



Effects of Aqueous Extract of *Piper guineense* on Wound Angiogenesis in Male Adult Guinea Pigs

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Abstract

Wound is a significant burden to healthcare and the discovery of substances with wound healing will be of great help to health practice. The use of plants extract has been found to be useful overtime. This study is aimed at analyzing the effects of aqueous extract of *Piper guineense* on wound angiogenesis. Eight adult male guinea pigs were used for the study. They were grouped into four groups of two animals each. The groups were designated as A, B, C & D. The Full thickness wound of approximately 4cm²-11cm² was caused on the dorsolateral region of the animals. Group D served as the control and received normal saline. The experimental groups B, C & D received different doses of drugs as follows: group A received 200mg/kg body weight of extract of *Piper guineense*, group B received 150mg/kg body weight of extract of *Piper guineense* while group C received 120/kg body weight of extract of *Piper guineense*. The wounds were dressed daily throughout the experimental periods. The administration was done orally for fourteen days. Twenty four hours after the last administration, tissue samples were collected for histological studies. The wound contraction was calculated and analyzed using SPSS. Histological result showed that angiogenesis was enhanced in the experimental groups when compared with the control. There was also improved fibroblast migration, reduced epithelialization and uniform rate of healing evidenced in the statistical analysis. *Piper guineense* stimulate angiogenesis in a healing wound and promotes fibroblast migration to the wound site thereby aiding healing.

1 Introduction

A wound is an injury or damage to a normal tissue as a result of a physical impact. It can also be said to be a loss or a compromise in the integrity of a normal tissue. The occurrence of a wound triggers the healing process. Wound healing is a complex, biological process which involves replacing a damaged tissue by a living one. The human adult wound healing process can be divided into four overlapping phases, which includes- the haemostatic phase, the inflammatory phase, the proliferative phase, and the remodeling phase¹.

Wounds that follow the normal physiological process of healing do not pose a threat to individuals; sometimes such wound can heal without medication. However some wounds fail to proceed

through an orderly and timely reparative process to produce anatomic and functional integrity of the injured site, these types of wounds are referred to as chronic or non-healing wound and usually it occurs in individuals who are at old age or have an underlying disease such as diabetes or obesity². Chronic wounds present a significant burden to healthcare services, health care professionals and the patients, consuming a large amount of human and financial resources^{3, 4}. According to the Australian wound management association an estimated 400 000 Australians have chronic wound at any time. This high incidence of wounds translates into a major burden on the healthcare system and the annual costs were estimated to be 3

billion dollars in 2005. In America 6.7 million patients affected costing an estimated 25 billion dollars annually. In Nigeria, even though there is limited information on the prevalence of wound and cost of management, the available data show that patients spend a lot in recovering from wound⁵.

On the part of the patients, it consumes almost every aspect of their daily lives as result of the pains associated with it. Sometimes individuals bearing wounds are immobilized while some are isolated due to the smelly and unpleasant nature of the wound⁶.

Angiogenesis occurs at the proliferative stage of wound healing. It is a critical component for processes of wound healing. It is defined as the formation of new capillaries from pre-existing blood vessels. The newly formed blood vessels participate in formation of granulation and provide nutrition and oxygen to growing tissues. They also provide a means of migration for the inflammatory cells, to the site of injury. When angiogenesis is insufficient, it can lead to impaired wound healing and chronic wound formation and as such substances that have the capacity to enhance angiogenesis in wound will be of help in wound healing therapy⁷.

Some approaches like the use of stem cells, creation of micro fluidic networks in organ constructs for implantation to wound site and the use of endothelial progenitor cells to stimulate wound angiogenesis etc, have been made to induce angiogenesis and effect wound healing, but despite all this chronic wounds remain significant healthcare problems. There is therefore a need to explore and create alternative pathways⁸.

