	20.00 ± 11.31	36.00 ± 16.97
Neutrophil	0.00 ± 0.00	74.00 ± 59.39	76.00 ± 107.48	152.00 ± 169.70
Mast cell	16.00 ± 22.63	2.00 ± 2.83	0.00 ± 0.00	0.00 ± 0.00
Eosinophil	16.00 ± 22.62	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelet	16.00 ± 22.63	0.00 ± 0.00	42.00 ± 59.40	0.00 ± 0.00
Day 16				
Macrophage	48.00 ± 5.66	50.00 ± 25.46	30.00 ± 42.42	30.00 ± 8.49
Lymphocyte	28.00 ± 5.66	18.00 ± 8.48	30.00 ± 42.42	14.00 ± 8.49
Fibroblast	42.00 ± 8.48	44.00 ± 16.97	46.00 ± 36.77	30.00 ± 14.14
Neutrophil	00.00 ± 0.00	0.00 ± 0.00	22.00 ± 31.11	64.00 ± 90.51
Mast cell	0.00 ± 0.00	4.00 ± 5.66	8.00 ± 11.31	0.00 ± 0.00
Eosinophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.00 ± 8.49
Platelet	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 21				
Macrophage	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocyte	0.00 ± 5.66	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Fibroblast	28.00 ± 39.59	350.00 ± 296.98	22.00 ± 2.82	68.00 ± 84.85
Neutrophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mast cell	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eosinophil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelet	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

PE propolis extract, *PG* propylene glycol, *SS* silver sulfadiazine, *UT* untreated group

Difference of values with superscript a is significantly higher than the differences between values with superscripts b and c

Wound edge edema and hyperemia are local signs of acute inflammation from days 0 to 3 of injury in clean wounds, and could be signs of wound infection and evidence of debridement challenges when these signs progress beyond day 3 of injury (Eyarefe et al. 2014). This observation may provide a rationale for the prolonged wound exudations and hyperemia observed between days 5 to 12 in the untreated group. The reduction in edema and surface hyperemia seen in wounds treated with silver sulfadiazine is in line with the use of silver sulfadiazine as a drug of choice in treating burn wounds due to