One of such ways includes the use of plant extracts; plants have immense for the management and treatment of wounds. Plants are used by tribal and folklore in many countries for the treatment of wounds and burns. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. These phytomedicine are not only cheap and affordable but are also safe. the presence of various life sustaining constituents in plants has urged scientists to examine these plants with a view to determine the potential wound healing properties the herbal extracts of plants such as *Alium cepa*, *Carica papaya*, and *Berberis lyceum* have been tested and found to accelerate wound healing.

In this study the effect of *Piper guineense* will be determined. *Piper guineense*, otherwise referred to as Climbing black pepper, Benin pepper, West African or bushpepper; belongs to the plant family called piperaceae⁹⁻¹¹. At least five species of the *Piper genus* are cultivated to produce what is generically called "pepper. The most common is *Piper nigrum* (blackpepper); others include *P. longum* (long pepper), *P.cubeba* (cubeb pepper) and *P. guineense* (West Africanor bush pepper). The pepper plants are either vines or climbing shrubs which can

grow up to fifty feet in length. The leaves are glossy; about six inches long^{12, 13}.

The Black pepper is the most pungent and flavorful of all types of peppers and is available as whole or cracked peppercorns or ground into powder. Studies have shown that apart from the use of these plants as spices and condiments, they have several other wide applications in the local treatment and management of many diseases. Indigenous people value the plants for their ethno medicinal uses as much as for spicing foods¹⁴.

P. guineense is used as anticonvulsant^{15, 16}. The fruits and leaves are used as spice for preparing soup for post-partum women. Powder from the dried fruits mixed with honey acts as carminative and relieves stomachaches¹⁷. Extract of black pepper has been reported to stimulate digestion of foods by stimulating secretion of digestive enzymes, pancreatic amylases, trypsin and chymotrypsin¹⁸ and is therefore used for treatment of digestive disorders. These various effects of piper guineense shows a possibility of a positive effect of it on wound healing and wound revascularization.

Therefore this work was aimed to determine the effects of topical aqueous extract of *Piper guineense* on angiogenesis in a healing wound.

2 Materials and Methods

2.1 Place of Study

This research was carried out in the Department of Human Anatomy, College of Health Sciences, Nnamdi Azikiwe University, Okofia Nnewi.

2.2 Experimental procedure

2.2.1 Preparation of extract

Clean and dried seeds of *Piper guineense* were purchased from a local market in Anambra Nigeria. The dried seeds were pulverized using Molino Victoria traditional grain mill (high hopper Ret 600009, Colombia), sieved and stored in air-tight bags. A 750.55g amount of the pulverized *Piper guineense* seed was obtained and was macerated with 1.5L of distilled water at room temperature. The preparation was filtered using muslin cloth after which it was concentrated using water bath at a temperature of 450 °C. After concentration, 229.80g of the dried extract was obtained and stored in the refrigerator at 40 °C until when it was used.

2.2.2 Experimental animals

Seven (7) adult male Guinea pigs weighing between 350g - 600g were used for this experiment. The animals were purchased from an Animal Farm in Umuokpu Village Awka, Anambra state. They were housed in ventilated cages, at 27 ± 2 °C, relative humidity 50 ± 15% and normal photo period (12h dark/12h light). They were allowed to acclimatize for a period two (2) weeks before carrying out the experiment at which their

weights were found to have increased to the range of 450-700g. Wood shavings were collected from sawmill and used for beddings. The beddings were changed at least twice per week throughout the period of acclimatization and three times per week during the experiment. They were fed twice daily with pelletized growers mash mixed with starters mash, vegetables and water mixed with vitamin supplement. The feed was bought from Nkwo Market, Nnewi, Anambra state.