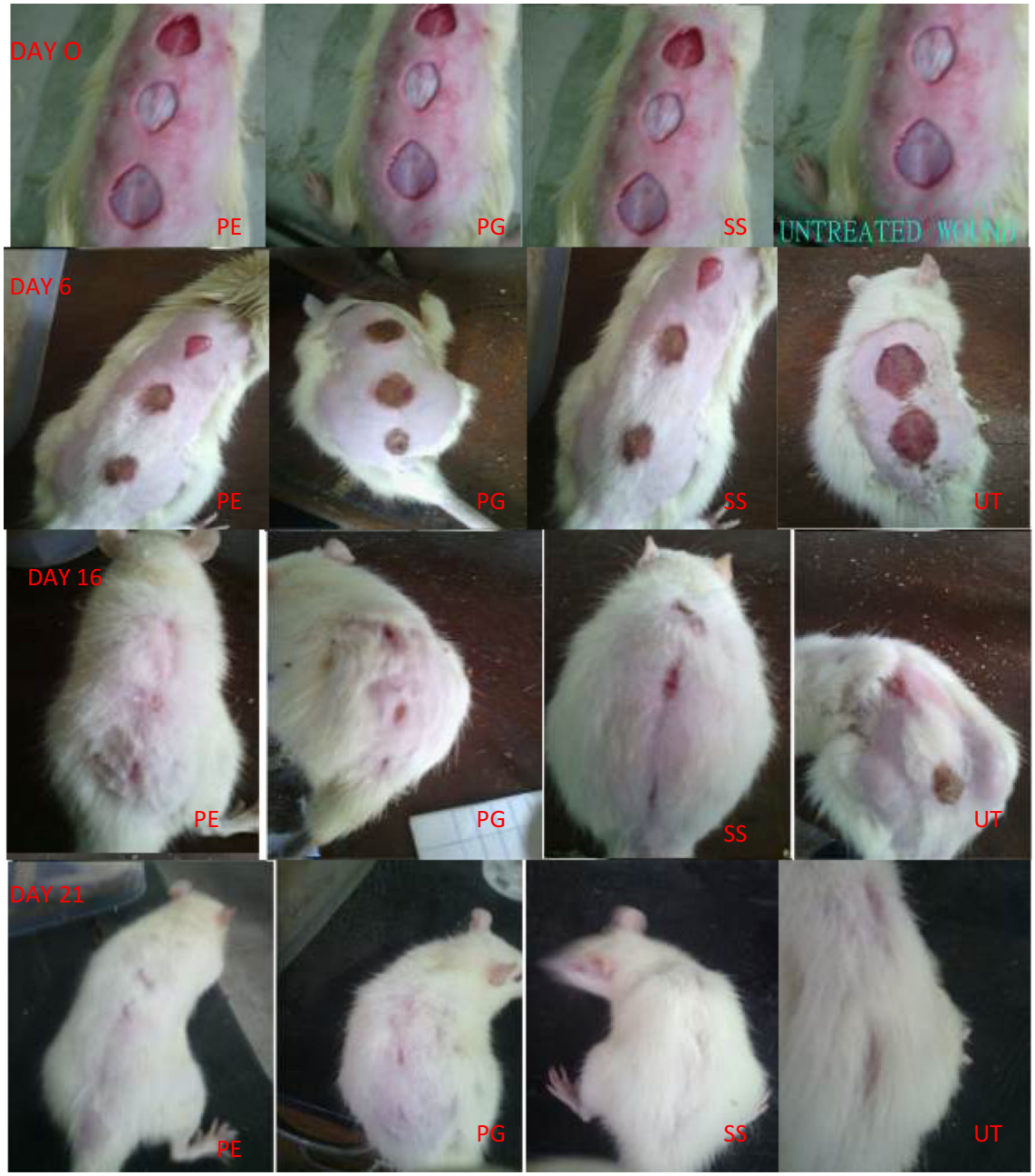

Fig. 1 Gross characteristics of the wounds with different treatments from days 0–21. The surface hyperemia of the wound was remarkably prominent in the PE-treated wound when compared to other treatment at day 0. The contraction was gradual in all the groups from day 16, but remarkable at in the PE- and SS-treated wounds

its wide spectrum of bactericidal activity against both gram-positive and gram-negative organisms (Miller et al. 2012).

Wound contraction was gradual in all the groups from day 0 to day 21. It was significantly higher between days 6 and 8 in the propolis extract group as well as the silver sulfadiazine group than in the untreated wounds. This could be traced to the ability of propolis to influence the production of transforming growth factor- alpha and beta 1 (TGF-α and TGF-β1) by immune cells which stimulates cell growth, mobilization of fibroblasts, and epithelial migration ((Martinotti and Ranzato 2015), as well as fibroblast transform to myofibroblasts (Patrick 2016). Wound contraction was slower in the untreated and propylene glycol groups due to inadequate production of granulation tissues as a result of extended inflammatory and debridement phases (Rosique et al. 2015).

The immunologic evaluation shows a significantly higher rate of inflammatory cellular infiltrate (neutrophils, macrophages, and lymphocytes) in the propolis extract group than in the silver sulfadiazine group on day 4. These tissues immune cellular infiltration must have aided in the autolytic