2.2.3 Wound creation

The wounds were created on the dorsolateral region of the animals, after shaving the hairs using a pair of scissors, the animals were anaesthetized with 2ml of lignocain local anaesthesia around the region of the skin where the wound is to be inflicted, using a pair of scissors and forceps, a portion of the skin was pinched and cut with scissors, after which the covering dermal and fascia was cut out until the panniculus adipose was exposed, creating a full thickness wound measuring approximately 4cm²-11cm². Then the animals were grouped randomly as follows:

Group A - Received 200mg/kg body weight of extract of *Piper guineense*

Group B - Received 150mg/kg body weight of extract of *Piper guineense*

Group C - Received 120mg/kg body weight of extract of *Piper guineense*

Group D - Control group and received normal saline

2.2.4 Drug administration/wound management

The working solution for the wound treatment was prepared at three different concentrations of 20%, 15%, and 12% for groups 1, 2 and 3 respectively. This was done by dissolving 20g, 15g and 12g of the extract in 100ml of water, to give 200mg/ml, 150mg/ml and 120mg/ml of extract preparation respectively. The solution was prepared at three days interval and stored in a refrigerator at 40c to ensure the potency is maintained and administered topically on daily basis using a 5ml syringe.

2.2.5 Wound dressing

The wounds were dressed immediately after the wound was caused. Daily dressing continued throughout the period of the experiment which lasted for fourteen (14) days. Normal was used to rid the wound of debris then the aqueous extract was applied topically on the wound. The wound was covered with gauze and held firmly with plaster.

2.2.6 Wound surface area/percentage rate of contraction

The wound surface was calculated by multiplying the cross diameter of the wound on daily basis, while the percentage rate of contraction was calculated by: $\times 100$

2.3 Histological Evaluation Sample Collection

Tissue sample was collected from the wound for biopsy on days 3, 6, 10, 14 from all the animal groups. They were fixed using the standard fixative of 10% formal saline and were kept in a container until processing of the tissues.

2.3.1 Dehydration of Tissues

The tissues were first immersed into a bath containing 70% alcohol and left overnight followed by transferring tissues into different baths containing 90%, 95%, absolute I,II and III for 2 hours each and then absolute IV overnight.

2.3.2 Clearing of the Tissues

On removal of the tissues from absolute alcohol, the tissues were passed through 3 changes of xylene (I, II, III) for 1 hour 30minutes each.

2.3.3 Impregnation of Tissue

On removal of the tissue from the clearing agent, they were immersed in a wax bath I for 2hours and wax bath II in a hot air oven for One hour.

2.3.4 Embedding of Tissue

Infiltration was done in molten paraffin wax at a temperature of 60°C for two hours each in two changes.

2.3.5 Sectioning

The embedded tissues were sectioned with a rotary microtome.

2.3.6 Staining

Haematoxylin and eosin method was used for staining after sectioning.

2.3.7 Microscopy/Cell Identification

The tissue slides were examined by a pathologist using a light microscope and the photomicrographs were taken

2.4 Statistical analysis

The mean and standard deviation for the percentage rate of contraction during administration was generated using the SPSS software, Version 22 (Chicago, IL, USA) and data were analyzed statistically. Comparison was done using one-way analysis of variance

3 Results

The topical administration of *Piper guineense* to animals exhibited that angiogenesis were enhanced in the experimental group compared to control group (Table 1).

The angiogenesis process in control and extract treated animals on different days are illustrated in Fig 1 to Fig 8 and table 2 to table 5. On fourteenth day of study, the extract treated animals at the dose of 200 mg/ml and 150 mg/ml exhibited very low inflammation with predominance of fibroblast and moderate angiogenesis. Consequently, the animal treated with extract

(120 mg/ml) demonstrated majorly fibroblast and moderate angiogenesis, while control animals have more of a scar tissue, mainly fibroblast with poor angiogenesis (Table 5 & Fig 7 & 8)

Table 1: Shows the mean and standard deviation for the rate of contraction from day 1-14