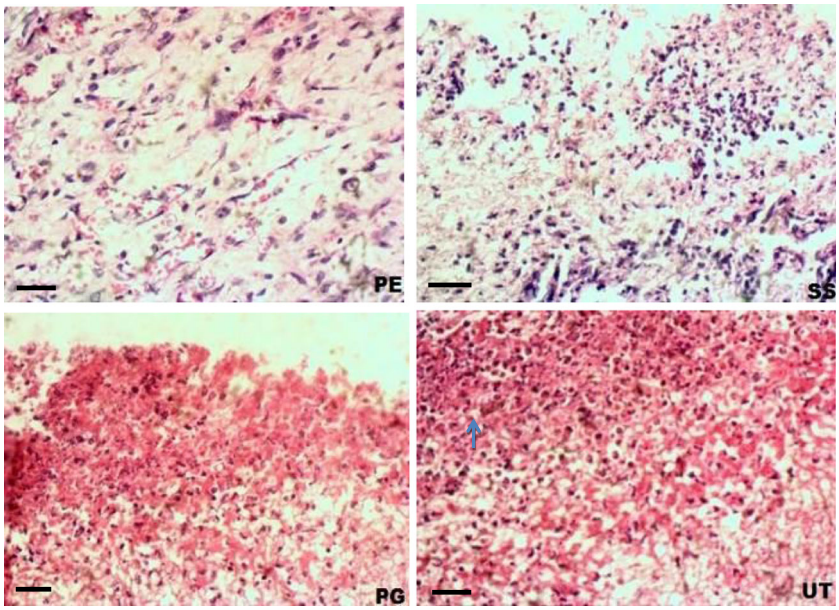

Fig. 2 Photomicrographs showing histologic section of all the groups on day 4 (hematoxylin-eosin stain, × 400). **PE** showing Inflammatory stage with diffuse cellular infiltrates, **SS** showing Inflammatory stage with diffuse acute cellular inflammation, **PG** showing early inflammatory stage with marked wound hyperemia and edema, and **UT** showing early inflammatory stage with marked wound hyperemia and acute inflammatory cells. Scale bar = 45 μm. PE, propolis extract; PG, propylene glycol; SS, silver sulfadiazine; UT, untreated group

debridement and growth of new tissues observed in this study, which has further authenticated, a previously observed propolis immune-stimulating potentials (Dimov et al. 1991). The Nigeria bee propolis is rich in flavonoids. Flavonoids such as naringenin in propolis have been observed to possess an immune-stimulatory property critical to wound healing (Martinotti and Ranzato 2015).

The number of macrophages and monocytes was significantly high in the PE group on day 4. Macrophages are perhaps the most important cells in the early phase of wound healing. In addition to phagocytosis of wound debris and bacteria, they release cytokines such as PDGF, which stimulates chemotaxis and proliferation of fibroblasts and smooth muscle cells (Patrick 2016). They also secrete substances that attract endothelial cells to the wound and stimulate their proliferation to promote angiogenesis (Landen et al. 2016). In studies in which experimental wounds are rendered monocytopenic, subsequent stages of fibroplasia and granulation tissue formation were impaired and the overall rate of wound healing was delayed. Inflammatory cellular response was significantly higher in the PE and SS groups when compared with the PG and UT groups on day 4 in this study. Leucocytes are critical to the cleansing of the wound site of bacteria and necrotic matter. They also release inflammatory mediators and bactericidal oxygen free radicals (Simon et al. 2014). Neutrophils and other inflammatory cells (macrophages, eosinophils, mast cells, and lymphocytes) regressed in the treated wounds on days 8 to 12 while the untreated group had neutrophilia from days 8 to 16 indicating an extension of the debridement phase which presented grossly as wound exudation and hyperemia