Rate of Contraction (%)	Group A	Group B	Group C	Group D (Control)	P-value
Day 1	0.00±0.00	0.00	0.00±0.00	0.00	
Day 2	11.89±4.69	13.11	8.05±5.25	0.00	0.413
Day 3	21.50±10.61	26.00	13.17±12.49	0.00	0.515
Day 4	25.99±12.71	37.78	17.99±6.78	19.00	0.551
Day 5	31.55±12.66	28.89	18.35±6.28	27.75	0.659
Day 6	29.94±17.30	37.78	29.18±1.76	36.00	0.913
Day 7	28.94±12.36	37.78	23.27±6.60	51.25	0.357
Day 8	33.38±1.56	33.33	27.42±8.02	61.00	0.114
Day 9	40.69±0.24	40.00	33.25±0.22	67.50	0.000
Day 10	45.90±7.53	44.00	44.15±4.64	79.00	0.109
Day 11	47.06±5.94	48.11	47.65±0.31	77.50	0.067
Day 12	51.73±12.54	58.44	55.35±3.20	79.75	0.323
Day 13	59.33±4.71	56.00	45.71±16.84	79.75	0.390
Day 14	57.97±7.90	56.00	58.19±3.79	87.75	0.135

Table 2: Day 3 of experiment/1st Biopsy

Group A (200mg/ml)	Highly inflammatory with poor angiogenesis
Group B (150mg/ml)	Moderately inflammatory, with adequate angiogenesis and a high number of fibroblast
Group C (120mg/ml)	Highly inflammatory
Group D (Normal Saline)	Highly inflammatory with incomplete re-epithelialization

4 Discussions

The key wound healing process is angiogenesis re-epithelialization, granulation tissue formation and remodeling. It is driven by dermal fibroblast, endothelial cells and keratinocytes and orchestrated by bioactive molecules including growth factors, their receptor and matrix molecules. Wound is a significant healthcare problem therefore researchers are in the quest for substances that can enhance healing. Angiogenesis as a stage of healing have been shown to be a very vital process and importantly, inhibition of angiogenesis has been shown to kdeter wound healing. Substances with angiogenic effect are therefore of great importance².