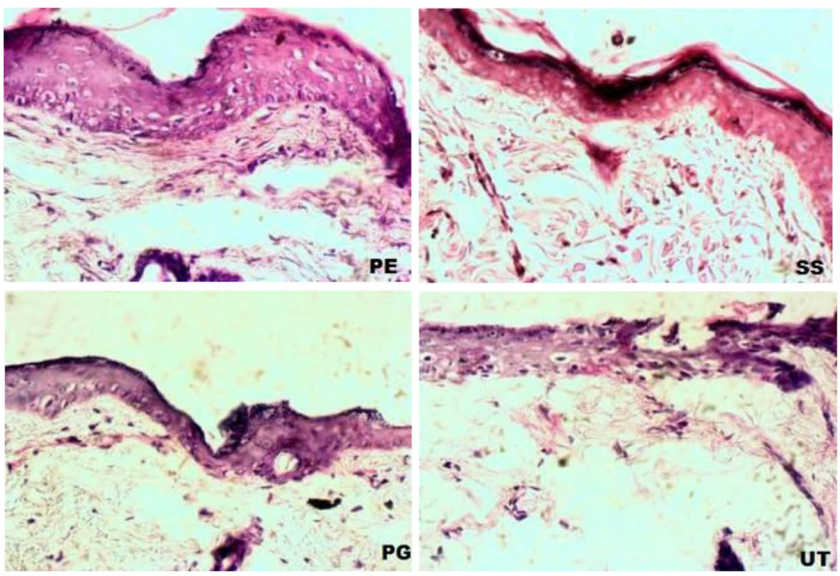

Fig. 3 Photomicrographs showing histologic section of all the groups on day 21 showing complete healing and epithelialization across the groups (hematoxylin-eosin stain, × 400). **PE** showing complete epithelialization and wound contraction, **PG** showing complete epithelialization and wound contraction, **SS** showing complete epithelialization and wound contraction, and **UT** showing complete epithelialization and wound contraction Scale bar = 45 μm. PE, propolis extract; PG, propylene glycol; SS, silver sulfadiazine; UT, untreated group

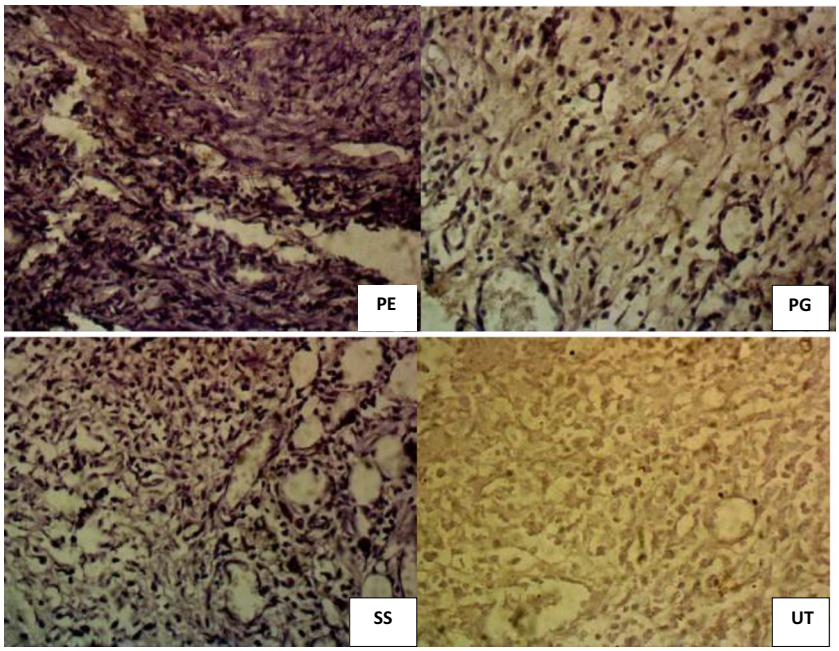

Fig. 4 Photomicrographs showing expression of epidermal growth factor at day 8 (ABC DAB/hematoxylin, × 400). PE, propolis extract; PG, propylene glycol; SS, silver sulfadiazine; UT, untreated group

Table 4 Evaluation of histologic wound healing indices on days 4, 8, 12, 16, and 21 across the groups

Histologic parameters	PE	SS	PG	UT
Day 4				
Granulation tissue	1.00 ± 0.10	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
Vascularization	1.00 ± 0.10	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.23
Fibro-elastic tissue	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Epithelialization	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 8				
Granulation tissue	3.00 ± 0.02^{a}	2.00 ± 0.04^{b}	1.00 ± 0.10^{c}	0.35 ± 0.26^{b}
Vascularization	2.00 ± 0.00^{c}	2.00 ± 0.00^{c}	1.00 ± 0.00^{c}	2.00 ± 0.01^{c}
Fibro-elastic tissue	1.00 ± 0.10^{b}	1.00 ± 0.20^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Epithelialization	$0.65.00 \pm 00^{c}$	1.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
Day 12				
Granulation tissue	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	1.00 ± 0.00^{c}	2.00 ± 0.00^{a}
Vascularization	1.00 ± 0.00^{b}	1.00 ± 0.06^{b}	1.00 ± 0.00^{c}	2.00 ± 0.00^{c}
Fibro-elastic tissue	2.00 ± 0.00^{b}	2.00 ± 0.00^{b}	1.00 ± 0.00^{c}	0.36 ± 0.06^{b}
Epithelialization	1.00 ± 0.65^{c}	2.00 ± 0.00^{b}	0.50 ± 0.10^{c}	0.35 ± 0.06^{b}
Day 16				
Granulation tissue	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}
Vascularization	1.00 ± 0.00^{c}	1.00 ± 0.00^{c}	1.00 ± 0.00^{c}	2.00 ± 0.00^{c}
Fibro-elastic tissue	2.00 ± 0.00^{c}	3.00 ± 0.00^{b}	2.00 ± 0.00^{b}	1.00 ± 0.00^{c}
Epithelialization	3.00 ± 0.00^{a}	3.00 ± 0.00^{b}	2.00 ± 0.00^{a}	1.00 ± 0.00^{b}
Day 21				
Granulation tissue	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Vascularization	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Fibro-elastic tissue	3.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
Epithelialization	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00

PE propolis extract, *PG* propylene glycol, *SS* silver sulfadiazine, *UT* untreated group

Difference of values with superscript a is significantly higher than the differences between values with superscripts b and c

Table 5 Immunohistochemical expression of the intensity of epidermal growth factor across the groups

DAYS after wound	UT	PE	SS	PG
4	73.72 ± 5.89	113.04 ± 4.87	107.32 ± 4.34	68.45 ± 3.44
8	80.73 ± 2.35^{c}	113.21 ± 2.12^{c}	134.35 ± 5.40^{b}	117.08 ± 5.29^{a}
12	57.27 ± 6.21^{c}	110.17 ± 2.44^{b}	147.25 ± 3.56^{b}	74.81 ± 4.35^{c}
16	87.96 ± 1.05^{a}	190.68 ± 6.39^{a}	163.03 ± 2.39^{a}	156.38 ± 3.33^{a}
21	96.48 ± 3.67	115.58 ± 3.45	115.37 ± 5.62	75.13 ± 6.60

PE propolis extract, *PG* propylene glycol, *SS* silver sulfadiazine, *UT* untreated group

Difference of values with superscript a is significantly higher than the differences between values with superscripts b and c

on days 5 to 12. This is evident of wound infection and debridement challenges showing that wound healing agents accelerate wound debridement and infection clearance which paves way for the proliferative phase (Zhao et al. 2016).

Granulation tissue consisting of fibroblasts, inflammatory cells, and angiogenic blood vessels in a matrix of fibronectin, collagen, glycosaminoglycans, and proteoglycans is a central event during the proliferative phase of wound healing (Landen et al. 2016). The propolis extract group had high granulation tissue on day 8 when compared with the silver sulfadiazine group. This can be attributed to the nourishing effects of polyphenols and flavonoids in propolis which enhances wound debridement and stimulates the release of cytokines and growth factors. Some of these substances includes caffeic acid, artepillin C, quercetin, and galangin compounds (Cushnie and Lamb 2005; Popova et al. 2010). The propylene glycol and untreated wound had a delay in granulation tissue formation. This could be due to the extended debridement phase. As wound matures, granulation tissue consisting mainly of collagen type III undergoes transformation into collagen type I, the number of blood vessels reduces, and the amount of fibro-elastic tissue increased.

The amount of fibro-elastic tissue on day 8 was significantly higher in the PE and SS groups than in the PG and UT groups. This indicates that type I collagen was more abundant in PE and SS groups. This is in agreement with a previous report that propolis contains active substances which are known to promote cell proliferation (Martinotti and Ranzato 2015). The observed enhanced blood vessel regression on days 12 and 16 in SS- and PE-treated wounds compared with the negative controls shows the ability of Nigerian bee propolis to accelerate wound healing by the conversion of fleshy and well vascular collagen type II granulation tissues to the less vascular collagen type I. Fibroblasts and its precursors such as collagen and elastin play an important role in restoration of tensile strength of wounded skin (Jorgensen et al. 1988). Other phytochemical contents of this propolis sample such as alkaloids which are known to possess analgesic properties and steroids known for their anti-inflammatory properties are important components needed for wound healing (Khan et al. 2011).