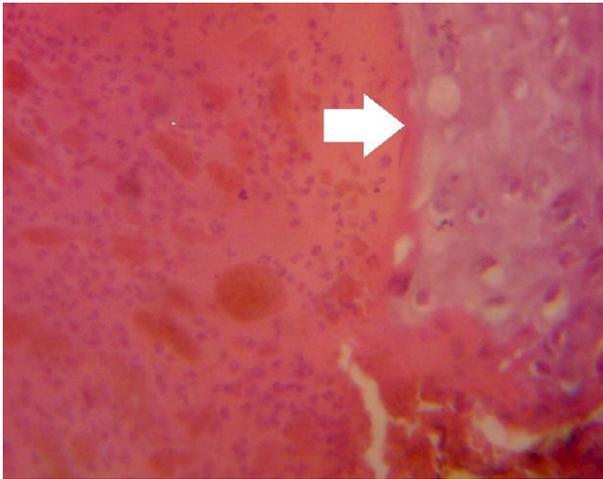


Fig 1: Photomicrograph of the control group on day 3, Mag X40, H&E stain

The Arrow Points towards the Epithelial Tissue Still Under Formation

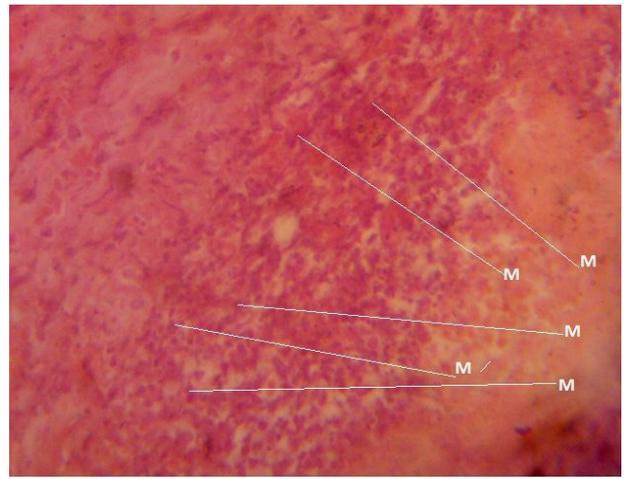


Fig 3: Photomicrograph of the control group on day 6 Mag X40 H&E stain

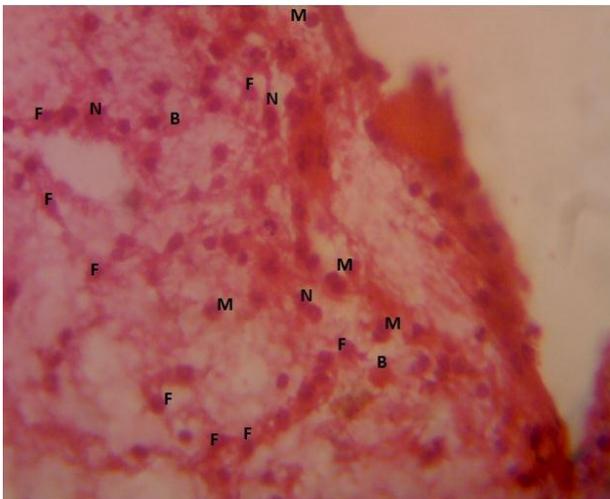


Fig 2: Photomicrograph of the experimental group on day 3. Mag X10 H&E staining

M Stands for Macrophages, N Stands for Neutrophils, B Stands for Blood Vessels, F Stands For Fibroblast

Table 3: Day six of experiment/2nd biopsy

Groups	Observation
Group A (200mg/ml)	Moderately inflammatory with poor angiogenesis
Group B (150mg/ml)	Adequate granulation tissue with adequate angiogenesis and a high number of fibroblast
Group C (120mg/ml)	Moderately inflammatory
Group D (Normal Saline)	Highly inflammatory

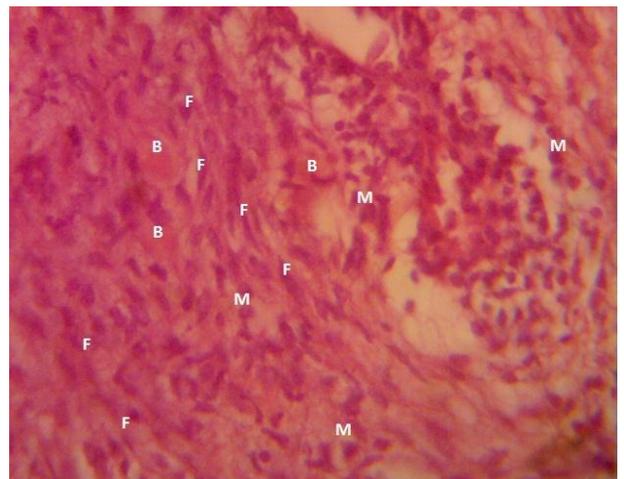


Fig 4: Photomicrograph of the experimental group on day 6, Mag X40, H&E stain

Table 4: Day 10 of experiment/3rd biopsy

Groups	Observation
Group A (200mg/ml)	High inflammation with moderate angiogenesis
Group B (150mg/ml)	Low inflammation with low angiogenesis and fibroblast deposition
Group C (120mg/ml)	Low inflammation with increased fibroblast and adequate angiogenesis
Group D (Normal Saline)	Moderate inflammation with predominance of fibroblast and poor angiogenesis

From the present study, on topical administration of *Piper guineense*, angiogenesis was seen to be enhanced in the experimental group as compared with the control, this is indicated in the fact that the experimental group especially the

ones with the higher doses was already undergoing angiogenesis at day 3 of the experiment while the control group was showing to be inflammatory without any sign of angiogenesis.

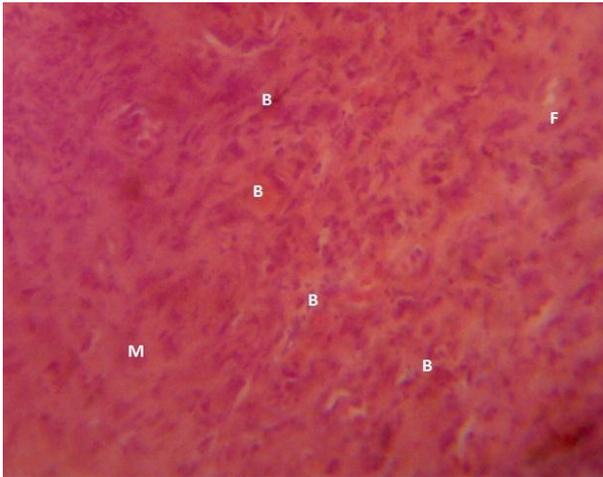


Fig 5: Photomicrograph of the control group on day 10, Mag X40 H&E stain

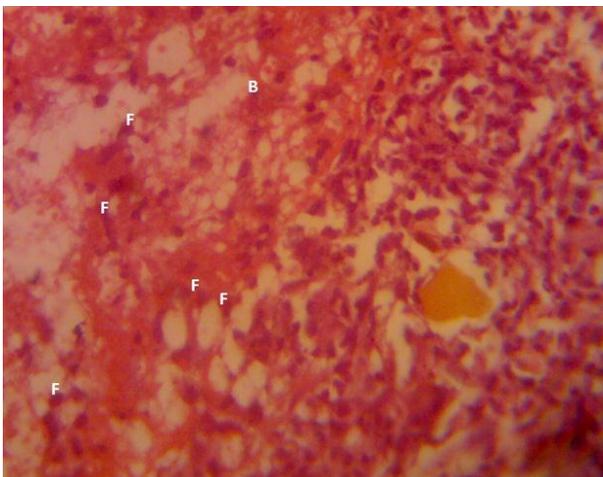


Fig 6: Photomicrograph of the Experimental Group on Day 10. Mag X40 H&E Stain

Table 5: Day 14 of Experiment/4th Biopsy

Groups	Observation
Group A (200mg/ml)	Very low inflammation with predominance of fibroblast and moderate angiogenesis
Group B (150mg/ml)	Very low inflammation with predominance of fibroblast and moderate angiogenesis
Group C (120mg/ml)	Majorly fibroblast and moderate angiogenesis
Group D (Normal Saline)	More of a scar tissue, mainly fibroblast with poor angiogenesis