The immunohistochemical expression of the intensity of epidermal growth factor (EGF) shows that EGF was consistently high in the SS and PE groups compared to the PG and UT groups. High levels of EGF in injured skin have been linked to increased wound epithelialization and contraction (Stortelers et al. 2002). Cytokines and growth factors play an essential role in modulating cell behavior during wound healing and they are usually secreted by leucocytes (Stortelers et al. 2002). The high levels of EGF recorded in the propolis-treated wounds, which is comparable to the silver sulfadiazine group, shows the ability of propolis to enhance the secretion of factors that are needed for wound healing. This immunomodulatory property of propolis helps mobilize immune cells to the wound which in turn aided the secretion of transforming growth factor-alpha and beta-1(TGF-alpha and beta 1) that was responsible for the secretion of epidermal growth factor (Bodnar 2013). EGF binds to the epidermal growth factor receptor (EGFR), a protein tyrosine kinase receptor, expressed on the majority of cells in the skin (Wong et al. 1999). Activation of epithelial growth factor receptor (EGFR) leads to stimulation of epithelial cell motility, proliferation, and migration of keratinocyte, endothelial cells, and fibroblasts (Bodnar 2013), thereby leading to epidermal and dermal regeneration (Wong et al. 1999). This provides the explanation for the early wound closure observed in the PE and SS groups.

The untreated group had the lowest EGF value. This could be ascribed to the delay in formation of granulation tissues and dryness of the wound. Decreased secretion of growth factors such as EGF has been associated with dry wounds (Zhao et al. 2016). This finding shows that application of Nigerian bee propolis extract increased wound healing rate and re-epithelialization of full thickness skin wounds in rats due to its anti-inflammatory, antioxidant, and tissue regenerative properties.

In conclusion, Nigerian bee propolis enhanced the healing of full thickness skin wounds in rats and could be recommended for the treatment of various types of wounds in humans and animals especially in low-income communities where propolis is affordable and available.

Acknowledgements The authors sincerely appreciate efforts of Mr. IC Mpama and Mr. AO Nwagbara during technical preparation of tissue samples.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Aga H, Shibuya T, Sugimoto T, Kurimoto M, Nakajima SH (1994) Isolation and identification of antimicrobial compounds in Brazilian propolis. Biosci Biotechnol Biochem 58:945–946

Atiyeh BS, Dibo S, Hayek SK (2009) Wound cleansing, topical antiseptics and wound healing. Int Wound J 6(6):420–430

Bodnar RJ (2013) Epidermal growth factor and epidermal growth factor receptor: the yin and yang in the treatment of cutaneous wounds and cancer. Adv Wound Care (New Rochelle) 2(1):24–29

Boudra A, Hamdi M, Amara K (2014) Role of propolis in osteointegration enhancement in allograft autoclaved implanted in femur of dogs. J Veterinar Sci Technol 5:173. https://doi.org/10.4172/2157-7579.1000173

De Castro SL (2001) Propolis: biological and pharmacological activities. Therapeutic uses of this bee product. Annu Rev Biomed 3:49–83

Cooper RA, Molan PC, Krishnamoorthy L, Harding KG (2001) Manuka honey used to heal a recalcitrant surgical wound. Eur J Clin Microbiol Infect Dis 20(10):758–759

Couto A (2001) How to process raw propolis into propolis extracts by the Food and Agriculture Organization of the United Nations. Under 'Value added products of the beehive' 2001. www.fao.org/teca.fao.org/pt-br/read/8580. Accessed 02 March 2016

Cushnie TPT, Lamb AJ (2005) Antimicrobial activity of flavonoids. Int J Antimicrob Agents 26(5):343–356

De Groot AC (2013) Propolis: a review of properties, applications, chemical composition, contact allergy, and other adverse effects. Dermatitis 24(6):263–282