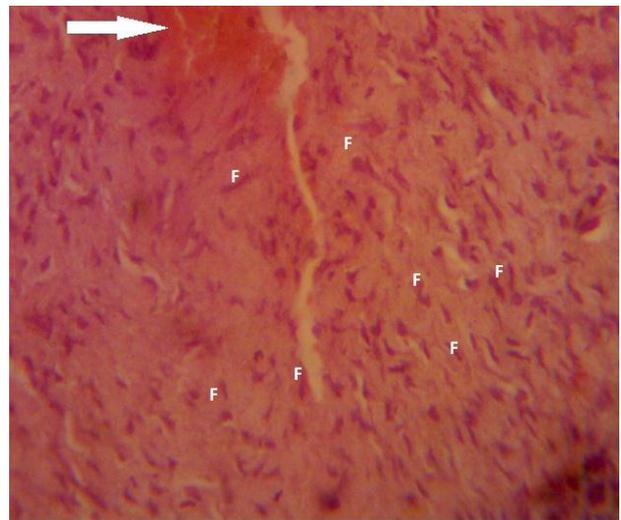


Fig 7: Photomicrograph of the control group on day 14 of the experiment

The arrow points towards the scar tissue

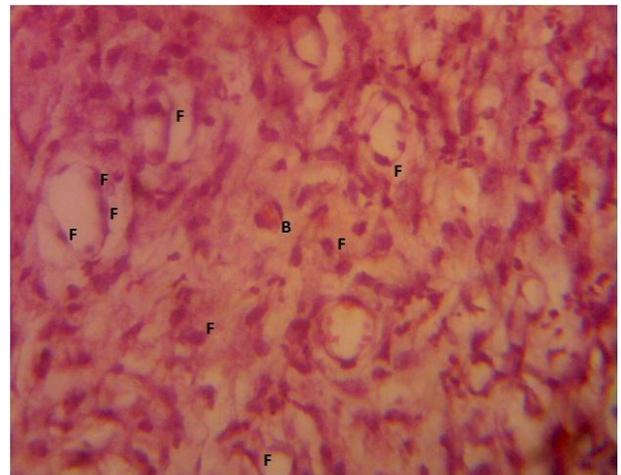


Fig 8: Photomicrograph of the experimental group on day 14 of the experiment

This supports the findings of Hazel *et al.*, (2003) that the herbal extract of *Calendula officinalis* enhanced angiogenesis¹⁹. In comparison with the stages of wound healing that have been earlier described, it can be seen that there was an enhancement in angiogenesis. In the normal wound healing process angiogenesis begins at the 4th day post wound²⁰ while in the present study, angiogenesis started on the 3rd day post wound. This stimulation of angiogenesis in the present experiment could be traceable to two phytochemicals present in *Piper guineense*, this includes steroid and triterpenes^{21,22}.

These two compounds have been earlier verified in the works of Della- loggia *et al.*, (1990) to have angiogenic effect and were verified in the same study to have up regulated vascular endothelial growth factor which is the soluble mediator for angiogenesis²³. The process of angiogenesis has been described to be regulated by low oxygen tension, growth factors and acidic wound environment²⁴. In the present study, the effect

of *Piper guineense* on angiogenesis can be related to any of these factors, it has already been shown that piper angiogenesis contains compounds which upregulates VEGF²³, in addition, *Piper guineense* has antioxidant effect, of which its topical administration may have created an environment favourable for angiogenesis to occur²⁵.

More so, from the result of this study, there was faster migration of fibroblast in the experimental group, indicated in the fact that at the third of the experiment fibroblast was present in the experimental group but was still absent in the control group until the 10th day. The migration of fibroblast has been described to be as a result of chemottractive gradient created by growth factor²⁶. *Piper guineense* may have acted as a chemoattractant causing the migration of these cells. This is in line with the findings of Fawehinmi *et al.*, (2008) which reports that plant extracts contain rich nutrient which acts in a growth factor-like manner, inducing the early migration of fibroblast²⁷.

Furthermore, the rate of healing in this study was found to be uniform on the analysis of data using one way anova, this contradicts previous findings which indicates that plant extract accelerate wound healing²⁸. From the records of the re-epithelialization in this experiment, it was observed that there was a retarded rate of re-epithelialization in the experimental group. This is indicated in the fact that as of the third day of the experiment the control group showed incomplete re-epithelialization while the experimental group did not, also at the fourteenth day of the experiment which was the end of the experiment, the control group showed complete re-epithelialization while the experimental group did not, this shows that the topical application of *Piper guineense* inhibits re-epithelialization of wound. The migrations of keratinocytes which are the chief cells of epithelialization are influenced by the nature of the wound environment and the protein composition of the wound²⁹.

However it is known that herbal extracts may contain endotoxins such as lipopolysaccharides which may hinder migration of keratinocytes. This therefore shows that *Piper guineense* inhibits epithelialization in as much as it induces angiogenesis and fibroblast migration. This is in line with the findings of that different phases of healing may react differently to a particular substance. The inhibition of epithelialization in the experimental group, formation of scar tissue in the control group at day 14 which was not seen in the experimental group and the uniform rate of healing amongst the experimental and control group indicates that *Piper guineense* had an anti-healing effect, particularly at the later stage of healing. The experimental group was seen to have resolved inflammation earlier than the control group, showing that there was a better progress in the experimental group at the earlier stage while the control group formed scar before the experimental group,

showing a better progress of the control group than the experimental group at the later stage of the wound^{29, 30}.

5 Conclusion

Piper guineense stimulates angiogenesis in a healing wound and promotes fibroblast migration to the wound site thereby aiding healing. *Piper guineense* does not have a significant effect on the rate of wound contraction and may also inhibit epithelialization of the wound

6 Conflict of interests

None declared

7 Author's contributions

NHC, ICV, EDN, ECD, Eao, CGU and AIO contributed equally for analysis/interpretation and manuscript preparation.

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