De Moura SA, Negri G, Salatino A, Lima LD, Dourado LP, Mendes J (2011) Aqueous extract of brazilian green propolis: primary components, evaluation of inflammation and wound healing by using subcutaneous implanted sponges. Evid Based Compl Alternative Med. Article ID 748283, 8 pages

Dealey C (1995) Common problems in wound care: caring for the skin around wounds. Br J Nurs 4(1):43–44

Devasagayam T, Tilak J, Boloor K, Sane K, Ghaskadbi S, Lele R (2004) Free radicals and antioxidants in human health: current status and future prospects. J Assoc Physicians India 52:794–804

Dimov V, Ivanovska N, Manolova N, Bankova V, Nikolov N, Popov S (1991) Immunomodulatory action of propolis. Influence on anti-infectious protection and macrophage function. Apidologie 22: 155–162

Duansak D, Somboonwong J, Patumraj S (2003) Effects of *Aloe vera* on leukocyte adhesion and TNF-alpha and IL-6 levels in burn wounded rats. Clin Hemorheol Microcirc 29:239–246

Dunford C, Cooper R, Molan P (2000) Using honey as a dressing for infected skin lesions. Nurs Times 96(14):7–9

Ejikeme CM, Ezeonu CS, Eboatu AN (2014) Determination of physical and phytochemical constituents of some tropical timbers indigenous to Niger Delta Area of Nigeria. Eur Sci J 10(18):247–270

Eyarefe OD, Amid SA (2010) Small bowel wall response to enterotomy closure with polypropylene and polyglactin 910 using simple interrupted suture pattern in rats. Int J Anim Vet Adv 2(3):72–75

Eyarefe OD, Fabiyi BO (2016) Wound healing potentials of aqueous pineapple (*Ananas comosus*) Extract – a Preliminary Report. Glob J Pharm 10(1):23–30

Eyarefe OD, Oguntoye CO (2016) Honey, an unexplored topical wound dressing agent in Nigerian veterinary practice. Sok J Vet Sci 14:8–17

Eyarefe OD, Alonge TO, Fayemi EO (2011) The incidence of intestinal obstructive diseases in selected veterinary clinics and hospitals in South Western Nigeria. Nig Vet J 32(1):36–39

Eyarefe OD, Ologunagba FM, Emikpe BO (2014) Wound healing potentials of natural honey in diabetic and non diabetic wistar rats. Afr J Biomed Res 17:15–21

Eyarefe OD, Idowu A, Afolabi JM (2015) Healing potentials of oral *Moringa oleifera* leaves extract and tetracycline on methicillin resistant *Staphylococcus aureus* infected wounds of Wistar rats. Niger J Physiol Sci 30:73–78

Eyarefe OD, Kuforiji DI, Jarikre TA, Emikpe BO (2017) Enhanced electroscalpel incisonal wound healing potential of honey in wistar rats. Int J Vet Sci Med 5:128–134

Fearnely J (2001) Bee propolis. Souvenir press Ltd, London

Fossum TW, Cheryl SH, Johnson AL, Schulz KS, Seim HB, Willard MD (2007) Surgical infection and antibiotic selection. In: Fossum TW, editor. In: Textbook of small animal surgery. Elsevier Science, Morsby Inc, St Louis Missouri, pp 79–89

Gavanji S, Larki B (2015) Comparative effect of propolis of honey bee and some herbal extracts on *Candida albicans*. J Veterinar Sci Technol 5(2):1–3. https://doi.org/10.4172/2157-7579.1000173

Gupta A, Kumar P (2015) Assessment of the histological state of the healing wound. Plast Aesthet Res 2:239–242

Jorgensen KD, Svendsen O, Greenough RJ Kallesen T, Goburdhun R, Skydsgaard K, Finch J, Dinesen B, Nilsson P (1988) Biosynthetic human growth hormone subchronic toxicity in rats and monkeys. Pharmacol Toxicol 62:329–333

Khan K, Karodi R, Sisoiquis A, Thube S (2011) Development of anti-acne gel formulation of Anthraquinone rich fraction from *Rubia cordifolia (Rubiaceae)*. Int J Appl Res Nat Prod 4(4):1–8

Khoo YT, Halim AS, Singh KKB, Mohamad NA (2010) Wound contraction effects and antibacterial properties of Tualang honey on full-thickness burn wounds in rats in comparison to hydrofibre. BMC Complem Alternative Med 10:48. https://doi.org/10.1186/1472-6882-10-48

Landen NX, Li D, Stahle M (2016) Transition from inflammation to proliferation: a critical step during wound healing. Cell Mo Life Sci 73(20):3861–3885

Martinotti S, Ranzato E (2015) Propolis: a new frontier for wound healing? BMC Burns Trauma 3(9):7. https://doi.org/10.1186/s41038-015-0010-z

Miller AC, Rashid RM, Falzon L, Elamin M, Zehtabchi S (2012) Silver sulfadiazine for the treatment of partial-thickness burns and venous stasis ulcers. J Am Acad Dermatol 66:159–165

Mirzoeva OK, Calder PC (1996) The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leukot Essent Fat Acids *55*(6):441–449

Molan PC (2011) The evidence and the rationale for the use of honey as a wound dressing. Wound Pract Res 19(4):204–220

Nguyen DH, Zhou T, Shu J, Mao JH (2013) Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity. Cancer InCytes M 2(1):1–6

Oguwike FN, Nwozor CM, Onwurah CN, Orjiewulu N, Olisah MC (2013) Comparative study on wound healing using potash-table salt mixture and honey on albino rats. Afrimedic J 4:29–32

Park YK, Alencar SM, Aguiar CL (2002) Botanical origin and chemical composition of Brazilian propolis. J Agric Food Chem 50(9):2502–2506

Patrick ES (2016) Skin wound healing. Plastic surgery medscape; www.emedicine.medscape.com, Retrieved 21 August 2017

Popova MP, Graikou K, Chinou I, Bankova VS (2010) GC-MS profiling of diterpene compounds in Mediterranean propolis from Greece. J Agric Food Chem 58(5):3167–3176

Prockop DJ, Kivirikko KI (1995) Collagens, molecular biology, diseases and potentials for therapy. Annu Rev Biochem ***64***:403–434

Qiao Z, Chen R (1991) Isolation and identification of antibiotic constituents of Propolis from Henan. Zhongguo Zhong 16(8): 481–482

Rosique RG, Rosique MJ, Farina JA (2015) Curbing inflammation in skin wound healing: a review. Int J Inflamm 25(1):73–78

Silva BB, Rosalen PL, Cury IA, Ikegaki M (2008) Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. Evid-Based Complement Alternat med 5(3):313–316

Simon PE, Outran HA, Romo III T, Pafford W, Pearson JM, Yalamanchili H, Zoumalan RA (2014) Skin wound healing. http://emedicine.medscape.com/article/884594-overview. Accessed 17 Dec 2016.

Simone-Finstrom M, Spivak M (2010) Propolis and bee health: the natural history and significance of resin used by honey bees. Apidologie 41(3):295–311

Stortelers C, Souriau C, van Liempt E, van de Poll ML, van Zoelen EJ (2002) Role of the N-terminus of epidermal growth factor in ErbB-2/ErbB-3 binding studied by phage display. Biochemistry 41(27): 8732–8741

Walker M (2009) Honeybees sterilize their hives, reporting life on earth. Earth news, BBC. http://news.bbc.co.uk/earth/hi/earth_news/newsid_8152000/8152574.stm. Accessed 22 June 2016

Wall IB, Davies CE, Hill KE, Wilson MJ, Stephens P, Harding KG, Thomas DW (2002) Potential role of anaerobic cocci in impaired human wound healing. Wound Repair Regen 10(6):346–353

Wong L, Deb TB, Thompson SA, Wells A, Johnson GR (1999) A differential requirement for the COOH-terminal region of the epidermal growth factor (EGF) receptor in amphiregulin and EGF mitogenic signaling. J Biol Chem 274(13):8900–8909

Zhao R, Liang H, Clarke E, Jackson C, Xu M (2016) Inflammation in chronic wounds. Int J Mol Sci 17(12):2085